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Immunological Detection of *Mercenaria mercenaria* in a Predator and Preparation of Size-Class Specific Antibodies

by

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Abstract. Successful culture of hard clams (*Mercenaria mercenaria*) requires high survivorship of seed stock during subtidal grow-out. This study was designed to identify natural predators of juvenile clams. Immunological techniques were used to identify *M. mercenaria* proteins in the guts of their natural invertebrate predators and to characterize antigen preparations (whole-organism extracts) of different size classes of clams. The grass shrimp *Palaemonetes vulgaris* was found to eat juvenile *M. mercenaria*. Immunoelectrophoretic separations and immunodiffusion tests of whole-organism extracts of *M. mercenaria* revealed unique antigens in the following size classes: veliger larvae, newly settled spat, juveniles, and adults.

INTRODUCTION

SHELLFISH PRODUCTION on the east coast of the United States is dominated by two commercially important bivalves, the American oyster *Crassostrea virginica* (Gmelin, 1791), and the hard clam, *Mercenaria mercenaria* (Linné, 1758). Because favorable conditions for growth of the hard clam exist in the relatively warm estuarine waters of South Carolina, a pilot scale mariculture facility was established to estimate growth and survivorship of seed clams emplaced subtidally in cages (MANZI *et al.*, 1980). Based on findings reported by numerous workers (*e.g.*, MACKENZIE, 1970, 1977; KRANTZ & CHAMBERLIN, 1978; WHETSTONE & EVERSOLE, 1978, 1981; KRAEUTER & CASTAGNA, 1980), it was reasonable to expect that some seed stock would be lost to predators during grow-out. Unprotected juveniles are known to suffer tremendous mortality soon after settlement in the natural environment (MILEIKOVSKY, 1974; HIBBERT, 1977), but such losses of newly settled spat have never been successfully measured directly in the field. Besides the obvious difficulties of sorting, quantifying, and identifying very small (150–200 μm) post-settlement individuals from sediment samples, the fragile young clams are also not easily identified in the guts of their potential predators. Loss of these small spat to their natural predators, then, is unlikely to be detected with conventional sampling and analytical techniques. This study was conceived to extend the use of an immunological method capable of detecting soluble proteins of *M. mercenaria* in stomachs of their natural marine predators, to identify heretofore suspected but undocumented predators upon this valuable species, and to characterize antigenic changes in the soluble proteins of *M. mercenaria* during its growth to marketable size.

MATERIALS AND METHODS

Preparation and Characterization of Antibodies

Target specimens (seed clams) were procured from Trident Seafarms Co., Charleston, SC, in eight size classes ranging from 0.6 to 16 mm total shell length (BROWN *et al.*, 1983). Clams that had been growing in tray culture in natural seawater for periods up to several weeks were sorted from their culture debris, separated by size, and immersed live in filtered seawater. The seawater was treated with antibiotics to reduce bacterial contamination, and the clams allowed to empty their stomachs of ingesta for 3 or 4 days at room temperature (20–22°C). Animals from each size class were then solubilized in ice-cold, buffered TES-saline and centrifuged at 3000 × g for 15 min to remove particulates. The whole-organism-extract supernates of each size class served as antigens for the preparation of antisera in New Zealand white female rabbits, according to the protocol of FELLER *et al.* (1979).

Antibodies were harvested from the rabbits by cardiac

puncture and assayed for titer and specificity in replicate using the double immunodiffusion micro-Ouchterlony technique in agarose gels (OUCHTERLONY, 1968). Further characterization of the antigen-antibody specificities was established using rocket-line and two-dimensional (crossed) immunoelectrophoresis separations in agarose (AXELSEN *et al.,* 1973). In the two-dimensional technique, antigenic components of a given size class are separated according to their electrophoretic mobilities prior to precipitin line formation in the second dimension with all mobilities relative to a bovine serum albumin (BSA) standard (AXELSEN & BOCK, 1972). These methods allow visualization of antigen-antibody precipitin line patterns shared by each size class of clams when antigens are reacted with either their homologous or heterologous antibodies. Immunological distances among the eight size classes of *Mercenaria mercenaria* were assessed by computing a matrix of cross-reactions of each antiserum based upon their relative similarities with the homologous reaction. A hierarchical clustering algorithm (BMDP2M; DIXON, 1981) was used to construct a dendrogram as in FELLER & GALLAGHER (1982).

