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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

It is with deep regret that THE BIOLOGICAL BULLETIN records the death, at his home in Woods Hole on February 6, 1978, of Dr. Donald P. Costello, Kenan Professor of Zoology Emeritus of the University of North Carolina, Chapel Hill, and our Managing Editor from 1951 to 1968. We know that many readers of, authors in, and editorial reviewers for THE BIOLOGICAL BULLETIN appreciate the extent to which the continued international standing of the journal during those 36 volumes, and even subsequently, depended upon his scholarly efforts.

Vol. 154, No. 1 February, 1978

THE
BIOLOGICAL BULLETIN

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STUDIES ON FUNCTIONAL MORPHOLOGY IN THE DIGESTIVE SYSTEM OF *OREASTER RETICULATUS* (L.) (ASTEROIDEA)

Reference: *Biol. Bull.*, **154**: 1–14. (February, 1978)

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Oreaster reticulatus (Fig. 1) is a large, conspicuous, and easily-recognized sea-star, abundant within its broad geographical range and probably of considerable ecological significance where it occurs. Curiously, however, the number of published papers dealing with aspects of the basic biology of this species is small. A brief note by Thomas (1960) describes its feeding habits; another, almost as brief, by Matthews and Lima-Verde (1968) suggests an ecological relationship between *Oreaster* and two species of *Panulirus* on the fishing-banks of Northeastern Brazil. The entire literature dealing with the internal anatomy of *O. reticulatus* appears to consist of a four-page paper with three plates by Tennent and Keiller (1911) and a brief abstract by Anderson (1967) reporting preliminary observations on the digestive system. Some special features of the internal anatomy of *Culcita*, a genus assigned to the Family Oreasteridae, were described and figured (rather sketchily) by Müller and Troschel (1842), whose drawing was reproduced by such later authors as Ludwig and Hamann (1899); but beyond this, published information on *Oreaster* and its relatives is scanty. The purpose of the present paper is to describe, in greater detail than that provided in previous accounts, the general morphology of the digestive system as a whole, noting particularly some interesting features of the pyloric stomach and related structures. This is intended as a contribution toward a broadly comparative survey of digestive systems among asteroids. Such a survey has never been undertaken; a step in this direction is provided by Anderson (1966), and a further contribution appears in Jangoux and van Impe (1971), but available data are still inadequate to permit comparisons in detail among representatives of many different families of sea-stars. Further, an attempt will be made to draw together published and unpublished descriptions of feeding behavior in *Oreaster*, and to correlate these with structural features of the digestive system. The results of histological studies on this system are to be published in a subsequent paper.

Paraphrasing a statement concerning *Oreaster* that he had published in 1902,

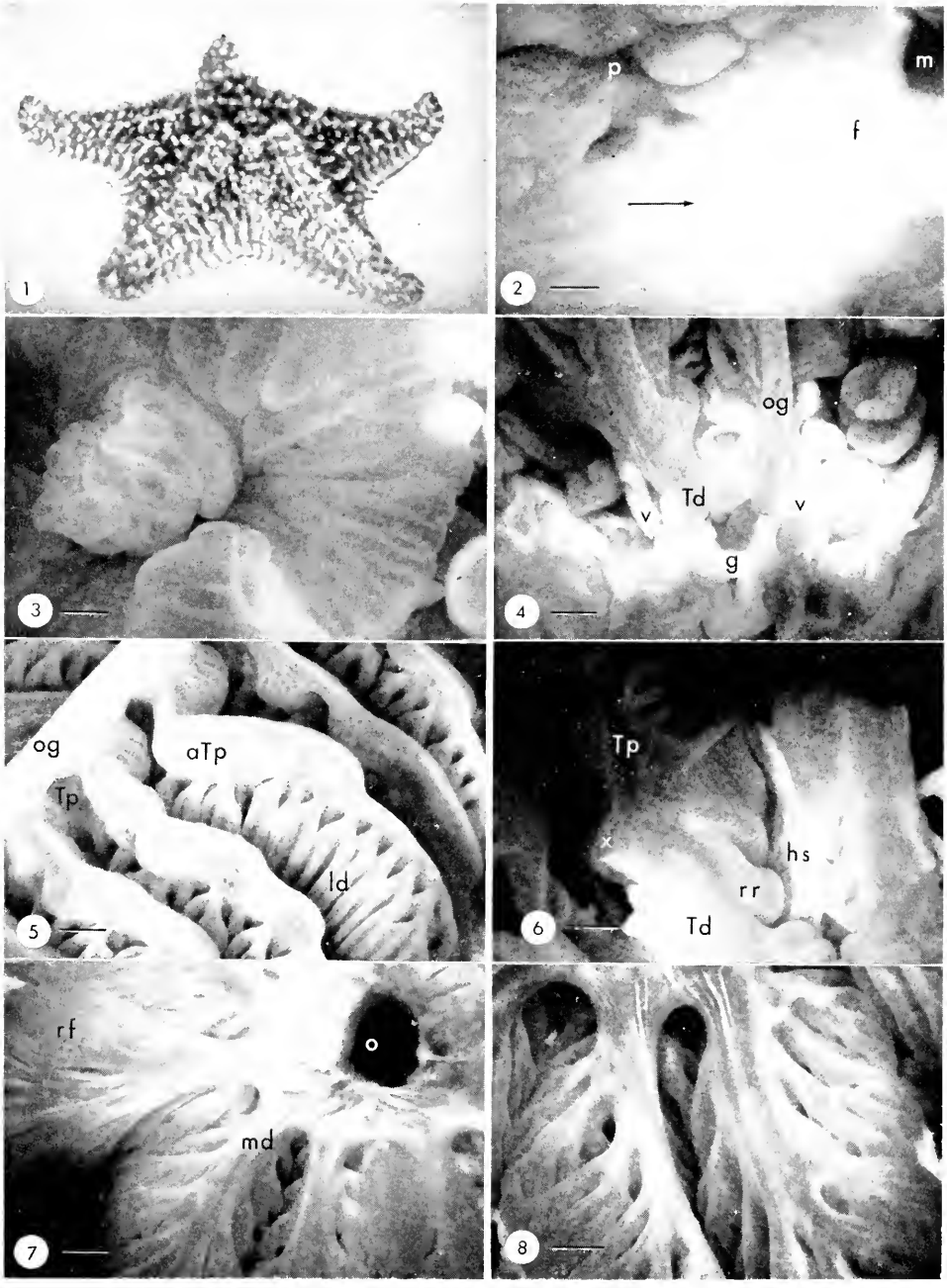

FIGURE 1. *Oreaster reticulatus*. The major radius of this specimen measured about 12 cm (from a color transparency by C. P. Anderson).

FIGURE 2. The floor of the cardiac stomach, internal view. The arrow indicates the dis-

H. L. Clark (1933, p. 22) says: "This is undoubtedly the best known of West Indian sea-stars, since it has been taken to many parts of the world in the past 175 years as a typical curio and souvenir of the region." It is a widely distributed species, being common, according to Downey (1973), in shallow-water grass and sand flats from Florida to Brazil; it occurs in the Cape Verde Islands and in Bermuda, and it is occasionally found as far north as Cape Hatteras. Overall, however, the scientific interest it has attracted is rather limited. A. Agassiz (1877) gives a meticulous and beautifully illustrated description of its skeletal morphology, and the species is, of course, treated in systematic and faunistic studies, such as those cited above, and others (among them Verrill, 1915, and Caso, 1961). Increased attention to other interesting features of this species seems long overdue.

Materials and Methods

Specimens were obtained from a commercial source in the Florida Keys. At the laboratory, they were maintained in large aquaria provided with running sea water and were fed periodically with crushed snails, shucked bivalves, and pieces of fish, all of which they ate. Under these conditions they remained in an evidently healthy, vigorous state until sacrificed for study. Animals to be dissected were first soaked until flaccid in a solution of $MgSO_4$ (8% in tap water). The specimens were all rather large, with major radii ranging from 10 to 14 cm. In such animals, as pointed out by Tennent and Keiller (1911), the body wall is exceedingly hard and tough, and gaining access to the internal organs is unusually difficult. The technique eventually adopted was as follows: using a strong, sharp scalpel (with a well-taped handle), a horizontal incision was made around the periphery of

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tinct line bounding the smooth, yellowish central area. Abbreviations are: *m*, mouth opening; *f*, floor; and *p*, a lateral pouch.

Figure 3. Pouches of the cardiac stomach, partially everted and not fully inflated with coelomic fluid. These show the typical asteroid branching gutter-patterns in the wall, here seen from the mucosal side.

Figure 4. Oral view of the origins of a pair of Tiedemann's ducts, and related structures. Abbreviations are: *g*, fibrous girdle, just above remnants of the cut wall of the cardiac stomach; *v*, vertical anchoring strands suspending the girdle from the roof of the disc; *Td*, Tiedemann's duct; and *og*, an oral gutter of Tiedemann's pouch.

Figure 5. Detail of a portion of a pyloric caecum, with associated structures, seen from below. Abbreviations are: *og*, oral gutter; *Tp*, striated side wall of the main Tiedemann's pouch; *aTp*, accessory Tiedemann's pouch; and *ld*, lateral diverticulum of a glandular pocket of the pyloric caecum.

Figure 6. Oral view of the proximal portions of two radial branches of the pyloric stomach, with related structures. Abbreviations are: *Td*, Tiedemann's duct, transected at *x*; *Tp*, Tiedemann's pouch; *rr*, radial reservoir of the pyloric stomach; and *hs*, horizontal connective-tissue sheet, which joins with the vertical anchoring strands to provide support for the roof of the pyloric stomach.

Figure 7. Aboral view of the roof of the pyloric stomach, after removal of the intestine, intestinal caeca, and rectum. Abbreviations are: *o*, opening from the pyloric stomach into the intestine; *rf*, roof folds in one of the paired fold-pattern sets; and *md*, main duct or channel of a fold-pattern, showing supposed hemal vessels in its roof.

Figure 8. A closer view, showing structural details of a pair of fold-patterns in the roof of the stomach.

For each figure, the scale bar shown represents 1 mm.

each ray, and the incisions in adjacent rays were joined by cutting through interradial structures. When the oral and aboral parts of the body wall were thus separated, the aboral wall was removed by carefully cutting all mesenteries and connective-tissue strands by which the viscera were suspended from it, and by transecting the gut just below the anus. The viscera were then floated up in the relaxing solution, the dissection continued, and the desired observations made. Anatomical details were photographed in freshly dissected, living specimens, using a 35 mm camera mounted on a dissecting microscope.

Observations and Results

The central cavity of the cardiac stomach has a smooth floor, continuous at the mouth with the external peristomial membrane (Fig. 2). The floor shows fine radial striations or wrinkles, and in all specimens examined it was of a yellowish color, contrasting with the generally brown cast of the rest of the stomach. A distinct boundary-line marks the margin of the floor, and just beyond it are ranged the regularly-spaced oral terminations of a large series of typical branching gutter-patterns in the wall of the stomach (Fig. 3). The wall is folded into a series of pouches, one set for each ray. In the fully retracted condition a set of these pouches lies above the proximal part of the ambulacral ridge pertaining to its radius in the floor of the disc, bounded on either side by an interradial septum. Each set of pouches comprises a thin-roofed median portion and a pair of complexly folded lateral pockets. The lateral pockets or pouches in adjacent rays are joined together by smooth walls, thrown into loose folds and passing around the interradial septa. These smooth walls, with inward extensions from the roofs of the several median pouches, continue upward to the aboral boundary of the cardiac portion of the stomach. This is more or less arbitrarily recognized as a slightly constricted region which in *Orcaster,* as in *Patiria* (Anderson, 1959), is marked by an encircling connective-tissue girdle related to the suspensor-retractor system to be described later.

The overlying pyloric stomach is tall in its oral-aboral dimension and is deeply divided radially in relation to the origins of the paired pyloric caeca to which it gives rise. The radial indentations, alternating with the pyloric caeca and their specialized appendages, are like those described in several other sea-stars. The similar condition found by Jangoux and van Impe (1971) in *Asterina, Henricia,* and *Echinaster* leads these authors to state that there is no pyloric stomach in the classical sense, but rather a characteristic structure which they call the "*complexe stomacal supérieure.*" For each pyloric caecum a separate duct originates from the stomach, arising just above the level of the girdle (Fig. 4); using the terminology applied to the similar structure in *Henricia* (Anderson, 1960, p. 377), this is referred to as Tiedemann's duct. In its proximal part each duct is a cylindrical tube, the lumen of which is set off by a distinct partition from the space above it. After proceeding a short distance, however, it opens out to form the oral gutter of a deep Tiedemann's pouch, the cavity of which is continuous aborally with the central duct of the pyloric caecum proper. This central, aboral duct (which, it will be understood, has no floor) gives off, alternately to right and left, side branches which are the ducts of a long series of typical glandular pockets, extending all the way to the distal end of the caecum. The side walls of these pockets are thickened

and folded vertically to form lateral diverticula. The most striking and conspicuous feature of these organs is the presence of an accessory or secondary pouch hanging below each lateral glandular pocket, in effect branching from the side walls of the main Tiedemann's pouch. They are provided with oral gutters which originate just above the main gutter, and they taper upward and outward to end a variable distance from the tip of each pocket. The walls of the secondary pouches are faintly marked by narrow, evenly-spaced, parallel vertical striations which give the appearance of separating adjacent channels in the lumen of the pouch. Such markings are present also in the side walls of the main Tiedemann's pouch. All of these features are shown in Figure 5. In all respects, the pyloric caeca and Tiedemann's pouches of *O. reticulatus* resemble very closely the corresponding structures in *Porania pulvillus*, as previously described (Anderson, 1961, 1966).

It was mentioned earlier that the pyloric stomach is deeply indented between the bases of the pyloric caeca. Alternating with the indentations are radiating branches of the stomach, corresponding in position with the Tiedemann's ducts just below them; their side walls taper outward and become continuous with the proximal ends of the main Tiedemann's pouches. Just proximal to the point of origin of the first of the lateral glandular pockets from the central aboral duct, at about the level where the tubular Tiedemann's duct opens to become the oral gutter, a change in the gross appearance of the side walls of the organ occurs. The line marking this change is interpreted as the boundary between Tiedemann's pouch and the radial branch of the pyloric stomach. Aborally, this branch receives the central duct of the pyloric caecum. In position and anatomical relationships (Fig. 6), it corresponds precisely to the structures observed in the digestive systems of *Henricia* and *Linckia* and termed "radial reservoirs" of the pyloric stomach (Anderson, 1960, 1966).

The roof of the pyloric stomach in *Oreaster* presents a specialized feature which has not, to my knowledge, been described in any other sea-star. Tennent and Keiller (1911) write of it as follows (p. 114): "Beneath the intestine, upon the surface of the stomach in each radius, is what appears at first sight to be a second set of five caeca, each made up of two parts. Further examination shows that these are merely pouches formed by the folding of the upper wall of the pyloric portion of the stomach. They involve the regions into which the ducts of the pyloric caeca open and have a narrow slit-like connection with the stomach." Unfortunately, these unique structures, so succinctly characterized, cannot be made out with certainty in Tennent and Keiller's plate showing an aboral view of the digestive system. As seen in Figure 7, the roof of the pyloric stomach has a relatively smooth portion surrounding the opening into the overlying intestine. Radiating from this central area are the five sets of "pouches" just described. This term seems inappropriate; the structures referred to are radially-arranged fold-patterns, like inverted grooves or gutters, in the roof of the pyloric stomach. In each pattern, the folds converge on a major channel leading toward the intestinal opening. Each member of a pair of fold-patterns lies above a radial reservoir, and the cavities of the fold-pattern and the reservoir communicate by way of the "narrow slit-like connections" mentioned by Tennent and Keiller. There are numerous conspicuous vessels, probably parts of the hemal system, running in the aboral walls of the main channels (Figs. 7, 8). Figure 9, a semidiagrammatic cross-section, shows the

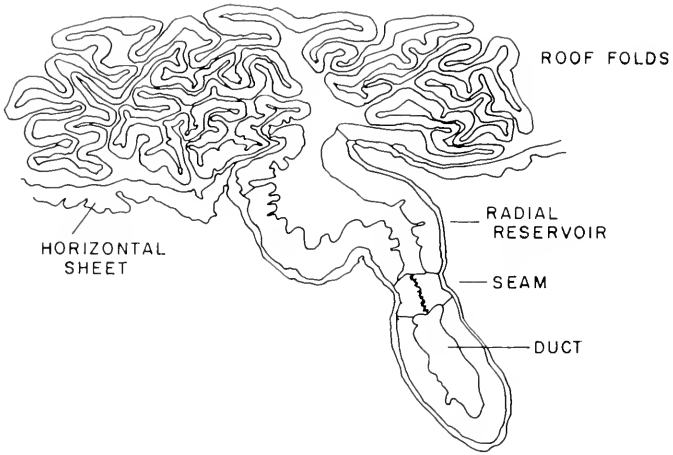

FIGURE 9. Semidiagrammatic cross-section of a radial portion of the pyloric stomach, showing the relationships between its component parts. This section is at a level proximal to the point at which Tiedemann's duct opens out to form the oral gutter of Tiedemann's pouch. The seam forming the partition between the duct and the overlying radial reservoir is maintained by permanent adhesion between cells in opposite walls. The figure was made by tracing, with some reconstruction, a projected histological section.

relationships between the fold-pattern, the radial reservoir, and the Tiedemann's duct pertaining to a single pyloric caecum. The close correspondence in position between the paired sets of fold-patterns and the paired pyloric caeca strongly suggests a significant functional relationship.

The intestine in *Oreaster* is a large, flattened, generally pentagonal organ. From its corners five flat prolongations extend outward in the interradii, crossing the roof of the pyloric stomach between adjacent sets of fold-patterns (Fig. 10). Each extension bifurcates as it reaches the interradial septum, and the ten branches thus formed become the very large and conspicuous intestinal caeca. The main duct gives off numerous bladder-like diverticula, and the whole organ, according to Tennent and Keiller, is capable of great distention. The intestinal caeca of *Oreaster* are very similar to those of *Culcita*, as illustrated by Müller and Troschel (1842); see also Ludwig and Hamann (1899, p. 585 and Plate IV).

The rectum is short, arising from about the center of the roof of the intestine and passing directly through the aboral body wall. The opening from the intestine into the rectum lies immediately above the passage leading from the pyloric stomach into the intestine (Fig. 11).

The complement of fibrous strands, sheets, and mesenteries developed in *Oreaster* to suspend and secure the digestive organs in the spacious body cavity, and to bring about retraction of the eversible parts, is very complex. Some account of this system will be helpful in understanding functional relationships.

The pyloric caeca are suspended from the aboral body wall by the usual paired, parallel mesenteries, which form continuous narrow sheets and enclose between them a long, tubular coelomic space above the central duct of each caecum. The mesenteries are unusually thick and tough in *Oreaster*, and they send short ex-

tensions laterally to suspend the glandular pockets of the caecum. Proximally, the mesenteries mingle with the fibrous bands by means of which the ducts of the intestinal caeca are hung from the roof of the disc, and become continuous also with a thick horizontal sheet which covers the roof of the pyloric stomach (Fig. 10). The sheet extends between the proximal ducts of the intestinal caeca, and it surrounds and attaches to the lower margins of the paired fold-patterns in the roof of the pyloric stomach. In each of the paired units, the level of attachment to the stomach is the line of transition between fold-pattern and radial reservoir (Figs. 6, 9, 10).

Additional suspension is provided for the digestive system by a pair of bands in each ray which originate on the aboral body wall and pass downward. Each of these vertical bands makes a connection with the mesentery complex at the edge of the horizontal sheet and then continues, passing lateral to a radial reservoir and joining the fibrous girdle encircling the cardiac stomach (Fig. 4). Strong bands, which I have termed "oral anchors," proceed from these junctions on the girdle to firm attachments alongside the proximal ambulacral ossicle in each ray (Figs. 12, 13).

In each radius, a group of glistening white extrinsic retractor strands arises from each side of the proximal end of the ambulacral ridge. From broad origins along the ridge, the fibers converge as they pass upward beside the pouches of the cardiac stomach and form three major branches (Fig. 13). One of these spreads over the roof of the median pouch, attaches to it along a line of insertion running radially, and sends further branches orally in its wall. The second distributes principally to the nearby lateral stomach pouch, where it bifurcates repeatedly (Fig. 14). Its branches, and those of the first major strand, give rise to the downward-coursing intrinsic retractor elements on and in the walls of the stomach, spreading out in patterns corresponding to those of the gutters mentioned earlier. These intrinsic strands are very similar in appearance and distribution to those designated "class 1" fibers in *Patiria* (Anderson, 1959). The third major extrinsic branch sends a few subsidiaries to nearby pouches and then passes directly to the girdle on the cardiac stomach, which it joins near the point of attachment of one of the vertical suspensory bands descending from the roof of the disc. I have designated this branch the "girdle retractor."

It is to be understood that all of the extrinsic retractor elements just described are paired; that is, in each ray there are two sets of the three main retractor branches, which arise and distribute symmetrically on either side of the axis of the ray.

Turning again to the intrinsic retractor strands, it is worth noting that in addition to the class 1 type previously described, other small branches are present. Considerable numbers of short, slender fibers originate on the folds and ridges of the lateral pouches and run horizontally, outside the wall of the stomach, before entering it again (Fig. 15). These strongly resemble the "class 2" fibers of *Patiria*. In the lowest part of the stomach groups of 3 to 12 thin strands emerge from folds related to the terminal gutter-patterns and run vertically, free in the coelom, to insertions on the outer surface of the smooth floor of the cardiac stomach (Figs. 16, 17). They are similar to the "class 3" fibers found in *Patiria* (Anderson, 1969). Precise correspondence between these fiber types cannot be firmly

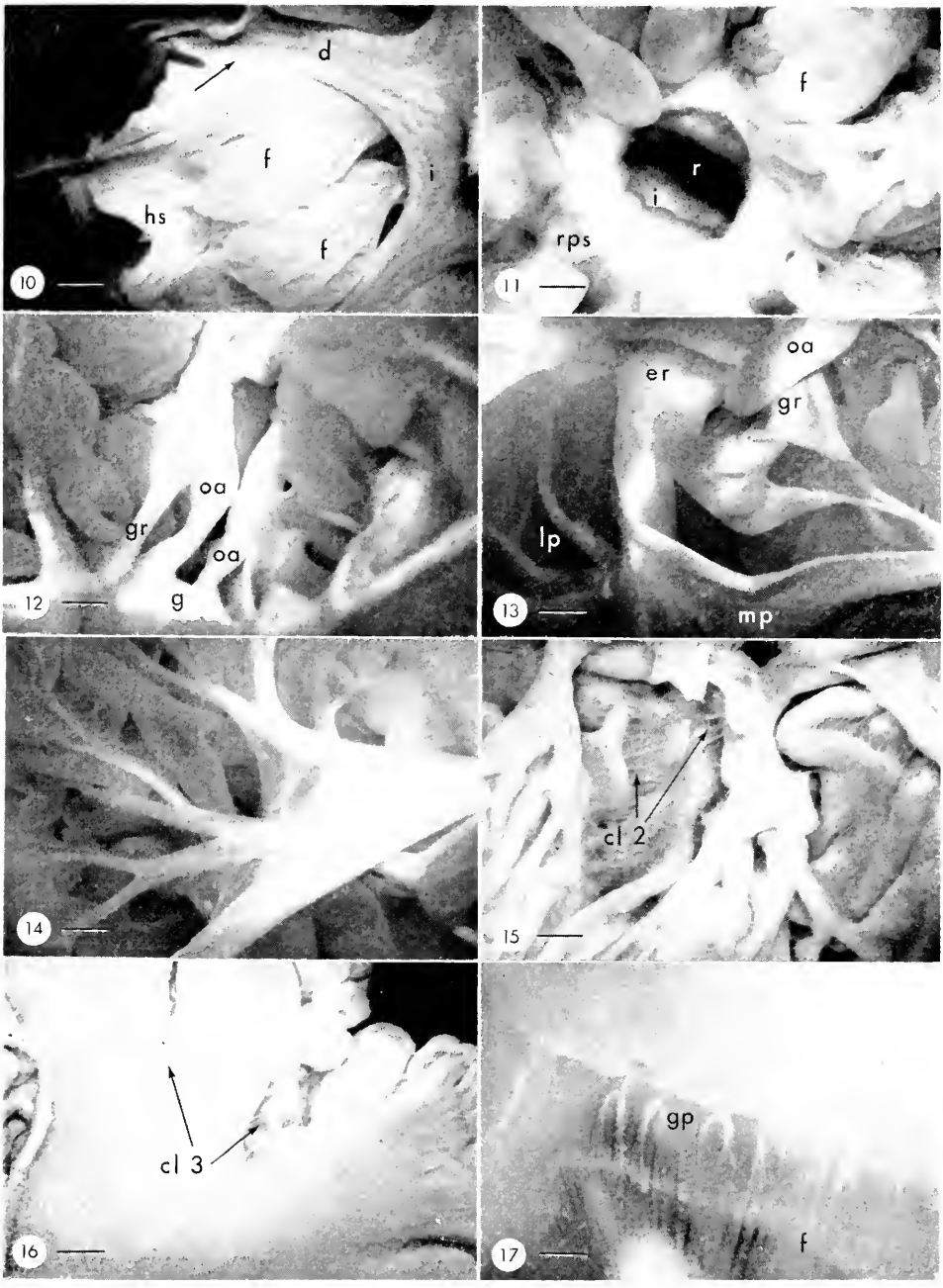

FIGURE 10. Aboral view of a portion of the roof of the pyloric stomach showing its relationship to the intestine and intestinal caeca. Abbreviations are: *i*, intestine; *d*, duct leading to a pair of intestinal caeca, proximal to its point of bifurcation; *f*, fold-pattern; and *hs*, horizontal connective-tissue sheet. The arrow indicates the margin of the duct where the hori-

established, however, without information on their histological characteristics. In *Patiria*, class 2 fibers are muscular, while those designated class 3 appear to consist of thin strands of connective tissue.

Discussion

The distinctive combination of special features presented by the digestive system of *O. reticulatus* suggests that this sea-star is capable of considerable versatility in its feeding habits. The unusually large, extensively eversible cardiac stomach, with its well-developed systems of anchoring and retracting fibers, is structurally and probably functionally similar to that of *Patiria* (Anderson, 1959). It seems primarily adapted for handling large pieces of food outside the body, in the manner characteristic of many carnivorous or omnivorous sea-stars. Observations made in the course of the present study, on specimens maintained in aquaria, confirm that *Oreaster* does envelop food in everted folds of the cardiac stomach. In feeding on a piece of fish, for example, the animal first dilates its mouth; several flattened, somewhat palmate lobes of the stomach (probably the lateral pouches described earlier) protrude in contact with the food and then surround it as they are further

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zontal sheet attaches and binds it down to the roof of the stomach. Above the arrow may be seen three of the many strands by which the duct is suspended from the roof of the disc.

Figure 11. Central portion of the roof of the pyloric stomach, as viewed from the oral side. Note the coarse folds (*f*) that hang down between the slit-like openings from the paired sets of fold-patterns. Other abbreviations are: *rps*, roof of the pyloric stomach; *r* rectum; and *i*, intestine. The anus opens immediately above the short rectum seen here.

Figure 12. Aboral view of a portion of the fibrous girdle (*g*) encircling the upper part of the cardiac stomach, showing two of the ten oral anchors (*oa*) that attach it to the proximal ambulacral ossicles, and a girdle retractor (*gr*) representing part of the extrinsic retractor system (see Fig. 13).

Figure 13. One of the paired sets of extrinsic retractor strands in a ray, showing the distribution of its principal branches. Abbreviations are: *er*, the main extrinsic retractor near its origin alongside the ambulacral ridge; *mp*, the branch that inserts principally on the roof of the median pouch of the cardiac stomach; *lp*, the branch that turns laterally under the preceding one and distributes to a lateral pouch of the cardiac stomach (see Fig. 14); and *gr*, a girdle retractor, the third major branch, whose stoutest portion runs directly to the girdle and attaches there. The remaining large strand (*oa*) is one of the oral anchors of the girdle (*cf.* Fig. 12).

Figure 14. External view of a portion of one lateral pouch of the cardiac stomach, showing the repeatedly bifurcating class 1 intrinsic retractor fibers distributing in the wall.

Figure 15. External view of a portion of a lateral pouch photographed after fixation in Bouin's fluid (to provide enhanced contrast). Some downward-coursing class 1 retractors are shown (*cl 1*), as well as a number of the slender class 2 fibers (*cl 2*) that stretch horizontally between adjacent folds of the stomach wall.

Figure 16. The floor of the cardiac stomach viewed from the coelomic side; specimen photographed after fixation in Bouin's fluid. Sets of slender fibers (*cl 3*) are seen running vertically from the gutter-patterns to attach outside the smooth floor of the stomach. These are provisionally identified as class 3 intrinsic retractor fibers.

Figure 17. Part of an everted vesicle of the cardiac stomach, seen from its mucosal side, showing part of the array of terminal branches of the gutter-patterns (*gp*) bordering the smooth floor of the stomach (*f*). Through the thin wall of the vesicle may be seen some of the slender, vertical, parallel strands of the supposed class 3 intrinsic retractors, attached to the coelomic side of the stomach wall.

For each figure, the scale bar shown represents 1 mm.

inflated with coelomic fluid. The characteristic branching gutter-patterns, with their associated intrinsic retractor strands, can be clearly seen on the vesicles of the stomach. The animal may remain with its stomach everted for several hours, as the food gradually disintegrates and the products of digestion are transported to the inner parts of the system. In the only published account of the feeding habits of *O. reticulatus* under natural conditions, Thomas (1960) reports having observed a specimen with its stomach everted over a small unattached sponge which appeared to have been partially digested. Thomas also cites an unpublished account by another observer who saw *Oreaster* feeding on a sponge. Further unpublished observations by Dr. Jerald Halpern (communicated to me by letter) confirm the fact that *O. reticulatus* consumes large pieces of detritus. Toponce (1973) reports that the related Eastern Pacific species *O. occidentalis* feeds on clumps of stony coral, or on bits of algae. All available evidence thus substantiates the supposition that might have been made on anatomical grounds alone: that *Oreaster* functions as a macrophagous carnivore or scavenger.

It is evidently capable of other modes of feeding as well. Thomas (1960) writes: "I have observed *Oreaster* many times with its stomach everted into small depressions in the coralline sand and *Thalassia* bottoms on which it lives. Examination reveals nothing either in the depression or in the stomach which might be of food value. Possibly any organic material close to the stomach wall is digested in this manner." Halpern (personal communication) also mentions having observed this type of feeding behavior in *Oreaster*. The phenomenon is interesting in view of the analogous behavior exhibited by *Patiria miniata*, as described by Anderson (1959). This species is very frequently seen in tide pools with its voluminous cardiac stomach fully everted, although no visible objects of food are enfolded by it. In an aquarium, the animal applies its everted stomach to the wall, as though digesting the film of microorganisms adhering to the glass. Anderson (1959) suggested that *Patiria* might be using its everted stomach as a flagellary-mucous feeding organ, to collect suspended particulate matter. Araki (1964) reports that under experimental conditions specimens of *Patiria* with everted stomachs are capable of rapidly removing organic compounds from solution in the surrounding water, and his suggestion is that the stomach is involved in this function. Although there appears to be some question as to the significance of dissolved organic matter in the overall nutrition of marine animals (Jørgensen, 1976), the thin-walled stomach seems ideally suited to mediate whatever exchange of materials may take place between sea water and the enclosed coelomic fluid. All things considered, one may justifiably conclude that whatever *Patiria* is doing with its stomach everted in the absence of visible, macroscopic food, *Oreaster* is probably doing something similar.

Further evidence of versatility in feeding is provided by the presence of highly specialized features in the digestive system above the level of the cardiac stomach. These include the very elaborate Tiedemann's pouches, the highly folded structures in the roof of the pyloric stomach, and the unusually large intestinal caeca. Such features as these are never found in strictly carnivorous sea-stars such as *Asterias* and its relatives; there, the pyloric stomach is small and simple, Tiedemann's pouches are lacking, and the intestinal or rectal caeca are strongly reduced. The

more highly specialized structures are characteristic of forms known to be, or suspected of being, microphagous particle-feeders. In all sea-stars, even carnivores, there is a consistent pattern of flagellary circulation through the digestive system. Tiedemann's pouches are interpreted as flagellary pumping organs, functioning to enhance the volume and velocity of water-flow through the gut in connection with the exploitation of suspended particulate matter as food (Anderson, 1960).

The remarkable anatomical similarity between the Tiedemann's pouches of *O. reticulatus* and those of *Porania* is significant in this regard. As long ago as 1915, Gemmill provided experimental evidence that *Porania* can be maintained without weight-loss for long periods (several months) with no food other than suspended particles. In describing Tiedemann's pouches in *Porania* many years later, Anderson (1961) called attention to their unusually complex structure, involving the development of many subsidiary pouches branching from the main one. The conclusion is unavoidable that this elaboration is related to the demonstrated ability of *Porania* to subsist on particulate food alone. It is of interest that Jangoux (1972) has described similar secondary Tiedemann's pouches in *Archaster angulatus;* they are present also in *Dermasterias imbricata* (Anderson, unpublished observations). It is perhaps not unreasonable to suggest that sea-stars with subsidiary or accessory Tiedemann's pouches are at least facultative particle-feeders.

Species in which Tiedemann's pouches are well developed characteristically possess much larger intestinal caeca than those lacking such structures. *Henricia* and *Patiria* both demonstrate this correlation to a considerable degree (Anderson, 1966), as do their relatives *Echinaster* and *Asterina* (Jangoux and van Impe, 1971). If the supposition is justified that Tiedemann's pouches are significantly related to a capacity for particle-feeding (Anderson, 1960), it is tempting to go one step further and suggest that well-developed intestinal caeca are also involved somehow in this function. Here again, *Porania pulvillus* provides a key example. The intestinal caeca of this species are very large indeed, and Gemmill (1915, p. 12) describes their rhythmic contraction and expansion, "sometimes with such activity as to suggest the systole of the auricular portion of a heart." According to Gemmill, *Porania* periodically inflates its gut with water, which is drawn in through the mouth and later expelled forcefully from the anus. Gemmill believed that the large and muscular intestinal caeca are responsible for the expulsion. Since the caeca lack any intrinsic mechanism for expansion, it seems likely that the pressure required to inflate them with water is provided by the large Tiedemann's pouches, whose centripetal currents converge on the roof of the pyloric stomach and enter the intestine.

The remarkably large, well-developed intestinal caeca of *Oreaster* and of its relative *Culcita* have been referred to earlier, and it will be recalled that Tennent and Keiller (1911) described the intestinal caeca as capable of great distention. They say further (p. 114): "Upon opening some specimens the caeca were found to be greatly distended. Upon stimulation they slowly contracted, the entire organ shrinking to about one-third of its former size. The contents were watery . . ." Although we lack for *Oreaster* any comprehensive series of observations on water-flow through the gut, and on filter-feeding, such as those provided by Gemmill (1915) for *Porania*, the structural similarities between the two forms strongly sug-

gest comparable functions; and direct evidence is not altogether lacking. Halpern, on the basis of unpublished observations, is convinced that *O. reticulatus,* in addition to its other modes of nourishing itself, is indeed a filter-feeder. His letter, previously referred to, states: "In the area I observed it in, it filter-feeds when there is a strong tidal current. There are many loggerhead sponges (*Spheciospongia vesparia*), and *Oreaster* often uses these as a purchase so as to be able to outstretch one, two, or even three arms. As the current slackens, they abandon this method of feeding. Both the fact of filter-feeding and the current acting as a stimulus have been confirmed (but not conclusively) by some preliminary laboratory experiments."

One further specialization in relation to water-movement in the gut, and possible particle-feeding, is represented by the folded structures in the roof of the pyloric stomach. Abundant wrinkles in this general area are known in a number of sea-stars; Tiedemann (1816) showed something of the kind, with associated vessels, in *Astropecten aurantiacus,* and according to Jangoux, Perpeet, and Cornet (1972) such structures are particularly well-developed in *Asterias rubens.* I know of no other species, however, in which strongly flagellated, radially folded patterns have been described, lying in such an obviously functional, oriented relationship between the radial reservoirs and the opening from the pyloric stomach into the intestine, as they do in *Oreaster reticulatus.* (I am informed by Dr. Michel Jangoux, however, that in an extensive series of unpublished observations on members of the Family Oreasteridae he has found similar structures in *Pentaceraster, Protoreaster,* and *Culcita*). In a dissected specimen of *Oreaster,* very rapid currents can be demonstrated, using dilute India ink, running through the fold-patterns from the central ducts of the pyloric caeca toward the intestine. In my interpretation, the fold-patterns in the roof of the pyloric stomach, the unusually large intestinal caeca, and the elaborate Tiedemann's pouches form a coordinated set of adaptations which enable *Oreaster* to utilize suspended particulate matter as a source of food. Interestingly, all of these features are present in other members of the Family Oreasteridae (Jangoux, Université Libre de Bruxelles, personal communication).

It is clear, however, from all the evidence, morphological as well as behavioral, that *Oreaster* is not exclusively, or perhaps even primarily, a particle-feeder, as *Henricia* may be (Anderson, 1960; Rasmussen, 1965). It should be borne in mind that even that celebrated, demonstrated particle-feeding species, *Porania pulvillus,* internally so similar to *Oreaster,* does not depend altogether on a particulate diet. Gemmill's statement (1915, p. 14) that "At the Millport Marine Station the *Porania* are never seen feeding on shell-fish, etc., or on their neighbors as other species readily do" seems to imply some such conclusion; but he goes on to say only that "ciliary feeding plays a part in the nutritional economy of *Porania.*" Recent studies by Ericsson and Hanssen (1973) have shown that *Porania pulvillus,* in fact, feeds on octocorals, brachiopods, and ascidians, both in its natural habitat and in aquaria.

It is to be hoped that similar studies may soon be made on the feeding biology of *Oreaster reticulatus,* to supplement incidental observations, and to determine the validity of conclusions that can now only be inferred from consideration of the comparative anatomy of the digestive system.

The dissections and observations on which this report is based, together with preliminary histological procedures, were carried out at the Mote Marine Laboratory, Siesta Key, Sarasota, Florida. It is a pleasure to express to the Director of the Laboratory, Dr. Perry W. Gilbert, my appreciation for his gracious hospitality, for excellent facilities generously provided, and for the valuable assistance of members of the staff, particularly Pat Bird and Susi Dudley. I am grateful also to Dr. Jerald Halpern and Dr. Michel Jangoux for providing details of their unpublished observations on *Oreaster* and its relatives.

Summary

This paper presents, with illustrations, a description of the digestive system of *Oreaster reticulatus*, a species for which such anatomical details have hitherto been unavailable. Special features of the digestive system include a large, highly eversible cardiac stomach with a particularly well-developed system of securing and retracting fibers; a highly specialized pyloric stomach giving rise to paired pyloric caeca, each of which is associated with an unusually elaborate Tiedemann's pouch featuring a series of secondary pouches branching off along its length; and a set of very voluminous intestinal caeca. By comparison with other asteroids for which anatomical details and feeding biology are known (especially *Patiria miniata* and *Porania pulvillus*), it is suggested that *O. reticulatus* is equipped for a variety of modes of feeding. The cardiac stomach is well adapted for the digestion of large pieces of food outside the body; it may also function as a flagellary-mucous particle-collector, as the similar organ of *Patiria* is thought to do. The specializations of the upper part of the digestive system are closely similar to corresponding organs in the known particle-feeding species *Porania pulvillus*, and it seems probable that *Oreaster* may use its Tiedemann's pouches and intestinal caeca to bring particle-laden water into the digestive system in a manner similar to that described for *Porania*. Such direct observations as are available on the feeding behavior of *O. reticulatus* tend to confirm the conclusions inferred on indirect, anatomical grounds.

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INTERACTION OF IONIZED AND UN-IONIZED AMMONIA ON SHORT-TERM SURVIVAL AND GROWTH OF PRAWN LARVAE, *MACROBRACHIUM ROSENBERGII*

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Ammonia is the principal excretory product of Crustacea (Hartenstein, 1970; Hochachka and Somero, 1973; Kinne, 1976), and its modes of toxicity as well as concentrations lethal to a variety of organisms have been well documented (Warren, 1962; Campbell, 1973). Ammonia exists in solution primarily as the NH_4^+ ion and the un-ionized NH_3 molecule, the proportions of which are highly pH-dependent. In this paper *ammonia* will refer to the sum of NH_4^+ and NH_3. *Un-ionized ammonia* will refer to the NH_3 molecule and *ionized ammonia* to the NH_4^+ form.

In the aquatic habitat, organisms rely on rapid diffusion of NH_3 across the gill membranes (Fromm and Gillette, 1968) or exchange transport of NH_4^+ with Na^+ (Maetz and Garcia-Romeu, 1964; Campbell, 1973; Mangum and Towle, 1977) to void themselves of this toxicant. Diffusion of NH_3 is a principal route of excretion because blood levels are normally much greater than ambient concentrations (see Kinne, 1976, for review). Fromm and Gillette (1968) reported that ammonia levels in the blood of trout are 9–40 times greater than in ambient water. Concentrations of ammonia in the blood of Crustacea range from 2 to 18 mg/liter (Myers, 1920; Florkin and Renwart, 1939; Mangum, Silverthorn, Harris, Towle and Krall, 1976), which are one to several orders of magnitude greater than concentrations in their habitat (Kinne, 1976). As external NH_3 concentrations increase, the rate of diffusion outward from an animal decreases and toxicity ensues when tolerable body loads are exceeded. Consequently, the toxicity of ammonia to aquatic organisms is generally credited to the NH_3 molecule (Ellis, 1937; Wuhrmann and Workers, 1948; Downing and Merkens, 1955; Spotte, 1970; Hampson, 1976), despite evidence that NH_4^+ adversely affects some physiological functions (Shaw, 1960; Maetz, 1972; Campbell, 1973).

The chemistry of ammonia in solution has been discussed by Whitfield (1974) and Colt and Tchobanoglous (1976). The proportion of total ammonia existing as NH_3 is dependent on temperature and ionic strength of the medium, but primarily on the pH of the solution (Warren, 1962; Trussell, 1972; Skarheim, 1973; Whitfield, 1974; Emerson, Russo, Lund and Thurston, 1975). Calculations by these authors show that the NH_3 fraction of ammonia increases as pH rises; an increase of one pH unit elevates the NH_3 concentration tenfold. As previously stated hypotheses have suggested, the toxicity of an ammonia solution should increase at higher pH values.

There has been little work done on the sensitivity of crustaceans to ammonia poisoning. During the course of this study only two reports were found which give

systematic evaluations of ammonia toxicity (based on mortality) to Crustacea (Anderson, 1944; Wickins, 1976), and a single study investigating the sensitivity of larval crustaceans to ammonia (Delistraty, Carlberg, Van Olst and Ford, 1977). Adverse effects of ambient ammonia on some physiological functions have also been reported by Shaw (1960), who found a significant reduction in sodium influx in the crayfish *Astacus pallipes*, and by Mangum *et al.* (1976), who reported reduced ammonia excretion rates in the blue crab, *Callinectes sapidus*. The exposure levels of ammonia in these experiments were high, 18 and 180 mg NH_4^+/liter, respectively, and may have been approaching lethal concentrations. However, the authors' interests were in impairment of physiological functions, and gross signs of stress or mortalities at these concentrations were not discussed.

It seems, therefore, that most researchers investigating the effects of ammonia on organisms tend to concentrate on one molecular form or another in designing and analyzing their work. On the one hand, those interested in concentrations lethal to fish and crustaceans underline the importance of the NH_3 species because of its ease in diffusing across membranes. Consequently, toxic levels of up to a few mg NH_3/liter may represent well over 100 mg NH_4^+/liter, especially at pH < 8.0. Such high ammonium ion concentrations may well contribute to observed mortality and should not be ignored.

On the other hand, physiologists concerned with the interactions between $Na^+ - NH_4^+$ in salt transport processes fail to address, first, the possibility that the NH_3 portion of the high total ammonia concentrations used (Carrier and Evans, 1976; Mangum *et al.*, 1976; Towle, Palmer and Harris, 1976) may constitute a severe stress to an organism or cellular system, thereby affecting a process that is thought to be NH_4^+-mediated only; and secondly, the effect that NH_4^+ inhibition of Na^+ transport or ammonia excretion may have on survival of organisms in different habitats.

The following study was performed to determine: first, concentrations of ammonia lethal to larval *Macrobrachium rosenbergii* in short-term exposures; secondly, roles and interaction of NH_3 and NH_4^+ in affecting toxicity using pH as a variable, and to analyze any observed interaction in light of possible physiological mechanisms; and thirdly, sublethal effects during short-term exposure using growth-reduction as the criterion of toxicity. An additional motive underlying this study was to gain information on ammonia toxicity that could be applied to general water quality requirements for crustaceans. This is particularly important since ambient ammonia concentrations in culture or holding water may often exceed levels recommended as safe (Spotte, 1970) despite extensive filtration.

Materials and Methods

Animals

Larvae were produced by second generation U. C. Davis brood stock initially obtained from Hawaii and Thailand. Broods were hatched and mass-reared in 80 liter glass aquaria with water circulated through biological filters. Water temperature and salinity were 27–28° C and 12‰ (Instant Ocean salts). Larvae were fed newly hatched *Artemia salina* nauplii and were used in tests from three to eight days after hatching.

Lethal toxicity bioassays

Static bioassays to assess ammonia toxicity were performed as described by Armstrong, Stephenson and Knight (1976a), for nitrite toxicity experiments wtih *Macrobrachium*. Fifteen larvae were placed in each 250 beaker containing 200 ml of test solution; the ratio of dry weight animal biomass (shrimp + *Artemia*) to volume of solution ranged from 3 to 17.5 mg/liter. Ammonia concentrations were made by serial dilution of reagent grade NH_4Cl (Mallinckrodt) for a concentration range of 1.0–320 mg ammonia/liter, spaced in threefold increments per decade. All amonia concentrations and controls at each pH were replicated, and the experiment was run twice with two broods of larvae.

Test solutions were renewed every 24 hr at which time larvae were transferred to new beakers, mortalities recorded, and fresh brine shrimp added to give a density of about 4–6 nauplii/ml. After the initiation of an experiment, mortalities were checked at 30 min, 1, 2, 4, 8, 16, and 24 hr, and three times in each subsequent 24 hr interval to the conclusion of 144 hr. In the first 24 hr death was defined as the cessation of heart beat and pulsing of the posterior intestine. Thereafter, the opaqueness commonly developed by moribund and dead larvae was used as the criterion of death (Armstrong *et al.*, 1976a; Armstrong, Buchanan, Mallon, Caldwell, and Millemann, 1976b).

Test water was maintained at 28° C (all beakers held in a single water bath) and 12‰ salinity. The photoperiod was 9D: 15L. Three pH values tested were 6.8, 7.6, and 8.4. Stock water of 12‰ was held in 20 liter carboys, aerted and adjusted frequently to desired pH with 1 M NaOH or HCl until levels stabilized which required several days prior to a test. The pH of test solutions was checked three times/24 hr period and adjusted with 0.1 M NaOH or HCl. Solutions to which high ammonia concentrations were added (> 100 mg ammonia/liter) required pH adjustment immediately. A Corning model 12 pH meter with Ag/AgCl and calomel electrodes, standardized with NBS type buffers, was used for measurements. Some investigations suggest inaccuracies in measuring pH of high ionic strength solutions on meters calibrated with low ionic strength buffers. Hansson (1973) stated such error could be 0.09 pH units at 20‰, and Whitfield (cited in Wickins, 1976) reported a 0.05 unit error at 35‰. Since our salinity was 12‰, we do not consider the possible error due to calibration with NBS type buffers to be significant. The mean pH values (calculated by converting pH to hydrogen ion concentration, averaging and then returning the means and standard deviation to pH units) were 6.83 ± 0.09, 7.60 ± 0.09, 8.34 ± 0.06 ($n > 100$ for each pH). These values were used to calculate un-ionized ammonia concentrations.

Beakers were not aerated; yet dissolved oxygen, measured with a Beckman O_2 Analyzer, exceeded 90% of saturation (7.3 mg/liter at temperature and salinity used) after 24 hr in all pH and ammonia concentrations. High dissolved oxygen values were due to the change of solutions every 24 hr, low biomass to volume ratio, and our procedure of stirring the solution of beakers several times a day to check for deaths.

Nitrite was measured in representative concentrations from all three pH values by a sulfanilamide-based colorimetric reaction (Federal Water Pollution Control Administration, 1969). At the end of 24 hr, the average nitrite concentration was

9.4 ± 3.3 μg NO_2-N/liter, which is several orders of magnitude lower than the incipient lethal level of 3 mg NO_2-N/liter reported by Armstrong *et al.* (1976a) for *Macrobrachium*.

Ammonia was measured with an Orion Ammonia Electrode Model 95–10 coupled with the Corning pH meter. Merks (1975) states that this probe loses accuracy with increasing salinity, and correction factors must be used. However, we made ammonia standards with fresh water and 12‰ sea water and found no difference in millivolt readings for the same ammonia concentrations in the two media. The average ammonia concentation at 24 hr in control beakers of all pH values was 0.45 ± 0.11 mg ammonia/liter. By the end of a 24 hr period the change in ammonia levels in test beakers was minimal. Average measured concentrations were 102% of time-zero nominal levels, indicating little volatilization or nitrification of the chemical during tests.

Growth experiments

Larvae of this warm water species grow rapidly, molting and gaining substantial weight in five to seven days (George, 1969; Armstrong *et al.*, 1976a). Therefore, documentation of sublethal effects by studying growth seemed feasible during short-term exposures. After establishing lethal concentrations of ammonia at each pH, identical bioassays were performed using two sublethal concentrations per pH. A time-zero sample of 25 larvae was dried at 70° C for 24 hr. Animals were then individually weighed on a Cahn Model 4700 automatic electrobalance, accurate to a few μg. Test animals were exposed as previously outlined and at the conclusion of a test were dried and weighed individually. A relative growth rate was calculated for each treatment with the formula of Waldbauer (1968): $G_R = P/TM$, where P is mean dry weight gain between sampling period, T is time between sampling period and M is mean individual weight over the sampling period. Two sublethal growth experiments were done: the first with five-day old larvae exposed to treatments for five days; and the second with three-day old animals exposed for seven days.

Statistical analyses

The effect of ammonia concentration on survival was investigated using a three-way analysis of variance. The effects of brood, pH, concentration and their interactions on the dependent variable, time to death for each larva, were analyzed. Effects of sublethal concentrations on growth were investigated with one-way ANOVA, by treating each pH-concentration combination as a separate factor. If a significant F value ($P < 0.01$) was obtained, treatment differences were contrasted by means of a Q value (Snedecor and Cochran, 1967; these authors regard this contrasting procedure as a conservative gauge of true differences). LC_{50} values (the concentration of toxicant lethal to 50% of the test organisms in a specified time period) were derived from log-probit plots of concentration *vs.* mortality. LT_{50} values (the time required for death of 50% of the organisms in a given concentration of toxicant) were obtained by probit analysis program BMD/O3S (Dixon, 1970).

Calculation of un-ionized ammonia: NH_3

The NH_3 fraction of the total ammonia measured is calculated from the general formula for bases (Albert, 1973):

$$[NH_3] = \frac{[\text{Ammonia}]}{1 + 10^{(pKa - pH)}} \quad (1)$$

The measurement, and possible inaccuracies, of ammonia and pH have been discussed. The pKa remains as the major variable of the equation and is influenced by physical conditions of the solution. Emerson *et al.* (1975) found the temperature dependence of the pKa value to be:

$$pKa = 0.09018 + 2729.92/T \quad (2)$$

$$T = \text{degrees Kelvin}$$

Equation (1) is based on an infinite dilution model for which the activity of an ion approaches its analytical concentration as the solute concentration approaches zero. For freshwater systems such a model is accurate. However, as the solute concentration (*i.e.*, salinity) of a solution increases, the activity of ions and uncharged species may be significantly different from their concentration. In turn, such changes will affect pKa values and, in the case of equation (1), will consequently change the concentration of un-ionized ammonia calculated.

The pKa values of the ammonia system in sea water have not yet been experimentally determined. Whitfield (1974) developed theoretical pKa values for sea water, but did not calculate them for salinities less than about 20‰. The salinity of our tests was 12‰ (ionic strength, $I = 0.242$) for which an appropriate pKa value was derived.

The acid dissociation reaction for ammonia in water is:

$$NH_4^+ + nH_2O \rightleftharpoons NH_3 \cdot nH_2O + H^+ \quad (3)$$

The equilibrium expression for this reaction is:

$$Ka = \frac{\{H^+\}\{NH_3 \cdot nH_2O\}}{\{NH_4^+\}\{H_2O\}^n} \quad (4)$$

where Ka = acidity equilibrium constant

$\{i\}$ = activity of the ith species

Rewriting equation (4) partially in terms of concentration

$$Ka = \frac{[NH_3]\gamma_{NH_3}\{H^+\}}{[NH_4^+]\gamma_{NH_4^+} \cdot \{H_2O\}^n} \quad (5)$$

where $\{i\} = \gamma_i[i]$

$[i]$ = concentration of the ith species.

Since the electrode method for determining pH measures the activity of the hydrogen ion rather than concentration, it is convenient to retain the $\{H^+\}$ term. Re-

writing equation (5)

$$\frac{Ka \cdot \{H_2O\}^n \gamma_{NH_4^+}}{\gamma_{NH_3}} = \frac{[NH_3]\{H^+\}}{[NH_4^+]} \quad (6)$$

The right hand expression is called the "mixed acidity equilibrium constant" (Stumm and Morgan, 1970).

Let

$$K'a = \frac{Ka \cdot \{H_2O^n \cdot \gamma_{NH}\}_4^+}{\gamma_{NH_3}} \quad (7)$$

Taking the $\log_{10}$ of both sides and making the substitution that $pK = -\log K$, the following equation results:

$$pK'a = pKa - \log \gamma_{NH_4^+} + \log \gamma_{NH_3} - n \log \{H_2O\} \quad (8)$$

The values used for the right hand terms are as follows: $pKa = 9.154$ (Emerson *et al.*, 1975); $-\log \gamma_{NH_4^+} = 0.140$ (Stumm and Morgan, 1970; Whitfield, 1974); $\log \gamma_{NH_3} = 0.008$ (Whitfield, 1974); $-3 \log \{H_2O\} = 0.008$ (Robinson, 1954). The pK'a calculated for 28° C and 12‰ was 9.310 and was used in equation (1).

Effect of NH_3 and NH_4^+

To test the hypothesis that NH_3 is solely responsible for ammonia toxicity, the concentration of total ammonia was varied with pH to achieve equal levels of NH_3 but unequal levels of NH_4^+. As an example, using equation (1) and pK'a = 9.31, it is calculated that 10.3 mg ammonia/liter (9.3 mg NH_4^+/liter) will give 1.0 mg NH_3/liter at pH 8.34. But at pH 6.83, 303 mg ammonia/liter (302 mg NH_4^+/liter) is required for the same concentration of NH_3. Survival was monitored to learn if these widely divergent NH_4^+ concentrations affected larvae.

Results

Analysis of variance of mortality data showed no significant effect due to brood or any interaction involving brood and therefore, all data were combined for computation of LC_{50} and LT_{50} values. There was a highly significant effect ($P < 0.01$) due to both pH and ammonia concentration and the interaction of these variables. However, during the four bioassays performed (lethal and sublethal), survival of control larvae at each pH always exceeded 85% and averaged 95% for 144 to 168 hr exposures.

The toxicity of ammonia over a range of identical concentrations was greatly influenced by the pH of the media. The 24 hr LC_{50} values were 200, 115 and 37 mg ammonia/liter at pH 6.83, 7.60 and 8.34, respectively (Fig. 1), and the sensitivity to ammonia remained greatest at higher pH values throughout the tests. By 144 hr the LC_{50} values at the same pH values had decreased to 80, 44, and 14 mg/liter; approximately a 2.7 fold decrease from 24 hr values (Fig. 1). At the test's conclusion, slopes of toxicity curves for ammonia in solution at pH 8.34 and 7.60 were approaching asymptotes indicative of incipient LC_{50} values (Sprague,

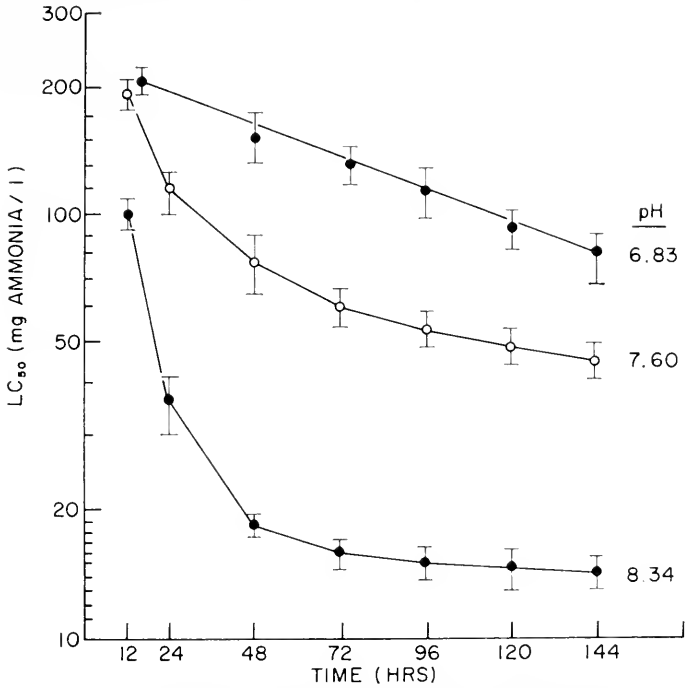

FIGURE 1. The toxicity of total ammonia to larval *M. rosenbergii* exposed in solutions of different pH. Bars are ± one standard deviation.

1969). However, no such decrease in the slope of the pH 6.83 toxicity curve had occurred, indicating longer tests were needed to estimate incipient concentrations.

The time to death for larvae held in solutions of equal ammonia concentration but different pH is shown in Figure 2. In 100 mg ammonia/liter, 50% mortality of larvae in solutions of pH 8.34, 7.60, and 6.83 occurred in about 9, 27, and 125 hr, respectively. Survival of larvae held in 32 mg/liter at pH 7.60 and 6.83 was nearly equal to that of controls. However, animals exposed to the same ammonia concentration at pH 8.34 were all dead by 48 hr (Fig. 2).

Un-ionized ammonia was not the exclusive toxic agent in these tests, and the NH_4^+ molecule apparently contributed to mortality also. When LC_{50} values were based on the concentration of NH_3 only (*i.e.,* normalized with respect to pH), there was no equality of the levels found to be toxic at specific time intervals (Fig. 3). In fact, larvae exposed to the lowest levels of NH_3 (pH = 6.83) were the most susceptible to toxicity due to the concomitantly high levels of NH_4^+ present (Table I). The proportions of NH_3 and NH_4^+ found to be toxic were inversely related as pH changed. Consequently, about five times less NH_3 was lethal in a given period at low pH compared to the high, but it was accompanied by six times more NH_4^+ ion (Table I).

The effect of NH_4^+ on survival times of larvae is further demonstrated when the response of groups in equal NH_3 concentrations at different pH values was compared. The LT_{50} values for larvae exposed to 0.98 mg NH_3/liter at pH 6.83

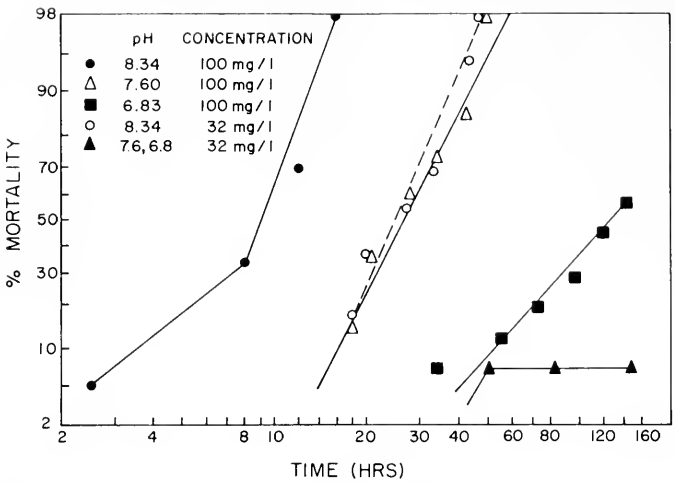

FIGURE 2. Cumulative percentage of mortality of *M. rosenbergii* larvae exposed to several combinations of ammonia and pH. Depicted are data for a single brood. Survival was adjusted to that of controls which averaged 95% at the end of an experiment.

and 8.34 were 9 hr and >144 hr, respectively, while the corresponding NH_4^+ concentrations were 319 and 9 mg/liter (Fig. 4). Animals exposed to 10.2 mg NH_3/liter survived twice as long as those exposed to 5.5 mg/liter, but the NH_4^+ concentration was 3.5 times higher in the latter case (Fig. 4).

Growth of *Macrobrachium* larvae was reduced in sublethal concentrations of ammonia and also seemed to be influenced by levels of NH_4^+ rather than NH_3. There was no significant effect of treatments in the first growth experiment. The initial mean weight was 52 ± 5 μg/larva and the final mean weight for all treatments was 77 ± 6 μg/larva, a 48% increase. In the second test, with smaller larvae exposed for a longer period, there was reduced gowth ($P < 0.01$) in solutions of 32 mg ammonia/liter at pH 6.83 and 7.60 (Table II). The initial weight of three-day old animals was 35 ± 6 μg each. At the test's conclusion, larvae of control groups weighed about 77 μg each (a 120% increase), while those in 32 mg/liter averaged 55 and 61 μg/larva (57% and 74% increases) in pH 6.83 and 7.60, respectively. These weights were significantly less than those of controls ($P < 0.05$,

TABLE I

Concentrations of ammonia toxic to M. rosenbergii *larvae expressed as both the NH_3 and NH_4^+ molecules.**

pH	24 hr LC_{50} (mg/liter)		144 hr LC_{50} (mg/liter)	
	NH_3	NH_4^+	NH_3	NH_4^+
6.83	0.66	199.34	0.26	79.74
7.60	2.10	112.90	0.80	43.20
8.34	3.58	33.42	1.35	12.65

* Total ammonia = $[NH_3] + [NH_4^+]$ and is depicted in Figure 1.

Q statistic) and were the only important differences found. Reduction in growth was not correlated with NH_3 concentrations. The relative growth rate (G_R) of controls was 0.108 g/(g dry body wt·day) (Table II). Larvae exposed to the highest NH_3 concentration of 0.98 mg NH_3/liter ($NH_4^+ = 9$ mg/liter) had a $G_R = 0.097$, while those exposed to 0.11 mg NH_3/liter ($NH_4^+ = 31.9$ mg/liter) had a $G_R = 0.063$ (Table II).

Discussion

The toxicity of ammonia to *Macrobrachium* larvae is inextricably linked to the pH of a solution, the total ammonia concentration present, and the proportions of that total which exist as either NH_3 or NH_4^+. Undoubtedly other factors, such as dissolved oxygen and salinity, could be varied from optimal levels to further complicate the story of ammonia toxicity to this crustacean.

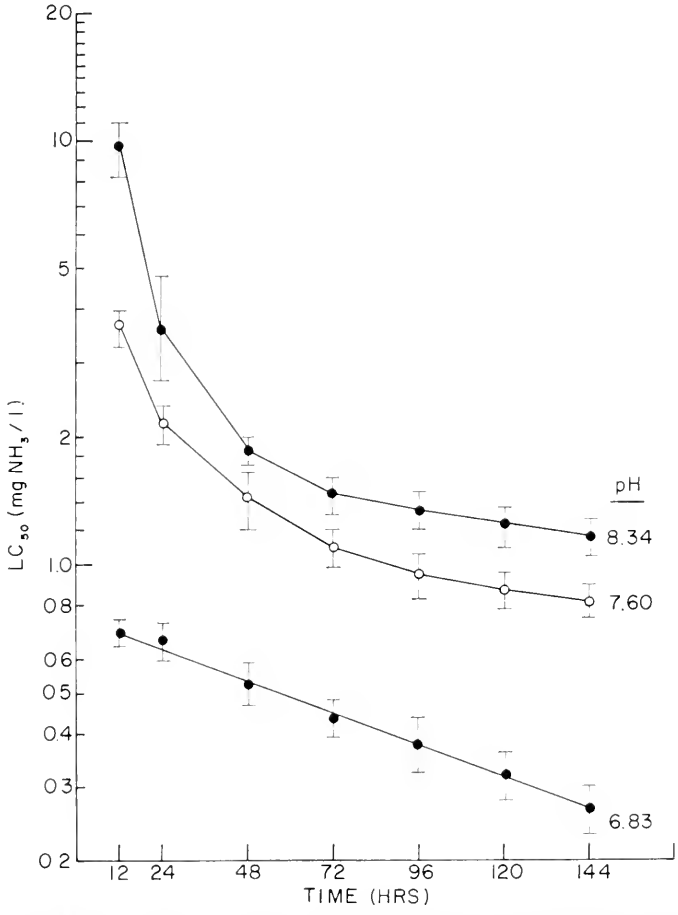

Figure 3. Concentrations of un-ionized ammonia (NH_3) causing 50% mortality in various time intervals. The NH_3 concentrations account for about 10%, 2%, and 0.3% of the total ammonia levels in the high to low pH values, respectively.

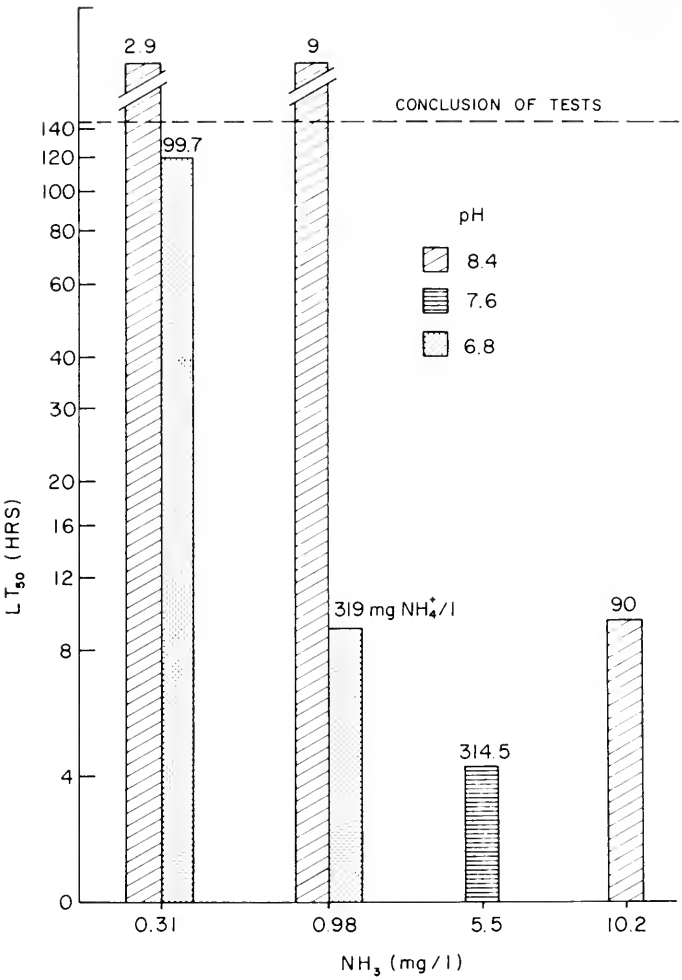

FIGURE 4. Time to 50% mortality of larvae exposed to several concentrations of NH_3 ammonia at different pH. At the top of bars are concentrations of NH_4^+. Bars exceeding 144 hr had survival equal to controls by the end of the experiment.

As is traditionally done in fish bioassays with ammonia, toxic concentrations derived from the present tests could not be normalized to pH variations by expressing results in terms of the NH_3 molecule only, because the NH_4^+ ion figured critically in causing stress. Toxic ammonia concentrations differ between the high and low end of the pH range tested, and are, we believe, determined by NH_3 at high pH and NH_4^+ at low pH values. At a pH of 8.34 the incipient LC_{50} value was estimated to be 14 mg ammonia/liter, which are NH_3 and NH_4^+ proportions of 1.35 and 12.65 mg/liter, respectively (Table I). Growth at this same pH was not inhibited by 10 mg ammonia/liter, indicating that an incipient lethal level is indeed about 12–14 mg/liter. In solutions of lower pH, more total ammonia is required

to cause toxicity, and the NH_3 fraction of these concentrations decreases exponentially with pH. Using growth as a sensitive gauge of stress, 32 mg ammonia/liter retarded development at both pH 6.83 and 7.60. The un-ionized NH_3 fraction at pH 6.83 is 0.11 mg NH_3/liter, only 0.3% of the total concentration and about 11 times less than the incipient LC_{50} value derived for pH 8.34. NH_4^+ ion accounts for nearly all ammonia present and is the species of ammonia probably responsible for toxicity at low pH values.

These observations may be combined in a model (Fig. 5) to describe differential ammonia toxicity caused by changes in pH. Water conditions shown in the model are those actually measured in these tests. Values for chemical factors in larval blood have been assumed based on literature data for adult and juvenile crustaceans. Blood osmolarity was estimated to be 500 mOsmol = 15.8‰ salinity based on determinations made with *M. rosenbergii* post-larvae (Armstrong and Nelson, unpublished data; Sandifer, Hopkins and Smith, 1975). Blood pH was chosen to be 7.55, 7.65, and 7.75 at corresponding water pH values of 6.83, 7.60, and 8.34 (from data of Johansen, Lenfant and Mecklenburg, 1970; Truchot, 1975; Weiland and Mangum, 1975; Mangum *et al.*, 1976). Total blood ammonia was taken to be representative of levels in control larvae, treated as described, before addition of toxic concentrations of ammonia to the ambient water. A concentration of 12 mg ammonia/liter of blood was assumed from data of Myers (1920), Florkin and Renwart (1939), Florkin and Frappez (1940), Gifford (1968), and Mangum *et al.* (1976). Sodium influx is depicted as relative magnitudes varying with ambient NH_4^+ concentrations. The pKa, 9.33, used to calculate un-ionized ammonia in the blood, was detennined for a salinity of 16‰, as previously outlined.

The model (Fig. 5) proposes that larvae exposed to ammonia at higher pH ($\approx$ 8.4) will be most affected by NH_3, which is nonpolar and can readily diffuse through biological membranes such as the gills (Warren, 1962). Of the total am-

TABLE II

Effect of ammonia on the relative growth rate of Macrobrachium *larvae held in water of different pH.*

pH	Total ammonia $NH_3 + NH_4^+$ (mg/liter)	Un-ionized ammonia NH_3 (mg/liter)	G_R* g/(g body wt·day)	Final mean** dry weight (±s.d.) μg/larva
6.83	0	0	0.107	77 (15)
	10	0.03	0.105	76 (11)
	32	0.11	0.063***	55 (11)
7.60	0	0	0.107	77 (16)
	10	0.20	0.113	81 (15)
	32	0.63	0.077***	61 (13)
8.34	0	0	0.109	78 (11)
	3.2	0.31	0.091	68 (12)
	10	0.98	0.097	71 (14)

* G_R = P/TM for dry wt. See text for explanation.

** Seven day exposure; initial mean dry weight = 35 ± 6 μg/larva; n = 19–23 larvae per group.

*** Significantly different from controls, $P < 0.05$.

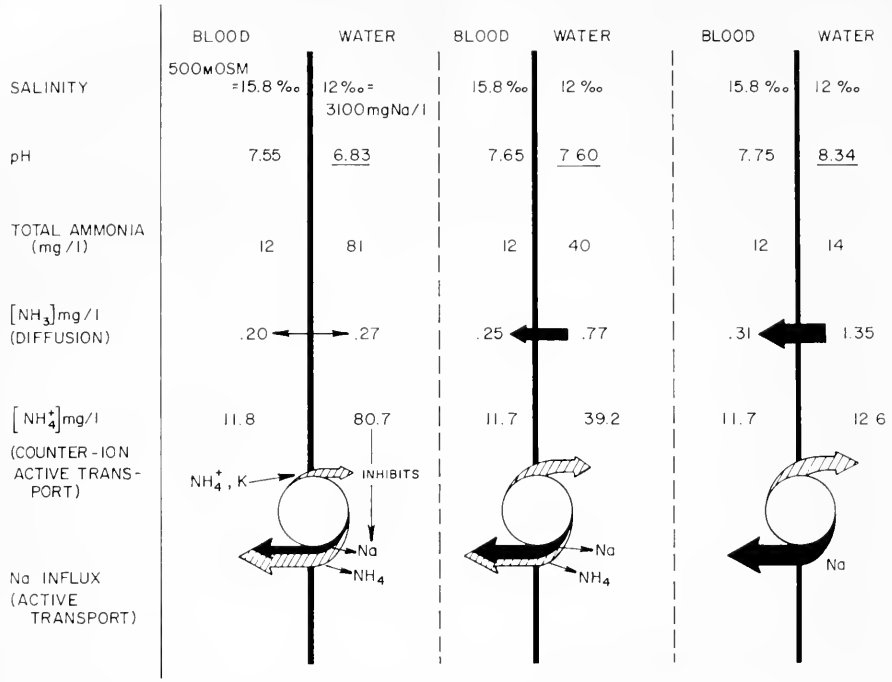

FIGURE 5. A proposed mechanism explaining the differential effects of NH_3 and NH_4^+ on *M. rosenbergii* larvae cultured in solutions of different pH. The values for blood salinity, pH, and total ammonia were estimated from literature data as described in the text. These conditions are assumed to be typical of larvae prior to addition of high ambient ammonia. The water ammonia levels are incipient lethal concentrations derived for the three pH values tested. Ammonia in water of high pH exists in relatively large quantities as unionized NH_3, which rapidly diffuses into larvae, increasing blood ammonia to toxic levels. In low pH solutions ammonia exists almost totally as NH_4^+. This ion is shown to compete with Na in active transport processes and toxicity ensues from osmoregulatory failure.

monia found toxic at high pH about 1.35 mg/liter or 10% exists as NH_3. This level exceeds that postulated for the blood by about four-fold, and consequently NH_3 would diffuse into animals. At a blood pH = 7.65 the molecule would be protonated to NH_4^+, thereby maintaining the NH_3 diffusion gradient inward. Body concentrations of ammonia would rise if alternate routes of excretion could not expel this surplus, and toxicity follow, perhaps *via* a mode described by Campbell (1973). Toxicity might include elevation of blood pH as NH_3 is protonated and a decrease in substrate for the tricarboxylic acid cycle as excess ammonia reverses the usual oxidation of glutamate (Campbell, 1973). Toxicity due to inward diffusion of NH_3 at high pH is rapid and caused mortality among test larvae in 2–18 hr (Fig. 2).

The deleterious effect of high ambient ammonia levels on an alternate route of ammonia excretion from the blood (nondiffusion) is the second component of the model. It is proposed that inhibition of sodium influx is a major factor contributing to ammonia toxicity at low pH. Larvae in water of pH 6.83 died in 81 mg

ammonia/liter, which is a NH_3 concentration of only 0.27 mg/liter. This water concentration is nearly equal to the blood level estimated and, even though the rate of diffusion of NH_3 outward is probably reduced, the decrease is apparently not serious. [Recall that 0.98 mg NH_3/liter at pH 8.34 caused no mortality (Fig. 4) or growth inhibition (Table II), yet this concentration certainly exceeded blood levels and should have established an NH_3 diffusion gradient inward.] Nearly all of the ammonia (80.7 mg/liter) exists as the NH_4^+ ion. By successfully competing with sodium ions, the NH_4^+ would both reduce the influx of Na^+, thereby diminishing body concentrations of this important salt, and also cause body levels of ammonia to rise by itself, riding the transport mechanism in or preventing metabolic NH_4^+ from riding it out. The resistance of the larvae to this form of osmoregulatory inhibition by NH_4^+ is apparently greater, and toxic manifestations do not develop as rapidly as when copious NH_3 diffusion inward (high pH) is operative. Mortality occurred in 40–140 hr at pH 6.83 (Fig. 2), and growth inhibition probably requires exposures of 5–7 days to be measurable with the larval stages used.

The hypothesis that toxicity at low pH is caused by inhibition of Na^+ transport by NH_4^+ (Fig. 5) has been based on several studies. Ammonium ion has long been suggested as a counter-ion for Na^+ transport (Krogh, 1939). Recently Mangum and Towle (1977) discussed the physiological roles of internal NH_4^+ in the euryhaline blue crab. They believe NH_4^+ aids in activating gill ATPase, serves as one counter-ion for sodium transport, aids in maintaining charge balance as it is excreted, and is an important form of ammonia in which this toxicant is eliminated from the body. In the external milieu, NH_4^+ can substantially reduce the influx of Na^+. Shaw (1960) found that 18 mg NH_4^+/liter caused an 80% decrease in Na^+ influx rates in the crayfish, *Astacus pallipes*. Inhibition of Na transport across gills by external NH_4^+ and stimulation of Na^+ uptake after intraperitoneal injection of NH_4^+ has also been documented for fish (Maetz and Garcia-Romeu, 1964; Carrier and Evans, 1976).

An interesting aspect of the Na^+–NH_4^+ transport system regards the affinity of the carrier mechanism for either molecule. Shaw (1960) found that the inhibition of sodium influx caused by ambient ammonium ion could be countered by increasing ambient sodium levels. Working with a freshwater crustacean in low levels of both Na^+ and NH_4^+, Shaw concluded that a concentration ratio of 10:1 favoring NH_4^+ must exist for inhibition of sodium transport to occur, and that the affinity of sodium for the transport site is greater than that of ammonium ion. The present experiments were done in 12‰ sea water or about 3100 mg Na^+/liter (Instant Ocean salt is 25.8% Na by weight based on manufacturer's analysis). Based on the concentrations of NH_4^+ found toxic (32–80 mg NH_4^+/liter), the NH_4^+ to Na^+ ratios in our tests were 0.01–0.02 : 1. Such low ratios for NH_4^+ imply that the ion has a greater affinity for the transport site than Na^+, contrary to Shaw's conclusion. This discrepancy might be partially explained by lower affinity of the transport mechanism for Na^+ in the euryhaline *Macrobrachium* than in the freshwater crayfish of Shaw's experiments. The K_m values for sodium transport may be tenfold greater in saline species than in similar freshwater forms (Prosser, 1973). Alternatively the low NH_4^+ : Na^+ ratios may indicate that NH_4^+ is causing toxicity in a manner other than inhibition of sodium movement.

It has been demonstrated in these studies that sufficiently high concentrations of NH_4^+ in water of low pH is lethal to crustacean larvae, even though the NH_3 concentration present may be sublethal. A model ascribes such toxicity to competitive inhibition of Na^+ transport. It is probably an over-simplification to attribute the toxicity of ammonia only to NH_3 at high pH and to NH_4^+ at low pH. There may be a contribution from each species at a total ammonia concentration found to be toxic, but we believe our model is accurate in assigning the bulk of toxicity to either NH_3 or NH_4^+ as the change in pH influences the ratios between them. Accordingly, we offer a caution for those studying ammonia-induced responses in organisms to consider the contribution from both NH_3 and NH_4^+ species in interpreting results. Relatively low but lethal concentrations of NH_3 may be accompanied by large amounts of NH_4^+, especially at lower pH values. High total ammonia levels used in some physiological experiments may represent near-lethal concentrations of NH_3, particularly at higher pH values. Mangum *et al.* (1976) reported that 10 mM = 180 mg ammonia/liter was used in tests on ammonia excretion. At a pH of about 7.8, this would equal 5.4 mg NH_3/liter, which is well within the range we found to be toxic (Table I).

Finally, some discussion of the results relative to water quality requirements of crustaceans is warranted. Whether the maintenance of animals is for long periods in commercial operations or for short acclimations prior to physiological experiments, water quality is an important variable that should be monitored and regulated. Ammonia concentrations found to be toxic in this study are in accord with other values reported at similar pH levels. Wickins (1976) found that 101 mg ammonia/liter (pH = 7.0) gave an LT_{50} of 24 hr for adult *Macrobrachium*. Further, growth was reduced 30–35% in concentrations of 0.19–0.39 mg NH_3/liter, which corresponds to a very high range of 20–41 mg NH_4^+/liter (pH = 7.2, pKa = 9.22 at his test conditions). Following from the results of the present study, we suggest that inhibition of growth resulted primarily from the NH_4 ion and not NH_3, as reported (Wickins, 1976). Anderson (1944) reported that *Daphnia magna* was immobilized in 16–24 hr when exposed to 46 mg ammonia/liter (no pH given); and an incipient LC_{50} for larvae of the lobster, *Homarus americanus*, was 37 mg ammonia/liter at pH = 8.1, salinity = 33.4‰ (Delistraty *et al.*, 1977). The incipient LC_{50} calculated for *Macrobrachium* larvae in water of pH 7.60 was 40 mg amomnia/liter.

These toxic concentrations are rather high and greatly exceed the "safe" level of 0.1 mg ammonia/liter recommended by Spotte (1970). Larvae in the present test survived 10 and < 32 mg ammonia/liter for seven days at pH = 8.34 and 6.83, respectively. Such levels would probably be injurious over long periods and an application factor, applied to the incipient LC_{50} values or concentrations inhibiting growth, would be needed to estimate safe levels. Sprague (1971) summarizes thought on this topic with the conclusion that 0.1–0.3 of an incipient LC_{50} value can predict safe concentrations. Such a criterion would predict as safe about 1 mg ammonia/liter at pH 8.34 and 3.2 mg/liter at the lower pH. However, the lack of mortality and sublethal growth inhibition at 10 mg/liter leads us to conclude that short-term exposure to rather high ammonia levels may not be damaging to *Macrobrachium*.

In general, the use of flow-through culture systems with water exchange ade-

quate to dilute excreted ammonia, or closed-systems with conditioned, nitrifying filters for detoxification should minimize the threat of ammonia toxicity for crustaceans. In our research culture facilities, the ammonia concentration in water passed through biological filters averages 0.5 mg/liter (pH $\approx$ 8.1), well below toxic levels reported in this study.

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Summary

1. The toxicity of ammonia to *Macrobrachium* larvae was tested at pH 6.83, 7.60, and 8.34, and the respective 144 hr LC_{50} values were 80, 44, and 14 mg ammonia/liter.

2. Toxicity of ammonia was not due solely to the NH_3 molecule. In solutions of different pH and equal NH_3 concentrations, survival was greatly reduced as NH_4^+ levels increased.

3. A model is proposed to explain the differential effect of ammonia as pH varies. At higher pH (8.4) toxicity results from copious diffusion of NH_3 into larvae. At lower pH (6.8) toxicity is thought to result from competitive inhibition of Na^+ transport by NH_4^+.

4. Retardation of growth was documented in sublethal concentrations of ammonia at 6.8 and 7.6. The average dry weight was about 26% less than that of controls ($P < 0.05$) after a seven day exposure.

5. Results are discussed relevant to the culture and maintenance of crustaceans, and it is concluded that ammonia will not pose a substantial threat in adequately managed systems.

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DESCRIPTIONS OF THE LARVAE OF *STICHASTER AUSTRALIS* (VERRILL) AND *COSCINASTERIAS CALAMARIA* (GRAY) (ECHINODERMATA: ASTEROIDEA) FROM NEW ZEALAND, OBTAINED FROM LABORATORY CULTURE

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In asteroids, as in other classes of echinoderms, there are two main types of development. Some species have indirect development with a free-swimming larva which may feed (planktotrophic) or not feed (lecithotrophic) in the plankton; other species have direct development, with no free stage, and eggs which may be brooded by the female starfish.

In species with indirect development and planktotrophic larvae, the first feeding stage is termed a bipinnaria. This generally develops into a brachiolaria larva which, at the end of development, attaches to the substratum and undergoes metamorphosis into the juvenile starfish. In lecithotrophic forms and those with direct development and even in some species with planktonic larvae, this sequence may be shortened by the omission of larval stages.

By the early part of this century a number of investigators had attempted to rear different asteroid species through these larval stages to metamorphosis, with varying degrees of success. Directly developing species often completed development, but planktotrophic species have proved more difficult to rear. This early work has been reviewed by Hyman (1955).

Since these early studies, there have been few detailed investigations of asteroid development, especially of species with indirect development and planktotrophic larvae. Chia (1968) has described the development of the brooding starfish *Leptasterias hexactis* (Stimpson); Birkeland, Chia and Strathmann (1971), the development of nonfeeding larvae of *Mediaster aequalis* Stimpson; Komatsu (1975), the development of the nonfeeding larvae of *Astropecten latespinosus* Meissner; and Atwood (1973), the development of *Echinaster echinophorus* (Lamarck). Of the species with planktotrophic larvae, Greer (1962) has described the development of *Pycnopodia helianthoides* (Brandt); Oguro, Komatsu and Kano (1976), the development of *Astropecten scoparius* Valenciennes; and Henderson (1969), Henderson and Lucas (1971) and Yamaguchi (1973), the development of *Acanthaster planci* (L.). Yamaguchi (1973) has also included some information on the development of *Linckia laevigata* (L.) and *Culcita novaeguineae* Müller and Troschel; and Crump (1969) has described some aspects of the development of the New Zealand starfish *Patiriella regularis* (Verrill). Strathmann (1971) has reared the larvae of *Patiria miniata* (Brandt) and *Evasterias troschelii* (Stimpson) part way through development and the larvae of *Luidia foliolata* Grube, *Pisaster ochraceus* (Brandt) and *Pycnopodia helianthoides* through to metamorphosis, but he did not describe the larval stages.

Coscinasterias calamaria (Gray) is widely distributed within the Indo-Pacific

and is probably the most common forcipulate starfish in New Zealand. *Stichaster australis* (Verrill) is endemic to New Zealand and is common on the exposed west coast of both the north and south islands.

Except for the early bipinnaria of *C. calamaria*, which Mortensen (1921) and Crump (1969) obtained from *in vitro* fertilized eggs, the larval stages of *C. calamaria* and *S. australis* are unknown. This paper reports on the methods used to rear the larvae of both species in the laboratory and the larval stages are described. Questions of breeding, settlement and post-larval development will be considered in later publications.

Materials and Methods

Adult starfish of *Stichaster australis* or *Coscinasterias calamaria* were collected from Maori Bay, on the west coast of Auckland, during the breeding season from August to February. The ovaries were removed in the laboratory and treated with a dilute solution (10^{-5} M) of 1-methyladenine (Kanatani, 1969) to obtain fertilizable eggs. A dilute sperm solution was prepared from small pieces of mature, excised testis. Immobile sperm were treated with a solution of EDTA in sea water to increase their activity. All sea water used was filtered with diatomaceous earth and glass fiber filters, to remove all extraneous matter over 1 μm diameter. All glassware used was washed in Pyroneg cleaning solution and soaked in dilute sodium hypochlorite solution before use.

Eggs obtained by the above method were washed in clean, filtered sea water and placed in a 500 ml beaker; a few drops of sperm solution were added and allowed to remain with the eggs for 5–10 minutes. Eggs were then filtered off in bolting cloth and washed in several changes of clean sea water and placed in 5 liter pyrex beakers at a concentration of approximately 10 per ml of water. Beakers were kept under natural light conditions at a temperature 20° C ± 1° C in a water bath or a temperature-controlled room.

It appears that some degree of water movement is important for the successful culture of planktotrophic asteroid larvae. Henderson and Lucas (1971) reared *Acanthaster planci* larvae in culture vessels held in shaking water baths, and Gemmill (1914) found that gentle water movements were necessary when rearing *Asterias rubens* L. In the present study, cultures were kept stirred with slowly revolving paddles at 10 rpm. It is possible that such movements help keep food organisms suspended in the water and would also prevent the larvae from congregating on the bottom of the culture vessel where they may be subject to greater risk of bacterial infection.

For the first two days, developing embryos were filtered off with bolting cloth every 12 hours and the water replaced. In addition, an antibiotic solution Crystamycin (Glaxo Lab.) was added to the cultures. This was added at a concentration of 25 International Units benzyl penicillin sodium and 0.25×10^{-4} g streptomycin sulphate per ml SW of the cultures. After 24 hours, the paddle was removed and any nondeveloping eggs allowed to sink to the bottom of the beaker. By this stage most developing embryos were at a free swimming blastula stage, maintaining themselves near the surface of the beaker. They were filtered off and placed in fresh

culture beakers at a concentration of 1 per 4 ml of sea water. Algal food was added to the cultures three days after fertilization for *C. calamaria* and four days after fertilization for *S. australis* larvae.

Every one or two days, samples of larvae were removed with a pipette to check the stage of development. These were preserved in Bouins or neutralized 4% formalin.

Every two days the water was changed and fresh food and antibiotic added. On days when the water was not changed, a sample of water was taken from cultures and the concentration of algal food cells present was determined with a Coulter counter (mean of three, 0.5 ml samples). Fresh algae were added to replace those cells removed by feeding larvae.

Algal foods consisted of unialgal (but not bacteria-free) cultures of the flagellates *Dunaliella primolecta* Butcher and *Isochrysis galbana* Parke and the diatom *Phaeodactylum tricornutum* Bohlin. These were grown in modified Guillard's medium (Lanigan, 1972) under constant illumination from overhead strip lights and at a constant temperature of 20° C. Samples of algae to be used as food were centrifuged at 10,000 rpm for 5 min; then the medium was poured off, and the algae were resuspended in filtered sea water. One ml samples of this solution were diluted with 50 ml filtered sea water, and the cell concentration determined with a Coulter counter (mean of three, 0.5 ml samples).

The concentration and type of algal food appears to be important in the culture of starfish larvae. Early in this study cultures were fed very high concentrations of algal species, often in excess of 50,000 cells/ml in the culture vessels. This proved detrimental, particularly in the case of *S. australis,* and few larvae in these cultures completed development and cultures had a high percentage of deformed larvae present. The stomachs of larvae were always packed with food but much of the faeces contained intact and undigested algal cells and in many cases the intestine appeared dark and swollen with a mass of consolidated faecal matter. It was not until food concentrations were reduced to the levels quoted below that a high percentage of normal larvae completed development. Although *Isochrysis galbana* was also used as a food, including mixtures of *I. galbana* with *D. primolecta* and *P. tricornutum,* the most satisfactory food for *C. calamaria* larvae was *Dunaliella primolecta* and for *S. australis, Phaeodactylum tricornutum.* Although other species were digested and some growth occurred, in very few cases was development completed and in many cultures a high percentage of larvae were deformed.

Algal food species were added to cultures at concentrations of 8000 cells per ml of culture of *D. primolecta* and 10,000–12,000 cells per ml for *I. galbana* and *P. tricornutum.* In cases when mixed algae were fed, the combined concentration of cells was kept at approximately 10,000 cells per ml.

Results

Spawning and fertilization

In *C. calamaria,* ovaries respond to treatment with 1-methyladenine from early to late in the breeding season (July–February). In *S. australis* ovaries only respond to this treatment late in the breeding season when the gonad is very ripe.

Kanatani (1969) also found 1-methyladenine to be more effective in stimulating spawning of *Astropecten aurantiacus* (Tiedemann) later in the breeding season.

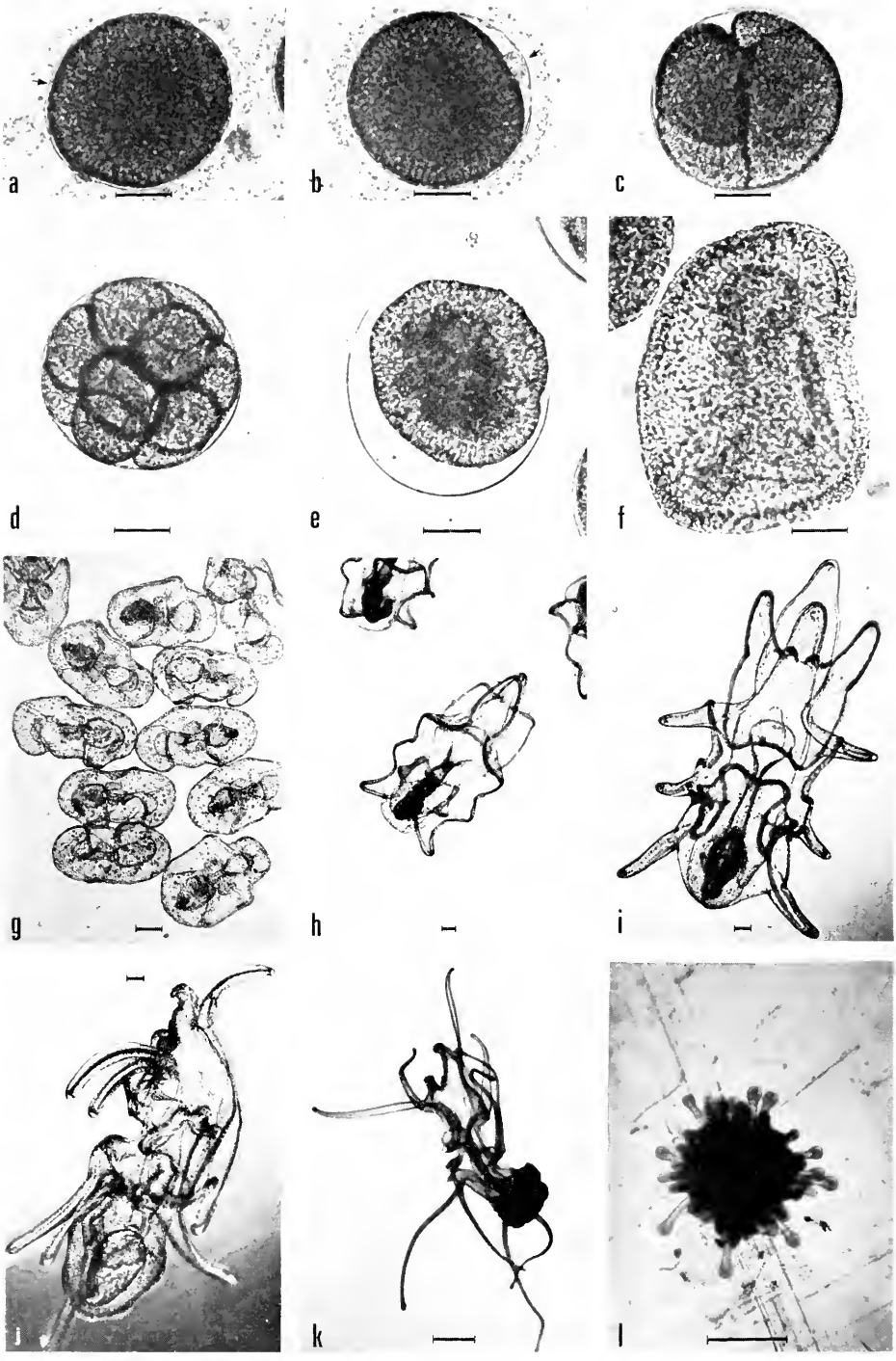
From 60 to 120 minutes (for *S. australis*) and 40 to 60 minutes (for *C. calamaria*) after being placed in this solution the ovary wall contracts, and mature ova are released from the oviduct and from any breaks in the ovary wall. Mature ova are spherical, almost translucent and the color varies from cream to light yellow in *S. australis* and from red to orange in *C. calamaria*. They are enclosed in a jelly layer 10–15 μm thick, and in *S. australis* have a total diameter of 120–140 μm and in *C. calamaria*, a diameter of 140–160 μm. On spawning, the germinal vesicle has either already disappeared or is breaking down rapidly.

The first indication of fertilization is a lightening in color of the surface of the egg, especially around the plasma membrane in the region where the perivitelline space will form (Fig. 1a). Within 30 seconds to 1 minute the fertilization membrane begins to lift, probably at the point of sperm entry. Elevation continues rapidly around the egg, normally being complete after 2–5 minutes, and a narrow perivitelline space of 3–5 μm is apparent. The fertilization membrane continues to expand until, after 5–6 minutes, there is a gap between the plasma and fertilization membranes of 15–20 μm. Within 5–10 minutes the first polar body is extruded (Fig. 2a, 1b) and the second polar body follows 1 to 1.5 hours later. At this stage the fertilized egg lies free within the fertilization membrane, the perivitelline space is about 15–20 μm in diameter, and the jelly coat has dissolved.

Embryogenesis

As is typical in echinoderms, cleavage is radial and holoblastic; however, the rate of embryonic development varies greatly for eggs from the same animal fertilized at the same time. The times given in the following description of the development of *S. australis* and *C. calamaria* are the average time after fertilization, for a particular stage to be reached, for the majority of embryos at 20° C.

From 1–2 hours (after fertilization), the first cleavage occurred (Fig. 2b), and after 3 hours the second and third cleavages were complete. At 5–8 hours developing embryos were at the 64–128 blastomere stage, the blastocoel now becoming obvious. At 16–18 hours the ciliated coeloblastula was revolving within the fertilization membrane (Fig. 2e), and at 20–22 hours the fertilization membrane ruptured releasing the developing embryo (Fig. 1e). At this stage typical embolic invagination was commencing at the vegetal pole. At 30–36 hours gastrulation was well advanced, and mesenchyme formation at the tip of the advancing archenteron was occurring (Figs. 2f and 1f). At 45–46 hours embryos were at the late gastrula stage, invagination being nearly complete. Left and right enterocoels were cut off from the tip of the archenteron, and stomodaeal invagination was well advanced. At 55–60 hours stomodaeum formation was complete, the gut was present as a narrow tube, but the bulbous stomach was not yet differentiated. The larva was now becoming dorsoventrally flattened and was beginning to assume the shape of the bipinnaria, 0.4 mm in total length (Fig. 2g). The left and right enterocoels were lying beside the gut, the left having put out a narrow evagination to the dorsal surface to form the hydropore. At 70–80 hours embryonic development was complete, with the stomach fully formed.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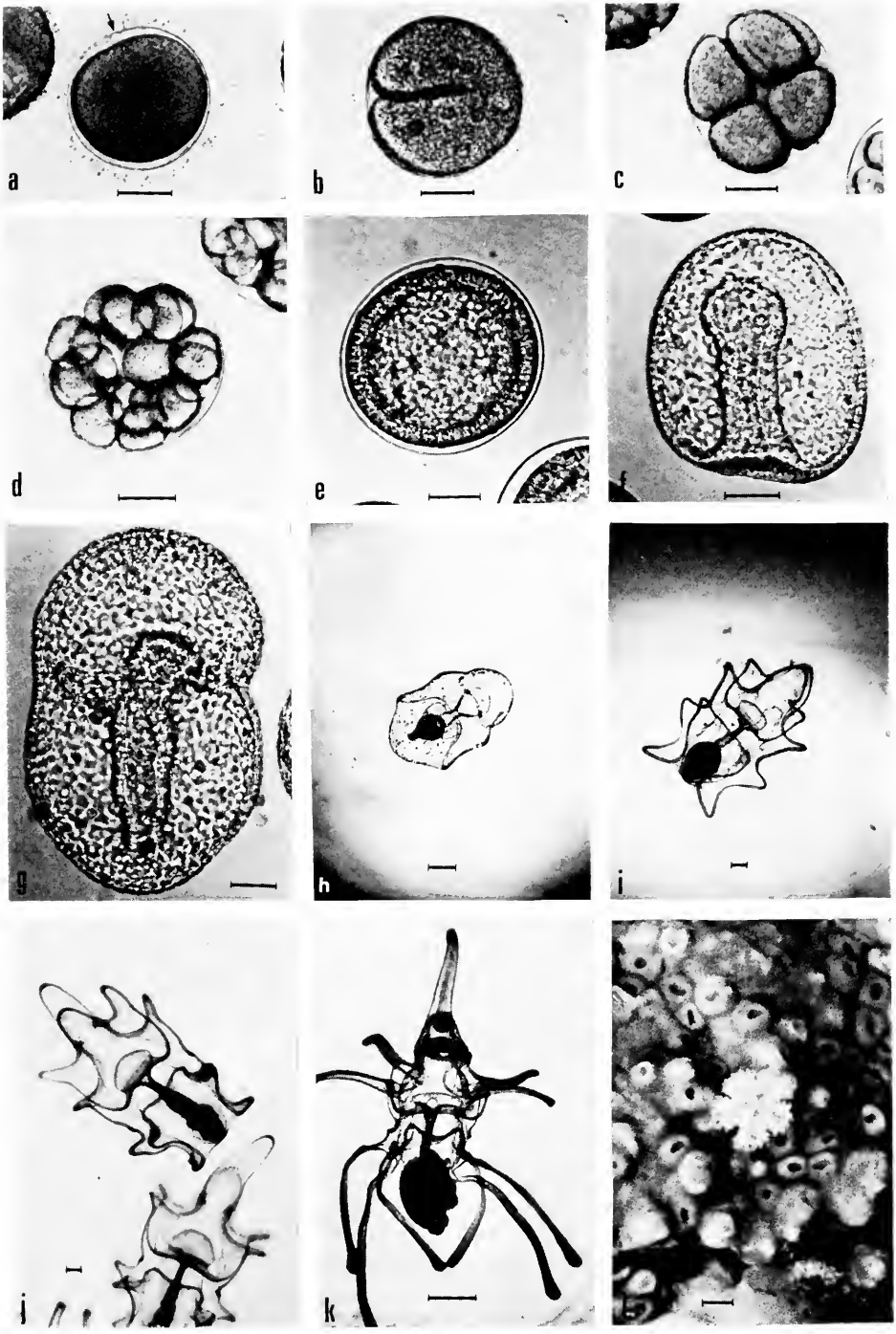
Development of the feeding larva

Although there are minor morphological differences, larval development in *S. australis* and *C. calamaria* is very similar and the following description applies to both species. As with embryonic development, the rate of development of feeding larvae in a particular culture shows considerable variation, and the times given for a particular stage to be reached are the average time after fertilization for the majority of larvae at 20° C. *C. calamaria* has a slightly faster rate of development than *S. australis,* and this difference becomes more pronounced as development proceeds. Sizes given are the total length of the larva.

The early bipinnaria stage (Fig. 1g, 2h) was reached 4 days after fertilization and the larvae of both *C. calamaria* and *S. australis* were 0.4–0.5 mm in length. Their major ciliated tracts, consisting of a preoral loop and postoral and lateral bands, were complete and the larvae were feeding. At 8 days (*C. calamaria,* 0.8–0.9 mm; *S. australis,* 0.9–1.0 mm) the left and right enterocoels were extending anteriorly and posteriorly. The ciliated tracts were becoming more complex and expanding in the regions where the processes would form. At 10–11 days (*C. calamaria,* 1.2–1.3 mm, Fig. 1h, 3a; *S. australis,* 1.0–1.1 mm, Fig. 2i, 4a) the enterocoels were continuing to grow anteriorly and posteriorly, the left often slightly better developed than the right. Posteriorly the ventral horn of the left posterior coelom was beginning to form. At 14 days for *C. calamaria* (1.7 mm) and 16 days for *S. australis* (1.2–1.3 mm) the preoral (paired), postoral (paired), anterior dorsal (paired), median dorsal (single), posterior lateral (paired) and posterior dorsal (paired) processes were beginning to form. Left and right enterocoels were joined, anterior to the stomodaeum and were growing into the median dorsal process (mdp). Posteriorly the enterocoels [termed the left posterior coelom (lpc) and the right posterior coelom (rpc), (Gemmill, 1914)] were beginning to surround the stomach and intestine. The ventral horn of the lpc was growing toward the rpc, ventral to the stomach and anterior to the intestine; and the lpc was growing around the posterior region of the stomach and intestine. In *C. calamaria* a dorso-ventral cleft was forming at the end of the mdp.

The late bipinnaria stage was reached at 16 days for *C. calamaria* (1.8–1.9 mm) and at 20 days for *S. australis* (1.6–1.7 mm, Fig. 2j). This stage was marked by the lengthening processes which in *S. australis* were becoming pigmented (brown) at the tips. The anterior coelom was advancing well up the mdp and in the most advanced specimens was growing out as two buds in the region where the posterior brachiolar arms would form. The posterior coelom had almost completed development around the stomach and intestine. A septum was forming separating the

FIGURE 1. Development of *Coscinasterias calamaria* (times given in a–k are the period after fertilization): a) egg immediately after fertilization, note the incipient fertilization membrane around the periphery of the plasma membrane (arrowed); b) 5 minutes, note the presence of a germinal vesicle (arrowed); c) 2 hours, 4 blastomere stage; d) 4 hours early blastula; e) 20–21 hours, the fertilization membrane ruptures releasing the ciliated coeloblastula; f) 36 hours, advanced gastrula; g) 4 days, ventral view of early bipinnaria; h) 11 days, bipinnaria, dorsal view; i) 18 days, ventral view of early brachiolaria; j) 20 days, brachiolaria, side view; k) 24 days, side view of well developed brachiolaria; and l) juvenile starfish 6 days after attachment to the substratum. Scale is 0.05 mm in a-f; 0.1 mm in g-j; and 0.5 mm in k-l.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left posterior from the left middle coelomic region, and a partial septum separating the right posterior from right middle coelomic region was also forming. In *S. australis* a dorso-ventral cleft was forming at the tip of the mdp.

The early brachiolaria stage [18 days for *C. calamaria* (2.1–2.2 mm, Fig. 1i) and 24 days for *S. australis* (2.1–2.3 mm)] was marked by the formation of rudimentary brachiolar arms, as outgrowths at the bases of the preoral processes and on the ventral tip of the mdp. The anterior coelom was bulging into these, and the adhesive disc was just becoming apparent on the ventral sides of the mdp, between the three brachiolar arms. The processes were now quite elongate (in *S. australis* with light brown pigmentation at their terminal ends). At 20 days for *C. calamaria* (2.5 mm, Fig. 1j) and 27 days for *S. australis* (2.4–2.5 mm) the brachiolar arms were lengthening and adhesive papillae were forming on their tips. The adhesive disc was now well-developed and five hydrocoel lobes were developing on the left posterior coelom. Posteriorly, the starfish primordium was apparent as five rounded outgrowths. At 24 days for *C. calamaria* (3.2–3.3 mm, Fig. 1k, 3b) and 31 days for *S. australis* (3.2 mm, Fig. 2k, 4b) the processes were very elongate and the brachiolar arms had also elongated and were pigmented yellow-brown in both *S. australis* and *C. calamaria*. Each terminated in a crown of adhesive papillae. The five hydrocoel lobes were now more symmetrically arranged on the oral side of the starfish primordium. Rudimentary spines and ossicles were forming on what would become the aboral surface of the yellow brown primordium. The primordium is dorso-ventrally orientated so that the oral side of the developing juvenile starfish is on the righthand side in Figures 3b and 4b.

At the late brachiolaria stage [27 days for *C. calamaria* (3.6–3.7 mm) and 38 days for *S. australis* (3.6–3.7 mm)], the starfish primordium was well-developed with pronounced ossicles and spines. In culture vessels larvae were often swimming near the bottom and if suitable substrata were presented would attach and undergo metamorphosis.

Metamorphosis

The process of metamorphosis is very similar in *C. calamaria* and *S. australis* and the following description applies to both species. As with development of the feeding larvae, metamorphosis proceeded at a slightly faster rate in *C. calamaria* than in *S. australis*. The following times are for the different stages of development after permanent attachment to the substratum by the adhesive disc.

From 0–6 hours the anterior portion of the larva bearing the ciliated processes, brachiolar arms, larval mouth, etc., gradually contracted and at the same time under-

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FIGURE 2. Development of *Stichaster australis* (times given in a–k are the period after fertilization): a) egg at 6 minutes, note the presence of a germinal vesicle (arrowed); b) 1.5 hours, the first cleavage is in progress; c) 3 hours, 8 blastomere stage; d) 6 hours, early blastula; e) 16 hours, the coeloblastula is revolving within the fertilization membrane; f) 36 hours, advanced gastrula; g) 60 hours, ventral view of very early bipinnaria—the gut is present as a narrow tube, but the bulbous stomach is not yet differentiated; h) 4 days, ventral view of early bipinnaria; i) 11 days, ventral view of bipinnaria; j) 20 days, ventral view of late bipinnaria; k) 31 days, well developed brachiolaria; and l) juvenile starfish 6 days after attachment to the substratum. Scale is 0.05 mm in a-g; 0.1 mm in h-j; and 0.5 mm in k-l.

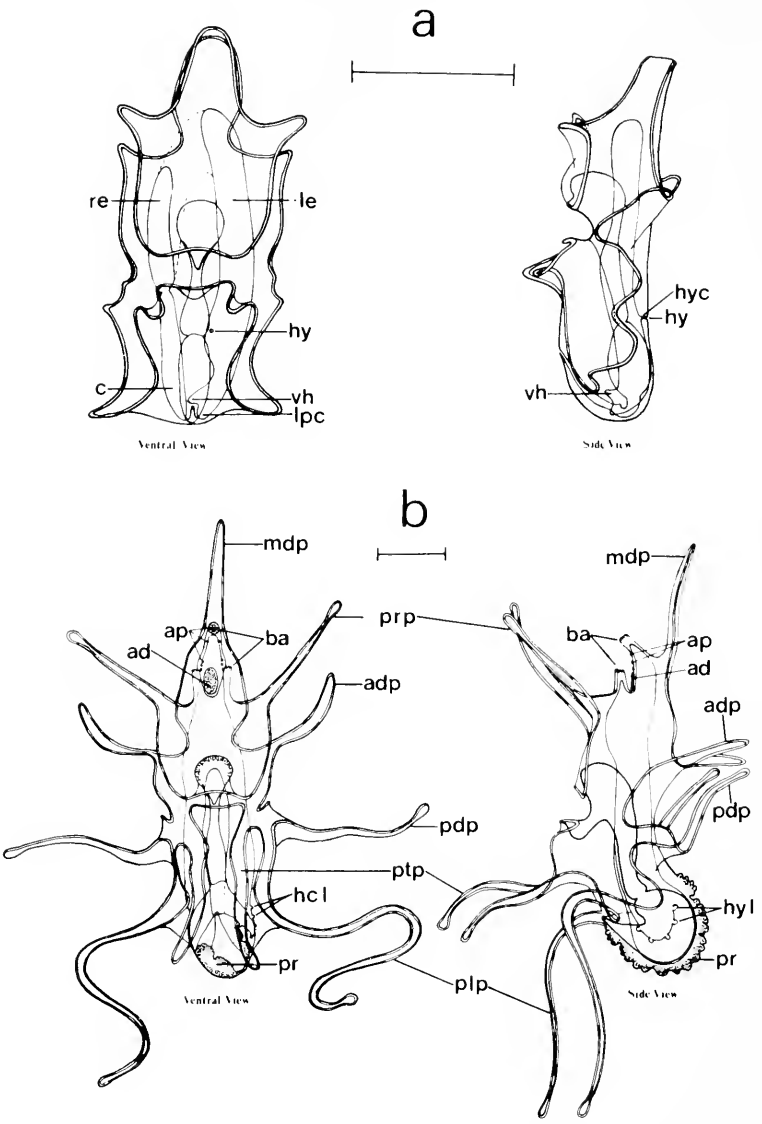

FIGURE 3. Larval stages of *Coscinasterias calamaria:* a) bipinnaria, 11 days after fertilization; b) well-developed brachiolaria 24 days after fertilization. Abbreviations are: ad, adhesive disc; adp, anterior dorsal process; ap, adhesive papillae; ba, brachiolar arms; hcl, hydrocoel lobes; hy, hydropore; hyc, hydropore canal; le, left enterocoel; lpc, left posterior coelom; mdp, median dorsal process; pdp, posterior dorsal process; plp, posterior lateral process; pr, primordium; prp, preoral process; ptp, postoral process; re, right enterocoel; and vh, ventral horn of left posterior coelom. Scale is 0.5 mm.

went torsion, bringing the oral side of the developing starfish primordium closer and parallel to the substratum. At 12 hours the ciliated processes were tightly coiled

and partially resorbed. The "larval notch" [gap between ray 1 and 5 where the larval body (now attachment stalk) was formed] was becoming smaller and yellow pigmentation of the primordium was darkening. At 23 hours shortening of the attachment stalk was continuing. The hydrocoel lobes had expanded somewhat and were lying more centrally on the oral side of the primordium. In *C. calamaria* division of each of the hydrocoel lobes into two pairs of tube feet had commenced

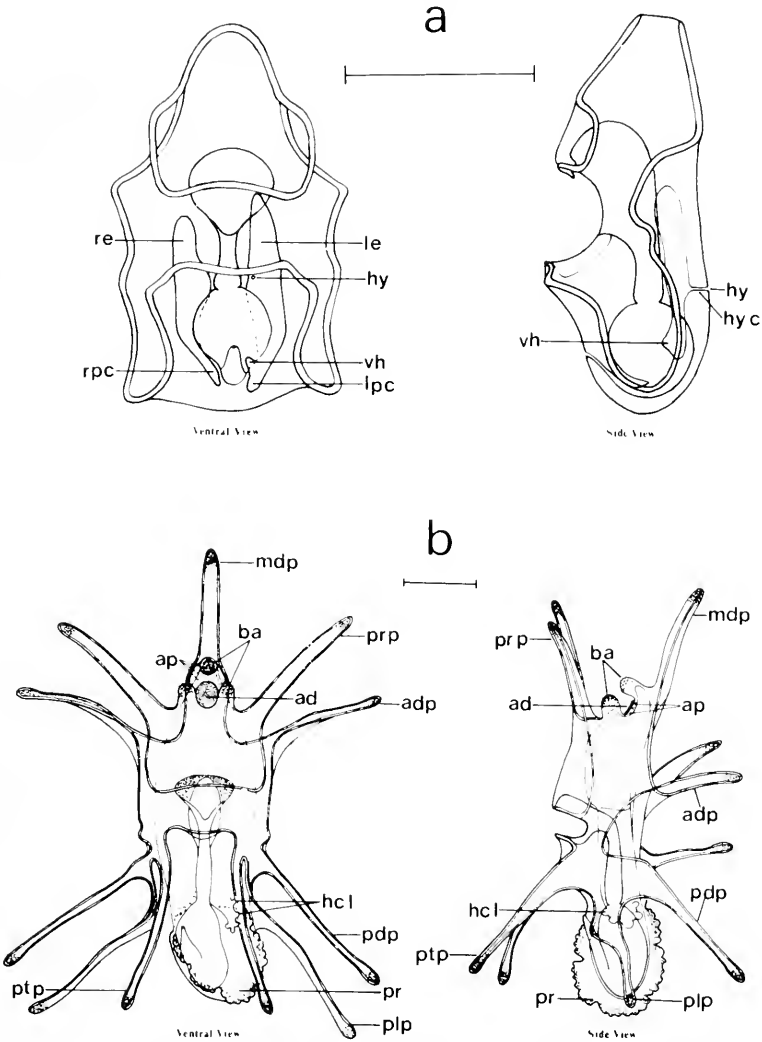

FIGURE 4. Larval stages of *Stichaster australis:* a) bipinnaria, 10 days after fertilization; b) well-developed brachiolaria, 31 days after fertilization. Abbreviations are: ad, adhesive disc; adp, anterior dorsal process; ap, adhesive papillae; ba, brachiolar arms; hcl, hydrocoel lobes; hy, hydropore; hyc, hydropore canal; le, left enterocoel; lpc, left posterior coelom; mdp, median dorsal process; pdp, posterior dorsal process; plp, posterior lateral process; pr, primordium; prp, preoral process; ptp, postoral process; re, right enterocoel; rpc, right posterior coelom; and vh, ventral horn of left posterior coelom. Scale is 0.5 mm.

but discrete podia were not yet obvious. At 27 hours the ciliated processes were fully resorbed. The 5 rays of the developing disc were symetrically arranged, and the larval notch was no longer obvious. Aborally the skeletal plates were starting to assume their adult arrangement of a central and 5 primary interradial and 5 terminal plates. Each bore 1 or 2 small spines. At 36 hours the juvenile starfish was pulled down close to the substratum, the ossicles were becoming larger and were covering a greater area of the aboral surface. In the development of the water vascular system, in *S. australis* each hydrocoel lobe was dividing into two pairs of podia. In *C. calamaria* development had proceeded further and small distinct podia and developing radial canals could be seen. At 52 hours separate podia and radial canals were obvious in *S. australis*. In *C. calamaria* podia were well formed and were attached to the substratum, and terminal tentacles had also developed. At 80 hours in *S. australis* podia were now attached to the substratum and terminal tentacles were present. In *C. calamaria* a red eyespot had formed at the base of each terminal tentacle. At 4 days red eyespots were forming at the base of each terminal tentacle in *S. australis*. In *C. calamaria* the obvious external changes of metamorphosis appeared complete. Podia were being used to move the juvenile against the restraining attachment stalk which was becoming thinner. At 5 days the obvious external changes of metamorphosis appeared to be complete in *S. australis,* and the podia were moving the juvenile starfish against the restraining attachment stalk. At 6 days for *C. calamaria* (Fig. 11) and at 6–7 days for *S. australis* (Fig. 21) the attachment stalk ruptured close to the point of attachment to the substratum, and the small juvenile starfish assumed free life. At this stage there were 5 primary rays, with 4 podia and 1 terminal tentacle per ray. The total diameter of the disc was 0.9 mm. The adult mouth had not yet formed. At 10–12 days formation of the adult mouth was complete, and the small juvenile starfish (0.95 mm in diameter) commenced feeding.

Discussion

The development of *S. australis* and *C. calamaria* follows closely the published descriptions of the development of other planktotrophic asteroid larvae. The most comprehensive of these descriptions is that given by Gemmill (1914) for *Asterias rubens*. *C. calamaria, S. australis* and *A. rubens* are all members of the family Asteriidae. The larvae of *C. calamaria* and *A. rubens* are very similar; *S. australis,* on the other hand, while showing the same general pattern of development as *A. rubens* and *C. calamaria,* exhibits slight differences.

S. australis larvae, for example, have more strongly pigmented and shorter processes and shorter and more rounded brachiolar arms than *C. calamaria* larvae. The slower growth rate may reflect differences in the laboratory culture conditions, rather than inherent differences in the biology of the two species.

The main structural differences which larval stages of *C. calamaria* and *S. australis* show when compared to *Asterias rubens* are: the former species do not develop a dorsal sac, the coelomic epithelium has few cilia, and there appears to be no movement of fluid within the coelomic cavities. In those species where it is present, the dorsal sac appears to exhibit rhythmic contractions, and, although it lacks an inlet or an outlet, it apparently maintains coelomic circulation within the

larvae by passing fluid through its walls (Gemmill, 1914). The observed lack of movement of the coelomic fluid in *C. calamaria* and *S. australis* may be due to the absence of this structure, and coelomic circulation in these species would appear to be unnecessary.

Larvae exhibiting structural abnormalities have been described by several authors (Gemmill, 1914; MacBride, 1896; and Newth, 1925). A double hydropore, single enterocoel, differing growth rates of enterocoels, and absence of the median dorsal process are common variations noted in the literature.

In the present study irregularities in development were found for both *C. calamaria* and *S. australis*. Variations in the growth rates of enterocoels (*i.e.,* one enterocoel expanding much faster than the other) were common in cultures of both species and would seem to be normal growth variations. In *C. calamaria* other irregularities in larval development were only rarely encountered. In some *S. australis* cultures, however, the absence of the median dorsal process or of one or other of the lateral processes occurred in up to 80% of the larvae. In other cultures, almost 100% of the larvae would develop normally. Abnormal larvae seemed to develop most commonly in those cultures fed on species other than *D. primolecta* for *C. calamaria* and *P. tricornutum* for *S. australis,* or in cultures fed a particularly high concentration of food. It would seem, therefore, that abnormal larvae are a result of unsuitable culture conditions, although it is possible some abnormalities have a genetic origin.

Culture conditions may also contribute to the wide variation in growth rates of larvae within a particular culture noted above. Similar variations in growth rates also occur in *Pycnopodia helianthoides* (Greer, 1962) and *Asterias rubens* (Gemmill, 1914) and are probably quite normal in planktotrophic larvae. In contrast, larvae of *Mediaster equalis,* a species with direct development, show little variation in size (Birkeland *et al.,* 1971), and it seems likely that lecithotrophic species, with their yolk reserves, have a much more synchronous development.

The internal reorganization of tissues at metamorphosis is complex and has been described fully by Gemmill (1914). The external changes that occur at metamorphosis in *S. australis* and *C. calamaria* parallel those described by Gemmill for *Asterias rubens,* except that in *A. rubens* three pairs of podia are formed per ray at the completion of metamorphosis, while in *S. australis* and *C. calamaria* two pairs are formed. This is, however, a minor point and is unlikely to reflect any major differences in internal structure. It, therefore, seems likely that metamorphosis in *S. australis* and *C. calamaria* follows the basic pattern described by Gemmill (1914) and further detailed description is unnecessary.

Many marine invertebrates have a planktonic dispersal phase in their life history, at the end of which occurs settlement and metamorphosis into the adult. Despite the complex structural reorganization that occurs, once settlement and the more obvious changes at metamorphosis commence, they are usually completed in a comparatively short time. For example, in barnacle cyprids the change from the larval to the adult form may be complete in 24 to as little as 8 hours. The reasons for this are fairly obvious. Demands on food reserves at this time are high and as changes from larval life to adult life are generally accompanied by a drastically altered diet, feeding must be quickly initiated again. Also, at this time the larva or

early juvenile must be very susceptible to predation. In view of this, it would appear somewhat remarkable that juvenile starfish do not break free from the substratum until six days after attachment by the brachiolaria. In starfish, however, the problem of food reserves is largely solved by resorption and re-utilization of larval structures as metamorphosis proceeds, the juvenile starfish generally being much smaller than the advanced brachiolaria. Although it would appear that the delicate, recently attached brachiolaria or partly metamorphosed juvenile is very vulnerable to predation, there is some evidence that the production of toxic substances, possibly saponins, deters predators. Personal observations have shown that some potential predators, such as polychaetes, avoid or quickly release searching larvae if they come into direct contact, and Yamaguchi (1975) has made similar observations.

Hyman (1955) noted the tendency for related asteroid species to hybridize, producing puzzling specimens. Gemmill (1914) found that a high percentage of oocytes of *Asterias rubens* were fertilized by *Marthasterias glacialis* (L.) sperm and *vice versa*, and a number of these oocytes proceeded to the blastula or gastrula stage. He also found that fertilization of small numbers of *A. rubens* oocytes could be achieved with sperm from other genera, although development did not proceed past the early cleavage stages. Gemmill (1916) also cross fertilized *Stichastrella rosea* (Müller) oocytes with *Marthasterias glacialis* sperm and found that development proceeded normally to the early bipinnaria stage in a small proportion of eggs. Lucas and Jones (1976) cross fertilized oocytes of *Acanthaster planci* and *A. brevispinus* Fisher with sperm of the other species and managed to rear the resulting larvae to adult starfish.

On one occasion, when ripe individuals of both *S. australis* and *C. calamaria* were present in the laboratory attempts were made to fertilize ova of the one species with sperm of the other. With *S. australis* oocytes and *C. calamaria* sperm a small number of oocytes were fertilized. Irregular cleavage produced a deformed blastula with a poorly formed blastocoel after 22 hours, and development did not proceed further. It was found that a higher percentage of *C. calamaria* oocytes were fertilized with *S. australis* sperm, and although the subsequent pattern of development was somewhat irregular in most oocytes, in a few development proceeded normally, and a well formed gastrula resulted 36 hours after fertilization. Unfortunately, a shortage of culture facilities did not allow continuation of this experiment. However, the development of some apparently normal gastrulae, plus the similar morphology of the larvae of *C. calamaria, A. rubens* and *S. australis,* all members of the Asteriidae, lends support to the suggestion of Oguro *et al.* (1976) that in some asteroids, developmental features are related to the systematic position of the species.

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Summary

1. Methods for the laboratory rearing of larvae of the starfishes *Stichaster australis* and *Coscinasterias calamaria* are described.

2. Larval development in *S. australis* and *C. calamaria* is very similar, although *C. calamaria* has a slightly faster rate of development. Fertilized eggs develop through a bipinnaria to a brachiolaria stage. Late brachiolaria larvae were present 38 days after fertilization in *S. australis* and 27 days after fertilization in *C. calamaria*.

3. As in the development of the feeding larvae, the process of metamorphosis is very similar in *S. australis* and *C. calamaria*. The time from attachment to the substratum by the late brachiolaria larvae to the completion of metamorphosis of the juvenile starfish is 6–7 days in *S. australis* and 6 days in *C. calamaria*.

4. Unfavorable culture conditions may have been the cause of abnormal larvae found in some cultures.

5. Larval development of *S. australis* and *C. calamaria* resembles closely that of other starfish species with indirect development, especially *Asterias rubens*. This may reflect the close taxonomic affinities of these three species.

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BIPHASIC PARTICULATE MEDIA FOR THE CULTURE OF FILTER-FEEDERS [1, 2]

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The principal conduit of nutrients between the primary producers and higher trophic levels in aquatic ecosystems is the micro-crustaceans. These herbivores which feed in nature on phytoplankton plus bacteria and fine detritus are, in turn, preyed on by various carnivores, such as small fish. While the trophic role of filter-feeding crustaceans in aquatic food chains has been extensively documented, little is known concerning their specific nutritional requirements. One reason for this deficiency has been the lack of artificial media which meet their specialized requirements as phagotrophs.

A new type of media in which nutrients are supplied as both particles and solutes (biphasic particulate media) has led to the establishment of a number of nutrient requirements for two of these crustaceans. The first chemically defined medium of this type allowed good survival and rapid growth from newborn to adult stages of the amphigonic race of the brine shrimp, *Artemia salina*, but the same medium supported growth only to juveniles for the parthenogenetic race (Provasoli and D'Agostino, 1969). A freshwater version of this medium for *Daphnia magna* gave similar results; growth to adult with only occasional progeny (Provasoli, Conklin and D'Agostino, 1970). While formulation of media supporting growth to adult stages is essential in defining cultural conditions and the major nutritional requirements, the lack of fertility of the animals indicated that these media were still nutritionally incomplete.

The missing fertility factors in filter-feeding crustaceans were studied, using the water flea *Moina macrocopa americana* which is viviparous, parthenogenetic, and has a much shorter life cycle. *Moina* was eventually grown for >200 germ-free consecutive generations on three artificial media—one of which is almost defined. This report describes the compounding of nutrient particles and discusses the possibility of using similar media to satisfy the phagotrophic requirements of other filter-feeding invertebrates.

[1] It has long been the policy of THE BIOLOGICAL BULLETIN not to accept methodological papers "which describe only a new technique or method" without extensive experimental results resulting from its use. In view of the difficulties encountered in earlier attempts at the axenic culture of filter-feeders, and the importance of these techniques to future studies in the physiology and productivity of a variety of aquatic invertebrates, it seemed appropriate to make an exception in this case—*Editor*.

[2] Work done in partial fulfillment of the requirements for Ph.D. degree at New York University. Supported by NSF grants GB-19143 and GA-33480 (Biological Oceanography).

Materials and Methods

The original culture of *M. macrocopa americana* was obtained from Dr. James Murphy of the Rockefeller University. Following his suggestion (Murphy, 1970), monoxenic cultures were maintained using the algal species, *Chlamydomonas reinhardii,* until an adequate artificial medium (E medium) was developed. Early testing of artificial media was complicated by the necessity of eliminating the algal cells. This was done by 5–10 consecutive transfers of several animals in sterile media containing starch particles. Ingestion of the particles cleared the gut of algal cells which were eliminated from the medium by the repeated dilutions. Once the E medium (supplemented with lipid-rich particles containing serum, egg yolk and

Table I

Artificial media

E. medium: basal medium 98 ml + 2 ml trigel particles + 0.2 ml egg particles; pH 7.6–7.8.

FP medium: basal medium 97 ml + 2 ml trigel particles + 1 ml FP particles; pH 7.6–7.8.

F1 medium: basal medium 97 ml + 2 ml SA gel particles + 1 ml FV particles; pH 7.6–7.8.

Particles

Trigel particles: 2 ml supply 15 mg egg albumin + 10 mg rice starch + 5 mg dry beef serum.

Egg particles: 0.2 ml supply 10 mg egg yolk + 2 mg vitamin E (type II, Sigma Co.) + 0.5 mg calciferol.

FP particles: 1 ml supplies 4.5 mg albumin fraction V + 3 mg vitamin E (type II) + 1.5 mg egg lecithin + 0.75 mg calciferol.

SA gel particles: 2 ml supply 15 mg egg albumin + 10 mg rice starch.

FV particles: 1 ml supplies 6 mg albumin fraction V + 1.5 mg egg lecithin + 1 mg BHT (butylated hydroxytoluene) + 1 mg calciferol + 0.5 mg β-carotene + 2 mg *dl*-α tocopherol + 1 mg palmitic acid + 0.5 mg oleic acid + 1 mg linoleic acid + 1.5 mg linolenic acid.

Common basal medium (per cent w or v/v)

KCl, 3 mg; $MgSO_4 \cdot 7\ H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_3PO_4, 2 mg; $Na_2SiO_3 \cdot 9\ H_2O$, 2 mg; metal mix PII, 1 ml (1 ml contains Na_2EDTA, 1 mg; Fe, 0.01 mg; B, 0.2 mg; Mn, 0.04 mg; Zn, 0.005 mg; Co, 0.001 mg); Fe (as $(NH_4)_2$ H citrate), 0.05 mg; glycylglycine, 50 mg, pH 8.0 [TRIS buffer (Sigma Co.) is toxic for *Artemia, Daphnia* and *Moina* at 50 mg%. TES buffer (Sigma Co.) is nontoxic at 100 mg% for *Moina*]; nucleic acid mix V, 2 ml (1 ml contains adenylic acid, 20 mg; guanylic acid, 10 mg; cytidylic acid, 10 mg; thymidine, 10 mg; dissolve in alkali, adjust to pH 8.0); DF 2, 1 ml (1 ml contains Tween 60, 2 mg; Tween 80, 2 mg; rutin, 0.5 mg; oxbile extract (Nutritional Biochem Co.), 1 mg; disperse and emulsify components; adjust to pH 8.0); Cholesterol, 0.6 mg (dissolved in 95% ethanol, squirted into boiling water, ethanol boiled off; forms fine crystalline precipitate); amino acids mix III, 1 ml (1 ml contains L-isoleucine, 10 mg; L-lysine HCl, L-glutamic acid, L-histidine base, L-threonine, L-methionine, L-leucine, L-valine, L-proline, 1 mg each; L-arginine base, L-tyrosine, L-serine, glycine, L-tryptophane, 0.5 mg each); vitamin mix M1B, 1 ml (1 ml contains thiamine HCl, 0.5 mg; nicotinamide, 1.5 mg; pyridoxine HCl, 0.2 mg; biotin, 0.06 mg; putrescine·2 HCl, 0.1 mg; Vitamin B_{12}, 0.002 mg; choline H_2 citrate, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.1 mg; Ca pantothenate, 4 mg); liver infusion L 25 (Oxoid, Flow Labs, Rockville, Md.), 70 mg (does not dissolve completely; upon autoclaving in medium forms a brown precipitate essential for growth). Adjust pH of basal medium to pH 7.6–7.8.

vitamins D_2 and E; Table I) was developed, it was used both as the maintenance medium and the control medium during further work on substitution of serum and egg yolk with more chemically defined particles. Transfer techniques used for the bacteria-free *Moina* studies were essentially those developed for *Artemia* nauplii (Provasoli, Shiraishi and Lance, 1959).

The general form of the media is presented in Table I. This type of biphasic media is similar to those developed for culturing *Artemia*. The liquid phase contains salts and trace metals, pH and metal buffers, amino acids, nucleic acids and a mixture of water-soluble vitamins. The solid phase is a slurry of fine (up to 30 μm) particles of proteins, carbohydrates, and lipids. The addition of lipid-rich particles proved necessary for continuous generations of *Moina*.

Particle preparation

SA gel. Dissolve completely 750 mg of egg albumin (2X cryst., Sigma Chem. Co.) in 30 ml H_2O before adding 500 mg of rice starch. The mixture is then homogenized in a Virtis homogenizer model "23" (container #16–117) for a few minutes using two straight blades at right angles (Virtis blade #2–16–108). The mixture is autoclaved (20 min at 20 lb), cooled, homogenized for another 5 min at medium to high speed and reautoclaved. Following a final homogenization, the suspension is diluted to 100 ml with H_2O resulting in a fine, milky-white liquid. Autoclaving the gel twice prevents reaggregation of the particles during storage and also during the autoclaving of the complete medium.

Trigel. Water is added drop-wise to 250 mg of dried beef serum, avoiding lumps which would stick to the container wall, until the serum is completely dissolved. The mixture is then brought to 30 ml with H_2O. Then 750 mg albumin and 500 mg rice starch are added, and the mixture is homogenized and autoclaved following the procedure detailed for the SA gel. The final appearance of the trigel is a fine light-brown suspension.

Egg particles. A fresh egg yolk, free of albumin, is transferred without breaking to a 30 ml beaker. The membrane is penetrated with a 5 ml pipette, and the yolk material sucked up. Three ml of yolk is allowed to flow from the pipette into a test tube (16 × 75 mm). Free flow insures more repeatability than blowing out since it avoids differing amounts of yolk coating the pipette wall. Add 150 mg of ergocalciferol to 0.6 ml of a tocopherol concentrate (α-tocopherol type II, Sigma Chem. Co.) and triturate with a glass rod until completely dissolved. After addition of 10 ml of H_2O, the mixture is emulsified on the "Vortex Genie" (Scientific Industries Inc., Queens Village, New York) at top speed. The mixture is transferred into a Virtis container 16–117, rinsing the test tube twice with 10 ml of H_2O each time and emulsified further with 3 min of homogenization with the double blades. The emulsion is heated in a water bath on a hot plate with constant stirring until coagulated in large flocs. The egg mixture is then put through two cycles of autoclaving, cooling, addition of 5 ml H_2O, and homogenization for 3 min. Finally, the mixture is diluted to 60 ml with H_2O. The resulting light yellow suspension is stored in a glass-stoppered bottle, flushed with N_2, and refrigerated. Even though autoclaved twice, the egg particles tend to aggregate on storage and must be thoroughly agitated before use.

FV particles. A more defined mixture of lipids was specifically tailored to the needs of *Moina* and replaced the serum and egg yolk supplements of the E medium. To compensate for the emulsifying properties of the egg yolk, egg lecithin is used. Add 75 mg of egg lecithin and 300 mg of albumin (Fraction V, Sigma Chem. Co.) to 25 ml of H_2O in a Virtis flask (16–115). This flask has an enlarged bottom with small fluting and a side-arm capped with a small rubber plug on the top of the enlarged bottom. The lipid solution is prepared separately in a short test tube. The dry solids are added first, in the following order: butylated hydroxytoluene (BHT), 100 mg; ergocalciferol, 100 mg; β-carotene, 30 mg; and palmitic acid, 100 mg. Then in order: dl-α-tocopherol, 0.2 ml; linolenic acid, 0.15 ml; linoleic acid, 0.1 ml; oleic acid, 0.05 ml; and 1.5 ml of acetone. Stirring with a glass rod and use of the "Vortex Genie" helps to dissolve the mixture completely. One ml of the lipid mixture is drawn into a small hypodermic syringe with a thin needle. The albumin and lecithin are homogenized thoroughly for 2–3 min at top speed (with the 2 straight blades) before the 1 ml of lipid mixture is slowly squirted into the Virtis container through the rubber cap covering the side-arm. Homogenization is continued for 8 min at top speed, followed by autoclaving and cooling. The appearance after autoclaving is not uniform: a thin skin of coagulated material overlaps the liquid containing a flocculent mass. The skin and the coagulum are mixed and resuspended with a glass rod, then homogenized for 5 min. The above procedure of autoclaving and homogenization for 5 min is repeated once more and the final volume brought to 100 ml. The final appearance is a brownish-red suspension of fine particles.

Carotene is difficult to dissolve and is replaceable with 0.03 ml retinol palmitate (Type IV, Sigma Chem. Co) resulting in a more homogeneous initial coagulum. Increasing the fat-binding albumin fraction V (>600 mg) inhibits the growth of *Moina*. However, we found recently that when egg albumin, which can be used in higher concentrations, is substituted for fraction V, the resulting particles are again more homogeneous. Initially 300 mg of egg albumin plus 75 mg of lecithin are homogenized together in 25 ml H_2O. After adding the fat solution to the mixture, it is homogenized for 5 min at top speed, then an additional 500 mg of egg albumin is added; followed with another 5 min homogenization. The emulsion from the Virtis container is transferred to a 600 ml beaker and coagulated in a boiling water bath with continuous stirring. Following this rapid coagulation, the lipid particle mixture is autoclaved and homogenized twice as outlined above and brought to 100 ml. All the lipid particle mixes are stored refrigerated in glass stoppered bottles which have been flushed with N_2.

FP particles. Another lipid particle was also successful in replacing the egg particle (medium FP, Table I, Conklin and Provasoli, 1977). Dissolve 225 mg of albumin fraction V in 20 ml H_2O, add 75 mg of egg lecithin in a Virtis flask with side arm; homogenize for 3 min at top speed. Then add, as above, 1 ml lipid mixture [0.15 ml α-tocopherol type II (Sigma Chem. Co.) + 37.5 mg ergocalciferol dissolved in 1 ml acetone] through the side arm and homogenize for 8 additional minutes; follow as for FV particles with 2 cycles of autoclaving, cooling, homogenizing and bring to 50 ml. The simpler FP medium may be useful for other filter feeders.

While the proportions in the medium of the SA gel, trigel, and the FV and FP particles may be varied to suit other filter feeders, modifications in the composition of the gels and lipid particles should not exceed the limited binding power of the albumin. To insure a good coagulation and protein binding and to avoid separation of the lipids, it is necessary to use a small amount of H_2O (20–30 ml) in the initial mixture that is homogenized and coagulated for the first time by heat or autoclaving. The particles thus produced are stabilized by the second autoclaving and after the final homogenization can be dispersed in a large volume of H_2O (50–100 ml or more) without changing their physical properties.

Results

The media are biphasic. The mineral base [minerals, trace metal mix, and glycylglycine (at pH 7.8)] was a modification of the medium formulated for *Daphnia magna* (D'Agostino and Provasoli, 1970) which proved satisfactory for rearing this cladoceran in dixenic culture on *Chlamydomonas reinhardii* and *Scenedesmus obliquus*.

Assuming that essential nutrients for *Artemia* might also be required by *Moina*, various combinations of amino acid, nucleic acid and vitamin mixtures were used, and various quantities and ratios of starch and protein were co-gelled into fine homogeneous particles. A striking difference was seen in protein : starch ratios. Specimens of *Moina*, as well as those of *Daphnia*, seem to prefer a more even ratio of protein : starch (P : S = 1 : 0.5–2.0) in contrast to *Artemia* which needs a high starch ratio (P : S = 1 : 5). On the contrary, the requirements for most water soluble nutrients were similar although adjustments in concentrations were necessary. Media at this stage did not support consecutive generations for *Moina macrocopa*. Some adults were produced but the sparse progeny did not reach adulthood.

Failure of satisfactory viability presumably was due to lipid deficiencies: many insects need several fatty acids and some require tocopherol for fecundity and all need sterols (Dadd, 1973). *M. rectirostris* produced males, females and ephippial eggs in bacterized cultures fed defatted yeast supplemented with olive oil and ergosterol (von Dehn, 1955); fertility of *Daphnia magna* under similar conditions was thought to be restored by vitamin E (Viehoever and Cohen, 1938).

Early attempts to supply lipids as emulsions did not prove very useful. Efforts were then directed toward producing lipid-rich solid particles. A particle made up of starch, protein and serum (trigel; Table I) permitted one or two more generations. Additions of ergocalciferol and the vitamin E concentrate in an egg yolk carrier resulted in a repeatable preparation of highly nutritious particles. The Sigma Chemical Co. "Type II" α-tocopherol, an equal-part mixture of α-tocopherol and a vegetable oil, supplied a convenient array of fatty acids and an antioxidant. Coagulated egg yolk, added primarily as a carrier for the vitamin E oil, presumably also supplied a number of nutrients; however, the egg yolk alone was poor or inhibitory. In this lipid-rich medium, *Daphnia magna* produced 5 or 6 successive parthenogenetic generations, while *M. macrocopa* continued to reproduce without decline in fertility.

A suitable lipid-rich particle (FV) was eventually devised with albumin frac-

tion V as the fat-acceptor and coagulant. This particle served to define the need of *Moina* for fatty acids, ergocalciferol and α-tocopherol. Details on nutritional requirements are given elsewhere (Conklin and Provasoli, 1977); it suffices to say that *Moina* also needs intact nucleic acids and water soluble vitamins and that the concentrations given for the F1 medium are close to optimal under our conditions (22–24° C, subdued light). All the solids used in biphasic media are a slurry of particles ranging up to 30 μm in diameter. When added to the media, the particles remain in suspension for several hours. For maximum efficiency of ingestion the particles are resuspended twice a day by shaking the test tubes on a "Vortex Genie"; the animals are not harmed by the vigorous mixing.

Media E and F1 allow a generation time of 4–6 days, clutches of 4–8 newborn, without decline in fertility for over 200 consecutive parthenogenetic generations. The effectiveness of each variable in the diet was gauged from the number of animals produced from a single female in a week, day 1 being the day when the female produced the first brood. The variability due to the time needed for the inoculated animal (newborn or young) to produce offspring was thus avoided. In 10 ml of complete medium at the end of day 7, the *Moina* population comprised three generations: the original female, females of the first and second clutch, and their combined progeny, *i.e.*, about 13 adults and close to 100 newborn. Growth and reproduction ceased in about two weeks when almost all the particles in the 10 ml of medium were ingested.

Discussion

This report on techniques for producing several kinds of nutrient particles is motivated by the hope that other researchers may formulate better particulate media and adapt this type of media to satisfy the particulate requirements of other filter-feeders. Protozoa, sponges, rotifers, molluscs and many crustacea are filter-feeders throughout life or at least in the early larval stages; some primitive chordates such as sea squirts and salps, and some fishes are also filter-feeders.

Our experience with *Artemia* and *Moina* and the work of Akov (1962) and Dadd (1972) on mosquito larvae, indicate that success in growing filter-feeders depends on two equally important factors: supplying all the essential nutrients for growth and reproduction, and compounding the media so that the nutrients are acceptable and readily available to the animals. The biphasic media for *Moina* satisfy both requisites and result in rapid growth, high fertility, and continuous parthenogenetic reproduction.

The compromise found experimentally effective was that the nutrients which are required in large amounts by crustaceans for rapid growth must be supplied as fine particles (*e.g.*, the amino acids as precipitated proteins and the energy sources as insoluble starch and/or fats). The soluble nutrients were added at noninhibitory concentrations and high enough to compensate for the poor uptake of solutes by crustaceans. Uptake through the thick chitinous exoskeleton, except for the areas used for osmoregulation, is apparently minimal; most of the uptake is through imbibition of water while ingesting the bolus and perhaps through anal uptake (Fryer, 1970). Stephens and Schinske (1961) found that of the 11 phyla tested, only the 6 crustaceans tried were unable to take up labeled amino acids from the

environmental water. Some uptake of palmitate and glucose (at 5–250 μCi, respectively) was shown by Sargent and Lee (1975), but evidently this uptake is not sufficient to support the nutritional requirements. We found that replacements of starch and protein particles with soluble carbohydrates and amino acid mixtures was partial and inefficient: growth rates were slowed 2–3× and the solutes had 1/20–1/60 × the efficacy of particulates for *Artemia*. Therefore, crustaceans may be considered as obligate phagotrophs.

On the contrary, the work of Stephens and Schinske (1961) and later work of Stephens (1975) and Wright and Stephens (1977) shows that soft-bodied marine invertebrates are able to take up and incorporate considerable amounts of dissolved carbon sources and amino acids at the very low concentrations present in sea water. Hence, the organic solutes in biphasic media could be taken up by the soft-bodied invertebrates, and if the rate of uptake is considerable it might be necessary to lower the concentration of the present media to compensate for the increased uptake of solutes. Yet, even for these "permeable" filter-feeders the need for particulates (phagotrophy) may be postulated, because filter-feeding is a very effective gathering process as indicated by the nutritional efficiency of particles over solutes.

While most filter-feeders living in oceanic (even coastal) waters depend mostly on phagotrophy, such an assumption may not be valid for environments high in soluble organic matter (*i.e.,* high domestic pollution, where death and decay of animals or plant blooms occur, and perhaps in aerobic detrital sediments). There, organisms utilizing phagotrophy as well as osmotrophy (effective uptake of solutes) would have a great advantage: they would not depend solely on the transformation of solutes into bacteria, being able to take up solutes directly. Rasmussen (1976) has brilliantly demonstrated that the freshwater ciliate *Tetrahymena pyriformis* is almost equally efficient as an osmotroph or a phagotroph and that the uptake of solutes by *Tetrahymena* is almost as efficient as bacterial uptake. Perhaps filter-feeders in organically-rich environments employ, in different degrees, the same strategy.

Biphasic media used with germ-free techniques could be a useful tool in rearing a variety of filter-feeders and in defining their nutritional requirements. They offer a new approach because of the great experimental versatility of preparing particles of different composition and ratios and of the possibility of supplying lipids more efficiently than with emulsions which are often inhibitory.

Once nutrient requirements are understood, it may be possible to remove the limitation of germ-free handling by using microencapsulation techniques (Jones, Munford, and Gabbott, 1974). Further improvements of biphasic media under both bacterized and bacteria-free conditions should lead to the definition of the nutritional requirements of ecologically and commercially important filter-feeders. Hopefully, this data can also be applied in the formation of efficient diets for a number of anticipated aquaculture species (Provasoli, 1976).

Summary

1. Over 200 parthenogenetic generations of the freshwater Cladocera *Moina macrocopa* were obtained aseptically in three artificial media.

2. The media have two phases: a liquid phase supplying mineral salts, water

soluble vitamins, nucleic acids, and a liver extract and a fine particulate phase made from coagulated proteins, starch and lipid factors.

3. The particulate phase supplies the bulk nutrients very efficiently; hence, this type of media may be useful for growing other filter-feeders.

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DEVELOPMENT OF THE DIMORPHIC CLAW CLOSER MUSCLES OF THE LOBSTER *HOMARUS AMERICANUS*. III. TRANSFORMATION TO DIMORPHIC MUSCLES IN JUVENILES

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The asymmetry observed in the chelipeds of many crustaceans presents interesting problems in a number of areas, including development, behavior, and neuromuscular physiology. While asymmetry is fixed in some animals (Przibram, 1931), there are a number of examples where it has been demonstrated that claw type can be "reversed." That is, loss of one claw, usually the larger or "crusher" will result in transformation of the remaining smaller claw, the "cutter," into a crusher. The regenerated claw will then become a cutter (Przibram, 1931; Hamilton, Nishimoto, and Halusky, 1976). Furthermore, in some of the species in which reversal has been demonstrated, it has also been shown that the claws are used for different behaviors (*e.g., Alpheus,* Przibram, 1931; *Calappa,* Shoup, 1968; Lewis, 1969; *Callinectes,* Hamilton *et al.,* 1976). Thus, it would be of interest to ascertain the mechanisms underlying both the morphogenetic changes which are manifested and also the possible central nervous system modifications which in some cases must also occur. There presently is little information regarding the neuromuscular physiology or development of any of the aforementioned crustaceans, either before or after reversal. Ritzman (1974) has described the neural mechanisms underlying closure of the large snapping claw in two species of *Alpheus,* but has not reported similar studies for the smaller "pinch-claw" or after claw reversal. While it is not yet known whether the muscle fiber populations differ between the two claws, they are certainly used differently in the behavioral repertoires of the animals (Darby, 1934). Thus the pinch-claw is not merely a miniature snapping claw which hypertrophies upon loss of the snapping claw.

Warner and Jones (1976) have studied muscle fiber properties in the dimorphic claws of *Macropipus depurator.* Although the stouter chela of this animal has a higher mechanical advantage than the smaller chela, there was no consistent difference between the muscle fiber populations found in each claw; both claws contained "slow" type fibers with sarcomere lengths of 6–10 μm.

In adult lobsters (*Homarus americanus*) the dimorphic claws contain closer muscles which have different populations of muscle fiber types (Jahromi and Atwood, 1971; Goudey and Lang, 1974). The fast acting cutter claw closer muscle is composed of over 60% short sarcomere (2–4 μm), fast fibers with the remainder being long sarcomere (6–12 μm), slow fibers. The slow acting crusher closer muscle has virtually all long sarcomere (>6 μm), slow fibers. Furthermore, in the cutter muscle, fast and slow fibers are regionally distributed on the inner aspect with fast fibers in the dorsal and central medial sections and slow fibers in the ventral sections (Lang, Costello, and Govind, 1977).

In larval lobsters the claws are identical and, indeed, the paired closer muscles have not differentiated into cutter and crusher types. In fact, the muscles are symmetrical in early larval animals (stages 1–2), being composed of 30–40% short sarcomere, over 50% intermediate and only 10% long sarcomere fibers. In the late larval lobsters (stage 3) there is a nearly equal distribution of short, intermediate and long sarcomere fibers (Lang, Govind and She, 1977). Thus, the transformation of the paired symmetrical muscles into cutter and crusher types must occur in postlarval (juvenile) forms.

In the larval stages (1–3) and early juvenile stages (4 and 5) the two claws are identical in external appearance, both being cutter-like (Herrick, 1896, 1911). A distinct change in external morphology of the paired claws into cutter and crusher claws is seen only at stage 7 or 8 when the cutter has a longer, more slender shape and the crusher has a larger blunt tooth (Herrick, 1896). It is reasonable to assume that the transformation in external character signals muscle fiber differentiation in the closer muscle. Muscle fiber types and their distribution in several juvenile stages were examined in this study and, while differentiation of the cutter muscle occurs as early as stage 6, that of the crusher is not usually completed until at least stage 13.

Materials and Methods

Newly hatched larval lobsters were obtained from the Massachusetts State Hatchery on Martha's Vineyard and reared in running seawater tanks at 20–23° C according to the methods of Hughes, Shleser and Tchobanoglous (1974). Their early development consists of three pelagic mysis (larval) stages. When they molt to the fourth stage they approximate their adult form, and during this stage, or the following one, they assume a benthic existence (Herrick, 1896). From the fourth stage onward, the juvenile lobsters were reared in individual trays (Lang, 1975) and their growth followed for periods up to two years.

Several animals were examined in the early or late period of the molting cycle. In the former case, animals were used within one or two days after a molt. In the latter case, two criteria were used to establish that lobsters were in the late part of the stage, *i.e.*, about to molt: first, when molting had occurred in animals that had simultaneously entered the same stage and had been kept under similar conditions; and secondly, the typical premolting behavior of failing to eat food put into the tray.

The claw closer muscles were fixed with Bouin's solution while the dactyl was in the fully open position. Methods for isolating muscle fibers and measuring sarcomere lengths have been previously described (Lang, Costello and Govind, 1977). The average sarcomere length for a fiber was established by measuring five consecutive sarcomeres in three separate myofibril bundles. Sarcomeres were sampled from the inner aspect of the closer muscle which was subdivided into nine sections. This partitioned the muscle laterally into dorsal, medial and ventral sections, and transversely into proximal, central and distal sections (Lang, Costello, and Govind, 1977). For some stage 4 animals the muscle was divided into only six sections by omitting a medial section and retaining only the dorsal and ventral sections. In most animals, ten fibers were inspected in each section giving a total sample of 90 fibers for each muscle; in four stage 4 animals, only 60 fibers were sampled from

the six sections for each muscle. It is estimated that each closer muscle contains 600–700 fibers; thus we are sampling approximately 13–15% of the total population. However, the extremely small size of the closer muscle may well have introduced significant errors in the sampling procedure. In a fourth stage animal, this muscle is 1.5 mm in length. Thus each sampling area is quite small, and there undoubtedly was some heterogeneity of the fiber population sampled for a given area. For this reason, the statistical test (Kolmogorov-Smirnoff two-sample test) was employed as a guide rather than the sole criterion for determining differences between sampled muscles.

Results

Herrick (1896) reported that the paired claws are symmetrical in external morphology in the first three juvenile (postlarval) stages (*i.e.*, stages 4 to 6) and subsequently differentiate into crusher and cutter claws from stage 7 or 8 onward. In stage 6 lobsters, one dactyl is always slightly longer than the other and is thus destined to be the cutter claw dactyl. By careful measurement, claw type in stage 6 could be unequivocally determined. Therefore, the first three juvenile forms, *i.e.*, stages 4, 5, and 6 and several later stages, namely, stages 11, 13, and 15, were examined. The results are summarized in Table I, in which the paired closer

Table I

Distribution of muscle fiber types in the paired claw closer muscles of juvenile lobsters.

Stage	Length of animal (rostrum to telson, cm)	Muscle fiber type based on sarcomere length (μm)					
		Claw I/Cutter			Claw II/Crusher		
		Short 4	Intermediate 4–6	Long 6	Short 4	Intermediate 4–6	Long 6
4 (early)*	1.2	35%	13%	52%	32%	15%	53%
4 (early)*	1.2	27	8	65	27	3	70
4*	1.25	35	3	62	17	3	80
4*	1.25	45	8	47	32	0	68
4	1.2	44	0	56	22	0	78
4	1.2	43	2	55	32	0	68
4	1.3	24	0	76	19	2	79
5 (early)	1.4	47	1	52	27	1	72
5	1.4	37	0	63	30	1	69
5 (late)	1.5	67	2	31	24	0	76**
6 (early)	1.5	59	0	41	18	0	82**
6	1.65	64	1	35	32	0	68**
6	1.7	58	0	42	6	2	92**
6	1.7	41	0	59	27	0	73
11	3.2	67	1	32	11	0	89**
11	3.3	79	0	21	34	2	64**
13	3.9	64	0	36	0	0	100**
15	5.5	82	0	18	4	0	96**

* Sixty muscle fibers sampled from each closer muscle; in all other animals, 90 fibers were sampled in each muscle.

** Closer muscles significantly different at 0.01 level (Kolmogorov-Smirnov two-sample test).

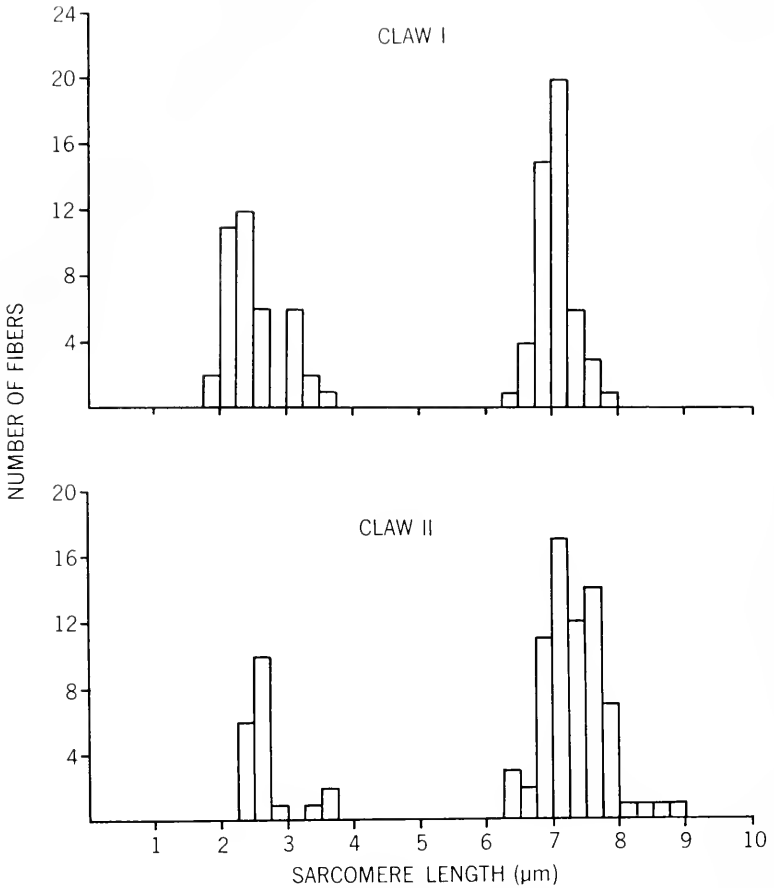

FIGURE 1. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of the paired closer muscles of a stage 4 lobster.

muscles are characterized according to the relative distribution of short, intermediate and long sarcomere muscle fibers. As in a previous paper (Lang, Govind, and She, 1977), muscle fiber types were characterized on the basis of sarcomere lengths since we have little information regarding their physiological properties. However, other things being equal, the fibers with short sarcomeres would contract more quickly than fibers with long sarcomeres, just on the basis of having more sarcomeres in series per unit length of fiber (Jahromi and Atwood, 1969; Josephson, 1975).

