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## EFFECT OF SALINITY ON INFECTION PROGRESSION AND PATHOGENICITY OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN)

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**ABSTRACT** The effect of salinity on *Perkinsus marinus*, a protozoan pathogen of the eastern oyster, *Crassostrea virginica* (Gmelin 1791) was investigated. Oysters parasitized by *P. marinus* were exposed in the laboratory to 6, 9, 12, and 20 ppt at a temperature ranging from 20–25°C, for an eight week period. Infection prevalence and intensity were assessed in samples (n = 25) from each treatment following 2, 4, 6, and 8 weeks of exposure and oyster mortality was determined daily. The pathogen persisted, at high prevalences, throughout the course of the experiment at all treatment salinities; however, *P. marinus* infection development was retarded at 12 ppt and did not progress at 6 and 9 ppt. Cumulative oyster mortalities progressively increased with increasing salinity and at the termination of the experiment were 9.1, 11.6, 21.1, and 27.8 percent at 6, 9, 12, and 20 ppt, respectively. A critical range for parasite pathogenicity apparently exists between 9 and 12 ppt. Although *P. marinus* is able to tolerate salinities as low as 6 ppt it is less virulent at salinities below 9 ppt.

**KEY WORDS:** *Perkinsus*, oyster, salinity

### INTRODUCTION

During the past three decades commercial oyster landings in Virginia have declined from an average of 3.5 million bushels per year prior to 1960, to a record low 0.1 million bushels in 1990–1991 (Virginia Marine Resource Commission landings data). This decline has been attributed to over fishing, declining water quality and disease (Hargis and Haven 1988). Factors that lead to disease epizootics in marine organisms are extremely complex and include biotic and abiotic parameters (Thorson 1969, Rohde 1982). For osmoconformers, such as the eastern oyster, *Crassostrea virginica*, salinity plays a major role in modulating its association with disease organisms (Hepper 1955, Bayne et al. 1978, Gauthier et al. 1990). Generally, oyster parasites have a narrower salinity tolerance than their host and are more common in high salinity areas (Hopkins 1956, Wells 1961, Andrews 1964, Farley 1975, Ford and Haskin 1982, Andrews 1983, Gauthier et al. 1990). Low salinity exposure (<10–15 ppt) often reduces the occurrence and the virulence of disease organisms. In the last decade salinity increases in Virginia's upper estuaries, resulting from four consecutive drought years (1985–1988), have caused an intensification of *Perkinsus marinus* (commonly known as Dermo), one of the Chesapeake Bay's most problematic oyster pathogens (Burreson and Andrews 1988, Burreson 1989). In response to increasing salinities in upper bay waters the parasite has spread to previously disease free seed areas and has had a severe impact on the oyster resource and industry. A more thorough understanding of the influence of salinity on the relationship between *P. marinus* and the eastern oyster will help elucidate the annual variability in the distribution and pathogenicity of this parasite and allow resource managers and oyster growers to forecast and perhaps avoid disease epizootics.

The influence of salinity on the activity of *P. marinus* has been the focus of numerous studies. Several investigators have documented a positive correlation between salinity and *P. marinus* infection intensity through field surveys (Mackin 1951, Mackin 1956, Andrews and Hewatt 1957, Soniat 1985, Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Powell et al. 1992). Oysters grown in high salinity areas (15–30 ppt) experi-

enced higher disease prevalence than those grown at lower salinities (<15 ppt). It has been suggested that the correlation between disease level and salinity is not a result of a limiting physiological effect on host or parasite but rather is due to the dilution of infective elements by freshwater inflow into the estuary (Mackin 1956, Ray 1954, Andrews and Hewatt 1957); however, disease development was retarded and oyster mortality was suppressed in infected oysters that were transplanted to a low salinity site (1–13 ppt) in the James River, Virginia (Andrews and Hewatt 1957) suggesting that salinity may have some physiological effect on the parasite.

Few laboratory studies investigating the effect of salinity on *P. marinus* have been conducted. Ray (1954) investigated the comparative development time of *P. marinus* in artificially infected oysters maintained at high (26–28 ppt) and low (10–13.5 ppt) salinity in closed aquaria. The parasite tolerated the low salinity treatment; however, development of infection and subsequent mortalities of oysters were delayed relative to the high salinity group. Similarly, Scott et al. (1985) found lower mortality in oysters held at 8–10 ppt than in oysters held at 21–25 ppt. Inhibition of *P. marinus* zoosporulation by low salinity (5–10 ppt) has been documented in vitro studies conducted by Perkins (1966) and Chu and Greene (1989).

Studies to date have greatly enhanced our understanding of the influence of salinity on the relationship between *C. virginica* and *P. marinus*; however, further investigations under controlled conditions are needed to substantiate and elaborate current knowledge. The experiment reported here investigated the effect of low salinity exposure (6, 9, and 12 ppt) on established infections of *P. marinus*.

### MATERIALS AND METHODS

Approximately 900 oysters (60–110 mm) were collected 5 May 1989 from Deep Water Shoal, the uppermost natural oyster reef in the James River, Virginia. Immediately following collection the oysters were placed in two trays and suspended from a pier at the Virginia Institute of Marine Science in the lower York River, Virginia. The oysters remained at this location until mid Septem-

ber 1989, during which time they acquired *P. marinus* infections. The mean daily salinity at the site during the exposure period ranged from 14–22 ppt and the mean daily water temperature ranged from 19–27°C. On 14 September 1989 the oysters were transferred to the laboratory and cleaned of fouling organisms. Three replicate samples ( $n = 25$ ) were analyzed for *P. marinus* intensity and prevalence.

Oysters serving as uninfected controls were collected 8 September 1989 from Ross' Rock located in the upper Rappahannock River, Virginia. At the time of collection a sample of 25 oysters was examined for *P. marinus* prevalence and intensity.

The laboratory portion of the experiment was conducted at the Virginia Institute of Marine Science Eastern Shore Laboratory, Wachapreague, Virginia. Oysters were randomly assigned to one of four salinity treatments; a high salinity (20 ppt) control treatment and three low salinity treatments, 12, 9, and 6 ppt. Five replicate, 50 liter, polypropylene tanks, each containing 30 oysters were established at each salinity treatment. One tank containing 30 uninfected control oysters was also established at each salinity treatment.

All oysters were conditioned to salinity treatments so that no greater than a 5 ppt change in salinity was experienced in a 24 hour period. Water of the desired treatment salinity was prepared daily by diluting filtered sea water (pumped from Finney Creek) with fresh well water in 44 gallon plastic containers. The sea water was filtered through a series of filters including a 25 micron bag filter, two sand filters containing sand and activated carbon, and a 1 micron bag filter. Filtration removed seston, ensuring that food availability did not vary between treatments, and reduced the possibility of exposure to *P. marinus* and other oyster parasites which may have been present in influent water. Aquaria water was aerated and changed daily. Mean daily water temperature was 23.3°C ( $\pm 1.9$  s.d.).

Oysters were fed a commercial algal diet (Diet A, Coast Oyster Co., WA) daily. An aliquot of the algal mix (2.5 ml) was diluted with 250 mls of filtered sea water and added to each aquaria. The food source was adequate on the basis of feces and pseudofeces production by most individuals and by the overall condition of uninfected oysters and oysters with low level infections (i.e. firm and opaquely colored tissue and well developed gonads).

The experiment was conducted for a period of eight weeks. Oyster mortality was recorded daily. All gaping oysters were removed from aquaria and examined for disease organisms. Random samples of live oysters, five from each replicate tank, were taken from each treatment group on day 14, 28, 42, and 56. The oysters were shucked and *P. marinus* prevalence and intensity were determined using thioglycollate culture of rectal, gill, and mantle tissue; infection intensities were rated as negative, light, moderate, and heavy (Ray 1952). Diagnosis of other oyster parasites [*Haplosporidium nelsoni* (Haskin, Stauber, and Mackin), *Bucephalus cuculus* McGrady and *Nematopsis ostrearum* Prytherch] was by routine paraffin histology of tissue fixed in Davidson's AFA. Parasite intensity and prevalence in the control groups were evaluated only at the termination of the experiment.

On day 28 of the experiment, 25 oysters from each low salinity (6, 9, and 12 ppt) treatment group were transferred to 20 ppt in order to determine if infections that may have become subpatent would reappear upon exposure to high salinity. Mortality of the transferred oysters was followed daily for the remainder of the experiment and at the termination of the experiment all live oysters were analyzed for *P. marinus* and other parasites. Control oysters

were not treated similarly; hence, we did not have an appropriate control to assess the solitary effect of the salinity change on the survival of oysters transferred from the low to high salinity conditions.

Cumulative mortality was determined for each treatment replicate on day 14, 28, 42, and 56. In order to adjust for samples removed from each replicate, mortality was calculated as follows. Interval mortality, mortality occurring between sample dates (day 1–14, 15–28, 29–42, 43–56), was determined for each replicate group by dividing the number of oysters dying during an interval by the number of oysters that were alive at the beginning of the interval. Interval mortality was then multiplied by the proportion of survivors of the previous interval (1–cumulative mortality of preceding interval) to yield the adjusted interval mortality. Successive cumulative mortalities were then determined by summing adjusted interval mortalities and preceding cumulative mortalities.

Differences in mean cumulative mortality and mean prevalence between treatment groups and through time were determined by a two factor analysis of variance (ANOVA). Differences in mean cumulative mortality and mean prevalence between treatment groups on each sample date and on data collapsed across time were determined by a one factor ANOVA. When significant differences were found a Student-Newman-Keuls (SNK) test for multiple comparison among means was performed (Zar 1984). Prior to analysis the dependent variable was arcsine transformed and evaluated for compliance to the test assumptions. Normality was examined using a Komogorov-Smirnov goodness of fit test and homoscedasticity was evaluated with a Cochran's C test (Sokal and Rohlf 1981, Zar 1984).

A hierarchical log-linear test (log-likelihood ratio test) was utilized to detect differences between salinity treatments and through time in the distribution of oysters within the four *P. marinus* intensity categories (Sokal and Rohlf 1981). All tests were judged significant at an alpha level of 0.05. Computations were made on a Prime computer using a SPSSX statistical package.

## RESULTS

The mean prevalence of *P. marinus* in oysters sampled at the initiation of the experiment was 80% ( $\pm 8\%$  s.d.) and infection intensity did not vary greatly between replicates (Figs. 1 and 2). Prevalence of *P. marinus* in oysters sampled from treatment groups on day 14, 28, 42, and 56 ranged from 76% to 100% (Fig. 1). A two factor analysis of variance indicated that the effect of salinity on prevalence was significant ( $P = 0.031$ ), while the effect of time and the interaction of salinity and time were not significant ( $P = 0.285$  and  $P = 0.915$  respectively). Prevalence, however, did not significantly differ among treatment groups on any sample date (day 14  $P = 0.3910$ , day 28  $P = 0.9446$ , day 42  $P = 0.1752$ , day 56  $P = 0.1538$ ). A significant difference in mean prevalence between treatment groups was observed when data was collapsed across time ( $P = 0.0235$ ). A SNK test revealed a significant difference only between 9 ppt and 12 ppt treatments. *Perkinsus marinus* was not detected in control oysters sampled at the initiation of the experiment; however, the parasite was present at low prevalences in live control oysters sampled at the termination of the experiment (0% at 20 ppt, 4% at 12 ppt, 12% at 9 ppt, and 0% at 6 ppt).

The effect of salinity on infection intensity was significant ( $P = 0.0338$ ). Oysters maintained at 6 and 9 ppt had a higher total number of negative and light infections and a lower total number

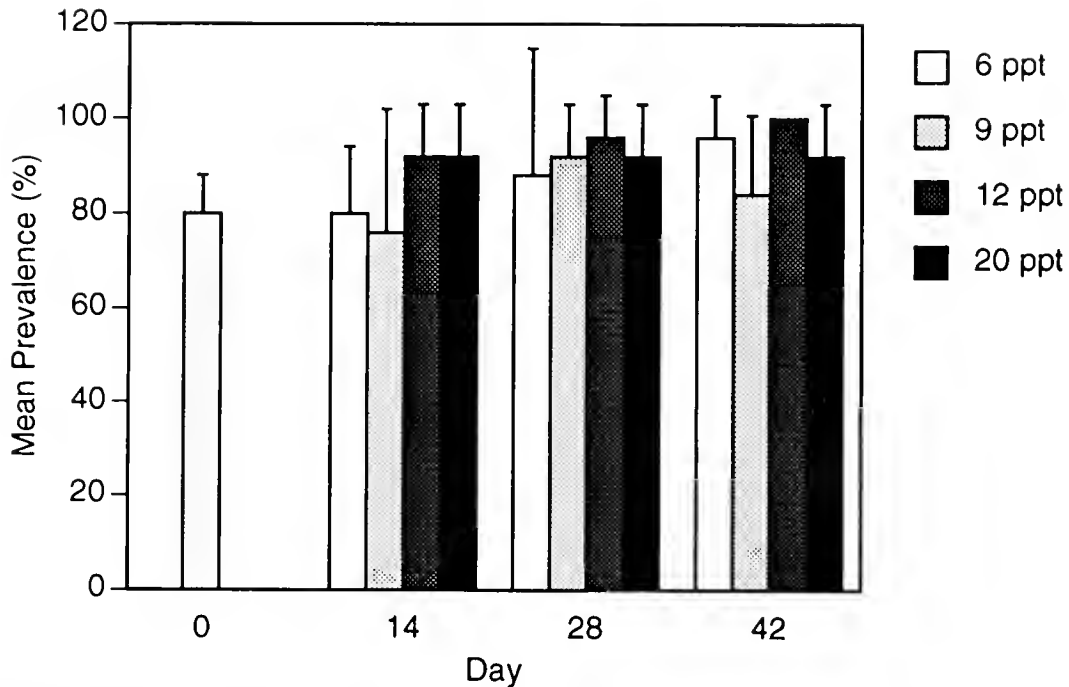


Figure 1. Mean prevalence ( $\pm 1$  standard deviation) of *P. marinus* in oysters sampled from each treatment group at the initiation of the experiment (day 0) and after 14, 28, 42, and 56 days of exposure to treatment salinities. Day 0 mean prevalence is based on three samples of 25 oysters, all other means are based on five replicate samples of 5 oysters.

of moderate and heavy infections than oysters held at 12 ppt and 20 ppt (Fig. 3). On day 14 there were relatively large differences between treatments in the number of light and heavy infections (Fig. 2). Differences in infection intensity between treatment groups were not as great as the experiment progressed and the number of oysters within each infection category (negative, light, moderate, and heavy) did not significantly differ through time ( $P = 0.0624$ ). The interactive effect due to salinity and time was not significant ( $P = 0.7087$ ).

Despite the high prevalence of *P. marinus* at all four salinity treatments a marked difference in mortality was observed. Mean cumulative mortality progressively increased with increasing salinity (Fig. 4). Mean cumulative mortalities at 6, 9, 12, and 20 ppt were respectively: 0.7%, 2.0%, 2.0%, and 7.3% on day 14; 1.5%, 6.8%, 10.1%, and 17.7% on day 28; 2.5%, 8.8%, 16.4%, 21.1% on day 42; and 9.1%, 11.6%, 21.1%, 27.8% on day 56. The effects of salinity and time on cumulative mortality were highly significant ( $P < 0.0001$ ) while the interactive effect of salinity and time was not significant ( $P = 0.8907$ ). Treatment means significantly differed on days 14 and 28 ( $P < 0.0281$  and  $P < 0.0037$ , respectively) but did not significantly differ on days 14 and 56 ( $P < 0.0956$  and  $P < 0.0607$ ) (Fig. 4).

Oysters transferred to high salinity, 20 ppt, following a 28 day low salinity treatment experienced a much higher mortality rate than those remaining continuously at the original treatment salinity. The mortality began soon after the transfer and continued until the termination of the experiment (Fig. 5).

Mortality of the uninfected control oysters was as follows: 4% at 20 ppt, 12% at 12 ppt, 4% at 9 ppt, and 0% at 6 ppt. Three of the five dead control oysters were infected by *P. marinus*. All three infections were light.

Histological analysis revealed the presence of *H. nelsoni*, *B. cuculus*, and *N. ostrearum* in 3%, 4%, and 20%, respectively, of

the total number of live oysters sampled. In general, as the experiment progressed the prevalence of all three parasites declined (Table 1). Prevalence of *H. nelsoni* at 6 and 9 ppt declined from an initial mean prevalence of 12% to 0% within the first 14 days of the investigation and remained below 4% for the remainder of the experiment. *Haplosporidium nelsoni* was present in only 3 of 73 gaping oysters that were examined histologically. In agreement with thioglycollate cultures, *P. marinus* was present in 100% of the dead oysters examined histologically. Ninety percent of the dead oysters had moderate to heavy *P. marinus* infections.

## DISCUSSION

Previous investigations have indicated that low salinity suppresses oyster mortality caused by *P. marinus* (Andrews and Hewatt 1957, Ray 1954, Scott et al. 1985). This investigation substantiates their results and further extends our understanding of this relationship by defining 9–12 ppt as a critical range for *P. marinus* activity. Oyster mortality at 6 and 9 ppt was reduced by more than 50% compared to oysters maintained at 12 and 20 ppt. At the end of the experiment mean cumulative mortality of oysters at 6 ppt was 67% lower than at 20 ppt. Additionally, oyster mortality was delayed at 6, 9, and 12 ppt relative to 20 ppt. Oysters exposed to 20 ppt began dying soon after the initiation of the experiment and continued to die through the duration of the experiment. An abundance of advanced infections in the dead oysters at 20 ppt indicates that infections were progressing during the course of the study. The pattern at 12 ppt was similar although the onset of mortality was slightly delayed relative to the 20 ppt group. Mortality of oysters at 6 and 9 ppt primarily occurred during the final two weeks of the experiment, presumably as a result of advanced infections which were present at the start of the experiment.

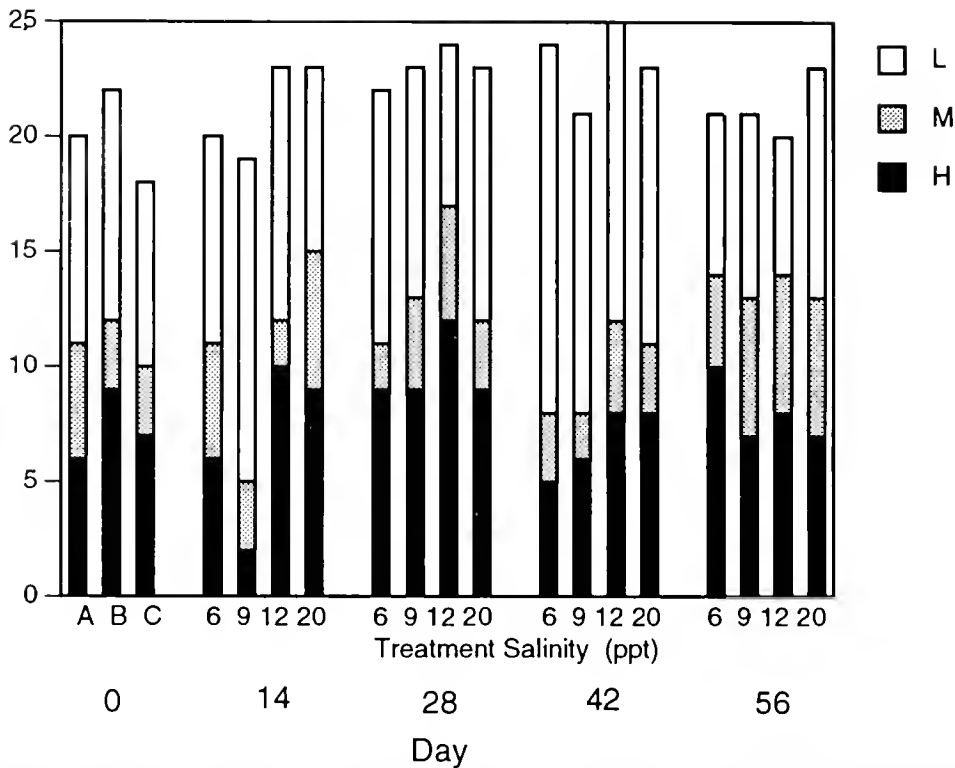


Figure 2. *Perkinsus marinus* infection intensity (H = heavy, M = moderate, and L = light) in oysters sampled at the initiation of the experiment (day 0) and after 14, 28, 42, and 56 days exposure to treatment salinities (6, 9, 12, and 20 ppt). Day 0 replicates are designated as A, B, and C. Sample size for the 12 ppt treatment group on day 56 was 20, all other samples consisted of 25 oysters.

Enhanced survival was not a permanent attribute of oysters exposed to low salinity. When transferred to high salinity, the oysters died at a relatively high rate compared to those continuously held at their original salinity. The sharp increase in mortality most likely reflects increased multiplication of the parasite in response to more favorable conditions. It is also possible that the change in salinity may have created additional stress thereby increasing mortality of oysters which had already been weakened by disease.

Although exposure of infected oysters to low salinity reduced oyster mortality, a concomitant decrease in *P. marinus* prevalence was not observed. Unlike *H. nelsoni*, which is readily eliminated from the oyster after a two week exposure to salinities less than 10 ppt (Ford 1985), *P. marinus*, once established in the eastern oyster, can tolerate salinities as low as 6 ppt for a period of at least 56 days at temperatures exceeding 20°C.

Infection intensities were also indicative of a lack of *P. marinus* expulsion. Had low salinity induced expulsion, a coincident decline in parasite intensity would have been observed in sampled oysters. A striking decrease in parasite intensity was not observed at any treatment; however, low salinity did prevent, or at least delay development of infections to pathogenic levels. Infections at 6 and 9 ppt did not significantly change during the experiment while infections at 12 and 20 ppt progressed and caused mortality within the first few weeks. Advanced infections were more numerous in oysters maintained at 12 and 20 ppt than in oysters held at 6 and 9 ppt. Statistical analysis suggest that infection intensity did not significantly change through time at any treatment. However, it is important to note that as the experiment progressed the number of oysters sampled from the high salinity groups having

advanced infections is obscured by the high mortality of oysters having advanced infections. Many oysters from the 12 and 20 ppt groups perished early in the experiment, as a result of moderate to heavy infections, and were not included in subsequent samples. Hence, the actual number of advanced infections at 12 and 20 ppt is not reflected in the statistical analysis. Development of *P. marinus* in the Ross' Rock "uninfected" control oysters may be attributed to infections which were present but undetectable at the initiation of the experiment. *Perkinsus marinus* has been detected in subsequent samples of native oysters from Ross' Rock. However, it is also possible that the infections resulted from infective cells received in incoming water during the experiment. Since the number of "new" infections is relatively small, we do not believe their presence confounds the results of this investigation.

Differences in survival between *P. marinus* infected oysters maintained at high and low salinity have been attributed to differences in infective cell densities associated with estuarine circulation dynamics (Mackin 1961, Andrews and Hewatt 1957) and to physiological differences in oysters exposed to different salinities (Scott et al. 1985). Since infective cell density was not variable in this investigation, it is presumed that the salinity effect observed reflects a physiological response of host and/or parasite. The effect of salinity on physiological aspects of *P. marinus* and *C. virginica* has been the focus of few studies. Perkins (1966) and Chu and Greene (1989) have shown that salinities from 5–10 ppt exert a direct effect on the parasite by inhibiting zoosporulation. Other studies have indicated that the oyster's defense mechanisms may be altered by environmental conditions such as temperature and salinity. The salinities examined here are well within the range of tolerance of *C. virginica* (Castagna and Chanley 1973) but may

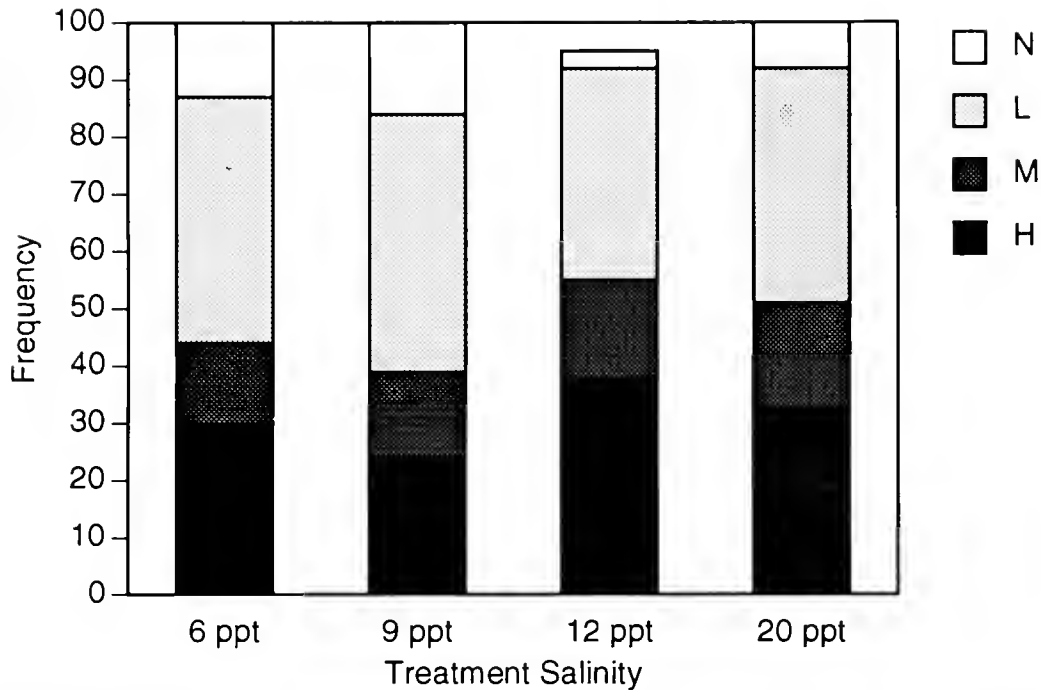


Figure 3. Infection intensity of *P. marinus* in oysters exposed to 6, 9, 12, and 20 ppt. Frequencies are based on the total number of live oysters sampled during the experimental period (day 14, 28, 42, and 56 samples are pooled). Infection levels are designated as negative (N), light (L), moderate (M), and heavy (H).

significantly effect hemocyte activities. Fisher and Newell (1986) and Fisher et al. (1987) have shown that hemocyte activities such as locomotion rate, ability to spread, and adherence capacity are reduced by elevations in salinity in *C. virginica* and *Ostrea edulis* under both acute and acclimated conditions. While the role of hemocytes in the oyster's defense of *P. marinus* has not been clarified, it seems apparent that salinity influences their activity.

Whether a physiological response of host, parasite, or a combination of both, the results of this investigation indicate that salinity has a significant effect on *P. marinus* pathogenicity at tem-

peratures exceeding 20°C. While this discussion has focused on salinity effects the importance of temperature should not be neglected. Temperature is believed to be the most important environmental factor regulating the geographic distribution and seasonal activity of *P. marinus* in the Chesapeake Bay (Andrews and Hewatt 1957, Ray 1954). This experiment was conducted at temperatures within the range most favorable to multiplication of the parasite (Andrews and Hewatt 1957, Ray 1954). Dissimilar results may be observed at other temperatures. Kinne (1964) states that

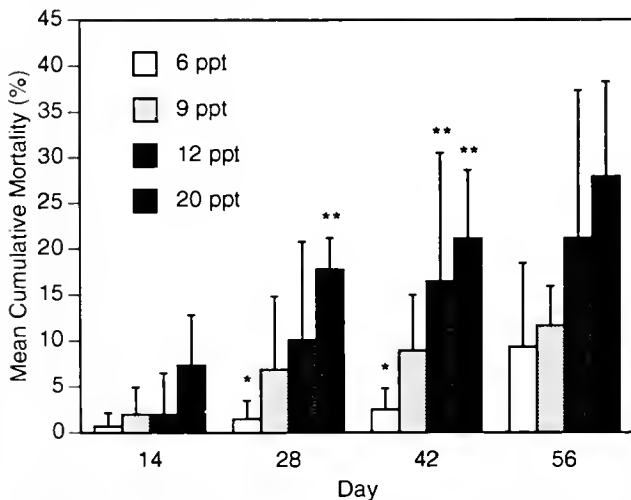


Figure 4. Mean percent cumulative mortality ( $\pm 1$  standard deviation) of oysters exposed to 6, 9, 12, and 20 ppt following 14, 28, 42, and 56 days of treatment (means are based on five replicate oyster groups). Treatment means denoted by a single asterisk significantly differ ( $P < 0.05$ ) from those denoted by a double asterisk.

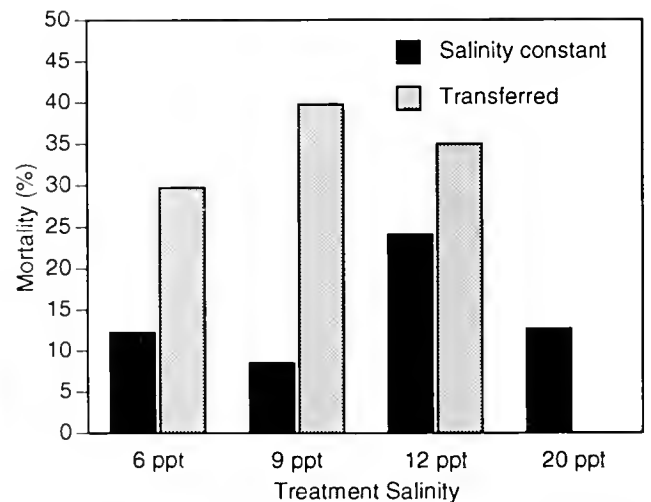


Figure 5. Percent mortality of oysters transferred to 20 ppt after 28 days exposure to 6, 9, and 12 ppt in comparison to those maintained at a constant salinity of 6, 9, 12, and 20 ppt. Percent mortality for each group is calculated for day 29–56. Values shown for groups in which the salinity was constant represent the means of five replicate groups. Transferred groups were not replicated.



TABLE 1.  
Parasite prevalence (%) based on histological diagnosis.

Day	Sample	Parasite Prevalence			
		<i>H. nelsoni</i>	<i>B. cuculus</i>	<i>N. ostrearum</i>	<i>P. marinus</i>
0	A	16	24	20	24
	B	16	8	24	20
	C	4	8	20	8
14	20 ppt	8	8	28	44
	12 ppt	0	4	8	24
	9 ppt	0	8	20	16
	6 ppt	0	0	8	16
28	20 ppt	12	8	12	32
	12 ppt	8	0	16	48
	9 ppt	4	8	0	16
	6 ppt	0	0	8	8
42	20 ppt	0	0	16	48
	12 ppt	8	8	4	40
	9 ppt	0	0	8	56
	6 ppt	0	4	16	44
56	20 ppt	8	4	0	56
	12 ppt	0	8	4	44
	9 ppt	0	4	4	48
	6 ppt	0	0	4	48

Day 0 replicates are designated as A, B, and C. N = 25 for all data sets except day 56 12 ppt where n = 20.

temperature can enlarge, narrow, or shift the salinity range of an individual. While some organisms tolerate subnormal salinities better at the lower part of their temperature range others exhibit a reciprocal salinity/temperature tolerance in which the range of salinity tolerated is widest at optimal temperatures and vice versa (Kinne 1964). Additionally, Feng and Stauber (1968) suggested that host defense activities as well as parasite multiplication, metabolite production, and nutritional requirements can be altered by

thermal elevations and depressions. A delicate balance between host and parasite exists and alterations in temperature can affect the outcome of host-parasite interactions (Feng and Stauber 1968). Environmental factors that stress the parasite and enhance host defense activities could shift the host-parasite balance to favor the host. In order to further elucidate the influence of environmental conditions on interactions between *C. virginica* and *P. marinus* investigations focusing on the synergistic effects of temperature and salinity are required. The combined effect of temperature and salinity may prove to be more important than either factor alone.

It is evident from this investigation that once *P. marinus* is established in natural oyster populations, it will not be eradicated by low salinity (6–12 ppt) over a relatively short time frame (<56 days). Nor will a temporary translocation of infected oysters to low salinity followed by a return to high salinity abate the pathogen. Oyster growers may be able to avoid disease mortality, however, by maintaining oysters in areas having salinities that are consistently below 9 ppt. Oysters maintained at 12 ppt are likely to experience mortality comparable to oysters maintained at 20 ppt. High mortalities in oysters that were transferred to high salinity following a period of low salinity exposure, suggest that increases in salinity that occur in areas normally having low salinities will allow infections to rapidly develop and cause mortality. In order to reduce or avoid oyster mortality it is essential that disease levels and salinities of oyster grounds be closely monitored, and be taken into consideration when making decisions regarding harvesting and management strategies.

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## DYNAMICS IN LOUISIANA'S OYSTER INDUSTRY AS PORTRAYED THROUGH STATE AUCTIONS, 1987-92

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**ABSTRACT** The American oyster (*Crassostrea virginica*) forms the basis for one of Louisiana's most valuable shellfisheries. Most of the state's production is derived from private leases equalling more than 300 thousand acres. Since 1987, the state has annually auctioned those leases in arrears for nonpayment of annual rental fees. This paper provides an analysis and related discussion of the auction data for the 1987-92 period.

**KEY WORDS:** auctions, Louisiana, leasing, oyster

### INTRODUCTION

Louisiana's oyster fishery (*Crassostrea virginica*) is primarily a leased-based industry (Keithly et al. 1992). More than 300 thousand acres are currently under lease for oyster production, and poundage taken from these leases averaged about 9.2 million pounds annually during the 1980s (National Marine Fisheries Service, unpublished data).

In addition to the private grounds, Louisiana also maintains considerable acreage devoted to public seed grounds and oyster seed ground reservations. These public grounds include the most productive natural reef area east of the Mississippi River (see Fig. 1), and encompass some 896 thousand acres in total (Perret et al. 1991). Vermillion Bay, west of the Mississippi River, is also a major public seed ground. The state periodically seeds these public grounds and the fishermen, in the fall season, will transport and bed the seed on their individual leases.

Leases used for the purpose of producing oysters are rented from the state on an annual basis for a duration of 15 years. The annual rental rate was one dollar per acre prior to 1980, and in subsequent years, two dollars per acre. Procedures for a lease cancellation in the event of default of rental payments are described in La.R.S. 56:429: "The failure of the tenant to pay the rent punctually on or before the first of each January, or within thirty days thereafter, ipso facto and without demand or putting in default, terminates and cancels the lease and forfeits to the department all the works, improvements, betterments, and oysters on the leased water bottoms. The department may at once enter on the water bottoms and take possession thereof. Such water bottoms shall then be open for lease to the highest bidder."

While provisions of La.R.S. 56:429 have been law for more than 30 years, the non-payment situation was minimal prior to the early to mid 1980s and, hence, no auctions were held prior to this period. By the mid 1980s, however, the delinquent payment sit-

uation had escalated to the point of mandating an auction as provided by statute. Some of the reasons speculated for the increased delinquent payment situation were: (1) the annual rental fee doubled from one dollar to two dollars per acre in 1980, (2) there was a shift away from the once traditional oyster producing areas, making leases in these areas no longer productive (see Van Sickle et al. 1976), (3) the decline in the oil-and-gas related activities in Louisiana's coastal area during the early to mid 1980s resulted in reducing oyster damage compensation payments, and (4) the sharp decline in Louisiana's coastal economy during the early-to-mid 1980s, tied to the decline in oil-and-gas related activities, resulted in reduced income which could be devoted to rental payments for nonproductive oyster acreage. Interestingly, the lease default situation escalated during a period of above average production from private leases in Louisiana and record real value of production. Annual production from private leases during the 1980s, for instance, was 675 thousand pounds above that reported in the 1970s, or about 8%, and because of a substantial increase in the real dockside price of Louisiana produced oysters, the real value of production during the 1980s was almost 40% above that reported during the 1970s (Keithly et al. 1992).

The first public auction in reference to La.R.S. 56:429 was held in April 1987, and an auction was held in March or April each year thereafter. Since the first auction and through the most recent auction held in March 1992, a total of 764 leases were offered. This paper provides an analysis of the auction data from 1987-1992.

### RESULTS

#### *Physical Characteristics*

#### **Number of Leases Offered and Taken**

As noted, 764 leases were offered at auction between 1987 and 1992, or about 10% of the total number of leases under ownership

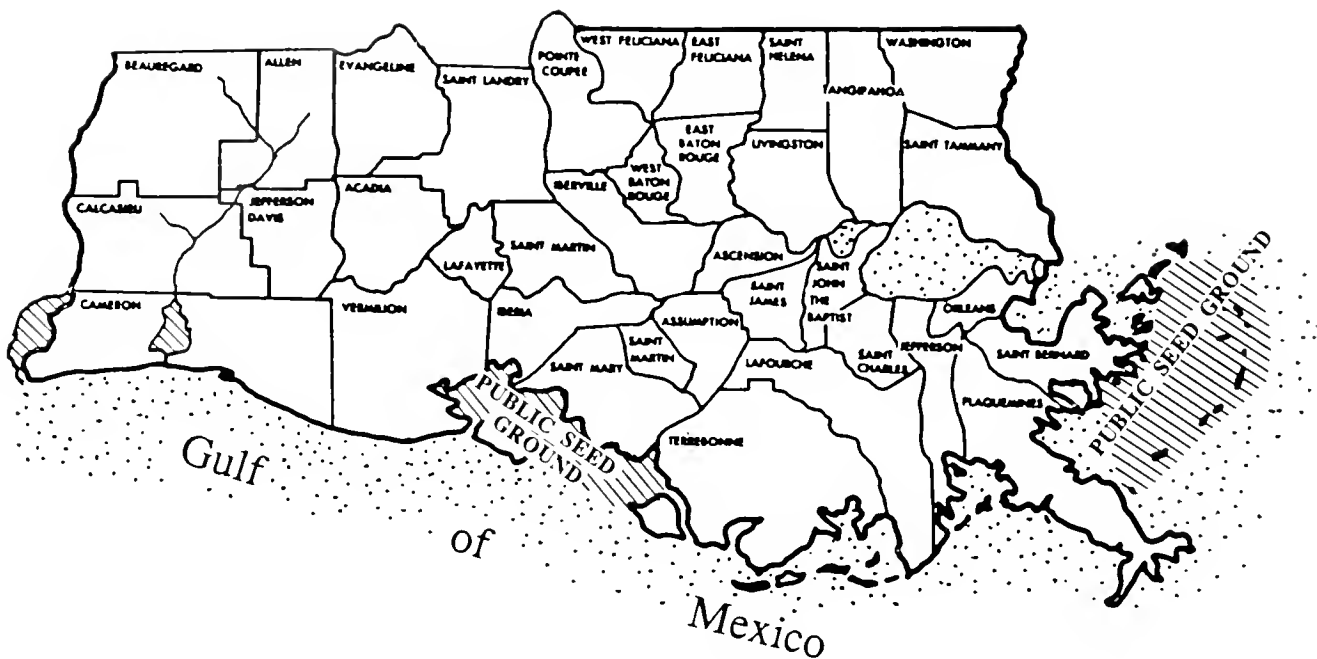


Figure 1. Louisiana Public Seed Grounds.

as of mid-1992.<sup>1</sup> As indicated in Figure 2, with the exception of the first auction in 1987, the number of leases offered for auction increased during each year in which the auction was held. The number of leases included in this first auction, however, is misleading to the extent that it included cancellations dating back to 1975. Overall, 136 of the 164 leases put up for auction in 1987 were canceled during the 1980s, with 21 of the cancellations occurring in 1986 and another 57 in 1987.<sup>2</sup> In all but the 1987 auction, the number of leases auctioned reflected the number canceled in that year.

A particularly sharp increase in leases offered for auction was observed in the latest two years of analysis with the 242 leases offered in 1992 representing three times the number reported as recently as 1990. The extremely sharp rise in 1992 may be related to the \$19.2 million 1991 dockside value of Louisiana's oyster harvest which was the lowest since 1983 when evaluated in current dollars and the lowest since 1981 when adjusted for inflation. This decline in value came despite a sharp increase in leased acreage during the 1980s (see Keithly et al. 1992).

Leases opened for auction had minimum bid requirements of two dollars per acre per year in default plus interest. Thus, for example, a ten acre lease defaulted upon in 1985 and opened for auction in 1987 required a minimum bid of sixty dollars plus accrued interest.<sup>3</sup> Annual auctions were held since 1988. Leases

auctioned since 1988 required minimum bids of \$2.20 per acre (\$2.00 rental plus \$0.20 interest).

Almost 70% of the 764 leases offered for auction during the study period, or 524 in total, were taken. The remaining 240 leases did not receive bids. On a yearly basis, the percentage of leases taken ranged from less than 45% in 1989, when 33 of the 78 leases offered were taken, to more than 80% in 1988, when 53 of the 66 offered leases were taken (Fig. 2). With the exception of 1989, the percentage of leases taken at auction has declined in each year since 1988. In 1988, for instance, 80.3% of the leases offered at auction were taken. The percentage fell to 76.5% in 1990, 73.7% in 1991, and 66.5% in 1992. The abnormally low proportion of leases taken at the 1989 auction (42%) reflects, as discussed below, the abnormally large size of leases canceled that year.

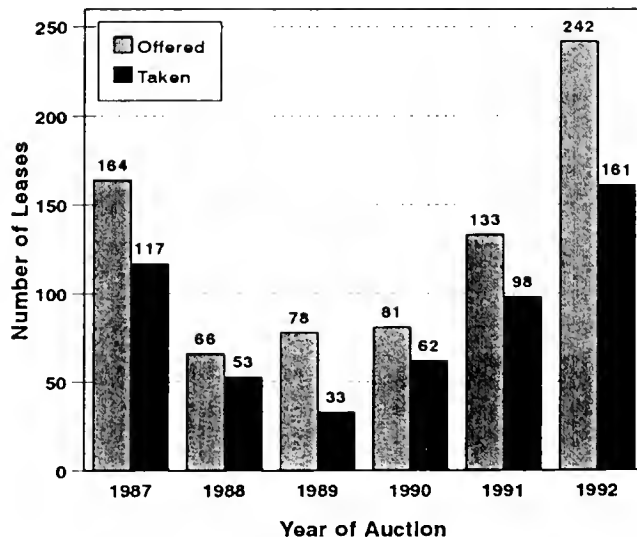


Figure 2. Number of Leases Offered and Taken at Annual LDWF Auctions, 1987-92.

<sup>1</sup>The number of leases and total acreage devoted to oyster production in Louisiana changes daily as new leases are issued or combined with existing leases. The number of leases and acreage lease size discussed in this paper are approximations as of June 1992.

<sup>2</sup>Prior to 1986, no more than ten leases were canceled in any one year, except for 1980. In 1980, the year in which rental fees increased from \$1.00 to \$2.00 per year, there were 30 cancellations.

<sup>3</sup>An exception to the minimum bid requirement involved those leases defaulted upon prior to 1980. Among this small group of leases, minimum bid requirement was one dollar per acre per year through 1979 and two dollars per year thereafter, plus accrued interest.

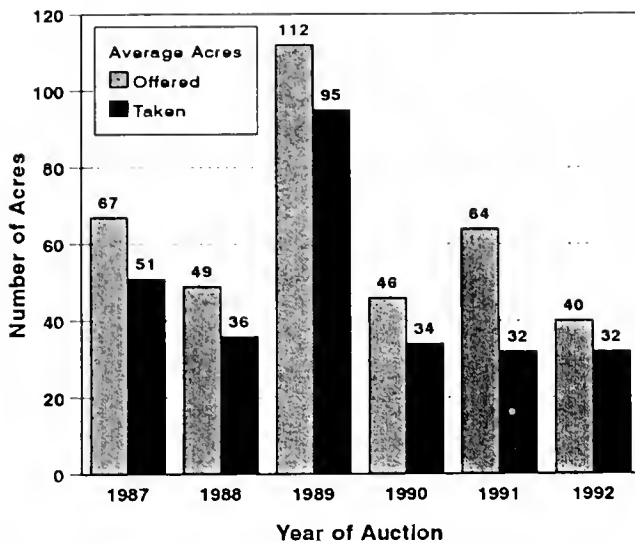


Figure 3. Average Number of Acres Among Leases Offered and Taken at Annual LDWF Auctions, 1987-92.

#### Size of Leases Offered and Taken

Average size of leases offered at auction during the study period was 59 acres compared to about 33 acres (as of mid 1992) for the industry in total. As indicated in Figure 3, the average size ranged from a low of 40 acres in 1992 to a high of 112 acres in 1989. Total acres offered at auction during the study period equalled almost 45 thousand, or about 13% of total acreage leased.<sup>4</sup> On average, 7,470 acres were offered each year at auction, with a range from 3,454 to 10,972 (Fig. 4).

The average size of leases taken at auction was consistently smaller than among those offered (Fig. 3). In 1987, for instance, size of leases taken averaged 51 acres compared to 67 acres among leases offered. The average size of leases taken compared to leases offered ranged from 50% (1991) to 85% (1989) and averaged 73% during the six year period. The proportion of acreage taken at auction ranged from 36.0% in 1989, when 3,148 of the 8,755 acres offered at auction were taken, to a high of 55.8% in 1990, when 2,087 of the 3,737 acres offered were taken (Fig. 4).

The lease size, as documented in Table 1, is an important determinant as to whether a particular lease is taken at auction. During 1987-92, for example, 564 leases, or 73.8% of the total number of leases offered at auction, were  $\leq 50$  acres. Among these 564 leases, 427 or 75.7% received at least the minimum bid. Of the 94 leases ranging from 51 to 100 acres in size, 57.5% were taken. Only 41.9% of the leases 101-500 acres in size were taken while less than a third of the leases  $> 500$  acres offered at auction were taken.

The negative relationship between the size of lease and its probability of being taken at auction is, in all likelihood, a function of (1) the amount of unproductive acreage associated with larger leases and (2) the risk of large monetary losses that would be incurred if a large lease proves to be unproductive. With respect to the first point, larger leases may have relatively few productive areas. Hence, potential investors may be unwilling to pay the required delinquency fees for relatively few productive acres.

With respect to the second point, there is a certain amount of risk, or uncertainty, related to bidding on acreage of which little or nothing is known. As such, there is likely to be reluctance in bidding on the larger leases, vis-a-vis smaller leases. The transfer of leases between willing parties also exhibited the small lease phenomena. The average size of leases transferred during 1980-89 was 46.5 acres (Keithly et al. 1992).

#### Area

Of the 45 thousand acres offered for auction during 1987-92, approximately 23% (10,467) was located in Plaquemines Parish (see Fig. 1). By comparison, Plaquemines Parish accounts for about 40% of the state's total leased acreage (128 thousand acres) and 4.8 million pounds of the state's 9.2 million pound annual production from private leases during the 1980s. Of the 10,467 acres offered at auction which were in Plaquemines Parish, 7,232 (69%) were subsequently taken. This proportion is considerably higher than that reported for the state in total (48%).

St. Bernard Parish based leases represent less than a quarter of Louisiana's total leased acreage. However, this parish accounted for 24.9 thousand of the 44.8 thousand acres (56%) offered for auction during 1987-92. Furthermore, only 8.7 thousand of these 24.9 thousand acres, or 35.1%, were taken at auction, i.e., received at least minimum bid. This is well below the state average. It is evident from these figures that St. Bernard leases face a challenging production environment.

Terrebonne Parish, with 15% of the state's leased acreage leases (51.8 thousand acres), had only three percent of the acreage offered at auction during 1987-92. The remaining acreage opened at auction included almost three thousand acres in both Iberia and Vermillion Parishes, 2.3 thousand acres in Lafourche Parish, and a lesser number of acres in Jefferson, St. Tammany, St. Mary, and Orleans parishes.

The relatively large number of leases canceled in St. Bernard parish is consistent with observed activities in Louisiana's oyster industry. For example, while there has been a large decline in the productivity per leased acre throughout Louisiana's oyster industry during the past three decades, this decline has been especially apparent in St. Bernard Parish. Estimated production per leased

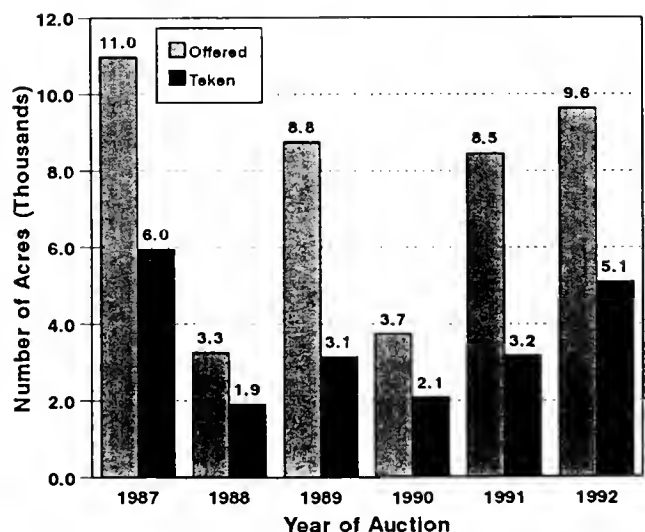


Figure 4. Total Number of Acres Offered and Taken at Annual LDWF Auctions, 1987-92.

<sup>4</sup>Some of the acreage may have been auctioned more than once. Due to the change in the number assigned to each lease, it is impossible to determine the extent of this.

TABLE 1.

Selected information pertaining to Louisiana Department of Wildlife and Fisheries water bottom lease auctions by size of lease, 1987-92 average.

Size of Lease (acres)	Leases Offered	Leases Taken	Percent of Leases Taken (\$/acre)	Sales Price of Leases Offered (\$/acre)	Sales Price of Leases Taken
≤50	564	427	75.7	10.63	15.23
51-100	94	54	57.5	6.01	10.22
101-500	93	39	41.9	1.56	4.08
>500	13	4	30.8	1.03	3.01

Source: Compiled from unpublished data maintained by Louisiana Department of Wildlife and Fisheries, Oyster Division.

acre at the state level, for instance, fell from 108 pounds annually during 1960-64 to 30 pounds during 1985-89, a decline of almost 75%. In St. Bernard Parish, however, the decline was almost 90%, from 70 pounds per acre to only eight pounds per acre. In Plaquemines Parish the decline was only about 60% (96 pounds per acre to 37 pounds per acre) which approximated that observed in Terrebonne Parish (97 pounds to 40 pounds). St. Bernard's close proximity to the state's largest public seed grounds was evidently of no advantage (see Fig. 1). As noted by Perret et al. (1991), the effectiveness of the public seed grounds in the St. Bernard area has declined by some 60 to 65 percent through time as a result of salt water intrusion, accelerated by the Mississippi River Gulf Outlet (MRGO). This channel, a man-made deep-water structure built in the 1960s, is approximately 75 miles in length and connects the open Gulf of Mexico waters to the Port of New Orleans. It traverses St. Bernard Parish and as noted by Dugas (1979), resulted in pronounced salinity changes in the MRGO surrounding areas upon its completion. These salinity changes destroyed many productive oyster beds in the area and, according to Dugas, led to an inland shift of the oyster growing area. Among other things, this inland shift resulted in the growing areas being closer to domestic sources of pollution which has resulted in periodic and permanent closures by health officials.

#### Auction Prices

The average sales price of leases taken at auction varied substantially on an annual basis, as indicated in Figure 5. To some

extent, this variation is commensurate with differences in average lease sizes from year to year. In 1989, for instance, when leases taken averaged a record 95 acres, the average bid per lease taken was a record \$577. Since 1989, however, leases taken have consistently averaged from 32 to 34 acres each, yet the sales price has ranged from \$157 to \$378. When evaluated on a per acre basis, the sales price of leases taken at auction varied from a low of \$4.66 per acre to a high of \$11.92 per acre (Fig. 6). Overall, the average sales price of leases taken at auction declined steadily from 1987 through 1990 but increased considerably in the subsequent two years.

When leases not taken at auction are included in the analysis, the average sales price per acre declined considerably due to the large proportion of leases not receiving at least the minimum bid (Fig. 7). The average price per acre, however, has increased steadily since 1989 with the 1992 sales price of \$6.32 per acre being more than 75% above that reported in 1991 and almost three times that reported in 1989.

As indicated in Table 1, there exists a strong negative relationship between the size of a lease and its per acre sales price. This relationship was also apparent among leases transferred (see Keithly et al. 1992). Leases taken at auction which were ≤50 acres, for example, received \$15.23 per acre compared to \$10.22 among leases of 51-100 acres, \$4.08 among leases 101-500 acres, and \$3.01 among leases in excess of 500 acres. In other words, leases of ≤50 acres taken at auction went for about five times as much as those >500 acres when evaluated on a per acre basis.

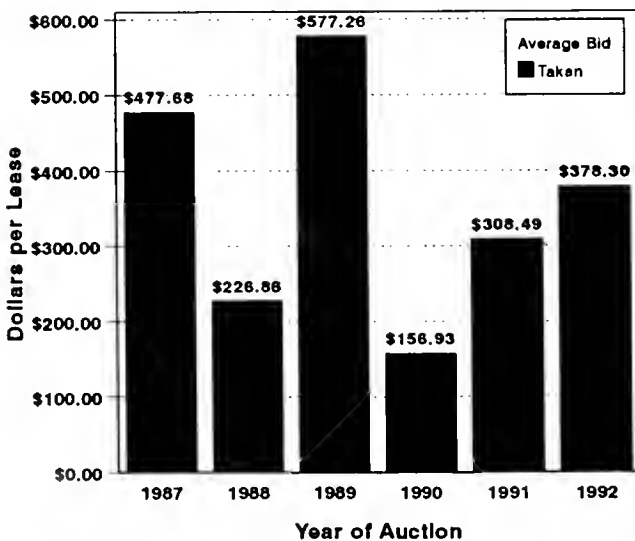


Figure 5. Average Bid Per Lease Taken at LDWF Auctions, 1987-92.

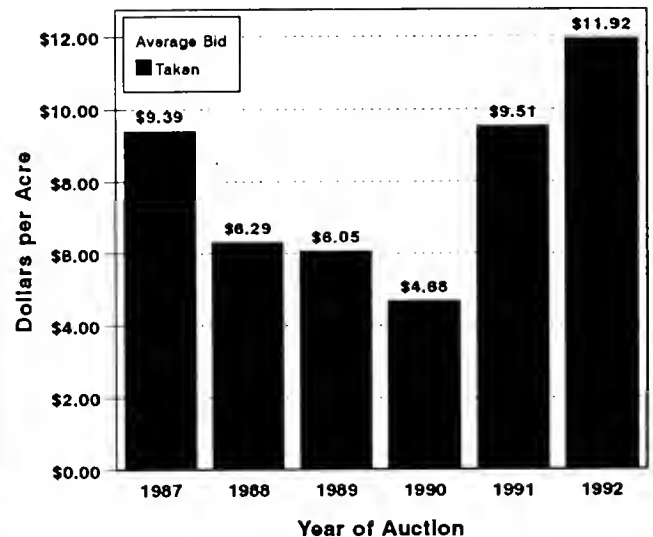


Figure 6. Average Bid Per Acre Among Leases Taken at Annual LDWF Auctions, 1987-92.

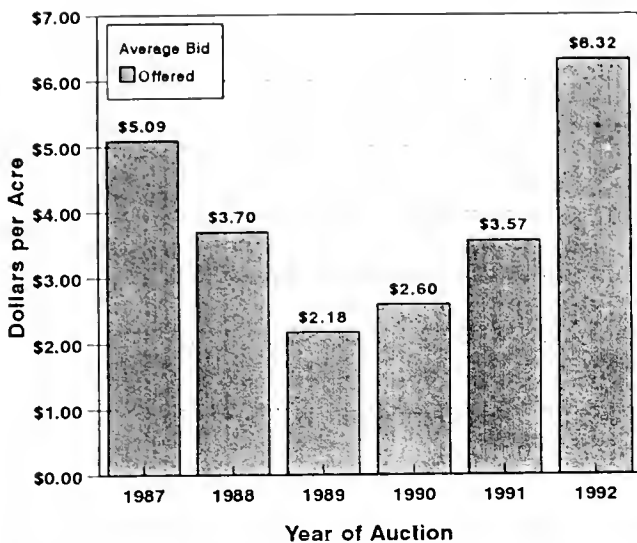


Figure 7. Average Bid Price Per Acre Among Leases Offered at Annual LDWF Auctions, 1987-92.

When leases not receiving the minimum bid are included in the analysis, the average sales price of leases  $\leq 50$  acres was more than ten times that of leases  $> 500$  acres. This increase reflects the greater proportion of larger leases not receiving minimum bid requirements.

Among leases taken at auction, those in Terrebonne Parish received the highest per acre bid (\$23.89). This was followed by Lafourche Parish (\$14.85), Plaquemines Parish (\$11.93), and Jefferson Parish (\$10.85). Lowest per acre bids among leases taken were received in St. Bernard Parish (\$4.43), Iberia Parish (\$3.34), and St. Mary Parish (\$2.99). The relatively low price observed for auctioned leases in St. Bernard Parish is consistent with deterioration of leases in that area.

## DISCUSSION

Several salient features were highlighted by the analysis of Louisiana's oyster lease auction data. One of these features is that a considerable proportion of Louisiana's leased acreage, about 10%–15% of the total, was valued at less than two dollars per acre among those individuals and companies who voluntarily relinquished their claim to the property by failure to pay annual rental fees. Of the almost 45 thousand acres that reverted back to the state for nonpayment of rental fees, however, about 45% was subsequently taken at auction.

The cancellation and subsequent purchase of oyster producing grounds at auction raises the issue of why this occurs. There are at least three plausible answers to this question. First, speculation likely plays a major role in any decision to relinquish property rights and/or to rent additional property. As noted by Perret and

Chatry (1988) "[f]ishermen not only lease areas which are currently productive, but they also hold leases in areas which may become productive as salinity conditions change." As such, what may be considered an exceedingly high risk by one individual (company), i.e., he relinquishes his rights to a given lease, may be considered an acceptable risk by another individual (company). The lease holder, however, evidently can not identify prospective buyers of the lease, since the cost of searching for prospective buyers is exceedingly high relative to potential benefits. A transfer via sale not being possible, the lease holder cancels the lease. The lease is then subsequently offered at auction.

A second explanation to the issue of cancellation and subsequent purchase of a given lease at auction relates to location. There are multiple lease holders in Louisiana. They are only subject to a one-thousand acre maximum. In some instances individuals (companies) may possess marginally productive leases far away from their major producing leases. It may not be profitable for these individuals (companies) to manage and harvest these leases due to the long distance which would need to be travelled. Other individuals (companies) who control leases in close proximity to the lease may, however, find such activities profitable. As such, it may be relinquished by the original owner only to be purchased at auction by an individual who maintains other leases in its general vicinity. A direct transfer may not occur because of lack of knowledge about availability or failure to agree on terms.

A final explanation for the cancellation and subsequent reissuing of oyster leases at auction relates to issue of absentee ownership in Louisiana's oyster industry. It is generally recognized that some of Louisiana's oyster lease holders are not active participants in the oyster industry and, as such, do not have current information on the productivity of all the leases under their ownership. Thus, they may relinquish certain leases they believe to be no longer productive. Active participants, recognizing the productivity in the area, may then purchase these leases at auction.

A second feature highlighted by the analysis reflects the observed increase in the number of leases being canceled through time and the related decline in the proportion of these leases subsequently being taken at auction. Both of these situations suggest continued deterioration in Louisiana's oyster lease-based businesses.

Another feature gleaned from the analysis reflects the low demand for the larger leases, vis-a-vis smaller leases, when evaluated on a per acre basis. As noted, this lower demand likely reflects increased monetary risk associated with the purchase of larger leases.

Finally, the analysis indicated that leases canceled, at least to some extent, were related to areas of declining productivity. This was found to be particularly the case in St. Bernard Parish.

## ACKNOWLEDGMENTS

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## ESTIMATION OF OYSTER SHELL SURFACE AREA USING REGRESSION EQUATIONS DERIVED FROM ALUMINUM FOIL MOLDS<sup>1</sup>

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**ABSTRACT** A method is described for estimation of surface area of shells of the American oyster, *Crassostrea virginica* (Gmelin 1791), as an alternative to direct measurement of surface area with aluminum foil molds. It is based on computation, from a small sample of shells, of the equation for regression of area of aluminum foil molds of shells on area enclosed within tracings of the shell outline. Area of other shells is then predicted from their shell outline area using the equation. Accuracy of the regression method in spatfall studies was established using data from shellstring collectors suspended in the Piankatank River, Virginia. For the most part, differences between foil mold area of individual shellstring shells and the area predicted from regression equations were small, and spat densities on individual shells, as computed from foil mold area and from regression-predicted area, were almost identical.

**KEY WORDS:** *Crassostrea virginica*, larval settlement, spatfall, oyster shells, surface area, aluminum foil

### INTRODUCTION

Quantitative field studies of settlement of oyster larvae (spatfall) on shell cultch of the same species is hampered by difficulty in measurement of shell surface area (Butler 1954). For that reason settlement data have been presented most frequently as number of spat per shell or per oyster (e.g., Singarajah 1980, Haven and Fritz 1985, Morales-Alamo and Mann 1990, Adams et al. 1991); those data, however, cannot be compared with each other or with other data because they lack shell dimensions.

Some investigators have estimated shell surface area from the dimensions of the longer and shorter axes of the shell (Lunz 1954, Carreon 1973), from the weight of paper cutouts of shells (McNulty 1953) or from shell height (Galtsoff 1964, Marcus et al. 1989). Those methods, however, failed to account for shell shape and texture. Other investigators avoided the problem by using alternate materials with flat surfaces and square corners (e.g., Kennedy 1980, Osman et al. 1989, Kenny et al. 1990).

Healy (1991) made direct surface area measurements of oysters using aluminum foil molds that accounted for shell shape and surface texture. Foil had been previously used to measure surface area of corals (Marsh 1970), stones (Shelly 1979), and the bivalve mollusc *Donax serra* (Donn 1990). Whereas Donn (1990) and Healy (1991) prepared foil molds of each animal in their studies, a technique is presented here for estimation of the surface area of shells of *Crassostrea virginica* (Gmelin 1791) that reduces time and tediousness because it does not require a foil mold of every shell examined. Shell surface area is predicted from the equation for regression of actual (foil mold) area on the area enclosed by a tracing of the shell perimeter outline; Marcus et al. (1989) measured the area within the shell perimeter outline to validate their area estimates but apparently did not consider shell shape and texture.

### MATERIALS AND METHODS

#### Source of Oyster Shells

Area measurements using aluminum foil were made on random samples of *C. virginica* shells from a natural oyster reef in the

James River, Virginia (referred to as reef shells), and from refuse piles at local oyster-packing houses (house shells). Regression equations were computed for three samples: a 1983 sample of 48 mixed reef and house shells, a 1983 sample of 68 reef shells, and a 1990 sample of 80 house shells. Attached organisms were scraped off reef shell surfaces before foil molds were made.

The 1990 sample of house shells came from stock used to construct shellstrings deployed in the Piankatank River, Virginia, as part of a spatfall monitoring program (Barber 1990), and the equation derived from those shells was used to predict surface area of shellstring shells. Shellstring collectors were described by Haven and Fritz (1985).

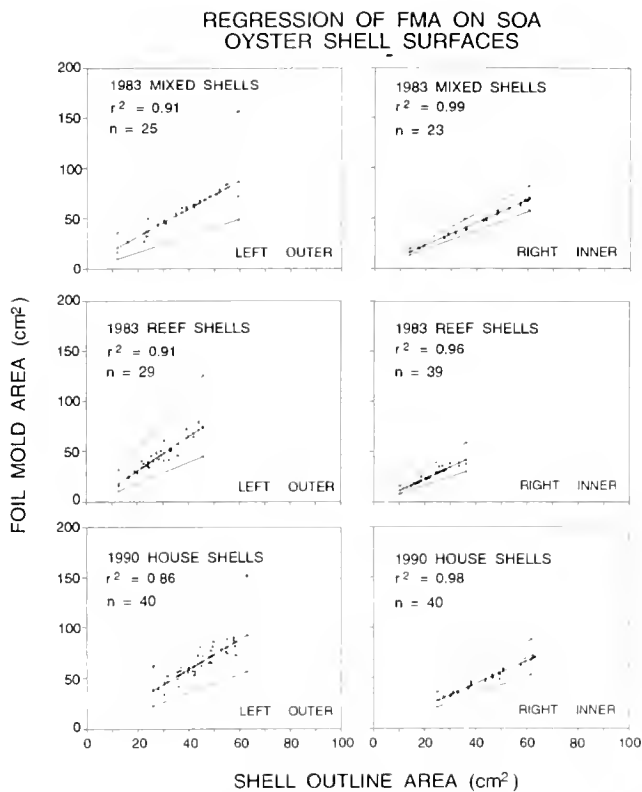
#### Foil Mold Preparation and Area Measurements

Molds were made by pressing aluminum foil over the shell surface and molding it over mounds and ridges and into depressions and crevices. The mold of the inner surface included the ligament furrow in the left valve and the buttress and umbonal cavity in the right valve. The foil was smoothed out continuously during the molding process to avoid pleating. Excess foil extending over the shell edge was trimmed and the mold removed from the shell without distorting mold shape. Slits were cut into the mold from the margin inward and carefully flattened out, concave surface down. The outline of the flattened mold was traced on paper and area of the tracing measured with an electronic digitizing planimeter; this area will be referred to as the foil mold area (FMA). Shell outline area (SOA) was also measured with the planimeter from a tracing on paper of the perimeter outline of each shell.

#### Accuracy of FMA Measurements

The accuracy of FMA measurements was evaluated by comparison with another measure of true surface area based on division of the shell surface into 1-cm segments across the long axis of the shell and addition of the segment areas. Length of the lines between segments was measured with a cotton string following shell contours and surface area computed using the equation for the Trapezoidal Rule (Britton et al. 1965). Lohse (1990) also measured the area of *Mytilus edulis* valves directly by adding segmental areas.

<sup>1</sup>Contribution No. 1788, Virginia Institute of Marine Science, School of Marine Science, The College of William and Mary.



**Figure 1.** Line and 95% prediction interval for the regression of FMA (foil mold area) on SOA (shell outline area) in three different samples of oyster shells. Lack of symmetry of prediction intervals is due to conversion of computed values from logarithms to original form. Mixed shells were a mixture of reef and house shells. Left: outer = outer surface of left valve; Right: inner = inner surface of right valve.

#### Reproducibility of FMA Measurements

Reproducibility of FMA measurements was tested by replicating the process 10 times for the outer surface of each of two shells and computing the coefficient of variation (CV). The outer surface was selected for this test because it is more uneven and complex than that of the inner surface, thus providing a more rigorous test. One of the shells was a very convex left valve with outer surface deformations originating from another oyster previously attached to it; the other shell was a relatively flat right valve.

#### Regression Equations

Equations for regression of FMA on SOA were computed by the least-squares method after logarithmic transformation of the data to correct for heterogeneity of variance. Shell surface area was then predicted from those equations for a multiple number of SOA measurements. Use of the same regression equation to make multiple predictions precludes application of the usual prediction interval (Tiede and Pagano 1979, Snedecor and Cochran 1980). In its place, a prediction interval given by Snedecor and Cochran (1980) was computed.

#### Accuracy of Predicted Surface Areas

Accuracy of surface area predictions was tested by comparing FMA of shellstring shells with the area predicted from the regres-

sion of FMA on SOA (the regression-predicted area, or RPA). Spat densities on the shellstring shells as derived from FMA and as obtained from RPA were also compared.

## RESULTS

#### Accuracy of the Aluminum Foil Mold Measurement

There was a high correlation between FMA and the area obtained from the sum of the segmental areas of the shell; the coefficients of determination ( $r^2$ ) for the outer and inner surfaces were 0.99 and 0.98 in a mixed sample of 20 reef and 20 house shells which ranged from 10.27 to 70.64 cm<sup>2</sup> in SOA. The absolute percent difference between the two types of measurements for individual shells ranged between 0.1 and 9.6 (mean = 3.0; standard deviation (SD) = 2.4) for the outer surface and between 0.1 and 13.6 (mean = 4.8; SD = 3.8) for the inner surface.

Mean surface areas obtained by the two methods were almost identical: for the outer surface, 45.2 cm<sup>2</sup> (SD = 20.8) by the foil mold method and 45.5 cm<sup>2</sup> (SD = 21.2) by the sum of segmental areas; for the inner surface they were 36.9 cm<sup>2</sup> (SD = 16.9) and 36.6 cm<sup>2</sup> (SD = 16.8), respectively. The coefficient of variation for ten FMA replications of the outer surface of each of two individual house shells was very low (1.2 and 1.4), indicating that this technique is highly reproducible.

#### Regression of FMA on SOA

There was a strong correlation between FMA and SOA in each of three shell samples analyzed (Fig. 1, Table 1). All coefficients

**TABLE 1.**

Equations for the regression of foil mold area (Y) on shell outline area (X) in shells of *Crassostrea virginica* from three sources.

Source of Shells Valve and Surface	Regression Equation ( $\log \hat{Y} = a + \log X$ )	$r^2$
1983 Mixed Reef & House Shells:		
Left Valve (n = 25)		
Outer Surface:	$\log \hat{Y} = 0.249 + 0.954 \log X$	0.91
Inner Surface:	$\log \hat{Y} = 0.171 + 0.949 \log X$	0.96
Right Valve (n = 23)		
Outer Surface:	$\log \hat{Y} = 0.072 + 1.039 \log X$	0.97
Inner Surface:	$\log \hat{Y} = 0.115 + 0.964 \log X$	0.99
1983 Reef Shells:		
Left Valve (n = 29)		
Outer Surface:	$\log \hat{Y} = 0.057 + 1.094 \log X$	0.91
Inner Surface:	$\log \hat{Y} = 0.038 + 1.047 \log X$	0.96
Right Valve (n = 39)		
Outer Surface:	$\log \hat{Y} = 0.051 + 1.047 \log X$	0.91
Inner Surface:	$\log \hat{Y} = 0.006 + 1.038 \log X$	0.96
1990 House Shells:		
Left Valve (n = 40)		
Outer Surface:	$\log \hat{Y} = 0.153 + 1.004 \log X$	0.86
Inner Surface:	$\log \hat{Y} = 0.085 + 0.994 \log X$	0.95
Right Valve (n = 40)		
Outer Surface:	$\log \hat{Y} = -0.012 + 1.086 \log X$	0.95
Inner Surface:	$\log \hat{Y} = 0.093 + 0.967 \log X$	0.98

Reef shells collected from Wreck Shoal in the James River Virginia; house shells, origin unknown, were obtained from shucking-house refuse piles in Virginia. Logarithms to the base 10.  $\hat{Y}$  = fitted Y, i.e., estimated Y (RPA in text).

TABLE 2.

Cumulative percent frequency distribution of the difference (in percentages, sign ignored) between foil mold area (FMA) and regression-predicted area (RPA) for individual oyster shells from shellstrings suspended in the Piankatank River in 1990 (predictions based on 1990 house shells).

Pct. Difference	Left Valves				Right Valves			
	Outer Surf.		Inner Surf.		Outer Surf.		Inner Surf.	
n	Pct.	n	Pct.	n	Pct.	n	Pct.	
<5.00	15	53.6	18	64.3	4	25.0	12	75.0
5.01-10.00	6	75.0	9	96.4	5	56.3	3	93.8
10.01-15.00	5	92.9	1	100.0	5	87.5	0	93.8
15.01-20.00	1	96.4			2	100.0	1	100.0
20.01-25.00	1	100.0						
n	28		28		16		16	
Mean	6.5		4.2		9.1		4.1	
SD	5.3		2.8		5.1		4.7	

SD = standard deviation.

of determination were higher than 0.86. Prediction intervals for the regression lines were very wide because a value of 500 was used for the number of future predictions in the equation from Snedecor and Cochran (1980). Figure 1 only includes the data for inner surface of right valves and outer surface of left valves because they represent extremes of shell flatness and concaveness (or convexity), respectively; regression data for the other two valve-surface combinations were intermediate in prediction interval width.

#### Comparison of Area and Spat Density Estimates

Differences between FMA and RPA were either all or mostly all under 15% for both surfaces of left and right valves in individual shells from Piankatank River shellstrings (Table 2). The same was true for shellstring shells used in the James River in 1983 (R. Morales-Alamo and D. S. Haven, unpublished data). Means for FMA and RPA were very close in each of the four groups of Piankatank River shellstring shells (Table 3).

Spat densities computed for individual shellstring shells using the two area estimates were identical or nearly identical in most shells (Table 3). Mean spat densities for each shellstring were identical in 6 of the 8 surface comparisons and very similar in the other two. The large variation around these means is associated with variations in larval settlement between shells in the same shellstring and not with variation in area estimates.

#### DISCUSSION

Surface area measurements of oyster shells using aluminum foil molds provide the closest approximation to true surface area of any technique proposed to date because they are the only ones that account for variations in shell shape and texture among individual shells. Their accuracy was demonstrated here by comparison with the sum of shell segmental areas.

Direct foil measurement of every shell examined (as done by Donn 1990 and Healy 1991) is the most desirable option. However, in studies that involve large numbers of shells, as in exten-

sive spatfall monitoring programs, that method would require an inordinate amount of time and effort. The same would be true in studies involving natural reef shells because direct measurement with foil molds would require preliminary removal of fouling organisms. The regression method presented here is a suitable alternative that would substantially reduce time and effort investment because few actual foil mold measurements are required. A maximum of 40 each of the right and left valves would be satisfactory to derive a regression equation; tracing shell outline and measurement of the area enclosed for all other shells is done relatively quickly.

Use of direct foil mold measurements or predictions made from regression of FMA on SOA solves some of the problems associated with substrate suitability in larval settlement studies with oysters: (1) they provide dimensional measures of spat density, unlike data presented in terms of spat per shell, (2) they make it unnecessary to use, just for dimensional purposes, alternate materials that may be potentially less attractive than oyster shells to oyster larvae (Kennedy 1980), and (3) they permit comparison of settlement on oyster shells with settlement on alternate materials when such comparisons are required. They also offer the option of making counts on several measured small areas of the shell surface, instead of on the whole shell, when the number of spat is extremely large. The mean of those counts would be comparable to those made on whole shells.

Regression of FMA on SOA may be characterized by wide predictive intervals, depending on the valve and surface being analyzed, which would ordinarily handicap use of the regression for prediction purposes. In actual practice, however, percent differences between individual FMA and RPA were for the most part small and when tested in spatfall studies, their effect was inconsequential: spat density values for Piankatank River shellstring shells were almost identical regardless of whether the area measured with foil mold or the area predicted from regression was used. In that context, therefore, it is acceptable to ignore the wide predictive regression intervals.

A drawback of methods based on foil molds is that a foil mold of the outer surface of an oyster shell cannot account for surface areas inside very small depressions, crevices and pits on the shell surface. They may, thus, underestimate the total area available to settling oyster larvae in heavily-pitted shells. That, however, is not a serious problem when house shells are used because their outer surfaces are relatively unblemished. Old shells from natural reefs are usually heavily pitted and the problem created by that condition must be acknowledged when surface area estimates are made using foil molds.

Investigators using shellstrings in spatfall studies have often stated that they used shells of similar size (Lutz et al. 1970, Kennedy 1980, Singarajah 1980). Although those data may present an adequate picture of relative spatfall at different stations and in different years, absence of dimensional units considerably reduces confidence in comparisons with other data. Adoption of the technique presented here, as an alternative to direct foil mold measurements of all shells, would be advisable in spatfall studies that use whole oyster shells as collection substrate. Refinement of the method for improved accuracy is possible by using very flat right valves and examination of only the inner surface. Differences in size and shape of shells from different geographic locations and environments require computation of separate regression equations in subsamples from each of those populations to ensure the highest accuracy of predictions based on the equations.

TABLE 3.

Surface area of oyster shells and density of spat in shellstrings suspended in the Piankatank River, VA.

V	Outline Area (cm <sup>2</sup> )	Shell Surface Area (cm <sup>2</sup> )				No. Spat and Density (No./cm <sup>2</sup> )					
		Outside Surface		Inside Surface		Outside Surface			Inside Surface		
		FMA	RPA	FMA	RPA	No. Spat	Dens. (FMA)	Dens. (RPA)	No. Spat	Dens. (FMA)	Dens. (RPA)
Exposure Period: 16-23 Aug 1990											
Palace Bar (n = 12)											
L	36.33	54.62	52.42	43.03	43.24	8	0.15	0.15	8	0.19	0.19
L	41.99	67.88	60.62	46.10	49.94	14	0.21	0.23	7	0.15	0.14
R	45.57	64.74	61.56	49.02	49.77	2	0.03	0.03	9	0.18	0.18
R	30.67	40.73	40.05	32.91	33.94	7	0.17	0.17	10	0.30	0.29
L	31.22	50.95	45.02	37.51	37.19	12	0.24	0.27	10	0.27	0.27
R	48.86	61.67	66.41	52.59	53.24	16	0.26	0.24	13	0.25	0.24
R	60.15	79.67	83.22	64.77	65.09	3	0.04	0.04	6	0.09	0.09
R	47.50	57.94	64.40	51.31	51.80	9	0.16	0.14	6	0.12	0.12
R	40.27	58.78	53.83	42.88	44.16	5	0.09	0.09	11	0.26	0.25
L	55.41	82.18	80.09	66.63	65.78	13	0.16	0.16	3	0.05	0.05
L	61.06	85.66	88.29	71.26	72.45	7	0.08	0.08	4	0.06	0.06
L	52.77	78.93	76.26	65.41	62.67	10	0.13	0.13	9	0.14	0.14
Mean	45.98	65.31	64.35	51.95	52.44	8.8	0.14	0.14	8.0	0.17	0.17
SD	10.30	13.92	15.26	12.47	12.01	4.4	0.07	0.08	2.9	0.09	0.08
Burton Point (n = 12)											
Mean	39.17	59.22	56.20	47.04	46.38	5.0	0.09	0.09	3.0	0.07	0.07
SD	7.90	14.31	11.93	10.46	9.63	4.2	0.07	0.07	2.3	0.05	0.05
Exposure Period: 23-30 Aug 1990											
Palace Bar (n = 10)											
L	49.97	75.38	72.19	57.90	59.36	22	0.29	0.30	32	0.55	0.54
L	69.60	100.38	100.69	89.92	82.52	30	0.30	0.30	31	0.34	0.38
L	60.56	100.97	87.56	74.98	71.86	7	0.07	0.08	52	0.69	0.72
R	45.01	55.49	60.74	53.76	49.18	41	0.74	0.67	29	0.54	0.59
R	39.13	58.86	52.18	44.22	42.95	9	0.15	0.17	25	0.57	0.58
L	48.93	74.50	70.69	62.62	58.14	10	0.13	0.14	30	0.48	0.52
L	35.92	55.62	51.83	43.58	42.76	5	0.09	0.10	25	0.57	0.58
L	53.26	70.33	76.97	59.36	63.25	23	0.33	0.30	55	0.93	0.87
R	39.83	66.45	53.19	53.01	43.69	13	0.20	0.24	22	0.42	0.50
L	37.42	62.98	54.00	46.51	44.53	1	0.02	0.02	9	0.19	0.20
Mean	47.96	72.10	68.00	58.59	55.82	16.1	0.23	0.23	31.0	0.53	0.55
SD	10.90	16.63	16.79	14.53	13.74	12.6	0.21	0.19	13.6	0.20	0.19
Ginney Point (n = 10)											
Mean	47.89	66.09	66.73	56.01	54.03	3.5	0.06	0.06	18.8	0.34	0.35
SD	10.90	15.66	14.43	10.00	10.91	3.3	0.06	0.06	7.6	0.12	0.13

Key to abbreviations: n = Number, V = Valve, L = Left, R = Right, SD = Standard Deviation.

Individual data for Palace Bar strings and means only for two other stations. Areas given as measured from aluminum foil molds (FMA) and as obtained from the regression equation of foil mold area on shell outline area for a sample of the house shells used to construct the shellstrings (RPA). Spat density computed using both surface area values.

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## DEVELOPMENT OF DISEASE CAUSED BY THE PARASITE, *PERKINSUS MARINUS* AND DEFENSE-RELATED HEMOLYMPH FACTORS IN THREE POPULATIONS OF OYSTERS FROM THE CHESAPEAKE BAY, USA

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**ABSTRACT** The development of infection caused by the protozoan parasite, *Perkinsus marinus* (Dermo) and some specific potential defense-related cellular and humoral components in oysters collected from three geographic areas, Deepwater Shoal of James River (DW), Wachapreague (WP), and Mobjack Bay (MJ) were examined over time. Oysters were maintained in estuarine water (salinity = 20 ppt) or in water at a salinity similar to the ambient salinity of the collection sites. Oysters were sampled at the initiation of the experiment (day 0), day 35, and day 100 to determine defense-related parameters and disease prevalence and intensity. All populations experienced a significant increase in *P. marinus* infection prevalence and intensity from the initiation of the experiment to the termination of the study. Oyster mortality differed between oyster populations. None of the DW oysters perished while cumulative mortalities for WP at 32 ppt and 20 ppt and MJ oysters were respectively, 23, 25, and 35%. The experimental oyster populations demonstrated significant differences with respect to cellular and humoral defense-related variables. As the study progressed, the mean number of total hemocytes declined in the WP and MJ populations and increased in the DW population. The percentage of granulocytes in DW oysters was consistently higher than other populations. DW oysters also had the highest concentrations of protein and lysozyme. This pattern persisted throughout the experimental period. Oyster condition index significantly decreased during the course of the study in all populations except the DW oysters at 10 ppt. Results suggest that the increase of hemocyte number and higher percentage of granulocytes, and lysozyme concentration in DW oysters may have contributed to the high (100%) survival rate of this population. Salinity may have affected disease development. Disease prevalence and intensity tended to be lower in the WP oysters maintained at low salinity than those maintained at high salinity. In the DW population, unexpectedly, oysters maintained at 20 ppt had lower infection prevalence and intensity than oysters maintained at 10 ppt. Salinity induced, to some extent, changes in certain hemolymph components: lysozyme concentration tended to be higher in oysters maintained at low salinity than those maintained at high salinity. Increase in percentage of granulocytes was also observed in WP oysters after transferring to a salinity lower than ambient salinity.

**KEY WORDS:** oyster disease, hemolymph factors, *Perkinsus marinus*

### INTRODUCTION

Disease-induced mortality in eastern oysters (*Crassostrea virginica*) caused by two parasites, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) is one of the factors contributing to the decline in oyster harvest in the Chesapeake Bay, U.S.A. Previously, disease pressure from *H. nelsoni* has been more intense on oysters than that from *P. marinus*. Because of its current expanded distribution and increase in abundance in waters of the Chesapeake Bay, *P. marinus* is now considered more significant than *H. nelsoni* as an oyster pathogen (Andrew 1988, Burreson 1989). It has been well documented that prevalence and intensity of *P. marinus* infections in oysters are related to milieu salinity (e.g., Soniat 1985, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al. 1990, Paynter and Burreson 1991). Significant growth reduction due to *P. marinus* infection in oysters raised in habitats of different salinity in the Chesapeake Bay has been reported by Paynter and Burreson (1991).

Hemocyte activities and lysozyme concentrations of eastern oysters have been reported to change seasonally (Fisher et al. 1989, Feng and Canzonier 1970, Chu and La Peyre 1989) and to be affected by salinity (Fisher 1988, Chu and La Peyre 1989, Chu et al. In review). Increased salinity suppressed hemocyte spreading and locomotion. Hemolymph lysozyme concentration in oysters was negatively correlated with salinity in oysters (Chu et al. In review).

The purpose of this study was to compare the development of disease caused by *P. marinus* in oysters collected from three dif-

ferent salinity habitats of the lower Chesapeake Bay and to determine if any changes occurred in some measurable cellular and humoral components in these oysters during the course of disease development.

### METHOD AND MATERIALS

#### Experiment

To encompass the natural salinity range of oysters in the lower Chesapeake Bay, oysters were collected from 3 locations: a low salinity site, Deep Water Shoal of James River (DW, ambient temperature = 22.5°C, salinity = 10 ppt), a high salinity site, Burtons Bay, Wachapreague (WP, ambient temperature = 19.5°C, salinity = 32 ppt), and a moderate salinity site, Mobjack Bay (MJ, ambient temperature = 20.0°C, salinity = 20 ppt), in early October 1990. Oysters were cleaned of fouling organisms and a hemolymph sample was withdrawn from 30 oysters from each population to measure initial total hemocyte count (TC), percent of granulocytes (PG) and protein and lysozyme concentrations. Oysters were then sacrificed to determine initial condition index (CI = dry meat weight/dry shell weight × 100, Lucas and Beninger 1985) and to examine for *P. marinus* infection (Ray 1952, 1966).

Sixty oysters from each population were maintained in 250 l static fiber-glass tanks at 22 ± 1°C and at conditions indicated below. Oysters from MJ (N = 60) were maintained in filtered (1µ filter) estuarine water (York River water, YRW, salinity = 20 ppt). Oysters from DW and WP were each divided into 2 groups

(60 oysters/group/tank); one group of the oysters was maintained in filtered YRW; the other group was maintained in water adjusted to ambient salinity (i.e. 10 ppt for DW oysters, 32 ppt for WP oysters). Oysters were fed daily with an algal diet (a mixture of *Pavlova lutheri*, *Isochrysis galbana* and Tahitian *Isochrysis galbana*). Water was changed every other day. The experiment was terminated in the middle of January, 1991 (100 days after experiment initiation). Thirty five days after the initiation of the experiment and at the end of the experiment, subsamples of oysters (N = 20 oysters, 35 days after initiation, N = 30 oysters at the end of the experiment) from each group were sampled for TC, PG, protein, lysozyme and CI measurement and *P. marinus* diagnosis.

#### Total and Differential Counts and Preparation of Sera

Hemolymph from individual oysters was withdrawn with a syringe from the adductor muscle sinus through notches in the shell and placed in micro test tubes in an ice bath. Total and differential (number of granulocytes and agranulocytes) hemocyte counts were obtained on each hemolymph sample using a hemocytometer. Differential counts were expressed as percentage of granulocytes (PG =  $100 \times$  number of granulocytes/total hemocytes). To determine protein and lysozyme concentrations in oyster serum (cell-free hemolymph), serum of each hemolymph sample was separated from hemocytes through centrifugation ( $400 \times g$  at  $4^\circ C$  for 10 min). Serum was withdrawn and stored in a freezer ( $-20^\circ C$ ) for subsequent protein and lysozyme measurement.

#### Protein and Lysozyme Measurements

Lysozyme concentration was determined spectrophotometrically according to the method of Shugar (1952) and Chu and La Peyre (1989). Cell-free oyster serum (0.1 ml) was added to 1.4 ml of the bacterial (*Micrococcus lysodeikticus*) suspension and the decrease in the absorbance was recorded at 450 nm on a Shimadzu UV 600 spectrophotometer for 2 minutes. All measurements were duplicated and were taken at room temperature ( $21 \pm 1^\circ C$ ). Recorded lysozyme activities were converted to lysozyme concentration using egg white lysozyme as a standard. Standard curves at different salinities were constructed by dissolving egg white lysozyme in a balanced salt solution of appropriate salinities (i.e. 10, 20, and 32 ppt), assuming that reactivity of oyster lysozyme and egg white lysozyme were similar if assayed in buffer of the same salinity.

Serum protein was measured by the method of Lowry et al. (1951) using bovine albumin as a standard. Ten  $\mu l$  of a cell-free hemolymph sample from individual oysters was used for the serum protein measurement.

#### Perkinsus Assay

The thioglycollate assay described by Ray (1952, 1966) was used for *P. marinus* diagnosis. Rectal tissue was removed from each oyster and incubated in thioglycollate medium for 4–5 days. Intensity of infection was ranked from 0 (negative) to 5 (heavily infected) based on the relative number of stained *P. marinus* hyphospores contained in the tissue smear.

#### Statistical Analysis

One factor analysis of variance (ANOVA) and Student-Newman-Keuls test were used to compare total hemocyte counts (TC) and percentage of granulocytes (PG), protein (P) and lysozyme concentrations, condition index, and prevalence and in-

tensity of *P. marinus* infection between population groups and between different salinity treatments of the same population (i.e. DW and WP oysters). Data were  $\text{Log}_{10}$  or Arcsine transformed whenever data showed a large variance. Differences were considered statistically significant at  $P \leq 0.05$ . Linear correlation (Pearson correlation analysis) was calculated between the measured variables, condition index, serum protein and lysozyme concentrations, and *P. marinus* infection intensity.

## RESULTS

The infection prevalence and intensity of oysters sampled from DW, WP, and MJ populations on day 0, day 35, and day 100 are shown in Figure 1. At the beginning of the experiment, prevalences in DW, WP, and MJ oyster samples (N = 30/population)

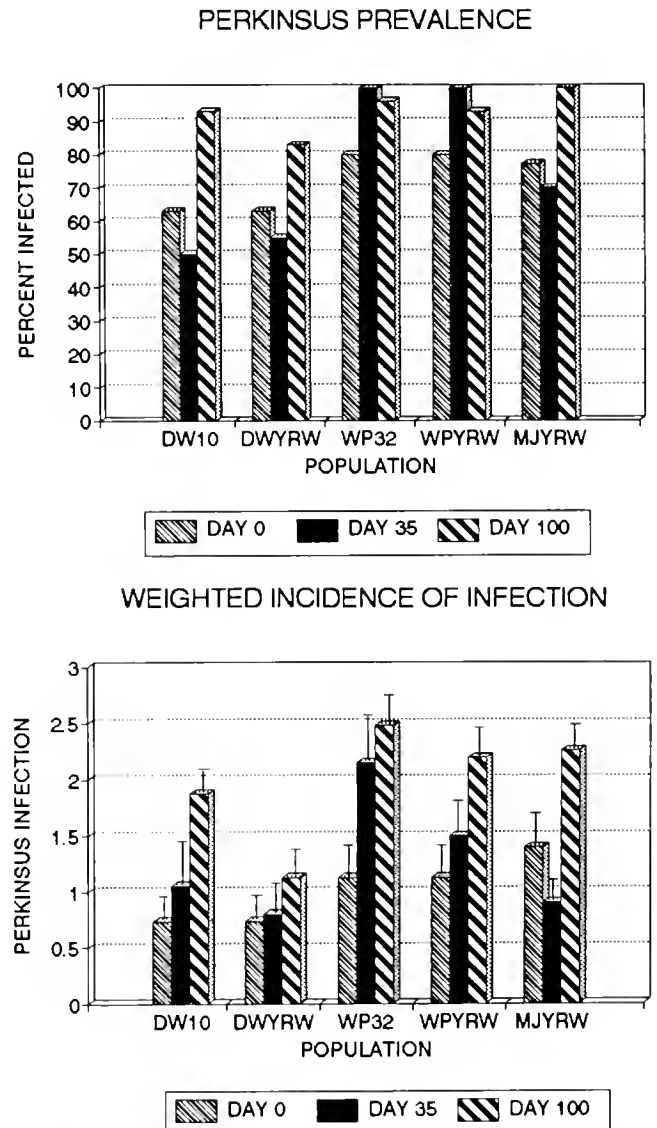


Figure 1. *Perkinsus marinus* prevalence and weighted incidence in DW (Deep Water Shoal, James River), WP (Burtons Bay, Wachapreague) and MJ (Mobjack Bay) oysters at day 0 (N = 30), day 35 (N = 20) and day 100 (N = 30). DW10 = DW oysters at 10 ppt water, DWYRW = DW oysters in York River Water, WPYRW = WP oysters in York River Water, WP32 = WP oysters at 32 ppt water, MJYRW = MJ oysters in York River Water.



were 63, 80, and 70%, respectively (Fig. 1). Infection intensities expressed as weighted incidence (WI = sum of disease code numbers/number of oysters) in WP and MJ oysters were significantly higher than in DW oysters (Fig. 1). *P. marinus* prevalence in oysters sampled on day 35 (N = 20/group) were 50% in DW at 10 ppt (DW10), 55% in DW at YRW (20 ppt, DW20), 70% in MJ, 100% in WP at 32 ppt (WP32) and at YRW (20 ppt, WP20). Weighted incidence increased in both WP and DW populations and decreased in the MJ oysters at day 35. At the termination of the experiment, prevalence in oyster samples (N = 30/group) were 93, 83, 96, 93 and 100% in DW10, DW20, WP32, WP20 and MJ populations respectively. All population groups experienced a significant increase in *P. marinus* infection prevalence and intensity from the initiation to the termination of the experiment, a period of 100 days. Generally, as in the beginning of the experiment, at the end of the experiment, DW oysters maintained relatively lower *P. marinus* weighted incidence than WP and MJ oysters. At all sampling dates, DW20 oysters had significantly lower ( $P < 0.05$ ) weighed incidence than all other groups of oysters. The DW oysters maintained in YRW (20 ppt) had lower prevalence and weighted incidence than those maintained at 10 ppt. Only four DW20 oysters developed moderate to advanced (level 3-5) infections. Disease prevalence did not appear to differ in WP20 and WP32 oysters, but disease intensity was lower in the former than the latter at both day 35 and day 100.

Oyster mortality differed among populations (Fig. 2). During the course of the study, none of the DW oysters perished. Cumulative mortalities in WP at 32 ppt, WP at 20 ppt, and MJ groups were 23, 25, and 35%, respectively.

At the initiation of the experiment, mean TC was significantly higher in WP and MJ oysters than in DW oysters (Fig. 3). However, mean PG was much higher ( $P < 0.05$ ) in the DW oysters than in the other oyster populations. As the study progressed, the mean TC declined in the WP and MJ groups and increased in the DW20 group. In the DW20, WP20 and MJ groups, the final mean TC differed significantly from the mean TC at day 0. No signif-

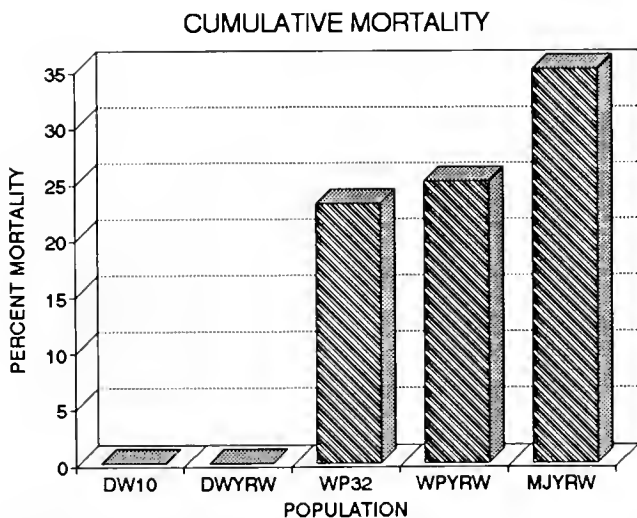


Figure 2. Cumulative mortality of DW (Deep Water Shoal, James River), WP (Burtons Bay, Wachapreague) and MJ (Mobjack Bay) oysters at the termination of the experiment. DW10 = DW oysters at 10 ppt water, DWYRW = DW oysters in York River Water, WPYRW = WP oysters in York River Water, WP32 = WP oysters at 32 ppt water, MJYRW = MJ oysters at 20 ppt in York River water.

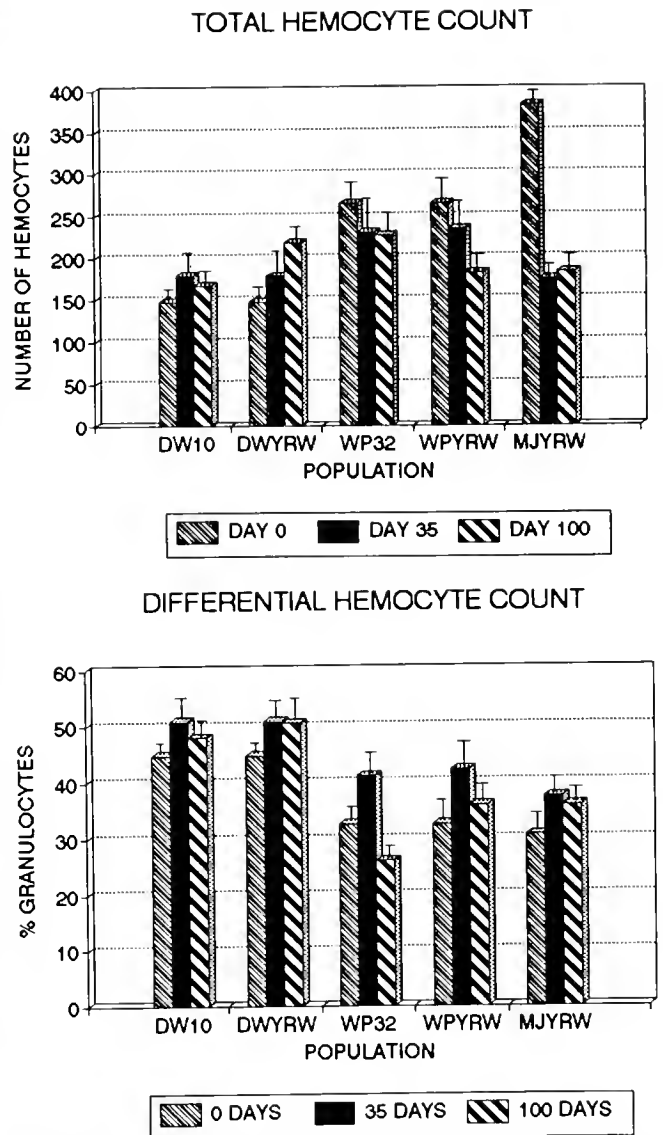
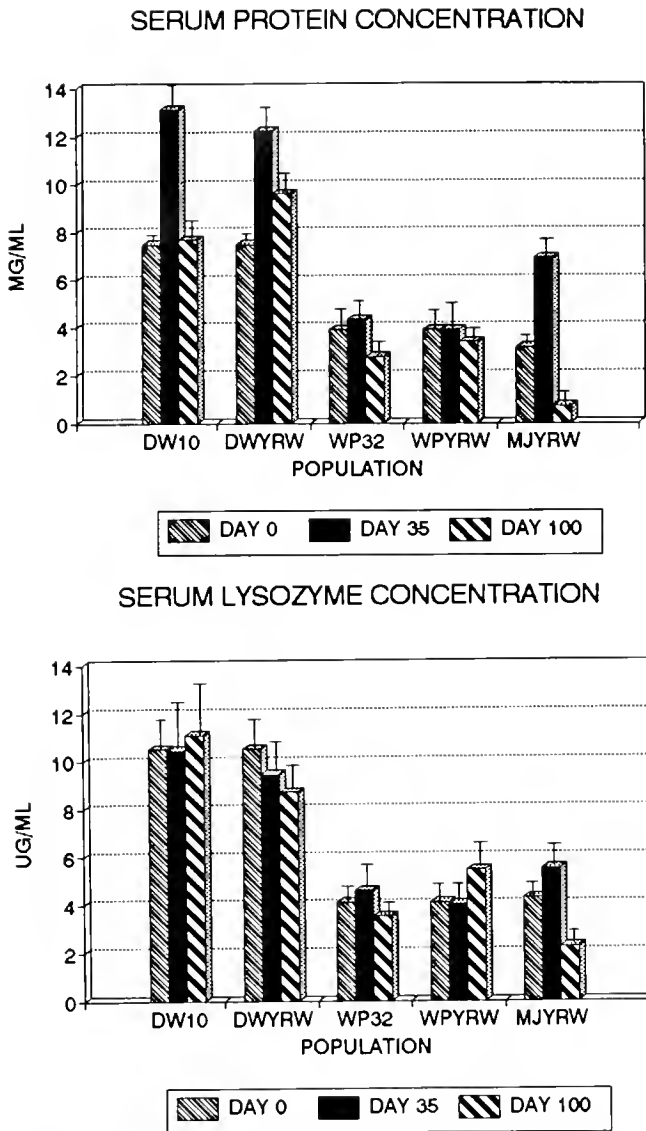


Figure 3. Mean total hemocyte counts and percentage of granulocytes ( $\pm$ SE) in DW (Deep Water Shoal, James River), WP (Burtons Bay, Wachapreague) and MJ (Mobjack Bay) oysters at day 0 (N = 30), day 35 (N = 20) and day 100 (N = 30). DW10 = DW oysters at 10 ppt water, DWYRW = DW oysters in York River Water, WPYRW = WP oysters in York River water, WP32 = WP oysters at 32 ppt water, MJYRW = MJ oysters at 20 ppt in York River water.

icant change in mean TC was observed over time in the DW10 and WP32 oysters. A trend of increasing TC with time was noted in the DW groups, although differences were not statistically significant. Generally, DW oysters had the highest PG over the course of the study. Generally, in both WP and DW oysters, no significant difference was observed in both TC and PG between salinity treatments.

Serum protein and lysozyme concentrations differed among the three oyster populations (Fig. 4). Concentrations of lysozyme and protein were significantly lower ( $P < 0.05$ ) in the WP and MJ than in DW populations on day 0 (Fig. 4). No significant difference in lysozyme or protein concentration was observed between MJ and WP oysters. This pattern persisted throughout the experimental period; DW oysters had the highest ( $P < 0.05$ ) concentration



**Figure 4.** Mean hemolymph protein and lysozyme concentrations ( $\pm$ SE) in DW (Deep Water Shoal, James River), WP (Burtons Bay, Wachapreague) and MJ (Mobjack Bay) oysters at day 0 (N = 30), day 35 (N = 20) and day 100 (N = 30). DW10 = DW oysters at 10 ppt water, DWYRW = DW oysters in York River Water, WPYRW = WP oysters in York River water, WP32 = WP oysters at 32 ppt water, MJYRW = MJ oysters at 20 ppt in York River water.

of protein and lysozyme on all sample dates. The concentrations of these two serum components fluctuated between sample dates. Within the DW populations, oysters sampled at day 35 had a significantly higher protein concentration than those sampled at day 0 and day 100; but lysozyme concentration in DW oysters did not change significantly through time. Protein concentration in MJ oysters also peaked at day 35 and differed significantly from both initial and final sample concentrations. Both lysozyme and protein concentrations in MJ oysters declined from day 35 to day 100; MJ oysters sampled at day 100 had the lowest protein and lysozyme concentrations. Protein and lysozyme concentration in the WP population did not differ significantly over time. WP oysters did not differ from MJ oysters in protein and lysozyme concentration except at day 35. At day 35, MJ oysters had significantly higher

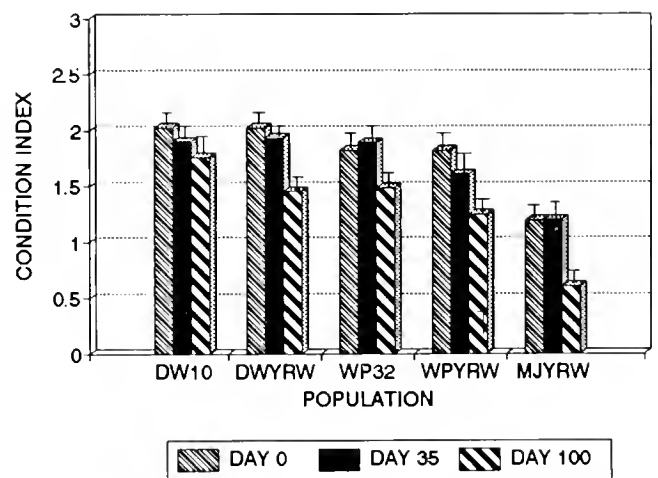
protein concentrations than WP oysters. Although insignificant statistically, lysozyme concentrations tended to increase in WP20 oysters and to decrease in DW20 oysters. The mean protein concentrations in DW20 oysters was higher ( $P < 0.05$ ) than DW10 oysters. The lysozyme concentrations in DW10, MJ, and WP32 oysters sampled at the termination of the experiment were negatively correlated with infection intensity.

Oyster condition, as indicated by condition index (Fig. 5), was significantly lower at the end than the beginning of the experiment in all population groups except the DW10 group. When Pearson correlation analysis was performed on data pooled from each group, it revealed that the condition index of DW10, WP32, WP20 and MJ oysters was negatively correlated with *P. marinus* infection intensity; condition index of DW20, WP32, WP20, and MJ oysters were positively correlated with serum protein concentrations.

## DISCUSSION

Oysters from the upper James River, in areas such as Horsehead and Deep Water Shoal, are quite vulnerable to both *P. marinus* and *H. nelsoni* (Andrews 1984, Ford and Haskin 1987, Andrews 1988, Barber et al. 1991, Burreson 1992) but have remained relatively disease free because of prevailing low salinity (Andrews 1988, Burreson 1989, 1990, 1991). Mobjack Bay of the lower York river is an endemic area for both *P. marinus* and MSX. Progeny from survivors of the 1960 MSX epizootics in Mobjack Bay were shown to be less susceptible to MSX than seed oysters from the James River (Andrews 1971, Andrews 1984). Until 1990, oysters from Wachapreague had a low incidence of *P. marinus* and low mortality caused by *P. marinus* (Burreson 1990, 1991). The three oyster populations under investigation may be genetically different. However, they displayed a similar response to *P. marinus*. Almost all oysters (83 to 100%) from each popu-

## CONDITION INDEX



**Figure 5.** Mean condition index ( $\pm$ SE) in DW (Deep Water Shoal, James River), WP (Burtons Bay, Wachapreague) and MJ (Mobjack Bay) oysters at day 0 (N = 30), day 35 (N = 20) and day 100 (N = 30). DW10 = DW oysters at 10 ppt water, DWYRW = DW oysters in York River Water, WPYRW = WP oysters in York River water, WP 32 = WP oysters at 32 ppt water, MJYRW = MJ oysters in York River Water.

lation were infected by the parasite when the experiment was terminated (Fig. 1). Oysters from Mobjack Bay were found to be less susceptible to *H. nelsoni* than oysters from James River (Andrews 1984), but results of the present study indicates that they are equally susceptible to *P. marinus* as are oysters from the James River.

Results of the present study confirm that *P. marinus* can endure low salinity (Chu and Greene 1989, Ragone 1991, Burreson 1990, 1991). At the beginning of the experiment, oysters from Deep Water Shoal of James River began with lower disease prevalence (63% at day 0 and 50% at day 35) and intensity than both WP and MJ oysters. The increased disease prevalence in DW oysters at ambient salinity (10 ppt) at the end of the experiment is, apparently, a result of disease transmission between infected and uninfected oysters maintained in the same tank. Thus, it is clear that low salinity did not restrict disease transmission among oysters. Under continuous disease pressure, salinity of 10 ppt did not inhibit the progress of disease development. It has been shown that *in vitro*, only salinities lower than 6 ppt restrained *P. marinus* sporulation from prezoosporangia (Perkins 1966, Chu and Greene 1989).

Oysters from Deep Water Shoal of the James River (DW oysters) and WP oysters may have responded differently to the low salinity treatment. It is surprising to note that DW oysters maintained at ambient salinity (10 ppt) had higher *P. marinus* weighted incidence than those DW oysters at 20 ppt, whereas placing WP oysters at a salinity (20 ppt) lower than ambient salinity (32 ppt) reduced, relatively, the weighted incidence in these oysters. It is not known whether this difference is based on genetic dissimilarities between DW and WP oysters or whether it was an artifact. Further study is needed to verify this result. Restraint of disease progress has been noted in DW oysters infected by *P. marinus* maintained at low salinity (<12 ppt) (Ragone 1991, Chu unpublished results).

High disease prevalence and relatively higher disease intensity in WP and MJ oysters at the beginning of the experiment (Fig. 1) may account for the high cumulative mortality in these two populations of oysters. The WP oysters had the highest weighted incidence when the experiment was initiated and at day 35, 100% disease prevalence was recorded for this group. The lower disease prevalence and intensity in MJ oysters at day 35 than at day 0 may be a result of high mortality in this group. The deceased oysters of this group could have been heavily infected by the parasite. Unfortunately, no tissue was able to be recovered for *P. marinus* diagnosis. The lower disease prevalence in DW oysters at day 35 than at day 0 was unexpected. These results are unexplained.

Previous studies (Ray 1954, Andrews and Hewatt 1957, Scott et al. 1985, Ragone 1991) have demonstrated that low salinity delayed and/or reduced mortality induced by *P. marinus*. In the present study, differing mortalities were not observed between low and high salinity treatments in WP oysters and no deaths occurred in DW oysters maintained at a salinity of 20 ppt (which was much higher than the ambient salinity, 10 ppt) even though prevalence was 63%, initially.

There were obvious differences in cellular and humoral components among the three populations of oysters. The variation in these components which presumably reflect different genetic and habitat backgrounds of the oysters, may account for the different survival rates among these oysters. The DW oysters started not only with higher differential hemocyte counts, serum protein and lysozyme concentrations, but also with higher condition index. No

mortality occurred in DW oysters while 23 to 35% cumulative mortality was noted for WP and MJ oysters during the experiment (Fig. 2). The greater quantities of hemolymph components and higher condition index of DW oysters may indicate that DW oysters were healthier than WP and MJ oysters at the beginning of the experiment, thus surviving the disease.

Although total hemocyte counts (TC) in DW oysters were low on day 0, as the experiment progressed, TC significantly increased in DW oysters at 20 ppt and TC tended to increase in DW oysters at 10 ppt. In contrast, there was a significant decrease of TC in the MJ group, which had the highest cumulative mortality (35%, Fig. 2). It has been suggested that the increase of hemocyte number (hemocytosis) in oysters is a response to parasitism (Ling 1990). Nevertheless, greater hemocytosis was observed in MSX-resistant than in MSX-susceptible oysters when infected by *H. nelsoni* (Ling 1990). Percentage of granulocytes was inherently high in DW oysters (Fig. 3). The higher concentration of granulocytes in DW oysters is probably habitat-associated. The DW oysters were from a habitat with lower ambient salinity than WP and MJ oysters. Fewer granulocytes were found in oysters from high salinity than from low salinity locations (Fisher and Newell 1986). However, it is interesting to note that the DW oysters which were transferred to 20 ppt also maintained a similar level of PG as those at 10 ppt. The increase of TC and relatively high PG in DW oysters may have provided a physiological advantage (increased disease tolerance).

Exposing oysters infected with *P. marinus* to low salinity significantly increased their lysozyme concentrations and a positive correlation was found between lysozyme concentration and the survivorship of the oysters (La Peyre et al. 1990, Ragone 1991). Lysozyme concentration in DW oysters was persistently higher than in MJ and WP oysters and as infection become more intensified at the end of the experiment, the level of lysozyme in these oysters remained high. In contrast, the lysozyme concentration in WP oysters stayed low and a significant decrease ( $P = 0.003$ ) in lysozyme level was found in MJ oysters when the experiment was terminated.

It is known that salinity affects lysozyme activity in oysters (La Peyre and Chu unpublished results). Lysozyme concentration decreases with increased salinity (Chu et al. In review). The higher lysozyme concentrations in WP oysters at 20 ppt than the WP oysters at ambient salinity is a result of salinity effect. The same explanation can be applied to the decrease of lysozyme concentration in DW oysters maintained at a salinity higher than ambient salinity.

It has been well-documented that the parasites, *H. nelsoni* and *P. marinus*, induce significant changes in growth, reproduction, and certain physiological functions of the oyster (e.g. Feng and Canzonier 1970, Newell 1985, Ford 1986, Barber et al. 1988, Ford 1988, Ford and Figeras 1988, Chu and La Peyre 1989, Ling 1990, Chintala and Fisher 1991, Barber et al. 1991, Burreson 1991, Paynter and Burreson 1991). Generally, parasitism depressed growth and feeding rate, reduced tissue and hemolymph protein, and impaired gonadal development at the gametogenesis stage. Decline in condition index was found in all groups of oysters at the end of this experiment, although the decrease of condition index observed in the DW oysters at 10 ppt was statistically insignificant. The observation that hemolymph protein concentrations were higher on day 35 than on day 0 in DW and MJ oysters was unusual. A possible interpretation is that the observed higher protein concentrations in DW and MJ oysters at day 35 resulted

from the relatively lower disease prevalence in DW and MJ oysters at day 35 (50–55% for DW oysters and 70% for MJ oysters) than day 0 (63% for DW oysters and 77% for MJ oysters). Although the difference was statistically insignificant, the protein contents in uninfected oysters in these 2 groups were found to be slightly higher than in infected oysters at day 35. Moreover, disease weighted incidence (WI) was lower in MJ oysters (0.9) at day 35 than at day 0 and WI remained relatively low in DW oysters at day 35 (0.8–1.1), thus depletion of hemolymph protein was not observed. It has been noted that hemolymph protein reduction occurred only in oysters heavily infected by the parasite *H. nelsoni* (Ling 1990). However, decrease of protein took place in all oysters at day 100, particularly in the MJ groups which had the highest cumulative mortality.

In summary, based on the infection prevalence and intensity at the end of the experiment, the three populations of oysters under investigation showed a similar response to *P. marinus*. Since no mortality was observed for the DW oysters, the higher hemolymph cellular and humoral components and condition index of these oysters may be an indication of better physiological fitness, which

provides them with greater tolerance of infection and prolonged survival. Salinity induced changes in certain hemolymph factors (i.e. lysozyme and PG). Salinity may have affected disease development, but results between populations were inconsistent.

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## CONTROL OF OVERSET ON CULTURED OYSTERS USING BRINE SOLUTIONS

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**ABSTRACT** The Haskin Shellfish Research Laboratory (HSRL) has a long standing and continuing program in oyster genetics and breeding. These various research projects generate many progeny groups—future brood stocks—that must be reared to maturity. Ironically one of the worst scenarios arises when there is a large natural set of oyster spat (*Crassostrea virginica*, Gmelin 1791) on the brood stocks (of *C. virginica*). Fast growing natural set may be mistaken for slow growing brood stock oysters. Preliminary experiments in 1990 indicated that overset might be controlled simply and efficiently by immersing animals in a concentrated brine solution; such treatments in 1990 resulted in 89–100% mortality of <1 mm spat. In 1990 field tests, overset on brood stocks was reduced to 3 spat/oyster using 200 ppt immersions compared to 22 spat/oyster in controls. In 1991 we refined the parameters for effective brine dips. First, we tested survival of oysters (potential substrate for overset) immersed for 2, 5, or 10 minutes in 200 ppt brine followed by either 3 or 6 hours aerial exposure. For juveniles (<1 yr old), cumulative mortalities ranged from 3–6% compared to 5% in controls; for adults (2–3 yr old), 2–4% died after brine immersion and 2–3% died in controls. Second, we tested survival of hatchery set oyster spat immersed in 200 ppt brine. For spat with shell lengths <5.0 mm and immersed in brine for 2, 5, or 10 min, 57%, 70% and 83% died after 3 hr aerial exposure and 64%, 85%, and 86% died after 6 hr aerial exposure. Control mortality averaged about 23% in both 3 and 6 hr aerial exposures. For larger spat immersed in brine for 10 minutes, cumulative mortality was 47% and 88% for 3 and 6 hr aerial exposure, respectively, and 22% and 32% for controls. Results of 1990 field tests and 1991 experiments demonstrate that brine immersions will be effective and save considerable labor.

**KEY WORDS:** oyster, overset, fouling, aquaculture, brine, fouling control

### INTRODUCTION

The Haskin Shellfish Research Laboratory (HSRL) has a long standing and continuing program in oyster genetics and breeding. Our various research projects generate many genetically distinct groups used for future evaluation or to serve as brood stock. These groups need constant attention to keep them clear of fouling organisms, especially *Polydora* spp. Ironically one of the worst scenarios arises when there is a large natural oyster set (*Crassostrea virginica*, Gmelin 1791) on the future brood stocks (of *C. virginica*). Elsewhere in Delaware Bay this set is welcomed, but experimental groups, heavily set with spat, can quickly become overgrown by the overset. Besides impeding growth, fast growing natural set may be confused with yearling brood stock, potentially contaminating the specialized gene pool(s) of, for example, resistant strains. In the past overset spat were removed by scraping oysters individually, which is both tedious and inefficient.

Various brine (salt) dips have been used to control oyster "enemies" such as squirts (*Mogula* sp.), boring sponges (*Cliona* sp.), and starfish (*Asterias* sp.) (Shearer and MacKenzie, 1961). Perhaps not surprisingly, there is little precedence for the use of brine dips to control oyster set, although Shearer and MacKenzie (1961), while testing the species above, observed mortality in 10–20 mm oysters at high brine concentrations. Preliminary experiments conducted in 1990 at HSRL indicated that overset might be controlled by treatments in concentrated brine solutions followed by some period of aerial exposure.

Results of these preliminary experiments showed that adult oysters survived well (1–2% mortality) when treated for 10 minutes in a 200 ppt brine solution, followed by 3, 6, or 12 hour aerial exposure. These treatments also resulted in 89–100% mortality of spat measuring 1 mm or less in shell length. Consequently, a field test was conducted in 1990 on oysters heavily set by Delaware Bay native spatfall. Three wire mesh trays, each containing overset adult oysters (age 3–5 yrs), were dipped in 200 ppt brine for 10

minutes; three similar trays were dipped in ambient seawater (22 ppt). Dips were followed by a three hour aerial exposure. Fifty days after treatments, numbers of spat on oysters were counted in both dipped and control trays: oysters in dipped trays averaged 3 spat per oyster; in controls—22 spat/oyster.

Yet other variables associated with this treatment were unexplored. The objective of the research reported here was to refine the dip procedure so that we might incorporate it as a tool in the routine maintenance of our stocks. Variables explored were (1) tolerance of adults to brine treatments of various durations, and aerial exposures; (2) optimum treatments for removing recently set spat at several durations and aerial exposures, and size related mortality; and (3) a field test of spat covered oysters.

### MATERIALS AND METHODS

Two experiments and a field test were designed to better define the parameters of brine treatments. Treatments consisted of immersing oysters or spatulated cultch contained in wire mesh trays in 200 ppt brine solution for various lengths of time, followed by either a 3 or 6 hour aerial exposure. We chose 200 ppt brine for two principal reasons: First, preliminary work with brine dips indicated that 200 ppt brine seemed as effective as saturated brine (~300 ppt). Secondly, and more important to our experimental design, saturation of brine solutions is relative, depending on factors such as temperature and humidity. It is almost impossible to achieve uniformity in saturation from experiment to experiment. Aerial exposures were conducted in a shaded area to normalize for differences in the intensity of sunlight over the course of the experiments. Aerial exposures were conducted at ambient outside temperatures, ranging from 21–28°C.

#### Experiment 1

Since it is imperative that dip treatments not harm adult oysters, the first experiment was conducted to test the effect of im-

mersion for 2, 5, or 10 minutes in a 200 ppt brine solution on survivorship for yearlings and for older adults (age: 2–3 yrs). Dips were followed by an aerial exposure of either 3 or 6 hours in a shaded location. For each of the two year class groups (age 1 and age >1), 16 groups of 50 oysters were tested; two replicates for each immersion duration (2, 5, and 10 minutes) × aerial exposure period (3 or 6 hours) combination (12 groups). Controls consisted of two replicates each of a 10 minute dip in ambient sea water followed by a 3 or 6 hour aerial exposure (4 groups). A total of 1600 adult oysters were used. Each group was examined 2, 4, and 6 days post treatment to assess survival.

### Experiment 2

The second set of experiments was conducted to test the effect of concentrated brine solutions on survivorship of recently set spat. We wanted to determine the maximum size that spat can be efficiently removed with a brine dip. Eyed larvae cultured in the hatchery were set on prepared shells of the Atlantic sea scallop, *Placopecten magellanicus* (Gmelin, 1791). Spat were reared in the hatchery for 1–3 weeks. Prior to treatments, individual spat were measured to the nearest 0.1 mm and surrounded by a numbered, pencil drawn circle. This made it possible to determine post-exposure mortality for individual spat. Two size classes of spat were tested: <5.0 mm (mean 2.3 mm; range 0.9–5.0 mm) and >4.0 mm (mean 6.1 mm; range 4.0–11.6 mm). Brine concentration for all dips was 200 ppt.

For spat <5 mm, 18 groups of 50 spat were tested: two replicates for each immersion duration (2, 5, and 10 minutes) × exposure period (3 or 6 hours) combination (12 groups). Controls consisted of one immersion for each duration (2, 5, and 10 minutes) × aerial exposure period (3 or 6 hours) combination (6 groups). A total of 900 spat were used.

For spat >4 mm, 6 groups of 50 spat were tested: two replicates and one control were dipped for 10 minutes and exposed to air for either 3 or 6 hours. A total of 300 spat were used. In both size classes (<5 and >4 mm) survival of individual spat was assessed 2, 4, and 6 days after treatment.

### Field Test

For the final experiment we had planned a large scale field trial on trays of adult oysters that were fouled by native set. Ironically, spatfall in the summer of 1991 was particularly light at our Cape

Shore grow out site. (It was non-existent in 1992). For the sake of completeness, a brine dip trial was conducted in October, 1991. The spat included for the test had set over the period from mid-July to mid-September and were larger (mean 11.3 mm; range 4.7–22 mm) than those spat tested in Experiment 2.

For the field test, 500 adult oysters with spat were randomly divided into 10 groups of 50 oysters each. The number of spat/oyster was counted and 10 spat were measured to the nearest 0.1 mm on 10 oysters from each of the 10 groups. Five trays were then dipped for 10 minutes in 200 ppt brine, followed by a 6 hour aerial exposure in the shade; five control trays received the same treatment except were dipped in ambient salinity (22 ppt) sea water. Six days after the dip, the number of spat/oyster and shell length of spat were again estimated. Mortality of adult oysters was also recorded.

### Statistical Analyses

All data were analyzed with the computer program SYSTAT (Wilkinson, 1990). Survival data were arcsine transformed prior to statistical analysis (Sokal and Rohlf, 1981). All comparisons, except one, were tested by two-way ANOVA with replication. Independent factors were exposure (3 or 6 hours) and dip duration. The exception was the experiment on spat >4 mm. Here a one-way ANOVA was run on controls (3 and 6 hour exposures pooled) and the two treatment (10 min × 200 ppt dip for 3 or 6 hour exposure).

TABLE 1.

Cumulative survival (percent) 6 days following treatment of either yearling (<1 year old) or adult oysters (2–3 years old).

Age (years)	Brine (ppt)	Duration (min)	Aerial Exposure (hours)	
			3 hr	6 hr
<1	22 (control)	10	95	95
<1	200	2	94	97
<1	200	5	95	96
<1	200	10	96	97
2–3	22 (control)	10	98	99
2–3	200	2	97	97
2–3	200	5	98	97
2–3	200	10	96	97

Mean of two replicates.

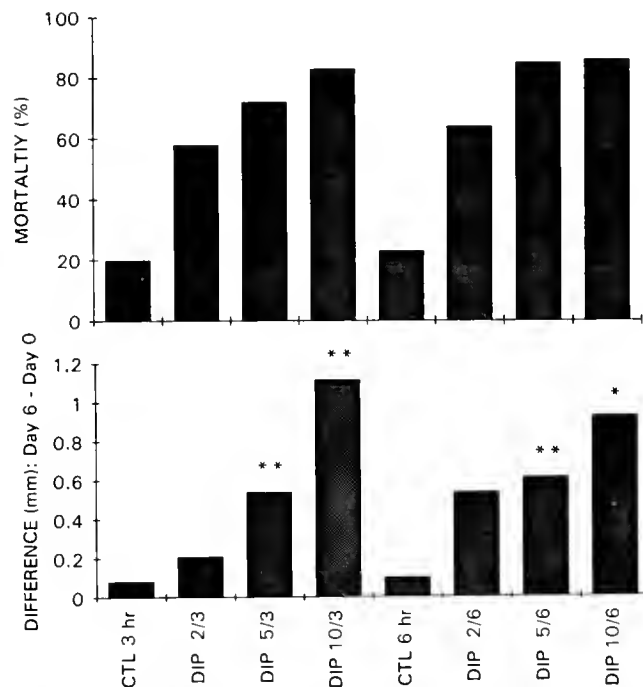


Figure 1. Top—Mean (of all replicates) cumulative mortality of spat (1–5 mm) 6 days after exposure to various brine treatments. Bottom—Difference in mean size of spat (1–5 mm) between day 0 and day 6 after treatment with various brine dips. Differences in means between day 0 and day 6 were tested by Student's t-test \*— $P < 0.05$ ; \*\*— $P < 0.01$ . Four histograms on left (3 hour aerial exposure): CTL 3 hr—22 ppt for 10 min; DIP 2/3—200 ppt for 2 min; DIP 5/3—200 ppt for 5 min; DIP 10/3—200 ppt for 10 min. Four histograms on right (6 hour aerial exposure): CTL 6 hr—22 ppt for 10 min; DIP 2/6—200 ppt for 2 min; DIP 5/6—200 ppt for 5 min; DIP 10/6—200 ppt for 10 min.



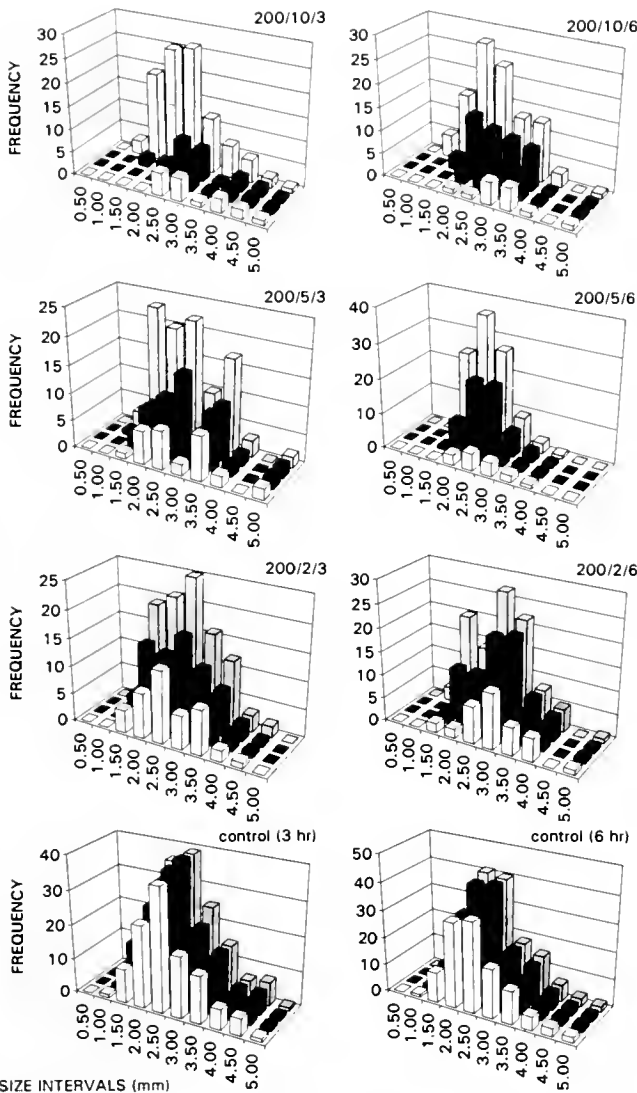


Figure 2. Frequency distribution histograms of spat (1–5 mm) in 0.5 mm size intervals on days (from back to front) 0, 2, 4, and 6 for various brine treatments. Left (3 hour aerial exposure), from bottom to top: control 3 hr—22 ppt for 10 min; 200/2/3—200 ppt for 2 min; 200/5/3—200 ppt for 5 min; 200/10/3—200 ppt for 10 min. Right (6 hour aerial exposure), from bottom to top: control—22 ppt for 10 min; 200/2/6—200 ppt for 2 min; 200/5/6—200 ppt for 5 min; 200/10/6—200 ppt for 10 min.

RESULTS

Experiment 1

Brine dips had no appreciable effect on survival of adult oysters for dip duration ( $P = 0.93$ ), aerial exposure ( $P = 0.506$ ), or the interaction of the two ( $P = 0.857$ ). Cumulative mean survival after 6 days ranged from 94–98% in treated groups compared to 97% for the controls (Table 1). We therefore felt that none of our treatments were harmful to adult brood stock.

Experiment 2

*Spat* < 5 mm Overall, the principal effect of brine dips on spat < 5 mm (mean 2.3 mm; range 0.9–5.0 mm) was high, selective mortality (Fig. 1, top). For 3 hour aerial exposure, mean mortality

of spat 6 days after treatment for 2, 5, and 10 minute dips in brine was 58%, 70%, and 83%, respectively, and 28%, 18%, and 24% for respective controls. In spat exposed to air for 6 hours, mean mortality for 2, 5, and 10 minute dips in brine was 64%, 85%, and 86%, respectively, and 38%, 22%, and 8% for respective controls. ANOVA demonstrated a significant effect due to dip duration ( $F_{3,10} = 32.9, P < 0.001$ ) but not for aerial exposure ( $F_{1,10} = 1.03, P = 0.335$ ) or interaction ( $F_{3,10} = 0.43, P = 0.734$ ). An *a posteriori* test (Tukey’s HSD) demonstrated that all treatments were significantly different from the control (Tukey’s HSD pairwise comparison, maximum  $P = 0.002$ ); 10 minute dips differed significantly from 2 ( $P = 0.041$ ), but not 5, minute dips.

Mortality in all groups, including controls progressed over the course of the 6 days of observation (Fig. 2). All size classes under 5 mm experienced some mortalities, but mortality was size dependent. For each treatment, we compared the mean size of spat before the brine dip with the mean size of spat 6 days afterward. Mean spat size was significantly larger 6 days after treatment (Student’s *t*-test, day 6 vs day 0) in brine dips for 5 and 10 min for both 3 and 6 hour aerial exposure (Figure 1, bottom). The obvious interpretation is small spat are more susceptible to brine dips than larger ones. (There was no significant difference among treatment groups at day 0: 2-way ANOVA, minimum  $P = 0.114$ ).

*Spat* > 4 mm For testing spat over 4 mm (mean 6.1 mm; range 4.0–11.6 mm), we used the best treatment from previous tests: 10 min dip in 200 ppt brine with either 3 or 6 hour exposure. In this experiment, the 6 hour exposure caused higher mortality than the 3 hour exposure, but not significantly (Tukey’s HSD

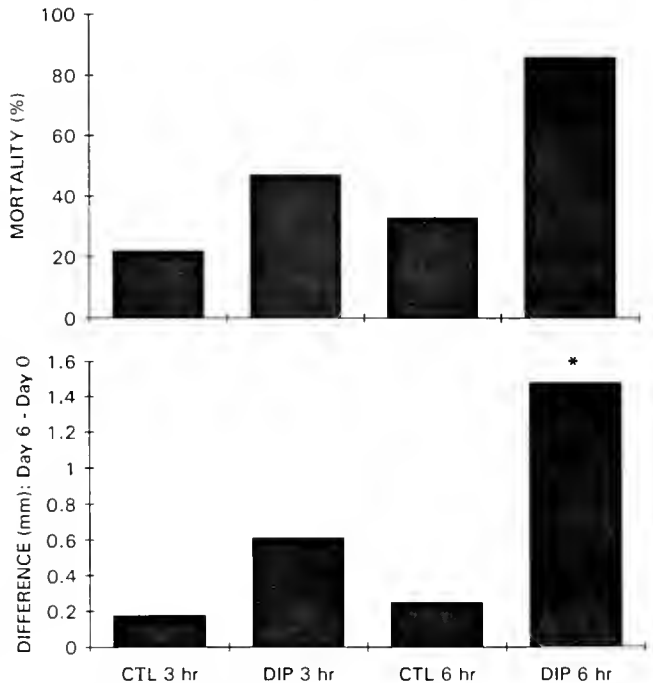


Figure 3. Mean (of all replicates) cumulative mortality of spat (4–12 mm) 6 days after exposure to various brine treatments. Bottom—Difference in mean size of spat (4–12 mm) between day 0 and day 6 after treatment with various brine dips. Differences in means between day 0 and day 6 were tested by Student’s *t*-test \*— $P < 0.05$ . Two histograms on left (3 hour aerial exposure): CTL 3 hr—22 ppt for 10 min; Dip 3 hr—200 ppt for 10 min. Two histograms on right (6 hour aerial exposure): CTL 6 hr—22 ppt for 10 min; DIP 6 hr—200 ppt for 10 min.

pairwise comparison,  $P = 0.053$ ) probably due to high variability in the data from the shorter exposure (Fig. 3, top). For 3 hour aerial exposure, mortality of spat 6 days after treatment was 36% and 58% for brine dips, and 22% for its control. For 6 hour exposure, mortality was 84% and 92% for brine dips, and 32% for controls.

Mortality in controls occurred mostly during the first two days, perhaps corresponding to handling, whereas mortality in brine dips occurred gradually over the 6 day observation period (Fig. 4). As found in the experiment using smaller spat, mortality was size dependent. We compared the mean size of spat before the brine dip with the mean size of spat 6 days afterward in each treatment. Mean spat size was significantly larger 6 days after treatment in brine for the 6 hour aerial exposure only (Fig. 3, bottom). (There was no significant difference among treatment groups at day 0:  $F_{2,3} = 0.083$ ,  $P = 0.963$ ). Judging from the data shown in Figure 4, it appears that the size cutoff for these dips is about 7.5 mm. That is, a 10 min dip in 200 ppt brine, followed by a 6 hour aerial exposure will kill mostly those spat less than 7.5 mm; also, the smaller the spat, the higher the mortality.

#### Field test

Brine dips conducted on large spat (mean 11.3 mm; range 4.7–22.0 mm) in a small scale field test in October 1991 had no effect on overset. For these larger spat, there was no effect due to brine dips on mean number of spat/oyster 6 days after treatment. Mean number of spat per oyster decreased 30% in controls, but only 40% in treatments ( $F_{1,8} = 1.07$ ,  $P = 0.332$ ). Adult survival 6 days post treatment was slightly higher in treated groups (94%) than in controls (90%), confirming that dip treatments are not injurious to adults.

#### DISCUSSION

The removal of overset from our brood stocks has been an ongoing maintenance problem at HSRL. Heretofore, overset was removed by scraping oysters individually. Very small spat were eliminated with a wire brush; older spat, by scraping with an oyster knife. Both of these methods are tedious and inefficient. Also, scraping often damages the growing edge of the oyster shell.

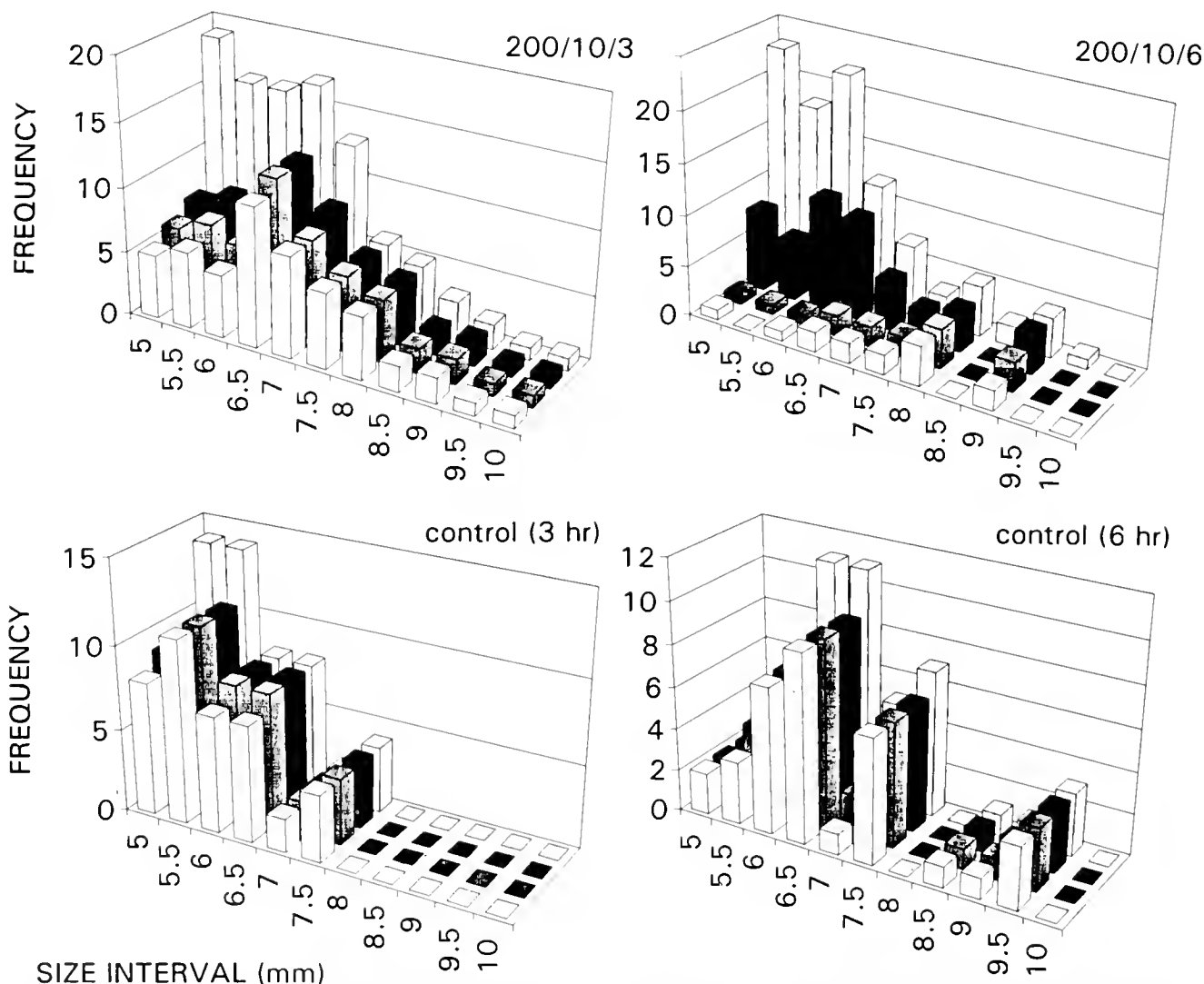


Figure 4. Frequency distribution histograms of spat (4–12 mm) in 0.5 mm size intervals on days (from back to front) 0, 2, 4, and 6 for various brine treatments. Left (3 hour aerial exposure), from bottom to top: control—22 ppt for 10 min; 200/10/3—200 ppt for 10 min. Right (6 hour aerial exposure), from bottom to top: control—22 ppt for 10 min; 200/10/6—200 ppt for 10 min.

The same problem would pertain to commercial grow-out anywhere natural set occurs. As a means of removing oyster overset on containerized oysters, for example in wire trays or in plastic mesh bags, brine dips are attractive for their efficiency and low cost, and would be amenable to large scale application.

Preliminary experiments conducted in 1990 indicated that overset might be controlled in this way, followed by some period of aerial exposure. Initially, aerial exposure was conducted in unshaded areas for 12 to 24 hours so that oysters could be returned at the next low tide or the next day's low tide. Early in the summer, when temperatures were moderate and when adult oysters were healthy (i.e., prior to spawning, disease pressures, food limitations, etc.), unshaded protracted aerial exposures were innocuous. Later in the summer when temperatures were higher and adult oysters less hardy, these same exposures began to cause higher mortalities. Fortunately, our preliminary trials and the data shown here demonstrate that only short aerial exposures, on the order of 3–6 hours, are required to kill overset. With such a short aerial exposure, brine treatments could be conducted within a tidal cycle, in most cases.

Immersion in brine solutions has been used in the past to remove fouling organisms from oysters. Loosanoff (1958) reported killing various soft-bodied invertebrates as well as egg cases of the oyster drill *Eupleura caudata* using 300 ppt brine solutions. MacKenzie & Shearer (1961) reported that from 87 to 98% of the mud blister worm *Polydora websteri* were killed using a 10–15 minute dip in a saturated salt solution, followed by 15 or more minutes of air drying. In another study, Shearer & Mackenzie (1961) reported 100% mortality of boring sponges (*Cliona celata*), starfish (*Asterias forbesi*), and tunicates (*Molgula manhattensis*) after immersion in 180 ppt brine solution for 10 minutes, followed by a 1 hour aerial exposure. When *Crassostrea virginica* spat measuring 10–20 mm in shell length were subjected to this treatment, 2.3% had died 14 days post treatment. More recently, Arakawa (1980) reported elimination of up to 59% of the fanworm

*Hydroides elegans* using a 60 minute dip in saturated brine. Finally, Dealeris et al (1988), while investigating alternative treatments to prevent the bio-deterioration of wood lobster traps by the wood-boring bivalve *Xylophaga atlantica*, found that a 30 second dip in saturated brine resulted in 99% mortality of the bivalve. The studies above shared the goal of removing fouling organisms, but these organisms, unlike the oyster, were incapable of isolating their soft-body parts from the treatment. Treatments to kill oyster spat must necessarily be more rigorous. This is apparent from the data of Shearer and Mackenzie (1961): treatments that lead to 100% mortality in boring sponges, starfish, and tunicates caused only 2.3% mortality in *C. virginica* spat.

Our tests in 1991 revealed that up to 86% of oyster spat measuring 5.0 mm or less can be removed using immersion for 10 minutes in a 200 ppt brine solution, followed by a 3 or 6 hour aerial exposure. These treatments did not hurt adults. In spat between 4 and 12 mm, up to 88% were removed with the same treatment, followed by a 6 hour exposure. However, for large spat (5–22 mm), removal by brine dip becomes ineffective. We conclude, based on the results of our preliminary field trials and the data shown here, that best results can be obtained from treatment of 5–10 min in 200 ppt brine, followed by a 6 hour aerial exposure. But of key importance is treating spat at a very small size, below 5 mm. Better yet, treatments should be most effective if they begin within days of the overset.

#### ACKNOWLEDGMENTS

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## OBSERVATIONS ON THE PEARL OYSTER FISHERY OF KUWAIT

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**ABSTRACT** The pearl oyster fishery of Kuwait was monitored daily from January 1989 to May 1990. Landings of pearl oysters in 1989 totalled 287 tons with a market value of U.S. \$1.0 million. Commercial pearls (>3 mm) were estimated to be present in one of every 4200 oysters. Most of the pearl oysters landed were new recruits with hinge lengths between 40–56 mm. There was a curvilinear relationship between total weight and size of oysters (length) and the sex ratio approached 1:1. Spawning occurs throughout the year, with a spat settlement peak in early fall. Over the size range examined there was no relationship between the size of oysters and the size of pearls and subsequent resource management strategies are discussed.

**KEY WORDS:** pearl, oyster, *Pinctada radiata*, fishery

### INTRODUCTION

Thriving from historic times until the 1930s, the traditional pearl oyster fishery in the Arabian Gulf was large and revered, furnishing about 80% of the world production of natural pearls, which were famous for their excellent shape and quality (Bowen 1951). Lorimer (1915) described the various pearl oyster banks in the Arabian Gulf (Fig. 1) and estimated the average yearly export values of pearls and mother-of-pearl (shells) to be Pounds Sterling 561,353 and 269,788, respectively, for the period 1873 to 1905. The annual catch for the entire Arabian Gulf was approximately 35,000 tons, a conservative estimate calculated from literature reports of catch rate, number of boats and number of fishermen (Lorimer 1915, Villiers 1969).

Bowen (1951) described the early pearl diving techniques and discussed various aspects of the industry. Pearl fishing in the Gulf was performed originally only during summer, May to September. Except for occasional inclement weather, diving was a continuous operation over this period. The traditional fishery declined steadily from 1930 onwards because of a world recession, the introduction of Japanese cultured pearls, and later with the discovery of oil in the area. In the late 1940s most people deserted the pearl industry for more lucrative oil-related positions.

In the late 1960s, pearl fishing was revived with the introduction of modern diving equipment, such as air compressors and speedboats. Since 1980, pearl oyster fishing is practised year round in Kuwait. A pearl oyster market was re-established in Kuwait in 1982, and the first catch statistics were reported for a five-month period in 1983 (Almatar et al. 1984). The present pearl oyster market of Kuwait is based exclusively on natural pearls from *Pinctada radiata* (Leach), (Khamdan 1988). In the Arabian Gulf this species has variously been referred to as *P. margaritifera* (Steininger 1968, Anderlini et al. 1981, Almatar et al. 1984), *P. fucata* (Mohammad 1976) and *P. radiata* (Sadig and Alam 1989). The objective of this report is to review pearl oyster landings,

describe size composition and frequency of pearl occurrence and discuss resource management strategies in light of the present findings.

### MATERIALS AND METHODS

Individual boat fishing activity and catches were monitored daily at the single pearl oyster market in Kuwait by interviewing fishermen in the market place. Fishing effort was calculated by multiplying the number of boats by average number of diving hours; the latter was estimated via interviews and direct observation.

Monthly size frequency distribution of the oyster hinge length (HL) were obtained from samples (200–300 oysters) purchased twice a month. Allometric measurements (maximum dorso-ventral height, (DVM), total oyster weight and wet meat weight) were obtained from subsamples. Shell measurements were measured to the nearest 0.1 mm using Vernier calipers. Oysters were cleaned of external fouling material and wiped dry before weighing to the nearest 0.1 g. Oyster meats were shucked from the shell and weighed individually. Sex was determined by gonad color: females were yellow-orange throughout study and mature males were milky white when sexually active or brown-yellow in the resting stage. Oysters of undetermined sex were recorded as immature. Wet mounts of gonads were conducted frequently to confirm sex.

### RESULTS

#### *The Current Fishery*

The diving fleet during this study consisted of 25 speedboats (3–8 m OAL), most with a single diver. Eleven major pearl oyster beds, varying in size from one to several square kilometers (10–20 m deep), were scattered within the fishing grounds (Fig. 2). An average of six 30-minute dives per day per diver were conducted using hookah air supply systems between 8 a.m. and 12 noon. Divers hand-picked oysters and placed approximately 6 kg in a

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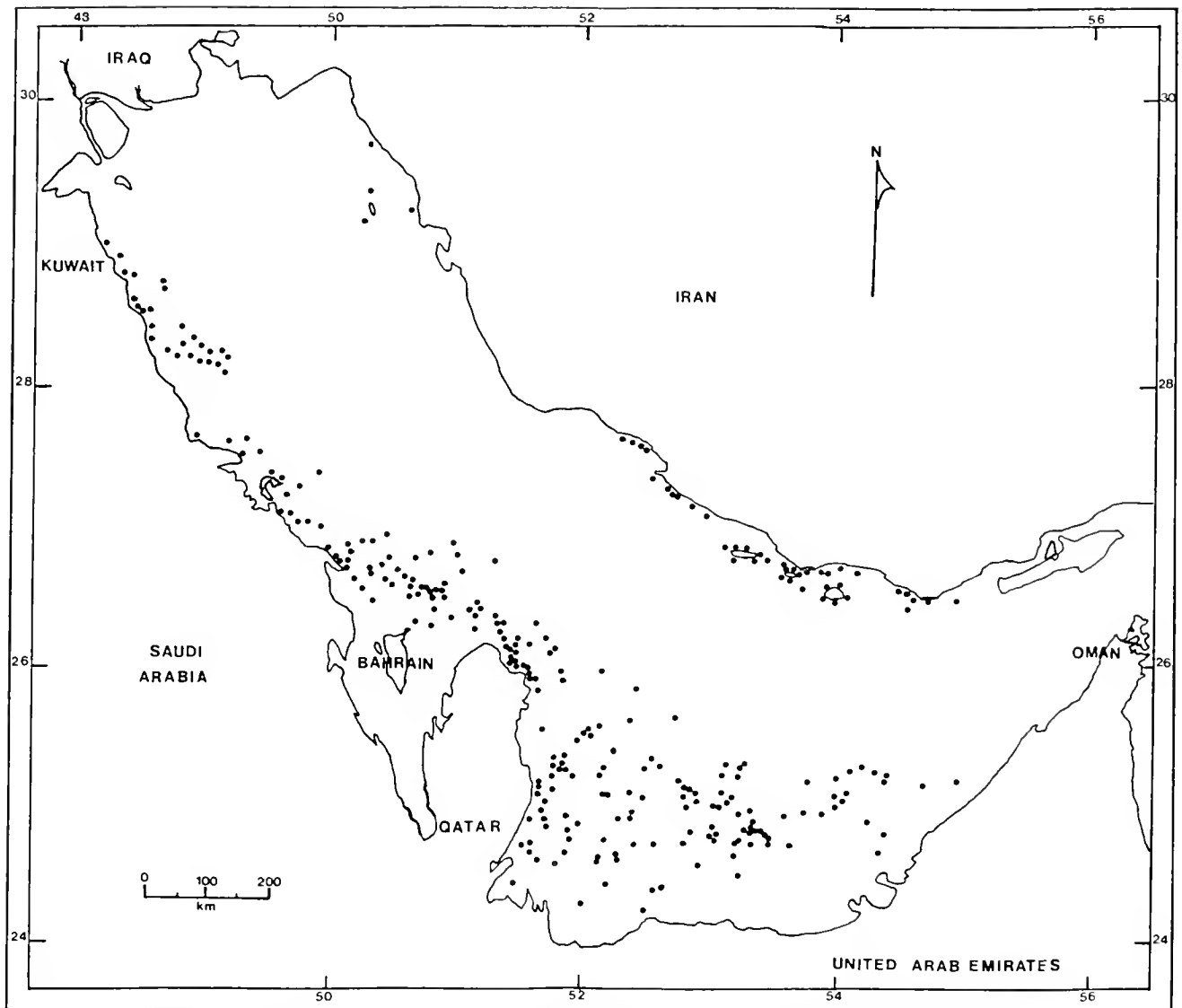


Figure 1. Location of traditional pearl oyster beds in the Arabian Gulf (from Lorimer 1915).

mesh bag. Unsorted oysters were sold to buyers at the market who later opened the oysters to retrieve any pearls which were subsequently resold.

#### Catch Statistics and Fishing Effort

The mean daily landing of pearl oysters in 1989 was 865 kg, and varied from 146 kg in January to 1716 kg in July (Fig. 3). Landings in 1989 totaled 287 tons or about  $6.3 \times 10^6$  oysters, worth approximately U.S. \$1.0 million.

Landings varied directly with effort; highest effort occurred between June and October. The poor weather/diving conditions between December and March accounted for the lowest effort (Fig. 3). The average catch per hour of diving (CPUE) in 1989 was  $37 \pm 17.4$  kg ( $n = 12$ ); this is a slight overestimate since some diving boats occasionally carried more than one diver. CPUE was lowest in January 1989 and highest in July 1989. Earlier data from 1983 also showed that landings and CPUE increased steadily from May to September 1983 (Fig. 3) (unpublished data).

#### Size Composition

Total shell and meat weight, wet flesh weight and hinge length (HL) are presented by size groups in Table 1. Quarterly size frequency histograms are shown in Figure 4. The HL of the majority of pearl oysters were unimodal and ranged between 40–56 mm. Oysters less than 40 mm HL were landed throughout the year, but were most abundant in fall and winter.

A linear relationship exists between HL and maximum height (DVM) measurement:

$$\text{DVM} = -16.863 + 1.619 (\text{HL}) \quad (r^2 = 0.79; n = 120)$$

The size-weight data (Fig. 5) are best described by curvilinear relationships of the form  $Y = aL^b$  (where Y is total weight in g and L is length in mm) as follows:

$$\begin{aligned} \text{Log (Wt)} &= -5.655 + 4.253 \log (\text{HL}) \\ (r^2 &= 0.78; n = 120) \end{aligned}$$

$$\begin{aligned} \text{Log (Wt)} &= -4.246 + 3.228 \log (\text{DVM}) \\ (r^2 &= 0.97; n = 120) \end{aligned}$$

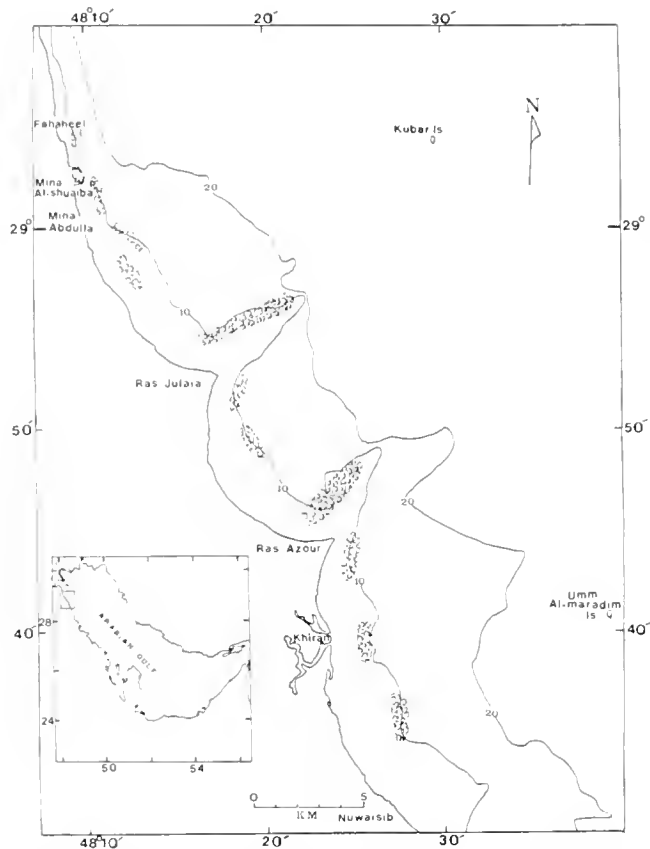


Figure 2. Location of major pearl oyster heds in Kuwait waters; shaded areas offshore indicate oyster beds.

### Pearl Harvest

Ninety-six of 4414 oysters sampled (2.2%) bore one or more pearls. Oysters with multiple pearls accounted for 17.7% of all pearl-bearing oysters. All pearls recovered from the study were too small ( $1.53 \pm 0.88$  mm;  $n = 132$ ) to be of commercial value. Table 2 displays pearl harvest by size and location over a range of oyster sizes. Pearls found in the mantle were significantly larger (Student's *t*-test:  $t < 0.05$ ) than those in the gonad. No pearls were found in oysters with HL less than 40 mm and there was no significant correlation between oyster size and pearl size ( $r^2 = 0.003$ ,  $df = 130$ ) from the oysters examined. However, the probability of pearl occurrence increases with size of oysters. Three percent of oysters less than 58 mm HL contained pearls whereas the frequency increased to 5 percent for those over 58 mm HL.

From the  $5.9 \times 10^6$  oysters landed from June 1989 to January 1990, only 400 large pearls (>4 mm) and 984 small pearls (3–4 mm) were sold in the market. Thus, the probability of landing a commercial-sized pearl is one in 4200. This estimate is slightly skewed because a few pearls were sold outside the oyster market.

### Maturity

Figure 6 reveals that both sexes matured at the same size (50 mm HL) and there was no evidence of a sex change with size in *P. radiata*, as has been reported for other species (Tranter, 1958). Sex ratio over the period of sampling approached 1:1. Because of

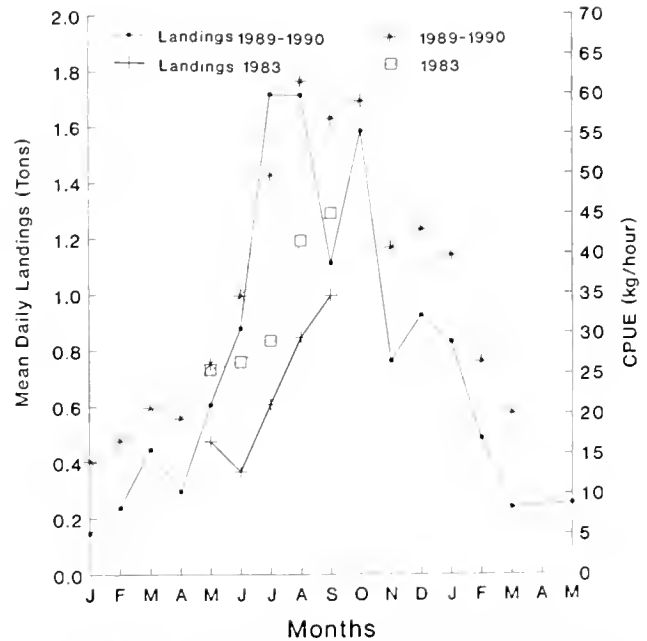


Figure 3. Mean monthly landings and mean monthly catch per unit of effort (CPUE) of *P. radiata* for 1989 and from January to May 1990. Data from May to September 1983 are also shown for comparison. CPUE is defined as amount (kg) of oyster harvested per hour.

the high growth rate of oysters, maturity is probably reached in the first year, and for those spawned in early spring, possibly during the first six months (Tranter 1958; Rose et al. 1990). The presence of small oysters (<40 mm HL) throughout the year indicates that spawning is continuous with the greatest activity occurring in the summer and late fall (Fig. 4).

### DISCUSSION

Compared with the harvest rates of pearl oysters in other tropical areas, Kuwaiti waters are highly productive (Pragasam and

TABLE 1.

Total weight and wet flesh weight (mean  $\pm$  standard deviations) in relation to 2 mm size intervals of HL for subsample of *P. radiata* landings

HL (mm)	n	Total Weight (g)	Flesh Weight (g)
22–23	1	1.17 —	0.34 —
24–26	1	8.51 —	2.51 —
30–32	3	3.45 $\pm$ 1.64	0.96 $\pm$ 0.40
33–35	2	7.28 $\pm$ 3.11	2.10 $\pm$ 0.74
36–38	5	8.35 $\pm$ 2.54	2.38 $\pm$ 0.41
39–41	4	13.83 $\pm$ 2.16	3.90 $\pm$ 0.46
42–44	8	23.63 $\pm$ 14.18	7.23 $\pm$ 4.33
45–47	22	34.46 $\pm$ 10.40	10.22 $\pm$ 2.91
48–50	14	44.23 $\pm$ 18.35	13.62 $\pm$ 5.24
51–53	16	49.20 $\pm$ 18.58	15.92 $\pm$ 6.66
54–56	18	64.08 $\pm$ 19.05	21.89 $\pm$ 5.60
57–59	16	60.85 $\pm$ 14.72	21.25 $\pm$ 3.35
60–62	9	64.14 $\pm$ 25.56	24.08 $\pm$ 5.32
63–65	1	81.83 —	29.75 —

(n = number of oysters).

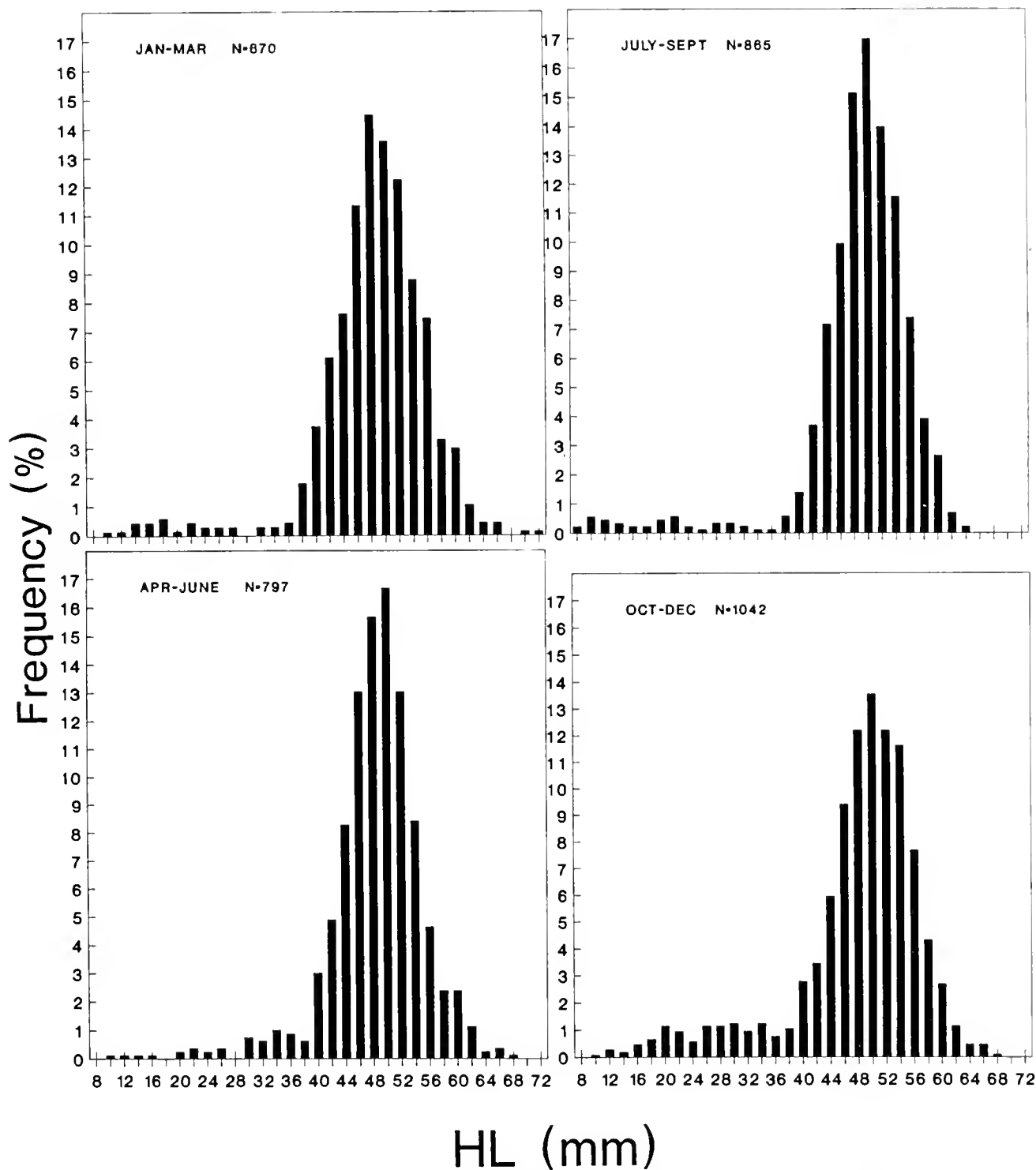


Figure 4. Quarterly hinge length frequency distribution at 2 mm intervals of *P. radiata* collected from landings in the pearl oyster market during 1989 and early 1990.

Dev 1987; Easwaran et al. 1969; Dybdahl and Rose 1986). Indeed, catch data from this study are relatively high—780 oysters or 37 kg per hour of diving. Direct observation and interviews indicate that our CPUE calculations may have been overestimated by 25 percent at most.

Due to the high annual harvest rates, the fishery in Kuwait relies heavily on recruitment of young oysters. Narayanan and Michael (1968) reported a growth rate of *P. vulgaris* in the Gulf of Kutch of 38.4 mm HL in the first year while Jeyabaskaran et al. (1983) reported a growth rate of *P. fucata* in the Gulf of Mannar



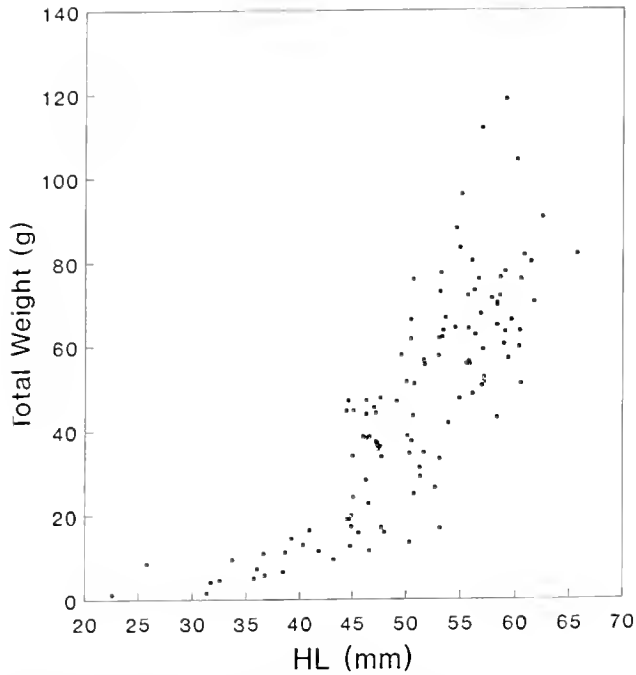


Figure 5. Total weight versus HL for *P. radiata* collected throughout the course of the study. The length-weight relationship is  $W = 0.00000221 L^{4.253}$  ( $r^2 = 0.78$ ,  $n = 120$ ).

of 41.2 mm in its first year. Nayar et al. (1992) concluded that the growth of *P. radiata* in Bahrain waters was higher than the growth of pearl oysters in the Gulf of Mannar (corroborates unpublished data of the present study). It appears that the majority of the commercial Kuwaiti catch is composed of 0+ or 1+ year-classes.

Although increased effort yields increased landings, other factors that affect landings are poorly understood. Annual fluctua-

TABLE 2.

Number, size (mean diameter  $\pm$  s.d.) and location of pearls found in relation to 2 mm HL intervals of pearl oysters.

HL (mm)	Number Oysters Searched	Number Oyster with Pearls	Pearl Location			
			Mantle		Gonad	
			n	size (mm)	n	size (mm)
<40	127	0	—	—	—	—
41	376	1	3	1.0 $\pm$ 0.3	0	—
45	355	4	3	1.4 $\pm$ 0.7	1	0.5
47	501	13	10	1.8 $\pm$ 1.0	8	1.3 $\pm$ 0.3
49	572	11	12	2.3 $\pm$ 1.7	2	1.0 $\pm$ 0.1
51	626	15	10	1.1 $\pm$ 0.8	7	1.2 $\pm$ 0.4
53	564	17	14	1.9 $\pm$ 0.8	8	1.3 $\pm$ 0.3
55	391	7	7	1.6 $\pm$ 0.3	2	1.1 $\pm$ 0.2
57	294	7	6	2.0 $\pm$ 0.8	6	1.4 $\pm$ 0.8
59	188	10	0	—	13	1.5 $\pm$ 0.9
61	106	6	9	1.1 $\pm$ 0.1	5	1.1 $\pm$ 0.2
63	52	3	1	2.6	2	1.8 $\pm$ 0.1
65	28	1	0	—	1	2.0
>67	8	1	0	—	1	2.5

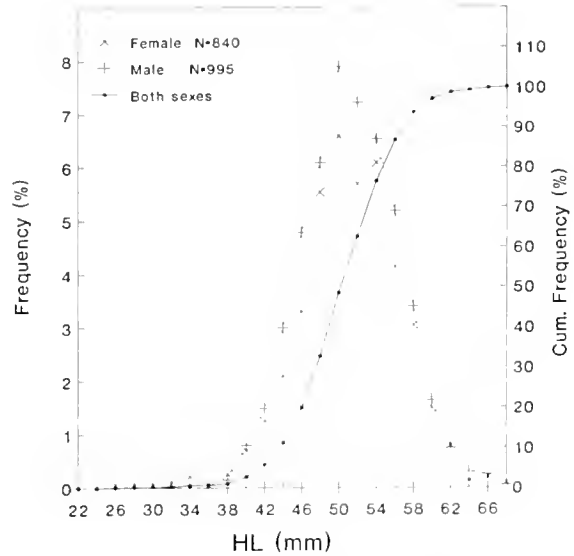


Figure 6. Percent HL frequency distribution of male and female and cumulative HL frequency distribution of combined sexes for *P. radiata* collected during 1989 and early 1990.

tions and sources of spat settlement are virtually unknown. Extensive beds in Saudi Arabian waters could be significant sources of spat, since they lie within protected zones near oil wells. There are no data to suggest that the present fishing pressure (about 1000 hours per month) is sustainable. Ongoing data collection subsequent to the Gulf War (May–November, 1992) indicates no apparent change in fishing pressure, but there is a slight drop in catch volume. This decreased volume may be due to overharvest or environmental damage to oyster beds caused by oil spills and combustion products of oil fires during the war.

The objective of managing the pearl oyster fishery is not to maximize the landings of oysters, but rather to maximize the value of pearls—via increased numbers or sizes of pearls. The present study found no relationship between pearl size and oyster size over the range 40–68 mm HL and agrees with earlier findings for the same species (Almatar et al. 1984). Large valuable pearls (>3 mm) observed in the market (not taken from our subsamples) were not found in unusually large oysters. This study did find an increase in occurrence of pearls in larger (>58 mm HL) versus smaller (<58 mm HL) oysters. Studies of other species have also related pearl yield to age of oyster (Pearson 1933; Easwaran et al. 1969). Results of this study indicate that the total value of pearls could be increased if the fishery were managed to promote harvest of oysters greater than 58 mm HL.

#### ACKNOWLEDGMENTS

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# ULTRASTRUCTURAL STUDY OF GAMETOGENESIS IN THE FRENCH POLYNESIAN BLACK PEARL OYSTER *PINCTADA MARGARITIFERA* (MOLLUSCA, BIVALVIA). I—SPERMATOGENESIS.

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**ABSTRACT** Ultrastructure of the germinal cells is described throughout the spermatogenesis, in the French Polynesian black pearl oyster, *Pinctada margaritifera* (L., 1758) var. *cumingii* (Jameson 1901). Special emphasis is given to the spermatozoon structure description. Abnormal spermatogenesis and processes of degeneration and resorption of residual germinal cells are also reported. Male germinal cells present a centripetal evolution in the acini. Germinal cells deriving from a same germinal lineage, are connected among themselves and among one auxiliary cell by cytoplasmic bridges. The mature sperm of this species is of the primitive type, with a short acrosome and without axial rod. The spermatozoa are 45–50  $\mu\text{m}$  long. Midpiece contains two centrioles along with satellites bodies and four or five mitochondria.

**KEY WORDS:** spermatogenesis, ultrastructure, mollusc, bivalve, *Pinctada margaritifera*

## INTRODUCTION

Initial study of spermatogenesis of the commercially important black pearl oyster was carried out with light microscopy on Australian specimens (Tranter 1958) and on French Polynesian specimens (Thielley 1989).

In French Polynesia, the black pearls provide the major source of exportation revenues for the Territory. As a result of this economic importance, a wide program of research focused on the biology of this species has recently been carried out.

The present study is part of this program and describes the ultrastructure of the germinal cells including normal and abnormal spermatogenesis with emphasis on the spermatozoon.

## MATERIALS AND METHODS

*Pinctada margaritifera* adult specimens were collected monthly between April 1990 and December 1990, from the natural stock and from a farm at Takapoto atoll's lagoon (Tuamotu, Archipelago French Polynesia).

Samples of male gonad were fixed for 3 hours in 3% glutaraldehyde in 0.4 M cacodylate buffer (pH 7.4; 570 mosM). The tissues were then washed in the buffer solution and postfixed for one hour in 1% osmium tetroxide in the buffer. After dehydration by ethanol, the pieces were embedded in Spurr resin. Semi thin sections (1  $\mu\text{m}$ ) were stained with toluidine blue. Ultrathin sections (600  $\text{\AA}$ ) were contrasted with uranyl acetate and lead citrate and examined under a JEOL TEM 200 transmission electron microscope. A few sections collected on gold grids, were treated for the detection of glycogen (Thiery and Rambourg 1974).

## RESULTS

The male germinal cells are gathered in acini and present a centripetal evolution.

### 1. Normal Spermatogenesis

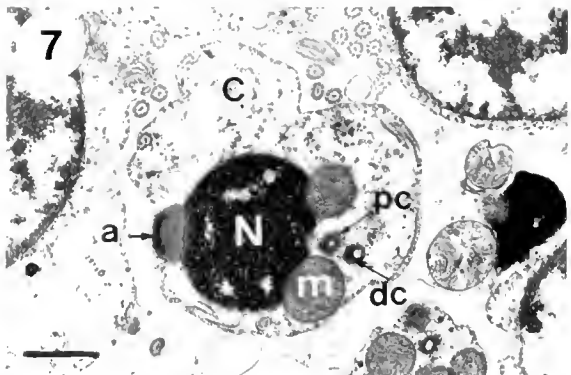
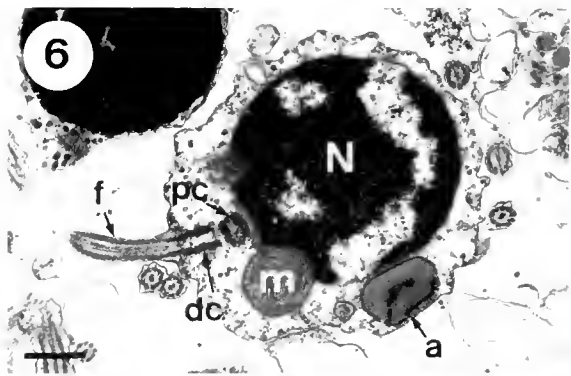
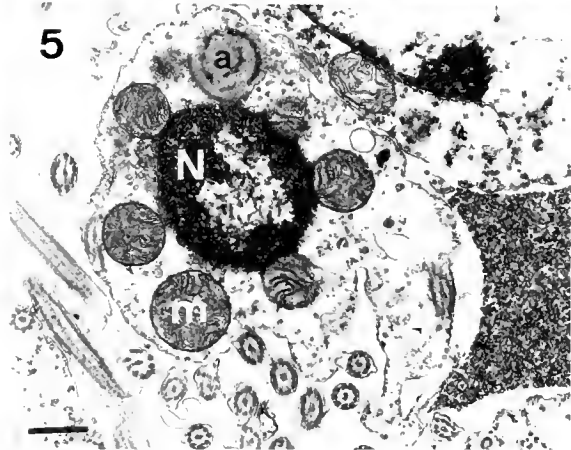
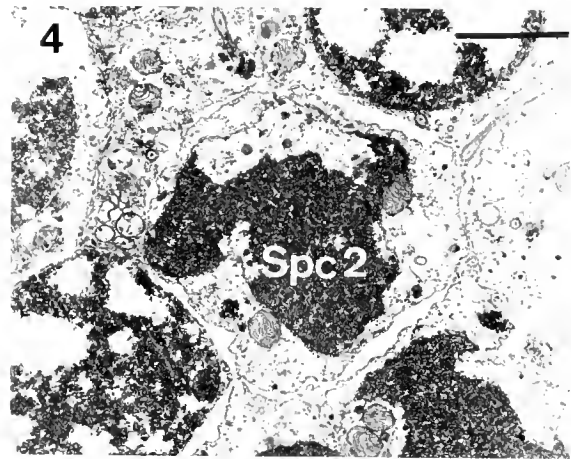
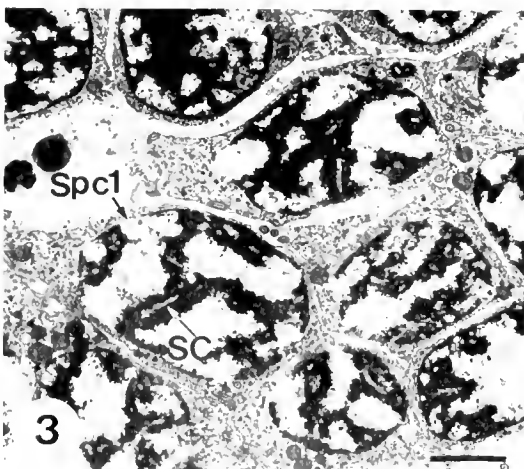
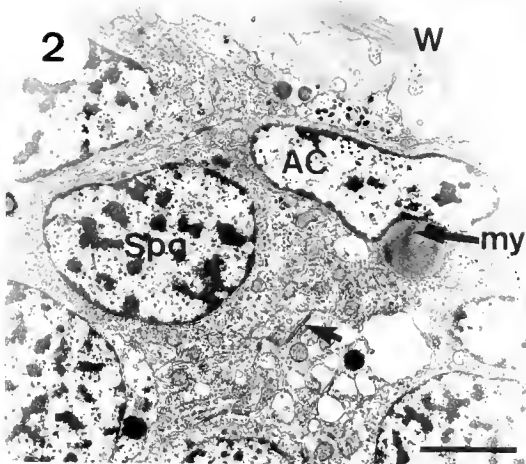
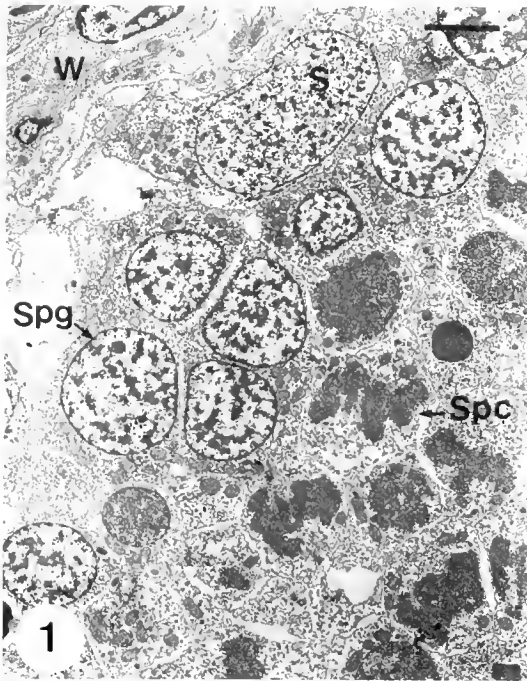
#### Spermatogonia Stem Cells

The spermatogonia stem cells stick largely to the acinus wall (Fig. 1). They are oval in section, with an average size of 14  $\mu\text{m}$   $\times$  8  $\mu\text{m}$ . Their nucleus can reach 9  $\mu\text{m}$  in length and 5  $\mu\text{m}$  in width. The chromatine is uniformly dispersed in small aggregates, and gives a fine granular aspect. One or two nucleoli are present. In their abundant cytoplasm, a large number of mitochondria, often oval in section, are gathered in two heaps at two poles of the cell. Rough endoplasmic reticulum, a few dictyosomes, ribosomes and few dense inclusions are present.

#### Spermatogonia

Primary and secondary spermatogonia are very similar in aspect. They often adhere to the acinus wall. The spermatogonia are spherical or oval and measure about 7  $\mu\text{m}$  in length. The nucleus is 5  $\mu\text{m}$  in diameter, containing a single nucleolus and small clumps of electron dense chromatine. Their cytoplasm is reduced and contains some dictyosomes, osmiophilic inclusions, a large number of mitochondria, ribosomes and endoplasmic reticulum (Figs. 1, 2).

Auxiliary cells, adhering to the acinus wall, can be observed between the spermatogonia (Fig. 2). Their nucleus is elongated, about 5  $\mu\text{m}$  in length and 1.5  $\mu\text{m}$  in width. They present a scattered chromatin essentially peripheral. The cell is polymorphic and notably emits cytoplasmic digitations that infiltrate between the spermatogonia. Their cytoplasm contains mitochondria, a lot of glycogen particles, endoplasmic reticulum and sometimes myelinic formations and dense lysosomal formations. Desmosome-like junctions can be observed between auxiliary cells and spermatogonia. One auxiliary cell can bridge several spermatogonia deriving from the same germinal lineage. These spermatogonia are connected between themselves by cytoplasmic bridges.



## Spermatocytes

Primary and secondary spermatocytes lie at the periphery of the acini, within groups of 10 to 20 synchronous cells.

### Primary spermatocytes:

The cells have a similar size to spermatogonia. Their cytoplasm also contains a complement of organelles very similar to them. In addition, centrioles can be sometimes observed. The nucleus undergoes complex morphologic changes during the early meiotic prophase. Distinguishable stages of prophase are:

*Leptoten stage:* the chromatin is set out in very dense clusters. Some vacuoles appear in the 5  $\mu\text{m}$  wide nucleus.

*Zygoten and pachyten stages:* the zygoten stage is characterized with the rise of synaptonemal complexes formation. As soon as these formations are fully completed all along the chromosome, the spermatocyte enters the pachyten stage. Electron-dense chromatin forms a network, where synaptonemal complexes can be observed. The nucleus size increases to 6  $\mu\text{m}$  in diameter (Fig. 3).

*Diploten-diacinese stage:* the nucleus has a similar size as in the previous stage, but chromatin appears more condensed. Intranuclear vacuoles can be distinguished.

### Secondary spermatocytes:

The average size of the secondary spermatocyte is about 5  $\mu\text{m}$ . Its nucleus has a variable shape and the nuclear envelope is not easily observed. The highly dense chromatin fills in most of the space in the nucleus. Its cytoplasm contains some mitochondria, ribosomes, vacuoles and golgi bodies (Fig. 4).

## Spermatids

The size of the spermatid is about 4  $\mu\text{m}$ . Its nucleus is spherical and about 2  $\mu\text{m}$  in diameter. Condensation of the chromatin takes place throughout the spermatogenesis. During the process, the few clear areas between the chromatin masses reduce in size and then disappear. The spermatid cytoplasm contains ribosomes, a single dictyosome, mitochondria and two centrioles. In the early stages of spermatid development, the mitochondria forms a collarette all around the nucleus (Fig. 5). They gather towards the basal pole of the future spermatozoon and fuse into only four or five voluminous mitochondrial spheres.

The centrioles appear at the basal pole and move to an orthogonal position (Fig. 6). Very soon, the distal centriole produces a caudal flagellum. The acrosomal vesicle appears at the basal pole. Originally spherical, about 0.6–0.7  $\mu\text{m}$  in diameter, it becomes flat and then slightly incurved against the nucleus when it migrates to the apical pole, where it takes a half sphere shape. Two major

different electron-dense regions are discernable within the acrosomal vesicle (Fig. 7).

During spermiogenesis, most of the cytoplasm evaginates from the maturing spermatids and is eliminated as free masses into the acinus lumen.

## Spermatozoa

The spermatozoon of *Pinctada margaritifera* is of the primitive type according to Franzen (1983). It is 45–50  $\mu\text{m}$  in length. It can be divided into three parts: sperm head consisting of the nucleus and acrosome, middle piece consisting of two centrioles and mitochondria, and tail.

The acrosome is invaginated at its adnuclear surface and forms a conical structure which is 0.9  $\mu\text{m}$  in diameter and 0.5  $\mu\text{m}$  in height (Fig. 8). The acrosome consists of three major electron-dense materials. One has an electron-low density and makes up the enlarged basal part of the cone. The apical part of the acrosome is formed of an highly electron dense material that includes a lamellar structure (Fig. 9). A third substance of intermediate density covers the both other materials, constituting so the walls of the acrosomal cone. Between the plasma membrane and the cone, as well as in the central lumen of the acrosome, a fine granular material accumulates.

The spherical electron-dense nucleus is 1.7  $\mu\text{m}$  in diameter, and presents a large anterior invagination, 0.3  $\mu\text{m}$  in depth, where fine granular material accumulates (Fig. 8), and a smaller posterior invagination, 0.2  $\mu\text{m}$  in depth (Fig. 4).

The midpiece contents a ring of four or five mitochondria of about 0.8  $\mu\text{m}$  in diameter (Figs. 11, 12) around two centrioles (Fig. 10).

The two centrioles are connected to each other at right angles, and show the classical nine triplets of microtubules. The proximal centriole is joined to the nuclear envelope by a satellite body found in the post-nuclear fossa (Fig. 14). The distal centriole forms the basal body of the flagellum. It is connected to the plasma membrane by radiating satellite bodies (Figs. 10, 14). Granules of glycogen are detected by the reaction of Thiery, essentially between mitochondria but also around the nucleus (Fig. 13).

The flagellum is about 45  $\mu\text{m}$  long and shows the classical structure of nine external and one internal microtubule doublets (Fig. 15).

## 2. Abnormal Spermatogenesis

Abnormal cells are more or less numerous according to the specimens. Plurinuclear cells can be observed at most of stages of the spermatogenesis. Up to six nucleus appear more particularly at the spermatogonia stages (Fig. 16).

**Figure 1.** Section through an acinus. Acinus wall (W); stem cell attached to the acinus wall (S); spermatogonia (Spg); primary spermatocytes in metaphase (Spc). Bar = 3  $\mu\text{m}$ .

**Figure 2.** Auxiliary cell (AC), attached to the acinus wall (W). Myelinic formation (my); spermatogonia (Spg); desmosome-like junction (arrow). Bar = 2  $\mu\text{m}$ .

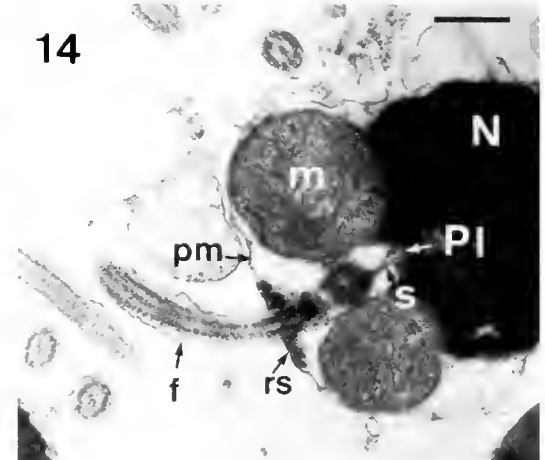
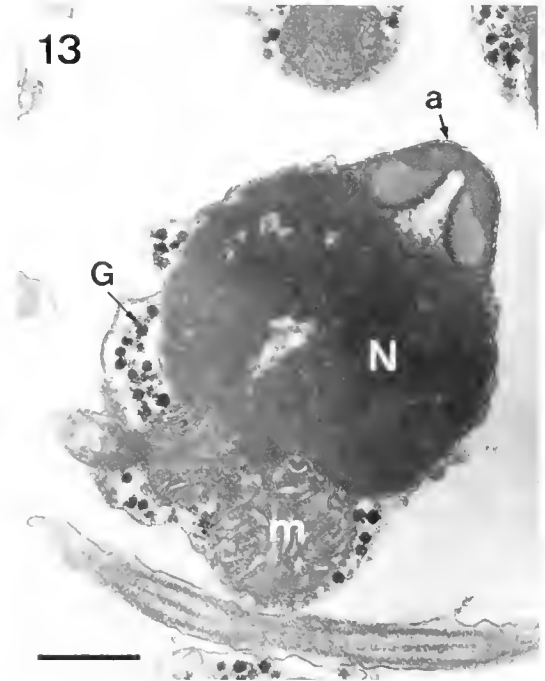
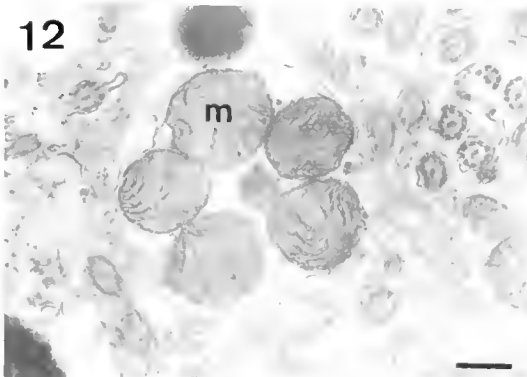
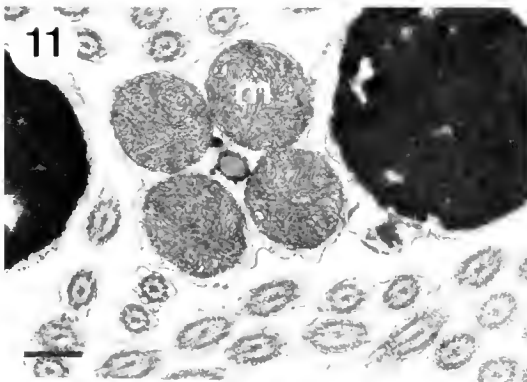
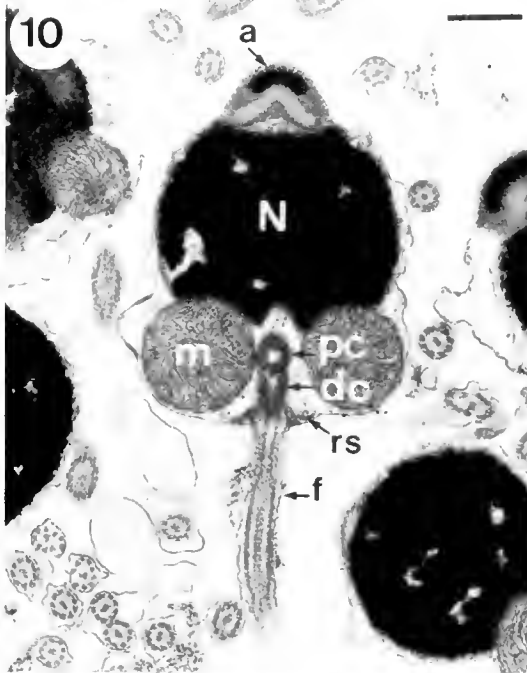
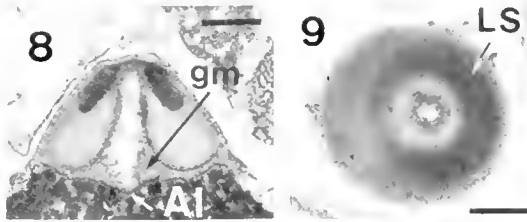
**Figure 3.** Primary spermatocytes (Spc1) in zygoten-pachyten stage characterized by the presence of synaptonemal complexes (SC). Bar = 2  $\mu\text{m}$ .

**Figure 4.** Secondary spermatocyte (Spc2). Bar = 2  $\mu\text{m}$ .

**Figure 5.** One of the early stages of spermatid development. Numerous mitochondria (m) form a collarette all around the nucleus (N). Acrosomal vesicle (a) is spherical. Bar = 500 nm.

**Figure 6.** Spermatid. Mitochondria (m) are in the basal pole around proximal centriole (pc) and distal centriole (dc) which begin to elaborate a caudal flagellum (f); acrosomal vesicle (a); nucleus (N). Bar = 500 nm.

**Figure 7.** One of the last stages of the spermatid. Mitochondria (m); proximal centriole (pc); distal centriole (dc); acrosomal vesicle (a) in the apical pole of the future spermatozoon; cytoplasm (c) is still abundant; nucleus (N). Bar = 1  $\mu\text{m}$ .



Binucleated spermatocytes and spermatids are frequent (Figs. 17, 18).

An intracytoplasmic flagellum was observed in some spermatids (Fig. 19) and more scarcely in the spermatozoa (Fig. 20). Occasionally, two flagellum complexes were observed inside the same plasma membrane (Fig. 21).

### 3. Gametic Degeneration and Resorption

Male germinal cell degeneration can occur at any developmental stages. Main degeneration aspects are caryolyses and cytoplasm alterations. The nucleus can present an hypercondensation of its chromatin (Fig. 22) or sometimes diffused chromatin with lysis of the nuclear envelope (Fig. 23). Main alterations of the cytoplasm are numerous vacuoles, huge lysosomal inclusions and altered mitochondria. Such aspects of male germinal cells degeneration make it difficult for their classification into a particular cellular type. The degenerative germinal cells, more or less degraded, can be driven out by the genital duct. Some residual cells can also be resorbed *in situ*: macrophage cells (12–15 µm in length) are often observed inside the acini (Fig. 24). Degenerative cells and residual bodies of the gametes, can be phagocyted by these cells.

### DISCUSSION

The processes of spermatogenesis described in *Pinctada margaritifera* are similar to other studies reported on other bivalves molluscs (Hodgson and Bernard 1986, Dorange and Le Penneec 1989). Four or five large mitochondria may be the result of a fusion of smaller mitochondria (Dorange and Le Penneec 1989, Hodgson and Bernard 1986).

The spermatozoon of *Pinctada margaritifera* is typically of the primitive type (Franzen 1983). The spermatozoon type is in direct relation with oocyte's reproduction and morphology (Franzen 1983). According to this author, spermatozoa of the primitive type are usually associated with species having external fertilization and small oocytes. Such is the case in the *Pinctada margaritifera* species.

The spermatozoon head differs in size, form and structure from the one described in many other bivalves in T.E.M. In the Mytilidae species, the acrosome structure seems to be more complex, particularly with the presence of an axial rod (Bourcart et al. 1965, Hodgson and Bernard 1986). This axial rod is also present in *Crassostrea virginica* (Daniels et al. 1971) and *Crassostrea angulata* (Gutierrez et al. 1978).

In *Chama macerophylla* and *Spisula solidissima* spermatozoa, Hylander and Summers (1977) report the presence of two major constituents of the acrosomal vesicle: an electron-dense acroso-

mial material as the "basal ring", and a less dense homogeneous material in the central and anterior portion of the acrosome. The acrosome of *Pinctada margaritifera* contains three major materials of different electron density, but, unlike the two previous species, the electron dense material occupies the apex of the acrosome and the less dense zone forms the basal ring of the conical acrosome.

In many mollusc species, the acrosome shows a lamellar structure (Popham et al. 1974, Dorange and Le Penneec 1989, Franzen 1983). This type of structure has been observed in *P. margaritifera*. According to Hylander and Summers (1977), the acrosome structure can be correlated with the oocyte vitelline envelope.

Accumulation of granular material around the acrosome and especially in the central lumen, has often been described in many bivalve species (Hodgson and Bernard 1986, Dorange and Le Penneec 1989, Popham et al. 1974, Hylander and Summers 1977). We also observed this granular material in *P. margaritifera*, which possibly binds the acrosomal vesicle to the nuclear envelope, according to Popham et al. (1974).

Franzen (1983) describes the midpiece as a stable structure in bivalve molluscs. The number of mitochondria is variable between and inside species. The spermatozoa of *Mytilus galloprovincialis* and *Aulacomya* described by Hodgson and Bernard (1986), present five or six mitochondria. *Crassostrea virginica* spermatozoon has four (Daniels et al. 1971). *Mytilus perna* has five mitochondria, very rarely four (Bourcart et al. 1965). Hodgson and Bernard (1986), in *Choromytilus meridionalis* and Dorange and Le Penneec (1989) in *Pecten maximus* observed four mitochondria, rarely five. According to the latter authors, the presence of five mitochondria is abnormal. In *Pinctada margaritifera*, four or five mitochondria were observed, with a 1/1 ratio. Therefore, it is difficult to conclude that four or five mitochondria give abnormal or normal spermatozoon.

A satellite body found in the postnuclear fossa, as a connection between the proximal centriole and the nuclear envelope, has been described by Popham et al. (1974) in *Bankia australis* and *Bankia carinata* and by Daniels et al. (1971) in *Crassostrea virginica*. A similar structure is observed in *Pinctada margaritifera*. The presence of this satellite body has not been reported in *Mytilidae* by Hodgson and Bernard (1986) and Bourcart et al. (1965). Franzen (1983) in his study of three Bivalve species and Dorange and Le Penneec (1989) in *Pecten maximus* did not describe this structure.

Otherwise, satellite bodies form connections between the distal centriole and the plasma membrane, at the basal part of the distal centriole. This structure is widely described by many authors about many species (Popham et al. 1974, Dorange and Le Penneec 1989, Franzen 1983).

Our study and other observations on bivalves spermatozoon

Figure 8. Longitudinal section through the acrosome showing the three major electron-dense materials. Anterior invagination (AI) where fine granular material (gm) accumulates. Bar = 300 nm.

Figure 9. Transverse section through the acrosome showing the three major electron-dense materials and lamellar structure (LS). Bar = 200 nm.

Figure 10. Longitudinal section of spermatozoon. Acrosome (a); nucleus (N); mitochondria (m); proximal centriole (pc); distal centriole (dc); radiating satellite bodies (rs); flagellum (f). Bar = 500 nm.

Figure 11. Transverse section through the midpiece showing four mitochondria (m). Bar = 400 nm.

Figure 12. Transverse section through the midpiece showing five mitochondria (m). Bar = 400 nm.

Figure 13. Longitudinal section through a spermatozoon. Granules of glycogen (G) are detected by the reaction of Thiery. Nucleus (N); mitochondria (m); acrosome (a). Bar = 500 nm.

Figure 14. Longitudinal section through the midpiece showing a satellite body (s) in the posterior invagination (PI) and radiating satellite bodies (rs) connected to the plasma membrane (pm); flagellum (f); noyau (N). Bar = 500 nm.

Figure 15. Transverse section through a flagellum showing the classical structure 9 external and 1 internal microtubule doublets. Bar = 50 nm.



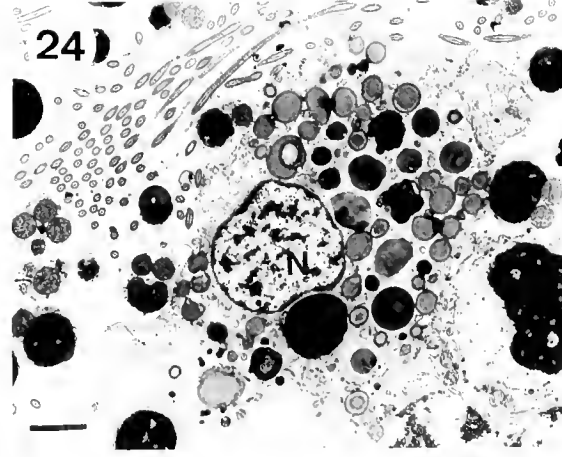
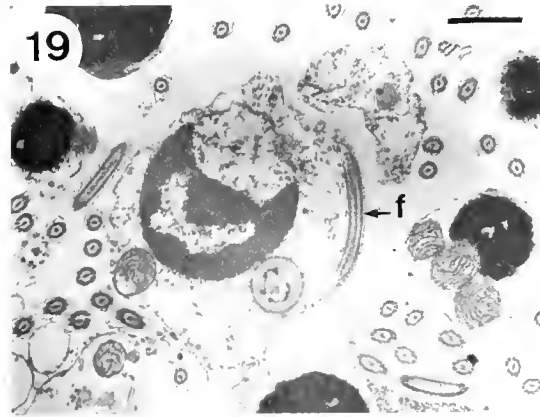
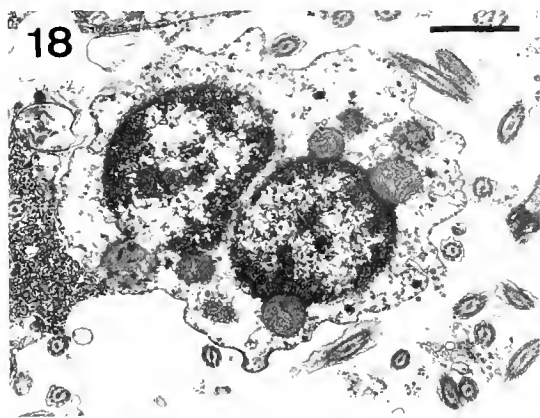
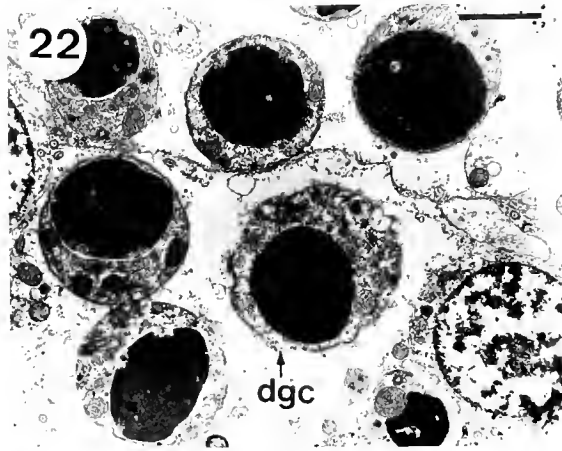
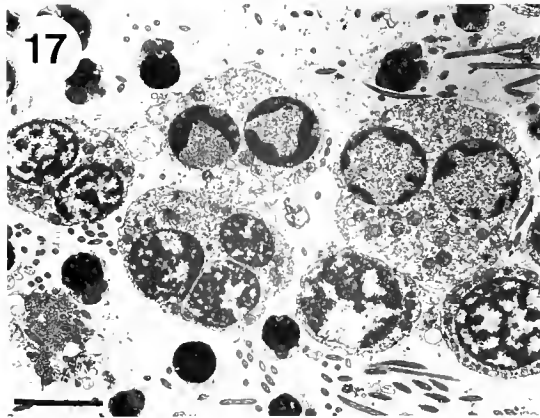
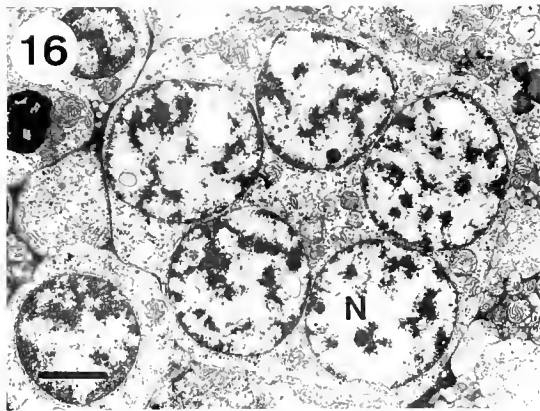




Figure 16. Multinuclear spermatogonium. Nucleus (N). Bar = 2  $\mu$ m.

Figure 17. Multinuclear spermatocytes. Bar = 4  $\mu$ m.

Figure 18. Binuclear spermatid. Bar = 1  $\mu$ m.

Figure 19. Spermatid with intracytoplasmic flagellum (f). Bar = 1  $\mu$ m.

Figure 20. Spermatozoon with intracytoplasmic flagellum. Bar = 500 nm.

Figure 21. Transverse section through a flagellum with two flagellum complexes. Bar = 200 nm.

Figure 22. Degenerating male germinal cells (dgc) showing an hypercondensation of their chromatin. Bar = 2  $\mu$ m.

Figure 23. Atretic spermatozoon. Flagellum (f). Bar = 500 nm.

Figure 24. Macrophage. Nucleus (N). Bar = 2  $\mu$ m.

structures, show that many differences in general morphology and structure are evident between the different species of the same family. Our results are in accordance with earlier studies that suggest the ultrastructure of the sperm can be used for identification purposes, and represents a significant taxonomic and phylogenetic criterion (Franzen 1983; Hodgson and Bernard 1986; Daniels et al. 1971; Popham et al. 1974).

Multinuclear cells are reported during gamete evolution in *Pecten maximus* by Dorange and Le Penneec (1989) and in *Mya arenaria* by Allen et al. (1986). Dorange and Le Penneec (1989) have also observed atypical spermatozoa with intracytoplasmic flagellum. According to Fain-Maurel (1966) and Dohmen (1983), these abnormalities are probably the result of accidental deviations in spermatogenesis, for example with abnormal multiplication of centrioles that can give numerous flagella, rather than the result of a pathological condition. In *Pinctada margaritifera*, degenerating multinuclear cells are frequently observed, but bicephal spermatozoa have never been noted.

In the same way, spermatozoa with intracytoplasmic flagellum are often observed in advanced degenerative stages. These observations lead us to think that abnormal cells are rapidly eliminated

and such degenerating cells might be driven out by the genital orifice.

In the acini, phagocytes have frequently been recorded in bivalves studies (Dorange and Le Penneec 1989; Mathieu 1987). These macrophages are thought to be the result of a differentiation from hemocytes. Such transformation of hemocytes into macrophages has been followed by Houtteville (1974) in *Mytilus edulis*.

With presence of lysosomal inclusions in certain auxiliary cells, also reported in *Pecten maximus* (Dorange and Le Penneec 1989), we can suppose that these cells can also be involved in the resorption of degenerative germinal cells. In the both types of resorption *in situ*, the products of cellular lysis can be recovered by the organism. Recuperation of this material is possible by absorbing cells in the gonoducts or digestive tract (Dorange and Le Penneec 1989).

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## INVESTIGATIONS INTO THE TRANSMISSION OF PARASITES OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LAMARCK, 1819), DURING QUARANTINE INTRODUCTION TO CANADIAN WATERS

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**ABSTRACT** The potential impact of bay scallop *Argopecten irradians* (Lamarck) parasites on commercially important bivalve species in Canadian Atlantic waters was assessed using two transmission experiments. The first was a parallel flow-through system passing water from the bay scallops over five species of native bivalves. The second was a synchronous spawning of infected bay scallops and uninfected blue mussels *Mytilus edulis*, to determine if larval bivalves are more susceptible to parasite transmission than adults. Zoospores of *Perkinsus karlssoni* were observed adhering to D-stage larvae of bay scallops approximately 48 hours post-spawning, suggesting this to be the method of transmission. Surface sterilization of fertilized bay scallop ova with 1% iodophor for 15 minutes failed to destroy the zoospores. No evidence of transmission of bay scallop parasites to adults of other species was found during the ten month experimental period. Results of the second experiment are inconclusive. No *P. karlssoni* zoospores were seen among the larvae, and no tissue-stages have been detected subsequently in the exposed mussels.

**KEY WORDS:** scallop, *Argopecten*, parasites, transmission, quarantine

### INTRODUCTION

The bay scallop *Argopecten irradians* (Lmk), occurs in the shallow tidal lagoons of the northeastern United States but does not occur naturally north of Maine. Due to interest in this species as a candidate for aquaculture, it was introduced to Canada in 1979 when broodstock were held in quarantine on Prince Edward Island (PEI) (Townshend and Worms 1983). Histological examination revealed rickettsial and chlamydial infections (Morrison and Shum 1982, 1983) which were monitored closely over the next 4-6 generations to determine their significance to both the bay scallops and native species. Since the rickettsial and chlamydial infections declined over this period, and native species were found to harbour similar prokaryotes, the F4 generation of bay scallops was released in 1983 for grow-out at specific sites around PEI (Townshend and Worms 1983). The transplanted seed grew well during the summer and autumn but did not survive the winter. Further generations of bay scallops were maintained in low numbers by overwintering broodstock in hatcheries while potential aquaculture sites were evaluated (Mallet and Carver 1987, 1988). In 1987, a commercial enterprise began growing and marketing adult bay scallops, thus stimulating interest in their culture as a cash crop. By 1989, commercial quantities of seed were produced at private hatcheries in Nova Scotia for grow-out in PEI.

In accordance with regional guidelines for introduction and transfer of live aquatic organisms, samples of bay scallop broodstock and seed (2 mm long) were checked in May 1989, prior to transfer to PEI. Nothing of concern was found in the spat, however a previously undescribed apicomplexan parasite, *Perkinsus karlssoni* (McGladdery et al. 1991) was found in the broodstock. Re-examination of histological sections from the original bay scallops introduced in 1979 revealed the same parasite which had been marked by a strong hemocyte encapsulation response. No similar parasite has been observed in native molluscs from Atlantic Canada. This information, together with histological evidence of the

same parasite in bay scallops from Rhode Island (Karlsson 1991), indicated that the parasite had persisted in hatchery bred populations for at least 10 generations.

Since *P. karlssoni* is related to the known oyster pathogen *Perkinsus marinus*, concern was raised about its potential for transfer to native bivalves. An additional observation that seed retained in the hatchery developed *P. karlssoni* infections similar to those in stocks which had been in open water suggested that the infection had been transmitted either in the egg or during the few minutes that newly-spawned gametes were exposed to infected broodstock. No other perkinsiid species has been reported to transmit directly from infected broodstock to their offspring. Transmission in other perkinsiids, where known, is reported as being from moribund hosts to neighbouring hosts, i.e., lateral proximal transmission (Ray and Chandler 1955, Andrews 1965, Goggin et al. 1989). Although individual parasites have been infrequently observed within bay scallop ova (Karlsson 1991) it is unlikely these ova maintain their viability. Infected ova are associated with an extensive hemocyte infiltration, and the parasite occupies a significant proportion of the cell volume.

In April 1989, shortly before the discovery of *P. karlssoni*, staff from the Department of Fisheries & Oceans, Canada, introduced a second bay scallop broodstock from Cape Cod, U.S.A. This introduction was in response to concern that repeated breeding from small numbers of broodstock had resulted in genetic impoverishment (Dr. M. Helm, pers. comm.). Figure 1 outlines the chronology of events in the present study in relation to those reported by McGladdery et al. (1991).

### MATERIALS AND METHODS

Wild bay scallops (n = 123) were harvested in April 1989, at Osterville, Cape Cod, Massachusetts, and transported to the quarantine laboratory at DFO, Halifax, for disease screening in accordance with ICES Guidelines (Turner 1987). Thirty specimens were submitted for bacteriological and virological examination as

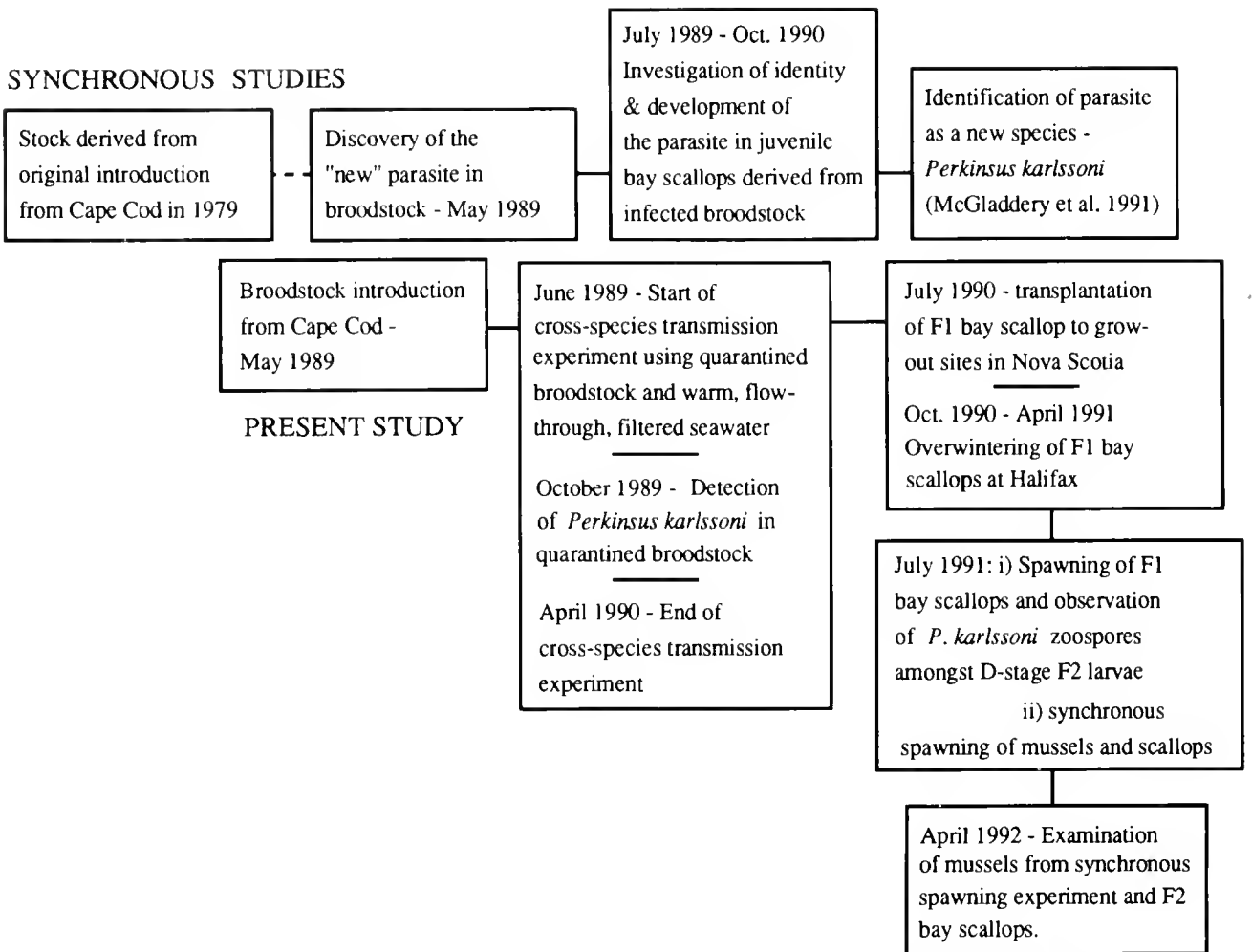


Figure 1. Chronology of events associated with the present bay scallop study and synchronous studies described in McGladdery et al. (1991).

soon as they arrived in Halifax. Tissue samples were also extracted for microscopic examination and thioglycollate culture. The remainder were cleaned of macroscopic fouling organisms and placed in quarantine for conditioning and breeding. Sea water for the quarantine laboratory is drawn from Halifax Harbour at a depth of 20 m, filtered through gravel, sand, and activated charcoal filters, and passed through heat exchangers. Temperatures in the experimental tanks were maintained by blending water from the different temperature lines. Waste water was injected with gaseous chlorine to give a minimum concentration of 3 ppm for 30 minutes.

#### Transmission Experiment #1

In June 1989, the newly introduced bay scallops were placed in wooden, mesh-bottom trays floating in a 1000 litre tank of flowing sea water at 17°C ( $\pm 1^\circ\text{C}$ ). The 1000 litre tank was fitted with air lifts at either end which discharged water into the floating trays to maintain circulation and ensure adequate aeration. A third air lift discharged water from the tank into one of two shallow, 200 litre tanks which held samples of the test species. A second 200 litre tank (the control) was fed seawater at the same temperature directly from the laboratory supply, thereby isolating it from water which had passed through the bay scallop holding tank (Fig. 2). After a

one-week acclimation period and initial histological examination of the stocks being used, 150 eastern (American) oyster (*Crassostrea virginica*), 150 edible (European) oyster (*Ostrea edulis*), 50 soft-shell clams (*Mya arenaria*), 150 mussels (*Mytilus edulis*), and 50 giant sea scallops (*Placopecten magellanicus*) were divided between the two 200 litre tanks. Bivalves in all three tanks were fed cultured strains of *Chaetocerus gracilis* and *Isochrysis galbana*, augmented as required with commercially prepared spray-

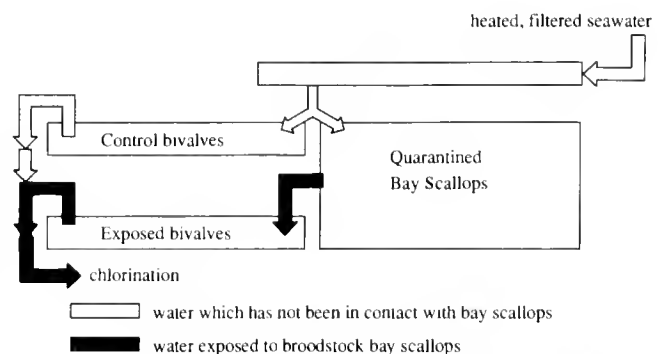


Figure 2. Diagram of the quarantine holding facilities for Transmission Experiment #1.

TABLE 1.  
Collection schedule for Transmission Experiment #1.

Species:	July 10 1989	Aug 10	Sep 14	Oct 26	Nov 30	Jan 16 1990	Feb 23	Apr 04	Aug 15	Total
<i>Osirea edulis</i>	10	10(c) 10(e)	10(c) 10(e)	10(c) 10(e)	5(c) 5(e)	10(c) 10(e)	5(c) 5(e)	10(c) 10(e)	14(e)	144
<i>Crassostrea virginica</i>	10	10(c) 10(e)	10(c) 10(e)	10(c) 10(e)	5(c) 5(e)	10(c) 10(e)	5(c) 5(e)	2(c) 10(e)	3(e)	125
<i>Mytilus edulis</i>	10	10(c) 10(e)	10(c) 10(e)	10(c) 10(e)	5(e)	10(c) 10(e)	5(c) 5(e)	9(c) 10(e)	1(e)	125
<i>Mya arenaria</i>	2	5(c) 5(e)	5(c) 5(e)	—	—	5(c) 4(e)	5(c)	2(c)	—	38
<i>Placopecten magellanicus</i>	5	5(c) 5(e)	5(c) 5(e)	5(c) 5(e)	3(e)	1(c) 1(e)	2(e)	—	—	42
Total	37	80	80	70	28	71	37	53	18	474

Key: (c) = control animals held in water bypassing the bay scallop holding tank; (e) = exposed animals held in a tank fed by effluent water from the bay scallop holding tank.

dried *Tetraselmis* sp. (Cell Systems Ltd<sup>1</sup>). Bay scallops were fed to excess to maintain breeding condition, while the other species were fed a maintenance ration only.

At approximately monthly intervals (Table 1) exposed and control specimens were selected at random, a 3 mm transverse section of the tissues was excised, preserved in modified Davidson's solution (Howard and Smith 1983), paraffin-infiltrated, sectioned and stained with Harris's Hematoxylin and Eosin for light microscopy.

Bay scallops were removed from the tanks, as required for an experimental breeding program, individually heat stimulated, and spawned in a separate spawning facility within the quarantine laboratory. These scallops were subsequently returned to the transmission experiment. On one occasion, water in the bay scallop holding tank rose to 20°C and stimulated a mass spawning. The scallops then required about 4 weeks reconditioning at 17°C before experimental spawning could recommence. Breeding continued throughout the summer. The last spawning was in October 1989, after which tissues from the remaining 10 scallops were excised and processed for histology (Table 2) and thioglycollate culture.

Water flow to the holding tank with the "exposed" experimental animals was switched to a blended laboratory supply at 17°C and sampling continued until the last exposed and control animals were removed for light-microscopical examination in August, 1990.

#### Spawning Observations

Bay scallop broodstock were spawned in quarantine as described above. The resultant F<sub>1</sub> spat were retained in the quarantine system until July 1990 at which time they were examined and transferred to 6 sites around Nova Scotia for growth to market size. In November 1990 they were returned to the Halifax Laboratory. Fifty bay scallops of the F<sub>1</sub> generation were conditioned for spawning in a 1000 litre tank (T = 18°C; S = 30 ppt) for approximately 8 weeks, beginning in February 1991. They were fed cultured algae consisting of *Chaetoceros muelleri*, *Isochrysis galbana* (Tahitian strain), *Thalassiosira* sp., and *Tetraselmis* sp. At week nine, 20 of the broodstock were selected for batch-spawning, producing approximately 10 million eggs. Eggs and sperm were

examined microscopically in order to count the eggs, assess fertilization rate and determine presence or absence of *P. karlssoni* zoospores. Water samples from holding tanks which did not contain bay scallops (control tanks) were also checked for *P. karlssoni*.

A second spawning (March 1991) produced fertilized eggs which were surface-disinfected with a 1% iodophor solution for 15 minutes. They were rinsed and allowed to develop normally. Unfortunately the experiment terminated 10 days post-spawning due to a technical malfunction. A further spawning was induced at the end of July, and the larvae raised and planted out as before. Eggs from this spawning were not surface-sterilized. These F<sub>2</sub> bay scallops were returned to the Halifax Laboratory for overwintering. In April 1992, tissue samples were collected from these bay scallops for histological examination and thioglycollate culture (Table 2).

#### Transmission Experiment #2

In July 1991, mature adult blue mussels, *Mytilus edulis*, were spawned at the same time as bay scallops, and the larvae from both species were reared together. No bay scallop spat survived past metamorphosis, but the mussels thrived, and a sample was examined histologically and using thioglycollate culture in April 1992 (Fig. 1).

## RESULTS

#### Parasites of Bay Scallop Broodstock, 1989

Microscopic examination and tissue culture in thioglycollate medium (Mr J. W. Cornic, pers. comm.) of the bay scallop broodstock on arrival in Halifax from Cape Cod in May 1989 revealed no evidence of perkinsiid infections. Infections by rickettsia-like organisms were observed (Fig. 3, Table 2), but these were not identical to those reported from the specimens originally introduced in 1979 (Morrison and Shum 1982, 1983).

Five bay scallops examined in October 1989 showed extensive tissue lesions identical to those observed in bay scallops examined by McGladdery et al. (1991) and attributed to *Perkinsus karlssoni* (Apicomplexa: Perkinsea). All five scallops were also positive for perkinsiid parasites, using thioglycollate culture of soft-tissue samples. The last 10 bay scallops remaining from the quarantined

<sup>1</sup>No longer available

TABLE 2.  
Parasite prevalence (%) in bay scallops, *Argopecten irradians*, examined during the present study.

Date	May 89	Oct 89	Nov 89	April 90	Nov 90	July 91	Apr 92
Generation	Quarantine Broodstock	Quarantine Broodstock	Quarantine Broodstock	F1 Juveniles	F1 Adults	F1 Broodstock	F2 Adults
Sample Size	30	5	10	12	7	9	30
<i>Perkinsus karlssoni</i>	0%	100%	100%	0%	100%	89%	57%
<i>Pseudoklossia</i> -like coccidian	0%	0%	0%	0%	0%	89%	0%
Gill ciliates	0%	0%	0%	0%	57%	0%	0%
Gill rickettsias	20%	0%	0%	0%	0%	0%	0%
Digestive tubule rickettsias	3.3%	0%	0%	0%	14%	0%	0%

broodstock were collected at the end of November and yielded the same results (Table 2). Since bay scallops normally die shortly after spawning, post spawning mortality could not be attributed conclusively to infection by *P. karlssoni*. Spawning broodstock, despite parasite loads, were apparently healthy when sacrificed for histological examination.

#### Transmission Experiment #1

There was no evidence from either histology or thioglycollate culture of transmission of *P. karlssoni* from the bay scallop to the "exposed" specimens, despite the likelihood that the broodstock

were already infected when introduced in May (even though the parasite was not detected until later). Similarly there was no evidence of transmission to native species of any of the rickettsial or chlamydial organisms observed in the bay scallop broodstock examined in May, 1989 (Tables 3–7). Rickettsia-like inclusions were observed in exposed samples of edible oyster, eastern oyster and giant scallop, however, histologically identical inclusions were also found in samples collected prior to exposure and in controls. All other parasites and prokaryote inclusions observed are commonly found throughout Atlantic Canada in the species examined (McGladdery 1990, McGladdery and Stephenson 1991, Morrison

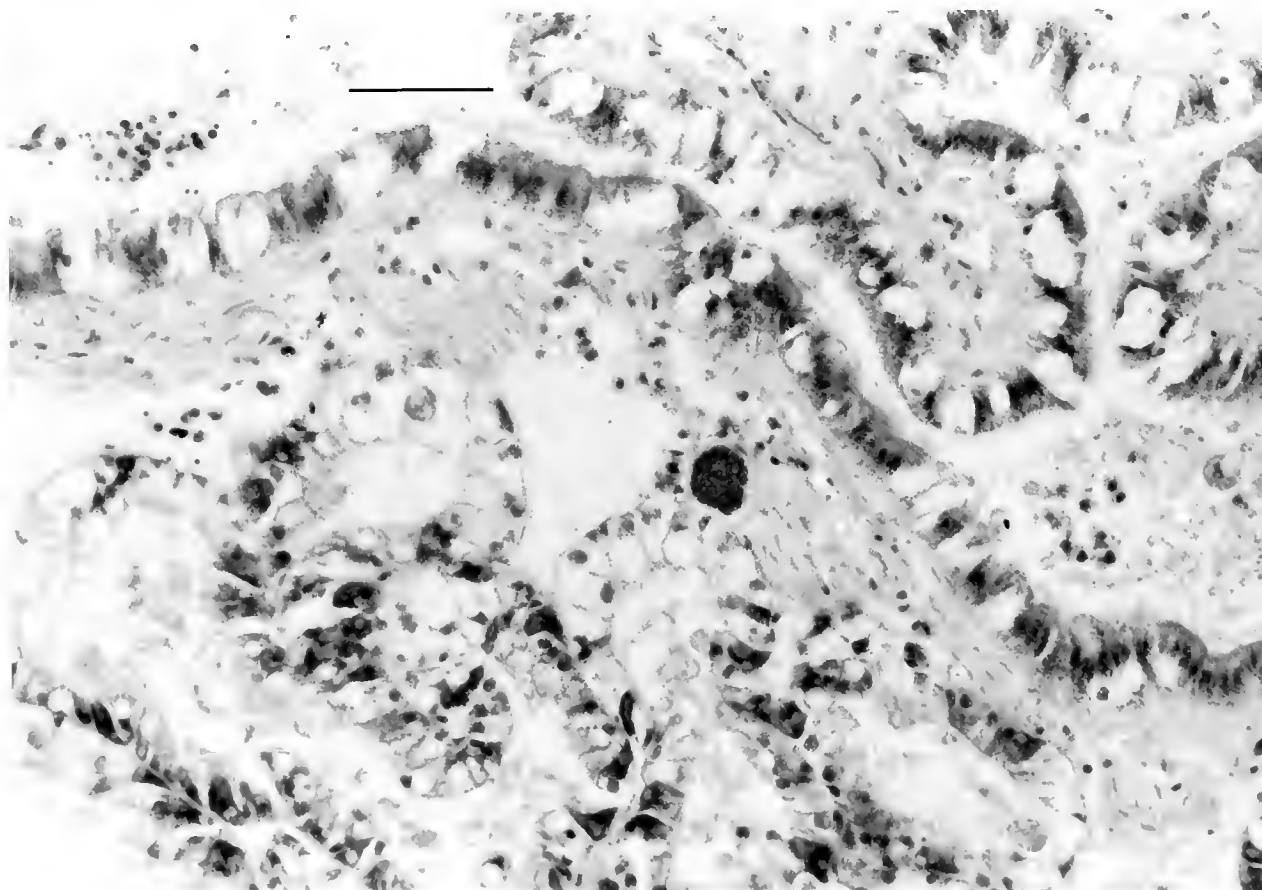


Figure 3. Intracellular *Rickettsia*-like inclusion bodies in quarantine bay scallop, *Argopecten irradians*, from Cape Cod. (Scale bar = 50  $\mu$ m).

TABLE 3.

Histological observations from control blue mussels, *Mytilus edulis*, and blue mussels exposed to effluent water from bay scallops, *Argopecten irradians*.

Observation		July 1989	August	September	October	November	January 1990	February	April	August
<i>Mytilus edulis</i> : Control		Time Zero								
Sample size		10	10	10	10	0	10	5	9	0
Internal Turbellaria	%P	0.0	10.0	0.0	0.0	—	0.0	0.0	0.0	—
	I		1.0							
	A		0.1							
<i>Ancistrum mytili</i>	%P	0.0	0.0	0.0	10.0	—	0.0	0.0	0.0	—
	I				1.0					
	A				0.1					
Sphenophryid-like gill-ciliate	%P	0.0	0.0	0.0	0.0	—	0.0	20.0	0.0	—
	I							1.0		
	A							0.2		
<i>Mytilus edulis</i> : Exposed										
Sample size		—	10	10	10	5	10	5	10	1
Internal Turbellaria	%P	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	I									
	A									
<i>Ancistrum mytili</i>	%P	—	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
	I			1.0						
	A			0.1						
Sphenophryid-like gill-ciliate	%P	—	0.0	0.0	20.0	0.0	0.0	0.0	0.0	0.0
	I				1.0					
	A				0.2					

Key: %P = prevalence; I = intensity (mean number of parasites per tissue section of infected individuals) and A = abundance (mean number of parasites per tissue section for all individuals in a sample).

and Shum 1983) and show no correlation with areas used to culture bay scallops since 1982.

A decrease in prevalence (percentage of histological sections containing evidence of infection) of rickettsial-like inclusions in bay scallops, edible oysters and giant sea scallops was observed within two months of being placed into the quarantine facility. This may be due to the relatively small numbers examined or, possibly, reflect a trend similar to that observed during the 1979 introduction of bay scallops, where the number of rickettsia-like lesions declined over time (Townshend and Worms 1983). Eastern oysters showed no distinct decline in similar lesions, with a 10%

prevalence being observed in the last sample examined in April 1990. Although mass mortality of bay scallops has been attributed to infection by these prokaryotes (Leibovitz 1989), no pathology was associated with any of the infections listed in Tables 3–7. Moreover, nearly all specimens showed evidence of feeding prior to being collected.

*Bay Scallop Spawning Observations*

Prior to spawning the F<sub>1</sub> generation bay scallops, a sample of 9 was examined in July, 1991. All showed extensive *P. karlssoni*

TABLE 4.

Histological observations from control edible oysters, *Ostrea edulis*, and edible oysters exposed to effluent water from bay scallops, *Argopecten irradians*.

Observation		July 1989	August	September	October	November	January 1990	February	April	August
<i>Ostrea edulis</i> : Control		Time Zero								
Sample size		10	10	10	10	5	10	5	10	0
Rickettsia-like inclusions	%P	30.0	20.0	20.0	0.0	0.0	0.0	0.0	0.0	—
Gymnophallid-like metacercaria	%P	0.0	0.0	0.0	0.0	0.0	0.0	20.0	0.0	—
	I							1.0		
	A							0.2		
<i>Ostrea edulis</i> : Exposed										
Sample size		—	10	10	10	5	10	5	10	14
Rickettsia-like inclusions	%P	—	10.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0
Gymnophallid-like metacercaria	%P	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	I									
	A									

Key: %P = prevalence; I = intensity (mean number of parasites per tissue section of infected individuals) and A = abundance (mean number of parasites per tissue section for all individuals in a sample).

TABLE 5.

Histological observations from control eastern oysters, *Crassostrea virginica*, and eastern oysters exposed to effluent water from bay scallops, *Argopecten irradians*.

Observation		July 1989	August	September	October	November	January 1990	February	April	August
Control (Sample size)		10	10	10	10	5	10	5	2	0
Rickettsia-like inclusions	%P	10.0	10.0	0.0	0.0	20.0	0.0	0.0	0.0	—
Internal Turbellaria	%P	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	I	1.0								
	A	0.1								
<i>Ancistrocoma</i> -like	%P	10.0	10.0	20.0	10.0	40.0	0.0	10.0	0.0	—
digestive gland ciliate	I	1.0	1.0	1.0	6.0	2.5		61.0		
	A	0.1	0.1	0.2	0.6	1.0		12.2		
Sphenophryid-like	%P	10.0	10.0	20.0	10.0	20.0	50.0	80.0	0.0	—
gill-ciliate	I	3.0	2.0	1.0	2.0	9.0	3.8	7.0		
	A	0.3	0.2	0.1	0.2	0.45	1.9	5.6		
Exposed (Sample size)		—	10	10	10	5	10	5	10	3
Rickettsia-like inclusions	%P	—	0.0	20.0	0.0	0.0	0.0	0.0	10.0	0.0
Internal Turbellaria	%P	—	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	I		1.0							
	A		0.2							
<i>Ancistrocoma</i> -like	%P	—	10.0	40.0	10.0	20.0	0.0	10.0	20.0	0.0
digestive gland ciliate	I		14.0	1.75	1.0	1.0		5.0	5.5	
	A		1.4	0.7	0.1	0.2		1.0	1.1	
Sphenophryid-like	%P	—	0.0	10.0	10.0	20.0	60.0	80.0	60.0	66.7
gill-ciliate	I			1.0	1.0	1.0	6.0	18.7	17.3	41.0
	A			0.1	0.1	0.2	3.6	15.0	10.4	27.3

Key: %P = prevalence; I = intensity (mean number of parasites per tissue section of infected individuals) and A = abundance (mean number of parasites per tissue section for all individuals in a sample).

lesions (Fig. 4, Table 2) as well as heavy kidney infection by a *Pseudoklossia*-like coccidian (Fig. 5, Table 2). *Pseudoklossia*-like coccidians have been found in bay scallops descended from the original introduction (McGladdery 1990), but this was the first observation of this parasite in the progeny from the 1989 introduction. Following spawning of the F<sub>1</sub> broodstock, zoospores of *P. karlssoni* were detected among the F<sub>2</sub> larvae (Figs. 6, 7).

Surface sterilization of fertilized bay scallop eggs with 1% iodophor appeared to have no effect on the parasite: free swimming zoospores were observed among, and possibly attached to the surface of healthy D-stage larvae 48 hours post treatment, and subsequently during larval development. Unfortunately problems with the heating system caused the loss of these larvae after 10 days, but development to that time had been normal.

No zoospores were seen among the larvae from the final breeding trial in late July, 1991, suggesting that they might be parasite-

free. These F<sub>2</sub> spat were outplanted in late August. In November 1991 stock from this outplanting were returned to the Halifax Laboratory where they were maintained overwinter. Tissue samples collected in April 1992 from these bay scallops were infected with *P. karlssoni* (Table 2).

#### Transmission Experiment 2

Results of the mussel-scallop larval rearing trial are equivocal. The bay scallop larvae did not survive past metamorphosis in the same tanks with the mussels, and no *P. karlssoni* zoospores were detected among the growing mussel larvae. The mussels were examined histologically in April 1992 following grow-out in open water. There was no evidence of transmission of *P. karlssoni* or *Pseudoklossia* to the mussels. Thioglycollate culture of tissues from the same mussels was negative for perkinsiid protozoans.

TABLE 6.

Histological observations from control giant sea scallops, *Placopecten magellanicus*, and giant sea scallops exposed to effluent water from bay scallops, *Argopecten irradians*.

Observation		July 1989	August	September	October	November	January 1990	February	April
<i>P. magellanicus</i> : Control		Time Zero							
Sample size		5	5	5	5	—	1	—	—
Rickettsia-like inclusions	%P	80.0	20.0	40.0	0.0	—	0.0	—	—
<i>P. magellanicus</i> : Exposed									
Sample size		—	5	5	5	3	1	2	—
Rickettsia-like inclusions	%P	—	60.0	20.0	0.0	0.0	0.0	0.0	—

Key: %P = prevalence; I = intensity (mean number of parasites per tissue section of infected individuals) and A = abundance (mean number of parasites per tissue section for all individuals in a sample)



TABLE 7.

Histological observations from control soft-shell clams, *Mya arenaria*, and soft-shell clams exposed to effluent water from bay scallops, *Argopecten irradians*.

Observation	July	August	September	October	November	January	February	April
<i>Mya arenaria</i> : Control	Time Zero							
Sample size	2	5	5	—	—	5	5	2
Gymnophallid-like metacercaria %P	0.0	0.0	0.0	—	—	0.0	20.0	0.0
I							2.0	
A							0.4	
<i>Mya arenaria</i> : Exposed	—	5	5	—	—	4	—	—
Gymnophallid-like metacercaria %P	—	0.0	0.0	—	—	0.0	—	—
A								

Key: %P = prevalence; I = intensity (mean number of parasites per tissue section of infected individuals) and A = abundance (mean number of parasites per tissue section for all individuals in a sample).

### DISCUSSION

Histological examination and thioglycollate culture of bay scallop broodstock introduced in May 1989 revealed no sign of *P. karlssoni* or *Pseudoklossia*-like protozoans (Table 2), however, rickettsia-like organisms were found which initiated cross-species transmission studies. Five months later (October 1989), a second sample of the quarantined bay scallop broodstock revealed 100% prevalence of *P. karlssoni*. This reinforces previous reports (McGladdery et al. 1991) stating that certain stages of *P. karlssoni*

may not be detected by routine histology or thioglycollate culture. This cryptic period can be shortened at elevated water temperatures (17–20°C), but no attempt was made to increase the temperature above 17°C during the transmission experiment. Conditions may, therefore, have been inadequate for transmitted *P. karlssoni* to develop in the native species to a detectable stage by the end of the ten month experiment. It is known that *P. marinus* can escape routine detection in eastern oyster, *Crassostrea virginica*, for a full year (Ray 1954, Andrews 1965). Experiment 1 was not repeated since a longer (two year) experiment commenced in November 1990 at

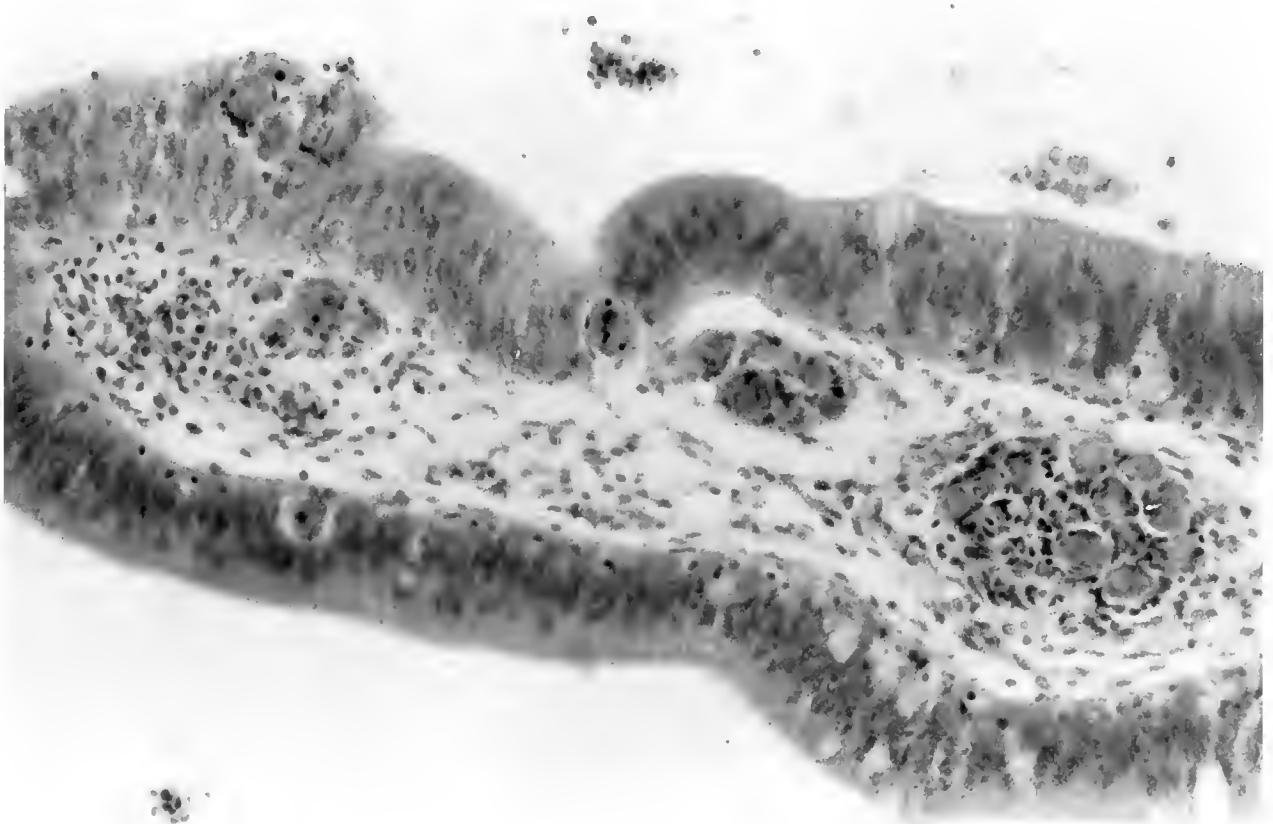


Figure 4. Tissue lesions containing *Perkinsus karlssoni* in quarantine bay scallop, *Argopecten irradians*, from Cape Cod. (Scale bar = 50  $\mu$ m).