Detection of Potential Predators

Experimental grow-out cages belonging to Trident Seafarms Co. were examined for macrobenthic predators at their high subtidal (−0.1 m, MLW) sites on Oak Island on 29 October 1980. The cages had been in place for five days and contained juvenile *Mercenaria mercenaria* in the size range 14–18 mm, a size well known to be essentially immune to predation even in open, unprotected sediments. No potential predators were found either on or within the pea gravel of any of the newly emplaced cages. Some other cages in the vicinity of the new ones (in place for approximately one year—Dr. J. Manzi, personal communication) were also examined for potential clam predators by divers. A variety of invertebrates was taken from the cages which, at this time, no longer contained any clams, nor were their containment screens intact. Sediment samples taken in replicate with 2.5-cm diameter cores to a depth of 5 cm were collected at random from the area surrounding both the old and new Oak Island cages and screened through a 250-μm mesh.

Several small grow-out cages (1 × 1 × 0.3 m) were emplaced subtidally in February, 1981 by Dr. J. Manzi near the Trident Seafarms Co. shore facility on Folly Beach, SC. They contained *Mercenaria mercenaria* ranging in size from 3.0 to 7.0 mm. A single cage containing clams was examined on 23 March 1981 for potential predators; the restraining mesh was not completely intact, and the cage contained numerous invertebrates, including the grass shrimp *Palaemonetes vulgaris* (Say, 1818) and the mud snail *Ilyanassa obsoleta* (Say, 1822). All organisms collected from the cage were frozen on dry ice immediately after collection.

Immunological analysis of the stomach contents of suspected predators involved dissection of the gut from individual specimens, microscopic examination for visually identifiable remains, and solubilization of the gut mass in TES-saline using a chilled mortar and pestle (FELLER *et al.,* 1979; FELLER, 1984). The solubilized proteins from within the gut were then analyzed by double immunodiffusion on 25 × 75 mm glass slides coated with agarose. The fluids (15 μL) were placed in a central well surrounded by antibody wells, each containing an antiserum to suspected prey—in this case different size classes of *Mercenaria mercenaria.* Antiserum to the predator itself, if available, was also used as a test control to ensure that any precipitin lines formed were due to proteins from the gut contents rather than sloughing of the predator's gut wall proteins. When control antisera were not available, an antiserum to the most closely related taxon was used. If none of these were available, it was assumed that gut wall proteins did not mask the observed reactions. This typically did not cause problems, because very few precipitin lines due to prey were observed in any of the predator guts tested.

RESULTS

Characterization of *Mercenaria mercenaria* Antigens by Immunodiffusion

Antisera were successfully prepared to antigens from eight size classes of *Mercenaria mercenaria* (Table 1). Antibody titer, the reciprocal of the highest dilution of an antiserum that gives a detectable reaction with its homologous antigen, was between 128 and 512 for size classes B and D–H, but only 64 for the two antisera prepared using antigens with the lowest protein concentrations, A and C. The number of precipitin lines produced by double immunodiffusion in replicated homologous double immunodiffusion self-reactions increased with increasing clam shell length, but there was considerable overlap among the size classes in the numbers of self-reaction precipitin lines formed (Figure 1). The veligers (size class A) and spat (B and C) had similar and significantly fewer numbers of homologous precipitin lines than the other size classes of older clams (D–H) which had similar numbers of lines (d.f. = 7,122; $P < 0.001$ by single-classification ANOVA). This immunological overlap reflects the amount of overlap in shell length among the size classes themselves and was entirely expected despite differences in mean weight per individual or protein concentrations among the eight size classes (Table 1). Antisera were unique to the extent that most of the cross-reactions between a given antiserum and antigens from heterologous size classes were not as extensive (did not produce as many precipitin lines) as the identity or self-reaction from that antiserum (Table 2). Exceptions to this include size classes E, F, and G; both E and F contained 6.0-mm individuals, and group G contained individuals similar in size to group F (Table 1). The cross-reactions involving antisera to these three

Table 1

Size-class composition of *Mercenaria mercenaria* antigen preparations.