In stages 4 and 5, as the paired claws cannot be separated into cutter and crusher types from external morphology, their closer muscles are labelled as Claw I and Claw II (Table I). In these cases the muscle with the higher percentage of short sarcomere fibers was regarded as belonging to Claw I. In stage 6, and subsequent stages, the paired claws are externally identifiable by their dimorphic appearance and their muscles were classified as cutter and crusher types (Table I).

Hence, for the paired muscles in Table I the dual heading Claw I/Cutter and Claw II/Crusher is used.

In most of the early juvenile stages, the closer muscle was examined at some undetermined point during the intermolt of that stage. In several animals the muscle was fixed several hours after it had molted into that stage (early) or a few hours before it might have molted into the next stage (late).

Stage 4

At the molt to stage 4 the lobster assumes its general adult form but the claws are both cutter-like in external morphology (Herrick, 1896, 1911). Except for animals newly molted to the fourth stage, each of the paired closer muscles of fourth stage lobsters is composed largely of two distinct populations of muscle fibers, namely short sarcomere (< 4 μm) and long sarcomere (> 6 μm) fibers (Table I). The bimodal distribution is clearly seen in a frequency histogram of fiber types in a stage 4 lobster (Fig. 1). The short sarcomere fibers exhibit a mode at 2.5 μm and long sarcomere fibers at 7 μm; there is a distinct lack of intermediate fibers. Intermediate fibers are present, however, in the early fourth stage, where they make up approximately 10% of the population. Even this is a significant change from the third larval stage where they make up half the total fiber population (Lang, Govind, and She, 1977). Their disappearance in the early fourth stage, and its correlation with the appearance of long sarcomere fibers, will be discussed below.

It is evident that all stage 4 animals examined have closer muscles with a substantial number of short sarcomere fibers (Table I). However, in no case did they contribute less than 17% or more than 45% of the total population. In this regard, neither claw exhibits a closer muscle characteristic of the adult condition in which short sarcomere fibers constitute over 60% of the population (cutter claw) or long sarcomere fibers constitute virtually the entire population (crusher claw).

Owing to the small size of the claws in this and other early postlarval stages, it is somewhat difficult to rely on the data in regard to a possible regional distribu-

TABLE II

Regional distribution of fiber types in claw closer muscles of juvenile lobsters.

Stage	Number	Muscle fiber type based on sarcomere length (μm)											
		Dorsal area						Ventral area					
		Cutter/Claw I*			Crusher/Claw II			Cutter/Claw I			Crusher/Claw II		
		4	4–6	6	4	4–6	6	4	4–6	6	4	4–6	6
4**	4	48%	5%	47%	40%	5%	45%	15%	2%	83%	12%	12%	76%
4	3	37	3	60	49	—	51	2	—	98	1	—	99
5	2	43	2	55	58	—	42	7	—	93	3	—	97
5 (late)	1	97	—	3	37	—	63	27	7	66	3	—	97
6	4	84	—	16	22	—	78	9	1	90	—	—	100
11–13	4	99	—	1	7	—	93	23	1	76	1	1	98

* For stages 4–6, Claw I is that which has the larger percentage of fast fibers.
** Claws sampled using six regions, all others sampled with nine regions.

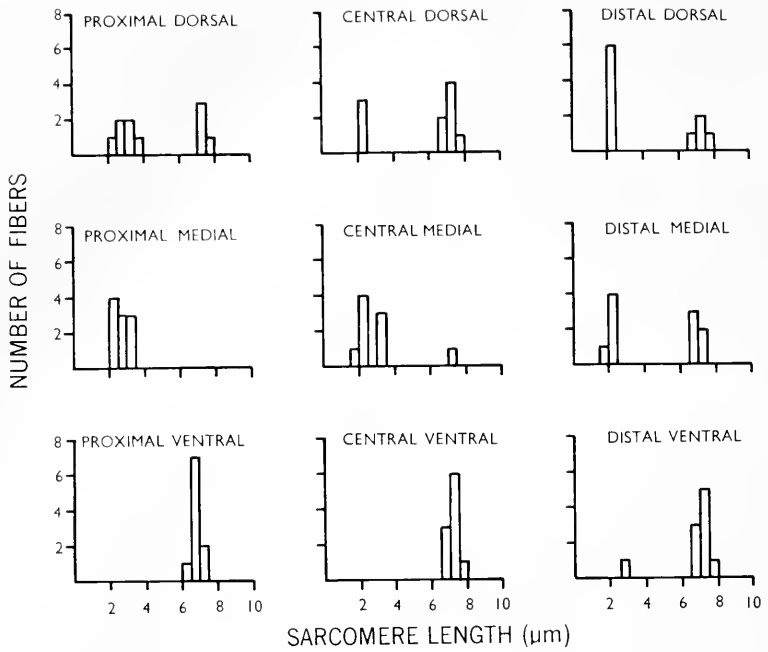

FIGURE 2. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution pattern on the inner aspect of a Claw I closer muscle in a stage 4 lobster.

tion of fiber types. Rather, the sampling technique is meant to provide a survey of muscle fibers from all areas of the claw. In general, however, a pattern emerged that was consistent among the seven pairs of claws examined (Table II). In the ventral areas, long sarcomere fibers (sarcomeres > 6 μm) comprised 88% of all fibers sampled. In the three pairs of claws where nine areas were sampled, this distribution was even more striking. Here, where the three ventral areas consisted of the bottom one third of the muscle (as opposed to the bottom half when six areas were used), long sarcomere fibers comprised over 98% of the sample (Fig. 2). In contrast, long sarcomere fibers comprised about 50% of the sample taken in the dorsal areas for both the six and the nine region sampling technique.

Stage 5

In stage 5, the claws are still morphologically identical, but the latter part of this stage may signal the transitional period between the symmetrical claws of previous stages and asymmetrical claws of subsequent stages. In animals from early and mid-fifth stage, the muscle fibers again are largely distributed into two distinct populations of short sarcomere (< 4 μm) and long sarcomere (> 6 μm) fibers (Table I). However, both claws from each animal have fewer than 50% short sarcomere fibers; thus, there is no apparent differentiation of claw type. In fact, the claws appear essentially similar to those in fourth stage animals with the

exception of the presence of a larger proportion of fibers with sarcomere lengths in the range of 8–11 μm.

In the one animal sampled during the late fifth stage there was a striking change in the population of muscle fibers in one of the claws (Table I). Claw I of this animal contained 67% short sarcomere fibers, approximately the condition of the adult cutter claw. Given the variability of the fiber populations and the limited sample from the late fifth stage, a definitive conclusion regarding the transition of the cutter claw must await further sampling during this period of growth.

The regional distribution of fiber types within stage 5 closer muscles was similar to that observed in stage 4 animals. Ventral fibers were primarily long sarcomere (91%), while dorsal fibers were about equally divided between short and long sarcomere (Table II). Of interest, however, is the observation regarding

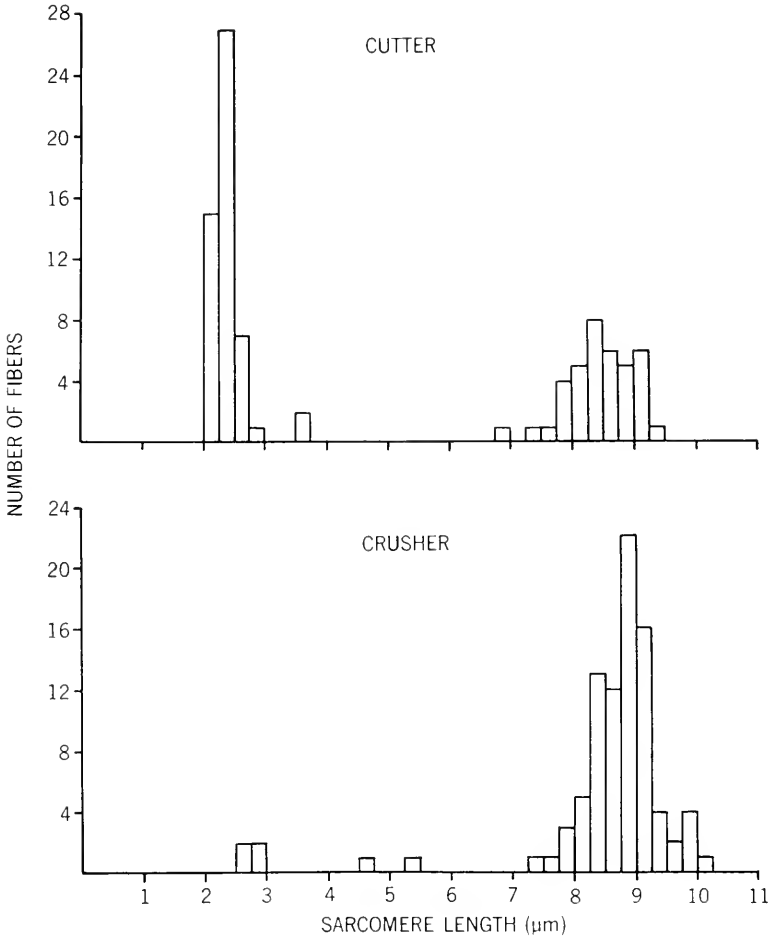

FIGURE 3. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of cutter and crusher closer muscles of a stage 6 lobster.

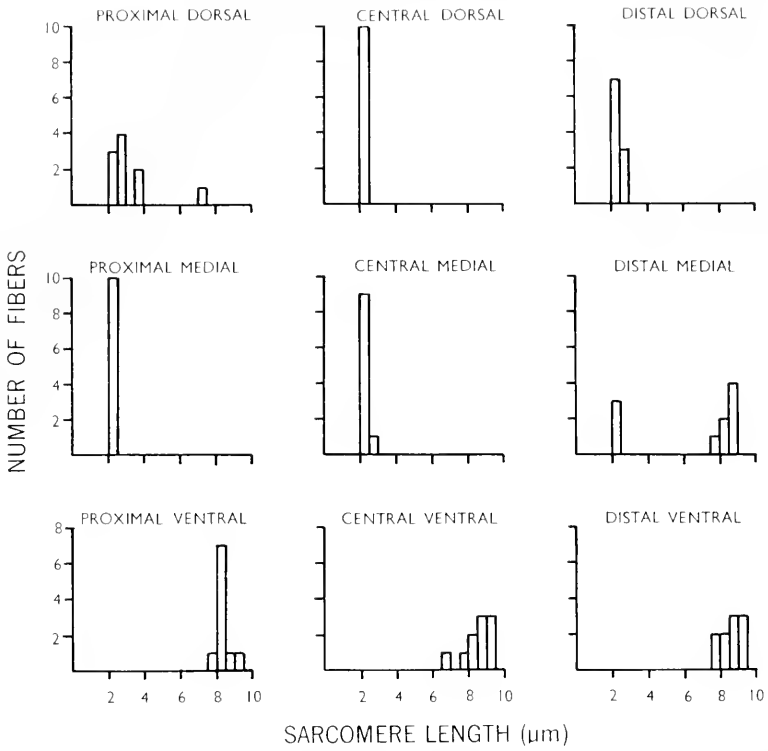

FIGURE 4. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution pattern on the inner aspect of a cutter closer muscle in a stage 6 lobster.

the distribution of fiber types in the stage 5 animal sampled just prior to molt. In the dorsal area of the cutter claw, short sarcomere fibers now predominate, as in later stages (Table II). However, in the other claw, the presumptive crusher, there is a decrease in the relative number of short sarcomere fibers in the dorsal area.

Stage 6

During stage 6 one of the pair of closer muscles usually has a majority of short sarcomere fibers, while the other has a majority of long sarcomere fibers (Table I; Fig. 3). In addition, careful measurements of the claw at this stage revealed that the dactyl of the former was usually longer than the dactyl of the latter, an invariant characteristic of the cutter claw in all later stages. Certainly the claw with a large proportion of short sarcomere fibers in the closer muscle resembles the adult cutter claw and may therefore be regarded as having already differentiated into this form.

In one of the sixth stage animals examined, there were fewer than 50% short sarcomere muscle fibers in the putative cutter (Table I). It is uncertain whether

this represents the variability normally present in claw development or whether it merely represents sampling variability. From the available evidence, the latter seems a likely possibility. The only significant regional variation for this claw occurred in the medial region where the sample revealed equal distribution between short and long sarcomere fibers. Among the other three sixth stage cutter claws, the medial region invariably contained at least twice as many short sarcomere fibers as long sarcomere fibers (Fig. 4).

As in previous stages, the ventral regions of both claws are primarily composed of long sarcomere fibers (Table II). However, in stage 6, there is a striking change in the distribution of fibers in the dorsal regions. In the cutter claw the majority (84%) of dorsal fibers have short sarcomeres, while in the crusher claw the majority (78%) of fibers have long sarcomeres. Thus, the pattern of distribution of fiber types clearly resembles the adult pattern for the cutter claw (Lang, Costello and Govind, 1977), while that for the crusher claw is approaching the adult distribution.

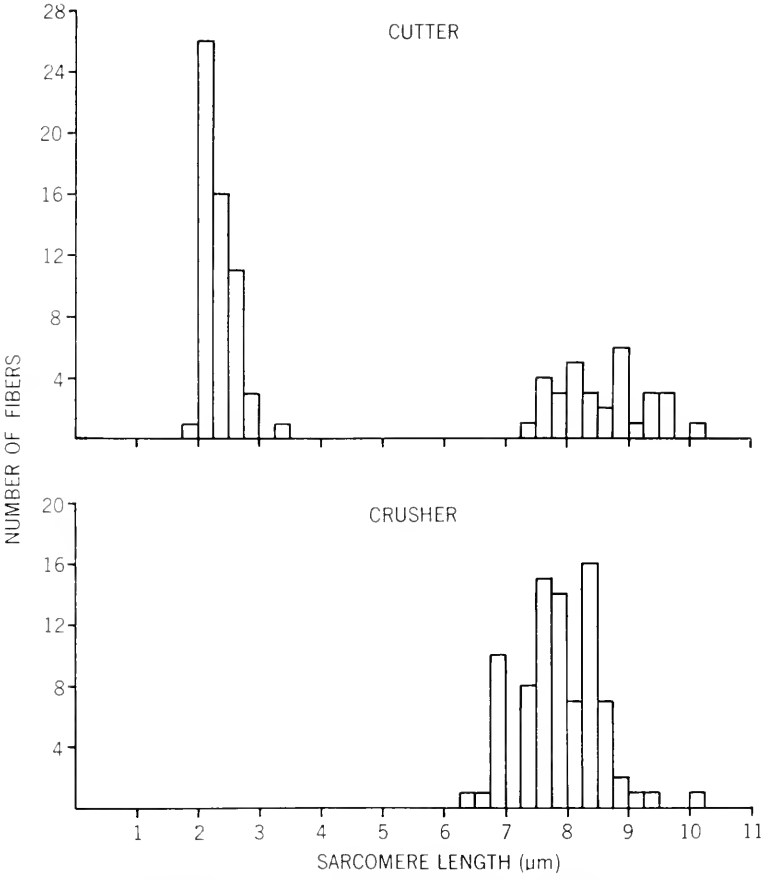

FIGURE 5. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of cutter and crusher closer muscles of a stage 13 lobster.

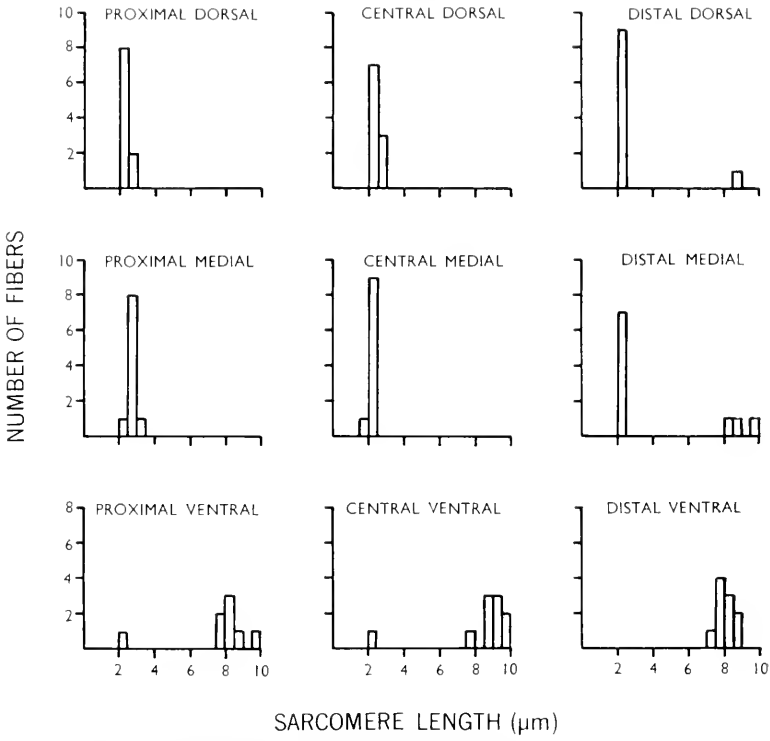

FIGURE 6. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution pattern on the inner aspect of a cutter closer muscle in a stage 13 lobster.

Stages 11–15

The characteristic dimorphic external morphology of the claws is discernible by stage 8 or 9 and is very distinct by stage 11. At this point, the cutter claw closer muscle has assumed the adult pattern of over 60% short sarcomere fibers (Table I; Fig. 5). The crusher claw on the other hand is still in the process of completing the transformation to the adult pattern. There may be short sarcomere fibers present, even as late as stage 16 (Goudey and Lang, 1974), but these never amount to more than 35% of the total. Indeed, the number of fast fibers is usually small and in some animals they may be absent completely (Table I).

The regional distribution first manifested in stage 6 is still evident (Table II). The dorsal region of the cutter is now virtually all short sarcomere fibers (Fig. 6), while that in the crusher is composed of nearly all long sarcomere fibers. However, there has been a change in the ventral region of the cutter claw. Short sarcomere fibers now comprise 23% of the population, typical of that found in the adult (Lang, Costello and Govind, 1977).

DISCUSSION

During the larval (stages 1–3) and early postlarval (stages 4–5) period, the two claws of the lobster are identical from all external appearances, and it is not

until the sixth stage that their asymmetry is evident. The claw closer muscles follow a similar time course of change from the symmetrical to the asymmetrical condition. In larval animals, the closer muscles are very similar, each having virtually identical muscle fiber populations. The same is true in the first two postlarval stages up until the end of the fifth stage. At that time, or during the sixth stage, the transformation to the asymmetrical state occurs.

In light of the timing of the transformation of the closer muscles, it is worth reconsidering the work of Emmel (1908) on claw "reversal" in the lobster. He showed, and we have confirmed (in preparation) that claw type is not established in the fourth stage. Normally either claw has an equal probability of being a crusher or cutter. However, removal of one claw during the fourth stage will always result in the remaining claw developing into a crusher. Emmel (1908) also observed that this was true during the early fifth stage (within a day or two after molting) but not in the later part of the fifth stage or thereafter. In the latter cases, removal of a claw did not influence the remaining claw, as it would become a cutter or a crusher with equal probability. The present result at the late fifth stage correlates well with this observation. At the time when the claws have the ability to "reverse", they are essentially symmetrical. Just after the ability to reverse is lost, the muscles become asymmetrical, one assuming the characteristics of the adult cutter claw. The mechanisms responsible for the loss of reversal and the fixation of claw type are unknown but perhaps are amenable to experimental analysis.

It is of interest to note the occurrence of and changes in the regional distribution of the muscle fiber types. Previous studies on larval and adult lobster closer muscles suggested that short and long sarcomere fibers had a tendency to be prevalent in certain areas (Lang, Costello and Govind, 1977; Lang, Govind and She, 1977). In larval muscle (Lang, Govind and She, 1977) as in the fourth stage, the claws are essentially similar in the composition and location of muscle fiber types. Thus in the fourth stage, the dorsal area has an equal proportion of short and long sarcomere fibers while the ventral areas averaged over 90% long sarcomere fibers. In the sixth stage and perhaps as early as the late fifth stage, these patterns change dramatically. In the cutter claw the short sarcomere fibers increase in prevalence until they comprise virtually the entire sample from the dorsal area of stage 11–13 animals. The ventral area of the cutter exhibits little change during this growth period. On the other hand, the crusher claw muscle fibers exhibit a different pattern in the dorsal area. Here, the short sarcomere fibers present in stages 4 and 5 are replaced by long sarcomere fibers over the next 6–8 molts. The ventral fibers, which are long sarcomere fibers in stage 4, remain thus in subsequent stages.

What influences the dimorphism of the closer muscles such that short sarcomere fibers are added and long sarcomere fibers lost in the cutter muscle and *vice versa* in the crusher muscle? The two excitatory motor axons to each muscle may influence the differentiation of muscle fiber types particularly as the axons are themselves differentiated into a fast and a slow (Wiersma, 1955, 1961); the former has a larger diameter and hence a faster conduction velocity than the latter. In the cutter claw the fast axon evokes rapid (20–40 msec) closure of the claw with a single stimulus, while the slow axon causes a tonic contraction only at higher frequencies of stimulation (Wiersma, 1955; Govind and Lang, 1974). The fast and slow axons in the crusher claw are, however, "slower" versions of their

counterparts in the cutter claw so that the fast axon cannot evoke a mechanical response to single stimuli but can produce a small twitch (500 msec) to a pair of stimuli (Govind and Lang, 1974). There is, thus, some correspondence between the type of motor axons and muscle fiber composition in each closer muscle. The adult cutter closer muscle has a bimodal distribution of fast and slow muscle fibers which matches the fast and slow axons. The crusher muscle has a unimodal distribution of slow muscle fibers which matches the "slower" versions of fast and slow axons in this muscle. The differentiation of muscle fiber types may therefore be influenced by its innervating motor axon through some type of neurotrophic influence as has been demonstrated in vertebrate muscle (for reviews see Guth, 1968; Harris, 1974; Gutmann, 1976).

Considering the striking differences in morphology of the chelipeds and the physiological properties of their closer muscles, it is evident that the claws must be used for different behaviors. This is true both for the pair of claws in the adult as well as for the claws in larval and juvenile animals as compared to the adult (Lang, Govind, Costello and Greene, 1977). Thus, it would be of interest to determine the physiological properties of the motor neurons controlling the chelipeds during growth. Studies in this direction are in progress.

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Summary

1. The two chelipeds of the adult lobster are asymmetrical with respect to their external morphology, neuromuscular physiology and utilization in behavior; however, they are not genetically fixed in terms of placement or handedness.

2. The differentiation of muscle fiber types was studied in the cutter and crusher claw closer muscles in the early juvenile stages of the lobster *Homarus americanus*. Muscle fibers were characterized on the basis of sarcomere length.

3. In contrast to the adult lobster, where the claw closer muscles are asymmetric, the closer muscles of the stage 4 lobster are nearly symmetric; both short and long sarcomere muscle fibers are present in each claw and both fiber types have an identical regional distribution within the closer muscle.

4. By stage 6 one of the muscles differentiates into a cutter muscle with over 60% short sarcomere fibers and a distinct regional distribution of short and long sarcomere fibers. The other claw closer muscle slowly loses its short sarcomere muscle fibers and is transformed into a crusher claw, usually by stage 13–15.

5. The change of the closer muscles from a symmetric to an asymmetric condition is correlated with the loss in ability for the claws to undergo a "reversal" rather than with the external appearance of the claw which becomes differentiated several molts later.

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THE ANNUAL REPRODUCTIVE CYCLE OF AN APODOUS HOLOTHURIAN, *LEPTOSYNAPTA TENUIS*: A BIMODAL BREEDING SEASON [1]

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Although representatives of the class Holothuroidea display interesting and diverse reproductive habits (Hyman, 1955), their seasonal reproductive biology has received little direct investigation. Studies have been done on the aspidochirotes, *Stichopus japonicus* (Tanaka, 1958), *Holothuria floridana* and *H. mexicana* (Engstrom, 1970), and *H. scabra* (Krishnaswamy and Krishnan, 1967); the dendrochirotes, *Sclerodactyla* (=*Thyone*) *briareus* (Turner, 1966) and *Cucumaria pseudocurata* (Rutherford, 1973); and the apodid, *Rhabdomolgus ruber* (Menker, 1970). However, only the studies by Tanaka (1958) and Engstrom (1970) have presented detailed histological data concerning the annual gonadal cycle. There appears to be no similar histological study of reproduction in any apodous holothurian.

McCrary (1969) studied the seasonal occurrence over a three-year period of the planktonic larvae of *Leptosynapta tenuis* (Ayres), a common synaptid of the east coast of the United States. She concluded that this species had "two discrete breeding seasons" on the North Carolina coast. The present study describes the bimodal reproductive cycle and gonad histology of a North Carolina population of *Leptosynapta tenuis* and the relationship between spermatogenesis and oogenesis in this hermaphroditic (Costello and Henley, 1971) holothurian.

Materials and Methods

Specimens of *Leptosynapta tenuis*, an infaunal species, were collected by digging in the sediments at Wrightsville Beach, North Carolina, at approximate monthly intervals from June, 1972, through August, 1973, with an additional collection in March, 1974. At each collection 4 to 25 holothurians were taken. The sample sizes were influenced by the quality of the low tides, and therefore numbers of collected individuals varied from month to month. However, only 4 of the 17 samples produced less than 10 specimens.

Whole gonads were fixed in Bouin's solution (Galigher and Kozloff, 1971) or in Susa's fixative (Humason, 1962). Paraffin (56–58° C, melting point) or Steedman's polyester (Steedman, 1960) were used for embedding. Sections were cut at 6 to 10 μm and stained in Heidenhain's hematoxylin (Galigher and Kozloff, 1971).

Twenty to fifty post-pachytene oocytes encountered sequentially in each sectioned gonad were measured in nucleolar section in order to determine their size

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frequencies. The position of each measured oocyte was carefully noted to avoid measuring the same cell twice. Since most of the cells were somewhat elliptical in outline, the diameter was calculated by averaging the long and short axes (Holland, Grimmer, and Kubota, 1975). Nonsectioned gonads were observed through a dissecting microscope, oocytes being measured with an ocular micrometer. Mean oocyte diameters were calculated for each animal, ranked in groups at 10-micron intervals from less than 20 μm to 200 μm (at which size they are spawned), and used to construct average frequency polygons for each collection date. The polygons were shaded to show the relative contribution of sectioned oocyte measurements to the total sample. The data were pooled to determine the mean oocyte diameter and standard deviation for each collection.

The testicular portion of each gonad was assigned to one of five stages based on cell types present, and a gonad maturity index (MI), similar to one used by Yoshida (1952) for work on the echinoid *Diadema setosum,* was devised here for testicular maturity of the ovotestis of *L. tenuis.* The index is calculated by the formula, MI = [1 (number of animals in stage I) + 2 (number of animals in stage II) + . . .]/ total number of animals staged that month.

Mean weekly sea water temperatures were furnished through the courtesy of the International Nickel Company, Inc., at Wrightsville Beach.

Results

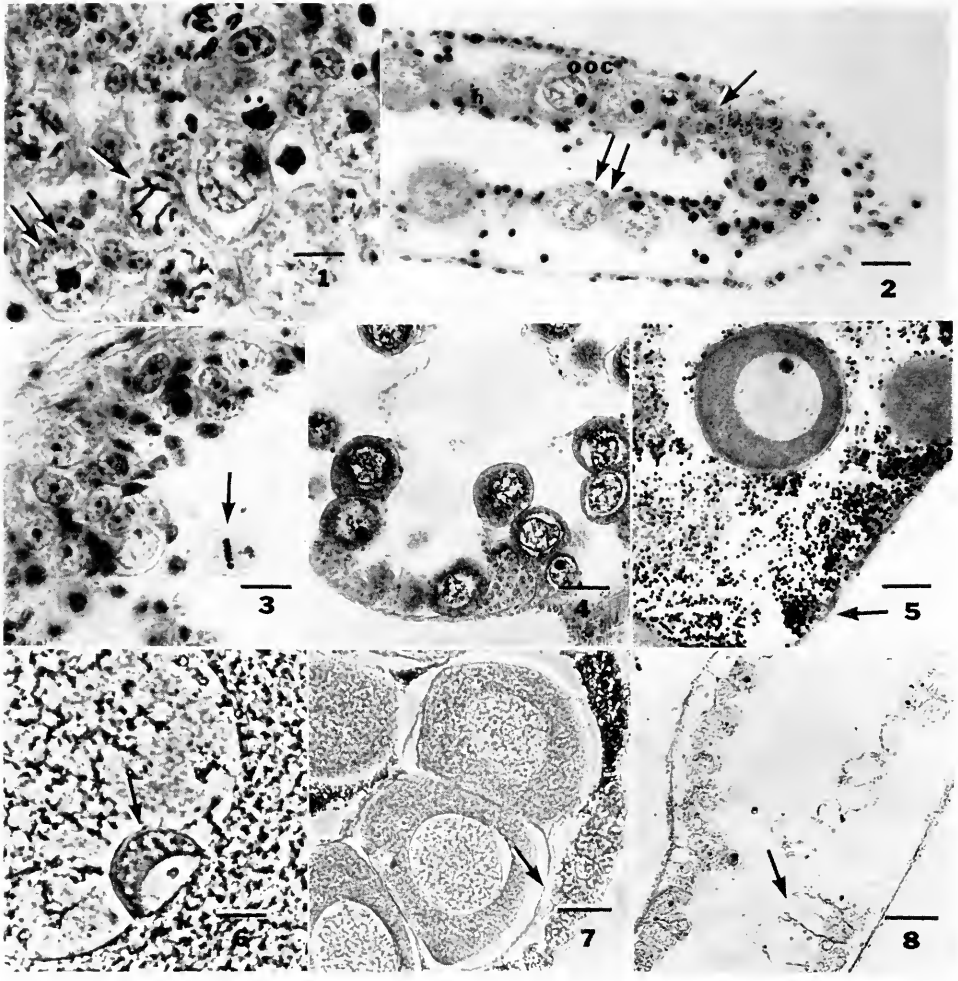
Gonad morphology

The gonad of *Leptosynapta tenuis* is composed of dichotomously dividing tubules. The degree of branching depends on the reproductive condition of the animal. In reproductively active animals the tubules are proliferated, while in inactive or spawned out individuals the tubules are shrunken and only a few branches remain. The right and left halves of the gonad unite in the dorsal mesentery, and the ciliated gonoduct is short, opening to the outside through a small papilla situated between two dorsal tentacles.

In *L. tenuis* there appears to be no specialization in gonadal tubules to contain *only* male or female sex cells. Testicular tissue is present between maturing oocytes and most tubules contain abundant testicular tissue as well as ovarian tissue.

Oogenesis

The gonads of 72 animals were sectioned; all contained post-pachytene, primary oocytes at least 10 μm in diameter, with a distinct nucleolus in a large germinal vesicle (Figs. 1–6). During the course of oogenesis several nuclear and cytoplasmic changes occurred. Presumed oogonia were characterized by their small diameter (less than 10 μm) and their strongly basophilic nuclear granules (Fig. 2). These cells lacked a single, distinct nucleolus. Presumably, after a number of mitotic divisions (Fig. 3), oogonia gave rise to primary oocytes. The primary oocytes underwent a series of chromatin transformations during the meiotic prophase stages of leptonema through pachynema (collectively called the spireme stages). In these stages the chromosomes were distinct because they had condensed and were seen as thick, individual threads in the clear nucleoplasm (Fig. 1). In the later



FIGURES 1–8. Heidenhain's hematoxylin stained, sectioned gonads of *Leptosynapta tenuis* showing stages in oogenesis.

FIGURE 1. Spireme oocyte (arrow) and young post-pachytene oocyte (twin arrows) with prominent nucleolus. Scale is 10 μm.

FIGURE 2. Oogonia (arrow), oocytes (ooc), and follicular cell nuclei (twin arrows); stage II testicular tissue. Scale is 20 μm.

FIGURE 3. Mitotic oogonium (arrow). Scale is 10 μm.

FIGURE 4. Diplotene oocytes; stage III testicular tissue. Scale is 50 μm.

FIGURE 5. Small post-pachytene oocyte (arrow) in a May 31 specimen. The large oocyte has diffuse, homogeneous chromatin in the germinal vesicle; stage I testicular tissue. Scale is 50 μm.

FIGURE 6. Nucleolus in germinal vesticle; phase contrast. Scale is 10 μm.

FIGURE 7. Follicular membrane (arrow) surrounding oocytes; phase contrast. Scale is 50 μm.

FIGURE 8 . Ruptured follicular membrane (arrow) indicating recent shedding of mature oocytes; stage II testicular tissue. Scale is 50 μm.

spireme stages a "bouquet" configuration, in which the chromosomes were arranged in the nucleus with their free ends directed toward one pole, was sometimes observed. Subsequently, the thick chromatin threads decondensed, the nucleolus grew prominent, the germinal vesicle enlarged, and the cell became a post-pachytene oocyte. These young oocytes had a large germinal vesicle approximately 65% of the diameter of the cell, and a nucleolus approximately 20% of the cell's diameter. The chromatin was strongly basophilic and apparently double stranded (diplonema) (Fig. 4). As the oocytes grew, both the nucleus and nucleolus enlarged more slowly, so that the final ratios of nucleus and nucleolus diameters to oocyte diameter were approximately 50% and 10%, respectively. Also, large oocytes were characterized by a diffuse, granular, and lightly staining chromatin (Fig. 5). Large nucleoli clearly showed an outer basophilic, vesicular, cup-shaped portion and a lighter inner portion (Fig. 6). The nucleolus was eccentric and rested against the nuclear membrane.

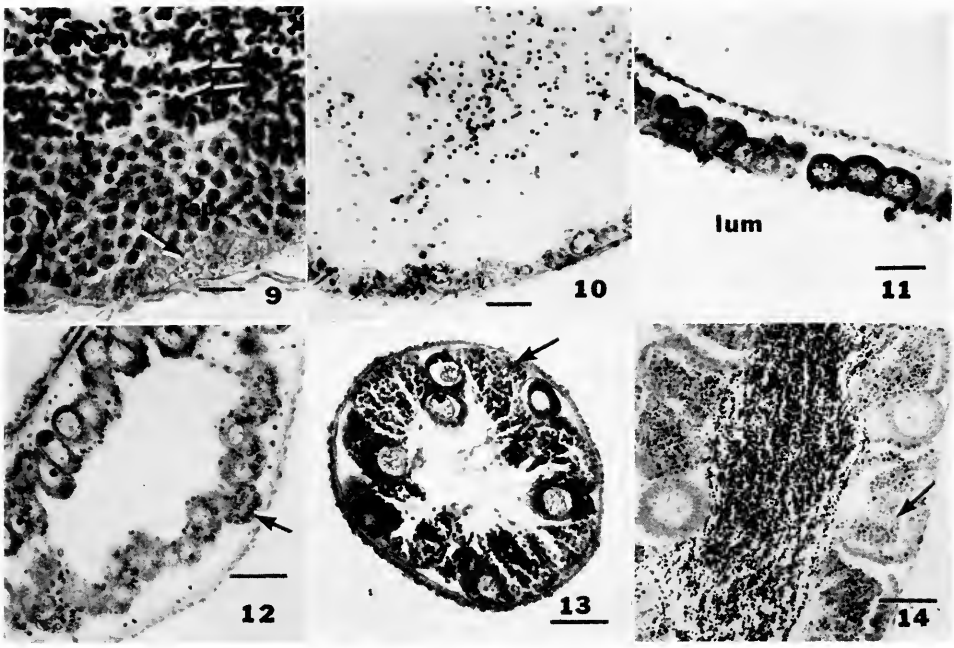
No further changes were observed in the morphology of the oocytes, which were spawned at approximately 200 μm. Maturation divisions take place during spawning and fertilization in holothurians (Kume and Dan, 1968), therefore stages later than primary oocytes were not present in the gonads of *L. tenuis*.

Developing oocytes were arranged in single layers and surrounded by a flat follicular epithelium (Fig. 2) with prominent ovoid nuclei 3 by 6 μm in diameter. As the oocytes grew, they bulged into the gonadal lumen, carrying with them their follicular epithelium (Fig. 7). Eventually oocytes broke free from their surrounding epithelium (Fig. 8), presumably through the muscular activity of the gonadal wall and were shed.

Spermatogenesis

Testicular tissue was distinguishable in 48 of the 72 animals sectioned, although developing oocytes were present in all of them. Those which did not exhibit spermatogenic stages were either spawned out individuals or animals collected during the winter months when gametogenesis was at a low point. Presumably, spermatogonia were present throughout the year, but were possible to distinguish from oogonia only by their position within testicular tissue and only during the testicular growth phase starting in early spring. Spermatogonia (Fig. 9) were 10 μm in diameter with a nucleus approximately 6 to 7 μm in diameter, having granular chromatin. These spermatogonia gave rise to primary spermatocytes (Fig. 9), which contained a nucleus 5 μm in diameter with darkly staining granular chromatin. Occasionally, primary spermatocytes were seen in metaphase preparing to divide into secondary spermatocytes. However, most of the spermatocytes observed were similar in size and appearance; these were probably primary spermatocytes. Secondary spermatocytes and spermatids were not observed in the same numbers as primary spermatocytes, presumably because those stages were of short duration. The spermatozoon (Fig. 9) had a rounded head approximately 2 to 3 μm in diameter, a small midpiece, and a flagellum which, in gonad smears, appeared to be 50 μm long.

To quantify testicular development, the testicular portion of each gonad was assigned to one of five stages. In stage I, sperm had been shed, although a few



FIGURES 9–14. Heidenhain's hematoxylin stained, sectioned gonads of *Leptosynapta tennis* showing testicular stages and spermatogenesis.

FIGURE 9. Testicular region of gonad with presumed spermatogonia (arrow), spermatocytes (spc), and spermatozoa (twin arrows). Scale is 10 μm.

FIGURE 10. Stage I testicular region with relict spermatozoa in the gonadal lumen. Scale is 20 μm.

FIGURE 11. Gonad in stage II testicular condition; gonadal lumen (lum). Scale is 50 μm.

FIGURE 12. Stage III testicular tissue (arrow) between oocytes. First indication of spermatogenesis for 1973 occurred in February. Scale is 50 μm.

FIGURE 13. Stage IV testicular tissue (arrow). Spermatozoa have not yet appeared in the gonadal lumen. Scale is 50 μm.

FIGURE 14. Stage V testicular tissue (arrow). Spermatozoa fill the lumen. Scale is 50 μm.

residual sperm may have remained in the lumen (Fig. 10), and the spermatocyte layer had disappeared. In stage II, the testicular components of the gonad were inconspicuous (Fig. 11). Any spermatogonia which may have been present were not distinguishable from oogonia. Beginning spermatogenesis marked stage III (Fig. 12), in which there were clumps of spermatogonia and spermatocytes two or three cells thick scattered around the gonad periphery between oocytes. Advanced spermatogenesis, with a thick spermatocyte layer of intensely stained cells, was characteristic of stage IV (Fig. 13). Spermiogenesis, however, was not very advanced, as evidenced by a patent lumen. Stage V was the mature stage (Fig. 14). A thick spermatocyte layer persisted, but the lumen was packed with spermatids and spermatozoa. Gonad smears produced actively swimming sperm. As spermatogenesis proceeded in stage V, the spermatocyte layer was reduced, so that

following spawning only small areas remained identifiable as testicular tissue, and stage I was restored.

Comparisons within each individual between testicular development and mean oocyte diameter, regardless of the time of year, showed a clear relationship (Fig. 15). Animals with stage II testicular tissue had small oocytes averaging 30 μm in diameter. Stages III and IV were found in animals with an average oocyte diameter of 38 and 51 μm, respectively. A wide range of oocyte diameters (60 to 160 μm) was present along with stage V testicular tissue. Most animals with stage I testicular regions had a mean oocyte diameter of 23 μm, although two animals also had oocytes larger than 120 μm in diameter. These latter probably had spawned their male gametes, but still had not spawned all their oocytes.

These last data suggest that *Leptosynapta tenuis* is a simultaneous hermaphrodite, developing and shedding both sperm and oocytes within the same reproductive period. Oocytes were present throughout the year, but spermatogenic stages seemed to be transitory. It appears that each animal underwent spermatogenesis rather quickly, so that sperm were shed when the oocytes were large but still growing (Fig. 15). The oocytes were then shed somewhat later.

Annual reproductive cycle

In 1972–1973, *Leptosynapta tenuis* bred at two different times of the year at Wrightsville Beach, North Carolina. This population spawned in the spring and again in the fall, as indicated by the bimodal occurrence of mature primary oocytes over the year (Fig. 16). Mean oocyte diameters of the population reached peaks in June and October, 1972, and May and August, 1973. The development of

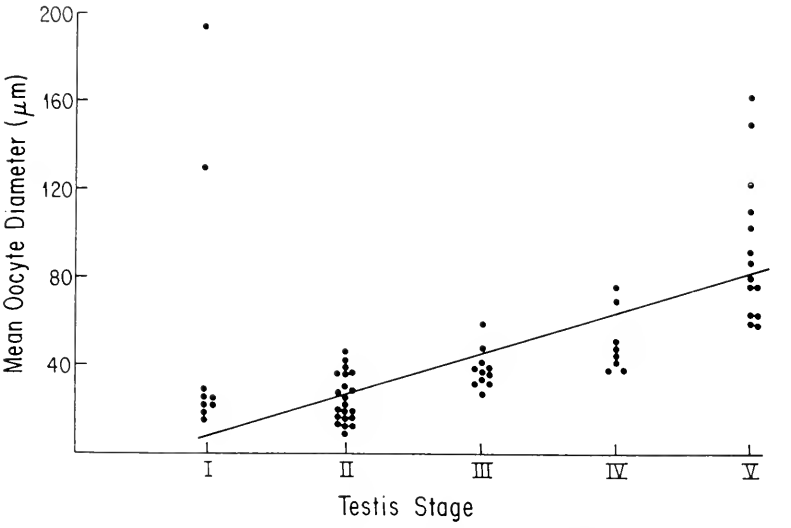


FIGURE 15. Comparison between ovarian and testicular development in the ovotestis of each animal: $y = -9.69 + 18.38x$, $n = 61$; $r = 0.79$, $P < 0.01$. (The two individuals in testis stage I having oocytes of 128 μm and 190 μm were omitted from the correlation and regression calculations.) These data are from sectioned material only.

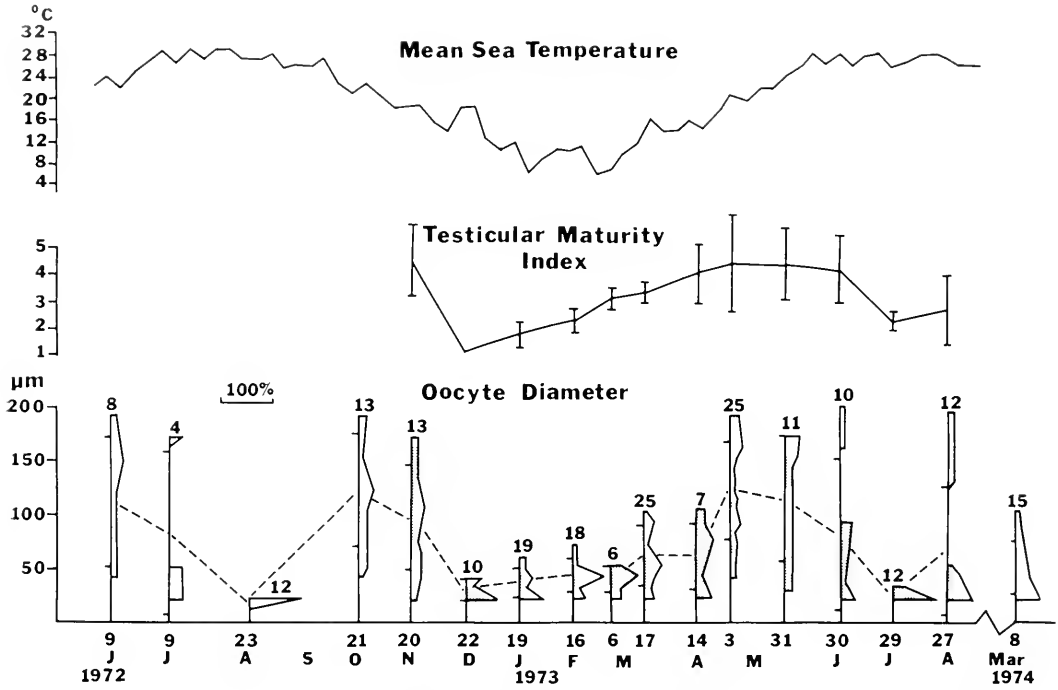


FIGURE 16. Average size-frequency polygons of post-pachytene oocytes in the population. Each polygon was constructed using the mean oocyte diameters from the number of individuals shown above the polygon. Combined measurements of sectioned and whole oocytes were used to construct the polygons which are shaded to show the relative contribution of sectioned oocyte measurements to the total sample. Population mean oocyte diameters are shown by the broken line. Standard deviations for most collections are indicated by horizontal lines intersecting the polygons. Testicular maturity indices (with standard deviations shown) are correlated with the mean oocyte diameters for the population ($r = 0.84$, $P < 0.01$). Mean weekly sea temperatures are also plotted.

testicular regions of the gonad also followed a bimodal pattern throughout the year (Fig. 16) and correlated well with the mean oocyte diameters ($r = 0.84$, $P < 0.01$). A mid-summer low of reproductive activity is indicated by the absence of mature oocytes from the August, 1972, sample and the absence of both mature oocytes (Fig. 16) and spermatozoa (Table 1) from the July, 1973, collection. (Mature gametes were also absent from December, 1972, through March, 1973.) These findings support the conclusion of McCrary (1969), based on larval occurrence in the plankton, that this species has two discrete breeding periods each year.

A striking difference existed between the range of oocyte diameters in the population for most collections and the range present in single individuals. Although a full range of oocyte diameters occurred in the *population* during the spawning periods, no *individuals* had such a range. On the average, each animal's oocyte diameters were within a range of ±23% of its mean oocyte diameter (Green, 1976). A few animals (two in November, two in May, and one in August, 1973) had two size classes of oocytes within the same gonadal tubules. The smaller

oocytes were at least 100 μm smaller than the large oocytes, except in one specimen from November, where the difference was approximately 60 μm between size classes. However, the small oocytes in these cases represented a small part of the total oocytes observed and were found singly near larger oocytes (Fig. 5). The fate of the small oocytes (that is, whether or not they reach maturity or degenerate) is not known.

One animal from the October, 1972, collection appeared to have recently spawned some of its oocytes. Some of its gonadal tubules were empty, while others had fully developed oocytes present in single file in proximal regions of the tubules. These latter tubules were just wide enough (ca. 200 μm) to accommodate the mature oocytes. During oocyte growth the tubules may attain a diameter of 700 μm. Presumably, muscles in the gonadal walls contract to push the oocytes through. This individual from October apparently had not finished spawning for the year when it was collected. This observation raises a question of whether one animal completes its spawning all at once, or whether it spawns several times during a specific time interval. Newly formed post-pachytene oocytes (less than 40 μm) were not observed in October, perhaps indicating that the oogenic cycle was involved with oocyte growth rather than the production of new oocytes.

Reproductive activity decreased during November, and the small oocytes present may have been representatives of the 1973 generation. Unidentified cells along with degenerating oocytes in the gonad may be evidence of phagocytosis of unspawned oocytes (Tanaka, 1958; Engstrom, 1970). Degenerating oocytes and unidentified cells were observed in a few other months as well, but this observation was made so infrequently that its significance cannot be judged. In November the gonads also contained dividing spermatocytes and mature spermatozoa.

By December 22, no mature oocytes were observed in the gonads. Residual sperm (Fig. 10) and debris of degenerating oocytes were present in the gonadal lumina. Although spermatogenesis had ceased, there were many spireme oocytes representing a new ovarian cycle.

TABLE I

Number of Leptosynapta tenuis *in each testicular stage per sample. Sectioned material from the collections of November 21, 1972, through August 27, 1973, was used to calculate the maturity index.*

Date	Stage I	II	III	IV	V	Maturity index	±s.d.
Nov. 21, 1972		1			4	4.4	±1.3
Dec. 22	5					1.0	±0
Jan. 19, 1973	2	3				1.6	±0.5
Feb. 16		4	1			2.2	±0.4
March 6		1	4			2.8	±0.4
March 17			4	1		3.2	±0.4
April 14		1		3	1	3.8	±1.1
May 3	1				4	4.2	±1.8
May 31		1		1	3	4.2	±1.3
June 30		1		2	2	4.0	±1.2
July 29		7	1			2.1	±0.3
August 27	1	1	1	1		2.5	±1.3

The January-to-early-March phase of the reproductive cycle was characterized by slow but steady gametogenesis. Oocyte growth occurred slowly through early March (Fig. 16), the population mean increasing by less than 15 μm. Spireme oocytes were present throughout this period, which was dominated by the emergence of new oocytes. Residual spermatozoa were still present in January, but were not seen in later winter specimens. By February there was evidence of a new spermatogenic cycle (Fig. 12), but most testicular regions were still in the recovery (II) stage (Table 1).

By the middle of March the "quiescent" period of oocyte growth seemed to have ended with the maximum oocyte diameters reaching 100 μm. Most testicular regions had meiotic spermatocytes. In April, spireme oocytes were not seen as frequently as in early March, indicating that growth of oocytes rather than production of new oocytes was predominant. For the first time in 1973, mature actively swimming spermatozoa were observed in gonad smears.

The height of reproductive activity was reached by May 3. Mature oocytes and spermatozoa were present, with some gonads already showing spawned out testicular regions. One animal with small oocytes (30 μm) from May 31 appeared to have recently shed mature oocytes, owing to the presence of a ruptured follicular membrane (Fig. 8). Neither May collection revealed emerging post-pachytene oocytes (no spireme stages observed), oogenesis still being dominated by growth of oocytes. Two individuals contained two distinct oocyte size classes separated by more than 100 μm. Since the smaller oocytes were larger than any that were observed in July, the possibility exists that two generations of oocytes, widely separated in size, matured and were spawned within a single breeding period, in this case, the spring period. Deteriorating oocytes were not observed, but the possibility that the second size class of oocytes degenerated cannot be eliminated at this time.

In addition to nearly mature oocytes, numerous small oocytes were present in June (Fig. 16), indicating that production of post-pachytene oocytes had occurred. Spermatozoa and spermatocytes continued to be present in late June.

The first half of the annual breeding season apparently ended between June 30 and July 29. The July specimens resembled those of the previous August, all animals having small, thin gonads, approximately one fifth of their maximum diameter. Oocytes were less than 30 μm in diameter, and the presence of numerous spireme oocytes signaled the proliferation of new oocytes for the autumn reproductive period. The animal in July with the largest oocytes (mean diameter of 26 μm) also had the most advanced testicular stage (III), although testicular portions in most animals were in the recovery (II) stage (Table 1).

Twenty-nine days later on August 27, a wide range of gametogenic stages was again present in the population (Table 1 and Fig. 16). Some of these specimens apparently had spawned already, and the reproductive activity of the population appeared headed for a second peak representing the autumn breeding period.

Evidence that the year-to-year reproductive patterns are similar is shown by the data from March, 1974 (Fig. 16); these data are comparable to those of the previous March.

Discussion

Leptosynapta tenuis appears to breed twice during the year in North Carolina, with a mid-summer cessation of reproduction occurring in July or August. Only Krishnaswamy and Krishnan (1967) have suggested a bimodal breeding season for any other holothurian. They reported that *Holothuria scabra* had breeding peaks in July and October in southern India. However, individuals with mature gonads were found in collections between July and October. In contrast to *H. scabra*, only young oocytes were found in the gonads of *L. tenuis* during August, 1972, and July, 1973. In addition, McCrary (1969) reported an absence of the planktonic larvae of *Leptosynapta* during the same months in her three-year study of the plankton on the North Carolina coast.

Factors governing gametogenesis and spawning have not been elucidated for holothurians, although salinity (Krishnaswamy and Krishnan, 1967) and temperature (Tanaka, 1958) have been proposed as regulatory factors. It is logical to assume that a reproductive cycle, such as *L. tenuis* seems to exhibit, could be regulated by external factors. At Wrightsville Beach, salinity values were erratic throughout each year, but mean weekly seawater temperatures were similar in 1972 and 1973 (Fig. 16). Furthermore, the apparent mid-summer spawning hiatus coincided with the highest temperatures in each year. The data of McCrary (1969) reveal a similar relationship. Clearly, experimental work on the effects of temperature on gametogenesis in this species is needed to clarify what relationship, if any, exists.

The assumption of a bimodal breeding season for *L. tenuis* requires that gametogenic growth, which occurred over a five-month period during the winter and early spring, could also reach completion in as little time as one month or less in summer or fall. The data (Fig. 16) indicate that this is, indeed, the case. On July 29, only small oocytes (30 μm or less) were observed in the population, but on August 27, 29 days later, mature oocytes (200 μm) were found. The increase of 170 μm in oocyte diameter is equivalent to a 19.1% per day increase in volume (calculated by the instantaneous relative growth rate method of Brody, 1945). There are no data in the literature concerning these growth rates in holothurian oocytes, but there are reports of oocyte growth rates for the echinoid, *Strongylocentrotus purpuratus* (Gonor, 1973) and the crinoid, *Comanthus japonica* (Holland, Grimmer, and Kubota, 1975). However, these species required almost a year for oocyte maturation, and they spawned only during one short interval each year. Only the winter growth rate of *Leptosynapta tenuis* oocytes (approximately 4% per day) was comparable to the rates for *S. purpuratus* and *C. japonica*.

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Summary

1. Gonads of *Leptosynapta tenuis* were examined histologically, and gametogenesis in this apodid holothurian is described.

2. *L. tenuis* is a simultaneous hermaphrodite. Each animal produces and sheds spermatozoa and oocytes during the same breeding season, although sperm seem to be shed somewhat earlier than oocytes.

3. Testicular maturity indices and oocyte diameter measurements revealed a bimodal annual reproductive cycle in a North Carolina population sampled at monthly intervals over a fifteen-month period. Breeding occurred for about three months both preceding and following a month, or so, of breeding inactivity in July or August.

4. The mid-summer absence of mature gametes in the population coincided with the highest seawater temperatures, suggesting a temperature regulated gametogenic cycle.

5. The high oocyte growth rate leading to the second spawning season in the autumn is perhaps five times that of the winter rate.

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DEVELOPMENT OF *AMPHIOPLUS ABDITUS* (*VERRILL*) (ECHINODERMATA: OPHIUROIDEA). II. DESCRIPTION AND DISCUSSION OF OPHIUROID SKELETAL ONTOGENY AND HOMOLOGIES [1]

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The descriptive studies of the last century, meticulous and seemingly irrefutable, sometimes conceal serious flaws. The comprehensive scheme of ophiuroid skeletal anatomy of Ludwig (1878, 1881, 1899, 1901), for example, is presented in numerous treatises (*e.g.*, Bather, Gregory, and Goodrich, 1900; MacBride, 1906; Cuénot, 1948; Hyman, 1955; Spencer and Wright, 1966) as a cornerstone for theories of echinoderm systematics and phylogeny. In the present paper, Ludwig's deductions and observations on ophiuroids are compared and contrasted with aspects of the anatomy and ontogeny of *Amphioplus abditus*, and a new interpretation of the ophiuroid skeleton is suggested. In addition, it is shown that juveniles of *A. abditus* undergo such drastic changes in skeletal morphology during ontogeny, that different developmental stages might easily be mistaken for the young of other genera. The implications of these transformations for systematics of the amphiurids are considered. Terminology employed for this treatment is based on accepted names of structures in the adult ophiuroid, except for "buccal scale" (*cf.*, Hyman, 1955; Thomas, 1962). The nomenclature and abbreviations are summarized in Figure 1.

Materials and Methods

Amphioplus abditus is a burrowing amphiurid with direct development. Its demersal, lecithotrophic embryos develop within a fertilization membrane that rests on the sediment (Hendler, 1977). Specimens were collected and treated as previously described (Hendler, 1977). Postlarval stages were reared in vessels of sediment, partly immersed in a running seawater system to maintain the cultures near field temperatures. Juveniles were collected from Beebe Cove, Noank, Connecticut, and Wild Harbor, West Falmouth, Massachusetts.

Skeletal development was studied using whole or dissected specimens which were dehydrated, cleared, and mounted in "Permount." The hard-parts were examined with both regular illumination and polarized light, and Rose Bengal or Grenacher's Borax Carmine were used to stain the water-vascular system (Humason, 1967).

Results

Larval skeleton

A triangular granule appears in both posterior angles of the 24-hour embryo, and each granule grows to a tetraradiate spicule within five hours. By 33 hours,

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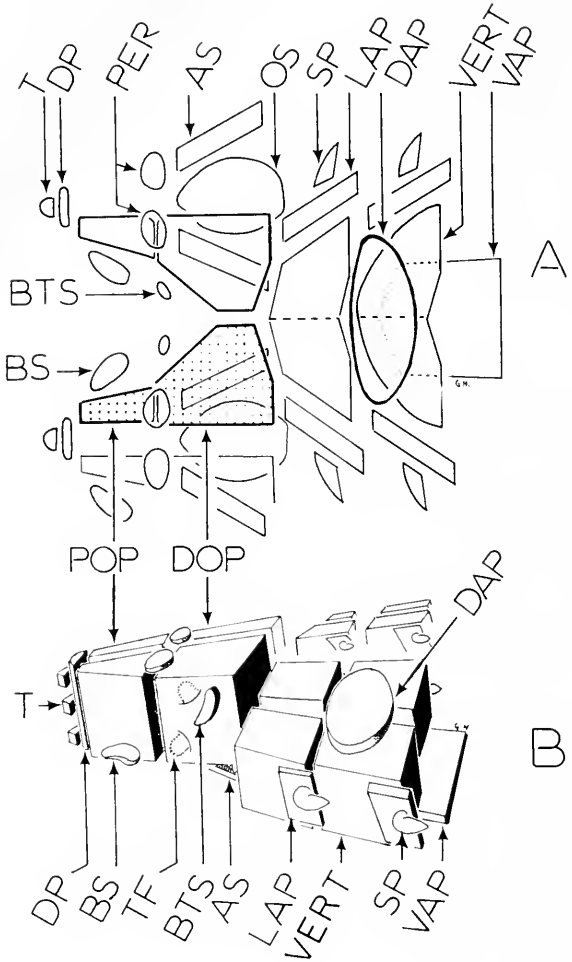

FIGURE 1. A. Diagrammatic portion of an ophiuroid jaw frame, in dorsal view with disc removed; mouth opening is to the left. The base of one arm and two half-jaws are illustrated; ambulacra of the vertebrae are separated by dashes; plates of the jaw shown as unfused pieces. Stippled area = oral plate = half-jaw = proximal oral plate + distal oral plate. B. Diagrammatic three-dimensional representation of an ophiuroid oral frame section with disc removed; mouth opening is to the left. An entire jaw and proximal bases of two arms are shown. Ambulacra of each jaw and vertebra are shown as unfused plates. The proximal and distal oral plates of the jaw are modified and abradially directed arm-vertebrae (ambulacra); ambulacral plates from *two* arms comprise each jaw. AS represents adoral shield (adambulacral-2); BS, buccal scale; BTS, first buccal tube-foot scale; DAP, dorsal arm-plate; DOP, distal oral plate (ambulacral-2); DP, dental plate; LAP, lateral arm-plate (adambulacral); OS, oral shield; PER, peristomial plate; POP, proximal oral plate (ambulacral-1); SP, arm spine; T, tooth; TF, buccal tube-foot; TP, terminal plate; VAP, ventral arm-plate; and VERT, vertebra (ambulacrum).

the four points of the spicule lengthen and give rise to lateral and terminal branches while several short, branching extensions accumulate at the nexus of the primary

rods (Fig. 2). Resorption of the larval skeleton normally begins around 57 hours (Fig. 2), and between 74 and 84 hours, as the embryonic arms disappear, the

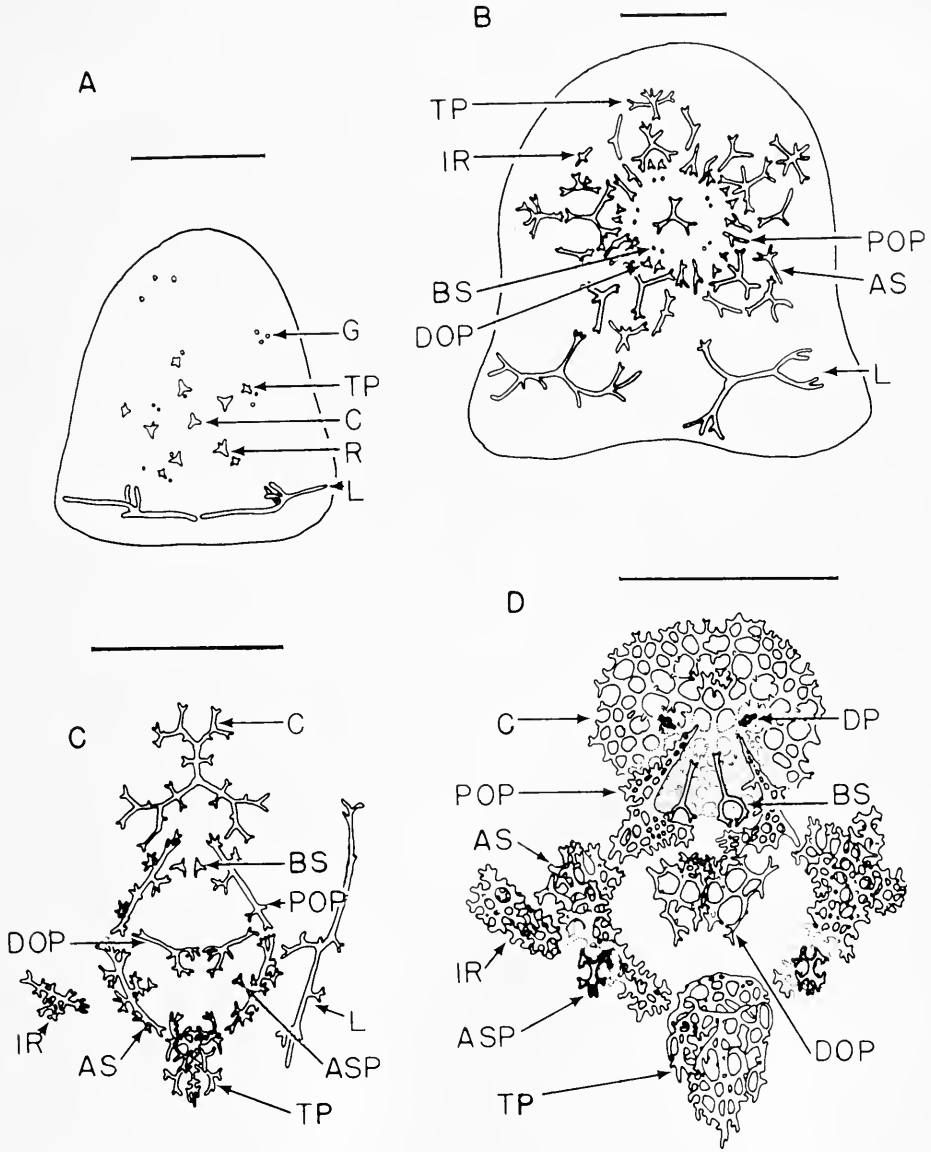

FIGURE 2. Diagrams showing approximate arrangement and relative size of developing skeletal elements (in ventral view). C and D show only one radial portion of the embryo: A, late triangular embryo, 35 hr; B, early star-disc stage, 55 hr; C, late star-disc stage, 73 hr; and D, newly hatched juvenile, 96 hr. Scale line is approximately 0.1 mm. Abbreviations as in Figure 1, and ASP represents adoral shield spine; C, central plate; G, granules of presumptive spicules; IR, interradial-1; L, larval skeleton; R, radial plate; and TP, terminal plate.

TABLE I

Chronology of skeletal element formation.

Time	Disc diameter (mm)	Aboral surface	Oral surface
35 hr		Radial plates, central plates, terminal plates	
35–42 hr			Adoral shields, proximal oral plates (POP)
45–55 hr			Distal oral plates (DOP)
48 hr			Interradial 1 (initial)
74 hr			Adoral shield spines
84 hr			Dental plates
96–147 hr			Interradial-1 (2nd-5th)
117 hr			Ventral arm-plate-1 (VAP)
110–147 hr	0.3		Teeth
55 days			Lateral arm-plate-1 (LAP)
55–72 days			Vertebrae, lateral arm-plate spines, ventral arm-plate-2
Number of arm segments			
2–3 (<5 months)	0.4		Dorsal arm-plate-1 (DAP), madreporite, oral shields
3–4 (8 months)		Radial shields	
5	0.7	Interradial-2	Infradental papillae, ventral disc scales
7		Peristomials	Second teeth
8			First buccal tube foot scales
9	0.9		Genital scales
15			Genital plates
17	1.1		Third oral papillae, fourth oral papillae (from adoral shield spines)
20	1.3		
>30	1.6		
≫30	1.9	Interradial muscle scales, dorsal disc scales	Tentacle scales, accessory scales
>41	2.3		
≫38	2.5		
82	3.5		

branched skeletons dwindle to straight pieces with furcate tips and then are lost (Fig. 2).

The larval skeleton of *Amphioplus* compares with that of the typical ophiopluteus, the four major branches corresponding to the body, posterolateral, anterolateral and postoral rods of planktonic ophiuroid larvae. The body rods of *A. abditus* bear branched tips which may be homologous to transverse and end rods, even though the tips do not articulate. However, the general shape and pattern of secondary branching of the skeleton varies between specimens, and for each individual the skeleton in one arm is larger or more complex than its counterpart in the other arm.

It is noteworthy that by 35 hours of development separate portions of the larval

skeleton, but not the ophiuroid skeletal elements, have distinct and different angles of extinction under polarized light. In other words, a larval skeletal element, which grows from a tetraradiate spicule, does not act as a single calcite crystal but is composed of irregular segments with different crystallographic orientations. This condition, unusual for echinoderm skeletons, may be an effect of a peculiarity in the coordination between the skeleton-depositing cells.

Ophiuroid skeleton

Between 30 and 35 hours, the radial, central, and terminal plates appear and regroup from a sagittal plane to form concentric rings on the ventral surface of the embryo (Fig. 2, Table I). Judging from the relative size (breadth rather than mass) of the spicules in slightly older specimens, the radial, central and terminal plates are produced in that order.

Minute, granular rudiments of the proximal oral plates and adoral shields are visible by 35 hours (Fig. 2). By 42 hours the adoral shield rudiments are larger than the proximal oral-plate rudiments, and the spicule is triradiate. Some 45-hour specimens have granular rudiments of the buccal scales and the distal oral plates.

By 55 hours the embryonic ophiuroid rudiment is pentaradiate, and its skeletal plates are arranged as successive stacks of concentric rings. In the dorsal-most plane are central and radial plates; terminal plates, shaped like stick figures with outstretched arms, are more ventral and on the perimeter of the disc; the branched proximal oral plates and adoral shields are, respectively, proximal and distal to the center of the disc and at a level below the terminals; and buccal scale granules and triradiate distal oral plates are near the ventral surface (Fig. 2).

In some specimens, after 45–55 hours of development, a single triradiate spicule, a rudiment of the initial aboral interradial plate (interradial-1), appears at the edge of the ophiuroid rudiment, ventral to the terminal plates and at the right side of the embryo in the interradius anterior to the right larval skeleton. Interradial-1 plates do not appear in the other four interradii until 96 hours of development.

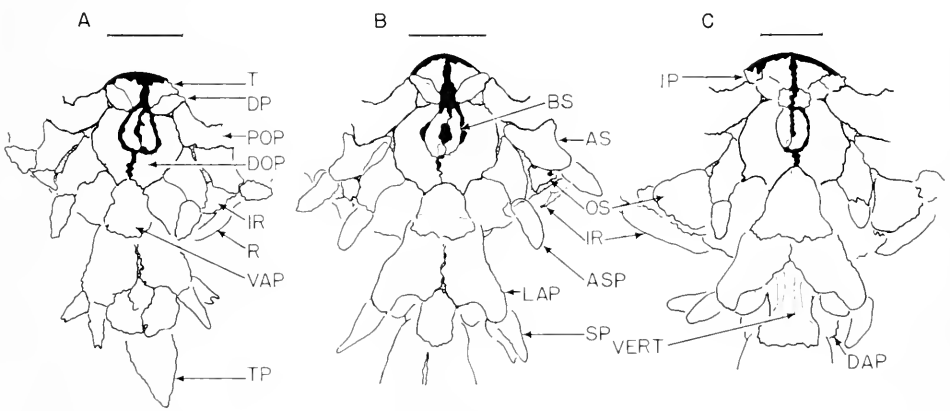

FIGURE 3. Portion of the disc, in ventral view, for different stages of development: A, 1-2 arm-segment stage; B, 3 arm-segment stage; and C, 5 arm-segment stage. Abbreviations as in Figures 1 and 2; and IP represents infradental papilla; R, radial plate. Scale line is 0.1 mm. Dotted lines separate DOP and POP as seen in polarized light.

Triradiate rudiments of the adoral shield spines appear at 74 hours, and by 84 hours the dental plates materialize as spicules proximal to the five jaws (Figs. 2 and 3). From 90 to 110 hours the skeletal elements of hatching juveniles become denser and approach their final form (Figs. 2 and 3).

By 96 hours, there are triradiate spicules, rudiments of the first interradial plates, in a plane beneath the radial plates (except for the radius with a precocious interradial-1). By 147 hours, these spicules form small plates with multiple branches; by 23 days they are larger and nearly perpendicular to the radial plates, and they then migrate to the dorsal surface of the disc.

The adoral shield spines move to the shield and grow beyond the edge of the disc. At the time of hatching, the juvenile has its dorsal surface shingled by a rosette of overlapping primary plates and around the disc there are spike-like, protruding terminal plates and adoral shield spines. Shortly before hatching (about 167 hours) on the ventral surface of the disc, the distal end of the proximal oral plate enlarges and fuses with the distal oral plate (the net-like stereom of the latter remains distinctive even after fusion). Pairs of oral plates articulate to form jaws by 230 hours.

Several days after hatching (by 196 hours) the first series of teeth, initially sparsely-branched triradiate spicules, form at the proximal surface of the dental plate, but they do not take their final massive, blunt-tipped form until the 9 to 17 arm-segment stage of development.

Rudiments of the ventral arm-plates, already present at 117 hours, become dense, fenestrate plates with an elongate pentagonal shape by 13 days. Opacity of the ventral arm-plates and the appearance of lateral arm-plates by 55 days obstruct examination of the vertebrae, although slender, unfused vertebral ossicles are still discernable until 72 days when the first arm spines and second series of ventral arm-plates appear. The first dorsal arm-plates are added to the arm before three months.

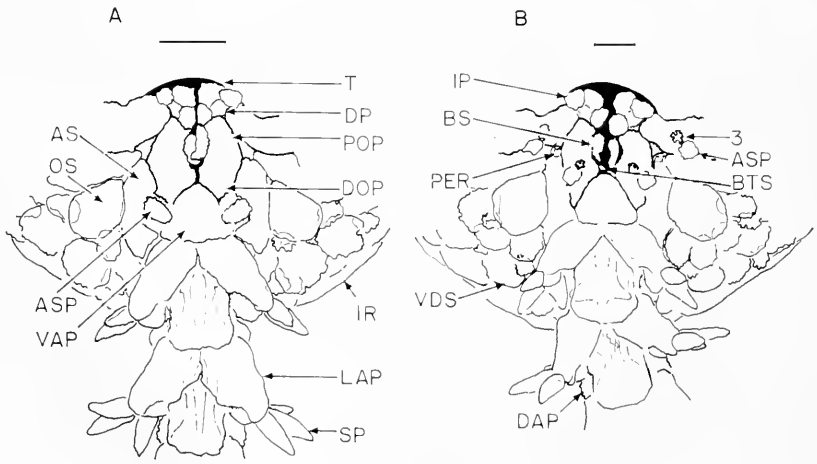

FIGURE 4. Portion of the disc,, in ventral view, for different stages of development: A, 9 arm-segment stage; and B, 17 arm-segment stage. Abbreviations as in Figures 1 to 3; and 3 represents third oral papilla; VDS, ventral disc scales. Scale line is 0.1 mm. Dotted lines separate DOP and POP.

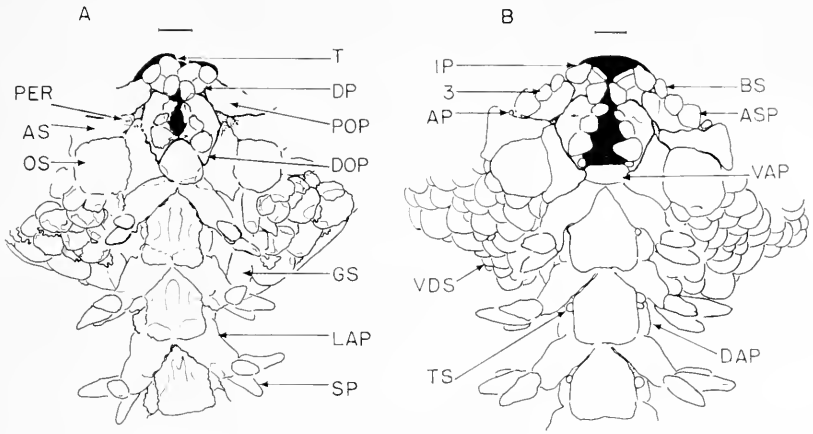

FIGURE 5. Portion of the disc, in ventral view, for different stages of development: A, 21 arm-segment stage; and B, >30 arm-segment stage. Abbreviations as in Figures 1 to 4; and AP represents accessory papillae; GS, genital scale; TS, tentacle scale. Scale line is 0.1 mm. Dotted lines separate DOP and POP.

As each new arm segment is added a ventral arm-plate appears before the lateral arm-plates and arm spines, and a dorsal arm-plate finally forms to cap the segment. The arm-plates and spines take a form characteristic of the adult by the 17 arm-segment stage.

Rudiments of the oral shields and madreporite do not appear until the third arm-segment begins to form (by five months). They form *in situ* on the oral surface of the disc, proximal to the interradial-1 series (Fig. 3). Even so, the stone and pore canals associated with the madreporite are present before the two arm-segment stage. By eight months there are four arm-segments, and radial shields appear at the base of the arm, on the ventral surface of the disc.

An exact chronology cannot be assigned for subsequent skeletal developments, as they were deduced using a growth series organized from material in field collections (Table I; Figs. 3, 4, and 5). There is no important change in the armature of the disc until the 5 arm-segment stage, when pairs of infradental papillae appear distal to the dental plates (Fig. 3). The most striking superficial change at this time is the addition of minute scales on the ventral surface of the disc, which separate the oral shields and interradial-1 plates. Interradial-2 plates erupt between the radials, isolating the radial plates from the interradial-1 on the dorsal surface of the disc (Figs. 3, 4, and 5). The central primary plate and the radial plates continue to grow until the disc diameter is 5 mm and then diminish in size with further growth. This allometric pattern is species-specific (Hendler, 1973).

There are three important series of minute plates within the disc. Reticulate peristomial plates are added by the 7 arm-segment stage on the inner surface of the jaw between the proximal and distal oral plates (Fig. 6). They probably arise as single spicules at an earlier stage, but there is no doubt that the peristomial plates and buccal scales are present at the same time (Fig. 6). By the 8 arm-segment stage, pairs of tiny plates form within the disc, distal to the buccal scales, and just proximal to each first ventral arm-plate. These plates will be referred to below as

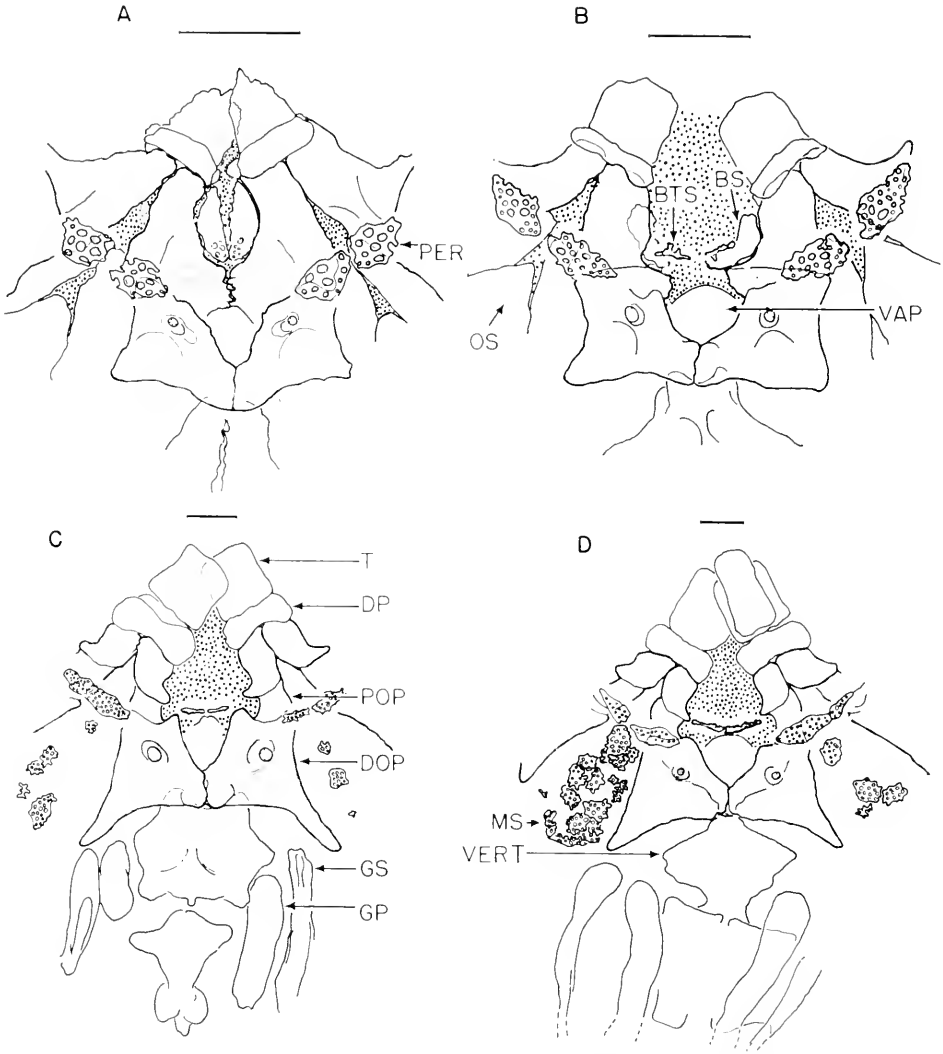

FIGURE 6. Portion of the oral frame, in dorsal view, with disc removed, for different stages of development: A, 7 arm-segment stage; B, 13 arm-segment stage; C, >30 arm-segment stage; and D, >41 arm-segment stage. Abbreviations as in Figures 1 to 4; and GP represents genital plate; GS, genital scale; MS, interradial muscle scale. Scale line is 0.1 mm. Note that buccal scales, which lie on the oral surface, cannot be seen in C or D. Dotted lines separate DOP and POP.

the first buccal tube-foot scales (corresponding plates have been treated as ventral arm-plates or homologues of ambulacral plates by various authors).

By the 9 arm-segment stage, due to growth of the adoral shield, the adoral shield spine sits near the center of the shield plate (Fig. 4). It then acts as a "tentacle scale" for the second buccal tube-feet, analogous to the minute plates on

the ventral surface of the arm that shield the contracted tube-feet (tentacles). The buccal scales transform from crescentric scales to blunt spines. The madreporite exhibits an external perforation for the hydropore. The genital scales were seen in a 9 arm-segment individual; and genital plates were seen in a specimen of 15 arm-segments, but, undoubtedly, both originated at an earlier stage.

By the 17 arm-segment stage the rudiments of the third oral papillae appear on the distal surface of the oral plates (Fig. 4). The spines of the adoral shield migrate to form additional oral papillae (the fourth oral papillae). Thus, by the 21 arm-segment stage, each row of oral papillae consists of a block-like infradental followed by a spine-like papilla (formed by a buccal scale), a third papilla (chronologically the youngest), and a large distal papilla (formed by an adoral shield spine) (Fig. 5). Oral shields of adjacent jaws are initially tightly appressed, but the first ventral arm-plates, which begin migrating into the buccal area by the 5 arm-segment stage, spread apart the adoral shields.

When more than 30 arm-segments have formed, the oral plates begin to take a definitive shape with enlarged lateral extensions for the insertion of the external interradial muscles and a deep cleft for the water-vascular ring (Fig. 6). In addition, a scattering of small thin plates accumulates on the surface of the interradial muscles (Fig. 6). The paired peristomial plates lengthen and then degenerate (or even fuse?), becoming fragile scales like those on the interradial muscles. The rudimentary skeletal elements proximal to the ventral arm-plates (first buccal tube-foot scales) form reticulate pieces perpendicular to the axis of the arm. By this time tentacle scales have appeared on many arm-segments, one scale per tube-foot.

The first buccal tube-foot scales located within the oral cavity surpass the size of the peristomial plates; in larger specimens they form thin, boomerang-shaped elements beside the inner oral papillae. While the peristomial plates stop growing and/or degenerate, the scales over the interradial muscles grow larger and more numerous, obscuring the peristomials. The interradial muscle scales finally form a medial band on the muscles in the adult (especially dense on the muscle above the madreporite) (Fig. 6).

On the dorsal surface of the disc, the number of tiny scales separating the primary and interradial plate continues to grow, but two series of interradial plates remain distinct. The interradial-1 plates remain on the edge of the disc until the specimens have at least 82 arm-segments (disc diameter = 3.5 mm), and in larger animals they are left on the dorsal surface of the disc as the rate of disc growth increases centrifugally. On the ventral surface of the disc, small accessory scales (the fifth oral papillae) form distally to the fourth oral papillae, and additional accessory scales may appear in later stages of growth (Fig. 5).

Discussion

The derivation of the oral frame and the armament of oral papillae bordering the buccal cavity are discussed in detail, as these are the features of *A. abditus* that best illustrate the systematics of amphiurids (the largest family of ophiuroids) and the affinities of the echinoderm classes. The apical rosette of primary plates, radial shields, and the madreporite will be discussed first, since these structures figure prominently in evaluations of echinoderm phylogeny.

In the ophiuroids the larval skeleton plays no part in the formation of the apical system (central and radial primary plates), since the larval skeleton of ophiuroids is resorbed, as in *A. abditus,* or sometimes "discarded" (Mortensen, 1931; Olsen, 1942; Hendler, 1975). In echinoids, on the other hand, the larval skeletal pieces generate the genital and terminal apical plates (Onoda, 1931). The origin of both the larval skeleton and apical plates has been thought to provide evidence for a close affinity between the echinoids and ophiuroids. However, the divergence seen in the ontogeny of ophiuroids and echinoids implies that there is nonhomology of the larval skeleton and/or the apical plates of the two classes.

Regardless of discrepancies in homology between ophiuroids and echinoids, the central plate of ophiuroids appears to be homologous to the central plate of asteroids. Apart from this characteristic, the similarities between the discs of ophiuroids and asteroids are few. Prominent structures of the ophiuroid disc, such as radial shields, oral shields, and genital plates and scales, seem to be ophiuroid specializations without homologues in asteroids.

Each radial shield (and the other major skeletal elements) of *A. abditus* and other ophiuroids originates from a single element (not by fusion of scales as suggested in Spencer and Wright, 1966). The radial shields (and genital plates and scales) reappear when the disc of *Amphioplus* regenerates, but the primary plates (loci of disc scale formation) never are replaced (personal observation). This morphogenetic difference between the central plate and radial shields indicates that the primary plates of ophiuroids are atavistic structures that are recapitulated during ontogeny but are not necessary for the growth of the disc.

Even structures, such as the madreporite, which have been judged to show close affinities between the ophiuroids and asteroids, point up differences between the classes. Although in the recent ophiuroids an oral shield acts as the madreporite, some primitive ophiuroids possess a madreporite but lack oral shields (Spencer and Wright, 1966). It is possible that a madreporite formed of an oral shield is an innovation in the recent ophiuroids, but a different structure held the water-pore in the ancient ophiuroids. In other words, the madreporites may be nonhomologous in recent ophiuroids, ancient ophiuroids, and asteroids. Furthermore, the "precocious interradial" plate and early formation of the hydroporic canal observed in *A. abditus* could be vestiges of the primitive madreporite.

The madreporite of asteroids is usually dorsal, and ophiuroids generally have a ventral madreporite. Hence, the relationship of these two classes has been gauged by comparing the point of origin of the ophiuran madreporite: whether on the ventral hemisome (indicating lack of affinity with asteroids), or by way of migration from the dorsal surface (indicating a close relationship) (Ludwig, 1881; Mortensen, 1912, 1921; Murakami, 1940, 1941). In fact, all recent ophiuroids reported to have a ventrally migrating madreporite may actually have a "precocious interradial." For example, Murakami (1940) described a dorsal to ventral migration of the madreporite in *Axiognathus* (= *Amphipholis*) *squamatus,* but his illustrations show that the "madreporite" is identical to the "precocious interradial" of *A. abditus;* so the true madreporite of *Axiognathus* must originate at its final, ventral location. This suggests that formation of the madreporite *in situ* on the ventral surface is a characteristic distinguishing recent ophiuroids from asteroids. But, without more information, the precocious interradial of ophiuroids cannot be

considered a homologue of the interradial plate, which acts as a madreporite, in recent asteroids.

Just as the superficial structures of the disc discussed above, such as the apical plates, radial shields and madreporite, suggest drastic distinctions between classes of echinoderms, basic differences in the formation of the oral frame further emphasize the divergence between ophiuroids and asteroids. In the ophiuroid embryo the oral frame is produced by a rearrangement of the paired, serial, skeletal elements that fuse to form "vertebrae" inside each arm. At the base of the arm, instead of forming vertebrae, the elements fuse with corresponding pieces in the neighboring arm, making a connecting bridge between adjacent arms that projects into the oral cavity as a tooth-bearing jaw. Half of each bridge is an "oral plate" comprised of elements from a single arm. Each jaw consists, in effect, of two oral plates (half-jaws) and accompanying elements from two contributing arms.

In his studies of this oral frame system, Ludwig (1878, 1881, 1889, 1901) homologized the ophiuroid and asteroid oral frames. He considered the proximal oral plates of the ophiuroid jaw (the proximal portion of each half-jaw) to be the first adambulacral plates, the adoral shields as the second adambulacrals, and the lateral arm-plates as serially analogous adambulacrals. He described the fusion of the proximal and distal oral plates during ontogeny and claimed the distal oral plates to be the second ambulacrals, serially homologous to the vertebrae of the arm. Ludwig believed the peristomial plates developed from the early-forming buccal scales and felt they represented the first ambulacrals of the jaw. This relationship between the peristomial plates and the buccal scales, and their identification as the first ambulacral plates, are both erroneous. Consequently, Ludwig's analogies between ophiuroid and asteroid jaws are not legitimate.

Zur Strassen (1901) pointed out that the peristomials (0 to 3 in number in different species) were not paired in each jaw as proper ambulacrals must be. Moreover, he observed that buccal scales, which Ludwig proposed to be the rudiments of peristomial plates, were present concurrently with, and hence unrelated to, peristomial plates. In the species that zur Strassen studied, the peristomials persist and the buccal scales are resorbed. In contrast, in *A. abditus* the peristomial plates are lost and the buccal scales become oral papillae. These observations indicate a basic flaw in Ludwig's scheme, calling into question the identity of the first plate of the ambulacral series and the affinities of the oral plate elements and buccal scales.

Just as Ludwig proposed, the adoral shield is adambulacral and serially homologous with the lateral arm-plates, as indicated by its position in relation to the arm and its transitory possession of a spine (Simroth, 1876; Fewkes, 1887; Murakami, 1937). The distal oral plate, clearly associated with the water-vascular system, is obviously an ambulacral plate, but the identity of the proximal oral plate is an unresolved problem. Here it is suggested that the proximal oral plate, rather than the peristomial, is the first ambulacral plate of the jaw.

Alone, the association of two pairs of tube-feet with the jaw indicates that two elements of the oral plate are ambulacral, and there are only two parts of the jaw *per se*, the proximal and distal oral plates. The attachment of the proximal oral plate in a series with the distal oral plate suggests that it is an ambulacral element, but the buccal tube-feet of *A. abditus* penetrate only the distal oral plate, suggesting

that the proximal oral plate is not ambulacral. But, just as the adoral shields are homologous to lateral arm-plates, although the orientations of the shields and plates are different, the obliquely oriented proximal oral plates may be ambulacral even though they are bypassed by the water-vascular system. The separation of the proximal oral plate and the water-vascular system may simply result from the formation of buccal tentacles in reverse order in *A. abditus* and other ophiuroids (Müller, 1851; Krohn, 1851; Apostolides, 1882; Grave, 1900; MacBride, 1906; Mortensen, 1921; Fell, 1941, 1946) and the inward migration of the buccal scales and marked dorsal rotation of the ventral arm-plates.

Thus, the jaw of ophiuroids must constitute two transformed arm-segments which are highly modified and "incomplete." In the first "segment" there is an ambulacral proximal oral plate, but the identity of the adambulacral plate is problematical, and homologues of accessory plates are lacking. The second "segment" consists of the distal oral plate (ambulacral), adoral shield (adambulacral), and the ventral arm-plate. There seems to be no unequivocal homologue of the adambulacral-1 in the oral frame of recent ophiuroids. As explained above, the peristomial plates are obviously secondary structures (not adambulacrals). The buccal scales, however, might be adambulacrals, but without corroborating evidence they cannot be considered a homologue of the adambulacral plates in the first modified arm-segment. The occurrence in primitive ophiuroids of a typical jaw structure, and the presence of two pairs of oral tentacles and a modified proximal oral plate (Sollas and Sollas, 1912; Schuchert, 1915; Spencer, 1951), demonstrate both the ambulacral nature of the proximal oral plates and a basic class-wide concord in ophiuroid jaw morphology. Besides the ambulacral affinity of the proximal oral plate, the fossil record suggests a recent origin for the peristomial plates and the buccal scales, as oral papillae and buccal scales appear to be absent in ophiuroids prior to the Silurian (Spencer and Wright, 1966). Such negative evidence must be weighed against the high probability of preservational bias, especially when minute elements such as these papillae are concerned.

The ambulacral nature of the proximal oral plate makes the ophiuroid jaw arrangement consistent with that in the "somasteroid" taxa, while in other asteroids the proximal element of the jaw is an adambulacral homologue (Fell, 1963; Turner and Dearborn, 1972). Besides this major contrast between the classes, differences, such as the fusion of different ambulacrals—numbers two and three in somasteroid forms (Fell, 1963), numbers one and two in the ophiuroids—and no fusion in the asteroids, also reflect the considerable divergences between ophiuroids and asteroids. Clearly, in light of the differences in homology of the oral frame, the differences in ontogeny and morphogenesis of the madreporite and primary and interradial plates, and the unique structures of ophiuroids such as radial shields and genital plates and scales, the relationship of asteroids and ophiuroids begs reevaluation.

The examination of skeletal ontogeny has revealed discrepencies in the phylogeny of the echinoderm classes, but ontogeny of the oral armature can indicate systematic relationships within the family Amphiuridae. Therefore, before discussing the systematics of the amphiurids, the origins of the oral papillae are reviewed.

In *A. abditus* the infradental and third oral papillae and the fifth (accessory) papillae originate *in situ*. They are secondary structures (*i.e.*, not of the ambu-

lacral-adambulacral skeletal groundwork). In contrast, the second and fourth papillae migrate to their ultimate positions while undergoing a transformation of shape and function.

The fourth (distal) papillae originate as formidable spines on the adoral shield and are used initially for locomotion and balance, but they are dwarfed and relocated during growth of the oral frame and ultimately are retained as diminutive scales on the oral plate. Adoral shield spines of other species develop as in *A. abditus,* disappear during development, or even take a peripheral position on the disc (Mortensen, 1933b; Murakami, 1941; Schoener, 1967).

In *A. abditus,* the only species of ophiuroid whose buccal scales have been traced through development to the adult stage, the buccal scales of the juvenile are among the first-formed and most prominent skeletal elements. It is remarkable that they initially close the oral gap but as their growth slows in relation to that of the oral plate, they "sink" into the oral slit and become the diminutive second oral papillae. In contrast, the buccal scales are resorbed during development in *Axiognathus* (= *Amphipholis*) *japonicus* and *Axiognathus* (= *Amphipholis*) *squamatus* (Ludwig, 1899; zur Strassen, 1901; Sollas and Sollas, 1912; Mortensen, 1912, 1913, 1933a, 1938; Murakami, 1937, 1940, 1941; Guille, 1964). Whether all ophiuroid species have buccal scales is not known.

Since the second oral papillae arise from the buccal scales in *Amphioplus,* and they differ from tentacle scales in their time of origin and mode of growth, they should not be called "oral tentacle scales" (*cf.,* A. M. Clark, 1970). The term "buccal scales" may be used for the early-forming "spoon-shaped" plates of the buccal gap whether or not they are resorbed in the adult; but the term "second oral papillae" should be used instead of "oral tentacle scales" for adult amphiurids.

It is not certain whether both sets of buccal tube-feet have tentacle scales. The accessory scales (fifth oral papillae) associated with the second buccal tube-feet, which are obviously tentacle scales, arise long after the other four oral papillae and, as the individual grows, they increase in number to a maximum of more than five per tube-foot (Hendler, 1973). There are calcareous elements, proximal to the first ventral armplates in *A. abditus* which form during the 7- to 9-arm-segment stage, that may be tentacle scales of the first buccal tube-feet. They resemble internal buccal elements described in other species (Ludwig, 1878; Mortensen, 1912; zur Strassen, 1901; Sollas and Sollas, 1912). If these elements are not tentacle scales, then the first buccal tube-feet lack scales.

From the early appearance of infradental and distal oral papillae in *A. squamatus* and the putative juveniles of *Amphioplus acutus,* it has been inferred that these species pass through an "*Amphiura*" stage (H. L. Clarke, 1914; Mortensen, 1936). Such phylogenetic speculations are vulnerable, being based on a single character which (in the case of *Amphiura* oral armature) could easily be paedomorphic or secondarily reduced. Juveniles of *Axiognathus* or *Amphioplus* may resemble early stages of *Amphiura* or even *Amphiodia,* but it cannot be assumed that these genera recapitulate an "*Amphiura* stage" before homologues of the oral papillae of all amphiurid genera are delineated.

On the basis of their oral armature, the amphiurids may be divided provisionally into two categories: those, like *Amphiura* and *Amphioplus,* with the second oral papillae located high on the oral plate; and those, like *Amphiodia* and *Amphipholis*

or *Axiognathus,* that lack second oral papillae. It is shown above that the buccal scale develops into the second papilla in *A. abditus.* However, zur Strassen (1901), Murakami (1940), and others described resorption of the buccal scale in *Axiognathus* (= *Amphipholis*) species, and these findings have been confirmed in *Axiognathus squamatus* from New England (personal observation). Thus, it is predicted that *Amphiodia* and *Amphipholis* (like *Axiognathus*) resorb the buccal scales, while *Amphiura* species (like *Amphioplus*) retain the scales as the second oral papillae.

The adoral shield spines develop in different ways in different families and genera, but it would be interesting to see whether the adoral shield spines of amphiurids always become distal oral papillae, demonstrating uniformity in the ontogeny of the amphiurid jaw. The adoral shield spine of *Axiognathus japonicus* develops identically to its homologue in *A. abditus,* but whether oral papillae of all amphiurids are homologous remains to be seen. The ontogeny of *A. abditus* supports A. M. Clark's (1970) contention that *Amphioplus,* bearing five oral papillae, occupies a central position among the amphiurids such that the oral formulae of other genera are derived by a simplification of the *Amphioplus* oral armature. Further studies are needed, however, to decide whether this complex oral structure is a primitive or an advanced trait.

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Summary

1. *Amphioplus abditus* has a vestigial two-piece larval skeleton that has portions with different crystallographic orientations. The larval skeleton is resorbed and, unlike that of echinoids, it does not act as a center of formation of the plates of the adult. The major skeletal elements of the adult develop from single (usually triradiate) spicules, and there is a uniform crystallographic orientation within each plate.

2. The radial shields, adoral shields, genital plates and genital scales are ophiuroid specializations without homologues in the asteroids. Ophiuroids can regenerate radial shields but not the apical primary plates (the latter are probably atavistic structures).

3. The madreporite and oral plates, generally thought to migrate from the dorsal surface of the disc, originate *in situ* on the ventral surface of *A. abditus*. A dorsolateral plate, probably confused with the madreporite in past studies, is a precociously formed interradial-1. The formation of a "precocious interradial plate" could be a vestige of the primitive ophiuroid madreporite. In fact, the madreporites of asteroids, ancient ophiuroids, and recent ophuroids may not be homologous.

4. The origin of each of the oral papillae is described. Buccal scales, previously (and incorrectly) thought to develop into peristomial plates, form the second oral papillae in *A. abditus*. Consequently, the second oral papillae of amphiurids should not be considered "oral tentacle scales". The true tentacle scales are cryptic structures within the buccal cavity.

5. The oral papillae of the different amphiurid genera are probably homologous. Judging from differences in the oral frame, there are probably two major amphiurid groups composed of taxa which retain the buccal scales as oral papillae (*Amphioplus* and possibly *Amphiura*), and those like *Axiognathus* (and possibly *Amphipholis* and *Amphiodia*) which resorb the buccal scales.

6. A new system of homologues is suggested for the plates of the ophiuroid oral skeleton. The proximal oral plate is considered the ambulacral portion of the first modified arm-segment and buccal scales may be the first pair of adambulacrals. The distal oral plates (ambulacral), adoral shields (adambulacral), and the first ventral arm-plate (within the buccal slit) compose the second transformed arm-segment of the oral frame. This pattern of homology, together with the dissimilarities between ophiuroid and asteroid discs constitute important differences between the ophiuroids and asteroids.

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A GENERIC REVISION OF THE BRACKISH-WATER SERPULID *FICOPOMATUS* SOUTHERN 1921 (POLYCHAETA: SERPULINAE), INCLUDING *MERCIERELLA* FAUVEL 1923, *SPHAEROPOMATUS* TREADWELL 1934, *MERCIERELLOPSIS* RIOJA 1945 AND *NEOPOMATUS* PILLAI 1960

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In the last half century, five monotypic serpulid genera have been described exclusively from brackish waters, and Pillai (1960) has united four of them in the subfamily Ficopomatinae (see below). One of these, *Mercierella*, has received the attention of many biologists in various fields of research. There has been considerable confusion about the identity of two of the species, namely, *Mercierella enigmatica* and *Neopomatus uschakovi* (see lists of synonyms in this paper). This confusion, and the view of the senior author that it was unlikely that similar specializations in the brackish habitat were evolved by five different but evidently closely related genera, made a review necessary. Preliminary work was done by the senior author and later elaborated by the junior author as partial fulfillment of his postgraduate studies.

Materials and Methods

The greater part of the material came from the collections of the British Museum (Natural History) and of the senior author, from whose material small series have been presented to other museums. Several institutions sent material as gifts or loans.

The photographs were taken by Mr. E. van der Vlist and other staff at the Zoölogisch Laboratorium, Rijksuniversiteit, Utrecht. Drawings of opercula were made by using a drawing-prism. In order to make camera-lucida drawings, it was necessary to separate the setae and uncini. This was achieved by putting entire animals in a few drops of 10% KOH for 12–24 hours and subsequently squashing them in glycerin-gelatin. Some figures were drawn from tufts of setae, extirpated from the animals, and preserved in glycerin-gelatin. All figures were drawn by the junior author.

Measurements, unless stated otherwise, are given in mm; meristic values are based upon counts in ten specimens minimally, unless otherwise stated (*e.g.*, n = 3). References in synonymies preceeded by "[?]" indicate a questionable identification in our view. The following abbreviations are used for collections: AHF, Allan Hancock Foundation, University of Southern California, Los Angeles; AM, the Australian Museum, Sydney; AMNH, the American Museum of Natural History, New York; BMNH, British Museum (Natural History), London; MNHN, Musée Nationale d'Histoire Naturelle, Paris; RMNH, Rijksmuseum van Natuurlijke Historie, Leiden; SME, Station Marine d'Endoume, Marseille; tHU,

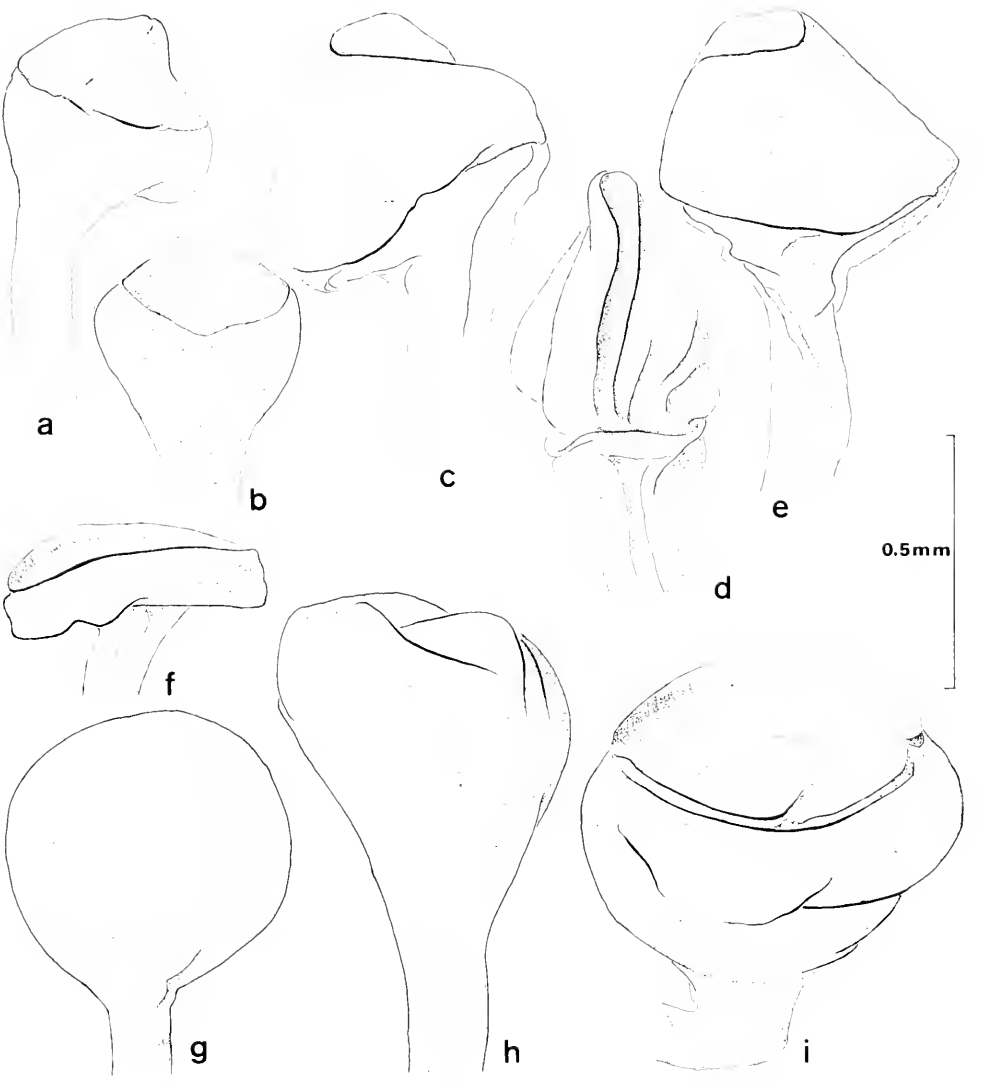

FIGURE 1. Opercula, different orientations. The specimens represented in a-e are *Ficopomatus macrodon* from Taléh-Sap; the series shows the possible development from young to old. The specimens in f–i are *F. miamiensis: f*, syntype from Miami; g, from Curacao; h–i, from Barbados, with and without horny plate. All are to the same scale.

collection of H. A. ten Hove, Utrecht; USNM, National Museum of Natural History, Smithsonian Institution Washington (formerly United States National Museum); ZMA, Instituut voor Taxonomische Zoologie, Zoologisch Museum, Amsterdam; and ZMU, Laboratorium voor Zoölogische Oecologie en Taxonomie, Zoologisch Museum, Utrecht.

Taxonomy

Genus *Ficopomatus* Southern, 1921

Type-species: *Ficopomatus macrodon* Southern, 1921, by monotypy. Gender: masculine. Synonyms: *Mercierella* Fauvel, 1923, type-species: *M. enigmatica* Fauvel, 1923, by monotypy; *Sphaeropomatus* Treadwell, 1934, type-species: *S. miamiensis* Treadwell, 1934, by monotypy; *Mercierellopsis* Rioja, 1945, type-species: *M. prietoi* Rioja, 1945, by monotypy; *Neopomatus* Pillai, 1960, type-species: *N. uschakovi* Pillai, 1960, by original designation.

Original diagnosis: "Modified setae present on the first thoracic segment, having blades provided with very stout teeth. Beneath the blades is a transverse row of more than two teeth. Uncini with relatively few teeth, the lowest of which is in the form of an elongate bifid spine. Ventral abdominal setae geniculate. Operculum fig-shaped, without any outgrowths" (Southern, 1921, p. 655).

Emended diagnosis: Tube white, gradually increasing in diameter toward anterior end and semicircular in cross-section. One or three keels sometimes present.

Thoracic segments seven, with six uncinigerous. Collar setae coarsely serrated and limbate. Remaining thoracic setae limbate. Thoracic uncini saw-like, exceptionally partly rasp-like, with six to twelve teeth visible in profile, including anterior gouged tooth. Uncinigerous tori placed in two, nearly parallel rows. Abdominal setae geniculate with denticulate edge. Abdominal uncini saw- or rasp-like, with one to four rows of teeth, six to fourteen teeth visible in profile, including anterior gouged tooth. Posterior abdominal segments without capillary setae and without dorsal glandular area.

Operculum consisting of bulbous fleshly part, terminated by horny plate; peduncle smooth, without filaments or wings, inserted just below left branchial lobe, near medial line. No pseudo-operculum present.

Collar not lobed, with entire edge, continuous with thoracic membranes which are united ventrally on anterior abdominal segments. Branchial filaments arranged in two semicircles, not united by branchial membrane. Pair of ventral mouth-palps absent.

Key to species of *Ficopomatus*

1. Operculum not "spiny" (Fig. 1) 2
   Operculum "spiny" (Fig. 2) 3

2(1). Operculum with conical horny cap with dorsal furrow (Fig. 1a–e); tube usually with median keel (Fig. 5e) ..... *F. macrodon*
   Operculum without horny endplate, or with slightly concave one (Fig. 1f–i); tube without median keel (Fig. 5a, b) ..... *F. miamiensis*

3(1). Operculum distally convex, "spines" curved outwards (Fig. 2a–d); thoracic membranes fused dorsally ..... *F. uschakovi*
   Operculum distally concave, "spines" curved inwards (Fig. 2f–i); thoracic membranes not fused dorsally ..... *F. enigmaticus*

Discussion: As already stated by Southern (1921, p. 655) the main character separating *Ficopomatus* from all other known serpulid genera is the peculiar shape of the special collar setae. A presumed difference in collar setae and presence of

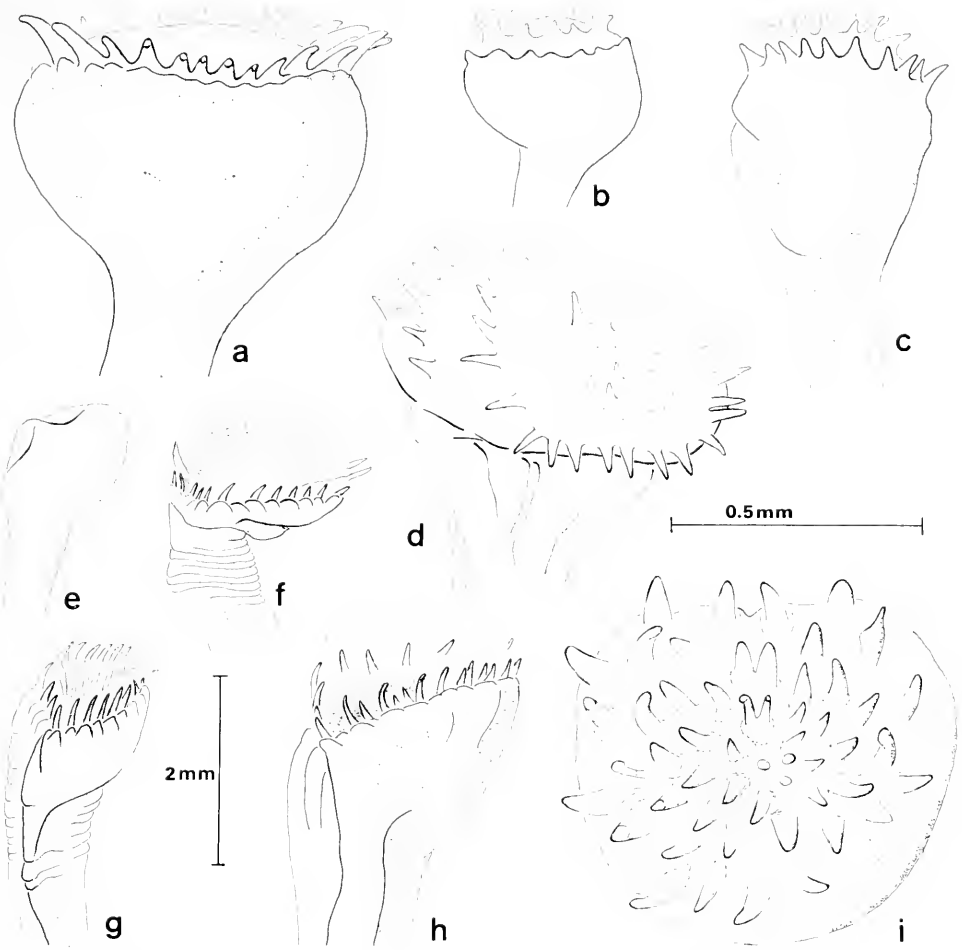

FIGURE 2. Opercula, different orientations. The specimens represented in a–d are *Ficopomatus uschakovi:* a–c, from Guadalcanal; d, paratype of var. *lingayanensis* from Luzon. The specimens in e–i are *F. enigmaticus:* e–h, from the Netherlands; i, from Uruguay. Scale in f, g, h is 2 mm; in remaining figures, the scale is 0.5 mm.

spines on the operculum were the main reasons for Fauvel's (1923, p. 429) proposing the new genus *Mercierella* for his specimens. Erroneously, Treadwell (1934, p. 340) counted only six thoracic setigers, which, along with the presumed difference in collar setae, was his main reason for erecting *Sphaeropomatus* . Rioja (1945, pp. 412–413) acknowledged the similarity of his *Mercierellopsis* with *Sphaeropomatus,* but thought it to be different in the number of thoracic setigers and in the presence of a horny endplate on the operculum of the former (not mentioned for *Sphaeropomatus* by Treadwell, although found to be present on his material). Similar reasons finally led Pillai (1960, p. 33) to describe a fifth genus, *Neopomatus,* although he recognized the similarities among four of the five genera by creating the subfamily Ficopomatinae for them (1960, p. 35). All differential diagnoses,

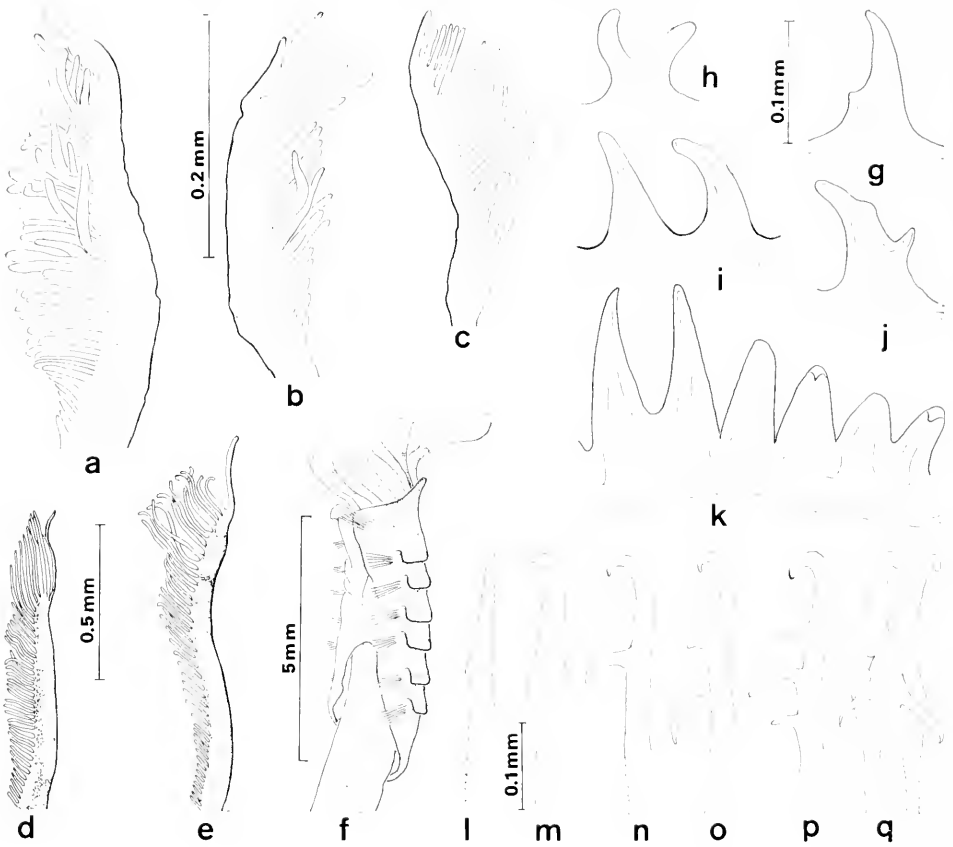

FIGURE 3. Branchial filaments (a–e); thorax (f); and variability in opercular spines (g–q). The specimens represented are: a, f–k, *Ficopomatus uschakovi* from Guadalcanal; b, *F. macrodon* from Taléh-Sap; c, *F. miamiensis* from Jamaica; and d, e, l–q, *F. enigmaticus* from the Netherlands.

given by these authors, were based upon literature only.

Straughan (1966, p. 145) held the opinion that ". . . there is a continuous cline between isolated populations between Sydney, where the brackish water serpulids are typical of *Mercierella,* and Brisbane, where the brackish water serpulids are typical of *Neopomatus* . . ." For that reason she synonymized *Neopomatus uschakovi* with *Mercierella enigmatica.* This has been refuted by Hartmann-Schröder (1971) and Pillai (1971). Although both authors report an enormous infraspecific variability, they still maintain a generic separation of the two species. Pillai (1971) redescribed the genera, with the omission of *Mercierellopsis.*

Thus, most authors agree that there are many similarities in this group, but that there are five different genera based on differences in denticulation of collar setae, the presence of spiny or nonspiny opercula, differences in shape of collar and thoracic membranes, and presence or absence of "peristomes" on the tubes (collar-like rings which indicate the position of former peristomes).

With regard to the differences in collar setae, a considerable variation in their shape may occur within one species, even in one specimen (*e.g.*, ten Hove, 1974, Figs. 4–9, for *Hydroides norvegica* Gunnerus). Extreme variability in opercula was noted by ten Hove (1970, Figs. 77, 121, 123) for *Spirobranchus polycerus* (Schmarda) and (1975, plates I–III) for *Pseudovermilia occidentalis* (McIntosh). Collar and thoracic membranes are thin, fleshy structures; their shape is dependent on the method of preservation. The presence or absence of "peristomes" may depend upon environmental conditions (Hartmann-Schröder, 1967, p. 454, for *Mercierella enigmatica*); a further example of variability of tubes, to the extent that within one species "peristomes" may be present or absent, is given by ten Hove (1973, plates I–II) for three species of *Sclerostyla* and (1975, plate VII) for *Pseudovermilia occidentalis*.

Considering that the differences between the five "genera" under discussion are certainly not greater than the above-mentioned examples of variability, and considering the striking similarities summarized in our emended generic diagnosis, it does not seem realistic to consider them as distinct. Further arguments for synonymizing these genera will be given in the various discussions following the species descriptions. In our opinion, the differences between *Ficopomatus* and other serpulid genera are too small to justify a distinction on the subfamily level. Therefore, we suggest that the subfamily Ficopomatinae Pillai (1960, p. 35) be withdrawn from recognition.

All species of the genus *Ficopomatus* may occur as solitary individuals or in dense aggregated masses. A discussion of the possible causes of mass occurrence has been given by ten Hove (1978).

Ficopomatus capensis Day (1961, pp. 552–553, Fig. 17 h–n; 1967, pp. 810–812, Fig. 38.5 j–n) definitely cannot be included in the genus, as emended above. From the figures and description it more probably should be placed in *Chitinopoma* Levinsen, emended (Zibrowius, 1969), *Chitinopomoides* Benham, or *Pseudochitinopoma* Zibrowius. Since these three genera are mainly characterized by the microstructure of the setae and uncini, the generic position of Day's species can only be established after a careful comparison of material of the genera concerned.

The occurrence of wide, flaring "peristomes" on the tubes of fossil serpulids was thought to be of generic diagnostic value by some palaeontologists. This has been disputed by Hartmann-Schröder (1967, p. 452). To our knowledge, "peristomes" may occur in species of the genera *Chitinopomoides* Benham, *Crucigera* Benedict, *Filograna* Oken, *Josephella* Caullery and Mesnil, *Metavermilia* Bush, *Pseudovermilia* Bush, *Serpula* Linnaeus, and *Vermiliopsis* Saint-Joseph. The tubes of *Serpula narconensis* Baird, as figured by McIntosh (1885, plate 54, Fig. 5) are very similar to those of *Ficopomatus enigmaticus*. Judging by the figures and measurements, *Mercierella? dubiosa* Schmidt (1951, p. 80, Fig. 4) might belong to *Filograna*. For similar reasons, the tube of *M. roveretoi* Schmidt (1951, p. 80, Fig. 5) is strongly reminiscent of a yet undescribed species of *Serpula* from the Caribbean. Regenhardt (1961) erected a genus, *Proliserpula*, containing species with "peristomes" and suggested a connection with *Mercierella*. In our opinion, most of his species resemble recent species of *Pseudovermilia*, as well as *Filograna*, *Serpula* and *Vermiliopsis*. *Mercierella* (?) *dacica* Dragastan (1966, pp. 147–150, Figs. 1–3) resembles not only *Josephella*, but also some calcareous algae.

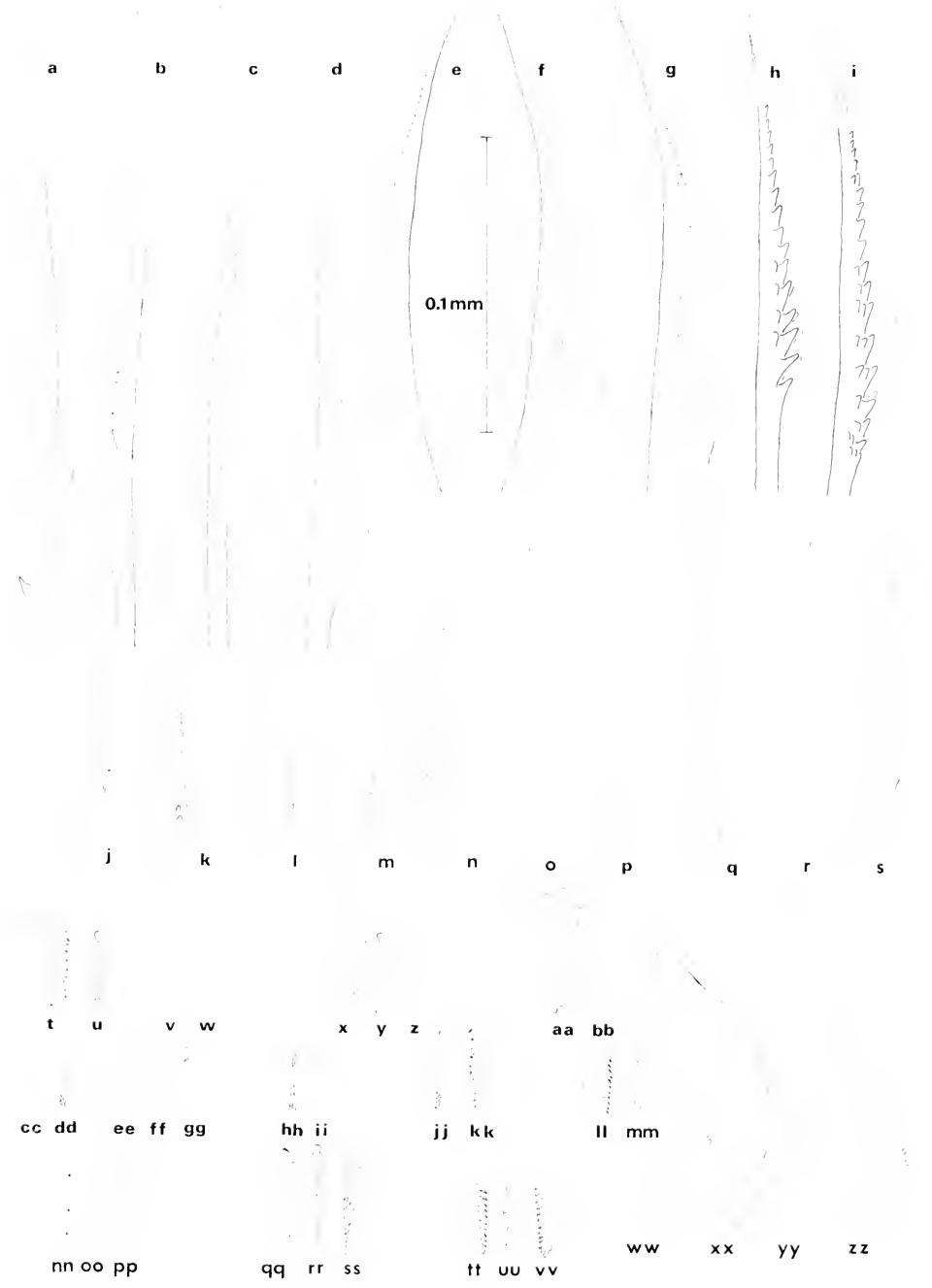

FIGURE 4. Collar setae are shown in the series, a–n, displaying all kinds of intergradations between a single row of teeth and a partial triple row; however, a prominent smooth gap be-

Ficopomatus macrodon Southern 1921

Figures 1a–e; 3b; 4e–g, o–p, t–u, cc–dd, ww; 5e

Serpulid—Annandale 1916, p. 100 [Siam, Taléh-Sap; material studied by us].

Ficopomatus macrodon Southern, 1921, pp. 655–659, plate 30, Fig. 27 A–M [India, Cochin Backwater; extensive description]; Rioja, 1924, pp. 168–169 [no new data; comparison with *F. enigmaticus*]; McIntosh, 1926, pp. 405–423, plate 13, Fig. 3, plate 15, Fig. 3 [India, Chilka Lake; no new data; anatomical description of operculum]; Hartman, 1959, p. 575 [name only]; Pillai, 1971, pp. 115–116, Fig. 8A–F [Ceylon, Tambalagam Lake; description and comparative study].

[?]*Ficopomatus macrodon*—Fauvel, 1931, p. 1069 [India, Madras Coast, Ennur Backwater; name only, could be *F. uschakovi;* see discussion below]; Fauvel, 1932, pp. 248–249 [India, Madras Coast, Ennur Backwater; Sunderbans; Taléh-Sap, Gulf of Siam, Stats. 11, 17, 21, 29, and 32; description; some material of Stat. 32 studied by us; see discussion below]; Fauvel, 1953, pp. 473–474, Fig. 248 c–l [India, Madras Coast, Ennur Backwater; Cochin Backwater; Chepparan; Sunderbans; Taléh-Sap, Gulf of Siam; description; see discussion below].

Ficopomatus—Pillai, 1960, pp. 32–35 [comparative study; diagnosis].

non*Ficopomatus* sp.—Hill, 1967, pp. 303–321 [Nigeria, Lagos; name only; most likely abnormal *F. uschakovi;* see discussion below].

Mercierella enigmatica—Nelson-Smith, 1967, p. 54 (in part?) [the palaeotropical records most probably are of *F. macrodon* or *F. uschakovi;* the diagnosis and figures are of *F. enigmaticus*].

[?]*Mercierella enigmatica*—Ganapati, Lakshmana Rao, and Nagabhushanam, 1958, pp. 197–206 [India, 17°N; 83°E; name only, material is probably *F. macrodon* or *F. uschakovi*].

Material Studied. Thailand: Taléh-Sap, Annandale Collection, Stat. 32 (five isolated specimens and three small pebbles with seven specimens in tubes [BMNH 1938: 5: 7: 89–91]; ten specimens identified by P. Fauvel and H. Zibrowius [MNHN]).

Tube: The tube is shining white, semicircular in cross-section. Collar-like rings of former peristomes were absent in the material studied. Most of the tubes have a high and sharp median keel (Fig. 5e); however, in a few tubes it is indistinct.

Branchiae: The branchial filaments arise from paired lobes and number about six (5–7; n = 5) on the left and seven (6–7; n = 5) on the right. They are arranged in two semicircles and are not connected by a branchial membrane. The filaments are shorter ventrally, their tips being free of pinnulae to a greater or lesser extent (Fig. 3b).

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tween proximal coarse bunches of teeth and distal series of teeth is nowhere present. Posterior setae are shown as follows: o–s, other thoracic setae; t–bb, thoracic uncini; cc–vv, abdominal uncini (cc–gg, jj–kk, nn–pp, anterior segments; qq–ss, middle segments; hh–ii, ll–nm, tt–vv, posterior segments); and ww–zz, abdominal setae. Species represented are: *F. enigmaticus* from the Netherlands, a–d, s, aa–bb, nn–vv, zz; *F. macrodon* from Taléh-Sap, e–g, o–p, t–u, cc–dd, ww; *F. miamiensis* from Barbados, h–i, q, v–w, ee–ii, xx; and *F. uschakovi* from Java, j; from Guadalcanal, k–n, r, x–z, jj–nm, yy. All are to the same scale.

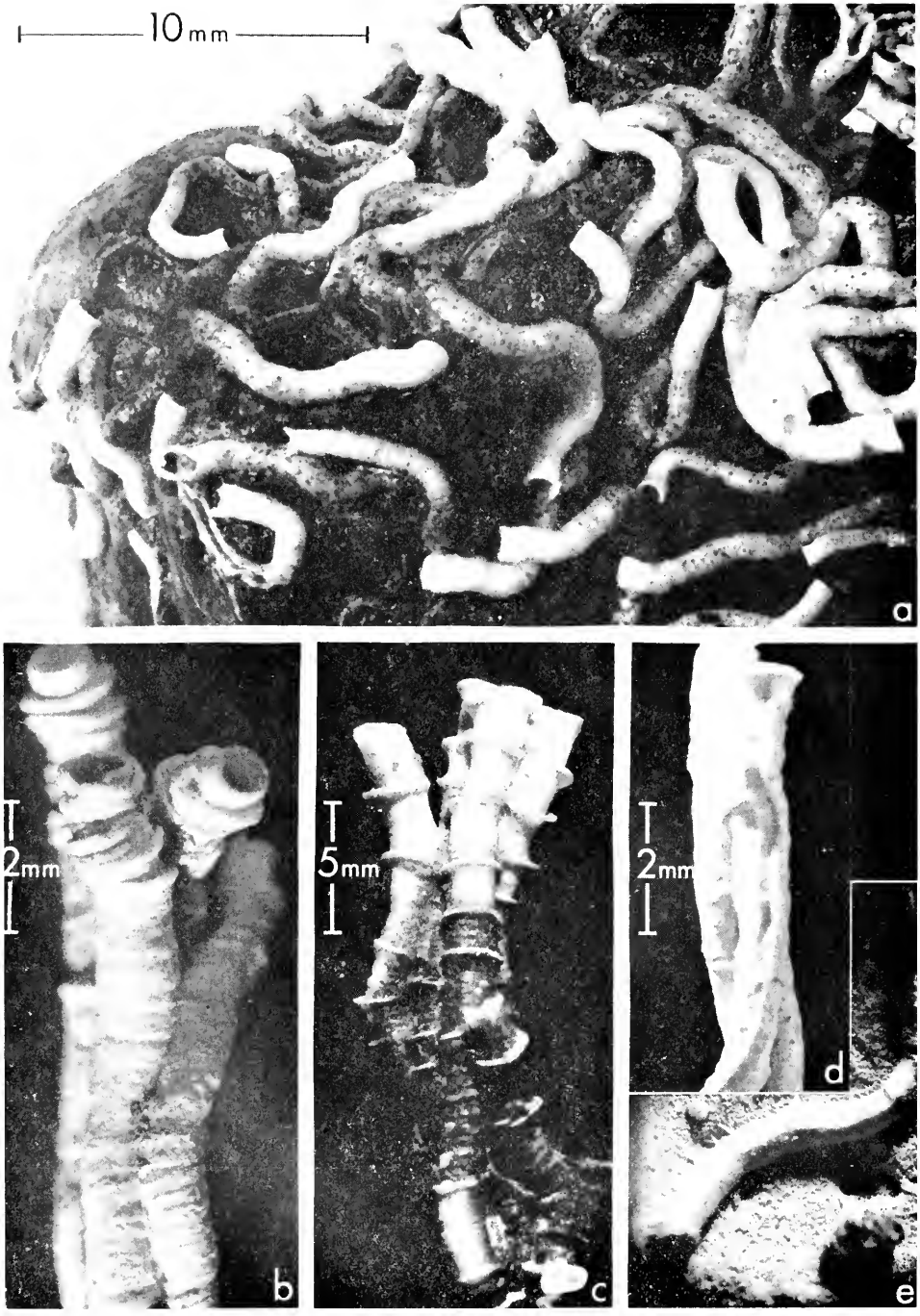

FIGURE 5. Tubes of *Ficopomatus*: a, b, *F. miamiensis* from Barbados, showing differences between two populations from Holetown river pool and from one-half mile north of Bellairs Institute; c, *F. enigmaticus* from the Netherlands; d, *F. uschakovi* from India, showing three longitudinal keels; and e, *F. macrodon* from Taléh-Sap, showing one longitudinal keel.

Peduncle: The peduncle is smooth, sometimes faintly wrinkled, especially just below the bulb of the operculum. It is subtriangular in cross-section with a shallow dorsal groove. There is a gradual transition from peduncle to opercular bulb (Fig. 1a–b).

Operculum: The operculum is a fleshly bulb, terminated by a more or less conical horny cap, which has a dorsal furrow (Fig. 1a–e). The thickness of the horny plate is positively correlated with increasing length of the cone.

Collar and thoracic membranes: The collar is rather high, not lobed, and has an entire edge. It is continuous with the thoracic membranes, which are united ventrally on the anterior abdominal segments.

Thorax: The thorax has seven segments, six of which are uncinigerous. The bundles of collar setae contain only a few setae of two types: coarsely serrated ones (Fig. 4e–g; see discussion below) and limbate ones. Subsequent fascicles of setae are larger and are in two nearly parallel rows, containing limbate setae only (Fig. 4o–p). The thoracic uncinigerous tori are arranged in two nearly parallel rows, with about 70 ($n = 2$) uncini per torus. The thoracic uncini have a single row of teeth; however, directly above the most anterior gouged tooth, there are one or two transverse rows of two or three teeth (Fig. 4t–u). There are ten to twelve ($n = 4$) teeth visible in profile. Thoracic uncini from the first row do not differ essentially from those in the last row.

Abdomen: Owing to the scanty and incomplete material, the number of abdominal segments and uncini per row could not be determined. The abdominal uncini are all rasp-like, with three to four rows of small curved teeth; about 13–15 teeth ($n = 3$) are visible in profile, including the anterior gouged one (Fig. 4cc–dd). The bundles of abdominal setae consist of two to five geniculate ones (Fig. 4ww).

Size: The length, including operculum, is at least up to 7 mm, but cannot be given more exactly owing to the incompleteness of the specimens; the width of the thorax is usually 0.5–0.7 mm ($n = 4$). The branchiae and the operculum may account for one-fifth of the entire length of the animal.

Discussion: Unfortunately the type material of *F. macrodon* was not available, and the material studied was in poor condition. Yet at least 20 collar setae have been studied.

The diagnostic value of the microstructure of the collar setae has been overstressed by several authors, which, along with some other variable characteristics, has resulted in five different genera. According to Southern (1921, p. 656, Fig. 27D–E) collar setae of *F. macrodon* have a transverse row of teeth, and a series of very coarse teeth distally, separated by a smooth gap. However, Pillai (1971, p. 116, Fig. 8D–F) indicated that the above mentioned gap occasionally may be absent. In our material this gap, if present at all, was not prominent. Thus, in all probability there is a complete cline from long smooth gap to continuous series of teeth. Consequently, this cannot be used as a distinguishing characteristic. Moreover, Hartmann-Schröder (1971, Fig. 7c–d) gives a similar variation in the collar setae of *F. uschakovi*. A considerable variation in shape and arrangement of teeth is given for *F. enigmaticus* by Rioja (1924, Fig. 16–19) and Hartmann-Schröder (1967, Fig. 4) and for *F. uschakovi* by Pillai (1960, Fig. 12C–D, I–K; 1965, Fig. 23G–I). Our studies also show a considerable variation in the microstructure of

the collar setae (Fig. 4a–n) and, after studying more than one hundred slides, each with about eight special setae, we still are incapable of distinguishing the species by their collar setae alone.

F. macrodon can be distinguished from other species in the genus by its peculiar thoracic uncini. In our opinion this feature is insufficient to justify a generic distinction (cf., *Pseudovermilia babylonia,* ten Hove, 1975, p. 96).

The operculum of *F. macrodon* resembles those of some specimens of *F. miamiensis* in having a horny endplate without spines. However, the shape of this endplate is different for both species (cf., Fig. 1).

Fauvel (1931, 1932, 1953), cited by Nelson-Smith (1967, p. 64), mentions both *F. macrodon* and *Mercierella enigmatica* from Madras. Fauvel (1932, p. 248) states, "These *Ficopomatus* tubes are rather square in section with three dorsal ridges, . . . but the animals enclosed in them are typical *Ficopomatus*". For *Mercierella enigmatica,* Fauvel (1932, p. 250) reports, "Its tube is cylindrical, . . . is neither ridged nor enlarged at the entrance." However, a re-examination of part of his material, from this locality, labelled *Mercierella enigmatica,* showed tubes with three prominent ridges, containing *F. uschakovi.* To our knowledge, tubes of *F. macrodon* generally have one longitudinal ridge only. It is evident that Fauvel's description is confusing, the more so since he figures European material (1953, p. 475, Fig. 249), and, therefore, his identifications need to be checked.

According to Annandale (1916), the type-locality Taléh-Sap is the same as the inland sea of Singgora (also spelled Sengora or Songhkla). It is located on the Malayan peninsula, on the Gulf of Siam (see Fig. 6). It appears that *F. macrodon* occurs in brackish waters adjacent to the Gulf of Bengal and the Gulf of Siam.

Ficopomatus miamiensis (Treadwell, 1934)

Figures 1f–i; 3c; 4h–i, q, v–w, ee–ii, xx; 5a–b

Sphaeropomatus miamiensis Treadwell, 1934, pp. 338–341, Figs. 1–5, 9 [Florida, Miami River; description; syntypes studied by us; see discussion below]; Hartman, 1956, p. 300 [Florida, Indian and Miami Rivers; description; material studied by us]; Hartman, 1959, p. 599 [name only]; Pillai, 1971, pp. 116–119, Figs. 8G–H, 9 A–F [studied same material as Hartman (1956); extensive description and comparative study; see discussion below]; Lacalli, 1977, pp. 300–303, Fig. 2 [embryological study; material studied by us].

Mercierellopsis prietoi—Rioja, 1945, pp. 411–417, plates 1, 2 [Mexico, Tecolutla (Gulf of Mexico); extensive description; material apparently lost; see discussion below]; Hartman, 1951, p. 120 [no new data; short diagnosis; see discussion below]; Hartman, 1954, p. 416 [name only]; Hartman, 1959, p. 582 [name only; see discussion below].

Mercierella enigmatica—Nelson-Smith, 1967, p. 54 (in part?) [Curaçao is listed in the distribution of *F. enigmaticus;* this record most probably should be referred to *F. miamiensis;* the diagnosis and figures are of *F. enigmaticus*].

Material studied. United States of America: Florida, Miami River, 17 May 1933, from carapace of freshwater shrimp, *Macrobrachium jamaicense* (Herbst), Capt. John W. Mills coll. (18 specimens, syntypes, tubes; USNM 20074, 20075, 20077; AMNH 2167; tHU 210); Florida, tributary of Indian River, Undersea Institute of

America (six specimens, many tubes; AHF; tHU 218); Florida, Vero Beach, adjacent to Indian River, artificial ponds at Entomological Research Center, 22 and 26 March 1963, 11 June 64 (40 specimens, tubes; USNM 54335-6); Florida, Miami, Coral Gables Canal, 25 March 1969, M. L. Jones coll. (one specimen; USNM 54337); Florida, northwest coast, St. Mark Wildlife Refuge, near St. Mark Lighthouse (30° 05′ N; 84° 12′ W), 15 Dec. 1976, 26 Feb 1977, on submerged tree limbs in brackish water ponds, salinity 11‰, P. G. Johnson coll. (28 specimens, tubes; USNM 54795; tHU 258); Louisiana, Lake Pontchartrain, mouth of industrial canal, salinity 2.5–3‰, M.A. Poirrier coll. (eight specimens, tube fragments; USNM 54794; tHU 255). Jamaica: Great Saltpond, entrance at Fort Clarence, 8 May 1973, P. Wagenaar Hummelinck coll., Stat. 1681, 0–1 m depth (50 specimens, many in tubes; BMNH 1976: 916–942; tHU 234). Barbados: Holetown River, pool near bridge, 18 Feb. 1964, P. Wagenaar Hummelinck coll., Stat. 1444 (four specimens and pebbles with tubes; RMNH 10706; tHU 235); about 1 km North of Bellairs Institute, closed lagoon, encrusting on roots and dead branches, April-May 1975, T. Lacalli coll. (six specimens, tube fragments; tHU 225). Curacao: Bottom of H.N.L.M.S. LUYMES, after one month in Caribbean waters, 9 May 1970, H. A. ten Hove coll. (15 specimens and 55 others in tubes; RMNH 10707; SME); Schottegat, east of Rijkseenheid Boulevard, opposite Zeelandia, 20 Sept. 1970, 11 Sept. 1975, H. A. ten Hove coll., Stats. 2065, 2065a, limestone boulders in sandy mud, *Caulerpa,* 10–20 cm depth (many specimens, many tubes; tHU 254). Belize [= British Honduras]: Salt Creek, approximately 8 km north of Stann Creek, 16 May 1977, M. L. Jones coll., in channel among mangroves, about 1 m depth, on living *Isognomon alatus,* temperature 32°C, salinity 31‰ (four specimens, many tubes; USNM 54980; tHU 259). Panama: Canal Zone, Pacific Third Lock, 16 April 1972, C. E. Dawson, D. L. Pawson, W. J. Byas, M. L. Jones colls., USNM Panama Survey Stat. 87–1, cobbles, rocks, on shelf adjacent to road (24 specimens: USNM 52743; tHU 233); Canal Zone, Pacific coast, Upper Miraflores Lock chamber, 26 Aug. 1974, C. E. Dawson, M. L. Jones, H. W. Kaufman, J. Rosewater colls., USNM Panama Survey Stat. 203, lock chamber walls (three specimens; USNM 54977–9).

Tube: The tube is shining white, exceptionally dull and roughened, semicircular in cross-section. There are no longitudinal ridges or keels. Normally collar-like rings, as in *F. enigmaticus,* are absent (Fig. 5a). However, in the populations from Barbados (1 km north of Bellairs Institute), Florida (Vero Beach), and Panama (Stat. 203), wide flaring "peristomes" are present (Fig. 5b).

Branchiae: The branchial filaments arise from paired lobes and number about seven (6–9) on the left and eight (6–10) on the right. They are arranged in two semicircles and are not connected by a branchial membrane. The filaments are shorter ventrally. The two rows of pinnulae become shorter toward the ends of the filaments, which are free of pinnulae to a greater or lesser extent (Fig. 3c).

Peduncle: The peduncle is smooth, sometimes faintly wrinkled, and circular or subtriangular in cross-section. There is a gradual transition between peduncle and opercular bulb (Fig. 1h).

Operculum: The operculum is spherical to fig-shaped (Fig. 1g–h), sometimes with a horny end-plate (Fig. 1f, i), which may be flat or slightly convex (see discussion below). The operculum never has spines.

Collar and thoracic membranes: The collar is high, not lobed and has an entire edge. It is continuous with the thoracic membranes, which are united ventrally on the anterior abdominal segments.

Thorax: The thorax has seven segments, six of which are uncinigerous. The bundles of collar setae contain only a few setae of two types: coarsely serrated ones (Fig. 4h–i) and limbate ones. Subsequent bundles of setae are larger and are in two nearly parallel rows, containing limbate setae only (Fig. 4q). Thoracic uncinigerous tori are arranged in two nearly parallel rows with up to 55 uncini per torus. The uncini along the entire thorax have a single row of seven (6–8) curved teeth, the most anterior one is gouged and apparently bifurcated (Fig. 4v–w).

Abdomen: The number of abdominal segments is usually about 40 (23–58, $n = 7$). The anterior two or three segments are apparently without setae or uncini. The following segments have very few uncini (five to ten). The number of uncini per row slowly increases to about 30 in the middle of the abdomen, then slowly decreases towards the pygidium (about three). The abdominal uncini of the anterior segments are partly rasp-, partly saw-like, in such a way that within a single uncinus both conditions may occur (Fig. 4ee–gg). About eight to ten teeth are visible in profile, including the anterior gouged tooth. The uncini of the posterior segments are smaller and rasp-like, with three to four rows of small curved teeth, with about 12 teeth visible in profile, including the anterior gouged one (Fig. 4hh–ii). The bundles of abdominal setae consist of three (sometimes one or two) geniculate ones (Fig. 4xx).

Size: The length, including the operculum, is about 7 mm (2.5–11). The width of the thorax is about 0.8 mm. The branchiae and the operculum usually account for one-sixth (sometimes up to one-third) of the entire length of the animal.

Discussion: As stated above, Treadwell's (1934) original description is not entirely correct; his main errors are the six thoracic setigers (in reality seven) and the entirely fleshly operculum. The opercula of 14 (out of 18) syntypes did show a horny endplate (Fig. 1f). Of about 200 specimens studied in this respect, 20% had a well-developed endplate. The endplate sometimes is difficult to see, looking more or less like a fleshy brim. Generally, however, the endplate is missing altogether.

We have the impression that there is no relation between presence or absence of endplate and the size of the specimens. Pillai's (1971, Fig. 9A–C) figures are based upon collapsed opercula without endplates (material re-examined). Although Rioja did not leave a collection (according to a personal communication from Dr. Maria Elena Caso, Instituto de Biologia, Universidad Nacional de Mexico), his figures and description of *Mercierellopsis prietoi* (1945, pp. 411–417, Figs. 1–20) are excellent, and show the conspecificity with *F. miamiensis* beyond doubt.

In contradistinction to all previous descriptions, the tube may show wide flaring "peristomes" (Fig. 5b).

Possibly Mörch's (1863, p. 353) remark on the occurrence of serpulid tubes on leaves of a freshwater plant from St. Thomas should be referred to *F. miamiensis,* although some spirorbids can occur in the brackish habitat too.

As far as is yet known, *F. miamiensis* is restricted to Atlantic tropical and subtropical areas in northern and middle America, and a more or less isolated locality

at the Pacific end of the Panama Canal (Fig. 6). In the brackish waters of Uruguay and Argentina, it is replaced by *F. enigmaticus*. It would be interesting to to know if this is the case, too, in northern America. Since the only record of *F. enigmaticus* from the northern Gulf of Mexico is from the bottom of a boat, it is uncertain if this represents a permanent population.

Ficopomatus uschakovi (Pillai, 1960)

Figures 2a–d; 3a, f–k; 4j–n, r, x–z, jj–mm, yy; 5d

[?] Serpuliden-Röhren Ehlers, 1918, p. 250 [Aru Islands; empty tubes; see discussion below].

Mercierella enigmatica—Fauvel, 1931, p. 1069 (in part?) [India, several localities; name only; the record of Ennur Backwater is *F. uschakovi* and, perhaps, *F. macrodon,* as well; see discussion of latter species]; Fauvel, 1932, pp. 249–251 [India, Madras coast, Ennur Backwater; description; material studied by us; see discussion below]; Allen, 1953, pp. 308, 311, 315 (in part) [Australia, from Noosa, Queenland to Carnarvon, Western Australia; name only; see discussion below]; Fauvel, 1953, pp. 474–476, *not* Fig. 249a–o [India, Ennur Backwater; description; most likely same material as above, 1932; see discussion below]; Rullier, 1955, pp. 288–289 [Ivory Coast, Abidjan; name only; material from same locality studied by us; see discussion below]; Dew, 1959, pp. 29–31, *not* Fig. 8A–H (in part) [Australia, several localities; description; material from Queensland (Townsville and Noosa) is *F. uschakovi,* specimens from other localities are *F. enigmaticus*]; Straughan 1966, pp. 139–146, Figs. 2, 3b–d (in part) [Australia, several localities; Brunei; Ceylon; some of this material studied by us; Straughan's Figs. 3a and 3e are *F. enigmaticus;* see discussion below]; Rullier, 1966, pp. 95–104 (in part) [Dahomey, Cotonou; other references to *F. uschakovi* in Rullier's listing are cited by us in this synonymy]; Sandison and Hill, 1966, pp. 235–250 [Nigeria, Lagos; name only; see discussion below]; Day, 1967, p. 812 (in part?) [South Africa, Natal; diagnosis; should be checked since locality is in tropical region]; Hill, 1967, pp. 303–321 [Nigeria, Lagos; name only; see discussion below]; Nelson-Smith, 1967, p. 54 (in part?) [the paleotropical records most probably are of *F. macrodon* or *F. uschakovi;* the diagnosis and figures are of *F. enigmaticus*]; Straughan, 1967, pp. 25–40 [Australia, Queensland, Brisbane River; ecological study]; Straughan, 1968, pp. 59–64, plates 1, 2, 3A (in part) [Australia, several localities; Straughan's plate 3B is *F. enigmaticus;* see discussion below]; Gibbs, 1971, p. 203 [Solomon Islands, Guadalcanal, Lunga Point and Komimbo Bay; short diagnosis; material studied by us]; Straughan, 1971, pp. 169–175 [Australia, Queensland, North Pine River; ecological study; see discussion below]; Straughan, 1972, pp. 93–136 [Australia, Queensland, Brisbane River; ecological study; see discussion below].

[?] *Mercierella enigmatica*—Day, 1951, pp. 65–66 [South Africa, St. Lucia Estuary; name only; should be checked since locality is in tropical region]; Ganapati, *et al.,* 1958, pp. 197–206 [India, 17° N 83° E; name only; material is probably *F. macrodon* or *F. uschakovi*]; Kirkegaard, 1959, p. 105 [Nigeria, Lagos, Victoria Beach; name only; see discussion below].

non *Mercierella enigmatica*—Mesnil and Fauvel, 1939, pp. 37–38 [Kei Islands, *Siboga* Exped. Stat. 260, 90 m; one empty tube; see discussion below].

[?] *Ficopomatus* sp. Hill, 1967, pp. 303–321 [Nigeria, Lagos; name only; most likely abnormal *F. uschakovi;* see discussion below].

Neopomatus uschakovi Pillai, 1960, pp. 28–32, Figs. 10H, 11A–H, 12A–H, plate I, Figs. 1, 2 [Ceylon, Panadura River Estuary, Madu Ganga Estuary, Ratgama Lake; description; holotypes studied by us]; Hartman, 1965, p. 80 [name only]; Pillai, 1965, p. 172 [Indonesia, Surabaja; East Java; Madura; name only]; Pillai, 1971, pp. 118–123, 127, Figs. 9G, 10A [Ceylon, several localities; description; comparative study]; Zibrowius, 1973, p. 64 [synonymy; useful discussion].

Neopomatus uschakovi var. *lingayanensis* Pillai, 1965, pp. 170–172, Fig. 23A–I [Philippine Islands, Luzon, Lingayan Gulf and other localities; description; some paratypes studied by us].

Neopomatus ushakovi [sic]—Hartmann-Schröder, 1971, pp. 7–27, Figs. 2, 3, 5, 7b–d, 11–14 [several paleotropical localities; partial revision, synonymy].

Neopomatus similis Pillai, 1960, pp. 32–33, Fig. 12I-M, plate II, Fig. 1 [Ceylon, Negombo Lagoon; description, holotype studied by us]; Hartman, 1965, p. 80 [name only].

Neopomatus similis var. *rugosus* Pillai, 1960, pp. 33–35, plate II, Fig. 2 [Ceylon, Negombo Lagoon; description; holotype studied by us]; Hartman, 1965, p. 80 [name only].

Material studied. Sri Lanka [= Ceylon]: Panadura River Estuary, 6 Jan. 1957 (Holotype of *N. uschakovi;* BMNH 1959: 4: 14:7); Maha Alamba, Negombo Lagoon, 18 Feb. 1959, T. G. Pillai coll. (holotype of *N. similis* var. *rugosus* and small tube on a pebble; BMNH 1959: 4: 14: 14); Ratgama Lake, 28 Feb. 1959, coconut petiole with tubes attached, T. G. Pillai coll. (*ca.* 25 specimens; BMNH 1959: 4: 14: 19); Cuming coll., specific locality unknown, tubes on gastropod shells (one dried operculum, empty tubes; BMNH 1965: 31: 4–5; at least 110 years in BMNH, identified by H. Zibrowius, 1972). India: Madras Coast, Ennur Backwater, Annandale coll. (four specimens in tubes, BMNH 1938: 5: 7: 92–94; also many specimens, some in tubes, MNHN; as *M. enigmatica* by P. Fauvel; as *Neopomatus* sp. by G. Hartmann-Schröder; as *N. uschakovi* by H. Zibrowius. Indonesia. Java: Specific locality unknown, 1904, P. Serre coll. (many specimens in tubes on barnacles; MNHN; as *M. enigmatica* by P. Fauvel; as *N. uschakovi* by H. Zibrowius). Philippines: Luzon, Lingayan Gulf, T. G. Pillai coll. (five paratypes of *N. uschakovi* var. *lingayanensis;* BMNH 1965: 53: 19–28). Solomon Islands. Guadalcanal: Komimbo Bay, 19 July 1965, at mouth of freshwater creek, above MTL and Lunga Point, 9 Sept. 1965, in brackish lagoon at LWM, P. E. Gibbs coll. (*ca.* 70 specimens, some in tubes; BMNH 1970: 830/831; as *M. enigmatica* by P. E. Gibbs, as *N. uschakovi* by H. Zibrowius). Australia. New South Wales: Yamba, 1 Sept. 1950 and Queensland: Townsville, 26 Dec. 1950, and Noosa, 1 March 1951, B. Dew coll. (10 specimens; BMNH 1955: 11: 1: 116; AM W-3777-9, 3781; as *M. enigmatica* by B. Dew; as *N. uschakovi* by H. Zibrowius and T. G. Pillai). Nigeria. Lagos: Jan. 1954 (11 specimens and many others in tubes; BMNH 1954: 3: 4: 1–50; as *M. enigmatica;* as *Neopomatus* sp., by G. Hartmann-Schröder and as *N. uschakovi* by H. Zibrowius). Ivory Coast. Abidjan: June 1955, M. Fox coll. (many specimens in tubes on pieces of

wood; BMNH 1955: 11: 1: 1–30; as *M. enigmatica;* as *Neopomatus* sp. by Hartmann-Schröder, and as *N. uschakovi* by H. Zibrowius. Netherlands. Noordwijk: on wood cast ashore on beach, 13 Oct. 1974, A. W. Lacourt coll. (many tubes and dried opercula, RMNH 07274, tHU 213).

Tube: The tube is shining white, sometimes the older parts are covered with a brownish layer of algae, presumably. It is semicircular in cross-section. At irregular intervals it bears more or less prominent collar-like rings, which indicate successive positions of the peristome. Usually there are three keels (Fig. 5d), of which the median is high and sharp, the lateral ones may be smaller; sometimes they are faint or lacking. The keels are less conspicuous toward the mouth of the tube.

Branchiae: The branchial filaments arise from paired lobes and number about eight (5–10) on the left and nine (6–11) on the right. They are arranged in two semicircles and are not connected by a branchial membrane. The filaments are shorter ventrally. The two rows of pinnulae become larger toward the end of the filaments, which is free of pinnulae to a greater or lesser extent (Fig. 3a).

Peduncle: The peduncle is smooth, sometimes faintly wrinkled, especially just below the bulb of the operculum. It is circular to subtriangular (the latter near the opercular bulb) in cross-section. There is a gradual transition from peduncle to opercular bulb, however, slightly more abruptly than in *F. enigmaticus* (*cf.* Fig. 2a with 2f).

Operculum: The operculum usually is spherical and radially symmetrical, sometimes with bilateral symmetry. It usually has a convex, slightly horny plate distally, which sometimes may be lacking. This end-plate is bordered by one to four (exceptionally up to eight) rows of small denticulations (Fig. 2a–d), curved outward. The denticulations ("spines") of one row may be either fused with or completely separated from each other. Sometimes the rows of "spines" are incomplete or irregular. The "spines" are randomly placed in a few specimens, and, exceptionally, cover the endplate. "Spines" with small outgrowths sometimes occur (*cf.* Fig. 3g, j with h, i, k).

Collar and thoracic membranes: The collar is rather high, not lobed and has an entire edge. It is continuous with the thoracic membranes, which are fused dorsally (Fig. 3f) and are united ventrally on the anterior abdominal segments. Exceptionally, there are specimens in which the thoracic membranes are not fused dorsally (one of the approximately 200 specimens studied).

Thorax: The thorax has seven segments, six of which are uncinigerous. The bundles of collar setae contain only a few setae of two types: coarsely serrated ones (Fig. 4j–n) and limbate ones. Subsequent bundles of setae are larger and are in two nearly parallel rows, containing limbate setae only (Fig. 4r). The thoracic uncinigerous tori are arranged in two nearly parallel rows, with up to 75 uncini (n = 5) per torus in large animals (Lunga Point). The uncini along the entire thorax have a single row of seven to nine curved teeth, the most anterior tooth gouged, apparently bifurcated (Fig. 4x–z).

Abdomen: The number of abdominal segments is usually about 40 (18–46, n = 7). The anterior two or three segments are apparently without setae or uncini. The following segments have very few uncini (three to four). The number of uncini per row slowly increases to about 45 in the middle of the abdomen, then

slowly decreases toward the pygidium (three to six). The abdominal uncini are rasp-like along the entire abdomen, with two rows of curved teeth anteriorly, two to three rows posteriorly; anteriorly about 10–12 teeth are visible in profile, including the anterior gouged one, posteriorly about 13 smaller ones (Fig. 4jj–mm). The bundles of abdominal setae consist of one or two to three geniculate ones (Fig. 4yy).

Size: The length, including the operculum, is quite variable. In a population from Lunga Point the length is about 10 mm (6–12 mm); the specimens from Komimbo Bay, however, are not longer than 5 mm (2–5 mm, n = 5). The width of the thorax is about 1 mm in the large specimens, about 0.4 mm in the small ones. The branchiae and the operculum usually account for one-quarter of the entire length of the animal.

Variations: Special attention should be given to a form differing in operculum, described by Pillai (1965) as *Neopomatus uschakovi* var. *lingayanensis*. This usually has a bilaterally symmetrical operculum, with a cluster of one to four spines on the endplate, in the center of the ring(s) of denticulations (Fig. 2d).

Discussion: The holotype is in poor condition, apparently having been dry. The species has been confused with *F. enigmaticus;* however, Pillai (1971) and Hartmann-Schröder (1971) have already clarified this confusion and indicated that the species are geographically separated—*F. enigmaticus* occurs in subtropical/temperate areas, *F. uschakovi* in the paleotropical region. The results of our research support this opinion (Fig. 6).