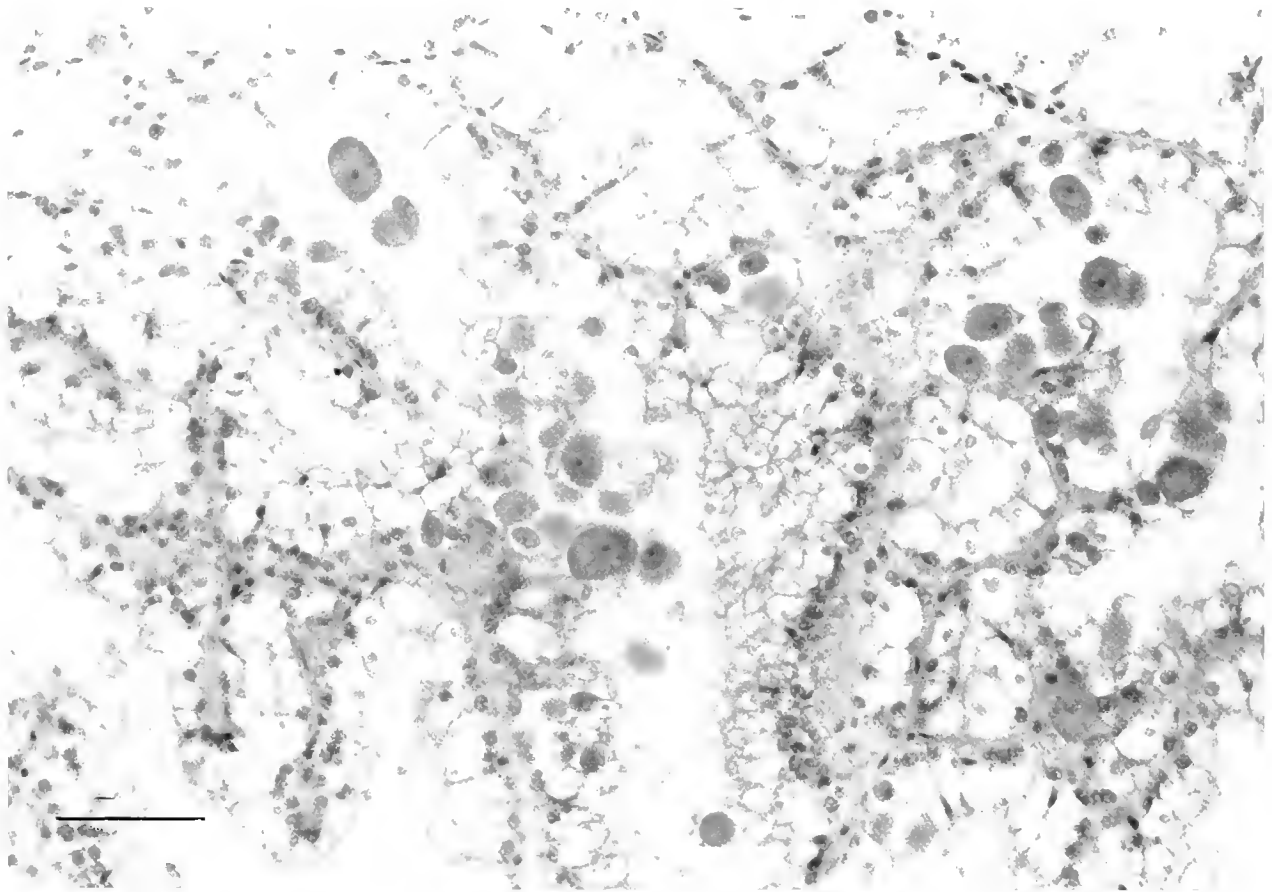


Figure 5. Kidney coccidia (*Pseudoklossia* sp.) in F<sub>1</sub> generation bay scallops, *Argopecten irradians*. (Scale bar = 50  $\mu$ m).

the Atlantic Veterinary College (AVC), in which bay scallops will be held in a closed-circulation system and in the same tanks as several native species (Dr. R. J. Cawthorn, pers. com.).

McGladdery et al. (1991) suggested that slight variations in

epidemiology and histological appearance of *P. karlssoni*, compared to other perkinsiid species, may have been due to ten years of transmission via hatchery-manipulated spawning. The lesions in quarantined broodstock imported directly from Cape Cod (Fig. 3),

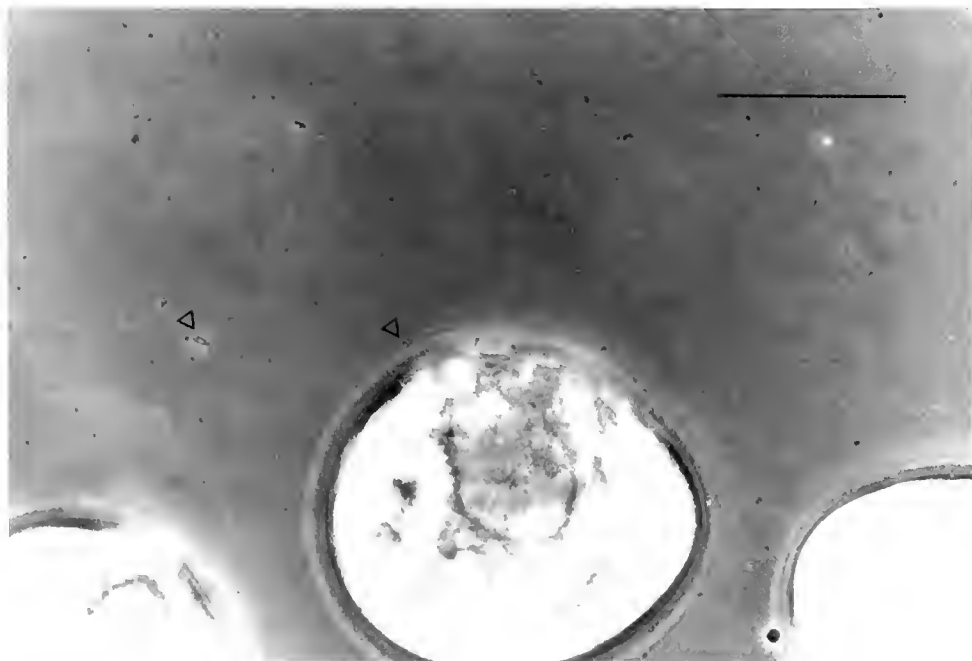


Figure 6. *Perkinsus karlssoni* zoospore attached to surface of D-stage bay scallop larva.

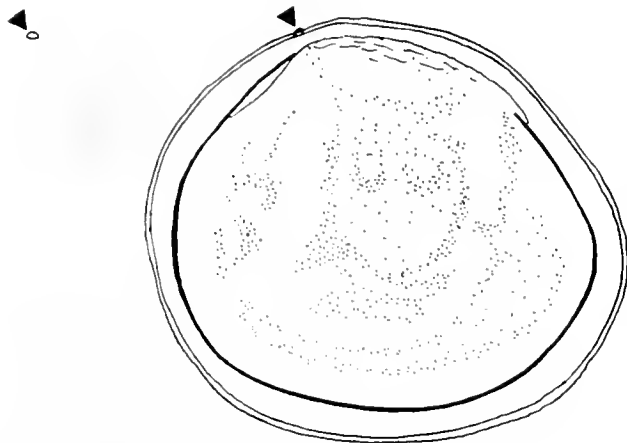


Figure 7. Diagrammatic representation of *Perkinsus karlssoni* zoospore attached to surface of D-stage bay scallop larva.

however, were identical to those observed in hatchery-bred stock. Moreover, *Perkinsus* infected material generously provided by Karlsson (pers. comm.) from Rhode Island bay scallops is indistinguishable from specimens collected from Canadian bay scallops descended from either the 1979 or 1989 introductions.

Precautions taken during spawning in 1989 to minimize the likelihood of transmitting parasites from broodstock bay scallops to their offspring involved removal of the adults from the dishes containing their spawn and the subsequent raising of the fertilized eggs in a separate section of the quarantine facility. Clearly, however, transmission of *P. karlssoni* to the  $F_1$  generation still occurred. The observation of zoospores among healthy D-stage larvae, reinforces the suggestion of McGladdery et al. (1991) that persistence of the parasite in the previously introduced stock was due to exposure of offspring to infected adults during breeding. The possibility of transmission via infected ova is discussed below. The question of the timing of transmission and its linkage to spawning is especially important for bay scallop culture in Canadian waters, where spawning is confined to hatcheries. The fact that bay scallops are spawned separately from other bivalve species within these hatcheries may reduce the impact of this parasite on native species if transmission is found to be possible.

The lower proportion of parents having the parasite during the early part of the spawning season suggests that there may be some lateral transmission between adult scallops. Alternatively, the parasite may persist in low numbers until the bay scallop grows and only proliferates to a detectable level with the maturation of the gonad. Under artificial conditions the parasite was observed in the tissues of immature scallops (<20 mm shell height) (McGladdery et al. 1991), however, no such development has been observed in bay scallops growing in ambient Canadian waters.

The question of transmission of bay scallop parasites to native bivalve species and the timing of such transmission is at least partially answered. Experiment 1 showed no evidence of transmission to other species held directly downstream of infective bay scallops. Scallop which spawned, accidentally, upstream from "exposed" native species, as well as moribund and dead scallops, were left in situ between June and October in order to enhance any potential for cross-species transmission (Ray 1954, Andrews 1965). Progeny from induced spawning were subsequently shown to be infected indicating that within-species transmission had taken place.

Initial observations of *P. karlssoni* zoospores adhering to holding-dish surfaces and demonstrating negative buoyancy suggested

that the flow-through system might not have been optimal for testing the transmission potential of this parasite. Subsequent detection of zoospores throughout the water column in tanks used for conditioning  $F_1$  broodstock, however, demonstrated that zoospores could be carried from tank to tank, with the upwelling system helping maintain the zoospores in suspension. The "exposed" bivalve species showed no evidence of transmission of any of the bay scallop parasites during the nine months of the experiment (July 1989 to April 1990). The additional 14 edible oysters, 3 eastern oysters and one blue mussel, maintained for a further 4 months, also showed no sign of infection attributable to exposure to the bay scallops.

Breeding in quarantine failed to prevent transmission of the *Pseudoklossia*-like coccidian from one generation to the next, although this has evoked less concern than the persistence of *P. karlssoni*, due to the widely-held belief that most *Pseudoklossia* species are non-pathogenic (see review by Lauckner 1983). Recently, however, Cawthorn et al. (1991) reported a mass mortality of experimentally held bay scallops caused by an unusually heavy infection of the same *Pseudoklossia*-like parasite. The appearance of this coccidian in  $F_1$  bay scallops reinforces the question of the efficacy of sub-sampling quarantined broodstock and breeding in quarantine as methods for preventing parasite introduction. These *Pseudoklossia*-like parasites are commonly found in bay scallops from the eastern US (Getchell 1991, Karlsson 1991) but have also been reported from bay scallop descendants of the original (1979) Canadian introduction (McGladdery 1990). No similar coccidians have been reported from native bivalve species.

Regardless of the pathogenicity of these parasites, there is a need to reassess disease screening protocols and techniques, especially for introduction of species for which little base-line information is available. For example, the 1979 introduction of bay scallops into Canadian waters preceded publication of reviews of parasites and diseases of scallops (including bay scallop) (Leibovitz et al. 1984, Getchell 1991, Karlsson 1991). Moreover, the assumption of disease-free status of  $F_1$  generations produced from broodstock found to be "parasite-free" by current diagnostic techniques may be erroneous, at least for certain bivalve parasites.

Since the  $F_1$  broodstock were found to be infected by two different species of protozoan, the biflagellate zoospores found among the  $F_2$  generation spat were examined carefully and compared to samples from bay scallops which had shown no evidence of the *Pseudoklossia*-like infection (McGladdery, et al. 1991). The zoospores were identical, and no other zoospores or oocyst-like stages were detected among the 8-month old juveniles. The biflagellate zoospores observed were, therefore, assumed to be exclusively of *P. karlssoni*. Observation of the zoospores but not the zoosporangia of *P. karlssoni* among the  $F_2$  spat leaves the precise mechanism of infection open to speculation. The zoosporangia of *P. marinus* and *P. atlanticus* develop within the host tissue, and it is from these that the motile zoospores are released (Perkins 1976, Azevedo 1989). Bay scallops spawned in Canadian hatcheries are only in contact with their spawn for up to 4 hours, indicating that zoospore release may occur during that period. Alternatively, the zoosporangia may be released from infected broodstock tissues and the zoospores emerge later. Histological sections of the infected  $F_1$  broodstock showed a marked localization of *P. karlssoni* around the mantle margin and other surface epithelia. Some of the mantle lesions appear to open to the outside of the scallop, but there was no evidence of zoospore release (Figure 4). Karlsson (1991) observed *P. karlssoni* inside individual ova. A large proportion of the egg volume was displaced by the protozoan which

casts doubt on the viability of infected ova and trans-ovarian transmission. No infected ova were observed in the present study. Additional evidence for extra-cellular transmission is that the infective stages of all *Perkinsus* species described to date are motile, biflagellate zoospores (Perkins 1976, Azevedo 1989, Goggin et al. 1989).

The possibility that bivalve larvae may be the most susceptible age group for *P. karlssoni* transmission was tested, based on the observation from this experiment that motile, adhesive, zoospores are present among D-stage larvae. Synchronous spawning of bay scallops and blue mussels, however, revealed no evidence of cross-species infection. Scallops from the same broodstock grown under similar conditions were infected. Cross-species transmission has been demonstrated experimentally for other species of *Perkinsus* (Goggin et al. 1989), although host-specificity appears to be the rule in the wild (Ray 1954).

The ability of *P. karlssoni* zoospores to survive the 15 minute 1% iodophor treatment may have been achieved by avoiding exposure inside a zoosporangial stage, or it may indicate that the zoospores themselves are highly resistant. Tissue stages of other

species of *Perkinsus* have been reported to withstand 6 ppm chlorine treatment for up to two hours, although free prezoosporangia (stage prior to expansion into the zoospore-containing zoosporangium) lasted less than 30 minutes in the same treatment (Goggin et al. 1990). Surface sterilization using this concentration of chlorine would kill the bay scallop ova. Investigation of alternative treatments is required.

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## GAMETOGENIC CYCLE OF THE CHILOE SCALLOP (*CHLAMYS AMANDI*)

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**ABSTRACT** The gametogenic cycle of the Chiloé scallop *Chlamys amandi* from Hueihue Bay, Chiloé Chile, was examined for one year. Chiloé scallops were collected at 4-5 week intervals between October, 1989 and December, 1990. Histological sections of the gonad were prepared and the gonadal index was determined. A semiannual spawning cycle was observed; i.e., scallops were in an active or ripe stage throughout most of the year. Spawning and spat were observed in conjunction with high food availability.

**KEY WORDS:** *Mollusca*, *Bivalvia*, *Pectinidae*, reproduction histology

### INTRODUCTION

Reproductive cycles of marine bivalves are comprised of a gametogenic phase, spawning, larval development and growth. The cycle may be annual, semiannual or continuous, depending upon the species and location (Sastry 1979). Well documented patterns of energy storage and utilization are often associated with these cycles, although the role of endogenous and exogenous factors and their interactions in the synchronization of gamete development and release within a population are still not fully understood (Bayne 1976, Sastry 1979, Mac Donald and Thompson, 1986, Barber and Blake 1991).

Several environmental factors may influence the timing of reproduction in bivalve molluscs. The most commonly cited are water temperature, food availability and tidal influence (Sastry 1966, Machell and De Martini 1971). Many authors have attempted to explain reproductive timing in bivalves primarily in terms of water temperature and its variation with latitude (Loosanoff 1937, Ropes and Stickney 1965, Newell et al. 1982, Sastry 1966, 1970, Malachowsky 1988).

The gametic production in several species of marine bivalves requires a great deal of energy suggesting a close relationship between the reproductive cycle and energy available for growth (Bayne 1985, Mac Donald and Thompson 1986). Time of spawning may also be related to food availability. Most bivalves tend to spawn during periods when food is available for developing progeny and for replenishing the energy adults spend in spawning (Bayne 1976). It is possible therefore that temporal and quantitative differences in the food supply have a greater influence on the reproductive cycle than water temperature or latitude (Emmett et al. 1987).

For restocking or mariculture purposes it is important to know the life cycle of the target species, and documentation of the reproductive cycle is one logical step in determining when recruitment might occur. The Chiloé's scallop (*Chlamys amandi*) is an unexploited, but potentially valuable resource. However, to date no research has been conducted on the biology or life history of this species. The present paper is a brief study describing the

gametogenic cycle and its relationship to temperature, salinity and food availability.

### MATERIALS AND METHODS

#### *Environmental Parameters and Phytoplankton Analysis*

Temperature and salinity were measured monthly at the culture site (20 m in depth) Hueihue Bay (41°54'S: 73°31'W) in Chiloé Island, Chile (Fig. 1) using a YSI Model 33 SCT meter. For phytoplankton, samples were taken with a net having a Kitahara mesh size of 100 µm. Phytoplankton were fixed in 5% formalin and subsequently decanted. Decanted phytoplankton volume was measured in a graduated test-tube (0.1 ml accuracy) and considered as an indirect index of primary productivity.

#### *Gonad Index Analysis*

Monthly samples of 28 to 30 gonads were used for determination of Gonad Index (GI), prior to fixation. At this sample size, the standard error of the mean Gonad Index remained below 4%, which was regarded as highly precise (see Table 3). The following relationship was used to determine the Gonadal Index

$$GI = \frac{(\text{fresh wet weight of gonad})}{(\text{fresh wet weight of soft parts})} \times 100$$

The high average values of GI are coincident with gonadal maturity. Minimal average values following high average values are considered as indicating of spawning (Akaboshi and Illanes 1983).

#### *Histological Procedures*

The gonads were removed from 440 scallops at monthly intervals from October 1989 to November 1990. Samples ranged from 28 to 30 individuals. Shell height ranged from 3.8 to 5.2 cm and shell length ranged from 4.5 to 6.0 cm. Scallops were cultured in both lantern nets and pearl-nets suspended at 15 to 20 m depth in Hueihue Bay.

Gonadal tissue was fixed in Hollande Bouin (picric-formol-

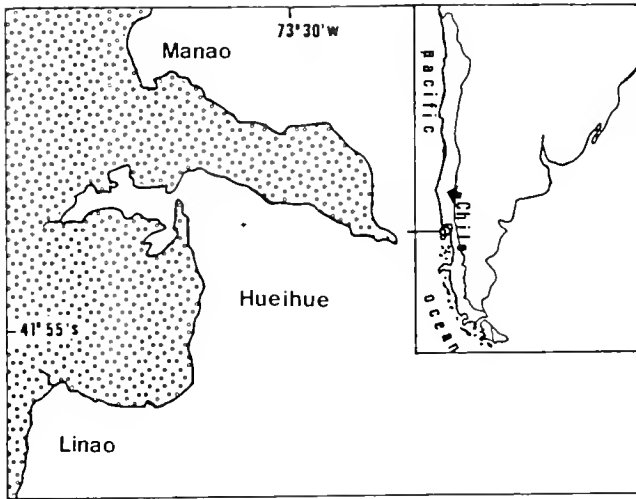


Figure 1. Map showing the culture site at Hueihue Bay in Chiloé Island Chile.

acetic plus cupric II acetate mixture) (Ganter and Jolles 1970) for 24 hours. The samples were then dehydrated using a series of decreasing ethanol solutions. The embedded tissue was sectioned at 5  $\mu$ m to 7  $\mu$ m and placed on slides. Tissue was processed using a series of increasing ethanol solutions and sections were stained with hematoxylin-eosin (Humanson 1962).

Gonadal tissue was qualitatively examined following the schemes of Ropes and Stickney (1965), Aviléz y Lozada (1975), Ramorino (1975) and Malachowsky (1988) to assess developmental stage.

The definition of each stage (active, ripe and spent) is stated in Table 1. Examples of each stage are pictured in Figs 1a, 1b, 1c, 2a, 2b, 2c. Photomicrographs were taken with Standard Leitz and Nikon microscopes.

## RESULTS

The Chiloé scallop *Chlamys amandi* is a gonochoristic species, commonly found inhabiting gravel or sand bottoms and scattered in small beds along the coast in depths of 15 to 30 m. This species is found exclusively in the South of Chile (Chiloé Island).

*Environmental Parameters and Phytoplankton* On Hueihue

Bay water temperature at 20 m depth ranged from 11.6°C in October 1989 to a maximum of 13.9°C in February 1990. A high temperature period of over 13°C was recorded during the summer months (January to March (1990)). Temperature during all other months was about 11°C (see Table 2).

Salinity measured (20 m depth) in Hueihue Bay fluctuated between 32 and 33.5 ppm throughout the study period (see Table 2).

The phytoplankton data obtained during this study are presented in Fig. 3. Densities (measured in volume) recorded during this study are presented in Table 2 and considered as an Indirect primary productivity index.

A seasonal fluctuation in phytoplankton was recorded during summer months (December 1989 to March 1990), for Autumn and Winter months (April to September 1990) relatively low values were observed but for October, newly values were increasing.

*Gonad Index* The mean gonad index (GI) values for the study period are presented in Table 3 and Fig. 4. Major peaks were observed in October 1989, and January, and August 1990. All these peaks were followed by decreases in GI, representative of spawnings events.

*Gametogenic Cycle* The Chiloé scallop showed a semiannual gametogenic cycle summarized in figs 5a, 5b, 5c. Scallops were either in an active (Fig. 5a), ripe (Fig. 5b) or spent phase (Fig. 5c) throughout the year. Microscopic observations of male and female gametic conditions revealed a tendency to maintain an active regeneration of the gametes during the year. Active males were found from October (1989) to December (1989), February to June (1990) and November to December (1990), in female scallops active stage was observed in all months sampled with exception of September 1990.

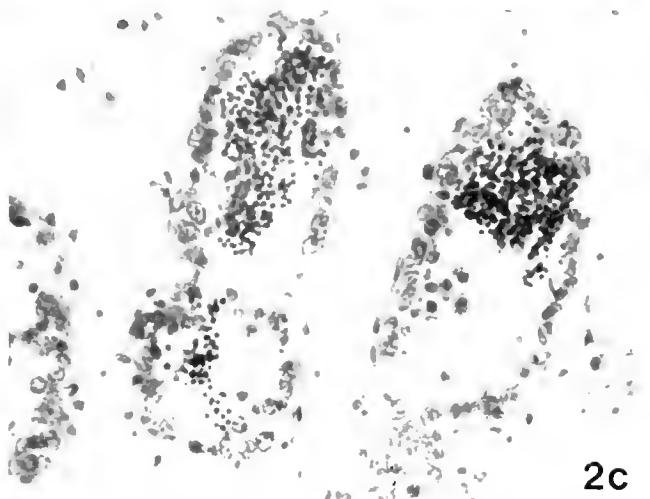
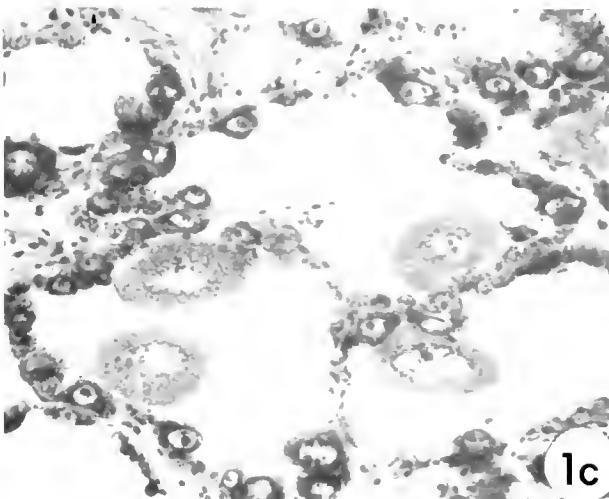
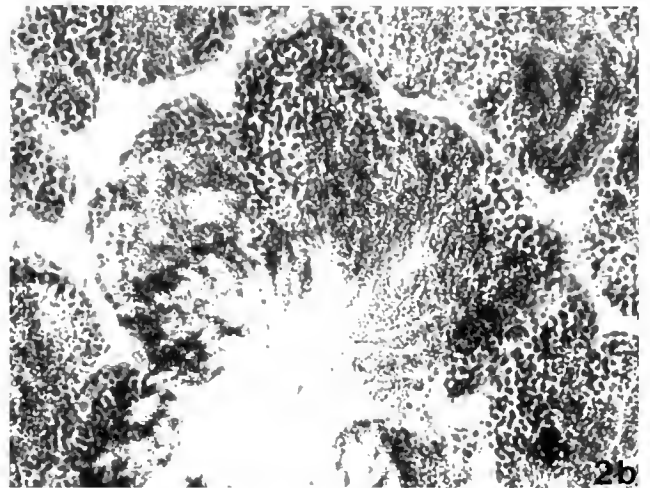
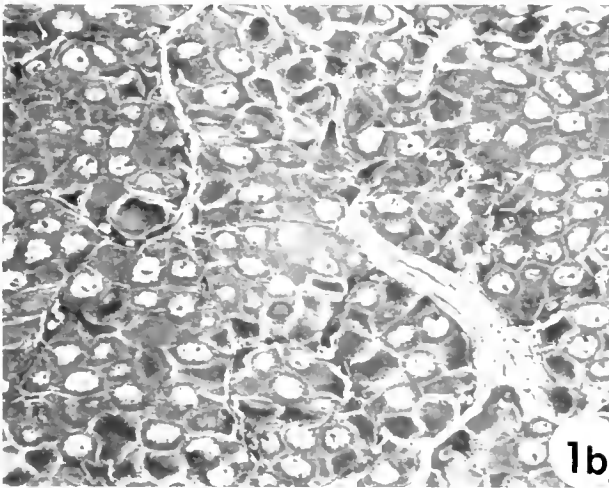
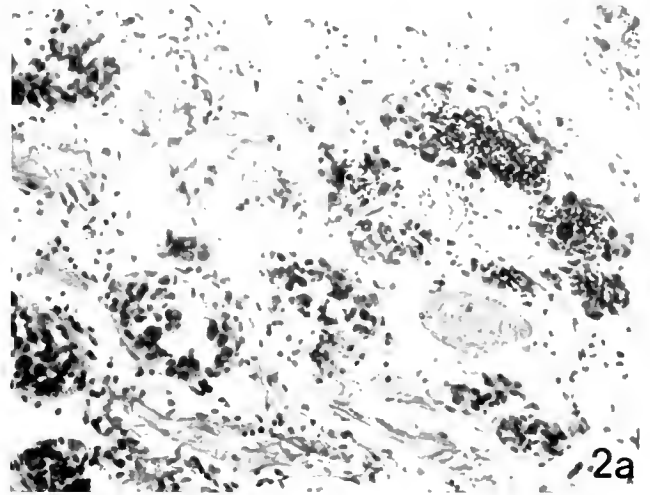
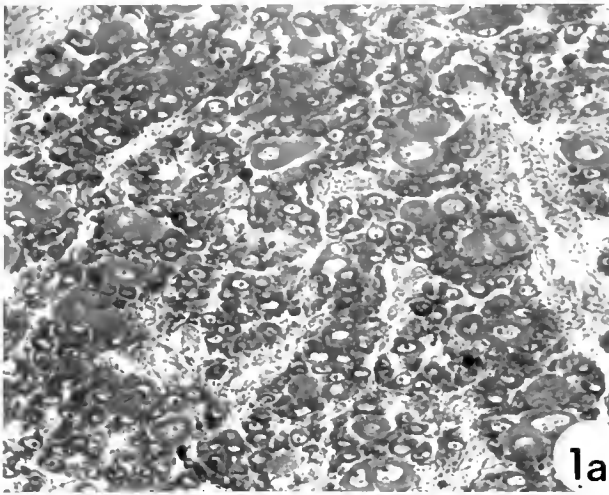
Ripe individuals of both sexes were found throughout the year. However ripe females were most numerous in February, June and September (1990). Meanwhile the lowest values of ripe female during the sampled period were seen during November–December 1989 and February 1990.

Spent stage was recorded in female during November–December 1989, May, September and November–December 1990. Spent male were recorded in both November–December 1989 and November–December 1990. That is four resting periods for females, including two short resting phases during May and

TABLE 1.  
Principal histological characteristics of different gonadal maturity stages.

Female	Male
<u>Active</u> (Fig. 1A) The active phase is characterized by the presence of ova in all stages of development, from oogonia on the follicle wall to stalked oocytes characterized by a large nucleus. Some fully developed oocytes are also free in the lumen.	(Fig. 2A) Stem cells, spermatogonia, spermatocytes, spermatids and a few spermatozoa are present extending from the follicle wall to the center of the lumen.
<u>Ripe</u> (Fig. 1B) The ripe ovary exhibits distended follicles with detached mature oocytes, their cytoplasm contains large amounts of yolk platelets of different sizes. Only a few stalked oocytes remain.	(Fig. 2B) The follicles are distended, the lumen is filled with mature spermatozoa. Spermatogonia, spermatocytes and spermatids are found on the follicle wall.
<u>Spent</u> (Fig. 1C) The follicles are empty except for developing oogonia lining the walls. Some follicles show free oocytes in the lumen.	(Fig. 2C) The follicles are collapsed or decreased in size. A few follicles contain a small amount of unspent spermatozoa in the lumen. Spermatogonia and spermatocytes are found on the follicle wall.





Photomicrographs of gonadal sections of female and male *Chlamys amandi* Fig. 1A. active female (80 $\times$ ), 1B. ripe female (80 $\times$ ), 1C. spent female (200 $\times$ ), 2A. active male (200 $\times$ ), 2B. ripe female (320 $\times$ ), 2C. spent (320 $\times$ ), female.

September 1990 and two notorious during November–December 1989 and 1990. Male appears with seasonal resting period during November–December each year (1989 and 1990).

Developing oogonia were present in the follicular walls of females in the spent phase indicating that redevelopment was occur-

ring after spawning (Fig. 1c). Males in the spent phase usually retained a few spermatozoa in a small number of seminal tubules (Fig. 2c).

*Larvae and spat* It is possible that the Chilóe scallop may spawn many times throughout the year in Hueihue Bay. At least

TABLE 2.

Month		Temperature °C	S.D. ±	Phytoplankton cm <sup>3</sup> /m <sup>3</sup>	S.D. ±	Safinity ppm	S.D. ±
October	1989	11.16	0.61	4.86	1.85	32.78	0.55
November	1989	11.33	1.02	6.67	2.35	33.40	0.26
December	1989	11.17	0.32	2.10	0.90	33.83	0.42
January	1990	13.28	1.64	5.13	4.57	33.18	0.66
February	1990	13.90	1.47	1.96	0.81	32.90	0.44
March	1990	13.50	0.54	2.43	0.98	32.73	0.31
April	1990	11.90	0.12	2.00	0.10	33.05	0.29
May	1990	11.70	0.22	0.34	0.26	33.22	0.37
June	1990	11.15	0.31	0.30	0.14	33.03	0.13
July	1990	10.66	0.29	0.56	0.26	32.92	0.22
August	1990	10.65	0.10	1.00	0.86	33.03	0.33
September	1990	10.50	0.16	4.80	2.55	32.83	0.30
October	1990	10.47	0.21	0.20	0.08	32.73	0.32
November	1990	11.15	0.34	0.55	0.45	32.87	0.34
December	1990	12.22	0.32	4.40	1.10	33.12	0.32

Temperature and Phytoplankton values (included Standard Deviation) recorded for the study period at Hueihue Bay, Chiloé, Chile.

four times are suggested by the recorded resting periods. Veliger larvae of the Chiloé scallop were found in plankton samples in November 1989, January, February, September and December 1990. Settlement may be dependent on certain environmental conditions (food, temperature). Spat settled on onion-bags collectors were found in late November 1989, and late February and September (1990), and early December.

#### DISCUSSION

The histological changes in the gonadal cycle of female Chiloé scallops (*Chlamys amandi*) collected from October 1989 to December 1990 showed that maturity and spawning were occurring in a semiannual pattern. Males were more or less continually spawning throughout the year. Similar observations have been described by Borden (1928), Naidu (1970) and Du Paul et al. (1989) for *Placopecten magellanicus*.

In bivalves the reproductive cycle is generally closely linked to several environmental factors. The most commonly cited are water temperature, food availability, tidal influence and depth (Sastry 1966, Machell and DeMartini 1971, MacDonald and Thompson 1985a).

In Hueihue Bay the water temperature was highly constant during several months of the study period, but there was a marked

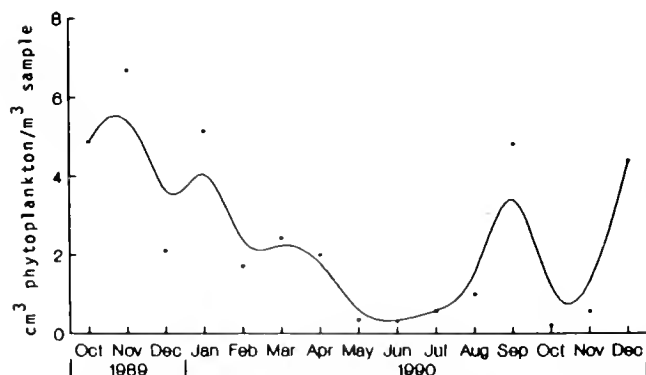


Figure 3. Phytoplankton represents cm<sup>3</sup> of phytoplankton decanted for each m<sup>3</sup> of sample.

seasonal peak during 1990 summer months just when gonads were in ripe stage. However, in spring 1990 when the gonads were ripe again, the water temperature was relatively low. Because high temperature appeared related to the ripe phase rather than the active phase, it is possible that another environmental factor was influencing gonad growth and gametogenesis. Gonadal development of Chiloé scallop is probably initiated when temperature is low, but food availability was high. Low temperature does not inhibit gametogenesis, at least for *Chlamys amandi*, (this study) *Placopecten magellanicus* (Thompson, 1977) and *Pecten novaezelandiae* (Bull, 1976).

Abundance of food has been generally associated with breeding period of marine invertebrates and thought to ensure adequate nutritional availability for planktotrophic larvae (for review see Thorson 1950, Giese 1959 and Sastry 1966). More recent studies have emphasized the importance of food availability (for review see Bayne and Newell 1983, Broom and Mason 1978, Emmett et al. 1987).

In the Chiloé scallop, gonad growth appears to coincide with periods in which there are high levels of food available in Hueihue

TABLE 3.

Month		G.I. 100%	S.D. ±
October	1989	12.5	2.90
November	1989	6.08	1.97
December	1989	8.12	1.01
January	1990	14.6	2.63
February	1990	10.24	3.82
March	1990	10.84	4.06
April	1990	8.62	3.98
May	1990	7.35	3.31
June	1990	9.03	2.72
July	1990	7.32	1.99
August	1990	10.56	2.87
September	1990	8.27	2.06
October	1990	5.05	1.63
November	1990	5.78	1.14
December	1990	2.53	1.54

G.I. values recorded (included S.D.) for the studied period at Yaldad Bay.



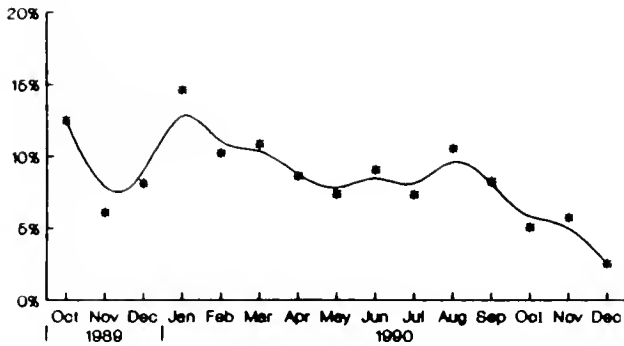


Figure 4. Variation of Gonadic Index for the sampled period.

Bay. Thus it is probable that food supply is more related to gonadal development in *Chlamys amandi* than temperature.

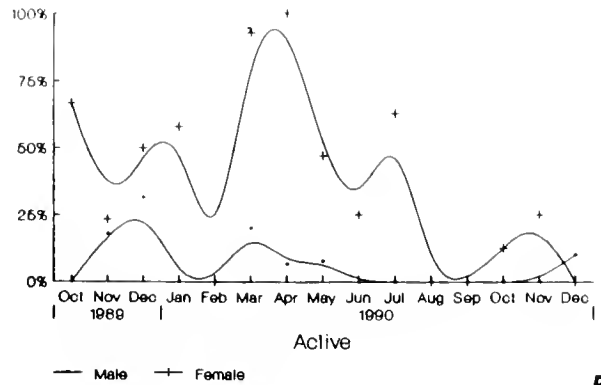
Observations reported by Sastry (1966) for bay scallops *Aequipecten irradians* exposed to various temperatures during the period of gonad growth, without food supply, showed a decrease in both gonad and digestive gland index. This shows that additional food is essential for gonad growth. Emmett et al. (1987) conclude that temporal and quantitative differences in food supply has a greater influence on reproductive cycles than water temperature or latitude.

Time of spawning may also be related to food availability. Most bivalves tend to spawn during periods when food is available for developing progeny (Bayne 1976). Disalvo et al. (1984) induced spawning in *A. purpuratus* in winter at 13°C by simply rinsing a concentrate of phytoplankton into the culture tanks. Thus Wolff (1988) suggests that high temperature, although favouring maturation and spawning, might be less critical for a successful spawning than food availability. In *Chlamys amandi* the spawning time also appeared related to high food levels rather than water temperature in Hueihue Bay.

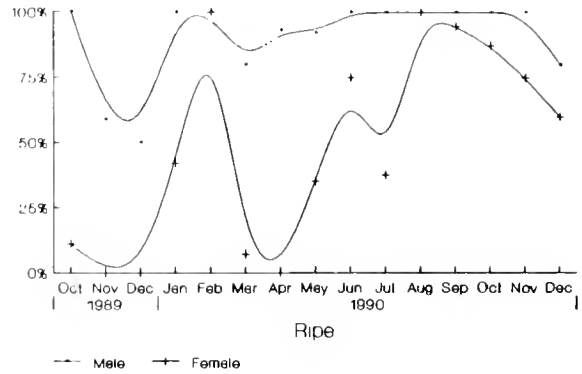
The presence of larvae of *Chlamys amandi* registered in November (1989), January, February, April, September and December (1990) may be explained by the timing of female spawning (mainly spring and fall) and a food availability.

The spat which settled during late November 1989, late February, April, September and December (1990) coincided with high food values and female spawning peaks.

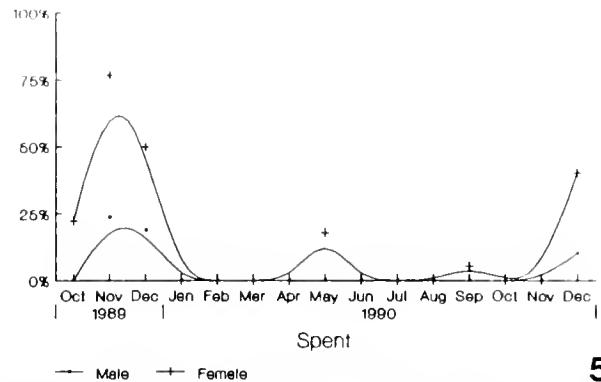
Success and rate of larval development of many marine species that have a planktonic larval stage are affected by physical and endogenous parameters. Among physical parameters, temperature is probably the most frequently investigated because it can be easily manipulated and has a significant effect on growth and survival (Davis and Calabrese 1964, Lought and Ganon 1973, Tettelbach 1979, Falmagne 1984, Wolf 1988). These and another studies have shown that growth to settling size, and therefore completion of the larval period, generally is more rapid as temperature increases to some optimum level, and then declines with further temperature increases (Bayne 1983). The presence of lar-



5a



5b



5c

Figure 5A,5B,5C. Gametogenic phases of Chiloé scallop, the values represents the percentage frequency of scallop in each phase.

vae of Chiloé scallops under favorable temperatures (over 11°C) after the spawning time agree with these suggestions.

ACKNOWLEDGMENTS

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## DISSEMINATED SARCOMAS OF SOFT-SHELL CLAMS, *MYA ARENARIA* LINNAEUS 1758, FROM SITES IN NOVA SCOTIA AND NEW BRUNSWICK

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**ABSTRACT** Over a one year period, 896 soft-shell clams from 22 locations along the Bay of Fundy coast of New Brunswick and Nova Scotia and the Eastern shore of Nova Scotia were examined for the possible occurrence of sarcomas. Biopsies revealed disseminated sarcomas in clams from five sites around the Bay of Fundy; the first records for Canadian waters. The data were insufficient to show whether there was any correlation between incidence of sarcomas and either pollution or recent declines in clam abundance.

**KEY WORDS:** sarcoma, neoplasia, soft-shell clam, *Mya*

### INTRODUCTION

Disseminated sarcomas have been reported in 15 species of marine and estuarine bivalve molluscs world-wide (Peters 1988) including the soft-shell clam, *Mya arenaria* Linnaeus, a species of importance to east coast fisheries in Canada and the U.S. These sarcomas consist of abnormal anaplastic cells that have a distinctive appearance; being enlarged and rounded, with large, hyperchromatic, often lobed nuclei containing one or more prominent nucleoli, surrounded by little cytoplasm. Usually, mitoses are common in these cells, which are found throughout the tissues, including the blood vessels. This distribution, together with immunocytological evidence (Smolowitz and White 1992) indicate that the neoplastic cells are of haemocytic origin. However, neoplastic cells have different antigens from normal haemocytes, suggesting that they may not have the same origin (Reinisch et al. 1983).

Disseminated sarcomas have been reported in soft-shell clams along the Atlantic coast of the United States from the Hudson River to the northern part of Maine, near the U.S./Canada border (Brousseau 1987, Brown et al. 1976, 1977 and 1979, Gardner et al. 1991, Peters 1988, Reinisch et al. 1984, Sherburne and Bean 1983, Yevich and Barszcz 1977), but not hitherto from Canadian waters. No sarcomas were reported in clams from Chesapeake Bay until 1979. These are believed to be derived from clams transferred from New England to Chesapeake Bay after hurricane Agnes decimated local stocks in 1972. Only isolated cases were reported until 1983, when the numbers reached epizootic proportions of up to 90% (Farley 1969, 1989, Farley et al. 1986a, 1986b, 1991). This relatively recent appearance and sudden increase of the numbers of affected clams indicates that an infectious etiology is involved, and a virus similar to a B-type retrovirus has been reported in Rhode Island clams with disseminated sarcomas (Cooper, Brown and Chang 1982a, Oprandy et al. 1981, Oprandy and Chang 1983).

Sarcomas have been found in relatively unpolluted as well as polluted areas; and there does not seem to be a clear-cut correlation between prevalence of the disease and presence of pollutants (Farley 1989, Mix 1986, Reinisch et al. 1984, Yevich and Barszcz 1977). There are, however, some indications that stress can enhance the spread of the infectious agent (Brown 1980, Peters

1988), which may explain the occurrence of more neoplasias at some sites where clams have been exposed to oil (Yevich and Barszcz 1977); herbicides (Gardner et al. 1991) or mixtures of pollutants (Reinisch et al. 1984); or where clams contain high levels of the pesticide chlordane (Farley et al. 1991) or polynuclear aromatic hydrocarbons (Brown et al. 1979).

There have been few studies of neoplasias in bivalves in Canada. Haematopoietic neoplasms were found in mussels *Mytilus edulis* from several sites along the shore of Vancouver island (Mix 1986), and in the truncate soft-shell clam *Mya truncata* and the chalky macoma, *Macoma calcarea* from Baffin Island (Neff et al. 1987). On the east coast "Malpeque disease", originally reported in 1915 from Malpeque Bay, Prince Edward Island, killed over 90% of the eastern oyster *Crassostrea virginica* in 3 years. The disease spread to other parts of P.E.I. between 1915 and 1937, causing similar epidemics (Drinnan and Medcof 1961). In 1955 the disease had spread to the mainland, and by the end of 1960 all major areas where oyster beds in New Brunswick and west of Cape George in Nova Scotia had experienced epizootics. Moribund oysters contain secondary invaders, and many agents, including bacteria, *Hexamita* sp. and fungi have been suggested as the etiologic agent of Malpeque disease (Logie 1958). However, Drinnan et al. (1960-61) described cells similar to the neoplastic cells of disseminated sarcomas in diseased oysters. It was not known at the time whether these cells were normal oyster cells or pathogens, but it seems likely that they were the cause of the mortalities in view of more recent work on neoplasias in bivalves. It is interesting that it was possible to rebuild oyster populations in the stricken areas of Nova Scotia and New Brunswick by transplanting P.E.I. oysters that had survived the epizootic and were resistant to the etiologic agent (Drinnan and Medcof 1961). Clams resistant to sarcomas were also found in Rhode Island, where sarcomas were chronic in some parts of the population, and showed remission in others (Cooper et al. 1982a). Oysters in the Bras d'Or lake, Cape Breton Island were never infected by Malpeque disease, but when moved to areas where it had spread developed disease symptoms and suffered high mortalities (Drinnan and England 1965). Apparently soft-shell clams in the areas where there were diseased oysters did not show typical symptoms and were considered to be unaffected. There have been no published studies of sarcomas in soft-shell clams from Canada, so in 1985 it

TABLE.

Geographical locations, site number, sampling dates, and number of specimens found to have disseminated sarcomas.

Location	Site Number	Date Collected	Number Positive by Stage			% Positive
			Early	Intermediate	Advanced	
Lepreau harbour	1	16.12.1985				0
Bocabec Bay	2	16.12.1985				0
St. Croix River	3	16.12.1985				0
Lepreau harbour	1	18.12.1985				0
Thorne's Cove	4	3.1.1986				0
Yarmouth area	5	3.1.1986				0
Advocate	6	6.8.1986	2	3	2	21.9
Lower Five Islands	7	6.8.1986	3	2	5	31.3
Smith's Cove	8	6.8.1986				0
Upper Clements	9	6.8.1986				0
Cook's Beach	10	6.8.1986	1	1		6.3
Pottery Creek	11	20.8.1986	1	1		6.3
Magaguadavic	12	20.8.1986				0
L'Etete	13	20.8.1986				0
Lepreau harbour	1	20.8.1986		1		3.1
Stuarttown	14	20.8.1986				0
McCanns Cove	15	20.8.1986				0
Goat Island	16	10.9.1986				0
Cole Harbour	17	26.11.1986				0
Oak Point, Annapolis	18	9.12.1986				0
Clementsport	19	9.12.1986				0
Thorne's cove	4	9.12.1986				0
The Joggins, Annapolis	20	9.12.1986				0
Economy Point	21	20.1.1987				0
Economy Point	21	20.1.1987				0
Advocate	6	20.1.1987				0
Five Islands	22	22.1.1987				0
Five Islands	22	23.1.1987				0

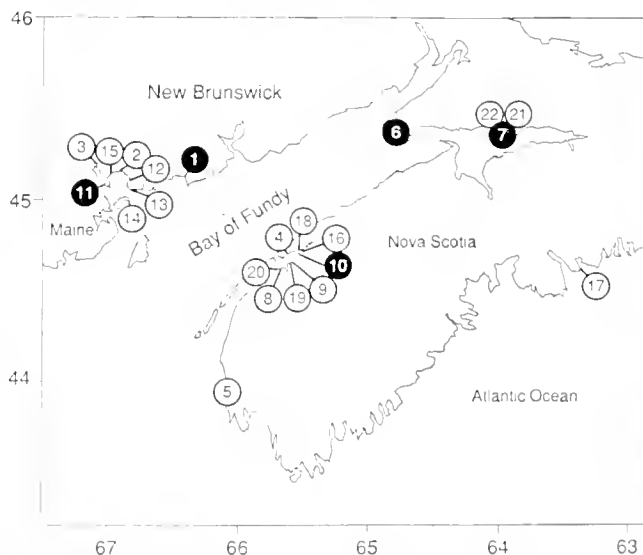
was decided to monitor sites close to the U.S. border, to determine if sarcomas were present in Canadian waters.

#### METHODS

Sampling was restricted to 22 sites in New Brunswick and Nova Scotia; most being in the Bay of Fundy (Table, Map). Most samples of clams were obtained from commercially harvested clam flats. Some of these flats are subject to closure to harvesting as a result of high faecal coliform counts in the overlay waters. The coliforms may originate from populated areas, farmland or wild animals such as gulls, and do not usually appear to stress the clams. The second sample from Lepreau harbour (Site 1) was taken one day after a diesel oil spill which resulted in some clam flats having to be closed due to detectable hydrocarbon odours. Thorne's Cove (Site 4) is in the Annapolis Basin, where the clam population had been depleted by overfishing. Clams were sampled from Oak Point (Site 18), near Goat Island, because there was an unexplained disappearance of clams between July 1986 and April 1987 (Prouse et al. 1988, Rowell and Woo 1990, Rowell in press). Clams were sampled from two sites at Five Islands (Site 22) because one was open for harvest, and one was closed because of contamination by faecal coliforms.

The sample from each site consisted of thirty-two clams, ranging in length from 2.9–8.5 cm, except for one of the Economy Point sites, where no small clams could be found, so only sixteen large clams were taken. Clams were biopsied using an *in vivo*

bleeding technique slightly modified from that described by Farley et al. (1986a). This technique detects neoplastic cells circulating in the haemolymph (Cooper et al. 1982b), and is quicker than preparing histological sections. Cooper et al. (1982b) found that 94% of diseased clams were detected using the bleeding technique,



Map. Locations and site numbers of sampled sites. Solid circles indicate the sites of positive cases.

compared to the number found to be positive from histological sections. The accuracy was greater as the disease progressed, being 66–71% in clams with a light infection, rising to 100% in more advanced infections. Farley et al. (1986b, 1989) found that the biopsy technique was more sensitive than examination of histologic sections, the latter only being reliable for the more advanced stages. Since this survey was conducted with limited resources only the biopsy technique was used, without histological comparisons.

Blood samples were put into eight-chambered tissue-culture slides (Lab-Tek, Miles Scientific, Illinois, U.S.A.) rather than a single chamber attached to a standard slide as used by Farley et al. (1986a). The former produces a smaller area of cells from each sample for observation, but more samples can be processed at one time, and a poly-L-lysine coating is not necessary to ensure adherence of neoplastic cells (Dr. R. A. Sonstegard, pers. comm.). We also used ambient sea-water filter-sterilized through a 0.45  $\mu$  membrane filter instead of artificial sea-water. The fixing and staining procedures were as described by Farley, and positive samples were staged according to the percentage of neoplastic cells into: early (0.01–0.9%), intermediate (1–49%) and advanced (50–100%) stages (Farley et al. 1986a).

## RESULTS

Neoplastic clams were found in the early, intermediate and advanced stages (Table). The neoplastic cells in the intermediate and advanced stages were very distinct from normal haemocytes, being large (3–7  $\mu$ m in diameter) with a round or oval nucleus containing clumps of chromatin, and a distinct nucleolus (Fig.). Nuclei from the early stages had a similar appearance, but were less distinct because they were only about 3–4  $\mu$ m in diameter. Neoplastic cells were found in 7 clams from Advocate, 10 clams from Lower Five Islands, 2 clams from Cooks Beach, 2 clams from Pottery Creek and 1 clam from Lepreau Harbour (Map). All positive cases were found in August. Two of the cases at Advocate were advanced, three intermediate and two early. Five of the cases at Lower Five Islands were advanced, two intermediate and three early. One case at Cook's Beach and Pottery Creek was early,

one intermediate, and the case at Lepreau Harbour was intermediate.

## DISCUSSION

Having discovered that neoplasias were present in the Bay of Fundy, it had originally been intended to extend this study to soft-shell clams from other sites around the Maritime Provinces of Canada, to sample at different times of year, and to use histological sections to verify the occurrence of neoplasias found using the bleeding technique, and to study the course of development of neoplastic cells. Unfortunately, lack of time and loss of personnel made this impossible. However, although limited in scope, this study clearly demonstrates the presence of sarcomas in soft-shell clams at several sites around the Bay of Fundy.

In other studies of disseminated sarcomas it has been found that prevalence differs among sites, and may vary seasonally. Usually the prevalence is low, as found here, although epizootics may occur (Peters 1988). In Chesapeake Bay, the epizootics seemed to develop in the fall and reach more advanced stages by spring. Laboratory-held animals having these sarcomas have experienced 100% mortality, and in the field few sarcomas were found from June to August, indicating that infected clams had died (Farley et al. 1986b, Farley 1989). The reason for this cycle is unknown (Peters 1988). The cycle in the Bay of Fundy seems to be different, because no neoplastic cells were found in December or January, although 52% of the clams were sampled during these months; and all infected animals were found in August. A sample size of 30 animals gives a 95% chance of detecting one or more infected specimens when the detectable infection rate is 10% or more in a population over 100,000 (Ossiander and Wedemeyer, 1973). Neoplastic cells in the intermediate and advanced cases are easily seen using the biopsy method, so the detection efficiency of these cases should be high, although early infections, which are more difficult to detect, could be present during the winter. Most mortalities from Malpeque disease occurred in summer and early fall (Needler and Logie 1947), so possibly colder winter temperatures in Canada delay the progress of disseminated sarcomas in bivalves. Further studies of sarcomas in soft-shell clams at different times of the year are needed, to see if there is a seasonal variation in the progression of the disease.

The sample sizes from Lepreau harbour (Site 1) were not big enough to establish any correlation with the presence of oil from the oil spill and neoplasias, although one neoplastic clam was found 8 months after the clam flats were contaminated by oil.

In the present study it was not possible to link mortalities to the occurrence of neoplasias, but this kind of correlation is difficult since so many factors can cause large mortalities among soft-shell clams. For example, reductions in numbers of clams have been attributed to silting of clam-beds, harvesting methods, overfishing, predation by the green crab, *Carcinus maenas*, the clam drills *Lunatia triseriata* and *L. heros*; and flounders and gulls (Hart 1954 and 1955, Needler 1947 and 1953, Emerson et al. 1990, Robinson and Rowell 1990). At the Oak Point site in the Annapolis Basin (Site 18), where there had been an unexplained reduction in the clam population, we found no neoplasias. The decline has since been shown to be due to the synergistic effects of predation by the nemertean worm *Cerebratulus lacteus* Verrill, and silting which apparently prevented larval settlement. The silting probably resulted from a change in hydrographic conditions as a consequence of the installation of a tidal power facility (Prouse et al. 1988, Rowell and Woo 1990, Rowell in press).

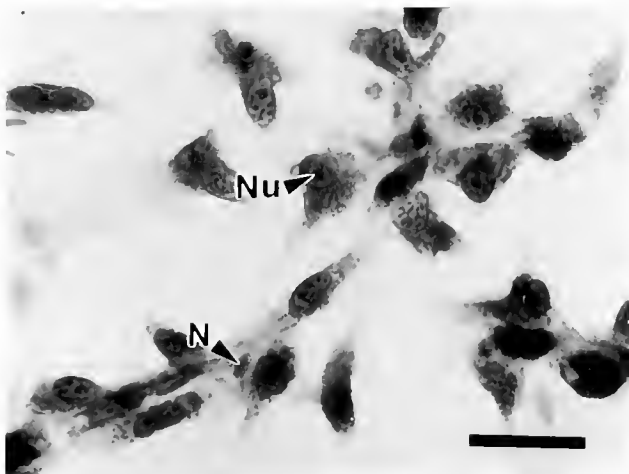


Figure. Neoplastic cells from a clam with a heavy infection. Abbreviations: Nu—nucleolus of neoplastic cell, N—nucleus of normal haemocyte. Bar = 20  $\mu$ m.

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Mr. A. Farley's advice was invaluable in carrying out this work, and he also sent a slide of a biopsy from a clam with a sarcoma and verified some of our slides. Mr. S. Sherburne also provided valuable comments, and sent us slides, and micrographs of sectioned material of clams with sarcomas. Members of the

Inspection Division, Fisheries Operations Branch and Mr. P. Woo of the Habitat Ecology Division, Biological Sciences Branch obtained and delivered samples, and co-workers in the Fish Health Unit looked after samples, processed biopsies, and provided advice and supplies. Mr. J. Black set up the computer program for the map. Mr. T. W. Rowell provided a thorough review of the manuscript, and provided helpful advice.

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## GONADAL COMPARISON OF MASCULINIZED FEMALES AND ANDROGYNOUS MALES TO NORMAL MALES AND FEMALES IN *STROMBUS* (MESOGASTROPODA: STROMBIDAE)

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**ABSTRACT** Gonad and other reproductive tissues were taken from masculinized females and sexually undeveloped individuals of the West Indian fighting conch, *Strombus pugilis*, and from two masculinized female queen conch, *S. gigas*, for comparison to normal males and females of their respective species. Masculinized females were indistinguishable from normal females, except for the presence of a small, deformed verge which resembled, microscopically, that of a normal male. Sexually undeveloped specimens did not possess a verge or an egg groove. Microscopic examination of the gonad tissue revealed some undeveloped, inactive spermatogenic tissue, showing that these individuals were androgynous males.

**KEY WORDS:** *Strombus*, masculinization

### INTRODUCTION

The Strombidae are tropical marine gastropods with the majority of species found in the Indo-Pacific region (Abbott 1960). The family is represented in the Caribbean by six species found: *Strombus gigas*, the queen conch, *S. costatus*, the milk conch, *S. raninus*, the hawkwing conch, *S. gallus*, the rooster-tail conch, *S. alatus*, the Florida fighting conch, and *S. pugilis*, the West Indian fighting conch. All conch are gonochoristic and exhibit sexual dimorphism: males have a penis (termed "verge") and females have an egg groove.

Intersexes can occur in any gonochoristic species due to genetic or environmental causes or a combination of genetic-environmental interactions. For example, meiotic nondysjunction of sex-determining chromosomes can lead to a variety of intersexes, and mitotic non-dysjunction can cause mosaics, as found in *Drosophila melanogaster* (see Ayala and Kiger 1984). Chemical toxins are known to cause imposex in dog whelks (Bryan et al. 1988, Gibbs et al. 1988) and masculinization in mosquitofish (Howell et al. 1980). Intersexes are found during transition from one sex to the other in those species that change sex. There are also cases of hermaphrodites occurring, at low percentages, in dioecious spe-

cies (e.g. brachiopods: Culter and Simon 1987, clams: Ropes 1982).

Individuals have been found in several species of *Strombus* that exhibit hermaphroditic characteristics in that they possess both an egg groove and a verge (R. S. Appeldoorn, pers. comm.; pers. obs.). The verge was, however, dysfunctional due to underdevelopment and deformity, often being multilobed. Kuwamura et al. (1983) described a similar condition in female *S. luhuanus* but assumed that this secondary sexual characteristic was a "clitoris" rather than any abnormality. Botero (1984) depicted a male *S. gigas* with three verges that appear to be of functional size but did not mention whether an egg groove was also present. No studies were ever done to determine the extent of the intersexual condition found in strombids.

This study was undertaken to determine the incidence and clarify the nature of intersexual strombids. The study concentrated on masculinized female *Strombus pugilis* taken from two populations in the La Parguera area of Puerto Rico, and includes two androgynous males. Two masculinized female *S. gigas* specimens were also found in this area and included for study.

### METHODS

Specimens for study came primarily from two populations of *Strombus pugilis*, designated CdP and MIG, that were separated by 1.5 km. Both inhabited muddy-sand bottoms, at a depth of 10

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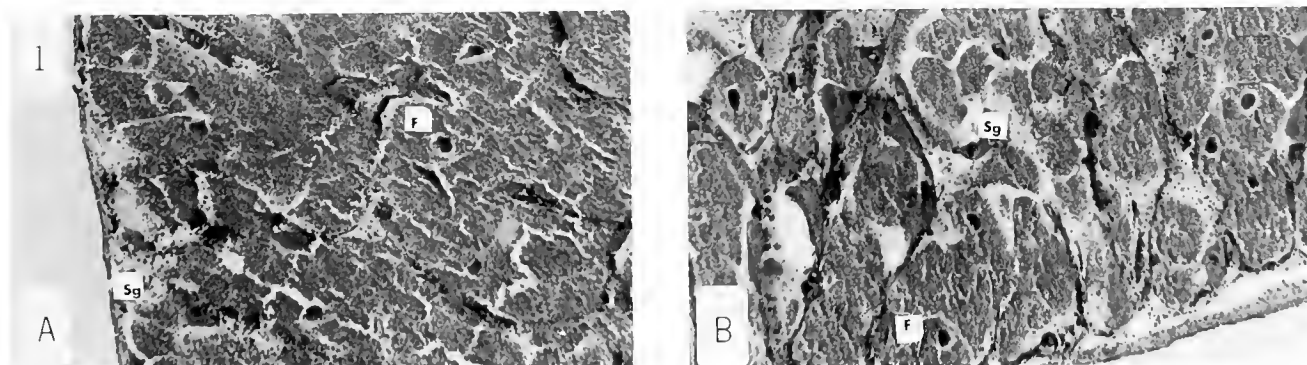


Figure 1. Histological section through A) normal and B) masculinized female *Strombus pugilis* ovaries ( $\times 100$ ); F—follicles, Sg—signet cells.

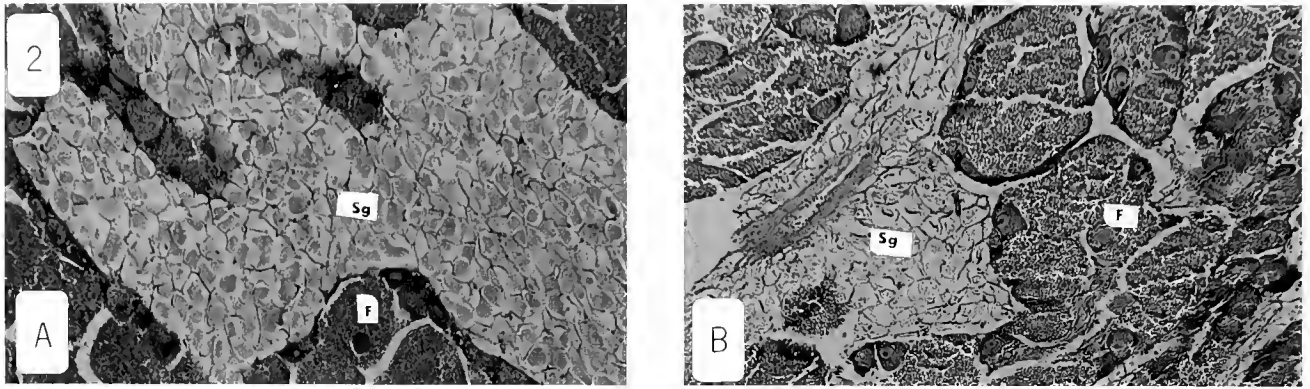


Figure 2. Histological section through A) normal and B) masculinized *Strombus gigas* ovaries ( $\times 100$ ); F—follicles, Sg—signet cells (signet cells in B) appear empty and partially collapsed as spawning had just been completed at time of capture.

meters, with mixed patches of algae such as *Halimeda*, *Ulva*, and *Penicillis*. *Strombus pugilis* were also examined from a third population from which conch were taken as part of another study (Sanders 1988); this population inhabited a mangrove bay with similar bottom characteristics (maximum depth 4 meters).

Samples of conch were collected haphazardly from both populations and brought back to the laboratory for processing. They were kept in a free-flowing holding tank for 1–7 days, as needed. All animals were sexed by observing the foot when extruded from the shell. Intersexes, which possessed both an egg groove and small verge, were separated from the rest of the sample; excess males and females not required for study were returned to their respective field sites. Two specimens of *Strombus gigas* were found that possessed both an egg groove and verge. Also, included for study were two adult *S. pugilis* from the CdP population that lacked secondary sexual development altogether.

All specimens were dissected to examine internal anatomy before processing. Gonads were excised from both *Strombus gigas* specimens and ten *S. pugilis* intersexes taken from the MIG population as well as one asexual specimen, and fixed in Davidson's solution prior to histological processing. Normal males and females of both species (six of each sex) were included for comparison. Tissues were dehydrated in 95% ethanol and embedded in paraffin. Serial sections were cut 6–10  $\mu\text{m}$  in thickness and mounted on albuminized slides. Staining was with hematoxylin and eosin according to Harris' regressive method in Howard and Smith (1983). Tissues from the reproductive tracts of some *S. pugilis* intersexes were also processed in the same way except that

they were fixed in Bouin's solution prior to dehydration and embedding. The slides were then examined using light microscopy to determine sexual condition and state.

Several *Strombus pugilis* intersexes were kept in a holding tank with normal individuals prior to processing. They were observed to copulate with normal males and to spawn. Egg masses were weighed and examined for abnormalities. Several masses were allowed to hatch in aerated aquaria in order to get an idea of the fertility and viability of the spawn. Intersexual individuals were also observed in their natural habitat.

## RESULTS

All intersex conch were normal females in terms of reproductive ability. Reproductive tracts were completely feminine in nature except for the development of a dysfunctional verge on the foot where it would normally be found in a male (see Reed, in press a). The verges of such *Strombus pugilis* females never approached the size of a normal male's (25 mm) and were often deformed by splitting into two or more. These verges ranged in size from nibs of no more than 2 mm to a well-developed 12 mm. There was no interference with egg laying, as the verge developed from the lip of the egg groove, rather than from the interior. All egg masses spawned by such females were normal in appearance and weight ( $10.05 \pm 5.72$  g,  $n = 10$ , compared to  $9.78 \pm 5.84$  g,  $n = 10$  for normal females), and hatched within five days, with no apparent differences in hatchability between the two groups.

Figure 1 shows histological sections of the ovaries of a normal

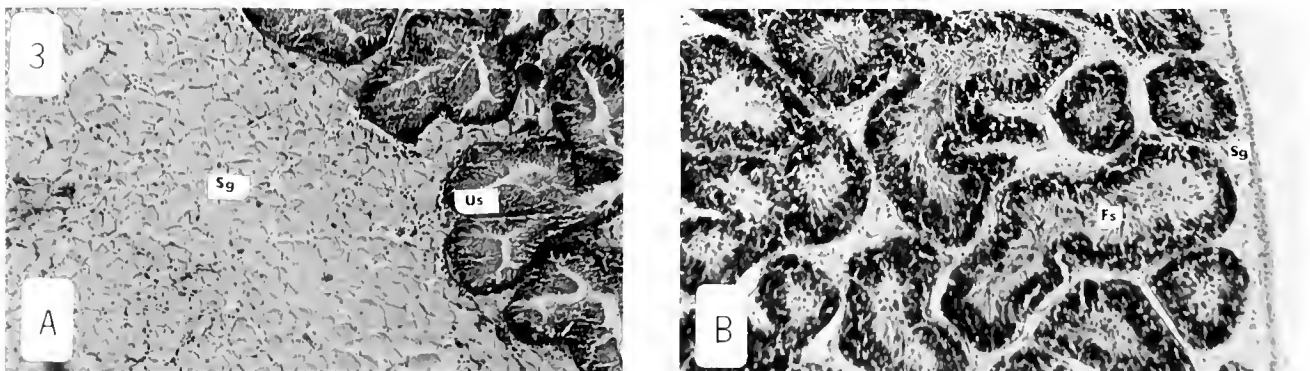


Figure 3. Histological section through A) sexually undeveloped and B) sexually developed male *Strombus pugilis* testes; Us—undeveloped sexual tissue, Fs—mature sperm free in lumen, Sg—signet cells.

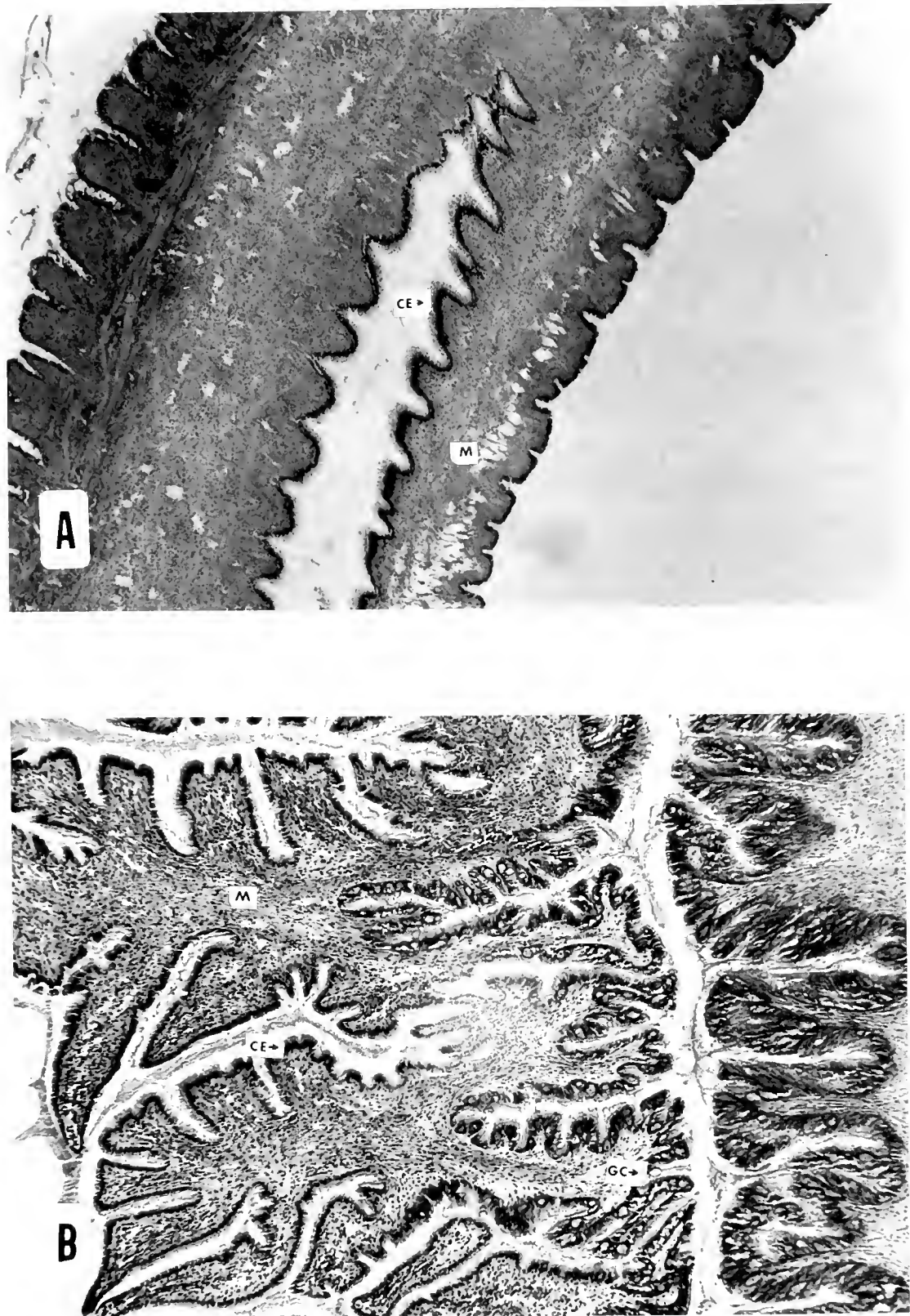


Figure 4. Histological section through the verges of A) masculinized female *Strombus pugilis* and B) male *S. pugilis* for comparison ( $\times 100$ ); CE—ciliated epithelium, GC—gohlet cell, M—muscle.

female (A) and a masculinized female (B) *Strombus pugilis*. There are no differences in the sexual or connective tissues. Both females were reproductively active at the time of capture.

Comparison of the gonads (Fig. 2) of a normal female *Strombus gigas* (A) to that of a masculinized specimen (B) revealed no differences in the sexual tissue. Note, however, that the signet cells of the masculinized female appear empty and partially collapsed, indicating that she had just completed spawning at the time of capture (she was found within 30 cm of a freshly-laid egg mass). Her verge was split into two at the base, and both measured 35 mm (normal male, 60 mm). Both verge tips resembled those of a normal mature male in appearance and structure, except for size (see Reed in press b), as did that of the other specimen.

Figure 3 shows histological sections through the gonads of the sexually undeveloped specimen (A) and a normal male (B) *Strombus pugilis* for comparison. The undeveloped specimen is a male, with typical testicular tissue (Egan 1985), but there is no spermatogenesis taking place. No feminine sexual tissue was found in either the gonads or other reproductive organs. Internal examination revealed the presence of an undeveloped prostate gland. One other asexual specimen found was kept in an aquarium for a year. Eventually, minor verge growth did begin, indicating that both these specimens were sexually retarded males.

Histologically, the verge of a masculinized female is no different from that of a normal, mature male (Fig. 4), except for underdevelopment. Goblet cells are not visible in the section shown for A, but all cell types can be found in both masculinized female and male verges. Other reproductive glands were indistinguishable from those of a normal female (see Reed in press c) and are not reproduced here. Thus, these females are only masculinized, rather than true intersexes. Masculinized females were found in the mangrove bay population but were not further studied.

The incidence of masculinization was 2.3% in the CdP colony and 13% in the MIG colony (see Table 1). The sex ratio was found to differ significantly ( $p < 0.05$ ) from 1:1 in both populations when masculinized females were excluded from the calculations, showing a deficit of males in both populations.

No masculinized female *Strombus costatus* have been found as yet, although over 500 individuals have been examined. Neither have other aberrant types such as the three-pronged male of Botero (1984) been found in any of the species sampled.

#### DISCUSSION

Anatomically and histologically, all masculinized females examined were reproductively normal females. None of their verges ever approached functional size, and were often deformed. This small verge development is similar to that of immature *Strombus gigas*, *S. costatus*, and *S. pugilis* males (see Appeldoorn 1988). None of these individuals were ever observed acting in any way as a male, such as attempting to copulate with another female. These

TABLE 1.

Sex ratios and incidence of masculinized females in the two populations of *Strombus pugilis*.

Population	Males	Normal Females	Masculinized Females
MIG	254 (31%)	416 (56%)	104 (13%)
CdP	542 (44%)	660 (54%)	29 (2%)

females spawned normally and their condition did not in any way interfere with reproductive activities. The correct term for this phenomenon in female conch is masculinization.

The incidence of masculinization was not confined to just one population, but was found in three separate colonies that have spatial and geographic barriers between them. Chemical mutagens have been found that cause gastropods to develop both male and female reproductive tissues (Bryan et al. 1988, Gibbs et al. 1988) when present in the water; however, in the case of these *Strombus pugilis* colonies, there is no likely source of chemical contaminants in the area, and no way to determine if exposure possibly occurred during the planktonic or juvenile stage.

Masculinization has been found in female mosquitofish (Howell et al. 1980) subject to chemical effluents; however, males were affected as well in that they exhibited precocious sexual development. It is not known whether males of the *Strombus pugilis* populations studied developed precociously or not, as these populations consist entirely of mature individuals. Masculinization has been recorded in other normally gonochoristic species as well (e.g. brachiopods; Culter and Simon 1987) with no apparent cause. In *S. pugilis*, at least, this condition is limited to morphological development that does not interfere with normal behavior or reproduction, but does appear to affect growth as masculinized females were larger than normal females which, in turn, were larger than males (see Reed in press a). The latter indicates that some genetic factor is involved, such as non-dysjunction of the sex chromosomes which leads to a variety of aberrant sexual conditions and morphological differences (Ayala and Kiger 1984). Preliminary electrophoretic observations (Reed and Juste, unpubl. data, cited in Reed 1992) revealed differences in enzymatic expression between males, and normal and masculinized females, further supporting the hypothesis of a genetic basis for sex determination in conch, aberrations of which can lead to masculinization and sexual retardation.

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## SIZE DIFFERENCES BETWEEN SEXES (INCLUDING MASCULINIZED FEMALES) IN *STROMBUS PUGILIS* (MESOGASTROPODA: STROMBIDAE)

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**ABSTRACT** The discovery of masculinized female *Strombus pugilis* provided a third morph which allowed sexual dimorphism in size to be studied in more detail. Masculinized females were found to be larger in overall size than normal females which were in turn larger than males. The difference in size among morphs was not due to differences in shape but only to growth to a larger average size. Lip thickness did not differ among the morphs indicating that masculinized females grow to a larger size than normal females which grow larger than males in the same amount of time. The apparent cause is a genetically-based sex determination system, operating in this species.

**KEY WORDS:** *Strombus*, dimorphism, masculinization

### INTRODUCTION

There appears to be sexual dimorphism in size for most strombid species (Abbott 1960). Two Indo-Pacific species studied showed females to be longer on average than males (Abbott 1949). For *Strombus gigas*, females are known to be larger than males based on shell length frequencies, and may also be broader (Randall 1964, Alcolado 1976, Blakesley 1977). Robertson (1959) mentions that females are larger than males in *S. costatus* and *S. raninus* but presents no data. *Strombus pugilis* and *S. alatus* also apparently show size dimorphism (Colton 1905, Goodrich 1944). No studies have been done that examine whether size differences between males and females are based on differences in shape, age at maturation, or rates of shell and tissue production, and whether sexual dimorphism in general is controlled by environmental and/or genetic factors.

Conch are characterized by the formation of a flaring lip on the outer edge of the last body whorl of the shell at maturity. Growth

in length ceases at this point, and may only decrease, due to abrasion, but never increase. Full sexual maturity is not attained until after lip formation. The conch continues to add shell material to the lip such that it becomes quite thick as the conch ages.

Masculinized female strombids, those that possess both an egg groove and a small verge, have been found (Kuamamura et al. 1983, Reed, in press, R. S. Appeldoorn, pers. comm.). Reed (1992) found several colonies of *Strombus pugilis* which had large numbers of such females. Masculinized females offer the opportunity to look at sexual dimorphism in strombids in more detail. No comparison of masculinized females has ever been done on the basis of size due to a paucity of specimens. The presence of a third sexual morph may allow hypotheses to be developed that explain not only the proximal causes of sexual dimorphism, but possibly their underlying causes as well.

This study was undertaken to characterize sex-based size differences in *Strombus pugilis*.

### METHODS

Two populations of *Strombus pugilis*, designated CdP and MIG for identification, were studied off the southwest coast of Puerto

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TABLE 1.

Means and standard deviations for the MIG population of *Strombus pugilis* (prob. level: 0.05).

Measurement	Males (n)	Normal Females (n)	Masculinized Females (n)
LENGTH (mm)	79.5 <sup>a</sup> ± 4.1 (45)	82.8 <sup>ab</sup> ± 4.2 (59)	87.6 <sup>b</sup> ± 2.6 (79)
LIP (mm)	5.1 ± 0.7 <sup>a</sup> (46)	4.7 ± 0.5 <sup>ab</sup> (53)	4.8 ± 0.7 <sup>b</sup> (67)
WIDTH (mm)	36.4 <sup>a</sup> ± 3.1 (51)	38.0 <sup>ab</sup> ± 2.5 (61)	39.6 <sup>b</sup> ± 2.4 (79)
SPIRE (mm)	26.2 <sup>a</sup> ± 1.5 <sup>a</sup> (47)	28.0 <sup>a</sup> ± 2.1 <sup>a</sup> (59)	28.5 ± 2.2 (80)
SHELL wt. (g)	76.6 <sup>a</sup> ± 11.5 (50)	86.2 <sup>ab</sup> ± 11.9 (62)	93.9 <sup>b</sup> ± 10.7 (7)
ANIMAL wt. (g)	12.7 <sup>a</sup> ± 2.2 (50)	14.3 <sup>ab</sup> ± 2.3 (62)	17.4 <sup>b</sup> ± 2.2 (7)
TOTAL wt. (g)	89.5 <sup>a</sup> ± 13.0 (51)	100.4 <sup>ab</sup> ± 13.4 (62)	111.3 <sup>b</sup> ± 11.5 (7)
WIDTH/LENGTH	0.46 ± 0.03 (45)	0.46 ± 0.03 (59)	0.46 ± 0.03 (79)
ANIMAL/SHELL	0.16 ± 0.02 (50)	0.16 ± 0.02 (62)	0.16 ± 0.02 (7)

<sup>a</sup> Significant difference between males and normal females.

<sup>b</sup> Significant difference between normal and masculinized females.



TABLE 2.  
Means and standard deviations for the CdP population of *Strombus pugilis* (prob. level: 0.05).

Measurement	Males (n)	Normal Females (n)	Masculinized Females (n)
LENGTH (mm)	79.0 ± 5.0 (10)	78.8 <sup>b</sup> ± 3.2 (12)	86.3 <sup>b</sup> ± 4.5 (28)
LIP (mm)	5.6 ± 0.7 (9)	5.3 ± 0.6 (10)	4.9 ± 0.6 (23)
TOTAL wt. (g)	76.4 <sup>a</sup> ± 7.9 (9)	86.0 <sup>ab</sup> ± 10.2 (12)	100.8 <sup>b</sup> ± 11.1 (28)

<sup>a</sup> Significant difference between males and normal females.

<sup>b</sup> Significant difference between normal and masculinized females.

Rico in the vicinity of La Parguera. Samples of conch were collected haphazardly from both populations and brought back to the laboratory for processing. They were kept in a free-flowing holding tank for 1–7 days, as needed.

All animals were sexed by observing the foot when extruded from the shell. Masculinized females were separated from the rest of the sample; excess males and females not required for study were returned to their respective field sites.

The MIG population was chosen for size comparison due to the disappearance of the CdP colony, most likely due to fishing, before sufficient samples were collected for analyses (only a limited amount of data is presented). Measurements taken were LENGTH, length of the shell from siphonal canal to tip of spire (mm), LIP, thickness of lip midway along shell (mm), WIDTH, width of the body whorl (mm), SPIRE, height of the spire (mm), TOTWT, total weight of live animal and shell (g), and ANIMWT, weight of animal after removal from shell (g). SHELLWT, weight of shell only (g), was determined by subtraction of ANIMWT from TOTWT. Some measurements were not possible due to shell damage, especially in cases of broken lips and spires.

Data were processed using the SYSTAT package on an IBM PC. Analyses of variance were used to test for differences among the three morphs. Tukey's HSD multiple comparison test was used to distinguish which morphs differed. In cases where variances were not homogeneous among morphs, separate variance t-tests were used to test for differences between means in a pair-wise fashion. For all statistical analyses, the significance level chosen was 0.05.

## RESULTS

All means and standard deviations are presented in Table 1 for the MIG population. Data collected on CdP individuals prior to their disappearance are presented in Table 2. Comparisons between populations and sexes within population should be interpreted with reservation for those cases where sample size was small.

In the MIG population, males were smaller than females, which were smaller than masculinized females. Males were significantly shorter in LENGTH and SPIRE, and lighter in all weights (TOTWT, ANIMWT, SHELLWT) than females, which were in turn shorter in LENGTH and SPIRE, and lighter in all weights than masculinized females. In the CdP population, masculinized females exceeded normal females in LENGTH and TOTAL weight.

LIP was not significantly different among the sexes within the MIG colony or within the CdP colony, but was between the two populations. CdP conch had thicker lips than MIG conch indicating that the colonies were different in overall collective age. The CdP colony was comprised of smaller animals than the MIG col-

ony. Ratios of shell width to length and animal weight to shell weight were not significantly different among sexes in the MIG colony.

## DISCUSSION

Size dimorphism is apparent between *Strombus pugilis* sexes, as has been noted in several other strombid species (Abbott 1949, 1960). The longer overall shell length, also reflected in spire height, implies that females grow faster than males (Abbott 1960, Alcolado 1976, Webber 1977). Females are also heavier in tissue weight indicating they grow to a larger body size than males. Masculinized females were found to be larger than normal females in all respects, even though they are functional females and are characterized only by small verge development, much like that of immature males (see Appeldoorn 1988), but with severe deformation (verge development does not account for differences in weight as its weight is negligible).

Lip thickness does not vary significantly among males, females, and masculinized females, indicating they are of the same age class and probably derived from the same stock. Extra shell weight, thus, cannot be accounted for by excess shell deposition with age, but rather by a larger shell in general. Females were found to be broader than males, as Colton (1905) found in his study. However, the ratios of shell width to length and animal weight to shell weight were not significantly different, indicating consistency in shape among the three groups. Consistent differences in length, width, and weight, among the three groups, coupled with similarities in shape and age, indicate that sexual dimorphism arises from differential rates of productivity.

The most probable explanation for this gradation in size between the sexes is genetic. A heterotic effect can account for masculinized females growing faster than males. This phenomenon has long been exploited in agricultural breeding programs to produce animals and plants that grow faster and larger in a shorter time period than others (Mitton and Grant 1984). In such a case, masculinized females would have to be more heterozygous than normal females, which would be more heterozygous than males, and could best be explained if sex-determining chromosomes are present. Heterosis in the female would result in faster growth to a larger size, and the presence of an extra dose of genetic material in masculinized females could cause further heterotic effects. Preliminary electrophoretic observations (Reed and Juste, unpubl. data, cited in Reed 1992) indicate that masculinized females do have extra genetic material, not possessed by normal females.

## ACKNOWLEDGMENTS

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# SPATIAL STRUCTURE OF THE PINK SHRIMP *PANDALUS BOREALIS* KRØYER, 1838 FROM THE FAR-EASTERN SEAS AS PROVED BY METHODS OF POPULATION GENETICS AND MORPHOMETRICS

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**ABSTRACT** Polymorphic allozyme loci GPI, PGM, MDH, FDH and 11 morphological traits were examined in 12 samples of the pink shrimp from the Sea of Japan, the Okhotsk Sea, and the Bering Sea to determine the specific population structure. Data obtained suggest within-sea-basin genetic homogeneity and, vice versa, statistically significant heterogeneity among shrimp samples from different seas. Three major clusters based on allele frequency data each representing a different sea were seen on a dendrogram. The discriminant and factor analyses used for morphological classification of shrimp individuals and populations support the results of genetic investigation. It is assumed that every sea in general is inhabited by genetically homogeneous local shrimp population, which in this case is an equivalent of Mendelian population. Differences in shrimp morphology within the seas give us an opportunity to suppose an existence of subpopulation structure on this level as well.

**KEY WORDS:** pink shrimp, *Pandalus*, genetics, population structure

## INTRODUCTION

The pink shrimp *Pandalus borealis* Krøyer is an important commercial fisheries in many countries of the Pacific and Atlantic basins. This has stimulated detailed studies of its distribution, population recruitment, growth rates and other features of the life cycle (Butler 1964, Ivanov 1972, Balsiger 1979, Shumway et al. 1985). Population structure of this species still remains insufficiently studied. Today the knowledge of population structure is considered to be a key for organizing a rational fishery and understanding the plasticity of stock reaction against fishing. Data on genetic composition of the pink shrimp cohorts from the Bering Sea, the Sea of Japan and the Barents Sea were presented but largely in Russian literature (Kartavtsev et al. 1991a). Morphometric investigations of the pink shrimp population structure were performed mostly in the Barents Sea (Kuznetsov 1964, Briazgin 1970, Briazgin, Rusanova 1974, Berenboim 1978, Teigsmark 1983) and without application of modern multivariate statistical approaches.

Here we summarize earlier published genetic data on the Far Eastern seas (Kartavtsev et al. 1991a) in combination with morphometric data using multivariate statistical analysis. Such analysis was presented in oral form but only an abstract has been published (Kartavtsev et al., 1990).

## MATERIALS AND METHODS

Shrimp samples were taken in 1987 and 1988 from the catches of off-bottom trawling in the Sea of Japan (JS), the Okhotsk Sea (OS) and the Bering Sea (BS). The exact geographic coordinates of samples were given earlier (Kartavtsev et al. 1991a). The distribution of the 12 samples is shown in Fig. 1. We managed to perform individual genotyping in only one (OS1) of the three OS samples.

Electrophoretic studies of enzymes were conducted in starch gel (14–15%). Tris-EDTA-boric buffer, pH 8.1 (Korochkin 1977) and tris-EDTA-maleate buffer, pH 7.4 (Shaw, Prasad 1970) were

used. Four enzymes from over 50 screened appear to be polymorphic and were included in the analysis: 1) Glucose phosphate isomerase (GPI, EC 5.3.1.9, abbreviation of locus is the same as enzyme), 2) phosphoglucomutase (PGM, EC 2.7.5.1), 3) malate dehydrogenase (MDH, EC 1.1.1.37), 4) formaldehyde dehydrogenase (FDH, EC 1.2.1.1). More detailed information on the electrophoresis and staining are given earlier (Kartavtsev et al. 1991a,b).

For each individual 11 traits of external body morphology were measured ( $\pm 1$  mm): 1) carapace length (CL), 2) body length (BL), 3) carapace width (CW), 4) width of pleura of the second abdominal segment ( $S_2W$ ), 5) length of the left scaphocerite (LSL), 6) length of the right scaphocerite (RSL), 7) telson length (TL), 8) length of the left exopodite of the uropode (LUEL), 9) length of the right exopodite of the uropode (RUEL), 10) length of the left endopodite of the uropode (LUENL), 11) length of the right endopodite of the uropode (RUENL). Ten indices-ratios were included in the analyses as well. To continue the numbering we indicated them in the following way: 12) CL/BL, 13) LSL/BL, 14) RSL/BL, 15)  $S_2W/BL$ , 16) CW/BL, 17) TL/BL, 18) LUEL/BL, 19) RUEL/BL, 20) LUENL/BL, 21) RUENL/BL.

When performing morphological analysis the following principles were taken into account:

1. Traits complex should represent different morpho-functional structures.
2. All individuals should be characterized with identical set of traits.
3. Normalized traits, i.e. divided by BL, should minimize size variability of individuals (allometry) and has their own meaning.
4. Estimates of differences should be based on biologically homogeneous material, i.e. considered sex dimorphism, and age variability.

Statistical analysis was performed mainly using BMDP software (Dixon 1982), which permit all necessary transformations,

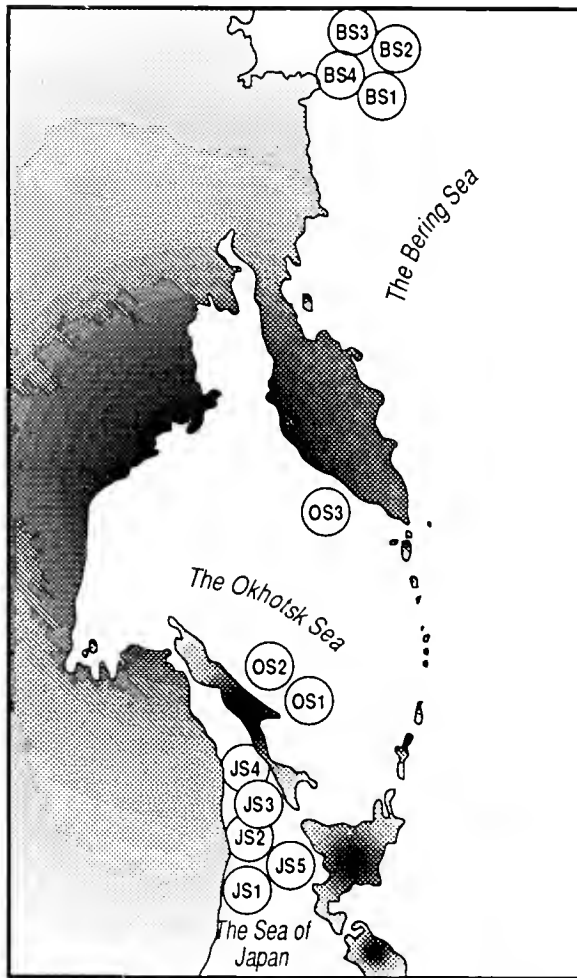


Figure 1. Map showing location of the pink shrimp *Pandalus borealis* samples in the Far Eastern seas. JS1-JS5 = samples from the Sea of Japan, OS1-OS3 = samples from the Okhotsk Sea, BS1-BS4 = samples from the Bering Sea.

standardizations and normalizations. Some other details of morphological analysis are given elsewhere (Kartavtsev et al. 1993).

## RESULTS AND DISCUSSION

Genetic data presented below in a schematic form to outline main concept concerning the population genetic variability in the pink shrimp cohorts. More thorough genetic examination are given elsewhere (Kartavtsev et al. 1991a,b).

For each of the four studied loci, all samples showed a close agreement between observed and expected Hardy-Weinberg equilibrium frequencies of genotypes the  $\chi^2$  did not exceed the critical levels (Table 1). At the studied loci allele frequencies within any sea were rather similar but they greatly differ between the sea basins. Examination of allele frequencies confirm this suggestion (Fig. 2).

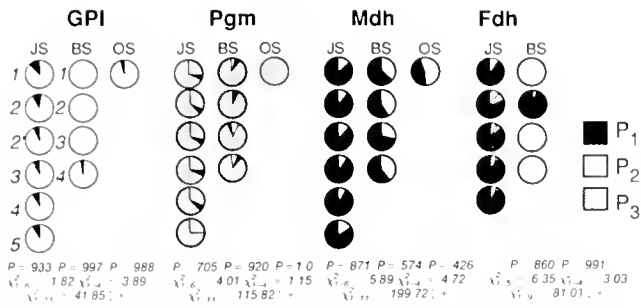
Taking into account the observed variability of allele frequencies in the area, we can assume that shrimp samples collected from the same sea basin are genetically homogeneous (Fig. 2). These results are in accordance with the above mentioned Hardy-Weinberg equilibrium in the individual shrimp samples (Table 1). Moreover, we can speak about the equilibrium between gametic (allele) and genotype frequencies in total shrimp samples from

TABLE 1.

Allele frequencies at the GPI, PGM, MDH, FDH loci in samples of the pink shrimp *Pandalus borealis* and  $\chi^2$ -values of goodness of fit of the observed and expected genotype frequencies.

Sample	N	Allele Frequency			$\chi^2$
		p1	p2	p3	
Locus GPI					
JS1	82	0.079	0.921	—	0.57
JS2	113	0.049	0.951	—	0.32
JS2'	70	0.050	0.936	0.014	0.69
JS3	117	0.064	0.927	0.009	—*
JS4	26	0.077	0.923	—	—*
JS5	49	0.071	0.929	—	0.24
ΣJS	457	0.063	0.934	0.003	4.22
OS1	42	0.012	0.988	—	0.00
BS1	51	—	1.000	—	—
BS2	88	—	1.000	—	—
BS3	55	—	1.000	—	—
BS4	96	—	0.990	0.010	0.00
ΣBS	290	?	0.996	0.004	1.47
Locus PGM					
JS1	84	0.047	0.292	0.661	1.61
JS2	113	0.014	0.265	0.721	2.25
JS2'	70	0.014	0.257	0.727	2.27
JS3	116	0.018	0.284	0.698	1.84
JS4	26	—	0.346	0.654	2.71
JS5	43	—	0.244	0.756	1.36
ΣJS	452	0.018	0.276	0.706	7.44
OS1	15	—	—	1.000	—
BS1	51	0.019	0.069	0.912	0.38
BS2	87	0.046	0.040	0.914	—*
BS3	55	—	0.055	0.945	3.72
BS4	95	0.032	0.052	0.916	0.82
ΣBS	288	0.028	0.052	0.920	—*
Locus MDH					
JS1	65	0.815	0.185	—	2.20
JS2	113	0.889	0.111	—	0.34
JS2'	70	0.871	0.129	—	1.56
JS3	117	0.897	0.103	—	3.34
JS4	26	0.885	0.115	—	0.40
JS5	49	0.847	0.153	—	0.02
ΣJS	440	0.873	0.127	—	1.53
OS1	27	0.426	0.574	—	0.75
BS1	15	0.598	0.402	—	1.68
BS2	88	0.534	0.466	—	0.22
BS3	55	0.655	0.345	—	0.06
BS4	96	0.552	0.448	—	3.11
ΣBS	290	0.574	0.426	—	1.68
Locus FDH					
JS1	26	0.923	0.019	0.058	0.23
JS2	78	0.820	0.052	0.128	3.68
JS2'	70	0.836	0.079	0.085	6.69
JS3	108	0.875	0.051	0.074	2.62
JS4	25	0.920	0.040	0.040	0.15
ΣJS	307	0.860	0.057	0.083	5.58
BS1	47	1.000	—	—	—
BS2	85	0.988	—	0.012	0.01
BS3	54	1.000	—	—	—
BS4	92	0.984	0.005	0.011	0.01
ΣBS	278	0.991	0.002	0.007	0.01

Note: Asterisk means that  $\chi^2$ -values were not calculated because of insufficient digital filling of some cells in the frequency table; N = number of studied animals; JS1-JS5 = shrimp samples from the Sea of Japan; OS1 = the sample from the Okhotsk Sea; BS1-BS4 = samples from the Bering Sea; ΣJS and ΣBS = total samples for the corresponding seas.



**Figure 2.** Variability of predominant allele frequencies at the MDH, GPI, PGM and FDH loci in the pink shrimp *Pandanus borealis* among three Far-Eastern seas. Size of sectors in the circles shows the variability of frequencies of three alleles. The variability of fastest allele (P<sub>1</sub>) is indicated with black color, the intermediate allele (P<sub>2</sub>) with white color, and slowest allele (P<sub>3</sub>) with shading. Arabic figures show the number of samples. JS, OS, BS = shrimps from the Sea of Japan, the Okhotsk Sea, the Bering Sea. Locations of samples over the area shown in Fig. 1. For the JS samples 2 and 2'—is a large sample split into two sub samples when analyzed for allele and genotype frequencies. Below circles are the mean values of predominant allele frequencies and  $\chi^2$ -test of their heterogeneity (Workman, Niswander 1970) for the different population complexes: JS =  $\chi^2_{1-6}$ , BS =  $\chi^2_{1-6}$ , JS + OS + BS =  $\chi^2_{1-11}$ , JS + BS =  $\chi^2_{1-9}$ , ++ = P < 0.001.

both the Sea of Japan and the Bering Sea (Table 1). The differences in allele frequencies of samples from the same sea collected in different years are not statistically significant. In the Sea of Japan samples were taken in 1987 and 1988 and studied over all 4 loci (Table 1, JS5 and JS1–JS4). These results are in good agreement with different data set (Kartavtsev et al. 1991b). Four samples from the Bering Sea taken in its western part in 1988 ( $\bar{p} = 0.920 \pm 0.011$ , Kartavtsev et al. 1991a) and the sample taken in 1972 in the offshore waters of Alaska ( $p = 0.924 \pm 0.009$ ; Johnson et al. 1974) did not differ at PGM—the only comparable locus. Data presented above agree well with a postulate that in time stability of allele frequencies is a valuable feature for large and self reproducing populations (Mattler, Gregg 1972), which is one of main statements of Hardy-Weinberg law.

Analysis of the whole set of samples reveals the opposite tendency in spatial variability of allele frequencies. For each of the four studied loci, a statistically significant heterogeneity of allele frequencies was observed (Fig. 2).

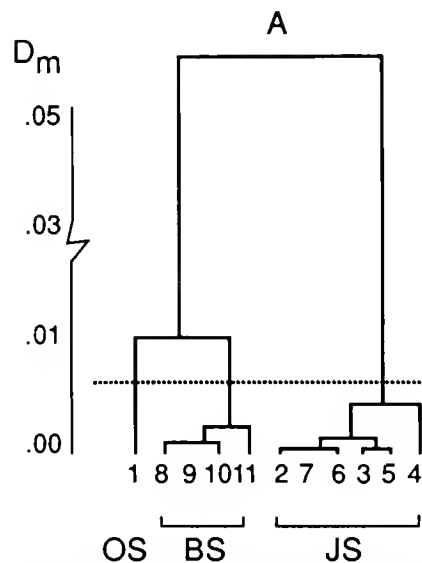
The amount of genetic differentiation within and between population units show an order or more increase from first to second level in three different scales: D<sub>st</sub>, F'<sub>st</sub>, and D<sub>m</sub> (Nei 1987). Averages at GPI, PGM, and MDH, (compared for the whole set of samples) were obtained for the three statistics above: JS – D<sub>st</sub> = 0.0009, F'<sub>st</sub> = 0.0031, D<sub>m</sub> = 0.0029 ± 0.0037; BS – D<sub>st</sub> = 0.0011, F'<sub>st</sub> = 0.0046, D<sub>m</sub> = 0.0006 ± 0.0032; JS + OS + BS – D<sub>st</sub> = 0.0160, F'<sub>st</sub> = 0.0506, D<sub>m</sub> = 0.0298 ± 0.0165.

Relationship between the samples in a scale of genetic distances may be presented in a graphic form (Fig. 3). On the basis of both the intuitive cluster division and an exact approach, which uses the formation of step value clusters (Rao 1980), the main conclusions drawn from the analysis of these dendrograms are identical: 1. There are three main clusters in the dendrograms which include the JS, BS and OS shrimps. 2. Samples from the same sea basin form sufficiently homogeneous clusters (Fig. 3).

By analyzing the variation distributions of morphological traits of the pink shrimp samples we found that mean values of over-

whelming majority of both the traits and the indices were different for females (F), males (M) and hermaphroditic (FM) individuals. As an example, the differences in body length and the S<sub>2</sub>W/BL index values are illustrated (Table 2). The integral differences in complexes of traits between females, males and hermaphrodites were also high-valued. This suggests the necessity of separate morphological analysis of three groups F, M, FM but inference that our pooled data on genotypic variation are representative taking into account absence of difference among these groups in allele frequencies (Kartavtsev et al. 1991a,b). For shortness let us consider the data on morphological variability and among sample differences in females—the most representative group in our material. The complexes of traits and indices chosen for comparison of the shrimp populations form 16 correlation clusters under step value r = 0.9; 7 of them belong to the traits complexes and 9 to indices (Fig. 4). As a whole, the differences between the shrimps of three studied seas are statistically significant for both traits and indices complexes (Table 3).

The results of discriminant analysis give a clear view on the ratio of intra- and inter-basin morphological differences. The distribution of individuals, taken from three Far Eastern seas, as the values of canonical variables (CV<sub>1</sub> and CV<sub>2</sub>, which estimate in the discriminant analysis the main integral information about differentiation of the elements classified) is shown in Fig. 5. In that case the analysis included 10 indices and individuals were combined according to their belonging to three seas: JS, OS and BS. Coordinates of mean values of CV<sub>1</sub> and CV<sub>2</sub> are indicated by circled numbers (Fig. 5). Total discriminative accuracy or the average accuracy of the classification of an individual to its group (sea) was 99.1%. Clusters of JS and BS do not overlap at all at CV<sub>1</sub> values. The JS and OS clusters are overlapping in the projections of individual values of the CV<sub>1</sub> axis by 5.7%, and to BS-OS clusters overlapping by 65.6% (overlapping here is a percent of



**Figure 3.** Dendrogram showing the integral differences in allele frequencies at the PGM, GPI, MDH loci between the samples of the pink shrimp *Pandanus borealis*. Along the axis—minimal unbiased genetic distance (Nei 1978). 1 = sample from the Okhotsk Sea (OS1), 2–7 = samples from the Sea of Japan (JS5, JS1–JS4), 8–9 = samples from the Bering Sea (BS1–BS4). Dotted line shows the step value D<sub>m</sub> = 0.006 defined at the level of mean standard errors values in the populations of JS and BS (see text).

TABLE 2.

An example of comparison of two morphological traits (BL, S<sub>2</sub>W/BL) in males (M), females (F) and hermaphrodites (FM) of the pink shrimp *Pandalus borealis*.

Compared Traits	BL			S <sub>2</sub> W/BL		
	M	F	FM	M	F	FM
Mean	93.66	104.75	101.11	13.03	15.36	13.79
SD	13.12	11.28	7.21	1.15	1.27	1.16
SE	1.05	0.67	0.99	0.13	0.08	0.16
Sample size	77	286	53	77	286	53
Max	116.0	146.0	122.0	20.0	18.7	18.5
Min	67.0	82.0	79.0	10.4	12.2	11.4
'M-F		7.39 (p < 0.0t)			14.61 (p < 0.01)	
'M-FM		4.16 (p < 0.01)			8.68 (p < 0.01)	
'F-FM		3.04 (p < 0.01)			8.40 (p < 0.01)	

Note: SD = standard deviation; SE = standard error; BL = body length; S<sub>2</sub>W/BL = ratio of abdominal segment width to body length; t = Student's statistics; Max, Min = maximal and minimal value of traits; \* = P < 0.01.

individuals having the same CV values or laid in the scope of intermediate values of this variable in two compared clusters). Thus, as it was previously shown by allozyme data, shrimps from the Okhotsk Sea are closer to those from the Bering Sea than to those from the Sea of Japan.

Using for sample grouping of individuals by means of the method of canonical variables, belonging as stated above to discriminant analysis, we can obtain a spatial distribution of the corresponding vector values without an *a priori* assumption of the existence of high hierarchical level, i.e. the sea basin. As a result of the analysis of indices complex we manage to get more detailed

understanding of features and degree of phenotypic differentiation of shrimps. Under this approach there were formed by a natural way the following clusters: BS88, JS88, OS87 and JS87 (Fig. 6). Indices and traits itself are not mutually complementary because of weak correlation of most of them (see Fig. 4) and make somewhat different contribution to the diagnostics of samples. In particular the samples taken in the offshore waters of the Eastern Sakhalin (ES) and the Western Kamchatka (WK) are clearly discriminated by the traits complex. In view of this we repeated the discriminant analysis according to the above mentioned scheme but combine the traits and indices complexes (Fig. 7). As we see the discriminative power of the method increased and OS87 cluster separated into two—ES87 and WK87 (Fig. 7). It is important that when using this approach the accuracy of inter-basin discrimination remains the same, and equal on the average 97.2%, while it is impossible to distinguish individuals from different samples of the same sea basin in most cases.

However, in contrast to allozymic data showing the gene pool homogeneity of populations from each sea, the morphological traits were not indicative of a full uniformity of shrimps within the same sea. For example, in the above mentioned discriminant analysis the accuracy of the classification of the individuals to the JS4

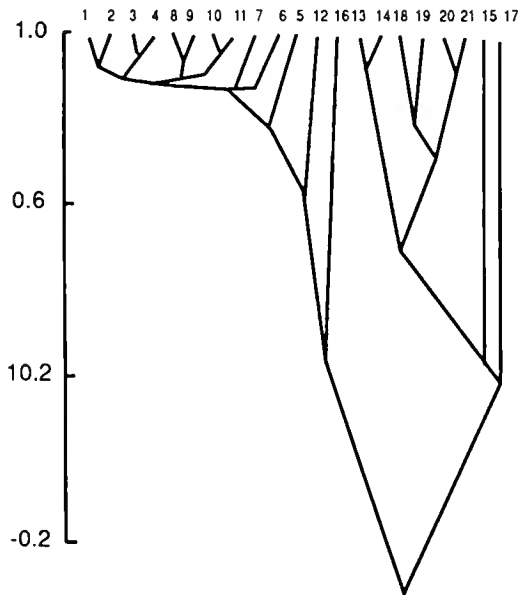


Figure 4. The correlation clusters of the studied traits and indices of the pink shrimp *Pandalus borealis*. On the axis the values of the correlation coefficient are shown. Designations of traits from 1 to 11 were as follows: 1) CL, 2) BL, 3) CW, 4) S<sub>2</sub>W, 5) LSL, 6) RSL, 7) TL, 8) LUEL, 9) RUEL, 10) LUENL, 11) RUENL. Indices were designated in the following way: 12) CL/BL, 13) LSL/BL, 14) RSL/BL, 15) S<sub>2</sub>W/BL, 16) CW/BL, 17) TL/BL, 18) LUEL/BL, 19) RUEL/BL, 20) LUENL/BL, 21) RUENL/BL. Explanation of the abbreviations are given in the section Materials and Methods.

TABLE 3.

Evaluation of the differences between the vectors of mean values as shown by the traits and indices complexes of the pink shrimp *Pandalus borealis* from three Far Eastern seas.

1. Traits (1–11)

Sea Basin	JS	OS	BS
JS	—	29.15	51.32
OS	89.59*	—	21.15
BS	290.81*	62.89*	—

2. Indices (12–21)

Sea Basin	JS	OS	BS
JS	—	29.56	41.09
OS	93.77*	—	22.28
BS	238.61*	67.94*	—

Note: Above the diagonals are D<sup>2</sup>-Makhalanobis distance, below it Fisher's F values, \* = P < 0.001. Abbreviations of the sea basins as before.

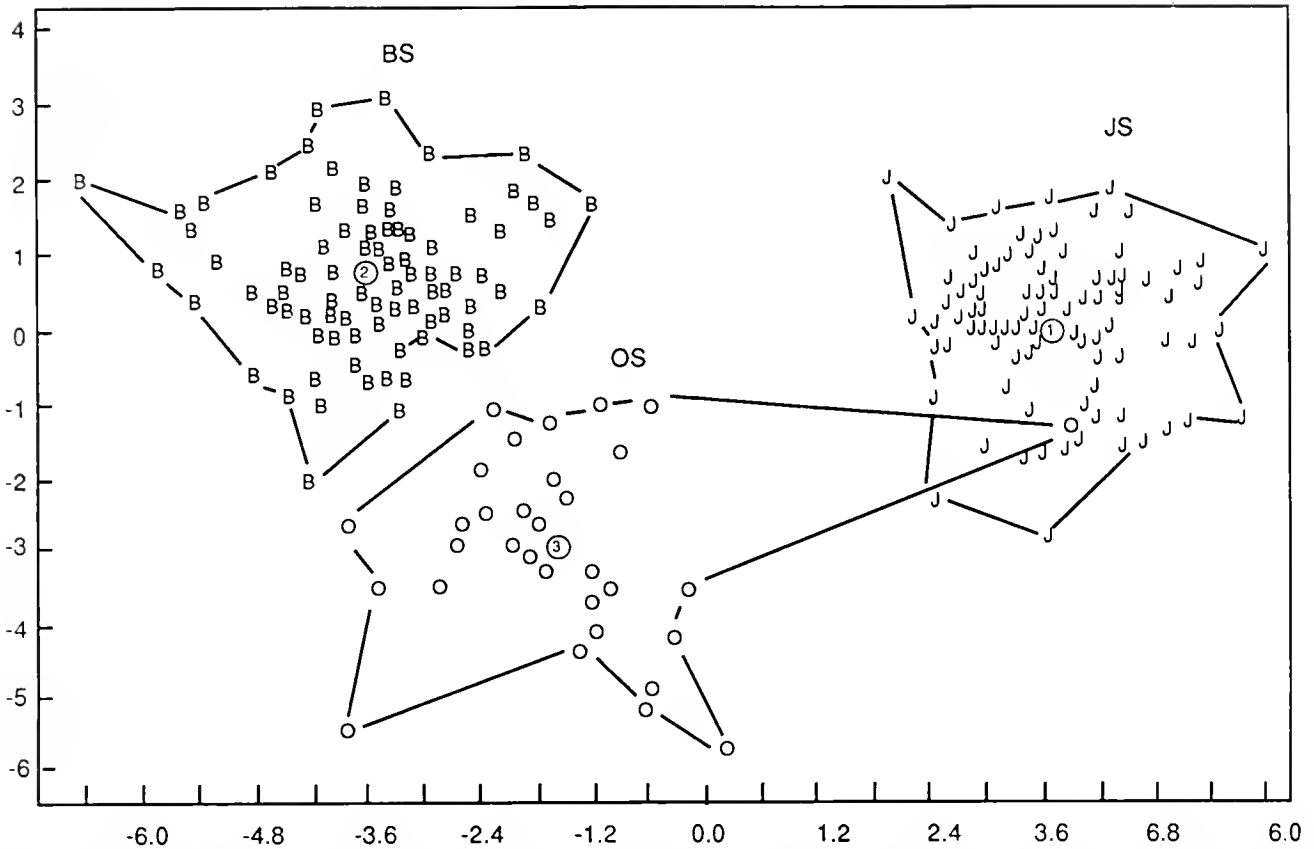


Figure 5. Female pink shrimp *Pandalus borealis* distribution at the first two canonical variables (CV) for the Sea of Japan (JS), the Okhotsk Sea (OS), and the Bering Sea (BS). Numbers in circles are mean values of CV<sub>1</sub> and CV<sub>2</sub> coordinates for the three seas. Each letter (B, O, J) corresponds to a classified individual from the given sea. The broken lines show the borders of clusters from these seas.

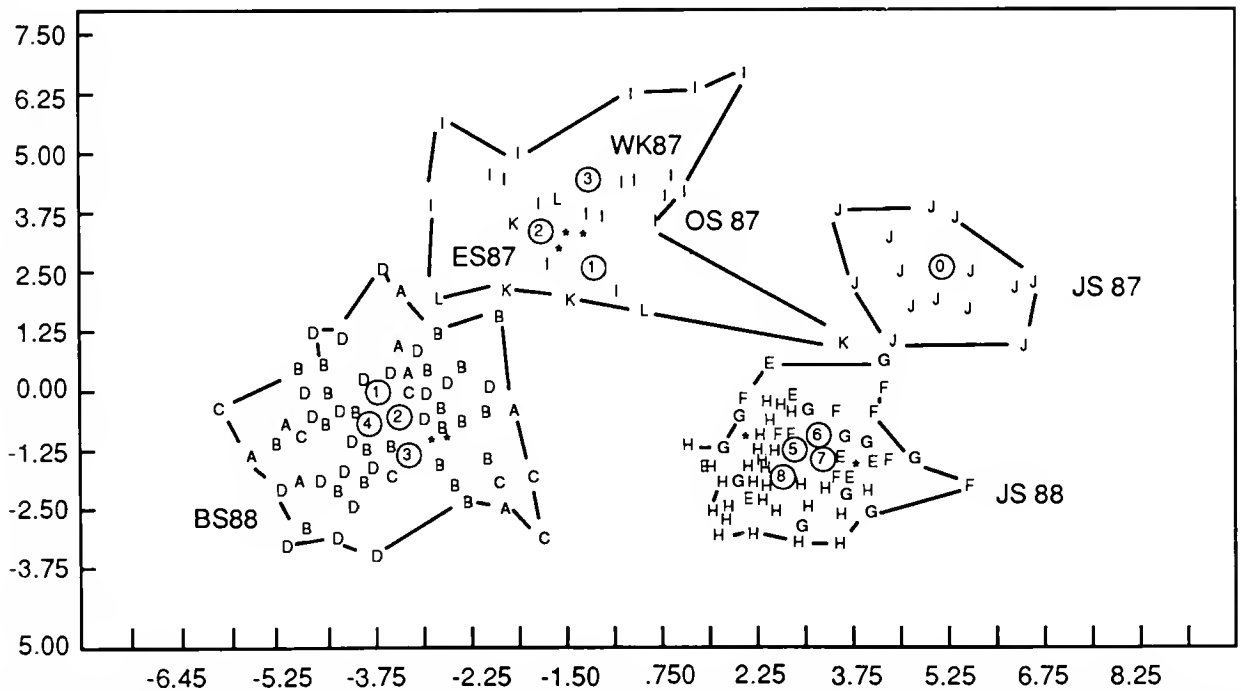


Figure 6. Female pink shrimp *Pandalus borealis* distribution on two canonical variables (CV) as exemplified by the for-sample, for-individual discriminative procedure based on 10 indices complex. The spread in values of CV for individuals and samples is shown by the corresponding letters: BS88—A, B, C, D.; JS88—E, F, G, H.; JS87—I; ES87—K, L. The asterisks indicate an overlap of two or more pairs of values. Other signs and abbreviations are identical to those in Fig. 5.

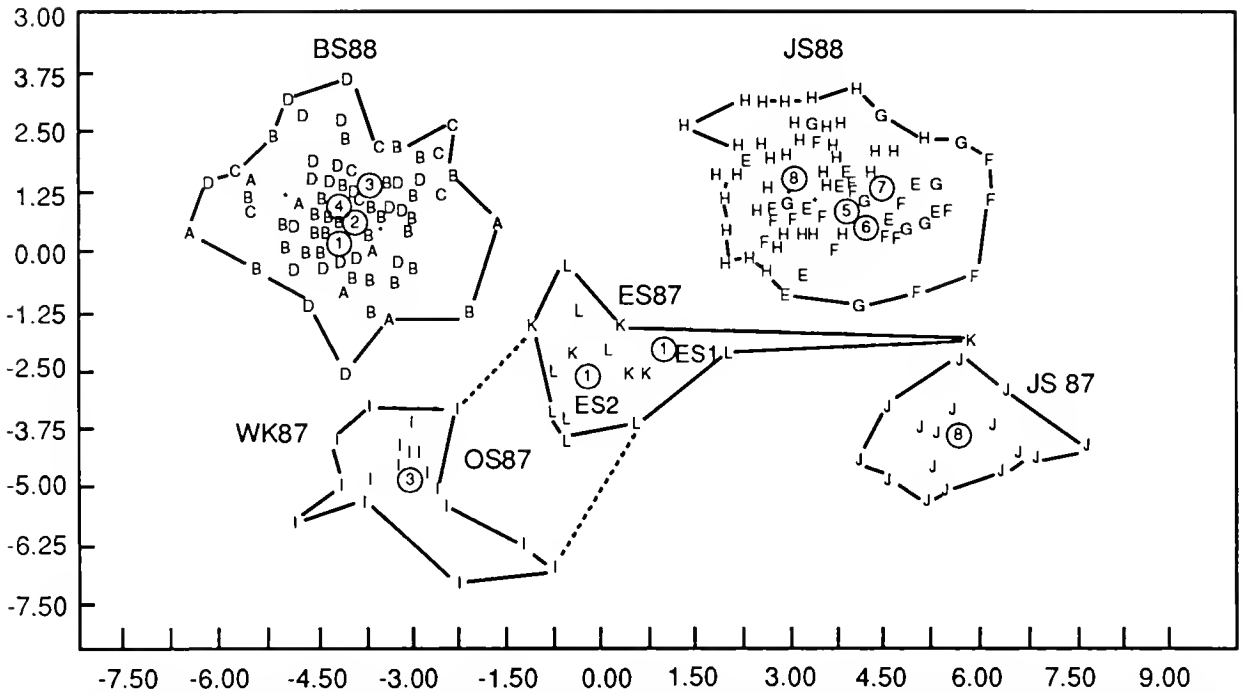


Figure 7. Female pink shrimp *Pandalus borealis* distribution on two canonical variables (CV) as exemplified by the for-sample, for individual discriminative procedure based on the combine morphological traits and indices complex. The signs and abbreviations are the same as in Fig. 5-6.

sample as to "its own" ones reached 87.5%, to the JS2 sample—73.5%. Taking into account the statistically significant differences of traits complex between some samples from the same sea, it is possible to speak about the intrapopulation differentiation of the pink shrimp. Evidently morphological differentiation of native shrimp cohorts is based on the effect of disruptive local selection at the larval and early juvenile developmental stages, the differences in the growth conditions at biotopes occupied by separate groups and due to the known certain territorial residency of adult individuals. Such morphological and ecological differentiation was also observed for shrimps from the Barents Sea (Berenboim 1978, 1982, Teigsmark 1983). The differences in morphological traits of shrimps may also depend on age structure of cohorts (Skuladottir et al. 1978).

From the information presented above on variability and similarity/distance data both for allele frequencies and morphological traits between the samples of the pink shrimp *Pandalus borealis* from three Far-Eastern seas two main conclusions can be drawn:

1. Local groups of individuals or cohorts of shrimps from the same sea are genetically homogeneous and, evidently, they are the members of the same Mendelian population.
2. Neighboring seas are inhabited by local populations which are basically different genetically and phenetically.

How do these conclusions correspond to the previously known data on population structure of the pink shrimp? As it was mentioned above, we and other authors investigated population structure of this species in the Barents Sea. It was proposed that in the Barents Sea there is only one superpopulation of the pink shrimp (Berenboim 1982). Our data on genetic composition of shrimp cohorts in this sea as well as in the Bering Sea and in the Sea of Japan (Kartavtsev et al. 1991a,b) correspond very well to this notion. Combined analysis presented here support all major statements made above. In general it is possible to claim that large, weakly differentiated populations are a common phenomenon among marine invertebrates species with a long-term planktonic larva, including crustaceans (Hedgecock et al. 1982).

Data presented in the paper do not infer that in other populations of the species their structure will be identical. For example in fjords or other regions with restricted gene flow and (or) differentiating natural selection more complex division is possible. In any case observed morphological differentiation within the sea basin should be taken into consideration in fishing of the pink shrimp populations.

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## PREDATION BY THE CRAB, *CANCER OREGONENSIS* DANA, INSIDE OYSTER TRAYS

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**ABSTRACT** *Cancer oregonensis* is a predator of sub-market size oysters (*Crassostrea gigas*). Crabs enter oyster trays as megalops larvae between May and October, and attain a carapace width (CW) of 30 mm within a year. Despite its small size, *Cancer oregonensis* has powerful chelae; molar teeth and sharp tips are well adapted for crushing and puncturing oysters. In laboratory experiments the largest *C. oregonensis* (43 mm CW) was able to open market size oysters larger than 60 mm in length, while even a 20 mm wide crab consumed oysters 30 mm in length. Medium size crabs (20-35 mm CW) consumed an average of one young oyster (20-40 mm in length) per day.

A field experiment was set up in which 15 trays, each containing  $315 \pm 23$  seed oysters, received 5, 2 or 0 newly settled *C. oregonensis*. Ten months later the average survival of oysters in the two crab treatments was 63% and 69% versus 90% for the control treatment. We recommend that crabs be manually removed during sorting operations.

**KEY WORDS:** crab, oyster culture, predation, *Cancer oregonensis*, *Crassostrea gigas*

### INTRODUCTION

Crabs have been identified as major predators in shellfish culture on the shores bordering the Atlantic and the Gulf of Mexico (Menzel and Hopkins 1955, Parsons 1974, Walne and Davies 1977, Dare et al. 1983). Quayle (1988) recognized five species of Northeastern Pacific crabs as potential predators on the Pacific oyster, *Crassostrea gigas* (Thunberg): *Hemigrapsus nudis* (Dana), *Hemigrapsus oregonensis* (Dana), *Cancer magister* (Dana), *Cancer productus* (Randall) and *Cancer gracilis* (Dana). We now add *Cancer oregonensis* (Dana) to this list. While the crabs listed by Quayle (1988) primarily attack newly-planted oysters on the seabed, *C. oregonensis* feeds on a wide size range of oysters inside suspended trays.

*Cancer oregonensis* is found in the subtidal and low intertidal zones from the Bering Sea to Santa Barbara, California (Hart 1982). *Cancer oregonensis* is a small crab, attaining a maximum carapace width (CW) of 45 mm (Morris et al. 1980). Megalops larvae settle in interstitial habitats such as rock crevices, mussel beds, barnacle patches, kelp hold-fasts, bumper tires on floating docks, and oyster trays (Hart 1982, Orensanz and Gallucci 1988, personal observation). While larger *Cancer* species leave their nursery habitats as adults, *C. oregonensis* remain in these refuge-rich habitats their entire life (Orensanz and Gallucci 1988). *Cancer oregonensis* is an opportunistic forager, feeding on barnacles, snails, bivalves, worms and algae (Knudsen 1964, Behrens Yamada, personal observation). Peak settlement of megalops larvae occurs during late spring and early summer (Jamieson and Phillips 1988, Lough 1975). Growth is rapid with some females attaining sexual maturity by the fall, just a few months after settlement (Orensanz and Gallucci 1988).

The small size of *C. oregonensis* megalops (2 mm CW; DeBrosse et al. 1989) allows them to enter oyster trays through the 6 mm holes provided for water circulation. Tray-raised oysters are thinner-shelled, and thus more susceptible to crab predators than intertidally raised oysters (C. Sanford, Innovative Aquaculture Products Ltd., Lasqueti Island, British Columbia, personal communication). Of all the crab species that settle inside oyster crabs,

*C. oregonensis* has the most powerful chelae for its size (Lawton and Elnor 1985, Behrens et al., in preparation). With stout molar teeth on the occlusal surfaces and pointed tips, the chelae appear well adapted for crushing and puncturing growing oyster (Figure 1). *C. oregonensis* is common at oyster farms off the west coast of Vancouver Island, in the northern Hood Canal, northern Puget Sound and in the Strait of Georgia where salinity remains high throughout the year. Oyster growers from these areas report predation rates on young oysters exceeding 40% and as high and 90%.

The objectives of this study were:

- 1) To determine the maximum size at which Pacific oysters are vulnerable to *C. oregonensis* of a given size.
- 2) To determine the feeding rates of these crabs on oysters in the laboratory.
- 3) To quantify predation damage of known densities of crabs inside oyster trays.
- 4) To make recommendations for crab control.

### MATERIALS AND METHODS

#### 1) Critical Size of Oysters

Laboratory trials were set up to determine the largest oyster a given size *C. oregonensis* could crush. Crabs and oysters were obtained from Westcott Bay Sea Farms, San Juan Island, Washington and transported to Oregon State University where they were kept in recirculating sea water at 14°C with 12 hour light:dark cycle. Sixteen crabs of either sex, ranging from 11-43 mm CW, were placed inside individual plastic sandwich boxes (5 × 15 × 15 cm) with mesh sides to allow for water circulation. Four single oysters ranging from 12 to 40 mm length were offered to each crab. Consumed oysters were replaced by slightly larger ones, while non-feeding crabs received oysters of a smaller size range. Containers were monitored three times a week from February 11 to March 6, 1991. Feeding trials were repeated with 17 fresh crabs from April 10 to May 28, 1991. All feeding crabs (N = 28) were sexed and the average of the two largest oyster eaten per crab was plotted against crab CW.

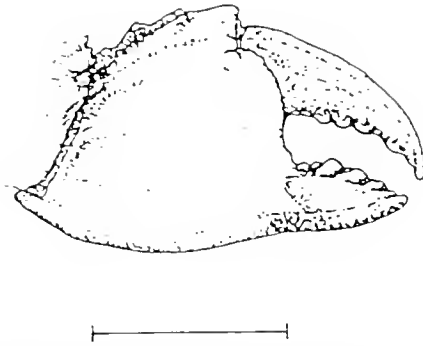


Figure 1. Right cheliped of *Cancer oregonensis* (32 mm carapace width). Scale bar = 10 mm.

## 2) Laboratory Feeding Rates

Feeding rates of crabs were determined in water tables with an open sea water system at the University of Washington Friday Harbor Laboratories, and in a re-circulating sea water system at Oregon State University (Table 1).

In a preliminary trial, 16 large crabs (30–40 cm CW) of either sex and 60 oysters ranging from 27 to 40 mm length were introduced into a sea water table (150 × 150 × 20 cm; water temperature = 14°C) at Friday Harbor Laboratories and covered with a sheet of black plastic on August 28, 1990. The number of oysters eaten in the first 9 hours was noted. Fifty more live oysters were then added to the water table and the number of oysters eaten in the subsequent 20 hours was determined.

In the next trial, 5 small crabs (19 to 28 mm CW) and 10 small oysters (21–36 mm in length) were introduced into each of 5 large plastic boxes (21 × 21 × 9 cm) in a water table (water temperature of 15°C) at Friday Harbor Laboratories. Boxes were checked daily for 5 days and consumed oysters replaced.

The subsequent trial was carried out with the same crabs at two locations. At Friday Harbor 32 crabs ranging from 17 to 44 mm CW were placed inside individual plastic sandwich boxes containing 4 oysters each. Crabs smaller than 30 mm received oysters ranging from 15 to 40 mm in length, while larger crabs received 30 to 50 mm oysters. Boxes were kept in a water table at 15°C. Feeding boxes were checked every day from June 24 to June 28 1991 and consumed oysters replaced. On June 29 all feeding crabs were transported inside a cooler to Oregon State University. For

the next 3 days crabs were fed cracked oysters and allowed to acclimate to the new conditions (water temperature = 16°C). Feeding trials resumed July 3 and continued until July 18. This time the boxes were monitored every second day. Daily feeding rates were determined for each feeding crab.

## 3) Crab Predation Inside Oyster Trays

To assess the predation pressure of *C. oregonensis* on oysters under natural conditions, we set up an experiment inside oyster trays at Westcott Bay Sea Farms on August 29, 1991. Fifteen Mexican oyster trays (56 × 57 × 7.5 cm) each received 3 liters of seed oysters (mean number per tray = 315; standard deviation = ±23), ranging in length from 28 to 35 mm. Either 5, 2 or 0 juvenile *C. oregonensis* (10 to 20 mm CW) were added to each tray.

Survival of oysters, growth of oysters and crabs, and settlement of juvenile crabs were monitored on October 12, 1991, February 7, and June 22, 1992. An average daily consumption rate per crab was estimated for all 10 crab trays by taking the number of dead oysters (difference between the number of live oysters at the beginning and the end of the experiment), subtracting 31 (the average number of dead oysters in a control tray) and dividing by the mean number of crabs in a tray (total of initial number and final number divided by 2) and by 297 d. The arcsine transformation was used on percent oyster survival before performing ANOVA on treatment effect (Sokal and Rohlf 1981).

## RESULTS

### 1) Critical Size of Oysters

The average length of the largest two oysters consumed by crabs of various CW is given in Fig. 2. No sex difference in crushing ability was detected. Since oysters vary in shape, length should not be interpreted as an absolute measure of critical size. Nevertheless, *Cancer oregonensis* of all sizes are able to crush and feed on oysters longer than their own carapace width. Thus, a crab of 20 mm carapace width can successfully attack oysters 30 mm in length, while the largest crab can open market size oysters (>60 mm).

### 2) Laboratory Feeding Rates

Over short time periods crabs are capable of consuming over 3 oysters (within their critical size range) per day. An average long-

TABLE 1.

Feeding rates of *Cancer oregonensis* in laboratory trials at Friday Harbor Labs (FHL) and Oregon State University (OSU).

Trial	Experim. Units	# crabs/unit	Crab CW (mm)	Oyster Length (mm)	Duration	Feeding Rate		(#/day/crab)
						Mean	(SD)	Maximum
FHL 14°C	1 water table	16	30–40	27–40	9 h 20 h	5.5 2.0		
FHL 15°C	5 large boxes	5	19–28	21–36	5 d	0.62	(0.25)	1
FHL 15°C	19 boxes	1	17–29	15–40	4 d	1.06	(0.76)	4
	13 boxes	1	31–44	30–50	4 d	0.77	(0.68)	3
OSU 16°C	16 boxes	1	17–29	15–40	15 d	1.16	(0.50)	3
	9 boxes	1	31–44	30–50	15 d	0.99	(0.18)	3

For details on experimental design see text.

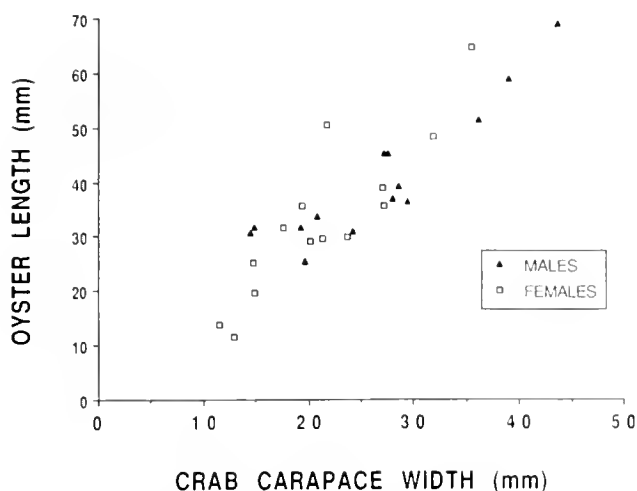


Figure 2. Average of two largest oysters crushed by crabs of various carapace width. No significant difference was found between male and female crabs.

term feeding rate of 1 oyster per day, however, is more realistic in that this rate includes data from molting crabs that ceased feeding for up to 6 d.

### 3) Crab Predation Inside Oyster Trays

Survival of experimental crabs inside oyster trays was 77% over the 297 days of the experiment. Since crabs died at various times throughout the year, no significant regression between number of surviving crabs and number of dead oysters was found. Their average carapace width was 23 mm in October, 26 mm in February and 30 mm in June. At termination of the experiment the oysters had attained an average length of 60 mm.

*Cancer oregonensis* open oysters by progressively chipping away at the shell margin or, more commonly, by puncturing the shell. Small, rapidly-growing oysters with fragile shells and thin lips are particularly vulnerable.

The average feeding rate of crabs inside the 10 oyster trays was estimated to be 0.09 (standard deviation =  $\pm 0.05$ ) oysters per crab per day. This value is one order of magnitude lower than those observed in laboratory trials. One reason for this discrepancy is that throughout the field experiment, the average oyster was over twice the carapace width of the average crab.

Survival of oysters inside control trays was significantly greater than in trays containing an initial 2 or 5 crabs (ANOVA for arcsine transformed percentages:  $F = 12.939$ , d.f. = 2,  $p < 0.001$ ) (Figure 3). Survival in control trays ranged from 88 to 93% while trays with crabs ranged from 37 to 81%.

Only 3 newly-settled juveniles entered the 15 trays between August 29 and October 12, while none were recovered in February. On June 22, eighteen newly-settled *Cancer oregonensis* (3–5 mm CW) were recovered.

## DISCUSSION

*Cancer oregonensis* has the potential to be an important predator inside suspended oyster trays. Since tray-raised oysters are thin-shelled, they do not attain an absolute size refuge from these powerful crab predators as is the case for sea bottom-reared oysters on Atlantic shores. Eggleston (1990) and Elner and Lavoie (1983) found that American oysters (*Crassostrea virginica* (Gmelin))

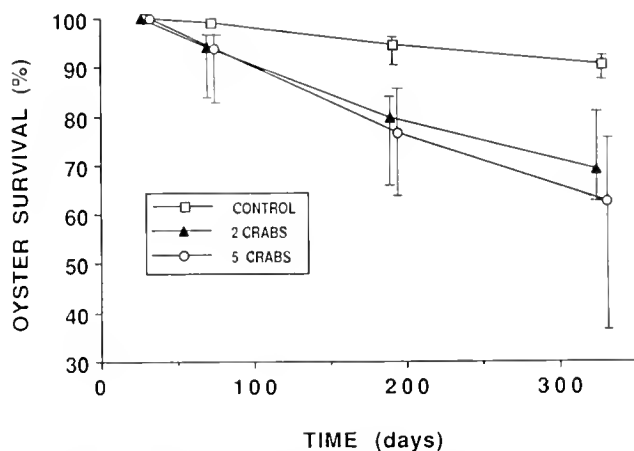


Figure 3. Mean survival rate (%) of seed oysters inside trays with and without crabs. The range of values are indicated by error bars.

larger than 30 mm in length were rarely opened by lobster (*Homarus americanus* (Milne-Edwards)), rock crab (*Cancer irroratus* (Say)) or blue crab (*Callinectes sapidus* (Rathbun)). The largest *C. oregonensis*, however, can open market size oysters for up to 60 mm in length, while even 20 mm CW crabs consume 30 mm length oysters.

At an average consumption rate of 0.1 oyster per day, five crab inside a tray could eat 120 oysters in the 8 mo during which the oysters are normally kept in trays. That represents a 40% reduction in oyster survival and profit. The winter and spring of 1992 were unusually mild, with oysters growing and surviving well. In years when oysters grow more slowly, crabs would gain a size advantage over the oysters and could cause more devastating effects than we measured.

*Cancer oregonensis* megalops larvae with a carapace width of 2 mm (Lough 1975) can easily enter the 6 mm diameter holes of Mexican oyster trays. Once inside, the larvae metamorphose into first stage crabs of 3 mm CW (Orensanz and Gallucci 1988, and personal observation). Oyster trays are ideal crab habitats, with abundant food and protection from predators such as the octopus. In addition to oysters, growing crabs can feed on fouling organisms such as algae, sponges, tunicates, sea cucumbers, gunnels, barnacles and mussels.

Settlement of *C. oregonensis* megalops larvae off the west coast of Vancouver Island occurs from April to August, with a peak abundance in late June (Jamieson and Phillips 1988). Our observations suggest that the settlement peak in 1991 occurred during late June, but that in June 1992 it occurred two weeks earlier. In our experimental oyster trays, we observed some newly settled *C. oregonensis* between late August and early October, none between October and February, and a moderate settlement during June 1992. While Lough (1975) reports some megalops larvae in Oregon plankton samples during the winter, the chance of a commercially important settlement to occur from October to April appears low.

### Recommendations for Crab Control

Since *C. oregonensis* attack oysters larger than their own carapace width, and since newly-settled crabs become oyster predators within 3 months, crabs of all sizes should be removed from oyster trays. Growers at Skerry Bay on Lasqueti Island, use a freshwater bath to rid their oyster trays of sea stars and crab pred-

ators (C. Sanford, personal communication). *Cancer* crabs, are osmoconformers (Dehnel and Carefoot 1965) and are thus intolerant of low salinities. Smaller *C. oregonensis*, with higher surface area to volume ratios, would be especially susceptible to fresh water baths.

Growers at Westcott Bay Sea Farms manually remove larger crabs (>20 mm in CW) from their trays when oysters are sorted. They report an increase in the survivorship of young oysters since this predator control measure was started four years ago (B. Peoples, personal communication). Crab predation, however, remains a problem during the winter when oysters grow more slowly and trays are not checked as frequently. Since crabs continue to feed during the winter, a special effort should be made to rid trays of all crabs during the last oyster sorting operation in the fall.

#### ACKNOWLEDGMENTS

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## INTERSEX AUSTRALIAN RED CLAW CRAYFISH (*CHERAX QUADRICARINATUS*)

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The Australian red claw crayfish has recently been considered a candidate for commercial aquaculture in the United States because of characteristics, such as large size, ease of reproduction, multiple spawnings, high fecundity, gregarious behavior, and higher percentage of tail meat than red swamp crayfish (*Procambarus clarkii*) (Jones 1990, Medley et al. 1991, Rouse et al. 1991). In 1989 and 1990, red claw juveniles were purchased from commercial hatcheries in Queensland, Australia, and Missouri, USA, respectively. These animals and their progeny were used in aquaculture production experiments conducted from May to October, 1990, at the Alabama Agricultural Experiment Station, Fisheries Research Unit, Auburn University, Alabama. During experimental culture, some red claw were noted to possess both male and female secondary sexual characteristics. Normally, crayfish are distinguished by distinct dimorphic secondary sexual characteristics. Males have two genital openings at the base of the fifth pereopods and females have two genital openings at the base of the third pereopods. Intersex crayfish or "pseudohermaphrodites", are characterized by aberrant secondary sexual characteristics of both male and female (Sokol 1988, Huner and Barr 1991).

In production trials, red claw crayfish averaging 3.7 g were stocked into three, 0.02-ha, fertilized ponds at a rate of 1/m<sup>2</sup> and fed hay at a rate of 500 kg/ha/mo (Medley 1991). The crayfish were cultured for 165 days. Average survival (mean  $\pm$  SE) of red claw at harvest for the three 0.02-ha earthen ponds was 85.5  $\pm$  4.6%. At harvest, crayfish were sampled (n = 326), sexed, and weighed. Total average weight (n = 513) for harvested crayfish was 70.0  $\pm$  1.9 g (mean  $\pm$  SE). Mean weights among the three production ponds were not significantly different ( $P > 0.05$ ). Red swamp crayfish averaging 3.3 g were also cultured under conditions identical to those used to raise red claw crayfish.

At harvest, no red swamp crayfish possessed aberrant secondary sexual characteristics (n = 116). Females with male secondary sex characteristics have been reported for red swamp crayfish, but this type of pseudohermaphroditism is not common (Huner and Black 1977). To date, only one case of true hermaphroditism has been documented in red swamp crayfish (Huner and Black 1977). Pseudohermaphroditism is more common among other native American crayfish of the genera *Cambarus* and *Orconectes* (Turner 1935, Huner and Barr 1991).

Among red claw the following combinations of gonopore placement were observed: (1) one male opening on right or left side and two normally-positioned female openings, (2) one male opening on right or left side and one female opening on opposite side, (3) one male opening on right side and one female opening on right side, (4) two normally-positioned male openings and one female opening on right or left side, (5) two normally-positioned female and male openings. Individuals with only one male or one

female genital opening were also found, but were not classified as intersex.

Of the above intersex types, combination (1) was the most common, comprising 31% of sampled intersex crayfish, whereas combination (3) was the least common, comprising only 2% of intersex crayfish. Normal males occurred most frequently in the culture ponds, comprising 60% of sampled crayfish, while intersex crayfish were the least common comprising 17%.

Average weights among the red claw crayfish sexes were significantly different ( $P < 0.01$ ). Mean weights for male, female, and intersex crayfish from the three ponds were 75.7 g, 58.2 g, and 77.8 g, respectively, with a mean square error (MSE) of 3.6. Intersex crayfish were larger than females ( $P < 0.01$ ), whereas no significant difference could be detected between males and intersex crayfish ( $P > 0.05$ ) (Fisher's protected LSD).

Although intersex individuals do occur in several species of Australian parastacids (Sokol 1988, Lake and Sokol 1988), nothing has been mentioned about this phenomenon in red claw crayfish. Several studies have been conducted with red claw crayfish that present information on sexual differentiation, reproduction, and biology (Sammy 1988, Jones 1990, Merrick and Lambert 1991), but none of these specifically mentioned the presence of intersex red claw crayfish. Merrick and Lambert (1991) gave a brief mention of intersex Australian crayfish possessing both male and female openings, and functioning as males.

Whether intersex red claw are functional hermaphrodites or in a transitional phase between sexes is not certain. Turner (1935) mentioned that a crayfish cannot be considered a true hermaphrodite unless both testicular and ovarian tissue are present. Of the intersex crayfish sacrificed and examined internally, one had a complete testis on one side and what appeared to be undeveloped ovarian tissue on the opposite (Fig. 1). However, in the absence of histological, anatomical, and endocrinological data, we cannot address the reasons for the observed intersex situation reported



Figure 1. Dorsal view of dissected red claw crayfish showing ovarian tissue (o) on right and testicular tissue (t) on left.

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here. Determination of whether the occurrence of intersex crayfish is a genetic or hormonal phenomenon, or a condition triggered by environmental factors will require further study.

Since red claw exhibit intersexuality, there may be merit to sex-reversal using hormone treated feeds similar to those used for

cultured fishes such as tilapia (*Oreochromis* spp.) (Clemens and Inslee 1968, Pandian and Varadaraj 1988). Production of all-male populations would eliminate spawning in grow-out ponds thus allowing red claw to divert more energy to growth. Male red claw are also larger, on average, than females.

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## EVALUATION OF MICROBIAL INDICATORS FOR THE DETERMINATION OF THE SANITARY QUALITY AND SAFETY OF SHELLFISH

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**ABSTRACT** Shellfish consumed either raw or partially cooked have been implicated in the transmission of viral gastroenteritis and hepatitis A. The effectiveness of bacterial indicators to signal the presence of human pathogenic viruses has been questioned. Earlier viral assays made it impractical to monitor shellfish for viral contaminants. There exists a need for rapid and sensitive assays for human enteric viruses to ensure the sanitary quality of shellfish. Sample collections of hard-shell clams (*Mercenaria mercenaria*) were taken from approved, conditionally approved and prohibited shellfishing areas in Narragansett Bay, Rhode Island between July 1989 and May 1990. Clams were assayed for poliovirus and other microbial indicators (total coliforms, fecal coliforms, *Clostridium perfringens*, enterococci and male-specific bacteriophage) to evaluate their usefulness as viral indicators. Of these indicators, bacteriophage were most consistently recovered from each of the collection areas, and enterococci were recovered with the least frequency. Poliovirus was detected in clams from the conditionally approved and prohibited area primarily during the fall and winter months. On one occasion in the prohibited area, the coliform standards for water and shellfish were not exceeded, although poliovirus was detected by a hybridization probe assay. A viral indicator system based on bacteriophage levels would require further development and evaluation to determine the correlation of specific human enteric viruses and phage. New advances in nucleic acid technology may soon enable routine monitoring of shellfish for enteric viruses.

**KEY WORDS:** male-specific bacteriophage, poliovirus and hybridization probe

### INTRODUCTION

Shellfish have been widely recognized as a means of transmission of foodborne enteric disease since early this century, when a number of serious shellfish associated typhoid fever outbreaks were reported (Guzewich and Morse 1985). Edible bivalve molluscs of the class Pelecypoda (oysters, clams, and mussels) are the only molluscan shellfish of commercial importance for which sanitary controls are currently required (Metcalf 1975). Although the National Shellfish Sanitation Program (NSSP) bacterial indicator system has decreased the incidence of shellfish-associated enteric disease, its efficacy as a reliable indicator for protecting against the presence of human enteric viruses is questionable (Wait et al. 1983). One of the principal concerns with the present indicators and standards are that coliform bacteria are much more sensitive to chlorine than are a number of human enteric viruses, such as hepatitis A virus (Engelbrecht and Greening 1978). Also, the survival of certain human enteric viruses in environmental water, during the winter months, is substantially greater than that of coliforms.

During the last several decades, viral infections appear to account for the majority of foodborne illnesses in the U.S. During 1982 there were 103 well-documented cases of gastroenteritis associated with the consumption of raw shellfish involving 1,017 individuals in New York. The predominant etiological agent was determined to be Norwalk virus (Guzewich and Morse 1985,

Morse et al. 1986). Other outbreaks of viral gastroenteritis and hepatitis A related to the consumption of raw or partially cooked shellfish have been reported (Gill et al. 1983, Portnoy et al. 1975, Richard 1985) as well. There is good likelihood that the incidences of individual cases and isolated outbreaks of shellfish-associated viral illnesses are significantly underreported.

All viruses known to be normally transmissible through foods are derived from the human intestine (Blackwell et al. 1985). The discharge of both treated and untreated sewage into waterways, being utilized as sources of seafood, has gained much attention in regard to viral contaminated shellfish (Gerba and Goyal 1978, Landry et al. 1983). All species of commercially important shellfish have been shown to enteric viruses from environmental seawater during routine feeding activities (Metcalf et al. 1980). Since ordinary wastewater treatment does not always completely remove or disinfect such viruses, there is a need to be able to assess the efficacy of current indicators and standards.

Currently, there is no one organism that can be considered to be the ideal indicator. Since it is impractical, indeed impossible to test for each individual bacterial or viral pathogen, the use of an alternative indicator, one that best correlates with the survivability and occurrence of the most resistant human enterovirus is probably the most practical means to ensure the sanitary quality of shellfish. The feasibility of using other indicator organisms such as fecal streptococci (Berg and Metcalf 1978) *Clostridium perfringens* (Emerson and Cabelli 1982) and bacteriophage (Havelaar et al. 1986) have been discussed. Assays involving the detection of enteroviruses in shellfish by cell culture (Bemiss et al. 1989, Idema

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et al. 1991) and by the use of hybridization probes (Bruce et al. 1989, Jiang et al. 1986, Margolin et al. 1986) have been evaluated.

The objective of this study was to determine the levels of and compare the relationships between bacterial indicators, male-specific bacteriophage, and poliovirus found in shellfish collected from approved, conditionally approved, and prohibited waters.

## MATERIALS AND METHODS

### *Shellfish and Water, Collection and Handling*

Hardshell clams (*M. mercenaria*) for this study were harvested from Narragansett Bay, Rhode Island. Samples were collected at approximately one month intervals. Clams were obtained with a long handled shellfish-rake from approved, conditionally approved, and prohibited waters and held in the polypropylene bags on ice. Samples from each of the three collection areas were taken from approximately the same sites over the course of the study. Clams were not segregated by size prior to analyses; therefore, large sized and also those typically eaten raw ('little necks') were analyzed together. The clams were divided into two equal portions, one assayed for poliovirus, and the other assayed for male-specific bacteriophage (MSB) and other bacterial indicators. Surface water samples were obtained at each site when shellfish were harvested. Water samples were collected in sterile, 500 ml, polypropylene screw cap bottles (Nalgene Laboratories Inc., Rochester, NY), and were held on ice until examined in the laboratory. Water samples were analyzed for total coliforms and fecal coliforms.

### *Microbiological Analyses*

#### (i) Shellfish

Approximately 10 clams were used in each analysis. Clams were scrubbed with a sterile brush, opened, and the entire contents (meat and liquor) were placed in sterile blender jars (Waring Corp., Corning, NY). Samples were blended at high speed for two minutes and held on ice (up to 60 minutes) until assayed.

Total and fecal coliform densities in shellfish were determined by a most-probable-number (MPN) procedure, using lauryl tryptose broth (Difco) as the selective enrichment medium prescribed in Recommended Procedures (American Public Health Association 1970). Fecal coliforms were confirmed in EC-MUG medium (Difco) (Rippey et al. 1987). Enterococci densities were determined by a 5-tube MPN procedure, utilizing azide dextrose broth (Difco) as the selective enrichment medium. Confirmation of tubes exhibiting growth was carried out at 24 and 48 hours; all positive tubes were streaked onto membrane filters (HC filters; Millipore Corp., Bedford, MA) placed onto Me (Levin et al. 1975) agar as previously described (Dufour 1980) with indoxyl- $\beta$ -D-glucoside (Sigma, St. Louis, MO). The modified Me plates were incubated for 24 hours at 41°C, and tubes positive for enterococci were confirmed by the presence of blue growth along the streaks. The levels of *C. perfringens* in shellfish were determined by an iron milk MPN procedure (Abeyta 1983). MSB levels were determined using a modified double-agar-overlay procedure (Cabelli 1988), utilizing *E. coli* strain (HS[pFamp]R). Plaques were counted after 18 to 24 hours of incubation at 35°C. MSB densities were calculated per 100 g of shellfish; determined by the number of plaques per volume of supernate assayed times the total volume

of supernate obtained times 100 g divided by the number of g of homogenate examined.

#### (ii) Water

Samples were analyzed utilizing a multiple tube fermentation technique with lauryl tryptose broth as the selective enrichment medium (Difco), according to the Recommended Procedures (American Public Health Association 1970). All tubes exhibiting gas production were confirmed for coliforms in brilliant green lactose bile broth (Difco) and for fecal coliforms in EC broth (Difco).

### *Poliovirus Elution from Shellfish Meats*

Clams were scrubbed with a sterile brush, opened, and 200 g of meat was transferred to a stainless steel canister (Omni Corporation, Waterbury, CT). Two hundred ml of elution medium, consisting of 3% beef extract, 3.2% NaCl and 90 mM glycine, at pH 9.5, was added to the sample (Deleon et al. 1986). The sample was homogenized using a Omni-Gen homogenizer (Omni-Gen), the pH checked and adjusted to 9.5 with 1 N NaOH, then centrifuged (10,000  $\times$  g for 10 minutes) (Beckman model J2-21M, Fullerton, CA). The supernatant was decanted, pH adjusted to 7.0 with 1 N HCl, and then divided into two aliquots. One aliquot (non-flocculated) was used for direct analysis by a hybridization probe and cell culture. The second aliquot was concentrated by acid precipitation (flocculated) (Katzenelson et al. 1976) prior to analysis by probe and cell culture techniques. The pellet generated by flocculation was resuspended in 0.1 M Na<sub>2</sub>PO<sub>4</sub> buffer at pH 9.5. The sample pH was checked and adjusted to 9.5 with 1 N NaOH, mixed for 5 minutes, and then centrifuged (10,000  $\times$  g for 10 minutes). The supernate was adjusted to pH 7.0 and the final volume adjusted to 30 ml.

### *Phenol Chloroform Extraction of Viral Nucleic Acid*

Viral nucleic acid was liberated from both the flocculated and non-flocculated samples as follows; approximately 50 ml of the non-flocculated and 10 ml of the flocculated sample were individually mixed with phenol:chloroform:isoamyl alcohol (25:24:1). Samples were vortexed for two minutes, centrifuged (10,000  $\times$  g for 10 minutes), and the aqueous phase was removed and transferred to new phenol:chloroform:isoamyl. The original tube was extracted two more times by the addition of diethyl-pyrocabonate (DEPC) treated water, and each time the aqueous phase was removed and transferred to new phenol:chloroform:isoamyl. The aqueous phases from the original tubes were extracted until there was a minimal amount of protein present. Residual phenol was removed by chloroform extractions followed by an ether extraction to remove the residual chloroform. Filtered air was passed through the sample using DEPC treated pipet tips to evaporate off the remaining ether. The samples were applied to a Genescreen Plus hybridization membrane (New England Nuclear) using a vacuum manifold dot blot apparatus (Bio-Rad, Richmond, CA). Two ml of extracted sample was applied to each well, the membranes were baked in an 80°C incubator for two hours.

### *Hybridization Probe Preparation and Hybridization*

Fragments of poliovirus cDNA, from poliovirus cDNA (bp 115-7440) (kindly supplied by David Baltimore) cloned into the Pst I site of pBR322 and transformed in *E. coli* HB-101 were used as the probe. Following amplification, the recombinant plasmid

was isolated (Maniatis et al. 1989). The insert was excised from the vector by performing a Pst I digest (Boehringer Mannheim Corp., Indianapolis, IN). Two bands corresponding to poliovirus cDNA, the 1174 base pair and the 1689 base pair bands were excised from the gel and purified by either a commercially available kit (Gene Clean II, La Jolla, CA) or by electroelution (Schleicher and Shuell, Keene, NH). These fragments were used as the probe in this study.

The cDNA fragments were labeled with  $^{32}\text{P}$  dCTP using a random primer extension labeling kit (New England Nuclear, Boston, MA). Probe activity was determined by a scintillation counter, activities of  $5 \times 10^8$  to  $1 \times 10^9$  were obtained.

Membranes containing the extracted nucleic acid samples were first prehybridized and then hybridized in heat sealed poly bags as described in Gene Screen Plus protocols (New England Nuclear, Boston, MA). The membranes were prehybridized at  $42^\circ\text{C}$  for 2 hours in a reciprocating water bath and hybridized for 36 hours at  $42^\circ\text{C}$  in the same solution, except for the addition of  $10^6$  to  $10^7$  cpm of the heat denatured radiolabeled probe. The membranes were washed twice in a solution of  $2\times$  sodium chloride/sodium citrate (SSC) 1% sodium dodecyl sulfate (SDS) for 15 minutes with constant agitation at room temperature, and once in  $2\times$  SSC/0.1% SDS with constant agitation at  $52^\circ\text{C}$ . The membranes were then air dried, and placed in a cassette with Dupont Cronex intensifying screens and Kodak XAR-5 film for 36 hours at  $-70^\circ\text{C}$ .

#### Cell Culture Analysis

The viral assay was performed using a continuous cell line of Buffalo Monkey Green Kidney (BGM) cells. Cells were grown in minimal essential media (MEM) (Sigma) supplemented with 8% fetal calf serum, 292 mg/L glutamine, 0.075% sodium bicarbonate, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 ug/ml kanamycin and 25 U/ml mycostatin. Three  $75\text{ cm}^2$  tissue culture flasks with confluent monolayers of BGM cells were each inoculated with 3 ml of sample. Adsorption of virus was allowed to proceed for 2 hours at  $37^\circ\text{C}$ , flasks were rocked every 15 minutes. Fol-

lowing adsorption, the cells were washed with phosphate buffered saline (PBS) and overlaid with maintenance medium. Flasks were incubated at  $37^\circ\text{C}$  and examined periodically for the presence of cytopathic effects (CPE) up to 14 days following inoculation. Flasks that exhibited CPE were confirmed by passage to new monolayers of BGM cells.

## RESULTS

Samples of surface waters and clams from Narragansett Bay were obtained during the period of July 1989 to May 1990. A total of 9 collection trips were made for each of the three different shellfish classification areas. The bacteriological quality of clams and their overlying waters from the approved area are presented in Table 1, from the conditionally approved area in Table 2, and from the prohibited area in Table 3.

Water from the approved area exceeded the total coliform standard (70/100 ml) once (April), and the fecal coliform standard (14/100 ml) was exceeded in another collection (May). Water quality for the majority of the conditionally approved area samples exceeded the coliform and fecal coliform levels found in the approved area (note: this area was conditionally closed during all but the 8/30/89 sample collection). From the conditionally approved area, 6 of 9 water samples exceeded the total coliform standard for approved areas, whereas 5 of 9 samples exceeded the fecal coliform standard. Coliform and fecal coliform NPNs from the prohibited area were greater than levels found for both the approved and conditionally approved areas. In the prohibited area 8 of 9 of water samples exceeded the coliform standard, and 7 of 9 samples exceeded the fecal coliform standard.

One of 8 clam samples obtained from the approved area and the conditionally approved area exceeded the fecal coliform market guideline (230/100 g), whereas 2 of 8 prohibited area samples exceeded the guideline.

*Clostridium perfringens* was detected in the prohibited area with the greatest frequency, and levels there remained detectable throughout all the collection times. Overall, levels of *C. perfrin-*

TABLE 1.  
Microbial indicator levels in waters and clams from the approved area.

Sample Date	Water <sup>a</sup>		Clams					Poliovirus			
	Total Coliforms	Fecal Coliforms	Total Coliforms <sup>b</sup>	Fecal Coliforms <sup>b</sup>	<i>Clostridium perfringens</i> <sup>b</sup>	Enterococci <sup>b</sup>	Male-specific Bacteriophage <sup>c</sup>	Probe		Cell Culture	
								Eluent	Floc	Eluent	Floc
7/31/89	<1.8	<1.8	nd <sup>d</sup>	nd	nd	nd	nd	-	-	na <sup>e</sup>	-
8/30/89	<1.8	<1.8	20	20	140	<20	15	-	-	na	-
10/3/89	<1.8	<1.8	110	110	200	<20	3	-	-	na	-
11/7/89	49	7.8	68	68	<20	<20	127	-	-	na	-
1/10/90	2	2	20	20	<20	<20	7	-	-	na	-
2/12/90	9.3	2	<20	<20	<20	<20	14	-	-	na	-
3/13/90	<1.8	<1.8	<20	20	<20	<20	15	-	-	na	-
4/16/90	130	2	20	78	790	<20	<16	-	-	na	-
5/31/90	39	17	140	1,300	2,200	<20	83	-	-	na	-

<sup>a</sup> MPN per 100 ml.

<sup>b</sup> MPN per 100 g.

<sup>c</sup> Densities per 100 g calculated from plaque counts.

<sup>d</sup> Not determined.

<sup>e</sup> Not analyzed due to toxicity.

TABLE 2.  
Microbial indicator levels in waters and clams from the conditionally approved area.

Sample Date	Clams							Poliovirus			
	Water <sup>a</sup>		Total Coliforms <sup>b</sup>	Fecal Coliforms <sup>b</sup>	<i>Clostridium perfringens</i> <sup>b</sup>	Enterococci <sup>b</sup>	Male-specific Bacteriophage <sup>c</sup>	Probe		Cell Culture	
	Total Coliforms	Fecal Coliforms						Eluent	Floc	Eluent	Floc
7/31/89	4.5	<1.8	nd <sup>d</sup>	nd	nd	nd	nd	-	-	na <sup>e</sup>	-
8/30/89	240	49	78	<20	1,300	20	25	-	-	na	-
10/3/89	33,000	2,300	5,400	790	2,400	110	501	+	-	na	-
11/7/89	79	11	490	140	20	68	180	+	-	na	-
1/10/90	2	2	<20	<20	20	<20	38	-	-	na	-
2/12/90	920	220	<20	<20	78	<20	322	+	-	na	-
3/13/90	<1.8	<1.8	<20	<20	<20	<20	147	-	-	na	-
4/16/90	2,400	790	68	<20	110	<20	198	-	-	na	-
5/31/90	79	33	790	110	490	<20	3	-	-	na	-

<sup>a</sup> MPN per 100 ml.

<sup>b</sup> MPN per 100 g.

<sup>c</sup> Densities per 100 g calculated from plaque counts.

<sup>d</sup> Not determined.

<sup>e</sup> Not analyzed due to toxicity.

*gens* were seen to decrease from the prohibited to conditionally approved to the approved area, and the latter exhibited the most samples with levels in clams below the detectable limits. Enterococci densities were generally below detectable levels throughout all the collection times in all the areas. The highest levels of enterococci were detected in clams from the prohibited area, and densities appeared to decrease in clams from the conditionally approved area. No enterococci were detected in any clam samples from the approved area.

The occurrence of MSB and results obtained for poliovirus in the approved area are shown in Table 1; Table 2 shows the results for the conditionally approved area; and those obtained for the prohibited area given in Table 3. Phage levels detected were greatest in the prohibited area, and these were notably higher than those

detected in the conditionally approved area; the lowest levels detected were found in the approved area. MSB in clams (per 100 g) were detected in 7 of 8 samples examined from the approved area. In the conditionally approved and prohibited area MSB (per 100 g) were detected in all samples analyzed.

In the approved area, hybridization probe results for poliovirus for both the non-flocculated and flocculated sample portions, were negative at all times. In the conditionally approved area, 3 of 9 non-flocculated clam samples were positive for viral nucleic acid, by probe analysis, whereas none of the flocculated samples were found to be positive. In the prohibited area, 4 of 9 non-flocculated samples were found positive by the probe assay, but only 2 of these 4 were positive using the flocculated samples.

Cell culture analyses of clam samples for poliovirus were per-

TABLE 3.  
Microbial indicator levels in waters and clams from the prohibited area.

Sample Date	Clams							Poliovirus			
	Water <sup>a</sup>		Total Coliforms <sup>b</sup>	Fecal Coliforms <sup>b</sup>	<i>Clostridium perfringens</i> <sup>b</sup>	Enterococci <sup>b</sup>	Male-specific Bacteriophage <sup>c</sup>	Probe		Cell Culture	
	Total Coliforms	Fecal Coliforms						Eluent	Floc	Eluent	Floc
7/31/89	>1,600	1,600	5,400	nd <sup>d</sup>	5,400	460	3,042	-	-	na <sup>e</sup>	-
8/30/89	>1,600	>1,600	9,200	1,100	2,400	230	465	-	-	na	-
10/3/89	49,000	2,300	>16,000	3,500	3,500	3,500	5,078	+	+	na	-
11/7/89	1,100	170	790	220	490	230	1,027	+	-	na	-
1/10/90	49	6.8	20	<20	140	<20	1,036	+	-	na	+
2/12/90	130	2	<20	<20	110	<20	994	+	+	na	-
3/13/90	490	130	<20	<20	78	<20	2,700	-	-	na	-
4/16/90	22,000	2,300	1,700	45	330	140	1,180	-	-	na	-
5/31/90	350	49	2,200	93	3,500	<20	124	-	-	na	-

<sup>a</sup> MPN per 100 ml.

<sup>b</sup> MPN per 100 g.

<sup>c</sup> Densities per 100 g calculated from plaque counts.

<sup>d</sup> Not determined.

<sup>e</sup> Not analyzed due to toxicity.

formed using the flocculated portions only. since the non-flocculated portions were toxic to the BGM cells. Results of clams from the approved and conditionally approved area failed to detect cytopathic effects (CPE) with any of the samples. One of the 9 samples from the prohibited area was found to cause CPE on the BGM cells.

### DISCUSSION

Periodic outbreaks of non-bacterial gastroenteritis and hepatitis A have indicated that the current means of evaluating the sanitary quality of shellfish and their harvesting waters requires re-evaluation. This study compared several bacterial indicators and MSB, to the occurrence of poliovirus in shellfish collected from approved, conditionally approved, and prohibited areas over about a one year period. Poliovirus was used because of a recently developed nucleic acid probe technique, its ease of detection by cell culture techniques, and the generally higher degree of poliovirus prevalence in sewage, relative to that expected for other enteric viruses. Earlier studies, (Margolin, unpublished results), demonstrated that this hybridization probe assay was able to detect virus with a sensitivity comparable to cell culture. The nucleic acid hybridization assay for poliovirus permitted analysis of shellfish non-flocculated portions directly. This could not be done with cell culture due to the toxic effects of the non-flocculated portion on BGM cells. Consequently, all samples evaluated by cell culture were further processed by flocculation of the sample. Although flocculation adequately reduces sample toxicity, it also provided results with a reduced level of detectable poliovirus, indicating the procedure is not 100% efficient for poliovirus recovery. Water samples were analyzed for coliforms only, as this currently is a monitoring tool in determining the sanitary quality of shellfish harvesting areas.

As expected, the approved area exceeded the coliform standard the least number of times. Results for the conditionally approved area show a wide variation in coliform levels, likely due to the affects from rainfall events which occurred prior to many of the collection periods. During most of the sample collections (all collection times except 8/89), the area was temporarily (conditionally) closed due to excess rainfall. Samples taken from the prohibited area usually exceeded the coliform standard. However, in one instance (January), coliform levels detected in waters from the prohibited area were found to be acceptable, while the shellfish non-flocculated portion was positive for poliovirus by the hybridization probe. The advent of molecular detection techniques, has greatly enhanced our ability to detect specific types of bacteria and viruses. Future modifications for probe procedures should focus on minimizing virus loss during sample processing. Since viruses appear to have a longer survivability time at lower temperatures than vegetative bacteria, and since standards based on levels of bacteriophage do not exist, it seems advisable to verify the sanitary conditions of shellfish, particularly those in conditionally managed areas, using a multifaceted approach which includes assays for viruses.

*Clostridium perfringens* is a spore forming, obligate anaerobe. This organism is widespread in the environment and is not solely of fecal origin. Compared to the coliform bacterial indicators, *C. perfringens* spores have a significantly longer survival time in estuarine and are less susceptible to environmental stresses. The levels of *C. perfringens* spores in the prohibited area were markedly higher than those found in the conditionally approved and approved areas. Compared to the coliforms and enterococci, *C. perfringens* levels are less severely affected during the colder

months in the conditionally approved and prohibited areas. In the approved area *C. perfringens* levels were similar to the coliform indicators, in that levels were undetectable during the colder months (November–March). While levels of *C. perfringens* spores were detectable more often than the coliforms or enterococci, there is no reliable relationship between *C. perfringens* and the occurrence of human enteric viruses.

Enterococci levels were significantly lower than those found for coliforms, *C. perfringens* and MSB. In the approved area enterococci densities were below the assay detection limit at all times. The lower levels of enterococci determined in this study are likely reflective of the lower numbers found in wastewater effluent. These data demonstrate that enterococci levels would no further ensure the sanitary quality of shellfish than the present coliform system.

The hybridization probe assay to detect coliform was performed with both the non-flocculated and flocculated sample portions. Non-flocculated portions were found to be positive more often than the flocculated samples, and in no instances were flocculated samples found positive where non-flocculated portions were negative. This indicates that the flocculation procedure is not 100% efficient in recovery of virus particles, loss of virus could occur resulting in loss of sensitivity. Considering the low level of viral contamination expected in the clams, it appears preferable to rely on results from non-flocculated portions to yield the greatest degree of sensitivity.

All clam samples were assayed for poliovirus by cell culture. Comparison of the hybridization probe and cell culture results for the non-flocculated portion was not possible, due to sample toxicity on BGM cell monolayers. Therefore, only the flocculated portions of the clam samples were examined by cell culture. Flocculation followed by resuspension in phosphate buffer was sufficient in detoxifying the samples for cell culture analysis. In the approved area none of the flocculated samples were positive by cell culture, this correlates with the hybridization probe results for this area. Similar results were obtained for flocculated samples from the conditionally approved area, in that all cell culture and hybridization probe assay results for poliovirus were negative. In the prohibited area, there was only one sample (January) that showed CPE in cell culture however, this sample was probe positive with the non-flocculated sample portion, but not with the flocculated portion. One explanation for the discrepancies between tissue culture and probe analysis would be that the lower virus concentrations in the flocculated sample were below the limits of detection for the nucleic acid hybridization assay.

MSB have received consideration as indicators of enteric viral pathogens. Though not regularly detected in fresh fecal material, this group of bacterial viruses are consistently present in sewage and sewage polluted waters (Debartolomeis and Cabelli 1991). Also, some members of the MSB group have been shown to be as resistant to disinfection by chlorination as Norwalk virus (Keswick et al. 1985). Relating to the occurrence of other indicators in this study, MSB were the only indicators consistently detected at all of the collection sites. However, MSB were present in all waters, including approved waters precluding their use in a simple presence/absence test, since this would probably result in the unnecessary closure of safe shellfish beds. Further data is needed to determine if there is a correlation between certain levels of MSB and the presence of human enteric viruses, also to establish a predictive index and protective MSB standards. Such studies are essential before MSB is considered as an indicator organism without excessively restricting shellfish waters.

In summary, the indicators in this study exhibit widely ranging results. Enterococci exhibited the lowest numbers throughout all of the collection sites and periods, followed by the fecal coliforms and total coliforms. These vegetative bacterial indicators are greatly affected by environmental stresses, such as water temperature, low nutrients, and salinity. *C. perfringens* and the MSB were present at greater frequencies than the coliform and enterococci groups, with MSB being detected more often and at greater levels than any of the other indicators.

The use of hybridization probes in this study demonstrates an alternative technique to detect the presence of enteric viruses in clams. Even so, the direct detection of viral nucleic acid by probe analysis appears to require improvements in its detection limits. Detection of low levels of viruses in the shellfish is compounded by the low efficiency of viral recovery from clam meats. Additionally, the procedures used in this study are time-consuming and labor-intensive.

The results of this study support those reported by others, which suggest that bacterial indicators and standards while serving to adequately protect against bacterial pathogens in shellfish, they do not reliably predict the presence of human enteric viruses. The development of better extraction procedures and rapid, inexpensive, automated molecular techniques, may soon allow for the direct detection of most if not all pathogens potentially present in shellfish. Consequently, a more complete approach in evaluating shellfish sanitary quality and the safety of shellfish is currently needed. This includes indicator organisms along with analyses to detect certain viral pathogens directly using a molecular based assay.

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**ABSTRACTS OF TECHNICAL PAPERS**

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**HISTORICAL PERSPECTIVES—SHELLFISH BIOLOGY SEMINAR/MILFORD AQUACULTURE SEMINAR.** Walter J. Blogoslawski, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

In May 1992, the federally-sponsored Joint Subcommittee on Aquaculture published a report, "Aquaculture in the United States: Stocks, Opportunities, and Recommendations", which stated that United States aquaculture production for 1990 exceeded 860.8 million pounds with a value of \$761 million dollars. This is a four-fold increase in production from 1980. The aquaculture industry accounts for more than 290,000 jobs with a total economic impact of \$8 billion dollars. In recognition of the importance of aquaculture to the northeast, the Milford Laboratory has sponsored shellfish biology seminars since 1975.

The first meeting of the Milford Shellfish Biology Seminar occurred as a technical exchange between staff at the Milford Laboratory and shellfish managers of the F. M. Flower Company, Bluepoints Company, and Long Island Oyster Farms. There were nine industry attendees and six Milford staff. In subsequent years the forum broadened in technical scope, covering topics of algal rearing, genetics, and water quality control, and organized as a technical exchange from Government scientists to shellfish industry representatives. Since 1980, the scope of the seminar has further expanded to include presentations by aquaculture scientists from Sea Grant, academia, and other state and federal agencies, as well as the commercial aquaculture companies.

The purpose of these annual gatherings of shellfish biologists was to share current ideas and innovative methodologies in shellfish research. This year the name was changed from "shellfish" to "aquaculture" seminar to focus attention on other marine species of commercial interest. For example, cultured marine Atlantic salmon, a new multi-million dollar industry in the Northeast, will soon exceed in value that of wild-harvested Maine lobsters.

Under a multi-species approach, innovation and flexibility can be applied to the northeast aquaculture industry. The industry can adopt new methods, use disease-resistant strains, or new species of animals, thereby offering greater value in the marketplace. Some of these adaptations, including the use of genetically manipulated or new species of oyster broodstock, are the focus of this year's meeting. We will also discuss possible causes of juvenile oyster mortalities, effects of shellfish disease, pollutants and noxious bloom organisms on shellfish, and effects of diseases of cultured fish.

**DEVELOPMENT OF HIGH SURVIVAL RESISTANT LINES IN OYSTERS USING MSX-DISEASE RESISTANT STRAINS.** Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

Since 1958 Rutgers has been breeding American oysters for

resistance to MSX-disease. Rutgers maintains a commitment to continuing these strains and to strategies for further improvements in the American oyster. The advent of Dermo in the northeast has caused us to reevaluate the future and role of the MSX-disease resistant (RR) strains. RR oysters are not resistant to Dermo. Additionally, because genetic variability in RR stocks has been constrained through intense selection pressure and population bottlenecks, they may be *more* susceptible. Almost certainly, there is enough loss of genetic variability in RR oysters to question the wisdom of selecting for Dermo resistance from any one of the RR strains per se. Our progress toward creating two new strains of resistant oysters is reported here. These strains are collectively to be called High Survival Resistant Lines (HSRL). From June 10 to July 21, 1992, we produced two geographic races of HSRL: a Delaware Bay (DB HSRL) and a northeast race (NE HSRL). Broodstock for DB HSRL comprised four strains of resistant oysters, three years old, and wild stock from Delaware Bay. The broodstock populations have been exposed to Dermo pressure for only one generation. Constituent populations for NE HSRL included two succeeding generations of Long Island RR strains (BLA and CLA), and F. M. Flowers, Inc. and Ocean Pond, Inc. varieties of the BLA line. We did not introduce Long Island wild stock genes into NE HSRL. Founder populations were produced by controlled matings among (but not within) each constituent population. Matings were made for both DB HSRL and NE HSRL using as many pairs as possible from each constituent population. Five founder sub-populations for each race (DB or NE) were produced, but we lost one of the NE sub-populations in the hatchery stage. Through a series of matings among (but not within) strains, we produced a total of 2388 families comprising the five DB HSRL sub-populations and 3287 families comprising the five NE HSRL sub-populations. The reason for subdividing the lines is so that each sub-population can be cross bred to other sub-populations (but not to itself) in future generations. Such a crossing scheme prevents matings between closely related individuals. Also, because the high survival line comprises five sub-populations, high effective population sizes are maintained, minimizing genetic drift. Breeding adjacent sub-populations reciprocally (but not to themselves) will produce a new generation ( $F_1$ ) also consisting of five sub-populations in each geographical race.

**INTERACTIVE SPREADSHEET ON THE ECONOMICS OF OYSTER FARMING.** Lee Anderson,<sup>1</sup> Dave Jones,<sup>2</sup> and Standish K. Allen, Jr.,<sup>2</sup> College of Marine Studies, University of Delaware, Newark, DE 19716 and <sup>2</sup>Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

From 1990–1992, NCRI sponsored a research program at the Haskin Shellfish Research Laboratory, "Oyster grow-out techniques for the mid-Atlantic: a Delaware Bay model". In our project, we explored intensive (rack and bag) aquaculture using "cultchless", MSX-disease resistant spat. The overall goal of our

demonstration project was to determine the economic feasibility of oyster grow-out using rack and bag culture of MSX-resistant, cultchless oysters. Specifically, we wanted to estimate the biological parameters and the economic feasibility of oyster farming for this area. The principal question we address here: How do the biological parameters affect the economic model? What are the sensitive features of the model? We developed a computer based spreadsheet program in the user friendly spreadsheet environment of Microsoft Excel (computer adaptation to Excel was done by Ken Cooper, Kingston, WA). The program is demonstrated in this poster session. Profitability of a commercial venture depends on a myriad of factors. For aquaculture, these factors include biological characters as well as more classic ones such as labor, capital, etc. In this program we have tried to incorporate all the economic elements that we have identified over the course of our pilot scale oyster farm. We have also tried to keep the spreadsheet as flexible as possible, enabling the operator to experiment with the economics of a "Ma-and-Pa" operation or with the economy of scale of a corporate giant. We have tried to incorporate several features to the economic analysis that are not immediately obvious to a novice. For example, on the east coast of the US, overwintering oysters, even in the mid-Atlantic, is a critical consideration. In this program we provide a worksheet for figuring these costs as part of the overall costs. The purpose of this program is to provide the prospective oyster farmer with a preliminary estimate of the profitability of a particular operation. Basically, the program is designed to demonstrate if a particular aquaculture project is a good investment. From a financial point of view, does it make sense to tie up one's money and time in the enterprise?

**OPTIMIZING METAMORPHOSIS AND SURVIVAL FOR LAB STUDIES OF *MULINIA LATERALIS*.** Ann Arseniu, Lyu Suifen, and Standish K. Allen Jr., Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

In 1969, Calabrese described the dwarf surfclam, *Mulinia lateralis*, as the "molluscan fruit fly", an observation still meaningful today. Its value to our lab is the short generation time (60–90 days) depending on conditions that allows genetic studies. For example, we are using *Mulinia* as a model system for the investigation of gynogenesis in bivalves. In general, *Mulinia* is easy to raise in the lab. But optimizing the conditions for rearing *Mulinia* to adulthood are important to eliminate excessive selection of lab stocks and for rearing rare genetic variants to adulthood. We investigated the effect of sediment on the rate of metamorphosis by rearing seven replicate crosses simultaneously. The seven replicate cultures were reared for eight days and each was split between 15 culture containers with or without sediment. Sediment did not increase the rate of metamorphosis significantly; replications with sediment had slightly fewer spat than those without. However, clams measured two weeks after settlement were significantly larger and there were fewer dead. We then compared the survival

and growth of newly set juveniles in either static seawater, static seawater with air, or flowing seawater. Flowing seawater (~2ℓ/hour) produced significantly more and larger juveniles after 2 weeks. Our conclusion from these studies is that conditions that mimic natural conditions for *Mulinia* are preferable to standard hatchery techniques for bivalves. Studies to maximize survival from juveniles to adulthood are ongoing.

**THE ROLE OF RESEARCH AND DEVELOPMENT IN A COMPETITIVE DOMESTIC AQUACULTURE INDUSTRY.** Sebastian Belle, New England Aquarium, Central Wharf, Boston, MA 02110.

Aquaculture is an increasingly technical field. Artisanal culture methods are rapidly evolving into a science. Research and development plays a critical role in this transition. The impact of research and development on a viable domestic aquaculture industry is discussed. The U.S. experience is compared with that of other countries. Pure and applied research are characterized and their relative contributions to commercial development examined. Research strategies in the private and public sectors are compared. The relationship between initial innovation, commercialization and competitive viability is discussed.

**DETECTION OF FISH PATHOGENS.** Joseph Buttner,<sup>1</sup> Pei Chang,<sup>2</sup> Paul Bowser,<sup>3</sup> Frank Hetrick,<sup>4</sup> Philip McAllister,<sup>5</sup> Bruce Nicholson,<sup>6</sup> and Paul Reno,<sup>7</sup> <sup>1</sup>Department of Biological Sciences, SUNY College at Brockport, Brockport, NY 14420; <sup>2</sup>Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; <sup>3</sup>Department of Avian and Aquatic Animal Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; <sup>4</sup>Fish Disease Laboratory, Department of Microbiology, University of Maryland, College Park, MD 20742; <sup>5</sup>NFRHL, U.S. Fish and Wildlife Service, Kearneysville, WV 25430; <sup>6</sup>Aquatic Animal Health Laboratory, Maine Animal Health Laboratory, University of Maine, Orono, ME 04469; <sup>7</sup>Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

Diseases, particularly bacterial and viral, cost the United States aquaculture industry millions of dollars in losses annually. Good management practices can avoid many disease problems, but not all. When a disease problem appears, an accurate and prompt diagnosis is essential to initiate an effective and appropriate corrective action. Current disease diagnostic procedures almost always require sacrificing the fish. Non-destructive techniques are being developed and evaluated for by the Northeastern Regional Aquaculture Center. Investigators have used blood, ovarian fluid, or mucous and survival-surgery procedures to obtain kidney and liver specimens. Quick and accurate immunological assay methods, recombinant DNA technologies, and viral plaque assays are being developed.

**POSSIBLE EUTROPHICATION-RELATED ENHANCEMENT OF THE MICROBIAL LOOP IN LONG ISLAND SOUND AND CONSEQUENCES FOR SHELLFISH.** **Gerald M. Capriulo,<sup>1</sup> Robert Troy,<sup>1</sup> Marcelo Morales,<sup>1</sup> Kathleen Beddows,<sup>1</sup> Helen Budrock,<sup>1</sup> Gary Wikfors,<sup>2</sup> and Charles Yarish,<sup>3</sup>** <sup>1</sup>Environmental Science Department, State University of New York Purchase, NY 10577; <sup>2</sup>National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460; <sup>3</sup>Department of Ecology and Evolutionary Biology, University of Connecticut, 641 Scofieldtown Road, Stamford, CT 06903.

Laboratory and field studies have shown that changes in water column chemistry related to absolute levels and relative ratios of important nutrients, alter the species composition of water column plankton communities towards smaller, microbial forms. In marine/estuarine waters, nitrogen, and to a lesser extent silicate, levels control phytoplankton growth, and N/P and N/Si ratios affect species composition. Therefore, anthropogenic inputs of N stimulate excessive algal growth, and select for smaller-sized phytoplanktonic species. A fundamental question arising from this is why the excess algal and other biomass is not enhancing secondary production of a quality leading to finfish and shellfish production in coastal systems such as Long Island Sound. We believe the answer to this question lies in the fact that food web dynamics in the western Long Island Sound have been anthropogenically shifted toward a microbial loop dominated system as compared to the more traditional food web dynamics of the central to eastern Long Island Sound. If such a fundamental shift has occurred in the western Sound, then both finfish and shellfish production are being negatively affected. We herein report preliminary results of an ongoing, comprehensive, baseline data study, in which we are comparing microbial loop and related planktonic food web dynamics in the western versus central Long Island Sound.

**CONTROL OF OVERSET ON CULTURED OYSTERS USING BRINE SOLUTIONS.** **Gregory A. Debrosse and Standish K. Allen, Jr.,** Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

HSRL has a long standing program in oyster genetics and breeding. One of the worst scenarios for maintenance of broodstocks is overset by native oysters. Preliminary experiments in 1990 indicated that overset might be controlled simply by immersing animals in a concentrated brine solution; such treatments in 1990 resulted in 89–100% mortality of <1 mm spat. In 1990 field tests, overset on broodstocks was reduced to 3 spat/oyster using 200 ppt immersions compared to 22 spat/oyster in controls. In 1991 we refined the parameters for effective brine dips. First, we tested survival of oysters (potential substrate for overset) immersed for 2, 5, or 10 minutes in 200 ppt brine followed by either 3 or 6 hours aerial exposure. For juveniles, cumulative mortalities

ranged from 3–6% compared to 5% in controls; for adults, 2–4% died after brine immersion and 2–3% died in controls. Second, we tested survival of hatchery set oyster spat immersed in 200 ppt brine. For spat with shell lengths <5.0 mm and immersed in 200 ppt brine for 2, 5, or 10 min, 57%, 70% and 83% died after 3 hr aerial exposure and 64%, 85%, and 86% died after 6 hr aerial exposure. Control mortality averaged about 23% in both 3 and 6 hr aerial exposures. For larger spat immersed in 200 ppt brine for 10 minutes, cumulative mortality was 47% and 88% for 3 and 6 hr aerial exposure, respectively, and 22% and 32% for controls. Results of 1990 field tests and 1991 experiments demonstrate that brine solutions will be effective and save considerable labor.

**JUVENILE OYSTER MORTALITY STUDIES—1992: HISTOPATHOLOGY, PATHOLOGY, EPIZOOTIOLOGY.** **C. Austin Farley and E. J. Lewis,** National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Studies of cytology, pathology, and population characteristics were conducted in relation to mortalities of Long Island Sound hatchery-reared juvenile oysters. Studies included major mortality periods of July–September in both 1991 and 1992. Data have been analyzed and support information reported previously by others suggesting size and temperature in relation to onset of disease and mortality. Dead oysters typically were less than 30 mm in length (mean 16–20 mm). Depending upon water temperature, mortalities in oysters occurred 3 to 8 weeks after being transplanted from the hatchery and maintained in trays in the nursery. Oysters from the nursery experienced 4–66% mortality with conchiolin deposition. Representative oysters from each spawning batch kept in the hatchery, in 25- $\mu$ m filtered ambient water diluted with high salinity well water, suffered 0–8% mortalities with conchiolin deposition. Epizootiology studies of variously treated juvenile oyster populations further suggest that an infectious entity is responsible for mortalities. As in our earlier studies, histological tissues revealed the presence of small, round intracellular bodies in lesions of the mantle epithelium in 60–90% of populations experiencing >50% mortality. We believe these bodies to be a parasite, not autophagic vacuoles or necrotic host cells as others have suggested. Tissues stained with Feulgen picromethyl blue revealed that many of these bodies possess multiple dense staining Feulgen-positive structures resembling developmental life cycle stages of protists, particularly ciliates.

Intracellular parasites with protistan characteristics were found by electron microscope studies. Mitochondria with tubular cristae, small nuclei, indications of a pellicle in some, and suggestions of endogenous budding similar to that seen in suctorian ciliates were seen. Similar intracellular organisms were seen in large commensal ciliates in spaces between the mantle and shell, suggesting a possible carrier host role. These large ciliates would not pass a 25- $\mu$ m filter, explaining the protection of comparable populations held in the hatchery.

**RECENT OUTBREAKS OF DERMO DISEASE IN THE NORTHEAST NEW INTRODUCTIONS OR CLIMATE CHANGE?** Susan E. Ford, Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

Since 1990, the protozoan *Perkinsus marinus*, cause of Dermo disease in the eastern oyster, *Crassostrea virginica*, has appeared in numerous northeastern areas where it had not previously been detected or caused mortality. Hypotheses offered to explain the phenomenon include recent introductions by movement of infected oysters from southern waters; the appearance of a new low temperature tolerant strain of the parasite; and a change to a more favorable environment.

Importation of a large number of *P. marinus*-infected oysters into Delaware Bay during the 1950s failed to establish a self-sustaining parasite population, which declined after importations ceased. Occasional findings of infected oysters over the following 35 years, however, suggest that the parasite remained in the Bay, but at undetectable levels.

The *P. marinus* introductions of the mid-1950s occurred during a period of average or below average temperatures. In contrast, the 1990–1992 epizootic coincided with a period, beginning in January 1990 and lasting until March 1992, in which monthly mean air temperature in southern New Jersey, adjacent to Delaware Bay, was consistently higher than average, often by several degrees Celsius. Similar temperature deviations were recorded in more northern sites where epizootics of *P. marinus* have occurred for the first time. Above average winter temperatures appear to correlate better with *P. marinus* outbreaks than do high summer temperatures.

It is difficult to believe that infected oysters have been introduced suddenly into multiple locations from New Jersey to Massachusetts, including sites condemned for the harvest of shellfish, over the last two or three years. Historical records do, however, show that for many years during the century oysters were moved from south (where *P. marinus* is enzootic) to north along the Atlantic coast. The simplest explanation consistent with historical knowledge and current observations is that extraordinarily high temperatures beginning in 1990 stimulated the proliferation of existing small foci of infection, which may have been present, but undetected, for years.

**STUDIES OF JUVENILE OYSTER MORTALITY ON LONG ISLAND SOUND, NY IN 1992.** Susan E. Ford,<sup>1</sup> Francisco J. Borrero,<sup>2</sup> and Walter J. Blogoslawski,<sup>3</sup> <sup>1</sup>Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349; <sup>2</sup>State University of New York, Marine Science Research Center, Stony Brook, NY 11794; <sup>3</sup>National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

A study of juvenile oyster mortality was conducted over the summer of 1992 at two sites on Long Island, New York: F. M. Flower and Sons Oyster Co. (north shore of Long Island) and the Bluepoints Co. (south shore of Long Island). Major objectives of the study included determining whether mortalities were associated with a particular broodstock (either a genetic problem or a source of pathogen transmitted to offspring) or growout site, whether mortalities could be stimulated by experimental temperature elevation, and to document the association of tissue and shell abnormalities (or their absence) with mortalities (or their absence) in the various experimental treatments.

High mortalities occurred at the F. M. Flower and Sons Oyster Co. even though a new broodstock (wild oysters from the Thames River, CT) was submitted for Oyster Bay, NY stock, offspring of which had suffered high mortalities in 1990 and 1991. Mantle lesions were found in oysters just before mortalities began but no evidence of a pathogenic protozoan was found. Oysters maintained inside the F. M. Flower and Son Co. hatchery at elevated temperature (25°C) in a mixture of well water and 25- $\mu$ m filtered bay water, supplemented with cultured algae, did not experience unusual mortalities. Wild spat from Connecticut (1991 year class) placed in Oyster Bay did not experience typical juvenile oyster mortalities, but some individuals were found to have abnormal conchiolin ring deposits. No losses occurred at the Bluepoints Co. site in offspring of both Oyster Bay, NY and Thames River, CT broodstock.

Results of this and previous studies lead us to conclude that broodstock is not the problem and to suspect that affected juvenile oysters are reacting to a toxin, probably of bacterial or microalgal origin, which irritates the mantle edge causing abnormal shell matrix secretion, tissue damage, and eventual death.

**REPRODUCTIVE GENETICS OF TRIPLOID CRASSOSTREA GIGAS.** Ximing Guo and Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Department of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

*Crassostrea gigas* has been variously proposed as a replacement or supplement species for *C. virginica* in several east coast situations. Triploids potentially offer a "safe" way to test *C. gigas* in the field. Are triploid *C. gigas* sterile? The genetics of reproduction in triploid Pacific oyster, *Crassostrea gigas*, was examined in matings between diploids (D), triploids (T), and their reciprocal crosses (D  $\times$  T and T  $\times$  D). Meiotic metaphases were examined in eggs of diploid and triploid eggs. Ploidy of embryos of all matings were determined by karyology and flow cytometry. Sperm from triploids showed a single distribution of DNA content at 1.49c, as determined by flow cytometry; no haploid peaks were observed. Before fertilization, eggs from diploids had ten synapsed chromosomes. In eggs from triploids, chromosome numbers varied considerably within and among females: some were completely synapsed to form ten trivalents, but most had between 11–13 trivalent and bivalent chromosomes. Gametes from trip-

loids were capable of fertilization and fertilization was about the same in all groups, probably limited only by the maturity of gametes. After fertilization, eggs of triploids went through two meiotic divisions, releasing two polar bodies. Ploidy of embryos from the four types of matings was determined by both flow cytometry and karyology to be  $2n$  for  $D \times D$ ,  $2.5n$  for  $D \times T$  and  $T \times D$ , and  $3n$  for  $T \times T$ . Survival to D-stage was about the same in all crosses, ranging from 32–66%. Survival to seven days post-fertilization was 40% for  $D \times D$ , 0.5% for  $D \times T$ , 8% for  $T \times D$ , and 0.4% for  $T \times T$ . Percent metamorphosis to spat was 23% for  $D \times D$ , 0.001% for  $D \times T$ , 0.058% for  $T \times D$ , and 0.0% for  $T \times T$ .

**EFFECT OF TREMATODES ON EAST COAST POPULATIONS OF THE BLUE MUSSEL, *MYTILUS EDULIS*.** Robert E. Hillman, Battelle Ocean Sciences, 397 Washington Street, Duxbury, MA 02332.

During the course of histological examinations of gonadal development of *Mytilus edulis* populations for the National Oceanic and Atmospheric Administration's (NOAA) Mussel Watch Project, apparent gonadal abnormalities were observed in connection with the presence of trematodes in the tissues. In most cases, either the gonadal follicle tissues were disorganized and did not progress past an early stage of development, or no gonads were present at all. These observations prompted a more detailed look at the effects of the trematode infestations on the mussel populations being monitored for the NOAA study. This paper is a preliminary report on the distribution and effects of the trematodes in mussel populations on the U.S. east coast monitored over a seven-year period.

A total of 49 sites have been sampled once annually from Maine to Delaware since 1986, although not each site has been sampled every year. At this point, no attempt has been made to thoroughly identify the species involved in the infestations, but there are at least two types of trematodes involved. The most common type, by far, appears to infest all tissues except the foot, and is responsible for much of the observed abnormal gonad development. The second type is found almost exclusively in the foot and does not appear to affect gonadal development. The intensity of infestation is highest in the New York Bight and Long Island Sound region. Gonadal abnormalities are from 10 to 26% higher at sites where trematode infestations occur than at sites where there were no observed trematodes.

**RELATIONSHIP OF ENVIRONMENTAL CONTAMINANTS TO OCCURRENCE OF NEOPLASIA IN *MYTILUS EDULIS* POPULATIONS FROM EAST AND WEST COAST MUSSEL-WATCH SITES.** Robert E. Hillman, Battelle Ocean Science, 397 Washington Street, Duxbury, MA 02332.

Over 8,000 mussels, *Mytilus edulis*, collected for the National Oceanic and Atmospheric Administration's Mussel Watch Project from approximately 80 sites along the U.S. east and west coasts from 1986 through 1991 were analyzed for levels of organic and

inorganic contaminants, and examined histologically for evidence of neoplasia. Neoplasias were found in mussels from 6 east and 12 west coast sites. With the exception of 2 germinomas, all neoplasias were disseminated neoplasias. Significantly higher concentrations of combustion-related and total PAHs, *cis*-chlordane, pesticides, and cadmium were found at east coast sites where neoplasias occurred than at sites where no neoplasias were found. Arsenic was found at higher concentrations at non-neoplasia sites than at neoplasia sites. On the west coast, significant differences were observed for combustion-related and total PAHs, total PCBs, and lead. Cadmium, chromium, and mercury were higher at non-neoplasia sites than at neoplasia sites. A step-wise regression analysis was performed to determine those contaminants whose concentrations significantly affected the presence of neoplasia. On the east coast, the negative effect of arsenic was overwhelmingly significant compared to the effects of all other contaminants. On the west coast, combustion-related PAHs contributed to high probabilities of neoplasia, while chromium and indene-based pesticides were significantly negatively correlated with the occurrence of neoplasia.

**CYTOLOGICAL AND CYTOGENETIC EXAMINATION OF GAMETOGENESIS IN TRIPLOID *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA GIGAS*.** Ya-Ping Hu and Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Box B-8, Port Norris, NJ 08349.

*Crassostrea gigas* has been variously proposed as a replacement or supplement species for *C. virginica* in several east coast situations. Triploids potentially offer a "safe" way to test *C. gigas* in the field. Are triploid *C. gigas* sterile? Gametogenesis of triploid *C. gigas* were compared to a triploid *C. virginica* group with both flow cytometric and histological techniques. A total of 118 *C. virginica* and 144 *C. gigas* were sampled from early May to August, 1992, at bi-weekly intervals. No haploid sperm were recorded in any male triploid, but all male triploids were capable of producing 1.5 N aneuploid sperm. Overall the gonadal development and gamete production in both male and female triploids were significantly decreased in terms of quality and quantity relative to diploids. The present study also documented a correspondence between the male gamete type (histology) and the relative DNA content (flow cytometry).

**PARASITES OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*.** John D. Karlsson, Rhode Island Coastal Fisheries Laboratory, 1231 Succotash Road, Wakefield, RI 02879.

Parasites observed during histological examination of approximately 2500 bay scallops, *Argopecten irradians* Lamarck, collected between 1983 and 1985 from wild populations in two coastal ponds in Rhode Island, are reported. In addition to a review of previously reported bay scallop diseases, occurrence of these diseases in Rhode Island waters is documented, and several

newly discovered disease conditions are described, including two ovarian parasites which appear to cause parasitic castration.

#### **OCCURRENCE OF *OSTREA EDULIS* IN RHODE ISLAND.**

**John D. Karlsson** and **Arthur R. Ganz**, Rhode Island Coastal Fisheries Laboratory, 1231 Succotash Road, Wakefield, RI 02879.

In recent years a number of European oysters, *Ostrea edulis*, have been found in Rhode Island waters. There is evidence that observed occurrences result from larval settlement rather than the release of post-metamorphic animals.

#### **A SIMPLE METHOD FOR THE *IN VITRO* CULTURE OF**

***PERKINSUS MARINUS*. S. J. Kleinschuster** and **S. L. Swink**, Haskins Shellfish Research Laboratory, Rutgers University, Box B-8, Port Norris, NJ 08349.

Cells tentatively identified as *Perkinsus marinus* were originally identified as a contaminant of primary tissue culture explants of visceral ganglia from *Crassostrea virginica*. Following sterile isolation, various mixtures of Leibowitz's medium (L-15), oyster hemolymph and fetal calf serum (FBS) were tested for cell growth potential. The osmolarity of each constituent was adjusted to 750 mOs/kg by the addition of sea salts. The pH of each medium and/or constituent was then adjusted to 7.6 and sterilized when necessary.

As might be expected, cells cultured with a high proportion of hemolymph in the medium (50%), displayed vigorous propagation. Alternately, those cells cultured with a high proportion of L-15 and/or FBS tended to attain sporangial morphology with many daughter cells. Anomalous morphology of cells was common in cultures with high L-15/FBS concentrations, especially in older cultures. Original cultures were subcultured several times over several months and the medium exchanged (50%) weekly. Cultures so obtained were challenged with sterile oyster tissue which became infected within 2–3 weeks. Additionally, cells from challenged and infected tissue formed hyphospores and tested positive upon exposure to Lugol's solution following culture in fluid thioglycolate. The optimum medium for growth and differentiation in this study consisted of 5.0 mg taurine, 50.0 mg glucose, 30.0 mg galactose, 50.0 mg fructose, 50.0 mg trehalose, 300.0 mg lactalbumin, 100.0 mg yeast extract, 1.0 ml vitamin mixture (Sigma Chemical Co.), 0.1 ml lipid mixture (Sigma Chemical Co.), 20.0 ml FBS (Sterile Systems Inc.) and 80.0 ml L-15. Various aliquots of hemolymph may be substituted for L-15. The authors wish to thank Dr. F. O. Perkins for his invaluable assistance with this study.

**OVERVIEW OF THE OPERATIONS OF ATLANTIC LITTLENECK CLAMFARMS. Kenneth P. Kurkowski**, Atlantic LittleNeck ClamFarms, P.O. Box 12139, Charleston, SC 29422.

Atlantic LittleNeck ClamFarms is a fully integrated commercial aquaculture company located south of Charleston, S.C. It began production in mid-1991, with facilities consisting of a

15,000-ft<sup>2</sup> hatchery building, an 11,000-ft<sup>2</sup> nursery, a 1,500-ft<sup>2</sup> field house and an 8,000-ft<sup>2</sup> fabrication shop. Seawater from Folly Creek is settled for 36 hours and filtered to 1 micron for the hatchery. It is further pasteurized prior to use in algal culture. Three-thousand broodstock clams are kept at 19°C in the conditioning system to provide for year-round spawning. Clams selected for spawning are suspended in trays in a larval tank at 28°C and allowed to spawn for two hours before being removed. After seven days in larval culture the pediveligers are transferred to downwellers in the post-set system for three to five weeks. The algal system produces 13,000 liters daily for feeding larvae, post-set and broodstock. Thirty-million 1-mm seed are transferred to either a land-based or pond nursery monthly. Two to four months in passive upwellers yield 200 million 4 to 6-mm clams ready for field growout in clam pens annually. Initially the clams are stocked at 1,000 per ft<sup>2</sup>. They are harvested 10 months later at 20–25 mm and replanted at 80 per ft<sup>2</sup>. Sixteen months later 140 million 50-mm clams will be harvested annually. Upon harvest, the clams are depurated for 48 hours and tested for heterotrophic bacteria by our own certified shellfish lab before being sent to market.

**PRELIMINARY RESULTS OF LABORATORY ATTEMPTS TO TRANSMIT A DISEASE AFFECTING JUVENILE OYSTERS IN THE NORTHEASTERN UNITED STATES. Earl J. Lewis, Jr.** and **C. Austin Farley**, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Oxford Laboratory, Oxford, MD 21654.

Since the late 1980s, juvenile oysters from areas in northeastern United States have experienced heavy mortalities. As yet, the cause of these mortalities has not been resolved, although many possible causes have been hypothesized. Our hypothesis is that this is an infectious disease process, with mortalities possibly resulting from pathology associated with a protistan parasite. Based on this, transmission experiments were designed to determine if the disease could be transmitted under controlled laboratory conditions. Preliminary results of ongoing experiments support this hypothesis.

Depending upon water temperature, Maryland hatchery-reared oysters held in recirculating aquaria showed mortalities with characteristic heavy conchiolin deposition within 3 to 7 weeks of exposure to infected oysters from Long Island Sound. Cumulative mortality in experimentally challenged oysters ranged from 40% (18°C) to 74% (24°C). Associated conchiolin deposition was present in 26% of dead oysters held at 18°C, compared to a high of 40% at 24°C. No indications of dinoflagellates were evident in water samples examined upon completion of the study. "Little round bodies" resembling what we have considered previously to be a parasite were observed in "gapers" processed for histology.

No conchiolin or comparable mortalities were observed in control animals.

Gross symptoms of the disease were observed to recur in sur-



vivors of the 1990 and 1991 mortalities when held in aquaria for 10 months.

**THE DIFFERENTIAL EFFECTS OF THREE HEAVY METALS ON PARTICLE FILTRATION AND AMINO ACID UPTAKE BY THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*.** Wenyu Lin and Michael A. Rice, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; Paul K. Chien, Department of Biology, University of San Francisco, San Francisco, CA 94117.

The effects of copper, cadmium and zinc on rates of particle filtration and glycine uptake by *Crassostrea gigas* were studied. Constant filtration rates were induced in oysters by irrigating the mantle cavity with flowing seawater from a peristaltic pump at a rate of 2.5  $\ell/h$ . The filtration rate (volume of water completely cleared of colloidal carbon per unit time) by control oysters was 26.60  $\text{m}\ell/\text{gh} \pm 7.68$  (SD). Filtration rates decreased with increasing concentrations of cadmium and zinc. In lower concentrations of copper (8–16  $\text{mg}/\ell$ ) filtration rates were significantly higher than the control, but higher copper concentrations reduced filtration. Influx of glycine is characterized by Michaelis-Menten Kinetics with  $J_{\text{max}}$  and  $K_t$  values of 1.85  $\mu\text{mol}/\text{gh}$  and 33.7  $\mu\text{M}$  respectively. The degree of inhibition of glycine uptake in oysters exposed to metals was in the order of copper > cadmium > zinc. At 128  $\text{mg}/\ell$  copper, glycine uptake was reduced to 10.5% of the control. The rate of glycine uptake by filter feeding bivalves is highly dependent on water pumping rate. The volume-specific glycine transport (amount of glycine transported/unit volume of seawater completely cleared of colloidal carbon) by control oysters in 1  $\mu\text{M}$  glycine concentrations was 1.03  $\mu\text{mol}/\ell$ . The volume-specific glycine transport remained constant at increasing zinc concentrations, and declined at increasing copper concentrations, suggesting differential effects of the metals on particle filtration and the amino acid carriers. The apparent volume-specific glycine transport increased to 2.14  $\mu\text{mol}/\ell$  in 128  $\text{mg}/\ell$  cadmium. This volume-specific transport which was greater than the glycine concentration in the medium suggests that there may have been uptake of cadmium-complexed glycine by the oysters.

**“NON-TOXIC” DINOFLAGELLATE BLOOM EFFECTS ON OYSTER CULTURE IN CHESAPEAKE BAY: PRELIMINARY RESULTS.** Mark Luckenbach,<sup>1</sup> Sandra Shumway,<sup>2</sup> and Kevin Sellner,<sup>3</sup> <sup>1</sup>Virginia Institute of Marine Science, College of William & Mary, Wachapreague, VA 23480; <sup>2</sup>Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575; <sup>3</sup>Benedict Estuarine Research Laboratory, The Academy of Natural Sciences, Benedict, MD 20612.

Dinoflagellate blooms appear to be increasing in frequency, magnitude and duration in Chesapeake Bay. During 1992 we documented dinoflagellate blooms of unprecedented intensity and distribution in the southern portion of Chesapeake Bay. Though the species involved in these blooms are generally termed nontoxic

(from a public health perspective), their effects on suspension-feeding bivalves, including oysters, may be anything but benign. As the oyster aquaculture industry continues to grow in Chesapeake Bay, the effects of these blooms may become increasingly important.

Field and laboratory experiments were conducted to evaluate impacts of several dinoflagellate bloom species on the feeding, growth and survival of *Crassostrea virginica*. Hatchery-spawned oysters from a single cohort were deployed in off-bottom culture at twelve locations exhibiting varying degrees of bloom development, and growth and survival monitored. Laboratory experiments of 4 to 6 weeks duration were used to evaluate growth and survival of juvenile oysters fed monocultures of dinoflagellates and the diatom *Thalassiosira weissflogii*. Flow cytometry was used to determine grazing rates in short-term feeding trials, and fecal and pseudofecal composition to assess utilization of dinoflagellates by oysters. Results from both the field and laboratory suggest that growth and survival of juvenile oysters are affected by these dinoflagellate blooms. Our findings indicate potentially significant impacts on oyster culture in this region as a consequence of dinoflagellate blooms.

**NORTHEASTERN REGIONAL AQUACULTURE CENTER: AN UPDATE.** Victor J. Mancebo, University of Massachusetts Dartmouth, Research 201, North Dartmouth, MA 02747.

The Northeastern Regional Aquaculture Center (NRAC), headquartered at the University of Massachusetts Dartmouth, is one of five Regional Aquaculture Centers (RACs) established by the U.S. Congress. Funded by the U.S. Department of Agriculture at an annual level of approximately \$750,000, and representing 12 states and the District of Columbia, NRAC develops and sponsors cooperative regional research, development and extension projects in support of the aquaculture industry in the northeastern United States.

A Board of Directors representing the region's aquaculture industries, academic institutions, and government agencies provides overall direction and management of NRAC. NRAC programs, like those of all the RAC's are industry-driven, i.e., industry communicates research and technology transfer priorities to NRAC through bi-annual industry summits and through NRAC's 12-member Industrial Committee. A 12-member Technical Committee provides technical oversight for NRAC's projects. Projects supported by NRAC are developed and carried out by Cooperative Regional Work Groups with researchers, extension specialists and industry representatives working together with multi-state and multi-institutional participation on each project. Projects are evaluated annually for achievement of technical and industry objectives.

NRAC has recently completed four major projects on genetic improvement and manipulation of oysters, finfish economics, and the development of a regional aquaculture extension program. Termination reports are being prepared and relevant findings will be

disseminated. Ten regional projects are ongoing with areas including genetic manipulation of striped bass, domestication of striped bass, government regulations affecting aquaculture, finfish nutrition, commercial field trials of selected oyster strains, fish health, water quality and waste management, marketing options, oyster larval development and a regional industry situation and outlook report. Three smaller projects designed to avail of rapid response funds are also ongoing. NRAC is currently reviewing three projects for 1993 funding including an economic impact study of government regulations, a followup regional extension project, and a computer communication network. A project on quality assurance in aquaculture will soon be developed for funding in 1993. Total NRAC funding commitment to projects in progress or pending is approximately \$1.8 million. NRAC also publishes "Northeastern Aquaculture", a quarterly newsletter highlighting NRAC projects and other topics of interest to the northeastern aquaculture community.

**SHRIMP CULTURE IN THE PHILIPPINES: BIRTH OF THE INDUSTRY.** Victor J. Mancebo, University of Massachusetts Dartmouth, Research 201, North Dartmouth, MA 02747.

Production of farmed tropical shrimp underwent explosive growth in the decade of the 80s. A major contributing factor to the growth of the industry was the successful larval rearing and development of feeds for *Penaeus monodon* which occurred in Taiwan and was led by Dr. I-Chiu Liao.

In 1978 a successful technology transfer for *P. monodon* hatchery, grow-out and feed-production methodology took place from Taiwan to the Philippines. The technology transfer was negotiated by Dr. Liao, President, Enterprise Corporation in Taiwan and San Miguel Corporation in the Philippines. Following a time lag for site selection and construction, a show-case facility was built in the Philippines and the first successful harvest demonstrating Taiwan technology took place in 1982. At the time of the first harvest the total production of *P. monodon* in the Philippines was estimated at 1,000–1,500 metric tons. Within six years (1988) production of *P. monodon* had increased to approximately 40,000 metric tons, an increase largely attributed to San Miguel's technology demonstration and dissemination.

**AQUACULTURE IN THE NORTHEAST REGION OF NMFS.** Harold C. Mears, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Regional Office, One Blackburn Drive, Gloucester, MA 01930.

The genesis of aquaculture research in the Northeast Region of the National Marine Fisheries Service (NMFS) was prior to Reorganization Plan No. 4 of 1970 at which time the Bureau of Commercial Fisheries was renamed NMFS, and transferred to the Department of Commerce. The first permanent assignment of a full-time biologist and plans to establish a laboratory in Milford, Connecticut, occurred in 1931. The development of methods for commercial shellfish cultivation began in 1944. During that de-

cade, federal biologists established procedures for conditioning eastern oysters to ripeness, inducing spawning and fertilization, rearing larvae, determining food requirements, and growing newly-set spat. Activities at this facility have continued on a variety of subjects including natural diets, genetics, disinfection techniques for hatchery water, and culture methods for eastern oysters, bay scallops, and Atlantic surfclams.

During the past 25 years, approximately \$18 million have been devoted by NMFS-administered programs to aquaculture-related research. In cooperation with state fishery resource agencies, academia, the fishing industry, and other private interest, NMFS grant programs have supported projects covering a range of activities from bay scallop culture techniques to eastern oyster shell planting to assessment of hatchery wastewater systems. During 1964–1986, under the Commercial Fisheries Research and Development Act (P.L. 88–309), aquaculture was divided among six primary categories: marine fish and shellfish culture (71.8%); environmental monitoring (10.7%); aquaculture systems (7.8%); freshwater fish and invertebrate culture (3.8%); restoration/fisheries enhancement (3.2%); and processing technology (2.7%).

Aquaculture-related activities have also included NMFS participation and responsibilities in the areas of habitat conservation, trade and industry services, and fishery restoration programs. The NMFS Strategic Plan calls for the reduction of impediments to U.S. aquaculture, and a re-evaluation of the NMFS' role in achieving that goal. An internal Task Force is currently conducting that assessment.

**ISOLATION OF INFECTIOUS PARTICLES HAVING REVERSE TRANSCRIPTASE ACTIVITY AND PRODUCING HEMATOPOIETIC NEOPLASIA IN *MYA ARENARIA*.**

Daniel J. Medina, Department of Medical Oncology, Yale University, New Haven, CT 06510; Gregory E. Paquette, Eileen C. Sadasiv, and Pei W. Chang, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

The causative agent of hematopoietic neoplasia (HN) of soft shelled clams, *Mya arenaria*, has not been defined, though most investigators agree that the disease is caused by a small transmissible agent.

Oprandy and Chang (1983) isolated a transmissible agent from HN clams. The agent measured 100 nm and had a buoyant density of 1.18 g/cm<sup>2</sup>. When it was passed through a 450-nm filter, it induced HN in clams after a latent period of 2 months. The virus has been reisolated from moribund clams experimentally infected with the virus and reinjected into normal clams which subsequently developed neoplasia, with the same virus again isolated. Oprandy described the agent as a retrovirus, based upon the morphology of the isolated particles. However, electron micrographs of clam tissues and neoplastic hemocytes have not been able to demonstrate budding virus particles and the PTA-stained particles found in purified preparations have not shown the surface pep-

lomers which can be characteristic of retrovirus. Attempts to unequivocally demonstrate retroviral presence in cells has not been successful so far.

Since retrovirus can demonstrate reverse transcriptase (RT) activity, we have tested purified virus for the presence of RT. RT has been found. It was active at 6°C and was inactive above 25°C.

Neoplasia was accompanied by metabolic alterations: increases in uric acid, aspartate transaminase and triglycerides; decreases in hemolymph urea. The neoplastic hemocyte cell membranes showed differences in lectin binding proteins, indicating a change in cell surface glycoproteins.

**DIFFERENCES IN THE SALINITY TOLERANCE MECHANISMS BETWEEN CHESAPEAKE BAY AND ATLANTIC COAST OYSTER: GENETICS OR DISEASE-INDUCED EFFECTS ON MITOCHONDRIAL METABOLISM.** Sidney K. Pierce, University of Maryland at College Park, Department of Zoology, College Park, MD 20742.

*Crassostrea virginica* from Florida to Cape Cod respond to increased external salinity by increasing intracellular concentrations of several amino acids, primarily taurine, and the quaternary amine, glycine betaine. Chesapeake Bay oysters from several populations use different amino acids, primarily glycine and alanine, and in addition, do not synthesize glycine betaine in response to high salinity stress. Since the synthesis of both the amino acids and glycine betaine occurs in the mitochondria, we have been comparing isolated mitochondrial metabolism of Bay and Atlantic oysters. The respiratory coupling ratios (RCR) of Bay oysters is always higher than in Atlantic oysters, regardless of biochemical substrate. Bay oyster RCRs are highest with  $\alpha$ -ketoglutarate, while malate is preferred by Atlantic mitochondria. In addition, mitochondria from low salinity adapted oysters take up choline (glycine betaine precursor) faster than high salinity adapted oysters and Atlantic mitochondria take it up faster than Bay mitochondria. The synthesis of glycine betaine is faster in high salinity adapted Atlantic oysters. We are currently measuring synthesis in Bay oyster mitochondria. These differences in amino acid production, RCRs and glycine betaine metabolism indicate major biochemical differences between the mitochondria of the two oyster groups. Since all of our Bay oysters are likely parasitized with Dermo, it is not clear if the differences are due to genetics, the presence of the parasite or some other environmental factor.

**FOOD LIMITED GROWTH AND CONDITION INDEX IN CRASSOSTREA VIRGINICA AND ARGOPECTEN IRRADIANS.** Robert B. Rheault, Spatco, Ltd., 264 Foddering Farm Rd., Narragansett, RI 02882.

Oysters (*Crassostrea virginica*) and bay scallops (*Argopecten irradians*) were held in flumes with flowing seawater pumped from Point Judith Pond for six weeks in August and September of 1992. Flow to each flume was held constant and ration available was continuously monitored with flow-through fluorometry. The

experiment was designed to define the relationships of growth and condition index (CI) to both the available and consumed ratios. These relations can then be used to derive a horizontal seston flux model for oyster growth. Filter feeding rapidly depleted downstream food concentration in the flumes reducing fluorescent particulate material by 90% or more. Downstream oysters and scallops responded to the depletion in available ration with significant decreases in incremental growth and condition index.

Simultaneous experiments were conducted in nearby bottom cages to evaluate the growth and CI response to seston depletion caused by varying the initial planting density in a commercial aquaculture setting. Oysters planted in mesh bags at high densities (6.8–10.5 kg/m<sup>2</sup>) demonstrated significantly lower CI and growth of the animals planted at 2.7–3.6 kg/m<sup>2</sup>.

Data indicate the possibility of a synergistic feeding interaction between scallops and oysters when cultured in close proximity. Scallops grown with oysters appear to grow faster than when grown with scallops alone.

Individual condition index response to shell irritations caused by *Polydora websterii* (mud blisters), 'unexplained juvenile oyster mortality syndrome' and 'bag scars' will also be discussed. Oysters with bag scars or survivors of the 'unexplained mortality' had significantly elevated (33% higher) CI over normal or *Polydora* infested animals.

**IPNV ANTIBODY AS A MEANS OF DETECTION OF POSSIBLE VIRUS CARRIAGE IN ATLANTIC SALMON SURVIVING VIRUS CHALLENGE.** Eileen C. Sadasiv, Pei W. Chang, and Wenyu Lin, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Prudent aquacultural practice suggests the use of salmonid broodstock which have not been exposed to certain infectious agents which might be capable of causing high levels of mortality in progeny. Certification of broodstock to be free of the pathogens can require sacrifice of a portion of the stock. This study was undertaken to determine the feasibility of non-lethal pathogen detection methods. We propose that antibody can be an indicator of infection or exposure.

Atlantic salmon *Salmo salar* L (AS) from presumptively virus-free broodstock were raised in aquaria supplied with deep well water and acclimated to 6°, 10° and 16°C. A total of 96 fish, aged either 17 or 27 months were used. Fish initially had no antibody and no virus was isolated from them. They were immersed in 10<sup>3</sup> TCID<sub>50</sub> of tissue culture produced (CHSE) infectious pancreatic necrosis virus, strain WB (IPNV) per ml of water and monitored for 193 days. Fish at all three temperatures produced a similar level of antibodies, with some retardation noted at lower temperatures. IPNV was found to persist in the kidneys of fish having circulating antibody, along with measurable levels of what appears to be virus-specific antibody, as detected by both virus neutralization in tissue culture and by ELISA.

Testing of a limited number of sexually-mature AS has shown variation in antibody. In at least four of five ocean-returned post-spawn AS, antibody was detected at higher levels than that found in similarly aged hatchery-held AS.

**IMPACT OF HARMFUL ALGAL BLOOMS ON SCALLOP CULTURE AND FISHERIES.** Sandra E. Shumway, Bigelow Laboratory for Ocean Sciences, Department of Marine Resources, West Boothbay Harbor, ME 04575; and Allen D. Cembella, National Research Council, 1411 Oxford Street, Halifax, Nova Scotia, Canada B3H 3Z1.

Harmful algal blooms occur worldwide and their associated phycotoxins are accumulated by filter-feeding bivalve molluscs. Since only the adductor muscle of scallops has been traditionally marketed, scallops are not usually included in routine monitoring programs. A renewed interest in marketing both whole and "roe-on" scallops from various geographic regions along with intensified aquaculture ventures in areas prone to toxic blooms have provoked public health concerns regarding the safety of this resource.

Our studies have focused on the sequestering and biotransformation of phycotoxins in scallops. Our results, coupled with a review of historic data, indicate that: 1) toxins are not distributed evenly throughout the scallop tissues; most toxin is usually concentrated in the mantle and digestive gland; 2) some scallop tissues, e.g. digestive glands and mantles remain highly toxic throughout the year; 3) toxicity varies considerably ( $\pm 43.5\%$ ) between individual animals collected in the same area; 4) no correlations could be made between toxicity levels in gonadal tissue and other tissues.

Scallop culture and commercial fisheries can thrive in areas prone to toxic algal blooms if only the adductor muscle is utilized. Safe marketing of "roe-on" scallops is feasible only under strict regulatory regimes. Marketing of mantles or whole scallops poses a high risk to public health and should only be undertaken after extensive monitoring. Scallop mariculturists should be acutely aware of the potential risks associated with phycotoxins. Further, public health guidelines with particular emphasis on toxin levels in individual tissues is necessary if scallops are to be marketed whole or in conjunction with tissues other than adductor muscles.

**VIABILITY AND GENETIC EFFECTS ON OYSTER EMBRYOS EXPOSED TO BACTERIA AND EFFLUENT FROM DISEASED JUVENILE OYSTERS FROM A LONG ISLAND HATCHERY.** Sheila Stiles and Walter Blogoslawski, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

Viability and cytogenetic effects were investigated in oyster embryos exposed to disease-causing organisms isolated from a commercial hatchery having high mortalities of juvenile oysters

(*Crassostrea virginica*). *Vibrio* bacteria isolated from TCBS media as yellow and green colonies at different concentrations, as well as a seawater wash from diseased juvenile oysters, were used to challenge embryos. No normal larvae developed after 48 hours in cultures at highest concentrations of bacteria; some normal larvae developed at lower concentrations, however, these were smaller than control larvae.

In these assays, vibrios from some colonies appeared more toxic than others. Although a greater percentage of embryos developed into normal larvae in the seawater wash than in the cultures challenged with bacteria, mortality was higher than in the cultures challenged with bacteria. Cytogenetic and cytological observations showed delayed and arrested development as evidenced by elevated frequencies of non-dividing cells in embryos. Results suggest that selective mortality occurs early in oysters after exposure to bacteria or wash water from diseased oysters.

**EFFECTS OF A *PROROCENTRUM* ISOLATE UPON THE OYSTER, *CRASSOSTREA VIRGINICA*: A STUDY OF THREE LIFE-HISTORY STAGES.** Gary H. Wikfors,<sup>1</sup> Roxanna M. Smolowitz,<sup>2</sup> and Barry C. Smith,<sup>1</sup> <sup>1</sup>National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460; <sup>2</sup>LMAH, School of Veterinary Medicine, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, MA 02543.

Evidence that some strains of the dinoflagellate genus *Prorocentrum* are harmful to shellfish has been obtained from both field and laboratory studies. Our previous laboratory exposures of one *Prorocentrum minimum* isolate (strain EXUV) to hard clams and bay scallops demonstrated clear differences in responses of the two bivalves; hard clams survived but did not grow, whereas scallops experienced complete mortality in 1–4 weeks. Histological evidence suggested effects of an enterotoxin upon scallops. The present study was undertaken to determine possible toxicity of cultured *P. minimum* (EXUV) to several life-history stages of the eastern oyster: embryos, feeding larvae, and juveniles.

Embryos exposed to whole EXUV cells, spent medium from EXUV cultures, and filtrates from heat-killed and sonicated cells showed no differences from controls in survival, development, or histology (light and electron microscopy). Forty-eight-hr larvae were fed EXUV alone and as a  $\frac{1}{3}$  or  $\frac{2}{3}$  portion of a mixed ration with *Isochrysis sp.* (strain T-ISO); controls of T-ISO alone and unfed larvae also were included. Differences in survival and growth were obtained, with larvae fed 100% EXUV performing only slightly better than unfed larvae; no EXUV-fed larvae survived to set. *P. minimum* EXUV cells were filtered poorly, relative to T-ISO; some ingestion, but limited digestion was noted by epifluorescence microscopy. Mixed diets produced intermediate results. Histologic examination revealed clear differences between unfed, T-ISO-fed, and EXUV-fed larvae. EXUV-fed larvae

showed more development than unfed animals, but not the vigorous development nor the cellular lipid reserves of T-ISO-fed larvae. Digestive glands of EXUV-fed larvae contained a very distinct phagolysosomal/residual body. Post-set oysters (ca. 3 mm) were evaluated in the same treatments as larvae. Oysters fed 100% EXUV produced abundant pseudofeces for 3 wk, following which well-formed fecal strands were seen; oysters fed T-ISO filtered normally. After 6 wk, no mortalities were noted, and slight growth

was obtained in most treatments. Differences in histologic appearance and condition of the digestive system were again observed.

In summary, although acute toxicity of *P. minimum* EXUV to oysters was not found, there was strong evidence for nutritional deficiency or interference with digestion. This study underscores the great variation in pathological effects that a single dinoflagellate can produce in different life-history stages and different bivalve species, i.e., oysters, clams, and scallops.



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## PARASITES AND DISEASES I

**TRIPLOIDS FOR FIELD TESTS? THE GOOD, THE BAD, AND THE UGLY.** Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

Interest and controversy surround the "proposal" to introduce *Crassostrea gigas* to the east coast, putatively, to bolster the ailing oyster industry. Yet there is no empirical data on how *C. gigas* would perform here. Key is whether or not *C. gigas* are resistant to Dermo, or MSX-disease, or both. For the latter two questions, field exposure seems necessary. Even for ecological issues, the reliability of data extrapolated from land-based experiments is questionable. The GOOD: Triploids, because they are reproductively incapacitated, provides a way to "safely" test *C. gigas* with little or no risk of reproduction. Use of F<sub>1</sub>, or greater, progeny reduces the risk of disease. Data show that triploids produce gamete types that vary little among individuals and that crosses using these gametes behave in predictable ways, all suggesting that the risk is estimable. The BAD: Recent evidence also suggests that there may be some spontaneous chromosome loss in triploids as they age. This surprising result means that analysis of individuals before field planting will be essential, perhaps yearly. And individual testing means a relatively small sample size, precluding pilot scale tests. The UGLY: There is no clear consensus on whether field tests using triploids should be approved; guidelines for approval of such tests are vague and variable; it is difficult to establish the distinction between an introduction for research purposes and a full scale release. This paper considers these points in view of the present crisis on the east coast oyster fishery.

**COMPARATIVE PHYSIOLOGY OF *CRASSOSTREA VIRGINICA* AND *C. GIGAS*: GROWTH, MORTALITY, AND INFECTION BY *PERKINSUS MARINUS*.** Bruce J. Barber,\* Dept. of Animal, Veterinary & Aquatic Sciences, University of Maine, Orono, ME 04469; R. Mann, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Hatchery-produced oysters (the eastern oyster, *Crassostrea virginica*, and the Pacific oyster, *C. gigas*), of the same age were held in quarantined flumes which received raw water from the York River, VA. From July 1991 to December 1993, growth and mortality were compared for experimental (dosed with *Perkinsus marinus*) and control (undosed) groups of both species.

Both prevalence and intensity of *P. marinus* infections were greater in *C. virginica* than in *C. gigas*. The experimental *C. virginica* group had 100% prevalence (with heavy infections) by August 1992; maximum prevalence in the experimental *C. gigas* group was 80%, and only 1 heavy infection was found the entire study. Overall mortality of *C. gigas* (76%) was greater than that of *C. virginica* (45%); however, only mortality of *C. virginica* was related to infection by *P. marinus*. In December 1992 (at age 20

months), mean shell height of *C. gigas* (55 mm) was significantly greater ( $P \leq 0.05$ ) than that of *C. virginica* (41 mm). Shell height was lower in the experimental group compared to the control group of *C. virginica* but not of *C. gigas*.

Thus *C. gigas* is more tolerant of *P. marinus* and grows faster than *C. virginica*, but may be less well adapted to environmental conditions prevailing in lower Chesapeake Bay.

**THE EFFECT OF WINTER TEMPERATURE AND SPRING SALINITY ON *PERKINSUS MARINUS* PREVALENCE AND INTENSITY: A LABORATORY EXPERIMENT.** Eugene M. Burrenson\* and Lisa M. Ragone Calvo, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The role of low temperature and low salinity in controlling *P. marinus* was investigated under laboratory conditions which simulated typical and extreme winter and spring environmental conditions. Oysters (*Crassostrea virginica*) infected with *P. marinus* were collected from the upper James River, VA in December 1991, individually marked and analyzed for *P. marinus* by hemolymph assay. The oysters were then subjected to a sequential treatment of various temperature and salinity combinations. In the first phase oysters were placed in recirculating seawater systems at 10 ppt and low temperature (1°C and 4°C). Half of the oysters were treated at each temperature for 3 weeks and the other half were held for 6 weeks. In the second phase the oysters were gradually warmed to 12°C, adjusted to one of three salinities (3, 6, and 15 ppt), and held for 2 weeks. Finally, all oysters were gradually adjusted to 25°C and 20 ppt and maintained for 4 weeks to determine if any observed declines in prevalence or intensity resulting from prior treatment were permanent. At the end of each phase *P. marinus* prevalence and intensity was assessed using hemolymph assay. Control oysters were maintained at 15°C and 15 ppt during treatment phase 1 and 2 and adjusted to 25°C and 20 ppt in phase 3.

Low temperature exposure, alone, did not significantly effect *P. marinus* prevalence or infection intensity. However, declines in prevalence and intensity, relative to initial levels were observed after 2 weeks at 12°C and 3, 6, and 15 ppt. *Perkinsus marinus* prevalence and intensity in control oysters significantly increased as the experiment progressed. These results suggest that low winter temperatures have little effect on the annual abundance of *P. marinus* within an estuary, while springtime depressions in salinity are very important.

**OVERWINTERING INFECTIONS OF *PERKINSUS MARINUS* IN CHESAPEAKE BAY OYSTERS.** Eugene M. Burrenson and Lisa M. Ragone Calvo,\* Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The scarcity of overwintering infections of *Perkinsus marinus* in Chesapeake Bay oysters has long puzzled investigators. Typi-

cally, prevalence of the pathogen declines in winter and infections are not easily disclosed by routine diagnosis using tissue cultured in thioglycollate medium (FTM). It is unknown whether cryptic stages of the parasite are harbored in the oyster during winter or whether elimination occurs; hence, the actual abundance and relative contribution of overwintering infections to subsequent summer prevalences is unclear.

The objective of this investigation was to determine the nature and abundance of overwintering *P. marinus* infections. Infected oysters were placed in a tray and suspended from a pier in the lower York River, VA in November 1991. Every six weeks from November 1991 through May 1992 oysters ( $n = 25$ ) were removed from the tray, examined for *P. marinus* by hemolymph analysis, gradually warmed in individual containers to 25°C and held for one month. After the incubation period, which permitted the development of very light and/or cryptic parasite stages to detectable levels, the oysters were reanalyzed for *P. marinus* by both hemolymph and tissue cultures in FTM. A second group of 25 oysters was sacrificed on each date, diagnosed using tissue FTM cultures, and examined for cryptic stages using immunoassays.

Prevalence of *P. marinus* gradually declined from 100% in November 1991 to 32% in April 1992. Incubation of oysters at 25°C always resulted in an increase of *P. marinus* prevalence and intensity, suggesting that the parasite was more abundant than FTM cultures indicated. Immunoassay did not reveal the presence of cryptic stages, although it was generally more sensitive than FTM diagnosis. *Perkinsus marinus* appears to overwinter at very light intensities in a high proportion of oysters. These infections are likely to be an important cause of summer mortalities.

**PERKINSUS MARINUS AS A SOURCE OF OYSTER MORTALITY IN COASTAL LAGOONS IN TABASCO, MEXICO.** Eugene M. Burreson,\* Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; Victor Vidal-Martinez and Raul Simá-Alvarez, Centro de Investigación de Estudios Avanzados del IPN Unidad Mérida, C. P. Mérida, Yucatan, Mexico.

Periodic oyster mortality in coastal lagoons in Tabasco, Mexico in the southern Gulf of Mexico was attributed to the Mexican oil industry because of a previous small-scale oil spill near Mecoacan Lagoon. In an attempt to identify the cause of the oyster mortality the Mexican oil company Petroleos Mexicanos (PEMEX) funded CINVESTAV-IPN Unidad Mérida to conduct a study that included pathology, effects of various pollutants and other water quality studies. This situation is very reminiscent of the sequence of events in Texas in the late 1940s that led to the discovery of *P. marinus*.

As part of the PEMEX-funded study a survey of oyster beds was conducted in Mecoacan and Carmen y Machona lagoons in October, 1992. Subsequent thioglycollate culture diagnosis revealed the presence of *Perkinsus* in all beds sampled. Prevalence

ranged from 60% to 100% and weighted prevalence ranged from 0.5 to 3.1. Previous samples from July, 1992 processed only for paraffin histology revealed prevalences of at least 50% and the presence of one extremely high *Perkinsus* infection. Immunoassay analysis of the Mexican samples using an anti-*P. marinus* antibody were positive. These results suggest that at least some of the oyster mortality in Mexico could be attributed to *P. marinus*, but more intensive areal and temporal surveys are necessary before the effect of this pathogen can be determined with certainty.

**EVALUATION OF PERKINSUS MARINUS QUANTIFICATION TECHNIQUES USING FLUID THIOGLYCOLLATE MEDIA.** David Bushek, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

Accurate quantification of parasite burden is critical for comparing host resistance, especially when resistance is a matter of degree. *Perkinsus marinus* loads can be estimated by culturing tissue or hemolymph samples in fluid thioglycollate media. Infections are ranked in tissue samples whereas hemolymph samples are enumerated. The accuracy and sensitivity of these methods was checked against total body burden across seasons. Oysters were collected throughout the year from Delaware Bay beginning in July, 1992. *Perkinsus marinus* levels estimated with both techniques were regressed on total body burden. Correlations improved as parasitism peaked in the population, but variability was high:  $r^2 = 0.63$  with tissue, 0.4 with hemolymph. Higher correlations with tissue apparently resulted from lumping infections into categories.

Neither technique is sufficient when total body burden estimates must be determined accurately or when infection levels are low. Tissue samples are recommended for routine diagnostics because they are quick, easy and moderately accurate. Hemolymph samples are only recommended when the oyster cannot be sacrificed. Contribution # K-32100-1-93 NJAES.

**CHEMOTHERAPY OF PERKINSUS MARINUS-INFECTED OYSTERS: A TWO WEEK BATH TREATMENT EXPERIMENT WITH AMPROLIUM, CYCLOHEXIMIDE, MALACHITE GREEN, AND SULFADIMETHOXINE.** Gustavo W. Calvo\* and Eugene M. Burreson, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

A repeated measures design was used to determine the effect of chemical baths on reducing *P. marinus* infections in oysters. To that end, 300 oysters were collected from Pt. of Shoals in the James River in September and maintained in a static renewal tank with 1  $\mu\text{m}$  filtered York River water (temperature 20°C, salinity 20 ppt) at VIMS for one week. During that time, oysters were labelled and screened individually for *P. marinus* using the hemolymph technique. Then, 180 oysters with known infection in-



tensities were selected and assigned to 10 L aquaria in groups of 10.

There were 8 chemical treatments (amprolium and sulfadimethoxine as 100 mg/L and 10 mg/L baths, and cycloheximide and malachite green as 10 mg/L and 1 mg/L baths) plus 1 untreated control treatment all run in duplicate. Chemicals were mixed with microalgae and added to aquaria, at the time of water renewal, every other day for 2 weeks. Dilution water consisted of 1  $\mu$ m filtered York River water (warmed and maintained at 20°C, and 20 ppt). Oysters were fed every day. After the 2 week treatment, *P. marinus* diagnosis was performed on a second hemolymph sample and on a combined rectum, gill and mantle sample taken from each oyster.

Pre-treatment and post-treatment infection intensities in hemolymph samples were compared by Wilcoxon's signed rank test. Only oysters exposed to 10 mg/L of cycloheximide showed a significant decrease in infection levels. Tissue samples also revealed a higher proportion of oysters with lower infection intensities in the group exposed to 10 mg/L of cycloheximide than in the control or any other group. These results suggest that cycloheximide is effective in reducing *P. marinus* infections in oysters. Use of cycloheximide, however, is mostly restricted to laboratory applications.

**PERKINSUS MARINUS SUSCEPTIBILITY IN EASTERN (CRASSOSTREA VIRGINICA) AND PACIFIC (CRASSOSTREA GIGAS) OYSTERS: TEMPERATURE AND SALINITY EFFECTS.** Fu-Lin E. Chu,\* Carrie S. Burreson, Aswani Voley, and Georgeta Constantin, Virginia Institute of Marine Science, School of Marine Science, College of William & Mary, Gloucester Point, VA 23062.

Susceptibility of *Crassostrea virginica* to *Perkinsus marinus* was compared with diploid and triploid (2N and 3N) *C. gigas* at 10, 15, and 25°C in the first experiment and at 3 salinities, 3, 10, and 20 ppt, in the second experiment. In both experiments, oysters were challenged twice with *P. marinus* trophozoites. The temperature effect experiment was terminated 68 days after 1st challenge and 27 days after 2nd challenge by *P. marinus*. The salinity effect experiment was terminated 50 days after 1st challenge and 34 days after 2nd challenge by *P. marinus*. Results revealed that at 15 and 20°C, infection prevalence was higher in challenged *C. virginica* than in challenged 2N and 3N *C. gigas*. But at 10°C, challenged 3N *C. gigas* had a prevalence higher than challenged 2N *C. gigas* and *C. virginica*. In all salinity treatments, prevalence was higher in challenged *C. virginica* than challenged 2N and 3N *C. gigas*. Weighted prevalence increased with temperature and salinity and was highest in *C. virginica* groups. Since, in both experiments, much higher infection prevalence and intensity were found in non-challenged *C. virginica* than in non-challenged 2N and 3N *C. gigas*, part of the recorded prevalence and intensity in *C. virginica* may be attributed to the hidden infection from the field. High mortality occurred in both 2N and 3N *C. gigas* during temperature and salinity acclimation and at the 25°C and 3 ppt treatments.

**THE QUANTITATIVE EFFECTS OF PERKINSUS MARINUS ON REPRODUCTION AND CONDITION IN THE EASTERN OYSTER, CRASSOSTREA VIRGINICA.** Dawn E. Dittman,\* Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Dermo disease, caused by *Perkinsus marinus*, is responsible for high oyster mortality in many areas along the East Coast. The evidence that *P. marinus* causes a decrease in reproduction before death has been ambiguous. This study examines the effect of infection by *P. marinus* on reproduction and condition index of live oysters.

Known-age susceptible oysters were exposed to *P. marinus* in 1990 and 1991. Sixteen samples of 40 to 50 animals were taken at two to four week intervals. The animals were prepared for histological analysis and cultured for *P. marinus* using standard techniques. *P. marinus* infection level was assigned to three categories: none, light, and advanced. Condition index was calculated and percent gonad area was measured using an image analysis system. The data were analyzed using a Multivariate analysis of variance model.

There was no significant effect on reproduction in the first year when infections were light during the reproductive period. In 1991 the percent gonad area of the individuals with advanced infections was significantly lower than that of individuals with no infections and in most cases was lower than in individuals with light infections. In all of the samples the condition index of oysters with advanced infections was lower than that of the uninfected oysters, and in most cases lower than that of the individuals with light infections. The results show that infection by *P. marinus* has a significant negative impact on the reproduction and the condition index the oyster before death. This is NJAES Publication No. 32501-K-1-93.