Sizeclass[1]	No. indiv.	Shell length size range (mm)	Total wet wt. (mg)	TES-buffer (mL)	Protein conc. (mg/mL)	Comments
A[2]	10,000	0.2–0.3	300.0	10.0	1.4	veligers
B[2]	400	2.0–5.0	100.0	6.0	3.7	starved 3 days
C	1466	0.6–3.5	4.3	6.0	1.3	ground whole
D	484	3.0–5.0	12.5	10.0	2.8	ground whole
E	320	5.0–6.0	11.9	9.5	3.1	ground whole
F	197	6.0–9.9	5.6	9.5	4.1	ground whole
G	53	10.0–16.6	1.3	5.5	4.1	foot muscle
H[3]	3	70.0–75.0	7.6	23.0	3.5	foot muscle

[1] A = veligers; B, C = newly set spat; D, E, F, G = juveniles; H = adult.

[2] Provided by J. W. Ewart, Hatchery Manager, University of Delaware; these veligers had been fed *Isochrysis* aff. *galbana* (T ISO) and *Thalassiosira pseudonana* 3H.

[3] From North Inlet, South Carolina.

groups were strong enough with antigens from adults (size class H) that neither D, E, F, nor G antisera could be considered size class specific. Cross-reactions among the A, B, C, and H size classes were all lower than the homologous reactions based upon maximum numbers of lines observed (Table 2). Thus, it was possible, using double immunodiffusion tests, to distinguish among the following four size classes of *M. mercenaria*: veliger larvae (A), newly settled spat (B, C), juveniles (D, E, F, G), and adults (H) or "chowders." A dendrogram of immunological similarity based on data in the cross-reaction matrix of Table 2 also reflects these differences between size classes (Figure 2).

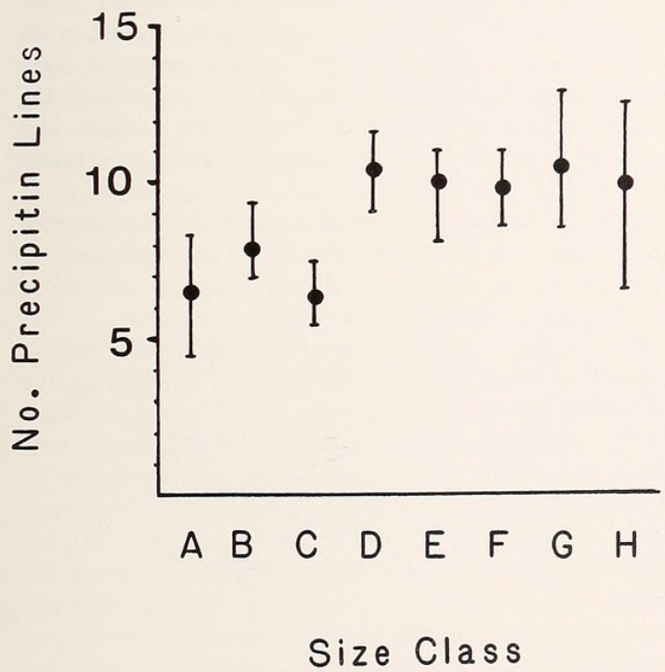

Figure 1

Mean number (±95% confidence limits) of precipitin lines observed in homologous antigen-antibody reactions for each size class of *Mercenaria mercenaria* defined in Table 1.