In eastern Australia the northern boundary of *F. enigmaticus* and the southern one of *F. uschakovi* lies just north of Sydney, according to the material studied by Pillai (1971) and by us. In western Australia it cannot as yet be defined exactly; the population in Swan River is *F. enigmaticus,* the material mentioned by Allen (1953, p. 308) from Carnarvon might be *F. uschakovi,* since this locality lies within the tropics.

The distributions of *F. enigmaticus* and *uschakovi* indicated above suggest that it is unlikely that both species will occur together in an entirely tropical area. In juvenile specimens of *F. enigmaticus, miamiensis* and *uschakovi,* opercula may have no horny parts. Therefore, *Ficopomatus* sp., as cited by Hill (1967) from Nigeria, most likely is abnormal *F. uschakovi* (see Zibrowius, 1973, p. 64).

The exact boundaries between both species in Africa cannot be given, since there are considerable gaps in the known distributions.

Mesnil and Fauvel's (1939, pp. 37–38) record of an empty tube of *Mercierella enigmatica* from a depth of 90 m off the Kei Islands is very doubtful. Unfortunately, the material could not be traced, but, since many genera show tubes with "peristomes," it is more likely that this tube belonged to a different genus than that the tube was deposited two miles offshore by streams. On the other hand, the diagnosis and locality of Ehlers' (1918, p. 250) record of empty serpulid tubes from a river on the Aru Islands indicate that these tubes most likely are *F. uschakovi.*

Our record of *F. uschakovi* from the Netherlands most probably can be explained by the brisk local trade in tropical wood, and, therefore, has not been included in Figure 6.

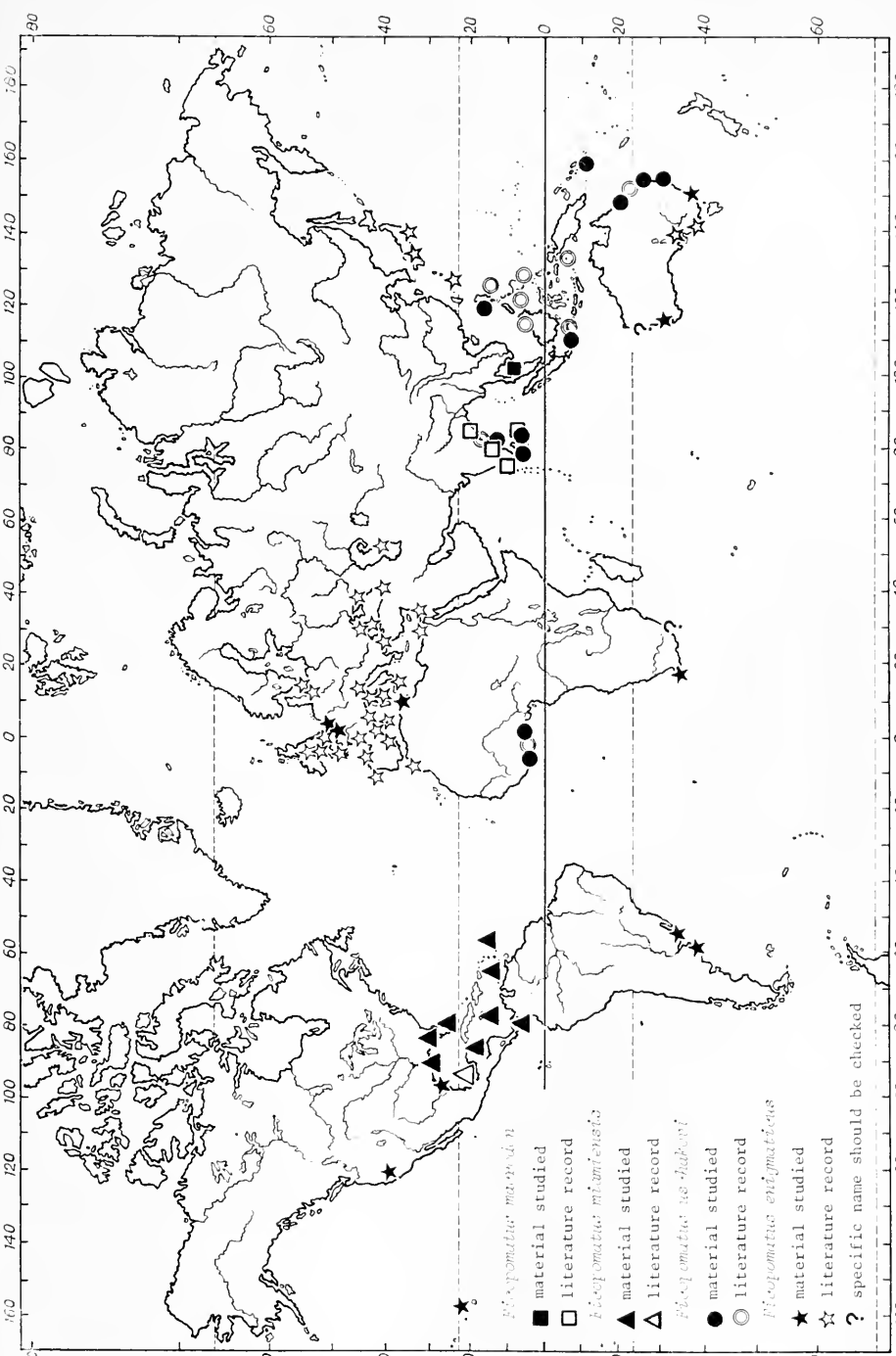

FIGURE 6. Distribution of the species of the genus *Ficopomatus*.

Ficopomatus enigmaticus (Fauvel, 1923)

Figures 2e–i; 3d–e, 1–q; 4a–d, s, aa–bb, nn–vv, zz; 5c

Due to the large number of literature citations of this species, the following represents a selected synonymy and is comprised of those papers which have a special bearing on taxonomic problems and those in which material studied by us has been mentioned. It should be emphasized that *Mercierella enigmatica* from tropical regions as reported by Allen (1953), Day (1951, 1967), Dew (1959), Fauvel (1931, 1932, 1953), Ganapati, *et al.* (1958), Gibbs (1971), Hill (1967), Kirkegaard (1959), Mesnil and Fauvel (1939), Nelson-Smith (1967), Rullier (1955, 1966), Sandison and Hill (1966), Straughan (1966, 1967, 1968, 1971, 1972), at least partly, belong to one of the other three species and can be found in their respective synonymies above.

Mercierella enigmatica Fauvel, 1923, pp. 424–430, Fig. 1a–o [France, Canal de Caen; description; syntypes studied by us]; Fauvel, 1931, pp. 1068–1069 (in part) [several localities; name only; the record of India, Ennur Backwater is probably *F. uschakovi* or *F. macrodon*]; Rioja, 1931, pp. 420–424, plates 137–139 [Spain, Gandia; extensive description; account of infraspecific variability] Fauvel, 1933, pp. 185–193 [several localities; short description]; Monro, 1938a, pp. 311 and 313 [Uruguay, Arroyo de las Brujas, Canelones; name only; material studied by us]; Monro, 1938b, p. 624 [Western Australia, Pelican, Swan River; name only; part of this material studied by us]; Hartman, 1952, p. 64 [Texas, Rockport; diagnosis; material studied by us]; Allen, 1953, pp. 308, 311, and 315 (in part) [Australia, from Noosa, Queensland, to Carnarvon, Western Australia; name only; see discussion of *F. uschakovi* above]; Cognetti 1953, pp. 36–40, Fig. 1a–n [Italy, Toscana; variability of operculum]; Day, 1955, p. 448 [South Africa, several localities on the Cape; name only; part of this material studied by us]; Dew, 1959, pp. 29–31, Fig. 8A–H (in part) [Australia, several localities; the material from Queensland (Townsville and Noosa) is *F. uschakovi*; specimens from other localities are *F. enigmaticus;* description; part of this material studied by us]; Hartman, 1959, p. 582 [name only]; Pillai, 1960, p. 33 [comparison with other species]; Vuillemin, 1964, pp. 514–527, plates 1–5 [Tunisia, Lac de Tunis; extensive description of opercular variability]; Vuillemin, 1965, 554 pages, many figures [Tunisia, Lac de Tunis; thesis on their biology]; Hartman, 1966, p. 238 [Hawaii, Oahu, Honolulu, Alai Wai Canal near Waikiki; diagnosis; material from same locality studied by us]; Rullier, 1966, pp. 95–104 (in part) [list of localities to 1964, from the literature; tropical records probably are *F. uschakovi*]; Straughan, 1966, pp. 139–146, Fig. 3a, e (in part) [Australia, several localities, and California, Berkeley; specimens in Figs. 2 and 3b–d are *F. uschakovi;* see discussion above]; Day, 1967, p. 812 (in part) [South Africa, several localities on the Cape; diagnosis; the record from Natal (tropical) should be checked for it may be *F. uschakovi*]; Hartmann-Schröder, 1967, pp. 421–456, Figs. 1–24 [Europe, several localities; monograph of the species]; Nelson-Smith, 1967, p. 54, Figs. 49–50 [Southwestern United Kingdom; diagnosis; tropical localities in the distribution most probably represent other species, see discussions above]; Straughan, 1968, pp. 59–64, plate 3B (in part) [Australia, several localities; the material on plates 1, 2, and 3A are *F. uschakovi;* see discussion above]; Wolff, 1969, pp. 85–92, Figs. 1–6 [Southwest-

ern Netherlands; extensive description; part of material studied by us]; Hartmann-Schröder, 1971, pp. 7–27, Figs. 1, 4, 6, 7a, 8–10, 15–17 [Mediterranean, several localities, Black Sea, and Australia, New South Wales; partial revision and synonymy]; Orensanz and Estivariz, 1971, pp. 106–108, Figs. 47–56 [Argentina, several localities; diagnosis; part of this material studied by us]; Pillai, 1971, pp. 120–125, Fig. 10B–H [United Kingdom, Radipole Lake, Weymouth; description; comparative study of the four species]; Zibrowius, 1973, pp. 62–64 [useful discussion]; Hove, ten 1974, pp. 45–48 [Southwestern Netherlands; name only; material studied by us]; Kajihara, Hirano and Chiba, 1976, pp. 363–366 [Japan, Hamana-ko; name only]; Bailey-Brock, 1976, p. 73 [Hawaii, Oahu, several localities; name only; part of material studied by us].

Material studied. France: Canal de Caen, 19 Sept. 1922, L. Mercier coll. (three syntypes; BMNH 1928: 4: 26: 16–17). Netherlands: Vlissingen, inner harbor, L. de Wolf coll. (empty tubes; tHU 78, ZMU; as *M. enigmatica* by W. J. Wolff); Vlissingen, Keersluisburg, 6 April 1972 and 25 Sept. 1973, on piling near power station, about 1 m deep, H.A. ten Hove coll. (very many specimens, tHU 169, 191, ZMA V. Pol. 2615, SME). Tunisia: Lac de Tunis, 1969, B. Hotman coll. (many specimens; tHU 85; as *M. enigmatica* by H. Zibrowius). South Africa: Cape Town, Milnerton Estuary (two specimens, 20 tubes; BMNH 1952: 8: 10: 1; as *M. enigmatica* by J.H. Day). Australia: Western Australia, Pelican, Swan River, 17 Aug. 1935, D. L. Serventy coll. (two specimens and others in tubes; BMNH 1938: 10: 31: 29–32; as *M. enigmatica* by C. C. A. Monro and H. Zibrowius); New South Wales, Sydney, Tempe, Cooke's River, B. Dew coll. (fragmentary specimen, clusters of tubes; BMNH 1955: 9: 2: 1–20; as *M. enigmatica* by N. Tebble). United States of America: Texas, Rockport, 28 Sept. 1951, fouling on bottom of boat, G. Gunter coll. (two specimens; AHF); California, Oakland, Lake Merritt, 1931 (ten specimens, 20 tubes; tHU 232; as *M. enigmatica* by P. Fauvel); Hawaii, Oahu, Honolulu, Alai Wai Canal near Waikiki, J. H. Bailey-Brock coll. (three specimens and several others in tubes; tHU 163; as *M. enigmatica* by J. H. Bailey-Brock). Uruguay: Las Brujas, Canelones, 25 July 1937 (many specimens in tubes; BMNH 1937: 10: 15: 1–10; as *M. enigmatica* by C. C. A. Monro and H. Zibrowius). Argentina: Buenos Aires (prov.), Albufera de Mar Chiquita, desembocadura del Canal 7, 12 Oct. 1968, J. M. Orensanz coll. (32 specimens; tHU 150).

Tube: The tube is white, sometimes covered with a brown layer, presumably algae. It is semicircular to circular in cross-section. At irregular intervals it often bears wide, flaring, sometimes faint, collar-like rings indicating the successive positions of the peristome (Fig. 5c). Solitary or juvenile tubes sometimes have a faint median keel (see Cognetti, 1954, Fig. 1).

Branchiae: The branchial filaments arise from paired lobes and number about seven (5–9) on the left and eight (7–10) on the right. They are arranged in two semicircles and are not connected by a branchial membrane. The filaments are somewhat shorter ventrally. The two rows of pinnulae become larger towards the ends of the filaments, which are free of pinnulae to a greater or lesser extent (Fig. 3d–e).

Peduncle: The peduncle is smooth, sometimes faintly wrinkled, especially below the bulb of the operculum (Fig. 2g); it is subtriangular in cross-section with

a shallow dorsal groove (Fig. 2f). There is a gradual transition between peduncle and opercular bulb (Fig. 2f).

Operculum: The operculum is fig-shaped, usually bilaterally symmetrical with a distal eccentrically placed concavity. The concave part generally has a horny plate, bordered by one to four rows of spines, curved inward (Fig. 2f–h). The rows of spines may be incomplete dorsally, or somewhat irregular (Fig. 2h). The spines are randomly placed in a few specimens (Fig. 2i). Exceptionally the operculum lacks spines (Fig. 2e). The spines sometimes have one to three short radial accessory spines (Fig. 31–q).

Collar and thoracic membranes: The collar is high, not lobed and has an entire edge. It is continuous with the thoracic membranes which are united ventrally on the anterior abdominal segments.

Thorax: The thorax has seven segments, six of which are uncinigerous. The collar setae are of two types: coarsely serrated (Fig. 4a–d) and limbate. Subsequent bundles of setae are larger and are in two nearly parallel rows, containing limbate setae only (Fig. 4s). The thoracic uncinigerous tori are arranged in two nearly parallel rows, with up to 90 uncini per torus. The uncini along the entire thorax have a single row of six to seven curved teeth; the most anterior tooth is gouged, apparently bifurcated (Fig. 4aa–bb).

Abdomen: The number of abdominal segments is usually about 60 (29–84; $n = 7$). The anterior two or three segments are apparently without setae or uncini. The following segments have relatively few uncini (21–35), the number per row increasing rapidly in the anterior one-third of the abdomen (80–120), then slowly decreasing towards the pygidium (3–20). The abdominal uncini of the anterior segments have a single row of curved teeth (six to seven), including the anterior gouged tooth; the uncini of the posterior segments are smaller, with two rows of small curved teeth, with 10–12 teeth visible in profile, including the anterior gouged one (Fig. 4nn–vv). The bundles of abdominal setae consist of two to five geniculate ones (Fig. 4zz).

Size: The length, including the operculum, is usually about 20 mm (7–44). The width of the thorax is about 1 mm (0.9–1.2). The branchiae and the operculum usually account for one-sixth of the entire length of the animal.

Discussion: *Ficopomatus enigmaticus* is mentioned in well over 150 papers, in various fields of research. We want to emphasize that some important ecological works have been based upon incorrectly identified material. Therefore, the results of this research can be evaluated only after a careful comparison with the distributional data, given in this paper (Fig. 6).

Records from Japan (Okayama Pref., Kojima Lake; Tokyo, Sumida River; Ryukyu Islands, Ishigaki-jima, Kabin Bay) have been confirmed by an excellent unpublished figure by M. Imajima.

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Summary

The brackish water serpulid genera *Mercierella, Mercierellopsis, Neopomatus* and *Sphaeropomatus* are synonymized with *Ficopomatus,* including four species: *F. enigmaticus, F. macrodon, F. miamiensis* and *F. uschakovi.* The geographical distributions of the species are illustrated, and the confused identity of tropical specimens has been clarified, at least in part. The generic position of *Ficopomatus capensis* is discussed. Fossil records of *Mercierella* and related genera most probably do not belong to the genus *Ficopomatus.*

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LIFE CYCLE, DISTRIBUTION AND ABUNDANCE OF *CARCINONEMERTES EPIALTI*, A NEMERTEAN EGG PREDATOR OF THE SHORE CRAB, *HEMIGRAPSUS OREGONENSIS*, IN RELATION TO HOST SIZE, REPRODUCTION AND MOLT CYCLE

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The conceptualization of the host as a microenvironment for the symbiont (Pavlovski, 1934) provided the seed for the growth of the field of parasite ecology. However, the difficulties involved in quantifying the biology of two disparate organisms, host and parasite, have impeded the study of host-symbiont systems from an ecological perspective. As hosts, arthropods lend themselves well to symbiont population studies. Cyclical events through the course of successive molt cycles impose many restrictions on aspects of host growth and reproduction. Thus, many host life history events are discrete and amenable to quantification.

In this study populations of the nemertean egg predator, *Carcinonemertes epialti* Coe, 1902 were monitored in relation to the biology of its host, *Hemigrapsus oregonensis* (Dana, 1851), a common intertidal shore crab along the west coast of North America. Adult specimens of *C. epialti* are only found within or adjacent to egg masses of female crabs. They live in mucoid tubes of their own construction. Since *C. epialti* adults feed on crab eggs, host reproductive conditions are a primary factor in the worm's biology.

The nonfeeding juvenile portion of the nemertean's life cycle is spent ensheathed on the exoskeleton of host crabs of either sex. Newly molted crabs lack nemerteans. The nemertean population of the previous instar is shed at ecdysis. Nemertean transmission through a host molt cycle should result in increasing nemertean density on the host with advancing stages in the molt cycle. Furthermore, crabs with longer intermolt cycles, such as large crabs, should tend to have more nemerteans. Thus, *C. epialti* is regarded as a population of animals disseminating through a habitat which consists of systematically renewed substrates, crab exoskeletons. Access to the host cuticle can be partially estimated by a size and sex-specific determination of the host's molt stage. Several studies have compared the biology of epizoic organisms with the general pattern of the host molt cycle. Barnacles have been used to estimate the molting frequency of lobster (*Nephrops*) hosts (Barnes and Bagenal, 1951). The barnacle *Trilasmis* reproduces more frequently than the average spiny lobster host intermolt duration, assuring continual replenishment of epizoic populations (Bowers, 1968). Apostome ciliates excyst and initiate feeding at host ecdysis (Trager, 1957; Bradbury and Trager, 1967). Peritrichous ciliates swarm at ecdysis of the gammarid amphipod host (Fenchel, 1965). The bryozoan, *Triticella koreni*, metamorphoses only on the cuticle of a recent postmolt *Calocaris* (Thalassinidea) (Ström, 1969), and the colony produces embryos just prior to the annual molt of the host (Ström, 1969;

Eggleston, 1971). Total abundance of epizoic hydroids, bryozoans and barnacles is greater on crabs (*Bathynectes*) in late postmolt (C_{1-3}) plus intermolt (C_4) molt stages than on early postmolt (A_1–B_2) stages (Lewis, 1976). The present study, employs the molt-staging scheme of Drach (1939; Drach and Tchernigovtzeff, 1967) to examine epibiont-host synchrony in detail.

The intricate exoskeletal morphology of an arthropod offers unique opportunities for studies of habitat preference and utilization. Here is a habitat which is varied, yet standardized. A given pit or groove varies in a manner that lends itself to a reasonably simple quantitative description. Studies of habitat exploitation and selection for epizoic forms on crustacean hosts have rarely (Walker, 1974) reached the level of sophistication demonstrated in studies of water mites on aquatic insects (Efford, 1965; Mitchell, 1967, 1968; Lanciani, 1970, 1971; Davids, 1973).

Although egg predators of crustaceans are common (Kuris, 1971), few population studies have been conducted. Humes (1942) and Hopkins (1947, 1970) describe the life cycle of *Carcinonemertes carcinophila* on the blue crab, *Callinectes sapidus,* in detail. Wickham (1977) describes a new distinctive species of *Carcinonemertes* from *Cancer magister* and indicates (Wickham and Fisher, 1978) that it is responsible for considerable brood mortality in this commercially important species.

Other than the original description from the kelp crab *Pugettia producta* (Coe, 1902) and a host (*Euphylax dovei*) and range extension to Payta, Peru (Humes, 1942), *C. epialti* is unstudied. *Carcinonemertes epialti* occurs on *H. oregonensis* at 13 localities from Bahia San Quintín, Baja California, Mexico, to Page's Lagoon, British Columbia, Canada (Kuris, 1971). The geographic distribution on the Pacific Coast of North America appears to be continuous between these two localities.

Materials and Methods

Field studies were conducted at Campbell Cove, Bodega Harbor, Sonoma County, California, during 1969–1971. Additional collections were made during the summers of 1973–1975. Material for some studies was sometimes collected elsewhere in Bodega Harbor. *Hemigrapsus oregonensis* was collected monthly from randomly placed removable substrate traps (sampling program detailed in Kuris, 1971), and the nemertean populations were censused. Supplementary host samples were collected by hand and at random without regard to size, sex or reproductive condition.

During April-May, 1969, and June-July, 1970, a survey of 26 populations of *H. oregonensis* was conducted along a transect from Bahia San Quintín, Baja California, Mexico, to Ucluelet, Vancouver Island, British Columbia, Canada. These collections, of 75–150 adult crabs each, greatly extended the geographic range of *C. epialti.* Station records are given in Kuris (1971).

From all crabs the following was recorded: carapace width to 0.1 mm, taken with a vernier caliper at the notch immediately anterior to the third lateral spine; sex; and molt cycle stage according to the scheme of Drach (1939) and Drach and Tchernigovtzeff (1967). Criteria and techniques used for molt stage assignment are given in Kuris (1971). For ovigerous female crabs the embryogenic process

was divided into 20 egg development stages based on cell number, amount of yolk remaining and appearance of various embryonic structures (Kuris, 1971).

All hosts were sampled by inspection of the external surface of the exoskeleton. Special attention was paid to the branchial chamber, the sternal-abdominal furrow and the pereiopod axillae. As the crustacean exoskeleton is a very complex but highly standardized structure, site specificity of *C. epialti* was detailed by subdividing the host's surface into 150 potential sites on male crabs and 160 sites on female crabs. Adult nemerteans and their eggs were only observed on ovigerous female crabs. Adult nemerteans were removed, then sized and sexed using a compound microscope.

Transmission of juvenile nemerteans was tested experimentally. Lightly infested hosts were examined daily, and all visible nemerteans were removed. These hosts were regarded as clean when no nemerteans were recovered on three successive days. One group each of three individual cleaned males, females with ripe ovaries (pre-ovigerous) or ovigerous females with broods in an early stage of embryogenesis was exposed to a single heavily infested (10 + nemerteans) male crab. Thus, four crabs, one infested, and three cleaned, were confined together in perforated 14 mm × 10 mm × 4 mm hard plastic boxes maintained in running sea water. Controls for each of these three combinations were run simultaneously by using a cleaned male in place of the infested male. A gravel substrate and a few small rocks for cover were provided in each transmission box. Crabs were fed *Ulva* every other day. All crabs were marked individually by a tattoo method (Kuris, 1971). The transmission experiments were conducted in July and August, during the period of peak nemertean abundance.

The number of nemerteans on the infested and clean crabs were recorded on the experimental days 7 and 14. The experiments were terminated on experimental day 14, at which time the crabs were dissected and exhaustively searched for the presence of nemerteans. The entire transmission experiment was replicated once.

Results

Host specificity

In the Campbell Cove, Bodega Harbor, study area, *Carcinonemertes epialti* is regularly found on *Hemigrapsus oregonensis,* with *H. nudus* serving less frequently as a host. In the lower reaches of the intertidal, *Carcinonemertes* species also occur regularly on *Cancer antennarius, C. anthonyi,* and *C. productus* juveniles and adults.

Important negative records based on hundreds of observations include *Pachygrapsus crassipes,* and the anomurous crab, *Petrolisthes cinctipes.* No nemerteans were ever found on the surface of 45 juvenile *Pugettia producta* from the shallow subtidal regions adjacent to the study area. A search of about 50 female ovigerous *P. producta* from elsewhere along the Sonoma coast produced a single positive record (recovered by R. I. Smith and examined by the author). However, *P. producta* from the Santa Barbara Channel is more frequently infested by *C. epialti.*

Life cycle

Extrusion of an egg mass by a nonovigerous female crab signals the start of the reproductive phase of the nemertean life cycle. Juvenile nemerteans ensheathed on

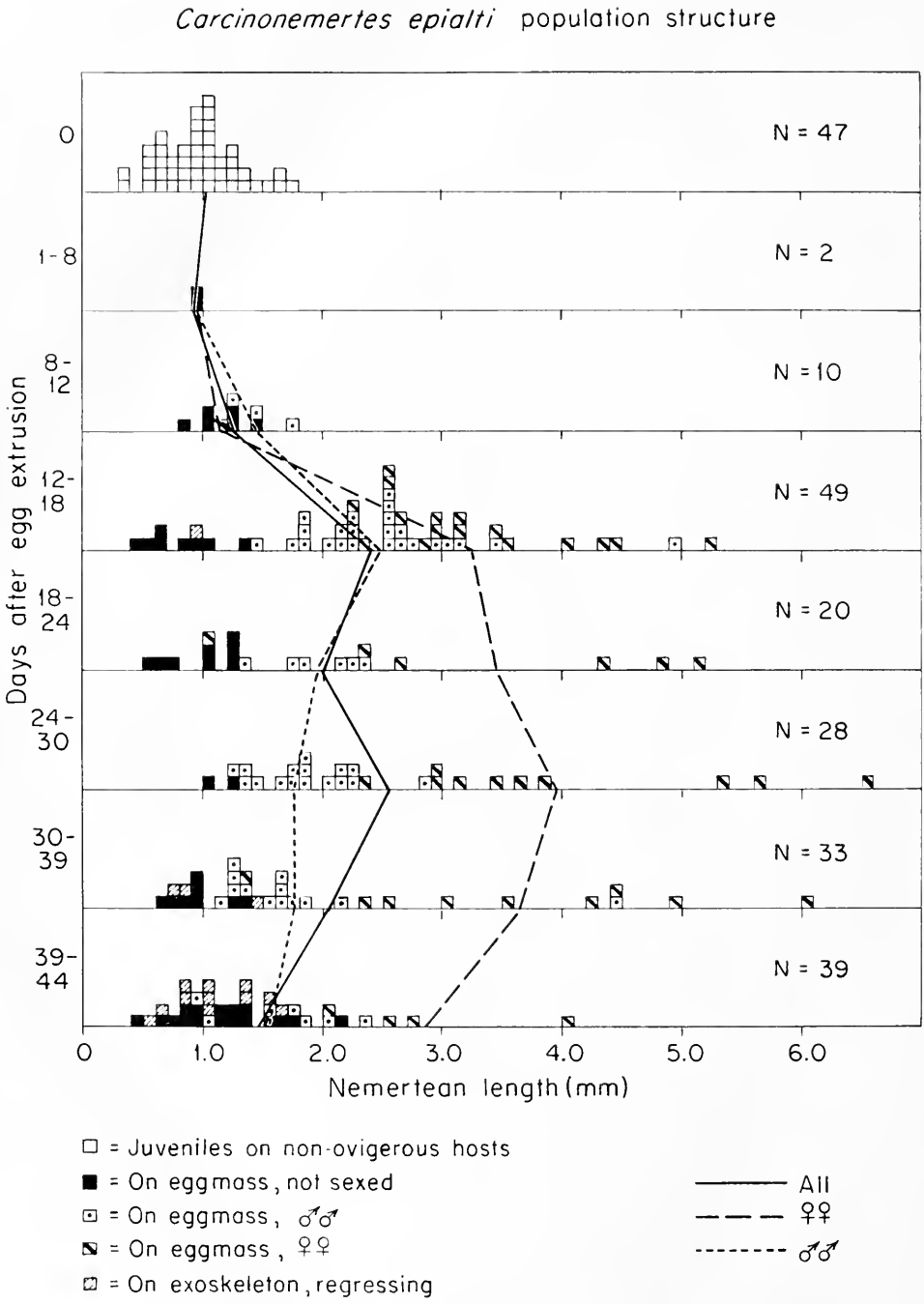


FIGURE 1. Size-frequency histogram, representing the population structure of *Carcinonemertes epialti* during the period of host egg development. Average sizes of sexable males (short dash line), females (long dash line) and the entire sample (solid line) are superimposed on histogram.

regions of the exoskeleton remote from the host egg mass exsheath and migrate to the pleopods or sternal surface of the thorax and abdomen. Here each individual constructs and inhabits a mucous tube. Occasionally, both sexes may be found within the larger female tube. A gravid *C. epialti* female deposits her eggs in the posterior portion of her mucous tube. No nemertean egg tubes are found on crabs with embryogenesis less advanced than initiation of thoracic limb development which is reached 18 days after host oviposition (Kuris, 1971). Embryogenesis advances until thoracic limb buds are large before hatching nemertean eggs are observed. At 10–12° C thoracic limb bud development takes 8 days (Kuris, 1971). This interval estimates the duration of nemertean embryogenesis. Nemertean oviposition may proceed for the next 25 days, until host eclosion. Nemertean egg hatching may continue five days subsequent to the hatching of the crab eggs.

Growth of the nemerteans, on the host's egg mass from the time of host egg deposition, is rapid (Fig. 1). From the average juvenile length of 1.0 mm, female worms grow to an average adult size of 3.9 mm 24–30 days after the host becomes ovigerous. Males grow more slowly, reaching an average of 2.5 mm 18–24 days after egg extrusion. Thirty days from the time of egg deposition, some of the nemerteans begin to regress, even to leave the egg mass and retire to the other sites on the crab. By the time the zoeae hatch, the average sexable adult females are 2.8 mm; the males, 1.6 mm. Both of these values probably over-estimate the size of adult worms, as regression below 1.5 mm makes sexing difficult. The average size of all worms on egg-bearing females reaches a maximum of 2.5 mm by 30 days after egg deposition, and then declines to 1.4 mm by the time the host's brood hatches, 44 days after having become ovigerous.

The nemertean's modified (Hyman, 1951) pilidium larva appears to be planktonic for an unknown period of time. Ultimately, this dispersal stage must settle on a crab host and transform to a juvenile.

Crab hosts of either sex, mature or not, may become infested with juvenile *C. epialti*. However, only when ovigerous or pre-ovigerous crabs are infested may the life cycle be completed.

Some of these larval and juvenile nemerteans reach pre-ovigerous female hosts. The sites occupied by juveniles on such crabs are similar to those occupied by juveniles on nonovigerous hosts. However, a day or two after a pre-ovigerous host undergoes oviposition, virtually all the juvenile nemerteans ensheath and migrate to the vicinity of the host's egg mass. Here the nemerteans begin a period of rapid growth, sexual differentiation and maturation. Copulation presumably occurs when the male nemertean enters the female's mucous sheath.

As the host's eggs near the date of hatching, the nemerteans cease to grow (Fig. 1). Some worms leave the egg mass, frequently migrating to sites within the branchial chamber of the host. The anterodorsal surface of the host's branchial chamber is frequently occupied by these worms. Here they ensheath, decrease in size, and become reproductively inactive. Soon they are indistinguishable from juvenile nemerteans. Some of the post-reproductive worms may die rather than regress. Large, seemingly moribund individuals are seen shortly after eclosion of the host brood. These worms may merely be undergoing negative growth, however. Whether secondarily reduced, post-reproductive worms are capable of another reproductive period is unknown.

TABLE I

Experimental transfer of juvenile C. epialti *from heavily infested male crabs (donors) to uninfested male and female (ovigerous and nonovigerous) hosts.*

Experimental combination	Number of hosts	Number of worms removed prior to start	Number of worms observed on day			Number of worms after dissection	Number of worms unaccounted for	Mean worm density on recipients day 14
			0	7	14			
Donors	2	—	54	31	14	22	18	—
Recipient males	6	3	0	7*	11	14	—	2.3
Donor control	1	0	0	0	0	0	0	—
Recipient male controls	3	6	0	1	1	1	—	0.3
Donors	2	—	100	75	57	69	19	—
Recipient nonovigerous females	6	4	0	1	6	12	—	2.0
Donor control	1	0	0	0	0	0	0	0
Recipient nonovigerous female controls	3	5	0	3	3	3	—	1.0
Donors	2	0	115	105	68	75	12	—
Recipient ovigerous females	6	2	0	5	12**	14	—	4.0
Donors control	1	0	0	0	0	0	0	—
Recipient ovigerous female controls	3	3	0	2	2*	2	—	1.0

* One recipient died prior to observation date, data for day 14 based on 2 donors and 5 recipients.

** One donor died prior to observation date, data for day 14 based on 1 donor and 3 recipient crabs.

Based upon microscopic examination of the gut, host eggs appear to be the only source of nutrition for the adult worms. Juvenile worms appear to have empty digestive tracts. Thus, it is not surprising to find that growth of juvenile worms on male hosts, juvenile females, or nonovigerous adult females is quite restricted. The size range of juvenile nemerteans from these sources is only 0.4 to 1.6 mm. These juveniles are considered to be essentially phoretic on the host crab. Since the newly-hatched larvae are 0.2 mm long, little food seems to be required for transformation to the juvenile form, and subsequent maintenance on the host exoskeleton. The source of energy for this maintenance remains unknown.

Transmission

It is likely that there are two modes of transmission for *C. epialti*. The free-swimming larval stage facilitates interhost transmission. Incidents of direct contact between hosts, followed by transferral of juvenile worms, may also enable transmission to occur. Direct transmission was tested experimentally.

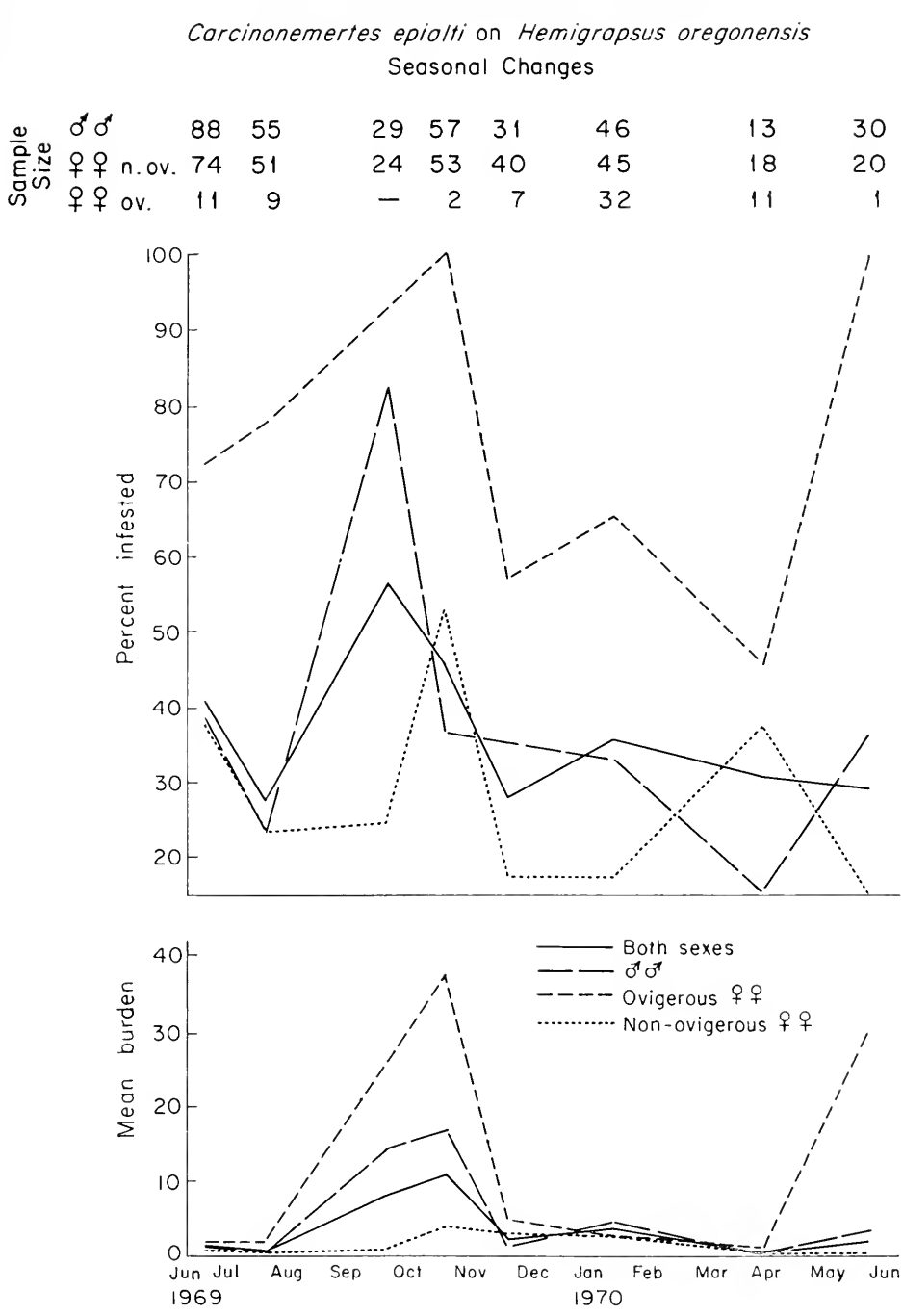


FIGURE 2. Seasonal pattern of the percentage of infestation (top) and mean burden (mean density per host) (bottom) for all hosts, ovigerous females, nonovigerous females and males over 8 mm, at Bodega Harbor.

Table 1 shows that significant transfer (G-test, day 14, $P < 0.05$) of juvenile nemerteans may occur between hosts and suggests that ovigerous hosts may elicit more transferrals than nonovigerous hosts. The occasional nemerteans seen on the externally cleaned and unexposed control crabs are probably derived from sites hidden within the host's branchial chambers, inaccessible to the removal-trapping method of cleaning crabs.

Infestation frequency and nemertean density

Seasonal variation. For 741 crabs greater than 8.0 mm wide, the overall infestation rate for 1969–70 at Bodega Harbor was 36.3%. Burden, the mean density per host (including uninfested hosts), was 3.96.

Through the sample year, the infestation level of *C. epialti* on *H. oregonensis* varied from 28% to 57%. Figure 2 shows that the overall infestation rate actually remained steady at 30–40%, except for the September and October samples, which reached 57% and 46%, respectively. This rise occurs when the host population is reproductively inactive while undergoing the final ecdysis prior to the onset of the winter anecdysial period (Kuris, 1971). The maturation of large juvenile and prepuberty female hosts also occurs at this time; so virtually all the crabs over 8.0 mm are adults in the coming winter reproductive season. The nemertean density per host reflects this pattern, rising to an October peak density of 11 worms per host. Excepting the autumn samples, worm burden ranges from 0.5 to 4.0 worms per host. Surprisingly, the peak period of crab reproduction, November to February (Kuris, 1971), is the interval of lowest nemertean density (Fig. 2).

Host reproduction. The importance of host reproduction to population dynamics of *C. epialti* is seen in Figures 2 and 4. In all seasons the frequency of ovigerous female crabs harboring nemerteans is higher than that of nonovigerous females or males. With the exception of the January sample, this is also true of the average nemertean density per host crab over 8 mm.

As host eggs proceed through embryogenesis, an increase in nemertean prevalence and density might be expected on ovigerous crabs (Table II). The percentage of infestation increases slightly, from 62.9% of broods in embryogenic stages to 73.7% of broods in late stages, with slight fluctuations at intermediate stages. Burden ($\bar{b}$) remains essentially the same (4.2–4.4) from early through late middle egg development stages. However, $\bar{b}$ then rises sharply, to 8.11 in late stage broods.

During host embryogenesis the worms anchor their mucous sheaths and feed while protruding from the open end. The nemerteans are able to feed on the eggs if their sheath is entwined among the host egg mass or is attached to the abdominal appendages or the sternal surfaces of the abdomen and the thorax. On nonovigerous adult female crabs only 73.3% of the nemerteans are found in the vicinity of the egg mass. However, almost immediately after deposition of the host's brood, 95.9% are near the egg mass. This distribution pattern remains almost constant for the first 26 days of host embryogenesis. Towards the end of the brooding period, a gradual withdrawal to other sites is evident (Table II). Only 64.7% of the nemerteans remain at sternal locations on post-ovigerous female crabs.

Host molt cycle. Figure 3 shows the changes in nemertean burden on similar-

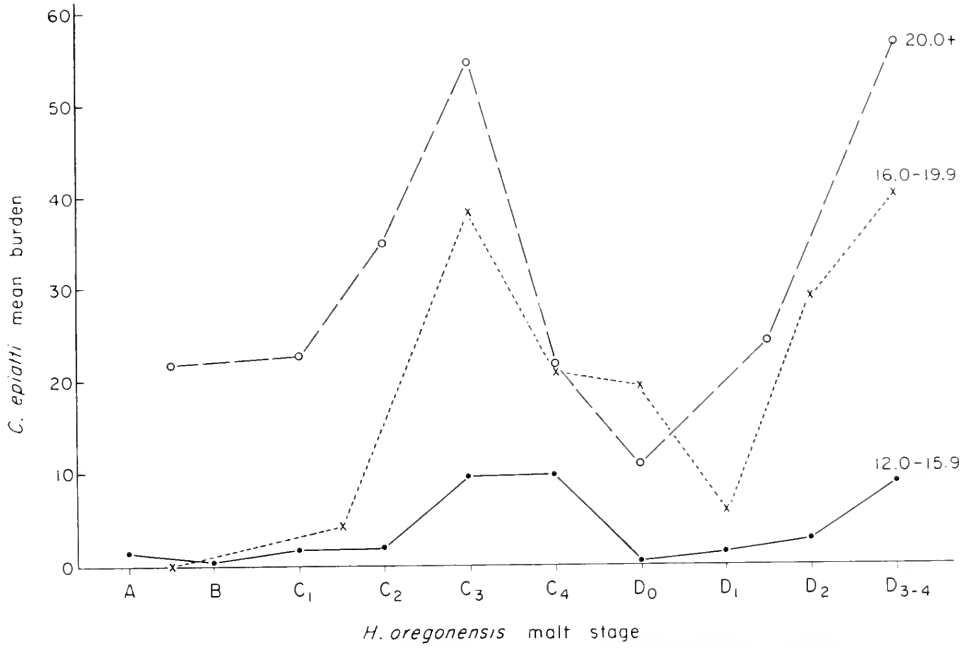


FIGURE 3. Average burden (mean density per host) of *Carcinonemertes epialti* on different sized male *Hemigrapsus oregonensis* in different stages of the molt cycle.

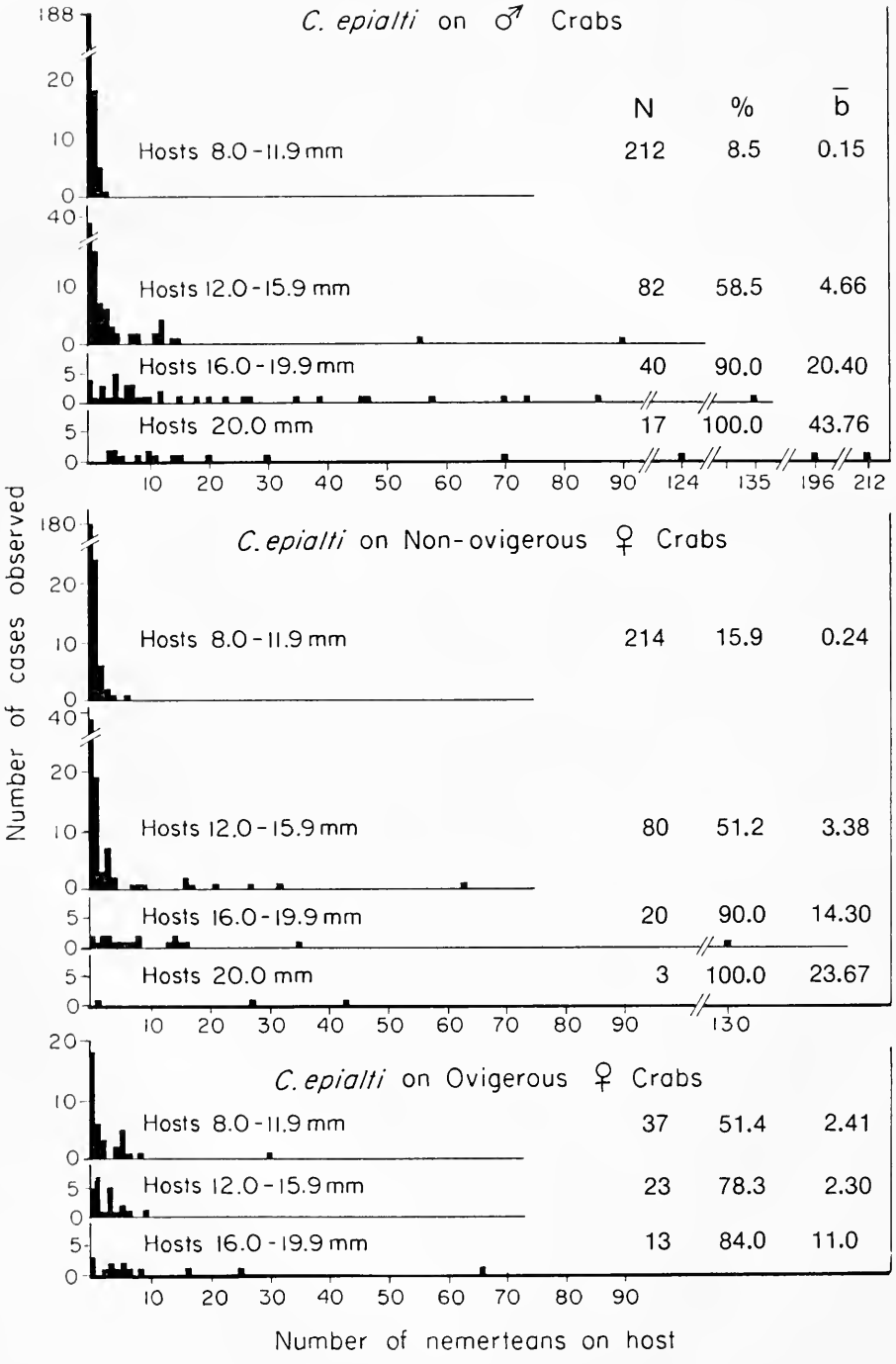
sized male hosts, with successive molt stages. The average burden is seen to rise sharply from Drach molt stages A to C_3 or C_4. However, from C_4 or D_0 to D_1 there is a sharp drop in average density per host; this is followed by an equally sharp rise in late premolt, D_2-D_4.

Host size. Figure 4 shows that both nemertean incidence of infestation, and the average burden, increase dramatically with increasing host size. Ovigerous females, while showing some size effects (Fig. 4 bottom), do not show as sharp an increase in average density with increasing size as do males and nonovigerous females. The incidence of infestation among different size classes is significant

TABLE II

Percentage infestation (%i), average burden ($\bar{b}$), and site preferences of C. epialti *on ovigerous crabs through the course of embryogenesis. Post-ovigerous crabs are also included.*

Egg development stages (grouped)	n	%i	$\bar{b}$	Percentage of nemerteans on egg mass and on thoracic and abdominal sterna	Percentage of nemerteans at other sites
Early (1 to 12 days)	35	62.9	4.2	95.9	4.1
Early middle (13 to 26 days)	58	74.1	4.4	95.7	4.3
Late middle (27 to 37 days)	38	76.3	4.3	88.1	11.9
Late (38 to 43 days)	19	73.7	8.1	73.2	26.8
Post-ovigerous (duration uncertain)	11	72.7	17.0	64.7	35.3


C. epialti on ♂ Crabs
N
%
b̄
Hosts 8.0-11.9 mm
212
8.5
0.15
Hosts 12.0-15.9 mm
82
58.5
4.66
Hosts 16.0-19.9 mm
40
90.0
20.40
Hosts 20.0 mm
17
100.0
43.76
C. epialti on Non-ovigerous ♀ Crabs
Hosts 8.0-11.9 mm
214
15.9
0.24
Hosts 12.0-15.9 mm
80
51.2
3.38
Hosts 16.0-19.9 mm
20
90.0
14.30
Hosts 20.0 mm
3
100.0
23.67
C. epialti on Ovigerous ♀ Crabs
Hosts 8.0-11.9 mm
37
51.4
2.41
Hosts 12.0-15.9 mm
23
78.3
2.30
Hosts 16.0-19.9 mm
13
84.0
11.0
Number of cases observed
Number of nemerteans on host

(G-test, $P < 0.001$) for males and nonovigerous females and for ovigerous females ($P < 0.05$).

Within each size class there is no significant difference between the three reproductive classes except for the 8.0–11.9 mm size class ($P < 0.005$). Relatively high levels of infestation among small (8.0–11.9 mm) ovigerous females account for both the generally high infestation rate of ovigerous females compared with males and nonovigerous females, and the significant difference in incidence seen among the reproductive categories for the smallest size class.

Two changes in the pattern of nemertean abundance occur as host size increases. The frequency of uninfected crabs drops sharply (91.5% to 0.0%) with increasing size. Also, the frequency of heavy infestations, $\bar{b} > 9$, increases strongly, from 0.2% to 65.0%. These trends with increasing size result in contagious distribution patterns; the variance to mean ratio (coefficient of dispersion) greatly exceeds one in all groups over 12 mm. Even in the 8–12 mm size classes, the coefficient of dispersion is over one, indicating that these samples are also clumped. Tests for goodness of fit (χ^2, Sokal and Rohlf, 1969) result in highly significant differences from the expected Poisson distribution in all 4 mm size classes for male, ovigerous female and nonovigerous female hosts.

Site specificity

The distribution of nemerteans is analyzed here for male crabs only. Nemerteans on nonovigerous crabs have a similar pattern of occurrence, differing only in the details of female *versus* male sternal anatomy.

On male crabs fifty of the sixty investigated sites harbored *C. epialti* with some regularity. The most frequented sites include the anterior face of the arthrodial membrane at the base of the coxa of the fourth walking leg, the posterior face of the equivalent membrane of the cheliped, and the ventral angles of the axillae between the second and third, and third and fourth walking legs. Other points at the bases of the limbs were only slightly less commonly utilized as sites. In general, locations in the second axilla, between the first and second walking legs, were the least commonly inhabited sites on the limb bases. In the sternal-abdominal furrow, the anterior sternal sutures of the thorax, the bases of the copulatory pleopods, and the anterior segments of the abdomen were often frequented. In the gill chamber, the fourth and fifth thoracic epimera and the vicinity of the pericardial sacs were commonly utilized sites.

A single case of internal infection of *H. oregonensis* by *C. epialti* was observed. Three juvenile nemerteans were found in the posterior portion of the host's intestine. Occasionally, juvenile nemerteans are wedged deeply into the apodemes originating from the branchial region of the thorax. Superficially, these resemble internal infections. Exsheathed juveniles are occasionally found actively wandering about the host surface.

FIGURE 4. Size frequency histogram for *Carcinonemertes epialti* on different size classes of 351 male, 317 nonovigerous and 73 ovigerous female *Hemigrapsus oregonensis*. N. is the sample size; %i is the percentage infested; and $\bar{b}$, mean burden, is mean density per crab (including uninfected crabs). For all male crabs %i = 36.0%, $\bar{b} = 5.70$; for all nonovigerous females %i = 30.3%, $\bar{b} = 2.14$; for all ovigerous females %i = 65.8%, $\bar{b} = 3.90$.

TABLE III

Site utilization by C. epialti *on male* H. oregonensis *at different host sizes. All densities of worm infestation are included. See text for descriptions of site regions I–IV. The percentage of nemerteans in a region is in parentheses; b is the number of nemerteans; i is the number of hosts infested.* $G_H = 93.268$, $P < 0.005$; *an* a posteriori *STP shows that the two smallest and three largest size classes constitute homogeneous sets.*

Host size group (in mm)	b at sites				Σb	i	Σb / i
	I	II	III	IV			
8–11.9	4 (5.6)	3 (4.2)	64 (88.9)	1 (1.3)	72	44	1.64
12–15.9	28 (4.1)	80 (11.6)	566 (82.1)	15 (2.2)	689	83	8.30
16–19.9	136 (7.5)	418 (22.9)	1243 (68.1)	27 (1.5)	1824	68	26.82
20+	182 (7.5)	578 (24.0)	1614 (66.9)	39 (1.6)	2413	76	31.75
	350	1079	3487	82	4998	271	18.44

To examine the relationship between host size and site specificity, potential sites were grouped into four regions. (I) branchial chamber-pericardial; (II) sternal-abdominal furrow; (III) interlimb axillae; (IV) miscellaneous (mouthparts, exposed body surface). Table III indicates that only region II shows a progressive increase in the percentage of site utilization of *C. epialti* with increasing host size. The heterogeneity G-test statistic (G_H; Sokal and Rohlf, 1969) is highly significant; size-specific site utilization is not homogeneous. As there is significant heterogeneity among size classes, an *a posteriori* test by a simultaneous test procedure (STP) of the different sizes for goodness of fit (Sokal and Rohlf, 1969) is used to locate the source of the heterogeneity. The results of the STP using the G-statistic indicate that a highly significant difference in the frequency of the nemertean at certain sites occurs between small ($\leq$ 15.9 mm) and large ($\geq$ 16.0 mm) crabs. Apparently, some of the sites in the sternal-abdominal furrow are not available to the nemerteans on small crabs. Presumably due to spatial considerations, these sites become available on crabs over 16.0 mm.

Changes in site utilization in relation to nemertean density were analyzed in a similar fashion to the site-host size relationship. Infested crabs were apportioned

TABLE IV

Shift in site preference with changes in nemertean density on H. oregonensis. *All sizes of infested hosts included. Site regions I–IV are described in text. The percentage of nemerteans in a region is in parenthesis; b is the total number of nemerteans; i is the number of hosts infested.* $G_H = 110.186$, $P < 0.005$; *an* a posteriori *STP disclosed these homogeneous sets: a) 1–10, 11–19, 20–49, b) 20–49, 100+, c) 1–10, 11–19, 50–99.*

Range of worm burdens	I	II	III	IV	Σb	i	Σb / i	Mean host size
1–10	47 (8.2)	90 (15.6)	435 (75.5)	5 (0.0)	577	156	3.70	15.6
11–19	19 (5.0)	67 (17.7)	286 (75.7)	6 (1.6)	378	29	13.03	18.0
20–49	88 (8.5)	217 (20.9)	720 (69.2)	15 (1.4)	1040	31	33.55	19.8
50–99	57 (5.0)	180 (15.8)	876 (77.0)	24 (2.1)	1137	15	75.80	18.8
100+	139 (7.5)	525 (28.2)	1163 (62.6)	32 (1.7)	1859	12	154.92	20.0

among five nemertean density classes (Table IV) without regard to host size; G_H is highly significant. An *a posteriori* STP was performed to locate the source of the heterogeneity. The STP shows that crabs having 20–40 and 100+ nemerteans (Table IV, homogeneous set b) have significantly more nemerteans on region II and fewer on region III than occur at other worm densities (homogeneous set c). Homogeneous set a shows that there is some overlap between sets b and c at these sample sizes.

Discussion

The common occurrence of *Carcinonemertes epialti* on *Hemigrapsus oregonensis* contrasts with its occasional presence on *H. nudus*, its scarcity on *Pugettia producta*, and its absence on *Pachygrapsus crassipes*. The infrequent infestation of *Pugettia producta* (= *Epialtus productus*) by *C. epialti* is of interest, as this is the type host (Coe, 1902). At least along the Sonoma coast, the specific name *epialti* is an unfortunate choice. All the ovigerous specimens of *P. producta* from this region are infested with several hundred turbellarian egg predators of an undescribed species [*Monocelis?* (Sakaji, personal communication)]. The inadequately documented record of *C. epialti* on *P. producta* (Boolootian, Giese, Farmanfarmaian and Tucker, 1959) is probably a misidentification of the undescribed turbellarian. The size (1–2 mm), and the activity, "gliding continually" (p. 219), both fit the turbellarian, and decidedly not *C. epialti*.

The small worms, found on *Cancer magister* by MacKay (1942) and probably misidentified as leeches (Sindermann and Rosenfield, 1967), are most likely the *Carcinonemertes* species to be described by Wickham (1977). Specific identification of the *Carcinonemertes* found on other *Cancer* species awaits further study (Wickham, personal communication).

Humes (1942) observed that 20 of the 26 host records for *Carcinonemertes* spp. available to him were for portunid crabs (including the Peruvian portunid *Euphylax dovei* as a host for *C. epialti*). He considered the littoral Portunidae to be the principal hosts for these worms due to their habits, abundance, and habitat preferences. The host specificity records for *Carcinonemertes* on the Pacific coast of North America, suggest that neither portunids, nor the behavioral and habitat characteristics typically associated with the swimming crabs, are necessarily associated with nemertean infestations.

The Carcinonemertidae are considered to exhibit little host specificity (Humes, 1942). However, the negative records on *Pachygrapsus crassipes*, despite the habitat overlap of these crabs with heavily infected host species, suggests that host specificity does play a part in governing the distribution of *C. epialti*.

A comparison of the life cycle of *C. epialti* with *C. carcinophila* (Humes, 1942; Hopkins, 1947) shows some important differences. The juvenile stage of *C. carcinophila* is found almost exclusively on the gills of the nonovigerous host. This site may indicate a decreased opportunity for interhost transfer of juvenile worms during casual contact. However, frequent transfer during the mating act seems feasible since copulation, followed by a post-mating embrace, is a lengthy process in portunid crabs (Hartnoll, 1969), principal hosts of *C. carcinophila*.

The fate of the post-reproductive adult nemertean is another potentially distinctive species difference. In *C. carcinophila* the adult worms retire to the gill chamber

of the host upon hatching of the crab's brood (Humes, 1942; Hopkins, 1947, 1970). Here they can be distinguished from pre-reproductive juveniles by their bright red color. The principal western Atlantic host, *Callinectes sapidus,* ceases to molt upon reaching adulthood (Van Engle, 1958), and thus the nemerteans are never shed after the host's eggs hatch. Hopkins (1970) feels that they also return to the host's egg-mass during the next ovigerous period. Since Hopkins (1947) describes the post-reproductive worms in the gill chambers as "large," there is an indication that the post-reproductive worms do not regress to the size of the juvenile worms upon their return to the gills.

Both juvenile transfer and larval settlement are regarded as important factors of a transmission model accounting for the distribution of nemerteans on host crabs. It is proposed that larval settlement of a short-lived larval phase accounts for the occasional occurrence of heavily infested crabs. Since *Hemigrapsus oregonensis* spends long periods of time aggregated under covering rocks (Kuris, unpublished mark recapture study), they may occasionally encounter a dense larval swarm in the small volumes of water with restricted circulation in this confined habitat. The considerably greater surface area of larger crabs available for settlement, as well as the occurrence of additional suitable sites for juvenile worms on such crabs, may result in most of the heaviest infestations being found on these crabs. As most large crabs are males, this would also account for the more frequent occurrence of large numbers of juvenile nemerteans on males.

Transferral of juvenile nemerteans from infested to uninfested hosts through accidental and mating contact may be the means by which most crabs with low nemertean burdens (1 to 10) become infested, as such contacts are of short duration (Knudsen, 1964; Kuris, 1971). Also, most juvenile nemerteans are usually surrounded by a thin mucous sheath; for host transfer to occur they must escape the sheath. Thus, only a small percentage of the population of nemerteans are available for contact transmission at a given instant.

The laboratory transmission experiments indicate that juvenile transferral occurs between donor males and recipient males, nonovigerous females, and ovigerous females. Perhaps such transfers also occur with donor nonovigerous females. However, it seems likely that transfers from ovigerous female crabs are much less likely to occur. Thus, ovigerous females come to have a much higher mean percentage of infestation, 65.8%, than do either males, 35.6%, or nonovigerous females, 30.3%. That this difference is a result of transfer interactions rather than more favorable conditions for larval settlement is shown by the higher frequency of low nemertean burdens on the ovigerous females than on the other classes of hosts (Fig. 4). Were larval settlement to be enhanced on ovigerous females, then the frequency of the heavy nemertean burdens, presumed to be due to larval transmission, would be greater on these females.

Most large crabs, especially those over 16.0 mm, are infested without regard to reproductive state. Thus, only small crabs show the effect of the accumulation of transferred juvenile nemerteans as an increase in the percentage infestation (Fig. 4). If larval nemerteans do not settle preferentially, then the nemertean burden of egg-bearing crabs is increased over nonovigerous crabs only by the number of nemerteans gained by juvenile transfer. In accordance with the transmission model, an increase in the number of nemerteans per ovigerous host over the period

of host embryogenesis is seen (weakly) in Table II, since ovigerous crabs gain but presumably do not lose nemerteans through contact transferral.

The increase in nemertean burden during postmolt stages is also in accord with the nemertean transmission model. However, the intermolt decline and premolt rise in worm abundance for all host size classes indicates that factors other than simple accretion of nemerteans through time are operating. Perhaps male and nonovigerous females lose nemerteans through contact transfer to ovigerous crabs. Crabs avoid contact during postmolt, and copulation is perhaps limited to C_4–D_1 in male crabs (Kuris, 1971). Also, selective transmission to postmolt crabs might give the nemerteans a better chance to locate a pre-reproductive female, or a precopulatory male. However, preferential settlement on these stages would not account for the equally dramatic rise in the abundance of nemerteans in D_2–D_4.

The strongly host-size dependent distribution of *C. epialti* does not appear to be due to a nemertean build-up over time on large crabs with long intermolt intervals. Nemertean populations fluctuate over the intermolt period when crab size (and molt cycle duration) is held constant. More likely, crab size directly influences nemertean burdens. Large crabs have relatively more sites to offer nemerteans, and can accommodate larger nemertean populations; also preferred nemertean sites are more spacious on large crabs and can support more worms per site.

If the size of the host influences the availability for nemertean habitation of certain sites on the host's exoskeleton, then the percentage of nemerteans on relatively unavailable site should rise as host size increases. Such is the case (Table III). However, nemertean density also influences site occupancy (Table IV). Crowding at high density may result in some individuals occupying suboptimal sites. However, those density classes (20–40, 100+) having the greatest proportion of worms at the presumably less preferred sites of region II also have larger mean host sizes (Table IV).

Examination of the interaction between the effects of intermolt interval, site availability and site preference suggests that all three effect the distribution of *C. epialti*. However, the increase in site availability with increasing host size seems to be the most important factor determining site occupancy.

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Summary

1. The geographic range of *Carcinonemertes epialti* has been greatly extended. The worms are found from Bahia San Quintín, Baja California, Mexico, to Page's Lagoon, Vancouver Island, British Columbia, Canada.

2. New host records for *C. epialti* include *H. oregonensis*, and *H. nudus*. It is rare on its type host *Pugettia producta*. Specimens of *Carcinonemertes* of uncertain affinities are also found on *Cancer antennarius, C. anthonyi* and *C. productus*.

3. *Carcinonemertes epialti* adults are egg predators on ovigerous hosts. Growth, demography and abundance are described in relation to the embryogenic stage of the host brood at Bodega Harbor, California.

4. Nonfeeding juveniles are ensheathed on individuals of both host sexes over 8.0 mm carapace width.

5. Transmission experiments show that contact transfer of juvenile nemerteans from males to other hosts may occur.

6. The percentage of infestation and mean density peak in autumn on *H. oregonensis* at Bodega Harbor.

7. Ovigerous female hosts are more frequently infested with *C. epialti*, particularly at small host sizes, than are male or nonovigerous female hosts at Bodega Harbor. However, average worm density on ovigerous females is low.

8. Mean density of *C. epialti* rises through late postmolt, declines during intermolt and rebuilds to a high level in late premolt *H. oregonensis* from Bodega Harbor.

9. Large crabs have a higher percentage of infestations and mean densities per infection than do small crabs. Nemerteans are more frequently found in the sternal-abdominal furrow and less frequently in the limb axillae on large crabs.

10. A model of *C. epialti* transmission and site occupancy is proposed, incorporating the influence of host size, sex, reproductive state, embryogenesis, molt cycle stage and molt cycle duration of *H. oregonensis* at Bodega Harbor. Site availability increases with host size. At higher densities the juvenile nemerteans increasingly occupy less preferred sites. Transferral of juvenile nemerteans occurs and is considered responsible for the high frequency of low infestation levels. Ovigerous females are more likely to be infested but with low density infestations.

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THE EFFECT OF pH ON OXYGEN CONSUMPTION AND ACTIVITY IN THE BATHYPELAGIC MYSID *GNATHOPHAUSIA INGENS*

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The physical and chemical properties of the ocean at a given depth are relatively stable; however, there are appreciable depth-related gradients of many parameters. Among these are temperature, pressure, and oxygen concentration. The effects of these gradients on the physiology of midwater species has only recently begun to be investigated (Teal and Carey, 1967; Teal, 1971; Smith and Teal, 1973, Childress, 1969, 1971, 1975; Quetin and Childress, 1976). Among the most dramatic are the changes in oxygen concentration associated with the oxygen minimum zones that exist at intermediate depths in most of the world's oceans (Schmidt, 1925; Sewell and Fage, 1948; Banse, 1964). Gradients of pH values are associated with oxygen minima, and pH values may range from 8.3 at the ocean's surface to 7.5 or less in the oxygen minimum (more than a six-fold increase in acidity; Park, 1968). Although the importance of blood pH is well known, there is virtually no information available on the metabolic effects of water pH. The crustacean, *Gnathophausia ingens*, which resides in the oxygen minimum layer, was chosen for the study of this problem. This shrimp is a lophogastrid mysid whose respiratory and circulatory adaptions to low oxygen have been extensively studied (Childress, 1968, 1969, 1971, 1975; Belman and Childress, 1976). This report examines the effect of pH on oxygen consumption, oxygen removal from the respiratory stream, and activity in *G. ingens*.

Materials and Methods

Specimens of *Gnathophausia ingens* were collected from basins off the coast of Southern California at depths of 400 to 900 meters with a midwater trawl. The animals were transported to the laboratory in aerated containers maintained at approximately 5° C. The mysid *G. ingens* was chosen as an experimental animal, because it can be maintained in the laboratory for long periods of time (Childress, 1971). All of the experimental animals were sexually immature, of undetermined sex, and had a wet weight between 3 and 13 g.

Oxygen consumption

Oxygen consumption rates for the mysid *Gnathophausia ingens* were measured in much the same way as Childress (1971). Animals were placed in a water-jacketed respirometer maintained at 5.5° C and covered to prevent the entrance of light. The rate of change of the partial pressure of oxygen in the respirometer

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was continuously monitored with a Clark-type oxygen electrode (Clark, 1956). The electrode was calibrated in air-saturated and nitrogen-saturated sea water (5.5° C) before and after each experiment. Any experiment in which the nitrogen calibration changed noticeably or the air calibration changed by more than 2% was not used.

In determining the effect of pH on respiration, two experimental procedures were followed. The first tested the effect of pH on oxygen consumption at low oxygen partial pressures. It was also used to estimate the limitation of activity by oxygen availability. This procedure required that an animal placed in the respirometer at a specified pH be allowed to consume all the oxygen present. The experiments that followed this procedure lasted approximately ten hours, depending on the size of the animal. Oxygen electrodes used in these experiments were calibrated in sea water of the same pH as the experiment. The second procedure was designed to show any short-term effect of pH on activity or rate of oxygen consumption without the stress of low oxygen. This procedure involved changing the water in the respirometer at five hour intervals and alternating sea water of pH 7.9 with that of either pH 7.1 or 8.7. These pH values were chosen because a tolerance over this range would also indicate a tolerance to fluctuations in pH which might occur in the environment. A total of five water changes was usually made during a single run. The oxygen electrodes used in these experiments were calibrated at pH 7.9 and the effect of pH on the calibration later determined. The difference in the calibrations was always less than 0.5% and was not subtracted in calculations of the respiratory rate.

In order to maintain constant pH throughout an experiment, it was necessary to buffer the sea water. Tris(hydroxymethyl)aminomethane (final concentration 20 mg/liter) adjusted to the required pH with either HCl or NaOH and diluted with distilled water so as to be isosmotic with salt water was found to be sufficient. To determine the effect of buffering the sea water, experiments of approximately four hours duration were done in both buffered and unbuffered sea water. During this period, the pH of the unbuffered water did not change greatly. Oxygen consumption rates in the buffered and unbuffered water were not significantly ($P \geq 0.1$, $n = 8$) different.

Bacterial growth was minimized by the addition of streptomycin sulfate (20 mg/liter) to the sea water. The remaining bacterial oxygen consumption was estimated by measuring the rate of oxygen consumption in the respirometer for 6–12 hours after the animal was removed. These rates were constant and always less than 5% of the total measured rate. The bacterial rates were subtracted in calculating the oxygen uptake rates of the animals.

Oxygen extracting ability

The ability of *G. ingens* to extract oxygen from sea water at different partial pressures was calculated from measurements of the oxygen content of sea water before and after passing through the gills. To measure oxygen in the exhaled water, an animal's head was placed in a plastic vial while a collar, cut from a rubber balloon, sealed the animal to the vial. The collar was loosely placed so as to not compress the carapace, and the flow of water through the vial was checked with a nontoxic dye. A microcathode (0.0152 mm diameter platinum cathode) oxygen

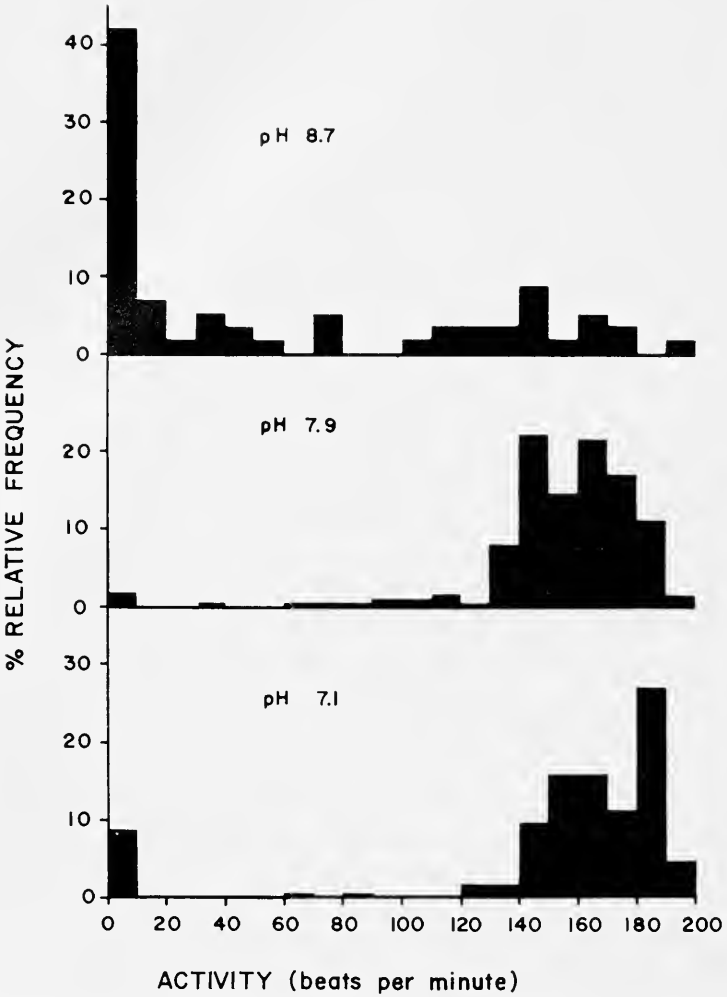

FIGURE 1. The relative frequencies of constant activities lasting at least ten minutes at pH 7.1, 7.9, and 8.7. There were 66 observations at pH 7.1, 206 at pH 7.9, and 57 at pH 8.7.

electrode, chosen because it is insensitive to stirring, was placed inside the vial, while another electrode in the bath recorded the oxygen content of inhaled water. A stirrer was placed in the bath to both stir the electrode and to keep the water evenly mixed.

Constant pH was maintained during this experiment by adding Tris(hydroxymethyl)aminomethane (10 mg/liter) to the water before the experiment. An experiment consisted of placing an animal in water of a specified pH and reducing the partial pressure of oxygen to approximately 6–10 mm Hg by bubbling nitrogen through the water. Constant stirring of the bath caused the partial pressure of oxygen to slowly return to approximately 100 mm Hg over a period of five hours. The pH of the water was then changed by slowly adding small aliquots of either

HCl or NaOH. Each animal was subjected to water of three different pH values, the order being changed for different animals to avoid "placement errors". The oxygen electrodes could be removed from the apparatus without disturbing the animal and were calibrated with each change of pH.

Activity

Activity was continuously recorded in all experiments. Individuals of *G. ingens* were held by the carapace in plexiglass "racks" (Quetin, Mickel, and Childress, 1978). A light-emitting diode light source and miniature photoresistor, both cast in clear epoxy, were placed opposite one another across the pleopods of an animal. The photoresistor functioned as one arm of a Wheatstone bridge. Each pleopod beat interrupted the light beam unbalancing the Wheatstone bridge, and thus generated an electrical pulse. The pulses were time-averaged by a cardiotachometer and recorded on a potentiometric chart recorder.

Results

Activity and pH

The activity of the mysid *Gnathophausia ingens* was affected by pH. The distributions of constant activities, lasting at least ten minutes, from 40 runs on eight individuals is presented in Figure 1. As shown, the activity of animals in water of pH 7.1 and 7.9 was remarkably constant. At these pH values, individuals either swam at 140 to 190 beats per minute or did not swim. At pH 8.7 individuals more frequently did not swim and were generally less active than at the other pH values.

Individuals placed in water of pH 8.7 were observed to perform extensive cleaning behavior. This behavior consisted of repeated wiping of the antennae and mouthparts with the first several pairs of pereiopods. During cleaning activity, the animal usually decreased its swimming activity and this most likely accounts for the trend toward lower and more variable activity at the higher pH.

Pleopod beat was not affected by oxygen concentration, and most animals continued swimming at relatively high rates for 15–30 minutes after the oxygen concentration in the respirometer became unmeasurably low.

Oxygen consumption and activity

Individuals of *G. ingens* were found to be capable of a wide range of oxygen consumption rates. Activity of individual *G. ingens* had a profound effect on oxygen consumption rate (Fig. 2). During a single run, rates could vary as much as ten-fold, from approximately 10 μl O_2/(g wet weight·hr) to 100 μl O_2/(g wet weight·hr). Most of this variation could be attributed to "spontaneous" changes in the animals' activity. Changes in activity during an experiment could completely mask any less subtle responses to the other parameters being tested. For this reason activity was recorded in all experiments.

Measurements of changes in oxygen consumption for short term changes in activity were difficult to make due to the lag in oxygen consumption with an increase in activity. Oxygen consumption rates were, therefore, calculated for periods

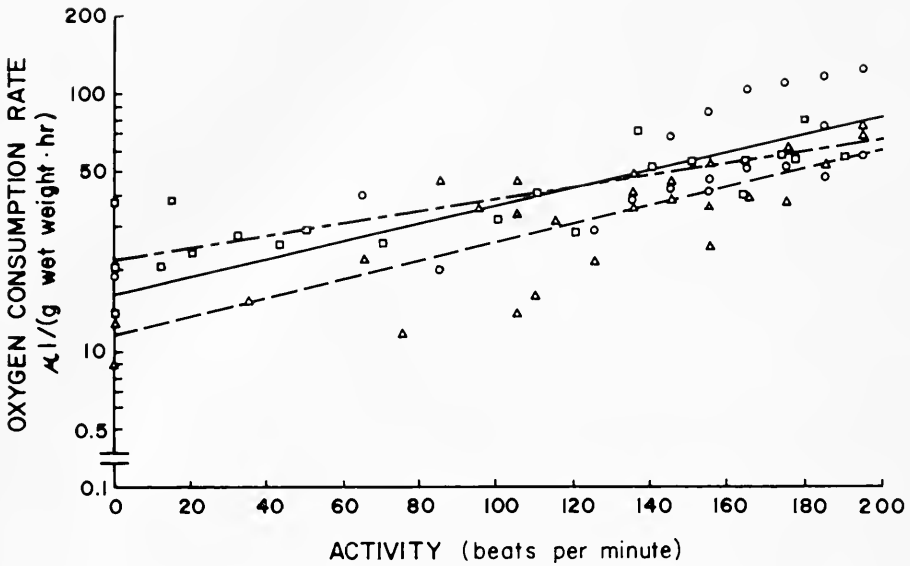

FIGURE 2. The relationship between activity (x, pleopod beats/minute) and oxygen consumption rate [y, μl O_2/(g wet weight·hr)] in *Gnathophausia ingens*. The regression line for pH 7.1 is log y = 1.2244 + 0.0035x, has an r of 0.737 and is represented by the solid line. The data points at pH 7.1 are represented by circles. The regression line for pH 7.9 is log y = 1.0884 + 0.0036x, has an r value of 0.851 and is represented by the uniformly dashed line. The data points at pH 7.9 are represented by triangles. The regression line for pH 8.7 is log y = 1.3571 + 0.0024x, has an r value of 0.836 and is represented by the line of long and short dashes. The data points at pH 8.7 are represented by squares.

of constant activity, sustained for at least ten minutes and at partial pressures of oxygen above 20 mm Hg. By measuring rates at oxygen partial pressures above 20 mm Hg, it was assured that the animals were above their critical partial pressure of oxygen (Childress, 1971).

The relationship of respiratory rate to activity is presented in Figure 2. As shown, the relationship was found to be semi-logarithmic rather than linear. This indicates that the amount of oxygen consumed per pleopod beat increases with the rate of pleopod beat. The mean respiratory rate of nonswimming animals, including all pH values, was 19.3 μl O_2/(g wet weight·hr), n = 32, s.d. = 14.5. The rates for animals swimming at 140 to 190 pleopod beats per minute, taken from Figure 2, are from 39.1 to 77.5 μl O_2/(g wet weight·hr).

Oxygen consumption and pH

The effect of pH on respiration in *G. ingens* was studied in eight individuals from 3.9 to 9.3 g wet weight, in a series of 40 runs. The large variation in respiratory rate due to changes in activity made it difficult to choose a representative value for the respiratory rate of an animal during an experiment. For this reason, the relationship between activity and respiration for all animals was compared at three pH values (Fig. 2). Respiratory rates for each animal were grouped according to

activity (*e.g.*, all rates at activity 100 to 110 pleopod beats per minute) and the means taken. Each point on Figure 2 is the mean rate for one animal at the mean activity and pH.

Analysis of the regression line of respiration on activity for the three pH values showed no significant ($P > 0.1$, F-test) difference between the slopes of the lines. A test for homogeneity of variance, however, showed that the variance at pH 8.7 was significantly larger ($P < 0.05$, Bartlett's test) from that at pH 7.9 and pH 7.1. Due to this difference in variances the data could not be pooled, and therefore a regression line for each pH is shown (Fig. 2). The slopes of all the regression lines are significantly ($P < 0.001$, *t*-test) different from zero.

The effect of pH on oxygen uptake at oxygen partial pressures of 10–30 mm Hg was studied in five animals. The mean respiratory rates and standard deviation in water of pH 7.1, 7.9, and 8.7 are, respectively: 34.33 ± 15.5, 30.07 ± 20.12, 27.16 ± 16.08 $\mu l\ O_2/(g\ wet\ weight \cdot hr)$. No significant ($P > 0.1$, *t*-test) difference between these means was found.

The critical partial pressure of oxygen (P_c) for *G. ingens* was found to be unaffected by pH (Fig. 3). The P_c values for 11 individuals at three pH values all fell within the 95% confidence interval for the relationship between regulated oxygen consumption rate and P_c found for *G. ingens* (Childress, 1971).

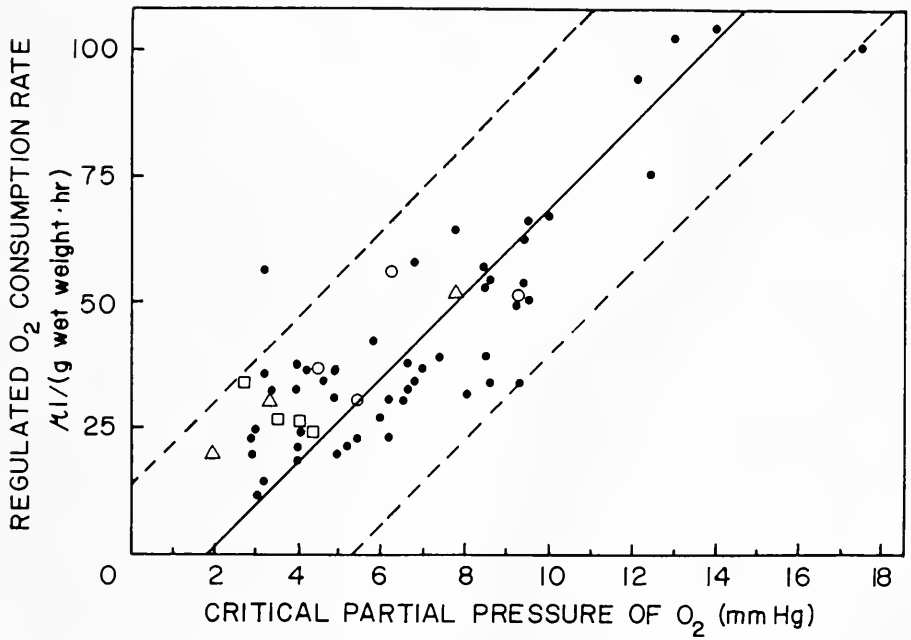

FIGURE 3. The relationship between oxygen consumption and P_c in *Gnathophausia ingens*. The regression line ($x = 4.973y + 1.798$, solid line), 95% confidence intervals for an individual x at a given y (dashed lines) and the solid circles which these relationships describe are taken from Childress (1971). These observations were made at pH values of approximately 8 to 8.3. The data points from the present study are represented by triangles for pH 7.1, circles for pH 7.9 and squares for pH 8.7.

TABLE I

The ability of G. ingens *to extract oxygen from water, at three pH values, expressed as the percentage of oxygen content of inhaled water. Values are means for percentage of oxygen extracted at oxygen partial pressures of 10–40 mm Hg and 40–80 mm Hg. The numbers in parentheses are the number of observations followed by standard error of the mean.*

pO_2 (mm Hg)	pH of observations					
	7.1		7.9		8.7	
	10–40	40–80	10–40	40–80	10–40	40–80
Animal						
1	62.8 (6, 3.6)	50.6 (9, 0.7)	51.7 (11, 3.9)	38.3 (10, 1.9)	58.4 (4, 2.6)	46.3 (13, 3.1)
2	70.5 (5, 3.6)	32.5 (5, 2.1)	58.7 (8, 5.6)	25.4 (10, 2.6)	58.5 (7, 3.6)	26.6 (18, 2.1)
3	52.7 (5, 9.4)	54.8 (12, 1.4)	37.2 (13, 3.2)	46.0 (19, 0.8)	30.4 (4, 4.2)	48.7 (21, 2.1)
4	51.6 (9, 6.2)	42.3 (13, 1.2)	39.8 (12, 3.5)	37.8 (19, 1.5)	21.9 (7, 2.6)	36.7 (18, 2.3)
5	72.5 (19, 1.2)	63.2 (17, 2.2)	45.7 (12, 2.3)	43.8 (22, 1.8)	6.0 (13, 3.2)	52.6 (21, 1.0)
6	71.9 (22, 1.4)	67.4 (10, 1.8)	53.9 (5, 4.6)	51.3 (14, 3.0)	64.3 (19, 1.8)	49.7 (19, 2.3)

Oxygen extracting ability and pH

The ability of individual *G. ingens* to extract oxygen from water was measured in 18 runs with six individuals ranging in size from 5–8 g wet weight. The results for the six animals are summarized in Table I. The values in Table I are means of the values for the percentage of O_2 extraction in the ranges of oxygen partial pressures of 10–40 mm Hg and 40–80 mm Hg. Standard errors given in the table are not meaningful because, as can be seen in Figure 4, the percentage extraction declines continuously at higher oxygen partial pressures. Therefore, standard errors would indicate the range of values for the percentage utilization over the oxygen concentrations tested, rather than the variability of sampled values. This would overestimate the variability found. While individual shrimp differed in the absolute values for the percentage oxygen extraction, general trends for responses to both changes in pH and oxygen concentration could be found. The results from a representative experiment are shown in Figure 4.

The typical response was moderate extraction (25–55%) at partial pressures of oxygen above 40 mm Hg. As the partial pressure of oxygen further declined, the percentage extraction increased to 75–85% and in some cases reached a peak at oxygen partial pressures of 10–15 mm Hg and then declined with a further decrease in oxygen concentration. This result is similar to that found by Childress (1971).

No statistical difference could be found between the values for the percentage O_2 extraction at pH 7.9 and 8.7 ($P > 0.1$), but each of the *G. ingens* that was studied extracted a significantly ($P < 0.05$, Mann-Whitney test) greater percentage of oxygen in water of pH 7.1 than in water of pH 7.9 or pH 8.7. As Figure 4 shows, increased O_2 extraction at pH 7.1 occurred over the whole range of oxygen partial pressures and apparently was not related to the stress of low oxygen.

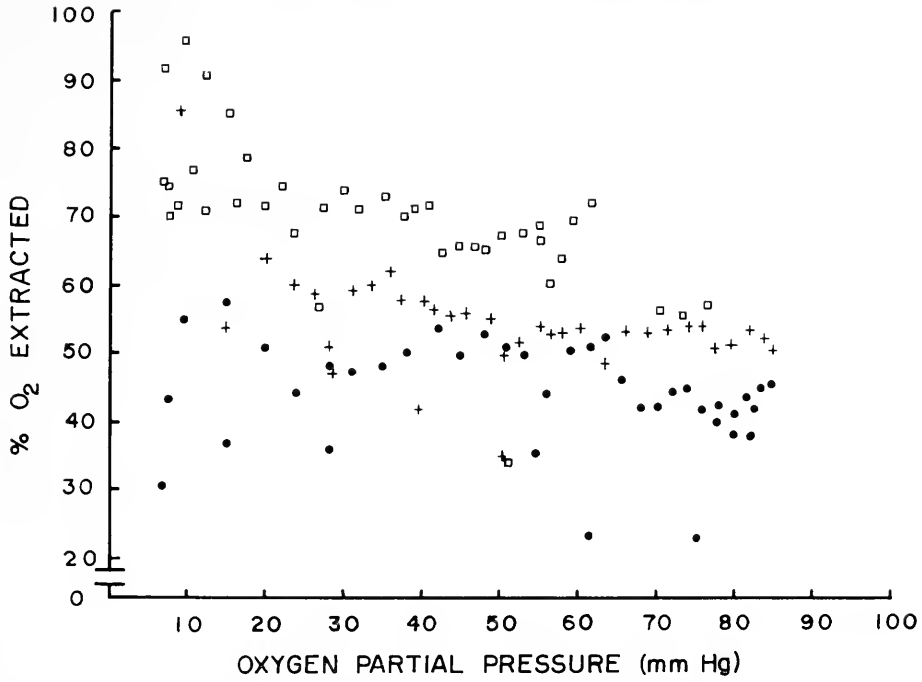

FIGURE 4. The oxygen extracting ability of a single *G. ingens*, at three pH values, expressed as the percentage of oxygen in inhaled water: squares, pH 7.1; solid circles, pH 7.9; and crosses, pH 8.7.

DISCUSSION

The relationship between activity and metabolism has been only slightly investigated in crustaceans. In one study of the mysid, *Mysis relicta,* it has been concluded that activity during vertical migration has no effect on metabolism (Foulds and Roff, 1975). Another study concludes that oxygen consumption of euphausiids remains the same regardless of whether the animal is swimming or not (Lasker, 1966). On the other hand, Halcrow and Boyd (1967) found a linear relationship between oxygen consumption and swimming activity in the amphipod *Gammarus oceanicus,* and Ivlev (1963) found a semi-logarithmic relationship between oxygen consumption and swimming velocity in the shrimp *Leander adspersus.* The data collected in this study show a quite significant relationship between activity and metabolic rate in *Gnathophausia ingens.* The exponential relationship between rate of pleopod beat and oxygen consumption in this species is comparable to that found for the relationship between rate of cirral beat and oxygen consumption in the barnacle *Balanus balanoides* (Newell and Northcroft, 1965). Clarification of the nature of the relationship between swimming velocity and oxygen consumption in *G. ingens* awaits the determination of the propulsive efficiency of the pleopod beat. It is clear from this study however, that the rate of aerobic metabolism in this species is quite closely related to its activity level. Furher, activity certainly constitutes a major fraction of the overall energy usage of this entirely pelagic species.

At the relatively high activities exhibited by individuals during experiments, respiratory rates could range from 30 to 80 μl O_2/(g wet weight·hr). Whether these rates of activity can be maintained in the oxygen minimum layer can be calculated by assuming an oxygen concentration of 0.25 ml/liter and a ventilation flow rate of 240 ml/(g wet weight·hr) (Childress, 1971). With O_2 extraction of 85%, individuals of *G. ingens* can extract enough oxygen to sustain rates of activity of 138 and 172 pleopod beats per minute at pH 7.1 and pH 7.9.

The most striking aspect of the data on this species is that there are no dramatic effects of pH on its respiratory processes. That is, this shrimp seems to regulate its metabolism over a wide range of pH values. For example, the relationship between activity and oxygen consumption is unaffected by pH. Further, pH appears to have little effect on activity at the two lower and environmentally more realistic values. Level of pH also apparently does not alter the ability of this species to regulate its oxygen consumption at the low environmental oxygen concentrations where it normally lives. This is shown by the fact that the relationship between critical partial pressure and regulated oxygen consumption rate found by Childress (1971) is unaffected by pH over the range tested.

The one case where pH had a strong effect involved the extraction of oxygen from the respiratory stream. The data show quite clearly that the percentage of O_2 extraction is 10% to 30% higher at pH 7.1 as compared to 7.9 and 8.7. Since the utilization at pH 7.1 is elevated at both high and low oxygen partial pressures, this is apparently not the result of a stress on the oxygen uptake systems of the animal forcing it to increase its extraction to maintain a constant oxygen consumption. Paradoxically this higher percentage of extraction at low pH apparently does not improve the ability of *G. ingens* to regulate its oxygen uptake (Fig. 4). This implies that there is a loss in effectiveness of uptake at some other site as a result of the lower pH. The question of how the percentage of extraction of this species can be higher at limiting oxygen partial pressures at pH 7.1 as compared to pH 7.9 and pH 8.7, however, is still left unanswered. Further studies on the tolerance of vertically-migrating animals to low pH may be interesting because pH may be as important a factor as oxygen in limiting the distribution of some species in relation to low oxygen regions.

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Summary

1. Pleopod beat of *G. ingens* was unaffected by pH at pH 7.1 and pH 7.9 but was lower at pH 8.7 due to increased cleaning activity.

2. The relationship between oxygen consumption rate, and pleopod beat was found to be semi-logarithmic.

3. The relationship between oxygen consumption rate and activity was unaffected by pH in the range of pH 7.1 and pH 8.7.

4. The per cent O_2 extraction of oxygen by *G. ingens* was not statistically different at pH 7.9 and pH 8.7, but was greater at pH 7.1.

5. The ability of *G. ingens* to regulate its oxygen consumption was unaffected by pH in the range studied.

6. Since the increase in per cent O_2 extraction at pH 7.1 does not improve the ability of *G. ingens* to regulate its oxygen uptake, it appears that there is a loss in effectiveness elsewhere in its respiratory system at this pH.

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SEPARATION AND PARTIAL PURIFICATION OF CENTRAL NERVOUS SYSTEM PEPTIDES FROM *LIMULUS POLYPHEMUS* WITH HYPERGLYCEMIC AND CHROMATOPHOROTROPIC ACTIVITY IN CRUSTACEANS [1, 2]

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Thirty-six years ago a substance in the central nervous system (CNS) of the chelicerate arthropod *Limulus polyphemus* was shown to possess chromatophorotropic activity when tested on the mandibulate arthropod *Uca pugnax* (Brown and Cunningham, 1941). More recent studies have demonstrated that CNS extracts from *Limulus* are also chromatophorotropic in a variety of other decapods, including both brachyuran and natantian species (Fingerman, Bartell, and Krasnow, 1971; Herman and Dallmann, 1975). Other experiments have shown that arthropod molting hormones (ecydsones) are present and active in *Limulus* (Krishnakumaran and Schneiderman, 1970; Jegla, Costlow and Alspaugh, 1972; Winget and Herman, 1976). The existence of both CNS material with crustacean neurosecretory hormone activity and ecdysones in this species suggests that *Limulus* might also produce other substances with arthropod hormone activity. If so, neuroendocrinological studies of *Limulus* could be of major importance in attempts to understand the basic properties and evolution of arthropod neuroendocrine regulatory mechanisms. Against this background a series of studies were conducted testing the effects of CNS extracts from *Limulus* in known arthropod neurosecretory hormone bioassays. During this work the existence of a CNS substance causing hyperglycemia in the freshwater crayfish, *Orconectes immunis*, was discovered. Initial studies on this substance, and evidence demonstrating that it is not the above-mentioned chromatophorotropin, are presented below.

Materials and Methods

Adult specimens of *Limulus polyphemus*, obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, were maintained without feeding in Instant Ocean artificial sea water at 12° C. Specimens of *Orconectes immunis*, obtained from Trans-Mississippi Biological Supply, St. Paul, Minnesota, were maintained in dechlorinated tap water aquaria at 12° C and fed Gainesburger dog food three or four times a week. Specimens of *Uca pugilator*, supplied by Gulf Specimen Co., Panacea, Florida, were held in Instant Ocean sea water aquaria at 18° C and fed Gainesburger dog food weekly. Eyestalks were removed from specimens of *Orconectes* and *Uca* 24 hr prior to experiments.

Central nervous systems from *Limulus* were removed by ventral dissection,

[1] Supported by the University of Minnesota Graduate School and USPHS grant HD-07336.

[2] Some of this research was part of a Ph.D. thesis submitted by P. D. Pezalla to the University of Minnesota.

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cleaned of adhering non-CNS material, weighed to the nearest mg and either immediately homogenized, or frozen on dry ice for lyophilization. Extracts were prepared with ethanol, acetone, 0.1 and 1.0 N acetic acid, and 0.1 M ammonia. The typical extraction protocol was as follows. The CNS was placed in a volume of solvent and thoroughly homogenized in a Potter Elvejem homogenizer at 4° C. The homogenate was then centrifuged for 15 min at 12,100 × *g* in a Sorval Superspeed RC2-B refrigerated centrifuge run at 4° C. The supernatant was saved, while the pellet was re-extracted three to four times to a total of 20 volumes. The pooled supernatants were then boiled for three min and recentrifuged as mentioned above. The crude extract was either used immediately or lyophilized for storage at −20° C. In most cases, the lyophilized residues were redissolved in distilled water at concentrations appropriate for injections or column chromatography. In some experiments crude extract was made 10^{-3} M with thiodiglycol (Sigma). Variations from the above procedure are cited in the text.

Assays of the CNS chromatophorotropin from *Limulus*, hereafter referred to as LUC, were conducted as previously described (Herman, 1975). In brief, the melanophore response of 5–10 eyestalkless female *Uca* to 10 μl aliquots of extract or solvent was observed, the control values subtracted from the experimental values, and the mean response per *Uca* calculated in chromatophore units.

Assays of CNS hyperglycemic activity from *Limulus* were performed on eyestalkless, mixed sex *Orconectes* randomly assigned to individual containers holding enough dechlorinated tap water to just cover the carapace. Injections of 50–100 μl of solvent or extract were made with disposable 1 ml tuberculin syringes fitted with 25 gauge needles. Hemolymph samples, withdrawn from the ventral abdominal or cephalothoracic sinus, were assayed for glucose by the Glucostat (Worthington Biochemicals) method (Meites, 1965) or for total carbohydrate by the Anthrone (Sigma) method (Chaykin, 1966). A Beckman DB-G spectrophotometer, set at 540 nm for Glucostat and 620 nm for Anthrone, was used for all colorimetric determinations. Glucose was used as the standard for both assay procedures.

Chromatography of LUC and experiments concerning both LUC and the hyperglycemic factor were conducted at 4° C on a Sephadex G-25 Fine (Pharmacia) column, 1.5 × 90 cm, equilibrated with 1.0 N acetic acid made 10^{-3} M with thiodiglycol. The flow rate was 25 ml/hr and 2.5 ml fractions were collected. Absorbancy of all fractions was read at 280 nm. The fractions were lyophilized and redissolved in distilled water. The column was calibrated with lysozyme (14,000), ACTH (4,570), glucagon (3,600) and bacitracin (1,400), all from Sigma.

The hyperglycemic factor was further chromatographed on a Sephadex G-50 Fine (Pharmacia) column, 2 × 40 cm, equilibrated with 0.1 N acetic acid made 10^{-3} M with thiodiglycol. This column was run at 4° C with a flow rate of 4.6 ml/hr, and fractions of 4.6 ml were collected. The fractions from this column were treated as previously mentioned with the exception that in some experiments the lyophilized fractions were redissolved in 0.1 N acetic acid. (Several experiments demonstrated that 50 μl of this solvent were not hyperglycemic in crayfish and thus did not interfere with the hyperglycemic assay.) This column was calibrated with bovine serum albumin (68,000), chymotrypsinogen (25,000), lysozyme (14,000) and glucagon (3600), all from Sigma.

LUC and the hyperglycemic factor were tested for susceptibility to some or all

TABLE I

Effect of CNS extracts from Limulus *on* Orconectes *hemolymph glucose.*

Material injected	Hemolymph glucose (mg %)
CNS equivalents	
0.04 (8)	8.7 ± 2.3
0.08 (4)	12.1 ± 4.4
0.20 (17)	19.2 ± 2.2
Solvent control (14)	3.6 ± 0.4

N in parentheses; experimental duration = 1 hr.

of the following enzymes: pepsin, protease, trypsin, chymotrypsin, thermolysin, and lysozyme (all from Sigma).

For each enzyme, extracts were incubated in the appropriate medium (enzyme concentration = 20 mg/ml) for 16 hr at 37° C. The reactions were terminated by boiling the reaction mixture for 5 min, after which the reaction mixtures were centrifuged to remove denatured enzyme. Extract without enzyme and enzyme without extract controls were subjected to the same conditions. The following buffers were used (Shepard, 1975): pepsin, 0.1 N acetic acid (pH 2.8); protease, 0.02 M HEPES-KOH (pH 7.5) containing 0.1 M calcium chloride; trypsin, 0.05 M Tris (pH 8.2) containing 0.01 M calcium chloride; chymotrypsin, 0.08 M Tris (pH 7.8) containing 0.1 M calcium chloride; thermolysin, 0.5 M Tris (pH 8.5) containing 0.005 M calcium chloride; and lysozyme, 0.1 M phosphate, pH 7.0.

The data are reported as mean ± s.e.m. Some of these data were analyzed by Student's *t*-test; the term significance in this report refers to statistical significance in this test at the 5% level or better.

RESULTS

Effects of CNS extracts from Limulus *on* Orconectes *hemolymph carbohydrates*

Initial studies tested CNS extracts from *Limulus* for hyperglycemic activity in *Orconectes*. The results of a typical experiment, using acetone extracts of fresh *Limulus* CNS, are summarized in Table I. Significant elevations of hemolymph glucose were obtained with as little as 0.04 CNS equivalent/crayfish, and larger doses produced substantially higher reponses. Analysis of total hemolymph carbohydrate before and after injection yielded similar results; in one such experiment *Limulus* CNS extracts (0.20 CNS equivalent/animal) produced a 130.0 ± 17.3% increase in 10 crayfish, while injections of solvent or muscle extract into 20 animals elevated total carbohydrate by only 32.0 ± 7.5%. Comparable experiments have been performed several times over a period of 2 yr using horseshoe crabs and crayfish obtained in both summer and winter. These studies invariably demonstrated that *Limulus* CNS extracts contained material capable of rising *Orconectes* hemolymph total carbohydrate and glucose levels.

The above results are duplicated with acetic acid extracts of *Limulus* CNS; ethanol and ammonia extracts also cause significant responses, but hemolymph glucose increases are quantitatively less impressive. Hyperglycemic activity can

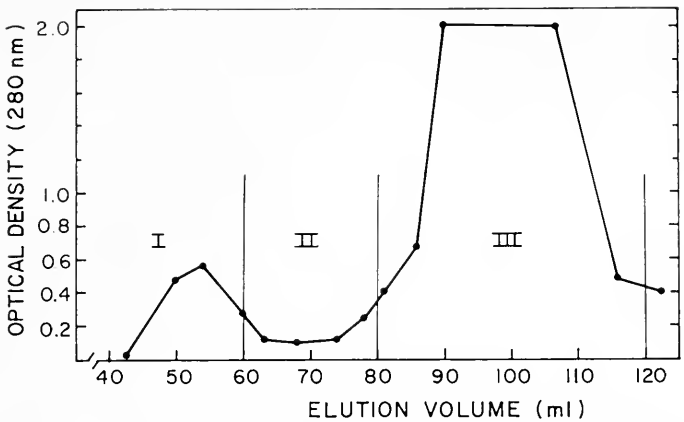

FIGURE 1. Chromatography of crude CNS acetic acid extracts from *Limulus* on Sephadex G-25 (fine). The extracts consisted of 3500 mg of CNS (wet weight) extracted as described and concentrated to 3 ml. The column was equilibrated with 1.0 N acetic acid; flow rate, 25 ml/hr; fraction volume, 4 ml; total volume, 130 ml; void volume, 54 ml. The absorbancy of each fraction was read at 280 nm (solid line). The fractions were pooled and assayed for hyperglycemic and melanophore dispersing activity (Table II).

usually be extracted from fresh or lyophilized CNS, but acetone extracts of lyophilized material have no effect. For convenience we have designated the active material in CNS extract LHGF, for *Limulus* hyperglycemic factor.

Separation of LHGF and LUC on Sephadex G-25

Preliminary gel filtration experiments (Fingerman *et al.*, 1971) indicated that LUC would elute in the last half of the elution volume on a Sephadex G-25 column. We therefore decided to attempt to separate LHGF and LUC by means of such a column. Concentrated *Limulus* CNS acetic acid extract was applied to the column, and 4 ml fractions were collected, pooled (as indicated in Fig. 1), lyophilized, and redissolved in distilled water for bioassay. The results were obvious (see Table II); LHGF, but not LUC, was present in Fraction I, which corresponded to the void volume. Neither activity was present in Fraction II, while Fraction III exhibited only LUC activity. From these experiments it was concluded that LHGF is excluded from Sephadex G-25 columns.

Preliminary characterization of LHGF

The preceding results clearly indicated that LHGF and LUC were separate substances. It was therefore decided to further characterize both LHGF and LUC to demonstrate their nonidentity.

Experiments were undertaken to determine the stability of LHGF. It was observed that unboiled acetic acid extracts of *Limulus* CNS are unstable at room temperature, with the majority of the hyperglycemic activity lost within 3 hr. This loss in activity could be prevented by brief boiling of the crude extract, or by storing the crude extract of 0° C. In addition, treatment of the crude extract with hydrogen peroxide (final concentration = 1%) reduced hyperglycemic activity by

TABLE II

Effects of Sephadex G-25 fractions of CNS extracts from Limulus *in* Orconectes *and* Uca.

Fraction tested	*Uca* response*	*Orconectes* response**
I	3.0 ± 1.8 (10)	86.0 ± 13.0 (10)
II	1.6 ± 1.1 (10)	17.0 ± 6.0 (10)
III	23.0 ± 3.0 (10)	22.5 ± 3.0 (10)
Solvent controls	0.0 (10)	16.5 ± 4.5 (10)

* Mean net chromatophore response; 10 μl of pooled fraction injected.

** Percentage of increase in total carbohydrate 60 min after injection; 100 μl of pooled fraction injected.

about one-third in 1 hr. On the basis of the above findings we now routinely boil the crude extracts briefly, centrifuge, and add thiodiglycol to a final concentration of 10^{-3} M. In addition, all extractions and chromatographic separations are performed at 4° C.

The next concern was to estimate the molecular weight of the LHGF *via* gel filtration. Initial separation of LHGF and LUC on Sephadex G-25 clearly demonstrated that a larger grade Sephadex was required; G-50 was selected. The results

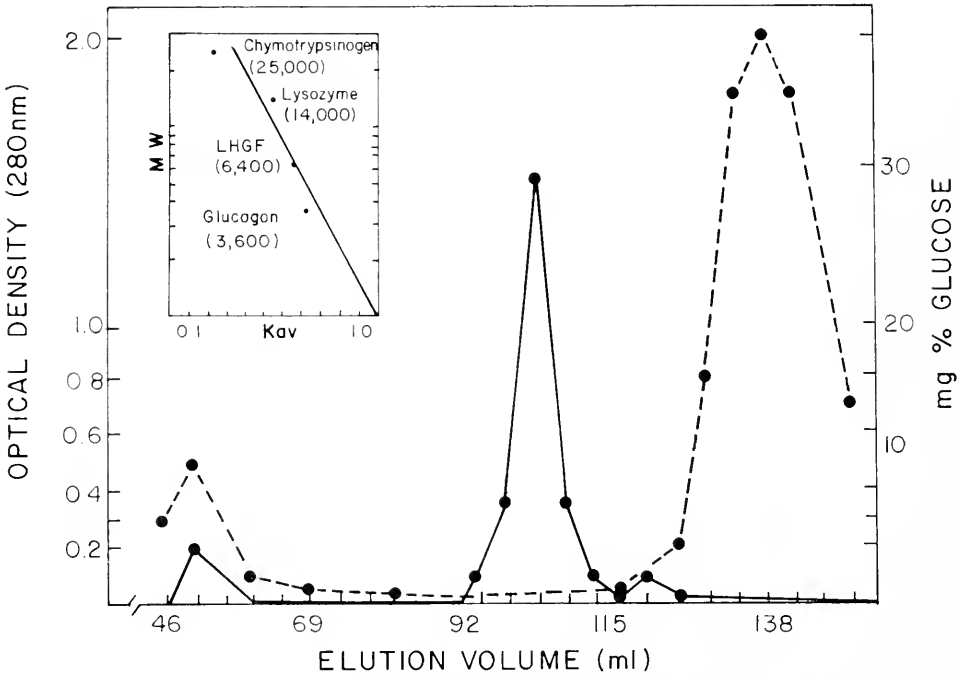

FIGURE 2. Chromatography of crude CNS acetic acid extracts from *Limulus* on Sephadex G-50 (fine). The column was equilibrated with 0.1 N acetic acid; flow rate, 4.6 ml/hr; fraction size, 4.6 ml; total volume, 140 ml; void volume, 51 ml. The absorbancy of each fraction was read at 280 (dashed line). The hyperglycemic activity of each fraction was tested on three animals per fraction (solid line). Insert shows G-50 calibration curve.

of a typical G-50 experiment are shown in Figure 2. On this column LHGF eluted as a single symmetric peak with an estimated molecular weight of 6400.

Active fractions from the G-50 runs were next treated with various proteolytic enzymes. Incubation of crude extracts with pepsin or protease resulted in a decrease in LHGF activity of 92.4% and 79.7%, respectively, while incubation with trypsin had no effect on activity.

Preliminary characterization of LUC

Several experiments were conducted on LUC to determine its stability, susceptibility to various proteolytic enzymes, and molecular weight. As reported elsewhere (Brown and Cunningham, 1941), brief boiling of crude extracts had no effect on activity. However, LUC did appear to be susceptible to oxidation. The combined results of experiments conducted at both 20° C and 37° C showed that untreated extracts held for 20 hr lost 32.9% of the original activity. In addition, treatment with thiodiglycol prevented this loss, while treatment with hydrogen peroxide lead to a 63.9% loss in activity. In view of these results, all subsequent extracts were

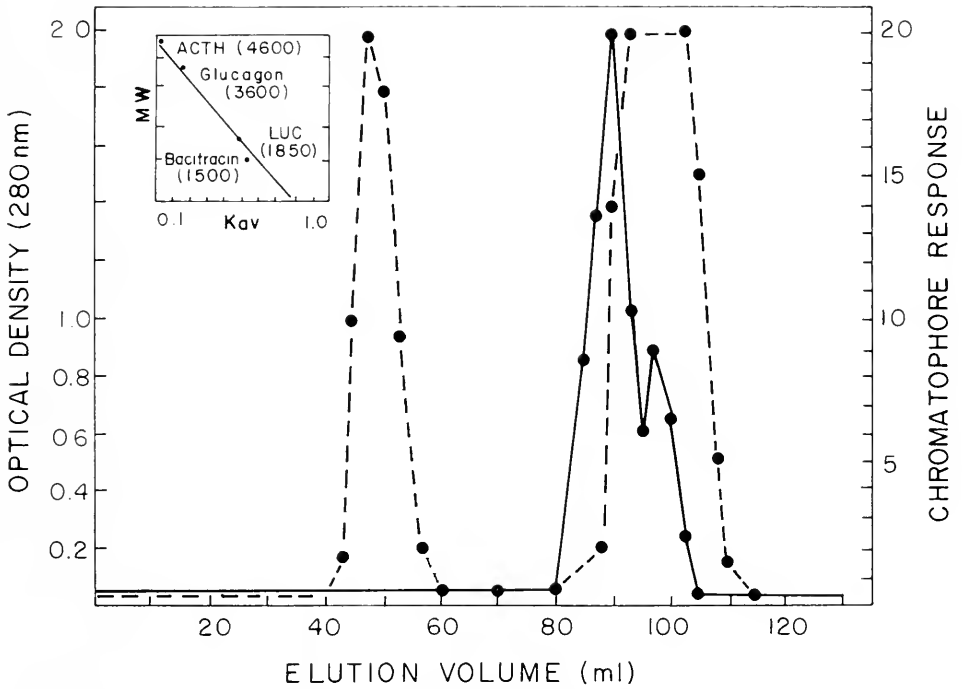

FIGURE 3. Chromatography of crude CNS acetic acid extract from *Limulus* on Sephadex G-25 (fine). The column was equilibrated with 1.0 *N* acetic acid; flow rate, 25 ml/hr; fraction volume, 2.5 ml; total volume, 134 ml; void volume, 46 ml. The crude extract consisted of 350 mg CNS (wet weight) extracted as described and concentrated to 2.5 ml. The absorbancy of each fraction was measured at 280 nm (dashed line). The chromatophorotropic activity of each fraction was tested on 18 animals/fraction and the response depicted (solid line). Insert shows G-25 calibration curve.

made 10^{-3} M with thiodiglycol. Treatment of crude *Limulus* CNS extracts with various enzymes, namely protease, pepsin, chymotrypsin, trypsin, and thermolysin resulted in a mean decrease in LUC activity of $92.8 \pm 1.4\%$, while the glycosidase lysozyme was without effect.

Molecular weight determination was done on a calibrated Sephadex G-25 column. As indicated in Figure 3, a major peak of activity eluted with an estimated molecular weight of 1,850 trailed by a secondary peak of activity. The presence of this latter peak, also noted by Fingerman *et al.* (1971), suggests the possible existence of more than one substance with LUC activity in the CNS of *Limulus*.

Discussion

These experiments have demonstrated the existence of two dissimilar substances with hormonal activity in crustaceans in *Limulus polyphemus* CNS extracts. One of these substances, the previously unreported LHGF, apparently has a molecular weight of about 6,400 daltons. In addition, it appears to be heat stable, inactivated by hydrogen peroxide, sensitive to some proteolytic enzymes, and unaffected by incubation with trypsin. These data collectively indicate that LHGF is a polypeptide. LHGF is clearly hyperglycemic in *Orconectes*, but lacking in melanophore pigment dispersing activity in *Uca*. The second substance(s) is the previously known chromatophorotropin, LUC. These studies have added to existing knowledge of this substance by demonstrating an apparent molecular weight of 1850 daltons, an estimate in agreement with earlier studies (Fingerman *et al.*, 1971). In addition, it has been shown for the first time that LUC is susceptible to a variety of proteolytic enzymes, including trypsin, and to the oxidizing agent hydrogen peroxide. Previous reports have demonstrated that LUC is heat stable (Brown and Cunningham, 1941; Herman, 1975). The total available data indicate that LUC is a peptide; it is chromatophorotropic in several crustaceans (see Herman and Dallman, 1975), but lacks hyperglycemic activity in *Orconectes*. On the basis of the above results, it can be concluded that *Limulus* CNS extracts contain at least two distinct peptides with different hormonal activity in crustaceans.

These data provide a basis for comparison of the properties of LHGF and LUC with those of known crustacean neurosecretory hormones. The available biological and chemical evidence indicates the existence of more than one decapod hyperglycemic hormone (Kleinholz and Keller, 1973; Kleinholz, 1976). Studies to determine the interspecific effect of various decapod hyperglycemic hormones (Keller, 1969) indicate little cross reactivity among the major suborders of decapods. However, partial chemical characterization of the hyperglycemic hormones from *Cancer magister, Pandalus jordani* and *Orconectes limosus* suggests chemical similarity; *i.e.,* all appear to have molecular weights of about 7,000 daltons, all are heat labile and susceptible to some proteolytic enzymes, and at least some appear to be resistant to trypsin and inactivated by hydrogen peroxide (Kleinholz, Kimball and McGarvey, 1967; Kleinholz and Keller, 1973; Kleinholz, 1976). LHGF also appears to fit into this basic scheme, with the notable exception that it is apparently heat stable. Decapod melanophore pigment dispersing hormones (MDH) currently seem to be less heterogeneous than the hyperglycemic hormones; they appear to be biologically similar, heat stable, susceptible to proteolytic enzymes and

oxidation, and to have molecular weights of about 2,000 daltons. The studies of Kleinholz (1976) suggest that the MDHs may possess a structure comparable to that of the distal retinal pigment hormone characterized by Fernlund (1976). The existing data therefore suggest that LUC and LHGF both resemble known crustacean hormones of comparable biological activity. Unfortunately, since only the distal retinal pigment hormone of *Pandalus borealis* has been totally characterized (Fernlund, 1976), the question of the identity, or nonidentity, of these various molecules will not be resolved until more complete structural data are available.

While the activity of LHGF and LUC in decapods is evident, the role of these peptides in *Limulus* remains enigmatic. LUC cannot act on integumentary chromatophores in the horseshoe crab, since this species lacks such chromatophores. Similarly, we have been unable in several attempts to demonstrate a hyperglycemic effect of partially purified LHGF in *Limulus*.

It is becoming more evident that the neuroendocrine system of *Limulus* is deserving of further study. This species produces and uses ecdysones (Jegla *et al.*, 1972; Winget and Herman, 1976), and it possesses at least two neurosecretory hormone-like peptides active in mandibulates. It is certainly reasonable to expect that further studies on this species will be of major importance in our attempts to understand the neuroendocrinology of chelicerate arthropods and the evolution of arthropod neuroendocrine systems.

We wish to express our appreciation to William Sparkes and Louisa Moore for their contributions to this research.

Summary

1. Crude extracts of Limulus CNS cause hyperglycemia in *Orconectes immunis* and expand chromatophores in *Uca pugilator*.

2. The hyperglycemic action is due to a previously unknown polypeptide (LHGF) with an estimated molecular weight of 6400 daltons. LHGF is inactivated by hydrogen peroxide, pepsin, and protease, but unaffected by trypsin and brief boiling.

3. The chromatophorotropic activity is due to the previously reported substance, LUC. LUC is shown to be a peptide with an approximate molecular weight of 1850 daltons; it is inactivated by hydrogen peroxide, protease, pepsin, trypsin, chymotrypsin, and thermolysin.

4. LUC and LHGF activity can be readily separated by gel filtration on a Sephadex G-25 column.

5. The similarity of LUC and LHGF to known crustacean hormones is discussed.

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DEVELOPMENT OF THE EOLID NUDIBRANCH *CUTHONA NANA* (ALDER AND HANCOCK, 1842), AND ITS RELATIONSHIP WITH A HYDROID AND HERMIT CRAB

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Two aspects of the biology of the eolid nudibranch *Cuthona nana* (Alder and Hancock, 1842) are examined here. The development of *C. nana* was studied because poecilogony (different developmental patterns within a species) was suspected. The distribution and behavior of *C. nana* was investigated because of the nudibranch's specialization on a sedentary prey species which is effectively mobile due to its commensal relationship with hermit crabs.

In 1971 cultured egg masses of *Cuthona nana* developed into actively swimming, planktotrophic veligers; whereas egg masses cultured in 1973 produced lecithotrophic, nonswimming veligers that metamorphosed within a day or two of hatching (Harris, Wright, and Rivest, 1975). Poecilogony may occur in some opisthobranchs (Berrill, 1931; Rasmussen, 1944; Franz, 1970, 1971), but this phenomenon is rare among marine benthic invertebrates and needs to be investigated further.

In the study reported here, egg masses cultured initially in the presence of the adult's food, *Hydractinia echinata* Fleming, 1828, developed only in the nonpelagic lecithotrophic mode. In an attempt to induce alternate modes of development, temperature, adult nutrition and exposure to *H. echinata* were manipulated on different egg masses and embryogenesis and metamorphosis were followed. Field data are compared with laboratory observations.

The ecology of *Cuthona nana* involves a species-specific predator-prey association with the colonial hydroid, *Hydractinia echinata*, commonly found on gastropod shells occupied by pagurid crabs (Fig. 1). *Hydractinia echinata* is a dioecious hydroid consisting of a basal mat from which arise gastrozooids, gonozooids, and defensive dactylozooids (Hyman, 1940). The motile nature of hermit crabs gives the hydroid's substrate a mobility that presents potential settlement problems for the veligers or newly metamorphosed juveniles of *C. nana*, and possible prey-locating difficulty for adult nudibranchs. Information from the literature, laboratory and field observations, and experiments reveals how the behavior and life histories of the hydroid, nudibranch and hermit crab are inter-related.

Materials and Methods

Specimens of *Cuthona nana*, their egg masses, and hermit crabs in mollusc shells bearing *Hydractinia echinata* were collected by scuba diving in Gosport Harbor at the Isles of Shoals (43° 59′ N; 70° 37′ W), *ca.* 10 km off the New Hampshire coast. Most of the collecting was done at the depth of 3–12 m in Haley's Cove, an

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area within the harbor that had the highest concentrations of hermit crabs with *H. echinata* colonies. Monthly field observations and collections were made from January, 1974, to July, 1975, excluding June through August, 1974. The hermit crabs, hydroid colonies, and nudibranchs were maintained at 11–13° C in a recirculating seawater system. Within two days of their collection, the colonies of *H. echinata* were examined under a dissecting microscope and the numbers and lengths of *C. nana* individuals found on each shell were recorded. The ciliated epithelium of the nudibranchs gave them a slight iridesence in contrast to the hydroid, so that even very small nudibranchs (<0.5 mm) could be seen among the polyps.

Egg masses laid in the laboratory were isolated in small dishes containing 50 ml of sea water and incubated at 11–13° C. The sea water used for culturing was collected in Gosport Harbor and filtered through a 0.45 μm Millipore filter. The culture water was initially changed daily, but in later experiments it was changed every two or three days with no effect on development. At intervals of six to twenty-four hours, the egg masses were temporarily transferred in drops of sea water to microscope slides and observed under a compound microscope using transmitted or reflected illumination. Egg masses collected in the field were cultured at the temperature at which they were collected, which ranged from 4–13° C.

The normal mode of development for *Cuthona nana* eggs cultured at 11–13° C was determined initially, then the effect of variations in temperature, adult nutrition, and the presence of *H. echinata* was tested. Specimens of *C. nana* and *H. echinata* colonies were kept in dishes of aerated sea water at 4, 8, or 16° C. Deposited egg masses were isolated as above and incubated at the same three temperatures. At 16° C, successful development was obtained only when the water was changed at least twice daily. Egg masses from starved adults were isolated at 11–13° C and their development followed.

Other specimens of *Cuthona nana* were kept in compartmentalized trays with flowing sea water. Shells covered with *H. echinata* were included with some specimens of *C. nana*. The growth of individual nudibranchs could thus be followed, the availability of food controlled, and the number of egg masses laid by particular individuals monitored.

Although the behavior and distribution of the early postlarval stage of *C. nana* could not be directly studied in the field, the distribution of juveniles on *H. echinata* colonies was noted monthly and two field experiments were conducted to test for the presence of planktonic *C. nana* veligers. In the first, a float was anchored 3 m off the bottom of Haley's Cove in 8 m of water on February 24, 1974. Pairs of *H. echinata*-covered shells were suspended at 1, 2, and 3 m off the bottom to determine if *C. nana* veligers, should they be capable of swimming, would settle directly on an *H. echinata* colony. These hydroid colonies had been collected approximately two weeks earlier, and had been examined under the dissecting microscope to remove all specimens of *C. nana*. The second experiment involved anchoring a 1 × 1 × 0.5 m open-bottomed cage covered with 0.25 inch nylon mesh on the sand near the float. The hermit crabs initially enclosed by the cage were removed before nine hermit crabs bearing *H. echinata*-covered shells were placed inside. These colonies had also been cleaned of *C. nana*. It was hypothesized that planktonic *C. nana* veligers might settle near *H. echinata* colonies before metamorphosing

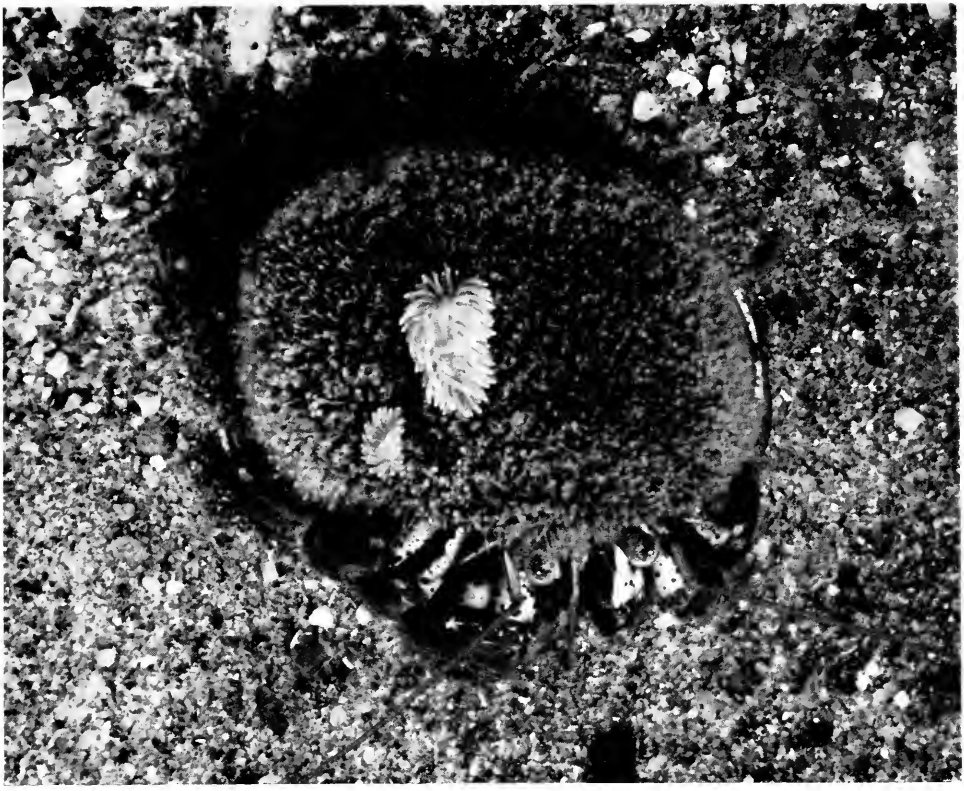

FIGURE 1. Two specimens of the eolid nudibranch *Cuthona nana* feeding on the colonial hydroid *Hydractinia echinata* covering a gastropod shell occupied by the hermit crab *Pagurus acadianus*. The larger nudibranch is about 14 mm in length.

and climbing onto the hydroid. The shells suspended from the float or confined inside the cage were changed at two week intervals until May 29, 1974. Each time, the hydroid colonies were examined for nudibranchs immediately upon return to the laboratory, and again one and two weeks later.

RESULTS

Development

Egg mass. Cuthona nana is reproductively typical of opisthobranchs in that it is a reciprocally copulating hermaphrodite that deposits eggs within a gelatinous stroma. The spawn of *C. nana* has been described and illustrated by Harris *et al.* (1975). The largest egg masses collected in the field or laid in the laboratory were 10 mm in diameter and contained about 1500 eggs. However, most egg masses observed were considerably smaller, averaging 450 eggs. Nudibranchs raised in the laboratory from immaturity to death laid up to 16 egg masses. The first and last few egg masses laid were smaller than average, but most contained 300–600 eggs. Individuals separated after copulation laid up to six egg masses before a substantial number of unfertilized eggs were produced.

TABLE I

Normal table of development for Cuthona nana *eggs incubated at 11–13°C.*

12 hours	First division	6–10 days	Cilia and shell develop
16 hours	Second division	15 days	Mantle withdrawn half-way
20 hours	Third division	16 days	Propodium first appears
36 hours	Morula	18–21 days	Hatching
3–5 days	Gastrulation	20–23 days	Metamorphosis

Development to hatching. Early development in *Cuthona nana* is similar to that described for other opisthobranchs (Casteel, 1904; Pelseneer, 1911; Thompson, 1958). Table I gives normal development time for eggs cultured at 11–13° C. At oviposition, the white eggs within their individual ovate capsules average 160 μm in diameter. Spiral cleavage produces a stereoblastula whose vegetal side begins to flatten at the end of the second day of development. Gastrulation results in a cup-shaped gastrula, with the ventral blastopore becoming asymmetrical before closing during the fifth day. Typically, the polar bodies adhere to the animal pole through gastrulation.

By the end of the sixth day a shell cap covers the posterior end of the embryo (Fig. 2a). The shell increases in size as the shell gland (now the mantle fold) spreads anteriorly. Two anal cells of disputed function (see Bonar and Hadfield, 1974) appear in front of the mantle fold on the right, ventro–lateral surface of the embryo, while anteriorly the velar lobes and foot are enlarging. The locomotor cilia elongate and rock the embryo within the egg capsule. The rate of shell formation exceeds the speed at which the mantle fold migrates anteriorly, so that a lumen (the perivisceral cavity) develops in the posterior end of the shell (Fig. 2b–c). The visceral mass is compact and opaque, occluding the anterior opening of the developing shell. The retractor muscle is visible within the perivisceral cavity, but shows no signs of contracting during shell formation. A group of cells surround its origin just dorsal and to the left of the shell apex. The anal cells remain visible for a time in front of the mantle fold but disappear before the shell becomes complete on the tenth day. Torsion in *C. nana* does not involve a 180° twist of the cephalo-pedal elements with respect to the shell; these parts differentiate in their post-torsional positions. The movement of the anal cells may be the only ontogenetic evidence of torsion (Thompson, 1962).

During shell growth the foot elongates ventrally and becomes heavily ciliated mid-ventrally and at the tip, but not laterally. Its dorsal surface has an operculum by the ninth day and several long, stiff compound flagella protrude from the tip. At this time, a ciliated subvelar ridge begins to develop. The visceral organs grow posteriorly into the perivisceral cavity, eventually filling the entire posterior end of the shell except for the dorsal mantle cavity. Due to the yolk content and opacity, it is difficult to discern individual organs.

When secretion of the larval shell is complete, the mantle fold at the shell aperture becomes thinner and less dense. By the eleventh day, it begins to withdraw posteriorly along the inner surface of the shell. The degree to which the velar lobes can be retracted into the mantle cavity depends on the position of the withdrawing mantle fold. Initially, the velum cannot be accommodated by the

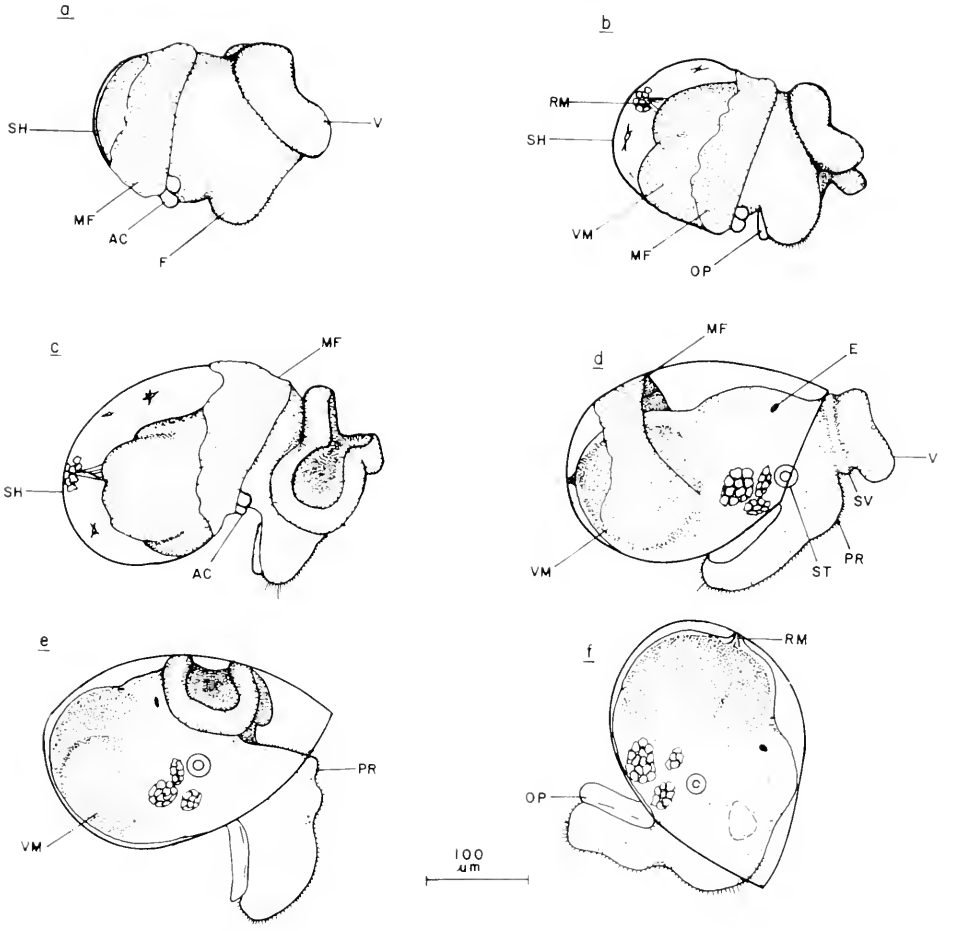

FIGURE 2. Late larval development and early metamorphosis in *Cuthona nana:* a–c) veliger with developing shell; d) veliger with complete shell and mantle withdrawn back along inside of the shell; e) late veliger retracted into the shell at the stage of development at hatching; and f) early metamorphosis after loss of the velum. AC indicates the anal cells; E, the eye; F, the metapodium; MF, the mantle fold; OP, the operculum; PR, the propodium; RM, the retractor muscle; SH, the shell; ST, the statocyst; SV, the subvelar ridge; V, the velum; and VM, the visceral mass. Velar locomotor cilia not shown.

mantle cavity, and only when the mantle has regressed three quarters of the way to the apex of the shell (Fig. 2d) can the velar lobes be entirely withdrawn. Normally, the foot is never fully retracted with the operculum closing the shell opening. Chemical irritants such as alcohol cause the veliger to retract beyond the normal restrictions of the mantle fold or even to draw the foot into the shell, but only at concentrations that kill the larva.

Two red eyespots become visible on the fifteenth day. A day later, the propodium begins to form, just ventral to the mouth (Fig. 2d). At this stage the mantle has migrated two-thirds of the way back from the shell aperture. The sub-

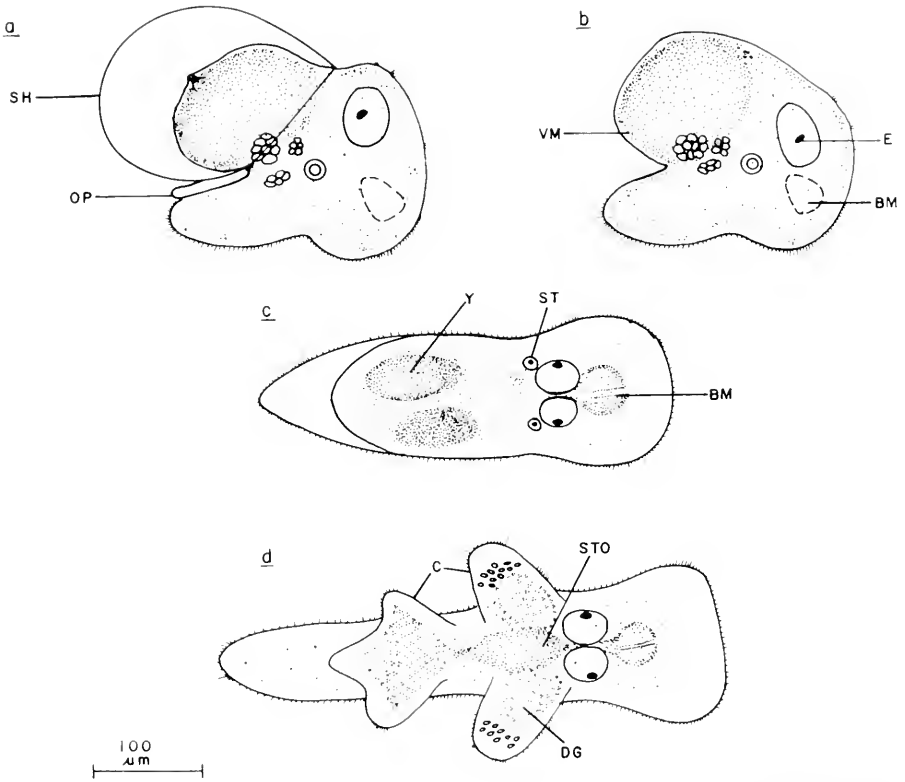

FIGURE 3. Late metamorphosis and early postlarval development in *Cuthona nana:* a) shell loss; b) newly emerged juvenile; c) elongated juvenile; and d) juvenile with four primary cerata. Stages a-c occur in 20–23 days after oviposition at 11–12° C. Growth to stage d occurs in another 2–3 weeks in the presence of abundant food. BM indicates the buccal mass; C, two cerata; DG, the digestive gland; E, the eye; OP, the operculum; SH, the shell; STO, the stomach; VM, the visceral mass; and Y, yolk concentrations in the visceral mass.

velar ridge is well-developed and heavily ciliated. By the eighteenth day the mantle has reached the shell apex, and fuses with the epithelial layer covering the visceral mass. The veliger is attached to the shell only around the origin of the larval retractor muscle, and possibly ventrally. In another day the propodium is fully developed (Fig. 2e), and muscular activity in the foot is evident. The velar locomotor cilia continue to beat almost continuously, but the veligers move about very little within the capsules. The visceral organs are still densely packed with yolk, while the foot and velum have become progressively less opaque.

Hatching. The rate of development varies slightly among siblings, although there is no noticeable relationship between position in the egg mass and developmental rate. The hatching of larvae from an egg mass is usually spread over several days. At 11–13° C, a few veligers typically begin escaping from their capsules late on the eighteenth day after oviposition. Cracks develop and radiate throughout the egg capsule causing its collapse. Although at this time the uniseriate

radula possesses two distinct teeth, it is not used to rupture the capsule wall. Furthermore, the physical activity of the veligers does not change markedly prior to hatching. It is thus unclear what causes the breakdown of the capsules. Also, hatching from the capsules does not depend on the integrity of the egg mass. Usually the gelatinous stroma of the egg mass deteriorates before the eggs hatch, but when incubated in still, filtered sea water this outer covering often remains intact. The process of hatching is identical in either situation.

The behavior of newly hatched veligers depends on their relative stage of development. Veligers may hatch prior to the complete development of the propodium. These larvae initially lay on their sides with the velum extended and velar cilia beating. Although the cilia may gently rock the veliger, or even lift the anterior end off the substratum so that it faces upward, a veliger was never seen to swim up into the water. When the propodium is more fully developed, the veligers roll over and crawl slowly about, with the velum only partially extended. Veligers that hatch with a well-developed propodium immediately adhere to the bottom and begin crawling. Movement may continue for up to two days, but activity progressively decreases.

Metamorphosis. Within a day or two of hatching, metamorphosis begins. The veligers cease crawling and remain in a semi-contracted state, with the shell held nearly vertically and the head just inside the shell aperture. The first noticeable morphological change is the loss of the velar lobes. The beat of the locomotory cilia becomes increasingly erratic. Cells bearing these cilia are cast off from the velum. In individuals still held within the egg mass stroma, these cells do not accumulate but appear to be ingested, as are homologous cells shed during metamorphosis in *Phestilla sibogae* (Bonar and Hadfield, 1974). The rest of the velar lobe is resorbed during the next several hours, until only a swelling remains protruding slightly from the dorsum.

During this period, loss of contact between the larval body and shell continues ventrally until only the retractor muscle attachment remains (Fig. 2f). The operculum is still attached to the dorsal surface of the metapodium but becomes progressively detached distally, allowing increased flexibility of the foot. Activity of the retractor muscle diminishes. By the time the velar lobes are resorbed, mechanical and nonlethal chemical stimuli do not elicit further withdrawal of the larva into the shell. Should the larva become dislodged from the substrate, the pedal cilia slowly spin it about until a foothold is regained.

Shell loss begins when a strong, continuous contraction of the retractor muscle breaks its connection with the shell. This occurs only if the foot is firmly attached to the substratum. For several hours after the connection is broken, the remnant of the origin of the retractor muscle may be visible as a small lump of cells or thickening in the epidermal layer. In some metamorphosing larvae this lump is not seen, possibly due to a more contracted state of the retractor muscle after it detaches from the shell. With the retractor muscle attachment severed, the larvae are free to crawl out of the shell (Fig. 3a), a process that may take five to eight hours in still water. Loss of the shell may be greatly accelerated by water currents, because the visceral mass is compact and equal to or just smaller in diameter than the shell aperture. The operculum may adhere to the shell when it is cast off or is lost separately.

The visceral mass of the young shell-less juvenile initially appears as a distinct hump (Fig. 3b). Within a day it fuses with the cephalo-pedal elements, thereby flattening the nudibranch dorso-ventrally. Elongation of the body continues until the juvenile measures about 0.32 mm in length (Fig. 3c). The developing buccal mass possesses three to four teeth in the uniseriate radula and a pair of weak jaws. The body is generally a translucent white color, with the red eyes clearly visible near the cerebral ganglia. The visceral mass is cream-white, indicating that yolk still remains. Cilia densely cover the ventral epithelium, but are sparse on the dorsum.

The effect of different temperatures on development. The development of eggs laid and maintained at 4, 8, or 16° C differed from those kept at 11–13° C only in the length of time until metamorphosis. Egg size did not vary with temperature. Metamorphosis occurred within 50–55 days at 4° C, 34–36 days at 8° C, and 16–17 days at 16° C. *Cuthona nana* may not tolerate temperatures much above 16° C, for specimens maintained at that temperature suffered a high rate of mortality. Eight adults placed with food at 16° C died within 10 days. Only 5–30% of the eggs from spawn laid at 16° C or transferred to that temperature from 11–13° C immediately following oviposition developed normally through metamorphosis. In contrast, nearly all of the eggs laid and maintained at 11–13° C reached metamorphosis. Nine egg masses collected in the field and incubated at the temperature at which they were collected (4–13° C) developed and metamorphosed normally, with their rates of development varying according to the temperature, and they are identical with those observed for eggs raised in the lab at similar temperatures.

The effect of adult nutrition and the presence of Hydractinia echinata *on larval development.* Hatching and the events of metamorphosis proceeded sequentially, unaffected by the presence of *H. echinata* during any stage of development. The development of embryos in egg masses incubated in a dish with *H. echinata* and those exposed to the hydroid only before or after hatching did not differ from the development of those egg masses kept isolated. The integrity of the spawn mass had no effect. The development and metamorphosis of larvae from egg masses that had broken down, exposing the capsules directly to the water, was similar to those in egg masses that remained intact.

Adult specimens of *C. nana* kept without food in Millipore-filtered sea water continued to lay egg masses for up to ten days. The size and activity of the adults progressively diminished during that time, but some starved individuals survived for 17 days. They laid several egg masses in the first few days of isolation, but the frequency of oviposition and the number of eggs per spawn decreased with time. Mean egg diameter did not vary, and the rate and events of larval development and metamorphosis proceeded normally in the presence or absence of *H. echinata.*

Postlarval development to the adult stage. In the absence of *H. echinata,* the newly metamorphosed juveniles crawl about constantly. They are negatively geotactic and positively phototactic, crawling up the sides of the dishes and toward unidirectional illumination or to the apex of rocks or shell fragments placed in their dishes. When *H. echinata* is added, movement is directed toward the hydroid. However, in the absence of food, activity decreases. Starved postlarvae become motionless within two weeks of metamorphosis, but movement increases rapidly when *H. echinata* or water exposed to the hydroid is added. Postlarvae survived

for five to six weeks without food at 11–13° C and ten weeks at 4° C, and were then still capable of feeding and growing if *H. echinata* was made available. Postlarvae from four egg masses laid by starved adults at 11–13° C survived up to six weeks without food. As the juveniles are starved, the visceral mass becomes less intensely colored. Teeth are added to the radula until there are five to eight, but no more are formed unless feeding begins.

Cuthona nana is attracted to *H. echinata* almost immediately after metamorphosis, but feeding is initially very slow if it occurs at all. In animals that crawl onto *H. echinata* shortly after metamorphosis, the orange color of the hydroid does not appear in the digestive gland of the nudibranchs for two or three days. However, juveniles that are not given food until four or five days following shell loss begin feeding immediately and color appears in the digestive gland within 24 hours. Development of the postlarval buccal or digestive structures necessary for feeding may therefore continue for several days following shell loss.

Cuthona nana will readily feed on any part of *H. echinata* colonies, as well as released eggs, planula larvae, or metamorphosing planulae. Young nudibranchs are sometimes much smaller than the hydranth they are feeding on, especially the gastrozooids. These polyps are very distensible and may reach a length of 5 mm or more and an oral disc diameter of more than 0.75 mm. Many of the prey items ingested by the gastrozooids are much larger than the newly metamorphosed nudibranchs, but recently collected and presumably well-fed colonies of *H. echinata* do not attempt to ingest the small eolids. On such colonies maintained in still water, postlarvae are often seen on the manubrium of gastrozooids. Small nudibranchs sometimes elicit a defensive response by *H. echinata;* the tentacles of several nearby polyps are brought down on top of them, but the hydroid's nematocysts apparently do no harm. In contrast, hydroid colonies starved for several weeks will eat recently metamorphosed *C. nana* juveniles. The nudibranchs are not killed before ingestion, and will survive if immediately removed from the gastrocoel of the hydranth. Those removed an hour or more later are dead and partly digested.

Table II summarizes post-metamorphic growth in *C. nana*. Growth is initially slow, even with an abundance of *H. echinata*. Dorsal enlargements, indicating the rudiments of the first pair of cerata, do not appear for two weeks after metamorphosis. The second pair of ceratal buds develop posterior to the first pair within another five days (Fig. 3d). New cerata develop at an increasingly rapid rate, with their pattern of appearance like that described for *Cuthona adyarensis* by Rao (1961). The uniseriate radula also grows in size, with new teeth being distinctly larger than the first five or six. Eventually *C. nana* adults may attain a length of 28 mm with 250 cerata and 27 radular teeth, but nudibranchs measuring 17–22 mm with 23–24 radular teeth are more common.

Development and growth varies substantially in individuals from the same egg mass, so that some mature several weeks before others. The white ovotestis first becomes visible through the body wall when the nudibranchs are 8–10 mm long. Anterior acini of the ovotestis develop first, and maturation proceeds posteriorly. Nudibranchs smaller than 10 mm have never been seen to copulate, and they are usually longer than 12 mm before they lay eggs. In the laboratory, specimens of *C. nana* raised on healthy *H. echinata* on a gastropod shell remained on that colony until they were at least 10 mm long, regardless of the presence or absence of a

TABLE II

Postlarval growth in Cuthona nana *in the presence of abundant* Hydractinia echinata *at 11–13°C.*

Time from metamorphosis	Length in mm	Characteristics
2 weeks	0.5	Eight to ten radular teeth; first pair of cerata
3 weeks	0.75	Eleven to twelve radular teeth; third pair of cerata; rhinophore primordia
4–5 weeks	1.0	Fifteen radular teeth; fourteen cerata; first heart beat; oral tentacles appear
6–7 weeks	4.0	Thirty to thirty-five cerata
9–10 weeks	8.0	Nineteen to twenty-four radular teeth
11 weeks	12.0	Shortest time observed for an individual to mature and lay an egg mass

hermit crab in the shell. At this time, if alone, *C. nana* leaves the hydroid colony in search of a mate. When a nudibranch measuring only 10–12 mm mates for the first time, it usually resumes feeding before laying egg masses.

Harris *et al.* (1975) reported that *Cuthona nana* adults do not lay egg masses on *H. echinata* colonies. They noted that since the nudibranchs do not die after laying eggs, they probably find new hydroid colonies. Subsequent laboratory observations were made on *C. nana* confined with hermit crabs bearing *H. echinata*-colonized shells. The nudibranchs invariably left the hydroid to lay egg masses and consistently returned to the colonies to resume feeding. The hermit crabs often remained motionless long enough for the nudibranch to find and crawl onto the hydroid. This pattern of leaving the *H. echinata* to deposit spawn, and then returning, continued until the nudibranchs died. At 11–13° C, adult specimens of *C. nana* survived in this fashion for six to eight weeks, while those kept at 4° C lived for over three months.

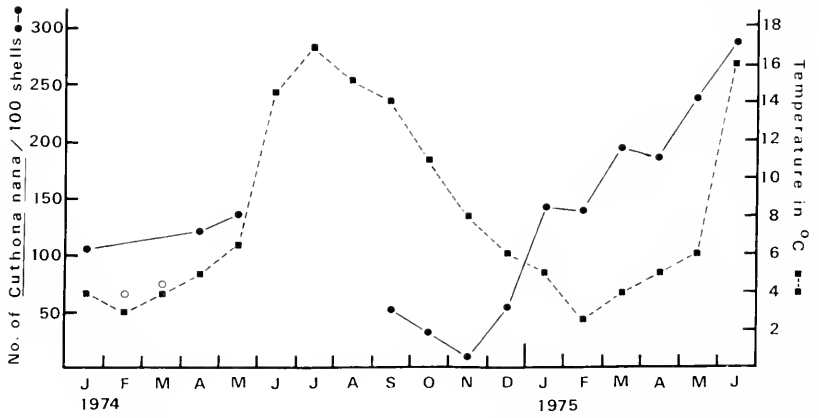

FIGURE 4. Number of *Cuthona nana* per 100 *Hydractinia echinata* colonies and surface water temperatures from January, 1974, to June 1975. Open circles refer to collections outside of Haley's Cove. No collections were made in June, July and August, 1974. See text for details.

Two generations of *Cuthona nana* were raised in the laboratory at 11–13° C. The nudibranchs survived well in the recirculating seawater systems where the salinity varied from 30 to 38‰. The shortest life cycle observed took 14 weeks from egg to egg.

Ecology

Cuthona nana was found to be more abundant in Gosport Harbor than previously reported (Harris *et al.,* 1975). Both juvenile and adult individuals were most common from January through June and least abundant in September through December (Table III). The number of *C. nana* observed in February and March, 1974, were considerably below that seen in those months during 1975. Foul weather in February and March, 1974, forced collection outside of Haley's Cove, the area where the density of pagurid crabs with *Hydractinia echinata* colonies was highest in Gosport Harbor. Laboratory studies have shown that the growth rate of *C. nana* at water temperatures observed during these months (3.5–4.5° C) is extremely slow. Therefore, the number of nudibranchs found in April and May, 1974, when the temperature reached only 6.5° C, indicates that nudibranch densities within Haley's Cove during the previous two months were much higher than in the collections outside Haley's Cove. Nudibranch numbers fluctuated asynchronously with temperature (Fig. 4). The population of *C. nana* was growing in size during the coldest months and decreased sometime during the summer. The average size of the nudibranchs collected varied little, for the number of adults and juveniles varied synchronously (Table III). Egg masses were seen in the field during every month of this study except for September and November, 1974. In October, 1974, only one egg mass was found, and only two were found in December. Reflecting the greater number of adults, egg masses were more abundant during the spring months with up to eight seen during a forty-minute dive.

Data on the distribution of *C. nana* less than 5 mm in length are included in Table III to show monthly differences in the numbers of juveniles and to evaluate seasonal fluctuations in reproduction and recruitment. Since young nudibranchs normally do not leave an *H. echinata* colony until they are about 8–10 mm in length, the 5 mm length was considered a conservative upper size limit for examining the distribution of nonreproductive juveniles. Such juveniles were likely to be found on the first hydroid colony they had occupied.

The numbers of *C. nana* juveniles fluctuated from a spring high of 94 in May, 1974, down to a fall low of 7 in November, 1974, and up to 242 per 100 hydroid colonies in June, 1975 (Table III). These juveniles were not evenly distributed over the population of *H. echinata* colonies. In the months when most abundant, young nudibranchs outnumbered the hydroid colonies collected, yet they were found on only 48–57% of them. Thus, if one small nudibranch was present on an *H. echinata* colony, chances were good that there were more. Four or five juveniles per colony were not uncommon, and much higher numbers were occasionally found. In May, 1974, one colony collected possessed 17 juveniles less than 3.5 mm long. A colony examined in June of 1975 carried 29 *C. nana* juveniles. During the fall months when *C. nana* was least abundant, *H. echinata* colonies with two or more young nudibranchs were still more frequently encountered than those with just one.

Cuthona nana juveniles were also not randomly distributed over the *H. echinata*

TABLE III

Data on Cuthona nana *from monthly collections of* Hydractinia echinata-*covered hermit crab shells from Gosport Harbor.*

Month	Number of *C. nana* per 100 shells*		Number of *C. nana* by size classes			Average size of *C. nana* in mm	Number of hermit crab shells examined	Percentage of shells with *C. nana*		Average number of *C. nana* <5 mm per infested shell
	<5 mm	Total	<5	5–10	>10 mm			<5 mm	Total	
Jan. 1974	90	107	26	2	3	3.1	29	41	59	2.2
Feb.**	24	67	5	8	1	6.1	21	14	43	1.6
Mar.**	9	56	2	6	4	8.5	22	9	36	1.0
Apr.	89	121	34	8	4	3.5	38	38	42	2.8
May	94	138	76	19	17	4.5	81	44	59	2.1
Sept.	46	51	31	4	0	2.6	68	24	25	1.9
Oct.	19	31	13	6	2	4.0	67	10	25	1.8
Nov.	7	11	4	1	1	4.4	55	7	11	1.0
Dec.	50	53	29	1	1	1.8	58	31	34	1.6
Jan. 1975	128	144	32	1	3	2.9	25	48	56	2.7
Feb.	123	139	54	4	3	2.9	44	52	61	2.4
Mar.	152	193	70	12	7	3.7	46	54	72	2.8
Apr.	143	188	80	18	7	3.4	56	57	71	2.5
May	180	238	88	15	14	2.8	49	55	65	2.7
June	242	285	138	10	15	2.4	57	53	70	4.6
										$\bar{X} = 2.7$

* These columns were obtained by adjusting the number of *C. nana* found in the monthly samples to 100 hermit crab shells so that population size fluctuations would be more visible.

** Collections during these months were made outside of Haley's Cove, and the number of *C. nana* found was lower than expected for Haley's Cove. See text for details.

colonies. The observed distribution of nudibranchs over the hydroid colonies collected each month was compared with a random distribution using a chi-square analysis. The differences were significant at the 0.05 level for the Haley's Cove samples for all months except October, November, and December, 1974. Thus, during the months when most abundant, the juveniles were nonrandomly distributed over the H. *echinata* colonies.

Two observations suggest that recruitment of *C. nana* on hydroid colonies is from benthic juveniles rather that pelagic veligers. First, no juveniles were found on either the caged pagurids or the suspended *H. echinata* colonies that were placed in the field during a period with high *C. nana* egg production and a growing population (February through May, 1974). In the laboratory, starved colonies of *H. echinata* had consumed veligers and postlarvae of *C. nana*. Therefore, the hydroid colonies recovered from the float and cage were tested to determine if the experimental manipulations had starved them to a point where they might have eaten any *C. nana* veligers or postlarvae they had contacted. Veligers and juvenile nudibranchs were not preyed upon when placed on the gastrozooids of experimental colonies shortly after being brought back from the field.

Secondly, *C. nana* juveniles smaller than 3 mm in length were found predominantly on the ventral half of the hydroid colonies collected. The gastrozooids of *H. echinata* are more numerous and longer around the ventral periphery of the

colony. These polyps sweep over the surface of the substrate as the hermit crab moves about. In the laboratory, *H. echinata* colonies swept over the bottom of dishes containing *C. nana* postlarvae picked up many of the small nudibranchs by a mechanism that probably involves the hydroid's nematocysts. These collected nudibranchs then reoriented and began feeding on the hydranths. By the time they had grown to a length of 5 mm, they may have moved half way around the colony. Since the location of nudibranchs less than 3 mm in length is close to the site of first contact with the colony, the ventral position of the smallest *C. nana* individuals on the collected *H. echinata* colonies supports the hypothesis that the nudibranch reaches the hydroid by being swept up from the bottom and not by settling onto the hydroid from the plankton.

In the field, most individuals of *C. nana* were observed on *H. echinata* colonies, but during the late spring months adult nudibranchs were often found crawling over the sand, rubble, or loose pieces of algae. Occasionally groups of two to four were seen, either copulating or depositing egg masses, but most were isolated individuals. Five adult nudibranchs found singly on the bottom or on a hydroid colony were returned to the laboratory and maintained in isolation. In every case, fertile egg masses were subsequently laid, indicating that the adults had copulated previously.

In late April and May, 1974, large specimens of *C. nana* were discovered in the cage. During each of four two-week periods, four to six nudibranchs with average lengths of 16 mm had crawled onto the hydroid colonies, and several more were seen in and around the cage. The presence of adults of *C. nana* on the caged *H. echinata* demonstrates the nudibranch's mobility and capacity for finding new colonies.

The pagurid population in Gosport Harbor consisted of *Pagurus acadianus* Benedict, 1901, and *P. arcuatus* Squires, 1964. Both were abundant down to a depth of twelve meters. *Pagurus acadianus* was found more commonly on the cleaner sand and *P. arcuatus* in the siltier, more cobble-strewn areas, but there was considerable intermixing. Observations during numerous dives indicated that the distribution of these hermit crabs changed continuously, such that denser concentrations were found in different areas of Haley's Cove on successive dive dates. Grant (1963) also found that populations of *P. acadianus* in the shallow subtidal were transient in nature, indicating a high degree of mobility. The feeding behavior of this pagurid has not been described, but it appears to be similar to that of the omnivorous European species, *P. bernhardus* (Orton, 1927; Gerlach, Ekstrøm and Eckardt, 1976). Food of *P. acadianus* consists partly of moribund invertebrates and pieces of algae, but predominantly of detrital material and small organisms captured by using the chelae to shovel sediment into the mouth parts where it is sifted. The hermit crabs remained stationary much of the time, sifting sediment or actively fanning the water with the maxillipeds and maxillae, possibly filter-feeding as in *P. bernhardus* (Gerlach *et al.*, 1976). *Pagurus acadianus* broods its eggs on abdominal pleopods until the zoeal stage. Females were seen in March, 1974, to release the zoeae by protruding three-fourths of the way out of their shells and waving their egg-laden pleopods. In cases where the shell aperture was lined by *H. echinata,* some zoeae were caught and eaten by the gastrozooids. Of all the hermit crabs collected bearing shells colonized by *H. echinata,* 98% were *Pagurus acadianus.* Like the European *P. bernhardus* (Jensen, 1970), *P. acadianus* prefers

shells covered with the hydroid over clean shells (Grant and Ulmer, 1974). In contrast, *P. arcuatus* preferentially selects naked shells (Grant and Ulmer, 1974). (*Pagurus pubescens* in Grant and Pontier, 1973, and Grant and Ulmer, 1974, was actually *P. arcuatus;* personal communication from W. Grant, 1975.) Whereas empty, clean gastropod shells were commonly seen in Gosport Harbor during the present study, unoccupied *H. echinata*-covered shells were rare.