**PHYSIOLOGICAL AND IMMUNOLOGICAL MEASURES OF APALACHICOLA BAY OYSTERS DURING A ONE-YEAR PERIOD.** William S. Fisher\* and James T. Winstead, U.S. Environmental Protection Agency, Center for Marine and Estuarine Disease Research, Environmental Research Laboratory, Gulf Breeze, FL 32561; Leah M. Oliver, Technical Resources Inc., Gulf Breeze, FL 32561; Patrice Edwards, Center for Environmental Diagnostics and Bioremediation, University of West Florida, Gulf Breeze, FL 32561.

Most physiological and immunological measures of oyster health are influenced by changes in salinity and temperature. To apply such measures in assessment of oyster health requires knowledge of variations introduced by temperature and salinity patterns. A year-long study was performed on oysters (*Crassostrea virginica*) from two subtidal, unpolluted, commercially-harvested sites in Apalachicola Bay, Florida. Oysters were collected monthly and multiple endpoints measured for each organism. Physiological measures included gonadal index and state of

maturation, condition index, tissue structure indices and hemolymph protein levels. Immunological measures included hemocyte morphology, mobility, phagocytic capacity and superoxide production as well as hemolymph lectin and lysozyme content. Parasite burdens and infection levels of *Perkinsus marinus* were quantified.

Results demonstrated high variability for most endpoints, with seasonal (temperature) cycles in evidence and relatively rapid responses to salinity events. Correlations among certain immunological endpoints support current hypotheses of immunological fitness. It is concluded that assessment of oyster health requires a continuous monitoring scheme for each site under consideration to reduce potential misinterpretation of results.

**SPORES OF HAPLOSPORIDIUM NELSONI (MSX): FINDINGS AND SPECULATIONS.** Susan E. Ford\* and Robert D. Barber, Rutgers University, Institute of Marine and Coastal Sciences, Haskin Shellfish Research Laboratory, Box B-8, Port Norris, NJ 08349.

The apparent rarity of spores produced in oysters infected with *Haplosporidium nelsoni*, cause of MSX disease, led to hypotheses that another host is involved in the life cycle. In contrast to previous studies, which found spores in <1% of infected adult oysters, we report that infected spat have a high probability (>50%) of producing the spore stage. Advanced infections nearly always result in sporulation. In 1988, 30–35% of spat in lower Delaware Bay produced spores, whereas, that the figure has been only 5% in the last 4 years (1989–92). Up to  $1.5 \times 10^6$  mature spores have been found in a single spat.

We have also found spores morphologically identical (by light microscopy) to those of *H. nelsoni*, ingested by oysters throughout Delaware Bay. Their presence in oyster guts during the summer coincides with the infective period for *H. nelsoni*. We estimate that the concentration of spores in the water processed by oysters must be several hundred per liter to account for their numbers in the digestive tract.

Although annual spat sets are temporally and spatially variable, data from 35 years of sampling in Delaware Bay lead us to estimate that spat density is about  $100 \text{ m}^{-2}$  in an "average" year ( $10^{10}$ – $10^{12}$  total in the Bay). If the ingested spores are *H. nelsoni*,  $10^9$  to  $10^{10}$  spat would be required, each producing  $10^6$  spores, to yield estimated concentrations in Delaware Bay during summer. Five percent of the total estimated spat in the Bay would somewhat exceed this number. We do not know how long spores remain viable, how long they are present in the water column, and our estimates have not taken into account potential loss of spores from the estuary in current outflow, loss from the water column through biodeposition, or destruction by microbes in the sediment. The calculations suggest that spat could produce enough spores to serve as a primary host; nevertheless, the possibility of an alternate host still cannot be excluded.

**IN VITRO CONTINUOUS CULTURE OF PERKINSUS MARINUS TROPHOZOITES: OPTIMIZATION OF THE METHODOLOGY.** Julie D. Gauthier\* and Gerardo R. Vasta, Center of Marine Biotechnology, University of Maryland, Baltimore, MD 21202.

A continuous pure culture of the oyster parasite *Perkinsus marinus* was accomplished in a variety of DMEM (Dulbecco's Modified Essential Medium, currently used in our laboratory for hybridoma culture) based media at  $26 \pm 2^\circ\text{C}$  with no added  $\text{CO}_2$ . DMEM was dissolved in 23 ppt artificial sea water and 15 mM HEPES (final pH 7.4) and 100 U/ml each Penicillin-G and Streptomycin sulfate added. The effect of supplements including oyster serum (0.1–50.0%), fetal bovine serum (FBS) (0.1–20%) and HAM's F-12 Nutrient Mixture (1:1 or 1:2 DMEM:HAM's F-12) was investigated and the formulations optimized. Oyster hemocytes harboring large numbers of *P. marinus* trophozoites were washed in a high antibiotic sea water solution (4,000 U/ml Penicillin + 5000 ug/ml Streptomycin sulfate) and plated at equal density in different media formulations. Growth was determined by direct counting and  $^3\text{H}$ -thymidine incorporation. Further optimization of culture conditions (supplement additions, seeding density and frequency of medium changes) was accomplished by adapting image analysis methodology. Optimal conditions at present time include the addition of 5% oyster serum to the three following formulations: 20% FBS/DMEM, 10% FBS/1:1 DMEM:HAM's F-12 or 1:2 DMEM:HAM's F-12 (Serum-free). The cultured parasite proliferates by multiple fission and/or budding at an estimated doubling time of 24 hrs within the first 72 hrs. Light and electron microscopy and serology demonstrate that the cultured forms are morphologically and biochemically identical to the freshly isolated ones. The cultured trophozoite enlarges in thiolglycollate medium and stains dark blue in Lugol's solution, both diagnostic for *P. marinus*. Virulence of the *in vitro* cultured parasite was determined by two biweekly injections of washed cultured trophozoites ( $\sim 2 \times 10^5$  cells) into uninfected oysters (Mook Seafarms, Inc., ME). After 4–5 weeks, all experimental oysters were heavily infected based on diagnostic tests on rectal, mantle and hemolymph tissues, whereas controls (receiving only sea water injections) remained uninfected. [Supported in part by Sea Grant Award NA90AA-D-SG063 to GRV and Sea Grant Traineeship to JDG/GRV.]

**GENETIC DIFFERENTIATION AMONG STRAINS OF DISEASE CHALLENGED OYSTERS.** John E. Graves\* and Jan R. McDowell, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) was used to determine levels of genetic variation and differentiation within and among 4 strains of Eastern oyster bred for resistance to MSX and dermo, and their respective source populations. Purified mtDNA from up to 20 individuals per sample was analyzed with 13 informative restric-

tion endonucleases to produce individual composite genotypes. The distribution of composite mtDNA genotypes was compared among samples from the source populations and the second generation of each challenged strain. Samples from all source populations exhibited modest levels of within-sample variation but no significant genetic differentiation was found among the source samples. In contrast, the distribution of mtDNA genotypes differed significantly among the 4 challenged strains, as well as between each challenged strain and its respective source sample. Different mtDNA genotypes, not represented in the source samples, occurred in relatively high frequencies in each of the challenged strains. The marked genetic differences between source samples and challenged strains, which occurred over 2 generations of selective breeding, could either be the result of intense selection pressure (disease resistance) or more likely, genetic drift.

**CHEMICAL INHIBITION OF *PERKINSUS MARINUS* IN AN *IN VITRO* TEST.** George E. Krantz,\* Maryland Department of Natural Resources, Cooperative Oxford Laboratory, Oxford, MD 21654.

A rapid diagnostic test for oyster parasites, recently developed at the Cooperative Oxford Laboratory, utilizes thioglycolate culture media in polystyrene tissue culture plates to detect *Perkinsus marinus* cells circulating in oyster hemolymph. This test was modified to serve as an *in vitro* assay system to detect chemical compounds that exhibit inhibitory activity toward the enlargement of *P. marinus* cells in the thioglycolate media. The assay system detected 16 organic chemicals and 2 inorganic salts that had inhibitory activity. Cellular changes of treated *Perkinsus* are described, and trypan blue vital stain confirmed that certain cellular changes resulted in death of the enlarging *Perkinsus* hypospores.

Application of minimum reactive concentrations of chemical compounds in oysters has failed to alter the infection levels of *Perkinsus* and induced high levels of mortality in host oysters. Present studies utilizing lower concentrations of chemicals may be helpful in evaluating the therapeutic value of long-term exposure of sublethal concentrations of reactive chemicals.

**PROPAGATION OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* *IN VITRO*.** Jerome F. La Peyre,\* Mohamed Faisal, and Eugene M. Burreson, School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The protozoan *Perkinsus marinus* causes mortalities of the eastern oyster, *Crassostrea virginica*. Attempts to propagate *P. marinus* in commercially available media have failed. We developed a culture medium (JL-ODRP-1) that contain most of the known constituents of hemolymph. Using this medium, we were able to propagate a protozoan (designated *Perkinsus-1*) resembling *P. marinus* from the heart tissue of an infected oyster. This organism adapted well to culture conditions, divided by schizogony-like processes, and has been subcultured 11 times. *Perkinsus-1*

was similar in morphology to histozoic stages of *P. marinus*, reacted with anti-*P. marinus* antibodies, and was infective to susceptible oysters.

Several attempts to use the visceral mass as a rich source of *P. marinus* merozoites for *in vitro* cultivation were unsuccessful due to excessive bacterial and protozoal contamination. By incubating the visceral mass first in fluid thioglycollate medium, isolating and purifying the prezoosporangia, and incubating them in JL-ODRP-1, numerous continuous cultures of *P. marinus* were initiated. Two types of divisions were observed in cells cultured according to this procedure: progressive cleavage and successive bipartition that resulted in the formation of flagellated cells.

The success achieved in propagating *P. marinus* will permit further study of the pathobiology and control of this pathogen.

**POPULATION MODELS TO EVALUATE IMPACT OF DISEASES AND MANAGEMENT OPTIONS FOR THE JAMES RIVER OYSTER FISHERY.** Roger Mann,\* School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Population models which quantify the impacts of biological and environmental variation on sequential life history stages of the oyster allow identification of factors which can be manipulated to alleviate disease related mortality and facilitate management of oysters as a resource for commercial exploitation. To date such models have been limited by a lack of methods to quantify several life history stages, especially larval production and survival. I present current data for a project designed to produce a quantitative description of the oyster population of the James River, Virginia in terms of the following components: standing stock, size specific fecundity, egg viability, larval survival and retention by frontal systems, availability of substrate, success of metamorphosis, post settlement growth, and post settlement losses to disease and predation. Both fecundity and egg viability vary temporally and are strongly influenced by the prevailing salinity, as is the prevalence and intensity of disease. Manipulation of the budget components illustrate the utility and possible limitations of management options that exist for the commercial resource.

**THE OYSTER DISEASE RESEARCH PROGRAM OF THE NATIONAL MARINE FISHERIES SERVICE (NMFS): AN OVERVIEW.** Harold C. Mears,\* National Marine Fisheries Service, Gloucester, MA 01930.

The Oyster Disease Research Program, administered by the National Marine Fisheries Service, is assessing research and management issues associated with the impact of shellfish diseases on the eastern oyster (*Crassostrea virginica*). The Program has funded investigations by state management agencies, colleges, and universities, in addition to several workshops and symposia. Thirty three peer-reviewed projects, at an average funding level of \$88,400, have been awarded on a competitive basis since 1990. Several of these studies are exploring the potential factors respon-

sible for the demise of the eastern oyster in Chesapeake Bay. Work has been conducted on topics such as disease transmission and resistance, diagnostic techniques, environmental modeling, and a social/economic assessment of the oyster industry.

Funding complements Federal financial support for oyster research from other sources including Sea Grant, the National Coastal Resources Research and Development Institute, and the U.S. Department of Agriculture. The NMFS Program is unique in that it requires coordination of research and management projects with the concerned State fishery agencies responsible for shellfish management. Accordingly, the Program promotes the use of scientific findings and state-of-the-art biotechnology in the development of practical approaches for state authorities to manage eastern oysters impacted by disease in Atlantic coastal waters. An overview of completed projects, currently funded research, and the current status of the Oyster Disease Research Program will be presented.

**UTILIZATION OF A GEOGRAPHICAL INFORMATION SYSTEM (GIS) FOR THE TIMELY MONITORING OF OYSTER POPULATION AND DISEASE PARAMETERS IN MARYLAND'S CHESAPEAKE BAY.** Gary F. Smith\* and Stephen J. Jordan, Maryland Department of Natural Resources, Cooperative Oxford Laboratory, Resources, Oxford, MD 21654.

The parasites *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) have over the past several years caused high mortality to Maryland's Chesapeake Bay oysters. An impediment to the timely management utilization of oyster disease and population monitoring data has been in the quantity and complexity of the information collected. This situation has resulted in data not being fully utilized and or availability greatly lagging collection date. Integration of data input and analysis programs with a PC based commercial GIS system has shown promise in improving oyster monitoring of disease and population parameters.

Initiation of a comprehensive annual oyster survey in 1990 geared to GIS applications has allowed site specific and regional representation of all available oyster data in a geographic context on the bay. Management oriented capabilities have been developed to allow user based queries combined with statistical analysis in a user friendly format.

**INFECTIVITY AND PATHOGENECITY OF TWO LIFE STAGES, MERONT AND PREZOOSPORANGIA OF *PERKINSUS MARINUS* IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*.** Aswani K. Volety\* and Fu-lin E. Chu, Virginia Institute of Marine Science, School of Marine Science, The College of William & Mary, Gloucester Point, VA 23062.

Two experiments were conducted to compare the infectivity and pathogenicity of two life stages, namely, meronts (trophozoites) and prezoosporangia of the parasite, *Perkinsus marinus* in eastern oysters (*Crassostrea virginica*). Partially purified trophozoites or prezoosporangia at a dose  $5 \times 10^3$ /oyster were injected into the shell cavity of the oyster. Prevalence and intensity of *P.*

*marinus* infection in oysters were determined 15, 25, 40 and 65 days, for the first experiment, and 20, 40, 50, 65 and 75 days, for the second experiment, after inoculation with infective particles. Condition index, serum protein and lysozyme were also measured. In the first experiment, *P. marinus* infection was first detected in the groups of oysters challenged by prezoosporangia. However, at the end of the experiment, prevalence and intensity of infection were higher in the groups of oysters exposed to trophozoites. In contrast to experiment 1, in the second experiment, infection was first detected in the groups of oysters challenged with trophozoites. Results from experiment 1 indicate that there was a decrease in condition index in all treatments, including control at the end of the experiment. A significant decrease was also observed at the end of the experiment in the serum protein in the groups challenged with prezoosporangia ( $P < 0.055$ ). Lysozyme concentrations did not show any significant change over the course of the experiment. Lower condition index and serum protein values in the groups challenged with prezoosporangia compared with the groups challenged by trophozoites at the end of the experiment, may suggest a higher energetic demand on these oysters.

## GENERAL BIOLOGY

**CONTRASTING FORAGING TACTICS OF TWO PREDATORS OF JUVENILE BAY SCALLOPS, *ARGOPECTEN IR-RADIANS*, IN THE EELGRASS CANOPY.** V. Monica Bricelj,\* Susan Bauer, and Shino Tanikawa-Oglesby, Marine Sciences Research Center, State University of New York, Stony Brook, NY 11794-5000.

As an extension of earlier work, we demonstrate that above-ground attachment to eelgrass, *Zostera marina*, provides juvenile ( $\leq 15$  mm) bay scallops with significant refuge from both non-swimming and swimming (portunid) crabs. However, we identify two common predators in Long Island, NY bays, which readily prey on scallops in the upper eelgrass canopy: xanthid mud crabs, *Dyspanopeus sayi*, and northern puffer fish, *Sphoeroides maculatus*. Both species may be important in controlling early recruitment of scallops, before they relocate to the bottom.

Puffers, as visual predators, exhibited a 6-fold reduction in feeding activity at night, but consumed scallops at high rates ( $44$   $5$  mm scallops  $\text{hr}^{-1}$   $7.4$  cm fish $^{-1}$ ) during daytime laboratory experiments. In contrast, mud crab consumption of scallops in the upper canopy increased significantly at night due to the crabs' increased nocturnal climbing activity, presumably an adaptive response to reduced predatory risk from finfish. Furthermore, in the presence of mud crabs, scallop survival in the upper canopy was greatest at low eelgrass densities ( $200$  shoots  $\text{m}^{-2}$ ) in both field and laboratory experiments. This unexpected result was explained by the crabs' reduced climbing effectiveness in low-density eelgrass. Laboratory mud crab predation rates were an order of magnitude lower than those of puffers. Field, predator-exclusion experiments provided more realistic measures of predation pressure

on scallops by natural populations of *D. sayi*. About 86% of the variability in scallop survival among cages could be explained by differences in the abundance of mud crabs  $\geq 15$  mm in carapace width, which comprised  $\leq 51\%$  of the total mud crab population at the study site.

**ROLE OF FECAL ELIMINATION DURING UPTAKE AND DEPURATION OF 65Zn AND 109Cd IN THE HARD CLAM, *MERCENARIA MERCENARIA*.** Albert F. Eble,\* J. Ramsbottom, and B. Burkhardt, Department of Biology, Trenton State College, Trenton, NJ 08650-4700.

Clams were collected at Shark River, Belmar, NJ and acclimated in all-glass aquaria for 10 days in sea water adjusted to 28‰ salinity and 22°C. Animals were fed mixtures of *Isochrysis galbana* var. *Tahiti* and *Chaetoceros calcitrans* thrice weekly during acclimation and for the balance of the experiment.

Radionuclides were dissolved in sea water at 3  $\mu\text{Ci/L}$ . Every three days during uptake (15 days) and depuration (33 days), 5 clams were sampled for feces, kidneys, hemocytes and hemolymph. Samples were counted in a crystal scintillation spectrometer for 30 minutes.

Both 65Zn and 109Cd showed similar uptake and depuration kinetics in hard clams: (1) fecal elimination was the major route of disposal during uptake, Days 1–6; (2) renal elimination surpassed fecal elimination by Day 9 of uptake and remained the major pathway of radionuclide elimination during the balance of the uptake period as well as during depuration; (3) hemocytes rapidly accumulated radionuclides during the uptake period and maintained high levels of activity throughout depuration.

**DEVELOPMENT OF CONFLUENT MONOLAYERS FROM TISSUES OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.** Mohamed Faisal,\* Jerome F. La Peyre, and Morris H. Roberts, Jr., Department of Environmental Sciences, School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

Because of the quiescence of cells under *in vitro* conditions, no immortal cell lines of oyster or any other bivalve molluscs have been developed. Many pathobiological investigations, however, could be performed if confluent monolayers of oyster cells were produced and maintained. In the present study, several attachment factors such as collagenase (types I, II, and IV), fibronectin, laminin, gelatin, poly-D-lysine, poly-L-lysine, and vitronectin were tested for their ability to promote the attachment and spreading of oyster cells in tissue culture plates.

Poly-L-lysine and poly-D-lysine induced a rapid attachment of the cells. Moreover, clumping of cells, a common problem in culturing oyster cells, was prevented. The cells were, however, unable to spread on the coated plates. In contrast, fibronectin promoted slow attachment of the cells but with strong spreading. A combination of both poly-L-lysine and fibronectin gave the best

results and confluent monolayers of spread oyster cells were obtained. We also found that covering the cell surface with a thin layer of 0.5% low melting point agarose prevented the cell migration without affecting cell viability. The best results were obtained using the heart and mantle tissue.

**OXYGEN UPTAKE, OXIDANT PRODUCTION, AND LUMINOL-ENHANCED CHEMILUMINESCENCE BY HEMOCYTES OF EASTERN OYSTERS.** Frank E. Friedl\* and Marvin R. Alvarez, Department of Biology, University of South Florida, Tampa, FL 33620.

To determine whether oysters produce biocidal reactive oxygen species, oxygen and hydroperoxide metabolisms of hemocytes of the Eastern Oyster, *Crassostrea virginica*, from Tampa Bay, Florida were investigated. Cell suspensions show an uptake of oxygen (about 1.3 nmoles  $\text{min}^{-1}$ ) partially inhibitable by cyanide and azide (Friedl and Alvarez, *Aquaculture*, 107:125, 1992). Using a sensitive fluorescence method, an endogenous hydrogen peroxide production, proportional to cell number is detectable, and added Concanavalin A or Zymosan increases the amounts of  $\text{H}_2\text{O}_2$  found (ibid.). Hemocyte suspensions also exhibit an endogenous luminol-enhanced chemiluminescence which is greatly increased over a period of 1–2 hours by zymosan addition. Endogenous luminol-enhanced light production is not limited to hemocytes, since other tissues such as excised mantle and gill show it to various degrees. *In vitro* catalase activity in oysters is easily measurable, but peroxidase activity with guaiacol was not found. However, peroxidase can be detected cytochemically using diaminobenzidine in hemocyte preparations. From the above data it appears that hemocytes in particular have oxidative metabolisms that respond to stimulants and are capable of producing potentially biocidal oxidants. Whether these oxidants are meaningful in cellular defense remains to be demonstrated. (Research supported in part by Florida Sea Grant Program).

**INVESTIGATION OF PHYSIOLOGICAL PARAMETERS OF BLACK ABALONE WITH WITHERING SYNDROME.** Gunadi Kismohandaka,\* Carolyn S. Friedman, Wendy Roberts, and Ronald P. Hedrick, University of California, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923; Michael P. Crosby, NOAA Sanctuaries and Reserves Division, 1825 Connecticut Ave NW, Ste 714, Washington, D.C. 20235.

Withering syndrome (WS) has spread widely among populations of black abalone, *Haliotis cracherodii*, along the Channel Islands and in Diablo Cove, California. The causative agent(s) of WS have not been identified and early detection of the disease is not possible. As a result of these facts and the paucity of information on abalone physiology, we have initiated studies to determine physiological differences between healthy black abalone and those with WS. By understanding the difference(s) in measured parameters we are attempting to identify which metabolic system(s) may be affected by these unknown agent(s) and/or identify

possible markers of early stages of WS. Every three months we examined rates of food consumption, respiration, ammonia excretion and fecal production of apparently healthy black abalone from Ano Nuevo Island and black abalone from Santa Rosa Island where WS occurs. Results indicate that abalone suffering from WS consumed 4.4 times less kelp, 1.2 times less oxygen and excreted 3.8 times more ammonia per gram wet weight than did healthy abalone. These data suggest that we can measure physiological abnormalities in abalone with WS and may be able to identify early signs of the disease.

**STUDIES OF THE SPORADIC RELEASE OF EPITHELIAL CELLS BY THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*.** Tracy Potter,\* Bruce A. MacDonald, and J. Evan Ward, Department of Biology, University of New Brunswick, Saint John, NB, Canada E2L 4L5.

During the course of feeding studies in the field, we documented that large numbers of ciliated and nonciliated cells (6–12  $\mu\text{m}$ ) were released by adult sea scallops during the summer months. An electronic particle sizer was used to distinguish these cells from suspended particles and to determine which individuals were releasing the cells. Tissue samples were collected from within the mantle cavity of animals known to have released cells and others that apparently had not. Scanning electron microscopy has confirmed that these cells are epithelial and are released from at least 4 different tissues: gill, mantle, gonad, and labial palp. Gill filaments in some individuals, known to have released cells, were devoid of cilia including those that comprise the lateral, laterofrontal, and frontal tracts. The loss of these ciliated cells has obvious implications for the animals' ability to capture and transport food particles. Parallel feeding studies and SEM analyses conducted during the past year have shown that the sloughing of these cells is not a common event, but may be a response to some external stimulus.

**ABALONE WITHERING SYNDROME AT SAN NICOLAS ISLAND, CALIFORNIA.** Joan L. Ruediger\* and Glenn R. VanBlaricom, University of California, Santa Cruz, and U.S. Fish and Wildlife Service, Santa Cruz, CA 95064.

Abalone withering syndrome (WS) has been linked to mass mortalities of black abalones (*Haliotis cracherodii* Leach) in the California Islands since 1986. WS apparently was absent from San Nicolas Island (SNI) until April 1992, when it was first observed at the island's westernmost point. We have since surveyed for WS at six intertidal study sites distributed around the Island. In May 1992 we found WS at low frequencies (<3%) at two of five sites. In July and August 1992 we found WS at four of six sites, again at low frequencies (<6%). In October and December 1992 distribution of WS was unchanged, but frequencies were higher (3–13%) at three of six sites. The first substantial reductions in abalone density (up to 50%) were observed in October at four of six sites and continued in December at three of six sites. More surveys were done in February and May 1993.

**MORPHOMETRIC MATURITY AND AGGREGATIVE MATING BEHAVIOR OF TANNER CRAB, *CHIONOECETES BAIRDI* (DECAPODA:MAJIDAE), SAMPLED BY SCUBA AND SUBMERSIBLE.** Bradley G. Stevens,\* J. Haaga, and J. E. Munk, National Marine Fisheries Service, Kodiak, AK; W. E. Donaldson, Ak. Dept. of Fish and Game, Kodiak, AK.

Paired male and female Tanner crabs *Chionoecetes bairdi* were collected from shallow (<13 m) and deepwater (>150 m) environments by scuba and submersible, respectively. Pubescent paired females representing a single instar with mean size of 80.9 mm CW were restricted to shallow water, whereas paired multiparous females ( $\bar{x}$  = 91.1 mm CW) occurred primarily in a large, deep-water mating aggregation. All males were larger than their female partners (mean ratio M:F = 1.37). Male crabs exhibited size-selectivity for pubescent females, but not for multiparous females, which were limited in size range. Grasping males represented at least three different instars, with mean size of 114 mm CW, and 99% (of 176) were morphometrically mature, i.e., had large claws. Fifty percent were morphometrically mature at a size of 99.1 mm CW. These data support the hypothesis that morphometric maturity is a pre-requisite for functional maturity (the ability to mate in wild populations) in male Tanner crabs.

## REPRODUCTION AND RECRUITMENT

**INSTANTANEOUS REPRODUCTIVE EFFORT OF THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA*, IN GALVESTON BAY, TEXAS MEASURED BY A PROTEIN A IMMUNOPRECIPITATION ASSAY.** Kwang-Sik Choi\* and Eric N. Powell, Department of Oceanography; Donald H. Lewis, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843.

Instantaneous reproductive rate of a field population of American oysters was measured in Galveston Bay, Texas over a 12 month period using  $^{14}\text{C}$  leucine as a tracer, rabbit anti-oyster egg IgG as the primary antibody, and protein A cell suspension as an antibody adsorbent. A weight-based gonadal-somatic index (GSI) was calculated from single ring immuno-diffusion assays (SRID) using rabbit anti-oyster egg serum and 1.5% agarose in barbital buffer. A mathematical model was developed to calculate the rate of egg protein production using  $^{14}\text{C}$ -leucine incorporation and the specific activity of free leucine. The calculated egg production rate ( $\mu\text{mol leucine hr}^{-1}$ ) was then used to estimate the number of days required for gonadal development prior to spawning (DS).

Gonadal production (G) was much higher in April and August than any other sampling period. SRID used in the quantitation of oyster eggs and histology indicated that most oysters collected during those two months were ready to spawn. Gonadal production was negatively correlated with oyster size and *Perkinsus marinus* infection intensity during those two months. DS varied from a few weeks to a month during this time. DS increased to many



months in the winter and during mid-summer, indicating negligible gonadal development during these periods. Accordingly, fewer days were required to prepare for spawning during the spring and fall spawning peaks than during the non-spawning season or during mid-summer. DS of oysters collected in April and August was also negatively correlated with oyster size and *Perkinsus marinus* infection intensity. Faster instantaneous reproductive rates found in smaller oysters indicated that small oysters may spawn more frequently than large oysters do, although the total number of eggs produced is smaller than the number produced from large oysters. Instantaneous rates of gonadal production tended to be negative during July and October, times when gonadal resorption is occurring after spawning has occurred.

**A MODELING STUDY OF THE ENVIRONMENTAL AND BEHAVIORAL FACTORS CONTROLLING THE VERTICAL DISTRIBUTION OF OYSTER LARVAE.** Margaret M. Deksheniks,\* Eileen E. Hofmann, and John M. Klinck, Center for Coastal Physical Oceanography, Crittenton Hall, Old Dominion University, Norfolk, VA 23529; Eric N. Powell, Department of Oceanography, Texas A&M University, College Station, TX 77843.

A vertical and time-dependent model, which includes physiological and behavioral responses to *in situ* environmental conditions, has been developed for larvae of the American Oyster, *Crassostrea virginica*. The larval size spectrum, which extends from egg to spat, is divided into six size classes. Within each size class, larval growth is regulated by temperature, salinity, food concentration and turbidity. The behavioral responses of the larvae to changes in environmental conditions are included through temperature effects on the larval swimming rate and salinity effects on the percent time the larvae spend swimming or sinking. Parameterizations, of larval growth and behavioral responses, are based upon laboratory and field observations. A series of simulations were performed to test the effects of temperature and salinity controls on the vertical distribution of oyster larvae. Salinity increases, which typically occur during flood tide, result in an upward movement of oyster larvae in the water column. Subsequent decreases in salinity during the ebb tide produce decreased swimming times and an increased sinking time. These behaviors result in positioning the larvae at or near the bottom of the water column during ebb tide. These simulations suggest that larval responses to changes in salinity may be an important process by which oyster larvae are retained within the estuarine environment.

**ESTIMATING THE SURVIVAL OF DELAWARE BAY OYSTER LARVAE WITHIN AND BETWEEN YEARS.** S. R. Fegley,\* Corning School of Ocean Studies, Maine Maritime Academy, Castine, ME 04420; J. N. Kraeuter, S. E. Ford, and H. H. Haskin, Haskin Shellfish Research Laboratory, Rutgers Univ., Port Norris, NJ 08347.

Extensive abundance records, based on landings or monitoring programs, commonly exist for commercially important species.

Unfortunately, these records, which can cover different stages of the species life history and are often available over long periods of time or from many different regions, usually reveal very little about the population dynamics of the target species for one of several reasons.

As an illustration of this problem, replicate, surface and bottom water samples have been collected every summer since 1953 to estimate the abundances of larvae of the eastern oyster (*Crassostrea virginica*) during the period when larvae are present over the eastern two-thirds of Delaware Bay. The oyster larvae in each sample were further enumerated into one of five developmental stages. This information should be sufficient to estimate directly the survival of oyster larvae in a season by following the fate of each discrete spawning event through each developmental stage. However, logistic and financial constraints prevent taking a sufficient number of samples either temporally or spatially to provide sufficient resolution to make direct estimates in any year and in almost any location.

We will present some of the life history information that can be extracted from these larval monitoring records, the level of confidence in this information, and the means of making statistical comparisons. This is Rutgers University N.J.A.E.S. contribution # K-32406-1-93.

**A JUVENILE CRITICAL STAGE IN THE DUNGENESS CRAB (*CANCER MAGISTER*) LIFE HISTORY.** Robert A. McConnaughey,\* Alaska Fisheries Science Center, National Marine Fisheries Service, 7600 Sand Point Way N.E., Seattle, WA 98115; David A. Armstrong, School of Fisheries, University of Washington, Seattle, WA 98195.

Substantial and unexplained variations in abundance characterize U.S. west coast populations of Dungeness crab. A paradigm has emerged which attributes this pattern to variable survival of cohorts during the ('critical') pelagic larval phase. Supporting studies, including our own, are largely statistical comparisons between commercial fishery landings and time-lagged environmental conditions for the larvae. In this study, however, we have used data from systematic trawl surveys of juvenile *C. magister* abundance along the Washington coast to demonstrate that substantial readjustments to year class strength can occur during the first (0+) year of benthic life. Monthly (May–September) estimates of 0+ abundance did not correlate with subsequent estimates of both 0+ and 1+ abundance for the five (1983–1987) year classes studied. Not until the 1+ stage was there consistency in year class strength from month to month. These findings suggest that *C. magister* year class strength established during the egg-larval stage may be modified and, as such, that the life history can be considered as a series of critical stages, each of which may influence future fishery production. Expression of the 0+ critical stage may be infrequent and may depend on density-dependent effects associated with dominant year classes and anomalous environmental conditions.

We argue that relative stability of year class strength after the 0+ stage reflects a size-based refuge from predation.

**COASTAL ADVECTIVE PROCESSES AND RECRUITMENT VARIABILITY IN DUNGENESS CRAB (*CANCER MAGISTER*) POPULATIONS.** Robert A. McConnaughey,\* Alaska Fisheries Science Center, National Marine Fisheries Service, 7600 Sand Point Way N.E., Seattle, WA 98115; David A. Armstrong, School of Fisheries, University of Washington, Seattle, WA 98195; Barbara M. Hickey, School of Oceanography, University of Washington, Seattle, WA 98195; Donald R. Gunderson, School of Fisheries, University of Washington, Seattle, WA 98195.

A conceptual model is proposed that relates *C. magister* year class strength to variable advection during the pelagic larval phase and restrictive juvenile habitat requirements. Systematic trawl surveys were conducted along the southern Washington coast during 1983–1988. Abundance of new recruits varied 40-fold and settlement was confined to a relatively narrow ( $\leq 15$  km) band along the coast and in estuaries. Analysis of Ekman and geostrophic flow indicated that strong (weak) settlement was associated with relatively weak (strong) northward transport and, to a lesser degree, strong (weak) landward transport during the preceding 4- to 5-month larval period. A similar analysis, using time-lagged and discretized landings data from Washington (1951–1990), corroborated these hypotheses. Persistent landward and net northward flow characterized the circulation of near-surface waters during the larval periods studied (1947–1986). This suggests that larvae are retained nearshore after hatching and that Washington *C. magister* populations receive a significant fraction of recruits from southern (upstream) sources. In addition, substantial numbers of Washington larvae may be advected northward and lost from the California-Oregon-Washington coastal system. A mechanism for progressive seaward transport of larvae through ontogeny (the species paradigm) was not apparent.

**PROTEIN, CARBOHYDRATE AND LIPID LEVELS ASSOCIATED WITH METAMORPHIC SUCCESS IN LARVAE OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.** Kennedy T. Paynter,\* Christopher Caudill, and Donald Meritt, Horn Point Environmental Laboratory, Cambridge, MD 21613; Scott Gallager, Woods Hole Oceanographic Institute, Woods Hole, MA 02543; Dennis Walsh, Aquacultural Research Corporation, Dennis, MA 02638.

Metamorphic success, measured as the proportion of pediveliger larvae which successfully become spat, is typically low in many hatcheries in the Chesapeake Bay region and especially in Maryland. Survival rates of pediveliger larvae to 5 mm spat average less than 5% at the Horn Point Hatchery of the University of Maryland. These rates are quite low compared to several hatcheries in the Northeast which usually get 35 to 50% of pediveligers to 5 mm spat. Although many hypotheses have been proposed,

many researchers believe that unsuccessful pediveligers lack certain nutritional or biochemical stores critical for surviving the stressful, nonfeeding metamorphic process. In order to test this hypothesis, various broods of larvae were transferred at different stages of development between the Horn Point hatchery and the Aquacultural Research Corp. (Dennis, MA) which usually experiences 50% metamorphic success with its oyster larvae.

A series of experiments were conducted in which oyster larvae were produced at both sites from broodstock representative of each facility. Subsequently a series of larval transfers were conducted so that the effects of broodstock, spawning site, culture site (D-hinge through pediveliger) and setting site could be assessed. Samples of the larvae were taken every other day during development and after setting for determination of gross biochemical stores (protein, carbohydrate and lipid). Differences in setting success and levels of biochemical stores were closely associated with the site at which the animals were raised, not where they were set. Supported by the Northeast Regional Aquaculture Center.

**GROWTH OF MICROCULTCHED AND REMOTE SET OYSTERS IN COASTAL WATERS OF ALABAMA.** David Rouse,\* Department of Fisheries, Auburn University, Auburn, AL 36849; Richard Wallace and Scott Rikard, Auburn University Marine Extension and Research Center, Mobile, AL 36615.

Larval oysters, *Crassostrea virginica* were set on microcultch and whole oyster shell using remote setting techniques in July 1991. Both groups of oysters were placed on racks in Portersville Bay along the southwest coast of Alabama. After a one-month nursery phase, the remote set oysters were spread on plastic mesh trays placed on the bay bottom at three sites along the coast of Alabama between Mobile Bay and Mississippi. Oysters set on microcultch were maintained in bags on racks. After 16 months, remote set oysters averaged more than 82 (range = 57–110) in height while cultchless oysters averaged 71 mm (range = 49–99).

**LABORATORY STUDY OF REPRODUCTION IN *ARGOPECTEN VENTRICOSUS*.** Janel R. Villalaz,\* Centro de Ciencias del Mar y Limnología, Universidad de Panama, Panama.

A laboratory study was carried out in Delaware to observe changes in reproduction of *Argopecten ventricosus* (Sowerby, 1842) by using relative dry weight changes in gonads, digestive gland, mantle-gill and adductor muscle. During 58 days, two combinations of monocultures (50:50) of C-ISO and CH-1 were added daily to tanks with filtered and aerated seawater. The study showed that *A. ventricosus* increased significantly in total weight by 30 days in high phytoplankton densities. Gonadal dry weight increased significantly after 40 days at high food ration, but gonadal index declined. The digestive gland declined sharply in dry weight under high and low phytoplankton densities, possibly suggesting that this organ was providing energy for reproduction. The adductor muscle index was higher at a high than at a low food



ration. This study is a contribution to the reproductive biology of *A. ventricosus* and mariculture of the tropical scallop.

## PARASITES AND DISEASES II

**HEMOCYTE RESPONSES IN *CRASSOSTREA VIRGINICA* INFECTED WITH *PERKINSUS MARINUS*.** R. S. Anderson,\* L. L. Brubacher, and L. M. Mora, Chesapeake Biological Laboratory, University of Maryland System, Box 38, Solomons, MD 20688; K. T. Paynter, Department of Zoology, University of Maryland System, College Park, MD 20742; E. M. Burreson, Virginia Institute of Marine Science, School of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062.

The circulating hemocytes provide mollusks with their main line of defense against pathogens. These cells produce cytotoxic reactive oxygen intermediates (ROIs) that mediate killing of pathogens and/or cell injury to adjacent host tissue. In order to better understand the immune response to *P. marinus* infection, total hemocyte count (THC) and ROI production/10<sup>6</sup> hemocytes were determined in individual oysters with known levels of hemolymph infection. Total ROI generation was quantified by phagocytically-induced, luminol-augmented chemiluminescence (CL) assays. Oysters were deployed at sites in the Wye River, Choptank River, and Mobjack Bay, and were sampled at three intervals during spring–fall 1992. *P. marinus* infection appeared earlier and progressed most rapidly in Mobjack Bay oysters, but was also present in oysters from the other sites.

Salinity differences at the sites (~13–20 ppt) had little effect on THC or CL responses. At all sites THC values for uninfected (Un) and lightly infected (L) oysters were not significantly different; however THC for L < moderately (M) < heavily (H) infected oysters. The CL response of the hemocytes also increased with the intensity of infection: Un ≈ L < M < H. Therefore the THC and CL differences observed, whether between experimental groups or sample times, could be explained by intragroup differences in frequencies of oysters with advanced infections. It appears that progression of this infection is characterized by hemocyte recruitment and activation, expressed as increased ROI generation. The increased oxidant load may contribute to the pathogenesis of the disease via tissue damage, but ROI production alone is ineffective in controlling the infection.

**DISEASES OF SPOT PRAWNS (*PANDALUS PLATYCEROS*) CAUSED BY AN INTRACELLULAR BACTERIUM AND A HEMATODINIUM-LIKE PROTOZOA.** Susan M. Bower,\* Gary R. Meyer, and Jim A. Boutillier, Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, B.C., Canada, V9R 5K6.

The cause of stained prawn disease (SPD) with clinical signs of black discoloration of the cuticle especially around the edges of body segments was identified as a *Rickettsia*-like infection of the

fixed macrophages. This disease was found in prawns from some localities of Howe Sound, British Columbia. The distribution of SPD within the vicinity of Howe Sound has not changed since it was first detected in 1989. However, the prevalence has declined to about 4% from a record high of about 15% in July 1990 and March 1991. The high prevalences of infection were found in areas where the prawn populations were showing signs of reduced productivity. Laboratory studies indicated that SPD can be transmitted vertically by cannibalism and via the water (exposure to screened (2 mm pore size) effluent from infected prawns) and remained infectious for at least 10 days of storage at about –10°C. About 50% of the prawns that fed on infected prawns (both fresh and after being frozen) and 25% of the prawns exposed to contaminated water became infected. In all cases, infected prawns began to die about 2 months after being exposed. Recently, a *Hematodinium*-like protozoan was identified as the cause of a new disease that turned infected prawns opaque and the haemolymph milky. In late September 1992, the disease seemed to be confined to prawn stocks from the middle section of Malaspina Strait between Texada Island and mainland British Columbia. Gross signs of infection were observed in about 2% of the prawns. However, histological examination indicated that an additional 18% of the prawns had subclinical infections. Studies to determine the impact of this parasite on prawns are in progress.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: INDUCTION OF STRESS PROTEINS.** Drew C. Brown\* and Brian P. Bradley, Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21228; Kennedy T. Paynter, Department of Zoology, University of Maryland, College Park, MD 20742.

Stress proteins are common to all organisms. Some such as the 70 kDa heat shock protein (HSP70), respond to many stressors while other respond only to specific stressors. HSP70 increases in oyster hemocytes with increasing *Perkinsus* infection intensity. To follow the induction of HSP70 during the natural course of infection in the field, samples were taken from oyster groups deployed in floating trays at low, moderate and high salinities. The samples were taken monthly, frozen in the field on dry ice and returned to the laboratory for analysis. Soluble proteins from the mantle were run on SDS-PAGE, and either silver stained for total protein or transferred to nitrocellulose membrane, probed with antiHSP70, visualized with an alkaline phosphatase reaction and quantified using densitometry. Within group HSP70 levels showed little variation, supporting the contention that only a few animals are needed to assess the levels of HSP70 in a given group. The time course through the summer and fall showed increasing levels of HSP70, strongly correlated with *Perkinsus* infection, at the high salinity site. HSP70 levels in oysters from the low and moderate salinity sites exhibited little trend.

To examine the induction of stress-specific stress proteins, oys-

ters (0.5 g) were exposed to salinity, temperature and anoxic stress in the laboratory, labelled with  $^{35}\text{S}$ -methionine and processed as above. Autoradiographic analysis was used to determine which proteins were induced or shut down by the stresses. A 55kDa was identified which increased with increasing salinity but not with increasing temperature. A 19 kDa protein was induced by salinity but decreased after 48 hr anoxia. Finally, a 35kDa protein decreased in abundance with increasing temperature at 10‰ but not at 30‰. Supported by the NOAA Oyster Disease Research Program.

**DETECTION, ISOLATION, AND HOST SPECIFICITY OF MIKROCYTOS MACKINI, THE CAUSE OF DENMAN ISLAND DISEASE IN PACIFIC OYSTERS CRASSOSTREA GIGAS.** Dominique Hervio,\* Susan M. Bower, and Gary R. Meyer, Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, B.C., Canada V9R 5K6.

To date, Denman Island disease has been found in 10 localities in British Columbia, Canada. Affected *Crassostrea gigas* have green focal lesions on the surface of the body, mantle and palps. To confirm the etiology, examination of stained tissue imprints for *Mikrocytos mackini* was more sensitive and rapid than preparing and screening histological sections. The seasonal occurrence of *M. mackini* in the field severely curtailed the amount of work that could be conducted. A method based on successive centrifugations on sucrose gradients (utilized for the purification of related protozoa such as *Bonamia* spp. and *Marteilia* spp.), was developed to isolate *M. mackini* from infected tissues. The resulting large numbers of microcells were injected into healthy oysters, thus, allowing the propagation of the parasite in the laboratory year round. The results of 10 experiments indicate that 61.5% to 100% of the oysters became infected with *M. mackini* and some oysters were heavily infected within 3 to 6 weeks after the inoculation. The techniques of injecting microcells was used to examine the host specificity of *M. mackini*. Preliminary results suggest that the eastern oyster (*Crassostrea virginica*) and the flat oyster (*Ostrea edulis*), are more sensitive to *M. mackini* than *C. gigas*. At the end of the 11 week experiment, the prevalence of infection was 100%, 92% and 55% for *C. virginica*, *O. edulis* and *C. gigas* respectively, with the intensity of infection much higher in the first two species. These results have to be confirmed by field studies, but they emphasize the potential impact that this disease could have on oyster production world wide if precautions are not taken during the movements of oyster stocks. Research supported by a Lavoisier Grant (French Ministry of Foreign Affairs).

**PATHOGENESIS OF DISSEMINATED NEOPLASIA IN EASTERN PACIFIC MYTILUS TROSSULUS.** James D. Moore\* and R. A. Elston, Battelle Marine Sciences Laboratory, 439 West Sequim Bay Road, Sequim, WA 98382.

Disseminated neoplasia of Eastern Pacific *Mytilus trossulus* is a progressive, often fatal disease found at prevalences up to 43%

in natural mussel populations. DNA content analyses demonstrated that neoplastic cells in mussels from Washington, Oregon and British Columbia have a distinct  $G_0G_1$  DNA content level of either tetraploid or approximately pentaploid. The tetraploid and pentaploid forms appear to arise from discrete transformation events which result in independent pathogenetic sequences. We have found that both forms of neoplasia are transmissible to conspecific mussels by whole cell injection, and that ploidy form is uniformly maintained in recipients. Mitotic indices, % S phase, and reactivity with a monoclonal antibody to 'proliferating cell nuclear antigen' each demonstrated high rates of neoplastic cell cycling compared to normal tissues.

Ultrastructural observations and cross-reactivity of *Mytilus* neoplasia-specific monoclonal antibodies with normal tissues suggests that both neoplastic cell forms have a connective tissue origin. Commercial monoclonal antibody probes for mammalian cytokeratins, vimentin, desmin, leukocyte common antigen, and epithelial membrane antigen were found to lack reactivity with normal or neoplastic *Mytilus* tissue.

Supported in part by a Frederik B. Bang Scholarship in Marine Invertebrate Immunology (administered by the American Association of Immunologists), and a doctoral fellowship from the Northwest Organization for College and University Science.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER CRASSOSTREA VIRGINICA: FEEDING AND METABOLISM.** Roger I. E. Newell,\* Christine J. Newell, and Ken P. Paynter, Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21631; Gene Burreson, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Eastern oysters are highly susceptible to infection by the parasite *Perkinsus marinus* which causes the oyster to cease growing and eventually die. This disease progression suggests that the parasite may interfere with routine physiological functions, as has been shown to occur with another major oyster parasite, *Haplosporidium nelsoni*. Thus, we hypothesized that oysters infected with *P. marinus* may have a reduced food intake, an elevated metabolic rate and decreased assimilation efficiencies compared with uninfected oysters. In a laboratory experiment, however, in which oysters were infected with differing numbers of *P. marinus*, there were no significant changes in either the rate of oxygen consumption or clearance rate.

In June 1992, oysters were transplanted to three locations within Chesapeake Bay with differing ambient salinity regimes and consequent differences in *P. marinus* infection intensities. Oysters at two sites became infected during the summer. In August, at the high salinity site, experimental oysters ceased growing shell, and in September exhibited a 35% mortality rate as a consequence of these infections. We could detect no differences in oxygen consumption, clearance rate, or assimilation efficiency (measured using the Conover ratio technique) between infected

and uninfected oysters at each of these locations. Ongoing studies are further investigating the mechanisms whereby *P. marinus* exerts its deleterious effects on oysters.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: INTRODUCTORY OVERVIEW.** Kennedy T. Paynter\* and Christopher Caudill, Department of Zoology, University of Maryland, College Park, MD 20742; Eugene M. Burreson, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

An interdisciplinary research project was initiated in 1992 to study the physiological effects of *P. marinus* infection on the Eastern oyster, *Crassostrea virginica*. Seven principal investigators from 5 academic campuses in Maryland and Virginia participated in the project. Physiologies examined were physiological energetics including clearance rates and oxygen consumption, hemocyte function, free amino acid accumulation, mitochondrial function, and stress protein induction.

Oysters were deployed at three sites in Chesapeake Bay to expose them to high, moderate and low salinities and the various prevalences of *Perkinsus marinus* associated with those sites. Samples from each site were provided to the various collaborators at predetermined stages of growth and infection. Growth, mortality, and condition index were monitored in the animals at each site biweekly. As expected, the oysters grew well until they became infected. Infection prevalences became high at both the low and high salinity sites while remaining low at the moderate salinity site. The disease progressed more rapidly at high salinity resulting in more intense infections even though final prevalences were similar at low salinity. Mortality was low until September and October when cumulative mortality reached about 35% in the group deployed at high salinity but remained low at the low and moderate salinity sites. Growth, mortality, condition index, and infection intensity and progression in the field were associated with the physiologies measured in the laboratory. Supported by the NOAA Oyster Disease Research Program.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: EFFECTS ON CELLULAR FREE AMINO ACID LEVELS.** Kennedy T. Paynter\* and Sidney K. Pierce, Department of Zoology, University of Maryland, College Park, MD 20742; Eugene M. Burreson, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

The Eastern oyster, *Crassostrea virginica*, is an osmoconforming bivalve which regulates intracellular free amino acid concentrations to maintain cell volume in response to changes in ambient salinity. This important ability allows the oyster to inhabit brackish water estuaries such as the Chesapeake Bay where many other species cannot survive. Oyster cells, like those of most other euhaline bivalves, accumulate free amino acids (FAA) when the

salinity increases and expel FAA when the salinity decreases. The accumulation of FAA is the result of a specific set of metabolic shifts which first causes the production of alanine from glucose, followed by glycine production and later proline production. After many weeks of high salinity acclimation, taurine becomes the major intracellular osmotic effector replacing alanine, glycine and proline.

Oysters acclimated to low salinity were deployed at high and low salinity sites in May. Gill and mantle tissues from 5 oysters were excised and quick frozen on dry ice in the field daily for 10 days after transfer and biweekly thereafter. *P. marinus* infection intensity was determined for each oyster sampled. Intracellular FAA followed a typical accumulation pattern after the hyperosmotic shift and appeared to reach stable acclimated levels 8 to 10 weeks after transfer. However, several amino acid concentrations changed once the oysters became infected with *P. marinus*. Taurine levels were significantly reduced in infected groups and the magnitude of reduction was positively correlated with infection intensity. These results suggest that the cell volume control mechanism in oysters may be impaired by *P. marinus* infection, and the oysters ability to tolerate salinity variation may be reduced. Supported by the NOAA Oyster Disease Research Program.

**SEVERAL MITOCHONDRIAL FUNCTIONS IN CHESAPEAKE BAY OYSTERS ARE DIFFERENT IN ATLANTIC OYSTERS: DISEASE OR GENETICS?** S. K. Pierce, L. A. Perrino, and L. M. Rowland-Faux, Department of Zoology, University of Maryland, College Park, MD.

*Crassostrea virginica* from Florida to Cape Cod respond to increased external salinity by increasing intracellular concentrations of several amino acids, primarily taurine, and the quaternary amine, glycine betaine. Chesapeake Bay oysters from several populations use different amino acids, primarily glycine and alanine, and in addition, do not synthesize glycine betaine in response to high salinity stress. Since the synthesis of both the amino acids and glycine betaine occurs in the mitochondria, we have been comparing isolated mitochondrial metabolism of Bay and Atlantic oysters. The respiratory coupling ratios (RCR) of Bay oysters is always higher than Atlantic oysters, regardless of biochemical substrate. Bay oyster RCRs are highest with  $\alpha$ -ketoglutarate, while malate is preferred by Atlantic mitochondria. In addition, mitochondria from low salinity adapted oysters take up choline (glycine betaine precursor) faster than high salinity adapted oysters and Atlantic mitochondria take it up faster than Bay mitochondria. The synthesis of glycine betaine is faster in high salinity adapted Atlantic oysters. We are currently measuring synthesis in Bay oyster mitochondria. These differences in amino acid production, RCRs and glycine betaine metabolism indicate major biochemical differences between the mitochondria of the two oyster groups. Since all of our Bay oysters were likely parasitized with Dermo, it is not clear if the differences are due to genetics, the presence of the parasite or some other environmental factor. (Supported by NOAA and NSF)

### FLOW CYTOMETRIC ENUMERATION AND ISOLATION OF IMMUNOFLUORESCENT *PERKINSUS MARINUS* CELLS FROM ESTUARINE WATERS.

**Bob S. Roberson\*** and **Tong Li**, Department of Microbiology, University of Maryland, College Park, MD 20742; **Christopher F. Dungan**, Maryland DNR, Cooperative Oxford Laboratory, Oxford, MD 21654.

Particles suspended in water samples from both Chesapeake Bay, and from laboratory aquaria containing moribund, *Perkinsus marinus*-infected oysters, were concentrated and double fluorochrome-labeled for flow cytometric analysis and fluorescence activated cell sorting (FACS). Pathogen cells were fluorescein-labeled using specific antibodies; cell DNA was propidium iodide-labeled by incubation with this nucleic acid fluorochrome in the presence of RNAase. Flow cytometric analyses utilized antibody fluorescence, DNA fluorescence, size (forward angle light scatter), and cellular complexity (90° light scatter) to differentiate cell populations within water samples. Water samples from aquaria seeded with infected oysters were used to determine analytical parameter value ranges characterizing pathogen cells, and provided the first observation of pathogen cells disseminated from infected hosts. Compositions of differentiated sample cell populations were confirmed by FACS, followed by microscopic evaluation of sorted cell populations. Following confirmation of discriminating analytical parameter value ranges, pathogen cell abundance estimates were made for aquarium water samples, using gated counts. Counted cells were sorted and population homogeneity was independently confirmed by microscopic enumeration. These methods are currently being applied to analyses of environmental water samples collected throughout the past year, for the purpose of generating accurate seasonal estimates of actual pathogen abundances in estuarine waters endemic for dermo disease.

## AQUACULTURE, ECOLOGY AND MANAGEMENT

### OVER EXPLOITATION AND SIGNS OF RECOVERY: ANALYSIS OF AN OFFSHORE WHELK FISHERY.

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Whelk trawling is an alternative fishery in South Carolina for commercial shrimp fishermen who harvest whelks to supplement earnings during closure of shrimp season. Using gear similar to shrimp fishing, trawling for subtidal knobbed and channeled whelks (*Busycon carica* and *B. canaliculatum*) started fifteen years ago and production peaked in 1982 at 32,000 U.S. bushels. A trend of increasing shrimp landings began in 1984; however, the dockside value of shrimp decreased, primarily due to imports. In addition, the advent of recreational shrimp baiting in the State has further eroded revenue received by commercial shrimp fishermen,

making the whelk fishery more critical, and in some cases, a necessary alternative source of income.

Results of a mark and recapture study illustrate that offshore whelks remain within a relatively small area. Further, whelks are particularly vulnerable to over exploitation since *Busycon* grows slowly, has a relatively large minimum breeding size and a long life span. By establishing a minimum harvest size, mandating reporting requirements, limiting the fishing season and restricting exploitation in certain offshore waters, the whelk fishery is beginning to show signs of increased production and possible recovery.

### EFFECTS OF INITIAL CLAM SIZE AND TYPE OF PROTECTIVE MESH NETTING ON THE SURVIVAL AND GROWTH OF HATCHERY-REARED INDIVIDUALS OF *MYA ARENARIA* IN EASTERN MAINE.

**Brian F. Beal\***, Division of Science and Mathematics, University of Maine at Machias, 9 O'Brien Avenue, Machias, ME 04654.

A field test was conducted at a low intertidal site located at the mouth of the Chandler River near the town of Jonesboro, Maine from 23 June 1990 to 13 June 1991 to assess the fate of two discrete sizes of hatchery-reared soft-shell clams, *Mya arenaria*, ( $\bar{X}_{Large} = 11.8 \text{ mm} \pm 0.145 \text{ SE}$ ,  $n = 237$ ;  $\bar{X}_{Small} = 8.5 \text{ mm} \pm 0.084 \text{ SE}$ ,  $n = 185$ ) in the presence and absence of predation. Clams were produced at the Beals Island Regional Shellfish Hatchery, a stock enhancement and management program that produces ten million soft-shell clam juveniles (8–12 mm) annually for ten Downeast Maine coastal communities. Sixty 1-m<sup>2</sup> wooden frames (width = 25 cm) with attached 35 cm legs were pushed into a mudflat so that approximately 12 cm protruded up through the sediments. Within each frame, six open, sediment-filled, plastic enclosures (15 cm diameter × 15 cm deep) were dug into the sediment so that 1 cm protruded. Small clams were seeded into three of the enclosures as were large clams. This design resulted in 360 experimental units, or enclosures. Ten of the sixty frames each remained completely open so that clams in the open enclosures within each frame were susceptible to predators. The remaining fifty frames each received one of five netting treatments: 1/8<sup>th</sup>-inch, 1/4-inch, or 1/2-inch flexible material, and 1/4-inch or 1/2-inch heavy, or extruded netting (Internet, Inc.).

Initial size was an important predictor of clam survivorship. Small clams within open frames had a mean survival of 63.9% ± SE 4.53,  $n = 10$  which was significantly lower ( $P < 0.05$ ) than the mean survival of large clams exposed to predation: 77.8% ± SE 3.01,  $n = 10$ . Similarly, when results from all protected frames were combined, smaller clams had significantly ( $P < 0.01$ ) poorer survivorship than larger clams ( $\bar{X}_{Small} = 80.6\% \pm 2.77 \text{ SE}$ ,  $n = 50$ ;  $\bar{X}_{Large} = 87.2\% \pm 2.76 \text{ SE}$ ,  $n = 50$ ). Green crabs (*Carcinus maenas*) did enter some of the meshed frames; however, protected clams had significantly higher survival rates than unprotected animals ( $P = 0.003$ ). For clams protected, type of netting (flexible vs. extruded) had no effect on survival ( $P = 0.715$ ) and no detectable differences in survival were noted with respect to net

aperture. Once transplanted to the field, hatchery-reared *Mya* leave distinct checks in their shell that uniquely mark initial transplant size. Relative growth was uninfluenced by presence or absence of netting ( $P = 0.314$ ) and type of netting as well as specific aperture size also did not affect growth. During the year-long test, both sizes of clams grew approximately 18.5 mm. Small clams reached an average shell length of  $26.9 \text{ mm} \pm 0.198 \text{ SE}$  ( $n = 60$ ) whereas large clams attained a maximum length of  $30.5 \text{ mm} \pm 0.201 \text{ SE}$  ( $n = 60$ ). Natural recruits settled into experimental units during the experiment. The average number of recruits that settled into frames protected with mesh netting and subsequently survived to be counted in June, 1991 was  $10.72 \pm 0.017 \text{ SE}$  ( $n = 50$ ) or  $606/\text{m}^2$ . An average of only  $3.73 \text{ recruits} \pm 0.333 \text{ SE}$  ( $n = 10$ ), or  $211/\text{m}^2$ , were found within units inside open frames. These results suggest that protecting clams from predators is economically and biologically effective. Even if clams are protected, initial size will play an important role in determining survival. To minimize costs associated with field-transplanting hatchery-reared soft-shell clams between 8–12, a ½-inch flexible netting should be used.

**GROWTH OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, IN FLOATING RAFTS IN NORTH CAROLINA.** Bonnie L. Brown, Department of Biology, Virginia Commonwealth University, Richmond, VA 23284-2012; Arthur J. Butt, Chesapeake Bay Program Office, Virginia Water Control Board, Richmond, VA 23230; Kennedy T. Paynter, Department of Zoology, Univ. of Maryland at College Park, College Park, MD 20742.

A study was conducted during 1992–1993 to provide information on major factors affecting successful cultivation of commercial quantities of Eastern oyster in North Carolina and to enhance the state of knowledge regarding the physiological effects of disease on one strain of oysters selectively bred for rapid growth. The selectively bred group of Eastern oyster, *Crassostrea virginica*, was derived from native Maryland oysters. Oysters from the Cape Lookout region of North Carolina were raised along side these selectively bred oysters for comparison.

Study sites selected on the basis of environmental quality, salinity and ease of access were located in Pamlico Sound (average 10 ppt) and Bogue Banks (average 31 ppt). Floating trays were employed as culture containers to limit exposure to predators, siltation and other consequences of benthic existence. Data collected included oyster growth, condition index, mortality, level of infection with Dermo, temperature, and salinity. Despite chronic Dermo infection, growth rate at the low salinity site was approximately 8.4 mm per month while growth at the high salinity sites averaged 9.0 mm per month. Initial post-introduction mortality at all sites was 1%. Subsequent mortality due to predators, fouling and disease averaged less than 1% per month. Oysters introduced at an average size of 10 mm in the spring of 1992 required approximately 8 months to reach average harvest size of 76 mm in

the high salinity sites and more than 12 months at the low salinity site. Under these conditions the selectively bred oysters grew more rapidly than the native oysters.

**ON FARM COMPUTER PROGRAM FOR MUSSEL FARMS.** T. Jeffrey Davidson,\* Rod McFarlane, and Judy Clinton, Atlantic Veterinary College, U.P.E.I., 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3.

The blue mussel (*Mytilus edulis*) industry in Atlantic Canada has grown tremendously in recent years. To provide mussel producers with a decision support tool which will allow more effective farm management decisions, an 'on farm' computer program is being developed. This program will also incorporate data available from shellfish processing plants. The program includes an 'in water' inventory control system, and a system to monitor farm production and efficiency.

The inventory control system will include visual representation of the farm lease using Geographical Information System (GIS) technology. Information available to the producer for each long-line will include: location and status (empty, collector, socks); average size of mussels; date deployed and harvested; origin and size of the seed stock; and type, size and stocking density of sock used.

Production and efficiency information available will include the length of time to market size in relation to: origin and size of the seed stock; type and size of the sock used; stocking density; and time of socking (spring vs. fall). Percent of market size mussels per sock will be compared to: stocking density; and origin and size of seed stock. This data will be available to the producer in written or graphic form.

**FACTORS OF MESH SIZE, STOCKING SIZE, STOCKING DENSITY AND ENVIRONMENT WHICH AFFECT GROWTH AND SURVIVAL OF *MERCENARIA MERCENARIA* (LINNAEUS, 1758) IN A MARICULTURAL GROWOUT APPLICATION IN COASTAL GEORGIA.** Dorset H. Hurley\* and Randal L. Walker, Shellfish Research Laboratory, Marine Extension Service, University of Georgia, Savannah, GA 31416.

Growth and survival of the hard clam were tested against stocking density, seed size, growout bag mesh diameter and benthic environment in a maricultural application in Georgia. Clams were stocked in commercially used oyster bags  $1.0 \times .05 \text{ m}^2$ . The bag mesh diameters were 3 mm, 6 mm and 12 mm. Clam densities were 250, 325, 500, 675, 750, 975, 1500, 2025 and 2250 per bag. Clam stocking sizes were 4.7 mm, 6 mm, 9.5 mm and 13.7 mm. Benthic environments differed from sand, oyster drift-mud composite and silty mud. All experiments were conducted from Oct 26, 1991 to Aug 17, 1992.

Significant differences in growth were noted in high density stockings of 2025 and 2250 versus moderate stockings of 750 to

1500. Denser stocking numbers resulted in a reduced growth rate. Survival differences between groups were equal.

Stocking densities of 250, 325, 500, 675 and 750 clams per bag versus bag mesh diameters of 3 mm and 6 mm showed significantly greater survival in the 6 mm, however, growth rates in the 3 mm mesh were significantly higher than in the 6 mm mesh bags for all densities. Both 3 mm and 6 mm mesh clams stocked at 750 showed a decreased growth rate as compared to the other stocking densities.

Survival and growth between all treatments exhibited higher survival and increased growth with an increase in stocking size between equal density groups.

Environmental differences between growout sites as a factor of benthic substrate yielded only one of five sites with lower survival (49%) as compared to the remaining four sites (70%–79%). Growth rates between sites were all significantly different ranging from 18.4 mm clams on a sand bottom to 25.5 mm clams on a mud/silt substrate. Clams were checked monthly by Satilla Sea Farm personnel to render mutually beneficial data for both research and industrial objectives.

**DEVELOPMENT OF THE CHUB LADDER OYSTER CULTURE METHOD.** Philip S. Kemp, Jr.,\* UNC Sea Grant Marine Advisory Service, P.O. Box 3146, Atlantic Beach, NC 28512; Alfred J. J. Evans, Tipper Tie Inc., P.O. Box 866, Apex, NC 27502.

During 1991–1992 an intensive effort was made to develop a method for culturing shellfish using new materials and techniques. The project was a joint effort between UNC Sea Grant Marine Advisory Service and Tipper Tie Inc., a private corporation. A description is given of the evolution of the project from first ideas to final product: the chub ladder.

The chub ladder method employs assembly line techniques and is suitable for commercial scale culture of oysters. Chub ladders are fabricated on-shore where seed oysters are placed in tubular mesh containers (chubs) which are clipped at the ends to two parallel stabilizer ropes in a ladderlike fashion (the chubs being the steps of the ladder). Floatation is included in each chub along with the oyster seed so that the entire apparatus floats at the water surface. Initial results show up to 45% market size (>76 mm) oysters after 4 months of growth from 22 mm seed and up to 68% of 22 mm seed grew to market size after 6 months. Specific management methodology is described.

**EFFECTS OF HURRICANE ANDREW ON LOUISIANA'S OYSTER RESOURCES.** W. S. Perret,\* R. Dugas, J. Rousel, and C. Boudreaux, Louisiana Department of Wildlife and Fisheries, Baton Rouge, LA 70898.

Hurricane Andrew crossed the central Louisiana coast just east

of Atchafalaya Bay, August 25–26, 1992, passing through the state's most productive oyster grounds. Sustained winds near the center of this storm were 130 mph for several hours, causing Gulf water storm surges. Resettlement of displaced marsh sediment and accompanying vegetation killed live oysters and destroyed suitable oyster habitat.

In July, prior to the storm, oyster density samples were taken on all of the State's public oyster grounds as part of the regular sampling program. At that time, oyster densities in the area where the storm would go ashore were the highest observed in the state. The week following the storm, density samples were conducted to determine the extent of oyster damage. Mortalities were severe on all public grounds along the central coast. In addition to the impact studies on the public oyster grounds, a sampling program was initiated to estimate damages on privately owned leases. Dredge samples were taken across a grid system from Vermilion Bay to the Mississippi River where concentrations of oysters were known to occur. Mortalities exceeded 25% in most of the impacted areas and often exceeded 75%.

**HEALTH ASSESSMENT OF OYSTER REEFS IN GALVESTON BAY, TEXAS.** Junggeun Song\* and Eric N. Powell, Department of Oceanography, Texas A&M University, College Station, TX 77843-3146.

The condition of oyster (*Crassostrea virginica*) populations in the Galveston Bay system was evaluated at the level of the community and the individual in late spring 1992. Fifty one sites were chosen based on the salinity regime, previous studies, use by the oyster fishery, and nearness to the Houston Ship Channel. The community-based indices included the total volume of shells collected, the number of boxes, oyster size frequency, and the abundance of oyster predators and competitors. The individual indices included a weight/volume condition index, condition rating according to Mackin's code, sex determined with smear slides, gonadal content by immunological probe, and *Perkinsus marinus* prevalence and infection intensity. *Perkinsus marinus* prevalence was unusually low at most sites, ranging from 0 to 30%, except for four sites where prevalences were between 60 and 90%, confirming that a flood-induced salinity decrease in spring of 1992 lowered parasite abundance and disease incidence well below the long-term mean. Highest prevalences were observed from oysters in West Bay where salinity remained high. Closer inspection of negatives using total body counts revealed that the very low prevalences estimated by Ray's method were partly due to the underestimation of very light infections (false negatives). Actual prevalences were higher by 20 to 93%. The proportion of female oysters among market-sized oysters was highly variable, ranging from 0 to 0.9. Mussel abundances and drill abundances were diametrically distributed, suggesting some predational control on mussel populations.



## HARMFUL PHYTOPLANKTON AND SHELLFISH INTERACTIONS

**AN INTERSPECIFIC COMPARISON OF PARALYTIC SHELLFISH POISONS IN MARINE BIVALVES: ANATOMICAL AND SPATIO-TEMPORAL VARIATION IN TOXIN COMPOSITION.** Allan D. Cembella\* and Nancy I. Lewis, Institute for Marine Biosciences, National Research Council, Halifax, Nova Scotia, Canada B3H 3Z1; Sandra E. Shumway, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575 USA.

Marine bivalve molluscs can accumulate paralytic shellfish poisoning (PSP) toxins through filter-feeding on blooms of toxic dinoflagellates, specifically, *Alexandrium* spp. on the Atlantic coast of North America. To determine the seasonal variation in PSP toxin composition in various anatomical compartments, in-shore and offshore populations of the sea scallop *Placopecten magellanicus* and the surf clam *Spisula solidissima*, two bivalve species noted for their prolonged toxin retention, were sampled periodically over two consecutive years in the Gulf of Maine. Individuals ( $n = 8$ ) were fractionated into tissue compartments (digestive gland, adductor muscle, gill and mantle), plus siphon and foot for clams, and gonads for scallops, for the determination of toxin composition (molar% and  $\text{nmol g}^{-1}$ ) by high-performance liquid chromatography. The calculated toxicity ( $\mu\text{g STXeq } 100 \text{ g}^{-1}$  shellfish tissue) confirmed the results of parallel mouse bioassays which indicated the distribution of toxicity among the tissues, but did not exactly track the bioassay over a seasonal time scale. For both scallops and surf clams, substantial differences in the relative amounts of PSP toxins were more evident among various tissue compartments, than were seasonal variations and geographical differences between populations. Analysis of PSP toxin profiles from an representative isolate of *Alexandrium tamarense* from the Gulf of Maine supported previous findings that the toxin composition in bivalves may differ considerably from that of the dinoflagellate. A pronounced shift in the toxin profile from the less potent N-sulfocarbamoyl toxins (C1/C2), which dominate in the dinoflagellate, to higher toxicity carbamate derivatives (e.g., GTXs, NEO, and STX) was apparent. The hypothetical basis for metabolic and physico-chemical toxin conversion processes and selective retention mechanisms which may differ among bivalve species will be compared.

**DOMOIC ACID IN THE PACIFIC RAZOR CLAM *SILIQUA PATULA*.** Ann S. Drum,\* Terry L. Siebens, Eric A. Crecelius, and Ralph A. Elston, Battelle Marine Sciences Laboratory, Sequim, WA 98382.

In the Fall of 1991 domoic acid was discovered in coastal Pacific razor clams *Siliqua patula* in Washington and Oregon states at levels higher than acceptable for safe human consumption, thereby forcing a closure of the recreational harvest. Tissue

distribution data, based on HPLC analysis, indicated the clams maintained these elevated levels from fall through early summer of 1992 in the edible muscular tissues (mantle, siphon, adductor muscles, and muscular foot) with concentrations of toxin averaging from 23.3 to 50.7  $\mu\text{g} \cdot \text{gm}^{-1}$ . The concentration in the non-edible tissue types (gill, digestive gland, gonad, and siphon tip) ranged from trace amounts to 8.4  $\mu\text{g} \cdot \text{gm}^{-1}$ . Clams that were dissected into edible and non-edible pooled portions contained  $36.4 \pm 22.6$  and  $13.7 \pm 7.6 \mu\text{g} \cdot \text{gm}^{-1}$ , respectively. On an additional sampling date, clams were sampled fresh or were frozen before sampling. The concentration in the edible portion of the fresh clams averaged  $16.8 \pm 11.6 \mu\text{g} \cdot \text{gm}^{-1}$ , while the blood and dissection fluids contained only trace amounts of toxin. The frozen edible portion domoic acid mean concentration was  $12.6 \pm 6.9 \mu\text{g} \cdot \text{gm}^{-1}$  with meltwater levels reaching  $4.2 \mu\text{g} \cdot \text{gm}^{-1}$  and the dissection fluid containing up to  $10.0 \mu\text{g} \cdot \text{gm}^{-1}$ . Clams collected in December 1991 with elevated levels of toxin ( $47.9 \pm 12.7 \mu\text{g} \cdot \text{gm}^{-1}$ ) that were held on inland seawater for 3 months maintained this level of contamination ( $44.3 \pm 19.8 \mu\text{g} \cdot \text{gm}^{-1}$ ). Razor clams from Alaska held under identical conditions during this time period did not contain detectable levels of toxin. Razor clam tissues collected in 1985, 1990, and 1991 revealed only trace levels of toxin.

**DOMOIC ACID IN WESTERN WASHINGTON WATERS.** Rita A. Horner\* and James R. Postel, School of Oceanography, University of Washington, Seattle, WA 98195.

In late October 1991, razor clams, *Siliqua patula*, living in the surf zone on Pacific coast beaches in Washington and Oregon were found to contain domoic acid with levels in the edible parts as high as  $154 \mu\text{g g}^{-1}$  wet weight of shellfish meat. As a result, the recreational and commercial harvest of the clams was closed. Subsequently, domoic acid was found in the viscera of Dungeness crabs, *Cancer magister*, in coastal waters of California, Oregon, and Washington and this important commercial fishery was closed for several weeks. Domoic acid levels in razor clams remained above the harvest closure level of  $20 \mu\text{g g}^{-1}$  at least until May, 1992. In the summer of 1992, domoic acid was found in trace amounts in mussels and oysters in the inland waters of northern Puget Sound.

Members of the diatom genus *Pseudonitzschia* H. Peragallo, *P. pungens* (Grunow) Hasle f. *multiseries* (Hasle) Hasle and *P. australis* Frenguelli, that may produce domoic acid, have been recognized in western Washington waters and elsewhere on the west coast. Their distribution is not well-known, probably because they often have been misidentified. However, they appear to be relatively common and may be abundant. The presence of these potentially toxic diatoms signals a new problem with regard to toxic phytoplankton and public health on the west coast. All locations where *Pseudonitzschia* blooms have occurred are areas where commercial or state finfish or shellfish aquaculture sites are lo-

cated, hence the need for regular phytoplankton monitoring and additional shellfish monitoring to ensure that seafood is safe for human consumption.

**BALLAST WATER AND SEDIMENTS AS MECHANISMS FOR UNWANTED SPECIES INTRODUCTIONS INTO WASHINGTON STATE.** J. M. Kelly, School of Marine Affairs, University of Washington, Seattle, WA 98195.

Ballast water and sediments from bulk cargo carriers have been implicated in the transfer of a diverse assortment of non-native species to near-shore marine environments worldwide. Dinoflagellate cysts present in discharged ballast sediments are believed to be responsible for the recent introduction of PSP-causing algal blooms and the subsequent disruption of the shellfish culture industry in Tasmania.

Examination of ballast water and sediments from Japanese woodchip carriers arriving at the ports of Tacoma and Port Angeles revealed the presence of living mollusc larvae, crustacea, macroalgae and numerous species of diatoms and dinoflagellates. With up to 20,000 metric tonnes of water and several cubic yards of sediment present in a cargo hold, the threat of introduction of harmful algae, pathogens, predators and resource competitors is realized. However, interviews with ships' officers indicated that at least some practice ballasting and deballasting procedures that may decrease the risk of introduction, such as offshore ballast loading, open-ocean exchange of ballast water and discharge of ballast sediments off-shore. Recent international, national and state policy efforts designed to prevent introductions via ballast discharge will be discussed.

**“NON-TOXIC” DINOFLAGELLATE BLOOM EFFECTS ON OYSTER CULTURE IN CHESAPEAKE BAY.** Mark Luckenbach,<sup>\*1</sup> Sandra Shumway,<sup>2</sup> and Kevin Sellner,<sup>3</sup> <sup>1</sup>Virginia Institute of Marine Science, Wachapreague, VA 23480, <sup>2</sup>Bigelow Laboratory, West Boothbay Harbor, ME 04575, <sup>3</sup>The Academy of Natural Sciences, Benedict, MD 20612.

Dinoflagellate blooms appear to be increasing in frequency, magnitude and duration in Chesapeake Bay. During 1992 we documented dinoflagellate blooms of unprecedented intensity and distribution in the southern portion of Chesapeake Bay. Though the species involved in these blooms are generally termed non-toxic (from an anthropogenic perspective), their effects on suspension-feeding bivalves, including oysters, may be anything but benign.

Field and laboratory experiments were conducted to evaluate impacts of several dinoflagellate bloom species on the feeding, growth and survival of *Crassostrea virginica*. Hatchery-spawned oysters from a single cohort were deployed in off-bottom culture at twelve locations exhibiting varying degrees of bloom development, and growth and survival monitored. Laboratory experiments of 4 to 6 weeks duration were used to evaluate growth and survival of juvenile oysters fed monocultures of dinoflagellates and a dia-

tom. Flow cytometry was used to determine grazing rates in short-term feeding trials. Results from both the field and laboratory suggest that growth and survival of juvenile oysters are affected by these dinoflagellate blooms. Our findings indicate potentially significant impacts on oyster culture in this region as a consequence of dinoflagellate blooms.

**EFFECT OF THE TEXAS BROWN TIDE ON *MULINIA LATERALIS* POPULATIONS AND FEEDING.** Paul A. Montagna,\* Dean Stockwell, and Greg Street, The University of Texas at Austin, Marine Science Institute, P.O. Box 1267, Port Aransas, TX 78373.

In 1990, there was an unusual brown tide bloom of an aberrant Chrysophyte sp. in Baffin Bay and Laguna Madre near Corpus Christi, Texas. The bloom was coincident with the complete loss of shellfish in Baffin Bay, and a dramatic reduction in Laguna Madre. The dominant clam, *Mulinia lateralis*, disappeared for about one year. We performed a series of experiments to determine if *M. lateralis* disappearance was related to feeding and assimilation of the brown tide. Radioactive tracers were used to compare feeding on brown tide, *Isochrysis galbana*, *Dunaliella tertiolecta*, and *Heterocapsa pygmaea*. At low cell concentrations ( $<100,000$  cells  $\cdot$  ml<sup>-1</sup>), *M. lateralis* grazing rates (cell  $\cdot$  h<sup>-1</sup>) increased with concentration and was not different among microalgal species. At higher concentrations grazing rates continued to increase on brown tide, but remained the same on the other microalgal species. This indicates that *M. lateralis* did have a different functional response to brown tide at bloom concentrations (500,000–6,000,000 cells  $\cdot$  ml<sup>-1</sup>). Assimilation efficiency by *M. lateralis* was about the same on all species of microalgae. The high grazing and assimilation rates of brown tide by *M. lateralis* indicate it is more likely that the bloom could have been due to loss of the clam population, rather than a negative trophic effect of the brown tide.

**FACTORS CONTROLLING PARALYTIC SHELLFISH POISONING (PSP) IN PUGET SOUND, WASHINGTON.** Jack Rensel, School of Fisheries, HF-15, University of Washington, Seattle, WA 98195.

PSP has spread throughout much of Puget Sound, Washington since the mid 1970's. Now all but parts of southern Puget Sound (SPS) and all of central and southern Hood Canal (CHC and SHC) are affected by PSP. The initial spread of PSP has been traced to major physical events, but lack of PSP in most of SPS and all of CHC and SHC has not been investigated until this study. Monitoring and experimental data suggest the lack of surface and subsurface (10 m) nitrogen in the unaffected areas prevents *Alexandrium catenella* growth.

In August of 1991 and 1992, filtered water from the surface and subsurface depths of CHC did not support any growth of *A. catenella* in the laboratory. However, growth of *A. catenella* oc-



curred with water from the same depths in SHC, although PSP has never been reported in that area. Slow estuarine-flow transport in CHC coupled with a seasonal lack of nitrogen in surface and subsurface waters forms a barrier to the passage of *A. catenella* cells to the more nutrient-rich SHC. Correlation analysis showed that PSP toxicity in mussels was related to elevated subsurface nitrogen concentrations and water temperature above 13°C. Increased nitrogen discharge from rapid urbanization and non-point sources could lead to annual PSP problems in areas presently unaffected by PSP, unless preventative measures are taken.

**EFFECTS ON THE OYSTER *CRASSOSTREA VIRGINICA* CAUSED BY EXPOSURE TO THE TOXIC DIATOM *NITZSCHIA PUNGENS* F. *MULTISERIES*. D. L. Roelke,\* G. A. Fryxell, and L. A. Cifuentes.**

Domoic acid (DA) is produced by some diatom species and has become a problem to the shellfish industry. *Nitzschia pungens* f. *multiseries*, a known DA producer, has been discovered in Galveston Bay, Texas. The region produces a large part of the total national oyster harvest. The threat of contamination or mortality to the oyster fishery due to *N. pungens* f. *multiseries* was investigated by performing feeding experiments with *Crassostrea virginica* using clonal cultures of the diatom. Emphasis was placed on oyster feeding behavior, tissue toxicity, and depuration.

*C. virginica* readily fed on cell concentrations greater than natural blooms. The percentage of cells filtered from the seawater was consistently around 80%. The filtration rate ranged from 0.01 to 2.02 liters hr<sup>-1</sup>. These variables along with oyster openness were not effected by the cell concentration, cell toxicity, or total toxicity of *N. pungens* f. *multiseries*. There were no detrimental effects observed to *C. virginica*.

Whole body analyzes showed DA accumulation ranging between 1 and 2 µg g<sup>-1</sup>. The "gut" had five times the toxicity of the adductor muscle and the gills/mantle/labial pulps tissue fractions. Approximately 70% of the total DA in the oyster resided in the "gut." Oysters showed no correlation between whole body toxicity and whole body weight. Whole body depuration of DA from *C. virginica* was slow over a 72 hour period (14%).

Domoic acid outbreaks may not be confined to the coasts of North America. Two persons in the USA exhibited signs of ASP after eating smoked Korean oysters. Monitoring of several brands of this product was conducted, and domoic acid was not detected.

**FISHERIES MANAGEMENT AND TOXIC PHYTOPLANKTON; THE RAZOR CLAM EXAMPLE. Donald D. Simons\* and Dan L. Ayres, Washington State Department of Fisheries, Coastal Field Station, 48A Devonshire Road, Montesano, WA 98563.**

In last decade, the popular razor clam (*Siliqua patula*) fishery on the coast of Washington State has undergone major turmoil following the devastation of the razor clam stocks by the pathogen NIX (Nuclear Inclusion X). In recent years, a conservative ap-

proach of regular short seasons began to return management of the fishery to a more predictable and positive condition.

Then, in November of 1991 the Washington State Department of Health informed the Washington State Department of Fisheries that razor clams being harvested by recreational users contained high levels of domoic acid. The fishery was closed by emergency order. This incident began an unprecedented period, in the sixty-plus year history of the razor clam recreational fishery, of extremely close monitoring of razor clam tissue from numerous locations for levels of domoic acid. In September 1992, just as domoic acid levels had reached a safe level, routine testing uncovered a new problem. Razor clam tissue was found to contain dangerously high levels of saxitoxin (the toxin responsible for Paralytic Shellfish Poisoning). Razor clams were now being monitored not only for domoic acid but also saxitoxin. This monitoring continues today. These incidents have resulted in closed or delayed fisheries, leaving the fishery users confused and angry. In addition new levels of cooperation are developing between Washington State Departments of Fisheries and Health, the US Food and Drug Administration, The National Park Service, various Indian Tribes and the fishery users, members of the public at large.

***PSEUDONITZSCHIA AUSTRALIS* FRENGUELLI AND OTHER TOXIC DIATOMS FROM THE WEST COAST OF THE U.S.A.: DISTRIBUTION AND DOMOIC ACID PRODUCTION. M. C. Villac,\* G. A. Fryxell, F. P. Chavez, and K. R. Buck, Department of Oceanography: Texas A&M University, College Station, TX 77843-3146.**

Awareness of the phycotoxin domoic acid (DA), the cause of Amnesic Shellfish Poisoning (ASP), reached the United States west coast in the fall of 1991. Levels of DA in razor clams, mussels, and Dungeness crabs led to the closure of fisheries along the coasts of California, Oregon, and Washington. The diatom *Pseudonitzschia australis* was found to produce DA, and was observed from Southern California to the mouth of the Columbia River (San Diego, Newport, Monterey Bay, Coos Bay, and Ilwaco) during the DA outbreak. High cell concentrations and DA in phytoplankton net hauls were detected in Monterey Bay. A survey based on net haul samples and identification in SEM, carried out in Monterey Bay during the fall of 1992 showed that not only *P. australis* but other *Pseudonitzschia* spp. were present, including the DA producers *P. pungens* f. *multiseries* and *P. delicatissima*. There was no report of DA outbreak in the Bay in 1992. Clones of *P. australis* from Monterey Bay, Coos Bay and Ilwaco were established in 1991 (15°C; salinity = 30; 96 µE/m<sup>2</sup>s in 12:12 hrs light:dark), and tested for domoic acid production (HPLC-UV detector; 250 µl injection volume; detection threshold of 0.01 µg/ml). Trace amounts of the neurotoxin were detected in late exponential phase (0.08–0.49 pg/cell), but not in two control cultures tested under continuous light. Tests for DA production in 1992 clones of *P. pungens* f. *multiseries* and *P. delicatissima* from Monterey Bay are underway.

## NON-TRADITIONAL SHELLFISHERIES

**MANAGEMENT OF THE COMMERCIAL FISHERY FOR SPOT PRAWNS (*PANDALUS PLATYCEROS*) IN BRITISH COLUMBIA.** Bruce E. Adkins,\* Department of Fisheries and Oceans, Pacific Region, Fisheries Branch, South Coast Division, 3225 Stephenson Point Road, Nanaimo, B.C., Canada V9R 1K3.

The commercial fishery for spot prawns (*Pandalus platyceros*) underwent a rapid expansion in the late 1970's which necessitated the development and implementation of a system of management to prevent recruitment overfishing of these stocks. This system of management involved onboard sampling of commercial catches of spot prawns, estimating the proportion of the female cohort in the stocks and comparing those results to predetermined monthly levels of spawner abundance. Fishery closures are based on the results of commercial catch sampling. In the mid-1980's minimum size limits and size selective trap requirements were implemented in this fishery to prevent or reduce growth overfishing as well as a seasonal closure during the larval hatching period was put in place to control the expanding effort in this fishery. In 1990 limited entry was also introduced as a control on effort.

Results of commercial catch sampling in select areas are presented and catch, effort and CPUE are compared between areas having varying degrees of in-season management. The effectiveness of the current method of managing the commercial spot prawn fishery is discussed and recommendations for alternate or modified methods of management are presented.

**THE INTERTIDAL CLAM FISHERY IN BRITISH COLUMBIA; A FISHERY UNDER REVIEW.** Frances V. Dickson,\* Department of Fisheries and Oceans, Suite 420-555 West Hastings Street, Vancouver, B.C., Canada V6B 5G3.

Intertidal clam fisheries in British Columbia have been managed as a common property resource. Increased market demands and prices resulted in an exponential increase in numbers of harvesters, effort and landings. With the harvest of accumulated stocks the fishery is now dependent on variable annual recruitment. This has impacted local employment opportunities, recreational use of beaches and upland owners' enjoyment of the foreshore. As well, maintenance of year round markets has become more difficult. There are also increasing numbers of beach closures due to sewage contamination. Opportunities to expand clam culture are dependent upon access to wild beaches.

Management of intertidal clam fisheries is now being reviewed with all stakeholders with the view to introducing changes that will benefit local communities including coastal Native Indian Bands and maximize socio-economic benefits from the clam resource.

**MANAGEMENT OF THE BRITISH COLUMBIA ABALONE FISHERY: A SQUARE PEG IN A ROUND HOLE.** Sue Farlinger\* and Greg Thomas, Department of Fisheries and Oceans, Prince Rupert, B.C., Canada.

The northern abalone harvest was prosecuted as commercial dive, native food, and sports fisheries in British Columbia (B.C.). Commercial landings increased rapidly in the late 1970's then declined through quota management to low levels prior to closure of the fishery following the 1990 season. Numerous management strategies were applied beginning in 1977; many were not effective and led to a conservative and highly regulated individual vessel quota (IQ) coupled with a minimum size limit. The total annual quota prior to closure was 47 t. Reductions in annual quotas led to consolidation of IQ's on fewer vessels. Price increased at a greater rate than the consumer index making the fishery more lucrative for legal and illegal participants. Stock assessment involved bi- and triennial dive surveys to provide indices of abundances from which quotas could be estimated, as well as review of harvest patterns and CPUE from harvest logs. Factors confounding stock abundance estimates were changes in diver experience and quota system effects on fishing strategy. The mean length of landed abalone remained well above the minimum size limit during the years prior to closure. Strategies for management of the abalone fishery are presently being reviewed including enhancement and territorial leasing options.

**DIG A DUCK—THE COMMERCIAL GEODUCK CLAM FISHERY IN BRITISH COLUMBIA.** Rick Harbo,\* Department of Fisheries and Oceans, Pacific Region, Fisheries Branch, South Coast Division, 3225 Stephenson Point Road, Nanaimo, B.C., Canada V9R 1K3.

The geoduck clam, *Panope abrupta*, has been fished in B.C. since 1976. There are 55 licences following limitation in 1979. Annual landings peaked at 5735 t in 1987 and have decreased to an annual quota of 2433 t in 1993. Area quotas are based on a 1% annual yield of virgin biomass estimates.

A management program with individual equal licence quotas began in 1989 with the support of industry. The management costs paid by licence holders were \$495 k in 1993. Individual quotas are equal, determined by dividing the sum of the annual area quotas by 55, the number of licences. Vessel quotas are 97,500 lb. in 1993 and several quotas (up to 5) may be "stacked" on a vessel. The program includes area quotas and area selection by licence holders, a three year rotational fishery, designated landing ports and validation of landings.

Each landing is validated by a contracted port observer to monitor the individual and area quotas.

**"KNOB COD"—MANAGEMENT OF THE COMMERCIAL SEA CUCUMBER FISHERY IN BRITISH COLUMBIA.** Steve Heizer,\* Department of Fisheries and Oceans, Pacific Region, Fisheries Branch, South Coast Division, 3225 Stephenson Point Road, Nanaimo, B.C., Canada V9R 1K3.

The California sea cucumber, *Parastichopus californicus*, has been fished commercially in B.C. since 1980. Recently there has been an increase in utilization of the skin as a dried product as well

as the traditional frozen muscle strip products. There are currently 84 licences following limitation in 1991. Annual landings peaked at 1922 t in 1988 and have decreased to an annual quota of 238 t split or eviscerated weight (650 t, round weight) in 1993. Area quotas are arbitrary and precautionary. Some area quotas have been reduced based on apparent declines in catch per unit effort. Rotational fisheries have been set in the south coast, fishing areas once every two or three years.

A management program with individual, equal licence quotas is to be considered for 1994 with the support of industry. A share of the management costs will be paid by licence holders. Each landing will be validated by a contracted port observer to monitor the individual and area quotas.

Some vessels are issued "P" licences to process their own catch at sea, in the north coast only where processing facilities have been limited in the past.

Studies are underway to standardize units of catch reporting. The sea cucumbers are delivered live and whole or split and drained.

**CONTINUING STUDIES OF GREEN URCHIN GROWTH AND RECRUITMENT NEAR KODIAK, ALASKA.** J. Eric Munk\* and R. A. MacIntosh, NMFS, Alaska Fisheries Science Center, P.O. Box 1638, Kodiak, AK 99615.

The fishery for green sea urchins (*Strongylocentrotus droebachiensis*) around Kodiak Island experiences annually fluctuating landings due primarily to the discovery and subsequent harvest of new beds. Past work suggests these urchins attain their full reproductive capacity (and yield) at approximately 50 mm test diameter and 3.5 years of age. A reduction in test growth rates at this size and age, however, limits the information gained from examining size-frequency distributions.

Annual size-frequency monitoring of an urchin bed at Chiniak has shown a strong yearclass settled in 1990. This has been the only significant recruitment at this site in the past six years and has substantiated past estimates of size at age for juvenile urchins. Harvests here in 1989 and 1990 combined with poor recruitment for the previous 3 years has substantially reduced the frequency of large urchins. This may allow tracking of the '90 yearclass to a size larger than usual (50–55 mm) and provide needed information on the growth of older, slower growing urchins.

**EXPLOITATION IN NATURAL POPULATIONS: A CASE STUDY OF A "NEW" FISHERY.** Catherine A. Pfister,\* Department of Zoology, NJ-15, University of Washington, Seattle, WA 98195; Alex Bradbury, Washington State Department of Fisheries, Point Whitney Shellfish Laboratory, 1000 Point Whitney Road, Brinnon, WA 98320.

The red sea urchin, *Strongylocentrotus franciscanus*, a conspicuous member of subtidal communities in the north Pacific, has been exposed to intense harvesting for the first time only in the last decade. Data from Washington state (U.S.A.) indicate that current

levels of harvest may cause negative rates of population change. These population data, combined with analyses of commercial diver exploitation patterns suggest that current harvest levels are not sustainable.

Since the red sea urchin fishery is only one of a number of growing "non-traditional" fisheries, it is a model for problems managers will face throughout the world. Because many new fisheries have no history of catch-effort data or even population censuses, information available for management is minimal. Focusing on the red sea urchin, we use bootstrap analyses to estimate adequate sample sizes for inferences about population growth and make further recommendations on the design of data collection. We find that conclusions from catch-effort logbook data may be at odds with urchin census data. Thus, for new fisheries where extensive censuses are often unavailable, catch-effort data should be interpreted with caution.

**THE SOFT-SHELL CLAM FISHERY IN THE CANADIAN MARITIMES: AN INDUSTRY IN CHANGE.** Shawn M. C. Robinson,\* Department of Fisheries and Oceans, Invertebrate Fisheries Section, Biological Station, St. Andrews, N.B., Canada E0G 2X0.

The fishery for the soft-shell clam (*Mya arenaria*) on the east coast of Canada is currently in a state of change. This fishery has a long history of exploitation (several thousand years) and landing records date back to the late 1800's. Harvesting methods have not changed appreciably for decades as individual fishermen still harvest the clam at low tide using clam forks. Regulations in the fishery are comprised of a minimum size limit of 44 mm (1.75 inches), licensing of commercial diggers, a daily bag limit for recreational diggers, and compliance with the public health inspection standards for paralytic shellfish poisoning (PSP) and coliform bacteria. There is some harvesting being done in moderately contaminated areas for depuration.

Landings in the clam industry have decreased over the last decade. Although cycles in landings have been noted in the past, the recent decline in landings are due more to the closing of the clam flats due to contamination by coliform bacteria than by over-harvesting. The possible directions of the industry in the future will be discussed.

**FISHERIES MANAGEMENT IMPLICATIONS OF NEW GROWTH AND LONGEVITY DATA FOR PINK (*CHLAMYS RUBIDA*) AND SPINY SCALLOPS (*C. HASTATA*) FROM PUGET SOUND, WASHINGTON.** Robert E. Sizemore\* and Lynn Y. Palensky, Washington State Department of Fisheries, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

Diver and trawler harvest of pink (*Chlamys rubida*) and spiny scallops (*C. rubida*) is permitted as an experimental fishery in Washington State. Growth data, vital to developing an effective fisheries management plan, was available from British Columbia

but not from Puget Sound. Juveniles were collected from the San Juan Islands and suspended in lantern nets in Dabob Bay for growout. Growth and mortality of individual scallops and pink scallops recruits were followed for several years.

Spiny scallops had the higher rate of growth. Pink scallops had no mortality over the observation period. Spiny and pink scallops recruiting to the harvest reach minimal size (2") in June and October, respectively.

Implications for fishery management will be discussed.

**MANAGEMENT OF AN EXPANDING RED SEA URCHIN FISHERY IN BRITISH COLUMBIA.** Greg Thomas,\* Department of Fisheries and Oceans, Prince Rupert, B.C., Canada.

The commercial dive fishery for Red Sea Urchin, *Strongylocentrotus franciscanus*, began in southern waters of British Columbia in 1970 and has been expanding rapidly since 1983. Annual landings increased from 1000 tonnes in 1983 to 6700 tonnes in 1991 and exceeded 10,000 tonnes in 1992 (preliminary data). Increases in fishing activity in recent years can be attributed to the development of a fishery in northern waters. The primary objective of management is the conservation of Red Urchin stocks, while secondarily optimizing harvest and maximizing economic return. These objectives have been addressed through a combination of a minimum size limit (100 mm), area quotas, and seasonal closures in southern waters. A management strategy based on minimum and maximum size limits (100 mm and 140 mm) and rotational area openings has been tested in northern waters, but a catch ceiling was introduced in 1993 in the face of high catches and poor compliance by fishermen. To restrict fishing effort, the number of licences issued annually was limited to 108 in 1991. Further management options under consideration include Individual Vessel Quotas and Marine Protected Areas.

## INTEGRATED PEST MANAGEMENT

**IMPACTS ON BENTHIC INVERTEBRATE COMMUNITIES CAUSED BY AERIAL APPLICATION OF CARBARYL TO CONTROL BURROWING SHRIMP IN WILLAPA BAY, WA.** Kenneth M. Brooks,\* Pacific Rim Mariculture, 644 Old Eaglemount Road, Port Townsend, WA 98368.

The broad spectrum pesticide carbaryl is used to control burrowing shrimp (*Callinassa californiensis* & *Upogebia pugettensis*) on oyster beds in Willapa Bay and Gray's Harbor Washington. These shrimp can liquify the substrate making it too soft and unstable to support oyster culture. In addition to providing a substantial portion of U.S. oyster production, these estuaries are important nurseries for numerous valuable fisheries. An understanding of the short and long term impacts to the invertebrate food web is essential to developing an Integrated Pest Management Plan for long term shrimp control while maintaining the estuaries other important ecological functions.

Epibenthic and benthic invertebrates were sampled, by

epibenthic pump and modified van Veen dredge, two days before and two, fourteen, and fifty-one days following aerial application. Results indicate significant short term impacts to arthropods on a species specific basis. Some important salmonid prey species suffer significant decreases immediately following application. Other, closely related species appear very tolerant. Within 51 days most populations recovery to or exceed pre-spray numbers. However, some species did not recover within the period of observation. This information is essential to developing a long term integrated pest management program to control burrowing shrimp with minimal impacts on non-target species.

**A PROPOSAL TO TAKE A CLOSER LOOK AT BURROWING SHRIMP RECRUITMENT TO OYSTER CULTURE AREAS IN WASHINGTON COASTAL ESTUARIES.** Brett R. Dumbauld,\* Washington State Department of Fisheries, P.O. Box 190, Ocean Park, WA 98640; David A. Armstrong and Kristine L. Feldman, School of Fisheries, University of Washington, Seattle, WA 98195.

The pesticide carbaryl has been used to control the mud shrimp *Upogebia pugettensis* and ghost shrimp *Neotrypaea californiensis* on oyster culture grounds in Washington State coastal estuaries for 30 years. Current efforts to find alternative control measures and develop an integrated pest management plan for these shrimp will ultimately fail unless the estuarine recruitment process of these deep burrowing crustaceans is addressed. Even if growers are able to find alternative means of eliminating adult shrimp from their beds, new recruits are supplied annually from a pool of larvae that exists seasonally in the nearshore coastal plankton. Without some attention to the timing and nature of this recruitment event, all efforts including continued use of carbaryl will be temporary solutions to the problem. We have initiated a study which will elucidate the role of benthic shell including live oysters in various configurations as physical barriers to recruitment and as habitat for predators which control survival of small recruits. We also expect to examine the effects of culture methods such as harvest dredging as potential disturbance mechanisms. Preliminary data indicate that juvenile Dungeness crab (second to sixth instar, 10–30 mm CW) are active predators of newly settled shrimp. These crab are up to 50 times more abundant in intertidal shell habitat than on open shrimp dominated mudflats and may therefore greatly influence shrimp survival.

**BURROWING SHRIMP RECRUITMENT TO INTERTIDAL SHELL HABITAT: SUBSTRATE SELECTION, POST-SETTLEMENT SURVIVAL, AND THE IMPACT ON SHELL LONGEVITY.** Kristine L. Feldman,\* David A. Armstrong, and David B. Eggleston, School of Fisheries, WH-10, University of Washington, Seattle, WA 98195; Brett R. Dumbauld, Washington State Department of Fisheries, Willapa Lab, P.O. Box 190, Ocean Park, WA 98640.

In April 1992, the United States Army Corps of Engineers constructed eight hectares of intertidal shell habitat to enhance

survival of 0+ (young-of-the-year, YOY) Dungeness crab, *Cancer magister*, in Grays Harbor Estuary, Washington. Intertidal shell serves as critical refuge for 0+ crab, and crab densities are significantly greater in shell than in mud habitats. However, the constant turnover of sediments through burrowing activity of ghost shrimp, *Neotrypaea (Callinassa) californiensis*, and mud shrimp, *Upogebia pugettensis*, can reduce sediment compaction and alter soft-bottom species assemblages. To assess the potential impact of burrowing shrimp on the long-term success of intertidal shell mitigation, we quantified patterns of adult distribution and larval settlement at two shell-mitigation sites within the estuary. Laboratory experiments also examined the impact of 0+ crab predators on recruitment success of infaunal shrimp in shell.

Monthly surveys of small-scale test plots in 1991 indicated that the burrowing activities of infaunal shrimp were partially responsible for the loss of crab habitat through shell subsidence. This often occurred within one month after shell placement. Results from 1992 indicate that 0+ shrimp are over four times more dense in open mudflats than shell habitats (58 vs 13 shrimp · m<sup>-2</sup>). Laboratory experiments conducted on crab foraging behavior and predation rates on 0+ shrimp in shell habitat indicate that a single YOY crab (18–22 mm carapace length) can eat as many as 12 shrimp within a 24-hour period. Although the two sites chosen for full-scale mitigation are devoid of high adult shrimp densities (e.g., <40 shrimp · m<sup>-2</sup>), it is uncertain whether continued recruitment of 0+ shrimp will degrade the quality of these shell habitats over time or whether, as the results of laboratory experiments conducted in 1992 suggest, 0+ crab resident in the shell will reduce the abundance of settling shrimp through predation.

**APPROACHES TO THE BIOLOGICAL CONTROL OF ZEBRA MUSSELS.** Daniel P. Molloy, Biological Survey, New York State Museum, Albany, NY 12230.

This paper examines what role biological control techniques may play in the integrated pest management of zebra mussels, *Dreissena polymorpha*.

*Predators:* Numerous organisms are known to prey on zebra mussels, but each would appear to be of little usefulness in actual control projects. Predators, however, have been reported to significantly reduce localized field populations of zebra mussels and could play an important role in the long-term reduction of zebra mussels in lakes, rivers, etc.

*Parasites:* Very little research has been conducted on zebra mussel parasites. A recent study in the Netherlands, however, has reported a severe and apparently lethal protozoan infection. Future use of parasites as biocontrol agents can not be dismissed. In terms of environmental impact, parasites are ideal control agents since they have been fine-tuned through evolution to be host specific and thus should cause negligible nontarget problems. Parasites, however, often have complex growth requirements and elaborate life cycles; these two characteristics can represent formidable obstacles toward economical mass production—a requirement for commercialization.

*Toxin-Producing Microbes:* A third and novel approach to developing a biological control method for zebra mussels is the screening of microorganisms (primarily bacteria) to find strains that are selectively lethal to these mussels. The microorganisms screened are not truly invasive parasites of zebra mussels, but rather microbes which are fortuitously lethal to zebra mussels when the mussels are exposed to artificially high densities of these microbes or their metabolic byproducts. Once a promising strain is found, these microbes can often be economically mass produced in vitro—a characteristic which can lead to their rapid commercialization. Such a screening process has a clear record of success in the development of microbial insecticides and may well prove valuable for zebra mussel control also. Laboratory data on lethality of bacterial stains will be presented.

**AN INTEGRATED PEST MANAGEMENT PLAN FOR THE CONTROL OF BURROWING SHRIMP POPULATIONS ON OYSTER BEDS IN SOUTHWESTERN WASHINGTON STATE.** John L. Pitts,\* Aquatic Farm Program, Washington State Department of Agriculture, Olympia, WA 98504-2560.

Two species of burrowing shrimp (*Neotrypaea californiensis* and *Upogebia pugettensis*) occur in Pacific Coast estuaries with varying impacts on oyster and clam culture. The Pacific Coast currently produces more than a third of the nation's oysters, therefore, pest infestation is of regional and national concern.

The carbamate pesticide carbaryl (Sevin) is the only effective tool currently approved for control of excess shrimp populations on oyster beds in Washington State. Objections by some crab fishers and environmentalists has led to the development of a multifarious Burrowing Shrimp Control Committee (BSCC). The BSCC developed an Integrated Pest Management Plan (IPMP) designed to evaluate alternative pest control methods and implement a plan using suitable strategies which allow continued oyster culture. Alternative methods identified include alternative culture techniques, mechanical control, enhancement of shrimp predators, electrofishing, and modification of carbaryl application. Critical timing for shrimp control requires additional study. A three year non-target species impact study commenced in 1992. Agriculture engineers are currently exploring alternative culture methods and modification of terrestrial pest control methods. Economic Threshold Determination studies are needed to determine "trigger" points for shrimp control maximum efficiency.

## ALASKAN SHELLFISH INDUSTRY PANEL

**PROMISE AND CONSTRAINTS OF SHELLFISH AQUACULTURE IN ALASKA.** Raymond Ralonde, Marine Advisory Program School of Fisheries and Ocean Sciences University of Alaska; James Cochran, Mariculture Coordinator, Fisheries Rehabilitation Enhancement and Development Division Alaska Department of Fish and Game; Jeff Hetrick, President of the Alaska

Shellfish Growers Association; **Manny Soares** and **Mike Ostasz**, Seafood Section, Division of Environmental Health, Alaska Department of Environmental Conservation; **Janet Burleson**, Coordinator Alaska Coastal Management Program, Alaska Department of Natural Resources.

In 1989, Alaska Senate Bill 514 revitalized the shellfish culture industry by improving aquatic farm permit processing. The changed regulations have caused an influx of permit applications that resulted in 72 aquatic farms. The new shellfish culture industry faces major challenges. The State of Alaska has conservative species import regulations, and does not have an operating shellfish hatchery, requiring farmers to buy spat from hatcheries outside the state. High operating cost, lack of a track record, and inexperience of the farmers makes financing difficult.

The Pacific oyster is an attractive species for aquaculture in Alaska because it grows very well on the abundant, high quality food. Cold, clean water also prevents bacterial contamination extending the shelf life, and retards sexual maturation resulting in high quality half shell oysters being available year around. Blue mussels, and scallops are also being cultured at experimental levels. Littleneck clams, urchins, abalone, and seaweeds are potential farm candidates. Each of these species has its own set of constraints and promises for aquaculture.

The constraints to shellfish aquaculture in Alaska may seem substantial, but the prospects for success are rapidly improving. Technological innovations are being developed to address some of the constraints, and construction of an Alaskan shellfish hatchery is receiving substantial attention.

Alaska holds a major advantage not found in other states, pristine water quality. Currently, Alaska is the only state with no restricted waters for shellfish harvest. While the amount of non-restricted and open shellfish harvest areas around the United States are decreasing, Alaskan marine aquaculture is expanding. Pristine water quality, technological improvements, and high sanitation standards will make Alaskan shellfish a viable industry.

## BIVALVE FEEDING AND NUTRITION

**VERTICAL GRADIENTS IN GROWTH OF JUVENILE BAY SCALLOPS, *ARGOPECTEN IRRADIANS*, IN RELATION TO FLOW AND SESTON CHARACTERISTICS IN EELGRASS MEADOWS.** **Francisco J. Borrero\*** and **V. Monica Bricelj**, Marine Sciences Research Center, State University of New York, Stony Brook, NY 11794-5000.

The presence and characteristics of eelgrass affect the hydrodynamic conditions of shallow (<4 m depth) subtidal bays in eastern Long Island, NY, which provide a nursery habitat for bay scallops, *Argopecten irradians*. We conducted a 2-year study with hatchery-reared juvenile scallops to assess the effects of 1) presence or absence of eelgrass, 2) vertical position (0, 15, 35 and 75 cm above-bottom), and 3) characteristics of the eelgrass bed (sed-

iment type and canopy height), on a) scallop growth, b) flow regime, and c) seston quality and quantity (total dry weight, and concentrations of chlorophyll *a*, phaeopigments and organics). Growth in dry weight of soft tissues, and individual growth in shell height were determined for scallops held in pearl nets, as well as survival of tethered scallops to predation, and the effect of net enclosure on growth and flow conditions at various elevations.

There were significant vertical gradients in scallop growth, both in areas with and without eelgrass, but steeper gradients (up to 3-fold differences in tissue weight) were observed within eelgrass beds than on unvegetated substrate. Scallop growth was greater at 35 or 75 cm above-bottom than at 0 to 15 cm. Most pronounced vertical differences in growth occurred at sites with lowest current velocity, characterized by taller eelgrass and finer-grained sediments. At each site, seston characteristics changed dramatically across sampling dates, and were strongly affected by wind and tidal conditions. However, vertical gradients in scallop growth generally correlated with those in seston concentrations (higher levels at 0–15 cm than at 35–75 cm above-bottom), and flow conditions. This study demonstrates that scallops derive significant benefits in growth by maintaining an above-ground position on eelgrass during their early life history. These results can be exploited in the selection of estuarine microhabitats for bay scallop cultivation.

**MICROCAPSULES AND SUSPENSION-FEEDERS—AN UPDATE.** **Christopher J. Langdon**, Hatfield Marine Science Center, Newport, OR 97365.

This paper will review several important steps that have recently occurred in the use of microcapsules in feeding studies with marine suspension-feeders.

Firstly, modified methods for preparing cross-linked, protein-walled capsules have resulted in a capsule type that is non-toxic and digestible by marine bivalves. Protein-walled capsules have been successfully used to examine protein requirements of mussels (*Mytilus edulis trossulus*). Growth experiments with mussels have also indicated that supplements of protein capsules can be successfully used to improve the nutritional value of protein-poor algae. Encapsulation of either amylose or maltodextrin mixed with protein did not significantly affect delivery of protein to mussels.

Secondly, delivery of low-molecular weight, water-soluble nutrients to suspension-feeders has been facilitated by improvements in the preparation of lipid-walled capsules. *In vitro* leakage experiments demonstrated that highest encapsulation and retention efficiencies were obtained with capsule walls primarily consisting of triglycerides, such as tripalmitin. Feeding experiments with the mysis stage of shrimp (*Penaeus vannamei*) indicated that larvae were able to ingest and digest tripalmitin-walled capsules, release encapsulated <sup>14</sup>C-glucose from the capsules and incorporate <sup>14</sup>C into their body tissues. Lipid-walled capsules, combined with either protein-walled capsules or micro-gel particles, offer a means of delivering complete artificial diets to suspension-feeders.



**MECHANISMS OF PARTICLE TRANSPORT AND INGESTION IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.** Roger I. E. Newell,\* Horn Point Environmental Laboratory, University of Maryland System, Cambridge, MD 21631; J. Evan Ward and Bruce A. MacDonald, Department of Biology, University of New Brunswick, Saint John, NB, Canada E2L 4L5; Raymond J. Thompson, Marine Sciences Research Laboratory, Memorial University of Newfoundland, St. Johns, NF, Canada A1C 5S7.

Suspension-feeding processes in the eastern oyster *Crassostrea virginica* were studied *in vivo* using video endoscopy. Analysis of our observations indicates that many of the previously published concepts of particle transport in this species are inaccurate, probably because they were based on results obtained from surgically altered specimens. Our observations clarify the following fundamental aspects of particle handling: 1) particles trapped by the gills are transported toward the labial palps by both mucociliary (marginal groove) and hydrodynamic (basal groove) processes, 2) the labial palps accept particles from the marginal food groove that are bound in mucus strings and from the basal groove that are in a slurry, 3) the labial palps reduce the viscosity of mucus strings, and disperse and sort entrapped particles, 4) particles are ingested in the form of a slurry, and 5) ciliary activity at the buccal region is independent from that on the palps, hence enabling oysters to clear particles from suspension and produce pseudofeces without ingesting any particles. We will use video images to illustrate fundamental processes of particle-handling applicable to most bivalve suspension feeders and discuss how accepted theories of particle handling need to be modified.

**PHYTOPLANKTON STOCKS AND THE FUTURE OF THE GALVESTON BAY OYSTER FISHERY.** Eric N. Powell,\* Elizabeth Wilson-Ormond, and Mathew Ellis, Department of Oceanography, Texas A&M University, College Station, TX 77843; Eileen E. Hofmann and John M. Klinck, Center for Coastal Physical Oceanography, Crittenton Hall, Old Dominion University, Norfolk, VA 23529.

Phytoplankton standing stocks and water turbidity have steadily declined in Galveston Bay over the last twenty years. Food supply for the oyster populations is primarily phytoplanktonic in Galveston Bay; accordingly food supply has steadily declined over the last twenty years. Declining turbidity, however, may have spared the effect of declining food supply by increasing filtration and ingestion efficiency. A time-dependent population dynamics model was used to determine the possible future effects of continuing declines in phytoplankton standing stocks and turbidity. All simulations assumed that the documented rate of decline in phytoplankton stocks and bay turbidity would continue at the rate observed over the last twenty years.

Three different temporal sequences of mortality were used in the model; continuous mortality throughout the year; mortality concentrated in the winter, simulating the effect of the fishery; and

mortality concentrated in the summer, simulating the effect predators and disease. All three conditions produced qualitatively similar results. Oyster populations maintained an increasing or level population density for 12 to 14 years as declining turbidity spared the effect of declining food supply, then declined rapidly to near-extinction in 2 to 4 years. The temporal sequence of mortality affected the outcome in only a minor way. Our simulations suggest that near-extinction of oyster populations can occur in less than 4 years in Gulf of Mexico bays once thresholds in population dynamics are crossed, and that, were the observed declines in phytoplankton stocks and bay turbidity to continue, oyster populations in Galveston Bay could crash near or just after the year 2000.

**IN SITU MEASUREMENTS OF BIVALVE SUSPENSION-FEEDING: COMPARISON BETWEEN RATES OF SCALLOPS AND MUSSELS.** J. Evan Ward\* and Bruce A. MacDonald, Department of Biology, University of New Brunswick, Saint John, NB, Canada E2L 4L5.

During the past three years, we have been conducting laboratory and field research for the Ocean Production Enhancement Network (OPEN). One of the objectives of OPEN is to combine oceanographic data with studies on scallop (*Placopecten magellanicus*) physiology to produce an integrated carrying capacity model for aquaculture sites in Atlantic Canada. Little is known, however, about the relationships between the quantity and quality of suspended particles, scallop feeding activity and growth rates. In contrast, more information is available on the blue mussel (*Mytilus edulis*). Therefore, we designed studies to simultaneously study feeding responses in scallops and mussels.

The natural food supply of bivalves consists of a complex mixture of organic and inorganic particles. Because it is difficult to realistically predict the animals' feeding response to this food supply from measurements of particle uptake made only in the laboratory, we developed a new method to measure rates *in situ*. By continuously recording physical conditions and measuring food quality during our feeding studies, we were able to correlate changes in behaviour with fluctuations in environmental variables. This will allow us to describe predictive relationships for scallop feeding and growth, applicable to many habitats in Atlantic Canada.

**FOOD AVAILABILITY TO NATURAL OYSTER POPULATIONS: FOOD, FLOW AND FLUX.** E. Wilson-Ormond\* and E. N. Powell, Department of Oceanography, Texas A&M University, College Station, TX 77843; E. E. Hofmann and J. M. Klinck, Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529.

Food availability to natural oyster populations is dependent upon the quantity of food present, water flow speed, and oyster density. Field experiments were conducted (Confederate Reef, Galveston Bay, TX) to determine the temporal variability in the food concentration and water flow speed on scales consistent with

oyster feeding. Results indicate that the amount of food ( $\text{mg l}^{-1}$ ) available to the population is highly variable on temporal scales as short as 3 hr. Water flow speeds ( $\text{cm s}^{-1}$ ) are also quite variable, however, they tend to cluster about a narrow range of slower speeds. The resultant food fluxes ( $\text{mg cm}^{-2} \text{ s}^{-1}$ ) indicate that natural populations experience a highly variable food supply. Rapid water flow can compensate for low food concentration by resulting in an overall higher flux of food, while slow flow typically results in low flux regardless of the concentration of food. These results suggest that in some cases, water flow speed is more important than food concentration in determining the amount of food available to the population.

A mathematical model of oyster energetics was employed to further assess the role of water flow in determining productivity in natural oyster populations. Simulation results suggest that oyster productivity is higher under conditions of rapid flow because of increased food availability due to a higher flux of food particles. Slower water flow can result in food depletion due to over-filtration and can ultimately reduce productivity. Productivity was better estimated in simulations using the variable food supply as compared to the average food supply. The latter consistently over-estimated productivity. Therefore, the short-term temporal variability in available food is an important factor affecting oyster feeding and productivity.

## GENETICS AND BREEDING

**EFFECTS OF GROWOUT DENSITY ON HERITABILITY OF GROWTH RATE IN THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA* (LINNAEUS, 1758).** John W. Crenshaw, Jr.,\* Shellfish Research Laboratory, University of Georgia, Savannah, GA 31416; Peter B. Heffernan, Martin Ryan Marine Science Institute, University College Galway, Galway, Ireland; Randal L. Walker, Shellfish Research Laboratory, University of Georgia, Savannah, GA 31416.

Realized heritability for increase in rate of growth in the northern quahog, *Mercenaria mercenaria*, was calculated, under conditions of moderate growout density (<90 per sq. ft.), independently for two lines. For one line, Group A, a mean estimate of heritability of 0.402 was obtained. For two replicates of Group B, a mean heritability estimate of 0.123 was calculated. The latter estimate is likely an under-estimate. Group A and B estimates are each based upon a single generation of selection in which a standardized selection differential (i) of 1.525 standard deviations was employed, representing a selection intensity of nearly 15.9%. When Select and Control progeny of Group A were maintained in growout at high densities (>350 per sq. ft.), Control progeny grew at significantly greater rates than Selects, thus resulting in negative estimates of heritability.

Clam stocks were collected in House Creek, Little Tybee Island, Wassaw Sound, in coastal Georgia, U.S.A. Same-age cohorts of  $F_1$  progeny were established in April and May of 1986.

Progeny were reared in the laboratory in ambient filtered sea water with food provided by Wells-Glancy cultured phytoplankton until December, 1986 when they were transferred to growout cages in an intertidal creek. Care was exercised to prevent any size culling. Selection was carried out for  $F_1$  cohorts in March, 1988 (Group A) and May, 1989 (Group B). Select and Control parental groups were identical in number, the latter randomly chosen from the entire population. Spawns of the Control and Select Group B  $F_1$  parents occurred on June 13 and 14, 1989, respectively; Group A parents, Control and Select, were induced to spawn on July 6 and 7, 1989, respectively. Clam progeny were transferred from nursery to growout cages September 12, 1990, and density reduced on April 1, 1991. Realized heritabilities were calculated on September 5, 1991 for clams of Group A, and for Group B, because of slower development on March 16, 1992. Measurements of clams maintained in high density growout were taken at the latter date.

**THE SUITABILITY OF LAND BASED EVALUATIONS OF *CRASSOSTREA GIGAS* AS AN INDICATOR OF PERFORMANCE IN THE FIELD.** Gregory A. Debrosse\* and Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

Besides disease resistance, there are a host of ecological questions regarding the suitability of *Crassostrea gigas* for introduction to the mid-Atlantic. Are tank based comparisons of survival, growth, disease, etc. suitable for estimating the performance of *C. gigas* in the field? In June 1991, equal numbers of spat from three crosses—WFLA (*Crassostrea virginica*), YWAA (*C. gigas* form Miyagi), and XJPNA (*C. gigas* form Hiroshima)—were split into two replicates and reared in upwellers for the first summer and in a land-based tank the second. After the first season, *C. virginica* had the highest mortality (65%, 36%, and 13% for WFLA, YWAA, and XJPNA, respectively) and average spat size was about 30% greater in both *C. gigas* groups. For the second year, the 3 crosses were transferred to a 4200 gallon tank; two replicates of WFLA were also placed in Delaware Bay. Cumulative mortality for the second season (through November, 1992) was WFLA—60%; YWAA—73%; XJPNA—93%; and WFLA (Delaware Bay)—37%. YWAA grew fastest followed by XJPNA and WFLA; however WFLA grown on the tidal flats were larger than all tank reared groups. All oysters in the tank were infested with *Polydora websteri*, *C. gigas* heavily and WFLA lightly; WFLA (Delaware Bay) were virtually free of infestation. These data indicate that tank-based comparisons are not likely to be a true measure of performance in the local environment. Publication No. K-32100-2-93 NJAES.

**GONADAL NEOPLASIA IN *MERCENARIA MERCENARIA*, *M. CAMPECHIENSIS* AND THEIR HYBRIDS.** Arnold G. Eversole,\* Department of Aquaculture, Fisheries and Wildlife, Clemson University, Clemson, SC 29634-0362; Peter B. Heffernan, Marine Extension Service, University of Georgia, Savannah, GA 31416.



*Mercenaria mercenaria*, *M. campechiensis* and hybrids (*M. mercenaria* ♀ × *M. campechiensis* ♂; *M. campechiensis* ♀ × *M. mercenaria* ♂) cultured in waters near Charleston, South Carolina were sampled from September 1987 through October 1988. Histological preparations revealed neoplastic cells identified as germinoma in the lumen of gonads. Invasive stages of neoplasia were found in a few samples. Gonadal neoplasia was observed in 42% of the 400 hard clams examined. Occurrences and intensities of gonadal neoplasia were the highest during the warmer months (May–July, and September). Shell lengths of those clams with neoplasia were similar to that of clams diagnosed as non-neoplastic. Neoplasia occurred more frequently in those clams which were sexed as indeterminate compared to male and female clams. Occurrences and intensity levels were higher in the hybrids than in either *M. mercenaria* or *M. campechiensis*. Hybrids (n = 40) collected in July 1992 had occurrences and intensities significantly higher than those levels observed in samples from four years earlier.

**ASSESSING REPRODUCTIVE STERILITY OF TRIPLOIDS: ANEUPLOID LARVAE PRODUCED FROM CROSSES BETWEEN TRIPLOID AND DIPLOID *CRASSOSTREA GIGAS*.** Ximing Guo\* and Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

*Crassostrea gigas* has been variously proposed as a replacement or supplement species for *C. virginica* in several east coast situations. Triploids potentially offer a "safe" way to test *C. gigas* in the field. Are triploid *C. gigas* sterile? The genetics of reproduction in triploid Pacific oyster, *Crassostrea gigas*, was examined in matings between diploids (D), triploids (T), and their reciprocal crosses (D × T and T × D). Ploidy of embryos of all matings were determined by karyology and flow cytometry. Sperm from triploids showed a single distribution of DNA content at 1.49c, as determined by flow cytometry; no haploid peaks were observed. In eggs from triploids, chromosome numbers varied considerably within and among females, but most had between 11–13 trivalent and bivalent chromosomes. Ploidy of embryos from the four types of matings was determined by both flow cytometry and karyology to be 2n for D × D, 2.5n for D × T and T × D, and 3n for T × T. Survival to 7 days post-fertilization was 40% for D × D, 0.5% for D × T, 8% for T × D, and 0.4% for T × T. Percent metamorphosis to spat was 23% for D × D, 0.001% for D × T, 0.058% for T × D, and 0.0% for T × T. These data suggest that it may be possible to estimate the reproductive likelihood of triploid oysters in the field. Publication No. K-32100-4-93.

**SECOND HERITABILITY ESTIMATE OF GROWTH RATE IN THE SOUTHERN BAY SCALLOP, *ARGOPECTEN IRRADIANS CONCENTRICUS* (SAY, 1822).** Peter B. Heffernan\*<sup>1,2</sup> and Randal L. Walker,<sup>1</sup> <sup>1</sup>Shellfish Re-

search Laboratory, Marine Extension Service, University of Georgia, P.O. Box 13687, Savannah, GA 31416-0687, \*<sup>2</sup>Martin Ryan Marine Science Institute, University College Galway, Galway, Ireland.

Realized heritability for growth rate in the southern bay scallop, *Argopecten irradians concentricus*, was estimated to be 0.368. This estimate is based upon a single generation (F<sub>4</sub>) of selection in which a standardized selection differential (i) of 1.4225 standard deviations was employed, and a response to selection, also in standard units, of 0.523 was obtained. A previous estimate of heritability for growth rate with the F<sub>1</sub> generation was reported to 0.206 (Crenshaw *et al.* 1991). The current results support (1) our earlier contention that the F<sub>1</sub> generation heritability estimate for growth rate was a considerable underestimate, probably due to age differences (selects 20 days younger) at the time of measurement (2) the adjusted F<sub>1</sub> upper limit of heritability estimated at 0.498.

The parental broodstock were obtained in St. Joseph Bay, west of Apalachicola, Florida in 1987 and spawned to produce the parental offspring for the selection program in October 1987. The first selection process was carried out in October 1988, with the growth response of the F<sub>1</sub> offspring being compared to yield the first heritability estimate in 1989. A second selection pressure was applied to this generation. Due to rearing difficulties and low survival a heritability calculation was not possible for the F<sub>2</sub> generation (1990), nor could selection pressure be applied to this generation. The F<sub>3</sub> generation yielded good survival and this was used to carry out the third selection process (1991), with their offspring (F<sub>4</sub>) being used to calculate the second heritability estimate (October 1992).

**THE EFFECT OF PARENTAL RELATEDNESS ON PROGENY GROWTH AND VIABILITY IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*.** Ami E. Wilbur\* and Patrick M. Gaffney, Graduate College of Marine Studies, University of Delaware, Lewes, DE 19958.

The degree of parental relatedness has long been thought to affect progeny fitness, and inbred offspring frequently demonstrate reduced fitness (inbreeding depression). Reductions in offspring fitness may also occur when parents are too distantly related (outbreeding depression). We investigated the effect of parental relatedness on offspring growth and survival in the bay scallop (*Argopecten irradians*), a simultaneous hermaphrodite capable of self-fertilization. This species displays extensive physiological, morphological and genetic variation throughout its geographic range which has led to the recognition of three subspecies (*A. irradians irradians*, *A. irradians concentricus*, *A. irradians amplicostatus*). Twenty families whose parents represented at least two degrees of relatedness were produced. Each family consisted of a mixture of selfed and outcrossed offspring. The outcrossed offspring were the products of cross fertilization between individuals of either the same subspecies (11 families) or different sub-

species (9 families). The resultant families were sampled at 3, 6, and 9 months of age. Sampled individuals were typed using protein electrophoresis to assess parentage and determine the relative viability of the progeny types. The effect of parental relatedness on growth was also assessed.

## WEST COAST AQUACULTURE

**THE EFFECTIVENESS OF PREDATOR EXCLUSION TUBES FOR GROWOUT OF THE GEODUCK CLAM, *PANOPEA ABRUPTA*.** Dwight W. Herren,\* Washington State Department of Fisheries, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

Although hatchery techniques have been developed to produce geoduck clam seed, field trials for seed growout have met with limited success. Yields of less than 1% lead to a predator study and then to testing various predator exclusion devices.

This report describes the success of using biodegradable fiber pulp tubes to protect geoduck seed from predation. Implication of this technique for geoduck culture are discussed.

**ABALONE CULTIVATION TECHNIQUES.** Thomas B. McCormick,\* McCormick & Associates, 323 E. Matilija St. #112, Ojai, CA 93023.

Commercial abalone farms throughout the world are now producing abalone to meet increasing market demands as wild fisheries continue to decline. Total world abalone landings peaked in the 1970's at 20,000 metric tons (mt). Since that time landings have declined by 7,500 mt to 12,500 mt in 1990, a loss of over \$200–300 million.

The cultivation of abalone can be said to have started in 1935 when a Japanese researcher, S. Murayama, fertilized abalone eggs and successfully raised the larvae and early juvenile abalone. Large scale hatchery techniques were developed in the 1970's when reliable methods for spawning and early juvenile rearing were developed. Today a variety of techniques are used to raise numerous species of abalone. Some of these techniques will be discussed in this presentation.

**HATCHERY TECHNIQUES OF THE ROCK SCALLOP (*CRASSADOMA GIGANTEA*) LARVAE IN THE PUGET SOUND REGION, WASHINGTON.** Walter Y. Rhee,\* School of Fisheries, University of Washington WH-10, Seattle, WA 98195.

Rock scallops (*Crassadoma gigantea*) have an adductor muscle yield of 40 to 50% of the wet tissue weight, larger than any other scallops known. The goal of this research is to establish the best hatchery techniques in response to the growing interest by the Washington shellfish farmers to culture rock scallops. In this research, the optimum techniques to maintain broodstock, spawn broodstock, fertilize, and to rear the larvae to metamorphosis up to 1 mm spat were sought to increase yield of scallop spat. Results

from this research indicate a flow-through system over a closed system for higher survival rate in maintaining broodstock; injection of serotonin (0.2 ml of  $2 \times 10^{-4}$  M) into the adductor muscle or gonads to be the most efficient method of spawning; and 18 degrees Celsius to be the optimum temperatures for rearing larvae to metamorphosis.

**THE SUMINOE OYSTER—CANDIDATE FOR THE HALF-SHELL TRADE?** Anja M. Robinson\* and Christopher J. Langdon, Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

Over the last four years, research funded by the National Coastal Resources Research and Development Institute at the Hatfield Marine Science Center, Oregon State University, has focused on development the aquaculture of the Suminoe oyster *Crassostrea rivularis*. This species was probably introduced to the West coast, USA, with importations of Pacific oysters (*Crassostrea gigas*) from Japan. We successfully reared Suminoe oyster larvae on algal diets containing diatoms of the genus *Chaetoceros* and at salinities of 15 to 20 ppt. Usually 20 to 25% of larvae (initially present in cultures) successfully metamorphosed to produce spat. Percent set was significantly increased by exposure of competent larvae to  $2 \times 10^{-4}$  M epinephrine, which also resulted in production of cultchless spat. These hatchery procedures have been successfully adopted by a commercial hatchery.

Laboratory growth experiments indicated that both Pacific and Suminoe juvenile oysters grew fastest at a salinity of 25 ppt, and there was no evidence that juvenile Suminoe oysters were more tolerant of lower salinities than Pacific oysters. Pacific oysters grew faster than Suminoe oysters when planted at most of the grow-out sites tested on the West coast, as determined by increase in shell length and dry tissue weight. The commercial value of the Suminoe oyster will depend on both its good flavor and its attractive appearance on the half-shell.

**SUBSTRATE ADDITIVE STUDIES FOR DEVELOPMENT OF HARDSHELL CLAM HABITAT.** Douglas S. Thompson\* and Walt A. Cooke, Washington State Department of Fisheries, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

The Washington State Department of Fisheries is developing new hardshell clam habitat through beach graveling. At Oakland Bay near Shelton, WA, two gravel treatments and a control are being used to evaluate clam recruitment, growth and survival and to investigate potential impacts on epibenthic and infaunal organisms. The treatments are a 10 cm layer of 6–19 mm gravel; a 10 cm layer of a 50/50 mixture of 6–19 mm gravel with crushed oyster shell. Each treatment was replicated three times. The test plots are  $15 \times 30$  m and were sampled before graveling and annually thereafter using a  $10 \times 15$  cm PVC core. Sediment cores were sieved through nitex screens. All clams were enumerated, weighed and counted. Other invertebrates were classified and

counted. Species diversity and taxa richness increased on both treatments compared to the control. Clam recruitment was highest on the control plots, however the clams did not survive past 25 mm. Survival was best on the gravel + shell plots 64.71% compared to 49.90% for the gravel plots. Clam growth was similar on both gravel and gravel + shell plots. The best treatment will be used to develop a 1.6 ha production plot for recreational and tribal use.

## POSTER SESSION

**OVERWINTERING HATCHERY-REARED INDIVIDUALS OF *MYA ARENARIA*: A FIELD TEST OF SITE, CLAM SIZE AND INTRASPECIFIC DENSITY.** Brian F. Beal,\* Division of Science and Mathematics, University of Maine at Machias, 9 O'Brien Avenue, Machias, ME 04654.

Soft-shell clam landings in Maine have declined by 65% during the past ten years. Since 1987, the Beals Island Regional Shellfish Hatchery has produced ten million 8–12 mm soft-shell clam (*Mya arenaria*) juveniles each year for stock enhancement of commercial flats in ten of Maine's coastal communities. Manipulative field experiments using hatchery-reared individuals have repeatedly shown that clams seeded to flats at the end of the first growing season have poor overwinter survival (usually less than 30% between November and the following April) on those flats where ice scours the top few cm of mud. Because it is impossible to forecast whether a certain flat will be scoured, a more efficient approach would be to hold the clams over the Winter so that they can be seeded the following Spring. Clams can not be overwintered at the Hatchery due to space and food limitations nor can they be kept at their floating nursery site due to the chance of being removed by icebergs.

A field investigation was conducted during the Winter of 1991–1992 to determine an effective strategy to overwinter soft-shell clam seed. Approximately 3.2 million clams produced at the Hatchery during the 1991 season were divided into three size classes:  $\bar{X}_{\text{Large}} = 11.5 \text{ mm} \pm 0.085 \text{ SE}$ ,  $n = 269$ , [6.5/ml];  $\bar{X}_{\text{Medium}} = 8.2 \text{ mm} \pm 0.060 \text{ SE}$ ,  $n = 344$ , [17.1/ml];  $\bar{X}_{\text{Small}} = 4.3 \text{ mm} \pm 0.056 \text{ SE}$ ,  $n = 449$ , [75.0/ml]. Small clams were added to overwintering units which consisted of six 18-inch  $\times$  18-inch  $\times$  3-inch wooden-framed subunits covered with 1/2-inch extruded mesh netting. Subunits, rigidly connected to each other, were arrayed vertically with 3–4 inch spacing between levels. Clams were placed inside nylon window screen zippered bags at one of three volumes (densities): 250/ml (18,750 clams), 500/ml (37,500 clams), and 750/ml (56,250 clams). One bag was added to each subunit. Replicate units containing clams at one density were deployed in November, 1991 at two subtidal sites near Beals Island and were retrieved during April, 1992. Sites were chosen based on differences in water depth and exposure to storm events. Units were arranged so that all levels (subunits) were submerged at all times and the lowest level was always 1 to 1.5 m off the

bottom. Large clams at one of two volumes (1 1/8<sup>th</sup> L [4,500 animals] or 2 1/4 L [9,000 animals]) were added to each level of two units/site. Medium clams at one of two densities (1 L [11,000 individuals] or 2 L [22,000]) were added to each level of three units/site.

No apparent difference in survivorship between sites existed for large and medium size clams. Combined mean survival rate for these clams was  $97.7\% \pm 0.36 \text{ SE}$ ,  $n = 9$ . Mean survival for small clams was not site dependent and was  $67.8\% \pm 4.40 \text{ SE}$ ,  $n = 10$ . At the more protected site, small clams in the highest density had significantly higher survival rates ( $75.3\% \pm 2.29 \text{ SE}$ ,  $n = 12$ ) than animals at either of the two lower densities ( $60.3\% \pm 5.19 \text{ SE}$ ,  $n = 24$ ). Clams in the uppermost level of the overwintering units at the more protected and shallow water site, had lower survival rates than those nearer the bottom. Exposure to air and windchill during periods of low spring tides may have contributed to this outcome. Using these techniques, soft-shell clams can be economically overwintered only if survivorship in the field to market size is greater than 50%.

**THE EFFECTS OF AIRLIFT CIRCULATION ON THE SPACIAL DISTRIBUTION OF *CRASSOSTREA GIGAS* LARVAE SET ON STRUNG CULTCH IN CIRCULAR TANKS.** Fred S. Conte,\* Michael N. Oliver, and Heidi A. Johnson, Department of Animal Science, University of California, Davis, CA 95616.

Hatchery produced *C. gigas* larvae were set on punched and strung, oyster and scallop shell cultch in "standard" circular commercial setting tanks and tanks modified to use an airlift system. The airlift system was used in attempts to improve circulation within the shell pack, isolate the heating element from the cultch, and to remove the effect of air bubbles within the shell pack on larval distribution and setting patterns.

The standard tanks used a series of drilled 1.9 cm PVC<sup>®</sup> pipes placed in parallel rows across the bottom of the tank. Each row was plumbed into a single, vertical, forced-air, delivery pipe. Sea water was heated with a side-mounted, thermo-statically controlled, stainless steel immersion heater.

Modified tanks used a circular screen to support the shell pack 10.16 cm from the bottom and four 7.62 cm PVC<sup>®</sup> airlifts extending through the screen and terminating at the water surface with 45° elbows that directed the water current to the tank's center. A 30.48 cm diameter airlift with slots cut into the top supported the immersion heater and directed water flow toward the tank sides.

Tagged stringers of cultch were placed in each tank, and following set and a 30-day hardening period the stringers were retrieved and the spat counted. Statistical analysis of the spacial distribution and larval setting patterns within tanks were performed on both shell type, shell surface, position, and orientation. Results demonstrated improved spacial distribution of set and increased set within the modified tanks over that of the standard setting tank.

**STATUS AND TRENDS ANALYSIS OF OYSTER REEF HABITAT IN GALVESTON BAY, TEXAS.** Matthew S. Ellis,\* Jung Song, and Eric N. Powell, Department of Oceanography, Texas A&M University, College Station, TX 77843-3146.

A new technique was utilized to determine the status and trends of oyster populations in Galveston Bay, Texas. An acoustic profiler was used to differentiate substrate type, a fathometer to assess bottom relief and a global positioning system to accurately establish position. Sediment characteristics and reefal features were interpreted from the acoustic profiler chart record according to the amount of return generated. We were able to distinguish oyster reef from mud, sand and shell hash. Occasional ground-truthing was required to distinguish reef from clam beds and coarse shell hash. The bathymetry, sediment type and geographic position data were computerized and processed for use by a Geographic Information System (GIS) to produce the maps. We used Arc/Info software to produce maps covering the majority of Galveston Bay, Trinity Bay, East Bay, and West Bay. Reefal area was compared to that determined in the late '60s and early '70s by the Texas Parks and Wildlife Department. The amount of oyster reef and oyster bottom recorded in this study is substantially higher than that depicted on the TPWD charts. Differences can be attributed to our improved methodology and new reef formation in the 20 yr since the TPWD study was completed. The oyster reefs of Galveston Bay can be divided into naturally occurring reef and reef that has originated through man's influence. In many areas of the bay, reefs originated through man's influence (e.g. spoil banks, oil and gas field development, oyster leases, modifications in current flow) account for 80 to 100% of the entire reefal area.

**GENETIC STRUCTURE OF BRACKISH WATER CLAMS (*RANGIA* SPP.).** David W. Foltz,\* Department of Zoology & Physiology, Louisiana State University, Baton Rouge, LA 70803-1725; Shane K. Sarver, Rosenstiel School of Marine & Atmospheric Science, University of Miami, Miami, FL 33149-1098.

Two congeneric species of brackish-water clams that are sympatric in the northern Gulf of Mexico, *Rangia cuneata* (Sowerby, 1831) and *Rangia flexuosa* (Conrad, 1839), were analyzed for variation at 20 allozyme loci. The genetic distance between Atlantic and Gulf Coast populations of *R. cuneata* was relatively small (Nei's unbiased genetic distance was 0.065), so there was no evidence for the existence of a complex of sibling or semi-species. *R. cuneata* has greatly increased its abundance in the mid-Atlantic coast of the USA within the last 100 years, due either to colonization from southern populations or to expansion of indigenous populations. Either of the above processes might have been expected to result in lower genetic variation in Atlantic coast populations of *R. cuneata* compared to Gulf coast ones, but heterozygosity did not vary significantly across the sampled range of this species. Also, the average variance in allele frequency across *R. cuneata* populations, measured by Wright's  $F_{ST}$  statistic, was not significantly greater than in *R. flexuosa*, whose sampled range was

much smaller and restricted to the northern Gulf of Mexico. Whether the lack of pronounced differentiation along the sampled range of *R. cuneata* is due to high levels of gene flow among long-established indigenous populations or to recent expansion of southern populations into the mid-Atlantic coastal region cannot be determined from available data.

**A COMPARISON OF METHODS FOR IDENTIFYING MOLLUSCAN HEMOCYTES.** Susan E. Ford\* and Kathryn A. Alcox, Rutgers University, Institute of Marine and Coastal Sciences, Haskin Shellfish Research Laboratory, Box B-8, Port Norris, NJ 08349.

There is much disagreement over the number of hemocyte sub-populations in bivalve molluscs. Uncertainty arises because of differences in definition among researchers as well as variability associated with location, season, and health status among individuals. We compared three methods for identifying hemocyte sub-populations in eastern oysters: light microscopy (description and size), Coulter counter (size), and flow cytometry (relative size and density, and fluorescent staining).

Hemolymph from the adductor muscle of individual oysters was examined by each method. Three types of granular hemocytes (large and small refractive [highly granular]; and non-refractive [few granules]); agranular hemocytes; and small cells with almost no cytoplasm ("mostly nuclei") were identified by microscopy. In samples measured by Coulter counter, a maximum of two "population" peaks was recorded—primarily in oysters with a high proportion of granular hemocytes. Single peaks were more likely to be associated with a high proportion of agranular hemocytes.

On the flow cytometer, forward light scatter estimates of size never showed more than one clear peak and frequently displayed none at all. Ninety-degree light scatter (log scale), a measure of density or granularity, showed a maximum of two clear peaks. Three populations, however, were usually present when forward scatter was plotted against 90° scatter. The two major groups represented granular and agranular cells. The third, a group of small very dense cells, were probably the "mostly nuclei" group. Using acridine orange, a fluorescent dye that stains granules red and nuclei green, we were able to distinguish between granular and agranular cells. We are as yet unable to clearly differentiate among the three granular hemocyte types.

**IDENTIFICATION OF A SUMMER MORTALITY-RESISTANT POPULATION OF BLUE MUSSELS IN THE MAGDALEN ISLANDS (QUÉBEC, CANADA).** Jean Gaudreault and Bruno Myrand,\* Direction de la Recherche Scientifique et Technique, MAPAQ, Cap-aux-Meules, Canada G0B 1B0.

Spat from 4 different Magdalen Islands' populations were placed in pearl-nets and transferred to 5 growing sites in November '89. In June '90 some of these mussels were placed in mesh cages (6 cages per stock-site combination) at an initial density of 50 ind./cage. During the next 2 years cages were changed monthly

between June and November to minimize fouling. On these occasions, dead and live mussels were counted and measured. In November, cages from each of the 20 combinations were randomly selected and some of the mussels used for length-flesh dry wt and length-shell wt regressions.

There were only minor differences between the sites compared to the huge stock effects caused by differential survival rates. All sites combined, stocks from the Amherst lagoon (BHA) and from the Bay of Pleasant (BP) exhibited higher survival rates (92.3% and 87.4% respectively) than stocks from the Great Entry (GE) and the House Harbour (HAM) lagoons (21.2% and 41.8% respectively) in November 1990. In November 1991 only mussels from BHA maintained a high survival rate of 82.1%. The survival rate of the 3 other stocks ranged between 5.7% and 11.1%. Thus mussels from the BP stock experienced a mass mortality at all sites during their second year in cages.

The estimation of the commercial production per cage reflected closely these highly variable survival rates. For example, the commercial production was almost 10 times higher in BHA cages (170.7 g/cage) than in GE ones (17.4 g/cage) in November 1990 and 7 times higher in November 1991 (439.3 g/BHA cage vs 62.3 g/GE cage). So, this experiment clearly demonstrates that the use of spat from BHA in state of the currently used GE and HAM ones could provide a cheap and easy solution to the summer mortality problem.

**REPRODUCTION OF SEA SCALLOPS (*PLACOPECTEN MAGELLANICUS*) AND ISLAND SCALLOPS (*CHLAMYS ISLANDICA*) IN THE MAGDALEN ISLANDS.** M. Giguere,<sup>1</sup> G. Cliche,\*<sup>2</sup> and S. Brulotte,<sup>1</sup> <sup>1</sup>Institute Maurice-Lamontagne, Ministère des Pêches et Océans, Mont-Joli, Québec, Canada G5H 3Z4, <sup>2</sup>Ministère de l'Agriculture, des Pêcheries et de l'Alimentation, Îles-de-la-Madeleine, Québec, Canada G0B 1B0.

The culture of scallops requires an adequate knowledge of the reproductive cycles of the indigenous scallop species in order to optimize the spat supply. The reproductive cycles of sea scallops and Island scallops were studied over an 18 month period in the Magdalen Islands by periodically examining the gonosomatic index and by histological staging. Sea scallop spawning is concentrated between the end of August and the middle of September in the lagoon and on their natural beds. On the Island scallop bed spawning occurs from July to September. The histological sections indicate gonad ripeness from June onward for the two species, but also reveal differences in gamete development which would lend to optimizing the collection of sea scallop spat.

**POTENTIAL OF HEMOCYTES TAKEN FROM VARIOUS BODY LOCATIONS OF THE EASTERN OYSTER TO INTERACT WITH FOREIGN MATERIALS.** Dale S. Mulholland and Frank E. Friedl,\* Department of Biology, University of South Florida, Tampa, FL 33620.

The immune system of *Crassostrea virginica*, as that of other

molluscs, depends on innate, primitive mechanisms, rather than on antibodies. A major defense against bacterial and other invasion thus rests on the capacity of hemocytes to phagocytose, encapsulate, and/or remove foreign particles and organisms.

For this study, hemocytes were withdrawn from hemolymph sinuses, and extracted from shell liquor and solid tissues, then allowed to contact a "lawn" of fluorescent microspheres. Hemocytes from all body regions were capable of attachment and/or phagocytosis, but extent of this activity, scored by using an epillumination fluorescence microscope, varied markedly according to the source of the hemocytes. About 29% of hemocytes from the adductor sinuses and 23% from the pericardial cavity phagocytosed spheres, whereas less than 10% of those from the body wall did so. Those from gills and palps scored about 13%, hemocytes from the mantle surface 16%, and those from the mantle edge and the shell liquor about 18%. These results suggest that hemocytes differ in their potential to respond to non-self depending on their locations within, upon, or about the animal.

**ECOSYSTEM MONITORING STUDIES IN COASTAL GEORGIA.** F. X. O'Beirn,\* Shellfish Research Laboratory, Marine Extension Service, University of Georgia, P.O. Box 13687, Savannah, GA 31416-0687; P. B. Heffernan, Martin Ryan Marine Science Institute, University College Galway, Galway, Ireland; R. L. Walker, Shellfish Research Laboratory, Marine Extension Service, University of Georgia, P.O. Box 13687-0687, Savannah, GA 31416.

As part of an ecosystem monitoring program at the Sapelo Island Estuarine Research Reserve, oyster recruitment was estimated at three sites on the Duplin River, Georgia. Samples were taken on a monthly basis for the duration of the spawning season (May–October). At two of the three sites, hydrological data (temperature, salinity and pH) were also gathered on a continuous basis. Allied to this, the data collected was also compared to data retrieved from a concurrent study in Wassaw Sound, Georgia, some sixty miles north of the Sapelo system. Recruitment was first recorded in Wassaw Sound at the end of May and in the Duplin River at the end of June. Peak recruitment occurred in August at both sites. Overall, the highest recruitment recorded was at the site adjacent to Sapelo Sound on the Duplin River, with 628 spat/0.01 m<sup>2</sup> collected on one collector. Recruitment was highly variable at both locations. From a managerial or commercial perspective, the results of the monitoring indicate that precise preliminary studies need to be carried out before any successful conservation policies or programs of natural spat collection should be implemented.

**TOXIC DIATOMS IN WESTERN WASHINGTON WATERS.** James R. Postel\* and Rita A. Horner, School of Oceanography, University of Washington, Seattle, WA 98195.

A new marine biotoxin, domoic acid, was discovered in the fall of 1987 when more than 100 people became ill after eating cultivated mussels from Prince Edward Island, eastern Canada. For the

first time, a diatom, *Pseudonitzschia pungens* (Grunow) Hasle f. *multiseries* (Hasle) Hasle was identified as the toxin producer. Domoic acid killed brown pelicans and cormorants in Monterey Bay, central California, in September, 1991. The toxin was traced through the bird's food source, anchovies, to a bloom of another diatom, *P. australis* Frenguelli.

In late October, 1991, razor clams living in the surf zone on Pacific coast beaches in Washington and Oregon contained domoic acid and the commercial and recreational harvest was closed. Domoic acid levels were still high in May 1992. Other molluscan shellfish, including oysters grown commercially in coastal embayments, and mussels, were not toxic. However, the important commercial fishery for Dungeness crabs in California, Oregon, and Washington was closed in early December 1991 when domoic acid was found in their viscera.

Several species of *Pseudonitzschia* have now been found in western Washington marine waters including Puget Sound. While domoic acid levels have been high in razor clams and crabs on the open coast, levels in commercial bivalves have not been high enough to cause closures in the inland waters of Puget Sound. Photographs are presented to enable observers to distinguish among several forms of *Pseudonitzschia* that have been present in these waters at least since 1990. Some data on their distributions are also shown.

**CLAM PRODUCTION IN IRELAND.** Elizabeth T. Rice, Point Whitney Shellfish Laboratory, 1000 Point Whitney Rd., Brinnon, WA 98320.9899.

The two clam species currently of commercial importance in Ireland are: (a) the Native clam—*Tapes decussata* and (b) the Manila clam—*Tapes semidecussata*.

#### THE NATIVE CLAM

Most of the wild populations of native clam have arisen due to sporadic settlements. Harvesting of clams in the past has largely been on the West and North-West coasts of Ireland. However the populations are very susceptible to overfishing and recover very slowly. As they fetch a high price in the market place, attempts have been made to culture them. To date these attempts have not been very successful. New stocks of a related species also exist but they have not been exploited commercially.

#### THE MANILA CLAM

In recent years, there has been increasing interest in the cultivation of an exotic species, the Manila clam, in Irish coastal waters. Initial culture trials showed much faster growth and better survival of these clams. Due to high prices for the Manilas in the late 1980's (IR£6/kg), a number of firms invested, so projects were initiated all around the coast of Ireland.

In Ireland, unlike many other European countries, there is no natural settlement of the Manila clam, so all seed requirements must be obtained from hatcheries.

The culture of the Manila therefore involves: production or purchasing of seed in or from hatcheries, holding in a nursery system

for about a year and the planting out of clams, at the beginning of their second year, onto sheltered shores.

Strains of Manila clam are now been produced in Ireland which resemble closely both the shape and colour of native clams.

**FLOW CYTOMETRIC ANALYSIS OF HISTOZOIC PERKINSUS MARINUS CELLS.** Bob S. Roberson\* and Tong Li, Department of Microbiology, University of Maryland, College Park, MD 20742; Christopher F. Dungan, Maryland DNR, Co-operative Oxford Laboratory, Oxford, MD 21654.

Methods developed for analysis of fluorochrome-labeled *Perkinsus marinus* cells in estuarine water samples were adapted for diagnostic analysis of infected oyster tissues by flow cytometry. Both hemolymph and visceral tissue homogenates from infected oysters whose infection status had been previously determined by traditional fluid thioglycollate medium assays, were analyzed. Prior to flow cytometry, oyster tissues or homogenates were subjected to enzymatic digestion, differential centrifugation, and double fluorochrome staining. Fluorescein labeling of pathogen cells was accomplished using specific antibodies; propidium iodide labeling of DNA was accomplished in the presence of RNAase. Pathogen cells were discriminated using characteristic ranges for the cytometric parameters of fluorescein and propidium iodide fluorescence intensities, size (forward angle light scatter), and cellular complexity (90° light scatter). Fluorescence activated sorting (FACS) of cell populations recognized as *P. marinus* permitted microscopic comparison of sorted cell morphologies to those of immunostained pathogen cells in histological sections of infected oyster tissues. Enzymatic treatment of sampled pathogen cells did not significantly compromise the intensity of antibody labeling; and sorted pathogen cell morphologies represented the entire range of cell morphotypes labeled *in situ*.

**COMPARISON OF 16S-LIKE rDNA OF CRASSOSTREA VIRGINICA AND HAPLOSPORIDIUM NELSONI.** Nancy A. Stokes\* and Eugene M. Burrenson, Virginia Institute of Marine Science College of William and Mary, Gloucester Point, VA 23062.

The life cycle of the oyster pathogen *Haplosporidium nelsoni*, or MSX, has yet to be elucidated, thus hindering laboratory research and development of disease management. We have purified genomic DNA from *C. virginica* and *H. nelsoni* in order to exploit DNA technology for the identification of MSX. The polymerase chain reaction (PCR) was employed to amplify 16S-like rDNA from the genomic DNAs by utilizing primers that are complementary to conserved regions of eukaryotic 16S-like rDNA. We obtained PCR products of approximately 1800 base pairs, which were cloned into plasmid vectors. Through restriction endonuclease analysis several enzymes were found that cut the 16S-like rDNA at one or two sites, yielding fragments which were suitable for subcloning. Dideoxysequencing was performed on all the clones and the 16S-like rDNA of both oyster and MSX was char-

acterized and compared to other small subunit RNA sequences in GenBank. The variable regions of MSX which are non-conserved are currently being tested for specificity and sensitivity as suitable hybridization probes for pathogen identification.

**AGE, GROWTH RATE, AND SIZE OF THE SOUTHERN SURF CLAM, *SPISULA SOLIDISSIMA SIMILIS* (SAY, 1822).** R. L. Walker,\* Shellfish Research Laboratory, Marine Extension Service, University of Georgia, P.O. Box 13687, Savannah, GA 31416-0687; P. B. Heffernan, Martin Ryan Marine Science Institute, University College Galway, Galway, Ireland.

The age, growth rate, and size of the southern surf clam, *Spisula solidissima similis*, was determined by shell sectioning techniques for clams collected from beach drift off Wassaw Island, Georgia (Atlantic coast) and Cape San Blas, St. Joseph Bay, Florida (Gulf of Mexico coast). The shell sectioning results for the Georgia population was validated by analysis of monthly size-frequency data for a field population collected from St. Catherines Sound, Georgia. The southern surf clam deposited a single age band during the summer months at both sites. A distinct alternating pattern of translucent to opaque to translucent zones in the shell was evident for clams from both sites. The translucent zone is formed from May to October while the opaque zone is formed from November to April. The annual band occurs within the translucent zone. According to the von Bertalanffy growth regressions, maximum size estimates of 76 mm and 135 mm for Georgia and Florida surf clam populations, respectively, are predicted. In Georgia, surf clams obtained a maximum shell length of 74 mm and were aged to a maximum of 4 years, as compared to 106 mm in shell length and 5.5 years for clams from Florida. In Georgia, the majority of surf clams (92%) collected from beach drift lived to a mean age of 1.5 years; whereas, clams from Florida tended to survive to a mean age of 3.5 years. Clam cohorts collected from St. Catherines Sound grew to 48 mm in 1990 and 47 mm in 1991 in 1.5 years before dying. Southern surf clams from Georgia were found to differ in age, growth rate, and size from a population from the Gulf coast of Florida, and both greatly contrasted from that of the northern surf clam, *Spisula solidissima* which grow to 226 mm and has a lifespan of 37 years.

**SUSPENSION-FEEDING MECHANISMS IN BIVALVES: RESOLUTION OF CURRENT CONTROVERSIES USING ENDOSCOPY.** J. Evan Ward,\*<sup>1</sup> Peter G. Beninger,<sup>2</sup> Bruce A. MacDonald,<sup>1</sup> and Raymond J. Thompson,<sup>3</sup> <sup>1</sup>Department of Biology, University of New Brunswick, Saint John, NB, Canada E2L 4L5; <sup>2</sup>Département de biologie, Université de Moncton, Moncton, NB, Canada E1A 3E9; <sup>3</sup>Marine Sciences Research Laboratory, Memorial University of Newfoundland, St. John's, NF, Canada A1C 5S7.

The mechanism of suspension-feeding in bivalves has been the subject of controversy for several years. The debate centers around whether particle processing is accomplished via mucociliary or hydrodynamic action. Evidence for and against these two processes has previously been based on studies of isolated structures and dissected specimens.

In recent years, endoscopic examination and video image analysis has enabled *in vivo* observations of the pallial organs of intact bivalves. Using this technique, we studied the feeding processes on gills of four species of bivalves, and discovered that the two currently debated hypotheses regarding feeding are not mutually exclusive and can be combined into a unifying model. Both mucociliary and hydrodynamic mechanisms function concurrently at different sites on the gill, thereby optimizing particle transport and minimizing particle loss. The importance of mucus in the normal feeding process of bivalves was confirmed. These novel findings refute results of previous studies that have used surgically invasive techniques, and emphasize the importance of making observations on morphologically intact specimens.

**PRODUCTION OF DOMOIC ACID BY *PSEUDONITZSCHIA AUSTRALIS* ISOLATED FROM THE SOUTHWESTERN OREGON COAST FOLLOWING AN ASP OUTBREAK IN FALL 1991.** Sheree J. Watson, and Nicole M. Apelian. Oregon Institute of Marine Biology, University of Oregon, Charleston, OR 97420.

The first detection of domoic acid on the West coast was in September of 1991 in Monterey Bay, California. Domoic acid was traced through the deaths of pelicans and cormorants to ingestion of anchovies whose gut contents contained diatom valves of *Pseudonitzschia australis*. *P. australis* has since been identified as a domoic acid producer in cultures isolated from the Monterey Bay bloom.

In the fall of 1991 domoic acid was detected in razor clams in the surf zone off Oregon and Washington coasts. Levels remained high throughout the spring. Water samples from the Coos Bay area in December 1991 and January 1992 detected no dominant taxa in the phytoplankton community. Since then, one of the cultures raised from isolations made in December of 1991 in the Coos Bay area has tested positive for domoic acid production. Preliminary light microscopy identification has indicated the culture producing domoic acid is *P. australis*. Further testing is in process to test other cultures isolated from this area for domoic acid production. A major ASP outbreak has not occurred in Oregon since 1991, and thus we cannot verify in retrospect whether *P. australis* identified here is responsible for the domoic acid production in the 1991 outbreak, but these results do suggest that the responsible organism is the same as the Monterey Bay species.



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**COVER PHOTO:** Northern shrimp, *Pandalus borealis*, Krøyer, 1878. Photo by Jim Rollins.

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## MYSTERIOUS DEMISE OF SOUTHERN CALIFORNIA BLACK ABALONE, *HALIOTIS CRACHERODII* LEACH, 1814

GARY E. DAVIS

Abalones, large gastropod mollusks of the genus *Haliotis*, inhabit coastal waters worldwide. Many people consider them gastronomic delicacies: the Forum restaurant in Hong Kong had no lack of customers for \$400 (US) abalone dinners in 1991 (Cross 1991). Massive midden deposits in southern California attest to extensive use of abalones by aboriginal peoples for several thousand years. The modern California fishery began in the mid-nineteenth century with exploitation of intertidal black abalone, *H. cracherodii*, by Chinese immigrants. By 1879, annual harvest approached 2,000 metric tons (mt). Socio-economic factors shifted harvest to subtidal species in 1900. After serially depleting four subtidal species (*H. corrugata*, *H. rufescens*, *H. fulgens*, and *H. sorenseni*), the fishery returned to black abalone in 1968. Black abalone bolstered generally failing commercial landings in the early 1970's with nearly 900 mt a year, but the relief lasted only

a few years. In the mid-1980's, southern California black abalone fishery landings began to decline, populations dwindled, and abalone began dying mysteriously. In less than five years, black abalone that had dominated rocky intertidal zones at densities of more than 100 m<sup>-2</sup> virtually disappeared from most of their former range south of Pt. Conception.

Southern California yielded thousands of tonnes of abalones annually for over a hundred years. In spite of the application of modern fishery management practices based on considerable knowledge of abalone biology, black abalone populations crashed! What happened? Why did populations that sustained centuries of harvest suddenly collapse? Did the environment change from pollution or habitat alteration? Did the massive 1982-83 el Niño trigger a cascade of ecological effects? Did disease sweep through the dense aggregations? Were reproductive stocks finally reduced



Figure 1. A black abalone *Haliotis cracherodii* from the California Channel Islands with symptoms of the Wasting Syndrome which is responsible for the population collapse there claiming over 90% of the populations since 1985.

beyond their capacity to replace annual harvest? Could it happen elsewhere?

This collection of papers describes patterns of the recent population collapse (Richards and Davis, VanBlaricom et al.), population trends from 1975–91 (Miller and Lawrenz-Miller), and an investigation of a coecidian parasite suspected of causing abalone mortality (Friedman et al.). Together with a description of with-

ering syndrome (Haaker et al. 1992) and tests of epidemiological hypotheses (Lafferty and Kuris in press), these papers constituted the core of a symposium held at the Western Society of Naturalists annual meeting in 1991 to raise awareness in the scientific community about the situation. The ultimate cause(s) of the population collapse are still unknown, in spite of considerable efforts by many scientists, fishery managers, and abalone harvesters.

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## DISCOVERY OF WITHERING SYNDROME AMONG BLACK ABALONE *HALIOTIS CRACHERODII* LEACH, 1814, POPULATIONS AT SAN NICOLAS ISLAND, CALIFORNIA

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**ABSTRACT** We report the first discovery, in April 1992, of abalone withering syndrome (WS) among intertidal populations of black abalones (*Haliotis cracherodii* Leach) at San Nicolas Island (SNI), California. Small samples of apparently healthy and apparently diseased individuals were collected from SNI and examined in the laboratory. Epizootic suctorian protozoans, renal coccidia, sporocysts of gregarine protozoans, and foci of rickettsia-like prokaryotes were found in subject abalones, but none could be definitively implicated as the cause of WS symptoms. Pathologies of apparently diseased abalones were limited and unremarkable, and virological studies were negative. WS symptoms in SNI abalones remain unexplained, although a possible role for toxic contaminants has not been ruled out. Field surveys for WS were conducted at SNI during spring and summer 1992. WS was present at most sites at low frequency (6% or less). WS was not observed along the northeast quadrant of the SNI shoreline. Initial activity of WS at SNI appeared to be concentrated at the west end of the island. Rate and pattern of spreading around the island were unclear because of generally low WS frequencies.

**KEY WORDS:** black abalone, California, *Haliotis cracherodii*, San Nicolas Island, withering syndrome

### INTRODUCTION

Black abalones (*Haliotis cracherodii* Leach) are common in the intertidal zone at San Nicolas Island (SNI), California. Populations are strongly aggregated in patches with local densities often ranging from 10 to 100 individuals/m<sup>2</sup>. Black abalone populations have been relatively stable at SNI since at least 1981 despite commercial and recreational fisheries and the reintroduction of natural predators (Rathbun et al. 1990, VanBlaricom in press).

Abalone withering syndrome (WS) is characterized by atrophied pedal musculature, epipodial discoloration, and diminished responsiveness to tactile stimuli. Affected individuals suffer abnormally high mortality rates as a result of the syndrome, and possibly as a result of greater vulnerability to dislodgement by waves or attack by scavengers and predators (Haaker et al. 1992). The cause of WS is unknown. WS was first recognized at Anacapa

Island, California, in 1986 (Haaker et al. 1992). To date, WS appears to be confined to California waters.

The development and spread of WS has threatened black abalone populations and fisheries on the islands off southern California. Afflicted populations have experienced high mortalities, sometimes approaching local extinction, over periods as brief as a few months (Tissot 1991, Davis et al. 1992, Haaker et al. 1992). By December 1991 WS had been observed at all of the southern California islands except SNI and Santa Catalina. To our knowledge there have been no efforts to date to search for WS at Santa Catalina, where densities of black abalones were low, compared to the other southern California islands, prior to the development of WS (Haaker, personal communication). The apparent absence of WS at SNI was reported by one of us (GRVB) at the 1991 Annual Meeting of the Western Society of Naturalists in Santa Barbara, California. The finding was encouraging, raising the possibility of

unafflicted source stocks for future restoration of black abalone populations at other islands.

VanBlaricom (in press; unpublished data) studied dynamics of dense black abalone populations in nine permanent study sites (Fig. 1) at SNI from winter 1981 through winter 1992. More than  $2 \times 10^5$  black abalones were examined during the study. Evidence of WS was not observed, although a few animals ( $N_{\text{total}} < 10$ ) were found in weakened condition over the span of the study. Haaker (personal communication) specifically searched for diseased abalones at a rocky intertidal site near the extreme western end of SNI in April 1990, 1991, and 1992 (Fig. 1). None of the searches produced evidence of the presence of WS.

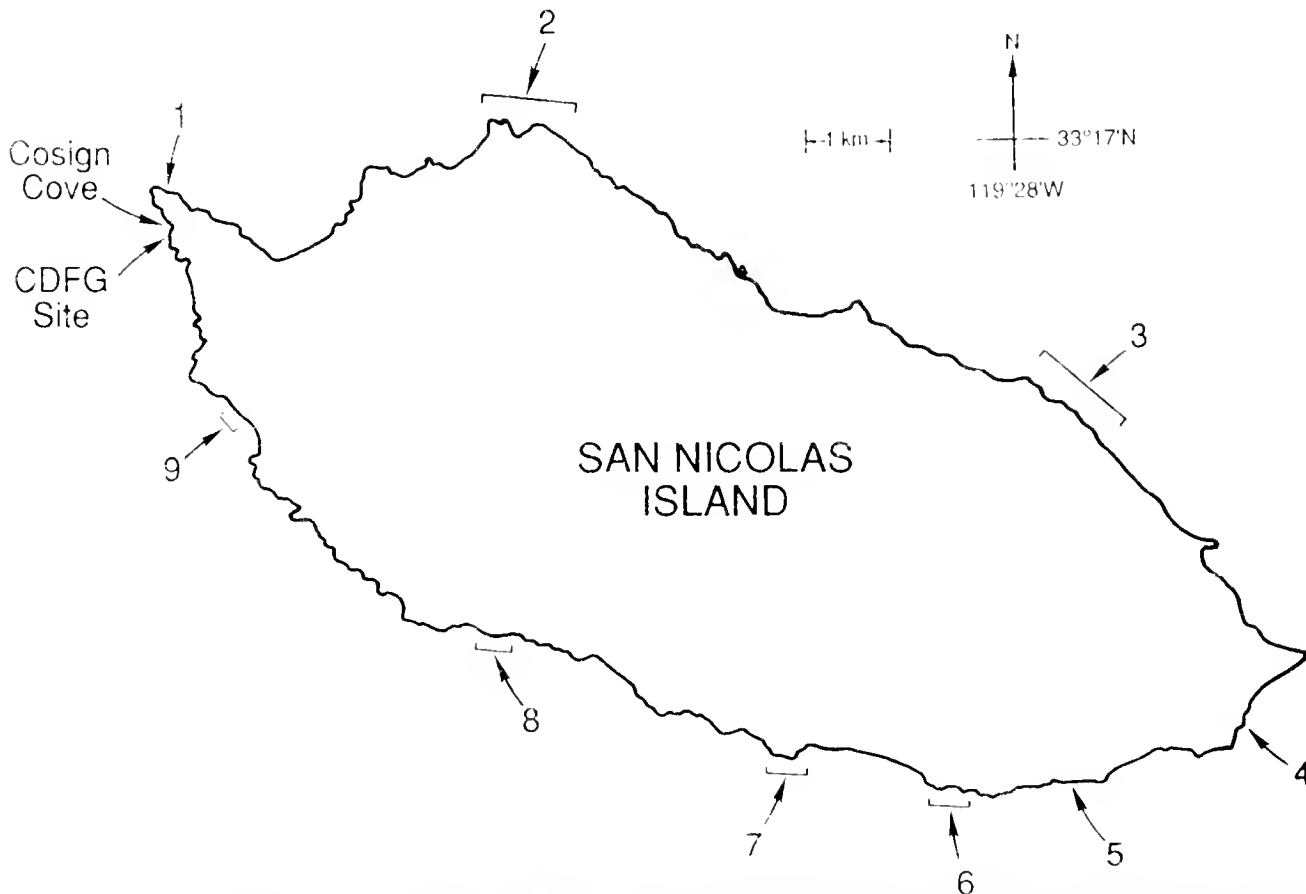
On 10 April 1992 two of us (JLR & DDW) examined 225 arbitrarily selected black abalones in a rocky intertidal area known informally as Cosign (not Cosine) Cove (Fig. 1), located about 200 m north of Haaker's site. Thirty-five (15.6%) of the abalones were found to have symptoms of WS. Here we describe the results of laboratory examinations of apparently diseased and apparently healthy black abalones from SNI. In addition, we present results of field surveys of the frequency and distribution of WS at SNI in spring and summer 1992.

#### MATERIALS AND METHODS

Nine apparently diseased (ADBA) and five apparently healthy (AHBA) black abalones were collected at Cosign Cove on 10 April 1992 and were sent to the Fish Pathology Laboratory of the University of California at Davis. Abalones were weighed (total

[tw] and shell [sw] weights, in gm), measured (maximum shell diameter [ $d_m$ ], in mm), and sexed. Mantle scrapings and gill squashes were observed by phase-contrast microscopy. Selected tissues from six ADBA and three AHBA were placed in Davidson's solution (Shaw and Battle 1957) and processed for routine paraffin histology. Deparaffinized  $5 \mu$  sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. Tissues from two AHBA and three ADBA, the latter with advanced clinical symptoms of WS, were stored at  $-70^\circ\text{C}$  for virological studies. Subsequently, tissues from the stored AHBA and ADBA were separately pooled and processed for routine virology (Amos 1985). Cell lines from brown bullhead, bluegill fry-2, and epithelioma papillosum cyprini were individually inoculated with homogenates from each pool and stored at  $20^\circ\text{C}$  for 2 weeks, after which all cells were passed blindly and incubated as above for 5 weeks. Cells were screened for cytopathic effects once or twice weekly.

During the late spring and summer of 1992 we searched for quantitative evidence of the distribution and frequency of WS at eight of VanBlaricom's (in press) nine permanent study sites at SNI (Fig. 1; site 8 was not sampled because of the presence of breeding pinnipeds). We used two methods, the removal test and the pull test. In the removal test, pry tools were used to detach abalones from the rock surface in arbitrarily selected patches. Detached animals were assessed for responsiveness, epipodial color, and for the extent to which the foot filled the shell aperture. Individuals were scored as WS victims if a) epipodial color (nor-



**Figure 1.** Map of study locations at San Nicolas Island (SNI), California. Numbered locations are the long-term study sites of VanBlaricom (in press). "CDFG Site" is the study location of Haaker (see text). Cosign (not Cosine) Cove is the site of initial discovery of withering syndrome among black abalone populations at SNI.

mally black) was faded; b) the animal did not attempt to right itself; c) the animal was unresponsive to tactile stimuli, and d) the foot filled <75% of the shell aperture. In the pull test, shells of arbitrarily selected individuals were gently tapped, stimulating maximum adhesion to the rock surface. After a pause of several seconds, an effort was made to pull the abalones from the substratum by hand without pry tools. Individuals afflicted with WS generally can be detached easily by hand, while healthy abalones cannot be detached. Based on our extensive field experience with black abalone studies, we assumed a probability of zero for successful removal of healthy abalones from the substratum with the pull test.

**RESULTS**

The average condition (C) of the abalones ( $C = \{tw - sw\}/tw$ ) did not differ between AHBA ( $C = 0.52$ ) and ADBA ( $C = 0.53$ ). However, slopes of bivariate plots of  $tw$  and  $d_m$  differed substantially between groups (AHBA:  $tw = (7.74 \{d_m\}) - 634$ ;  $r^2 = 0.89$ ; ADBA:  $tw = (4.04 \{d_m\}) - 263$ ;  $r^2 = 0.95$ ). In addition, pedal muscles of ADBA were visibly atrophied relative to those of AHBA. Although sample size was too small for legitimate statistical analysis, differences in bivariate plots suggested that AHBA typically weigh more per unit length than ADBA. This relation will be tested with additional study.

Phase-contrast microscopy of gill squashes and mantle scrapings from three ADBA revealed the presence of numerous suetorian protozoans. One AHBA also had suetorian protozoans on the gills. However, numbers of suetorians were insufficient in any sample to account for the clinical symptoms of the ADBA. Coccidia, endemic to California waters (Friedman 1990, Haaker et al. 1992, Steinbeck et al. 1992), were observed within both nephridia of all abalones examined. Sporocysts of gregarine protozoans were observed within gill, muscle, kidney, or digestive tissues of ADBA and AHBA. Foci of rickettsia-like prokaryotes were observed within epithelia of the digestive tract of two ADBA. Cytopathological changes in abalones associated with these organisms were limited to hypertrophy of the infected cells. The protozoan and prokaryotic organisms found in the abalones are commonly observed in invertebrates of California waters (Friedman et al. 1989, Friedman 1990, Haaker et al. 1992). Field and laboratory studies suggest that the renal coccidian is not pathogenic to abalones (Friedman 1990, Friedman et al., in preparation, Haaker et al. 1992). The pathogenicities of the rickettsia-like prokaryotes and gregarine protozoans in abalones are unknown.

Pathological changes in abalone tissues were limited to autolysis of the digestive gland in four of six ADBA, and increased numbers of serous (brown) cells in muscle tissues of ADBA as compared with AHBA. In all AHBA and half of the ADBA serous cells were localized primarily within the large and small hemal sinuses of the pedal muscle. However, we observed brown cells dispersed throughout the foot muscle in three of six ADBA. Infiltration of hemocytes and formation of brown cells in affected tissues have been observed in oysters with advanced infections of *Perkinsus marinus* (Mackin 1951), and in oysters recovering from Delaware Bay Disease (Farley 1968). Both pathological changes were observed in only two ADBA. Autolysis of the digestive tissues may indicate extreme morbidity, or may be an artifact of fixation (Luna 1968). Because of the extremely weak and atrophied condition of the ADBA, the homogeneous autolysis of the digestive gland of the ADBA, and the lack of autolysis of digestive tissues of AHBA, we attribute observed autolysis to extreme morbidity. No other parasites or pathological changes were observed in stained tissue sections.

Virological studies produced entirely negative results. No cytopathologic effects were observed in any cells inoculated with homogenates from AHBA or ADBA.

We found evidence of WS at sites 1, 2, 5, 7, and 9 at SNI (Table 1). Frequency of WS was low (6% or less) at all sites surveyed, and apparently substantially lower than the frequency observed at Cosign Cove in April 1992. Our data suggest that WS is most prevalent in abalones at the west end of SNI. Frequencies of occurrence at other sites on SNI are too low to permit confident assessment of patterns of spreading of WS.

**DISCUSSION**

All laboratory results suggest that black abalones collected at SNI were suffering from WS and not another infectious agent. The etiology of WS remains unknown. Diagnosis of WS is based on clinical symptoms and the lack of an identifiable infectious agent (Haaker et al. 1992, Friedman et al. in preparation). Toxicological analyses were not performed on the samples. Toxic contaminants may be considered a possible alternative explanation for the poor health of ADBA from SNI. At this writing (October 1992), we have been unable to return to Cosign Cove for quantitative sampling because of perceived risks of disturbance to seasonally breeding seabirds and pinnipeds.

We suggest two alternative interpretations of the field data. It is possible that WS is chronically present at low frequency in black abalone populations at SNI. Our discovery of WS in 1992 may have been a consequence of increased awareness and search effort. More likely, in our view, is the possibility that WS has appeared at SNI only recently, and was not overlooked prior to April 1992. In either case three alternative outcomes can be considered. First, it is possible that WS will not persist at SNI, and will have no measurable effect on black abalone populations. Second, WS may persist at SNI, but at frequencies too low to influence abalone population dynamics. Third, WS may increase in frequency and distribution, ultimately causing significant declines in numbers of black abalones at SNI. Data from the six California islands with recognized outbreaks of WS indicate that the third alternative is the most likely outcome (Haaker et al. 1992). The existence of a

**TABLE 1.**

**Frequencies of withering syndrome (WS) in black abalones sampled from eight rocky intertidal sites at San Nicolas Island. Percentages indicate animals with WS. Site locations are as indicated in Figure 1. All dates are in 1992. Sampling details are provided in text.**

Site	Date	Removal test		Pull test	
		N	% WS	N	% WS
1	31 July	—	—	100	1.0
2	7-8 May	61	0	—	—
2	1 August	—	—	100	1.0
3	7 May	61	0	—	—
3	31 July	—	—	100	0
4	19 May	78	0	100	0
4	1 August	—	—	100	0
5	20 May	100	1.0	100	0
6	20 May	100	0	100	0
7	6 May	111	1.8	—	—
7	2 August	—	—	200	3.0
9	7 May	78	2.6	—	—
9	31 July	—	—	100	6.0

large longitudinal data base for permanent sites at SNI, and our continued frequent sampling of the sites, will allow a quantitative assessment of the alternatives we have considered. Continued study will permit measurement of intensity and scale in the development of WS, direct linkage of the characteristics of WS with fluctuations in density of black abalones, and accumulation of additional data on the physiological and cytological characteristics and consequences of the syndrome.

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## EARLY WARNINGS OF MODERN POPULATION COLLAPSE IN BLACK ABALONE *HALIOTIS CRACHERODII*, LEACH, 1814 AT THE CALIFORNIA CHANNEL ISLANDS

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**ABSTRACT** Abundance and distributions of selected rocky intertidal organisms were monitored in fixed plots at 10 sites within Channel Islands National Park, California from 1985 to 1992. While abundances of barnacles (*Balanus*, *Tetraclita*, and *Chthamalus*), mussels (*Mytilus californianus*) algae (*Pelvetia fastigiata*, *Hesperophycus harveyanus*, and *Endocladia muricata*), and owl limpets (*Lottia gigantea*) remained relatively stable, black abalone populations declined precipitously, with less than 10% of the 1985 levels present in 1992. At the southeastern islands, in the warm waters of the Californian Province, 90% of the abalone died between 1985 and 1988, and the proportion of large individuals among survivors increased as the population declined to less than 1% of its 1985 level. In contrast, populations at the northwestern islands, in the cold water of the Oregonian Province, declined gradually, until after the southeastern islands' populations crashed and were closed to commercial harvest in 1991. The proportion of large individuals declined as abundance dropped at the northwestern islands, implicating harvest as a contributing factor in the decline there. In both Provinces, recruitment of juvenile abalone virtually ceased when adult populations dropped below 50% of their initial abundance. Withered and weak abalone were frequently observed, suggesting an infectious agent. No single cause for the mass mortalities has been found to date.

**KEY WORDS:** black abalone; *Haliotis cracherodii*; mass mortality; California

### INTRODUCTION

Black abalone, *Haliotis cracherodii*, are important structural components of rocky intertidal communities in southern California. They are slow growing, long-lived, occupy extensive areas, and constitute a large portion of the consumer biomass. These large herbivorous gastropod mollusks range from central Baja California, Mexico to southern Oregon and subsist largely on drifting fronds of giant kelp *Macrocystis pyrifera* and other algae (Cox 1962, Leighton and Boolootian 1963, Ault 1985, Douros 1987).

Black abalone have played an important role in a large and valuable California fishery for thousands of years starting with the Chumash Indians (Glassow 1980). Chinese immigrants started the modern fishery in the mid-19th century, but black abalone harvest virtually ceased when the fishery shifted to subtidal species in 1900 (Cox 1962, Cicin-Sain et al. 1977). As southern California stocks of subtidal pink, *H. corrugata*, red, *H. rufescens*, and green *H. fulgens*, abalone declined in the early 1970's, black abalone once again became a major component of the harvest, comprising 32% to 60% of the total landings from 1972–1988 (Tegner 1989, Dugan and Davis 1993).

We monitored black abalone population dynamics in Channel Islands National Park as part of a Rocky Intertidal Ecological Monitoring program (Richards and Davis 1988). This paper describes the collapse of black abalone populations at the California Channel Islands from 1985–1992.

The eight California Channel Islands lie 20–100 km off the coast in two groups of four, stretching from Point Conception to San Diego (Fig. 1). The four northern islands lie along the transition zone between two major biogeographic provinces (Seapy and Littler 1980, Murray et al. 1980). To the north and west, the Oregonian province is characterized by high biological productivity resulting from upwelling off the mainland coast at Point Conception, local upwelling around the islands, and eddies from the California Current system (Owen 1980). The Californian province, a warm temperate system, dominates the southern group of islands and extends northward during El Niño years.

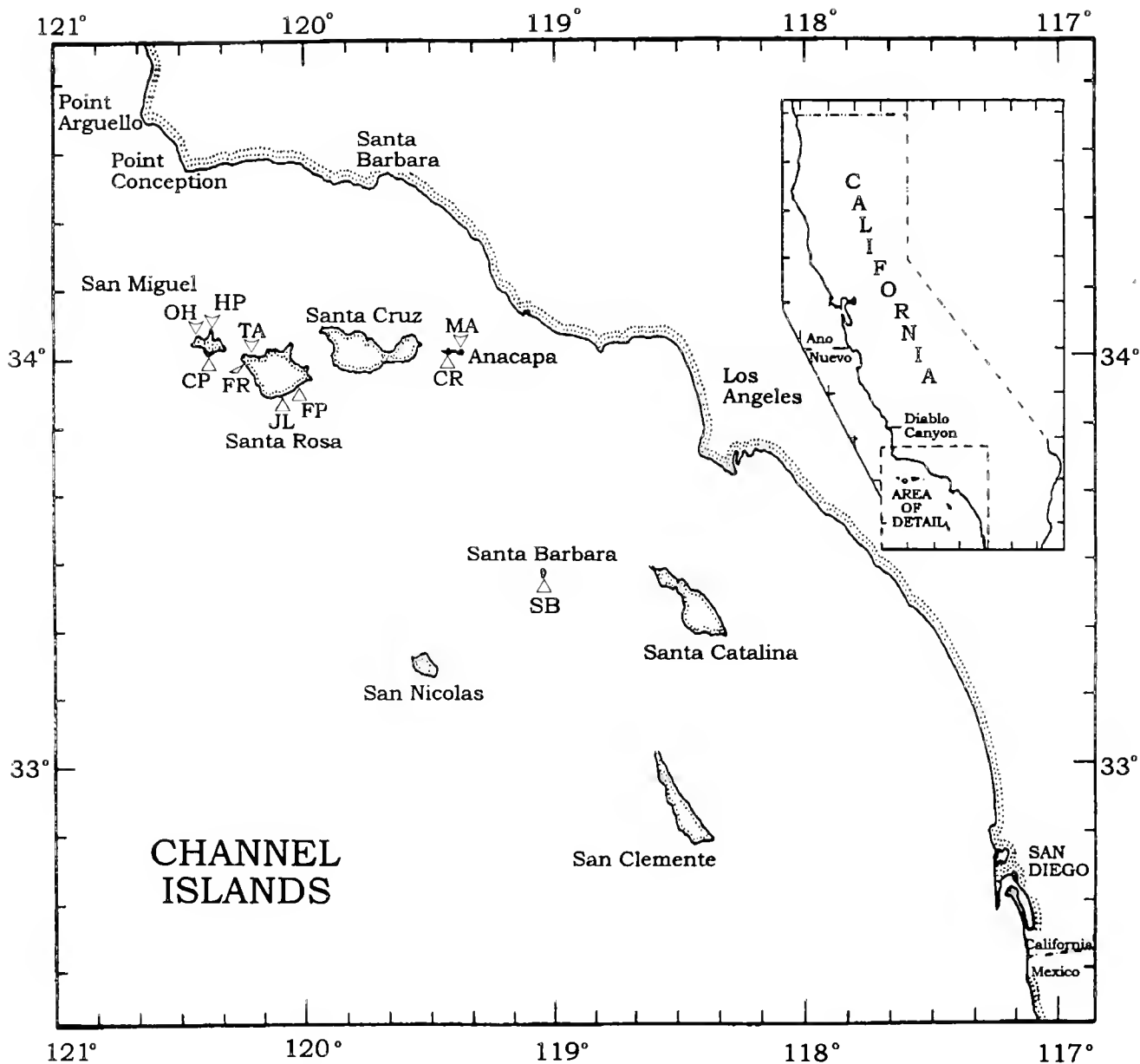
### METHODS

A variety of techniques were used to measure population dynamics of selected marine organisms as part of the long-term ecological monitoring program at Channel Island National Park (Davis 1989). Black abalone were monitored at ten sites on Anacapa, Santa Rosa, San Miguel, and Santa Barbara Islands (Fig. 1). Monitoring sites were established between 1985 and 1988 at areas selected for their high black abalone abundance.

Black abalone abundance and size distribution were measured in fixed plots. At each location, rocky habitat was stratified into areas of high and low abalone densities. Five plots were randomly chosen from 10 areas within the high density strata. The 10 areas represented nearly all the suitable habitat within a 100 m section of coast. Some plots were contiguous and none were more than 50 m apart. The 50 plots in this study ranged from 1–11 m<sup>2</sup>, and were established to assure that each plot included a minimum of 30 abalone, most plots contained about 100 abalone (Richards and Davis 1988). Plot corners were marked with bolts fixed to the rocks. During monitoring each spring (March–May) and fall (October–December), all of the abalone inside each plot were counted and measured to the nearest millimeter. Sizes are reported in four size classes; juveniles (<45 mm), adults smaller than the sport harvest limit (45–126 mm), sport harvestable adults (126–145 mm), and commercial harvestable adults (>145 mm).

Ground cover of dominant taxa was determined at each site biannually in 20, 50 × 75 cm, fixed plots distributed in four zones characterized by rockweeds, *Pelvetia fastigiata* and *Hesperophycus harveyanus*, turfweed, *Endocladia muricata*, barnacles, *Balanus*, *Tetraclita*, and *Chthamalus*, or California mussels, *Mytilus californianus*. Owl limpet, *Lottia gigantea*, abundance and sizes were monitored in fixed plots at four of the sites on Santa Rosa and San Miguel Islands. At Johnson's Lee and at Ford Point, owl limpets were monitored in five, 50 cm radius circle plots at each site. At Crook Point and Otter Harbor, owl limpets were monitored within three abalone plots at each site.

At four sites where abalone shells noticeably accumulated on



#### OREGONIAN PROVINCE

OH - Otter Harbor  
 HP - Harris Point  
 CP - Crook Point

#### TRANSITION ZONE

TA - Talcott  
 FS - Fossil Reef  
 JL - Johnson's Lee  
 FP - Ford Point

#### CALIFORNIAN PROVINCE

CR - Cat Rock  
 MA - Middle Anacapa  
 SB - Santa Barbara Island

Figure 1. California Channel Islands black abalone study sites censused 1985–1992.

the beach (Cat Rock, Talcott, Fossil Reef, and Harris Point), abalone shells were counted and removed from the beach to document on-site mortality. Shells were measured, and freshness and marks indicating predation were noted.

#### RESULTS

Table 1 summarizes seasonal abalone abundance in the fixed plots from 1985 through 1992. Ninety percent of the black abalone initially present were gone by 1992 at all sites. Only sites on San

Miguel Island still had appreciable black abalone populations at the end of 1992.

While black abalone populations crashed, other elements of the intertidal community showed little change. Rockweeds and turf-weed covered about 50% of the rock surfaces, varying from 25% to 85% among sites. Algal cover appeared relatively stable within normal limits of variation at most locations. California mussel abundance declined at Harris Point, Johnson's Lee, and Middle Anacapa. Predation from a dramatic increase in *Pisaster* sea star

TABLE 1.  
Average density of black abalone (per square meter) in fixed plots, 1985–1992.

	Season and Year <sup>a</sup>															
	S'85	F'85	S'86	F'86	S'87	F'87	S'88	F'88	S'89	F'89	S'90	F'90	S'91	F'91	S'92	F'92
Californian province																
Cat Rock	27.4	24.4	19.1	17.2	15.2	9.8	6.3	3.1	2.2	0.8	0.3	0.2	0.1	0	0	0
Middle Anacapa	74.2	77.5	68.7	56.4	42.3	13.7	7.8	3.4	0.9	0.5	0.1	0.2	0.2	0.2	0.2	0
Santa Barbara Is	9.2	8.6	10.4	8.7	10.1	9.5	8.8	2.8	1.3	0.4	0.2	0.1	ND <sup>b</sup>	0	<0.1	0
Transition Zone																
Ford Point		34.7	28.2	25.5	13.0	4.5	1.7	1.0	0.7	0.3	0	0.2	0	0	0	0
Johnson's Lee		52.8	63.1	57.2	51.6	32.7	24.1	18.6	15.6	6.5	4.6	1.4	0.8	0.5	0.5	0.2
Talcott			14.5	14.7	12.6	13.8	12.8	11.4	9.1	7.6	3.7	2.5	1.8	1.2	1.2	0.4
Fossil Reef							29.2	26.9	17.9	9.3	5.8	2.1	1.8	1.2	0.3	
Oregonian Province																
Harris Point	17.4	21.4	18.6	23.2	19.0	19.3	16.1	22.4	17.2	20.2	18.5	15.1	15.3	14.9	14.5	16.0
Otter Harbor	33.3	33.5	28.5	29.8	28.5	31.3	27.2	28.8	26.7	27.4	27.7	24.2	14.8	7.3	5.0	2.9
Crook Point	47.0	37.4	38.1	30.2	27.7	22.9	21.2	16.6	15.4	15.0	11.6	13.3	11.3	8.2	4.5	1.8

<sup>a</sup> S = Spring (March–April), F = Fall (October–December).

<sup>b</sup> No data.

abundance appeared to be the cause of mussel decline at Johnson's Lee, but not at the other two sites. Increased use of the intertidal zone by hauled-out California sea lions correlated with a general decline in ground cover at Santa Barbara Island. Owl limpet populations at Johnson's Lee and Ford Point remained relatively stable, with a slight increase in abundance between 1988 and 1992, while they declined slightly at Otter Harbor. At Crook Point, owl limpets declined steadily from 1986, and by 1992 only about 10 percent remained.

**Geographical Pattern of Decline.** The first dramatic declines in black abalone abundance occurred in 1986 on south facing reefs in the Californian Province, at Ford Point and Cat Rock. Initially the number of abalone dropped more than 30% in the first 12 months but continued until more than 99% were gone by 1991. In early 1987, the rapid declines spread to the north side of Anacapa, Johnson's Lee, and west into the Oregonian province at Crook Point, San Miguel Island. At Middle Anacapa Island the total number of abalone in monitoring plots dropped from 551 to 5 ( $78 \text{ m}^{-2}$  to less than  $1 \text{ m}^{-2}$ ) between fall 1985 and spring 1989, and showed no recovery through 1992. In 1988, populations at Santa Barbara Island and Harris Point, San Miguel Island declined rapidly, followed in 1989 by marked declines at Talcott, Santa Rosa Island. San Miguel Island populations generally declined slowly until 1991, when abundance dropped sharply. The Otter Harbor population appeared healthy until late 1990, when it underwent a rapid decline during 1991–1992. At Crook Point, on the south side of San Miguel Island, abundance declined slowly but steadily, about 10% per year (low compared to 60% per year on Santa Rosa Island). Harris Point abalone abundance dropped in spring 1988 with the appearance of moribund abalone, but it recovered and retained nearly 90% of the 1985 densities through 1992.

**Size Distributions of Survivors.** Initial size distributions were similar at all sizes, but changed with time (Table 2). Initial mortality occurred equally in all size classes, but final survivors in the Californian Province tended to be the largest adults, while in the Oregonian Province the larger, harvestable-sized abalone disappeared, leaving smaller survivors.

**Shell Accumulations.** Black abalone shells accumulated on adjacent beaches as populations declined. Shells of all size classes were found during counts, though shells smaller than 40 mm were

rarely found. Most of the shells in the early counts were fairly fresh indicating recent mortality, and showed no obvious indications of predation, such as drill holes or chipped edges.

The highest shell count at Cat Rock occurred in fall 1988, with over 400 shells accumulating on the beach in seven months. Live abalone abundance dropped by 50% during that time, while only 11% of the 1985 population remained. At Fossil Reef, nearly 2,000 shells were collected from a small cobble beach in the first half of 1989. The population rapidly declined during that time, and most of the shells were fresh. Shells of all sizes were found (20–156 mm). As populations declined, shell counts dropped also. Large, weathered shells were more common than fresh shells in later counts.

Shell accumulations on adjacent beaches supported direct observations that the accelerated mortality was not confined to the monitoring plots. Marked shells placed on different sections of Fossil Reef indicated that shells from the reef adjacent the beach were the main source of accumulated shells.

**Abalone Condition.** By fall 1987, it was apparent that a mass mortality of black abalone was occurring along the south side of Santa Rosa Island and at Anacapa Island. In addition to declines in abundance of live, attached abalone and accumulations of empty shells, we found numerous dead or weak individuals. At Santa Rosa Island, we collected 34 shells in spring 1987, 11 still had significant amounts of tissue attached. Weak animals could be pulled off the rocks easily by hand. Many appeared shrunken, only partially filling their shells. Muscle and gonadal tissue were greatly reduced. The term Withering Syndrome (WS) was subsequently applied to describe affected animals (Haaker et al. 1992). While healthy abalone were generally quite active when handled, WS abalone were lethargic and slow to respond to stimulus. WS abalone also had bluish-green foot muscles, quite different than the normal cream color.

It appeared that WS abalone were more susceptible to predation by sea stars and crabs. We often found weak abalone lying below a rock, foot up, where it had just fallen off a vertical rock surface. We observed some pecking by western gulls and other birds on moribund abalone, but generally, birds seemed to ignore them. Large numbers of dead abalone washed up on the beaches, and shells that were broken or had holes in them indicated that impacts from wave action hastened mortality.

TABLE 2.  
Black abalone size frequency distributions (percent) in fixed plots, 1985-1992.

Size Classes (mm)	Year							
	1985	1986	1987	1988	1989	1990	1991	1992
Californian Province								
<45	6	8	5	2	5	8	0	0
45-126	78	77	79	86	73	42	67	50
127-145	16	15	16	12	22	50	33	50
>145	1	0	0	1	0	0	0	0
Number	1933	1604	994	375	65	12	3	2
Transition Zone								
<45	1	3	2	2	4	3	3	5
45-126	77	71	68	67	66	70	81	78
127-145	21	25	29	31	30	25	16	16
>145	1	0	1	0	0	1	0	0
Number	1202	2291	1592	1812	1854	713	227	88
Oregonian Province								
<45	7	5	4	5	5	5	4	5
45-126	70	70	71	73	73	78	85	91
127-145	23	25	24	22	22	17	11	4
>145	1	0	1	0	0	0	0	0
Number	2687	2542	1956	2055	1870	1721	1188	833

We sent moribund abalone to the Veterinary Medical Laboratory at the University of California at Davis, the California Department of Fish and Game Fish Disease Laboratory, and the California State Pesticide Laboratory for analysis, and there were no remarkable findings (Friedman 1991, Davis et al. 1992). Pycnogonids were found on moribund abalone on Santa Cruz (D. Kushner pers. comm.) and Santa Rosa Islands. These small crustaceans were on all parts of the abalone mantle, generally favoring the head and mouth area or the tentacle groove. Pycnogonids were usually associated with a small lesion in the epidermis. From a sample of 50 abalone (15 healthy, 35 unhealthy) from two sites on Santa Rosa Island, we found that 50% of the apparently healthy abalone had pycnogonids on them, while 100% of the abalone considered to be in poor health had pycnogonids.

#### DISCUSSION

Mass mortalities of abalone of the sort described here are unprecedented. More than 99% of the black abalone vanished from Anacapa, Santa Barbara, and Santa Rosa Islands in less than five years, while other mollusks and plants remained unchanged at the same sites. Tissot (1988) reported that the Santa Cruz Island black abalone population disappeared about the same time as the Anacapa and Santa Rosa Island populations (Davis 1988, Richards 1988). Black abalone began disappearing at San Clemente and San Nicolas Islands in 1990 and 1992 respectively (P. Haaker pers. comm. and G. Vanblaricom et al. 1993). About the time the decline at Anacapa was noticed, a similar mass mortality occurred within Diablo Cove near Avila Beach on the central California Coast (Steinbeck et al. 1992). Only abalone within the warm thermal plume of a large power plant were affected at Diablo Cove. Black abalone observed in surveys near Point Conception appeared healthy through 1992 (P. Haaker unpubl. report).

The rates of decline observed in this event showed two patterns (Fig. 2). Early declines in the Californian Province were initially rapid and caused by losses of all sizes of abalone, with survivors tending to be the largest animals in the population (>126 mm). In contrast, initial declines in the Oregonian Province were relatively

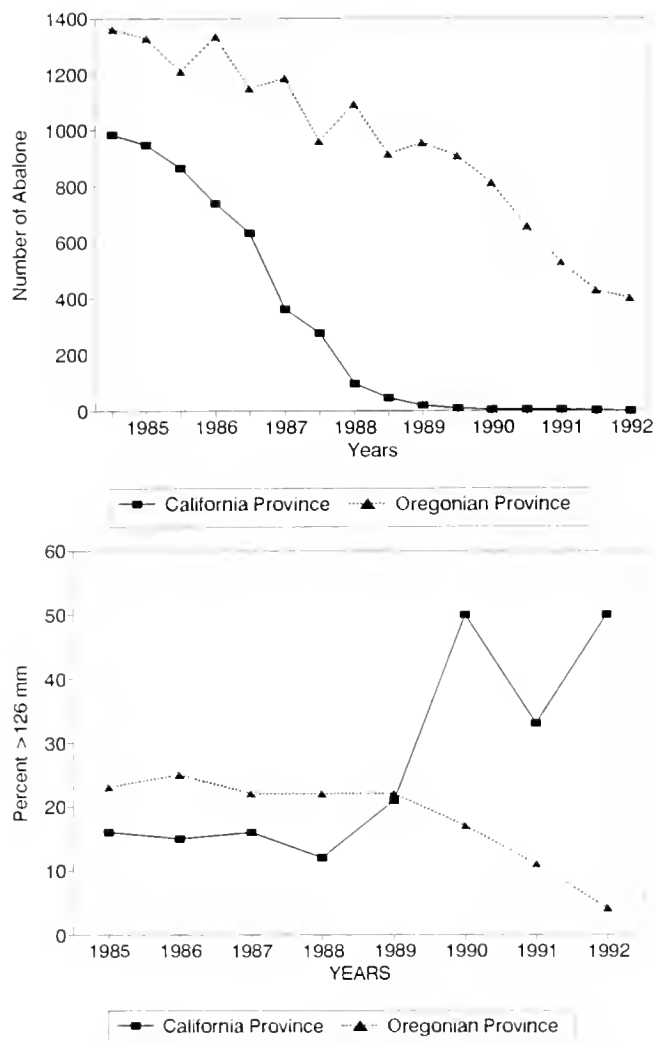
gradual, and survivors tended to be smaller than 126 mm. In 1990, after 90% of the populations elsewhere were gone and the state of California closed Santa Barbara, Anacapa, and Santa Cruz islands to further commercial harvest, population declines in the Oregonian Province began to accelerate. In both provinces, recruitment of juvenile abalone dropped drastically, or ceased completely, when the adult population dropped to less than half its initial density (Fig. 3). We suggest that these observations indicate more than one factor caused the black abalone population declines described here.

A similar black abalone mass mortality was reported along the mainland coast at Palos Verdes Peninsula in southern California in the late 1950's (Cox 1962). This mass mortality was attributed to starvation, precipitated by loss of a neighboring kelp bed that was destroyed by a large El Niño event. Cox (1962) reported recovery of withered abalone transplanted to areas with abundant drift kelp. Other instances of abalone mass mortality also have been attributed to starvation (MacGinitie and MacGinitie 1966, Tanaka et al. 1986). Moribund WS abalone taken from the Channel Islands in the 1980's and provided kelp to satiation in laboratories showed no recovery (Haaker et al. 1992, Steinbeck et al. 1992).

In 1983, just prior to onset of the abalone decline in the Californian Province, southern California experienced the largest El Niño event ever recorded, with warm, nutrient poor water and 100-year storms (Tegner and Dayton 1987). Extensive kelp canopies, a primary source of black abalone food, were lost to storms in 1982-83. Canopy recovery was inhibited by low seawater nutrient levels and grazing by increased sea urchin populations. Nevertheless, when black abalone mortality peaked in 1987 in the Californian Province, many nearby kelp forest canopies were growing back; and by the time mortality peaked in the Oregonian Province in 1990, kelp canopies were at pre-1983 levels. It seems unlikely that starvation alone caused the declines (Davis et al. 1992).

Populations of several sea stars were also severely reduced by a wasting disease in the early 1980's (Richards et al. 1993). The sea star disease was apparently caused by bacteria (Schroeter and



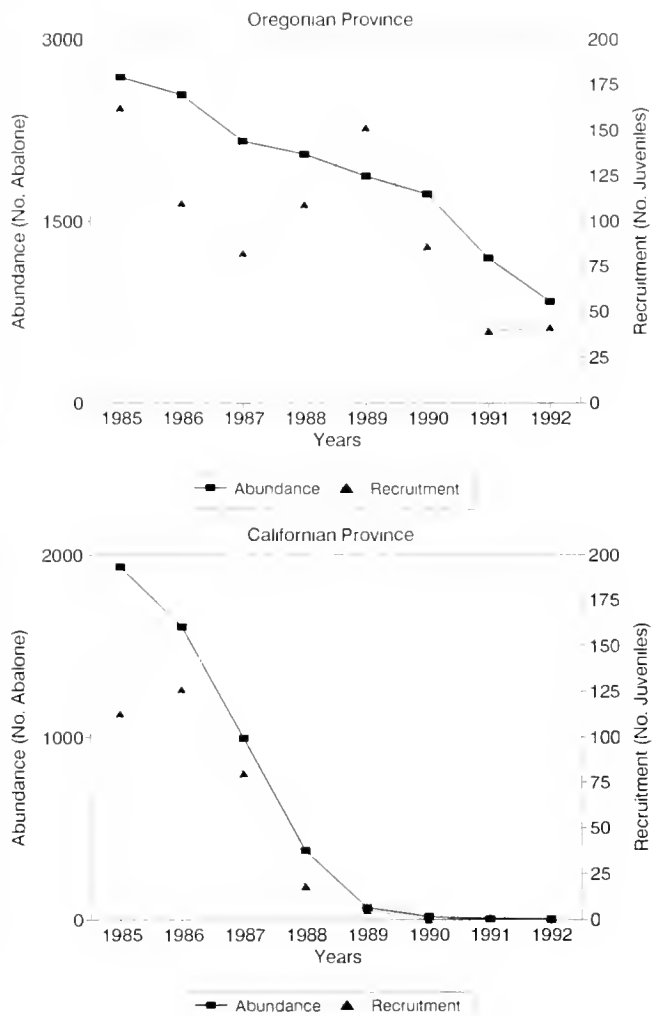


**Figure 2. A:** Abundance of black abalone in Californian and Oregonian Province sites censused 1985–1992. **B:** Proportion of large black abalone (>126 mm) surviving in fixed plots in the Californian and Oregonian Provinces 1985–1992.

Dixon, 1988) and still affects southern California sea star populations when water temperatures exceed 16°C. The apparent spread of WS mortality from warm Californian Province waters into cooler Oregonian Province waters suggest that elevated sea temperatures may be a factor in this black abalone mass mortality, but laboratory experiments were inconclusive (Steinback et al. 1992, Haaker et al. 1992).

The presence of withered abalone, shell accumulations, and differential survival of large, legally-harvestable abalone indicate that fishery harvest did not cause the declines in the Californian Province. However, the lack of large survivors in the Oregonian Province, and the delayed onset of rapid decline there, suggest that harvest may have contributed to the decline there.

The apparent spread of mass mortality from Anacapa, Santa Cruz, and the south side of Santa Rosa Islands may indicate an infectious agent (Lafferty and Kuris 1993). However, the onset of rapid mortality at San Miguel and northwestern Santa Rosa Island appears to have been caused by harvest (perhaps intensified by loss of other stocks) that increased the rate of loss in a population already in decline for several years. It did not represent the sudden incidence of *in situ* mortality. The adult population at San Miguel



**Figure 3. A:** Black abalone abundance and juvenile recruitment (<45 mm) Oregonian Province. **B:** Black abalone abundance and juvenile recruitment (<45 mm) Californian Province.

Island was declining in 1986, and we saw WS abalones there in 1987, but annual recruitment buffered the population decline until 1990 (Fig. 2a). It is not possible to identify the onset of mass mortality in 1985, since monitoring only began in that year and observations of Withering Syndrome are anecdotal before 1987. No infectious agent has been found to date, and the coccidian parasite implicated earlier seems to be prevalent in abalones throughout California and was found in populations with no indication of increased mortality (Friedman 1991, Haaker et al. 1992, Steinbeck et al. 1992).

The long-term ecological consequences of abalone loss in these rocky intertidal communities are not yet apparent. We observed that sand castle worms, *Phragmatopoma californica*, and scaled tube-snails, *Serpulorbis squmigeris*, invaded much of the space vacated by dying black abalone at many sites. The ensuing competition for space may be a factor in the declining numbers of owl limpets at Crook Point. Although the causes of black abalone population demise are not clear, it will be decades before black abalone are space-dominant elements of rocky intertidal communities on the California Channel Islands as they were in the early 1980's.

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## LONG-TERM TRENDS IN BLACK ABALONE, *HALIOTIS CRACHERODII* LEACH, 1814, POPULATIONS ALONG THE PALOS VERDES PENINSULA, CALIFORNIA

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**ABSTRACT** Since 1975, 16 annual surveys of the densities and individual sizes of the black abalone, *Haliotis cracherodii*, have been made at four intertidal sites along the Palos Verdes Peninsula. Density declines have occurred at all sites with the average density over the four sites going from 2.8 m<sup>-2</sup> (range: 1.0-6.8) during the period of 1975 to 1979 down to 0.03 m<sup>-2</sup> (range: 0.0-0.2) from 1987 to 1991. Even with the majority of the individuals in the populations being reproductive-sized through the mid-1980's, there has been a recruitment failure. The first-year size class has declined substantially: 233 recruits were censused at all sites from 1975 to 1980, but only 33 were found from 1981 to 1991.

Although the reasons for the declines have not been directly studied, food supply is not a likely factor since giant kelp cover around the Peninsula was extremely low in the mid-1970's, had increased 10-fold by the early 1980's, and has remained high. However, there has been a large increase in the abundance of intertidal sea urchins (over 3-fold at three sites), a potential competitor. In addition, predation (including human harvesting) and factors related to warm sea water temperatures, such as the disease associated with the abalone declines in the Channel Islands, should be considered as potential reasons for the decline here.

**KEY WORDS:** abalone, intertidal, population, gastropod, *Haliotis cracherodii*

### INTRODUCTION

Black abalones, *Haliotis cracherodii* Leach, have long been a common inhabitant of the rocky intertidal zone in southern California. Their shells have been found in American Indian middens (Cox 1962; Douros 1985; Raab 1992) and commercial harvesting by the Chinese and Japanese thrived in the late 1800's and early 1900's (Bonnot 1940). Although not generally considered to have high-quality meat, black abalones have been commercially harvested most of this century, especially in the past 20 years in the Channel Islands (Ault 1985).

Our interest in black abalone populations along the Palos Verdes Peninsula started in 1975 when Los Angeles County completed its acquisition of the private-access Abalone Shore Club. Since the County planned to open Abalone Cove to the public, we were interested in monitoring abalone and some echinoderm populations here to follow any changes that might be associated with increased access to the tidepool areas, as has been found for some intertidal species along the Peninsula (Ghazanshahi et al. 1983). In addition to two areas at Abalone Cove, we censused two other sites on the Peninsula that had restricted public access because of the long hikes necessary to reach them. Although we initially felt that the latter two sites would serve as "low human impact" controls, our experience over the ensuing years indicated that this was not necessarily the case for black abalones. Even though the initial objective changed, the study became one of the rare long-term studies of intertidal zone populations.

We have censused these four sites each spring since 1975 (with the exception of 1985) for population density and size class composition for the black abalone, *Haliotis cracherodii*, the purple sea urchin, *Strongylocentrotus purpuratus* (Stimpson), the ochre star, *Pisaster ochraceus* (Brandt), and the bat star, *Patiria miniata* (Brandt). This paper presents the results for black abalones and speculates on some possible explanations for the changes noted during the 16-year duration of the study.

### SITES

Two of the four sites on the Palos Verdes Peninsula are located at Abalone Cove, Rancho Palos Verdes, Los Angeles County (33°44'N, 118°22'W) and are separated by approximately 0.8 km of rocky beach. The Point Vicente site is 2.8 km NW of Abalone Cove and Portuguese Bend is 1.7 km to the SE. The Abalone Cove sites (I and II) are very accessible, a five-minute walk from a large parking lot. The other two sites can be reached only by walking along rugged trails for at least 20 min. The substratum at Abalone Cove I and Point Vicente is primarily boulders with some larger rocky outcrops. Abalone Cove II and Portuguese Bend have long rock ledges oriented parallel to the coast and contain smaller, loose boulders. Abalone Cove appears to be less wave-exposed than the other two sites, which face the general direction of the swells.

### METHODS

Each site was censused annually starting in 1975, with the exception of 1985, during a spring tide series from late February to early June. At each site, permanent axes for a grid were established and 30 quadrats (1 m × 1 m) were randomly placed within the grid. Each grid was approximately 60 m long (parallel to the shoreline) and 20 m wide, encompassing habitat from the lower intertidal to the upper middle intertidal zones. Within a quadrat, all *Haliotis cracherodii* were counted and measured in place for greatest shell length.

### RESULTS

#### *Black Abalone Density*

Figure 1 presents the changes in black abalone density since 1975. The trends in all sites have been the same: during the early years, there were relatively high densities with some fluctuations, followed by declines until black abalones are rare or nonexistent in

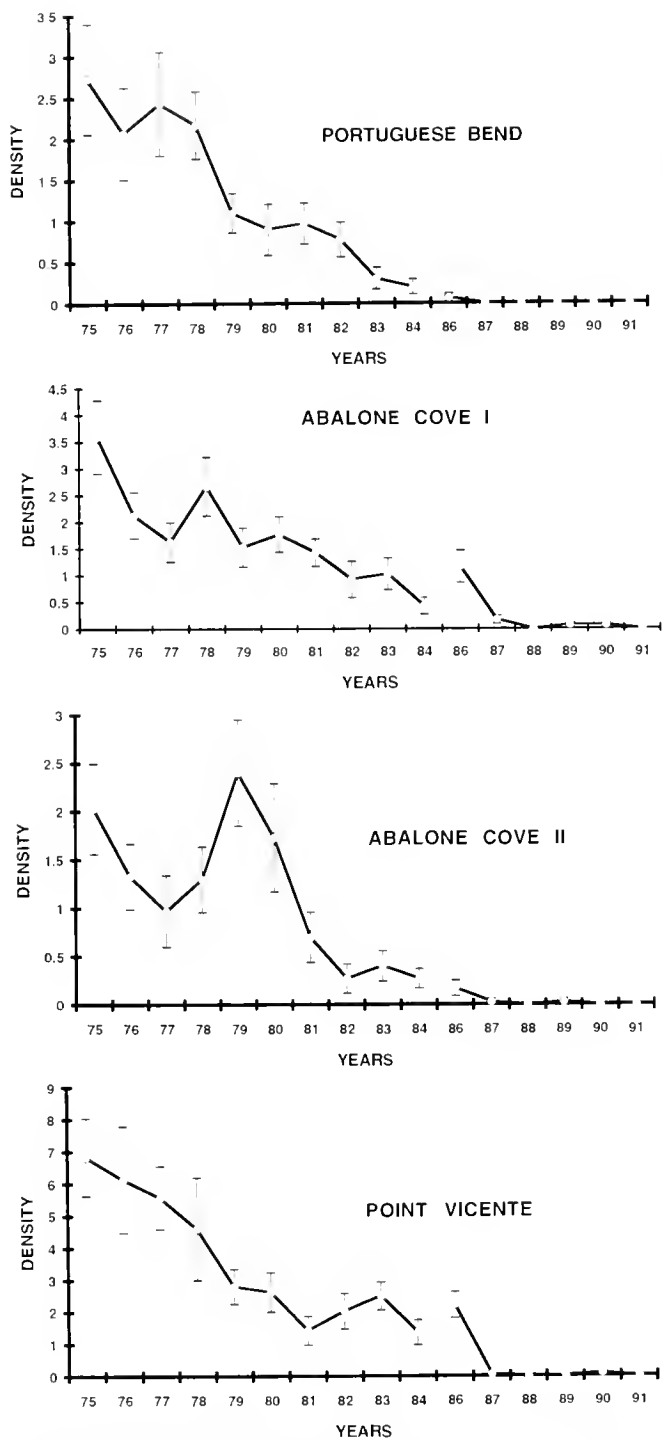


Figure 1. The annual density (number per m<sup>2</sup>) of black abalones (±SEM) at each of the four sites.

the sample areas. The average density over the four sites decreased from 2.8 m<sup>-2</sup> (range: 1.0–6.8) during the period of 1975 to 1979 down to 0.03 m<sup>-2</sup> (range: 0.0–0.2) from 1987 to 1991. In recent years, we have searched for abalones in likely habitats outside of the quadrats; but rarely have we found any individuals.

An analysis of the equality of the slopes of the regression lines for density (transformed by logarithms to the base 10) versus years suggests that the rates of decline are similar among the sites ( $F = 2.03, P > 0.10$ ).

*Black Abalone Sizes*

Figure 2 presents the median size of the individuals censused and the number of individuals that are <2.5 cm in shell length, which should represent individuals about one year old or younger (Leighton and Boolootian, 1963). A median size of five centimeters or more indicates that the population has half or more of the

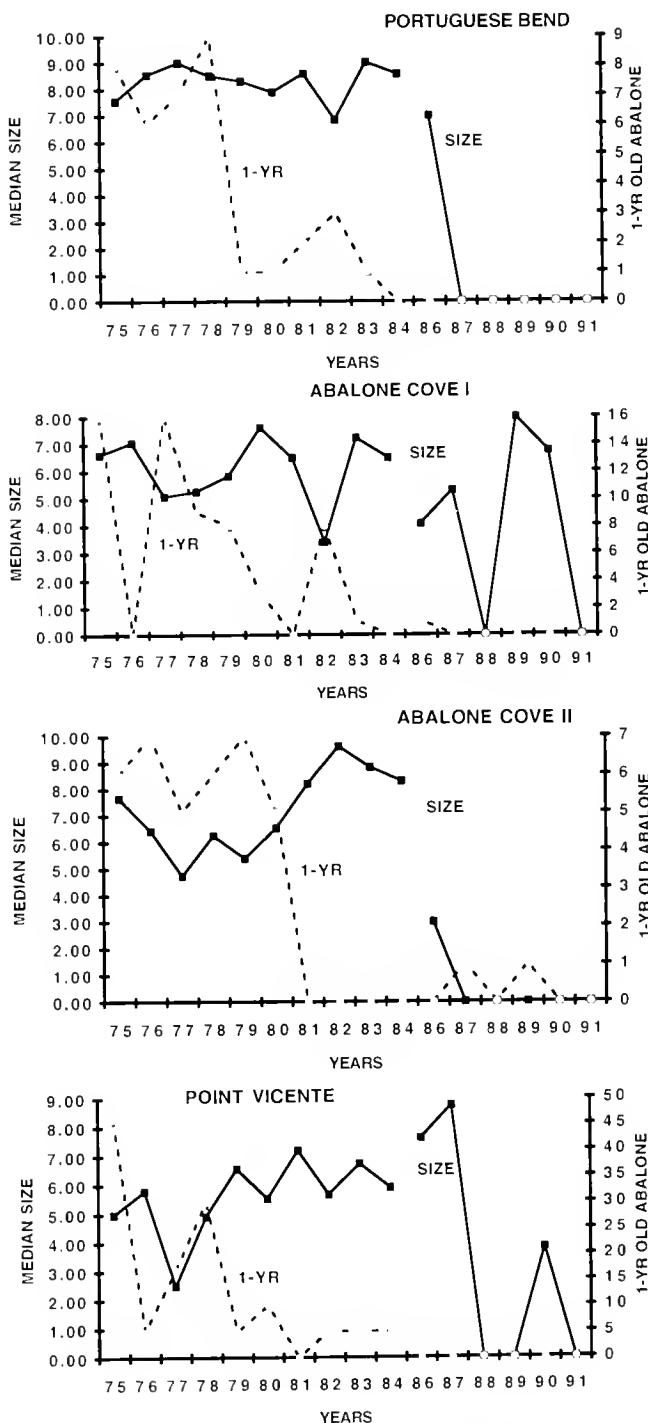


Figure 2. The number of one-year old or younger black abalones (shell length <2.5 cm) and the median size (cm) of individuals sampled at each of the four sites. Note: where the median size line touches the X-axis, there were either one or zero individuals found.

members in the reproductive size classes (Leighton and Boolootian, 1963).

Recruitment has obviously declined, especially since 1980, even though for most years through 1984, half or more of each population consisted of reproductive-sized individuals.

## DISCUSSION

Our data indicate that the black abalone populations along Palos Verdes Peninsula have suffered a severe decline in numbers since 1975. Without data on densities previous to this, we cannot assess whether our initial values represented high, medium, or low densities for these areas. Our densities are at the extreme low end of the range reported by Douros (1987) for black abalones on Santa Cruz Island:  $<1$  to  $126 \text{ m}^{-2}$ . The fact that the declining trend in density occurs early in three of our sites suggests that the populations were already in a state of decline in 1975. The brief increase in density at Abalone Cove II in the late 1970's (Fig. 1) coincides with the establishment of Abalone Cove as an ecological reserve and this site is shoreward of the small giant kelp bed (*Macrocystis pyrifera* (Linnaeus), that was being maintained with transplanted kelp and sea urchin control measures by the California Institute of Technology and California Department of Fish and Game kelp project personnel. However, whatever factors might have contributed to this local density increase could not sustain it.

Earlier studies on the Peninsula suggested that the black abalones here might not be extremely healthy (Cox 1962; Leighton and Boolootian 1963). Cox (1962) mentions that black abalones collected in 1956 at White Point, about 5.9 km southeast of Portuguese Bend, had "shrunk" bodies compared to individuals from Santa Catalina Island. In his study, the input of sewage at White Point and the loss of offshore giant kelp beds were mentioned as potential reasons for the poor condition of the abalones.

Leighton and Boolootian (1963) studied black abalones at Flat Rock at the northwestern limit of the Peninsula. They noted that, in addition to the 1959 Flat Rock population being underweight compared to a population at the northwestern end of Santa Monica Bay (Pt. Dume), no mature gonads were found during the year's study. Since the decline in offshore kelp beds was well underway along the Peninsula by the late 1950's with the last major bed disappearing off Flat Rock in 1958 (North, 1967), the populations of abalone on the Peninsula may have been suffering as early as then.

One indication of a population in a declining state is the drop in numbers of recruits to the population. In a randomly selected group of black abalones collected in April 1959 at Flat Rock, Leighton and Boolootian (1963) found 18 of 115 individuals (15.6%) in the one-year or younger (shell length  $<2.5$  cm) size class. The numbers in our populations of this size class were variable among sites and among yearly surveys in the early years (Fig. 2) but are similar to this previous study. This size class has declined substantially over the years, signaling a recruitment problem: a total of 233 recruits were censused at all sites from 1975 through 1980, but only 33 were found from 1981 through 1991. It is interesting that Tissot (1988) found only six black abalones (estimated from his published graph) less than two centimeters long in his April 1984 sample ( $n = 112$ ) and none in the May 1985 sample ( $n = 60$ ) from a boulder field near the city of Laguna, about 63 km to the southeast of Abalone Cove. Although he did not sample under every boulder in his transects, this population may also have had low recruitment at that time.

Even though the direct causes of the decline in these black abalone populations have not been studied, we offer some speculations on potential causes in the following section.

## Speculation on Causes of the Decline

### Food Supply

Although diatoms are eaten by black abalones and, when in abundance, can support growth, larger individuals show a preference for macroscopic algae (Leighton and Boolootian 1963). Assuming that the abalone food supply suffered with the demise of the offshore kelp beds, individuals could have sustained a low level of nutrition by grazing diatoms. In turn, abalone abundance should have increased as the availability of drift *Macrocystis* increased in the late 1970's with the return of the offshore kelp beds (Fig. 3).

However, the data do not support this: linear regression analyses produced significant negative slopes ( $P < 0.10$  for Abalone Cove II;  $P < 0.05$  for the other sites) for the relationship between abalone density and the average hectares of kelp cover at all sites from 1975 through 1987, the last year when any numbers of abalone occurred at our sites.

### Potential Competitors

Work by Tegner and Levin (1982) on growing red abalones and sea urchins together indicates that these abalones grow more slowly in the presence of sea urchins when food is scarce, suggestive of a competition for food. If *Strongylocentrotus purpuratus* in the intertidal can have a similar effect on nearby black abalones during times of food deprivation, then competition for food and, perhaps, space may have been a factor in the black abalone decline along the Peninsula before the kelp came back.

Our data on the *Strongylocentrotus purpuratus* populations at these sites (Miller and Lawrenz-Miller, unpublished) indicate a substantial increase in all the study areas; e.g., comparing densities for 1975 with 1991: Abalone Cove I, 13 to  $39 \text{ m}^{-2}$ ; Abalone Cove II, 25 to  $41 \text{ m}^{-2}$ ; Portuguese Bend, 19 to  $89 \text{ m}^{-2}$ ; and Pt. Vicente, 11 to  $98 \text{ m}^{-2}$ . Such large increases are probably in response to the assumed increase in drift kelp; however, the decline in abalones may have also provided more ecological room for sea urchins. The current high densities of urchins may limit the access of abalones, especially the smaller ones, to food and space.

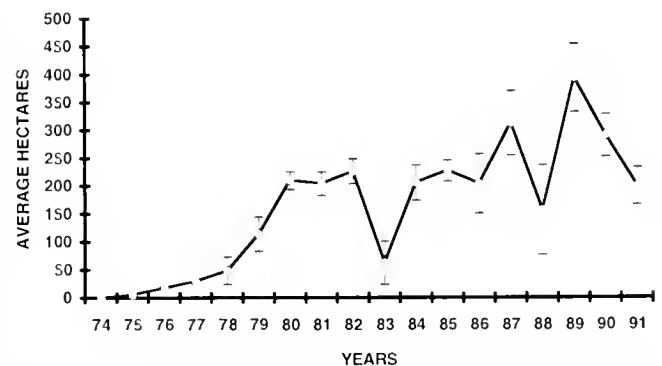


Figure 3. The annual average ( $\pm$ SEM) number of hectares of giant kelp, *Macrocystis pyrifera*, covering the sea surface off the Palos Verdes Peninsula, based on aerial photographic data supplied by the California Department of Fish and Game. All sample sizes are 4, except: 1989 ( $n = 2$ ), 1975 and 1986 ( $n = 3$ ), and 1983 ( $n = 5$ ).

A different type of potential competitor for space are the colonies of tubes of the sand castle worm, *Phragmatopoma californica* (Fewkes), that cement rocks to the substratum, eliminating the undersides of rocks as a refuge for mobile invertebrates (Connell et al. 1988). Qualitatively, we have observed at the Portuguese Bend site that most of the rocks, under which small abalones used to be found, are now cemented to the substratum by these worms. Increases in *Phragmatopoma* at the other sites have not been noticed. It is also possible that these filter-feeding worms could consume veliger larvae or gametes of the black abalone.

### Predation

Black abalones should face two different suites of predators: plankton-feeding animals preying on the free-swimming larvae and large animals feeding on rock-dwelling juveniles and adults.

Along with the increase in kelp beds offshore, there should be an increase in plankton-feeding fishes and invertebrates associated with the kelp (Coyer 1984; Gaines and Roughgarden 1987; DeMartini and Roberts 1990). Although black abalone populations normally exist inshore from kelp beds, the kelp increase along the Peninsula may have resulted in increased predation on an already reduced stock of abalone larvae. If this were important in influencing the abalone density, we would expect a decline in the number of settling abalones.

As Figure 2 shows, the numbers of one-year old and younger individuals drop to negligible values at all of the sites by the early 1980's, corresponding to the build-up of the kelp beds. Regression analyses found there to be a significant negative relationship between these numbers and the average hectares of kelp cover ( $P < 0.10$  for Abalone Cove I;  $P < 0.05$  for the other areas). However, there are no direct data on the increase in predation on abalone larvae; and larval abundance and settlement success probably is influenced by other factors also, such as temperature, competitors for space, the production of gametes by adults, etc. The tremendous increase in sea urchin density (Miller and Lawrenz-Miller, unpublished) could cast some doubt on this idea of increased larval predation, unless the sea urchin larvae have better defenses against filter-feeding predators (Cowden et al. 1984).

Predation on juveniles and adults attached to the rocks involves a number of different predators (Cox 1962), of which octopuses (Tissot 1988) and humans can be the more devastating in southern California. Although octopus density could have grown with the potential increase of its prey associated with the build-up of the giant kelp, there are no data on this.

Studies on the black abalone shells in American Indian middens on the offshore islands in southern California (Douros 1985; Raab 1992) suggest that the combination of sea otter and human predation on *H. cracherodii* in the past had a significant effect on reducing their density and shifting size distributions toward the smaller sizes. Human predation in recent times on the settled abalones along the Palos Verdes Peninsula has not been formally documented; however, there is ample anecdotal evidence of its importance.

We interviewed Captain Tim Sawyer, a warden for the California Department of Fish and Game who worked the Peninsula from 1979 to 1982. He remembers that it was common to contact people with a "few hundred" black abalones in their possession. In one month, he wrote 93 citations for abalone poaching, with most resulting in a \$500 to \$600 fine (at \$25 plus \$5 per abalone). His review of the Fish and Game records indicated that 34 abalone poaching citations were written for 1985, 46 in 1986, 3 in 1987,

and none in 1988. Although some of the decrease in citations may have been due to fewer patrolling hours, this trend corresponds to the decline in the abalone populations that we observed (Fig. 1).

Our observations were that, although it was more difficult for people to reach the Portuguese Bend and Point Vicente sites, the people that we saw at these sites were usually there to exploit the marine life, especially abalone. It was for this reason that we abandoned the idea of these two sites serving as controls for the "easy access" Abalone Cove sites.

There are available data on the pounds of commercially caught black abalones, almost all of which are landed in southern California (Ault 1985; Oliphant et al. 1990; Peter Haaker, personal communication). Figure 4 presents the annual estimated numbers (using the California Department of Fish and Game conversion factor of 11.34 kg for 12 black abalones) of commercially harvested black abalones. The low numbers in the early years probably reflect the lower preference for this species until other abalone species became less common. Not only have there been large numbers of reproductive-sized individuals harvested over the past 24 years, but there also has been a serious decline in numbers harvested in the past few years. Assuming no major decrease in fishing effort, this pattern probably reflects the decline in populations noticed in the Channel Islands (Richards and Davis 1993). Although most of these individuals were probably collected on the off-shore islands, these populations may represent potential sources of larvae for the Palos Verdes Peninsula.

### Temperature

Temperature has been shown to have direct effects on various aspects of abalone life: egg fertilization and development, larval growth and settlement, and growth and feeding of juveniles and adults (see references in Ault (1985)). However, there could also be indirect influences of temperature, such as on the susceptibility of individuals to disease or the toxic effects of pollution. For instance, elevated temperatures in the Sea of Cortez have been associated with mass mortalities of seastars due to disease (Dungan et al. 1982). Although not much work has been done on the effects of temperature on black abalones, it would not be unreasonable to expect them to show the same trends as other abalone species, except their intertidal lifestyle may mean higher temperature tolerances or optimal ranges.

Figure 5 presents average yearly sea surface temperatures taken by the Los Angeles County Sanitation District off Palos Verdes Peninsula. The temperatures from 1976 through 1987 (median = 17.0°C), when the black abalone populations declined to almost

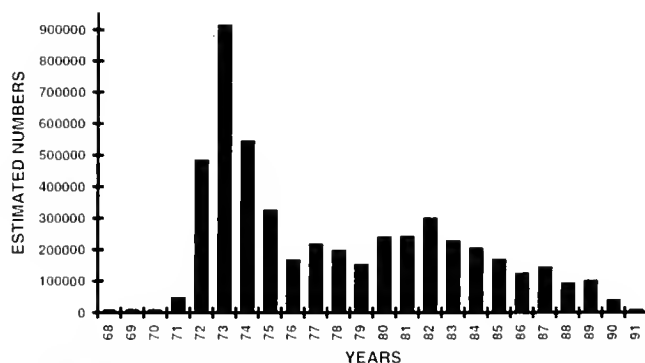


Figure 4. The annual estimated numbers of black abalones commercially harvested in California, based on data supplied by the California Department of Fish and Game.

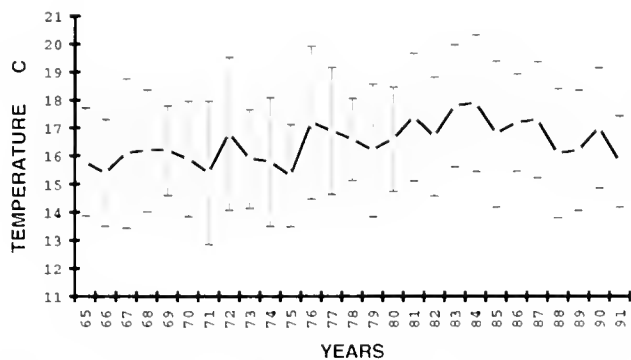


Figure 5. The annual average ( $\pm$ SEM) sea surface temperatures ( $^{\circ}$ C) taken off Palos Verdes Peninsula, based on data supplied by the Los Angeles County Sanitation District ( $n = 12$  for each year).

nothing, are significantly higher than those for 1965 through 1975 ( $15.9^{\circ}$ C;  $U$ -test,  $P < 0.001$ ). In fact, five years in the 1980's had average annual temperatures  $1^{\circ}$ C or more higher than those of the late 1960's.

Regression analysis indicated that both density and number of one-year old and younger abalones exhibit negative relationships with temperature during the 1975 to 1987 period when abalones were present ( $P < 0.10$  for recruits at Portuguese Bend;  $P < 0.05$  for all other analyses).

The disease associated with the black abalone declines in some areas of the Channel Islands (see papers in this issue) may be an indirect result of elevated sea temperatures reducing the resistance of black abalones to infectious agents. We observed no abalones in a moribund state during any of our censuses; however, the steep drops in numbers between 1986 and 1987 at Abalone Cove I (35 individuals to five) and Point Vicente (67 to two) do coincide with the observed outbreak of the "withering syndrome" on the Channel Islands to the north. Although we cannot dismiss disease as a contributor to the decline along the Peninsula, the density decline had started long before the presence of a disease was noticed anywhere in southern California.

#### Pollution

At this point, it cannot be completely ruled out that pollutants from the offshore discharge of sewage at White Point and in Santa Monica Bay may have been detrimental to the health of black abalones. Young et al. (1981) found elevated metals in black abalones near the White Point sewage outfall on the Peninsula, but

work must be done to assess any effects on black abalone reproduction. Hunt and Anderson (1989) have demonstrated that zinc and sewer effluents can have negative effects on red abalone (*H. rufescens* Swainson) larval development and metamorphosis, so there might be similar problems for black abalones living close to sewage outfalls. On the other hand, we note that the kelp has made a significant comeback and we have noticed that another large intertidal archeogastropod, *Lottia gigantea* Sowerby, is common at all of the sites.

#### CONCLUSIONS

The 16 annual surveys of the densities of black abalones at four sites along the Palos Verdes Peninsula indicate that these populations have crashed. Although we do not have data to indicate directly the causes of the decline, the fact that the rate of the decline in density is similar for all sites suggests that whatever factors have contributed, have done so in a more or less equal fashion.

Our size data reveal that there has been a recurring recruitment failure at least as early as 1980, in spite of there being mature-sized individuals in the populations (Fig. 2). However, the problem may be that the density of reproductive individuals went below some critical number necessary to produce enough gametes for a significant number of larvae to settle. This has been demonstrated for *H. rubra* Leach in Australia where reduced recruitment density occurred in experimental plots where adults had been removed (Prince et al. 1988). But, we do not have the critical data on whether the decline in black abalone adults and young was due to predation (on the adults and/or the larvae) or other factors such as competition, disease, or environmental changes.

There is not a good understanding of the larval portion of the black abalone life cycle in terms of time and space in the plankton. However, studies done in Australia suggest that some abalone species have very localized recruitment (Prince et al. 1987, 1988). If this were true for black abalones, we would predict that the population along the Palos Verdes Peninsula will take many years to recover, especially since the human predators are still foraging in the intertidal zone.

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## TRANSMISSIBILITY OF A COCCIDIAN PARASITE OF ABALONE, *HALIOTIS* SPP.

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**ABSTRACT** Renal coccidian infections developed in seed red abalone, *Haliotis rufescens*, after 5–7 mo of exposure to infective waters at the Fish and Game Marine Culture Laboratory in Monterey County, California. Similar infections developed in cohort seed abalone after 3 mo of exposure to infective waters in a barrel culture system located in an embayment near Pt. Hueneme, California. In the experimental trials the coccidian was directly transmitted from red abalone to pinto abalone after 10.5 mo of cohabitation. One hundred percent of the pinto abalone that shared aquaria with infected red abalone had coccidian infections after 17 mo of cohabitation, while no control abalone developed infections with coccidia. No change in the condition of the abalone or mortality resulted from natural or experimental infections with coccidia.

**KEY WORDS:** coccidian; kidney; direct transmission; red abalone; *Haliotis rufescens*; pinto abalone; *H. kamschatkana*; California; British Columbia

### INTRODUCTION

Renal coccidia were discovered during an investigation of a mass mortality of black abalone, *Haliotis cracherodii* (Leach 1814), in Diablo Cove, California (Steinbeck et al. 1992) and the Channel Islands off Southern California (Friedman 1991, Haaker et al. 1992). Upon further examination Friedman (1991) determined that the six species of abalone sampled throughout California had morphologically indistinguishable renal coccidia. These included the following species: black (*H. cracherodii*), red (*H. rufescens* Swainson 1822), pink (*H. corrugata* W. Wood 1828), green (*H. fulgens* Philippi 1845), flat (*H. walallensis* Stearns 1899) and pinto abalone (*H. kamschatkana* Jonas 1845). Microscopic examination of infected kidney tissues suggested that transmission of the parasite may be direct due to the presence of both asexual and sexual life stages including sporulated oocysts of the coccidian within the kidneys (or nephridium) of a single host (Friedman et al. In preparation, Steinbeck et al. 1992). In the study that follows the question of whether the renal coccidian was transmissible directly or indirectly via infective waters to seed abalone was examined. Also, transmission directly from infected red abalone to pinto abalone from British Columbia (B.C.), Canada that were free of renal coccidia was tested.

### METHODS

**Abalone.** Seed red abalone, *Haliotis rufescens*, that were free of renal coccidia were obtained from a culture facility in Pt. Hueneme, California. The seed ranged in size from 3.55 mm to 11.90 mm and weighed an average of 0.10 grams. Cultured red abalone that were reared at the California Department of Fish and Game (CDFG) Marine Culture Laboratory (MCL), Monterey, California

were used as the source of coccidia in all studies. The red abalone ranged in size from 61.6 mm to 89.2 mm and weighed an average of 65.05 grams. Wild pinto abalone, *H. kamschatkana*, from Vancouver Island and the Queen Charlotte Islands, B.C., Canada, were donated by the Canadian Department of Fisheries and Oceans. The pinto abalone ranged in size from 90.9 mm to 130.1 mm and weighed an average of 179.33 grams. In this study the relationship of weight to length of individual abalone was used to determine their condition.

**Histology.** All mortalities and samples were fixed in Davidson's solution (Shaw & Battle 1957) and processed for routine paraffin histology (Luna 1968). Deparaffinized 5  $\mu$ m sections were stained with Harris' hematoxylin and eosin (Luna 1968) and viewed by light microscopy. Parasites were enumerated using the following scale at 200 times magnification: (0+) no coccidia, (1+) 1–10 coccidia per field of view, (2+) 11–100/field, (3+) 101–1000/field and (4+) >1000 per field or all cells infected with coccidia.

**Seed Experiment.** We examined whether the coccidia were transmissible to seed red abalone via infective waters either directly from abalone or indirectly from an intermediate host inhabiting waters adjacent to MCL. Sixty seed abalone were weighed, measured, sampled and examined by histology upon initiation of the study. Abalone were randomly divided into six groups of 140 animals and were held in 12.5 l aquaria receiving flow-through seawater (31 ppt). The experiment at the MCL consisted of three duplicate treatments. Two groups of animals received unfiltered seawater at  $13 \pm 1^\circ\text{C}$  and each week were fed *Macrocystis pyrifera* (kelp) that had been rinsed in freshwater prior to feeding as the experimental treatment (T1). The remaining four aquaria received 1  $\mu$ m-filtered seawater at  $13 \pm 2.5^\circ\text{C}$  as the negative con-

control treatments (T2 and T3). Of these control aquaria, two received *M. pyrifera* each week that had been rinsed in fresh water (as above) prior to feeding (T2). Animals in the final two aquaria were fed 3 times per week with a prepared dry diet (T3) that was formulated and donated by Dr. Karen Norman-Boudreau (Sebastopol, California). A fourth group of animals not held at the MCL and referred to as 'treatment four' (T4) consisted of two barrels in Pt. Hueneme harbor (PHH) that each contained 2500 abalone (that were cohorts to those in T1-3). The abalone in T4 received *M. pyrifera* weekly that had been rinsed in sea water prior to feeding. Water temperatures at this site ranged from 13-18°C during the period of experimentation.