The presence of cross-reactions among antisera to, and antigens from, *Mercenaria mercenaria* indicated that many of the size classes shared common antigenic proteins. To visualize these antigenic similarities, rocket-line electrophorograms were prepared in 1% agarose gels. These tests essentially confirmed the immunological identities of the size classes discussed above, and in nearly all cases the numbers of precipitin lines observed in double diffusion tests were the same as observed in the rocket-line comparisons. As a further check on the specificity of the separate antisera, two-dimensional (crossed) electrophorograms were prepared on which the precipitin line patterns of both self- and cross-reactions could be visualized.

The existence of both common and unique antigenic components among each of the separate size classes of

Table 2

Maximum number of precipitin lines observed in micro-Ouchterlony double immunodiffusion tests using homologous (on the diagonal) and heterologous reactions between antibodies to and antigens of eight size classes of *Mercenaria mercenaria* (A–H defined in Table 1).

	Antigens (whole-organism extracts)							
	A	B	C	D	E	F	G	H
Antisera								
(A)	9	7	6	8	7	7	8	7
(B)	7	10	6	8	10	9	5	9
(C)	7	6	9	5	6	4	5	7
(D)	7	11	8	14	13	12	10	10
(E)	7	9	7	11	11	8	11	11
(F)	7	9	9	11	10	12	9	12
(G)	10	11	10	9	12	12	13	13
(H)	8	10	9	9	10	7	12	13

Mercenaria mercenaria was demonstrated using the two-dimensional immunoelectrophoretic separation technique. All possible antigen-antibody reactions were compared, but to illustrate the basic principle, identity and cross-reactions involving the B and G size classes are shown in Figure 3. Not only do the precipitin-line peak patterns show which components are common to both antigens, but the heights of the peaks also reflect the different protein concentrations comprising each antigen-antibody complex (KENNY & FOY, 1975). Comparison of electrophoretic mobilities of each precipitin line relative to BSA's migration in the first dimension (where BSA's migration distance from the antigen well equals unity) also complemented the visual comparisons by establishing which peaks were unique or common to a given antigen-antibody reaction pair. For example, the antiserum for spat produced seven precipitin peaks of identical electrophoretic mobility with both spat and juvenile antigens, indicating that these antigenic components are common to both age classes (Figure 3).

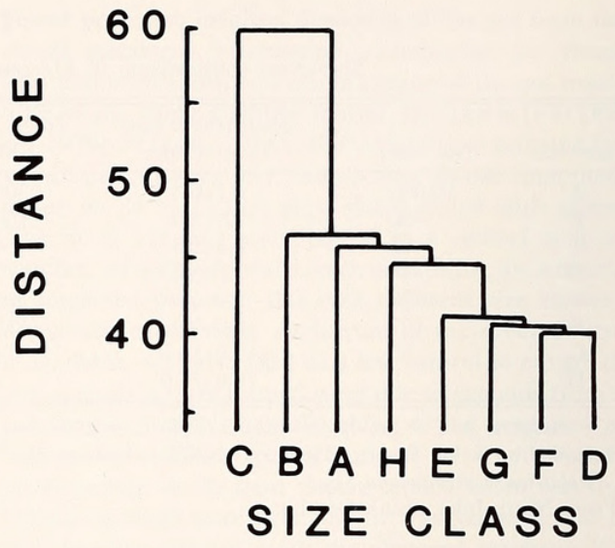

Figure 2

Dendrogram of immunological similarity (based on Euclidean distance) among antisera to the eight size classes of *Mercenaria mercenaria* defined in Table 1.

Detection of Potential Predators

Having established the relative specificities and sensitivities of the antisera to individual size classes, it was possible to use them to detect the presence of specific *Mercenaria mercenaria* proteins in the stomachs of potential predators in the field.

Amphipods of the genera *Melita* (n = 12) and *Corophium* (n = 15) collected from the year-old floating pens at Oak Island on 20 October 1980 did not contain any *Mercenaria mercenaria* protein, nor did any of the snapping shrimp *Alpheus* sp. (n = 8) or any of several nereid polychaetes examined. Apparently no clams were available for ingestion in these old cages, and none were visible (as previously noted). Organisms collected by cores from around the newly placed pens (containing 18-mm clams in pea gravel) included a typical assemblage of low intertidal or high subtidal invertebrates in areas of compact oyster shell debris—nereid and phyllodocid polychaetes, turbellarians, nematodes, and a few harpacticoid copepods. The only member of this potential predator community that contained bivalve protein was a single specimen of *Nereis* sp. (12 mm total length), but it was not *M. mercenaria* protein; it was tentatively identified as *Crassostrea virginica* protein. The newly emplaced clams were apparently not accessible to predators, nor were any predators seen in, on, around, or under the new pens.