The feeding of juvenile nudibranchs had little noticeable effect on the hydroid colonies; the regeneration rate of the hydranths approximated the predation rate. Adult nudibranchs, however, cleared patches among the hydranths, leaving only the basal mat. In such cases, the first polyps eaten were often regenerating while the nudibranch was still enlarging the patch.

Discussion

The pattern of development of *Cuthona nana* eggs remained constant over a variety of conditions during this study. Incubation of egg masses collected in the field throughout the year and those laid in the laboratory under various conditions of temperature and adult nutrition yielded veligers which invariably metamorphosed without a pelagic stage. Developmental rate varied inversely with temperature. The times to hatching obtained at 4, 8, 11–13, and 16° C fall close to the regression line of Spight (1975, Fig. 1) for prehatching period *versus* temperature for other opisthobranchs. These results further support his thesis that time to hatching can be estimated with reasonable accuracy from taxonomic affinity and temperature alone.

The presence or absence of *Hydractinia echinata* had no noticeable effect on the rate or sequence of events in the development of *C. nana*. Egg size and subsequent development were unaltered by differences in adult nutrition; starved animals simply laid fewer eggs. In contrast to *Mytilus edulis* (Bayne, 1972; Bayne, Gabbott, and Widdows, 1975), there was no increase in abnormal development in eggs from starved adults. Furthermore, starved postlarvae from starved adults developed as fast and survived as long (four to six weeks at 11–13° C) as starved postlarvae produced by well-fed adults. *Hydactinia echinata* apparently plays no role in inducing metamorphosis in *C. nana,* as *Electra pilosa* does for *Aldaria proxima* (Thompson, 1958).

Two schemes that categorize opisthobranch development have been presented in the literature. Thompson (1967) formed three categories distinguishing opisthobranchs by feeding type and place of metamorphosis. His development-types 1, 2, and 3 refer to species with pelagic planktotrophic, pelagic lecithotrophic and nonpelagic lecithotrophic ("direct") development, respectively. *Cuthona nana* falls between development-types 2 and 3 in that it does not possess a pelagic lecithotrophic larva, nor does it hatch out of the capsule at a post-veliger benthic stage. Because of the ecological significance of its nonpelagic development, it should be classified as having development-type 3. In this category Thompson (1967) included *Cuthona pustulata,* which like *C. nana* hatches out of the capsule as a veliger, but remains within the stroma of the egg mass until metamorphosis (Roginskaya, 1962).

Tardy (1970) presented a classification scheme for the Nudibranchia that primarily segregated them on the basis of protoconch type, which he felt represented

basic ontogenetic differences such as different origins of the adult dorsal epidermis. Species with a spiral protoconch were categorized as having type 1 development, while type 2 referred to those species possessing an inflated protoconch. There are at least two exceptions to Tardy's scheme. First, Bonar and Hadfield (1974) and Bonar (1976) have reported that the dorsal epidermis of *Phestilla sibogae* was derived from the lateral surfaces of the larval foot and not from the floor of the mantle cavity as thought by Tardy for type 2 nudibranchs. Secondly, whereas Tardy felt that all nudibranchs with inflated protoconchs underwent torsion after the shell was complete, in *Cuthona nana* structures develop in their post-torsional positions. Additional studies are needed on the origin of the adult dorsal epidermis and differences in the expression of torsion within the Opisthobranchia.

The field data support the laboratory observations of nonpelagic development in *Cuthona nana*. A pelagic veliger might have settled on the experimental colonies suspended in the water column or enclosed by the cage, but this was not observed. Table III shows that from January to June, 1975, juveniles of *C. nana* were found on only about one-half of the hydroid colonies collected, even though the nudibranchs greatly outnumbered the colonies. The uneven nonrandom distribution observed during the late winter and spring months is what would be expected if recruitment to the *C. nana* population was from simultaneous colonization by clustered benthic juveniles, with the distribution of these clusters being determined by the deposition sites of egg masses.

Predatory benthic marine invertebrates that are relatively nonmotile as adults and lack pelagic larval stages are faced with the problems of prey location and of dispersal. From field and laboratory observations, it is concluded that the postlarvae of *Cuthona nana* 'find' an *Hydractinia echinata* colony much the same way as the hydroid's planulae 'find' a clean hermit crab shell. The gonozooids on female hydroid colonies produce large orange-red eggs that are fertilized when released, drop to the bottom and develop within two or three days into a planula with an enlarged anterior end possessing numerous secretory cells (Bunting, 1894; Van de Vyver, 1964, 1967). A healthy colony covering an hermit crab shell may release several hundred eggs in a season. The resulting planulae remain benthic and crawl slowly about in a turbellarian-like fashion, being positively phototactic and somewhat negatively geotactic (Schijfsma, 1935; Cazaux, 1961; Van de Vyver, 1964). The planulae do not actively search for a clean hermit crab shell; it is the hermit crab's activity, either its locomotion or feeding movements, that bring the two in contact (Schijfsma, 1935). The planulae adhere to the shell with the anterior end. A single polyp is initially formed, then a basal mat grows out over the shell as new polyps are added. The gastrozooids develop primarily around the ventral side of the shell, where they capture small invertebrates on the surface of the substrate during the hermit crab's travels and feeding movements (Christensen, 1967; Harris *et al.*, 1975). Postlarvae of *Cuthona nana* also get picked up by these gastrozooids. Just as with the planulae, the positive phototaxis and negative geotaxis of the nudibranchs keep them in the open on the substrate surface. Such positioning increases the chances that the postlarvae will be swept up by an *H. echinata* colony should an hermit crab bring one by.

The mobility of the hermit crabs is likely to be an important factor in the spread of *Cuthona nana* and *Hydractinia echinata*, for neither species has an actively dis-

persing larva. An adult nudibranch feeding on an *H. echinata* colony will be carried across the bottom as the hermit crab wanders, and may be taken tens of meters before it leaves the colony to find a mate or lay eggs. The next hydroid colony it climbs onto will be carried in a direction and distance independent of the previous ones. Large *Pagurus acadianus* in deeper water (below 17 m) have been seen in *Lunatia* or *Buccinum* shells bearing *H. echinata* and *C. nana*. These large crabs are quite noticeable because of their size, but are relatively rare. Their occasional presence in frequently observed areas indicates they probably travel long distances. They are sometimes seen in shallow water where small hydroid-colonized hermit crab shells are more numerous. By visiting different hermit crab concentrations, these large hermit crabs may provide a means for colonizing new areas and a mechanism of genetic communication between physically distant populations of *C. nana*. Other crab species may also be important; *C. nana* was collected on *H. echinata* growing on the legs and ventral side of a *Cancer borealis* in 18 m of water. Hermit crabs also act as colonization vectors for the direct developing *Crepidula convexa* (Hendler and Franz, 1971).

Cuthona nana may also disperse by rafting on pieces of dislodged algae. Wave-dislodged algae occasionally blanket the bottom in shallow areas of Gosport Harbor. Nudibranchs that climb onto the algae or egg masses deposited there could be carried off by storms or current changes. Data from seabed drifters indicate that the local average water speed is from 0.07 km/day (Loder, Anderson, and Shevenell, 1973) to 0.2 km/day (Graham, 1970). Thus at 4° C, juveniles having developed and metamorphosed on a piece of drifting algae could travel 8.75 to 25 km before starving.

The interspecific association between *Pagurus acadianus* and *Hydractinia echinata* is mutually advantageous. The hermit crab's shell provides a suitable substrate for the hydroid, which in turn makes the shell more desirable for *P. acadianus* and less so for *P. arcuatus* (Rees, 1967; Grant and Pontier, 1973; Grant and Ulmer, 1974). *Pagurus acadianus* will even occupy shells smaller than their preferred size range if these shells are colonized by *H. echinata* (Grant and Pontier, 1973). The hydroid can increase the effective size of the shell by growing beyond the lip, so the hermit crab needs to change its shell less frequently (Harris *et al.*, 1975; Jensen, 1975). *Hydractinia echinata* may act as a deterrent to predation on the pagurid crabs. Grant and Pontier (1973) found that *Cancer irroratus* did not feed on *P. acadianus* occupying shells with *H. echinata* colonies. However, during the present study a few starved specimens of *C. borealis* and *Carcinus maenus* did feed on *P. acadianus* in hydroid-covered shells, although most did not.

The hermit crab's mobility may provide a means of escape from overpredation for *Hydractinia echinata*. The hydroid colonies are perennial and once established, may persist for years (Sutherland, 1975; personal observation). *Cuthona nana* preys on the hydroid by eating the polyps, leaving the basal mat intact. When an adult nudibranch leaves an hydroid colony to search for a mate or deposit eggs, the colony will be carried away, descreasing its chances of being preyed upon by that nudibranch again. During the spring and early summer months, when large specimens of *C. nana* were most common, the majority of the hydroid colonies collected possessed patches devoid of polyps due to grazing by *C. nana*, the only significant local predator of *H. echinata*. However, never was a colony collected that had

more than half of its polyps eaten and usually the grazed areas showed signs of regeneration. *Cuthona nana* thus appears to simply crop *H. echinata* colonies and not kill them, with the perennial colonies regenerating lost polyps. Similarly, *Dendronotos iris* feeds on just a few tentacles of *Cerianthus* sp., not killing the anemone which presumably replaces the lost tentacles (Wobber, 1970).

Precuthona peachi (Alder and Hancock, 1847) has been reported to feed on *Hydractinia* growing on hermit crab shells in Europe (Farran, 1903; Swennen, 1961; Christensen, 1977). Several workers (L. Harris, T. Gosliner, T. E. Thompson, G. Brown; personal communications) consider *P. peachi* to be a junior synonym of *Cuthona nana*. Christensen (1977) recently reported that *P. peachi* in Sweden produced actively swimming planktotrophic veligers. These larvae survived unfed for 14 days without metamorphosing in the presence of *Hydractinia*. Christensen reported an egg diameter for *P. peachi* of 100 μm and a development time to hatching of 20–26 days at 7–9° C. This compares with the respective values determined during the present study on *C. nana* of 160 μm and 31–34 days at 8° C, with the veligers remaining benthic and metamorphosing immediately after hatching. Different modes of development have been previously reported for *C. nana* by Harris *et al.* (1975) who found planktotrophic development in 1971 and nonpelagic lecithotrophic development in 1973 in egg masses laid by individuals collected off the New Hampshire and Maine coasts. These differences may have resulted from observations on two virtually indistinguishable species, or *C. nana* may indeed possess two modes of development with the factors that influence the developmental pattern remaining enigmatic.

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Summary

1. The larval development, metamorphosis, and postlarval growth of the eolid nudibranch, *Cuthona nana*, is described. Hatching occurred within 19 days at 11–13° C. The lecithotrophic veligers remained nonpelagic and proceeded to metamorphose within another two days.

2. Adult nutrition did not affect egg size or subsequent development and metamorphosis.

3. Embryogenesis, hatching, and metamorphosis were unaffected by the presence or absence of the adult nudibranch's prey, the hydroid *Hydractinia echinata*.

4. Different temperatures altered the rate of development and of metamorphosis but not the type of development. Egg masses collected in the field and incubated at the temperature at which they were collected invariably produced nonpelagic lecithotrophic veligers which then metamorphosed.

5. Newly metamorphosed specimens of *C. nana* survived for up to six weeks at 11–13° C and ten weeks at 4° C in the absence of *H. echinata*.

6. In the presence of abundant food, specimens of *C. nana* deposited fertile egg masses within 11 weeks after metamorphosis at 11–13° C, and continued feeding and ovipositing for two months.

7. *Cuthona nana* feeds specifically on *Hydractinia echinata*, which in Gosport Harbor is found predominantly on shells occupied by *Pagurus acadianus*. As the hermit crabs move about, postlarvae of *C. nana* are swept up by the gastrozooids of *H. echinata*, are not eaten by the polyps but reorient and feed on hydroid tissue.

8. Nonpelagic development in *C. nana* appears to result in a patchy distribution of postlarvae on the bottom, and an uneven, nonrandom distribution of young nudibranchs on the *H. echinata* colonies.

9. *Cuthona nana* does not kill the *H. echinata* colonies it preys upon, but only crops some of the polyps before leaving the colony to find a mate or deposit eggs. Lost polyps are subsequently regenerated.

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THE
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INCREASE IN RANGE OF TEMPERATURE TOLERANCE BY ACCLIMATION IN THE COPEPOD *EURYTEMORA AFFINIS*

Reference: *Biol. Bull.*, **154**: 177–187. (April, 1978)

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Adaptation to temperature change is an obvious requirement for the survival of a temperate species whose habitat is subject to seasonalities. The response of a population to environmental stress depends on the time and intensity of the stress. Slobodkin and Rapoport (1974) suggest that if one level of response (for example physiological) is sufficient to meet the environmental challenge, the next level (for example change in gene frequency) need not be invoked.

The calanoid copepod, *Eurytemora affinis* (Poppe), found in the Chesapeake Bay in temperatures ranging from 0 to 30° C, has many generations per year; hence, it could adapt to this variability in temperature either on an individual or population level. If the range of individual tolerance were sufficient, the species might not need to change genetically through the year. Bradley (1975) found that individual copepods could tolerate the 0–30° C range for short periods.

The question addressed in this paper is whether individuals can become adapted physiologically to a wider range of temperatures (acclimation). This paper also explores the effects of temperature, salinity, sex and stage of development on acclimation to high temperature, the effects of temperature and sex on acclimation to low temperature, and the relationship between tolerances to high and low temperature.

Materials and Methods

Specimens of *Eurytemora* used in some of the experiments were descended from animals collected from Bear Creek in the upper Chesapeake Bay in winter. In other experiments, on salinity effects and on survival, the animals originated in the middle reaches of the Patuxent River, Maryland, in late spring. The reason for

the different collection sites was the availability of specimens. Only one source of animals was used for each experiment. The experiments on heat tolerance, except those on salinity effects, were done in water from the Patuxent River with near zero salinity. Those on cold tolerance were done in 5‰ water from Bear Creek. Salinity was measured with a refractometer.

Acclimation is defined in this paper as the increase in temperature tolerance of individuals following exposure to a temperature closer to the extreme temperature, whether high or low.

Tolerance to high temperature was measured using the shock-recovery assay (Bradley, 1975), permitting data to be obtained on individual copepods. The measurements were highly repeatable with test-retest correlations of around 0.8, and were also closely related to survival time at high temperatures (Bradley, 1976). Single animals in 2 ml of water in test-tubes were immersed in an aquarium held constant at 34.5° C using a heating-stirring unit. No temperature gradients in the aquarium were detected, and the temperature in the test-tubes reached 34.5° C within 90 seconds. Time to succumb (TS), or enter a coma, and time to recover (TR) were observed during a 30 min exposure of all test animals to 34.5° C. Animals were considered comatose when rotating and agitating the vial failed to rouse them. Recovery was noted at the first movement. The assay period was 30 min, and the measures of tolerance were combined in an index $30 + TS - TR$, which could range from 0 to 60, the higher number indicating the greatest tolerance. All animals were exposed to the 34.5° C temperature for 30 min, and those failing to recover while exposed to this temperature were given a TR of $30 - TS$, the index becoming 2TS in these cases. No attempts were made to distinguish between coma and lethality, but both TS alone and the index $(30 + TS - TR)$ were closely related to survival time at 30° C and higher (Bradley, 1976).

Similar methods were used to test tolerance to low temperatures. In the latter case, animals were placed in an aquarium at 0.5° C, and all were removed after 10 min (whether succumbed or not). A majority of animals did become comatose before 10 min, and recovery could be more easily observed at room temperature. Animals not succumbing at all were arbitrarily scored 50, the remainder using the index described above. The next largest score was 40 (animals succumbing at 10 min, recovering immediately), since animals became comatose at or before 10 min or not at all, so setting the maximum tolerance at 50 rather than 60 reduced the discontinuity of tolerance scores.

In the present study 12 animals and all treatment groups were included in each run. Each set of experiments was done by the same observer. In the case of heat tolerance, variance between replicate runs was treated as error variance, since no interactions between treatment and run were detected. The net effect of ignoring runs was to make the tests of variables more conservative because of the increased error variance. In the case of cold tolerance, differences between runs were quite large, due to difficulties in controlling the low temperature at exactly 0.5° C. So run variance (and interaction) were included in the analyses of variances of cold tolerances.

Tolerance was also observed as longevity in two constant temperatures and in a

slowly increasing temperature. Acclimation was observed in these cases as the increase in duration of activity in the test temperature regime of animals previously exposed to an intermediate temperature. Half the animals tested in increasing temperatures were maintained at 24° C for 24 hr before the temperature was raised from 24° C to 31° C in 30 min. The remainder were kept at 15° C. Only males were included in these experiments in increasing temperatures. All the animals were then exposed to a temperature of 31° C initially, which was increased 1° C every 30 min. The test animals were continuously monitored and the times when each animal succumbed and could not be roused were noted. In this case, the end-

TABLE I

Increased temperature tolerance of animals raised at 10° C and exposed to 18° C and 24° C for three periods of time. Body of table gives mean tolerances (in min) to high temperature measured by the shock-recovery assay described in Methods.

Mean temperature tolerances

		Time of exposure		
		2 day	4 day	7 day
Exposure temperature				
10° C (control)	♀	7.5	9.3	5.8
	♂	5.8	7.0	5.8
18°C	♀	14.0	12.8	7.3
	♂	9.0	8.8	10.8
24° C	♀	29.0	40.8	36.6
	♂	21.0	22.8	16.5
		8 animals per mean, 144 total		

Variance analyses for each sex

	Mean squares	
	Females	Males
Time of exposure		
Days at 10° C	24.5	8.3
18° C	63.2	19.0
24° C	284.3	166.4*
Temperature of exposure		
18° C, 24° C *vs.* 10° C	3792.5*	1190.3*
18° C *vs.* 24° C	7575.2*	1344.1*
Within subclass	159.7	21.0
Total variances	312.3	57.1

* $P < 0.01$.

TABLE II

Increased high temperature tolerances of males raised at 20° C compared to 10° C and exposed to three temperatures for two days. Body of table gives tolerances measured by the shock-recovery assay. All animals recovered when raised at 20° C and two of 24 recovered when raised at 10° C.

	Mean temperature tolerance	
	Raised at 10° C	Raised at 20° C
Exposure temperature		
10° C	6.7	31.7
18° C	10.0	43.4
24° C	25.0	47.3
	8 animals per mean	9 animals per mean

	Variance analyses	
	Mean squares	
	Raised at 10° C	Raised at 20° C
Exposure temperature		
18° C, 24° C *vs.* 10° C	622.0*	1117.9*
18° C *vs.* 24° C	900.0*	68.4
Within subclass	39.4	102.5
Total variances	102.2	140.7

* $P < 0.01$.

point may not have been death itself, but tantamount to death, since recovery did not occur in the increasing temperature.

The relationships between heat and cold tolerance were measured as correlations between observations on the same animals assayed for cold tolerance and heat tolerance 5–6 hr apart on one day. Both assays were repeated the next day, thus two assays for heat tolerance and two for cold tolerance were done on each animal.

When the data in each of the experiments were analyzed, the sexes were treated separately. This was done because of the observed differences between the means and variances of temperature tolerances of the two sexes.

RESULTS

Acclimation to increased temperature occurs in *Eurytemora affinis* (Table I). The set of animals exposed to 18° C or 24° C prior to testing were significantly more tolerant than those kept at 10° C, their rearing temperature. The largest effect was clearly in animals exposed to 24° C, since they were significantly more tolerant than those exposed to 18° C. Time of exposure had a relatively small effect, although it was significant in males exposed to 24° C. Females appeared to acclimate more than males, even proportionally. This can be seen from the changes

in mean tolerance, especially at 24° C. Furthermore, of the 16 animals (out of 144) recovering within 30 min of the temperature shock or failing to succumb at all, 14 were female and 2 were male. Females also seem to be more subject to environmental influences other than exposure temperatures, as indicated by the variances within treatments. These variances were 159.7 for females and 21.0 for males. The greater variance between females is consistent with their greater response to exposure temperature.

By comparison with the low rates of recovery in animals raised at 10° C (above), animals raised at 20° C almost always recovered from the temperature shock (Table II). In this experiment on rearing temperature, progeny from the same stock as above were raised at 20° C and tested after exposure to 10, 18, and 24° C as before. Only males were tested in this case. The results in Table II clearly show the increase in tolerance of the animals raised at 20° C, regardless of

TABLE III

Increased high temperature tolerance of animals raised at 10° C and exposed to 18° C and 23° C for 3 hr and 20 hr. Body of table gives tolerances measured by the shock-recovery assay.

		Mean temperature tolerance	
		Exposure time	
		3 hr	20 hr
Exposure temperature			
10° C (control)	♀	10.0	12.0
	♂	9.0	13.0
18° C	♀	11.5	20.5
	♂	9.5	14.0
23°C	♀	15.0	51.0
	♂	15.5	17.0
		4 animals per mean	4 animals per mean

	Variance analyses for each sex and exposure time			
	Mean squares			
	♀ at 3 hr	♂ at 3 hr	♀ at 20 hr	♂ at 20 hr
Exposure temperatures				
23° C, 18° C *vs.* 10° C	28.2	32.7	1504.2**	10.7
23° C *vs.* 18° C	24.5	72.0*	1860.5**	18.0
Within subclass	7.9	11.8	83.5	63.9
Total variances	11.2	19.2	374.2	54.9

* $P < 0.05$.
** $P < 0.01$.

TABLE IV

Increased high temperature tolerance at higher salinities following acclimation at two temperatures and two salinities for 24 hr. Body of table gives tolerances measured by shock-recovery assay. Animals in Experiment C were shocked at 33.5° C; the others at 34.5° C.

Mean temperature tolerances

	Experiment								
	A			B			C		
	Exposure Temp.	Salinity		Exposure Temp.	Salinity		Exposure Temp.	Salinity	
		0‰	13‰		0‰	13‰		0‰	13‰
♀	20° C	14.8	30.3	13° C	12.3	27.8	11° C	14.4	18.4
♂		11.0	23.8		8.3	12.3		5.8	9.4
♀	24° C	19.8	27.8	23° C	13.2	24.2	23° C	22.6	33.0
♂		13.8	18.8		8.0	11.5		11.6	26.2
	4 animals per mean			6 animals per mean			5 animals per mean		

Variance analyses for each sex

	Mean squares					
	Experiment					
	A		B		C	
	♀	♂	♀	♂	♀	♂
Between temperatures	6.3	5.0	12.0	2.0	649.8*	638.4*
Between salinities	552.3**	315.1**	1053.4**	84.4	259.2	414.1*
Interaction	56.3	60.1	30.4	0.4	51.2	151.3
Within subclass	58.1	28.0	76.0	32.0	90.0	86.9
Total variance	87.5	47.8	113.7	31.6	126.3	136.5

* $P < 0.05$.
** $P < 0.01$.

what temperature they were exposed to later. Thus, acclimation, and adaptation to an elevated temperature, can occur during development, and the effect of a low temperature during development cannot be completely overcome by subsequent exposure to a higher temperature.

Having shown that acclimation occurred, even during development, the next question was whether a short exposure time would suffice. Table III shows that as little as 3 hr at 23° C and certainly less than 24 hr were required for acclimation to occur. There is some evidence that males acclimate earlier and less than do

females. This was indicated also in the earlier data in Table I. The same animals were tested each time, and the correlation between measured tolerances was 0.48 ($P < 0.01$).

All the experiments reported so far were done in water with no detectable salinity. Temperature tolerance was shown earlier to increase when animals collected at 0‰ were placed in higher salinity (Bradley, 1975), so it seemed reasonable to test for an effect of salinity on acclimation. The results of three experiments are shown in Table IV. None of the three experiments gave much indication that acclimation was influenced by salinity. In the first two experiments (A and B) the shock temperature was too high; but even when there was sufficient variation in tolerance (C), no evidence of interaction between exposure temperature and salinity was found.

The data in Table IV cannot be directly compared to those in Tables I and II, since the source of the animals differed and the experiments in Table IV were done almost 9 months later. However, tolerances of females were again higher, as were the variances among females within treatment as discussed earlier.

Additional experiments on acclimation to high temperatures were done using two other criteria, survival times at constant high temperatures and times until complete inactivity of animals in slowly increasing temperatures. In the former experiments, females survived longer than males at both 32° C and 33° C, but there was no evidence of acclimation in animals exposed to 25° C for 24 hr. Mean survival times of females ranged from 9.3 to 13.0 hr and of males ranged from 3.7 to 9.5 hr, depending mainly on test temperature.

There was evidence of acclimation, when time to inactivity was the criterion. Animals exposed to 24° C for 24 hr remained active significantly longer (146–151 min) than did animals kept at 15 hr (74–103 min), when tested in a temperature increasing slowly from 31° C. The temperatures at immobilization were 35.3 to 35.7° C and 32.9° C, respectively.

The reasons for the inconsistency between these two experiments is not clear. In the second set of experiments the total time of observation was less than 2.5 hr, allowing a more accurate measurement of longevity. The stress due to temperature probably was greater in the second experiments, perhaps allowing more accurate expression of the effects of acclimation.

Acclimation to decreased temperature also occurs in *Eurytemora affinis,* although less rapidly than to increased temperatures. Two sets of data were obtained, one set from animals tested for tolerance following exposure to 4, 10, and 15° C for 24 hr and a second set from different animals exposed to 4, 10, and 15° C for 60 hr (Table V). There is clear evidence for acclimation to low temperatures, especially after 60 hr. There also seems to be more acclimation in males by 24 hr and more in females by 60 hr, which is consistent with the inference from Tables I and II that males acclimate earlier and less than do females. However, the sexual dimorphism in degree of acclimation was much less for cold tolerance than for heat tolerance. Finally, the four variances (mean squares) within run and exposure temperature in Table V taken as crude measures of physiological variance, are consistent with the more immediate and smaller flexibility of males.

TABLE V

Increased low temperature tolerance of animals raised at 15° C and exposed to 10° C and 4° C; one set exposed for 24 hr and another for 60 hr. Indices of tolerance are in body of table.

Mean temperature tolerance

		Exposure time	
		24 hr	60 hr
Exposure temperature			
15° C (control)	♀	23.6	31.8
	♂	20.9	22.8
10° C	♀	24.6	35.4
	♂	27.3	23.8
4° C	♀	28.3	50.0
	♂	29.2	35.3
		16 per mean	16 per mean

Variance analyses for each sex

	Mean squares			
	24 hr		60 hr exposure	
	♀	♂	♀	♂
Exposure temperatures				
4° C *vs.* 10° C, 18° C	187.1*	276.8*	3381.4**	1928.0**
10° C *vs.* 15° C	8.0	331.6*	207.1	9.1
Runs	2084.8**	522.4**	137.1	775.1**
Runs × temps.	349.9**	121.5*	231.4	125.2
Within subclass	29.5	51.3	138.6	75.8
Total variance	204.4	101.1	207.2	160.2

* $P < 0.05$.
** $P < 0.01$.

One question raised by these results is whether hot and cold tolerances are similar or different characters. Earlier indications (Bradley, 1975) were that animals resistant to high temperatures tended to be resistant to cold temperatures. To test the relationship more formally, tests of heat and cold tolerances were done twice on 24 animals on successive days. All the correlations except one were positive, eight of twelve were significant. Test-retest correlations (heat-heat, cold-cold) averaged 0.45 for males and 0.70 for females, which were only slightly higher than the average correlations between cold and heat tolerances (0.32 and 0.54, respectively). Hence, there is no evidence that tolerance was biased in one direction in each animal. In other experiments where both tolerances were measured,

correlations were sometimes low but were never negative when averaged over the experiment.

Discussion

Acclimation to high temperatures occurs probably quite quickly (<24 hr), being completed in a few days, perhaps more slowly and certainly more extensively in females than in males. Exposure to increased temperature during development also leads to increased tolerance in adults, beyond what could be achieved by exposure beginning at the adult stage. Apparently, changes affecting temperature tolerance occur during development and are only partially reversible in the adult stage. Although salinity affects temperature tolerance, there is no evidence that acclimation is greater at higher salinity. Acclimation to low temperatures also occurs, but less rapidly than to high temperatures. Effects of sex on acclimation to cold temperatures are also less marked.

The results on acclimation to high temperatures agree with the observations of others. Levins (1969) found that most of the thermal acclimation of *Drosophila* species took place in the first 12 hr. Bowler (1963a) also found that acclimation in the crayfish, *Astacus pallipes*, occurred rapidly and was completed in about two days. Vernberg and Moreira (1974) reported that males of the copepod species *Euterpina acutifrons* had a lower metabolic rate at 15° C than females when both had been acclimated at 25° C. However, males were smaller and the metabolic (respiration) rates were not adjusted for body size. According to the data of McLeese (1956), from a study of the effects of salinity, acclimation, and oxygen tension on lobster survival, there appeared to be little effect of salinity on acclimation.

Data in this study also suggest that acclimation (at least to high temperature) is more easily detected using coma tolerance rather than survival as the criterion. Survival time was not increased following exposure to 25° C, compared with 15° C. Where coma tolerance was the criterion, whether in a shock temperature (Tables I–IV) or in slowly increasing temperature, the data indicate that significant acclimation did take place. Heinle (1969) also reported that thermal tolerances of *E. affinis*, measured as survival in constant environments, was not increased in animals exposed to 20 or 25° C, compared with animals exposed to 10 or 15° C. Hamby (1975) found that acclimation of a marine snail, *Littorina littorea*, significantly shifted the temperature at which the animal entered heat coma but affected the lethal temperature very little. He concluded that the nervous system of *Littorina* was most vulnerable to thermal extremes, as is the case with other poikilotherms (Prosser, 1973).

The influence of acclimation on the nervous system (and so on coma tolerance) is indicated by the results of Baldwin and Hochachka (1970) who showed that different variants of acetylcholinesterase were present in the brains of trout acclimated to different temperatures. Other reported responses to exposure to higher temperature were lowered temperature-specific respiration rates in a toad (Fitzpatrick and Atebara, 1974), lower temperature-specific respiration rates and heart rates in limpets (Markel, 1974), and alterations in enzyme systems donating energy required in the functions of tissue "cation pumps" (Bowler, 1963b).

Having noted the agreement with other results and described possible mechanisms, the question remaining is how such ability to acclimate (individual flexibility) is maintained (or how it arose), when no individual copepods are exposed to the whole range of temperatures in the Chesapeake Bay (0 to 30° C). Daily fluctuations in temperature, together with diurnal migration may be sufficient for physiological flexibility to be an important trait, which is maintained by natural selection. Another (complementary) hypothesis is that tolerances to high and low temperatures are much the same trait genetically. They appear to be related phenotypically, as shown previously (Bradley, 1975, 1976) and reported again in this paper. Thus, the flexibility observed may be the result of natural selection for tolerance to extremes. Such selection would be relaxed in intermediate temperatures, but never reversed. One problem with this explanation is that in several experiments large additive genetic components of variance in temperature tolerance have been observed, which should not be the case if selection is always in the same direction (Bradley, 1978).

Even if there were a single explanation for the flexibility observed, the reasons for the greater flexibilities or acclimation in females are not at all obvious. Female specimens of *Eurytemora* do not store sperm much beyond the first egg sac (Heinle and Flemer, 1975), although such storage may occur occasionally. If males are required for each mating, and there is only a short interval between fertilization and hatching, there is no obvious reason why males should be less tolerant and less flexible than females at high temperatures.

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Summary

The copepod, *Eurytemora affinis,* was tested for its ability to recover from short exposures to a high temperature (temperature tolerance). Animals kept at a warm temperature for several hours or days before the test increasd in tolerance (acclimation). Females showed higher tolerance and acclimation than males. Temperature tolerance was greater at a higher salinity (13‰ *vs.* 0‰), but acclimation was not. Analogous tests were done at low temperatures. Acclimation to cold temperature also occurred, but more slowly. Sexual differences were less marked than for heat tolerance. When tested on the same animals, heat and cold tolerances seemed to be positively related traits.

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THE INFLUENCE OF CONSTANT AND CYCLIC ACCLIMATION TEMPERATURES ON THE METABOLIC RATES OF *PANOPEUS HERBSTII* AND *UCA PUGILATOR* [1, 2]

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Temperature is one of the major physical factors influencing the metabolic rates of intertidal invertebrates (Newell, 1975; Vernberg and Vernberg, 1972). Most previous studies on the respiratory metabolism of intertidal organisms have been conducted at constant temperatures and have utilized organisms acclimated to constant temperatures. Although these studies have led to many insights into the influence of temperature on respiratory adaptations, they may not describe the metabolic response of animals subjected to fluctuating thermal environments typical of those normally encountered in nature. Hence, a question can be raised concerning the value of the previous data from these studies at constant temperature in the analysis of the ecological energetics. To understand the significance of respiration in energy transfer in an ecosystem requires accurate estimates of oxygen consumption rates. This paper reports the results of a study on the comparative influence of constant and cyclic acclimation temperatures on the respiratory metabolism of two intertidal crabs which are common in South Carolina estuaries, the mud crab, *Panopeus herbstii* (Milne-Edwards), and the fiddler crab, *Uca pugilator* (Bosc).

Published data dealing with the influence of cyclic thermal environments on the physiology of marine invertebrate animals is limited, especially for respiratory metabolism. Earlier Kahn (1965) studied the effects of cyclic temperature on the growth of copepods while observations on larval crab growth under cyclic thermal regimes were reported by Costlow and Bookhout (1971), Christiansen and Costlow (1975), and Sastry and Vargo (1977). The influence of cyclic temperature on survival of crab larvae (Costlow and Bookhout, 1971; Sastry and Vargo, 1977) and of grass shrimp (Thorp and Hoss, 1975) has been reported. Sastry and Vargo (1977) recorded the metabolic response of larval crabs to cyclic temperatures, as did Humphreys (1975) for the nonmarine wolf spider. Widdows (1976) and Bayne, Widdows, and Worrall (1977) have reported on the influence of cyclic temperatures on the physiology of a bivalve, *Mytilus edulis*. Some investigators have reported that the metabolic response of marine animals is different depending on whether the temperature was increasing or decreasing (Van Winkle, 1969, for the mud snail, *Nassarius obsoleta;* Vernberg and Vernberg, 1966, for the fiddler crab, *Uca pugnax*).

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Materials and Methods

Mud crabs, *Panopeus herbstii*, were collected from intertidal oyster beds in the tidal creeks of the North Inlet Estuary near Georgetown, South Carolina. Fiddler crabs, *Uca pugilator*, were collected from nearby salt marshes where they are abundant on sand beaches. These species were selected because each has distinctive ecological requirements and both are present in great numbers. Thus, it is possible to compare responses of intertidal crabs from different habitats to determine any commonality of response by intertidal temperate zone animals.

The crabs were brought into the laboratory, washed in salt water, and placed in numbered partitioned plastic boxes containing 35‰ sea water. Then the boxes of crabs were kept in Revco Environmental Chambers in which light and temperature could be controlled. The crabs were fed every two days, and, after feeding, the crabs were placed in a clean box with fresh sea water. After seven days of acclimation to a constant temperature, the respiration rate of the crabs was determined using a Gilson Differential Respirometer. These results served as the baseline value against which measurements determined under fluctuating conditions were compared. Oxygen consumption was computed as $\mu l\ O_2/(hr \cdot g$ dry weight), corrected to standard temperature and pressure.

After completing the initial metabolic determinations, the environmental chambers were programmed so that the animals would experience a once daily cyclic temperature regime where the previous constant acclimation temperature was the maximum temperature and the minimum temperature was 10° C. A daily 10° C thermal change was selected as this degree of fluctuation is not uncommonly experienced by these animals throughout much of the year. The change in temperature followed a square wave with about an hour of elapsed time before a new stabilized temperature was reached. After thermal cycling had started, respiration rates were measured on days 3, 6, 9, and 15–22. At the end of the experiments the crabs were dried in an oven at 105° C. Although different photoperiods have been shown to influence the metabolic response of crabs (Dehnel, 1958), the photoperiod regime was the same for animals exposed to both constant and fluctuating thermal experiments.

For simplicity of experimental design and to compare the relative effect of only thermal regimes, photoperiods were selected which corresponded to those the

Table I

The various experimental conditions and number of organisms for each experiment.

Cyclic temperature range (° C)	Photoperiod (L:D)	Constant acclimation temperature	Cyclic acclimation (days)	N
5–15 (*Panopeus*)	8:16	15	3, 6, 9, 16	27
10–20 (*Panopeus*)	12:12	20	3, 6, 9, 19	25
15–25 (*Panopeus*)	14:10	25	3, 6, 9, 16	25
20–30 (*Panopeus*)	14:10	30	3, 6, 9, 15	27
5–15 (*Uca*)	8:16	15	3, 6, 9, 16	28
10–20 (*Uca*)	12:12	20	3, 6, 9, 22	24
15–25 (*Uca*)	14:10	25	3, 6, 9, 18	25
20–30 (*Uca*)	14:10	30	3, 6, 9, 15	23

organisms typically experience at the different thermal ranges. For example, low temperatures normally occur during periods when the day length is short; while, in contrast, long days and high temperatures are usually coincident. A summary of the experimental conditions is given in Table I. The standard statistical techniques of Steel and Torrie (1960) were used to determine means, standard errors, and confidence intervals.

Results

Panopeus herbstii showed no statistically different change in its respiratory response at the low and high temperature ranges of 5–15° C and 20–30° C, with oxygen consumption rates the same after constant and cyclic acclimation (Fig. 1 and Table II). However, the oxygen consumption response to the middle acclimation temperatures (10–20° C and 15–25° C) varied with acclimation time. After 19 days exposure to cyclic temperature acclimation, the 10–20° C group was consuming about the same amount of oxygen as it did after acclimation to constant temperature. However, a statistically significant ($P < 0.001$) decrease in metabolic rate occurred between day 6 and day 9 followed by a significant increase between day 9 and day 19. These responses resulted in a U-shaped metabolic curve. Animals exposed to the fluctuating temperature range of 15–25° C showed a significant metabolic decrease ($P < 0.05$) by day 3 and another decrease ($P < 0.01$) by day 6. The metabolic rate remained constant after this time until day 16 when the experiment was discontinued.

After exposure to three temperature ranges (5–15° C, 10–20° C, and 20–30° C) *Panopeus* had the same oxygen consumption rate before and after exposure to the cyclic thermal acclimation period. In contrast, those crabs exposed to 15–25° C had a significantly lower metabolic rate ($P < 0.001$) than animals exposed to a constant temperature of 25° C (Fig. 2). Animals exposed to cyclic temperatures

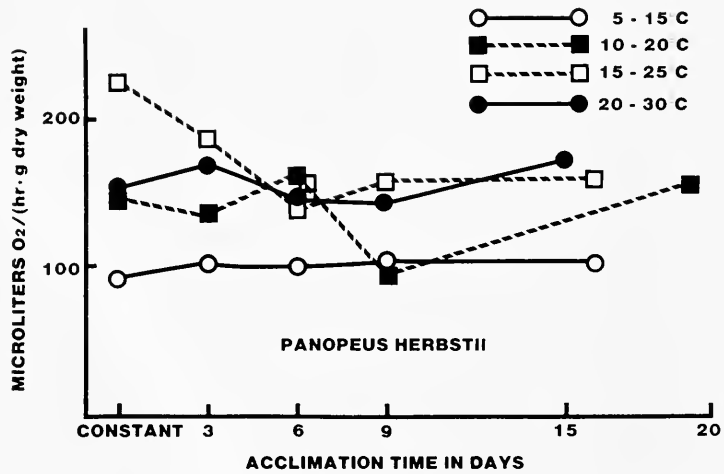

Figure 1. Oxygen consumption of *Panopeus herbstii* after constant acclimation (constant) and after varying lengths of time under the influence of cyclic temperatures.

TABLE II

Statistical analysis of metabolic-temperature responses of Panopeus *and* Uca *exposed to fluctuating temperatures for different periods of time (NS indicates that means are not significantly different).*

Panopeus

Days	Temperature range (° C) 5–15	10–20	15–25	20–30
0 *vs.* 3	NS	NS	$P < 0.05$	NS
3 *vs.* 6	NS	NS	$P < 0.01$	NS
6 *vs.* 9	NS	$P < 0.001$	NS	NS
9 *vs.* last	NS	$P < 0.001$	NS	NS

Uca

Days	Temperature range (° C) 5–15	10–20	15–25	20–30
0 *vs.* 3	NS	$P > 0.001$	NS	NS
3 *vs.* 6	NS	$P > 0.05$	NS	NS
6 *vs.* 9	$P > 0.01$	NS	NS	NS
9 *vs.* last	$P > 0.001$	NS	$P > 0.001$	$P > 0.05$

for 16–19 days exhibited an excellent degree of metabolic-temperature regulation in that the metabolic rate was the same at 20°, 25°, and 30° C. In contrast, when exposed to constant temperature, the metabolic-temperature curve showed an increase in metabolism with increasing temperature until a high stressful thermal

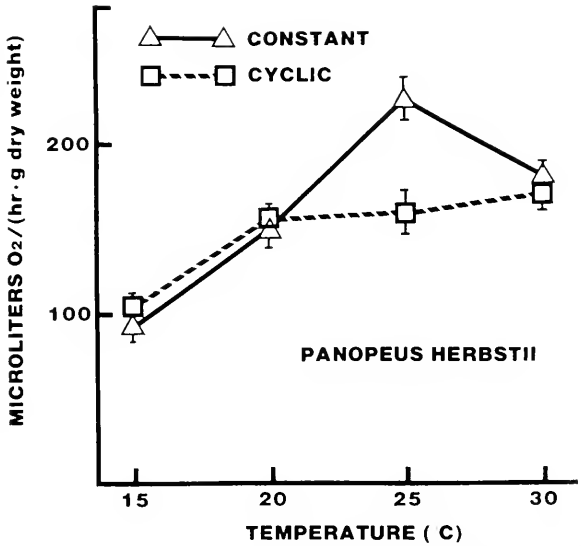

FIGURE 2. A comparison of the oxygen consumption of *Panopeus herbstii* after constant and cyclic acclimation temperatures. Vertical brackets are the standard errors of the means.

TABLE III

Q_{10} values for metabolic rates of Panopeus *and* Uca *exposed to constant and cyclic thermal regimes.*

Thermal temperatures	Constant thermal regime		Cyclic thermal regime	
	Panopeus	*Uca*	*Panopeus*	*Uca*
15–20	2.6	1.5	2.3	1.2
15–25	2.5	1.1	1.6	1.2
20–25	2.3	1.0	1.1	1.2
20–30	1.1	1.2	1.1	1.5
25–30	<1.0	1.5	1.2	1.9

point (30° C) was reached and the rate decreased. *Panopeus herbstii* is killed at temperatures slightly over 30° C. These responses are better expressed as changes in Q_{10} values (Table III), in that the Q_{10} is less than one over the range of 25–30° C, but at all other temperature ranges the Q_{10} is greater than one. The Q_{10} values of animals maintained at constant temperature are higher than those of animals subjected to a cyclic thermal regime, except for those at thermal ranges involving 30° C. Of particular interest, animals exposed to a cyclic thermal regime did not show the same level of metabolic depression as did animals maintained at constant temperature. This response could have survival value to the *Panopeus* population.

The oxygen consumption of *Panopeus herbstii* with response to size and oxygen concentration has been investigated by Leffler (1973). This study utilized constant conditions, but a similar measurement technique. He showed that oxygen consumption was influenced by size following the general relationship expressed by von Bertalanffy (1957) and Hemmingsen (1960). Also, oxygen consumption in *P. herbstii* dropped in rough proportion to the oxygen level of the medium. Neither size nor oxygen concentration factors should have influenced our findings since the crabs were of approximately the same size (0.5–2.0 grams dry weight), and the crabs were partially exposed during the metabolic determinations in the Gilson respirometer. The oxygen consumption values reported by Leffler (1973) were of the same order of magnitude as those determined in our study, but exact conversion from Leffler's to our work is impossible since he used a lower salinity (22‰) than we (35‰). Recently Dimock and Groves (1975) have shown that the oxygen consumption of *Panopeus herbstii* is influenced by temperature and salinity combinations. Their observations of oxygen consumption are slightly lower than ours at the two most comparable temperature and salinity combinations (10° C and 30‰; 25° C and 30‰). The reduced metabolic values of Dimock and Groves' data are probably the result of using a full range of size classes, thus a larger average size being used in their studies.

The respiratory responses of *Uca pugilator* to increasing cyclic temperature acclimation time were different than those of *Panopeus* and were highly variable (Fig. 3). Interspecific differences were noted in that the fiddler crabs had the higher metabolic rate at each temperature. This result is consistent with the earlier finding of Vernberg (1969) that crabs which exhibit a high level of locomo-

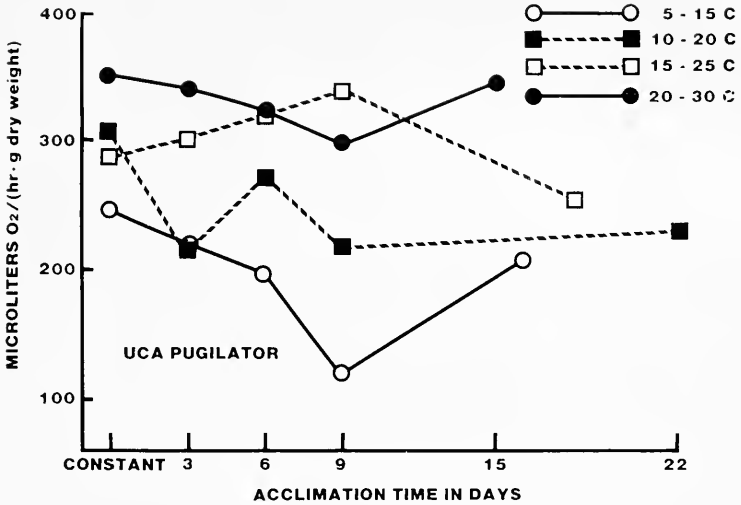

FIGURE 3. Oxygen consumption of *Uca pugilator* after constant acclimation (constant) and after varying lengths of time under the influence of cyclic temperatures.

tor activity tend to have a higher metabolic rate than a more lethargic species. *Uca* is an active crab at low tide when it can be seen darting about, while *Panopeus* is more secretive, hiding among the oyster shells. Also, some specimens of *Uca* are more temperature tolerant than *Panopeus;* an exposure to 30° C is less stressful on their metabolic response.

Following different exposure times to fluctuating temperatures, significant differences in means of metabolic rate were observed at each thermal regime (Table II). In contrast, significant differences occurred at only two of the four temperature ranges for *Panopeus*. However, when comparing the initial metabolic rate with the rate at the end of the exposure to fluctuating temperatures, only at 20° C was there a statistically significant difference with the rate being lower. After extended cyclic temperature acclimation, specimens of *Uca* acclimated to the warmest range (20–30° C) consumed significantly more oxygen than the other three groups which had similar rates (significant at the 95% confidence level). This trend suggests that specimens of *Uca* are less sensitive to thermal change than are those of *Panopeus*. Further evidence for this statement is that the Q_{10} values for *Uca* tend to be lower than those for *Panopeus* (Table III).

A graphic representation of the oxygen consumption rates of crabs maintained at constant and cyclic temperature is shown in Figure 4. The metabolic rate of *Uca pugilator* showed significant differences between constant- and cyclic-temperature-acclimated crabs at 20° C (95% level), 25° C (92% level), and 15° C (93% level), but not at the other temperature of 30° C. The metabolic response of *Panopeus* varied significantly only at 25° C. In the case of both species, the oxygen consumption rate was lower in animals subjected to cyclic temperatures.

The metabolic responses of *Uca pugilator* have been extensively investigated by Vernberg (1969). Constant-temperature-acclimated specimens of *U. pugilator* from the present study exhibited the same acclimation curve and metabolic rates as

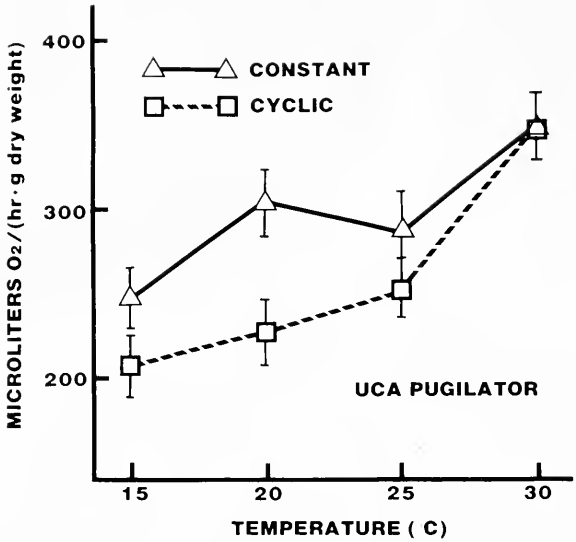

FIGURE 4. A comparison of the oxygen consumption of *Uca pugilator* after constant and cyclic acclimation temperatures. Vertical brackets are the standard errors of the means.

crabs from North Carolina. The cyclic-temperature-acclimated specimens of *U. pugilator* showed a depressed response compared to that of constant-temperature-acclimated crabs.

DISCUSSION

The experiments described here offer evidence that the metabolic rate of intertidal organisms, as measured by oxygen consumption, is influenced differently by constant and cyclic temperature acclimation regimes. The evidence is most striking in *Panopeus* exposed to the 15–25° C cycle and *Uca* exposed to 5–15°, 10–20°, and 15–25° C cycles. Other workers have demonstrated differences in various physiological response that could be correlated with variations in the type of thermal acclimation regime.

Thorp and Hoss (1975) determined that cyclic temperatures decreased survival of the grass shrimp, *Palaemonetes pugio* and *P. vulgaris*, at low salinities (5‰) and low cyclic temperatures (7–13° C) when compared to shrimp kept at constant temperatures under the same salinity. In contrast, the pupfish, *Cyprinodon nevadensis*, acclimated to cycling temperatures demonstrated a greater tolerance to both high and low temperature than animals acclimated to a constant temperature (Feldmeth, Stone, and Brown, 1974). This increased scope of thermal tolerance could have survival value to animals occupying habitats which characteristically are subjected to wide daily and seasonal thermal changes.

Cyclic temperatures may influence survival and development of larvae. Costlow and Bookhout (1971) found that survival of the larval mud crab, *Rhithropanopeus harrisii*, was about the same for a cycled temperature as for a constant temperature equal to the mean of the cycled temperatures over the range of 10–30° C, but

larvae maintained at a cycle of 30–35° C survived better than those at either 30° or 35° C. The time required to complete metamorphosis at the warmer cycles was influenced by the high temperature in the cycle at all salinities tested (Christiansen and Costlow, 1975). However, the results of these two studies at higher temperatures are different, possibly the result of variation in survival of crab larvae from hatch to hatch. More recently, Sastry and Vargo (1977) found that larvae of a decapod crustacean, *Cancer irroratus*, showed a greater survival rate when reared under a suitable amplitude and rate of temperature change than larvae maintained at comparable constant temperatures.

Cyclic temperature may influence sublethal responses of organisms, but not always in an apparent, predictable way. Hoffman (1974), working with crickets, reported that varying day-night temperature cycles do not accelerate physiological functions, except for life span and egg production.

Thorp and Hoss (1975) determined the oxygen consumption rate of two species of grass shrimp after acclimation to constant temperatures (7° and 10° C) and cyclic temperatures (7°–13° C) at salinities of 5, 20, and 35‰. Both species of shrimp utilized more oxygen when acclimated to constant temperature (10° C) and 35‰ than cyclic temperatures of 7–13° C and 35‰. This result is similar to our work in that our crabs were acclimated to 35‰, and when differences in oxygen consumption were observed, cyclic temperatures depressed oxygen consumption.

Unlike our results, the respiration rate of spiders maintained on a cyclic temperature regime was higher than animals kept at a constant temperature (Humphreys, 1975). This response may be correlated with an increase in growth and development rates of this species. However, since the resting metabolic rate is higher when kept on cyclic thermal regimes, the organisms must be more efficient in extracting energy from their food or they must eat more in order to grow. *Mytilus edulis*, a bivalve, utilizes a different strategy in adapting to cyclic temperatures. It reduces the amplitude of both oxygen consumption and filtration rate (Widdows, 1976). Specimens of *Panopeus* and *Uca* respond metabolically in a similar manner, at least at intermediate temperatures. One result of reducing the standard metabolic rate is to conserve energy which can be used for other functional activities necessary for an organism to successfully compete and survive. This is particularly important for these crabs in that the cyclic thermal ranges at which they demonstrate reduced rates of oxygen uptake are those in which these organisms are most active during most of the year.

Based on the results of this study it seems that any previous estimate of the role of oxygen consumption in energy budgets of a species and/or a community might be in error, unless the influence of cyclic temperature on respiration has been determined.

We would like to thank Bill Murtiashaw for his valuable assistance in the metabolic measurements and acclimation procedures. Stuart Stevens and Bill Johnson provided data processing assistance.

Summary

The comparative influence of acclimation to constant and cyclic temperatures on the metabolic rates of the mud crab, *Panopeus herbstii*, and the fiddler crab, *Uca pugilator*, was observed. Although interspecific differences were observed, cyclic acclimation temperatures significantly depressed oxygen consumption in the 15°–25° C temperature range in both species when compared to rates of animals subjected to constant acclimation rates. Since this depression of metabolic rates occurs over that portion of the yearly temperature range within which the animals are most active, it is suggested that these organisms utilize energy more efficiently when subjected to natural cyclic temperature conditions than when subjected to constant temperature environments. This difference in metabolic data would be significant in analyzing the role of the yearly energy budgets of crabs in ecosystem energetics.

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MULTIPLE NUCLEI DURING EARLY OOGENESIS IN *FLECTONOTUS PYGMAEUS* AND OTHER MARSUPIAL FROGS

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In the great majority of amphibians yet investigated the occurrence of oocytes with more than one nucleus or more than a single meiotic figure is exceptional (Humphries, 1956, 1966; Parmenter, Derezin and Parmenter, 1960; Humphrey, 1963). In the tailed frog *Ascaphus truei*, however, Macgregor and Kezer (1970) found that oogenesis regularly involves oocytes with eight nuclei, all but one of which disappear before the final stages of oogenesis. When a related species, *Leiopelma hochstetteri*, was investigated by Robinson, Stephenson and Stephenson (1973), only a single binucleate oocyte was found among 26 oocytes examined. In the present paper the occurrence of multinucleate stages as a regular feature of oogenesis in several genera of marsupial frogs of South America is reported. The multinucleate condition is associated with the early stages of oogenesis; in large, yolky oocytes only one nucleus is present.

Most of the marsupial frogs are inhabitants of the humid forests of South America. In these frogs the aquatic larval stages are reduced or eliminated altogether, a phenomenon associated with the fact that the female carries the embryos on her back, either within a pouch of integument or in shallow depressions of the skin. The genera *Flectonotus*, *Gastrotheca*, and *Amphignathodon* are characterized by pouches, while in *Fritziana*, *Cryptobatrachus*, *Stefania* and *Hemiphractus* the embryos are carried in depressions of the skin. For a list of these frogs with locality and references of taxonomic interest see the work of Duellman (1976).

Relatively little information is available regarding reproduction and development in marsupial frogs. The relationship between mother and embryos has been studied in some detail in *Gastrotheca riobambae*, a species that carries the embryos in the pouch up to the tadpole stage (see del Pino, Galarza, de Albuja, and Humphries, 1975, for references). Among the species of *Gastrotheca* that carry the embryos to the young froglet stage, *Gastrotheca ovifera* is the best known (see Mertens, 1957, for a description of its life history and references).

This report describes some features of oogenesis in 33 species of marsupial frogs that correspond to the described species of *Amphignathodon* and *Hemiphractus* as listed by Duellman (1976), to two (of three) species of *Flectonotus*, one (of six) species of *Stefania* and 20 (of 32) species of *Gastrotheca*. In addition, the ovaries of three unnamed species of *Gastrotheca* were also analyzed. The peculiarities of oogenesis have been studied in more detail in *Flectonotus pygmaeus*, *Gastrotheca ovifera* and *Gastrotheca* sp., an unnamed species from Venezuela, since in these instances both living and preserved specimens were available. Living specimens of *G. marsupiata*, *G. excubitor*, *G. mertensi* and *G. plumbea* were also

available, but only in limited numbers. Study of other frogs was restricted to museum specimens, with the exception of *G. riobambae*, in which the ovary had been studied previously (del Pino and Sánchez, 1977).

Materials and Methods

Specimens examined and laboratory care of living frogs

Flectonotus pygmaeus, *Gastrotheca ovifera* and *Gastrotheca* sp. were collected at Estación Biológica de Rancho Grande, Maracay, Estado de Aragua, Venezuela in November 1975 and transported alive to the laboratory in Quito. In addition, several specimens preserved in 10% formalin or in Bouin's picro-formol were available for study. *Flectonotus pygmaeus* gives birth to advanced tadpoles, while *G. ovifera* and *Gastrotheca* sp. give birth to froglets. For this analysis the ovaries

Table I

Size of ovarian oocytes of Flectonotus pygmaeus *at various reproductive stages.*

Reproductive stage	Oocyte diameter (μm)	Number of large oocytes			Number of embryos in the pouch
		Left ovary	Right ovary	Total	
Juvenile					
Without pouch	500	4	7	11	
	500				
Open pouch	1200	0	5	5	
	1500				
	2000	3	3	6	
	2500	3	6	9	
Adult					
Closed pouch	3000	4	9	13	
	3000	6	5	11	
	3000	5	3	8	
Pregnant					
4 days	800	3	3	6	6
8 days	1000	6	7	13	8
18 days	500				6
20 days*	1500	3	2	5	7
20 days	2000	4	5	9	8
23 days	2000	6	6	12	11
28 days	500				7
At birth	2000	1	11	12	11
	2000	1	5	6	5
	2000	1	4	5	8
	2500	3	5	8	7
	2000	4	5	9	6
After birth					
2 days	1500	6	2	8	7
3 days	500				8
4 days	1500	2	3	5	6
Mean ± s.d.		3.5 ± 1.8	5.0 ± 2.3	8.5 ± 2.8	7.4 ± 1.7

* Frog was killed 20 days after ovulation; mating did not occur.

of 25 *F. pygmaeus* females (Table 1) and those of five *G. ovifera* and two *Gastrotheca* sp. were studied.

Juvenile specimens of *F. pygmaeus* were kept in a humid terrarium at 20° C (range 17–21° C) and were fed *Drosophila* once or twice a day. The frogs lived for nearly a year. Reproductive activity occurred spontaneously after about five months of captivity. Three females became pregnant; a fourth deposited eggs but did not mate. In the latter case, the eggs were deposited on the wall of the terrarium. One of these eggs was fixed with Bouin's picro-formol for cytological examination. Under laboratory conditions, the period of incubation in the pouch lasts about 29 days; advanced tadpoles then emerge from the pouch and metamorphose after about 30 days. Incubation in the pouch of this species is considerably abbreviated in comparison with the situation in *G. riobambae*, where development in the pouch lasts nearly four months (del Pino *et al.*, 1975).

Most of the museum specimens that were examined belong to the Museum of Natural History of the University of Kansas (KU). Other specimens belong to The American Museum of Natural History (AMNH), Museo de la Estación Biológica de Rancho Grande (EBRG), the Field Museum of Natural History (FMNH), Museo de la Fundación Miguel Lillo (MFML), the Museum of Zoology of the Louisiana State University (LSUMZ) or the Museum of Natural History of the University of Southern California (USC). Information regarding the museum specimens is presented below.

AMPHIGNATHODON: *A. guentheri* KU-164228, Ecuador, Pichincha: 5 km ESE Chiriboga, 2010 m.

CRYPTOBATRACHUS: *C. fuhrmanni* KU-169378, Colombia, Norte de Santander, 32 km W Sardinata, 1050 m.

FLECTONOTUS: *F. fissilis* KU-92240, Brazil, Guanabara: Rio de Janeiro, Tijuca.

GASTROTHECA: *G. argenteovirens* KU-144123, Colombia, Cauca: Road to Pacific coast from El Tambo, 2170 m. *G. cavia* KU-148534, Ecuador, Imbabura: Laguna de Cuicocha, 2890 m. *G. ceratophrys* KU-77016, Panamá, Darien, Laguna, 820 m.; AMNH-90984. *G. christiani* MFML-02117, MFML-02117-5, Argentina, Jujuy, Abra de Cañas V. Grande. *G. cornuta* KU-169394, Colombia, Cauca: La Costa, El Tambo, 1000 m. *G. excubitor* KU-163135, KU-163140, Perú, Abra Acanacu, 25 km NNE Paucartambo, 3520 m. *G. gracilis* MFML-01972, Argentina, Tucumán, Road Tafí del Valle km 41. *G. griswoldi* KU-138221, Perú, Junin, Mayupampa, 21 km N La Oroya, 3400 m; KU-138227, Perú, Junin, Comas 3220 m. *G. lojana* KU-138234, Ecuador, Loja, 10 km W Loja 2500 m; KU-142608, Ecuador, Loja, 5.5 km W Loja, 2330 m. *G. marsupiata* KU-138399, Perú, Huancavelica: Huancavelica 3780 m; KU-139187, Perú, Cuzco, 14.5 km S Paucartambo, 3450 m. *G. mertensi* KU-140386, Colombia, Nariño: La Victoria, 2700 m. *G. microdisca* KU-154610, Brazil, São Paulo: 10 km NW Caraguatatuba 500–750 m. *G. monticola* KU-138402, Ecuador, Azuay: Girón 2240–2500 m; KU-142610, Ecuador, Loja: Saraguro, 2510 m. *G. ochoai* KU-138668, Perú, Puno: Ollachea, 53 km W. Macusani, 2800 m. *G. ovifera* KU-125372, Venezuela, Distrito Federal de Caracas; KU-13338, Venezuela, Aragua: Quebrada, 0.5 km E. Res. Sta. at Rancho Grande, 1075 m. *G. peruana* KU-138444, Perú, Huanco 5 km NE La Union 3100 m; KU-138494, Perú, Cajamarca: Cajamarca, 2800 m; KU-138526, Perú, Ancash, Chavin de Huantar 3230 m. *G. plumbea* KU-132414, Ecuador, Cotopaxi: Pilaló 2460–2580 m; KU-164230, Ecuador, Pichincha: 9.5 km NW Nono 2530 m. *G. testudinea* KU-163276, Perú, Ayacucho: Tutumbaro, Rio Piene, 1840 m. *G. weinlandii* KU-146042, Ecuador, Morona Santiago: Rio Piúntza, 1830 m. *Gastrotheca* sp., FMNH-39889. *Gastrotheca* sp., LSUMZ-32049. *Gastrotheca* sp., EBRG-48240, Venezuela, Aragua: Res. Sta. at Rancho Grande.

HEMIPHRACTUS: *H. bubalus* KU-169426, Colombia, Putumayo: 10.3 km W El Pepino, 1440 m. *H. fasciatus* KU-93503, Panamá, Altos de Pacora; KU-116353, Panamá, San

Blas: Camp Summit, 300–400 m. *H. johnsoni* USC-716, Perú. *H. proboscideus* KU-123139, Ecuador, Napo: Santa Cecilia, 340 m. *H. scutatus* KU-147118, Ecuador, Morona Santiago: Rio Piúnza, 1400 m.

STEFANIA: *S. scalae* KU-167222, Venezuela, Bolivar: Paso de El Danto, El Dorado-Sta. Elena de Vairén road, km 117–119 (100–1150 m); KU-16239, KU-167248, Venezuela, Bolívar: El Dorado-Sta. Elena de Vairén road, km 112, 860 m.

Cytological procedures

The nuclei of ovarian oocytes were observed in both living and histological preparations. Oocytes from 100–500 μm are somewhat transparent and can be studied intact. Observations were made on such oocytes using a depression slide or a standard slide on which the coverglass was slightly elevated. Individual nuclei were observed similarly with phase contrast microscopy after rupturing the oocyte in Ringer's solution or a 5 : 1 mixture of 0.1 M NaCl and 0.1 M KCl.

Ovarian tissue fixed in 10% formalin or in Bouin's picro-formol, as well as that from museum material, was embedded and cut into sections of 10 μm thickness and stained with Harris' hematoxylin. Alcoholic eosin yellow was used as the counterstain. Some preparations were made using the standard Feulgen procedure.

The number of nuclei per oocyte was estimated by counts in the sectioned material. The section interval for the counts was decided by measurements of nuclear diameter. Since the size of nuclei within a single oocyte is highly variable at some stages, the estimates of total nuclear number are subject to considerable error. Sections of museum material were unsuitable for nuclear counts, thus only rough appraisals were made in those cases.

Ovaries of two specimens of *F. pygmaeus* were labeled with ^{3}H-uridine. Each frog received an intracoelomic injection of 40 μCi of ^{3}H-uridine (New England Nuclear, specific activity 26.7 Ci/mmole) in amphibian Ringer's solution and was sacrificed 24 hours later. Pieces of ovary were fixed in Bouin's picro-formol or in 3% glutaraldehyde in a phosphate buffer at pH 7.4. The tissues were embedded in Paraplast and cut into sections of 10 μm thickness. After incubation in 10% trichloracetic acid at room temperature for one hour the slides were coated with photographic emulsion (Ilford Nuclear Research emulsion type K-2, in gel form) and stored for 20 days at 4° C. Following development, the slides were stained with Harris' hematoxylin and alcoholic eosin yellow.

Results

Oogenesis in Flectonotus pygmaeus

The ovary of *F. pygmaeus* produces relatively few mature eggs per breeding period (Table I), but each egg accumulates a considerable store of yolk and reaches a final diameter of about 3 mm. The right ovary generally contains more large oocytes than the left (Table I), and sometimes the left ovary appears to be absent altogether. There are, however, individual differences, and in some frogs the left ovary is the larger one.

In the mature ovary there are large, yellowish-white yolky oocytes of 1500 to 3000 μm diameter, a number of previtellogenic oocytes of about 500 μm diameter, and smaller oocytes and oogonia. Following ovulation, oocytes pass through the

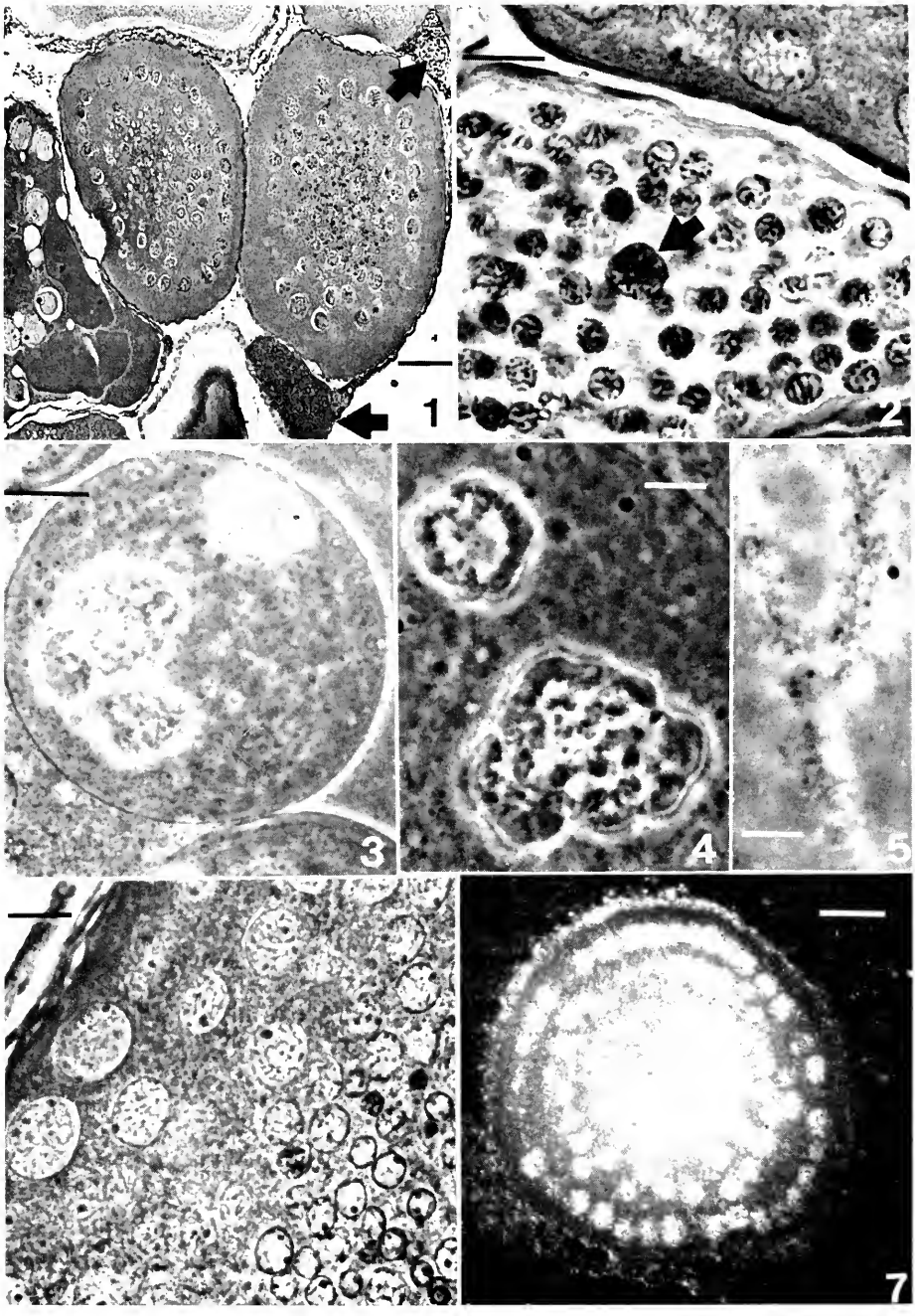

FIGURE 1. Cross section of the ovary of *Flectonotus pygmaeus*. The two centrally located oocytes show nuclei of various sizes, with the larger nuclei distributed towards the periphery and the smaller toward the center of the oocyte. In the oocyte on the left, most nuclei have

oviducts and become covered with a thin coat of jelly similar to that in *Gastrotheca riobambae* (del Pino *et al.*, 1975). During amplexus the eggs are fertilized as they leave the cloaca and are then moved into the pouch; however, the details of the process are not yet known. The oocytes of intermediate size grow rapidly in the mother's ovaries during the period that embryos are being incubated in the pouch and by the time of tadpole birth these oocytes are vitellogenic and about 2 mm in diameter (Table I). Only after birth of the tadpoles do the oocytes attain their mature size of about 3 mm.

The time required for the growth of oocytes from the initial stages to ovulation is not known. Vitellogenesis, however, seems to occur in a matter of a few months, since the oocytes of frogs kept in captivity grew in about five months from 500–800 μm diameter to their mature size. It seems likely that in nature the period of ovarian growth might be shorter.

Oogonial divisions and various stages of oogenesis are easily observed in the adult ovary (Figs. 1, 2). Small oocytes contain from about 1000 to 3000 nuclei (Table II); in larger oocytes the number of nuclei decreases gradually (Figs. 8, 9) until, at later stages, only one nucleus remains (Figs. 10, 11). Early oogenesis occurs within chambers referred to as cysts, following the terminology of King (1908). Oogonial mitoses within cysts appear to be synchronous, but division of the cytoplasm does not always accompany nuclear division; thus occasional multinucleate oogonia are observed. Fusion of the cells of a cyst appears to produce the multinucleate oocyte (Fig. 2). Assuming that all the nuclei in a given cyst are the division products of an original oogonial cell, there are about 11 rounds of mitosis involved in the formation of the multinucleate aggregate that becomes the oocyte. The aggregate measures about 100 μm in diameter and contains some 2000 nuclei (Table II). There are large differences in the number of nuclei from one aggregate to another, but the size of the nuclei is generally uniform; however, there are a few exceptionally large nuclei which may be polyploid (Fig. 2).

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disappeared, and the cytoplasm is not homogeneous. Cysts (arrows) are found frequently. Bar represents 100 μm.

FIGURE 2. Cross section of a cyst in the ovary of *F. pygmaeus*. The size of the nuclei is mostly uniform, but an occasional large nucleus (arrow) is seen. Cell membranes were present between the oogonia of the cyst, but these seem to disappear as the oocyte forms. In the oocyte, nuclei begin to enlarge; two nuclei in the edge of an oocyte can be seen in the upper portion of the figure. The increased size is evident. Bar represents 20 μm.

FIGURE 3. Nucleus from a living preparation of a multinucleate oocyte of *F. pygmaeus*. Note the very large and irregular aggregates of nucleoli. This nucleus had lampbrush chromosomes. In the same oocyte there were other nuclei of smaller size. Bar represents 20 μm.

FIGURE 4. Higher magnification of nucleolar aggregates from a nucleus of a multinucleate oocyte of *F. pygmaeus*. These aggregates are comparable to those of Figure 3. Bar represents 5 μm.

FIGURE 5. Lampbrush chromosome from a living preparation of a nucleus from a multinucleate oocyte of *F. pygmaeus*. Bar represents 5 μm.

FIGURE 6. Cross-section of an oocyte of *F. pygmaeus* with nuclei of various sizes. Large nuclei are located towards the periphery and small nuclei are clustered in the center. Lampbrush chromosomes are found in the larger nuclei. Nucleoli can be seen in most nuclei. Bar represents 20 μm.

FIGURE 7. Autoradiogram showing the incorporation of ^{3}H-uridine into the nuclei of a multinucleate oocyte of *F. pygmaeus*. There was incorporation in both large and small nuclei and in the nuclei of follicle cells. Bar represents 50 μm.

TABLE II

Average number of nuclei in the ovarian oocyte. The number in parentheses indicates the number of oocytes analyzed. In oocytes of 250–500 μm diameter, only the large nuclei were counted (see text).

Diameter of oocyte (μm)	Nuclear diameter (μm)	Number of nuclei/oocyte (± s.d.)	Volume oocyte/ volume nuclei (± s.d.)
	Flectonotus pygmaeus (nine frogs)		
100– 190 (6)	6.1	2013 ± 961	4.1 ± 1.8
200– 290 (9)	13.9	1484 ± 541	3.9 ± 1.8
300– 390 (4)	21.5	877 ± 95	4.4 ± 0.7*
460 (2)	28.5	818	4.9
950 (1)	180	3	49.0
1200–1300 (3)	393–274**	1	86.3
1500 (5)	390–289	1	114.2
2000–3000 (3)	511–186	1	1186.3
	Gastrotheca ovifera (one frog)		
200– 250 (2)	30	194.5	1.9
300– 400 (2)	32	411.5	2.9
410– 500 (5)	40	481.6	3.6
510– 570 (3)	40	390.0	6.7
	Gastrotheca sp. (one frog)		
200– 300 (4)	33	71.0	9.1
300– 400 (2)	40	77.0	10.3
500– 550 (3)	33	125.7	32.7
680 (1)	40	107.0	45.9

* The volume of 2000 small nuclei ($650 \times 10^3\ \mu^3$) was added to the nuclear volume of each oocyte since small nuclei were not counted.

** Largest and smallest diameters of nuclei of oval shape.

Once meiosis begins, there are no more mitotic divisions within a given cyst. The beginning of meiosis is characterized by moderate enlargement of the oocyte and its nuclei. The nuclear size is generally uniform and the distribution of nuclei seems to be random, except in oocytes with few nuclei; in the latter case most nuclei are located toward the periphery. Inside each nucleus the chromosomes are visible as fine threads, and there are one to a few nucleoli of rounded shape. Elimination of nuclei may begin at this early stage, judging by the presence of numerous small, pycnotic nuclei that stain darkly with hematoxylin; these are found among more normal appearing nuclei located centrally in the oocyte (Figs. 1, 6).

As the oocyte grows, there develop conspicuous differences in nuclear size, correlated with their location in the oocyte. Nuclei located toward the periphery enlarge to a greater extent than those located more centrally. These differences can be detected in oocytes of 250 μm diameter and are soon obvious. In oocytes of 300 μm the largest nuclei, located just beneath the cortex, measure about 20 μm;

nuclei located somewhat deeper are smaller; those located in the center are the smallest (6 to 7 μm) (Figs. 1, 6). Large and medium size nuclei contain chromosomes in the lampbrush state (Fig. 5); in smaller nuclei the chromosomes are visible as fine threads but other characteristics could not be determined. The nucleoli of each nucleus seem to associate into a few large masses of irregular shape, nucleolar aggregates (Figs. 3, 4), which are common to all nuclei, except possibly those that are pycnotic. Growth of the oocyte is accompanied by further enlargement of the peripheral nuclei (Table II) and a decrease in their number. Correlated with the smaller number of large nuclei there seems to be an increase in the number of medium size nuclei and small nuclei. The latter nuclei are very abundant in oocytes of 300 to 700 μm diameter and are clustered in the center of the oocyte (Fig. 6); they are so numerous and closely packed that they are impossible to count, but their number seems to vary. Owing to the difficulty of counting the centrally located nuclei, only the larger nuclei were counted in oocytes of 250 to 500 μm diameter (Table II). The decrease in total number of nuclei during this period is thus more gradual than is suggested by the figures in Table II, which shows exclusively the decrease in the number of larger nuclei. Indeed, the number of nuclei appears to remain rather constant from the formation of the oocyte up to a size of about 400 μm and possibly later. In one oocyte of 390 μm diameter, for example, the number of small nuclei was estimated to be about 2500 with a mean diameter of 8.6 μm; in the same oocyte there were about 750 large nuclei with diameters of about 25 μm.

In oocytes of 700 to 800 μm the chromosomes are no longer in the lampbrush condition and cannot be seen in most nuclei. All nuclei, including the smallest ones, contain large and conspicuous nucleolar aggregates of irregular shape. In oocytes of 800 to 900 μm, the number of large and small nuclei decreases markedly (Figs. 8, 9); during this process the large nuclei decrease somewhat in volume and become pycnotic before finally disappearing. Associated with nuclear disappearance there are changes in the appearance of the cytoplasm, presumably the result of the addition of nuclear material (Figs. 1, 8, 9). This modified cytoplasm is originally distributed unevenly in the oocyte, but the several areas of differing appearance eventually join and give a more homogeneous character to the cytoplasm. Numerous vesicles are formed slightly later and the oocyte eventually becomes almost filled with them. When only 1 to 10 large nuclei remain in the oocyte, yolk platelets begin to appear. At this time the diameter of the oocyte is approximately 1000 μm.

The remaining nuclei enlarge greatly and reach a diameter of about 180 μm. These large nuclei occupy the central region of the oocyte, in contrast to the peripheral location of large nuclei during earlier stages. After oocytes reach about 2 mm in diameter, only one nucleus is found. This germinal vesicle is originally spherical and is located near the center of the cell (Fig. 10); as vitellogenesis proceeds, it moves toward the periphery of the oocyte and becomes ovoid (Fig. 11). It is considerably larger than any of the preceding nuclei.

In the last of the multiple nuclei and in the germinal vesicle there are sometimes accumulations of basophilic material just inside the nuclear envelope (Fig. 11); the nature of this material is unknown. In each of the final nuclei, the nucleolar aggregates become very large before disappearing (Fig. 10); these seem to be re-

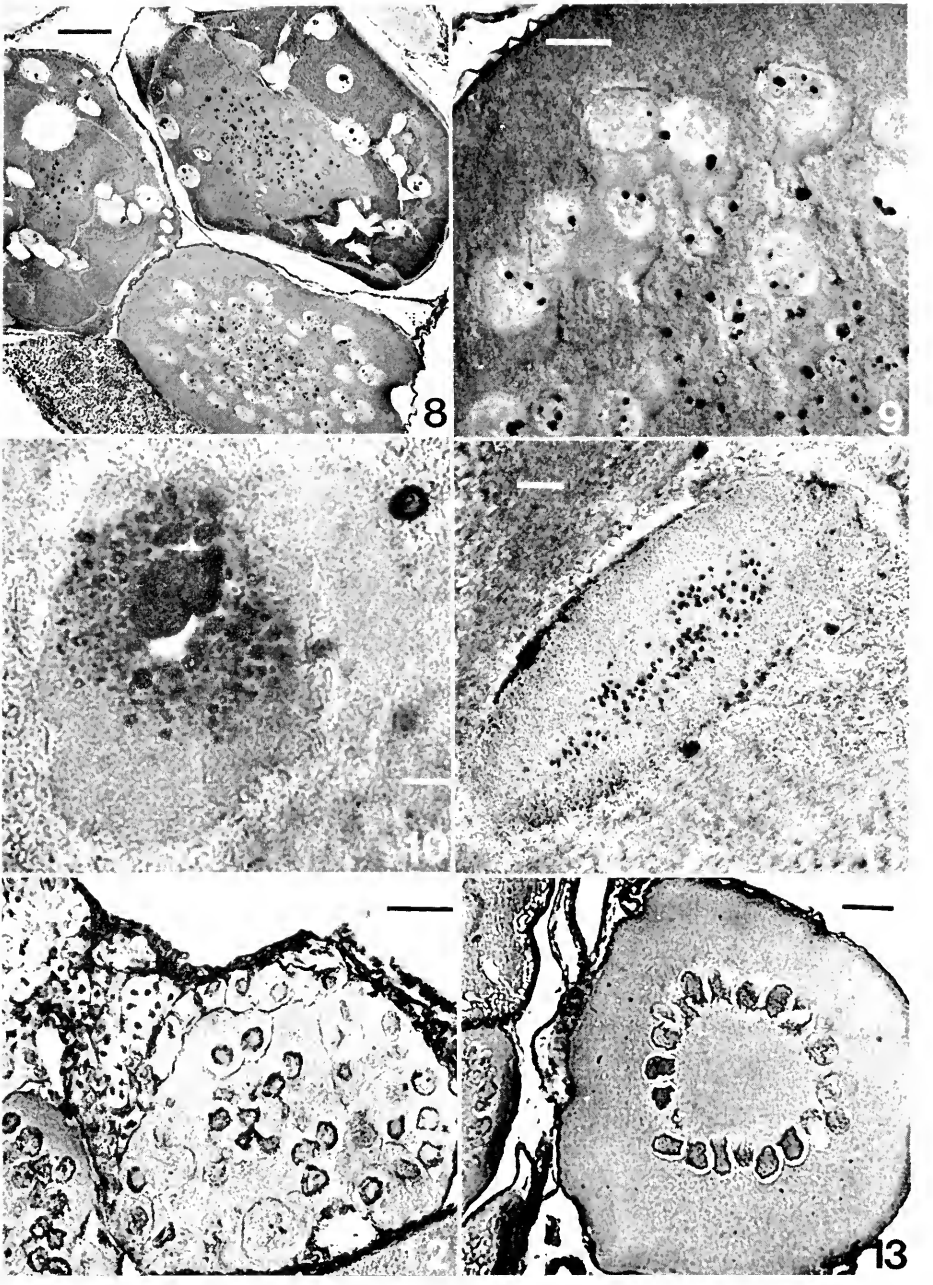

FIGURE 8. Cross section of ovary of *F. pygmaeus*. In the two upper oocytes there is great reduction in the number of nuclei. Large nuclei shrink and become pycnotic before disappearing. Both oocytes show also the uneven distribution of cytoplasm modified by nuclei. In the lower portion of the figure there is a multinucleate oocyte with nuclei of various sizes and a follicle of atresia. Bar represents 100 μm.

placed gradually by small ovoid corpuscles that might be nucleoli. In addition, there are many spherical corpuscles, much smaller than the usual nucleoli (Fig. 11). Both types of corpuscles are present in the germinal vesicle of the largest oocytes. The chromosomes of this stage were not found.

The yolk-filled cytoplasm of large ovarian oocytes and ovulated eggs contains numerous transparent round vesicles that are distributed almost uniformly. The animal pole of the egg is almost devoid of both vesicles and yolk granules; it is within this zone that the germinal vesicle is located. After ovulation, the germinal vesicle could not be found in the only such egg that was available, suggesting that ovulation and germinal vesicle breakdown occur at about the same time.

Small nuclei of large oocytes gave a strong Feulgen reaction, while lampbrush chromosomes, nucleoli and nucleolar aggregates of larger nuclei reacted weakly. Although nucleolar aggregates did not ordinarily give a positive reaction, there was sometimes a positive reaction from large bodies that seemed to be nucleolar aggregates. In the germinal vesicle, only the ovoid corpuscles reacted with Feulgen, and these only faintly.

All nuclei in oocytes up to about 500 μm in diameter incorporated ^{3}H-uridine (Fig. 7). Uridine incorporation was detected in association with chromosomes during the early stages of meiosis; later, however, uridine seemed to be incorporated almost exclusively by the nucleoli. In large oocytes, where the nuclear number was greatly decreased, there was slight or no incorporation of label.

Nuclear changes during oogenesis in Gastrotheca ovifera *and other marsupial frogs*

During oogenesis the oocyte becomes multinucleate in *G. ovifera* and other species of marsupial frogs (Table III), but the number of nuclei per oocyte in these frogs is smaller than that in *F. pygmaeus* (Table II). In *G. ovifera* the nuclear number reaches about 500, presumably the result of approximately nine mitotic divisions of the original oogonium; in *Gastrotheca* sp. (Venezuela) there are only about 100 nuclei, corresponding to fewer than seven divisions. The number of nuclei in oocytes of other species with multinucleate oocytes (Table III) was not counted, but it seems comparable to that of *G. ovifera* or *Gastrotheca* sp., with the exception of *Hemiphractus johnsoni*, which has fewer nuclei. There was a maximum of four nuclei per oocyte in the only ovary of this species that was examined.

FIGURE 9. Detail of the changes in large nuclei prior to disappearance. The cytoplasm adjacent to nuclei becomes modified and the nuclei shrink before becoming pycnotic. Bar represents 50 μm.

FIGURE 10. Cross section of the nucleus from a mononucleate oocyte of *F. pygmaeus*. This nucleus has enlarged greatly and shows very large aggregates that will be replaced later by smaller corpuscles. Bar represents 25 μm.

FIGURE 11. Cross section of the single germinal vesicle of an oocyte of *F. pygmaeus*. There are ovoid corpuscles towards the center and very abundant spherical entities of smaller size. The chromosomes could not be seen. Note the basophilic accumulations of material just inside the nuclear envelope. Bar represents 25 μm.

FIGURE 12. Cross section of a cyst in the ovary of *Gastrotheca ovifera*. Most oogonia are mononucleate; these will fuse to give a multinucleate oocyte with about 500 nuclei. Bar represents 50 μm.

FIGURE 13. Cross section of an oocyte of *G. ovifera*. The enlarged nuclei are distributed in a layer. Nuclei are absent from the central region. Bar represents 100 μm.

TABLE III

Type of oogenesis and development at birth in marsupial frogs.

Species	Egg diameter (mm)	Development	
		Tadpole	Froglet
Mononucleate type of oogenesis			
Cryptobatrachus			
C. fuhrmanni			+
Flectonotus			
F. fissilis	3	+	
Gastrotheca			
G. argenteovirens			
G. cavia	3	+	
G. christiani	4		+
G. excubitor	6		+
G. gracilis	3	+	
G. lojana			
G. marsupiata	2.5	+	
G. mertensi	5		+ (1)
G. monticola		+	
G. ochoai	5		+
G. peruana	3	+	
G. plumbea	4		+ (1)
G. riobambae	3	+	
G. testudinea	3.5		+
G. sp. (LSUMZ-32049)			+
G. sp. (FMNH-39889)	4		+
Hemiphractus			
H. bubalus			+ ?
H. fasciatus	7		+
H. proboscideus			+
H. scutatus	10 (2)		+
Multinucleate type of oogenesis			
Amphignathodon			
A. guentheri			+
Flectonotus			
F. pygmaeus	3	+ (3)	
Gastrotheca			
G. ceratophrys	8		+
G. cornuta			+
G. griswoldi	8		+
G. microdisca			+
G. ovifera	8		+
G. weinlandii			+
G. sp. (Venezuela)	7		+
Hemiphractus			
H. johnsoni			+
Stefania			
S. scalae	9		+
Total		12	21

Number of species: 33. (1) Young are born as froglets or towards the end of metamorphosis. (2) Egg diameter from Trueb, 1974. (3) Born as advanced tadpole.

In *G. ovifera,* oogonial divisions within a cyst are apparently synchronous, as in *F. pygmaeus,* and most oogonia contain a single nucleus (Fig. 12). Oogonia with 2 to 4 nuclei are rare, in contrast to *F. pygmaeus,* where many oogonia seem to be multinucleate. In *G. ovifera* the oocyte seems to be formed by fusion of mononucleate oogonia of a cyst. With the onset of meiosis, nuclei enlarge, form lampbrush chromosomes and later, conspicuous nucleolar aggregates. Most nuclei, and possibly all of them, become arranged toward the peripheral region of the oocyte, forming one or two layers of nuclei (Fig. 13). The nuclei of the internal layer are slightly smaller than those toward the periphery. In addition, there are a few pycnotic nuclei of small size that are found just internal to the larger nuclei. Pycnotic nuclei have also been observed in the periphery and sometimes in the central region of the oocyte. In most cases, however, there are no nuclei in the central part.

As the oocyte grows, there is a gradual disappearance of nuclei and, toward the beginning of vitellogenesis, there is apparently a period of more rapid nuclear disappearance. The onset of vitellogenesis is accompanied by changes in the cytoplasm of the oocyte that are comparable to the changes described for *F. pygmaeus. Gastrotheca ovifera* produces very large oocytes (approximately 8 mm diameter) and it seems that the oocyte contains multiple nuclei until a large size is attained: oocytes of about 2 mm in diameter do not have yolk platelets and still have numerous large nuclei.

Eleven of the 33 species of marsupial frogs examined (Table III) are characterized by the presence of multiple nuclei in the oocyte during the early stages of oogenesis. Although the formation of the oocyte in some of these species was not observed, those that were studied show similarities to *F. pygmaeus* or *G. ovifera.* In the majority, the nuclei become arranged toward the peripheral region of the oocyte and enlarge. The central region does not have nuclei in most instances, but occasionally a few small pycnotic nuclei can be seen. Each nucleus contains lampbrush chromosomes and nucleolar aggregates. The number of nuclei decreases gradually as the oocyte grows; the multinucleate condition, however, is prolonged into the vitellogenic period, as in *G. ovifera.*

Discussion

There is little doubt that the multiple entities described here as nuclear are either nuclei or derivatives of nuclei. The presence of visible chromosomes in many of the nuclei and the incorporation of labeled uridine attest to this view, as do ultrastructural studies (in preparation) which reveal the presence of a typical nuclear envelope. What is unclear is whether the nuclei all contain chromosomes and whether the number of chromosomes in each is the same. The extreme variation in size of the nuclei suggests that the chromosome number may not be the same, or that the condition of the chromosomes may vary from the large nuclei to the small. Although no information is available as to the DNA content of these multinucleate oocytes or the DNA content of individual nuclei, the situation suggests a massive overall amplifiation of the oocytes' DNA, considerably greater than that occurring in the eight-nucleate oocytes found in *Ascaphus trui* (Macgregor and Kezer, 1970). All nuclei are presumably engaged in synthesizing RNA, at least prior to the final stages where only a single germinal vesicle or a few large nuclei are present. It appears, then, that these multinucleate oocytes represent a remark-

able case of a sort of endopolyploidy of high degree, even if the nuclei contain abnormal chromosome complements.

We have no explanation to propose for the widespread occurrence of multinucleate oocytes in marsupial frogs; moreover, we have been unable to find a clear relationship between the occurrence of the multinucleate condition in these species and such characteristics as the final size of the egg or the pattern of reproduction. However, there does seem to be some correlation between the multinucleate condition, production of unusually large eggs, and development of young to the froglet stage in the maternal pouch. A notable exception is *F. pygmaeus,* in which the eggs do not seem unusually large and in which the young are kept in the pouch only until they are advanced tadpoles. An egg of 3 mm, however, might be considered large for such a small frog (30 mm snout-vent length), since other frogs with multinucleate oogenesis measure from 40 to 80 mm (snout-vent length). Some frogs with mononucleate oogenesis, however, are of similar size and produce eggs that are as large as those of species with multinucleate oocytes. In both the multinucleate and mononucleate groups there are species that give birth to tadpoles and species that give birth to froglets (Table III). One generalization that may be important is that the marsupial frogs, regardless of nuclear number during oogenesis, produce eggs that are very large by usual amphibian standards, but very few in number. Furthermore, reproduction seems to be exceptionally efficient: almost all eggs produced appear to be fertilized and undergo development. In *F. pygmaeus,* for example, the number of large eggs in the ovary is small, and corresponds closely to the number of embryos in the pouch.

The total nuclear volume increases considerably during early oogenesis, but the concomitant great increase in oocyte volume results in what seems to be a rather constant ratio between the two during the previtellogenic period (Table II). No firm conclusions can be reached, however, since the estimations of volume are subject to large error and since there is also great variability in the number of nuclei per oocyte. During the vitellogenic phase, oocyte growth is not paralleled by an increase in nuclear volume; thus, the ratio of oocyte to nuclear volume increases greatly toward the end of oogenesis (Table II). Little information is available regarding ratios between oocyte volume and nuclear volume in the Amphibia, but in the mononucleate oocytes of *Rana pipiens* (Parmenter *et al.*, 1960) the ratio seems to increase earlier than in *F. pygmaeus.* In any case, however, the multinucleate condition obviously results in an enormous nuclear surface area, particularly in oocytes of *F. pygmaeus,* with their large number of centrally located nuclear entities.

The multinucleate condition in the oocytes described in this report differs in several notable ways from that in *Ascaphus truei* (Macgregor and Kezer, 1970). The maximum number of nuclei in *Ascaphus* oocytes is usually eight, although many ovaries contain a few oocytes with sixteen nuclei (Kezer, personal communication); the condition arises from failure of cytokinesis to occur following nuclear division. In *F. pygmaeus, G. ovifera,* and *Gastrotheca* sp., however, the number of nuclei reaches the hundreds, and the condition seems to originate through the disappearance of cell membranes between adjacent cells within a cyst. Another difference is the fact that in *Ascaphus* the multinucleate condition persists until late

oogenesis, while in at least some of these marsupial frogs the multinucleate condition lasts only until about the time yolk formation begins. In both situations, however, the multiple nuclei contain chromosomes in the lampbrush condition, and there are similar nucleolar features. In neither situation is there good evidence as to the mechanism by which the number of nuclei is finally reduced to one.

This work would have been impossible without help in the provision of living and preserved specimens of these frogs and without the availability of museum materials for analysis. For the collaboration we received, we acknowledge with gratitude the help of the following persons: Dr. William E. Duellman, who provided specimens, arranged loans from various museums and arranged a visit to the Museum of Natural History of the University of Kansas for E. M. del Pino. His recent herpetological work in South America was supported by grants NO. GB-42481 and DEB76-09986 from the National Science Foundation. Dr. Raymond F. Laurent from Fundación Miguel Lillo, Tucumán, República Argentina, provided working space at that institution for E. M. del Pino and allowed the study of museum specimens of *Gastrotheca*. Mr. Scott J. Maness from Estación Biológica de Rancho Grande, República de Venezuela, provided living and preserved specimens from that area. Dr. Gonzalo Medina Padilla, Jefe de la Oficina de Fauna del Ministerio de Agricultura y Cria, gave the corresponding permits for the collection and export of frogs from Venezuela. Dr. Richard P. Seifert and Florence Hammet Seifert aided in the collection of frogs from Venezuela.

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Summary

The occurrence of multinucleate stages during oogenesis appears to be widespread in the marsupial frogs of South America. In some species the number of nuclei or nucleus-like entities per oocyte is estimated to be as high as 2000, but the number in other species may be considerably lower; some species have only a single oocyte nucleus. In all cases it seems that only a single nucleus remains as the oocyte approaches maturity. The situation suggests a massive general amplification of the genome of the multinucleate oocytes that is much greater than has yet been reported. Possible relationships between the occurrence of the multinucleate condition and such features as egg size and reproductive pattern are discussed, but no final conclusions can be made on the basis of the evidence presently available.

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EVIDENCE FOR A NONINTESTINAL NUTRITIONAL MECHANISM IN THE RHYNCHOCOELAN, *LINEUS RUBER* [1]

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The majority of the members of the phylum Rhynchocoela are found free-living in the benthos of the intertidal and subtidal zones. These worm-like organisms are described as acoelomate Bilateria with a complete digestive system (Hyman, 1951). Coe (1943) described the rhynchocoelans (nemerteans) as carnivorous in their feeding behavior, and in some littoral communities these organisms are apparently the most abundant predators (Roe, 1970).

The function and morphology of the digestive system of a number of rhynchocoels has been extensively examined with regard to particulate food digestion by Gontcharoff (1948), Jennings (1960, 1962), Jennings and Gibson (1969) and Gibson (1970) utilizing light microscopy and histochemical techniques.

The ability of numerous free-living marine invertebrates to remove organic solutes, present in relatively low concentrations, from the environment has been extensively investigated by Stephens and his associates (*e.g.,* Stephens, 1964; Reish and Stephens, 1969). Similar absorption phenomena for small molecular weight organic nutrients have been identified in the rhynchocoelan, *Lineus ruber,* by Fisher and Cramer (1967). This paper extends those preliminary reports and indicates that the epidermal free surface is the major site for absorption of these nutrients from their environment.

Materials and Methods

Source and maintenance of animals

Specimens of *Lineus ruber* (5 to 8 cm) were collected at low tide in the vicinity of Manomet Point, Massachusetts, and maintained in the laboratory in running sea water at 20–24° C. Animals were starved while being maintained in artificial sea water lacking organic solutes (Cavanaugh, 1964), and all animals shipped to Rice University for study were held in Instant Ocean (34‰; Aquarium Systems, Inc.). All experimental animals were provided with a coarse sand substrate of sufficient depth to cover the bottom of the holding containers.

Solute accumulation techniques

Rhynchocoelans, being vermiform animals, were manipulated, as described below, after the methods of Fisher and Read (1971); however, artificial sea water

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was substituted for the balanced saline used by those authors for elasmobranch parasites. On removal from preincubation in artificial sea water at 20° C for 30 minutes to 1 hour, individual samples of at least five organisms were carefully blotted on hard surfaced filter paper and transferred to ^{14}C-galactose, ^{14}C-glucose, ^{14}C-leucine or ^{14}C-alanine containing incubation mixture. Unless otherwise stated, the incubation time and temperature were two minutes and 20° C. Following the incubation period the groups of worms were removed from the radioactive mixture, quickly rinsed three times in large volumes of artificial sea water, blotted, weighed on a torsion balance, and placed in 2.0 ml of 70% ethanol. Worm carcasses were extracted in the ethanol with intermittent shaking for at least 18 hours at room temperature (22–26° C). Aliquots of the extraction ethanol were dried on ringed-copper planchettes and counted in a low background gas-flow counter for 10 minutes or 10^4 counts, whichever process ended first. The original incubation solution was diluted 1 : 100 with 70% ethanol and counted in a similar manner to determine the specific activity of the test medium. Ethanol extracted worms were dried to constant weight in aluminum foil tares at 100° C and weighed on an analytical balance. Total water within worms of a single group was estimated from wet weight and dry weight comparisons. These data were used to calculate the internal radiolabeled substrate's concentration represented by μmoles/ml worm water.

Chemical assays

Polysaccharide was determined by the phenol : H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers and Smith (1956) and the modified anthrone method of Dimler, Schaffer and Wise (1952). Glucose was estimated by the glucose oxidase method utilizing the Glucostat Special (Worthington Biochemical Corporation). Protein was isolated by trichloroacetic acid (TCA) precipitation of homogenates of worm carcasses and estimated by the colorimetric method of Lowry, Rosebrough, Farr and Randall (1951).

Chromotagraphic analysis of worm extracts was accomplished using the following solvents on Whatman #1 paper: first) N-butanol : ethanol : acetone : water = 50 : 40 : 30 : 20 (descending) (Gray and Frankel, 1954); secondly, N-butanol : propionic acid : water = 63 : 31 : 44 (descending) (Crowley, 1963); and thirdly, 1-propanol : ethyl acetate : water = 7 : 1 : 2 (ascending) (Baar, 1954). Carbohydrates were visualized on developed chromatograms with the alkaline silver nitrate reagent of Trevelyan, Procter and Harrison (1950). Radioactive areas were localized on chromatograms using a gas-flow scanner. These areas were eluted from chromatograms by a technique similar to that of Dimler *et al.* (1952), and the eluates were reduced to dryness *in vacuo* at 40° C. The redissolved residue was co-chromatographed with authentic standards in the above solvents for positive identification.

Ultrastructural techniques

Specimens of *Lineus ruber* were placed in 4° C, 0.12 M monobasic sodium phosphate-sodium hydroxide buffer at pH 7.4 containing 6% glutaraldehyde plus 3% sucrose. Subsequently the partially fixed worms were cut into cross sections about 1 mm thick and returned to the fixative for three to six hours. Fixation was

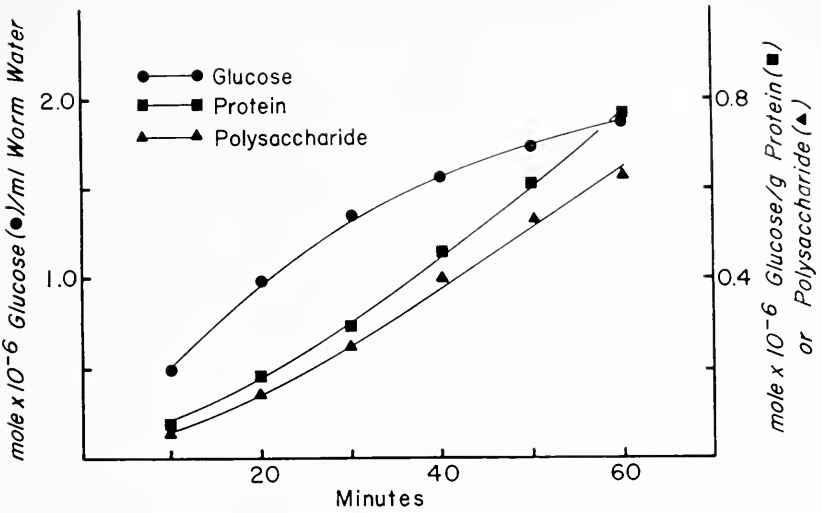

FIGURE 1. The accumulation of 10^{-4} M external glucose into free pool, proteins and polysaccharide of *L. ruber* with time. Glucose in the free pool was determined by glucose oxidase, while calculations of glucose incorporated into protein and polysaccharide were based upon specific activity of radioglucose. Data is based on the mean of four samples per time interval.

terminated by washing in buffer containing 5% sucrose. After washing in buffer for 12 to 18 hours, the tissue was post-fixed for 45 minutes in 1% osmium tetroxide plus 1% sucrose in the same buffer. Post-fixed tissue was rinsed in tap water, dehydrated through a series of ethanol and propylene oxide solutions and embedded in Epon Epoxy 812 (Shell Chemical Co.). Thin sections cut on diamond knives were mounted on bare copper grids, stained with lead and uranium salts and viewed in the Philips 300 electron microscope.

RESULTS

Permeation of glucose

Lineus ruber incubated in sea water containing 10^{-4} M glucose rapidly accumulated that hexose into the free pool within the worm. When extracts were examined, the concentration of glucose inside the worm determined by chemical analysis was ten times that in the external medium after an incubation period of 20 minutes, and within 60 minutes the internal concentration was approximately 17 times the original concentration in the surrounding sea water (Fig. 1). The initial "free" glucose in the worms was less than 2×10^{-4} mole of "worm water." If trace amounts of ^{14}C-glucose (0.1 μCi/μmole) were added to the incubation medium, both protein and polysaccharide fractions of the nemerteans became labeled and the amount of incorporation of the carbon from hexose into these large molecular weight compounds increased up to at least 60 minutes (Fig. 1).

It could be argued that, because these worms possess a digestive system, glucose was ingested from the surrounding sea water and accumulation was occurring across the intestinal epithelium. If these worms were obtaining labeled glucose by

swallowing the sea water medium, one would expect nonuniform distribution of radiocarbon along the linear dimension of the worm body; *i.e.*, during short term incubation periods more label would be absorbed into tissues near the mouth and anterior end of the animal, and, as the length of the incubation period increased, the label would proceed down the gut toward the anus. Individual rhynchocoels were incubated for varying periods of time in sea water containing 10^{-4} M glucose-^{14}C. At the termination of the incubation period the worms were rinsed in sea water, placed in an extended position on a thin glass plate resting on solid CO_2, and frozen immediately. Each worm was cut into 0.5 cm pieces and each section was individually extracted in ethanol for determination of the radioactivity along the length of the nemertean. The data in Table I indicate that there was uniform distribution of label along the length of the nemertean following different incubation periods. In no case was there any difference in the amount of radiocarbon in either end of the worm which suggests that over the time period examined, movement of sea water into the gut *via* the mouth and/or the anus is not a significant factor in determining the distribution of accumulated glucose. These data also suggest that glucose accumulation is occurring over the entire surface of the worm and that there is no difference in the rate of glucose influx along the body of the rhynchocoelan. Confirmation that the worms were not ingesting the incubation medium was obtained by ligaturing ten specimens of *Lineus ruber* posterior to the mouth and anterior to the anus with 4/0 silk suture prior to the incubation in the radioactive substrate. No difference in distribution of accumulated radioglucose could be observed between ligatured and the unligatured control animals (Table II). Amounts of radioglucose/body section is similar in control and ligatured animals (Table II).

The initial rate of glucose accumulation was examined during two-minute incubations using glucose concentrations ranging over several orders of magnitude. Glucose influx exhibits initial rate saturation kinetics as a function of concentration

TABLE I

Distribution of ethanol-soluble counts/minute along the body of Lineus ruber *after incubation for different periods. Initial external glucose-^{14}C concentration is 10^{-4} M; incubation temperature, 20° C.*

Number of section	counts/minute				
	1 min	2 min	4 min	8 min	16 min
1 anterior	90	160	201	340	873
2	73	171	260	339	734
3	89	163	220	370	759
4	69	181	229	310	819
5	91	170	248	319	840
6	83	190	271	357	839
7	94	167	209	348	790
8	71	159	219	301	763
9	75	179	210	359	793
10	84	187	254		779
11	70		220		810
12 posterior	61				791

TABLE II

Distribution of ethanol-soluble counts/minute along the body of ligatured and unligatured Lineus ruber. *Initial external glucose-^{14}C concentration is 10^{-4} M; incubation time, 5 minutes at 20° C.*

Number of section	$\bar{x}$ counts/minute (± standard error of the mean)	
	Ligatured	Without ligature
1 anterior	263 ± 47	258 ± 47
2	243 ± 39	261 ± 51
3	199 ± 61	241 ± 32
4	231 ± 27	229 ± 26
5	246 ± 43	263 ± 59
6	271 ± 59	237 ± 23
7	251 ± 47	231 ± 31
8	220 ± 17	243 ± 35
9	237 ± 30	240 ± 40
10	246 ± 38	221 ± 26
11	251 ± 53	279 ± 60
12 posterior	239 ± 37	264 ± 61
	n = 10	n = 15

(Fig. 2). Galactose uptake followed a similar entry pattern (Fig. 2). The accumulation of glucose is inhibited by galactose (66% inhibition) and three glycosides: phlorizin (93% inhibition), quercetin (71% inhibition) and ouabain (29% inhibition). Phlorizin, the β-glucoside of phloretin and β-D-glucose, is a potent inhibitor of glucose permeation in a number of systems (see Crane, 1960). The permeation of glucose was not inhibited by other hexoses, or di- and trisaccharides including levulose, mannose, N-acetylglucosamine, cellobiose, maltose, trehalose, sucrose and melibiose. Amino acids, fatty acids and organic acids also failed to

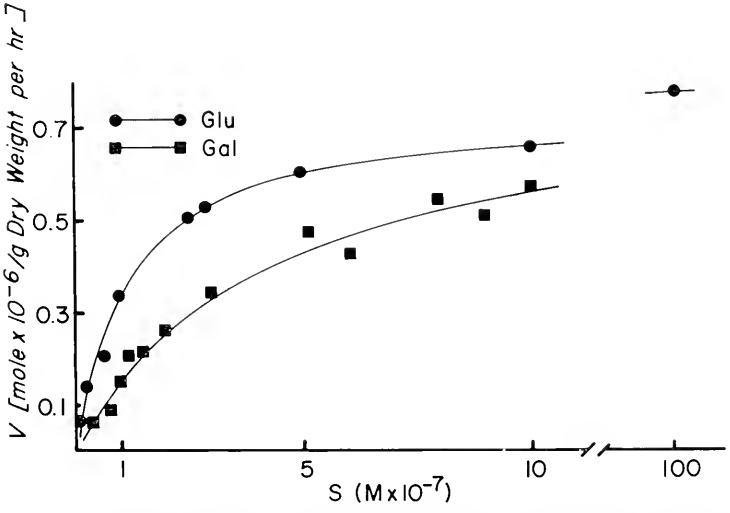

FIGURE 2. The effect of external substrate concentration on the permeation of glucose and galactose into the free hexose pool. Data is based on the mean of three samples per concentration of hexose.

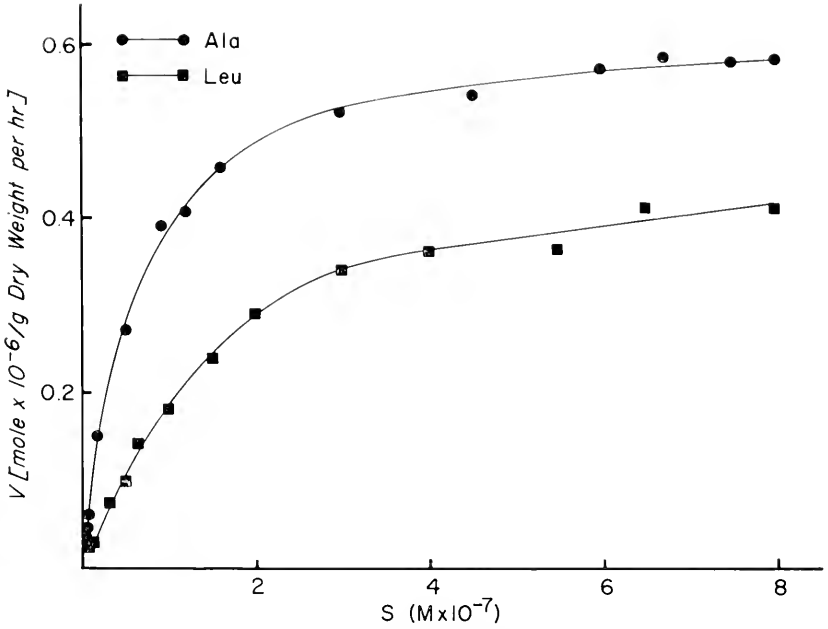

FIGURE 3. The effect of external substrate concentration on the accumulation of alanine and leucine into free pool of *L. ruber*. Data is based on the mean of three determinations per concentration of amino acid.

inhibit glucose permeation. In these experiments the initial external glucose concentration was 10^{-4} M; and the inhibitors, 10^{-3} M. All incubations were carried out for two minutes at 20° C.

Chromatographic examination of ethanolic extracts of worms after two-minute incubation in radioglucose revealed that there was very little (<0.5%) metabolism of the glucose during the relatively short incubation period. If nemerteans are post-incubated in sea water without glucose following a two-minute incubation in radioglucose, there is no chromatographically identifiable glucose "leakage" into the efflux medium. There are, however, traces of succinate which appear in the surrounding medium during this second incubation period.

Permeation of alanine and leucine

The uptake of alanine and leucine by *Lineus ruber* also followed saturation kinetics (Fig. 3). The fate of the accumulated alanine was further examined using long-term incubations to follow the possible appearance of radiocarbon into protein. The results of this experiment, expressed as μmoles/g protein is seen in Figure 4. The radiocarbon skeleton from alanine was incorporated into protein during the 60-minute experimental period. Longer incubation times were not examined.

The accumulation of alanine and leucine were not influenced by 50:1 ratios of carbohydrates, organic acids or fatty acids; however, the uptake of those substrates was inhibited by some amino acids examined (Table III). Diabasic and dicarboxylic amino acids, the imino acid proline, and the sulfonic acid derivative, taurine,

TABLE III

Inhibition of alanine and leucine by other l-amino acids. External substrate concentration is 5×10^{-5} M; *inhibitor concentration,* 2×10^{-3} M; *incubation time, 2 minutes at 20° C.*

Inhibitor	Inhibition of alanine permeation (%)	Inhibition of leucine permeation (%)
Alanine	97	49
β-Alanine	11	0
Arginine	0	0
Aspartic acid	0	0
Glutamic acid	0	0
Glycine	64	39
Isoleucine	19	53
Leucine	53	94
Lysine	0	0
Methionine	47	61
Proline	0	0
Taurine	0	0
Valine	21	49

did not influence the uptake of either alanine or leucine. The mutual inhibition of uptake by leucine and alanine suggested that those amino acids may compete for entry through the same membrane site.

Chromatographic examination of extracts from two-minute incubations indicated that there were no detectable metabolites of either alanine or leucine in the free pool of solutes within the worm bodies. Similar examination of the incubation medium revealed that there were no metabolites of these amino acids excreted into the external medium during the two-minute exposure to the isotopes.

Morphology of the epidermal and epithelial surfaces

Two interfaces for the accumulation of organic solutes exist on most free-living, aquatic metazoans: the epidermal covering and the epithelia lining the digestive

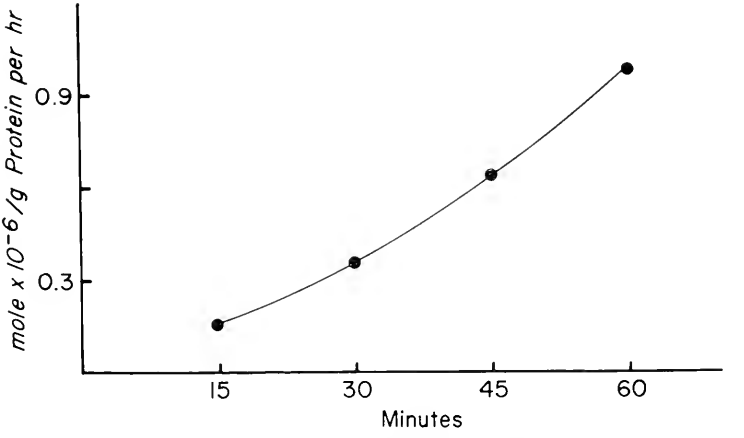

FIGURE 4. The effect of time on the incorporation of 10^{-5} M external alanine into protein of *L. ruber*. Data based on the mean of four samples per time interval.

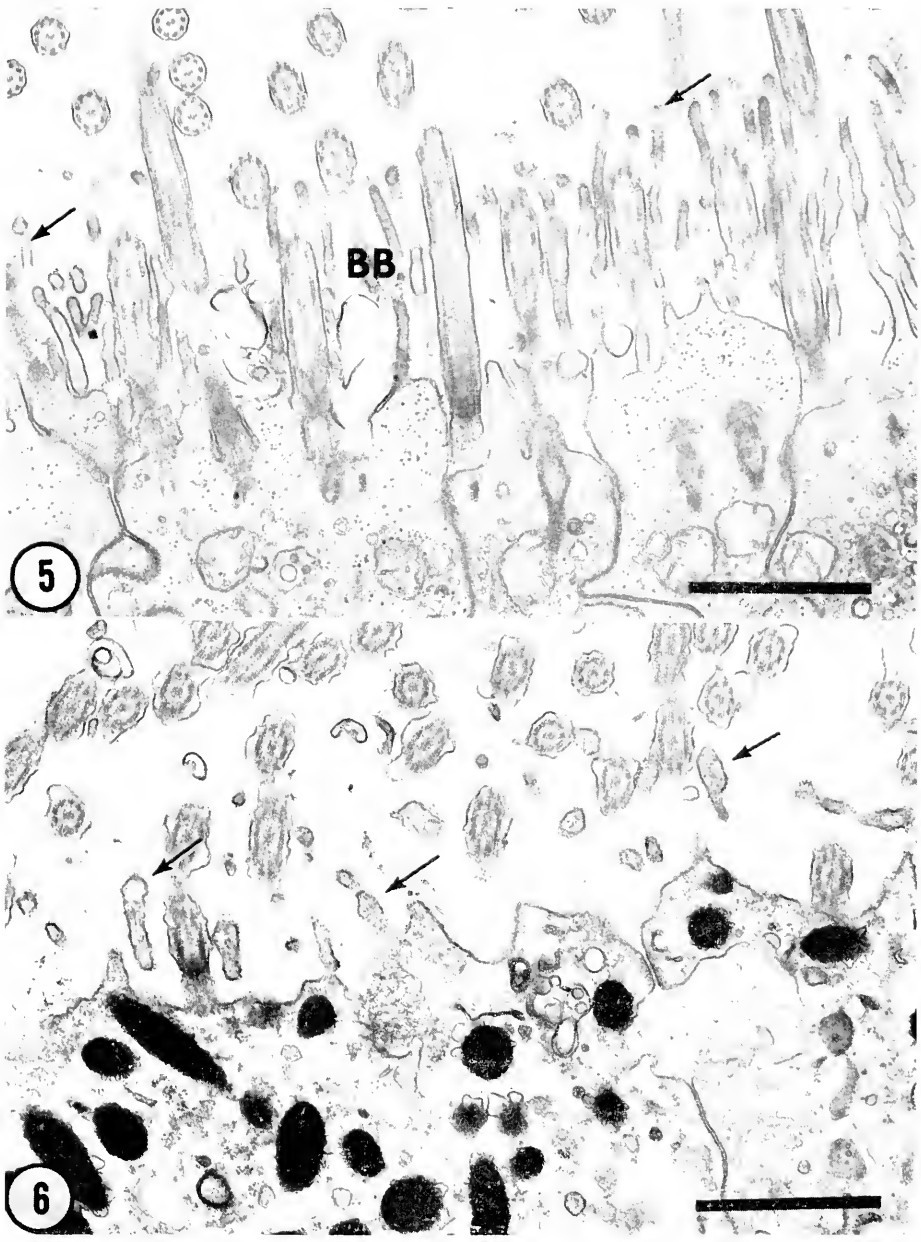

FIGURE 5. Ultrastructure of the external surface of *Lineus ruber* epidermis. Apical cytoplasm and plasmalemma of the free surface possess cilia which extend beyond the outer limits of the brush border (BB), defined by the tips of the microvilli (arrows). Note the close spacing of adjacent microvilli. Bar equals 1 μm.

FIGURE 6. Ultrastructure of the luminal surfaces of *Lineus ruber* gut. Apical cytoplasm and plasmalemma of the intestinal free surface possess a sparse population of microvilli (arrows) projecting from the intestinal surface. Note that these microvilli are not arranged in a parallel array as observed at the epidermal surface. Bar equals 1 μm.

system. Both epithelial surfaces of *Lineus* were examined for structural specialization which could account for the uptake of the dissolved compounds. The free surfaces of the gut epithelium and of the epidermis possess microvilli and cilia amplifing their free surface (Figs. 5, 6). However, the microvilli are far more numerous and regularly distributed on the apical surfaces of epidermal cells than they are on the corresponding surfaces of the intestinal epithelial cells (Fig. 5). The close register of microvilli at the epidermal surface resembles that described for the brush borders present at the surfaces of organs and tissues modified for absorptive function. In contrast to the epidermis, the distribution of microvilli at the surface of the intestinal epithelium in *Lineus* is relatively sparse and less consistently organized (Fig. 6) resembling the surfaces where the function of surface amplification is not well understood (*i.e.,* vertebrate trachea, Steinman, 1968; or the trematode miracidium, Wilson, 1969).

Discussion

There are diverse opinions on the role of dissolved reduced carbon compounds as a source of energy for marine metazoans. Stephens (1967) pointed out that the concept of the utilization of dissolved organic material in the nutrition of aquatic animals is not new and that Pütter (1908a, b) first advanced the idea of their importance. Later Krogh (1931) dismissed this notion and concluded that there was no substantial evidence to support Pütter's hypothesis.

Investigators, too numerous to completely list here, have since shown that various soft-bodied marine invertebrates can remove dilute organic solutes from sea water (*i.e.,* Stephens, 1968), some at concentrations as low as 3×10^{-9} M (Goreau, Goreau, and Yonge, 1971). Johannes, Coward and Webb (1969) criticized the methodology involved in most uptake studies using amino acids because the net efflux of free amino acids has seldom been measured. The simultaneous movements of such compounds into and out of biological systems is well documented. Wilbrandt and Rosenberg (1961) and Johannes *et al.* (1969) stress that there is a net loss of free amino acids during most uptake experiments involving radioactive substrates. However, the significant increase of internal free glucose in *Lineus ruber* incubated in 10^{-4} M glucose suggests that the amount of glucose lost by efflux is relatively small in comparison to the accumulated glucose available to the worm's metabolism. Therefore, some organic solutes available in the organism's environment could serve as a significant source of nutrition for this nemertean.

During the two-minute incubation period used to determine initial rates of glucose accumulation, there are no metabolites of glucose excreted into the incubation medium, and less than 0.5% of the radiocarbon inside the worm is identifiable as nonglucose moieties. During a longer incubation, however, there is metabolism of glucose to succinate. This latter metabolite subsequently appears in the external sea water, and the concentration of this acidic end product increases with extended incubation periods. Absorbed glucose is readily incorporated into the nemertean's polysaccharide and almost equivalent amounts of radiocarbon are incorporated into the proteins of the worm body. Preliminary analysis of the TCA precipitable fraction indicates that the radiocarbon is present primiarily as alanine with a small amount of aspartic acid suggesting that one pathway of glucose metabolism in *L. ruber* may resemble those reported for a number of parasitic platyhelminths

(Von Brand, 1966). These data also strongly indicate that this hexose absorbed from the surrounding sea water is, in fact, serving as an energy source for this organism. It should be emphasized that the concentration of glucose used in these experiments is consistent with the values reported for dissolved carbohydrate in oceanic waters (Wangersky, 1952; Wangersky and Guillard, 1960; Walsh, 1965a, b, 1966; Walsh and Douglass, 1966).

Absorbed alanine enters the metabolic systems of the worm, since it is readily incorporated into the TCA precipitable fraction during incubation periods of moderate length. Although preliminary in nature, these data indicate that amino acids from the surrounding sea water can and do serve as a source of amino-nitrogen for protein synthesis. The concentration of substrates used in these experiments are also within the range of those reported for oceanic and estuarine waters (Adams and Richards, 1968; Belser, 1959 and 1963; Chau and Riley, 1966; Siegel and Degens, 1966; Webb and Johannes, 1967).

Stephens and Schinske (1961) described the removal of amino acids from sea water by numerous invertebrates belonging to eleven phyla. In their experiments only arthropods failed to remove such solutes from the surrounding water. The presence of a hard, acellular, relatively impermeable cuticle on the exterior of arthropods and an epidermis on soft-bodies marine invertebrates suggests that the epidermis may be the site for absorption of some soluble substances from sea water. The observed morphology of the external surface of *Lineus ruber* is typical of many of those soft-bodied organisms. MacRae (1967) has also found brush border microvilli amplifying the epidermal surface area in contact with sea water on Turbellaria; Lloyd (1969) and Lane (1963) on molluscs; Little and Gupta (1968) and Norrevang (1965) on pogonophorans; Potswald (1971) on annelids; and Menton and Eisen (1970) on echinoderms. Except for the cilia present among the brush border's microvilli in some invertebrate epidermises, these brush borders resemble those on tissue surfaces known to possess high rates of transport of amino acids and monosaccharides, such as the vertebrate intestine, the proximal tubule of the kidney and the tegument of tapeworms (reviewed by Lumsden, 1975). The resemblance of these surfaces is also consistent with the hypothesis that the epidermis covering *Lineus ruber* may be the site of absorption of nutrient molecules present in sea water. The epithelium of the digestive system in *Lineus ruber* also possesses microvilli. These could serve as a second site of solute absorption from sea water, even though they are fewer in number on the intestinal surface than those present on the epidermis. Jennings (1969) suggests that the intestinal epithelium serves to phagocytize partially digested material from the lumen of the gut rather than as a primary surface for nutrient solute transport. In line with that suggestion, our experiments, involving the use of ligatured and unligatured nemerteans, indicates that the epidermis investing this worm is the most important, if not the sole route, in solute feeding.

In a preliminary report, Fisher and Cramer, (1967) suggested that the membrane transport of solutes represented a new feeding mechanism in the phylum Rhynchocoela. We have shown that glucose enters *L. ruber* by a mediated process. Our data and that of Fisher (unpublished) suggest that the accumulation of glucose is competitively inhibited by galactose. Three glucosides also inhibit glucose

permeation; however, amino acids, fatty acids and organic acids are without effect. This accumulation process for glucose can be described as an active transport system (Fisher, unpublished). The facts that the concentration of chemically determined glucose inside the worm is greater than that in the external sea water, that a stereoisomer of glucose inhibits uptake and that glucose is accumulated against a concentration gradient also support the notion that this is a mediated process.

Alanine and leucine also enter *L. ruber* by a mediated process which demonstrates saturation kinetics. Entry of these compounds is inhibited by other neutral amino acids; however, acidic and basic amino acids, as well as proline and taurine, do not inhibit the uptake of alanine and leucine. Our data and that of Fisher (unpublished) indicate that the uptake of alanine is competitively inhibited by leucine.

The undiminished accumulation of glucose in ligatured and nonligatured animals, the incorporation of glucose into polysaccharide, the synthesis of amino acids from hexose with subsequent incorporation into protein, the incorporation of an absorbed amino acid into protein fraction, and the consistency of the epidermal morphology with other tissues which are known to transport solutes, strongly support the notion that this surface of *Lineus ruber* serves as a functional feeding mechanism, capable of metabolite accumulation from sea water in its littoral habitat.

Summary

1. *Lineus ruber* rapidly accumulates glucose from sea water into free pools within the worm concentrating the hexose to 17 times the original external concentration (10^{-4} M) in one hour.

2. Accumulated glucose, alanine and leucine are incorporated into protein, and additional glucose is incorporated into polysaccharide. No free glucose, alanine or leucine is effluxed during two minutes; however, succinate, derived from glucose, is detectable in the external medium when the incubation time is extended.

3. The demonstration of saturation kinetics for both glucose and galactose, the partial inhibition of glucose entry by galactose and inhibition of glucose accumulation by phlorizin, quercetin and ouabain is consistent with specific sites of glucose transport.

4. Similar kinetics for both alanine and leucine accumulation, their mutual competition for entry and the inability of carbohydrates, organic acid, and fatty acids to influence the uptake of alanine and leucine is consistent with specific transport sites for neutral amino acids.

5. Comparison of glucose accumulation by whole ligatured and unligatured worms, as well as along the length of unligatured worms, indicates that a majority of the sites of entry available to glucose in the worm's environment is through its epidermis.

6. Ultrastructural examination of free epidermal and gut luminal surfaces reveal that each is bounded by a plasmalemma with a surface area expanded by microvilli. The surface area of epidermis is greatly increased by numerous microvilli arranged in the form of a brush border and is greater than the analogous surface region of the gut. Presence of a brush border is characteristic of tissues with high rates of transport function.

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ANTENNULAR CHEMOSENSITIVITY IN THE SPINY LOBSTER, *PANULIRUS ARGUS*: STUDIES OF TAURINE SENSITIVE RECEPTORS [1]

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A recent study of the antennular chemosensory system in *Panulirus argus* showed that the low molecular weight fractions of extracts of several potential food organisms duplicated the receptor activity elicited by the total unfractionated extracts (Ache, Fuzessery and Carr, 1976). Further, in at least one of the above extracts, the amino acids were shown to account for a large portion of the activity with taurine being the single most stimulatory amino acid (Johnson and Ache, 1978). Taurine emerges as an effective stimulant in other crustacean studies as well (Case, 1964; Crisp, 1967; Ache, 1972; Shepheard, 1974; Carr and Gurin, 1975; Fuzessery and Childress, 1975; Allison and Dorsett, 1977). Taurine sensitive receptors, with response thresholds as low as 10^{-10} M, occur on both the lateral and medial antennular filaments of the spiny lobster (Fuzessery, in preparation). The present study examines the molecular specificity of taurine sensitive receptors by comparing the stimulatory capacity of taurine with that of taurine analogs, derivatives, and structurally related compounds. The results indicate that antennular taurine receptors of *P. argus* are characterized by a narrow and consistent specificity similar to that of the taurine endoreceptors of diverse organisms.

Materials and Methods

Excised antennular filaments were fitted with a Sylgard sleeve over their proximal end, and inserted into a tubular stimulating chamber. The sleeve separated fluid in the stimulating chamber from a second compartment containing about 10 ml of *Panulirus* saline (Mulloney and Selverston, 1974) into which the filament's proximal end projected. The preparation was perfused with oxygenated *Panulirus* saline introduced under pressure through a tapered glass capillary inserted in the cut distal tip of the filament. Axons were exposed for recording by cutting the articular membrane between the fourth and fifth most proximal segments of the filament and removing the cuticle in the manner of removing insulation from a wire. Care was taken to place minimal stress on the axon bundle during this process. Receptor activity was recorded extracellularly using a monopolar plati-

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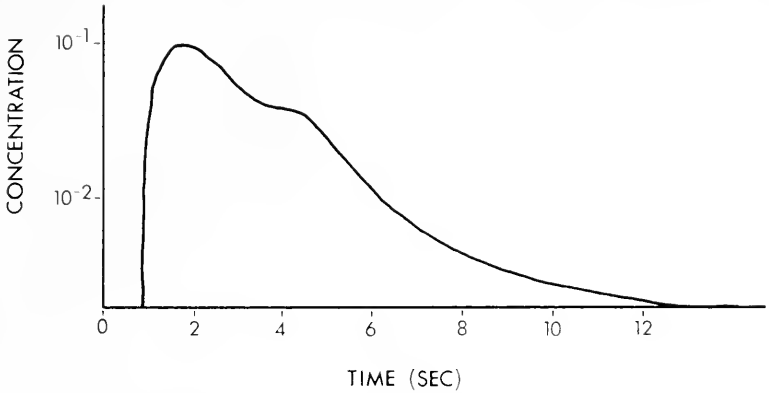

FIGURE 1. Temporal profile of 50-μl stimulant pulse as monitored by densitometry.

num-iridium hook electrode referenced against an Ag-AgCl pellet submerged in the 10 ml saline bath. Signal amplification and display involved standard electrophysiological instrumentation. All activity was stored on magnetic tape for subsequent analysis.

Reagent-grade artificial sea water (ASW, MBL formula) continuously entered the stimulating chamber at the filament's proximal end and flowed distally over the filament at a rate of 10 ml/min. Fifty-microliter pulses of test stimulant were pipetted into this carrier flow of ASW through a port 2 cm upstream from the preparation. Figure 1 shows the temporal profile of a stimulus pulse, measured by monitoring a pulse of methylene blue with a densitometer located at the midpoint of the tubular compartment.

All compounds employed in the study were obtained from commercial sources and used to prepare 10^{-4} M stock solutions in ASW. These were frozen until needed, thawed and serially diluted with ASW to the required test concentrations. All solutions were tested at the pH (7.5) and temperature (*ca.* 22° C) of the carrier ASW flow.

The general protocol in each experiment was to search for single taurine sensitive neurons while stimulating with 10^{-5} M taurine. Nerve bundles containing taurine sensitive units were sub-divided until only the taurine sensitive unit remained, or the taurine sensitive unit could be clearly discriminated from background multiunit activity. Single units were identified as such by consistent amplitude, configuration, regularity of interspike interval and relative response latency. Unless otherwise indicated, the entire group of compounds tested in an experiment was applied to each taurine sensitive receptor. Taurine was applied at the beginning, midpoint and end of each test series. Any loss of activity in response to the final taurine application voided that test series. The application sequence of test compounds was randomized. A 30-sec period followed the introduction of each test solution, during which time the filament was flushed vigorously with two 1-ml injections of ASW. Preliminary trials indicated 30 sec was sufficient time for full receptor recovery at the stimulant concentrations used. Procedural details unique to specific experiments are included in Results.

Response parameters of maximum impulse frequency, number of impulses/response and response duration were quantified by playing taped responses through a window discriminator and electronic counter (Haer 7400 series). The transformed output was displayed on a storage oscilloscope in the form of a post-stimulus time histogram of the impulses/100 msec over the duration of the response. Maximum impulse frequency was determined by observing the greatest number of impulses collected in a single 100 msec time interval. The number of impulses/response was determined as the sum of the impulses in all time intervals over the duration of the response. The index of relative stimulatory capacity (RSC) used in this study to compare stimulants was calculated as the number of impulses/response elicited by a given compound divided by the number of impulses/response elicited by taurine × 100. Hence, the RSC value for taurine on each receptor is 100. In a few cases where chemoreceptors were spontaneously active, an index of average baseline activity in the absence of chemical stimulation was calculated and subtracted from the activity elicited by test compounds in that individual receptor.

Results

Preliminary tests of taurine—dose/response relationships

Taurine was tested over a concentration range of 10^{-11} to 10^{-4} M on 18 lateral and 18 medial filament receptors. The average values for maximum impulse frequency, impulses/response and response duration are shown in Figure 2. Maxi-

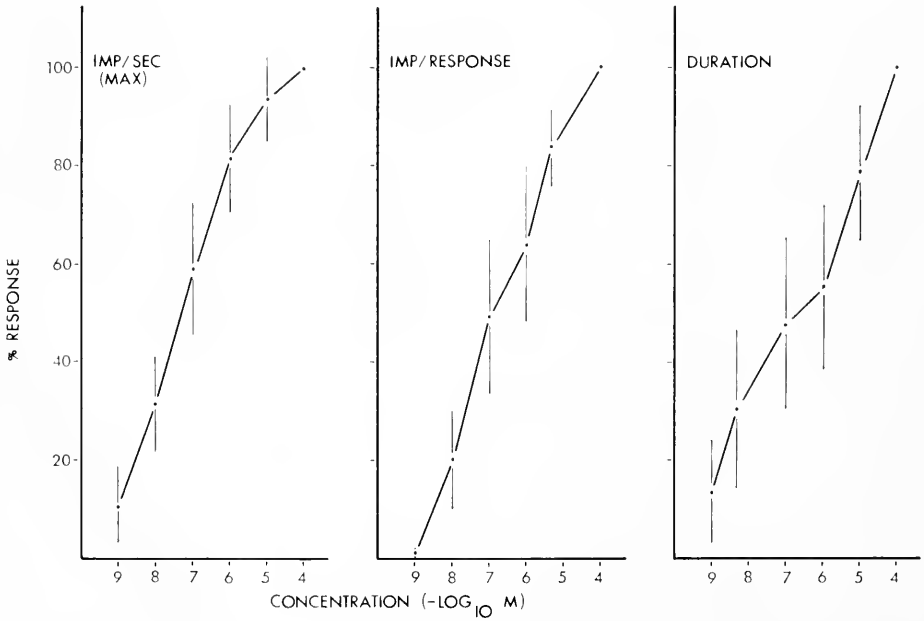

Figure 2. Average dose/response relationships given by 36 antennular receptors to taurine stimulation. Ordinate indicates percentage of maximum response, *i.e.*, that to 10^{-4} M taurine.

TABLE I

Relative stimulatory capacity (RSC) of eight taurine analogs and derivatives tested on 21 taurine sensitive receptors. All compounds were tested at 10^{-5} M. Value of taurine response is an arbitrary 100 in all cases. Blanks indicate no response. RSC values are based on total impulses/response.

Compound	Receptor number																				$\bar{X}$*	s.d.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
β-Alanine	66	82	49	49	67	14	14	36	47	40	55	59	73	57	5	79	47	43	80	94	46	52	±23
Hypotaurine	56	82	71	51	17	3	15	26	40	52	46	44	38	30	5	71	46	41	74	72	25	43	±23
2-Aminoethyl phosphonic acid																				43		2	± 9
Aminomethyl sulfonic acid																11	21	45				4	±10
Cysteic acid																13			10			1	± 3
Hydroxyethane sulfonic acid																				25	12	2	± 6
Ethane sulfonic acid																			14			1	± 3
2-Chloroethane sulfonic acid																						0	

* $\bar{X}$ = average RSC value. For individual receptors, an RSC value of zero was recorded when there was no response to a compound.

mum impulse frequency began to plateau at concentrations of 10^{-5} and 10^{-4} M, while the total number of impulses and the response duration increased regularly over the entire concentration range. The large standard deviations of each parameter reflect in part variations in sensitivity among receptors. Individual threshold concentrations ranged from 10^{-8} to 10^{-10} M. As subsequent data will indicate these deviations also reflect variations in the slopes of the dose/response curves that are characteristic of individual receptors. Based on these findings, a standard test concentration of 10^{-5} M taurine was chosen to insure a strong yet nonsaturating response from all receptors.

Specificity of taurine sensitive receptors

The stimulatory capacity of taurine (= 2-aminoethyl sulfonic acid) was compared with that of three analogs and five related sulfonic acids. All compounds were tested at 10^{-5} M on each of 21 taurine sensitive receptors on the lateral and medial filaments. Calculations of the relative stimulatory capacity (RSC) for each compound on each receptor are summarized in Table I. A comparison of the RSC values reveals that only taurine and its carboxylic and sulfinic acid analogs, β-alanine and hypotaurine, stimulated all receptors.

Other comments on data in Table I are presented below following the presentation of some additional results.

To further define response specificity, thirteen additional compounds, structurally-related to taurine, were tested at 10^{-5} M on each of 18 taurine sensitive lateral- and medial-filament receptors. The resulting RSC values are presented in Table II. Structural formulae of compounds that were tested are shown in Figure 3. Conclusions concerning receptor specificity are summarized below.

1. Compounds with one terminal basic group and one terminal acidic group separated by two carbon atoms were most effective. Taurine and its analogs, hypotaurine, β-alanine and 2-aminoethyl-phosphonic acid all meet these structural requirements. Though less stimulatory than taurine, the analogs hypotaurine and β-alanine stimulated all receptors and their RSC values with individual receptors were consistently similar. The phosphonic acid analog was dramatically less effective and elicited a response from only one of the 21 receptors tested (Table I).

2. Compounds with one terminal basic group and one terminal acidic group separated by more than two carbon atoms were also effective although the RSC values decreased with the distance of separation of the charged groups. This is illustrated in Table II by comparing the RSC values of the following: β-alanine > γ-amino-n-butyric acid (GABA) > 5-aminovaleric acid > 6-aminocaproic acid. Note also that two isomers of GABA, 2-aminobutyric acid and 3-aminobutyric acid, with nonterminal amine groups, are markedly less effective than GABA.

3. Compounds with a terminal basic group and a terminal acidic group separated by only one carbon atom (rather than two carbon atoms) were markedly less effective. This is shown in Table I by the low incidence of receptor stimulation and the low RSC value of aminomethyl sulfonic acid (AMS). Note that AMS, like taurine, has terminal amine and sulfonic acid groups. Later in the report data are presented to show that the AMS analog, glycine, as well as other α-amino acids are virtually ineffective in taurine sensitive receptors.

TAURINE AND ANALOGS

$H_2N-CH_2-CH_2-SO_3H$
Taurine (VA)

$H_2N-CH_2-CH_2-SO_2H$
Hypotaurine (VA)

$H_2N-CH_2-CH_2-CO_2H$
β-Alanine (VA)

$H_2N-CH_2-CH_2-PO_3H_2$
2-Aminoethylphosphonic acid (I)

OTHER SULFONIC ACIDS

$H_2N-CH_2-SO_3H$
Aminomethyl sulfonic acid (SA)

$CH_3-CH_2-SO_3H$
Ethane sulfonic acid (I)

$HO-CH_2-CH_2-SO_3H$
Hydroxyethane sulfonic acid (I)

$Cl-CH_2-CH_2-SO_3H$
2-Chloroethane sulfonic acid (I)

$HO_2C-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CH_2-SO_3H$
Cysteic acid (I)

COMPOUNDS WITH NON-TERMINAL BASIC GROUPS

$CH_3-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H$
2-Aminobutyric acid (I)

$CH_3-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CH_2-CO_2H$
3-Aminobutyric acid (I)

$H_2N-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H$
2,3-Diaminopropionic acid (I)

$H_2N-CH_2-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H$
2,4-Aminobutyric acid (I)

$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO_2H$
α-Amino acids (I)

COMPOUNDS WITH TERMINAL BASIC AND ACIDIC GROUPS

$H_2N-CH_2-CO_2H$
Glycine (I)

$H_2N-CH_2-CH_2-CH_2-CO_2H$
γ-Aminobutyric acid (A)

$H_2N-(CH_2)_4-CO_2H$
5-Aminovaleric acid (A)

$H_2N-(CH_2)_5-CO_2H$
6-Aminocaproic acid (SA)

$H_2N-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-CO_2H$
γ-Amino-β-hydroxybutyric acid (SA)

$H_2N-CH_2-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CO_2H$
β-Aminoisobutyric acid (A)

$H_2N-\underset{\displaystyle NH}{\underset{\|}{C}}-NH-CH_2-CO_2H$
Guanidoacetic acid (I)

$H_2N-\underset{\displaystyle NH}{\underset{\|}{C}}-NH-CH_2-CH_2-CO_2H$
Guanidopropionic acid (I)

$H_2N-CH_2-CH_2-\underset{\displaystyle O}{\underset{\|}{C}}-NH-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CO_2H$
β-Alanylalanine (I)

$H_2N-CH_2-CH_2-\underset{\displaystyle O}{\underset{\|}{C}}-NH-CH_2-CO_2H$
β-Alanylglycine (I)

FIGURE 3. Structural formulae of compounds tested on taurine sensitive receptors. Indices of relative activity are as follows: (VA), very active; (A), active; (SA), slightly active; (I), virtually inactive.

TABLE II

Relative stimulatory capacity (RSC) of 16 compounds structurally-related to taurine tested on 18 taurine sensitive receptors. All compounds were tested at 10^{-5} M. Value to taurine is an arbitrary 100 in all cases. Blanks indicate no response. RSC values are based on total impulses/response.

Compound	Receptor number																		$\bar{X}$*	s.d.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
Hypotaurine	76	88	38	82	53	30	99	58	41	50	63	73	70	56	77	89	54	32	63	±20
β-Alanine	43	67	48	82	27	45	76	71	59	44	51	64	74	64	79	81	63	29	59	±17
γ-Amino-n-butyric acid (GABA)						8	4	12	12		5	27	28	29	30	21	32	19	13	±12
β-Aminoisobutyric acid										3	5	10	12	9	24	13	63	12	8	±15
5-Aminovaleric acid												10	18	8	20	19	25	17	7	± 9
DL-γ-Amino-β-hydroxybutyric acid												5	12	5	10	9	16	10	4	± 5
6-Aminocaproic acid													18	1	3	7	21	10	3	± 7
Guanidoacetic acid															8		17	6	2	± 4
DL-3-Amino butyric acid															6	9		6	1	± 3
β-Guanidopropionic acid																	19	15	2	± 6
DL-2-Aminobutyric acid																		1		
Glycylglycine																		1		
β-Alanylalanine																		1		
β-Alanylglycine																		1		
2,3-Diamino-propionic acid																		1		
2,4-Aminobutyric acid																		1		

* $\bar{X}$ = average RSC value, see footnote to Table I.

4. Taurine derivatives lacking the basic amine group were markedly less effective. This is shown by the low incidence of receptor stimulation and the low RSC values of ethane sulfonic acid, hydroxyethane sulfonic acid and chloroethane sulfonic acid (Table I).

5. The addition of a neutral side chain decreased the effectiveness of a compound. γ-Amino-β-hydroxybutyric acid differs from GABA by having a hydroxyl group and yet has a much lower RSC value (Table II). Likewise, β-aminoisobutyric acid differs from β-alanine by having a methyl group and yet has a much lower RSC value.

6. Compounds with an *alpha*-amine group in addition to a terminal amine group were virtually ineffective. Note in Table II that 2,3-diaminoproprionic acid and 2,4-aminobutyric acid are virtually inactive, whereas the closely related compounds, β-alanine and GABA, have marked activities.

7. Compounds in which the terminal basic group is a guanido group rather than an amine group were far less effective. This is shown in Table II by the very low RSC values of guanidoacetic and β-guanidopropionic acid.

8. Two dipeptides containing the stimulatory amino acid β-alanine were ineffective thereby suggesting that activity is lost when the carboxyl group is involved in a peptide bond (Table II). Likewise, the presence of two acidic groups apparently negates activity as shown by the ineffectiveness of cysteic acid (Table I).

The data in Table II also indicate the existence of a distinct relationship between the average RSC value of a compound and the number of receptors responding to that compound. Hence compounds with higher RSC values elicited responses from a larger percentage of the receptors. This relationship implies strongly that the "taurine receptors" have a consistent and predictable specificity and thus appear to comprise a distinct receptor class. Regarding this specificity, no differences were observed between taurine sensitive receptors present on the lateral or the medial antennular filaments.

Additional tests of receptor specificity

In order to gain further insight into the restricted specificity of these cells, 12 α-amino acids, 3 organic acids, and the quaternary amine, glycine betaine, were tested at a concentration of 10^{-5} M on additional taurine sensitive receptors. Table III shows that individual compounds were applied to 5 to 65 receptors and that none of the new compounds cited above elicited responses. As in Tables I and II, the taurine analogs included in this test-series stimulated all receptors, whereas GABA and β-aminoisobutyric acid stimulated a large percentage of them. RSC values were not computed because in this phase of the study all of the compounds were not tested on all of the receptors. The inability of all α-amino acids to activate taurine sensitive receptors strongly supports the preceding results which indicated that amine groups in the *alpha* position reduced effectiveness. The ineffectiveness of the organic acids tested supports the earlier conclusion that stimulatory molecules require both positively and negatively charged atoms.

Quantitative effects of stimulatory compounds

Whereas the RSC values presented earlier for various compounds showed a consistent ranking with individual receptors, considerable variations were apparent

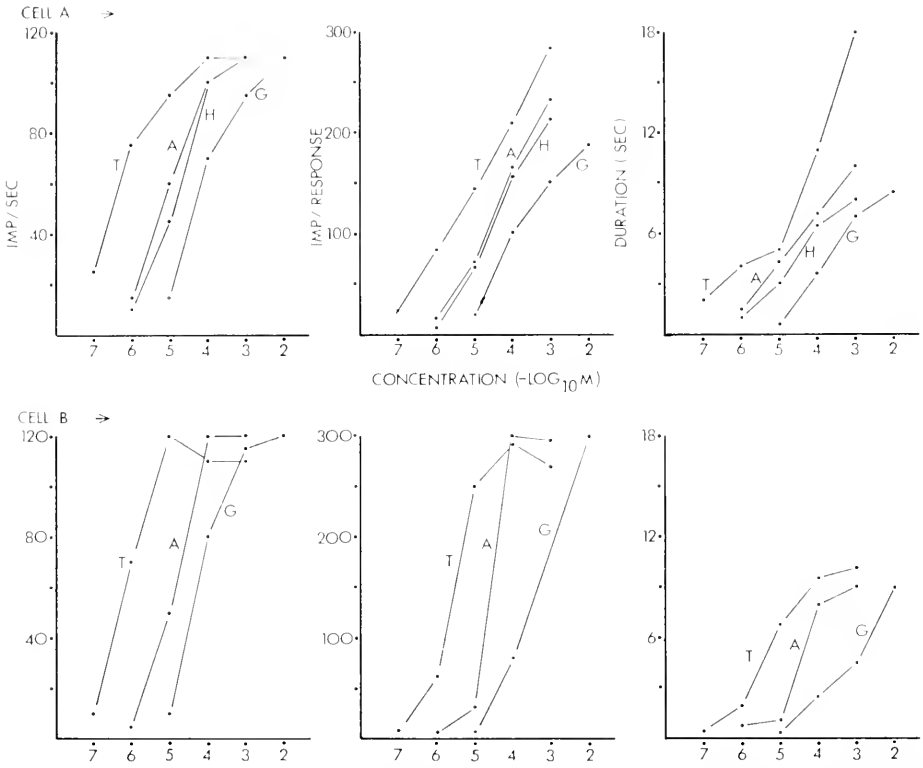

FIGURE 4. Three concentration-dependent response parameters (maximum impulse frequency, total impulses, and response duration) of two receptors stimulated with taurine (T), β-alanine (A), hypotaurine (H) and γ-amino-n-butyric (G).

in the RSC values obtained with individual compounds on different receptors. For example, β-alanine was usually the second more stimulatory compound, yet its RSC values ranged from 5 to 94 (Table I). To explore the basis of this variability, a detailed evaluation was made of three response parameters of two receptors tested with graded concentrations of the four most stimulatory compounds (Fig. 4). In both receptors, the concentration functions of the three parameters described a series of roughly parallel curves. Also in both receptors, maximum impulse frequency reached a maximum value and did not increase at higher concentrations. In the slower adapting receptor (Fig. 4A), impulses/response and response duration continued to increase with concentration; while in the more rapidly adapting receptor (Fig. 4B), these parameters reached maximum values at approximately the same concentration as frequency. This variation between the slow and fast adapting receptors likely results from the mode of stimulus introduction which was a pulse with an exponential dilution profile (see Fig. 1). Hence, as concentration increased, the period during which the pulse remained at a suprathreshold concentration also increased, thereby prolonging the response of the slow-adapting receptor.

In addition to the variations cited above, individual receptors also varied in sensitivity and in the profile of their dose-response curves. Note that the concentration function of impulses/response rises more sharply in the receptor represented in Figure 4B than that in Figure 4A. Individual variations in sensitivity indicate that response to the standard test concentration (10^{-5} M) will not occupy the same relative position on the dose-response curve of each receptor. In less sensitive receptors, a 10^{-5} M concentration of a given compound may be close to the threshold concentration. In very sensitive receptors, a 10^{-5} M concentration may be close to the plateau concentration. To return to the example of variation in the individual RSC values of β-alanine, it can be inferred that in a very sensitive, rapidly adapting receptor, the test concentration of 10^{-5} M may be near the plateau concentrations of both taurine and β-alanine, resulting in approximately equal RSC values. Conversely, in a less sensitive receptor, the test concentration may be near the threshold concentration of β-alanine, resulting in a very low RSC value. This inherent variability among receptors underscores the necessity of comparing RSC values only in cases where all compounds are applied to each receptor in the test population. These factors may also explain why GABA, the fourth most stimulatory compound, did not activate all receptors (Tables II and III). In less sensitive receptors, the test concentration may be below the threshold concentrations for GABA (see also Fig. 4).

That the three response parameters detailed in Figure 4A, B describe a series of roughly parallel curves suggests that these compounds effect impulse generation in

TABLE III

Sensitivity of taurine sensitive receptors to taurine analogs, α-amino acids and other compounds. All compounds were tested at 10^{-5} M.

Compound	Number of receptors tested	Number of receptors activated	Receptors activated (%)
L-α-Alanine	43	0	—
β-Alanine	33	33	100
α-Aminoisobutyric acid	8	0	—
β-Aminoisobutyric acid	8	5	63
γ-Amino-n-butyric acid	15	14	93
L-Aspartic Acid	30	0	—
Citric acid	10	0	—
L-Glutamic acid	19	0	—
Glycine	65	0	—
Glycine betaine	43	0	—
Hydroxy-L-proline	7	0	—
Hypotaurine	9	9	100
L-Isoleucine	7	0	—
L-Leucine	12	0	—
L-Lysine	8	0	—
Propionic acid	19	0	—
Succinic acid	19	0	—
Taurine	65	65	100
L-Tryptophan	7	0	—
L-Tyrosine	7	0	—
L-Valine	5	0	—

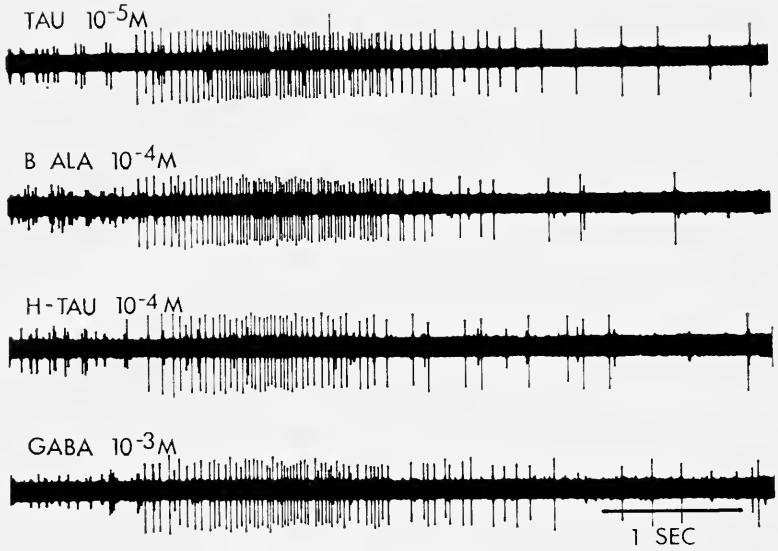

FIGURE 5. Response of single receptor to four stimulants with concentrations adjusted to elicit essentially equal-intensity responses. Time bar is 1 sec. Tau represents taurine; β-ala, β-alanine; H-Tau, hypotaurine; and GABA, γ-amino-n-butyric acid.

a manner mimicking the concentration function of a single compound. A less stimulatory compound effects receptor response in the same manner as a more stimulatory compound applied at a lower concentration. The functional implication is that the receptor response elicited by taurine at 10^{-5} M would be very similar to that of β-ala at 10^{-4} M and GABA at 10^{-3} M, as suggested by Figure 5.

DISCUSSION

These results indicate that the antennules of the spiny lobster, *Panulirus argus,* possess taurine sensitive receptors with a narrow and consistent response specificity. Previous electrophysiological studies of crustacean chemoreception indicate that individual receptors exhibit differential specificities to amino acids and related compounds (Laverack, 1964; Case, 1964; Ache, 1972; Shepheard, 1974; Fuzessery and Childress, 1975). The two studies dealing most thoroughly with receptor specificity (Case, 1964; Shepheard, 1974) provided values of the relative activity of compounds obtained by pooling results from the entire population of receptors tested. This practice treats all receptors as being effectively monotypic with respect to specificity. Moreover, in the above studies all compounds were not applied to each receptor in the test population. The latter procedure is essential for an analysis of both the specificity and the inherent variability of individual receptors. However, regarding the taurine sensitive receptors analyzed in the current study, some corroborating evidence is present in the study by Shepheard (1974) on another decapod crustacean, *Homarus americanus.* In a case in which 43

amino acids and related compounds were applied to a single receptor, only taurine and β-alanine were stimulatory.

It is important to emphasize that our current documentation of the distinct specificity of taurine receptors in *P. argus* was made possible largely by our early recognition of the extreme sensitivity of these receptors to taurine. This recognition led to our decision to work with a dilute (10^{-5} M) standard test concentration. As shown clearly in Figure 4, the apparent specificity of a receptor becomes less distinct as the test concentration is increased. The failure of earlier workers to detect receptor classes with distinct specificity in crustaceans may be due to using high stimulant concentrations (*ca.* 10^{-2} M).

Taurine sensitive receptors with a somewhat similar specificity to those found in antennules of *Panulirus argus* have been reported in endoreceptors serving a variety of functions. In the examples cited below, note that the activity of taurine was mimicked by the analogs hypotaurine and β-alanine but, in the instance where tested, not by the phosphonic acid analog. Also, in cases where tested, the taurine receptors were markedly less responsive to α-amino acids. Taurine is effective in suppressing induced heart seizures in dogs, and this action is most effectively mimicked by β-alanine, hypotaurine and GABA but not by glycine or α-alanine (Barbeau, Tsukada, and Inoue, 1976). Induced arrhythmia in dogs is suppressed by taurine but not by ethanesulfonic acid and other compounds lacking both basic and acidic groups (Welty, Read and Byington, 1976). In an active transport system in human blood platelets, taurine uptake is inhibited competitively by β-alanine and hypotaurine but not by the phosphonic acid analog (Grant and Nauss, 1976). Similarly, taurine uptake by rat brain slices is inhibited competitively by hypotaurine and β-alanine but not by α-amino acids (Kaczmarek and Davison, 1972; Lähdesmäki and Oja, 1973).