All mortalities and animals that were sampled from both locations during the experiment were weighed, measured (maximum length) and processed for histology. Ten abalone per aquaria (MCL) and 17 abalone per barrel (PHH) were sampled every 6-8 wk after initiation of the study. The abalone in T4 were not sampled at the 8, 10 and 13 mo intervals of the study. The experiment was terminated after 15 mo when the remaining abalone at MCL and 30 abalone per barrel (PHH) were sampled and examined by histology.

**Cohabitation Experiment.** Transmission of the coccidia from infected red abalone to pinto abalone during cohabitation was examined. Two replicate aquaria contained 35 pinto abalone as negative controls. An additional two aquaria had 35 red abalone as positive controls, and the final two aquaria contained 25 pinto and 10 red abalone as the experimental treatment. All animals were held at the Bodega Marine Laboratory (Bodega Bay, California) in 135 l aquaria receiving 1 µm-filtered flow-through seawater (31 ppt) at 13 ± 1°C. Each week abalone received *Macrocyctis pyrifera*, *Neroecystis luetkeana* and/or *Eggregia menziesii* that had been soaked in a 100 ppm iodine solution for 15 min and then rinsed in freshwater prior to feeding. All mortalities and abalone sampled during the experiment were weighed, measured and processed for histology. Five pinto abalone from each experimental and negative control aquaria and 5 red abalone from the positive control aquaria were sampled at 3, 6 and 17 mo after initiation of the study. The experiment was terminated after 18.5 mo when the remaining abalone were sampled and examined by histology.

## RESULTS

**Seed Experiment.** All (except one) control abalone were free of renal coccidian infections throughout the study. A single abalone from T2 (freshwater-rinsed kelp) sampled upon termination of the study had a 1+ (mild) coccidian infection in the left kidney. At the time this sample was taken the average length and weight of abalone in T2 were 30.22 mm and 3.53 g, respectively.

In group T1 coccidian infections were observed in the left kidney of 10-30% of the abalone sampled beginning 7 mo after initial contact with unfiltered seawater. At this time the average length and weight of the animals was 19.7 mm and 0.934 g, respectively. Infections in the T1 group were mild to moderate (1-2+) in abalone sampled after 7 mo, moderate to heavy (2-4+) in those sampled after 8 mo and heavy thereafter (10-13 mo). Infections in the right kidney were not observed until termination of the study when 43% and 55% (X = 49%) of the abalone remaining in the two replicate aquaria of T1 had moderate to heavy (2-4+) coccidian infection in one or both kidneys.

Mild (1+) coccidian infections were observed in the left and/or right nephridium of 29% of the abalone sampled from T4 3 mo after introduction into infective waters. At this time the average length of the abalone was 14.7 mm and the average weight was

0.371 g. The proportion of abalone with renal coccidian infections increased between each subsequent sample. Abalone introduced into Pt. Hueneme harbor showed moderate to heavy (2-4+) infections in the left kidney of 47% of the abalone sampled after 5 mo and in 97% of those sampled after 7 mo. A single abalone sampled after 7 mo also had a mild (2+) coccidian infection in the right nephridium. All abalone sampled had moderate to heavy (2-4+) infections in one or both nephridia after 12 mo of culture in Pt. Hueneme Harbor.

Abalone survival was high in all treatments and was 98% and 97% in T1, 95% and 94% in T2, and 95% and 91% in T3. No abalone that died during the study had coccidian infections. Survival, though not quantified was also high for T4 (J. McMullen, personal communication). Abalone condition was determined using a graphic relationship of weight to length (Fig. 1A-D) and was similar in all treatments. Growth, however, as shown by an increase in weight and length, differed between the treatments. The abalone in T4 that were fed kelp and maintained in an embayment where water temperatures ranged between 13-18°C grew to the largest size relative to those in all treatments at the MCL Table 1, Fig. 1D). Animals in T2 that received kelp and 1 µm-filtered seawater grew the most of those held at MCL (Fig. 1B). Abalone in T1 that received unfiltered seawater and kelp grew to an intermediate size (Fig. 1A) and abalone in T3 that received a prepared diet and 1 µm-filtered seawater grew the least (Fig. 1C).

**Cohabitation Experiment.** All red abalone sampled throughout the experiment had moderate (1-2+) to heavy (3-4+) coccidian infections in one or both nephridia. None of the control pinto abalone developed coccidian infections during the study. No coccidia were detected in pinto abalone after 3 and 6 mo of cohabitation with red abalone. A single pinto abalone that died after 13.5 mo of cohabitation with red abalone had a moderate (2+) coccidian infection in both nephridia. No other pinto abalone died during the 18.5 mo of the study. Approximately 30% of the experimental pinto abalone sampled after 17 mo of cohabitation had moderate (1-2+) coccidian infections in one or both kidneys; the remaining 70% had heavy infections (3-4+). Upon termination of the study (18.5 mo) all remaining pinto abalone had moderate to heavy infections with coccidia. The condition of the pinto abalone did not change upon infection with coccidia (Fig. 2A). The condition of the red abalone remained unchanged during the study (Fig. 2B). However, the red abalone grew substantially, while the pinto abalone showed little growth during the experiment.

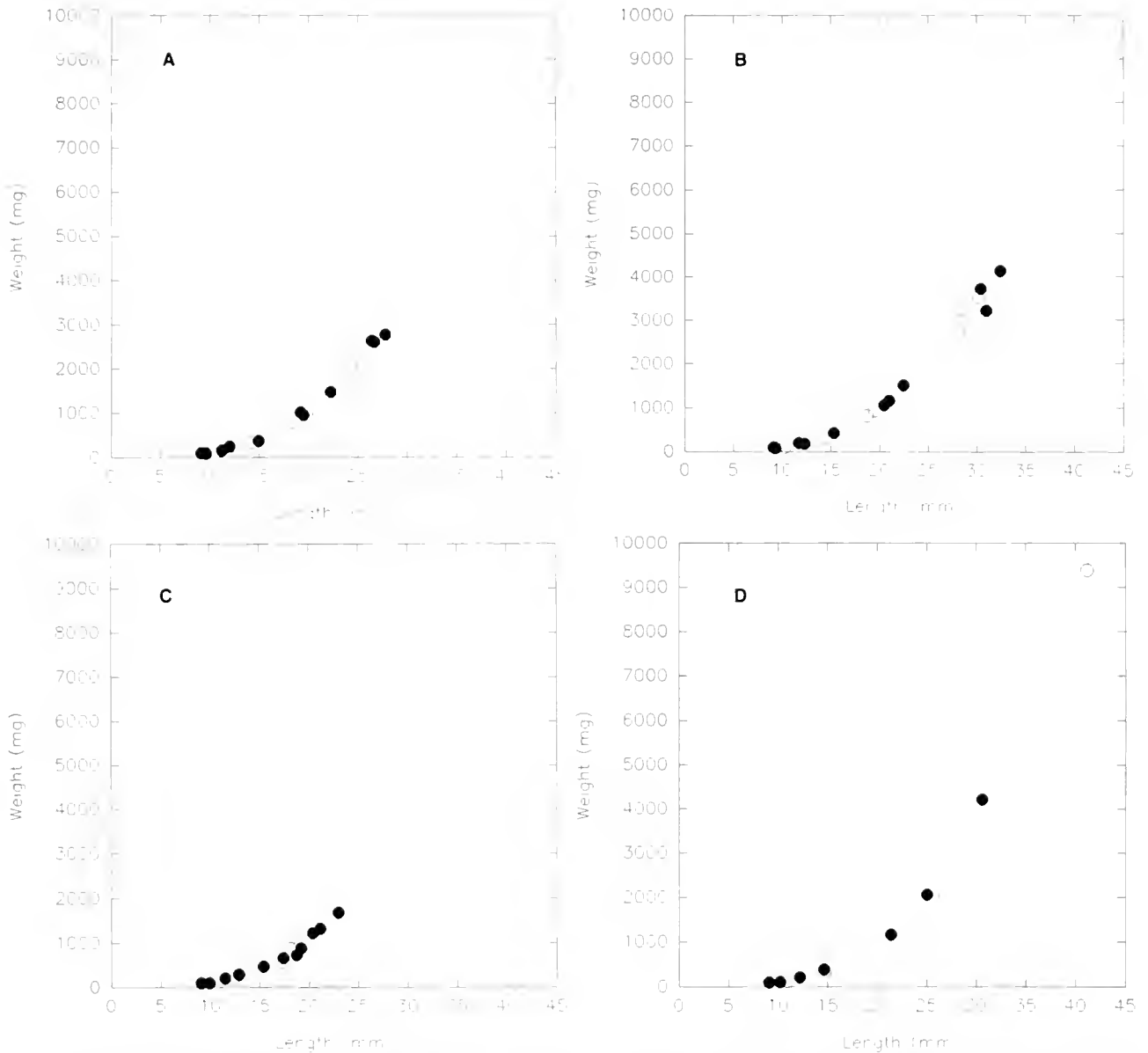
## DISCUSSION

Both direct and indirect transmission of coccidian parasites between their aquatic hosts, such a fish, have been demonstrated

TABLE 1.

This table indicates the average increase in weight and length of juvenile red abalone in the four treatments of the seed study one year after initiation.

Treatment	Initial		Increase in	
	Weight (mg)	Length (mm)	Weight (mg)	Length (mm)
1	100	9.11	2235	16.35
2	100	9.11	3240	20.25
3	100	9.11	1005	10.53
4	100	9.11	6695	26.76

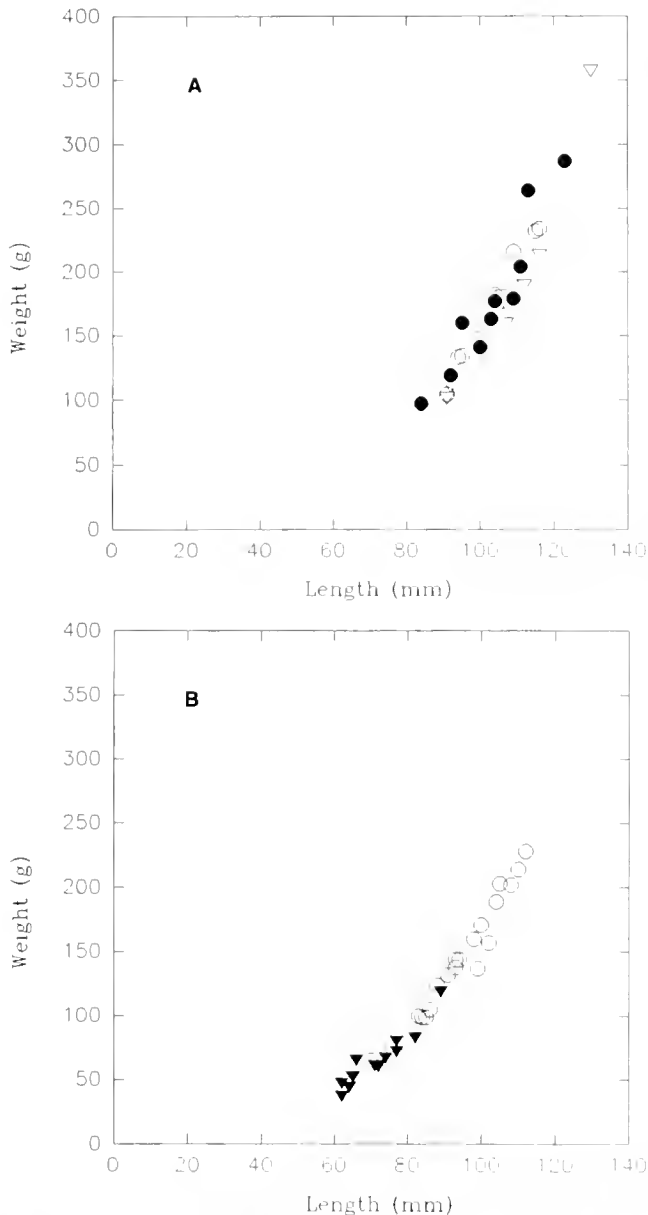


**Figure 1.** The relationship of weight (mg) and length (g) for red abalone in the seed experiment is illustrated by this graph. Each point on a graph represents the average weight and length for a sample of 10 abalone at a particular sampling period. Data from animals in treatments 1–4 are represented in Figures 1A–1D, respectively. The open squares represent one replicate and the filled squares represent the 2nd replicate group in each of the four treatments.

(Steinhagen and Korting 1988). Aquatic invertebrates, such as tubificid worms (Molnar 1979), mysid crustacea (Landau et al. 1975) and grass shrimp (Solangi and Overstreet 1980) have been reported to serve as intermediate or paratenic hosts of coccidia to which finfish serve as the definitive host. Direct transmission of coccidia between marine molluscan hosts has not been reported. Unsuccessful experimental transmission of coccidia has often been attributed to the lack of an intermediate host necessary for completion of a parasite's life cycle (Kent and Hedrick 1985). Successful direct transmission of coccidia in which a marine mollusc serves as the definitive host has not been reported. We have shown through natural exposures and an experimental trial that the renal coccidian of abalone is transmissible directly via a waterborne stage. Additionally, direct transmission of the coccidian from red to pinto abalone was demonstrated by a cohabitation trial in the laboratory.

Results from the seed experiment indicate that under the conditions used in this study, infections with coccidia developed within 3–7 mo after introduction into infective waters. The more rapid infection of animals in T4 (after 3 mo) relative to those in T1 (after 7 mo) may be density-dependent (Cheng 1986) due to higher numbers of abalone and/or exposure of abalone to greater numbers of infective stages in T4 than in T1. Infection may also be temperature-dependent (Cheng 1986) and this may influence the duration between exposure and infection and may explain the disparity in timing of initial infections between the two treatments. Abalone in T4 (Pt. Hueneme harbor) experienced temperatures between 13–18°C, while those at the MCL received seawater at 13 ± 1°C.

Microscopic examination of stained tissue sections revealed that coccidian infections tended to develop initially in the left and then spread to the right nephridium within 4–8 mo after initial



**Figure 2.** This graph illustrates the relationship of weight (g) and length (mm) for abalone in the cohabitation study. A. Pinto abalone sampled upon initiation of the study are depicted using ( $\nabla$ ). Control pinto abalone sampled 17 mo after initiation of the study are shown using ( $\bullet$ ), while pinto abalone sharing aquaria with red abalone for 17 mo are illustrated using ( $\circ$ ). B. The weight to length relationship of red abalone in the presample are depicted by ( $\blacktriangledown$ ) and those sharing aquaria with pinto abalone for 18.5 mo are shown using ( $\circ$ ).

infection. This pattern of infection may be attributed to the unidirectional flow of pericardial fluid into the left kidney where glucose and electrolytes are resorbed followed by passage of this fluid (primary urine) into the right kidney where nitrogenous wastes are removed and urine is formed and excreted (Andrews 1985, Daguzan 1981, Harrison 1962).

The more rapid growth of abalone in T4 relative to those in T1–3 may be attributed to higher water temperatures (13–18°C and 13°C, respectively) and better husbandry methods at Pt. Hueneme than at MCL. Abalone reared at water temperatures approaching

18°C grow more rapidly than those grown in lower water temperatures (J. McMullen, The Ab Lab and E. Ebert, California Department of Fish and Game, personal communications). The poor growth of abalone in T3 relative to all other treatments may have been due to a nutritional deficiency in the prepared diet, poor palatability of the diet, or to feeding of inadequate amounts of the prepared diet. The cause of the intermediate growth of animals in T1 relative to those in T2 and T3 is unknown. The significance of the differences in abalone growth in the three treatments at the MCL is not known. The low prevalence (10–30%) of animals with coccidia in T1 during the first nine of ten samples suggests that renal infections with this coccidian did not influence growth of the abalone in T1.

Results from the cohabitation study suggested that the renal coccidian was directly transmissible between red and pinto abalone and, thus, has a homoxenous life history. The coccidian infections had no observable negative effects on the pinto abalone (Fig. 2A). This may indicate that the coccidian is not a serious pathogen. Alternately, the duration of the infections may have been too short or the health of the abalone may not have been compromised sufficiently to have been measured by the physical parameters we examined. Ruff and Reid (1977) illustrated the importance of the nutritional and physiological condition of the host in the pathogenesis of coccidian infections in higher vertebrates. These factors may also be integral in the pathogenesis of coccidiosis in marine invertebrates. Coccidia have been observed in both healthy and moribund abalone in California (Friedman 1991, Haaker et al. 1992). No statistical association was found between abalone condition and intensity of coccidian infection in apparently healthy and moribund abalone collected from the field (Friedman 1991). Coccidia have been observed in a variety of marine molluscs (Kudo 1966, Cheng 1967, Morado et al. 1984). These infections appeared to be nonlethal but were associated with abnormal behavior of the infected host on at least one occasion. Morado et al. (1984) observed numerous native littleneck clams, *Protothaca stamina*, that were heavily parasitized with renal coccidia lying on the surface rather than burrowed in the substratum. The pathogenicity of coccidia in marine organisms has not been studied thoroughly and may account, in part, for the lack of association between presence of the coccidia and disease or mortality.

The homoxenous life history of this parasite differs from most previously reported coccidian infections in marine molluscs (Cheng 1967, Morado et al. 1984). Kudo (1966) describes most coccidia that infect marine molluscs as being heteroxenous including many members of the genera *Aggregata*, *Hyaloklossia*, *Pseudoklossia*, *Merocystis* and *Myriospora*. Morado et al. (1984) reported a coccidian from the native littleneck clam, *Protothaca staminea*, similar in morphology to the coccidian from abalone (Steinbeck et al. 1992, Friedman et al. in preparation) with the exception of the number of sporozoites per sporocyst (4 in clams and 2 in abalone) and the presence of a resting stage or cyst that has not been observed in abalone. All life stages of the coccidian in the clams were observed within a single host as evidenced by histological examination. However, transmission of the coccidian in littleneck clams has not been examined. More information on the taxonomy, host specificity, transmissibility and pathogenicity of marine coccidia is warranted due to the increased interest in marine aquaculture and conservation of native species. The ability by both the aquaculture industry and resources managers to make informed management decisions requires this information.

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## DISTRIBUTION, SHELL GROWTH AND PREDATION OF THE NEW ZEALAND OYSTER, *TIOSTREA* (= *OSTREA*) *LUTARIA* HUTTON, IN THE MENAI STRAIT, NORTH WALES

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**ABSTRACT** A population of the New Zealand oyster, *Tiostrea lutaria*, at Tal-y-foel in the Menai Strait was surveyed during June–July 1992. Oyster density was highest in the immediate vicinity of the Ministry of Agriculture, Fisheries and Food (MAFF) experimental shellfish beds where this species had been introduced in 1963, but a few isolated oysters occurred up to 0.5 km from this locality. Intertidal and subtidal populations showed clear differences in size composition. Analysis of size frequency distributions using the method of Bhattacharya (1967) indicated that these populations could be broadly resolved into two (intertidal) and four (subtidal) overlapping size classes.

The age of individual oysters was determined from annual growth lines in acetate peel replicas of polished and etched sections of the shell. Although growth rates of intertidal and subtidal oysters were similar during the first few years of growth these populations exhibited different Von Bertalanffy growth constants ( $K = 0.597 \pm 0.398$  &  $0.299 \pm 0.068$ , respectively) and attained a different asymptotic length ( $L_{\infty} = 79.89 \pm 17.77$  &  $108.48 \pm 9.56$  mm, respectively). The maximum age of subtidal oysters (8 yrs) was also greater than that of intertidal oysters (5 yrs).

Laboratory predation experiments showed that whilst crabs, *Carcinus maenas* and *Cancer pagurus*, fed voraciously on the Pacific oyster *Crassostrea gigas*, they were reluctant to feed on *T. lutaria*, particularly when both species were presented simultaneously. Video recordings of foraging crabs suggested that this reluctance to feed on *T. lutaria* was due to mechanical difficulties associated with prey handling.

**KEY WORDS:** oyster, *Tiostrea lutaria*, growth, predation

### INTRODUCTION

For centuries the native flat oyster, *Ostrea edulis*, has been gathered from around the coast of the British Isles where there has been a long established fishery (Spencer 1990). However, over the past century there has been a steady decline in landings of this species due mainly to disease, pollution, and spatfall failure which have resulted in the devastation of the indigenous stocks (Yonge 1960, 1970). Various attempts have therefore been made to improve the dwindling oyster fishery by introducing non-native species such as the Pacific oyster, *Crassostrea gigas*, and the eastern (= American) oyster, *Crassostrea virginica*. Experimental growth trials of several bivalve species have been undertaken by the Ministry of Agriculture Fisheries and Food (MAFF) at the Conwy laboratory in North Wales. In 1963 *Tiostrea* (= *Ostrea*) *lutaria* was introduced from New Zealand, where it supports an important commercial fishery and held in quarantine in the Conwy laboratories (Walne 1979). These oysters were induced to spawn and after the resulting spat had been diagnosed to be free of parasites and disease, these were transferred to the experimental shellfish beds at Tal-y-foel in the Menai Strait, N. Wales. Oysters subsequently became established as a result of successive natural spatfalls. In New Zealand *T. lutaria* is typically found in areas of clear water and strong tidal flow.

Oysters belonging to the genus *Tiostrea* have characteristic larvae with none of the distinctive features of other ostreid larvae (Chanley and Dinamani 1980). The larvae of the New Zealand oyster, *T. lutaria*, like those of the Chilean oyster, *Tiostrea* (= *Os-*

*trea chilensis* are incubated throughout almost the entire larval period and the free swimming stage is usually measured in minutes or hours (Millar and Hollis 1963, Walne 1963). *T. lutaria* and *T. chilensis* are now thought to be conspecific with the latter name apparently taking priority (Buroker et al. 1983).

Since its introduction, *T. lutaria* has become an established component of the local fauna at Tal-y-foel, yet little or no information is available regarding this population. In this paper we present some data concerning the distribution, growth characteristics and vulnerability of *T. lutaria* to crab predation.

### MATERIALS AND METHODS

Samples of *Tiostrea lutaria* were collected from Tal-y-foel at the south-western end of the Menai Strait (Fig. 1). Oysters occur predominantly in a discrete band at low water of spring tides and extend into the shallow subtidal zone. The shore at this site is protected by a sandbank, Traeth Gwyllt, which separates the Tal-y-foel inlet from the main tidal channel. Here a tidal current of 1–2 knots, which flows parallel to the shore, is associated with a tidal rise and fall of about 6 m on spring tides and 3 m on neap tides. Approximately 300 oysters were collected in June 1992 from an intertidal site adjacent to the MAFF experimental shellfish beds and 100 oysters from the subtidal zone in the same area in July 1992; all oysters were deep-frozen until required.

A transect line was established (Fig. 1 A–B) and estimates of the density of oysters were made at intervals along its length from ten random 0.1 m<sup>2</sup> quadrats. The length, (umbo-rim axis), of all oysters was measured with vernier calipers and population size frequency distributions constructed. These distributions were then separated into their component size classes using the method of Bhattacharya (1967).

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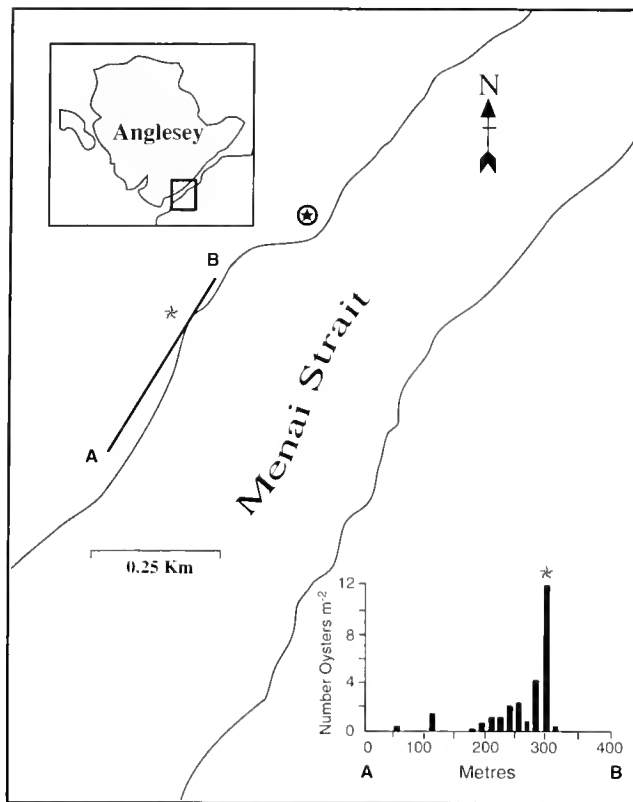


Figure 1. Location of the study site at Tal-y-foel in the Menai Strait, North Wales. Inset bottom right, shows the distribution of oysters along transect A-B; (\*) indicates the central location of the MAFF experimental shellfish beds, (⊗) the location of a commercial Pacific oyster farm.

Although oysters generally lack any clear annual growth checks on the shell surface, growth lines deposited in the umbonal region are known to have an annual periodicity (Richardson et al. 1993). Using these annual lines, the ages of 50 intertidal oysters and 10 subtidal oysters, were determined. The umbo region of each shell was embedded in resin and acetate peel replicas prepared of the cut, ground, polished and etched surfaces following the methodology described by Richardson et al. (1993). The number of annual growth lines in acetate peels was counted under the light microscope.

The distance between individual growth lines and/or the growing edge of the umbo (Fig. 3) was measured using a micrometer eyepiece. Because age had to be estimated from annual lines in the umbonal region, shell length at any given age could not be measured directly. However, the relationship between umbo length ( $L^1$ ) and overall shell length ( $L$ ) was established by measuring umbo length and overall shell length in a range of oysters of different sizes. Shell length of oysters of any given umbo length could thus be estimated from the following regressions:

$$\text{Intertidal population } L = 26.7 + 6.88 L^1 \quad (r^2 = 0.716)$$

$$\text{Subtidal population } L = 28.6 + 7.12 L^1 \quad (r^2 = 0.698).$$

Using these relationships growth curves for intertidal and subtidal populations were constructed and the Von Bertalanffy growth constant ( $K$ ) and maximum shell length ( $L_\infty$ ) determined for each population using an asymptotic regression program (Allen 1966).

Sub-samples of 50 oysters each containing a representative size range of the intertidal and subtidal populations were selected and their shells cleaned of any encrusting organisms. Shell length, height and width (Fig. 3) were measured to the nearest 0.1 mm and the soft tissues removed by briefly placing each oyster into boiling water. Shells and wet flesh (blotted dry) were weighed separately

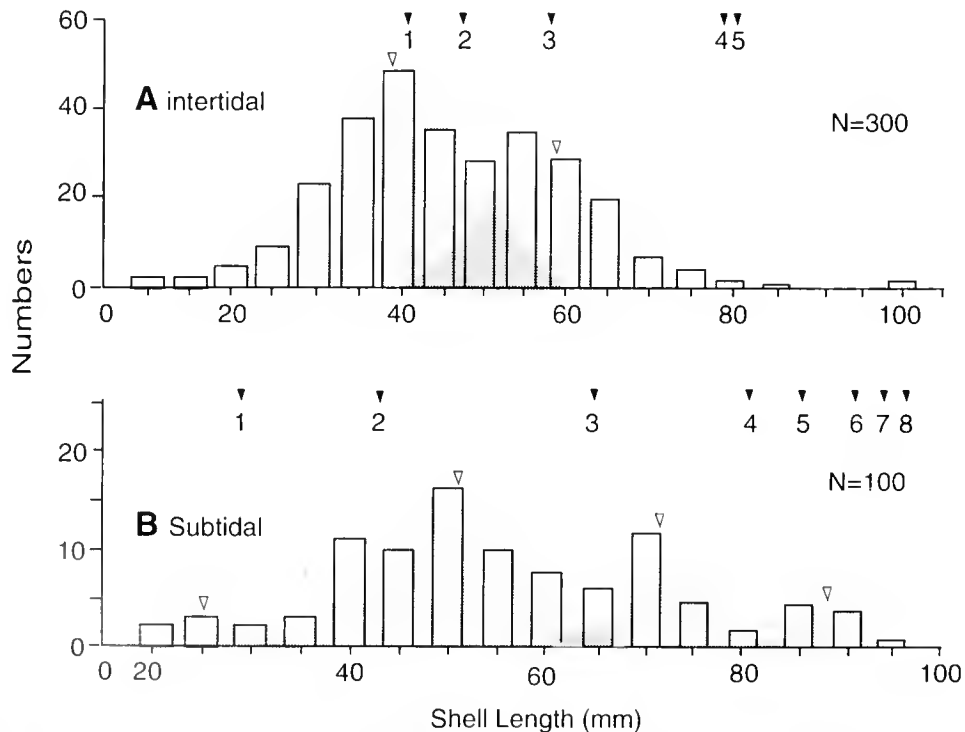


Figure 2. Size frequency distributions of (A) intertidal and (B) subtidal, populations of *T. lutaria* at Tal-y-foel. Size classes (shaded) fitted using the method of Bhattacharya (1967); open symbols denote mean values of individual size classes, solid symbols the estimated age (yrs) determined from shell growth lines in population subsamples.



and the flesh then oven dried to constant weight at 60°C. All weighings were made to the nearest 0.01 g on a top loading balance. The data were then examined for evidence of differential growth between various growth parameters by testing each pair of size variables  $x$  and  $y$  for their fit to the allometric equation  $y = ax^b$  where  $a$  &  $b$  are constants. When written in its linearised logarithmic form this becomes  $\log y = \log a + b \log x$ . The constants ( $a$  &  $b$ ) were estimated by regression analysis and pairs of size variables between the two populations compared using two way analysis of variance with a covariate.

Shore crabs, *Carcinus maenas*, 42–75 mm carapace width (CW), and edible crabs, *Cancer pagurus*, 40–126 mm (CW), were collected from the low shore in the Menai Strait. These were kept individually in 50 L aquaria supplied with running seawater at ambient temperature (range 16–17°C). All feeding experiments used only male crabs to avoid any potential bias that might arise through minor sexual differences in chelal morphology and feeding behaviour. Hunger levels were standardised by starving crabs for 48 h before each experiment. Feeding behaviour using *Crassostrea gigas* and *Tiostrea lutaria* as food was studied under regimes of unlimited and limited food availability, the latter by presenting crabs of three different size ranges (see Table 2) with five oysters in each of four size categories. These were placed at random over the floor of the aquarium and the number of oysters in each size class which had been consumed was monitored daily for 14 d. None of the oysters which were eaten were replaced. Similar experiments assessed the effects of diets with unlimited prey. Here, however, the experiments were monitored daily and any oysters which had been consumed were replaced by ones of similar size so as to maintain constant prey availability. Prey handling behaviour of individual crabs was studied in a darkened room using an infra-red camera connected to a time-lapse video recorder.

## RESULTS

The distribution and abundance of intertidal *Tiostrea lutaria* at Tal-y-foel are shown in Fig. 1. The substratum along transect A-B consisted mainly of small stones and empty bivalve shells overlying a layer of compacted sediment. To the north and south of the MAFF shellfish beds, the substratum progressively changes to mud and sand. Population density of oysters along this transect varied markedly. The highest mean density, 11 individuals  $m^{-2}$ , occurred close to the MAFF experimental site ( $\star$ ), with a sharp decline in density either side of this location. *T. lutaria* frequently occurred in clumps, typically with several oysters attached to individual shells or stones. In addition to *T. lutaria* numbers of the mussel, *Mytilus edulis*, and the Pacific oyster, *Crassostrea gigas* were also common along the transect. No *T. lutaria* were found north of the transect line where the sediment consisted of fine mud, although a few individuals were found attached to the supporting structures of oyster cages at a commercial Pacific oyster farm located about 0.5 Km beyond the transect ( $\otimes$  Fig. 1). Similarly, no *T. lutaria* were found south of the transect where the substratum consisted predominantly of sand.

Length-frequency distributions of the two *T. lutaria* populations are illustrated in Fig. 2. The largest oysters sampled from the intertidal and subtidal populations measured 100 mm and 95 mm in shell length, respectively. However, the intertidal population contained substantially more smaller (<50 mm in shell length) individuals, 56% compared with only 31% in the subtidal population. Most of the largest (>80 mm) oysters occurred subtidally

(Fig. 2B). Using the method of Bhattacharya (1967), the intertidal and subtidal length frequency distributions could be resolved respectively into either 2 or 4 overlapping size classes.

Figure 3B shows a diagrammatic radial section through the shell and umbo region of *T. lutaria* whilst Fig. 3C illustrates the typical appearance of four growth lines in an acetate peel of the umbo region of a subtidal oyster. The well defined annual growth lines in this region were used to estimate age, and Von Bertalanffy growth curves for the intertidal and subtidal oyster populations were fitted to these data (Fig. 4). In both populations, size increased rapidly during the first three years but thereafter growth slowed down particularly amongst the intertidal oysters. The estimated maximum age of oysters varied from 5 years intertidally to 8 years for the subtidal population. Both populations exhibited different growth constants and a different asymptotic length ( $K = 0.597 \pm 0.398$  &  $0.299 \pm 0.068$  and  $L_\infty = 79.89 \pm 17.77$  &  $108.48 \pm 9.56$  mm for the intertidal and subtidal populations respectively). Thus, whilst the structure of these oyster populations comprised different size classes these did not correspond closely to the average size of oysters estimated from shell sections (Fig. 2). Regression analysis for various combinations of size parameters in the two *T. lutaria* populations are shown in Table 1. None of the growth coefficients ( $b$ ) departed significantly from isometry indicating that these oysters maintain geometric similarity with increasing body size. Moreover, when pairs of size variables were compared using a two-way analysis of variance with a covariate, no significant differences could be detected between the intertidal and subtidal populations (Table 1).

Figure 5 shows the results of experiments in which *Carcinus maenas* and *Cancer pagurus* were fed a restricted diet of either *C. gigas* or *T. lutaria*. Two salient points emerge from this particular series of experiments. Firstly, very few *T. lutaria* were consumed by *C. maenas* or *C. pagurus* whereas both crabs fed extensively on *C. gigas*. Moreover, *C. maenas* ate *T. lutaria* only when most of the small *C. gigas* had been consumed (Fig. 5). Secondly, when feeding on *C. gigas*, crabs first selected the smaller size classes of prey and only after these had been substantially depleted did they proceed to attack progressively larger oysters. Similar results were obtained when these crabs were fed unlimited diets of oysters (Fig. 6). Again, relatively few *T. lutaria* were consumed, particularly by *C. maenas*, whilst the preferred size range of *C. gigas* was broadly related to crab size. When *C. maenas* and *C. pagurus* were each presented with an unlimited diet consisting of equal numbers of *C. gigas* and *T. lutaria* of similar size, both crabs fed extensively on *C. gigas*, whereas none of the *T. lutaria* were eaten (Table 2). Nevertheless, video recordings of foraging crabs clearly revealed that individual *T. lutaria* were repeatedly handled by both species despite their inevitable rejection. Moreover, exposed flesh of *T. lutaria* was readily accepted whenever this was offered to hungry crabs suggesting that the reluctance to feed on this species is due largely to mechanical difficulties associated with shell crushing. Table 2 further reveals the close relationship that exists between the size of the crab and that of its preferred prey.

## DISCUSSION

In the British Isles there have been few studies on the biology and cultivation of *T. lutaria* since its introduction in the early 1960's (Walne 1979, Utting 1987). The first consignment of New Zealand oysters reared at Conwy and set out in the Menai Strait, died as a result of the severe winter of 1962/63 and only 30% of

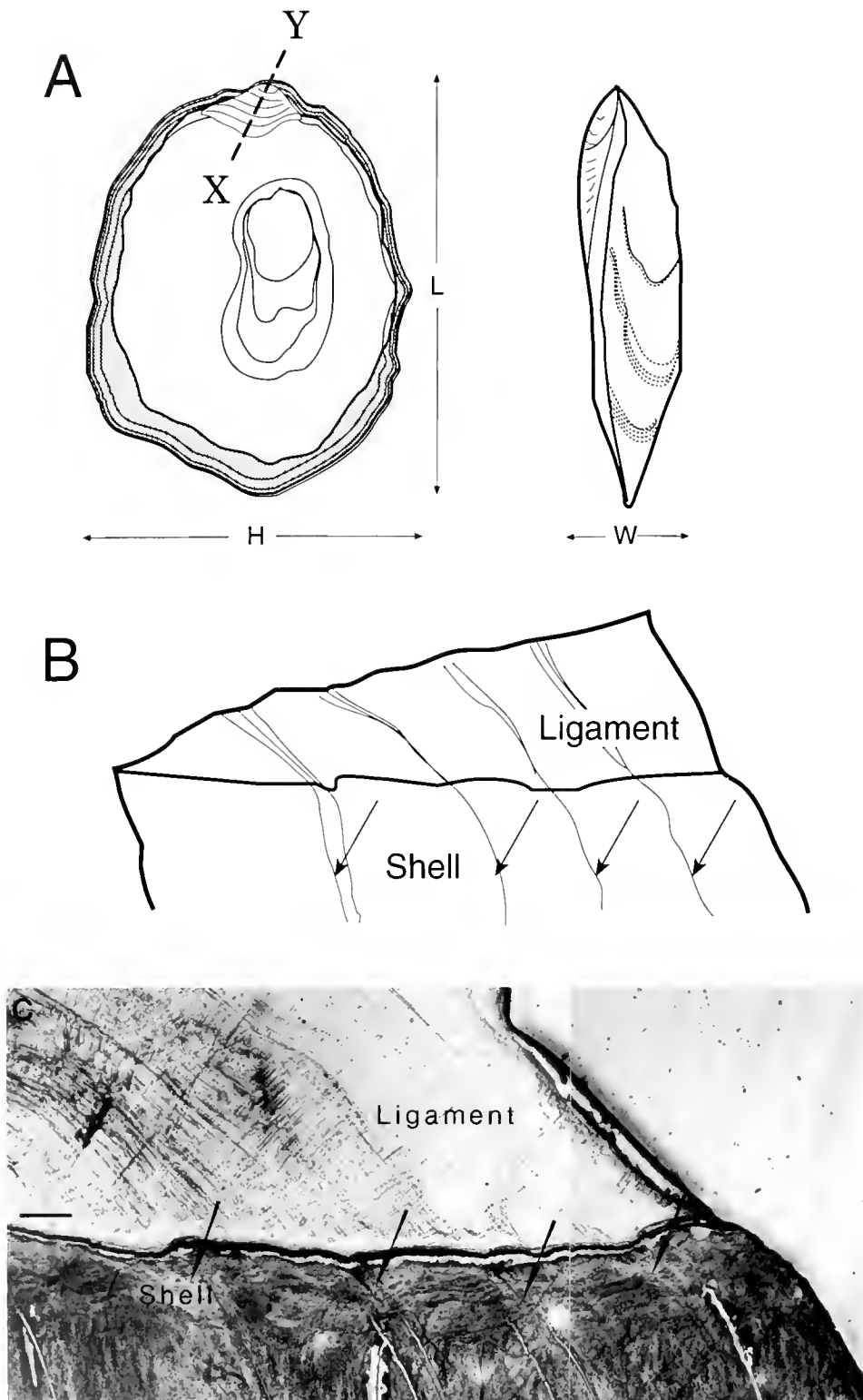


Figure 3. A) Shell of *T. lutaria*, H = Height, L = Length, W = Width and x-y = axis of section through the umbo region of the shell, B) Radial section through the umbo and C) Photomicrograph of an acetate peel replica of a section through the umbo of a subtidal oyster collected in July 1992; four clear growth lines (arrows) are marked. Scale bar = 100  $\mu\text{m}$ .

a second batch which was transferred to Tal-y-foel in 1970 survived. Nevertheless, during the following summer juvenile oysters were observed attached to adult shells, indicating that a successful natural settlement had occurred.

Our survey of the Menai Strait at Tal-y-foel during the summer of 1992 showed that *T. lutaria* was widely distributed over half a kilometer of a narrow band of shoreline with the highest densities occurring close to the MAFF experimental shellfish beds where

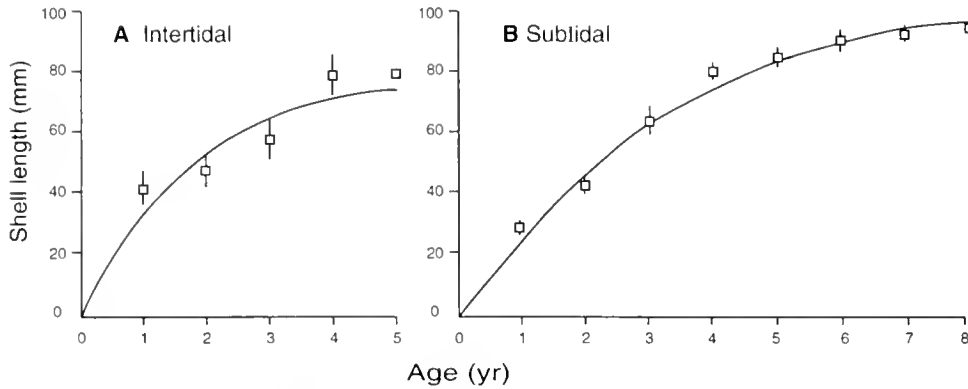


Figure 4. Population growth rates of A) intertidal and B) subtidal, populations of *T. lutaria* at Tal-y-foel. Values are means ( $\pm$  SD). Curves fitted using the Von Bertalanffy growth equation:

Intertidal,  $Lt = 79.89 (1 - \exp(-0.597t))$ ;

Subtidal,  $Lt = 108.48 (1 - \exp(-0.299t))$ , where  $Lt$  is shell length (mm) at time  $t$ .

this population had originally been introduced. Beyond this site the density of *T. lutaria* declined rapidly due to the lack of suitable settlement substratum, though individuals were found attached to the supporting structures of commercial oyster trays at a nearby shellfish farm. The localised distribution of *T. lutaria* in the Menai Strait presumably reflects the limited dispersal ability of this brooding oyster with most settlement occurring close to the adult population. In New Zealand Hickman (1987) found that *T. lutaria* settled abundantly on natural and artificial surfaces placed close to adult oysters. Cranfield (1968 a,b) showed that the larvae of *T. (=Ostrea) lutaria* were released at an advanced stage of development and settled soon after their liberation. Minimal spatfall occurred away from the adult stock. Some of the oysters collected during the present study were extensively covered by juvenile oysters (<10 mm) which had presumably crawled away upon their release and settled directly on the adult shells.

Size-frequency distributions within some bivalve populations are characteristically polymodal with each mode representing an individual year class. By estimating the mean size of these modal distributions the mean population growth rates can be estimated. This, however, is usually possible only when the period of recruitment to the population is relatively restricted and where growth rates of individuals within each cohort are fairly uniform (Cerrato 1980). Where annual recruitment is more extended and individual

growth rates more variable, size classes may overlap to such an extent that size frequency distributions are of little or no value for estimating population growth rate (Richardson et al. 1990). Even when size-frequency analysis is used, it can provide only a measure of the average growth of individuals within the population and such estimates may have been substantially modified by size-specific mortality.

When analysed by the Bhattacharya method, the intertidal and subtidal *T. lutaria* populations at Tal-y-foel could be resolved respectively into two and four relatively distinct but overlapping size classes. Although it is conceivable that these may represent dominant cohorts within these populations, our analyses of internal shell growth lines indicate that at least five year classes are present in the intertidal population and as many as eight in the subtidal population. Some of these year classes, particularly those comprised of older oysters may, however, be only poorly represented within these populations. Furthermore, the mean size-at-age estimated from growth lines does not correspond closely with that obtained from analysis of population structure (Fig. 2). Annual growth lines have been observed in shell sections of many bivalves, e.g. *Mya arenaria* (Brousseau 1979), *Arctica islandica* (Ropes & Murawski 1983), *Modiolus modiolus* (Anwar et al. 1990) and *Ostrea edulis* (Richardson et al. 1993) and have been widely used to study growth rates and population structure from

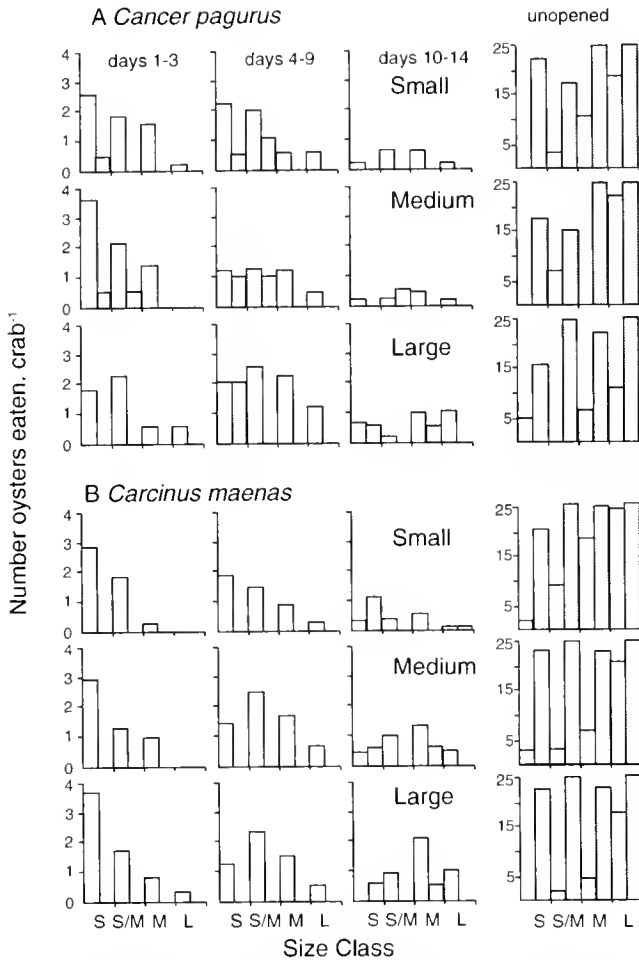
TABLE I.

Regression constants for various combinations of size parameters in *Tiostrea lutaria* from Tal-y-Foel in the Menai Strait.

Dependent Variable (y)	Independent Variable (x)	Regression Constants							Comparison of:	
		Intertidal			Subtidal					
		a <sup>1</sup>	b <sup>2</sup>	r <sup>2</sup>	a	b	r <sup>2</sup>	F (slopes)	F (intercepts)	
Height	Length	-0.362	0.852	0.664	-0.558	0.948	0.840	0.800	1.060	
Width	Length	-0.019	0.984	0.925	0.052	0.930	0.915	0.880	0.490	
Height	Width	-0.312	0.984	0.685	-0.470	0.939	0.778	0.408	0.690	
Shell weight	Length	-3.749	2.842	0.926	-4.001	2.949	0.953	0.440	0.820	
Dry flesh weight	Length	-5.581	3.067	0.911	-5.963	3.254	0.881	0.730	0.980	
Total body weight	Length	-3.820	2.941	0.940	-3.790	2.873	0.954	0.230	0.010	

y and x are the dependent and independent variables, respectively in the equation  $\log y = \log a + b \log x$

<sup>1</sup> and <sup>2</sup> are the regression constants a and b.



**Figure 5.** Numbers of *C. gigas* (open columns) and *T. lutaria* (shaded columns) consumed by 3 size classes of A) *C. pagurus* and B) *C. maenas*. Five crabs in each size class were fed a restricted diet of either *C. gigas* or *T. lutaria* and all experiments run for 14 d; the number of oysters unopened at the end of the experiment is also indicated. Size classes (mm) of *C. gigas* and *T. lutaria* (in parenthesis) denoted as small (S) <40 (<10); small-medium (S/M) 40–49 (11–44); medium (M) 50–69 (45–54); large (L) >70 (>50).

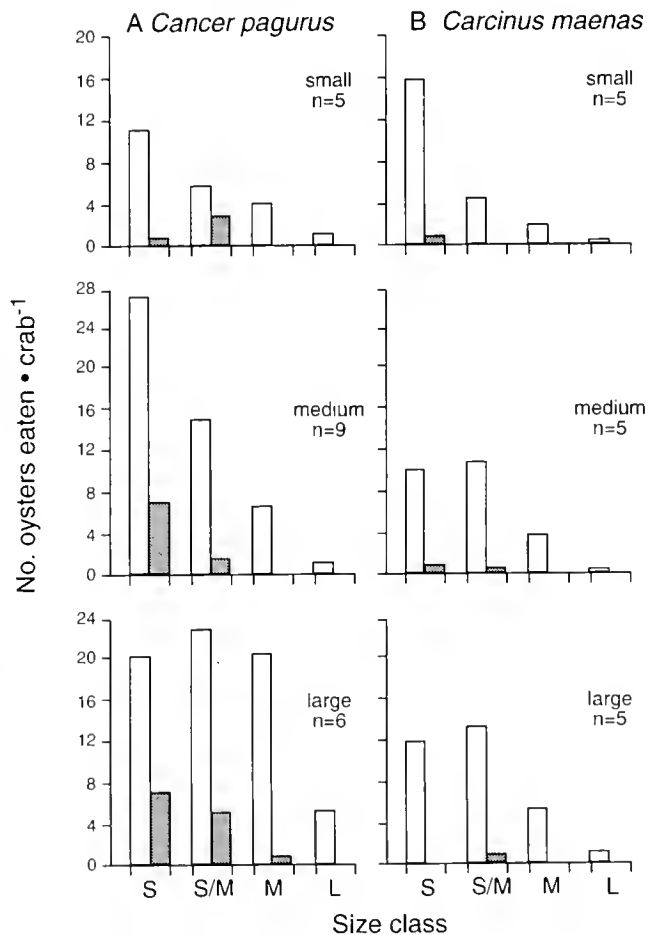
single population samples. The ability to use these growth lines to measure growth rates is especially valuable, particularly in habitats where regular sampling is not possible, for example offshore production platforms (Richardson et al. 1990).

Growth rates of the intertidal and subtidal populations of *T. lutaria* were similar over the first few years of life, both populations achieving mean lengths of 60–65 mm by their third year; thereafter growth of intertidal oysters slows appreciably, resulting in a much lower asymptotic size. The largest oyster recorded during this investigation was a four year old intertidal individual measuring 100 mm.

The growth rate of the Menai Strait *T. lutaria* population is broadly similar to that reported for *Ostrea edulis* populations from the Fal Estuary in Cornwall and the Blackwater Estuary in Essex, both of which attained shell lengths of approximately 55–60 mm after 3 years of growth (Richardson et al. 1993). However, both these populations lived longer and achieved slightly smaller body size (9 & 14 years and 91 & 76 mm, respectively) than *T. lutaria* at Tal-y-foel. Whilst *T. lutaria* might be considered a suitable alternative commercial species to *Ostrea edulis*, both of these

species are known to be susceptible to *Bonamia ostreae* (Utting 1987), a disease that has devastated the flat oyster, *Ostrea edulis*, industry in Europe (Grizel et al. 1988).

Various species of crabs are known to be major predators of shellfish including oysters (e.g. Parsons 1977, Krantz & Chamberlin 1978, Dare et al. 1983, Bisker and Castagna 1987). Whilst many of these crabs attack the smaller size classes (Elner and Lavoie 1983, Quayle 1988, Eggleston 1990) others feed on a wide size range of oysters. Yamada (1993) for example showed that large (43 mm CW) *Cancer oregonensis* was capable of opening *C. gigas* over 60 mm in length whilst even small (20 mm CW) crabs consumed 30 mm oysters. Medium-sized crabs (20–35 mm CW) each consumed an average of one young oyster (20–40 mm) per day. Thus, assuming a very modest consumption rate of 0.1 oysters · crab<sup>-1</sup> · d<sup>-1</sup>, Yamada (1993) estimated that the presence of 5 crabs within an experimental tray could effectively reduce oyster survival by ≈40% over the 8-month period during which these oysters are normally kept in suspended trays. In the present investigation *C. maenas* and *C. pagurus* were capable of feeding on large (>70 mm) *C. gigas*, though smaller size classes were preferred even by the largest crabs. The main point to emerge from our laboratory feeding experiments is the reluctance of these two crab species to feed on *T. lutaria*, even though the exposed flesh of this oyster is readily ingested. In this context, it is inter-



**Figure 6.** Numbers of *C. gigas* (open columns) and *T. lutaria* (shaded columns) consumed by 3 size classes of A) *C. pagurus* and B) *C. maenas*. All crabs were fed an unlimited diet of either *C. gigas* or *T. lutaria* and each experiment run for 14 d. Size classes of oysters used as in Fig. 5.

TABLE 2.

Consumption of oysters by three size classes of (A) *Cancer pagurus* and (B) *Carcinus maenas* when presented with a mixed diet of *Crassostrea gigas* and *Tiostrea lutaria* under conditions of constant prey availability. Individual crabs were supplied simultaneously with 20 *C. gigas* and 20 *T. lutaria*, five from each size class; all experiments were run for 14 d.

Crab species/size	n	Number of oysters eaten · crab <sup>-1</sup>				Total	<i>T. lutaria</i> Total <sup>1</sup>
		Small (<40 mm)	Small-medium (40–49 mm)	Medium (50–69 mm)	Large (>70 mm)		
(A) <i>C. pagurus</i>							
small (43–59 mm CW)	4	12	2	1	0	15	0
medium (60–89 mm CW)	4	7	12	3	0	22	0
large (90–127 mm CW)	4	5	6	10	3	24	0
(B) <i>C. maenas</i>							
small (41–55 mm CW)	3	27	12	7	3	49	0
medium (56–65 mm CW)	3	25	25	10	4	64	0
large (66–75 mm CW)	3	11	15	15	5	46	0

<sup>1</sup> Individual size classes omitted as no *T. lutaria* were eaten.

esting to note that Cranfield (1968b) could find no evidence that the mortality of young *T. lutaria* in New Zealand was due to predation by either crabs or starfish. Video recordings of foraging crabs strongly suggest that the apparent inability of crabs to open *T. lutaria* is related to mechanical difficulties associated with prey handling. Thus, the relatively flat, roughly ovate shell of *T. lutaria* may be more difficult to grip than the deeply cupped and highly ornamented shell of *C. gigas*. It is clear that both *C. maenas* and *C. pagurus* selectively predate *C. gigas* rather than *T. lutaria*. The question now is how, and on what basis, does this selection occur?

#### ACKNOWLEDGMENTS

We are grateful to Dr. S. Utting and Mr. B. E. Spencer, Ministry of Agriculture, Fisheries and Food, Fisheries Laboratory, Conwy, North Wales for their help and suggestions during the course of this work and for their constructive comments of this manuscript. We thank Mr. R. Snyder for collecting the sample of subtidal oysters and Dr. R. Jones for supplying *C. gigas* used in the predation experiments. E.M.H.Al-R. is also grateful to the Bahrain Centre for Studies and Research for financial assistance.

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## THE STATUS OF THE KUMAMOTO OYSTER *CRASSOSTREA SIKAMEA* (AMEMIYA 1928) IN U.S. COMMERCIAL BROOD STOCKS

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**ABSTRACT** Long-standing confusion about the taxonomic status of the Kumamoto oyster has recently been resolved by demonstration of concordant molecular and reproductive-trait differences between *Crassostrea sikamea* (Amemiya 1928) and the closely related Pacific oyster *C. gigas* (Thunberg). Concern for the status of Kumamoto oyster brood stocks in the U.S. oyster-culture industry stems from reported contamination of these stocks with Pacific oysters and failure to find native populations of *C. sikamea* in Japan. Two commercial brood stocks of Kumamoto oysters were surveyed for allozyme and mitochondrial DNA markers that allow discrimination of Kumamoto and Pacific oysters. Pacific oysters were detected in both Kumamoto stocks, in one case, even after careful culling on the basis of shell morphology. Interspecific hybridization was also detected. Inadvertent admixture of Pacific oysters in Kumamoto brood stocks can result in hybridization because Pacific oyster sperm can fertilize Kumamoto oyster eggs. Hybridization and introgression thus pose potential threats to the integrity of Kumamoto oyster stocks in North America. Another threat to these stocks is loss of genetic diversity owing to random genetic drift in very small hatchery-propagated populations. Random changes in allozyme frequencies between generations of one Kumamoto oyster stock imply an effective population size of only 5.4. Steps should be taken both to eradicate Pacific oysters and interspecific hybrids from North American Kumamoto brood stocks and to retard the erosion of genetic diversity within these brood stocks.

**KEY WORDS:** Kumamoto oysters, Pacific oysters, North American stocks, hybridization, species mixture, genetic drift, mitochondrial DNA, allozymes, gametic compatibility

### INTRODUCTION

Three species of cupped oysters have been introduced from Japan to the west coast of North America for cultivation, the Pacific oyster *Crassostrea gigas* (Thunberg), the Kumamoto oyster *C. sikamea* (Amemiya 1928), and the Suminoe oyster *C. ariakensis* (Wakiya 1929), formerly *C. rivularis* (status currently being revised, Eugene V. Coan, pers. comm.). The Pacific oyster comprises the vast majority of west coast oyster production, while the Kumamoto oyster commands a sizeable share of the half-shell trade. Hatchery methods for breeding the Suminoe oyster have been determined (Robinson and Langdon 1993), but there is no commercial production yet. Recently, the Pacific and Kumamoto oyster were found to differ at seven nucleotide positions in a portion of the mitochondrial gene coding for the large subunit ribosomal RNA (Banks et al. 1993a). This observation, coupled with concern among U.S. oyster growers that Kumamoto oyster hatchery seed might be contaminated with Pacific oysters, prompted us to re-investigate the taxonomic status of the Kumamoto oyster (Hedgecock and Robinson 1992, Banks et al. 1993b).

Previous evidence for specific distinction of the Kumamoto and Pacific oysters includes differences in shell morphology (Amemiya 1928, Ahmed 1975), salinity tolerance (Amemiya 1928), growth rate (Amemiya 1928, Numachi 1978), egg size (Amemiya 1928, Numachi 1978), reproductive season (Numachi 1978, Robinson 1992), allozymes (Buroker et al. 1979), and most critically, gamete compatibility (Numachi 1958, cited in Numachi 1978). On the basis of fertilization tests, Numachi defined two types of Kumamoto oysters, A and B, from Ariake Bay, Kyushu, Japan. Type B oysters were fully interfertile with all *C. gigas* populations tested. Sperm from Type A males, however, were

unable to fertilize eggs from Type B or *C. gigas* oysters. On the other hand, eggs from Type A females could be fertilized by Pacific oyster sperm and by concentrated suspensions of Type B Kumamoto oyster sperm. We have recently confirmed Numachi's observations; sperm from Kumamoto oysters cultured on the U.S. west coast cannot fertilize eggs from the common, Miyagi variety, Pacific oysters cultivated in North America, but the reciprocal cross produces viable offspring (Banks et al. 1993b).

Numachi's finding that two morphologically similar but reproductively isolated Pacific oysters were sympatric in the Kumamoto region of Japan went largely unappreciated. Imai and Sakai (1961), for example, must have used Type B Kumamoto oysters in crossbreeding experiments showing complete interfertility of oysters from the Kumamoto, Hiroshima, Miyagi, Hokkaido Prefectures, but they make no mention of Numachi's work. North American malacologists and oyster culturists have likewise regarded the Kumamoto oyster as a geographical variety of the Pacific oyster (Woelke 1955, Quayle 1988). Previous confusion about the biological species status of *C. sikamea* in North American commercial oyster stocks has been resolved by the compelling concordance of diagnostic differences in mtDNA and allozymes with the one-way gametic segregation described by Numachi (Banks et al. 1993b).

More than correct taxonomy may be at stake. Recent attempts to find *C. sikamea* in Ariake Bay, Japan, have yielded only specimens with the allozyme or mtDNA profiles of *C. gigas* (Ozaki and Fujio 1985, Banks et al. 1993b). Of great concern, therefore, is the status of Kumamoto stocks in North America, which may be threatened by hybridization with Pacific oysters and by very small effective sizes of commercial brood stock populations. We report here the results of a genetic survey of two major independent lineages of Kumamoto oysters in the U.S. commercial oyster culture industry. Diagnostic allozyme and mtDNA markers are used to discriminate species, and changes in allozyme frequencies be-

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tween generations within a stock are used to measure genetic drift and estimate the effective population number.

## MATERIALS AND METHODS

**Stocks.** Two distinct lineages of Kumamoto oysters that have been propagated in the U.S., but whose histories (numbers of founders, dates of introduction, etc.) are poorly documented, were sampled. One, represented by a stock belonging to Taylor United Co., Shelton, WA, was derived from stocks imported from Japan in the late 1970s by Oregon Oyster Co. and initially propagated by Oregon State University researchers at the Hatfield Marine Science Center, Newport, OR (A. Robinson, pers. comm.). By 1991, however, the Taylor United stock appeared, on the basis of shell morphology, to comprise "Kumamoto-like" and "gigas-like or hybrid" oysters (D. Robertson and K. Cooper, Taylor United Co., pers. comm.). Four specimens from each group were shipped to the Bodega Marine Laboratory for molecular testing in July 1991; an additional 41 "Kumamoto-like" and 14 "gigas-like or hybrid" specimens from this same stock were subsequently sent to the BML in February, 1992. A second lineage of Kumamoto oysters is represented by stocks belonging to Coast Oyster Co. (now Coast Seafoods Co., Bellevue, WA), which were imported and propagated independently of the OSU stocks. Both four year-old adults ( $N = 34$ ) and spat ( $N = 30$ ), were obtained from Coast Oyster Company's facilities at Humboldt Bay, CA, in June, 1991. Allozyme data for the Coast adults and mtDNA data for selected individuals used in experimental crosses were previously reported by Banks et al. (1993b).

**Allozyme Electrophoresis.** Electrophoretic methods and nomenclature have been described previously (Hedgecock and Sly 1990, Banks et al. 1993b). Thirteen loci were scored for the Coast Oyster Co. 1991 spat: *Acon-1*, *Acon-2*, *Adkin*, *Gpi*, *Idh-1*, *Idh-2*, *Lap-2*, *Tap-2*, *Mdh-1*, *Mdh-2*, *Pgm*, *Sdh*, *Sod-1*. Four loci, *Aat*, *Idh-1*, *Idh-2*, and *Mpi* are diagnostic (*sensu* Ayala and Powell 1972) for the Kumamoto and Pacific oysters (Buroker et al. 1979, Banks et al. 1993b). These loci, together with *Mdh-2*, which appeared to be diagnostic for Coast Oyster Co. Kumamoto oysters (Banks et al. 1993b), were scored for 56 specimens in the Feb. 1992 sample from Taylor United. *Mpi* and *Idh-2* were not scored for the initial Taylor sample.

**Mitochondrial DNA Typing.** Methods for extracting DNA and typing mitochondrial haplotypes of the Kumamoto and Pacific oysters were described by Banks et al. 1993a. Polymerase chain reactions (PCR) using two primers, A and B, that amplify a 319 base-pair (bp) segment of oyster 16SrDNA were done on the first eight Taylor specimens. For 50 specimens in the second Taylor series, multiplex PCR reactions were made using primers A and B plus a third internal primer, E, which in combination with primer A specifically directs the amplification of a 246 bp DNA fragment from *C. sikamea* templates only. PCR products were incubated with the restriction endonuclease *Dra I*, according to manufacturer's directions (USBiochemical), electrophoresed in 3% NuSieve/agarose gels with 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator. The resulting RFLP gel patterns are diagnostic because *Dra I* cleaves the 319 bp PCR product from *C. gigas* but not that from *C. sikamea* into two fragments of 141 and 178 bp (Banks et al. 1993a). For the 50 individuals from the second Taylor sample, PCR products were blotted on replicate nylon membranes in a vacuum, dot-blot apparatus, cross-linked to the membranes by exposure to UV light, and hybridized against species-specific oligonucleotide probes Cg,

Cs, Dg, and Ds, where C and D denote two nucleotide sites in the 16S rDNA sequence at which the Pacific and Kumamoto oysters differ and g and s denote *C. gigas*-specific and *C. sikamea*-specific oligonucleotide probes, respectively, as described by Banks et al. (1993a). Redundancy of maternal-species diagnosis at seven nucleotides in the 16SrDNA gene, which is provided by the combination of multiplex PCR, restriction endonuclease digestion, and hybridization with oligonucleotide probes, eliminates any possibility that intra-specific polymorphism could result in false identification of any specimen (see Banks et al. 1993a).

**Analysis of Temporal Change.** The Coast Oyster Co. hatchery used their 1987 class of adult Kumamoto oysters to produce the 1991 spat (J. Donaldson, pers. comm.). Samples from these two year classes permit an analysis of genetic change between parent and offspring generations for this commercial stock by methods described in detail elsewhere (Hedgecock and Sly 1990, Hedgecock et al. 1992, and references therein). The analysis is based on the inverse relationship between observed temporal change in the frequencies of alleles and the effective size of an isolated population,  $N_e$ :

$$E(\hat{F}) \approx t/(2N_e) + 1/(2S_0) + 1/(2S_t),$$

where  $E(\hat{F})$  is the expected variance, owing to random drift of allelic frequencies, between an initial sample (taken without replacement) of  $S_0$  individuals and a second sample of  $S_t$  individuals taken (without replacement) after an interval of  $t$  generations. Rearrangement of this equation yields an estimator of the effective population number:

$$\hat{N}_K = t/(2[\hat{F} - 1/(2S_0) - 1/(2S_t)]),$$

where  $\hat{N}_K$  and  $\hat{F}$  denote estimators of the parameters  $N_e$  and  $E(\hat{F})$ , respectively. In this study,  $t = 1$ , and  $1/(2S_0)$  and  $1/(2S_t)$  are harmonic mean sample sizes per locus, weighted by numbers of independent alleles per locus, for the 1987 adult and 1991 spat population samples, respectively. Variances of allelic frequencies between adult and spat samples were calculated for the 13 allozyme loci assayed in the Coast Oyster Co. stock (the  $\hat{F}$ 's in Table 2). These variances are standardized to eliminate the effect of differences in initial allelic frequencies and then averaged across loci, weighting by the number of independent alleles at each locus, to yield an estimate,  $\hat{F}$ , of  $E(\hat{F})$  (Hedgecock et al. 1992). If allozymes are selectively neutral, then  $13\hat{F}/E(\hat{F})$  is distributed as a chi-square variable with 13 degrees of freedom corresponding to the number of independent loci sampled in this case. Agreement of the observed distribution with the chi-square distribution provides a test of the assumption of selective neutrality, as well as a means for calculating confidence limits on  $\hat{N}_K$ . An independent test of selective neutrality compares the actual loss of alleles over time to that predicted by population genetic theory assuming  $N_e = \hat{N}_K$ .

## RESULTS

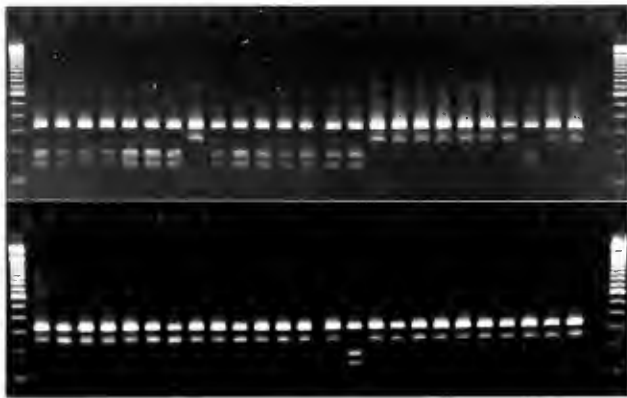
**The Taylor United Stock.** All four "Kumamoto-like" specimens in the first of two samples from this stock had molecular genetic markers diagnostic of *C. sikamea*, i.e. they were homozygous, *Aat*<sup>92/92</sup> and *Idh-1*<sup>95/95</sup>, and yielded mtDNA PCR products that could not be cut with *Dra I*. Three of the individuals were homozygous, *Mdh-2*<sup>107/107</sup> like Coast Oyster Kumamoto oysters sampled previously (Banks et al. 1993b), but one was homozygous for the most common Pacific oyster allele, *Mdh-2*<sup>100</sup>. The four specimens in the "gigas-like or hybrid" category appeared to be evenly divided between those two possibilities. Two were ho-



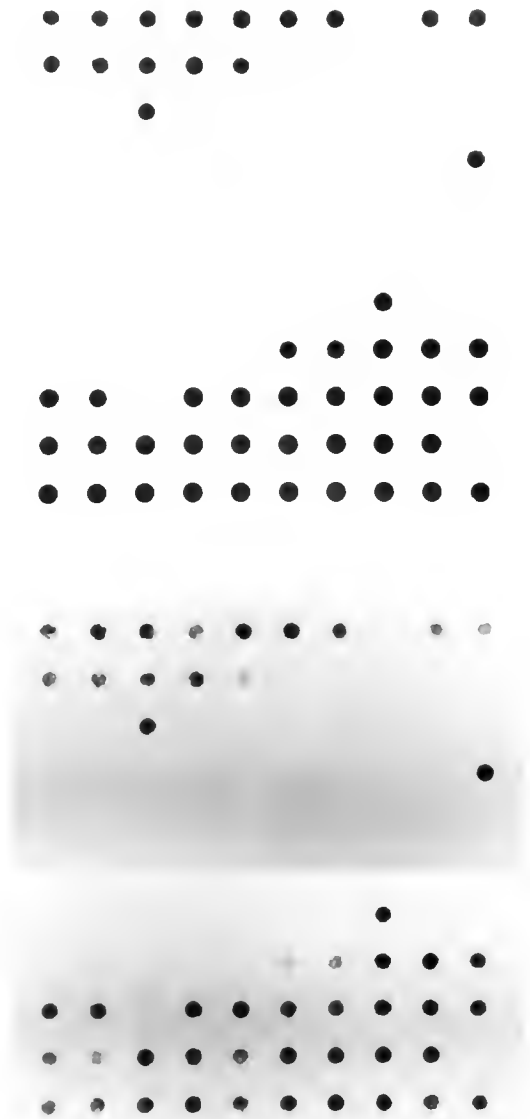
mozygous for the most common Pacific oyster alleles at *Aat*, *Mdh-2*, and in one case, *Idh-1*; both of these oysters also yielded mtDNA PCR products that were digested by *Dra I*. The other two were heterozygous for diagnostic alleles at the *Aat* and *Idh-1* loci and had *C. sikamea* mtDNA haplotypes (*i.e.*, PCR products un-cleaved by *Dra I*) expected from successful fertilizations of *C. sikamea* eggs by *C. gigas* sperm. One of these individuals was homozygous, *Mdh-2*<sup>100/100</sup>, while the other was heterozygous, *Mdh-2*<sup>100/107</sup>.

In the second series of samples from this stock, 14 were classified morphologically as "gigas-like" and 41 were classified as "Kumamoto-like"; one small, unclassified oyster attached to a "gigas-like" oyster was also studied. All 14 "gigas-like" individuals had molecular markers diagnostic of *C. gigas*. They were each homozygous for the *Idh-1*<sup>100</sup> and *Mpi*<sup>100</sup> alleles; 13 of 14 were homozygous for the *Mdh-2*<sup>100</sup> allele and one was a *Mdh-2*<sup>100/107</sup> heterozygote. Genotypes at the more polymorphic *Aat* locus were typical for *C. gigas* and none was homozygous for the *Aat*<sup>92</sup> allele that is fixed in *C. sikamea*. The mtDNA typing results for these "gigas-like" individuals are given in Figs. 1 and 2 (Fig. 1, upper panel, samples in lanes 2–8, 10–16; positions 1–7, 9–15 in each of the four panels in Fig. 2). The small oyster attached to the shell of one of these Pacific oysters proved to have markers diagnostic of *C. sikamea* (Fig. 1, upper panel, sample in lane 9; position eight in each of the four panels in Fig. 2).

Of the 41 "Kumamoto-like" oysters in the second Taylor series, 38 had molecular marker diagnostic of *C. sikamea*, 2 had markers diagnostic of *C. gigas*, and one was an apparent hybrid. Specimens classified as *C. sikamea* yielded the Kumamoto-specific 246 bp product in multiplex PCR (Fig. 1, upper panel, lanes 17–23, 25, 26; lower panel, all lanes except 16 and 20) and PCR products that hybridized to probes Cs and Ds, but not Cg and



**Figure 1.** Photograph of two agarose gels containing products from 50 PCR amplifications of mitochondrial DNA (mtDNA) coding for large subunit ribosomal RNA from Pacific and Kumamoto oysters (*Crassostrea gigas* and *C. sikamea*, respectively). PCR products were partially digested with the restriction enzyme *Dra I* prior to electrophoresis, and the gels were stained with ethidium bromide. Lanes 1 and 28 in each gel contain a 100 base pair (bp) ladder of DNA standards; lane 27 in each gel contains a no-template PCR control reaction. The bright, 319 bp band in all other lanes corresponds to the full-length product obtained from oyster mtDNA with primers A and B (see Methods; Banks et al. 1993a). *Dra I* cleaves the Pacific, but not the Kumamoto oyster PCR products into 141 and 178 bp fragments (top gel: lanes 2–8, 10–16, 24; bottom gel: lane 16). A third primer in each PCR reaction (see Methods; Banks et al. 1993a) directs the synthesis of a 246 bp product from *C. sikamea* templates only (top gel: lanes 9, 17–23, 25, 26; bottom gel: lanes 2–15, 17–26).



**Figure 2.** Photographs of four dot-blot hybridizations of the same 50 PCR products as in Fig. 1 (arrayed in five rows of ten in each panel). Panels 1–4 (top to bottom) were hybridized with probes Cg, Cs, Dg, and Ds, respectively, where C and D denote two nucleotide sites in the 16SrDNA sequence at which Pacific and Kumamoto oysters differ and g and s denote *C. gigas*-specific and *C. sikamea*-specific oligonucleotide probes, respectively. PCR products from individuals 1–7, 9–15, 23 and 40 hybridize to Cg and Dg (panels 1 and 3), while PCR products from individuals 8, 16–22, 24–39, 41–40 hybridize to Cs and Ds (panels 2 and 4).

Dg (Fig. 2, positions 16–22, 24–39, 41–43, and 45–50 in all four panels). These same specimens were also homozygous for the following diagnostic allozymes: *Aat*<sup>92</sup> (N = 38), *Idh-1*<sup>96</sup> (N = 38), *Idh-2*<sup>95</sup> (N = 25; 13 not scored), and *Mpi*<sup>96</sup> (N = 38). At the *Mdh-2* locus, 18 individuals were homozygous for the 107 allele, 4 were homozygous for the 100 allele, and 17 were heterozygous for these two alleles (Table 1). Including the four individuals from the first sample, frequencies of the 100 and 107 alleles in the Taylor United stock are estimated to be about 0.3 and 0.7, respectively; the *Mdh-2* genotypic proportions are in agreement with Hardy-Weinberg-Castle expectations (goodness-of-fit chi-square

TABLE 1.

Frequencies of *Mdh-2* genotypes in wild and cultivated populations of *Crassostrea sikamea* and *C. gigas*. Data for Japanese *C. sikamea* are from Buroker et al. (1979); data for *C. gigas* are pooled from Buroker et al. (1979) and Banks et al. (1993b). Genotypic frequencies are those expected under Hardy-Weinberg-Castle equilibria, rounded to the nearest integer.

	<i>Mdh-2</i> Genotypes			Total No. individuals
	100/100	100/107	107/107	
<i>sikamea</i> (Coast stock)	0	3	60	63
<i>sikamea</i> (Taylor stock)	4	17	21	42
<i>sikamea</i> (Japan)	69	9	0	78
<i>gigas</i> (pooled)	261	1	0	262

= 0.9, 1 d.f.). Table 1 compares *Mdh-2* genotypic frequencies for *C. gigas*, native *C. sikamea* and the Coast and Taylor hatchery stocks of *C. sikamea*. The two individuals classified as *C. gigas* in this group yielded mtDNA PCR products that were cut by *Dra I* (Fig. 1, upper panel, lane 24; lower panel, lane 16) and that hybridized to probes **Cg** and **Dg** but not **Cs** and **Ds** (Fig. 2, positions 23 and 40 in all four panels). They were also homozygous for Pacific oyster diagnostic allozymes (*Idh-2* was not scored in one individual). Finally, the apparent hybrid yielded a *C. sikamea*-specific PCR product (Fig. 1, lower panel, lane 20), which hybridized to probes **Cs** and **Ds** but not **Cg** and **Dg** (Fig. 2, position 44 in all four panels). This individual was also heterozygous for the diagnostic or most common *C. sikamea* and *C. gigas* alleles at *Aat*, *Idh-1*, *Idh-2*, *Mdh-2* and *Mpi*.

**The Coast Oyster Stock.** Of the thirty Kumamoto hatchery spat sampled from this stock in 1991, one was diagnosed as *C. gigas* by allozyme genotype. Allelic frequencies for 13 allozymes for the remaining 29 individuals are given in Table 2. Together with allozyme frequencies reported previously for adults of this same stock (Banks et al. 1993b), these data allow calculation of a mean temporal variance of allelic frequencies,  $\bar{F} = 0.1399$ , which in turn yields an estimate of effective stock size,  $\hat{N}_K = 5.4$ . The 95% confidence range for  $\hat{N}_K$  is from 2.5 to 11.8. Of the total of 40 alleles observed at these loci in the adult generation, only 30 remain in the sample of spat; numbers of alleles remaining and lost in this comparison of adults and offspring are not significantly different than those expected, 33.0 and 7.0, respectively, in a model of random genetic drift in a population of effective size equal to 5.4 (goodness-of-fit chi-square = 1.040, 1 d.f.). The distribution of standardized temporal variances at individual loci is not significantly different from the chi-square distribution expected under random genetic drift (Fig. 3).

#### DISCUSSION

The Kumamoto oyster *Crassostrea sikamea* (Amemiya 1928) can be unambiguously discriminated from the Pacific oyster *C. gigas* (Thunberg) on the basis of molecular markers and gametic compatibility. Although re-evaluation of the taxonomic status of the Kumamoto oyster has been carried out on stocks cultivated in North America, results are in accord with earlier studies made on Japanese native populations. Previously observed differences between the two oysters in reproductive traits (Amemiya 1928, Numachi, 1958 cited in Numachi, 1978) and allozymes (Buroker et al. 1979) are shown to be congruent for North American stocks

TABLE 2.

Variances in allelic frequencies,  $\bar{F}$ , for 13 allozyme-coding loci between two year classes of Coast Oyster Co. Kumamoto oysters.

Locus	Allele	1987	1991	$\bar{F}$
<i>Acon-1</i>	(N)	(25)	(29)	0.0400
	100	0.980	1.000	
	97	0.020	0.000	
<i>Acon-2</i>	(N)	(22)	(18)	0.1533
	109	0.045	0.000	
	103	0.045	0.000	
	100	0.545	0.861	
	97	0.364	0.139	
<i>Adkin</i>	(N)	(34)	(22)	0.1154
	105	0.029	0.000	
	103	0.235	0.068	
<i>Gpi</i>	(N)	(18)	(29)	0.1879
	105	0.306	0.069	
	100	0.639	0.879	
	94	0.056	0.052	
<i>Idh-1</i>	(N)	(30)	(29)	0.0
	95	0.983	0.983	
	92	0.017	0.017	
<i>Idh-2</i>	(N)	(30)	(29)	0.3186
	95	0.800	0.983	
	93	0.200	0.017	
<i>Lap-2</i>	(N)	(18)	(28)	0.2340
	103	0.250	0.071	
	101	0.139	0.000	
	100	0.611	0.857	
<i>Mdh-1</i>	(N)	(34)	(29)	0.1184
	108	0.029	0.121	
	100	0.971	0.879	
<i>Mdh-2</i>	(N)	(34)	(29)	0.0150
	107	0.985	0.966	
	100	0.015	0.034	
<i>Tap-2</i>	(N)	(18)	(13)	0.0073
	102	0.111	0.077	
	100	0.806	0.846	
	98	0.083	0.077	
<i>Pgm</i>	(N)	(34)	(9)	0.1731
	103	0.044	0.167	
	100	0.206	0.000	
	96	0.456	0.556	
	90	0.118	0.000	
	86	0.088	0.167	
82	0.088	0.111		

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TABLE 2.  
continued

Locus	Allele	1987	1991	$\hat{F}$
<i>Sdh</i>	(N)	(34)	(25)	
	106	0.000	0.020	
	104	0.000	0.080	
	100	0.897	0.900	
	97	0.088	0.000	
	93	0.015	0.000	0.1015
<i>Sod-1</i>	(N)	(33)	(18)	
	100	0.803	0.972	
	90	0.197	0.028	0.2670

(Banks et al. 1993b). Moreover, newly observed differences in mitochondrial DNA sequences, which are also congruent with allozyme and reproductive-trait differences, permit rapid and accurate diagnosis of maternal species lineage (Banks et al. 1993a).

Oysters collected from the Kumamoto area in recent times have all been *C. gigas* by molecular tests (Ozaki and Fujio 1985, Banks et al. 1993b). Interestingly, some of these had the shallow, fluted, purple-streaked, shells typical of the Miyagi-type Pacific oyster, while others resembled the smaller, deeper, wrinkled shell morphology of true *C. sikamea*. The latter may represent the endemic form of *C. gigas*, Numachi's (1978) Type B Kumamoto oyster. Until a more systematic search reveals relict native populations of *C. sikamea*, the only Kumamoto oysters known to exist are those cultivated in the U.S. Our survey of the two major lineages of Kumamoto oysters propagated in North America highlights two

potential threats to the integrity of these genetic resources, perhaps to the survival of this oyster species. The first is admixture and hybridization with Pacific oysters, leading possibly to progressive introgression of Pacific oyster genes into Kumamoto oysters brood stocks. The second is erosion of genetic diversity from random genetic drift within small, hatchery-propagated populations.

Reports from several U.S. growers that Kumamoto oyster hatchery seed contained significant numbers of Pacific oyster "weeds" aroused concern about contamination of commercial brood stocks (Hedgecock and Robinson 1992). We document here admixture and/or hybridization of Pacific and Kumamoto oysters in the two commercial brood stock lineages. For one stock, admixture of Pacific oysters and possible hybrids was initially suggested by examination of shell morphology (D. Robertson and K. Cooper, Taylor United Co., pers. comm.). Molecular diagnoses subsequently showed that 42 of 45 individuals classified morphologically as Kumamoto oysters were indeed *C. sikamea*, but that one was a hybrid and two were Pacific oysters. Two additional hybrids were detected in a group classified morphologically as "gigas- or hybrid-like" and an unclassified *C. sikamea* was found attached to a larger "gigas-like" individual. Of the 69 Kumamoto oysters sampled from Coast Oyster Co. stocks, only one spat was diagnosed as *C. gigas*; the remaining adults and spat typed as pure *C. sikamea*.

Results for the Taylor United stock suggest that commercial breeders can, on the basis of shell morphology, fairly reliably discriminate the Kumamoto oyster *C. sikamea* from the Miyagi strain of Pacific oyster typically grown in North America. Yet, diagnosis of brood stock must be absolutely correct in order to preserve the specific integrity of Kumamoto oyster stocks. Sperm from *C. gigas* can fertilize eggs from *C. sikamea* so that accidental admixture of the two species in mass spawnings of commercial brood stock could lead to hybridization. We infer from our finding of three hybrid individuals that hybridization has indeed occurred in commercial spawns of supposed Kumamoto brood stocks. All three Pacific X Kumamoto hybrid oysters were heterozygous for species-diagnostic allozyme markers but had *C. sikamea* mitochondrial DNA haplotypes, the composite nuclear and mitochondrial hybrid genotype expected on the basis of the one-way gametic compatibility between these species.

The fate of hybrids in commercial stocks is presently unknown. Pacific X Kumamoto hybrids seem morphologically to be quite variable. Some are evidently indistinguishable from pure Kumamoto oysters. Unless hybrids can be excluded from Kumamoto brood stocks, there is potential for progressive introgression of Pacific oyster genes into Kumamoto stocks. In this regard, there is need to assess compatibilities of gametes from interspecific hybrids, both in crosses among themselves and in backcrosses to the parental species. If hybrid sperm is unable to fertilize the Pacific oyster egg, hybrids would be falsely diagnosed as pure Kumamoto oysters by the cross-fertilization test that otherwise unambiguously discriminates Kumamoto from Pacific oyster males (Numachi 1958 cited in Numachi 1978, Hedgecock and Robinson 1992, Banks et al. 1993b).

The diversity of genetic resources within commercial stocks of Kumamoto oysters may be threatened by random genetic drift, as shown for numerous aquaculture hatchery stocks (Waples and Teel 1990, Hedgecock et al. 1992). Substantial genetic change occurred between the '87 and the '91 year classes of Coast Oyster Co. Kumamoto oyster stocks, as estimated from allelic frequencies at 13 loci in samples taken from the Humboldt Bay, CA,

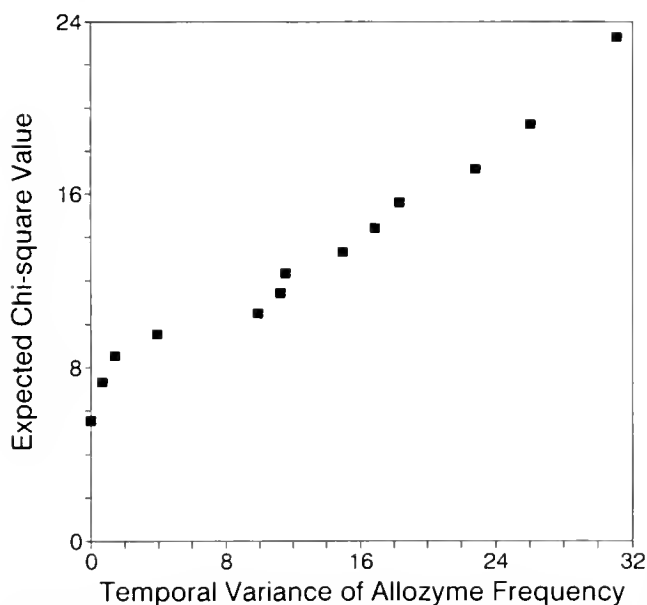


Figure 3. Probability plot of standardized variances of allelic frequencies at 13 allozyme-coding loci between two generations of a Kumamoto oyster commercial hatchery stock. Observed temporal variance on the x-axis is plotted against the corresponding expected value from the chi-square distribution with 13 d.f. on the y-axis (see Hedgecock et al. 1992 for details of the method). The linearity of the plot suggests that the observed variances are distributed as chi-square variables, as expected under random genetic drift in the absence of selection.

production grounds. Assuming that we randomly sampled parental and offspring generations, we estimate, from average temporal variance of allelic frequencies, that the effective size of this stock is only 5.4. That the genetic changes in this stock represent random genetic drift is evidenced by (1) agreement between observed and expected numbers of alleles lost and retained in the '91 year class and (2) agreement between the distribution of drift variances for individual loci and a chi-square distribution with 13 degrees of freedom (Fig. 3; Hedgecock et al. 1992). With an effective number of 5.4, the Coast stock of Kumamoto oysters is expected, from population genetics theory, to lose a little more than one-tenth of its genetic diversity each generation and to become rapidly inbred. Inbreeding in small, unpedigreed populations is likely to reduce greatly the long-term productivity and persistence of the stock, as appears to have happened in the very small lines of the American oyster *C. virginica* bred for resistance to MSX (Vrijenhoek et al. 1990, Hedgecock et al. 1992, P. Gaffney, pers. comm.). Loss of genetic diversity through random genetic drift is especially worrisome in the case of the Kumamoto oyster since it cannot be counteracted by importation of fresh brood stock from extant natural populations.

Divergence of the two major U.S. brood stocks of Kumamoto oysters at the *Mdh-2* locus, from each other and from the native population sampled by Buroker et al. (1979), may have resulted from random genetic drift in both hatchery and natural populations (Hedgecock et al. 1992, Hedgecock 1993). The frequency of the "fast" allele at this locus (*104* in Buroker et al. 1979; *107* in Banks et al. 1993b) was 0.058 in the native Kumamoto population, 0.7 in the Taylor stock and 0.98 in the Coast stock (Table 1). This allele has been detected only once in *C. gigas* samples (N = 262), a frequency of 0.002; thus, *Mdh-2* is a diagnostic locus (*sensu* Ayala and Powell 1972) for the Coast Oyster Kumamoto oyster stock, but not for the Taylor United Kumamoto oyster stock.

We believe that three steps should be taken to conserve the Kumamoto oyster in North America. First, *Crassostrea sikamea* (Amemiya 1928) should be adopted by North American malacologists and the U.S. oyster industry as the scientific name for the Kumamoto oyster. The specific status of this animal is well supported by several concordant lines of evidence indicating reproductive isolation and evolutionary genetic divergence. Use of the correct scientific name should promote the recognition and conservation of this unique species, which, as far as is now known, survives only in the U.S. oyster industry.

Second, in view of their importance to the overall conservation of the species, the species integrity of commercial Kumamoto oyster stocks should be safeguarded (Hedgecock and Robinson 1992). With currently available diagnostic methods, pure Kumamoto brood stock can be identified by a progeny testing scheme

involving: (1) strict selection of brood stock candidates with Kumamoto morphology and growth history; (2) non-destructive (thermal) induction of spawning; (3) testing of sperm for inability to fertilize Pacific oyster eggs; (4) testing of larvae from controlled pairwise crosses for mitochondrial DNA haplotype; (5) testing of these same progeny at an early juvenile stage for allozyme genotype; (6) conservation of brood stock whose progeny are diagnosed as pure Kumamoto and culling of those individuals whose progeny carry Pacific oyster genes. Commercial hatcheries should at least practice steps 1 to 3, and on this basis, step 6.

Development of species-diagnostic nuclear DNA markers would improve the efficiency of brood stock testing. The maternal lineage of an individual can be identified by PCR and mtDNA typing of eggs or progeny as soon as one day post-fertilization or of tissue biopsy samples from the brood stock oysters themselves. Yet, mtDNA typing cannot distinguish pure Kumamoto oysters from Pacific male X Kumamoto female hybrids, the most likely contaminants in brood stocks, because both carry the Kumamoto mitochondrial genome. Discrimination of hybrids from pure Kumamoto oysters can only be made by testing for paternally inherited Pacific oyster genes. Allozymes provide such markers, but they cannot be reliably identified in individuals before a young spat stage and they require destructive tissue sampling. There is need, therefore, to develop PCR methods for nuclear DNA markers, which would facilitate identification of paternal species lineage at the larval stage or in biopsy tissue samples from individual adults.

Finally, genetic diversity within commercial brood stocks of the Kumamoto oyster needs to be monitored and conserved through improved brood-stock and hatchery-management practices. Genetic drift and inbreeding in aquatic hatchery populations is made possible by the very high fecundities and very large variances in family sizes of aquatic animals (Hedgecock and Sly 1990, Hedgecock et al. 1992). Breeders should take steps to increase the number of individuals that contribute offspring to a brood stock population and to equalize the reproductive contributions of those individuals in order to reverse the tendency towards small effective population numbers.

#### ACKNOWLEDGMENTS

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## SIZE, AGE AND GROWTH OF THE BLACK-LIP PEARL OYSTER, *PINCTADA MARGARITIFERA* (L.) (BIVALVIA; PTERIIDAE)

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**ABSTRACT** Shell dimensions of farmed *Pinctada margaritifera* were examined to determine the best indicators of age and growth. Length-frequency analyses and morphometric ratios were also examined from shells taken from the wild. Samples from different lagoons and different depths were compared. Growth in shell diameter and heel depth is highly variable. Dorsoventral measurement is the best indicator of growth performance in younger shells, but heel depth is a better overall indicator of age. The rate of increase in shell thickness may be slower in deep water, which might result in better quality pearls.

**KEY WORDS:** size, age, growth, *Pinctada*, pearl oyster, shell

### INTRODUCTION

The black-lip pearl oyster, *Pinctada margaritifera* (Linnaeus, 1758), occurs throughout the Indo-Pacific region, but reaches its greatest abundance in the atoll lagoons of French Polynesia and the Cook Islands in the South Pacific. In the Cook Islands the species occurs naturally in the larger lagoons of Manihiki, Penrhyn and Suvarrow.

A lucrative black pearl culture industry has recently become established in Manihiki. The culture techniques are based on those developed in French Polynesia (Coeroli et al. 1984). Juveniles are collected from natural stocks, or from spat-collectors made of local hardwood, nylon rope, gauze or plastic sheet strung on sub-surface lines. Adults are drilled, tied with wire or monofilament nylon, and suspended from platforms, rafts, or lines. Pearl-seeding operations commence when oysters are about two years of age, with pearls produced 18 to 24 months after implantation.

Growth rates are useful as indicators of oyster fitness. Faster growth reduces the lengthy grow-out time and incubation period of the pearls. Pearl production and quality have been assessed in some culture trials (Alagarswami 1987). Mizuno (1983) and Coeroli and Mizuno (1985) used a coefficient of pearl quality with *P. margaritifera*. A "strong relationship" (ibid, p 551) was found between shell growth rate and thickness of the nacre coating on the pearls, validating the use of shell dimensions as indicators of pearl production potential. Comparisons of shell growth rates under different conditions could therefore be an easy way to optimize site selection and evaluate farm management strategies.

Shell size, morphological ratios, or growth rings can be used as indicators of age or growth history, but have been poorly described for *P. margaritifera* (Service de la Pêche 1970, Coeroli 1983, Coeroli et al. 1984). Morphometric studies on *P. margaritifera* shells are reported from French Polynesia (Coeroli et al. 1984), the Hawaiian Islands (Galtsoff 1931), the Andaman Islands (Alagarswami 1983), and Manihiki (Hynd 1960). Hynd (ibid) used shell morphology to confirm the genetic homogeneity of stocks in Manihiki, but only Galtsoff (1931) examined the relationship between morphometrics and growth. There is conflicting evidence of annual growth rings in pearl shells. Pandya (1976), and Jeyabaskaran et al. (1983) reported growth rings for *P. fucata* from

India, but Chellam (1978) found none. Hynd (1955) found no growth rings in *P. maxima* from Western Australia, but rings have recently been noted in some shell sections (Rand Dybdahl, pers. comm.).

This study examined different measurements on *P. margaritifera* shells of known age. These results are then used to identify the best criteria for determining age and for monitoring growth in this species. Shells from different depths and different lagoons are compared.

### MATERIALS AND METHODS

#### Study Area

The atolls of Manihiki, Penrhyn and Suvarrow are located in the Northern Group of the Cook Islands, between Samoa and the Tuamotu Archipelago of French Polynesia, in the central Pacific Ocean (Fig. 1).

#### Age Determination

Pearl oysters were collected by pearl farmers from spat-collectors over a number of years, and cultured under identical conditions at the same location on a private farm near Tauhunu village, Manihiki. The average age of each cohort was calculated from farm records (Table 1).

Two sets of measurements were obtained. The first set of measurements was made on 16 pearl oysters in 1986. These animals were sacrificed so that internal and external shell dimensions could be compared. Only external shell measurements were examined in more detail in 1987 from a further 183 live oysters from six age-groups.

Dorsoventral measurement (DVM) was taken to the nearest mm using calipers from the umbo to the furthermore continuous edge of the non-nacreous border, excluding digitate growth processes (Fig. 2). The internal nacre width was taken from the umbo to the most distant edge of nacre. Heel depth, from the dorsal edge of the hinge-line to the deepest point of abutance of the valves, was measured externally with the sharpened probe end of the calipers.

Shell sections were examined for growth rings using dissecting and compound microscopes. Striations in the hinge-ligament, the underlying nacre, the byssal notch, and on the anterior ear were often obscure, and gave inconsistent counts for individual speci-

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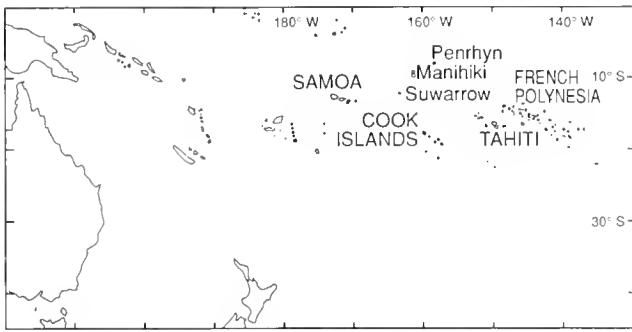


Figure 1. Locality of the Cook Islands.

mens. Concentric striations in the periostracum gave the most consistent counts.

On some shells, the striations in the periostracum were abraded, overgrown, or faint, particularly close to the umbo. Counts in the umbo region on nine undamaged valves were therefore made (Table 2) to allow extrapolation for shells with faded lines near the umbo. As the non-nacreous border was often broken, or striations were obscure or overlapping, counts were only made to the nacre edge. Counts varied on individual valves, and at least three counts were made across each shell until a consistent value was obtained.

#### Comparative Morphology and Length-Frequencies

Samples for morphological comparisons were collected from two depths in Manihiki: one in deep water (30 to 36 m) and one in shallower water (18 to 24 m). Both were from the same location on the southern side of Rahea pinnacle reef, near the center of the lagoon. Samples were also collected in the open lagoon of Suwarow from the shallow sill reef directly inside the passage (15 to 25 m depth). Intensive SCUBA searching removed all available oysters from within an area of reef to minimize potential size biases. DVM, heel depth, and the ratio of DVM/heel depth were compared between shallow and deep samples in Manihiki and with the Suwarow sample.

Length-frequency data from earlier survey results were also analysed by use of Wetherall plots (Wetherall 1986). Requisite assumptions of distinct spawning seasons, constant yearly recruitment, and consistent growth year-to-year within age-classes (Pearson and Munro 1991, K. Allen, pers. comm.) are less critical with long-lived, heavily fished stocks. All Wetherall regressions were calculated for a minimum size at first capture of  $L_c = 110$  mm. This also approximates mean size at first recruitment (from *in-situ*

TABLE 1.

Spat-fall records from Manihiki (Williams' farm).

Set Number	Soak Period	Heaviest falls or median date	Age (Years) (as of 12/86)
1	11/4/82–23/7/83	12/82 (median)	4.0
2	6/8/83–31/5/84	8/83–9/83 (spring set)	3.25
3	6/8/83–31/5/84	2/84–3/84 (autumn set)	2.75
4	17/7/84–16/5/85	12/84 (median)	2.0
5	30/7/85–23/5/86	2/86 (autumn set)	0.75

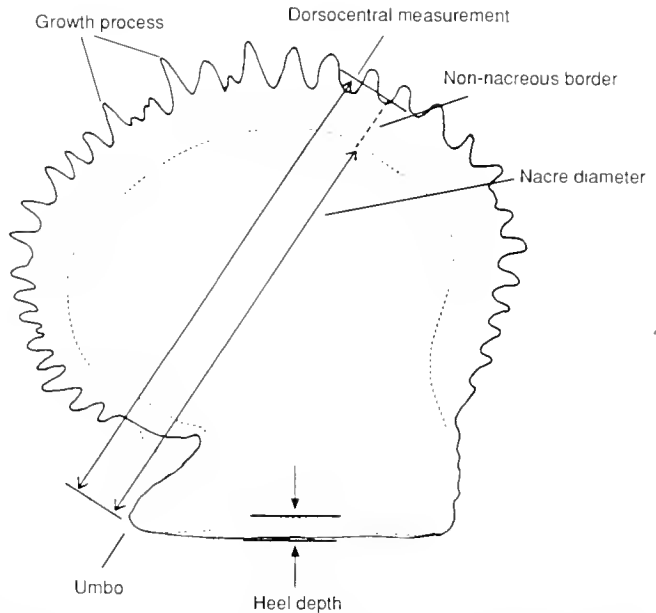


Figure 2. Dimensions used for measurement of shells. Nacre diameter can only be measured internally. Dorsocentral measurement and heel depth can be measured externally on live animals.

studies in Manihiki: Sims 1992b), as oysters may be fished as soon as they are visible to divers.

Estimates of population parameters from Wetherall plots were compared between the three lagoons and between the deep and shallow samples in Manihiki. Penrhyn and Manihiki plots used data from earlier surveys (1985), as this contained no size-selective biases (Sims 1990, 1992a). Manihiki data were separated into shallow (18 to 27 m) and deep (27 to 36 m) samples, based on the depth range of the survey quadrats. The data from the same 1985 survey in Suwarow provided only a small sample size. The specimens collected from Suwarow for morphometric study were therefore also used for Wetherall plots. There was less likelihood of any size-selective bias in Suwarow, where the unfished oysters were larger and more visible to divers.

## RESULTS

#### Age Determination

In the preliminary evaluation, shell measurements and counts of striations differed widely between the left and right valves of individual oysters. External dorsoventral measurement (DVM) differed by as much as 11 mm, with up to 3.0 mm differences

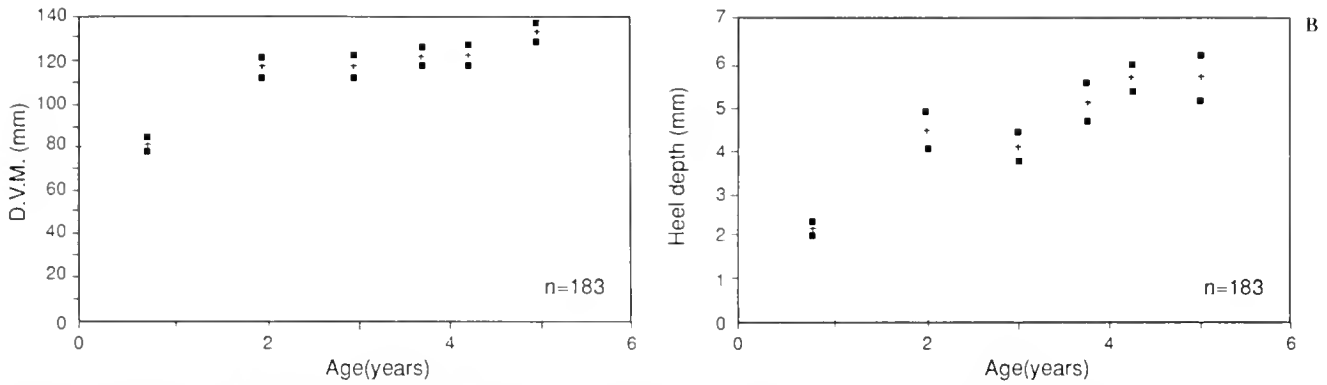
TABLE 2.

Mean numbers of striations in umbo region of shells, relative to the distance from the umbo.

Distance (mm)	Number of Striations		
	Mean $\pm$ 95%	S <sup>2</sup>	Range
20	9.4 $\pm$ 1.0	1.9	7–12
30	14.1 $\pm$ 1.2	2.8	10–18
40	17.9 $\pm$ 1.4	3.7	13–24

(n = 9 clear, countable shells)





**Figure 3. A:** External DVM against known age. Means and 95% confidence limits; n = 183. Growth in DVM is rapid for the first two years, then slows. **B:** External heel depth against known age. Means and 95% confidence limits; n = 183. Growth in heel depth is more constant over the first five years.

between heel depths. Nacre DVM and striation counts showed less disparity between valves. Averages for each measurement or count showed an increase with age, but with wide variation (Sims 1990). Counts of shell striations were highly variable ( $r = 0.25$ ), and were therefore an inappropriate measure of age.

Heel depth increased linearly from 2 to 4 years ( $r = 0.60$ ;  $p < 0.05$ ). Internal nacre provided a better linear fit to age than external DVM, but cannot be used on live animals. Internal nacre measurements were therefore not continued.

Both heel depth and external DVM increase with age (Spearman Rank Correlation:  $p < 0.001$ ). DVM increases by around 10 mm per year (Fig. 3a;  $p < 0.001$ ), and heel depth by around 0.8 mm per year (Fig. 3b;  $p < 0.001$ ).

DVM growth slows in older oysters, but heel depth growth continues at a similar rate. The ratio of DVM/heel depth therefore decreased with age ( $p < 0.001$ ; Figs. 4 and 5). Comparison of DVM/heel depth ratios between samples relies on coarse statistical tests (ANOVAs compare mean ratios for all oysters). Differences in DVM for each heel depth class between Suwarrow and Manihiki were therefore not statistically different, despite apparent differences in Fig. 6.

**Comparative Morphology**

At Rahea, shells from deep water were larger than those from shallower water (Table 3). No differences in heel depths were

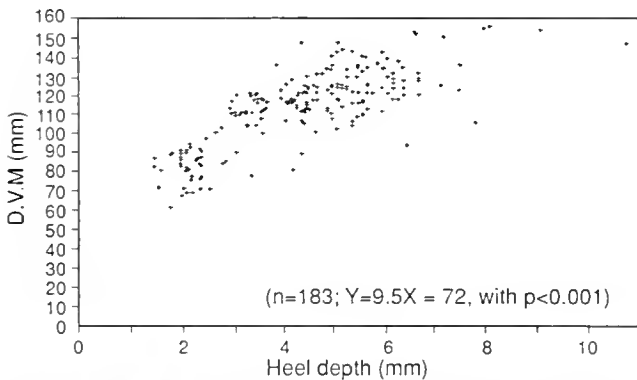
found. For any one heel depth class, shells from the deeper sample have a larger mean DVM (Fig. 7). Deep water shells therefore either grow faster in diameter or slower in thickness. Age or growth differences are also reflected in the greater mean DVM and heel depths from Suwarrow, compared with those from all depths in Manihiki (Table 3).

Wetherall plots of shallow and deep samples in Manihiki are similar (Fig. 8a and b, and Table 4). Between lagoons, however, values of  $L_{\infty}$  (average maximum size) and  $Z/K$  (ratio of total mortality over the von Bertalanffy growth co-efficient,  $K$ ) were different (Fig. 9a-c).  $Z/K$  was significantly different between Suwarrow and Penrhyn, yet the  $Z/K$  ratio for Manihiki was similar to both Penrhyn and Suwarrow (Table 4).

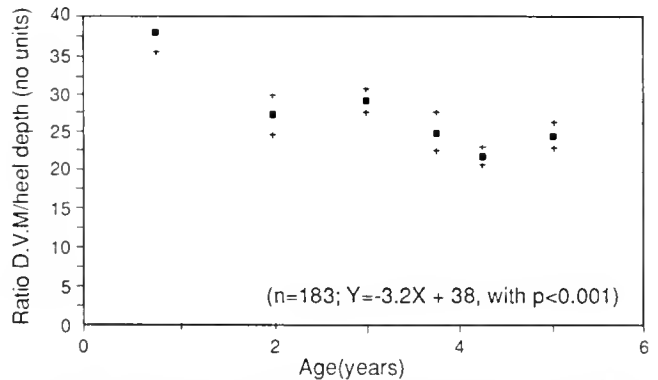
**DISCUSSION**

Growth varied widely within age-groups, even under identical culture conditions. Erratic effects of decreases in shell diameter (Sims 1990) and inherent genetic differences are both probably responsible. Highly variable growth in *P. margaritifera* shell diameters is also reported elsewhere (Nicholls 1931, Nasr 1984, Coeroli et al. 1984). The inconsistent relationships of DVM and heel depth to age suggest that growth conditions may vary from year to year. Results from growth trials from different years should therefore be compared cautiously.

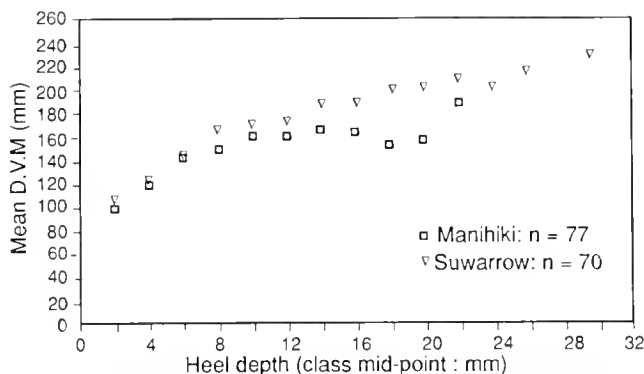
DVM/heel depth ratios can indicate differences in either age structure or relative growth rates, but actual growth data are



**Figure 4. DVM plotted against heel depth reveals a tight linear relationship. Individual shell measurements; n = 183,  $Y = 9.5X + 72$ ;  $p < 0.001$ .**



**Figure 5. The ratio of DVM/heel depth plotted against age. Means and 95% confidence limits; n = 183,  $Y = -3.2X + 38$ ;  $p < 0.001$ . The ratio of DVM/heel depth is relatively constant after the second year.**

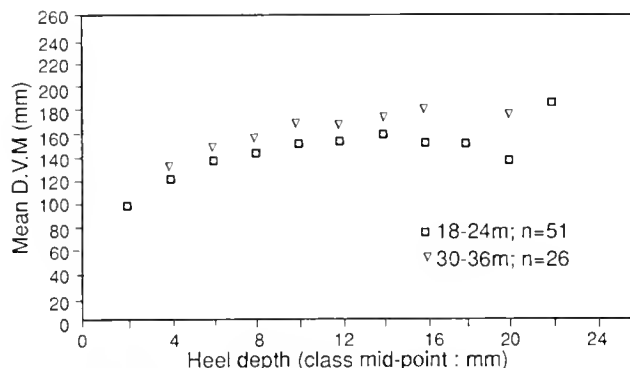


**Figure 6.** Mean DVM for each heel depth class from Manihiki (all depths,  $n = 77$ ) and Suwarrow samples ( $n = 70$ ). Suwarrow oysters have relatively larger DVMs for each heel depth class.

needed to fully understand these results. For example, it is difficult to interpret alone the larger size (mean DVMs) of shells from deeper water, compared with shells of the same heel depth class from shallow water. Results from tag-remeasure trials reported elsewhere, however, indicate slower DVM growth in deep water; fishing patterns also suggest older oysters occur in deeper water (Sims 1990). The similar heel depths must therefore be due to slower thickening of the shells in deeper water, produced by thinner or fewer layers of nacre.

Similarly, the greater mean DVM and heel depths from Suwarrow may reflect either age or growth differences. The Suwarrow stock is unfished and presumably older. Mean DVM for each heel depth class was also consistently larger in Suwarrow (Fig. 6), suggesting faster growth in shell diameter or slower growth in shell thickness.

Slower DVM growth in deep water may be due to suspended silt interfering with filtering or feeding mechanisms, or the stresses of smothering by sediment. The secretion of thinner layers in deep



**Figure 7.** Mean DVM for each heel depth class from deep (30–36 m,  $n = 26$ ) and shallow (18–24 m,  $n = 51$ ) samples, Raheia, Manihiki. Deep oysters have relatively larger DVMs for each heel depth class.

water may be commercially important, as thin nacre layers reportedly produce better colour and luster on pearls (Matsui 1958, T. Fujii, pers. comm.). Faster nacre deposition is more desirable early in the pearl production cycle, and shallow water culture is better then. However, these results suggest that seeded oysters could be placed in deep water for short "finishing-off" periods before harvest to improve the quality of the final nacre coats on the pearl.

Differences in heel depth growth rates between deep and shallow samples imply that heel depth is not always a reliable indicator of age. However, heel depth is elsewhere considered "indispensable (to) age determination" (Tranter 1958a, p 136), growing at a constant rate throughout the life of the oyster, irrespective of environmental conditions (ibid. 1958b, 1959). Heel depth is an inconvenient and destructive measurement to use in the field, as the oyster's byssus has to be detached from the substrate, rendering it more vulnerable to predation. DVM is more responsive to environmental influences, and may be a less reliable indicator of age than heel depth, but it is a better index of growth and is easier to use in surveys.

The different levels of fishing in each lagoon are apparent in the differences in average shell sizes and  $L_{\infty}$  and  $Z$  estimates from Wetherall analyses. The greater  $Z/K$  value for Penrhyn compared to Suwarrow was probably due to fishing of the stock, rather than different growth rates or natural mortalities. Manihiki has similar  $Z/K$  values to the other lagoons (Table 4) as faster growth or lower

**TABLE 3.**

Shell dimensions and shape compared between deep and shallow samples in Manihiki lagoon and Suwarrow lagoon.

	Mean	n	Manihiki	
			Deep	Shallow
<b>D.V.M.</b>				
Suwarrow	190.4	70	0.032*	0.000*
Manihiki Deep	160.7	26		0.002*
Manihiki Shallow	146.3	51		
<b>Heel Depth</b>				
Suwarrow	12.7	70	0.020*	0.002*
Manihiki Deep	9.4	26		0.833
Manihiki Shallow	9.2	51		
<b>D.V.M./Heel Depth</b>				
Suwarrow	17.4	70	0.323	0.089
Manihiki Deep	19.0	26		0.636
Manihiki Shallow	20.1	51		

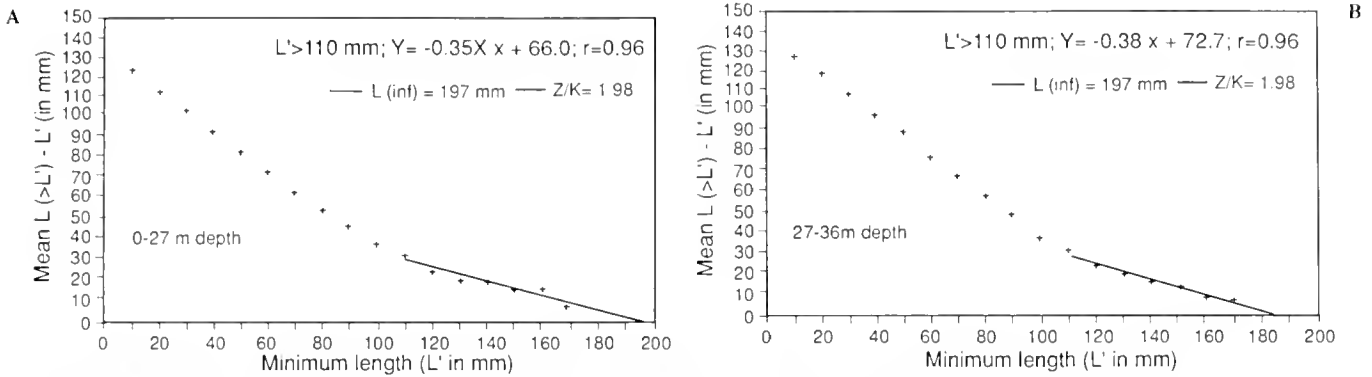
Ho: The dimensions are the same between samples; \* = significant difference:  $p < 0.05$ , for series of one-way ANOVAs. DVM is larger in Suwarrow than for both the Manihiki samples. The deep sample DVM is larger than the shallow sample in Manihiki. Heel depth is larger in Suwarrow than in both the Manihiki samples, but heel depth is the same for deep and shallow in Manihiki.

**TABLE 4.**

Wetherall plots compared between deep and shallow in Manihiki lagoon, and between Manihiki, Suwarrow and Penrhyn lagoons.

	$L_{\infty}$ (mm)	$Z/K$
<b>Depth comparisons: Manihiki</b>		
Shallow range (18–27 m)	197	1.98
Deep range (27–36 m)	189	1.60
<b>Lagoon comparisons: all depths</b>		
Manihiki lagoon	192**	1.67
Suwarrow lagoon	266***	1.10
Penrhyn lagoon	241	1.82**

Comparison of plot intercepts shows  $L_{\infty}$  from Manihiki is smaller than from Suwarrow and Penrhyn, and  $L_{\infty}$  from Suwarrow is larger than from Penrhyn. Comparison of plot slopes shows that  $Z/K$  from Suwarrow is smaller than from Penrhyn. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$



**Figure 8.** Wetherall plots from deep and shallow water samples in Manihiki lagoon. No significant differences are apparent. **A:** Shallow water (18–27 m), from 1985 survey data.  $L_{\infty} = 197$  mm,  $Z/K = 1.98$ . **B:** Deep water (27–36 m), from 1985 survey data.  $L_{\infty} = 189$  mm,  $Z/K = 1.60$ .

natural mortality in this more enclosed atoll may balance the heavier fishing there.

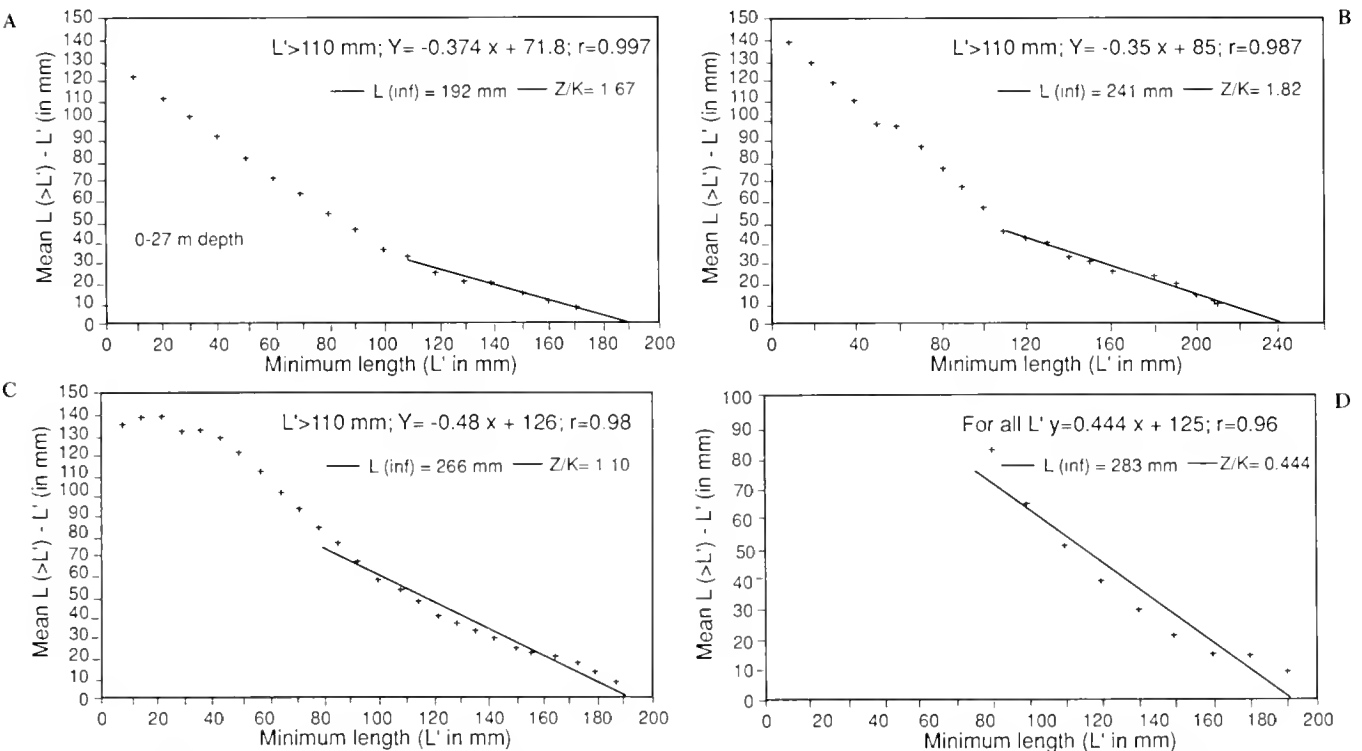
Wetherall plots of *P. margaritifera* length data from Pearl and Hermes Reef, in the Northwest Hawaiian Islands (Galtsoff 1933; Figure 9d:  $L_{\infty} = 283$  mm, and  $Z/K = 1.25$ ) also gave high  $L_{\infty}$  and low  $Z/K$  values, similar to Suwarrow. Pearl and Hermes Reef is also an open atoll with virtually unfished stocks.

**CONCLUSIONS**

There is much inherent variability in growth. Although heel depth usually increases linearly, it can also be affected by envi-

ronment. Heel depth is therefore not completely reliable as an indicator of age. DVM is more responsive to environmental influences and is the best indicator of growth performance. It is also the best measure for use *in situ*. The ratio of DVM over heel depth is not by itself a reliable index of growth or age and can only be used to indicate potential differences between samples. Taken together, these results provide guidelines for future studies of shell growth and population dynamics of *P. margaritifera*.

Differences in fishing pressure, natural mortality or growth rates between lagoons are inferred from differences in mean sizes and Wetherall plots. It appears that shells show a slower rate of increase in thickness in deep water than in shallow water. This



**Figure 9.** Wetherall plots from samples in Manihiki, Penrhyn and Suwarrow lagoons, and from Pearl and Hermes Reef, in the North-West Hawaiian Islands. **A:** Wetherall plot: Manihiki, all depths, from 1985 survey data.  $L_{\infty} = 192$  mm,  $Z/K = 1.67$ . **B:** Wetherall plot: Penrhyn, all depths, from 1985 survey data.  $L_{\infty} = 241$  mm,  $Z/K = 1.82$ . **C:** Wetherall plot: Suwarrow, all depths, data from SCUBA and free-diving searches, 1986.  $L_{\infty} = 266$  mm,  $Z/K = 1.10$ . **D:** Wetherall plot: Pearl and Hermes Reef, data from Galtsoff (1933).  $L_{\infty} = 283$  mm,  $Z/K = 0.44$ .

may be due to thinner layers of nacre being deposited in deep water. Deeper farming may therefore improve final pearl quality.

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## A DETERMINATION OF *IN VIVO* GROWTH RATES FOR *PERKINSUS MARINUS*, A PARASITE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN, 1791)

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**ABSTRACT** *Perkinsus marinus*, a protozoan parasite of oysters (*Crassostrea virginica*), exerts a significant controlling influence on oyster population dynamics over much of its range. Annual mortalities are typically estimated at greater than 50% of the host population. The rate of DNA synthesis in *P. marinus* was measured by following the rate of incorporation of <sup>14</sup>C-aspartic acid under field conditions. The DNA content in each *P. marinus* hypnospore was approximately 1 pg. The growth rate of *P. marinus* in the oyster host is dependent upon *P. marinus* population density. When the parasites occur at densities of greater than 10<sup>4</sup> cells g dry wt oyster<sup>-1</sup>, *P. marinus* exhibited an increase in population doubling time. At low cell density, doubling times of 1 to 10 hr were obtained. Doubling time increased to >10<sup>4</sup> hr at near-lethal infection levels. Very little mortality of *P. marinus* occurred during the experiment; thus the immune system was not active against *P. marinus* infection during the summer months. One important consequence of the growth dynamics of *P. marinus* is the importance of the parasite in controlling its own population levels. Infection intensity in the summer was controlled by the feedback of *P. marinus* cell density on doubling time. Because our data suggest that many oyster populations routinely exist a few doublings from death, epizootics must be produced by mechanisms, not well understood, that destabilize this delicate balance.

### INTRODUCTION

*Perkinsus marinus* is a protozoan parasite of oysters infecting 50% to 100% of the oysters in populations of the southern Atlantic and Gulf of Mexico coasts of the United States (Craig et al. 1989; Wilson et al. 1990; Andrews and Hewatt 1957). The geographic range of this protozoan extends from Delaware Bay to at least as far south as the coast of Texas and Puerto Rico (Perkins 1987). Annual mortality rates in this region typically exceed 50% of the adult oyster population (Ray 1954; Ray and Chandler 1955; Mackin 1962). Epizootics in Chesapeake Bay are a primary cause of the decline in the mid-Atlantic oyster fishery (Mann et al. 1991).

Mackin et al. (1950) described the histopathology of *P. marinus* infection in detail. Tissue inflammation of the oyster is characteristic of the early stages of infection. Host cell lysis occurs as the infection progresses. Infected oysters often exhibit a reduction in growth rate and slowed reproductive development (Menzel and Hopkins 1955; White et al. 1988; Wilson et al. 1988).

The extent and severity of the energetic drain of *P. marinus* parasitism on its host is partially dependent upon the size of the parasite population. Population size is determined by the difference between the rates of cell division and cell mortality. Although *P. marinus* has been studied for decades, the growth and mortality rates of this organism are poorly known. Choi et al. (1989) estimated a generation time for *P. marinus* of approximately 6 to 7 hr and concluded that decreases in oyster growth rate and reproductive output exhibited by parasitized oysters could be caused by the drain on the oyster's energy resources by the parasite population.

The annual cycle of *P. marinus* infection includes low infection intensities in the winter, a rise in infection intensity during the spring as the temperature warms, high infection intensities and mortality in the summer and early fall, and then a decline in infection intensity as the temperature cools in the fall (Mackin 1962; Soniat 1985; Crosby and Roberts 1990). Accordingly, the population growth rate of *P. marinus* exceeds the mortality rate, ascribed to the oysters immune system, in the warmer half of the

year. To what extent this annual cycle is due to changes in *P. marinus* growth rate or *P. marinus* mortality rate is unknown. An important first step is the development of a method for the *in vivo* measurement of growth rate and mortality rate of *P. marinus*. Here, we describe such a method and report the first *in vivo* measurements of *P. marinus* generation time and mortality rate in its oyster host, *Crassostrea virginica*.

### METHODS

Growth rates of single-celled organisms are typically determined by measuring the time required to achieve a doubling of population size. The rate of nucleic acid synthesis, determined from the rate of uptake of radiolabelled pyrimidines, purines or other nucleic acid precursors (e.g. Lovell and Konopka 1985; Robarts and Wicks 1989; Reimann et al. 1990), has often been used because the concentration of DNA per cell is relatively constant. *In vivo* measurement of the doubling time of *P. marinus* requires the development of a method to quantify the rate of DNA synthesis by *P. marinus* in its oyster host. This depends first on the separation of the parasite from the host and second on the purification of parasite DNA. Ray (1952) detected *P. marinus* infection by incubating oyster tissue in fluid thioglycollate medium (FTM). When infected oyster tissue was placed into FTM, the *in vivo* stages of *P. marinus* develop into hypnospores without reproduction so that hypnospore number approximates actual cell number (Ray 1954; Stein and Mackin 1957; Perkins and Menzel 1966). This characteristic provides a mechanism for the quantitative separation of the parasite from its host. The DNA can then be purified from the hypnospores.

#### *Isolation of Perkinsus marinus Hypnospores*

*Perkinsus marinus* hypnospores were obtained from infected oysters using the culture technique developed by Ray (1966). After shucking, the oysters were homogenized in a Brinkman Polytron tissue homogenizer at low speed (3). Homogenized oyster tissue, one meat per 100 ml FTM in capped 125 ml Erlenmeyer

flasks, was incubated in the dark at room temperature for 2 weeks. The FTM was fortified with mycostatin and chloramphenicol (Ray 1966). After 2 weeks, FTM-containing hyphospores and oyster tissue was centrifuged at  $10,000 \times g$  for 15 min. The pellet was resuspended in phosphate-buffered saline (PBS) (0.15 M NaCl, 0.003 M KCl, 0.01 M phosphate, pH 7.3) and centrifuged at  $10,000 \times g$  at least 3 times to remove all FTM from the pellet.

To separate the hyphospores from the oyster tissue, the oyster-hyphospore pellet was ground in a hand-held Pyrex tissue grinder to assure the release of all hyphospores from the oyster tissue. The clearance of the tissue grinder,  $>0.15$  mm, was chosen to be larger than the diameter of most hyphospores (10–200  $\mu\text{m}$ ; Stein and Mackin 1957; Ray 1952). The hyphospore-tissue mixture was digested using 3 ml 0.25% trypsin for 6 hr at  $37^\circ\text{C}$  (Perkins and Menzel 1966). After digestion, 1 ml 10% sodium dodecylsulfate was added, the digestion solution mixed by inversion 4 times, and then incubated for 30 min at  $37^\circ\text{C}$ . The digest was heated again for 20 min in a  $50^\circ\text{C}$  oven immediately prior to mixing with Percoll. The hyphospores were then separated from the oyster tissue digest by centrifugation on self-generated continuous density gradients of isosmotic Percoll. The density of the Percoll used to create the gradients varied from 15% to 40% (100% Percoll diluted with 0.15 M NaCl) depending on meat condition. The density used for each oyster sample was determined after a test separation of an aliquot of each homogenate on gradients of differing densities. The layer containing hyphospores was identified microscopically using Lugol's iodine to stain the spores. Ten ml Percoll of the chosen density and 2 ml oyster tissue digest were placed in a 15-ml Corex centrifuge tube. The Percoll-digest mixture was centrifuged at  $9,000 \times g$  for 30 min.

Following centrifugation, the layer containing hyphospores was removed from the Percoll with a Pasteur pipette. The purified spores were resuspended in PBS and the Percoll residue removed by centrifuging the sample and discarding the supernatant. The hyphospores were washed with PBS repeatedly under the identical protocol until all Percoll was removed. The total number of hyphospores present in each oyster was determined from an aliquot counted using a hemocytometer.

#### Extraction of DNA

The hyphospore samples were chilled on ice for 30 min, then ruptured by a 10-min exposure to ultrasonic vibrations using a Sonifier cell disrupter set at intensity 2 with a 60% pulse interval or by using a French press (for the DNA characterization studies). Ethidium bromide was used to assess the adequacy of DNA release. When isolating small quantities of DNA from individual oysters for analysis of radioisotope incorporation, crude calf thymus DNA (0.5 mg) was added to each sample to minimize the damage of DNase on the *P. marinus* DNA and to aid in precipitation.

The extraction protocol began with a sequential digestion with 200 mg chitinase for 12 hr at  $24^\circ\text{C}$ , followed by 1 mg Proteinase K for 12 hr at  $37^\circ\text{C}$ , and then 200 mg of RNase A for 1 hr at  $37^\circ\text{C}$ . Deproteinization of the samples was accomplished in two steps. First, the samples were treated with 1 ml of cetyltrimethylammonium bromide (10% CTAB/0.7 M NaCl) for 10 min at  $65^\circ\text{C}$  (Murray and Thompson 1980). Then, 1 volume of phenol/chloroform/isoamyl alcohol (24:24:1) was added to each sample and the sample mixed by inversion for 15 min. The sample was then centri-

fuged at  $900 \times g$  for 30 min to separate the aqueous layer containing the DNA from the interfacial layer containing the hyphospore debris and the lower layer containing phenol and chloroform.

The aqueous layer was recovered without disturbing the interfacial layer using a Pasteur pipette. DNA was precipitated from the aqueous layer by the addition of 1 ml 3 M sodium acetate and 2 volumes of 100% EtOH. The EtOH mixture was incubated for 12 hr at  $0^\circ\text{C}$ , then centrifuged for 30 min at  $8,000 \times g$  at  $4^\circ\text{C}$ . The DNA and the sodium acetate, which acts as a carrier to assist small amounts of DNA to precipitate, adhered to the sides and the base of the centrifuge tube. The ethanol was decanted and the sample was dried. The DNA was redissolved in distilled water for samples destined for liquid scintillation counting and in 0.05 M Tris-HCl/NaCl buffer for samples used for quantifying DNA.

The DNA from cells lysed with the Sonifier was examined for degradation by electrophoresing 2 ml of DNA on a 1% agarose gel using  $\lambda$ -phage DNA as a standard. The gel was stained with ethidium bromide and examined under ultraviolet light. To insure that all RNA was removed from the DNA preparation with the RNase A, two samples of *P. marinus* DNA, one prepared with RNase and one prepared without RNase A, were electrophoresed on a 1% agarose gel containing ethidium bromide.

#### Analysis of DNA

##### Purity of Preparation

*Perkinsus marinus* hyphospores were isolated from several dozen oysters and a subsample counted using a hemocytometer. The hyphospores were ruptured using a French Press to minimize shearing of the DNA that can take place with sonication. The DNA was purified using the previously described protocol, however no crude calf thymus DNA was added. Purity was estimated spectrophotometrically by measuring the absorbance at 260 nm relative to 280 nm ( $A_{260}/A_{280}$  ratio).

##### Quantification of DNA Content

Samples were dissolved in 0.05 M Tris-HCl/NaCl buffer. Calf thymus DNA (Hoefer) was used as the standard. A fluorescent dye, ethidium bromide, which is a bifunctional intercalating compound that binds specifically to DNA (Markovits et al. 1979), was added in excess ( $500 \text{ ng ml}^{-1}$ ) to each calf thymus standard, to a blank and to a 3 ml aliquot of each *P. marinus* DNA sample. The mixtures were allowed to equilibrate for 30 min. The fluorescence intensity of each mixture was measured at 370 nm excitation and 620 nm emission in a Shimadzu RF 5000U spectrofluorophotometer programmed for high sensitivity, 1-sec time interval, 10-nm band width and auto response. The technique assumes that the DNA standard and *P. marinus* DNA are similar in the number of binding sites (Markovits et al. 1979).

##### In Vivo Incorporation Using Thymidine and Aspartic Acid

Preliminary experiments were performed to determine if the injection of  $^3\text{H}$ -thymidine or  $^{14}\text{C}$ -aspartic acid would result in the labeling of *P. marinus* DNA. Oysters were collected from Confederate Reef in Galveston Bay and transferred to aquaria containing aerated artificial seawater with a salinity of 20‰. Each oyster was notched and  $20 \mu\text{Ci } ^3\text{H}$ -thymidine or  $2.5 \mu\text{Ci } ^{14}\text{C}$ -aspartic acid injected intramuscularly. Each time-series group of injected

oysters was placed into a separate aquarium. After the required time period, the oysters were shucked, blotted dry and the wet meat weights measured. The oysters were homogenized and placed into FTM fortified with antibiotics. The formation of hypnospores in FTM is not believed to involve cell division and/or reproduction (Ray 1954; Perkins and Menzel 1966) so that no DNA synthesis should occur; however, to minimize the possibility that  $^3\text{H}$ -thymidine or  $^{14}\text{C}$ -aspartic acid would be incorporated into DNA during FTM incubation, 3 mmoles unlabeled thymidine or 3.75 mmoles unlabeled aspartic acid were added to each flask (in addition to the aspartic acid already present in FTM). Following a 2-week incubation, the hypnospores were isolated and the DNA extracted. The extent of incorporation of the radiolabelled thymidine or aspartic acid into the *P. marinus* DNA was measured by dissolving the DNA in 7 ml of double-distilled water to which was added 14 ml of Soluscent A scintillation fluid (National Diagnostics). Each sample was counted 4 times for 50 min each in a liquid scintillation counter. Quench was calculated using an internal standard (Gordon 1980).

#### In Vivo Growth Experiments Using Aspartic Acid

Oysters were collected from Big Slough near Aransas Pass, Texas, on July 23, 1991. The oysters were transferred to an outside flowing saltwater pond at the Port Aransas Marine Laboratory of the University of Texas. Water was pumped continuously directly from Aransas Pass. Accordingly, the oysters had access to the food normally present in the bay water near their original habitat. Salinity was 17‰ and the temperature ranged from 28°C at night to 32°C during the day. The days were sunny and no rainfall occurred during the 5-day experiment.

A rock saw was used to make a v-shaped notch in each oyster valve. Each oyster was injected intramuscularly with 2.5  $\mu\text{Ci}$   $^{14}\text{C}$ -aspartic acid. Ten oysters were placed in each of six dive bags and returned to the saltwater pond. One dive bag was removed after 1, 5, 10, 24, 60, and 120 hr. The oysters were shucked within 5 min, the meats weighed and homogenized. A 500- $\mu\text{l}$  aliquot was reserved for trichloroacetic acid (TCA) treatment and the remainder placed into FTM fortified with antibiotics and containing 3.75 mmoles unlabeled aspartic acid.

The hypnospores were isolated following a 2-week incubation in FTM and the DNA extracted. The extent of incorporation of the radiolabelled aspartic acid into *P. marinus* DNA was measured by dissolving the DNA in 7 ml double-distilled water and mixing with 14 ml Soluscent A scintillation fluid (National Diagnostics). Each sample was counted 4 times at 50 min each in a liquid scintillation counter. Quench was checked using an internal standard.

A 500- $\mu\text{l}$  aliquot of oyster homogenate, frozen immediately following homogenization, was added to 500  $\mu\text{l}$  20% TCA, incubated at 4°C for 30 min, then centrifuged. The quantity of  $^{14}\text{C}$ -aspartic acid incorporated into oyster tissue was determined by dissolving the pellet in 2 ml Solusol (National Diagnostics) for 12 hr at 55°C. Glacial acetic acid (500  $\mu\text{l}$ ) was added to reduce chemiluminescence. Soluscent A (15 ml) and water (3 ml) were added to the scintillation vial prior to counting.

The aspartic acid in each TCA soluble fraction was measured using a lithium citrate elution system and *o*-phthalaldehyde as the detecting compound. The quantified free aspartic acid was collected in a fraction collector and transferred to a 7-ml plastic scintillation vial. Soluscent A (5 ml) was added prior to measurement on the liquid scintillation counter.

## MODEL DESCRIPTION

### Perspective

Because of the varying concentrations of the labeled and unlabeled free amino acid pool during the time course, as described in the Results section, specific activity was not a constant during the experiment. Accordingly, calculation of *P. marinus* growth rate and generation time required a more sophisticated mathematical approach than would be necessary in a constant perfusion experiment. We assume that growth always involves an increase in cell number and that mortality involves the loss of DNA. Accordingly, an increase in DNA observed by the incorporation of labeled aspartic-acid carbon into DNA implies growth and cell division. Given sufficient time after the label was introduced to permit a significant reduction in the specific activity of the precursor pool, a decrease in labeled aspartic-acid carbon in the DNA on a per cell basis implies growth using unlabeled precursors. A decrease in the amount of labeled aspartic-acid carbon in the population, however, implies true mortality.

Because the precursor pool can only be measured on a per oyster basis, that is because the tissue and cellular distribution of *P. marinus* cells and the labeled and unlabeled aspartic acid pools are unknown, growth rates of *P. marinus* can best be calculated for the entire *P. marinus* population. Although variability in specific activity among tissues and cell types probably occurs, *P. marinus* is widely distributed in oyster tissues and exists both intercellularly and intracellularly under most infection intensities so that population-level calculations should be relatively accurate. In order to calculate the instantaneous rate of *P. marinus* growth, the measured rate of incorporation of labeled aspartic acid must be corrected by the specific activity of the free aspartic acid pool. Stated mathematically,

$$dD/dt = [dD^*/dt] [f(t)/f^*(t)] \quad (1)$$

where  $D$  is the amount of aspartic acid incorporated into *P. marinus* DNA (moles population $^{-1}$ ),  $D^*$  is the amount of labeled aspartic acid incorporated into *P. marinus* DNA (dpm population $^{-1}$ ),  $t$  is time,  $f$  is the amount of free aspartic acid in the oyster (moles g dry wt $^{-1}$ ), and  $f^*$  is the amount of labeled free aspartic acid in the oyster (dpm g dry wt $^{-1}$ ). As aspartic acid loses 25% of its labeled carbon during metabolism into pyrimidine nucleosides, estimates of the amount of aspartic acid incorporated into DNA included this correction. We assume throughout that all  $^{14}\text{C}$  in DNA was in labeled pyrimidines and that all labeled pyrimidine molecules inherited  $^{14}\text{C}$  from all aspartic acid-derived carbons.

The experimental protocol necessitates that the amount of labeled free aspartic acid,  $f^*$ , be a function of time because a constant perfusion technique was not used. As most animals undergo a stress response to experimental manipulation which results in changes in the free amino acid pool, the amount of free aspartic acid present,  $f$ , is also likely to be a function of time. As a result, evaluating equation (1) first requires an evaluation of  $f(t)$  and  $f^*(t)$ .

Most pulse-labeling experiments utilize a number of replicates to permit calculation of the mean effect and the variation about the mean. Such experiments are based on the assumption that all individuals are initially equivalent, to the extent permitted by the normal stochastic variation about the mean. Experiments of *P. marinus* growth, however, frequently do not meet this assumption because infection intensity, measured as cell density (cells g dry

wt oyster<sup>-1</sup>), cannot be known a priori and because cell density affects cell growth (as described later). [Note that the thioglycolate method of Gauthier and Fisher (1990) requires a minimum of 7 days incubation in FTM, a time span permitting more than 20 population doublings under optimum conditions, so that animals cannot be sorted into cell density classes prior to an experiment.] Accordingly, no true replicates exist in this set of experiments, except for those individuals fortuitously having similar *P. marinus* cell densities (not cell numbers) as identified a posteriori, and the mean value for the population is not necessarily meaningful for interpretation. Consequently, equation (1) must be solved separately for each individual or cell density group identified a posteriori.

The problem posed as equation (1) cannot be solved separately for every individual or cell density, however, unless one individual or initial cell density can be followed over an entire time course. The former would require more sensitive analytical methods than available today; the latter would require an inordinately large number of individuals to be sacrificed at every experimental time. Nevertheless, components of equation (1) can be solved for single individuals or cell density classes provided that the specific rates of some components can be assumed to be common to all individuals. This approach permits in situ experiments to be conducted with a reasonable number of individuals.

The specific rates which are important in the solution of equation (1) are those controlling the loss of labeled aspartic acid from the free aspartic acid pool, changes in concentration of the free aspartic acid pool, the rate of uptake of free aspartic acid carbon into the *P. marinus* DNA pool, and the rate of loss of aspartic acid-derived carbon from the *P. marinus* DNA pool. As the rates of uptake and loss from the *P. marinus* DNA pool are unlikely to be equivalent in all individuals, we must assume that the rates controlling the specific activity of labeled aspartic acid [ $f(t)/f^*(t)$ ] are equivalent. This assumption is reasonable because the loss of labeled aspartic acid probably involves diffusional and metabolic processes common to most individuals and the change in the free aspartic acid pool involves a stress response to a manipulation common to all individuals. In particular, the amount of labeled aspartic acid used in the formation of *P. marinus* DNA is small relative to the amount injected. Accordingly, variations in *P. marinus* growth rate had little effect on the total available labeled aspartic acid. Furthermore, the concentration of both the labeled and unlabeled free amino acid pools did not vary significantly with *P. marinus* cell density (MANOVA,  $P = 0.57, 0.21$ , respectively; time-cell density interaction,  $P > 0.8$ , both cases) so that time-dependent variations in pool size were independent of *P. marinus* infection level.

#### Calculation of Specific Activity—The Labeled Free Aspartic Acid Pool

We assume that the loss of labeled aspartic acid from the free aspartic acid pool is a first-order process. Accordingly,

$$df^*/dt = -kf^* \quad (2)$$

where  $k$  is the first-order rate constant (time<sup>-1</sup>). Comparison of the results obtained by evaluating equation (2) with the measured values shows that equation (2) does not adequately describe the change in labeled free aspartic acid in the free aspartic acid pool over the experimental time course. Assuming that the labeled aspartic acid exists in two separate pools, however, is much more satisfactory. Hence,

$$df_1^*/dt = -k_1f_1^* \quad (3)$$

and

$$df_2^*/dt = -k_2f_2^* \quad (4)$$

where  $f_1^* + f_2^* = f_{\text{total}}^*$ , the measured value.

The two-pool model does not necessarily imply that only two pools exist or that the pools are continuously discrete. Failure of equation (2) to adequately predict the measured results requires a multipool model if the processes are first-order. Experience indicates that equations (3) and (4) are good curve fitting routines and frequently adequately fit data from multiple pools (e.g. Powell et al. 1991). Accordingly, in using equations (3) and (4), we do not necessarily conclude anything about the processes determining the time course of labeled aspartic acid except that a multiple pool model is required.

We solved equations (3) and (4) using the boundary conditions  $t = t_0$  at  $f^*(t) = f_0^*(t)$ . We cannot generally set  $t_0$  equal to zero, the time of injection, because, initially, the specific activity would be controlled by processes affecting the distribution of the label throughout the animal as well as tissue-specific metabolism. Accordingly, the experimental protocol necessitates that  $t_0$  be the time of the first sampling (1 hr). As a consequence, data from the first sampling cannot be used to evaluate any subsequent process rate. That sampling only defines the metabolic milieu at the beginning of the measured time course.

Solving equations (3) and (4) yields

$$f^*(t) = f_1^*(t) + f_2^*(t) = f_{0_1}^*e^{k_1(t_0-t)} + f_{0_2}^*e^{k_2(t_0-t)} \quad (5)$$

We define  $f_0^* = f_{0_1}^* + f_{0_2}^*$  as the mean amount of labeled aspartic acid observed in the first sampling period. The two first-order rate constants,  $k_1$  and  $k_2$ , and the fraction of the labeled free aspartic acid in each pool,  $f_{0_1}^*/f_0^*$  and  $f_{0_2}^*/f_0^*$ , were obtained iteratively by computer by searching for the values yielding the best fit to the observations using a chi-square-type error term to evaluate the goodness-of-fit.

As stated earlier, if the assumption is made that the specific rates are equal among all individuals, that is that the specific rates describe a process common to all individuals, then the value for  $f^*(t)$  for any individual can be obtained by solving equation (5) using the value of  $f_0^*$  for that individual. In essence, this assumes that the variation between individuals is produced by the efficiency of injection—some animals received more label than others—rather than the processes controlling loss after injection. Choi et al. (in press) obtained data suggesting that the success of injection is the primary determinant controlling the variation in the amount of labeled free amino acid available for use in metabolism during experiments of this kind.

#### Calculation of Specific Activity—The Free Aspartic Acid Pool

The free aspartic acid pool was not stable during the experimental time course. Free aspartic acid was high in concentration initially and then declined over the first 10 hr of the experiment. As the experiment extended beyond the first 24 hr, aspartic acid rose again.

The free amino acid pool is governed by a balance between the addition of amino acid from protein breakdown or assimilative processes and the loss of amino acid by anabolic and catabolic processes. Accordingly, and assuming that all processes are again of first-order



$$df/dt = \text{production terms} - \text{loss terms} = k_3P(t) - k_4f \quad (6)$$

where  $P(t)$  is the precursor pool,  $f$  is the unlabeled aspartic acid pool, and  $k_3$  and  $k_4$  are specific rates.  $P(t)$  can be considered to result from a first-order reaction analogous to equation (2), where

$$dP/dt = -kP \quad (7)$$

Once again, evaluation of equation (6) generally showed that a one pool model was inaccurate. A simple two pool model also failed. Inspection of the data shows that the increase in aspartic acid observed toward the end of the time course probably began at 10 hr, rather than at the experiment's inception. A two pool model taking into account this time offset provided an accurate description of the measured values:

$$df_1/dt = k_3P_1(t) - k_4f_1 \quad (8)$$

and

$$df_2/dt = k_5P_2(t) - k_6f_2 \quad (9)$$

where  $df_2/dt = -k_6f_2$  for  $t = 0, 10$ ; and where  $f_1 + f_2 = f_{\text{total}}$ , the measured value. We assume  $f_1/f_2 = P_1/P_2$ . Once again, the same caveats and assumptions apply as were discussed in the evaluation of  $f^*$  and the equations for  $P_1$  and  $P_2$  are of the form of equations (3) and (4). Once again, we solved the equations using the boundary conditions  $t = t_0$  at  $f(t) = f_0(t)$  and set  $t_0$  to be the time of the first sampling (usually 1 hr). Equation (8) yields

$$f_1(t) = f_{01}e^{k_4(t-t_0)} + [k_3P_{01}/(k_4 - k_3)] [e^{k_3(t+t_0-t_0)} - e^{k_4(t+t_0-t_0)}] \quad (10)$$

where  $t_{00} = 0$ . Equation (9) yields a similar solution with the exception that  $f_2(t = 0, 10) = f_{02}e^{k_6(t_0-t)}$  and  $t_{00} = 10$ . The four first-order rate constants ( $k_3, k_4, k_5, k_6$ ) and the fraction of free aspartic acid in each pool,  $f_{01}/f_0$  and  $f_{02}/f_0$ , were obtained iteratively by computer by searching for the values yielding the best fit to the observations using a chi-square-type error term to evaluate the goodness-of-fit.

The values of  $P_1$  and  $P_2$  are unknown. Obtaining an adequate fit necessitated that  $P_{\text{total}}$  be equivalent to about 50 times the highest value of free aspartic acid measured ( $f_{\text{total}}$ ). To the extent that this is inaccurate, the specific rates calculated ( $k_3, k_4, k_5, k_6$ ) will not reflect those rates actually present. These values, then, are relative to the estimated protein pool size. However, as these rates were obtained from observations of the free aspartic acid pool and as equations (8) and (9) are used solely to estimate  $f$ , the use of specific rates scaled to an unknown protein pool size still provides an accurate estimate of the concentration of free aspartic acid at any particular time. Moreover, the requirement of the model for  $P_{\text{total}} \approx 50 \cdot (f_{\text{total}})$  suggests that the increase in free aspartic acid after 10 hr drew upon a large protein pool.

#### The Specific Rates of Aspartic Acid Uptake and Loss from *Perkinsus marinus* DNA

The amount of labeled aspartic acid measured in *P. marinus* DNA is the net of two processes: free amino acid incorporation during DNA synthesis and free amino acid release during DNA degradation associated with cell mortality. Stated mathematically, for the population (not the cell)

$$dD^*/dt = \text{growth terms} - \text{loss terms} = k_{gd}f^*(t) - k_{ld}D^* \quad (11)$$

where  $D^*$  is the amount of labeled aspartic acid in *P. marinus*

DNA and  $k_{gd}$  and  $k_{ld}$  are the specific rates governing the rates of DNA synthesis and degradation, respectively. Solving (11) yields

$$D^*(t) = D_0^*e^{k_{ld}(t-t_0)} + k_{gd} \left\{ \frac{f_{01}^*}{(k_{ld} - k_1)} [e^{k_1(t-t_0)} - e^{k_{ld}(t-t_0)}] + \frac{f_{02}^*}{(k_{ld} - k_2)} [e^{k_2(t-t_0)} - e^{k_{ld}(t-t_0)}] \right\} \quad (12)$$

Equation (12) was used to calculate the specific rates ( $k_{gd}, k_{ld}$ ) for each individual. We obtained  $f_{01}^*$  and  $f_{02}^*$  for each individual using equation (5), and the specific  $f^*$  measured for each individual. The specific rates,  $k_1$  and  $k_2$ , and the fractional division of the protein pool in equation (5) were all estimated from the mean values of  $f^*$ . Once again, we assume that the specific rates,  $k_1$  and  $k_2$ , are common properties of all individuals.

To solve equation (12) for  $k_{gd}$  and  $k_{ld}$ ,  $D_0^*$  must be known, however  $D_0^*$  cannot be known for most individuals, all except those sampled at  $t = t_0$ , because an individual can only be measured once during the time course. Accordingly, we estimated the value of  $D_0^*$  for any individual using the ratio of the mean value of  $D^*$  at the first sampling ( $D_0^*$  by definition) to that at any other sampling and assumed that this ratio was common among all individuals. Because this aspect of the process must be a property of the cell, rather than the population, we obtained the ratio after normalization to a per cell basis. Accordingly,

$$D_0^* = [\bar{D}^*(t = t_0)] / \bar{D}^*(t = t_1) D_1^*(t = t_1) \quad (13)$$

We further assumed the first term in equation (11),  $D_0^*e^{k_{ld}(t-t_0)} \approx D_0^*$ . We then obtained  $k_1$  and  $k_2$  using equation (11) by iterative search using the measured value of  $D^*(t)$ .

In practice, the variability introduced by population density-induced variation in cell growth rate made the accurate estimation of  $D_0^*$  very difficult. The resulting calculation must, therefore, be treated cautiously. Reducing this error would require better control on the variability in infection intensity of the experimental population.

#### The Amount of DNA Production (D)

Following the arguments for equation (11),

$$dD/dt = k_{gd}f(t) - k_{ld}D \quad (14)$$

Solving equation (14), and recalling that  $f(t)$  is the product of two pools, yields

$$D(t) = D_0e^{k_{ld}(t-t_0)} + k_{gd} \left\{ \frac{f_{01}}{(k_{ld} - k_4)} [e^{k_4(t-t_0)} - e^{k_{ld}(t-t_0)}] + \frac{[k_3P_{01}/(k_4 - k_3)] \{ [1/(k_{ld} - k_3)] (e^{k_3(t-t_0)} - e^{k_{ld}(t-t_0)}) \} + [1/(k_{ld} - k_4)] (e^{k_4(t-t_0)} - e^{k_{ld}(t-t_0)}) \}}{[k_3P_{01}/(k_4 - k_3)]} + \frac{f_{02}}{(k_{ld} - k_6)} [e^{k_6(t-t_0)} - e^{k_{ld}(t-t_0)}] + \frac{[k_5P_{02}/(k_6 - k_5)] \{ [1/(k_{ld} - k_5)] (e^{k_5(t-t_0-10)} - e^{k_{ld}(t-t_0-10)}) \}}{[k_5P_{02}/(k_6 - k_5)]} + [1/(k_{ld} - k_6)] (e^{k_6(t-t_0-10)} - e^{k_{ld}(t-t_0-10)}) \} \right\} \quad (15)$$

Recall that equation (14) depends on a two-pool model for estimating free aspartic acid that includes a time delay of 10 hr for the second pool. Accordingly, at  $t \leq 10$  hr, Equation (15) becomes

$$D(t) = D_0e^{k_{ld}(t-t_0)} + k_{gd} \left\{ \frac{f_{01}}{(k_{ld} - k_4)} [e^{k_4(t-t_0)} - e^{k_{ld}(t-t_0)}] + \frac{[k_3P_{01}/(k_4 - k_3)] \{ [1/(k_{ld} - k_3)] (e^{k_3(t-t_0)} - e^{k_{ld}(t-t_0)}) \}}{[k_3P_{01}/(k_4 - k_3)]} + [1/(k_{ld} - k_4)] (e^{k_4(t-t_0)} - e^{k_{ld}(t-t_0)}) \} + \frac{f_{02}}{(k_{ld} - k_6)} [e^{k_6(t-t_0)} - e^{k_{ld}(t-t_0)}] \right\} \quad (16)$$

Because we are interested in the amount of DNA production since the first sampling ( $t_0$ ), the first term in equations (15) and (16) ( $D_0 e^{k_1(t_0-t)}$ ) can be discarded. The remaining terms include the specific rates previously calculated and the values for the pool sizes  $f_0$  and  $P_0$ . The latter two are determined for individuals as described previously for  $f_0^*$ .

#### Estimation of Doubling Time

The amount of DNA in one *P. marinus* cell is 1 pg. Assuming aspartic acid labeled both pyrimidines at a specific activity equivalent to the free aspartic acid pool, then the amount of DNA potentially labeled was  $0.5119 \text{ pg cell}^{-1}$ ; these two nucleosides account for 51% of DNA weight assuming a 50/50 adenine/cytosine ratio. We converted to a molar basis using ( $2 \cdot 669 \text{ g mole}^{-1}$ ), where 669 is the molar weight of the two pyrimidines and the factor of two is necessitated by two moles of aspartic acid being necessary, one for each pyrimidine. These conversions give us the aspartic acid-derived portion of the DNA molecule in moles  $\text{cell}^{-1}$  which could then be converted to moles  $\text{population}^{-1}$ , the currency of our calculations. If we then assume that  $dD/dt$  from equation (1) =  $d\text{DNA}/dt$ , each defined on a molar basis, then doubling time (T) is

$$T = [\text{DNA}]/(d\text{DNA}/dt) = [\text{DNA}]/(dD/dt) \quad (17)$$

where DNA is taken as  $1 \text{ pg cell}^{-1}$  converted as previously described.

#### Calculation of Oyster Production

Our protocol did not distinguish between  $^{14}\text{C}$ -aspartic acid incorporated into oyster or *P. marinus* tissue; however, even at high cell densities, *P. marinus* biomass is comparatively small. Furthermore, cell density did not significantly affect the amount of  $^{14}\text{C}$ -aspartic acid incorporated into tissue protein (MANOVA,  $P = 0.81$ ; time-cell density interaction term,  $P > 0.9$ ). Thus, oyster tissue protein accounted for most of the  $^{14}\text{C}$ -aspartic acid in the TCA precipitate.

The amount of  $^{14}\text{C}$ -aspartic acid incorporated into oyster tissue is the net of two processes: free aspartic acid incorporation during tissue synthesis and free aspartic acid release during degradation of proteins. The equation is

$$dS/dt = \text{growth terms} - \text{loss terms} = k_{gs}f(t) - k_{ls}S \quad (18)$$

where  $S$  is the amount of aspartic acid in the oyster tissue and  $k_{gs}$  and  $k_{ls}$  are the specific rates controlling the rates of molecular synthesis and degradation, respectively. The calculation of production of oyster tissue was derived in the same manner as the calculation of DNA production using equations analogous to equations (11)–(17).

## RESULTS AND DISCUSSION

#### Isolation of *Perkinsus marinus* Hypnospores

The technique developed to separate *P. marinus* hypnospores from oyster debris produced samples of hypnospores which were free of contamination from oyster debris and other parasites when examined using light microscopy, but did not damage the hypnospores.

#### Extraction of DNA

A major difficulty in the extraction of *P. marinus* DNA was lysing of the hypnospore. The spore could not be lysed by conventional methods, lysozyme or a detergent such as SDS (Marmur 1961). Sonication of the spores successfully ruptured the spore coat. The DNA measured in the growth experiments was obtained using a Sonifier cell disrupter to lyse the cells, however sonication is not the most desirable method for lysis because DNA can be damaged by shearing. Shorter periods of sonication were tried to minimize damage to the DNA, but the majority of cells were not ruptured by sonication of less than 10 min. Fortunately, any degradation of DNA that did occur did not prevent its extraction and purification. Inasmuch as some degradation of the DNA might have occurred, the DNA used for the measurement of cellular DNA content was obtained by cell lysis in a French Press to minimize damage to the nucleic acids.

Electrophoresis was used to assess the condition of the DNA. If the sonicated DNA was degraded, it would appear as a smear in the agarose gel. The *P. marinus* DNA appeared as a discrete band in the agarose gel following electrophoresis, indicating that the DNA was not extensively degraded. The DNA sample prepared without RNase showed a distinct band of RNA in the agarose gel following electrophoresis, but the DNA sample prepared with RNase lacked the RNA band. Thus the RNase digestion step effectively removed RNA from the DNA preparation.

In developing the extraction method described, the effects of sonication and multiple extractions on the purity and quantity of extracted DNA was assessed. Re-extraction of the protein interfacial layer following the initial phenol/chloroform/isoamyl alcohol treatment did not result in the release of additional DNA into the aqueous layer nor was significant DNA lost during the extraction technique, as determined by the ethidium bromide reaction.

#### Analysis of DNA

##### Purity of DNA Preparation

An absorbance ratio ( $A_{260}/A_{280}$ ) to 1.8 of 1.95 is considered to indicate a pure preparation of DNA, although the source of the DNA can affect the ratio (Schy and Plewa, 1989; Maniatis et al., 1982; Rodriguez and Tait, 1983). Aromatic amino acids in proteins have an absorption peak at 280 nm, so protein contamination of a DNA sample will cause the ratio to fall below 1.8. A ratio of greater than 2.0 would indicate the presence of RNA and/or denatured DNA (Schy and Plewa, 1989). Absorbance ratios ( $A_{260}/A_{280}$ ) for the *P. marinus* DNA samples were below 1.8, suggesting some protein contamination (Table 1). However, these ratios also indicate that the preparations were free of RNA and that the DNA was not substantially denatured. Re-extraction of precipitated DNA did not increase the purity of the preparation and may have decreased the yield.

TABLE 1.

Spectrophotometric absorbances of calf thymus standard and two replicate *Perkinsus marinus* DNA preparations.

Sample	$A_{260}/A_{280}$
Calf thymus standard	1.902
<i>P. marinus</i> DNA	1.277
<i>P. marinus</i> DNA	1.312

The DNA concentration in our samples was below 30  $\mu\text{g ml}^{-1}$ . Schy and Plewa (1989) found that the  $A_{260}/A_{280}$  ratio declines markedly with a decrease in DNA concentration below 30  $\mu\text{g ml}^{-1}$ . Accordingly, the low  $A_{260}/A_{280}$  ratios may also have originated in the DNA concentration used rather than from contaminants in the preparation. The limited amount of DNA that could be extracted did not permit us to distinguish between these two alternatives.

#### Quantification of DNA Content

Estimates of the average DNA content per cell (pg) yield a DNA content of a single hypnospore in the range 0.9–1.1 pg cell $^{-1}$  (Table 2). The DNA content of *P. marinus* is comparable to other protozoa (Table 3). One source of uncertainty is the number of DNA copies present in a hypnospore. One *P. marinus* cell produces one hypnospore (Ray 1954; Stein and Mackin 1957), but hypnospores produce multiple zoospores (Perkins and Menzel 1966; Azevedo et al. 1990) and, thus, should have multiple DNA copies. Comparison to other protozoa (Table 3) suggests that this might be the case; our values are higher than many protozoa, but they do fall within the range of values. Hundreds of zoospores are usually released from one hypnospore, however (pers. comm., anon. reviewer). A DNA content of 1 pg cell $^{-1}$  suggests that no DNA multiplication pursuant to zoospore formation had occurred in our samples because the amount of DNA is too low. Thus, in our calculations, we assume one copy per hypnospore. If DNA multiplication pursuant to zoospore formation did occur prior to or during hypnospore formation in FTM, however, later estimates of doubling times *in vivo* would overestimate or underestimate the true value depending upon the timing of multiplication.

#### *In Vivo* Experiments with Thymidine and Aspartic Acid

A series of preliminary experiments was run using labelled thymidine and aspartic acid to determine the usefulness of aspartic acid as the labelling compound. Aspartic acid is a precursor of pyrimidines, contributing three carbons to the nitrogenous bases of thymidine and cytosine (Fig. 1). Aspartic acid offered a more promising approach than thymidine because the aspartic acid pool is a rather large component of the amino acid pool in oysters (Powell et al. 1982), thus the concentration of the labeled compound is buffered by a large unlabeled pool. In addition, the ease of measurement of aspartic acid facilitated the tracking of specific activity during the experimental time course and the alternative pyrimidines are usually broken down and resynthesized in eukaryotes prior to incorporation into DNA (Gutteridge and Coombs 1977). The only contribution aspartic acid can make to the synthesis of purines in protozoa is a single nitrogen atom (Fig. 1). Therefore the  $^{14}\text{C}$  content of DNA should come from the pyrimidines. Although a possibility exists that the labelled carbon lost as  $^{14}\text{CO}_2$  during synthesis of pyrimidines or metabolic degradation

TABLE 2.

Quantification of *Perkinsus marinus* DNA using the ethidium homodimer fluorimetric assay and calf thymus DNA as the standard.

	DNA sample I	DNA sample II
DNA 7-ml sample $^{-1}$ (ng)	4025	2730
Number of hypnospores	$3.55 \times 10^6$	$2.98 \times 10^6$
DNA cell $^{-1}$ (pg)	1.1	0.9

TABLE 3.

The DNA content per cell for protozoa.

Organism	DNA (pg cell $^{-1}$ )	Reference
<i>Astasia longa</i>	1.52	Neff (1960)
<i>Eimeria tenella</i>	0.73	Wang and Stotish (1975)
<i>Entamoeba histolytica</i>	0.45	Gelderman et al. (1971)
<i>Euglena gracilis</i>	2.9	Brawerman et al. (1960)
<i>Plasmodium berghei</i>	0.05	Gutteridge and Coombs (1977)
<i>Pneumocystis carinii</i>	0.22–0.34	Gradus et al. (1988)
<i>Tetrahymena pyriformis</i>	13.6	Scherbaum (1957)
<i>Toxoplasma gondii</i>	0.10	Gutteridge and Coombs (1977)
<i>Trichomonas gallinae</i>	0.40	Mandel and Honigberg (1964)
<i>Trichomonas vaginalis</i>	0.53	Mandel and Honigberg (1964)
<i>Trypanosoma cruzi</i>	0.077	Gutteridge and Coombs (1977)
<i>Trypanosoma equipderum</i>	0.077	Gutteridge and Coombs (1977)
<i>Trypanosoma gambiense</i>	0.077	Gutteridge and Coombs (1977)
<i>Urostyla caudata</i>	1.057	Pigon and Edstrom (1959)

of aspartic acid may be incorporated into other molecules associated with the DNA, this contribution was assumed to be negligible due to the rapid turnover of the  $\text{CO}_2$  pool in oysters. Aspartic acid catabolism was minimized by the aerobic conditions maintained during the experiments (Collicutt and Hochachka 1977) and the use of continuously fed animals for the experiment.

We report selected results of several preliminary experiments with thymidine for comparison to the later experiments with as-

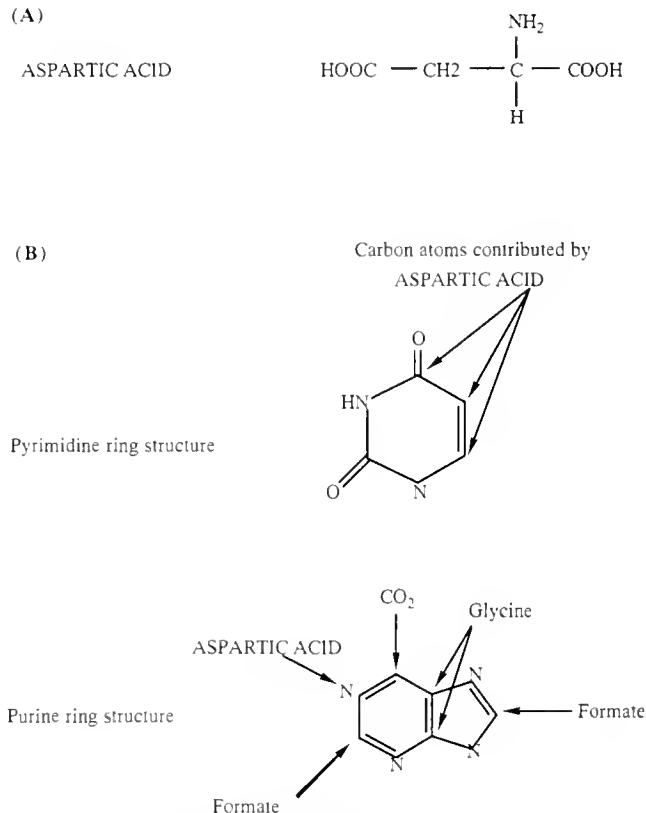


Figure 1. A: the structure of aspartic acid; B: the sources of the carbon molecules used in the synthesis of the pyrimidine ring and the purine ring, with reference to aspartic acid.

partic acid. The thymidine and aspartic acid-based experiments both showed the following important characteristics.  $^3\text{H}$ -thymidine and  $^{14}\text{C}$ -aspartic acid were incorporated into DNA and the amount of each incorporated showed an increasing trend over the first 10 hr of the time course (Fig. 2). However, a large variation existed between replicates at any one time. The amount of DNA labeled per cell declined as cell population density increased (Fig. 3). This density effect, noted in both the  $^3\text{H}$ -thymidine and  $^{14}\text{C}$ -aspartic acid experiments, explains much of the variation at any single time during the experimental time course. Longer-term experiments with either precursor demonstrated little loss of DNA, suggesting little cell mortality and remobilization of *P. marinus* DNA by oyster DNases or removal by exomigration of hemocytes (Fig. 4). Accordingly, aspartic acid proved to be an adequate replacement for thymidine in these experiments.

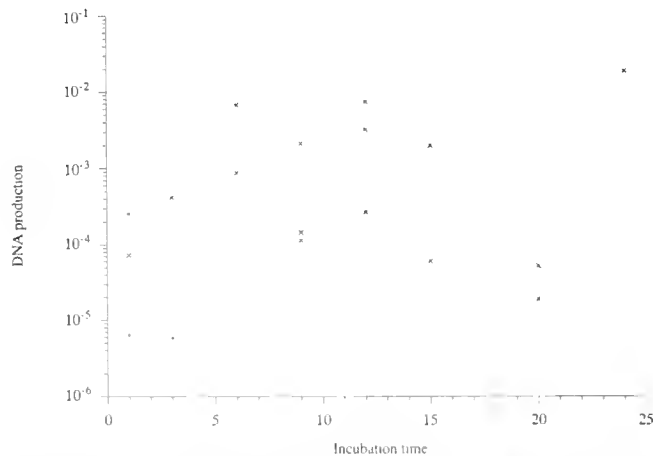
#### *In Vivo* Growth Experiment Using $^{14}\text{C}$ -Aspartic Acid

The growth experiment was performed under conditions as similar to the natural environment as possible. As expected from the preliminary laboratory experiments, *P. marinus* DNA gradually became labeled over the time course of the experiment. Figure 5 shows the expected effect of cell density on incorporation rate that was observed in the thymidine experiments previously described, demonstrating that this was not an artifact of laboratory conditions.

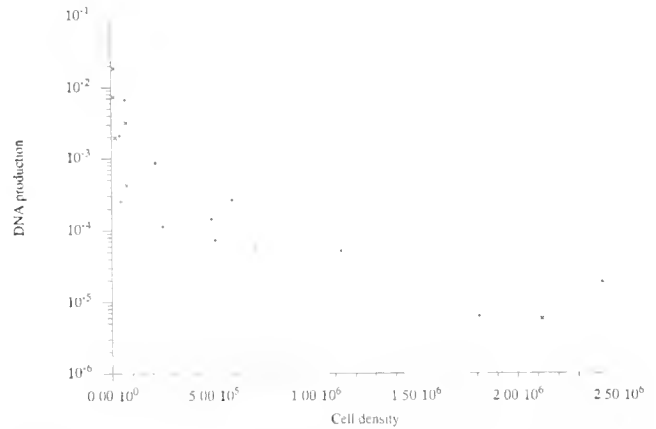
#### Specific Activity

Unfortunately, neither the pool of labeled aspartic acid nor the pool of unlabeled aspartic acid remained constant over the experimental time course (MANOVA, time effect, both cases  $P < 0.001$ ). As a constant perfusion protocol could not be run, the concentration of  $^{14}\text{C}$ -aspartic acid declined during the experimental time course (Fig. 6). The pool of unlabeled aspartic acid also varied during the 120-hr time course (Fig. 7).

Oysters, like most invertebrates, may respond to long-term stress by elevating their free amino acid pool as protein breakdown occurs (Powell et al. 1982, 1984; Koenig et al. 1981). A change in feeding rate would also affect the aspartic acid pool. The con-



**Figure 2.** Typical results of a laboratory time course experiment utilizing  $^3\text{H}$ -thymidine to monitor DNA production by *Perkinsus marinus*. DNA production in dpm  $^3\text{H}$ -thymidine incorporated cell $^{-1}$ ; incubation time in hr.



**Figure 3.** Incorporation of  $^3\text{H}$ -thymidine into *Perkinsus marinus* DNA as a function of *P. marinus* cell density (cells g wet wt oyster $^{-1}$ ), in a laboratory experiment. DNA production in dpm  $^3\text{H}$ -thymidine incorporated cell $^{-1}$ .

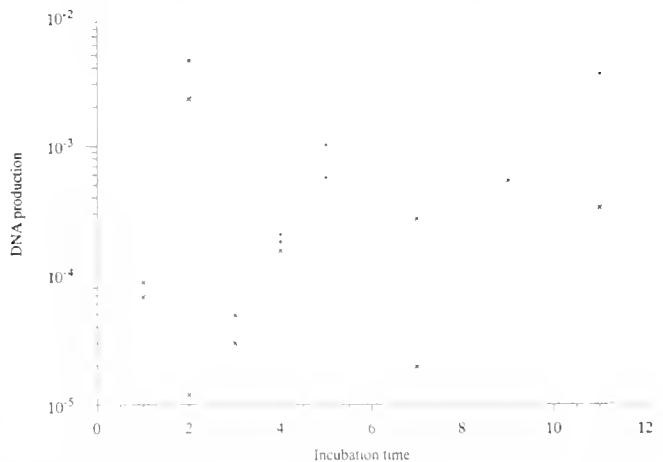
centration of aspartic acid was higher after 1 hr than at 5, 10 or 24 hr and then began a second and more significant increase at 60 hr which continued throughout the remainder of the time course. As environmental salinity did not change substantially during the 120 hr, these changes in aspartic acid concentration either indicate that the experimental animals' health varied—protein breakdown occurred as a result of injection and the oysters' health began to deteriorate at 60 hr and continued throughout the remainder of the time course—or the rate of feeding changed so that the amount of assimilated aspartic acid varied during the time course.

#### Tissue Growth

The rate of oyster tissue growth,  $k_{gs}f(t)$  typically was 1 to 10 times greater than the rate of tissue degradation,  $k_{1s}S$ . Therefore, the oyster experienced net tissue growth during the experiment (Fig. 8).

#### Cell Growth and Mortality

Figure 9 illustrates that the amount of aspartic acid incorporated into *P. marinus* DNA,  $D(t)$ , increased with time. The rate of



**Figure 4.** Changes in the amount of  $^3\text{H}$ -thymidine present per cell and incubation time over an extended time course. DNA production in dpm  $^3\text{H}$ -thymidine incorporated cell $^{-1}$ ; incubation time in days.

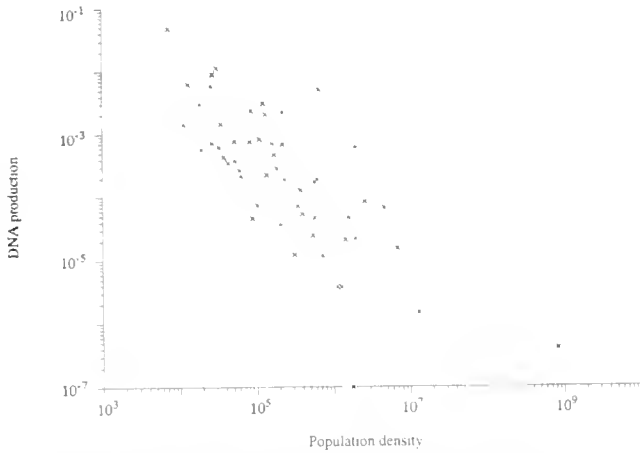


Figure 5. The relationship between the incorporation of  $^{14}\text{C}$ -aspartic acid into *P. marinus* DNA and *P. marinus* cell density during a 120-hr time course experiment. DNA production in  $\text{dpm cell}^{-1}$ ; Population density in  $\text{cells g dry wt oyster}^{-1}$ .

DNA synthesis,  $k_{\text{gd}}f(t)$ , was  $10^1$  to  $10^{17}$  times greater than the rate of DNA degradation,  $k_{\text{id}}D$ . Recall that net growth is the difference between DNA production,  $k_{\text{gd}}f(t)$ , and DNA loss,  $k_{\text{id}}D$ . The samples with  $k_{\text{gd}}f(t)/k_{\text{id}}D$  values of  $10^1$  suggest some *P. marinus* mortality; these occurred only after 60 and 120 hr of incubation and represented a small minority of the oysters used. The samples that had  $k_{\text{gd}}f(t)/k_{\text{id}}D$  values of  $10^2$  to  $10^{17}$  indicate such a low rate of mortality that mortality had only a minor effect on population doubling times. These negligible mortality rates occur at all incubation periods and in most oysters.

The *P. marinus* mortality rates were used to calculate the time required to decrease each population by half (assuming no population growth). The halving times ranged from  $10^4$  to  $10^{19}$  hr. Therefore, a *P. marinus* population experiencing no growth and the maximum mortality rate would experience 50% mortality in approximately 415 days. For all practical purposes, then, under the conditions of the experiment, no *P. marinus* mortality occurred. In effect, the oysters' immune systems were incapable of successfully protecting the oyster from parasite proliferation in this

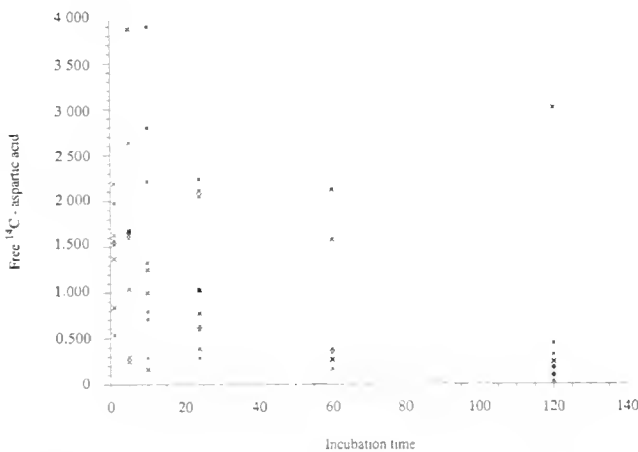


Figure 6. The change in the concentration of free  $^{14}\text{C}$ -aspartic acid ( $10^6$   $\text{dpm g dry wt oyster}^{-1}$ ) and incubation time (hr) during the 120-hr time course of the in vivo growth experiment.

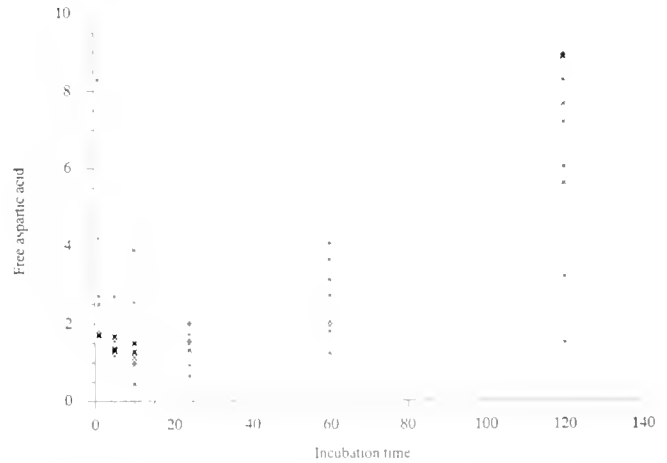


Figure 7. The change in the concentration of free aspartic acid ( $\mu\text{mole g dry wt}^{-1}$ ) and incubation time (hr) during the 120-hr time course of the in vivo growth experiment.

experiment, as expected under the high temperatures ( $30^\circ\text{C}$ ) characteristic of the summer season when the experiment was run.

#### Doubling Time

Figure 10 illustrates the dependency of doubling time on *P. marinus* cell density. As the parasite population density increases, the time required for the population to double in number also increases according to the power relationship:

$$\log_{10}(\text{doubling time}) = 0.014954 e^{[0.47364 \log_{10}(\text{cell density})]} \quad (19)$$

for cell densities from  $10^4$  to  $10^9$   $\text{cells g dry wt oyster}^{-1}$ ; doubling time in hr ( $R = 0.69$ ). No data exist below a cell density of  $10^4$   $\text{cells g dry wt oyster}^{-1}$ . The data suggest, however, that a population that is not substrate limited can double in less than 10 hr (the dashed line in Fig. 10). Thus minimum doubling time is likely in the range of 1 to 10 hr and population density effects probably become important at or near the lowest cell density measured by us, about  $10^4$   $\text{cells g dry wt oyster}^{-1}$ . A 1 to 10 hr doubling time is well within the range typical of single-celled organisms (e.g.

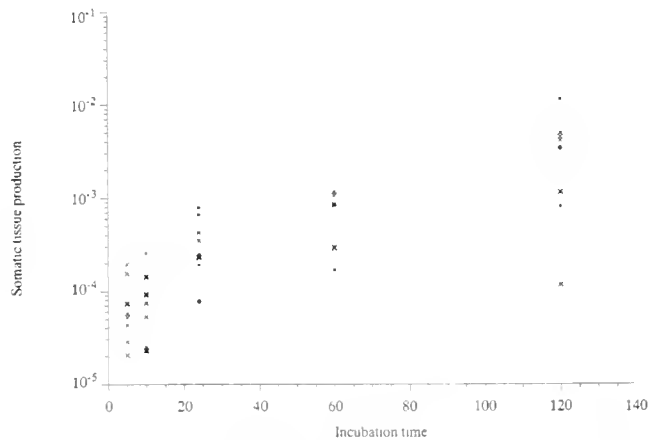
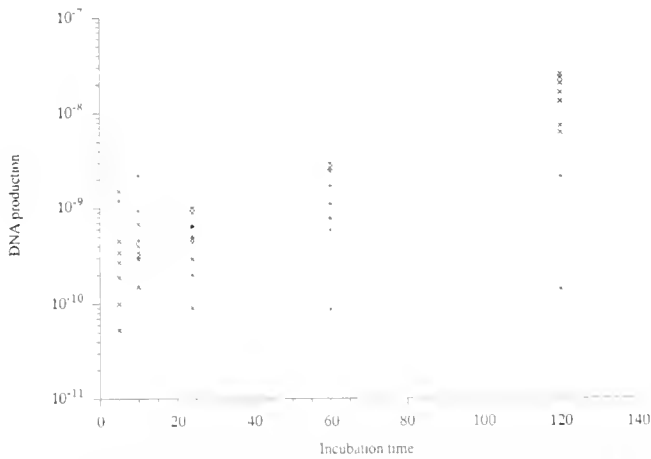


Figure 8. Results of model calculations of oyster tissue production,  $S(t)$ , ( $\text{mmole aspartic acid incorporated g dry wt}^{-1}$ ) versus incubation time (hr) based on the incorporation of  $^{14}\text{C}$ -aspartic acid.



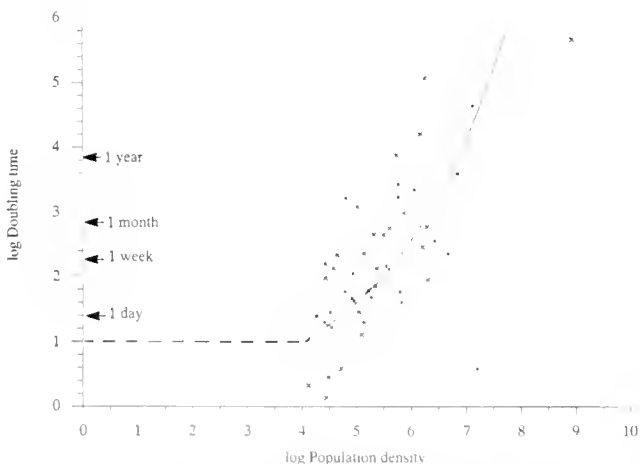
**Figure 9.** Results of model calculations for *Perkinsus marinus* DNA production,  $D(t)$  (mmole aspartic acid cell<sup>-1</sup>), versus incubation time (hr) based on the incorporation of <sup>14</sup>C-aspartic acid into *P. marinus* DNA.

Lovell and Konopka 1985) and comparable to rates measured for tumor cells (Casciari et al. 1992).

Protozoan generation times are mostly a function of the temperature and food availability of their environment (Laybourn-Parry 1987). For endoparasitic organisms, the host's body is their environment and their source of food. Many organisms experience an increase in growth rate as the temperature of their environment increases towards the upper range of their tolerance. Chu and Greene (1989) determined that *P. marinus* undergoes the most rapid development at a temperature of 28°C. Our growth experiment was performed at an average daily temperature of 30°C. Therefore, the growth rates we measured should be near the maximum rate.

#### Population-Level Effects

The generation time for a *P. marinus* population is affected by the density of the population. As the density of the parasites increases in the "closed" environment within an oyster, the para-

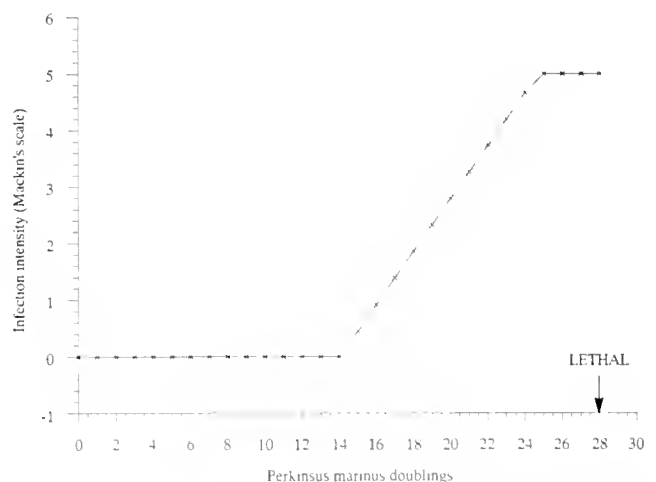


**Figure 10.** The effect of cell density ( $\log_{10}$  cells g dry wt oyster<sup>-1</sup>) on doubling time ( $\log_{10}$  hr). The dashed line is an approximate minimum doubling time for the population at low density.

sites deplete the energy resources of the oyster at an increasing rate. Eventually, the *P. marinus* population becomes so large that further growth of the parasites is limited by decreased food availability (Choi et al. 1989). The decrease in growth rate of the densest *P. marinus* populations is evident in Figure 10, which shows that the doubling time of a population of  $10^4$  parasites g dry wt oyster<sup>-1</sup> is approximately one day but the doubling time of a population of  $10^7$  parasites g dry wt oyster<sup>-1</sup> is approximately one year. These estimations include negligible mortality of *P. marinus*.

This virtual cessation of *P. marinus* growth at high infection intensities is vital to the survival of infected oysters during periods of high temperature. *P. marinus* infection intensities are commonly measured on the basis of the semiquantitative numerical scale from 0 (uninfected) to 5 (heavily infected) based upon examination of FTM-incubated oyster tissue samples (Mackin 1962). Infected oysters collected along the Gulf of Mexico coast during the summer months often exhibit *P. marinus* infection intensities of 3 and 4 (Soniati 1985; Quick and Mackin 1971; Ray 1954), which respectively correspond to population densities of  $2 \times 10^6$  to  $1.7 \times 10^7$  cells for a 1-g dry wt oyster.

Figure 11 illustrates the number of *P. marinus* generations (doublings) required to reach a given infection intensity on Mackin's scale for a 1-g dry wt oyster, assuming infection is initiated by a single cell and no mortality. Lethal infection intensities can develop from measured (false) negative infection intensities after 12 to 14 generations. [Infection intensities of  $\leq 10^3$  cells g wet wt oyster<sup>-1</sup> frequently generate false negatives (Choi et al. 1989)]. If the *P. marinus* populations in these infected oysters were doubling in only one day, the parasites would reach lethal cell densities in only a few days from an infection intensity of 3 or 4 at summer temperatures. Oysters along the Texas coast typically experience water temperatures of 30°C or higher for several months during the summer and early fall. If the generation time of *P. marinus* did not slow as infection intensities increased, infected oysters with infection intensities of 3 to 4 would be rare even in populations expe-



**Figure 11.** The number of *Perkinsus marinus* doublings required to reach the indicated level of infection on Mackin's scale assuming that infection was initiated by a single cell. Values are for a 1-g dry wt oyster. Choi et al. (1989) found that the first 12 to 14 doublings produced cell densities that would frequently produce false negatives when the standard technique of Ray (1966) was used. Lethal level is only approximate.

riencing heavy mortality because only a few days would be required to achieve a lethal infection level from an infection intensity of 3.

Thus oysters survive over the summer because doubling times increase. One possible explanation for the failure to find the development of *P. marinus* resistance in oysters is that oyster populations of varying resistance as measured by *P. marinus* growth and mortality rates, given long enough, will approach mean infection intensities of 3 to 4 simply because the decline in *P. marinus* growth rate produced by cell density overrides all other effects on doubling time.

One important consequence of the growth dynamics of *P. marinus* is the feedback of parasite density on population growth rate which tends to stabilize summer infection intensities in the range of 3 to 4 on Mackin's scale (moderate to moderately-heavy infections). Because an increased food supply would feed not only the oyster, but also decrease doubling time, the interplay of food supply and environment may be crucial in producing an epizootic.

Our data suggest that many populations routinely exist a few doublings from death for many months and that epizootics must be produced by mechanisms, not well understood, that destabilize this delicate balance.

#### ACKNOWLEDGMENTS

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## ENVIRONMENTAL EFFECTS ON THE GROWTH AND DEVELOPMENT OF EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN, 1791), LARVAE: A MODELING STUDY

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**ABSTRACT** The effects of temperature, food concentration, salinity and turbidity on the growth and development of *Crassostrea virginica* larvae were investigated with a time-dependent mathematical model. Formulations used in the model for larval growth are based upon laboratory data. Simulations were done using temperature conditions characteristic of Laguna Madre, Galveston Bay, Apalachicola Bay, North Inlet and Chesapeake Bay. These simulations show that the duration of the planktonic larval phase, which is determined by larval growth rate, decreases at lower latitudes in response to warmer water temperatures. Also, oysters in the more southern locations have a longer spawning season during which the oyster population can produce more larvae. Simulations were done for Galveston Bay and Chesapeake Bay using idealized time series of food supply that included higher concentrations in the spring, summer or fall. Additional simulations considered the effects of increased food supply in both spring and fall seasons. The results show that shifting the period of enhanced food supply from March-April to April-May, when temperatures are warmer, reduces the minimum larval planktonic period from 44 to 34 days. Shifting the fall bloom from August-September to September-October, however, does not appreciably change the minimum larval planktonic period. The final set of simulations considered the effect of low salinity events and turbidity on the planktonic period of the larvae of *Crassostrea virginica*. By imposing a simulated low salinity (5 ppt) event of one month duration in August, the larval planktonic time is increased by about 39% over normal August salinities. Turbidity concentrations less than  $0.1 \text{ g l}^{-1}$  result in slightly decreased planktonic times. These model results show clearly the importance of ambient environmental conditions in determining the planktonic time of larvae of *Crassostrea virginica*, and hence their ultimate recruitment to the adult oyster population.

### INTRODUCTION

The failure to obtain a significant correlation between brood-stock size and yearly spatfall success in many species, including the eastern oyster *Crassostrea virginica*, indicates that adult fecundity and/or larval survival are as important as adult abundance in determining the viability of the population (Prytherch 1929, Loosanoff and Engle 1940, Olson and Olson 1989). Understanding the basic causes of the large year-to-year variation in spatfall success at any site (Loosanoff 1966, Kenny et al. 1990) and the apparent latitudinal gradient in adult population stability (persistence and resilience) (Powell et al., in press), requires that the interaction of environmental factors on oyster reproduction and larval survival be examined over a wide range of environmental conditions.

The timing and intensity of spawning of *Crassostrea virginica*, is influenced by a variety of factors, some of which are temperature, salinity and food supply. A recent modeling study (Hofmann et al. 1992) showed that, for conditions representative of mid-latitude bays, the timing of the spring increase and fall decrease in water temperature relative to the spring and fall phytoplankton blooms can significantly alter the pattern, frequency and intensity of spawning in an oyster population. Depending upon the juxtaposition of the spring temperature and food supply increase, the first spawning may occur any time from April to June. The timing of the final fall spawn is equally as variable. The key spawning pulses, which account for the majority of the reproductive effort, may also occur at widely different times during the spawning season in response to variations in environmental conditions. As a consequence, the environment experienced by larvae of *Crassos-*

*trea virginica* may encompass a wide range of temperature, salinity and food conditions.

Once the larvae are spawned, recruitment to the adult population is determined by the survivability of the larvae in the plankton. Survivorship can be expected to be inversely correlated with larval life span because most factors controlling mortality, like predation, should be functions of the time of exposure, namely larval life span. The time spent in the plankton is determined by the larval growth and developmental rates which are significantly affected by environmental conditions.

Loosanoff and Davis (1963) and Loosanoff (1965) showed that temperature and food concentration were the two primary environmental variables affecting the development of *Crassostrea virginica* larvae. Additional studies demonstrated that salinity (Butler 1949, Davis 1958, Davis and Calabrese 1964, Ulanowicz et al. 1980), turbidity (Davis 1960, Carriker 1986, Huntington and Miller 1989), and oxygen content (Widdows et al. 1989) also affect larval growth and survival. These studies, while providing insight into the factors controlling larval growth, typically considered only one or two environmental factors. However, in the environment it is the combined effect of all environmental factors that determines the growth, development and ultimate survivorship of the larvae.

To investigate the interaction of environmental factors on the growth and development of oyster larvae, we developed a time-dependent numerical model that combines the effects of food concentration, temperature, salinity and turbidity on the growth and development of oyster larvae. Formulations for larval growth and development are taken from laboratory experiments and are combined with time series of monthly-averaged food, temperature,

salinity and turbidity measurements from several bays along the east coast of the U.S. and the Gulf of Mexico, ranging from Chesapeake Bay to the Laguna Madre.

The model was used to simulate oyster larval growth and development over a range of latitudes in response to varying environmental conditions. Simulations are presented that illustrate the importance of the timing of events, such as the occurrence of the spring bloom in relation to increasing water temperature, to the survival and potential recruitment success of the larvae. The results of this study, while specific to the larvae of *Crassostrea virginica*, have relevance to any organism whose life history contains a planktonic larval stage. The conclusions from this study relate to the more general questions concerning the processes that determine larval survivability and ultimately recruitment success.

The following section presents the formulations that were used to model the growth and development of the oyster larvae. The simulations presented in the results section are designed to illustrate the isolated effect of temperature as well as the combined effects of temperature, food, salinity and turbidity on larval growth and development. These results are followed by a discussion and summary.

## MODEL

### Larval Development

Before describing the larval growth and development model, it is first useful to discuss the characteristics of the larval life history that are important to the model. Stafford (1913) and Galtsoff (1964) present measurements of larval development (measured in  $\mu\text{m}$ ) at  $24^\circ\text{C}$  as a function of time. These data sets, when normalized by total developmental time at  $24^\circ\text{C}$ , allow construction of a growth curve that expresses larval development as a fraction of total developmental time (Fig. 1). The representation of larval growth as a fraction of total developmental time standardizes the growth curve. In this way, the variability in total developmental time, resulting from development at different temperatures is eliminated. This approach assumes that larval oyster development is equi-proportional, which means that a given stage persists for the same fraction of total development independent of temperature. However, the duration of a given stage will vary with temperature.

For the first 8% of its development the oyster larva is non-feeding. Larval growth during this time is supported by a small energy reserve which is sufficient for the larva to increase in its

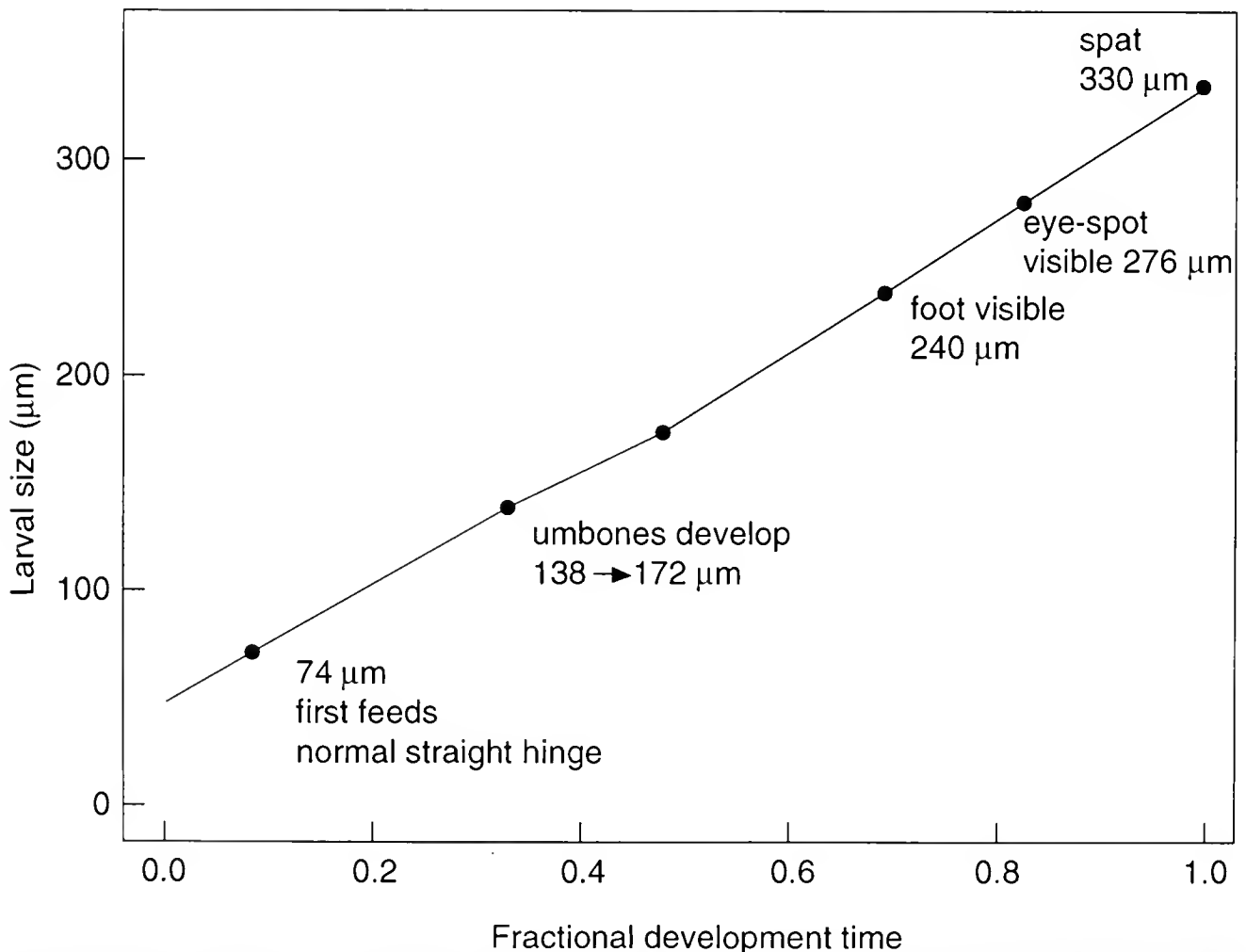


Figure 1. Larval development expressed as a fraction of total developmental time. The sizes given for the larval developmental stages represent average population values. Data used to construct the figure are from Galtsoff (1964) and Stafford (1913). Developmental times were measured at  $24^\circ\text{C}$  and 26.5 ppt. Major changes in larval development are indicated.

length dimension about 20  $\mu\text{m}$  (Galtsoff 1964, Stafford 1913). The larva first feeds when it measures 74  $\mu\text{m}$  (Yonge 1960, Galtsoff 1964). After it begins feeding, larval growth rate is determined by *in situ* environmental conditions. Settlement occurs when the larva measures 300 to 350  $\mu\text{m}$  (Galtsoff 1964).

#### Governing Equation

The larval model includes the effects of temperature, salinity, food concentration and turbidity on larval growth and development. Stated mathematically:

$$\frac{dS}{dt} = \text{growth}(\text{food}, \text{size}) * \text{tsfactor} * \text{turbef} \quad (1)$$

where  $S$  is larval size [a length measurement; anteroposterior distance in  $\mu\text{m}$  (Carriker 1979)]. The increase in larval size over time is determined from measurements that relate ambient food concentration and larval size to growth rate. This growth rate is then modified by the ambient temperature and salinity (*tsfactor*) and turbidity effects (*turbef*). The effect of hypoxia on larval development (Widdows et al. 1989) is not included in the model because observations to adequately describe this effect on larval growth and development are lacking for the environments considered in this study. Also, in most of the bays used in this study, prolonged periods of low oxygen do not occur. The measurements and relationships used to formulate the terms on the right side of equation (1) are described below. Equation (1) was solved numerically using an Euler method with a time step of one day.

#### Growth Rate

Food availability has a major effect on the growth rate of the larvae of *Crassostrea virginica* (Loosanoff and Davis 1963, Loosanoff 1965). In many growth models constructed for planktonic organisms (e.g., Steele and Frost 1977, Hofmann and Ambler 1988) the effect of available food is obtained from relationships between ingestion rate and ambient food concentration. The ingested food is then apportioned with an energetics-based approach to satisfy requirements for growth, development, reproduction and other metabolic responses. For the larvae of *Crassostrea virginica*, some feeding rates and energetics measurements are available (Baldwin and Newell 1991, Chrétiennot-Dinet et al. 1991). However, these measurements are not sufficient to allow derivations of relationships that include a range of environmental conditions, e.g., temperature effects on ingestion rate. Therefore, an approach that does not depend explicitly on relationships for individual metabolic processes was used to obtain larval growth and developmental rates.

Rhodes and Landers (1973) measured larval growth rates at 28°C and 26 ppt, for several food concentrations and for larval sizes that ranged from 74.2 to 255  $\mu\text{m}$ . These laboratory measurements were linearly interpolated to obtain larval growth rates at intermediate sizes and food concentrations (Fig. 2). The food concentrations shown in Figure 2, encompass the full range of values that larvae experience in the environment. The growth rate at 255  $\mu\text{m}$  was assumed to apply for larval sizes from 255 to 330  $\mu\text{m}$  (settlement size), for all food concentrations.

The larval growth rates given in Figure 2, show low growth rates at low food concentrations at all sizes. Maximum growth rates occur at larval sizes of 105 to 135  $\mu\text{m}$ , at food concentrations

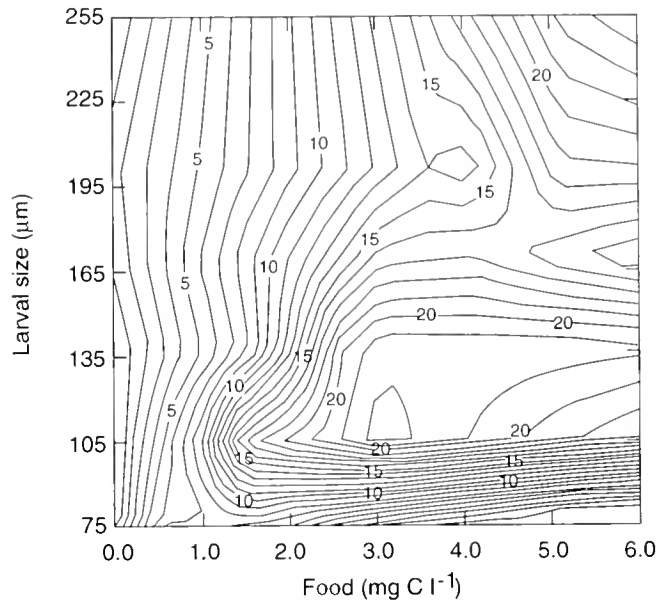


Figure 2. Effect of varying food concentration (at 28°C and 26 ppt) on larval growth rate, as a function of larval size. The contours represent larval growth rate in  $\mu\text{m d}^{-1}$ . Contour interval is 1.0  $\mu\text{m d}^{-1}$ .

of 3.0  $\text{mg C l}^{-1}$ . The growth rates are used to specify the growth term on the right hand side of equation (1) for a given larval size and ambient food concentration.

#### Temperature-Salinity Effects

Davis (1958) and Davis and Calabrese (1964) present measurements of oyster larval growth rate in  $\mu\text{m d}^{-1}$  for a range of temperatures (17.5 to 32.5°C) and salinities (7.5 to 27.5 ppt). These data were linearly interpolated to obtain larval growth rates at intermediate temperature and salinity values.

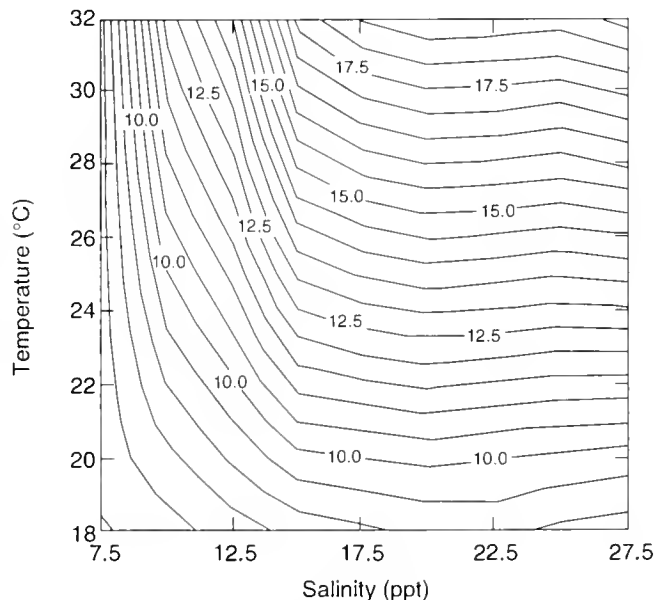


Figure 3. Temperature and salinity effects (at optimal food concentration) on larval growth rate. The contours represent larval growth rate in  $\mu\text{m d}^{-1}$ . Contour interval is 0.5  $\mu\text{m d}^{-1}$ .

TABLE 1.

Fractional change in larval growth rate at specific salinities and temperatures. See text for details.

Temperature °C	Salinity (ppt)						
	5.0	7.5	12.5	17.5	22.5	27.5	32.0
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	0.0	0.47	0.52	0.56	0.58	0.55	0.55
20	0.0	0.48	0.57	0.63	0.63	0.62	0.62
22	0.0	0.49	0.63	0.72	0.73	0.72	0.72
24	0.0	0.49	0.68	0.81	0.82	0.82	0.82
26	0.0	0.49	0.73	0.90	0.92	0.92	0.92
28	0.0	0.49	0.78	0.99	1.01	1.02	1.20
30	0.0	0.49	0.83	1.08	1.10	1.11	1.11
32	0.0	0.49	0.88	1.18	1.20	1.21	1.21
35	0.0	0.49	0.88	1.18	1.20	1.21	1.21

The general features of the temperature and salinity effects on larval growth rate are as expected (Fig. 3). At low salinities and temperatures the larval growth rate is low. As temperature increases, larval growth rate increases at all salinity values. At all temperatures, salinities of 17.5 to 25 ppt. result in slightly in-

creased larval growth rates. This suggests that salinities in this range are optimal for the growth of larvae of *Crassostrea virginica*.

The upper and lower bounds of the temperature and salinity effects on growth rate (Fig. 3) were extended to 15°C, 0 ppt and 35°C, 32 ppt respectively, to encompass the range of possible values to which the larvae might be exposed. Larvae kept at or below 15°C show no growth, while larvae maintained at temperatures of 17.5°C show minimal growth (Davis and Calabrese 1964). By assuming zero growth at 15°C and using the measured growth rate at 17.5°C, the larval growth rates between 15 and 17.5°C were obtained by linear interpolation. Below 15°C, larval growth rate is assumed to be zero. A drastic reduction in larval growth occurs at temperatures greater than 35°C, but not before (Davis and Calabrese 1964). Therefore, the upper limit for temperature was set at 35°C. The larval growth rates were extended to 35°C by using the measured value at 32°C, across all salinities. This assumes that larval growth rate is constant between 32 and 35°C.

Larvae of *Crassostrea virginica* show no growth at salinities below 5 ppt, and minimal growth at 7.5 ppt (Davis 1958). Therefore, larval growth rate is assumed to be zero between 0 and 5 ppt, and growth rates between 5 and 7.5 ppt were obtained by linear interpolation using the measured value at 7.5 and zero growth at 5

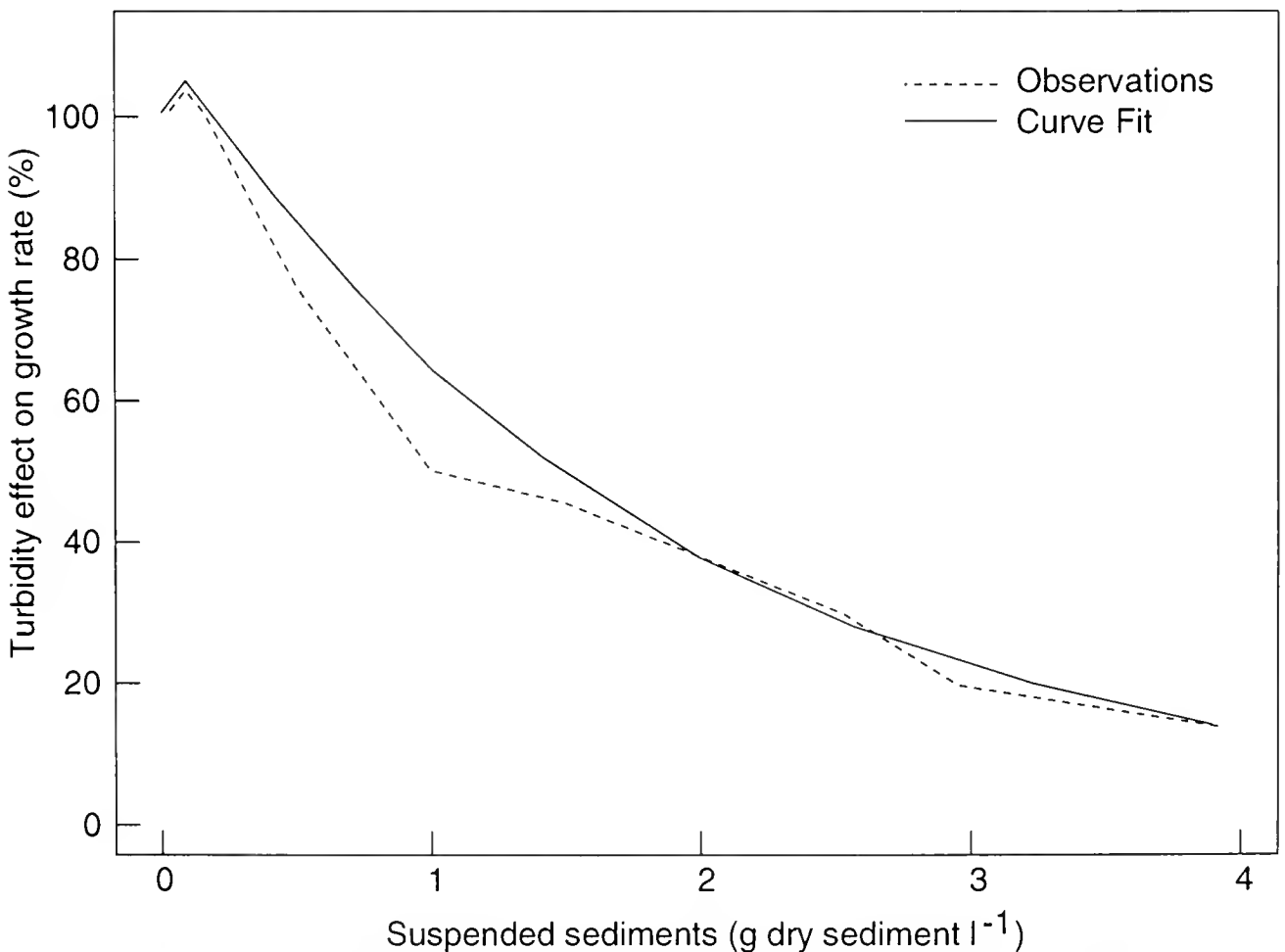


Figure 4. The effect of turbidity on growth rate of *Mercenaria mercenaria* larvae. Dashed line is constructed from measurements given in Davis (1960) and Huntington and Miller (1989). Solid line represents the curve fit to these data.

TABLE 2.

Characteristics of the monthly-averaged temperature time series used in the model. All temperatures expressed in °C. Spring warming and fall cooling were assumed to occur when temperature increased and decreased to 20°C, respectively.

Bay	Minimum Temperature	Maximum Temperature	Average Temperature	Spring Warming	Fall Cooling
Chesapeake Bay <sup>1</sup>	1.0	26.0	14.9	May 1	Sept 15
North Inlet <sup>2</sup>	9.8	28.2	19.2	May 1	Oct 3
Apalachicola Bay <sup>3</sup>	8.9	26.7	20.4	April 20	Nov 15
Galveston Bay <sup>4</sup>	10.0	27.0	19.8	April 20	Nov 11
Laguna Madre <sup>3</sup>	12.2	29.2	22.9	March 4	Nov 24

<sup>1</sup> Berg and Newell 1986, <sup>2</sup>Crosby and Roberts 1990, <sup>3</sup>Powell et al. 1992, <sup>4</sup>Soniat and Ray 1985

ppt. Above 27.5 ppt larval growth rate was held constant at the rate for 27.5 ppt for all temperatures. This assumes a constant salinity effect on larval growth rate at salinities between 27.5 and 32 ppt.

In order to modify the larval growth rates shown in Figure 2 by temperature and salinity effects, the growth rates shown in Figure 3 were normalized by the temperature (28°C) and salinity (26 ppt) value at which the food dependent growth rates were obtained. The resultant values (Table 1) scale the larva growth at any temperature or salinity relative to that at 28°C and 26 ppt. This normalization assumes that temperature and salinity effects are equivalent across all size classes and at all food concentrations, as is true for juvenile and adult oysters (Powell et al. 1992).

#### Turbidity

Laboratory studies have shown that suspended sediment concentrations greater than 0.1 g dry sediment l<sup>-1</sup> produce a reduc-

tion in growth rate of *Mercenaria mercenaria* larvae (Huntington and Miller 1989). However, sediment concentrations below this value result in an enhancement of larval growth rate (Davis 1960, Huntington and Miller 1989). Assuming that the measurements given for *Mercenaria mercenaria* in Davis (1960) are representative of the growth response of *Crassostrea virginica* larvae to turbidity, a relationship relating turbidity effects to larval growth rate was obtained as:

for turbidity values <0.1 g l<sup>-1</sup>

$$turbef = m * turb + c \quad (2)$$

for turbidity values >0.1 g l<sup>-1</sup>

$$turbef = be^{B(turb - turb0)} \quad (3)$$

where *turb* is the suspended sediment concentration in g dry wt l<sup>-1</sup>. The first relationship gives the fractional enhancement of larval growth rate, with *m* and *c* equal to 0.542% (g dry wt · l<sup>-1</sup>)<sup>-1</sup> and 1.0%, respectively. The second relationship gives the frac-

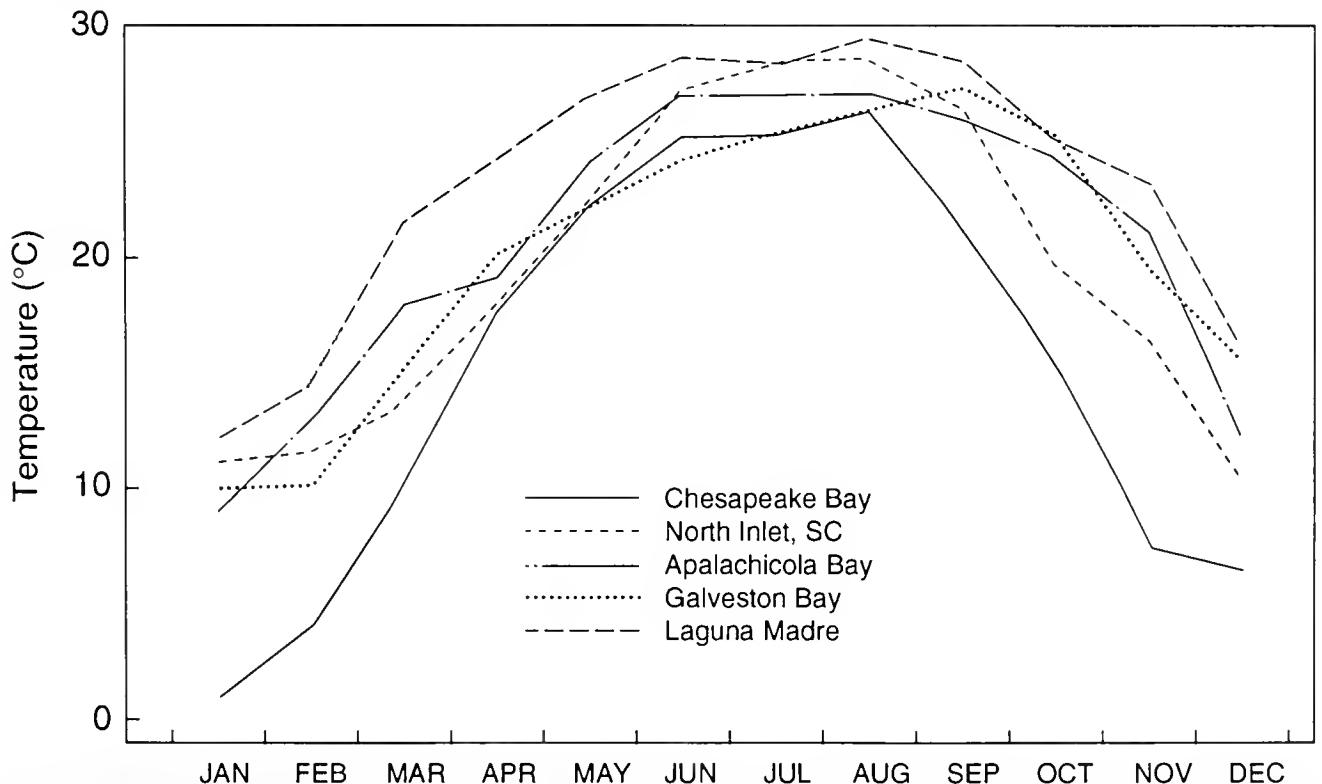
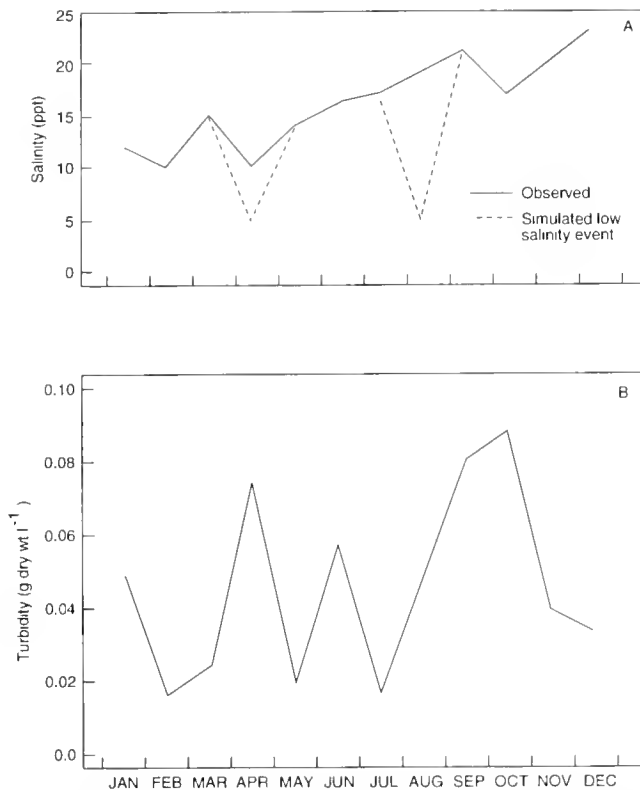


Figure 5. Monthly-averaged temperature time series for five different bays. Temperature values are plotted at the middle of each month. See Table 2 for literature citations for the source of these data.



**Figure 6.** A: Monthly-averaged salinity values from Galveston Bay, Texas measured by Soniat et al. (1984). Salinity values are plotted at the middle of each month. The dashed lines represent simulated low salinity events imposed in mid-April and mid-August. B: Monthly-averaged turbidity values from Galveston Bay, Texas measured by Soniat (1982). Turbidity values are plotted at the middle of each month.

tional decrease in larval growth at higher turbidity concentrations, where the values of  $b$ ,  $\beta$  and  $turb\theta$  are 0.375%, 0.5 (g dry wt l<sup>-1</sup>)<sup>-1</sup>, 2.0 g dry wt l<sup>-1</sup>, respectively. These relationships are used to specify the fractional change in larval growth rate in equation (1). The correspondence between equations (2) and (3) and the observations is shown in Figure 4.

### Environmental Forcing

#### Temperature

The temperature distributions used as input to the model consisted of monthly-averaged time series from five bays along the east coast of the United States and the Gulf of Mexico (Table 2, Fig. 5). All of the temperature time series extend for one year. In general, all time series show the temperature variations that are expected for temperate mid-latitude bays. The spring increase in temperature and the fall decrease in temperature occurs later in the spring and earlier in the fall respectively, in the more northerly bays (Table 2).

#### Salinity and Turbidity

The salinity time series used in the model is from Galveston Bay, Texas (Fig. 6a), which has been chosen to be representative of a temperate latitude bay in a majority of the simulations presented in this paper. Salinity in Galveston Bay tends to be low (less than 15 ppt) during spring months as a result of increased

freshwater discharge. During summer and fall months, salinity increases. Maximum salinities of about 20 to 25 ppt usually occur in August and persist throughout the fall. These trends are typical of most estuarine systems.

On occasion, estuarine systems are influenced by short-term periods of freshwater discharge. This may occur in the spring, for example, in response to spring storms. To simulate the effects of this type of event, the Galveston Bay salinities were modified by imposing a low salinity event, which decreases to 5 ppt and then increases back to the normal salinity level over a one month period, on April 15th and on August 15th. These modifications were imposed, so that the effects of low salinity events on larval growth could be investigated.

The monthly-averaged turbidity values (Fig. 6b) used in the model are also from Galveston Bay, Texas (Soniat 1982). These values range from 0.005 to 0.088 g dry sediment l<sup>-1</sup>, with maximum values occurring in the spring and fall. These measured turbidity values are below the concentration at which larval growth is inhibited (cf. Fig. 4).

#### Food Concentration

Phytoplankton biomass (and production) in estuarine systems exhibits considerable seasonal variability in terms of when maxima may occur. For example, in Chesapeake Bay, chlorophyll maxima have been observed to occur as distinct spring or fall blooms (Harding et al. 1986), as a spring or fall bloom (Malone et al. 1986, Malone et al. 1988), or as a summer maxima (Malone et al. 1988). Similar variability in the seasonal distribution of phytoplankton biomass maxima have been observed in Galveston Bay (Wilson, unpub. obs.).

The wide temporal range over which maxima in phytoplankton biomass occur could have considerable impact on survival of oyster larvae, which depend on this for food supply. To test this effect, idealized time series, in which the timing of the maximum in food supply was varied, were used to specify environmental food concentrations. These time series include a single maximum in food supply in spring (Fig. 7a), summer (Fig. 7b), and fall (Fig. 7c) as well as maxima in the spring and fall (Fig. 7d). The range chosen for the food values in these time series is based upon that observed for Galveston Bay (Soniat and Ray 1984). The yearly-integrated food supply is the same for all the time series that include a single maximum. The double maxima time series gives a slightly higher (14%) yearly food availability.

As a comparison, a food supply time series was constructed from observations reported in Soniat and Ray (1984) from the western central portion of Galveston Bay (Fig. 7e). This time series shows a maximum in food supply during summer months (May to September). More recent observations (Wilson, unpub. obs.) also show a summer maximum in food supply for this region of Galveston Bay. Malone et al. (1988) suggested that a summer maximum in phytoplankton productivity may be a general characteristic of mid-latitude, partially-stratified estuaries.

## RESULTS

#### Model Verification

Observations on the effect of temperature on total oyster larval developmental time given in Davis and Calabrese (1964) provide an independent check on the simulated larval developmental times. These observations (Fig. 8) are in agreement with developmental times obtained at a specific temperature from laboratory culture experiments for Chesapeake Bay oyster larvae (Dupuy

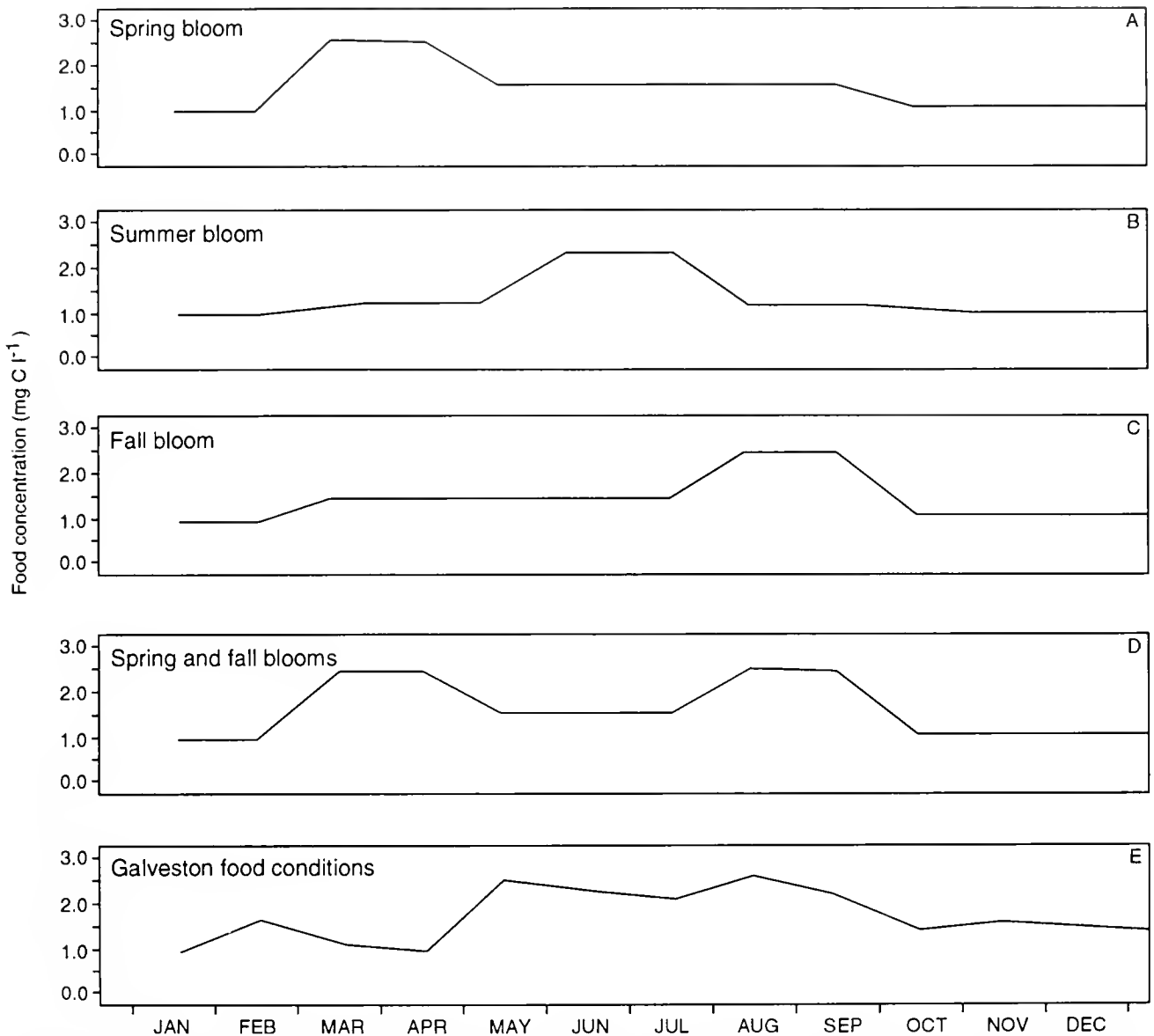


Figure 7. Idealized and measured time series used to specify the ambient food concentration for the larval model in  $\text{mg C l}^{-1}$ . A: Spring bloom in March-April. B: Summer bloom in June-July. C: Fall bloom in August-September. D: Spring bloom in March-April and fall bloom in August-September. E: Monthly-averaged food concentrations measured for Galveston Bay by Soniat and Ray (1984).

1977). The observed developmental times shown in Figure 8 can be used to obtain a relationship from which total developmental time in days,  $D$ , at a specific temperature,  $T$ , can be estimated:

$$D = ae^{-\alpha(T-T_0)} \quad (4)$$

The base temperature,  $T_0$ , was chosen to be  $24^\circ\text{C}$ . The values of the coefficients  $a$  and  $\alpha$  are 25 days and  $0.1099^\circ\text{C}^{-1}$ , respectively. This relationship assumes optimal salinity and food conditions. A comparison of the developmental times estimated from equation (4) and the observed developmental times is given in Figure 8.

Numerous simulations were run with constant and idealized environmental time series to ensure that the larval developmental response was correct. One such simulation used the temperature and salinity ( $24^\circ\text{C}$  and 26.5 ppt) conditions that correspond to those used in the laboratory experiments from which Figure 1 was generated. Galtsoff (1964) did not report the food concentration used in these experiments; however, given the developmental

times, it is unlikely that the larvae were food limited. Therefore, the food concentration in the simulation was held constant at an optimal value of  $3 \text{ mg C l}^{-1}$  (Fig. 2). For these environmental conditions, the total simulated developmental time was 25 days. The total time obtained from equation (4) is 25 days.

The importance of food supply for the growth and development of oyster larvae is emphasized when comparing simulations using the previous temperature and salinity conditions ( $24^\circ\text{C}$  and 26.5 ppt) for a range of food concentrations. The larval developmental time extends to 37 days for food concentrations of  $2 \text{ mg C l}^{-1}$ . Doubling the food concentration to  $4 \text{ mg C l}^{-1}$ , gives a larval period of 23 days, which is a 38% reduction over the previous.

The larval developmental curve obtained from the simulation using a food concentration of  $2 \text{ mg C l}^{-1}$  (Fig. 9a) is similar to the measured developmental curve (Fig. 1). Larval growth rate is rapid through the first 20% of development (after first feeding), which corresponds to a time of rapid increase in length. Larval

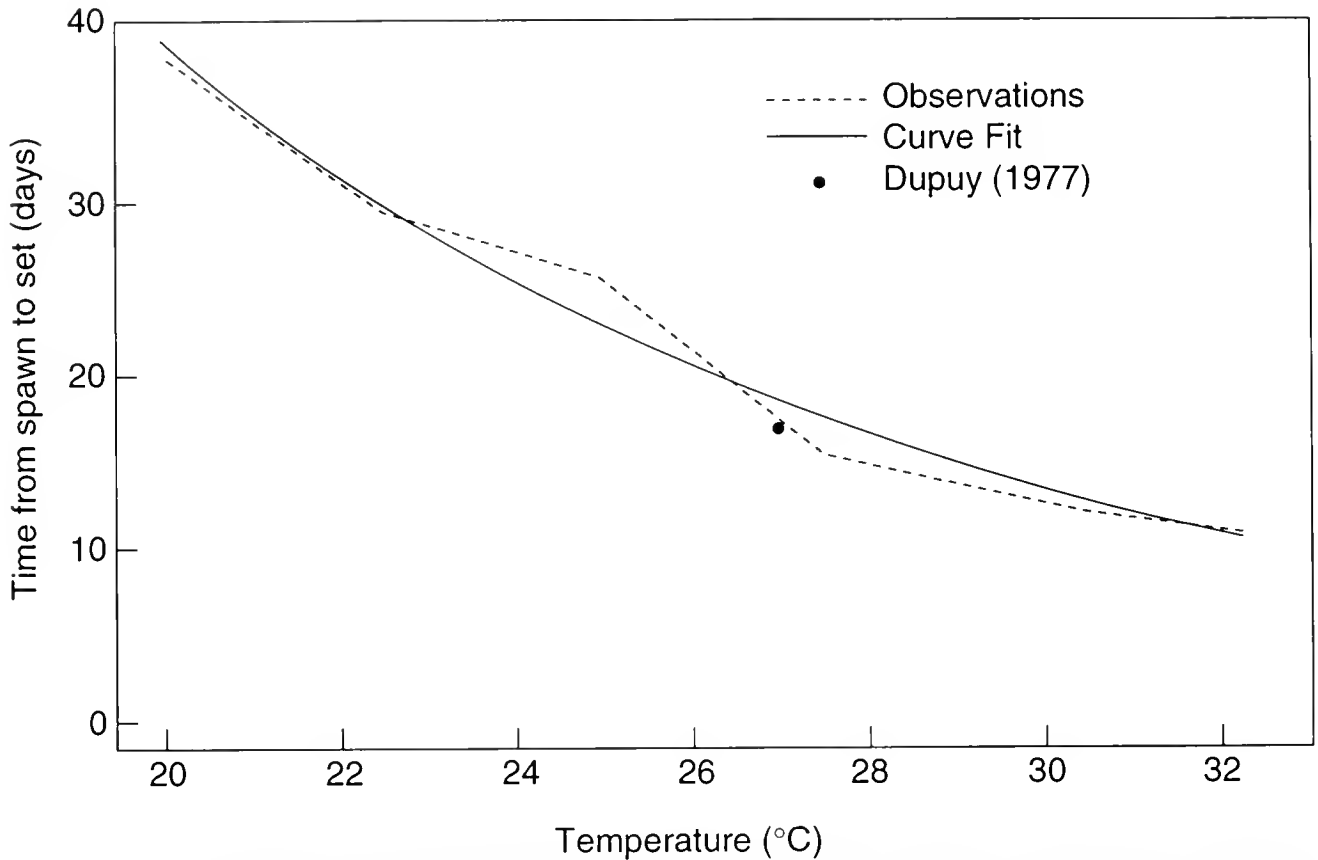


Figure 8. The effect of temperature on total development time of oyster larvae. The dashed line represents data from Davis and Calabrese (1964). The solid line represents the curve fit to these data using equation (4). The filled circle represents larval development time measured by Dupuy (1977).

growth rate decreased markedly between 138 and 172  $\mu\text{m}$  and continued to decrease until the larvae metamorphosed at 330  $\mu\text{m}$ .

The pattern of larval growth rate and increase in size is similar when temperature, salinity and food concentrations (26°C, 19 ppt, 2.5  $\text{mg C l}^{-1}$ ) measured in Galveston Bay, Texas in August are input into the model (Fig. 9b). Overall, the characteristics of the simulated larval development correspond to developmental curves derived from laboratory measurements. The primary difference is that larval growth rate is higher, which results from higher temperatures in Galveston Bay. These comparisons show that the model given by equation (1) adequately describes oyster larval growth and development. Therefore, the model was used to test hypotheses concerning the effects of temperature, food availability, low salinity events and turbidity on oyster larval development. The results of these simulations are given in the following sections.

#### Temperature

The first series of simulations considered temperature effects on oyster larval development. The other environmental conditions were assumed to be optimal; a constant salinity of 24 ppt, food concentrations that include a spring bloom (Fig. 7a) and zero turbidity. The monthly-averaged temperature time series from the five bays (Fig. 5) were used to specify ambient temperature conditions, which allows the comparison of temperature effects on larval development across a latitudinal gradient as well as seasonal effects within specific bays. The simulations were initialized by

introducing larvae on the last day in March and every 10 days thereafter. Simulations were ended when the larvae either attained, or failed to attain, the size of 330  $\mu\text{m}$  at which metamorphosis occurs.

The time from spawn to metamorphosis (Fig. 10) shows differences within individual bays as well as between bays. The largest range in total planktonic time occurs in Chesapeake Bay. Larval planktonic time decreases with decreasing latitude (Table 3). In the summer months, the larval planktonic times in different bays are similar, varying only from 14 to 20 days. The three southernmost bays show similar trends in planktonic life span even into the fall, with Laguna Madre consistently having larvae with the shortest planktonic life span. However, the fall planktonic life spans increase dramatically from Laguna Madre to Chesapeake Bay. The practical result of this trend is that the last settlement occurs progressively later in the fall from north to south. The simulated spawning seasons for each bay are in agreement with spawning seasons defined from field studies (Table 3).

#### Food Availability

In Galveston Bay, Texas, water temperatures begin to increase in March and reach 20°C in April (Fig. 5; Table 2). A spring bloom in March-April may coincide with this warming. The larval development, occurring in response to these temperature and food conditions and a constant salinity of 24 ppt, results in the planktonic times shown in Figure 11a. The minimum time from spawn to set is 44 days in early April, when increased food is available



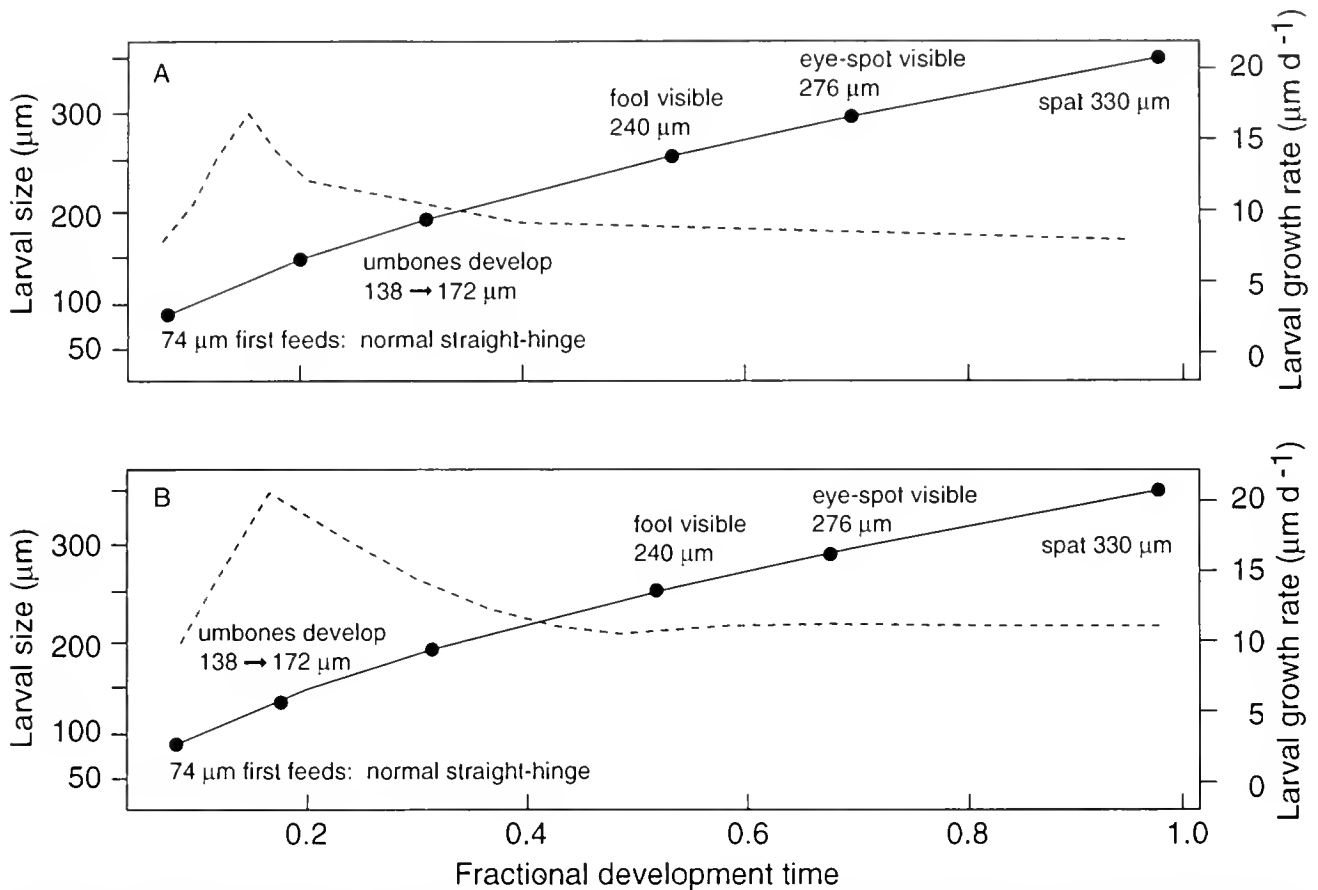


Figure 9. A: Simulated development (solid line) and growth rate (dashed line) for larvae exposed to environmental conditions of; 24°C, 26.5 ppt, 2 mg C l<sup>-1</sup> food, and zero turbidity. B: Simulated development (solid line) and growth rate (dashed line) for larvae exposed to temperature, salinity and food conditions typical of Galveston Bay, Texas, and zero turbidity.