The Folly Beach cage collections of predators on 23 March 1981 were examined for the presence of *Mercenaria mercenaria* proteins in three specimens of the spionid polychaete *Streblospio benedicti* (Webster, 1879), six *Ilyanassa obsoleta,* two unknown errant polychaetes, and 22 specimens of *Palaemonetes vulgaris*. The *P. vulgaris* stomachs were separated according to total shrimp length (from tip of rostrum to end of telson) into small (<20 cm), medium (20–25 cm), and large (26–27 cm) groups. Each of these groups of potential predators was homogenized in saline after visual analysis (dissecting microscope at 50×) of individual organism's gut smears revealed only the presence of amorphous material and fluids.

Gut contents from the three groups of *Palaemonetes vulgaris* were tested for the presence of *Mercenaria mercenaria* proteins using antisera to four size classes, C–F inclusive. Because antiserum to *P. vulgaris* was unavailable for use as a control for these immunoassays, antiserum to *P. pugio* was used instead. The control reaction between antiserum to *P. pugio* and antigens from *P. vulgaris* produced eight distinct precipitin lines, whereas the cross-reaction between any one of the four *M. mercenaria* antisera and either *P. pugio* or *P. vulgaris* antigens produced a maximum of only two lines. Presence of *M. mercenaria* in *P. vulgaris* gut contents would thus be indicated by existence of more than two precipitin lines in the immunoassays, as an empty gut would produce the same number of lines as occur in the control cross-reaction.

The combined gut contents of five small *Palaemonetes vulgaris* produced four precipitin lines with anti-D, the antiserum to juvenile *Mercenaria mercenaria* in size class D. The combined gut contents of 13 medium shrimp produced three lines with anti-C, seven lines with anti-D, and three lines with anti-E. No more than two precipitin lines were produced in tests of the four large *P. vulgaris* whose gut contents were combined into one group for analysis. As an additional check that the lines observed were due to the presence of *M. mercenaria* in the shrimp guts, immunoassays were run with antiserum to size class D and known antigens of the same *M. mercenaria* size class adjacent to wells containing the shrimp gut contents. These tests produced lines of identity between the gut contents and the *M. mercenaria* antigens, thus confirming that the grass shrimp had indeed eaten *M. mercenaria.*