The inhibitory effect of GABA (= 4-aminobutyric acid) on crustacean stretch receptors is most effectively mimicked by 3 and 5 carbon chain amino acids with terminal amine groups, *i.e.*, β-alanine and 5-aminovaleric acid. 6-Aminocaproic acid was less effective, and glycine was essentially without effect. Taurine was less effective than its carboxylic acid analog, β-alanine (Robbins, 1959; Edwards and Kuffler, 1959). As in the present antennular system, the latter workers reported that the addition of neutral side chains reduced effectiveness, and that the presence of both the acidic and the basic groups were essential. In general, the GABA system appears to resemble the present one, differing primarily in that the ideal separation of opposite charges is three, rather than two, carbon atoms.

Similarities in the apparent specificity of both internal and external taurine receptors lend support to the concept that systems for molecular recognition, once evolved, may be preserved and used in a variety of functions, ranging from solute uptake and regulation to chemical sensing, synaptic transmission and others (Kittredge, Takahashi, Lindsey, and Lasker, 1974; Lenhoff, 1975). In the future we hope to provide a detailed model of the antennular receptor site for taurine. However, the presentation of such a model must await the testing of several additional analogs and derivatives that are not available commercially and hence must be specially synthesized.

Panulirus argus is a predator/scavenger that feeds on a variety of molluscs, arthropods, echinoderms and fish (Herrnkind, VanDerwalker and Barr, 1975).

Analyses of tissue extracts of marine molluscs, arthropods, echinoderms and fish show that the taurine concentration ranks from first to fifth in the total pool of free amino acids (Carr, 1976; Carr, Blumenthal and Netherton, 1977). Taurine is certainly the most abundant β-amino acid in most marine animals. According to Awapara (1976 p. 1), "taurine exists uncombined and distributed throughout the animal kingdom in a manner almost unparalleled by any known small organic molecule." Therefore, it is clear that taurine receptors could be expected to provide sensory information on the proximity of an array of suitable food organisms. Although the taurine analogs, hypotaurine and β-alanine, are also very stimulatory to the antennular taurine receptors, both of these compounds occur in only minor concentrations in the tissues of most organisms (Sturman, Hepner, Hofmann, and Thomas, 1976; Awapara, 1976). Hence, one must assume that the potential chemosensory role of these other stimulants is far less than that of taurine.

It is of special interest that taurine receptors are very insensitive to α-amino acids, particularly since these compounds are present in high concentration in the tissues of many marine animals. The antennular chemosensory system appears to be so constructed that a portion of the total receptor population responds to a single, ubiquitous β-amino acid, and is functionally insensitive to other commonly occurring amino acids. Perhaps the significance of this finding resides in the fact that α-amino acids are common constituents of sea water, occurring at individual concentrations of 10^{-7} to 10^{-9} M (Duursma, 1965). Comparable concentrations of taurine have not been reported. A plausible speculation may be that dissolved α-amino acids produce a chemical "white noise" against which chemosensory-based discrimination must occur. Taurine receptors would be unaffected by ambient α-amino acid levels, and therefore may provide less ambiguous information regarding the proximity of potential prey.

In the present antennular system, taurine appears to comply with Beets' (1971) definition of a nonideal mono-osmatic odorant, *i.e.*, a single compound which activates a single receptor at lower concentrations than other compounds within the specificity of that receptor. In addition, when one considers the chemical composition of the natural foods of *P. argus*, taurine is the only compound that we have tested which is likely to be present in sufficient concentrations to activate these receptors. From a functional standpoint, the antennular taurine receptors can be considered specialist receptors which may serve to monitor the presence of a single compound. This is particularly significant in that it is one of the few cases in which specialist receptors have been identified which may play a role in the mediation of feeding behavior, and the first documentation of such receptor organization in crustacean chemoreceptors.

Summary

1. Taurine sensitive receptors in the antennules of the spiny lobster, *Panulirus argus*, were identified electrophysiologically.

2. Recordings from single receptors revealed a narrow and consistent specificity when tested with taurine, taurine analogs and derivatives, and structurally related compounds.

3. Taurine was the most stimulatory compound tested. Threshold concentrations for 36 individual receptors ranged from 10^{-8} to 10^{-10} M.

4. The taurine analogs, hypotaurine and β-alanine, were also very effective but the phosphonic acid analog of taurine was ineffective.

5. Regarding receptor specificity, receptor stimulation was greatest with compounds having single terminal basic (amine) and acidic groups separated by two carbon atoms. Compounds having terminal basic and acidic groups separated by three to five carbon atoms were also active. However, activity decreased with the distance of separation of charged groups.

6. *Alpha*-amino acids and compounds with terminal basic and acidic groups separated by only one carbon atom were virtually ineffective.

7. Receptor stimulation was markedly less with structurally related compounds that either lacked a terminal amine group, had additional amine or acidic groups, or had neutral side chains.

8. Dose/response relationships of four differentially stimulatory compounds (taurine, hypotaurine, β-alanine and γ-aminobutyric acid) applied to single receptors were compared and found to describe a series of roughly parallel lines. This implies that a less stimulatory compound effects receptor response in the same manner as a more stimulatory compound applied at a lower concentration.

9. The possible role of taurine in food finding, and the similarity of the specificity of antennular taurine receptors and taurine endoreceptors identified in various organisms are discussed.

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LARVAL DEVELPOMENT OF THE RARE BURROWING MUD SHRIMP *NAUSHONIA CRANGONOIDES* KINGSLEY (DECAPODA: THALASSINIDEA; LAOMEDIIDAE)

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Thompson (1903) discovered unusual larvae in the plankton of Woods Hole, and after comparing postlarvae obtained from planktonic late stage larvae with adult specimens of *Naushonia crangonoides,* he was able to attribute the planktonic larvae to this species. Adults of *Naushonia crangonoides* are known only from the Woods Hole region (Williams, 1974), but larvae similar to Thompson's have been taken in Delaware Bay (Deevey, 1960), Narragansett Bay (Hillman, 1964), and Chesapeake Bay (Sandifer, 1972; Goy, 1976). Larvae from Chesapeake Bay show a number of differences from the description given by Thompson. Moreover, at least one other species of the genus is known from western Atlantic waters (Rathbun, 1901; Gurney and Lebour, 1939).

The account of the early development of *N. crangonoides* as given by Thompson is incomplete in description and in illustrations, making identifications of planktonic larvae difficult. The purpose of the present study is to provide a redescription of the larval development of *Naushonia crangonoides,* and to review larval characters in the family Laomediidae.

Materials and Methods

First stage larvae of *N. crangonoides* were taken in plankton collections off Cape Henry, Virginia, U.S.A. (36° 56′ 45″; 76° 00′ W) on June 30, 1976 and July 28, 1976. When brought to the laboratory, the larvae were immediately placed individually into compartmented plastic trays with 25 ml of 25‰ artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio, U.S.A.). Freshly hatched *Artemia salina* nauplii (San Francisco strain) were added to the compartments daily as food. All zoeae were placed in a darkened incubator at 25° C, receiving light only 1 to 2 hr/day when the water was changed and new food provided.

Postlarvae and juveniles, as they reached these stages, were transferred to 8.0 cm diameter culture dishes (125 ml capacity) containing 75 ml of 25‰ sea water and substrate in the form of fine sand or fine mud, to facilitate observation of burrowing behavior.

A daily record of molting, mortality, and sequence of larval stages was kept. Larvae and exuviae of known history were preserved in 70% ethyl alcohol. To obtain more material for comparisons of appendages, dead animals were slowly heated in 5% KOH for approximately ten minutes to remove tissue from the exo-

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skeleton. These specimens and all casts from molted animals were stained in either Mallory's Acid Fuchsin Red or Chlorazol Black E (1% in 70% alcohol). The dissection of the appendages was done in lactic acid, followed by mounting in glycerin jelly. Drawings were made with the aid of a camera lucida; measurements were made with the aid of a stage micrometer. Total length of larvae, postlarvae, and juvenile stages was measured from the tip of the rostrum to the most posterior margin of the telson, and excluded all telson processes and setae. Length of carapace was measured from tip of the rostrum to the posterolateral margin of the carapace.

Duration refers to the time spent in a given stage by zoeae that survived the molt to the succeeding stage. The term *stage* is used here to refer to the intermolt phase of larval development.

Results

First stage specimens of *Naushonia crangonoides* reared under laboratory conditions reached the postlarval stage after six or seven zoeal intermolts. Length measurements for each prejuvenile stage, range and mean duration data for these stages are given in Table I.

Of 18 first stage larvae collected from plankton, four died without molting and four of the remainder reached postlarva. A third and a fourth stage larva were also collected from plankton and produced postlarvae after molting several times. Of the six postlarvae obtained in the laboratory, only two molted to the next stage, one of them reaching fifth juvenile instar in 53 days after metamorphosis.

The intermolt period is approximately seven days for the first two molts after postlarva and approximately 14 days for each succeeding molt. The animal increases in total length by less than 0.5 mm with each successive molt.

Postlarvae were observed to feed on *Artemia* but not on detritus provided. Juveniles following the postlarva fed only on detritus and not on *Artemia*. No burrowing behavior was observed.

Table I

Range and mean duration, total length and carapace length of the larval stage of Naushonia crangonoides.

Stage	Number of specimens molting to next stage	Range of duration (days)	Mean duration (days)	Number of specimens measured	Total length (mm)	Carapace length (mm)
I	13	5–8*	5.24*	3	2.3– 2.6	0.8–0.9
II	11	3–5	3.73	2	2.9– 3.4	0.9–1.2
III	11	3–7	3.82	2	4.5– 5.0	1.5–1.8
IV	13**	3–6	4.76	2	6.3– 6.6	1.8
V	11	3–9	4.73	7	7.1– 8.0	2.1–2.5
VI	7	3–12	9.28	2	7.8– 8.5	2.2–2.7
VII	2	6–7	6.50	2	9.6–10.4	2.8–3.7
PL	2	3–4	3.50	6	4.5– 4.8	2.7–2.9

*First stage zoeae were collected in the plankton so this duration is probably an underestimate.
** Includes two planktonic specimens.

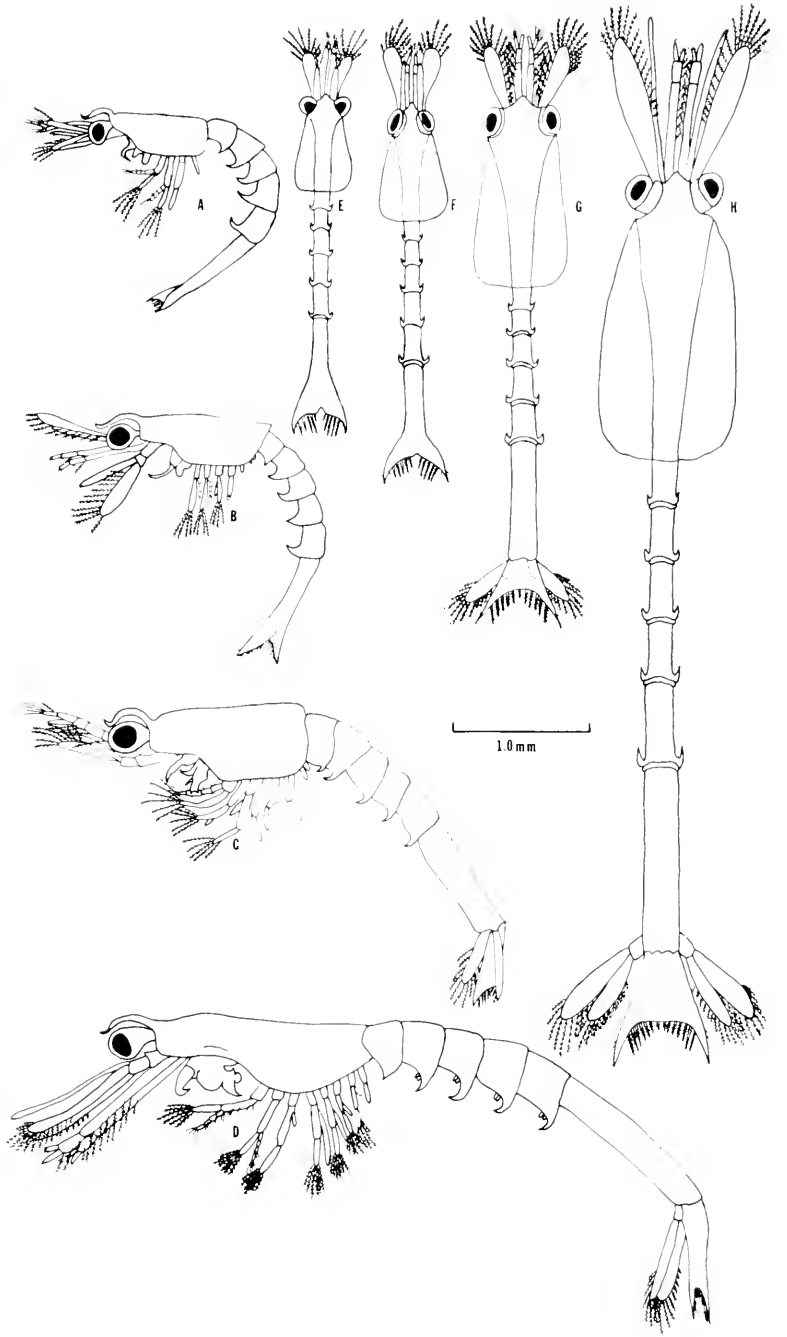

FIGURE 1. *Naushonia crangonoides:* lateral view of zoeal stages I (A), II (B), III (C), and VI (D); dorsal view of zoeal stages I (E), II (F), III (G), and VI (H).

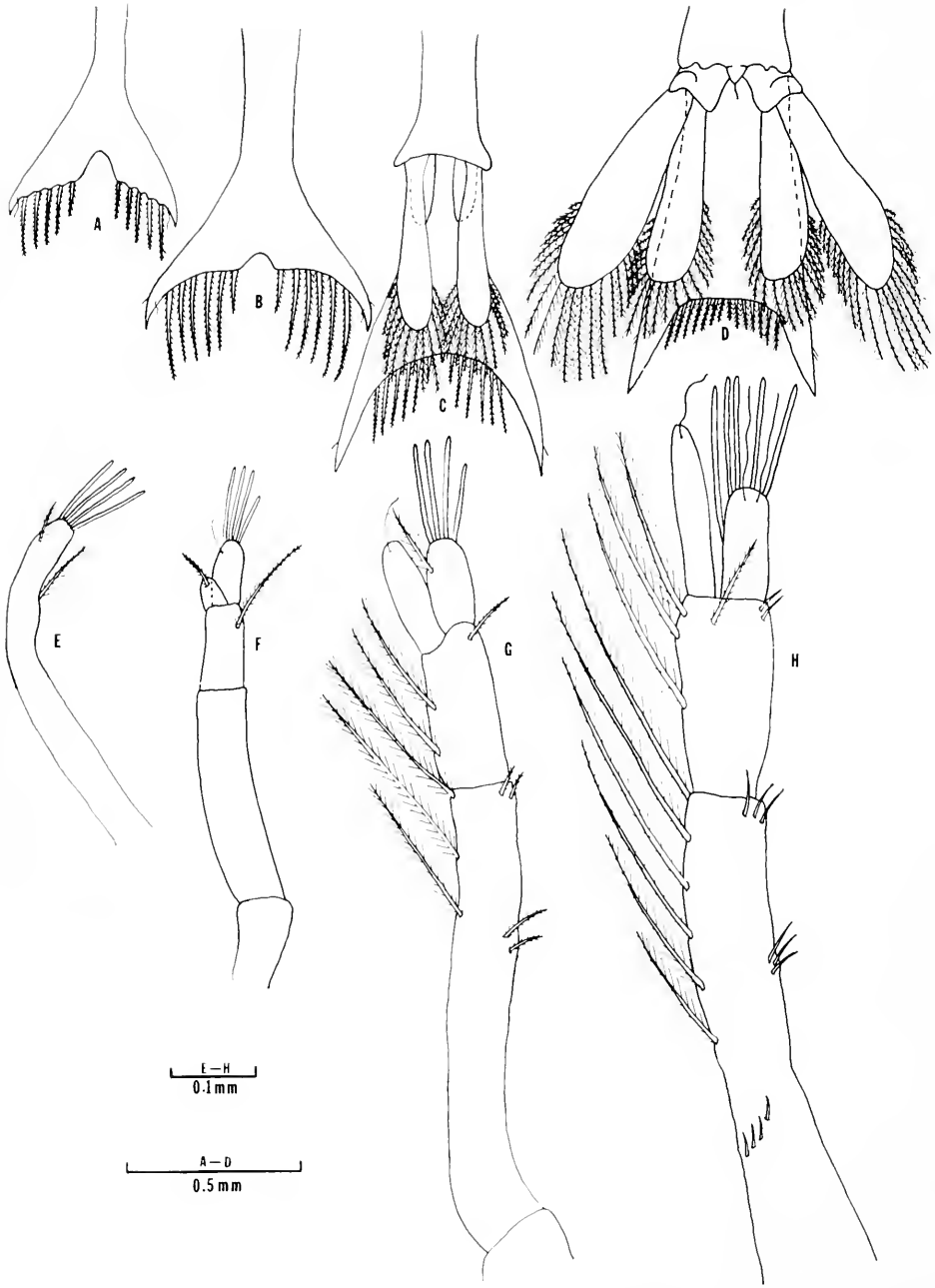

FIGURE 2. *Nanshonia crangonoides:* telson of zoeal stages I (A), II (B), III (C), and VI (D); antennule of zoeal stages I (E), II (F), III (G), and VI (H).

First zoea (*Figs. 1A, E*)

Rostrum small, slender, upturned at end. Carapace smooth with no spines forming short "neck" forward of mandibles. Eyes sessile. Abdomen without spines but somites modified with small procurved pleural hooks. Hooks only imperfectly developed on first somite in earlier stages; completely absent on sixth somite in all stages. Sixth somite fused to telson. Telson (Fig. 2A) triangular, with deeply notched posterior margin, bearing five pairs articulated plumose setae; pair of external spines; pair of fine hairs representing reduced second telson process.

Antennule (Fig. 2E) uniramous extending beyond rostrum, slightly longer than antenna; bearing four large aesthetascs, one small plumose seta terminally, and a long plumose seta subterminally.

Antennal endopodite articulated to basipodite (Fig. 3A) bearing three terminal plumose setae. Antennal scale narrow, oval, bearing ten plumose setae on medial margin, most distal being smallest. Basipodite with spine at base of scale.

Mandibles (Figs. 3E, 3F) asymmetrical, left one sickle-shaped, bearing on inner surface of base four stout teeth; inner apex an erect, serrate plate bearing seven teeth. Mandible on right side conical with inner surface of base bearing stout process having four teeth and serrate plate. Paragnath (Fig. 3e) on left side transformed into slender sickle; remaining in this form throughout larval development.

Maxillule (Fig. 4A) with unsegmented, unarticulated endopodite with three terminal setae. Coxal endite bearing two stout spines and two setae terminally and one seta subterminally, while basal endite bearing three setae terminally and one subterminally.

Maxilla (Fig. 4E) with three inner lobes; proximal lobe of coxal endite apparently absent, but distal lobe present, bearing one seta. Four setae on proximal and distal lobes of basal endite. Endopodite reduced, bearing four terminal setae. Scaphognathite small, without proximal extension, bearing five short plumose setae.

First maxilliped (Fig. 4I) with four setae along medial margin of basipodite. Exopodite bearing four long plumose natatory setae. Endopodite with four segments; setation proximal to distal, 2–1–2–4. This formula unchanged in later stages.

Second maxilliped (Fig. 5A) with exopodite bearing four plumose natatory setae. Endopodite four-segmented, setation proximal to distal 0–0–2–4. This formula unchanged in later stages.

Third maxilliped (Fig. 5E) two-jointed rudiment without setae.

The chromatophores are mostly red, small, numerous, and dispersed over the whole animal. Smaller yellowish chromatophores blend with the red ones to produce an overall orange or ruddy tint. Concentrations of red pigment are found on the antennule and the ventral surface of the carapace, abdomen, and telson. There is a yellow cast on the antennae, mandibles, maxillae, the medial surface of the maxillipeds, and the dorsal surfaces of the carapace, abdominal somites, and telson. This pattern is also typical of later larval stages. By the postlarval stage, the animal is almost colorless with the ruddy background color largely lost, and the red chromatophores very contracted.

FIGURE 3. *Naushonia crangonoides:* antenna of zoeal stages I (A), II (B), III (C), and VI (D); mandibles of zoeal stage I, left (E), right (F), and left paragnath (e); left and right mandibles of zoeal stages II (G, H), III (I, J), and VI (K, L).

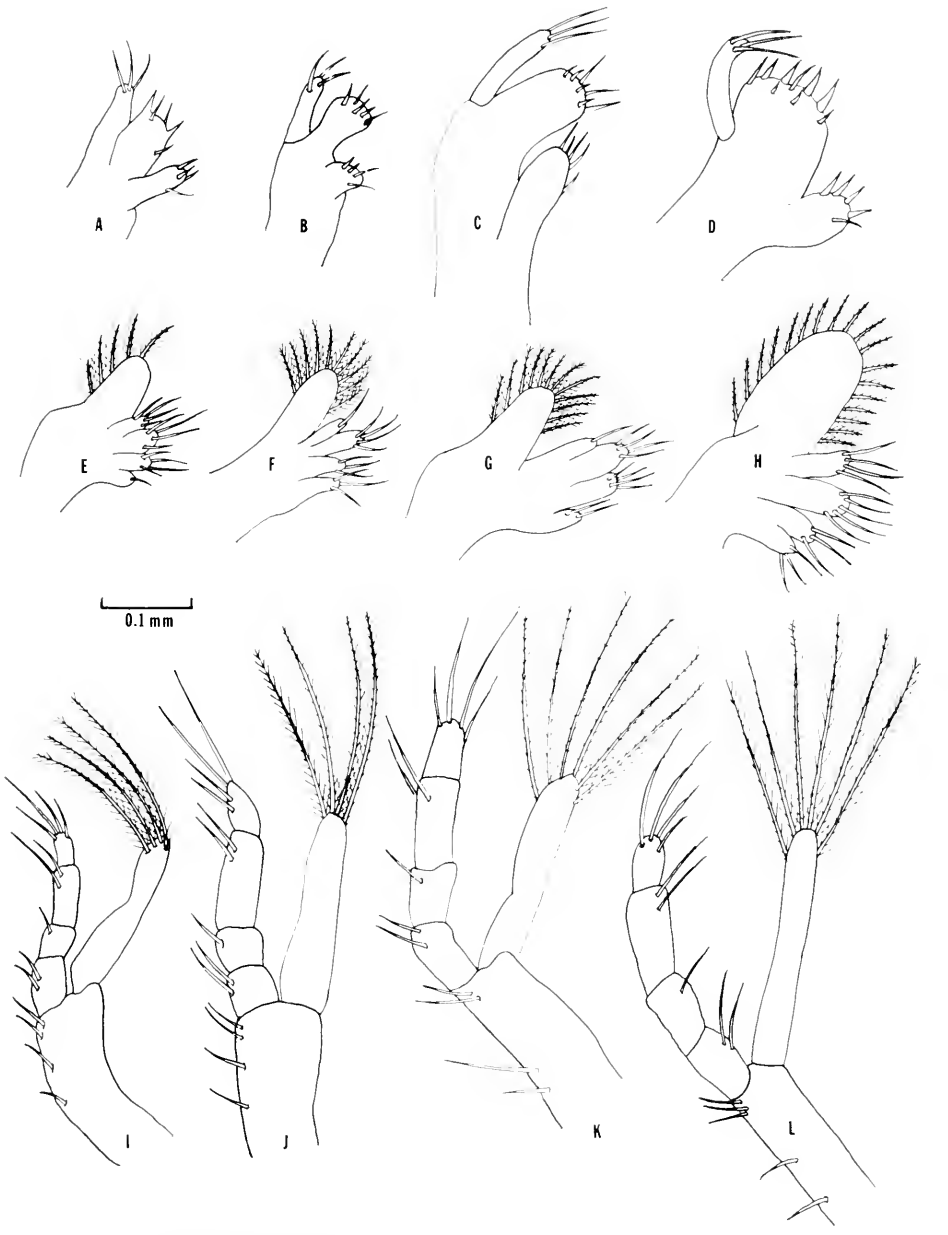

FIGURE 4. *Naushonia crangonoides:* maxillule of zoeal stages I (A), II (B), III (C), and VI (D); maxilla of zoeal stages I (E), II (F), III (G), and VI (H); first maxilliped of zoeal stages I (I), II (J), III (K), and VI (L).

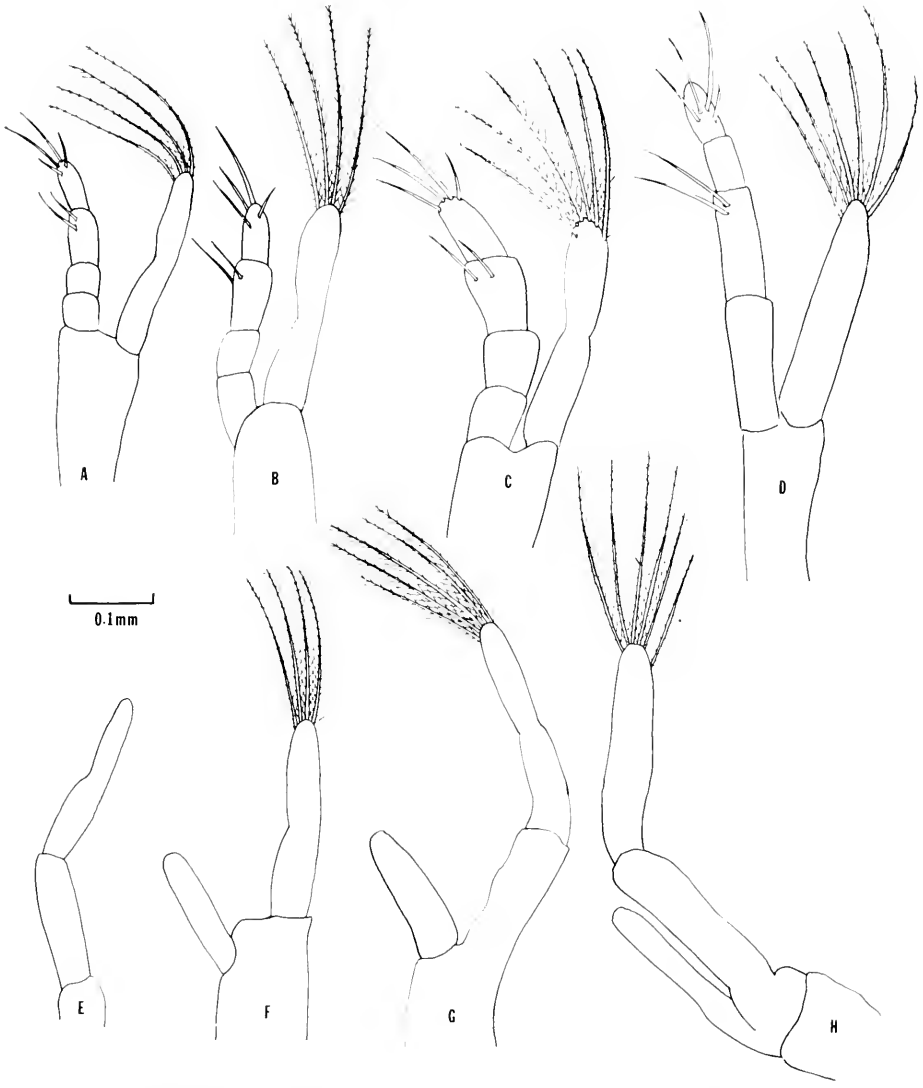

FIGURE 5. *Naushonia crangonoides:* second maxilliped of zoeal stages I (A), II (B), III (C), and VI (D); third maxilliped of zoeal stages I (E), II (F), III (G), and VI (H).

Second zoea (Figs. 1B, F)

General form of carapace and abdomen as in first zoea, except eyes mobile. Telson notch (Fig. 2B) less pronounced, with one minute lateral spine on each external spinous process. Spine 2 reduced to thalassinid hair and rather indistinct. Either five or six pairs of plumose setae on posterior margin of telson.

Antennular peduncle (Fig. 2F) three-segmented and biramous, with bud of

inner flagellum carrying a small plumose seta; bud of outer flagellum bearing four or five aesthetascs and fine setule. A long plumose seta located medially on distal segment.

Armature of antennal endopodite (Fig. 3B) a single minute fine setule. Antennal scale with 11 or 12 plumose setae medially; two spines on basipodite at base of scale and endopodite.

Ten teeth on serrate plate of mandibles (Figs. 3G, 3H); stout spinous teeth reduced to two.

Maxillule (Fig. 4B) unchanged except endopodite now articulated.

Maxilla (Fig. 4F) with three setae on coxal endite, three and four on proximal and distal basal endites, respectively. Endopodite with one seta; scaphognathite with ten plumose setae on margin.

First and second maxillipeds (Figs. 4J, 5B) unchanged in setation but slightly larger.

Third maxilliped (Fig. 5F) consisting of exopodite bearing four plumose setae, and rudimentary endopodite subterminally on basipodite.

Chelipeds (first pereiopods) uniramous, two-jointed rudiments without setae.

Second, third, and fourth pereiopods uniramous buds.

Third zoea (Figs. 1C, G)

Sixth abdominal somite distinct, uropods developed. Exopodite of uropod bearing 12 to 16 plumose setae, endopodite a bud without setae. Posterior margin of telson straighter, no indentation (Fig. 2C); one minute lateral spine on each external spinous process, but thalassinid hair absent; seven or eight pairs of plumose processes.

Antennular peduncle (Fig. 2G) three-segmented and biramous, bud of inner flagellum slightly longer than outer, bearing minute setule terminally. Bud of outer flagellum with four aesthetascs terminally and a short plumose seta subterminally. Distal segment bearing two long plumose setae on inner margin and short plumose seta terminally on outer margin. Middle segment bearing three long plumose setae on inner margin and two sets of two short plumose setae on outer margin.

Antenna (Fig. 3C) with a terminal minute setule on endopodite; antennal scale with 15 to 17 plumose setae medially. Two spines on basipodite at base of scale and endopodite.

Mandibles (Figs. 3I, 3J) unchanged but larger.

Endopodite of maxillule (Fig. 4C) still unsegmented and bearing three to five setae terminally. Basal endite bearing three to four short setae and four short spines; coxal endite bearing two to four setae terminally, one subterminally.

Maxilla (Fig. 4G) with two or three setae on coxal endite, four to six setae on each lobe of basal endite. Endopodite with one seta; scaphognathite bearing 12 to 15 plumose setae.

First maxilliped (Fig. 4K) with three or four setae along medial margin of basipodite, exopodite with five or six long plumose setae. Endopodite four-segmented; setation, proximal to distal, 2–1–2–4.

Second maxilliped (Fig. 5C) with setation of exopodite increased to five or six.

Third maxilliped (Fig. 5G) with five or six natatory setae.

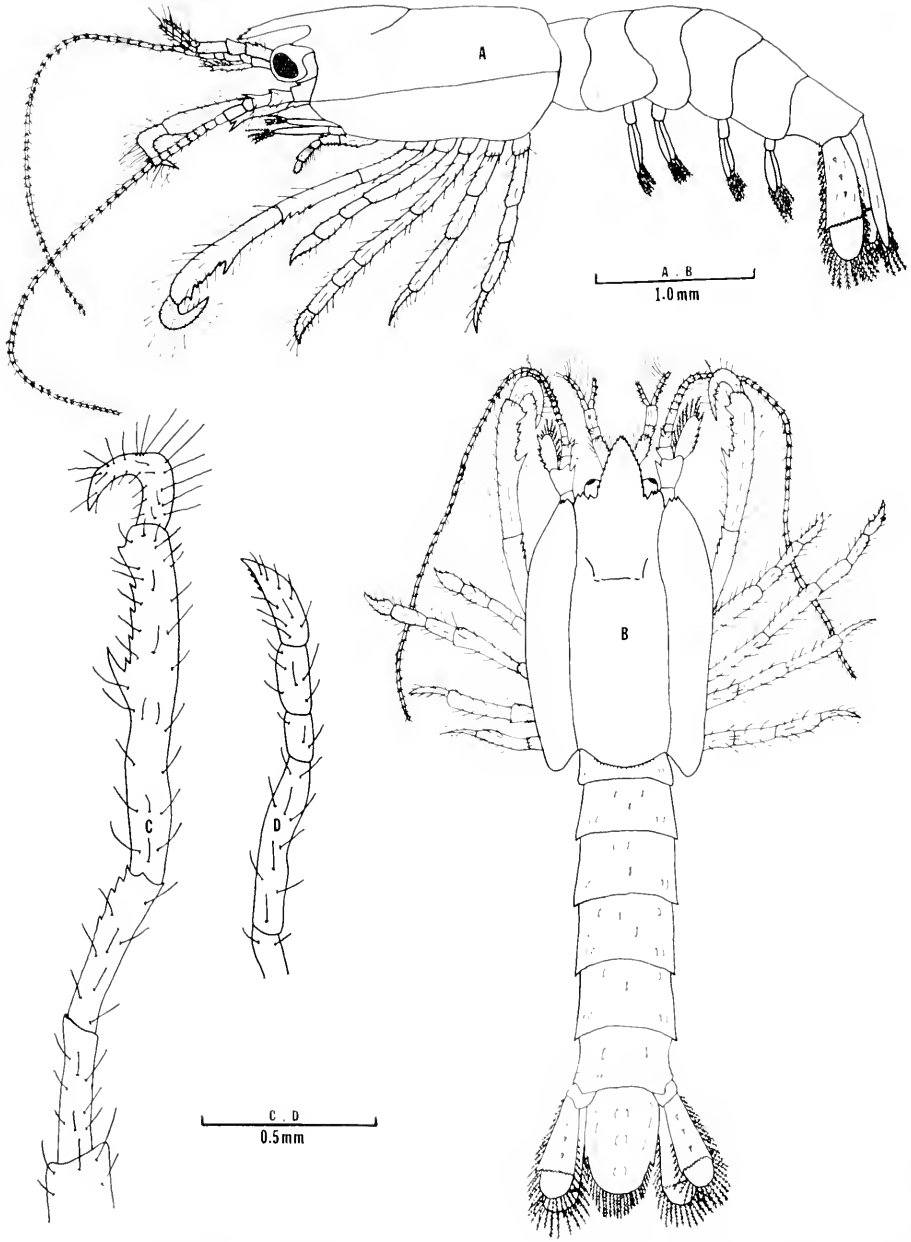

FIGURE 6. *Naushonia crangonoides:* postlarva, lateral view (A), dorsal view (B), first pereiopod (C), and second pereiopod (D).

First, second, and third pereiopods biramous without functional exopodites. Fourth and fifth pereiopods still uniramous buds.

Fourth zoea

Exopodites on first three anterior pereiopods now functional. Endopodite of uropod articulated with 9 to 12 plumose setae; exopodite bearing 15 plumose setae. Telson unchanged, but each external spinous process now bearing two minute lateral spines.

Antennular peduncle three-segmented; bud of inner flagellum bearing a fine minute setule and extending further than outer flagellum. Bud of outer flagellum bearing five aesthetascs, three short plumose setae terminally, and one long plumose seta subterminally on inner margin. Distal segment bearing two long feathered setae on inner margin and a short feathered seta terminally on outer margin. Middle segment with four long plumose setae on inner margin and two sets of two short plumose setae on outer margin.

Antennal endopodite extending beyond antennal scale with terminal setule; scale bearing 16 to 18 plumose setae.

Mandibles unchanged but larger.

Endopodite of maxillule may have up to six setae. Basal endite with six to nine spinous processes, while coxal endite with three to six.

Maxilla (Fig. 7K) bearing three to five setae on each of three inner lobes. Endopodite bearing two to three setae; scaphognathite with 13 or 14 plumose setae.

First, second, and third maxillipeds unchanged

Exopodites of first, second, and third pereiopods functional, with 6–6–5 long plumose setae, respectively. Fourth and fifth pereiopods uniramous buds.

Fifth zoea

Principal distinguishing feature: all pereiopods except fifth now bearing functional exopodites. Exopodite of uropod with 22 to 28 plumose setae; endopodite with 15 to 23 plumose setae. Telson spinous process unchanged; posterior margin straighter than in preceding stage.

Antennular peduncle two-segmented, bud of inner flagellum bearing fine minute setule and slightly longer than outer. Bud of outer flagellum bearing only five aesthetascs. Distal segment of peduncle bearing four or five long plumose setae on inner margin and two small plumose setae terminally on outer margin. Proximal segment with four or five long plumose setae on inner margin and three sets of shorter plumose setae (3–2–2) on outer margin.

Antennal endopodite without terminal minute setule. Antennal scale shorter than endopodite bearing 19 or 20 plumose setae medially.

Mandibles larger, otherwise unchanged.

Endopodite of maxillule unchanged. Basal endite with six to nine short processes; coxal endite with five or six short setae.

Maxilla with three setae on coxal endite, three to five on lobes of basal endite. Endopodite with three or four setae and scaphognathite with 15 to 22 plumose setae.

First, second, and third maxillipeds unchanged, but larger.

FIGURE 7. *Naushonia crangonoides:* postlarval appendages: telson (A), antennule (B), antenna (C), antennal scale (c), mandible (D), maxillule (E), and maxilla (F).

Exopodites of first three pereiopods unchanged, but fourth pereiopod biramous with five long plumose setae on exopodite; fifth pereiopod uniramous and rudimentary.

Pleopods present on second to fifth abdominal somites as minute buds, hardly discernible.

Sixth zoea (*Figs. 1D, 1H*)

Most distinguishing feature: pleopods nonfunctional and without setae, but now larger and biramous. Five animals out of six molted to postlarvae from this stage. Exopodite of uropod with 20 to 22 plumose setae; endopodite with 17 to 20 plumose setae. Telson (Fig. 2D) with two minute lateral spines on each external spinous process and seven pairs of plumose spines on posterior margin.

Antennular peduncle (Fig. 2H) two-segmented, bud of inner flagellum unchanged. Bud of outer flagellum bearing two fine setules in addition to five aesthetascs. Distal segment bearing three long plumose setae on inner margin, one long plumose seta medially and terminally, and two small plumose setae terminally on outer margin. Proximal segment with six long plumose setae on inner margin, two sets of three short feathered setae on outer margin, and four plumose setae medially, proximal to base.

Antennal endopodite unchanged. Antennal scale with 20 plumose setae medially (Fig. 3D).

Number of teeth on serrate plate of mandibles (Figs. 3K, 3L) increased from 10 to 14.

Maxillule (Fig. 4D) with four terminal spines and one subterminal spine on coxal endite, five to nine processes on basal endite, endopodite with three to five setae.

Maxilla (Fig. 4H) with setation of lobes on endites essentially unchanged. Endopodite bearing three or four setae; scaphognathite with 18 or 19 plumose setae.

Setation of maxillipeds (Figs. 4L, 5D, 5H) unchanged.

Pereiopods unchanged.

Pleopods now biramous but lacking setae.

Seventh zoea

This stage was seen in four animals: two died in this stage, one molted to postlarva, and the fourth molted three additional times within 30 days but never reached postlarva.

Exopodite and endopodite of uropod each bearing 25 plumose setae. Telson in some specimens having single medial spine in place of medial pair.

Antennular peduncle two-segmented, bud of outer flagellum now bearing six aesthetascs. Distal segment of peduncle now bearing four long plumose setae on inner margin.

Marginal setae of antennal scale increased to 22.

Serrate plate of mandibles with 12 to 14 teeth.

Endopodite of maxillule unchanged. Basal endite bearing stout spines; coxal endite with six short setae terminally and two subterminally.

Maxilla with two to four terminal setae on coxal endite, four to six terminal on proximal lobe of basal endite, and four or five terminal setae on distal lobe. Endopodite bearing three setae. Scaphognathite with 16 to 18 plumose setae.

First and second maxillipeds unchanged, but larger.

Endopodite of third maxilliped bearing three short spines subterminally on outer margin.

Exopodites of first four pereiopods bearing six long plumose setae on terminal segment. Endopodite of first pereiopod slightly swollen with slight fissure discernible under high power (trace of chela?).

Fifth pereiopod uniramous, very elongate and slender, bearing minute spine on apex.

FIGURE 8. *Naushonia crangonoides:* postlarval appendages: first maxilliped (A), second maxilliped (B), and third maxilliped (C).

Pleopods on second to fifth abdominal somites, biramous, with single long plumose setae on each exopodite.

Postlarva (*Figs. 7A, 7B*)

Carapace with strong supra-antennal spine, linea thalassinica and cervical groove strongly marked. Eyes visible from above. Rostrum with lateral serrations. Telson (Fig. 7A) with small lateral spine and 42 plumose setae on posterior margin. Serrate transverse sutures on endopodites and exopodites of uropods ending with external spine. Endopodite bearing three spines dorsally and 44 plumose setae (in each of two specimens), while exopodite also with three dorsal spines but 48 plumose setae.

Peduncle of antennule (Fig. 7B) consisting of four segments extending far beyond front of eye. Proximal segment bearing numerous setae around terminal edge; second segment with six setae and a prominent spine on inner margin, seven other setae dorsally. Next two segments with four setae and eight setae, respectively, on ventral surface. External flagellum of five segments each bearing one aesthetasc and two setae, except penultimate segment with two aesthetascs and three setae. Four-segmented inner flagellum with two setae on three proximal segments and four setae on terminal segment.

Second segment of antennal peduncle bearing ovate antennal scale (Figs. 7C, 7c) with six lateral teeth, six lateral setae, and 15 plumose setae on inner margin. Second, third, and fourth peduncular segments with spine distally on inner margins. Flagellum of approximately 48 segments, most bearing setae distally.

Mandibles (Fig. 7D) symmetrical, cutting edge with four small teeth. Palp developed, unsegmented, bearing minute seta terminally.

Endopodite of maxillule (Fig. 7E) with minute seta terminally, rarely with seta proximally. Basal endite rounded and bearing two long terminal plumose setae and 14 short spines. Coxal endite with nine short spines.

Maxilla (Fig. 7F) showing proximal lobe of coxal endite for the first time, with three long and eight short setae; distal lobe of coxal endite and proximal lobe of basal endite each with eight setae, distal lobe of basal endite with 12 setae. Unsegmented endopodite bearing one long and four short setae terminally, two setae subterminally. Scaphognathite with 37 plumose setae on outer margin and 15 minute setae on inner margin.

First maxilliped (Fig. 8A) with two-lobed basipodite, proximal lobe bearing 13 plumose setae, distal lobe bearing 12 marginal and 12 submarginal plumose setae. Endopodite three-segmented with terminal segment not expanded without setae. First segment with 11 feathered setae, second (middle) segment with three feathered setae on outer margin. Exopodite three-segmented with widened proximal segment bearing 10 plumose outer setae. Second segment lacking setae, distal segment with two long plumose terminal setae. Elongate epipodite bearing long plumose terminal seta.

Basipodite of second maxilliped (Fig. 8B) with three long plumose setae on inner margin, heavily serrate epipodite on outer margin. Endopodite four-segmented with 4–0–4–10 setae, proximally to distally. Exopodite two-segmented

Table II

Comparison of some postlarval characters of Naushonia crangonoides *and* Naushonia portoricensis.

	N. crangonoides	*N. portoricensis*
Rostrum	No lateral teeth	Lateral tooth on each side
Rostral apical process	Absent	Present
Linea thalassinica	Distinct	Faint
Small, anterior tubercle on eye	Absent	Present
Ischium of mxp_3	Serrate	Smooth
Arthrobranch mxp_1	Present	Absent
First pereiopod	Does exceed eyes	Does not exceed eyes
Tooth of propodus of p_1	Two large, four small	One large, inner
Dactyl of p_1	$\frac{1}{4}$ length	$\frac{1}{2}$ length
Marginal setae of telson	42	22
Suture of uropodal endopodite	Complete	Incomplete

with eight short setae on first segment and four long plumose terminal setae on second segment.

Basipodite of third maxilliped (Fig. 8C) with numerous short plumose setae, one long plumose seta. Epipodite consisting of serrate-margined mastigobranch with two plumose setae, rudimentary podobranch. Endopodite five-segmented, ischium serrate distally, bearing four short plumose setae. Number of setae on other segments as follows: four on inner margin and two on outer margin of second segment; three on inner margin and three terminally on third segment; six on inner margin, seven subterminally, and four terminally on fourth segment; and six terminally with five shorter setae dispersed randomly on fifth segment. Exopodite two-segmented, with four long plumose terminal setae.

Chelipeds (Fig. 6C) slender, symmetrical extending well beyond cornea, held stiffly in front of living animals during locomotion. Propodus with two large, four smaller inner teeth; dactyl slender and falcate.

Second to fifth pereiopods slender, short, very similar in structure, except for dactyls. Second and third pereiopods (Fig. 6D) with robust dactyls bearing five small stout spines on their inner margins, dactyls of fourth and fifth pereiopods elongate, slender, and lacking stout spines.

Pleopods on abdominal somites two to five, biramous, lanceolate. Second and third pleopods with eleven and thirteen plumose setae on endopodites and exopodites, respectively; fourth pleopod with nine and ten setae, fifth pleopod with seven and nine setae on endopodites and exopodites, respectively. No appendix interna or appendix masculina present.

Juvenile stages

In the first few molts after the postlarval stage, neither length of the specimens nor their morphology changes drastically. There seems to be a gradual development of adult characteristics. A detailed description of the fifth stage juvenile and its appendages and comparison of this juvenile with adult *Naushonia* will be presented elsewhere (Goy and Provenzano, in preparation).

Discussion

Since *N. crangonoides* can reach postlarva in six zoeal stages, the seventh stage seems not to be essential in the larval life history of *Naushonia*. Thompson found the equivalent of the seventh stage in the plankton, and we obtained it four times in the laboratory. One of the seventh stage zoeae in the present study molted an additional three times within 30 days, but failed to metamorphose. Studies on other larvae show that in more than 15 families of decapods, the number of larval instars preceding metamorphosis is variable (see Knowlton, 1974, for review).

The pattern of change in mean duration as development proceeds in this species (Table I) is similar to that observed in some other decapods. The first stage is longer than the succeeding several stages, but towards the end of development duration increases again (for example, Provenzano, 1968; Robertson, 1968). The extent of distribution of this pattern is not yet established, but it probably occurs only in species with moderately long larval development, and possibly only in laboratory-reared larvae. Relatively long duration of the first stage may be related to the feeding behavior or yolk supply of this stage, while the lengthening of later stages over intermediate stages may be related to the energy demands for tissue growth and preparation for metamorphosis.

Adults of *Naushonia crangonoides* are known only from Massachusetts at Bass River, Vineyard Sound, and Elizabeth Islands (Williams, 1974). Larvae believed to belong to *N. crangonoides* have been collected from the Woods Hole area during July, August, and September (Fish, 1925); in Delaware Bay from August to October (Deevey, 1960); in Narragansett Bay in August (Hillman, 1964); and in Chesapeake Bay from August to September (Sandifer, 1972; Goy, 1976). In the last two collections, first stage larvae of *Naushonia* were most numerous especially near the baymouth. The presence of early larval stages suggests a breeding population of *N. crangonoides* somewhere near the mouth of Chesapeake Bay.

The first stage larva of *Naushonia crangonoides* has not yet been hatched from ovigerous adults. Our identification of this larval series is based on morphological similarities between the fifth juvenile stage and adult specimens of *Naushonia crangonoides* and *N. portoricensis* from collections in th U.S. Nationeal Museum (Goy and Provenzano, in preparation).

Thompson (1903) made no mention of the thalassinid hairs on the telson in the first and second larval stages of *N. crangonoides*. These are extremely minute and he probably overlooked them. He also apparently overlooked the minute lateral spines on the external spinous process of the telson of the third to the seventh zoea. Thompson mentioned that no traces of the cheliped could be found even in his fifth stage larva, but close examination of our seventh zoea, equivalent to his fifth, showed under high power a slight fissure, probably a trace of the chela. Discrepancies in setation and number of spines or teeth on certain appendages of Thompson's larvae and those of the present study probably can be attributed to normal variation within the species. This is also probably true of the size differences found. The zoeae of Thompson were usually much larger than those obtained by us in the laboratory. Thompson's second stage larva had a total length of 4.0 mm, whereas our largest second stage zoea was only 3.4 mm. The largest third stage larva attained was 5.0 mm, while Thompson's equivalent stage was over 5.0 mm and its

first pereiopod had an exopodite functional as a swimming organ. The fourth and sixth stages we obtained in the laboratory were omitted from Thompson's description entirely. His fourth stage is equivalent to the fifth stage in the present study, and his fifth stage is essentially the same as the seventh zoea that we obtained in culture.

Larval stages related to or belonging to *Naushonia* have been found off Samoa and the Great Barrier Reef (Gurney, 1938); off Bermuda (Gurney and Lebour, 1939); and off New South Wales (Dakin and Colefax, 1940). The larvae reported from Samoa and the Great Barrier Reef by Gurney have the essential characters of *Naushonia,* but are quite distinct from *N. crangonoides.* Both Gurney (1938) and Dakin and Colefax (1940) suggested that Gurney's larvae may represent a genus other than *Naushonia.* The first stage larva from Samoa agrees fairly closely with *N. crangonoides* but differs in the absence of pleural spines on the abdomen and the possession of a papilliform process on the fifth somite. The Barrier Reef specimen was believed to be a fourth stage larva of the Samoan species; it also lacks the abdominal pleural spines and has no exopodite on the fourth pereiopod. Gurney and Lebour (1939) stated that if these same larvae belong to *Naushonia,* they could possibly be zoeae of *N. perrciri* known from the Red Sea.

The zoeae that bear the closest resemblance to those of *N. crangonoides* were collected from New South Wales by Dakin and Colefax (1940). They described six zoeal stages in their species of *Naushonia* and recorded a sixth stage larva just about to metamorphose, in which they could observe the postlarval telson. The differences between that species and *N. crangonoides* are slight; the telson is shaped differently, and the endopodite of the third maxilliped in later stages bears a long seta, missing in *N. crangonoides.*

The developing telson of the postlarvae observed by Dakin and Colefax differs from that of *N. crango*noides in having only 20 setae on its posterior margin (42 in *N. crangonoides*) and no evidence of an external spine.

The larvae collected off Bermuda were believed by Gurney and Lebour (1939) to belong to *Naushonia portoricensis* based on similarities between the postlarva and adults of that species. These larvae closely resemble those of *N. crangonoides* but differ from *N. crangonoides* in the relatively smaller size of *N. portoricensis,* differently shaped telson, and a stronger pleural spine on the first abdominal somite. Gurney and Lebour also described a postlarval stage and first juvenile stage for what they believed to be *N. portoricensis.* Their postlarva differs from ours (Table II).

Larvae of the other two genera of the Family Laomediidae are known. Certainly identified larva of *Jasea* have been described for two species, *J. nocturna* (Claus, 1884; Cano, 1891; Bouvier, 1914; Caroli, 1924), and *J. novaezealandiae* (Gurney, 1924; Wear and Yaldwyn, 1966). The long-necked *Lucifer*-like larva of *J. nocturna* was given the name trachelifer by Brooks (1889). The postlarva of this species was obtained in the laboratory from metamorphosed last stage zoeae (Caroli, 1924; Tattersal, 1938). Wear and Yaldwyn (1966) described the complete larval life history of *Jasea novaezealandiae* from plankton material and devised keys to separate larval species of *Jasea* and first postlarval stages of *J. novaezealandiae, J. nocturna, Naushonia crangonoides,* and *N. portoricensis.*

A sixth stage larva collected by Kurian (1956) in the Adriatic Sea and attributed to *Jaxea* seems to bear a closer resemblance to larvae of *Naushonia* than to those of *Jaxea*. The rostrum of Kurian's larva has a "double curve," and the end of the rostrum does not reach the extremity of the eye, characteristics of all known *Naushonia* larvae. The telson has 12 posterior marginal plumose setae with two small spines on the outer margins of the lateral prolongations of the telson; the telson also has the posterior corners drawn into curved processes. This is similar to the telson of the *Naushonia* sp. found by Dakin and Colefax (1940) and that of *N. portoricensis* (Gurney and Lebour, 1939). The pleural hooks are absent on the sixth abdominal somite of Kurian's larva as in all known *Naushonia* larvae. They are present on the larvae of *Jaxea nocturna* and *J. novaezealandiae*. The pleural hooks are also reduced on the first abdominal somite, which is characteristic of *N. crangonoides* larvae. The second antenna of this Adriatic species is also very similar to that of *N. crangonoides* larvae. This larva found by Kurian is probably only a stage V. Its size of 5.7 mm fits in the range of 5.0 to 8.0 mm total length from known *Naushonia* stage V larvae, whereas it is rather small for that of the known *Jaxea* stage V larvae, which range from 10.7 to 12.5 mm in total length.

The larvae of the genus *Laomedia* are known from the first stage zoeae hatched from an ovigerous *Laomedia astacina*. Sakai and Miyake (1964) described these larvae and compared them with zoeae of *Naushonia* and *Jaxea*. In their paper on *L. healyi*, Yaldwyn and Wear (1972) believed larvae described by Dakin and Colefax (1940) from Sydney harbor might be zoeae of *Laomedia healyi*. To test this hypothesis, they asked Dr. Sakai for larval material of *L. astacina*, but from the description and illustrations of their borrowed material one can see they mistakingly described a larva of *Upogebia* instead of *Laomedia*. We consider the description by Sakai and Miyake to represent the true first stage zoea of *Laomedia astacina*.

The first stage larvae of the three genera of Laomediidae can be distinguished. *Laomedia* differs from the others in having a telson formula of $6 + 6$ rather than $7 + 7$, in lacking the thalassinid hair on the telson, in having only five apical processes on the antennule rather than six, and in not having a conspicuous rostrum. *Naushonia* can be distinguished from *Jaxea* by having an upturned, short rostrum rather than a straight or long rostrum.

All known larvae of Laomediidae can be distinguished from other decapod larvae by the procurved pleural hooks on at least four abdominal somites and by the asymmetrical mandibles (left mandible and paragnath drawn out into sickle-shaped structures).

We thank Steve Morgan, who provided the first stage zoeae from plankton cruises on the R/V LINWOOD HOLTON, and Dr. Raymond Manning for making possible examination of specimens of *Naushonia crangonoides* and *Naushonia portoricensis* from the United States National Museum. This work was supported by National Science Foundation grant DEB76–11716.

Summary

1. Larval stages captured from plankton in Chesapeake Bay were reared in the laboratory to and beyond metamorphosis and were determined to be those of the mud shrimp, *Naushonia crangonoides,* known as adults only from the area of Woods Hole.

2. The postlarval stage in *Naushonia* may be reached after six or seven zoeal stages. Descriptions and illustrations of the zoeal stages and the postlarva are presented.

3. Larvae are compared with others known for the genus and the Family Laomediidae and distinctive specific and generic characters are discussed.

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REPRODUCTION IN THREE SPECIES OF INTERTIDAL BARNACLES FROM CENTRAL CALIFORNIA

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The reproductive biology of eastern Atlantic barnacles has been studied extensively, with emphasis on the role of temperature in regulating reproduction (*e.g.*, Crisp, 1950, 1954; Patel and Crisp, 1960a; Barnes, 1963; Barnes and Stone, 1973). However, there has been much less work on cirripedes elsewhere in the world. This paper compares the reproductive cycles and brood production of three species of intertidal barnacles abundant in central California: *Chthamalus fissus* Darwin, 1854; *Balanus glandula* Darwin, 1854; and *Tetraclita squamosa rubescens* Darwin, 1854 (hereafter called *T. squamosa* in this paper).

On the west coast of North America the role of barnacles in the structure of intertidal communities has been stressed (*e.g.*, Connell, 1970; Dayton, 1971), but the reproductive cycles of only two intertidal and one subtidal species have received attention. *Balanus glandula* from British Columbia and southern California broods primarily in the cold winter and spring months but may show minor brooding activity in summer (Barnes and Barnes, 1956). On the other hand, *Pollicipes polymerus* has a variable reproductive cycle with a limited summer brooding season in Washington which increases in length to central California (Hilgard, 1960; Lewis, 1975), while brooding activity in southern California peaks in winter with 30% still brooding in summer (Straughan, 1971). *Balanus pacificus*, a subtidal species in southern California, broods at high frequencies year-round showing no correlation with temperature (Hurley, 1973). Thus, in these species the relationship of reproduction with temperature is complex. The role of temperature or other environmental factors as proximal cues synchronizing brooding has not been studied experimentally in species of cirripedes on the west coast of North America.

In this paper brooding and nutrient storage cycles are compared in populations of the three species of barnacles occurring in the warm-water discharge canal of a large power plant and in adjacent areas of ambient temperature. Aspects of the regulation of these cycles by temperature, photoperiod, and food availability are investigated experimentally. The size and number of broods produced during a season are estimated so that the patterns of reproductive effort of these three species can be compared with other cirripedes.

Materials and Methods

This study was conducted at the Pacific Gas and Electric Company fossil-fuel power plant at Morro Bay, California (35° 22′ 30″ N, 120° 52′ 30″ W). This 1030 megaWatt plant uses ocean water for once-through cooling, discharging a

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plume with an isotherm 5°C above ambient of about 0.6 to 3.0 acres surface area. Continuous temperature records (see Fig. 1) were taken from "Ryan" temperature recorders positioned next to the intake screens and the outfall next to the discharge tubes. Both recorders were at about mean lower low water, corresponding to the approximate intertidal level of the sampled barnacles.

Field data were gathered from populations of *Chthamalus fissus, Balanus glandula,* and *Tetraclita squamosa* occurring in the warm-water outfall and in adjacent control areas of ambient temperatures in the Morro Bay harbor channel. Collecting trips were made at about monthly intervals from November, 1972, to January, 1975. *C. fissus* is small (< 8 mm basal diameter) and common in the high intertidal Zone 1 of Ricketts and Calvin (1968) from San Francisco to Baja California. *B. glandula* grows to about 20 mm in diameter and is common in the upper mid-intertidal Zone 2 from Alaska to Baja California. *T. squamosa* attains a maximum diameter of 50–60 mm and is found in lower mid-intertidal Zone 3 from San Francisco to Baja California. Although these zonal distributions are characteristic, the three species are often found together in Zone 3, and every effort was made to collect samples from equivalent tidal levels (0 to +1.0 feet above mean lower low water) to minimize effects of differences in exposure and feeding time.

For each species, barnacles of haphazard sizes over the entire range available were selected for processing. Each barnacle was examined for brooded embryos, ripeness of ovary, and ripeness of the male reproductive system. Brooding frequencies were calculated for barnacles known to be reproductively mature. Ovaries were staged "ripe" when they had large quantities of yolky material bulging into the mantle chamber, or "not ripe" when little or no yolk was present. The male reproductive system was staged as "ripe" or "not ripe" according to the presence or absence of seminal vesicles discernibly filled with white seminal fluid. The basal diameter along the rostral-carinal axis and the following dry weights were determined for each barnacle: the opercular valves; the body (soma only, excluding ovary, retractor muscles, and tissue lining the mantle cavity); brooded egg mass; and, in some cases, the ovary (including retractor muscles and tissue lining the mantle cavity).

Egg numbers per brood were counted using a Model A Coulter Counter modified to count all the eggs in each brood. The eggs of each brood were dissociated with protease in sea water, fixed in formalin, and run through the counter. The length and width of a few eggs in each brood were recorded, as was the dry body weight of the parent.

For laboratory experiments barnacles were maintained on small rocks continuously submerged in vigorously aerated sea water under constant photoperiod and temperature conditions. Barnacles were fed *ad lib* with dense suspensions of *Artemia salina* nauplii, augmented in some cases with cultures of *Dunaliella* sp. All three species were maintained many months using these techniques. Temperature experiments were conducted at 11.5° or 20° C, representing winter ambient and outfall water temperatures, respectively. Photoperiods used represented the long (14L: 10D), intermediate (12L: 12D), and short (10L: 14D) day-lengths occurring at Morro Bay.

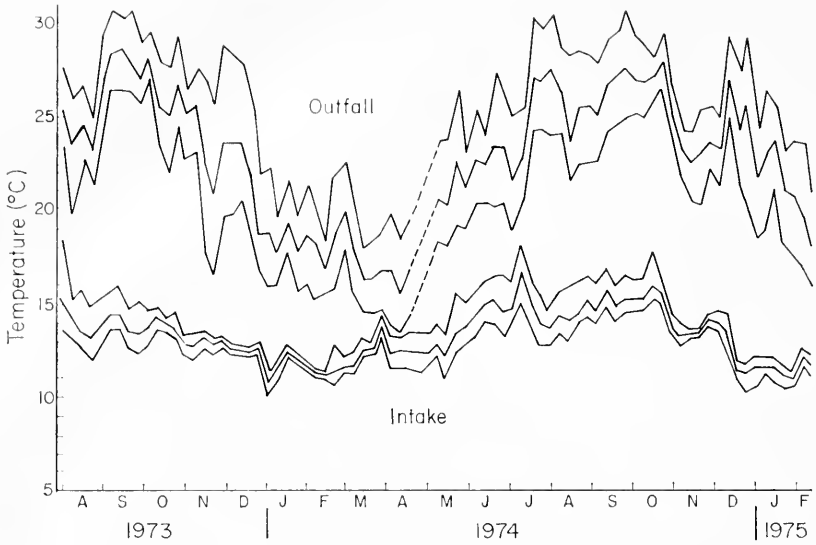

FIGURE 1. Temperature records for outfall and intake at Morro Bay power plant, plotting weekly high, mean, and low temperatures averaged from six-hour intervals. Recorders were positioned at about mean lower low water.

To estimate the length of time embryos were brooded, egg lamellae judged freshly deposited were removed from barnacles, broken into small clumps of embryos, and held in the laboratory under constant temperature and photoperiod until they hatched or reached late developmental stages judged ready to hatch. Sterilized sea water for these *in vitro* brooding-time experiments was treated with antibiotics, continuously aerated, and frequently changed.

RESULTS

Brooding cycles

Brooding frequencies for outfall and control populations of the three species are shown in Figure 2. The control populations of *Chthamalus fissus* brooded during a long summer season from about March or April to October. In peak periods from June to September, 50–75% of the sample were brooding, but low levels of about 10% brooding often occurred during the "off" season. Although the brooding frequencies of outfall and control samples of *C. fissus* were often quite different at any given month, there was no consistent difference in the overall brooding cycle timing or amplitude from the two areas. Broods in all developmental stages were found in both populations at all times of the year.

The control populations of *Balanus glandula* brooded embryos in winter and spring from about December or January to May, with about 60–80% of the control population brooding during peak months. Occasional low levels (about 5%) of brooding occurred in fall months, but the onset of the brooding cycle was abrupt. The samples from the warm-water outfall consistently had a lower percentage of

brooding, and the onset of brooding was delayed one or more months in both 1972–73 and 1974–75, but not in 1973–74. The samples at the onset of the brooding period each year showed a high frequency (about 95%) of broods in early developmental stages, suggesting that the deposition of the first brood was quite synchronous in both populations. Subsequent samples in the brooding season did not reflect any synchrony, and all developmental stages of brooded embryos were found.

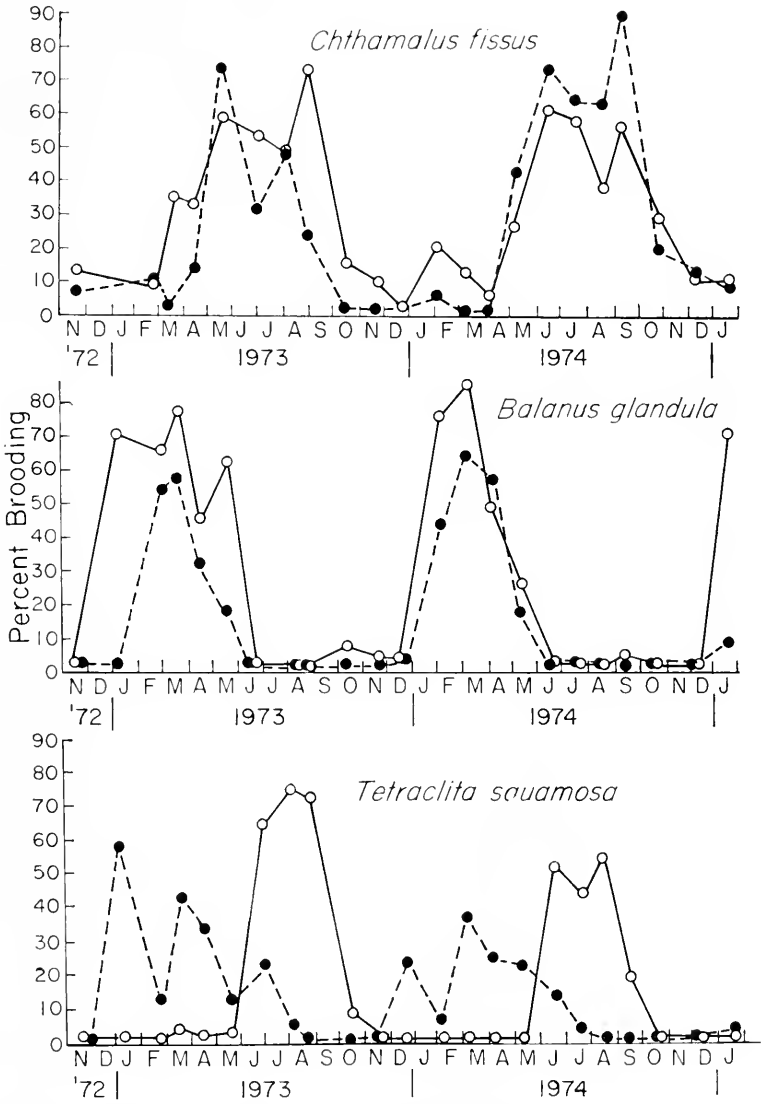

FIGURE 2. Cycles of brooding frequencies: outfall, solid symbols; control, open symbols. Sample sizes are: *Chthamalus fissus* = 50; *Balanus glandula* = 60; *Tetraclita squamosa* = 60 ≥ 18 mm basal diameter.

The control population of *Tetraclita squamosa* brooded during summer from about June through September. The onset of brooding was sharp, and 40–75% of the population brooded embryos during peak months. The population in the warm-water outfall began brooding in or near December and continued at erratically variable levels through spring into early summer, diminishing in June or July when the control population was reaching peak activity. The brooding cycle of the outfall population was thus about six months out of phase with the control and more variable in activity, of longer duration but with lower peak brooding frequencies than the control population. Samples from the control population at the onset of the brooding season showed a high frequency (about 80%) of broods in early developmental stages, indicating a synchrony of deposition of the first brood. This synchrony was not found in subsequent samples in the season, and it was not found at all in the samples of the outfall population.

Brooding frequencies as a function of size were calculated for each species. There was no significant change in brooding activity with size in *Chthamalus fissus;* any individual above 2 mm basal diameter (about 2 months old; Hines, 1976) was judged to be mature. No individual of *Balanus glandula* less than 5 mm basal diameter was available for sampling during months of brooding activity, since they had all grown to at least that size by December (about 6 months old; Hines, 1976). Above 5 mm there was no significant change in brooding activity with size, so all individuals were considered mature by the time the populations began to brood in the winter. In *Tetraclita squamosa*, however, barnacles less than 6 mm basal diameter did not brood. They began to mature at about 12 mm, and became fully mature at about 18 mm in diameter (at about two years old; Hines, 1976). Above 18 mm in size there was no significant change in brooding activity.

Laboratory experiments on brooding

Comparisons of brooding cycles in the warm-water outfall and control populations suggested that for *Chthamalus fissus* temperature is not an important factor regulating brooding, since the cycles of the two populations are similar. In *Balanus glandula* the delayed and lower percentages of brooding in the outfall population suggested that temperature is important in regulating both the timing

TABLE I

Laboratory brooding experiment. Chthamalus fissus *and* Balanus glandula *collected from both the outfall and control populations were maintained in the lab from October 12 to December 30, 1973: photoperiod, 12L:12D; food,* Artemia *nauplii fed* ad lib; *temperatures, 11.5° or 20° C. Brooding frequencies for the lab barnacles and the field populations at the end of the experiment are shown.*

	11.5° C		20° C		Field 12/20/73	
	Control	Outfall	Control	Outfall	Control	Outfall
Chthamalus fissus	49%	42%	46%	40%	14%	13%
N =	58	56	38	62	50	50
Balanus glandula	75%	61%	10%	8%	0%	0%
N =	77	76	80	80	60	60

TABLE II

Laboratory brooding experiment on Chthamalus fissus *maintained in the laboratory from September 17 to October 28, 1974: photoperiod, 12L:12D; temperature, 12° C. Brooding frequencies as a function of increasing food doses of* Artemia salina *nauplii are shown.*

Food dose (ml)	0	10	31	58	100
Brooding (%)	15	18	33	48	74
N	103	66	98	87	66

and intensity of brooding, and that cold temperature is necessary for reproduction to proceed normally. In *Tetraclita squamosa* the pronounced shift in the brooding cycle of the outfall population suggested that temperature is important in regulating reproduction in this species also, but that warm temperatures are required for reproduction. A series of laboratory experiments was conducted to test these hypotheses and to investigate more fully the roles of temperature, photoperiod, and food availability in regulating reproduction. Results of these experiments will only be summarized here; further details and complete data are available from Hines (1976).

Brooding in *Chthamalus fissus* is regulated directly by food availability, and feeding with *Artemia salina* nauplii in the laboratory elicited high brooding frequencies (Table I) during periods when brooding activity and food levels in the field were low (Icanberry and Adams, 1974). The increased brooding response to food in the laboratory was rapid (within about 2 weeks), and the frequency of brooding was directly proportional to the size of the food dosage (Table II). Temperature (11.5° or 20° C) and photoperiod (10L:14D, 12L:12D, or 14L:10D) did not affect brooding in *C. fissus* in the laboratory during any season. For *Balanus glandula* cold temperature (11.5° C) induced early brooding in the laboratory during late fall and early winter, and warm temperatures (20° C) tended to inhibit it (Table I). However, cold temperature in the laboratory did not induce brooding in late summer to early fall even though *B. glandula* appeared ripe then; nor did cold temperature in the laboratory extend the brooding period into summer. Photoperiod (10L:14D, 12L:12D, 14L:10D) did not affect brooding activity during any season in *B. glandula*. Although the 6-month shift in the brooding cycle of *Tetraclita squamosa* in the outfall population strongly suggests that warm temperatures stimulate brooding, *T. squamosa* did not brood in the laboratory under any of the conditions tested (combinations of 11.5° or 20° C with 10L:14D, 12L:12D, 14L:10D and several food regimes), even during the time the field populations were brooding. Individuals appeared ripe with yolk in the laboratory, but the stimulus for brooding seemed missing.

Brood and egg size

Regressions of dry brood weight *vs.* dry body weight with 95% confidence intervals for slopes and intercepts are: *C. fissus:* $y = (0.718 \pm 0.039) \times + (0.035 \pm 0.030)$, $n = 391$, $r = + 0.855$; *B. glandula:* $y = (1.58 \pm 0.093) \times - (0.962 \pm 0.526)$, $n = 363$; $r = + 0.868$; and *T. squamosa:* $y = (1.21 \pm 0.066) \times - (8.86 \pm 1.82)$, $n = 248$, $r = + 0.908$. Brood weight is quite variable in all three species;

however, there was no significant difference in brood weight/body weight regressions between outfall and control population or between broods occurring early or late in the respective brooding season of any of the three species (slopes and intercepts are not different at the 0.20 level). Because brood weight is positively correlated with body size and because there is a large size range of barnacles both within each species and between species, the slope of these regressions is taken as a relative measure of the brood size for each species. By this measure *Balanus glandula* has the largest relative brood size (1.58); *Tetraclita squamosa* puts out an intermediate brood (1.21); and *Chthamalus fissus* has a comparatively small brood (0.718).

Numbers of eggs in thousands per brood as a function of dry body weight are given in the following regressions showing the standard errors of the slopes and intercepts: *C. fissus:* $y = 2.54\ (\pm 0.34) \times - 0.28\ (\pm 0.27)$, $n = 24$, $r = 0.85$; *B. glandula:* $y = 1.72\ (\pm 0.13) \times - 0.37\ (\pm 0.98)$, $n = 33$, $r = 0.92$; and *T. squamosa:* $y = 0.75\ (\pm 0.08) \times - 2.05\ (\pm 1.15)$, $n = 33$, $r = 0.87$.

Size of the ovoid-shaped eggs of each species are given by their length and width at the first naupliar stage before hatching: $130 \times 95\ \mu$ for *Chthamalus fissus;* $245 \times 175\ \mu$ for *Balanus glandula;* and $340 \times 195\ \mu$ for *Tetraclita squamosa.* These dimensions varied only by about $\pm 5\ \mu$ within each species.

Body weight relative to opercular weight

The regressions of body weight relative to opercular valve weight for each outfall and control sample were computed. The intercepts of all these regressions were all near zero, and changes in the slopes were interpreted as measures of fluctuations in body weights of the barnacles. The body weights of the three species fluctuated erratically throughout the year, but there was no discernible seasonal cycle of body weight for any of the three species (Fig. 3). The control samples had consistently higher body weight to opercular valve weight ratios than outfall samples for all three species, except for the second year of data for *Balanus glandula* where there was no difference between the two populations. The body weights are not large compared to brood weight in all species, and it is hard to see how any sizeable quantity of nutrients could be stored there. Variability of body weights in monthly samples may reflect differences other than stored nutrients, *e.g.,* gut contents. Furthermore, differences between outfall and control samples could have been due to differences in opercular valve weights. Because the opercular valves are much heavier than the bodies, small increases in calcium deposition at the higher temperatures in the outfall could have accounted for the smaller body weight to opercular valve weight ratios. The body weight cycles did not correlate with cycles of male reproductive systems of any of the species. It was therefore decided that body weights do not provide good reflections of nutrient storage patterns for these three species.

Male reproductive system

Cycles of the male reproductive systems of the three species are shown in Figure 4. Nearly all specimens of *Chthamalus fissus* have ripe male reproductive tracts

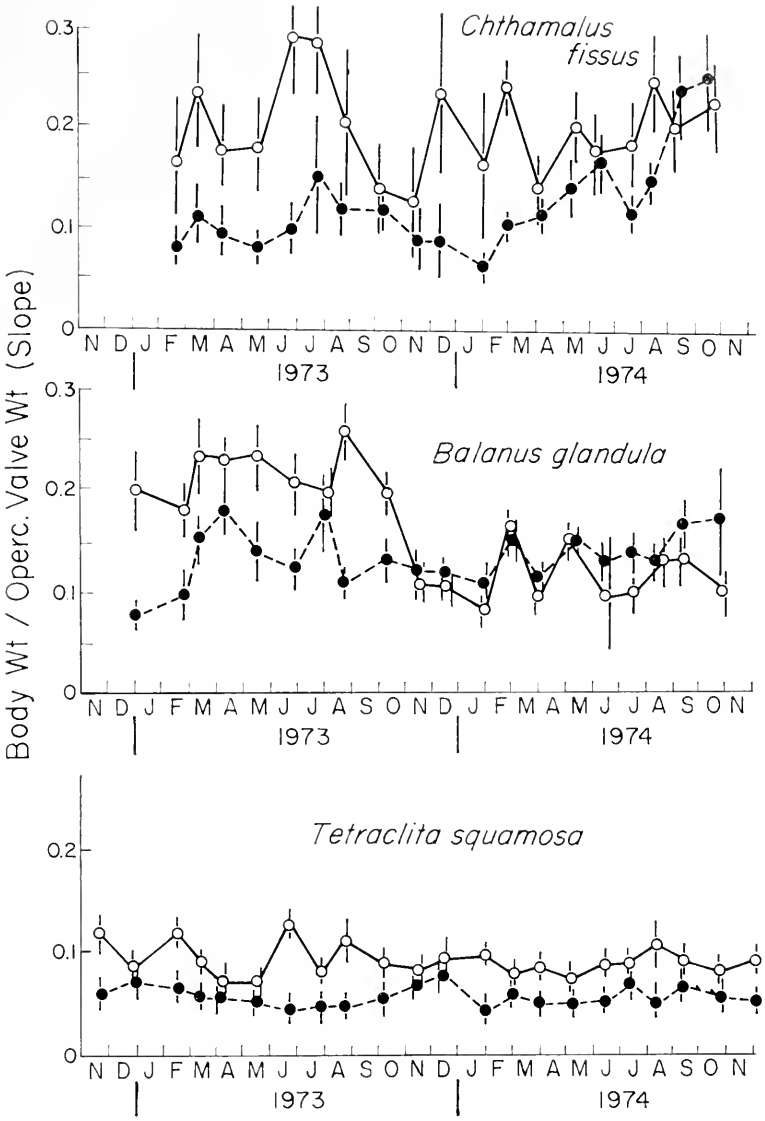

FIGURE 3. Body weight fluctuations. Slopes and 95% confidence limits for regressions of body weight *versus* opercular valve weight are plotted. Outfall population is represented by solid symbols; control population, open symbols.

year-round, with no significant difference between outfall and control populations. *Balanus glandula* had a definite cycle of the male system, developing in the fall from September to November, remaining ripe during the brooding season from December to May, and rapidly becoming quiescent in summer from June to August. Both populations were very synchronous and similar each year, although the control

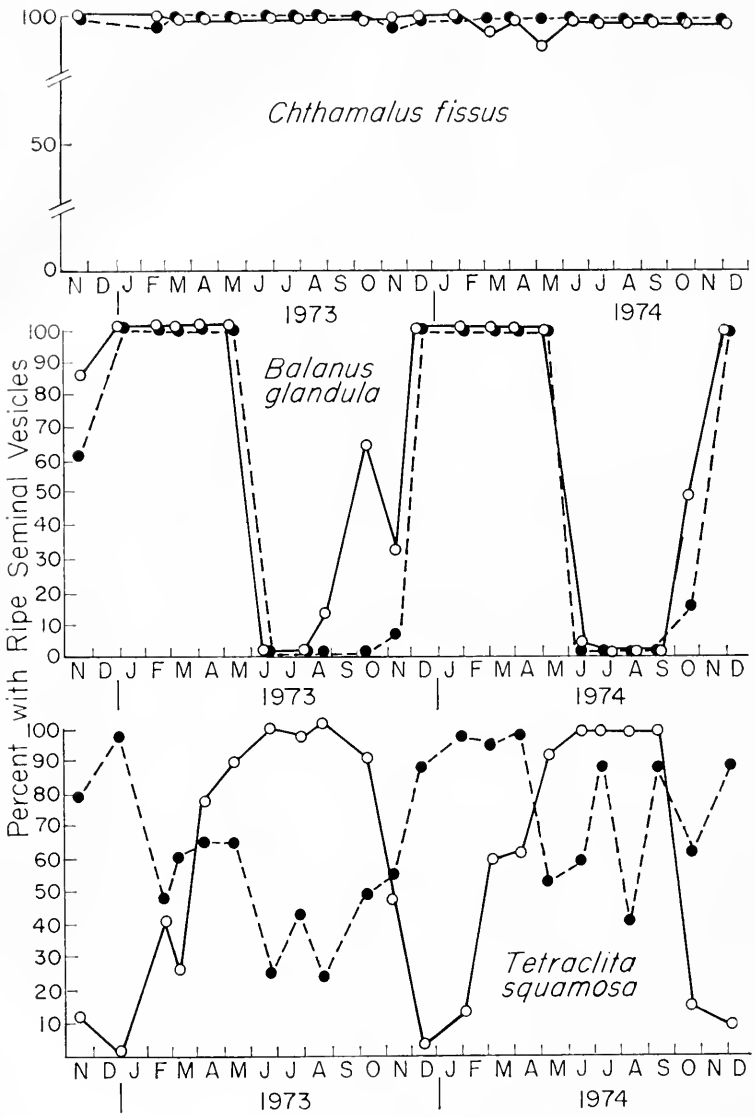

FIGURE 4. Cycles of male reproductive systems: outfall, solid symbols; control, open symbols. Sample sizes are: *Chthamalus fissus* = 50; *Balanus glandula* and *Tetraclita squamosa* = 60.

population became ripe slightly in advance of the outfall population. In *Tetraclita squamosa* the control population showed a distinct cycle of the male system with peak activity occurring from about April to October and low frequencies of ripe individuals from November to February, when development of the male tracts began again. In contrast, the outfall population tended to be at peak frequencies

of ripeness during winter, with erratic intermediate levels of activity during the rest of the year. The warm-water outfall obviously had a major disruptive effect on the cycle of development of the male system in this species.

Ovarian cycles and ovary weight-body weight regressions

Cycles of ovarian ripeness are shown in Figure 5. Ovarian development in *Chthamalus fissus* showed the same cycle as that of brooding frequency, except that

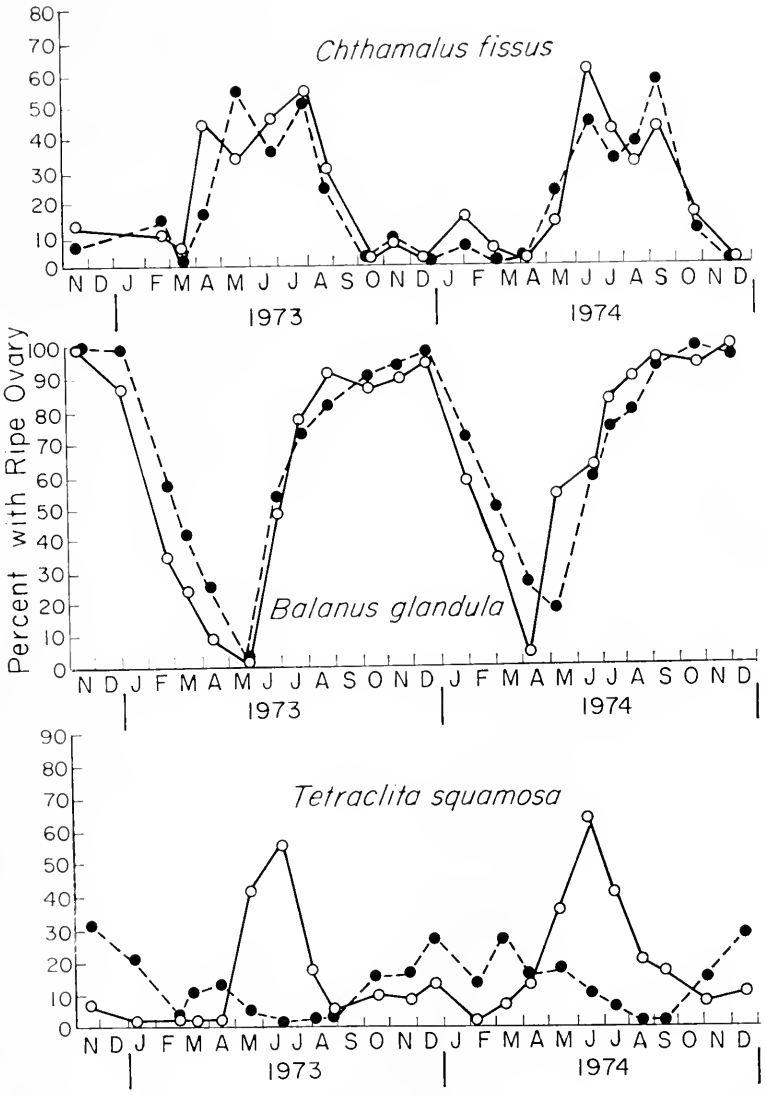

FIGURE 5. Ovarian cycles: outfall, solid symbols; control, open symbols. Sample sizes are: *Chthamalus fissus* = 50; *Balanus glandula* and *Tetraclita squamosa* = 60.

the summer peaks in ovary development did not reach as high frequencies. As in brooding frequencies, there were no consistent differences between outfall and control cycles of ovarian ripeness. The ovaries of *Balanus glandula* ripened rapidly in the summer from June to August. Nearly the whole population was ripe from August through December or January when brooding began. The frequencies of ripe individuals dropped during the brooding period to a low in April and May. The cycles for outfall and control populations were nearly identical, except that the control population appeared to have spent its ovaries somewhat in advance of the outfall population, which might be expected since it began brooding sooner. The ovarian cycle of the control population of *Tetraclita squamosa* showed that these barnacles became ripe in the late spring from April to May, slightly in advance of the brooding period, and percentages of ripe individuals dropped precipitously in July and August when the summer brooding peak was reached. Peak frequencies of ripe individuals roughly corresponded to the peak brooding frequencies attained. However, the warm-water outfall population had only low frequencies of ripe ovaries during a long period from October to May or June, encompassing the same time as the brooding period. This population had virtually no ripe individuals in the summer months.

To estimate the number of broods for which the ovary stored nutrients, ovary weights were analyzed during months when each species was maximally ripe (June, 1973, for *Chthamalus fissus;* December, 1973, for *Balanus glandula;* and June, 1974, for the *Tetraclita squamosa* control population), and also during months when they were least ripe (December, 1973, for *C. fissus;* May, 1973, for *B. glandula;* and

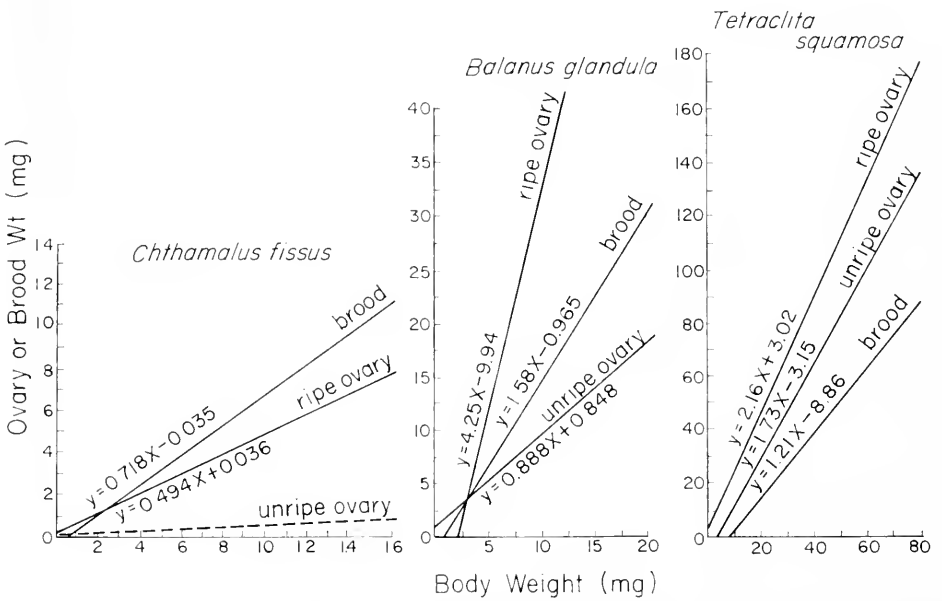

FIGURE 6. Regressions of ripe ovary weight, unripe ovary weight, and brood weight *versus* body weight. See text for further explanation.

February, 1974, for *T. squamosa* controls). The latter series of ovary weights were used as a baseline for "unripe ovary weight", because some of the "ovary" was actually retractor muscles and tissue lining the mantle chamber. Ripe ovaries are extremely diffuse and ramify through the compartment walls, so that some ovarian tissue always remained uncollected when scraped out for weighing. The regressions for "ripe" and "unripe" ovary weight *versus* body weight, as well as the brood weight *versus* body weight regressions, are shown in Figure 6. In *C. fissus* the unripe ovary was essentially nonexistent, and no tissue could be collected for weighing; as the ovary ripened, it increased up to a weight about equal to the brood size, indicating that not more than one brood was prepared at a time. In *B. glandula* the unripe ovary and associated tissues were significant in size and the ovary showed an increase of about 2.5 times the brood weight when ripe. This indicated that yolky material for a minimum of three broods and possibly more was stored in the ovary, considering that much yolky ovarian tissue was left behind in the shell during collection. In *T. squamosa* although both ripe and unripe ovaries and the associated tissues weighed more than the brood, ripe ovaries were only slightly larger than unripe ovaries. Therefore, although these tissues were potentially sizeable storage areas in *T. squamsa,* not much nutrient storage in the form of yolky material detectable by weight-change actually occurred—at most one brood was prepared in advance.

Time per brood and number of broods per season

Preliminary estimates of the time each brood was retained in the mantle chamber were made for *Chthamalus fissus* and *Balanus glandula* by keeping barnacles moist, but not submerged, in a 12° C cold room. Periodic samples of barnacles were inspected for the developmental stages of brooded embryos. Since copulation did not occur and no new broods were laid down unless the barnacles were submerged, only already existing broods continued to develop without hatching. In *C. fissus,* 100% of the broods were judged ready to hatch in about two weeks. In *B. glandula,* all broods were ready to hatch after one month, but the adults were in poor condition after this much time without submergence. *Tetraclita squamosa* could not be maintained unsubmerged for any extended period.

Patel and Crisp (1960b) showed that brooding time determined *in vitro* corresponded well with *in vivo* times for several species of barnacles. To get a better estimate of the time per brood, freshly deposited eggs were removed from the mantle chambers of each species and maintained *in vitro*. *C. fissus* nauplii did not hatch *in vitro* but development appeared to continue normally to a darkly pigmented, eyed nauplius stage which was judged ready to hatch by comparison with the most advanced embryos brooded in the field. The reason hatching did not occur is not known, and whether the proper stimulus required parental presence, or some environmental factor, or both, was not investigated. At 12° C, *C. fissus* nauplii were judged to be ready to hatch in 14 days (s.d. = ± 3 days; n = 21 broods). At 19° C development time for *C. fissus* was 12 days (s.d. = ± 2 days; n = 15 broods). Embryos of *B. glandula* readily developed and hatched *in vitro*. At 12° C time to hatching was 27 days (s.d. = ± 6 days; n = 24 broods), and at 19° C *B. glandula* hatched in 22 days (s.d. = ± 4 days; n = 15 broods). *T. squamosa*

TABLE III

Reproductive effort estimated as the yearly weight allocation to egg production. Length of brooding season divided by the incubation time per brood is the maximum number of broods per year. The number of broods per year times the slope of the brood weight versus body weight regression is brood weight/body weight per year.

	Chthamalus fissus	*Balanus glandula*	*Tetraclita squamosa*
Length of season	8 months	6 months	4.5 months
Time per brood	0.5 month	1 month	1.5 months
Maximum number broods per year	16	6	3
Brood wt/body wt	0.718	1.58	1.21
Brood wt/body wt per year	11.49	9.48	3.63

nauplii hatched in 40 days (s.d. = ± 8 days; n = 20 broods) at 12° C and 30 days (s.d. = ± 7 days; n = 13 broods) at 19° C. In summary, "normal brooding time" (*i.e.*, at 12° C) was estimated at about 0.5 months for *C. fissus* ($Q_{10} = 1.25$); 1.0 month for *B. glandula* ($Q_{10} = 1.34$); and 1.5 months for *T. squamosa* ($Q_{10} = 1.51$).

To calculate a maximum number of broods per year produced by the control populations of each species, the length of the brooding season was divided by the estimated time required for each brood, yielding about 16 broods per season for *Chthamalus fissus,* six for *Balanus glandula,* and three for *Tetraclita squamosa* (see Table III). This calculation makes the assumption that there is no delay between broods. Probably at least a short period between broods in fact occurs, because less than 100% of the barnacles were brooding at any given time. However, there is strong circumstantial evidence that each species puts out several broods in rapid succession, as shown by Crisp and Davies (1955) and Patel and Crisp (1960a, b) for other species. The length of the brooding season for each species in the present study was clearly much longer than the developmental time for each brood, and most barnacles had ripe ovaries remaining while still brooding. Embryos of all stages of development were found throughout the brooding season of each species, and occasionally a new brood was present while advanced embryos from a previous brood remained. Thus, the assumption that there was minimal delay between broods appears reasonable.

Any delay between broods would tend to decrease the number of broods produced in a season. For example, a lag of only 4–5 days between broods in *Balanus glandula* would reduce from six to five the estimate of the number of broods produced over the 6-month season, and this short lag could result in 15–20% of the population not brooding at any given time. On the other hand, any factor, such as temperature higher than 12° C, which shortens the brooding time would tend to make the production of more broods possible. It is thus very difficult to estimate the number of broods produced by the outfall populations. *B. glandula,* for example, tends to have a delayed, shorter brooding season in the outfall with a lower percentage of the population brooding at any given time, but the higher temperatures probably shorten the time each brood is retained. *B. glandula* in the control population thus produces a minimum (based on storage) of three and a maximum (based on brooding time) of six broods per season. It is difficult to estimate a

minimum number of broods per season for *Chthamalus fissus* and *Tetraclita squamosa,* because they do not store yolk for more than one brood at a time.

Patterns of nutrient storage and reproductive effort

A schematic model of the patterns of nutrient storage during the year is presented in Figure 7, showing proportional changes in ovary size relative to brood size for each species. Broods in *Chthamalus fissus* are deposited as soon as enough nutrients are accumulated. As plankton production increases during spring and

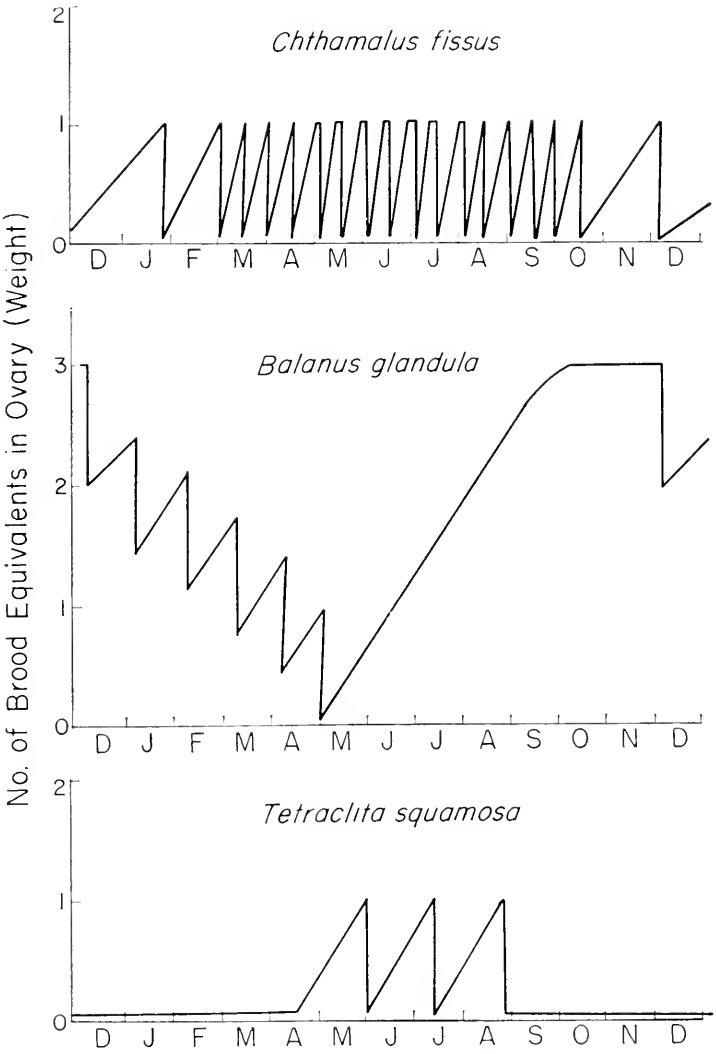

FIGURE 7. Schematic model of patterns of nutrient storage in ovary. Amount of yolky material equivalent to the weight of a brood is shown over a one year cycle for each species.

summer, the rate of yolk accumulation increases and broods are produced more frequently, until brood production is limited by the time required for brood incubation. In contrast, *Balanus glandula* rapidly stores nutrients for at least three broods during the summer and remains ripe until cold temperatures induce brooding. As broods are released during winter and spring, more nutrients are probably added as the barnacles feed until they are spawned out in May after up to six broods. *Tetraclita squamosa* produces only three broods in the summer, and only yolk for one brood at a time is accumulated.

Comparisons of the reproductive efforts of the three species estimated as brood weight relative to body weight per year are shown in Table III. For each species the estimated maximum number of broods produced per season is multiplied by the slope of the regression of brood weight on body weight to calculate the total size-specific brood weight expended during the year. *Chthamalus fissus* has the highest proportional reproductive effort—11.49 times the body weight produced as broods; *Balanus glandula* expends an intermediate but large amount—9.48 times the body weight; and *Tetraclita squamosa* has the lowest weight allocation to brood production—3.63 times the body weight.

Discussion

Species of barnacles may be grouped into roughly five categories based on a spectrum of reproductive patterns: first, a boreo-arctic pattern in which a single large brood is incubated over winter; secondly, a pattern shown by a few cold-temperature species which produce a small number of broods in winter and spring; thirdly, a variable pattern found in several warm-temperature and subtropical species which produce many small broods during summer; fourthly, a possibly different summer pattern of producing only a few broods, as demonstrated here by *Tetraclita squamosa;* and fifthly, a pattern of brooding throughout the year. Some species may show different patterns of reproduction in different parts of their geographic range.

The boreo-arctic species *Balanus hameri, B. balanus,* and especially *B. balanoides* are perhaps the best understood and have the most precisely timed, least complex pattern of brood production. These barnacles store nutrients during a refractory summer period when brooding cannot be induced (Barnes, 1963). In *B. balanoides* copulation occurs in late fall, cued by a low temperature threshold modified by photoperiod (Barnes, 1963), and in *B. balanus* and *B. hameri* copulation occurs in mid-winter (Crisp, 1954; Barnes and Barnes, 1954). A single large brood is incubated over winter, and naupliar release is synchronized with the spring diatom bloom by chemical agents produced by the parents and/or the diatoms (Crisp, 1956; Barnes, 1957). Clearly, the adaptive significance of this precise timing is based on the predictability of the marked seasonal changes in temperature, photoperiod, and productivity of northern latitudes.

Verruca stroemia and *Balanus glandula* in cold temperate waters exemplify a different pattern of brood production during winter and spring. *V. stroemia* is similar to the borea-arctic species in that it produces a major brood in the winter which is synchronous throughout the whole population, and which is released about

the time of the spring diatom increase (Barnes and Stone, 1973). However, unlike the boreo-arctic species, minor broods are subsequently produced asynchronously during spring and summer. Barnes and Barnes (1956) found brooding cycles for *B. glandula* at British Columbia and southern California similar to that reported here for central California. The northern population showed a shorter period of high brooding frequency from January to March, with low frequencies occurring erratically during spring and summer, while their southern population showed a broader season of high brooding frequencies from November to May. The length of the season reported here for Morro Bay is intermediate from December or January to May. Since the present laboratory experiments and the delay of brooding in the warm-water outfall clearly show that the initiation of brooding in *B. glandula* is regulated by cold temperature, the longer brooding season of the southern populations is hard to explain. The brooding season of the Morro Bay population was not extended with laboratory manipulations. In any case, based on cycles of brooding frequency, Barnes and Barnes (1956) suggest *B. glandula,* like *V. stroemia,* produces a single major winter brood followed by a second minor brood in spring. At Morro Bay at least three and as many as six broods are produced, with the first being nearly synchronous in winter and the others following at about monthly intervals through spring. This pattern apparently times the settlement of many larvae in late spring and early summer, when warming temperatures and high food availability are optimal for growth (Hines, 1976), and this pattern distributes the chance of reproductive success over several broods.

Several species of cirripedes in warm-temperate and subtropical regions characteristically produce numerous small broods in rapid succession during summer. The length of the brooding season is usually broadly defined by temperature, and during the season the production of broods is only limited by food availability for restoring the ovary and by the temperature-dependent development rate of the brood in the mantle chamber (Crisp, 1950; Patel and Crisp, 1960a, b). The incubation time per brood as a function of temperature has been measured for a number of species (Patel and Crisp, 1960b), allowing a calculation of about 13–22 broods produced in *Chthamalus stellatus* and 10–25 broods in *Balanus amphitrite denticulata* during spring and summer. *C. fissus* in the present study produces about 16 broods. Although its brooding season is limited by food availability rather than temperature, its reproductive pattern fits in this category. This seasonal pattern is adaptive for quick, opportunistic response to short-term changes in the environment, while minimizing the energtic cost of any single brood if conditions turn bad. It also disperses sibling larvae and improves chances of colonization (Strathmann, 1974).

In the present study *Tetraclita squamosa* produces only about three broods in summer, each incubated for a long period. This is contrary to the pattern in which those species that reproduce in summer tend to produce numerous, frequent broods, while those that put only a few broods seem to be northern species which breed in winter and/or spring. The early reproduction of *T. squamosa* in the warm-water outfall strongly suggests that brooding is cued by warm temperature, although further study is needed since brooding of this species could not be induced in the laboratory. *T. squamosa* grows to a size large enough to attain immunity from

many predators and compete successfully for space at low tidal levels (Hines, 1976). It does not become reproductively mature until it has grown to about 18 mm in diameter, and brooding occurs during the productive summer period. This would minimize the amount of energy diverted from growth to reproduction during critical periods of small size and low food availability.

Elminius modestus and *Balanus pacificus* brood continuously year-round, and both species grow and become reproductively mature very quickly (Crisp and Davies, 1955; Hurley, 1973). *E. modestus* produces a brood about every two weeks during summer; but the rate of brood production is markedly reduced during the cold winter months when food availability is low, resulting in about 12–20 broods per year. *B. pacificus* produces about 23–33 broods per year with no obvious seasonal cycle. Both species are characterized as colonizers, with *E. modestus* undergoing a rapid range expansion since its introduction in Europe and *B. pacificus* settling subtidally on newly bared substrate (Crisp and Davies, 1955; Hurley, 1973).

Some species of cirripedes with wide latitudinal distributions exhibit variable reproductive cycles. For example, *Pollicipes polymerus* shows a variable reproductive season from Washington to southern California. The northern populations brood in summer (Lewis, 1975), and the length of the season increases to the south in northern and central California (Hilgard, 1960; C. Hand, J. Standing and J. Rutherford, personal communication). Farther south at Morro Bay brooding occurs at erratically high frequencies year-round (Hines, unpublished), and at Santa Barbara in southern California peak brooding activity occurs in the winter with at least a 30% brooding frequency in the summer (Straughan, 1971). *Balanus amphitrite denticulata* has a brooding season limited by temperatue from June to August in Great Britain (Patel and Crisp, 1960b), while the brooding of *B. amphitrite communis* in India appears restricted from September to June by salinity and food availability (Pillay and Nair, 1972). *Balanus crenatus* in northern British waters has a boreo-arctic pattern of producing a single large winter brood (Barnes and Barnes, 1968), but it may produce a second, spring brood elsewhere in Great Britain (Patel and Crisp, 1960b), while subtidal populations in central California produce numerous small broods year-round with no obvious seasonal cycle (personal observations). These species with variable reproductive cycles deserve much more study, because they may provide valuable insights into the way proximal environmental cues regulate reproduction under different conditions.

Comparisons of reproductive effort in barnacles require estimates of both the size as well as number of broods produced per year. Because published data on brood weights for barnacles have not been available before the present paper, Barnes and Barnes (1968) compared relative brood volumes of a variety of barnacle species as a next best indicator of brood size. For each species they calculate a product (NV) as a measure of the brood volume relative to the size of the barnacle. ("N" is the increase in numbers of eggs per brood per 50 micrograms dry body weight and "V" is the volume in 10^{-6} ml of an ellipsoid calculated from the length and width of the "egg" at the first naupliar stage.) Brood sizes of barnacles from their calculations fall into three categories: first, boreo-arctic species (including *B. balanoides*, *B. balanus*, and *B. crenatus*) with very large broods, NV = 1500–3500; secondly,

temperate and subtropical species (including five species of *Balanus,* two species of *Chthamalus,* and one each of *Octomerus, Tetraclita,* and *Elminius*) with intermediate but much smaller broods, NV = 100–500; and thirdly, a few species (including two species of *Pollicipes,* one *Chthamalus* and one *Verruca*) with very small broods, NV = 30–60. From their data, *B. glandula* (NV = 292) is included in the second group and *C. fissus* (NV = 47) is in the third; no data are given for *T. squamosa.* From the regression of egg numbers per brood *versus* dry body weight and from the dimensions of the eggs for the Morro Bay populations, comparable NV values can be calculated for *C. fissus* (61) and *B. glandula* (265), and an additional value for *T. squamosa* (199).

Barnes and Barnes (1968) proposes the product (NVB) of the number of broods produced per year (B) times the NV value of a species. They suggest that since boreo-arctic species produce only a single brood and warm-water species many broods, NVB values are roughly equal for all species and the "metabolic efficiency of egg production" in barnacles is constant. However, without measurements of the amount of food available to different species and calculations of their assimilation rates and energy budgets, the term "metabolic efficiency" is misleading, and "reproductive output" or "reproductive effort" are better terms to describe the data. The reproductive efforts of the Morro Bay populations computed similarly as a function of volume are: NVB = 979 for 16 broods in *Chthamalus fissus;* NVB = 1589 for six broods and 795 for three broods in *Balanus glandula;* and NVB = 597 for three broods in *Tetraclita squamosa.* By this measure *B. glandula* has the largest reproductive output of the three species. This ranking does not correspond with the estimates of yearly weight allocation to eggs presented here, although at only three broods per year *B. glandula* would be intermediate between *C. fissus* and *T. squamosa.* Moreover, neither these NVB values nor the estimates of reproductive effort based on weight support the hypothesis that the relative reproductive output of barnacles is constant. Although the NVB value for *B. glandula* at six broods per year is in the low range of values for boreo-arctic species, *C. fissus* would have to produce eight and *T. squamosa* four to five additional broods per year to approach the NVB values of *B. balanus* or *B. crenatus.* In fact, the constancy of reproductive effort proposed for barnacles would not be expected (see Stearns, 1976). In species which produce many small broods small NVB variations would be equivalent to a difference of a brood or two, and this may be very significant ecologically. The unanswered critical questions center on why boreo-arctic species have such a very large reproductive output and on the environmental causes for small, but important variations in reproductive effort in barnacles.

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Summary

1. The reproductive cycles and brood production of *Chthamalus fissus, Balanus glandula* and *Tetraclita squamosa* from central California are compared. *C. fissus* produces about 16 small broods from March through October. *B. glandula* produces three to six relatively large broods from December or January to May. *T. squamosa* incubates only about three intermediate-sized broods from June through September.

2. Brooding in *C. fissus* is regulated by food availability, and yolk for no more than one brood is stored at a time. Feeding in the laboratory elicited high brooding frequencies during periods when brooding activity and food levels in the field were low, and the frequency of brooding was directly proportional to the size of the food dosage. Temperature and photoperiod did not affect brooding frequencies. *B. glandula* rapidly stores nutrients in the ovary for about three broods during summer. Cold temperatures induce early brooding in the laboratory during late fall and early winter, and the population in the warm-water outfall showed delayed and lower brooding frequencies. Photoperiod did not affect brooding in *B. glandula*. *T. squamosa* in the warm-water outfall brooded six months earlier than the control population, suggesting warm temperatures are required for reproduction. Yolk for only one brood at a time is stored in *T. squamosa*.

3. Comparisons of reproductive efforts estimated as brood weight relative to body weight per year show that *C. fissus* has proportionally the largest brood production; *B. glandula* an intermediate but large amount; and *T. squamosa* the smallest reproductive output.

4. It is proposed that species of barnacles may be grouped into five categories based on major patterns of reproductive timing and brood production. The three species in the present paper show three of these patterns. The reproductive effort of these three species is compared with other cirripedes.

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ADAPTATIONS TO INTERTIDAL DEVELOPMENT: STUDIES ON *NASSARIUS OBSOLETUS* [1]

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Egg capsules of marine invertebrates are often described as "protective" (Carriker, 1955; Hunt, 1966; Mileikovsky, 1971; Sverdrup, Johnson and Fleming, 1942), but the question of what the capsules protect against has rarely been addressed. The possibility that the egg capsules of the intertidal mud snail, *Nassarius obsoletus,* evolved as an adaptation to reproduction in the intertidal zone is considered in this study.

Although many marine gastropod and polychaete species deposit egg capsules or egg masses in the intertidal zone, the consequences of intertidal development to the developing young have not been examined. Houbrick (1973, p. 883) noted that the egg masses of *Cerithium variable* are "frequently exposed to sun and air during low tide," and "appear resistant to desiccation." Similarly, the jelly masses of the polychaete *Marphysa* are presumed to protect embryos from the sun at low tide (Aiyar, 1931), and the egg masses of *Littorina littoralis* (= *L. obtusata*) are said to protect developing embryos from desiccation (Fretter and Graham, 1962, pp. 389–390), but data in support of these suppositions are not given. Egg masses of the salt marsh pulmonate *Melampus bidentatus* can be desiccated for long periods of time, apparently without interference to embryonic development (Holle and Dineen, 1957). Whether the egg mass protects the embryos or whether the embryos themselves are especially tolerant of desiccation was not determined.

Spawning in the intertidal zone does not necessarily imply that developmental stages are resistant to intertidal stresses. Several authors have suggested that encapsulated invertebrate embryos are susceptible to desiccation (Spight, 1975; Kohn, 1961) and osmotic stress (Carriker, 1955; Gibbs, 1968). The walls of egg capsules from *Urosalpinx cinerea* are said to be freely permeable to a variety of organic and inorganic solutions, organic salts, and dyes (Carriker, 1955; Galtsoff, Prytherch and Engle, 1937).

Nassarius obsoletus deposits fertilized eggs in capsules affixed to firm substrates in the parental habitat (Dimon, 1905). The capsules, figured by Scheltema (1962), are approximately 1.5 mm high and contain 30 to several hundred eggs (Costello and Henley, 1971). The encapsulated embryos develop in the intertidal zone for approximately one week, after which time veliger larvae emerge from the capsules to continue their development in the plankton for at least several more weeks before metamorphosing to the benthos (Scheltema, 1962). While encapsulated, embryos are potentially exposed to intertidal stresses, the most obvious of which is desiccation. Kanwisher (1957) reports that relative humidities of 40% are com-

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monly observed in the intertidal zone. Successful development in the intertidal zone may require egg capsules which retard water loss at low tide, embryos capable of tolerating extensive dehydration, or preferential placement of the capsules in high-humidity situations. Alternatively, substantial pre-hatching mortality may occur. These possibilities were considered through determinations of desiccation tolerances of encapsulated *N. obsoletus* embryos, rates of water loss from *N. obsoletus* egg capsules relative to rates of water loss from capsules of the subtidal species *N. trivittatus,* and studies of the adult egg-laying behavior of *N. obsoletus.*

Materials and Methods

Egg capsules of *Nassarius obsoletus* were removed from *Fucus* and eel grass collected at the Barnstable mudflats on Cape Cod, Massachusetts. Egg capsules of *N. trivittatus* were deposited in the laboratory by adults dredged from Buzzard's Bay, Massachusetts. Undamaged capsules of both species were sorted into two age groups before each experiment, based on the extent of anatomical differentiation of the enclosed embryos visible at a magnification of 25×. Capsules containing "early" embryos (no velum pigmentation or shell visible) were distinguished from those containing "advanced" embryos (distinct shell and easily discernible velum pigment). Only capsules completely full of eggs and containing embryos of a single age class were included in experiments.

Desiccation tolerance of encapsulated embryos was examined at two relative humidities, approximately 0% and 75% as determined with a Honeywell portable hygrometer. These relative humidities were achieved by covering the bottoms of glass jars with anhydrous $CaSO_4$ or a saturated solution of NaCl in distilled water (O'Brien, 1948), respectively. Age-sorted *N. obsoletus* capsules were spooned into perforated, plastic petri dishes, and most of the adhering water quickly blotted away. From 50 to 209 egg capsules were spooned into each dish. Large clumps of capsules were broken up into smaller groups, but no attempt was made to isolate each individual capsule. Each dish was suspended above desiccant in a jar which was then sealed. All experiments were conducted at room temperature, 22–23° C. The air in each jar was stirred by rocking the dish of capsules every fifteen minutes. At pre-determined intervals, one dish from each age class was removed from a jar, capped with a perforated top, and submerged in running sea water (approximately 30‰). One dish of egg capsules from each age class served as a control for its age group and was not subjected to desiccation, being placed in flowing sea water at the start of the experiment. Capsules suspended over distilled water for the entire exposure period served as additional controls; relative humidity within these jars was 100%, as determined with a Honeywell portable hygrometer. After treatment, the dishes of egg capsules were submerged in flowing sea water and examined periodically to assess survival. Tolerance of the stress was indicated by the eventual escape of veligers from the capsules. Capsules do not open spontaneously; a specialized hatching substance produced by the embryos is required for escape to occur (Pechenik, 1975).

Additional experiments were conducted to determine the effect of repeated exposures to low humidity air on pre-hatching mortality. Groups of capsules were

subjected to 75% relative humidity for either 0.5 hr or 2 hr each day, until hatching was completed after about 9 days.

The rates of water loss from intertidal *N. obsoletus* egg capsules were compared with rates of water loss from the morphologically similar, but primarily subtidally-deposited capsules of *N. trivittatus* (Scheltema and Scheltema, 1964). The magnitude of the differences found should reflect the degree to which *N. obsoletus* capsules are specifically adapted for their intertidal deposition. Ten to 12 min after the addition of $CaSO_4$ desiccant to the weighing chamber of a Cahn Electrobalance, a single egg capsule was blotted dry, dropped onto the weighing pan, and the weighing chamber was quickly resealed. Capsule weight was determined at 30 sec intervals for 15 min, or until the weight stopped changing between readings.

Data were obtained for 40 egg capsules, ten capsules for each of the two age groups for both species. The rate of weight loss was found to be constant for all capsules for the first 8.0 min after the start of each experiment. Hence, the rate of weight loss was computed from the weight change observed during this interval. Capsule weight at 1.5 min after the initiation of the experiment was taken as the "initial" weight. Since the rate at which water can be lost from a closed container is a function of exposed surface area, proportional to $(\text{weight})^{2/3}$, the data were adjusted using the following expression before statistical comparisons were made between groups of capsules: adjusted rate = (mg lost/30 sec)/(initial capsule weight)$^{2/3}$. This manipulation substantially reduced variability in the data. Rates of weight loss discussed in the text are unadjusted rates, unless otherwise indicated.

The placement of *N. obsoletus* egg capsules with respect to substrate orientation was examined in the laboratory. Adults were collected from Quissett Harbor and Barnstable Harbor, Massachusetts, and held in aerated aquaria. Snails were fed every two to three days on chopped *Mercenaria mercenaria* tissue. A rectangular, plastic container with one curled edge on the upper surface was placed on the bottom of each tank, completely submerged in sea water, and the number of capsules deposited upon each surface of the container was determined on nine occasions over a 2-week period. All capsules were removed from the container after each observation, so that the extent of deposition on any surface was never limited by the area available.

The extent to which egg capsules are protected from exposure to low humidity air in the field was estimated by spraying exposed clumps of *Fucus* with blue enamel paint during low tide at Quissett Harbor and Eel Pond. The spray paint should have reached only those capsules exposed to the air, while capsules in more sheltered, high-humidity sections of the *Fucus* should have remained untouched by the paint. The *Fucus* clumps were then detached from their rocks and the numbers of blue, "speckled" (exhibiting from one to several small blue spots), and unpainted capsules on the algae were determined. The position of each capsule with respect to the basal 2 cm of algae was also recorded.

Results

Desiccation tolerance of encapsulated embryos

Egg capsules of *N. obsoletus* did not afford substantial protection against desiccation. At 0% relative humidity, mortalities of at least 60% were sustained after

TABLE I

Mortality of encapsulated early-stage N. obsoletus *embryos after single exposures to 75% relative humidity.*

Treatment	Number of capsules	Exposure time (hr)	Mortality (%)
Sea water control	209	0	0.5
100% relative humidity control	111	5	0.0
75% relative humidity	156	1	0.0
75% relative humidity	123	2	3.3
75% relative humidity	126	2.5	1.6
75% relative humidity	151	3	2.6
75% relative humidity	141	3.5	12.5
75% relative humidity	167	4.5	10.2
75% relative humidity	169	5	15.7

a single exposure of less than one hour (Fig. 1). Substantial mortality of early-stage embryos resulted after only 15 minutes of exposure. Controls exhibited less than 5% failure to hatch. Even at 75% relative humidity, 12% of the capsules failed to release larvae after a single 3.5 hr exposure, while control mortalities were less than 0.5% (Table I). Clumping together of the egg capsules in the petri dishes probably accounts for the lack of increased mortality between 2 and 3 hours, since clumps would retain moisture longer than individual capsules. Although mortalities did not exceed 3.3% at 75% relative humidity for single exposures of less than 3.5 hr, daily exposures for considerably shorter periods, 0.5–2 hr, resulted in mortalities of approximately 15% (Table II).

Advanced-stage encapsulated embryos were significantly more tolerant of desiccation stress than were early-stage embryos (Fig. 1; $P < 0.05$, as tested by Chi-square). This indicates that age-related changes occur either in the water-retaining ability of the capsules or in the tolerance of the embryos themselves, as considered below.

Rates of water loss from egg capsules

There were no statistically significant differences in adjusted rates of water loss from capsules of different ages, for either *N. obsoletus* or *N. trivittatus,* as analyzed

TABLE II

Mortality of encapsulated N. obsoletus *after daily exposures to 75% relative humidity. Capsules contained early-stage embryos at start of experiment.*

Treatment	Number of capsules	Exposure time (hr)	Mortality (%)
Control	72	0	4.2
Control	131	0	3.0
100% relative humidity	122	1	4.1
100% relative humidity	50	2	0.0
100% relative humidity	143	2	0.0
75% relative humidity	63	0.5	17.5
75% relative humidity	73	2	12.3

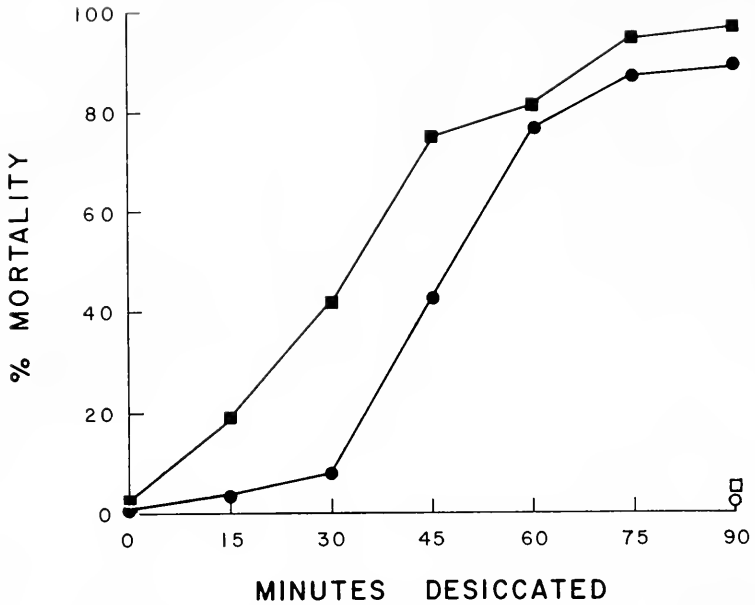

FIGURE 1. Desiccation tolerances of encapsulated embryos of *N. obsoletus*. Experiments were conducted over $CaSO_4$ desiccant. Circles represent data from capsules containing late-stage embryos ($N = 455$ capsules). Squares represent data from capsules containing early-stage embryos ($N = 293$ capsules). Open symbols indicate data from control capsules held at 100% relative humidity for the full 90 minutes.

by analysis of variance ($F_{N.\ trivittatus} = 1.94$; $F_{N.\ obsoletus} = 2.62$; $P > 0.1$; $N = 20$ capsules for each species). Since the water-retaining ability of the capsule itself does not change with age, age-related improvements in desiccation tolerance are attributable to a change in embryonic tolerance, possibly due to the development of the embryonic shell.

Egg capsules of both species lost weight at a constant rate for at least the first 8 minutes of observation. One-way analysis of variance revealed no statistically significant differences in the adjusted rates of water loss from egg capsules of the two different species ($F = 1.07$; $N = 20$ capsules for each species; $P > 0.1$). Thus, the water-retaining ability of the intertidally-placed *N. obsoletus* capsule is essentially identical to that of the subtidally-placed *N. trivittatus* capsule.

Rates of water loss (weight/unit time) from capsules similar in initial weight were essentially identical, regardless of capsule age or identity. Larger capsules lost weight more rapidly than smaller capsules, as expected. The relationship between the rate of weight loss (Y) and the weight of the capsule at 1.5 minutes after the start of the experiment (X) is given by the equation $Y = 0.027 + 0.015X$, as calculated by linear regression analysis (Fig. 2). The mean rate of weight loss from the 40 egg capsules was 0.044 mg/30 sec ± 0.005 (mean ± s.d.). The differences in rates of water loss between egg capsules were due primarily to differences in the sizes of the capsules; the correlation coefficient (r) between rate of

weight loss (mg/30 sec) and "initial" egg capsule weight was 0.82 ($F = 80.85$; $N = 40$ experiments).

Placement of egg capsules in the laboratory

Capsules were not deposited randomly on the plastic containers (Table III). The differences in the numbers of capsules received by each surface of the container are significant at the 0.01 level, as tested by analysis of variance (d.f. = 11,96; $F = 4.7$). Statistical comparisons reveal two behavioral phenomena associated with capsule deposition. Low thigmokinesis (Fraenkel and Gunn, 1961) is revealed by particularly heavy deposition along edges, under the curled edge, or underneath the platform, regions maximizing the amount of contact stimulation of the foot of the depositing female. There is also an orientation component, specifically a preference for depositing while hanging. The fewest capsules were attached to the bottom and outside top surfaces of the container. The correlation coefficient (r) between the number of capsules deposited and available surface area on the different surfaces of the container was 0.36, indicating that placement preferences were not related to the amount of surface area available.

Capsules were never deposited above the water line in laboratory aquaria, suggesting that capsule deposition in the field occurs only when the substrate is submerged.

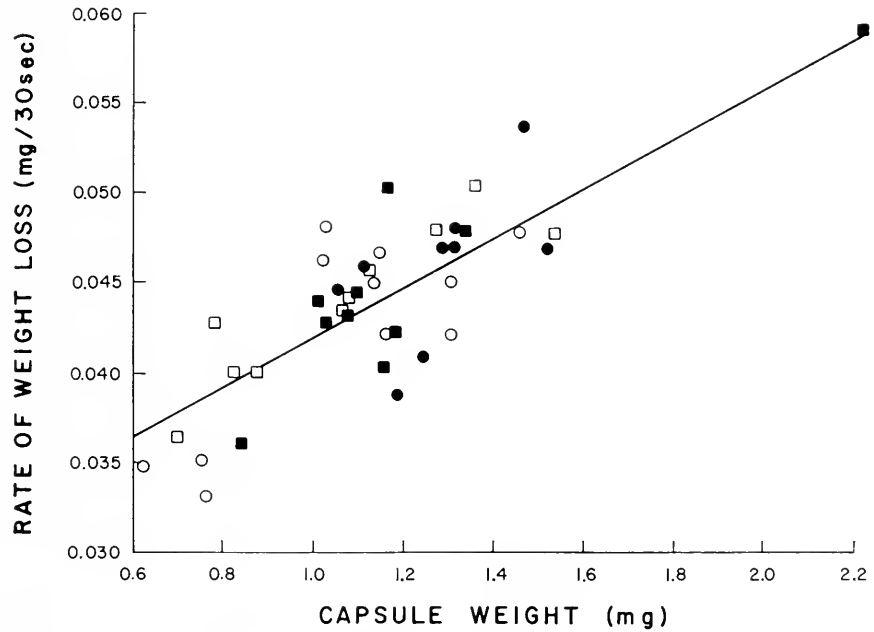

FIGURE 2. Rate of weight loss for desiccating egg capsules of *N. obsoletus* (squares) and *N. trivittatus* (circles) as a function of "initial" capsule weight. Open symbols represent data obtained from capsules containing early-stage embryos, while solid symbols represent data obtained from capsules containing advanced-stage embryos.

TABLE III

Distribution of N. obsoletus *egg capsules deposited on plastic containers in the laboratory.*

	Quissett Harbor adults	Barnstable Harbor adults
Total capsules deposited	855	501*
Percentage of capsules deposited on:		
Bottom	1.6	2.0
Back	18.5	4.7
Sides	9.8	6.1
Top (inside)	16.0	17.2
Top (outside)	1.2	0.0
Edges (inside)	21.9	64.0
Edges (outside)	11.0	5.0
Under curled edge	20.0	1.0

* This container was improperly anchored. An additional 101 capsules were deposited on the underside of the container.

Placement of capsules in the field

Due to the low thigmokinetic component of the adult egg-laying behavior, one expects to find more capsules deposited near the holdfast of *Fucus* than upon other portions of the algae, owing to the closeness of individual strands in the holdfast area relative to other sections of the algae when submerged. One would also expect most of the capsules to be deposited on the undersides of the strands, due to the apparent preference for hanging while depositing.

Capsules on *Fucus* collected at Quissett Harbor were located primarily in the region of the holdfast, as predicted (Table IV). Although this did not hold true for Eel Pond *Fucus* on a strictly numerical basis, many more capsules were deposited in the holdfast region than elsewhere on the algae when the relative surface areas (estimated by dry weight) available for deposition of capsules were considered. One hundred capsules/gram algae were found in the region of the holdfast and 11.6 capsules/gram algae were found elsewhere, based on examination of four plants, and counts of 68 and 235 capsules in the basal and distal regions of the algae, respectively.

The pattern of paint on egg capsules was similar for tufts of *Fucus* sprayed at both locations at low tide, so that only the results from Quissett Harbor are presented here (Table IV). Most of the capsules were at least partially protected from exposure to low humidity air, fewer than 8% of the capsules being entirely

TABLE IV

Distribution and color of 583 N. obsoletus *egg capsules recovered from* Fucus *spray-painted at low tide at Quissett Harbor, Massachusetts.*

	Holdfast	Nonholdfast
Deposited in zone	69.0%	31.0%
Blue capsules	7.7%	4.4%
Unpainted capsules	8.0%	13.3%
Speckled capsules	84.3%	82.3%

exposed at low tide. Speckled capsules, which generally exhibited only one or two small spots of paint, can probably be considered as being protected.

The dearth of firm substrate available to the large *N. obsoletus* population on the Barnstable mudflats results in the attachment of capsules to all available surfaces; suitable macro-algae are literally covered with capsules. Since much of this material is completely exposed to air at low tide, pre-hatching mortality here may be high. Dried and shrunken *N. obsoletus* egg capsules are frequently encountered. Of 664 egg capsules collected at the end of a low tide at Barnstable early in August and held in flowing sea water in the laboratory, 28% failed to release veligers. As most of these capsules contained early-stage embryos when collected and because repeated exposure to low humidity air increases mortality significantly, as demonstrated above, 28% is probably a minimum estimate of the total pre-hatching mortality that would have occurred had the embryos been allowed to complete their development in the field.

Discussion

Caution must be used in relating experimental data on embryonic desiccation tolerances and rates of water loss from egg capsules to actual events in the field. Relative humidities of 0% are unlikely to occur normally, although relative humidities of 40% are not uncommon in the intertidal zone (Kanwisher, 1957). Experiments conducted at 0% relative humidity demonstrate two important points, however. First, encapsulated embryos of *N. obsoletus* are more susceptible to desiccation stress than are the embryos of *Melampus bidentatus* (Holle and Dineen, 1957). The apparently lower susceptibility of *M. bidentatus* embryos to desiccation is surprising; although the egg masses are deposited high in the intertidal zone, an accumulation of detritus around them probably prevents their desiccation (Russell-Hunter, Apley and Hunter, 1972). Secondly, the egg capsules of *N. obsoletus* are no more effective in retaining water than are capsules of the subtidal *N. trivittatus*. Bayne (1968) obtained similar results with the egg capsules of the intertidal gastropod *Nucella lapillus*. Rates of water loss [mg/(min·unit surface area)] from egg capsules of this species were nearly identical with those from the normally submerged spawn of both the opisthobranch *Aplysia punctata* and the basommatophoran *Lymnea stagnalis*.

It is not possible to predict the actual extent of pre-hatching mortality from the experimental data presented here. The impact of desiccation in the field will vary with humidity, temperature, duration and degree of exposure, wind velocity, and the thickness of any boundary layer that may be present above the capsules. The important points are that substantial pre-hatching mortality of *N. obsoletus* occurs in the laboratory after even a single, short exposure to 75% relative humidity, and that daily exposure to desiccation results in significantly greater mortality than that observed after a single exposure. Substrates literally encrusted with *N. obsoletus* egg capsules are often found completely out of the water at low tide on the Barnstable mudflats. In such cases, pre-hatching mortality must be high, since the egg capsules are not effective in preventing water loss and the embryos are not particularly tolerant of dehydration. Spight (1975) reported pre-hatching mortalities of

approximately 40% for intertidally-deposited *Thais lamellosa* egg capsules; desiccation was a major cause of this mortality.

There is, therefore, no evidence that gastropod egg capsules are specifically adapted for placement in the intertidal zone, and it seems unlikely that egg capsules evolved as adaptations to intertidal stresses.

Protection of the encapsulated embryos of *N. obsoletus* seems dependent upon adult behavior. Because capsules are placed on the undersides of *Fucus,* they are kept moist by the blanketing effect of the seaweed above them. Detailed experimental work on egg capsule placement preferences is lacking for most marine invertebrates (Meadows and Campbell, 1972), but the protection of early developmental stages through adult spawning behavior appears widespread in marine gastropods. Kudinsky (1972) claims that eggs of the prosobranch gastropod *Testudinalia tessellata* are laid preferentially in situations where they are spared direct exposure to sunlight. The egg capsules of *Conus spp.* (Kohn, 1961), *Cypraea* spp. (Crovo, 1971), shallow-water columbellid gastropods (Bandel, 1973), *Urosalpinx cinerea* (Carriker, 1955), and *Bembicium auratum* (Anderson, 1962) are deposited on the undersides of rocks, presumably to prevent desiccation (Anderson, 1962; Kohn, 1961), and the archaeogastropod *Neritina virginea* is said to deposit its capsules preferentially in crevices (Andrews, 1935).

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Summary

1. The extent to which reproduction of the intertidal mud snail, *Nassarius obsoletus,* is adapted to the intertidal environment was examined in an attempt to understand the adaptive significance of egg capsules in the life history.

2. Contrary to expectation, laboratory studies on desiccation tolerance of encapsulated embryos and rates of water loss from egg capsules failed to reveal any adaptation to intertidal development. Fifteen minutes of desiccation over $CaSO_4$ caused as much as 20% mortality of *N. obsoletus* embryos, and daily 0.5 hr exposures to 75% relative humidity killed 17.5% of the embryos. Egg capsules of *N. obsoletus* and those of the subtidal *N. trivittatus* lost water at essentially equal rates.

3. Protection of the developing embryos seems dependent upon adult behavior. Adults tend to deposit egg capsules into microenvironments where the embryos are probably spared exposure to desiccation stress at low tide. Fewer than 8% of the capsules examined at Quissett Harbor, Massachusetts, were fully exposed to desiccation.

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DIFFUSIONAL WATER PERMEABILITY IN SELECTED MARINE BIVALVES

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The intertidal habitat presents many severe problems to the organisms living in this particular environment. Cyclic fluctuations in salinity, oxygen levels and temperature occur, and these changes in the physical parameters of this environment are amplified with increasing height above the sublittoral zone. Organisms which inhabit the intertidal area have undergone certain adaptations which increase their survival potential in the face of a constantly changing environment.

In crustaceans, the permeability of the body surface to water and ions can be correlated with the animals' particular habitat (Lockwood, 1962; Herreid, 1969a, b). In this case, sublittoral crustaceans are more permeable than littoral species, which in turn are more permeable than estuarine species. Pieces of exoskeleton from crustaceans which are osmoregulators, and for the most part intertidal, are less permeable than osmoconformers, which are generally sublittoral (Gross, 1957). In barnacles, the resistance of the organism to desiccation is a function of its vertical zonation level, *i.e.*, the higher the species in the intertidal zone, the more resistant it is to desiccation (Foster, 1971). Some intertidal crustaceans apparently are capable of controlling the permeability of the exoskeleton and decrease water permeability in response to decreased external salinity (Smith, 1970; Lockwood, Inman and Courtenay, 1973).

Physiological adaptations to some environmental stress situations have also been demonstrated in intertidal molluscs. Resistance to desiccation in certain intertidal gastropods has been correlated with vertical zonation (Brown, 1960), and the sequence of thermal death points in some intertidal gastropods has also been correlated with zonation level (Broekhuysen, 1940). Physiological adaptations at the tissue level have also been demonstrated in intertidal bivalve molluscs (Vernberg, Schlieper and Schneider, 1963). In this case bivalve gill tissue from intertidal animals could withstand a wider range of salinity changes than could sublittoral animals as determined by gill ciliary activity. Behavioral adaptations of intertidal bivalves include closing of the valves during periods of osmotic stress (Krogh, 1939).

A question arises as to whether or not the tissues of intertidal bivalve molluscs display the same adaptive water permeability characteristics as intertidal crustaceans, *i.e.*, a decrease in water permeability with increasing height in the intertidal zone; or whether the distribution of intertidal bivalves is influenced mainly by other environmental factors independent of salinity (Pilgrim, 1953). In addition, can specific intertidal bivalves alter the diffusional water permeability of their tissues in response to an osmotic stress, as is apparently the case in crustaceans? These

problems were investigated in a series of marine bivalves by measuring the rate of movement of tritiated water across the isolated mantle tissue.

Materials and Methods

The diffusional water permeability across isolated mantle tissue was determined in eight different lamellibranch species obtained from different collection sites in New England. Water samples were also collected from each collection site and total salinity determined by potentiometric chloride titration. Specimens of *Placopecten magellenicus* (Gmelin) and *Modiolus modiolus* (L.) were both collected from waters off Mount Desert Island, Maine. *Placopecten* was collected from a depth of 10 m, while *M. modiolus* was found at a depth of 2 to 3 m, in both cases the salinity of the water was approximately 32‰. Specimens of *Spisula solidissima* (Dillwyn) were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. *Spisula* was found approximately 2 m deep on a sandy bottom in water with an average salinity of 33‰. This population of *Spisula* was never exposed at low tide. Specimens of *Mercenaria mercenaria* (L.) were collected from Narragansett Bay, Rhode Island, in water 0.5 to 2 m deep with muddy-sand substratum and having a salinity range from 24 to 31‰. Two different groups of *Mytilus edulis* (L.) were collected from the rocky shores off Jamestown, Rhode Island. One group was located low in the intertidal zone (ELWN), while the other group was found sublittorally in approximately 5 m water. The salinity of the water at this site ranges from 26 to 32‰. Specimens of *Modiolus demissus* (Dillwyn), *Crassostrea virginica* (Gmelin) and *Mya arenaria* (L.) were all collected from Sippiwissett salt marsh near Woods Hole and specimens of *Anodonta* sp., also used in this study, were obtained from a local biological supply house. Mantle water permeability, unless indicated otherwise, was measured in freshly collected animals.

The mantle was used in these studies because of its relatively simple structure, an epithelial sheet with two cell layers (Neff, 1972), and the ease with which it can be removed from the animal. The mantle was excised from the animal by cutting the adductor muscles to open the valves and then removing the central portion of the mantle from one of the valves where it was not attached. The mantles were always covered with sea water during dissection and experimental procedure, except for *Anodonta* for which a Ringer's solution was used (Istin and Kirschner, 1968).

The isolated mantle was used to separate a diffusion chamber into two compartments of 10 ml each, the diameter of the exposed tissue being 0.5 cm. The diffusional water permeability (P_d) of the isolated mantle was determined by adding 10 to 50 μCi tritiated water (THO) to one compartment and monitoring its rate of appearance in the other compartment. Both compartments were constantly stirred by bubbling air through the medium. Aliquots (100 μl) of the medium in the second compartment were taken at various time intervals, placed in a scintillation vial with 15 ml Aquasol (New England Nuclear) and counted in a liquid scintillation counter. Each aliquot removed during the course of an experiment was replaced with an equal volume of fresh medium in order to maintain constant volume.

Samples of the medium in the compartment to which THO was initially added were also counted. Since identical solutions are on both sides of the mantle and it is assumed that the concentration of THO added to the first compartment remains constant for the duration of the experiment, P_d can be calculated from the following relationships: first, specific activity (SA) = $[THO]/[H_2O]$; secondly, tritiated water flux, J_{THO} = slope of the linear portion of the flux curve/mantle surface area; thirdly, water flux, $J_{H_2O} = J_{THO}/SA$; and fourthly, diffusional water permeability, $P_d = J_{H_2O}/[H_2O]$. Mantle thickness was determined by freezing isolated pieces of tissue in an acetone-dry ice bath, then measuring the thickness of broken pieces of the frozen mantle through a microscope with a calibrated ocular micrometer.

Specimens of *Mytilus edulis,* collected by the MBL Supply Department from Lucas Shoal off of Martha's Vineyard at a depth of approximately 9 m, were used in another series of experiments designed to determine whether or not this particular bivalve species is capable of altering its tissue water permeability in response to an induced environmental osmotic stress. Approximately fifty individuals were randomly divided into two groups after the mantle water P_d was initially determined for five individuals. One group of animals was maintained in ten liters of aerated, full-strength sea water, while the other group was maintained in an equal volume of 70% sea water. The water for both groups of animals was changed daily. Mantle

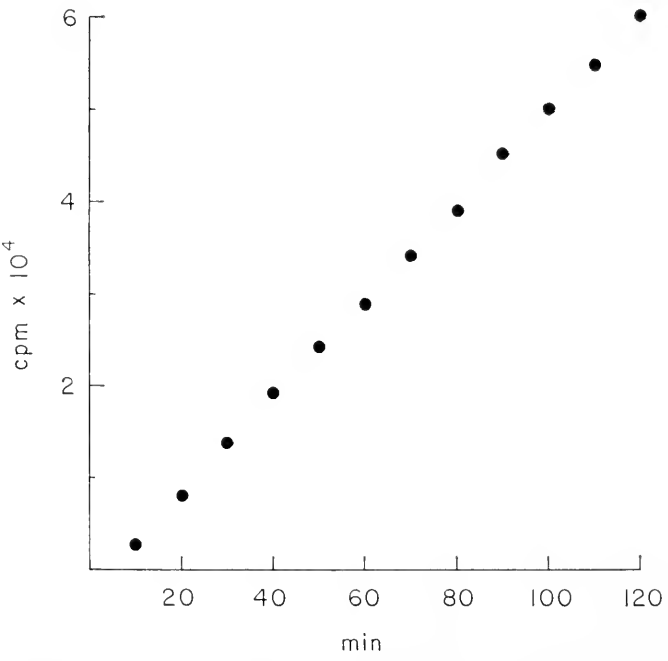

FIGURE 1. Unidirectional, tritiated water movement across an isolated piece of *Mercenaria mercenaria* mantle tissue. The total counts per minute appearing in the bath are presented as a function of time.

water P_d was determined for animals from both groups at various time intervals after the start of the equilibration period.

In all of the experiments in this study, no selection for size was made of the animals used, except that each animal had to be large enough to yield a piece of mantle tissue which would cover the hole between the chambers. All experiments were performed at room temperature, 19 to 20° C. In most instances this temperature was higher than the environmental temperature the animals were collected at, but most likely this temperature change has a minimal effect on the measured mantle water P_d. For example, the diffusion of water between 10 and 20° C is not greatly influenced by an increase in temperature, $Q_{10} = 1.04$. The results are presented as the mean (number of determinations) ± the standard error of the mean.

Results

A representative THO flux curve is shown in Figure 1. THO was added to one side of the flux chamber separated into two compartments by the isoated mantle tissue, and its rate of appearance followed on the other side of the chamber. Since the amount of isotope added to the first compartment is relatively large, and in the time course of these experiments it decreases only slightly (less than 5%), the flux curve is linear after an initial lag period. The observed lag period in these experiments, 3 to 10 minutes, is most likely due to the initial time required for a constant specific activity to be established in the intracellular compartments of the isolated mantle tissue and did not vary in the different species examined in this study.

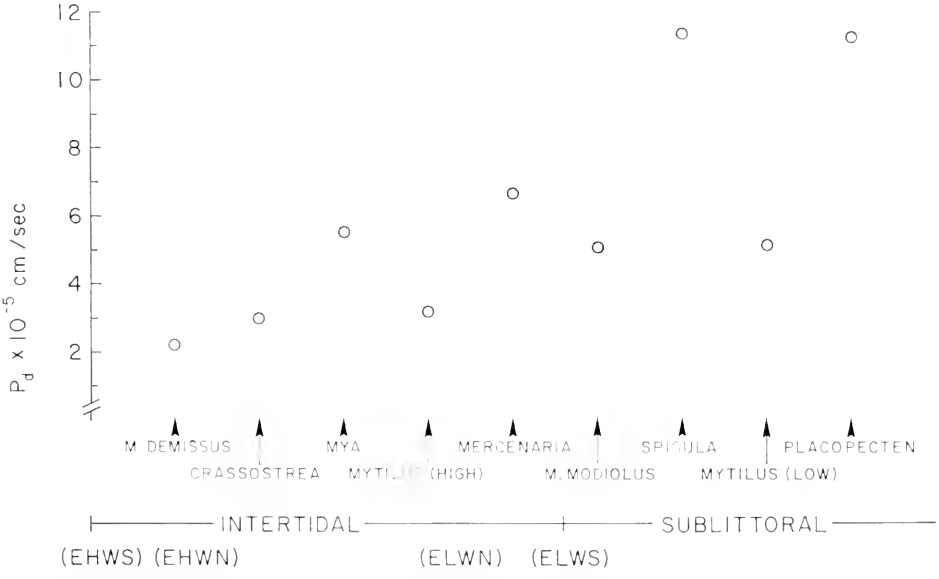

Figure 2. Calculated diffusional water permeabilities (P_d) for isolated mantle tissue from a series of lamellibranch molluscs as a function of their relative distribution to each other.

TABLE I

Mantle thickness in a series of bivalves in which the diffusional water permeabilities were also determined.

	Mantle thickness mm
Modiolus demissus	1.15 ± 0.06 (12)
Mytilus edulis (high)	1.28 ± 0.09 (6)
Mercenaria mercenaria	0.76 ± 0.05 (10)
Modiolus modiolus	1.04 ± 0.07 (7)
Spisula solidissima	0.73 ± 0.02 (6)
Anodonta sp.	0.68 ± 0.05 (7)

Calculated diffusional water permeabilities for eight different marine lamellibranch species are presented in Figure 2 as a function of their distribution in relation to one another. These P_d values are as follows: *Modiolus demissus*, 2.2 ± 0.18 (12); *Crassostrea virginica*, 3.01 ± 0.28 (7); *Mya arenaria*, 5.51 + 0.19 (9); *Mytilus edulis* (high), 3.21 ± 0.61 (6); *Mercenaria mercenaria*, 6.69 ± 0.25 (10); *Modiolus modiolus*, 5.08 ± 0.24 (7); *Spisula solidissima*, 11.40 ± 0.67 (6); *Mytilus edulis* (low), 5.18 ± 0.45 (8); and *Placopecten magellanicus*, 11.29 ± 0.07 (5) $\times 10^{-5}$ cm/sec. In addition the diffusional water permeability for the isolated mantle tissue of a representative freshwater bivalve, *Anodonta* sp., was found to be 6.17 ± 0.22 (7) $\times 10^{-5}$ cm/sec.

Measurements of the thickness of the mantles used in some of these permeability determinations are given in Table I. There is apparently no correlation between the thickness of the mantle tissue and its measured water P_d. For example, there is no real significant difference between the thickness of mantle tissue used from *Mytilus edulis*, *Modiolus demissus* and *Modiolus modiolus* in these studies, although there is a considerable difference in their water permeabilities. This apparent independence between tissue thickness and water permeability may be accounted for if the tissue itself is nonhomogeneous as far as water movements are concerned. That is, permeability is a positive function of thickness in a given system only if the resistance to diffusive movement is constant throughout its entire thickness. Alternatively, one thin rate limiting diffusion barrier may be in series with the thicker, more permeable tissue. In this case, the measured tissue P_d would be apparently independent of the thickness of the tissue (Prusch and Benos, 1976).

An interesting question arises from this study concerning the ability of intertidal bivalves to alter their tissue water permeability in response to osmotic stress, such as has been previously reported in some crustaceans (Herreid, 1969a). In Figure 2, the mantle water P_d is presented for two groups of *Mytilus edulis* from the same general area, one collected intertidally and the other found sublittorally. Those animals found higher intertidally had a significantly lower tissue water P_d than those found in the sublittoral zone. This would suggest either that the two groups of mussels represent different physiological races or that this animal is capable of altering its tissue permeability in response to an osmotic or desiccation stress situa-

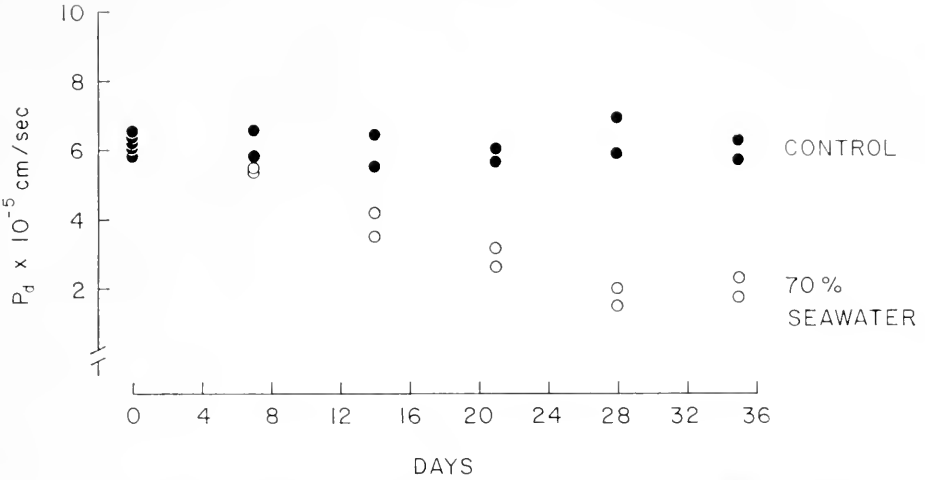

FIGURE 3. Diffusional water permeability as a function of time in *Mytilus edulis;* solid points represent animals maintained in full strength sea water; and open points; animals maintained in 70% sea water.

tion. In experiments with a different population of *Mytilus edulis* in the Woods Hole area, it was found that these animals could indeed decrease their tissue permeability in response to an altered osmotic environment (70% sea water) as is shown in Figure 3. Those animals maintained in full-strength sea water maintained a constant mantle water P_d for the duration of the experiment, 35 days, at about 6×10^{-5} cm/sec, while those in 70% sea water slowly decreased their mantle water P_d during this time period to about 2×10^{-5} cm/sec.

DISCUSSION

Diffusional water permeabilities in a series of lamellibranchs, as measured across the isolated mantle tissue, can be correlated with the habitat of the animal (Fig. 2). The animals used in this study can be divided into several groups: high intertidal or estuarine, low intertidal, and sublittoral. In general, the diffusional water permeability of the mantle decreases with increasing osmotic or desiccation stress to which the animal is exposed. That is, *Modiolus demissus* and *C. virginica,* which are found high in the salt marsh, are much less permeable to water than are *P. magellanicus* and *S. solidissima,* which are located in relatively deep water. Organisms which are exposed intermittently or for brief periods of time, such as *Mya, Mercenaria* or *M. modiolus,* have intermediate diffusional water permeabilities.

In this particular study, two different groups of *Mytilus edulis* were used. One group was distributed on rocks low in the intertidal zone, approximately ELWN, while another group at the same site (Jamestown, Rhode Island) was found sublittorally. As can be seen in Figure 2, water P_d in the isolated mantle tissue from these two groups of animals is significantly different, with animals from the higher-zoned area having the lower water permeability. This not only indi-

cates that diffusional water permeability in bivalve molluscs is influenced by vertical zonation level, but that some bivalves may be capable of altering the water permeability of their tissues in response to increased environmental osmotic or desiccation stress situations. Changes in water permeability in response to increased osmotic stress has already been demonstrated in several crustaceans (Herreid, 1969a; Lockwood *et al.,* 1973) and in *Limulus* (Hannan and Evans, 1973).

The possibility of short term changes in tissue water P_d in bivalves was investigated in what presumably was a homogenous population of *Mytilus edulis* collected from deep water off of Martha's Vineyard. These animals were split into two groups, one maintained in sea water and the other in 70% sea water. Those animals maintained in full-strength sea water maintained a constant water P_d, while those animals in 70% sea water demonstrated a slow, steady decrease in tissue water permeability which leveled off at a new steady state value (2×10^{-5} cm/sec) approximately 24–28 days after the initiation of the equilibration period (Fig. 3). This indicates that tissue water permeability can be altered in at least *Mytilus edulis.* Since *Mytilus edulis* is incapable of any significant degree of ionic or osmotic regulation (Potts, 1954), then what is the physiological significance of this permeability change? It may simply be that those specimens of *Mytilus,* which are located in areas subjected to alterations in the osmotic and ionic environment, decrease the rate of tissue equilibration in response to these environmental alterations by decreasing their tissue permeability.

Anodonta, a freshwater lamellibranch, has a diffusional water permeability which is also intermediate between high and subtidal species. In a similar study with crustaceans, Rudy (1967) found that *Astacus,* a freshwater crayfish, had the lowest water permeability found in a series of decapod crustaceans ranging from marine to freshwater species. Why this is not the case in a similar series of lamellibranchs used in this study is not known, but may be related to the maintenance of an extremely low osmolality of the hemolymph in these organisms (Potts, 1954). Since these animals maintain themselves only slightly hyperosmotic to their environment in comparison with other freshwater invertebrates, their osmotic problems are correspondingly decreased, and they may not need to reduce their tissue water permeability further in order to maintain their osmotic equilibrium.

Without regard to the actual mechanism by which water moves across the isolated mantle, but assuming the mechanism of water movement across the mantle tissue is the same in the different bivalves used in this study, reduction of tissue water permeability could be brought about in one of several different means. These would include reduction of exposed, permeable surfaces, increasing tissue thickness or changes in the chemical composition of the tissue, among other possibilities. An organism could reduce the total surface area of permeable tissues, thereby reducing its overall permeability. This mechanism has been utilized by some crustaceans in which it has been noted that there is a reduction in gill area per unit weight going from subtidal to intertidal species, the gill being the most permeable structure in crustaceans (Gray, 1957). This noted reduction in crustacean gill area may also be dependent in part upon the availability of oxygen. That is, proceeding from the subtidal to the estuarine and terrestrial environments there is an increase in the availability of oxygen, and therefore an animal could carry out its respiratory

functions with less gill surface area. Intertidal bivalves have most likely not resorted to a reduction in gill surface area as a means to decrease their total surface permeability, even though the gills make a major contribution to the total exposed surface area in these animals. The gill in these animals is used for filter-feeding, as well as maintaining a respiratory function, and with a reduction in the time available for this type of feeding higher in the intertidal area, reduction in gill surface area would most likely be counter-productive.

Alternatively, an organism could reduce the diffusional water permeability of a given tissue by increasing the thickness of the tissue. In a series of different bivalve mantles, there was no correlation between measured mantle thickness and diffusional water permeability (Table 1). In addition, there was no change in lag time (Fig. 1) across the mantles with different permeabilities, which is what could be expected if decreases in P_d were brought about by increased tissue thickness. Apparently then, molluscs have not utilized this possibility to decrease tissue permeability.

Without changes in the physical dimensions of a given tissue, changes in diffusional water permeability could be brought about by changes in the chemical composition of the tissue. For example, the water permeability of artificial bilayer membranes is influenced by lipid composition (Cass and Finkelstein, 1967; Granziani and Livne, 1972). Exposure of the blue crab *Callinectes* to decreased osmolality results in an increase in lipid synthesis in the gill (Whitney, 1974). *Callinectes* is an estuarine organism and is capable of withstanding large changes in external salinity, accomplished in part apparently by decreasing its water permeability. The spider crab *Libinia* on the other hand is a sublittoral animal incapable of any great degree of osmoregulation. When this animal was exposed to lowered salinities, there was no change in gill lipid synthesis. Differences in diffusional water permeability across the mantle tissue of bivalves from different habitats may then reflect differences in the lipid composition of the tissue. That is, there may be an increase in the lipid/protein ratio in the mantle tissue with increasing exposure to osmotic stress resulting in decreased diffusional water permeability.

Adaptations to water problems in other intertidal molluscs include structural, behavioral, and physiological processes. The structure of the shell in the European limpet *Patella* is correlated with their intertidal distribution (Davies, 1969). Higher zoned animals have higher shells with a smaller circumference than lower zoned animals. This effectively reduces the surface area of the higher zoned animals. Davies also suggested that there may be differences in the water permeability of the mantle tissue of these limpets. The false limpet *Siphonaria pectinata* has no ability to osmoregulate but can tolerate salinities between 20 and 40‰. Salinity variations outside of the tolerance range cause the animal to contract the foot musculature creating a seal between the shell and substrate, effectively shutting out the external environment (McAlister and Fisher, 1968). Wolcott (1973) claims that the most important adaptation of *Acmaea digitalis* to environmental stress situations is the secretion of a mucous sheet between the shell margin and substratum, again sealing off the external environment, a situation analogous to the secretion of the epiphragm in terrestrial snails (Machin, 1968).

Although biotic factors, such as competition, behavior, predation, etc., probably

play a major role in the vertical distribution of intertidal animals (Wolcott, 1973), abiotic factors also influence the intertidal distribution of these animals (Newell, 1970). This present study suggests that the ability of certain groups of animals to adapt physiologically to specific environmental stress situations may also influence their distribution.

Supported in part by grant NS-09090.

Summary

1. The diffusional water permeability of the isolated mantle tissue from a series of marine, and one freshwater, species of lamellibranch molluscs was determined.

2. The water permeability of the mantle tissue was generally correlated with the habitat of the organism, permeability decreasing with increasing height above the sublittoral zone.

3. Evidence is presented that a given intertidal lamellibranch species, *Mytilus edulis,* is capable of altering its tissue water permeability when presented with changes in external osmolality.

4. The observed differences in tissue water permeability from different animals are not due to change in the physical dimensions of the tissue, but may be the result of changes in the chemical composition of the tissue.

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THE LIGHT-DARK CYCLE AND A NONLINEAR ANALYSIS OF LUNAR PERTURBATIONS AND BAROMETRIC PRESSURE ASSOCIATED WITH THE ANNUAL LOCOMOTOR ACTIVITY OF THE FROG, *RANA PIPIENS*

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During the course of study on intestinal calcium transport in the frog *Rana pipiens,* it became apparent that maximal transport activity in April to June occurred during the nocturnal hours (Robertson, 1976). This period of increased physiological activity of the digestive system may be related to feeding behavior, but at present few studies have been made to determine the activity patterns of the leopard frog. Observations of frogs as a group in temperate zones show that activity is present during the day as well as at night, depending upon the species (Wright and Wright, 1949). At lower latitudes, as in Panama, *Bufo marinus* is active primarily during the day (Park, Barden and Williams, 1940); however, Jaeger, Haitman and Jaeger (1976) noted in the Panamanian frog, *Colostethus,* a bimodal activity pattern with maximums at 0830 and 1630 hr. This activity pattern was coincident with the crepuscular activity of the primary food source, dipterans and coleopterans.

With respect to the Ranidae group, *Rana temporaria* displays higher locomotor activity at night (Chugunov and Kuznetsov, 1972), while *Rana esculenta* is active throughout the day (Kuznetsov, Chugunov and Brodskii, 1972). Dole (1972) noted that newly metamorphosed specimens of *Rana pipiens* were more active after nocturnal rains. The food gathering activity patterns of adult *Rana pipiens* may be inferred from the stomach contents which contain those insects most commonly available at various times of the feeding season (April to October) (Whitaker, 1961; Linzey, 1967). Diet appears to be a reflection of the availability of various insects rather than preference and includes species of Coleoptera (beetles, weevils), Hymenoptera (ants), and Homoptera (aphids, leafhoppers). Of these, beetles (Oldryd, 1960) and ants (Skaife, 1961) display increased activity at night.

When food is made available at a preset period of time, the bullfrog, *Rana catesbeiana,* exhibits increased activity presumably in anticipation of food availability, suggestive of a "conditioned" response (van Bergeijk, 1967).