(Table 4). Later in April and May, planktonic time increases, then decreases into the summer months, and increases again in the fall. The shorter times initially are the result of increased food, which enhances larval growth rate. Throughout the remainder of the year developmental time is controlled primarily by temperature in this simulation.

Moving the spring bloom to April and May, so that it occurs after the spring increase in temperature, results in significantly decreased planktonic times relative to the earlier bloom. Once the increased food is no longer available, larval development and planktonic time are once again primarily temperature controlled.

Imposing a bloom, in June and July, when temperatures average 24 to 25°C, results in planktonic times of 28 days (Table 4). An early to mid-summer maximum in food supply results in long planktonic times in the spring and fall and reduced times in the mid to late summer (Fig. 11b). Similar patterns in larval planktonic time are obtained with the Soniat and Ray (1984) food time series.

A planktonic bloom in August-September coincides with the time when temperatures in Galveston Bay are still elevated. The combination of warm temperatures and enhanced food availability result in 25 day planktonic periods (Table 4). As the food availability decreases and the waters cool into the fall months, larval development slows and planktonic times are longer (Fig. 11c). The occurrence of a bloom in September-October extends the period of minimum planktonic time further into the fall, offsetting

the decrease in temperature (Fig. 11c). The enhanced food concentrations produce increased larval growth rates into the fall similar to the introduction of a bloom in August-September.

A year in which spring and fall blooms coincide with the spring and fall temperature increases results in planktonic times shown in Figure 11d. In this case, the impact of the spring bloom is minimal because of cooler water temperatures. Increased food availability coupled with higher fall temperatures results in a dramatically shorter planktonic period of 25 days in August and September as compared to the 44 day planktonic period in the spring (Table 4).

As a comparison, the monthly-averaged Chesapeake Bay temperatures (Fig. 5) were used with the six idealized food time series to obtain larval planktonic times for a more northern bay. Salinity was held constant at 24 ppt and turbidity was zero. The results of these simulations (Table 4) show that shifting the spring bloom has little effect on reducing planktonic times in Chesapeake Bay because of the cooler spring temperatures that characterize this bay. A bloom in June and July in Chesapeake Bay results in the shortest planktonic period of 27 days. While a bloom during the same time frame in Galveston Bay does result in an abbreviated planktonic period, the shortest larval planktonic periods occur in Galveston Bay in August when the bay temperatures exceed the June and July values.

Blooms that occur early in the fall, after warming occurs, have more of an effect on reducing larval planktonic times than the

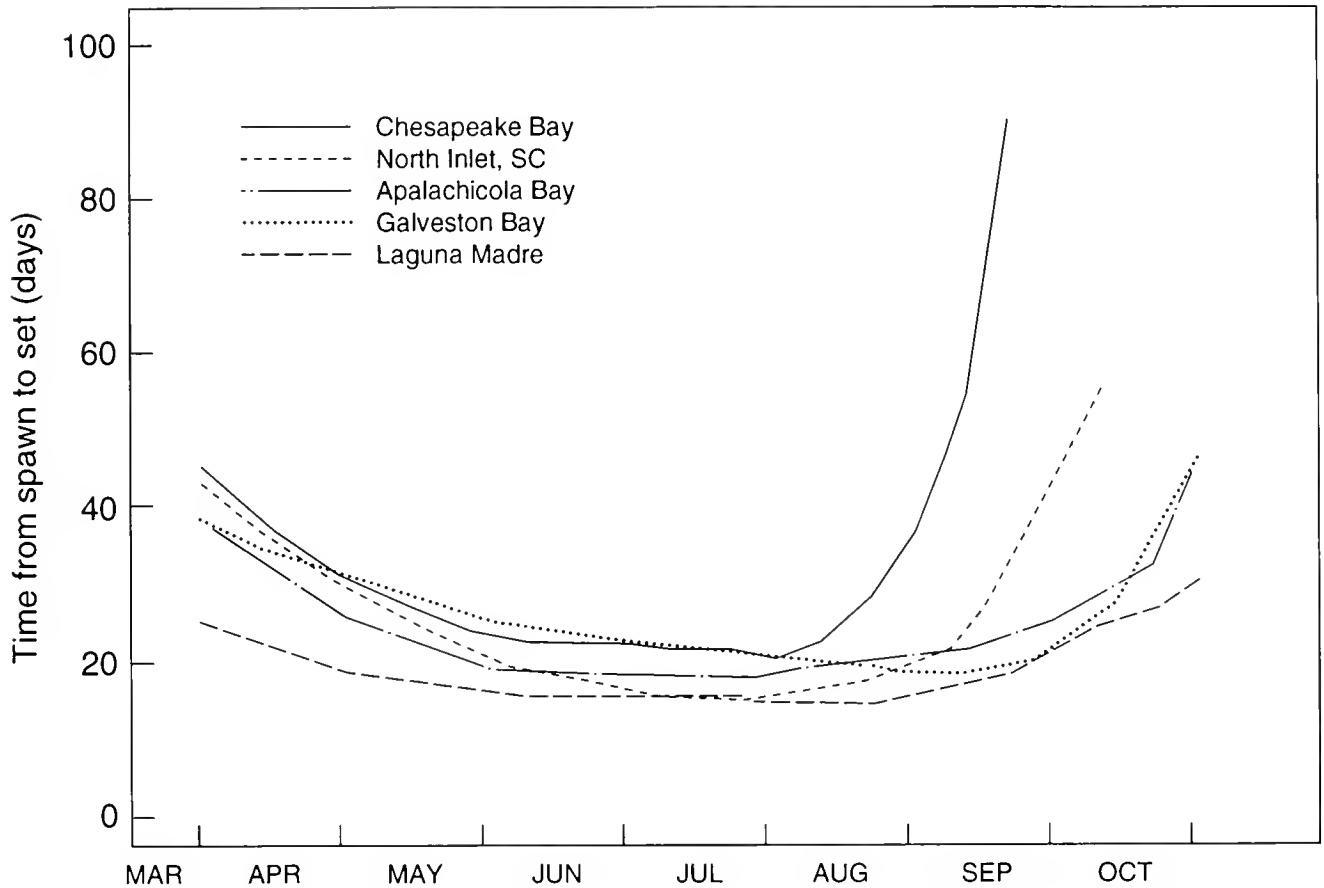


Figure 10. Simulated planktonic time from early spring to late fall for oyster larvae exposed to temperature time series for the five indicated bays.

spring blooms. Consistently, the maximum larval period in the Chesapeake Bay is April to May, irrespective of the timing of the maximum food availability. Galveston Bay by contrast tends to have maximum larval planktonic times in the fall. This difference arises from the delay in spring warming in Chesapeake Bay relative to Galveston Bay. However, the average larval planktonic time in Chesapeake Bay is somewhat shorter than that for Galveston Bay. The earlier fall cooling in Chesapeake Bay (Fig. 5) shortens the period during which fall settlement can occur. Hence, the longer planktonic times that can occur in Galveston Bay in the fall are not possible in Chesapeake Bay. Therefore, the planktonic

time in Chesapeake Bay averaged over a spawning season tends to be slightly shorter.

#### *Galveston Bay Food, Salinity and Turbidity Conditions*

The simulated larval planktonic times obtained using temperature, food and salinity conditions from Galveston Bay, Texas (Fig. 12a), show extended larval planktonic periods in the spring and fall, with abbreviated larval periods during the summer months (Table 5). More rapid growth, resulting in a shorter planktonic period, is observed in the summer months when temperatures are higher and food availability is greatest.

TABLE 3.

Summary of temperature effects on larval developmental times from five bays. The duration (days) and month during which minimum and maximum larval planktonic times occur in each bay are shown. Also shown are the average larval planktonic times (days) and the time span (months) from first set to the last viable fall set.

Bay	Minimum Larval Period (days: month)	Maximum Larval Period (days: month)	Average Larval Period (days)	First to Last Set (months)
Chesapeake Bay	20: Aug	89: Sept	32.2	July to early October <sup>1</sup>
North Inlet	15: July	55: Oct	25.7	May to October <sup>2</sup>
Apalachicola Bay	18: June-Aug	46: Nov	24.2	April to November <sup>3</sup>
Galveston Bay	18: Sept	46: Nov	25.9	April to November <sup>3</sup>
Laguna Madre	14: Aug	30: Nov	18.5	April to November <sup>3</sup>

<sup>1</sup> Andrews 1954, <sup>2</sup>Lunz 1954, <sup>3</sup>Hopkins 1955

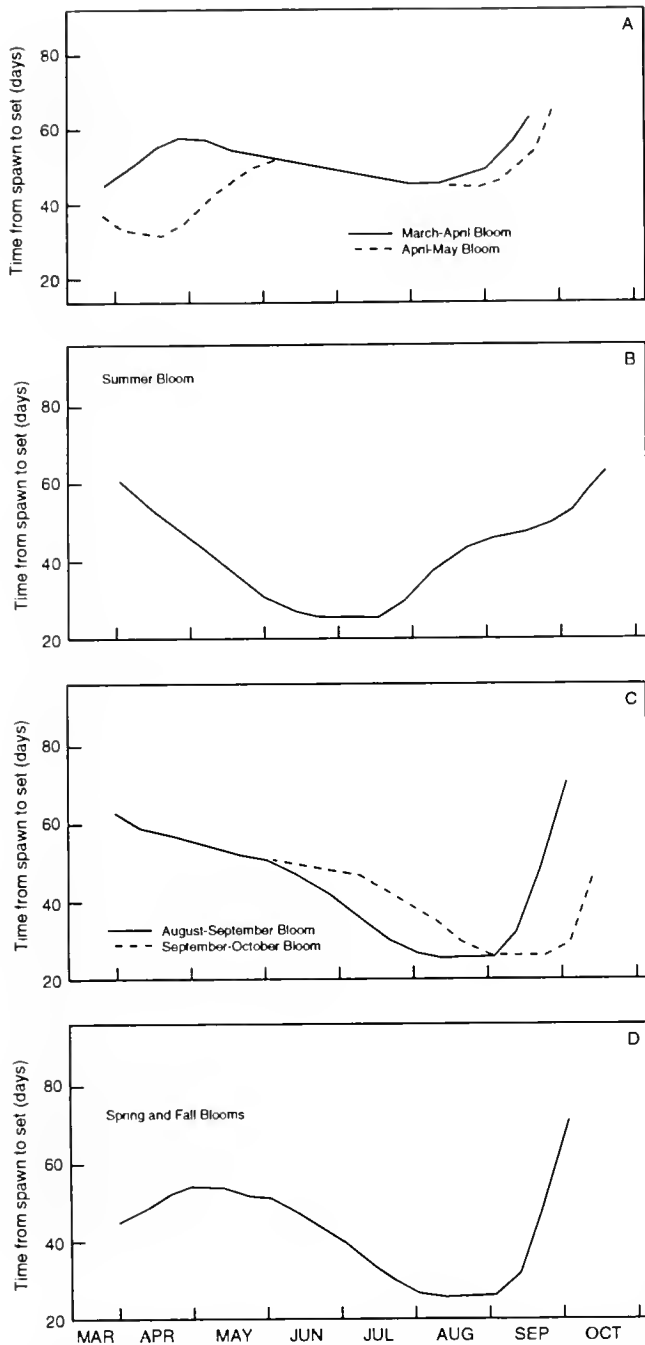


Figure 11. Simulated planktonic time for oyster larvae exposed to food conditions in which the maximum food supply occurred in; A: March-April and April-May blooms. B: June-July. C: August-September and September-October blooms. D: March-April and August-September blooms.

Imposing a simulated low salinity event (Fig. 6a), in August (Fig. 12a) significantly alters the amount of time the larvae are in the water column. Reducing the salinity in August from 19 ppt to 5 ppt, and back to normal levels, decreases the larval growth rate and correspondingly increases planktonic time from 25 days (at 19 ppt) to a maximum of 38 days during the low salinity event.

Imposing a simulated low salinity event in April (Fig. 12a) also extends the time the larvae are in the water column. However, normal April salinities are 12 ppt, and planktonic times produced

by this salinity are on the order of 52 days. A spring low salinity event only increases the April planktonic period by about 4 days, as compared to the extension of the larval period by 13 days that occurs during the low salinity event in August.

Similarly, a small change in simulated larval planktonic period is observed when turbidity values characteristic of Galveston Bay are included (Fig. 12b; Table 5). However, the effect of turbidity in this case *increases* the larval growth rate, thereby decreasing the amount of time the larvae are in the water column. The turbidity levels from Galveston Bay (Soniata and Ray 1984) are all below  $0.1 \text{ g dry wt l}^{-1}$  and these low sediment concentrations enhance larval growth rates by a small factor (Fig. 4). While the larval planktonic period is abbreviated by the Galveston turbidity levels, it is only decreased by a maximum of 4 days in the late fall. The turbidity values used in these simulations are relatively low. With increases in turbidity levels an extension of the larval planktonic period can be expected.

## DISCUSSION

### Temperature Effect

Oyster larvae can tolerate a wide range of temperatures. However, variability within this range can have a major effect on larval physiology. The major trend observed in the temperature simulations, the warmer the temperatures (below lethal temperature) the shorter the larval time span, is a trend already well documented for oyster larvae (Davis and Calabrese 1964, Dupuy et al. 1977).

However, the simulations of planktonic time span show that the implication of this is that the average larval life span, the minimum, and particularly the maximum larval time periods decline in length with decreasing latitude. The major difference in larval planktonic time between the bays used in this study occurs in the fall. Of the five simulated bays Chesapeake Bay cools earliest in the fall, therefore this bay has the shortest time window within which a viable fall set can occur each season. In a bay like Laguna Madre, where temperatures are elevated late into the fall, a potential remains for a viable set as late as the first week of November.

This effect of the temperature on larval life span across a latitudinal gradient has been documented in field studies. The first spawning of oysters in Long Island Sound and Milford Harbor, Connecticut was observed to occur in the first week of July (Loosanoff and Engle 1940). By the middle of July, oysters in these areas in shallow and moderately deep sites were half or more than half spawned. The majority of the oysters completed spawning early in August; however, oysters at deep-water sites continued to spawn until early September. In contrast, *Crassostrea virginica* populations in the southern regions of the Gulf Coast have been observed to spawn in April or earlier, with setting occurring from April through November (Hopkins 1955). Thus, for Milford Harbor oyster larvae, a three month time window exists within which a viable set may occur; whereas, this time frame is extended to eight months along the Gulf Coast. This provides oysters five additional months within which successful recruitment to the adult population is possible.

### Timing of Food Availability

The Galveston Bay and Chesapeake Bay simulations that include the effects of food concentration show that this environmental variable can have an important effect on oyster larval growth

TABLE 4.

Summary of the effect of food availability on larval periods in Galveston Bay (GB) and Chesapeake Bay (CB). The duration (days) and month of the minimum and maximum larval planktonic times are shown for each bay. Also shown are the average larval planktonic times (days) for each bay. The Galveston Bay simulation results that were obtained using the food supply time series given in Soniat and Ray (1984) are denoted by S&R.

Bloom Condition	Min. Larval Period		Max. Larval Period		Av. Larval Period	
	GB	CB	GB	CB	GB	CB
March–April	44: April	39: July	60: Sept	59: April–May	48.6	43.9
April–May	34: April	34: May	63: Oct	49: May	44.6	39.9
June–July	28: June–July	27: June	60: April	66: April	42.3	39.8
S&R	25: August	—	54: Oct	—	34.2	—
Aug–Sept	25: Aug–Sept	27: Aug	69: Oct	64: April–May	43.4	40.1
Sept–Oct	25: Sept–Oct	34: Aug	62: April	64: May	43.1	42.5
Spring and Fall Bloom	25: Aug–Sept	27: July	69: Oct	59: April	41.5	39.3

rate and hence planktonic time span. Increased food concentrations in spring months before water temperatures increase have little effect on larval planktonic time. However, if increased food occurs with or following the spring warming, planktonic time is reduced. The effect of both summer and fall blooms in both bays is to increase growth rates and thus decrease planktonic time. This effect occurs independent of the timing of the bloom because of the warmer temperatures that are found at these times of the year.

Moreover, unlike the spring bloom case, the positive effect of a late fall bloom on shortening larval life span overrides the lengthening effect of the initial decrease in fall temperature. Dramatically shorter larval time spans are the result.

Overall then, increased food concentration in the fall has a larger effect on larval growth rate than does increased concentrations in the spring or summer in Galveston Bay. The effect of increased food in the spring, summer or fall is to reduce larval planktonic times for the period surrounding the bloom. This latter point is of particular importance because increased spawning by the adult oyster populations occurs in response to increased food concentrations (Hofmann et al. 1992). Preparation for spawning by the adult oysters takes several weeks to two months depending on temperature and food supply (Hofmann et al. 1992, Choi et al. 1989). Thus, larvae will likely appear in the water column in the later stages of a bloom. Hence, the period of co-occurrence of adequate food and optimal temperatures could be shorter for the oyster larvae than for the adult population. Certain spawns may be doomed to failure by dropping temperatures that dramatically extend larval time spans and, consequently, decrease larval survivorship. Spawns later in the spring, in the summer months, or early in the fall that coincide with increased food conditions will result in the shortest planktonic time, thereby increasing survivorship to settlement by limiting losses to predation or advection from the system.

#### Other Environmental Factors

Salinity concentration and distribution in estuarine environments arises from the combination of tidal effects, freshwater run-

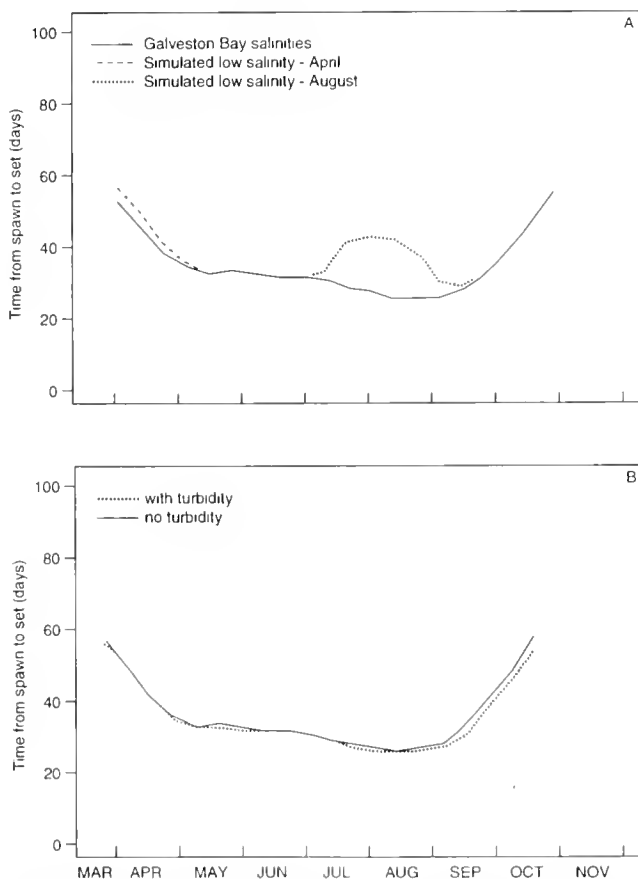


Figure 12. A: Simulated planktonic times produced by Galveston Bay conditions and idealized low salinity events imposed in April and August. B: Simulated planktonic times for Galveston Bay conditions with (dashed line) and without (solid line) the effects of turbidity.

TABLE 5.

Summary of minimum and maximum larval planktonic times (days) and month of occurrence for the simulations that used Galveston Bay environmental conditions.

	Minimum Larval Period (days: month)	Maximum Larval Period (days: month)	Average Larval Period (days)
Galv temp, saltn, food	25: Aug	54: Oct	35.9
Low salinity, April	25: Aug	56: April	36.5
Low salinity, August	28: Sept	54: Oct	37.4
Turbidity	25: Aug	55: April	34.1

olf and river inputs. As a result, the salinity environment encountered by oyster larvae can vary considerably over short (e.g., tidal) or long (e.g., seasonal) time scales. One feature of estuarine environments is that they experience extended periods of low salinity water that result from increased freshwater inputs. Episodes of low salinity are considered to be beneficial to adult oyster populations because they result in lower disease prevalence and decreased predator densities (Ray 1987). On the basis of simulation results, Hofmann et al. (1992) observed that a decrease in salinity (as long as salinities remain above 5 ppt) has considerably less effect on adult oyster populations than does a small change in temperature or food concentration. However, the larval simulations indicate that extended periods of low salinity have a pronounced effect on larval growth rate. Larval growth is slowed, under prolonged conditions of low salinity, thus extending the time required for development to settlement size.

These modeling results are indirectly supported by field observations. Abbe (1988) observed that higher oyster larval recruitment in the central Chesapeake Bay was related to periods of sustained salinity higher than 16 ppt. In general, the fair recruitment events observed between 1976 and 1979 coincided with high salinity conditions; whereas, poor recruitment years were characterized by low salinity. Above average recruitment in the central Chesapeake Bay in 1980–1982 and 1985 also coincided with periods of high salinity.

Furthermore, Ulanowicz et al. (1980) used forty years of observations of fishing effort, spat production, salinity, water and air temperatures and precipitation to construct a multivariate model for production of annual harvest of oysters in the central Chesapeake Bay. This analysis showed that sustained high salinity was a dominant factor affecting spat production, with spat production increasing with increasing salinity. Hence, the frequency and spatial distribution of low salinity water may be a factor in determining settlement patterns of oyster larvae.

The final environmental variable considered in this modeling study was turbidity. Larvae of *Crassostrea virginica* are exposed to the varying turbidity levels that characterize estuarine environments. For the Galveston Bay conditions used in this study, turbidity concentrations were below those that adversely effect larval growth rate. In fact, the low levels provide an enhancement of growth rate which shortens larval planktonic time. However, sustained periods of high turbidity can reduce larval growth rates. In contrast to salinity, where larvae were more sensitive than the

adults, turbidity exerts a lesser impact on larvae than it does on the adult populations where filtration efficiency is adversely affected (Hofmann et al. 1992). However, if increased turbidity levels were to coincide with other environmental conditions that slow larval growth rate (e.g., reduced food, cold temperatures, low salinity) then turbidity could be a factor determining the survivorship of oyster larvae.

#### SUMMARY

The simulations that consider only temperature effects on the growth and development of larvae of *Crassostrea virginica* provide a range of minimum and maximum planktonic times for specific bays across a latitudinal gradient. The implication of these results is that the period during which bivalve larvae are available for recruitment to adult populations decreases with increasing latitude. The addition of food concentration shows the importance of this environmental variable in regulating larval growth and development. As was found for adult oyster populations (Hofmann et al. 1992) the timing of food availability relative to water temperature is important in determining larval planktonic time and hence the survivability of larvae. The addition of the effects of salinity and turbidity also modify the time required for oyster larvae to reach settlement size.

Throughout development and over a spawning season larvae of *Crassostrea virginica* are exposed to varying conditions of temperature, food concentration, salinity and turbidity. It is the cumulative effect of all these environmental variables that determines larval survivorship. Therefore, management strategies for an oyster fishery must be broad enough to include habitat effects on larval survivorship, which ultimately determines recruitment to the adult population.

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## GAMETOGENIC CYCLE OF THE SOUTHERN SURFCLAM, *SPISULA SOLIDISSIMA SIMILIS* (SAY, 1822), FROM ST. CATHERINES SOUND, GEORGIA

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**ABSTRACT** The reproductive cycle of the southern surf clam was investigated for the first time in the southeastern USA using specimens collected from St. Catherines Sound, Georgia. Monthly (January 1990–July 1991) trawl samples were obtained from a site (7–11 M depth) north of St. Catherines Island. Specimens (N = 30/sample) were measured for shell length (SL) and processed for histology. Qualitative and quantitative assessments of gonadal samples were performed. The unimodal gametogenic cycle began in September–October, with a rapid period of development through November [male gonad index (G.I.) = 4.00, female G.I. = 4.25] followed by a plateau through January (female) or February (male) prior to final maturation by March–April. Females [ $4.47 \pm .13$  (SE),  $4.48 \pm .12$ ] achieved significantly higher G.I. levels (ANOVA,  $p = .009$ ,  $.001$ ) than males ( $4.06 \pm 0.6$ ,  $4.28 \pm .125$ ) in 1990 and 1991, respectively. Peak maturity levels were significantly higher for both sexes during 1991 than 1990 (ANOVA, males  $p = .0165$ ; females  $p = .0004$ ). Spawning was from March–May (female) and April–May 1990 (male) and April–June 1991 (both sexes). Sex ratios were 1:1 (Chi-squared  $p = .08$ ). There was no relationship observed between shell length (SL) and stage of sexual maturity. In three monthly samples (from 17), size differences were detected with the females significantly (ANOVA) larger on each occasion (November 1990,  $p = .0005$ ; December 1990,  $p = .0236$ ; January 1991,  $p = .0355$ ).

**KEY WORDS:** *Spisula*, reproductive cycle, gametogenesis, surf clam, image analysis

### INTRODUCTION

The southern surf clam, *Spisula solidissima similis* (Say 1822), is a potential species for aquaculture inhabiting the marine waters of southeastern U.S.A. This subspecies occurs from Massachusetts to Florida and around the Gulf of Mexico to Texas (Abbott 1974). *S. s. similis* has been aged to 5.5 years obtaining a maximum size of 106 mm in the coastal waters of the Gulf of Mexico (Walker and Heffernan, in manuscript) as compared to the northern surf clam, *Spisula solidissima* (Dillwyn) which lives for 31 years off the coast of New Jersey (Jones 1981) and attains a size of about 170 mm. In Georgia, clams rarely live beyond 1.5 years and obtain a size of 76 mm (Walker and Heffernan, in manuscript).

Early attempts to document the reproductive cycle of the surf clam using gonad distention (Westman and Bidwell 1964), and excision of gametes (Allen 1951, 1953; Schechter 1941) were followed by Ropes (1968a), whose histological examination of gonads of New Jersey surf clams over a 3.5 year period represents the most comprehensive study to date. Ropes (1968a) found both annual and bi-annual cycles in offshore clams collected at depths below the thermocline of 18 to 32 M. Jones (1981) observed only annual cycles over a period of two years for clams collected at depths of 18 to 32 M from Island Beach, New Jersey.

In this study, the reproductive cycle of the southern surf clam was investigated for the first time using specimens collected from St. Catherines Sound, Georgia. The objective was to describe the reproductive cycle of the southern surf clam, *S. s. similis* were

collected from lower latitudes and much shallower depths (7–11 M) than those conducted in the same species in the northern part of its range (Ropes 1968a, Jones 1981, Sephton 1987).

### MATERIALS AND METHODS

Monthly (January 1990–July 1991) trawl samples were obtained from a site (7–11 M depth) north of St. Catherines Island, Georgia. After collection, clams were kept overnight in aerated-sea water collected from the sampling location. Specimens (N = 30/sample) were measured for shell length (i.e., anterior-posterior measurement) to the nearest 0.5 mm with vernier calipers. Prior to processing, a mid-lateral (cross-sectional) gonadal tissue sample (ca 1 cm<sup>2</sup>) was dissected from each clam. The gonadal sample was held in Davidson's fixative for 48 hours under refrigeration, tissues were rinsed with 50% EtOH and then transferred to 70% EtOH (Howard and Smith 1983). Tissue samples were dehydrated in an alcohol series, cleared in toluene and embedded in paraplast. Tissue sections (5  $\mu$ m) were cut and stained with Harris Hematoxylin and Eosin Y (Howard and Smith 1983).

#### Qualitative Reproductive Analysis

Gonadal preparations were examined with a Zeiss Axiovert 10 microscope (20 X), sexed, and assigned to a developmental stage as described by Ropes (1968a). Staging criteria of 1 to 5 were employed for Early Active (EA = 3), Late Active (LA = 4), Ripe (R = 5), Partially Spawned (PS = 2) and Spent (S = 1). These categories are only approximations of gonadal development which is a continuous process and distinctions between stages are not always clear, Ropes (1968a) and Jones (1981). The monthly Gonadal Index (G.I.) for both sexes was determined by multiplying the number of specimens ascribed to each category score, sum-

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ming all those values and dividing this figure by the total number of clams analyzed.

**Quantitative Reproductive Analysis**

Quantitative analysis of gonad preparation was carried out using Color Image Analyzed Densitometry Microscopy system housed at the Skidaway Institute of Oceanography, Savannah, GA. Stained slides were viewed on an Olympus BHT microscope from where the images were captured by a Hitachi Model DK-7000SU-3 Chip CCD color camera and were then viewed on a Trinitron color video monitor, field area = .638 mm<sup>2</sup>. The image analyzer (IM-3000 software, Analytical Imaging Concepts) is capable of carrying out detailed area measurements and statistical analysis of features detected within the blue level thresholds (operator-controlled). Two fields per specimen were analyzed to ensure detection of within specimen variations in gametogenic development.

Females were analyzed for percent gonad (i.e. percentage area occupied by follicular walls and gametes as apposed to other tissues in the field), percent of gonad area occupied by oocytes, oocyte number per field and mean oocyte diameter. An operator controlled marker was used to edit non-gonadal tissue (i.e., intestines and blood vessels) in the evaluation of percent gonad per field. The same areas were taken from each section a marker being employed to minimize the effect of non gonadal tissue. Egg number was manually counted from the Trinitron screen, and the diameter of nucleolated oocytes was measured directly on the

screen. Microscopic measurements of nucleolated oocytes (N = 15/female) were done on a compound microscope, at different stages of development, to validate the image analysis measurements where low numbers of nucleolated oocytes were seen per field. Males were analyzed for percent of gonad occupied by spermatogenic stages and for percent of spermatogenic stages consisting of spermatozoa.

Mean individual values for each data category were calculated by the image analyzer. Mean monthly values were then computed and used in the quantitative assessment of reproduction. Sex ratios were tested against a 1:1 ratio with Chi-square tests (Steel and Torrie 1960). Statistical analysis (Analysis of Variance) was applied to various quantitative data sets (mean-value points), in order to validate or reject conclusions drawn from general patterns.

Sea surface and bottom water temperature and salinity at the sampling site, were monitored on a monthly basis.

**RESULTS**

A detailed insight into the gametogenic cycle of *S. s. similis* population was ascertained from a combination of qualitative and quantitative data gathered during the study period (January 1990–July 1991). Monthly qualitative assessment of reproductive condition are illustrated in Fig. 1. From this data, it is apparent that there was one spawning, each year, in spring.

**Qualitative Results**

*S. s. similis* showed a unimodal gametogenic cycle beginning in September–October (EA) with a rapid development through

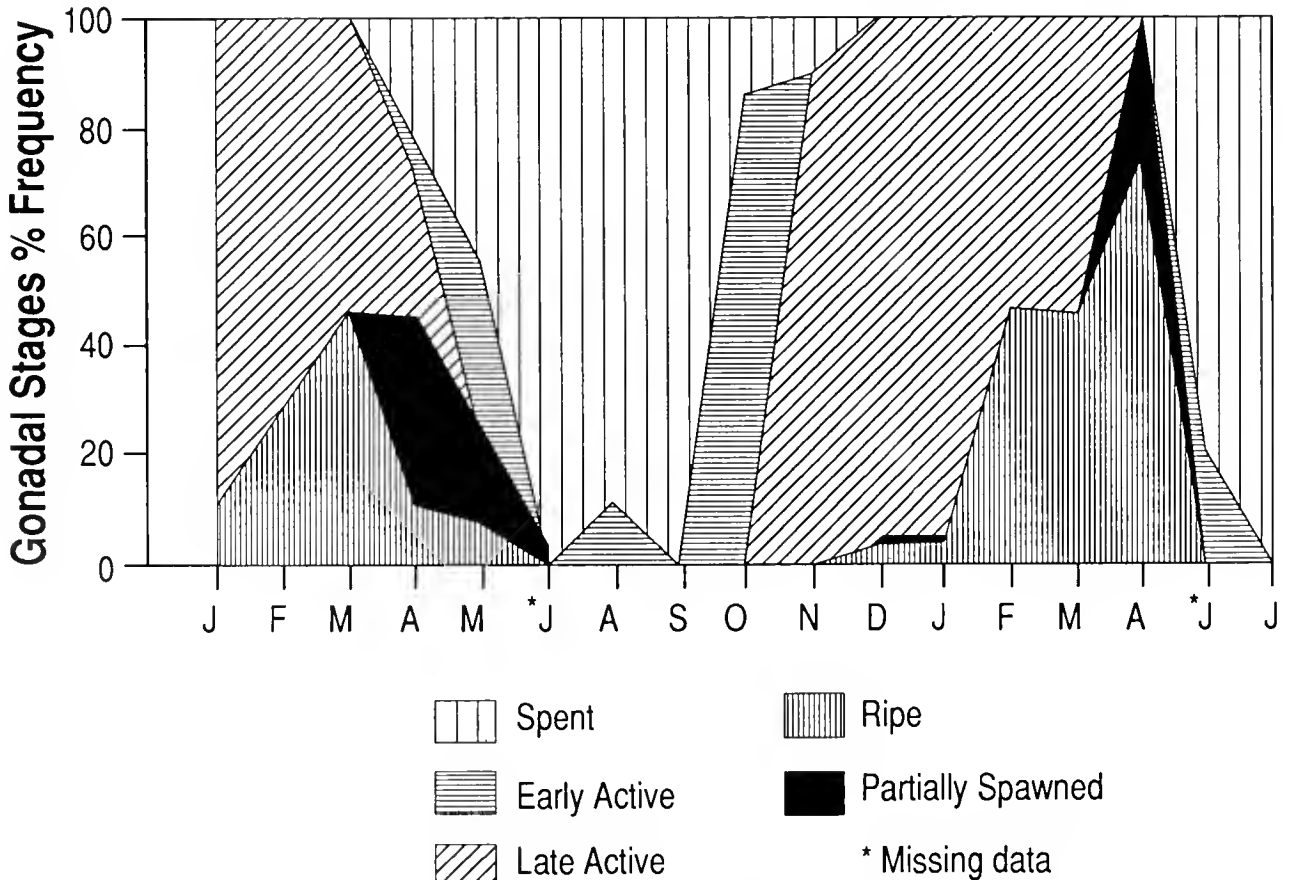


Figure 1. Percentage of surf clams (*Spisula solidissima similis*) collected from St. Catherines Sound, Georgia (1990–1991) in each phase of the reproductive cycle during this study.



November (LA; male G.I. = 4.0, female G.I. = 4.25) followed by a plateau through January (female), February (male) prior to final maturation by March–April (R) (Fig. 1). Females [ $4.47 \pm .13$  (SE),  $4.48 \pm .12$ ] achieved significantly higher G.I. levels (ANOVA,  $p = .009$ ,  $p = .001$ ) than males ( $4.06 \pm .06$ ,  $4.28 \pm .125$ ) in 1990 and 1991, respectively (Fig. 2). Peak maturity levels were significantly higher for both sexes in 1991 than 1990 (ANOVA, males  $p = .0165$ ; females  $p = .0004$ ). Spawning was from March–May (female) and April–May (male) in 1990, and April–June (both sexes) 1991 (Fig. 2).

#### Quantitative Results

The percent gonad area data had a temporal pattern similar to that of G.I. but differed from G.I. data that the males showed a higher percent gonad areas (ANOVA,  $p = .005$ ; 1990, ANOVA,  $p = .004$ ; 1991) in both years (Figs. 3a; 4a). Male gonad area showed a decline from March–May [66.5%–26.5% ( $p = .0219$ )] in 1990; April–June [77.7%–9.1% ( $p = .0001$ )] in 1991 (Fig. 3a), which coincided with the decline in spermatogenic levels from March to May [54.0%–13.8% ( $p = .003$ )] in 1990; March to June [80.5%–4.8% ( $p = .0001$ )] in 1991. This decrease in percentage spermatogenic area and gonadal area was indicative of spawning. Both the gonad area (ANOVA,  $p = .0124$ ) and spermatogenic levels (ANOVA,  $p = .0026$ ) were higher in 1991. In December–January 1991, male gonadal area showed a decline with slight recovery in January–February and spermatogenic levels showed a sharp decline (December–February) (ANOVA,  $p = .002$ ) with a rising trend occurring again in March ( $p = .0142$ ) (Fig. 3b). This decline was not interpreted as being indicative of a major spawning event as most of the specimens examined were judged to be in the late active stage, with a few (4%) showing signs of maturation. Spawning was seen to occur in March–May 1990, March–June 1991 as evidenced by the decline in spermatogenic levels (Fig. 3b).

Females gonad area (Fig. 4a) which increased from October–November [14.8%–42.8%] and declined in November–December [42.8%–38.3% ( $p = .06$ )], increased again in December–January [38.3%–57.7% ( $p = .0026$ )]. Peak maturity was attained in January–February [52.1%–53.8% in 1990; 57.7%–66.4% in 1991] as seen in the female gonad area. Peak maturity levels were significantly higher for 1991 (ANOVA,  $p = .0006$ ). Significant differ-

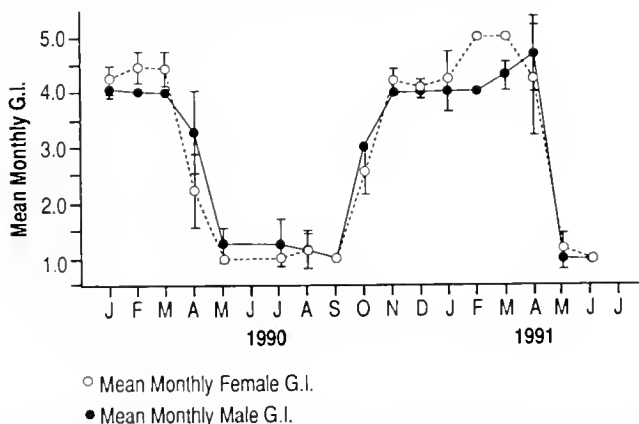


Figure 2. Qualitative mean monthly gonad index (G.I.) of *Spisula solidissima similis* collected at St. Catherines Sound (1990–1991). Error bars represent 2 S.E. around mean.

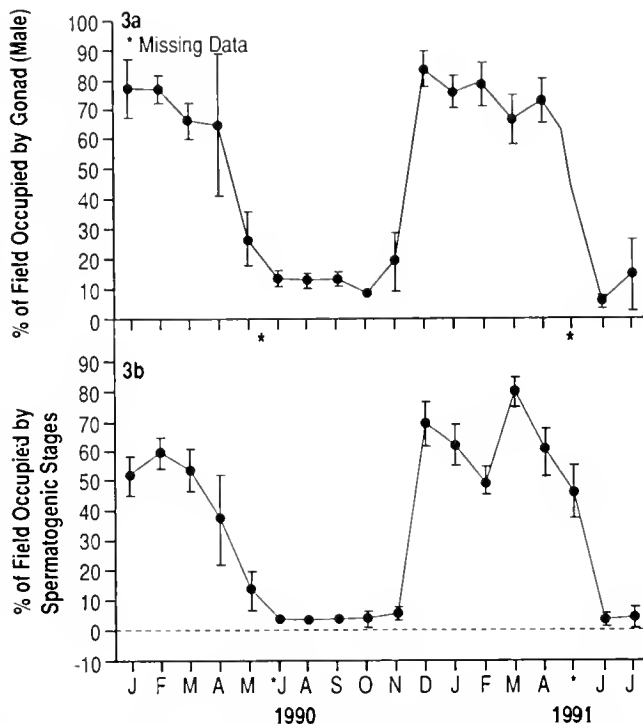
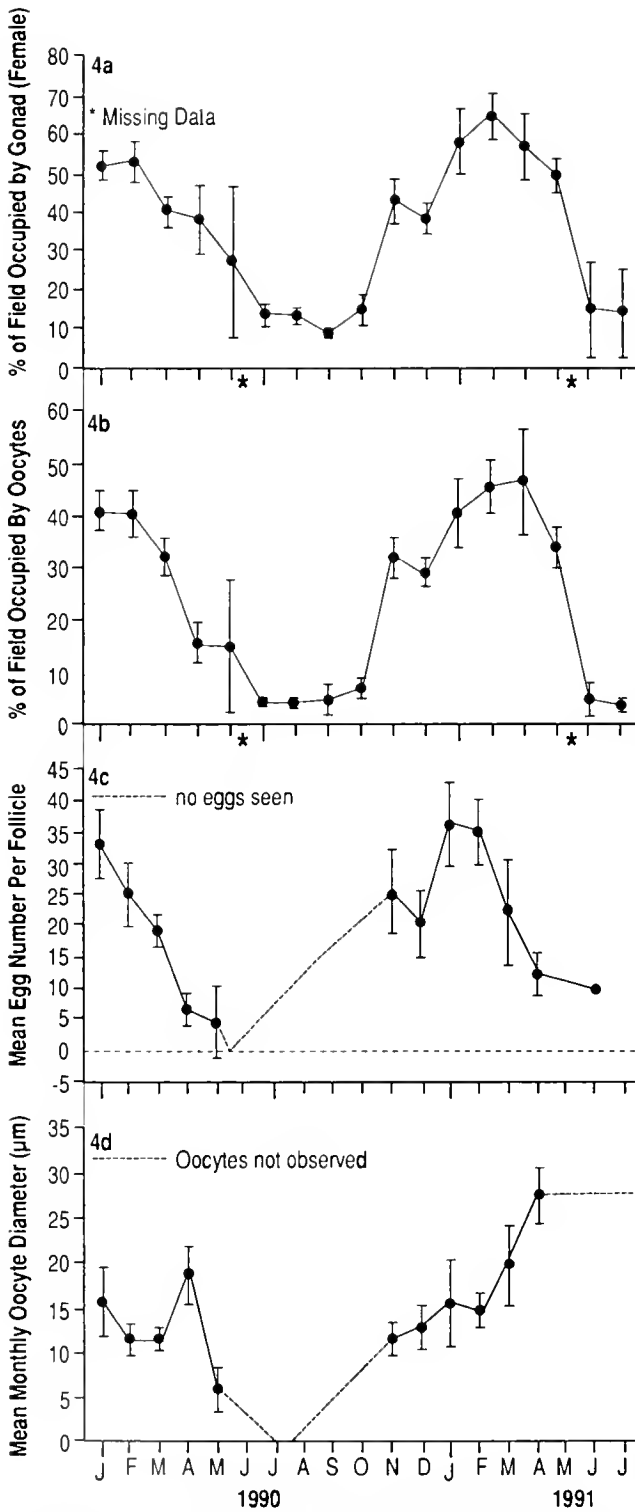


Figure 3. Composite of quantitative data (obtained using image analysis) representing the state of gonad condition for male surf clams (*Spisula solidissima similis*) collected at St. Catherines Sound, Georgia, 1990–1991. Error bars represent 2 S.E. around mean. a: Quantitative representation of percentage of field occupied by male gonad tissue. b: Quantitative representation of percentage of field occupied by spermatogenic stages.

ences between the two years were observed in percent area occupied by oocytes ( $p = .0032$ ) (Fig. 4b). Spawning occurred in March–April (33.2%–15.3%) in 1990 and March–June (49.5%–5.5%) in 1991, (ANOVA,  $p = .0126$ ;  $p = .0001$ , respectively) as evidenced by the significant decline in percentage oocyte area. The percentage of gonad tissue occupied by oocytes followed similar trends to gonad development, but 1991 was a more productive year in terms of percentage area occupied by oocytes (ANOVA,  $p = .0034$ ). The mean egg count per follicle also showed slightly higher levels in 1991, but the differences between the two years were not significant (Fig. 4c). The largest mean egg diameter values occurred in April each year [ $19 \mu\text{m} \pm 1.12$  (SE) in 1990;  $27.5 \mu\text{m} \pm .99$  in 1991] (Fig. 4d). The average number of values seen per field in the image analysis measurements were lower ( $N = 8/\text{field}$ ) as compared to the microscopic measures ( $N = 15$ ). The mean values of the microscopic data were higher and ranged from  $24 \mu\text{m} \pm 2.63$  to  $32 \mu\text{m} \pm 1.23$  in 1990 and 1991, respectively. Significant differences were observed between the two data sets ( $p = .03$ ) when egg sizes were largest. Percentage area occupied by oocytes decreased significantly from February to March 1990 [40.5%–32.2% (ANOVA,  $p = .0125$ )] and March–April 1991 [46.5%–36.1% ( $p = .0012$ )], indicating the onset of ripening (Giese and Pearse 1975, 1979). Data from all measurements support the hypothesis that spawning occurred in March–April 1990 and March–June 1991. Duration of the male spawning period was longer than that of females in both years, but the general development cycle was similar in both sexes.

Sex ratios were 1:1 (Chi-squared  $p = .08$ ). There was no



**Figure 4.** Composite of quantitative data (obtained using image analysis) representing the state of gonad condition for female surf clams (*Spisula solidissima similis*) collected from St. Catherines Sound, Georgia 1990–1991. Error bars represent 2 S.E. around mean. a: Quantitative representation of percentage of field occupied by female gonad. b: Quantitative representation of percentage of field occupied by oocytes. c: Mean number of eggs present per follicle. d: Mean monthly oocyte diameter of nucleolated oocytes present per, field analyzed.

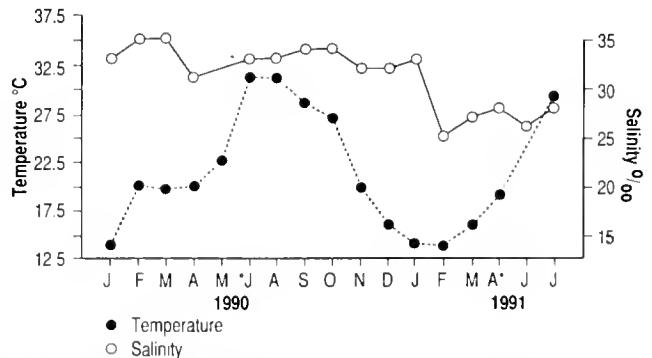
correlation between shell length and stage of sexual maturity. These animals show signs of sexual maturity at six months of age, reaching a mean size of  $27.1 \pm 5.5$  mm. In general color of the gonad, noted while shucking, was a pinkish-orange in ripe females and a creamy-yellow in males. In three monthly samples (from 17), size differences between sexes were detected with females being significantly (ANOVA) larger on each occasion (November 1990  $p = .0005$ ; December 1990  $p = .0236$ ; January 1991  $p = .0355$ ).

Water temperature and salinity showed similar cyclic patterns in both years of study (Fig. 5). Water temperatures ranged from 14 to 31°C in 1990 and from 14 to 29°C in 1991. Salinities ranged from 35‰ to 32‰ 1990 and from 25‰ to 33‰ in 1991. Spawning periods coincided with rising spring water temperature.

**DISCUSSION**

*S. s. similis* showed a unimodal gametogenic cycle as found in the northern surf clam, *Spisula solidissima*, but the timing of gametogenesis and spawning were different. In the northeastern U.S. waters, the surf clam reached its peak maturity in June–July (15–20°C) followed by spawning in late August (23°C) (Ropes 1968a, Jones 1981, Sephton 1987); whereas, *S. s. similis* reached its peak maturity in March–April (19.5–20°C in 1990, 19.5°C in 1991) and spawned from late March to early June (19.5–30°C in 1990, 19.5–29°C in 1991). The earlier timing of initiation, maturation and spawning (spring) of the southern surf clam may be linked to higher temperatures exhibited in the southeastern U.S. coastal waters (Fig. 5).

Aided with the reported quantitative data (Figs. 3a,b, 4a,b,c,d), we were able to test the percentage gonadal areas as evidenced by our per field sampling procedure. In 1991, the spermatogenic levels were significantly higher (Fig. 3b) than 1990 ( $p = .0026$ ). After a rapid increase in spermatogenic activity from November to December 1990, the percent spermatogenic area was seen to drop ( $p = .002$ ) from December–February 1991 (70% to 50%). Peak spermatozoan levels occurred in March [80.5% ( $p = .0142$ )] followed by a significant decrease from March to June [80.5%–4.8% ( $p = .0001$ )] which indicated the onset of major spawning. The earlier spermatozoan fluctuations (December–February) could not be supported as a major spawn as only 4% of the specimens encountered were staged as ripe, and no partially spawned specimens were seen. There could have been a minor spawn by the ripe animals due to fluctuations in levels of water temperature and salinity (Fig. 1, 3b, 4b). Ambient water temper-



**Figure 5.** Mean ambient water temperatures and salinity for the surf clam *Spisula solidissima similis* study site in St. Catherines Sound, Georgia 1990–1991.

atures remained fairly constant, December 1990 through February 1991 (16–17°C), but salinities dropped from January (33‰) to February (25‰) 1991 ( $p < .05$ ). The drop in salinity levels could have caused a minor spawn in males which mature earlier than females, but as these salinity readings were taken on a monthly basis this interpretation remains speculative.

In general females followed the same cyclical gametogenic pattern as the males. With ripening the percent gonadal area and oocyte area was seen to decline. Giese and Pearse (1975, 1979) reported this decline to be a common feature in marine invertebrates. During this decline in percentage gonad area and percentage oocyte area partially spawned specimens were encountered (as seen in Fig. 4a). The largest egg diameter was seen in April in both years which indicated the ripeness of the gonad, after which a decrease in egg diameter could be an indication of spawning in which the largest and the best eggs were released first into the environment. Egg diameter was smaller in *S. s. similis* (present study) than in *S. solidissima* Ropes (1968a).

It is not possible from the available data to give a reason for observed differences in gametogenic development in 1990–1991. Temperature and salinity could be one of the causative agents and one can only speculate on the effects of varying food availability. In temperate waters, reproduction is related to seasonal temperature variations (Fournier 1992) and stress induced changes in other environmental factors may effect gamete production (Bayne et al. 1982, Newell et al. 1982). Shell growth in all age classes of *S. s. similis* slowed during the summer months, and clams older than one year died (Walker and Heffernan, in manuscript) (Fig. 6). Surviving 0+ clams showed increased somatic growth and gametogenesis as the temperature began to drop (November–April, 15.5–18.5) (Fig. 5). When reproductive activity was at its peak (March–April), a marked reduction in somatic growth was seen (Fig. 6) which resumed after spawning. Jones et al. (1988) saw a decline in somatic growth with the onset of maturity in *Notospisula trigonella*, due to the resources being diverted toward gonadal growth. An increase in reproductive activity was also seen by Sasaki (1987) in *Spisula sachalinensis* in Sendai Bay, with a drop in water temperatures from 25°C–10°C. Giese (1959) and Sastry (1979) observed latitudinal differences in timing of the reproductive cycles of marine molluscs in general.

The application and reliability of image analysis techniques to surf clam gametogenesis is illustrated by the general strong agreement of qualitative and quantitative data, the exception to this pattern was where the G.I. values contradicted the percentage gonad area data. This could have been due to the fact that devel-

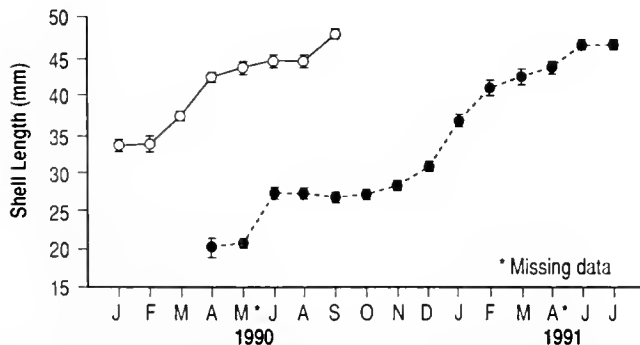


Figure 6. Surf clam (*Spisula solidissima similis*) collected at St. Catharines Sound, Georgia, monthly shell growth data (1990–1991). Error bars represent S.E. around mean.

opment is continuous and distinctions between stages are not clearly defined and, this subjective classification (Ropes 1968a) could be the cause of the higher index score observed in the G.I. data. Another factor which may account for the difference in levels in the two analyses may be due to limitations of the analysis system for quantifying male intrafollicular space (i.e. non-gamete area). In ripe females "empty" intrafollicular space is seen even when the follicle is fully distended; whereas, in ripe males the intrafollicular space decreases to a large extent and, the small spaces present could not be detected by the image analyzer (10×). Therefore, spermatozoa appeared to fill the whole follicle, thereby giving higher male gametogenic values. In the current study, there were instances of greater sensitivity to gametogenic events displayed by quantitative data (e.g., a decrease in spermatogenic levels, which could be indicative of a minor spawning event; decrease in percentage area occupied by oocyte as an indication of ripening) (Figs. 3b, 4b). Qualitative data did not differentiate these events showing a continuous maturation cycle. The G.I. values computed were gross values which showed the major changes in the cycle, minor fluctuations could not be recorded as the demarcations between stages were not sharp. Image analysis showed lower values in egg diameter as compared to the microscopic measurements which could have been due to the fact that fewer nucleolated eggs were seen and measured per field ( $N = 8$ ) as compared to the microscopic measurements ( $N = 15$ /female).

Temperature regimes have been shown by many marine invertebrate researchers to have a profound influence on gametogenesis (e.g. Orton 1920, Nelson 1928, Loosanoff 1937, Giese 1959, Ansell 1961, Loosanoff and Davis 1963, Porter 1964, Eversole et al. 1980, Manzi et al. 1985). Spawning in many bivalves has been slightly delayed by low temperatures (Ropes 1968a). Similar influence on *S. s. similis* is likely with the onset of gametogenesis, when temperatures drop and spawning takes place with the rise in temperature. The observed differences in the timing of the different phases in the reproductive cycle of the surf clam at different latitudes is probably due to local variations of environmental factors, with major ones being water temperature and food availability. As an external factor, temperature can exert a selective pressure in the determination of the breeding season of a species and its fluctuations act as external clues that synchronize the reproductive cycle of the species (Giese 1959, Fretter and Graham 1964, Giese and Pearse 1977). Tarifeño (1980) reported an increase in water temperature as triggering spawning in surf clams *Mesodesma donacium*, in Queule Beach, South Chile. Other estuarine invertebrates also show latitudinal variability. *Mulinia lateralis* exhibits high fecundity, rapid growth rate and early maturity (Calabrese, 1970), which is also seen in *Notospisula trigonella*, to ensure survival in a disturbance prone environment (Jones et al. 1988). In *Mulinia lateralis* (Calabrese 1970), there is a tendency for the clams to develop gametes and spawn progressively earlier in the season southward from northern Massachusetts. Bivalve mollusc such as *Mya arenaria* and *Mercenaria mercenaria* exhibit a change from a unimodal to a bimodal cycle with a decrease in latitude (Ropes and Stickney 1965, Brousseau 1978, Heffernan et al. 1989a). However, several other bivalves (i.e., *Geukensia demissa*, *Crassostrea virginica* and *S. s. similis*) showed unimodal gametogenic cycle in the southeastern U.S. waters (Heffernan and Walker 1989, Heffernan et al. 1989b). This could also be a reason for the shift in gametogenesis as seen in *S. s. similis* to ensure survival and growth of the cohort before the high summer water temperatures become potentially lethal for early life stages. A

shorter life span in *S. s. similis* (1.5 years) in Georgia: Walker and Heffernan (in manuscript), as compared to 31 years in New Jersey (Jones 1981), could be an adaptation to a stressful environment where growth and development are seen to be rapid to ensure survival in a stress prone environment.

The effect of salinity on gonadal maturation is unclear in this study. Where a correlation has been demonstrated in nature, it may reflect changes in nutrition, rather than salinity (Angell 1986). Given the lack of primary productivity data in this study, it is difficult to relate food abundance to gonadal development for this species. Furthermore, studies on the biochemical composition of surf clam tissue would be needed to interpret the conversion of reserve nutrients and nutrients derived from a food source into gamete material (Stephen 1980). In order to develop mariculture techniques for *S. s. similis*, the combined effects of temperature, salinity and nutrition have to be investigated for optimum management of "spawners" in the laboratory, facilitating optimal conditioning and maximum larval survival (Lannan et al. 1980). With this knowledge at hand, the naturally conditioned broodstock can be brought in from field populations for spawning purposes, thereby reducing hatchery costs.

The results show that *S. s. similis* male and female sex ratio was 1:1. This was also seen in *S. solidissima* by Sephton (1987), Jones (1981) and Ropes (1968a). Hermaphrodites were not encountered in this study. Ropes (1968b) observed only one her-

maphrodite from a sample of 2500 *Spisula solidissima*, confirming the view that occurrence of hermaphrodites is a rare event for this species. Color of ripe gonads could not consistently predict the sex of *Spisula*, but the results do support the findings of Schechter (1941) that female gonads are generally pinkish and male gonads cream colored. Rasmussen (1973) reported a distinct rosy color in the female gonads of *Spisula subtruncata*. Jones et al. (1988) in *N. trigonella* and Brousseau (1987) in *Macoma balthica* could determine the sex of ripe individuals by their color, while at other reproductive stages color of the gonad could not be used to determine the gender. In *Mulinia lateralis* (Calabrese 1969), the female gonad was seen to be red-to-orange, while the male gonad appeared white. Gustafson et al. (1987) reported an orange color in the ovaries of *Solemya reidi* when ripe and black when less than ripe, testes ranging from olive-green-to-white colored.

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## FLOWING SEAWATER AS AN INDUCER OF SPAWNING IN THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791)

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**ABSTRACT** Thermal shock and chemical stimulation by serotonin injection are the main methods used to induce spawning in bivalves. In this study, spawning was induced in the giant scallop *Placopecten magellanicus* by mechanical stimulation using flowing seawater. The ability of this new method to induce spawning in the giant scallop was investigated in both sexes. In males, this method was compared to serotonin injection. Mechanical stimulation of ripe giant scallops with flowing seawater was 93% and 100% effective in inducing spawning of males and females respectively. Chemical stimulation by serotonin injection into the gonads was 100% effective in inducing gamete release by males. Flowing seawater triggered spawning in females kept at 10°C and in males maintained between 6 and 10°C. Release of gametes occurred within 30–60 min of the mechanical stimulation of males and females; both kinds of gametes were released synchronously. The females spawned for about one hour while the males continued to spawn some hours after the treatment. The number of spermatozoa released with the mechanical stimulation of males at 10°C was about 10 times that obtained by serotonin injection, suggesting that mechanical stimulation was a strong inducer of spawning. Scallop oocytes obtained by mechanical stimulation were fertilizable by spermatozoa released using both chemical and mechanical stimulations. Mechanical stimulation with flowing seawater is a simple, rapid, and efficient method for inducing synchronous spawning of giant scallops. It is suggested that changes of seawater currents or pressures could play a role in the spawning induction of giant scallops in the natural environment.

**KEY WORDS:** flowing seawater, giant scallop, *Placopecten magellanicus*, serotonin, spawning

### INTRODUCTION

The giant scallop, *Placopecten magellanicus* (Gmelin 1791), is among the major shellfish in the Northwest Atlantic. This bivalve mollusc supports a valuable fishery in eastern Canada and New England, USA. It is likely the most important scallop species in the world from an economic viewpoint (Naidu 1991). Because of the wide fluctuations in giant scallop recruitment from year to year and the increased consumer demand for products of high quality, many attempts have been made to artificially propagate this species. A major problem with the establishment of its commercial aquaculture is the control of timing of spawning.

Several methods to induce spawning in bivalves have been reported, including temperature increases, addition of gametes or phytoplankton, and ammonia treatment. However, spawning is generally accomplished by thermal stimulation (Loosanoff and Davis 1963). This latter method has been previously used to induce spawning in the giant scallop (Culliney 1974).

Recently, it was reported that serotonin induced spawning in several bivalve species (Matsutani and Nomura 1982, Gibbons and Castagna 1984). Our initial results showed that intragonadal injection of serotonin was highly effective in inducing male giant scallops to spawn. Unfortunately, serotonin injections into female gonads were unreliable in inducing spawning. Moreover, alternative methods such as the addition of phytoplankton or sperm suspension were also unsuccessful in females.

Meanwhile, our observation that ripe male and female scallops were induced to spawn during the cleaning of their tanks with a vacuum pump prompted us to investigate the effect of flowing seawater as a new means of inducing ripe individuals to spawn. Thus, the aim of this study was to develop a simple, inexpensive, and efficient method to induce synchronous spawning of ripe adult giant scallops in order to control its reproduction in a hatchery.

### MATERIALS AND METHODS

Giant scallops, *Placopecten magellanicus*, were collected on the Lower North Shore of the Saint Lawrence River and Baie des Chaleurs, Quebec, Canada. Scallops were kept in 170-l tanks supplied with running seawater and conditioned by maintaining the temperature between 5 and 12°C. They were fed on an algal diet consisting of equal proportions of *Isochrysis galbana* and *Pavlova lutheri*; about  $50 \times 10^9$  algal cells were distributed daily in each tank.

Gonadal condition was evaluated in conditioned scallops using a method similar to that described by Devauchelle and Mingant (1991) for *Pecten maximus*. Ripe scallops with a shell length from 10 to 15 cm were used for the experiments. Spawning was induced in females which had been held at 8°C for at least one week prior to chemical or mechanical stimulations. The males were kept in running seawater between 5 and 8°C before spawning induction.

Chemical stimulation in one group of scallops was made by the injection of 0.4 ml of 2 mM serotonin (5-hydroxytryptamine) into the gonads (Gibbons and Castagna 1984). Control scallops were injected with 0.4 ml of 1  $\mu$ m-filtered seawater only. A maximum period of 3 h was allowed for the scallops to spawn.

In another group of animals, mechanical stimulation was used to induce spawning. Ripe scallops were put in a 30-l recirculating seawater downwelling system (Fig. 1). Female scallops were held in a cylinder with a 20  $\mu$ m screen attached to its base in order to collect the released eggs. Seawater was introduced from above and flowed at approximately 10 l per min with a magnetic drive pump. Male scallops were put in a transparent plastic basket in order to easily detect the released spermatozoa. Flow rates through the systems were controlled by a valve. Usually, the temperature was maintained at  $10 \pm 1^\circ\text{C}$ . Under these conditions, ripe scallops spawned within one hour. When the animals began to spawn, they

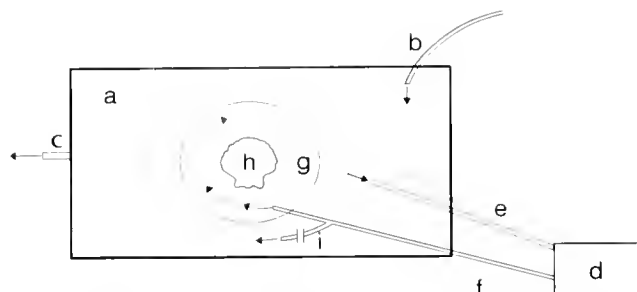


Figure 1. Diagram showing the device used to induce spawning of ripe giant scallops by flowing seawater. (a) plastic container filled with about 30 l of seawater; (b) delivery pipe of seawater; (c) outlet; (d) magnetic drive pump to generate the current of flowing seawater; (e) inlet and (f) outlet of seawater which flowed through the pump; (g) cylindrical plastic basket with a 20  $\mu\text{m}$  screen attached to its base to collect the released oocytes from the female scallop (h); (i) valve to control the seawater flow rate stimulating the scallop. Arrows indicate the direction of flowing seawater through the system. Running seawater flowed continually down through the screen attached to the cylinder (g) and holding the scallop (h). Temperatures were maintained at 6°C or 10°C by controlling the flow of seawater (b). A simpler device was used to induce the males to spawn by omitting the cylinder with the screen (g) and putting directly the male (h) into the container (a).

were transferred quickly into small individual containers where they continued to spawn. These small containers allowed the collection of concentrated gametes.

Gametes were filtered on Nitex screens and then washed with seawater. Oocytes were maintained in suspension at 10°C while the spermatozoa were kept on ice until use. Fertilization was initiated by the addition of spermatozoa to oocyte suspension of 5000 oocytes/ml; the final ratio of spermatozoa to oocytes was 40:1. Fertilized oocytes were allowed to develop until dividing embryos at 10°C. Some cultures were diluted to a concentration of about 100 larvae/ml and were left to develop until the veliger stage at 13°C.

## RESULTS

### Chemical Stimulation of Males

The results of initial attempts to induce sperm release by cold or heat shock were either unsuccessful or took several hours for the small percentage of responding animals. In order to shorten the time required to obtain the gametes, the males were injected in the testes with serotonin. Serotonin injection of ripe scallops induced the males to spawn with an efficiency of 100% while no males released spermatozoa in controls injected with seawater only (Table 1). Serotonin injections induced spawning of males incubated at temperatures varying between 5 and 10°C. Control males were injected at 5°C to avoid a spawning induction due to a heat shock. The experimental males clapped their shells together a few minutes after serotonin injection, but this behaviour was absent in the control males.

The males began to spawn within 1–2 hours after the injection of serotonin and they continued to release spermatozoa for several hours. The number of active spermatozoa released was usually about  $10 \times 10^9$  per male and in some cases it reached  $20 \times 10^9$ . Nevertheless, serotonin injections induced only a partial spawning in males because the testes were still rounded and plump following the spawning. In addition, the animal's survival was not affected by this type of chemical stimulation. Thus, chemical stimulation

TABLE 1.

Effects of chemical and mechanical stimulations in inducing giant scallop, *Placopecten magellanicus*, to spawn.

Gonadal Stimulation	Temperature (°C)	Number of Animals Treated	Spawning Individuals (%)
Seawater			
injection (Males)	5	10	0
Serotonin	5	5	100
injection (Males)	10	24	100
Flowing seawater	6	5	100
(Males)	10	9	89
Flowing seawater			
(Females)	10	22	100

was a very efficient method to induce male giant scallops to spawn.

### Mechanical Stimulation of Males

Despite its high efficiency, chemical stimulation required a delay of 2–3 hours before a substantial quantity of sperm was obtained. An alternative method, mechanical stimulation by flowing seawater, was tried and compared to serotonin injections (Fig. 1). The stimulation of ripe males by flowing seawater released gametes with 93% efficiency (Table 1). This stimulation was effective at temperatures ranging from 6 to 10°C. However, the spawning response was faster and stronger in males maintained at higher temperatures. At 6°C, males took about 2 hours to spawn while at 10°C they reacted within 30–45 min. At 10°C, the number of released spermatozoa was greater than at 6°C. In fact, flowing seawater induced plentiful release of sperm at 10°C,  $70\text{--}260 \times 10^9$  spermatozoa per male, suggesting that this stimulation was a strong inducer of spawning.

The numbers of spermatozoa were about one order of magnitude greater than those released by serotonin injections. As the males were kept at 5–8°C for at least one week before mechanical stimulations, they underwent the mechanical stimulation as well as a small thermal stimulation when they were transferred to 10°C for one hour. However, control males maintained at 10°C without mechanical stimulation showed a negligible reaction to this short and small thermal shock, suggesting that the spawning induction was mainly a consequence of the mechanical stimulation. These results demonstrated that mechanical stimulation was a better and faster method than thermal or even chemical stimulation to induce male spawning in the giant scallop.

### Mechanical Stimulation of Females

Initial experiments to induce female spawning by the addition of sperm or phytoplankton were fruitless, while the results with thermal shocks and serotonin injections were unreliable. Given the effectiveness of flowing seawater as an inducer of spawning in males, this method was applied to the females (Fig. 1). When ripe females were stimulated by seawater with a flow rate between 5 and 13 l/min at 10°C, they responded by spawning within one hour and often within 30 min. Mechanical stimulation had a 100% success rate with ripe females (Table 1). The efficiency and the time needed to induce the spawning in females by this method were similar to those previously observed in males.

When the females began to spawn, they were transferred into



small containers where they continued to release their oocytes for about one hour. The number of released gametes ranged from  $2 \times 10^6$  to  $26 \times 10^6$  eggs. In spite of these great amounts of released oocytes by mechanical stimulations, the same females respawed a few weeks later. The rapidity and the efficiency of mechanical stimulation to induce the females to spawn showed the usefulness of this method and its superiority over any alternative method.

Exposure of scallop oocytes obtained by mechanical stimulations to spermatozoa released either following serotonin injection or flowing seawater resulted in normal fertilization. The processes of meiotic maturation, embryogenesis, and development of scallop larvae up to veliger stage were similar for both methods to those described previously for gametes released after heat shock (Culliney 1974).

### DISCUSSION

Different methods were used in this study to induce spawning in the giant scallop *P. magellanicus* in our hatchery. Cold and heat shocks resulted in spawning of the giant scallops as reported previously (Culliney 1974). However, the efficiency of these methods was generally low and unpredictable, yielding, at best, 50% of slowly responding animals. On the other hand, chemical stimulation by serotonin injection into the gonads was very effective in all tested males. Similar observations have been previously reported in several bivalve species (Gibbons and Castagna 1984). However, preliminary attempts with female giant scallops showed that spawning induction by serotonin injection was unpredictable and poorly efficient.

To overcome these problems, a new and efficient method was developed which uses flowing seawater as an inducer of spawning in both sexes and was compared to serotonin injection which is also efficient in males. The effects of different temperatures were studied on the gamete yields by mechanical stimulations. Flowing seawater induced males and females to spawn within the range of temperatures investigated, 6–10°C, but with various speeds and 89–100% efficiencies. The optimal temperature was 10°C for both sexes. Interestingly, 10°C is also the best temperature for the fertilization and early development of scallop embryos (Desrosiers et al. 1993).

In males kept at about 6°C before the mechanical stimulation, a treatment at 10°C for 30–60 min could possibly induce a small heat shock. However, in control males transferred to 10°C without other stimulations, the release of spermatozoa was negligible, suggesting that mechanical stimulation was the main inducer of male spawnings. Nevertheless, synergistic effect of mechanical and

thermal stimulations cannot be excluded in these males. This double stimulation could explain the rapidity and strength of the spawning response in males. They spawned within 30–45 min and released about  $100 \times 10^9$  spermatozoa each, almost 10 times the number of spermatozoa released with serotonin injections. The mechanical stimulation was more efficient than serotonin injection in triggering a spawning response because animals of both sexes released their gametes synchronously and responded faster. Moreover, flowing seawater is less invasive than serotonin injection. Both mechanical stimulation by flowing seawater and chemical stimulation by serotonin injection induced the ripe adults to spawn only partially, allowing the use of the same individuals in subsequent experiments after a short reconditioning period, which is useful in aquaculture. However, the physiological pathways of spawning activated by flowing seawater in the giant scallop remain to be determined.

The ability of mechanical stimulation to induce spawning in both sexes of the giant scallop *P. magellanicus* suggests that flowing seawater may act as a trigger to release gametes in the natural environment as well. A 13-year study on the reproductive cycle of the giant scallop in New Brunswick, Canada, showed that the distribution of spawning events was significantly related to the lunar/tidal cycles (Parsons et al. 1992). Similar relationships also have been reported in other subtidal bivalves (Sastry 1979). Among the possible spawning factors which can be correlated with the tidal cycle, such as temperature, pressure, currents, and food levels, the results of this study suggest that pressure and/or current changes could be factors precipitating the spawning of giant scallops in the wild.

In conclusion, the results show that mechanical stimulations were efficient in inducing synchronous spawning of ripe male and female giant scallops *Placopecten magellanicus*. This simple and rapid method can be applied to individual or mass spawning experiments. We now use this method on a routine basis in our scallop research laboratory. Further experimentation should indicate whether this new method may also prove useful with other species in which spawning is hardly induced by conventional methods.

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## FATTY ACID DYNAMICS IN SEA SCALLOPS *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791) FROM GEORGES BANK, NOVA SCOTIA

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**ABSTRACT** This work presents a comprehensive analysis of the fatty acid components in the major organs of sea scallops *Placopecten magellanicus* (Gmelin) from Georges Bank as related to relevant biological information. About 50 different fatty acids were identified in scallop tissues. Regardless of variations according to lipid classes, organs and seasons, the major fatty acids were 16:0, 16:1n-7, 18:0, 18:1n-7, 18:4n-3, 20:5n-3 and 22:6n-3. Selected fatty acids, such as 16:1n-7 and 18:1n-9, were found to be good indicators of lipid transport between the lipid-rich digestive gland and the developing female gonad.

Two fatty acids, a C<sub>20</sub> monounsaturated acid and the isoprenoid acid trimethyltridecanoic (TMTD), exhibited unusual anatomical distributions in *P. magellanicus*. In particular 20:1n-11 was preferentially found in the phospholipids of the mantle and gills. TMTD was present exclusively in the triacylglycerol fraction of the digestive gland. TMTD is a byproduct of the degradation of chlorophylls, and was virtually absent in any other organ or lipid class. Due to the highly restricted anatomical distribution of TMTD, it has the potential for use as an indicator of an autotrophic source of reduced carbon and of the nutritional condition of the animal through estimation of triacylglycerol reserves.

The consistent level of 16:1n-7 as a proportion of total fatty acids in the digestive gland and gut contents, together with 18:1n-7 > 18:1n-9, suggests metabolic control and a specific role for the n-7 monoethylenic fatty acids, possibly as precursors of the unusual non-methylene-interrupted fatty acids.

Most polyunsaturated fatty acids (PUFA) also presented preferential anatomical and temporal distributions. Thus 16:4n-1, 18:4n-3, 20:5n-3 and 22:6n-3 reflected the seasonal fluctuations in the water temperature and the food supply on Georges Bank. Phospholipids in the digestive gland, female gonad, mantle and gills had different levels of fatty acid polyunsaturation, with the polyunsaturation index ranging approximately from 195 to 330. Nevertheless, it was found that changes in the polyunsaturation levels of these organs took place in parallel over time.

Temporal variations in the fatty acid profiles of the digestive gland and gut contents in sea scallops were consistent with the reported alteration of diatoms and microflagellates as the major local phytoplankters. On the other hand, changes in the content and composition of PUFA in the female gonad reflected the process of seasonal gamete differentiation and gonadal growth.

**KEY WORDS:** scallops, fatty acid dynamics, *Placopecten magellanicus*

### INTRODUCTION

The sea scallop, *Placopecten magellanicus*, is a bivalve mollusc (Bivalvia, Pectinidae) commonly occurring along the east coast continental shelf of North America from the north shore of the Gulf of St. Lawrence to Cape Hatteras (Posgay 1957, Bourne 1964). The major offshore Canadian fishery is located in the northern portion of Georges Bank, with a secondary fishing ground in the Bay of Fundy (particularly off Digby, Nova Scotia). Scallops are also fished in the southern Gulf of St. Lawrence, on the St. Pierre Bank, and in Port au Port Bay, Newfoundland (Bourne 1964). Georges Bank is one of the most biologically-productive areas in temperate latitudes (Riley 1982, O'Reilly et al. 1982), an important factor in the reproductive success of the scallop as well as for growth.

Lipids are extremely important biochemical components in ma-

rine organisms and are involved in many metabolic reactions, "from the more mundane to the more sophisticated" (Allen 1976). An extensive literature on the lipid composition of marine invertebrates exists (Joseph 1989), but only a few studies illustrate lipid storage dynamics in post-metamorphic marine invertebrates. This work presents a systematic study of the anatomical distribution and temporal variations of fatty acids of sea scallops from Georges Bank, and their relationship with food sources. In a companion paper (Napolitano and Ackman 1992), we have presented the anatomical distribution and temporal variations of lipid classes in *P. magellanicus* from the same location.

### MATERIALS AND METHODS

#### *Lipid Analysis. General Procedures*

Sea scallops, *P. magellanicus*, were collected in the Canadian sector of Georges Bank at approximately N41°35'09", W61°10'11". Specimens were captured during commercial fishing operations between August 1987 and October 1989 as previously described (Napolitano 1991). Scallops used for lipid analyses were transported live to the laboratory. Adult animals (shell height >10

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cm) were sexed and grouped into three sample lots of three to seven animals each, depending on availability. Digestive gland, gonad, adductor muscle, mantle, and gills were dissected and individually weighed. Samples of the contents of the scallop digestive gland cavity ("gut content") were obtained by freezing, splitting the digestive gland in half, and then removing the contents. Usually the lipids were extracted immediately but a few samples were stored at  $-35^{\circ}\text{C}$  for several days. Whenever possible and necessary, all procedures were carried out under a nitrogen atmosphere.

Scallop organ lipids were extracted with a mixture of chloroform:methanol (2:1 v/v) using a stainless steel Waring Blender, following the classical method described by Bligh and Dyer (1959). After extraction, the lipid extracts in the chloroform layer were washed with water, dried over sodium sulfate, concentrated, and stored in chloroform in screw-cap (Teflon-lined) glass vials at  $-35^{\circ}\text{C}$  under a nitrogen atmosphere. Small quantities of solvents were evaporated under a stream of nitrogen in glass centrifuge tubes placed in a water bath at  $40\text{--}50^{\circ}\text{C}$ . The evaporation of a large amount of solvent was conducted using a rotary evaporator under reduced pressure.

Fatty acid standards were purchased from Serdary Research Laboratories, London, Ontario. The organic solvents were A.C.S. reagent grade (Anachemia), redistilled in glass before use. The acids and other reagents were A.C.S. grade from Fisher Scientific Company (Canada).

#### *Lipid Class Separation for Fatty Acid Analysis*

Total lipids were separated by thin layer chromatography (TLC) on "Prekote" silica gel plates (20 cm  $\times$  20 cm, 200  $\mu\text{m}$  particle size, Applied Science Laboratories, College Park, PA). Before use the plates were cleaned by developing in ethyl acetate and activated by heating at  $110^{\circ}\text{C}$  for 30 min. Lipid mixtures were applied as chloroform solutions using a plate streaker (Applied Science Laboratories). Plates were developed in solvent-saturated glass tanks. The common developing solvent was hexane:diethyl ether:acetic acid (80:20:1; v/v/v). Lipids were visualized by spraying with a 1% 2',7'-dichlorofluorescein solution in ethanol and observation under UV light. Standards and standard mixtures were spotted alongside the samples to compare  $R_f$  values.

#### *Preparation of the Fatty Acids for Gas-Liquid Chromatography*

The individual classes in total lipids extracted from the scallop organs were recovered from preparative TLC plates by extraction of silica gel with chloroform-methanol prior to fatty acid methylation. Fatty acids in different lipid classes were converted to their respective methyl esters using 10%  $\text{BF}_3$ -methanol, following a modification of the method described by Morrison and Smith (1964). Total lipid extracts or individual lipid classes were redissolved in benzene (1 ml) in a 10 ml screw-capped (Teflon-lined) centrifuge tube. Then 1 ml of 10%  $\text{BF}_3$ -methanol was added to the tube, which was flushed with nitrogen and capped. This was then shaken thoroughly and heated at  $100^{\circ}\text{C}$  in a heating block for 1 h. After cooling the sample to room temperature, distilled water (2 ml) was added, the mixture shaken vigorously, and the top layer containing the methyl esters removed. The remaining mixture was again extracted with benzene (2  $\times$  2 ml). The combined benzene extracts were concentrated under a stream of nitrogen and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The solution was filtered or decanted and evaporated to dryness under a stream of nitrogen. The esters were

redissolved in hexane, which was the standard solvent for injection into the gas-liquid chromatograph.

#### *Gas-Liquid Chromatography of the FAME*

Analytical gas-liquid chromatography (GLC) of the FAME (fatty acid methyl esters) was carried out on a Perkin-Elmer Model 8420 (Perkin Elmer, Norwalk, CT) equipped with FID (flame ionization detection) and a bonded polyglycol (SUPELLOWAX-10) flexible fused silica capillary column (30 m in length  $\times$  0.25 mm ID, Supelco, Inc. Bellefonte, PA). Helium was the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was programmed as follows: an initial temperature of  $185^{\circ}\text{C}$  was maintained for 8 min; then the temperature was increased to  $240^{\circ}\text{C}$  at a rate of  $3^{\circ}/\text{min}$ . Retention times and area percentages were recorded on a Perkin-Elmer LCI-100 Laboratory Computing Integrator. Relative areas were converted to weight % amounts of fatty acids by correcting for the FAME FID responses (Ackman 1986, Ackman and Eaton 1978).

#### *Identification of the Fatty Acids*

FAME were identified by combinations (Ackman 1986) of the following procedures: a) Co-injecting the sample with authentic standards, or a FAME mixture of established composition, b) Silver nitrate-TLC followed by GLC of fractions, c) Plotting procedures, d) Comparing equivalent chain lengths (ECL) of fatty acids on chromatographic columns of different polarity, e) Catalytic hydrogenation of the sample over  $\text{PtO}_2$  and reanalysis for chain lengths, f) Checking molecular weights and mass spectra obtained by GLC/MS (gas-liquid chromatography/mass spectroscopy). The system used for this purpose was a Perkin-Elmer gas-liquid chromatograph Model 990 equipped with a SUPELLOWAX-10 capillary column interfaced directly into a Finnigan MAT 700 Ion Trap Detector (ITD) (Finnigan MAT, San Jose, CA).

Pseudoreplicate data for each sample consisting of three to seven pooled organs was treated statistically by a one way analysis of variance. Prior to this analysis, lipid content and fatty acid proportions were normalized through the arcsine transformation (Snedecor and Cochran 1980).

## RESULTS

#### *Fatty Acid Composition*

As in most marine organisms, a group of 10 to 15 components out of 50 identifiable fatty acids represented 80 to 90% of the total fatty acids in all organ and seasonal samples. Tables 1 to 4 present the detailed fatty acid compositions of digestive gland, female and male gonads, adductor muscle, mantle, and gills for the four seasons respectively. Fatty acids of the digestive gland and the female gonad have been separated into those from the two major constituents, i.e. triacylglycerols (TG) and phospholipids (PL). Since adductor muscle, mantle and gills contained very small amounts of TG, only the fatty acid composition of their isolated PL were tabulated. The male gonad was routinely analyzed only for phospholipid fatty acids, except for one case (fall sample, Table 3), when a conspicuous amount of TG was observed during TLC preparations.

Regardless of variations according to lipid class, organ and seasons, the major fatty acids in this pectinid were 16:0, 16:1n-7, 18:0, 18:1n-7, 18:4n-3, 20:5n-3, and 22:6n-3. Among these, 16:0 and 20:5n-3 were always very abundant in all the organs, each of

TABLE 1.

Selected fatty acids<sup>a</sup> in major organs in scallops (*P. magellanicus*) collected during the spring on Georges Bank.

Organ Lipid	Digestive Gland TG	Gonad Female		Gonad Male PL	Adductor Muscle PL	Mantle PL	Gills PL
		PL	TG				
TMTD <sup>b</sup>	1.54	tr	tr	tr	tr	tr	tr
14:0	3.49	1.77	2.90	1.74	2.74	2.31	2.05
16:0	12.56	18.11	23.66	19.98	24.18	21.48	19.33
16:1n-7	7.31	1.86	6.26	1.38	2.75	1.54	2.06
16:4n-1	3.13	0.35	1.09	0.13	0.08	0.41	1.06
17:0 <sup>c</sup>	1.36	1.64	0.89	0.48	0.79	1.09	1.11
18:0	2.42	5.24	2.51	3.68	5.30	7.76	7.21
18:1n-9	2.17	0.77	2.06	0.49	0.93	0.64	0.50
18:1n-7	5.75	2.07	5.93	2.57	5.26	4.70	3.92
18:2n-6	1.16	0.34	0.64	0.23	0.52	0.95	1.41
18:2n-4	1.51	0.37	1.37	0.28	0.66	0.42	1.41
18:3n-3	0.86	0.16	0.48	0.14	0.29	0.12	0.26
18:4n-3	5.50	4.01	5.69	1.65	2.82	1.23	1.77
18:4n-1	1.37	0.21	0.78	0.05	0.16	0.08	0.10
20:1n-11	0.33	1.76	0.63	0.97	0.81	2.49	5.02
20:1n-9	0.41	0.61	0.28	0.81	0.70	0.58	0.98
20:1n-7	0.78	0.51	0.83	0.61	0.72	0.77	1.07
20:4n-6	0.46	1.24	0.28	1.24	0.89	2.26	3.15
20:5n-3	36.46	28.74	25.08	24.58	20.72	15.17	14.44
21:5n-3	1.01	1.08	1.72	1.98	1.45	0.83	0.86
22:5n-3	0.33	1.10	0.40	3.76	1.06	0.83	1.00
22:6n-3	6.61	21.16	9.32	26.44	20.06	24.55	18.23

<sup>a</sup> Minor components identified (<1%) and not included in Table are 15:0; Iso16:0; pristanate (2,6,10,14-tetramethylpentadecanoic acid); 7-methylhexadecanoic acid; 16:1n-11, n-9, n-5; 16:2n-7, n-4; 16:3n-4, n-3; Aiso18:0; 18:1n-5; 18:3n-6, n-4, n-3; 20:0; 20:1n-7, n-5; 20:2NMID; 20:3n-6; 20:4n-3; 22:0; 22:1n-13, n = 11, n-9; 22:2NMID; 22:4n-6 and 22:5n-6.

<sup>b</sup> TMTD = trimethyltridecanoic acid; tr = trace.

<sup>c</sup> Includes phytanic (3,7,11,14-tetramethylhexadecanoic) acid

them showing an overall level higher than 15%, and in some cases they reached almost 50% of the total fatty acids when combined. Other fatty acids, 14:0, 16:4n-1, 18:2n-6, 20:4n-6, 21:5n-3 and 22:5n-3, were important but were almost always present below the 5% level. The scallop organs showed similar fatty acid patterns in samples from different seasons. A special case of marked differential anatomical distribution was displayed by the isoprenoid acid, 4,8,12-trimethyltridecanoic (TMTD). The identification of this branched-chain fatty acid was confirmed by a series of analytical techniques as described in Materials and Methods, which included GLC/MS. TMTD was present at conspicuous levels only in the TG fraction of the digestive gland (ca. 2.5%), and it was barely detectable in the TG and PL of other organs. This pattern of distribution of TMTD was consistent in all the samples analyzed including those from different seasons. Another isoprenoid fatty acid detected in most of the analyses was pristanic acid. However, no anatomical or lipid class-related preferential distribution was apparent for this acid. The third isoprenoid acid expected, phytanic, (Ackman et al. 1971), coincided with the peak of 17:0 and was not differentiated. This factor may account for some of the variations in the proportions reported for 17:0.

Palmitic acid (16:0) was present at different concentrations in the TG of the digestive gland and of the female gonad. The level of 16:0 in the TG of the digestive gland ranged between 12% and 17%, according to the season, while the proportions of this fatty acid in the female gonad TG were always between 19% and 25% of the total fatty acids. The concentrations of 16:0 in the female gonad and in the rest of the body PL were variable, but normally

<15%. A very high proportion of 16:0 was observed in the PL of the male gonad in the summer samples (Table 2).

In contrast with the different levels of 16:0 found between the TG of the digestive gland and of the female gonad, the concentrations of 16:1n-7 in the neutral lipids of these two organs were maintained at similar values (about 6.5%). There was also a marked difference in the 16:1n-7 content between the female gonad TG (~6.5%) and the PL of the same and the other organs, except for one adductor muscle sample PL (Table 4). Two other relatively minor unsaturated fatty acids showed a similar trend, 16:4n-1 (the major C<sub>16</sub> polyunsaturated component found in scallop tissues), and oleic acid (18:1n-9). The concentrations of these two fatty acids in the TG of digestive gland and female gonad were similar, and normally higher than in PL. The concentration of 16:4n-1 in the male gonad TG was also low (Table 3), and comparable with the values obtained for phospholipids in the other organs analyzed. Other C<sub>16</sub> PUFA, i.e. 16:2n-7, 16:2n-4, 16:3n-4 and 16:3n-3 were always at concentrations <1% of the total fatty acids. *Cis*-vaccenic acid, (18:1n-7), was also at relatively low concentrations in the female gonad PL. However, in contrast with 16:4n-1 and 18:1n-9, which showed low values in PL, 18:1n-7 was present at levels as high as 5.5% in the polar lipid fractions of most of the organs.

Among the important C<sub>18</sub> fatty acids, 18:0 showed a clear association with PL in all tissues. The major C<sub>18</sub> PUFA found in sea scallops, 18:4n-3, exhibited a consistent pattern of anatomical distribution. Relatively high proportions of 18:4n-3 (4–7%) were found in the TG of the digestive gland, and in both TG and PL of

TABLE 2.

Selected fatty<sup>a</sup> acids in major organs in scallops (*P. magellanicus*) collected during summer on Georges Bank (Abbreviations as in Table 1).

Organ Lipid	Digestive Gland TG	Gonad Female		Gonad Male PL	Adductor Muscle PL	Mantle PL	Gills PL
		PL	TG				
TMTD	2.07	tr	tr	tr	tr	tr	tr
14:0	3.22	0.92	3.79	1.59	0.21	0.07	1.17
16:0	12.02	14.02	23.94	30.54	13.69	11.10	16.25
16:1n-7	6.25	1.22	5.91	1.34	1.04	0.75	1.47
16:1n-5	0.45	0.56	0.61	0.63	0.47	0.51	1.51
16:4n-1	1.81	0.18	0.92	0.73	0.38	0.39	0.09
17:0 <sup>b</sup>	0.79	3.82	0.68	1.76	0.54	2.73	2.15
18:0	2.02	7.17	2.37	6.48	7.85	9.99	8.61
18:1n-9	2.57	0.69	2.54	0.51	1.18	0.85	0.92
18:1n-7	4.86	2.06	4.43	2.93	5.51	4.47	3.68
18:2n-6	1.36	0.29	0.98	0.27	0.31	0.36	0.25
18:2n-4	1.05	0.27	1.16	0.30	0.47	0.17	0.19
18:3n-3	1.17	0.23	0.94	0.33	0.27	0.41	0.39
18:4n-3	5.76	6.69	7.10	2.91	2.88	5.61	2.27
20:1n-11	0.41	2.24	0.52	0.82	1.52	4.81	8.82
20:1n-9	0.54	1.05	0.41	1.13	1.52	4.47	1.33
20:1n-7	4.86	0.66	0.76	0.81	1.30	1.70	2.05
20:1n-5	tr	0.36	tr	tr	tr	nd	1.32
20:4n-6	0.51	1.67	0.61	1.17	0.12	3.13	4.10
20:5n-3	33.52	23.4	20.89	20.26	23.55	15.25	11.79
21:5n-3	1.32	1.31	1.17	1.56	1.42	0.97	0.50
22:5n-3	0.40	1.23	0.51	2.45	1.96	1.50	0.73
22:6n-3	9.96	24.11	10.52	16.27	28.27	25.91	21.13

<sup>a</sup> Minor components identified (<1%) and not included in Table are 15:0; Iso16:0; pristanate (2,6,10,14-tetramethylpentadecanoic acid); 7-methylhexadecanoic acid; 16:1n-11, n-9, n-5; 16:2n-7, n-4; 16:3n-4, n-3; Aiso18:0; 18:1n-5; 18:3n-6, n-4, n-3; 20:0; 20:1n-7, n-5; 20:2NMID; 20:3n-6; 20:4n-3; 22:0; 22:1n-13, n = 11, n-9; 22:2NMID; 22:4n-6 and 22:5n-6.

<sup>b</sup> Includes phytanic (3,7,11,14-tetramethylhexadecanoic) acid.

the female gonad. However, in the adductor muscle, gills, and mantle the proportions of 18:4n-3 were reduced to 1–2% of the total fatty acids.

Other consistent fatty acid-organ associations were observed between 20:1n-11 and the gill PL, and to a minor extent with the mantle PL. The level of 20:1n-11 in most of the organs rarely exceeded 1%, while in the PL of gills and mantle it ranged from 3.5 to 8.8% and from 1.2 to 4.8% respectively. Gills and mantle also exhibited an enrichment in the other C<sub>20</sub> monounsaturated fatty acids (i.e. 20:1n-9 and 20:1n-7), but this association was less pronounced and less consistent.

The two major and more important PUFA in the sea scallops were 20:5n-3 (eicosapentaenoic acid or EPA) and 22:6n-3 (docosahexaenoic acid or DHA). These two n-3-fatty acids were present at very high concentrations, and often either one or the other was the major PUFA, depending on the organ or on the sampling time. EPA was extremely high (up to 36% of the total fatty acids) in the digestive gland, female and male gonads, and adductor muscle during all seasons. Maximum values of EPA were observed in the TG fraction of the digestive gland, and in both PL and TG of the female gonad. Mantle and gills also contained high proportions of EPA, but their levels (about 15% of the total) were consistently lower than the EPA in the digestive and reproductive organs.

DHA showed a clear association with the PL fraction in all organs and during all seasons. In general, the proportion of 22:6n-3 in TG was relatively low, with levels <10% of the total fatty acids, while typical levels of this fatty acid in PL were >20%. A remarkable observation, and an exception to the relatively low

levels of 22:6n-3 in neutral lipids, was shown by the one male gonad TG analyzed (Table 3). The concentration of DHA in the triacylglycerol fraction of the mature male gonad was 50% of the total fatty acids, while the phospholipids contained only 21.9%.

To summarize the results for the fatty acids in different organs and lipid classes for seasonal variations in the level of unsaturation they can be expressed as a "polyunsaturation index" (PUI = the summed products of PUFA weight percentages larger than 1 multiplied by the number of double bonds). Results for adductor muscle, mantle, gills, digestive gland and female gonad, are illustrated in the Figs. 1a,b and c. Interestingly, although each organ had different degrees of PL polyunsaturation, all showed similar trends throughout the seasons (Fig. 1a). In all cases there was a moderate (female gonad and gills) to marked (adductor muscle and mantle) increase of polyunsaturation from spring to summer, followed by an important decline in the fall, and another increase in winter. Minimum values in one lipid class in each organ corresponded to a maximum in the other lipid class, and vice versa (Figs. 1b and c). As a consequence of this complementary pattern of fatty acid variation, no net seasonal change could be observed in the PUI of the total lipids from separate samples of scallop female gonad (Fig. 1c).

#### Temporal Variations of Fatty Acids

To detect possible variations in the quality of food supply to scallops in Georges Bank, the total lipid fatty acid composition of the digestive gland and of its gut content were analyzed during the

TABLE 3.

Selected fatty acids<sup>a</sup> in major organs in scallops (*P. magellanicus*) collected during fall on Georges Bank (Abbreviations as in Table 1).

Organ Lipid	Digestive Gland TG	Gonad Female		Gonad Male		Adductor Muscle PL	Gills PL
		PL	TG	PL	TG		
TMTD	2.46	tr	tr	tr	tr	tr	tr
14:0	4.90	1.60	4.94	1.40	0.95	1.50	5.63
16:0	16.01	18.64	19.25	27.68	8.92	14.41	14.26
16:1n-7	10.17	1.57	6.53	1.37	2.46	1.61	3.12
16:1n-5	0.72	1.09	0.83	1.18	0.31	0.85	1.30
16:4n-1	1.07	0.40	1.26	0.04	0.13	1.51	0.41
17:0 <sup>b</sup>	0.79	0.90	0.70	0.95	0.40	0.91	1.08
18:0	2.24	5.27	3.56	4.21	1.56	6.72	6.81
18:1n-11	0.04	0.02	tr	nd	nd	tr	1.43
18:1n-9	6.42	0.81	0.41	1.19	2.58	2.73	1.17
18:1n-7	6.06	2.70	4.40	2.93	2.61	4.61	2.77
18:2n-6	1.62	0.74	0.87	0.29	0.84	0.90	1.31
18:3n-3	0.85	0.22	0.54	0.10	0.34	0.60	0.03
18:4n-3	3.79	3.21	3.12	1.40	0.81	2.27	2.67
20:1n-11	0.79	1.85	0.82	0.73	0.61	1.27	3.52
20:1n-9	1.01	1.07	0.56	1.52	1.27	1.22	1.26
20:1n-7	0.89	0.48	0.64	0.97	0.55	0.71	1.62
20:1n-5	tr	tr	nd	tr	tr	tr	1.18
20:4n-6	0.63	2.65	0.55	2.14	1.27	2.90	3.15
20:5n-3	21.64	21.19	15.98	19.07	11.93	16.45	14.96
21:5n-3	0.78	0.77	1.01	0.66	1.02	0.83	0.73
22:5n-3	0.49	1.20	1.09	2.90	5.34	1.54	0.64
22:6n-3	8.91	24.80	23.91	21.93	50.19	28.42	15.82

<sup>a</sup> Minor components identified (<1%) and not included in Tables are 15:0; Iso16:0; pristanate (2,6,10,14-tetramethylpentadecanoic acid); 7-methylhexadecanoic acid; 16:1n-11, n-9, n-5; 16:2n-7, n-4; 16:3n-4, n-3; Aiso18:0; 18:1n-5; 18:3n-6, n-4, n-3; 20:0; 20:1n-7, n-5; 20:2NMID; 20:3n-6; 20:4n-3; 22:0; 22:1n-13, n = 11, n-9; 22:2NMID; 22:4n-6 and 22:5n-6.

<sup>b</sup> Includes phytanic (3,7,11,14-tetramethylhexadecanoic) acid.

course of a year (Table 5). For simplicity, only major fatty acids, or those that could be indicators of certain algal groups through fatty acid input, are presented.

Five out of the eleven important fatty acids of the scallop digestive gland showed significant seasonal variations ( $p < 0.05$ ). These fatty acids were 16:0, the group of C<sub>16</sub> PUFA (mainly 16:4n-1), 18:0, 20:5n-3 and 22:6n-3. Palmitic acid (16:0) gradually increased its concentration in the digestive gland lipids from spring to winter. Although this increment was small, the concentration of 16:0 in the spring samples was significantly lower (11.3%) than those of the rest of the year (13.9% in winter). The group of C<sub>16</sub> PUFA showed a different trend by displaying a maximum concentration during the spring (3.5%) and a minimum during the fall (2.1%). Stearic acid (18:0) was another component showing a small but significant seasonal variation in the digestive gland lipids. The variation of 18:0 was similar to that shown by palmitic acid, consisting of a slight and gradual increment from spring (1.8%) to winter (3.1%). The important long-chain PUFA, 20:5n-3 and 22:6n-3, exhibited much larger seasonal changes. EPA was relatively high in the spring and summer samples (30.8 and 28.2% respectively) and its concentration gradually decreased during fall and winter (24.7 and 23.7% respectively). In contrast, DHA presented a minimum concentration in the digestive gland lipids during the spring (8.3%), and its level increased significantly in fall (13.9%) and winter (15.5%).

Table 5 also contains the fatty acid composition of the gut contents for the different seasons. These quantities represent single determinations of three pooled samples. Thus, it was not possible

to use this information alone to demonstrate statistically valid seasonal variation. Nevertheless, the seasonal changes observed for EPA and DHA in the lipids of the digestive gland are accompanied by identical trends in the fatty acids of their gut content. Most notable was the presence of TMTD at concentrations almost as high as those observed in the whole digestive gland (ca. 3% of the total fatty acids).

## DISCUSSION

### *Anatomical Distribution and Temporal Variation of Fatty Acids*

Overlooking some marked variations according to lipid class, organ, and seasons, the fatty acid compositions of the sea scallops reported here are similar to those accepted for most marine bivalves from similar cold water latitudes (Joseph 1982). Unfortunately, all of the information available on fatty acids of *P. magellanicus* refers exclusively to the edible adductor muscle (e.g. Gruger et al. 1964, Stansby and Hall 1967, Exler and Weihrauch 1977). These earlier reports show reasonable agreement with the fatty acid proportions of the major fatty acid components listed here for this organ. There are some discrepancies among these authors regarding the proportions of 16:0. Nevertheless, data in Tables 1 to 4, and in a complete study in Japan on seasonal, regional and anatomical variation of the fatty acids of the giant ezo scallop *Patinopecten yessoensis* (Hayashi and Yamada 1975), suggests that such discrepancies are primarily due to seasonal variations in the fatty acid composition of the adductor muscle rather than arising from major species differences.

TABLE 4.

Selected fatty acids<sup>a</sup> in major organs in scallops (*P. magellanicus*) collected during winter on Georges Bank (Abbreviations as in Table 1).

Organ Lipid	Digestive Gland TG	Gonad Female		Gonad Male PL	Adductor Muscle PL	Mantle PL	Gills PL
		PL	TG				
TMTD	3.05	tr	tr	tr	tr	tr	tr
14:0	5.78	1.57	3.88	1.66	2.16	2.43	1.28
16:0	17.67	16.91	25.15	19.54	19.16	21.08	16.39
16:1n-7	7.54	1.56	7.83	1.57	4.28	1.41	0.25
16:4n-1	1.37	0.07	0.54	0.19	0.01	0.17	1.33
17:0 <sup>b</sup>	0.90	2.02	0.90	2.24	0.80	0.75	1.04
18:0	2.76	5.80	2.18	4.37	5.23	8.15	5.28
18:1n-9	3.14	0.89	3.39	0.79	0.79	0.85	0.56
18:1n-7	5.27	2.37	6.26	3.08	4.28	4.47	3.08
18:2n-6	1.44	0.30	1.19	0.29	0.29	0.93	1.43
18:3n-3	1.41	0.25	0.78	0.23	0.22	0.28	0.66
18:4n-3	6.12	4.52	3.94	3.26	1.81	1.97	1.00
20:1n-11	0.69	2.62	0.95	2.25	1.00	3.07	6.20
20:1n-9	0.49	0.97	0.42	0.73	0.70	0.74	1.05
20:1n-7	0.99	0.72	0.78	0.65	0.69	0.71	1.00
20:4n-6	0.65	2.96	0.77	2.40	1.40	3.08	5.21
20:5n-3	20.86	18.17	16.32	18.86	22.11	13.35	13.41
21:5n-3	0.93	1.14	1.02	0.89	1.59	0.56	1.17
22:5n-3	0.39	1.10	0.58	1.63	2.03	0.56	0.85
22:6n-3	9.07	26.27	13.70	26.90	33.12	26.07	25.51

<sup>a</sup> Minor components identified (<1%) and not included in Table are 15:0; Iso16:0; pristanate (2,6,10,14-tetramethylpentadecanoic acid); 7-methylhexadecanoic acid; 16:1n-11, n-9, n-5; 16:2n-7, n-4; 18:3n-4, n-3; Aiso18:0; 18:1n-5; 18:3n-6, n-4, n-3; 20:0; 20:1n-7, n-5; 20:2NMID; 20:3n-6; 20:4n-3; 22:0; 22:1n-13, n = 11, n-9; 22:2NMID; 22:43n-6 and 22:5n-6.

<sup>b</sup> Includes phytanic (3,7,11,14-tetramethylhexadecanoic) acid.

Most studies of the fatty acids of other scallop species are based on a single sampling during the year. Thus, it was not always possible to discriminate in the literature reports between seasonal or species-specific differences. Reports of the fatty acid composition of adductor muscle or whole animals in a number of different scallop species, such as *Aequipecten irradians*, *Aequipecten gibbus*, *Patinopecten caurinus*, *P. magellanicus* (Krzeczkowski et al. 1972) and *Chlamys nipponensis* (Hayashi and Yamada 1973), show a strong homogeneity of the fatty acid composition in the Order Pterioidea as previously suggested by Joseph (1982). An exceptional composition was the rock scallop *Hinnites multirugosus* (now *Crassadoma gigantea*), which contained only 1.3% of 16:0, high proportions of both 16:1, 18:2, 18:3 and 20:1 (ca. 15% ea.), 25% 20:5, and only trace levels of 22:6n-3 (Phleger et al. 1978). Unusually, this analysis was conducted on a non-polar GLC column and some fatty acid identification problems may be responsible for the atypical fatty acid compositions. A detailed study of the adductor muscle, viscera and gonad lipids in *P. yessoensis* in Nemuro Bay, Japan (Tsuji and Nishida 1988) also presented results comparable to those for *P. magellanicus*, although their samples contained much higher proportions of EPA in the adductor muscle (ca. 30% of the total). The digestive glands of their samples of *P. yessoensis* also exhibited some other features not observed in *P. magellanicus*, including rather low proportions of DHA (1 to 7.4%), and a marked difference between males and females in the proportion of EPA.

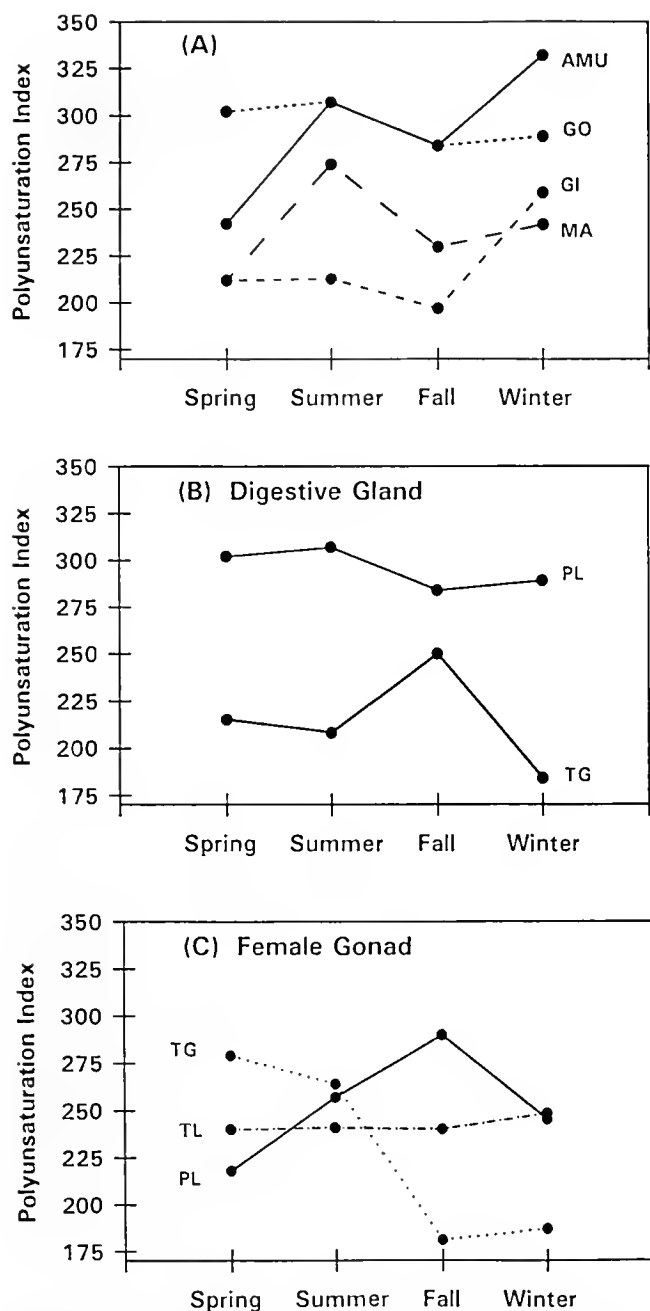
#### Trimethyltetradecanoic Acid

Some fatty acids were important only in certain organs, sometimes showing a clear trend during the year. The branched-chain

fatty acid TMTD exhibited an extreme case of restricted anatomical distribution. This isoprenoid fatty acid is a metabolic product of the degradation of phytol (*trans*-3,7,11,15-tetramethylhexadec-2-en-1-ol), the fatty alcohol side chain of chlorophylls (Blumer et al. 1964). At 33% of the weight of chlorophyll, phytol is an important component of the algal diet of filter-feeding bivalves. The presence of TMTD exclusively in the TG fraction of the digestive gland in sea scallops suggests that this fatty acid is a byproduct of phytol subjected to a specific catabolic route which may not involve an initial formation of phytanic acid (Prahil et al. 1984). Structurally, TMTD is a 16-carbon fatty acid with a straight 13-carbon chain, and it may deceive some enzymes into treating it as either a 14:0 or 16:0 fatty acid, both common in depot fats. The concentration of TMTD in the digestive gland of the sea scallop lipid reserves is at least two orders of magnitude larger than in the lipids of the other organs. Thus, one can infer that for some reason this fatty acid is definitely not transferred to the maturing female gonad along with other lipid material. Due to its unique accumulation in the TG fraction, we believe that TMTD is the most obvious fatty acid for use as an indicator of the animal's nutritional condition.

Interestingly, TMTD was also detected at relatively high levels in the gut contents of scallops (Table 5). This is likely to represent the immediate product of extracellular hydrolysis of phytol, and possibly an initial enzymatic transformation occurring in the water column (Bradshaw et al. 1990) or in the digestive cavity. The initial steps of the metabolic route in the degradation of phytol have been demonstrated to occur in higher animals in the vertebrate liver cells and liver homogenates, as well as in microbial communities (Hansen 1980). Thus, the existence of TMTD in the





**Figure 1.** Temporal variation of the "polyunsaturation index" in sea scallops *P. magellanicus* from Georges Bank; A: phospholipid fatty acids in the female gonad (GO), adductor muscle (AMU), mantle (MA), and gills (GI); B: triacylglycerol (TG) and phospholipid (PL) fatty acids in the digestive gland; C: total lipid (TL), triacylglycerol, and phospholipid fatty acids in the female gonad.

gut cavity of the scallop suggests, as in ruminants, the contribution of a microbial population inhabiting the animal digestive system, and acting on phytol extracellularly once it is hydrolyzed from the molecule of chlorophyll.

Although TMTD is present at a considerable concentration in the lipids of the sea scallop digestive gland (Tables 1 to 4), and is very likely to be part of lipids of other herbivorous bivalves, it is only rarely reported (Ackman et al. 1971, Joseph 1989). In the case of *P. magellanicus*, this is probably due, at least in part, to

the fact that single lipid classes of individual organs are not commonly examined, which results in the dilution of this fatty acid by the fatty acids of other organs in which it is barely present. In addition, the retention time of TMTD under standard GLC conditions leads to the peak falling among 14:1 isomers and near iso-15:0 (Ackman 1969); TMTD also follows closely after the usually large 14:0 peak in the popular polar GLC columns and may be simply included in 14:0 by persons not specifically interested in isoprenoid fatty acids.

#### Unsaturated Fatty Acids

The role of the digestive gland in molluscs is far from being fully established; this is reflected in the large number of names used in the literature for this organ (a problem reviewed by van Weel in 1974). Nevertheless, there is experimental evidence showing hemolymphatic transport of preformed metabolites, including both dietary lipids and lipids formed *de novo* (Pollero and Heras 1989) from the digestive gland to the developing gonads in molluscs (Vassallo 1973). The importance of the digestive gland of the bay scallop *Argopecten irradians concentricus* (Say) in the transfer of nutrients may differ from that in other marine bivalve species (Barber and Blake 1985). Seasonal changes in the anatomical distributions of some fatty acids of the scallop *P. magellanicus* reflect a close parallel between the digestive gland and the female gonad. For instance, it is possible to observe clear similarities in the distribution of some major and important fatty acids in the TG fraction of the digestive gland and the same lipid fraction of the female gonad (Tables 1 to 4). In contrast to the special case already described for TMTD (which is excluded from the female gonad), the concentrations of some unsaturated fatty acids in the digestive gland, such as 16:1n-7, 18:1n-7, 18:1n-9, 16:4n-1, 18:4n-3 and 22:6n-3 seem to be positively related to their concentrations in TG of the female gonad.

The high concentration of C<sub>20</sub> monounsaturated acids, especially 20:1n-11, in the gills of this scallop is another example of differential anatomical distribution of fatty acids. In comparison with the other lean organs (i.e. adductor muscle and mantle), gills have a much more delicate anatomical structure. Gill membrane lipids in marine invertebrates (Nevenzel et al. 1985) are densely ciliated structures known to contain lipids with a relatively low degree of unsaturation (Morris et al. 1987), but no previous studies on scallops make special reference to the enrichment in the 20:1 fatty acids shown in Tables 1-4. Other recent studies of lipids of aquatic invertebrates (Takagi et al. 1980, Stefanov et al. 1992) suggest that these isomeric fatty acids should be investigated more closely.

Molluscs (Joseph 1982, Napolitano et al. 1988a) and other invertebrate phyla (Paradis and Ackman 1977, Takagi et al. 1980, Joseph 1989), contain moderate to high proportions of C<sub>20</sub> and C<sub>22</sub> non-methylene-interrupted dienoic fatty acids (NMID). The significance of these uncommon fatty acids is not known (Zhukova 1986), although they resist autooxidation (Kaneniwa et al. 1988). Their apparent enrichment in the PL fractions of some animals has suggested a structural role (Paradis and Ackman 1975, Rabinovich and Ripatti 1991). Both C<sub>20</sub> and C<sub>22</sub> NMID have been observed in all organs and lipid classes of the sea scallops from Georges Bank eastern Newfoundland and from other locations (Napolitano et al. 1991, and references therein). In comparison with other members of the Class Bivalvia analyzed, the levels of NMID in *P. magel-*

TABLE 5.

Temporal variation of major or important fatty acids (w/w%) in the total lipids of the digestive gland (DG TL; mean  $\pm$  SD, n = 3) and gut contents (GC; pooled samples) of scallops (*P. magellanicus*) from Georges Bank.

	Spring		Summer		Fall		Winter	
	DG TL	GC	DG TL	GC	DG TL	GC	DG TL	GC
TMTD	3.9 $\pm$ 0.5	3.0	3.7 $\pm$ 1.0	3.6	3.0 $\pm$ 0.4	3.1	3.3 $\pm$ 0.1	1.4
14:0	2.8 $\pm$ 0.2	0.4	2.9 $\pm$ 0.2	4.2	3.3 $\pm$ 0.4	2.9	3.4 $\pm$ 0.2	3.2
16:0	11.3A $\pm$ 0.6	10.6	12.4 $\pm$ 0.7	14.3	13.0a $\pm$ 0.5	12.7	13.9a $\pm$ 0.3	12.5
16:1n-7	6.3 $\pm$ 0.7	5.4	6.7 $\pm$ 1.7	6.0	6.3 $\pm$ 1.7	6.0	5.7 $\pm$ 1.3	3.2
C16PUFA	3.5A $\pm$ 0.4	2.6	2.3 $\pm$ 1.1	3.6	2.1a $\pm$ 0.3	2.2	2.4 $\pm$ 0.7	1.0
18:0	1.8A $\pm$ 0.05	4.4	2.4aB $\pm$ 0.2	2.9	2.7a $\pm$ 0.3	2.7	3.1ab $\pm$ 0.3	2.8
18:1n-9	1.6 $\pm$ 0.01	2.7	2.7 $\pm$ 0.8	2.8	4.3 $\pm$ 0.8	3.3	1.8 $\pm$ 1.1	2.4
18:1n-7	4.1 $\pm$ 0.1	3.8	4.9 $\pm$ 0.4	3.6	4.4 $\pm$ 0.9	4.1	4.6 $\pm$ 0.7	3.0
18:4n-3	5.5 $\pm$ 0.5	4.7	6.1 $\pm$ 0.4	6.9	6.4 $\pm$ 0.9	4.3	5.5 $\pm$ 1.2	3.9
20:5n-3	30.8A $\pm$ 1.0	30.1	28.2 $\pm$ 4.0	24.3	24.7a $\pm$ 1.4	25.2	23.7a $\pm$ 2.4	19.4
22:6n-3	8.3A $\pm$ 0.3	11.1	11.1 $\pm$ 2.4	9.9	13.9a $\pm$ 1.5	10.0	15.5a $\pm$ 3.3	27.1

Analyses of variance for comparisons of digestive gland fatty acids between seasons: Aa Bb significantly different ( $p < 0.05$ ).

*lanicus* and other scallop species were very low (e.g. Pollero et al. 1979, Napolitano et al. 1988a, Besnard et al. 1989).

#### Temporal Variations of Polyunsaturation

The seasonal variations of the PUI in scallops from Georges Bank showed very interesting characteristics. It can be observed that the changes in polyunsaturation of the fatty acids of scallop organs (Figs. 1a,b and c) are dictated by the concentration of the two major components, i.e. EPA and DHA (Tables 1 to 5). Analogous and very reproducible variations in PUI of the phospholipid fractions were observed for the different organs of scallops.

It is well known that the fatty acid composition of phospholipids in both plant and poikilotherm animals changes with ambient temperature (Cossins and Lee 1985, Connolly et al. 1985, Bell et al. 1986, Carey and Hazel 1989), and also that the PL fatty acids in animals are, to a large extent, independent of the fatty acids supplied by diet (e.g. Fraser et al. 1989). The well known negative correlation between low ambient temperature and the level of unsaturation in fatty acids of PL appropriate for maintaining membrane fluidity (Spector and Yorek 1985) is depicted by the high PUI in the PL of the major scallop organs (Fig. 1a). Maximum seawater temperatures in Georges Bank during the fall (Walsh et al. 1987) are consistent with the minimum values of the PUI observed during this season. The relatively low value of the PUI detected in the late spring, and its rapid increase toward the summer, require, however, a different explanation, highlighting the need to consider the participation of other factors affecting the biochemical composition of the animal. The period of rising water temperature coincides with the phase of gamete proliferation and growth in this scallop population, and the same effect is reported for the Iceland scallop *Chlamys islandica* (Thorarinsdottir 1993). At the biochemical level, this maturation process is accompanied by the transport of lipid material from the digestive gland to the developing eggs (Vassallo 1973, Barber and Blake 1985), and it obviously results in an increased level of PUFA in that organ. As is shown in Fig. 1c, an increase in the polyunsaturation of PL during the period of active gamete proliferation precedes the increase in the polyunsaturation of the TG fraction during the fall-winter period of egg growth. Changes in the proportion of the major phospholipids (i.e. phosphatidylcholine and phosphatidyl-

ethanolamine) may also occur during sexual maturation, and these changes in turn would affect the overall PL fatty acid composition.

The changes in the PUI of the digestive gland in scallops from Georges Bank represent the combined processes of intensive feeding and sexual maturation (Fig. 1b). The seasonal changes of the PUI of the digestive gland also demonstrate the importance of analyzing individual lipid classes. While it is not possible to detect net changes in the level of polyunsaturation of the total lipids in the digestive gland, the analyses of PL and TG separately showed (as well as in the case of the female gonad [Fig. 1c]) opposite and complementary trends (Fig. 1b). In contrast to the pattern of seasonal variation of PL polyunsaturation found in most of the scallop organs (Fig. 1a), the PUI of the digestive gland triacylglycerides (Fig. 1b) rose to a maximum during the fall and showed a sharp drop in winter. This pattern of change is related to the periods of high (spring to fall) and relatively low (winter) primary phytoplanktonic production on Georges Bank (Loder and Platt 1985). In comparison with the other organs, the digestive gland in scallops has a relatively low intrinsic phospholipid content. Therefore, the high PUI for TG observed during the period of active feeding (spring to fall) reflected accumulation of the fatty acids of the marine phytoplankton rich in polar lipids and highly unsaturated fatty acids. A subsequent very dramatic change in the TG of the digestive gland (Fig. 1b) is shown by the drop in the PUI from late summer and fall to winter. This decline in the concentration of PUFA (mainly 20:5n-3 and 22:6n-3) coincides in time with the well documented transport of TG reserves from the digestive gland to the TG of the developing female gonad (Vassallo 1973).

#### Temporal Variations of the Fatty Acids in the Digestive Gland

It is already known that the animal TG reflect the lipid composition of the diet through fatty acid input (Sargent and Whittle 1981). One objective of our work was to detect seasonal variations of the fatty acid compositions of scallop lipids, which could be related to the ingestion of different food items (eg. diatoms, autotrophic flagellates or bacteria). Although most of the different types of food available to filter feeders contain many of the same common fatty acids, their proportions vary substantially from one type of food to another. The abundant literature on the fatty acid composition of marine diatoms, microflagellates and bacteria

(Ackman et al. 1968, Chuecas and Riley 1969, Holz 1981, Claustre et al. 1989, Napolitano et al. 1990) validates this approach by demonstrating that each group of food organisms has its characteristic fatty acid profile.

Chang and co-workers (1989) have recently illustrated their conclusions from *P. yessoensis* that the digestive gland of bivalve molluscs contains large lipid deposits. These lipid stores are not in the form of adipose tissue, as is characteristic of vertebrates, but in the form of intracellular drops of oil contained in specialized tubular cells (see also Robinson et al. 1981 and Barber and Blake 1985). Accordingly, fatty acids of the digestive gland, and of the gut contents of *P. magellanicus* from Georges Bank, were analyzed separately to identify major changes in the quality of the food supply during the seasons. It should be stressed that the total lipid, rather than the TG fraction, was analyzed in this case (Table 5). The reason for doing so is that the bivalve digestive gland incorporates a large number of food particles by the process of phagocytosis (Chang et al. 1989). Therefore, it was suspected that intact phytoplanktonic cells (including their membrane lipids) and other food items would be found within this organ. The vegetable PL and glycolipid fatty acids would therefore contribute substantially to the fatty acid characterization of the food.

Seasonal differences in five out of eleven important fatty acids from the scallop digestive gland as shown in Table 5, were of interest. In three of these, the C<sub>16</sub> PUFA group, EPA, and DHA, changes could be interpreted as being at least partially due to differences in the type of ingested food. A very suggestive, but small, increment in the concentration of 18:4n-3 in the digestive gland and gut contents of scallops was detected in the fall samples; it could be related to an enrichment of the scallop diet in autotrophic flagellates since 18:4n-3 is a major component in dinoflagellates and prymnesiophytes (Holz 1981, Volkman et al. 1981, Napolitano et al. 1988b). Microflagellates are also involved in the production of a fall phytoplankton bloom. While a number of diatom species (e.g. *Thalassiosira nordenskioldii* and *Chaetoceros* spp.) form the bulk of the local phytoplanktonic biomass on Georges Bank during most of the year, the dinoflagellate *Prorocentrum micans* is the dominant type during the fall (Cura 1987). DHA (22:6n-3) is another fatty acid characteristic of this last algal group. The results reported here (Table 5) demonstrated marked increases in the proportion of 22:6n-3 towards the winter especially in the very high concentration of 22:6n-3 in the gut content of the winter sample.

The trends in the variations of C<sub>16</sub> PUFA and 20:5n-3 agree with changes of the dominant algal groups of the phytoplankton on Georges Bank inferred from variations in the concentration of 22:6n-3 and 18:4n-3. EPA and C<sub>16</sub> PUFA are very abundant in diatoms (e.g. Napolitano et al. 1990); they are less common in microflagellates. The maximum concentrations of both C<sub>16</sub> PUFA (mostly 16:4n-1) and 20:5n-3 occurred during spring, and were a minimum during the winter (Table 5). These observations agree with those of Hayashi and Kishimura (1991) for EPA in TG of the hepatopancreas of *P. yessoensis*. Our observations certainly reflect the importance of the spring algal bloom, when diatoms are known to be the dominant algal group (Cura 1987).

This association between total lipid fatty acids and algal types is still apparent, although to a lesser extent, if comparisons are based on the TG fatty acids of the digestive gland instead of the total lipids (Tables 1 to 4). The proposed effect of different planktonic algae on the lipid composition of the scallop digestive gland is consistent with the results obtained during the analyses of sam-

ples of gut contents (Table 5). The total lipid fatty acids in scallop gut contents, for key compounds such as 16:4n-1, EPA and DHA, showed a fatty acid profile and a seasonal pattern of change very similar to that of the total lipids of the digestive gland (Table 5).

Our digestive gland fatty acid data (summarized in Table 5) show that 16:1n-7 and 18:1n-7 are remarkably consistent over the four seasons of the year. Unfortunately 18:1n-7 is missing from the comparison of fatty acids from samples and organs of Chilean scallop *Argopecten purpuratus* held in the laboratory or in the ocean (Martinez et al. 1992). In the fatty acids of ocean samples of juveniles, 16:1n-7 was 2.6–3.2%, versus 9.2–9.6% in laboratory samples fed the likely sources for this fatty acid, *Chaetoceros calcitrans* and *C. gracilis*, both rich in 16:1n-7 (Volkman et al. 1989). However the recent emphasis on EPA and DHA may be concealing the importance of monoethylenic fatty acids of the n-7 family. These fatty acids are key precursor components of one series of the two NMID fatty acids (see above) as noted for *C. gigas* by Thompson and Harrison (1992). The NMID are seemingly ubiquitous mollusc components (e.g. Ackman and Hooper 1973, Zhukova 1986, 1991, Napolitano et al. 1988a, Jeong et al. 1990, Fang et al. 1993). As yet there is not specific function for bivalves confirmed for these fatty acids, but 16:1n-7 may be involved as an essential precursor. The questions generated by Table 5 data are whether 16:1n-7 is accumulated selectively or opportunistically, and whether the excess of 18:1n-7 over 18:1n-9 in bivalve lipids is important in this context of a special role for n-7 monoenoic fatty acids.

Both field (Shumway et al. 1987) and laboratory (Cranford and Grant 1990) work have indicated that *P. magellanicus* can feed efficiently on particles of varied sizes and composition. If we assume that the fatty acids of both the digestive gland proper and the gut content in scallops are intimately related to the diet of the animal, then it is possible to draw some conclusions regarding the main sources of food for the scallop population in Georges Bank. Certain fatty acids (i.e. C<sub>15</sub> and C<sub>17</sub> branched-chain fatty acids and 18:1n-7) are typically very abundant in marine bacteria. They have been successfully used to trace the bacterial biomass in marine food webs (Gillan and Johns 1986, Bradshaw et al. 1991, Sargent et al. 1987), but are not especially important in the scallop gut contents, nor do they exhibit a particular trend during the year. Conspicuous microbial activity has been reported on Georges Bank; however the maximum bacterial biomass is known to be produced in spring and summer, precisely when the algal food is most readily available (Hobbie et al. 1987).

This study has clearly demonstrated that the main seasonal variations in the fatty acids of the digestive gland and the gut content involved PUFA typically associated with major algal groups (diatoms and flagellates). Information on fatty acids of the digestive gland, and the presence of large quantities of lipid reserves all year round (Napolitano and Ackman 1992), indicates that there is a continuous supply of photosynthetically produced organic matter for the scallop population on Georges Bank.

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# INFLUENCE OF BIOFILM ON SETTLEMENT OF SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791), IN PASSAMAQUODDY BAY, NEW BRUNSWICK, CANADA

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**ABSTRACT** The microscale influence of a biofilm on substrate selection by sea scallop larvae, *Placopecten magellanicus*, was determined using artificial monofilament collectors in Passamaquoddy Bay, New Brunswick. Biofilm coverage was greatest on rough textured monofilament collectors which had been previously conditioned. Substrates with a high biofilm coverage had a significantly greater scallop settlement than those with less coverage. We concluded that no differential mortality or differential larval availability occurred among treatments and that sea scallop larvae actively selected substrates with a high biofilm coverage. As one method of culturing sea scallops relies on natural spat obtained from artificial collectors containing monofilament, our findings suggest that allowing a biofilm to develop on collectors, by deploying and conditioning them prior to spatfall, can enhance scallop settlement.

**KEY WORDS:** spat settlement, biofilm, sea scallop aquaculture

## INTRODUCTION

Settlement and metamorphosis of benthic marine invertebrates has been shown to be influenced by many variables including light, flow, pressure, larval behavior, and substratum (Crisp 1974). Settlement can be passive and controlled by hydrodynamic variables (Eckman 1987) or active when larvae selectively choose a desirable substrate (Keough and Downes 1982). Larval settlement behavior can be induced at a distance from the substratum (meters or centimeters) or from stimuli resulting from direct contact with the substrate (Keough and Downes 1982, Le Tourneux and Bourget 1988). Variables influencing larval selection of a substratum include physical (e.g. substrate texture or substrate contour), biological (e.g. hydrozoans or biofilms), and chemical cues (Crisp 1974, Tamburri et al. 1992).

The sea scallop, *Placopecten magellanicus* (Gmelin, 1791), supports a valuable commercial fishery which has been exploited for over 100 years in both Canada and the United States (Naidu 1991). It is also the basis of a fledging aquaculture industry in eastern Canada. The aquaculture industry currently relies upon natural seedstock (spat) obtained by using artificial collectors made of "onion bags" filled with monofilament gill netting (Dadswell and Parsons 1991). Gill netting is used as a substrate in scallop aquaculture because it is widely available, inexpensive, easy to handle, and long-lasting.

Several studies have described scallop settlement on both artificial and natural substrates (Merrill and Edwards 1976, Minchin 1992). However, few field studies have experimentally examined variables which influence substrate selection using specific statistical designs. Culliney (1974) reported that sea scallop larvae, in the laboratory, were capable of choosing several physical substrates to settle upon. Similarly, Hodgson and Bourne (1988) and Foighil et al. (1990) found that the spiny scallop, *Chlamys hastata* Sowerby, and the Japanese scallop, *Patinopecten yessoensis* Jay,

respectively, preferentially settled and metamorphosed on fouled surfaces. Experiments attempting to elucidate variables which influence settlement of scallops under field conditions have been hindered by confounding factors, including differences in collector design, larval availability, mortality, substrate surface area, color, and chemical composition (Naidu et al. 1981, Eckman 1987, Thorarinsdóttir 1991, Ambrose et al. 1992).

The aim of this experimental field study was to determine whether sea scallop settlement was active and if it was influenced by substrate biofilm. We used an experimental design with substrate biofilm coverage as the independent variable, spat settlement as the dependent variable and collector design, type and quantity of material, duration of time, and water depth as constants.

## MATERIALS AND METHODS

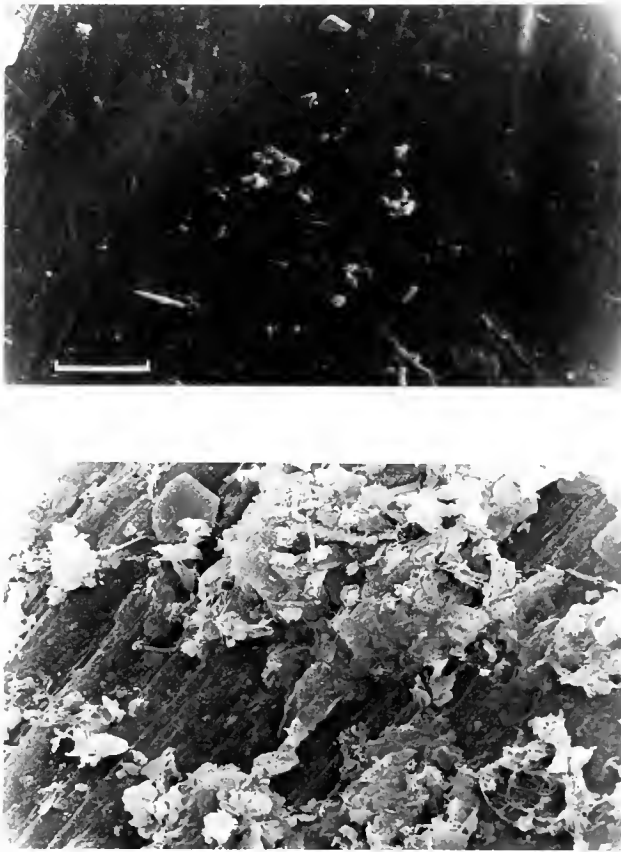
This study was conducted at an aquaculture site near Tongue Shoal, Passamaquoddy Bay, New Brunswick (45°04'N 67°01'W) during September 1988. The physical characteristics of this study site are reported by Dadswell and Parsons (1991) but briefly, Passamaquoddy Bay is a semi-enclosed bay with semidiurnal tides which have a maximum range of 8.3 m. Maximum current velocities were 25 cm · s<sup>-1</sup>, salinity was approximately 31 psu and bottom temperature 11°C during the late summer, at the aquaculture site.

Spat collectors were constructed of 500 g of light green nylon monofilament gill netting (twine size #14; 0.6 mm diameter) placed into 2 mm mesh Japanese onion bags (length × width = 75 × 35 cm). Collectors for all treatments were randomly attached, 75 cm apart, on a horizontal line by SCUBA divers. This horizontal line was 3 m off-bottom and perpendicular to the prevailing current.

We used a 2 × 2 factorial experimental design with substrate texture and substrate conditioning as the two factors. The two treatments for substrate texture were rough (R) and smooth (S). Smooth substrate was new monofilament while rough substrate

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**Figure 1.** A: SEM photograph of new (smooth) and unconditioned monofilament substrate. B: SEM photograph of old (rough) and conditioned monofilament substrate showing longitudinal grooves and biofilm (detritus, etc.). Scale bar = 10  $\mu\text{m}$ .

was 2 yr old monofilament which had been previously used for collection of sea scallop spat. The used monofilament had been rinsed and shaken clean in seawater and air dried for 4 mo, which removed all traces of detritus on the monofilament. The second factor was conditioning of the monofilament gill netting; the two treatments being whether the collectors were conditioned (C) or unconditioned (U). Rough and smooth monofilament collectors were conditioned, in the laboratory, by submerging the bags in 1 m diameter tanks (1 m deep); this allowed the development of a biofilm on the surface of the monofilament. The tanks were continuously supplied with seawater from Passamaquoddy Bay and held under ambient light conditions for 2 wk prior to deployment in the field. The seawater was initially passed through a sand filter

which removed particles  $\approx >50 \mu\text{m}$ ; thus no scallops settled onto the collectors while they were being conditioned. Twenty collectors, 5 replicates for each treatment (R/S  $\times$  C/U), were deployed September 14 during the period of peak sea scallop settlement (Dadswell and Parsons 1991).

The collectors were retrieved after 2 wk by SCUBA divers placing individual collectors into 253- $\mu\text{m}$ -mesh plankton nets. In the laboratory, the contents of each collector were emptied into a 100-L tub containing 30 L of filtered seawater, the monofilament was vigorously washed, and the contents filtered onto a 253- $\mu\text{m}$ -mesh screen. The contents were then examined and enumerated for scallop spat. A sample of 100 spat was measured, using an ocular micrometer, for each treatment. An initial and final sample of monofilament from each treatment were retained and preserved in 4% buffered formalin. The biofilm on the surface of these monofilament samples was photographed using a JOEL scanning electron microscope. The percent biofilm coverage was determined from ten replicates of a randomly selected sample for each treatment by using a digitizer to measure the areal proportion of biofilm coverage from the SEM photographs of the monofilament. Surface texture of the monofilament gill netting substrate was assessed according to the percentage of surface area containing a grooved or scratched appearance, by using a binocular microscope. The percent coverage data were arcsine transformed prior to statistical analysis. All statistical tests were performed using the SPSS statistical package and probability of type I error was set at 0.05.

## RESULTS

New monofilament gill netting had a clear, smooth surface whereas the used material was dull, rough and exhibited a series of longitudinal grooves and scratches along the filament (Fig. 1a & b). Monofilament gill netting that was conditioned had a significantly greater biofilm coverage (5 times more, Table 1) than unconditioned monofilament at the end of the test period (two-way ANOVA, Table 2). Likewise, rough substrates had greater biofilm coverage than smooth substrates (Table 1 and 2). However, there was a significant interaction between substrate conditioning and substrate texture.

A one-way ANOVA of biofilm coverage by the four treatments showed a significant difference ( $F_{3,16} = 70.06$ ,  $P < 0.001$ ), with rough conditioned collectors being significantly greater than smooth conditioned ones, which were significantly greater than rough and smooth unconditioned collectors (S-N-K test,  $P < 0.01$ ) (Table 1). There was no significant difference between the rough and smooth unconditioned collectors. The biofilm consisted of organic detritus, microalgae and bacteria (Fig. 1b).

The mean abundance of sea scallops in the collectors ranged

**TABLE 1.**

**Treatment type, percent coverage of biofilm, spat abundance, and shell height (mean and standard deviation, SD) of sea scallops settling on monofilament collectors deployed in Passamaquoddy Bay during September 1988.**

Treatment	N	Mean Percent Coverage	SD	Mean Number per Collector	SD	Mean Shell Height ( $\mu\text{m}$ )	SD
Rough & Conditioned	5	70.0 <sup>a</sup>	7.1	407.6 <sup>a</sup>	42.8	509.8 <sup>a</sup>	111.5
Smooth & Conditioned	5	49.1 <sup>b</sup>	10.5	335.4 <sup>b</sup>	50.2	508.2 <sup>a</sup>	129.2
Rough & Unconditioned	5	13.3 <sup>c</sup>	4.0	276.2 <sup>c</sup>	34.8	491.3 <sup>a</sup>	124.6
Smooth & Unconditioned	5	15.0 <sup>c</sup>	6.4	321.6 <sup>c</sup>	37.9	475.8 <sup>a</sup>	109.1

<sup>a,b,c</sup> Similar superscript letters denote no significant difference among treatment means for each variable: biofilm coverage, number of spat per collector, and shell height (S-N-K test,  $P < 0.01$ ).



TABLE 2.

Statistical summary of a two-way ANOVA of substrate conditioning and texture on biofilm coverage for monofilament gill net collectors deployed in Passamaquoddy Bay during September 1988.

Source of Variation	Sum of Squares	d.f.	F Value	P Value
Conditioning	1.28	1	166.93	0.001
Texture	0.08	1	9.94	0.006
Conditioning × Texture	0.10	1	12.89	0.002
Residual	0.12	16		

from 276 to 408 spat per collector (Table 1). Sea scallop settlement was significantly different among the treatments (ANOVA,  $F_{3,16} = 12.05$ ,  $P < 0.001$ ). Rough conditioned substrates (RC) had significantly greater sea scallop settlement than the smooth conditioned collectors (SC), which had greater settlement than the other two treatments (RU and SU) (S-N-K test,  $P < 0.01$ ) (Table 1). There was no significant difference between the rough and smooth unconditioned collectors.

Mean shell heights of spat ranged from 475.8 to 509.8  $\mu\text{m}$  and were not significantly different among treatments (ANOVA,  $F_{3,396} = 1.82$ ,  $P = 0.144$ , Table 1). The size frequency distribution of sea scallop spat for all treatment combinations (Fig. 2) were not significantly different (Kolmogorov-Smirnov test, Table 3).

#### DISCUSSION

A significant interaction was observed between substrate conditioning and texture with respect to biofilm coverage. An

ANOVA demonstrated that there was no significant difference in biofilm coverage for the unconditioned collectors (RU & SU) but there was a significant difference between the rough and smooth conditioned collectors (RC & SC). The surface of the rough conditioned collectors probably provided a better substrate for the fouling organisms compared to the smooth substrate resulting in an enhanced biofilm coverage. It was this difference in biofilm coverage between the conditioned substrates and no difference in the unconditioned ones that resulted in the significant interaction term.

Sea scallop settlement was significantly different among the treatments and settling intensity was reflected in the biofilm coverage, that is, scallop settlement was highest on collectors with the greatest biofilm coverage and lowest on collectors with a lesser coverage (Table 1). We hypothesize that behaviour mechanisms related to substrate surface are associated with larval sea scallop settlement. However, it first has to be demonstrated that there was no differential larval availability or differential mortality among the treatments. The size frequency distributions were not significantly different among treatments suggesting that sea scallop larvae were all from the same cohort. Further, all collectors were randomly assigned a position at the sampling site and all were within close proximity of each other ( $< 15$  m) within a well mixed water column, hence we suggest that the larval availability was spatially and temporally equal during this experiment. We also suggest that no differential mortality occurred among the treatments (see Keough and Downes 1982). The spat collectors were deployed in mid September, after the starfish settlement had occurred, thus no starfish (a potential predator of sea scallops) were found in any of the collectors. All collectors were of the same design and contained the same amount of monofilament per col-

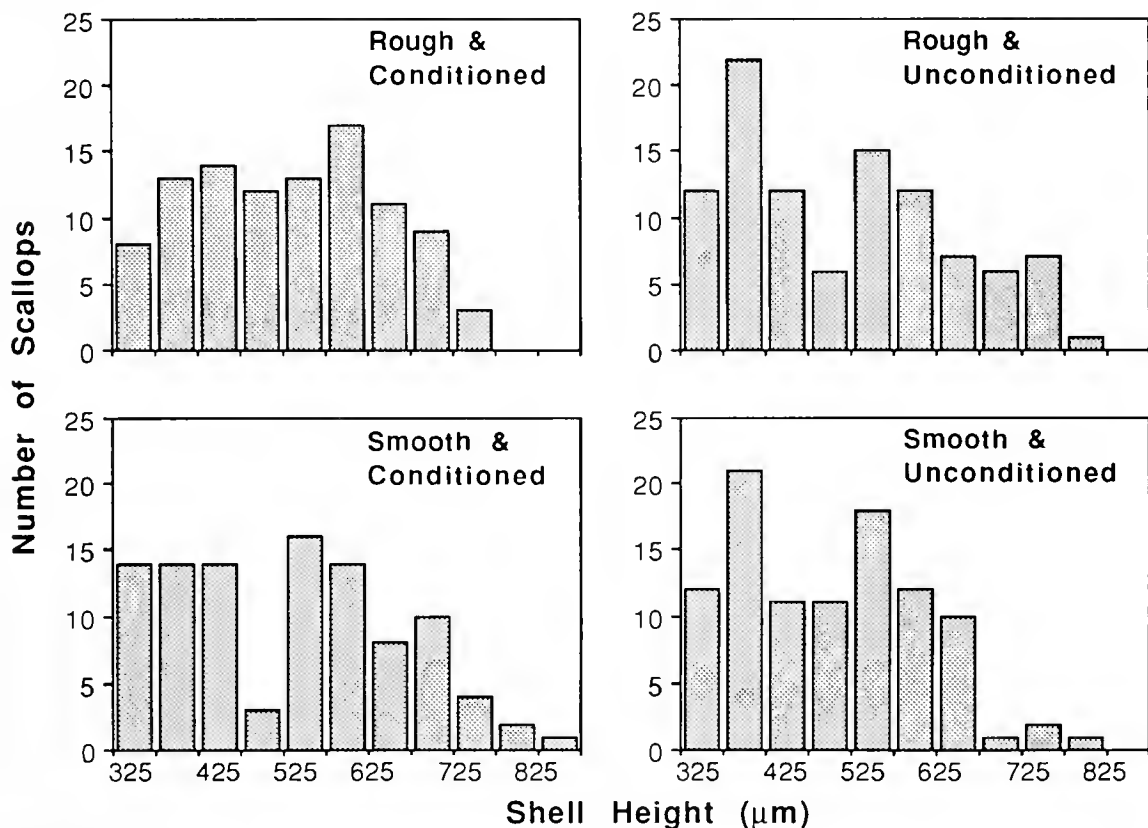


Figure 2. Size frequency histogram of sea scallop spat collected from Passamaquoddy Bay during September 1988 for each of the four treatments (N = 100 for each of the treatments).

TABLE 3.

Comparisons of size frequency distributions of sea scallops among all treatment combinations using the Kolmogorov-Smirnov test.

Combination	Z	
	Statistic	Probability
Rough Conditioned × Rough Unconditioned	0.919	0.367
Rough Conditioned × Smooth Conditioned	0.636	0.813
Rough Conditioned × Smooth Unconditioned	0.990	0.281
Rough Unconditioned × Smooth Conditioned	0.778	0.581
Rough Unconditioned × Smooth Unconditioned	0.778	0.581
Smooth Conditioned × Smooth Unconditioned	1.202	0.111

lector. Collectors, therefore, should have had the same water flow characteristics through the bags, hence having a similar amount of suspended food particles. Since scallops in all treatments exhibited similar growth rates (Table 1), we believe this condition was met. No dead scallop shells were found when the contents of the collectors were sorted and enumerated. Thus given that no differential larval availability or mortality occurred, we concluded that sea scallop settlement was active and larvae exhibited substrate selection in response to microscale heterogeneities in surface biofilm coverage. Larvae preferentially, although not exclusively, selected substrates with high biofilm coverage.

The presence of a biofilm, often consisting of bacteria, microalgae, and detritus (Hudon and Bourget 1981) has been shown to influence settlement in many species of benthic invertebrates (Tamburri et al. 1992, Tritar et al. 1992) but not all (e.g. tunicates, Crisp and Ryland 1960). Hodgson and Bourne (1988) have reported a preference for fouled surfaces in the spiny scallop *Chlamys hastata* and Foighil et al. (1990) found greater Japanese scallop (*Patinopecten yessoensis*) settlement on cultch which was fouled with diatoms compared to a clean substrate. The bay scallop, *Argopecten irradians* L., was induced to settle in response to a bacterial film and individual strains of bacteria could induce differing rates of settlement (Xu et al. 1991). However, bacterial films were not shown to influence settlement in the scallop *Pecten maximus* (L.) (Tritar et al. 1992).

Culliney (1974) describes sea scallop larvae as showing a general thigmotactic response. Since sea scallops are free-living benthic animals, we hypothesize that their requirements for substrate selection may not be as specific as sessile animals. Sea scallops settled on all monofilament treatments in this study, albeit

in higher numbers on the collectors with the greatest biofilm coverage. Monofilament gill net collectors are extensively used in the sea scallop aquaculture industry (Naidu et al. 1981, Dadswell and Parsons 1991, 1992). Sea scallops have also been reported to settle on a wide variety of substrates, both artificial and natural, including, sand grains, gravel, shell, filamentous hydrozoans and bryozoans, glass, buoys, red algae, and polyethylene sheets (Naidu 1970, Caddy 1972, Culliney 1974, Merrill and Edwards 1976, Larsen and Lee 1978, Naidu et al. 1981). The extent to which passive processes such as currents and substrate hydrodynamics influence the horizontal distribution or dispersal of scallop larvae over its pelagic development is uncertain, yet may be important (Robinson et al. 1992).

The influence of a biofilm on substrate selection by larval sea scallops has important implications for fisheries management and aquaculture practices. Knowledge of the recruitment processes of sea scallop includes an understanding of their requirements for settlement on natural substrates. Any determination of these requirements should consider the microscale heterogeneities ( $\approx 0.1$ – $1$  mm) of the physical and biological characteristics of the substrate. Aquaculture practices for the sea scallop in eastern Canada almost exclusively rely on natural spat supply obtained from artificial monofilament collectors. Site location, collector depth, quantity of monofilament, and starfish abundance have been reported to influence settlement intensity and survival (Naidu et al. 1981, Parsons et al. 1990, Dadswell and Parsons 1991, Robinson et al. 1992) and our findings suggest that by deploying and conditioning collectors prior to spatfall they will develop a biofilm on the monofilament which can increase scallop settlement. The abundance of monofilament gill netting in fishing communities and the use of what would otherwise be waste material would further reduce the equipment costs of conducting scallop aquaculture and aid in the recycling of waste monofilament.

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## SEASONAL AND DEPTH CHARACTERISTICS OF SCALLOP SPATFALL IN AN AUSTRALIAN SUBTROPICAL EMBAYMENT

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**ABSTRACT** Artificial collectors were placed in a subtropical embayment in Queensland, Australia, to study the settlement of scallop spat over a 16 month period. Five scallop species were collected: *Amusium japonicum balloti* (Bernardi), *Pecten fumatus* (Reeve), *Mimachlamys gloriosa* (Reeve), *Mimachlamys leopardus* (Reeve), and *Decapecten decapecten strangei* (Reeve). *M. gloriosa* and *P. fumatus* were the most abundant species recorded on collectors, with a mean of 272 (August 1991) and 502 (September 1990) spat per collector, respectively, during the sampling period in which spat settlement was most abundant. Few *A. j. balloti* spat were found on collectors despite the fact that *A. j. balloti* supports a major scallop fishery in the area. Settlement of *M. gloriosa*, *P. fumatus*, and *A. j. balloti* was greatest in the period winter to spring, while spat of *D. d. strangei* were most abundant during summer. Settlement of *M. leopardus* spat occurred throughout the collection period. There were no significant depth-related differences in spat settlement rates for any of the five scallop species. This study suggests conventional spat collectors are unsuitable for *A. j. balloti* spat, possibly as a consequence of the species' brief or non-existent byssal phase. Other scallop species which occur in Queensland waters, such as *P. fumatus*, may be more suitable for enhancement programs which rely on natural caught spat than *A. j. balloti*.

**KEY WORDS:** scallop spat settlement

### INTRODUCTION

The enhancement of scallop populations relies on hatchery reared spat or on spat from natural populations, collected using mesh bags placed in the water column. After a planktonic phase, most scallop larvae settle out of the water column and attach via a byssal thread onto suitable substrates for varying periods of time (Beninger and Le Penneec 1991). Some scallops remain attached to the substrate, while many species separate from their byssal thread to become free-living again. Mesh bag collectors take advantage of this byssal attachment phase. Planktonic scallop larvae which pass into the mesh bags may settle on and attach to the collector. Whilst attached, post larval scallops will continue to grow, becoming too large to escape from the mesh bags when they separate from their byssal thread. Scallops retained in the mesh bags may then be used in enhancement programs.

Most investigations into scallop enhancement have been based on commercially important temperate water species including *Pecten maximus* (L.) and *Chlamys operularis* L. (Brand et al. 1980) and *Pecten novaezealandiae* Reeve (Bull 1989). While scallop enhancement has been attempted in many countries (China, United Kingdom, New Zealand, United States), the most successful enhancement program has been in Japan, based on *Patinopecten yessoensis* (Jay). Spat of *P. yessoensis* are obtained from artificial hanging collectors placed at selected sites within bays or the open sea. The scallop spat are then either reseeded into beds on the sea floor or are on-grown in hanging cases (Ito and Byakuno 1989). In Australia, the most extensive investigation of scallop enhancement has occurred on the temperate water species *Pecten fumatus* (Hortle and Cropp 1987, Cropp 1989). The enhancement program of *P. fumatus* again relies on the collection of spat from wild populations of scallops. Studies on tropical and subtropical scallop species have suggested that the enhancement of *Argopecten circularis* (Sowerby) and *Chlamys nobilis* (Reeve) may be possible using artificial collecting techniques (Reyes 1986, Lou 1991).

Most interest in the enhancement of scallop populations in

Queensland has centered around the Queensland saucer scallop, *Amusium japonicum balloti*. This species is commercially fished in waters between 18° and 26°S with annual catches varying between 250 and 1500 tonnes of meat (Dredge 1988). These fluctuating catch rates and a perceived depletion of natural stocks by the commercial fishing industry (Dredge 1988), are the main reasons for interest in the enhancement of this species. *A. j. balloti* have been reported to grow to a shell height of 75 mm within 30 wks of settlement (Williams and Dredge 1981). This rapid growth rate, together with a relatively high meat value, makes *A. j. balloti* a potentially attractive species for enhancement.

The successful enhancement of scallop population relies on detailed knowledge of the reproductive biology and spat settlement patterns of the species. Placement of spat collectors, both spatially and temporarily, is a crucial factor in obtaining high spatfalls (Ambrose et al. 1992). In Queensland waters, operational factors constrained the location of spat collectors. Possible sites where collectors could successfully be deployed and then retrieved were limited by exposure to strong wind and sea fetch conditions, the existence of an active trawl fishery, and accessibility for collector maintenance. The majority of work on *A. j. balloti* has concentrated on its reproductive biology (Dredge 1981, Heald and Caputi 1981, Rose et al. 1988) and stock assessment (Dredge 1988). Sumpton et al. (1990) completed a preliminary study on the suitability of artificial collectors for retaining subtropical scallop species in Queensland. The study was conducted over a 6 mo period and was unable to demonstrate any clear seasonal patterns of settlement. Variations in settlement rates as a function of depth were recorded, but sample sizes were too small to allow statistical comparisons.

The present study extended this previous work by examining scallop spat settlement over a 16 mo period and quantified the pattern of scallop settlement with depth. The artificial collectors used were similar in construction to previous work, but had a smaller external mesh. Also, the suitability or otherwise of subtropical Queensland scallops as candidates for enhancement using wild caught spat, was examined in this study.

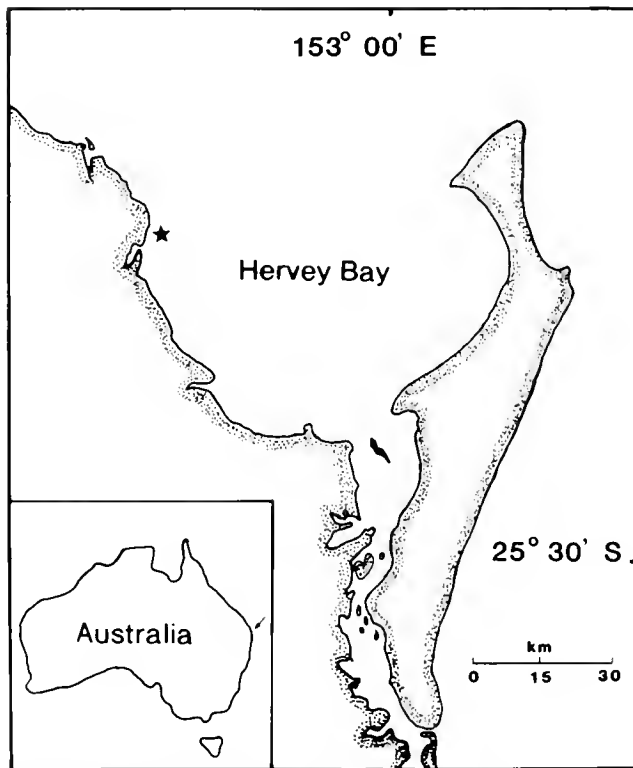


Figure 1. Hervey Bay, Australia, showing spat collector site ( $24^{\circ}53.8'S$ ,  $152^{\circ}31.0'E$ ), 2.8 km off the coastline.

#### MATERIALS AND METHODS

Artificial collectors were made from 2 mm mesh "minnow netting", sewn into bags 25 cm  $\times$  25 cm. The mesh bags were loosely filled with 3 polypropylene onion bags, with a mesh size of 5 mm. Six spat bags were attached individually at 2 m intervals along a buoyed and anchored rope. The first spat bag was attached 2 m from the bottom of the rope. Each set of collectors consisted of two such ropes, giving 2 replicate spat bags at each depth.

The collectors were deployed in Hervey Bay ( $24^{\circ}53.8'S$ ,  $152^{\circ}31.0'E$ ), 2.8 km off the coast, in a water depth of 14 m (Fig. 1). One set of collectors was deployed each month between July 1990 and August 1991. A total of 14 sets of collectors were deployed during the study. Each set of collectors remained at sea for approximately 2 mo before being retrieved. Thus, each set of collectors overlapped with the previous and following set by approximately 1 mo. Spat bags were frozen on retrieval until in-

spected in the laboratory. Spat were removed by washing the mesh bags over a 500  $\mu$ m sieve. The mesh bags were then inspected for any remaining scallop spat under a binocular microscope. After identification, the shell height of all scallop spat was measured to the nearest 0.1 mm using an eyepiece micrometer. Identification was based on Iredale (1939), Woodburn (1989) and Sumpton et al. (1990).

#### RESULTS

Over 12,000 scallop spat were collected during the study, and 5 species of Pectinidae were identified. Species collected included, *Amusium japonicum balloti*, *Pecten fumatus*, *Mimachlamys gloriosa*, *Mimachlamys leopardus*, and *Decapecten decapecten strangei*. Numerous other species were found in the collectors besides scallop spat. These included mussels, *Pinna fucata* (Gmelin) and *Pinna bicolor* (Gould), ascidians, polychaete worms, sponges and filamentous algae. The sets of collectors were deployed for a mean of 65.2 days, with a range of 54 to 79 days. Variation in duration of collector deployment was due to the difficulties in accessing the spat bags, normally as a consequence of bad weather. Most sets of collectors had some missing bags due to wind and wave action. Contents from missing samples were estimated using least squares regression (Young et al. 1992).

##### Spat Settlement with Depth

Settlement of spat occurred at all depths sampled (Table 1). Spat settlement as a function of depth and time was compared using a two way analysis of variance after data was transformed to natural logarithms. Month of collector retrieval had a significant effect on spat settlement for all species, but the effect of depth was not significant for any species (Table 2). Only *M. gloriosa* showed a significant month  $\times$  depth interaction ( $P < 0.01$ ).

##### Spat Settlement with Season

There was significant variation in the temporal distribution of spat numbers for all species of scallop collected (Table 2). A total of 34 *A. j. balloti* spat were collected during the study. Approximately 90% of these spat were found in collectors deployed during winter and early spring (Fig. 2). *A. j. balloti* spat found on collectors during winter and spring had shell heights ranging from 2.5 to 15.5 mm (Fig. 3).

Spat of *P. fumatus* were most abundant on collectors during winter and early spring (Fig. 2), with a mean of 502 spat per collector in September 1990, pooled across depths and replicates. *P. fumatus* spat were much less numerous on collectors retrieved

TABLE 1.

Proportion of scallop spat recorded on artificial collectors as a function of the collector distance from the sea floor, (pooled across sampling periods, n = number of spat collectors).

Species	(Total Nos. Collected)	Distance from the Sea Floor					
		2 m (n = 15)	4 m (n = 17)	6 m (n = 18)	8 m (n = 20)	10 m (n = 20)	12 m (n = 18)
<i>Amusium j. balloti</i>	(34)	0.04	0.08	0.08	0.12	0.28	0.40
<i>Pecten fumatus</i>	(4666)	0.10	0.20	0.19	0.19	0.16	0.15
<i>Mimachlamys gloriosa</i>	(8863)	0.15	0.17	0.20	0.14	0.14	0.20
<i>Mimachlamys leopardus</i>	(392)	0.15	0.26	0.22	0.14	0.13	0.10
<i>Decapecten d. strangei</i>	(339)	0.08	0.28	0.16	0.22	0.14	0.12

TABLE 2.

Analysis of variance for scallop spat settlement between months and depth: using 2 replicates per depth (Ln abundance per bag).

Significance of factors and interactions terms are indicated as: \*P > 0.05, \*P < 0.05, \*\*P < 0.01. Missing samples were estimated by least squares regression prior to analysis.

<i>Amusium j. ballati</i>			
Source	df <sup>1</sup>	Mean Squares	F
month	8	0.464	3.11**
depth	1	0.042	0.28 <sup>ns</sup>
month × depth	55	0.045	0.30 <sup>ns</sup>
residual	35	0.149	
<i>Pecten fumatus</i>			
Source	df <sup>1</sup>	Mean Squares	F
month	8	32.285	82.52**
depth	1	0.025	0.06 <sup>ns</sup>
month × depth	55	0.266	0.68 <sup>ns</sup>
residual	35	0.391	
<i>Mimachlamys gloriosa</i>			
Source	df <sup>1</sup>	Mean Squares	F
month	8	21.151	153.35**
depth	1	0.262	1.90 <sup>ns</sup>
month × depth	55	0.328	2.38**
residual	35	0.391	
<i>Mimachlamys leopardus</i>			
Source	df <sup>1</sup>	Mean Squares	F
month	8	3.081	5.89**
depth	1	0.556	1.06 <sup>ns</sup>
month × depth	55	0.576	1.10 <sup>ns</sup>
residual	35	0.523	
<i>Decapecten d. strangei</i>			
Source	df <sup>1</sup>	Mean Squares	F
month	8	2.413	6.02**
depth	1	0.103	0.26 <sup>ns</sup>
month × depth	55	0.241	0.60 <sup>ns</sup>
residual	35	0.400	

<sup>1</sup> Missing sample values reduce the degrees of freedom of the ANOVA. Residual df indicate that the test is still valid (residual df > 30).

from November 1990 to July 1991, with fewer than 5 spat per collector being recorded. On average, shell heights of *P. fumatus* which settled during samples with highest spat abundance (September, October 1990) were mostly small (mean 5.3, 6.2 mm, pooled across depths and replicates). Samples in 1991 with low spat abundance also had small shell heights (June, July 1991). 1991 samples which had high levels of spat settlement had larger sized spat, with mean shell heights of 9.0 mm (August 1991), 7.6 mm (September 1991) and 8.1 mm (October 1991) (Fig. 3).

*M. gloriosa* was the most abundant and most frequently collected scallop species. Peak settlement of *M. gloriosa* occurred on collectors retrieved during October 1990 and August 1991, although spat were common on all collectors retrieved during winter and spring (Fig. 2). Settlement of *M. gloriosa* occurred throughout the remainder of the year, however, numbers of spat collected were much lower, usually less than 20 spat per collector. *M. gloriosa* spat were small in sample retrieved in October 1990 and August 1991, with a mean shell height of 3.6 and 2.7 mm, re-

spectively (data pooled for each sample across depths and replicates, Fig. 3). Following these peak settlement periods, mean spat size generally increased to between 8 and 10 mm shell height.

*M. leopardus* was less abundant on collectors, with a total of 392 individuals being recorded during the study. Maximum settlement of *M. leopardus* spat occurred on collectors during May 1991, however, small numbers of *M. leopardus* spat were recorded throughout the year (Fig. 2). Mean shell height of *M. leopardus* varied between 2.2 and 7.0 mm (Fig. 3). Spat of *D. d. strangei* were most abundant in April 1991, with a mean of 18 spat per collector. Spat were considerably less abundant during the remainder of the year, averaging fewer than 5 spat per collector (Fig. 2).

## DISCUSSION

The tropical scallop *A. j. ballati* has characteristics such as rapid growth and high meat value (Hart in press) which make this species an attractive enhancement candidate. The previous study on the collection of *A. j. ballati* spat deployed 3 sets of collectors between August 1987 and January 1988 (Sumpton et al. 1990). Few *A. j. ballati* spat were recorded on collectors. The present study extended the deployment of collectors over a 16 mo period between July 1990 and October 1991 and also reduced the size of the outside mesh of the artificial collectors from 5 to 2 mm to increase the likelihood of retaining *A. j. ballati* spat. Relatively few *A. j. ballati* spat were recorded in the artificial collectors. Ninety percent of *A. j. ballati* spat were collected during winter months, with a small number also collected in early spring (Fig. 2). These settlement periods coincide with the June to October spawning season of *A. j. ballati* (Dredge 1981). The smaller spat size in winter (Fig. 2) and a known planktonic phase of 22 to 28 days (Rose et al. 1988) suggests that spawning started prior to the July settlement (or that increasing sea temperatures resulted in faster growth). Rose et al. (1988) found that newly settled *A. j. ballati* spat actively crawled over substrates without appearing to attach permanently. The absence of a byssal stage may account for the lack of *A. j. ballati* spat trapped within the collectors. If so, and as spat collecting relies on the byssal attachment of scallops to the collector, it appears that the use of conventional, artificial collectors is unsuitable for *A. j. ballati* spat. Alternatively, the spat collectors may have been deployed in a location of limited spatfall, even though a major fishery for *A. j. ballati* occurs in the area.

Spat settlement is probably determined by currents and tidal movements and can vary from year to year (Heald and Caputi 1981). Other studies suggest that scallop spatfall occur in proximity to adult beds (Ambrose et al. 1992, Joll 1987). Commercial scallopers have since reported significant catches of *A. j. ballati* in 1992 in the vicinity of where the spat collectors were located. Unless a more effective spat collector could be designed and a feasible site with a high spatfall could be located, any program to supplement natural stocks of *A. j. ballati* in Queensland would have to rely on hatchery reared spat. Hatchery techniques for *A. j. ballati* spat are still in the experimental culturing phase (D. Cropp, in press).

While artificial collectors were not successful at retaining useful numbers of *A. j. ballati* spat, other pectinid species were caught in substantial numbers and may have potential in enhancement programs. *P. fumatus* (= *Pecten alba* Tate) spat were abundant during late winter and early spring. This coincides with spat settlement periods reported for *P. fumatus* (Hortle and Cropp 1987, Sause et al. 1987). Spat of *P. fumatus* were more abundant

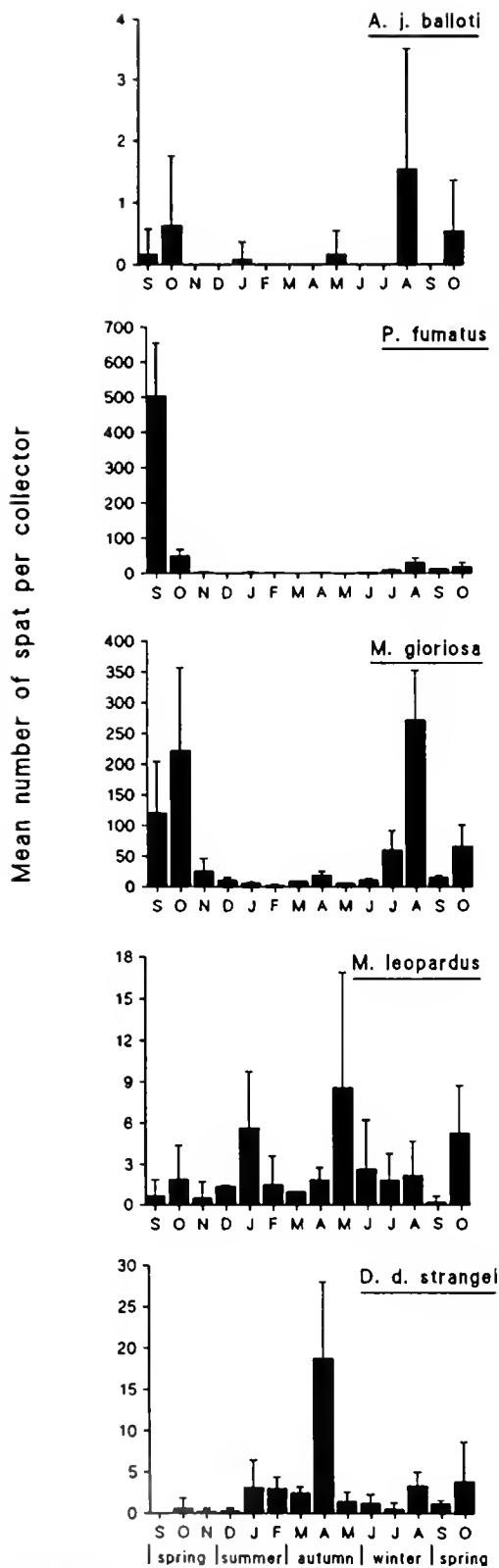


Figure 2. Mean number of scallop spat ( $\pm$ SD) on artificial collectors, pooled across depth and replicates for each sampling period (month retrieved), set in Hervey Bay, between July 1990 and August 1991.

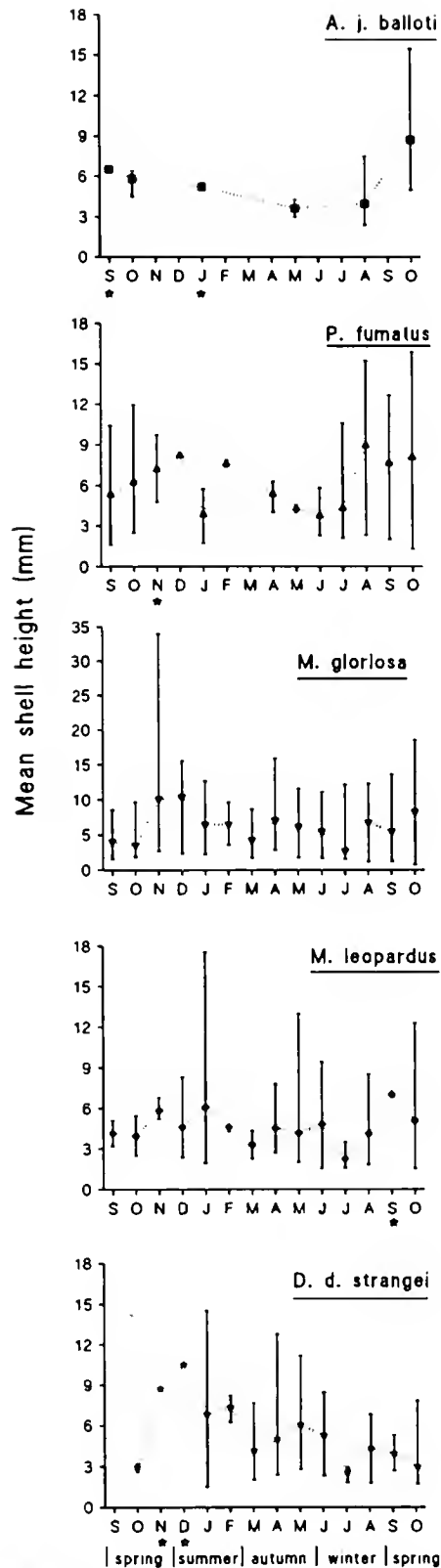


Figure 3. Mean shell height and range of scallop spat settled on artificial collectors set in Hervey Bay, pooled across depth and replicates for each sampling period (month retrieved), ★ indicates n = 1.



in 1990 than in 1991 (Fig. 2). Sumpton et al. (1990) reported very few *P. fumatus* on collectors deployed during winter and early spring in 1987. These year to year variations in observed spat settlement may be due to differences in collector location, time at sea, or the occurrence of a strong spawning pulse during 1990. *P. fumatus* has been reported as having major fluctuations in spat settlement between locations and years in temperate Australian waters (Jacobs 1983, Sause et al. 1987). Spat collection programs in Tasmania and Victoria reported that settlement and growth of *P. fumatus* spat varied with depth, the greatest spat settlement occurring on collectors placed at midwater (Hortle and Cropp 1987, Sause et al. 1987). More recent studies also suggest that *P. fumatus* spat abundance was positively correlated with depth, with more spat being present on the deep water collectors deployed at the beginning of the settlement season (Young et al. 1992). In the present study, spat settlement was only marginally greater in mid-water collectors than on those near the surface (Table 1) and such differences were not statistically significant for *P. fumatus*. Variation in spatfall of *P. fumatus* as a function of depth may possibly be explained through more directed, laboratory based studies. Results from the present study give no indication why such variation should occur.

*M. gloriosa* was the most commonly collected species, having major spat settlement during winter and early spring. This species was also common during the study by Sumpton et al. (1990), averaging 599 spat per collector during early spring. While this species had the best spatfall and grew quickly in the collectors to a shell height of 34 mm, very little is known about the biology of this species, including its maximum shell height. At present, *M. gloriosa* is not commercially fished anywhere in Australia, generally being too small to attract commercial interest. Before *M. gloriosa* could be considered as an enhancement candidate, a substantial amount of research into its growth, reproductive and population biology would be necessary.

*M. leopardus* and *D. d. strangei* were less numerous in collectors during 1990 and 1991 than reported by Sumpton et al. (1990). *D. d. strangei* was the only scallop species recorded which had a peak spat settlement period during autumn months (Fig. 2). Spat of *M. leopardus* did not show a distinctive seasonal settle-

ment pattern, but settled in collectors throughout the study. This is consistent with the suggestion of Sumpton et al. (1990) that spawning and settlement of spat may occur over a prolonged period for *M. leopardus*.

#### Suitability of scallop enhancement in Queensland

A successful enhancement program is based on a cheap and abundant supply of scallop spat. Limiting factors to the enhancement of *A. j. balloti* in Queensland include the lack of suitable spat collecting sites and the uncertainty of the ability of artificial collectors to retain *A. j. balloti* spat, given the poor collection rates reported to date, and the possibility that this species has a short or nonexistent byssal phase. Current hatchery work on *A. j. balloti* may provide an alternate means of obtaining large numbers of spat for an enhancement program. Several other pectinid species besides *A. j. balloti* settled on the artificial collectors, including *P. fumatus*. This species is already the subject of an enhancement project in southern Australia (Cropp 1989). Anecdotal comments from commercial fishers suggest that *P. fumatus* is abundant on trawl grounds in the study area during certain times of year, but this species is not commercially fished in Queensland. Given the presence of *P. fumatus* in the area, the present study suggests that *P. fumatus* may be a possible candidate for enhancement in subtropical Queensland waters. *M. leopardus* is also a possible candidate for enhancement as it has a history of being intermittently fished in Queensland. The relatively low numbers of *M. leopardus* spat collected suggest that other more abundant species would be better enhancement candidates. *M. gloriosa* was abundant in spat collectors, but the lack of knowledge about its biology and market potential precludes this species from consideration as a potential enhancement candidate until further research is completed.

#### ACKNOWLEDGMENTS

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## GROWTH OF THE CARIBBEAN SCALLOP *ARGOPECTEN NUCLEUS* (BORN 1780) IN SUSPENDED CULTURE

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**ABSTRACT** We quantified the growth of juvenile *Argopecten nucleus* (10 mm in shell length and height and probably 1–1.5 months old) suspended at 15–20 m in depth at Turpialito in the Golfo de Cariaco on 24 January 1990. Shell dimensions increased rapidly during the first months and near maximum size (45–50 mm) was attained in 6–7 months. In contrast, the mass of the shell, muscle and remaining somatic tissues increased slowly until late June, doubled in July and then showed little change thereafter. The rapid growth in July probably did not reflect changes in food availability but was possibly due to a temperature increase associated with decreased upwelling. The scallops began to reproduce in June, at ≈38–40 mm in shell length (≈6 months in age), and then reproduction continued until late September. Survival was high (90–96%) until late August, dropped exponentially during September and October and few individuals survived until November. Gamete production stopped in October. These observations suggest a life span of 8–10 months. This is the first report of the biology of *A. nucleus* and indicates that this scallop could furnish 3.53 g of muscle after 6–7 months in suspended culture. Sale of the whole animal should be possible, because of the favourable quality of various tissues and the attractive and robust shells.

**KEY WORDS:** *Argopecten nucleus*, caribbean scallops, growth, survival, aquaculture

### INTRODUCTION

Most scallops belong to the family Pectinidae which contains ≈400 living species of which ≈33 are of current or potential commercial interest (Brand 1991, Waller 1991). Scallop aquaculture is highly developed in Japan, where 250,000 tons of scallops are produced annually, and such techniques are now being introduced into the United States, Canada, Australia, China and other countries (Dore 1991). Associated with this commercial interest, the literature on the biology and ecology of scallops has expanded rapidly (Shumway 1991). However, few studies have examined the growth of tropical species, even though their growth is likely to be superior to that of temperate species. In the Caribbean region, the most well studied scallop is *Euvola (Pecten) ziczac* (Wilkens and Ache 1977, Wilkens 1981, Vélez and Lodeiros 1990, Vélez et al. 1987, 1990, Lodeiros et al. 1991, Freitas et al. 1993, Lodeiros and Himmelman, 1993) and consideration has also been given to *Lyropecten nodosus* in northeastern Venezuela (A. Vélez, personal communication).

The present study examines the growth of the Caribbean scallop *Argopecten nucleus*, a species which is rare in natural benthic habitats, but which readily recruits on to suspended structures. We quantify the rate of increase of the shell and major body components of juveniles placed in suspended culture in the Golfo de Cariaco, Venezuela. *Argopecten nucleus* is a functional hermaphroditic species having two sturdy and coloured convex valves. Its geographic distribution extends from southeastern Florida to the

Caribbean coasts of Colombia and Venezuela (Waller 1969). This is the first report on its biology.

### METHODS

Our growth studies were conducted from January to November 1990 at Turpialito in the Golfo de Cariaco, Estado Sucre, eastern Venezuela (Fig. 1). They were initiated using juvenile *Argopecten nucleus* with a mean shell length of 10.02 mm (SD 1.46), which had settled during the previous two months on pearl nets on which the scallop *Euvola ziczac* was being cultured (the pearl nets had been placed in the water on 17 November 1989). The scallops were transferred to other pearl nets (6 mm mesh) and suspended from a long line at 15–20 m in depth. We decreased the density of the scallops as their size increased following the recommendations of Ventilla (1982). Thus, they were set out on 24 January 1990 at a density of 320 individuals per pearl net, the density was reduced to 100 individuals per net on 25 February, to 33 individuals per net on 25 June and to 15 individuals per net on 25 September. During each reduction in density, we determined the proportion of living scallops and cleaned fouling organisms from the shells of the living scallops. The scallops were returned to the sea in new pearl nets.

At about monthly intervals, 15–20 living scallops were selected at random from the pearl nets for determinations of shell dimensions (height, length and width, as defined by Seed 1980) and of the dry mass of the shell, muscle, gonad and other tissues (drying at 70°C ≈2 d). Further, so that the organic content of the shell could be calculated, we determined the mass of the shell after ashing at 475°C for 6 h. The mass of the gonad as a percentage of the mass of the somatic tissues was used as a gonadal index.

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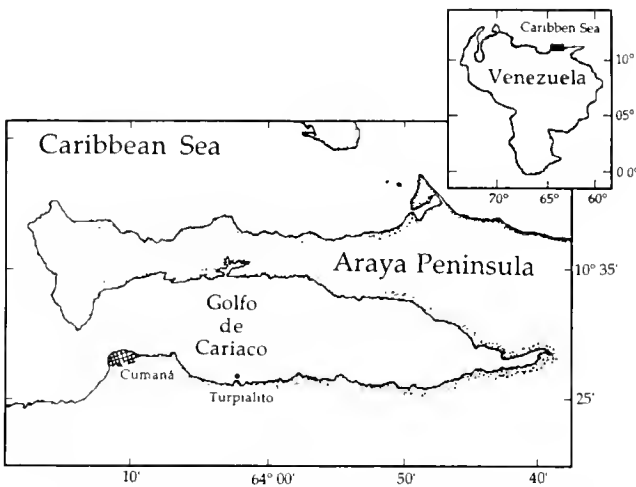


Figure 1. The Golfo de Cariaco, northeastern Venezuela, showing the location of study (●).

## RESULTS

### Shell Growth

Throughout the study, the rates of increase for shell length and height were virtually identical. Width increased at a slower rate but nevertheless showed accelerated growth during the same periods (Fig. 2A). Growth was most rapid between 24 January and 29 March 1990 ( $\approx 9.0$  mm of length or height and 4.5 mm of width per month), intermediate between 29 March and 27 July ( $\approx 4.5$  mm for the length and height and 2.5 mm of width per month) and slowest between 24 July and 25 October ( $< 2$  mm of length and height and  $< 1.5$  mm of width per month). The sigmoidal growth pattern can be described as follows:

$$\text{Length} = -42.5 + 36.4 \log \text{days} \quad (r = 0.99; P < 0.001).$$

The dry mass and organic content of the shell showed a slow and progressive increase from 24 January until 25 June, a two fold increase during July and then no significant change during the subsequent months (ANOVA, Sheffe F-test,  $P > 0.1$ ) (Fig. 2B).

### Growth of Somatic Tissues

The pattern of increase of the muscle and other somatic tissues closely followed that of shell dry mass ( $r^2 = 0.86$  for the muscle,  $P < 0.001$ ;  $r^2 = 0.94$  for the remaining tissues,  $P < 0.001$ ). There was a slow increase during the beginning of the experiment, a sharp increase during July 1990, and little change during the remaining months (Fig. 2C).

### Gonadal Development

The scallops were immature when they were first set out on 25 February 1990, with 40% of the individuals not having clearly defined gonads. One month later (23 March) all individuals had well-defined gonads and the mean gonadal index was 14.6% (Fig. 3). In May 1990, the mean index fell to 9.9%; however, this was entirely due to an increase in the muscle and remaining tissues which constituted the denominator of the index. The absolute mass of the gonads did not change (Fig. 2C). On 25 June 1990, the mean index was similar to that in May, however, the variation in gonadal size had increased greatly (Fig. 2C, 3). Three individuals (measuring 38–40 mm in shell length) with gonadal indices of  $< 10\%$  had spawned gonads (the gonadal tissues were watery and

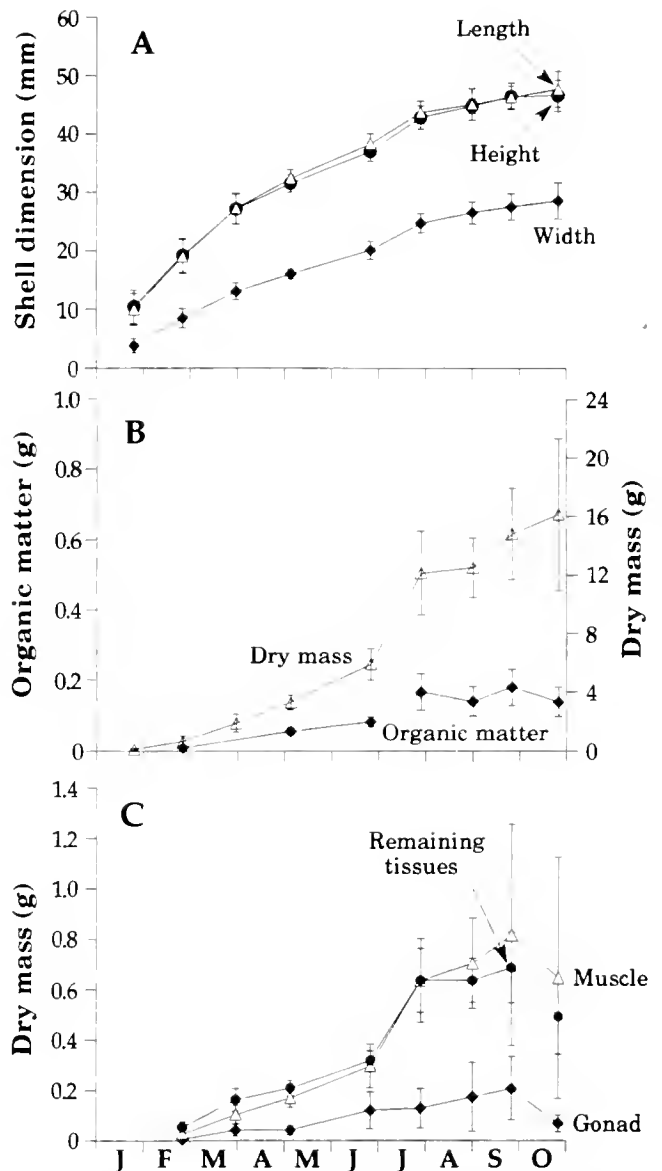


Figure 2. *Argopecten nucleus*. Seasonal changes in mean shell length, height and width (A), dry mass and organic content per individual of the shell (B), and dry mass of the muscle and other somatic tissues, as well as the gonad (C) for juveniles placed in suspended culture in 24 January 1990. The vertical bars represent 95% confidence intervals.

translucent in colour) and five others had gonads bulging with gametes and indices of  $> 18\%$ . The gonads also varied greatly in size and condition during July, August and September. Finally, in October, as all individuals had small gonads containing only traces of gametes, reproductive activities seemed to have stopped. Thus, the scallops began spawning in June, when they attained 38–40 mm in shell length. This corresponded to an age of about 6 months (assuming the scallops were 1 month old on 25 January). Reproduction seemed to be continuous in the following three months, although not synchronous amongst individuals, and then in October gamete production apparently terminated.

### Survival

Monthly survival was high (90–98%) during the first 7 months of the study (January 24 to August 30) and then dropped precip-

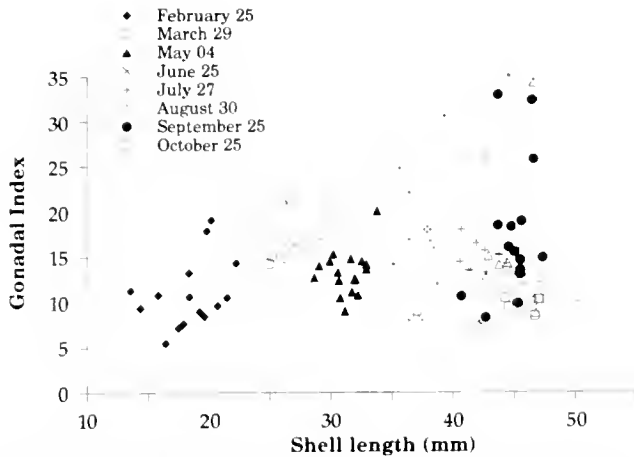


Figure 3. *Argopecten nucleus*. Relationship of the gonadal index to shell length. The data for different sampling dates are identified separately.

itously during September and October (70% and 39%, respectively). Observations of the five pearl nets which remained in November 24 indicated that only 7% of the individuals were alive.

#### DISCUSSION

Shell dimensions of *Argopecten nucleus* increase at a rapid rate during the first months in suspended culture and then at a progressively slower rate until the sixth or seventh month when they change little. By contrast, the mass of the shell, the muscle and other somatic tissues shows a different pattern. Growth is slow during the first 5 months in suspended culture, increases sharply during the sixth month and then is slow thereafter. Accelerated growth in shell dimensions preceding major increases in the mass of the shell and somatic tissues is also characteristic of numerous other bivalves (Sundet and Vahl 1982, Hilbish 1986, Borrero and Hilbish 1988, Côté et al. submitted, Lodeiros and Himmelman, 1993).

Gonadal development in *Argopecten nucleus* follows still another pattern. The major increase in gonadal mass occurs during the fourth and fifth month in suspended culture (May and June), thus preceding the sharp increase in the mass of the shell and somatic tissues in the sixth month (Fig. 2). The highly variable size and condition of the gonads from the fifth to the eighth month (June through September), as shell length increases from 38 to 48 mm, suggests that reproduction is continuous during this period: a proportion of individuals are spawning while others are recovering or producing gametes. Such continuous reproduction, once attaining maturity, is the classic pattern for invertebrates (Giese and Pearce 1974, Sastry 1979). However, it contrasts with most temperate molluscs where gonadal growth and spawning is relatively synchronous amongst individuals in any given population (Rand 1973, Mackie 1982). It also contrasts with the scallop *Euvola ziczac* in the Golfo de Cariaco where two synchronous spawnings occur each year (Brea 1986, Vélez et al. 1987, Lodeiros and Himmelman 1993).

Although environmental conditions were not monitored during our study, growth and mortality events can be compared to the general seasonal pattern in the Golfo de Cariaco (Moigis 1986, Ferraz-Reyes 1987, Okuda et al. 1978, Okuda 1981, Lodeiros

and Himmelman 1993). From about July or August until December or January, the water column is stratified with surface temperatures of 27–29°C. Then wind-driven upwelling begins and causes temperature to decrease to below 25°C. This continues with varying degrees of intensity until July or August. Primary productivity increases several-fold during the upwelling period (January to July). It is unlikely that the sudden doubling of tissue mass of *Argopecten nucleus* in July was caused by an increase in phytoplankton availability since July is near the end of the 4–5 month period of high primary productivity. On the other hand, it possibly coincided with an increase in temperature related to decreased upwelling, since this often occurs in July. Surprisingly, oceanographic conditions seem to have little effect on the reproduction of *A. nucleus*, since spawning was already taking place in June, when upwelling was likely occurring, and continued well into the period of strong thermal stratification. This contrasts with most invertebrates where temperature and food abundance strongly affect reproductive activity. For example, gonadal production in *Euvola ziczac* in the Golfo de Cariaco coincides with periods of low temperatures and increased food abundance and appears to end shortly after the water column becomes thermally stratified (Lodeiros and Himmelman 1993). These two species exemplify the divergence in reproductive strategies in tropical species.

Several observations indicate that adult *Argopecten nucleus* live for 8 to 10 months. Thus, mortality was low from January through August but increased sharply in September, October and November. If the thermal stratification of the water column in 1990 took place in July, it probably did not cause the mortality observed in September. However, it is possible that the stratification of the water column was delayed until late August or September in 1990, in which case it would have coincided with the onset of high mortality. One might predict that thermal stress would lead to a decrease in the size of the gonad, muscle and other tissues but no decrease was observed during August and September. A decrease in these tissues is suggested in October (Fig. 2C), but too late to be coupled with the onset of thermal stratification of the water column and associated reduced food abundance. Finally, we can virtually exclude the hypothesis that mortalities were caused by fouling since the scallops and pearl nets were cleaned regularly. Further, few organisms colonized the shells of *Argopecten nucleus*. In this respect *A. nucleus* differs from most other scallops (Duggan 1973, Wallace and Reinsnes 1985, MacDonald and Bourne 1989, Claereboudt et al. submitted), including *Euvola ziczac* in suspended culture in the Golfo de Cariaco (Lodeiros and Himmelman 1993). Thus, *A. nucleus* is a short-lived species which breeds in the latter half of its life. A short life span (<2 yr) and reproduction only during one period in the latter part of life is also reported for other species of *Argopecten* (Epp et al. 1988, Orensanz et al. 1991).

The available reports on *Argopecten* spp. indicate an increase in the rate of growth with decreasing latitude. Thus, growth is least for *Argopecten irridians* in North Carolina, increased for *A. gibbus* in the Florida and greatest for *A. nucleus* in our region. In our study, 10 mm *A. nucleus* which were placed in the suspended culture in the late January 1991 attained near maximum shell length and somatic tissue mass 6–7 month later. At this size, the wet mass of the muscle is 3.53 g and 128 scallops furnish 1 pound of meat. A similar muscle size is characteristic of the calico scallop *Argopecten gibbus* (Dore 1991). *A. nucleus* is further interesting as a commercial species because (1) the somatic tissues are light in colour, tender in texture and good tasting and (2) the shell

is robust, attractive and almost free of fouling organisms. Thus, sale of the whole animal should be possible. Studies are required to quantify the effects of environmental factors on growth and mortality, to determine whether growth patterns are similar when juveniles are set out in other periods of the year, and to determine optimal culturing techniques (type of cages, density of individuals per cage, depth of cages, etc.). Further, laboratory methods need to be developed for producing spat in the laboratory, to permit producing juveniles when desired and for genetic improvement of the species.

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## STRUCTURE OF A SCALLOP *ARGOPECTEN PURPURATUS* (LAMARCK, 1819) DOMINATED SUBTIDAL MACRO-INVERTEBRATE ASSEMBLAGE IN NORTHERN CHILE

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**ABSTRACT** The structure and biomass of the subtidal, macro-invertebrate assemblage of Tongoy Bay was analyzed from 255 samples taken by divers during the winter and summer periods of 1990 and 1991. The main purpose of the study was to assess the relative importance (in numbers and biomass) of the scallops within the assemblage and to look for functional relationships between scallops and associated species. Of 52 taxa found, the scallop *Argopecten purpuratus* was the dominant species (30% of total biomass) followed by the crab *Cancer polyodon*, the sea stars *Meyenaster gelatinosus* and *Luidia magellanicus* and the predatory snails *Xanthochorus* sp. and *Priene rude*. As shown by a cluster analysis, these 6 species (which present 70% of the biomass) are closely associated, suggesting a functional unit with the scallop as prey and the others as predators. This is confirmed by literature reports on the feeding behavior of the above predators. As the species abundance data conformed to a straight line the log-series model was applied and the diversity index  $\alpha$  was calculated based on the numbers of species ( $=7.5$ ). For comparison with published data from Independence Bay (Peru), located about 2000 km to the north of the study area, the Shannon-Wiener diversity index  $H'$  ( $=3.6$ ) and the index of species evenness  $J'$  ( $=0.64$ ) were also calculated. Species richness (58),  $H'$  (4.4) and  $J'$  (0.76) were higher for the macro-invertebrate assemblage of the Peruvian Bay, while the dominant species and their rank order seemed similar, indicating important functional similarities between the two bays. The biomass found in Tongoy Bay ( $26.4 \text{ g m}^{-2}$  wet wt, macrophytes excluded) is low when compared to reports from temperate zones and is also somewhat lower than that reported for the coast of Volta and Congo and West Africa. This low biomass in Tongoy Bay is explained by a heavy clandestine scallop fishery over the past years causing a two- to threefold decrease in scallop biomass and a concomitant biomass decrease of associated species. It is postulated that *Argopecten purpuratus* occupies a central role in the assemblage as a filter feeder that converts planktonic food into available prey biomass, and that is not fully replaceable by other species of the system. Scallops and associated species were found on gravel, sand and soft sand bottoms, but scallops, the sea star *M. gelatinosus* and the snail *P. rude* were more frequent on gravel, and the crab *C. polyodon* and the sea star *L. magellanicus* on soft sand grounds.

**KEY WORDS:** scallops, community structure, macrobenthos, predation

### INTRODUCTION

The scallop *Argopecten purpuratus* is the only commercially important pectinid species in the southeast Pacific upwelling system. It belongs to the *Argopecten* group, that evolved in the subtropical Caribbean/Atlantic region, from where it gave rise to a radiation of species into the Atlantic and Pacific (Waller 1969). Of about 10 recent species of the *Argopecten* group, only two persist in the Pacific: *Argopecten circularis* in Mexico and Ecuador and *Argopecten purpuratus* in Peru and Chile. Like other species of this group *A. purpuratus* is a "bay scallop", that can be found in shallow water from Paita ( $5^{\circ}\text{S } 81^{\circ}\text{W}$ ) in the north to Bahia Vicente ( $37^{\circ}\text{S}, 73^{\circ}\text{W}$ ) in the south. Among the most important scallop grounds are those located in Independence Bay (Peru) and Tongoy Bay (Chile), being separated by about 2000 km of coastline (Fig. 1). On the sandy bottoms of both bays, *A. purpuratus* is the dominant macroinvertebrate that has sustained a diving fishery for many decades. At present, fishing is closed in both bays as the resource is considered to be overfished. Clandestine fishing has continued, however, and fisherman report scallop densities as low as  $<0.1/\text{m}^2$ . Several studies have been carried out on the population ecology and dynamics of this scallop in Peru (Wolff and Wolff 1984, Wolff 1985, 1987, Mendo et al. 1987, Yamashiro and Mendo 1988) and Chile (Illanes et al. 1985), and a recently published report gives some additional information on the scallop species assemblage in Independence Bay (Mendo et al. 1987).

The purpose of the present study is to describe the structure of

the subtidal, macroinvertebrate assemblage of Tongoy Bay and to gain insight into functional relationships between the scallop and associated species. Specifically, we analyzed species richness, species abundance order, diversity and biomass and determined species associations conducting a cluster analysis. In addition we looked for summer/winter differences in the scallop assemblage structure and for correlations between substrate softness and abundance of scallops and associated species. The present study is of particular interest, as Tongoy Bay is becoming the center for suspended scallop culture in Chile and the structure of the macrobenthos assemblage is therefore most likely to change in the coming years due to the organic enrichment of the bay. The study thus provides the basis to assess future changes and to formulate adequate conservation policies.

### MATERIAL AND METHODS

#### Sampling and Processing

During the winter (July–October 1990) and summer (February–April 1991) periods, samples (132,123 respectively) were taken along transects covering the whole bay area (Fig. 1). Along each transect approx. 10 sample units (distance between sample units approx. 200 m) were taken by a scuba diver who collected all the epibenthic macrofauna  $> 10 \text{ mm}$  within 30 square meters, using a  $5.5 \text{ m} \times 5.5 \text{ m}$  metal frame that was lowered onto the seafloor from the anchored boat. This sampling unit was considered more

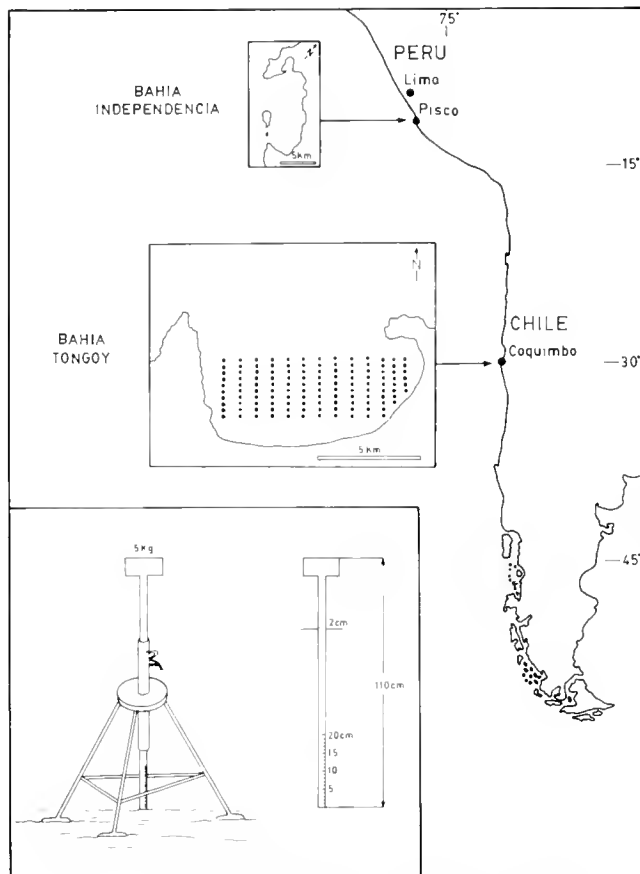


Figure 1. Study site and penetrometer used for the study.

appropriate than smaller units as it avoids too many zero counts at the low scallop densities in the bay ( $<0.1 \text{ m}^{-2}$ ) and as species associations are more likely to be detected. Sampling was restricted to a depth range of 7 to 25 m, where scallops are known to be most abundant. In addition, the diver measured the substrate softness using a "penetrometer", that had been constructed for this purpose. This instrument consists of a tripod and a iron-bolt of 2 cm diameter with a 5 kg weight on top that penetrates the sediment according to its softness (see Fig. 1). The samples were stored in plastic bags and transferred to the laboratory the same day for processing. All scallops were measured and weighted to the nearest 0.1 mm and 0.1 g respectively and the numbers and total weight of all the other species collected were also registered. Macroalgae biomass was roughly estimated on board using a bucket and a hand held balance. These estimates, however, were not included in our biomass per area estimates.

#### Data Analysis

#### Species Richness, Diversity and Biomass

Data from the winter and summer samples (255) were pooled and a rank order of species according to their corresponding biomass and numbers was established. As the data fell on a straight line using the natural logarithm of the abundances, the log-series model (Taylor et al. 1976) was applied and the model parameter  $\alpha$

(diversity index) was calculated by maximum likelihood using the following equation (Southwood, 1978):

$$ST = \alpha \ln(1 + N/\alpha), \quad (1)$$

where ST is total number of species and N is the total number of individuals sampled. Contrary to other numerical estimators of diversity (like  $H'$ , see below) this model also allows for a graphical representation of the relative importance of each species of the assemblage. In addition, and for the purpose of comparison with published data, we calculated the Shannon-Wiener diversity index ( $H'$ ) for the total number of species as well as Species evenness ( $J'$ ):

$$H' = - \sum (n_i/N) \log_2 (n_i/N) \quad (\text{Pielou, 1969}) \quad (2)$$

$$J' = H'/\log_2(S) \quad (\text{Pielou, 1969}) \quad (3)$$

where N is the total number of individuals, and  $n_i$  is the number of individuals of the  $i^{\text{th}}$  species; S is the total number of species found. Prior to the above procedure, we plotted the number of species found against the cumulative number of samples taken to see at how many samples the curve reached its maximum, thus verifying that our sample number was adequate for the determination of species richness.

In order to compare the species composition of Tongoy Bay with that reported for Independence Bay (Peru) by Mendo et al. (1987) we used Sørensen's index (Sørensen 1948) given by:

$$CC = 2C/(A + B), \quad \text{where} \quad (4)$$

C is the number of species shared in both areas and A and B are the total numbers of species in area A and B respectively

#### Scallop Dominance and Species Associations in the Winter and Summer Samples

We expressed the dominance of the scallop in the winter and summer samples by the following index "d":

$$d = B_{sc}/B_t \quad (5)$$

where  $B_{sc}$  is the total biomass of the collected scallops and  $B_t$  the total biomass of all specimens collected. This index was chosen because of its simplicity and as it is considered not to be influenced by species richness ST (Southwood 1978). For both winter and summer samples, a cluster analysis was performed from a species abundance (biomass) matrix using the program package SYSTAT. Euclidean distances were calculated and the Ward-linkage algorithm was used. A clustering of sample stations was also performed to look for regions of the bay with characteristic species associations. No evidence was found, however, that such areas exist (i.e. sample stations with species belonging to the same species clusters were scattered over the entire bay) which confirmed our initial assumption that the bay can be considered as a discrete habitat for this study. The "scallop clusters" determined from the winter and summer samples were further analyzed with respect to the biomass proportions of the component species and possible trophic relationships. Following Mendo et al. (1987) a simple "predation index" was calculated:

$$Pb/Sb \quad (6)$$

where Pb and Sb are the total predator and scallop biomasses respectively.



### Frequency of Occurrence of Scallops and Linked Species According to Substrate Type

Sample stations were classified according to substrate softness using the penetration depth of the penetrometer. The following categories were established: soft sand (penetration depth, p.d.: 12–16 cm) sand (p.d.: 7–11 cm) and hard sand or gravel (p.d.: 2–6 cm). Samples taken from the so classified sample stations then were analyzed separately for the frequency of occurrence of scallops and associated species.

## RESULTS

### Species Richness, Diversity and Biomass

At about 60 samples (= 24% of all samples taken, 1800 m<sup>2</sup>) the total number of species found in the study was reached (Fig. 2). The species rank order and their corresponding abundances (biomass and numbers) can be seen in Fig. 3. The species names are given in Table 1. Except for the scallop (first point), the (Ln)

biomass data (upper line) fit a straight line well ( $r = 0.9923$ ). A similar line is produced when (Ln) numbers are substituted for (Ln) biomass as a measure of abundance. These data also fit a straight line ( $r = 0.9956$ ), except for the first three species, whose points were therefore omitted for the calculation. Fig. 3 also contains the values calculated for the diversity index of the log-series model  $\alpha$ , the Shannon-Wiener index  $H'$  and the index of species evenness 'J'. Average macrobenthic biomass in Tongoy Bay (area sampled = 7650 m<sup>2</sup>) was estimated as 26.4 g m<sup>-2</sup> wet wt (macrophyte biomass not included).

### Scallop Dominance and Species Associations

Scallop dominance was similar between summer and winter ( $d = 0.27$  and  $0.33$  respectively). As seen in Fig. 4, scallop biomass and the biomasses of the crab *Cancer polyodon* and the sea stars *Meyenaster gelatinosus* and *Luidia magellanicus* was considerably higher in the summer samples. The gastropods *Priene rude* and *Xanthochorus sp.* had higher biomasses in winter and summer

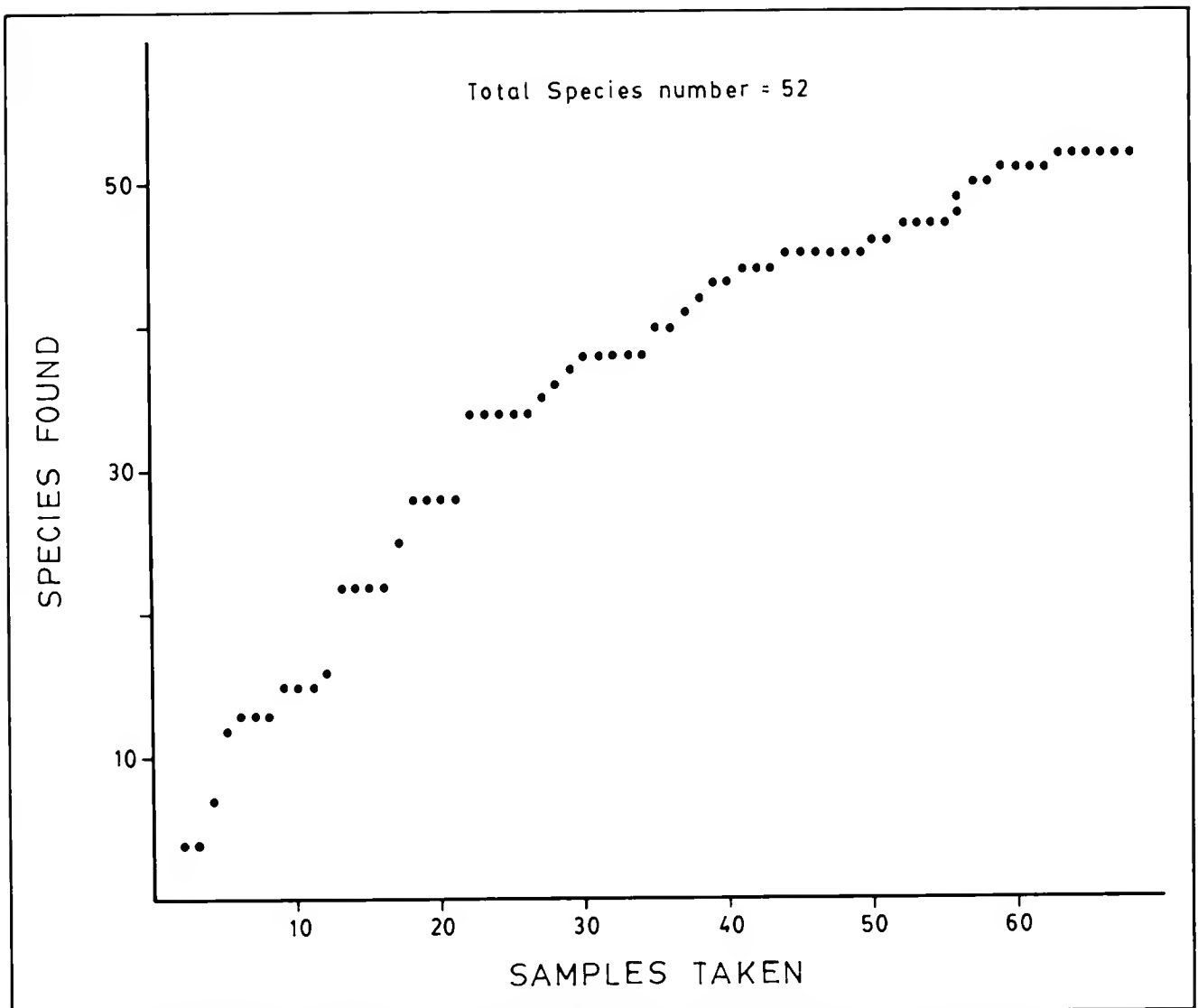


Figure 2. Number of samples taken versus cumulative species number (total number of samples taken was 255).

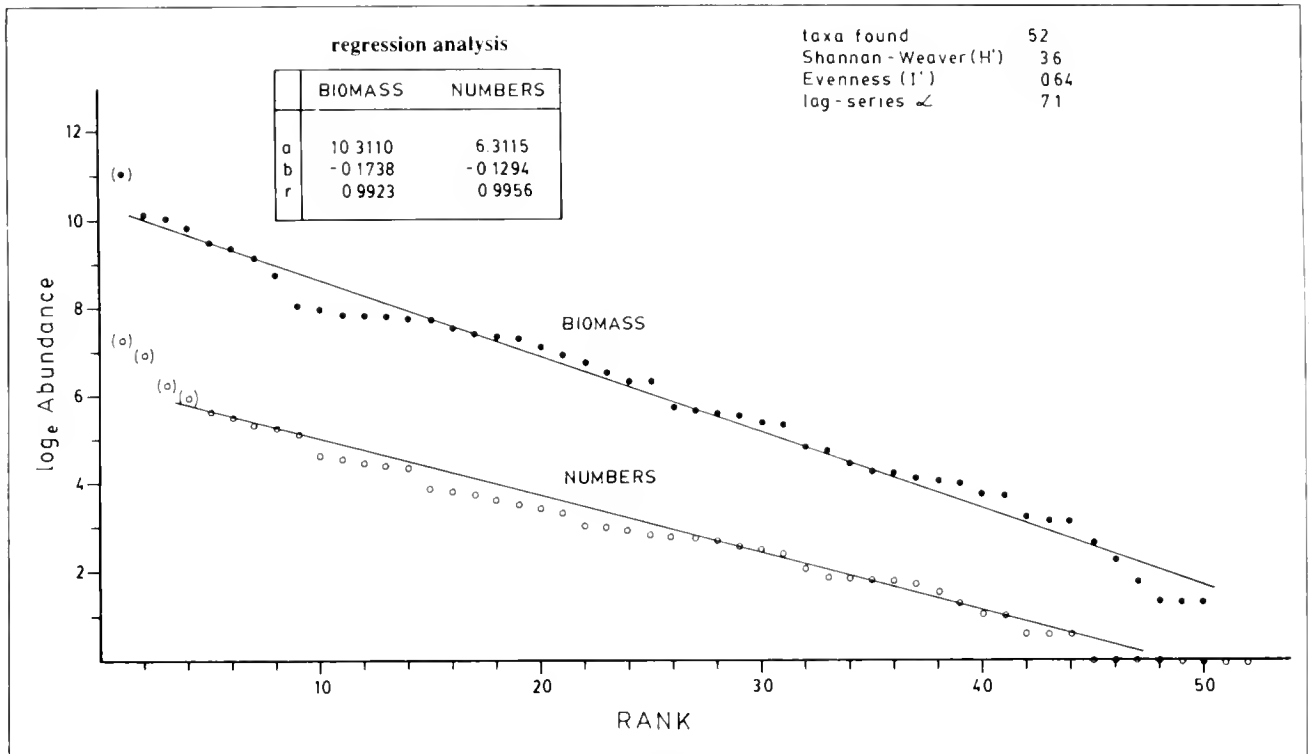


Figure 3. Rank order of species found (log-series model) for the biomass and numerical data along with their regressions (see also Table 1); Estimates for Evenness ( $I'$ ), Shannon-Weaver ( $H'$ ) and species richness are also given.

respectively. The frequency of occurrence in the samples remained similar for most of the species of the first 20 in biomass rank order. Exceptions were the gastropods *Xanthochorus sp.* and *Priene rude*, because they appeared significantly less frequently in the summer samples. Fig. 5 shows the dendrograms from the cluster analysis. The analysis was done with only the major species, which contributed about 90% to the total biomass in the winter and summer samples. The three species that are closest associated with the scallop in both clusters are the crab *C. polyodon*, and the sea stars *M. gelatinosus* and *L. magellanicus*. The snail *Xanthochorus sp.* follows next in the summer cluster, and is replaced in its position by the snail *P. rude* in the winter cluster. Fig. 6 illustrates these relationships together with the relative biomasses of the component species of the "scallop clusters" and gives the values calculated for their predation indices.

For comparison with the data of Tongoy bay, the species abundance data of Independence Bay (Peru) of Mendo et al. (1987) are given in Table 1 together with community indices calculated for both bays.

#### Frequency of Occurrence of Scallops and Associated Species According to Substrate Type

The frequency of occurrence of the 15 dominant species (in terms of biomass) according to substrate type is shown in Fig. 7. Except for the snail *Oliva peruana* (which was absent on soft sand and gravel) and the mussel *Aulacomya ater* (which was absent on gravel) all species occurred on all substrate types. Scallops were found most frequently on gravel (66.7%) but also appeared on

sand and soft sand (40%). Among the predators *C. polyodon*, *L. magellanicus* and *M. gelatinosus*, the first two species were more frequently encountered on soft sand, while the latter was more common on gravel. Among the predatory snails *Xanthochorus sp.* was equally distributed over all substrate types while *P. rude* was more common on gravel.

## DISCUSSION

### Species Richness, Diversity and Biomass

The species collection in the present study was directed towards the larger epibenthic macrofauna > 1 cm (visible to the diver). Small species and individuals are therefore likely to be undercollected. Indirect sampling methods with drags or the use of smaller sampling units by the diver would have avoided this bias but would have led to an undercollection of the sparsely distributed larger individuals which are important scallop predators. Species number did not increase after 60 samples (1800 m<sup>2</sup>) (Fig. 2) which demonstrates an adequate sampling to describe species richness. Parker (1963) gives a similar curve from a shell dredge survey on sand bottoms (11–36 m) in the Gulf of California that shows a steady increase of species number with each dredge sample (20 m<sup>2</sup>) yielding over 140 species after 9 samples (180 m<sup>2</sup>). The same author reproduces cumulative curves from boreal waters from Holme (1953) for Whitesand bay (water depth of 16.5 m), England, and from Petersen and Boysen-Jensen (1911) from Thisted breeding (water depth 27 m), Denmark, which level off at species numbers of about 35 and 15 respectively. These reports suggest that the species richness (52) found in Tongoy Bay for the depth

range 7–26 m lies between boreal and tropical waters. Mendo et al. (1987) hand-collected macrofauna in Independence Bay (Peru) as we did, and their results seem comparable to ours (they, however, sampled only 1 square meter at each of their 180 sample stations and do not report on the biomass of most of the species). They found a slightly higher species richness (58 taxa), despite the fact that only three years before their study (1982/83), a strong El Niño event had caused drastic changes in the macrofaunal species assemblage, i.e. mortalities of many species, immigration of others and an enormous scallop (*A. purpuratus*) proliferation (Wolff 1987, Arntz et al. 1988).

Species diversity (log-series  $\alpha$ ,  $H'$ ) and evenness ( $J'$ ) are also higher than in Tongoy Bay and Sørensen's similarity index of 0.51 (Table 1) indicates higher structural differences between the two habitats than when only judged by the species richness. These differences are most likely to be due to the more tropical position of Independence Bay and to Panamanian species that are absent in Tongoy Bay. The species registered in both bays and their rank order show notable similarities, however: both habitats share 8 of the first 20 species in numeric rank order and 6 of those species of Independence Bay are also among the first 20 species in biomass rank order in Tongoy Bay. Among these 20 species are the predatory snails *P. rude* and *Xanthochorus sp.* and the sea star *L. magallanicus* which form part of the "scallop cluster" of Tongoy bay. This suggests that there are important similarities in the functional relationships between the scallop (which is numerically the second and third most important species in Tongoy Bay and Independence Bay respectively) and associated species in both bays.

The average macroinvertebrate biomass of 26 g wet wt  $\cdot m^{-2}$  found in Tongoy Bay is low for subtidal sandy bottoms, when compared to temperate zones. A comparison with the literature is difficult because of the heterogeneity of sampling techniques used and the incompatibility of units. We shall try to compare assuming that 1 g Carbon represents about 19 g wet weight (Mills and Fournier, 1979). Sanders (1956) report 4.8 g C  $m^{-2}$  (about 91.2 g wet wt) for Long Island Sound, USA, Wolff & Wolff (1977) give values of 10 g C  $m^{-2}$  (190 g wet wt) for the Gravelingen estuary, Netherlands, and the macrobenthic biomass recorded in the Baltic Sea (1.7 g C  $m^{-2}$  corresponding to about 32.3 g wet wt) is higher than our biomass values in Tongoy bay. Sparck (1951) and Longhurst (1959) report similar values, however, for the coast of Volta and Congo and West Africa (30–40 g wet wt  $m^{-2}$  and 6.73–74.23 g wet wt  $m^{-2}$ ) and Buchanan (1958) gives values of 28–120 g wet wt  $m^{-2}$  for the coast of Ghana. Despite these similar values the question arises why the macroinvertebrate biomass in Tongoy bay is so low, considering that the bay is strongly influenced by a nearby upwelling center and regarded as highly productive (Alarcon 1975, Acuna et al. 1989).

Food does not seem to be a limiting factor for the filter feeding macrobenthos as the bay is known to have supported scallop densities of  $>30$  ind.  $m^{-2}$  (500 g wet wt) in past years. In Independence Bay (Peru) the El Niño event 1982/83 produced densities of  $>500$  ind./ $m^{-2}$  and biomasses of 5000–6000 g  $m^{-2}$  (Arntz et al. 1985) while primary production had not increased. This enormous scallop proliferation coincided with heavy mortalities of most of the scallop predators (Wolff 1987), which suggests that predation is important in keeping scallop densities low. This seemed confirmed by the post El Niño increase of predator biomass paralleled by a simultaneous reduction of scallop biomass (Mendo et al. 1988). However, while this mechanism could explain that predator

and scallop biomass are interdependent, it would not explain the low total macro-invertebrate biomass found in Tongoy. The answer may lie in a heavy clandestine scallop fishery that has intensified over the past years due to the high demand for seed scallops for the suspended cultures (Wolff and Alarcon, personal observations) leaving an average scallop population, that is 2–3 times reduced compared with previous "average" years (CIS.U. del Norte 1975, Viviani 1979). This is also confirmed by a low average scallop size found in the present study (59.1 mm) compared with the late seventies (85 mm reported by SERPLAC, 1978).

#### Scallop Dominance and Species Associations

Despite its low abundance (compared to past years), *A. purpuratus* is still the dominant macroinvertebrate (representing about 30% of the total biomass) which seems indicative of the above-mentioned interdependence of total epibenthic macroinvertebrate biomass with scallop abundance. The almost constant predation index (around 1.3) between the summer and winter samples (by significantly higher total macro-invertebrate biomass in summer) is a further indication of this.

In terms of biomass, *Cancer polyodon* and *Meyenaster gelatinosus* seem to be the most important predators (representing 17.8% and 16.7% of the other species), followed by *Luidia magallanicus* and the snails *Xanthochorus sp.* and *Priene rude* (which represent 9.5%, 8.5% and 4.5% of the remaining species respectively). It is notable that the 6 species of the scallop cluster represent 70% of the biomass of the 52 species found in the bay which corroborates their trophic relations. As cited by Parker (1963), a dominance of about 10 invertebrate species was also reported by Buchanan (1958) for the Gold Coast area of West Africa and by Longhurst (1957, 1958) off Sierra Leone to the north, while in the tropical Gulf of California such a dominance did not exist.

*C. polyodon* is known as a voracious predatory omnivore that is able to detect dense patches of prey, to aggregate quickly around these and to feed at high rates (Wolff and Cerda 1992). DiSalvo et al. (1984) reported that 1000 scallops (*Argopecten purpuratus*) of 30 mm shell length in an open cage were consumed in less than three days by this crab. *Meyenaster gelatinosus* is also known as an omnivorous predator and eats sea urchins, bivalves, other sea stars and crabs (Vasquez, per. com). Mendo et al. (1987) consider the sea star *Luidia magallanicus* and the snails *Xanthochorus sp.* and *Priene rude* as important predators of *A. purpuratus* in Peru, which is also coincident with our data through the position of these species in the scallop cluster. The muricid snail *Crassilabrum crassilabrum*, although not as abundant as the other predators and not identified as part of the "scallop cluster" might also prey on *A. purpuratus*.

Evidently, the above predators also feed on other species besides the scallop or on each other (known for *C. polyodon* and *M. gelatinosus*), but the scallop *A. purpuratus* occupies a central position in this assemblage for its abundance and functional role as a filter feeding species that converts planktonic food into available prey biomass. In addition, *A. purpuratus* is an extremely fast-growing, highly productive species (Wolff 1987), whose mobility allows its population biomass to be distributed over wide areas.

As the recruitment success of *A. purpuratus* is known to vary significantly between years (Wolff 1988), one would expect total macro-invertebrate biomass also to vary. At high scallop densities most of the energy leading to the predators supposedly travel through a short 3-step food chain (similar to the pelagic food chain

TABLE I.

(a) Species abundance data from Tongoy Bay (this study) and from Independence Bay (Mendo et al. 1987); (b) community indices calculated from these data.

Tongoy Bay				Independence Bay	
Species	Taxonomic group	Biomass (g)	Number	Species	Number
1. <i>Argopecten purpuratus</i>	Mollusca	61,712	1397	1. <i>Diopatra</i> sp.	80
2. <i>Cancer polyodon</i>	Crustacea	25,144	96	2. <i>Massarius gayi</i>	74
3. <i>Meyenaster gelatinosus</i>	Echinodermata	23,370	80	3. <i>Argopecten purpuratus</i>	60
4. <i>Aulacomya ater</i>	Mollusca	18,645	21	4. <i>Ophiactix kroyeri</i>	48
5. <i>Xanthochorus</i> sp.	Mollusca	13,249	263	5. <i>Crucibulum</i> spp.	40
6. <i>Luidia magellanicus</i>	Echinodermata	11,977	46	6. <i>Pagurus</i> spp.	34
7. <i>Tegula</i> sp.	Mollusca	9,573	1,085	7. <i>Tegula atra</i>	31
8. <i>Priene rude</i>	Mollusca	6,367	371	8. <i>Eurypanopeus transversus</i>	31
9. <i>Turritella cingulata</i>	Mollusca	3,132	516	9. <i>Mitrella</i> sp.	28
10. <i>Crucibulum quiriquiremae</i>	Mollusca	2,971	87	10. <i>Trophon</i> sp.	25
11. <i>Crassilabrum crassilabrum</i>	Mollusca	2,609	100	11. <i>Espongiarios</i>	25
12. <i>Raja</i> sp.	Chondrichthys	2,431	31	12. <i>Xanthochorus buxea</i>	22
13. <i>Arbacia dufruesmii</i>	Echinodermata	2,271	28	13. <i>Priene rude</i>	20
14. <i>Pagurus</i> sp.	Crustacea	2,270	190	14. <i>Luidia bellonae</i>	19
15. Anthozoa	Cnidaria	2,559	203	15. <i>Synalpheus</i> sp.	19
16. <i>Hepatus chilensis</i>	Crustacea	1,922	16	16. <i>Arbacia spatuligera</i>	18
17. <i>Cancer coronatus</i>	Crustacea	1,733	15	17. <i>Bursa ventricosa</i>	17
18. <i>Oliva peruana</i>	Mollusca	1,599	204	18. <i>Polynices otus</i>	17
19. <i>Thais chocolata</i>	Mollusca	1,531	10	19. <i>Majidae</i>	17
20. <i>Calyptrea trochiformis</i>	Mollusca	1,248	17	20. <i>Crepipatella dilatata</i>	16
21. <i>Ovalipes trimaculatus</i>	Crustacea	1,055	5	21. <i>Actinias</i>	15
22. <i>Diopatra</i> sp.	Polychaeta	888	1,636	22. <i>Hepatus chiliensis</i>	11
23. <i>Tagelus dombeii</i>	Mollusca	703	35	23. <i>Poliqueto 2</i>	10
24. <i>Nucella calcarlongus</i>	Mollusca	633	105	24. <i>Poliqueto 1</i>	10
25. <i>Semele solida</i>	Mollusca	631	13	25. <i>Fissurella</i> spp.	10
26. <i>Paraxanthus barbiger</i>	Crustacea	330	7	26. <i>Oliva peruviana</i>	9
27. <i>Gari solida</i>	Mollusca	287	2	27. <i>Malaguas</i>	8
28. Decapoda indet.	Crustacea	270	2	28. <i>Aulaconya ater</i>	8
29. <i>Homalaspis plana</i>	Crustacea	259	1	29. <i>Thais chocolata</i>	8
30. <i>Murcia gaudichaudi</i>	Crustacea	220	3	30. <i>Semele solida</i>	6
31. <i>Pseudochorystes sicarius</i>	Crustacea	218	7	31. <i>Asterina chilensis</i>	5
32. <i>Squilla mantis</i>	Crustacea	124	2	32. <i>Poliplacoforos</i> (chitones)	5
33. <i>Ovalipes catharis</i>	Crustacea	117	1	33. <i>Cancer porteri</i>	5
34. <i>Crepipatella dilatata</i>	Mollusca	84	8	34. <i>Ascidia</i>	4
35. <i>Grapsidae</i>	Crustacea	71	67	35. <i>Calyptrea trochiformis</i>	4
36. <i>Octopus vulgaris</i>	Mollusca	71	1	36. <i>Cynatum</i> sp.	4
37. <i>Crepipatella</i> sp.	Mollusca	62	18	37. <i>Tertrapigus niger</i>	3
38. <i>Venus antigu</i>	Mollusca	56	1	38. <i>Balanus</i> sp.	3
39. <i>Nassarius</i> sp.	Mollusca	55	245	39. <i>Cancer setosus</i>	3
40. <i>Plumnoides perlatus</i>	Crustacea	44	78	40. <i>Tegula tridentata</i>	2
41. <i>Taliepus dentatus</i>	Crustacea	42	2	41. <i>Cancellaria</i> sp.	2
42. <i>Pisoides edwardsi</i>	Crustacea	25	2	42. <i>Petrolisthes</i> spp.	2
43. <i>Perymytilus purpuratus</i>	Mollusca	23	2	43. <i>Heliaster helianthus</i>	2
44. Porifera	Porifera	23	2	44. <i>Hyatella solida</i>	2
45. <i>Chiton cummingsii</i>	Mollusca	14	44	45. <i>Calliostroma fonkii</i>	2
46. <i>Nudibranchia</i>	Mollusca	10	1	46. <i>Cancer edwardsii</i>	2
47. <i>Fissurella</i> sp.	Mollusca	6	14	47. <i>Cardita</i> sp.	2
48. <i>Loxechinus albus</i>	Echinodermata	4	7	48. <i>Platyxanthus orbigny</i>	1
49. <i>Eurypodius longirostris</i>	Crustacea	4	1	49. <i>Glycyneris ovata</i>	1
50. <i>Alpheus</i> sp.	Crustacea	4	22	50. <i>Sipunculidae</i>	1
51. <i>Tetrapigus niger</i>	Echinodermata	1	4	51. <i>Pilumnoides perlatus</i>	1
52. <i>Cancer edwardsii</i>	Crustacea	1	2	52. <i>Huevos de cefalopodos</i>	1
				53. <i>Pinnixa</i> spp.	1
				54. <i>Sinum cymba</i>	1
				55. <i>Caenocentrotus gibbosus</i>	1
				56. <i>Discinisca lamellosa</i>	1

continued on next page

TABLE I.  
continued

Tongoy Bay				Independence Bay	
Species	Taxonomic group	Biomass (g)	Number	Species	Number
				57. Nitra sp.	1
				58. Crassilabrum crassilabrum	1
Total (area: 7650 m <sup>2</sup> )		202,648	7,116	Total (area: 180 m <sup>2</sup> ) 799	
<b>b.</b>					
Tongoy Bay (Chile)			Independence Bay (Peru)		
Species richness, ST		52			58
Log-series diversity, $\alpha$		7,1			14,4
Shannon-Wiener, H'		3,6			4,4
Evenness, J'		0,64			0,76
Similarity (Sorensen), CC		0,51			0,51

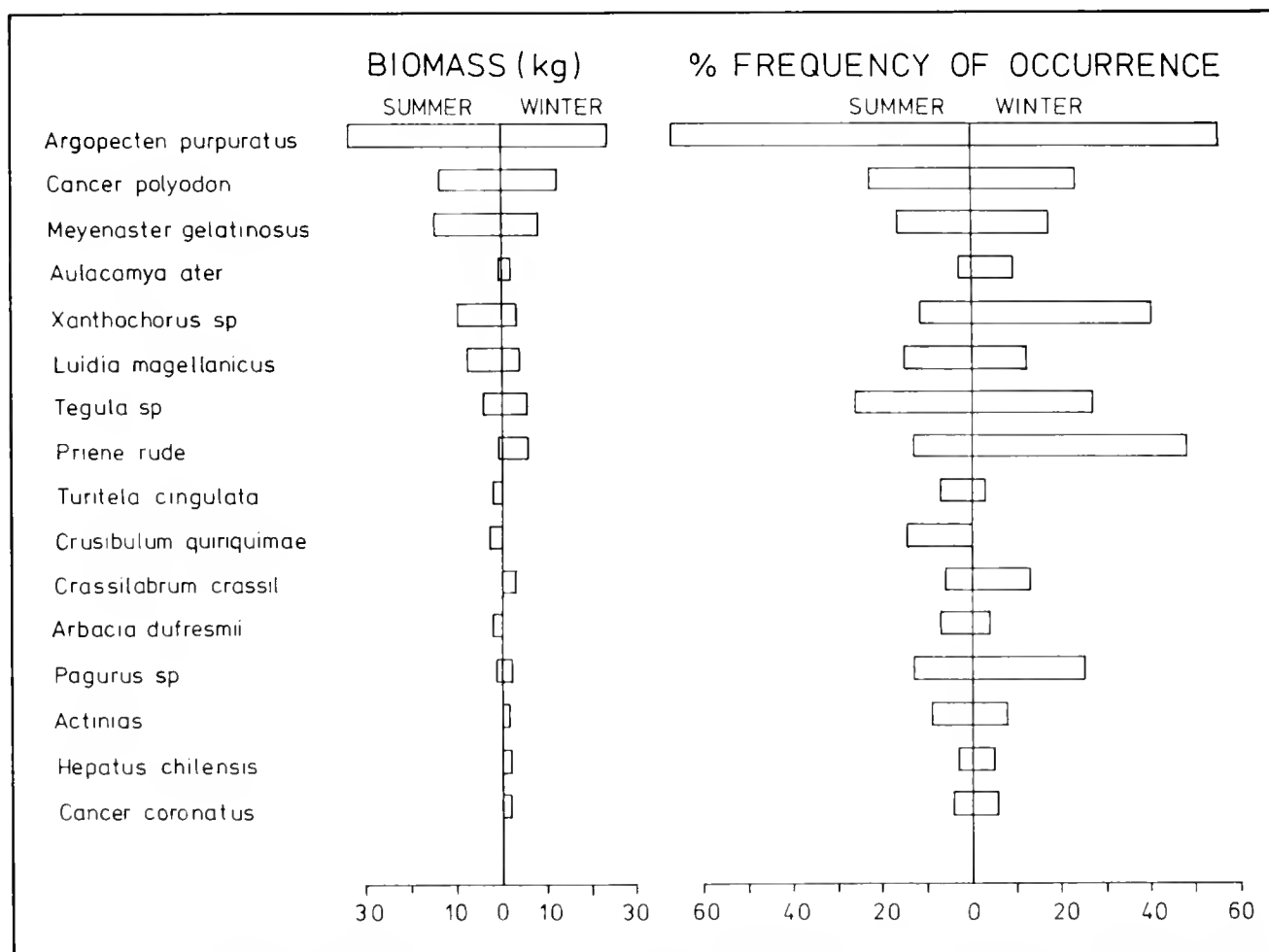


Figure 4. Biomass and frequency of occurrence of the 16 most important species (representing &gt;90% of total epibenthic biomass) in the winter and summer samples.

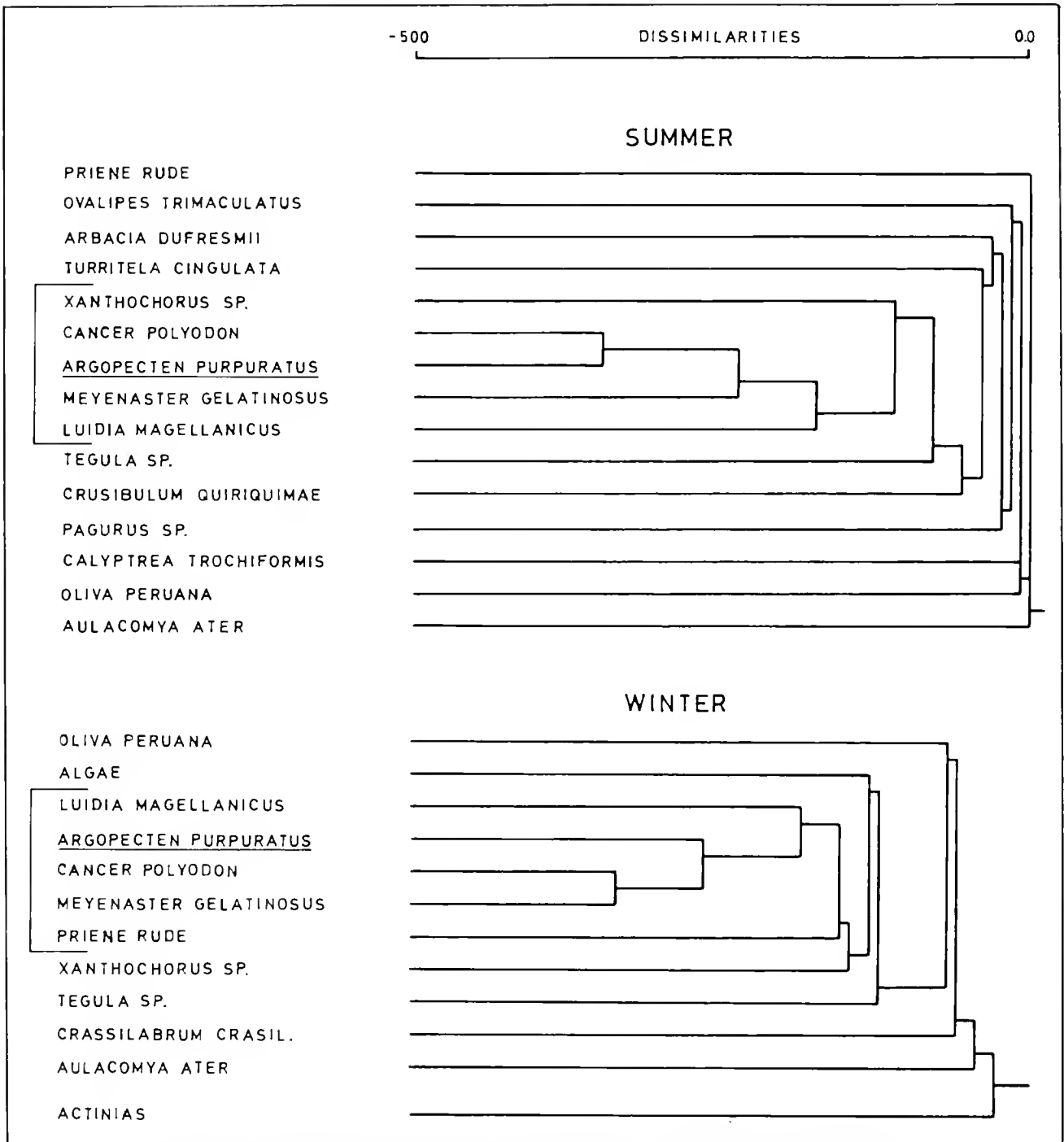


Figure 5. Cluster analysis for the summer and winter samples, with the scallop cluster in brackets.

in upwelling regions) while at low scallop densities, predators are likely to intensify the use of alternative prey, including individuals from the same species. A prolonged absence (or heavy decline in abundance) of scallops in these areas may cause a general decrease in macro-invertebrate biomass, as a central and primary food source is missing, a situation that seems to prevail in Tongoy Bay.

*Relation of Substrate Type with Frequency of Occurrence of Scallop and Associated Species*

The ubiquity of scallops on different bottom types has been reported previously in the literature (Olsen 1955 for *Notovola me-*

*ridionalis*; Ciocco 1983 for *Chlamys tehuelcha*; Roe et al. 1971 for *Argopecten gibbus*; Wolff 1985 for *A. purpuratus* among others). On the other hand, it has frequently been pointed out (Belding 1919, Dryer 1941, Marshall 1947, Wolff 1985 among others) that scallops preferably recruit on gravel grounds with abundant algae, which provide substrates to which they attach as larvae. From these "recruitment areas" many specimens migrate later on into relatively unstructured sandy bottom areas. Our study seems to confirm this as the frequency of occurrence of scallops was almost 70% on gravel (where algal biomass was also higher, Fig. 7) compared to only about 40% on sand and soft sand grounds.

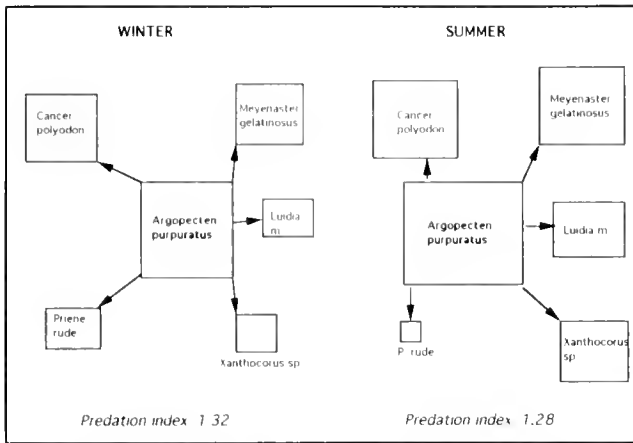


Figure 6. Diagrammatic representation of the biomass proportions and possible trophic interactions within the "scallop cluster" (box size is proportional to biomass).

The higher frequency of occurrence of *M. gelatinosus* on gravel and of *L. magellanicus* and *C. polyodon* on soft sand (Fig. 7) might be indicative for a certain competitive partition of the habitat between the former and the latter two species. The snails *Xanthochorus sp.* and *Priene rude* seem to be as ubiquitous as the scallop with no marked preference for a substrate type.

The present study represents a first attempt to describe the scallop dominated invertebrate assemblage in Tongoy Bay and to look for functional relationships between *A. purpuratus* and associated species. In order to quantify the trophic interactions within this assemblage, studies on food composition and consumption rates of the component species should follow.