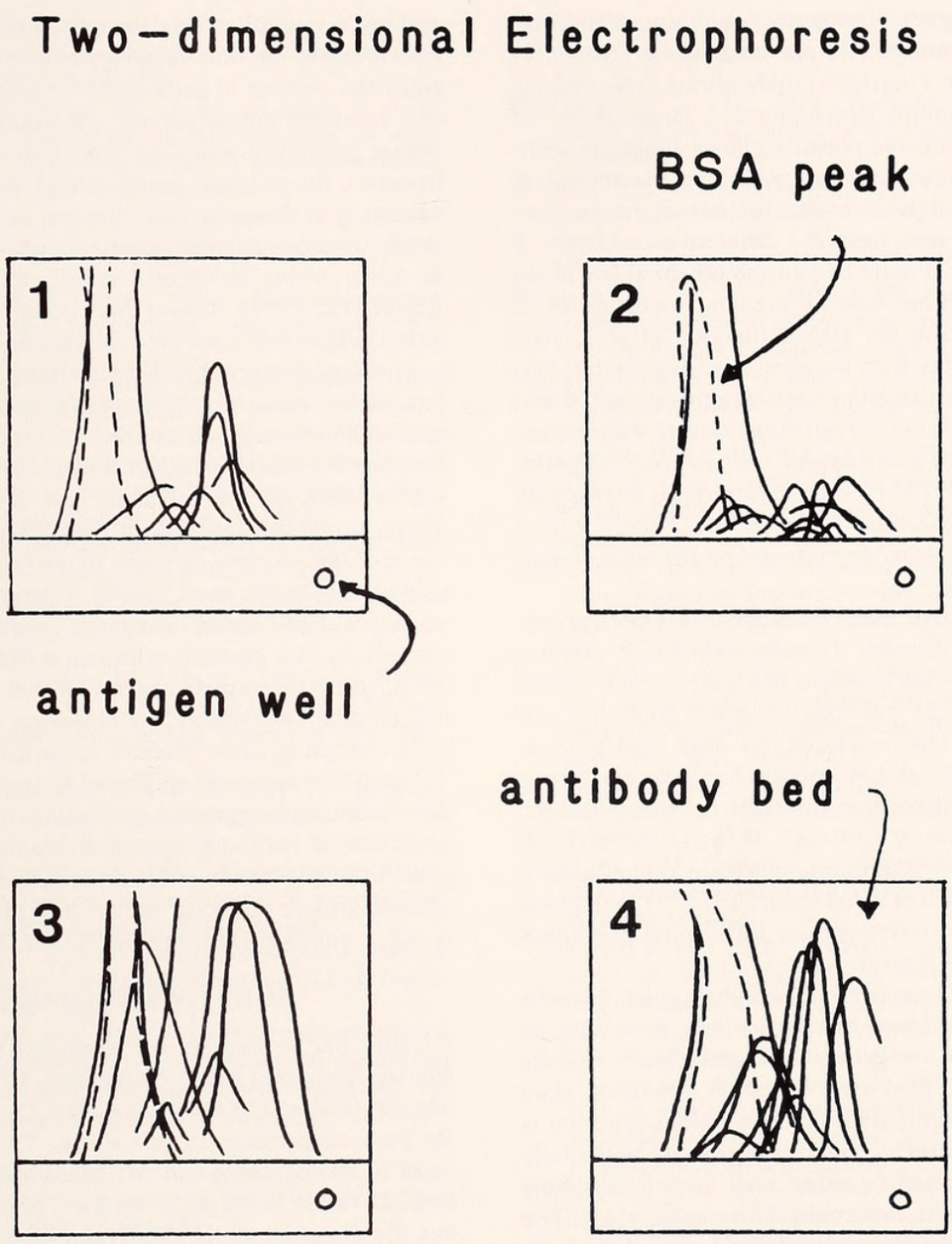

Figure 3

Schematics of two-dimensional immunoelectrophorograms for the homologous (1 and 4) and heterologous (2 and 3) reactions between antigens and antibodies to *Mercenaria mercenaria* size class B (spat) and G (juveniles). Antigens were separated for 1.25 h in the first (horizontal) dimension and then rocketed into a 20% (vol./vol.) antibody gel bed for 6.0 h, all at 2 Volts/cm with constant power at room temperature. Bovine serum albumin (BSA) was used as a marker to which all first dimension migration distances from the antigen well were referenced. Two microliters of anti-BSA (Miles Laboratories) were present in each antibody bed.

(1) Spat antigen into anti-spat bed (the homologous reaction).
(2) Spat antigen into anti-juvenile bed (the heterologous reaction).
(3) Juvenile antigen into anti-spat bed (the heterologous reaction).
(4) Juvenile antigen into anti-juvenile bed (the homologous reaction).

DISCUSSION

Because separations of antigenic proteins based on Fickian diffusion in agarose gels by double immunodiffusion are not strictly comparable to separations based on electrophoretic mobility, one cannot explicitly equate cross-reactions observed with these two techniques. However, both immunodiffusion and immunoelectrophoresis (either rocket-line or two-dimensional) are independent methodologies suitable for establishing unique immunological specificities of antisera.