In spontaneous or nonconditioned activity, amphibians appear to be sensitive not only to the general environment, such as season and rainfall (Gibbons and Bennett, 1974), but to solar and lunar cues for orientation and migration (Ferguson, Landreth and Turinipseed, 1965; Ferguson and Landreth, 1966; Landreth and Ferguson, 1966, 1967; FitzGerald and Bider, 1974). When maintained in a closed environment away from visual cues, the activity of salamanders (*Triturus*) appears to be modified by a lunar influence (Ralph, 1957), which may also influence physiological activity, such as oxygen consumption (Brown, Webb, Bennett

and Sanders, 1955). In an attempt to provide an overview of the diurnal activity of physiological processes of the digestive system, a year-long study was conducted on the spontaneous activity patterns of adult male *Rana pipiens* exposed to the change in outdoor ambient lighting conditions.

Material and Methods

Adult male specimens of *Rana pipiens* (Northern variety) of 40–60 g body weight were obtained commercially (Bay Biological, Canada) throughout the period of study from March, 1976, to February, 1977, with studies conducted in Syracuse, New York (Lat. 43° 03′ N). Frogs were unfed and maintained in a continuous change of fresh water (20–24° C) and exposed to outdoor ambient diurnal lighting conditions for one week prior to use. After this period, groups of 8–10 frogs were placed in the detection apparatus described below in a relatively quiet room. The frogs were exposed to southern ambient lighting (300–400 lux max). The surrounding yearly temperature was maintained at 19.3 ± 3.1° C and only significantly elevated above the yearly mean in September. Newly acclimated frogs were introduced into the apparatus at irregular intervals after 10–20 days. Since Brown, Webb and Macey (1957) had noted that barometric pressure may affect biological activity in amphibians, local barometric pressure at 1200 hr was recorded on a recording aneroid barometer throughout the course of study.

Detection apparatus

The spontaneous locomotor activity of groups of frogs was monitored by the detection of vertical water movement in an isolated translucent plastic chamber (33 × 23 × 10 cm). The apparatus consisted of a plastic float connected to a pivoted transverse rod through which a vertical water displacement of 2.0 ± 0.25 mm was multiplied by a factor of five. A contact switch at the end of the rod completed a circuit with an event marked on a strip recorder. The switch interval was adjusted daily to maintain a constant sensitivity.

Analysis of data

Locomotor activity (LA) was recorded as the number of events/hour and the number of events/day. Hourly variations were reduced by averaging the data for two hour periods. Further, the relative activity (per cent activity/two hour period) was calculated from the total number of events/day. Since lunar perturbations appeared to influence amphibian behavior (Ralph, 1957), identification of coherent patterns of locomotor activity was facilitated by the construction of isograms of the relative levels of activity within a lunar month. The mean relative activity pattern for a "typical lunar month" was calculated by deriving the mean relative activity (per cent/two hours) of corresponding lunar days over a three to four lunar month period. Lines were connected between equal levels of activity (5% intervals) to provide a "contour map" which would emphasize basic activity patterns.

The presence of a lunar influence during the diurnal and nocturnal periods was determined by a procedure described by Brown *et al.* (1955). High transit

(HT; time of moon at zenith) advances 50 minutes each day, and completes a cycle in about 29.53 days (synodical lunar month). The absolute levels of locomotor activity at HT for each day were then tabulated into a single column (Column 3) with the data of the subsequent time period shifted accordingly to the right. This procedure enhances any lunar effect associated with the time of HT that is superimposed upon the general level of activity. The times of transit were obtained from the *American Ephemeris and Nautical Almanac* (1976, 1977, pp. 52–67), and all time periods designated in Eastern Standard Time. Day of lunar month is designated as (NM + day).

Period analysis

A computer program was designed to identify and analyze the times of maximal activity with respect to a specific reference date. Since most activity was during the nocturnal period, the time of maximal locomotor activity (LA_{max}) was recorded as the time (hours) before (−) or after (+) 0000 hr. For example, a LA_{max} period of activity at 2000 hr was recorded as −4 (hr), while an activity peak at 0400 hr was recorded as +4 (hr). Initial observations suggested that the time of LA_{max} occurred at a different time each day but was repeated several days later at a similar time period. This could best be visualized as an oscillatory cycle with the function: $LA_{max} = M + \cos(\omega t - \phi)$, where the cyclic parameters are M (mean of all LA_{max} time values), A (amplitude of the oscillation), and ϕ (arcophase or phase angle, which is the day in the cycle in which LA_{max} occurs at the maximal positive hour after 0000 hr), after a specific reference date expressed in degrees. The reference date was taken as day 1 (NM + 1) of a lunar month on 4/1/76. The angular frequency $\omega = 360°/t$, where t = number of days in the cycle. For cyclic

TABLE I

Monthly locomotor activity levels of adult male frogs, Rana pipiens.

Month	N (Days)	Events/day ± s.e.m.	Temp. range ° C (min-max)	Barometric pressure (mm Hg)*
January	17	539 ± 78	15.0–18.0	756.4 ± 7.2
February	28	420 ± 60	15.5–18.0	759.5 ± 7.3
March	11	297 ± 14	15.0–17.0	761.2 ± 6.0
April	28	373 ± 26	16.0–18.5	762.3 ± 3.3
May	30	387 ± 52	18.0–20.0	760.0 ± 5.8
June	21	405 ± 50	20.0–23.0	762.8 ± 4.2
July	10	286 ± 30	22.0–23.0	760.2 ± 4.4
August	29	281 ± 34	22.0–23.0	763.3 ± 4.0
September	29	211 ± 36	23.0–26.0	762.5 ± 5.2
October	31	256 ± 29	20.0–22.0	762.0 ± 6.8
November	30	146 ± 31	17.0–20.0	760.5 ± 5.7
December	14	159 ± 45	15.0–18.0	759.7 ± 8.7
Total annual mean	278	313 ± 15	19.4 ± 3.1	761.0 ± 6.1

* Mean pressure at 1200 hr.

analysis of LA_{max}, the time of maximal activity was paired with the number of the day after the reference date. The data was then tested with the cosine function above with hypothetical wave functions of various periods where $t = 5$ to 90 days for the monthly analysis and cycles up to 360 days for all data collected for the entire year. The procedure was conveniently done through a linear transformation of the cosine time parameter in which the data was analyzed as a simple linear regression. The "best fit of the data" was expressed as the maximum positive value of the correlation coefficient (r) at a specific ϕ. This value at ϕ was obtained by calculating the values of r at 10° increments through the entire wave function of 360° for each hypothetical cycle. The resulting maximum values of r for the range of cycles represents a spectrum in which the t statistic was calculated and $P \leq 0.01$ was considered a significant cycle. Possible correlations of barometric pressure and the daily absolute levels of locomotor activity on the cyclic spectrum were conveniently analyzed by processing the data only for those days when the values were above or below the annual mean. All data expressed in the text are mean ± s.d. unless otherwise stated, and $P \leq 0.01$, determined from a simple t-statistic between values, was considered significant.

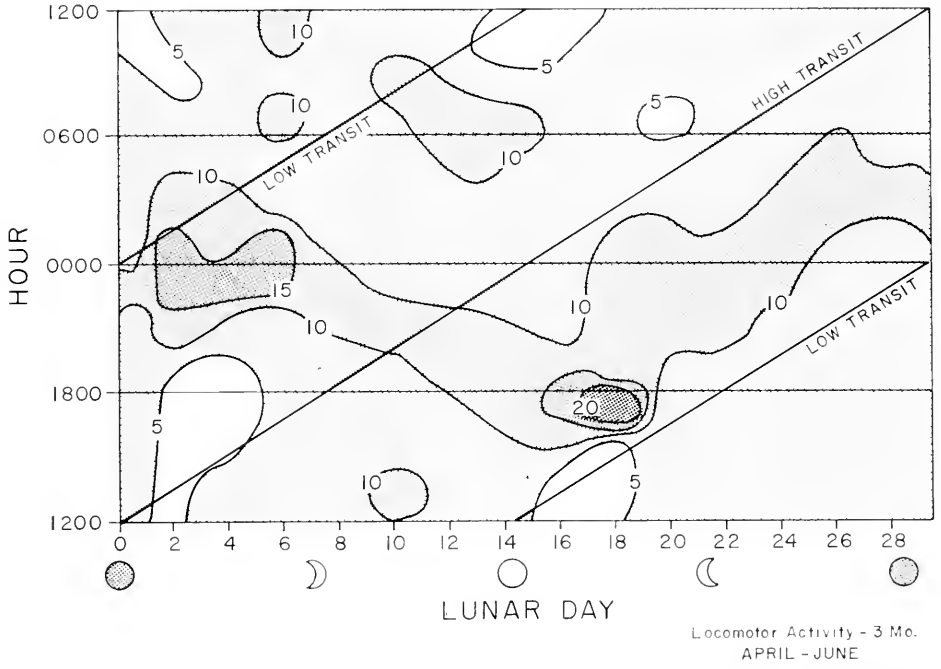


FIGURE 1. Isogram of relative locomotor activity (% activity/two hours) during April to June based upon the mean activity levels of corresponding lunar days over a three lunar month period. A coherent sinusoidal pattern is apparent which oscillates within the lunar month to reflect high activity at 0000 hr near New Moon which shifts to 1800 hr at a time of Full Moon. Additional activity at 0600 hr at Full Moon indicates a bimodal diel pattern. Diagonal lines represent times of high and low lunar transit.

Results

General activity patterns

Since 8–10 frogs were continuously monitored, the locomotor activity (LA) in this study is group activity in contrast to activity of individual frogs. Such activity throughout the year (total of 278 days) revealed changes in the relative levels of hourly activity from month to month which did not appear to be related to the absolute monthly activity level or the ambient temperature. The yearly mean temperature was maintained at $19.4 \pm 3.1°$ C and only significantly elevated above the mean in September (Table I). The mean daily activity levels in the calendar months of November, December and January showed significant deviations from the mean daily activity levels of 313 ± 115 events/day for the entire year. No significant correlation existed between the mean daily activity for each month and the corresponding mean monthly ambient temperature ($r = -0.395$) or the prevailing mean monthly barometric pressure ($r = -0.421$).

Chi square analysis of the relative hourly activity for each month showed no significant variation from "random" activity (8.3% activity/two hours) in the months from October through March, while a significant nonrandom light-dark response pattern of activity was apparent from April through September. Further analysis of this nonrandom period based upon monthly relative activity isograms

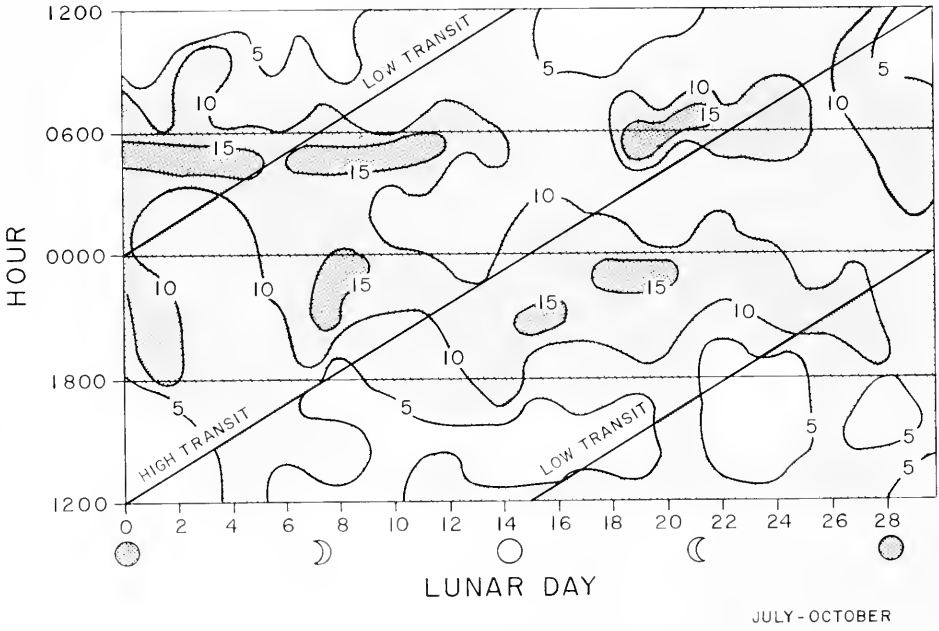


Figure 2. Isogram of relative locomotor activity (% activity/two hours) for a three lunar month period from July to October. Elevated activity (> 10%) is primarily nocturnal while > 15% activity is observed between 2100–0000 hr and 0400–0600 hr which reflects a bimodal diel activity pattern. Diagonal lines indicate time of high and low lunar transit.

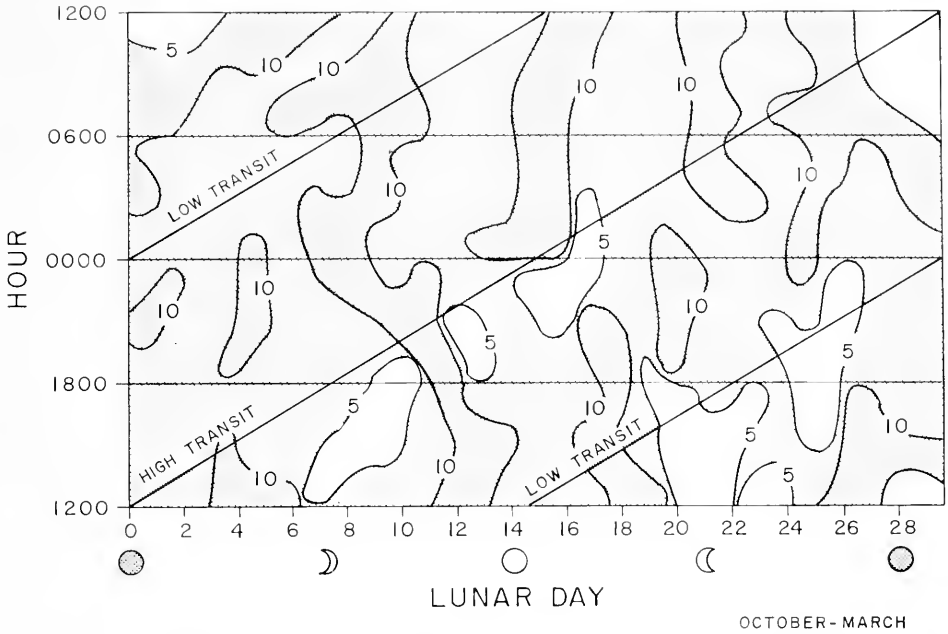


FIGURE 3. Isogram of relative locomotor activity (% activity/two hours) from October to March based on data of four lunar months. Activity is randomly distributed throughout the 24 hr period each day with no significant diurnal-nocturnal pattern evident. Diagonal lines indicate times of high and low lunar transit.

revealed a change in pattern during July. Thus, three major periods were identified, the first nonrandom period from April through June (79 days), a second nonrandom period from July to the first New Moon in October (90 days), and the third "random" period from New Moon in October through March of the following year (109 days).

The composite "lunar month" isogram constructed for the April to June period (three lunar months) revealed a coherent sinusoidal pattern with periods of increased LA (> 10%/two hours) near 0000 hr at New Moon (Fig. 1). This pattern shifted toward early evening hours during the progression of the lunar month to exhibit maximal LA at 1800 hr at Full Moon (NM + 15). In addition, a second period of increased activity was observed at 0600 hr which indicated a bimodal diurnal pattern. During the remainder of the month, the primary activity pattern returned toward 0000 hr and continued the shift to 0300 hr at the end of the lunar month. Thus, a single mode of activity appeared from NM + 16 to NM + 5 of the following lunar month and a bimodal activity pattern from NM + 6 to NM + 15.

The isogram based upon a composite of three lunar months of the second period (July through October) revealed a prominent bimodal diel pattern which was maximal at 0400–0600 hr with a minor maximum at about 2200 hr (Fig. 2). The major early morning maximum coincides with the time of sunrise (0430–0600 hr)

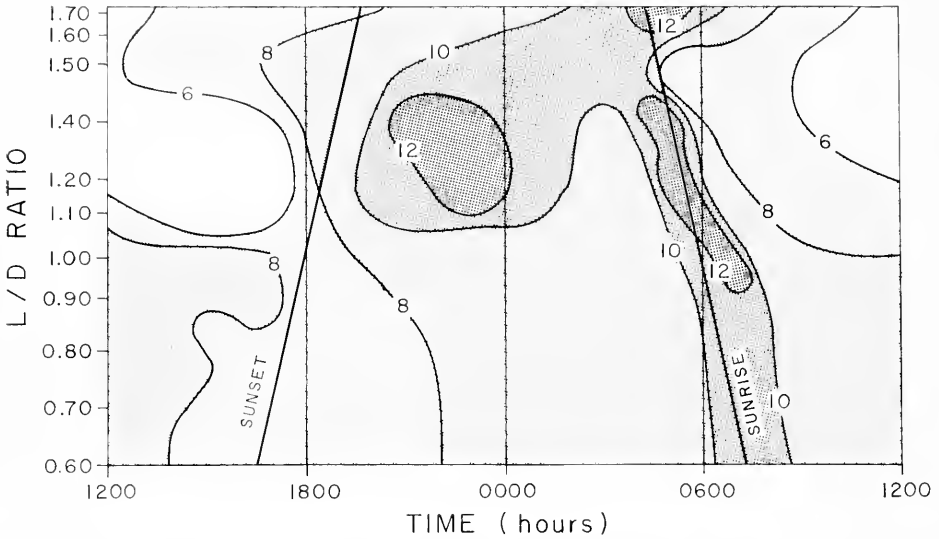


FIGURE 4. Isogram of relative locomotor activity (%/two hours) in adult male *Rana pipiens* as a function of the prevailing L : D ratio over the course of an entire year. During winter with an L : D ratio < 1.0, activity is not defined into a significant diurnal-nocturnal pattern although a nonsignificant increase is observed at sunrise. An L : D ratio between 1.0–1.45 is coincident with a significant increase in nocturnal activity between 2100–0000 hr and during the hours at sunrise. Above an L : D ratio of 1.5, frogs are most active at sunrise with little activity (< 6%) during daylight hours.

during this time of year. The sinusoidal pattern of the previous period did not appear to be dominant.

The third period (October through March), classified as random activity, was substantiated by the composite isogram based upon four lunar months (Fig. 3). No coherent daily pattern was apparent and the overall pattern was characterized by higher levels (> 10%/two hours) of activity present during the daylight hours, and low activity levels (< 5%/two hours) at night.

The change in pattern appeared to be related to the seasonal alterations in the L : D ratio as depicted in Figure 4. An isogram based upon the relative mean LA/two hours for each calendar month as a function of the prevailing L : D ratio revealed that random LA from mid-October to March occurred when the L : D ratio was < 1.0. As the L : D ratio increased to 1.0, increased LA was more apparent at time of sunrise and became maximal between 2100 and 0000 hr up to the time when the L : D ratio was < 1.45. Above this ratio the LA was predominately in the hours near sunrise with < 6% of the activity occurring during the mid-daylight hours.

Lunar influence

The relationship of lunar position on hourly LA was analyzed by columnating the absolute level of activity for each two hour segment during the time of High

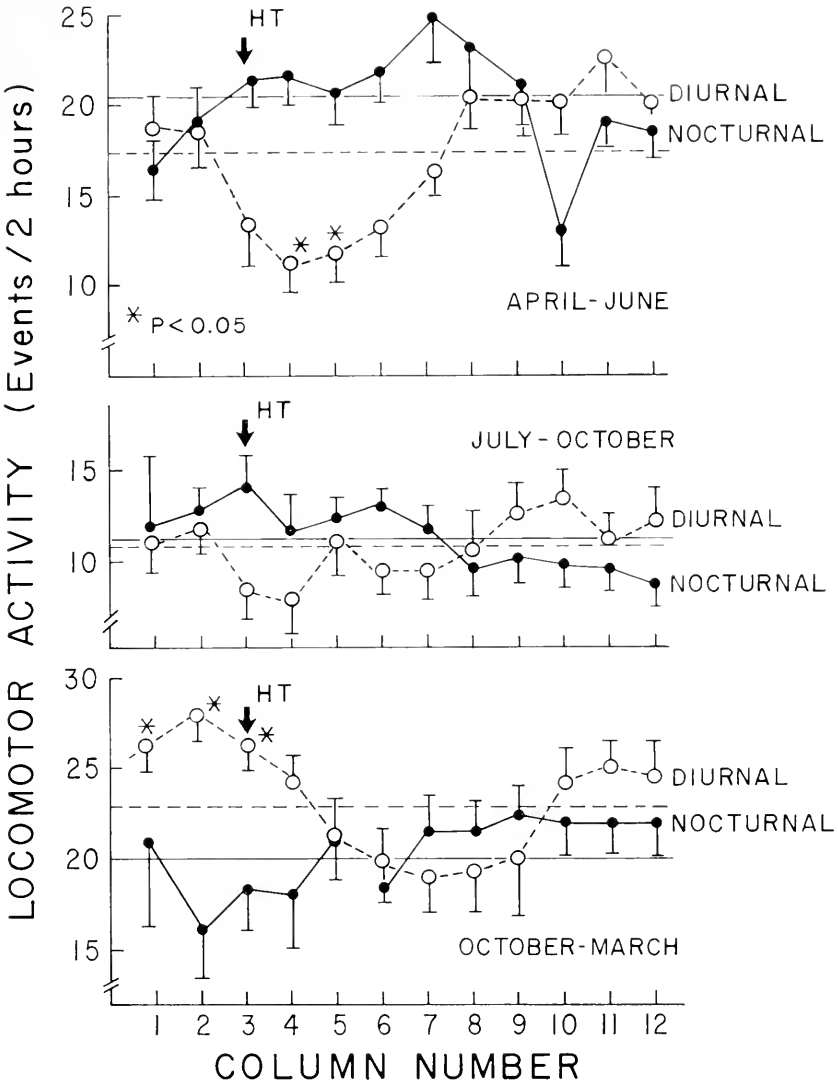


FIGURE 5. Relationship of high lunar transit (HT in column 3) on absolute levels of activity (number of events/two hours) during daylight (open circles with dashed line) and nocturnal (solid circles with solid line) hours during three major periods of the year. In April to June, HT during nocturnal period had no significant influence on activity level from nocturnal mean (solid line), while six hours after HT during diurnal period, activity was significantly decreased when compared to daily mean (dashed line). In July to October mean diurnal (dashed line) and nocturnal (solid line) activities were significantly lower than activity in preceding and subsequent monthly periods, and high transit was not associated with any significant change in mean hourly activity. In October to March mean nocturnal activity (solid line) was significantly below mean diurnal activity (dashed line) and activity four to six hours prior to HT during daylight was significantly above diurnal mean. Each point is mean ± s.e.m.

Transit (HT) (Column 3) when transit occurred either during the diurnal or nocturnal period (Fig. 5). During April, for example, HT advanced through the nocturnal period in 14 days, and through daylight in 16 days. For the entire April to June segment the time of HT was not coincident with any significant change in LA at any time during the dark from the mean nocturnal level of 20.4 ± 3.3 events/two hours. When transit occurred during daylight hours there was a significant decrease for six hours after HT below the diurnal mean of 17.3 ± 3.7 events/two hours. Further, the mean daylight level of activity was significantly below the nocturnal activity level.

High transit in July to October was not coincident with any hourly differential effect on diurnal or nocturnal activity levels, nor were the mean activity levels different from one another [$(11.3 \pm 1.9$ (nocturnal) *vs.* 10.9 ± 1.7 events/two hours (diurnal)]. During this period, the total number of events/day (254 ± 17 s.e.m.) was significantly below the yearly mean of 313 ± 15 s.e.m. events/day.

The association of HT with decreased activity observed in April-June was reversed during the period from October to March. When HT occurred during daylight there was a significant increase in LA six hours prior to transit above the mean level of 22.9 ± 3.9 events/two hours which was also elevated above the nocturnal mean of 20.2 ± 2.2 events/two hours.

Period analysis

The characteristics of the FORTRAN Program employed in this study are such that insignificant values of r will be generated if data cannot be fitted to any sine wave by least squares or if LA_{max} is a linear function (*e.g.*, LA_{max} occurs at the same time each day). When LA_{max} was analyzed across a spectrum of hypothetical wave lengths at five day intervals, the presence of significant ($P \leq 0.01$) oscillatory patterns appeared as peaks of increased values of r. This procedure performed on each of the three monthly segments and for the entire year revealed the presence of cycles indicative of coherent and organized behavior patterns. Further processing of data based upon the relative levels of barometric pressure and locomotor activity (events/day) above or below the annual mean (Table I) altered the values of r (and degree of significance) of specific cycles. Under these circumstances the phase angle (ϕ) was identical to each base cycle for the recalculation of r.

In April to June (Fig. 6) the base spectrum for all data indicated the presence of a 28 day and 50 day cycle. The 28 day cycle where $\phi = 0$, began each cycle on the first day of New Moon during this period, and became a more significant cycle when analyzed for those days of low barometric pressure (LBP); whereas the 50 day cycle which began 14 days into the lunar month was more prominent under conditions of high barometric pressure (HBP). On days of low locomotor activity (LLA) a slight shift in the cycle occurred with the increase in the correlation coefficient to $r = 0.641$ with other peaks at 15 and 60 days.

In July to October, two additional shorter cycles were noted in addition to those seen in April to June (Fig. 7). The 5 day cycle was more significant under conditions of LBP, while the 15 day cycle was more prominent when tested on days of HBP. As noted in the April to June spectrum, the 30 and 45 day cycles

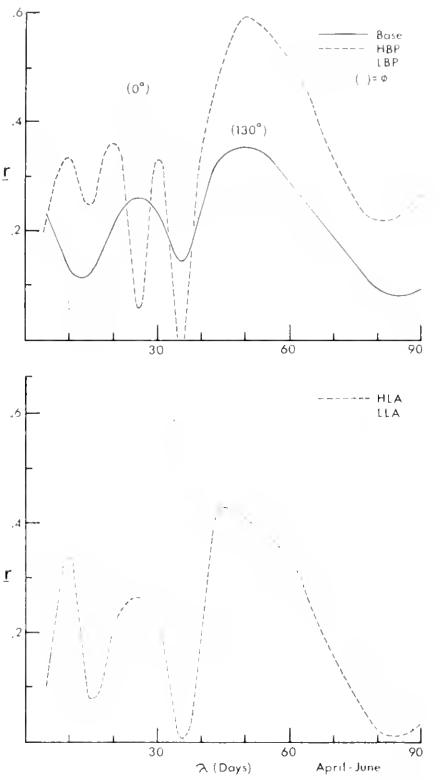


FIGURE 6. Upper graph shows a period analysis of times of LA_{max} during April to June depicted by the solid line indicating two significant cycles at 28 and 50 days. The resulting spectrum after analysis of data on days of low barometric pressure (LBP) at identical ϕ shows enhancement of the 28 day cycle, while on days of high barometric pressure (HBP) the 50 day cycle was the most significant. Lower graph shows a similar analysis on days in which activity was lower than the series mean (LLA) a 30 day cycle was most significant, whereas high locomotor activity (HLA) was associated with the 50 day cycle.

were more prominent under conditions of HBP. Examination of HLA revealed a significant enhancement of the 15, 25, and 50 day cycles, while the 5 day cycle was dominant on days of LLA.

For the period from October to March (Fig. 8) the base spectrum displayed cycles at 15, 30, and 50 days in which only the 50 day cycle showed a significant enhancement under conditions of lower than average barometric pressure. Higher than average locomotor activity was coincident with the 15 and 65 day cycles while LLA was associated only with the 30 day cycle.

When all data for the entire year was examined in the same manner (Fig. 9), only the significant cycles whose phase angle was relatively constant throughout the year could be detected. The significant base cycles present were those at 30, 55, 105, and 160 days. On days of HBP the 105 and 160 day cycles were most

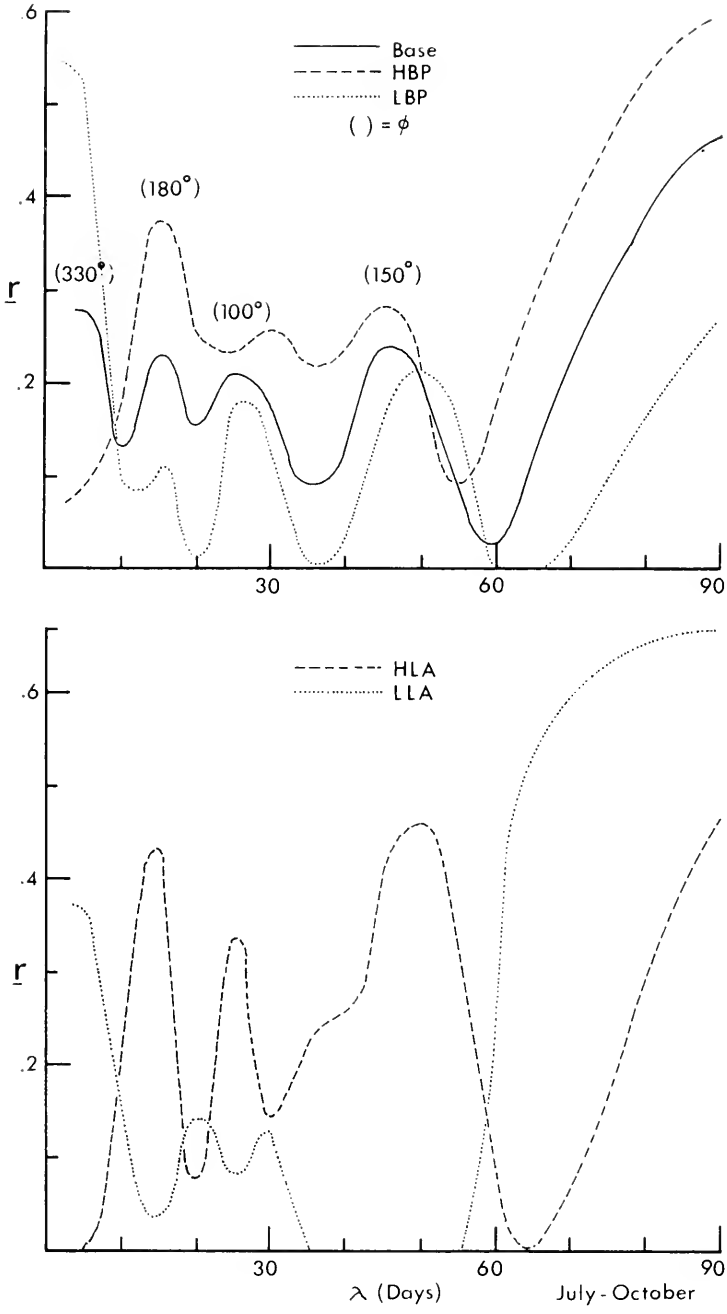

.6
.4
.2
r
Base
HBP
LBP
( ) = ϕ
(180°)
(330°)
(100°)
(150°)
30
60
90
HLA
LLA
λ (Days)
July - October

TABLE II

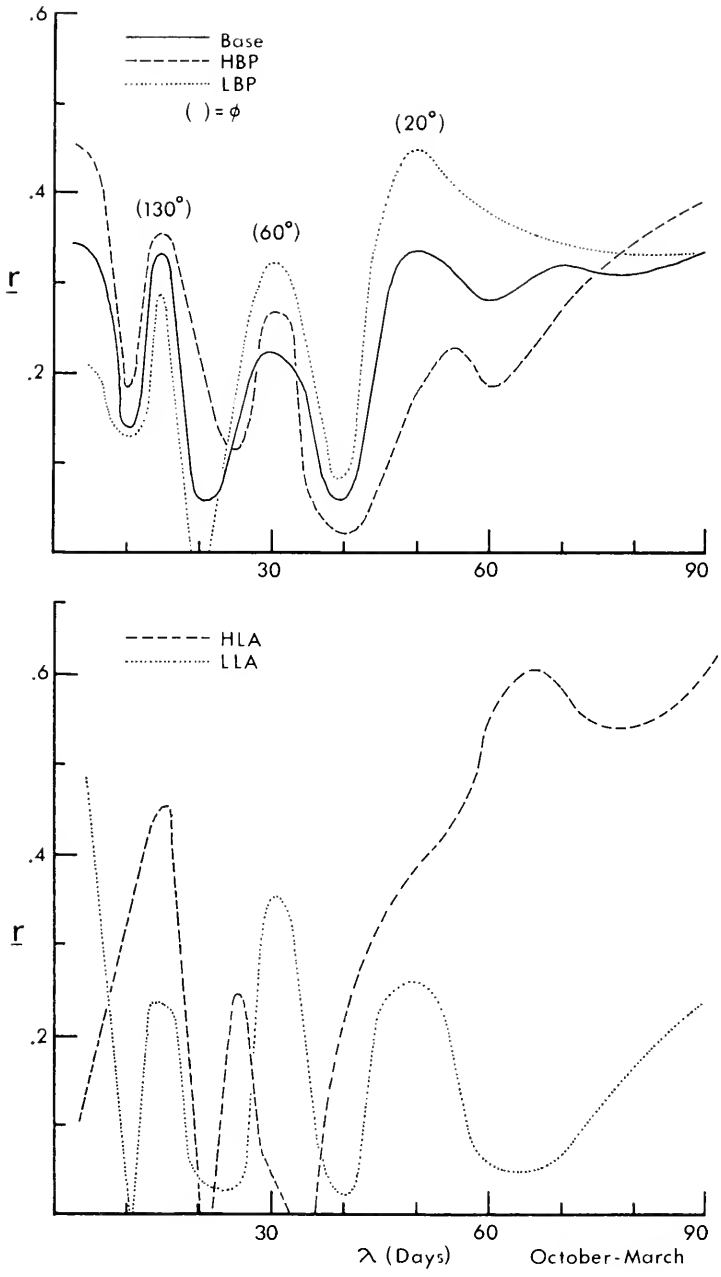
Parameters for significant monthly and annual locomotor activity cycles.

Time segment	Period (days)	ω	ϕ (Degrees)	ϕ (Day)	M	A	Related variables (see text)
April–June	15	24	210	9	−0.73	−1.98	LLA
	20	18	270	15	−1.00	−2.10	HBP, HLA
	30	12	30	3	−0.30	3.35	LBP, LLA
	50	7.3	100	14	−0.95	2.78	HBP, HLA
July–October	5	72	310	4	1.64	−3.30	LBP, LLA
	15	24	170	7	1.08	2.61	HBP, HLA
	27	13.3	120	9	1.15	2.05	LBP, HLA
	43	8.4	220	26	3.38	−2.09	LLA
	50	7.3	310	43	2.41	−3.49	HLA
October–March	15	24	110	5	1.36	5.69	HBP, HLA
	25	14.4	70	5	0.75	3.61	HLA
	30	12	40	3	2.88	2.80	LBP, LLA
	50	7.3	40	6	0.91	4.73	LBP
	65	5.5	160	29	1.59	7.05	HLA
Annual	30	12	52	4	2.25	2.16	LBP, LLA
	55	6.5	52	8	0.18	1.61	HLA
	92	3.9	307	78	2.86	−2.13	LBP, LLA
	105	3.4	60	17	0.09	2.95	HBP, HLA
	162	2.2	147	66	0.69	2.15	HLA
	360	1	110	110	1.91	1.49	LLA

dominant, whereas on days of LBP the 30, 60, and 90 day cycles were most significant. High LA increased the significance of the 55 and 105 day cycles, while LLA was associated with the presence of 30, 60, and 90 day cycles.

Parameters generated from the nonlinear analysis (Table II) are based upon the maximum values of r derived from the data based upon the relative barometric pressure and locomotor activity. These values show slight shifts in period and phase angle (ϕ) when compared to the base spectra (Figs. 6–9) where ϕ was held constant. It is apparent that values of M (mean of LA_{max} time values) for each of the cycles in the three monthly segments varied from negative to positive values, which indicates that they are probably oscillating on a larger period of about a year. This reflects the greater degree of LA_{max} activity prior to 0000 hr (negative values) in April to June and the gradual shift of activity after 0000 hr (positive values) in July to October with an apparent reversal during October to March.

FIGURE 7. Upper graph shows a period analysis of time of LA_{max} during July to October indicating by the solid line revealing several significant cycles at 5, 15, 27, and 43 days and larger periods (> 90 days). On days of low barometric pressure (LBP) the 5, 27, and 50 day cycles are dominant, whereas on days of high barometric pressure (HBP), the 15 day cycle was the most significant. Lower graph shows a similar analysis on days of low activity (LLA) revealing that the 5 day and larger periods were significant, while high activity (HLA) was associated with 15, 27, and 50 day cycles.


.6
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LBP
( ) = ϕ
(130°)
(60°)
(20°)
30
60
90
HLA
LLA
λ (Days)
October-March

The dispersion of activity throughout the day during this latter period is also reflected in the larger amplitude values.

The cycles that are dominant in the annual analysis are the result of two major fundamental frequencies which possess specific harmonic characteristics. The first set of cycles (alpha series) oscillates on M which ranges between 0.09–0.69 and is represented by the 55, 105, 162 day cycles. The phase angles indicate that the 55 and 105 day cycles are reinforced about every 105 days, and the 162 day cycle is reinforced by the 55 and 105 day cycles about every 323 days. Thus, the shortest fundamental period would be about 323 days in which the harmonics are 6ω, 3ω, and 2ω with the first synchronous date on November 15, 1976.

The second fundamental series of cycles (beta series) oscillates on M which ranges between 1.91–2.86. The fundamental period may be the 30 day cycle with multiples at $\omega/2$, $\omega/3$, and $\omega/12$ (Fig. 9), and the values of ϕ indicate that the two significant 30 and 90 day cycles are synchronized at days 32–34, 124, 214–216, and 304–308.

By χ^2 analysis the variables of barometric pressure and relative daily activity, which alter the significance of these cycles throughout the year, have a significant ($P = 0.01$) nonrandom association in which high barometric pressure is associated with increased daily activity and low pressure with low daily activity. These associative features are also dichotomous, since high barometric pressure and high activity is significantly associated ($P < 0.01$) with the alpha cyclic series, while low barometric pressure and low activity is associated with the beta cyclic series.

Discussion

The spontaneous group activity over the course of a year in adult male frogs under the conditions of this study show a correlation with the light-dark cycle, ambient barometric pressure and an exogenous lunar perturbation which appears to influence the time and relative level of locomotor activity. Each of these variable factors is present throughout the year, but the change in the L : D ratio appears to be the dominant influence. Absence of a significant diurnal activity pattern with a L : D ratio of < 1.0 indicates that the length of time of the nocturnal period may be the primary influence of increased nocturnal activity, since the reversed pattern was not apparent during the winter. With a decreasing dark period, activity shifts from primarily nocturnal to auroral at the time of the longest days in June. Bush (1963) noted that specimens of *Bufo fowleri* were inactive with a 4L : 20D cycle but became quite active as the L : D ratio was altered to 12L : 12D and 20L : 4D. Further, toads consumed more with the longer photoperiods. Physiological responsiveness of amphibians to photoperiod is variable, since the spawning period of

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Figure 8. Upper graph shows a period analysis of times of LA_{max} from October to March revealing basic cycles (solid line) at 5, 15, 30, and 50 days. Only the 5 day cycle was altered significantly under conditions of high barometric pressure (HBP), while the 50 day cycle was more dominant under low barometric pressure (LBP). Lower graph shows analysis of dominant cycles in periods of high locomotor activity (HLA) showing significant enhancement of the 15 and 65 day cycles, whereas a 30 day cycle was most significant with days of low locomotor activity (LLA).

Rana temporaria appears to be controlled by temperature and not by light (van Oordt and van Oordt, 1955); but the spermatogenic cycle in the salamander *Plethodon cinereus* is primarily regulated by photoperiod (Werner, 1969). The

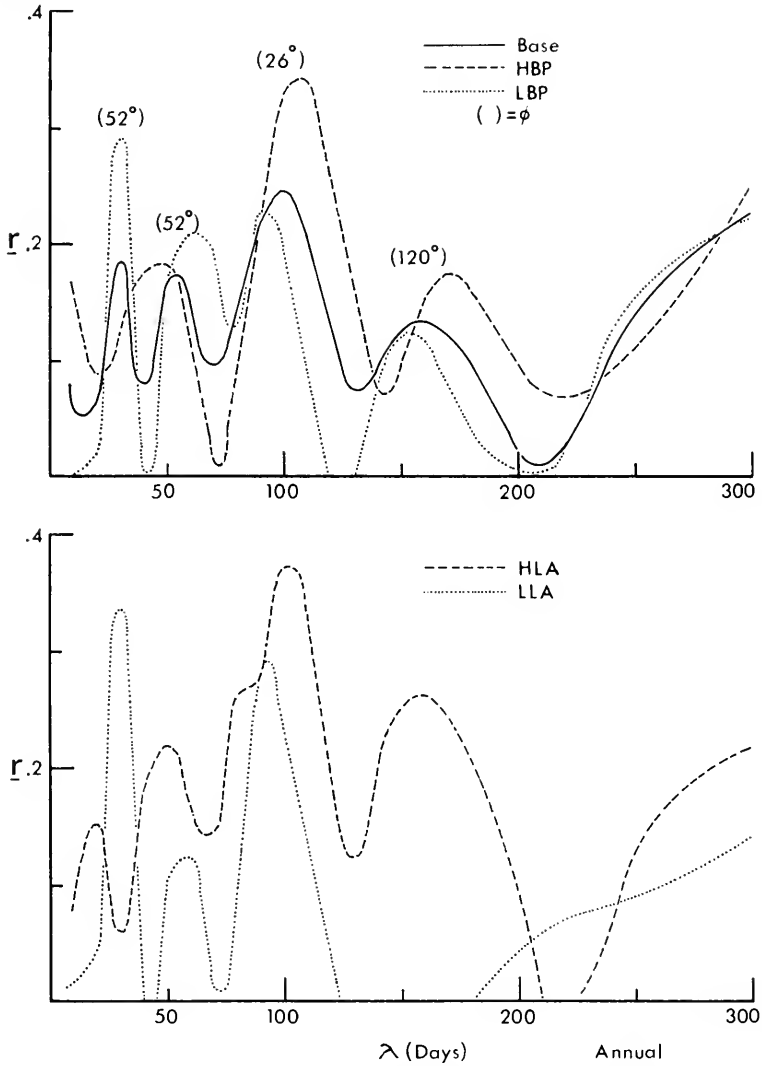

FIGURE 9. Upper graph shows a period analysis of all LA_{max} data collected throughout the year revealing major cycles at 30, 55, 105, and 160 days. Under conditions of lower than the yearly mean barometric pressure (LBP), significant cycles were apparent at 30, 60, 90, and 155 days, whereas with higher than the yearly mean barometric pressure (HBP) the dominant cycles were at 50, 105, and 175 days. Lower graph shows a similar analysis of those cycles most dominant when frogs were least active (LLA), present at 30, 60, and 90 days, while cycles apparent on days of high activity (HLA) were at 50, 105, and 165 days.

increase in nocturnal activity with the beginning of longer days may be an independent but synchonized part of the initiation of sexual activity after the spring equinox.

The influence of temperature on amphibian behavioral activity has been noted by several authors. Early observations by Torelle (1903) of specimens of *Rana virescens virescens* (*Rana pipiens pipiens*) found that at temperatures $< 10°$ C, frogs moved away from light; whereas with temperatures $> 30°$ C frogs were attracted to light. Higginbotham (1939) observed that with a 10° C rise in temperature there was a doubling to tripling of the amount of activity of toads (*Bufo americanus* and *Bufo fowleri*) but no alteration in the overall pattern of activity. *Bufo fowleri* also appears to be most active between 19.6–24.7° C in April to May (Martof, 1962) and consumes twice as much food with a 10° temperature rise from 21° C to 31° C (Bush, 1963). These responses in behavioral activity with change in ambient temperature is of importance in the interpretation of the present data. Over the course of a year the temperature range was held at 8° C and was within the limits observed by Martof (1962) where toads are most active. However, within the temperature range of the present study there was a nonsignificant, and somewhat negative relationship, between total daily activity and the prevailing temperature. Within any monthly period, the maximal temperature range was only 3° C, which was considerably less than the 10° C differential associated with variations in total activity of toads (Higginbotham, 1939; Bush, 1963). Thus, while it is possible that the slight variations in temperature could modify portions of the observed activity, clear relationships were not as apparent as those associated with the light-dark cycle, lunar frequencies and barometric pressure.

The exogenous lunar influence which has been described by others (Ralph, 1957; FitzGerald and Bider, 1974) to affect amphibian activity patterns was also apparent in the present study. Time of high transit was associated with an enhancing and depressing effect during daylight hours in winter and spring to early summer, respectively, and represents a possible mechanism to induce oscillatory patterns in activity as seen in the activity isogram for April to June. The patterns may be modified with the varying length of daylight hours. The fact that the activity isogram in the fall is not a replica of the pattern observed in the spring may reflect the reversal in activity levels of these animals during daylight to this exogenous influence.

The cyclic patterns of locomotor activity, as revealed by wave analysis, in the three major monthly periods and for the entire year appears to represent a consistent phenomenon. That is, the time of maximal activity each day occurred at a similar time period at a later date which was determined by the dominant cycle present. This is best exemplified by the lunar cycle which approximates 30 days (beta-series) and is apparent in the isogram of locomotor activity for April to June. But in addition to this more obvious lunar cycle, there are other cycles that are generated by lunar and solar movement which can give rise to tidal harmonics (Godin, 1972). The principal lunar constituent (M_2) has a period of 12.42 hours and other cycles "beat" against M_2 giving rise to a lunar fortnightly tide (13.66 days), a monthly modulation of 27.57 days and a solar semiannual tide of 182.9 days. Thirty-four separate tidal frequencies have been identified (Godin, 1972),

each of which can generate their own harmonics which can reinforce or dampen one another. The activity cycles which are observed in frogs may be affected by such tidal harmonics, the relative dominance of each reflected in the value of r at the optimal phase angle. For example, the characteristics of the alpha-series of cycles, with a fundamental period of 323 days, suggests that it may be related to the eclipse year of 346.6 days (a "beat" frequency of the synodic and draconitic months), since the first synchronous date of all harmonics was nine days after a lunar eclipse on 11/6–7/76. The next ideal synchronous date is 10/5/77 which is between two eclipses on 9/27/77 (lunar) and 10/12/77 (solar) (*American Ephemeris and Nautical Almanac*, 1976, 1977). Confirmation of such a relationship would require analysis of locomotor activity over a period of several eclipse years.

Of interest is that these cycles are present from October to March, a period characterized by continuous 24 hour activity in the absence of a defined diurnal pattern. Period analysis shows that they are quite similar to the cycles observed in April through June and thus appear to be independent of the light-dark cycle and may be similar to the lunar perturbations observed by Ralph (1957) in the salamander kept in continuous darkness. These oscillations may be expressed as overt activity patterns when the light-dark effect is significant to induce a defined nocturnal pattern. While it may be argued that these are statistical cycles and not overt, patterns can be identified in composite isograms. Further, they can be mathematically described as a first order approximation to a sine wave which allows a possible means of prediction (Table II).

An additional feature of the wave analysis is that other variables such as barometric pressure and relative levels of locomotor activity can be related to two fundamental cycles which might otherwise be difficult to detect. Brown *et al.*, (1955) noted that oxygen consumption in the salamander *Triturus* was inversely related to barometric pressure but not sharply defined. Even in the present study a significant inverse relationship can be derived between absolute daily lomomotor activity and absolute barometric pressure during May ($r = -0.0418$: $P = 0.02$), but it is not consistent at other months. The present analysis reduces the apparent "biological noise" by defining specific days and times of LA_{max} which can be correlated with prevailing barometric pressure.

The sensitivity of animals to atmospheric pressure changes has been observed in aquatic invertebrates such as *Carcinus* (Naylor and Atkinson, 1972) and to hydrostatic pressure as in the amphibian tadpoles, *Rana silvatica* and *Amblystoma* (Johnson and Flagler, 1951), which in both cases increase their activity with an increase in pressure. Conversely, in a terrestrial form such as a newly hatched chick, increased activity is associated with a decrease in barometric pressure (Bateson, 1974).

In the present study this apparent correlation of barometric pressure to activity level was specific for certain cycles. The relationship of these two variables is unclear, but lunar and solar movement also generates atmospheric tides in addition to marine tides (Siebert, 1961). The lunar component can induce a barometric variation of 0.001 millibars (0.025 mm Hg), while the solar component can induce a variation up to 0.03 millibars (0.76 mm Hg). Detection of such variations in

barometric pressure exclusive of the daily weather perturbations might induce the observed cyclic patterns. In view of the dichotomy of the alpha and beta series of cycles which are associated with specific variables, the resultant patterns of activity may reflect two independent receptor-effector behavioral systems.

In summary, the daily and annual solar light pattern appears to be related to the diurnal and nocturnal activity of the adult male frog, *Rana pipiens*, but it has activity patterns of specific periodicities which are similar to lunar periodicities. Additionally, there are significant correlations between these cyclic patterns with the prevailing barometric pressure and the level of locomotor activity. The general behavior patterns of this amphibian over the course of a year exhibits several correlates to basic geophysical forces. The significance of these relationships may be reflected in similar activity patterns of organisms, such as insects (see reviews of Harker, 1958; Corbet, 1960), which enter into the amphibian food chain. Such synchrony could increase the probability of successful feeding and ultimate survival. Variations in intestinal transport activity (Robertson, 1976) may reflect the general physiological cycling coherent with these food gathering processes.

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Summary

The spontaneous locomotor activity (LA) of adult male frogs (*Rana pipiens*, Northern variety) was monitored throughout the year in an apparatus which detected vertical water movements. Frogs exposed to the seasonal change in ambient light and maintained at a constant mean annual temperature of 19.3 ± 3.1° C exhibited significant correlations of activity to the light-dark cycle, barometric pressure and lunar perturbations. When the light : dark ratio was < 1.0 (October to March) frogs displayed "random" activity throughout the 24 hr period; but with the L : D ratio between 1.0–1.45 activity was primarily nocturnal between 2100–0000 hr and at sunrise, while with a L : D Ratio > 1.45 maximal activity occurred at sunrise. Activity also was correlated with time of lunar high transit (HT) where occurrence of HT during daylight hours in April to June was associated with depressed activity, while HT during daylight in October to March was coincident with elevated period of activity. Use of a FORTRAN IV Program to analyze time of maximal LA each day throughout the year revealed oscillatory behavior patterns with periods similar to lunar tidal cycles. An alpha-series of cycles (55, 105, and 162 day periods) were significantly associated and dominant on days of high barometric pressure (above the annual mean of 761 mm Hg) and characterized by high levels of activity (above the annual mean of 313 events/day). A beta-series (30, 60, 90 day cycles) was dominant on days of low barometric pres-

sure (< 761 mm Hg) and coincident with low levels of activity (< 313 events/day). Spontaneous activity of frogs apparently is not random, but reflects an association with basic geophysical forces which elicit a complex but definable behavior pattern.

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SEASONAL RESPIRATION IN THE MARSH PERIWINKLE, *LITTORINA IRRORATA*

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Intertidal invertebrates inhabit an environment which varies on a diurnal, tidal and seasonal basis. It is not surprising that the relationship between their metabolism and the environment may be complex. Adaptations to the intertidal environment by some invertebrates include a standard metabolic rate that is insensitive to a wide range of temperatures and an active metabolic rate that is temperature-independent, or thermo-neutral, within a zone centered about the ambient field or acclimation temperature (summarized by Newell, 1969, 1973, 1976). The relationship between metabolism and environmental variables may be obscured by other adaptations, such as the presence of diurnal or tidal metabolic rhythms (Sandeen, Stephens and Brown, 1954; Sandison, 1966; Shirley and Findley, 1978). Seasonal acclimatization and acclimation temperature may also greatly affect metabolic rate and range of the temperature-insensitive zone (Pye and Newell, 1973). Many metabolic studies of invertebrates have failed to consider these possible complexities of invertebrate metabolism or have not been of a long enough duration to investigate seasonal changes.

The marsh periwinkle, *Littorina irrorata* (Say, 1822), is widely distributed from New York to Texas (Bequaert, 1943) and is the most important gastropod in terms of biomass in the salt marshes of the Gulf of Mexico (Day, Smith, Wagner, and Stowe, 1973; Subrahmanyam, Kruczynski and Drake, 1976; Hamilton, 1976). Its well-studied life history (Bingham, 1972a, b; Alexander, 1976; Odum and Smalley, 1959; Shirley and Findley, 1978) and the concurrent investigation of its biochemical composition and body component indexes (Bistransin, 1976) are useful in understanding the snail's metabolic patterns. The snail is supratidal in habit, normally found on *Spartina* stems above the air-water interface. Although a number of respiration studies of intertidal gastropods have been reported (Bertness and Schneider, 1976; Coleman, 1976; Huebner, 1973; McMahon and Russell-Hunter, 1977; Newell and Pye, 1970a, b, 1971; Sandison, 1966, 1967), relatively few have been performed with supratidal marine snails. A trait of *L. irrorata* also meriting investigation is its ability to attach to a stem by means of a mucous holdfast and withdraw into its shell, supposedly to avoid unfavorable conditions. The effect of formation of mucous holdfasts on the metabolism of the snail has not previously been examined.

The investigation, therefore, focused on three main areas: first, the possibility of diurnal metabolic rate rhythms; secondly, seasonal changes in metabolic rate-

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temperature curves; and thirdly, the metabolic rate of snails in conditions which are conducive to mucous holdfast formation.

Materials and Methods

Snails were collected at monthly intervals for 15 months beginning in October, 1973, from a *Spartina alterniflora* salt marsh located 3.5 km northwest of Grande Isle, Louisiana. The snails were transported to the laboratory and maintained under salinity, temperature and photoperiodic regimen corresponding to measured field conditions (Table I). Meteorological data were obtained from the U. S. Coast Guard Station on Grande Isle in order to consider the effects of acclimatization temperatures on experimental results. Inasmuch as the snails are supratidal in habit, air temperature was considered to be of greatest importance and temperature means were determined from measurements made at three hour intervals for the duration of the study. Studies were initiated on the day that snails were collected and respiration measurements of the snails were usually completed within three to four days after collection. Individual oxygen consumption rates of 14 snails were measured for each uptake determination with a Gilson Differential Respirometer using standard manometric techniques. The largest snails present in the field ($\bar{x} =$ 152 mg dry tissue wt) were selected for all determinations. The 110 ml reaction vessels and snails were allowed to equilibrate for one hour prior to the initiation of measurements. Measurements were made for approximately one hour and corrected to STP. The reaction vessels were not shaken during the experiment. A paper wick and 0.5 ml of 30% KOH were placed in the sidearm of each vessel.

Three separate studies, each with different experimental procedures, were conducted. Studies 1 and 2 were conducted under water saturated conditions. Eight milliliters of artificial sea water (Instant Ocean) of the same salinity as in the field at the time of collection were placed in each reaction vessel. In study 1 the respiration of 14 snails was measured for a one hour period on alternate hours for 36 hours at a 5° C temperature increment $\pm$ 2.5° C of the field temperature at the time of collection. Study 2 consisted of a one hour measurement of respiration of snails during daylight hours at 5° C temperature increments from 5 to 45° C. Fourteen different snails were used for each temperature. In study 3 an attempt was made to induce the snails to form mucous holdfasts by subjecting them to low humidity conditions. Accordingly, no water was added to the vessels and additionally, mantle compartment water was removed by gently pushing the snail as far as possible into its shell after which the snail was dried. A drying tube filled with calcium sulfate was attached to the air intake of the respirometer, and the snails were exposed to the dry air overnight prior to respiration measurements. Measurements in study 3 were made at the same temperature as study 1.

Observations on the condition of the snails and formation of mucous holdfasts were noted after each respiration measurement. After completion of each experiment, the shell of each snail was cracked and the soft parts removed carefully by hand and dried to constant weight at 85° C.

Regression analyses of $\log_{10}$ oxygen consumption per animal against $\log_{10}$ dry tissue weight were determined for each experiment by the least squares method.

Inasmuch as the majority of regression equations were not significant ($P > 0.05$), mean weight specific oxygen consumption rates [μl $O_2/(g \cdot hr)$] and confidence intervals at the 95% level were calculated for all experiments. In study 1, analysis of variance of the randomized block design was performed to determine differences in respiration rates with respect to time of day. Further partitioning of the variance was tested by orthogonal comparisons. In study 2, Q_{10} values over 5° C temperature intervals were determined, and the significance of each was tested against the null hypothesis that $Q_{10} = 1.0$ using a modified t-test (Snedecor and Cochran, 1971). In study 3, regression equations, mean respiration rates and 95% confidence intervals were calculated separately for those snails that had formed mucous holdfast and those that had not.

One of the principal modifying agents of metabolism is activity, and several methods of coping with animal activity have been attempted to reduce scatter about the regression lines relating log metabolism to log body weight. One of the more recent methods used by Newell and associates (Newell and Northcroft, 1965; Newell and Pye, 1971; Newell and Roy, 1973) has been to separate active rates from standard rates solely on the basis of magnitude without correlation to animal activity. Although this method has been used with success in significantly reducing regression scatter, it ignores the activity-metabolism and individual variation (Barnes and Barnes, 1969; Coleman, 1976). The concept of metabolism increasing directly with activity is obvious. Difficulties in correlating measured activity with metabolism have, however, been encountered by several investigators (McFarland and Pickens, 1965; McLusky, 1973). Additional complications in *L. irrorata*

TABLE 1

Average rate of oxygen consumption (Q_{O_2}) *of* Littorina irrorata *over 24 hr, expressed as* μl $O_2/(g$ *dry wt*$\cdot hr)$; $\pm 95\%$ *confidence interval (C.I.)*

Month	Temperature ° C		Orthogonal day/night	$\bar{X}$ dry weight of snails (mg)	Oxygen consumption	
	Field	Expt.			Day $\bar{X} \pm 95\%$ C.I.	Night $\bar{X} \pm 95\%$ C.I.
Oct. 73	24	25	**	121	540.6 ± 35.0	615.0 ± 25.4
Dec. 73	19	15	**	148	199.5 ± 15.0	242.3 ± 13.6
Jan. 74	12	15	NS	160	245.4 ± 12.9	241.8 ± 10.9
Feb. 74	19	25	**	169	530.2 ± 29.3	622.3 ± 34.2
Mar. 74	19	25	**	201	561.1 ± 19.4	673.5 ± 37.5
Apr. 74	20	25	NS	193	586.6 ± 28.3	589.4 ± 38.9
May 74	23	30	**	203	649.4 ± 20.0	708.3 ± 27.1
Jun. 74	27	30	**	173	822.2 ± 37.4	1039.2 ± 75.8
Jul. 74	26	30	**	163	893.7 ± 63.3	968.7 ± 70.2
Aug. 74	27	30	**	175	735.0 ± 28.7	923.3 ± 45.5
Sep. 74	26	30	**	121	824.3 ± 34.5	921.5 ± 47.4
Oct. 74	21	30	**	127	542.4 ± 26.9	636.3 ± 34.5
Nov. 74	19	15	NS	144	236.4 ± 16.0	220.2 ± 11.4
Dec. 74	13	15	**	148	238.0 ± 11.5	264.9 ± 15.1

** $P < 0.01$.
NS = $P > 0.05$.

are the snail's positive phototropism (Bingham, 1972a) and a lower metabolic rate during light than dark (Shirley and Findley, 1978). Illumination of flasks to monitor activity was therefore avoided during dark conditions in this study. Movement of reaction flasks containing specimens was also avoided to preclude disturbing the specimens and increasing metabolism (Newell, Weiser and Pye, 1974; Aldrich, 1975). Since the snails' activities were not measured, all data were utilized in regression analyses and computations without arbitrarily assigning a basal or active rate. The term "basal" metabolic rate has a number of specific criteria which cannot be readily applied to poikilothermic invertebrates, especially gastropods, because of the plasticity of their oxygen consumption (Lewis, 1971; McMahon and Russell-Hunter, 1977; Russell-Hunter, 1964; Sandison, 1967). The data are hopefully indicative of natural metabolic patterns in study 1 and indicative of routine or normally active snails in the respiration-rate temperature study. McMahon and Russell-Hunter (1977) used a similar approach in their work with littoral snails.

The percentage of caloric content respired per day per snail was determined by month for the year 1974. The total volume of oxygen consumed per gram dry weight of snail per day at the average air temperature of each month was calculated, with adjustment for the increased night consumption for appropriate months. Oxygen consumption was converted to caloric values by means of an oxycaloric coefficient of 4.8 cal/ml O_2 (Crisp, 1971). The oxycaloric value was adjusted to that of the mean weight of snails for each month. The total dry weight of carbohydrate, lipid and protein per snail for each month from the same population sample used for the respiration investigation was obtained from the work of Bistransin (1976) and used to determine the total caloric value for the mean weight snail per month. Division of the caloric value of respiration per day by the total caloric content resulted in the percentage of total calories respired per day.

Results

One of the principal parameters which might be expected to influence metabolism is the acclimatization temperature. The average air temperature for each month, determined from measurements made at 3 hr intervals for the entire year, is listed on Table I.

Regression analyses of $\log_{10}$ oxygen consumption *versus* $\log_{10}$ dry tissue weight were significant ($P < 0.05$) in only 72 of 256 regression equations in study 1. Oxygen consumption, expressed as μl O_2/(g dry wt·hr) (Table I) was therefore not normalized from regression equations, but rather adjusted to per gram dry tissue wt. No pattern was found in the occurrence of significant regression equations according to time of day or year. The nine months with weight ranges of snails greater than 67 mg, with two exceptions, had the greatest number of significant regression equations.

Highly significant differences in respiration rates with respect to time of day were found by ANOVA in all months except February, 1974. Further partitioning of variance by orthogonal analysis demonstrated that highly significant increased

TABLE II

Monthly Q_{O_2} values of Littorina irrorata *expressed in μl O_2/(g dry wt·hr) ± 95% confidence interval.*

Month	Temperature ° C								
	5	10	15	20	25	30	35	40	45
Oct. 73		324 ± 42	311 ± 45	440 ± 74	578 ± 95	913 ± 80	936 ± 144	1134 ± 117	
Dec. 73	238 ± 31	304 ± 62	245 ± 31	451 ± 33	638 ± 53	1274 ± 473	1283 ± 190	1204 ± 156	
Jan. 74	140 ± 29	165 ± 36	203 ± 32	470 ± 52	881 ± 59	1086 ± 58	1340 ± 122	1466 ± 258	144 ± 33
Feb. 74	171 ± 14	227 ± 33	287 ± 33	308 ± 36	537 ± 101	565 ± 45	751 ± 88	1040 ± 140	134 ± 77
Mar. 74	116 ± 26	185 ± 47	291 ± 29	434 ± 43	279 ± 44	604 ± 75	740 ± 90	1030 ± 78	55 ± 39
Apr. 74	139 ± 18	274 ± 30	289 ± 28	418 ± 34	531 ± 91	640 ± 69	780 ± 97	1014 ± 114	102 ± 19
May 74	182 ± 32	224 ± 38	289 ± 37	411 ± 50	509 ± 126	673 ± 58	1138 ± 105	1167 ± 150	895 ± 404
Jun. 74	66 ± 21	211 ± 49	293 ± 60	413 ± 86	487 ± 55	795 ± 141	1087 ± 136	1059 ± 95	301 ± 213
Jul. 74	109 ± 35	291 ± 47	312 ± 49	487 ± 45	495 ± 105	960 ± 227	1343 ± 160	1377 ± 314	126 ± 61
Aug. 74	104 ± 21	133 ± 26	346 ± 47	419 ± 41	376 ± 85	604 ± 112	949 ± 134	1195 ± 170	995 ± 186
Sep. 74	237 ± 37	215 ± 25	484 ± 42	311 ± 60	505 ± 106	865 ± 151	1250 ± 154	1351 ± 202	157 ± 72
Oct. 74	169 ± 41	252 ± 31	312 ± 38	388 ± 41	547 ± 82	1097 ± 316	939 ± 109	1059 ± 271	
Nov. 74	228 ± 98	442 ± 52	206 ± 35	1323 ± 153	1242 ± 91	834 ± 64	1245 ± 239	1432 ± 95	953 ± 185
Dec. 74	157 ± 36	323 ± 38	230 ± 33	778 ± 114	803 ± 87	1228 ± 142	1583 ± 188	1619 ± 355	

TABLE III

Monthly Q_{10} values of Littorina irrorata *at 5° C temperature increments. ND indicates no data available.*

Month	Temperature ° C							
	5–10	10–15	15–20	20–25	25–30	30–35	35–40	40–45
Oct. 73		0.9	2.0	1.7	2.5	1.1	1.5	ND
Dec. 73	1.6	0.6	3.4	2.0	4.0	1.0	0.9	ND
Jan. 74	1.4	1.5	5.3	3.5	1.5	1.5	1.2	<0.1
Feb. 74	1.8	1.6	1.2	3.0	1.1	1.8	1.9	<0.1
Mar. 74	2.5	2.5	2.2	0.4	4.7	1.5	1.9	<0.1
Apr. 74	3.9	1.1	2.1	1.6	1.5	1.5	1.7	<0.1
May 74	1.5	1.7	2.0	1.5	1.7	2.9	1.1	0.6
Jun. 74	10.4	1.9	2.0	1.4	2.7	1.9	0.9	0.1
Jul. 74	7.1	1.2	2.4	1.0	3.8	2.0	1.1	<0.1
Aug. 74	1.6	6.8	1.5	0.8	2.6	2.5	1.6	0.7
Sept. 74	0.8	5.1	0.4	2.6	2.9	2.1	1.2	<0.1
Oct. 74	2.2	1.5	1.5	2.0	4.0	0.7	1.3	ND
Nov. 74	3.8	0.2	41.2	0.6	0.6	2.2	1.3	0.4
Dec. 74	4.3	0.5	11.4	1.1	2.3	1.7	1.0	ND

respiration rates occurred at night in 11 of the 14 months. No relationships between respiration rates and tidal cycle were evident.

Comparison of respiration rates in study 1 across all months at the same temperature is not possible, because of the different experimental temperatures. For those months with the same experimental temperatures, as February through April, 1974, at 25° C, and May through October, 1974, at 30° C, direct comparisons can be made. An increase in oxygen consumption rate occurred for both day and night readings for the period February through April, with the exception of the day rate in April. The increase in oxygen consumption rates continued for the period May through July, with the exception of the day rate in July. Oxygen consumption rates then declined for the period August through October, with the exception of the day rate in September. A comparison of the rates during the remaining months can be made by using the readings from November and December of 1974 and January of 1973, as all have experimental temperatures of 15° C. The decline continued through November, increased in December and remained at that rate in January. It may be presumed that experimental snails were acclimatized to field temperatures, making some degree of seasonal comparison possible. Increased respiration rates, and presumably activity, increased with increasing temperatures from late winter through midsummer. Oxygen consumption rates started declining, however, prior to a corresponding decline in ambient temperatures. The decline in rate of consumption continued from late summer through the fall months and early winter before starting to increase again.

In study 2, regression equations of $\log_{10}$ respiration rate *versus* $\log_{10}$ snail dry weight were significant in only 44 of 121 metabolic rate-temperature experiments. Monthly Q_{O_2} values, expressed as μl O_2/(g dry wt·hr) were therefore not norma-

TABLE IV

Monthly Q_{O_2} values of Littorina irrorata *in vessels with sea water (controls) and in low humidity, expressed at μl O_2/(g dry wt·hr) ± 95% confidence interval.*

Month	Temp. (° C)	Controls	Low humidity	
			− mucous holdfast	+ mucous holdfast
Dec. 73	15	257 ± 46	118 ± 69	171 ± 209
Jan. 74	15	244 ± 29	208 ± 77	215 ± 23
Feb. 74	25	538 ± 101	677 ± 160	
Mar. 74	25	279 ± 44	421 ± 81	400 ± 758
Apr. 74	25	533 ± 103	444 ± 46	455 ± 73
May 74	30	673 ± 58	336 ± 101	249
Jun. 74	30	795 ± 141	921 ± 153	
Jul. 74	30	960 ± 227	841 ± 167	
Aug. 74	30	604 ± 112	846 ± 1037	687 ± 148
Sep. 74	30	865 ± 151	879 ± 132	
Oct. 74	30	544 ± 81	498 ± 48	368 ± 179
Nov. 74	15	206 ± 35	300 ± 29	
Dec. 74	15	276 ± 53	219 ± 32	192 ± 155

lized from regression equations (Table II). The lowest temperature at which rates were measured, 5° C, had no significant regression equations. Similarly, the highest temperature, 45° C, had only two significant regression equations. The higher temperatures, 35° and 40° C, had the most significant rate *versus* weight regression equations: 9 and 8, respectively, for the entire study.

Although the snails were inactive at 5° C in all months, with the exceptions of May and September, the rate of consumption was higher during the colder months and lower during the warmer months. The trend was more obvious at 10° C, with highest consumption rates during the colder months. The snails were always in a heat coma at 45° C, although no snails died during the experiment. The O_2 consumption rates at 45° C frequently approximated those at 5° C during certain months. The relatively stable metabolic rate at intermediate temperatures might best be observed by examining Q_{10} values (Table III). For the months March through August, 1974 the Q_{10} values for the temperature range 20–25° C are not significantly different from 1.0, indicating a temperature insensitivity. Values of Q_{10} at 15–20° C and 25–30° C average around 2.0–2.5 over the year, a normal temperature response in poikilotherms.

Respiration rates of snails in vessels containing sea water and snails with and without mucous holdfasts in low humidity for all months are given in Table IV. No apparent relationship between mucous holdfast formation and oxygen consumption rates was evident. No significant difference in respiration rates was found between those with and without mucous holdfasts for snails in low humidity. Although significant differences often occurred between respiration rates of snails in low and high humidity, the relationship varied. During some months the Q_{O_2} values of snails in dry air would be significantly lower than those of snails in high humidity, while in other months the inverse was true. In addition to those mucous hold-

TABLE V

Energy budget for Littorina irrorata *for the year 1974. Dry weight index is given in g/100 g standard animal, and caloric content is modified from Bistransin (1976).*

Month	Temp. (° C)	Dry wt index	Mean dry wt (mg)	Caloric content (calories)	Cal/day respired	Percentage of caloric content respired per day
Jan.	12	5.31	160	554	3.6	0.65
Feb.	19	6.21	169	624	6.5	1.04
Mar.	19	6.08	201	711	10.2	1.43
Apr.	20	6.47	193	810	9.3	1.15
May	23	6.33	203	957	10.9	1.14
Jun.	27	5.71	173	737	16.1	2.18
Jul.	26	4.59	163	696	11.2	1.60
Aug.	27	5.99	175	747	11.7	1.56
Sep.	26	3.93	121	409	8.4	2.04
Oct.	21	4.43	127	407	6.7	1.65
Nov.	19	4.33	144	528	18.3	3.47
Dec.	13	5.47	148	572	5.2	0.92
$\bar{x}$	21	5.40	165	646	9.8	1.57

fasts whose formation were induced in study 3, the formation of mucous holdfasts also occurred in studies 1 and 2. A record of these mucous holdfasts was kept and most were formed during the winter months at the coldest experimental temperatures. None was formed at temperatures above 15° C, with the exception of two that were formed at 40° C in January, 1974. Of 74 mucous holdfasts that were formed in all determinations in studies 1 and 2, 63 were formed at 5 or 10° C, and 47 of those were formed in January and February of 1974.

Seasonal changes in the respiratory expenditure of energy by *Littorina irrorata* are given in Table V. The average dry weight of the population varied seasonally as the animals accumulated nutrient reserves for spawning, which probably occurred in two episodes: June to July and August to September (Bistransin, 1976). The average dry weight of snails cycled very closely with the average dry weight index of the same population as determined by Bistransin (1976). Caloric content cycled with the dry weight indexes and average dry weight. The average number of calories respired per day over the course of the study was 9.8, and the percentage of caloric content respired per day was 1.57. Both values cycled seasonally. The calories respired per animal per day cycled more closely with average air temperature than did the percentage of caloric content respired per day. This was principally due to concomitant changes in the dry weight index. The high respiratory loss in November is the result of the highest respiration rate of the year occurring at 20 and 25° C during that month.

DISCUSSION

A circadian rhythm of oxygen consumption, with higher rates of consumption during the night, was present in all months of the year. Further investigation of the rhythm of *L. irrorata* under various experimental conditions has demonstrated

that light is the phase-setting factor and that the rhythm can be shifted according to the light regime (Shirley and Findley, 1978). It is probable that the rhythm reflects changes in the activity of the snails, such as foraging, breeding and movements to more optimal conditions. A circadian rhythm of feeding-related activity is not likely, as *L. irrorata* feeds on the exposed marsh floor during low tide (Alexander, 1976; Bingham, 1972a). A tidal rhythm of activity might therefore be expected; however, no activity of *L. irrorata* is synchronous with the tides other than its moving up *Spartina* stems when covered by a rising tide (Bingham, 1972a). The stimulus to move down the stems and initiate feeding is apparently increased temperature, but feeding will proceed only if the marsh floor is exposed (Bingham, 1972a). Moreover, no tidal rhythm of respiration was detected in this study. The absence of tidal rhythms of activity in other supra and upper-littoral littorinid snails has been reported (Zann, 1973). The vagaries of the tide in the marshes of the northern Gulf of Mexico, with the wind often having a greater effect than lunar forces on tidal height, may help explain the lack of a tidal rhythm.

Respiratory rhythms that have been reported for other marine gastropods differ from that of *L. irrorata*. Sandeen *et al.* (1954) reported both diurnal and tidal rhythms in *Littorina littorea* and *Urosalpinx cinereus*. In both species, maximal respiration rates occurred in the hours following sunrise and sunset. The lowest rates occurred during the early morning hours, when the highest rates were found in this study. Sandison (1966) also reported a diurnal rhythm of respiration by *L. saxatilis* in water, *L. littorea* in air and a tidal rhythm of respiration in the latter species while it was in water. Sandison (1966) reported the highest rates for *L. littorea* to be between the hours of 800 to 1200. As he only measured rates for 12 hours of the day, the possibility of increased nocturnal respiration was not examined. Both the investigations by Sandeen *et al.* (1954) and Sandison (1966) measured the rates of groups of snails rather than the rates of individual snails, as was done in this study.

The adaptive significance of the circadian rhythm of respiration with respect to the biology of the snail is uncertain and warrants further investigation. One possible explanation is that increased nocturnal respiration, and presumably activity, may be related to predation. The snail may be more active at night when it is less susceptible to visually oriented predators, such as the blue crab, *Callinectes sapidus* (Hamilton, 1976). Certainly the amplitude of the rhythm is great enough to obscure relationships between oxygen consumption and experimental variables, and should be a consideration in metabolic experiments.

A seasonal comparison of respiration rates may also be made from the data of study 1. The increase in respiration rates at 25° C from February through April and likewise from May through July at 30° C may be due to warm temperature stimulation of metabolism. The subsequent decrease in respiration rates from August through October at 30° C occurs prior to the corresponding decrease in seasonal temperatures. The decrease in oxygen consumption rates, indicative of a seasonal change in metabolism, is perhaps related to changes in photoperiod (Dehnel, 1958). The increase in respiration rate in the winter at 15° C prior to an increase in ambient temperature is suggestive of cold temperature acclimatization.

The oxygen consumption rates of *L. irrorata* in study 2 differ notably from those of temperate species (McMahon and Russell-Hunter, 1977; Sandison, 1967). *Littorina irrorata* is active near its upper lethal temperature, while the temperate species are not. Also, the temperature at which *L. irrorata* enters heat coma is much higher than temperate littorinids (Sandison, 1967; McMahon and Russell-Hunter, 1977). The two-phased reaction of snails entering heat coma observed by Sandison (1967), which consisted of an initial rise in respiratory rate followed by an irregular fall, was not observed in *L. irrorata*. Low Q_{10} values for the entire year are present for the temperature range of 35–40° C. Although activity of the snails was not inhibited at 40° C during any season of the year, the low Q_{10} values suggest that 40° C is near the snails' upper limit of capacity adaptation. Lewis (1971) also found that activity was not inhibited in three species of tropical intertidal gastropods at 37° C. A seasonal increase in the upper limit of thermal tolerance reported for some temperate intertidal molluscs (Newell and Pye, 1970a) was not evident in *L. irrorata*.

One of the more interesting aspects of the respiration rates at various temperatures in study 2 is a plateau in oxygen consumption rates in the temperature range of 20–25° C, clearly demonstrated by the Q_{10} values being not significantly different than 1.0 for the months of March through August. This is perhaps a thermoneutral zone, or zone of temperature independence, for *L. irrorata* during these months. This narrow zone of metabolic homeostasis occurs near the average annual temperature at the collection site, approximately 20° C. Likewise, the range of the temperature independent zone approximates the average daily temperature range, 6.5° C. Daily temperature variations of 10° C or greater occurred less than 8% of the time. Since oxygen consumption rates were measured only at discrete 5° C increments, the actual temperature independent zone may be several degrees broader than the discernable 5° C zone. The zone may shift seasonally, as suggested by another group of low Q_{10} values present at 5–10° and 10–15° C in October, 1973 through January, 1974. Yet another set of low Q_{10} values is present in the 15–20° C range in August through October, 1974, and in the 10–15° C range in October through December, 1974. The lack of correlation between activity and oxygen consumption in this study may have made temperature independent zones less distinct. Most temperature independent zones reported for marine invertebrates have been restricted to standard metabolic rates (summarized by Newell, 1969, 1973), although other temperature independent zones have been reported for routine metabolic rates of intertidal snails (Bertness and Schneider, 1976).

The ability of littorinid snails to attach themselves to a substrate by means of a mucous holdfast and then withdraw into their shell has been considered a means by which they decrease exposure to unfavorable conditions. The relationship that salinity and relative humidity have on mucous holdfast formation in *L. irrorata* has been investigated (Bingham, 1972b). The effect of temperature on holdfast formation and the effect that holdfast formation has on metabolism has not been reported. Although it would seem that inactive snails attached by a mucous holdfast would have reduced metabolic demands, no significant difference was found between the respiration rates of snails in containers with sea water and those with

and without holdfasts in low humidity. The greater number of mucous holdfasts formed at low temperatures during the winter months suggests that temperature must be considered as an important factor in inducing holdfast formation, as well as relative humidity and salinity. W. A. Murphy (Tulane University, personal communciation) has found that snails form holdfasts more rapidly at various relative humidities at 10° C as compared to 20 and 30° C. The terrestrial snail *Otala lactea* is also more likely to become dormant and form epiphragms at low relative humidities and low temperatures than at high relative humidities and high temperatures (Rokitka and Herreid, 1975). Although no metabolic advantages were found for mucous holdfast formation in this study, presumably holdfast formation in *L. irrorata* serves the same functions that were reported by Vermeij (1973) for mucous holdfasts in other littorinid snails: to reduce water loss and contact between soft tissues and substrate; to obviate the need for a large water reservoir; and, to increase the degree of temperature regulation.

In previous productivity studies of *Spartina* marshes, the contribution of *L. irrorata* to community metabolism has been estimated (Day *et al.*, 1973; Odum and Smalley, 1959). Alexander (1976) measured the egestion rate of *L. irrorata* to be 145 g organic matter/($m^2 \cdot yr$). Day, Smith and Gayle (unpublished manuscript) have estimated the standing crop *L. irrorata* in Louisiana salt marshes to be 4.9 g/m^2. If our respiration data, Alexander's egestion data and the Day, Smith and Gayle's standing crop data are used, and annual energy budget for *L. irrorata* is calculated to be: 182.7 g organic matter/($m^2 \cdot yr$) total food intake, 4.9 g/m^2 standing crop, 9.8 g/($m^2 \cdot yr$) net organic production, 145 g organic matter/($m^2 \cdot yr$) feces production and 27.9 g organic matter/($m^2 \cdot yr$) lost to respiration.

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Summary

1. Respiration rates of *Littorina irrorata* were measured monthly for the period from October, 1973, through December, 1974. The study consisted of three main parts: first, hourly measurements of respiration rates at ambient field air temperature over a 36 hr time period; secondly, one-hour measurements of respiration rates at 5° C temperature increments from 5° to 45° C during daylight hours; and thirdly, one-hour measurements of respiratory rates under conditions conducive to mucous holdfast formation. Respiration rates were measured with a Gilson respirometer using standard manometric techniques.

2. A diurnal rhythm of respiration was found for 11 of the 14 months. Respiration rates during the night were significantly higher than during the day.

3. Snails were in thermal stress at 5° C and 45° C and their respiration rates were depressed. Respiration rates at 10° C were highest during the colder months, demonstrating inverse cold temperature acclimatization. The Q_{10} for the temperature range 20–25° C were not significantly different from 1.0 for the months March through August, suggesting thermal insensitivity or the presence of a thermo-neutral zone.

4. No apparent relationship between mucous holdfast formation and oxygen consumption was evident. Mucous holdfasts were formed most frequently during the winter months at the coldest experimental temperatures.

5. An annual energy budget of *L. irrorata* is calculated.

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TRANSEPIDERMAL ACCUMULATION OF NATURALLY OCCURRING AMINO ACIDS IN THE SAND DOLLAR, *DENDRASTER EXCENTRICUS* [1]

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Echinoids and asteroids exhibit a full range of feeding habits including carnivores, herbivores, detritus feeders and filter feeders. However, distribution of nutrients derived from digestion, whatever the feeding habit, appears to be slow and incomplete. Ferguson (1970) injected a mixture of ^{14}C-labeled amino acids into the stomach and perivisceral coelom of the starfish, *Echinaster,* and followed the subsequent distribution of labelled material using autoradiography. Translocation of nutrients throughout visceral and subepidermal regions was observed, but no labelling of epidermal tissue was evident at the end of 75 days. Conversely (Ferguson, 1967), ^{14}C-labeled amino acids supplied in the ambient medium were incorporated into epidermal tissues of starfishes, but there was little or no export of label from epidermis to subepidermal or visceral tissues. A comparable barrier to distribution of nutrients between epidermal and visceral tissues is discussed in the work Péquignat (1969, 1970), Péquignat and Pujol (1968), and Pearse and Pearse (1973) employing various echinoids and asteroids. The barrier is not necessarily complete. Slow translocation across the barrier is reported by some investigators. These observations agree well with the morphology of these echinoderm groups; the "circulatory" systems, though complex, do not appear to provide a well-organized morphological substrate for distribution of material to the epidermis.

Stephens and Schinske (1961) showed net influx of glycine from a rather concentrated solution into two species of starfishes. Since that time, a number of investigators have studied uptake of amino acids in echinoderms (*e.g.,* Stephens and Virkar, 1966; Fontaine and Chia, 1968; Clark, 1969; Dixit, 1973; Ahearn and Townsley, 1975). Most of this work used ^{14}C-labeled substrates and described kinetics of influx by radiochemical techniques and/or distribution of labeled material by autoradiography. However, Ferguson (1971) showed by direct chemical determination that there was a net influx of amino acids from a medium concentration of 37.5 μM into ten different species of starfishes from the Puget Sound area. This work did not permit estimation of rates of net influx but established the capacity of several of the forms employed to reduce ambient amino acid concentrations to extremely low levels at the end of a six-hour incubation period.

There is, thus, considerable evidence for uptake and utilization of amino acids by epidermal tissues in echinoids and asteroids. There is also considerable evidence

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that distribution of nutrients from the digestive system to superficial tissues is very slow. This has led Ferguson (1970) to suggest that epidermal tissues may derive much of their sustenance by direct influx of nutrients from the environment independent of the nutrition of visceral and subepidermal tissues. Péquignat (1970) frames a similar hypothesis, adding the possibility of cutaneous digestion and assimilation of larger organic substrates to account for support of epidermal structures.

The sand dollar, *Dendraster excentricus,* was selected as an experimental organism for two reasons. First, there are large populations of this organism readily available for study and the feeding behavior has been well described (Timko, 1976). Secondly, we wanted to work with an organism which lives in or on a soft substrate, since the occurrence and distribution of amino acids in such habitats has already been analyzed (Stephens, 1975) in relation to the nutrition of annelid infauna.

In the present work, data are presented on the following: kinetics of influx of ^{14}C-labeled amino acids from solution, kinetics of net influx of amino acids, levels of amino acid present in the microenvironment, availability of naturally occurring amino acids as assessed by net influx, estimates of energy metabolism of whole animals, and estimates of energy metabolism in isolated portions of the test. Collectively, this information allows an estimate of possible contributions of transepidermal transport of amino acids to the support of epidermal tissues.

Materials and Methods

Animals and sediment samples were obtained from two locations. Most of the material studied was obtained from a shallow water population in a lagoon at Point Mugu Naval Base near Port Hueneme, California. Some samples were taken from a population off Newport Beach at a depth of approximately 5 meters. Animals were maintained in aerated sea water at a temperature of 15° C. Sediment cores were taken using plexiglass coring tubes (25 mm internal diameter) and were analyzed promptly. Interstitial water from sediment was expressed through a Millipore filter (0.45 μm) under nitrogen at 10–20 psi.

Influx rates of amino acids were determined by measuring the disappearance of radioactivity from a solution containing the ^{14}C-labeled compound. Solutions were prepared in artificial sea water (Cavanaugh, 1956) prepared from reagent grade salts. Radioactivity was initially 20 μCi/liter ($2–5 \times 10^{-7}$ moles/liter depending on specific activity) with ^{12}C-amino acid added to obtain the desired concentration. Radioactivity was measured using a scintillation counter; samples of 0.5 ml were added to a toluene-based cocktail containing a detergent to solubilize the sample. Samples were acidified to drive off CO_2. Volumes to which animals were exposed ranged from 50 to 200 ml; air was bubbled slowly through the vessel to provide aeration and circulation.

Net flux of amino acids was followed using fluorescamine (North, 1975) to determine primary amines in solutions to which the animals were exposed. After sample preparation, fluorescence was measured using a Perkin-Elmer spectrophotofluorometer with an excitation wavelength of 390 nm and an emission wavelength of 480 nm. This procedure was also used for estimating levels of naturally occurring primary amines in interstitial water of sediment samples. Some of the

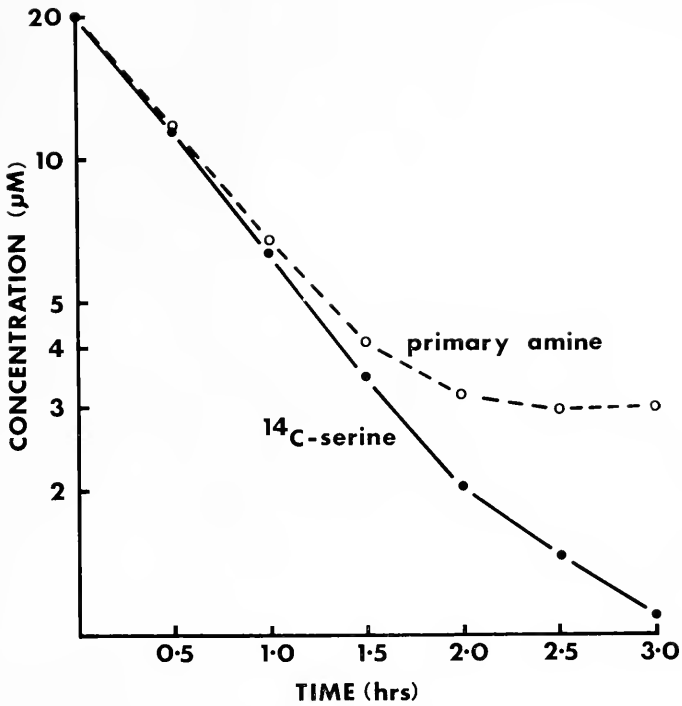

FIGURE 1. Removal of serine from 100 ml of a 20 μM solution by a specimen of *Dendraster*. The solid line shows decrease in radioactivity with time; the broken line shows decrease in primary amines estimated by the fluorescamine procedure.

latter measurements were repeated using an independent procedure based on o-phthalaldehyde (OPA). The procedure is based on that of Mendez and Gavilanes (1976) but employs a lower concentration of OPA (0.03 mg/ml rather than 0.3 mg/ml). It proved to be necessary to read samples and standards at a constant time after preparation.

Amino acids present in interstitial water before and after exposure to the animals were identified by thin layer chromatography. Seawater samples (5–10 ml) were desalted on Dowex–50, eluted off the column with 3 N NH_4OH and chromatographed as described by Clark (1968) with the following modifications. Spots (1–5 μl) were applied to the chromatogram using a glass capillary drawn to a tip diameter of approximately 50 μm which minimized spot diameter. Chromatograms, 10 cm × 10 cm, rather than 20 cm × 20 cm as supplied (Polygram CEL 300), were developed for approximately one hour in each dimension without scoring following the first solvent system.

Spots were located using OPA as a location reagent as follows. An OPA stock solution is prepared several hours prior to use by dissolving 30 mg OPA in 200 ml glass distilled water and adding a drop of 1 N NaOH. This stock is stable for one to two days. Just prior to use, the spray mixture is prepared consisting of 20 ml OPA stock, 20 ml absolute ethanol, 0.2 ml triethylamine and 0.01 ml 2-

TABLE I

Rates of disappearance of amino acids expressed as nmoles/(hr·cm²). Data for aboral surface and for both surfaces are combined. Average diameter 4.1–7.6 cm.

	Mean rate	Standard deviation	N
ala	42.0	2.7	2
asp	16.8	2.3	3
gly	45.8	5.0	6
glu	6.1	1.0	5
lys	25.4	3.8	5
ser	49.5	5.5	8
val	63.0	4.6	3
naturally occurring primary amines	24.9	7.6	5

mercaptoethanol. The spray mixture is stable for several hours. It is applied using a mist sprayer; approximately 5 ml suffices for a 10 cm × 10 cm plate. One to ten minutes after spraying, chromatograms are examined under a long wavelength UV-lamp. Most spots intensify on drying, but lysine fades. Detection levels for most amino acids range from 20 to 50 picomoles. However, 200 to 300 picomoles are required for location of some hydrophobic amino acids (*e.g.,* val, leu, ile). Chromatograms were photographed through a yellow filter (455 nm) using Kodak Tri-X film and diafine development (ASA 1600) at f5.6 and 0.5 second exposure. Schiltz, Schnackerz and Gray (1977) have recently described a comparable procedure. When chromatograms are prepared as described, ninhydrin can also be used as a location reagent with detection levels in the range of a few hundred picomoles.

Oxygen consumption was measured using a YSI oxygen electrode. Measurements of oxygen consumption as well as studies of influx and net flux of amino acids were carried out at a temperature of 20° C.

RESULTS

Figure 1 presents the results of a typical set of observations. A sand dollar, 7.2 cm average diameter, was placed in 100 ml of artificial sea water to which serine had been added at a concentration of 20 μmoles/liter. The solution also contained 2 μCi of ^{14}C-serine (UL). As indicated in the figure, radioactivity decreased rapidly with time as did the total primary amine in the solution estimated by the fluorescamine reaction. Disappearance followed first order exponential kinetics for the first hour, and the two curves are virtually identical. The rate of entry (disappearance of primary amine) at the initial concentration of 20 μM was 2.15 μmoles/hr. It proved to be best to relate rates to the surface area of the animals and express them as nmoles/(hr·cm²). This gave consistent results over the considerable size range of animals examined. For the case presented in Figure 1, uptake of serine from a 20 μM solution proceeded at a rate of 53 nmoles/(hr·cm²). If influx of ^{14}C is expressed in the same units, the rate from a 20 μM solution is 56.7 nmoles/(hr·cm²).

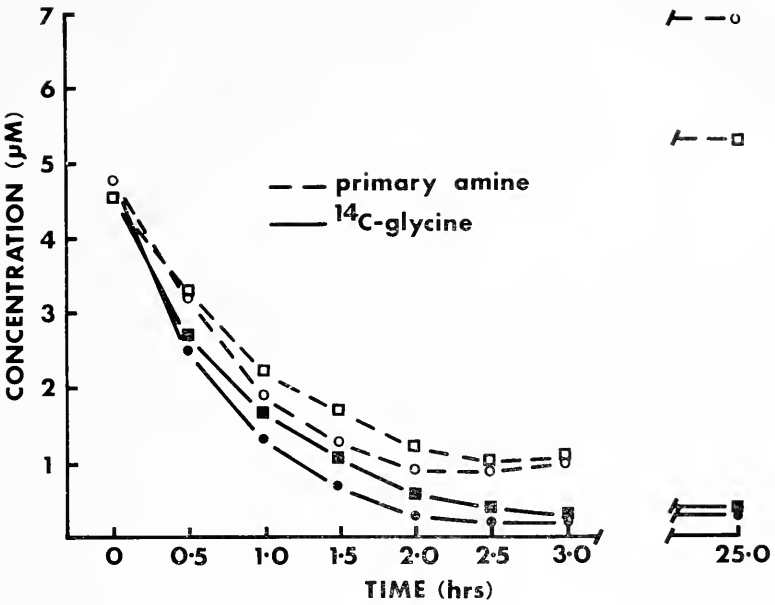

FIGURE 2. Removal of glycine from 100 ml of a 5 μM solution by two specimens of *Dendraster*. Data points for the two individuals are solid and open circles and solid and open squares respectively.

Results of experiments of this kind were quite repeatable. When animals were supported on glass rods to facilitate circulation of solution across the oral surface, observed rates of disappearance of an added amino acid were approximately doubled. Thus both surfaces of the animals seem to participate equally in removal of amino acid from dilute solution. Table I presents rates of disappearance of various amino acids. All rates are expressed as nmoles/(hr·cm^2) at an ambient concentration of 20 μM. It should be noted that the determinations of ^{14}C and of primary amines diverge at low concentrations (Fig. 1). Figure 2 shows data for two different animals offered ^{14}C-glycine at 5 μmoles/liter. Fluorescent material declined over the course of two hours to approximately 1 μmole/liter (glycine-equivalent concentration) and then slowly increased to levels of 5–7 μM over the course of the ensuing 23 hours. If animals were placed in a small volume of artificial sea water with no added amino acid, primary amines slowly increased and stabilized at similar final concentrations. These same levels were found in the aerated water in which groups of animals were kept over a period of days.

Figure 2 also shows that small amounts of radioactivity (6–8%) remained in the solution 25 hours after ^{14}C-glycine was supplied. In the case of other amino acids, for example serine, this effect was quite pronounced with as much as 15–20% of initial radioactivity persisting in solution at the end of 24 hours. The radioactivity does not appear to be in the form of serine; about 60% of the activity passes through a Dowex-50 column in the acid form, and TLC shows several spots that are unidentified but do not react as primary amines.

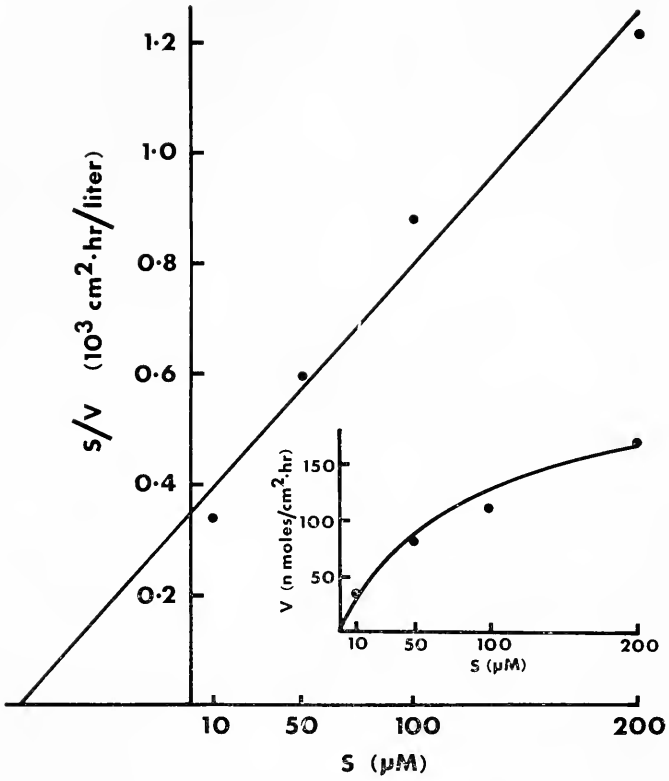

FIGURE 3. Removal of glycine from solution by *Dendraster* as a function of ambient concentrations. The insert graph presents rates determined at the concentrations indicated; the curve is a hyperbola fitted to the kinetic constants. The larger graph is a Woolf plot of the data. K_t is 74 μM V_{max} is 215 nmoles/(hr·cm²).

Figure 3 presents data relating influx (measured by disappearance of ^{14}C) to glycine concentration. The insert is a plot of influx as a function of ambient concentration. Kinetic constants were evaluated from the Woolf plot presented in Figure 3. The K_t was 74 μM and the V_{max} was 215 nmoles/(hr·cm²).

Dendraster does not take up glycylglycine from dilute solution. Animals were incubated for 24 hours in 20 μM glycylglycine with a trace amount of ^{14}C-glycine. Radioactivity in the medium decreased rapidly, as expected. Fluorescamine positive material (expressed as equivalent glycylglycine concentration) increased slightly during early incubation, presumably reflecting efflux of unknown primary amines. After 24 hours, levels had decreased to about 75% of the original concentration. This may reflect microbial activity or very slow uptake. In any case, uptake of glycylglycine either does not occur at all or is so slow as to be insignificant compared to uptake of neutral amino acids.

Naturally occurring primary amines in interstitial water were determined using the fluorescamine technique and, in some cases, OPA. Determinations using the

two procedures were in good agreement. Sediment cores were divided into 3 cm zones from the surface downward; water was expressed through a Millipore filter under N_2, and concentrations of primary amines expressed as glycine-equivalent concentration. We do not believe that the cores taken at 5 meters depth were undisturbed. They showed interstitial concentrations of 17 and 23 μmoles amines/liter, respectively, in the top 3 cm. Core samples could be taken in the immediate vicinity of the shallow water population with minimum disturbance of sediment organization. The samples showed great variability in primary amine content. Fifteen samples gave an averaged value of 115 μM in the interstitial water of the top 3 cm of the cores with a standard deviation of 60 μM. The range was 17–244 μM. Stephens (1975) also reports considerable variability in primary amine concentration in sediment cores. In general, primary amine concentration decreased with depth, also in agreement with Stephens (1975) and Crowe, Dickson, Otto, Colón and Farley (1977), though there were two cores which showed an increase at the 3–6 cm and 6–9 cm zones.

Observations were carried out on rates of influx using samples of naturally occurring primary amines from both collection sites. Although the samples were expressed from sediment which was collected as carefully as possible, it is likely that they were somewhat diluted during collection. Also, a period of several hours elapsed before it was possible to obtain interstitial water from sediment collected at the shallow water site. The initial concentrations for the two sets of observations were 14 μM (for the deeper population) and 33 μM. The results are presented in Table I, recalculated to present rates from an ambient concentration of 20 μM to facilitate comparison with rates for known amino acids. The correction was made assuming a linear relation between ambient concentration and influx over the relevant range (14–33 μM).

Figure 4 presents photographs of TLC, including a standard and samples of sea water before and after exposure to a sand dollar for 24 hours. The standard contained 250 picomoles of each amino acid. The sea water to which the animal was exposed was interstitial water which initially contained 33 μM primary amine as estimated by the fluorescamine procedure. Final concentration was 7 μM. Desalted samples representing 125 μl of the interstitial water before and after exposure were spotted and chromatographed. Figure 4 illustrates the marked decrease in neutral amino acids at the end of the exposure period. A larger amount of the post-exposure sample was spotted and chromatographed; spots were more intense, but the pattern was the same as that illustrated. Neither the initial nor the final sample chromatograph in Figure 4 should be interpreted as a complete inventory of primary amines in the interstitial water. Only 70–85% of primary amine as estimated by the fluorescamine procedure is retained on passage through a Dowex-50 column in the acid phase and subsequently eluted with NH_4OH, whereas the retention of a standard mixture of amino acids in sea water is virtually complete. Thus, some of the naturally occurring primary amine is not behaving as do most amino acids, is not present in our desalted sample, and hence is not represented on the TLC. As an example, taurine reacts with fluorescamine but is not retained on a Dowex column. However, primary amines which pass through the column were not identified.

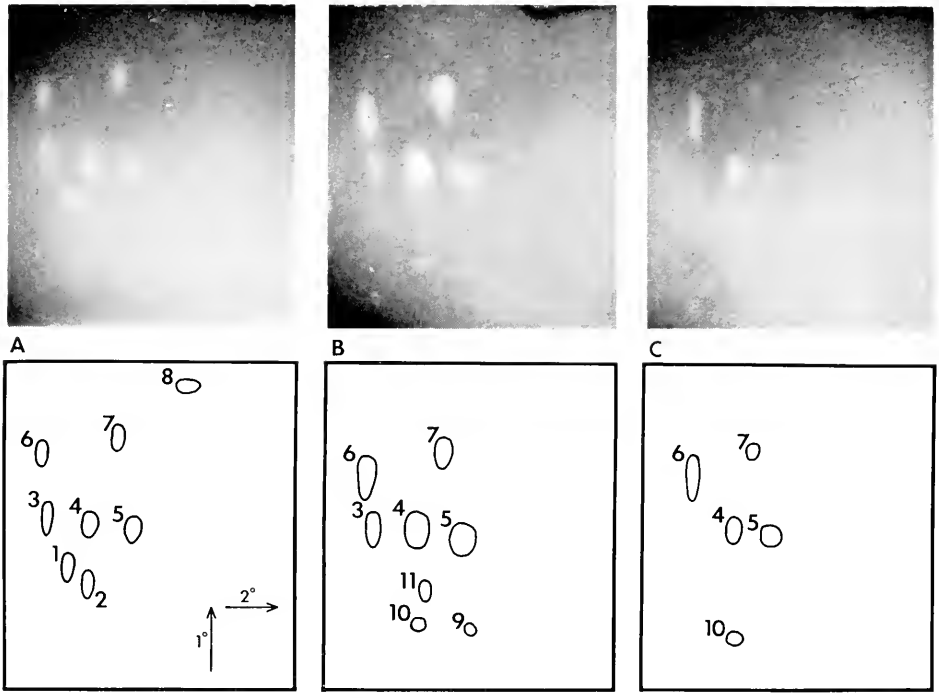

FIGURE 4. Thin layer chromatograms developed with OPA (see text for procedure). Amino acids are coded as (1) arg, (2) lys, (3) asp, (4) gly, (5) ser, (6) glu, (7) ala, (8) val, (9) his, (10) orn, (11) gln. **A** is a standard containing 250 picomoles of each of the first 8 amino acids, made in artificial sea water, desalted and run. **B** is a sample of interstitial water; total primary amine content is approximately 4.1 nanomoles. **C** is a sample of interstitial water after 24 hours exposure to a sand dollar; total primary amine content is approximately 875 picomoles. His, orn and gln are identified by Rf values from other standards.

Values for oxygen consumption were not found for sand dollars in the literature. The measurements presented in this study are intended to offer an approximate figure for oxygen consumption for comparison with measured rates of amino acid uptake. Two small animals (1.8 and 1.9 cm average diameters) consumed 4.6 and 4.7 μl O_2/(hr·cm^2); two large animals (6.15 and 5.75 cm average diameter) consumed 5.0 and 2.8 μl O_2/(hr·cm^2). In contrast to the relative constancy of oxygen consumption expressed per unit surface, oxygen consumption per unit weight decreased rapidly with size as would be anticipated. For the small animals, rates were 46.0 and 43.4 μl O_2/(g·hr); for the larger animals, they were 13.2 and 15.1 μl O_2/(g·hr). Despite the small sample size, it seems reasonable to accept the average figure of 4.3 μl O_2/(hr·cm^2) as an estimate of typical oxygen consumption at 20° C. Isolated portions of the aboral test survived well in aerated sea water for two to three days at 20° C as judged by general appearance and activity of pedicellariae. Oxygen consumption of two such portions of the test was 6.3 and 3.7 μl O_2/(hr·cm^2), respectively. The subdermal portion of the test was cleaned of adherent tissues, but the epithelium contributed to oxygen consumption; however,

it appears that the epidermis is responsible for a large fraction of the total oxygen consumption of the animals.

Discussion

Simultaneous measurement of influx and net influx of seven amino acids indicate that neutral amino acids (ala, gly, ser, val) are removed rapidly from dilute solution in ambient sea water by *Dendraster*. The amino acids asp and lys enter more slowly; glu is removed from solution very slowly, if at all (Table 1). Entry rates as estimated by disappearance of ^{14}C-labeled substrate and by chemical determination of total primary amine remaining in solution are comparable at ambient concentrations of 5 μM or more (Fig. 1). Thus, estimates of influx (^{14}C) reflect net influx (primary amine) at concentrations which are normally present in the habitat of the organism.

When *Dendraster* is placed in a fixed volume of sea water, an efflux of primary amines of unknown composition occurs until an apparent steady state is reached at an ambient concentration of 5–7 μM. Efflux appears to be slow compared with uptake of neutral amino acids. Thus, the pattern of primary amine concentration in the medium with time may show a decrease with a subsequent increase (Fig. 2).

Dendraster is capable of net accumulation from solution of some of the naturally occurring primary amines found in the interstitial water of sediment from its habitat. Rates of removal are approximately half the rates observed for neutral amino acids (Table I) when expressed in comparable units. Two explanations for this lower rate can be suggested. First, glu is present in interstitial water and therefore contributes to total primary amine but is relatively unavailable to the animal. Secondly, 15–30% of the primary amines in interstitial water are not retained on Dowex-50 in the acid phase and may represent material, some or all of which is unavailable for transepidermal uptake.

Comparison of the amino acids present in interstitial water before and after exposure to *Dendraster* shows a change in total primary amines and a change in pattern of amino acids present (Fig. 4). The changes are consistent with predictions based on experimental results with single amino acids. Thus, neutral amino acids are reduced, while glu is relatively unchanged; total primary amines are reduced to a stable level of 5–7 μM.

As noted, a portion of the primary amines normally present in interstitial water does not appear to behave as typical amino acid. Changes in the contribution of this fraction to total primary amines during exposure to *Dendraster* were not determined. Estimation of its concentration by difference before and after passing through a Dowex-50 column proved to be unsatisfactory. Presence of this unknown material also prohibits a complete description of the primary amines which appear in the medium in which animals are incubated.

Amino acids removed from solution by these animals apparently enter metabolic pathways. In general, acidification of the medium leads to a reduction in measured radioactivity of a medium sample after an animal has been exposed to a known labelled substance. This acid volatile radioactivity may be evidence for the production of $^{14}CO_2$, commonly found in experiments of this kind (Stephens, 1972).

The presence of radioactivity which is not acid volatile and which is not amino acid at the end of incubation experiments may be evidence of the presence of labelled metabolites lost from the animals. These have not been identified but are not primary amines.

The failure of *Dendraster* to remove gylcylglycine from dilute solution suggests that if epidermal digestion does occur in these animals, it is a slow process compared to transepidermal transport of amino acids. This is consistent with the very low protein digestion activities reported by Péquignat (1970), but other pathways of disappearance of glycylglycine cannot be excluded in these observations.

Possible bacterial contributions to the appearance of labeled, acid volatile and acid nonvolatile metabolites in the medium and to the disappearance of glycylglycine cannot be completely excluded. However, bacterial contributions to influx and net flux measurements are certainly small. Animals were incubated for 24 hours in penicillin (500,000 units/liter) and streptomycin (200 mg/liter) and rates of influx and net flux of lysine and serine compared to unincubated controls. No difference was observed. Such incubation would not inactivate all possible microbial contaminants, but one would anticipate some effect on rates if microbial activity plays a substantial role in these observations. Failure to observe influx of glutamate in *Dendraster* also suggests that the animal is the principal agent; there is no reason to expect that glutamate would not be metabolized as well as other amino acid substrates by a contaminant microbial population.

The potential contribution of transepidermal transport to the animals can be estimated by comparing rates of influx to an estimate of reduced carbon required to support oxidative metabolism. An approximate conversion factor to equate oxygen consumption with complete oxidation of a mixture of amino acids (1 ml O_2 = 1 mg amino acid) and an average molecular weight for amino acids of 100 can be used. Then, the average oxygen consumption of 4.3 μl $O_2/(hr \cdot cm^2)$ is equivalent to 43 nmoles amino acid/$(hr \cdot cm^2)$. The average influx of naturally occurring amines from interstitial water (Table I) is 24.9 nmoles/$(hr \cdot cm^2)$, a contribution of 58% of the material required to support oxygen consumption. This estimate is probably based on an overly conservative figure for the level of naturally occurring amines in the sediment. Only two of the fifteen cores analyzed from the habitat showed less than 50 μmoles primary amines (17,36 μmoles) in the 0–3 cm zone of the sediment. The average was 115 μM. Since the K_t for influx of gly was measured as 74 μM (Fig. 3), the presence of levels of primary amines in interstital water greater than the 20 μM used for this estimate would certainly lead to greater influx rates and an increased contribution to carbon requirements. In fact, it can be concluded that if the surface of *Dendraster* is exposed to levels of primary amines measured in 14 or our 15 samples ($\geq$ 35 μM), influx is sufficient to account for oxygen consumption.

This discussion assumes that the bulk concentration of primary amines measured in the interstitial water of the sediment is a measure of concentrations available at the surface of the animal. Stephens (1975) has reported increased primary amines in interstitial water as a result of irrigation by the annelid infauna. This may also be true for *Dendraster*. Alternatively, renewal of primary amines at the surface may be dependent on bulk flow of interstitial water and diffusion. Until this ques-

tion can be investigated, it should be re-emphasized that the conclusions of the preceding paragraph should be phrased conditionally.

Timko (1976) describes suspension feeding in *Dendraster*. When behaving in this fashion, about one-third of the anterior portion of the test is embedded in the sediment substrate. Clearly, only a portion of the test would be in contact with interstitial water of the sediment in this feeding mode. *Dendraster* also behaves as a prone deposit feeder according to Timko and other authors. In this feeding mode, the animal is often below the sediment surface and is fully exposed to interstitial water. Animals in both the inclined suspension feeding and prone deposit feeding orientation were observed in the shallow water population at Point Mugu Naval Base.

Timko (1976) concludes that *Dendraster excentricus* is primarily a suspension feeder. However, Chia (1969) reports that all the individuals in a population from Puget Sound, Washington, were completely buried at low tide. We suggest that *Dendraster* can supplement both suspension feeding and deposit feeding by influx of amino acids into the epidermis. This supplement would be small when the animals are behaving as inclined suspension feeders but would be large during deposit feeding. In our experiments, transepidermal influx of amino acids would support energy metabolism at ambient levels of primary amines greater than 35 μM; our measurements indicate these are realistic levels for prone deposit feeding animals buried in the superficial layers of the sediment.

Our data suggest that animals might indeed survive without taking in and digesting food, provided *Dendraster* has pathways for translocating nutrients from the epidermis to deeper tissues. However, it is more likely that transepidermal uptake of small organic compounds may contribute to the sustenance of the epidermis. If there is a barrier to translocation of nutrients in *Dendraster* comparable to that reported for other asteroids and echinoids, direct uptake of nutrients from the environment may play a large role in the nutrition of pedicellariae, spicules, podia and other epidermal structures. Our data suggest that the oxidative requirements of the epidermis represent a large fraction of the total requirements of the animal. However, levels of ambient primary amines (> 35 μM) adequate to support total oxidative metabolism are *a fortiori* adequate for the epidermal fraction thereof.

Summary

1. Influx of amino acids from dilute solution into the sand dollar, *Dendraster*, was measured by following the disappearance of radioactivity in the medium supplying known labeled substrates. Net flux was monitored simultaneously by following the decrease in primary amines in the medium fluorometrically. Rates of influx and net flux correspond closely at ambient concentrations greater than 5 μM.

2. *Dendraster* is capable of net accumulation of some of the primary amines normally found in the interstitial water of its sediment habitat.

3. A sensitive method for location of amino acids on thin layer chromatograms is described. Comparison of interstitial water before and after exposure to *Dendraster* shows a changed pattern of amino acids, as well as a decrease in total amino acids, which is consistent with measurements of rates of influx with single substrates.

4. Comparison of rates of influx of naturally occurring primary amines with the metabolic requirements of animals as estimated from their oxygen consumption indicates that *Dendraster* can acquire sufficient reduced carbon to account for its oxidative needs if its surface is exposed to naturally occurring primary amines at concentrations greater than or equal to 35 μM.

5. Primary amines in the interstitial water of sediment in the immediate vicinity of a shallow water population of *Dendraster* range in concentration from 17 to 244 μM ($115 \pm 60\ \mu$M).

6. *Dendraster* lives in an environment which is relatively rich in amino acids, and it possesses a transport system which can accumulate these compounds at rates sufficient to provide a significant supplement to other forms of feeding. These findings support the hypothesis that sustenance of epidermal structures of echinoids and asteroids may be relatively independent of translocation of nutrients from the digestive organs and may be based primarily on transepidermal influx of nutrients from the medium.

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OCCURRENCE AND GROUP ORGANIZATION OF ATLANTIC BOTTLENOSE PORPOISES (*TURSIOPS TRUNCATUS*) IN AN ARGENTINE BAY

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While the social behavior of many terrestrial mammals has been well described (see Wilson, 1975, pages 456–546, for a review), much less is known about the social organization of the several species of porpoises that inhabit all oceans of the world (Norris and Dohl, 1978a, provide a review). This lack of information results from the difficulty of remaining with a group of porpoises in the open ocean long enough to observe the details of porpoise behavior, and from the interference with the animals' behavior that a boat causes.

There are places where porpoises come close enough to shore to make observations from land feasible (Mitchell, 1975). Saayman, Bower, and Tayler (1972) described the activity cycles and movements of Indian Ocean bottlenose porpoises and Indopacific humpback porpoises by observations from South African cliffs; and Norris and Dohl (Norris, 1974; Norris and Dohl, 1978b) made similar observations on Hawaiian spinner porpoises from shore vantage points. In the present study, Atlantic bottlenose porpoises were observed from a 45-meter cliff located on the coast of south-Argentina. The purpose of this paper is to describe the seasonal pattern of occurrence, group stability, surfacing associations, and calving seasonality of these animals. These data represent a first step in understanding the social behavior of the bottlenose porpoise. Some information on the group stability of this porpoise population and the photographic technique used to gather these data has been presented elsewhere (Würsig and Würsig, 1977). The present paper is a more complete treatment of this material.

Materials and Methods

During a 21-month stay, from July 1974 through March 1976, at Golfo San José (42° 23′ S, 64° 03′ W), bottlenose porpoises, *Tursiops truncatus,* were observed as they periodically passed within one kilometer of a shore observation point (camp). To investigate the group composition and stability of this population, porpoises were identified by photographing the natural markings on the trailing edges of their dorsal fins (see Würsig and Würsig, 1977). Observations lasted from ten minutes to several hours, depending on the length of time that the porpoises stayed near shore. It was assumed that all porpoises were photographed when each animal was identified at least four times within the record of one photographic observation session.

In the present paper, *group* refers to 53 individually identified bottlenose porpoises which passed through the study area during a 21-month period. This group

is part of a larger population of unknown size. *Subgroup* refers to those animals of the group which passed by shore at any one time.

To assess surfacing associations of animals, a motordrive Nikon camera was used. This provided data not only on which individuals were present, but also on their dive times and on which individuals surfaced together. To collect this information, a photograph was taken each time one or more animals surfaced (up to 1.5 frames/sec could be taken). The camera clicks were recorded on magnetic tape, and comparison of times between photographs and the individuals recognized in those photographs provided individual dive times and a measure of whether any animals tended to surface at nearly the same time. The technique can be profitably used when the animals are close enough to the camera to allow for recognition of all individuals as they surface. The use of a 35 mm motordrive camera provided large negatives with the detail necessary for recognizing individual animals; yet the rate of picture taking was sufficient to photograph all animals as they surfaced. A ciné-camera technique for determining group size, deployment, and speed (but not recognition of individuals) was described by Tayler and Saayman (1972a).

Seasonal occurrence patterns were analyzed using analysis of variance (Sokal

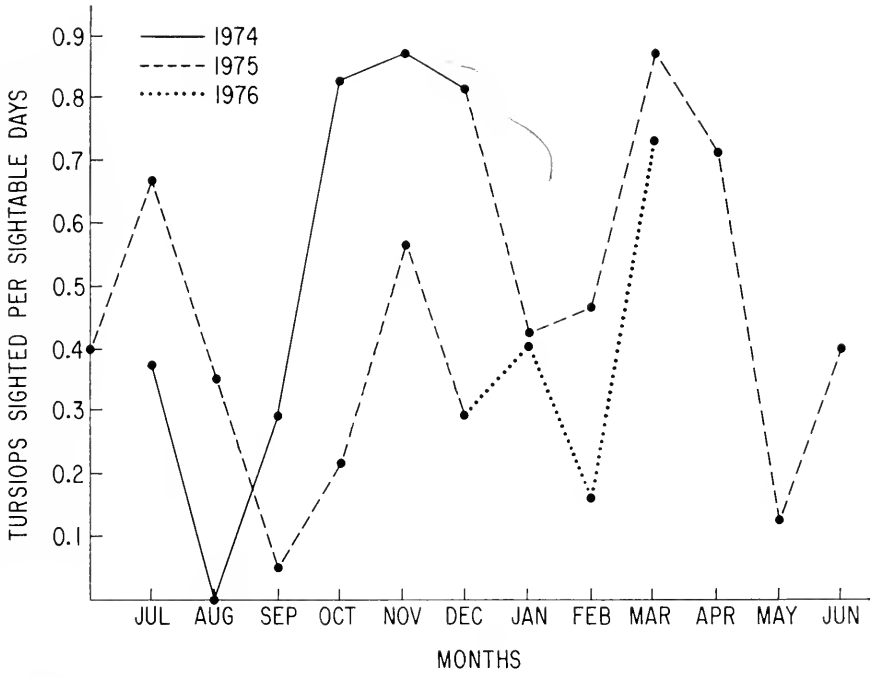

FIGURE 1. The fraction of the possible days each month on which bottlenose porpoises were sighted. The Y-axis represents the ratio of the number of days on which porpoises were sighted divided by the number of days each month with winds less than 20 km/hr. July, November, and March were months of maximum porpoise sightings; in September, February, and May they were sighted significantly less ($P < 0.001$, analysis of variance, Sokal and Rohlf, 1969, pages 204–249, and Rohlf and Sokal, 1969, pages 168–197).

and Rohlf, 1969; Rohlf and Sokal, 1969), surfacing associations were tested with a sampled randomization test (Sokal and Rohlf, 1969), and significance of calving seasonality was obtained with the Raleigh test, using the procedure described by Greenwood and Durand (1955).

Results

Seasonal occurrence pattern

On days with winds greater than 20 km/hr it was difficult to see or photograph porpoises. Of the 433 days with winds less than 20 km/hr, bottlenose porpoises were seen on 191 days, or 44% of the days on which observations were made. The number of days on which porpoises were sighted varied greatly from month to month. As Figure 1 shows, porpoises were seen near shore during 20 of the 21 months studied; August, 1974, was the only month without sightings. But the number of days on which they were sighted varied greatly from month to month: there was a peak of abundance about every four months, and this pattern was similar for the two years.

Subgroup composition and stability

Bottlenose porpoise subgroups were photographed on approximately 150 of the 191 sighting days. There are 35 days on which all individuals in a subgroup were photographed at least four times. Figure 3 shows which of the 53 known individuals were present on each of these 35 days. Only one subgroup was sighted during any one day. The number of individuals in a subgroup varied from eight to 22, with a mean of 14.9 (s.d. = 3.28, see Figure 2).

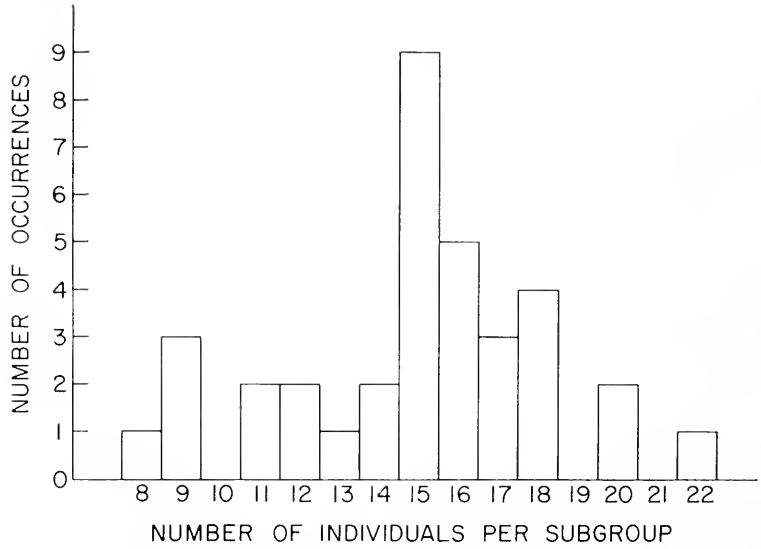

Figure 2. A histogram of subgroup sizes during 35 days on which all individuals present were recognized.

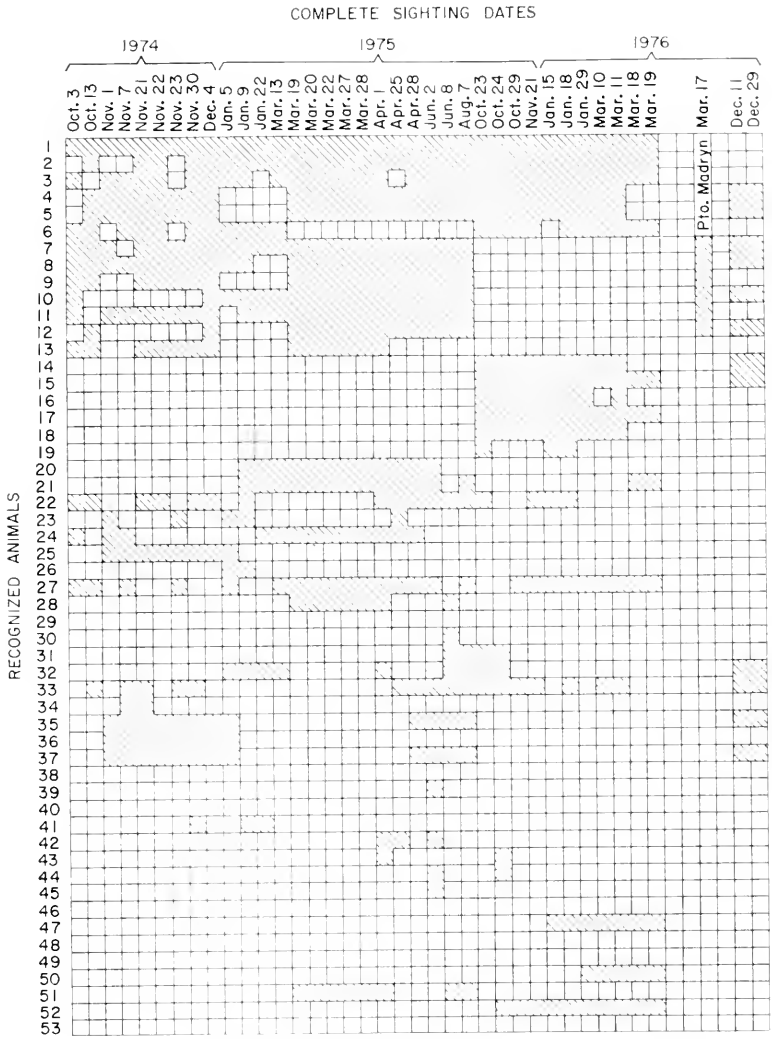

FIGURE 3. Specimens of *Tursiops* recognized during a 21-month continuous study period. Shaded blocks represent the presence of individuals during 35 complete sighting days (see text). The last three shaded columns on the right represent additional sightings of animals first at 300 kilometers removed from camp, and, secondly and thirdly, animals found near camp December, 1976.

As Figure 3 makes clear, this variation in subgroup size was the result of a continual flux of animals leaving and joining the subgroups. Nevertheless, some animals, namely #1–5, may be termed "core" animals since they were present throughout the study period. Animals #7–12 were present during the first ten months but subsequently disappeared during the month when animals #14–18 first appeared. In addition to these major changes in subgroup composition, other

individuals appeared and disappeared together. For example, #2, #9, #13, and #22 disappeared between October 13, 1974, and November 21, 1974, while #24 appeared during their absence. At that time as well, four others (#25 and its calf-#36, #35, and #37) appeared and stayed until January 5, 1975. From January 5, 1975, to March 19, 1975, five animals (#4 and its calf-#5, #9, #12, and #13) disappeared while once again an individual (#32) appeared in their absence. When these five reappeared and #32 disappeared March 19, two new animals appeared (#28 and #51). Porpoises #51, #28, and #13 disappeared again by April 25, 1975. Porpoise #6, which was absent from March 19 to October 23, 1975, reappeared with the large shift in individuals first documented on that date. A monthly summary of the presence and absence of 22 of the animals described above is shown in Figure 4. The appearance and disappearance of other animals was apparently not related to the presence or absence of conspecifics.

Porpoises #7–12, which disappeared from camp in September 1975, were spotted in March, 1976, over 300 kilometers from the study site. On December 11 and 29, 1976, after nine months of no observations, a spot-check of porpoises near

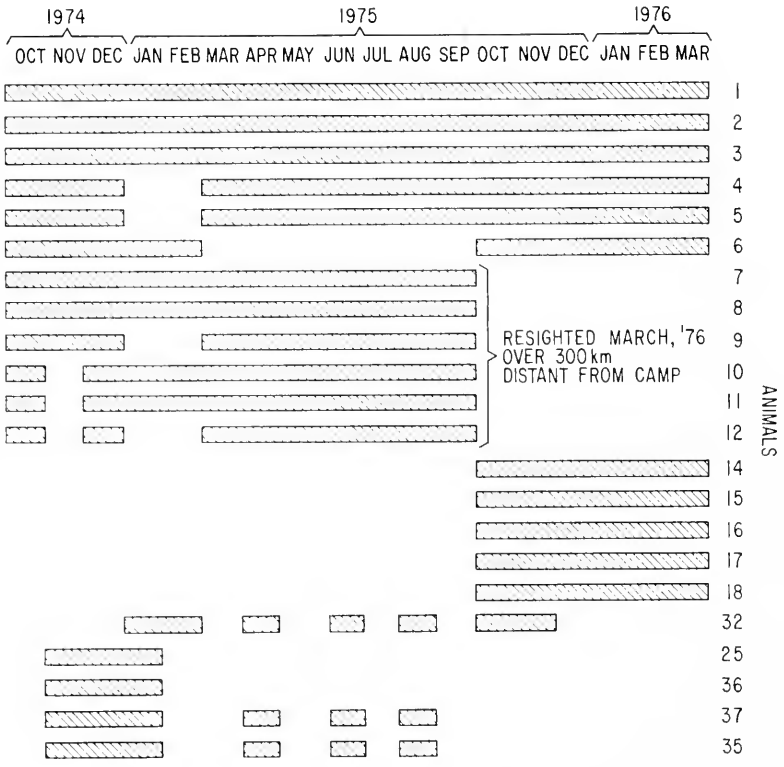

FIGURE 4. A month-by-month summary of the presence of 22 individuals which show interpersonal relationships in presence or absence within a subgroup. Differences in the summary and Figure 3 are due to consideration here of all sighting days, while Figure 3 represents only the 35 complete sighting days when all individuals were recognized.

TABLE I

Pairs of porpoises surfacing within three seconds of each other on two days in January, 1976 [(ns) = not surfacing significantly together; probability of random occurrence, $P < 0.025$, or $P < 0.001$**, sampled randomization test].*

January 18, 1976	January 29, 1976
#4–#5**	#4–#5**
#3–#52*	#3–#52(ns)
#2–#6*	(only #6 present)
#2–#52*	(only #52 present)
#27–#6*	#27–#6 (ns)
#6–#18*	#6–#18(ns)
#4–#27 (ns)	#4–#27*
(only #52 present)	#52–#50**

camp showed that four of these six animals were again in the original study area. At that time, 12 of the 53 known animals were present but no new animals were recorded. Interestingly, of the core unit of five animals, only #4 and #5 were present, suggesting that even the "core" may not be stable over a larger time period.

Surfacing associations and dive times

To determine whether there is any association between animals breathing together which might indicate a social bond, the surfacing pattern of porpoises was examined for two days in January, 1976. As Table I shows, while adult-adult surfacing associations occurred, they were not the same associations during the two sampled days, and they were never of greater significance than at the $P < 0.025$ level (sampled randomization test, Sokal and Rohlf, 1969, p. 633). Adult-calf associations, however, were observed for #4 and its calf (#5) on both days, and were highly significant ($P < 0.001$). Porpoise #52 and its calf (#50) were present on only one of the two sampled days (January 29, 1976), and their association at that time was also highly significant ($P < 0.001$). Except for adult-calf surfacing associations there was no detectable stable relationship in animals surfacing together (Table I) and animals appearing together in subgroups on different days (Fig. 3).

Because all individuals were recognized as they surfaced during the two sampled sessions, dive time data were accumulated as well. Individuals dove for a mean of 21.8 seconds, with little fluctuation from this mean (s.d. = 3.01). The small calf present on January 29, 1976, surfaced slightly more frequently than the overall mean (#50 = 17.8 sec/dive) and slightly more frequently than its presumed mother (#52 = 21.3 sec/dive).

Calving seasonality

Five calves were observed throughout the study, and two were reported after termination of the study (Table II). The mean number of calves per subgroup was 1.5 for the 35 sightings of completely-known groupings to pass by camp. On the average, 10% of a subgroup was composed of calves. Calves were born during

TABLE II

The estimated birthdates of seven calves, arranged by month. The two un-numbered calves were reported first seen in the months shown, after termination of the 21-month study. Note the absence of births May through October.

Months	Estimated dates of calf births
January	#21 (1975); #50 (1976)
February	none
March	#53 (1976); no number (1977)
April	#5 (1974); no number (1977)
May	none
June	none
July	none
August	none
September	none
October	none
November	#36 (1974)
December	none

spring, summer, and fall, with no births during winter (June-September). The births of seven calves during the six month period from November through April (see Table II) are nonrandomly clumped toward those months ($P < 0.02$, Raleigh test, Greenwood and Durand, 1955). There was no evidence of births for May through October.

DISCUSSION

Recognized individuals of bottlenose porpoises were found in the study area throughout the year and thus did not migrate with the changing seasons. True (1891) reported that specimens of *Tursiops* off Cape Hatteras, North Carolina, move toward the north in the spring and return south in the fall (see also Mead, 1975). Gunter (1942), however, reports that specimens of *Tursiops* in Texas waters show no seasonal migration; while Caldwell and Caldwell (1972) and Odell (1975) suggest a possible seasonally-related movement of this species off the southern tip of Florida. Apparently some bottlenose porpoise populations migrate and others, at least at times, do not. It is likely that the porpoises go where they can find food, as has been indicated for other species (Evans, 1971; Norris and Dohl, 1978a).

Although there was no evidence for seasonal migration in the Argentine study area, there was a four months cycle in the number of times that porpoises were sighted. Lows occurred in August-September, January-February, and May-June; and highs in July, November, and March. The near-shore surface water temperature in the study area varies from a July-August low of approximately 10.5° C to a January-March high of 17 to 18° C (personal observation). Since lows and highs of bottlenose porpoise presence occurred during both the low and high temperature periods of the year, as well as during intermediate water temperatures, occurrence pattern of this population does not appear temperature-dependent. In other areas of the world, this species is also found over a wide water temperature

range, from approximately 8° C to 30° C (Van Bree, University of Amsterdam, personal communication).

Subgroups which were seen from shore during the 21-month study varied in size and in composition of individuals from sighting to sighting. Nevertheless, five animals of a recognized group of 53 animals were consistently present when a subgroup was sighted. These five individuals were composed of a large adult (#1), two smaller adults (#2 and #3), and an adult (#4) with its calf (#5). Judging by size, it is possible that the large adult, #1, was a male and the other adults were females. This kind of association has been described for bottlenose porpoises in captivity (Tavolga, 1966; Caldwell and Caldwell, 1972), as well as in the wild (Caldwell and Caldwell, 1972; Irvine, University of Florida, personal communication). In the present study this supposition rests only on relative size of individuals and not on known sex.

In addition to the four adults and one calf which were consistently present, six individuals (#7–12) were present until September, 1975; and five individuals (#14–18) were present from September, 1975, to the end of the study. These two stable groupings were composed of all adults, with no calves or juveniles present. Caldwell and Caldwell (1972) hypothesized that such units may be composed of nonbreeding population members. They may travel together as do, for example, bachelor herds of elephants (Douglas-Hamilton and Douglas-Hamilton, 1975), but for the present population this can only be taken as a suggestion, in need of further study.

Perhaps most interesting in the present study was the apparent fluidity with which many individuals appeared and disappeared, causing a constant fluctuation in subgroup size and composition. These individuals were composed of adults of varying sizes, and of calves and juveniles. A similar situation in group size fluctuation exists in the Hawaiian spinner porpoise, *Stenella longirostris* (Norris and Dohl, 1978b). A possibly similar system in Florida bottlenose porpoises has been observed recently by Wells and Irvine (University of Florida, personal communication) and may be found to be quite common in coastal porpoise species as further population studies are made.

Such a fluidity in stucture surpasses the individual interchanges between known "open" groups of most terrestrial mammals (Wilson, 1975, pages 456–546). To conform to the standard notion of groups as relatively stable units, the 53 known individuals of the present population have been labeled *group*, while the units that periodically came by shore, consisting of 8 to 22 animals, have been termed *subgroups* (Würsig and Würsig, 1977). These flexible subgroups appear similar to the casual units found in chimpanzee (*Pan troglodytes*) society (Goodall, 1965; Reynolds and Reynolds, 1965; Hall, 1968; Nishida, 1968; Sugiyama, 1968). The possibility of similarity in group organization between *Tursiops* and *Pan* was first suggested by Tayler and Saayman (1972b). In chimpanzee society, it appears that this constant fluctuation in subgroup size is in direct response to irregular and patchy food availability, with small units when food is being sought and larger units in areas of greater food abundance (Reynolds, 1965). It is suggested that food availability may also be a primary determinant of subgroup size and stability in the present bottlenose porpoise population.

Because this study relied on sightings at a discrete point along the shoreline, little information about the group's total range was gathered. At least at times some of the animals travelled unexpectedly long distances, however. Six individuals were identified in a bay south of Golfo San José, separated from camp by over 300 kilometers, and nine months later four of these animals were again found near camp. Either the normal range of this population extends over so extensive a distance, or the individuals so observed had crossed into the new area. Similar distances travelled have been reported for pelagic porpoises (Perrin, 1975; Evans, 1974), but not for nonmigrating near-shore species.

Although some adult porpoises showed a tendency to surface together, this is not a long-term relationship and may shift from day to day. Just as terrestrial animals which have formed close social bonds do not in general exhibit synchronized breathing, porpoises not surfacing together may still be closely associated. However, porpoises, unlike terrestrial animals, must leave their underwater positions while surfacing to take a breath. As a result, very close animal associations may be reflected in the breathing-surfacing pattern. This appears to be the case in adult-calf associations. Porpoise #4 and its calf (#5) and #52 and its calf (#50) often surfaced together. In the #4-calf association, this relationship was still strong in December, 1976, approximately two and one-half years after #4's calf was born. Nevertheless, such association is not absolute. As the increased surfacing rate of #50 (17.8 sec/dive) over that of its presumed mother (#52 = 21.3 sec/dive) shows, the calf at times surfaced independently of the adult. When it did so, it was almost always seen moving ahead of the subgroup of animals, an apparently investigative or "play" behavior summarized for other species by Norris and Dohl (1978a). While Caldwell and Caldwell (1972) reported the same type of nonassociation to be present at times in captive porpoises, Irvine (University of Florida, personal communication) believes that it does not occur in a bottlenose porpoise population in the Sarasota-Bradenton area of central west Florida. A possible explanation of this difference in behavior may be the relatively undisturbed state of porpoises in the present study, unlike Florida populations which have been harassed by capture vessels and tourist boats. Thus, Florida porpoises may keep their young within the confines of the school during periods of possible danger such as the approach of a boat, while no such restriction appeared to apply to porpoises in Golfo San José. Instead, calves and subadults at times briefly left the side of the adult with which they normally surfaced and approached the investigator's boat without apparent caution.

Since bottlenose porpoises have an approximately 12-month gestation period (Sergeant, Caldwell, and Caldwell, 1973), the marked summer calving season within the present population also indicates an increase in mating activity at that time. Various investigators (Mead, 1975; Odell, 1975; Sergeant, Caldwell, and Caldwell, 1973; Evans, Navy Underwater Center, San Diego, personal communication, for *Delphinus delphis;* Nishiwaki, Nakajima, and Kamiya, 1965, and Harrison, Brownell, and Boice, 1972, for *Stenella attenuata*) have reported a tendency toward bimodal calving, with peaks in spring and fall. Ridgway and Green (1967) found anatomical evidence for mating peaks in spring and fall by an increase in testes weights of male *Delphinus delphis* and *Lagenorhynchus obliquidens* during these

two seasons. The present population may exhibit a similar mating and calving trend, with one calf first observed in November, and six others first seen from January through April. Why a bimodal calving peak appears to be present in various different species of toothed cetaceans is not known. In the present study, the late summer calving preference coincides with the highest water temperatures of the year. This higher ambient temperature, as in most terrestrial mammals and in pinnipeds and baleen whales, may be of physiological advantage to the newly-born young.

The present study demonstrates that by systematically photographing small groupings of coastal porpoises much can be learned about their organization and dynamics. This represents one of the first times that such an attempt has been reported (see also Würsig and Würsig, 1977), and it is hoped that more such studies, on different odontocete cetacean populations and on different species, will soon be made. In this manner, by observing coastal porpoises for long periods of time, long-overdue descriptions of natural populations—analogous to the recent flowering of primate research—may take place.

Dr. Charles Walcott provided encouragement and advice throughout the study, and Dr. Roger Payne supported all phases of the field work. Peter Tyack, Martin Hyatt, Russ Charif, Christopher Clark, Jane Moon, Hugo Callejas, and Carlos Garcia assisted with the gathering of field data. Mary Griswold Smith of the National Geographic Society arranged for generous help with 35 mm film, and gave advice on how best to utilize it. Jan Wolitzky and Steven Ferraro provided invaluable assistance with computer analyses, and Dr. Charles Walcott critically read the manuscript. This study was supported by contributions and facilities from the New York Zoological Society, by the Program for Neurobiology and Behavior of the State University of New York at Stony Brook, and by grants to R. Payne and C. Walcott from the Committee for Research and Exploration of the National Geographic Society. Melany Würsig assisted in all phases of the study.

Summary

1. During a 21-month study, individuals of *Tursiops truncatus* in Golfo San José, Argentina, exhibited a four month occurrence cycle, but showed no seasonal migration.

2. Subgroups numbering 8 to 22 animals included a small core unit of individuals which were consistently found together. Other animals appeared and disappeared in these subgroups on different days in a highly fluid manner which paralleled the open society of African chimpanzees, *Pan troglodytes*.

3. Some adults showed weak and changing surfacing associations with other adults. Calves consistently surfaced together with a particular adult, except during apparent play or investigative behavior, when calves left adults for brief periods. The mean dive time per animal was 21.8 seconds.

4. Six of seven calves were born in late summer. This calving peak coincided with the highest water temperatures of the year.

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THE
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RHEOTAXIS AND CHEMORECEPTION IN THE FRESHWATER SNAIL *BIOMPHALARIA GLABRATA* (SAY): ESTIMATION OF THE MOLECULAR WEIGHTS OF ACTIVE FACTORS

Reference: *Biol Bull.*, **154**: 361–373. (June, 1978)

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For many aquatic organisms chemoreception plays an important, often decisive role in the selection and location of diet items (for reviews see Kohn, 1961; Lenhoff and Lindstedt, 1974; Bardach, 1975). In many instances it has proved possible, using a suitable behavioral or physiological assay, to identify some of the chemicals which elicit these responses (see for example Carr, 1967; Carr and Chaney, 1976; Gurin and Carr, 1971; Hara, 1976, 1977; Lenhoff, 1968, 1969; Suzuki and Tucker, 1971; Pawson, 1977). Indeed, from a study of the relationships between chemical structure and biological activity Hara (1976, 1977), working with the rainbow trout, *Salmo gairdneri*, and Lenhoff (1968, 1969), with the marine hydra, *Hydra littoralis*, have been able to describe in some detail the physico-chemical properties of the receptor sites themselves. The long term object of the present investigation has been to arrive at a similar level of understanding of the chemosensory mechanisms employed by *Biomphalaria glabrata* (Say) (Planorbidae, Mollusca), a phytophagous freshwater snail found in South America, and parts of the Caribbean.

The need for a detailed study of chemoreception in this organism is of particular importance, for two reasons. First, while freshwater plants are known to be an important factor conditioning the habitats of many species of freshwater snails (Gaevskaya, 1969; Bovbjerg, 1965; Pimentel and White, 1959; Pip and Stewart, 1976) little is known about the chemosensory basis of such interactions. *Biomphalaria glabrata* is particularly suitable for such a study for it has been shown to orient both chemotactically (Etges, 1963a, b; Michelson, 1960; Townsend, 1973a, b, 1974) and rheotactically (Etges and Frick, 1966) to dilute solutions of various plant extracts. Secondly, many species of freshwater snails are of considerable economic and medical importance as intermediate hosts of digenetic trematodes parasitic in man or domestic animals. *Biomphalaria glabrata*, for example, is host to the human schistosome, *Schistosoma mansoni* (Sambon). Attempts to control the disease usually rely on the use of molluscicides to remove or reduce snail populations in areas where the risk of transmission is particularly

high (Webbe and Jordan, 1966; Farooq, 1973). There are, however, numerous problems associated with the use of conventional molluscicides, among these being their high cost (Berg, 1973; Ritchie, 1973) and ecological side effects (Shiff and Garnett, 1961; Ritchie, 1973). A detailed knowledge of the role played by chemoreception in determining the distribution of snails within habitats may lead to more efficient and acceptable methods of control. For example, if baits could be formulated which could lure the snails to sites where slow release molluscicides were present, a saving in both labor and cost might be achieved (Etges, 1963a, b; Cardarelli, 1977).

In this paper a preliminary study of the molecular weight characteristics of chemicals eliciting rheotaxis is described.

Materials and Methods

Methods

Reproductively mature specimens of a Venezuelan albino strain of *Biomphalaria glabrata*, weighing 400 ± 30 mg ($\bar{x} \pm$ s.d), were selected from laboratory cultures maintained in the manner described by Thomas (1973). These individuals were then kept in water (see below) at densities of 20/liter in small plastic buckets, maintained at 26° C, under a constant (12L : 12D) regime. Each container was thoroughly cleaned out each day and fresh water (ionic composition $KHCO_3$—0.037 mM; KNO_3—0.0495 mM; $NaHCO_3$—0.634 mM; $MgSO_4$—0.13 mM; $CaCl_2$—2 mM; preaerated to pH 7.8–8.2) and food (0.1 g fresh lettuce per snail per day) provided. Each snail was used in no more than one trial per day, and was conditioned in clean water for one hour before the test.

Test apparatus and assay procedure

The test arena consisted of a shallow perspex trough ($24 \times 24 \times 3$ cm) into which a translucent polythene cylinder (23 cm diameter $\times$ 3 cm deep) was fitted. The substrate was formed by a glass plate which could be removed, and thoroughly cleaned before each trial. Snails placed at the center of this plate were thus free to move within a cylindrically symmetric environment.

To create the flow, two jets of compressed air were directed in opposite directions around the edges of the container from a single T-tube held close to and parallel with the surface. The net effect was to draw a stream of water approximately 12–15 cm wide and with surface speed 0.75–1.5 cm/sec across the center from the opposite side of the arena. Flow was returned via the sides.

Only snails which were actively moving at the bottom of their buckets at the time of their test were used in this study. This was done in order to standardize, as far as was possible, the initial behavioral state of the tested individuals. Each snail was removed gently from its container and then held just above the center of the glass plate floor until the foot extended and adhered to it. Subsequent movement of the snails was plotted on tracing paper on the screen of a video monitor. This was connected to a camera observing the movement of the snail from beneath the arena.

All tests were conducted under conditions of diffuse illumination from above. In order to counteract any residual directional bias due either to phototaxis (Sodeman, 1973; Sodeman and Dowda, 1974) or starting configuration (shell orientation/flow direction) snails were always started with the shell parallel to a fixed axis, while the direction of the current was rotated, periodically, through 90°

Stimuli

Lettuce was obtained from local market gardens. Care was taken to ensure that no plants had been treated with pesticides in the interval two weeks before use. Commercially available wheatgerm (Jordan's Natural Wheatgerm, Holme Mills, Biggleswade, Bedfordshire) was used. All plant extracts were made on the eve of an experiment using a standard technique.

In the case of lettuce, 20 g (wet weight) of the outer leaves were homogenized for one minute in a laboratory blender in 50 ml of distilled water chilled to 50° C. This was filtered through a Whatman No. 1 paper, made up to 100 ml with more distilled water and centrifuged at 10,000 rpm for 30 minutes. The supernatant was then filtered through Whatman glass fiber papers GF/C and GF/F (nominal cut-off 0.7 μm) and stored at 5° C until the following day.

Wheatgerm extract was made using the same technique, but here the working strength was 1 g (dry weight)/100 ml, and the first filtering stage was omitted.

Ultrafiltrates were prepared using Amicon Diaflo® ultrafiltration membranes UM–05, UM–2, UM–10, PM–30, and XM–100A. According to Amicon Corporation (1974) these membranes retain microsolutes of molecular weights (mol wt) greater than 500, 1000, 10,000, 30,000, and 100,000, respectively. Although retention is actually a function of molecular size, configuration and charge, a nominal retention characteristic curve can be drawn for each membrane, and these are shown in Figure 1.

Ultrafiltration was carried out at 5° C under nitrogen (20–40 psi) using a 100 ml stirred cell. All membranes were flushed *in situ* with 200 ml distilled water before use. One hundred ml of extract was prepared as described above and passed through the cell overnight.

Stimulus solutions were made up immediately before use as standard extract dilutions of 750 ml of water preheated to 26° C. This filled the arena to a depth of approximately 1.5 cm. As soon as the flow pattern had stabilized, the test was begun. Controls were run in water to which no extract had been added. In order to prevent contamination, stimuli were used for only one test and then discarded, the apparatus being thoroughly rinsed between tests to remove any residual traces of chemical.

Statistics

All trails representative of a particular treatment were superimposed on tracing paper so that their starting positions coincided, and the flow directions were parallel. The individual trails were then scored in the following manner.

The overall trial direction was defined as the angle made between the flow direction and a vector joining the starting point and the place where the trial first cut an 8 cm radius boundary centered on the middle of the arena. These

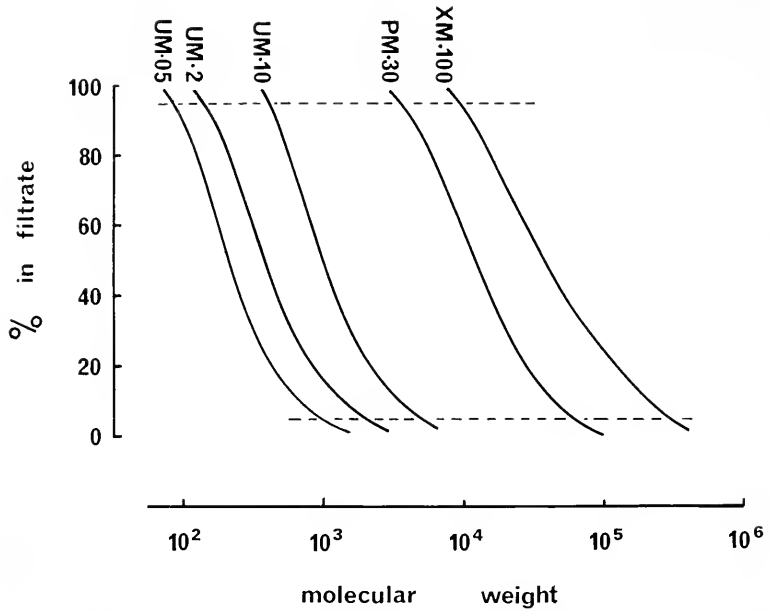

FIGURE 1. Molecular weight transmission charateristics of five Amico Diaflo® ultrafiltration membranes used in this paper. Data plotted in semi-logarithmic form. The ordinate shows the percentage of solute of given molecular weight transmitted by the membrane. Levels of the transmission (95% + 5%) are indicated by dotted lines. [*Replotted from Amicon Corporation publication* (1974, see Literature Cited).]

angles were measured to 50° accuracy using an anticlockwise convention and the flow direction as reference.

Test control data distributions were compared using the nonparametric tests for directional data developed and described by Watson (1962) and Mardia (1972). Overall estimates of the responses were obtained by treating each datum as a unit vector and calculating the resultant r (r, θ), where

$$\text{data set} = (\theta_i); \quad i = 1 \text{ to } n$$

$$r = \left[\left(\sum_{i=1}^{n} \sin \theta_i\right)^2 + \left(\sum_{i=1}^{n} \cos \theta_i\right)^2\right]^{\frac{1}{2}}/n$$

$$\theta = \tan^{-1}\left[\sum_{i=1}^{n} \sin \theta_i / \sum_{i=1}^{n} \cos \theta_i\right]$$

However, for the purpose of constructing graphs the scalar quantity r cos θ is more useful. This has a range of values from +1 to −1 and by convention has been taken as positive when the net movement is upstream (positive rheotaxis) and negative when the net movement is downstream (negative rheotaxis).

RESULTS

Typical data from a series of experiments in which lettuce extract (cultivar *Kenate*) was tested are shown in Figure 2. The data are shown in two forms.

To the top, the superimposed trails obtained for a given treatment are shown. Each trail is from a different snail, and in each case the movement was centrifugal, with current direction from 6 o'clock to 12 o'clock. It can be seen that lettuce extract tested at a concentration of 10 ml/liter produces a strong polarization in favor of upstream movement (positive rheotaxis), whereas the control snails exhibited very little directional bias. The difference between these two distributions was highly significant [$P < 0.001$, Watson's U^2 test, (Watson, 1962)]. Below these trails the same data are re-represented in the form of a circular histogram.

Figure 2 also shows what effect passing the extract through ultrafiltration membranes has on the activity of the solution. As can be seen the effect of membranes PM–30, UM–10, UM–2 and UM–05 was to produce a gradual reduction in the length of the resultant vector and an increase in scatter in the individual trail directions as the retention characteristic moved to progressively lower molecular weights. At this concentration (10 ml extract/liter) all the treatments produced positive responses which were significantly different from the controls [PM–30, UM–10, $P < 0.01$; UM–2, UM–05, $P < 0.05$; Watson's U^2 test (Watson, 1962)]. The activity of the UM–05 and UM–10 filtrates, however, was significantly less ($P < 0.01$ and 0.05, respectively) than that of the original extract.

The reason why membranes with such widely different characteristics merely produce a gradual, rather than an all-or-nothing effect on activity can be seen immediately from Figure 1. It is clear that there is a considerable overlap between the characteristics of the four membranes concerned. For any individual membrane the 5–95% transmission limits span a molecular weight range equivalent to at least one order of magnitude. Consequently, in order to be able to interpret the results of ultrafiltration, some method of calculating the attenuation produced by any given membrane must be found.

In Figure 3 the response magnitude-extract concentration profile for *Renate* lettuce has been plotted in semi-logarithmic form. Other forms of representation are possible, but this method was found to be the most successful for the purposes of linearizing the data (see Beidler, 1971). From the linear regression on these points, it is possible to calculate the concentration of lettuce extract which would produce a response equivalent in magnitude to the response produced by a given filtrate. For example, UM–10 filtrate tested at concentrations of 2 ml/liter produces a response which is equivalent in magnitude to that of lettuce extract tested at a concentration of only 0.4 ml/liter. Thus the UM–10 filtrate only contains 20% of the original activity. From Figure 1 it can be seen that the point of 20% transmission occurs for molecular weights of approximately 2000. The results of testing four different ultrafiltrates of *Renate* extract, each at two different concentrations, are shown in Figure 3 (see legend), and the molecular weight estimates obtained shown in Table IA. The range of this extract was 1000–10,000. However, since it has been shown elsewhere (Carr, Hall and Gurin, 1974) that stimulants from different sources may be characterized by different molecular weight spectrums, these results have been complemented with tests using a different cultivated variety of lettuce and with wheatgerm. Data from experiments involving lettuce (cultivar *Amanda*) are

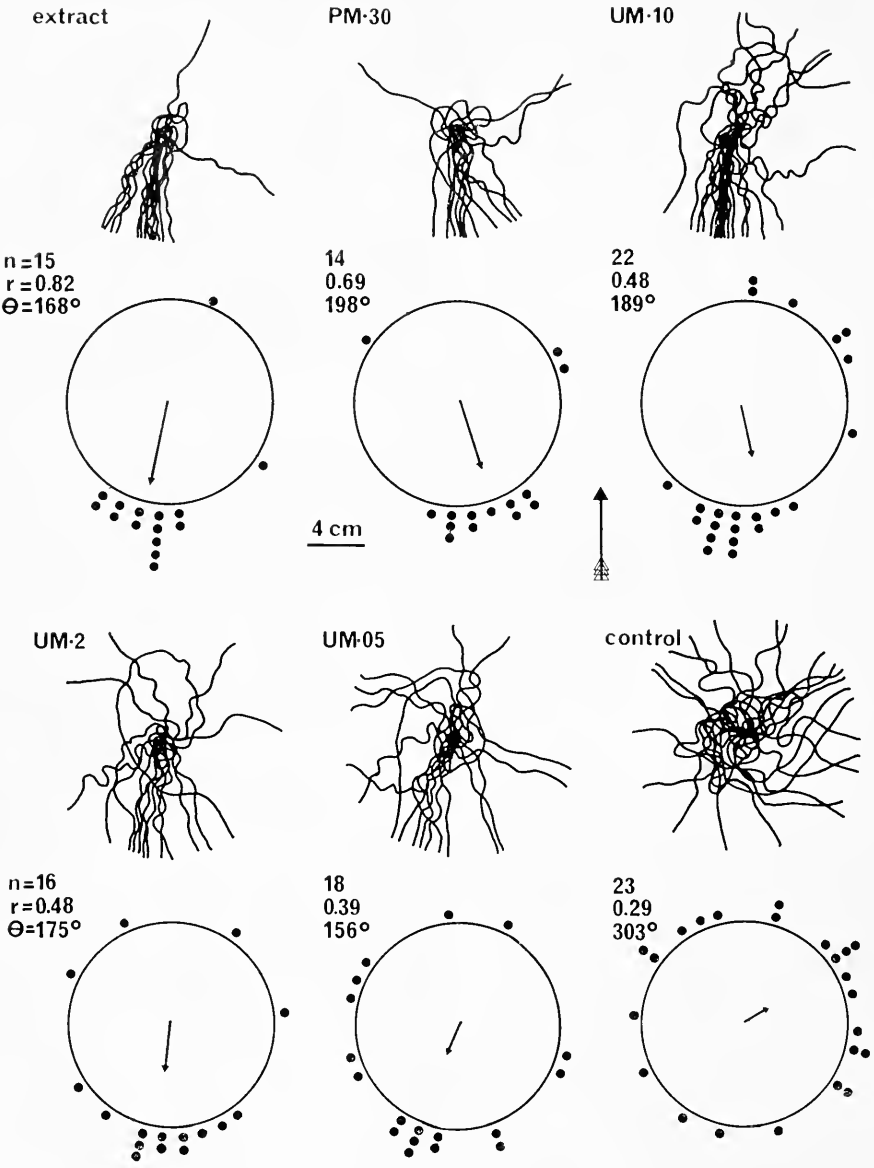

FIGURE 2. Trail data obtained using lettuce extract (cultivar *Renate*) and the effect produced by passing it through Diaflo® membranes. All stimuli were tested at concentrations of 10 ml extract or ultrafiltrate per liter. Control values were obtained using water alone. Each data set is shown in two forms. At the top the trails, starting at the center and radiating outward, of all the snails (n) tested in a given solution are shown superimposed. Below the same, data are represented in the form of a circular histogram. The vector in the center (r, θ) represents the magnitude of the resultant of the individual unit vectors obtained for each trial. A vector reaching the edge of the circle signifies a case in which all the snails

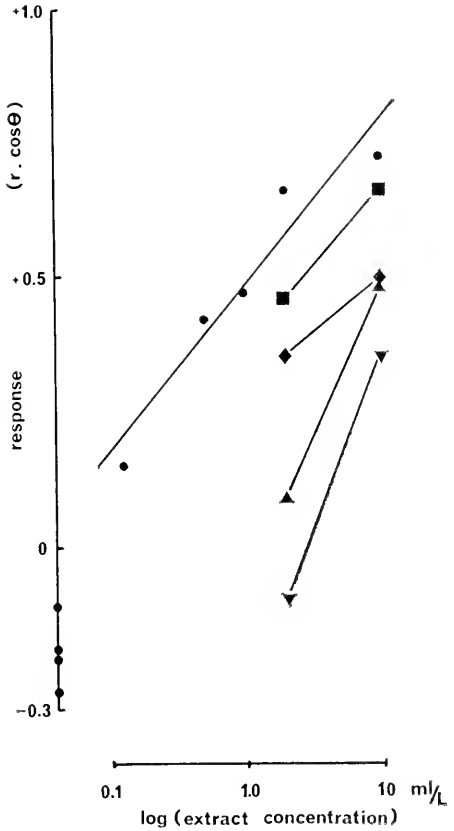

FIGURE 3. Response-concentration profile (circles) obtained for lettuce extract (cultivar *Renate*), together with the effects produced by various ultrafiltration treatments (other symbols). The projection of the resultant vector on the direction of flow (r cos θ) is used as an index of the response magnitude (see Methods). The regression equation for the lettuce extract data is r cos $\theta = 0.306$ log (concentration) $+0.48$. Each extract data point represents the resultant of 35–45 individual trials. Ultrafiltrate and control data points are averages of 15–25 trials. Squares represent PM–30 filtrate; diamonds, UM–10 filtrate; triangles, UM–2 filtrates; and inverted triangles, UM–50 filtrate. All filtrates were tested at concentrations of 2 ml and 10 ml/liter. Control values are shown on the ordinate.

also shown in Table IB. Exactly the same procedure was used here. The estimates arrived at using this extract agree well with those obtained using the *Renate* variety (Table IA). The range of values obtained was from 1000 to 6000. In contrast, however, estimates for the molecular weights of the attractants

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moved in the same direction. On the other hand, a vector of near zero length represents a case in which there was no bias and the direction of movement tended to be random. In each case the direction of flow is from 6 o'clock to 12 o'clock. Note the reduction in flow vector length and in the clustering of the trails which occurs as increasingly more retentive membranes are used.