#### ACKNOWLEDGMENTS

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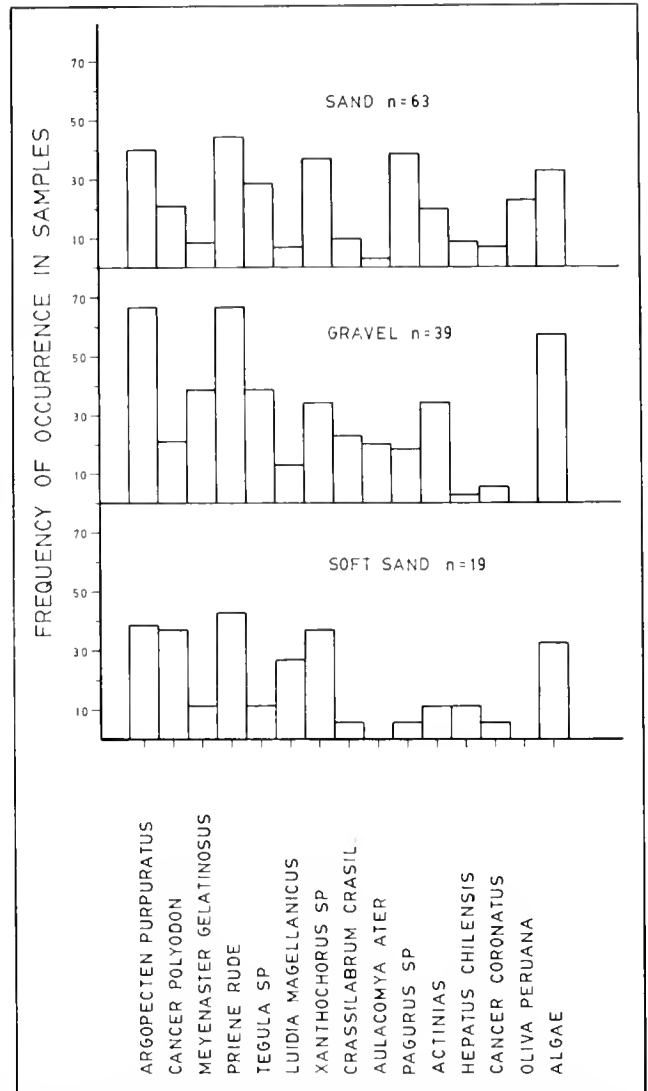


Figure 7. Frequency of occurrence of the most important taxa according to bottom type.

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## AN ASCETOSPORAN DISEASE CAUSING MASS MORTALITY IN THE ATLANTIC CALICO SCALLOP, *ARGOPECTEN GIBBUS* (LINNAEUS, 1758)

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**ABSTRACT** The Atlantic calico scallop, *Argopecten gibbus*, prior to December 1988, supported a fishery off the east coast of Florida with an annual production varying between 10 and 40 million pounds of adductor muscle meats. In a six week period from December, 1988 through mid-January, 1989 all harvestable stocks over the 2500 square mile fishing area were devastated. Stocks began to reappear in the summer of 1989. In January of 1991, as the population was rebounding and fishing had resumed, massive mortalities were again observed in the calico scallop stock; by February, 1991 the scallop population had again been reduced to negligible numbers. Histopathological examination of the scallops involved implicates an ascetosporan of the genus *Marteilia* as the primary cause of the observed mortalities.

**KEY WORDS:** *Argopecten gibbus*, *Marteilia*, scallop, pathogen, ascetosporan

### INTRODUCTION

The Atlantic calico scallop, *Argopecten gibbus* (Linnaeus 1758), was identified as a potential commercial species in the early 1960's but large scale fishing off Cape Canaveral, Florida did not begin until the introduction of mechanical processing in 1980 (Blake and Moyer 1991). Production levels have fluctuated greatly since that time but by the 1980's between 10 and 40 million pounds of adductor meats were processed annually from the 2500 square miles of fishing grounds located off Cape Canaveral. During this time period 5 processing plants each supported from 5 to more than 20 boats. Each boat typically made 5 to 7 fishing trips of 16 to 20 hours per week.

In December of 1988 fishermen began to report finding increased numbers of dead and dying scallops. By early January, 1989 there was evidence of widespread mortality. By the end of January, 1989 the population had been decreased to the point that no scallops could be found by either commercial or research trawlers. By the summer of 1989 the population had rebounded sufficiently for regular monthly sampling of the population to resume. Population levels became large enough for commercial fishing to resume by the beginning of 1990. There was no evidence of any further problems until January, 1991 when mortality was again observed throughout the calico scallop population. By the end of February, 1991 the scallop population had once again been reduced to minimal levels and commercial fishing was suspended. As of September, 1992 the population of calico scallops while increasing has not reached levels sufficient to enable the resumption of commercial fishing for this species in the Cape Canaveral area.

The causes of these mass mortalities could not be directly associated with physical environmental factors. Seasonal patterns of temperature, salinity and upwelling events over the Cape Canaveral scallop grounds have been measured since 1983 and no major deviations have been observed which could contribute to these mass mortalities. In an attempt to ascertain a biological cause of these mass mortalities, a histopathological analysis of calico scallops collected from 1983 to 1992 was made. This paper describes the results of these histopathological analyses.

### MATERIALS AND METHODS

Scallops were collected from the Cape Canaveral fishing grounds which extend from Daytona Beach south to Fort Pierce at depth ranging from 20 to 50 fathoms (Fig. 1). Sample collections were made using a variety of research and commercial vessels. All samples were collected with modified otter trawls. Commercial vessels tow two trawls concurrently, one off of each side, while research vessels typically tow a single trawl from the stern. Since 1983 samples were obtained when possible on a monthly basis. These routine collections were augmented occasionally both by intensive sampling on specific research cruises, and by additional collections made in response to abnormal events such as increased mortalities. Each sample contained 16 to 20 scallops when possible. The live scallops were fixed immediately for histological examination using Helly's fixative made with zinc chloride. After 1 to 2 hours in the fixative the animals were bisected using a mid-sagittal cut and returned to the fixative for a total fixation time of 20 hours. The tissues were then processed and embedded in paraffin using standard histological techniques (Barszcz and Yevich 1975). Sectioning was accomplished with a rotary microtome set at 6  $\mu$ m and the resulting slides stained either in Hematoxylin and Eosin (Luna 1968) or with Cason's Trichrome stain for connective tissue (Cason 1950). The finished slides were examined and photographed using a Zeiss Photomicroscope III.

### RESULTS

A total of 59 scallops collected over a 1 month period December, 1988 to January, 1989 were examined histologically during the first period of increased mortality. In all of these animals a protozoan parasite which we tentatively identified as belonging to the genus *Marteilia* was found in the digestive and basophilic epithelial cells of the tubules of the digestive diverticulum. Examination of more than 1000 scallops collected from 1983 until December, 1988 revealed no prior evidence of this parasite. From January of 1989 until July, 1989 no live scallops were located in the Cape Canaveral fishing area. In July, 1989 when it was again possible to obtain samples there was no evidence of the protozoan

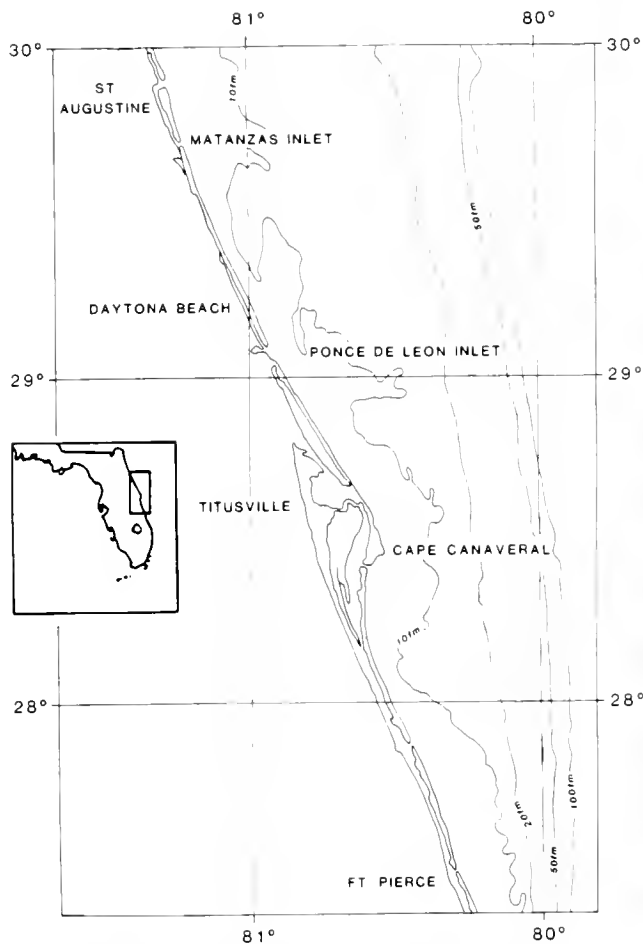


Figure 1. Map of the eastern coast of Florida. Calico scallops are normally located between Daytona Beach, Florida and Fort Pierce, Florida in depth ranging from 20 to 50 fathoms.

in any of the animals examined. Sampling continued from that point at approximately monthly intervals but no evidence of the protozoan parasite was detected until February, 1991. The first evidence of the return of the parasite was indications of increased scallop mortality reported by the fishermen. There was no evidence of infection in samples taken in early January but examination of 45 scallops obtained from three locations in February revealed that the protozoan was again present in some portion of the population. Infection levels of 86%, 27% and 69% were detected for scallops collected from the northern, central and southern areas, respectively, of the fishing grounds. Overall, 60% of the scallops collected during February, 1991 exhibited evidence of the protozoan parasite. As was the case in 1989 within a few weeks it was no longer possible to obtain additional samples due to the virtual elimination of the population and closure of the fishery until late 1991. Samples obtained since that time have failed to reveal any further evidence of the parasite. More than 1700 scallops, collected between 1983 and 1992, were examined histologically for evidence of infection by this protozoan parasite. Of these the parasite was observed only in scallops collected from December, 1988 to January, 1989 and in February, 1991. In both 1989 and 1991 almost 100% of the natural population died within 4 weeks of the appearance of the pathogen.

The digestive diverticulum of a healthy calico scallop and one

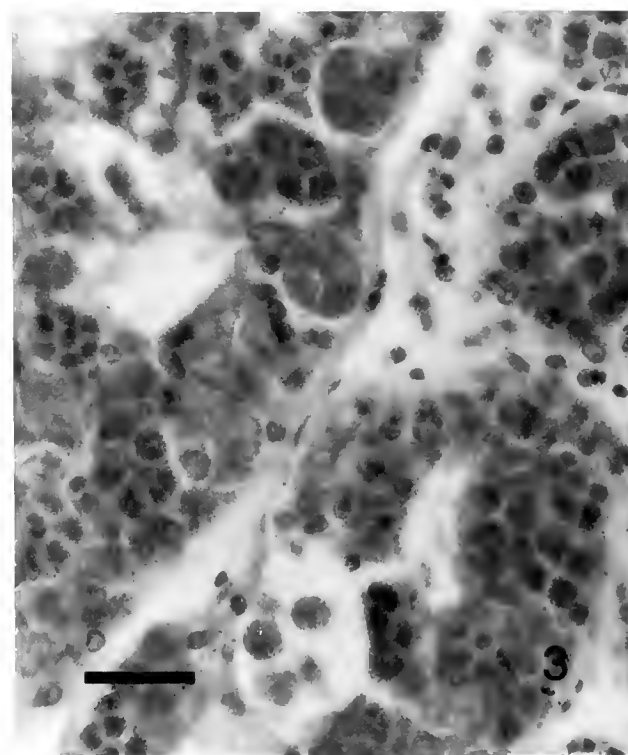
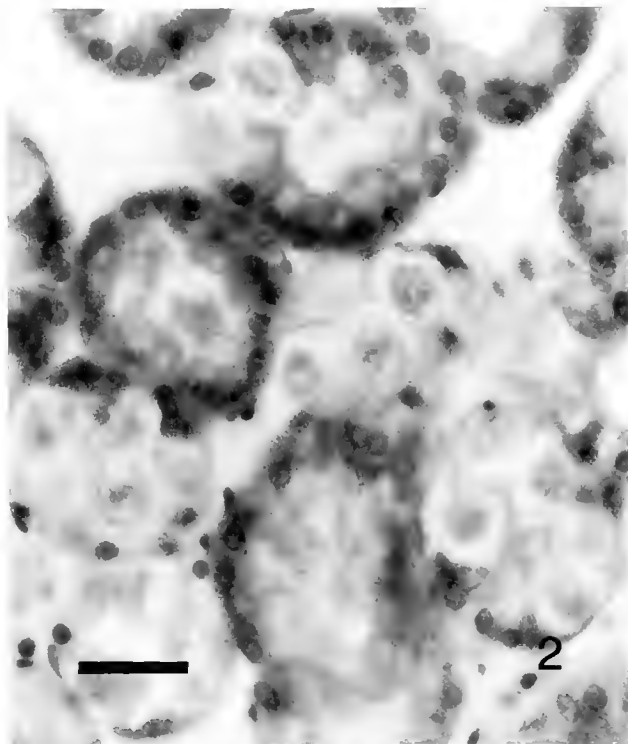


Figure 2. Digestive diverticulum showing the tubules of a healthy calico scallop collected off Cape Canaveral, Florida (haematoxylin and eosin stain; scale bar is 25  $\mu$ m).

Figure 3. Digestive diverticulum showing the tubules of a calico scallop collected off Cape Canaveral, Florida exhibiting a heavy infection by the *Marteilia* sp. ascetosporan (haematoxylin and eosin stain). The pathogen has completely filled the tubule epithelial cells but there is no evidence of invasion into surrounding tissue or host hemocyte response (scale bar is 25  $\mu$ m).

infected with the protozoan are contrasted in Figures 2 and 3. On the basis of light microscopy the parasite observed in the calico scallop appears to meet all of the descriptors that have been established for members of the genus *Marteilia* (Grizel et al 1974, Perkins 1976, Perkins and Wolf 1976, Figueras and Montes 1988), and so we shall remain consistent with the terminology that they have employed in describing this parasite. The lumens of the tubules in the digestive diverticulum of infected scallops are filled with sporangiosori each containing approximately 8 presporangia or sporangia. The sporangiosori are easily discerned with either Hematoxylin and Eosin staining or Cason's Trichrome stain. The Cason's stain shows the sporangia containing mature spores appearing pink against a blue background leading to rapid identification of animals containing the pathogen. The sporangia in turn appear to contain 3 or 4 spore primordia. The sporangiosori examined in histological sections have a mean length (the longest axis) of 17  $\mu\text{m}$  (range: 14 to 22  $\mu\text{m}$ ; N = 25). Plasmodia, the stage of the sporangiosori prior to the development of presporangia, were not observed in these samples.

The percentage of the digestive diverticulum exhibiting evidence of the pathogen varies, but it appears to be progressive. The most extensive infection appears to be found in those animals at or near death (adductor muscle shrunken in size, shells gaping and slow to respond to tactile stimulation, mantle slightly withdrawn from the shell edge) at the time of collection. At that point virtually 100% of the tubules are infected.

In those scallops exhibiting extensive infection, mature spores are also observed in the lumen of the intestine of the animal (Fig. 4). The spores, which range in size from 3.5 to 4.3  $\mu\text{m}$  in diameter, appear to be in the process of being excreted rather than

invading surrounding tissue. It is not known whether this is an attempt by the scallop to clear the pathogen, or if it is instead normal excretion of undigested spores as part of the life cycle of *Marteilia*.

No evidence could be found of the pathogen invading the surrounding epithelial cells or connective tissue. There was also no sign of hemocyte infiltration in response to the parasite. The parasite spores were occasionally seen mixed with food items in the gut indicating that ingestion may lead to the spread of the pathogen although the spores were not plentiful among the gut contents. The pathogen was observed only in the tubules of the digestive diverticulum or as spores within the gut or being excreted through the intestine.

The only other pathology observed was in those scallops in which large portions of the digestive diverticulum were infected by the pathogen. These animals exhibit evidence of catabolizing body tissue. This is particularly clear in the adductor muscle where extensive atrophy of the muscle bundles is evident. Figures 5 and 6 illustrate the differences in the adductor muscles of normal and heavily infected calico scallops. Scallops typically derive some of their energy requirements from utilization of adductor muscle tissue when unable to extract sufficient energy from food intake. There was no evidence that food levels were abnormal during either of the two epizootic episodes. The apparent inability of these heavily infected scallops to extract sufficient energy from available food levels to maintain routine metabolic energy costs may be due to the presence of the pathogen. The large numbers of spores located throughout the digestive tubules may have prevented the processing of ingested food as gut contents indicate feeding was occurring.

## DISCUSSION

We have identified the protozoan parasite present in the calico scallops as being an ascetosporan of the genus *Marteilia*. This determination was based upon a comparison with the published descriptions of the genus *Marteilia* and its constituent species (Perkins 1976, Perkins and Wolf 1976, Comps 1976, Comps et al. 1982, Comps 1985). Aspects leading to this conclusion include the formation of approximately 8 sporangia within the sporangiosori, the formation of 3 to 4 spore primordia within the sporangia, the presence of refringent bodies, the size of the respective stages, the location of the parasite within the tubules of the digestive diverticulum, the lack of hemocyte infiltration, and the epizootiological pattern observed. The failure to identify the presence of plasmodia which normally precede development of presporangial and spore stages is somewhat puzzling. It is possible that this stage was no longer present by the time scallops were sampled.

There are currently five species identified in the genus *Marteilia* (Figueras and Montes 1988). These species have been identified as pathogens primarily in oysters and mussels from Europe and Australia (Comps 1970, Wolf 1972, Alderman 1979).

The ascetosporan *Marteilia refringens* was the first species of *Marteilia* identified and studied (Comps 1970). *M. refringens* has been cited as the cause of mass mortalities in the edible oyster *Ostrea edulis* population of the Bretagne region of France since the early 1970's (Bonami et al. 1971, Grizel et al. 1974, Comps et al. 1975, Grizel 1983). The disease syndrome was initially termed Aber disease in reference to the estuaries in Bretagne where mortalities were first recorded, and was later named digestive gland disease in reference to the main infection site in the edible oyster (Figueras and Montes 1988).

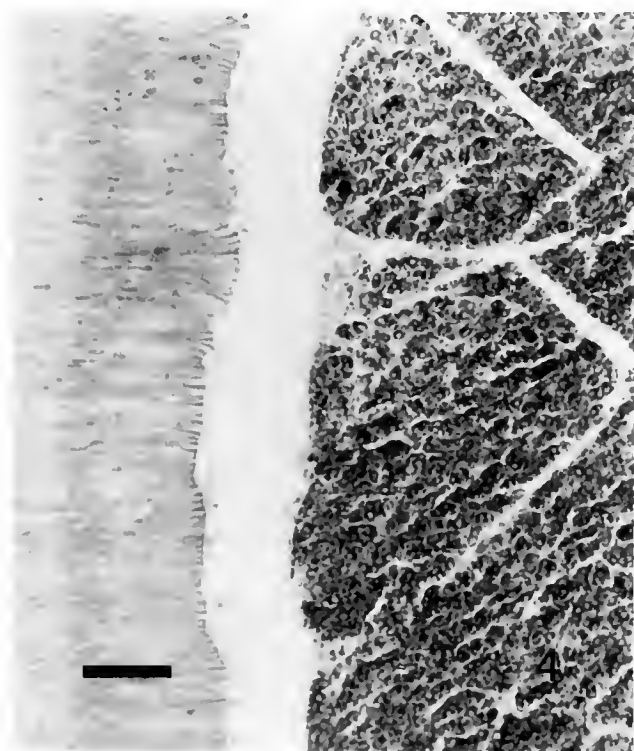


Figure 4. Intestine of a calico scallop exhibiting extensive occlusion of the lumen by mature spores of *Marteilia* sp. (Cason's Trichrome stain). There is no evidence of penetration into the intestinal wall nor of a host hemocyte response by the scallop (scale bar is 50  $\mu\text{m}$ ).

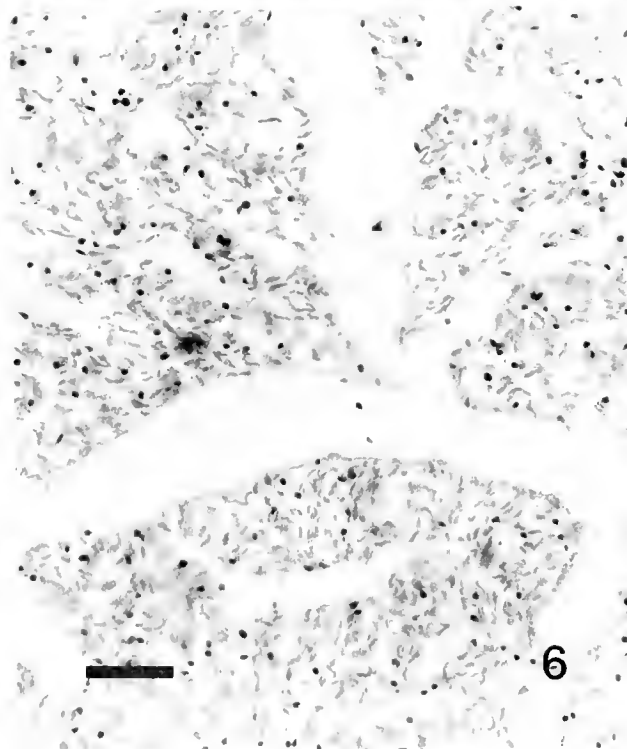
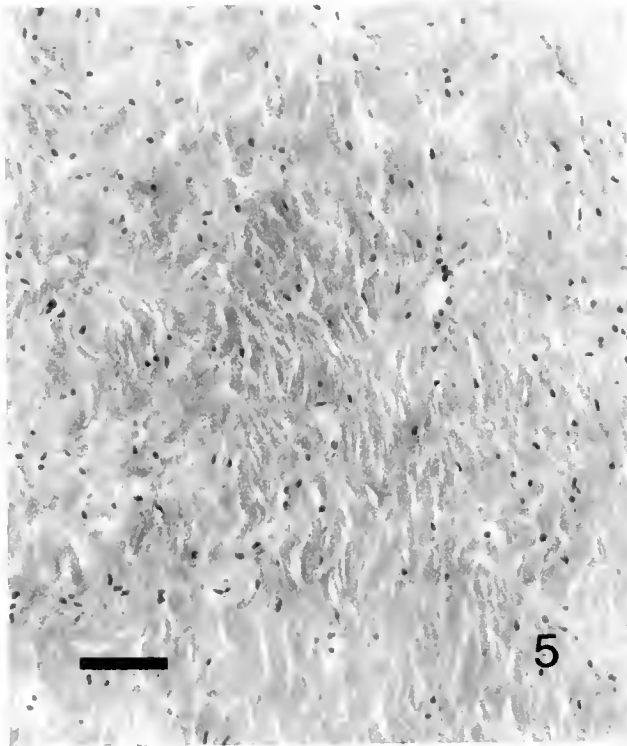


Figure 5. Adductor muscle tissue of a healthy calico scallop collected off Cape Canaveral, Florida (haematoxylin and eosin stain; scale bar is 50  $\mu$ m).

Figure 6. Adductor muscle tissue of a moribund calico scallop collected off Cape Canaveral, Florida infected by *Marteilia* sp. (haematoxylin and eosin stain). The muscle bundles exhibit extensive atrophy although the parasite was found predominately in the digestive diverticulum (scale bar is 50  $\mu$ m).

A tentative life cycle for *M. refringens* has been proposed by Grizel et al. (1974) and later revised by various authors (Lauckner 1983, Figueras and Montes 1988). As stated in Figueras and Montes (1988) primary infections are thought to occur by plasmidia in epithelia of the gut, the gills, or both. Sporangia mature in the lumina of the digestive diverticula and are discharged via the gut. How the edible oyster becomes infected and by what stage it becomes infected has not yet been determined. Experimental attempts to transmit the disease to healthy edible oysters in the laboratory have failed, although field experiments have been successful (Balouet 1979).

Aber disease results in a severe pathological response in edible oysters. Infected oysters become progressively emaciated, and the digestive gland becomes brown to pale yellow in color. When glycogen reserves have been depleted the mantle becomes translucent and shell growth ceases. The visceral mass also loses its pigmentation and may appear shrunken and slimy in heavily infected individuals (Figueras and Montes 1988).

In the edible oyster incidences of *M. refringens* infections as high as 100% have been reported for some of the estuaries in the Bretagne region of France (Bonami et al. 1971). Mortalities usually commence in May, peak in June through August, and diminish in the fall. Subclinical infections may persist throughout the winter and the surviving young plasmidia then reinitiate new clinical infections the following May (Balouet 1979). Factors influencing the timing of disease transmission are unknown. The potential spacial extent of disease transmission also is unknown. While it has been shown that transplanted oysters infected with *Marteilia* have transmitted the disease to indigenous stocks (Balouet 1979), Alderman (1979) has reported that *M. refringens* was able to become established in one site in Spain and infect indigenous oysters but was unable to cross 2 km of open water to infect an adjacent site. This despite the fact that the estuary is wholly marine, has little fresh water input and an adequate tidal exchange. Clearly there are factors involved which are not yet understood.

Three other species, *Mytilus edulis*, *Cardium edule*, and *Crassostrea gigas*, have been identified as possible hosts for *Marteilia refringens* (Comps et al. 1975, Gutiérrez 1977, Cahour 1979). The percentage of the populations exhibiting infection is quite low (2.0–10.0%) for these alternate species and the protozoan appears to have minimal effect upon them.

The second species of *Marteilia* discovered was *M. sydneyi*. This species has also been associated with massive mortalities in its hosts, the Sydney rock oyster, *Crassostrea commercialis*, and *C. echinata* in Australia (Wolf 1972, Perkins and Wolf 1976). No other species have been reported as possible hosts for this species. *M. sydneyi* appears to be especially virulent, with an incubation period of less than 60 days from early infection to death of the host (Figueras and Montes 1988). This is in contrast to *M. refringens* in which the edible oyster host does not exhibit mortality until perhaps one year or more after the initial infection. *M. sydneyi* has been linked with the loss of as much as 80% of the Sydney rock oyster during intense epizootic episodes in Queensland and New South Wales (Wolf 1972, 1979). Ultrastructural studies of this species has revealed that it typically contains 16 sporonts in the sporangiosorus (Perkins and Wolf 1976) instead of the 8 sporonts reported for the other four species of *Marteilia*.

*M. maurini* has been identified as a parasite in both *Mytilus galloprovincialis* (Comps et al. 1982) and *M. edulis* (Auffret and

Poder 1985). In *M. edulis* infection rates as high as 70% were observed. The infection rate for *M. galloprovincialis* is lower (35%) but it appears to cause an inflammatory response, massive mucus secretion and infiltration of hemocytes into the digestive diverticulum, responses which have not been reported in any of the other *Marteilia* hosts (Figueroa et al. 1991).

The remaining two species of *Marteilia* are each associated with only one host species to date. The parasite *M. lenghi* is found in the epithelium of the stomach in the digestive diverticulum of *Crassostrea cucullata* (Comps 1976). The most recently discovered species is *M. christenseni* a parasite found in the epithelium of the digestive diverticulum of *Scrobicularia piperata* (Comps 1985).

Delineation of *Marteilia* to the species level requires analysis of the ultrastructure utilizing electron microscopy. The transient nature of *Marteilia* in the calico scallop population, coupled with the extreme virulence of the pathogen which results in the decimation of the natural population in less than a month, has thwarted our efforts to obtain the necessary samples for ultrastructure analysis of this parasite. Some comparisons are, however, possible. It is possible to eliminate *M. sydneyi* because 16 sporonts are not observed in the sporangiosorus. We observed a sporangiosorus mean length of 17  $\mu\text{m}$  (range: 14 to 22  $\mu\text{m}$ ; N = 25). This is smaller than the mean sporangiosorus length reported for *M. refringens* of 21  $\mu\text{m}$  (range: 16 to 27  $\mu\text{m}$ ; N = 25) (Perkins and Wolf 1976) but not by a great deal. Since different fixatives were used the difference could be due to differing amounts of shrinkage.

The size of the mature spores is another comparison that can be made. The literature reports spore sizes for the 5 species of *Marteilia* as follows: *M. refringens* 2.6  $\mu\text{m}$ , *M. sydneyi* 2.7  $\mu\text{m}$ , *M. maurini* 2 to 3  $\mu\text{m}$ , *M. lenghi* 5 to 6  $\mu\text{m}$ , and *M. christenseni* 3.5 to 4.5  $\mu\text{m}$  when measured from histological sections (Perkins and Wolf 1976, Comps et al. 1982, Comps 1976, Comps 1985). The comparable figure from this research are 3.5 to 4.3  $\mu\text{m}$ . Based on this alone, *M. christenseni* would be the likely choice but the use of different fixatives in preparing the histological sections makes it impossible to rely too heavily upon such similarities. It should be noted that Perkins (1976) reported a range of 3.5 to 4.5  $\mu\text{m}$  for living, unfixed spores of *M. refringens*. Another possibility is that rather than extending the range and suitable hosts of a previously identified species we are dealing with an entirely new species.

Although the precise species remains undetermined, the sudden appearance of *Marteilia* in North American waters is the proximate cause for the epizootic disease which resulted in mass mortalities of the calico scallop in 1989 and 1991. It is possible that this parasite only becomes a factor when the animals are already experiencing stress due to other factors. The fact that we have no evidence pointing to this does not eliminate the possibility of synergistic effects from a variety of factors. Balouet (1979) has suggested that Aber disease, as with other shellfish diseases may be not so much a microbial disease as one arising from unfavorable physicochemical factors in the seawater. What these factors may be is entirely unknown. The pattern exhibited in the case of this epizootic disease follows the classic pattern of a marine shellfish population exposed to a pathogen with which it has had no previous experience and to which it was susceptible (Sindermann 1990). Although initial indications are that a smaller percentage of the population was involved in 1991 as compared to 1989 it is unclear at this point if any increased resistance has or will develop among the survivors or their offspring.

The rapid rebounding of the calico scallop population in 6 months as evidenced in 1989 may seem quite remarkable to those unfamiliar with the life history of this animal. The calico scallop has a total life span of only 18–24 months, reproductive maturity can be reached in as little as 71 days and spawning occurs both in the spring and in the fall (Miller et al. 1979, Blake and Moyer 1991). Scallops have a very high fecundity with each scallop producing 500,000 or more gametes. It therefore only takes the successful spawning of a relatively few scallops to generate millions of offspring. This in turn enables a very rapid increase in the size of the population in a very short period of time.

The *Marteilia* parasite appears to cause the death of the scallop by preventing it from obtaining sufficient nutrition from the water column possibly by interfering with the normal process of digestion in the tubules or by interfering with the biochemical transfer of stored nutrients. The presence of food within the gut indicates that the infected scallops were indeed still feeding but that the ingested food was not being digested perhaps due to disruption of normal physiological function of digestive epithelial cells. There is no evidence of inflammatory or other immunological responses by the scallop to the parasite. Death is too rapid however, especially in the heavily infected scallops, to be solely the result of starvation. What other factors may be involved are currently unknown.

The significant population reductions caused by *M. refringens* during epizootic episodes of Aber disease in *O. edulis* had extensive economic repercussions (Grizel 1983, 1985). The economic impact arose not only from the mortalities but also the loss of oyster tissue weight. A comparison of the total wet weights of healthy and diseased oysters showed that diseased oysters were lighter than healthy oysters by 25–30% for 18 month old oysters and 20–35% in two year old oysters (Morel and Tigé 1974, Figueroa and Montes 1988).

The mass mortalities reported in this paper have had a similar drastic economic effect upon the calico scallop industry in Cape Canaveral (Blake and Moyer 1991). Since the 1991 appearance of the parasite almost all of the producers have been forced out of business and the scallop fishing fleet which numbered upwards of 100 vessels each making 5 to 7 trips a week has been dispersed to other fisheries. The future viability of the calico scallop as a commercial species is unknown. It will however, in any case take years for production levels to return to the levels of the early 1980's even if there is no recurrence of the *Marteilia* parasite in the waters off Cape Canaveral. It is also unknown whether or not the calico scallop will develop increased resistance to this pathogen or whether chronic infections in the calico scallop will result in reduction of the size of the adductor muscle similar to the tissue loss that has been observed in the edible oyster.

This is the first reported incidence of *Marteilia* in North American waters as well as the first reported incidence of a member of the family Pectinidae serving as a host for this genus of ascetosporeans. We do not know the source of this protozoan. All of the histopathological evidence suggests that it was not present in calico scallops off Cape Canaveral prior to 1988. This suggests that it is a newly arrived species but the mode of its transportation is unknown. Shellfish relocation is not utilized in the calico scallop industry nor among any other shellfish aquaculture industries in the affected areas so that vector can be disregarded. There is considerable freighter traffic from all over the world using ports on Florida's east coast including Cape Canaveral. It is conceivable

that bilge waters from some of these freighters could have transported the protozoan into the area. While this is an easy speculation to make it is virtually impossible to either prove or disprove. It is also unknown if this parasite has infected other molluscan species of the western Atlantic or what other species from this area may serve as hosts.

#### ACKNOWLEDGMENTS

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## COMMERCIAL LENGTH, CATCH/EFFORT, AND LANDINGS OF SOFT-SHELL CLAMS (*MYA ARENARIA*) FROM AN UNDUG INTERTIDAL POPULATION AT MACHIASPORT, MAINE

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**ABSTRACT** An undug population of soft-shelled clams, *Mya arenaria*, in the Machias River at Machiasport, Maine, was opened to depuration digging on 33 low tides between 2/7/86-8/4/86. Information on catch/effort, and landings was recorded for all 33 tides. Commercial size information was collected on 18 tides. Diggers removed between 644-4,923 lbs/tide and a total of 65,692 lbs. The mean length of clams harvested per tide varied between 59.03-72.57 mm with an overall weighted mean of  $66.63 \pm 1.17$  mm ( $\bar{x} \pm 1SE$ ). The commercial length frequency distribution varied between 38-100 mm and 3.09% of the commercial catch was less than 2 in. (51 mm). An exponential relationship exists between mean commercial clam length harvested and the actual low tide level recorded at Cutler, Maine (U.S. Navy radio station). Clams were dug at a mean rate of  $64.99 \pm 1.11$  ( $\bar{x} \pm 1SE$ ) lbs/hour. When both digging and picking were employed, clams were harvested at a mean rate of  $108.63 \pm 4.99$  lbs/hr. Digging gear was described from measurements taken on 12 commercial hoes used in the harvest of *Mya arenaria* from the study area.

### INTRODUCTION

The soft-shelled clam, *Mya arenaria* is extensively harvested by commercial clam diggers along the Maine coast. Between 1975 and 1991, soft-shelled clam landings have varied between 1,546,000-7,835,000 lbs. The landed value of these clams increased to a maximum of \$12,132,000.00 in 1985 and then decreased to \$5,004,000.00 by 1991. Value per pound has increased from \$1.18 to \$3.52 during this period (R. Lewis,\* pers. comm.).

Occasionally, moderately polluted clam-producing areas are opened to commercial depuration digging after bacterial contamination has been reduced through pollution abatement. Clams harvested from these areas are closely monitored during harvest, transport, and the depuration process. The present study was undertaken because the opening of a "depuration" area, where harvesting had been prohibited for 27 years, offered a unique opportunity to collect very reliable information on commercial size, catch/effort, and landings from a previously undug intertidal population of *Mya arenaria*.

### METHODS

#### The Study Area

The 29 acre study area is located on the Machias River at Machiasport, Maine (Fig. 1). It is subjected to a mean tidal range of 12.6 ft (U.S. Dept. Commerce 1986). Sediments in this area consist primarily of silt-clay with some sand and the presence of sawdust is obvious. The portion of the study area to be dug on each tide was clearly marked with yellow stakes as required by law. No portion was dug more than once and some portions were not dug because clams were not present. The area was dug on 33 tides between 7 Feb 1986-4 August 1986 and the majority (32 tides) were dug between 9 May 1986 and 30 July 1986.

#### Sources of Digging and Depuration Information

An authorized representative, employed by the depuration plant and approved by the State of Maine, Department of Marine Resources (DMR) was responsible for overseeing harvesting activity in the depuration area, and the transportation of shellfish to the depuration plant. Information on the numbers of diggers, the number of pounds dug, and the digging time per digger, was obtained from this representative. Digging time was initiated the moment diggers disembarked from their boats and began digging, and concluded when they returned to their boats and departed. The estimate of digging time was simplified by the fact that most diggers embarked by boat, and arrived at and departed from the digging site as a unit. Therefore, digging time was usually the same for all diggers on a given tide.

Information on clam breakage within the plant and the value per bushel was obtained from the plant manager. Prior to 20 May 1986, the plant manager recorded breakage before and after clams were immersed in the depuration tanks. This was possible because only clams dug from the "depuration" area at Machiasport were being subjected to depuration at that time. After that date, it was only possible to obtain actual breakage information before depuration because clams from several depuration areas were being processed simultaneously and different lots were mixed together into shipping crates after 48 hours. We conservatively estimated the final clam breakage at half the initial breakage because depuration plant managers had already determined that the final breakage was approximately one half to one third the initial breakage (D. Thurlow,† and D. Trask,‡ pers. comm.). The value per bushel was recorded for both the depuration plant and the digger. The \$6 difference is a transportation fee from the digging site to the depuration plant. The value per pound is an estimate derived from the

†Thurlow, D., Depuration plant owner-operator, Thurlow Shellfish Inc., Scarborough, Maine 04074.

‡Trask, D., Depuration plant owner-operator, Superior Shellfish Inc., Searsport, Maine 04974.

\*Lewis, R. Marine Resources Scientist, Maine Dept. of Marine Resources, Augusta, Maine 04430, personal communication, April 1992.

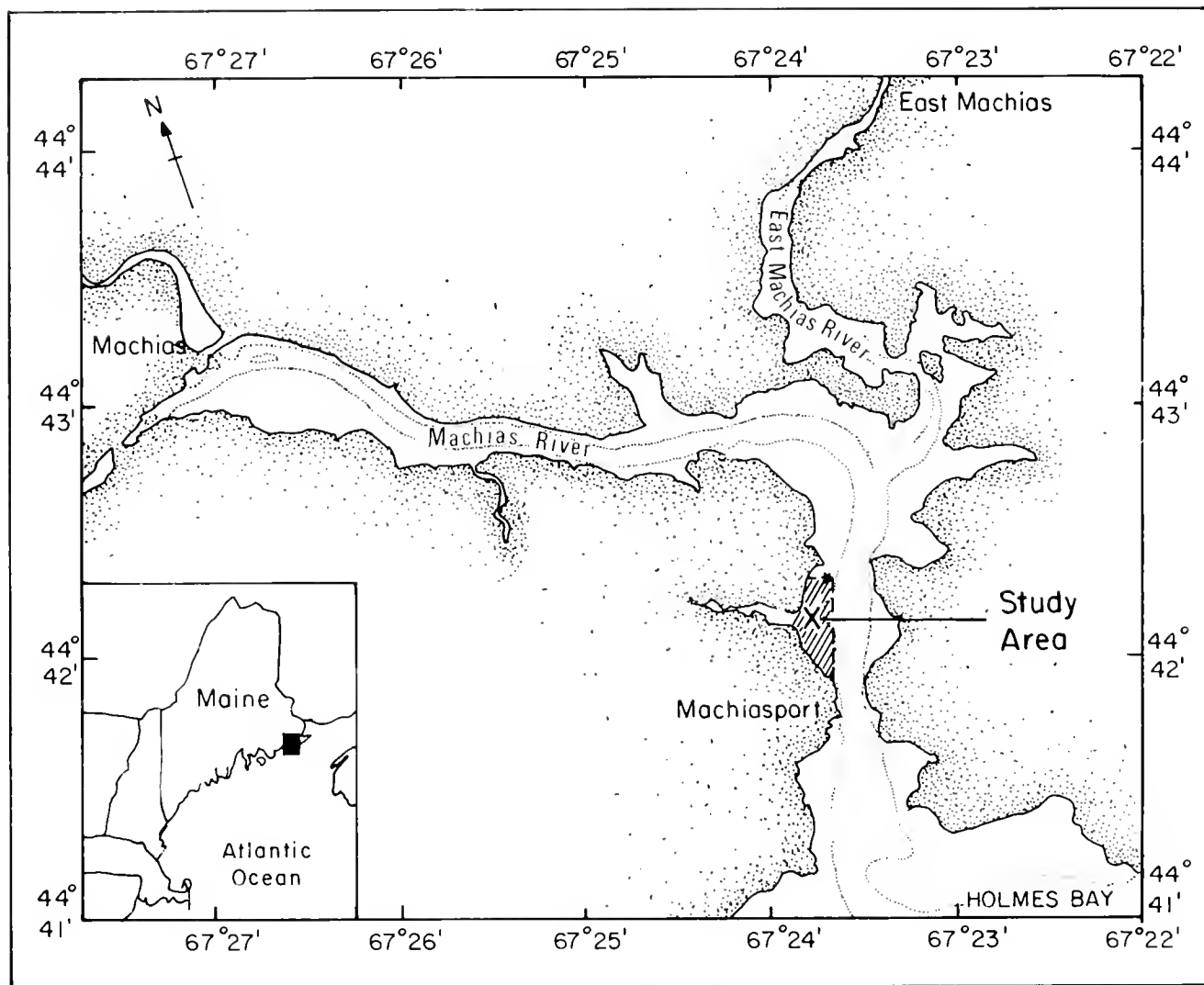


Figure 1. The study area at Machiasport.

value/bushel divided by 55 (deputation plant managers use a standard 55 lbs/bushel).

The numbers of bushels landed were derived from the total number of lbs landed divided by the actual weight per bushel for Machiasport clams. The value of  $53.58 \pm .28$  lbs/bushel ( $\bar{x} \pm 1$  SE) was derived on 5/14/86 after weighing 12 separate bushels of clams packed for shipment at the Searsport deputation facility.

#### Tide Levels

Tide gauge information required to establish the relationship between mean commercial clam size and the low tide height during harvest, was obtained from the U.S. Navy radio station at Cutler, Maine, approximately five and one-half nautical miles from the study area (Hubbard, §, pers. comm.).

#### Length Frequency Information

Commercial length frequency information was collected at the deputation plant on 18 of 33 tides dug. An attempt was made to collect length information for a range of low tide heights. Thirty-five clams were measured from each of 10, one-half bushel wire containers by systematically sampling every 1st, 2nd, 3rd, etc. container in the upper tier of clam containers immersed in the deputation tanks. The clam sample was removed from the corner of the clam containers, in clockwise fashion as individual containers were sampled.

#### Statistics

Estimates of the clam sample size required from each one-half bushel container, and the number of containers to be sampled, were derived using samples of deputation clams and the methodology of Snedecor and Cochran (1967). The results indicate that a sample of 35 clams from each one-half bushel container would yield an acceptable error of 5% about the mean. Measurements from 10, one-half bushel containers would yield an acceptable

§Hubbard, J. R., Chief, Tidal datum quality assurance section, Sea and Lake Levels Branch, U.S. Dept. of Commerce/NOAA, 11420 Rockville Pike, Rockville, Maryland 20852. pers. comm. Oct. 1986.



TABLE 1.  
Landing statistics collected from depuration facilities.

Landings Statistics									
1 1986 (Date)	2 Total # Diggers	3 Total Amt. Dug		4 Total Amt. Shipped		5 Breakage before Depur.		6 Breakage after Depur.	
		(Lbs.)	(Bush.)	(Lbs.)	(Bush.)	(Lbs.)	(Bush.)	(Lbs.)	(Bush.)
1. 2/7	15	2968	55.39	2411.5	45.00	371 est	6.92 est	185.5 est	3.46 est
2. 5/9	9	1614	30.12	1420.5	26.51	129 est	2.41 est	64.5 est	1.20 est
3. 5/12	11	1105	20.62	1004.5	18.74	67 est	1.25 est	31.5 est	0.63 est
4. 5/13	12	1520	28.37	1473.5	27.50	31 est	0.58 est	15.5 est	0.29 est
5. 5/14	10	1360	25.38	1277.5	23.84	55 est	1.03 est	27.5 est	0.51 est
6. 5/17	9	929	17.34	872.0	16.28	38 est	0.71 est	19.0 est	0.35 est
7. 5/19	14	1911	35.67	1795.5	33.51	77 est	1.44 est	38.5 est	0.72 est
8. 5/20	12	2135	39.85	2009.0	37.50	84 est	1.57 est	42.0 est	0.78 est
9. 5/22	14	2553	47.65	2419.5 est	45.16 est	89	1.66	44.5/est	0.83 est
10. 5/24	14	2621	48.92	2526.5 est	47.16 est	63	1.18	31.5 est	0.59 est
11. 5/26	12	2622	48.94	2521.5 est	47.06 est	67	1.25	33.5 est	0.63 est
12. 5/29	12	1531	28.57	1471.0 est	27.45 est	40	0.75	20.0 est	0.37 est
13. 5/30	12	1436	26.80	1401.5 est	26.16 est	23	0.43	11.5 est	0.21 est
14. 6/6	19	1394	26.02	1346.0 est	25.12 est	32	0.60	16.0 est	0.30 est
15. 6/7	16	1868	34.86	1811.0 est	33.80 est	38	0.71	19.0 est	0.35 est
16. 6/10	16	1519	28.35	1474.0 est	27.51 est	30	0.56	15.0 est	0.28 est
17. 6/12	15	1509	28.16	1464.0 est	27.32 est	30	0.56	15.0 est	0.28 est
18. 6/15	12	1064	19.85	1007.0 est	18.79 est	38	0.71	19.0 est	0.35 est
19. 6/17	17	1650	30.80	1581.0 est	29.51 est	46	0.86	23.0 est	0.43 est
20. 6/21	27	3418	63.81	3289.0 est	61.38 est	86	1.51	43.0 est	0.80 est
21. 6/23	25	4923	91.88	4785.0 est	89.31 est	92	1.72	46.0 est	0.86 est
22. 6/24	27	4344	81.08	4213.5 est	78.64 est	87	1.62	43.5 est	0.81 est
23. 6/25	28	3174	59.24	3078.0 est	57.45 est	64	1.19	32.0 est	0.60 est
24. 6/28	17	1771	33.05	1729.0 est	32.27 est	28	0.52	14.0 est	0.26 est
25. 6/30	23	1887	35.22	1857.0 est	34.66 est	20	0.37	10.0 est	0.19 est
26. 7/3	16	864	16.13	834.0 est	15.57 est	20	0.37	10.0 est	0.19 est
27. 7/6	10	664	12.02	620.0 est	11.57 est	16	0.30	8.0 est	0.15 est
28. 7/8	10	685	12.78	665.5 est	12.42 est	13	0.24	6.5 est	0.12 est
29. 7/20	17	1204	22.47	1160.5 est	21.66 est	29	0.54	14.5 est	0.27 est
30. 7/21	21	2263	42.24	2168.5 est	40.47 est	63	1.18	31.5 est	0.59 est
31. 7/22	27	3212	59.95	3060.5 est	57.12 est	101	1.89	50.5 est	0.94 est
32. 7/25	31	3155	58.88	2947.5 est	55.01 est	138	2.58	69.5 est	1.30 est
33. 7/30	17	839	15.66	813.5 est	15.18 est	17	0.32	8.5 est	0.16 est
		65692	1266.07	62508.5	1166.63	2122	39.53	1061.5	19.8

Columns 3, 4, 5, 6, 7 (Bushels) = Columns 3, 4, 5, 6, 7 (Lbs.) ÷ 53.58 Lbs/Bushel (Actual Weight/Bushel).

Column 4 = Column 3 - Column 7.

Column 7 = Column 5 + Column 6 or Column 3 - Column 4.

Column 8 = Column 7 ÷ Column 3.

Column 10 = Column 9 ÷ 55.00 Lbs/Bushel (Dealer's Weight/Bushel).

Column 11 = Column 3 (Lbs) × Column 10.

error of 10% about the mean. An error of 5% could be achieved by sampling from approximately 25 one-half bushel containers but sometimes that quantity was not available and the time required to sample from that many containers was considered impractical. Mean clam lengths ( $\pm 1$  SE) derived from approximately 350 clams measured on each of 18 tides sampled, and the overall weighted mean length (all samples combined) were also computed using the methodology of Snedecor and Cochran (1967). Ratios of two variables (catch/effort data expressed as lbs dug/hour or lbs dug and picked/hour) and overall ratio estimates conform to the methodology of Cochran (1963). The overall length frequency distribution was derived from length/frequency data collected from approximately 350 clams sampled on each of 18 tides. First, the total number of clams dug on each tide was estimated from knowl-

edge of the mean weight of 50 clam lots (obtained from each of 5 randomly selected one-half bushel containers submerged in the depuration tanks) and the overall weight of all clams landed on that tide. This value was then divided by the total number of clams sampled for length determination on each tide (approximately 350 clams) to compute a "rising factor." The numbers of clams in each length frequency size increment (mm) were then multiplied by the rising factor to derive an overall length frequency distribution for all clams dug on each of the 18 tides. Finally, all 18 corrected length frequency distributions were combined to derive an overall length frequency distribution. The numbers of individuals in each size increment were recorded as percent occurrence. The relationship of tide height to mean clam size harvested was established. The range of low tide heights encountered ( $-69.2$  cm

TABLE 1.  
continued

Landings Statistics								
7 Total Breakage		8 Breakage (%)	9 \$ Value/Bush.		10 \$ Value/Lb.		11 \$ Total Landed Value	
(Lbs.)	(Bush.)		(Digger)	(Depur. Fac.)	(Digger)	(Depur. Fac.)	(Digger)	(Depur. Fac.)
556.5	10.39	18.75	28.00	34.00	0.51	0.62	1513.68	1840.16
193.5	3.61	11.99	16.00	22.00	0.29	0.40	468.06	645.60
100.5	1.88	9.10	16.00	22.00	0.29	0.40	321.56	447.00
46.5	0.87	3.06	18.00	24.00	0.33	0.44	501.60	668.80
82.5	1.54	6.07	22.00	28.00	0.40	0.51	544.00	693.60
57.0	1.06	6.14	22.00	28.00	0.40	0.51	371.60	473.79
115.5	2.16	6.04	26.00	32.00	0.47	0.58	903.38	1108.38
126.0	2.35	5.90	26.00	32.00	0.47	0.58	1007.72	1238.30
133.5 est	2.49	5.23	26.00	32.00	0.47	0.58	1204.80	1480.48
94.5 est	1.76	3.61	28.00	34.00	0.51	0.62	1334.32	1620.25
100.5 est	1.88	3.83	28.00	34.00	0.51	0.62	1354.96	1625.64
60.0 est	1.12	3.92	28.00	34.00	0.51	0.62	779.28	949.22
34.5 est	0.64	2.40	30.00	36.00	0.55	0.65	783.27	993.40
48.0 est	0.90	3.44	32.00	38.00	0.58	0.69	811.05	963.13
57.0 est	1.06	3.05	32.00	38.00	0.58	0.69	1086.84	1290.62
45.0 est	0.84	2.96	34.00	40.00	0.62	0.73	938.89	1104.72
45.0 est	0.84	2.98	34.00	40.00	0.62	0.73	932.83	1097.45
57.0 est	1.06	5.36	34.00	40.00	0.62	0.73	657.74	773.74
69.0 est	1.29	4.18	34.00	40.00	0.62	0.73	1019.99	1199.88
129.0 est	2.41	3.77	34.00	40.00	0.62	0.73	2112.94	2485.82
138.0 est	2.58	2.80	34.00	40.00	0.62	0.73	3043.30	3580.36
130.5 est	2.44	3.00	34.00	40.00	0.62	0.73	2685.37	3159.27
96.0 est	1.79	3.02	36.00	42.00	0.65	0.76	2077.52	2423.78
42.0 est	0.78	2.37	38.00	44.00	0.69	0.81	1223.60	1416.80
30.0 est	0.56	1.59	40.00	46.00	0.73	0.84	1372.36	1578.22
30.0 est	0.56	3.47	44.00	50.00	0.80	0.91	691.20	785.45
24.0 est	0.45	3.73	44.00	50.00	0.80	0.91	515.20	585.45
19.5 est	0.36	2.85	44.00	50.00	0.80	0.91	584.00	622.66
43.5 est	0.81	3.61	46.00	52.00	0.84	0.95	1006.98	1138.26
94.5 est	1.76	4.18	46.00	52.00	0.84	0.95	1892.69	2139.44
151.5 est	2.83	4.72	46.00	52.00	0.84	0.95	2686.40	3036.80
207.5 est	3.87	6.58	46.00	52.00	0.84	0.95	2638.84	2982.74
25.5 est	0.48	3.04	46.00	52.00	0.84	0.95	701.66	793.19
3183.5	59.42	4.75					39767.63	46877.4

below and +57.3 cm above mean low water), was rescaled and numbered sequentially 0 (-70 cm) to 13 (+60 cm) to simplify fitting the data with linear, exponential, power, and logarithmic equations (Texas Instruments<sup>11</sup>). The best fit was obtained with an exponential equation. Both low tide heights and rescaled values (0-13) are presented on the x-axis in Figure 4.

## RESULTS AND DISCUSSION

### Landing Statistics

A complete summary of the landing statistics collected on each of the 33 tides dug is presented in Table 1. Table 1 shows that between 9-31 diggers dug on a given tide. These diggers removed between 644-4,923 lbs of clams per tide and a total of 65,692 lbs (1226.07 bushels).

Total breakage of clams within the depuration plant was approximately 3,183.5 lbs with a mean breakage per tide of 4.75%.

The high breakage (18.75%) reported on 2/7/86 probably resulted from freezing during transport and the long transportation distance between the harvesting area and the depuration plant at Scarborough, Maine. The relatively high breakage values reported during the month of May 1986 probably resulted from inadequate culling at the commercial harvesting site. Breakage was reduced to acceptable levels during June and July when culling practices improved. An overall breakage of 4.75% is about half the breakage of 10% reported by Medcof and MacPhail (1952) for clams transported in wooden hods to shucking plants. This difference is probably insignificant when the multitude of factors affecting breakage is considered. Dow et al. (unpublished)<sup>12</sup> reports that breakage of clams transported by diggers is affected by shell thickness, clam density, sediment type, weather, unfavorable digging conditions, and digger handling practices.

<sup>11</sup>Texas Instruments. Applied statistics. Solid state library module 1977.

<sup>12</sup>Dow, R. L., D. E. Wallace and L. N. Taxiarchis 1954. Clam breakage in Maine. Maine Dept. Sea and Shore Fish. Res. Bull. No. 15, Augusta, 4 p.

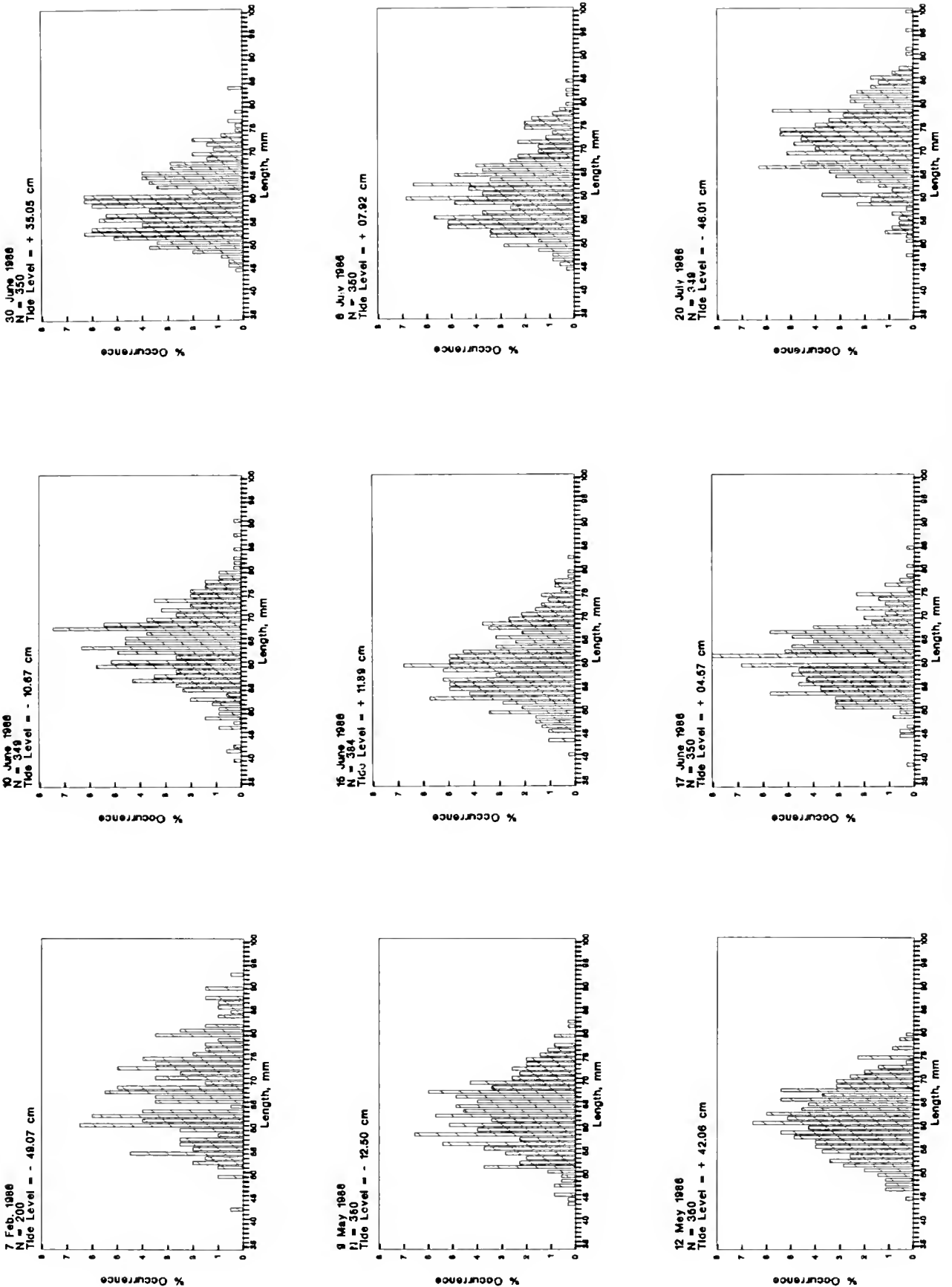


Figure 2. Commercial length frequency distributions from each tide sampled.

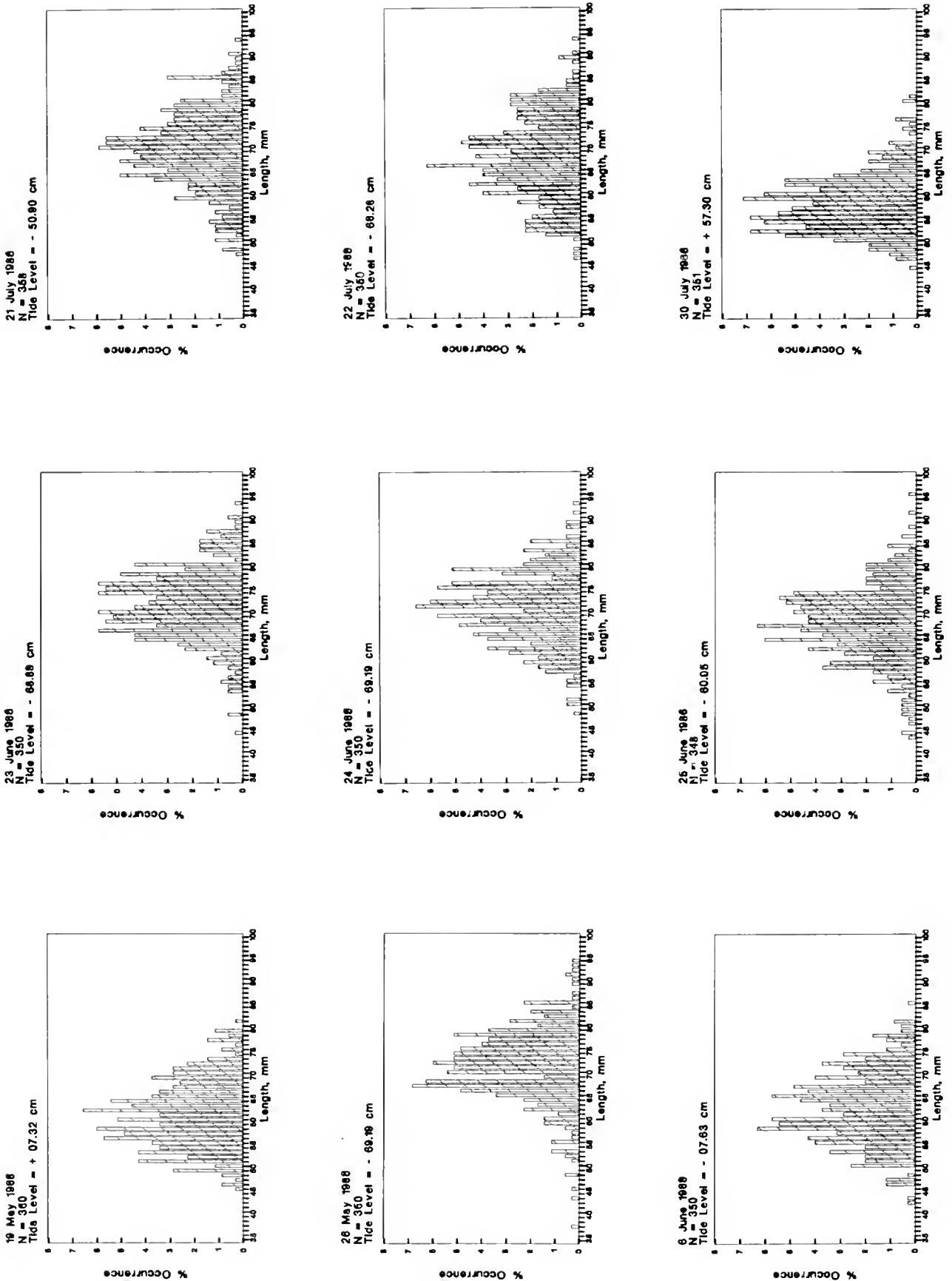


Figure 2. Continued.

Table 1 shows that the value per bushel varied over time. The relatively high value reported on 7 Feb. 1986 probably resulted from low clam availability. The low value reported at the beginning of May probably resulted from low demand for clams which were abundant. The value per bushel continued to climb throughout May, June, and July 1986 because of an increased demand for clams. The total landed value to the diggers was \$39,767.63.

#### Commercial Length Information

Length information was collected from commercial clams harvested on 18 tides between 7 Feb. 1986–30 July 1986 (Table 2). The mean length of these clams varied between 59.03–72.57 mm with a weighted overall mean ( $\pm 1$  SE) of  $66.63 \pm 1.17$  mm. Commercial length frequency distributions from each tide sampled, are presented in Figure 2. The normal distributions of these data are visually obvious. Length frequency distributions were corrected for the total clams dug on each tide and the results were combined. The overall length frequency distribution is presented in Figure 3. Figure 3 shows that the commercial length frequency

distribution varied between 38–100 mm with 3.09% of the commercial catch less than 2 inches in length (minimum legal size). Mean commercial clam lengths were plotted against actual low tide levels of +57.30 cm (+1.88 ft) to -69.19 cm (-2.27 ft) above or below mean low water in Figure 4. The highest correlation coefficient ( $\rho = .93494$ ) was obtained with an exponential regression. Figure 4 shows that, in an undug population, an inverse relationship exists between mean commercial clam size harvested and the level of the clam flat dug. Clam diggers apparently dig that portion of the flat immediately adjacent to the low tide level on each tide dug. Several investigators have reported a higher growth rate for *Mya arenaria* located near the low tide level (Newcombe 1936, Dow and Wallace 1961, Newell 1982a). This probably occurs because clams in this region are inundated longer and are therefore able to pump and obtain food over a longer period of time. Wilton and Wilton (1929) have also demonstrated that clams constantly submerged grow better than intertidal clams because they can feed constantly. Other factors which are frequently interrelated with intertidal height and which also affect growth (and size) include currents (Belding 1930), salinity (Belding 1930, Shi

TABLE 2.  
Mean length and catch/effort information collected from depuration facilities.

1986	Time of Low Tide*	Actual Tide Level**		Clam Length (mm)		Dig. Time Each Digger (Hrs.)	No. Diggers	Lbs. Dug/Hr.		No. Digger Pickers	Lbs. Dug-Picked/Hr.		
		(ft)	(cm)	$\bar{X}$	$\pm 1$ SE			$\bar{X}$	$\pm 1$ SE		$\bar{X}$	$\pm 1$ SE	
1.	2/7	1533	-1.61	-49.07	67.95	0.68	2.75	15	71.95	4.79	—	—	—
2.	5/9	0500	-0.41	-12.50	63.24	0.39	1.33	9	134.84	15.16	—	—	—
3.	5/12	0656	+1.38	+42.06	61.75	0.37	0.75	11	133.00	7.84	—	—	—
4.	5/13	0738	+1.09	+33.22	—	—	2.00	12	63.33	7.85	—	—	—
5.	5/14	0823	+0.52	+15.85	—	—	2.10	10	64.76	4.47	—	—	—
6.	5/17	1057	+0.90	+27.43	—	—	1.42	9	72.69	8.19	—	—	—
7.	5/19	1243	+0.24	+07.32	62.06	0.40	1.97	14	69.29	6.77	—	—	—
8.	5/20	1334	-0.37	-11.28	—	—	2.00	12	88.96	11.58	—	—	—
9.	5/22	1515	-0.87	-26.52	—	—	2.17	12	77.23	7.74	2	135.71	12.21
10.	5/24	0437	-2.34	-71.32	—	—	2.25	12	73.19	11.22	2	143.33	16.67
11.	5/26	0623	-2.27	-69.19	72.33	0.44	2.17	11	93.76	10.41	1	176.96	—
12.	5/29	0911	-0.73	-22.25	—	—	2.13	10	55.02	5.45	2	84.27	8.22
13.	5/30	1011	-0.19	-05.79	—	—	2.08	12	66.75	7.04	—	—	—
14.	6/6	0356	-0.25	-07.62	63.69	0.43	1.25	19	43.93	3.66	—	—	—
15.	6/7	0435	-0.35	-10.67	—	—	1.50	16	76.31	7.17	—	—	—
16.	6/10	0634	-0.35	-10.67	64.69	0.43	1.75	16	54.25	5.03	—	—	—
17.	6/12	0757	+0.36	+10.97	—	—	2.33	15	43.18	5.52	—	—	—
18.	6/15	1018	+0.39	+11.89	59.91	0.41	4.00	12	22.17	3.14	—	—	—
19.	6/17	1204	+0.15	+04.57	61.56	0.38	2.43	17	39.94	3.59	—	—	—
20.	6/21	0326	-2.10	-64.01	—	—	1.75	25	67.63	5.40	2	131.14	9.43
21.	6/23	0516	-2.26	-68.88	72.47	0.42	2.08	23	89.17	6.74	2	157.93	16.11
22.	6/24	0609	-2.27	-69.19	71.68	0.42	2.00	25	75.22	5.25	2	145.75	13.75
23.	6/25	0701	-1.97	-60.05	68.29	0.45	1.83	26	57.29	4.50	2	122.40	13.11
24.	6/28	0943	+0.03	+00.91	—	—	1.83	17	56.93	4.61	—	—	—
25.	6/30	1130	+1.15	+35.05	59.54	0.38	1.80	23	45.58	3.75	—	—	—
26.	7/3	1407	+1.98	+60.35	—	—	0.67	16	80.60	4.99	—	—	—
27.	7/6	0410	+0.26	+07.92	61.49	0.42	1.17	10	55.04	4.15	—	—	—
28.	7/8	0532	+0.23	+07.01	—	—	1.33	10	51.50	6.57	—	—	—
29.	7/20	0312	-1.51	-46.01	72.57	0.44	1.33	12	43.30	3.86	5	77.14	9.18
30.	7/21	0408	-1.67	-50.90	69.89	0.45	1.08	15	93.15	9.47	6	116.36	20.41
31.	7/22	0502	-2.24	-68.28	68.59	0.45	1.58	20	69.65	7.33	7	91.32	16.15
32.	7/25	0731	-1.23	-37.49	—	—	1.83	26	51.43	3.88	5	77.38	7.15
33.	7/30	1144	+1.88	+57.30	59.03	0.35	2.15	17	22.99	2.18	—	—	—
					66.63	$\pm 1.17$	1.84		64.99	$\pm 1.11$		108.63	$\pm 4.99$

\* Eastport corrected for Machiasport (EST).

\*\* Recorded from the Cutler gauging station.

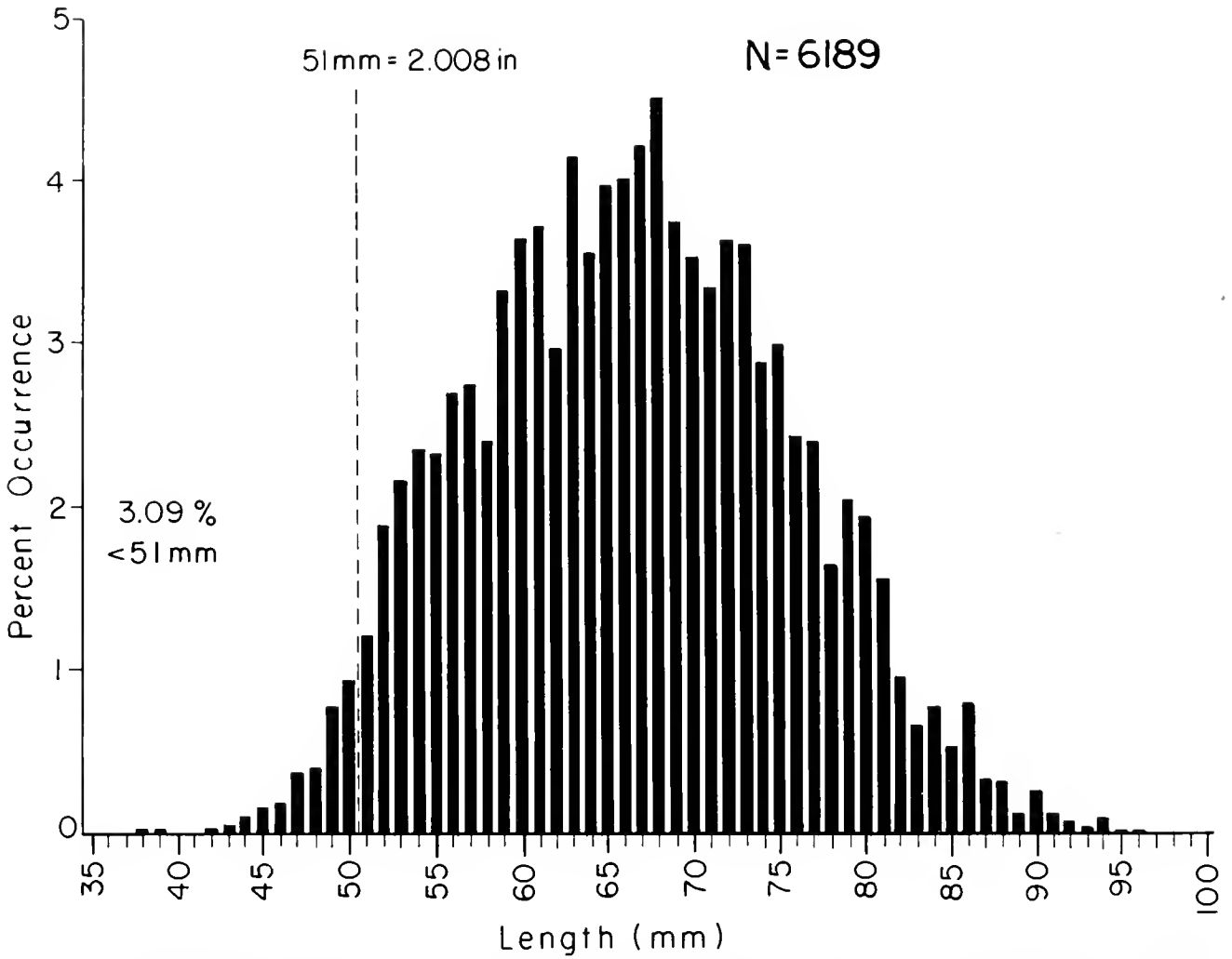


Figure 3. The corrected overall length frequency distribution for clams harvested during 18 tides from an undug flat at Machiasport, Maine.

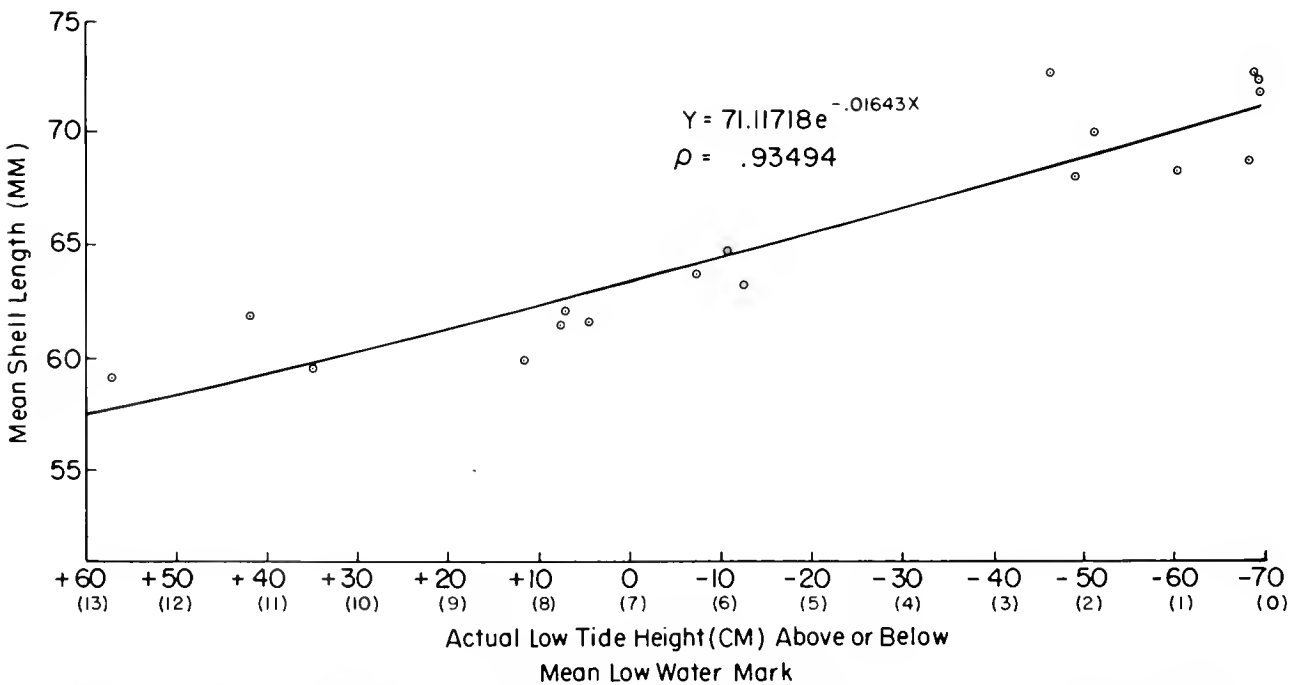


Figure 4. The relationship of mean commercial clam length to the actual low tide level above or below the mean low water mark.

TABLE 3.

A summary of measurements from clam hoes used in the harvest of clams from the Machiasport study area.

No. Hoes Measured	Tine Measurements ( $\pm 1$ SE)					Hoe Measurements ( $\pm 1$ SE)			
	Number	Length (cm)	Width (cm)	Shape	End	Width (cm)	Handle Length (cm)	Handle-tine Angle ( $^{\circ}$ )	Distance Handle-tine (cm)
12	4.50 $\pm 0.26$	24.13 $\pm 0.63$	1.43 $\pm 0.03$	100% Diamond	100% Round	20.68 $\pm 1.27$	32.88 $\pm 1.02$	55.17 $\pm 1.89$	29.41 $\pm 0.83$

1937, Swan 1952), and sediments (Kellogg 1905, Belding 1920, Newcomb 1935, Swan 1952, Dow and Wallace 1961, Newell 1982b).

#### Catch/effort Information

Table 2 shows that the average amount of time a digger dug on any one tide varied between 0.67–4.00 hours with an overall mean digging time per digger of 1.84 hours per tide. Clams were dug from the flat on 33 tides at a rate which varied between 22.17–134.84 lbs/hr. with a mean ( $\pm 1$  SE) of  $64.99 \pm 1.11$  lbs/hour. A combination of digging and "picking" on 12 tides yielded catch/effort values which varied between 77.14–176.96 lbs/hour with a mean ( $\pm 1$  SE) of  $108.63 \pm 4.99$  lbs/hour. The combination of digging and "picking" employed by some diggers was apparently a more effective method of harvesting clams.

Catch/effort data collected from "deuration" areas are not comparable to similar data collected from nonpolluted clam flats which are always open; deuration areas always produce above average yields/effort (Townsend 1985).

#### Clam Hoe Measurements

A description of the harvesting gear used by 12 of 23 diggers in the Machiasport study area on 30 June 1986, is presented in

Table 3. The description is probably typical of the gear used on all dates when deuration digging occurred because these same commercial diggers were employed throughout the study.

#### ACKNOWLEDGMENTS

We extend our appreciation to D. Trask (owner-operator of Superior Shellfish Inc., Searsport, Maine 04974) and D. Thurlow (owner-operator of Thurlow Shellfish Inc., Scarborough, Maine 04074) for recording information on numbers of diggers, lbs or bushels dug, value/bushel, breakage, and for allowing us to sample the deuration tanks for the length frequency distribution of the commercial catch. We also wish to thank J. Bridges (master digger for Superior Shellfish) for his estimates of digging time, T. Bridges for his explanation of digging and picking procedures, and J. Olson and B. Olson for informing local diggers of our various activities in the deuration area. Special appreciation is also extended to J. Rollins (Bigelow Laboratory) for photographic services, M. Hunter (DMR) for computer services, and P. Mancuso and L. Achor (DMR) for typing this manuscript. This research was conducted by the Maine Department of Marine Resources Research Laboratory, West Boothbay Harbor, Maine.

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## GROWTH AND MORTALITY OF NORTHERN QUAHOG, (LINNAEUS, 1758) *MERCENARIA MERCENARIA* IN PRINCE EDWARD ISLAND

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**ABSTRACT** Quahogs (*Mercenaria mercenaria*) from three locations in Prince Edward Island, Canada, were studied to determine growth and mortality levels in the northern limit of their natural geographical distribution. There were no significant differences among growth curves of these sites, although quahogs from Pownal Bay require an average of 13 years to reach legal commercial size (50 mm) compared to approximately 9 years in West River and Hillsborough River. A reciprocal transfer experiment showed that the differences in growth rates observed between quahogs from Pownal Bay and West River were site specific. Among environmental parameters evaluated from these two sites, chlorophyll *a* correlated well with growth. High mortality levels at the West River site are attributed to the presence of a nemertean worm, *Cerebratulus lacteus*.

**KEY WORDS:** quahog, *Mercenaria*, growth, mortality, reciprocal transfer, site evaluation

### INTRODUCTION

The commercial fishery for quahogs (*Mercenaria mercenaria*) in the Gulf of St. Lawrence, Canada, averaged 771 t annually between 1982 and 1992. The culture of quahogs in Atlantic Canada is still in the development phase and there are no estimates of production at this time. Most of the research and developmental work conducted has been concentrated in the southern portion (south of 47°30' lat.) of the Gulf of St. Lawrence, which represents the northern limit of the distributional range of *M. mercenaria*. Research on site evaluation, growth and mortality rates, culture techniques and other topics have been conducted in Atlantic Canada over the past 15 years to assist in the development of the culture of quahogs. Little is known, however, about the growth and mortality rates of wild quahogs and the factors influencing these two parameters in the southern Gulf of St. Lawrence. The purpose of this study is to evaluate growth rates of quahogs from three areas in Prince Edward Island, and to examine the potential genetic and environmental influence on growth and mortality of two different stocks of quahogs.

### MATERIALS AND METHODS

#### Study Sites

West River, Pownal Bay and Hillsborough River are located within a 10 km radius of Charlottetown in the central part of Prince Edward Island, and are three popular quahog fishing areas (Fig. 1). These three sites are part of the Hillsborough Bay system and have a tidal range of approximately 2-3 m. Pownal Bay is characterised as a large open inlet with a sandy bottom. The West River and Hillsborough River sites are partly closed estuaries with muddy bottoms. The vegetation at the three sites was similar with a mixture of eelgrass (*Zostera marina*) and sea lettuce (*Ulva lactuca*) as the predominant species.

#### Comparison of Natural Populations

In 1987, random samples were collected from each study site, at ten (10) stations divided equally along approximately 500 meters of shoreline and within water depths of 1 meter at mean low water (MLW). Each sample 1 m<sup>2</sup> was taken with a water suction

dredge which sampled to a depth of 15 cm. Live quahogs were enumerated and measured (shell length) to the nearest mm.

Sub-samples of fifty quahogs were collected from each site for age and growth rate determination. The right valve from each specimen was coated with epoxy glue and sectioned using a low speed geological saw. A thick (3 mm) section was made through the axis of maximum shell height. The age of each specimen was determined by enumerating the annuli on the valve margin and chondrophore.

Comparisons of growth rates among populations were done by comparing growth profiles estimated from polynomial regression analysis (Septon and Bryan 1990, Chouinard and Mladenov 1991). The independent variable (age) of the polynomial (cubic) regression was centered about the mean age of all samples used (mean X = 12 yr) to offset the effects of multicollinearity (Sokal and Rohlf 1981). Pairwise comparisons of growth profile were made by testing the equality of the independent data set regression coefficients with those of the combined data of the comparison.

#### Reciprocal Transfer

A reciprocal transfer experiment was performed to estimate the relative importance of genetical and environmental factors on growth and mortality of quahogs from the Pownal Bay and West River sites. One hundred quahogs, with a shell length between 35 and 45 mm, were collected from each site in May, 1990. Each specimen was tagged with a small (15 mm diameter) plastic disc attached to the right valve with epoxy glue. Fifty (50) quahogs from each site were then transferred to the alternate site while the remaining 50 were re-planted at their original site on a 1 m<sup>2</sup> unprotected experimental plot. Growth and mortality were evaluated in October, 1990, June and October, 1991. Each sampling exercise comprised of removing and measuring all the tagged quahogs from the experimental plots and replanting all the live specimens. In 1991, replicate water samples were collected in opaque containers one hour before low tide, once a month during the ice free season and analyzed for chlorophyll *a* and seston. Each sample was filtered within 6 hours of collection, on Whatman GF/F glass fibre filters with pore size ≈ 0.7 μm and frozen until analyzed. The seston filters were analyzed for Total Particulate Matter (TPM) after drying for 12 hours at 60°C, and Particulate Inorganic

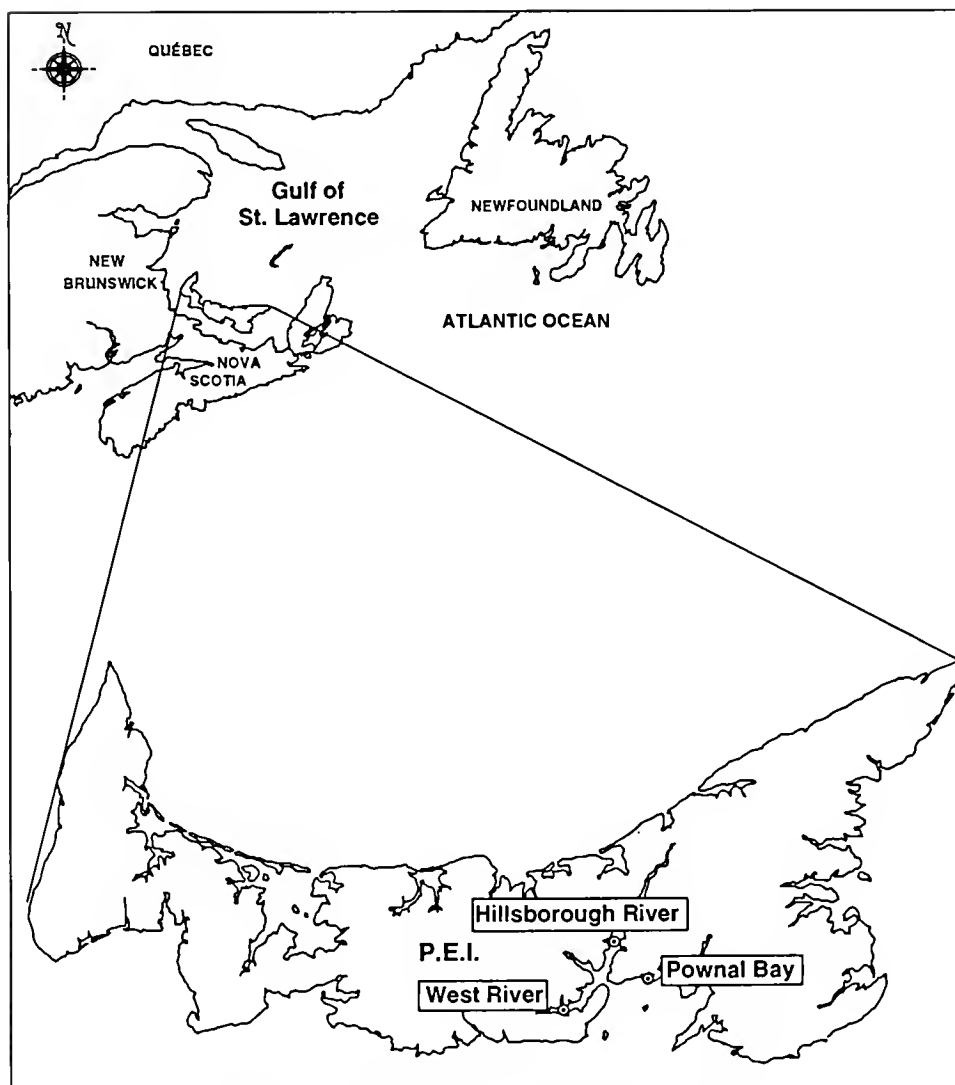


Figure 1. Sampling sites in Prince Edward Island, Canada.

(PIM)/Organic (POM) Matter after ashing at 450°C for 4 hours. Chlorophyll *a* levels were determined with a spectrophotometer using the method of Boto and Bunt (1978). Temperature and salinity were recorded during the water sampling. Sediment cores of approximately 15 cm deep were collected from each site and the sediment particle size distribution was analyzed according to Folk's methods (1968).

Growth data from this survey were analyzed using the repeated measures analysis of variance (ANOVA) from the General Linear Models (GLM) procedure (SAS 1982) to evaluate differences in shell length among treatments.

## RESULTS

### *Comparison of Natural Populations*

Quahog densities were significantly ( $p < 0.05$ ) greater in Pownal Bay with an average density of 16.4/m<sup>2</sup>, compared to 4.6/m<sup>2</sup> in West River and 5.8/m<sup>2</sup> in Hillsborough River. The size frequency distributions for the three populations showed a larger percentage of juvenile quahogs (length < 50 mm) in Pownal Bay with 95% of quahogs recovered smaller than 50 mm (legal size

limit) compared to 52% and 65% in West River and Hillsborough River respectively (Fig. 2).

A total of 105 quahogs were aged: 42 from Pownal Bay, 28 from West River and 35 from Hillsborough River. Pair-wise comparison of growth profiles resulting from the cubic polynomial regressions showed no differences ( $p > 0.05$ ) among quahogs from Hillsborough River, West River and Pownal Bay. Nonetheless, the resulting growth rate of quahogs from Hillsborough River was the highest with a Broody's growth coefficient (*k*) of 0.096 compared to 0.055 in West River and 0.033 in Pownal Bay (Fig. 3). From these data quahogs from Pownal Bay took an average of 13 years to reach the legal commercial size of 50 mm, compared to approximately 9 years for quahogs from West River and Hillsborough River.

### *Reciprocal Transfer*

The one hundred (100) quahogs collected from Pownal Bay for the reciprocal transfer experiment had an average length of 40.4 mm  $\pm$  1.73% (C.I. 0.95), while those from West River had an average length of 38.5 mm  $\pm$  1.32% (C.I. 0.95). Initial size variations among the 4 treatment groups of quahogs were not

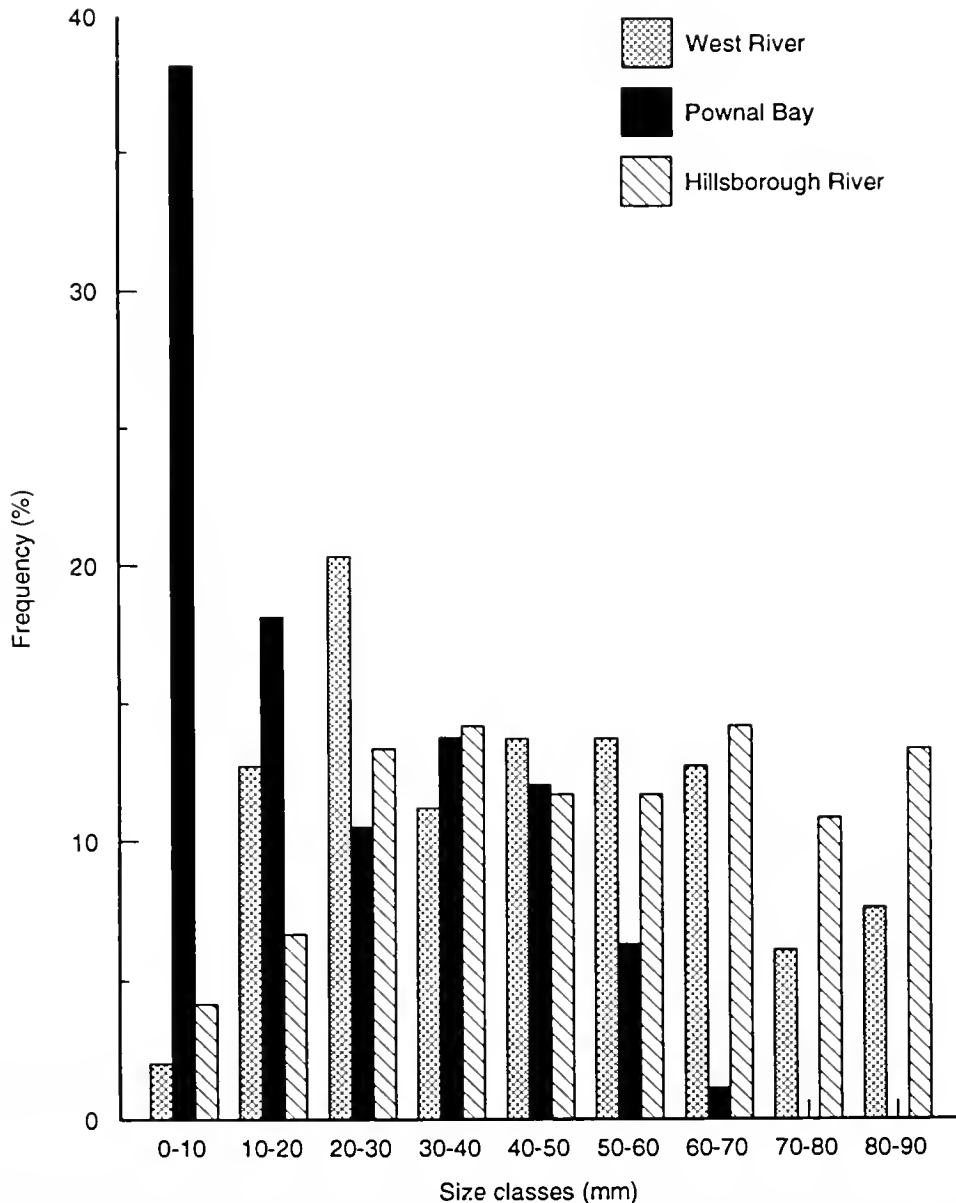


Figure 2. Size (length) class frequencies of quahogs from West River, Pownal Bay and Hillsborough River, Prince Edward Island.

significantly different ( $p > 0.05$ ). Specimens with incomplete growth records due to mortality or missing values in the course of the 18 month study were deleted from subsequent analysis on growth variations. Throughout the duration of this study quahogs planted in West River grew significantly ( $p < 0.05$ ) larger than those planted in Pownal Bay regardless of the source of the specimens thereby clearly showing the effect of environment influence over genetic influence (Fig. 4).

Several environmental factors were monitored at both sites during the 1991 field season (Table 1). The temperature profiles for the two sites were very similar throughout the 1991 field season, which ranged from lows of 8°C and 12°C in May and 6°C and 5°C in October, to highs of 24°C and 25°C respectively from West River and Pownal Bay. There was some variation in the salinity profiles between the two sites with West River having a lower salinity that ranged from 20 to 29 ppt compared to 22.5 to 30 ppt in Pownal Bay. The lower values recorded from West River may

be due to its larger drainage and catchment basin, and subsequent fresh water input in that system.

The sediment collected from the West River site had a significantly ( $p < 0.05$ ) higher silt/clay component (29.31%) and Total Organic Content (TOC) (3.74%), compared with the sediment from the Pownal Bay site (11.4% and 1.72%, respectively).

Seston estimates from Pownal Bay varied from 51 mg/l in July to 77 mg/l in May, while in West River they ranged from 62.7 mg/l in May to 408 mg/l in October. The July and October values from West River were significantly ( $p < 0.05$ ) higher than those from Pownal Bay. The overall percentage of particulate organic matter (POM) in the seston collected from the two experimental sites were approximately 18% in Pownal Bay and 14% in West River. Chlorophyll *a* estimates were also significantly ( $p < 0.05$ ) higher in West River than in Pownal Bay throughout the 1991 field season, with the October value from West River soaring to 15.8 µg/l.

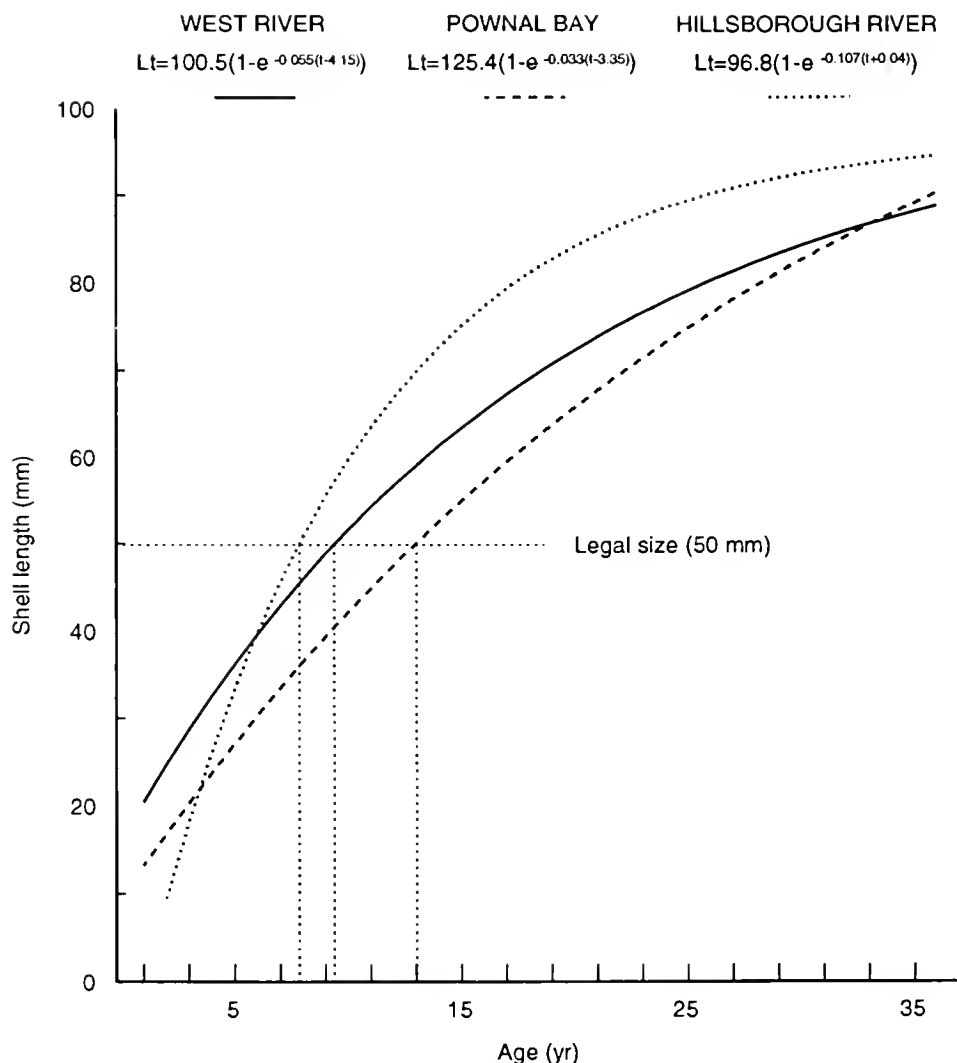


Figure 3. Growth curves of quahogs from West River, Pownal Bay and Hillsborough River. Also given are the parameters of the von Bertalanffy growth equations  $L_t = L_{\infty}(1 - e^{-K(t-t_0)})$ , where  $L_{\infty}$  is the mean asymptotic length,  $K$  is the Brody growth coefficient and  $t_0$  is the hypothetical age at which length is zero.

The overall survival levels were substantially higher in Pownal Bay than in West River (Table 2). In 1990, six months after the initial transplantation, survival was higher in the quahogs from Pownal Bay at both experimental sites. The highest mortality level was observed at the West River site, where 53.3% of the quahogs from West River died. In 1991, the best survival rates were again in Pownal Bay where over 76% of the quahogs from West River and Pownal Bay were found alive. At the West River study site over 50% of the quahogs from Pownal Bay survived while only 24% of those from West River survived.

#### DISCUSSION

The size frequency distributions from the three study sites (Fig. 2) suggest that the recruitment and fishing mortality is higher in Pownal Bay compared with those from Hillsborough River and West River. Although there are commercial fishery activities in the three studied areas, Pownal Bay receives a greater proportion of the overall fishing effort.

The higher recruitment pattern observed in Pownal Bay could be the result of one or more site specific biological or physical and chemical parameters. According to Scheltema (1974), the former has more impact than the latter. One of the striking biological parameters from the Pownal Bay site compared with the other two sites, is the higher density, which has been found by others to be negatively correlated to recruitment (Andre and Rosenberg 1991, Woodin 1980). The opposite was apparently demonstrated in our study. Other studies, however are more specific in their approach and have shown the relationship between density and recruitment in molluscs is better explained by looking at the density of adults (Best 1978, William 1980, Berthou and Glemarec 1988, Rice et al. 1989). The density of adult quahogs (shell length  $\geq 50$  mm) is estimated to be 2.46/m<sup>2</sup> in Pownal Bay, 2.20/m<sup>2</sup> in West River and 2.03/m<sup>2</sup> in Hillsborough River. This would suggest that recruitment in Pownal Bay is enhanced by factors other than density. Other biological parameters that are known to have an impact on recruitment, such as predator composition and abundance, were not evaluated in this study.

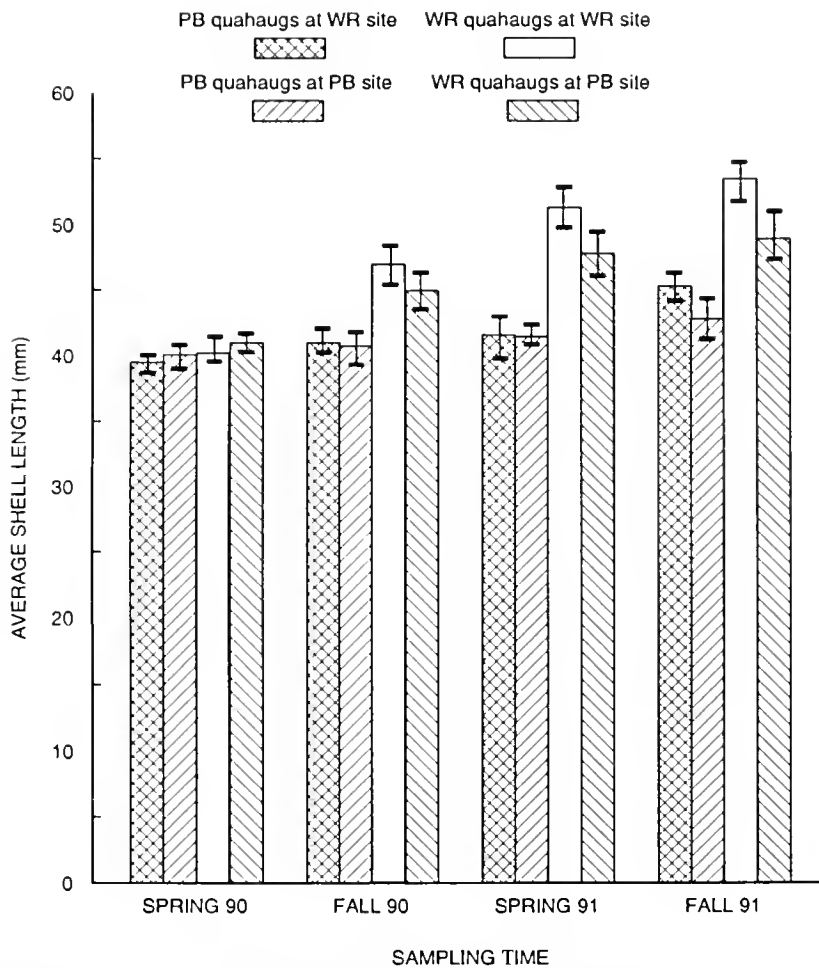


Figure 4. Average shell length of quahogs from West River (WR) and Pownal Bay (PB) used in the reciprocal transfer experiment.

Of the different physical and chemical parameters that have been shown to have an influence on recruitment, salinity, temperature, particulate inorganic matter (PIM), and substrate, were evaluated at Pownal Bay and West River in 1991. Salinity, temperature and PIM profiles from both sites were similar and well within reported tolerance ranges for larval and adult quahogs (Ta-

ble 1) (Malouf and Bricelj 1989). However, substrate was different between sites and may account for some of the differences in recruitment levels. Wells (1957) found that quahog abundance was negatively correlated to mud (silt) component in substrate, which was significantly higher in West River. Craig and Bright (1986) showed that the amount of shell debris in the substrate contributes

TABLE 1.

Environmental characteristics from the West River (WR) and Pownal Bay (PB) sites collected during the 1991 season.

Month	Temperature (°C)		Salinity (ppt)		TPM (mg/l)		POM (mg/l)		Chlorophyll <i>a</i> (µg/l)	
	PB	WR	PB	WR	PB	WR	PB	WR	PB	WR
May	9.0	11.5	24.0	20.5	77.0	62.7	13.0	9.7	1.28	4.75
June	16.0	16.0	28.0	29.0	67.7	70.7	11.0	11.0	0.39	1.57
July	20.5	22.0	30.0	27.0	51.0	78.9	9.0	13.0	0.82	2.81
August	24.0	25.0	25.0	22.0	65.3	69.7	1.7	11.7	0.86	3.66
September	18.0	18.0	25.0	25.0	73.7	85.0	13.3	13.0	0.55	1.41
October	8.0	7.0	23.0	20.0	62.7	408.0	11.0	48.0	2.13	15.84
Average	15.9	16.6	25.8	23.9	66.2	129.2	9.8	17.7	1.01	5.01
Std Error	5.8	6.1	2.4	3.3	8.4	124.9	3.9	13.6	0.57	4.98

TPM is the total particulate matter while POM is the particulate organic matter.

TABLE 2.

Survival levels of quahogs from West River and Pownal Bay, PEI.

Year	Site	Quahogs from West River			Quahogs from Pownal Bay		
		Dead	Missing	Live	Dead	Missing	Live
1990	West River	53%	5%	42%	5%	3%	92%
	Pownal Bay	13%	15%	72%	10%	33%	57%
1991	West River	56%	20%	24%	33%	16%	51%
	Pownal Bay	2%	21%	77%	9%	15%	76%

positively to quahog recruitment. The Pownal Bay area has a greater amount of shell debris, stemming from greater amount of fishing activity.

Age determination of quahogs from natural populations at the three study sites showed that quahogs from Pownal Bay require approximately four (4) additional years to reach the legal commercial size (50 mm) compared with those from West River and Hillsborough River (Fig. 3). The reciprocal transfer experiment showed substantially and significantly faster growth at West River compared to Pownal Bay, and thereby indicated that environmental influences were the cause of the differences in growth patterns between these two study sites. Environmental factors evaluated in 1991 showed that salinity, temperature, TPM and POM were similar between sites but chlorophyll *a* concentrations and sediment characteristics differed. Chlorophyll *a* concentrations were consistently higher at the West River site. Although chlorophyll *a* may not be a good measure of actual food quantity or quality, its higher concentration at the West River site and the general lack of differences in seston concentrations (TPM and POM) between sites indicates that the sestonic food quality was probably greater there compared to the Pownal Bay site. Similar observations of faster growth in molluscs correlated to higher concentration of chlorophyll *a* have been reported by Anders and Lopez (1988). Nonetheless, the use of chlorophyll *a* as an indicator of food quality for site evaluation is not clear since it can be greatly influenced by the specific composition of the algal biomass and the presence of noxious algae such as *Prorocentrum minimum* (Wikfors and Smolowitz 1993).

The published research on the effects of sediment characteristics on growth of quahogs is inconclusive with positive, negative or no correlations. Grizzle and Morin (1989) suggest that the effect of this factor on the growth of quahogs is dependent upon other environment variables while the overall trend among several field experiments is that silt-clay content is generally negatively corre-

lated with clam growth (Rice and Pechenik 1992). This trend, however, is not reflected by our data which shows that faster growth is observed in West River where the proportion of silt-clay and TOC are over two times higher than those in Pownal Bay. However, since it is recognized that single environmental variable should not be considered alone for site evaluation, it is possible that any negative effects on growth by higher sediment silt-clay content were offset by the generally greater food quality available to the quahogs at the West River site.

The high mortality level of quahogs from West River at the West River site observed during the first six months of the tagging experiment was originally thought to be associated with the experimental handling, where, for some unknown reason, these quahogs were in poor condition prior to being planted. In 1991, however, the experimental handling was ruled out as a possible cause for mortality differences between sites. The high mortality levels observed in quahogs both from West River and Pownal Bay at the West River site compared with those from the Pownal Bay site, was therefore indicative of a site specific factor. The abundance of known quahog predators at both sites, such as rock crabs (*Cancer irroratus*), mud crabs (*Neopanopeus sayi*) and moon snails (*Lunatia heros*), was not quantitatively evaluated in this study. Field observations of these known predators revealed no noticeable differences in their abundance. Native quahogs in West River outside of the experimental plots, however, were regularly found partly uncovered. When these quahogs were removed, the presence of a nemertean worm was often observed. This nemertean worm (*Cerabratulus lacteus*) has been the focus of some recent research by Rowell and Woo (1990), as a predator of bivalves (not including quahogs). The high abundance of *C. lacteus* in West River and its absence in Pownal Bay may partially explain the higher mortality levels observed in West River.

In conclusion, the differences in the population structure among the three sites seems to be associated to the substrate, while observed growth rates appear related to environmental factors, and more specifically nutrient levels, than to genetic influence. The mortality observed in this study is probably the result of an endemic predator in the West River study site which, if it can be conclusively attributed to *C. lacteus*, could have an important negative impact to the culture of quahogs in infested areas.

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## SEASONAL CHANGES IN THE GROSS BIOCHEMICAL COMPOSITION OF THE TURKEY WING *ARCA ZEBRA*, IN BERMUDA

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**ABSTRACT** The relationship between the reproductive cycle and storage or utilization of food reserves was examined in the tropical mussel *Arca zebra* in Bermuda, the northernmost part of its geographical range. Gross biochemical composition—total lipids, total carbohydrate and proteins—was determined for the pedal muscle, gonads and digestive gland over an annual cycle. Environmental variations in temperature and chlorophyll *a* were reflected in the seasonal fluctuations of gross biochemical composition. The turkey-wing mussel appeared to rely on a glycogen-based metabolism, with the pedal muscle as a principal storage organ. The reproductive strategy of *A. zebra* was one of mixed temperate and tropical tendencies. The first gametogenic cycle, leading to early summer spawning, derived most of its energy from the utilization of stored total carbohydrate reserves, accumulated over the winter months. Pedal muscle proteins were partially utilized as respiratory substrate as oocytes matured and energy demand increased. The second reproductive cycle was characterized by a direct reliance on ingested food, coinciding with increasing food availability and resulting in autumn spawning. Energy values, calculated indirectly from biochemical data, reflected differences in levels of stored reserves prior to the summer and autumn spawnings. Decreasing ambient temperature during late autumn and winter appeared to be a key factor inhibiting continuous reproductive activity.

**KEY WORDS:** *Arca zebra*, gross biochemical composition, reproduction, temperature, Bermuda

### INTRODUCTION

The turkey-wing mussel, *Arca zebra* (Swainson) (Family: Arcidae) is distributed in lower latitudes of the Atlantic Ocean, inhabiting Venezuela, Cuba, the Caribbean sea and Bermuda; the latter appears to be the northernmost limit of its geographical range. Despite commercial and recreational fishing of this species in the Caribbean (Mari et al. 1980) and Bermuda (Burnett-Herkes pers. comm.), little has been recorded on its biology. Insight into population growth and survival may be provided by knowledge of its reproductive cycle, and of the exogenous and endogenous factors regulating it.

Energy derived for vitellogenesis and gametogenesis may be obtained by the transfer and conversion of reserves from several body parts to the gonad, as seen for other bivalves; for example in *Pecten maximus* (Comely 1974); *Chlamys opercularis* (Taylor and Venn 1979); and *Macoma balthica* (Wenne and Styczynska-Jurewicz 1987). An alternative strategy is that of a direct dependence on food supply for gonadal development and maturation, as reported for *Chlamys septemradiata* (Ansell 1974) and *Placopecten magellanicus* (Couturier and Newkirk 1991, Thompson 1977). The preferred strategy varies from species to species coinciding with specific environmental conditions. The influence of seasonal variations in environmental factors on reproduction has been illustrated for several higher latitude bivalves (Seed and Brown 1975, Ansell and Bodoy 1979). More specifically, ambient food and temperature conditions have been shown to primarily control seasonal fluctuations in levels of reserves—i.e. proteins, carbohydrate and lipids—in the soft tissue of marine bivalves (Gabbott 1983, Wenne and Styczynska-Jurewicz 1987). As latitudes decrease and seasonal environmental fluctuations become less pronounced, the cyclical nature of gametogenesis and the cycles of storage and utilization of reserves may not be as marked as in higher latitude populations (Gabbott 1975); for example a more constant food supply may be reflected in less extreme changes in tissue composition, as observed in some studies (Ansell and Bodoy 1979).

This paper examines the influence of environmental conditions on the reproductive cycle of the tropical mussel, *Arca zebra*, inhabiting the northernmost part of its geographical range. Biochemical composition of separate organs, as opposed to whole tissue, is assessed over the course of a year, allowing the determination of nutrient storage sites and nutrient use during periods of reproduction, lack of available food or other conditions of stress, as illustrated for other bivalve species (Couturier and Newkirk 1991, Giese et al. 1967). The relative importance of utilization of stored reserves and that of direct reliance on food supply will thus be ascertained for the turkey-wing mussel, indicating its reproductive strategy in Bermuda. In this way, insight into the key factors regulating the reproduction, hence limiting the northern distribution, of *A. zebra* in Bermuda may be provided.

### MATERIALS AND METHODS

Adult turkey-wing mussels were collected by SCUBA in Harrington Sound, Bermuda, over a 12 months period from July 1988 to July 1989 (Fig. 1). Sea surface temperatures were recorded monthly during this period. The sampling site, on the south side of Rabbitt Island, was of easy access and supported a relatively substantial abundance of mussels between 10 and 14 m (Sarkis 1992).

Following collection, individuals were transported to the laboratory and maintained in running ambient seawater for at least 24 h to allow gut clearance. Mussels were scrubbed of all epiphytes and measured with vernier calipers ( $\pm 0.1$  mm); length was defined as that measured along the hinge line. Mussels with mean shell length of  $58.1 \pm 4.7$  mm (S.D.) ( $n = 180$ ) were used over the experimental period.

Gross biochemical composition was determined for the gonads, pedal muscle and digestive gland of the turkey-wing mussel. The first two organs were selected due to pronounced annual weight changes coinciding with spawning periods, unlike those measured for the adductor muscles (Sarkis 1992); furthermore, the proportion of pedal muscle (approx. 50% dry flesh weight) in *Arca zebra*, suggested its potential importance as a storage organ. The

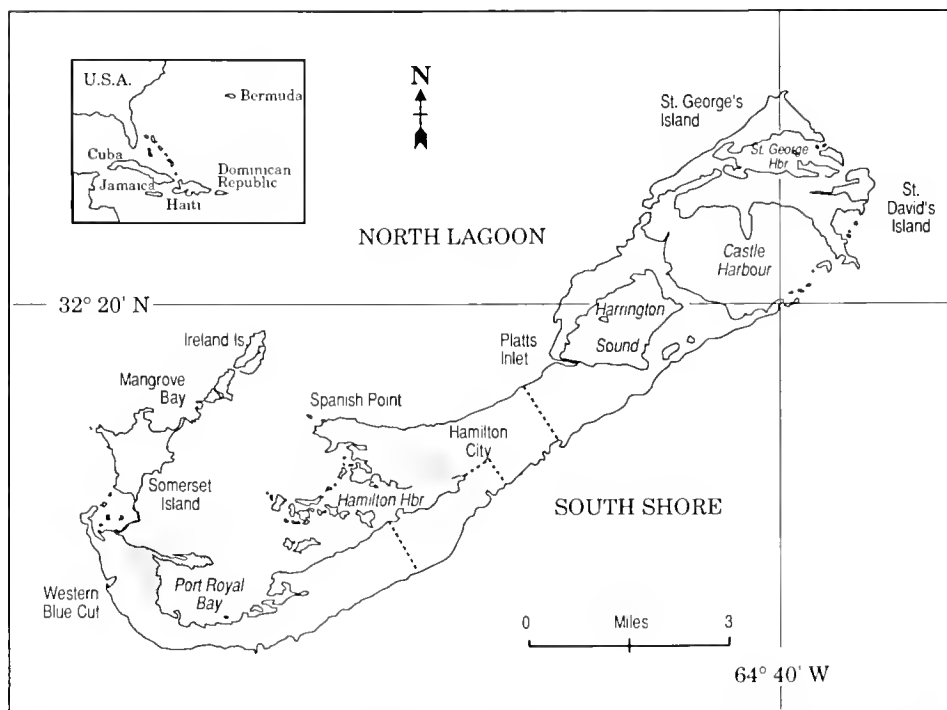


Figure 1. The Bermuda Islands (32°N, 64°W) showing the Harrington Sound study site (Sleeter, 1984).

digestive gland, easily separated from the gonads, was analysed in view of its reported contribution to production and ripening of eggs (Thompson et al. 1974). The pedal muscle, gonads and digestive gland of 10 individuals were dissected and freeze-dried until constant weight ( $\pm 0.001$  g); these were stored in a desiccator at  $-10^{\circ}\text{C}$  awaiting further analysis. The degradation of biochemical constituents should have been minimal during this storage period (M. Shick pers. comm.).

Total lipids, total carbohydrate and proteins were determined according to the methods outlined by Mann and Gallager (1985). 15–25 mg of dry tissue was homogenized in 3000–4000  $\mu\text{l}$  of distilled  $\text{H}_2\text{O}$  using a glass homogenizer. Homogenization time was standardized for each organ. Duplicates of each body component were homogenized separately and triplicates of each sample analysed for gross biochemical composition. Individuals were analysed independently and monthly results were pooled.

For the lipid assay, aliquots were extracted in 1:2 v/v chloroform:methanol followed by a second extraction of 2:1 v/v chloroform:methanol. Purification of the lipid containing chloroform layer was performed using 0.7% w/v NaCl solution and calibrated vs cholesterol. The disadvantage with this method was the possible charring of the lipid during the drying step which at times resulted in a suspension of floccular material on addition of  $\text{H}_2\text{SO}_4$  and gave unsatisfactory assays.

Carbohydrate and protein assays began with the extraction of the initial water homogenate with trichloroacetic acid (TCA) to give a final concentration of 5% w/v after mixing. After standing overnight at  $4^{\circ}\text{C}$  and centrifuging for 10 mins at approx 1000 g, the supernatant was removed for total carbohydrate assay. The data of Mann (1979) suggest that the major storage polysaccharide, glycogen, is extracted by cold 5% w/v TCA in homogenized bivalve tissue. Therefore, the cold extraction technique seemed adequate. Total carbohydrate content of the supernatant was assayed by the phenol-sulphuric acid method of Raymont et al.

(1963) using glucose as a standard. The protein content of the precipitate was assayed by the Folin-Phenol reaction of Lowry et al. (1951) using bovine albumen as a standard.

Growth changes were considered minimal for the size range of mussels analysed in the present work, such that the calculation of the composition of an animal of standard size, minimizing the complication introduced by major changes resulting from the growth of the animal, was not considered essential; individual weights were used for calculations in the present work and variations in biochemical composition within and among organs were compared in terms of the "absolute" weight of each constituent in mg present in each specific body division. This was found preferable to the expression of constituent changes as % tissue dry weight, since proportional values involve reciprocal relationships between two or more constituents, and may lead to misinterpretation of the data. However, for comparative purposes with other studies, biochemical composition was referred to as % tissue dry weight in some instances. Seasonal variations of each biochemical constituent, within a body component or among organs (mg) were compared with a one-way ANOVA (STATVIEW SE+ program) at  $p < 0.05$ .

Caloric content was calculated indirectly from the biochemical data using the following equivalents of  $35.3 \text{ kJ} \cdot \text{g}^{-1}$ ,  $20.1 \text{ kJ} \cdot \text{g}^{-1}$ , and  $17.2 \text{ kJ} \cdot \text{g}^{-1}$  for total lipids, proteins and total carbohydrate respectively (Beukema and De Bruin 1979). Individual energy values were converted to Joules, and presented for each body component; the sum energy value for the three organs was termed total energy value, but did not include energy comprised in the rest of the soft tissue.

## RESULTS

Sea surface temperatures of Harrington Sound fluctuated throughout the year (Fig. 2); minimum temperatures ( $17^{\circ}\text{C}$ ) were

recorded between November and January, and maximum readings (30°C) in July and August. As phytoplankton abundance is taken to represent food availability in the present work, an indication of food supply to the turkey-wing mussel may be provided by chlorophyll *a* levels. Mean values determined over a 5-year period by D. Connelly (unpublished) are illustrated in Figure 3. Despite large standard deviations indicating the variations from year to year, a gross trend showing minimum standing crop of chlorophyll *a* between February and June (0.44  $\mu\text{g chl } a \cdot \text{l}^{-1}$ ), gradually increasing during the summer months to a maximum in December (1.62  $\mu\text{g chl } a \cdot \text{l}^{-1}$ ) may be indentified. The time period at which this maximum occurs is known to vary widely among years in Bermuda's inshore waters, ranging between August and December (Von Bodungen et al. 1982).

Turkey-wing mussels spawned in June and September 1988, and July 1989. Cyclic changes occurring within each of the body components analysed over the course of a year are shown in Figures 4, 5 and 6.

#### Pedal Muscle

Total carbohydrate levels in the pedal muscle were higher than in other analysed body parts throughout the year (Fig. 4). Marked seasonal variations were recorded in the pedal muscle, increasing over the winter months to a maximum in March (103.23 mg); a gradual decrease was observed from March to July 1989, prior to the first annual spawn. Total carbohydrate levels were very low during the summer months (approx. 15 mg), as gametogenesis proceeded for the second time; levels remained low until December, with the exceptional increase in November (38.34 mg), coinciding with the phytoplankton "bloom," expressed in terms of chlorophyll *a* (Figs. 2 and 4). Differences in total carbohydrate levels measured between post-spawn July 1988 and spawning period of July 1989 may be attributed to yearly variations in levels of stored reserves.

Total lipids were not stored in very high levels in the pedal muscle, although fluctuations were significant throughout the year ( $p < 0.05$ ), increasing through the winter months to a maximum in March (29.13 mg) (Fig. 5). Minimum levels occurred after the July spawning period (6.84 mg), and remained relatively low through the summer.

Protein levels of the pedal muscle were consistently higher than those found in the gonads and digestive gland (Fig. 6), comprising

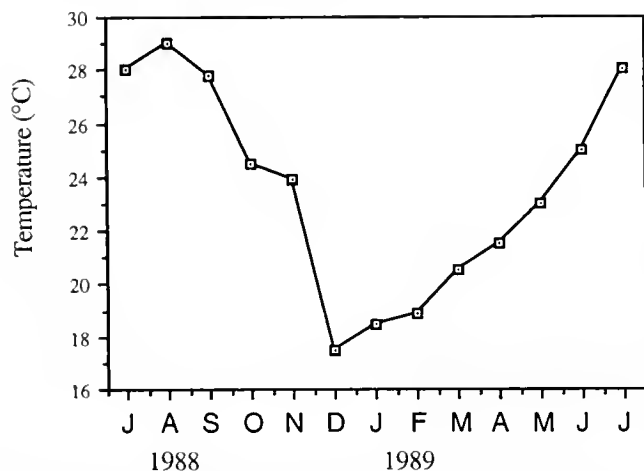


Figure 2. Sea surface temperatures in Harrington Sound, Bermuda.

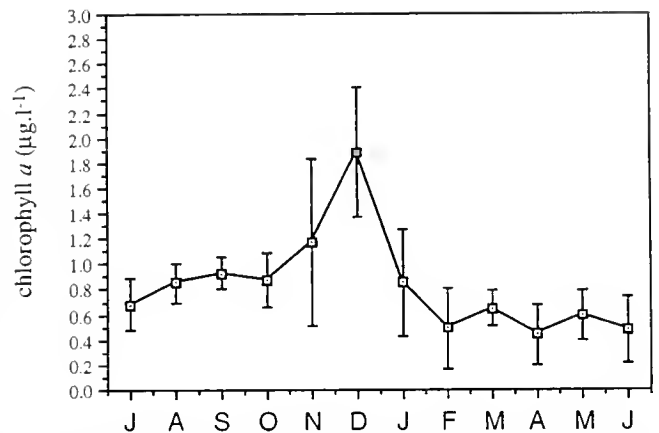


Figure 3. Mean and standard deviation of phytoplankton abundance, determined over a 5-year period in Harrington Sound (D. Connelly, unpublished).

the major proportion of biochemical constituents in the pedal muscle throughout the year. Exceptionally, total carbohydrate levels dominated in May and June; protein values ranged between 133.82 mg in March to 44.27 mg in May. The sudden significant decrease (approx. 90 mg;  $p < 0.05$ ) in protein levels during May and June prior to spawning suggested utilization of proteins at this time. Protein levels were high during the summer months, showing a slight (but non-significant) decrease in September (71.75 mg) when mussels spawned, and a second minimum in December (54.66 mg). Maximum levels were analysed during the winter months, 131.34 mg and 133.82 mg in February and March respectively.

#### Gonads

Low total carbohydrate levels were recorded in the gonads of *Arca zebra*, ranging from 0.41 mg in December to 21.39 mg in July 1988 (Fig. 4). Although significant fluctuations ( $p < 0.05$ ) occurred throughout the year, these were less marked than those of protein and lipids.

Total lipids gradually became the most important constituent of gonadic material as oogenesis proceeded, reaching a maximum in June of 46.38 mg, shortly before the first spawning (Fig. 5). At this time, as gonads ripened (May–July 1989), gonadal lipid levels exceeded those of the pedal muscle (17.39 mg) and digestive gland (28.45 mg). A subsequent post-spawn decrease in total lipid composition was seen in July and August 1988, followed by a second maximum level (25.64 mg) in September, at the time of the second spawning. A mean difference of 10 mg (not statistically significant) was calculated between gonadal lipid levels of mussels spawned in July and September.

Maximum levels of proteins were present prior to the autumn spawning period (64.56 mg in August; 62.44 mg in September), although variations among individuals were wide (Fig. 6). Gonads were relatively empty after the second spawning period, as reflected from generally reduced levels of biochemical constituents in November and December (Figs. 4, 5 and 6). The increasing level of lipids and proteins during February was associated with developing oocytes (Figs. 5 and 6) (Sarkis 1992). The decrease in protein levels in May (25.13 mg) and June (16.05 mg) resembled that seen in the pedal muscle, and occurred simultaneously with the completion of the first gametogenic cycle (Fig. 6).

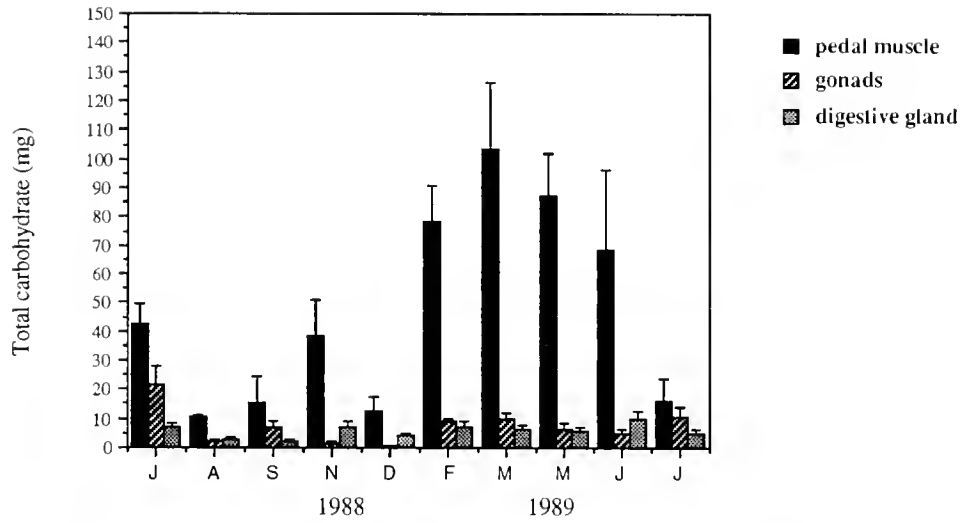


Figure 4. Total carbohydrate levels (mg) in the turkey-wing mussel, for three body divisions (mean  $\pm$  s.d.; n = 10 for each analysis).

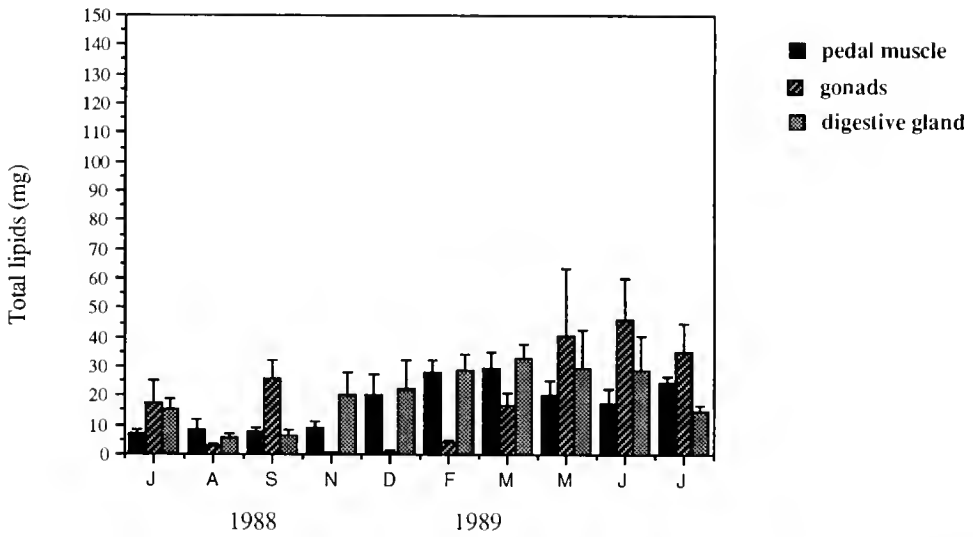


Figure 5. Total lipids (mg) in the turkey-wing mussel, for three body components (mean  $\pm$  s.d.; n = 10 for each analysis).

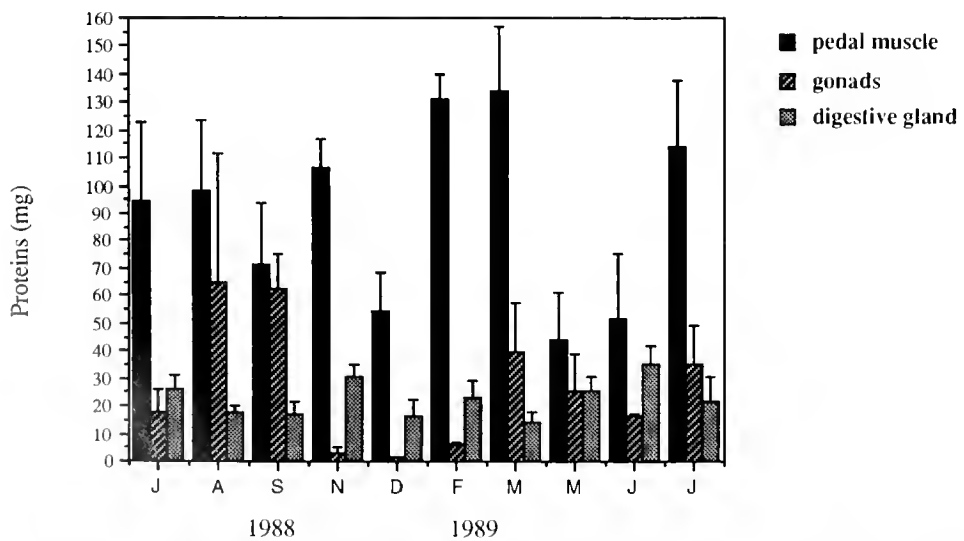


Figure 6. Protein levels (mg) in three body divisions of *Arca zebra* (mean  $\pm$  s.d.; n = 10 for each analysis).

### Digestive Gland

Total carbohydrate was least accumulated in the digestive gland, but showed significant seasonal fluctuations ( $p < 0.05$ ) ranging from 2.16 to 9.98 mg, with a mean value of 5.68 mg (Fig. 4). Total lipids increased gradually from November (19.95 mg) to March (32.77 mg), followed by an equally gradual decline prior to the first spawn (Fig. 5). Low lipid levels were analysed during the summer months (approx. 5.5 mg) continuing so until the second spawning period. The cyclical changes of proteins did not show any clear trends (Fig. 6). Maximum storage of this constituent occurred in November (30.55 mg), during the phytoplankton bloom, and in June (34.83 mg), prior to the first spawning period. Low protein levels were measured over the winter (14.83 to 23.30 mg). Lack of accumulation for all constituents was seen in the digestive gland during August and September, despite increasing food supply, illustrated by chlorophyll *a* levels (Fig. 3); mussels were undergoing a second gametogenic cycle at this time.

### Calorific Value

The energy value for each body component was determined separately (Table 1). Maximum energy values in the pedal muscle reached 5.49 kJ in March, whereas the maxima for gonads occurred in July 1989 (summer spawning period) (2.11 kJ), and September (autumn spawning) (2.28 kJ). A maximum energy value of 1.88 kJ was calculated for the digestive gland during June 1989, prior to the first spawning period. Minimum values for both pedal muscle and gonads occurred in December, 2.03 and 0.07 kJ respectively; and minimum digestive gland energy values (0.60 kJ) were determined as mussels recovered from their first spawning period and approached the second (August and September 1988). Changes in energy values of the pedal muscle were mainly attributed to total carbohydrate, whereas those of the gonads and digestive gland were mainly due to total lipids. A summation of the calorific values indicated minimum energy value of 3.30 kJ for turkey-wing mussels in December (Table 1). Total energy value was lower in the summer and autumn (July–Nov 1988) and higher during the winter and spring (Feb–July 1989). Spent mussels of July 1988 showed little difference in total calorific value (5.41 kJ) with individuals undergoing gametogenesis prior to the September spawn (mean of 4.86 kJ); this was a result of changes in energy values undergone by the pedal muscle and gonads. The simulta-

neous increase in caloric value for the three body components after December, and through the winter, suggested a high amount of stored reserves. Maximum energy value in March (8.59 kJ) was mainly attributed to high pedal muscle energy value (due to total carbohydrate); unlike in the spring and early summer months (May–July 1989), when much of the energy was mainly derived from lipids present in the gonads.

### DISCUSSION

Bermuda has a subtropical climate, with marked seasonal fluctuations in temperature and food availability (expressed as chlorophyll *a*). The extreme temperatures recorded in Harrington Sound are characteristic of the inshore waters of Bermuda (Fig. 2). These temperature variations are similar in range to those of more temperate areas; however, the maxima are close to those found in tropical latitudes (Gonzalez 1990, Grotta and Lunetta 1982), and the minima are considered the limit for the existing tropical fauna and flora (Creswell 1984). The presence of a phytoplankton "bloom," indicated by chlorophyll *a* levels, is also typical of more temperate rather than tropical systems (Fig. 2); this is in accordance with Beers et al. (1968), who found Bermuda to have more defined seasonal variations in production, and of greater magnitude, than the Caribbean. It becomes evident that Bermuda, and more specifically Harrington Sound, has temperate characteristics reflected by seasonal variations in temperature and food supply, but also tropical characteristics implied by the absolute values of these two parameters. Such variations may prove important factors in the regulation of the gametogenic and reproductive cycle, as well as in the storage and utilization of metabolic reserves in the turkey-wing mussel, *Arca zebra*.

There are two well-defined spawning periods for *Arca zebra*, early summer and autumn (Sarkis 1992); the first coincides with a temperature increase (Fig. 2) and the second with a phytoplankton "bloom" (Fig. 3). Seasonal changes in the reproductive cycle of *A. zebra*, characteristic of temperate species, are reflected in the respective biochemical composition of the three body components analysed. The presence of total carbohydrates and proteins mainly in the pedal muscle, and distribution of total lipids in the gonads imply a glycogen-based metabolism in *A. zebra*; this is expected since many bivalve species rely on glycogen as their energy reserve and both Gabbott (1983) and Leavitt et al. (1990) reported a similar strategy in members of the family Arcidae. Furthermore, the lipid content of the turkey-wing mussel—7% total tissue (Leavitt et al. 1990)—is closer to a species as *Mytilus edulis* with a glycogen-based metabolism (3.9–9.6%) than to one with a lipid-based metabolism such as *Macoma balthica* (8.4–36.4%) (Wenne and Styczynska-Jurewicz 1987). The metabolic processes preceding both spawning periods are discussed separately, in order to determine the turkey-wing mussel's strategy in deriving energy for vitellogenesis and gametogenesis.

### Summer Spawning

The accumulation of total carbohydrate in the pedal muscle during gametogenesis (February onwards), resulting in a maximum energy value in March, and its subsequent utilization as oocyte maturation proceeds (May–July), suggests a conversion of total carbohydrate to total lipids for the developing eggs (Fig. 4; Table 1). This transfer of reserves may be necessary due to a reduced food availability at this time (Fig. 3). This conclusion is supported by studies on oysters—*Crassostrea gigas* (Mann and Gallager 1985), and *Ostrea puelchana* (Fernandez Castro and de Vido de Mattio 1987)—, mussels (Gabbott and Bayne 1973) and

TABLE 1.

Seasonal changes of energy content in three body components of the turkey-wing mussel (kJoules), over a 12-month period (1988–1989) ( $n = 10$  for each value).

Month	Pedal Muscle	Gonads	Digestive Gland	Total Energy Value
July	2.88	1.34	1.19	5.41
August	2.45	1.42	0.60	4.47
September	1.98	2.28	0.60	4.86
November	3.12	0.12	1.43	4.67
December	2.03	0.07	1.20	3.30
February	4.97	0.42	1.61	7.00
March	5.49	1.55	1.55	8.59
May	3.10	1.76	1.64	6.49
June	2.83	2.04	1.88	6.75
July	3.43	2.11	1.04	6.58

The summation of these for each month is referred to as total energy value (kJoules).

pectinids (Ansell 1974, Comely 1974, Taylor and Venn 1979) where a similar use of carbohydrate reserves as respiratory substrate for both storage and gametogenesis was established. The comparable percentage composition of total carbohydrate in the pedal muscle of *Arca zebra* ( $2.7 \pm 1.6\%$  dry weight to  $16.4 \pm 2.4\%$ ; mean  $\pm$  s.d.) to that found in the main storage organ of other bivalve species—for example, 1.2–18.5% adductor muscle dry weight in *Chlamys opercularis* (Taylor and Venn 1979), or 10–16% of digestive gland-gonad complex dry weight in *O. puelchana* (Fernandez Castro and de Vido de Mattio 1987)—points to the importance of this body part as storage organ in the turkey-wing mussel. Furthermore, *A. zebra*'s pedal muscle possesses approx.  $3\times$  the calorific value of the digestive gland mainly due to total carbohydrate (Table 1), compared with  $2\times$  the difference between the adductor muscle and digestive gland for *Pecten maximus* (Comely 1974).

The increase in gonadal lipids for *Arca zebra* with the approach of spawning, and its decrease post-spawn, reflects oocyte development and release (Fig. 5). Energy value of the gonads reach a maximum prior to spawning, attributed to total lipids (Table 1). Large variations in total lipid levels in this body component, as seen for May ( $32.64 \pm 24.47$  mg), may reflect differences in sex and gametogenic stage of mussels analysed; such sexual differences were recorded by Ansell (1974) in *Chlamys septemradiata*, where females contained  $2\times$  as much lipid as males. In other organs of *A. zebra*, namely the pedal muscle, total lipids constituted a low proportion of the dry weight throughout the year ( $1.98 \pm 0.5\%$  to  $4.72 \pm 0.6\%$  dry weight); however, fluctuations in this constituent are apparent prior to the summer spawning. In the digestive gland, the gradual accumulation of total lipids (November and December) first coincides with the phytoplankton "bloom" (Figs. 3 and 5); thereafter, the continued increase in total lipids in February and March may be attributed to changes in composition of the phytoplankton species itself in Bermuda waters, assessed by Von Bodungen et al. (1982). Although phytoplankton abundance is taken to represent food supply to the turkey-wing mussel in the present work, other food sources may be of importance for this suspension-feeding bivalve, as illustrated in other bivalve species (Ansell 1974, Bayne and Scullard 1977); a more complete understanding of the food requirements of *A. zebra* may explain, more adequately, variations in digestive gland biochemical composition. The decline of digestive gland and pedal muscle lipids, as gametogenesis approaches completion (Fig. 5), implies the transfer of nutrients to the gonads. At this time, food availability is low (chlorophyll *a*), and the digestive gland, associated with controlling the distribution of assimilated food to other body components, may supply the energy demand of gametogenesis. Decreasing energy values of the digestive gland at this time further illustrates these processes (Table 1). Total lipids determined in the three body divisions totalled 39.04 mg for September 1989, a value within the range reported by Leavitt et al. (1990) for this species for total tissue (74.35 mg to 37.40 mg).

A clear trend in protein fluctuations is difficult to identify. However, reduced levels in the pedal muscle during May and June, coinciding with oocyte maturation, possibly suggest its utilization as a respiratory substrate during the last stages of gametogenesis (Fig. 6). At such a period of low food availability, the energy demand for the completion of gametogenesis may be too high for the sole reliance on total carbohydrate reserves in the pedal muscle; hence, the increased use of proteins by *Arca zebra* for body maintenance occurs. This enhanced utilization of proteins during periods of low food supply by the turkey-wing mussel

living in Bermuda is also reported in temperate species of oysters and mussels (Bayne, 1976). Maximum protein levels determined in *A. zebra*— $32.3 \pm 1.3\%$  in the pedal muscle,  $29.5 \pm 0.29\%$  in the gonads, and  $34.6 \pm 5.9\%$  in the digestive gland—were at the lower end of the range reported for other species; for example, proteins constitute approx. 25–45% of gonad dry weight in *Placopecten magellanicus*, and 35–50% of its adductor muscle (Couturier and Newkirk 1991); levels of 48–62% have been determined in the adductor muscle of the Pismo clam, and 30–40% in its digestive gland (Giese et al. 1967). Differences in protein assays used may partially account for these discrepancies and the reduction in protein levels during May and June may thus be accentuated; however, as all samples were processed and analysed in a similar manner, the trend is nevertheless present and interpreted as above.

The metabolic processes of storage and transfer of mainly total carbohydrate during gametogenesis in *Arca zebra*, associated with seasonal fluxes in food availability, is thus comparable to temperate species. Moreover, the rising temperature from April–July may also accelerate the conversion of total carbohydrate (glycogen) into lipid material necessary for egg maturation, suggesting a required threshold temperature for the transfer of reserves as demonstrated by Sastry and Blake (1971) for the bay scallop, *Argopecten irradians*.

#### Autumn Spawning

Gross biochemical composition of *Arca zebra* following the summer spawning period indicates low total carbohydrate reserves in the major storage organ (pedal muscle), as well as low levels of digestive gland total lipids, despite increasing phytoplankton biomass (Figs. 3, 4 and 5). This results in a reduced total energy value, and implies that energy for gametogenesis and vitellogenesis may not be derived from stored reserves (Table 1); the alternative is a direct reliance on ingested food, reflected in low digestive gland energy values and suggesting a rapid nutrient turnover of recently ingested food (Table 1). The rapid development and maturation of oocytes during the summer months, reflected in a second gonadal lipid peak in September, suggest a positive influence of environmental factors on gonad development of the turkey-wing mussel (Fig. 5). Exposure to high temperature for a specific time period, as *A. zebra* is subjected to during July and August ( $28 \pm .5^\circ\text{C}$ ), has been shown to favour reproductive activity for other bivalve species (Mann, 1979). Moreover, the rate of nutrient transfer from the digestive gland to the gonads may be accelerated by temperature (Sastry and Blake 1971) as well as increasing food availability (Thompson and Bayne 1972). These environmental conditions enable this tropical species to maximize the energy derived from ingested food for a second reproductive cycle. Furthermore, the process may derive additional energy following atresia; evidence for the latter was reported after the first spawning period (Sarkis 1992); products derived from oocyte lysis were suggested by Paulet and Boucher (1991) to be reprocessed for gametogenesis, or to be recycled to meet the demands of basal metabolism (Lowe and Pipe 1987). Proteins become at this period, the main respiratory substrate for *A. zebra*, and their utilization is reflected in the September decline in the pedal muscle (Fig. 6). The direct use of ingested food for gonadal development during the second reproductive cycle of *A. zebra* suggest that annual differences in the timing and success of this spawning period may be linked to phytoplankton biomass fluctuations.

Seasonal variations in energy values reflect processes preced-

ing each spawning period; high values illustrate the mobilization of stored reserves from February onwards, and low values coincide with the lack of stored reserves and dependence on food supply during the summer. Minimum values occur during gametogenic quiescence in late autumn and winter (Table 1). In a gross comparison of *A. zebra*'s calorific value with other species, it appears that the maximum energy value of the turkey-wing mussel is approx. 2× lower than that found in many temperate species, such as *Chlamys septemradiata* (Ansell 1974) and *Mytilus edulis* (Dare and Edwards 1975). This is a difficult comparison since energy values are dependent on the size of the animal analysed, and furthermore, total energy value of *A. zebra* reported in this work comprise only the pedal muscle, gonads and digestive gland. However, the large difference mentioned above is in accordance with Wafar et al. (1976) who found tropical species to have lower calorific content than temperate species, related to lower food abundance.

In conclusion, the reproductive strategy of *Arca zebra* in Bermuda appears to be one of mixed temperate and tropical tendencies. The build-up of food reserves during periods of food availability and subsequent utilization in the winter, illustrated by gross biochemical constituents dynamics in *A. zebra*, is characteristic of temperate bivalves (Gabbott 1983). On the other hand, rapid gonadal development (2 months), observed during the summer and favoured mainly by high temperatures, may be more typical of tropical systems. Both strategies appeared equally successful for the reproduction of this species, based on fecundities and propor-

tion of responding individuals following laboratory induction (Sarkis 1992). It may be speculated that in more southern waters, a more constant food supply may be reflected in less pronounced variations in mobilized and utilized reserves; and that direct dependence on food supply may be the preferred strategy favoured by constant high ambient temperatures. The absence of a third reproductive activity in October mussels, exposed to relatively high food availability, may be attributed to decreasing ambient temperature at this time; similarly, gametogenic quiescence during the winter months is associated with minimum temperature values. The role of temperature on *A. zebra*'s reproduction may thus explain the well-defined and short reproductive season of this tropical species in Bermuda, unlike that expected in lower latitude bivalves (Grotta and Lunetta 1982, Ansell and Bodoy 1979). Seasonal physiological measurements performed on *A. zebra* in Bermuda, indicating a reduction in energy intake attributed to low ambient temperatures, further support this conclusion (Sarkis 1992). Hence, it appears that environmental temperature, and more specifically its effect on the turkey-wing mussel's reproduction, is a key factor in the northernmost distribution of this species in Bermuda.

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## METAMORPHOSIS OF *CONCHOLEPAS CONCHOLEPAS* (BRUGUIERE, 1789) INDUCED BY EXCESS POTASSIUM

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**ABSTRACT** Planktonic larvae of *Concholepas concholepas* Bruguiere underwent complete metamorphosis in response to excess  $K^+$ . The effect was dose-dependent and optimal at approximately double the normal concentration of  $K^+$  in 0.45  $\mu$ m membrane-filtered seawater. Increasing concentration over 25 mM  $K^+$  produced a decline in survival, suggesting toxicity. Metamorphosis began with propodium attachment to the substratum and subsequent deciliation and destruction of the velum, followed by the emergence of cephalic tentacles from the larval shell margin. Velar loss, induced by  $K^+$ , began with the detachment of large ciliated cells at the velar margin. Field-collected larvae, from different localities along the Chilean coast, presented a positive response to excess  $K^+$ , and almost 80% of the larvae metamorphosed in 1 to 2 days. The induction of metamorphosis of *Concholepas concholepas* larvae by  $K^+$ , provides a useful biotechnological tool for the cultivation of this socio-economically important marine resource of the southeastern Pacific Ocean.

**KEY WORDS:** Molluscs, *Concholepas concholepas*, planktonic larvae, settlement, metamorphosis, potassium (ions)

### INTRODUCTION

*Concholepas concholepas* (Bruguiere 1789), known as stone shell, is a muricid gastropod inhabiting the subtidal and intertidal zones of the Peruvian and Chilean coast (Stuardo 1979). This species is the mollusc of greatest commercial importance in Chile (Castilla 1976, Castilla and Jerez 1986). This gastropod has been a traditional Chilean delicacy, but as exports have increased in recent years, pressure on the fishery has intensified. At present, the fishery is closed due to heavy overexploitation.

In view of the declining stocks of this important resource, strong measures, including rearing of juveniles for reseeding or complete mariculture systems, are becoming necessary. In the mariculture of stone shells, some bottlenecks need to be removed before the culture can become a reality. For example, the planktonic larval stage is more than 3 months long, and laboratory rearing has been considered very difficult (DiSalvo 1988). Therefore, most of the knowledge of stone shell larvae has been gained from studies of planktonic specimens. Different aspects of ontogeny and settlement of *Concholepas* larvae have been studied (Inestrosa et al. 1988, González et al. 1990, Brandan et al. 1990, 1992).

In several species of marine invertebrates, substratum-associated morphogenetic chemical cues have been found to be neurotransmitter-mimetic substances (Pawlik 1992, Rodríguez et al. 1993). Exogenous neurotransmitters can elicit metamorphic responses similar to those induced by natural cues, thus further implicating neuronal receptors in the initial processes (Bonar et al. 1990, Morse 1990). Because signaling in many receptor systems involves depolarization of sensory membranes, settlement of molluscan larvae may be induced by increasing the concentration of  $K^+$  in seawater (Baloun and Morse 1984). Several marine invertebrate species, including *Phestilla sibogae*, *Haliotis rufescens*, *Astraea undosa*, *Crepidula fornicata*, *Crepidula plana*, and *Ad-*

*alaria proxima*, respond to increased external  $K^+$  in seawater by going through metamorphosis (Yool et al. 1986, Pechenik and Heyman 1987, Todd et al. 1991). However, the efficacy of  $K^+$  is not absolute. Nell and Holliday (1986) found only 19% metamorphosis in the bivalve, *Saccostrea commercialis*, and Eyster and Pechenik (1987) did not find any effect of  $K^+$  on the induction of metamorphosis in *Mytilus edulis* larvae. Finally, Rittschof et al. (1986) found an inhibitory response to increased  $K^+$  concentration in larvae of the barnacle, *Balanus amphitrite* Darwin.

The aim of this work is to study the capacity of  $K^+$  ions to induce the settlement and metamorphosis of competent larvae of the prosobranch mollusc *Concholepas concholepas*, including an analysis of the metamorphic process. Preliminary results of this work have been presented elsewhere (Inestrosa 1991, Inestrosa et al. 1992).

### MATERIALS AND METHODS

#### Sample Collections

Larvae of *Concholepas concholepas* (average shell lengths between 1500 and 2000  $\mu$ m) were collected from plankton samples with a net of a minimum mesh size of 1 mm between August, 1990, and September, 1991, at three different localities along the Chilean coast: Coquimbo and Lagunillas (30°03'S; 71°20'W), Las Cruces (33°30'S; 71°30'W), and Valdivia (39°24'S; 73°13'W). The larvae were immediately transported, in 1 L plastic flasks containing seawater without microalgae, to the laboratory. The total time of transportation was less than 8 hours. The larvae were placed in small dishes with fresh seawater, and measured using a dissecting microscope fitted with an ocular micrometer. All larvae were measured several times in order to obtain an average length. Larvae were individually maintained in sterile culture flasks with 30 ml of 0.45  $\mu$ m millipore filtered seawater. Salinity was 30 parts per thousand. Temperature was maintained between 16 and 18°C, and seawater was changed daily.

#### Laboratory-Reared Larvae

Pre-competent larvae (n = 40) reared in the "Laboratorio Biológico Pesquero" of the Instituto de Fomento Pesquero (IFOP) in

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Putemún-Chiloé (42°25'S; 73°45'W), were also used in our experiments. These larvae were reared as described previously (Pinto 1992), and had an average shell length of 1400  $\mu\text{m}$ .

#### Induction of Metamorphosis by $\text{K}^+$

All larvae ( $n = 240$ ) were cultured in darkness at 16–18°C, without microalgae in 30 ml of incubation medium for the indicated time (see below). For induction assays, each larva was randomly assigned to a sterile culture flask with (up to 20 mM  $\text{K}^+$ ) or without an elevated KCl concentration (control: 9 mM  $\text{K}^+$ ). The number of larvae metamorphosed were scored at different times of continuous exposure. To study the effect of  $\text{K}^+$  concentration, 12 larvae were used per experiment and the data reported are averages of 3 different experiments ( $n = 36$ ) with 2 replicates per each  $\text{K}^+$  concentration. The standard error of the 3 experiments was less than 15% (Fig. 1). Loss of the velum, one of the major and irreversible morphological transformations occurring during the metamorphic process (Bonar and Hadfield 1974), was considered as the "metamorphic event" and was used to determine the end of the incubation period for each larva and the number of metamorphic larvae after each treatment. The medium was then removed, and larvae were maintained in fresh seawater with a normal KCl concentration (9 mM) until a full ring of conspicuously-rayed new shell was generated.

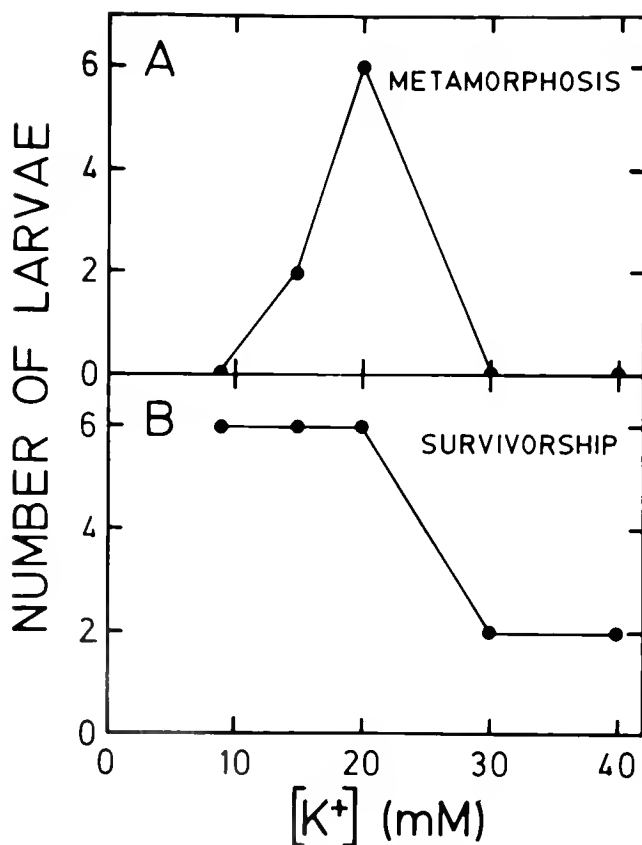


Figure 1. Effect of  $\text{K}^+$  concentration on the metamorphosis and survival of *Concholepas concholepas*. The number of larvae metamorphosed (A) or alive (B) were scored at 40 h of continuous exposure. The values corresponds to one representative experiment of the three carried out with larvae collected in Las Cruces. The standard error among the 3 experiments was less than 15%.

#### Video Recordings

Using an *in vivo* microscopy intravital system composed of a video camera mounted on a dissecting Ophthipot Nikon microscope, some larval structures, such as velum, cephalic tentacles, propodium, siphon, etc., and their localization in the living organisms, were monitored. Likewise, the effects of elevated  $\text{K}^+$  ions concentration on the behavior and larval activity, in groups of 6 to 8 competent larvae, from each locality studied, were observed. Larvae were illuminated obliquely using a fiber optic light; larval images were stored using a VHS video recorder and later replayed for qualitative analysis of their behavior.

#### Scanning Electron Microscopy of Ciliated Velar Cells

Competent larvae of *Concholepas concholepas* were fixed in 1% osmium tetroxide, dehydrated in ethanol and acetone and then critical point dried. They were shadowed with gold and observed in a Jeol JSM-25 II scanning electron microscope (SEM).

#### Kinetics of the Metamorphic Process

The different steps involved in stone shell metamorphosis, as well as the time course of each step, were studied in twelve competent larvae that were divided into 2 groups of 6 each. One of the groups was exposed to 20 mM  $\text{K}^+$ , and the other to control seawater (9 mM  $\text{K}^+$ ). Each larva was kept in a flask containing 30 ml of filtered seawater. Observations were done under a dissecting microscope every 3 to 5 h. The whole experiment was repeated twice with field-collected larvae from Valdivia and Las Cruces. In each case, the following behavioral and morphological traits were registered: fixation of the propodium to the substrate, deciliation, velum retraction, tentacle movement, and finally, velum loss.

## RESULTS

#### $\text{K}^+$ -induced Metamorphosis of Stone Shell Larvae

Planktonic larvae of *Concholepas concholepas* metamorphosed in response to increased external  $\text{K}^+$  in seawater in the absence of any natural source of inductive stimulation. Larval metamorphosis, measured as loss of the velum and emergence of the cephalic tentacles from the shell margin, was dose-dependent and showed an optimal response at 20 mM  $\text{K}^+$  (Fig. 1A). Increasing concentrations of  $\text{K}^+$  over 25 mM produced progressive evidence of toxicity as shown by the survival curve (Fig. 1B).

The effect of  $\text{K}^+$  was also assayed in planktonic larvae captured in different localities along the Chilean coast. As shown in Table 1, percentage of larvae that metamorphosed after 60 hours was very high with a total average of 80% (Coquimbo, Lagunillas, Las Cruces, Valdivia). However when the kinetics of the metamorphic induction by  $\text{K}^+$  was followed, it was clear that not all the field-collected larvae showed the same sensitivity to  $\text{K}^+$ , in fact, differences along the Chilean coast were observed. The first metamorphic events were registered 24 hours after  $\text{K}^+$  addition in larvae captured at Valdivia, 30 hours for Las Cruces and 46 hours for larvae collected in Coquimbo and Lagunillas. These results suggest that field-collected larvae in the North of Chile present a delayed response to  $\text{K}^+$  induction in comparison with those collected in the South of the country. Shell length of competent larvae that metamorphosed was in the range of 1600–2000  $\mu\text{m}$  with an average of 1700  $\mu\text{m}$ . Field-collected larvae of small size (1500  $\mu\text{m}$ ) were not induced to metamorphose, the same was true for

TABLE 1.

Induction of metamorphosis by  $K^+$  ion in competent larvae of *Concholepas concholepas* collected along the Chilean coast.

Localities	n	Metamorphic Larvae	
		- $K^+$ (%)	+ $K^+$
Coquimbo (IV Region)	20	10	90
Lagunillas (IV Region)	36	0	61
Las Cruces (V Region)	30	0	87
Valdivia (X Region)	22	0	90
Total	108		82

The average shell length of competent larvae that metamorphosed was 1700  $\mu\text{m}$  (range 1600–2000  $\mu\text{m}$ ). The induction to metamorphosis by  $K^+$  was evaluated after 60 hours of continuous exposure to the ion.

laboratory reared organisms of an average shell length of 1400  $\mu\text{m}$  ( $n = 40$ ). The same result was obtained by Pinto (1992) with more than 600 larvae of the same size. In this context, it is possible that the different time-course of metamorphic induction by  $K^+$  in larvae obtained from different localities, could be related to the size of these larvae. In fact the size-range for the larval population harvested in Coquimbo and Lagunillas (North) was 1500–1800  $\mu\text{m}$ . however for Valdivia (South) was 1900–2000  $\mu\text{m}$ . In the present study, captured larvae did not metamorphose in the absence of excess  $K^+$  for at least 10 days; after this period of time, some larvae metamorphosed spontaneously.

#### Detachment of Ciliated Cells from the Velum of $K^+$ -Treated Larvae

Detailed video analysis of the behavior of stone shell larvae induced to metamorphose by  $K^+$  showed an interesting pattern. In fact soon after the larval propodium attached to the plastic wall of the culture flask, the velum was partially retracted and cilia of the velar lobes began to beat asynchronously. Velar loss began with the detachment of large ciliated cells. Figures 2A and 2B show successive loss of two groups of ciliated cells from one velar lobe (see arrows in Fig. 2A,B). The large ciliated cells (Fig. 2C) were cast off intact from the velum, and their cilia continue to beat for at least 2 h after detachment (Fig. 2D,E). Usually, clumps of ciliated cells with beating cilia were observed in the culture flask. One or two hours later, few of these cells were found in the flask.

#### Kinetics of the Metamorphic Process in Stone Shell Larvae

Larvae of *Concholepas concholepas* displayed a rapid behavioral change in the presence of increased  $K^+$  concentration. Figure 3 shows a representative experiment, which indicated that the processes of propodial attachment to the substratum, deciliation, regression of the velum, and withdrawal of the cephalic tentacles from the larval shell, followed a precise temporal sequence. In all larvae, the first accurate indicator of metamorphic commitment was propodial attachment to the plastic wall of the flask, around five hours after exposure to high  $K^+$  concentration. Occasionally, larvae crawled over the flask wall, but in most cases they remained at the same spot of settlement until completion of metamorphosis. Some control larvae showed a transient contact with the substratum, but without propodial attachment (Fig. 3A). Concomitant with larval adherence,  $K^+$ -treated larvae began to lose ciliated cells from the columnar epithelium of the velum. The deciliation process was maximum around 12 hours after  $K^+$  exposure (Fig.

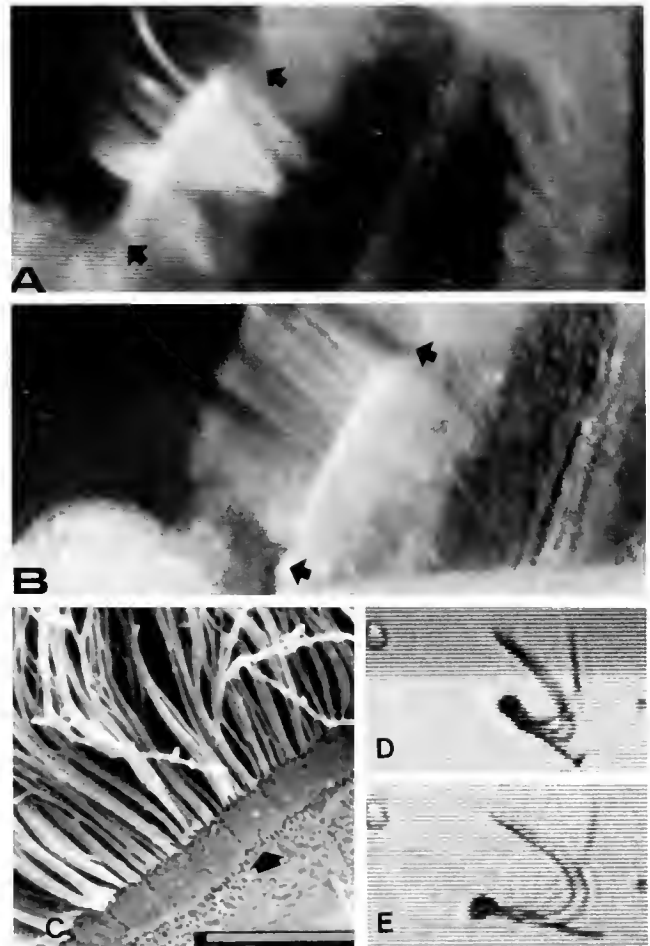


Figure 2. Deciliation of velar lobes in *Concholepas concholepas* triggered by  $K^+$ . A: shows a video picture of a velar lobule that has lost two groups of ciliated cells, indicated by arrows. B: shows the same velar lobule after movement of the cilia. The two groups of missing ciliated cells are more clearly appreciated (the whole photographic field is 300  $\mu\text{m}$ ). C: shows a scanning electron micrograph of the velar margin; the arrow indicates one large ciliated cell (scale bar = 40  $\mu\text{m}$ ). D and E: shows two consecutive pictures of a large ciliated cell with its cilia in movement. Note the two reference particles (15  $\mu\text{m}$ ) in both pictures.

3B). After deciliation, regression of velar lobes and movement of the cephalic tentacles was apparent (Fig. 3C). The tentacles were finally extended from the larval shell, and the velum was lost. The first metamorphic events (Fig. 3D) were observed around 24 hours after excess  $K^+$  exposure.

#### DISCUSSION

Results indicate that an increase in  $K^+$  concentration is an effective inducer of settlement of planktonic larvae of *Concholepas concholepas*. The effect was dose-dependent and optimal at approximately 20 mM  $K^+$  in seawater. Competent larvae (80%) collected along the Chilean coast were induced to metamorphose between 1 to 2 days after addition of this monovalent cation. Analysis of the metamorphic process allowed definition of both the temporal course as well as the steps involved.

Velar loss during stone shell metamorphosis showed that the deciliation process is similar to that reported for other molluscs

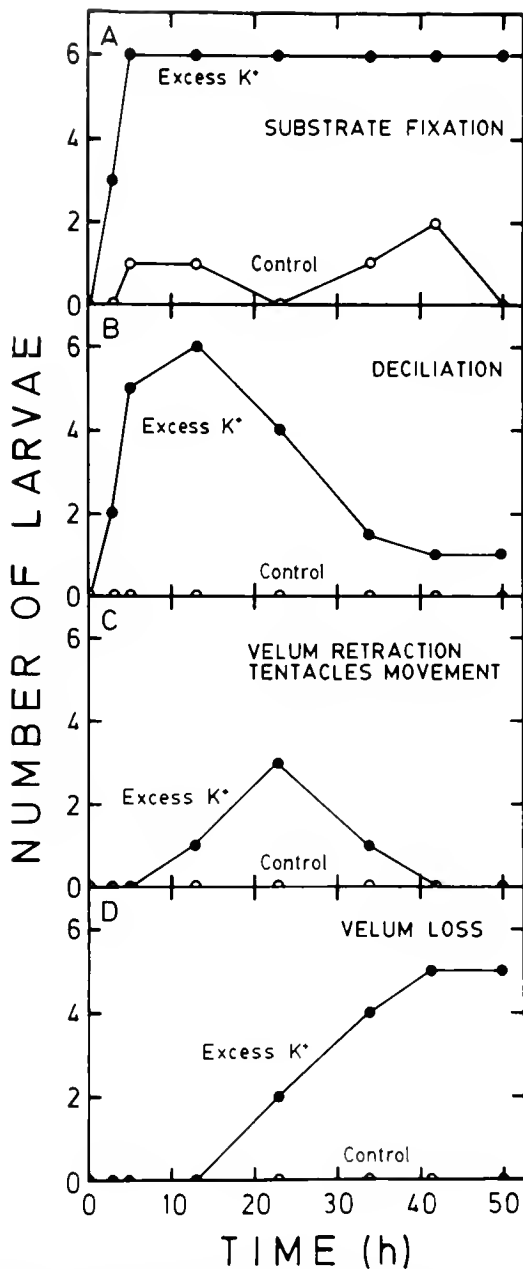


Figure 3. Sequence of metamorphic steps followed by competent larvae of *Concholepas concholepas* after induction to metamorphosed by excess potassium. Graph A: shows time of attachment to the substratum (wall of the plastic flask). Graph B: shows the curve for deciliation process. Graph C: indicates the timing of the third step: decrease in the velar lobe size and movement of tentacles. Graph D: shows the curve of velar loss and exit of cephalic tentacles from the shell, events which indicate that metamorphosis is over. For this experiment twelve competent larvae collected in Valdivia were divided in two groups of 6 each. One of the groups was exposed to excess of  $K^+$ . Each larva was kept in one flask containing 30 ml of filtered seawater. Observations were done under a dissecting microscope each 3 to 5 hours.

(Fretter and Graham 1962, Morse et al. 1980). In fact, groups of large ciliated cells localized in the periphery of the velar lobes were detached very early after larval attachment to the plastic substratum. This observation suggests that detachment of ciliated

cells from the velum is a primary response of the larvae in the chain of morphogenetic events triggered by exposure of competent larvae to  $K^+$ . Recent studies by Pires and Hadfield (1991) on the effects of oxidative breakdown products of catecholamines and hydrogen peroxide on metamorphosis of the nudibranch *Phetilla sibogae*, suggested that hydrogen peroxide could regulate the activity of a factor involved in epithelial cell adhesive interactions. The timing of the deciliation process in *Concholepas concholepas* was similar to that observed in competent *Haliotis* larvae exposed to GABA (Morse et al. 1980). Also, and because only a few ciliated velar cells were found in the flask after deciliation, most of them may have been eaten by the metamorphic larvae, a possibility previously suggested by Fretter and Graham (1962).

The induction of metamorphosis by potassium ions in a large number of species makes it a useful agent to be used in the cultivation of commercially important marine invertebrates (Rodríguez et al. 1993). The use of GABA, L-DOPA, and other inducers can be replaced by  $K^+$  ions which, at up to 20 mM, depolarize the epithelial membrane triggering metamorphosis (Pawlik 1992, Rodríguez et al. 1993).

Molluscan metamorphosis determines several morphological, physiological, and eventually, some biochemical changes (Bonar and Hadfield 1974, Hadfield 1984, Fenteany and Morse 1993). Recently the successful induction of settlement of *C. concholepas* by excess  $K^+$  has been used to characterize some of the molecular changes that take place during molluscan metamorphosis. Modifications in the pattern of protein synthesis, an increase in heparin-binding proteins, and a decrease in cyclic AMP levels have been found (Inestrosa et al. 1993). Of particular interest is the increase of [ $^{35}S$ ]-methionine incorporation in heparin-binding proteins because such macromolecules are usually related to mitogenic factors such as fibroblast growth factors (Lobb and Feet 1984). In fact, such growth-associated factors have been identified in the large muscular foot of *Concholepas concholepas* (Cantillana and Inestrosa 1993). Recent evidence indicates that high larval settlement rates of *Haliotis rufescens* occur on substrates of conspecific mucus containing some unknown inductive cue (Slattery 1992). It is possible that heparin-binding growth factors present in the mucus could be triggering the larval settlement of some molluscan species (Rodríguez et al. 1993).

The induction of settlement of larvae of *Concholepas concholepas* by  $K^+$  may be a useful tool for the cultivation of this commercially important species. In addition, use of  $K^+$  would allow determination of intrinsic larval molecular changes and factors associated with settlement in *Concholepas concholepas*.

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## COMPARATIVE LIFE HISTORY STUDIES OF TWO SYMPATRIC *PROCAMBARUS* CRAWFISHES

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**ABSTRACT** A comparative 12 month life history analysis was conducted on syntopic populations of *Procambarus clarkii* and *Procambarus zonangulus* from Hardin Co., Texas. Temporally burrowing, molting, reproduction, and surface water use were relatively similar between the species. *Procambarus zonangulus* gained more weight per unit length and obtained a greater maximum length than *P. clarkii*. Male *P. zonangulus* possessed significantly longer chelae than did *P. clarkii* counterparts, but no differences between females existed. Dietary differences existed between the species. *Procambarus clarkii* had more full guts than *P. zonangulus*, and female *P. clarkii* consumed more animal tissue than female *P. zonangulus*. Even though female *P. zonangulus* possessed greater weights and greater carapace lengths, their fecundity was significantly lower than that of *P. clarkii*.

**KEY WORDS:** *Procambarus clarkii*, *Procambarus zonangulus*, life history, ecology, behavior

### INTRODUCTION

This study analyzed the life history patterns of two closely related (Hobbs 1962, 1984), syntopic crawfish, *Procambarus clarkii* (Girard) and *P. zonangulus* Hobbs and Hobbs, collected simultaneously from a complex of sloughs and baygall breaks in Southeast Texas. Excellent, detailed life history studies have been conducted on *P. clarkii* (Penn 1943) and possibly on *P. zonangulus* (Albaugh 1973). *Procambarus zonangulus*, recently described by Hobbs and Hobbs (1990), is a member of a species complex which replaces *P. acutus acutus* west of the Mississippi River. Since Albaugh (1973) worked with several populations, it is possible that he worked with more than one species. Therefore, in this paper references to *P. acutus* (Girard) involving populations west of the Mississippi River will use the name *P. zonangulus*, but it must be realized that more than one species may be involved. The value in this study is that it clarifies specific life history traits in sympatric wild populations the two most important commercial species of crawfish in North America.

### STUDY AREA

The study area was located in Hardin County, Texas, (Long. 94°10'; Lat. 30°17') in hills bordering the Neches River. The study area included Massey Lake Slough, a permanent multi-channel system and an unnamed, intermittent slough which drain into the Neches River. Pools in the sloughs were 0.2 to 1.3 m deep, and the channels were 5 to 20 cm deep. Lying between the two sloughs were several baygall breaks. These breaks consisted of shallow depressions which easily filled with water during winter and spring floods or heavy rains. During high water the two sloughs become contiguous via water flowing from the unnamed slough through the baygall breaks and into Massey Lake Slough. This aquatic system was dominated by bald cypress, *Taxodium distichum* (L.) Rich, and tupelo, *Nyssa aquatica* L. The low hills surrounding the

sloughs and baygall breaks were covered by a mixed deciduous forest (*Quercus* and *Fagus*) with occasional stands of loblolly pine, *Pinus taeda* L. (extensive logging occurred after the study). The substrate along the sloughs and baygall breaks consists of a heavy clay which allows the breaks to hold water well into the summer and sometimes never dry up. As a result, herbaceous plants and grasses rarely grow in the breaks, but are common along the well drained sloughs.

### MATERIALS AND METHODS

Crawfishes were collected monthly from May 1984 to April 1985. No night time collections were made at the study site because of danger from poachers who frequented the area at night. Collections were made by seining (3.1 mm mesh) and with a dip net (6.3 mm mesh). Specimens were hardened in 10% formalin for 24 hr, washed 24 hr in water and preserved in 55% isopropyl alcohol. Measurements were made using a Mitutoyo dial caliper and an ocular micrometer. Measurements referring to length are carapace length (CL) measured from the rostrum tip to the posterior of the carapace. Wet weights were determined using a Mettler AE 100 analytical balance. Fecundity was determined by counting heavily yolked eggs that could be teased from the ovaries. Mean ova diameters were calculated using counts of 20 randomly selected, heavily yolked ova per specimen. Gut analyses were conducted by placing a slurry of material from the stomach on a slide and identifying 50 pieces as sand, vegetal, or animal tissue, or unknown material. When possible, the specific type of plant or animal material was identified.

Relationships involving length-weight, chelae-length or fecundity-length were analyzed using BMDP9R, an all possible subsets regression analysis package (Dixon et al. 1985). Regression analyses included linear and quadratic components. Interspecific analyses were conducted by comparing form I males, and juveniles and form II males, and females of one species against those of the other species. Descriptive statistics were calculated using SPSSx Frequencies statistical package (SPSSx 1983).

\*We regret to report the death of the senior author, a dedicated biologist (Bechler 1990)

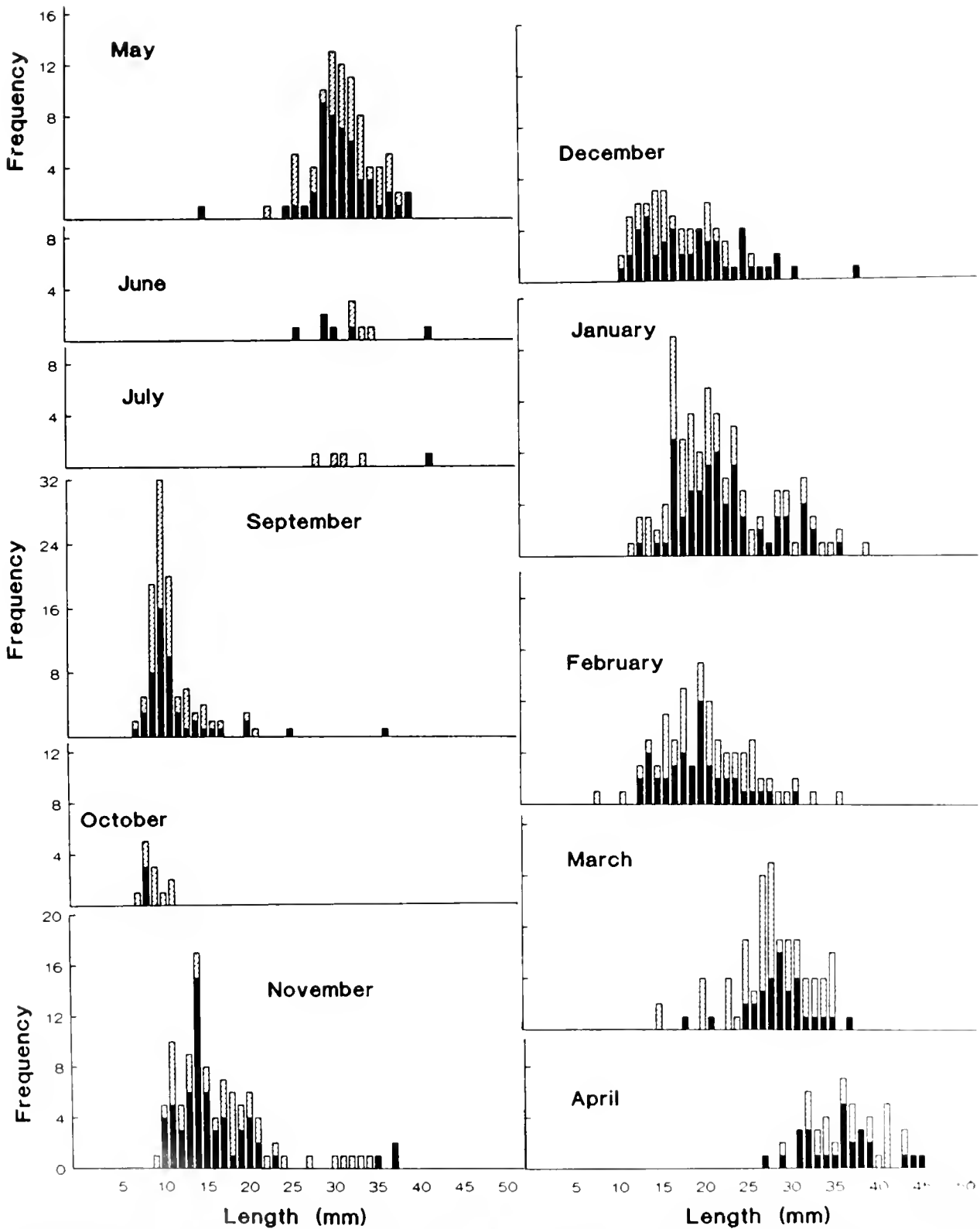


Figure 1. Length-Frequency Histograms for *P. clarkii* in 1984-1985 are given. All tick marks on vertical axes are in four unit increments except for September which is in eight unit increments. Males = solid bars, Females = slashed bars.



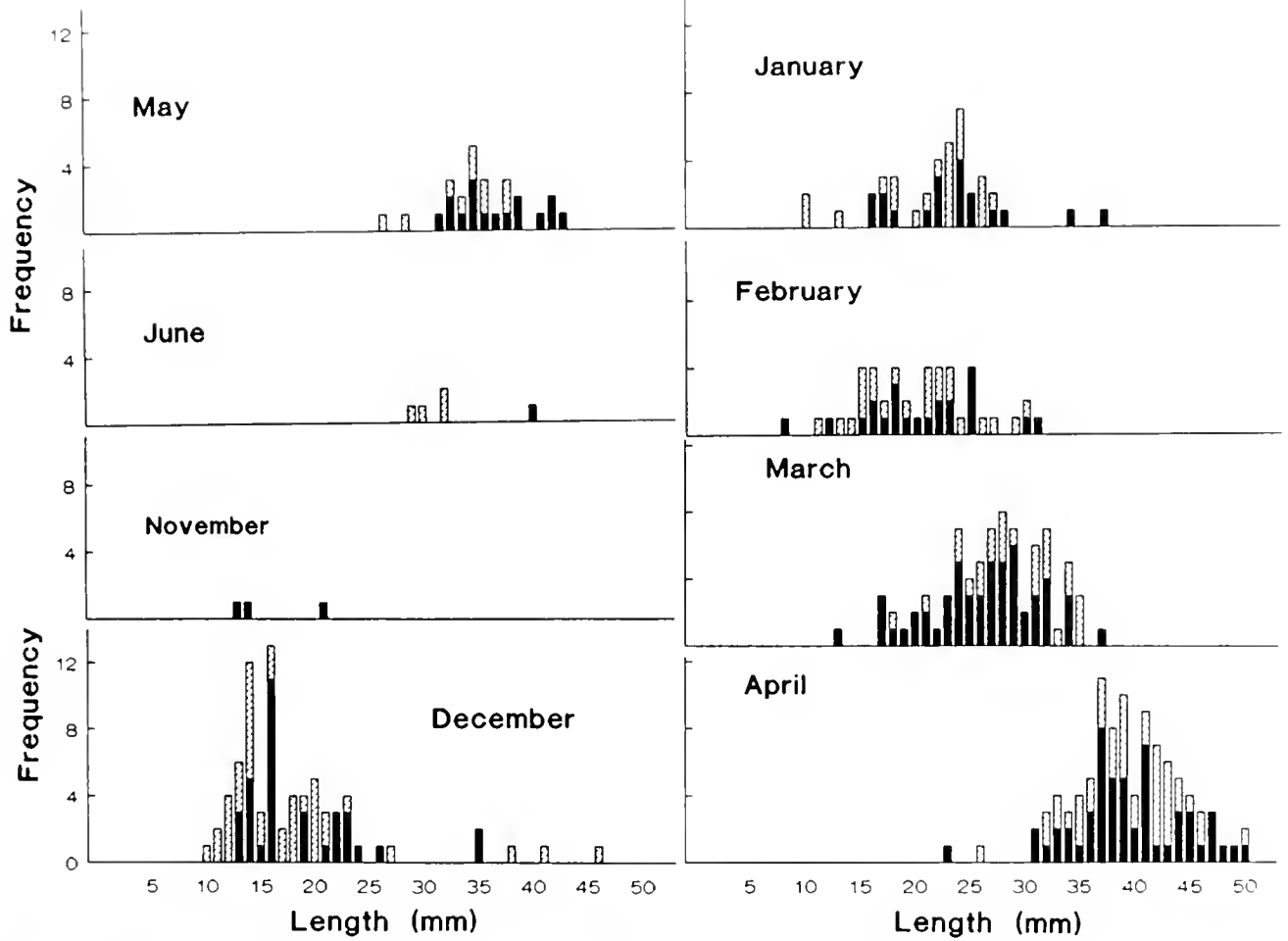


Figure 2. Length-Frequency Histograms for *P. zonangulus* in 1984-1985 are given. All tick marks on vertical axes are in four unit increments. Males = solid bars, Females = slashed bars.

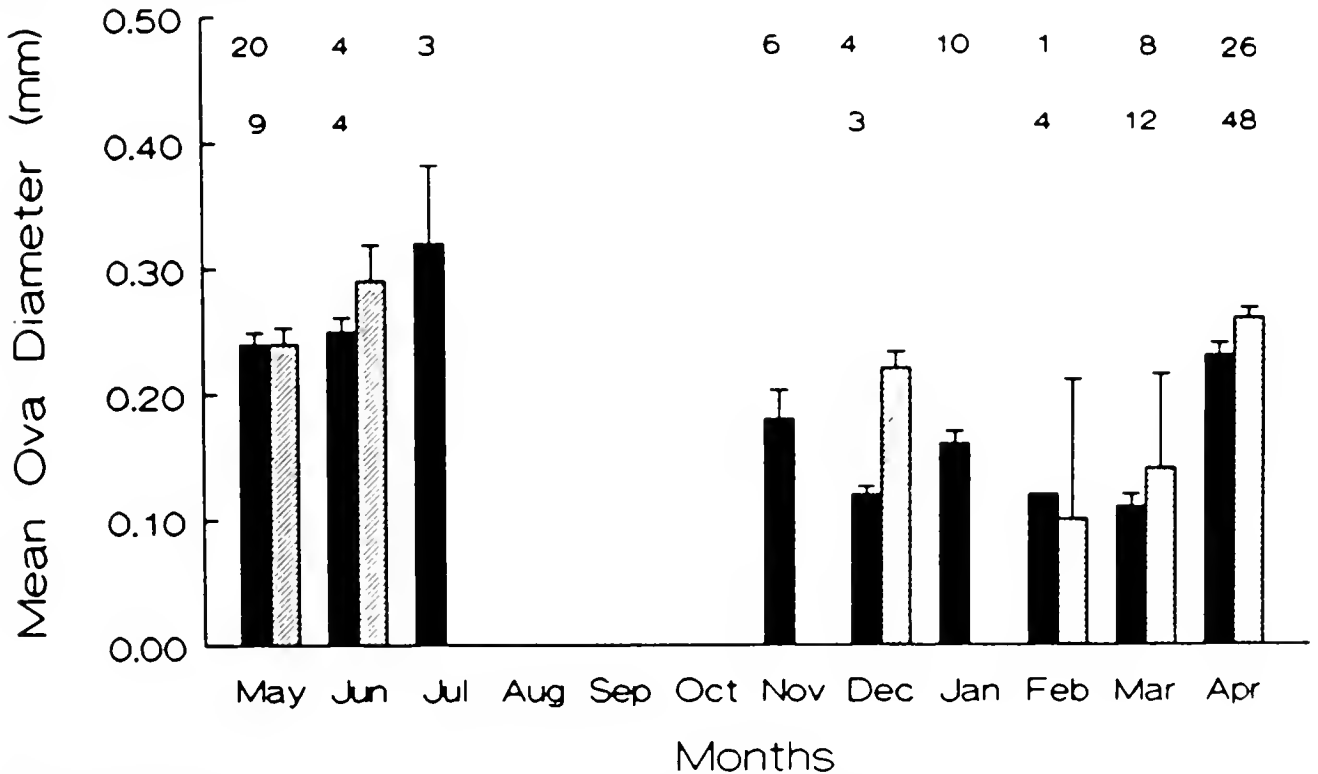


Figure 3. Mean Ova Diameters for *P. clarkii* (solid bars) *P. zonangulus* (slashed bars) for 1984-1985 are given. The top row of numbers above the bars indicate sample sizes for *P. clarkii* and the bottom row indicates sample sizes for *P. zonangulus*. Cross bars equal standard errors of the means.

## RESULTS

*General Ecology and Behavior*

Both species were collected from the sloughs and baygall breaks, but limited numbers of the two species occurred at various times of the year. Crawfish were present only in April and May in Massey Lake Slough which contained many predators that can limit the presence of crawfish (Stein and Magnuson 1976). Besides predators crawfish distribution in surface waters was strongly affected by desiccation. Summer, 1984, rainfall was limited, and the sloughs and breaks began to dry in June and July with breaks completely dry by August. September rains increased the flow of the unnamed slough, but the breaks remained dry until November. Figures 1 and 2 reflect the effects of desiccation as only 15 adult *P. clarkii* and five adult *P. zonangulus* were collected in June and July. Large numbers of juveniles and two adult *P. clarkii* were collected in September, but only 12 juveniles were collected as drought reoccurred in October. Except for three juveniles collected in November, *P. zonangulus* was not seen until December when large numbers of juveniles and a few adults were collected.

Confounding desiccation effects was the burrowing behavior of both species. Both species are tertiary burrowers that burrow during oviposition (Hobbs 1981). Oviposition, discussed below, began in July or August for both species, so sequestration in burrows during June and July would be expected. By late June *P. clarkii* became difficult to collect and *P. zonangulus* disappeared completely from surface waters.

In baygall breaks, adults and juveniles of both species utilized the available surface waters differently. Large specimens were captured in pools 30 to 50 cm deep. Juveniles hid in leaf litter around tree bases and baygall break perimeters in 5 to 10 cm of water.

*Population Structure and Growth*

Juvenile *P. clarkii* appeared in surface waters from September to February (Fig. 1). The smallest individual was 6.7 mm CL. Three juvenile *P. zonangulus* were collected in November and the large size of one individual, 20.4 mm CL, suggests that this individual came from a brood produced as early as September (Fig. 2). Otherwise, newly emergent juveniles appeared from December through March with the smallest being 7.8 mm CL.

After the initial emergence of young into surface waters, the monthly overall carapace length of both species increased steadily until April (Fig. 1 and 2). Sample sizes for May through July, 1984, are inadequate for both species, but suggest that rapid growth in the mean carapace length slowed in May as members of both species reached maturity.

Adults of both species were collected with newly emerging and maturing juveniles from September to January. The large size relative to the rest of the population indicated that these adults were from the previous generation. The fact that these adults were not larger than the largest specimens from May indicated that they were not from two previous generations. Therefore, few individuals of either species were living longer than 12 months. The few that lived past 12 months lived no more than 17–18 months. Females living 17 to 18 months could engage in a second breeding season.

Soft exoskeletons and the presence of gastroliths can serve as indicators of molting (Travis 1960, Rao et al. 1977, Huner et al. 1978). Specimens of *P. clarkii* and *P. zonangulus* with soft

exoskeletons and/or gastroliths were collected throughout the study period. The majority, 16 *P. clarkii* and 11 *P. zonangulus*, were collected in March and April. Six *P. clarkii* and five *P. zonangulus* were collected November through January. The only other time molting specimens were collected was in June when one *P. clarkii* and two *P. zonangulus* were collected. The paucity of molting specimens from June to August corresponds with the time period when the adults sequestered themselves for reproductive activities.

Males can be classified as form I or II based upon the cornification and structure of the copulatory stylets (Hobbs 1972). For the purpose of analysis, all males that were not form I were classified as juveniles/form II. Lengths of form I *P. clarkii* ranged from 29.0 to 44.5 mm CL (mean = 35.3 mm). The range for juveniles/form II males was 6.9 to 43.7 mm CL (mean = 20.7 mm).

Form I *P. zonangulus* ranged from 32.7 to 45.3 mm CL (mean = 39.8 mm) and juvenile/form II males ranged from 7.8 to 49.7 mm CL (mean = 25.1 mm). Maturation of the Hardin County population occurred between March and April such that only ju-

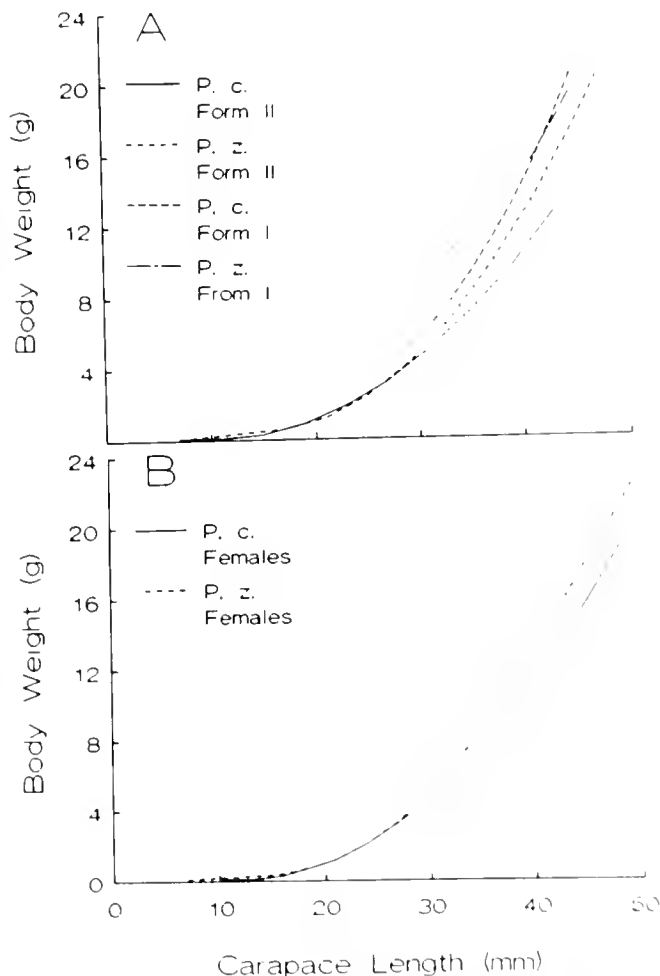


Figure 4. Regression lines for weight-carapace length are graphed for *P. clarkii* and *P. zonangulus*. Part A represents form I males (top two lines) and form II males (bottom two lines). Part B represents females. The length of the lines represents the range of the carapace lengths for each set of animals. Data points are not given. Statistics are given in Table 1 and the text. Form II includes both juvenile and form II males.

veniles were collected in March and form I males were only collected from April to June. *Procambarus clarkii* followed a similar strategy with form I males only being collected from April to June, except for a single form I male collected in December.

Females are considered sexually mature when ova diameters rapidly increase (Penn 1943, Smart 1962, Albaugh 1973, Boyd and Page 1978). Maximum egg size for both species in March was 0.17 mm; and both species showed a rapid ova diameter increase in April (Fig. 3) with a size range of 0.17 to 0.35 mm for *P. clarkii* and 0.15 to 0.35 mm for *P. zonangulus*. Therefore, females of both species with ova 0.18 mm or greater were considered sexually mature. Using this criterion the smallest sexually mature female *P. clarkii* was 28.2 mm CL (mean = 32.9 mm) and the smallest *P. zonangulus* was 30.8 mm CL (mean = 37.9 mm). Females of both species followed a pattern of sexual maturation similar to that of the males with maturation occurring in April.

Chi square analyses for 1:1 sex ratios for the entire year for each species were conducted. Neither *P. clarkii* (N = 747, X<sup>2</sup> = 1.297, P = 0.20, df = 1) nor *P. zonangulus* (N = 368, X<sup>2</sup> = 0.696, P > 0.30, df = 1) deviated from a 1:1 sex ratio.

Curvilinear relationships between carapace length and body weight were found for females and juvenile/form II males of both species (Fig. 4). Hence, separate quadratic polynomial models for females and juvenile/form II males were postulated for each species. Form I males produced a more complex pattern of length-weight relationships. Regression equations and relevant statistical information for the length-weight relationships for the two species are given in Table 1. Both juvenile/form II males and female *P. zonangulus* were significantly heavier than their *P. clarkii* counterparts. The greater weight of juvenile/form II *P. zonangulus* males resulted from a significantly higher quadratic coefficient (t = 12.87, P = 0.0001) associated with the regression equations. The greater weight of female *P. zonangulus* resulted from signif-

icantly higher coefficients for both the linear (t = 3.34, P = 0.001) and quadratic (t = 10.04, P = 0.0001) components of the regression equations.

No significant difference existed between the weights of form I males. *Procambarus zonangulus* males were heavier at shorter carapace lengths; however, the greater weight of *P. clarkii* at longer carapace lengths negated any differences between the species. *Procambarus clarkii* males have more robust claws than *P. zonangulus* males, especially the larger form I males. The more robust claws of larger form I *P. clarkii* in our population undoubtedly provided them with a greater weight than *P. zonangulus*. This factor negated weight differences that existed between smaller Form I males of each species.

Figure 5 depicts curvilinear relationships of chela length versus carapace length for five of six possible relationships. Only form I *P. clarkii* produced a linear relationship. Regression equations and statistics for chelae length versus carapace length are given in Table 1.

Regression analyses indicated that form I and juvenile/II males and female *P. zonangulus* possessed significantly longer chelae than did their *P. clarkii* counterparts. The greater chelae length of Form I *P. zonangulus* males and females resulted from higher intercept coefficients (Males: t = 5.05, P = 0.0001; Females: t = 3.30, P = 0.001). The significantly greater chelae length for juvenile/form II males was due to higher coefficients for the linear components (t = 2.71, P = 0.007) and the quadratic components (t = 4.84, P = 0.0001).

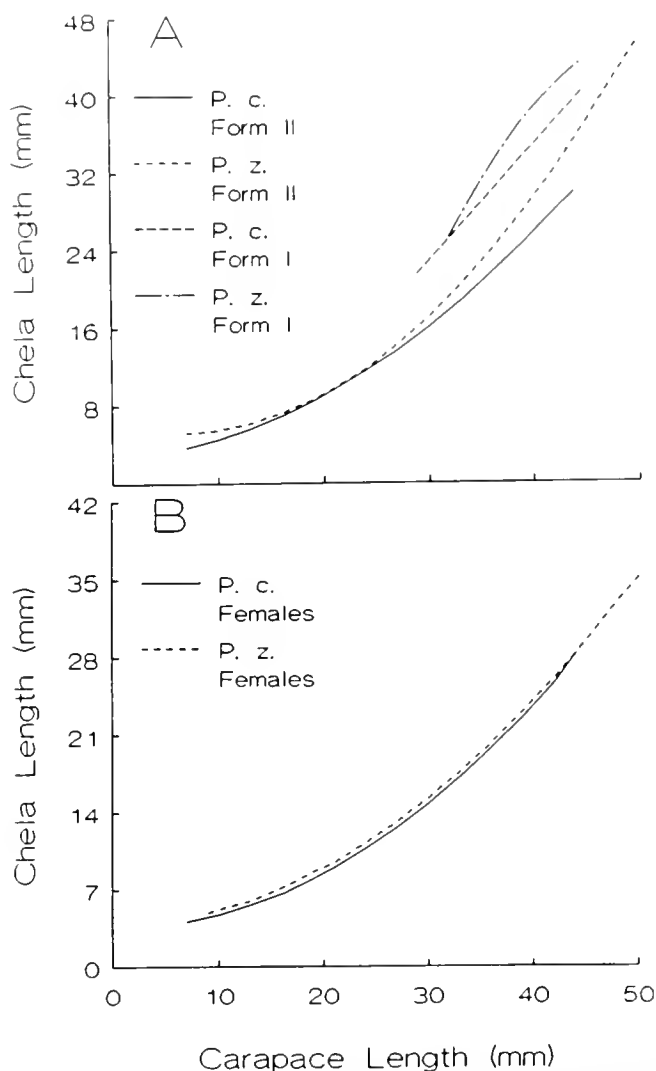
**Reproduction**

*Procambarus clarkii* produced significantly more eggs than *P. zonangulus* (Fig. 6, Table 1). At 35 mm CL, the mean carapace length for the two species, *P. clarkii* produced 212 more eggs than female *P. zonangulus*. This difference resulted from the fact that

**TABLE 1.**  
Regression analyses for weights, chelae lengths, and egg counts for *P. clarkii* and *P. zonangulus*.

	Juv/II	Y <sub>weight</sub> = 1.647 + 0.269X + 0.012X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>weight</sub> = 1.440 + 0.269X + 0.019X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Form I	Y <sub>weight</sub> = 11.727 + 1.016X + 0.030X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>weight</sub> = 13.032 + 0.705X + 0.030X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Females	Y <sub>weight</sub> = 2.045 + 0.340X + 0.015X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>weight</sub> = 2.045 + 0.361X + 0.020X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Juv/II	Y <sub>chela</sub> = 10.944 + 0.657X + 0.013X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>chela</sub> = 11.155 + 0.709X + 0.022X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Form I	Y <sub>chela</sub> = 0.971 + 1.198X	( <i>P. clarkii</i> )				
		Y <sub>chela</sub> = 35.390 + 1.489X - 0.047X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Females	Y <sub>chela</sub> = 11.679 + 0.650X + 0.013X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>chela</sub> = 12.150 + 0.650X + 0.013X <sup>2</sup>	( <i>P. zonangulus</i> )				
	Eggs	Y <sub>eggs</sub> = 477.0 + 16.995X + 1.383X <sup>2</sup>	( <i>P. clarkii</i> )				
		Y <sub>eggs</sub> = 265.3 + 16.995X + 0.469X <sup>2</sup>	( <i>P. zonangulus</i> )				
	<b>Body Weight</b>			<b>Chelae Length</b>			<b>Females</b>
	<b>Juv/II</b>	<b>Form I</b>	<b>Females</b>	<b>Juv/II</b>	<b>Form I</b>	<b>Females</b>	<b>Egg Count</b>
N	525	45	519	405	43	397	159
R <sup>2</sup>	0.975	0.834	0.982	0.955	0.936	0.960	0.469
F	65.856	1.673	70.959	41.142	13.324	5.870	34.817
Df	3,519	3,39	3,513	2,399	2,37	2,391	3,153
P	<0.01	>0.05	<0.001	<0.01	<0.01	<0.01	<0.001

Subscripts indicate the parameters regressed. Independent variable (X) represents carapace length. Juv/II represents the combined groups of juvenile and form II males.



**Figure 5.** Regression Lines for Chelae Length-Carapace Length relationships are graphed for *P. clarkii* and *P. zonangulus*. Part A represents form I males (top two lines) and form II males. Part B represents females. The length of the lines represents the range of carapace lengths for each set of animals. Data points are not given. Statistics are given in Table 1 and the text. Form II includes both juvenile and form II males.

*P. clarkii* had a significantly higher intercept ( $t = -7.88$ ,  $P = 0.00001$ ), than did *P. zonangulus*. Female *P. clarkii* also produce a wider range of ovarian egg numbers. The range of egg numbers for *P. clarkii* was 215 to 1,820 compared to *P. zonangulus* with a range 28 to 795.

Ova development for both species followed similar patterns (Fig. 3). Ova were smallest in February and March and rapidly increased in size during April. After April ova development increased steadily until June for *P. zonangulus* and July for *P. clarkii*. By August females of both species sequestered themselves for oviposition. As a result, no females with measurable ova were obtained until November, and newly emergent juvenile females were not measured due to their small size. From November to January large females of both species were captured, but only *P. clarkii* consistently possessed ova large enough to measure. Mean

ova size during these months was less than the period prior to sequestration, but larger than specimens seen in February and March. Some specimens contained no measurable ova, suggesting that they had completely spawned and/or reabsorbed their unspawned eggs. Other females contained unspawned eggs that were intermediate in size, suggesting failure to spawn with possible reabsorption or development of new ova.

#### Gut Analysis

A comparative gut analysis/species/sex was conducted on individuals exceeding 19 mm CL (Table 2). A greater percentage of male ( $N = 94$ ,  $df = 1$ ,  $X^2 = 7.301$ ,  $P < 0.01$ ) and female ( $N = 79$ ,  $df = 1$ ,  $X^2 = 20.213$ ,  $P < 0.001$ ) *P. clarkii* possessed food in their stomachs than did male and female *P. zonangulus*. An analysis was conducted on variation in the quantity of various food items eaten by each species. Male *P. clarkii* and *P. zonangulus* consumed about equal amounts of vegetal tissue, but *P. clarkii* consumed significantly less animal tissue, unrecognizable material, and more sand than did *P. zonangulus* ( $N = 87$ ,  $df = 3$ ,  $X^2 = 186.753$ ,  $P < 0.001$ ). Female *P. clarkii* consumed significantly more animal tissue than female *P. zonangulus* but less vegetal tissue, unrecognizable material, or sand ( $N = 75$ ,  $df = 3$ ,  $X^2 = 320.768$ ,  $P < 0.001$ ).

Qualitatively, identifiable items found in the stomachs included insect legs, compound eyes, butterfly scales, mayflies, amphipods, copepods, and other crawfish. Vegetal tissue included leaves, blue green algae, and diatoms. Vegetal tissues comprised the majority of each species' diet and animal tissue was next in abundance of nutritional items (Table 2).

#### DISCUSSION

The literature on *P. zonangulus*, originally *P. acutus* for specimens west of the Mississippi River (Hobbs and Hobbs 1990), and *P. clarkii* is extensive and suggests basic differences between the species. However, differences are implied since most studies dealt with allopatric populations. Sheppard (1974), Huner (1975), Romaine and Lutz (1989), and Niquette and D'Abramo (1991) are exceptions, but their studies did not examine all the same life history traits that our study did. Errors can occur if comparative conclusions are drawn from allopatric populations (Terman 1974). Because our populations were sympatric, if not syntopic (Pianka 1988), our results provide a clearer picture of how the two species responded to similar environmental conditions and each other in Massey Lake Slough.

While several minor differences involving behavioral responses to desiccation and reproduction were found, the major differences involved size and length-weight relationships, fecundity and diet. The greater carapace length, chelae length, and body weight of *P. zonangulus* suggests that it should be a superior competitor for food and lay more eggs than *P. clarkii*. However, the opposite was found in this study.

Body size (Bovbjerg 1953, Berrill and Arsenault 1984) and chelae size (Stein 1976) are key factors in intraspecific competition. Traits that confer intraspecific dominance often confer interspecific dominance (Grant 1972); and large crawfish species dominate smaller ones (Momot 1984). If these factors hold for *P. zonangulus*, then the lower rates of food consumption by male and female *P. zonangulus* and the lower rate of animal tissue

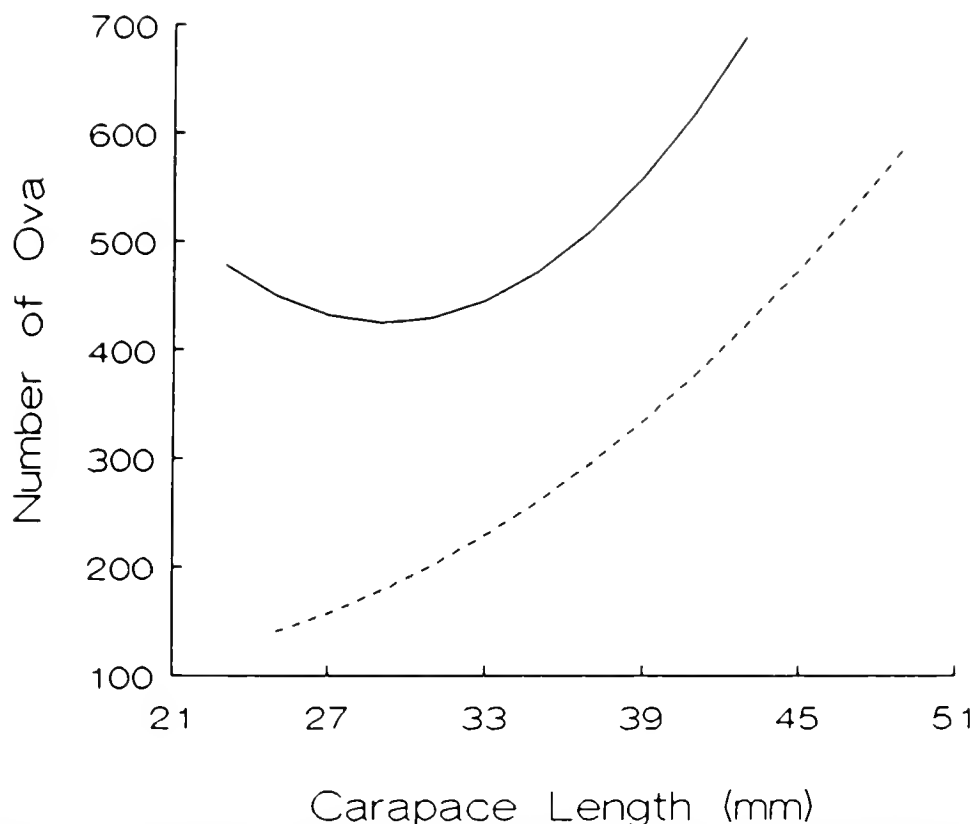


Figure 6. Regression Lines for Ova Number-Carapace Length relationships for *P. clarkii* and *P. zonangulus* are given. Elevation of the left hand side of the regression line for *P. zonangulus* results from a low sample size and several high ova counts. Data points are not given. Statistics are given in Table I and the text.

consumption by female *P. zonangulus* are in opposition to what one would conclude from the literature. However, two factors not examined in this study, apparent chelae size and foraging efficiency, might explain the observed differences.

Apparent chelae size was not quantified in this study. Quantitatively, *P. zonangulus* possessed longer chelae, but qualitatively *P. clarkii* possessed more robust chelae. The importance of this difference is not known, nor is it known which species is more efficient at using its chelae during interspecific aggression. Apparent chelae size may be a factor as evidence indicates that *P. zonangulus* males may view *P. clarkii* males as more dominant (Bechler et al. 1988).

The observed dietary differences might also result from differ-

ences in foraging efficiency. To the best of our knowledge, the crawfish literature contains no references which explicitly examine interspecific crawfish foraging efficiency as in this study.

The number of eggs produced by *P. clarkii* versus *P. zonangulus* per unit body length is also counter to what is predicted in the literature (Momot 1984), but was recognized by LaCaze (1966). The differences in fecundity can be best explained by historical constraint (Brooks and Wiley 1984). That is, past environments of one or both species have forced upon one or both species levels of fecundity not yet changed in response to recent selection pressures. Comparisons of the fecundity rates of *P. zonangulus* and *P. clarkii* from Massey Lake Slough to other populations indicates that both species have fecundity rates comparable to other referenced populations of the same species (Penn 1943, LaCaze 1966, Albaugh 1973). *Procambarus hayi*, a species similar to *P. zonangulus* (Payne 1972), also possesses a fecundity rate equivalent to that of the Massey Lake Slough population (Payne 1971). If proximal selection pressures were primarily responsible for the fecundity rates of individual populations, then it would be expected that different fecundity rates would exist for different populations, but this is not the case. Therefore, fecundity is best explained by historical factors.

TABLE 2.

The percentage of stomachs containing food and the percentage of each dietary item by sex by species. Statistical analyses are in the text.

	<i>P. clarkii</i>		<i>P. zonangulus</i>	
	Males	Females	Males	Females
Stomachs with food	92.6	94.9	81.8	72.3
Vegetal Tissue	69.8	71.6	69.7	76.0
Animal Tissue	11.7	13.2	16.5	6.2
Unknown Material	0.4	0.3	1.2	0.7
Sand	18.1	15.0	12.6	17.1

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## **OYSTER DISEASE RESEARCH (ODR) PROGRAM**

*Administered by the  
National Marine Fisheries Service,  
Northeast Region,  
1990 — 1992*

**An Overview Published in Cooperation  
with The National Shellfisheries Association**

December 1993





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## PREFACE

The Northeast Region of the National Marine Fisheries Service (NMFS), for a three-year period beginning in 1990, assumed technical oversight responsibilities for administration of the NMFS Oyster Disease Research (ODR) Program. This report provides an overview on the status of program accomplishments during that period.

Two major oyster pathogens, "Dermo" (*Perkinsus marinus*), which was first reported during the late 1940's, and "MSX" (*Haplosporidium nelsoni*), first reported in the mid-1950's, continue to adversely impact oyster resources in the Northeast. The ODR Program was initiated as a consequence of the increased prevalence of these disease agents, with resultant declines in stock abundance and commercial landings of the eastern oyster, *Crassostrea virginica*, along the Atlantic seaboard. Congressional appropriations during 1990–1992 for the conduct of research and management-related investigations totalled approximately \$3.2 million. Although congressional and public attention has focused primarily upon oyster resources in the Chesapeake Bay, the problems of disease and deterioration of the commercial fishery have had economic and biological repercussions in Delaware Bay as well, and extending northward to New England. The ODR Program has promoted the development and use of state-of-the-art biotechnology and in-depth social and economic assessments of the current industry to address the pressing issue of managing the impact of disease on East Coast oyster populations.

A portion of the early Program appropriations was transferred to the National Sea Grant College Program for co-sponsorship of workshops on oyster diseases and industry problems. The first was held at the Virginia Institute of Marine Science and emphasized oyster disease issues from a biological and technical perspective. A second workshop, in Annapolis, Maryland, addressed socio-economic, management, marketing, and other problems affecting the oyster industry. These meetings provided a basis for the identification of resource management and research needs, which were incorporated as program priorities in the 1990 and 1991 (ODR Program) Requests for Proposals (RFPs). A survey of researchers and resource managers in August 1991 confirmed the continued relevance of these needs, and provided the basis for minor adjustments in priorities. A third workshop was held in October 1991, again in Annapolis, to address the biology of the Pacific oyster, *Crassostrea gigas*, and the potential ecological risks and benefits if this species were introduced into coastal areas of the mid-Atlantic.

Thirty-three peer-reviewed projects, with an average funding level of \$88,400, have been awarded on a competitive basis under this Program since 1990. Funding was allocated during the three-year period among the following research categories: (1) Disease Resistance, 32%; (2) Oyster Stock Status, 19%; (3) Disease Diagnostics, 17%; (4) Pathogen Life-Cycle Studies, 14%; (5) Genetic Studies, 13%; and (6) Disease Transmission, 5%. To date, notable program accomplishments have included the successful establishment of cultural and diagnostic techniques to enable intensified studies of the oyster pathogen *Perkinsus marinus*, the

development of a socio-economic profile of the Northeast United States oyster industry, and the discovery of cell-surface antigens which will be used in future research to identify oyster stocks with natural genetic resistance to selected pathogens.

Complementary research under the ODR Program has also been supported at NMFS laboratories in Oxford, Maryland, and Milford, Connecticut. These investigations involved the development of rapid diagnostic methods for studying the prevalence and distribution of MSX and Dermo; the study of the *in vivo* osmotic effects of rapid salinity changes on oysters infected with MSX; an assessment of the causative agent for coastwide mortalities of hatchery-spawned juvenile oysters; and assessment of the pathological effects of toxicity from dinoflagellates on oyster survival.

The ODR Program was administered on the premise that the current problems imposed by disease on the eastern oyster cannot be answered by basic research alone and must be met with progressive and innovative research applied to management and conservation techniques. Accordingly, all proposals submitted for funding consideration were required to be submitted by, or coordinated with, agencies having state oyster resource management authority in the respective jurisdictions where project activities occurred. The integration of research and management concerns was continued through intensive exchange of ODR findings. This has been accomplished through the public availability and distribution of progress reports for each funded project, and presentation of technical papers at workshops like the Annual Shellfish Biology (Aquaculture) Seminar sponsored by the NMFS Laboratory in Milford, Connecticut, and during special sessions such as "Issues of Importance to Shellfisheries" held at the 1991 Meeting of the National Shellfisheries Association (NSA) in Portland, Maine, and more recently, in conjunction with the NSA at its 85th Annual Meeting during 1993 in Portland, Oregon. During the latter event, more than 20 principal investigators presented technical papers describing the results of research funded under the ODR Program. The following pages include the abstracts of these presentations, which were previously contained within the overall summary of abstracts for this Annual Meeting published in volume 12(1), pages 117–157, of the *Journal of Shellfish Research*.

Subsequent sections of this overview contain abstracts describing ODR-funded research conducted within the National Marine Fisheries Service, a listing of technical papers reporting individual project accomplishments to date as published in peer-reviewed journals (Table 1), and a summary of titles for external investigations funded during the period 1990–1992 (Table 2). Information concerning the availability of final progress reports for individual projects may be obtained from: National Marine Fisheries Service, State-Federal and Constituent Programs Division, One Blackburn Drive, Gloucester, MA 01930.

The contributions of the following NMFS personnel who contributed to the success of the ODR Program are gratefully acknowledged: Anthony Calabrese, Virginia Fay, Frederick Kern, and Carl Sindermann. We especially appreciate the assistance of the more than 75 technical reviewers from the state, academic,

TABLE 1.

Technical publications, to date, resulting from individual ODR Program project accomplishments.

- Allen, S. K. Jr., P. M. Gaffney, J. Scarpa & D. Bushek. 1993. Inviably hybrids of *Crassostrea virginica* (Gmelin) with *C. rivularis* (Gould) and *C. gigas* (Thunberg). *Aquaculture* 113:269–289.
- Allen, S. K. Jr. & P. M. Gaffney. 1993. Genetic confirmation of hybridization between *Crassostrea gigas* (Thunberg) and *Crassostrea rivularis* (Gould). *Aquaculture* 113:291–300.
- Anderson, R. S. In press. Modulation of blood cells mediated oxyradical production in aquatic species: Implications and applications. In: Molecular biological approaches to aquatic toxicology. Ed. by G. K. Ostrander and D. C. Matlins. Boca Raton, Florida: CRC Press.
- Anderson, R. S., K. T. Paynter & E. M. Bureson. 1992. Increased reactive oxygen intermediate production by hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. *Biol. Bull.* 183:476–481.
- Barber, R. D. & S. E. Ford. 1992. Occurrence and significance of ingested Haplosporidium spores in the eastern oyster, *Crassostrea virginica* (Gmelin, 1791). *J. Shellfish Res.* 11(2):371–375.
- Cheng, T. C., W. J. Dougherty & V. G. Burrell, Jr. 1993. Lectin-binding differences on hemocytes of two geographic strains of the American oyster, *Crassostrea virginica*. *Trans. Am. Microsc. Soc.* 112(2):151–157.
- Cheng, T. C. 1992a. Selective induction of release of hydrolases from *Crassostrea virginica* hemocytes by certain bacteria. *J. Invert. Patho.* 59:197–200.
- Cheng, T. C. 1992b. Requirement of a chelator during ionophore-stimulated release of acid phosphates from *Crassostrea virginica* hemocytes. *J. Invert. Patho.* 59:308–314.
- Cheng, T. C. In press. Oyster hemocytes: Form and functions. In: Biology, Culture and Management of the American Oyster. Ed by A. F. Able, V. S. Kennedy, and R. Newell. MD Sea Grant.
- Chu, F.-L. E. & J. La Peyre. In Press. *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters, *Crassostrea virginica*: Temperature effects. *Dis. Aquat. Organ.*
- Chu, F.-L. E. & J. La Peyre. 1993. Development of disease caused by the parasite, *Perkinsus marinus* and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. *J. Shellfish Res.* 12(1):21–27.
- Chu, F.-L. E., J. La Peyre & C. Bureson. In Press. *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters, *Crassostrea virginica*: I Salinity effects. *J. Invert. Patho.*
- Dougherty, W. J., T. C. Cheng & V. G. Burrell, Jr. 1993. Occurrence of the pathogen *Haplosporidium nelsoni* in oysters, *Crassostrea virginica*, in South Carolina. *Trans. Am. Microsc. Soc.* 112(1):75–77.
- Dungan, C. F. & B. S. Roberson. 1993. Binding specificities of mono- and polyclonal antibodies to the protozoan oyster pathogen *Perkinsus marinus*. *Dis. Aquat. Organ.* 15:9–22.
- Faisal, M. & J. La Peyre. In Press. Development of confluent monolayers from tissues of the eastern oyster, *Crassostrea virginica*. *J. Tissue Culture Methods.*
- Faisal, M. & J. La Peyre. Accepted. Decontamination of oyster tissue for long term culture using antibiotics and thermal treatment. *J. Tissue Culture Methods.*
- Ford, S. E., K. A. Ashton-Alcox & S. A. Kanaley. 1993. In vitro interactions between bivalve hemocytes and the oyster pathogen *Haplosporidium nelsoni* (MSX). *J. Parasit.* 79:255–265.
- La Peyre, J. F. & M. Faisal. In Press. Initiation of In vitro cultures of the oyster pathogen *Perkinsus marinus* (Ampicomplexa) with prezoosporangia. *J. Eukaryotic Microbiology.*
- La Peyre, J. F., M. Faisal & E. M. Bureson. 1993. In vitro propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *J. Eukaryotic Microbiol.* 40:304–310.
- Scarpa, J. & S. K. Allen, Jr. 1992. Comparative kinetics of meiosis in hybrid crosses of Pacific oyster *Crassostrea gigas* and Suminoe oyster *C. rivularis* with the American oyster *C. virginica*. *J. Exper. Zool.* 263:316–332.

TABLE 2.

Individual projects and recipients funded through the ODR Program, administered by the NMFS Northeast Region, 1990–1992.

1990	
Project Title	Recipient
“Studies on the Life Cycle of the Oyster Parasite <i>Haplosporidium nelsoni</i> (MSX)”	Rutgers University
“Cytogenetic and Electrophoretic Confirmation of Hybrid Diploid and Polyploid Crosses between the American and Pacific Oyster”	Rutgers U. & University of Delaware
“Development of a DNA Probe to Investigate the Life Cycle of <i>Haplosporidium nelsoni</i> (MSX)”	VA Institute of Marine Science
“A Comparison of Defense Capacity and Disease Resistance in Native and Non-native Oysters”	VA Institute of Marine Science
“A Profile of the Northeast United States Oyster Industry”	University of Maryland & VA Inst. of Marine Science
“The Role of Scavengers in the Transmission Dynamics of the Oyster Pathogen <i>Perkinsus marinus</i> ”	VA Institute of Marine Science
“Production of Mono- and Polyclonal Antibodies Specific for the Protozoan Oyster Pathogen, <i>Perkinsus marinus</i> , and Assessment of Their Utilities in Rapid Diagnostic Methods and Life History Studies”	Maryland Department of Natural Resources

TABLE 2.  
continued

1991	
Project Title	Recipient
"Sterility and Genetic Constancy in Triploid <i>Crassostrea gigas</i> . Evaluating the Suitability of Triploids for Ecological Testing"	Rutgers University
"Life Cycle Studies of <i>Perkinsus marinus</i> —Host Specificity"	VA Institute of Marine Science
"Disease Processes and Transmission Dynamics of <i>Perkinsus marinus</i> in American Oysters ( <i>Crassostrea virginica</i> )"	VA Institute of Marine Science
"A Physiological Approach to Understanding of Parasite ( <i>Perkinsus marinus</i> ) and Oyster ( <i>Crassostrea</i> spp.) Interactions: Pathological Effects and Disease Resistance"	VA Institute of Marine Science
"Development of an <i>In vitro</i> Cell System from the American Oyster <i>Crassostrea virginica</i> Tissues and the Use of this System in Isolation and Characterization of Oyster-Associated Viruses"	VA Institute of Marine Science
"Environmental Control of <i>Perkinsus marinus</i> and Elucidation of Overwintering Infection"	VA Institute of Marine Science
"An Analysis of Genetic Variation between and within Strains of the American Oyster Selected for Disease Resistance"	VA Institute of Marine Science
"Flow Cytometric Quantification and Analysis of <i>Perkinsus marinus</i> Cells Present in Estuarine Waters"	MD Department of Natural Resources
"Chemotherapy to Mitigate the Impact of Perkinsiasis (Dermo Disease)"	MD Department of Natural Resources
"Integrated Physiological Investigation of the Effects of Protozoan Parasitism in the Oyster, <i>Crassostrea virginica</i> "	University of Maryland
"Identification of Recognition Sites on Oyster Phagocytes and Oyster Parasites by Using Lectins"	Medical University of South Carolina
1992	
Project Title	Recipient
"Flow Cytometric Quantification and Analysis of <i>Perkinsus marinus</i> Cells Present in Estuarine Waters"	University of Maryland
"Potential Use of Immuno-Stimulants to Augment the Resistance of the Eastern Oyster <i>Crassostrea virginica</i> to Infection by <i>Perkinsus marinus</i> "	VA Institute of Marine Science
"A Stock-Recruit Model of the James River Oyster Fishery"	VA Institute of Marine Science
"Studies of Genetic Variation between and within Strains of the American Oyster Selected for Disease Resistance II. Analysis of Anonymous Nuclear Loci"	VA Institute of Marine Science
"Development of a DNA Probe to Investigate the Life Cycle of <i>Haplosporidium nelsoni</i> (MSX)"	VA Institute of Marine Science
"Life Cycle Studies of <i>Perkinsus marinus</i> —Host Specificity"	VA Institute of Marine Science
"Development of a Microcomputer-Based Geographic Information System (GIS) for the Visualization, Interpretation, and Analysis of MD Chesapeake Bay Oyster Disease and Population Information"	MD Department of Natural Resources
"American Oyster Stock Assessment in Maryland"	MD Department of Natural Resources
"In vitro Propagation of <i>Perkinsus marinus</i> "	MD Department of Natural Resources
"Integrated Physiological Investigation of the Effects of Protozoan Parasitism in the Oyster, <i>Crassostrea virginica</i> "	University of Maryland
"Resistance to <i>Crassostrea virginica</i> Races to <i>Perkinsus marinus</i> Isolates: A Foundation for Breeding and Management"	Rutgers University
"Life Cycle Studies of <i>Haplosporidium nelsoni</i> (MSX): Spores and Non-Oyster Hosts"	Rutgers University
"Relative Effects of Harvest Pressure and Disease Mortality on the Population Dynamics of the Eastern Oyster in Delaware Bay"	Rutgers University

private, and federal sectors who volunteered their time and expertise during the peer-review stages of the project selection procedures. Earle Buckley of the National Coastal Resources Research and Development Institute (NCRI), Victor Mancebo of the Northeastern Regional Aquaculture Center, and James McVey of the National Sea Grant College Program contributed valuable assistance during the technical review of proposals, as well as infor-

mation to facilitate the coordination of ODR funding decisions with related oyster disease research being funded under other state and federal programs.

Harold C. Mears  
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## Abstracts of ODR program papers, presented at NSA's 85th Annual Meeting, May 31–June 3, 1993, Portland, Oregon.

**TRIPLOIDS FOR FIELD TESTS? THE GOOD, THE BAD, AND THE UGLY.** Standish K. Allen, Jr., Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349.

Interest and controversy surround the "proposal" to introduce *Crassostrea gigas* to the east coast, putatively, to bolster the ailing oyster industry. Yet there is no empirical data on how *C. gigas* would perform here. Key is whether or not *C. gigas* are resistant to Dermo, or MSX-disease, or both. For the latter two questions, field exposure seems necessary. Even for ecological issues, the reliability of data extrapolated from land-based experiments is questionable. The GOOD: Triploids, because they are reproductively incapacitated, provide a way to "safely" test *C. gigas* with little or no risk of reproduction. Use of  $F_1$ , or greater, progeny reduces the risk of disease. Data show that triploids produce gamete types that vary little among individuals and that crosses using these gametes behave in predictable ways, all suggesting that the risk is estimable. The BAD: Recent evidence also suggests that there may be some spontaneous chromosome loss in triploids as they age. This surprising result means that analysis of individuals before field planting will be essential, perhaps yearly. And individual testing means a relatively small sample size, precluding pilot scale tests. The UGLY: There is no clear consensus on whether field tests using triploids should be approved; guidelines for approval of such tests are vague and variable; it is difficult to establish the distinction between an introduction for research purposes and a full scale release. This paper considers these points in view of the present crisis on the east coast oyster fishery.

**HEMOCYTE RESPONSES IN *CRASSOSTREA VIRGINICA* INFECTED WITH *PERKINSUS MARINUS*.** R. S. Anderson,\* L. L. Brubacher, and L. M. Mora, Chesapeake Biological Laboratory, University of Maryland System, Box 38, Solomons, MD 20688; K. T. Paynter, Department of Zoology, University of Maryland System, College Park, MD 20742; E. M. Burreson, Virginia Institute of Marine Science, School of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062.

The circulating hemocytes provide mollusks with their main line of defense against pathogens. These cells produce cytotoxic reactive oxygen intermediates (ROIs) that mediate killing of pathogens and/or cell injury to adjacent host tissue. In order to better understand the immune response to *P. marinus* infection, total hemocyte count (THC) and ROI production/ $10^6$  hemocytes were determined in individual oysters with known levels of hemolymph infection. Total ROI generation was quantified by phagocytically-induced, luminol-augmented chemiluminescence (CL) assays. Oysters were deployed at sites in the Wye River, Choptank River, and Mohjack Bay, and were sampled at three intervals during spring–fall 1992. *P. marinus* infection appeared earlier and progressed most rapidly in Mohjack Bay oysters, but was also present in oysters from the other sites.

Salinity differences at the sites (~13–20 ppt) had little effect on THC or CL responses. At all sites THC values for uninfected (Un) and lightly infected (L) oysters were not significantly different; however THC for L < moderately (M) < heavily (H) infected oysters. The CL response of the hemocytes also increased with the intensity of infection: Un = L < M < H. Therefore the THC and CL differences observed, whether between experimental groups or sample times, could be explained by intragroup differences in frequencies of oysters with advanced infections. It appears that progression of this infection is characterized by hemocyte recruitment and activation, expressed as increased ROI generation. The increased oxidant load may contribute to the pathogenesis of the disease via tissue damage, but ROI production alone is ineffective in controlling the infection.

**COMPARATIVE PHYSIOLOGY OF *CRASSOSTREA VIRGINICA* AND *C. GIGAS*: GROWTH, MORTALITY, AND INFECTION BY *PERKINSUS MARINUS*.** Bruce J. Barber,\* Dept. of Animal, Veterinary & Aquatic Sciences, University of Maine, Orono, ME 04469; R. Mann, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Hatchery-produced oysters (the eastern oyster, *Crassostrea virginica*, and the Pacific oyster, *C. gigas*), of the same age were held in quarantined flumes which received raw water from the York River, VA. From July 1991 to December 1993, growth and mortality were compared for experimental (dosed with *Perkinsus marinus*) and control (undosed) groups of both species.

Both prevalence and intensity of *P. marinus* infections were greater in *C. virginica* than in *C. gigas*. The experimental *C. virginica* group had 100% prevalence (with heavy infections) by August 1992; maximum prevalence in the experimental *C. gigas* group was 80%, and only 1 heavy infection was found the entire study. Overall mortality of *C. gigas* (76%) was greater than that of *C. virginica* (45%); however, only mortality of *C. virginica* was related to infection by *P. marinus*. In December 1992 (at age 20 months), mean shell height of *C. gigas* (55 mm) was significantly greater ( $P \leq 0.05$ ) than that of *C. virginica* (41 mm). Shell height was lower in the experimental group compared to the control group of *C. virginica* but not of *C. gigas*.

Thus *C. gigas* is more tolerant of *P. marinus* and grows faster than *C. virginica*, but may be less well adapted to environmental conditions prevailing in lower Chesapeake Bay.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: INDUCTION OF STRESS PROTEINS.** Drew C. Brown\* and Brian P. Bradley, Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21228; Kennedy T. Paynter, Department of Zoology, University of Maryland, College Park, MD 20742.

Stress proteins are common to all organisms. Some such as the 70 kDa heat shock protein (HSP70), respond to many stressors while other respond only to specific stressors. HSP70 increases in oyster hemocytes with increasing *Perkinsus* infection intensity. To follow the induction of HSP70 during the natural course of infection in the field, samples were taken from oyster groups deployed in floating trays at low, moderate and high salinities. The samples were taken monthly, frozen in the field on dry ice and returned to the laboratory for analysis. Soluble proteins from the mantle were run on SDS-PAGE, and either silver stained for total protein or transferred to nitrocellulose membrane, probed with antiHSP70, visualized with an alkaline phosphatase reaction and quantified using densitometry. Within group HSP70 levels showed little variation, supporting the contention that only a few animals are needed to assess the levels of HSP70 in a given group. The time course through the summer and fall showed increasing levels of HSP70, strongly correlated with *Perkinsus* infection, at the high salinity site. HSP70 levels in oysters from the low and moderate salinity sites exhibited little trend.

To examine the induction of stress-specific stress proteins, oysters (0.5 g) were exposed to salinity, temperature and anoxic stress in the laboratory, labelled with <sup>35</sup>S-methionine and processed as above. Autoradiographic analysis was used to determine which proteins were induced or shut down by the stresses. A 55kDa was identified which increased with increasing salinity but not with increasing temperature. A 19 kDa protein was induced by salinity but decreased after 48 hr anoxia. Finally, a 35kDa protein decreased in abundance with increasing temperature at 10‰ but not at 30‰.

**THE EFFECT OF WINTER TEMPERATURE AND SPRING SALINITY ON *PERKINSUS MARINUS* PREVALENCE AND INTENSITY: A LABORATORY EXPERIMENT.** Eugene M. Burrenson\* and Lisa M. Ragone Calvo, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The role of low temperature and low salinity in controlling *P. marinus* was investigated under laboratory conditions which simulated typical and extreme winter and spring environmental conditions. Oysters (*Crassostrea virginica*) infected with *P. marinus* were collected from the upper James River, VA in December 1991, individually marked and analyzed for *P. marinus* by hemolymph assay. The oysters were then subjected to a sequential treatment of various temperature and salinity combinations. In the first phase oysters were placed in recirculating seawater systems at 10 ppt and low temperature (1°C and 4°C). Half of the oysters were treated at each temperature for 3 weeks and the other half were held for 6 weeks. In the second phase the oysters were gradually warmed to 12°C, adjusted to one of three salinities (3, 6, and 15 ppt), and held for 2 weeks. Finally, all oysters were gradually adjusted to 25°C and 20 ppt and maintained for 4 weeks to

determine if any observed declines in prevalence or intensity resulting from prior treatment were permanent. At the end of each phase *P. marinus* prevalence and intensity was assessed using hemolymph assay. Control oysters were maintained at 15°C and 15 ppt during treatment phase 1 and 2 and adjusted to 25°C and 20 ppt in phase 3.

Low temperature exposure, alone, did not significantly effect *P. marinus* prevalence or infection intensity. However, declines in prevalence and intensity, relative to initial levels were observed after 2 weeks at 12°C and 3, 6, and 15 ppt. *Perkinsus marinus* prevalence and intensity in control oysters significantly increased as the experiment progressed. These results suggest that low winter temperatures have little effect on the annual abundance of *P. marinus* within an estuary, while springtime depressions in salinity are very important.

**OVERWINTERING INFECTIONS OF *PERKINSUS MARINUS* IN CHESAPEAKE BAY OYSTERS.** Eugene M. Burrenson and Lisa M. Ragone Calvo,\* Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The scarcity of overwintering infections of *Perkinsus marinus* in Chesapeake Bay oysters has long puzzled investigators. Typically, prevalence of the pathogen declines in winter and infections are not easily disclosed by routine diagnosis using tissue cultured in thioglycollate medium (FTM). It is unknown whether cryptic stages of the parasite are harbored in the oyster during winter or whether elimination occurs; hence, the actual abundance and relative contribution of overwintering infections to subsequent summer prevalences is unclear.

The objective of this investigation was to determine the nature and abundance of overwintering *P. marinus* infections. Infected oysters were placed in a tray and suspended from a pier in the lower York River, VA in November 1991. Every six weeks from November 1991 through May 1992 oysters (n = 25) were removed from the tray, examined for *P. marinus* by hemolymph analysis, gradually warmed in individual containers to 25°C and held for one month. After the incubation period, which permitted the development of very light and/or cryptic parasite stages to detectable levels, the oysters were reanalyzed for *P. marinus* by both hemolymph and tissue cultures in FTM. A second group of 25 oysters was sacrificed on each date, diagnosed using tissue FTM cultures, and examined for cryptic stages using immunoassays.

Prevalence of *P. marinus* gradually declined from 100% in November 1991 to 32% in April 1992. Incubation of oysters at 25°C always resulted in an increase of *P. marinus* prevalence and intensity, suggesting that the parasite was more abundant than FTM cultures indicated. Immunoassay did not reveal the presence of cryptic stages, although it was generally more sensitive than FTM diagnosis. *Perkinsus marinus* appears to overwinter at very

light intensities in a high proportion of oysters. These infections are likely to be an important cause of summer mortalities.

**PERKINSUS MARINUS SUSCEPTIBILITY IN EASTERN (*CRASSOSTREA VIRGINICA*) AND PACIFIC (*CRASSOSTREA GIGAS*) OYSTERS: TEMPERATURE AND SALINITY EFFECTS.** Fu-Lin E. Chu,\* Carrie S. Burreson, Aswani Voley, and Georgeta Constantin, Virginia Institute of Marine Science, School of Marine Science, College of William & Mary, Gloucester Point, VA 23062.

Susceptibility of *Crassostrea virginica* to *Perkinsus marinus* was compared with diploid and triploid (2N and 3N) *C. gigas* at 10, 15, and 25°C in the first experiment and at 3 salinities, 3, 10, and 20 ppt, in the second experiment. In both experiments, oysters were challenged twice with *P. marinus* trophozoites. The temperature effect experiment was terminated 68 days after 1st challenge and 27 days after 2nd challenge by *P. marinus*. The salinity effect experiment was terminated 50 days after 1st challenge and 34 days after 2nd challenge by *P. marinus*. Results revealed that at 15 and 20°C, infection prevalence was higher in challenged *C. virginica* than in challenged 2N and 3N *C. gigas*. But at 10°C, challenged 3N *C. gigas* had a prevalence higher than challenged 2N *C. gigas* and *C. virginica*. In all salinity treatments, prevalence was higher in challenged *C. virginica* than challenged 2N and 3N *C. gigas*. Weighted prevalence increased with temperature and salinity and was highest in *C. virginica* groups. Since, in both experiments, much higher infection prevalence and intensity were found in non-challenged *C. virginica* than in non-challenged 2N and 3N *C. gigas*, part of the recorded prevalence and intensity in *C. virginica* may be attributed to the hidden infection from the field. High mortality occurred in both 2N and 3N *C. gigas* during temperature and salinity acclimation and at the 25°C and 3 ppt treatments.

**DEVELOPMENT OF CONFLUENT MONOLAYERS FROM TISSUES OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.** Mohamed Faisal,\* Jerome F. La Peyre, and Morris H. Roberts, Jr., Department of Environmental Sciences, School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

Because of the quiescence of cells under *in vitro* conditions, no immortal cell lines of oyster or any other bivalve molluscs have been developed. Many pathobiological investigations, however, could be performed if confluent monolayers of oyster cells were produced and maintained. In the present study, several attachment factors such as collagenase (types I, II, and IV), fibronectin, laminin, gelatin, poly-D-lysine, poly-L-lysine, and vitronectin were tested for their ability to promote the attachment and spreading of oyster cells in tissue culture plates.

Poly-L-lysine and poly-D-lysine induced a rapid attachment of the cells. Moreover, clumping of cells, a common problem in culturing oyster cells, was prevented. The cells were, however, unable to spread on the coated plates. In contrast, fibronectin promoted slow attachment of the cells but with strong spreading. A combination of both poly-L-lysine and fibronectin gave the best results and confluent monolayers of spread oyster cells were obtained. We also found that covering the cell surface with a thin layer of 0.5% low melting point agarose prevented the cell migration without affecting cell viability. The best results were obtained using the heart and mantle tissue.

**ESTIMATING THE SURVIVAL OF DELAWARE BAY OYSTER LARVAE WITHIN AND BETWEEN YEARS.** S. R. Fegley,\* Corning School of Ocean Studies, Maine Maritime Academy, Castine, ME 04420; J. N. Kraeuter, S. E. Ford, and H. H. Haskin, Haskin Shellfish Research Laboratory, Rutgers Univ., Port Norris, NJ 08347.

Extensive abundance records, based on landings or monitoring programs, commonly exist for commercially important species. Unfortunately, these records, which can cover different stages of the species life history and are often available over long periods of time or from many different regions, usually reveal very little about the population dynamics of the target species for one of several reasons.

As an illustration of this problem, replicate, surface and bottom water samples have been collected every summer since 1953 to estimate the abundances of larvae of the eastern oyster (*Crassostrea virginica*) during the period when larvae are present over the eastern two-thirds of Delaware Bay. The oyster larvae in each sample were further enumerated into one of five developmental stages. This information should be sufficient to estimate directly the survival of oyster larvae in a season by following the fate of each discrete spawning event through each developmental stage. However, logistic and financial constraints prevent taking a sufficient number of samples either temporally or spatially to provide sufficient resolution to make direct estimates in any year and in almost any location.

We will present some of the life history information that can be extracted from these larval monitoring records, the level of confidence in this information, and the means of making statistical comparisons. This is Rutgers University N.J.A.E.S. contribution # K-32406-1-93.

**A COMPARISON OF METHODS FOR IDENTIFYING MOLLUSCAN HEMOCYTES.** Susan E. Ford\* and Kathryn A. Alcox, Rutgers University, Institute of Marine and Coastal Sciences, Haskin Shellfish Research Laboratory, Box B-8, Port Norris, NJ 08349.

There is much disagreement over the number of hemocyte sub-



populations in bivalve molluscs. Uncertainty arises because of differences in definition among researchers as well as variability associated with location, season, and health status among individuals. We compared three methods for identifying hemocyte subpopulations in eastern oysters: light microscopy (description and size), Coulter counter (size), and flow cytometry (relative size and density, and fluorescent staining).