Increasing immunogenicity of *Mercenaria mercenaria* antigens as a function of size reflects the more complex

nature of its antigenic components with increasing age (Figure 1). This ontogenetic phenomenon is reasonably well established for a variety of invertebrate taxa wherein the existence of unique developmental stage-specific or age-specific antigenic components allows immunochemical detection of these taxa in predators (BOREHAM & OHIAGU, 1978). Unique two-dimensional separation patterns seen for antigens from the different size classes of *M. mercenaria* (*e.g.,* Figure 3) point to potential use of the antibodies to detect age-specific predatory mortalities. If antigenic components are also a function of local food resources, then it may even be possible to develop habitat-specific antisera for the veliger age class of *M. mercenaria* and detect the routes of larval dispersal for this species. Notwithstanding the polymorphic traits of natural populations (*e.g.,* PESCH, 1974), such an approach has already been suggested by MENZIES & KERRIGAN (1978) for tracing routes of spiny lobster recruitment on the basis of their biochemical genetics.

The development of antisera capable of detecting minute quantities of *Mercenaria mercenaria* tissue proteins in the predator gut environment is a prerequisite for the use of immunological methods to detect otherwise unknown predators. The technique has been used successfully in both terrestrial and aquatic habitats, and previously unknown predator-prey linkages have usually been identified (BOREHAM & OHIAGU, 1978; CALVER, 1984; FELLER *et al.,* 1985). The immunological study of gastropod predation on oysters by MARSHALL (1977) also attests to the power of this technique for identifying previously unknown predatory species.

Finding that *Palaemonetes vulgaris* had eaten juvenile *Mercenaria mercenaria* was not particularly surprising, as these small shrimp are generalist feeders that typically tear and shred their food upon ingestion, rendering clam tissue visually unidentifiable. A more serious question is whether losses from such a small predator are potentially as great as those posed by other well known predators (drills, xanthiid crabs, asteroids, blue crabs, *etc.*). The Folly Beach cages were not sampled on any other dates; hence, it is unknown whether there were other predators present that could have ingested *M. mercenaria* from them. Casual observations of fauna in the area revealed the presence of several known predators on bivalves (*e.g., Callinectes sapidus* [Rathbun, 1896], *Urosalpinx cinerea* [Say, 1822], birds, and xanthiid crabs), so the potential for additional losses via predation from those small cages did exist.

The impact of large predators (whelks, drills, rays, and crabs) is known to be destructive on *Mercenaria mercenaria* populations (WALKER *et al.,* 1980), and preventive measures may be successful in restricting their access to cultures. A small motile predator such as *Palaemonetes vulgaris,* however, will be much more difficult to exclude, especially if it is small enough to go through protective meshes or screens. It is conceivable that the young of such a predator might gain access to a culture tray and grow amidst an unlimited food supply. Successful bivalve culture requires not only rapid growth at high stocking densities and absence of pathogens, but also high survivorship and favorable socioeconomic conditions. Most efforts to reduce predatory mortality have been basically physical in nature (mesh cages, gravel burial, *etc.*), but such methods have, in the past, been directed at preventing known predators from gaining access to cultures (*e.g.,* MENZEL & SIMS, 1964; ELDRIDGE *et al.,* 1976; CASTAGNA & KRAEUTER, 1977). Losses that occur in physically protected culture trays are typically assumed to be a result of mechanical damage, handling artifacts, innate morbidity, parasites, disease, or environmental stresses—little consideration had been given to previously unknown predators. Reasons for this are logical and obvious, for if it is not known what all the predators are, it is not possible to design protection from all of them. This is evidenced by the sporadic success of cages in protecting desirable organisms (MENZEL *et al.,* 1976; VIRNSTEIN, 1978). Identification of previously unknown predators enhances the probability that preventive measures can be taken to avoid them, either by emplacing grow-out trays in areas having low predator abundance, by removing specific predators, or by designing more effective exclosure devices.

Preventive measures employed by the Trident Seafarms Co. (mesh and aggregate protection) coupled with emplacement of relatively large individuals for grow-out appears to be effective in reducing predatory losses. Whether optimal growth can occur under these grow-out conditions is still a question (HADLEY & MANZI, 1984).

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