TABLE I

The responses obtained for various ultrafiltrates of lettuce and wheatgerm extracts, together with estimates of the molecular weights of the attractants. The method of deriving the "estimated concentration" and "percentage of activity left" are explained in the text. Where the response to the filtrate was either very similar to that of the original extract, or was very small these values were not calculated, but a minimum or maximum molecular weight estimate is given. Asterisks denote values obtained using retentates. Note how these compare with those obtained using filtrates.

Stimulus	Concentration (ml/liter)	Response (r. cos θ)	Estimated conc. (ml/liter)	Per cent activity left	Estimated mol wt
(A) Lettuce (cultivar *Renate*)					
UM-05 filtrate	2	−0.10	—	—	>2000
UM-05 filtrate	10	0.35	0.40	4	1000
UM-2 filtrate	2	0.09	—	—	>3000
UM-2 filtrate	10	0.48	1.1	11	2000
UM-10 filtrate	2	0.35	0.40	20	2000
UM-10 filtrate	10	0.48	1.1	11	3000
PM-30 filtrate	2	0.46	0.80	40	10,000
PM-30 filtrate	10	0.66	4.0	40	10,000
(B) Lettuce (cultivar *Amanda*)					
UM-2 filtrate	3	−0.04	—	—	>3000
UM-2 filtrate	8	0.26	0.56	7	2000
UM-2 retentate	8	0.56	8.0	100	>3000*
UM-10 filtrate	3	0.11	0.16	5	6000
UM-10 filtrate	8	0.26	0.56	7	4000
UM-10 retentate	8	0.48	4.0	50	1000*
(C) Wheatgerm					
UM-2 filtrate	8	−0.12	—	—	>3000
UM-10 filtrate	8	0.29	0.1	1.25	>10,000
PM-30 filtrate	2	0.47	0.4	20	30,000
PM-30 filtrate	8	0.56	0.8	10	40,000
XM-100 filtrate	2	0.80	—	~100	~10,000
XM-100 filtrate	8	0.77	4	50	40,000

in wheatgerm were, on the whole, higher (mol wt 10,000–40,000) than those obtained using lettuce extract (Table IC).

DISCUSSION

Before proceeding with a discussion of the results, it is important to view critically the methods used in this study. Ultrafiltration has been used extensively in studies of chemoreception as a means of removing or selectively attenuating specific molecular weight fractions from solutions containing stimulants (Carr, Hall and Gurin, 1974; Carr and Gurin, 1975; Carr, 1976; Carr and Chaney,

1976). However, the "cut-off points" for each ultrafiltration membrane, in fact, span a considerable range of molecular weights (Fig. 1). Thus, even if the stimulant chemicals were all of the same molecular weight, a membrane whose "cut-off point" lay above that weight would not necessarily remove the biological activity completely. The situation is further complicated by the possibility that the "stimulant" may, in fact, be a group of chemicals all with different molecular weights.

While bearing in mind that the published characteristics of each membrane are at best nominal and depend on a number of factors such as molecular charge, configuration and the presence of other solutes, the first difficulty may be overcome by relating the activity of an ultrafiltrate to the stimulus concentration-response magnitude profile of the original extract. This provides an estimate of the alteration produced by the given membrane and consequently a value for the molecular weight of the attractant. The second difficulty, the possible presence of a range of active molecules with different retention characteristics, can be overcome, to some extent, by using a range of membranes with widely different ultrafiltration properties. Membranes whose 50% retention points lies above the mean molecular weight of the attractants will provide estimates of this mean which are too high. Conversely, membranes whose 50% retention points lie below this mean will produce estimates which are too low. In general this tendency for ultrafiltration membranes with high molecular weight cut-offs to give higher estimates than those with low cut-offs is borne out by the results shown in Table I, although the effect is particularly obvious only for the wheatgerm data.

The results of this preliminary study of the characteristics of stimulants triggering rheotaxis clearly demonstrate that the factors involved are not simple compounds, such as amino acids, short chain organic acids or small sugars, but are substances having molecular weights in excess of 1000. An exact value is, however, for reasons given above, difficult to determine. While the estimates obtained for two varieties of cultivated lettuce are in agreement and provide a value somewhat below 10,000, all the estimates made for wheatgerm lie on or above this limit (Table I). It is unlikely then that the response is specific to a single chemical compound as has been found to be the case in some marine coelenterates (Lenhoff and Lindstedt, 1974). On the contrary, these differences suggest that some generalized property of a class of macromolecules is the active stimulus. It is interesting to note in this context that differences in stimulant molecular weights have also been found for the shrimp *Palaemonetes pugio* when tested with extracts made from a variety of marine invertebrates (Carr and Gurin, 1975).

In the past studies of chemoreception and food-finding behavior in aquatic organisms have stressed the role played by low molecular weight nitrogenous compounds. For example, sensitivities to amines and amino acids have been demonstrated in a number of marine and freshwater fish (for example Carr, 1976; Carr and Chaney, 1976; Hara, 1976, 1977; Pawson, 1977; Suzuki and Tucker, 1971), in marine Crustacea (Fuzessery and Childress, 1975; Laverack, 1963; Mackie, 1973), marine molluscs (Carr, 1967; Crisp, 1967; Jahan-Parwar, 1975) and a freshwater planarian (Coward and Johannes, 1969). Although in many instances the activity of food extracts is well accounted for by the presence

of these substances (see for example Carr, 1967, 1976; Carr and Chaney, 1976; Mackie, 1973; Pawson, 1977), it is becoming increasingly clear that compounds of larger molecular weights play an important stimulatory role in certain cases (see, for example, Ash, McClure and Hirsch, 1973; Carr, Hall and Gurin, 1974; Carr and Gurin, 1975; Gurin and Carr, 1971). For instance, Carr and his co-workers have shown that for the marine prosobranch, *Nassarius obsoletus*, macromolecules with properties consistent with those of proteins and peptides are the main active factors in extracts eliciting exploratory feeding behavior. In the fresh water planarian, *Dugesia dorotocephala*, the factors which elicit feeding behavior have molecular weights of between 700 and 2000 (Ash *et al.*, 1973).

Aquatic macrophytes and algae (Fogg, 1971; Hellebust, 1974; Wetzel and Manny, 1972) release large quantities of organic carbon into the surrounding water. It has been suggested that these chemicals may attract aquatic snails and be, in part, responsible for certain plant-snail associations observed in the field (Pip and Stewart, 1976). Natural plant exudates may also be responsible for the positive rheotactic movements which have sometimes been observed in field mark-recapture experiments performed with *B. glabrata* (Paulini, 1963; Pimentel and Ildefonson, 1957; Radke and Ritchie, 1961). They are certainly not simply responses to the presence of the currents themselves (Etges and Frick, 1966).

The majority of the material secreted by plants is made up of low molecular weight compounds such as glucose and glycollic acid (Hellebust, 1974; Wetzel and Manny, 1972) but polysaccharides, polypeptides and glycoproteins are also released (Fogg, 1971; Hellebust, 1974). These simpler compounds are, however, often photolabile and may be rapidly utilized by epiphytic organisms (Allen, 1976; Sepers, 1977; Wetzel and Manny, 1972). Since the rheotaxic response described here allows *Biomphalaria glabrata* to orient to distant sources of organic chemical, it is possible that such ecological pressures have favored the evolution of chemoreceptivity for larger, more stable molecules.

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Summary

1. Dilute solutions of lettuce and wheatgerm extracts trigger positive rheotaxis in the freshwater snail, *Biomphalaria glabrata*. This response can be used as the basis of a sensitive bioassay for characterizing and identifying the chemicals to which the snail is attracted.

2. Using ultrafiltration techniques a range of different molecular weight fractions could be attenuated or removed from these extracts. By comparing the activity of these solutions with that of the original extract an estimate of the molecular weight of the attractant could be made.

3. In both cases the molecular weights of the attractants were estimated as being greater than 1000. Those in the lettuce were estimated at lying between 1000

and 10,000; whereas for wheatgerm the values were slightly higher and lay between 10,000 and 40,000. The ecological significance of these results is discussed.

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ACID PHOSPHATASE DURING THE LIFE CYCLE OF THE NEMATODE, *PANAGRELLUS SILUSIAE*

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Since the late 1920's many theories have been suggested as possible explanations for aging, but little agreement seems to exist regarding its true nature.

Strehler (1962) proposed that aging must be universal, occurring in all old animals of a species, and essentially absent in the very young; time dependent, progressing gradually in an individual and in the population; intrinsic, due to the action of time on the biological system, rather than the result of a pathology or accident; and deleterious, unfavorably affecting the survival capacity of the individual organism in its normal environment.

Based on the concept that aging is a universal phenomenon among metazoans, Gershon (1970) considered the nematode to be suitable for aging studies, because: first, it is possible to obtain age-synchronized populations of nematodes and establish survival curves; secondly, the nematode's life-span and growth are not altered by up to 90% inhibition of DNA synthesis; and thirdly, they yield large populations under easily controlled environmental conditions, thus making them fit for biochemical investigations.

Since nematodes are eutelic organisms, cell division and turnover are negligible and most cells are already differentiated after hatching (Hyman, 1951), making any deteriorative processes leading to senescence more easily observable. The nematode chosen for this study was the free-living form, *Panagrellus silusiae*.

The lysosome has been implicated as part of the terminal lytic aging process (Brock and Strehler, 1968; Herold and Meadow, 1970; Hochschild, 1971). The purpose of this study was to investigate the lysosomal enzyme, acid phosphatase, and its isozyme patterns in the life of *P. silusiae*. The next step will be to relate these isozyme changes to a later study centering on aging.

Materials and Methods

The stock cultures of *Panagrellus silusiae* were maintained at 23–25° C. The growth medium was Gerber Mixed Cereal, which was mixed with distilled water in a weight to volume ratio of 1 : 5. Each culture was maintained for 14 days. A dilute antibiotic solution of 0.6 μg penicillin-G and 10 μg streptomycin/ml was added to the nematodes during subculturing to avoid contaminating the fresh cultures.

Panagrellus silusiae is an ovoviviparous animal with five larval stages. The first larval stage (L_1) is intrauterine, but the remaining stages (L_2, L_3, L_4 and adult) are free-living. The different stages were identified by using the average lengths of worms, based on the method of Gysels and van der Haegen (1962).

After sample collection, the nematodes were separated by age using the glass

microbead technique outlined by Samoiloff and Pasternak (1969). Since this procedure only separated the youngest free-swimming larval stage, it was necessary to obtain as many L_2 worms as possible. Therefore, seven-day-old cultures were placed in the dark for 16 hr prior to sample collection, to induce the nematodes to reproduce, since *P. silusisc* tends to copulate more often while in darkness, thus producing more L_2 larvae.

When later stages were studied, the L_2 larvae were allowed to molt at 23–25° C to a more advanced stage of the life cycle. Following the work of Chow and Pasternak (1969), the L_2 larvae were added to petri dishes containing 10 ml of clear 1% barley solution, so that the ensuing growth to maturation would be highly synchronous. In the barley solution, L_3 larvae were obtained in 24 hr, L_4 in 48 hr and adults in 72 hr.

The nematodes were also kept in the 1% barley solution until they were 10, 15, 20, and 25 days old, in order to study the aging adult worms. This part of the study was conducted at 5° C, which allowed the adults to age at a somewhat slower rate than normal, but not to reproduce. Therefore, new L_2 larvae could not be born into the age-synchronized cultures. Every 24 hr, one ml of fresh barley solution was added to the petri dishes in each experiment, to offer fresh nutrients.

The nematodes to be studied were concentrated by centrifugation and ground with a Foredom tissue grinder in an ice-cold container to minimize the denaturing of the isozymes. In each experiment, the protein content of the samples was determined using the method of Lowry, Rosebrough, Farr, and Randall (1951). Polyacrylamide gel electrophoresis, based on the methods of Davis (1964) and Ornstein (1964) was used in this study. The following technique changes were made. The bridge buffer used was an 0.01 M histidine-NaOH buffer of pH 7.5, which was suggested by Robinson (1972). The gels were 10% acrylamide and were run at 6° C at 4 mAmp/tube. The sites of acid phosphatase on the gels were determined using the reaction method of Barka (1961). Electrophoretic mobility (E_f) values were determined directly from the gels.

Densitometric tracings of the gels were made immediately after the end of incubation, at 515 nm. The relative activity of each peak was calculated by dividing the peak height by the μg of protein applied to the gel (Bolla, Weinstein and Lou, 1974).

Triton X-100, a detergent which disrupts lysosomal membranes, was utilized to determine the amount of membrane bound and unbound acid phosphatase in the different nematode stages. Modifying the procedure of Meany, Gahan and Maggi (1967), Triton X-100 was added to a mixture of the stages of *P. silusiae* using six different methods of introduction and the following concentrations: 0.1%, 0.5%, 1.0%, 2.5%, 5.0%, 10%, 25%, 50%, and 100%. The six procedures for adding the detergent are as follows: immediately before the nematodes were ground for electrophoresis; 10 min before grinding the nematodes; 10 min at 37° C before grinding the tissue for electrophoretic experimentation; immediately after grinding the nematodes; to pre-ground tissue 10 min before experimentation; and to pre-ground nematodes 10 min at 37° C before running electrophoresis. Each concentration of Triton X-100 was added to the worms in all six of the procedures. Each result compared with the appropriate control

of distilled water added to the homogenate, and equal portions of nematodes and detergent were used in each case.

To determine the quantity of acid phosphatase liberated in each trial, the Sigma total acid phosphatase test (Sigma Technical Bulletin No. 104, 1963, Sigma Chemical Company, St. Louis, Missouri) was run on a sample from each test, at 410 nm on a spectrophotometer. The percentage of transmittance was converted to Sigma units/ml of acid phosphatase, using a standard curve based on para-nitrophenol.

To determine which structures within *P. silusiac* contain acid phosphatase, a light microscopic study was done on each larval stage. The tissue was fixed in 1 : 10 commercial formalin for 1 hr, and dehydrated in an ascending series of ethanols following the procedure of Jensen (1962). The worms were infiltrated with paraffin, positioned in paraffin blocks and sectioned at 10 μ. The sectioned tissue was affixed to glass slides and stained using the acid phosphatase-lead sulfate procedure of Gomori (1952), which was modified by Jensen (1956). The control for this study was heat-killed tissue (*i.e.,* boiled in distilled water for 5 min) carried through the entire staining process.

Results

This study showed that there is a relationship between changes in acid phosphatase activity and life cycle stages in *Panagrellus silusiac*. After measuring the electrophoretic mobility (E_f) values of the stained bands on each gel, it was determined that ten separate and distinct isoenzymes actually existed. The L_2 and L_3 stages each showed four isozyme bands on each gel. Five separate isozymes were present in the L_4, and six distinct bands were found on the gels of the 6-day, 10-day, 15-day, and 20-day-old nematodes. The 25-day-old nematodes showed seven isozymes of acid phosphatase, which was the largest number present in any stage of the life cycle. Only two of the ten isoenzymes were present in all eight of the stages studied. The average E_f values are listed in Table I. Isozyme 10 always travelled beyond the tracking dye, therefore resulting in an E_f value of greater than 1.0.

Table I

Average electrophoretic mobility (E_f) values. A dashed line indicates an absence of the isozyme at that stage.

Stage	Band numbers									
	1	2	3	4	5	6	7	8	9	10
L_2	—	0.1176	—	0.2581	—	0.3521	—	—	—	1.1427
L_3	—	0.1077	—	—	—	0.3529	—	—	0.6452	1.1385
L_4	—	0.1176	—	—	—	0.3516	0.5156	—	0.6464	1.1406
6 days	—	0.1270	—	—	0.3143	0.3498	0.5178	—	0.6395	1.1471
10 days	0.0366	—	—	—	0.3171	0.3540	0.5143	—	0.6402	1.1427
15 days	0.0342	—	0.1370	—	0.3093	0.3501	—	—	0.6461	1.1398
20 days	0.0328	—	0.1313	—	0.3099	0.3521	—	—	0.6380	1.1406
25 days	0.0351	—	0.1405	—	0.3170	0.3513	—	0.5956	0.6453	1.1385

The isozymes of acid phosphatase stained in one of three ways on the gels. The bands appeared either red, faint red, or yellow in color. By conducting a Sigma total acid phosphatase test on each individual band, it was determined that each colored band was truly acid phosphatase. A piece of blank gel was used as a standard.

Densitometric tracings were made of the electrophoretic gels from each age group (Fig. 1 and 2). The relative activities of each separated isozymes were

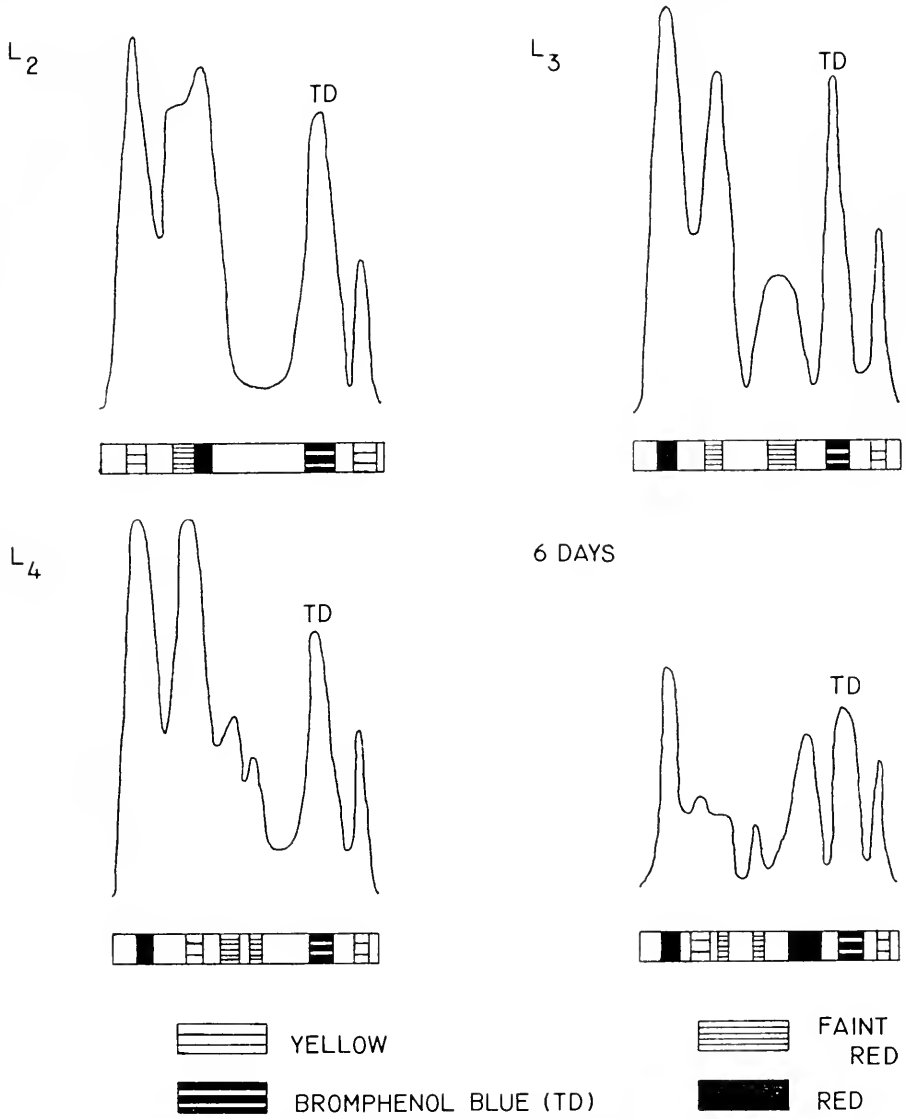

FIGURE 1. Densitometric record of acid phosphatase in L_2, L_3, L_4, and 6-day-old *Panagrellus silusiae*.

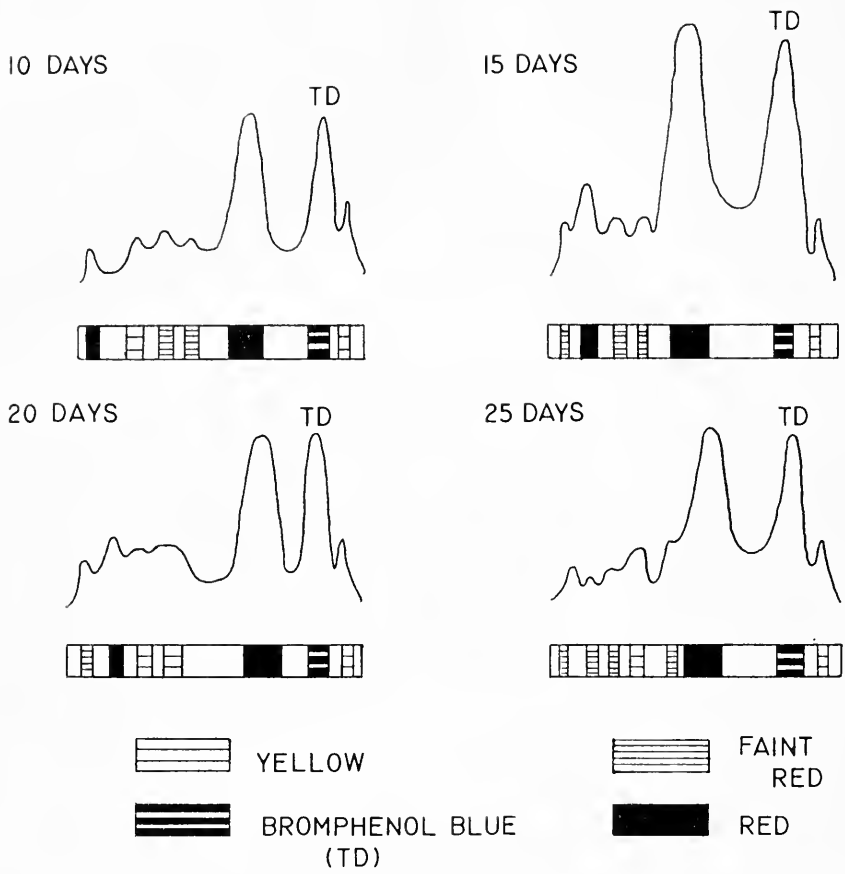

FIGURE 2. Densitometric record of acid phosphatase in 10-day, 15-day, 20-day, and 25-day-old *Panagrellus silusiae*.

calculated directly from the tracings. The values obtained appear in Table II. By studying these values and the densitometric tracings, it can be seen that the individual isozymes increase or decrease in relative activity in correlation with the life cycle stage of the nematode. However, as the nematode cycle progresses, there is a general decrease in the relative activity of the enzyme itself, while there is a concurrent increase in the number of isozymes present.

The results of the Triton X-100 study are listed in Table III. Method I (added and ground immediately) liberated no more than 2% acid phosphatase than the distilled water control, while method II (added and ground after 10 min) showed no increase in the level of acid phosphatase obtained. Method III (added and ground after 10 min at 37° C) and method IV (added immediately to ground tissue) yielded no more than a 3% increase in acid phosphatase activity. Method V (ground 10 min after it was added) showed no increase in the enzyme level, and method VI (gound 10 min after it was added at 37° C)

TABLE II

Relative activity of acid phosphatase (peak height/μg protein applied to gel). A dashed line indicates an absence of the isozyme at that stage.

Stage	Peak numbers									
	1	2	3	4	5	6	7	8	9	10
L_2	—	2.50	—	2.02	—	2.26	—	—	—	1.07
L_3	—	2.73	—	—	—	2.32	—	—	0.95	1.28
L_4	—	2.62	—	—	—	2.62	1.25	—	1.04	1.18
6 days	—	1.57	—	—	0.67	0.50	0.47	—	1.11	0.95
10 days	0.22	—	—	—	0.35	0.37	0.33	—	1.11	0.50
15 days	0.30	—	0.56	—	0.35	0.35	—	—	1.54	0.37
20 days	0.24	—	0.38	—	0.30	0.34	—	—	1.04	0.35
25 days	0.22	—	0.16	—	0.18	0.30	—	0.32	1.03	0.26

liberated no more than 1% acid phosphatase than the control. The results of these six trials indicate that only a negligible amount of acid phosphatase is bound to membranes within the cells.

In order to determine what structures within *Panagrellus silusiae* contain acid phosphatase, the nematodes were specifically stained for the enzyme, and studied using light microscopy. The controls of heat-killed tissue were run for each stage to prove that any staining was truly due to the presence of acid phosphatase and not to something in the staining process itself. In the experimental studies, any structure that appeared black in color contained acid phosphatase.

Only the digestive tract stained lightly in the L_2 stage. The L_3 stage stained lightly throughout the length of its body and the faint line of the intestine was again visible. This indicates that these two stages contain small amounts

TABLE III

Effect of concentration of Triton X-100 on acid phosphatase activities using p-nitrophenol as a substrate (values expressed in Sigma units/ml of acid phosphatase).

Conc. of Triton X-100	Method used					
	I Added and ground immediately	II Added and ground after 10 min	III Added and ground after 10 min (37°C)	IV Added immediately to ground tissue	V Ground 10 min after it was added (37°C)	VI Ground 10 min after it was added (37°C)
0.0%	0.40	0.34	0.39	0.54	0.45	0.51
0.1%	0.41	0.34	0.34	0.55	0.44	0.45
0.5%	0.42	0.32	0.38	0.57	0.39	0.44
1.0%	0.40	0.30	0.39	0.57	0.39	0.50
2.5%	0.35	0.27	0.41	0.52	0.40	0.50
5.0%	0.41	0.31	0.42	0.58	0.43	0.52
10%	0.35	0.29	0.40	0.49	0.44	0.48
25%	0.39	0.27	0.39	0.52	0.44	0.44
50%	0.40	0.31	0.34	0.50	0.40	0.47
100%	0.35	0.30	0.35	0.55	0.44	0.51

of the enzyme. In the L_4 stage, a large amount of staining occurred. For the first time, the esophagus and intestine stained darkly, indicating that large amounts of acid phosphatase were present. Also, the immature gonads, which begin to develop in this stage, stained positively for the enzyme. A number of structures stained in the adult stage, including the entire gastrointestinal tract, the fully developed reproductive system, the excretory canals and eggs within the bodies of sexually mature adult females. L_4 worms waiting to emerge from the bodies of adult females also stained lightly, indicating the presence of a small amount of acid phosphatase.

Discussion

For the data to be meaningful, both the unbound and membrane-bound isozymes of acid phosphatase have to be considered. Since the unbound isozymes could be assayed, Triton X-100 was selected to release those isozymes bound to membranes within the cells. Depending on the method of introduction and concentration of the detergent used, no more than 3% of the total acid phosphatase present in *P. silusiae* was found to be bound by membranes, meaning 97% of the enzyme could be assayed without the use of Triton X-100. Therefore, the amount of bound acid phosphatase was considered negligible, and the use of Triton X-100 was abandoned.

The results of the electrophoretic and densitometric studies indicate that there is a relationship between acid phosphatase levels and specific life cycle stages in *Panagrellus silusiae*. The changes exhibited by acid phosphatase probably result from the involvement of several molecular subunits (isozymes) in the activity of the enzyme.

By studying Table II, it can be seen that isozymes 1, 3, and 5 were only present in the adult stages, indicating they may be connected with the onset of maturity. Isozyme 2 appeared only in the larval stages, indicating involvement with the development of the nematode, instead of the later stages. Because isozyme 4 was only present in the L_2 stage, it seems to be related to some early development in the young larvae. Isozyme 6 was present throughout the entire life cycle. Its relative activity peaked in the L_4 stage, dropped by almost 80% in the molt to the adult stage, and continued to drop during the rest of the life cycle, indicating a greater involvement with early development than with maturation. Perhaps it is involved with the onset of gonadogenesis, since its activity peaks during the stage when this process begins. The seventh isozyme was present briefly in the middle of the life cycle, indicating that it is involved with the onset of development of some particular structures and disappears upon their completion. The eighth isozyme may be involved with the final aspects of aging since it appeared only in the 25-day-old nematodes. Isozyme 9 may be connected with the aging process, since its relative activity peaked in the 15-day-old worms. The tenth isozyme travelled beyond the tracking dye in every stage of the life cycle. This indicates that this isozyme might be of a molecular weight less than that of the bromphenol blue tracking dye, and may be involved in both the early and late stages, since it is always present. However, charge and other factors involved in electrophoresis might also have caused such an occurrence.

The number of isozymes of acid phosphatase increases during the life span in *P. silusiae,* but the relative activities of the different isozymes peak at different stages, while the overall enzyme activity decreases with maturation. These results are consistent with the findings of Erlanger and Gershon (1970) on *T. aceti* and Bolla *et al.* (1974) on *N. brasiliensis,* who concluded that these biochemical changes correlate with the morphological and physical changes that occur during the stages of development and aging throughout the nematode's life cycle.

The isozyme bands on the electrophoretic gels appeared either red, faint red, or yellow in color. The difference between the two shades of red is explained by the fact that those isozymes that stained faint red were always of a lesser activity than those that stained red on the same gel, indicating that there was less of each faint staining isozyme than of those which stained darker. The yellow bands are explained by MacIntyre (1971) who studied the staining reactions used in this research. He found that acid phosphatase ordinarily combines with two molecules of fast garnet, forming a red dicoupled colored complex. However, this usually spontaneous reaction sometimes does not occur to completion, which leaves the acid phosphatase in a yellow, monocoupled colored complex, with one molecule of fast garnet.

From the light microscopic study, it can be seen that the L_2 and L_3 stages show very little staining for acid phosphatase, indicating a low activity of the enzymes. However, the electrophoretic and densitometric studies indicated high levels of activity but few isozymes at these stages. Perhaps some isozymes may not be stained by the histochemical technique used. Due to these somewhat conflicting results, further study of these two larval stages is necessary. Complete staining of the gastrointestinal tract and developing gonads was obvious in the L_4 stage. In the adult stage, the digestive tract, excretory canals, reproductive system, and eggs and unborn L_1 worms in the bodies of mature females, all stained positively for acid phosphatase. This microscopic study therefore indicates that acid phosphatase is present in high concentrations in the digestive, excretory, and reproductive systems of *Panagrellus silusiae,* which is consistent with previous findings in other lower metazoans (Cesari, 1974).

Cristofalo, Parris and Kritchevsky (1967) hypothesized that with increased age, acid phosphatase activity gradually shifts the equilibrium in the cell away from the synthesis and towards catabolism, thus resulting in a general deterioration of the cells. The data indicates that there is a specific change in the isozymes of acid phosphatase which corresponds to the stages in the life cycle. Acid phosphatase isozymes appear to vary with the age of the nematodes, as discussed in the Gershon (1970) model.

Summary

This study showed that there is a relationship between acid phosphatase levels and life cycle stages in the nematode, *Panagrellus silusiae.* Ten different isozymes of acid phosphatase were separated electrophoretically. Relative activity peaked at different stages in the life cycle for the different isozymes. Later in the life cycle, there is a general decrease in the relative activity of acid phosphatase itself, while there is a concurrent increase in the number of isozymes present. At least

97% of the acid phosphatase in *P. silusiae* is soluble (unbound). Acid phosphatase appears to be present in large quantities in the entire gastrointestinal tract, the excretory canals, and the reproductive system of mature *Panagrellus silusiae*.

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MORPHOLOGY OF THE MOUTHPARTS OF LARVAL LOBSTERS, *HOMARUS AMERICANUS* (DECAPODA: NEPHROPIDAE), WITH SPECIAL EMPHASIS ON THEIR SETAE

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The mouthparts of decapod crustaceans exhibit a rich diversity of form. Such structural diversity is evident from the morphological descriptions of mouthparts which are generally included in reports of research on a variety of topics. Examples from the Nephropidae alone include morphological studies of adults (Herrick, 1911), descriptions of larval development (Santucci, 1926 and 1927; Wear, 1976), functional morphology of appendages (Farmer, 1974), and descriptions of new species (Holthuis, 1974). Descriptions of mouthparts are also featured in the substantial literature on larval development of non-nephropid decapods reared in the laboratory. A small, but representative, sampling of such papers might include those of Gonor and Gonor (1973a), who described and illustrated the larval mouthparts of several porcellanids; Bookhout (1972) and Roberts (1970, 1973), who described the mouthparts of pagurids; and Costlow and Bookhout (1959), Roberts (1969), Perkins (1972), and Bookhout and Costlow (1974, 1977), who described those of brachyurans.

So far as can be determined, no general review of the types of setae found in crustaceans has been published; in fact, few studies describing the setae of macrurans, or of decapods generally, have been reported. Huxley (1880) briefly mentioned setae in his classic work on the crayfish, *Astacus fluviatilis*. More recently, Thomas described the types and distribution of setae present on the adult (1970) and hatchling stages (1973) of another British crayfish, *Austropotamobius pallipes*, and Farmer (1974) studied the functional morphology of mouthparts in *Nephrops norvegicus*.

Surprisingly, there are but few such studies of the American lobster, *Homarus americanus*. While the excellent and extensive monographs of Herrick (1896, 1911) remain the major works on the morphology of this species, his treatment of the mouthparts of larval lobsters and their setae is rather brief; Herrick left much work to be done on these appendages.

It is the purpose of the present work to describe the types of setae found on the mouthparts of larval lobsters, to devise a scheme for their classification, to describe the distribution of the various setal types, and to present observations of interesting or previously overlooked features of the mouthparts themselves. Wherever possible, the structures described will be considered in relation to their possible functions. A further purpose is to follow sequential changes in the mouthparts and their setae which may take place in successive stages. The first three stages, the larvae, are strictly planktonic, while fourth-stage, postlarval lobsters begin to take up the benthic existence typical of adults. This drastic change of habitat is necessarily accompanied by a change of feeding habits, and it

seems inescapable that these changes should be reflected in significant structural alterations in the feeding apparatus. The detailed information on the structure of the feeding appendages presented in this study may prove helpful in attempts to devise a suitable artificial food for the culture of this commercially important food species.

Materials and Methods

Collection of specimens

The lobsters used in this study were collected at the Massachusetts State Lobster Hatchery and Research Station on Martha's Vineyard. Approximately forty specimens, hatched from several females, were examined.

Several external morphological features described by Hadley (1905), which could easily be observed with a dissecting microscope, were used to determine the stage of each specimen. Several thousand larvae are raised in each rearing tank at the Hatchery. In the early stages the intermolt period is relatively brief, and the larvae do not molt synchronously. It was therefore not practicable to separate early from late intermolt lobsters, and no attempt was made to identify stages in the molt cycle.

Procedures for light microscopy

Specimens for light microscopy were fixed in seawater Bouin's fluid (Humason, 1962) where they were stored until needed. Staining proved unnecessary. Unstained mouthparts were mounted on microscope slides in an aqueous mounting medium, either Salmon's polyvinyl lactophenol (Type A.2) (Gatenby and Beams, 1950) or polyvinyl pyrrolidone (Burstone, 1957). Preparations were observed with a compound microscope using brightfield and phase contrast illumination.

Procedures for scanning electron microscopy

Specimens for scanning electron microscopy were selected after fixation from a group of animals being prepared for a future study involving transmission electron microscopy. The fixation procedure was thus more elaborate than that customarily used for specimens to be studied by scanning microscopy; it was modified only slightly from the method employed by Walker (1976).

Specimens were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.0) at 6° C for 3.5 to 6.5 hours. The buffer contained a balanced salt solution comprising 30 mg/ml NaCl and 20 μg/ml $CaCl_2$ (McDonald, 1972). Specimens were post-fixed in 1% OsO_4 in buffer, washed in solutions of buffer with decreasing concentrations of NaCl, treated with 2% uranyl acetate, and dehydrated to 70% alcohol where they were stored. As needed, lobsters were hydrated in an alcohol series, dehydrated in an acetone series, and dried in a Sorvall critical-point drier using CO_2 to replace acetone. Mouthparts were dissected from whole, dried lobsters, mounted on aluminum stubs with Scotch double-coated tape (No. 666), and coated with gold/palladium in a Tecknics Hummer Coater. Preparations were observed with an International Scientific Instruments Mini-SEM at an accelerating voltage of 15 kV.

Preparation of line drawings

Line drawings of individual appendages were made by the "photo etching" method described by Yob (1973). Light micrographs of the mouthparts and setae to be drawn were taken using a compound microscope and were photographically enlarged to an appropriate scale. Features of the photographs to be retained in the drawings were outlined directly on the prints with a fine Rapidograph pen. When the inked lines were thoroughly dry, the background photographic images were bleached with an iodine solution and removed with fixer, leaving only the line drawings on white photographic paper.

Results

Relative positions and orientation of fourth-stage mouthparts

The first five pairs of appendages serving as mouthparts (mandibles through second maxillipeds) are flattened and make up a series of layers covering the mouth. The sixth pair, the third maxillipeds, are not flattened but extend anteriorly to act as grasping structures. Since the mouthparts lie roughly in the frontal plane and do not have the same attitude as most appendages of the lobster, and because they are flattened and layered, the terms *inner* and *outer* are most useful for describing features toward and away from the mouth, respectively. Most mouthparts have inner and outer surfaces; for example, the outer surface of the first maxilla is adjacent to the inner surface of the second maxilla, on the segment just posterior. Using the terms inner and outer, as well as medial (toward the path of food) and lateral, and proximal and distal, it should be possible to describe, compare, and understand the positions of the mouthparts and the structures found on them.

Types of setae

The construction of categories of setae is a useful aid in understanding the variety of setal types found on the mouthparts of larval lobsters. Such a classification is based largely on the external morphology of the setae, particularly the nature and distribution of the setules. In the hope of standardizing terminology, Thomas's (1970) system for naming the setae of *Austropotamobius pallipes* is used as the basis for naming the groups of setae described in the present work. Although Thomas's terms are used when possible, changes and additions have been made whenever appropriate. In their studies of larval crabs, Bookhout and Costlow (1974, 1977) have also followed Thomas's terminology for naming setae.

The setal complement of the first four stages of the lobster may be arranged into ten categories. Most categories contain noticeable variations in the form of the setae assignable to them, emphasizing the artificial nature of such a classification. In several categories the variation is sufficient to warrant subdivision into several types (each designated by a letter and a number); each type may, however, be considered a variation on the general plan of the category in which it has been placed. Within many of the categories several features of the setae were inconsistently observable. Apical pores, annulations, and bulbous swellings of the

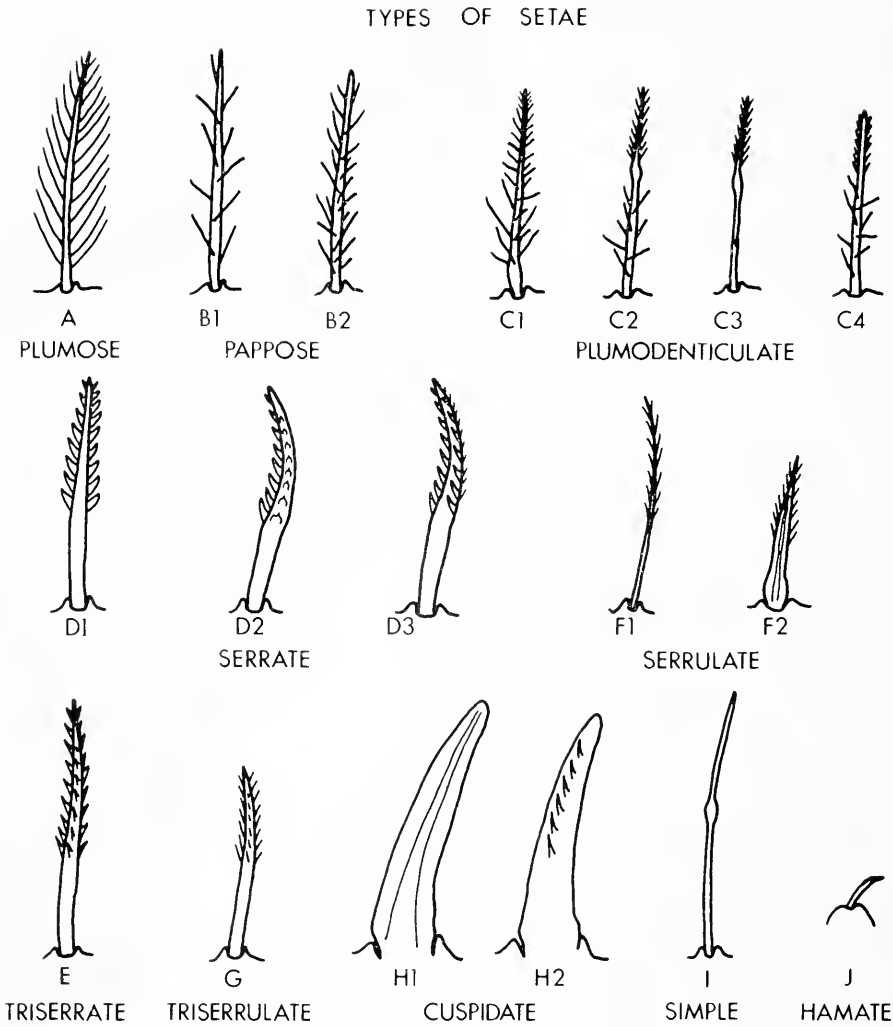


FIGURE 1. Diagrammatic representation of the types of setae found on the mouthparts of larval lobsters. (Refer to the text for a detailed explanation of the setal types.) Drawings are not to the same scale.

shaft are mentioned only when they are consistently or conspicuously present. The types of setae encountered in the present study, illustrated in Figure 1, will now be described.

Plumose, Type A. Plumose setae bear two distinct rows of long, fine setules along most of the length of the shaft. The setules may be more or less densely arranged, but the rows are always situated opposite each other, forming an angle of 180° (Fig. 19). These setae may be segmented by constrictions, or annulations, of the outer surface of the shaft which occur near the insertion on the shaft of some pairs of opposed setules (Fig. 24).

Pappose, Type B1. Typically pappose setae have long, fine setules similar to those of plumose setae. Instead of lying opposite each other, however, the setules are loosely arranged about the shaft in a seemingly random manner (Fig. 15).

Pappose, Type B2. Densely pappose setae are similar to the previous type but with setules more closely crowded on the shaft. (These are limited in distribution to a single tuft on the first maxilla of the fourth stage.)

The setae included in the plumodenticulate category, when examined with the light microscope, closely resemble those called plumodenticulate by Thomas (1970), but bear coarse or fine setules instead of denticulations. Plumodenticulate setae exhibit considerable variation (Figs. 1, 11).

Plumodenticulate, Type C1. The sparse setules of the proximal portion of these setae, arranged in the same manner as those on pappose setae, gradually give way to finer and more densely arranged distal setules.

Plumodenticulate, Type C2. The sparse setules of the proximal half, arranged in the same manner as the setules on pappose setae, are sharply separated from the finer, denser setules of the distal half. The setules are similar to those of type C1, but the transition from base to tip is abrupt. The two regions may be separated by a bulbous swelling of the setal shaft.

Plumodenticulate, Type C3. Although there are no setules on the proximal half of these setae, they have been included in the plumodenticulate category because of their resemblance to type C2. In fact, they would be identical to type C2 setae if proximal setules were present. The distal half of the shaft bears fine, densely packed setules. A bulbous swelling may lie midway along the length of the shaft.

Plumodenticulate, Type C4. This setal type bears proximal setules identical to those of type C1 and C2 but differs in having shorter, coarser setules distally. This type most closely resembles the basic plumodenticulate setae as described by Thomas (1970).

All three types of serrate setae are characterized by large, distinct, tooth-like setules along the distal half of the shaft. They setules are clearly arranged in two rows forming an angle of less than 180°.

Serrate, Type D1. These are typical serrate setae which fit the general description above and have no additional setules.

Serrate, Type D2. In addition to two rows of tooth-like setules, this group bears scale-like setules on the opposite side of the shaft (Figs. 20, 22).

Serrate, Type D3. A row of shorter, finer setules opposite the larger, tooth-like setules distinguishes this group from the other serrate setae (Fig. 18).

Triserrate, Type E. Triserrate setae bear three rows of typical serrate setules. The setules of all three rows are approximately equal in length (Fig. 17).

Serrulate, Type F1. The distal half of the shaft of serrulate setae appears to bear denticulations, or notches, when viewed with the light microscope. The scanning electron microscope reveals, however, that these can be short, fine, peg-like setules, arranged in two rows forming an angle of less than 180 . These setae

are quite similar to typical serrate (D1) setae, but are smaller and have shorter, finer setules (Fig. 16).

Serrulate, Type F2. Thicker walls of the shaft and a narrower lumen separate this type of serrulate setae from type F1. The subterminal pore is clearly visible. This type appears to be somewhat similar to the "teazel" setae described by Thomas (1970), but the setules are opposite and are not arranged in pappose fashion. They are found only on the first maxillae of the fourth stage.

Triserrulate, Type G. The short, fine, peg-like setules of the distal half of triserrulate setae are arranged in three rows (Fig. 22). They can be distinguished from triserrate (E) setae by their finer setules, and from serrulate (F1) setae by the presence of three rows of setules rather than two.

Cuspidate, Type H1. Cuspidate setae are large, somewhat conical, tooth-like setae. They are stout, with thick walls and relatively narrow lumen, and lack setules (Fig. 13).

Cuspidate, Type H2. These are cuspidate setae, similar to type H1, with sparsely arranged, fine, short, almost needle-like setules on the shaft (Fig. 21).

Simple, Type I. Simple setae are usually relatively long and thin and are without setules of any kind (Fig. 14). There may be a bulb midway along the length of the shaft. Several much shorter simple setae have been observed and are included in this category.

Hamate, Type J. These are small, short setae shaped like hooks and lacking setules. They were observed only on the epipodites of the maxillipeds.

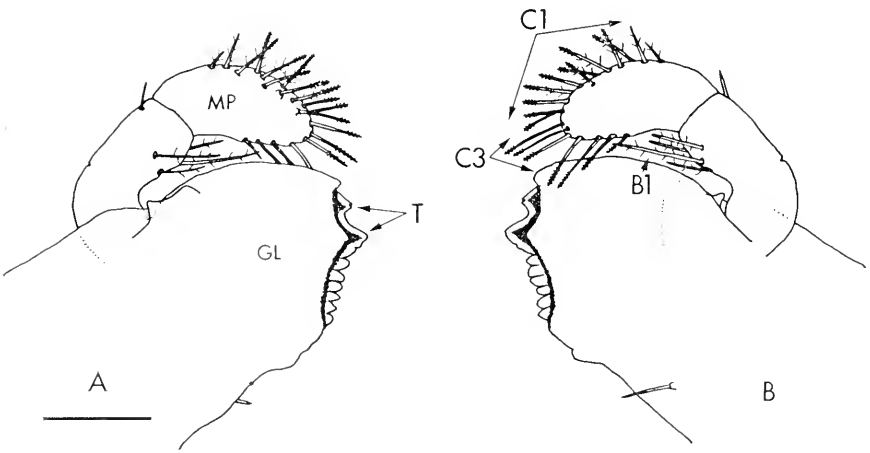


FIGURE 2. Mandible (left) of fourth-stage lobster, showing inner (A) and outer (B) surfaces. Abbreviations are: GL, gnathal lobe; MP, mandibular palp; B1, pappose setae; C1 and C3, plumodenticulate setae; I, simple setae; and T, two major teeth. Scale bar represents 0.25 mm. (Figures 2 and 4–9 are line drawings of representative mouthparts of fourth-stage lobsters showing the types of setae present and their distribution as well as the general morphology of each mouthpart. Two views of each mouthpart are illustrated, showing the inner and outer surfaces; these are *not* matched pairs of right and left mouthparts.)

Detailed descriptions of the mouthparts

Because the mouthparts of the fourth-stage lobster are more highly developed, show more features than corresponding appendages of earlier stages, and presumably resemble those of the adult more closely, the detailed observations of the fourth stage are presented at the beginning of the section on each mouthpart. Observations of the first three stages then follow and are compared to the situation found in the fourth stage. In this way major developmental trends and interesting differences between the earlier stages and the fourth stage can be noted. The descriptive account is accompanied by a series of line drawings of the fourth-stage mouthparts (Figs. 2–9) which serve not only to illustrate the general form of each appendage but also to map the distribution of the various types of setae. The drawings should also be referred to for details of setal distribution which have not been included in the text.

Mandibles. The most conspicuous feature of the mandible is a massive gnathal lobe at its distal end (Fig. 2). The medial surface of the gnathal lobe is responsible for the masticatory effect of the mandible, and it forms a cutting and grasping edge consisting of a series of teeth. The teeth of the fourth-stage mandible are blunt and rounded and can be seen with transmitted illumination to be heavily cuticularized. Each tooth is solidly sclerotized and lacks a lumen. The basal portion of the cutting edge usually bears at least six similar teeth (Fig. 10). Distally, one or two more massive teeth protrude past the tips of the smaller teeth.

The mandibles of fourth-stage lobsters are asymmetrical. The single large tooth of one side fits between two large teeth on the opposite mandible (Fig. 3). Distal to the large teeth is a smaller, less heavily cuticularized protuberance which is present on both sides.

The mandibular palp projects anteriorly from the base of the gnathal lobe on the lateral surface of the mandible. The palp of the fourth-stage lobster has three segments; the articulation between the first and second segments, however, is usually inconspicuous.

Almost all of the setae present on the mandible are found on the two distal segments of the palp. The second segment bears several pappose (B1) and simple (I) setae, while the terminal segment is heavily setose, bearing type C1 and C3 plumodenticulate setae (Fig. 11). Several setae with relatively thick walls and a narrow lumen were also observed on some specimens. These are similar to type F2 serrulate setae but have a terminal, rather than subterminal, apical pore. They resemble even more closely the setae Thomas called "teazels", and they are found in the same location.

The first-stage mandible differs significantly from that of the fourth stage. The teeth of the gnathal lobe are thinner, sharper, and more delicate, shaped like slender cones (Fig. 12). The basal portion of the cutting edge bears approximately ten to thirteen of these teeth with spaces between them. Each tooth has a relatively narrow, but clearly discernible, lumen and thick, cuticularized walls. In this regard they appear similar to some types of setae; however, they lack the basal socket characteristic of setae. At the proximal end of the cutting edge, on the inner surface, is a "pad" or dense field of small setae which are all directed medially, or toward the mouth (Fig. 12).

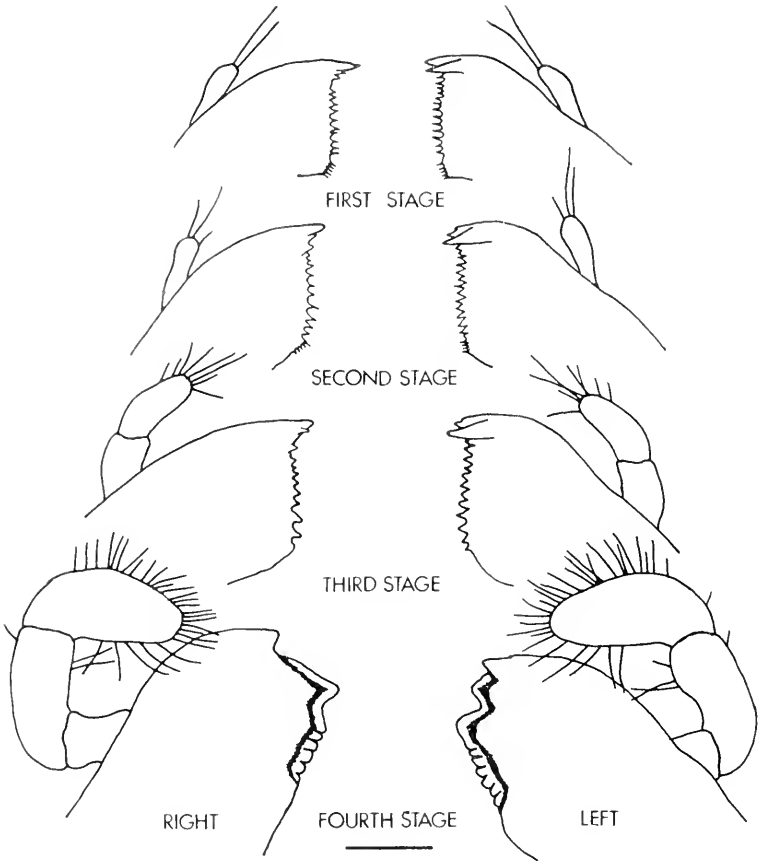


FIGURE 3. Outer-surface views of representative matched pairs of right and left mandibles, illustrating the developmental changes occurring in first- through fourth-stage lobsters. All drawings are to the same scale. Scale bar represents 0.25 mm.

Unlike the chelipeds, which are symmetrical until the fifth to eighth stage (Herrick, 1911, p. 266), the mandibles are asymmetrical through the fourth stage (Fig. 3). In all specimens of the four stages examined during this study, the distal end of the cutting edge on the right mandible bears a single large tooth and two smaller teeth associated with it (Fig. 12), while the left mandible bears two major teeth. It is interesting to note that a similar type of asymmetry can be seen in the mandibles of adult lobsters and has been reported for the adult mandibles of *Nephrops norvegicus* (Farmer, 1974) and *Austropotamobius pallipes* (Thomas, 1970).

Several trends can be seen in the mandible as lobsters pass from the first to the fourth stage. First is the tendency of the teeth on the gnathal lobe to become more heavily cuticularized, thickening the walls and decreasing both the size of the lumen and the space between teeth, until the pointed, comparatively delicate teeth of the first stage are transformed into the massive molars of the fourth stage

(Fig. 3). Second- and third-stage mandibles display intermediate conditions, although the most drastic change occurs between the third and fourth stages. The second trend involves increasing complexity of the mandibular palp (Fig. 3). The first-stage palp bears only two setae; these are probably pappose (B1), but the pattern of setules is not obvious. The articulations separating the three segments of the palp are barely discernible. In the second stage, three setae are present, clearly type C3 plumodenticulate; approximately ten setae of this type are present on the palp during the third stage. By the fourth stage, the terminal segment is covered by three types of setae, as previously described. The tendency, then, is an increase in the number and variety of setae present on the mandibular palp in successive stages.

Paragnaths. Paragnaths were noted in all four larval stages as rounded lobes protruding immediately posterior to the mouth. The surface facing the mouth is covered by a mat of extremely fine, closely-set, simple setae (Fig. 12). No significant changes appear during the larval stages.

First maxillae. The first maxilla is composed of a coxal endite, a basal endite, and an endopodite (Fig. 4). The medial edges of the endites are the food-handling surfaces, and this is reflected in their setal armature. The basal endite of the fourth stage is provided with two rows of stout, cuspidate (H1) setae along its medial edge (Fig. 13), which are obviously useful in manipulating food. The basal endite bears a row of type F1 serrulate setae on its outer surface and a row of five type F2 serrulate setae on its inner surface. A clump of pappose (B1) setae lies on the inner surface of the basal endite near the coxal endite, and a clump of three setae, noted in several specimens to be pappose (B1), plumodenticulate (C4), and serrulate (F1), lies at the anterior end. Several serrulate (C4) setae are scattered on the inner surface of this endite.

The medial edge of the coxal endite bears type F2 serrulate setae distally and typical pappose (B1) setae proximally. Like the basal endite, the coxal endite

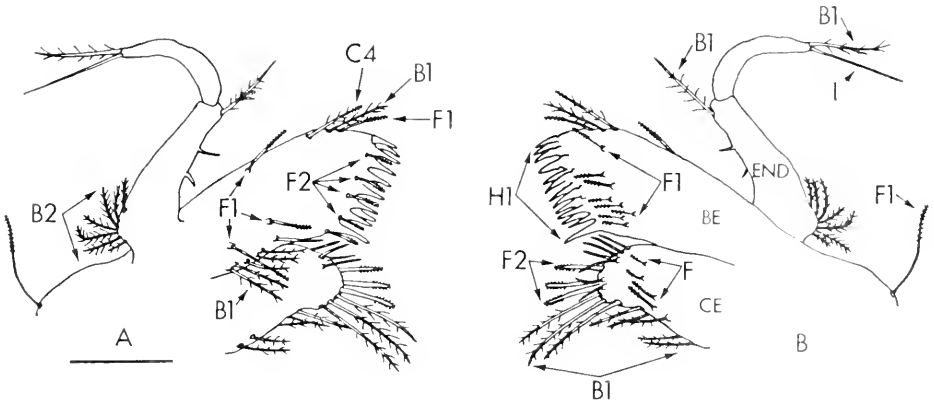


FIGURE 4. First maxilla (left) of fourth-stage lobster, showing inner (A) and outer (B) surfaces. Abbreviations are: BE, basal endite; CE, coxal endite; END, endopodite; B1, typical pappose setae; B2, densely pappose setae; C4, plumodenticulate setae; F1 and F2, serrulate setae; H1, cuspidate setae; and I, simple setae. Scale bar represents 0.25 mm.

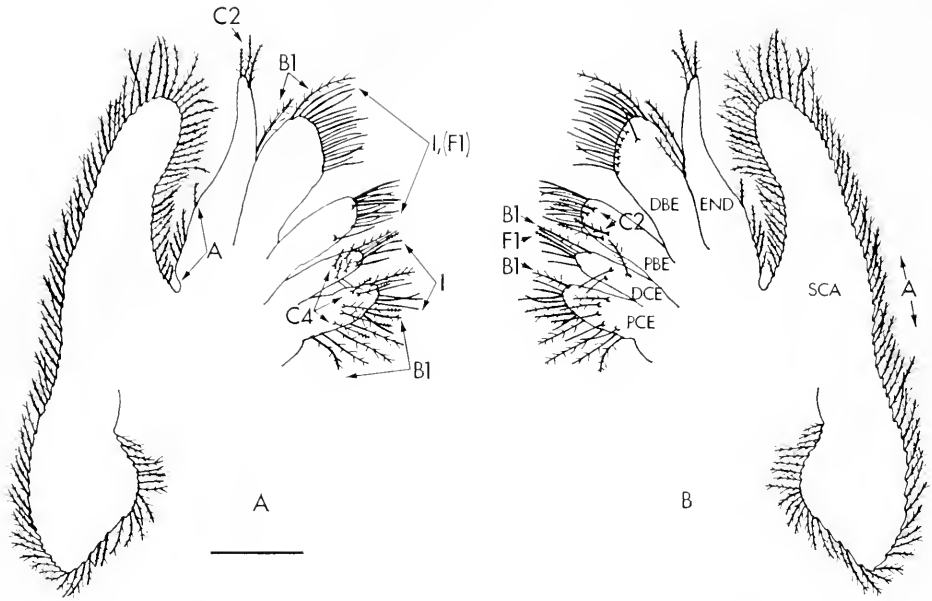


FIGURE 5. Second maxilla (left) of fourth-stage lobster, showing the inner (A) and outer (B) surfaces. (The number of plumose setae on the scaphognathite has been reduced slightly for clarity.) Abbreviations are: DBE, distal lobe of basal endite; PBE, proximal lobe of basal endite; DCE, distal lobe of coxal endite; PCE, proximal lobe of coxal endite; END, endopodite; SCA, scaphognathite; A, plumose setae; B1, pappose setae; C2 and C4, plumodenticulate setae; F1, serrulate setae; and I, simple setae. Scale bar represents 0.25 mm.

bears on its outer surface a row of type F1 serrulate setae behind the medial edge.

The two-segmented endopodite of the fourth stage bears typical pappose (B1) and simple (I) setae as well as a clump of densely pappose (B2) setae near the base of the first segment. This is the only location where type B2 pappose setae were observed.

The first maxilla of the first stage differs in several details. The most interesting of these involves the presence of rows of small, fine setules on the cuspidate (H2) setae of the basal endite. These setules are also present in the second and third stages. Here, as in several other instances to be noted below, the first, second, and third stage mouthparts have cuspidate setae with setules, while all of the cuspidate setae observed on the fourth stage mouthparts are simple.

The endopodite of this appendage in the first three stages has only a single segment. The number of setae at the tip of the endopodite appears to undergo a reduction, as stages one, two, and three have three to five pappose (B1) and plumodenticulate (C2) setae in that position instead of the two found in the fourth stage. The clump of pappose (B2) setae, so obvious at the base of the fourth-stage endopodite, is absent in the first two stages and represented by only three setae in stage three.

Second maxillae. A bilobed coxal endite, a bilobed basal endite, an endopodite,

and the scaphognathite constitute the second maxilla (Fig. 5). In the fourth stage the medial edge of the distal and proximal lobes of the basal endite bears mostly simple (I) and several serrulate (F1) setae, with one or two pappose (B1) setae at its distal end. The distal and proximal lobes of the coxal endite are armed with pappose (B1), plumodenticulate (C4), serrulate (F1), and simple (I) setae.

The one-segmented endopodite is only sparsely set with setae. Three type C2 plumodenticulate setae are borne on its tip, three pappose (B1) setae occur medially, and several plumose (A) setae are found laterally.

The scaphognathite is a long, flat structure formed by a fusion of the endopodite (anterior lobe) and epipodite (posterior lobe). It serves to pump water through the gill chamber and is aided in this function by a complete fringe of closely set plumose (A) setae.

Although the second maxilla follows the general pattern of increase in size and number of setae, it appears to undergo less drastic changes than the other mouthparts. This may be linked to the necessity for a well developed scaphognathite (for respiratory purposes) from the beginning of the lobster's free-swimming existence—the first stage. The endopodite of the first stage bears pappose (B1) setae in contrast to the types found here in the fourth-stage lobster.

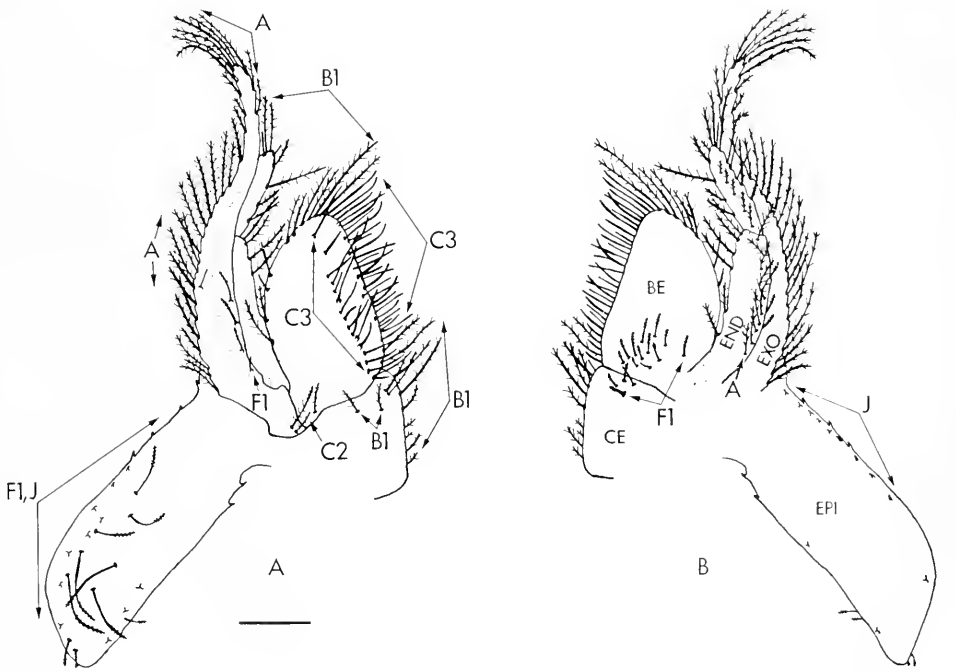


FIGURE 6. First maxilliped (right) of fourth-stage lobster, showing the outer (A) and inner (B) surfaces. (The number of plumose setae on the exopodite has been reduced slightly for clarity.) Abbreviations are: BE, basal endite; CE, coxal endite; END, endopodite; EXO, exopodite; EPI, epipodite; A, plumose setae; BI, pappose setae; C2 and C3, plumodenticulate setae; F1, serrulate setae; and J, hamate setae. Scale bar represents 0.25 mm.

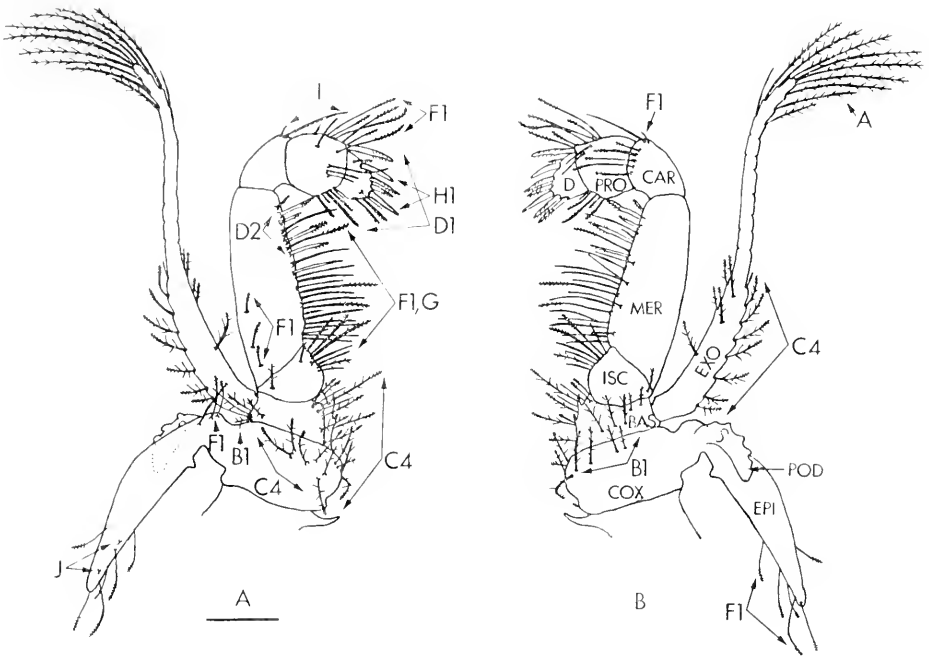


FIGURE 7. Second maxilliped (right) of fourth-stage lobster, showing the outer (A) and inner (B) surfaces. Abbreviations are: D, dactyl; PRO, propus; CAR, carpus; MER, merus; ISC, ischium; BAS, basis; COX, coxa; EXO, exopodite; EPI, epipodite; POD, podobranch; A, plumose setae; B1, pappose setae; C4, plumodenticulate setae; D1 and D2, serrate setae, I, simple setae; and J, hamate setae. Scale bar represents 0.25 mm.

First maxillipeds. Basal and coxal endites are the food-handling structures of the first maxillipeds (Figs. 6, 14). In the fourth stage most of the medial edge of the basal endite is occupied by type C2 plumodenticulate setae often with bulbs halfway along their length. Another row of type C3 setae lies parallel to the medial edge on the outer surface. A field of serrulate (F1) setae is present on the inner surfaces of the basal and coxal endites, and pappose (B1) setae fringe the lateral edge of the basis.

The two-segmented endopodite bears a long row of plumose (A) setae on its inner surface in addition to a row of pappose (B1) setae along its medial and lateral edges.

The exopodite of the first maxilliped is divided into two subequal portions, a basal segment and a distal flagellum comprising seven segments separated by constrictions, or superficial folds, of the exoskeleton. The lateral edge of the entire exopodite, the tip, and part of the medial edge of the flagellum are fringed by densely packed plumose (A) setae (Fig. 19). Several serrulate (F1) setae are found basally, near the medial edge.

The epipodite is a flattened structure which lies adjacent to the scaphognathite (of the second maxilla) and aids in the function of moving water through the gill

chamber. Several serrulate (F1) setae are scattered on its outer surface. Many short, hooked, hamate (J) setae are present on most of the surface of the epipodite and form a row along its lateral edge. Hamate setae are restricted in distribution to the maxillipeds and, when they are present, are found only on the epipodites of these mouthparts.

The first maxilliped undergoes relatively little change from the first to fourth stages besides an increase in size and in number of setae. The endopodite remains two-segmented throughout the larval stages. In the first stage, the exopodite is not divided into two regions and is fringed by plumose (A) setae only on its lateral edge; the coxa is almost bare with only two or three pappose (B1) setae distally. Hamate (J) setae are absent from the epipodite of the first and second stages. The third stage shows an increased number of setae on the coxa, a flagellum with three segments on the exopodite, and a row of hamate setae on the lateral edge of the epipodite.

Second maxillipeds. The second maxilliped is composed of a protopodite bearing an endopodite, exopodite, and epipodite (Fig. 7). The five-segmented endopodite, the food manipulating structure, is more elaborate than in any of the previously encountered mouthparts. The ischium and long merus project anteriorly, but the carpus, propus, and dactyl turn sharply toward the midline to give the endopodite the shape of an inverted L.

The dactyl and propus are heavily armed with a variety of setal types. The tip of the dactyl bears two stout, cuspidate (H1) setae each usually with a distinct annulation. Other setae present on the dactyl and propus include serrate (D1 and D2), serrulate (F1), and simple (I) types. Except for a row of serrulate (F1) setae on its inner surface, and a single simple (I) seta, the carpus is bare.

The merus is the longest segment of the second maxilliped, and it bears almost all of its setae on the medial edge. Serrulate (F1) and triserrulate (G) setae are present along the entire medial edge, although the distal end bears four or five scaled serrate (D2) setae (Fig. 20) and the proximal end several plumodenticulate (C4) setae. Serrulate (F1) and plumodenticulate (C2) setae are present on the small ischium.

The protopodite is distinguished by the conspicuous absence of the coxal and basal endites which are so prominent on the maxillae and first maxillipeds. The basis bears plumodenticulate (C4) and pappose (B1) setae. Plumodenticulate (C4) setae occur on the proximal portion of the exopodite, which extends from the basis. The distal flagellum is distinctly divided into approximately twelve segments and bears segmented plumose (A) setae near its tip.

Plumodenticulate (C4) and pappose (B1) setae also occur on the coxa. An epipodite extends from the coxa and bears several scattered serrulate (F1, with distinct annulations) and hamate (J) setae. A small, rudimentary podobranch protrudes from the base of the epipodite, near the coxa.

During the first stage, the dactyl of the second maxilliped bears at its tip a single cuspidate (H2) seta which has needle-like setules in two rows along the shaft (Fig. 21). The dactyl also bears two or three serrulate (F1) setae. Generally, fewer setae are present on the second maxilliped in the first stage than in the fourth. The row of setae on the inner surface of the carpus in the fourth stage

is represented in the first stage by a cluster of only three setae, and the medial margin of the merus, densely setose in the fourth stage, bears fewer setae in the first stage, including serrate (D2) setae with scales and serrulate (F1) and triserrulate (G) setae (Fig. 22).

The exopodite is faintly divided into two segments in the first stage, with only four or five setae at its tip. Hamate (J) and serrulate (F1) setae are not yet present on the epipodite. Contrary to Herrick's (1896) observations, a rudi-

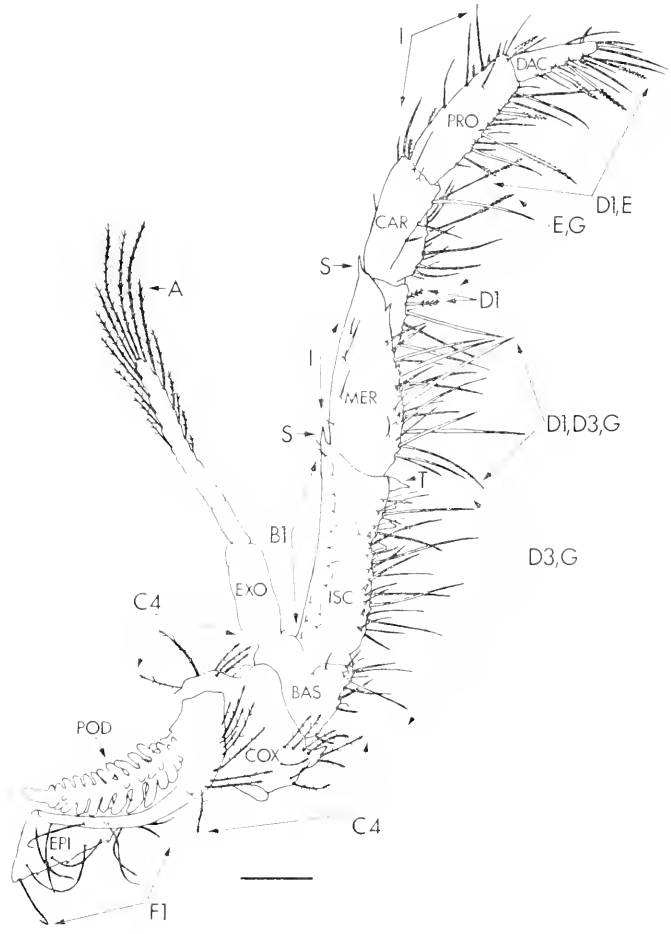


FIGURE 8. Third maxilliped (right) of fourth-stage lobster, showing the outer surface. Abbreviations are: DAC, dactyl; PRO, propus; CAR, carpus; MER, merus; ISC, ischium; BAS, basis; COX, coxa; EXO, exopodite; EPI, epipodite; POD, podobranch; S, spines at distal ends of ischium and merus; T, most distal tooth of the row of teeth on inner medial edge of ischium; A, plumose setae; B1, pappose setae; C4, plumodenticulate setae; D1 and D3, serrate setae; E, triserrate setae; F1, serrulate setae; G, triserrulate setae; I, simple setae; and J, hamate setae. Scale bar represents 0.25 mm.

mentary podobranch in the form of a small but distinct lobe is present near the base of the first-stage epipodite.

By the third stage, the single cuspidate seta at the tip of the dactyl has lost its setules. Also present on the dactyl are several setae with stout shafts but fine setules. It seems likely that one of these setae will lose its setules and become the second cuspidate (H1) seta, and that others are the forerunners of the serrate (D1) setae of the fourth stage. The exopodite has developed a flagellum of about six segments with plumose (A) setae at its tip and is similar to the exopodite of the fourth stage.

The podobranch is somewhat larger but remains a simple lobe in the third stage. Although the podobranch is still rudimentary by the fourth stage, the main axis has acquired secondary lobes typical of the trichobranch type of gill.

Third maxillipeds. The third maxillipeds, together with the mandibles, are the mouthparts most responsible for the mastication of food. The form of the third maxillipeds is similar to that of the second pair. A five-segmented endopodite, flagelliform exopodite, and lamellar epipodite extend from the protopodite (Figs. 8, 9).

In the fourth stage, although its lateral surfaces are almost bare, the endopodite is heavily setose on its medial surfaces and edges, the parts of the mouthpart which come into contact with food being passed to the mouth. The dactyl and propus are flattened laterally, so that each has medial and lateral surfaces and inner and outer edges, and bears serrate (D1), triserrate (E), and simple (I) setae.

The carpus, merus, and ischium would appear somewhat triangular in cross section and have three edges which can be considered inner medial, outer medial, and lateral. The edges define three surfaces: medial, outer lateral, and inner lateral (Fig. 23). The inner medial edge of the carpus is densely setose and bears serrate (D1), triserrate (E) (Fig. 17), and triserrulate (G) setae. Both inner and outer medial edges of the merus bear triserrulate (G) and serrate (D1 and D3) setae. The distal end of the lateral edge of the merus forms a stout spine pointing distally.

The ischium is the longest segment of the third maxilliped. Its outer lateral surface bears short pappose (B1) setae, some of which lie in a row along the axis of the segment. As in the merus, the distal end of the lateral edge of the ischium is extended into a substantial spine (Fig. 23). A row of serrate (D3) and triserrulate (G) setae and four or five small spines pointing distally (Fig. 8) are present on the outer medial edge. Of special interest is the nature of the inner medial edge of the ischium. This edge consists of a row of approximately fourteen stout teeth (Fig. 23). A row of up to seven simple (I) setae is present on the inner lateral surface, parallel to the row of teeth (Fig. 23).

The medial edge of the basis is an extension of the outer medial edge of the ischium and bears the same kinds of spines and setae. The basis gives rise to a typical exopodite divided into an unsegmented basal region and a flagellum of approximately twelve segments, most of which bear a pair of long, opposite plumose (A) setae (Fig. 24). Plumodenticulate (C4) setae are scattered over much of the coxa on both its inner and outer surfaces. The epipodite bears hamate (J) and serrulate (F1) setae, as well as a row of plumodenticulate (C4) setae on its

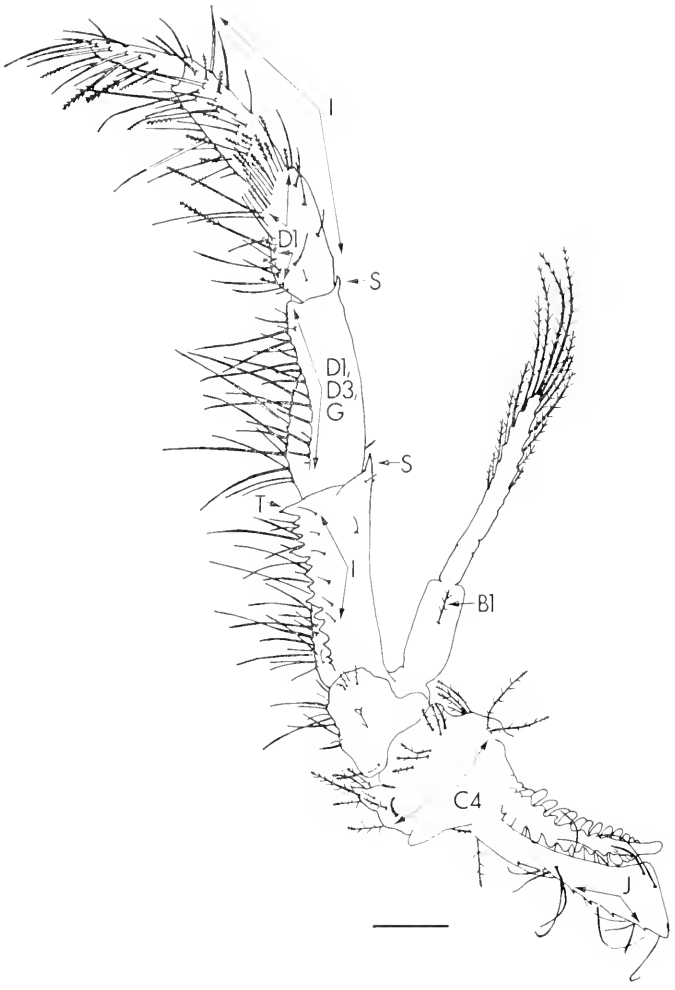


FIGURE 9. Third maxilliped (right) of fourth-stage lobster. View of the inner surface. Segments and abbreviations as identified in Figure 8. Scale bar represents 0.25 mm.

outer edge. Five rows of short filaments extend from the central axis of the well developed podobranch, which is much further advanced than that of the second maxilliped and clearly shows the trichobranch architecture.

The structure of the third maxilliped in the first-stage larva is generally similar to that of the fourth, although, as in other cases, it is less densely setose. Nevertheless, several significant changes do occur between the first and fourth stages.

The dactyl bears only about eight setae in the first stage, mostly of the serrulate (F1) type. There are several long setae at the tip of the dactyl, one of which is quite stout and simple (I) in most specimens. It is reminiscent of the single cuspidate seta at the tip of the first-stage second maxilliped but is longer and lacks thick walls and needle-like setules. The propus bears triserrate (E), serrulate

(F1), and serrate (D2) setae with scales. On the carpus can be found serrate (D1 and D2), serrulate (F1) and triserrulate (G) setae. The merus bears serrate (D1), serrulate (F1), and triserrulate (G) setae and is only indistinctly separated from the ischium. The setae on the endopodite are quite variable.

Herrick (1896, p. 196) implies that the prominent row of teeth on the inner medial edge of the ischium does not develop until the fourth stage. This was found to be incorrect. Most first-stage specimens examined already have a row of two to four rudimentary teeth. During the second and third stages approximately six teeth, rudimentary but increasingly substantial, are present. The fourth stage bears roughly fourteen formidable teeth (Fig. 23).

Several serrulate (F1) setae are present on the basis of the first stage. The flagellum of the exopodite comprises about eight segments, each with a pair of plumose (A) setae. The exopodites on the third maxillipeds of the first three stages function as swimming organs along with the exopodites of the five pereiopods. These are the only instances in which the exopodites of the mouthparts aid in swimming.

Few serrulate (F1) setae are borne on the coxa in the first stage. The epipodite may bear serrulate (F1), but no hamate (J), setae and has a podobranch with three or four rows of short filaments. It may be noted that the first-stage podobranch of the third maxilliped is better developed than the fourth-stage podobranch of the second maxilliped. Several hamate (J) setae are present on the second-stage epipodite. The podobranch of the third stage carries four rows of filaments around the central axis; this increases to five rows in the fourth stage.

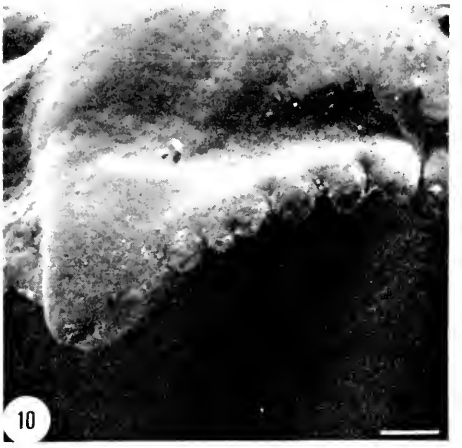
Discussion

Changes in the mouthparts which occur as lobsters pass through the larval stages generally include increases in the size of mouthparts and in the number of setae borne on them. Several other trends of particular interest, however, warrant further discussion.

It has previously been mentioned that the mandibles and third maxillipeds are the most important masticatory appendages. Observations of feeding behavior in an adult lobster reveal that the teeth on the ischium of the third maxillipeds are used to grasp one end of a string of food while the other end is held firmly between the mandibles. Downward movement of the third maxillipeds then causes the food to be stretched and torn as part of the process of mastication before it is swallowed.

The most striking change in the mandible during the larval stages is the development of the teeth of the cutting edge, in which the relatively delicate, seta- or spine-like teeth of the first stage are progressively transformed into the molars of the fourth stage. Similarly, a prominent change in the third maxilliped is the development of the teeth on the inner medial edge of the ischium. The few, insignificant teeth present in the first stage are replaced by approximately fourteen substantial teeth in the fourth stage.

Analyses of the stomach contents of hatchery- and aquaria-raised first through fourth stage lobsters by Williams (1907) and Herrick (1896) have shown the diet to comprise a variety of small planktonic organisms, including diatoms, bacteria,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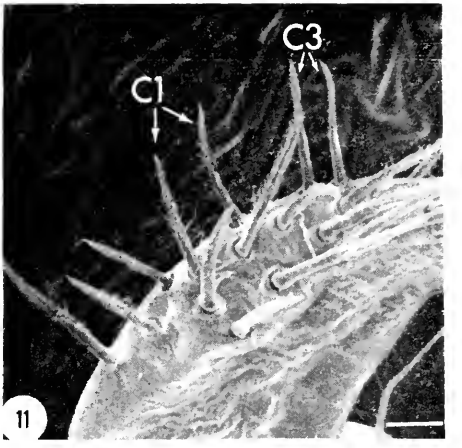
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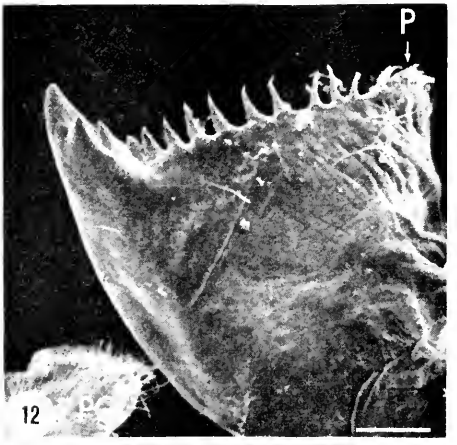
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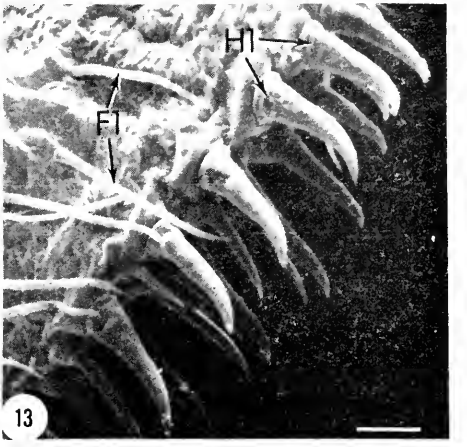
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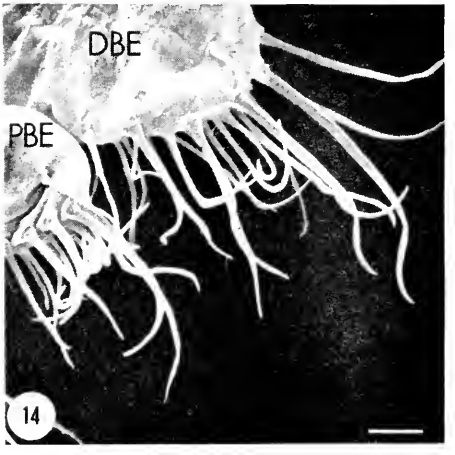
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copepods, filamentous algae, and parts of larval decapods (including lobsters). Studies of stomach contents of adult lobsters (Herrick, 1896; Weiss, 1970; Ennis, 1973) indicate that the diet includes crabs, isopods, sea urchins, sea-stars, snails, clams, polychaetes, fish, eelgrass, hydroids, ascidians, and ectoprocts. The change in diet which accompanies the lobster's assumption of the benthic habitat requires a corresponding change in mouthparts.

It is evident that the development of the mandibles and third maxillipeds enables lobsters to deal successfully with the more substantial food they encounter in the benthic environment, which they usually enter at the fourth or fifth stage. Furthermore, the coordinated development of the teeth on the mandibles and on the ischium of the third maxilliped emphasizes the coordinated manner in which these appendages function. Both features appear necessary for the food-manipulating process typical of the later stages. The development of what appear to be functional teeth on the ischium of the third maxilliped occurs at the time when the primary function of these appendages changes from swimming, in the first three stages, to feeding in all subsequent stages. The conclusions drawn from structural features should, however, be augmented by observations of living, feeding larval lobsters.

Larval lobsters may be usefully compared to the hatchlings of *Austropotamobius pallipes* described by Thomas (1973). First-stage hatchlings of this crayfish are less well developed than first-stage lobsters. They are attached to the pleopods

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FIGURE 10. Scanning electron micrograph of a fourth-stage mandible (left) illustrating one of the two major teeth and several smaller teeth of the cutting edge. Scale bar represents 25 μ. (Figures 10–24 are scanning electron micrographs illustrating the structure of the mouthparts and the setae they bear.)

FIGURE 11. Mandibular palp of a fourth-stage mandible (right). This view of the lateral surface of the terminal segment of the palp illustrates type C1 and C3 plumodenticulate setae. Notice the pappose setules on the proximal portion of the type C1 setae and the absence of setules on the proximal portion of the C3 setae. Scale bar represents 25 μ.

FIGURE 12. Gnathal lobe of a first-stage mandible (right, outer surface). The distal end of the cutting edge bears a single major tooth and two associated smaller teeth. The position of the "pad" of setae (P) can be seen at the proximal end of the cutting edge, although most of the setae in this field are on the inner surface and cannot be seen in this view of the outer surface. A paragnath (PG), covered with very fine, simple setae can also be seen. Scale bar represents 50 μ.

FIGURE 13. Two rows of cuspidate (H1) setae on the medial edge, and a row of serrulate (F1) setae on the outer surface, are visible on the basal endite of a fourth-stage first maxilla (right, outer surface). Scale bar represents 25 μ.

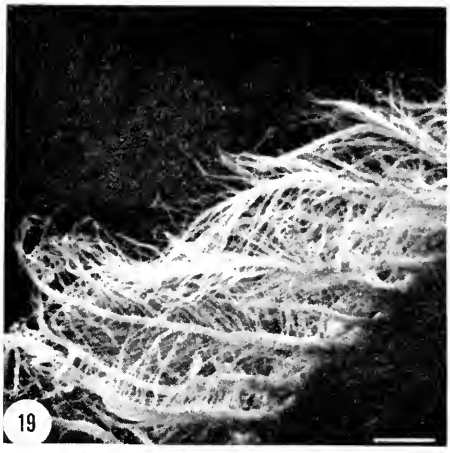
FIGURE 14. Distal (DBE) and proximal (PBE) lobes of the basal endite of a first-stage second maxilla showing mostly simple (I) setae on the medial edge. Scale bar represents 25 μ.

FIGURE 15. Pappose (B1) setae from the basal endite of a fourth-stage first maxilliped. Scale bar represents 25 μ.

FIGURE 16. Serrulate (F1) seta from the propus and carpus of a first-stage second maxilliped. Scale bar represents 10 μ.

FIGURE 17. A triserrate (E) seta on the carpus of a fourth-stage third maxilliped showing three rows of setules (arrows). Scale bar represents 10 μ.

FIGURE 18. A type D3 serrate seta on the outer medial edge of the ischium of a fourth-stage third maxilliped. The two rows of tooth-like setules can be seen as well as a third row of shorter, finer setules (arrow) which distinguish type D3 from the other serrate setae. Scale bar represents 10 μ.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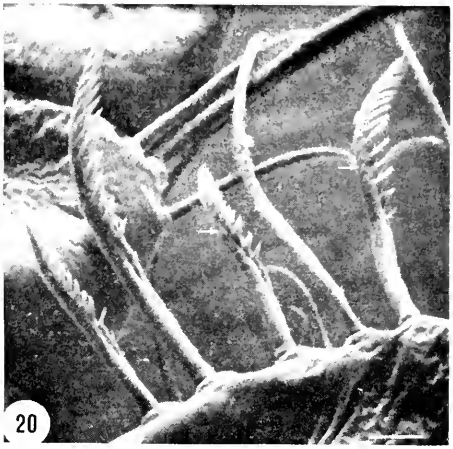
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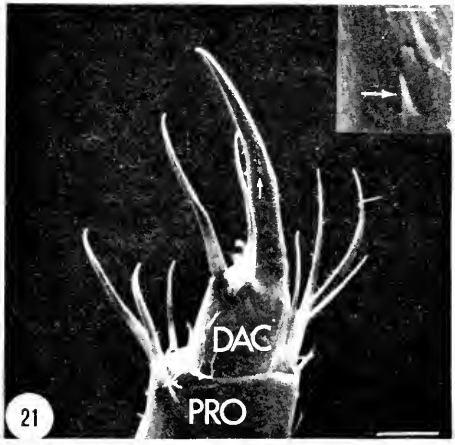
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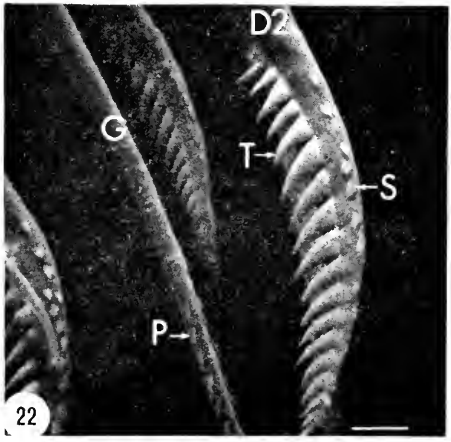
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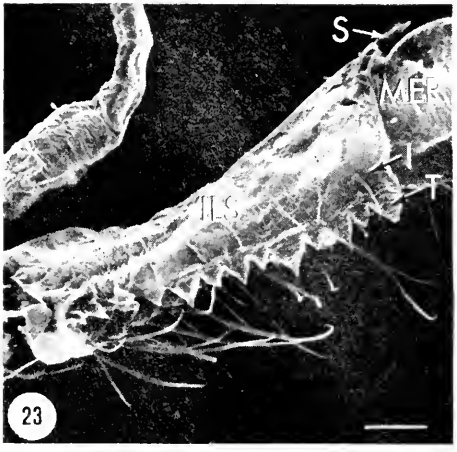
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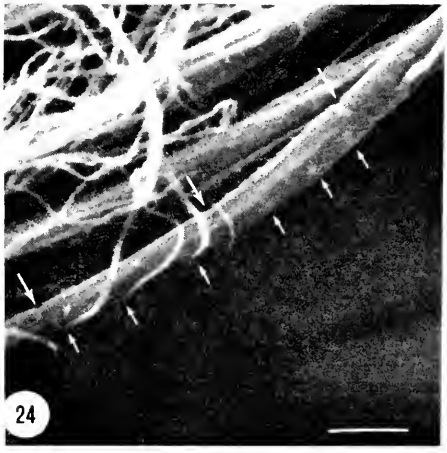
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of the mother by a thread of chitin and are not active feeders but survive on the remnant of the yolk. The mandibles reflect this condition: they have no teeth or molar processes and are lightly cuticularized. First-stage crayfish bear relatively few setae, and Thomas (1970) suggests that those that are present are all associated with the creation of respiratory currents, for example the fringe of setae on the scaphognathite. In contrast, first-stage lobsters are free-swimming, feeding organisms, with mouthparts adequately developed to handle appropriate food; the setae they bear are not restricted to respiratory current-production but presumably also play an important role in feeding.

Gonor and Gonor (1973), who studied setation in procellanid crabs, state that analysis of the variations in setal counts may be a useful tool for distinguishing among different populations of larvae from the same species. A similar problem has been approached by Rogers, Cobb and Marshall (1968) who used variations in size to distinguish larvae of inshore populations from those of offshore populations of *Homarus americanus*. The results of Gonor and Gonor (1973) suggest that comparisons of setation might provide additional criteria for making this distinction.

While the setae of decapods probably serve a variety of sensory functions, perhaps the most extensively studied is chemoreception. Most research in this area has been concerned with distance chemoreception (or smell) in the antennules, especially the structure and electrophysiology of the aesthetasc setae they bear. There has also been considerable interest in contact chemoreception (or taste) in the setae on the tips of the pereiopods, particularly from an electrophysiological viewpoint. Several studies of the pereiopods have also included evidence of mechanoreception. Few physiological studies have identified the setae acting as end organs or have even described the types of setae present in the region under investigation. Papers

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FIGURE 19. Dense fringe of plumose (A) setae on the lateral edge of the exopodite of a fourth-stage first maxilliped. Scale bar represents 25 μ.

FIGURE 20. Second maxilliped of a fourth-stage lobster (right) showing four type D2 serrate setae at the distal end of the medial edge of the merus. Arrows indicate the scale-like setules typical of type D2 setae. Scale bar represents 25 μ.

FIGURE 21. Tip of a first-stage second maxilliped illustrating the single, terminal cuspidate (H2) seta and the arrangement of serrulate (F1) setae on the dactyl (DAC) and propus (PRO). Arrow indicates row of setules on type H2 seta. Scale bar represents 50 μ. Inset: arrow indicates a single setule from a type H2 seta. Scale bar represents 5 μ.

FIGURE 22. Several type D2 serrate setae (from the merus of a first-stage second maxilliped) with tooth-like (T) and scale-like (S) setules. Also visible is a triserrulate (G) seta with three rows of peg-like setules (P). Scale bar represents 10 μ.

FIGURE 23. Ischium of a fourth-stage third maxilliped (left, inner view). The row of prominent teeth (T) on the inner medial edge of the ischium is clearly visible in this micrograph. A row of six simple (I) setae is present on the inner lateral surface (ILS), parallel to the row of teeth. The distal end of the ischium is extended into a substantial spine (S) which overlaps the merus (MER). The setae from the outer medial edge are visible behind the row of teeth. (Refer to Figs. 8 and 9 for orientation and terminology.) Scale bar represents 100 μ.

FIGURE 24. Plumose (A) setae on the tip of the exopodite of a fourth-stage third maxilliped. Several annulations of the setal shaft (large arrows) are clearly visible in this micrograph. Three pairs of setules extend from each section of the shaft between annulations. Small arrows indicate the points of insertion on the shaft of intact setules or setules which have broken. Scale bar represents 10 μ.

concerned with chemoreception in *Homarus gammarus* (=*H. vulgaris*) and *Homarus americanus* include the studies of Laverack (1963) and Shelton and Laverack (1968), on the response of chemoreceptors on the dactyl of the walking legs to various stimulants, and those of McLeese (1970), Ache (1972), Mackie (1973), and Shepheard (1974), who examined distance chemoreception and the sensitivity of chemoreceptors in the antennules.

Apparently, however, very little interest has been shown in chemosensory structures associated with the mouthparts. An exception is a paper by Shelton and Laverack (1970) who studied the adult mouthparts and pereiopods of the European lobster, *Homarus gammarus*. They state that all mouthparts and pereiopods bear chemoreceptive endings. While these investigators made no attempt to survey the various types of setae that might be involved in chemoreception, the type responsible for this function appears, from their illustrations, to be identical with the serrate setae described in the present work.

It is only to be expected that many of the setae on mouthparts are sensory, but the work of Shelton and Laverack (1970) appears to be the only instance in which this function has been experimentally demonstrated. If the serrate setae of the larvae of *Homarus americanus* are also chemosensory, one would expect them to be located in places where the mouthparts come into contact with food. This is, in fact, the case on the second and third maxillipeds where the serrate setae occur along the medial portions of the appendages. Although Shelton and Laverack (1970) found serrate setae on all six pairs of mouthparts of adult *Homarus gammarus,* setae of this type are present only on the second and third maxillipeds of larval *Homarus americanus.* This does not necessarily mean that chemoreception is limited to the second and third maxillipeds among the mouthparts; it is, more likely, an indication that other types of setae are also chemosensory.

Several other functions of setae, in addition to chemoreception and mechanoreception, have been reported. Bauer, studying the pandalid shrimp *Pandalus danae* (1975) and several species of caridean shrimps (1977), describes serrate setae on the third maxillipeds and pereiopods. He assigns to these setae a rasping function and emphasizes the role they play in grooming. This position creates a conflict: are the serrate setae rasping brushes used for grooming (Bauer) or are they chemosensory end organs used for tasting (Shelton and Laverack, 1970)? Bauer asserts (1975, p. 70) that "the complex tooth and scale setulation are primarily adaptations to rasping and scraping", although "all of these serrate setae could concomitantly be chemoreceptive as well." Roberts (1968) has also correlated serrate setae with cleaning processes in his study of hermit crabs, and Herrick (1911, Pl. XXXVI, Fig. 5) refers to the medially directed groups of setae on the endopodite of the adult third maxilliped as "cleaning brushes" and describes their use in cleaning the antennae of the lobster. It is possible that the serrate (and also serrulate, triserrate, and triserrulate) setae on the third maxillipeds of larval lobsters aid in grooming, but observations of grooming behavior in larvae are lacking.

Filter feeding has been described in a variety of decapods. Gonor and Gonor (1973b) describe the changes which take place in several porcellanid crabs as they pass from the carnivorous zoeal stages to the filter-feeding megalopa. Gerlach,

Ekström, and Eckardt (1976) found that the hermit crab *Pagurus bernhardus* is capable of removing nauplii of the brine shrimp (*Artemia*) and unicellular algae (*Dunaliella*) from suspension. Budd and Lewis (1977) report filter feeding in the crayfish *Orconectes immunis* which appears to have no elaborate modifications of the mouthparts for that purpose. The filter comprises setae on the second maxillae and first maxillipeds, and the exopodites produce water currents which carry particles through the filter apparatus. It will be recalled that Herrick (1896) and Williams (1907) found diatoms in the stomachs of larval lobsters (*Homarus americanus*); however, Williams (p. 176) thinks these are not taken as food, but rather enter the stomach "merely because it is impossible to avoid these omnipresent organisms." The possibility that diatoms and other very small particles of food are obtained by filtering the surrounding water deserves attention.

Some setae present on larval lobsters are of such form and are present in such positions as to suggest that their main function is to aid in the manipulation of food. Examples include the cuspidate setae present on the basal endite of the first maxilla and the cuspidate and serrate setae on the dactyl of the second maxilliped. It is easy to view them as spikes which give the mouthparts purchase so that they may better move food toward the mouth, and which at the same time aid in mastication.

Still another function of some types of setae is to extend the effective area of structures responsible for creating water currents. Plumose setae seem best adapted to this function. They fringe the scaphognathite of the second maxilla, which creates the respiratory current through the gill chamber, and the exopodites of the maxillipeds, which create (in the adult lobster, at least) currents effective in the removal of the debris of feeding. In the first three stages the exopodites of the third maxillipeds, along with those of the pereiopods, also serve the important function of swimming. Additionally, the fringe of plumose setae may serve the gasket-like function of sealing the space between the scaphognathite and the wall of the gill chamber, thereby preventing backflow during the pumping process.

A complete understanding of the functioning of the mouthparts of larval lobsters will require further investigation; the first step toward this end, however, is to understand the structure of the mouthparts and to appreciate the diversity of form of the setae they bear.

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Summary

1. This study provides a detailed account of the morphology of the mouthparts of larval lobsters (*Homarus americanus*) and the setae they bear. The results describe the types of setae found on the mouthparts, present a scheme for their classification, describe the distribution of the various setal types, and present observations of interesting or previously overlooked features of the mouthparts themselves.

2. A scheme of classification (based on the external morphology of the setae, particularly the nature and distribution of the setules) has been devised to describe and categorize the types of setae found on the mouthparts. The setal complement may be arranged into ten major categories, in some of which the variation is sufficient to warrant subdivision into several types of setae.

3. Detailed descriptions of the mouthparts and the distribution of their setae are presented and major developmental trends are noted. Changes in the mouthparts which occur as lobsters pass through the first four stages generally include increases in the size of mouthparts and in the number of setae they bear. Of special interest is the transformation of the comparatively delicate teeth of the first-stage mandible into the massive molars of the fourth stage. The coordinated development of teeth on the gnathal lobe of the mandible and on the ischium of the third maxilliped emphasizes the coordinated manner in which these appendages function. It is evident that the development of the mandibles and third maxillipeds enables lobsters to deal successfully with the more substantial food they encounter in the benthic environment, which they usually enter at the fourth or fifth stage.

4. The various functions and possible functions (for example, chemosensory, tactile, and mechanical) of the setae borne on the mouthparts are discussed in light of the available functional and physiological evidence. The possibility of filter feeding in lobsters, particularly in the constantly-swimming, planktonic larval forms, is considered.

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OSMOTIC AND IONIC REGULATION IN SEVERAL WESTERN ATLANTIC CALLIANASSIDAE (CRUSTACEA, DECAPODA, THALASSINIDEA)[1]

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Thalassinid mud shrimps of the genera *Callianassa* and *Upogebia* are frequently characterized as capable of ionic and volume regulation but incapable of osmotic regulation (Gross, 1957; Brown and Stein, 1960; Lockwood, 1962; Kinne, 1963). Studies by Zenkevich (1938), L. Thompson and Pritchard (1969), and Hill (1971), however, document osmoregulatory ability among upogebids. The assumed absence of this ability among callianassids is meanwhile supported by L. Thompson and Pritchard's (1969) studies of *Callianassa californiensis* and *C. filholi*. The recent report of strong ionic and osmotic regulation in *C. kraussi* from southern Africa (Forbes, 1974) constitutes the first evidence of such ability within the genus. However, other *Callianassa* species are also in some way adapted to low or varying salinities (Monod, 1927; Hedgpeth, 1950; Wass, 1955; Phillips, 1971; Rodrigues, 1971; LeLoeuff and Intes, 1974). Generalizations at the generic level must, therefore, await further physiological studies or perhaps be altogether abandoned until the systematic fate of the genus *Callianassa* Leach has been resolved; revisions proposed by de Saint Laurent (1973), for example, would partition *Callianassa* into six genera.

The present study compares osmotic adaptations of three species of Callianassidae from Louisiana and correlates these adaptations to local distributions. Specifically, salinity tolerance, osmotic regulation, and ionic regulation are reported. Despite the wide distribution of the species concerned, their trophic significance (Frankenberg, Coles, and Johannes, 1967), their potential as bait fisheries (Hailstone and Stephenson, 1961; Bybee, 1969), and the value of mud shrimp burrows in interpreting ancient environments (Weimer and Hoyt, 1964; Dewindt, 1974), basic understanding of their salinity tolerances and regulatory capacities is lacking.

Species concerned in the present study are *Callianassa jamaicense* Schmitt, 1935, *C. major* Say, 1818, and *C. islagrande* Schmitt, 1935, all of which fall within the subgenus *Callichirus* Stimpson, 1866. In a study of western African thalassinids, LeLoeuff and Intes (1974) note that *Callichirus* is frequently euryhaline and typically restricted to littoral waters in tropical latitudes. Habitats of *Callianassa* on the Louisiana coast are poorly documented, except in observations made on several coastal islands by Willis (1942); he notes predominance of *C. islagrande* on front beaches, interspersion of *C. islagrande* and *C. major* on ends of islands,

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and predominance of *C. jamaicense* on back sides of islands and in backbeach pools. Distributions are largely attributed to sediment characteristics as in a later study of *C. islagrande* and *C. jamaicense* on the Mississippi coast (Phillips, 1971).

North Atlantic coastal habitats of *C. major* are described by Lunz (1937), Pohl (1946), Weimer and Hoyt (1964), and Frankenberg *et al.* (1967); limited colonization of estuary mouths is noted, and *C. major* is usually reported from higher-salinity open beaches. Rodrigues (1971) suggests some tolerance to variations in salinity by *C. major* in Brazil but reports *C. jamaicense* to survive at the mouth of the Rio Caravelas. Hedgpeth (1950) notes *C. jamaicense* to inhabit estuarine mud flats on the Texas coast. Wass (1955) reports *C. jamaicense* from estuaries in northwestern Florida but lists *C. islagrande* only from the higher-salinity intertidal zone of Gulf beaches.

Materials and Methods

Studies were conducted from January, 1972, to December, 1974. Initially, distributional records were supplemented by collecting callianassids from all accessible localities. Collecting techniques included shoveling and sieving, coring with a "yabby pump" (Hailstone and Stephenson, 1961), and using a portable water jet to obtain specimens, much as described by Bybee (1969). Except for some *C. islagrande* taken by shovel and sieve, animals for experimental studies were collected by the water jet method as it was the most productive and least injurious to animals.

All specimens of *C. jamaicense* used in experimental studies were collected from the perimeter of a tidally influenced pond near the Louisiana Wildlife and Fisheries Commission Marine Laboratory on Grand Terre Island. To prevent injury to animals, each was placed into a perforated, plastic vial. An insulated ice chest containing water from the collecting site was used to transport animals to the laboratory.

Animals were maintained unfed in individual, perforated vials throughout acclimation periods. Early in the study, free-swimming animals were held in sea water (SW) without isolation, and over 90% of 140 *C. jamaicense* perished within two days of collection. Aggressive encounters between individuals in a common container resulted in mutilation and consequent bleeding which accounted for high mortality.

Within two days of collection, after water in the ice chest had equilibrated to room temperature (25 ± 1° C), the animals and vials were transferred to artificial SW equivalent (±1‰ salinity) to that from the pond. Two to three days were then allowed for attrition of animals injured during collecting. Ovigerous females, injured animals, and animals showing postmolt characteristics detailed by L. Thompson and Pritchard (1969) were not used in experimental studies.

Acclimation solutions were prepared by dilution of artificial SW with deionized water. Salinities were approximated with a refractometer. Animals were acclimated stepwise in 5‰ increments or decrements per day in the dark at 25° C with continuous aeration. Animals were maintained at the final acclimation salinity for nine days before blood was sampled.

One group of *C. jamaicense* was acclimated to 20‰ for nine days after which half were weighed and transferred directly to 3‰; the rest were weighed and transferred to 37‰. Animals were rinsed with deionized water and thoroughly blotted dry before being weighed to the nearest milligram. Five individuals were removed from each salinity extreme at timed intervals, rinsed, blotted and re-weighed; blood was then sampled and the animals were lyophilized to constant weight. The same rinsing and blotting procedures were followed with all animals from which blood was sampled.

Blood was obtained by puncturing the arthrodial membrane just posterior to the coxa of the fifth pereiopod; 20 μl were drawn for determination of osmotic concentration and another 20 μl were immediately diluted for ion analyses; squeezing of animals was avoided. Osmotic concentrations (mOsmol/Kg H_2O) of whole blood and acclimation media were determined with a Hewlett-Packard vapor pressure osmometer. An Aminco chloride titrator was used for chloride analyses. Sodium was determined with a Coleman flame photometer and magnesium with a Perkin-Elmer atomic absorption spectrophotometer.

Individuals of *C. major* used for experimental studies were collected from Grand Isle and Grand Terre Island and those of *C. islagrande* were taken from Isles Dernieres and Chenier Caminada. Acclimation of these species to salinities below 15‰ was in some cases attempted in 2.5‰ steps. Collecting, acclimating, blood sampling, and analysis techniques were otherwise as described for *C. jamaicense*. Direct transfers into 3 and 37‰ media were not attempted with *C. major* or *C. islagrande*.

Results

Distributions

As noted by Willis (1942) and Phillips (1971), distributions of the species studied are in part determined by substrate characteristics. *Callianassa jamaicense* is found most often in muddy substrates of back-beach ponds, estuarine flats, and tidal streams. Sandier substrates of beaches facing the open Gulf are the usual habitat of *C. major* and *C. islagrande*. However, lower salinities also typify most habitats of *C. jamaicense;* its distribution in Lousiana extends to well inside the 5‰ isohaline (Chabreck, 1972). Dense populations, with burrows exceeding 200/m², are found at 2 to 3‰ salinities near Johnson's Bayou and at 5 to 7‰ in the Lafourche Delta. On Grand Terre Island *C. jamaicense* occurs in salinities which vary seasonally from 6 to 28‰, and habitat includes bayward margins of Barataria Pass. Salinities at Barataria Pass commonly change by 10 to 15‰ over a period of a few hours (Hewatt, 1951).

By contrast, *C. major* and *C. islagrande* occur only in areas outside the 15‰ August isohalines of Chabreck (1972) and *C. islagrande* rarely occurs inside the 20‰ isohaline. *Callianassa islgrande* is the only callianassid found on Isles Dernieres front beaches which are bathed by high salinity coastal waters. Both *C. major* and *C. islagrande* are found on front beaches of Chenier Caminada and Grand Isle, but *C. major* predominates on the eastern portion of Grand Isle where salinities are less stable. On Grande Terre front beaches, which are inside the

20‰ isohaline (Chabreck, 1972), *C. major* is abundant and *C. islagrande* is uncommon.

During May, 1975, a mixed population of *C. major* and *C. islagrande* on Grand Isle was bathed by low salinity water (~7.0‰) for at least four days. Following the low salinities, numerous identifiable decomposing fragments of *C. islagrande* were found, but only *C. major* was found alive. Salinity of water issuing from *C. major* burrows ranged from 12 to 14‰. Of 40 *C. major* collected here half were held at a salinity of 7‰ and half were placed in artificial SW of 15‰ salinity. Those held at 7‰ were dead within two days, while most of those in 15‰ lived more than two weeks. Apparently, substrate interstitial water may adequately buffer *C. major* from some low overlying salinities, while *C. islagrande* succumbs under the same conditions. Populations of *C. islagrande* likely undergo mass mortalities where waters bathing beaches are subject to occasional extended periods of low salinity, as may be brought about by heavy rainfall, high rates of discharge from the Mississippi River, and the influence of winds and tides on water movement (Hewatt, 1951). The least vulnerable populations of *C. islagrande* are probably those on the front beaches of Isles Dernieres, Timbalier Island, and the Chandeleur Islands where salinity rarely falls to low levels.

Mortality, acclimation, and lower lethal limits

All experiments were completed within two to three weeks after animals were collected. Of the three species, *C. jamaicense* proved most hardy in the laboratory during and beyond this period, provided animals were isolated in individual vials. Mortality of *C. major* and *C. islagrande* during the first two to three days after collection ranged from 7 to 10%, probably from injuries during collection; during two to three weeks thereafter attrition ranged from 2 to 4% per week. Mortality of *C. jamaicense* seldom exceeded 2% during the first two to three days and

TABLE I

Survival during attempts to acclimate C. jamaicense, C. major, *and* C. islagrande *to low salinities.*

Species	Salinity transfer (‰)			Number at Start	Number surviving at final salinity		
	From	To	Step/day		Day 1	Day 3	Day 9
C. jamaicense	2.0	0.0	2.0	5	5	3	0
	15.0	2.0	5.0 and 3.0	10	10	9	9
	15.0	2.5	5.0 and 2.5	8	8	8	8
	20.0	3.0	17.0	50	50	50	50
	15.0	5.0	5.0	10	10	10	9
C. major	15.0	5.0	5.0	6	5	1	0
	15.0	7.5	5.0 and 2.5	12	9	4	0
	15.0	8.0	5.0 and 2.0	7	6	2	2
	15.0	10.0	2.5	20	20	15	14
C. islagrande	20.0	5.0	5.0	7	6	1*	1*
	20.0	10.0	5.0	7	5	2	1*

* Juvenile

TABLE II

Changes in wet weight during attempts to acclimate C. major *and* C. islagrande *to 5 and 10‰ media. (Per cent difference from original wet weight is given as mean ± standard error when more than one animal survived; numbers in parentheses for mortalities indicate numbers of animals surviving at the end of each day.)*

Species	Salinity transfer (‰)		Number of animals	Difference (%) from original wet weight				
	From	To		1 day	2 days	3 days	4 days	9 days
Mortalities:								
C. major	25	10	2	9.7 ± 0.8 (2)	15.3 ± 4.6 (2)	20.9 (1)	(0)	—
	10	5	5	15.5 ± 1.3 (5)	21.4 ± 3.0 (5)	21.2 (1)	(0)	—
C. islagrande	15	10	4	14.4 ± 1.4 (4)	17.7 ± 1.8 (3)	23.5 ± 4.5 (2)	31.2 (1)	(0)
	10	5	5	37.6 ± 3.5 (5)	42.6 (1)	(0)	—	—
Survivors:								
C. major	15	10	5	14.7 ± 1.5	10.9 ± 1.7	9.3 ± 1.2	4.8 ± 1.5	3.2 ± 0.7
C. islagrande	15	10	1*	13.3	9.0	2.7	0.4	0.4
	10	5	1*	16.9	12.3	11.9	5.6	3.2

* Juvenile

averaged < 1% per week under stable conditions for up to one month thereafter.

Of 180 *C. jamaicense* specimens collected in March and April, 1974, isolated in vials, and held unfed in static aerated aquaria at 25 ± 1° C, > 80% were still alive in November. Under similar conditions, < 40% of the *C. major* and 17% of the *C. islagrande* specimens survived beyond two months.

Lower and upper lethal limits of salinity were apparently not reached when *C. jamaicense* was acclimated to salinities from 2 to 45‰; mortalities were no more pronounced at extremes of salinity than at midrange salinities. Three specimens, transferred to deionized water after acclimation to 2‰ and sampling of blood, survived in excess of five days (Table I). No mortalities occurred during nine days after direct transfer of 50 *C. jamaicense* specimens from 20 to 3‰, and only one animal died during nine days after direct transfer of another 50 from 20 to 37‰.

The lower lethal salinity for *C. major* was attained just below 10‰ in several acclimation attempts (Table I). Although specimens were acclimated to 10‰ on several occasions, mortalities during nine days at the final salinity exceeded 25%; most deaths occurred during the first three days after the step from 12.5 to 10‰. Mortalities for *C. major* during acclimation to salinities from 12.5 to 40‰ did not exceed 10%. In an attempt to acclimate five specimens to 45‰, all animals died between the seventh and eighth days after transfer from 40‰.

Few specimens of *C. islagrande* were available for experimental studies and tolerance data are preliminary. Mortality was less than 10% during acclimation to salinities from 20 to 45‰. Below 20‰, acclimation was much less successful. Although one *C. islagrande* juvenile survived nine days at 5‰, and another survived nine days at 10‰, all attempts to acclimate adults to salinities ≤ 10‰ resulted in 100% mortalities within five days (Table I).

Weight changes were monitored during acclimation of some *C. islagrande* and *C. major* specimens to low salinities. Mortalities were preceded by substantial increases in wet weights, which suggests inability to regulate volume (Table II). Moribund animals under low-salinity stress had turgid abdomens

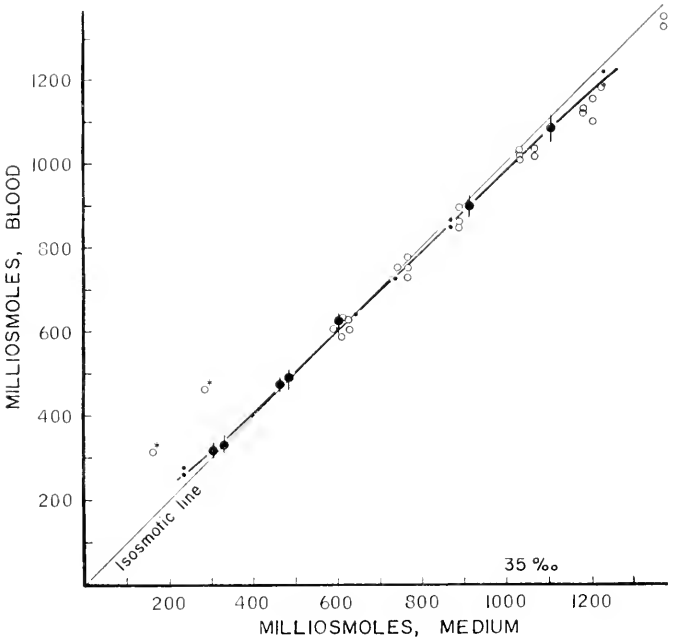

FIGURE 1. Blood osmotic concentration in acclimated *C. major* (solid circles) and *C. islagrande* (open circles) as a function of media osmotic concentrations. Each large solid circle is mean of 6 to 10 determinations; vertical lines indicate range; open circles and small solid circles are individual determinations. Heavy line is fitted to points for *C. major*. Asterisk denotes juveniles.

and branchiostegites which, by restricting movement and ventilation, probably caused respiratory stress. Those which survived low-salinity acclimation increased in weight initially, but began to regulate volume within three days; by the fourth day to ninth day of acclimation, wet weights returned to near original values.

Osmotic and ionic regulation

Blood of *C. major* and *C. islagrande* is nearly isosmotic to media over the entire salinity range in which these animals survive (Fig. 1). Slightly hyperosmotic values obtained for *C. major* at 8‰ salinity represent a low percentage of animals which survived at that extreme. Likewise, hyperosmotic values for *C. islagrande* at low salinities are from two juveniles which survived while nine adults died at these salinities.

Blood of *C. jamaicense* is hyperosmotically regulated at salinities $\leq$ 20‰ and shows little depression of osmolality at the lowest salinity extreme of 2‰ (Fig. 2). Slightly higher levels of hyperosmicity are maintained by January animals collected from a field salinity of 11‰ and temperature of 8° C than by August animals collected from 23‰ and 33° C. Blood is isosmotic to most media salinities $\geq$ 25‰ and very slightly hyposmotic at the upper-salinity extreme of 45‰.

Blood chloride is hypoionic in *C. major* and adult *C. islagrande* over the entire range of acclimation salinities, though less so at lower salinities (Fig. 3). Blood chloride was hyperionic in the two juveniles of *C. islagrande* which survived acclimation to 5 and 10‰ salinities. In *C. jamaicense* blood chloride is hypoionically regulated at salinities ≥ 20‰, isoionic at 15‰, and hyperionic at salinities < 15‰ (Fig. 3).

At salinities ≤ 20‰ blood sodium in *C. major* is isoionic to acclimation media (Fig. 4). Hypoionic regulation of sodium is exhibited at higher salinities, but not to levels as low as chloride. As with osmolality and chloride, sodium is hyper-regulated in the *C. islagrande* juveniles surviving low-salinity acclimation (Fig. 4). In *C. islagrande* adults acclimated to salinities from 25 to 45‰ blood sodium is hypoionically regulated to levels approximating those for *C. major*. Sodium levels in acclimated *C. jamaicense* are slightly hypoionic to media at salinities ≥ 25‰, near isoionic at 20‰, and markedly hyperionic at lower salinities from 15 to 2‰ (Fig. 5).

Blood magnesium in acclimated *C. major* is to some degree hyper-regulated at salinities ≤ 30‰ (Fig. 6); blood concentrations are maintained at about 6.0 mM/liter higher than media concentrations in salinities from 8 to 20‰. Hyper-regulation of magnesium is diminished at 30‰ and concentrations fall to slightly hypoionic levels at 37‰. In acclimated *C. jamaicense*, magnesium is slightly hyper-regulated

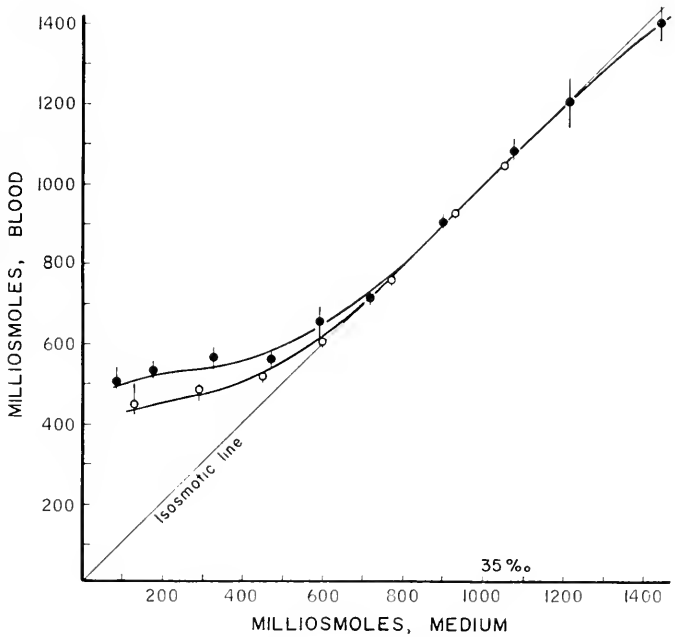

FIGURE 2. Blood osmotic concentration in *C. jamaicense* as a function of media osmotic concentrations after acclimation of summer (open circles) and winter (solid circles) animals at a media temperature of 25° C. Each open circle is mean of 5 to 6 determinations, and each solid circle is mean of 8 to 10 determinations; vertical lines indicate range.

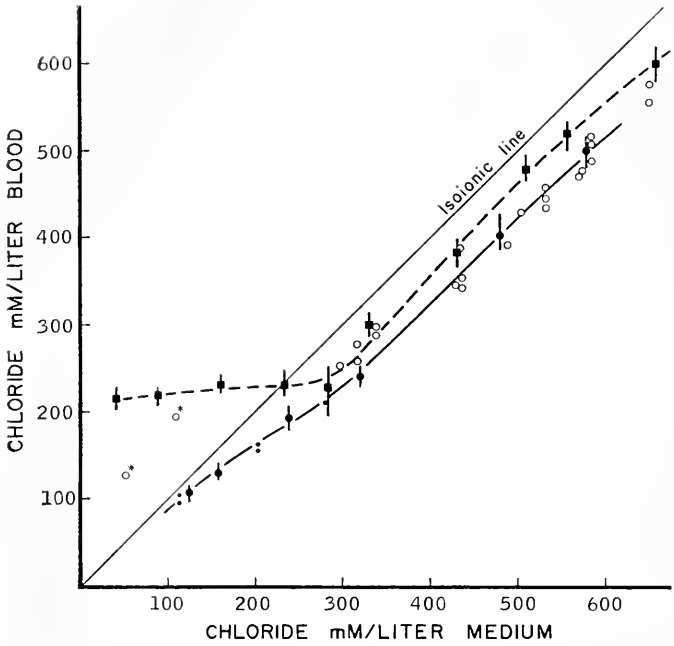

FIGURE 3. Blood chloride concentration in acclimated *C. jamaicense* (solid squares), *C. major* (solid circles) and *C. islagrande* (open circles) as a function of media chloride concentrations. Each solid square or large solid circle is mean of 6 to 10 determinations; each small solid or open circle is value for individual animal; vertical lines indicate range. Asterisk denotes juveniles.

at salinities from 10 to 25‰ and more markedly hyper-regulated below 10‰ (Fig. 6). Slight hypoionic regulation is exhibited by *C. jamaicense* at salinities $\geq$ 35‰. Blood magnesium for *C. islagrande* was not determined.

Osmoregulatory response of C. jamaicense *to dramatic salinity changes*

Temporal changes in body water, blood osmolality, and blood ion concentration were monitored following direct transfer of 20‰-acclimated animals to salinities of either 37‰ or 3‰. Body water increases slightly ($\sim$ 1%) during the first 12 hours after direct transfer to 3‰ medium but is maintained at levels equal to or slightly less than original values after 1 day (Fig. 7). Osmotic, chloride, and sodium concentrations of blood fall to near or just below stable concentrations during the first 12 hours in 3‰ medium (Figs. 8 and 9). Osmotic and sodium concentrations of blood show a slight undershoot after 12–24 hours, but at no time fall to the levels of the medium. Blood magnesium levels drop little over the first 6 to 12 hours, briefly recover, and then continue to drop at a decreasing rate over the entire nine day period (Fig. 10). Near stable levels of blood magnesium are achieved after four days, and concentrations are maintained above that of the 3‰ medium.

When *C. jamaicense* is directly transferred to 37‰ medium, body water de-

creases by ~ 3% over the first six hours and remains below original levels until the second day (Fig. 7). Osmotic, chloride, and sodium concentrations of blood increase markedly during the first day and continue to increase, at a decreasing rate, through day 9 (Figs. 8 and 9); near stable levels are attained by day 4. The levels of blood osmolality and sodium on day 9 approximate those of the 37‰ medium. Blood chloride remains slightly hypoionic to chloride concentrations of the medium through day 9. Blood magnesium increases slowly until the fourth day when it stabilizes at a level just below that of the medium (Fig. 10).

Discussion

Investigations by Teal (1958), Snelling (1959), Kinne (1963), and Barnes (1967) are among those which correlate osmoregulatory capacities of decapod crustaceans to their differential penetration of estuaries. Distributions of callianassids on the Louisiana coast also correlate with their osmoregulatory capacities and tolerance of dilute media. This is not to say that habitat preference is solely or even primarily determined by salinities. For example, despite its survival in varying salinities, *Emerita talpoida* is localized on wave-washed beaches by its feeding specialization (Bursey and Bonner, 1977). Devine (1966), Phillips (1971), and McLachlin and Grindley (1974) note the importance of substrate stability and composition in limiting distributions of burrowing thalassinids. How-

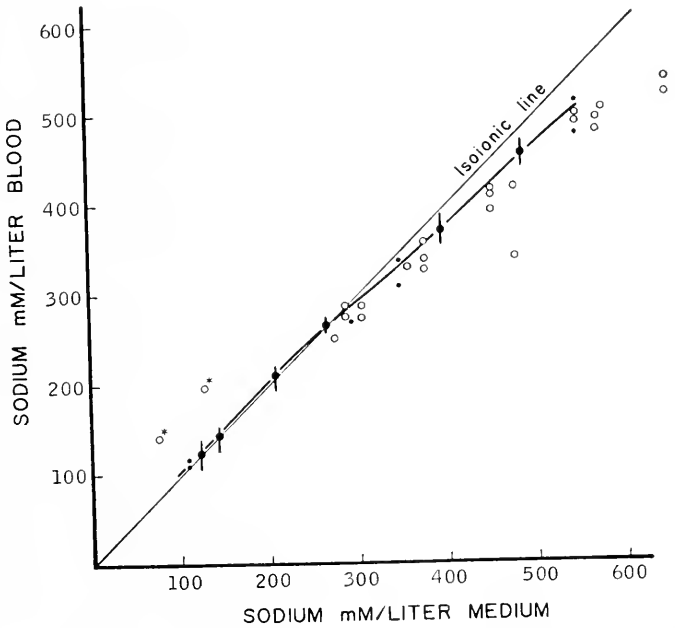

Figure 4. Blood sodium concentration in acclimated *C. major* (solid circles) and *C. islagrande* (open circles) as a function of media sodium concentrations. Each large solid circle is mean of 6 to 9 determinations; each small solid or open circle is value for individual animal; vertical lines indicate range. Heavy line is fitted to points for *C. major*. Asterisk denotes juveniles.

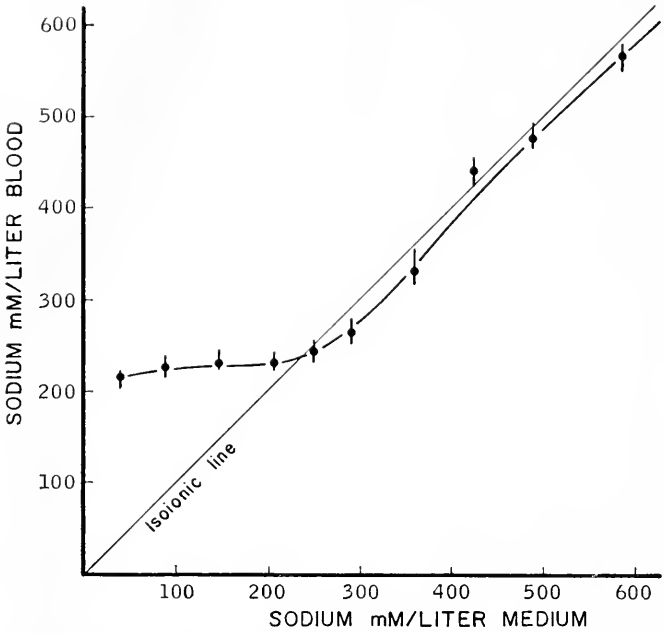

FIGURE 5. Blood sodium concentration in acclimated *C. jamaicense* as a function of media sodium concentrations. Each solid circle is mean of 7 to 10 determinations; vertical lines indicate range.

ever, both substrate and salinity are thought to limit penetration of *Callianassa australiensis* into estuaries (Hailstone and Stephenson, 1961).

The interaction of substrate and salinity accounts in part for distributions of Louisiana Callianassidae; for example, *C. jamaicense* survives at high salinities but is seldom taken above 25‰, because substrates in those areas of the coast are predominantly sand and therefore coarser than those in which Phillips (1971) reports it to burrow successfully. Conversely, *C. islagrande* is probably limited to transitory occurrence on Grand Terre Island and ends of other islands by fluctuating salinities, since sandy substrates in those areas differ little from substrates of high salinity beaches where *C. islagrande* is common.

Callianassa major and adult *C. islagrande* cannot osmoregulate but tolerate limited reductions of salinity. Similar findings are reported by L. Thompson and Pritchard (1969) for *C. californiensis* and *C. filholi*, which are likewise poikilosmotic but tolerate salinities down to ~ 10‰ and ~ 13‰, respectively. Osmoconformation and limited tolerance of dilute media are also reported for *C. affinis* by Gross (1957). It thus appears that the polystenohaline categorization, which was prematurely applied in general to *Callianassa* and *Upogebia* by earlier workers (Lockwood, 1962; Kinne, 1963), may be retained for at least five species of Callianassidae and probably for others which occupy similar habitats. However, some of these species are less stenohaline than others; the ability of *Callianassa major* to tolerate acclimation to 10‰ salinities while *C. islagrande* usually dies at

this salinity in part explains more frequent occurrence of *C. major* inside the 20‰ isohaline and its predominance on ends of coastal islands where salinities occasionally fluctuate. Preliminary evidence of low-salinity tolerance and slight hyperosmotic regulation in juveniles of *C. islagrande* suggests an ontogenic loss of tolerance and regulatory ability, although the two juveniles studied furnish an insufficient sample for firm conclusions. Juveniles of the hermit crab, *Pagurus bernhardus*, regulate volume in lower salinities than adults, and Davenport (1972a) suggests that the aperture of the nephropores in relation to body size limits this capacity in adults.

Tolerance of dilute media by *Callianassa major* and *C. islagrande* may prove of only short-term benefit for survival of populations. Hill (1971) notes that while *Upogebia africana* can tolerate a salinity of 1.7‰, it can only survive through a molt in a salinity $\geq$ 3.4‰. Although *C. major* and adult *C. islagrande* do not appear to osmoregulate (Fig. 1), their tolerance of dilute media may in part relate to accommodation of short-term increases in blood volume. The anterior portion of the abdomen is soft, and its elasticity may minimize mechanical effects of turgor. Davenport (1972b) suggests such an adaptation in *Pagurus bernhardus* and shows that with increased blood volume in low salinity, a larger proportion of the blood shifts from the thorax to the abdomen.

The degree of hypoionicity in blood chloride of *Callianassa major* and adult

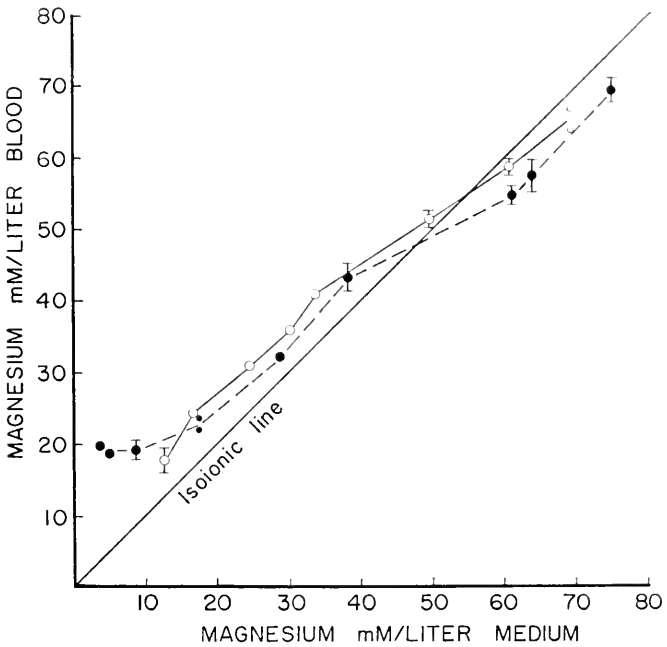

FIGURE 6. Blood magnesium concentration in acclimated *C. jamaicense* (solid circles) and *C. major* (open circles) as a function of media magnesium concentrations. Each large solid or open circle is mean of 6 to 10 determinations; vertical lines indicate standard errors where they exceed ±1.0; small solid or open circles are individual determinations.

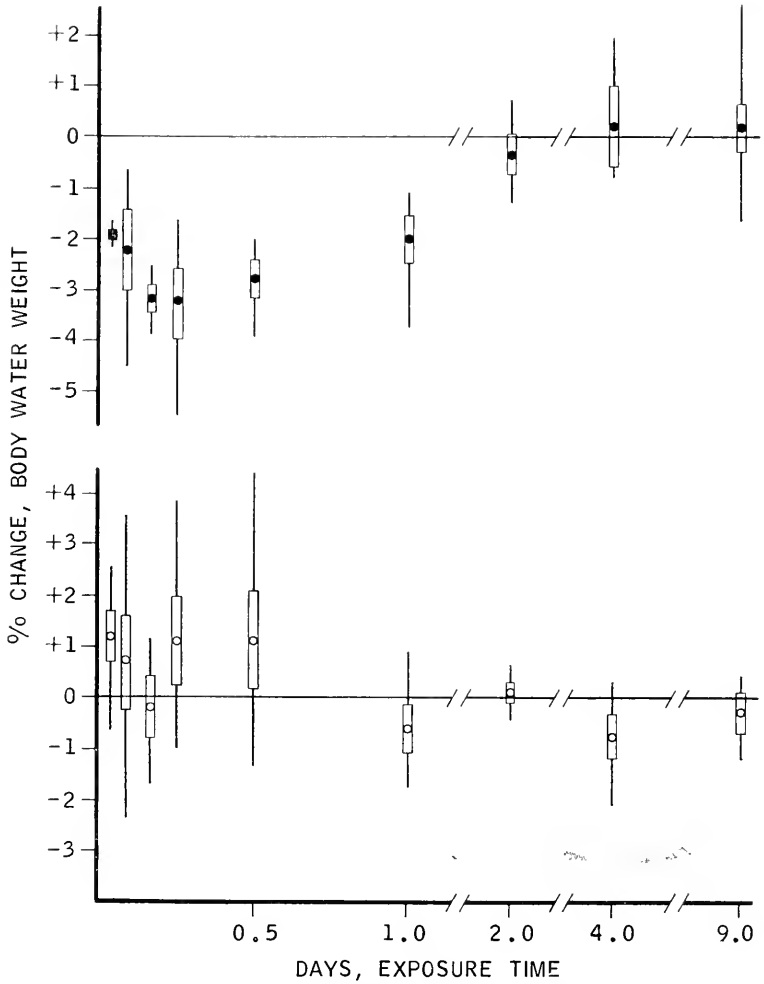

FIGURE 7. Percentage of change in weight of body water at timed intervals after direct transfer of *C. jamaicense* from 20‰ salinity to 3‰ (open circles) or 37‰ (solid circles). Each solid or open circle is mean of 5 determinations; rectangles indicate standard errors; vertical lines indicate range.

C. islagrande (Fig. 3) is very near that reported for *C. californiensis* by L. Thompson and Pritchard (1969). Some degree of ionic regulation is common to osmotically conforming crustaceans, but levels of blood chloride in such crustaceans are usually reported to approximate those of the media (Robertson, 1960; Potts and Parry, 1964). L. Thompson and Pritchard (1969) suggest that chloride hypoionicity may be attributable to a protein anionic component of blood in *C. californiensis*. However, as noted by Dall (1974), blood chloride is virtually equivalent to blood sodium at any given salinity despite the apparent difference when blood ion concentrations are plotted against media concentrations of the same ion. Hence,

where sodium and chloride in media are at normal SW ratios, sodium concentration being slightly less than that of chloride, equilibrium between the two ions is reflected in hypoionicity of chloride at any given medium concentration of chloride provided blood sodium is near or below sodium concentrations of the

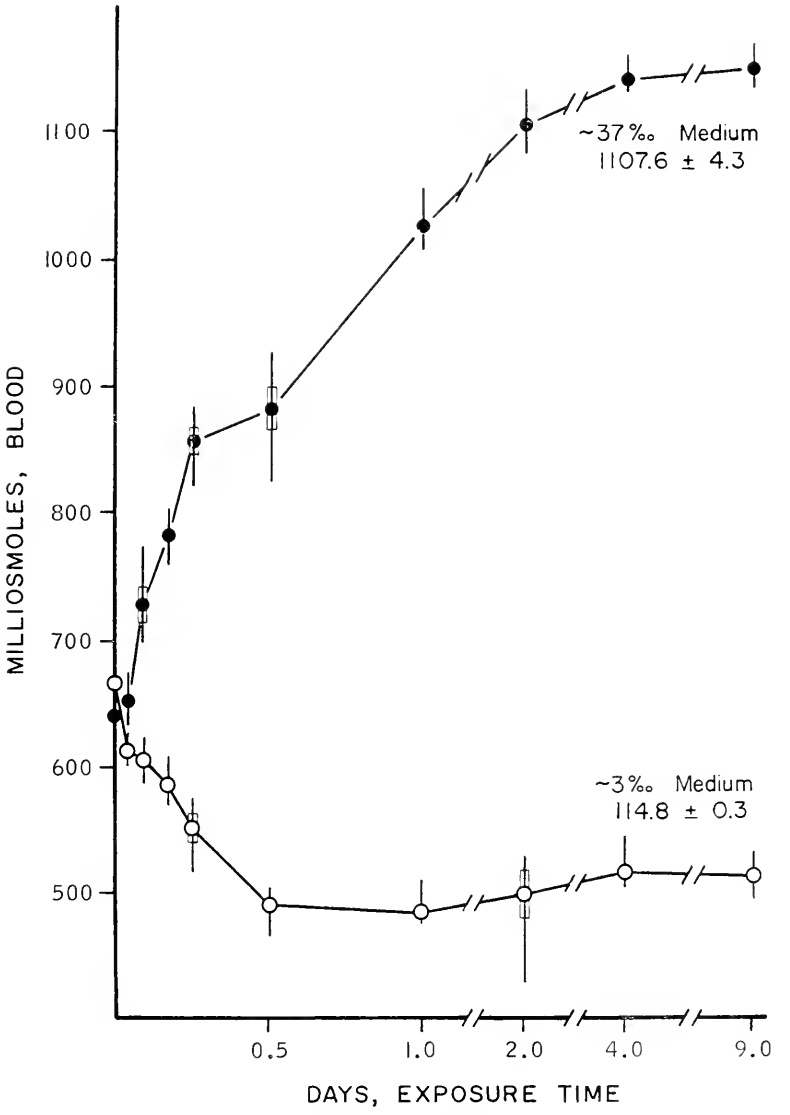

FIGURE 8. Blood osmotic concentration at timed intervals after direct transfer of acclimated *C. jamaicense* from 20‰ salinity to 3‰ (open circles) or to 37‰ (solid circles). Each solid or open circle is mean of 5 determinations; vertical lines indicate range; rectangles indicate standard errors where they exceed ±10. Figures beneath salinities indicate means and standard errors of media osmotic concentrations over 9-day period.

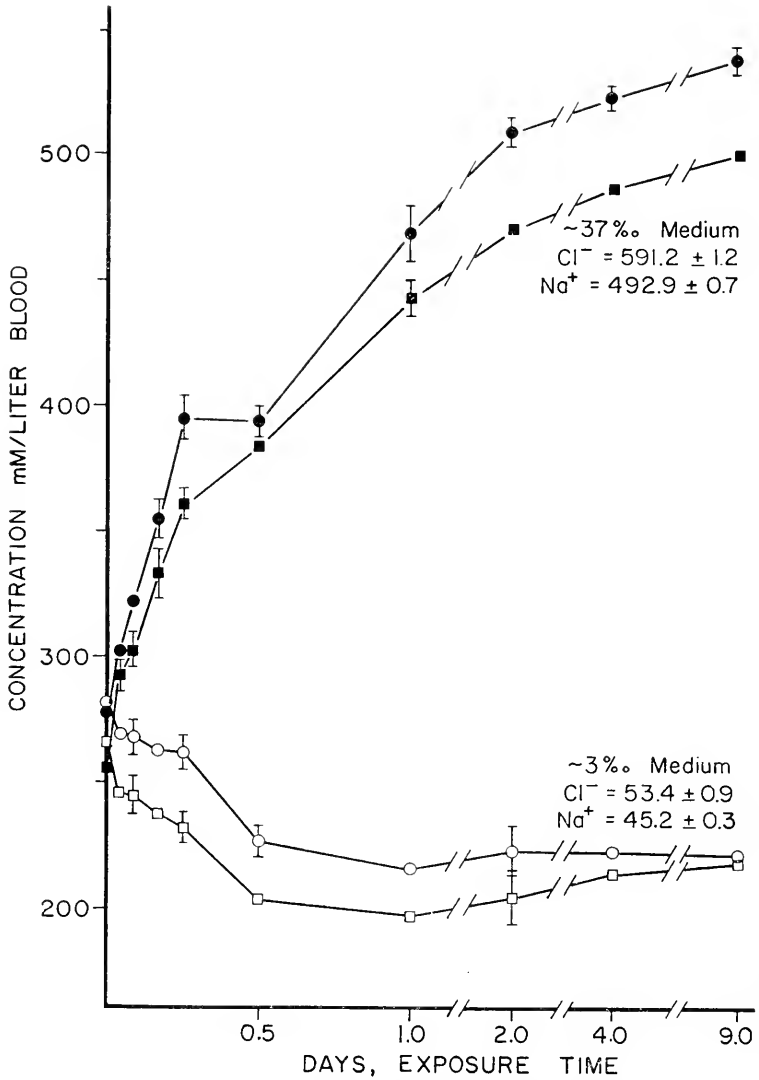

FIGURE 9. Blood chloride (open and solid circles) and sodium (open and solid squares) concentration at timed intervals after direct transfer of acclimated *C. jamaicense* from 20‰ salinity to 3‰ (open circles and squares) or to 37‰ (solid circles and squares). Each circle or square is mean of 5 determinations; vertical lines indicate standard errors where they exceed ±5. Figures beneath salinities indicate means and standard errors of media ion concentrations in mM/liter over 9-day period.

medium. Blood chloride in *Callianassa* (Fig. 3) exceeds blood sodium concentrations (Figs. 4 and 5) at each acclimation salinity and the degree to which it does so increases with increasing salinity, probably in electrochemical response to increased concentrations of magnesium and other cations. Blood sodium in *C.*

major and adult *C. islagrande* is equivalent to media concentrations ≤ 300 mM/liter and, much as blood osmolality (Fig. 1), drops slightly below equilibrium at the upper extremes of salinity. Blood sodium and osmotic concentrations respond similarly in acclimations of *C. californiensis*, but both sodium and osmolality of

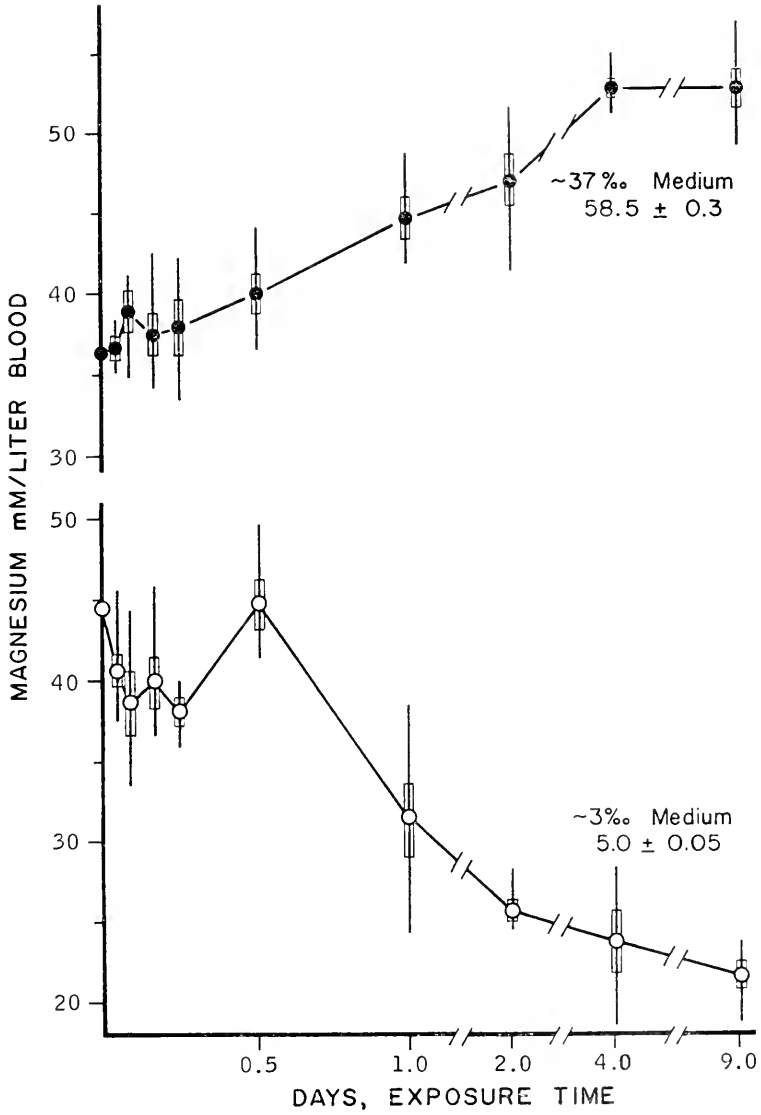

FIGURE 10. Blood magnesium concentration at timed intervals after direct transfer of acclimated *C. jamaicense* from 20‰ salinity to 3‰ (open circles) or to 37‰ (solid circles). Each solid or open circle is mean of 5 determinations; vertical lines indicate range; rectangles indicate standard errors. Figures beneath salinities indicate means and standard errors of media concentrations in mM/liter over 9-day period.

blood remain more nearly equivalent to media concentrations over the entire range of salinity (L. Thompson and Pritchard, 1969).

Most marine crustaceans strongly hyporegulate blood magnesium (Robertson, 1953). Exceptions to this rule include several brachyuran spider crabs, the primitive brachyuran, *Dromia vulgaris*, and the anomuran, *Lithodes maia*, in which relatively high blood magnesium is correlated with low levels of responsiveness attributed to magnesium interference with neuromuscular transmission (Robertson, 1960). *Callianassa major* and *C. jamaicense* also have high levels of blood magnesium and appear to hyper-regulate this ion at media concentrations < 50 mM/liter (Fig. 6). An advantage of high blood magnesium is suggested by its effects on oxygen binding in hemocyanins (Larimer and Riggs, 1964; Roxby, Miller, Blair, and Van Holde, 1974; Miller and Van Holde, 1974). Miller and Van Holde (1974) report a mean magnesium concentration of 48 mM/liter for *C. californiensis* at an unspecified salinity. Although high compared to that of most crustaceans, this value is well within the ranges of blood magnesium here reported for *C. major* and *C. jamaicense*. Specifically, Miller and Van Holde show that magnesium effects allosteric transitions in callianassid hemocyanin *in vitro*. By increasing oxygen binding (lowering P_{50}) high blood magnesium may be advantageous to thalassinids which burrow in hypoxic substrates. Such substrates are inhabited by *C. californiensis* on the Pacific coast (R. Thompson and Pritchard, 1969) and by the *Callianassa* species on the Louisiana coast (Felder, in preparation). Miller and Van Holde (1974) suggest that magnesium levels remain stable in *Callianassa;* this does not apply to *C. major* and *C. jamaicense* as blood magnesium, while somewhat regulated, varies markedly with changing salinity. Survival of these species in a hypoxic habitat could thus be influenced by interactions between salinity, ion balance, and oxygen availability.

Hyperosmotic regulation by *C. jamaicense* at low salinities, its tolerance of salinities $\leq 2‰$ and its ability to withstand abrupt changes in salinity with marked regulation of volume clearly support its categorization as euryhaline. Such capacities are well documented among upogebid Thalassinidea (Zenkevich, 1938; L. Thompson and Pritchard, 1969; Hill, 1971), but the South African *C. kraussi* is the only other species of the Callianassidae (*sensu* de Saint Laurent, 1973) in which hypersomotic regulation is reported (Forbes, 1974). The blood osmotic, sodium, and chloride concentrations in acclimated *C. jamaicense* (Figs. 2, 3, and 5) closely resemble those reported for *C. kraussi*. The deterioration of regulatory ability that Forbes reported in *C. kraussi* at lower extremes of salinity is not pronounced in *C. jamaicense*, probably because the lowest acclimation extreme used for *C. kraussi* is lower than that used for *C. jamaicense*.

The difference between summer and winter levels of hyperosmotic regulation in *C. jamaicense* (Fig. 2) likely reflects the lower field temperatures from which winter animals were collected; both field temperature and salinity were lower during winter collections. Lynch, Webb, and Van Engel (1973) and Charmantier (1975) list a number of studies documenting seasonal temperature effects upon blood osmotic and ionic concentrations in crustaceans. Higher blood osmotic and ionic concentrations occur in animals from colder water (Dehnel, 1962; Ballard and Abbott, 1969), even when, as in the present case, acclimations are

conducted at equivalent temperatures in the laboratory. Acclimation studies of *Callinectes sapidus* suggest that lower salinity could produce an effect opposite from that of low temperature, as blood osmotic concentration of acclimated crabs is lower when crabs are collected at low field salinity; however, in salinities < 15‰ blood osmotic concentrations of *Callinectes sapidus* depend little upon the direction from which the acclimation salinity is approached (Ballard and Abbott, 1969).

After direct transfers of *Callianassa jamaicense* from 20‰, the animals placed into 3‰ regulate volume nearer original levels than do those placed into 37‰, but in both cases volumes are near original levels after two days (Fig. 7). Limited data on weight changes of *C. major* and *C. islagrande* after less dramatic stepwise transfers to low salinities suggest much poorer volume control in those species (Table II). The means by which *C. jamaicense* controls volume and blood osmotic concentration is at present uncertain. Studies of urine in both hyperosmotically regulating (Forbes, 1974) and osmotically conforming (L. Thompson and Pritchard, 1969) Callianassidae show that, as in the great majority of euryhaline Crustacea (Potts and Parry, 1964), an isosmotic urine is produced by animals once acclimated to various salinities. However, urine volumes and osmolality are not reported during acclimation in either of these studies. Osmoregulatory functions of the antennal glands are suggested by increased urine volumes in the crab, *Carcinus maenas*, with decreased salinity (Binns, 1969) and by studies of the lobster, *Homarus americanus*, in which urine is near isosmotic to blood in animals fully acclimated to lowered salinity, but markedly hyposmotic during acclimation (Dall, 1970). Changes in permeability may also facilitate regulation of volume and blood osmolality, and such changes are documented in other euryhaline decapods subjected to dilute media (Capen, 1972; Spaargaren, 1975). Additionally, Heeg and Cannone (1966) describe an osmoregulatory diverticulum on the posterior mid-gut of grapsid crabs; a similar diverticulum is present in *Callianassa jamaicense, C. major*, and *C. islagrande*, although its function is unknown.

After direct transfer of *C. jamaicense* from 20‰ to 3‰ media, blood osmotic, chloride, and sodium concentrations are near new stable levels within 12 hours, but gradual changes in the sodium/chloride ratio continue to occur through day 9 (Figs. 8 and 9). Changes in blood osmotic and sodium concentrations of *C. jamaicense* are very nearly proportional over observed time increments after transfers to either 3‰ or 37‰ media. A similar close correlation between sodium and osmotic concentrations is reported in crustacean blood by other investigators (Colvocoresses, Lynch, and Webb, 1974) and such observations seem compatible with data indicating that the sodium transport system ultimately establishes the blood osmolality (Shaw, 1960). By day 9 after direct transfers, blood sodium/chloride ratios in *C. jamaicense* are higher at 3‰ than at 37‰ salinity. A similarly elevated sodium/chloride ratio is also observed after *C. kraussi* is acclimated to low salinity (Forbes, 1974).

Blood magnesium concentrations approach stable levels less rapidly than other ions after salinity transfers (Fig. 10). This may contribute to what Forbes (1974) describes as slower, smaller changes in blood osmotic concentrations after stabilization of blood sodium and chloride concentrations following salinity transfers of *C. kraussi;* Forbes (p. 310) speculates such changes could be asso-

ciated with "non-ionic osmotically active entities in the blood," but does not report divalent ion concentrations.