Hemolymph from the adductor muscle of individual oysters was examined by each method. Three types of granular hemocytes (large and small refractive [highly granular]; and non-refractive [few granules]); agranular hemocytes; and small cells with almost no cytoplasm ("mostly nuclei") were identified by microscopy. In samples measured by Coulter counter, a maximum of two "population" peaks was recorded—primarily in oysters with a high proportion of granular hemocytes. Single peaks were more likely to be associated with a high proportion of agranular hemocytes.

On the flow cytometer, forward light scatter estimates of size never showed more than one clear peak and frequently displayed none at all. Ninety-degree light scatter (log scale), a measure of density or granularity, showed a maximum of two clear peaks. Three populations, however, were usually present when forward scatter was plotted against 90° scatter. The two major groups represented granular and agranular cells. The third, a group of small very dense cells, were probably the "mostly nuclei" group. Using acridine orange, a fluorescent dye that stains granules red and nuclei green, we were able to distinguish between granular and agranular cells. We are as yet unable to clearly differentiate among the three granular hemocyte types.

**SPORES OF *HAPLOSPORIDIUM NELSONI* (MSX): FINDINGS AND SPECULATIONS.** Susan E. Ford\* and Robert D. Barber, Rutgers University, Institute of Marine and Coastal Sciences, Haskin Shellfish Research Laboratory, Box B-8, Port Norris, NJ 08349.

The apparent rarity of spores produced in oysters infected with *Haplosporidium nelsoni*, cause of MSX disease, led to hypotheses that another host is involved in the life cycle. In contrast to previous studies, which found spores in <1% of infected adult oysters, we report that infected spat have a high probability (>50%) of producing the spore stage. Advanced infections nearly always result in sporulation. In 1988, 30–35% of spat in lower Delaware Bay produced spores, whereas, that the figure has been only 5% in the last 4 years (1989–92). Up to  $1.5 \times 10^6$  mature spores have been found in a single spat.

We have also found spores morphologically identical (by light microscopy) to those of *H. nelsoni*, ingested by oysters throughout Delaware Bay. Their presence in oyster guts during the summer coincides with the infective period for *H. nelsoni*. We estimate that the concentration of spores in the water processed by oysters must be several hundred per liter to account for their numbers in the digestive tract.

Although annual spat sets are temporally and spatially variable, data from 35 years of sampling in Delaware Bay lead us to estimate that spat density is about  $100 \text{ m}^{-2}$  in an "average" year ( $10^{10}$ – $10^{12}$  total in the Bay). If the ingested spores are *H. nelsoni*,  $10^9$  to  $10^{10}$  spat would be required, each producing  $10^6$  spores, to yield estimated concentrations in Delaware Bay during summer. Five percent of the total estimated spat in the Bay would somewhat exceed this number. We do not know how long spores remain viable, how long they are present in the water column, and our estimates have not taken into account potential loss of spores from the estuary in current outflow, loss from the water column through biodeposition, or destruction by microbes in the sediment. The calculations suggest that spat could produce enough spores to serve as a primary host; nevertheless, the possibility of an alternate host still cannot be excluded.

**GENETIC DIFFERENTIATION AMONG STRAINS OF DISEASE CHALLENGED OYSTERS.** John E. Graves\* and Jan R. McDowell, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) was used to determine levels of genetic variation and differentiation within and among 4 strains of Eastern oyster bred for resistance to MSX and dermo, and their respective source populations. Purified mtDNA from up to 20 individuals per sample was analyzed with 13 informative restriction endonucleases to produce individual composite genotypes. The distribution of composite mtDNA genotypes was compared among samples from the source populations and the second generation of each challenged strain. Samples from all source populations exhibited modest levels of within-sample variation but no significant genetic differentiation was found among the source samples. In contrast, the distribution of mtDNA genotypes differed significantly among the 4 challenged strains, as well as between each challenged strain and its respective source sample. Different mtDNA genotypes, not represented in the source samples, occurred in relatively high frequencies in each of the challenged strains. The marked genetic differences between source samples and challenged strains, which occurred over 2 generations of selective breeding, could either be the result of intense selection pressure (disease resistance) or more likely, genetic drift.

**CHEMICAL INHIBITION OF *PERKINSUS MARINUS* IN AN *IN VITRO* TEST.** George E. Krantz,\* Maryland Department of Natural Resources, Cooperative Oxford Laboratory, Oxford, MD 21654.

A rapid diagnostic test for oyster parasites, recently developed at the Cooperative Oxford Laboratory, utilizes thioglycollate culture media in polystyrene tissue culture plates to detect *Perkinsus marinus* cells circulating in oyster hemolymph. This test was mod-

ified to serve as an *in vitro* assay system to detect chemical compounds that exhibit inhibitory activity toward the enlargement of *P. marinus* cells in the thioglycollate media. The assay system detected 16 organic chemicals and 2 inorganic salts that had inhibitory activity. Cellular changes of treated *Perkinsus* are described, and trypan blue vital stain confirmed that certain cellular changes resulted in death of the enlarging *Perkinsus* hypnospores.

Application of minimum reactive concentrations of chemical compounds in oysters has failed to alter the infection levels of *Perkinsus* and induced high levels of mortality in host oysters. Present studies utilizing lower concentrations of chemicals may be helpful in evaluating the therapeutic value of long-term exposure of sublethal concentrations of reactive chemicals.

**PROPAGATION OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* IN VITRO.** Jerome F. La Peyre,\* Mohamed Faisal, and Eugene M. Burreson, School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The protozoan *Perkinsus marinus* causes mortalities of the eastern oyster, *Crassostrea virginica*. Attempts to propagate *P. marinus* in commercially available media have failed. We developed a culture medium (JL-ODRP-1) that contain most of the known constituents of hemolymph. Using this medium, we were able to propagate a protozoan (designated *Perkinsus-1*) resembling *P. marinus* from the heart tissue of an infected oyster. This organism adapted well to culture conditions, divided by schizogony-like processes, and has been subcultured 11 times. *Perkinsus-1* was similar in morphology to histozoic stages of *P. marinus*, reacted with anti-*P. marinus* antibodies, and was infective to susceptible oysters.

Several attempts to use the visceral mass as a rich source of *P. marinus* merozoites for *in vitro* cultivation were unsuccessful due to excessive bacterial and protozoal contamination. By incubating the visceral mass first in fluid thioglycollate medium, isolating and purifying the prezoosporangia, and incubating them in JL-ODRP-1, numerous continuous cultures of *P. marinus* were initiated. Two types of divisions were observed in cells cultured according to this procedure: progressive cleavage and successive bipartition that resulted in the formation of flagellated cells.

The success achieved in propagating *P. marinus* will permit further study of the pathobiology and control of this pathogen.

**POPULATION MODELS TO EVALUATE IMPACT OF DISEASES AND MANAGEMENT OPTIONS FOR THE JAMES RIVER OYSTER FISHERY.** Roger Mann,\* School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Population models which quantify the impacts of biological and environmental variation on sequential life history stages of the oyster allow identification of factors which can be manipulated to alleviate disease related mortality and facilitate management of

oysters as a resource for commercial exploitation. To date such models have been limited by a lack of methods to quantify several life history stages, especially larval production and survival. I present current data for a project designed to produce a quantitative description of the oyster population of the James River, Virginia in terms of the following components: standing stock, size specific fecundity, egg viability, larval survival and retention by frontal systems, availability of substrate, success of metamorphosis, post settlement growth, and post settlement losses to disease and predation. Both fecundity and egg viability vary temporally and are strongly influenced by the prevailing salinity, as is the prevalence and intensity of disease. Manipulation of the budget components illustrate the utility and possible limitations of management options that exist for the commercial resource.

**THE OYSTER DISEASE RESEARCH PROGRAM OF THE NATIONAL MARINE FISHERIES SERVICE (NMFS): AN OVERVIEW.** Harold C. Mears,\* National Marine Fisheries Service, Gloucester, MA 01930.

The Oyster Disease Research Program, administered by the National Marine Fisheries Service, is assessing research and management issues associated with the impact of shellfish diseases on the eastern oyster (*Crassostrea virginica*). The Program has funded investigations by state management agencies, colleges, and universities, in addition to several workshops and symposia. Thirty three peer-reviewed projects, at an average funding level of \$88,400, have been awarded on a competitive basis since 1990. Several of these studies are exploring the potential factors responsible for the demise of the eastern oyster in Chesapeake Bay. Work has been conducted on topics such as disease transmission and resistance, diagnostic techniques, environmental modeling, and a social/economic assessment of the oyster industry.

Funding complements Federal financial support for oyster research from other sources including Sea Grant, the National Coastal Resources Research and Development Institute, and the U.S. Department of Agriculture. The NMFS Program is unique in that it requires coordination of research and management projects with the concerned State fishery agencies responsible for shellfish management. Accordingly, the Program promotes the use of scientific findings and state-of-the-art biotechnology in the development of practical approaches for state authorities to manage eastern oysters impacted by disease in Atlantic coastal waters.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*: FEEDING AND METABOLISM.** Roger I. E. Newell,\* Christine J. Newell, and Kennedy T. Paynter, Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD 21631; Eugene M. Burreson, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Eastern oysters are highly susceptible to infection by the parasite *Perkinsus marinus* which causes the oyster to cease growing

and eventually die. This disease progression suggests that the parasite may interfere with routine physiological functions, as has been shown to occur with another major oyster parasite, *Haplosporidium nelsoni*. Thus, we hypothesized that oysters infected with *P. marinus* may have a reduced food intake, an elevated metabolic rate and decreased assimilation efficiencies compared with uninfected oysters. In a laboratory experiment, however, in which oysters were infected with differing numbers of *P. marinus*, there were no significant changes in either the rate of oxygen consumption or clearance rate.

In June 1992, oysters were transplanted to three locations within Chesapeake Bay with differing ambient salinity regimes and consequent differences in *P. marinus* infection intensities. Oysters at two sites became infected during the summer. In August, at the high salinity site, experimental oysters ceased growing shell, and in September exhibited a 35% mortality rate as a consequence of these infections. We could detect no differences in oxygen consumption, clearance rate, or assimilation efficiency (measured using the Conover ratio technique) between infected and uninfected oysters at each of these locations. Ongoing studies are further investigating the mechanisms whereby *P. marinus* exerts its deleterious effects on oysters.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: INTRODUCTORY OVERVIEW.** Kennedy T. Paynter\* and Christopher Caudill, Department of Zoology, University of Maryland, College Park, MD 20742; Eugene M. Bureson, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

An interdisciplinary research project was initiated in 1992 to study the physiological effects of *P. marinus* infection on the Eastern oyster, *Crassostrea virginica*. Seven principal investigators from 5 academic campuses in Maryland and Virginia participated in the project. Physiologies examined were physiological energetics including clearance rates and oxygen consumption, hemocyte function, free amino acid accumulation, mitochondrial function, and stress protein induction.

Oysters were deployed at three sites in Chesapeake Bay to expose them to high, moderate and low salinities and the various prevalences of *Perkinsus marinus* associated with those sites. Samples from each site were provided to the various collaborators at predetermined stages of growth and infection. Growth, mortality, and condition index were monitored in the animals at each site biweekly. As expected, the oysters grew well until they became infected. Infection prevalences became high at both the low and high salinity sites while remaining low at the moderate salinity site. The disease progressed more rapidly at high salinity resulting in more intense infections even though final prevalences were similar at low salinity. Mortality was low until September and October when cumulative mortality reached about 35% in the group deployed at high salinity but remained low at the low and

moderate salinity sites. Growth, mortality, condition index, and infection intensity and progression in the field were associated with the physiologies measured in the laboratory.

**THE PHYSIOLOGICAL EFFECTS OF PROTOZOAN PARASITISM ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: EFFECTS ON CELLULAR FREE AMINO ACID LEVELS.** Kennedy T. Paynter\* and Sidney K. Pierce, Department of Zoology, University of Maryland, College Park, MD 20742; Eugene M. Bureson, Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

The Eastern oyster, *Crassostrea virginica*, is an osmoconforming bivalve which regulates intracellular free amino acid concentrations to maintain cell volume in response to changes in ambient salinity. This important ability allows the oyster to inhabit brackish water estuaries such as the Chesapeake Bay where many other species cannot survive. Oyster cells, like those of most other euryhaline bivalves, accumulate free amino acids (FAA) when the salinity increases and expel FAA when the salinity decreases. The accumulation of FAA is the result of a specific set of metabolic shifts which first causes the production of alanine from glucose, followed by glycine production and later proline production. After many weeks of high salinity acclimation, taurine becomes the major intracellular osmotic effector replacing alanine, glycine and proline.

Oysters acclimated to low salinity were deployed at high and low salinity sites in May. Gill and mantle tissues from 5 oysters were excised and quick frozen on dry ice in the field daily for 10 days after transfer and biweekly thereafter. *P. marinus* infection intensity was determined for each oyster sampled. Intracellular FAA followed a typical accumulation pattern after the hyperosmotic shift and appeared to reach stable acclimated levels 8 to 10 weeks after transfer. However, several amino acid concentrations changed once the oysters became infected with *P. marinus*. Taurine levels were significantly reduced in infected groups and the magnitude of reduction was positively correlated with infection intensity. These results suggest that the cell volume control mechanism in oysters may be impaired by *P. marinus* infection, and the oysters ability to tolerate salinity variation may be reduced.

**SEVERAL MITOCHONDRIAL FUNCTIONS IN CHESAPEAKE BAY OYSTERS ARE DIFFERENT IN ATLANTIC OYSTERS: DISEASE OR GENETICS?** S. K. Pierce, L. A. Perrino, and L. M. Rowland-Faux, Department of Zoology, University of Maryland, College Park, MD.

*Crassostrea virginica* from Florida to Cape Cod respond to increased external salinity by increasing intracellular concentrations of several amino acids, primarily taurine, and the quaternary amine, glycine betaine. Chesapeake Bay oysters from several populations use different amino acids, primarily glycine and alanine, and in addition, do not synthesize glycine betaine in response to high salinity stress. Since the synthesis of both the amino acids and glycine betaine occurs in the mitochondria, we have been com-

paring isolated mitochondrial metabolism of Bay and Atlantic oysters. The respiratory coupling ratios (RCR) of Bay oysters is always higher than Atlantic oysters, regardless of biochemical substrate. Bay oyster RCRs are highest with  $\alpha$ -ketoglutarate, while malate is preferred by Atlantic mitochondria. In addition, mitochondria from low salinity adapted oysters take up choline (glycine betaine precursor) faster than high salinity adapted oysters and Atlantic mitochondria take it up faster than Bay mitochondria. The synthesis of glycine betaine is faster in high salinity adapted Atlantic oysters. We are currently measuring synthesis in Bay oyster mitochondria. These differences in amino acid production, RCRs and glycine betaine metabolism indicate major biochemical differences between the mitochondria of the two oyster groups. Since all of our Bay oysters were likely parasitized with Dermo, it is not clear if the differences are due to genetics, the presence of the parasite or some other environmental factor.

**FLOW CYTOMETRIC ANALYSIS OF HISTOZOIC *PERKINSUS MARINUS* CELLS.** Bob S. Roberson\* and Tong Li, Department of Microbiology, University of Maryland, College Park, MD 20742; Christopher F. Dungan, Maryland DNR, Cooperative Oxford Laboratory, Oxford, MD 21654.

Methods developed for analysis of fluorochrome-labeled *Perkinsus marinus* cells in estuarine water samples were adapted for diagnostic analysis of infected oyster tissues by flow cytometry. Both hemolymph and visceral tissue homogenates from infected oysters whose infection status had been previously determined by traditional fluid thioglycollate medium assays, were analyzed. Prior to flow cytometry, oyster tissues or homogenates were subjected to enzymatic digestion, differential centrifugation, and double fluorochrome staining. Fluorescein labeling of pathogen cells was accomplished using specific antibodies; propidium iodide labeling of DNA was accomplished in the presence of RNAase. Pathogen cells were discriminated using characteristic ranges for the cytometric parameters of fluorescein and propidium iodide fluorescence intensities, size (forward angle light scatter), and cellular complexity (90° light scatter). Fluorescence activated sorting (FACS) of cell populations recognized as *P. marinus* permitted microscopic comparison of sorted cell morphologies to those of immunostained pathogen cells in histological sections of infected oyster tissues. Enzymatic treatment of sampled pathogen cells did not significantly compromise the intensity of antibody labeling; and sorted pathogen cell morphologies represented the entire range of cell morphotypes labeled *in situ*.

**FLOW CYTOMETRIC ENUMERATION AND ISOLATION OF IMMUNOFLOUORESCENT *PERKINSUS MARINUS* CELLS FROM ESTUARINE WATERS.** Bob S. Roberson\* and Tong Li, Department of Microbiology, University of Maryland, College Park, MD 20742; Christopher F. Dungan, Maryland DNR, Cooperative Oxford Laboratory, Oxford, MD 21654.

Particles suspended in water samples from both Chesapeake Bay, and from laboratory aquaria containing moribund, *Perkinsus marinus*-infected oysters, were concentrated and double fluorochrome-labeled for flow cytometric analysis and fluorescence activated cell sorting (FACS). Pathogen cells were fluorescein-labeled using specific antibodies; cell DNA was propidium iodide-labeled by incubation with this nucleic acid fluorochrome in the presence of RNAase. Flow cytometric analyses utilized antibody fluorescence, DNA fluorescence, size (forward angle light scatter), and cellular complexity (90° light scatter) to differentiate cell populations within water samples. Water samples from aquaria seeded with infected oysters were used to determine analytical parameter value ranges characterizing pathogen cells, and provided the first observation of pathogen cells disseminated from infected hosts. Compositions of differentiated sample cell populations were confirmed by FACS, followed by microscopic evaluation of sorted cell populations. Following confirmation of discriminating analytical parameter value ranges, pathogen cell abundance estimates were made for aquarium water samples, using gated counts. Counted cells were sorted and population homogeneity was independently confirmed by microscopic enumeration. These methods are currently being applied to analyses of environmental water samples collected throughout the past year, for the purpose of generating accurate seasonal estimates of actual pathogen abundances in estuarine waters endemic for dermo disease.

**UTILIZATION OF A GEOGRAPHICAL INFORMATION SYSTEM (GIS) FOR THE TIMELY MONITORING OF OYSTER POPULATION AND DISEASE PARAMETERS IN MARYLAND'S CHESAPEAKE BAY.** Gary F. Smith\* and Stephen J. Jordan, Maryland Department of Natural Resources, Cooperative Oxford Laboratory, Resources, Oxford, MD 21654.

The parasites *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) have over the past several years caused high mortality to Maryland's Chesapeake Bay oysters. An impediment to the timely management utilization of oyster disease and population monitoring data has been in the quantity and complexity of the information collected. This situation has resulted in data not being fully utilized and or availability greatly lagging collection date. Integration of data input and analysis programs with a PC based commercial GIS system has shown promise in improving oyster monitoring of disease and population parameters.

Initiation of a comprehensive annual oyster survey in 1990 geared to GIS applications has allowed site specific and regional representation of all available oyster data in a geographic context on the bay. Management oriented capabilities have been developed to allow user based queries combined with statistical analysis in a user friendly format.

**INFECTIVITY AND PATHOGENECITY OF TWO LIFE STAGES, MERONT AND PREZOOSPORANGIA OF *PER-***

***KINUS MARINUS* IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*. Aswani K. Volety\* and Fu-lin E. Chu, Virginia Institute of Marine Science, School of Marine Science, The College of William & Mary, Gloucester Point, VA 23062.**

Two experiments were conducted to compare the infectivity and pathogenicity of two life stages, namely, meronts (trophozoites) and prezoosporangia of the parasite, *Perkinsus marinus* in eastern oysters (*Crassostrea virginica*). Partially purified trophozoites or prezoosporangia at a dose  $5 \times 10^4$ /oyster were injected into the shell cavity of the oyster. Prevalence and intensity of *P. marinus* infection in oysters were determined 15, 25, 40 and 65 days, for the first experiment, and 20, 40, 50, 65 and 75 days, for the second experiment, after inoculation with infective particles. Condition index, serum protein and lysozyme were also measured. In the first experiment, *P. marinus* infection was first detected in

the groups of oysters challenged by prezoosporangia. However, at the end of the experiment, prevalence and intensity of infection were higher in the groups of oysters exposed to trophozoites. In contrast to experiment 1, in the second experiment, infection was first detected in the groups of oysters challenged with trophozoites. Results from experiment 1 indicate that there was a decrease in condition index in all treatments, including control at the end of the experiment. A significant decrease was also observed at the end of the experiment in the serum protein in the groups challenged with prezoosporangia ( $P < 0.055$ ). Lysozyme concentrations did not show any significant change over the course of the experiment. Lower condition index and serum protein values in the groups challenged with prezoosporangia compared with the groups challenged by trophozoites at the end of the experiment, may suggest a higher energetic demand on these oysters.

## Abstracts of ODR-funded research conducted by the National Marine Fisheries Service

**DEVELOPMENT AND APPLICATION OF RAPID DIAGNOSTIC TECHNIQUES IN THE STUDY OF OYSTER DISEASES.** C. Austin Farley, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Methodologies utilizing hemolymph (oyster blood) have been developed that allow for the rapid and accurate diagnosis of systemic oyster diseases such as MSX (*Haplosporidium nelsoni*) and "dermo" (*Perkinsus marinus*). The hemolymph is withdrawn from the large sinus of the oyster's adductor muscle and treated several ways: (1) Hemolymph diluted in a buffered saline solution is placed in temporary wet cell chambers and allowed to settle on a microscope slide. The cells on the slide are then chemically fixed and stained for microscopic examination. (2) Hemolymph is also placed in plastic culture wells, allowed to settle, and overlaid with antibiotic-fortified thioglycollate medium. After incubation for 3 days at room temperature, the surface fluid is removed, concentrated Lugol's iodine solution added, and each well examined for spore stages of *P. marinus* using an inverted microscope. The benefits of using these methods are: (1) diagnosis can be performed on living animals, permitting clinical studies of progressive disease; (2) the method is relatively rapid when compared with histology; and (3) expenses are low and equipment demands are modest, permitting field application. The Maryland Department of Natural Resources has utilized these methods for the past few years as part of their annual oyster disease survey of Chesapeake Bay. The techniques have also been applied to the study of diseases of other invertebrate species.

**JUVENILE OYSTER MORTALITY STUDIES—1992: HISTOPATHOLOGY, PATHOLOGY, EPIZOOTIOLOGY.** C. Austin Farley and E. J. Lewis, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Studies of cytology, pathology, and population characteristics were conducted in relation to mortalities of Long Island Sound hatchery-reared juvenile oysters. Studies included major mortality periods of July–September in both 1991 and 1992. Data have been analyzed and support information reported previously by others suggesting size and temperature in relation to onset of disease and mortality. Dead oysters typically were less than 30 mm in length (mean 16–20 mm). Depending upon water temperature, mortalities in oysters occurred 3 to 8 weeks after being transplanted from the hatchery and maintained in trays in the nursery. Oysters from the nursery experienced 4–66% mortality with conchiolin deposition. Representative oysters from each spawning batch kept in the hatchery, in 25- $\mu$ m filtered ambient water diluted with high salinity well water, suffered 0–8% mortalities with conchiolin deposition. Epizootiology studies of variously treated juvenile oyster populations further suggest that an infectious entity is responsible for mortalities. As in our earlier studies, histological tissues re-

vealed the presence of small, round intracellular bodies in lesions of the mantle epithelium in 60–90% of populations experiencing >50% mortality. We believe these bodies to be a parasite, not autophagic vacuoles or necrotic host cells as others have suggested. Tissues stained with Feulgen picromethyl blue revealed that many of these bodies possess multiple dense staining Feulgen-positive structures resembling developmental life cycle stages of protists, particularly ciliates.

Intracellular parasites with protistan characteristics were found by electron microscope studies. Mitochondria with tubular cristae, small nuclei, indications of a pellicle in some, and suggestions of endogenous budding similar to that seen in suctorian ciliates were seen. Similar intracellular organisms were seen in large commensal ciliates in spaces between the mantle and shell, suggesting a possible carrier host role. These large ciliates would not pass a 25- $\mu$ m filter, explaining the protection of comparable populations held in the hatchery.

**SHELLFISH HEALTH INSPECTIONS OF CHILEAN AND AUSTRALIAN OYSTERS.** Frederick G. Kern, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

In 1990, the Invertebrate Pathology Investigation began examining oyster samples shipped to the Oxford Laboratory from Chile and Australia in accordance with Memoranda of Understanding (MOUs) with the Food and Drug Administration and the National Marine Fisheries Service. Chile identified two designated areas and two species of oysters (*Ostrea chilensis* and *Crassostrea gigas*) to ship to the United States. Australia designated four areas and two species of oysters (*Ostrea angasi* and *C. gigas*) to be examined. Approximately 4000 Australian oysters and 1700 Chilean oysters were examined for parasites and diseases over a 2-year period. The examinations of the native Chilean oysters routinely resulted in the detection of high levels of the parasite *Bonamia* sp. which was indistinguishable from the organisms responsible for the dramatic loss of the European oyster, *Ostrea edulis*. None of the Australian oysters examined were determined to be infected by organisms on the International Council for the Exploration of the Sea (ICES) list of serious pathogens. However, Australian researchers have recently reported cases of *Bonamia* sp. in several *O. angasi* oysters obtained in other studies. Labeling requirements on shipments of live foreign molluscan shellfish have been incorporated into the MOUs with these governments. The labeling instructions are in the form of "NOTICE TO RECIPIENTS" that are designed to reduce the risk that undesirable organisms contaminate U.S. aquatic resources.

**PRELIMINARY OSMOCONFORMING STUDY OF THE OYSTER *CRASSOSTREA VIRGINICA*.** Earl J. Lewis, Jr., National Marine Fisheries Service, NOAA, Northeast Fisheries

Science Center, Cooperative Oxford Laboratory, Oxford, MD, 21654.

Oysters are known osmoconformers. As such, tissues are bathed in fluids of the same salinity as the surrounding water as long as the oyster is actively pumping water. Oysters are frequently subjected to changes in salinity by man and natural events such as storms. Also, researchers have shown that oysters exposed to low salinities purge themselves of *Haplosporidium nelsoni*, the cause of MSX disease. When salinity is used as a tool to deplete oysters of disease, or organisms harmful to consumption, it becomes necessary to understand how rapidly oysters respond to salinity changes to determine an appropriate depuration time. Adult oysters from the Tred Avon tributary of the Chesapeake Bay were tested for the time necessary to conform to changes in salinities. Blood samples were obtained, centrifuged to remove particulate material from the hemolymph, and osmolarity tested by freeze-point depression to determine when equilibration was attained with osmolarity of the surrounding water. Oysters held at 8 ppt salinity conformed to increased salinities of 12, 16, and 20 ppt at 22°C by the time the first reading was taken, 24 hours after salinity was increased. Oysters acclimated for 4 weeks at 22°C and salinities of 10, 12, 16, 20 and 25 ppt, then subjected to a salinity of 10 ppt, were found to conform within 8 hours of exposure. In exposing oysters to lower salinities, changes in blood osmolarity occurred rapidly with 80% to 100% of the change occurring within 4 hours. From this preliminary study, it appears that oysters can adapt to changes of 12 to 15 ppt salinity within 24 hours. The effect of disease on the oyster's ability to conform is unknown at this time.

**RESULTS OF LABORATORY ATTEMPTS TO TRANSMIT A DISEASE AFFECTING JUVENILE OYSTERS IN THE NORTHEASTERN UNITED STATES.** Earl J. Lewis, Jr. and C. Austin Farley, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Since the late 1980s, juvenile oysters from Maine, Rhode Island, New York, and Massachusetts have experienced heavy mortalities. As yet, the cause of mortalities has not been resolved, although many possible causes have been hypothesized. Our hypothesis is that this is an infectious disease, with mortalities possibly caused by pathology associated with a protistan parasite. Based on this, experiments were designed to determine if the disease could be transmitted under controlled laboratory conditions. Laboratory experiments demonstrated this to be a transmissible, temperature-dependent, waterborne infectious disease with an incubation period of 3 to 7 weeks. Depending upon temperature, Maryland hatchery-reared oysters challenged in recirculating aquaria showed heavy mortalities, abnormal, internal conchiolinous shell lesions, and small round intracellular inclusion bodies in mantle epithelium after 3 to 7 weeks of exposure to infected

oysters from Long Island Sound, NY. Cumulative mortality in experimentally infected oysters ranged from 40% (18°C) to 74% (24°C). Associated conchiolin deposition was present in 26% of dead oysters at 18°C, compared to a high of 40% at 24°C. No indications of dinoflagellates, believed by some to be the disease agent, were found in water samples examined upon completion of the study. No conchiolin, or comparable mortalities were observed in control animals. Gross symptoms of the disease were found to recur in survivors of the 1990 and 1991 mortalities after being held in aquaria for 10 months.

**1992–1993 EAST COAST OYSTER DISEASE SURVEY.** Earl J. Lewis, Jr. and C. Austin Farley, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Since the late 1980s, a new oyster disease has caused severe mortalities in cultured juvenile oysters in the northeastern United States from Maine south to New York. This juvenile oyster disease (JOD) is characterized by mortalities of sudden onset in oysters less than 30 mm in length, mantle recession, abnormal conchiolinous lesions inside shells, one abnormally cupped valve, spontaneous detachment of adductor muscle, and abnormal shell growth in survivors. There has been debate whether these gross characteristics are diagnostic for the disease, whether the conditions may be caused by other etiologies, and if characteristics persist in larger oysters. Oysters were sampled from 11 locations in 9 states from Maine to Louisiana. Sites were selected for anticipated presence of 1 or more disease problems, including the east coast JOD, *Haplosporidium nelsoni*, and *Perkinsus marinus*. Oysters 30 to 60 mm in length were grossly examined for mortality, mean size, conchiolinous shell lesions, severe shell checks, *Polydora*, *Cliona*, mantle recession, chalky shell lesions, and yellow discolorations on the interior shell. To date, 2756 oysters, mean length 44 mm, have been examined grossly. An additional 1450 oysters have been examined and processed for histological examination. Thus far, data support the belief that the combined gross characteristics of JOD are diagnostic for the disease. Seventy (3%) of the oysters examined demonstrated internal conchiolinous shell lesions of the type associated with JOD. Of these, 94% were found in oysters from areas affected by JOD. Another 2 oysters (3%) from Louisiana and Maryland had conchiolinous lesions associated with shell damage. Conchiolin in the remaining 2 oysters from Delaware is unexplained. Severe shell checks at 14 to 25 mm appear to be linked to oysters affected by JOD. There has not been an association of other diseases, or parasites with conchiolinous deposits.

**CROSS INFECTION STUDIES OF OYSTER "DERMO," PERKINSUS MARINUS, IN SOFTSHELL CLAMS, MYA ARENARIA.** Shawn M. McLaughlin, National Marine Fisheries Service, NOAA, Northeast Fisheries Science Center, Cooperative Oxford Laboratory, Oxford, MD 21654.

Recent increases in the prevalence of the parasite *Perkinsus marinus* ("dermo") in oysters, *Crassostrea virginica*, from the uppermost portions of the Chesapeake Bay have been followed by a concomitant increase in the presence of *Perkinsus* spp. in soft-shell clams, *Mya arenaria*. An experimental cross infection study was initiated to determine the relationship between oyster and clam "dermo." Fifty-eight softshell clams were diagnosed as "dermo" negative by blood thioglycolate culture methods. Half of the clams were injected with hemolymph collected from an oyster with an advanced case of "dermo." The remaining control clams were not injected. Both groups were held in separate recirculating aquaria at 16°C in Tred Avon River water at 15 ppt salinity. After 3 weeks, blood thios showed early stages of "dermo" in 4 (14%) of the injected clams and in none of the controls. At 6 and 10 weeks, the 4 clams no longer showed signs of the parasite with the blood culture technique. All clams were processed after 12 weeks for histology, and standard rectal thioglycolate cultures were performed. Six (21%) of the injected clams were diagnosed with "dermo," including only 1 of the 4 originally positive clams. In a repeat of the experiment, 50% (15/30) of clams injected with infected oyster hemolymph were diagnosed by rectal thios with "dermo" after being held for 7 weeks at temperatures ranging from 17–19°C at 15 ppt salinity. In an additional study, softshell clams were held for 7 weeks in a recirculating tank containing oysters with advanced cases of "dermo." No indirect transmission between infected oysters and uninfected clams occurred.

**EFFECTS OF A PROROCENTRUM ISOLATE UPON THE OYSTER, CRASSOSTREA VIRGINICA: A STUDY OF THREE LIFE-HISTORY STAGES.** Gary H. Wikfors,<sup>1</sup> Roxanna M. Smolowitz,<sup>2</sup> and Barry C. Smith,<sup>1</sup> <sup>1</sup>National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460; <sup>2</sup>LMAH, School of Veterinary Medicine, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, MA 02543.

Evidence that some strains of the dinoflagellate genus *Prorocentrum* are harmful to shellfish has been obtained from both field

and laboratory studies. Our previous laboratory exposures of one *Prorocentrum minimum* isolate (strain EXUV) to hard clams and bay scallops demonstrated clear differences in responses of the two bivalves; hard clams survived but did not grow, whereas scallops experienced complete mortality in 1–4 weeks. Histological evidence suggested effects of an enterotoxin upon scallops. The present study was undertaken to determine possible toxicity of cultured *P. minimum* (EXUV) to several life-history stages of the eastern oyster: embryos, feeding larvae, and juveniles.

Embryos exposed to whole EXUV cells, spent medium from EXUV cultures, and filtrates from heat-killed and sonicated cells showed no differences from controls in survival, development, or histology (light and electron microscopy). Forty-eight-hr larvae were fed EXUV alone and as a 1/3 or 2/3 portion of a mixed ration with *Isochrysis sp.* (strain T-ISO); controls of T-ISO alone and unfed larvae also were included. Differences in survival and growth were obtained, with larvae fed 100% EXUV performing only slightly better than unfed larvae; no EXUV-fed larvae survived to set. *P. minimum* EXUV cells were filtered poorly, relative to T-ISO; some ingestion, but limited digestion was noted by epifluorescence microscopy. Mixed diets produced intermediate results. Histologic examination revealed clear differences between unfed, T-ISO-fed, and EXUV-fed larvae. EXUV-fed larvae showed more development than unfed animals, but not the vigorous development nor the cellular lipid reserves of T-ISO-fed larvae. Digestive glands of EXUV-fed larvae contained a very distinct phagolysosomal/residual body. Post-set oysters (ca. 3 mm) were evaluated in the same treatments as larvae. Oysters fed 100% EXUV produced abundant pseudofeces for 3 wk, following which well-formed fecal strands were seen; oysters fed T-ISO filtered normally. After 6 wk, no mortalities were noted, and slight growth was obtained in most treatments. Differences in histologic appearance and condition of the digestive system were again observed.

In summary, although acute toxicity of *P. minimum* EXUV to oysters was not found, there was strong evidence for nutritional deficiency or interference with digestion. This study underscores the great variation in pathological effects that a single dinoflagellate can produce in different life-history stages and different bivalve species, i.e., oysters, clams, and scallops.



**PROCEEDINGS OF THE SPECIAL SYMPOSIUM: HARMFUL PHYTOPLANKTON AND SHELLFISH INTERACTIONS**

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## FACTORS CONTROLLING PARALYTIC SHELLFISH POISONING (PSP) IN PUGET SOUND, WASHINGTON

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**ABSTRACT** PSP has spread throughout much of Puget Sound, Washington since the mid 1970s. Now all but parts of southern Puget Sound and all of central and southern Hood Canal are periodically affected by PSP. There are important sport and commercial shellfish beds in these areas that could be threatened by further expansion of PSP. The initial spread of PSP has been traced to major physical events, but the lack of PSP in most of southern Puget Sound and all of central and southern Hood Canal has not been investigated. Monitoring and preliminary experimental data suggest that the low concentration of surface and subsurface (10 m) nitrogen in the unaffected areas prevents the growth of *Alexandrium catenella*. Increased nitrogen discharge from rapid urbanization and non-point sources could lead to PSP problems in areas presently unaffected by PSP, unless preventive measures are taken.

**KEY WORDS:** paralytic shellfish poison, *Alexandrium catenella*, nitrogen

### INTRODUCTION

The geographic distribution and intensity of paralytic shellfish poisoning (PSP) has increased in Puget Sound since the mid 1970s (Nishitani and Chew 1988, Washington Dept. of Health unpublished reports). In Puget Sound and adjacent marine waters, PSP is attributable to the chain forming, motile dinoflagellate *Alexandrium catenella* (Whedon and Kofoid) Balech. It is thought that most populations in Puget Sound originate from cysts in sediments and that some areas, known as breeding bays, are likely sources of blooms (Nishitani and Chew 1984).

Historically, PSP occurred on the open coast of Washington State and the Strait of Juan de Fuca (Fig. 1). By 1975, it occurred as far south as central Puget Sound near Seattle, although levels were not high enough to cause shellfish harvesting closures. In 1978 a major bloom occurred in the Whidbey Basin that spread south during a period of unusually large riverine discharge. This bloom was apparently exacerbated by an exceptionally deep surface layer of warm water (Erickson and Nishitani 1985).

Presently all but portions of southern Puget Sound (SPS) and all of central and southern Hood Canal (CHC and SHC) have had shellfish harvesting closures due to PSP (Fig. 2). This is particularly perplexing because live cells of the causative organism, *Alexandrium catenella*, and some low levels of toxin have been documented all the way into the southernmost areas of SPS, but only at trace levels (Saunders et al. 1982). The unaffected areas include some of the region's most productive sport and commercial hardshell clam and oyster-growing beaches. The further spread of PSP into these areas could have significant adverse effects on shellfish stocks, local economics and shellfish consumers.

Some of the factors that control the growth of *A. catenella* are discussed below. Cell toxicity and toxin composition may or may not be closely coupled to these factors (Boyer et al. 1987, Anderson et al. 1990).

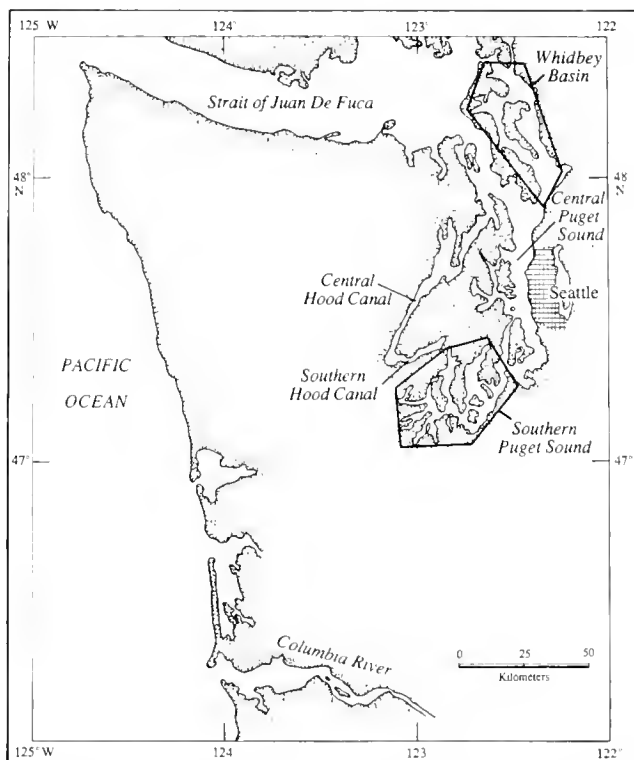
**Water Temperature:** Past work has suggested that a temperature of 13–14°C was the threshold for accelerated growth of *A. catenella* both in the laboratory (Norris and Chew 1975) and in field studies of a small bay near central Puget Sound (Nishitani et al. 1988). Many of the areas of Puget Sound not subject to recurring blooms of *A. catenella* apparently have adequately warm surface

waters, but the subsurface water temperature is likely to be more important because that is where the cells may congregate. Erickson and Nishitani (1985) hypothesized a possible relationship between exceptional PSP episodes and warm Puget Sound water associated with El Niño/Southern Oscillation events. In neighboring British Columbia, however, Gaines and Taylor (1985) concluded that elevated toxicity of shellfish was related to increased water temperature, although not closely.

**Physical Oceanography:** Portions of SPS and north Hood Canal have very infrequent PSP blooms and entry waters that are subject to relatively great vertical mixing due to semi-blocking sills. Vertical mixing is difficult to measure except indirectly through the use of physical and chemical measures, but it has been known for years that dinoflagellate populations generally do not prosper in mixing conditions. Hood Canal has relatively small but sustained riverine discharge throughout the year that produces vertical stability and a steady estuarine flow pattern, out at the surface and in at depth. The stability leads to intense spring diatom blooms and nutrient depletion of surface waters (Barlow 1958, Tetra Tech 1988, Rensel Associates and PTI Environmental Services 1991). SPS is less affected by riverine discharge, but has many shallow, poorly flushed bays and inlets where thermally caused stratification occurs during clement weather.

**Nutrients:** Despite some anecdotal evidence, there has been no definitive link established between coastal enrichment from nutrient pollution and PSP blooms worldwide (Smayda and White 1990). However, nitrogen is widely regarded as the most important macronutrient controlling phytoplankton growth in stratified or poorly flushed coastal-marine areas. Phosphorus may be limiting to algal growth in certain coastal zones subject to large amounts of nitrogen-bearing riverine discharge (Harrison et al. 1990). Although few nutrient-addition bioassays have been conducted in Puget Sound, most of the monitoring data point to nitrogen as the most likely growth-limiting macronutrient, when other factors are supportive for algal growth (Rensel Associates and PTI Environmental Services 1991).

Working with a local isolate of *A. catenella*, Norris and Chew (1975) found no growth limitation of cells exposed to 10, 30, and 40 µM-N. Normal Puget Sound values vary from 0 to about 35



**Figure 1.** Vicinity map of basins in Puget Sound, Hood Canal, Strait of Juan de Fuca and the Pacific Ocean adjacent to Western Washington.

$\mu\text{M-N}$ , but growth at less than  $10 \mu\text{M-N}$ , which is common in nutrient-sensitive areas and areas without regular PSP blooms, has not been investigated.

**Light:** Solar radiation is likely an important growth limiting factor for *A. catenella*, at least in the winter when photosynthetically usable radiation is minimal and in the summer when the depth to which cells can migrate and still maintain net photosynthesis is of importance. Subsurface chlorophyll *a* maxima indicate that phytoplankton cells in CHC and SHC often congregate near the pycnocline or nutricline at 5 to 10+ meters depth (Tetra Tech 1988), where light may be attenuated.

**Predation:** A few studies have shown that *A. catenella* cells may be relatively good food for some zooplankton. Erickson (1988) traced food-chain toxicity by feeding *A. catenella* to copepods, that in turn were consumed by coho, pink and chum salmon and Pacific herring. Huntley et al. (1986) found in the laboratory that the copepods *Calanus pacificus* and *Paracalanus parvus* rejected *A. tamarensis* as food, but consumed *A. catenella* at a normal feeding rate. There has been virtually no useful field work on this topic to corroborate the laboratory work, and toxicity of cells used in laboratory trials could vary greatly depending on genetic strain and varying environmental conditions. A naturally occurring dinoflagellate parasite *Amoebophyra ceratii*, is known to prey on *A. catenella* cells (Taylor 1968, Nishitani et al. 1985), but biological control of associated harmful blooms was rejected by Nishitani et al. (1988) because the parasite may also attack benign phytoplankton species.

**Other Factors:** There may be chemical factors or toxins that inhibit growth of *A. catenella*. For example, on the East Coast it

has been found that *A. tamarensis* rarely blooms in the hyper-eutrophic Raritan-Hudson Bays, but the cause is unknown (Mahoney et al. 1988). In Puget Sound naturally occurring toxins, metabolites or degradation products of phytoplankton or bacteria could be responsible for growth inhibition of *A. catenella*. For example, the relatively common dinoflagellates *Gymnodinium splendens* and *Ceratium fusus* have been closely associated with oyster larvae mortality in distal bays and inlets of Puget Sound (Cardwell et al. 1977, 1979). The mechanism of mortality and malformation of oyster larvae is unexplained, but could be due to chemical exudates. There has been no systematic survey of the phytoplankton species assemblage in these areas, so other species could be involved.

This paper focuses on the physical and chemical differences between PSP-affected and unaffected areas of Puget Sound. In particular the role of nitrogen supply and water column stability are discussed as key factors that could control PSP distribution in Puget Sound.

## METHODS

Hydrographic and nutrient data were obtained from the Washington Department of Ecology for the months of April to November, 1981–85 and are part of a routine monitoring program; their protocols and methods are discussed elsewhere (EPA 1991, Janzen 1992). Density information, i.e., degree of stratification or mixing, was extracted from several years of monthly surveys published by Collias et al. (1974). Shellfish toxin data were provided by the Washington Department of Health, Office of Shellfish Programs.

Field and laboratory data were from experiments with water samples collected in late August 1992 from Hood Canal. The field study involved collection of water samples using a float plane from the surface, 10 m and 30 m depths from central and southern Hood Canal. Preliminary work in 1991 and monitoring of shellfish in past years showed that *A. catenella* cells survived and caused toxicity in north Hood Canal, so that station was omitted in this work. Water samples were collected with a water bottle, iced, taken to the laboratory, filtered through GF/F filters the same afternoon and inoculated with 15 cells/ml of exponentially-growing *A. catenella* cells. The cells, from isolate GC84-40 originally from Quartermaster Harbor, were growing in HESNW enriched seawater medium (Harrison et al. 1980).

The cells were grown for about 3 weeks at  $14^{\circ}\text{C}$  with light equivalent to bright sunlight, about  $100 \mu\text{E m}^2 \text{ sec}^{-1}$ . Positions of culture flasks were altered every three days to normalize any possible differences in light intensity. Nutrient samples were collected in the field, in the initial cultures, and at the completion of the culture period for analysis using standard autoanalyzer methods. Cell counts were conducted using a Palmer-Maloney counting chamber having a volume of 0.1 ml. Six separate counts were done for each of three replicate water samples for each depth and location or laboratory sample.

## RESULTS

### Monitoring

Surface waters of central and south Hood Canal are the most nitrogen-depleted areas in Puget Sound (Table 1), (Rensel Associates and PTI Environmental Services 1991). Several areas in southern Puget Sound also have low average nitrogen concentration in surface waters during summer months. Increased subsur-

TABLE 1.

Subareas of Southern Puget Sound and Hood Canal ranked in order of increasing 10 m concentration of dissolved inorganic nitrogen (DIN,  $\mu\text{M-N}$ ) from June to August, 1981–1985.

Area/Subarea	Mean Surface DNA Concentration		PSP Presence/Absence and Water Column Status During Summer Months
	Surface	10 meter	
Hood Canal			
Centrl Hood Canal	1.9	2.7	Not Reported—stratified
North Hood Canal	5.0	12.1	Rare—mixing area
South Hood Canal	1.4	13.5	Not Reported—stratified
South Puget Sound			
Hammersly/Oakland Bay	4.2	3.7	Not Reported—variable
Totten Inlet	2.2	4.0	Extremely Rare—stratified
Eld Inlet	3.0	5.7	Not Reported—stratified
Pickering Passage	5.2	6.2	Not Reported—mixing area
Budd Inlet	2.7	7.3	Not Reported—stratified
Case Inlet	3.7	8.6	Rare—stratified
Dana Passage	6.4	10.0	Extremely Rare—mixing area
Carr Inlet	3.5	12.0	Frequent since 1988—stratified
Nisqually Reach	12.0	16.0	Occasional—mixing area
Other Areas of Puget South	average 11.5	range of ca	Occasional to Frequent PSP—variable
Combined N = 25 <sup>a</sup>		12 to 22	hydrographic conditions with intense mixing at sills and in channels

<sup>a</sup> Does not include other nutrient-sensitive areas not located in SPS or Hood Canal ( $n = 5$ ).

face (10 m) concentration of dissolved inorganic nitrogen (DIN) appears to correlate with the increased frequency of PSP in stratified areas not subject to sill-induced vertical mixing (Table 1). Certain channel areas with relatively high concentrations of nitrogen and semi-blocking sills, e.g., Nisqually Reach (Fig. 2), most likely have too much vertical mixing to support dinoflagellate populations. The one notable exception to the pattern is southern Hood Canal. This area has relatively high subsurface nitrogen concentrations during the summer, but no reports of PSP. This apparent anomaly is of particular interest and is discussed later in this paper.

The greater relative abundance of subsurface DIN in most south Puget Sound inlets may be the primary source of nitrogen for many autotrophic dinoflagellate populations during calm weather periods of the summer and fall. Cardwell et al. (1977, 1979) correlated maximum cell counts of certain harmful dinoflagellates in subsurface waters (3–10 m +) in some Puget Sound inlets with increased toxicity to shellfish larvae in bioassays. The dinoflagellates may either migrate to depth at night to obtain nutrients or perhaps continually stay near the nutricline, i.e., the layer of nutrient concentration discontinuity. The nutricline will generally be shallower than 10 meters in these restricted bays and inlets (e.g., central Budd Inlet; URS 1986) and may coincide with the thermocline depth.

#### Experimental

The bioassay results show a general correlation between initial nitrogen concentration, including nitrogen added with the inoculum, and final cell yield (Table 2,  $R^2 = 0.98$ , total  $df = 5$ ). The quantity of nitrogen in surface samples was significantly affected by the nitrogen carried over in the inoculum, but there was less effect on cultures grown with water from other depths. No apparent growth inhibition attributed to dissolved substances in the filtered water samples was evident. I tentatively conclude that if *A. catenella* cells were present in SHC water with this composition,

and able to migrate to nitrogen-rich depths, they should survive and grow. However, in CHC surface and subsurface waters, concentrations of nitrogen are normally too low in the summer to expect growth of *A. catenella* cells.

Initial and final N:P ratios were less than about 16:1 in all cases, and are normally suggestive of possible nitrogen limitation if the supply rate is low. Final nitrogen concentrations were very low, which further suggests that nitrogen limited the growth of the cells. Phosphorus concentrations are not included here for brevity, but were  $>1 \mu\text{M-P}$  in the initial samples and therefore not considered minimal compared to the nitrogen concentrations.

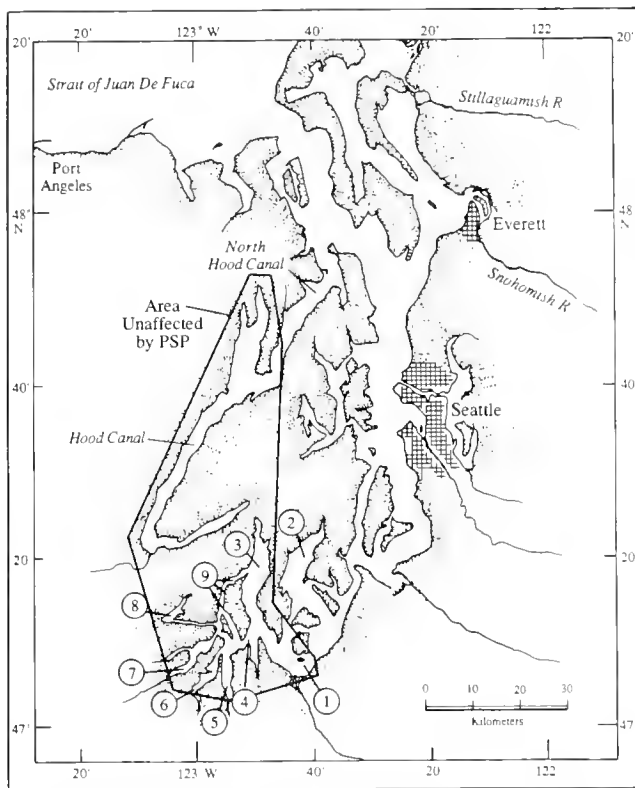
Subsurface (10 m) nitrogen concentrations were significantly different from the average summer conditions previously discussed. Nitrogen was much lower than expected at 10 m in SHC and much greater than expected in CHC. Under average summer nitrogen conditions, much greater growth would be expected in the subsurface waters of SHC and virtually no growth in subsurface waters of CHC.

#### DISCUSSION

The monitoring data and the relationship between cell yield and initial nitrogen concentration of the bioassay cultures point to a lack of nitrogen in surface and subsurface water as a major factor that limits the further spread of PSP into bays and inlets of Puget Sound otherwise suitable for *A. catenella*. The effects of water column mixing and turbulence in shallow or narrow channels could explain the general lack of PSP in areas replete with nitrogen, such as Nisqually Reach in SPS and North Hood Canal.

#### Nitrogen Half-saturation Constants

Although the threshold DIN concentration for growth limitation of *A. catenella* is unknown, it may be less than  $10 \mu\text{M DIN}$  (Norris and Chew 1975). If it is similar to or less than the mean value for many dinoflagellates of approximately  $8 \mu\text{M-N}$  (Bowie et al. 1985), then most of south Puget Sound and south and central



**Figure 2.** Map of Puget Sound and Hood Canal showing areas unaffected by PSP (inside solid line) and subareas of southern Puget Sound: 1) Nisqually Reach, 2) Carr Inlet, 3) Case Inlet, 4) Dana Passage, 5) Budd Inlet, 6) Eld Inlet, 7) Totten Inlet, 8) Hamersley Inlet/Oakland Bay, 9) Pickering Passage.

Hood Canal may have insufficient DIN in summer months to support growth of *A. catenella* at the surface or 10-meter depths.

Other dinoflagellates that are apparently more common throughout south Puget Sound (e.g., *Ceratium fusus* and *Gymnodinium splendens*) may be adapted to lower concentrations of DIN. For example, the half-saturation constant for nitrate uptake by *G. splendens* is  $1.0 \mu\text{M}$  at  $18^\circ\text{C}$  (Thomas and Dodson 1974). If those data are applicable to *G. splendens* in Puget Sound, the results could explain why this species occurs in the bays and inlets with very low surface and subsurface DIN concentrations that to date have not harbored populations of *A. catenella*. Determination of the actual half-saturation constant for *A. catenella* should be conducted for several clones of local isolates.

#### Hood Canal

The most plausible explanation why PSP isn't reported in SHC is a combination of physical and nutrient-supply factors. It is likely that weak, but sustained, estuarine outflow at the surface prevents live *A. catenella* cells from reaching the south end of the canal. The strong mixing zone at the entry to Hood Canal further discourages ingress of an intact "bloom" of *A. catenella* cells. The normal lack of nitrogen in both surface and subsurface waters of CHC effectively acts as a barrier to passage of live cells to the south. Although cells could be transported in at depth, light may be insufficient to sustain the cells, given the very slow transport rates (Cokelet et al. 1990). Cysts that form and fall into the relatively great depths of nearly 200 m in CHC where excystment cues

**TABLE 2.**

**Results of August 1992 bioassay with Hood Canal water. DIN in  $\mu\text{M-N}$ . Balanced molar N:P ratio for algae is approximately 16:1.**

	South Hood Canal	Surface	10 m Depth	30 m Depth
Initial Ambient DIN		0.13	0.86	26.9
Mean Cell Counts (cells/ml)		42.4	62.9	214.4
Standard Deviation		12.4	8.7	13.6
Initial DIN + Inoculum DIN		3.3	4.1	30.1
Initial N:P Ratio		0.1	0.4	9.1 <sup>a</sup>
Final DIN		1.3	1.1	0.29
Final N:P Ratio		8.8	3.7	3.8
Cell Yield/Nitrogen Use Ratio		21.2	21.0	7.2
<b>Central Hood Canal</b>				
Initial Ambient DIN		0.04	15.5	29.3
Mean Cell Counts (cells/ml)		45.5	148.1	243.4
Standard Deviation		7.7	18.9	41.3
Initial DIN + Inoculum DIN		3.24	18.7	29.3
Initial N:P Ratio		0.1	8.5	10.3 <sup>a</sup>
Final DIN		0.35	0.03	0.02
Final N:P Ratio		3.1	1.2	0.4
Cell Yield/Nitrogen Use Ratio		15.7	7.9	8.3

<sup>a</sup> Concentration of DIN sufficiently great to assume no nitrogen limitation and therefore N:P ratio not indicative of algal growth limitation.

from light or temperature changes are limited and endogenous clock driven excystment would result in cells germinating into deep, dark and relatively cold water. Given the available data, however, I can not rule out predation and growth inhibitors that could occur at other times, but I believe the previous explanation is more likely.

The deep water of south and central Hood Canal is subject to hypoxic conditions associated with slow rates of flushing (Cokelet et al. 1990) and intense spring plankton blooms of  $>40 \mu\text{g/L}$  chlorophyll *a* (Barlow 1958) that result in downward export of oxygen demanding organic matter. Several fish kills have been reported in past years, some due to recurring blooms of the harmful diatoms *Chaetoceros concavicornis* or *Ch. convolutus* (Rensel Associates and PTI Environmental Services 1991, Rensel 1992) and others due to the surfacing of hypoxic deep water that occurs in the late summer and fall (Collias et al. 1974). The most recent evidence in the past few years indicates that the bottom water hypoxia may be increasing in intensity and duration, and in some recent years is persistent throughout the year (Janzen and Eisner 1993). It is not known if this represents a continuing trend, or if it is the result of short or long term climate variation that has been found to affect flushing rates and other physical and biological properties of Puget Sound (Ebbesmeyer et al. 1989).

#### Southern Puget Sound

Although no experimental data were collected for southern Puget Sound, the hydrographic situation is similar in many ways to Hood Canal. That is, there are entry areas of turbulent mixing leading to restricted waters that become seasonally depleted of nitrogen in surface and subsurface waters. The exception, Carr Inlet, has relatively high subsurface nitrogen compared to other SPS inlets and has had PSP toxicity and some shellfish harvesting closures since 1988. It is much deeper than other SPS inlets, has relatively high concentrations of nitrogen in the deep water, and is contiguous with nutrient-rich mixing areas. Case Inlet had two

minor episode of PSP, but they were in the fall when nutrient-rich deep water may have been mixed to the surface by the prevailing south winds or from the normal, seasonal intrusion of deep, nutrient-rich seawater.

#### Future Spread of PSP in Puget Sound

Nitrogen supply rates, water column stability or vertical mixing during the summer appear to be the most influential factors preventing the further spread of PSP in Puget Sound. However, unusual meteorological or hydrographical conditions could cause extreme deviations from the average conditions discussed herein and lead to *A. catenella* blooms in previously unaffected areas. Other factors including predation could be responsible or partly responsible for controlling or limiting the spread of *A. catenella* cells.

Rapid urbanization, land clearing, logging and other potentially adverse land-use practices in the PSP-unaffected watersheds will generate significant amounts of nitrogen-bearing runoff. In general nitrogen compounds are more soluble and have less affinity for absorption to particles than inorganic phosphorus. However, six of eleven urban bays in Puget Sound have had significant recent increases in phosphorus concentrations (Tetra Tech 1988) despite use of phosphorus-reducing secondary sewage treatment. Nitrate data from SPS were inadequate for long-term trend analysis, but in general have declined for all of Hood Canal coincident

with increased percent dissolved oxygen saturation at 10 m. Together with possible increasing persistence of deep water hypoxia this suggests increasing eutrophication that could allow *A. catenella* cells to survive transport through CHC to prosper in the already nitrogen-enriched subsurface water of SHC.

There is a need to routinely monitor phytoplankton density and species assemblages in the PSP-unaffected areas to detect the early onset of conditions that would support the spread of PSP. We need additional bioassays to verify the preliminary results shown here, as well as determination of accurate nitrogen use dynamics for *A. catenella* in the laboratory. Most importantly, there needs to be new emphasis to identify and modify the primary causes of non-point pollution-caused eutrophication in nutrient sensitive areas of Puget Sound.

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## EFFECTS OF TOXIC DINOFLAGELLATES ON CLEARANCE RATES AND SURVIVAL IN JUVENILE BIVALVE MOLLUSCS

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**ABSTRACT** Feeding and survival experiments using unialgal cultures of the toxic dinoflagellates, *Alexandrium* (= *Protogonyaulax*) *tamarensis*, and *Gyrodinium aureolum*, were conducted on several species of juvenile bivalve molluscs. These experiments were designed to assess the potential impact of toxic algal blooms during the "grow-out phase" for the faster-growing juvenile stages. Mortality of juvenile bivalves after exposure to toxic dinoflagellates was dependent upon time after exposure and temperature during exposure, suggesting species specific patterns and an overall higher toxicity of *Gyrodinium aureolum* during both the winter and summer experiments. Feeding rates on unialgal cultures of toxic dinoflagellates during the winter of 1989 were uniformly low, and are correlated with the lower mortality observed in the survival experiments. Preference for the non-toxic microalgae, *Isochrysis* sp. was significant during these experiments for all bivalves except *Placopecten magellanicus*, which probably reflects more on the size of *Isochrysis* sp. and the functional morphology of the ctenidia of this species. Experiments conducted in the spring of 1990 reveal species-specific patterns which in some cases mirror the winter experiments. Other bivalve species show a significant preference for toxic dinoflagellates that is not always correlated with the survival experiments suggesting that some species can ingest and utilize toxic dinoflagellates without short-term effects.

**KEY WORDS:** toxic dinoflagellates, *Alexandrium*, *Gyrodinium*, *Mercenaria*, *Ostrea*, *Crassostrea*, *Argopecten*, *Mya*, *Mytilus*, *Placopecten*, *Geukensia*, clearance rates, survival

### INTRODUCTION

Many coastal marine habitats are affected by periodic blooms of toxic microalgae that can have a significant impact on the shellfish industry, and public health (Shumway 1990). Historically, the primary focus has been on toxic dinoflagellates responsible for paralytic shellfish poisoning (PSP) associated with filter-feeding bivalve molluscs that accumulate toxins in their tissues, and can lead to PSP in human consumers.

What of the effect of these toxic dinoflagellates on the shellfish themselves, and the potential for economic loss due to a decrease in growth or outright mortality of shellfish? Recent studies have clearly demonstrated that exposure to toxic dinoflagellates has a significant effect on many physiological processes that include changes in feeding rates, respiration rates, shell valve closure, mucous production, and altered cardiac activity (Shumway and Cucci 1987, Gainey and Shumway 1988, Shumway 1990).

Almost all previous work on the effects of toxic dinoflagellates has been carried out using adult bivalve molluscs. For either the grow-out phase of juveniles suspended in the water-column or the introduction of juveniles onto bottom sites, the potential for exposure to blooms of toxic dinoflagellates is high, while the biological effects of these exposures for juvenile bivalve molluscs is presently unknown. During the juvenile phase, weight-specific metabolism is high (Griffiths and Griffiths 1987), and there must be sufficient phytoplankton available to cover the energetic costs of routine maintenance and growth. Exposure of juvenile bivalve molluscs to toxic dinoflagellates during this period could potentially affect feeding and, therefore, rates of growth as does exposure of adult blue mussels to toxic dinoflagellates (Nielsen and Stromgren 1991).

Two dinoflagellates commonly associated with toxic blooms are *Alexandrium* (= *Protogonyaulax*) *tamarensis* and *Gyrodinium aureolum*. *Alexandrium tamarensis* is well documented as a worldwide source of PSP toxins in shellfish and PSP outbreaks in humans (Shumway 1990). Toxins associated with *A. tamarensis* may persist for months in the tissues of bivalves with unknown long-term consequences (Shumway and Cembella 1993, Cembella et al. in press). *Gyrodinium aureolum* has not been indicated in any outbreaks associated with human illness, but has been shown to cause mortalities in a number of shellfish species (Shumway 1990), and was recently associated with a massive shellfish kill in Maquoit Bay, Brunswick, Maine (Heinig and Campbell 1992). Widdows et al. (1979) demonstrated the direct cytotoxic effects of *G. aureolum* on adult *Mytilus edulis* when bloom concentrations of this dinoflagellate caused a decline in clearance rates and cellular damage to the gut after a short (<24 h) exposure. *Gyrodinium aureolum* has also been shown to inhibit feeding in the post-larvae of *Pecten maximus* and to cause mortalities in juvenile scallops (Lassus and Berthome 1988). The toxic effects of *G. aureolum* are not restricted to shellfish of commercial interest and affect a wide range of marine invertebrates and vertebrates (Cross and Southgate 1980, Shumway 1990).

Blooms of toxic *Alexandrium tamarensis* are a seasonal and annual occurrence in coastal Maine waters, and the incidence of *Gyrodinium aureolum* in these waters has recently increased. With the large investment in shellfish aquaculture in Maine we began an investigation on the effects of these toxic dinoflagellates on juvenile shellfish by examining the effects of bloom concentrations of *A. tamarensis* and *G. aureolum* on survival and feeding in eight species of commercially important juvenile bivalves. We present here the results of survival and feeding experiments using unialgal cultures of toxic dinoflagellates. In a subsequent paper, we will address the feeding of juvenile bivalves on natural assemblages of particles in conjunction with bloom concentrations of toxic dinoflagellates.

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## MATERIALS AND METHODS

Algal cultures were supplied from the Provasolli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences. *Alexandrium tamarens* (clone GT429) and *Gyrodinium aureolum* (clone PLY 497A) cultures were grown in mass cultures (20 l) using f/2 media at 15°C on a 14:10 light/dark photoperiod. Cells were harvested during exponential phase of growth.

### Survival Experiment

Short- and long-term mortality associated with exposure to the toxic dinoflagellates, *Alexandrium tamarens* and *Gyrodinium aureolum* was assessed in juveniles of eight species of commercially important shellfish: *Mercenaria mercenaria*, *Ostrea edulis*, *Crassostrea virginica*, *Argopecten irradians*, *Mya arenaria*, *Mytilus edulis*, *Placopecten magellanicus*, and *Spisula solidissima* obtained from Mook Sea Farm Inc., Damariscotta ME. All animals were scrubbed free of any epibionts, and maintained in unfiltered, flowing sea water from Boothbay Harbor, Maine prior to use in experiments. Animals were not fed any supplementary food prior to the experiments. Bloom concentrations of *A. tamarens* ( $10^5$  cells  $l^{-1}$ ) and *G. aureolum* ( $10^6$  cells  $l^{-1}$ ) were maintained for one week in 175 l tanks using unfiltered sea water containing natural seston. Control tanks with just natural sea water were run simultaneously for all experiments. Mortality was assessed in control and treatment tanks at one week and six weeks post exposure. The experiment was run in the winter (5°C) of 1989 and spring (10°C) of 1990. Percentage of mortalities obtained were arcsine transformed prior to a Chi-square analysis at the 5% significance level, that compared bivalves exposed to toxic dinoflagellates and natural seston against controls exposed to natural seston only. During the time course of this experiment no *A. tamarens* or *G. aureolum* cells were present in natural sea water (D. Jacobsen, personal communication).

### Feeding Experiments Using Unialgal Cultures of Toxic Dinoflagellates

Unialgal feeding experiments using bloom concentrations of *Alexandrium tamarens* and *Gyrodinium aureolum* were conducted on *Mercenaria mercenaria*, *Ostrea edulis*, *Crassostrea virginica*, *Argopecten irradians*, *Mya arenaria*, *Mytilus edulis*, *Placopecten magellanicus*, *Spisula solidissima*, and *Geukensia demissa* that were compared to feeding rates on *Isochrysis* sp. (clone TISO, [ $10^5$  cells  $l^{-1}$ ]). All animals were allowed to purge themselves in filtered sea water (0.7  $\mu$ m Gelman glass filter) for 24 h prior to being used in feeding experiments. These experiments were also conducted in the winter (5°C) of 1989 and Spring (10°C) of 1990. Individual specimens were placed in aerated glass beakers containing 40–100 ml of the algal culture in filtered sea water. Control vials, without animals, were run simultaneously to correct for algal cell division during the experiment. Experiments lasted for 1 h, with samples taken at the end of the experimental period. Samples were analyzed with a Coulter counter model ZM fitted with a 100  $\mu$ m orifice. Dry weights of soft tissues were obtained for all animals by constant drying at 60°C for 48 h. Clearance rates were calculated by the method of Coughlan (1969). Dry weight was used to normalize all data, while assuming a 100% retention efficiency for all algal species tested. Production of pseudofeces was not observed during these experiments. Differences in the weight specific clearance rates of total cells corrected for any cell division were evaluated using an ANOVA. No unequal variances

were detected using the  $F_{max}$  test for the ANOVA (Sokal and Rohlf 1981), and where significant treatment effects occurred, the Student-Neuman-Keuls (SNK) multiple comparison test was applied at the 5% significance level to identify individual differences among the data sets.

## RESULTS

No mortalities were noted after a one week exposure to *Alexandrium tamarens* or *Gyrodinium aureolum* during the winter of 1989 or spring of 1990 for any of the bivalve species tested. In 1989 no mortalities were noted six weeks after the one week exposure for bivalves exposed to *A. tamarens* while non-significant (Chi-square,  $P > 0.05$ ) mortalities were noted for *Crassostrea virginica* and *Ostrea edulis* in the spring of 1990 six weeks after the one week exposure period (Table 1).

For bivalves exposed to *Gyrodinium aureolum* there were significant mortalities of *Mercenaria mercenaria* and *Argopecten irradians* after one week, while after the subsequent six weeks significant mortalities were noted in *Crassostrea virginica* and *Spisula solidissima* (Table 1) suggesting strong, specific-specific differences in mortality for time after exposure to toxic dinoflagellates and ambient water temperature during exposure.

During the winter of 1989 the unialgal experiments (Fig. 1a) all showed a significant within species ANOVA ( $P < 0.001$ ) for feeding rates on the toxic dinoflagellates and the non-toxic microalgae

TABLE 1.  
Percent mortality of juvenile bivalve molluscs six weeks after a one week exposure to bloom concentrations of the toxic dinoflagellates, *Alexandrium* (-*Protogonyaulax*) *tamarens*, and *Gyrodinium aureolum*.

<i>Alexandrium tamarens</i>				
Bivalve Species	Winter 1989		Spring 1990	
		Chi-square		Chi-square
<i>Mytilus edulis</i>	NM	NS	NM	NS
<i>Crassostrea virginica</i>	NM	NS	4%	NS
<i>Ostrea edulis</i>	NM	NS	4%	NS
<i>Mercenaria mercenaria</i>	NM	NS	NM	NS
<i>Spisula solidissima</i>	NM	NS	NM	NS
<i>Argopecten irradians</i>	NM	NS	NM	NS
<i>Placopecten magellanicus</i>	NT		NM	NS
<i>Mya arenaria</i>	NT		NM	NS
<i>Gyrodinium aureolum</i>				
Bivalve Species	Winter 1989		Spring 1990	
		Chi-square		Chi-square
<i>Mytilus edulis</i>	4%	NS	NM	NS
<i>Crassostrea virginica</i>	NM	NS	68%	$P < 0.05$
<i>Ostrea edulis</i>	12%	NS	4%	NS
<i>Mercenaria mercenaria</i>	44%	$P < 0.001$	NM	NS
<i>Spisula solidissima</i>	8%	NS	16%	$P < 0.05$
<i>Argopecten irradians</i>	100%	$P < 0.001$	8%	NS
<i>Placopecten magellanicus</i>	NT		NM	NS
<i>Mya arenaria</i>	NT		NM	NS

Temperatures for winter 1989 and spring 1990 were 5°C and 10°C respectively. Percentages were arcsine transformed prior to a Chi-square analysis at the 5% significance level, and compared bivalves exposed to toxic dinoflagellates and natural seston against controls exposed to natural seston only. NM = no mortalities, NS = not significant, NT = not tested.

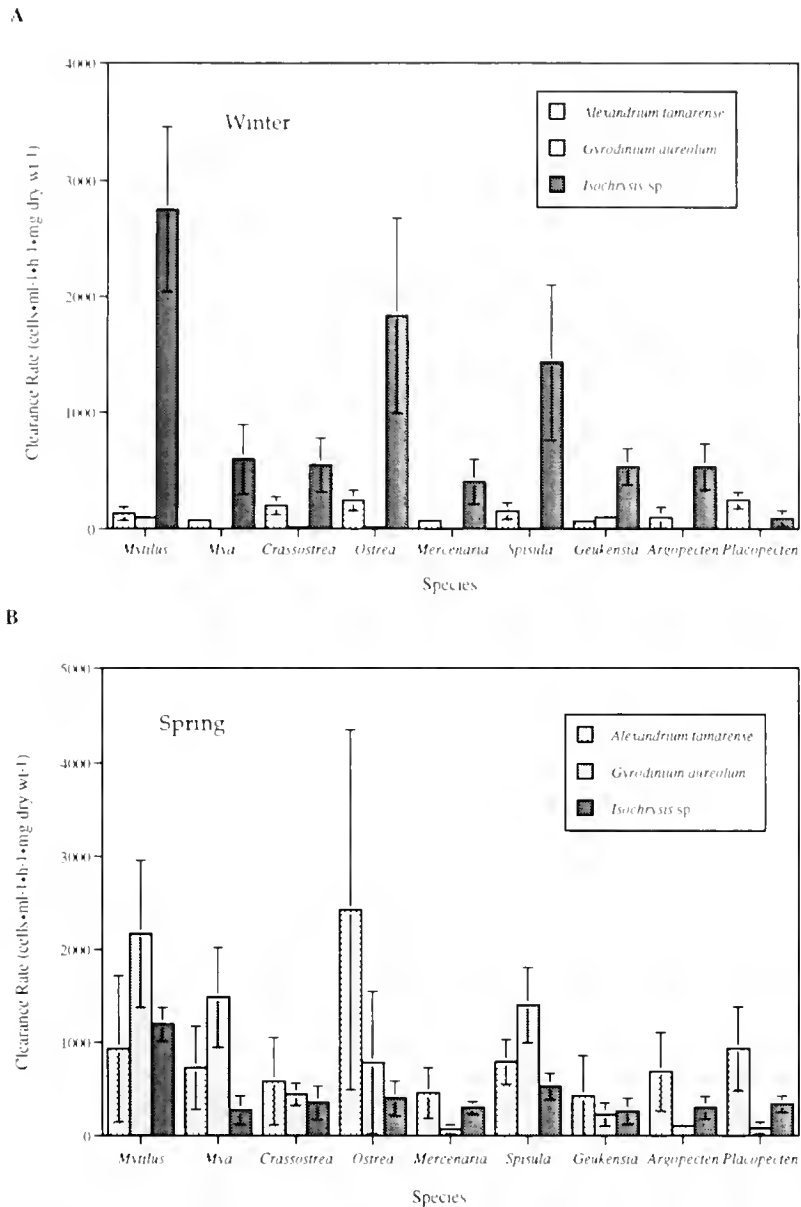


Figure 1. A: Clearance rates of nine species of juvenile bivalves fed pure cultures of *Alexandrium tamarense*, *Gyrodinium aureolum*, and *Isochrysis sp.* in the winter (5°C) of 1989. B: Clearance rates of nine species of juvenile bivalves fed pure cultures of *Alexandrium tamarense*, *Gyrodinium aureolum*, and *Isochrysis sp.* in the spring (10°C) of 1990.

*Isochrysis sp.* (Fig. 1a). In all species examined, except for *Crassostrea virginica* and *Placopecten magellanicus*, the post-hoc multiple comparison tests showed that the feeding rates on *Alexandrium tamarense* and *Gyrodinium aureolum* were grouped together (SNK;  $P > 0.05$ ), but were significantly lower than the feeding rate on *Isochrysis sp.* (SNK;  $P < 0.05$ ). For *C. virginica* and *P. magellanicus* the feeding rates between *A. tamarense* and *G. aureolum* were significantly different from one another (SNK;  $P < 0.05$ ), but still significantly lower than the feeding rates on *Isochrysis sp.* as reported for the other species of bivalves. It should be noted that for *P. magellanicus*, exposure to *G. aureolum* induced the production of copious amounts of mucus and cessation of feeding. Analysis of between-species differences in clearance rates of *Alexandrium tamarense* and *Gyrodinium aureolum* showed a significant ANOVA ( $P < 0.001$ ) for both species of

toxic dinoflagellates with multiple comparison testing partitioning the feeding rates of the bivalve species tested in three groups for *A. tamarense* and two groups for *G. aureolum* (Table 2).

Experiments in the summer of 1990 (Fig. 1b) showed a similar significant ANOVA ( $P < 0.05$ ) for the within bivalve analysis of feeding rates on the toxic dinoflagellates and *Isochrysis sp.* on all species tested except *Crassostrea virginica* and *Geukensia demissus* where no significant differences in clearance rates were detected (ANOVA:  $P > 0.05$ ). Multiple comparison testing on *Ostrea edulis*, *Argopecten irradians*, and *Placopecten magellanicus* all showed a similar pattern with clearance rates on *Isochrysis sp.* being significantly higher than the two species of toxic dinoflagellates, which were grouped together, using multiple comparison testing. Comparisons for *Mytilus edulis*, *Mya arenaria*, and *Spisula solidissima* all showed a distinctively different pattern

TABLE 2.

Groupings of bivalves from significant ( $P < 0.05$ ) post-hoc multiple comparison testing (SNK).

<i>Alexandrium tamarense</i>		
Bivalve Species	Winter 1989	Spring 1990
<i>Mytilus edulis</i>	B	B
<i>Mya arenaria</i>	C	B
<i>Crassostrea virginica</i>	A	B
<i>Ostrea edulis</i>	A	A
<i>Mercenaria mercenaria</i>	C	B
<i>Spisula solidissima</i>	B	B
<i>Geukensia demissus</i>	C	B
<i>Argopecten irradians</i>	C	B
<i>Placopecten magellanicus</i>	A	B
<i>Gyrodinium aureolum</i>		
Bivalve Species	Winter 1989	Spring 1990
<i>Mytilus edulis</i>	A	A
<i>Mya arenaria</i>	B	A
<i>Crassostrea virginica</i>	C	B
<i>Ostrea edulis</i>	C	A
<i>Mercenaria mercenaria</i>	B	B
<i>Spisula solidissima</i>	B	A
<i>Geukensia demissus</i>	A	B
<i>Argopecten irradians</i>	B	B
<i>Placopecten magellanicus</i>	B	B

Species with common letters exhibit equivalent rates of feeding.

where the feeding rates on *Gyrodinium aureolum* were significantly higher than feeding rates on *Alexandrium tamarense* or *Isochrysis* sp. which were grouped together using multiple comparison testing. Finally, *Mercenaria mercenaria* exhibited significant differences in feeding rates where *A. tamarense* and *Isochrysis* sp. were grouped together and exhibited higher rates of consumption of *A. tamarense* than *G. aureolum* (Fig. 1). Analyzing between-species differences in clearance rates for the spring of 1990 experiments again showed a significant ANOVA ( $P < 0.001$ ) for both species of toxic dinoflagellates with multiple comparison testing dividing the bivalve species tested into two groups for both *A. tamarense* and *G. aureolum* (Table 2).

## DISCUSSION

This study provides an initial assessment of the effects of two species of toxic dinoflagellates on survival and clearance rates in several species of juvenile bivalve molluscs. We were able to demonstrate that *Ostrea edulis* had significantly higher clearance rates of *Alexandrium tamarense* than did any other species of bivalve tested under spring conditions. These results are consistent with previous studies which showed that European oysters become toxic prior to any other species under field conditions, and selectively feeds on dinoflagellates under laboratory conditions (Shumway et al. 1985, Shumway et al. 1990). The feeding studies using *Gyrodinium aureolum* showed that *Mytilus edulis*, *Mya arenaria*, and *Spisula solidissima* exhibited the highest rates of consumption, while *Ostrea edulis* demonstrated feeding rates intermediate with the rest of the species examined.

The dinoflagellate, *Gyrodinium aureolum* has frequently been

associated with fish kills, especially salmonids (see Turner et al. 1989, Jones et al. 1982, Roberts et al. 1982). Since the late sixties, *G. aureolum* has also been implicated in massive kills of marine fauna including shellfish (Partensky et al. 1989). *Gyrodinium aureolum* is the dinoflagellate implicated in the massive shellfish kills which occurred in Maquoit Bay, Maine, in September of 1988 (Heinig and Campbell 1992); however, the specific cause of death was not verified.

It seems likely that *Gyrodinium aureolum* may have a cytotoxic effect on shellfish, unlike other toxic dinoflagellates (e.g. *Alexandrium tamarense*) that normally induce neurotoxic responses (Shumway and Cucci 1987). Previous studies of the impact of *G. aureolum* on shellfish biology have demonstrated mortality in juveniles (Erard-LeDenn et al. 1990, Tangen 1977, Lassus and Berthome 1988, Helm et al. 1974), reduced shell growth (Nielsen and Stromgren 1991), reduced clearance rates (Widdows et al. 1979, Shumway unpublished) and marked cellular damage to the gut (Widdows et al. 1979). Preliminary studies carried out with Dr. Antonello Novelli (University of Oviedo, Spain) on isolates of *Gyrodinium aureolum* Clone PLY 497 provided little evidence for a neurotoxin in this species. Further, recent studies by Turner et al. (1987), Partensky et al. (1989), Gentien and Arzul (1990) and Gentien et al. (1991) all indicate that *G. aureolum* produces toxins. Partensky et al. (1989) confirmed the presence of at least one fat-soluble cytotoxin, and Gentien and Arzul (1990) determined that the toxic action proceeds from two different processes which are possibly associated with two types of toxic compounds.

Finally, it has been demonstrated that the harmful effects of *G. aureolum* can be reversed if the animals are returned to clean seawater before permanent damage has taken place (Widdows et al. 1979, Erard-LeDenn et al. 1990).

Recent, unexplained mortalities of hatchery-reared, juvenile oysters (*Crassostrea virginica*) began coincidentally with a bloom of another closely related dinoflagellate, *Gymnodinium sanguineum*. Further, during a second bloom of this dinoflagellate, mantle lesions were noted in the oysters with no mortalities. This species of dinoflagellate has not been previously demonstrated to be toxic to bivalves and was not directly linked to the oyster mortalities (Bricelj et al. 1992); however, it does implicate yet another species of dinoflagellate in harmful effects on shellfish.

As in previous studies on the effects of toxic dinoflagellates on shellfish, our results indicate that responses are species-specific, and that feeding-rates were significantly lower in the winter than spring. These differences are likely caused by the lower temperatures experienced in the winter, but for animals in the field both a decrease in temperature and lower food resources would contribute to decreased rates of consumption. Although exposure to toxic dinoflagellates during winter is unlikely under normal conditions, the combined results from the survival and feeding studies presented here would encourage aquaculturists to focus their attention on the possibility that exposure of *Placopecten magellanicus*, *Spisula solidissima* and *Crassostrea virginica* to outbreaks of *Gyrodinium aureolum* during early spring and late fall, where temperature conditions are similar to those used in this study, could result in substantial mortality and cessation of feeding. Cessation of feeding for extended periods of time is likely to have an effect on survivability and the time it takes to produce a marketable product. Our studies on the feeding of juvenile bivalves on natural assemblages with toxic dinoflagellate blooms (Shumway et al. in preparation) should provide additional information on the effects of these blooms on feeding.

Shellfish toxicity associated with blooms of toxic dinoflagellates is not novel, and is largely well defined due to the economic and public health issues. The increase in the occurrence of these, and other, noxious algal blooms have serious implications for the development and success of aquaculture. Shellfish toxicity monitoring programs ensure public safety and maximize harvesting time of adults ready for the market, but tell us nothing about the effects on future harvests. Comprehensive studies on the effects of toxic microalgae on juvenile bivalves of commercial importance are long overdue, and are more important than ever as aquaculturists seed juveniles into coastal waters with hopes of an ever increasing yield in the future.

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## SURVIVAL OF LIVE *ALEXANDRIUM TAMARENSE* CELLS IN MUSSEL AND SCALLOP SPAT UNDER SIMULATED TRANSFER CONDITIONS

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**ABSTRACT** This preliminary study investigates the potential for inadvertent transfer of toxic or nuisance phytoplankton with shipments of bivalve spat between aquaculture sites. Spat of cultured mussels (*Mytilus edulis*, L.) and scallops (*Placopecten magellanicus*, Gmelin) when fed cultures of the toxic dinoflagellate *Alexandrium* (= *Gonyaulax*) *tamarense* Lebour were found to retain living cells. Live *A. tamarense* cells were recovered from rinse water after 6 hours in conditions designed to simulate trans-shipment. Use of the flagellate *Tetraselmis suecica*, Butcher, which has different chlorophyll constituents than *A. tamarense*, showed that gut retention time of *M. edulis* was 3.5–3.9 hr at 16°C, giving an indication of the minimum time that spat should be in a purging system to minimize the likelihood of transferring undigested *A. tamarense* cells. Rinse water from scallops fed *A. tamarense*, and pure cultures of *A. tamarense* repeatedly exposed to UV light in a recirculating system, showed no viable cells after 19.5 hours. The potential for transfer of living toxic phytoplankton in shipments of wild bivalve spat is clearly demonstrated, as is the use of purging systems as a possible solution to the problem.

**KEY WORDS:** phytoplankton, *Alexandrium*, survival, mussels, scallops

### INTRODUCTION

The increasing trade in shellfish spat, specifically of mussels (*Mytilus* sp.) and scallops (*Placopecten magellanicus*) for culture in areas distant from where they originated, has provoked some concern among shellfish growers and fisheries managers for the transfer of diseases and alien species. While regional fisheries policies in Atlantic Canada require that shellfish be examined for parasites and diseases prior to trans-shipment for culture purposes, the examination does not include any search for toxic or nuisance phytoplankton. Arguments for considering the possibility of transferring such organisms centre on real concerns for introducing alien species into local waters, and the desire to minimize the likelihood of blooms of toxic phytoplankton in areas not yet afflicted. There has been a world-wide increase in the variety, frequency and severity of toxic algal blooms (Shumway 1990) and increasing evidence of the inadvertent transfer and introduction of non-indigenous species, both microscopic and macroscopic (Hallegraeff and Bolch 1991, 1992, Smith and Kerr 1992). Introduction of the zebra mussel *Dreissena polymorpha* Pallas to the Great Lakes has caused much damage (Neary and Leach 1992) and the "Chinese Clam" *Potamocorbula amurensis* Schrenck, introduced to California from Asia is causing much concern (reviewed by Carleton 1992). Earlier recorded transfers include the slipper limpet, *Crepidula fornicata* L., transported among oysters from North America to Europe around 1880 (Barrett and Yonge 1958). Ford (1992) has discussed the issue of transmitting disease during commercial mollusc culture, and Carriker (1992) has reviewed the risks which generally accompany the introduction and transfer of molluscan species. Surprisingly, no-one has yet addressed the

transfer of free-living microscopic planktonic organisms entrapped in and among the shells of wild or cultured molluscs.

The likelihood may seem remote that small quantities of shellfish spat, each measuring at most 10 mm shell height, could contain sufficient numbers of phytoplankton cells to inoculate a stretch of ocean and start a bloom: most of the toxic or nuisance species encountered in the Canadian Atlantic Provinces are widely distributed, albeit at relatively low abundance. Nevertheless, even one cell capable of surviving and dividing has the potential to seed a bloom. It was not known whether such cells could survive a period of several hours in the mantle cavity, or in the drip-water of consignments of shellfish transferred from places where toxic phytoplankton are abundant to places where they are not.

Transfer of scallop spat between Passamaquoddy Bay, New Brunswick and Mahone Bay, Nova Scotia has been conducted now for several years as part of a development program for giant scallop culture (Dadswell and Parsons 1992). Scallop spat have also been shipped from Port au Port Bay, Newfoundland, where local hydrographic conditions favour their collection, to other areas of Atlantic Canada for grow-out. On Prince Edward Island, mussel farmers have collected wild spat in St. Peters Bay for grow-out elsewhere and there is already some trade in mussel spat from Northern New Brunswick to other locations on the mainland. The issue is not likely to be different for hatchery-raised spat since most spend some time before shipment in nursery systems using natural water which may or may not be augmented with cultured algae.

Passamaquoddy Bay is routinely closed to the harvesting of shellfish due to the high levels of paralytic shellfish toxin caused by the dinoflagellate *Alexandrium tamarense*. In addition, the diatom *Nitzschia pungens* Grunow var. *multiseries* Hasle, responsible for domoic acid poisoning, has been recorded in St Peters Bay,

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although not in extreme bloom conditions (Worms et al. 1991). While these and other algal species associated with shellfish toxins have been recorded at many locations in the Canadian Atlantic, (Shelley Hancock pers comm, Carver et al. 1992) there is clearly some potential for introducing a species into an area where it has not already been recorded, particularly if the shipping and receiving waters are geographically remote.

This paper presents a brief preliminary overview of the issue:

1. by exploring the survival of *A. tamarensis* in scallop spat during simulated transfer conditions;
2. by examining the gut retention time of small mussels and scallops to determine how long undigested cells of *A. tamarensis* might remain in the gut, and
3. by determining whether consignments of spat can be purged of viable phytoplankton cells by exposure in recirculating, UV-irradiated seawater.

## METHODS

### Experimental Animals and *A. tamarensis* Culture

Scallop spat (*P. magellanicus*), ranging in size from 8.3–13.2 mm shell height, were obtained through Dr. André Mallet, from the hatchery at Sandy Cove, Halifax County, Nova Scotia, and wild from Mahone Bay, through Dr. M. J. Dadswell, Chester, N.S. Mussel spat (*Mytilus* sp.)† ranging between 11.7–17.0 mm shell length were collected from samples of small, cultivated mussels held at the Halifax laboratory. Spat were held on screens in a large holding tank in coarsely filtered seawater drawn from Halifax Harbour at 4°C, and 28‰ salinity.

*A. tamarensis* (strain OK 875-1, obtained from M. Kodama, Kitasato University, Japan) was grown in 20 l polycarbonate carboys using filtered, autoclaved natural seawater enriched with Harrison's enrichment solution, silicate omitted (Harrison et al. 1980). Cultures were grown at 18°C, 100–150  $\mu\text{E m}^{-2} \text{s}^{-1}$  irradiance. At the time of the experiment the culture was in late exponential phase with some settling beginning to occur, but with large numbers ( $6.7 \times 10^6$  cells  $\text{l}^{-1}$ ) of healthy, active cells present.

### Survival of *A. tamarensis* in Spat

Groups of 20 mussel and wild scallop spat were exposed separately to live *A. tamarensis* at a concentration not less than  $2.6 \times 10^5$  cells  $\text{l}^{-1}$  for a few hours, removed from the water, and stored moist in a styrofoam box at 1°C. After 6 h storage they were rinsed briefly in 100 ml filtered seawater. Samples of the rinse water, pseudofaeces, and faeces were examined at 40 and 100 $\times$ . Live *A. tamarensis* cells were observed both free swimming and enmeshed in the mucus of the pseudofaeces. Spat were then placed in 100 ml of seawater for 20 minutes to allow the mantle cavity to be thoroughly irrigated, and for accumulated faeces and pseudofaeces to be washed clear. This water was then filtered through a 10  $\mu\text{m}$  Nitex screen to collect any *A. tamarensis*, faeces and pseudofaeces which had been expelled. The filter screen was placed in 25 ml of sterile, nutrient enriched seawater and incubated at 18°C and irradiance of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ , photoperiod 16:8 h L:D.

†Two species of mussels, *M. edulis* and *M. trossulus*, co-exist on the Atlantic coast of Nova Scotia (Varvio et al. 1988). They can not be distinguished by morphological characteristics at the size used in these experiments. For convenience this study refers to all mussels used as *M. edulis*.

### Uptake of *A. tamarensis* and Gut Retention Time

Gut retention time is generally determined by use of marker particles which are fed to experimental animals in a discrete pulse, then quantified in faecal material. The time at which 90% of the ingested particles have accumulated in faecal material is generally considered to be the gut retention time (Bayne et al. 1987, Hawkins et al. 1990).

This study uses the green flagellate *Tetraselmis suecica* as a marker particle to estimate the gut retention time of bivalve spat feeding on *A. tamarensis*, which can be achieved by pigment analysis of their faeces. *T. suecica* contains chlorophylls *a* and *b*, whereas *A. tamarensis* contains chlorophylls *a* and *c*. By evaluating the amount of chlorophyll *b* (indicative of the material from the marker particles) vs the amount of chlorophyll *c* (indicative of material from *A. tamarensis*), gut retention time can be estimated. *T. suecica* was grown in a 10 l carboy under standard laboratory conditions and harvested at a concentration of  $1.3 \times 10^6$  cells  $\text{ml}^{-1}$ .

Two days prior to experimentation, scallop and mussel spat were transferred to a tank with unfiltered flow-through seawater at 12°C. Over the next 6 hours, water temperature was slowly raised to 16°C. One day prior to experimentation, spat were fed a mix of cultured *Chaetoceros gracilis* and *Isochrysis galbani* at a concentration of  $4.8 \times 10^5$  cells  $\text{ml}^{-1}$  for approximately 30 min.

The experimental apparatus consisted of 5 plastic trays ( $9.5 \times 72.5 \times 5$  cm) each filled with 2 l UV-irradiated seawater filtered to 1  $\mu\text{m}$ . Recirculation of water in each tray was achieved with an air-lift system. Temperature was maintained at 16°C.

### Uptake of *A. tamarensis*

Three hours prior to experimentation, mussel and scallop spat were transferred to the plastic trays. Spat were contained in baskets (6 cm  $\times$  6 cm  $\times$  6 cm) constructed from 2.0 mm woven plastic mesh. Five spat were placed in each basket, and 3 baskets were placed in each plastic tray for a total of 15 animals per tray. Two trays contained mussel spat, two contained scallop spat, and one remained empty of animals to serve as a control.

After the three-hour acclimation period, 500 ml of seawater in each tray was replaced with 500 ml *A. tamarensis* culture to a final density of approximately 1100 cells  $\text{ml}^{-1}$ . The concentration of *A. tamarensis* was monitored over an 8 hr period by analyzing a 20 ml sample of seawater from each tray on a Coulter Counter. To limit settling of *A. tamarensis* at the bottom of the trays, the seawater was gently agitated at hourly intervals.

Behaviour of individual mussel and scallop spat was also observed under a dissecting scope at 4 $\times$  magnification. Two to four spat were placed in a petri dish with 15°C seawater and allowed 30 min to acclimate. Several drops of *A. tamarensis* culture were then added to the petri dish to a final concentration of approximately 60 cells  $\text{ml}^{-1}$ . Observations were made over the following hour.

### Gut Retention Time

Following the uptake experiments, spat were left overnight in the recirculating trays to continue feeding on *A. tamarensis*. One hour prior to delivery of the *T. suecica* marker, 500 ml fresh *A. tamarensis* culture was added to each tray.

Introduction of the marker cells was recorded as time zero. Thirteen (13) ml *T. suecica* culture was added to each of the 4 trays containing spat (final concentration =  $8 \times 10^6$  cells  $\text{l}^{-1}$ , a cell volume roughly equivalent to that of the *A. tamarensis* cells).



Spat were allowed to feed on the mixture of *A. tamarensis* and *T. suecica* cells for 1 hour, removed from the baskets, rinsed gently in filtered, UV-irradiated seawater, and transferred to a second set of 4 plastic trays filled with 2 l of 16°C UV-irradiated seawater and 500 ml *A. tamarensis* culture. The experiment did not include a control tray of spat exposed to *A. tamarensis* but not to *T. suecica*. This control was considered unnecessary: the seawater in the plastic trays was filtered to 1 µm and UV-irradiated, so there could be no background source of chlorophyll *b*. Pigment analysis of the *A. tamarensis* culture showed no chlorophyll *b*. Any chlorophyll *b* in the fecal pellets must have originated from the *T. suecica* marker cells.

At regular intervals over the next 6 hours, samples of accumulated faeces were taken from each tray, and the bottom of the trays were siphoned clean of uncollected debris. Samples were frozen for subsequent pigment analysis by high performance liquid chromatography (HPLC). Pigments were extracted in 90% acetone and separated using a 10 minute gradient of 80% methanol, 15% Milli-Q water and 5% ion pairing solution to 70% methanol and 30% acetone on a Beckman HPLC column. The amount of *T. suecica* marker present in each sample was determined by the formula:

$$\frac{[\text{chlorophyll } b + \text{breakdown products}]}{[\text{chlorophyll } c + \text{breakdown products}]}$$

with the amount of each pigment type present in each sample being calculated from the area of the chromatogram peaks. Since chlorophyll is degraded in the digestive tract, any detectable breakdown products of chlorophyll *b* and *c* (including phaeophorbides, phaeophytins, and chlorophyllides) were included in the calculations. All peak identifications on the chromatograms were made through the use of pigment standards.

#### Depuration

Following the clear indication that *A. tamarensis* would survive up to 6 hours in and among both mussel and scallop seed stored in cool, moist conditions, it was appropriate to determine whether some purging system could be devised which would effectively remove living *A. tamarensis* from the seed before planting out. Traditionally, shellfish have been cleansed (depurated) of faecal coliform contamination in recirculated water sterilized with ozone, ultraviolet light, or chlorine followed by dechlorination, (Blogasowski 1991). Of these, ultraviolet light is the most convenient and was used here.

A small scale recirculation unit was assembled comprising a Trojan TS 6012 UV-sterilizing system (Trojan Technologies, London, Ont.), a 30 l bucket of filtered (1 µm) sea water (maintained at 7°C), and a small submersible pump. Approximately 500 hatchery-bred scallop seed (mean shell height 11.00 mm) were placed in a mesh container in a 120 l tank containing  $5 \times 10^5$  cells l<sup>-1</sup> *A. tamarensis* at 9°C. After 2 hours, the container was removed and allowed to drip for a few minutes to remove excess water. Approximately 50 spat were removed, rinsed in filtered sea water and the rinse water examined for living *A. tamarensis*. The remainder were suspended in the 30 l bucket filled with filtered sea water and the pump and UV light switched on. Samples of water were taken at intervals over the following 4 hours and examined for living cells at 40×.

To determine the survival of *A. tamarensis* in the UV-recirculation system, a fresh supply of filtered (1 µm) sea water

was inoculated with *A. tamarensis* culture to a final concentration of  $1.7 \times 10^5$  cells l<sup>-1</sup>, and the unit set running. Cell counts were made by Coulter Counter, and observations of viability were made with a microscope at intervals over the next 19.5 hours.

## RESULTS

### *Survival of A. tamarensis in Mussel and Scallop Spat*

Cultures of *A. tamarensis* recovered from spat were examined after 2 days. Live, swimming cells were observed in cultures from both the mussel and the scallop samples. After 4 days, live *A. tamarensis* cells were still visible in both cultures but showed no signs of growth. Large numbers of small, chain-forming diatoms, ciliated protozoa, a round-celled, non-motile green alga, and a green flagellate resembling *T. suecica*, were also present, likely derived from the natural flora associated with the spat, or possibly as minor contaminants of the original culture. Densities of *A. tamarensis* were estimated at around 10–20 cells ml<sup>-1</sup>. After 6 days, live *A. tamarensis* cells were observed in only the culture derived from the scallops. The culture from the mussels contained many other cells of various species and no dinoflagellates could be seen in a 200 µl drop. After 10 days no living *A. tamarensis* were visible in 1 ml samples from either culture, which had been completely taken over by the green flagellate. No other species were visible. Decaying pigmented debris which might have been derived from *A. tamarensis* was observed in both cultures.

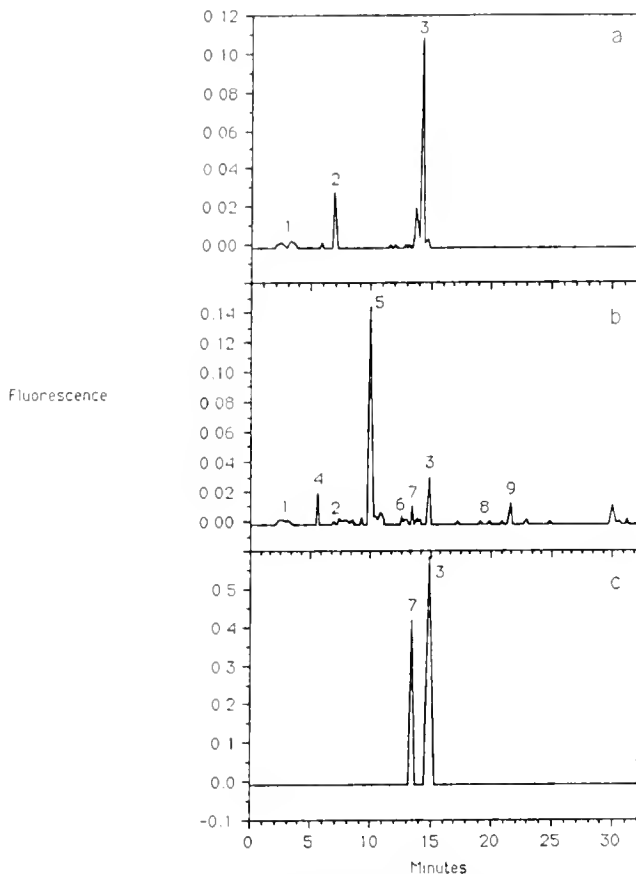
### *Uptake of A. tamarensis*

Microscope observations of the spat in petri dishes showed that mussel spat readily took *A. tamarensis* cells into the incurrent siphons and cleared the cells from suspension. Although many of these cells were rejected as pseudofaeces, pigment analysis of faecal pellets indicates that some cells were ingested and passed through the digestive system. The chromatogram of pigments extracted from *A. tamarensis* shows carotenes (Fig. 1a) which are also present in the pigments extracted from the mussel faeces (Fig. 1b). The chromatogram from *T. suecica* (Fig. 1c) shows neither of these pigment types. Scallop spat were not observed to be actively pumping when under the microscope, perhaps due to their size, or to light- or temperature stress. Scallops had been transferred from 4°C to 16°C only 2 days previously and may not have been fully acclimated.

Results from the Coulter Counter analysis of the uptake experiments clearly indicate uptake of *A. tamarensis* by mussel spat, but not by scallop spat (Fig. 2). The decrease in concentration of *A. tamarensis* in the scallop and control trays at 8 hours was likely due in part to settling of the cells, which were visible as a red streak across the bottoms of those trays. Cells may have also accumulated elsewhere in the apparatus.

### *Gut Retention Time*

The wild scallop spat did not produce any faeces during the course of the uptake experiment, so gut retention times were determined for mussel spat only. Both trays showed an initially high proportion of chlorophyll *b* in the faeces, followed by a sharp decrease after 2 hours (Fig. 3). The non-linear model  $y = \alpha K t e^{-Kt}$  (Bayne et al. 1984) was fitted to the data using an iterative least squares procedure. The time at which 90% of the chlorophyll *b* was passed through the mussels, the gut retention time, was then determined by integration of the curve. Gut retention time was cal-



**Figure 1.** Chromatograms of pigment content of: a: *Alexandrium tamarense*, b: faeces from mussels feeding on *A. tamarense*, and c: *Tetraselmis suecica*. Peaks were identified using standards as: 1-carotenes and xanthophylls, 2-chlorophyll c, 3-chlorophyll a, 4-chlorophyllide a, 5-phaeophorbide a, 6-phaeophorbide b, 7-chlorophyll b, 8-phaeophytin b, and 9-phaeophytin a.

culated to be 3.9 hr for sample tray 1 and 3.5 hr for sample tray 2. Furthermore, after 6 hr, 98.3% and 98.0% of the marker had passed through mussels in trays 1 and 2 respectively.

#### Depuration

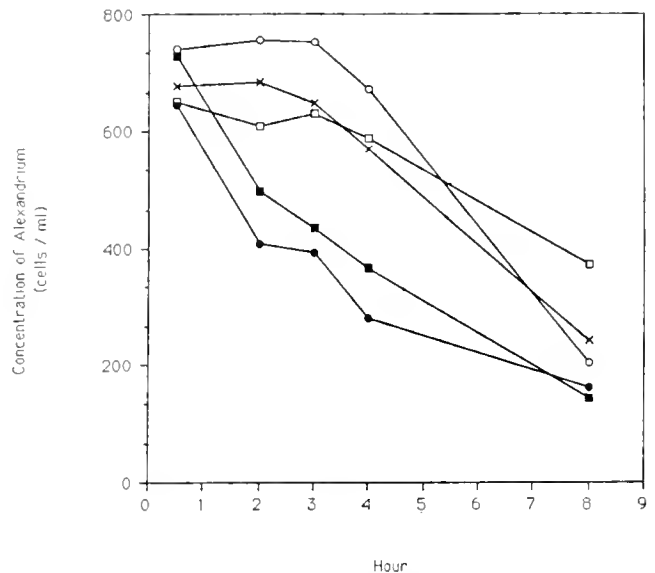
Five hundred hatchery-bred scallop spat feeding in the 120 l tank reduced the concentration of *A. tamarense* from 1500 to 40  $\text{ml}^{-1}$  over 2 hours, at which point they were transferred to the purging unit. However, rinse water from 50 spat examined at the time of transfer contained very few cells (ca.  $1 \cdot \text{ml}^{-1}$ ) indicating low concentrations of *A. tamarense* in the spat. After 2 and 4 hr circulation and irradiation, 200 ml water were filtered at 0.45  $\mu\text{m}$ , and resuspended in 1 ml water and examined at 40 $\times$ . No *A. tamarense* were seen.

Concentrations of *A. tamarense* in recirculating UV-irradiated water,  $1.73 \times 10^5 \text{ cells} \cdot \text{l}^{-1}$ , as determined by Coulter Counter, initially decreased, but later rose as cells began to fragment (Fig. 4). At time 3.25 hr a 200 ml sample of water was filtered and resuspended, and 100 cells examined; only 1/100 was motile. At 19.5 hr there were no living cells in a filtered 200 ml sample, all particles were cell fragments or otherwise broken and empty cells.

#### DISCUSSION

The major findings of this study are:

1. live *A. tamarense* can be recovered from spat of mussels

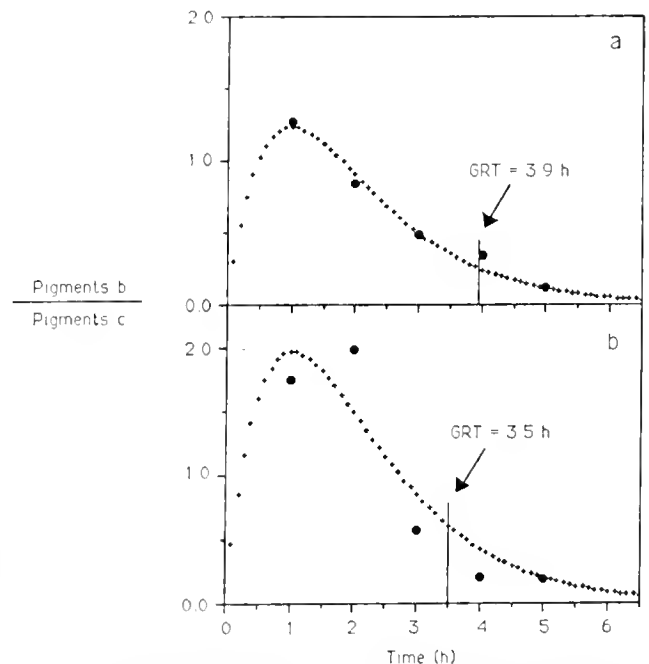


**Figure 2.** Removal of *Alexandrium tamarense* from feeding trays by scallop and mussels spat. —○— scallop tray 1, —□— scallop tray 2, —●— mussel tray 1, —■— mussel tray 2, —×— control tray.

and scallops after exposure to *A. tamarense* and storage in stimulated shipment conditions;

2. the gut retention time of small mussels under our laboratory conditions was relatively short (3.5–3.9 hr) with 98.0–98.3% of the gut marker being eliminated in the faeces within 6 hrs, and;
3. seawater contaminated with *A. tamarense* at a concentration of up to  $1.7 \times 10^5 \text{ cells} \cdot \text{l}^{-1}$  can be purified by UV irradiation in 6–12 hr.

Although live *A. tamarense* were recovered from rinse and drip



**Figure 3.** Observed • and predicted + values of the amount of organic marker present in faeces collected from mussels in tray 1 (fig. 2a) and tray 2 (fig. 2b). Gut retention times determined by integration of each curve were 3.9 and 3.5 hours respectively.

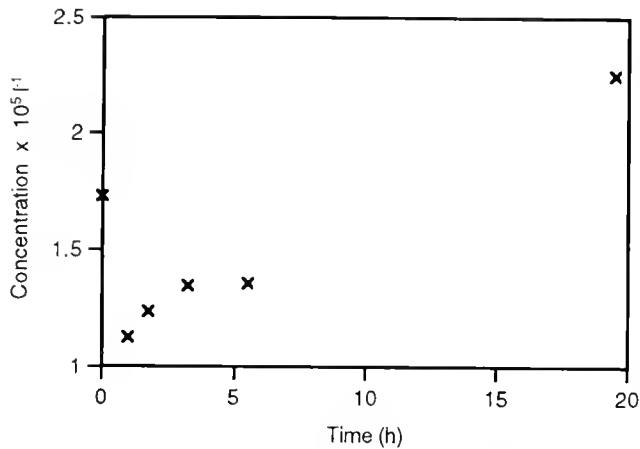


Figure 4. Coulter counts of *Alexandrium tamarense* cells in a recirculating depuration system.

water from the bivalve spat, they did not survive and reproduce in culture. The fact that *A. tamarense* did not survive in these cultures does not preclude their survival in the wild, nor in the laboratory under different conditions. These were small volume cultures which contained some vigorous competitors. It is speculated that many of the *A. tamarense* would be carried in water within the mantle cavity. Shellfish feeding on *A. tamarense* prior to harvest can release viable cells after transport. If water conditions were favourable, or if hypnozygote cysts were able to accumulate after several such transfer operations, a new bloom could be initiated.

Our second finding, that mussels have relatively short gut retention times under conditions similar to those of this study, indicates that depuration or purging procedures can be timed accordingly. It must be noted that gut retention time is highly variable (e.g. Bayne et al. 1984, 1987, 1988, 1989, Hawkins and Bayne 1984, Hawkins et al. 1990, Taghon 1981) and thus mussels or scallops kept under different conditions or collected at different times of the year could have different gut retention times. We suggest that 12 hours purging would be sufficient in most instances, although this should be verified using *A. tamarense* cysts. There is some evidence that bivalves react to an increased organic/inorganic ratio by increasing their feeding rate (Bayne, et al. 1984, 1988, Hawkins et al. 1990, Taghon 1981), thus it might be possible to shorten the time needed for purging.

It should be noted that in using the flagellate *T. suecica* as a marker for the dinoflagellate *A. tamarense*, a number of assumptions are made. Foremost of these is that the two particles are treated similarly in the digestive tract. It is possible that they are ingested at different rates, since *A. tamarense* is much larger (40 vs 10  $\mu\text{m}$  diameter), and likely has different chemical surface properties. However, differential ingestion rates of the two particles (i.e. pre-ingestive selection on ctenidia or labial palps) would not affect the accuracy of the technique provided some cells were indeed ingested.

If post-ingestive processing of the two particle types differs, the technique may become less accurate. Bricelj et al. 1984, have

shown that some bivalves can sort different algal species within the gut, eliminating less digestible species more rapidly. The digestibilities, and hence gut retention times, of the two species used here are not known, but given that both are hard-bodied algal species, it is reasonable to assume that *T. suecica* is treated more like *A. tamarense* than traditional marker particles such as bovine blood cells (Bayne et al. 1984), latex particles (Bricelj et al. 1984, Hummel 1985) or iron filings and plastic microtags (Jumars and Self 1986).

The technique also assumes the stability of chlorophyll *a* and *c* within the digestive tract are similar. In general one must use caution when using pigments as biogenic markers since they are chemically reactive (Hawkins et al. 1986, Hendry et al. 1987). The relative reactivity and stability of different pigments within the bivalve gut has not been studied extensively, but for the purposes of this investigation we have assumed that any differences in stability are insufficient to materially affect the principles discovered.

Our third finding is that holding transferred mollusc spat in UV irradiated, recirculated seawater is a potential means of purging them of toxic or nuisance phytoplankton. There is clear evidence among these experiments that scallops may not always pump and filter *A. tamarense* in experimental conditions. However, scallops do filter and eat toxic phytoplankton in the wild, witness the sometimes high levels of phycotoxins measured in them, and are therefore potential carriers of living phytoplankton between aquaculture sites.

The demands for top grade spat by shellfish growers are not unreasonable and are no different from those of any other farmer who wishes to access stock from proven sources when it is small and inexpensive, and grow it until it may be marketed profitably. Supplies of wild spat may not be easily available where a farmer wishes to operate, even though the species may already exist there in wild populations. This study clearly shows the potential for transferring living plankton with shellfish spat. While the numbers are likely less than would be transferred in ships' ballast, the fact that they will be delivered directly to shellfish farms, rather than industrial harbours adds an extra measure of concern. The risks of inadvertent transfer of toxic or nuisance phytoplankton can be minimized by some sort of purging regime, preferably before shipping. Living cells should be washed from the mantle cavities using recirculating seawater with appropriate, filters and UV-irradiation units. Results of this study suggest that 12 hours would likely be sufficient. Sludge retained in the holding tank should be sterilized before discharge if purging is conducted at the receiving site.

#### ACKNOWLEDGMENTS

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## ANATOMICAL DISTRIBUTION AND SPATIO-TEMPORAL VARIATION IN PARALYTIC SHELLFISH TOXIN COMPOSITION IN TWO BIVALVE SPECIES FROM THE GULF OF MAINE

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**ABSTRACT** Marine bivalve molluscs accumulate paralytic shellfish poisoning (PSP) toxins through filter-feeding on blooms of toxic dinoflagellates, specifically, *Alexandrium* spp. on the Atlantic coast of North America. To determine the seasonal variation in PSP toxin composition in various anatomical compartments, inshore and offshore populations of the sea scallop *Placopecten magellanicus* and the surfclam *Spisula solidissima*, two bivalve species noted for prolonged toxin retention, were sampled periodically over two consecutive years in the Gulf of Maine. Individuals were dissected into tissue fractions for the determination of toxin composition (molar% and nmol g<sup>-1</sup>) by high-performance liquid chromatography with fluorescence detection (HPLC-FD). The individual tissues included digestive gland, adductor muscle, gill and mantle, plus siphon and foot for clams and gonads for scallops. The calculated toxicity (µgSTXeq 100 g<sup>-1</sup> shellfish tissue) confirmed the distributional trend of parallel mouse bioassays performed upon the tissues, but did not match quantitatively the bioassay results over a seasonal time scale. Partitioning of PSP toxin components among various organs was markedly different for the two bivalve species. For both sea scallops and surfclams, substantial differences in the relative amounts of PSP toxins among tissue compartments and seasonal variation were more evident than were differences between geographical populations of the same species. Analysis of PSP toxin profiles from a representative isolate of *Alexandrium tamarense* from the Gulf of Maine supported previous findings that the toxin composition in bivalves may differ considerably from that of toxigenic dinoflagellates. A pronounced seasonal toxin shift from the less potent N-sulfocarbamoyl toxins (C1/C2), which dominate in the dinoflagellate, to higher toxicity carbamate derivatives (e.g., GTXs, NEO, and STX) was found in both bivalve species. Relative to sea scallops, surfclams have a much higher capacity for *in vivo* PSP toxin conversion to decarbamoyl analogues. Metabolic and physico-chemical mechanisms which may be involved in PSP toxin transformation are compared among bivalve species.

**KEY WORDS:** *Placopecten*, *Spisula*, PSP toxins, biotransformation, saxitoxin

### INTRODUCTION

The neurotoxins associated with paralytic shellfish poisoning (PSP) are among the most potent phycotoxins (toxins of algal origin) found in the marine environment. The accumulation of PSP toxins in suspension-feeding bivalves harvested in coastal zones constitutes a major public health risk to human consumers and has severely restricted the exploitation and development of both natural shellfish resources and aquaculture production. Although acute cases of PSP are relatively rare in advanced industrialized countries, due to the implementation of shellfish toxin monitoring programs and strict inspection procedures (Shumway et al. 1988, Cembella and Todd 1993), there have been few achievements in mitigating techniques to minimize toxin accumulation or to enhance detoxification of contaminated shellfish to levels below the acceptable regulatory limit (80 µgSTXeq [saxitoxin equivalents] 100g<sup>-1</sup>) adopted by many countries.

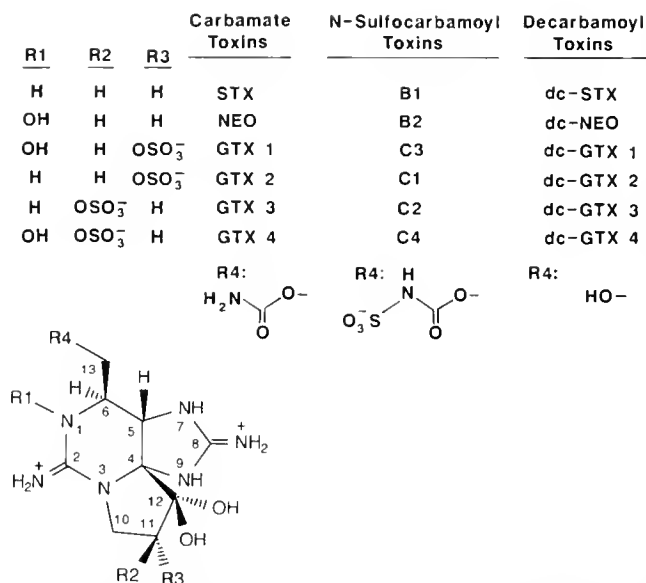
The shellfish toxicity associated with PSP has been a recurrent problem in the Gulf of Maine for several decades. As a result, a comprehensive shellfish toxicity monitoring program based upon the mouse bioassay procedure (AOAC 1984) has been in effect for nearshore waters of the coast of Maine since the 1970s (Shumway et al. 1988). A recent dramatic increase in PSP toxicity in key shellfish species from both the Canadian and American sectors of Georges Bank since 1989 (Watson-Wright et al. 1989, Shumway et al. 1993, White et al. 1993a), has seriously threatened the economic viability of the offshore 'roe-on' fishery for sea scallops,

*Placopecten magellanicus* and the harvest of whole surfclams, *Spisula solidissima*.

The present review incorporates recent evidence of PSP toxin accumulation and biotransformation in commercially-important natural populations of sea scallops and surfclams from the Gulf of Maine. The distribution of PSP toxins in inshore and offshore sea scallops in Maine coastal waters was compared with surfclams from an inshore site and populations from Georges Bank (40–43°N 66–70°W). Where relevant, comparative data on anatomical distribution and spatio-temporal variation in PSP toxin composition in other bivalve molluscs have been considered. Expressly excluded are detailed discussions of PSP toxin uptake kinetics, detoxification rates, resistance to deleterious effects of accumulated toxin, and other species-specific physiological responses to toxin exposure.

### PSP Toxins in Toxigenic Dinoflagellates

The PSP toxins comprise a suite of at least 18 naturally-occurring neurotoxic tetrahydropurine derivatives produced among several species of free-living planktonic marine dinoflagellates, including *Alexandrium* spp., *Pyrodinium bahamense* var. *compressum*, and *Gymnodinium catenatum* (Hall and Reichardt 1984, Taylor 1984, Oshima et al. 1990) (Fig. 1). These PSP toxin derivatives can be classified according to their chemical structure and specific potency in mammals (as sodium channel blocking agents); the carbamate toxins (GTX1-GTX4, NEO, STX) are the



**Figure 1.** Structures of PSP toxins found in toxigenic dinoflagellates and shellfish, which include carbamate, N-sulfocarbamoyl, and decarbamoyl derivatives. Saxitoxin = STX; neosaxitoxin = NEO; gonyaulaxins 1,2,3,4 = GTX1,2,3,4; dc- = decarbamoyl analogues.

most potent, whereas the N-sulfocarbamoyl derivatives (B1, B2, C1–C4) have a much lower specific toxicity. The decarbamoyl (dc-) analogues, of intermediate toxicity, are generally less abundant in toxigenic dinoflagellates, particularly *Alexandrium* spp., but they may be important toxin components in certain bivalve species (Sullivan et al. 1983).

No natural toxigenic dinoflagellate population or individual isolate in culture has been found to contain all naturally occurring PSP toxin analogues, and non-toxic variants of indistinguishable morphotypes are often found. In toxigenic varieties, a rather complex spectrum of PSP toxin derivatives may be produced at physiological equilibrium during exponential growth phase. In the absence of environmental stress, the toxin profile of *Alexandrium* cells is considered to be characteristic of the strain (Cembella et al. 1987, Anderson 1990). This conservative toxin profile (presumably fixed genetically) has been employed with some success to define geographical populations (Cembella et al. 1987, Oshima et al. 1989).

#### *Alexandrium* spp. Associated with PSP Toxicity in the Gulf of Maine

At the generic level, the organism(s) responsible for PSP toxicity in shellfish along the Atlantic coast of North America is no longer a matter of dispute. The taxonomic history of marine dinoflagellates implicated in PSP toxicity in this region is, however, convoluted and requires further clarification. The species causing PSP toxicity in the lower estuary and Gulf of St. Lawrence and in the Bay of Fundy was identified originally as *Gonyaulax tamarensis* (Needler et al. 1949), according to a description of this species from the type locality near Plymouth, UK. Later attempts to differentiate populations from eastern North America into varieties *sensu* Braarud (1945) (i.e., var. *excavata* versus var. *tamarensis*) proved ultimately to be unconvincing. After these varieties were elevated to species, subsequent alteration of the descriptions to include characters such as toxicity, bioluminescence and the presence of a ventral pore were used to redefine the species

(Loeblich and Loeblich 1975). Thus, the New England red-tide species was assigned to *G. excavata*. With the eventual recognition that these toxigenic species were not "true" *Gonyaulax* (reviewed by Taylor 1984), several alternative generic solutions (*Protogonyaulax*, *Gessnerium*, or *Alexandrium*) were proposed. Regardless of the respective merits of these morphotaxonomic treatments, now accompanied by biochemical and molecular genetic data (Scholin et al. 1993), it is clear the PSP toxicity in eastern North American waters can be attributed to species referable to the genus *Alexandrium* (Halim) emend Balech (1990), principally *A. excavata*, *A. tamarensis*, and *A. fundyense*.

Blooms of *Alexandrium* spp. associated with PSP toxicity are recurrent events along the coast of eastern North America, generally following the annual vernal warming (Hurst and Yentsch 1981), but populations tend to be sub-surface and visible water discolorations ("red-tides") are rarely (if ever) observed. Bloom initiation, development and dispersion appear to be largely driven by hydrodynamic factors involved in tidal mixing, upwelling, density stratification and longshore currents arising from geostrophic flow (Franks and Anderson 1992). In the Gulf of Maine, the respective contribution to PSP toxicity in shellfish attributable to localized blooms versus longshore transport of toxic vegetative cells remains to be established.

In any case, even cryptic *Alexandrium* blooms are capable of causing high toxicity levels in inter-tidal and neritic populations of bivalve shellfish, including clams, mussels, oysters, and scallops, in Maine coastal waters (Shumway et al. 1988). In a comprehensive review of PSP toxicity in scallops, Shumway and Cembella (1993) cited levels as high as 150,000 µgSTXeq 100 g<sup>-1</sup> in scallop digestive glands from the Bay of Fundy in eastern Canada, where *A. fundyense* is considered to be the source of the toxicity. The New England coastline is subject to periods of intense annual PSP toxicity in shoreline molluscs, with a gradual diminution in maximum toxicity, frequency and duration in toxic events towards the south. In the aftermath of the catastrophic meteorological events associated with Hurricane Carrie in 1972, apparently resulting in a major bloom dispersion, PSP toxicity has become endemic in Massachusetts, albeit at a generally reduced intensity since the original episode. There is some evidence that the net toxicity per cell in *Alexandrium* populations tends to decline from north to south along a latitudinal gradient, resulting from a shift in the toxin composition and a decrease in the amount of PSP toxin per cell (Maranda et al. 1985).

The exact identity and population dynamics of the organism responsible for this offshore toxicity on Georges Bank are currently unknown. A recent net sample from Georges Bank yielded cells of *Alexandrium tamarensis* (Shumway et al. 1993), a plausible candidate species as the cause of PSP toxicity in this region. However, the presence of *Alexandrium* spp. in the gut contents of surf clams from Georges Bank was not confirmed.

#### Net Accumulation of PSP Toxicity

Time-series data from shellfish toxin monitoring programs based upon the AOAC (1984) mouse bioassay have indicated both geographical and seasonal variation in net PSP toxicity among diverse bivalve species (reviewed by Quayle 1969, Prakash et al. 1971, Shumway et al. 1988, 1993, Shumway and Cembella 1993). Specifically, in sea scallops from the Gulf of Maine, wide seasonal fluctuations in toxicity have been reported (Bourne 1965, Jamieson and Chandler 1983, Watson-Wright et al. 1989, Gillis et al. 1991),

occasionally with the appearance of fall and winter maxima. All bivalve species known to accumulate PSP toxins exhibit marked differences in the distribution of toxicity among the various organs (Prakash et al. 1971, Blogoslawski and Stewart 1978, Maruyama et al. 1983). As PSP toxins are released after digestion of toxic cells in the viscera, the digestive system is invariably found to contain the highest toxicity levels immediately following exposure to toxic algal blooms. However, the kinetics of PSP toxin elimination and the sequestration of toxin in other organs follow a characteristic pattern in each bivalve species (reviewed by Shumway and Cembella 1993 for scallops). Both sea scallops (Medcof et al. 1947, Prakash et al. 1971, Jamieson and Chandler, 1983) and surfclams (White et al. 1993a, Shumway et al. 1993) are capable of prolonged retention of PSP toxins, thus they are suitable candidate species for comparative studies of long term changes in PSP toxicity and toxin composition.

Seasonal partitioning of PSP toxicity in various anatomical compartments was compared for individual tissues of adult surfclams from an inshore site at Head Beach, ME and offshore stations on Georges Banks (1990–91), and for sea scallops from inshore (20 m depth) and offshore (180 m depth) stations in the Gulf of Maine near Boothbay Harbor (1988–89) (Fig. 2). Tissues selected for both species included adductor muscle, mantle (rims), digestive gland (viscera) and gill. For scallops, gonads were dissected and analyzed separately; the prominent foot and the distal extension of the mantle (siphon) were analyzed as separate tissues for surfclams. Toxicity was determined by the mouse bioassay (AOAC 1984). The tissues of randomly selected individuals of surfclams ( $n = 6$ ) and sea scallops ( $n = 8$ ) were pooled for homogenization in 0.1 M HCl, followed by heating at 100°C (5 min), pH adjustment to 3.5–3.7, and centrifugation to clarify the supernatant. After intraperitoneal injection of 1 mL of tissue extract into adult white mice ( $n = 3$ ), toxicity was determined by interpolation of mouse death time within 15 min from the calibrated dose response table prepared by injection of purified STX.

For comparison with the mouse bioassay, two alternative methods of high-performance liquid chromatography with fluorescence

detection (HPLC-FD) (Sullivan and Wekell 1986, Oshima et al. 1989) were applied to toxin extracts of tissues from the same sites in the Gulf of Maine. The analytical methods were optimized to preserve the native toxin composition in the tissues by extraction in 0.1 M acetic acid without heating (Bricelj et al. 1990, 1991, Cembella et al. 1993). Net toxicity (in  $\mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) was calculated from toxin concentrations (in  $\mu\text{mol l}^{-1}$ ) measured by HPLC, based upon specific toxicity values (in  $\mu\text{gSTXeq } \mu\text{mol}^{-1}$ ) (Fig. 3) determined empirically from mouse bioassay calibration data using purified toxins (Sullivan et al. 1985, Oshima 1992). For scallop tissues, the 11-hydroxysulfate toxins GTX1 and GTX4 were combined for data analysis due to inconsistent epimerization.

The calculated toxicity results determined by HPLC generally reflected the toxicity trend of the corresponding mouse bioassays for populations of both bivalve species, although the bioassay values were usually substantially higher (Fig. 4). For both species, the summer toxicity peaks in digestive glands, which are inferred to indicate the occurrence of toxic blooms, were more pronounced in the bioassay results than for the HPLC-FD data. The mouse bioassays indicated as much as three-fold higher toxicity in surfclam digestive glands than the HPLC method and differences for inshore sea scallops were often even more dramatic. Since previous validations of the HPLC-FD method (Sullivan et al. 1985, Sullivan and Wekell 1986, Martin et al. 1990) have shown good correlations ( $r^2 \geq 0.9$ ) with the mouse bioassay when performed on extracts prepared according to the AOAC (1984) protocol, it is likely that the discrepancy was due primarily to differences in sample preparation.

In the AOAC (1984) bioassay procedure, extraction of toxins with hot 0.1 M HCl tends to increase net toxicity (known as Proctor enhancement) due to indeterminate hydrolysis of low potency N-sulfocarbamoyl toxins (C1–C4) to their GTX analogues (Fig. 1); some degradation of the low toxicity components B1 and B2 to the non-sulfated carbamate toxins STX and NEO, respectively, can also occur, resulting in increased toxicity (Hall and Reichardt 1984, Boyer et al. 1986) (Fig. 3). A chemically-induced shift in the ratios of  $\alpha$ - $\beta$ -epimers of the C-11 sulfated derivatives (GTX2/GTX3; GTX1/GTX4) is also expected, although this would have little effect on net toxicity. The efforts to avoid artificial toxin conversion in the extraction procedure for HPLC-FD

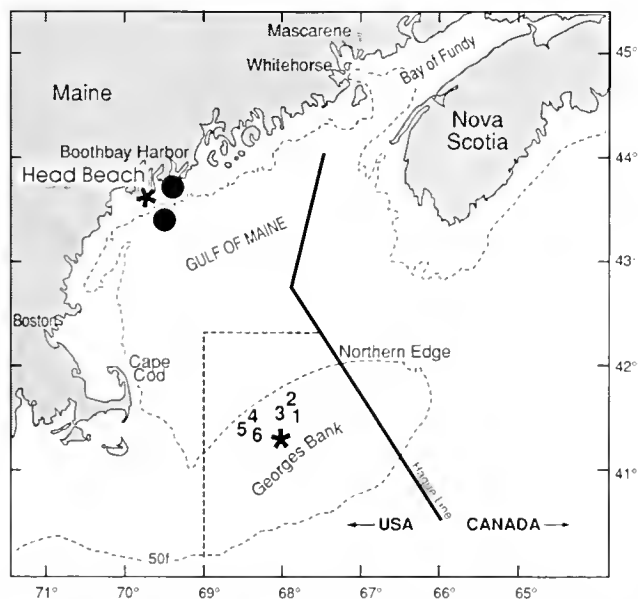


Figure 2. Map of primary sampling sites for sea scallops ● and surfclams \* in the Gulf of Maine.

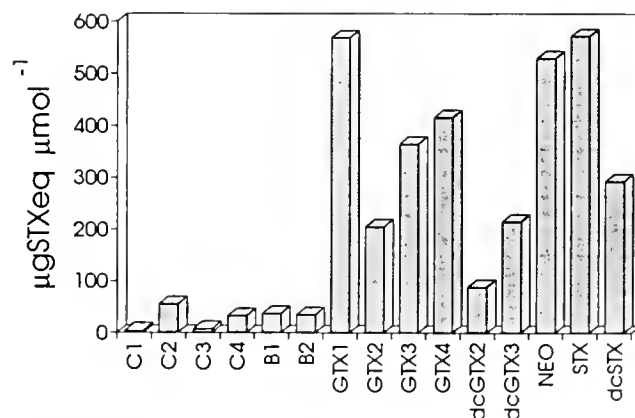
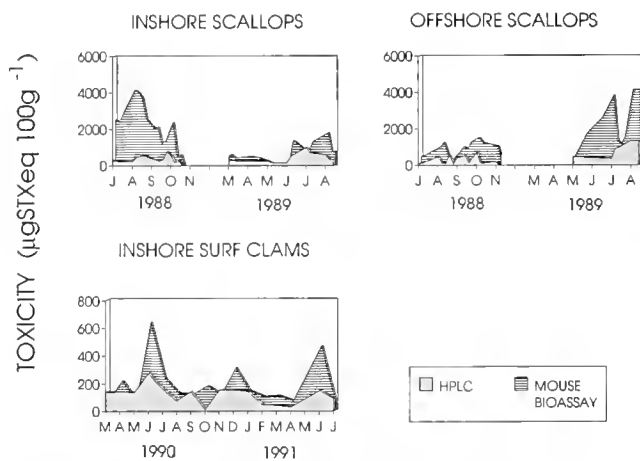


Figure 3. PSP toxin conversion factors for the calculation of specific toxicity ( $\mu\text{gSTXeq } \mu\text{mol}^{-1}$ ) based upon values determined empirically by mouse bioassays (mouse units [M.U.]  $\mu\text{mol}^{-1}$ ) (Oshima 1992), assuming 1 M.U. = 0.23  $\mu\text{gSTXeq}$ . The factor for B2 was calculated from a value given by Sullivan et al. (1985) in M.U.  $\mu\text{mol}^{-1}$ .





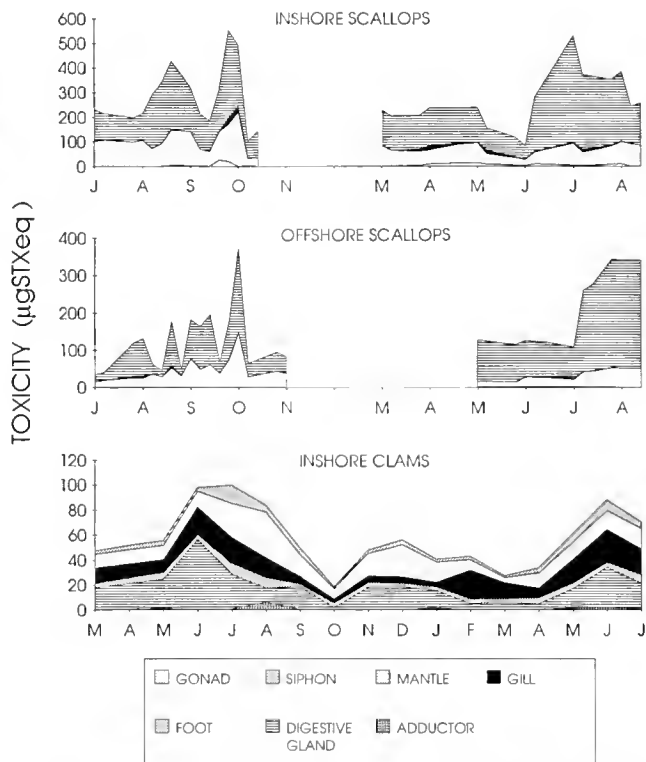
**Figure 4A,B.** Comparison of mean seasonal variation ( $n = 8$ ) in toxicity ( $\mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) in digestive glands of offshore and inshore scallops (A) and inshore surf clams from Head Beach (B), determined by AOAC mouse bioassay and calculated from HPLC-FD chromatograms using toxin specific conversion factors ( $\mu\text{gSTXeq } \mu\text{mol}^{-1}$ ).

analysis thus yield values representing "actual" toxicity rather than an approximation of "potential" toxicity. This explanation for the difference in net toxicity as determined by these alternative methods was supported further by the fact that the greatest discrepancies were almost invariably found during summer toxicity peaks when the relative contribution of the N-sulfocarbamoyl toxins (particularly C1/C2) to the total toxin body burden was at maximum, apparently indicating the recent ingestion of dinoflagellates rich in these derivatives.

On a weight-normalized basis ( $\mu\text{gSTXeq } 100 \text{ g}^{-1}$ ), toxicity in digestive glands from sea scallops was often much higher than in surfclams from the inshore site, according to the mouse bioassay data (Fig. 4). However, it is unwise to attribute much validity to this comparison, as the sampling dates did not overlap and the inshore sites for each species were in close proximity but not identical. Both analytical techniques revealed a winter peak in toxicity in inshore surfclams during November to February, which is difficult to explain in terms of conventional toxic bloom dynamics. Unfortunately, no samples of sea scallops were available during the winter from the Gulf of Maine to prove whether or not toxicity was present throughout the winter months, when toxic *Alexandrium* blooms are not expected to occur. Nevertheless, the substantial (although declining) body burden of toxicity in both scallop populations in the late fall, prior to the suspension of sampling for the winter, and elevated toxicity ( $>200 \mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) found in early spring when sampling was resumed (Fig. 5), offers circumstantial evidence that considerable toxicity persisted in digestive gland and mantle tissue during the winter.

This is consistent with mouse bioassay data acquired from 1985–87 for combined fractions (digestive gland, mantle, and gill) of sea scallops from Gulf of Maine sites (Shumway et al. 1988). Whereas high toxicity levels were maintained throughout the year in the offshore zone, the peak toxicity in inshore scallops occurred during early summer, with persistent toxicity extending into the fall and winter. Moreover, peak toxicity was reported previously to occur in scallops from the Bay of Fundy during fall and winter, in the apparent absence of toxic blooms (Bourne 1965, Jamieson and Chandler 1983).

In the present study, the general pattern of toxicity among



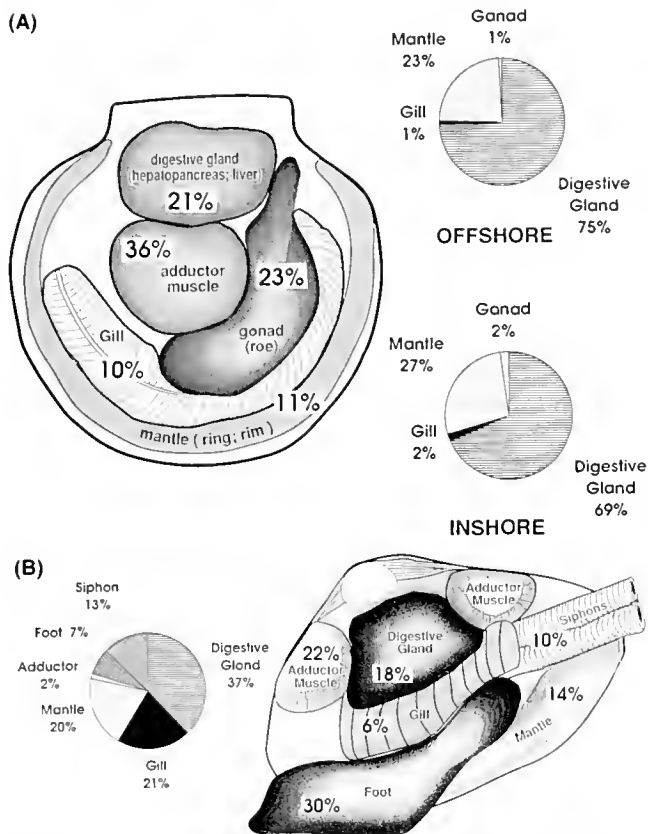
**Figure 5.** Seasonal variation in mean toxin burden ( $\mu\text{gSTXeq}$  per individual) in tissues of sea scallops (1988–89) and surfclams (1990–91) from the Gulf of Maine, calculated from HPLC-FD values.

various tissues, as determined by the HPLC-FD method, essentially substantiated that found previously for natural populations of both sea scallops (reviewed by Shumway et al. 1988 and Shumway and Cembella 1993) and surfclams (Shumway et al. 1993) using the AOAC mouse bioassay. For sea scallops, the typical rank order of toxicity burden ( $\mu\text{gSTXeq}$ ) throughout the year was as follows: digestive gland  $>$  mantle  $\geq$  gill  $>$  gonad  $\geq$  adductor muscle (Fig. 5), although mantles were briefly more toxic on a weight-normalized basis ( $\mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) than digestive glands during the post-bloom period in the fall. This toxicity hierarchy was supported by the corresponding mouse bioassay data for individual tissues (not shown), albeit that toxicity values for gills, gonads, and adductor muscles remained consistently below the bioassay detection limit ( $<58 \mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) throughout the two-year sampling period, except for a brief toxicity peak (maximum:  $426 \mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) in gonads from the offshore population in the summer of 1989.

For sea scallop populations, rapid increases in toxicity burden in digestive glands were usually accompanied by concomitant, but less dramatic, increases in toxicity in other organs, particularly in mantles (Fig. 5). The prominent rise in toxicity in digestive glands in the summer of 1989 was delayed by a month in the offshore zone, relative to the inshore population. The scallop populations exhibited a peak in toxicity of similar magnitude in all tissues during the fall of 1988.

During summer toxicity peaks, the distribution of PSP toxins among various organs was strikingly similar for inshore and offshore scallops; in excess of 95% of the total toxin load (nmol per individual organ) was partitioned into the digestive gland plus mantle tissues (Fig. 6). As expected, the relative contribution of





**Figure 6.** Schematic diagrams of soft tissue components of scallops (A) and surfclams (B) indicating the relative contribution (%) of each tissue to total body weight of inshore specimens during the summer. Adjacent pie charts indicate the relative toxin load (%) as a portion of total body toxin burden (nmol per individual) retained in each tissue averaged during peak toxicity periods in the Gulf of Maine. Adductor muscle toxicity for scallops is not shown, as levels were very low (<0.5 nmol g<sup>-1</sup>) and were not consistently detected.

digestive glands to total toxicity in scallops was maximized during the high toxicity periods in summer (Fig. 5), presumably due to short-term accumulation of toxic cells in the viscera. The brief inversion in weight-specific toxicity observed in the fall, with mantles appearing to be more toxic than digestive glands, was not reflected in the body burden calculation since the viscera formed a greater fraction of total body weight than mantles (Fig. 6).

As determined by HPLC-FD, the toxicity levels in surfclams collected offshore at stations on Georges Bank (Fig. 2) were generally higher than at the inshore Head Beach, ME site, confirming the results of previous mouse bioassays (Shumway et al. 1993). The pattern of toxicity at offshore stations was essentially similar to the inshore site and thus is not presented graphically. As in sea scallops, an increase in toxicity in the viscera of surfclams was accompanied by a prompt rise in total toxicity in other organs, but the seasonal distribution of toxicity among organs was quite different. In surfclams, the pattern of toxicity at the inshore site indicated a biphasic peak in digestive glands in the spring to fall of 1990, followed by high levels maintained throughout the winter, and a subsequent rise in the spring and early summer of 1991. The viscera constituted the most significant toxic component (as much as 50% of the body burden of toxicity at the peak) during the late spring to fall (Fig. 5). During this time, mantle and gill tissues

were approximately equal sub-dominant contributors to total toxicity, when calculated on the basis of toxin burden (µgSTXeq per individual organ) (Fig. 5 and 6). The tendency for bimodal toxin peaks in summer was less pronounced in other surfclam tissues. In fact, with the possible exception of the gills, toxin concentration maxima in other anatomical compartments occurred several weeks after the initial toxin increase in the digestive gland, when toxin concentration in the latter organ was actually declining. The precipitous decline in total toxin concentration in the digestive gland to levels below 0.5 nmol g<sup>-1</sup> in the fall was accompanied by a prolonged shift in the relative order of tissue toxicity such that gill and mantle tissues combined were more toxic than the viscera. During early winter, toxin burden in mantles surpassed that in the viscera, and in late winter and early spring the gills became the most toxic organ. The foot and siphon contained consistently less total toxicity than any of these tissues.

This distributional rank order of toxicity among surfclam tissues followed that found previously in other clam species. In feeding experiments using a high toxicity *Alexandrium* isolate from New England, Bricelj et al. (1991) found that at the peak of toxin accumulation, the viscera comprised 29% of the total soft tissue weight and accounted for >78% of total body burden of toxicity in the northern quahog *Mercenaria mercenaria*. In natural softshell clam populations from the Bay of Fundy, digestive gland toxicity was much higher than that in gills and gonads during toxic *Alexandrium* blooms, whereas during periods when blooms were absent, gills were approximately as toxic as digestive glands (Martin et al. 1990). In the *Spisula solidissima* detoxification experiments of Blogoslawski and Stewart (1978), the relative distribution of toxicity (mantle = gill > viscera > siphon > foot ≫ adductor) undoubtedly reflects the two month lag period between the occurrence of the toxic *Alexandrium* bloom and the subsequent harvest of the clams from the field, and an additional one month post-harvest depuration before mouse bioassays were performed.

The extended toxin retention characteristic of surfclams results in high levels of PSP toxins which are maintained throughout the winter and perhaps even sequestered cumulatively for years. The peak in winter toxicity in the Gulf of Maine, as evidenced by mouse bioassay data, remains, nevertheless, to be explained. This phenomenon cannot be attributed solely to bioconversion of toxins to more potent derivatives, since the HPLC-FD results showed a clear rise in total PSP toxin concentration during this period.

Although the peak concentration of PSP toxins (nmol g<sup>-1</sup>) in offshore scallop gonads was approximately twice that of their inshore counterparts, the much higher gonadal weight in inshore specimens (up to 10-fold) contributed more to total body toxin burden (Fig. 6). This is consistent with previous observations that gonads from deep water scallop populations from the Gulf of Maine are poorly developed and exhibit low fecundity (Barber et al. 1988, Schick et al. 1992). During peak toxicity periods, total toxicity in gonads was approximately equivalent to that in gills (Fig. 6), due to the larger relative contribution of gonads to total body weight of soft tissues. Attempts to establish a predictive index of toxicity in gonads by linear correlation with digestive gland toxicity were unsuccessful (Fig. 7), as also noted by Watson-Wright et al. (1989). Significant toxicity only occurred in gonads when levels in adjacent digestive glands were high, indicating an inefficient transfer of PSP toxins into reproductive tissues. No definitive quantitative relationship could be established with toxin levels in any other tissue compartment. This caveat against the use of such toxicity indices to infer PSP risk to public

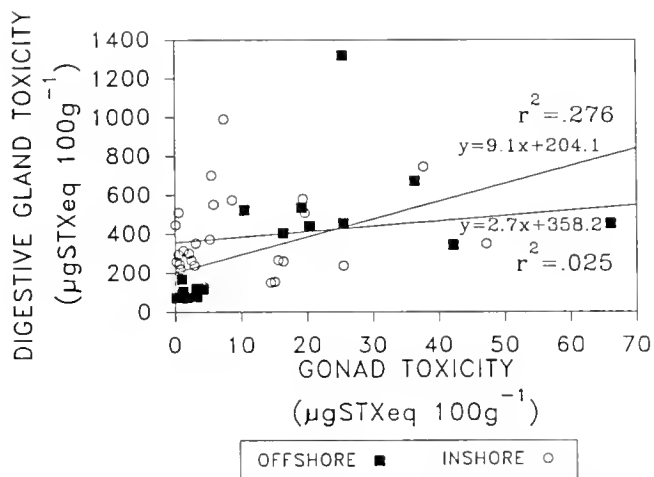


Figure 7. Linear correlation of PSP toxicity ( $\mu\text{gSTXeq } 100 \text{ g}^{-1}$ ) in digestive glands and gonads of inshore and offshore scallops from the Gulf of Maine, as determined by HPLC-FD.

health for the marketing of 'roe-on' scallops has been underscored in a previous review (Shumway and Cembella 1993).

Scallop adductor muscles were virtually free of PSP toxicity throughout the sampling period. A few specimens contained low amounts of PSP toxins ( $<0.5 \text{ nmol g}^{-1}$ ), particularly during the summer peaks in digestive gland toxicity. Toxicity levels never exceeded the regulatory limit ( $80 \mu\text{gSTXeq } 100 \text{ g}^{-1}$  shellfish tissue) and remained consistently undetectable by mouse bioassay. Adductor muscle toxicity never constituted more than 1% of the total body toxin burden, as confirmed by the HPLC-FD technique (Cembella et al. 1993). The reported decrease in net toxin content following incubation of PSP toxin fraction with adductor muscle homogenates (Shimizu and Yoshioka 1981) indicated the possibility of an active detoxification mechanism. In spite of a few reports of high toxicity in scallop adductor muscles (cited in Shumway and Cembella 1993), the risk of human PSP intoxication due to the consumption of adductor muscles which have been carefully dissected to avoid contamination by adjacent visceral tissues appears to be very remote.

For surfclams, toxin burden in adductor muscles was a significant portion of total body burden (up to 5%) only when total toxicity was declining from the summer maximum. According to the corresponding mouse bioassay data from Head Beach, the toxicity of adductor muscles and foot tissues never exceeded  $80 \mu\text{gSTXeq } 100 \text{ g}^{-1}$  throughout the sampling period (Shumway et al. 1993). Nevertheless, the fact that PSP toxins accumulate in adductor muscles of surf clams more readily than in those of scallops suggests that caution should be exercised in marketing this tissue.

#### Interspecific Differences in PSP Toxin Composition

Post-ingestion shifts in PSP toxin composition can be used to evaluate species-specific differences in toxin metabolism and elimination kinetics. Immediately following the ingestion of toxic cells, the PSP toxin spectrum in bivalves, particularly in the viscera, tends to reflect that of the toxigenic organism and thus can help to identify the source of the toxicity. If the species-specific nature of subsequent toxin bioconversions and elimination kinetics can be established based upon qualitative and quantitative PSP

toxin data, this information may also be useful in hindcasting toxic bloom events. Unfortunately, with a few notable exceptions (e.g., Chebib et al. 1993), corresponding data on toxin profiles and dinoflagellate bloom dynamics are largely absent from the literature.

Differences in PSP toxin composition between bivalve shellfish and ingested toxigenic dinoflagellates could arise via two alternative (but not mutually exclusive) mechanisms: 1) selective retention/elimination of specific toxins, and 2) biotransformations among toxin components within tissues. In practice, these mechanisms are difficult to distinguish as they may operate simultaneously, possibly even shifting toxin ratios in opposite ways. Toxin conversions may be mediated by a variety of biochemical and physico-chemical (e.g. pH, temperature) mechanisms. A schematic representation of PSP toxin conversions proposed for *in vitro* tissue homogenates and pure toxins under specified conditions is offered in Fig. 8.

Most of the arguments for selective binding of toxins in particular tissues, i.e., the high levels of STX associated with the siphons of butter clams, *Saxidomus giganteus* (Price and Lee 1971), are not based upon time-series data. The *in vivo* binding constants for specific PSP toxins in bivalve tissues have yet to be compared. The clearest evidence for biotransformation as opposed to selective retention is the *de novo* appearance of a toxin component in shellfish which was not present in the toxigenic dinoflagellate. Other inferential evidence of biotransformation would include an increase in the total body burden of a given toxin

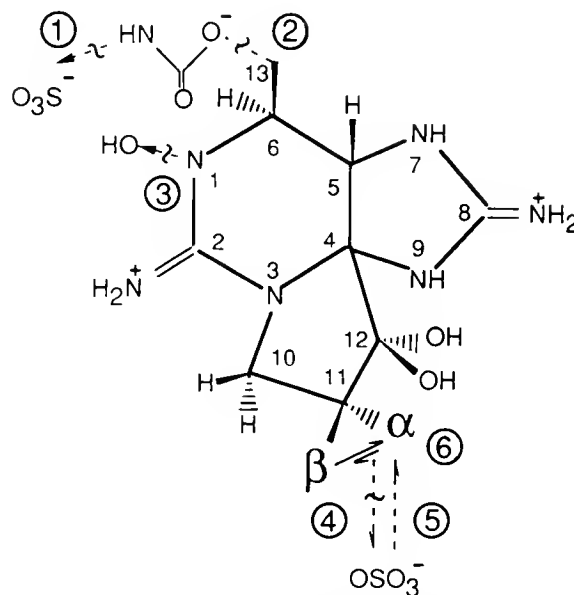


Figure 8. Schematic diagram of putative PSP toxin transformations in marine bivalve molluscs including conditions for their catalysis adapted from literature reports (Shimizu and Yoshioka 1981, Sullivan et al. 1983, Kotaki et al. 1985, Oshima 1992, 1993) and new empirical data (M.V. Laycock, N. Ross and A. D. Cembella). 1) low pH + heat or strong acid without heat; 'sulfatase' enzyme(?); amine N-sulfotransferase; 2) neutral pH + heat or strong acid + heat; 'carbamoylase' enzyme; 3) reductants (DTT, mercaptoETOH, glutathione, cysteine); marine bacteria; oxidoreductase enzyme(?); 4) reductants (DTT, mercaptoETOH, glutathione, cysteine); marine bacteria; 'sulfatase' enzyme(?); sulfohydrolase; 5) transferase enzyme(?); O-sulfotransferase via OH<sup>-</sup> intermediate; 6) epimerization at neutral or slightly acid pH.

while levels of other components are decreasing during detoxification. As catabolic processes are more likely to occur than elaboration of toxin structures during digestion, any apparent catabolic shift in toxin ratios, e.g. a decrease in the ratio of N-sulfocarbamoyl:carbamate toxins, would suggest that biotransformation is dominant over toxin accumulation.

Toxin conversion can lead to a net increase in PSP toxicity even as the total body toxin burden is dropping, making comprehensive modelling of toxin dynamics a daunting task. Early work on putative biotransformation of PSP toxins in bivalves was based upon discrepancies between the toxic fractions produced by cultured *Alexandrium* isolates and those found in PSP toxin-contaminated bivalves, such as softshell clams (Shimizu et al. 1975), butter clams (Oshima et al. 1977), and sea scallops (Oshima et al. 1977, Boyer 1980, Fix Wichmann et al. 1981) from the same geographical area. Toxin fractions were resolved by ion-exchange and thin layer-chromatography methods which are at best only semi-quantitative. In retrospect, even the qualitative results may have been compromised by inadvertent chemical conversions resulting from relatively harsh toxin extraction, the lack of purified reference toxins, and inadequate knowledge of the properties of the N-sulfocarbamoyl toxins.

Among analytical techniques capable of resolving and quantifying the individual toxin components in shellfish (Sullivan and Wekell 1986, Oshima et al. 1989) and toxigenic dinoflagellates (Cembella et al. 1987, Oshima et al. 1989), HPLC-FD has provided detailed insights into toxin kinetics and biotransformation in a number of bivalve molluscs. These species include blue mussels (*Mytilus edulis*), northern quahogs (*Mercentaria mercenaria*), European oysters (*Crassostrea gigas*), scallops (*Pecten maximus*), Manila clams (*Ruditapes philippinarum*), littleneck clams (*Protothaca staminea*), and butter clams (*Saxidomus giganteus*) (Sullivan 1982, Sullivan et al. 1983, Lassus et al. 1989, 1992, Bricelj et al. 1990, 1991, Beitler and Liston 1992). Several such studies have shown that the PSP toxin profiles in natural shellfish populations (Oshima et al. 1976, 1990, Maruyama et al. 1983, Martin et al. 1990, Cembella et al. 1993, Chebib et al. 1993) are related to those of the dinoflagellate populations associated with their toxicity, but significant deviations also occur. In controlled contamination experiments using toxigenic *Alexandrium* cultures fed to bivalves in experimental systems (Sullivan 1982, Lassus et al. 1989, 1992, Bricelj et al. 1990, 1991, Beitler and Liston 1990, Bricelj and Cembella 1993) these changes in toxin profile have been shown to be both species-specific and time-dependent through the toxin uptake and detoxification sequence.

The HPLC-FD chromatograms of PSP toxins from the Gulf of Maine field populations indicated that toxin conversions in sea scallops were more limited than for surf clams from a similar environment. For example, chromatograms of toxic extracts of sea scallop tissues often showed dominance of C-11 sulfated toxins GTX2 and GTX3 (Fig. 9) which persisted throughout the year. In contrast, chromatograms of PSP toxins in surf clams revealed that the complex mixture of gonyautoxins (GTX1-GTX4), which was an important mixture of gonyautoxins (GTX1-GTX4), which was an important mixture of gonyautoxins in the early spring in all tissues, diminished rapidly and was largely replaced by a dominant STX/dcSTX fraction within a few weeks. An alternative chromatographic method (Oshima et al. 1989) discriminated the C-toxin components from fluorescent artifacts and resolved dcSTX from STX (Fig. 10).

For comparison, the toxin composition of a cultured isolate of *Alexandrium tamarense* (GT429 [CCMP117], Bigelow Labora-

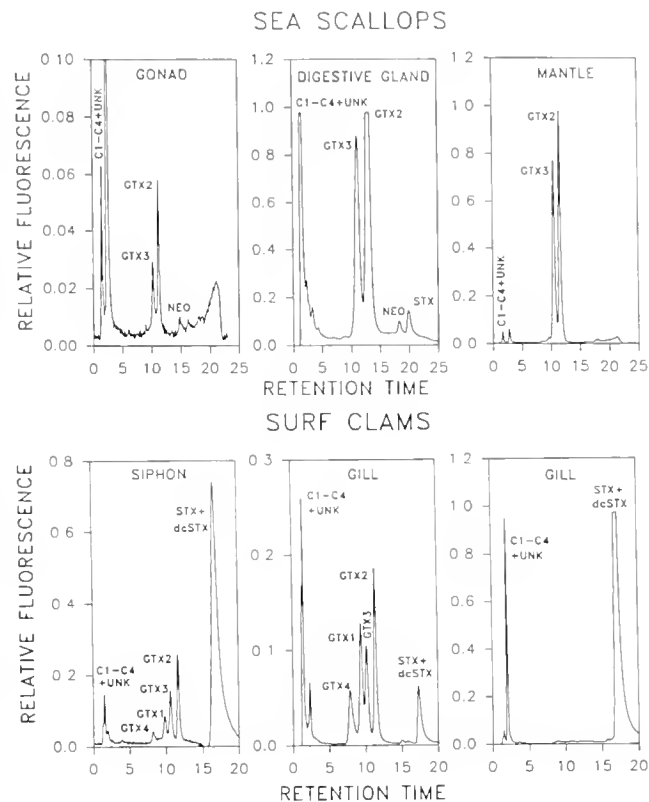


Figure 9. Representative HPLC-FD chromatograms of PSP toxin components in gonad, digestive gland, and mantle tissues of sea scallops during peak toxicity, and siphon and gill tissue sampled during and subsequent to a toxic bloom event. Toxins were resolved by binary gradient elution on a PVDBS resin column (Hamilton PRP-1), according to Sullivan and Wekell (1986).

tory for Ocean Sciences, Boothbay Harbor, ME) from Ipswich Bay, Gloucester, MA in the Gulf of Maine was also prepared by a method designed to preserve the integrity of the toxin spectrum and analyzed by HPLC-FD (Cembella et al. 1987, Bricelj et al.

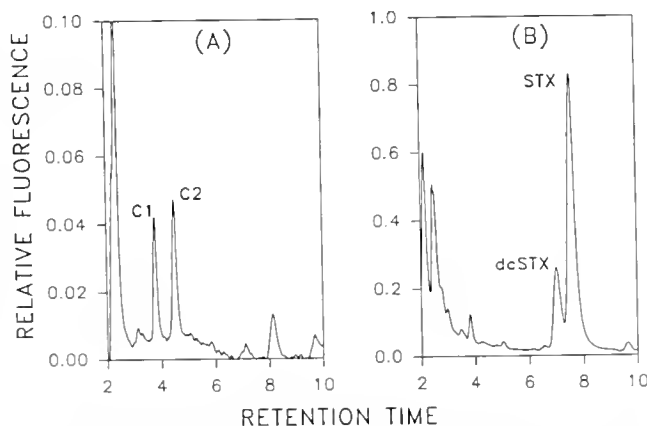


Figure 10. Representative HPLC-FD chromatograms of PSP toxin components showing resolution of N-sulfocarbamoyl toxins (C1/C2) in sea scallop gonads (A) and the separation of dcSTX from STX in surf-clam gills from an NEFDA offshore site on Georges Bank. Isocratic separations were performed on a C-8 silica-base column (Inertsil, GL Science), with minor modification of the method of Oshima et al. (1989).

1990, Cembella et al. 1993) (Fig. 11). The toxin profiles of cultured isolates generally reflect that of natural populations from which they were isolated (Cembella and Therriault 1989, Oshima et al. 1990) and the toxin profile in *Alexandrium* populations from the lower St. Lawrence estuary in Atlantic Canada was quite stable over a seasonal time-scale (Chebib et al. 1993). Analysis of the toxin profiles of other PSP toxin-producing dinoflagellates from the Gulf of Maine (A. Cembella, unpublished obs.) do show differences from isolate GT429 but the fundamental toxin hierarchy is maintained. In the absence of toxin compositional data from natural populations from the Gulf of Maine, isolate GT429 may be considered as a reasonable 'archetype' for the following discussion.

The epimerization of C-11 sulfated derivatives from the  $\beta$ -configuration (C2, C4, GTX3, GTX4), which tend to predominate in toxigenic dinoflagellates, to their corresponding  $\alpha$ -epimers (C1, C3, GTX2, GTX1) is commonly observed in shellfish extracts from dinoflagellate feeding studies (Bricelj et al. 1990, 1991, Oshima et al. 1990, Lassus et al. 1992) and following incubations of purified PSP toxins in crude shellfish tissue homogenates (Oshima 1993). Sullivan (1982) showed that epimerization of toxins C1/C2 and GTX2/3 was particularly evident in butter clams from a controlled feeding study, and in littleneck clams collected in late summer from Puget Sound, WA. No metabolic mechanisms need to be invoked for this conversion; epimerization can proceed spontaneously in dilute acid under mild conditions (Fig. 8). This epimerization follows thermodynamic equilibrium, with the  $\alpha$ -epimer as the more stable configuration. The ratio of  $\beta$ -to- $\alpha$ -GTX epimers (1.4:1) found in isolate GT429 is typical of that found in other PSP toxin-producing dinoflagellates. In sea scallops from the Gulf of Maine, with the exception of anomalies in the gonads from the offshore population during summer and early fall, the  $\alpha$ -epimer usually predominated, with a  $\beta$ -to- $\alpha$ -epimeric ratio that was typically  $<0.7:1$  in all tissues (Fig. 12a). In contrast, in the surfclams (Fig. 12b), an increase in the  $\beta$ -to- $\alpha$ -GTX epimer ratio to  $>1:1$  coincided with an overall rise in toxin content in most tissues during the summer, presumably linked to recent exposure to toxic blooms, whereas this ratio was less than unity during the late winter to early spring. Inexplicably, there was also a maximum in the  $\beta$ -to- $\alpha$ -GTX epimer ration during the winter toxicity peak (November to January) in all surfclam tissues, long after toxic blooms should have terminated.

The low potency N-sulfocarbamoyl toxins (C1-C4, B1, B2) are typically in higher relative abundance in toxigenic dinoflagel-

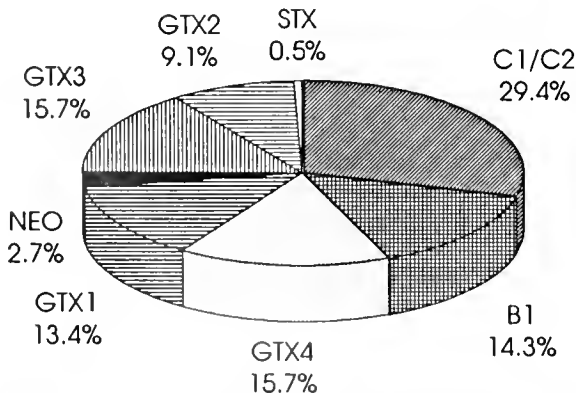


Figure 11. Relative toxin composition of *Alexandrium tamarensis* GT429, a representative isolate from the Gulf of Maine.

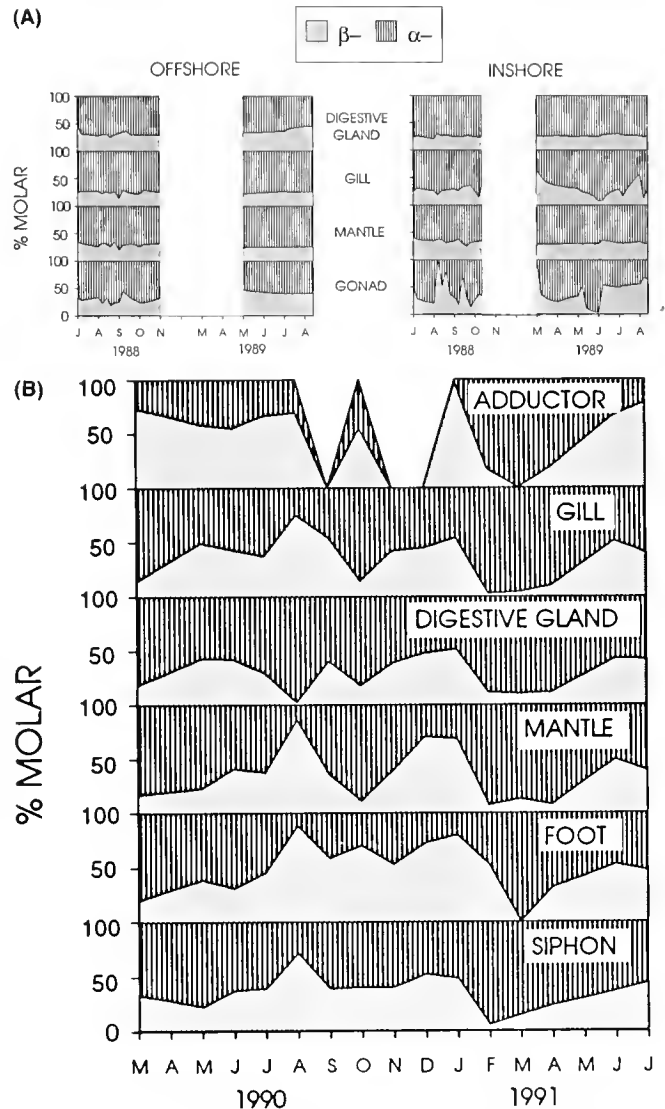


Figure 12A,B. Seasonal variation in mean relative composition (% molar) of  $\alpha$ - versus  $\beta$ -epimers of gonyautoxins found in tissues of offshore and inshore sea scallops (A) and surfclams (B) from the Gulf of Maine. For sea scallops, only the ratio of GTX2:GTX3 are shown; GTX1 and GTX4 were not included due to non-systematic variation in their respective concentrations.  $\alpha$ -epimers = GTX1, GTX2;  $\beta$ -epimers = GTX3, GTX4.

lates than in shellfish which sequester PSP toxins. With respect to both bivalve species in the present study, the dinoflagellate isolate from the Gulf of Maine was relatively much richer in toxins C1/C2 and B1 and contained less STX (Fig. 11). The N-sulfocarbamoyl:carbamate toxin ratio in surfclams differed radically from that of sea scallops; in surfclams, N-sulfocarbamoyl toxins were only found for short discrete periods associated with toxic blooms, yet these toxins were persistent throughout the sampling period in high relative abundance in sea scallops. Although the conversion from N-sulfocarbamoyl toxins to their high toxicity carbamate analogues can be effected physico-chemically (indeed this occurs to a large extent in the hot 0.1 M HCl extraction protocol for the AOAC mouse bioassay), the application of mild extraction procedures in time-series bivalve feeding and detoxification experiments (Bricelj et al. 1990, 1991, Lassus et al. 1992)

has demonstrated the role of metabolism in this process. Unfortunately, interpretation of some previous data on N-sulfocarbamoyl:carbamate ratios to infer toxin metabolism and kinetics in field studies (e.g., Martin et al. 1990) and in controlled laboratory studies (e.g., Lassus et al. 1989) is complicated by the relatively harsh extraction conditions employed, which resulted undoubtedly in some degradation of the N-sulfocarbamoyl components.

Reductive desulfation, resulting in the loss of the C-11 O-sulfate moiety has been reported in homogenates of certain tissues of sea scallops (Shimizu and Yoshioka 1981, Fix Wichmann et al. 1981). This reaction is the most credible explanation for the increase observed in the STX:GTX ratio during detoxification of intact bivalves, including blue mussels (Bricelj et al. 1990), butter clams (Sullivan 1982, Beitler and Liston 1990), European scallops (Lassus et al. 1992), Japanese scallops (Oshima 1991) and northern quahogs (Bricelj et al. 1991). Desulfation would also account for the appearance of STX in bivalves fed upon dinoflagellate cultures which do not contain this derivative. Reductive cleavage of the O-sulfate group at C-11 does not proceed readily under mild extraction conditions and has often been assumed to be regulated by a cryptic 'sulfatase' (sulfohydrolase) (Fig. 8). The possible role of marine bacteria in this conversion was reported by Kotaki et al. (1985) who noted the conversion of GTX2/3 to STX by *Pseudomonas* sp. and *Vibrio* sp. isolated from the digestive tract of certain shellfish. Recently, however, reductive cleavage of C-11 O-sulfate from GTX has been shown to occur with purified toxins and shellfish tissue homogenates in the presence of sulfhydryl reagents (e.g., dithiothreitol, mercaptoethanol, glutathione, cysteine, etc.) (Oshima 1993, M. V. Laycock, unpubl. obs.). It is not yet clear whether or not naturally-occurring sulfhydryl analogues are capable of mediating such toxin conversions *in vivo*. In any case, this reductive mechanism is substantially more active in surfclams than in sea scallops, as the high relative concentration of gonyautoxins which appeared during summer toxicity peaks did not persist for long in the toxin profile of the clam.

To date, efforts to confirm sulfotransferase activity in sea scallop digestive glands containing PSP toxins, which could convert STX to GTX by sulfation via a hydroxyl intermediate at C-11, have been unsuccessful (M.V. Laycock, pers. comm.). A specific N-sulfotransferase involved in the biosynthesis of GTX has been found, however, in the dinoflagellates *Alexandrium tamarense* and *Gymnodinium catenatum* (Oshima 1993). If such sulfotransferase enzymes are produced by toxigenic dinoflagellates, but not by shellfish, toxin conversion catalyzed by these enzymes may only be significant during the early stages of digestion in shellfish viscera while dinoflagellate cells remain intact and metabolically active. There is no biochemical evidence of massive shifts from STX, NEO or B1/B2 to C-11 sulfated derivatives from either field studies or laboratory feeding experiments on bivalves which cannot be linked plausibly to the ingestion of toxic dinoflagellates.

A decrease in the NEO:STX ratio in sea scallop homogenates, particularly of locomotory tissues, following incubation with partially purified PSP toxin fractions was interpreted by Shimizu and Yoshioka (1981) as *de facto* evidence of reductive loss of the N-1 hydroxy moiety. In controlled long term detoxification experiments with the Japanese scallop *Patinopecten yessoensis* (Oshima 1991), the relative decrease in GTX1 and GTX4 in mantle tissues, accompanied by an increase in GTX2 and GTX3, were also attributed to hydroxyl group reduction. It was assumed originally that this reduction is mediated enzymatically (Shimizu and Yoshioka 1981), but no specific enzyme has been identified. Ko-

taki et al. (1985) indicated that the conversion of GTX1/GTX4 to an epimerized mixture of GTX2/GTX3 and STX, and of NEO to STX, could be effected by marine bacteria. Recent work by Oshima (1993) showed that cleavage of the N-1 hydroxy group from GTX1, GTX4 and NEO in shellfish tissue homogenates, resulting in the formation of GTX2, GTX3, and STX, respectively, can occur in the presence of natural reductants such as glutathione and cysteine.

Numerous independent lines of evidence indicate that the *de novo* formation of significant quantities of decarbamoyl toxins, is a common although not universal capability of clam species. Sullivan (1982) found that over a four week detoxification period the apparent conversion of C2 was accompanied by the appearance of dcGTX and dcSTX in littleneck clams, whereas in butter clams only STX was present in the siphon. Among the many bivalve species screened by Oshima (1993), the capacity for enzymatic hydrolysis of the N-sulfocarbamoyl or carbamoyl moieties to form decarbamoyl toxins was found only in homogenates of two clam species from Japan, *Macra chinensis* and *Peronida venulosa*. In temperate waters where *Alexandrium* blooms are the reputed cause of PSP toxicity, the accumulation of decarbamoyl toxins appears to be largely restricted to certain clams. The possibility that decarbamoyl toxins could occur in softshell clams from eastern North American waters could not be confirmed in the seasonal field study of this species in the Bay of Fundy (Martin et al. 1990), since the HPLC-FD method used for toxin analysis (Sullivan and Wekell 1986) was unable to resolve decarbamoyl derivatives from their carbamate analogues. A least one decarbamoyl derivative (dcSTX) was identified recently in the hepatopancreas and tail muscle of the lobster, *Homarus americanus* from the Gaspé region of eastern Canada where PSP toxicity in bivalves is a chronic annual problem (Desbiens and Cembella 1993). Decarbamoyl toxins have also been found in marine species from the tropics, e.g., in planktivorous fish *Sardinella* sp. and green mussels *Perna viridis* (Oshima 1989), as well as in the bivalve *Spondylus butleri* and crabs (Harada et al. 1983). This decarbamoyl toxin accumulation may be due to direct dietary incorporation rather than biotransformation since dcSTX was identified in the dinoflagellate *Pyrodinium bahamense* var. *compressum* responsible for the PSP toxicity. Similarly, the presence of dcGTX and dcSTX in Tasmanian mussels can be linked to their occurrence in the dinoflagellate *Gymnodinium catenatum* from the same area (Oshima et al. 1989).

Incubation studies of purified PSP toxins using homogenates of *Protothaca staminea* provided circumstantial evidence that decarbamoylation was mediated by an endogenous enzyme in the shellfish digestive system (Sullivan et al. 1983). Current research has confirmed the existence of a 'carbamoylase' in digestive gland homogenates of *Protothaca* capable of converting the C-11 sulfated toxins GTX2/3 and C1/2 to decarbamoyl derivatives with a consistent efficiency of approximately 80% (molar yield) (M. V. Laycock, pers. comm.). The *Protothaca* enzyme exhibits an evident stereospecificity, catalyzing the conversion of GTX3 more readily than its epimeric pair GTX2, and yielding dcGTX efficiently from purified C-toxins. Sullivan et al. (1983) also noted that this decarbamoylation was effected against a broad spectrum of N-sulfocarbamoyl (B1, C1, C2) and carbamate (GTX2, GTX3, STX) substrates. The *in vivo* conversion of toxin C2 to dcGTX and dcSTX observed by Sullivan (1982) in field populations of *Protothaca* (but not in *Mytilus edulis* or *Saxidomus giganteus*) likely proceeded via direct decarbamoylation rather than by desulfation at N-21 to a GTX intermediate.

To date, in spite of repeated attempts using the enzyme assay protocol developed for *Protothaca* (N. Ross and A. Cembella, unpubl. obs.) we have not been able to detect 'carbamoylase' activity in homogenates of any tissues of adult surfclam specimens from a site in Maine where high PSP toxicity levels recur annually. Neither was enzyme activity found in juvenile surfclams obtained from a hatchery, thus not subjected to prior exposure to PSP toxins. Considerable dcGTX2 and dcGTX3 was produced in the course of controlled feeding experiments with juvenile *Spisula* exposed to an *Alexandrium* strain rich in GTX1-GTX4 (but containing no dcGTX or STX), yet no dcSTX was detected (Bricelj and Cembella 1993). Either the 'carbamoylase' activity in juvenile surfclams is specific only for the 11-OSO<sub>3</sub> derivatives (unlike the *Protothaca* enzyme), or immature specimens lack the capacity for reductive conversion of GTX to STX, as a precursor for dcSTX. This specificity question cannot be resolved simply with reference to the adult field specimens from the Gulf of Maine, since it is probable that some STX is acquired directly through ingestion of toxic dinoflagellates. The apparent lack of dcGTX in both inshore and offshore natural surfclam populations could be interpreted to indicate either greater 'carbamoylase' specificity than in *Protothaca*, or rapid and complete conversion of dcGTX to dcSTX via desulfation at C-11.

Unlike the surfclams where dcSTX constituted as much as 20% of the relative molar toxin composition in most tissues, decarbamoyl toxins were apparently absent from the toxigenic dinoflagellate and were found only at trace levels in scallop digestive glands and mantles from the Gulf of Maine (Cembella et al. 1993). Previously, only small amounts of decarbamoyl toxin, in the form of dcNEO (=GTX7) were detected in the viscera of Bay of Fundy scallops (Hsu et al. 1979). Thus, surfclams occupy an intermediate position in terms of their capacity for decarbamoylation reactions, as littleneck clams *P. staminea* from the Pacific coast of North America were reported to sequester virtually all of their PSP toxin load as decarbamoyl derivatives, specifically dcGTX and dcSTX (Sullivan et al. 1983).

Based upon evidence of temporal shifts in toxin profile, it is possible to construct a hierarchy among shellfish, in their relative capacity for PSP toxin modification. Compared to other species of Atlantic shellfish that have been analyzed, including scallops (Cembella et al. 1993), northern quahogs (Bricelj et al. 1991), softshell clams (Martin et al. 1990) and mussels (Bricelj et al. 1990, Chebib et al. 1993), surfclams appear to have the greatest capability for profound alteration of the toxin composition of ingested toxic dinoflagellates. Moreover, this is not merely a function of extended toxin residence time in this species, since the toxin profile in surfclams shifts rapidly after exposure to toxic dinoflagellates in the field (Cembella and Shumway 1993) and in controlled feeding experiments (Bricelj et al. 1993).

#### Anatomical and Spatio-temporal Distribution of Toxin Components

The differences in toxin profiles among individual tissues of surfclams and sea scallops reflect a differential capacity to sequester specific toxins (net transfer balance), superimposed upon tissue-specific differences in toxin catabolism rates. The few published studies on PSP toxin conversions using crude tissue homogenates of bivalve species (e.g., Shimizu and Yoshioka 1981, Oshima et al. 1993) have not yielded kinetic rate constants and have provided little information on the physiological significance

of *in vivo* biotransformations. While there were obvious qualitative differences in PSP toxin composition among various tissues of bivalves from the Gulf of Maine, and considerable seasonal variation, there was much less geographical variation in relative toxin amounts (% molar) within a species. It is therefore likely that the toxigenic blooms causing PSP toxin contamination in the Gulf of Maine produce a similar toxin spectrum. Furthermore, this suggests that the species-specific mechanisms responsible for toxin biotransformation and detoxification are functionally equivalent among bivalve populations capable of prolonged toxin retention, even though they may be radically divergent among species.

The PSP toxins identified among various surfclam tissues included carbamate toxins (GTX1-GTX4, NEO, STX), N-sulfocarbamoyl derivatives (C1/C2), and dcSTX (Cembella and Shumway 1993). In contrast to sea scallops where STX was only slightly enriched (relative to the representative dinoflagellate GT429), in surf clams STX was typically the dominant toxin on a relative

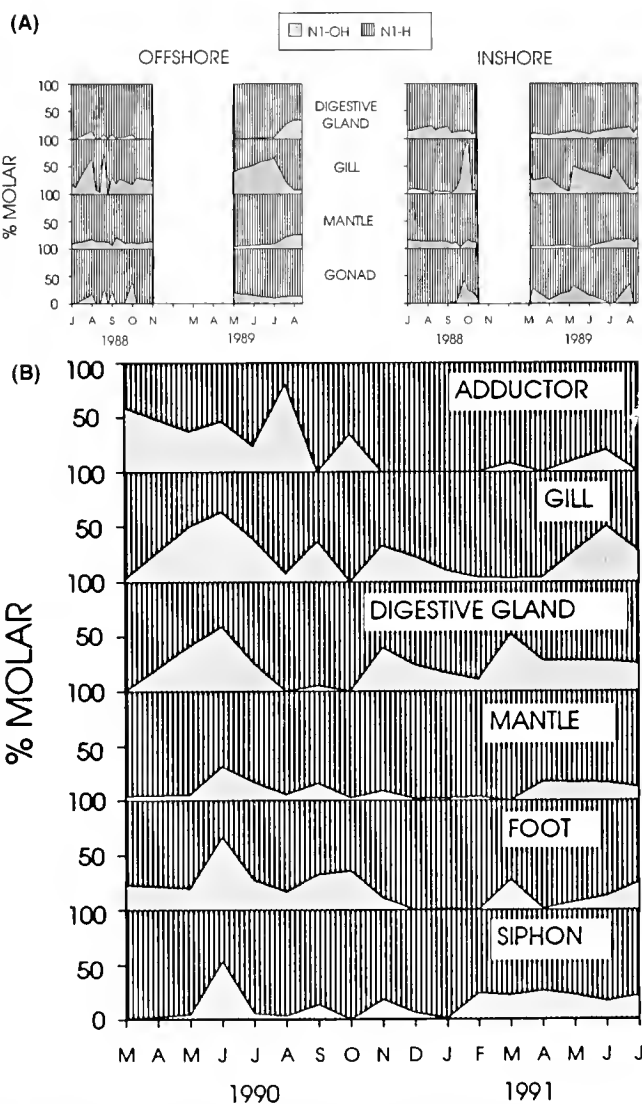


Figure 13A,B. Seasonal variation in mean relative composition (% molar) of N1-OH versus N1-H toxins found in tissues of offshore and inshore sea scallops (A) and surf clams (B) from the Gulf of Maine. N1-H = C1, C2, STX, dcSTX, GTX2, GTX3; N1-OH = NEO, GTX1, GTX4.



basis (% molar), except during summer toxicity peaks, when the toxin profile became more complex. The N-sulfocarbamoyl toxins were prevalent for short periods during toxicity peaks in digestive gland, gill, foot and siphon tissue, whereas they were barely registered in mantle and adductor muscles. During the summer toxicity maximum, the ratio of  $\beta$ -to  $\alpha$ -epimers of the C-11  $\text{OSO}_3^-$  derivatives (GTXs) rose in all tissues, except in the viscera, where there was strong evidence of epimerization (Fig. 12B).

The relative distribution of N-1 hydroxy derivatives also exhibited some seasonal variation among surf clam tissues; there was a prominent maximum in these toxins which corresponded temporally to the initial toxicity peak in early summer in all tissues (Fig. 13B). As overall toxin levels decreased following the winter toxicity peak (Fig. 5), the ratio of N-1 hydroxy toxins to total components also declined (Fig. 13B).

The origin of the winter toxicity maximum from November to January in digestive glands and mantles of surf clams (cryptic late-season bloom? sinking of senescent fall bloom? toxic benthic cysts?) is difficult to explain by invoking arguments based upon the toxin spectrum. Substantial amounts of dcSTX were accumulated in the fall, especially in gills, mantles and siphons, and these high relative levels were maintained throughout the winter and subsequent spring. Biotransformation alone cannot account for the winter toxicity increase; the relatively high levels of STX and dcSTX in the most toxic tissues (digestive gland, mantle, gills) indicated that substantial toxin catabolism had already occurred prior to the toxicity peak. The lack of significant N-sulfocarbamoyl toxins during the winter also suggests that "new" toxin was not introduced from cryptic winter blooms. Nevertheless, the shift towards an increase in the  $\beta$ - $\alpha$ -epimeric ratios of the C-11  $\text{OSO}_3^-$ -toxins and the relative increase in N-1 hydroxy toxins observed during early winter would support the proposed scenario that there was a exogenous toxin source at this time.

With reference to previous studies on other clam species, the fact that relative and absolute amounts of STX retained in the siphon were not dramatically elevated in surfclams was rather surprising. Early work on the PSP toxin content of butter clams

from Alaska (reviewed by Schantz 1984) tended to emphasize this organ as the major repository for STX. Although this is now seen as an oversimplification, given that the first efforts at toxin fractionation tended to regard STX as the sole PSP toxin component, subsequent work has not contradicted this observation. According to Sullivan's (1982) studies on natural butter clam populations from Puget Sound, WA and from controlled feeding trials, STX and dcSTX were retained primarily in the siphons. This was confirmed subsequently by Beitler and Liston (1990) who also found STX accumulation mainly in the siphon.

In sea scallops, the most important contributors to total toxin content in digestive glands were toxins GTX2 and C1/C2 throughout most of the year. The epimeric ratio of GTX2:GTX3 in sea scallops approximated 3:1 and did not exhibit much seasonal variation, especially in digestive glands and mantles (Fig. 12a). The N-1 hydroxy carbamate toxins (GT1/GTX4, NEO) represented >30% of the molar toxin composition in the Gulf of Maine dinoflagellate, yet these components were relatively less abundant in scallop populations (Fig. 13a). Apart from the occasional appearance of GTX1/GTX4, there was little variation in relative toxin composition in digestive glands from either scallop population within a given year.

The proportion of N-sulfocarbamoyl derivatives C1-C4 in scallop digestive glands was higher in 1989 than in the preceding year, but was less persistently elevated in the inshore population. The higher C1/C2 content and N-sulfocarbamoyl:carbamate ratio in digestive glands from the early spring of 1989, relative to the previous autumn, may indicate recent exposure to a toxic bloom. Unlike surfclams, where this ratio could be used to identify peaks in the occurrence of summer dinoflagellate blooms, the corresponding seasonal trend in the proportions of carbamate:N-sulfocarbamoyl toxins in scallop digestive glands was less clearly defined.

The typical toxin profile of scallop mantles was similar to that of the digestive gland, with the carbamate epimers GTX2 and GTX3 as the dominant toxins in both populations. There was a lesser contribution by toxins C1/C2 than in the digestive tissues, particularly during toxicity peaks. Trace quantities of N-sulfocar-

TABLE 1.

Mean coefficient of variation\* (S.D./ $\bar{X}$  as %) of weight-normalized toxicity ( $\mu\text{gSTXeq } 100\text{g}^{-1}$ ) for tissues of individual sea scallops (n = 8) and surfclams (n = 6) from populations in the Gulf of Maine.

	C1-C4	GTX1/GTX4	GTX2	GTX3	NEO	dcSTX	STX	Total
INSHORE SCALLOPS								
Digestive gland	37.6	73.1	52.6	57.3	63.6		73.9	53.0
Gill	27.2	111.2	90.2	98.6	43.7		111.6	58.6
Mantle	33.3	72.8	43.5	41.4	59.6		47.6	40.3
Gonad	67.1	27.6	138.4	146.8	77.0		47.9	122.0
OFFSHORE SCALLOPS								
Digestive gland	41.1	81.1	42.4	43.8	56.4		36.9	42.2
Gill	24.4	84.7	67.7	76.7	52.6		123.6	74.2
Mantle	32.9	73.4	49.7	50.5	50.8		56.7	45.7
Gonad	63.0	50.4	102.3	100.9	57.9		61.0	103.5
SURF CLAMS (Head Beach)								
Digestive gland	13.5	48.4	64.5	71.2	71.6	55.8	72.5	54.0
Gill	8.4	52.4	67.0	80.1	124.1	45.2	65.5	58.9
Mantle	20.1	99.7	50.9	63.9	51.1	46.6	62.8	54.8
Siphon	19.8	67.6	50.7	77.6	75.9	48.5	74.3	62.3
Foot	4.9	85.5	66.8	89.2	58.6	16.7	79.0	72.1
Adductor	5.9	57.5	65.4	84.3	14.8	9.4	102.2	93.6

\* Number of observations averaged: inshore scallops (n = 26); offshore scallops (n = 17); inshore surfclams (n = 15).

bamoyl toxins C3 and C4 were detected in both mantle and digestive glands during the summer. Virtually all toxin was present as carbamate derivatives in mantles in both scallop populations during 1988, and the N-sulfocarbamoyl:carbamate toxin ratio was consistently lower than in other tissues. That the N-sulfocarbamoyl fraction was not present in the mantle in strict equilibrium with the digestive gland suggests that these toxins are preferentially eliminated from mantles or are transformed in the digestive gland prior to export to the mantles.

Among all scallop tissues, the toxin profile in gills was the most erratic on a seasonal basis, and some geographical variation between inshore and offshore population was evident. During toxicity peaks, the principal toxin analogue in gills was NEO, although GTX2 and C1/C2 sometimes co-dominated. Averaged seasonally on a relative molar basis, toxins C1 + C2 were the most significant toxins in the inshore scallop population, usually comprising half of the total toxin content ( $\text{nmol g}^{-1}$ ). This was not the case for the offshore stocks, where NEO and GTX2 usually tended to dominate. Trace concentrations of C3 and C4 were found, particularly in association with high levels of toxin C2 occurring at maximum toxicity.

The PSP toxin profile in scallop gonads (when toxin was present) fluctuated seasonally and was dominated by C1/C2, GTX2 and GTX3, with the gonyautoxin components accounting for most of the toxicity. The N-sulfocarbamoyl toxin content in inshore scallop gonads was unusual in the summer in that significant amounts of toxin C4 were accumulated. The clearest evidence for biotransformation in scallop tissues from the Gulf of Maine was found in offshore gonads in 1989. Dominance of the C-toxin fraction in the spring shifted to a large relative increase in GTX2/GTX3 and a decrease in NEO accompanied by the appearance of STX in summer. This pattern is consistent with the loss of the N-21 sulfocarbamoyl moiety and reductive loss of the N-1 hydroxyl group. Since STX appeared only rarely in gonads, during summer when abundance in digestive glands was maximal, it is likely that transfer efficiency of this toxin analogue from surrounding tissues is rather low. There was no apparent systematic seasonal trend in the ratio of carbamate:N-sulfocarbamoyl toxins in gonads, yet shifts in this ratio closely corresponded to those occurring in digestive glands. The gonadal toxin composition was very similar to that of associated digestive glands in offshore scallops, particularly in 1988. In contrast, in inshore scallop gonads, toxins C1/C2 typically comprised a greater fraction of the toxin components than in either digestive glands or offshore scallop gonads. In gonads from the inshore population, there were wide fluctuations in the GTX2:GTX3 ratio (range:  $>9:1$  to  $<1:9$ ) in both years which did not appear to be linked temporally with toxic dinoflagellate blooms in an obvious manner. This discrepancy may be accounted for by the effects of gonadal maturation on toxin dynamics, as inshore scallop gonads are expected to be more active reproductively than their offshore counterparts (Barber et al. 1988).

As for surfclams, the adductor muscles of sea scallops were distinguished from other organs by the low relative abundance of N-sulfocarbamoyl toxins. When toxins were present in scallop adductor muscles, GTX2 and GTX3 were usually found, although occasionally trace levels of GTX1/GTX4 or STX were identified. As the HPLC-FD detection limits for GTX2/GTX3 are much lower than for STX and N-1 hydroxy derivatives, the spectrum of toxins reported here for weakly toxic scallop adductor muscles may be somewhat biased towards the highly fluorescent deriva-

tives produced from toxins GTX2/GTX3. That STX was not relatively abundant in adductor muscles could also indicate that this derivative is not readily transferred due to its high binding affinity for the viscera.

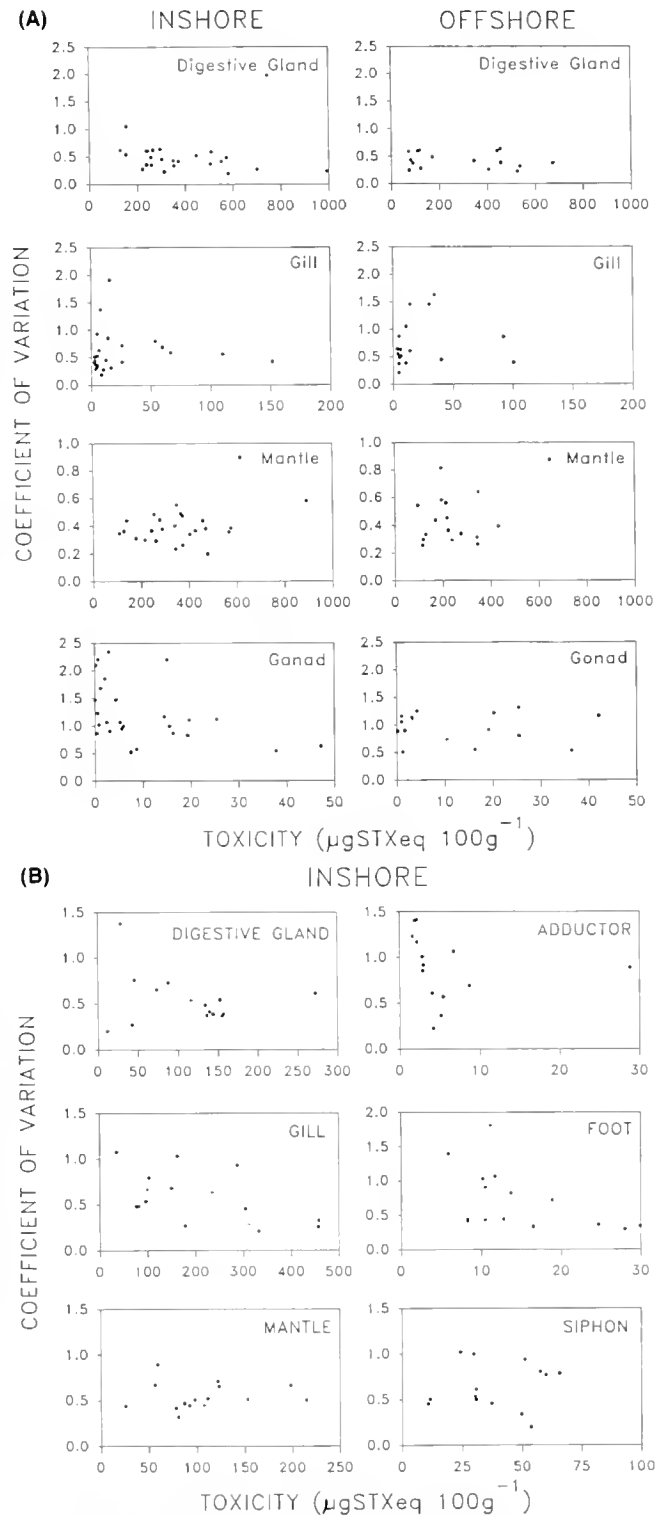


Figure 14A,B. Mean toxicity ( $\mu\text{gSTXeq } 100\text{g}^{-1}$ ) as determined by HPLC-FD versus the coefficient of variation (C.V. =  $S.D./\bar{X}$ ) in different organs of inshore and offshore scallops (A) and surfclams (B) from the Gulf of Maine.



### Variation in Toxin Composition among Individuals

Individual bivalves from the same location are known to vary widely in PSP toxicity, as determined by mouse bioassay (Gillis et al. 1991, White et al. 1993b and references cited therein). Some variation is attributable to imprecision in the mouse bioassay ( $\pm 20\%$  under optimal conditions) (Adams and Furfari 1984), but the large variation observed in natural populations cannot be explained only by analytical error. Biological and physical factors which could account for this localized variation in net toxicity include differences in size, age, reproductive status, rates of feeding, digestion and toxin transfer, as well as microzonal patchiness affecting exposure to toxic algal cells. The present study represents an attempt to determine the magnitude of toxin-specific variation in each tissue compartment, among individuals sampled simultaneously from the same site. The coefficients of variation (C.V.) for individual toxicity components and total toxicity are presented in Table 1. As might be expected, the highest C.V. values for total calculated toxicity corresponded to gonads for sea scallops and to adductor muscles of surfclams. Scallop adductor muscles were not represented due to insufficient toxicity data.

The mean variation in PSP toxicity was comparable to the high mean values determined previously for whole sea scallops (C.V. = 43.6%) and surfclams (C.V. = 48.6%) from offshore populations in the Gulf of Maine and approximated the range of variation among data sets based upon bioassay results (Shumway et al. 1993, White et al. 1993b). The highest toxin-specific variation was associated with GTX2 and GTX3 levels in scallop gonads.

Mouse bioassay toxicity data have indicated an inverse relationship between the C.V. and total toxicity (Shumway et al. 1993, White et al. 1993b), but except for adductor muscle and foot tissues of surfclams this tendency was not substantiated for toxicity determined by HPLC-FD (Fig. 14). Since the HPLC-FD method has lower detection limits than the mouse assay, the relationship observed for the bioassay may merely reflect a lower precision at low toxin levels rather than a biologically meaningful trend. When the toxin-specific C.V. was plotted against toxicity attributed to individual components in each tissue of sea scallops (data not shown), the inverse relationship between C.V. and toxicity was associated primarily with the N-1 hydroxy toxins GTX1 and GTX4 (Cembella et al. 1993). Since oxidized derivatives of the N-1 hydroxy toxins exhibit the lowest specific fluorescence yield among the PSP toxins, the HPLC detection limit is effectively higher for those toxins.

The high variation among individual bivalves illustrate a significant weakness in site-specific shellfish toxin monitoring data regardless of the analytical method applied. The present data strongly suggest that large sample sizes (i.e., number of individuals per extraction) are crucial to the accurate quantitation of toxicity in natural bivalve populations.

In summary, the seasonal variation and tissue compartmentalization of specific PSP toxin analogues were shown to be important parameters determining total toxicity in bivalves from the Gulf of Maine. Within a species, the toxin composition varied seasonally and among tissues to a greater extent than between populations. Examining the shifts in toxin composition ratios is useful in establishing the flux of PSP toxins among tissues, and may eventually assist in the formulation of dynamic models of toxin partitioning. Maximal PSP toxicity in the viscera of both sea scallops and surfclams appears to coincide with the seasonal occurrence of *Alexandrium* blooms in coastal waters of the Gulf of Maine. Peaks in certain toxin derivatives, such as N-sulfocarbamoyl toxins, are characteristic of recent exposure to toxic dinoflagellates and are useful in hindcasting toxic blooms. However, toxin biotransformation processes appear to occur on a time scale much shorter than the sampling intervals often selected for shellfish toxin monitoring. The development of effective toxin monitoring strategies and aquaculture site selection criteria should include a recognition of species-specific differences in the capacity for toxin retention and biotransformation.

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## BALLAST WATER AND SEDIMENTS AS MECHANISMS FOR UNWANTED SPECIES INTRODUCTIONS INTO WASHINGTON STATE

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**ABSTRACT** Examination of ballast water and sediments from bulk cargo carriers involved in the export of woodchips from Washington State to Japan was conducted to determine the potential for introduction of non-native species. The focus of this investigation was to determine if ballast sediments contained viable microalgae, and to identify ballasting practices which would allow for the transfer of organisms into local waters. Samples of ballast water and sediments collected from woodchip carriers entering the Ports of Tacoma and Port Angeles, WA were found to contain numerous viable organisms which survived the 11-15 day transoceanic voyage. Incubation of sediment sub-samples in nutrient-enriched seawater induced a proliferation of microalgae including various diatoms, dinoflagellates and phytoflagellates. These incubation trials suggest the presence of microalgae benthic spores and cysts. These life-stage characteristics are significant for introduced organisms, allowing them to remain viable for extended periods of time in unfavorable conditions. With up to 20,000 metric tonnes of water and several cubic yards of sediment discharged with each voyage, the threat of introduction of harmful algae, pathogens, predators and resource competitors is genuine. Decisions on where and when to take on and discharge ballast is made by ship personnel whose primary responsibilities are ship safety and economic efficiency. Interviews with ships' officers provided evidence that while at least some ships practice ballasting and deballasting procedures that may decrease the risk of introduction, all ships routinely discharge some volume of ballast water and sediments into local waters. Efforts to regulate ballast discharge need to consider the unique characteristics of the maritime industry and environment if they are to be effective.

**KEY WORDS:** ballast, ship discharge, introduced species, exotic species, harmful algal blooms, PSP, toxic phytoplankton

### INTRODUCTION

Ballast water and sediments from ships have been recently implicated in the transfer of a diverse assortment of non-native species to near-shore marine environments worldwide (Medcof 1975, Willan 1987, Carlton 1985, 1987, 1989, Williams et al. 1988, Hallegraeff et al. 1988a,b, Hallegraeff & Bolch 1992, Carlton & Geller 1993). Ballast water, used since the 19th century by cargo ships to ensure stability and seaworthiness, is now recognized as having a dual role, as an operational necessity and as a mechanism for the unintentional transfer of organisms. In the first comprehensive discussion of the role of ballast discharge as a mechanism for the transfer of marine species, Carlton (1985) concluded his seminal paper by stating that the continued discharge of ships' ballast water would act as "an international biotic conveyor belt" for marine organisms.

Bulk cargo carriers (primarily woodchip ships) have been the focus of investigations in Australia (Hallegraeff and Bolch 1992) and Oregon (Carlton and Geller 1993) for several reasons. These ships are unique in their design configuration and utilization of ballast tanks. As with other types of ships, several "dedicated" tanks are used to contain ballast water, these being the fore peak, aft peak and double bottom tanks. Unlike other types of ships, bulk cargo carriers routinely pump or gravitate additional ballast water into one of 5 or 6 large ("floodable") cargo holds, adding over one hundred thousand cubic feet of available ballast space (Fig. 1). For woodchip ships, these holds average 21 meters in depth and 15 meters in both length and width, contributing to the total volume of 20,000 metric tonnes (6.5 million gallons) of ballast water capacity per ship. Cargo holds filled with ballast water must be emptied (de-ballasted) and cleaned in preparation

for the loading of cargo. Cleaning includes the removal of sediments which are generally composed of woodchip debris, rust, and biotic and abiotic material entrained during ballasting, which settles out during the course of the voyage.

Although all other types of cargo vessels carry ballast water and associated sediments, only bulk cargo carriers using floodable holds are forced by operational necessity to fully empty and clean out these holds almost every time they load cargo (topping-off a partial load would be the exception). The bulk cargo trade is also unique in that ships are usually contracted to carry cargo for only one leg of each voyage, requiring them to travel "in ballast" approximately 50% of the time they are at sea. Bulk cargo carriers are used by countries with major processing industries to import large quantities of raw materials (woodchips, grain, coal, sugar, iron ore). Therefore, countries exporting bulk products can become the recipients of large amounts of foreign ballast discharge, as is the case with Australia (Jones 1991) and the U.S. (Carlton and Geller 1993).

Routine use of large volumes of ballast water and the required discharge of both ballast water and sediments while in port, make bulk cargo carriers worthy of investigation. In the late 1980's, ballast water was identified as the likely method of introduction into the Great Lakes of the zebra mussel, *Dreissena polymorpha* Pallas, the cladoceran crustacean, *Bythotrephes cederstroemi* and the European river ruffe, *Gymnocephalus cernuus* (Hebert et al. 1989). At the same time, ballast sediment became the object of concern in Australia, where toxic phytoplankton blooms were linked to sediments discharged from woodchip ships. Hallegraeff and colleagues provided evidence that blooms of the toxic dinoflagellate *Gymnodinium catenatum* Graham, which caused the closure of Tasmanian shellfish farms in 1986 and in subsequent years, were linked to sediments discharged from the ballast tanks of cargo ships (Hallegraeff et al. 1988b, 1989, 1990). Using historic plankton samples, cyst surveys, genetic analysis and sexual

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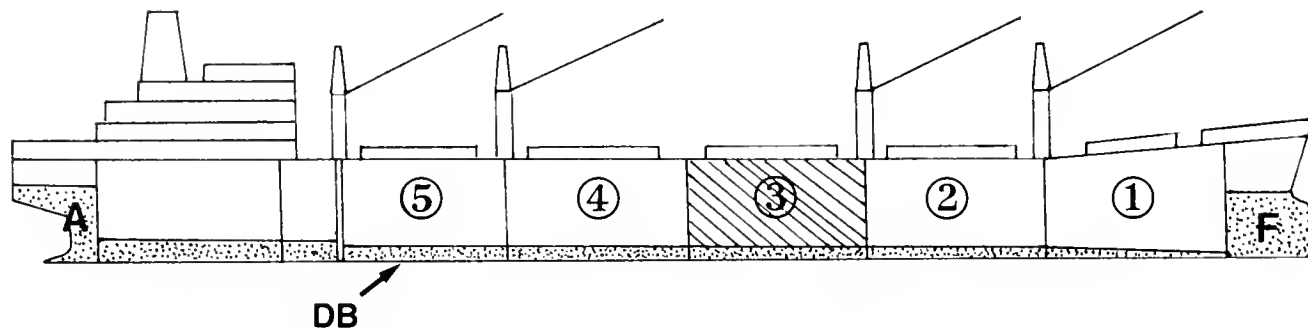


Figure 1. Lateral section of bulk cargo carrier showing segregated ballast tanks (fore peak, aft peak and double bottom) and cargo holds 1-5 of which hold 3 is "flooded" with ballast water.

compatibility experiments, further investigations suggested that blooms of *Gymnodinium catenatum*, *Alexandrium catenella* (Weldon et Kofoid) Balech and *Alexandrium minutum* Halim were all potentially related to the introduction of resting cysts from ballast sediments (Blackburn et al. 1989, Bolch & Hallegraeff 1990, Hallegraeff & Bolch 1991, Hallegraeff & Bolch 1992, Scholin & Anderson 1993). Hallegraeff proposed that "while the planktonic stages of diatoms and dinoflagellates show only limited survival during the voyage in dark ballast tanks, their resistant resting spores are well-suited to survive these conditions" (Hallegraeff 1993). The Australian Quarantine and Inspection Service recently completed a 4 year survey of 343 cargo vessels entering 18 Australian ports, confirming the prevalence of dinoflagellate cysts and diatom spores in ballast sediments (Hallegraeff and Bolch 1992). One woodchip ship, apparently having taken on ballast water in a Japanese port during a bloom, was found to contain an estimated 300 million toxic *Alexandrium* cysts in one tank (Hallegraeff & Bolch 1991).

The present study, conducted in 1991, was prompted by the observation that the same vessels which export woodchips from Australian ports to Asia also export woodchips from Washington State to Asia via the ports of Tacoma and Port Angeles. An actual voyage memo (Fig. 2) illustrates typical routing of ships which frequent ports in the Pacific woodchip trade. It was hypothesized, therefore, that the Pacific Northwest and Washington State might be at risk for introductions similar to those which have occurred in Australia. Although toxic phytoplankton and paralytic shellfish poison (PSP) episodes have occurred in Puget Sound for centuries (Rensel et al. 1989, Horner et al. 1990), many toxic species, including *Gymnodinium catenatum* and *Alexandrium minutum*, have not been reported in the Pacific Northwest.

To determine if Washington State could be the recipient of non-native organisms transported in ballast sediments from woodchip ships, two fundamental questions were posed:

1. do ships entering Washington ports carry ballast sediments containing viable organisms?, and
2. are these sediments being discharged into local waters?

#### MATERIALS AND METHODS

Woodchip ships were boarded at the Diashowa Chip Dock in Port Angeles and the Weyerhaeuser Company (Weyco) Chip Dock at the Port of Tacoma within 24 hours of arrival at berth. Each ship was visited on two consecutive days; first to sample ballast water and then to sample ballast sediments after de-ballasting. Personnel including the master, chief mate, and chief engineer were interviewed to obtain operational data on ballasting procedures. Crew

members, shipping agents, stevedores and longshoremen were informally interviewed to gather information on ballasting operations and general ship practices.

Ballast water samples were taken from those ships which arrived at berth with cargo holds containing ballast water, and hatches opened. Both whole samples and samples collected by plankton tow (53  $\mu$ m mesh net) were taken. Samples were stored on ice up to 12 hours prior to microscopic examination.

Sediment samples were collected in the hold by the crew, or from temporary storage containers placed on deck, by the author. Triplicate 250 ml samples were kept in opaque plastic bottles and held at 4°C until examination. Subsamples of 25 ml were sonicated for 2 minutes (Braun Labsonic homogenizer, intermediate probe, 100 watts) then screened through a 150  $\mu$ m Nitex sieve and collected onto a 20  $\mu$ m Nitex sieve. The resulting fraction was back-washed using autoclaved or sterile-filtered (0.2  $\mu$ m) seawater. Subsamples were examined using phase contrast and Nomarski optics at 40-1000 $\times$ .

		VOYAGE MEMO			
		=====			
NAME OF SHIP :		PORT : TACOMA			
NATIONALITY : PANAMA		DATE : JUL 13TH 1991			
VDY ND.	PDRT	ARRIVAL	DEPARTURE	REMARKS	
21.	SENDAI, JAPAN	NOV 13TH 1990	NOV 17TH 1990	DISCHARGING	
	NEWCASTLE, AUS	DEC 1ST 1990	DEC 3RD 1990	LOADING	
	LAUNCESTON, AUS	DEC 5TH 1990	DEC 7TH 1990	LOADING	
22.	SENDAI, JAPAN	DEC 30TH 1990	DEC 9TH 1991	DISCHARGING	
	NEWCASTLE, AUS	JAN 22ND 1991	JAN 24TH 1991	LOADING	
	LAUNCESTON, AUS	JAN 26TH 1991	JAN 30TH 1991	LOADING	
23.	TDYAMA, JAPAN	FEB 18TH 1991	FEB 22ND 1991	DISCHARGING	
	SAIKI, JAPAN	FEB 24TH 1991	FEB 24TH 1991	BUNKERING	
	LAUNCESTON, AUS	MAR 12TH 1991	MAR 13TH 1991	LOADING	
24.	NEWCASTLE, AUS	MAR 15TH 1991	MAR 17TH 1991	LOADING	
	SENDAI, JAPAN	MAR 31ST 1991	APR 05TH 1991	DISCHARGING	
	VANCOUVER, CANADA	APR 20TH 1991	APR 21ST 1991	LOADING	
25.	TACOMA, USA	APR 22ND 1991	APR 24TH 1991	BUNKERING N DISCHARGING	
	TDYAMA, JAPAN	MAY 18TH 1991	MAY 22ND 1991	DISCHARGING	
	COOS BAY, USA	JUN 04TH 1991	JUN 08TH 1991	BUNKERING N LOADING	
26.	SENDAI, JAPAN	JUN 25TH 1991	JUN 29TH 1991	DISCHARGING	
	TACOMA, USA	JUL 13TH 1991			

Figure 2. Voyage memo detailing passage of woodchip carrier from Asia to export ports in British Columbia, Oregon, Washington and Australia.

Aliquots of the fractionated subsample were inoculated into 2 ml of seawater-based GPM medium (Loeblich 1975) using 6 and 24-well tissue culture plates. Modifications to the GPM medium were made to test salinities of 18, 22 and 26 ppt. using 6 replicates per sediment sample. A pH-buffered silicate additive was added to selected cultures to support diatom growth. All samples were incubated at 20°C with overhead illumination of 180  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  from white fluorescent lights on a 14:10 h LD cycle for 2–10 weeks.

## RESULTS

### Ballasting Procedures Survey

All personnel interviewed emphasized that ballast water is essential for the safe and efficient handling of the vessel, both during ocean passage and while entering port. Used to control stability and trim of the vessel, ballast acts to balance stresses on the ship's hull, allows for steering within coastal waters and is integral to efficient fuel consumption. Decisions to take on and discharge ballast are complex, based on many criteria, and are executed unilaterally by the ship's officer or designated crew member. Criteria include, but are not limited to, weather conditions, port draft restrictions, loading equipment requirements at berth, scheduling, fees charged while at anchor or berth, maneuverability of the ship in harbor waters and quality of the ballast water source.

Of 6 ships sampled, 5 arrived at berth with one cargo hold containing ballast water (Table 1). The ship without ballast water had sailed from Vancouver B.C., Canada carrying 3 holds of woodchips, and arrived in Tacoma to load an additional 3 holds. This ship reportedly used "continuous" ballast exchange, starting 2 days prior to arrival in port. Two ships had voluntarily "exchanged" water taken on in Japanese ports with North Pacific water. In both cases the volume discharged and replaced was less than 50% of the original volume. The most common reason cited for exchange of ballast water during transit was to eliminate Japanese coastal water considered to be polluted. It was explained that the prompt removal of polluted water from the cargo hold minimized the time required to clean the holds in preparation for loading cargo. One crew commented that rough weather during transoceanic passage helped to scrub down the holds for them. The three other ships carried ballast water directly from their port of

departure in Japan, although one took on additional water while in transit through the North Pacific.

After deballasting, the crew or longshoremen descended into the hold to shovel sediment into containers, usually 55 gallon drums. In one case burlap sacks were used. Drums were hoisted by crane to the deck where they were held pending disposal. All of the ships sampled had sediment taken from the hold for disposal, with volumes ranging from 600–1900 liters. The sediments were eventually to be dumped overboard. When questioned, 4 of 6 officers stated that sediments would remain stored on deck until the ship was outside harbor waters before dumping. Where disposal was to occur varied, with officers citing distances up to 300 miles from port.

With the exception of new legislation affecting the Great Lakes, there is currently no U.S. regulation prohibiting the discharge of "clean" (not contaminated by oil or hazardous substances) ballast and accompanying sediments (see Bederman 1991 for discussion on laws governing exotic marine species and Bodansky 1991 for discussion on vessel-source pollution). When officers interviewed in this study were asked why they chose to hold sediments on board in preparation for off-shore disposal, none could cite an authority for this practice. Two possible explanations are suggested.

From interviews it was learned that masters from 3 of the ships were familiar with the voluntary control guidelines and inspections being conducted by the Australian Quarantine and Inspection Service. Of these, only one had experienced an inspection, but two others had heard of ships being boarded by authorities investigating the content of ballast water. While it is quite reasonable to suppose that knowledge of the Australian survey influenced some of these masters to take precautions in their disposal of sediments, a more probable cause is habit, based on existing policy in Japan regarding ballast discharge. Rule 24 of the Japanese Ports and Harbor Act prevents any ship from disposing of ballast, oil, coal or garbage within 10,000 meters (6.2 miles) from the boundary of the port area (Someya et al. 1991). In addition to being influenced by control efforts in other countries, some ship officers apparently continue home-port practices when in foreign ports.

Although the majority of officers made an attempt to dispose of sediments offshore, it was observed that sediments were also dis-

TABLE 1.

Voyage data and ballast activity of sampled woodchip ships entering the ports of Tacoma and Port Angeles, Washington.

Vessel ID	Port of Origin (Japan)	Voyage Length (days)	Open-ocean Exchange	Sediment Disposed Offshore
MB	Kushiro	11	yes	no
SS	Sendai	14	yes	no
PT	Kure	15 <sup>1</sup>	no	yes 60 miles
KM	Tagonoura	14	no	yes 1–2 days
TR	Iyomishima <sup>2</sup>	14	yes <sup>3</sup>	yes 1–300 miles
SM	Tagonoura	12	no	yes outside port

<sup>1</sup> Bad weather caused rough conditions and delays.

<sup>2</sup> Prior to arriving Tacoma this vessel loaded chips in Vancouver B.C.

<sup>3</sup> Crew stated that ballast tanks were continuously pumped for all voyages, commencing approximately two days before arrival in port.

TABLE 2.

Taxa observed in incubated ballast sediment samples collected from woodchip ships entering the Washington ports of Tacoma and Port Angeles.

Diatoms	Dinoflagellates	Phytoplankton
<i>Achnanthes</i> sp.	<i>Gymnodinium</i> sp.	<i>Eutroptiella</i> spp.
<i>Asterionella</i> sp.	<i>Protoperidinium</i> sp.	
<i>Bacteriastrum</i> sp.	<i>Scrippsiella</i> sp.	
<i>Odontella</i> ( <i>Biddulphia</i> ) sp.		
<i>Chaetoceros</i> spp.		
<i>Corethron</i> sp.		
<i>Coscinodiscus</i> spp.		
<i>Ditylum brightwellii</i>		
<i>Navicula</i> sp.		
<i>Nitzschia</i> sp.		
<i>Pleurosigma</i> sp.		
<i>Rhizosolenia</i> sp.		
<i>Skeletonema</i> sp.		
<i>Thalassiosira</i> sp.		

charged into port waters during deballasting of water, cleaning of the hold and subsequent cleaning of the ship decks. The hold cleaning process in particular involves hosing down the walls and floor using several hundred gallons of seawater, which is released directly into surrounding port waters.

#### *Sediment incubation trials*

The quantity and composition of ballast sediments varied greatly, with samples showing distinct differences in gross appearance, color, texture and water content. Samples of sediment taken from the ship which used burlap bags for containment were, as an example, desiccated compared to samples from 55 gallon drums. For all ships, examination of subsamples prior to and after sonication and fractionation revealed few recognizably live organisms. Half of the samples contained small numbers of live ciliates, varying in size from 5–30  $\mu\text{m}$ , while motile dinoflagellates were observed in one subsample. Sonicated and sieved samples revealed many identifiable objects (woodchip fibers, fecal pellets, copepod appendages, centric diatom frustules, silicoflagellate exoskeletons).

Incubation of prepared sediment subsamples resulted in a proliferation of organisms; taxa included pennate and centric diatoms, euglenoid flagellates, ciliates, and dinoflagellates (Table 2). All of these taxa have been reported in ballast sediment surveys conducted by Australian researchers. The quantity and variety of organisms present in the incubated samples were significantly greater than the non-incubated samples. The successful culture of three genera of dinoflagellates and numerous diatom species indicated the presence of cysts and spores in the sediments. The euglenoid flagellate, *Eutreptiella* spp., was cultured from the sediment samples from four ships.

Nutrient media were modified to test salinities of 22, 26 and 18 ppt for all sediments. No species were uniquely presented in salinities lower than 28 ppt. A pH-buffered silicate added to the GPM media provided more vigorous cultures of diatoms, but did not induce cultures of previously unobserved species.

#### *Water Samples*

Ballast water samples were taken from three ships. Salinity ranged from 28 to 32 ppt. Samples contained live zooplankton and phytoplankton including larval bivalves, gastropods, polychaetes, and fish as well as amphipods, isopods and copepods. These findings compared favorably with other surveys on ballast water. Notably, one ship which had exchanged water in the Pacific Ocean contained oceanic specimens, including *Pterosperma* spp. (Prasinophyceae).

### DISCUSSION

This study provides evidence that ballast sediments can act as a mechanism for the transport of microalgae into Washington State waters. It is expected that this holds true for most Pacific Northwest ports participating in the bulk shipment of raw materials to overseas ports. It cannot be concluded that the inoculation of port waters with foreign ballast water and sediments will necessarily result in established populations of non-native species. The majority of organisms entrained in ballast tanks will not survive a transoceanic voyage (Carlton 1985). However, the extensive investigations by the Australian government, and the present study provide support for the role of ballast sediments as a transport

mechanism for microalgae, and establish it as a potential pathway for both historical and future introductions.

With the exception of Australia, all ballast research efforts to date have focussed on ballast water, not ballast sediments. The present study was directed at answering the most basic questions regarding the possibility of transport of organisms in ballast sediments as they may occur in Washington state: Do ballast sediments contain viable organisms, and are they being discharged into coastal waters? With these questions having been affirmatively answered, the next set of questions that must be addressed include, which species of microalgae are being transported and what is the potential impact should they survive to become established populations, or merely give rise to a single bloom event? When approaching the issue of ballast introductions on its appropriate scale, which is global, this is not necessarily an easily executed task. Particularly in regard to phytoplankton, the current struggle to accurately identify organisms to the species level requires that recent tools in molecular technology, e.g. gene sequencing, must be used in conjunction with traditional morphology-based taxonomic methods to rigorously establish links between populations.

There is growing evidence that harmful algal blooms are increasing on a global level (see review by Hallegraeff 1993). In an effort to understand the cause of these episodes and to generate predictive models, researchers have focused on both natural and anthropogenic forces. Natural phenomena such as meteorological forces, atmospheric events and ocean dynamics have been implicated along with human-mediated impacts such as nutrient loading from agricultural practices, industry-generated pollution and global warming. The routine transport of millions of gallons of ballast water and sediments across oceans and within coastal areas must also be considered a probable factor in the global spread of toxic marine phytoplankton.

The protective life stages of many phytoplankton species represent a key feature of their potential for transport and fitness as introduced organisms. Spores and cysts provide an opportunity for some species of phytoplankton to remain viable in unfavorable conditions for varying periods of time. This characteristic merits special consideration when control options (i.e. biocidal treatments) are proposed for treating ballast discharge. Recent studies show that certain dinoflagellate cysts can survive autoclaving, treatment with strong acid and over 30 days of desiccation (Burkholder et al. 1991). Evidence that cysts can remain viable for several years implies that seeding may occur. The accumulation of benthic cysts is thought to be a factor in dinoflagellate blooms (Dale 1983, Anderson 1984). Many port areas in the U.S. may be considered relatively uninhabitable, limiting the suite of organisms which might persist. In this case, the Port of Tacoma waterway (Commencement Bay) is a Superfund site, and Port Angeles harbor is sandwiched between 2 large pulp mills. However, on-going efforts to rid these areas of pollutants along with the routine dredging and resulting aeration of sediment accumulations in port waterways could constitute a disturbed, and hence, a more favorable environment for a broad range of non-native species. The dynamics which permit biological invasions to occur are complex. It is impossible to presume the ability of a species to establish itself in a new habitat. In the end, we cannot predict or control which species will be selected by the environment to become an established population. It may be more worthwhile to expend efforts to reduce or eliminate the daily inoculation of our coastal waters by cargo ship ballast discharge.



Policy response to the problem of ballast introductions has been largely in reaction to two major invasion events. The invasion of the Great Lakes by the zebra mussel and the presence of the toxic dinoflagellate *Gymnodinium catenatum* in Australia prompted the affected countries (the U.S., Canada, and Australia) to consider restrictions on cargo vessels. This action catalyzed response by the Marine Environment Protection Committee (MEPC) of the International Maritime Organization (IMO), which, as a specialized agency of the United Nations, is charged with facilitating cooperation among member nations on technical matters relating to international shipping. On July 4 1991, IMO MEPC Resolution 50(31) "International Guidelines for Preventing the Introduction of Unwanted Aquatic Nuisance Organisms and Pathogens from Ships' Ballast Water and Sediment Discharges" was adopted. As an international resolution this agreement carries no weight as law, but provides a framework for member nations to create regulations for themselves. It provides guidance on procedures to minimize the risk from ballast introductions including the use of open-ocean exchange and land-based disposal of sediments. It also stresses education and safety as priorities.

On November 29, 1990 President Bush signed the "Non-indigenous Aquatic Nuisance Prevention and Control Act of 1990." While focused primarily on the zebra mussel invasion, the law does contain provisions for a national perspective on the issue of introduced freshwater and marine species. Because federal response has been limited, several states have developed legislation in attempt to minimize the risk of ballast associated introductions (Kelly 1992). In 1992 the state of California passed bill No. 3207 requiring incoming vessels to report ballast discharge activity and to encourage the use of the IMO guidelines. The states of Washington and Hawaii have also developed draft legislation aimed at preventing ballast introductions.

Shipping is inherently an international activity. Many would argue that any solution must be international in scope. Jurisdictional conflicts abound as one steps away from the terrestrial realm and faces the coastal environment and the maritime industry. However, Washington State, like many coastal states, depends heavily on its coastal resource. With a thriving aquaculture industry, lucrative commercial and recreational fishing resources, and a biologically rich coastal environment, the threat of introduced spe-

cies cannot be taken lightly. Aquaculturists, specifically the oyster industry, have long been labeled as one of the most prodigious distributors of non-native marine organisms (Elton 1958). Washington's oyster industry, now the largest in the country, has as its mainstay, the non-native Pacific oyster, *Crassostrea gigas* Thunberg. Yet there are several other reminders of introductions from historical oyster shipments which continue to plague both aquaculturists and natural resource managers, i.e., the salt marsh cordgrass, *Spartina alterniflora* Loisel and the Japanese oyster drill, *Ceratostoma inornatum* Recluz (Cheney and Mumford 1986).

There now exists a duality in current law. Aquaculturists who want to import non-native species for culture are asked to provide a statement of environmental impact, disease-free certification, quarantine of broodstock and evidence of acceptable health history before the state permits the shipment (Sizemore and Elston 1992). Yet every day, millions of gallons of ballast water and sediments, replete with a variety of live organisms are discharged into coastal waters, unrestricted by law.

There are some lessons to be learned from the aquaculture experience. Involvement of industry in policy development is vital. Education is essential. The tendency to over-regulate needs to be curbed to prevent oppressive and counter-productive laws. The maritime industry, in particular, presents unique challenges in that monitoring for compliance becomes a near impossibility. As shown in this research, the decisions of when to take on ballast and discharge ballast are almost unilaterally decided by the master of the ship. This autonomy must be factored into any effort to minimize introductions from ballast discharge. Only with the cooperation of the shipping industry can the threat of ballast introductions be minimized.

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## EFFECTS OF TWO BLOOM-FORMING DINOFLAGELLATES, *PROROCENTRUM MINIMUM* AND *GYRODINIUM UNCATENUM*, ON THE GROWTH AND SURVIVAL OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN 1791)

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**ABSTRACT** Laboratory experiments were conducted to investigate the effects of the dinoflagellates *Prorocentrum minimum* and *Gyrodinium uncatenum* on the growth and survival of juvenile eastern oysters, *Crassostrea virginica*. In separate experiments lasting 30 d and 18 d for *P. minimum* and *G. uncatenum*, respectively, the dinoflagellates were offered to the oysters in both unialgal and mixed diets (with the diatom *Thalassiosira weissflogii*). Eight diets were used in each experiment: (i) the dinoflagellate at bloom density, (ii) the dinoflagellate at 33% bloom density, (iii) the dinoflagellate at 5% bloom density, (iv-vi) the diatom at the above densities, (vii) 50% dinoflagellate bloom density + 50% diatom bloom density, and (viii) 5% dinoflagellate bloom density + 95% diatom bloom density.

*P. minimum* at bloom density resulted in 100% mortality of juvenile oysters within 14 d and at 33% bloom density it resulted in 43% mortality within 22 d. Diets containing 5% *P. minimum* density did not cause mortality and supported good shell growth. No mortality was observed among oysters fed *G. uncatenum* and diets which included this dinoflagellate resulted in significantly greater growth than diets of the diatom *T. weissflogii*.

**KEY WORDS:** dinoflagellates, oysters, *Crassostrea virginica*, *Prorocentrum minimum*, *Gyrodinium uncatenum*, growth, survival

### INTRODUCTION

Blooms of toxic and noxious algae are increasing worldwide in distribution, intensity and duration (Anderson, 1989; Cherfas, 1990; Smayda, 1990). While most of the attention focussed on toxic blooms has been related to species which pose public health risks, evidence is mounting that numerous species of algae, which apparently do not threaten human health, may nevertheless be noxious or harmful for bivalves (see Shumway, 1990; Shumway et al., 1990 and references therein).

In Chesapeake Bay, USA, no occurrences of PSP, DSP, or NSP have been recorded (VA Health Department) and the causative species (within the genera *Gymnodinium*, *Pyrodinium*, *Protogonyaulax*, *Dinophysis* and *Ptychodiscus*) have not been reported. Yet, substantial impacts on shellfish resources, particularly bivalve culture operations, have been observed. Anecdotal evidence from commercial hard clam (*Mercenaria mercenaria* (L.) and oyster (*Crassostrea virginica*) aquaculturists in Virginia suggests that dinoflagellate blooms in the late spring/early summer and in the late summer/early fall are responsible for widespread mortalities of juveniles (Cherrystone Aquafarms, Bagwell Enterprises, Intertidal Marine, pers., comm.). When they occur, late spring/early summer dinoflagellate blooms in this area are domi-

nated by *Prorocentrum minimum* (var. *mariae-lebouriae*) and late summer/early fall blooms by a *Gyrodinium-Coccolodinium-Gymnodinium* complex (Mackiernan, 1968; Zubkoff et al., 1979; Marshall, 1993; Sellner and Luckenbach, pers. obs.). At the Virginia Institute of Marine Science Oyster Hatchery, located on the York River estuary, Chesapeake Bay, Virginia, we have consistently observed impacts of dinoflagellate blooms on oyster reproduction, growth and survival. Conditioned adult oysters frequently do not spawn in the presence of bloom densities of *P. minimum*, and early larval development is impaired and high mortalities occur when *P. minimum* or the lysate from ruptured cells is present in larval culture tanks (V. Shaffer and M. Luckenbach, unpublished data). Juvenile oysters (from metamorphosis to ca. 2 cm shell height) within a land-based, flow-through nursery and an overboard floating nursery system exhibit little or no growth during the late summer bloom (M. Luckenbach, unpublished data).

The lack of quantitative evidence on the effects of these dinoflagellate blooms on oyster aquaculture in Virginia lead us to initiate investigations to (i) document the extent and composition of dinoflagellate blooms in the lower Chesapeake Bay, (ii) determine filtration and ingestion rates for selected bloom species in monocultures and in mixed diets, (iii) evaluate growth and survival of oysters feeding on two bloom-forming dinoflagellate spe-

cies, and (iv) quantify the effects of dinoflagellate blooms on oyster growth and survival in the field. This report addresses the third of these objectives by detailing results from laboratory experiments with juvenile oysters fed *P. minimum* and *G. uncatenum*.

### MATERIALS AND METHODS

Separate experiments were run to investigate the effects of the dinoflagellates *Prorocentrum minimum* and *Gyrodinium uncatenum* on the growth and survival of juvenile oysters. Both experiments were conducted at the Virginia Institute of Marine Science Oyster Hatchery using single cohort oysters ranging in shell height from 2.5 to 3.8 cm. These oysters were reared for 6 to 9 months in ambient waters of the Chesapeake Bay then transferred to the hatchery where they were maintained for at least two weeks prior to experiments on cultures of *Isochrysis galbana* (Tahitian strain) and *Thalassiosira pseudonana* (clone 3-H) and suspensions of *Thalassiosira weissflogii* (clone T.FLUV) paste.

Dinoflagellates were cultured in the hatchery using unialgal stock solutions. *P. minimum* (HP9001) was supplied by the University of Maryland Laboratory at Horn Point and *G. uncatenum* (CCMP1310) was obtained from the Provasoli-Guillard culture collection. Cultures from 227 L Kalwall tubes were used to inoculate 2460 L vats and the phytoplankton were allowed to reach bloom levels. Daily cell counts and/or in vivo fluorescence measurements were made on samples from these vats to determine cell densities and the species composition of the vat assemblage. Bloom densities ranged from  $8.9 \times 10^3$  to  $2.5 \times 10^5$  cells  $\cdot$  ml $^{-1}$  and  $1.6 \times 10^3$  to  $1.0 \times 10^4$  cells  $\cdot$  ml $^{-1}$  for *P. minimum* and *G. uncatenum*, respectively. Growth and survival experiments were conducted as long as these densities were maintained and contamination by other species was minimal. The diatom *Thalassiosira weissflogii* was cultured in the hatchery and centrifuged (15,000 rpm for ca. 4 hrs) to produce a paste; resuspended cells from this paste provide a diet known to support oyster growth in the laboratory.

Eight diets were used in each experiment (Table 1). Bloom density of the dinoflagellate varied daily within the limits specified above, while bloom densities of *T. weissflogii* were achieved by resuspending an appropriate quantity of paste in filtered water to match daily counts from the dinoflagellate culture. Reduced densities were achieved by diluting bloom suspensions with filtered estuarine water.

The experimental design was similar for each experiment. Seven (for the *P. minimum* experiment) or 10 (for the *G. uncatenum*

experiment) individually numbered and pre-measured oysters were randomly allocated to each of 24 20-L plastic containers. Three of these were assigned to each of the eight diets above; a fourth container for each treatment received the algae diet, but no oysters. Twenty L of the appropriate algae suspension were added to each container. Light aeration helped maintain algae in suspension and precluded the development of hypoxia in the experimental containers. Water was changed and new rations provided daily between 0800 and 1030 for all treatments throughout the duration of the experiments. Additionally, the 5% bloom treatments were changed and fed between 1730 and 1830 daily. Preliminary measurements had revealed that densities approximating 33–100% bloom levels could be maintained for 24 hrs, but that 5% bloom treatments were substantially grazed down within 12 hrs. The *P. minimum* experiment was initiated on June 16, 1992 and terminated on July 20, 1992; the *G. uncatenum* experiment was run from Jan. 12–30, 1993.

Containers were inspected daily for moribund oysters and any dead oysters were removed and replaced with live ones. Water temperature and salinity were measured daily and dissolved oxygen occasionally throughout the experiments. In vivo fluorescence measures were made at various times throughout the day from each treatment replicate. Grazing rates were estimated according to Coughlan (1969) using regressions established between cell counts and fluorescence (*P. minimum*:  $y = 11065x + 6920$ ,  $r^2 = 0.71$ ; *G. uncatenum*:  $y = 1215.6x + 31.5$ ,  $r^2 = 0.87$ ; *T. weissflogii*:  $y = 12344.4x + 0$ ,  $r^2 = 0.85$ ). The no-oyster control containers provided a means of accounting for passive deposition and reproduction growth of algae.

All oysters were photographed (right valve up) at the initiation and termination of the experiment (or sooner if mortality occurred). Photographs were digitized (International Imaging Systems, Model 75 Image Processor) and shell growth computed as the change of shell surface area, expressed as mm $^2 \cdot$  d $^{-1}$ . We analysed for differences in shell growth across diets and between containers within diet using a 2-way, nested analysis of variance followed by Tukey's a posteriori multiple comparisons tests where appropriate (Sokal and Rohlf, 1981).

### RESULTS

#### *Prorocentrum* Experiment

Water temperature within the experimental containers ranged from 19.8–30.5°C and salinity varied from 15 to 18 ppt during the course of the experiment. Dissolved oxygen levels varied from 3.6–8.0 mg  $\cdot$  L $^{-1}$  with lowest levels recorded in bloom concentrations in early morning readings. There was no indication of low D.O. induced mortality.

Grazing rates indicate that oysters fed at reduced rates on *P. minimum* relative to the diatom *T. weissflogii* in the unialgal diets (Table 2). Clearance rates in the mixed diets could not be estimated in this study because of the differing regressions between in vivo fluorescence and cell counts for the two species. An inverse relationship between grazing rate and cell density was observed for *P. minimum*, but not *T. weissflogii* (Table 2).

Forty-seven percent mortality occurred in the 100% *P. minimum* bloom treatment on day 11 of the experiment and by day 14 100% of the original oysters in that treatment had died (Fig. 1). Mortality in the 33% dinoflagellate bloom treatment began on day 10 and stabilized at 43% on day 22. No mortality was observed in any of the other diets (Fig. 1).

TABLE 1.

Algal diets used in each experiment with the dinoflagellates *Prorocentrum mariae-lebouriae* and *Gyrodinium uncatenum*. See text for cell concentration ranges at bloom densities.

Diet	Algae
I	100% bloom density, dinoflagellate
II	33% bloom density, dinoflagellate
III	5% bloom density, dinoflagellate
IV	100% bloom density, <i>T. weissflogii</i>
V	33% bloom density, <i>T. weissflogii</i>
VI	5% bloom density, <i>T. weissflogii</i>
VII	50% bloom density, dinoflagellate + 50% bloom density, <i>T. weissflogii</i>
VIII	5% bloom density, dinoflagellate 95% bloom density, <i>T. weissflogii</i>

TABLE 2.

Grazing rates on unialgal diets for (A) *Prorocentrum minimum* experiment and (B) *Gyrodinium uncatenum* experiment. Means and standard deviations are derived from estimates made for 3 replicate containers during 2–6 grazing periods per day for most days over the duration of the experiment; SD's represent variances between daily averages.

Algae	Diet	Mean Grazing Rate ( $L \cdot O_y^{-1} \cdot Hr^{-1}$ )	SD
A. <i>P. minimum</i>	100% bloom	0.030	0.074
	33% bloom	0.063	0.140
	5% bloom	0.117	0.159
<i>T. weissflogii</i>	100% bloom	0.301	0.224
	33% bloom	0.379	0.198
	5% bloom	0.388	0.233
B. <i>G. uncatenum</i>	100% bloom	0.051	0.115
	33% bloom	0.338	0.235
	5% bloom	0.310	0.039
<i>T. weissflogii</i>	100% bloom	0.252	0.145
	33% bloom	0.240	0.121
	5% bloom	0.224	0.105

Shell growth varied significantly among dietary treatments ( $F = 17.06$ ,  $p < 0.0005$ ), but not among containers within treatments ( $F = 1.40$ ,  $p = 0.150$ ). Rank orderings of treatment means revealed a surprising pattern with the diets containing 5% bloom concentrations of *P. minimum* having the greatest growth (Fig. 2). Tukey's multiple comparisons tests indicated clear differences between the growth rates on diets with minimal *P. minimum* densities (diets III, V, VI & VIII) and those on diets with high *P. minimum* densities (I & II).

#### *Gyrodinium* Experiment

Water temperature varied from 20.5–27.5°C and salinity ranged from 8–14 ppt. Dissolved oxygen levels ranged from 6.98–9.64  $mg \cdot L^{-1}$ .

Grazing rate estimates reveal that *G. uncatenum* and *T. weissflogii* were both consumed in the unialgal treatments; again, graz-

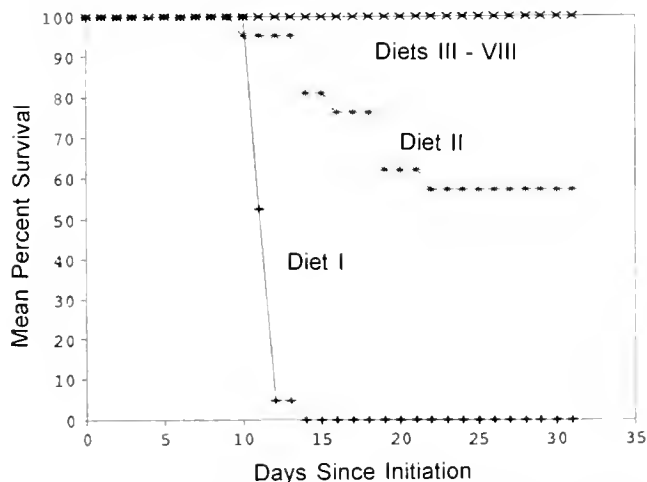


Figure 1. Survival of *Crassostrea virginica* in unialgal and mixed diets with *Prorocentrum minimum* and *Thalassiosira weissflogii*. Diet designations are as in Table I. Data are for oysters placed in each diet at the initiation of the experiment only and do not include replacement oysters.

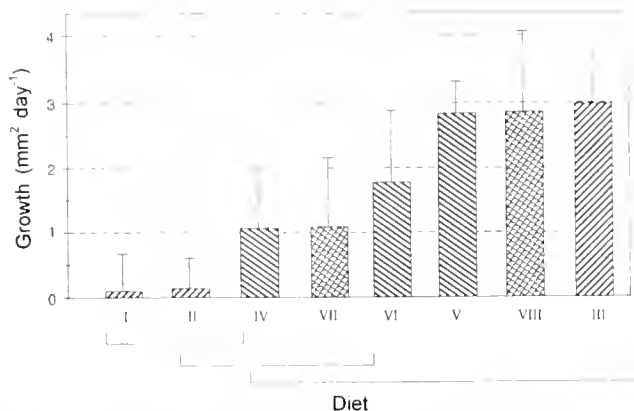


Figure 2. Growth of *Crassostrea virginica* on unialgal and mixed diets with *Prorocentrum minimum* and *Thalassiosira weissflogii* expressed as changes in surface area of the right valve per day (see text). Diet designations are as in Table I. Error bars represent one standard deviation of the mean. Growth rates on diets not connected by a line are significantly different (experiment-wise error rate  $< 0.05$ , Tukey's multiple comparisons test).

ing in mixed diets could not be estimated (Table 2). Lower clearance rates were observed in the bloom density of the dinoflagellate than in the reduced densities, but again no relationship between cell density and clearance rate was noted with the diatom (Table 2).

One oyster died in the 50/50 mixture of *G. uncatenum* and *T. weissflogii* (diet VII) on day 2 of the experiment. No further mortality occurred in any of the treatments during the feeding trials with *G. uncatenum*.

Significant variation in shell growth occurred between algal diets ( $F = 15.36$ ,  $p < 0.0005$ ), but not between replicate containers within a diet ( $F = 1.31$ ,  $p = 0.19$ ). Shell growth was greater in the diets which included *G. uncatenum* than in those lacking the dinoflagellate and the greatest growth was observed on the 33% bloom diet (Fig. 3).

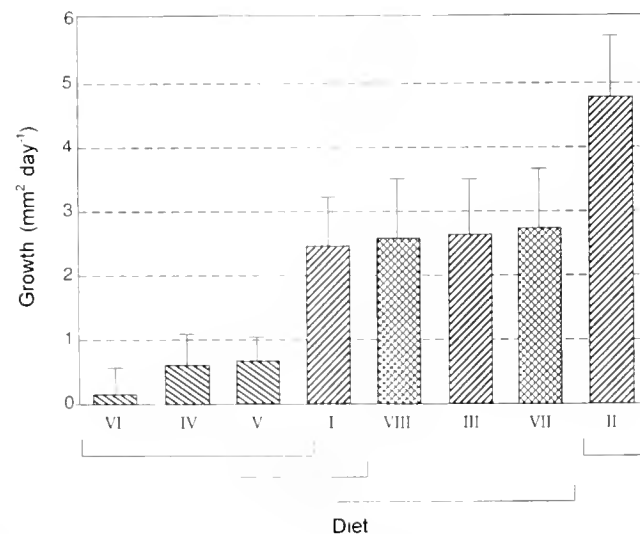


Figure 3. Growth of *Crassostrea virginica* on unialgal and mixed diets with *Gyrodinium uncatenum* and *Thalassiosira weissflogii* expressed as changes in surface area of the right valve per day (see text). Symbols as in Fig. 2.

## DISCUSSION

*Prorocentrum minimum* and *Gyrodinium uncatenum* are frequent late spring and summer bloom-forming members of the phytoplankton community, respectively (Tyler and Seliger, 1978; Tyler, et al., 1982) and, as such, could potentially provide an abundant food supply for production in suspension feeding bivalves. Dinoflagellates have proven to be excellent substrates for elevated egg production and growth rates in planktonic copepods versus other foods such as diatoms (e.g., Paffenhofer, 1976; Morey-Gaines, 1979; Smith and Lane, 1985; Kleppel et al., 1991). Alternatively, bloom levels of each dinoflagellate could inhibit oyster feeding and/or growth via cell-induced feeding problems or development of hypoxic-anoxic habitats shutting down the oyster.

Juvenile oysters responded differently in this experiment to blooms of the two dinoflagellate species, dying at bloom and 33% bloom densities of *P. minimum* and growing well on *G. uncatenum*.

Positive clearance rates and the observations of fecal production indicate that *P. minimum* was ingested by oysters, but standard deviations on the order of the means indicate considerable daily variation. Mean grazing rates for *P. minimum* and *T. weissflogii* reported here are lower than those observed by Sellner et al. (in press). Using similar-sized oysters (shell height: 2.5–3.8 cm) in grazing experiments conducted between 21.5 and 25.0°C, with approximate concentrations of  $10^4$  cells  $\text{mL}^{-1}$ , Sellner et al. reported values of 1.95 and 3.73  $\text{L} \cdot (\text{oyster h})^{-1}$  for juvenile oysters fed on the dinoflagellate and the diatom, respectively. Unlike the present study, they used individual oysters in short-term feeding experiments (0.5–1.0 h) and analysed samples only for oysters which actually fed. The values reported in this study are means which reflect periods of non-feeding by some oysters. Additionally, high variability in daily densities for a given diet in these experiments would also increase variability in the grazing rates. For example, "bloom" levels of *P. minimum* ranged from  $8.9 \times 10^3 - 2.5 \times 10^5 \text{ L}^{-1}$ . Oysters may alter filtration rates accordingly, increasing filtration at lower cell densities. The principal utility of the clearance rates reported here lies in confirming that dinoflagellates were indeed cleared from suspension.

Mortality in the bloom and 33% bloom concentrations of *P. minimum* is presumed to be the impact of harmful effects of the dinoflagellate and not secondary low dissolved oxygen effects. The lowest D.O. levels measured near dawn in the bloom treatment was 3.6  $\text{mg} \cdot \text{L}^{-1}$ , above the lethal limit for juvenile oysters. At 5% bloom densities *P. minimum*, both in a unialgal diet and in combination with *T. weissflogii*, did not cause mortality and supported good growth. The rank ordering of mean growth rates in this experiment (see Fig. 2) suggests that at low densities *P. minimum* may support growth as well or better than *T. weissflogii* alone. The harmful impacts, if any, of longer-term exposure to reduced concentrations of *P. minimum* cannot be assessed from the present study.

Limited data are available on harmful effects of other strains of *P. minimum* to oysters and other shellfish. Wickfors et al. (1993) have reported that the EXUV strain of *P. minimum* did not support larval development and supported only minimal juvenile growth in the eastern oyster. However, they observed no mortality of juvenile oysters over a six-week period and suggested that nutritional deficiency or digestive interference, rather than acute toxicity, was responsible for the observed patterns. This strain of *P. minimum* is also a poor food source for juvenile hard clams, *Mercenaria mercenaria*, but it is apparently highly toxic to juvenile bay scallops,

*Argopecten irradians* (Lamarck, 1819) (Wickfors and Smolowitz, 1992). *P. minimum* has been implicated in the mortality of adult oysters on the French Atlantic coast (Lassus and Berthome, 1988). Nakazima (1965a; 1965b; 1956c; 1968) credited *P. minimum* with causing outbreaks of shellfish poisoning in *Tapes japonica* (Gmelin, 1791) which have been lethal to humans.

*G. uncatenum*, alone and in mixed diets with *T. weissflogii*, supported oyster growth which was greater than or equal to the diatom alone. Growth on *T. weissflogii* alone was lower in the experiment with *G. uncatenum* than in the one with *P. minimum* (compare diets IV, V & VI in Figs. 2 & 3), presumably a result of lower rations in the former experiment. "Bloom" levels for the diatom were achieved by matching cell concentrations with daily counts from the dinoflagellate cultures; since *G. uncatenum* bloom densities were generally lower than those for *P. minimum*, lower concentrations of the diatom were offered in the former.

On day 2 of the *G. uncatenum* experiment one oyster died in the 50/50 dinoflagellate/diatom diet. This was presumably not in response to the diet, since no other deaths occurred during the experiment. The 18 d duration of the experiment was set by our ability to maintain bloom levels of *G. uncatenum*; it is possible that longer term exposures might have produced other effects. The apparent lack of toxic impacts of *G. uncatenum* on juvenile oysters suggests that field observations of oyster mortalities and reduced growth during late summer/early fall blooms in the lower Chesapeake Bay are the result of other dinoflagellate species. Two major components of these blooms *Cochlodinium heterobatum* and *Gymnodinium splendens* have been reported to be toxic to oysters (Woelke, 1961; Cardwell et al., 1979; Ho, and Zubkoff, 1979).

Species-specific and density-dependent effects of bloom-forming dinoflagellates on *C. virginica* will warrant further attention by fishery resource managers and aquaculturists in Chesapeake Bay. Shumway (1990) noted that there are few practical options for reducing bloom impacts on shellfish culture, but that early warning systems are requisite for the continued growth of shellfish aquaculture. Variable responses of oysters to bloom-forming species in this region point to the need for reliable monitoring in support of aquaculture to track bloom composition and development.

In summary, *P. minimum* proved an unsatisfactory food at elevated levels, reducing filtration rates and elevating mortality in juvenile oysters. However, highest growth in a food mixture with minimal *P. minimum* levels suggests that oysters might conceivably exact substantial grazing pressure on low dinoflagellate densities, retarding development of late spring blooms of this taxon as well as enhancing oyster production. Spatial decoupling of oysters and low *P. minimum* densities in the spring could release *P. minimum* from oyster predation, permitting bloom development, and, in turn, yield poor food environments for subsequent oyster growth.

In contrast, *G. uncatenum* was an acceptable food for juvenile oysters, supporting high growth rates at near bloom levels ( $33\%$  of  $0.05 - 0.3 \times 10^4$  cells  $\cdot \text{ml}^{-1}$ ). As discussed for copepods (see references above), this dinoflagellate supports highest growth, exceeding that observed with the normal "good" food, the diatom *Thalassiosira weissflogii*. Spatial overlap of this dinoflagellate and oyster populations would be expected to lead to substantial losses of *G. uncatenum* to herbivory. Frequent *G. uncatenum* blooms on the Bay might therefore reflect the paucity of healthy oyster populations in the Bay over the last decade.

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## EXPERIMENTAL STUDY OF THE EFFECTS OF A TOXIC MICROALGAL DIET ON FEEDING OF THE OYSTER *CRASSOSTREA GIGAS* THUNBERG

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**ABSTRACT** The aquaculture industry is often faced with the problem of toxic microalgal blooms that can cause human poisoning after contamination of cultivated shellfish. The oyster *Crassostrea gigas*, because of its particular sensitivity to dinoflagellates that produce paralytic toxins, was chosen as a model for study of the absorption of different monospecific algal diets of varying toxicity. A continuous, flow-through system was used to expose batches of shellfish successively to nontoxic and toxic diets of *Alexandrium tamarense* (7 200 ng STX eq. per 10<sup>6</sup> cells). The same experiment was repeated with other batches for cultures of *Scrippsiella trochoidea* (nontoxic) and *Alexandrium minutum* (500 ng eq. STX per 10<sup>6</sup> cells). The results indicate that the clearance rate decreases in the order *Scrippsiella trochoidea* > *A. minutum* > nontoxic *A. tamarense* > toxic *A. tamarense*. It was difficult to determine the exact nature of the physiological process enabling oysters to perform selective feeding since particle size probably interfered with toxin effects. However, *C. gigas* would seem to prefer very toxic *Alexandrium* cells during absorption in the digestive tube, perhaps because of higher nutritive value or easier digestibility.

**KEY WORDS:** *Crassostrea gigas*, ecophysiology, nutrition, paralytic toxins, *Alexandrium tamarense*, *Alexandrium minutum*

### INTRODUCTION

Contamination of bivalves with paralytic shellfish poison (PSP)-producing toxic algae in natural or experimental conditions has often been studied with regard to the kinetics of toxin accumulation and distribution to each organ (Shumway and Cucci, 1987; Gainey and Shumway, 1988; Lassus et al., 1989; Shumway, 1990; Bricelj et al., 1990, 1991). However, the direct effects of these Protista on bivalve metabolism, particularly the filtration rate and assimilation, are less known. Studies of selective nutrition (Loosanoff, 1949; Cucci et al., 1985; Shumway et al., 1985a, b) and the effects of the dinoflagellate *Protogonyaulax tamarensis* (= *Alexandrium tamarense*) on the behavior and physiology of bivalve mollusks (Gainey and Shumway, 1988) have demonstrated that the animals respond differently to varying food sources. Thus, there is no single response to toxic microalgae, but instead species-specific responses. Moreover, it has been suggested that mollusks periodically exposed to toxic blooms would develop mechanisms allowing them to feed on microalgae without risk of death which has led to significant geographical variations for the same shellfish species (Twarog and Yamaguchi, 1974; Bricelj et al., 1991).

Species-specific behavioral and/or physiological responses to toxic dinoflagellates range from none (*Mytilus edulis* in Maine; Shumway and Cucci, 1987) to complete isolation from the environment or even fleeing [moving away by clicking valves in the case of *Placopecten magellanicus*, or digging into the sediment in the case of *Mercenaria mercenaria* (Shumway, 1989)]. Responses vary according to the toxic strain present, the bivalve species and the geographical environment, as well as among individuals in a given locality.

Oysters (*Crassostrea* spp.) are highly sensitive to neurotoxins and show more negative reactions than resistant bivalves. Nevertheless, these reactions are quite variable and sometimes contrary,

particularly for valve-closing reflexes (Ray and Aldrich, 1967; Dupuy and Sparks, 1968; Sievers, 1969) and clearance rates (Twarog and Yamaguchi, 1974; Shumway and Cucci, 1987). With respect to feeding habits, it has been shown (Shumway and Cucci, 1987) that *Ostrea edulis* and *Crassostrea virginica* tend to reject a toxic strain of *Protogonyaulax tamarensis* (= *Alexandrium tamarense*) in the form of pseudofeces. However, there are no complete data on ingestion and absorption rates or the absorption efficiency of oysters fed with toxic microalgae (Shumway et al., 1990). Accordingly, ecophysiological studies of toxic dinoflagellate effects on commercial bivalves would not only improve our understanding of this phenomenon but contribute to a better definition of risk evaluation criteria, especially for the introduction of new species into sensitive areas. Our purpose in this study was to evaluate the real impact of paralytic poison producers by conducting continuous flow-through experiments using a reputedly sensitive indicator, the oyster *Crassostrea gigas*, in the presence of several strains of dinoflagellates with variable PSP toxicity but comparable cell diameter (to minimize effects due to cell size).

### MATERIALS AND METHODS

Oysters (*Crassostrea gigas* Thunberg), each 60 to 70 g in total weight (soft tissue dry weight 1 to 3 g), were collected in April and May in Bourgneuf Bay (western Atlantic coast of France) in an area not exposed to toxic algal blooms and then acclimated for a week at 16°C in aerated 35-liter tanks (8 individuals per tank). During this period, the oysters were fed daily with a culture of the diatom *Thalassiosira weissflogii* (Grunow) Fryxell and Hasle, and tank water was changed every two days.

Four experiments were successively conducted with each of the four unialgal diets: nontoxic (Plymouth Strain, U.K.) and toxic (Onagawa strain, Japan) *Alexandrium tamarense* Taylor (Balech), nontoxic *Scrippsiella trochoidea* (Stein) Loeblich III and toxic *A.*

*minutum* Halim. The same batch of oysters was exposed successively to non-toxic and toxic *A. tamarensis*, and a different group to *Scrippsiella* and *A. minutum*. Toxic profiles for *A. tamarensis* and *A. minutum* are given in Table 1. Overall toxicities, expressed as ng eq. STX per  $10^6$  cells were respectively 7 200 for *A. tamarensis* and 500 for *A. minutum*. This acclimation period was assumed to reduce, as much as possible, the effect of food particle size change at the beginning of the experiment. The oysters were progressively acclimated to experimental conditions for a day by placing them individually in aerated 6 4-liter tanks and feeding them with a mixture of *T. weissflogii* and the nontoxic dinoflagellate (*A. tamarensis* Plymouth or *S. trochoidea*).

Mean algal concentrations for the different experiments (Table 3) were based on field values observed during red tide phenomena (Delgado et al., 1990; Boni et al., 1983) and intended to favor pseudofeces production (Deslous-Paoli et al., 1992).

Behavioral (valve activity) and nutritional effects were studied in these various microalgal treatments. Nutritional effects were evaluated by filtration rates (indirect measurement of phytoplankton cell concentrations in water) and determination of consumption, ingestion and absorption as expressed by the quantity of matter or seston (indirect measurement of total and organic seston weights in water and direct measurement of biodeposit production: pseudofeces and feces). Particulate organic matter (POM) and particulate inorganic matter (PIM) were estimated by weighing algal cells collected on Whatman GF/C filters first at 60°C and then again, after combustion, at 450°C. Cell concentrations were determined using a Multisizer particle counter (Coultronics) equipped with a 100  $\mu\text{m}$  aperture probe.

The use of the same species of oysters previously studied in a semi-open system (Anon., 1987) and exposed successively to non-toxic and toxic strains facilitated comparison of the effects of the different diets. Natural 35‰ salinity sea water flowed into the 6 experimental 1 l boxes from an 87-l tank maintained at experimental temperature (16°C), and supplied (by an electroswitch) with sea water stored in a 30 m<sup>3</sup> outside tank.

Before being introduced (input) into the experimental boxes, flowing sea water was mixed with algal culture in a homogenization chamber to obtain a steady algal concentration in each box. Water flows for each experimental species ranged from 4.2 to 5.2 l · h<sup>-1</sup>, and mean microalgal sizes from 26.9 to 36.3  $\mu\text{m}$ . (Table 2). Residual sea water (output) containing toxic algae, was trapped in a tank filled with sodium hypochlorite. The following parameters were used to quantify the effects of the different diets:

1. percent retention R, such that  $R = (C1 - C2)/C \times 100$
2. filtration (or clearance) rate F, such that  $F = R \times D = [(C1 - C2)/C1] \times D$  (Vahl., 1972)

TABLE 1.

Toxic profiles (in percent of toxin) of the two algal strains used for experimental contamination of *C. gigas*. (in: Ledoux, 1991 and Erard-Le Denn, 1991).

	<i>Alexandrium tamarensis</i>	<i>Alexandrium minutum</i>
C toxins	77.2	traces
GTX 4	12.9	—
GTX 1	6.2	—
GTX 3	1.6	62.2
GTX 2	0.7	37.7
Neo STX	1.4	—

TABLE 2.

Mean cell diameter ( $\mu\text{m}$ ) determined from particle size distribution (Multisizer) and range for the 4 experimental dinoflagellates, and the corresponding water flow rate through the oyster feeding chambers (in l · h<sup>-1</sup>).

Experimental Species	Mean Diameter	Range (min-max)	Flow Rate
<i>Alexandrium tamarensis</i>			
Plymouth (nontoxic)	36.3	20-48	4.2
<i>A. tamarensis</i> (MOG 835)	30.9	20-40	4.6
<i>Scrippsiella trochoidea</i>	26.9	16-35	5.2
<i>A. minutum</i> (toxic)	29.5	14-35	4.6

3. consumption C, such that  $C = (\text{POM input} - \text{POM output}) \times D$

4. ingestion I = C - PF, and absorption Ab = I - F

where C1 = phytoplankton cell concentration (cells · l<sup>-1</sup>) at the control tank outlet; C2 = cell concentration at the experimental tank outlet; D = flow within the tanks in l · h<sup>-1</sup>; PF = hourly production of pseudofeces in mg POM · h<sup>-1</sup>; and F = hourly production of feces in mg POM · h<sup>-1</sup>. The units of the different parameters measured were as follows: R in %, F in l · h<sup>-1</sup>, C in mg · l<sup>-1</sup> and Ab in mg · h<sup>-1</sup>. Hourly clearance rate measurements were done. All results are expressed as weight-specific values, F/W for 1 g dry weight of oyster meat as determined after 48 h of lyophilization.

The algal strains used were provided by the Plymouth Marine Biology Laboratory (*S. trochoidea*, nontoxic *A. tamarensis* and the diatom *T. weissflogii*) and the University of Sendai, Japan [(toxic *A. tamarensis* (MOG 835)]. The culture of toxic *A. minutum* was isolated at the IFREMER Center in Brest, France. The dinoflagellates were cultured at 16°C and 37‰ salinity with a 12:12 photoperiod (2,500 lux) in ES medium (Provasoli et al., 1966). In these conditions, they attained concentrations ranging from 35,103 to 60,103 cells · ml<sup>-1</sup> in 15 to 25 days. Dinoflagellate cultures were used at the end of the exponential phase for each experiment.

## RESULTS

Experimental seston concentrations (mg · l<sup>-1</sup>) were the same for toxic and nontoxic *A. tamarensis* but greater for *S. trochoidea* than *A. minutum* (Table 3). In general, the percentage of POM relative to total dry matter in the dinoflagellate cells (PIM + POM) was high and in the same range for all 4 strains. Differences in food supply in mg/million of cells were due essentially to cell size and volume: *A. tamarensis* was larger than *S. trochoidea* or *A. minutum* and thus had a relatively higher PIM + POM value.

Oyster behavior sometimes varied among individuals, or over the time for a single individual during the experiments. For this reason, aberrant values for relatively inactive individuals were excluded in order to maintain a homogeneous population. However, all individuals reacted similarly upon contact with the more toxic species (MOG 835): regular and violent valve clicking for an hour, then slight and nearly continual opening during the rest of the experiment. Valve closure reactions to the less toxic species (*A. minutum*) were less pronounced.

Mean clearance rates (Fig. 1) showed statistically significant differences at the 1% level between toxic (0.04 l · h<sup>-1</sup>) and nontoxic (0.26 l · h<sup>-1</sup>) *A. tamarensis* as well as between *S. trochoidea* (0.86 l · h<sup>-1</sup>) and *A. minutum* (0.32 l · h<sup>-1</sup>). In both trials, the

TABLE 3.

Values for suspended particulate dry matter concentrations in the 4 strains of microalgae fed to experimental oysters (PIM = particulate inorganic matter, POM = particulate organic matter).

Species	Mean Supply in cells/ml	Mean Seston Supply in mg/l		Mean Algal Dry Weight and Organic Content in mg/million Cells	
		PIM + POM	POM	PIM + POM	POM
<i>Alexandrium tamarense</i>					
Plymouth	1,970	8.22	6.41	4.2	3.2
<i>A. tamarense</i> (MOG 835)	2,445	6.65	5.76	2.7	2.5
<i>Scrippsiella trochoidea</i>	9,130	11.42	10.14	1.2	1.1
<i>A. minutum</i>	4,000	7.62	6.38	1.9	1.6

clearance rate for the toxic species decreased sharply during the first hour of contact, even reaching the null level for the more toxic strain (MOG 835).

Biodeposition after 6 hours showed markedly higher mean production of feces as well as slightly to markedly higher mean production of pseudofeces in nontoxic as compared to toxic strains (Fig. 2). Mean consumption (with standard deviation) was greater for the nontoxic species, i.e.,  $1.53 \pm 1.10$  vs  $0.74 \pm 0.92$   $\text{mg} \cdot \text{h}^{-1}$  respectively for nontoxic and toxic *A. tamarense*, and  $5.30 \pm 1.57$  vs  $0.43 \pm 0.33$   $\text{mg} \cdot \text{h}^{-1}$  for *S. trochoidea* and *A. minutum*. Finally, the net values expressed in terms of mean ingestion and absorption efficiency were determined for the 4 experimental monospecific diets (Fig. 3). When the consumption percentage was expressed as 100% for each strain, the ingestion rate differed only slightly or not at all between toxic and nontoxic diets, though it was lower for the less toxic *A. minutum* (60.3%) than for the more toxic MOG 835 (87.2%). Conversely, absorption yields (in %) indicated marked differences between the absorption rate for toxic *A. tamarense*, which was much higher than that for the nontoxic strain, and the rate for *A. minutum* which is much lower than that of *S. trochoidea*. The percentage of absorption efficiency ( $A/I \times 100$ , Table 4) gave similar results.

#### DISCUSSION

In the oyster *C. gigas*, exposure to toxic PSP-producing dinoflagellates led to disturbed behavioral response (particularly in valve activity) independent of food particle size. Moreover, excessive valve activity was more marked upon exposure to the more toxic strain of *A. tamarense* than to the less toxic *A. minutum*. These data confirm the results of Dupuy and Sparks (1968) who observed the same reaction (closing of valves, sometimes with vigorous clapping) when *C. gigas* was exposed to *Gonyaulax washingtonensis*. Likewise, Shumway and Cucci (1987) noted initial withdrawal of *C. virginica* in the presence of *Protogonyaulax tamarensis*.

Moreover, particle filtration decreased in the presence of toxic algae, sometimes becoming null for the most toxic strain after the first hour of exposure, whereas filtration was comparable to that of the nontoxic control at the beginning of the experiment. This sharp drop might have been due to pre- or post-ingestion detection by the oyster of the toxic nature of the alga, leading to reduced filtration within a short time period (less than an hour). Dupuy and Sparks (1986) also observed such decreased clearance and pumping in *C. gigas* exposed to *G. washingtonensis*, and Shumway and Cucci (1987) confirmed these findings for *C. virginica* fed with *G. tamarensis*.

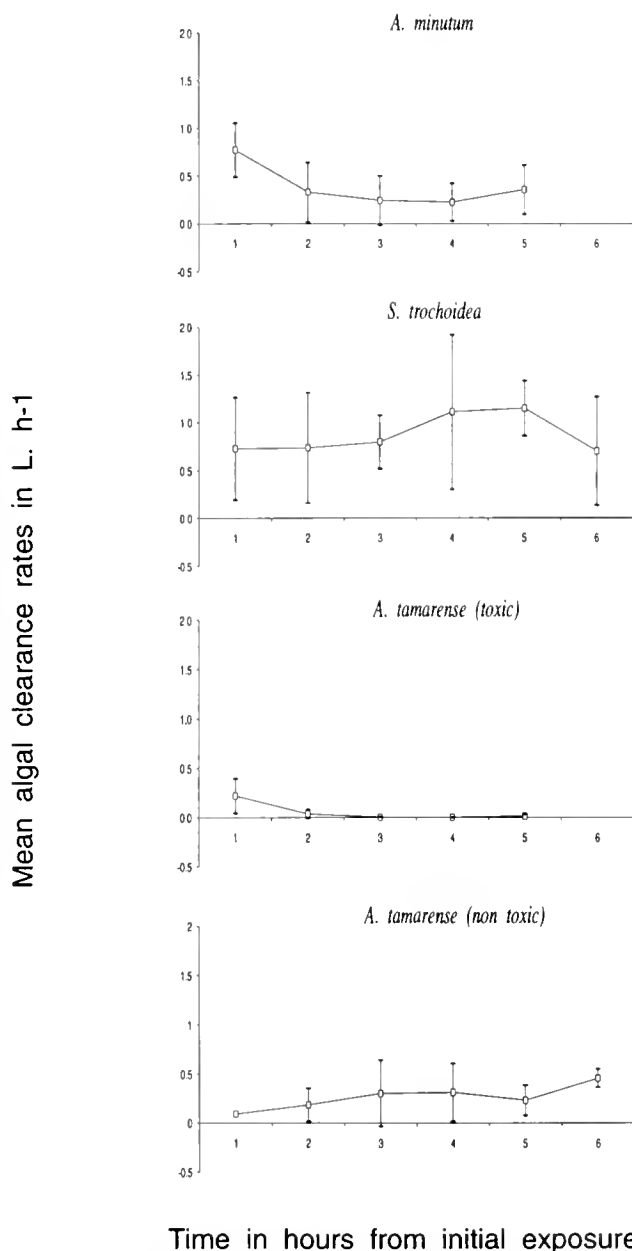


Figure 1. Mean filtration rates (standard deviations for the six individuals are indicated by a vertical bar) for the 4 microalgal strains in the oyster, *Crassostrea gigas* tested during 6 hours of experiments.

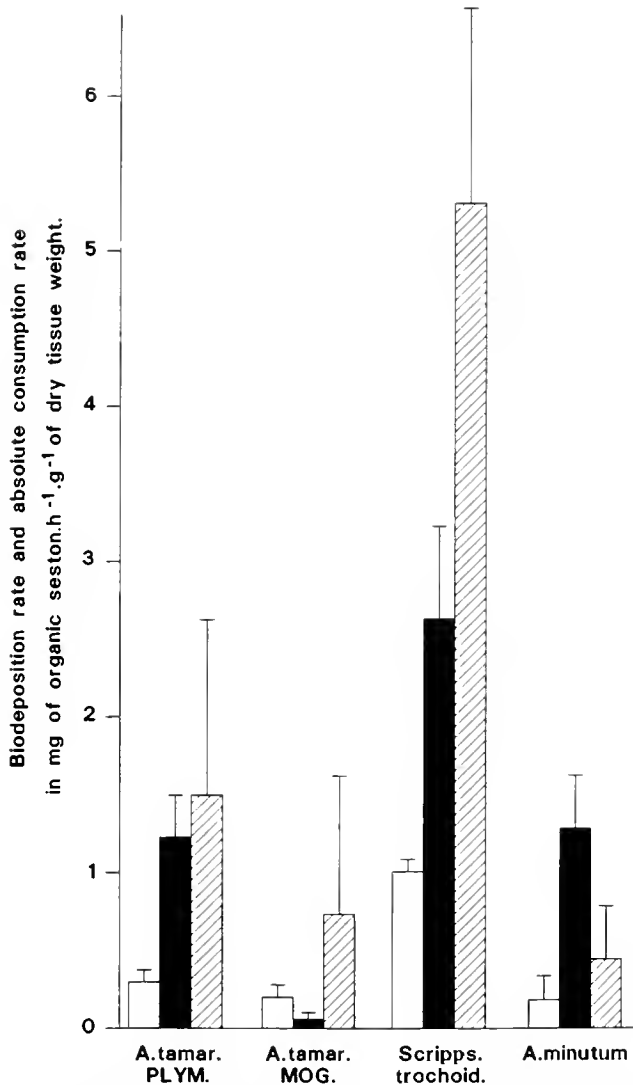


Figure 2. Mean time-averaged production of pseudofeces (white bars) and feces (dark bars), and absolute consumption rate (striped bars) in mg of particulate organic material (POM) per hour and gram of oyster soft-tissue dry weight (GDW) for different microalgal diets. Standard deviations for the six individuals are represented by a vertical bar.

Biodeposit production was generally lower than that previously measured in the natural environment or the laboratory (Deslous-Paoli et al., 1990), probably because of the greater size of dinoflagellates (28–35  $\mu\text{m}$ ) compared to that of the diatoms or flagellates used in aquaculture (12–20  $\mu\text{m}$ ). The quantity of feces produced actually depends on the quality and quantity of food available (Razet et al., 1990). The fact that *S. trochoidea* cell supply was greater than that of *A. minutum* may account for the higher feces production. Conversely, as cell supplies were the same and pseudofeces production comparable for toxic and nontoxic *A. tamarensis*, the difference in feces production could not have been related to the quality of the algae, but clearly reflected a difference in filtration rates.

These data and inverted microscopy observations of biodeposit production seem to confirm that there is a selection of organic matter on the labial palps, with mineral matter being rejected in

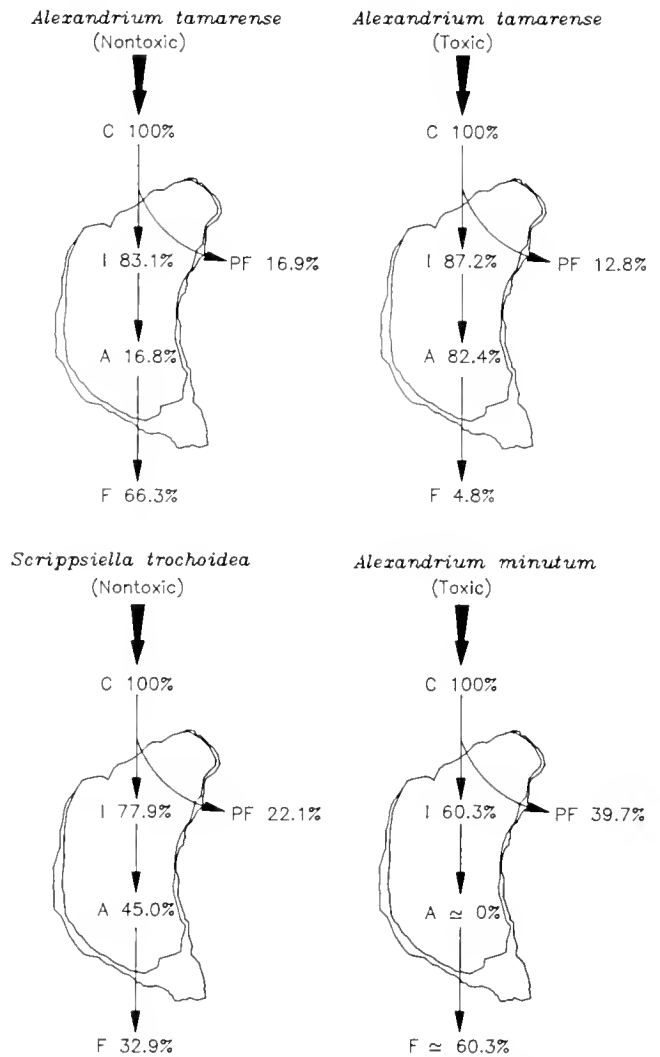


Figure 3. Simplified drawings representing the mean ingestion and absorption yields obtained with the 4 monospecific microalgal diets. C = consumption expressed as 100% for each experiment. I = ingestion, PF = pseudofeces, Ab = absorption, F = feces.

pseudofeces (Shumway et al., 1985b; Razet et al., 1990). The proportion of empty thecae appeared greater in pseudofeces, whereas numerous intact algal cells were found in feces.

The toxic nature of *A. minutum* would seem to account for the lower absorption of this species than of *S. trochoidea*. However, for *A. tamarensis* absorption was much greater for the highly toxic MOG 835 strain than for the nontoxic strain (despite equivalent food value) or even for *A. minutum*. Thus, preferential selection of

TABLE 4.  
Percentage of absorption efficiency (A/I  $\times$  100) of *C. gigas* for the different algal diets used. Mean time-averaged values.

Microalgal Diet	Absorption Efficiency
<i>A. tamarensis</i> (nontoxic)	16.5
<i>A. tamarensis</i> (toxic)	94.6
<i>A. minutum</i> (toxic)	0
<i>S. trochoidea</i> (nontoxic)	61.2

the highly toxic alga would seem to occur in the digestive tube, possibly because this strain is easier to digest. Shumway et al. (1985b) noted that *C. virginica* ingested but did not absorb the nontoxic dinoflagellate *Prorocentrum*. However, Shumway and Cucci (1987) observed no particular reaction by the oyster in the presence of *P. tamarensis*. This toxic dinoflagellate was filtered and rejected in pseudofeces and feces. Unfortunately, no data on ingestion and absorption efficiency are available.

In our study, *C. gigas* seemed to make use of the very low amounts of toxic *A. tamarensis* consumed as food at the very beginning of the experiment. These individuals had supposedly not come in contact with toxic dinoflagellates and thus not developed adaptive mechanisms allowing them to use the toxic cells as food (Twarog and Yamaguchi, 1974). Despite its toxicity, *A. tamarensis* might have particular nutritional value for *C. gigas* during early exposure, even without prior adaptation. Subsequently, after the toxicity is detected by the labial palps, the oyster seems to withdraw partially and no longer consume these cells. However, the detection process for *A. minutum* is apparently different. At first, the oyster seems to consume but not absorb this dinoflagellate, possibly because its food value is low, and then subsequently avoids it because of its toxicity.

Bricelj et al. (1990) studied *Mercenaria mercenaria* exposed to two strains of *Alexandrium* of varying toxicity. This shellfish is peculiar since it is not reported to accumulate paralytic toxins in

nature and is not directly sensitive to saxitoxin. In an experiment with a monospecific diet of the less toxic strain, *M. mercenaria* ingested and absorbed this dinoflagellate. However, it ingested very little of the more toxic strain, and only in the presence of a nontoxic diatom as supplement. The authors conclude that a toxin-recognition mechanism was involved.

Our preliminary results would seem to confirm this hypothesis for the oyster *Crassostrea gigas*. Ultrastructural studies should indicate the mechanisms by which oysters select food particles, and experiments at other times of the year may provide additional data related to the influence of different physiological states on uptake of toxic cells.

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## RESEEDING EFFORTS AND THE STATUS OF BAY SCALLOP *ARGOPECTEN IRRADIANS* (LAMARCK, 1819) POPULATIONS IN NEW YORK FOLLOWING THE OCCURRENCE OF "BROWN TIDE" ALGAL BLOOMS

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**ABSTRACT** The bay scallop, *Argopecten irradians irradians* (Lamarck 1819), comprised a multimillion dollar fishery in Long Island, New York waters prior to the first occurrence of *Aureococcus anophagefferens* algal blooms in 1985. Three successive years of these "brown tides" caused extensive mortality of adult scallops and severely limited larval recruitment; the impact of the brown tide was magnified by the short lifespan of the bay scallop. By the fall of 1988 virtually no native stock remained in the Peconic Bays and the New York fishery was essentially eliminated.

Extensive reseeded of hatchery-reared scallops was initiated in the Peconic Bays by the Long Island Green Seal Committee in 1986. Twenty-mm seed free-planted in late October/early November survived at one of three sites to spawn in July 1987. *Aureococcus* bloom conditions which coincided with this spawning apparently prevented successful recruitment. Twenty-mm seed planted in mid-September 1987 experienced complete mortality within one month; shell fragments implicated crabs as the primary cause of mortality. In mid-October 1988, 30-mm scallops were seeded at six sites. Mean survival until the following summer ranged from 0-12%. Spawning of these surviving scallops is thought to have produced 25% of the scallop set which occurred throughout eastern Peconic Bays in 1989; the rest is attributed to a relict population which survived east of the Peconic Bays. Heavy recruitment was observed in 1990, suggesting that scallop populations were recovering. Optimism was tempered in 1991, however, when adult stocks suffered high mortality, probably from a shell-boring parasite, *Polydora* sp., and a summer brown tide impacted scallop recruitment. The present status of bay scallop populations and the fishery in Long Island waters is precarious.

**KEY WORDS:** bay scallop, *Argopecten irradians*, reseeded, brown tide, fishery, New York

### INTRODUCTION

The bay scallop, *Argopecten irradians*, is the focus of a prized fishery in embayments and coastal areas of the United States Atlantic and Gulf coasts. The geographical ranges of the two most important subspecies, *A. i. irradians* and *A. i. concentricus*, historically have been given as Massachusetts—New Jersey, and New Jersey—Georgia and western Florida—Louisiana, respectively (Clarke 1965, Abbott 1974). In the 1930's, however, Atlantic bay scallop populations suffered dramatic reductions in many areas following the decimation of eelgrass (*Zostera marina*) beds by the wasting disease (Dreyer and Castle 1941); bay scallop landings also plummeted (McHugh 1989). In some areas, e.g. Chesapeake Bay, scallop populations have never recovered (Orth and Moore 1982).

In recent years, bay scallop populations have suffered further declines. In Connecticut and New Jersey, no commercial landings have been reported since 1966 and 1974, respectively (McHugh 1989). Bay scallops are now scarce in Rhode Island (J. Karlsson, Coastal Fisheries Laboratory, pers. comm.). In North Carolina, the first recorded red tide (a bloom of *Gymnodinium breve*) in the state decimated stocks of *Argopecten irradians concentricus* in 1989 (Summerson and Peterson 1990).

The major objectives of this paper are:

1. to provide an overview of the status of *A. i. irradians* populations and the fishery in New York waters following the initial appearance of brown tide (*Aureococcus anophagefferens*) algal blooms in 1985 and
2. to summarize efforts to reseed embayments of Eastern Long Island, New York with hatchery-reared scallops between 1986-1991.

### THE NEW YORK BAY SCALLOP FISHERY PRIOR TO BROWN TIDES

The fishery for bay scallops in New York is concentrated in the embayments toward the eastern end of Long Island (Fig. 1). The focal points of the commercial fishery historically have been Northwest Harbor (NWH), Orient Harbor (OH) and Flanders Bay (FB) in the Peconic Bay system. Additional landings sporadically come from along the south shore (Shinnecock (SB), Moriches (MB), and Great South Bays (GSB)) and from Oyster Bay (OB) and Huntington Harbor (HH) along the north shore of Long Island (Fig. 1). Prior to 1985, the bay scallop fishery employed between 400-600 full-time baymen (Anonymous 1985) and was valued at around US \$2 million (Rose 1987). For many baymen, the bay scallop harvest comprised about 2/3 of their yearly income prior to 1985 (P. Wenzel unpub. data).

Between 1968 and 1984, commercial bay scallop landings in New York ranged from 93,000 to 678,000 lbs of meats (Fig. 2). Such variations in landings, which reflect stock sizes, are considered normal for bay scallops. Belding (1910) showed that in successive years at a given location the population size may be high, crash to nothing, and then return to a high level. These fluctuations are probably due to variability in recruitment and are accentuated by a life cycle in which adults generally spawn once during the usual 18-22 month lifespan (Belding 1910, Karney 1991).

### IMPACT OF BROWN TIDES ON SCALLOP POPULATIONS, 1985-1988

"Brown tides" were first observed in Narragansett Bay, Rhode Island, in Barnegat Bay, New Jersey, and in the Peconic, Shin-

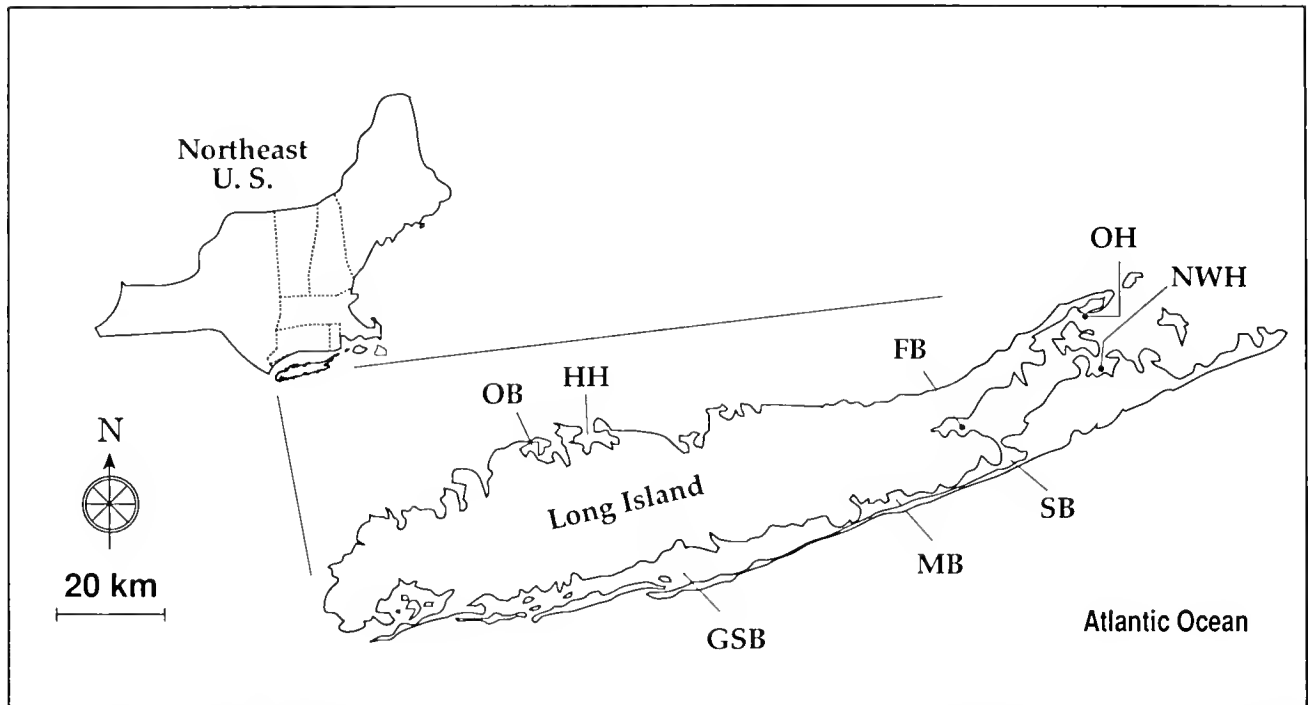


Figure 1. Map of Long Island, New York, showing the major embayments discussed in the text: OB = Oyster Bay; HH = Huntington Harbor; GSB = Great South Bay; MB = Moriches Bay; SB = Shinnecock Bay; FB = Flanders Bay; OH = Orient Harbor; NWH = Northwest Harbor. The embayments between the north and south forks of eastern Long Island comprise the Peconic Bay system. (●) = stations for *Aureococcus* cell counts plotted in Fig. 3. Inset shows the location of Long Island in the Northeast United States.

necock, Moriches and Great South Bays, New York between May–July 1985 (Casper et al. 1987). These blooms were later confirmed to be dominated by a previously undescribed chryso-phyte, *Aureococcus anophagefferens* (Sieburth et al. 1988).

Brown tide was recorded in the Peconic Bays during July and August of 1985. After a comprehensive monitoring program was implemented (Nuzzi and Waters 1989), concentrations of *Aureococcus* greater than  $2 \times 10^5$  cells/ml (see following discussion) were observed in FB between late May–late August 1986 and in NWH and OH between early June–early August 1986 (Fig. 3). In 1987, the brown tide bloom occurred later but persisted much longer in FB (July through December). In NWH and OH, concentrations  $> 2 \times 10^5$  cells/ml were recorded in 1987 between July–October, and July–August, respectively (Fig. 3). Recorded cell concentrations between 1985–87 were generally highest at the western end of the Peconic Bays and declined towards the east (Nuzzi and Waters 1989).

Previous studies have shown that brown tides impacted bay scallop populations in three ways: by directly causing scallop mortality, by interfering with spawning and recruitment of successive scallop year classes, and by causing shading and subsequent mortality of eelgrass (*Zostera marina*), a preferred habitat for bay scallops.

Adult bay scallops are thought to have essentially starved during bloom conditions, not because of the small size, indigestibility or poor nutritional quality of *Aureococcus* cells, but due to some toxic property which appears to inhibit normal feeding (Tracey 1988, Bricelj et al. 1989, Gallagher et al. 1989). Inhibition of algal grazing appears to be caused by direct contact with *Aureococcus* cells, not by extracellular dissolved exudates (Tracey 1988, Gallagher et al. 1989, Ward & Targett 1989). The minimum *Au-*

*reococcus* concentration which is considered harmful to bay scallops is thought to be  $\sim 2 \times 10^5$  cells/ml (Bricelj & Kuenster 1989). When bloom conditions persist long enough, scallops probably deplete their energy reserves and then die; this effect was also believed to be the cause of mussel mortalities in Narragansett Bay (Tracey 1988). Juvenile bay scallops may succumb more quickly than adults because of lower energy reserves, but this has not been demonstrated experimentally. Adult (1+ yrs) scallops which survived the brown tide in 1985 recovered rapidly and grew well during the fall (Bricelj et al. 1987).

The brown tide severely impacted scallop recruitment between 1985–87 (Siddall and Nelson, 1986; Casper et al. 1987) probably because larvae succumbed to starvation (Gallagher et al. 1989) or

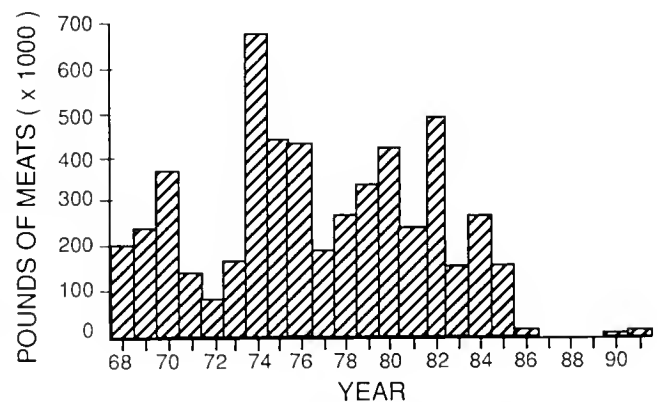


Figure 2. Commercial bay scallop landings from New York waters, 1968–91. Data from T. Drumm, New York State Dept. of Environmental Conservation.



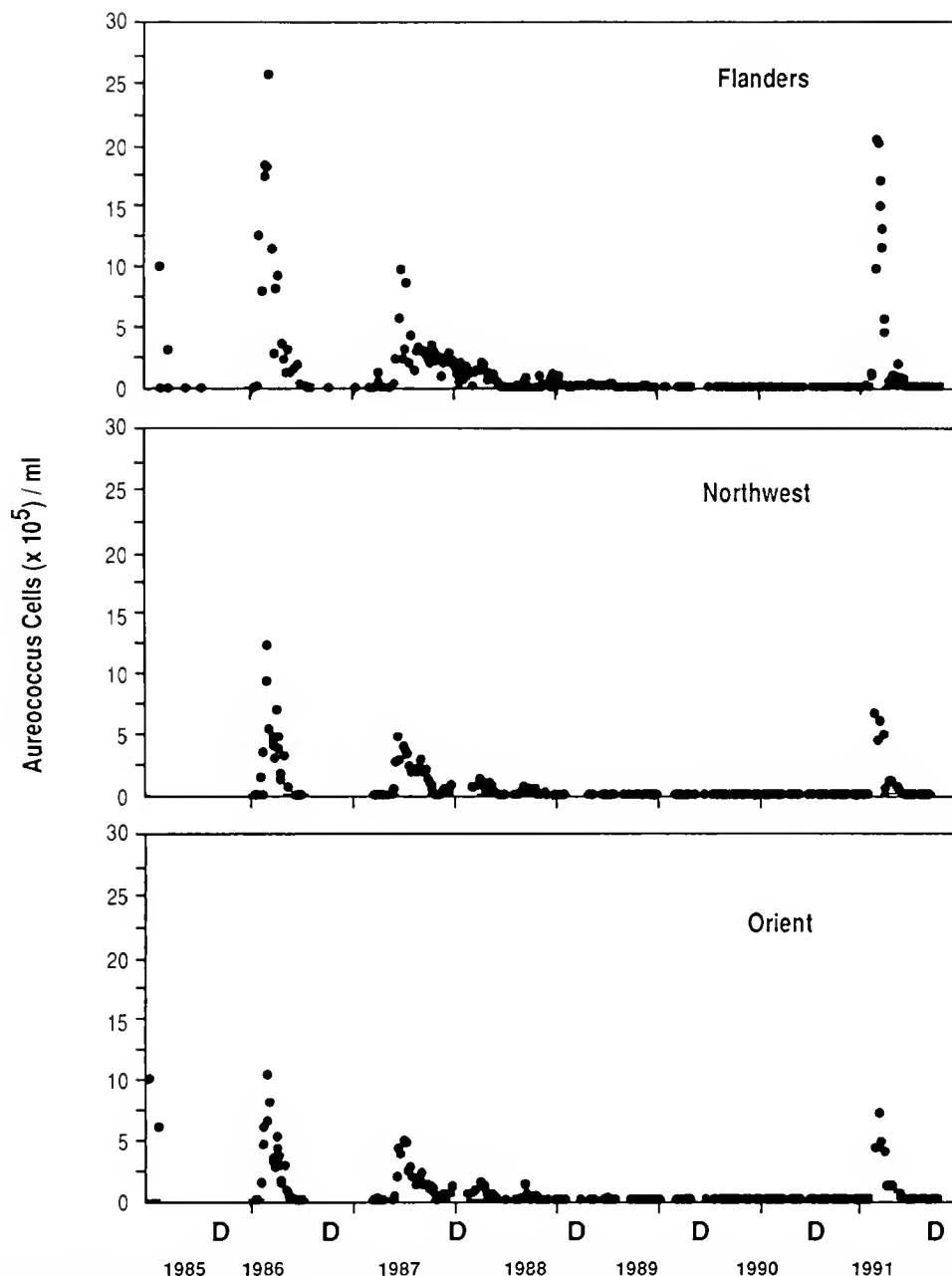


Figure 3. Brown tide (*Aureococcus anophagefferens*) concentrations at Flanders Bay, Northwest Harbor and Orient Harbor, 1985–91. D = December. Data from R. Nuzzi, Suffolk County Dept. of Health Services.

because spawning was inhibited or delayed until after the normal late May—late August spawning period (Bricelj et al. 1987). Such a delay in spawning may have subjected larvae to temperatures at which survival is reduced (Tettelbach and Rhodes 1981).

The observed reduction in the biomass of *Zostera marina* in the Peconic Bays due to the brown tide (Cosper et al. 1987) may have indirectly impacted scallop populations because this is a preferred settlement site for larval bay scallops (Eckman 1987). Eelgrass also serves as a spatial refuge for attached juveniles from some crustacean predators (Pohle et al. 1991).

Based on several hundreds of hours of dredging surveys conducted by baymen while harvesting scallops during Fall 1985, it was estimated that scallop mortality due to the brown tide was

approximately 95% in FB, 50% in NWH, and 10% in OH (Wenzel 1987). These data were compiled by examining ratios of live scallops and cluckers (dead scallops with shell valves still connected at the hinge) present in scallop dredges. The differential rates of mortality paralleled the observed W-E gradient in *Aureococcus* cell counts (Fig. 3). Tracey et al. (1989) have suggested, however, that for *Mytilus edulis* which were monitored in the Peconic and Great South Bays the observed toxicity of brown tide did not directly parallel *Aureococcus* concentrations.

While bay scallops historically had been distributed throughout the Peconic Bays prior to the brown tide in 1985, the observed distribution in 1986 was greatly contracted (Fig. 4). No scallop seed (0+ yrs) were detected during extensive surveys by baymen

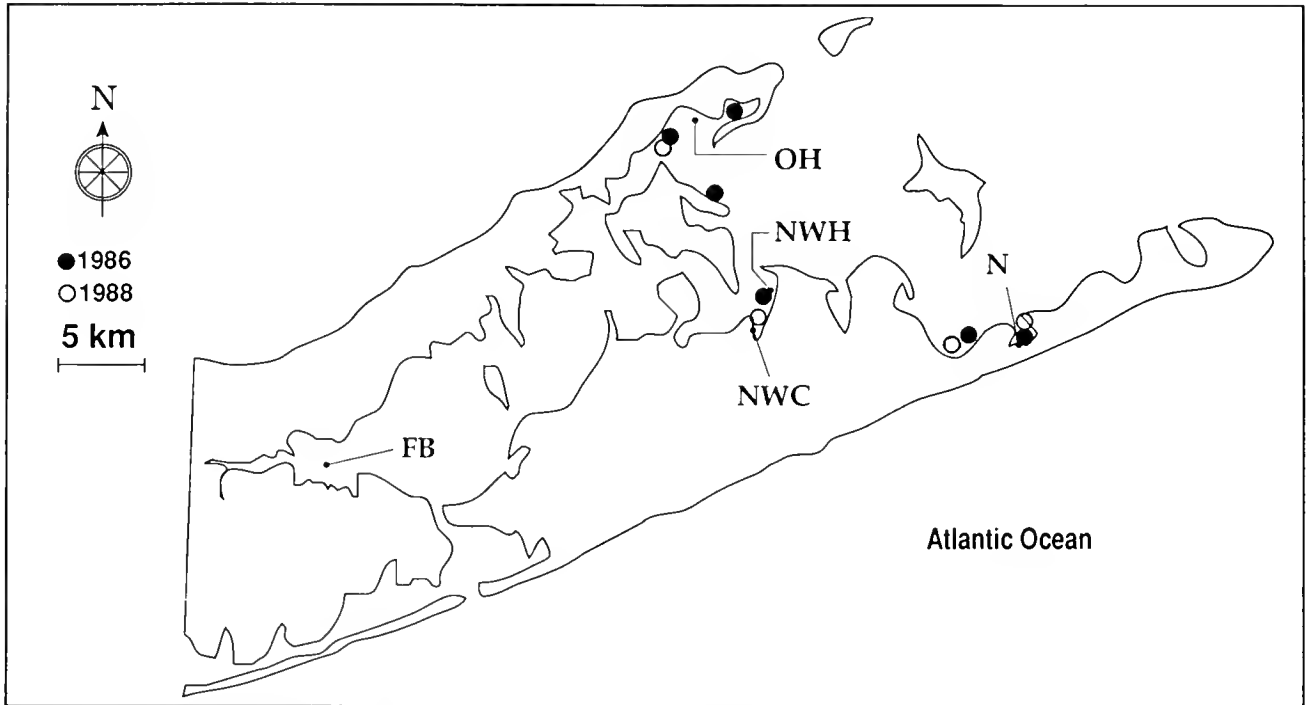


Figure 4. Reported sightings of natural bay scallops in the Peconic Bays during 1986 (●) and 1988 (○). FB = Flanders Bay; OH = Orient Harbor; NWH = Northwest Harbor; NWC = Northwest Creek; N = Napeague Harbor.

in FB in fall 1985 or spring 1986; some juvenile scallops were observed in 1985 in NWH and OH, with the bulk of these occurring in the latter area. The scallop set in 1985 probably occurred late, because seed were not observed until December and they were very small (~10 mm in shell height, as measured along a tangent from the umbo to the ventral margin). Normally, *A. i. irradians* seed of this size are first noticed in August and reach a mean height of 40–50 mm by December (P. Wenzel unpub. data).

Scallop populations declined further in 1986 with the reappearance of brown tide. By September 1986, no live scallops could be found in FB or NWH. In OH, low numbers of normal sized adults (~60 mm) were found at this time, but most adults (confirmed by the presence of an annual growth ring) were only 30 mm. These small adults probably represented individuals which set late in 1985, grew for a short time in 1986 before ceasing growth during the brown tide (June–August in OH), and then began to grow again after it subsided. A small set of scallops was observed by baymen in OH in fall 1986. The commercial scallop harvest for 1986 was only 13,000 lbs, down dramatically from the 174,000 lbs harvested in 1985 (Fig. 2).

Following an extended brown tide bloom in 1987, no scallop recruitment was observed in the Peconic Bays. By fall 1988, it appeared that populations of bay scallops in the Peconic Bays were virtually eliminated except for some relict stock which appeared to persist in OH and Northwest Creek (Fig. 4).

The impact of the 1985–87 brown tides on Peconic Bay scallop populations was magnified because of the short life history of the species. *A. i. irradians* normally die at an age of 18–22 months, although in unexploited populations perhaps 20% survive to spawn a second time at an age of 2 years (Belding 1910). Because there were no apparent sanctuaries for bay scallops from the brown tide in the Peconic Bays, and because the brown tide occurred for 3

years in succession and scallop recruitment apparently failed in each of these years, the brood stock size declined to the point where the link to future generations was virtually eliminated by fall 1988. At this time it appeared doubtful that the Peconic Bay scallop populations could rebound on their own.

Commercial bay scallop landings in New York for 1987 and 1988 totalled 373 and 250 lbs of meats, respectively (Fig. 2). The 1988 harvest was virtually all from the Napeague Harbor area (Fig. 4) (T. Drumm, New York State Dept. of Environmental Conservation, pers. comm.). Here, a relict population of bay scallops persisted through the 1985–87 years, presumably because *Aureococcus* cell counts remained lower than in the Peconic Bays (R. Nuzzi, Suffolk County Dept. of Health, pers. comm.).

#### BAY SCALLOP RESEEDING EFFORTS

Peter Wenzel and Steven Latson of the Long Island Green Seal Committee (LIGSC), a baymen's organization comprised primarily of members from the five easternmost towns on Long Island, conceived and initiated plans in 1985–86 to transplant hatchery-reared bay scallops into the Peconic Bays following the decline of natural populations due to the brown tide. The basic premise was to transplant juvenile (seed) scallops during the fall months (when they are available from hatcheries) so that they would mature and survive to the next summer to spawn naturally. Thus, transplanted scallops would serve as supplemental brood stock rather than for direct augmentation of the fishery.

LIGSC ordered seed scallops to be ready for transplanting in fall 1986. During the first year, scallops were obtained directly from hatcheries in Massachusetts, Maine and New York. The majority of seed obtained in subsequent years was comprised of scallops first spawned in Maine and then raised to planting size by a facility in New York.

Reseeding sites were selected on the basis of several criteria:

historical productivity of the area for scallop harvests, abundance of predators (particularly starfish and crabs), bottom characteristics, degree of exposure of the area to prevailing NW winter winds which can strand scallops on adjacent beaches (see Kelley 1981), and anticipated larval dispersion after spawning. The latter consideration was based on a computer simulation model developed by Siddall et al. (1986). For later transplants, the potential impact on scallop survival of burial by shifting sediments in winter was also considered (Tettelbach et al. 1990). Scallops used for transplantation were either free-planted or held in cages.

Approximately 930,000 seed scallops, averaging 20 mm in size, were free-planted on 27 October and 10 November 1986 (Table 1) at FB, NWH and OH (Fig. 5). Planting was accomplished by broadcasting seed by hand from a boat transiting the selected area. None of these reseeded scallops are thought to have survived to the following summer at the FB or OH sites. However, ~67% of the transplants at NWH were estimated by divers to have survived to mid-late July 1987, at which time gonadal indexes indicated that these scallops spawned (Smith 1987). Unfortunately, the brown tide bloomed here within 1–2 weeks of this time. No seed were observed on spat collectors or in dredging surveys conducted by baymen in NWH during fall 1987.

Approximately 583,000 20-mm scallops were free-planted at NWH and OH in mid-September 1987 (Table 1). Divers observed complete scallop mortality within 1 month; shell fragments implicated crabs as the primary cause of the mortalities.

A total of 115,000 scallops (mean size = 20–30 mm) was placed in 100 cages at two sites in the FB area on 25 September and 1 October 1987 (Fig. 5). These were moved west to a nearby site in late November/early December in the hope of reducing losses from starfish, which were observed to consume scallops by everting their stomachs through the single layer mesh walls of the cages. By 22 February 1988, however, 100% of the scallops in cages were dead. These continued mortalities were attributed to unexplained 'winter mortality' as well as predation by starfish.

Different strategies were adopted for the reseeded program in 1988. On the basis of the heavy predation observed on 20-mm seed planted in 1987 and earlier studies of crab predation on scallops (Tettelbach 1986), larger seed (30 mm) were utilized in 1988

with the expectation that predation rates should be lower. A lower planting density was also used (~10/m<sup>2</sup>) (Table 1), with the hope that predation intensity might be lower (Boulding and Hay 1984). A greater number of planting sites (six) was also selected, after extensive SCUBA surveys, with the hope that with more sites there would be a greater chance that some scallops would survive to spawn in at least one area. Reseeding in 1988 was conducted between 19–26 October. (Considerations and recommendations for scallop reseeded programs are further detailed in Wenzel et al. 1993).

Fortuitously, the brown tide did not bloom to lethal concentrations in the Peconic Bays from summer 1988 through 1990 (Fig. 3), which improved the potential for success of the reseeded program. By late June 1989, scallop survival at the six planting sites in NWH and OH ranged from 0–12% (Table 1). The Hallock Bay (HB) site near the northeastern tip of Long Island had the highest survival rate, followed by the Alewife Creek (AC) site in NWH (6%) (Fig. 5).

A spatially extensive scallop set was observed in the Peconic Bays, primarily in the eastern portion, during summer–fall 1989 (Fig. 6). By late October, seed density averaged ≤3 scallop seed/m<sup>2</sup> in NWH; mean seed height was 40–50 mm. The highest observed density was 12.0 seed/m<sup>2</sup> in the NE corner of NWH. No seed was reported from FB.

Krause (1992) performed comparative electrophoretic analyses of tissue proteins from 1989 Peconic Bay seed, 1988 transplanted scallops, and 1988 natural stock from Napeague Harbor and found that approximately 25% of the natural scallop set which occurred in the Peconic Bays in fall 1989 resulted from spawning of LIGSC-transplanted scallops which had survived from October 1988 to the following summer. This exciting result probably provides the best documentation of successful scallop reseeded in the United States. The rest of the 1989 set was attributed to the surviving population in the Napeague area, less ~0.7% which may have been derived from relict natural scallops in the Peconic Bays (Krause 1992).

A scallop transplant was again carried out by LIGSC during fall 1989. A total of approximately 429,000 30–40 mm scallops (Table 1) was planted at nine sites (Fig. 5). Growth of hatchery-reared

TABLE 1.

Summary of bay scallop reseeded efforts conducted by the Long Island Green Seal Committee in the Peconic Bays, New York, 1986–89. FB = Flanders Bay; NWH = Northwest Harbor; OH = Orient Harbor; C = Cutchogue Harbor; ERI = East Side of Robin's Island.

Year	Reseeded Areas	Planting Date(s)	Water Temperature (°C) at Planting	Initial Planting Density (#/m <sup>2</sup> )	Total # Scallops Planted (1000's)	Mean Scallop Size at Planting (mm)	Estimated Survival (%) to Following June	
1986	FB	10, 18 Nov		49	248	15–20	0	
	NWH	27 Oct		73	372		67	
	OH	10, 18 Nov		73	372		0	
1987	NWH	18 Sep		56	284	20	0	
	OH	17 Sep		59	300		0	
1988	NWH (3 sites)	26 Oct	12.0	9	200	30	0, 0, 6	
	OH (3 sites)	19 Oct	13.5	10	170		0, 0, 12	
1989	NWH (3 sites)	27 Nov	5.6	9	100	~26	0, 0, 0	
	OH (2 sites)	27 Nov	5.6	11	100		0, 0	
	FB (2 sites)	1, 6 Dec	1.4, ~1.0	5, 11	121		~40, 26	0, 0
	C	6 Dec	2.9	11	54		~26	0
	ERI	6 Dec	2.2	11	54			0



spawned during summer–fall 1990 and produced one of the heaviest sets in recent memory. Between December 1990 and early April 1991 mean densities of 45–50 mm seed were as high as 20–21/m<sup>2</sup> in some areas of NWH and OH (Table 2). The 1990 scallop set was most concentrated in the eastern Peconic Bays, but seed also were confirmed from the central Peconic, Flanders, and Shinnecock Bays (Fig. 6).

While the LIGSC reseeding program was proceeding, eastern Long Island towns also had been conducting scallop transplants since 1986 (Table 3, Fig. 5). Little monitoring of scallop survival was done for the town transplants and thus it is difficult to quantify the extent to which these activities may have contributed to scallop sets observed since 1989.

#### 1991: POLYDORA AND THE RETURN OF BROWN TIDE

Optimism over the heavy scallop set during 1990 and conjectures of a "pre-brown tide" harvest for fall 1991 were tempered by two major events during 1991: the discovery of extensive parasitic infections of scallop shells by a boring polychaete worm, *Polydora* sp., and the return of brown tide bloom conditions to the Peconic Bays.

*Polydora* infestation of scallop shells was first noticed in January 1991 during our surveys of natural bay scallop populations. Subsequent surveys conducted through March 1991 revealed that 100% of the 1773 scallops sampled at a total of eight sites in Orient and Northwest Harbors (Fig. 6) were infested. Infestation levels ( $\neq$  worms/shell) appeared to vary from one site to the next and smaller scallops seemed less affected than larger ones. Shells of many larger individuals were so brittle they could be snapped in half as easily as ridged potato chips.

Infestations of bay scallops by *Polydora ciliata* have been reported by Turner and Hanks (1959) in Massachusetts and Russell (1973) in Rhode Island. The latter author suggested that *Polydora* infestations may be epidemic in nature and that *Argopecten irradians irradians* is probably not a preferred host. He further suggested that there was no evidence that extensive mortality of bay scallops resulted from *Polydora* infestations.

It is unclear whether *P. ciliata* was the species which infested Peconic Bay scallops, but it appears that extensive scallop mortality did result from worm infestations in 1991. At the Alewife Creek (AC) site in NWH (Fig. 6), mean density of live scallops in March 1991 was significantly lower ( $t = 4.07, p < .001$ ) than in

June (Table 2). The latter sampling was done just prior to a brown tide bloom. At the time of this sample, cluckers were found in high numbers (4.0/m<sup>2</sup>), an indication of recent mortality. Eleven of 50 cluckers (22%) and 1 of 72 live scallops (1.4%) at this site had holes ~1–2 cm in diameter in the middle of the dorsal valve, at or near the point at which the adductor muscle attaches to the shell. This type of shell damage is rarely seen in the field (S. Tettelbach pers. obs.). We suggest that heavy *Polydora* infestations lead to weakened scallop shells (see Bergman et al. 1982) and that holes in the dorsal valve subsequently result from the contracting force generated by the adductor muscle of the scallop when it forcefully closes its valves. This explanation is supported by observed development of dorsal shell holes in live scallops with heavy *Polydora* infestation levels which were held in predator-free nets in the laboratory after collection from NWH. Holes in the dorsal valves of scallops observed in the field may also have resulted from predatory attacks by crabs.

Surveys of three other locations in summer–fall 1991 revealed that scallop densities were significantly lower than in winter 1990–91 (Table 2). While brown tide cannot be ruled out as a cause of adult scallop mortality during summer 1991, the relatively short duration (~1 mo) of the bloom and the absence of high numbers of cluckers (0.45/m<sup>2</sup>) at the AC site (Fig. 6) on 22 August 1991 following the subsidence of the bloom suggest that it was not a major cause of adult scallop mortality.

In contrast, the 1991 brown tide appeared to have interfered with the normal timing of scallop recruitment in the Peconic Bays. *Aureococcus* concentrations exceeded  $2 \times 10^5$  cells/ml in the Peconic Bays between 18 June and 16 July (Fig. 3); this occurred at the time when scallops are historically in peak spawning condition (Bricelj et al. 1987). Seed were not reported in the Peconic Bays until November–December 1991 and they were very small (<10–15 mm) at this time. This suggests that spawning of adult scallops was delayed until after the brown tide bloom had subsided.

Two emergency plans for scallop transplantation and reseeding were conceived and conducted jointly by NYSDEC, LIGSC and Cornell Cooperative Extension of Suffolk County (CCE) in fall 1991 prior to the discovery of the natural set. First, 80 bushels of natural scallop seed (~80,000) were collected from NWH and subsequently transplanted to three areas less affected by the brown tide in 1991: Lake Montauk (30 bu), Shinnecock Bay (30 bu), and Moriches Bay (20 bu). A second effort involved reseeding the

TABLE 2.

Comparison of bay scallop densities at Peconic Bay sites, Winter 1990–91 vs. Summer–Fall 1991. All densities were determined through *in situ* suction dredge sampling of scallops in 1-m<sup>2</sup> quadrats, except at the Alewife Creek site on 12 June 1991 when densities were determined by visual counts of scallops in 1/4-m<sup>2</sup> quadrats.

Site	Sampling Dates		Scallop Density [Mean (SD); # quadrats]		t-value	p-level
	Winter	Summer–Fall	Winter	Summer–Fall		
E. Marion	21 Dec, 2 Jan	23 Jul	13.2 (5.3); 17	0.3 (0.6); 3	9.69	p < .001
Greenport	8, 15 Mar	24, 31 Jul, 2 Aug	10.9 (6.1); 21	1.6 (1.2); 70	6.95	p < .001
N. Orient Harbor	29 Jan, 8 Feb	2 Oct	21.5 (10.9); 11	1.0 (1.2); 26	6.22	p < .001
off Alewife Creek	25 Mar	12 Jun	20.0 (11.1); 11	5.8 (6.9); 50	4.07	p < .001
E. Shelter Is.	8, 11, 15 Feb		7.1 (5.7); 31			
W. Shelter Is.	29 Mar, 1 Apr		16.8 (9.4); 13			
Sag Harbor	1, 4 Mar		8.4 (5.5); 26			
Barcelona Neck	18, 26, 28 Mar		5.6 (3.2); 37			

TABLE 3.

Summary of bay scallop reseeded efforts conducted by towns of Eastern Long Island, New York, 1986–91.

Town	Reseeding Year(s)	Total # of Scallops Planted (1000's)	Mean Scallop Size (mm) at Planting	Reseeding Sites
East Hampton	1986	100 <sup>a</sup>	13.5	Lake Montauk (LM)
	1986–90	10–40 per yr	20–30	Lake Montauk, Northwest Creek (NWC), Napeague Harbor (N), Three Mile Harbor (TMH), Accabonac Harbor (A), Hands Creek (HC)
Riverhead	1989	5	40	East Creek (EC)
	1990	1,000	40	Flanders Bay (FB)
	1991	300	40	Great Peconic Bay (GPB)
Shelter Island	1986–91	100 per yr	20–30	Coele's Harbor (CH), West Neck Harbor (WNH)
Southampton	1989	60	30	Shinnecock Bay (SB), Tiana Bay (TB), Sag Harbor Cove (SHC), Noyack Creek (NC), North Sea Harbor (NSH), West Neck Creek (WNC), Cold Spring Pond (CSP), Red Creek (RC)
	1990	123 <sup>b</sup>	25–30	All of the above, except Red Creek
	1991	50	25–30	"
	1991	601 <sup>a</sup>	13.5	Hallock Bay (HB)
Southold	1986	601 <sup>a</sup>	13.5	Hallock Bay (HB)
	1991	68	20–30	Hallock Bay

<sup>a</sup> Transplants done jointly by Cornell Cooperative Extension of Suffolk County (C. Smith) and Town of Southold (J. McMahon).

<sup>b</sup> Includes ~20,000 scallops planted in Shinnecock Bay jointly by CCE and Long Island University.

Peconic Bays with 15–25 mm scallops obtained from a hatchery in Maine. The six reseeded areas, which each received 50–55,000 scallops, included two sites in NWH and OH and one site in FB and Cutchogue Harbor (C) (Fig. 5). All transplants were done on 7 November. The effect of these two programs is unknown as little monitoring was done subsequently.

The opening of the 1991 scallop season in the Peconic Bays was delayed to early October so that adult scallops would be allowed to grow further and possibly spawn after the brown tide subsided. The 1991 commercial harvest totalled 15,100 lbs of meats, up 41% from the 1990 harvest of 10,700 lbs, but far below pre-brown tide harvests (T. Drumm pers. comm.).

### CONCLUSIONS

The status of bay scallop populations and the fishery in Long Island waters is precarious. *Aureococcus anophagefferens* now appears to be a persistent component of the Peconic Bay ecosystem which, under appropriate circumstances, can reach bloom proportions. While reseeded of the bays with hatchery-reared scallops appears to have been somewhat successful in accelerating natural repopulation processes, the western Peconic Bays have not experienced any substantial set of scallops since 1985 and overall Peconic populations are well below historical levels. The spectre of the brown tide and the parasite *Polydora* continue to loom as threats to a full recovery of the resource.

### ACKNOWLEDGMENTS

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## DWARF SURFCLAM *MULINIA LATERALIS* (SAY, 1822) POPULATIONS AND FEEDING DURING THE TEXAS BROWN TIDE EVENT

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**ABSTRACT** In 1990, there was an unusual brown tide bloom of an aberrant Chrysophyte sp. in Baffin Bay and Laguna Madre near Corpus Christi, Texas. Coincident with the bloom was a dramatic loss of shellfish in Baffin Bay and Laguna Madre. The dominant clam, *Mulinia lateralis*, disappeared for about two years. We performed a series of experiments to determine if disappearance of *M. lateralis* was related to negative feeding interactions with the brown tide organism. Radioactive tracers were used to compare feeding rates on brown tide, *Isochrysis galbana*, *Dunaliella tertiolecta*, and *Heterocapsa pygmaea*. At low cell concentrations ( $<1,000$  cells  $\cdot$  ml $^{-1}$ ), *M. lateralis* grazing rates (cell  $\cdot$  h $^{-1}$ ) increased with concentration and were similar among the microalgal species. At higher concentrations, grazing rates on *Isochrysis* were inhibited, but remained the same for the other microalgal species. Assimilation efficiency by *M. lateralis* was lowest on *Heterocapsa*, and was about the same for the three other species of algae. The high grazing and assimilation rates of brown tide by *M. lateralis* indicate that the loss of the clam population was not likely caused by a negative trophic effect of the brown tide. Other bloom factors, e.g. reproductive effects or toxic effects, may have contributed to the concomitant loss of the clam population and the occurrence of brown tide. It is also possible that non-bloom factors, e.g. natural population variability increased predation pressure, could have caused the population loss. The reduced populations of filter feeders could have been partially responsible for conditions conducive for the brown tide bloom.

### INTRODUCTION

A monospecific bloom of a small chrysophyte alga began in January 1990 in Baffin Bay, Texas. This bloom caused water discoloration and is called a "brown tide." The bloom is chronic; it remains intense after 3 years, waning only during the winter months. The organism is an unknown species. It is a Type III Chrysophyte, 4–5  $\mu$ m in diameter, similar (yet different) to *Aureococcus anophagefferens* and *Pelagococcus subviridis* (Stockwell et al. 1993). Chlorophyll content in the water column was about 10  $\mu$ g  $\cdot$  l $^{-1}$  before the bloom, and it increased to 80  $\mu$ g  $\cdot$  l $^{-1}$  during the apex of the bloom (Stockwell et al. 1993). Bottom light levels decreased 80% to 20% due to diffraction by the dense particulate matter in the water column (Dunton personal communication). The general ecological trends during the brown tide were a decrease in mesozooplankton (Buskey and Stockwell 1993), fish larvae (Holt personal communication) and benthic abundance and diversity (Montagna and Kalke in preparation). One interesting coincidence was a dramatic reduction in abundance of filter-feeding mollusks.

Other brown tides are known to have had catastrophic effects on bivalves (Shumway 1990). Effects have ranged from reproductive or recruitment failures (Bricelj et al. 1987, Tracey 1988), to adverse effects on feeding (Bricelj and Kuenstner 1989, Tracey 1988, Tracey et al. 1988) to a toxic effect (Draper et al. 1989, Tracey et al. 1990; Gainey and Shumway 1991). Mass mortalities of shellfish were usually reported. Although specific mechanisms for the mortality are difficult to ascertain, it is possible that one, or a combination of these effects is causing the population declines.

The dominant bivalve in the brown tide area, *Mulinia lateralis*, practically disappeared for 2 years. This caused great concern about the dominant finfishery, because *M. lateralis* is the predominant food source for black drum, *Pogonias cromis* (Martin 1979).

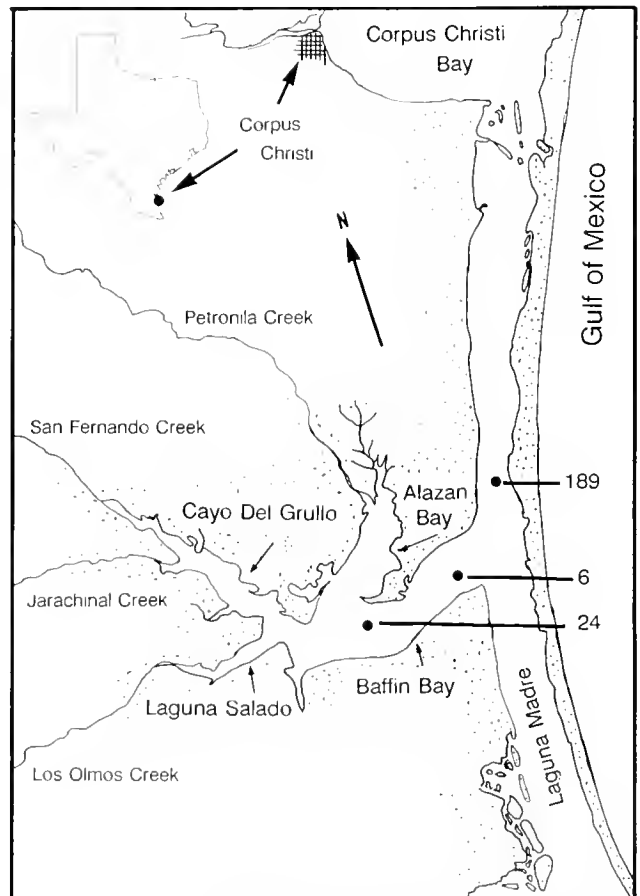


Figure 1. Study area.

TABLE 1.  
Algae used in the two feeding experiments.

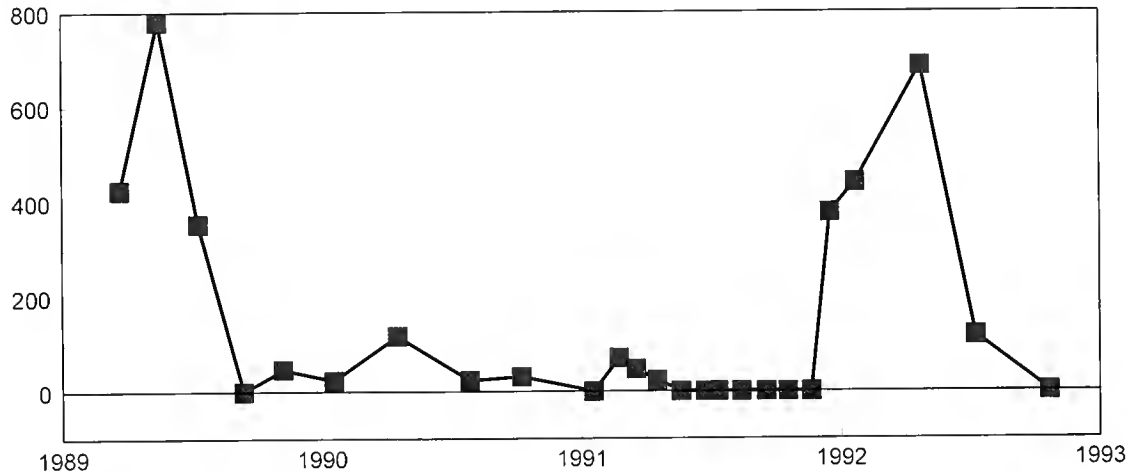
Division	Species	Cell Volume ( $\mu\text{m}^3 \cdot \text{cell}^{-1}$ )	Cell Biomass ( $\text{pg C} \cdot \text{cell}^{-1}$ )	Expt. 1 ( $10^3 \text{ cells} \cdot \text{ml}^{-1}$ )	Expt. 2 ( $10^3 \text{ cells} \cdot \text{ml}^{-1}$ )
Chrysophyte	Brown tide	33.5	3.685	2710	666
Chrysophyte	<i>Isochrysis galbana</i>	65.5	7.205	2450	765
Chlorophyte	<i>Dunaliella tertiolecta</i>	524	57.64	1045	322
Pyrrophyte	<i>Heterocapsa pygmaea</i>	720	79.20	142	32

The general concern was that there might be a major alteration of the ecosystem since carbon was tied up in a primary producer that was not being transferred into the food webs (Buskey and Stockwell 1993). Similar major ecological changes to the subtidal community occurred in Long Island, New York embayments experiencing *A. anophagefferens* blooms (Cosper et al. 1987). When *M. lateralis* reappeared in 1992, we initiated a feeding experiment to determine if the brown tide was causing feeding-related problems to the clam. We also document population change of *M. lateralis* during this period.

#### MATERIALS AND METHODS

*Study Sites.* The brown tide started in January 1990 in Alazan Bay, Laguna Salada, and Cayo de Grullo, which are three tertiary bays of Baffin Bay (Fig. 1). Each of the tertiary bays is fed by small creeks and rivers that contribute freshwater inflow intermittently in this drought-prone region. On average, evaporation exceeds river inflow, so these bays are often hypersaline. Salinity in Baffin Bay ranged from 40–60‰ during 1989, the year preceding the brown tide (Whitledge 1993). Four stations have been sampled

#### A. Abundance (n/m<sup>2</sup>)



#### B. Population Structure (frequency)

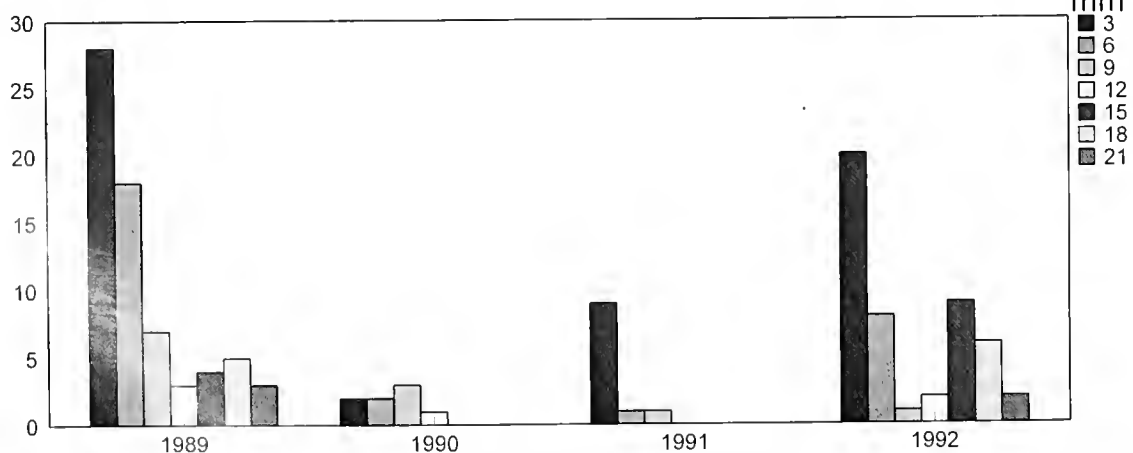


Figure 2. Population dynamics of *Mulinia lateralis* in Baffin Bay and Laguna Madre from 1989 to 1992. A. Average abundance at all stations sampled. B. Size structure of populations.

continuously since March 1988 (Fig. 1). Two of these stations are located near Markers 6 and 24 in Baffin Bay in open bay, mud bottoms at a depth of about 3 m. Two other stations are located west of marker 189 in the Intracoastal Waterway in the Laguna Madre. One of these stations is located in a seagrass bed, and the other in an adjacent unvegetated habitat. The brown tide did not reach the Laguna Madre until June 1990.

**Population Study.** Sediment at the four stations was sampled with core tubes held by divers. The tube was 6.7 cm inner diameter, and three replicates were taken within a 2-m radius. Sediment was sectioned at depth intervals of 0–3 cm and 3–10 cm. *Mulinia* was rarely present in the lower depth stratum. Samples were preserved with 5% buffered formalin. All macrofauna were extracted with 0.5 mm sieves, identified, and counted, but are reported elsewhere (Montagna and Kalke in prep.).

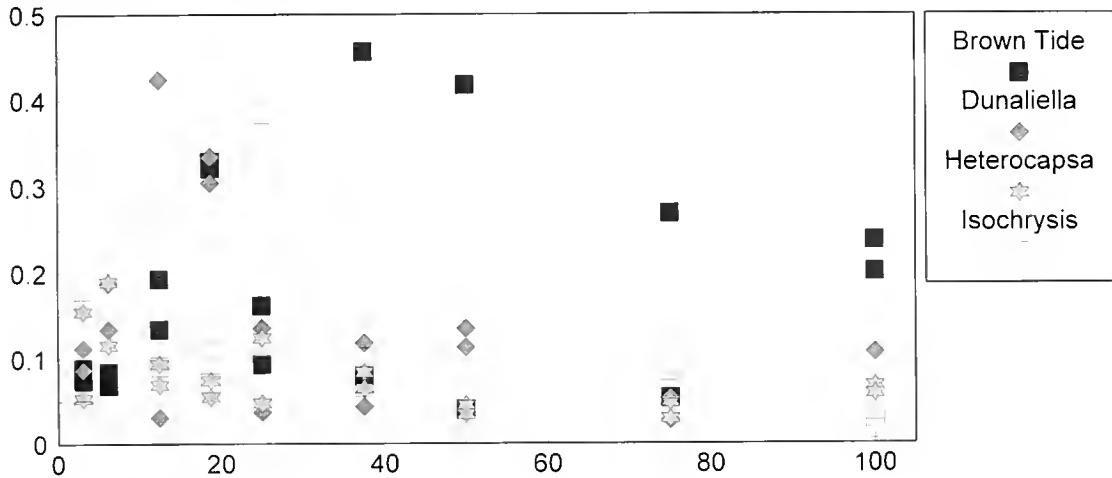
**Feeding Experiments.** Two experiments were performed where algae were pre-labeled with <sup>14</sup>C and fed to clams. The goal of the first experiment was to determine if there were functional responses of clam feeding rates to various algal concentrations. The goal of the second experiment was to determine if the algae were being assimilated. Four species of algae were used in each experiment (Table 1). The algae were prepared from stock cultures

maintained at the University of Texas Marine Science Institute. A comparative approach was used to determine if responses to brown tide was different from responses to other algae that were not suspected of being poor food sources to the clams.

The first experiment was performed May 28, 1992. The brown tide was harvested from the field ( $2.71 \times 10^6$  cells · ml<sup>-1</sup>), and was monospecific. A stock culture was made for each algal species and incubated with <sup>14</sup>C-bicarbonate overnight. Each stock culture was diluted to the following concentrations relative to the original: 100%, 75%, 50%, 37.5%, 25%, 18.75%, 12.5%, 6.25%, and 3.125%. The initial specific concentration of label was determined for each algal dilution (DPM<sub>algae</sub>). Clams of similar size were collected ( $n = 72$ , mean length = 11.7 mm ± 1.0 mm SD, mean wet weight = 323 mg ± 82 mg SD). Each clam was offered 24 ml of algae in a sterile 50-ml centrifuge tube. After 1 h, the clam was harvested, rinsed with 1% HCl, and placed in 0.3 ml of Soluene tissue solubilizer for 24 h. Samples were counted by liquid scintillation spectrophotometry in 20 ml Insta-Gel (DPM<sub>clam</sub>). The grazing rate fraction ( $F$ ) was calculated by the following formula:

$$F = \text{DPM}_{\text{clam}} / (\text{DPM}_{\text{algae}} \times \text{Incubation Time}) \quad (1)$$

**A. Measured Rate (1/h)**



**B. Predicted Rate (1/h)**

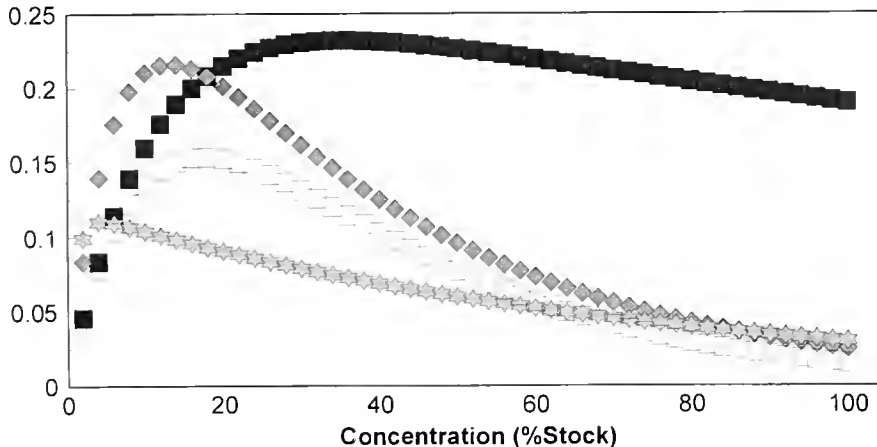


Figure 3. Grazing rate,  $F$  ( $\text{h}^{-1}$ ), versus dilutions of stock cultures (% dilution of stock culture). A. Measured grazing rates for all four algal species. B. Predicted grazing rates by fitting raw data to the inhibition model.

The units of the grazing rate fraction are in  $\text{h}^{-1}$ . The feeding rates were normalized in various ways.  $F$  was multiplied by the number of cells offered to calculate feeding as  $\text{cells} \cdot \text{h}^{-1}$  ( $I_{\text{cell}}$ ):

$$I_{\text{cell}} = F \times \text{cell concentration} \times 24 \quad (2)$$

This number was multiplied by the cell carbon content to calculate biomass grazed per h as  $\mu\text{g C} \cdot \text{h}^{-1}$  ( $I_C$ ):

$$I_C = I_{\text{cell}} \times (\mu\text{g C} \cdot \text{cell}^{-1}) \quad (3)$$

The clearance rate ( $I_{\text{clear}}$ ) was calculated as the volume of water swept clear of cells per unit time ( $\text{ml} \cdot \text{h}^{-1}$ ):

$$I_{\text{clear}} = I_{\text{cell}} / \text{cell concentration} \quad (4)$$

The grazing rate data ( $F$ ,  $I_{\text{cell}}$ ,  $I_C$ , or  $I_{\text{clear}}$ ) were fitted to a feeding rate inhibition model. The model assumes that grazing ( $I$ ) increases exponentially as a function ( $k$ ) of food concentration (cells or C) to some maximal value ( $I_m$ ), and at high food concentrations the maximal value of feeding is inhibited as an exponential function ( $d$ ):

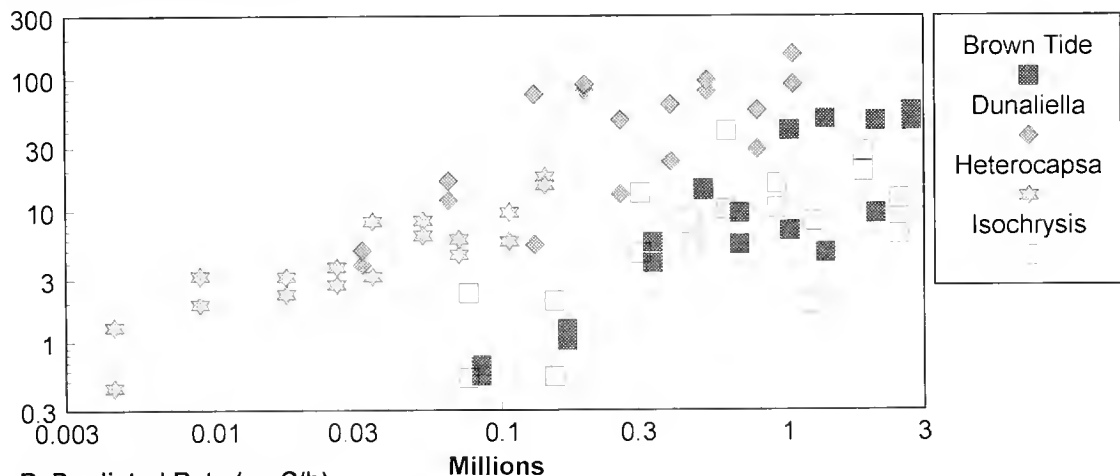
$$I = I_m (1 - \exp^{-k \times \text{concentration}}) \exp^{-d \times \text{concentration} / I_m} \quad (5)$$

The second experiment was performed June 2, 1992. The brown tide was harvested from the field ( $0.666 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$ ), and was monospecific. A stock culture was made for each algal species and incubated with  $^{14}\text{C}$ -bicarbonate overnight, and the initial specific concentration of label was determined for each algal stock ( $\text{DPM}_{\text{algae}}$ ). Clams of similar size were collected ( $n = 36$ , mean length =  $7.4 \text{ mm} \pm 0.6 \text{ mm SD}$ , mean wet weight =  $374 \text{ mg} \pm 63 \text{ mg SD}$ ). Each clam was offered 24 ml of algae in a sterile 50-ml centrifuge tube. After 2 h, the clams were moved to 10 ml of an unlabeled culture of *Thalassiosira* and allowed to feed and depurate label for 2 h. There were 9 replicates for each algal treatment. At the end of the incubation, the clams were harvested, feces collected by filtration, and the culture media retained to trap respired  $^{14}\text{CO}_2$ . The media were acidified with 0.1 ml of 3M HCl to convert bicarbonate to carbon dioxide, then the carbon dioxide was trapped on a strip of filter paper that was impregnated with 0.15 ml of phenylethylamine (Hobbie and Crawford 1969). All sample types were counted by liquid scintillation spectrophotometry in 20 ml Insta-Gel. Total label uptake is calculated as the sum of the label in all three compartments:

$$\text{DPM}_{\text{total}} = \text{DPM}_{\text{clam}} + \text{DPM}_{\text{feces}} + \text{DPM}_{\text{respired}} \quad (6)$$

The percentage of the label in each compartment is calculated.

### A. Measured Rate ( $\mu\text{g C/h}$ )



### B. Predicted Rate ( $\mu\text{g C/h}$ )

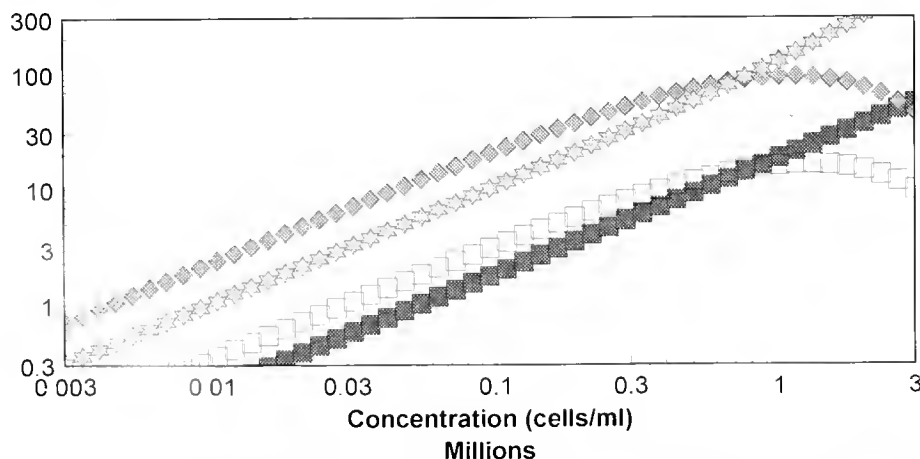


Figure 4. Carbon consumption rate,  $I_C$  ( $\mu\text{g C} \cdot \text{h}^{-1}$ ), versus cell concentrations ( $10^6 \text{ cells} \cdot \text{ml}^{-1}$ ). A. Measured grazing rates for all four algal species. B. Predicted grazing rates by fitting raw data to the inhibition model.

Assimilation of the label is calculated as the sum of incorporated and respired label:

$$\% \text{assimilation} = (\text{DPM}_{\text{clam}} + \text{DPM}_{\text{respired}}) / \text{DPM}_{\text{total}} \quad (7)$$

## RESULTS

In the Baffin Bay-Laguna Madre ecosystem, *Mulinia lateralis* usually recruits in the spring and has low densities during other seasons. In 1989, before the bloom, populations were dense (Fig. 2A), and there was a large spectrum of different sized individuals (Fig. 2B). During the years 1990 and 1991, when the brown tide bloom was at its greatest extent, population densities decreased to near extinction. The spring abundance peaks were very low, indicating a poor recruitment year. Large members of the population (>10.5 mm) were lost. When the population rebounded during 1992, large sized organisms were again present (Fig. 2B).

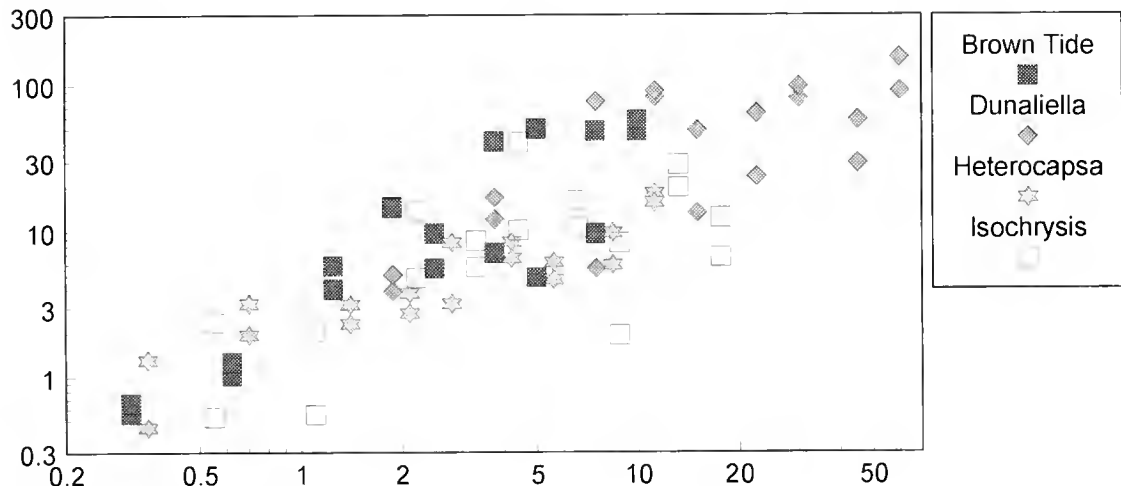
Feeding rates (equations 1–4), as a function of algal concentration, were measured in the first experiment (Figs. 3–7). These rates were fitted to the inhibition model (equation 5) and estimates for the three parameters were calculated (Table 2). The grazing rate fraction,  $F$ , increased for all four algal species to concentrations of stock culture of about 20–35%, and then declined (Fig.

3A). When fitted to the inhibition model, it appeared that maximal grazing rates were reached at the concentrations corresponding to 35% of the stock solution for brown tide, and 20% for two of the algal species: *Dunaliella* and *Isochrysis* (Fig. 3B). Grazing rates on *Heterocapsa* are best at the lowest concentrations (about 5% of stock). Surprisingly, inhibition of grazing rates at high stock culture concentrations was the least for brown tide. The stock cultures were started at very different densities (Table 1).

Grazing rates are presented in four other ways. The biomass consumed ( $I_C$ ) and clearance ( $I_{\text{clear}}$ ) rates were plotted versus the concentration of cells offered (cells  $\cdot$  ml $^{-1}$ ) and the biomass offered ( $\mu\text{g C} \cdot \text{ml}^{-1}$ ) (Figs. 4–7). These rates and concentrations varied over several orders of magnitude, so are shown on logarithmic scales. The number of cells consumed ( $I_{\text{cell}}$ ) generally had the same shaped curves as the biomass consumed ( $I_C$ ), so are not shown. Parameters fit to all grazing models are shown in Table 2.

Biomass consumed ( $I_C$ ) varied over four orders of magnitude from about 0.4 to 150  $\mu\text{g C} \cdot \text{h}^{-1}$  (Fig. 4A). Consumption rates increased with cell concentration offered (Fig. 4A). Inhibition ( $d$ ) at the cell concentrations measured is obvious for *Dunaliella* and *Isochrysis* (Fig. 4B). Initial uptake rates ( $k$ ) are very different for all four species. Maximal grazing rates ( $I_m$ ) were highest for *Du-*

### A. Measured Rate ( $\mu\text{g C/h}$ )



### B. Predicted Rate ( $\mu\text{g C/h}$ )

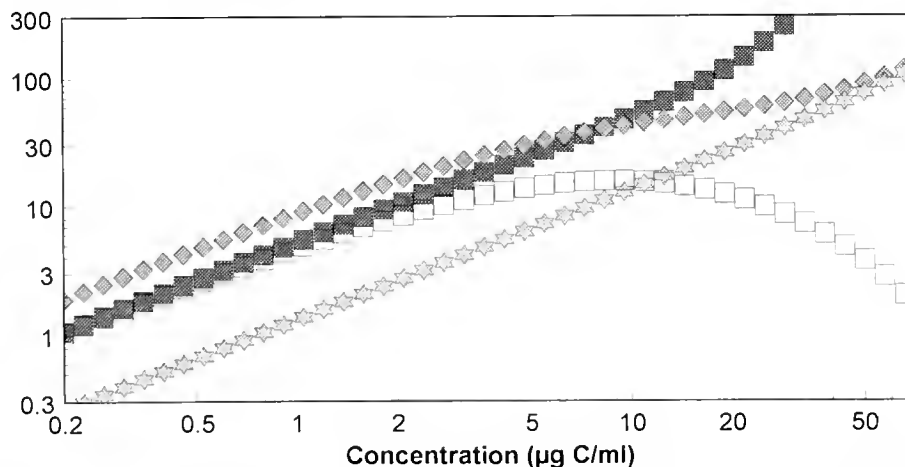
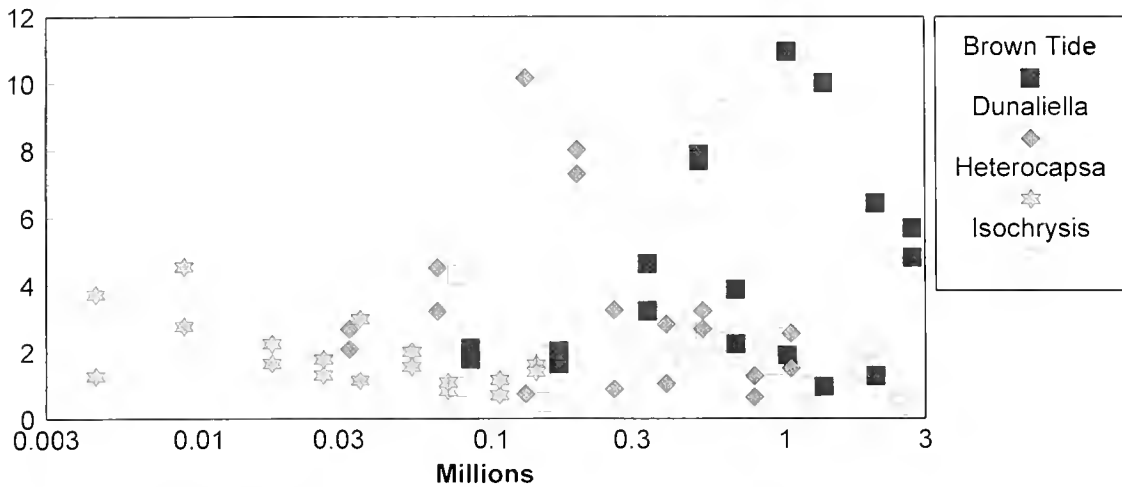


Figure 5. Carbon consumption rate,  $I_C$  ( $\mu\text{g C} \cdot \text{h}^{-1}$ ), versus carbon concentrations ( $\mu\text{g C} \cdot \text{ml}^{-1}$ ). A. Measured grazing rates for all four algal species. B. Predicted grazing rates by fitting raw data to the inhibition model.

## A. Measured Rate (ml/h)



## B. Predicted Rate (ml/h)

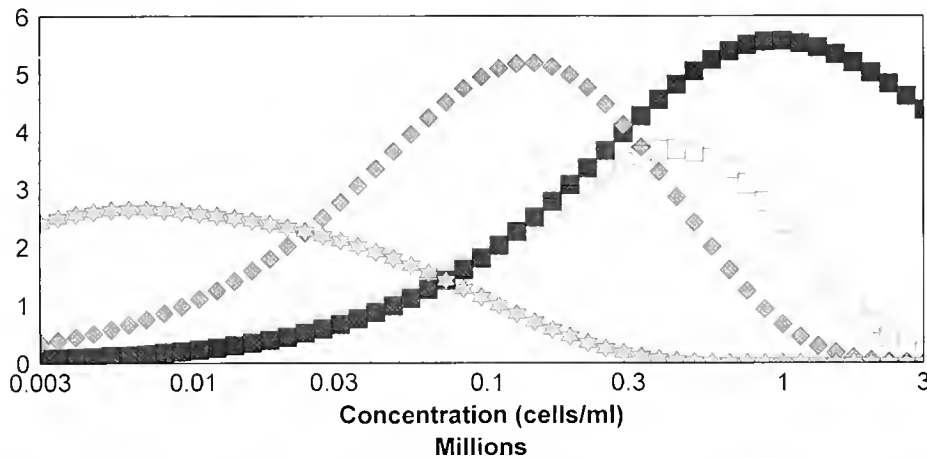


Figure 6. Clearance rate,  $I_{\text{clear}}$  ( $\text{ml} \cdot \text{h}^{-1}$ ), versus cell concentrations ( $10^6 \text{ cells} \cdot \text{ml}^{-1}$ ). A. Measured grazing rates for all four algal species. B. Predicted grazing rates by fitting raw data to the inhibition model.

*naliella* (Fig. 4A), but the simulation indicates *Heterocapsa* also would have high maximal rates at high cell concentrations (Fig. 4B).

The different sizes of the algae means that different amounts of carbon were offered in each experiment (Table 1). This can be corrected for by presenting grazing rates versus the concentration of carbon offered ( $\mu\text{g C} \cdot \text{ml}^{-1}$ ) (Fig. 5A). Again, inhibition ( $d$ ) at the carbon concentrations measured were obvious only for *Isochrysis* and to a lesser extent *Dunaliella* (Fig. 5B). Maximal grazing rates ( $I_m$ ) were highest for brown tide, *Heterocapsa*, and *Dunaliella*. Initial uptake rates ( $k$ ) were similar for three species: brown tide, *Dunaliella* and *Isochrysis*, which were higher than the rate for *Heterocapsa* (Fig. 5B).

Clearance rates ( $I_{\text{clear}}$ ) generally had different shaped curves than the feeding rate curves. Clearance rates generally decreased with increased food offered. Peak feeding rates ( $I_m$ ) occurred at different cell concentrations for all four species of algae (Figs. 6A and 6B). Inhibition ( $d$ ) was high for *Dunaliella* and *Isochrysis*, but low for brown tide. Initial clearance rates ( $k$ ) were highest for *Heterocapsa* and *Dunaliella*.

The shapes of the curves were similar when clearance rate is plotted against biomass offered (Fig. 7A). Inhibition ( $d$ ) for all

algal species was greatest for *Isochrysis* and *Heterocapsa* (Fig. 7B). Maximal clearance rates ( $K_m$ ) were greatest for brown tide and *Dunaliella*. Initial clearance rates ( $k$ ) were similar for all species.

The fate of algal carbon consumed was determined in the second experiment (Table 3). In general, assimilation rates are high, but this may be due to the short depuration time (2 h). Similar assimilation, respiration and defecation rates were found for brown tide, *Dunaliella*, and *Isochrysis*. *Heterocapsa* had the lowest assimilation rate, and highest defecation rate, indicating that this alga was not being utilized as efficiently as the other species.

## DISCUSSION

*Mulinia lateralis*, of the family Mactridae, is an extremely hardy species, ranging from Prince Edward Island, Canada to Yucatan, Mexico and in salinities from 5 ppt to 80 ppt (Parker 1975). It is considered an opportunist, because it can colonize rapidly after a disturbance event such as dredging or heavy rain (Flint and Younk 1983, Flint et al. 1981). It is abundant in the low salinity zones of Gulf coast bays (Harper 1973, Montagna and Kalke 1992). In the current study area, the Baffin Bay-Laguna

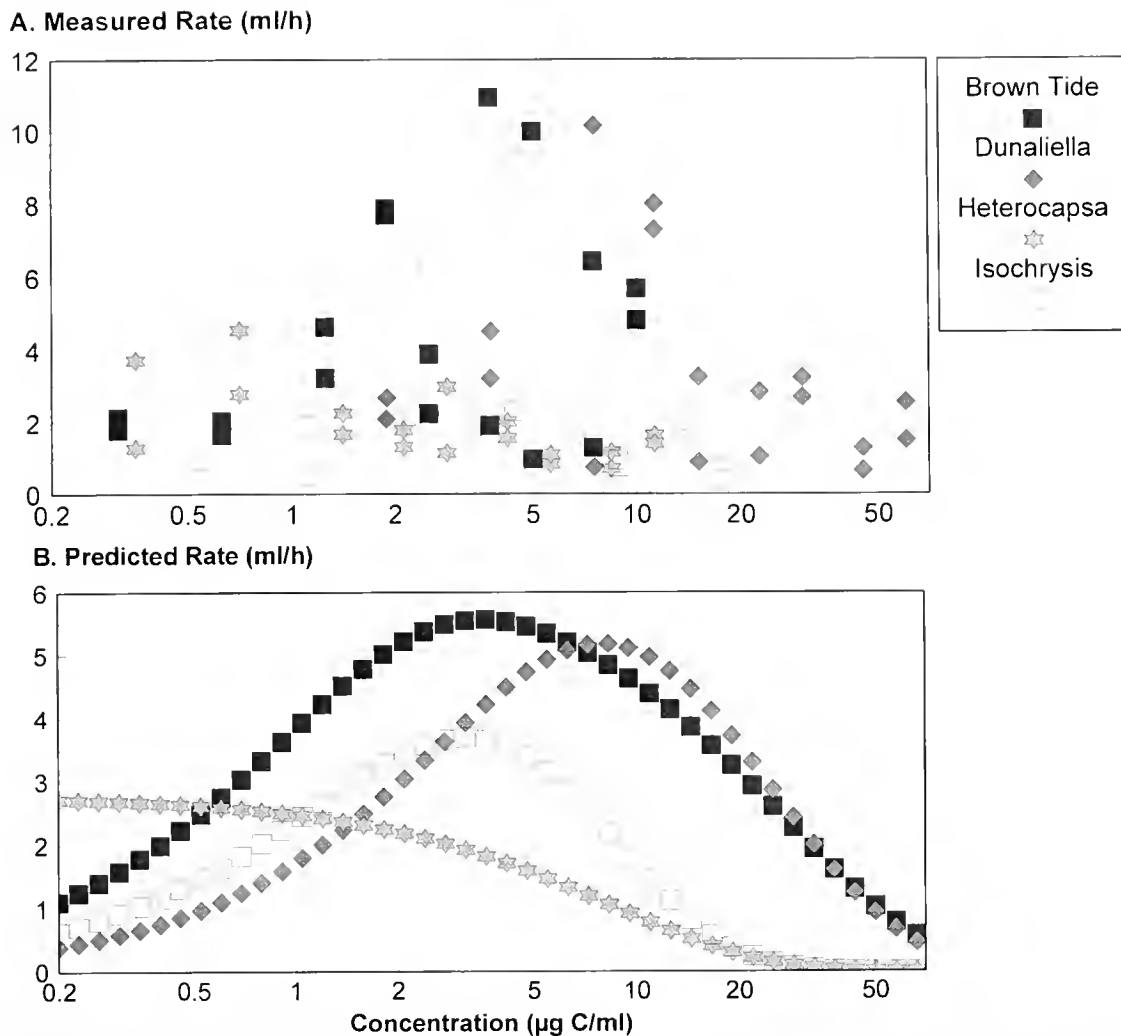


Figure 7. Clearance rate,  $I_{\text{clear}}$  ( $\text{ml} \cdot \text{h}^{-1}$ ), versus carbon concentrations ( $\mu\text{g C} \cdot \text{ml}^{-1}$ ). A. Measured grazing rates for all four algal species. B. Predicted grazing rates by fitting raw data to the inhibition model.

Madre ecosystem (particularly Alazan Bay). *M. lateralis* is the most abundant and widespread mollusk (Martin 1979, Cornelius 1984).

*Mulinia lateralis* spawning appears greatest in the spring in Baffin Bay and Laguna Madre (Fig. 2). However, it can have a continuous period of setting from a single spawning cycle from May through November in the Tred Avon River, Maryland and Chesapeake Bay (Shaw 1965, Holland et al. 1977). In Alazan Bay, Texas, Cornelius (1984) observed juveniles in all months except December, and Poff (1973) observed year-round spawning in Trinity Bay on the northern Texas coast. In San Antonio Bay, on the Central Texas coast, *Mulinia* population peaks occurred predominantly between January and April from 1987 through 1992 (Montagna unpublished data). It has a very short generation time and is capable of successfully spawning at 3 mm in length, which is approximately 60-days old (Calabrese 1969a). Embryo survival and development occurs over a wide range of salinity and temperature ranges. *Mulinia* develop into normal larvae throughout the salinity range of 15 to 35 ppt and the temperature range of 10 to 30°C (Calabrese 1969b).

*Mulinia* is an important food item for bottom feeding organisms, e.g., the black drum (Pearson 1929, Breuer 1957, Simmons

and Breuer 1962, Martin 1979) and to the greater and lesser scaup ducks (Cronan 1957). Large rafts of scaup ducks were observed in upper San Antonio Bay, Texas in November 1988 corresponding to densities of  $15,000 \cdot \text{m}^{-2}$  of *Mulinia lateralis* (Kalke personal observation).

These three factors (wide-spread distribution and high densities, rapid population growth, and importance as a food source to fish and wildlife) indicate that *Mulinia* is an important species in the Laguna Madre-Baffin Bay ecosystem. There was great concern about the integrity of the ecosystem when the brown tide occurred, because brown tides may have negative effects on shellfish.

The brown tide bloom was most intense for two years, 1990–1991. During this time cell concentrations averaged  $1.9 \times 10^6 \cdot \text{ml}^{-1}$  (Stockwell et al. 1993). During these two years, *Mulinia* populations suffered from very poor recruitment (Fig. 2). In both these years, there was a very small spring peak. This could be a coincidence, but the occurrence of brown tide occurred with the reduction of *Mulinia* populations. *Mulinia* populations declined during the last quarter of 1989 prior to the brown tide. The decline in late summer and fall appears to be a normal cycle that occurred in all four years. The low density in late 1989 is consistent with the trends found in earlier studies of Cornelius (1984). Although *Mulinia* ap-

TABLE 2.  
Parameters fitted for grazing rates using the inhibition model. The variables are designated as Y versus X.

Variables	Alga	R <sup>2</sup>	I <sub>m</sub>	k	d
F × %stock	Brown tide	74%	2.75 × 10 <sup>-1</sup>	9.21 × 10 <sup>-2</sup>	1.02 × 10 <sup>-3</sup>
	<i>Isochrysis galbana</i>	63%	4.51 × 10 <sup>-1</sup>	5.49 × 10 <sup>-2</sup>	1.54 × 10 <sup>-2</sup>
	<i>Dunaliella tertiolecta</i>	73%	3.70 × 10 <sup>-1</sup>	1.36 × 10 <sup>-1</sup>	1.00 × 10 <sup>-2</sup>
	<i>Heterocapsa pygmeae</i>	85%	1.19 × 10 <sup>-1</sup>	9.66 × 10 <sup>-1</sup>	1.63 × 10 <sup>-3</sup>
F <sub>cell</sub> × cells · ml <sup>-1</sup>	Brown tide	79%	4.55 × 10 <sup>8</sup>	1.10 × 10 <sup>-8</sup>	2.22 × 10 <sup>-7</sup>
	<i>Isochrysis galbana</i>	65%	3.67 × 10 <sup>6</sup>	1.47 × 10 <sup>-6</sup>	1.03
	<i>Dunaliella tertiolecta</i>	80%	6.99 × 10 <sup>5</sup>	1.38 × 10 <sup>-5</sup>	-6.41 × 10 <sup>-1</sup>
	<i>Heterocapsa pygmeae</i>	91%	1.17 × 10 <sup>9</sup>	1.14 × 10 <sup>-9</sup>	1.02 × 10 <sup>-9</sup>
F <sub>cell</sub> × μg C · ml <sup>-1</sup>	Brown tide	79%	6.54 × 10 <sup>8</sup>	2.07 × 10 <sup>-3</sup>	1.00 × 10 <sup>-8</sup>
	<i>Isochrysis galbana</i>	65%	3.67 × 10 <sup>6</sup>	2.04 × 10 <sup>-1</sup>	1.43 × 10 <sup>5</sup>
	<i>Dunaliella tertiolecta</i>	74%	8.00 × 10 <sup>6</sup>	5.41 × 10 <sup>-3</sup>	1.81 × 10 <sup>4</sup>
	<i>Heterocapsa pygmeae</i>	91%	1.31 × 10 <sup>9</sup>	1.28 × 10 <sup>-5</sup>	1.10 × 10 <sup>-8</sup>
F <sub>C</sub> × cells · ml <sup>-1</sup>	Brown tide	79%	2.31 × 10 <sup>6</sup>	7.97 × 10 <sup>-12</sup>	1.59 × 10 <sup>-2</sup>
	<i>Isochrysis galbana</i>	64%	1.05 × 10 <sup>6</sup>	3.30 × 10 <sup>-11</sup>	8.25 × 10 <sup>-1</sup>
	<i>Dunaliella tertiolecta</i>	76%	9.48 × 10 <sup>5</sup>	2.44 × 10 <sup>-10</sup>	8.63 × 10 <sup>-1</sup>
	<i>Heterocapsa pygmeae</i>	91%	1.58 × 10 <sup>6</sup>	6.52 × 10 <sup>-11</sup>	-3.41 × 10 <sup>-1</sup>
F <sub>C</sub> × μg C · ml <sup>-1</sup>	Brown tide	79%	2.56 × 10 <sup>1</sup>	2.10 × 10 <sup>-1</sup>	-2.03
	<i>Isochrysis galbana</i>	65%	2.64 × 10 <sup>1</sup>	2.04 × 10 <sup>-1</sup>	1.03
	<i>Dunaliella tertiolecta</i>	80%	4.03 × 10 <sup>1</sup>	2.40 × 10 <sup>-1</sup>	-6.41 × 10 <sup>-1</sup>
	<i>Heterocapsa pygmeae</i>	91%	1.08 × 10 <sup>2</sup>	1.20 × 10 <sup>-2</sup>	-9.37 × 10 <sup>-1</sup>
F <sub>clear</sub> × cells · ml <sup>-1</sup>	Brown tide	74%	6.60	3.40 × 10 <sup>-6</sup>	9.03 × 10 <sup>-7</sup>
	<i>Isochrysis galbana</i>	63%	1.09 × 10 <sup>1</sup>	2.23 × 10 <sup>-6</sup>	1.51 × 10 <sup>-5</sup>
	<i>Dunaliella tertiolecta</i>	73%	8.89	1.33 × 10 <sup>-5</sup>	2.31 × 10 <sup>-5</sup>
	<i>Heterocapsa pygmeae</i>	85%	2.85	6.79 × 10 <sup>-4</sup>	2.75 × 10 <sup>-5</sup>
F <sub>clear</sub> × μg C · ml <sup>-1</sup>	Brown tide	74%	6.59	9.22 × 10 <sup>-1</sup>	2.44 × 10 <sup>-1</sup>
	<i>Isochrysis galbana</i>	63%	9.00	3.73 × 10 <sup>-1</sup>	1.49
	<i>Dunaliella tertiolecta</i>	73%	8.88	2.26 × 10 <sup>-1</sup>	3.99 × 10 <sup>-1</sup>
	<i>Heterocapsa pygmeae</i>	85%	2.77	9.77 × 10 <sup>2</sup>	3.22 × 10 <sup>-1</sup>

pears to have a cyclical life cycle in south Texas estuaries, it seems certain that recruitment and densities were unusually low in 1990–1991 during the peak of the brown tide bloom.

*Mulinia* is known to have cyclical life cycles, so it is difficult to prove that low abundances are directly related to the brown tide. Population declines could have been caused by the brown tide in at least three different ways: reproductive failure, feeding inhibition, or a toxic effect. Each of these events has been observed numerous times in shellfish in coincidence with other brown tide blooms on the east coast of the U.S. (Tracey 1988, Tracey et al. 1988, Gainey and Shumway 1991). The main goal of this study was to determine if feeding was affected adversely. The test is to determine if inhibition of grazing rates occurred at high densities as indicated by high values of the parameter *d*. Two other parameters of interest are the initial grazing rate (*k*) and the maximal grazing rate (*I<sub>m</sub>*). Finally, food must be assimilated to be utilized.

The main approach in this study was comparative; therefore, the major assumption was that if feeding on brown tide was similar

to that of other species, then there was no adverse feeding effect. Since the other algal species are kept in culture at the University of Texas Marine Science Institute to provide feed for animal cultures, this is not an unreasonable assumption.

There are many different ways of calculating a feeding rate and normalizing it to comparable units. Each different calculation and unitization would yield different interpretations. The basic measurement was the percent of label removed at each dilution level of a stock culture (Fig. 3). The concentrations of the stock cultures were at different levels of peak densities. When the dilutions were made, the result was experiments at differing concentrations for each species. The experiment was designed to determine how feeding varied as a function of food concentration. Dilutions were normalized to cell concentration to elucidate differences in cell concentration. Cell sizes among the four algae also differed. Although there are interspecific differences, generally bivalves retain larger particles more efficiently than smaller particles (Mohlenberg and Riisgard 1978). Differences in sizes of cells were ac-

TABLE 3.  
Fate of algal <sup>14</sup>C in the second experiment. Average (± standard deviation) for n = 9.

Species	Incorporation	Respiration	Defecation	Assimilation
Brown tide	68% (±8)	8% (±5)	24% (±6)	78%
<i>Isochrysis galbana</i>	69% (±10)	11% (±3)	20% (±9)	82%
<i>Dunaliella tertiolecta</i>	64% (±10)	13% (±4)	23% (±7)	79%
<i>Heterocapsa pygmeae</i>	59% (±11)	11% (±3)	20% (±10)	73%



TABLE 4.  
Summary of results of the feeding experiments.

Variables	$I_m$	$k$	$d$
$F \times \% \text{stock}$	Bt > (Du = Is) > He	(Du = He) > (Bt = Is)	(Du = Is) > He > Bt
$F_C \times \text{cells} \cdot \text{ml}^{-1}$	(Du = He) > (Bt = Is)	Du > He > Is > Bt	Is > Du > (Bt = He)
$F_C \times \mu\text{g C} \cdot \text{ml}^{-1}$	Bt > (Du = He) > Is	Du > (Bt = Is) > He	Is > Du > (Bt = He)
$F_{\text{clear}} \times \text{cells} \cdot \text{ml}^{-1}$	(Du = He) > (Bt = Is)	He > Du > (Bt = Is)	Is > (Du = He) > Bt
$F_{\text{clear}} \times \mu\text{g C} \cdot \text{ml}^{-1}$	(Bt = Du) > He > Is	He > Bt > (Is = Du)	Is > (Du = He) > Bt

Variables are designated as Y versus X in Figures 3–7 respectively.

Abbreviations used: Bt = brown tide, Du = *Dunaliella*, Is = *Isochrysis*, and He = *Heterocapsa*.

counted for by normalizing the ingestion rates by cell-carbon content ( $I_C$ ) (Figs. 4 and 5). Generally, the functional response is that feeding rates increase with increased food concentrations (Figs. 4 and 5). Clearance rates are the volumes of water swept clear, and, in general, this rate decreases as the food concentration increases (Figs. 6 and 7).

The strongest inhibition of grazing rates is observed when the fraction removed ( $F$ ) is plotted against the dilution series (% stock) (Fig. 3). The least amount of inhibition occurred with brown tide. This may be partially due to culture artifacts. At the time of this study, we had not learned how to culture the brown tide, therefore field populations were collected and used in the feeding experiments. The three cultured species had strong inhibition in experiments up to 20% of the stock solution and the brown tide sample showed only minor inhibition at full strength samples. This could be due to the build up of algal metabolites in the cultures, which are known to inhibit bivalve feeding (Ward and Targett 1989).

The cell density in the brown tide samples was high enough to discover the inhibition response. Tracey (1988) did not see feeding rate inhibition in *Mytilus edulis* until brown tide concentrations were at  $10^5 \text{ cells} \cdot \text{ml}^{-1}$ . The concentrations of brown tide offered in this study were up to  $2 \times 10^6$  (Table 1, Figs. 4 and 6).

Results appear to be different among the different ways to plot the data (Table 4), but some trends were consistent. *Isochrysis* was the only alga to have its feeding rate consistently inhibited by high food concentrations (Table 4). The only alga to have a low assimilation rate was *Heterocapsa* (Table 3). Brown tide never had the lowest maximal feeding rates ( $I_m$ ) or initial rates ( $k$ ) (Table 4). In all cases, brown tide did not appear to negatively affect feeding by *Mulinia*.

*Mulinia* appears to have the potential to control phytoplankton blooms. Clearance rates were near  $10 \text{ ml} \cdot \text{h}^{-1}$  at peak brown tide densities of  $10^6 \text{ cell} \cdot \text{l}^{-1}$  (Fig. 6A), thus at prebloom densities ( $800 \cdot \text{m}^2$ , Fig. 2A) *Mulinia* could clear  $8 \text{ l} \cdot \text{h}^{-1}$ . The average water column depth in the Baffin Bay-Laguna Madre ecosystem is 1.2 m (TDWR 1983); therefore, the clams associated with each square meter of sediment could clear the overlying water column in 150 h or about 6–7 days. Microzooplankton were common before the brown tide, but also nearly disappeared. Microzooplankton consumption decreased from 95% of the phytoplankton

production to 5% consumed per day (Buskey and Stockwell 1993). With the loss of both the microzooplankton and the bottom filter feeding animals, there was almost no filtering capacity in the bay during the peak of the brown tide bloom, between 1990 and 1991.

Although we did not find negative feeding effects on adults, it is possible that there would be negative effects on juveniles or larvae. The east coast brown tide did cause negative feeding and locomotory behavior on scallop larvae (Gallagher and Stoecker 1989). We used individuals (6–12 mm) in the middle-size range (Fig. 2) in this study.

If feeding inhibition did not occur, then something else may have caused the population declines. It is well known that *Mulinia* population sizes have large natural variability, but brown tides on the east coast have been toxic to bivalves and have caused declines in reproductive potential. It is not known if these kinds of effects occur with *Mulinia*. Another possibility is increased predation pressure. When *Mulinia* populations declined in Texas, we were most concerned about how this would effect the food web that supported the black drum fishery. The black drum population has been increasing over the last five years, and reached 20-year record levels during the brown tide (Larry McEchron, Texas Parks and Wildlife Department, personal communication). It is possible that *Mulinia* populations were wiped out by the high populations of the predatory black drum. If this is true, there might have been a trophic cascade that led to conditions favorable for a bloom. In a trophic cascade, the predator reduced populations of the herbivore, which, in turn, allowed the primary producer populations to bloom uncontrollably. Since *Mulinia* feeds well on the brown tide alga, a trophic cascade is a plausible hypothesis for (at least partially) explaining the mollusk population declines and the occurrence of the bloom.

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## DOMOIC ACID IN THE PACIFIC RAZOR CLAM *SILIQUA PATULA* (DIXON, 1789)

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**ABSTRACT** In the fall of 1991 domoic acid was discovered in coastal Pacific razor clams *Siliqua patula* (Dixon, 1789) in Washington and Oregon states at levels higher than acceptable for safe human consumption, thereby forcing a closure of the recreational harvest. Tissue distribution data indicated the clams maintained these elevated levels from fall through early summer of 1992 in the edible muscular tissues (mantle, siphon, adductor muscles, and muscular foot) with concentrations of toxin averaging from 23.3–50.7  $\mu\text{g/g}$ . The concentration in the non-edible tissues (gill, digestive gland, gonad, and siphon tip) ranged from trace amounts to 8.4  $\mu\text{g/g}$ . Clams that were dissected into edible and non edible pooled portions contained  $36.4 \pm 22.6$  and  $13.7 \pm 7.6$   $\mu\text{g/g}$ , respectively. On an additional sampling date, clams were sampled fresh or were frozen whole before sampling. The concentration in the edible portion of the fresh clams averaged  $16.8 \pm 11.6$   $\mu\text{g/g}$ , while the blood and dissection fluids contained only trace amounts of toxin. The domoic acid concentration of the frozen edible portion averaged  $12.6 \pm 6.9$   $\mu\text{g/g}$  with meltwater levels reaching 4.2  $\mu\text{g/g}$  and the dissection fluid containing up to 10.0  $\mu\text{g/g}$ . Clams collected in December 1991 with elevated levels of toxin ( $47.9 \pm 12.7$   $\mu\text{g/g}$ ) that were held on Strait of Juan de Fuca seawater for three months maintained this level of contamination ( $44.3 \pm 19.8$   $\mu\text{g/g}$ ). Razor clams from Alaska held under identical conditions during this time period did not contain detectable levels of toxin. Razor clam tissues collected in 1985, 1990, and the summer of 1991 revealed only trace levels of toxin.

**KEY WORDS:** domoic acid, razor clam, *Siliqua patula*, neurotoxin

### INTRODUCTION

In the late fall of 1987 there was an outbreak of human shellfish poisoning on Prince Edward Island (PEI), Canada. The toxin identified in this outbreak was domoic acid, a neurotoxic amino acid not previously found in shellfish (Wright et al., 1989). The cultured shellfish consumed was the blue mussel, *Mytilus edulis* (Linnaeus 1758), and the source of domoic acid was identified as the diatom *Nitzschia pungens* Grunow forma *multiseries* Hasle (Bates et al., 1989). Due to the primary neurological symptoms of confusion, disorientation, and permanent memory loss, the name Amnesic Shellfish Poisoning (ASP) was adopted for the syndrome (Perl et al. 1990, Todd 1990). In November 1991, domoic acid was found for the first time in coastal Pacific razor clams, *Siliqua patula*, in Washington and Oregon states.

Razor clams are a recreational shellfish in Washington with an estimated 250,000 people harvesting up to 14 million clams per year in limited seasons (Lassuy and Simons 1989). When razor clams from Washington showed domoic acid contamination above 20  $\mu\text{g/g}$ , a closure of the recreational season was declared by the Washington Department of Fisheries.

Throughout the winter and spring of 1991–1992, Washington razor clams maintained levels of toxin higher than 20  $\mu\text{g/g}$ . Fortunately, razor clams from Alaska, which were uncontaminated with domoic acid, were being held at our laboratory on Sequim Bay which is removed from the open Pacific coast and where domoic acid has not been detected (Fig. 1). Furthermore, Washington clams contaminated with domoic acid were brought to our laboratory during the winter and spring of 1991–1992, held in the same manner as the Alaskan clams, and were periodically sampled during this time. Frozen historical samples were also available, including samples collected in late summer 1991 from Washington coastal beaches prior to domoic acid detection. Although not originally sampled for this purpose, these clams were available for domoic acid analysis.

The Pacific razor clam at maturity can measure 150 mm in

length and weigh 250 g when harvested. The freezing and thawing of clams is a common practice before selecting muscular tissues to eat. Prior to this study, there was no information on domoic acid distribution in razor clam tissues or methods for sampling. Development of sampling techniques and tissue distribution data are required for further studies, including use of selective tissue types for harvest or analysis, and analysis of frozen historical samples. The tissue distribution pattern of domoic acid in tissues may also indicate pathways of domoic acid metabolism in the clam.

The purpose of this paper is to examine: 1) the distribution of domoic acid in razor clam tissues, 2) the effect of freezing on domoic acid concentration in the clam, 3) the depletion of domoic acid in contaminated coastal clams maintained on domoic acid-free seawater, and 4) the levels of domoic acid in historical samples of razor clam tissues.

### MATERIALS AND METHODS

#### *Tissue Distribution and Freezing*

Pacific razor clams, *Siliqua patula*, were collected from Copalis Beach, Washington (Fig. 1) during a period when elevated levels of domoic acid were present in the clams. Clams were dug the morning of the sampling dates and transported live to the Battelle Marine Sciences Laboratory (MSL) in Sequim, Washington. Before dissecting or freezing the clams, the seawater that was present in the siphon or body cavity was drained from the clam and the shell surface was blotted dry. All clams were measured for greatest shell length and, if required for the study, tissues were dissected and weighed prior to freezing at  $-20^{\circ}\text{C}$ . Tissue samples were later thawed and homogenized in their original sample container to include all fluids in the analysis.

#### *Sample Group #1*

On May 18, 1992, 2 lots of 10 clams were sampled. In the first lot, 10 clams were processed individually upon arrival at the lab-

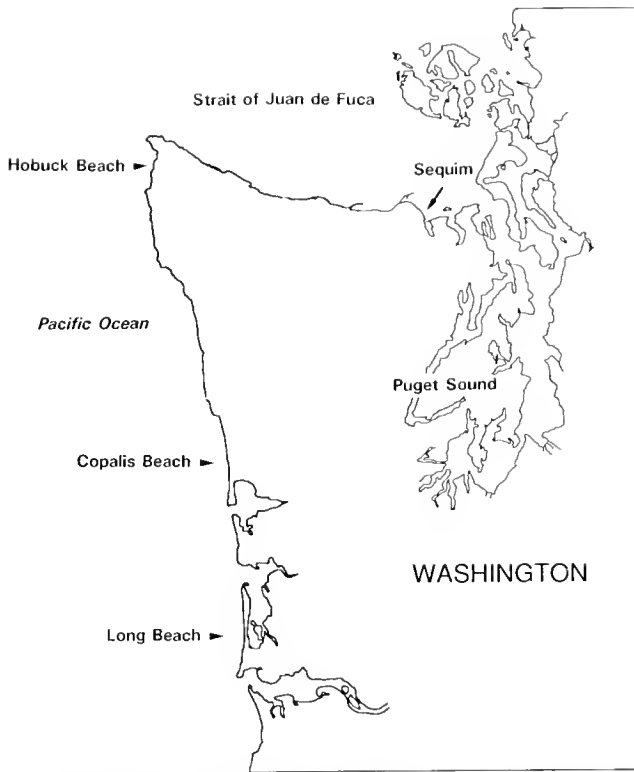


Figure 1. Location of Washington state coastal beach sampling areas and the Battelle Marine Sciences Laboratory on the Strait of Juan de Fuca.

oratory. Before dissecting the clam, a sample of blood was withdrawn from the intersiphon vessel with a 22-gauge needle. This was frozen in 50 ml conical tubes as either whole blood or as pelleted cells ( $300 \times g$  for 5 min) generating two blood samples from each clam. Eight tissues were then dissected from the clam (Fig. 2), including the siphon tip with all pigmented tissue, the adductor muscles, and an approximately 0.5 cm section from the midpoint of the siphon. The mantle tissue sample consisted of a

rectangular piece spanning the area from the hinge to the external mantle fringe. The tip of the muscular foot, measuring approximately  $0.5 \times 2.5$  cm when contracted, was carefully dissected to exclude gonadal tissue. The digestive gland was carefully cleaned of any external attached organs including esophagus and gonadal tissue.

Tissues were placed directly into individual petri dishes. After all samples were collected from an individual clam, the tissues were minced by the scissoring action of two scalpel blades. Tissues were transferred to tared 50 ml conical centrifuge tubes and tissue weights were recorded. Most tissue samples weighed between 1 and 2 g. For mantle and siphon, up to 2.5 g was collected. Wet mounts of gonad smears were examined to determine the sex of clams. Tissues were kept on ice as they were collected before freezing at  $-20^{\circ}\text{C}$ .

To represent the preparation of clams for human consumption, clams in the second lot on May 18 were dissected into pooled edible and non-edible tissue groups. All soft tissue present in the clam was included in the pooled samples with the dissection fluids excluded from the samples. The edible portion of the clam contained mantle, siphon without the tip, adductor muscles, and muscular foot. All remaining tissues were included in the non-edible sample.

#### Sample Group #2

On June 4, 1992, 30 adult razor clams of approximately the same length as those processed on May 18th were sampled. Twenty of these clams were analyzed for domoic acid concentration and 20 were dissected for tissue weight distribution data. An additional 10 smaller clams were processed for domoic acid content.

The 20 adult clams for domoic acid analysis were randomly divided into 2 groups of 10 clams, with 1 group frozen before sampling tissues and another group processed fresh. The clams that were processed fresh were measured upon arrival and a 2 ml sample of whole blood was collected from the siphon sinus. The clams were then dissected and tissues were pooled into edible and non-edible portions, as above. The clams were processed in indi-

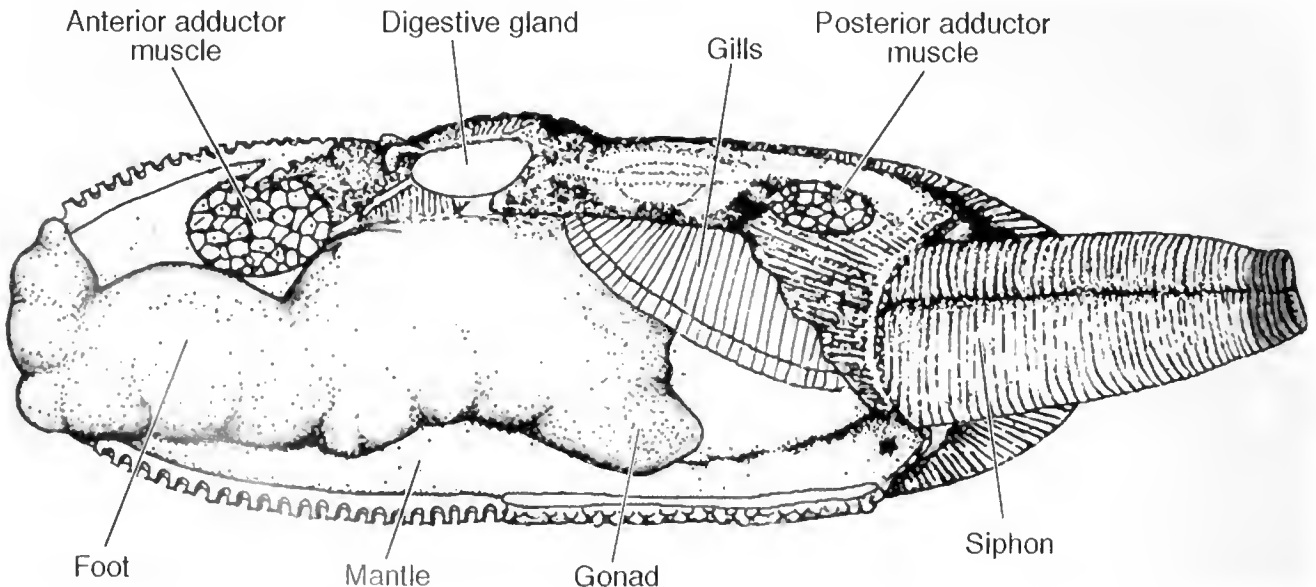


Figure 2. Tissues of the Pacific razor clam selected for domoic acid analysis.

vidual trays and the dissection fluid was sampled before including it with the non-edible sample. Wet mounts were made from the gonad tissue to determine the sex of the clams. The 40 samples for domoic acid analysis generated from these 10 clams were frozen at  $-20^{\circ}\text{C}$ , and thawed for analysis on June 5, 1992.

The second group of 10 adult clams for domoic acid analysis was frozen at  $-20^{\circ}\text{C}$  upon arrival in individual dissection trays. The frozen clams were removed from the freezer on June 15, 1992, and thawed at room temperature for approximately 1 hour in the trays. After thawing, the meltwater from the clams was sampled for domoic acid analysis. Each tray was cleaned and the clam was then dissected into edible and non-edible portions. The dissection fluid was sampled and then discarded. The 40 tissue samples collected were prepared without additional freezing for analysis the same day.

A third set of adult clams was sampled to determine tissue weight distributions in the clam. Adductor muscles, siphons without the pigmented tip, mantles, and muscular foot tissues were dissected from the clams in their entirety and weighed individually. These four tissue groups comprise the edible portion of the clam. The remaining non-edible tissues were combined and weighed without including the dissection fluids.

The final group of clams sampled on June 4 consisted of 10 juvenile clams 83–100 mm in length. These smaller clams were sampled for domoic acid in pooled edible and non-edible tissue groups prior to freezing.

#### *Laboratory Maintained Clams*

##### **Sample Group #1**

On July 9, 1991, razor clams were collected from Clam Gulch, Alaska, and transported to the Battelle MSL in Sequim, Washington. The clams were placed into holding tanks containing beach sand approximately 70 cm in depth with ambient, 8–14°C, raw Sequim Bay seawater flowing over the sand allowing the clams to either bury or feed at will. The clams were undisturbed until they were removed from the tanks on April 29, 1992, and frozen whole at  $-20^{\circ}\text{C}$ . For domoic acid content, the whole frozen clams were thawed on July 1, 1992, and the edible tissue portions were dissected for analysis.

##### **Sample Group #2**

Two sets of Washington clams were collected from Copalis Beach, Washington, and were transported to the laboratory and held in flow-through sand tanks as described above. The first set was collected on December 17, 1991, with ( $n = 5$ ) clams frozen upon arrival at the laboratory, and the remaining clams placed into a holding tank. This set was subsampled periodically during February and March 1992, and whole clams were frozen at  $-20^{\circ}\text{C}$ . The second set of clams from Copalis Beach, Washington, was collected on March 17, 1992, with 11 clams frozen upon arrival and the remaining maintained as above. This set was also subsampled in the following months of April and May with whole clams frozen at  $-20^{\circ}\text{C}$ . The clams in the above sample sets were thawed on July 10, 1992, and the edible tissue portion was dissected for domoic acid analysis.

#### *Historical Samples*

On August 7, 1991, two sets of razor clams were collected from Washington coastal beaches, including Long Beach, a south-

ern coastal beach, and Copalis Beach, a centrally located beach. On August 9, 1991, razor clams were collected from a northern coastal beach, Hobuck Beach (Figure 1). The clams were collected and transported to the MSL laboratory the same day where they were measured and selected tissues were dissected and placed in individual containers and frozen at  $-20^{\circ}\text{C}$ . For domoic acid analysis the muscular foot was sampled as a representative tissue for toxin contamination.

On February 26, 1990, a set of 10 clams was collected from Copalis Beach, Washington, and transported to our laboratory where the siphon and mantle tissues were dissected and pooled prior to freezing. These clams were not measured upon collection, but the tissue weights and small siphon size indicate the clams were less than 120 mm in length. Prior to domoic acid analysis the siphon tips were removed.

A set of 13 clams was collected on November 13, 1985, and frozen whole at  $-20^{\circ}\text{C}$ . Upon thawing and measuring the clams on August 5, 1992, the edible portion was dissected for domoic acid analysis.

#### *Extraction and Analytical Procedure*

Domoic acid analysis was performed following the National Research Council of Canada's Institute for Marine Biosciences (NRC/IMB) Technical Report #64: "A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples" (Quilliam et al., 1991). For extracting samples of up to 2.5 g, 4 ml of methanol was added to the sample and the total volume was adjusted to 10 ml with deionized water, homogenized for 2–3 min at three quarter to full speed using a Tissue Mizer, centrifuged 10 min at approximately  $3000 \times g$ , and 5 ml of the supernatant was collected. The extraction procedure for the larger edible and non-edible pooled samples consisted of adding an equal volume of deionized water to the sample and homogenizing for 3 to 4 min. From this homogenate 8 ml was subsampled, 8 ml of methanol was added, the final volume brought to 20 ml with DI  $\text{H}_2\text{O}$ , the sample was centrifuged 10 min at  $3,000 \times g$ , and 5 ml of the supernatant was reserved as above.

After solid phase extraction (SPE), the domoic acid was eluted with a 1.0 M citrate buffer solution (10.51 g citric acid monohydrate and 12.61 g ammonium citrate brought to a final volume of 100 ml with 10% acetonitrile). The pH was then adjusted to 4.4 with approximately 6 ml of 12% ammonium hydroxide. Two ml of this buffer was added to the SPE cartridge and eluted into an auto-sampler vial at a rate of one drop per second. Samples that were not analyzed immediately were stored in a dark refrigerator at approximately  $4^{\circ}\text{C}$ .

For HPLC analysis, an auto sampler (Waters W15P 710B) was set to deliver a 20  $\mu\text{l}$  injection volume to the system (Waters Guard Pak™ precolumn module (Guard Pak™,  $\mu\text{Bondapak}^{\text{TM}}$  C18) and a Supelcosil™ LC-PAH HPLC Column (Simicron 25 cm  $\times$  4.6 mm ID). A running flow rate of 1.50 ml/min was adopted using an Applied Biosystems 1400A Solvent Delivery System running at 206–212 bar. The mobile phase was 10% acetonitrile in deionized water with 0.1% trifluoroacetic acid. The detector (Applied Biosystems 1783 A Absorbance Detector-Controller) was set to 242 nm with a range of 0.005. The working system, including the column, was not under temperature control and the retention time of the domoic acid under these conditions was 8 min 25 sec  $\pm$  15 seconds and was recorded on a Shimadzu CR601 Chromatopac integrator.

A quantitative standard of domoic acid (DACS-1, 89 µg/mL, NRC MACSP) was serially diluted to establish the retention time and to calibrate the system. One level was used to check system function before beginning each set of samples. Quantification was based on peak height and results are reported in µg of domoic acid per g of wet tissue. Analytical quality assurance was maintained by analyzing reference and replicate standards for each set of 20 samples or less. These sets included one blank, one duplicate prepared from the current sample group, a reference tissue with a known concentration of domoic acid, and a standard dilution of domoic acid analytical solution.

#### Statistical Analysis

A t-test was used to compare the total domoic acid concentration between the edible and non-edible portions of the clams using the natural logarithm of concentration to stabilize the variances between classes. One-way analysis of variance was used to compare domoic acid concentrations in fresh, frozen, and juvenile clams; again, the natural logarithm transformation was used to stabilize the variances. Linear regression of total domoic acid concentration versus holding date was used to determine if there was a significant decrease in concentration during the first and second depuration experiments.

## RESULTS

### Tissue Distribution and Effects of Freezing

#### Sample Group #1

When the clams that were dissected into individual representative tissue types in May 1992 were analyzed, the concentration of domoic acid in the four edible tissues types (mantle, adductor muscles, siphon, and muscular foot) was significantly higher ( $p < 0.001$ ) than the levels present in the non-edible tissues (gill, gonad, digestive gland, and siphon tip) (Table 1). The average concentration of toxin in these 4 edible tissue types was 12 times higher than the average of the 4 non-edible tissues. The muscular foot contained the highest levels of domoic acid present in the clam (mean =  $50.7 \pm 26.5$  µg/g) followed by the adductor muscle, the siphon and the mantle. The non-edible gill and the diges-

TABLE 1.

Summary of domoic acid content in edible and non-edible tissue types.<sup>1</sup>

Tissue Type	Domoic Acid Concentration µg · g <sup>-1</sup>	
	Mean ± S.D.	Range
<i>Edible</i>		
Siphon	28.9 ± 22.4	7.5–84.9
Adductor	39.4 ± 18.6	20.2–78.4
Mantle	23.3 ± 11.7	10.1–48.2
Foot	50.7 ± 26.5	20.7–99.0
<i>Non edible</i>		
Gill	0.4 ± 0.9	ND <sup>2</sup> –2.9
Gonad	8.4 ± 3.9	3.9–15.7
Digestive Gland	0.4 ± 0.4	ND <sup>2</sup> –1.2
Siphon Tip	4.1 ± 5.0	1.0–18.2

<sup>1</sup> Average length of clams was  $136 \pm 7$  mm, all groups  $n = 10$ .

<sup>2</sup> Domoic acid was not detected.

tive gland contained only trace amounts of toxin while the average siphon tip and gonad concentrations were  $4.1 \pm 5.0$  µg/g and  $8.4 \pm 3.9$  µg/g, respectively. The whole blood and blood cell samples from two clams were analyzed and domoic acid was not detected.

Table 2 shows the results from the second set of clams in Sample Group 1, that were divided into edible and non-edible portions, representing the tissues consumed and discarded when the clam is prepared for human consumption. The concentration of domoic acid in the edible portion was  $36.4 \pm 22.6$  µg/g. The remaining tissues contained a mean of  $13.7 \pm 7.6$  µg/g.

#### Sample Group #2

The levels of domoic acid in the adult clams collected on June 4 were  $16.8 \pm 11.6$  µg/g in the edible tissues and the non-edible tissues contained  $13.2 \pm 9.0$  µg/g. After freezing the clams, the edible tissue average concentration was  $12.6 \pm 6.9$  µg/g and the non-edible portion contained  $7.3 \pm 3.7$  µg/g domoic acid. The meltwater collected upon thawing the clams contained toxin in eight of the ten clams, averaging  $1.4 \pm 1.5$  µg/g, while the dissection fluids contained a mean concentration of  $4.6 \pm 2.9$  µg/g. In the fresh clams, the whole blood sampled before dissecting the clam, and the fluids released when dissecting the clam, did not contain detectable levels of toxin. The edible and non-edible concentrations of domoic acid in the juvenile clams were  $4.3 \pm 1.4$  µg/g and  $3.9 \pm 1.0$  µg/g, respectively. The average concentration found in the juvenile edible tissues was significantly less ( $p < 0.001$ ) when compared to the average adult edible tissue concentration (Table 3).

#### Relative Proportion of Edible Tissues by Weight

The clams that were sampled to assess the relative proportion of the representative tissue types present in the edible portion of the clam reveal the average weights of the muscular foot, mantle, siphon, and adductor muscles represent 4, 59, 29 and 8%, respectively. The total amount of tissue present in the clam is divided into 53% edible with the remaining 47% non-edible tissue (Table 4).

#### Toxin Depletion in Laboratory Maintained Clams

The two sets of Washington beach clams that were held on Sequim Bay seawater maintained elevated levels of domoic acid in their edible tissues. The first set of clams, collected on December 17, 1991, arrived at the laboratory with a mean concentration of  $47.9 \pm 12.7$  µg/g. This level of contamination persisted through the final sampling dates of March 11 and 12, 1992, with a mean concentration of  $44.3 \pm 19.8$  µg/g. The second set of clams, which included a wider range in size, collected on March 17, 1992 with initial levels of  $30.7 \pm 14.6$  µg/g, contained a domoic acid average concentration of  $33.5 \pm 18.5$  µg/g when sampled two months later, during May 5 through 11, 1992 (Table 5). Razor

TABLE 2.

Summary of domoic acid in pooled edible and non-edible portions.<sup>1</sup>

Domoic Acid Concentration µg · g <sup>-1</sup>	Tissue Group	
	Edible	Non-edible
Mean ± SD	36.4 ± 22.6	13.7 ± 7.6
Range	9.5–79.6	3.2–25.5

<sup>1</sup> The average length of the clams was  $123 \pm 9$  mm, all groups  $n = 10$ .

TABLE 3.  
Summary of domoic acid concentration in juveniles, fresh and frozen adult razor clams.<sup>1</sup>

Domoic Acid Concentration $\mu\text{g} \cdot \text{g}^{-1}$	Clam Length mm	Analytical Fraction				
		Dissection Fluids	Melt Water	Blood	Edible Portion	Non-Edible Portion
FRESH ADULT CLAMS						
Mean $\pm$ SD	134 $\pm$ 10	ND <sup>2</sup>	NA <sup>3</sup>	ND <sup>2</sup>	16.8 $\pm$ 11.6	13.2 $\pm$ 9.0
Range	122–148				4.9–36.3	2.4–29.9
FROZEN ADULT CLAMS						
Mean $\pm$ SD	137 $\pm$ 5	4.6 $\pm$ 2.9	1.4 $\pm$ 1.5	NA <sup>3</sup>	12.6 $\pm$ 6.9	7.3 $\pm$ 3.7
Range	130–144	ND <sup>2</sup> –10.0	ND <sup>2</sup> –4.2		1.6–23.7	0.9–13.4
FRESH JUVENILE CLAMS						
Mean $\pm$ SD	92 $\pm$ 6	NA <sup>3</sup>	NA <sup>3</sup>	NA <sup>3</sup>	4.3 $\pm$ 1.4	3.9 $\pm$ 1.0
Range	83–100				1.7–6.6	2.0–5.2

<sup>1</sup> All groups n = 10.

<sup>2</sup> Domoic acid concentration was not detected.

<sup>3</sup> Analytical fraction was not available.

clams from Alaska maintained in the laboratory during this time and sampled on April 29, 1992, did not have measurable levels of domoic acid.

#### Historical Samples

The clams from Washington coastal beaches sampled in August 1991 contained low concentrations of domoic acid. The muscular foot toxin concentrations of clams from northern, central, and southern beaches were  $1.3 \pm \mu\text{g/g}$ ,  $1.6 \pm 1.1 \mu\text{g/g}$  and  $1.4 \pm 1.2 \mu\text{g/g}$ , respectively. The mantle and siphon tissues from clams sampled in early 1990 contained  $2.6 \pm 0.4 \mu\text{g/g}$  toxin, and the edible tissue portion from the 1985 late autumn samples contained  $0.9 \pm 0.7 \mu\text{g/g}$  (Table 6).

## DISCUSSION

#### Distribution of Domoic Acid in Clam Tissues

##### Edible Tissues

This study indicates that the Pacific razor clam concentrates domoic acid in its muscular tissues. This is in contrast to the blue mussel, *Mytilus edulis*, which posed a health threat in Canada, where virtually all of the domoic acid was present in the digestive gland (Wright et al., 1989). Novacek et al. (1992) demonstrated that domoic acid was in the gut lumen of contaminated mussels while insignificant intracellular levels of domoic acid were present in the digestive gland. The lack of domoic acid in the razor clam digestive glands indicates that the clams had not been feeding on a domoic acid source when sampled but, due to lack of knowledge

of the uptake and depuration of domoic acid in razor clams, the level and duration of toxin exposure remains unknown. The presence of domoic acid in the muscular tissues of the razor clam could demonstrate differences in uptake and depletion patterns among bivalves.

In the razor clam, the average concentrations in representative tissues indicate that the adductor muscle and muscular foot have the highest concentrations of domoic acid. This distribution pattern, however, is not consistent among clams, with the remaining edible tissues, siphon and mantle, containing the highest levels of toxin in some clams. The weight distribution data show that the muscular foot and adductors, often the most highly toxic tissues, comprise only 12% of the edible tissues by weight. However, the individual variability in toxin retention pattern within all edible tissues does not support the selective consumption of edible tissues to avoid accumulations of toxin.

##### Non-Edible Tissues

The gill and digestive gland tissues contained only trace amounts of domoic acid in the razor clams examined in this study. These tissues are accumulation sites of the toxin in the mussel (Novacek et al., 1991). The lack of domoic acid in these tissues suggests the razor clams on the Washington coast were not being exposed to domoic acid when sampled for this study.

The non-edible siphon tip is normally discarded before eating the razor clam. It was included in the tissue distribution analysis of the razor clam because in the case of toxins involved in paralytic shellfish poisoning (PSP), the butter clam, *Saxidomus giganteus* (Deshayes, 1839), of the Pacific Northwest has been known to

TABLE 4.  
Summary of the distribution of edible and non-edible tissues in the razor clam.<sup>1</sup>

Tissue Weight g	Tissue Type				
	Foot	Mantle	Siphon	Adductor	Non-Edible
Mean $\pm$ SD	1.8 $\pm$ 0.3	23.6 $\pm$ 2.5	11.6 $\pm$ 1.6	3.2 $\pm$ 0.7	35.0 $\pm$ 3.8
Range	1.4–2.5	20.7–27.9	8.1–13.6	2.2–4.5	28.0–41.3

<sup>1</sup> The average length of the clams was  $122 \pm 3\text{mm}$ , all groups n = 10.

TABLE 5.  
Summary of the domoic acid content of clams of held on inland seawater.<sup>1</sup>

	Domoic Acid Concentration $\mu\text{g} \cdot \text{g}^{-1}$	Sampling Date				
		Dec 17	Feb 12, 13	Feb 22, 23	Mar 4	Mar 11, 12
COPALIS Group #1	Mean $\pm$ SD	47.9 $\pm$ 12.7	50.4 $\pm$ 9.9	45.7 $\pm$ 18.8	53.8 $\pm$ 19.7	44.3 $\pm$ 19.8
	Range	31.0–60.8	39.8–66.3	20.5–66.2	36.8–75.4	15.5–57.6
	n =	5	5	8	3	4
	Length mm					
	Mean $\pm$ SD	131 $\pm$ 7	134 $\pm$ 5	136 $\pm$ 6	127 $\pm$ 2	137 $\pm$ 5
	Range	124–142	128–140	125–144	125–129	130–141
	Domoic Acid Concentration $\mu\text{g} \cdot \text{g}^{-1}$		Mar 17	Apr 7–10	May 5–11	
COPALIS Group #2	Mean $\pm$ SD		30.7 $\pm$ 14.6	33.6 $\pm$ 12.60	33.5 $\pm$ 18.5	
	Range		8.0–50.3	13.7–60.7	18.2–61.4	
	n =		11	11	7	
	Length mm					
	Mean $\pm$ SD		129 $\pm$ 12	127 $\pm$ 11	122 $\pm$ 11	
	Range		105–144	111–144	110–138	

<sup>1</sup> The Alaska clams maintained in the laboratory during this time and sampled on April 29 1992 did not have detectable levels of domoic acid. n = 19, and the average length was 123 mm  $\pm$  9 with a range of 111–136 mm.

concentrate toxins in the siphon (Beitler 1988). It has been suggested that this is a possible defense mechanism used by the clam to inhibit predation (Kvitek and Beitler 1991). The average value of domoic acid in the siphon tip of the razor clams was 4.1  $\pm$  5.0  $\mu\text{g}/\text{g}$  while the remainder of the siphon tissue contained a mean of 28.9  $\pm$  22.4  $\mu\text{g}/\text{g}$ . Furthermore, the levels in the siphon tissue were not consistently elevated above the concentrations found in other muscular clam tissues (Table 1). These results do not support the concept that razor clams selectively concentrate domoic acid in the siphon as an anti-predation mechanism.

#### Clam Size and Toxin Content

The results of domoic acid analysis reveal uniformly lower levels of toxin in the tissues of smaller clams when compared to adult clams of the same sampling date (Table 3). Two general size

groups of clams were collected and analyzed for domoic acid in May and June, with average lengths of either 135 mm or 92 mm. From studies correlating length to growth rings in the shell of the clam, an estimate of the age of the Copalis Beach clams can be made (Lassuy and Simons 1989). Using this method, the group of smaller clams with a mean length of 92 mm would be approximately 2-year-old juveniles. The average length of clams at 1 year would be approximately 25 mm. The larger clams sampled in this study were estimated to be 4–9 years old.

The interval between 1 and 2 years of age is clearly a significant growth period for the razor clam based on the increase in shell length. The lower levels of toxin seen in the 2-year-old clams could result if the clams accumulated the toxin in their first year during a fall phytoplankton bloom, and through increased tissue mass in the following year, effectively decreased their tissue concentration in the second year of growth. In the case of a more

TABLE 6.  
Summary of the domoic acid content of historical razor clam samples.

Sampling Date	Domoic Acid Concentration $\mu\text{g} \cdot \text{g}^{-1}$	Sampling Site		
		Hobuck Beach	Copalis Beach	Long Beach
August 7, 9 1991	Mean $\pm$ SD	1.3 $\pm$ 1.3	1.6 $\pm$ 1.1	1.4 $\pm$ 1.2
	Range	ND <sup>1</sup> –4.1	ND <sup>1</sup> –3.0	0.6–3.4
	n =	10	10	5
February 26 1990	Mean $\pm$ SD		2.6 $\pm$ 0.4	
	Range		1.6–2.9	
	n =		10	
November 13 1985	Mean $\pm$ SD		0.9 $\pm$ 0.7	
	Range		ND <sup>1</sup> –2.0	
	n =			

<sup>1</sup> Domoic acid levels were not detected



persistent of continuing toxin exposure period for the second year class, the lower levels could be due to differences in uptake rates, or an increased domoic acid clearance rate, as observed in juvenile blue mussels (Novaczek et al., 1992).

Within the group estimated to be 4-to-9 years old, individual clams do not show a positive correlation between domoic acid content and clam length. This supports the hypotheses of either limited exposure to domoic acid over the adult life of a clam, or cycles of uptake and depuration of the toxin.

#### *Effect of Freezing Clams*

#### **Meltwater and Dissection Fluids**

Results of this study demonstrate that freezing clams for a minimal length of time can cause domoic acid release from tissues into fluids draining from the clam during its thawing and cleaning. This was not the case in freshly dissected clams where neither the whole blood prior to dissection, nor the fresh dissection fluids contained domoic acid at detectable levels. However, in clams frozen prior to sampling, the levels of domoic acid in these liquids in some individuals approached the concentration remaining in the clams' edible tissues. The presence of domoic acid in the meltwater, or fluids drained passively from the clams upon thawing, demonstrates that the freezing process releases toxin. The fluids that drain from a razor clam during thawing and dissection can be of considerable volume in relation to the tissue weight of the clam and would remove significant amounts of toxin, thereby reducing total tissue burden.

#### **Edible Tissues**

The accumulation of toxin in meltwater and dissection fluids after freezing affects edible tissue concentration of toxin as determined by analysis prior to, and after freezing. These results demonstrate that this loss of toxin, resulting from a freeze-thaw step prior to analysis, would considerably limit accurate reporting of live clam toxin concentrations. If a freeze step is necessary prior to analysis, tissues should be dissected from a live clam and then frozen with the entire sample and any melt water released after thawing included in the tissue analysis.

#### *Depletion of Toxin in Razor Clams*

#### **Field clams**

The mean concentration of domoic acid in the edible tissues for December 1991, March 17, May 18, and June 4, 1992, were  $47.9 \pm 12.7$ ,  $30.7 \pm 14.6$ ,  $36.4 \pm 22.6$  and  $16.8 \pm 11.6$   $\mu\text{g/g}$ , respectively. The apparent loss of toxin during this period could be due to beach temperatures (influenced by changes in coastal ocean currents, tide cycles, sun exposure, and air temperatures), salinity changes and reproductive development, all of which affect the metabolism of the clam. In mussels, changes in temperature influenced the depuration rate of domoic acid while salinity changes did not (Novaczek et al. 1992). It should be noted that in the present study the sample groups were not controlled for clam size nor were they consistently sampled relative to tidal zone, and that

the clams from December and March were frozen prior to analysis while the May and June samples were processed upon arrival.

#### **Laboratory Maintained Clams**

Clams collected from the Washington coast, when domoic acid levels were elevated in their tissues, maintained these levels for 8–12 weeks when held on laboratory seawater. Clams from Alaska held under the same conditions from July 1991 through April 1992 remained uncontaminated by toxin. The lack of toxin in the Alaska clams held at our laboratory, during this time of discovery of toxin in the Washington and Oregon coastal clams, indicates the source organism and/or the desirable conditions for a domoic acid toxic bloom were not present in inland waters. These results demonstrate the retention of domoic acid in the edible tissues of the razor clam for a minimum of three months. The retention of domoic acid in field clams substantiates the hypothesized fall 1991 exposure to domoic acid, and reveals the impact a single uptake event can have on this valuable shellfish resource.

The retention rate in the razor clam is much longer than that of toxic mussels which depurate domoic acid in several days when the source of toxin is no longer present (Novaczek et al., 1992). With the increased tissue accumulation and retention patterns seen in the razor clam, further study of uptake, retention, depuration and physiological pathways of domoic acid needs to be conducted.

#### *Historical Samples*

The clams from 1985 that were collected in November, the month in 1991 when domoic acid was first detected in razor clams, reveal measurable but very low levels of toxin in the edible tissues. The clams from February 1990 also contain minimal levels of toxin in the pooled edible tissues. These clams could have maintained elevated levels of domoic acid, from a previous fall exposure, as demonstrated in this study. Clams that were sampled from a northern, a central, and a southern beach in August 1991, three months prior to the detection of domoic acid in razor clams, contained levels less than 4  $\mu\text{g/g}$  of toxin in the muscular foot.

Although the historical samples are limited in number, and there was a loss of toxin during frozen storage and processing, these studies suggest that the levels observed in these samples from previous years were significantly lower than those detected in the episode of 1991–92.

The results of this study, which contribute to the understanding of tissue distribution, retention, and historical occurrence of domoic acid in the razor clam, also point out the lack of knowledge concerning this potential public health threat. More information is needed about the source of toxin, the clams' exposure history and the effect the toxin has on the clams, metabolic pathways of toxin uptake and depuration in the clam, and influences of environmental factors on the presence of domoic acid in the environment and marine organisms.

#### **ACKNOWLEDGMENTS**

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## RETENTION OF DOMOIC ACID BY PACIFIC RAZOR CLAMS, *SILIQUA PATULA* (DIXON, 1789): PRELIMINARY STUDY

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**ABSTRACT** Domoic acid concentrations up to 160  $\mu\text{g g}^{-1}$  shellfish meat were reported in razor clams on the Washington/Oregon coasts in the fall of 1991. Toxin levels in the clams remained above the regulatory closure level of 20  $\mu\text{g g}^{-1}$  for at least 6 months. In summer, 1992, razor clams, averaging about 10  $\mu\text{g g}^{-1}$  of domoic acid toxin, were maintained under laboratory conditions to determine how long it would take them to be free of the toxin. Periodically, edible (foot, siphon, and mantle) and non-edible (gill, digestive gland, and gonad) parts were tested for domoic acid. After 86 days, toxin levels remained near the original levels, but at least one clam in each group of six tested contained ca 22  $\mu\text{g g}^{-1}$  reflecting the clam-to-clam variability in their natural habitat. It appears that razor clams are able to depurate domoic acid in their natural environment, but may maintain a low level of domoic acid for long periods.

**KEY WORDS:** Domoic acid, razor clams, retention time, *Pseudonitzschia* spp., *Siliqua*

### INTRODUCTION

Domoic acid is a toxic, neuroexcitatory amino acid produced by several species of marine red algae (all members of the Order Ceramiales), and three species of the diatom genus *Pseudonitzschia*. Its discovery in diatoms in 1987 (Bates et al. 1989) was the first known incident of diatoms producing biotoxins that can cause human health problems. This toxin differs from other phytoplankton-produced toxins, e.g., saxitoxins that produce paralytic shellfish poisoning, because it acts on the central nervous system, causing short-term memory loss, coma, and even death. At the present time, there is no known antidote.

In the fall of 1991, razor clams (*Siliqua patula* Dixon) living in coastal beaches of Washington and Oregon became contaminated with domoic acid levels in the edible parts (i.e., foot, siphon, and mantle) as high as 160  $\mu\text{g g}^{-1}$ , necessitating the closure of an important recreational and commercial fishery. Domoic acid concentrations remained above the U.S. Food and Drug Administration regulatory closure level of 20  $\mu\text{g g}^{-1}$  for at least six months, but even after levels fell below the closure level, extensive clam-to-clam variability was present (Wekell et al. in press).

No information was available on the retention time or depuration of domoic acid by razor clams and the data for other bivalve molluscs is scant. In blue mussels, *Mytilus edulis* L., depuration was rapid, with 17% (Wohlgeschaffen et al. 1992) or 50% (Nowaczek et al. 1992) of the toxin being eliminated within 24 hr. Depuration is also rapid in soft-shell clams, *Mya arenaria* L. (Gilgan et al. 1990). However, deep-sea Atlantic scallops, *Placopecten magellanicus* (Gmelin), appear to retain domoic acid in their digestive glands for months (Gilgan et al. 1990). Thus, our

preliminary study was designed to determine the depuration rate of domoic acid by razor clams.

### METHODS

Forty-five razor clams were collected from Twin Harbors beach, Washington, (Fig. 1) on 28 July 1992. Following instructions from Washington Department of Fisheries personnel, narrow-bladed spades were used to minimize destruction and stress on the clams. The clams were put into buckets of seawater with bags of ice floated on top and transported to the University of Washington, School of Oceanography, in Seattle, WA. At the laboratory, each clam was wrapped with rubber bands about 4 mm wide to simulate the pressure of sand in its natural environment, then placed on a 2.5 cm mesh plastic grid in a 570 L, temperature-controlled aquarium filled with filtered, UV-treated Puget Sound seawater, obtained from the Seattle Aquarium. No domoic acid producing diatoms or red algae were present in the water. Water in the aquarium was aerated using three, 2.5 cm air diffusers and two aquarium power heads that pump about 750 L of water per hour. Seawater was recirculated over a sand filter and through a cooling bath before being returned to the aquarium. About 100 L of fresh seawater were added each month. The plastic grid held the clams above the bottom and minimized reingestion of fecal material. Water temperature was maintained near 11°C and salinity at 28 PSU (practical salinity units).

The clams were fed a concentrated algal diet (packaged by Coast Seafood, Inc., Quilcene, WA, for feeding newly-set oyster seed), consisting of an axenic culture of *Thalassiosira pseudonana* (Clone 3H) concentrated by centrifugation, preserved, and packaged. About 30 ml of concentrate were mixed with seawater, shaken, and added to the aquarium each day. The filtering system was turned off for approximately 3 hours following the addition of

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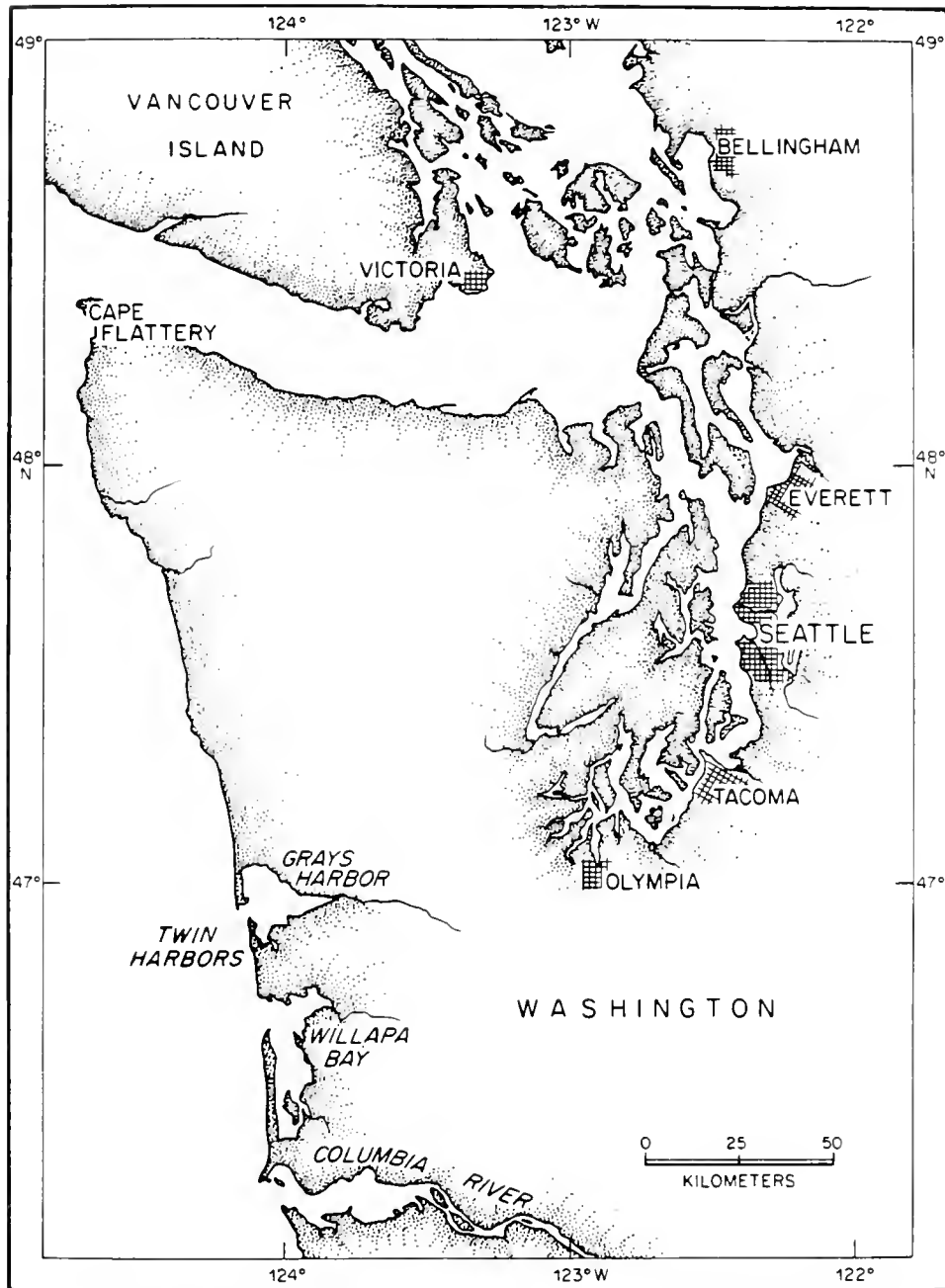


Figure 1. Map of western Washington with location where razor clams were collected.

the food to allow the clams to feed. When the filtering system was turned on, any remaining food particles were removed. The aquarium was checked daily and dead, or obviously stressed, animals removed. Mortality during the experiment was about 50%.

Six clams were frozen immediately after collection for determination of the initial domoic acid concentration. On 19 August and at 2–4 week intervals thereafter, six clams were frozen until domoic acid analysis by the National Marine Fisheries Service. Before analysis, clams were thawed and individual clams separated into edible (foot, mantle, siphon, adductor muscles) and non-edible or visceral (gill, digestive gland, and gonad) parts. Samples were weighed and analyzed using HPLC with a diode

array detector set at 242 nm (Quilliam et al. 1991) combined with a solid phase extraction clean-up procedure (Hatfield et al. in press).

## RESULTS

Initial domoic acid levels in the razor clams were relatively low, averaging only  $12.3 \mu\text{g g}^{-1}$  in the edible and  $6.2 \mu\text{g g}^{-1}$  in the non-edible portions, well below the closure level of  $20 \mu\text{g g}^{-1}$  (Table 1). Domoic acid in the edible parts decreased somewhat in the first three weeks of the experiment, then increased slightly to  $9.2 \mu\text{g g}^{-1}$  at day 76 before dropping again in the last 10 days (Fig. 2a). In the non-edible portions, domoic acid increased to day

TABLE 1.

Average domoic acid concentration and weight of razor clams during the experiment. Domoic acid is the average for six clams except on day 86, when it is the average for three clams. The whole domoic acid level is based on weight fractions. The domoic acid dose = domoic acid x weight; the non-edible fraction =  $Wt_{\text{nonedible}}/Wt_{\text{whole}}$ .

Time (days)	Domoic Acid			Weight			Domoic Acid Dose			Non-Edible Fraction
	Edible	Non-Edible	Whole	Edible	Non-Edible	Whole	Edible	Non-Edible	Whole	
0	12.3	6.2	10.7	75.8	28.1	103.9	932	174	1106	0.27
21	7.9	8.7	8.2	40.8	20.1	60.9	322	175	497	0.33
35	8.3	8.3	8.3	61.2	34.5	95.7	508	286	794	0.36
76	9.2	12.4	10.3	57.5	29.1	86.6	529	361	890	0.34
86	5.6	10.4	7.2	53.9	28.0	81.9	302	291	593	0.34

76 before dropping  $2 \mu\text{g g}^{-1}$  in the last 10 days (Fig. 2a). For whole clams, domoic acid dropped slightly during the first 21 days, leveled off between 21 and 35 days, and then increased slightly from 8.3 to  $10.3 \mu\text{g g}^{-1}$  over the next 31 days (Fig. 3).

Initial weights for the clams averaged 76 g for the edible and 28 g for the non-edible portions (Fig. 2b, Table 1). After 21 days, the weight was 40.8 g in the edible and 20 g in the non-edible portions, increasing after 35 days to 61 g and 34.5 g, respectively. The clams maintained similar, relatively higher weights to the end of the experiment. The initial average weight for the whole clams was 104 g (Fig. 2b). The average weights of both the whole animals and the edible and non-edible parts appeared to drop by day 21. (Fig. 2b, 3), but this could have been because smaller animals were inadvertently selected at that time for domoic acid testing. Assuming that this is so, then the average whole clam weight dropped about 22 g during the experiment, with the weight drop being only in the edible portion.

## DISCUSSION

Due to permitting problems, we were unable to obtain clams until mid-summer 1992, nine months after the initial domoic acid contamination. Consequently, initial domoic acid levels in the clams were relatively low, being only  $12.3 \mu\text{g g}^{-1}$  in the edible portions and  $6.2 \mu\text{g g}^{-1}$  in the non-edible portions. After 86 days, the length of the experiment, the average domoic acid level in whole clams was not appreciably lower, only  $2.6 \mu\text{g g}^{-1}$ , but the highest levels,  $10.4 \mu\text{g g}^{-1}$ , were in the non-edible portions and the lowest levels,  $5.6 \mu\text{g g}^{-1}$ , were in the edible parts (Fig. 2a, Table 1). Further, there was a drop of  $3.6 \mu\text{g g}^{-1}$  in the edible portions and  $2 \mu\text{g g}^{-1}$  in the non-edible portions during the last 10 days of the experiment. On average, weight of the whole clams dropped about 22 g. Reasons for the weight loss may be that we did not provide enough food or that the food was not similar enough in nutritional value to the surf diatoms that are their normal food source and thus the clams were utilizing body tissues.

After day 76, the health of the clams deteriorated rapidly. As a result, we had only three clams for the final domoic acid analysis. We attribute the relatively large decrease in domoic acid in both the edible and non-edible parts to this deterioration. Domoic acid is water soluble and presumably would diffuse out of cells that are damaged. Another possibility is that bacteria or catabolic activity may have metabolized the domoic acid. If the day 86 data point is eliminated, there seems to be a trend to domoic acid loss, possibly concomitant with tissue loss.

Our results suggest that razor clams rid themselves of domoic acid to a relatively low level but do not become completely free of the toxin. Drum et al. (1993) found that razor clams, collected in December 1991 with toxin levels near  $50 \mu\text{g g}^{-1}$ , maintained that level for three months. A second collection of razor clams in spring 1992, with somewhat lower toxin levels, also maintained that level for several months (Drum pers. comm. 1993). Our clams, obtained in the summer of 1992, showed a similar trend. Thus, clams, collected at about three-month intervals, rid themselves of substantial amounts of domoic acid in their natural habitat, but clams held in captivity for three months maintained their original domoic acid levels. Why this difference? Drum et al. (1993) held their clams in a flow-through seawater system with water entering directly from Sequim Bay; their food source was whatever was present in the Bay and, in winter, the phytoplankton population would be expected to be sparse. Our clams were held in filtered seawater and fed a prepared algal mix. They were not re-contaminated by the seawater in which they were kept. Thus the question, are clams in their natural habitat better able to get rid of domoic acid because of something in that environment, e.g., surf action? It is also possible that the clams in the laboratory experiments were not feeding or not obtaining enough food and either stopped or slowed their metabolism so the domoic acid was not excreted.

Moreover, Wekell et al. (in press) reported that domoic acid levels in razor clams from the Twin Harbors area were highest in December 1991 with  $147 \mu\text{g g}^{-1}$  in the edible portion. Within four months, the level dropped to  $40 \mu\text{g g}^{-1}$ , and by June 1992, about seven months later, the level was the  $12 \mu\text{g g}^{-1}$  reported here for day 0.

Average domoic acid concentrations for each group of clams tested may not reflect the true distribution of domoic acid in the clams because at least one clam from each batch averaged nearly three times the batch average concentration. Further, at the beginning of our study, the highest average domoic acid concentration,  $12 \mu\text{g g}^{-1}$ , was in the edible portion of the clams, but by day 76, the highest average concentration,  $10 \mu\text{g g}^{-1}$ , was in the non-edible portion.

Razor clams harvested between 1985 and 1990, obtained from local harvesters and either home-canned or frozen, had low levels of domoic acid, i.e.,  $<11 \mu\text{g g}^{-1}$  (Wekell et al. in press). However, clams were not tested for domoic acid before 1991 because domoic acid was not known to be a human health problem until 1987 and then only in eastern Canada. Therefore, it is possible that the organism(s) producing domoic acid has been present in Wash-

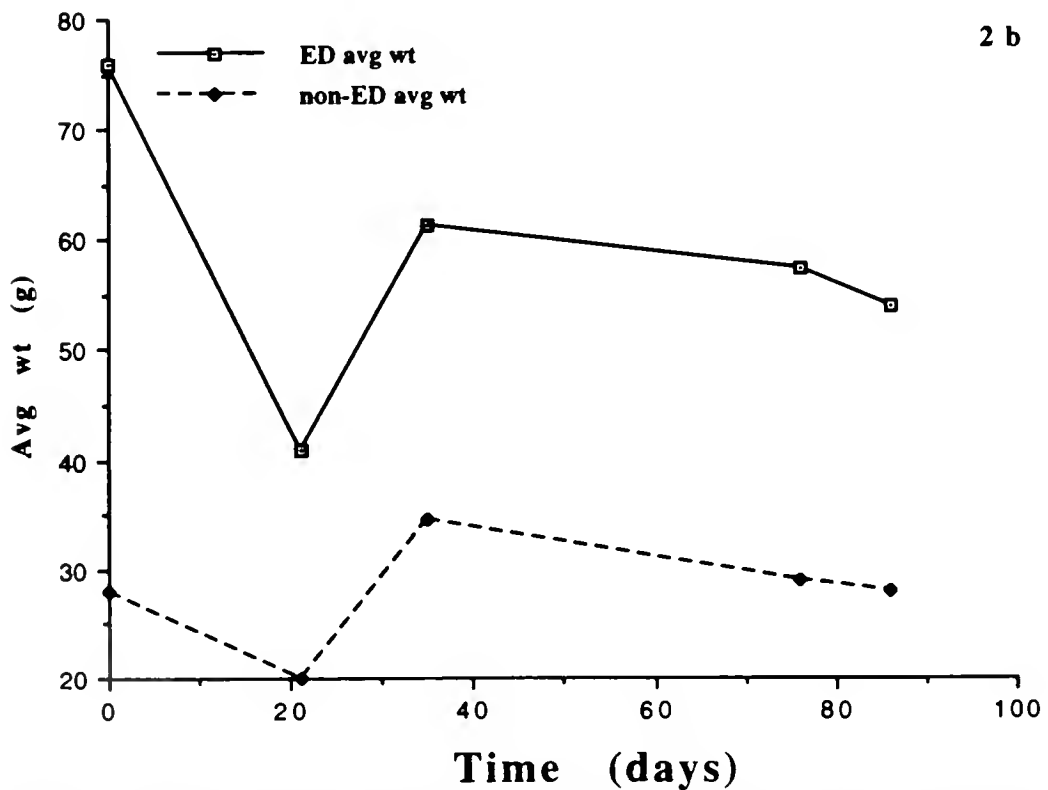
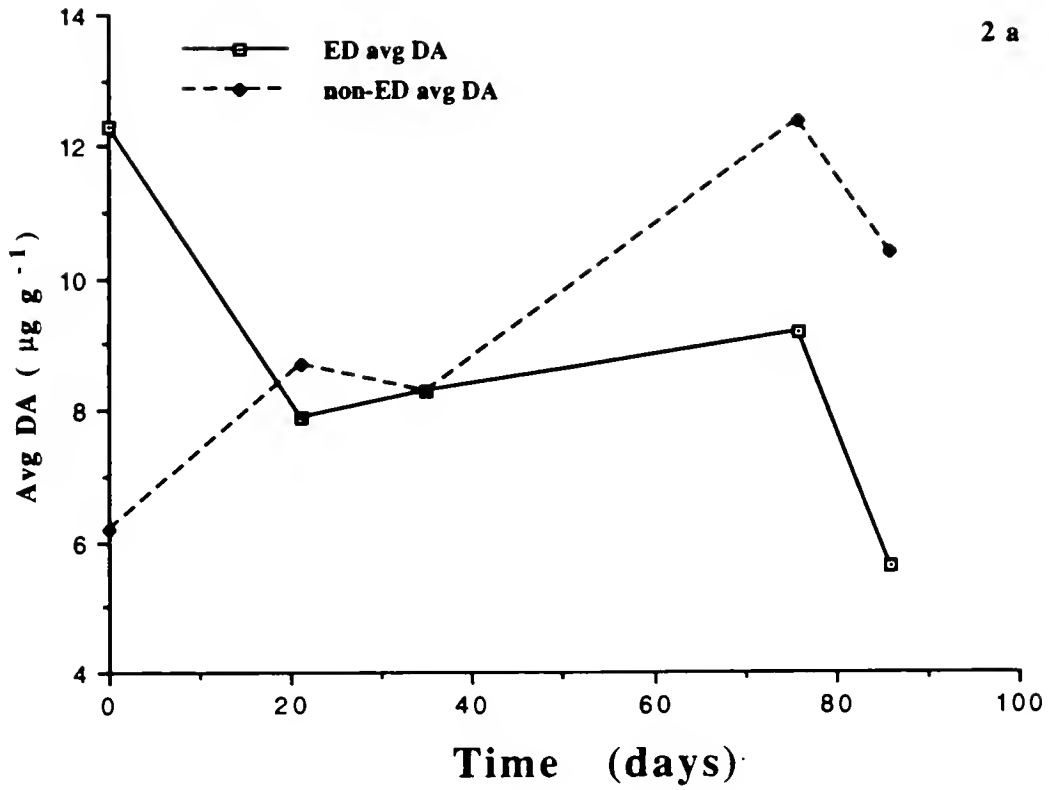


Figure 2. Average domoic acid levels ( $\mu\text{g g}^{-1}$ ) and weights (g) of edible and non-edible portions of the razor clams.

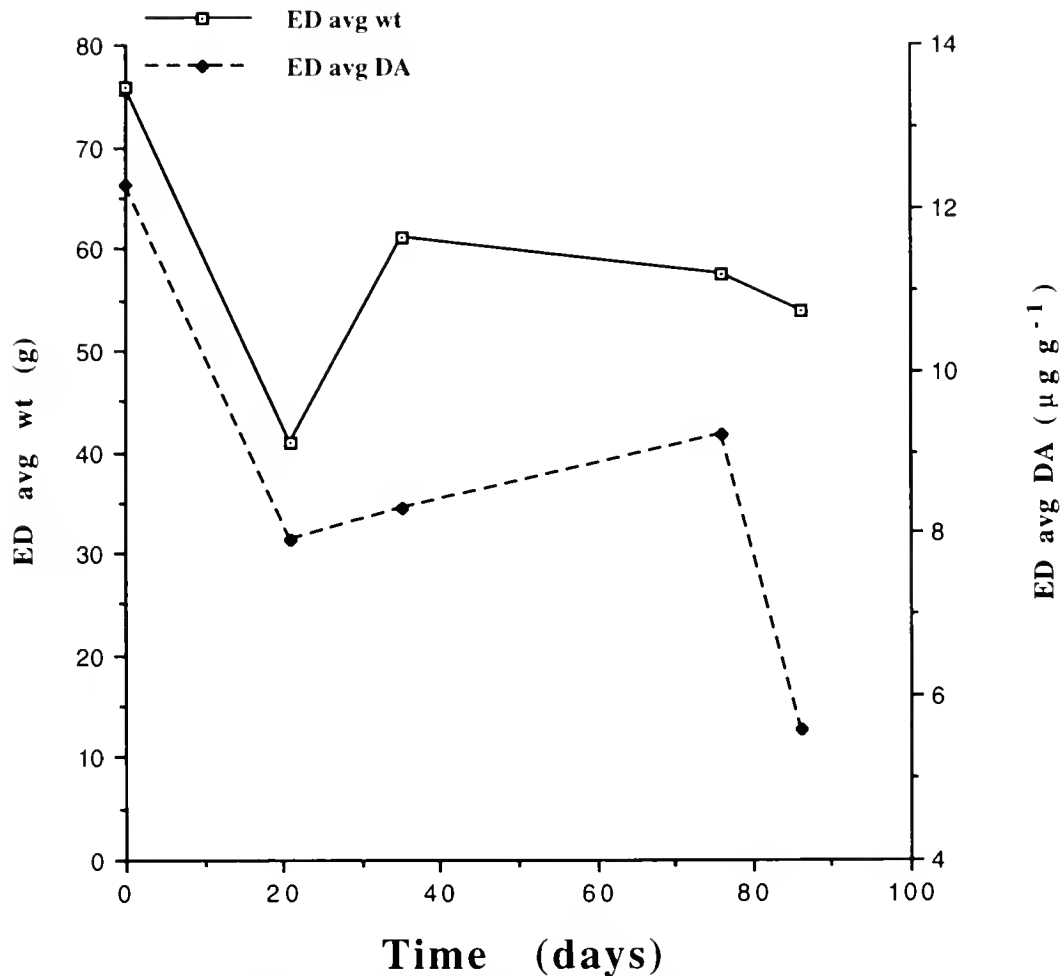


Figure 3. Average domoic acid levels ( $\mu\text{g g}^{-1}$ ) and weights (g) of whole razor clams.

ington/Oregon coastal waters for some time, but little is currently known about the geographic distribution of the diatoms producing domoic acid because they are difficult to identify, often needing scanning electron microscopy for positive identification.

No phytoplankton samples are available from the open Pacific Ocean off Oregon or Washington before the razor clams became toxic in 1991, so it is not known for certain what organism(s) produced the domoic acid in Oregon and Washington. However, domoic acid has been obtained in cultures of *Pseudonitzschia australis* Frenguelli isolated from Monterey Bay, CA, and near the mouth of the Columbia River in Washington (Garrison et al. 1992; G. A. Fryxell and C. Villac pers. comm. 1992). Phytoplankton samples collected about 5 miles off Grays Harbor, WA, in late May, 1992, contained both *P. australis* and *P. pungens* (Grunow) Hasle (Horner and Postel In press). It is not known if they were producing domoic acid at that time, but toxin concentration in the razor clams was still above the closure level.

Moreover, it is not known under what circumstances the organism(s) produces domoic acid, how it enters the food web, what effect, if any, it exerts on commercially utilized species, or how long these species retain the toxin. The problem is compounded because razor clams living in the surf zone normally obtain most of their food from a special community of diatoms, e.g., *Gonioceros* (*Chaetoceros*) *armatum* (T. West) H. & M. Per., *Asteri-*

*onellopsis* (*Asterionella*) *socialis* (Lewin & Norris) Round, and *A. glacialis* (Castr.) Round, that live only in the surf zone (Lewin and Norris 1970; Lewin 1974). Only a few dead or dying cells of *P. australis* were present in the surf-zone diatom community in November 1991 during the domoic acid incident (Horner and Postel 1993), but this does not preclude their having been present earlier, possibly in late October, when the clams must have become contaminated.

Thus, our study raises more questions than it answered. For example, why do clams held in captivity retain their toxin levels over long, e.g., three month, periods? Why do domoic acid levels apparently not go to zero? Does this mean it will remain in the area for long periods? Are residual levels present in the sediments where the clams live? If so, will clams continually be re-contaminated? Why were domoic acid levels higher in the edible portions at the beginning of the experiment and in the non-edible portions at the end? Were the clams losing body mass? Were they unhealthy or unduly stressed under our experimental conditions? Why do some clams retain more domoic acid than others?

What was the source of the domoic acid? Was it produced by species of the genus *Pseudonitzschia* as was the case at Prince Edward Island and California? If so, which species? Are these species commonly present in Washington/Oregon coastal waters? Do they produce domoic acid at some times and not at others? If

so, why? Do local or regional, short or long-term, weather patterns contribute to environmental or diatom physiological conditions that promote domoic acid production? Is it possible that other species, presently not known to produce domoic acid, are the culprits? If so, are these species regularly present in coastal waters and, if so, have they always produced domoic acid, but perhaps in lower concentrations? Are the surf-zone diatoms involved? And finally, we now know that at least three diatom species that may produce domoic acid are present and sometimes abundant in U.S. West Coast waters. Will there be another domoic acid incident in the future? We think it is highly likely.

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## PSEUDONITZSCHIA AUSTRALIS FRENGUELLI AND RELATED SPECIES FROM THE WEST COAST OF THE U.S.A.: OCCURRENCE AND DOMOIC ACID PRODUCTION

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**ABSTRACT** Awareness of the threat of the phycotoxin domoic acid, the cause of Amnesic Shellfish Poisoning (ASP), reached the U.S.A. west coast in the fall of 1991. Domoic acid in razor clams, mussels, and Dungeness crabs led to the closure of fisheries along the coasts of California, Oregon, and Washington. The death of pelicans that had fed on contaminated anchovies in Monterey Bay, California, set off the alarm by mid-September. The diatom *Pseudonitzschia australis* Frenguelli, detected in high concentrations in Monterey Bay at that time, was found to be a source of domoic acid. The present survey shows that, during the fall of 1991, *P. australis* and other *Pseudonitzschia* spp. were also observed in other sites on the west coast from Southern California to the mouth of the Columbia River (Newport, Coos Bay, and Ilwaco). In the fall of 1992, besides *P. australis*, other *Pseudonitzschia* spp. were present in Monterey Bay: *P. americana* and *P. pungens*, along with the known domoic acid producers *P. delicatissima*, *P. pungens* f. *multiseries*, and *P. pseudodelicatissima*. There was no report of a domoic acid outbreak in the Bay in 1992. There is strong evidence from the literature that, except for *P. americana*, all *Pseudonitzschia* species found in 1991 and 1992 have been part of the diatom community of the U.S.A. west coast at least since the 1940's. The study of their distributional patterns can provide a predictive tool for the future onset of potential harmful blooms, and hence help protect the consumer and the seafood industry. Clones of *P. australis* from Monterey Bay, Coos Bay and Ilwaco were established in 1991, and clones of *P. australis*, *P. americana*, *P. delicatissima*, *P. pungens*, and *P. pungens* f. *multiseries* from Monterey Bay were established in 1992. Domoic acid was detected in *P. australis* (0.02–0.4 pg · cell<sup>-1</sup>) and in *P. pungens* f. *multiseries* (0.06–1.5 pg · cell<sup>-1</sup>) while *P. americana*, *P. delicatissima*, and *P. pungens* tested negative. The low toxicity found for these *Pseudonitzschia* clones may be attributed to testing the cell contents only and to growth and harvesting conditions in the lab. The implications of background levels of domoic acid to shellfish contamination in the field and, therefore, to long-term exposure of low concentrations of this toxin to consumers have yet to be explored.

**KEY WORDS:** Amnesic Shellfish Poisoning-ASP, U.S.A. west coast, toxic diatoms, *Pseudonitzschia*, domoic acid

### INTRODUCTION

Awareness of the presence and on-going threat of the phycotoxin domoic acid, the cause of Amnesic Shellfish Poisoning (ASP), reached the U.S.A. west coast in the fall of 1991. In September 1991, Monterey Bay, California, was the focus of a domoic acid outbreak that caused the deaths of pelicans (*Pelecanus occidentalis* Ridgway) and cormorants (*Phalacrocorax penicillatus* [Brandt]) in Santa Cruz. High levels of the toxin were found in the stomach content of affected birds and in the viscera of local anchovies (*Engraulis mordax* Girard), their main food source (Fritz et al. 1992; Work et al. 1993). Based on field and culture evidence, the diatom *Pseudonitzschia australis* Frenguelli was identified as the domoic acid producer at that time (Buck et al. 1992; Garrison et al. 1992). By late October of the same year, domoic acid was detected in razor clams (*Siliqua patula* Dixon) harvested on Oregon and Washington beaches, and by early December, Dungeness crabs (*Cancer magister* Dana) from California, Oregon and Washington were also found to be contaminated with the toxin (Wood and Shapiro 1993). Closure of commercial and recreational fisheries resulted. Only a few cases of mild gastrointestinal disorder and one complaint of memory deficit were reported (Anonymous 1991).

The first known ASP event was reported from Prince Edward

Island (P.E.I.), Canada, in the fall of 1987. Comparing the domoic acid outbreak on the U.S.A. west coast with the ASP event that took place in P.E.I. (Table I), there are some parallels and many differences (Wright et al. 1992; Wood and Shapiro 1993). The common features were 1) the presence of domoic acid in marine organisms, 2) a diatom in the genus *Pseudonitzschia* (also considered a section of the genus *Nitzschia*) was the source of the toxin, 3) the toxic diatom blooms were during the fall season, and 4) there was a great impact on the fisheries due to closures. On the other hand, the west coast event seems to be more complicated in that 1) domoic acid was not only detected in the guts, but also in the tissues of several organisms, 2) razor clams showed a very slow depuration rate, 3) there is a reasonable possibility that more than one toxic diatom (or some other domoic acid source) might be involved, and 4) sampling was sparse and it is not known whether there were several simultaneous blooms or one bloom that was somehow displaced along the coast seeding different areas.

The Canadian experience shows that, even with recurrent blooms of the toxic diatom in the fall of the following years (1989–1991), consumers and the mussel industry can be protected by a very successful management strategy based on shellfish and phytoplankton monitoring programs (see below). Harvest of cultured mussels at present exceeds 1987 levels, and public confidence on this product is high (Wood and Shapiro 1993). To manage Canadian fisheries, authorities and the scientific community in Canada have recognized that "it is essential to develop a sound un-

TABLE 1.  
Comparison of the ASP event in eastern Canada, 1987, and the ASP event on the U.S.A. west coast, 1991.

AFFECTED AREA	eastern Prince Edward Island, Canada	U.S.A. West Coast (California, Oregon, and Washington)
ASP SYNDROME	107 cases of human poisoning (vomiting, diarrhea, seizures, short-term memory loss); 3 deaths	a dozen cases of mild gastrointestinal symptoms and one complaint of memory deficit
VECTORS	blue mussel	anchovies, razor clams, Dungeness crabs
DEPURATION RATES	$50 \mu\text{g} \cdot \text{g}^{-1}$ to $<5 \mu\text{g} \cdot \text{g}^{-1}$ over 72 hours	razor clams: $47.9 \mu\text{g} \cdot \text{g}^{-1}$ to $44.3 \mu\text{g} \cdot \text{g}^{-1}$ over 3 months
BLOOM LOCATION	mouth of the Cardigan River	Monterey Bay, California
BLOOM CHARACTERISTICS	<ul style="list-style-type: none"> <li>• from mid-October to early January, 1987</li> <li>• max: <math>1.5 \times 10^7</math> cells <math>\cdot \text{L}^{-1}</math></li> <li>• recurrence: 1988 to 1991 (DA detected, but no ASP cases)</li> </ul>	<ul style="list-style-type: none"> <li>• from mid-September to late November, 1991</li> <li>• max.: <math>6.7 \times 10^5</math> cells <math>\cdot \text{L}^{-1}</math></li> <li>• recurrence of species (not bloom density): 1988 to 1990 (no DA detected, nor ASP cases)</li> </ul>
SOURCE OF DOMOIC ACID	<i>Pseudonitzschia pungens</i> f. <i>multiseries</i>	<i>P. australis</i>
OTHER KNOWN DOMOIC ACID PRODUCERS IN THE AFFECTED AREA	<i>Amphora confreaeformis</i> , <i>P. delicatissima</i>	<i>P. delicatissima</i> , <i>P. pungens</i> f. <i>multiseries</i> , <i>P. pseudodelicatissima</i>

Bates et al. 1989, Wright et al. 1989, Maranda et al. 1990, Perl et al. 1990, Smith et al. 1990b, Todd 1990, Anonymous 1991, Smith et al. 1991, Buck et al. 1992, Novaczek et al. 1992, Wright et al. 1992, Drum et al. 1993, Villac et al. 1993, Wood and Shapiro 1993.

Understanding of the factors that influence the species composition of phytoplankton communities, especially the toxigenic species'' (Budgen et al. 1993). Once the presence of species is detected, the study of their distributional patterns and correlation with environmental parameters can provide a better predictive capability for the onset of a potentially harmful bloom, and hence protect the consumer and the seafood industry.

The urgent need to understand more about the implication of phytoplankton populations in the bird kill at Monterey Bay, and the reports of domoic acid contamination of shellfish from California, Oregon, and Washington, led us to sample several sites on the west coast of the U.S.A. during the fall of 1991, and to take part on an intensive sampling program carried out in Monterey Bay in the fall of 1992 (Figure 1). The short-term objectives of the

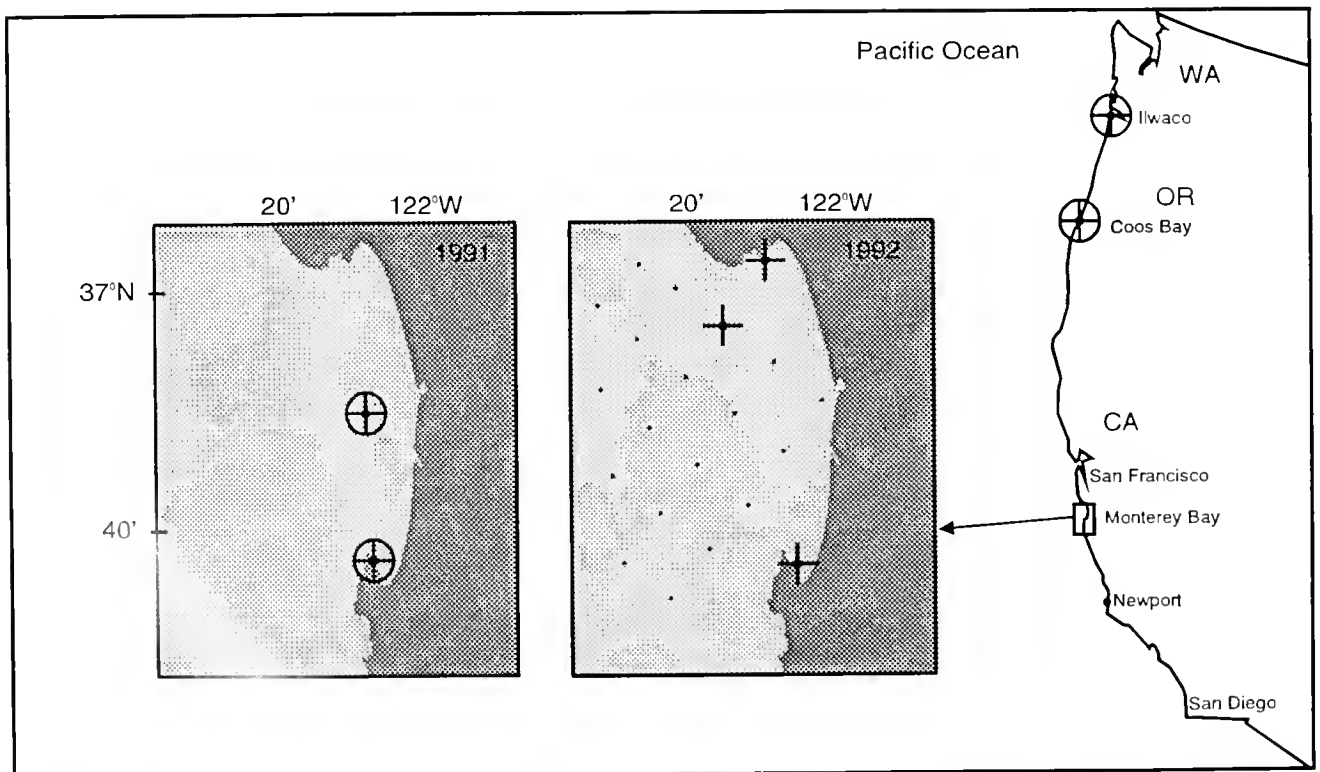


Figure 1. U.S.A. west coast and detail of Monterey Bay (inserts). Stations at which phytoplankton net samples were taken and (●) preserved for species identification, (○) frozen for domoic acid analysis, and (+) kept alive for isolation of target species.

field trips, whose results are presented here, were to ascertain the presence of seed stocks of target species of *Pseudonitzschia* in the localities visited and to isolate cultures of the organisms for testing domoic acid production in the laboratory.

## METHODS

Sampling sites visited during the fall of 1991 were Monterey Bay, California, in early November, and Newport, California, Coos Bay, Oregon, and Ilwaco, Washington, in mid-December. In late September and mid-November 1992, sampling was carried out in Monterey Bay (Figure 1).

### Species Identification

Field samples were collected with a 35  $\mu\text{m}$  net (surface tows) from on-board ship and land-based sites and preserved with 2% glutaraldehyde. Permanent Hyrax light microscope and scanning electron microscope mounts were prepared with net samples cleaned of organic matter (Hasle and Fryxell 1970, Simonsen 1974).

A diatom can be readily identified as a *Pseudonitzschia* by the overall shape and type of colony formation (elongate, needle-shaped cells, connected by overlapping tips into stepped chains). At the species level, however, the study of the fine structure of the cell wall is required. The identification of the species followed the description provided by Hasle (1964, 1965), and their nomenclature updated (Hasle 1993).

### Culture Isolation and Maintenance

Living samples were also collected using a 35  $\mu\text{m}$  net, and *Pseudonitzschia* chains were promptly isolated after collection. Single chains (made up of siblings) were picked up with a glass micropipet and rinsed repeatedly in sterile seawater enriched with  $f/2$  nutrients (Guillard 1975, using stock solutions from Fritz Chemical Co.). Batch cultures (nonaxenic) were maintained at 15°C, at salinity 30, and at 96  $\mu\text{E}/\text{m}^2\text{s}$  in a 12:12 light:dark cycle. Aliquots of cultures were harvested and processed for identification as described above.

### Domoic Acid Analysis

Domoic acid (DOM) analysis of cultures and net hauls followed the protocols established by Quilliam et al. (1989) and Dickey et al. (1992). For cellular DOM analysis from cultures, 18-mL aliquots were centrifuged in test tubes for 10 min at ca. 2000 rpm, the supernatant removed, and the volume made up to 10 mL with Milli-Q water. The samples were then sonicated for 15 min at 80 W (Branson B-12 ultrasonic cleaner) to release DOM and cell debris removed by filtration through a 0.45  $\mu\text{m}$  cellulose membrane (Millipore Corp.). The filtrate was then evaporated (Savant-SVC200), re-dissolved in 2 mL of 10% acetonitrile, and filtered by a 0.2  $\mu\text{m}$  polycarbonate membrane (Poretics Corp.). Domoic acid was analyzed by high-performance liquid chromatography with ultraviolet detection at 242 nm (ISCO, V4). A 25 cm  $\times$  4.6 mm I.D. Hypersil Ods C18 5 mm column (Alltech) was used with a gradient of 5% to 25% acetonitrile with 0.1% trifluoroacetic acid for 20 min (flow rate: 1.0 mL  $\cdot$  min<sup>-1</sup>; detection threshold: 7.5 ng). Sigma-DOM dissolved in 10% acetonitrile was used as a standard. Domoic acid per cell was calculated by dividing the DOM concentration by the number of cells, and cell number assessed by the settling technique (Utermöhl 1958). Aliquots for DOM analysis and counts were harvested at the same time

(cultures in late log phase or in senescence), the latter preserved with 2% glutaraldehyde. Aliquots of some of the surface net tows were frozen for DOM analysis. The samples were later thawed, sonicated, filtered (0.2  $\mu\text{m}$ ), and analyzed for DOM concentration; DOM per cell was calculated by settling a known volume from a preserved aliquot of the same net haul.

## RESULTS AND DISCUSSION

### Occurrence of *Pseudonitzschia* spp.

In 1991, during the domoic acid outbreak, *Pseudonitzschia australis* was found in all sampling sites visited; *Pseudonitzschia pungens* f. *multiseries* was present in Coos Bay, Monterey Bay, and Newport; *P. pungens* (Grunow in Cleve & Moller) Hasle was detected in Ilwaco, Monterey Bay and Newport; *P. delicatissima* (Cleve) Heiden in Heidin & Kolbe, *P. pseudodelicatissima* (Hasle) Hasle, and *P. subpacificica* (Hasle) Hasle were found in Monterey Bay and Newport. In the fall of 1992, *P. americana* (Hasle) G. A. Fryxell, *P. australis*, *P. delicatissima*, *P. pungens*, *P. pungens* f. *multiseries*, *P. pseudodelicatissima*, and *P. subpacificica* were noted in Monterey Bay.

*Pseudonitzschia* species have been observed along the U.S.A. west coast as early as the 1920's (Allen 1922, 1924, Gran and Angst 1931, Cupp and Allen 1938, Cupp 1943, Hasle 1965, 1972, Buck et al. 1992—just to mention a few studies). Only *P. americana* can be considered at present as not previously reported for these waters. Although most previous records cannot be verified in the electron microscope (except for Hasle 1965, 1972, and Buck et al. 1992), there is strong evidence based on drawings, descriptions, and distributional patterns, that all other species found in 1991 and 1992 have been part of the diatom community of these shores at least since the 1940's, except for *P. australis*, whose first record can only be confirmed from the 1960's. All reliable records of *P. seriata*, however, are restricted to north of 40°–45°N (Hasle 1972). This suggests that some historical records that have been identified as *N. seriata* (= *P. seriata*) from more southerly regions (Allen 1922, 1924, Gran and Angst 1931, Cupp and Allen 1938) could have been a misidentification of other species of the "seriata complex" *sensu* Hasle (1965), such as *P. australis* and/or both forms of *P. pungens*. Some records that refer to what was identified at the time as *N. delicatissima* (Gran and Angst 1931, Cupp 1943), could have included *P. delicatissima*, *P. pseudodelicatissima* and *P. cuspidata* (Hasle) Hasle.

Of all *Pseudonitzschia* species found, *P. australis*, *P. pungens* f. *multiseries*, *P. delicatissima* and *P. pseudodelicatissima* have been reported to be able to produce domoic acid (Villac et al. 1993). Based on the Canadian experience, it is expected that blooms of toxic diatoms and risk of ASP events can recur on the U.S.A. west coast as well. Little is known about the overall distributional patterns and seasonality of the *Pseudonitzschia* spp. on these shores, but there are some valuable data for what was considered the main "hot spot" of the domoic acid outbreak in 1991. Buck et al. (1992) present the autecology of *P. australis* for Monterey Bay for the period of 1989–91. They show that peaks of *P. australis* occurred in the fall of the years studied; however, it was not dominant in the plankton during the same period in 1992.

Potential domoic acid producers were all present in the fall of 1992 in Monterey Bay. Domoic acid was detected in phytoplankton net tows taken in the bay for that period (<10 pg  $\cdot$  cell<sup>-1</sup>, P. Walz, pers. comm.). Farther north on the coast, Horner and Postel (1993) reported the presence of *P. pungens* f. *multiseries* in Puget

Sound, Washington, in July 1992, concomitant with low levels of domoic acid ( $<5 \mu\text{g} \cdot \text{g}^{-1}$ ) in blue mussels and oysters (*Crasostrea gigas* Thunberg). *Pseudonitzschia australis* was also found in several sites in the Puget Sound area, and it was abundant off Grays Harbor in May 1992 (Horner and Postel 1993). No *Pseudonitzschia* blooms were reported for the west coast during 1992 (c.a.  $10^6 \text{ cells} \cdot \text{L}^{-1}$  is here considered bloom concentration).

Environmental variability due to long-term meteorological/oceanographic fluctuations may account for interannual variations in biological systems. This is especially true for the eastern Pacific Ocean, which is directly influenced by fluctuations such as those caused by El Niño (Barber and Chavez 1983, Fiedler et al. 1992). Avaria and Muñoz (1987) have found that *P. australis* was among the most quantitatively important diatoms in Chilean coastal waters during non-El Niño years. The fall of 1991 was immediately prior to the 1992–1993 California El Niño, considered to be a moderate one (Murray et al. 1992). Planktonic communities could have responded to El Niño conditions with changes in their composition and relative abundance.

Although *P. australis* was traced as the only source of the toxin in the outbreak of 1991, the presence of other potential domoic acid producers certainly pose a risk. Data on the ecology of *P. delicatissima* and *P. pseudodelicatissima* in relation to bloom formation is not available, but *P. pungens* f. *multiseriis* has been studied intensively in the field and in culture (Villac et al. 1993). One can speculate that upwelling of cold water with high nitrogen concentrations (such as that found in Monterey Bay) may stimulate the increase of *P. pungens* f. *multiseriis* populations. This diatom species seems to have affinity for colder waters, because it can become dominant in P.E.I. by mid-October (Smith et al. 1990a), and its population has increased in Galveston Bay, Texas, following cold fronts during winter and spring (Fryxell et al. 1991, Dickey et al., 1992). A succession of events preceded blooms of *P. pungens* f. *multiseriis* in P.E.I. (Bates et al. 1989, Smith et al. 1990b): it started with a long dry summer and nutrient limitation in the water in the early fall, followed by heavy rains which led to runoff and a nitrogen pulse. The study of bloom dynamics followed by phytoplankton monitoring may thus provide a few day warning before toxicity is detected in shellfish as it happened in P.E.I. (Smith et al. 1990a).

The widespread domoic acid outbreak on the west coast indicates that blooms are not only a function of local dynamics, but also may depend on large-scale circulation and transport patterns (e.g., Franks and Anderson 1992). On one hand, the California Current system links ecosystems from the shores of Alaska to southern California (Hickey 1979); but, on the other, local environmental conditions may partially regulate the phytoplankton population dynamics of specific sites (e.g., Monterey Bay upwelling system, the mouth of the Columbia river). Besides the large geographical extension of contaminated shellfish, the fact that high levels of domoic acid ( $>20 \mu\text{g} \cdot \text{g}^{-1}$ ) were detected in razor clams from Oregon and Washington until the spring and summer of 1992 (Drum et al., 1993) is of great concern. In this regard, toxin concentration/depletion rates in shellfish are also important (see below)

#### Toxicity of *Pseudonitzschia* spp.

Phytoplankton net tows taken in Monterey Bay during the fall of 1991 tested positive for domoic acid (9.1 and 20.28

$\mu\text{g} \cdot \text{cell}^{-1}$ ). Those from Coos Bay and Ilwaco were negative. The concentrations found in Monterey Bay were in the range of what was detected by Buck et al. (1992) for the same period of time (from 3 to 31  $\mu\text{g} \cdot \text{cell}^{-1}$ ). The relative abundance of phytoplankton cells in the net hauls from Monterey Bay shows that *P. australis* contributed with 99% of the whole community. Yet, for Coos Bay and Ilwaco, the relative abundance of *P. australis* was less than 1% of the phytoplankton.

Clones of *P. americana*, *P. australis*, *P. delicatissima*, *P. pungens*, and *P. pungens* f. *multiseriis*, were isolated and tested for domoic acid concentration (Table 2, Figure 2). Most clones of *P. australis* tested positive for domoic acid (0.02–0.4  $\mu\text{g} \cdot \text{cell}^{-1}$ ) but some did not (MB-7, MB-14, CV-15, CV-16). From the clones that tested positive, some of them presented domoic acid peaks that did not allow for quantification (ORI-7, ORC1, ORC2, MB-10, MB-39). All clones of *P. pungens* f. *multiseriis* tested positive for domoic acid (0.06–1.5  $\mu\text{g} \cdot \text{cell}^{-1}$ ), while clones of *P. americana*, *P. delicatissima*, and *P. pungens* tested negative.

TABLE 2.

Domoic acid analysis for clones of *Pseudonitzschia* spp. isolated from different sites on the U.S.A. west coast in the falls of 1991 and 1992 (all clones isolated by M. C. Villac).

Clone (species)	Origin	Domoic Acid ( $\mu\text{g} \cdot \text{cell}^{-1}$ )
ORI-1 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.06
ORI-2 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.4
ORI-3 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.3
ORI-4 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.2
ORI-5 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.3
ORI-6 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	0.1
ORI-7 ( <i>P. australis</i> )	Ilwaco, WA / 14 Dec 91	+
ORC-1 ( <i>P. australis</i> )	Coos Bay, OR / 12 Dec 91	+
ORC-2 ( <i>P. australis</i> )	Coos Bay, OR / 12 Dec 91	+
MB-1 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	0.1
MB-7 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	nd
MB-8 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	0.02
MB-10 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	+
MB-14 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	nd
MB-27 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	0.08
MB-29 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	0.1
MB-30 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	0.1
MB-39 ( <i>P. australis</i> )	Monterey Pier, CA / 1 Nov 91	+
CV-15 ( <i>P. australis</i> )	St. Cruz Pier, CA / 14 Nov 92	nd
CV-16 ( <i>P. australis</i> )	St. Cruz Pier, CA / 14 Nov 92	nd
CV-17 ( <i>P. australis</i> )	St. Cruz Pier, CA / 14 Nov 92	0.3
CV-18 ( <i>P. australis</i> )	St. Cruz Pier, CA / 14 Nov 92	0.1
CV-19 ( <i>P.p. multiseriis</i> )	Monterey Pier, CA / 15 Nov 92	0.06
CV-22 ( <i>P.p. multiseriis</i> )	Monterey Pier, CA / 15 Nov 92	0.3
CV-26 ( <i>P.p. multiseriis</i> )	Monterey Pier, CA / 15 Nov 92	1.5
CV-2 ( <i>P. americana</i> )	Monterey Bay, CA / 1 Oct 92	nd
CV-3 ( <i>P. delicatissima</i> )	Monterey Bay, CA / 1 Oct 92	nd
CV-5 ( <i>P. pungens</i> )	Monterey Bay, CA / 1 Oct 92	nd
CV-7 ( <i>P. pungens</i> )	Monterey Bay, CA / 1 Oct 92	nd
CV-23 ( <i>P. pungens</i> )	Monterey Bay, CA / 1 Oct 92	nd

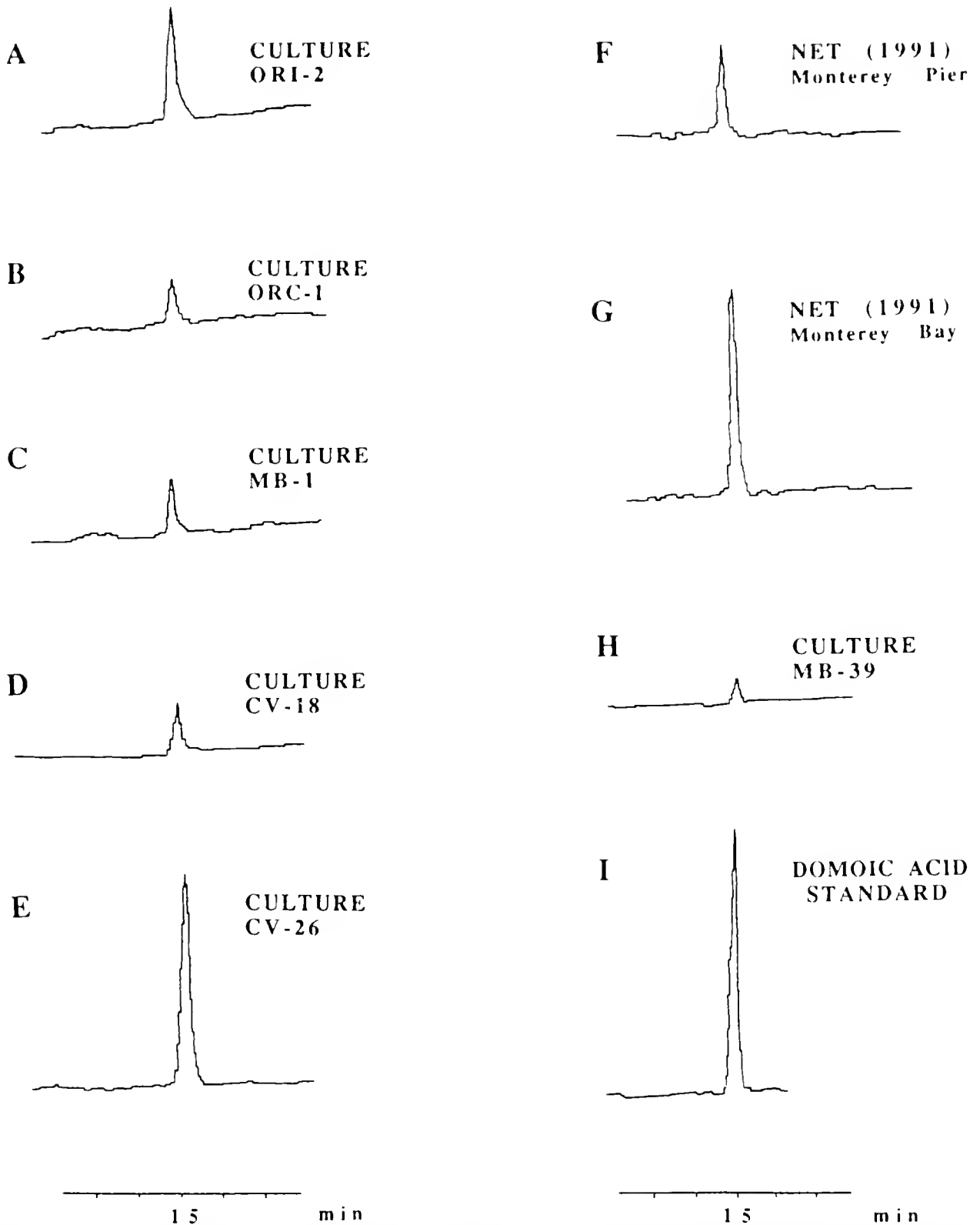


Figure 2. HPLC chromatograms of domoic acid concentration (all samples concentrated  $9\times$  during preparation, except for standard and net hauls). *Pseudonitzschia australis* cultures isolated in 1991 from A) Ilwaco, B) Coos Bay, C) Monterey Bay, and in 1992 from D) Monterey Bay. E) *P. pungens* f. *multiseriis* culture isolated in 1992 from Monterey Bay. F), G) net hauls collected in the fall of 1991 in Monterey Bay. H) Example of sample considered positive for domoic acid, but not quantified. I) Sigma domoic acid standard at  $0.55 \mu\text{g} \cdot \text{mL}^{-1}$ .

Previous records of toxin production by *P. australis* were demonstrated only in two clones from Monterey Bay isolated in the fall of 1991 (Garrison et al., 1992). Toxicity of *P. pungens* f. *multiseries* was previously reported only from clones from the Atlantic coast of North America (Bates et al. 1989, Villareal et al. 1993) and from clones from Galveston Bay, Gulf of Mexico (Reap 1991).

Domoic acid production in cultures of *P. pungens* f. *multiseries* has been intensively studied (e.g., Subba Rao et al. 1988, Bates et al. 1989, Bates et al. 1991, Reap 1991, Douglas and Bates 1992, Hargraves et al. 1993, Lewis et al. 1993, Wang et al. 1993). Toxin production in batch cultures of *P. pungens* f. *multiseries* may vary depending on growth conditions and on growth stage (Bates et al. 1991): It requires cessation of cell division, availability of nitrate or other nitrogen source during stationary stage, and the presence of light; extracellular domoic acid increases with time in the growth medium, eventually exceeding cellular domoic acid. Variability in domoic acid concentration can be detected among clones (Bates et al. 1989, Reap 1991), and an overall decrease in toxicity may be expected over a period of a year or more in culture (G. A. Fryxell, unpublished data). Data on domoic acid production in cultures of *P. australis* are scarce (Garrison et al. 1992), and the dynamics of toxin production are yet to be investigated. Comparison of toxicity levels from different studies should take into account harvesting time and methods of sample preparation for domoic acid analysis (cellular domoic acid as opposed to whole sample domoic acid).

Domoic acid levels of *P. pungens* f. *multiseries* clones from Monterey Bay are in the lower range of values found for clones from P.E.I. (1.0 to 20.0  $\text{pg} \cdot \text{cell}^{-1}$ ; Bates et al. 1989) and from Galveston Bay (0.31 to 19.67  $\text{pg} \cdot \text{cell}^{-1}$ ; Reap 1991). Nevertheless, it is important to point out that the domoic acid concentrations reported for these Canadian and Texan clones include cellular and extracellular domoic acid, that is, the values presented as  $\text{pg} \cdot \text{cell}^{-1}$  might have been overestimated. Later reports on cellular domoic acid production only, also from Canadian clones of *P. pungens* f. *multiseries*, show values that do not exceed 10  $\text{pg} \cdot \text{cell}^{-1}$  (Bates et al. 1991) or 2  $\text{pg} \cdot \text{cell}^{-1}$  (Smith et al. 1993).

Domoic acid production measured in our cultures of *P. australis* are lower than those determined by Garrison et al. (1992, 12  $\text{pg} \cdot \text{cell}^{-1}$  and 37  $\text{pg} \cdot \text{cell}^{-1}$ ). Our clones were initially maintained on a 24 hour light cycle, and a preliminary domoic acid test under this light regime carried out shortly after the isolation date came out negative (Roelke et al. 1992). After it was transferred to a 12:12 light:dark cycle regime, this clone of *P. australis* (MB-1) tested positive for domoic acid; the same happened with clone ORI-4. Although exposure to light is necessary for toxin production in cultures of *P. pungens* f. *multiseries*, an alternation of light and darkness may also play a role. Experiments to test the influence of light regime on domoic acid production are underway. Another possibility for low domoic acid values might be that, in our cultures, the toxin could have already been released to the medium at the time of sampling and/or that a source of nitrogen was no longer available (assuming *P. australis* has the same dynamics as *P. pungens* f. *multiseries*). A third possibility is that, with time, *P. australis* might also lose the capability of domoic acid production. Finally, we have to consider that variability among clones should be expected.

*Pseudonitzschia delicatissima* tested negative under our conditions. Considering that the previous record has shown only trace

amounts of domoic acid in culture ( $5 \times 10^{-3} \text{pg} \cdot \text{cell}^{-1}$ ; Smith et al. 1991), further experiments on toxicity of this species will require testing of distinct growth conditions and/or a lower threshold to detect small toxin concentrations (FMOC derivatization; Pocklington et al. 1990).

#### Trophic Interactions

Domoic acid is an amino acid compound that interferes with glutamate receptors in the brain, causing continuous stimulation that leads to the destruction of the neurons; it is heat stable and water soluble (Bird et al. 1989, Wright et al. 1989). Domoic acid has shown to be harmful, even lethal, to man (Perl et al. 1990), birds (Fritz et al. 1992), monkeys and rodents (Todd 1990). Possible toxicity effects of domoic acid to mussels, oysters, clams, Dungeness crabs, or anchovies are not known when the toxin is present over some as yet undetermined threshold level. The regulatory guide line for human consumption ( $<20 \mu\text{g} \cdot \text{g}^{-1}$ ), established during the first ASP event by the Health and Welfare Ministry of Canada, was based on estimates of the amount ingested by those hospitalized with acute intoxication. Effects of chronic low level ingestion of domoic acid are not known. If toxicity can be accumulative, then it is of even greater importance to understand the mechanisms that determine the availability of the toxin (phytoplankton dynamics), and its concentration and fate in shellfish, fish, and organisms of higher trophic levels.

Toxin contamination of shellfish is species-specific; it may also depend on the shellfish organ, and/or on the amount of cells available to the animals (Shumway 1990). Only few shellfish species have been tested for domoic acid concentration and depuration rates under controlled conditions (see Table 1 for data on blue mussels and razor clams). Although domoic acid was shown to depurate from mussels fairly rapidly (Novacek et al. 1992), this is not the case for razor clams (Drum et al. 1993) and the oyster *Crassostrea virginica* (Gmelin) (Roelke 1993). The bulk of domoic acid resided in the gut of the blue mussel and of the oyster, while for razor clams, higher domoic acid levels concentrated in the edible muscular tissues and lower levels in the non-edible tissue parts. Dungeness crabs accumulated the toxin mostly in the viscera, although it can enter meat during cooking if the crabs are not eviscerated previously (Wood and Shapiro 1993). Finally, domoic acid was found not only in the viscera of anchovies but also in the fish muscle (Fritz et al. 1992).

One can expect that low values of domoic acid can be intoxicating if depuration rates are very low, which is the case for razor clams. Therefore, the constant presence of domoic acid producing diatoms at low densities, that is, potential chronic low level exposure to the toxin, might result in long-term high concentration in the clam. It is not clear, however, how razor clams were contaminated, since *Pseudonitzschia* species do not normally contribute to the surf-zone diatom community. The contamination of Dungeness crabs also requires further investigation, considering that they are primarily carnivorous (Stevens et al. 1982). New sources of domoic acid and trophic links in the chain related to toxin transfer/accumulation are yet to be found.

Buck et al. (1992) pointed out that mortality of pelicans in the Central California coast during the autumn of 1971, 1976 and 1981 have been reported and, although there is no report on the cause of these mortalities, the possibility that they were the result of domoic acid intoxication cannot be ruled out. Moreover, razor clam tissues collected in 1985 and 1990 revealed trace levels of the toxin (Drum et al. 1993), which indicates that domoic acid pres-

ence on the U.S.A. west coast have gone unnoticed for many years. In this context, since the effects of low level ingestion of domoic acid are unknown, one has reason to question the effectiveness of the present Canadian regulatory guideline for human consumption ( $20 \mu\text{g} \cdot \text{g}^{-1}$ ).

#### *Pseudonitzschia* spp. and Harmful Algal Blooms

*Pseudonitzschia* species are widely distributed diatoms, but their life histories and population dynamics are poorly understood. The potentially toxic species probably have a wider global distribution than is presently reported (see Hasle 1972, Fryxell et al. 1990, Villac et al. 1993), because misidentification is very common when careful morphometrics and electron microscopy are not available. Therefore, it is not surprising that several species of *Pseudonitzschia*, including the domoic acid producing taxa, were present on the U.S.A. west coast in 1991–92 and in historical records.

Evidence is accumulating that the apparent increase of harmful algal blooms is a spreading phenomena that might be linked to human activities (Anderson 1989, Smayda 1990, Hallegraeff 1993). The global increase of algal blooms may be due, in part, to increased awareness of toxic species, to an increase in eutrophication of coastal waters, to unusual climatological conditions, to the artificial dispersal of phytoplankton species (ballast waters or transplanted shellfish or seagrass), and to increased utilization of coastal waters for aquaculture—organisms are more vulnerable to noxious blooms than wild stocks. *Pseudonitzschia* spp. often contribute to the diatom community considered as the "hidden flora", that is, those that are always present, despite changes in environmental conditions. Environmental changes may stimulate growth of "hidden flora" to detectable levels and to bloom concentrations

(Taylor 1990). A major difficulty, however, is to distinguish between natural fluctuations and anthropogenic changes.

Phytoplankton blooms have been known since biblical times and shellfish toxicity associated with them has been recognized for centuries (Shumway 1990). "It has taken too long for the general recognition that these are global problems concerning human health and economic growth" (Taylor 1990). During the domoic acid outbreak on the U.S.A. west coast in 1991, hundreds of pelicans died and set off the alarm; a cooperative effort is required now to save human lives and money in the future. Emphasis should be placed in short-term approaches such as the monitoring of both shellfish and phytoplankton, the study of accumulation and fate of domoic acid through the food web, and international regulations for seafood harvesting and marketing. In addition to that, however, longer-term studies on phytoplankton dynamics are of paramount importance to understanding causes, predicting occurrences, and mitigating effects of toxic diatom blooms.

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## A NOTE ON DOMOIC ACID IN CALIFORNIA COASTAL MOLLUSCS AND CRABS

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Domoic acid poisoning in humans was first recognized in November 1987, following an outbreak of gastrointestinal and neurologic illness in persons who had eaten cultivated mussels from Prince Edward Island in eastern Canada containing 300 to 1200 parts per million (ppm) of domoic acid (Perl et al. 1990, Teitelbaum et al. 1990). More than 100 persons became ill with a syndrome characterized by nausea, vomiting, abdominal cramps, diarrhea, severe headache, loss of short term memory, and a number of other less common symptoms. The source of domoic acid (DA) in the mussels was determined to be an intense bloom of the diatom *Pseudonitzschia pungens* forma *multiseries* the month before (Addison and Stewart 1989). Following this incident, the regulatory limit for DA in shellfish was set at 20 ppm.

Since the Canadian epidemic of domoic acid poisoning, unpublished data and some published reports (Haya et al. 1991, Dickey et al. 1992, CDHS, 1992, Garrison et al. 1992, Buck et al. 1992) have shown that DA can be found in various other North American coastal waters.

In September 1991, an epidemic of domoic acid poisoning killed hundreds of brown pelicans and Brandt's cormorants in Monterey Bay, California (Work et al. 1993). No human illnesses were reported in this case. This was the first documentation of DA poisoning occurring outside of Atlantic Canada, as well as the first documentation of DA being found in herbivorous finfish (anchovies) and being produced by the phytoplankton *Pseudonitzschia australis*. Since this episode, the California Department of Health Services, supported in part by the U.S. Food and Drug Administration, has regularly monitored for domoic acid in California seafood.

Between the end of October 1991 and July 1993, a total of 1182 bivalve shellfish samples were analyzed for domoic acid (787 mussel, 349 oyster and 46 clam samples). Overall, 53 (4.5%)

were found to be positive, with the maximum concentration being 47 ppm in mussels, 1.9 ppm in oysters and 29 ppm in razor clams. The highest concentrations were observed during November and December, 1991, in mussels from Monterey Bay and razor clams from Humboldt County, approximately 300 miles north of Monterey Bay. Low concentrations of DA have now been identified in mussels from each of California's fifteen coastal counties, and in oysters from most commercial growing areas in the state. There has been no demonstrable seasonality to these low level occurrences of DA. More pronounced seasonality has been observed in *P. australis* abundances in Monterey Bay (Garrison et al. 1992, Buck et al. 1992).

In addition to DA being found in bivalves and anchovies, it also has been found in both rock and Dungeness crabs harvested along the California coast. In crabs, the highest concentrations of DA occur in the viscera with only small amounts translocated into the meat during cooking. DA in crab viscera is generally higher than in bivalves; however, none of the crabs tested to date contained sufficient DA to pose a human health concern. The findings of DA in crab viscera have not correlated with what has been observed with nearshore bivalve molluscs.

Based on twenty months of sampling, it seems apparent that at least low level concentrations of domoic acid may be found in a number of marine species anywhere along the California coast, at any time, and that offshore fisheries data (e.g., crab and anchovy) are not reliable indicators of potential toxicity in nearshore bivalve molluscs. Conversely, nearshore bivalves are not good indicators of offshore fishery toxicity. The ultimate significance of these findings is not clear, and more testing is being conducted. However, the findings are reported now in an effort to facilitate the growing understanding of the occurrence of domoic acid in North America.

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# THE NATIONAL SHELLFISHERIES ASSOCIATION

The National Shellfisheries Association (NSA) is an international organization of scientists, management officials and members of industry that is deeply concerned and dedicated to the formulation of ideas and promotion of knowledge pertinent to the biology, ecology, production, economics and management of shellfish resources. The Association has a membership of more than 1000 from all parts of the USA, Canada and 18 other nations; the Association strongly encourages graduate students' membership and participation.

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**COVER PHOTO:** Black abalone *Haliotis cracherodii* in the intertidal at the California Channel Islands. (Photo by Gary Davis; see series of papers beginning on p. 183)

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