Evolution of hyperosmotic regulation in *C. kraussi* is attributed to the unique flood-influenced salinity gradient in southern African estuaries (Forbes, 1974); similar conditions occur in other areas including coastal estuaries of the Northern Gulf of Mexico (Hewatt, 1951; Barrett, Tarver, Latapie, Pollard, Mock, Adkins, Gaidry, White, and Mathis, 1971). Euryhalinity may be characteristic of a phyletic stock, rather than of an isolated species or genus and probably is a very conservative physiological adaptation once acquired (Hedgpeth, 1957); Ortmann (1902) furnishes examples of such phyletic stocks among crustaceans for the now freshwater Atyidae and the Palaemonidae which occur in marine, estuarine, and freshwater habitats. Since *Callianassa kraussi* and *C. jamaicense* share the conservative character of euryhalinity, further examination of their phylogenetic proximity may prove interesting. However, phylogenetic interpretations must be made with caution; osmoregulatory ability may be a conservative trait once acquired, but it could have been acquired independently following separation of ancestral stocks. Lockwood and Croghan (1957) suggest that only 700 years were required for development of a separate race of Baltic isopods which now possesses distinctly greater powers of osmotic and ionic regulation than its ancestral stocks.

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Summary

Osmotic and ionic regulatory capacities of callianassid mud shrimps, *Callianassa jamaicense, C. major,* and *C. islagrande,* are correlated to their distributions on the Louisiana coast. *Callianassa jamaicense* burrows in muddy estuaries where salinity may commonly fall to < 5‰, but *C. major* and *C. islagrande* usually burrow in sandy beaches bathed by higher salinities. Lower lethal limits of salinity are < 2‰ for *C. jamaicense,* 7–8‰ for *C. major* and probably just below 15‰ for adult *C. islagrande.* After exposure to low salinity *C. jamaicense* exhibits better volume control than the other two species. Blood osmotic, sodium, and chloride concentrations in *C. jamaicense* are regulated near stable levels at acclimation salinities beneath ~ 20‰ but those of *C. major* and *C. islagrande* are not. Blood magnesium is slightly hyper-regulated by *C. jamaicense* at most acclimation salinities < 25‰ and more markedly hyper-regulated at salinities < 10‰; it is also slightly hyper-regulated by *C. major* at acclimation salinities < 30‰.

After direct transfer of *C. jamaicense* from 20‰ salinity to 3‰ salinity, blood osmotic, sodium, and chloride concentrations fall slightly but approach stable concentrations within 12 hours; blood magnesium concentration falls less rapidly. When *C. jamaicense* is transferred from 20 to 37‰, blood osmotic, sodium, and chloride concentrations increase markedly during the first day and continue to

slowly increase through day 9; blood magnesium increases to a near stable level by day 4.

Differences in osmoregulatory capacities, along with substrate preferences, appear to limit distributions of Callianassidae on the Louisiana coast. With one exception, previous studies suggest that osmoregulatory ability does not occur in this group. The present report of osmoregulatory ability in *C. jamaicense* documents a second exception.

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LARVAL REARING, METAMORPHOSIS, GROWTH AND REPRODUCTION OF THE EOLID NUDIBRANCH *HERMISSENDA CRASSICORNIS* (ESCHSCHOLTZ, 1831) (GASTROPODA: OPISTHOBRANCHIA)

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In the eolid nudibranch, *Hermissenda crassicornis* (Eschscholtz, 1831), neural pathways responsive to light, chemosensory stimuli, and gravitational stimuli converge within the circumesophageal nervous system (Alkon, 1974, 1975, 1976). These convergence points, as defined by intracellular recordings, may be important for choice behavior and behavioral modification as demonstrated for this animal. Maintenance conditions, primarily light-dark cycle, temperature, and diet, had to be carefully controlled when analyzing both the behavior and the nervous system of *Hermissenda*. The goal of the present study was to establish a laboratory strain of *Hermissenda* to provide animals of known history for these studies, and for studies on behavioral and neural development in these three sensory pathways.

Hermissenda, a monotypic genus, is widely distributed along the west coast of North America (Lance, 1966; MacFarland, 1966). Field observations (Yarnell, 1972; Birkeland, 1974) indicate that *Hermissenda*, although preying primarily on coelenterates, has a broader diet than most nudibranchs. *Hermissenda* and its egg masses appeared on fouling panels exposed for one month at a time throughout the year in Monterey Bay, California (Haderlie, 1968). Year-round availability of eggs and adults and a relatively broad diet in the adult stage simplify cultivation of *Hermissenda*.

Materials and Methods

Reproductive periodicity

Weekly shipments of *Hermissenda* were obtained from Mr. Michael Morris, Sea Life Supply, Sand City, California. Ten animals (2.5 + cm in length) were removed from each week's shipment from May, 1976, to May, 1977. Animals were incubated separately on a 12L: 12D schedule at an average sea water temperature (± s.d.) of 14.1° ± 1.8° C, which approximates the mid-point of the annual temperature range occurring in the natural habitat (9°–18° C; Haderlie, Mellor, Minter, and Booth, 1975). A daily record was kept of the number of each set of ten animals that deposited an egg mass.

Fecundity measurements

Fifty newly-arrived animals of widely varying sizes (73–3204 mg) were weighed underwater on a Mettler PN323 balance, immediately after each deposited

its first egg mass. The number of eggs per egg mass was estimated by multiplying the length of the egg string in mm by the average number of embryos per mm.

Egg diameter and egg capsule size were measured through a Zeiss Universal microscope with a calibrated ocular micrometer. All computer analyses of data on growth and reproduction were performed on a PDP 11/10 computer, using standard statistical packages.

Diet experiments

Forty individuals (ten per diet) were maintained until death on one of four locally available diets: frozen squid mantle muscle (*Loligo pealii*), mussel (*Mytilus edulis*), tunicate (*Ciona intestinalis*), minus the tunic, and an alternating diet consisting of one day of squid, then mussel, then tunicate, etc.

Weighed animals were placed singly in numbered 7 × 4 cm plastic snap-top vials which were perforated with slits for water exchange, and maintained at a sea water temperature of 12°–14° C. An excess of food was provided fresh daily. Weight, days survived, number of egg masses laid and whether eggs developed normally were recorded for each animal on each diet.

Larval rearing

Egg masses were incubated at 13°–15° C in 0.22 μm Millipore-filtered (MPF) sea water. On day 5 or 6 following oviposition, the veligers were liberated by teasing apart the egg mass. Cetyl alcohol flakes sprinkled on the surface of larval cultures prevented larvae from becoming entrapped in the surface film (Hurst, 1967). The rearing method was adapted from that developed for aplysiid larvae by Switzer-Dunlap and Hadfield (1978). A similar method was employed by Harrigan and Alkon (1978) to rear the opisthobranch molluscs, *Elysia chlorotica* Gould, 1870 and *Haminoea solitaria* (Say, 1822).

Larvae were cultured at a concentration of three per ml in covered one-liter Pyrex beakers filled with 800 ml of culture water (0.22 μm MPF sea water containing 5 ppm chloromphenicol). Cultures were maintained on a 12L : 12D cycle at an average temperature of 13.8° ± 1.2° C. Larvae were transferred three times a week to clean culture water. Cultures were fed daily and stirred to resuspend food and veligers.

Cultures were initially fed equal amounts of *Isochrysis galbana* and *Monochrysis lutheri* at a final concentration of 3.0×10^4 cells per ml and the larger flagellate *Chroococcus salina* (strain 3C) at a final concentration of 7.5×10^3 cells per ml. Food concentration was gradually decreased as the cultures aged. Algal cultures were bacteria-free, and were grown in 100 ml aliquots according to the methods of Guillard (1975).

Juveniles were fed for one week on the hydroid species (provided by Sea Life Supply) on which they metamorphosed. They were then fed only on tunicate (*Ciona intestinalis*) obtained from Cape Cod waters. Body lengths, measured when the animals were fully extended, were taken weekly; body weights were taken occasionally.

Results

Reproductive periodicity

Fertile egg masses, which produced normal veligers, were obtained every week of the year from sets of ten animals collected from Monterey Bay, California. Chi-square analysis of an R × C contingency table indicated no significant interaction between the number of animals laying eggs per week and the month of the year the eggs were obtained ($P > 0.99$, df = 33). *Hermissenda* did not exhibit seasonal periodicity in egg-laying in the laboratory.

Over the one-year sampling period 79% of the total number of animals tested (n = 490) deposited at least one egg mass. Thirty-one per cent of the animals laying one egg mass produced a total of 2, 3, or 4 egg masses within the one-week test period.

Characteristics of the egg mass

Hermissenda deposits its egg masses, while or pink, in a tight counter-clockwise spiral. Structure of the egg mass is further described by Hurst (1967). Diameters of the first egg mass deposited in the laboratory by the adults (73–3204 mg body weight) ranged from 0.24 cm to 3.62 cm. Average egg mass diameter increased linearly with adult weight (polynomial regression, $P < 0.01$, df = 49). The number of eggs estimated per egg mass (see Methods) varied from 6.9×10^3 to 1.0×10^6.

The number of eggs per egg capsule increased with adult weight ($P < 0.01$) from one to an average of nine eggs for adults greater than 500 mg. Eggs are packed one per capsule for adults weighing less than 500 mg. The average egg diameter was 65.4 ± 1.2 μm (n = 70, 7 adults). Egg diameter was not a function of the number of eggs per capsule. Egg capsule length increased significantly with number of eggs per capsule (one-way ANVAR, $P < 0.01$) (Table I).

Larval development

Veligers hatch in 5–6 days at 13°–15° C. Unsculptured shells are of about $\frac{3}{4}$ whorl and belong to Thompson's Type I (Thompson, 1961). Average shell length and width at hatching is 105.9 ± 6.3 × 75.4 ± 4.8 μm (n = 25). *Hermissenda* has an obligatory veliger stage of at least 34 days. Metamorphosis is delayed

Table I

Relationship between capsule size and number of eggs per capsule. Each number represents average length and width (μm) of 100 capsules, 20 from each of five adults.

Eggs/capsule	Length × width (μm) (±s.d.)
1	110.3 ± 10.0 × 76.2 ± 4.4
2	145.5 ± 5.8 × 102.3 ± 6.1
3	157.0 ± 9.9 × 112.8 ± 3.8
4	175.4 ± 8.5 × 126.4 ± 7.1
5	185.8 ± 10.1 × 141.9 ± 10.9

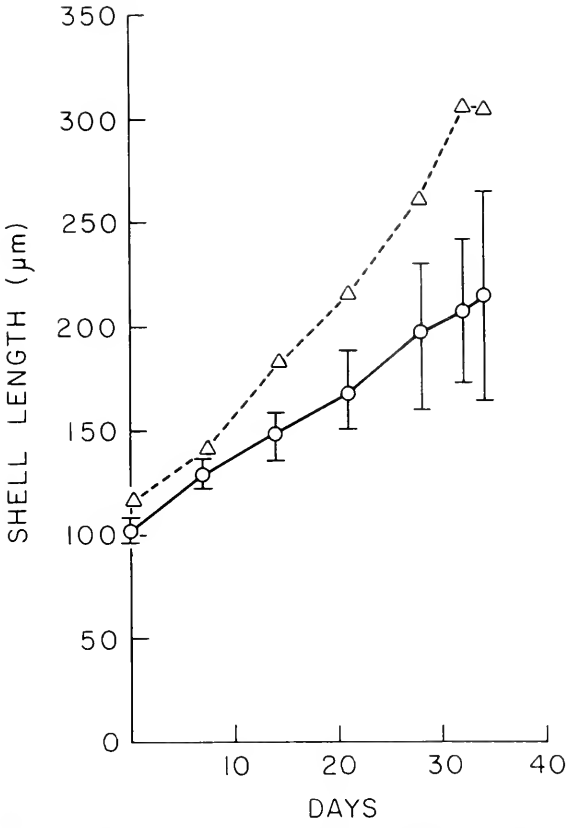

FIGURE 1. Growth of veligers in terms of shell length. Vertical bars represent one standard deviation. Dots represent average shell length; triangles represent the size of the largest individual measured.

by about 2–4 days after maximum shell length (310.4 ± 9.8 μm, n = 11) is attained.

Veligers which were competent to metamorphose were recognized by the following criteria: presence of eyes, shell length of at least 300 μm, enlargement of the foot and development of the propodium, reduced swimming activity, the veliger remaining on or near the bottom, and the presence of a tooth at the base of the shell aperture. The average shell length of a sample of veligers did not accurately reflect the size of the largest individuals. Figure 1 illustrates shell growth in veligers from three replicate cultures.

On day 34 post-hatching, competent veligers crawled immediately on the thecate hydroid, *Obelia longissima*, and on an unidentified thecate hydroid from California. Competent veligers also crawled on the related species, *Obelia geniculata*, collected from Woods Hole, Massachusetts. The velum is lost during the first 12–24 hours after crawling begins. In the next 12–24 hours the larva slowly crawls out of its shell. During shell exit one pair of tentacle buds and two pairs of cerata buds grow out of the dorsal surface. The operculum is discarded at

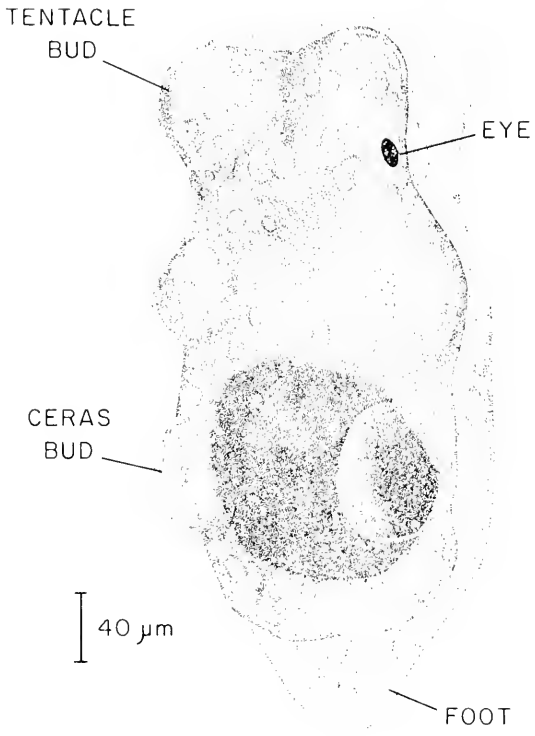

FIGURE 2. Newly metamorphosed *Hermissenda*. A pair of tentacle buds and two pairs of cerata buds are visible.

metamorphosis. The newly-metamorphosed animal measures about 400 μm in length (Fig. 2). The body is still divided into a dorsal visceral mass and a ventral foot, and the larval digestive system is visible. By four to five days post-metamorphosis the distinction between foot and visceral mass is lost and the juvenile has begun to feed on hydroid tissue.

Metamorphosis occurred only in veligers that reached full development between days 34–58 post-hatching, although individuals settling after day 50 soon died. Although larvae may survive up to 76 days, there was little or no shell growth after day 58.

Diet

Survival of *Hermissenda* through metamorphosis was low. Addition to a larval diet of *Isochrysis galbana* and *Monochrysis lutheri* (5 μm cell diameter) of the larger flagellate *Chroococcus salina*, strain 3 C (10–11 μm cell length) increased the percentage of metamorphosis from 1 to 5%. Increasing the concentration of *Isochrysis* and *Monochrysis* did not improve the percentage of metamorphosis, nor did feeding *Chroococcus* alone.

Post-metamorphic stages, however, were easily maintained in the laboratory.

Variation in diet significantly affected both growth rate of adults and number of days survived, but not number of egg masses laid. Diets containing tunicate, either alone or in combination with squid and mussel, gave the best growth and survival (Table II).

Initial average weights of four groups of ten small wild *Hermissenda* each varied from 299 mg to 509 mg. Average weight gains on each of the four diets were: 195.8 ± 362.1 mg (squid); 1218.0 ± 1514.3 mg (mussel); 2680.5 ± 1121.1 mg (tunicate); and 2752.3 ± 1268.4 mg (alternating diet). Animals survived significantly longer on tunicate-containing diets than on either squid or mussel (t-test, $P < 0.01$, df = 19). Mean number of days survival on the two tunicate-containing diets was 63.9 days (range = 34–122 days).

The total number of egg masses produced did not vary significantly between diets (Table II). There was no significant correlation between an individuals growth rate on any diet and the total number of egg masses produced by that individual. However, there was a significant positive regression of number of egg masses produced on days survived, all diets combined (polynomial regression, $P < 0.01$, df = 40) (Fig. 3).

Growth rate and reproduction in five F1 adults

From day 1 to day 70 post-metamorphosis increase in body length (on a tunicate diet) was approximately linear, averaging 0.82 ± 0.11 mm per day. The growth rate slowed to 0.35 ± 0.17 mm per day between days 71–120 post-metamorphosis. The largest individual attained a length of 81.7 mm, nearly equalling the length of the largest *Hermissenda* obtained from the field, 90 mm. After day 120 food intake decreased and the animals began to shrink. Death occurred between 116–137 days post-metamorphosis ($\bar{X}$ = 128 days).

The average life-span of a laboratory-reared *Hermissenda* encompasses approximately 163 days (35 day veliger stage plus 128 day adult stage), confirming that *Hermissenda* is a subannual species.

Hermissenda was not observed to self-fertilize. No egg masses were deposited by F1 adults, which were maintained separately, until three animals were allowed to copulate on day 65 post-metamorphosis (total egg masses = 28 from first copulation to death). Two isolated individuals deposited 2–3 sterile egg masses each between days 95–133 post-metamorphosis.

Fertile egg masses were deposited in the laboratory by wild specimens of *Hermissenda* as small as 73 mg, and motile sperm were observed in squash prepa-

TABLE II

Growth rate, survival, and egg mass production for ten specimens of Hermissenda *on each of four diets.*

Diet	Average growth rate mg/day	Average days survived	Average egg mass production
Squid mantle	10.1 ± 11.0	28.0 ± 11.4	2.0 ± 1.8
Mussel	31.4 ± 26.7	45.4 ± 14.8	3.7 ± 2.4
Tunicate	58.4 ± 35.6	65.0 ± 25.3	3.5 ± 3.9
Alternating	55.3 ± 14.9	62.9 ± 7.0	1.9 ± 1.6

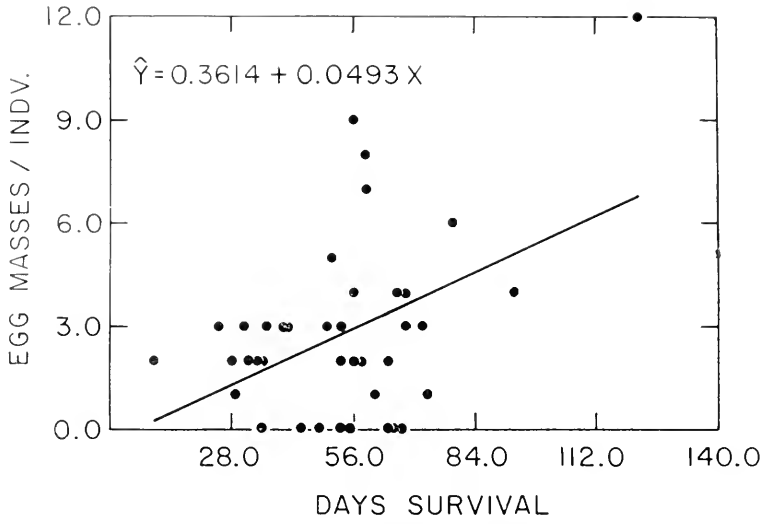

FIGURE 3. Regression of number of egg masses deposited on days survival.

rations from wild individuals weighing 34 mg (1.12 cm body length). Egg production in individuals from wild populations is estimated to begin at about 1.5 months post-metamorphosis and continue until death at 5–8 g, four months post-metamorphosis. Both the total number of egg masses produced and the age at which egg-laying commences depend on age at initial copulation.

DISCUSSION

Hermissenda crassicornis is one of several nudibranch species which have been reared through metamorphosis in the laboratory (Bonar and Hadfield, 1974; Thompson, 1958, 1962, 1967; Tardy, 1970; Perron and Turner, 1977; Harris, 1975). Harris (1975) and Perron and Turner (1977) have successfully reared nudibranch species having planktotrophic (feeding) larvae from egg to egg. Other nudibranch species reared have been either lecithotrophic or direct developers. *Hermissenda* has a longer obligatory planktotrophic stage, 34 days, than either *Phestilla melanobranchia* Bergh 1874 (Harris, 1975) or *Doridella obscura* Verrill (Perron and Turner, 1977).

The length of the veliger stage in *Hermissenda* is similar to that reported for five species of Pacific aplysiid opisthobranchs, 30–34 days (Kriegstein, Castellucci, and Kandel, 1974; Switzer-Dunlap and Hadfield, 1978). Switzer-Dunlap and Hadfield (1978) observed a plateau in shell growth before metamorphosis in four aplysiid species similar to that noted in *Hermissenda*. As adults, three of the four above mentioned aplysiid species were reported to grow to a larger size in the laboratory than in the field. No specimens of *Hermissenda* fed in the laboratory have exceeded the maximum size of wild individuals.

Stages in the life cycle of *Hermissenda* follow in the same sequence as the seven general life history stages listed by Bonar and Hadfield (1974): hatching,

competency to metamorphose, velum loss, shell and operculum detachment and loss, and sinking of the visceral mass into the foot. The seventh stage, the pseudovermis stage, is eliminated in metamorphosing specimens of *Hermissenda* which grow tentacle buds and cerata buds as they crawl out of the shell.

The life cycle of *Hermissenda*, as observed in the laboratory, follows the pattern described for other hydroid-eating nudibranchs by Thompson (1964) and Clark (1975). Animals used in the present study came only from the Monterey Bay population; however, reported sizes of eggs and egg capsules, and structure and size of the egg masses deposited by individuals from other parts of the species range are within the range of values reported here (Hurst, 1967; O'Donoghue and O'Donoghue, 1922).

The most variable factor observed in populations of veligers and adults was growth rate. In the veliger stage part of this variation may have been due to culture conditions. Growth of larvae may have been inhibited by the antibiotic used, chloramphenicol, known to inhibit protein synthesis in eukaryotes as well as bacteria (Pestka, 1975), or the larval diet may have been suboptimal for many veligers.

Large laboratory populations of *Hermissenda* can be maintained on the tunicate *Ciona intestinalis*, which is commonly found in Cape Cod waters. Year-round availability of *Ciona* and ease of collection gives it an advantage over the normal field diet, which consists primarily of numerous coelenterate species, as well as tunicates. A mixed diet did not markedly improve growth or survival over the single item tunicate diet.

In *Hermissenda*, individuals which have the fastest growth rates are also the largest adults. A program of selective breeding of *Hermissenda* will concentrate, at least initially, on selection for fast growth rates. High selection pressure is already exerted on the laboratory population in terms of survival in the specific culture conditions utilized, and because adults are reared on a diet of only tunicate.

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Summary

1. *Hermissenda crassicornis* is a subannual nudibranch species that reproduces year-round.

2. There is a significant positive relationship between adult weight, diameter of the egg mass, estimated number of eggs per egg mass, and average number of eggs per capsule.

3. There is a planktonic veliger stage of 34 days minimum at 13°–15° C.

4. Larvae metamorphose on at least three species of hydroids.

5. To develop in reasonable numbers to a state competent to metamorphose

veligers require a diet that includes phytoplankton of larger cell size (10–11 μm) than the commonly used *Isochrysis* and *Monochrysis* (5 μm).

6. Although *Hermissenda* feeds on a wide variety of sessile invertebrate species in the ocean, a diet of tunicate alone (*Ciona intestinalis*) promotes good growth and survival in the laboratory.

7. Egg mass deposition is initiated only after first copulation, except in the last month of life, and continues from about one-month post-metamorphosis to death, at about four months post-metamorphosis. Generation time (egg-to-egg) may be as short as 2.5 months.

8. A laboratory strain of *Hermissenda* is being established to provide animals of known history for research on the neural correlates of behavior. Animals, at least initially, are being selected for fast growth rate.

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CAPACITY FOR BIOSYNTHESIS OF PROSTAGLANDIN-RELATED COMPOUNDS: DISTRIBUTION AND PROPERTIES OF THE RATE-LIMITING ENZYME IN HYDROCORALS, GORGONIANS, AND OTHER COELENTERATES OF THE CARIBBEAN AND PACIFIC

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The search for new marine sources of physiologically potent chemicals of interest to biology, and potential utility to medicine, agriculture, industry, and research, has in many cases been hindered by the lack of analytical procedures of sufficient generality, rapidity, and adaptability to use in the field. This has been particularly so in the case of the hormone-like prostaglandin-related compounds (PGRCs), which are now known to include a large and confusing multiplicity of prostaglandins (PGs), prostacyclins, thromboxanes, and prostaglandin-endoperoxides (Karim and Rao, 1975; Hamberg, Svensson and Samuelsson, 1975, Pace-Asciak and Wolfe, 1971; Johnson, Morton, Kinner, Gorman, McGuire and Sun, 1976).

Very high levels of the prostaglandins PGE_2, PGA_2, and certain of their related isomers have been found in different clonal populations of the Caribbean gorgonian, *Plexaura homomalla* (*Anthozoa: Gorgonacea*) (Weinheimer and Spraggins, 1969; Weinheimer, 1974; Light and Samuelsson, 1972; Schneider, Hamilton and Rhuland, 1972). This finding generated considerable interest in the potentials for development and conservation of this gorgonian as a major medical resource (Bayer and Weinheimer, 1974), although commercial interest in this fragile and slowly growing species (Kinzie, 1974; Himman, Anderson and Simon, 1974; Jordan, Castanares and Ibarra, 1978) has been supplanted by recent improvements in synthetic methods for the production of some of the prostaglandins. The full extent of the distribution and potential resource of PGRCs from the marine invertebrates, as well as the functions of the PGRCs in these animals, remain largely unknown, however. There are well over a hundred prostaglandins and other PGRCs now recognized, with newly identified members of this family being discovered at an exponential rate (Karim and Rao, 1975). Assays are further complicated by the fact that these compounds are for the most part highly unstable (under physiological, aqueous, and aerobic conditions), and possess overlapping spectra of physical and biological properties, thus necessitating resolution and analysis by complicated and specialized techniques (Schneider, 1976; Salmon and Karim, 1976).

All of the PGRCs, however, are synthesized from a common (and unstable) intermediate: a prostaglandin-endoperoxide (PGEP) (Hamberg and Samuelsson, 1973, 1974). The enzyme-complex catalyzing the rate-limiting step in the biosynthesis of this central intermediate is known as prostaglandin-endoperoxide syn-

thetase (also known as prostaglandin synthetase or fatty acid cyclo-oxygenase) (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976). Although several techniques for the assay of this enzyme are available (Samuelsson, Granström, Green, Hamberg and Hammarström, 1975; Salmon and Karim, 1976), use of these assays for the direct measurement of a tissue's maximal capacity for PGEP synthesis (and thus, the total capacity for subsequent biosynthesis of PGRCs) has been complicated by the pronounced autocatalytic and autodestructive activities of the enzyme during such procedures (Miyamoto *et al.*, 1976; Lands and Rome, 1976). Such marked deviations from simple first-order kinetics result in a complex, nonlinear proportionality of the reaction with respect to both time and the amount of enzyme present, thus limiting the usefulness of these techniques for comparative assessments of relative PGRC biosynthetic capacities.

The PGEP synthetase-catalyzed reaction is markedly stimulated by hydrogen peroxide (H_2O_2), and the enzyme from many invertebrate sources appears to generate this activator autocatalytically during the course of its normal reaction (Morse, Duncan, Hooker and Morse, 1977, 1978). Addition of exogenous hydrogen peroxide (or addition of a hydrogen peroxide *generating system*) rapidly activates PGEP synthetase; the rate of the reaction catalyzed by this enzyme is then easily measured, and is directly proportional to the amount of the enzyme present. Based upon this finding, a rapid and convenient spectrophotometric micro-assay for PGEP synthetase (Takeguchi and Sih, 1972) was modified, especially adapting it for use in the field by inclusion of a stable enzymatic H_2O_2-generating system. Using this technique to measure the levels of PGEP synthetase in a variety of marine coelenterates from the Caribbean and Pacific, especially high specific activities of this enzyme were found in several of the plexaurid *Gorgonacea* (including *P. homomalla*), in three species of "*Hydrocorallia*" (*Milleporina* and *Stylasterina*), and in two species of *Hydroida*; significant levels of the enzyme were also found in species belonging to other orders, as well.

Materials and Methods

Specimen collection

Marine coelenterates were obtained from both the Caribbean (Bonaire, Netherlands Antilles; July–August, 1976; 1–30 m depth) and the Pacific (Santa Barbara Channel, California; August, 1976–July, 1977; 0–15 m depth). Small samples of tissue (generally < 5 g, including associated skeletal and substrate material) were collected from these marine species and sealed, *in situ*, in sparate polyethylene bags of sea water (*ca.* 100–200 ml); these were brought ashore for prompt assay. Freshwater hydroids were obtained from the Carolina Biological Corporation. Only fresh, live specimens were used for all assays reported here. Identification and classification of species were made according to Hyman (1940), Bayer (1961), Roos (1971), Boschma (1956), Smith (1971), Smith and Carlton (1975), Durham and Bernard (1952), Johnson and Snook (1955), and Allen (1976).

Preparation of extracts

All samples of marine species were rinsed with sea water after separation from associated substrate and other biota as necessary. Tissue was removed from

the samples of *Scleractinia, Milleporina* and *Stylasterina* by scraping the skeletal material with a scalpel, and irrigating with a small volume of chilled *tris*-hydroxymethylaminomethane (Tris)-HCl buffer (10 mM, pH 7.1, 0° C); all other samples were minced (at 0° C) to facilitate homogenization.

Small samples of weighed tissue (0.1–0.5 g) were homogenized (0° C) in 1–3 volume-equivalents of the above Tris-Cl buffer, using a small glass or Teflon Dounce homogenizer. Particulate material and debris were removed by brief low-speed centrifugation, and the extracts held at 0° C for immediate assay.

Assays

The catalytic activity of PGEP synthetase was measured using a modification (Morse *et al.*, 1977) of the technique originally developed by Takeguchi and Sih (1972). This assay spectrophotometrically monitors the obligatory co-oxidation of the colorless aromatic cofactor, *L*-epinephrine, as it is converted to the intensely red adrenochrome product. The assay-mixture (1 ml, 20–23° C) contained Tris-Cl buffer (10 mM, pH 7.1), arachidonic acid as substrate and *L*-epinephrine as cofactor (each at 1 mM), with the extract to be assayed and other additions as indicated in the text. The course of the reaction was monitored as the rate of change in optical absorbance at 480 nm. For use in the field, assays were performed with a Bausch and Lomb Mini-Spectrophotometer (weight *ca.* 200 g) and stopwatch; assays performed in the laboratory made use of a Gilford recording spectrophotometer. Assays of the same homogenates performed in parallel with these two instruments were found to agree within ± 9%.

Where indicated (Table I), extracts were heated at 90° C for 10 min prior to assay, to denature enzyme protein. Also as indicated, catalase was added at 0.1 μg/ml; phenylcyclopropylamine, aspirin, indomethacin, acetaminophen, DDTC, and EDTA were added at 1 mM concentration as shown.

Aliquots of extracts were stored frozen, and subsequently assayed for protein concentration by the method of Lowry, Rosebrough, Farr and Randall (1951). Specific activities are expressed as the change in absorbance (at 480 nm) in the assay mixture per minute per mg of added protein.

Chemicals

Tris and Tris-Cl (pre-equilibrated to yield pH 7.1 at 10 mM, 22° C, *L*-epinephrine, glucose oxidase and catalase were obtained from the Sigma Chemical Co.; H_2O_2 (30%, stablized) was obtained from Mallinckrodt, and diluted just before use. Diethyldithiocarbamate (DDTC, sodium salt) and ethylenediaminetetraacetic acid (EDTA, tetrasodium salt) were from Fisher Chemical Corporation; all other chemicals were reagent grade. All solutions were prepared with distilled water.

Results

That the "prostaglandin A_2 synthetase complex" of *P. homomalla* is activated by 1 M NaCl (Corey, Washburn and Chen, 1973) was verified in this study, using the spectrophotometric assay for the PGEP synthetase reaction; this activation was found to be a general (although somewhat variable) property of the

TABLE I

Properties of PGEP synthetase in extracts of Allopora porphyra. *PGEP synthetase activity was assayed in 5 μl aliquots of a freshly prepared extract (8.8 mg protein/ml) of* Allopora porphyra *as described in the text, with alterations as specified. Both the maximal rate of the enzyme-catalyzed reaction (in the presence of 1 M NaCl) and the initial rate (in the presence of 0.6 mM H_2O_2) were measured; results are the averages (±s.d.) of duplicate determinations normalized to values obtained with the respective complete assay mixtures. The maximal rate of the reaction (+NaCl, measured after* ca. *5 min) corresponds to 0.27 μmole epinephrine oxidized per minute; the initial rate in the complete system with H_2O_2 was 0.24 μmole/min.*

Assay mixture	Relative activity (%)	
	Maximal rate (with NaCl)	Initial rate (with H_2O_2)
Complete system	100 ± 8	100 ± 6
Omit activator (NaCl or H_2O_2)	41 ± 5	2 ± 1
+ catalase	0 ± 0	0 ± 0
+2x Extract	198 ± 4	208 ± 9
Omit extract	0 ± 0	0 ± 0
+Heated extract	0 ± 0	0 ± 0
Omit arachidonic acid	38 ± 3	54 ± 2
+Phenylcyclopropylamine	3 ± 0	0 ± 0
+Aspirin	84 ± 6	54 ± 6
+Indomethacin	44 ± 5	16 ± 2
+Acetaminophen	61 ± 2	62 ± 7
+DDTC	0 ± 0	4 ± 1
+EDTA	133 ± 5	140 ± 12

enzyme from most of the coelenterates assayed. No such salt-stimulation of PGEP synthetases was observed in active extracts from marine echinoderms, molluscs, or fishes, however.

As the spectrophotometric assay affords a means for continuously monitoring the progress of the enzymatic reaction, the effect of salt upon the coelenterate PGEP synthetase coud be studied in more detail. Addition of NaCl increases both the maximal (autocatalytic) rate and the final yield of the reaction by *ca.* 2–3 fold; there is no significant effect of salt on the slow *initial* rate, however. Final yield of the reaction is limited, in part, by an enzymatic, autoinhibitory process, and not by depletion of substrate. [Similarly complex autocatalytic and autoinhibitory processes also have been observed in kinetic analyses of the reaction catalyzed by PGEP synthetases from a variety of mammalian sources (*e.g.*, Lands and Rome, 1976; Miyamoto *et al.*, 1976).]

The data in Table I illustrate the properties of the PGEP synthetase in an extract of the Pacific "hydrocoral," *Allopora porphyra* (Hydrozoa: Stylasterina), a species especially rich in this enzyme. Similar properties were found for the PGEP synthetases in extracts of *P. homomalla* (Anthozoa: Gorgonacea), *Millepora* spp. (Hydrozoa: Milleporina), and *Sertularia turgida* and *Hydractinia milleri* (both Hydrozoa: Hydroida); thus, the data in Table I are generally representative of the PGEP synthetases from those coelenterates which contain significant quantities of this enzyme (cf. Table III).

As seen in Table I, the maximal rate of the autocatalytic reaction is stimulated *ca.* 2.5-fold by 1 M NaCl. By continuously recording the change in absorbance

during the spectrophotometric assay, this maximal rate of the salt-stimulated enzyme-catalyzed reaction can be determined with a high degree of accuracy, and is proportional to the amount of extract added. This same maximal rate (± 15%) can be obtained—with no autocatalytic lag—by providing hydrogen peroxide as activator in place of NaCl in the complete assay mixture. Maximal stimulation of the enzyme from coelenterate tissues was found to occur at approximately 0.6 mM H_2O_2; this is close to the value of 0.3 mM previously found to give optimal stimulation of PGEP synthetase from eggs of the abalone, *Haliotis rufescens* (Morse *et al.*, 1977, 1978). As expected, the peroxide-stimulated reaction is completely inhibited by the addition of purified catalase, an enzyme which rapidly and specifically decomposes the added H_2O_2 to water and oxygen. More significant, however, is the observation that both the autocatalytic activation, and all catalytic activity, seen in the absence of exogenous peroxide (± NaCl) are completely inhibited by a small concentration of catalase. This observation, also made with the PGEP synthetase from other marine invertebrates (Morse *et al.*, 1977, 1978), indicates that both the activity and autocatalytic activation of the enzyme from these sources normally depend upon the (autocatalytic) generation of H_2O_2 by the PGEP synthetase itself.

Both the maximal rate of the salt-stimulated reaction and the initial (= maximal) rate of the peroxide-stimulated reaction are absolutely dependent upon a heat-labile factor (presumably enzyme) in the added extract (Table I). Dependence upon the added substrate, arachidonic acid, is only partial and widely variable from extract to extract, presumably reflecting the variable presence of endogenous lipid substrates in the crude extracts. Enzymatic activity in the presence of either NaCl or H_2O_2 is inhibited to various extents by the pharmacological anti-inflammatory, analgesic and/or antipyretic agents phenylcyclopropylamine, aspirin, indomethacin, and acetaminophen; these agents are known to inhibit PGEP synthetases from a variety of different organisms and tissues with widely varying efficiencies (Lands and Rome, 1976). Phenylcyclopropylamine is most efficient, of these, at inhibiting the coelenterate enzyme; it had been observed previously that the salt-activated enzyme from *P. homomalla* was relatively insensitive to indomethacin, although lower concentrations of that agent than used in the present study had been employed (Corey *et al.*, 1973). Our data indicate, however, that the initial rate of the reaction catalyzed by the peroxide-activated coelenterate enzyme is significantly more sensitive to inhibition by aspirin and indomethacin than is the maximal rate achieved after autocatalytic activation in the presence of salt. As with the PGEP synthetases from other sources (Morse *et al.*, 1977; Letellier, Smith and Lands, 1973), the coelenterate enzyme is strongly inhibited by diethyldithiocarbamate (DDTC), a chelator strongly specific for copper. The addition of EDTA, a chelator which is specific for heavy metals other than copper, results in a slight but significant increase in catalytic activity. These latter observations suggest that copper may play some essential role in the coelenterate PGEP synthetase, as it does in many other oxygenases (Morse *et al.*, 1978); traces of other heavy metals appear to cause slight inhibition, which may be relieved by addition of EDTA.

The peroxide-stimulated reaction was further adapted for use in an assay which could be performed conveniently under field conditions, by replacement of

the H_2O_2 with a stable, enzymatic H_2O_2-generating system. As seen in Table II, a simple enzymatic system (consisting of glucose oxidase and its substrate, *D*-glucose) can be incorporated in the spectrophotometric assay for the continuous production of H_2O_2 (and glucuronic acid) *in situ*. The purified and concentrated glucose oxidase, which is inexpensively available from several commercial sources, proves to be fairly stable; such preparations can tolerate several weeks in transit without refrigeration, with little significant loss in activity. Using the "coupled assay" shown in Table II, with optimal concentrations of glucose and glucose oxidase replacing the direct addition of H_2O_2, the measured activity was found to exhibit dependence upon added coelenterate extract and substrate, and sensitivity to inhibitors, closely parallel to results obtained with the simple H_2O_2-stimulated reaction shown in Table I.

Using this convenient and readily portable assay procedure, the relative PGEP synthetase levels were measured in extracts of fresh, live tissue from a variety of coelentrates of the Caribbean and eastern Pacific (Table III). In addition to the very high levels of this enzyme found in *P. homomalla*, high or significant specific activities were found in four other species of plexaurids and *Gorgonia ventalina* (all Gorgonacea), the solitary Scleractinian, *Coenocyathus bowersi*, the Caribbean Antipatharian ("black coral") *Antipathes atlantica*, the Hydroids *Hydractinia milleri* and *Sertularia turgida*, and four species of *Millepora* and *Stylasterina* (the "Hydrocorallia"). Enzymatic activities from all of these sources were found to be dependent upon H_2O_2. The low specific activities measured in the other species assayed actually reflect lower concentrations of the enzyme, rather than the presence of some inhibitor of its activity, as no significant inhibition was detected upon mixing any of the extracts (of *all* species tested) with extracts of *Plexaura*, *Millepora*, or *Allopora*.

The distribution of the enzyme in the plexaurid Gorgonacea and in the "Hydrocorallia" (*Millepora* and *Stylasterina*) appears to be of some general significance, although few other taxonomic or physiological correlates of this distribution are apparent. It should be noted that if *Muricea* is included in the Plexauridae, as according to Bayer (1961), high levels of PGEP synthetase may not be entirely characteristic of this family; however, others have classified this genus

TABLE II

"Coupled assay" with endogenous generation of H_2O_2 by glucose oxidase. PGEP synthetase activity was assayed in an extract of Allopora porphyra *as described in Table I, except that the otherwise complete system contained "activator" as specified. Results are the averages (±s.d.) of duplicate determinations, normalized to the value obtained in the presence of H_2O_2 at optimal concentration.*

Activator	Initial Rate (%)
H_2O_2 (0.6 mM)	100 ± 1
None	2 ± 1
Glucose (5 mM)	2 ± 1
Glucose Oxidase (10 μg)	14 ± 6
Gluc. (5 mM) + Gluc. Ox. (10 μg)	115 ± 3
Gluc. (50 mM) + Gluc. Ox. (10 μg)	82 ± 7
Gluc. (5 mM) + Gluc. Ox. (100 μg)	83 ± 6

TABLE III

Species-distribution of PGEP synthetase in coelenterates. Specimens were collected and assayed as indicated; specific activities are the averages (±s.d.) of results from two or more separate colonies measured in the "coupled" assay with endogenous generation of H_2O_2 (5 mM glucose + 10 μg/ml glucose; cf. Table II). Collection sites are: P, Pacific; C, Caribbean; f.w., freshwater. An asterisk denotes species with high specific activity of PGEP synthetase. In all cases in which significant activity (≥0.1) was detected, proportionality of activity with added extract, and dependence upon H_2O_2 were determined (as in Tables I and II). Absence of inhibitors in all extracts was verified as described in the text.

Family	Species	Collection Site	PGEP Synthetase (Specific Activity)
(Anthozoa: Octocorallia)			
Plexauridae	*Plexaura homomalla*		
	(var. *homomalla*)	C	* 9.0 ± 1.8
	(var. *kükenthali*)	C	* 9.0 ± 2.2
Plexauridae	*Plexaura flexuosa*	C	* 8.5 ± 1.6
Plexauridae	*Pseudoplexaura flagellosa*	C	* 5.8 ± 1.5
Plexauridae	*Eunicea tourneforti*		
	(var. *tourneforti*)	C	* 3.0 ± 1.2
	(var. *atra*)	C	* 3.2 ± 0.3
Plexauridae	*Plexaurella dichotema*	C	* 2.0 ± 0.6
Gorgoniidae	*Gorgonia ventalina*	C	* 1.6 ± 0.3
Gorgoniidae	*Pseudopterogorgia americana*	C	≤0.1 ± 0
Gorgoniidae	*Eugorgia rubens*	P	0.3 ± 0.1
Gorgoniidae	*Lophogorgia chilensis*	P	≤0.1 ± 0
Gorgoniidae	*Filigella mitsukurii*	P	≤0.1 ± 0
Muriceidae	*Muricea californica*	P	≤0.1 ± 0
Clavulariidae	*Clavularia* sp.	P	0.3 ± 0.1
Virgulariidae	*Stylatula elongata*	P	≤0.1 ± 0
Virgulariidae	*Acanthoptilum gracile*	P	0.2 ± 0.1
Renillidae	*Renilla köllikeri*	P	≤0.1 ± 0
(Anthozoa: Hexacorallia)			
Seriatoporidae	*Madracis decactis*	C	0.2 ± 0.1

as belonging to a separate group (as indicated in Table III). No significant differences were observed between two subspecies each of *P. homomalla* (var. *homomalla* and var. *kükenthali;* Table III), *Eunicea tourneforti* (var. *tourneforti* and var. *atra;* Table III), or *Allopora porphyra* (vars. red *vs.* orange; cf. Ostarello, 1973), when these pairs were collected and assayed in parallel. Similarly, no significant differences were observed (in parallel collections and assays) between PGEP synthetase levels in male and female colonies of dioecious species such as *Plexaura*, *Millepora*, or *Allopora*.

Corey and Washburn (1974) had previously shown that the PG synthetase complex of *P. homomalla* resides in the tissue of the animal, rather than in its symbiotic zooxanthellae. In view of the suggestions made by them and others (Corey and Washburn, 1974; Gonzalez, 1978) that photosynthetic products of the zooxanthellae may neverthless contribute to (or control) the biosynthesis of PGRCs in coelenterates, it was of interest to determine the relative activities of PGEP synthetase from colonies of the same species exposed to widely differing regimes of illumination. However, we have found that colonies of *Millepora alcicornis* collected from depths of 1 m and 30 m (the extremes of its depth-

distribution which we observed), when assayed in parallel, showed no significant differences in specific activity, thus suggesting that photosynthetic activity may have little direct influence over the synthesis or activity of the rate-limiting enzyme, PGEP synthetase.

Discussion

In their studies of the PG synthetase from *P. homomalla*, Corey *et al.* (1973) found apparently complete dependence of activity upon added NaCl, whereas our assays detect only a 2–3 fold stimulation in extracts of this and other coelenterates. Possible reasons for the difference between these observations include the fact that Corey *et al.* measured the final yield of the overall enzymatic synthesis of PGA_2, whereas we have measured the rate of the reaction catalyzed by PGEP synthetase alone. Also, Corey *et al.* measured the final cumulative activity in extracts which had been stored frozen, whereas our assays were performed with specimens which had been freshly collected and live immediatly prior to assay. In fact, the activity of the PGEP synthetase complex was found in this study to be only partially stable in frozen tissues, with samples variably losing 50–80% of their activity when kept at − 30° C for two months.

Table III—*Continued*

Family	Species	Collection Site	PGEP Synthetase (Specific Activity)
Acroporidae	*Acropora palmata*	C	≤0.1 ± 0
Agariciidae	*Agaricia agaricites*	C	0.2 ± 0
Agariciidae	*Agaricia fragilis*	C	0.2 ± 0.1
Faviidae	*Diploria labyrinthiformis*	C	≤0.1 ± 0
Trochosmiliidae	*Meandrina meandrites*	C	≤0.1 ± 0
Trochosmiliidae	*Dendrogyra cylindrus*	C	0.3 ± 0.1
Eupsammidae	*Ballanophyllia elegans*	P	0.3 ± 0.1
Astrangidae	*Astrangia lajollaensis*	P	0.5 ± 0.2
Caryophylliidae	*Coenocyathus bowersi*	P	* 3.3 ± 0.4
Anthopleuridae	*Anthopleura elegantissima*	P	≤0.1 ± 0
Anthopleuridae	*Anthopleura xanthogrammica*	P	≤0.1 ± 0
Actiniidae	*Tealia crassicornis*	P	≤0.1 ± 0
Sagartidae	*Corynactis californica*	P	≤0.1 ± 0
Antipathidae	*Antipathes atlantica*	C	* 3.7 ± 0.5
Antipathidae	*Antipathes rhipidion*	C	0.2 ± 0.1
(Hydrozoa)			
Bougainvilleidae	*Hydractinia milleri*	P	* 9.8 ± 2.1
Tubulariidae	*Tubularia crocea*	P	≤0.1 ± 0
Eudendriidae	*Eudendrium californicum*	P	0.2 ± 0
Hydridae	*Pelmatohydra pseudoelegactis*	f.w.	≤0.1 ± 0
Hydridae	*Chlorohydra viridissima*	f.w.	≤0.1 ± 0
Sertulariidae	*Sertularia turgida*	P	* 11.2 ± 2.9
Campanulariidae	*Clytia bakeri*	P	≤0.1 ± 0
Plumulariidae	*Aglaophenia struthionides*	P	≤0.1 ± 0
Milleporidae	*Millepora alcicornis*	C	* 10.3 ± 2.4
Milleporidae	*Millepora complanata*	C	* 8.8 ± 3.0
Milleporidae	*Millepora squarrosa*	C	* 8.3 ± 4.3
Stylasteridae	*Allopora porphyra*	P	* 4.6 ± 2.2
Chondrophorae	*Velella velella*	P	≤0.1 ± 0

The PGEP synthetase reaction stimulated by salt remains autocatalytic, and thus, difficult to measure; assays monitoring the yield of PG products have proven unreliable for accurate and comparative quantitations of enzymatic activity (Corey *et al.*, 1973; Samuelsson *et al.*, 1975; Miyamoto *et al.*, 1976; Lands and Rome, 1976). Using the spectrophotometric assay with a continuously recording spectrophotometer, however, reliable determinations of the rate of the autocatalytic, salt-stimulated reaction catalyzed by PGEP synthetase in coelenterate extracts were obtained. This maximal rate is directly proportional to the amount of extract added (Table I), and is thus useful for comparative quantiations of enzyme activity.

Previous work from this laboratory has demonstrated that the PGEP synthetases from a variety of marine invertebrates can be activated by hydrogen peroxide; this activation proceeds with immediate elimination of the autocatalytic lag in the PGEP synthetase-catalyzed reaction, and thus makes possible the convenient quantitation of the enzyme with simple first-order kinetics (Morse *et al.*, 1977, 1978). Similar activation (with H_2O_2 in place of NaCl; see Table I) makes possible the direct and convenient quantitation of the enzyme from a wide variety of marine coelenterates.

That H_2O_2 is apparently generated by the enzyme reaction itself, and is thus responsible for the autocatalytic activation (observed in the absence of added peroxide), is indicated by the finding that the addition of *catalase* (0.1 μg/ml) to the reaction-mixture ($\pm$ 1 M NaCl) completely eliminates both autocatalytic activation and all catalytic activity of the enzyme in extracts of the coelenterates *Plexaura, Pseudoplexaura, Antipathes, Millepora,* and *Allopora.* Similar evidence has been found for the enzyme from marine molluscs and echinoderms (Morse *et al.*, 1977, and unpublished observations), and thus appears to reflect a general property of the reaction-mechanism of this enzyme from many invertebrate species. A role for copper at the active site of these enzymes has been postulated in the generation of H_2O_2 (Morse *et al.*, 1978), and is, in part, supported by the sensitivity of these enzymes to the copper-chelator, DDTC (Table I; Morse *et al.*, 1977, 1978). In these respects, as well as in the relatively low sensitivities to the anti-inflammatory drugs which are potent inhibitors of the mammalian enzymes, the properties of the PGEP synthetases from the marine invertebrates differ from those of the enzymes from mammalian sources (see also Corey *et al.*, 1973).

From a practical point of view, there are several advantages which use of the peroxide-stimulated reaction affords over measurement of the salt-stimulated reaction. Accurate measurements of the maximal autocatalytic rate of the salt-stimulated reaction require sophisticated electronic equipment for continuous monitoring and recording of the spectrophotometric assay. In contrast, the initial rate of the (first-order) peroxide-stimulated reaction can be measured readily in the field, with a simple spectrophotometer (or colorimeter) and stopwatch. H_2O_2 itself is unstable in dilute solution, and in concentrated form (or as the solid, *e.g.*, sodium peroxide) is both caustic and potentially explosive, and thus subject to internationally regulated precautions in transport. However, the peroxide-stimulated reaction can be further adapted to use in the field by replacement of H_2O_2 with a stable enzymatic H_2O_2-generating system (Table II). When used

with miniaturized and highly portable spectrophotometric equipment, this procedure makes convenient and reliable assays under field conditions possible, allowing comparisons of the specific activities of PGEP synthetase from live, freshly collected specimens of a variety of coelenterates from the Caribbean and eastern Pacific.

Use of these procedures has confirmed the identification of *Plexaura homomalla* as a species exceptionally rich in PGEP synthetase (Table III; Corey *et al.*, 1973; Weinheimer and Spraggins, 1969; Bayer and Weinheimer, 1974). In addition, this study has identified several related plexaurids, as well as certain other Gorgonacea, "Hydrocorallia", Antipatharia, Scleractinia, and Hydroida as species warranting further investigation as sources of potentially great PGRC biosynthetic activity. Although little systematic pattern is discernible in the distribution of the high levels of PGEP synthetase observed, it may be significant that all of the hydrocoral species tested (three *Millepora*, one *Stylasterina*) were found to have exceptionally high levels of this enzyme. Since the total productivity of Pacific and Atlantic species (particularly of the tropical hydrocorals) thus identified far exceeds the relatively low productivity of the Caribbean gorgonian *P. homomalla* (Hinman, 1974; Jordan, *et al.*, 1978), these findings may serve to relieve and diversify pressure for exploitation upon this latter and potentially threatened species.

Marine coelenterates are the phylogenetically simplest organisms in which significant levels of PGEP synthetase thus far have been found. Such activity was not detected in several species each of freshwater Protozoa and marine Porifera. Specific activities of enzyme in the most active coelenterate extracts (Table III) exceed those found in mammalian reproductive tissues, although they are about 50% lower than the highest values found in the eggs of abalone, *Haliotis* spp., and the urchins, *Strongylocentrotus* and *Lytechinus* spp. (Morse *et al.*, 1977, and unpublished observations). Although data implicate this enzyme in the control of reproductive processes in both abalones (Morse *et al.*, 1977, 1978) and urchins (Jensen and Morse, unpublished observations), there is as yet no information regarding the physiological functions of the especially active PGEP synthetases of the marine coelenterates. Similarly, the final (PGRC) products of the enzyme from these sources, with the exception of those from *P. homomalla,* remain to be identified.

The apparent distribution of PGEP synthetase activity found in the marine coelenterates (Table III) might reflect some pattern of seasonal variation, perhaps in reproductive or other specialized functions and/or tissues. However, no such seasonal variation has been detected in samples of five of the Pacific species (*Allopora, Sertularia, Lophogorgia, Muricea,* and *Tealia*) collected and assayed at intervals throughout the year. It is possible, then, that the high levels of PGEP synthetase characteristic of certain species may reflect a role in some fundamental process such as the regulation of ion- and water-transport, as originally suggested by Christ and Van Dorp (1972).

Alternatively, the potent PGRCs in these species might play some role in defense against predation or parasitism, or in specialized aggressive or prey-securing functions. The effectiveness of the PGRCs from molested and damaged colonies of *P. homomalla* in causing severe irritation and other symptoms of intoxication in human collectors has been documented previously (Brooks and

White, 1974). For a discussion of the many physiological functions in which postaglandins and PGRCs have been implicated, the reader is referred to the recent comprehensive reviews edited by Karim (1975, 1976).

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Summary

A convenient and reliable assay is described for PGEP synthetase, the rate-limiting enzyme determining the total capacity for biosynthesis of prostaglandin-related compounds. Results of such assays, performed with fresh specimens under both field and laboratory conditions, newly identify several marine coelenterate species as potentially important resources of PGRCs for research and possible development. Properties of the typical marine coelenterate PGEP synthetase, and the reaction which this enzyme catalyzes, have been further characterized.

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ADDITIONAL EXPERIMENTS ON THE BEHAVIOR OF BUDS IN THE ASCIDIAN, *APLIDIUM MULTIPLICATUM*

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In the colonial ascidian, *Aplidium multiplicatum*, the strobilae produced in the abdomen and postabdomen migrate through the tunic and approach the regenerating thorax (their mother zooid) to form a common cloacal system with it (Nakauchi, 1966a, Nakauchi and Kawamura, 1974a). In a previous paper (Nakauchi and Kawamura, 1974b), a series of experiments were undertaken by the authors to study the mechanism by which the buds move in the "right" direction, and by which the buds and mother zooid form a system.

Three kinds of experiments were described in the previous paper: first, destroying the mother zooid; secondly, pulling out the mother zooid; and thirdly, pulling out the mother zooid together with the tunic covering it. The results of these experiments suggested the possibility that a substance secreted by each mother zooid diffuses through the tunic and attracts the growing buds.

In order to confirm the existence of the attractant and to determine the time and site of its secretion, four additional experiments were designed.

Material and Methods

A colonial ascidian, *Aplidium multiplicatum*, was used (see Nakauchi and Kawamura, 1974a). The experiments were done at the Usa Marine Biological Station of Kochi University, from March to June, 1974, at a sea water temperature of 18–22° C. For details of culture method and treatment of colonies prior to operations, see Nakauchi and Kawamura (1974b). The four experiments described in this report are numbered consecutively with those of the previous paper (Nakauchi and Kawamura, 1974b; Experiments I, II and III).

Experiment IV

The results of Experiment II and III suggested that the attractant is secreted from a budding (mother) zooid, and it remains in the tunic for a while even after the removal of the zooid. Experiment IV was designed to determine whether the substance is secreted only by budding zooids or whether it is also produced by nonbudding zooids. It is known in polycitorines (Oka and Watanabe, 1961) and in polyclinids (Freeman, 1971) that the removal of the thorax of a zooid is followed by strobilation of the abdominal region within one or two days. So, in this experiment the thorax of a grown zooid (a prospective mother zooid) was cut off, and the behavior of the experimentally-produced buds, which lack a mother zooid from the first, was followed. If the substance is secreted into the tunic even in the nonbudding period, the produced buds would aggregate near the place where

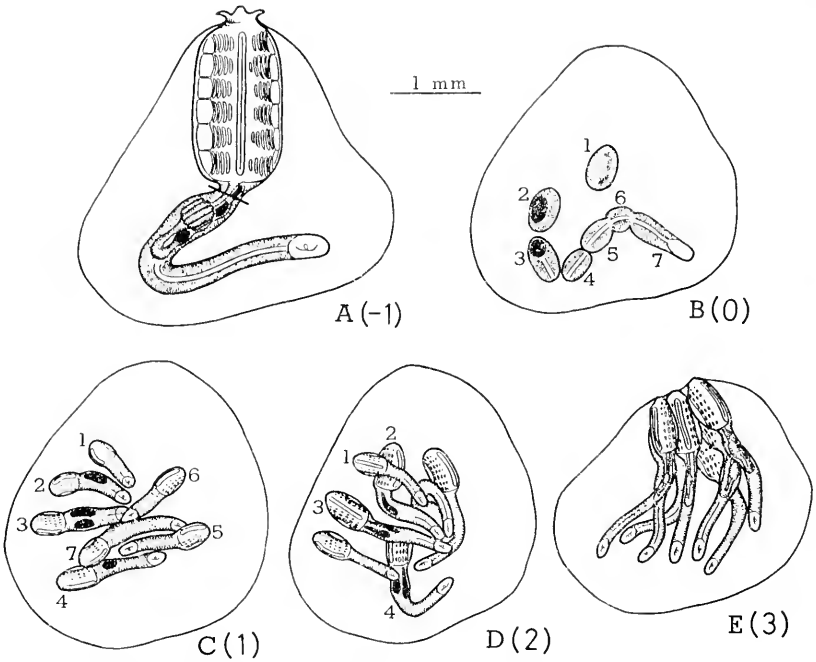

FIGURE 1. Behavior of buds in Experiment IV. Successive stages (A–E) in a typical case are viewed from the ventral side. The small arabic numbers identify individual buds in order from anterior to posterior, and the figures in parentheses indicate the number of days before or after budding.

the thorax had been located; if it is not, the grown buds should not aggregate at any definite place. They might aggregate at various places by chance, or open to the exterior independently without grouping. A total of 18 operations of this type were made.

Experiment V

In Experiment IV the growing buds arranged themselves near the place where the prospective mother zooid had been located. Therefore, in Experiment V the thorax of a grown zooid was cut off, together with the tunic surrounding it (Fig. 3A). By this procedure it was hoped to eliminate the attracting influence of the prospective mother zooid. A total of eight operations of this type were made.

Experiment VI

In normal budding, growing buds arrange themselves around the atrial aperture of mother zooid. It is plausible, therefore, that the attractant is most actively secreted by the epidermis around the aperture. So, in Experiment VI the anterior tip of the mother zooid was removed within one day after strobilation. At the

same time, all the buds but one were also removed, and the behavior of the remaining bud was followed. A total of nine operations of this type were made.

Experiment VII

The result of Experiment IV suggested that the attractant is secreted not only by budding zooids but also by nonbudding zooids. Consequently, Experiment VII was designed to find out whether a grown (nonbudding) zooid has the potency to attract buds produced by other zooids in the same colony. For the convenience of observation, all the zooids but two were removed from the colony. After the operation the two remaining zooids came together, and a small colony consisting of only two zooids was formed. It was known that budding in a colony does not occur synchronously. In the present case, therefore, one zooid was expected to make buds earlier than the other, and we could hope to study the attractive influence of a nonbudding zooid to buds produced by the other.

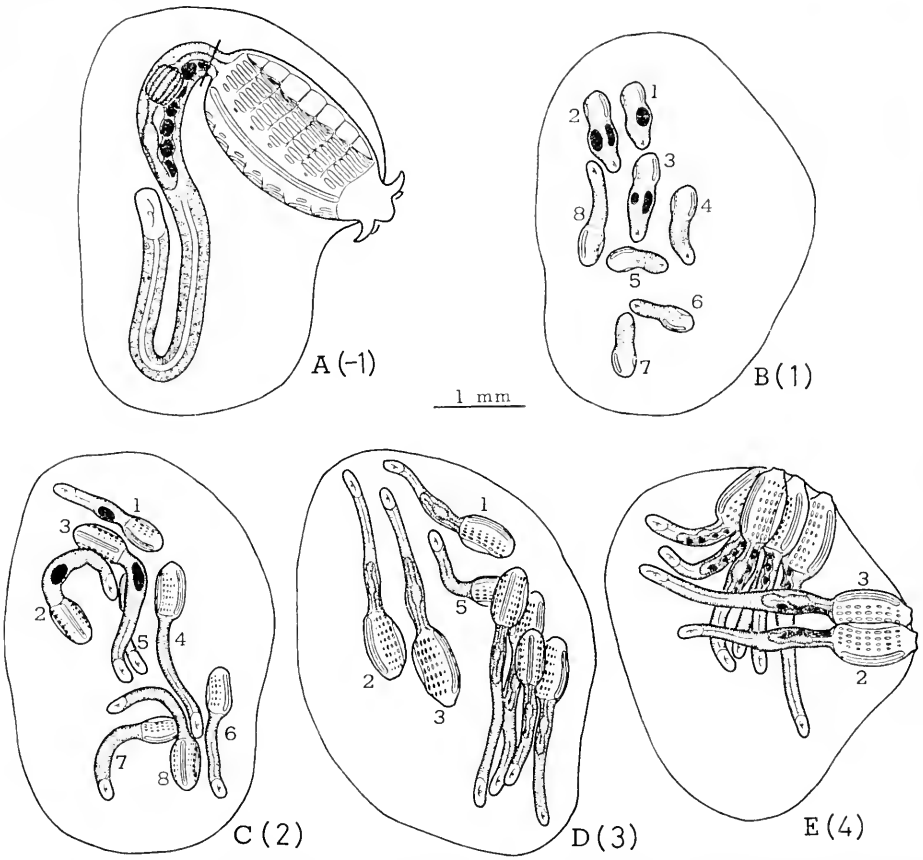

FIGURE 2. Behavior of buds in Experiment IV. Successive stages (A–E) in one of the minor cases are viewed from the ventral side. Bud identification numbers and days before or after budding are indicated as in Figure 1.

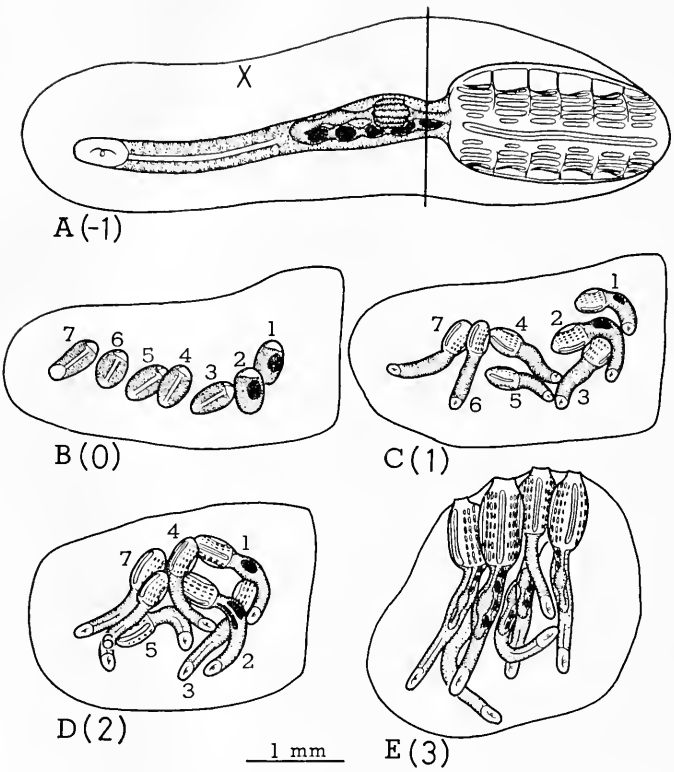

FIGURE 3. Behavior of buds in Experiment V. Successive stages (A–E) in a typical case are viewed from the ventral side. Bud identification numbers and days before or after budding are indicated as in Figure 1.

RESULTS

Experiment IV

In 14 cases out of 18, a single common cloacal system was formed by grown buds more or less near the place where the thorax (prospective mother zooid) had been located (Fig. 1). In the remaining four cases growing buds formed two groups and finally made two systems (Fig. 2). Even in the cases in which only one system was formed, the behavior of buds was somewhat different from that in usual budding. Buds lacking their mother moved more irregularly than in usual budding for about two days after budding (Fig. 1C–D). As a rule, the buds which had been originally located apart from the removed thorax needed more time to find the right direction than those located near the thorax. Following this stage the growing buds grouped to form a common cloacal system.

Experiment V

In five out of eight cases observed, one common cloacal system was formed by new zooid, while in the remaining three cases two systems were formed. Irrespec-

tive of the number of systems formed, the site of the common cloacal aperture did not appear to be influenced by the location of the prospective mother zooid which had been removed with its tunic before budding. That is, the systems did not

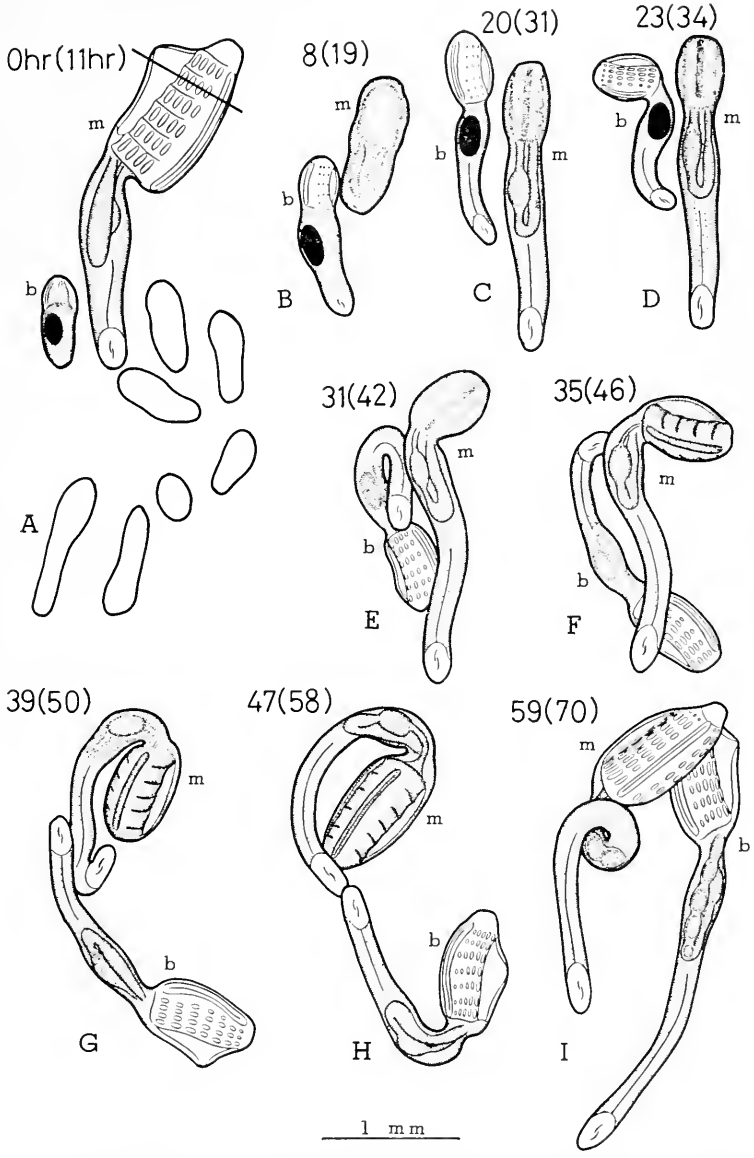

FIGURE 4. Behavior of an injured mother zooid and one remaining bud (Experiment VI), successive stages (A–I), in ventral view. The outline of the original tunic is omitted in this figure only. Time shown outside parentheses indicates the time after the operation in hours. Time shown in parentheses indicates the time after budding in hours. Abbreviations are: b, bud; m, mother zooid.

arise close to the cut surface of the tunic (Fig. 3). In most cases in which one system was formed, the new common cloacal aperture formed lateral to the position of the middle of the mother zooid's abdomen before strobilation (point "X" on Fig. 3A).

Experiment VI

In all nine cases the behavior of the single remaining bud looked strange. In eight cases the bud first moved forward, then turned and approached the abdomen or postabdomen of the mother zooid. This was followed by a complicated behavior of both bud and mother, the behavior of one apparently affecting the behavior of the other. After this, mother and bud arranged themselves side by side and finally made a common cloacal system. In the remaining exceptional case, the bud moved away from its mother zooid, and each opened to the exterior independently.

Figure 4 shows one of the major cases. The mother zooid contracted strongly after the operation; its thorax remained contracted for about 20 hours, while its new heart began to beat faintly about 8 hours after the operation. The bud moved toward the mother's thorax during the first 20 hours, then began to turn and finally pointed in the opposite direction (Fig. 4E). After this the bud moved toward the posterior end of the mother. On the other hand the mother, which had been turning very slowly, began a complicated behavior as the bud came near. Mother and bud changed their position as if they were affected by each other (Fig. 4, F–H), and they finally arranged themselves side by side (Fig. 4I) and made a system.

Experiment VII

Figure 5A shows two zooids left in the tunic, in which Zooid A is making ten buds. It was desired to eliminate the attracting influence of the thorax of Zooid A in order to see the attractive effect of Zooid B upon the buds produced by Zooid A. Thus, the thoracic bud of Zooid A was extirpated from the tunic. Four buds (3, 4, 9, and 10) were also cut off for the convenience of the observation. After these operations, Bud 1 and Bud 2 gradually moved toward the place where their mother had been located, and then they began to form a new system by themselves (Fig. 5B–D). On the other hand, Buds 5, 6, 7, and 8 approached the thorax of Zooid B (Fig. 5B); however, Zooid B made nine buds two days after Zooid A had budded. Of the nine buds of Zooid B, five buds (*1*, *2*, *3*, *5*, and *6*) were incorporated into the system which was being formed by Buds 5, 6, 7, and 8 of Zooid A. The remaining four buds were, however, attracted by Buds 1 and 2 of Zooid A, and finally formed a system with them. As shown in Figure 5E, two systems were formed in a colony, each of which consisted of zooids of two different origins.

Discussion

The results of Experiments IV and V are consistent with the hypothesis that a substance secreted from the thoracic region of the mother diffuses through the tunic and attracts buds. It is plausible that in Experiment IV the substance

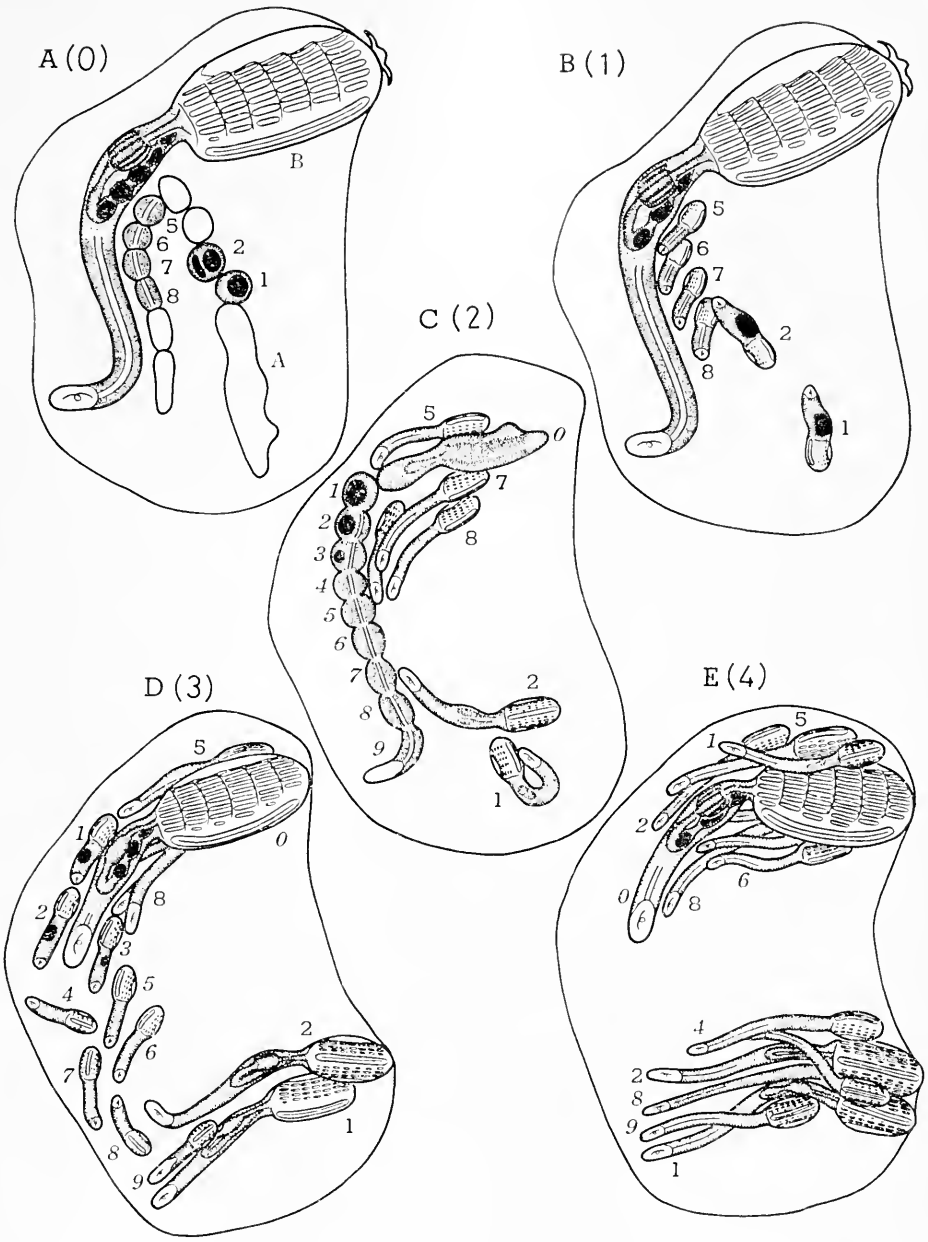

FIGURE 5. Behavior of buds in Experiment VII, successive stages (A–E), in ventral view. Time shown in parentheses indicates the time in days after budding of zooid A. The buds of Zooid A are identified by upright arabic numbers, and those of Zooid B by sloping ("italic") numbers.

remaining in the tunic after removal of the mother's thorax attracted the buds. This suggests that the period of secretion is not restricted to the time of budding. It is also likely that in Experiment V the removal of the adjacent tunic, as well as the maternal thorax, caused the elimination of the substance, and the buds became free of the influence of the mother. On the other hand, the buds appear to have influenced each other, seeing that they formed a system independent of the position of the "prospective mother zooid".

The result of Experiment VI is susceptible to various interpretations. The behavior of buds which have temporarily moved from the thoracic region of the injured mother may be explained at least in two ways. One is that the buds "dislike" the substance secreted by the wounded thorax, and they moved away from the vicinity of the wound. Another possibility is that the attractant is constantly secreted all over the body surface, although secretory activity shows an anteroposterior gradient with its high point at the most anterior region. When the anterior end is removed, the injury is accompanied by a reduction in secretory activity of the region, and the density of the attractant in the posterior region may become higher than that at the anterior. This temporary reversal of the polarity may last only until regeneration of the lost anterior end is completed. If this supposition is the case, one would expect the bud to move posteriorly at first, and then, after the recovery of the original polarity, to move anteriorly. Judging from the behavior of buds in other experiments, the latter case seems probable.

In Experiment VII it was shown that a bud is attracted not only by its mother but also by another grown (nonbudding) zooid or by a developing bud of another zooid. Because all the zooids in a colony originate from an oozooid, every zooid has the same set of genes. Consequently, zooids in a colony appear to have many characters in common. In other words, they lack individuality in many features. Therefore, an attractant secreted by one zooid may naturally attract all the buds in a colony regardless of their origin. The behavior of Buds 1 and 2 of Zooid A is understandable if we presuppose an attractant which had been secreted by the thorax of Zooid A and had diffused into the tunic before the thorax was removed. This result coincides with that of Experiment III shown previously (Nakauchi and Kawamura, 1974b).

Experiments are being undertaken to get more direct evidence of the existence of the attracting substance. The authors do not necessarily postulate a substance which has evolved specifically for the purpose of attraction. On the contrary, it is likely that growing buds are attracted by some metabolite produced by their mother zooid.

Setting aside the possible existence of an attracting substance, the movement of developing buds is known in many colonial ascidians. In polyclinids, most of which form common cloacal systems, Brien (1936) seems to have been the first to describe the movement of buds and its relation to system formation. Movement of buds is known even in colonial ascidians which form no systems (*Polycitor mutabilis*, Oka and Usui, 1944; *Metandrocarpa taylori*, Abbott, 1953, and Newberry, 1965; *Archidistoma aggregatum*, Nakauchi, 1966b; *Symplegma reptans*, Sugimoto and Nakauchi 1974; *Ritterella pulchra*, Nakauchi, 1977). Among these forms the buds of *Ritterella pulchra* can turn as much as 180°. We suggest that

buds of colonial ascidians have some ability to move through the tunic and to change direction of movement. Some colonial ascidians may have exploited this ability for the purpose of system formation. In *Metandrocarpa* it is known that the common vascular system in the tunic plays an important role in the movement of buds (Newberry, 1965). Nothing is known of the mechanism of bud movement in polyclinid ascidians which have no common vascular system. However, buds and developed zooids are sometimes observed to contract and expand, and it is likely that this action is involved in the movement of zooids. The fact that all the buds and developed zooids can move forward only (Nakauchi and Kawamura, 1974a) may be a clue for solving this problem.

It is our pleasant duty to express our hearty thanks to all the members of the Usa Marine Biological Station, Kochi University, in which the study was carried out. We also thank Professor D. P. Abbott of the Hopkins Marine Station, Stanford University, for his encouragement and critical reading of the manuscript.

Summary

In a previous paper by the authors, it was suggested that the behavior of growing buds, which form a common cloacal system, is affected by a substance which is secreted by the mother zooid and diffuses through the tunic.

Four sets of experiments were made to confirm the existence of the substance, and to get more information about the attractant. In the first set, the thorax of a grown zooid was removed before budding, and artificial strobilation was induced. In this case the buds lacked the mother zooid from the first. In the second, the thorax of a grown zooid was removed before budding, together with the tunic covering the thorax. In the third, the anterior tip of a mother zooid, thought to be a center of secretion, was removed. In the fourth, the experiment was designed to show whether a bud is attracted only by its mother and sisters or also by other zooids in the same colony. After these operations the behavior of buds was followed.

The results supported the existence of the attractant. They suggested that the time of secretion is not restricted to the period of budding, that the site of secretion is not restricted to a special region of the zooid, and that a bud is attracted not only by its mother but also by any other zooid in the same colony.

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SEASONAL BURROWING BEHAVIOR AND ECOLOGY OF *APORRHAIS OCCIDENTALIS* (GASTROPODA: STROMBACEA)

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The mesogastropod family Aporrhaidae is represented by only three living species comprising the genus *Aporrhais*. *Aporrhais pespelecani* (L.) and *A. serresiana* (Michaud) are restricted to the eastern Atlantic and have been studied by Yonge (1937). [See Fretter and Graham (1962) for a general account of the natural history of these species.] Locomotion in *A. pespelecani* has been examined by Weber (1925) and by Haefelfinger (1968). *Aporrhais occidentalis* (Beck) ranges from Labrador to Massachusetts in the western Atlantic (Johnson, 1930) and is found in depths of water from 10–2000 m (Clarke, 1962). Little information is available on *A. occidentalis*.

Aporrhaids are of particular interest to malacologists because they are the most primitive members of the superfamily Strombacea which includes the widely distributed and conspicuous tropical genera *Strombus* and *Lambis*. According to Cox (1960) and Zittell (1913), aporrhaids first appeared in the Jurassic as the earliest representatives of the Strombacea, and, on the basis of shell structure, Morton (1956) considers *A. occidentalis* to be the most primitive living aporrhaid. As is typical of most members of the Strombacea, the shell of *Aporrhais* is subject to age-dependent changes in morphology. The expanded and thickened outer shell lip of adults is absent in juveniles.

The Aporrhaidae, as well as the related but less ancient Struthiolariidae (Morton, 1951), are known to burrow in soft marine sediments, and Schafer (1972) has commented on the importance of *Aporrhais* in reworking the substrate. Yonge (1937) described the burrowing behavior of *A. pespelecani* and *A. serresiana* under laboratory conditions and concluded that these gastropods are specialized for burrowing in muddy gravel and only rarely move about on the surface of the substrate. Barnes and Bagenal (1952) examined dredged specimens of both species and found that the shells of adult snails were frequently encrusted with barnacles, bryozoans and polychaete tubes. Based on this evidence, they suggested that *Aporrhais* spends more time on the surface of the mud than was previously thought.

The SCUBA techniques used in the present study of *A. occidentalis* have permitted *in situ* tagging experiments and observations on the burrowing of these gastropods in their natural habitat.

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Materials and Methods

During 1973–1976 a population of *A. occidentalis* was studied in 17 m of water at the Isles of Shoals off Portsmouth, New Hampshire (42° 59′ N, 70° 37′ W). The size structure and density of this population was determined through quantitative bottom sampling using SCUBA transects and an epibenthic sled (Hessler and Sanders, 1967).

In April, 1975, individual snails were tagged so that their movements both upon and within the substrate could be followed from month to month. Nylon fishing line was used to affix numbered plastic tags to the shell spires of 20 male and 20 female specimens of *A. occidentalis*. The highly visible tags were buoyant and floated 5–8 cm above the mud at all times. The tagged animals were placed around a cinder-block anchor from which 10 m transect lines were extended in the four compass directions. The transect lines were marked at 1 m intervals so that the snails could be located within the resulting grid system. From May, 1975, to May, 1976, monthly SCUBA dives were made on this site. In addition to daytime observations, night dives were made in summer and winter. Data were taken on the location of tagged snails within the grid system and on whether or not these animals were epifaunal or infaunal.

During each monthly dive, bottom water temperatures were recorded with a hand-held mercury thermometer and notes were taken on the occurrence of potential predators within the transect area. Specimens of *A. occidentalis* were collected each month and preserved for subsequent gut content analyses. Untagged animals were normally used for this purpose, tagged snails being sacrificed only when no others could be found. Empty *A. occidentalis* shells brought up in dredge hauls or found during SCUBA dives were examined for evidence of predation.

In the laboratory, adults and juveniles of *A. occidentalis* were maintained in a flowing seawater system. Burrowing, feeding and copulation were observed and attempts were made to determine the effects of different water temperatures on burrowing behavior.

Results

Specimens of *A. occidentalis* were first observed by the author at the Isles of Shoals during a SCUBA dive in March, 1973. The animals were fully exposed on the level muddy bottom and seemed to be grazing on a thin brown film which covered the substrate. This film was later examined and found to consist of high concentrations of the benthic diatom *Pleurosigma* sp., as well as the decaying remains of several species of macroalgae. Gut content analyses revealed that this material was indeed being ingested along with some sand, sponge spicules and empty foraminifera tests. The shells of these snails were not encrusted with sessile organisms except that the shells of older specimens of *A. occidentalis* were frequently riddled by the boring spionid polychaete *Polydora commensalis* Andrews.

A series of thirty 1 m × 15 m SCUBA transects run at the study site in April, 1974, yielded a total of 28 epifaunal specimens of *A. occidentalis*. Twenty-one of these animals were mature adults with well-developed outer shell lips, while the remaining seven were juveniles ranging in shell length from 20–45 mm. Epi-

benthic sled hauls taken in the same area contained large numbers of juvenile *A. occidentalis* not seen during the SCUBA transects. Ten 0.5 m × 15 m sled hauls yielded 40 young snails and only four adults. Therefore, most of the juveniles in this population were infaunal, while the adults were epifaunal. Laboratory observations over a three year period also showed that juveniles burrow more rapidly and spend more time in the substrate than do adults. Sediment samples taken in April, 1975, contained early post-metamorphic *A. occidentalis* juveniles measuring 1.2–1.5 mm in shell length. Similar sediment samples taken in October, 1975, contained no juveniles smaller than 6.5 mm.

In both 1973 and 1974 the population of *A. occidentalis* at the Isles of Shoals disappeared from the surface of the mud by August and did not reappear until the following February. Although dredging carried out during the winter of 1974–1975 showed that the snails had burrowed at the study site, tagging experiments begun in April, 1975, provided more detailed and quantitative data on seasonal burrowing behavior.

Figure 1 shows the percentages of tagged specimens of *A. occidentalis* found burrowing each month from May, 1975, through April, 1976. Figure 1 also includes monthly bottom water temperatures. Numbers of burrowing animals are expressed as percentages because the total number of snails found each month (both infaunal and epifaunal) varied as a function of water clarity and the time available for searching. Also, the number of tagged snails diminished over time as animals were sacrificed for gut content analyses or were lost due to predation or other factors.

Virtually all of the tagged animals were infaunal from August through October. In November, all of the males remained infaunal but eight of the ten females counted were epifaunal. In December and January the entire population was again infaunal. Most of the tagged *A. occidentalis* were found crawling about on the surface of the substrate from February through June, and 40% were epifaunal in July. Except during the month of November, there were no obvious differences in burrowing behavior between male and female snails.

The results of gut content analyses performed on specimens of *A. occidentalis* collected at the study site suggest seasonal changes in feeding behavior correlated with burrowing. From August through January, all animals had empty stomachs and intestines. Epifaunal snails collected from February through July were actively feeding and had full guts. Furthermore, each of these animals had a well-developed crystalline style in its style sac. Crystalline styles were never found in animals with empty guts.

Gut content analyses were also performed on specimens of *A. occidentalis* collected in deep water by the United States National Marine Fisheries Service and made available by Dr. Roland Wigley of the Woods Hole, Massachusetts, office of the NMFS. Three specimens (two females and one male) dredged from 174 m (42° 05′ N, 69° 50′ W) in November, 1958, had empty guts. Six specimens (three males and three females) collected from 242 m (43° 19′ N, 67° 45′ W) in June, 1961, had full guts.

Because field observations were made only at monthly intervals, it was impossible to obtain detailed data on the mobility of epifaunal snails. From May through July, 1975, when most specimens of *A. occidentalis* were actively feed-

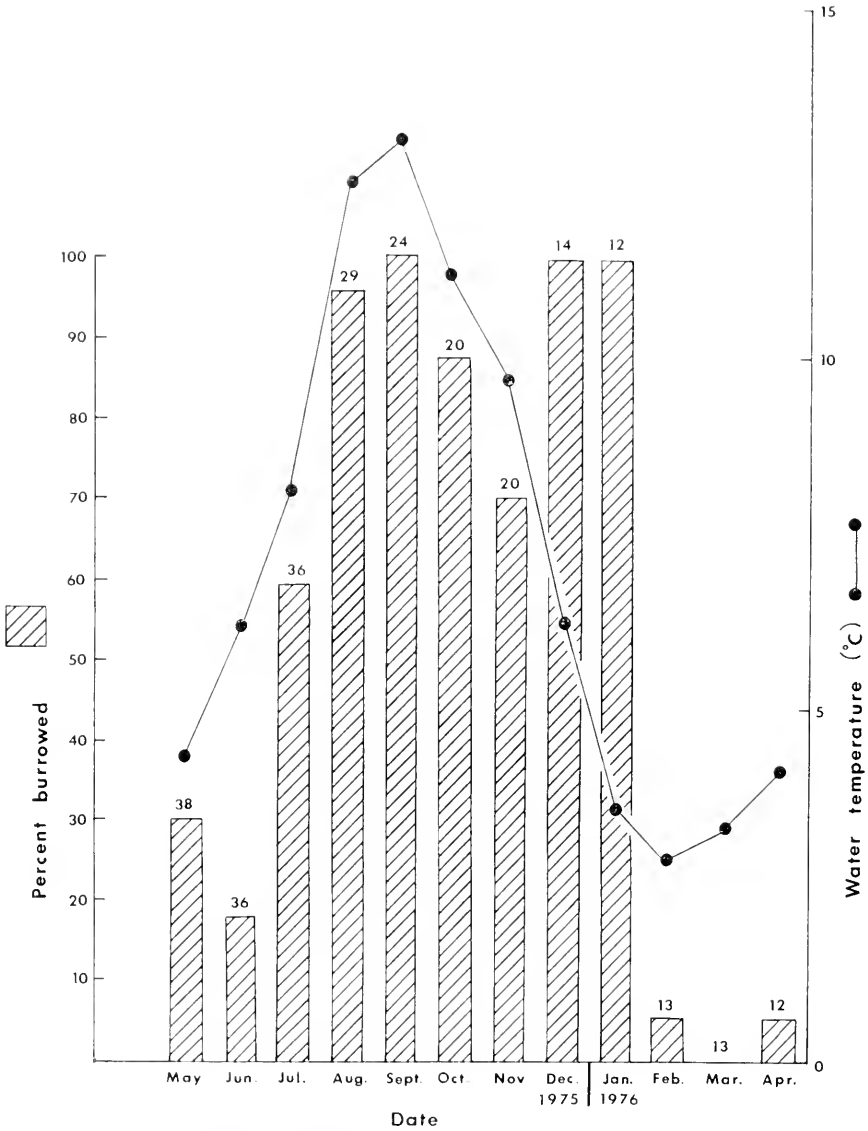

FIGURE 1. Percentages of tagged *A. occidentalis* found burrowing each month at the Isles of Shoals study site. Numbers over histogram bars indicate the total number of tagged snails counted each month. Bottom water temperatures are represented by connected dots. Temperatures represent single measurements taken during monthly dives.

ing on the surface of the substrate, no animal was observed to move more than 10 m from one month to the next. When the population of tagged *A. occidentalis* became infaunal in August, 29 of the original 40 snails were still within the limits of the transect lines. The 11 animals not counted in August may have wandered

away from the study area. However, it is also possible that they were carried off by predators or had lost their numbered tags. From August through January, with the exception of November, no movements of individual snails were noted from month to month. Observations made during night dives showed that although *A. occidentalis* is more active at night than in the daytime during its epifaunal period (February–July), burrowed snails during August–January do not emerge from the substrate at night.

Although copulation was never observed in the field, specimens of *A. occidentalis* kept in the laboratory frequently copulated at night during March and April.

Potential predators on *A. occidentalis* include the carnivorous gastropod *Colus stimpsoni* Morch, the crab *Cancer irroratus* Say, and possibly the molluscivorous wolf fish *Anarhichas lupus* L. *Colus stimpsoni* is present at the Isles of Shoals study site throughout the year and preys on a variety of gastropods. Although *C. stimpsoni* was not observed actually feeding on *A. occidentalis* in the field, instances of predation did take place in the laboratory. *Aporrhais occidentalis* shows a distinct escape response (accelerated locomotion) to the presence of *C. stimpsoni* (Perron, 1978). The crab *C. irroratus* was active at the study site from July through November. Several instances of attempted predation on *A. occidentalis* were observed in the field, and in one case, a crab was seen grasping the numbered tag of a burrowed *A. occidentalis* and pulling the snail from the substrate. In the laboratory, crabs readily devoured juvenile *A. occidentalis* by progressively cracking away the shell aperture until the soft parts were exposed. However, even large specimens of *C. irroratus* (carapace width 6 cm) were rarely able to feed on an adult *A. occidentalis* with well-developed outer shell lips.

In Table I the 71 empty *A. occidentalis* shells collected haphazardly over the course of a year at the Isles of Shoals are classified according to types of visible shell damage. Shells showing crab damage all had apertures which were chipped away in the manner observed in the laboratory and as described and figured by Vermeij (1976). Five of the adult *A. occidentalis* shells showing crab damage had previously been weakened by infestations of the boring polychaete *Polydora commensalis*. Shells so badly crushed that they were reduced to fragments may have been attacked by fish or crabs. Finally, undamaged empty shells may indicate predation by *Colus stimpsoni* or some undetermined cause of mortality.

Laboratory attempts to influence the seasonal burrowing behavior of *A. occi-*

TABLE I

The condition of empty A. occidentalis *shells collected over a one year period at the Isles of Shoals study site. During the same period 143 live animals (60 adults and 83 juveniles) were found.*

Type of shell damage	Number of shells		Probable predator
	Adult	Juvenile	
Chipped outer lip	6	41	Crab
Crushed	2	4	Fish or crab
No damage	11	20	Predatory gastropod

dentalis by manipulating water temperature were unsuccessful. Twenty active epifaunal adult snails collected in March, 1976, were split into two groups and kept at water temperatures of 4–7° C and 13–16° C, respectively. No differences in behavior were noted between the two groups, and all 20 animals remained epifaunal until the experiment was terminated after two months. Specimens of *A. occidentalis* kept in the laboratory for long periods of time tended to become less active and more infaunal. Such animals were also subjected to differing temperature regimes, but no resultant changes in burrowing behavior were observed.

Discussion

The results of the experiments reported here show that the specimens of *A. occidentalis* in the population studied alternate between periods of epifaunal feeding activity and infaunal nonfeeding quiescence. Although tagging data are available only for the year 1975–1976, SCUBA observations during the preceding two years indicate that seasonal burrowing is a regular occurrence in this gastropod. Since *A. occidentalis* has such an extensive bathymetric range, it may not be reasonable to assume that the shallow water Isles of Shoals population is typical of the species as a whole. However, gut content data from specimens collected in deeper water (174–242 m) conform precisely to the pattern observed in the Isles of Shoals population.

The observations of Barnes and Bagenal (1952) on dredged *A. pespelecani* are consistent with a seasonal burrowing pattern similar to that of *A. occidentalis*. The shells of *A. pespelecani* collected by Barnes and Bagenal in April were covered with small newly set barnacles, while "enormously elongated" barnacles were found on specimens dredged in late July. The presence of live barnacles indicates that these *A. pespelecani* were epifaunal during the spring and summer months. Barnes and Bagenal also reported that the shells of dredged juvenile *A. pespelecani* were nearly always free of encrusting organisms. Their suggestion that juveniles spend more time burrowed than do adults is supported by the field and laboratory observations in the present study.

The data in Figure 1 suggest a possible relationship between water temperature and burrowing. At the Isles of Shoals study site, specimens of *A. occidentalis* emerge from the substrate when water temperatures are at their lowest, and remain active until warming takes place during the summer. However, laboratory experiments failed to provide evidence for a causal relationship between temperature and burrowing. Furthermore, since *A. occidentalis* ranges to a depth of 2000 m where seasonal temperature fluctuations are small (Rokop, 1974), temperature would seem an unlikely coordinator of seasonal burrowing. Further research will be necessary to identify the environmental factor or factors which control burrowing in *A. occidentalis*.

Similarly, the available data are not sufficient to explain the role of seasonal burrowing in the life history of this gastropod. It is tempting to suggest that *A. occidentalis* avoids predation by *C. irroratus* by burrowing at the time of year when the crab is most active. Jeffries (1966) has shown that the temperature optimum of *C. irroratus* is approximately 14° C and that these predators become less active and move to deeper water during cold winter months. Nevertheless,

this explanation for the seasonal burrowing of *A. occidentalis* seems questionable when one again considers that the Isles of Shoals population is at the shallow water end of a bathymetric range which extends into the thermally stable depths where predators are presumably not affected by seasonal temperature fluctuations.

Although little is known about reproduction in *A. occidentalis,* Johansson (1948) has studied the reproductive system of *A. pespelecani,* while Lebour (1933) has observed the eggs and larvae of this eastern Atlantic species. *Aporrhais pespelecani* eggs are small (0.25 mm) and are deposited singly or in small groups. The larvae are planktotrophic and undergo considerable growth in the plankton before settling (Lebour, 1933; Thorson, 1946).

The eggs and larvae of *A. occidentalis* have never been reported. However, young benthic animals with shells measuring 1.2–1.5 mm collected by the author in April, 1975, were nearly identical to early post-metamorphic juveniles of *A. pespelecani* figured by Lebour (1933). Since no juveniles smaller than 6.5 mm were taken in October, 1975, it is possible that the breeding season of *A. occidentalis* is similar to that of *A. pespelecani,* with egg laying taking place in early spring (February–March) and larvae settling in April and May (Lebour, 1933). If this is the case, then the reproductive cycle of *A. occidentalis* may consist of a build up of energy reserves during the epifaunal feeding period followed by conversion of this energy into gonad development during the period of infaunal quiescence. The presence of epifaunal nonfeeding females in November is perplexing and may indicate that oviposition takes place at this time rather than in the spring.

Aporrhais is not unique among the Strombacea in possessing burrowing habits. The Struthiolariidae, which probably evolved directly from the Aporrhaidae (Morton, 1951), remain infaunal for long periods while feeding by a ciliary mechanism similar to that of the burrowing, nonstrombacean mesogastropod, *Turritella* (Yonge, 1946). However, there is no suggestion in the literature that burrowing in the Struthiolariidae is seasonal, and unlike *Aporrhais,* these gastropods certainly continue feeding while burrowed. The strombid *Terebellum terebellum* (L.) is also known to be an active burrower (Abbott, 1962), but, again, year round studies have not been carried out.

A seasonal burrowing cycle similar to that of *Aporrhais* has been described for the tropical strombid gastropod *Strombus pugilis* L. by Percharde (1968, 1970). Percharde reports that colonies of *S. pugilis* off the island of Trinidad in the Caribbean burrow in November, cease feeding, and do not resume normal activity until March or April. At the end of this infaunal period the males emerge from the substrate first, while the females remain burrowed for a time and lay their eggs. Percharde (1970) also presents data suggesting similar burrowing behavior in *S. alatus* Gmelin and *S. raninus* Gmelin.

Recent studies by Berg (1974) and Perron (1978) have pointed out the marked homogeneity of locomotory behavior patterns within the Strombacea from the primitive *Aporrhais* to the more highly evolved *Strombus* and *Lambis.* Until year round *in situ* studies have been carried out on additional members of the Strombacea, it will not be possible to determine how pervasive the trend toward seasonal burrowing may be within this superfamily. Nevertheless, the similarities in burrowing habits between *A. occidentalis* and *S. pugilis* probably

reflect the conservative nature of behavioral evolution within the morphologically diverse Strombacea.

I thank Larry Harris and Ruth Turner for their encouragement and support during this project. Technical assistance was provided by Cynthia Mroch. Also, much of the field work could not have been done without the diving assistance of Brian Rivest, Barry Spracklin, Alan Hulburt and Paul Lavoie. Special thanks are due Ned McIntosh, captain of the University of New Hampshire research vessel, JERE A. CHASE.

Summary

1. SCUBA observations and *in situ* tagging experiments were carried out on a population of *Aporrhais occidentalis* during 1973–1976. Seasonal changes in burrowing behavior were quantified by determining the percentage of tagged snails found burrowing each month. Gut content analyses were performed at monthly intervals to determine if the intensity of feeding activity fluctuates seasonally. Empty *A. occidentalis* shells were collected and examined for evidence of predation.

2. Specimens of *A. occidentalis* alternate between periods of epifaunal activity and infaunal quiescence. Tagged snails tended to remain burrowed from August through January, but were active on the surface of the substrate from February until late summer. Gut content analyses showed that the snails fed actively during their epifaunal period, but ceased feeding while burrowed.

3. Laboratory attempts to influence burrowing behavior by manipulating water temperature were unsuccessful.

4. Published observations on eastern Atlantic species of *Aporrhais* suggest that seasonal burrowing behavior may be characteristic of the genus.

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FOOD-RESOURCES AND THE INFLUENCE OF SPATIAL PATTERN ON FEEDING IN THE PHORONID *PHORONOPSIS VIRIDIS*

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The Phoronida are a coelomate phylum of vermiform, lophophorate tube-dwelling organisms. Although the phylum consists of but two genera and some eleven species (Emig, 1974), all resident in shallow marine waters (Hyman, 1959), it is possibly of great phylogenetic and ecological importance. Indeed, phoronids may well represent the most primitive of living deuterostomes (Zimmer, 1964) and the ancestoral stock of the lophophorates (Valentine, 1973; Farmer, Valentine and Cowen, 1973). Ecologically, phoronids are often important in the structure of soft-sediment and fouling communities in that they may monopolize primary space (Ronan, 1975), a potentially limiting resource as in the rocky intertidal region. Despite the significance of the Phoronida, it remains a relatively obscure phylum which has not attracted the attention of many investigators. Hyman (1959) has reviewed the literature pertaining to phoronid biology. Work in English on the Phoronida has emphasized systematics (Marsden, 1959; Emig, 1974), developmental biology (Rattenbury, 1953; Zimmer, 1964, 1967), and genetics (Ayala, Valentine, Barr, and Zumwalt, 1974); phoronid ecology has received little attention (MacGinitie, 1935; Johnson, 1959; Ronan, 1975).

This paper examines the spatial pattern, feeding, and food-resources of the phoronid *Phoronopsis viridis*, a large phoronid with a pale green lophophore which inhabits intertidal localities in west coast embayments. In the past it has been assigned to *Phoronopsis viridis* (Hilton, 1930) based on specimens from Morro Bay, California, but it has also been synonymized with *P. harmeri* (Pixell, 1912) described from Vancouver Island, British Columbia (Marsden, 1959). Zimmer (University of Southern California, personal communication) believes the Canadian and Californian populations are specifically distinct, with the California form to be called *P. viridis;* I shall employ this name, although there is no work on geographic variation and relationships are uncertain.

Materials and Methods

Study site

The study was conducted in Bodega Harbor, California (38° 19′ N, 123° 03′ W), a small marine coastal embayment located 100 kilometers north of San Francisco, California. The harbor is quite shallow (maximum depth, 4.0 m at low water). At mean lower low water (MLLW = 0.0 ft) extensive tidal flats, which occupy about 60% of the harbor, are exposed. The harbor is a depositional environment. Without periodic maintenance dredging, the harbor would revert to a lagoon.

Within the harbor there is a 0.5 mile^2 sand flat which is posted and maintained as a marine life refuge by the University of California Bodega Marine Laboratory. Phoronid and sediment collections were taken within the refuge and just to the north of the refuge. Feeding observations and nearest-neighbor (N-N) measurements were made within the refuge boundaries.

Field census and nearest-neighbor relations

The intertidal distribution and abundance of *Phoronopsis* were determined by hand excavation of square meter holes along two transects. The transects were roughly parallel to each other from MLLW to the mean higher high water (MHHW) mark (120 cm above MLLW). The distance between transect stations was 10 meters; the longer transect A had twice as many stations as transect B. Care was taken to establish the transect stations in areas known to be free from clam digging which can greatly modify the spatial pattern of *Phoronopsis*. During excavation, all phoronid tubes were separated from the sedimentary matrix and their numbers recorded. Phoronid numbers were estimated at 95% of the counted numbers of tubes because about 5% of the tubes in dense aggregations are known to be vacant (Ronan, 1975).

Nearest-neighbor measurements were made along transect A, at stations 3, 5, and 7, following methods proposed by Clark and Evans (1954). Spacing measurements were not possible at station 1 because phoronid tube apertures were occluded by flocculent seston which thickly mantled the depositional interface. Higher in the intertidal, the spatial pattern of *Phoronopsis* was easier to determine since the small holes produced by the animal at the sediment-water interface (SWI) remain open at low water. Because individuals of this species aggregate in clusters of up to thousands per m^2 throughout the study area, all N-N measurements are within cluster distances. At each sampling station, three 25 cm^2 frames fitted with clear plastic inserts were randomly dropped and the area occupied by the largest cluster circumscribed with a rectangle. Within the rectangle, the position of each animal was recorded on the plastic with a felt tip marker. Since only inhabited tubes had open apertures, cluster population density was accurately determined by counting the dots on the plastic. For all animals, distance to N-N was estimated as the distance to the nearest mm between the centers of the dots.

Feeding observations

Low intertidal sites (2 m^2), each estimated to contain more than 17,000 *Phoronopsis,* were selected for detailed underwater feeding observations. The study sites were adjacent to transect A and separated from each other by 5 m. About six hours were spent underwater on various occasions observing phoronid feeding behavior.

As a phoronid lophophore is small and held close to the SWI, it is best to view it from the side. Feeding observations were made by SCUBA diving with a heavy weight belt and tethering to a short line anchored in the sediment nearby. Height measurements were made on nine clustered phoronids every 15 minutes over a 60-minute period.

Food resources

Early observations indicated that a feeding animal positions its lophophore within the turbid near-bottom layer of water (Ronan, 1975). The location of the feeding appendage is reflected in the animal's stomach contents in that the ingested materials primarily represent items resuspended from the SWI. To confirm this impression, food selection in relationship to the animal's available food was quantified by examining the food-resources of the SWI and the water column.

A large diameter (5 mm) pipette was used to collect seston (skeletal material, mineral grains, and organic particles) from the SWI around the tubes. The seston was preserved in 90% alcohol and examined under a dissecting microscope. Using the criteria of Johnson (1974), seston material was classified by particle type. Mineral grains were categorized by size and the presence or absence of encrusting organic matter. Loose aggregates of fine-grained minerals bound in an organic matrix were termed floc (organic-mineral) aggregates. Firm organic-mineral aggregates in the form of pellets, or fragments, were classified as either *Phoronopsis* feces (which are distinctive) or other fecal matter. The remaining material was listed as either plant fragments, pollen, diatoms, or small metazoans (copepods, nematodes, ostracods, etc.). Mineral grains were measured with an ocular micrometer. Particle type abundance categories (Johnson, 1974) were used to express the abundance of different fractions of the food-resources available to the organism.

During the same period (Sept.–Oct., 1975) in which phoronids were collected for stomach content analysis, plankton was also collected by towing a 0.25 m plankton net with 0.333 mm mesh size twice through the water with the base of the net no more than 10 cm above the bottom for two 15-minute periods. The entire sample was analyzed and particle type abundance categories (which were calculated by averaging the two samples) were used to express the relative abundance of the plankton species available to *Phoronopsis*.

Stomach contents

Twenty specimens of *Phoronopsis* were removed from their tubes, preserved in 90% alcohol, and the ingested material collected from the stomach. After the stomach fractions were washed in distilled water to remove adhering mucus, they were examined under magnification. The methods described above for analyzing particle fractions were employed to determine abundance of particle types and size distributions for the stomach samples.

The results of the analysis of abundance of different particle types are expressed as percentage of particle abundance. Whitlatch (1974) suggests the use of this measure in determining food selection because it reflects the relative amounts of different particles available in the environment of an organism.

Electivity coefficients of different particle types selected by *Phoronopsis* were determined using the statistic of Ivlev (1961). The statistic is calculated as $E' = (r_i - p_i)/(r_i + p_i)$. For the ith food type, r_i equals the percentage ingested and p_i is the percentage of that food type available in the environment. The coefficient is bounded and symmetrically distributed about zero ($E' = 0$ indicates

nonselective feeding; $-1 \leq E' < 0$ indicates avoidance; and $0 < E' \leq 1$ indicates feeding preference).

Phoronid fecal pellets were collected from the field with a small-diameter (2 mm) pipette, washed and disaggregated in sea water on a 250 μm sieve, and the contents examined under magnification. Particle size analyses of disaggregated feces were performed in distilled water.

Results

Abundance and spatial distribution

Figure 1 shows the number of *Phoronopsis* excavated from meter-square quadrats along the two transects. In the intertidal zone, phoronids are aggregated in discrete clusters that are separated from other clusters by intervening open

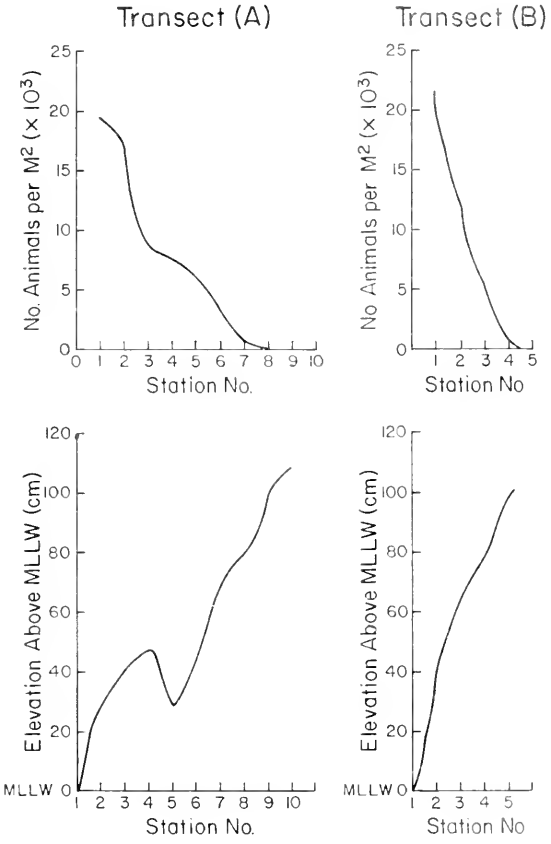

Figure 1. A and B (above) show the number of *Phoronopsis viridis* removed from square-meter excavations along two parallel intertidal transects; and (below) station elevations with respect to distance above mean lower low water. The sampling interval between stations was 10 m.

spaces where density is low. The clusters were largest and the densities greatest within firm sediments with a median particle diameter less than 250 μm. Along the transects which traversed fine sediment only, cluster density ranged from 21,422 phoronids/m^2 at MLLW to zero at the highest intertidal stations; their upper limit roughly corresponded to the mean lower high water (MLHW = 100 cm above MLLW) line. The mosaic of irregular clusters and intervening open spaces was most obvious near the animals' upper limit where clusters were small (range: 3–67 phoronids) and their boundaries distinct. The most dense and continuous clusters were around MLLW, where aggregate clusters containing up to 150,000 phoronids and covering up to 10 m^2 occurred.

Phoronids were not distributed evenly throughout the low and mid-intertidal, however. *Phoronopsis viridis* was absent or rare in lenses of loosely packed sediment with a median particle diameter greater than 250 μm. In an elliptical bed of coarse sand (5 × 30 m) along transect A, located eight meters shoreward from the low tide line, cluster density declined from 12,116 phoronids/m^2 at the periphery of the bed to 0/m^2, 1.5 meters into the interior of the coarse substrate. A smaller lens-shaped (5 × 8 m) body of coarse sediment occurred in the mid-intertidal. In this substrate, the cluster density of phoronids was very low, ranging from 9370 animals/m^2 at the periphery to 0 animals/m^2 at a distance of 0.5 m into the interior. Although the number of animals per square meter always declined in loose coarse sediment, the phoronids remain aggregated in small, tight clusters.

Table I shows the N-N frequency distribution for 2616 *Phoronopsis* in nine intertidal clusters composed of from 75 to 481 individuals. All of the measurements were made in fine sediment prior to the excavation of stations 3, 5, and 7 along transect A. The mean distance to N-N was 5.4 ± 2.8 (s.d.) mm with a range of 1–25 mm. The mode is slightly less than the mean, and the distribution has a long tail of distances greater than the mean.

N-N analysis of the dispersion pattern of *Phoronopsis* indicates the distribution

TABLE I

Frequency distribution of distances between individuals for 2616 Phoronopsis viridis *in nine intertidal aggregations.*

Distance (to nearest mm)	Frequency	Per cent	Cumulative percentage
1	92	3.52	—
2	183	7.00	10.52
3	401	15.33	25.85
4	516	19.72	45.57
5	360	13.76	59.33
6	341	13.04	72.37
7	293	11.20	83.57
8	125	4.78	88.35
9	136	5.20	93.55
10	33	1.26	94.81
11–13	79	3.01	97.82
14–16	36	1.37	99.19
>17	21	0.80	99.99

of individuals within clusters departed significantly from randomness. A mean R ($\bar{R}$) value for 9 clusters (cluster size 75–481 individuals; $N = 2616$) of 0.64 indicates that N-N were only slightly more than half as far apart as expected under conditions of randomness ($P < 0.01$). The mean N-N distance of 5.4 mm was roughly half the space required to expand two adjacent lophophores completely, without their impinging each other. Individuals crowded together in this manner were observed to stratify their lophophores vertically by some individuals extending their trunk above the SWI. Because the body wall is flexible, a phoronid can spread the lophophore away from neighbors by bending the extended trunk. Small N-N distances often produced an array of "tall" and "short-standing" phoronids as the individuals in a cluster maneuver for space to expand their lophophores. However, few ($< 11\%$) individuals of *Phoronopsis* were packed within 3 mm of each other. At such close distances, even a stratification of feeding appendages failed to provide for full expansion of adjacent lophophores; the N-N distances were so slight that an expanded lophophore would abut against the trunk of a neighboring "tall-standing" individual. Therefore, the distance from the lophophore of one individual to the trunk of an adjacent individual provides a measure of the lower limit below which feeding space cannot be reduced by a stratification of lophophores.

The lophophore

The action of the lophophore was observed microscopically in laboratory-maintained *Phoronopsis*. Currents produced by cilia on the tentacles bring water and suspended particles down within the loop of the lophophore and then out between the tentacles. The mechanics of particle capture, rejection, and food transport were found to be as described for *Phoronis vancouverensis* by Strathmann (1973).

There was no diel periodicity in feeding; animals fed continuously during tidal submergence. This observation was expected, since variations in food availability are not to be expected. Although the lophophore is perpetually bombarded by small particles cascading along the SWI, the animal is sensitive to disturbance and has a well-developed escape response. Contact between the tentacles of the lophophore and large tumbling fecal pellets and detritus produced a partial folding of the lophophore and retraction of the trunk. Predatory strikes at the feeding appendage by the nudibranch *Hermissenda crassicornis* resulted in a rapid folding of the lophophore and retraction into the tube. Only rarely was a large lophophore completely cropped by *Hermissenda;* most strikes removed only a few tentacles from the lopophore. However, a small phoronid can lose its entire lophophore to *Hermissenda* as well as to fishes which can remove even a large phoronid from its tube (Ronan, in preparation).

Feeding in clusters

Clustered phoronids were observed to sporadically vary the height of their expanded lophophores. The structure of the stratification undergoes continuous modification as the animals raise, lower, and interfinger their feeding appendages (Table II).

TABLE II

Sequential underwater measurements of the height of Phoronopsis viridis *above the sediment-water interface.*

Phoronid no.	Time (min)					
	0	15	30	45	60	$\bar{x}$
1	15	0	8	13	17	11
2	24	24	20	22	25	23
3	18	23	12	0	9	12
4	19	21	6	0	14	12
5	14	8	8	5	9	9
6	22	22	16	8	3	14
7	9	0	11	14	9	9
8	17	17	24	19	19	19
9	24	20	25	16	0	17
Mean height* of cluster	18	15	14	11	12	

* Height measurements were made by inserting a transparent metric ruler directly in front of the animal and recording the distance from the sediment-water interface to the base of the lophophore. The mean distance to nearest-neighbor was 4.9 mm with a range of 2–13 mm.

The longest trunk extension noted was 25 mm with the lophophore extending another 6 mm above the trunk. The trunk is flexible so than an animal may bend away from and expand the feeding appendage above surface obstructions. Periodic height adjustments maintain the stratification and safeguard against impingement between neighboring lophophores.

Seston composition

Analysis of the food-resources of the SWI revealed that three particle types averaged over 69% of the potential available food: small (< 100 μm) encrusted mineral grains, floc aggregates, and *Phoronopsis* fecal pellets (Table III). Small encrusted mineral grains were usually the most abundant particle type. The encrusting material varied in its consistency and degree of adherence. When the organic matter attached to mineral grains is stained with the periodic acid-Schiff (PAS) histological reagent, it characteristically gives a strong positive reaction, thus suggesting the encrusting material is largely carbohydrate (Johnson, 1974; Whitlatch, 1974). Small encrusted mineral grains plus floc aggregates, the second most abundant particle type, together averaged more than 56% of the available particulate material.

Floc (organic-mineral) aggregates comprised the second most abundant particle type in the samples examined. Floc material consists of very fine-grained mineral matter, incorporated into an amorphous organic matrix (Johnson, 1974; Whitlatch, 1974). Not all floc material is the same. Some aggregates were rich in mineral matter, tightly bound by the matrix material. Other floc material consisted of a loose indistinct matrix with few bound particulates. From extensive staining experience, Johnson (1974) and Whitlatch (1974) conclude that

the matrix of organic-mineral aggregates is largely carbohydrate. Floc material was always abundant in harbor water samples collected from just above the SWI and was especially abundant in samples collected from the low intertidal zone and tidal channels. Rhoads (1973) has reported that different types of floc material may differ in floc bulk density and ease of resuspension.

Fragmented fecal pellets of *Phoronopsis* were the third major particle type. Intact pellets are spindle-shaped rods up to 7 mm in length, which are rich in silt and clay. Natural decomposition of the mucous envelope which binds a pellet produces many stringy fecal fragments. There was a strong morphological resemblance between naturally decomposing phoronid fecal matter and the floc material complexed with mineral grains (organic-mineral aggregates).

All three common categories of seston (small encrusted mineral grains, floc aggregates, and fecal pellets) were resuspended by tidal currents and wind-driven waves. Hence they were readily available to *Phoronopsis*. The remaining particulate material consists of large mineral grains (> 100 μm), plant detritus (fragments of *Ulva expansa* and *Zostera marina*), living diatoms, pollen, and a variety of small metazoans (copepods, ostracods, nematodes, etc.).

Plankton composition

During fall sampling, there was a plankton bloom in the harbor. A pair of daytime plankton tows from about 10 cm above the phoronid bed contained approximately 35% dinoflagellates (*Ceratium* sp. and *Gonyaulax* sp.), 12% centric diatoms (two species each of *Chaetoceros sp.* and *Coscinodiscus sp.*), 10% harpacticoid copepods, 7% *Cancer* crab zoea, 6% ostracods (? *Cylindroberis sp.*), and 4% hydromedusae (*Polyorchis sp.*). The remaining living material consisted mostly of pennate diatoms (1.9%) and small flagellates (1.3%).

The tow also contained two types of organic detritus that constituted about 18.8% of the samples; amorphous strings and balls of organic matter and *Zostera marina* fragments averaged 11 and 7% of the samples, respectively.

TABLE III

Particle type abundance of seston sampled near the tubes of* Phoronopsis viridis, *as mean percentages.*

Particle type	$\bar{x}$
Mineral 100–200 μm encrusted	7.0
Mineral 100–200 μm not encrusted	6.6
Mineral <100 μm encrusted	35.0
Floc aggregates	21.1
Plant detritus	3.1
Pollen grains	1.0
P. viridis fecal fragments	13.4
Other fecal matter	7.1
Living diatoms	3.5
Small metazoans	2.1

* Seston is defined as inorganic detritus and organic (living and nonliving) particles. Mean percent abundance was determined by counting and averaging 200 particles at each of eight sampling stations.

TABLE IV

Electivity coefficients of seven most abundant particle types in the stomach of Phoronopsis viridis *(data averaged for 20 animals).*

Particle type	Electivity
Mineral 100–200 μm (encrusted)	−0.18
Mineral 100–200 μm (not encrusted)	−0.15
Mineral <100 μm (encrusted)	+0.11
Floc aggregates	+0.08
Dinoflagellates	+0.05
P. viridis feces	+0.03
Diatoms	−0.20

Stomach contents

Only six items were routinely present in the stomach of *Phoronopsis.* Positive electivity values suggest a preference for small (< 100 μm) encrusted mineral grains (Table IV). Within this category, 35–75 μm mineral grains were thickly encrusted with loosely adhering organic matter. The electivity data also indicate a preference for floc aggregates along with planktonic dinoflagellates. In 60% of the animals examined, floc aggregates of silt- and clay-sized materials occupied over one-third the volume of the stomach. Small dinoflagellates were selected most often.

A strong avoidance was displayed for mineral grains larger than 100 μm. Organic encrustations, which increase both the sphericity and effective diameter of the particles, further reduced the electivity of large mineral grains. Avoidance of particles in the 100–220 μm range probably is due to either the inability of the frontal cilia on the tentacles to transport the particles or an upper limit to the size of material which can be ingested.

Fecal pellets

The seston and plankton ingested by the animal is defecated at the SWI as easily fragmented fecal pellets. Embedded in the fine-grained mucous matrix were mineral grains (50–90 μm) and an occasional pollen grain or still motile ciliate. The common nudibranch, *Hermissenda crassicornis,* was observed to ingest large numbers of phoronid fecal pellets. The importance of fecal material as a food source for invertebrates has been demonstrated by Newell (1965) and Johannes and Satomi (1966). They have shown that the bacteria which decompose feces are more important nutritionally than the waste material present.

Some of the phoronid fecal material is incorporated into the sediment by numerous small burrowing metazoans which disaggregate and intermix fecal material with the surface sediment. Floc, or organic-mineral aggregates, is probably produced mainly by the mixing of decomposing phoronid (or other) fecal material and sediment. Unmixed fecal material accumulates in surface depressions (ripple troughs, ray feeding pits, etc.), decomposes, and becomes flocculent seston. Resuspension of this material makes it available for ingestion by *Phoronopsis.*

Discussion

In recent years a number of studies have been made of the distribution patterns of benthic species. Most studies suggest that distributions tend toward aggregation and that random or uniform distributions seldom occur in marine (*e.g.*, Clark and Milne, 1955; Angel and Angel, 1967; Warner, 1971) or terrestrial environments (Greig-Smith, 1964; Pielou, 1969). Surprisingly, although a number of soft-sediment species are known to form dense aggregations, particularly brittle stars (Warner, 1971; Broom, 1975; Wilson, Holme, and Barrett, 1977), there is little statistical information on the distribution of individuals within such aggregations.

The present study provides detailed statistical information on *Phoronopsis,* which forms dense aggregations in the intertidal region. Detailed sampling has shown that the population exhibits a clumped distribution whose degree of aggregation remains relatively constant with changes in intertidal elevation and population density. This close association between nearest neighbors produces a pattern of tight clusters.

These results differ from those reported by Johnson (1959) who has used another N-N measure (Clark and Evans, 1955) to examine the spatial pattern of *Phoronopsis.* His results indicate that individual animals tend to be distributed evenly within clusters. Further, he suggests that this pattern of dispersion reflects the minimum distance between individuals necessary for feeding, but he reports no N-N distances nor does he mention a stratification of feeding appendages.

In general, invertebrates that commonly form large, dense aggregations are animals that spend much of their time suspension feeding (*e.g., Ophiothrix fragilis,* Warner, 1971; *Dendraster excentris,* Timko, 1975; *Spisula solida,* Ford, 1925; *Ampelisca* spp., Mills, 1967). This emphasis on feeding activity means that they are continually placed in situations that expose them to disturbance and probably make them highly susceptible to predators. Although a close association between phoronids creates spacing problems among themselves for expansion of the lophophore during feeding, clustering may be an adaptation to predation: when N-N distances are small and lophophores stratified, a close association between individuals can limit the number of animals available to the predatory nudibranch *Hermissenda crassicornis.* I have observed that the sudden retraction of a lophophore creates a disturbance that is transmitted to neighboring animals either by collision of overlapping lophophores or by the generation of sudden perceptible pressure waves that can produce multiple retraction of lophophores. Although the clusters are noncolonial aggregations, this imperfect wave of withdrawal that spreads over part of the cluster produces a response that makes the cluster less vulnerable to predation. Without the response, escape of *Phoronopsis* would depend upon contact with a crawling predator such as *Hermissenda,* which could more easily forage through the cluster.

A dense assemblage of *Phoronopsis* can also stabilize sediment and limit burrowing of large errant infauna which are potentially destructive to the phoronids. In areas of natural contact between the thallassinid sandshrimp *Callianassa californiensis* and *Phoronopsis,* the burrowing activity of the shrimp can act to set the upper limit of *Phoronopsis* intertidal range (Ronan, 1975). This type of

interaction in which one population is limited while the other is not has been termed "amensalism" (Odum, 1971). While there is no evidence of shrimp predation at low population densities of *Phoronopsis,* manipulated tubes are frequently found at unnatural depths, and occasionally tubes are found to be actually broken with pieces of tube offset and/or rotated on opposite sides of *Callianassa* burrows. Former occupants of broken and disoriented tubes were found to be living free in the sediment in the process of building new tubes in contact with the water column. This nonpredatory but potentially destructive interaction with *Callianassa* constitutes a form of "substrate amensalism" that operates at low tube densities to restrict the intertidal distribution of *Phoronopsis.* However, when *Phoronopsis* densities are high and N-N distances small, the numerous tubes buttress the sediment and constitute a subsurface obstruction to some large burrowing organisms. Dense clusters of *Phoronopsis* are only rarely undermined by foraging *Callianassa* (Ronan, in preparation).

Cluster formation may, therefore, permit *Phoronopsis* to coexist in sandflats with an established errant infauna which it might not otherwise successfully inhabit. However, cluster formation also could have other advantages: first, the proximity of large numbers of adults could insure gamete fertilization during the breeding season; and secondly, clustering may have even more subtle, advantageous effects on feeding. The feeding currents of an individual may work better with other individuals nearby. Aggregated feeding currents may possibly modify localized water flow with the clusters acting as "food funnels" for the accumulation of both resuspended and planktonic food material. The thick seston layer which develops within phoronid clusters, but not in the open spaces between clusters, may be a manifestation of the funneling effect.

Previous reports of diet composition and selectivity in the Phoronida are lacking. However, there are studies which are pertinent to the present work. Whitlatch (1974 and personal communication) has shown that the polychaete *Pectinaria gouldii* concentrates organic material found in the sediment by preferentially ingesting large encrusted mineral grains, fecal material, and floc aggregates. He suggests that there are probably several major sources of the organic material that encrusts mineral grains and forms low-density floc aggregates (terrigenous input, plant debris, decomposing fecal material, and metabolites of plankton and bacteria) and food value differences may depend upon the original source, state of decay, and number of times the material has passed through an animal gut. Further, he has demonstrated that the feeding of *Pectinaria* channels large amounts of organic material to the SWI where it can become available to other organisms. At the depositional interface, the combined effects of bioturbation and tidal energy create a constant upwelling and recycling of organic material from the sediment into the water column (Rhoads, 1973). The data on size selectivity and diet presented in the present report show that resuspended encrusted mineral grains, fecal pellets and floc materials, and plankton are of trophic significance to a suspension-feeding phoronid. The continuous feeding and stable generalized diet are undoubtedly important factors which have allowed *Phoronopsis* to attain great abundance in shallow water coastal embayments.

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Summary

1. In the intertidal zone of Bodega Harbor, California, the phoronid, *Phoronopsis viridis,* aggregates in clusters often composed of thousands of tightly aggregated individuals (up to 150,000/m^2). Within a dense cluster, there is a spacing problem for expansion of the lophophores. When nearest-neighbor distances are small, a stratification of feeding appendages is a workable solution to the spacing problem, allowing simultaneous expansion of clustered feeding appendages.

2. Suspension-feeding specimens of *Phoronopsis* expand their lophophores and collect food items from the turbid near-bottom layers of water. Comparison of ingested items with material collected where the phoronids feed indicates a preference for small (< 100 μm) organic encrusted mineral grains, floc aggregates, and fecal material, all resuspended from the depositional interface. Also taken to a lesser extent are plankton bloom species, such as diatoms and dinoflagellates.

3. The fact that *Phoronopsis* forms dense assemblages in the intertidal zone has consequences when the community structure of sandflat areas is considered. Although it is probable that no single factor can explain aggregation in *Phoronopsis,* two possible factors, constituting strong selection pressures for cluster formation, are relative immunity from disturbance by large burrowing infauna and protection from predation by crawling predators.

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THE LIFE CYCLE OF *CORYMORPHA* (= *EUPHYSORA*) *BIGELOWI* (MAAS, 1905) AND ITS SIGNIFICANCE IN THE SYSTEMATICS OF CORYMORPHID HYDROMEDUSAE

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The systematic interrelationships of medusae and polyps in the hydrozoan family Corymorphidae are, as yet, unclear in their details. The metagenic nature of the life cycle of *Corymorpha nutans* Sars was among the first such cycles described (Sars, 1835), but progress toward a unified systematics of the family has been slow. In its original usage, the polyp genus *Corymorpha* included a heterogeneous mixture of several separate evolutionary lines. Kramp (1949) proposed that polyps previously collected under this name were of at least two lineages, one associated with the medusa genus *Euphysa* Forbes (1848) and the other associated with the medusa genus *Steenstrupia* Forbes (1846). He resurrected the polyp genus *Heteractis* (Allman, 1872) for polyps which differ from typical *Corymorpha* by their permanently capitate oral tentacles, their strongly contractile moniliform aboral tentacles, their lack of a parenchymous diaphragm separating the hypostome from the hydrocaulus, their replacement of a basal tuft of root-filaments by a belt of papillae in the upper hydrocaulus, and by differences in perisarc structure (Kramp, 1949, p. 185). A compelling argument for the subdivision of the family was the observation that the medusae produced by *Heteractis* polyps were invariably species of *Euphysa* and that the only medusa known from typical *Corymorpha* polyps was a *Steenstrupia*. However, since all *Euphysa* medusae had not been linked to *Heteractis* polyps and all metagenic *Corymorpha* polyps had not been associated directly with *Steenstrupia* medusae, Kramp retained a dual classification system under which the specific name was shared by both life cycle stages, but the polyp and medusae retained their "classical" generic designations. This taxonomic device was accepted by some systematists (*e.g.*, Russell, 1953), but has been disputed by others (Rees, 1957; Naumov, 1960; Brinckmann-Voss, 1970). Despite differences of opinion on matters of nomenclature, the separation of the family Corymorphidae into distinct lines is generally accepted. Indeed, Rees (1957) recognized four sub-families (Euphysinae, Corymorphinae, Boreohydrinae, and Branchiocerianthinae), thereby re-establishing the Euphysinae of Haeckel (1879) to emphasize the distinctiveness of the *Euphysa* (= *Heteractis*) line.

Within this systematic framework, the position of the genus *Euphysora* has

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remained totally obscure. Since its erection by Maas in 1905, this medusa genus has had a complex taxonomic history. It has at various times been combined with either *Euphysa*, *Corymorpha* (as *Steenstrupia*), or both. Hartlaub (1907) immediately reassigned the type species, *Euphysora bigelowi* Maas, to the genus *Corymorpha* within the subgenus *Euphysa;* and Mayer (1910) combined *Euphysora* and *Euphysa* into *Corymopha*. Vanhöffen (1911) and Browne (1916) thereupon retained *Euphysora*, but little more than a decade later Uchida (1927) assigned *E. bigelowi* to *Euphysa*. The following year Kramp (1928) argued for the retention of *Euphysora*, further suggesting that the genus was more closely allied to *Corymorpha* than to *Euphysa*. While demonstrating the distinctness of the polyp stages of *Euphysa* and *Corymorpha*, Kramp (1949) did not speculate further on the position of *Euphysora*.

The ambiguity in the systematic position of *Euphysora* arose for three reasons. First, the criteria upon which Maas (1905) based this new genus were felt by some workers to be arbitrary and susceptible to individual interpretation (Mayer, 1910). Secondly, the genus as currently constituted (Kramp, 1961) may be a heterogeneous mixture of species (Kramp, 1948). Finally, and most significantly, the polyp stage has not been described for any species of *Euphysora* (Kramp, 1961).

In this paper the life cycle of the type species, *Euphysora bigelowi*, is described. On the basis of this life cycle it is necessary to revise the nomenclature of *Euphysora* to reflect a close alliance with the higher Corymorphines and to continue the recent trend of elimination of dual classification in hydrozoans (Rees, 1957; Naumov, 1960; Brinckmann-Voss, 1970). The implications of this revision on the systematic positions of other members of the medusa genus are also discussed.

Materials and Methods

Five sexually mature corymorphid medusae, later identified as *Euphysora bigelowi*, were collected in a plankton tow in Monterey Bay, on September 24, 1973. The net (0.5 m diameter) was towed on a weighted 15 m line at very slow speed for about 30 min, and was on the bottom during part of the tow (some sediment was recovered with the sample).

The medusae were returned to the laboratory, fed brine shrimp nauplii, and left in a small finger bowl at about 14° C for several days. They died shortly thereafter and disintegrated. At this time, however, metamorphosing larvae were noticed on the bottom of the bowl and the sea water was replaced. Within a week these larvae had completed metamorphosis into eight polyps. Several polyps were transferred to the Bodega Marine Laboratory for culture; the remainder were maintained at Stanford.

The first evidence of gonosome development in the Stanford culture was on October 17, 1973, and the first medusa was liberated on December 5, 1973. The Stanford culture deteriorated shortly thereafter, and the line was lost in January, 1974. Attempts to rear young medusae on brine shrimp nauplii were unsuccessful. The descriptions which follow are based on a preserved polyp, five newly released medusae preserved within 24 hr of liberation, notes made on living polyps and

medusae, and extensive photographic records of various stages. The polyp and five newly released medusae have been deposited at the National Museum of Natural History (#56762 and #56760).

Results

Adult medusa

An adult medusa is shown in Figure 1c. The medusae were corymorphid in morphology with three short simple tentacles and one long tentacle which differed from the others in form as well as size. All tentacles were hollow. The length of the bell ranged up to 5.0 mm. When extended, the manubrium reached the umbrellar margin and sometimes protruded slightly beyond. The edges of the mouth were armed with nematocysts. The apical projection was produced as a conical process with an apical canal extending about two thirds of the way to the tip. The primary tentacle was as long as the bell and was studded with as many as eight (possibly nine) subterminal nematocyst bulbs along its length. The largest specimen had a large, club-shaped terminal bulb which may have represented a small terminal bulb and a ninth subterminal bulb in the process of division. All subterminal nematocyst bulbs were adaxial in orientation. The remaining three tentacles were short and simple, and were not armed with nematocyst bulbs, but with scattered nematocysts. The two tentacles adjacent to the primary tentacle, the "lateral" tentacles (Kramp, 1928), were twice as long as the one opposite. The morphology of these medusae was well within the range of variation of previous descriptions of *Euphysora bigelowi* (Maas, 1905; Browne, 1916; Uchida, 1927; Kramp, 1928).

Zoogeographic records of *Euphysora bigelowi* indicate a wide, warm-water distribution. Since its original collection in the Malay Archipelago (Maas, 1905), its known distribution has been extended to include the Indian Ocean (Browne, 1916), northeastern Australia (Kramp, 1953), and southeastern Japan (Uchida, 1938; Yamazi, 1958). It ranges westward across the Pacific Ocean to the Palau Islands (Uchida, 1947) and has been reported from Chile (Kramp, 1952). Kramp (1968) later suggested that the Chilean record might be erroneous, although it now appears less suspect. The collection of *Euphysora bigelowi* in Monterey Bay, California, is rather surprising and is inconsistent with all previous records except that of Chile. The Monterey Bay collection represents a substantial range extension (of about 8,000 km) into the northeastern Pacific Ocean.

In comparing the various descriptions of *Euphysora bigelowi* with the California material, it is clear that there is extensive morphological variation in this species, both within and between populations. Characters which have proven to be quite variable are the presence or absence of the apical canal, the relative lengths of the three secondary tentacles, and the relationship between bell height and the number of nematocyst bulbs on the principle tentacle. Populations from the Malay Archipelago (Maas, 1905) included animals with and without apical canals; and Kramp (1928) reported variation for specimens from the Sunda Strait (the predominant type there was lacking the canal). Other descriptions

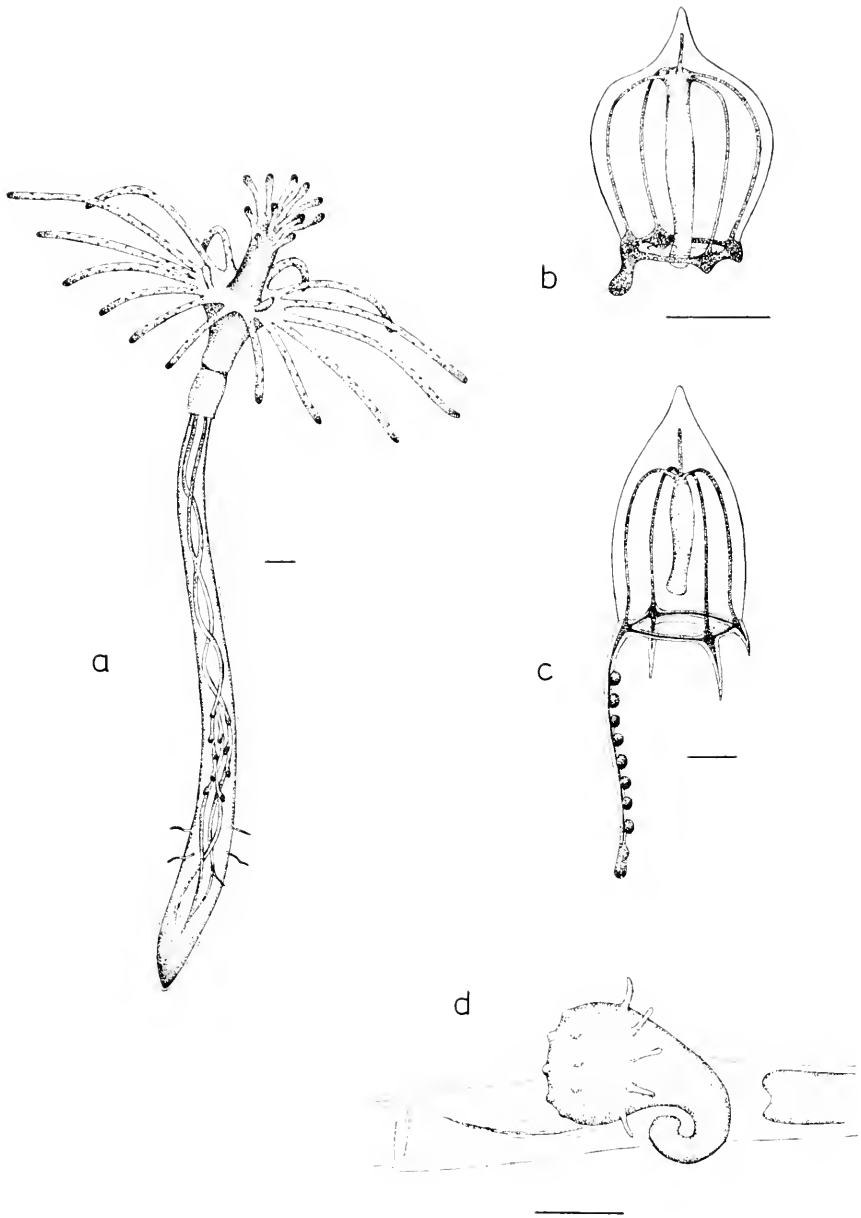

FIGURE 1. Stages in the life cycle of *Corymorpha* (= *Euphysora*) *bigelowi:* a, composite drawing of the mature polyp illustrating its general aspect and emphasizing characteristic features; b, the newly released medusa; c, the adult medusa; d, polyp reproduction by frustulation (the fragmented base of the parental polyp is shown to the right of the metamorphosing bud). Scale bar is 1.0 mm for (a) and (c) and is 0.5 mm for (b) and (d).

(*e.g.*, Browne, 1916; Uchida, 1927) indicate the complete absence of apical canals in animals from the Indian Ocean and off Japan. The California specimens, in contrast, all had well-developed apical canals. Variation in the relative lengths of the three secondary tentacles is also substantial. Kramp (1928) tabulated the relative lengths of the "lateral" and "opposite" secondary tentacles for the Sunda Strait specimens. His analysis indicated that the opposite tentacle is shorter than the other two in small specimens (1.5 mm high), but that its relative length increases with medusa size and may eventually exceed the lateral tentacles in length (in 2.25 to 3 mm high medusae). In contrast, Browne (1916) noted that in his small specimens the three secondary tentacles were of equivalent length, but that in the larger specimen (4 mm high) the opposite tentacle was much shorter than the lateral tentacles. In the California specimens the opposite tentacle was substantially shorter than the other two, even in the largest (5 mm high) medusa (Fig. 1c). There appears to be differential development of the secondary tentacles, with variation among populations. For this character our specimens are more like those from the Indian Ocean than those from the Sunda Strait. A third morphological feature showing substantial variation is the number of nematocyst bulbs on the principal tentacle. This character seems to be related to medusa height (Browne, 1916; Kramp, 1928), but the degree to which the number of bulbs increases per unit change in medusa height seems to vary among populations. For example, a 4.0 mm high medusa from the Indian Ocean had 11 nematocyst bulbs (Browne, 1916), whereas a 2.25 mm medusa from the Sunda Strait had 21 bulbs, and one individual 1.5 mm high had 31 (Kramp, 1928). Uchida (1927) illustrates a 3.5 mm medusa with 26 subterminal bulbs. For this character the California sample is more similar to that from the Indian Ocean than to the Sunda Strait or Japanese collections. Variation in the three characters does not appear to be correlated. The California specimens resemble those from the Indian Ocean with regard to the lengths of the secondary tentacles and the number of nematocyst clusters on the primary tentacle, but in one group the apical canal was uniformly lacking and in the other it was uniformly well-developed. The use of these characters in delineating the genetic relationships between populations in different parts of the species range will probably not be very productive.

Morphology of the polyp

The following description was made from a polyp grown in the laboratory until preservation on December 12, 1973, and from notes and photographs of live polyps in culture. The preserved specimen is 13 mm high and about 1 mm wide at its widest point. The hypostome is 3 mm high and also about 1 mm wide. There is considerable variation in dimensions depending upon the state of expansion in live individuals. Figure 1a illustrates the general aspect of the polyp and emphasizes some of the characteristic morphological features. Figure 2a shows the hypostome and the early gonosome of a mature polyp.

Among individuals there are between 15 and 20 aboral filiform tentacles (beset with scattered nematocysts) in a single whorl. These tentacles are apparently not very contractile; our photographs do not include any in which the aboral

tentacles are substantially contracted despite the use of intense lighting and the occasional addition of brine shrimp nauplii during photographing. In mature polyps there are up to 35 oral tentacles (with scattered nematocyst batteries) set in irregular rows on the hypostome. Although the oral tentacles are not distinctly capitate, they may be somewhat thickened at their tips, particularly in young polyps. A diaphragm separates the hypostome from the polyp body (Fig. 1a). The hydrocaulus is enclosed in a thin, membranous perisarc which is attached to an annular ring of thickened ectoderm slightly below the diaphragm. In some specimens the perisarc extended beyond the base of the hydranth in the form of a thin tube. The body is slightly inflated at its base to a width of about 1.5 mm. Anchoring rootlets with inflated tips and varying in width between 25 and 50 μm arise from prominent endodermal canals which are visible in the hydrocaulus (Fig. 1a). The medusa buds are mounted in clusters on inflated pedicels which arise from the hypostome between the oral and aboral tentacles, but much nearer the aboral tentacles (Fig. 2a). These pedicels are not very long (1 to 2 mm) and are not highly branched (Fig. 2b).

Gonosome development and the newly liberated medusa

Gonosome development was first observed about two weeks after larval metamorphosis. Subsequent development of the medusa buds was substantially slower than initiation of the gonosome, and the first medusa was not released until about six weeks after the gonodendra were first visible.

The gonodendra develop asynchronously on the polyp, several stages of progression being found on the same hydranth. The earliest structure is a simple tubular projection from the hypostome immediately above the aboral tentacles. This projection elongates and branches, the medusa buds forming at the termini of each branch (Fig. 2b). The differential development of the primary tentacle of the medusa takes place during attachment to the gonodendra and the manubrium swells to occupy most of the subumbrellar cavity (Fig. 2c). Approximately a day before liberation the attached medusa falls below the whorl of aboral tentacles (Fig. 2d) by which time the medusa is contractile, but is not rhythmically pulsating. It is released (or breaks free) with an incompletely formed apical chamber at a size of about 1.3 mm high by 1.2 mm wide.

The newly released medusa (Fig. 1b) is colorless except for pale yellow tentacle bulbs. The manubrium is tubular and extends to the velar opening or slightly beyond. The apical canal is variable in development, extending from one-sixth to two-thirds of the way to the tip of the apical projection. This range of variation is found even between individual medusae released from the same polyp. The tip of the apical projection has small papillae on its surface. Only one tentacle is developed to any appreciable degree, the others being reduced to conical projections. The primary tentacle bears a club-shaped terminal nematocyst bulb, but is lacking the subterminal adaxial bulbs of older medusae. Nematocysts are present on all tentacles, but are lacking on the exumbrellar surface.

The asynchronous development of the gonosome results in a prolonged period of medusa liberation. One polyp was censused daily and produced a total of 34 medusae over a period of three weeks. Newly released medusae were not

seen to feed on brine shrimp nauplii and did not live for more than a few days.

The newly released medusae are similar in size to the smaller individuals described from plankton collections by Kramp (1928) and Browne (1916). It is of interest to note that in these collections small individuals (1.25 to 1.5 mm high) have subterminal nematocyst bulbs on the primary tentacle which are absent on the newly released *Euphysora bigelowi* medusae. In *Corymorpha nutans* the annular subterminal bulbs are developed even before liberation from the polyp (Russell, 1953).

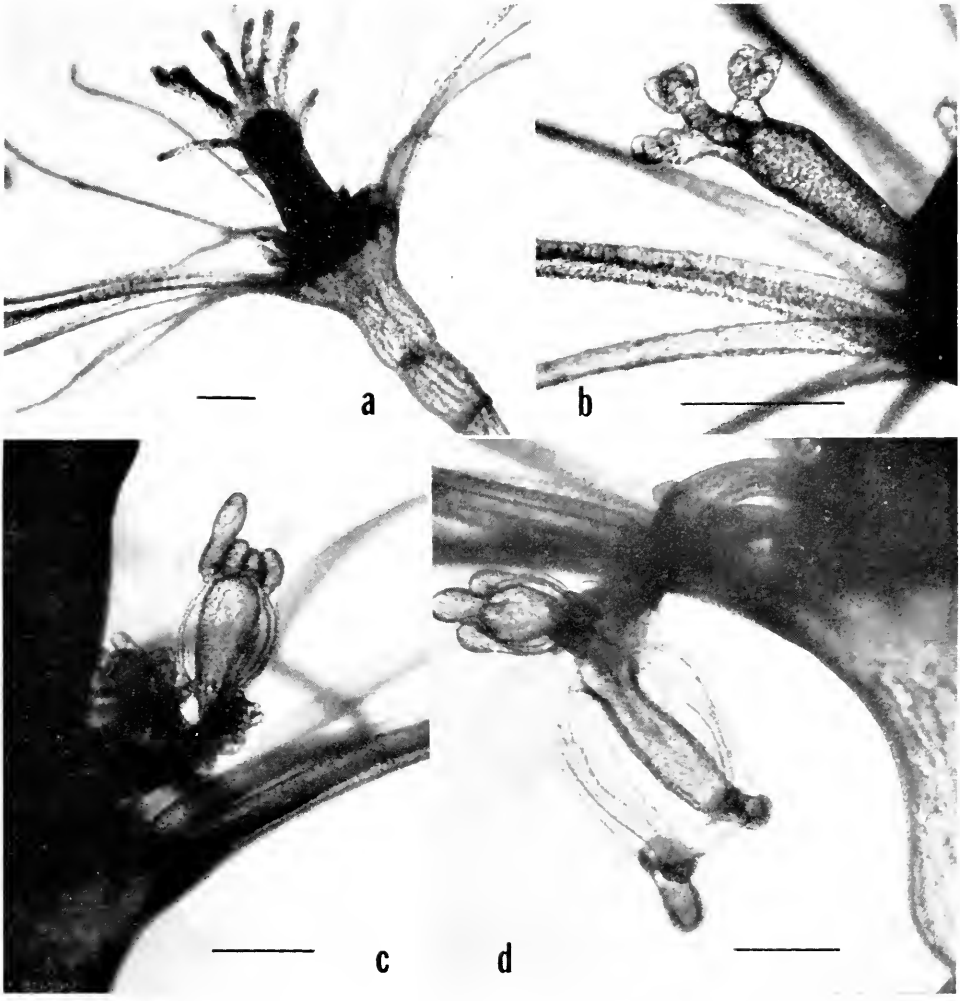

FIGURE 2. The development of the gonosome of *Corymorpha* (= *Euphysora*) *bigelowi:* a, lateral view of the hypostome of a polyp with early gonodendra; b, immature gonophore with inflated pedicel and developing medusa buds; c, medusa buds in advanced state of development (note the enlarged manubrium and primary tentacle); d, medusa just prior to release. Scale bar is 0.5 mm.

TABLE I

Cnidom of Corymorpha (= Euphysora) bigelowi, *with measurements in microns.*

Stage	Stenoteles (large)	Stenoteles (small)	Microbasic mastigophores	Desmonemes	Anisorhizas*
Polyp					
Oral and aboral tentacles	13–16 × 8.5–10	7–8 × 4.5–5	7.5–8.5 × 3–4	4–6 × 3.5–4.5	
Newly released medusa					
Tentacles	11–15 × 9–12	8–10 × 7–8	4.5 × 9.5	6.5–9 × 3.5–5.5	
Adult medusa**					
Primary tentacle	13–14 × 11–12	7.5 × 7		5 × 10	
Secondary tentacle	11 × 13	8–9 × 7			11 × 12
Lips of mouth	12–14 × 10–12	6 × 7			
Umbrella		7 × 9	3 × 8		9 × 10

* No fired nematocysts of this type were closely examined.
** Measurements from photographs of tissue squashes.

Cnidom

Data on the sizes, types, and locations of nematocysts present in different stages of the life cycle are given in Table I, and selected types are illustrated in Figure 3. Four types of nematocysts were found: stenoteles, microbasic mastigophores, desmonemes, and probably anisorhizas. These four types have been previously reported in two related species, *Corymorpha nutans* Sars and *Ectopleura dumortieri* (Van Beneden) (Russell, 1938; Weill, 1934). In *Euphysa aurata* Forbes the heteronemes and desmonemes have not been found and atrichous haplonemes are present (Rees, 1957).

Mode of polyp asexual reproduction

Asexual polyp reproduction was observed once. The terminal portion of the base of the polyp detached from the remainder of the hydrocaulus, and within a few days this fragment began development of both oral and aboral tentacles and broke free of the parental perisarc (Fig. 1d). This mode of reproduction has been termed "frustulation" by Kramp (1948) to emphasize its relationship to transverse fission and its distinctness from true budding. It is apparently a normal process of reproduction in several species of *Euphysa* (*e.g.*, Miles, 1937) but has not been reported for *Corymorpha* (Kramp, 1949) in which other forms of asexual reproduction, such as polyp development from root filaments, are observed (Ikeda, 1910; Kramp, 1949).

DISCUSSION

The polyp reared from *Euphysora bigelowi* demonstrates the following structural features which are characteristic of the genus *Corymorpha:* an irregularly

arranged cluster of oral tentacles, a single whorl of aboral tentacles with scattered (as opposed to annular) nematocyst batteries, an annular diaphragm, rooting filaments borne only in the lowermost part of the hydrocaulus, gonophores borne in clusters on pedicels (gonodendra), and well developed endodermal canals in the lower part of the hydrocaulus. Indeed, the parenchymous diaphragm and endodermal canals are considered to be characteristic of advanced and highly specialized members of the genus (Kramp, 1949). However, the polyp of *E. bigelowi* also shares certain features with *Euphysa*-type polyps. The initially somewhat capitate oral tentacles of *E. bigelowi* apparently do not become completely filiform in older polyps and are intermediate between *Corymorpha* and *Euphysa* in this regard. The curious mode of asexual reproduction by frustulation (Fig. 1d) has been reported in *Euphysa* (Miles, 1937) but not in *Corymorpha* (Kramp, 1949). The perisarc of *E. bigelowi* is reminiscent of *Euphysa* both in its attachment to the upper region of the hydrocaulus and in its extension beyond the base of the hydrocaulus. We do not know the extent to which peculiar environmental factors of our culture conditions may have influenced the expression of these characteristics, or the degree of phenotypic plasticity in this species. Modes of budding in hydro-

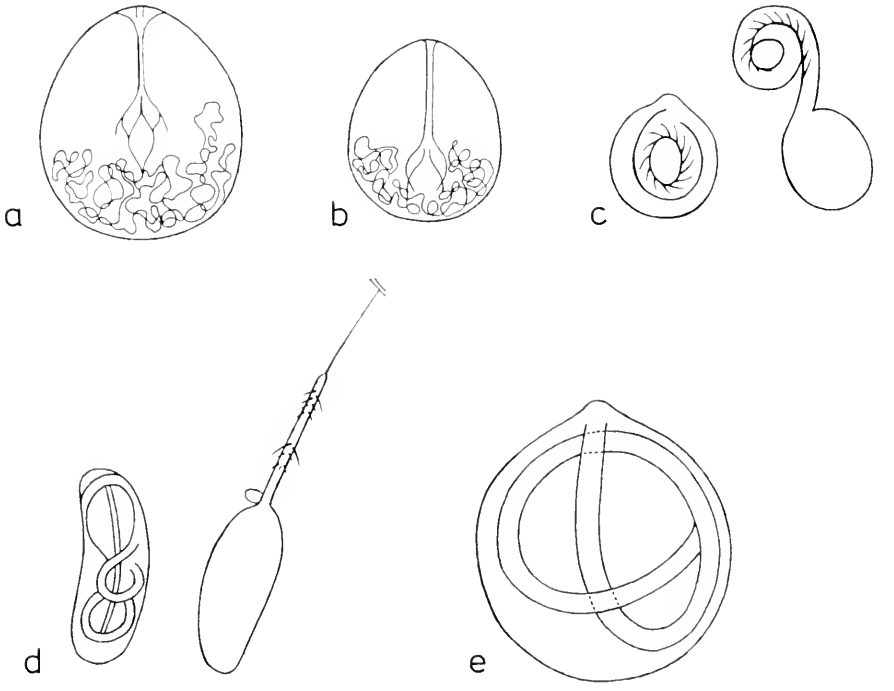

FIGURE 3. The cnidom of *Corymorpha* (= *Euphysora*) *bigelowi:* from aboral tentacle of polyp (a–c)—a, large stenotele, b, small stenotele, c, undischarged and discharged desmoneme; d, undischarged and discharged microbasic mastigophore from oral tentacle of polyp; e, anisorhiza (?) from secondary tentacle of adult medusa. Scale bar is 10 μ.

zoans are quite variable, and bizarre forms can be produced under unnatural culture conditions (Sassaman, 1974).

The substantial similarities of the reared polyp to *Corymorpha* far outweigh in significance the minor deviations from the typical form; thus, the polyp can be relegated to the genus *Corymorpha*. *Corymorpha nutans* is the only other metagenic polyp in the genus whose medusa is known. Following recent efforts in eliminating the dual classification system which has bedeviled hydrozoan systematics from its inception (Rees, 1957; Naumov, 1960; Brinckmann-Voss, 1970), it is deemed appropriate to refer to both the polyp and the *Euphysora bigelowi* medusa as *Corymorpha bigelowi,* since *Corymorpha* (Sars, 1835) precedes *Euphysora* (Maas, 1905).

Since this revision is based on the type species of the genus *Euphysora,* and the genus is believed to be a heterogeneous mixture of species (Kramp, 1948), other medusae previously assigned to *Euphysora* are of an uncertain status. These species are *E. gracilis* (Brooks, 1882), *E. annulata* (Kramp, 1928), *E. furcata* (Kramp, 1948), *E. gigantea* (Kramp, 1957), *E. normani* (Browne, 1916), and *E. valdiviae* (Vanhöffen, 1911). This assemblage includes species which resemble *C. bigelowi* in having unbranched primary tentacles (*E. annulata* and *E. gracilis*), species with branched primary tentacles which lack subterminal nematocyst bulbs (*E. furcata, E. gigantea,* and *E. valdiviae*), and two species (*E. valdiviae* and *E. normani*) with exumbrellar nematocyst tracts. This latter condition may be a more primitive condition than is typical in *Corymorpha* (Rees, 1957). Ultimate resolution of the systematic positions of these various species will require additional life cycle data.

It is uncertain whether or not the polyp of *C. bigelowi* has been found in nature. No polyps similar to *C. bigelowi* are known from central California. Extensive hydroid collections by the Allan Hancock Foundation Expeditions (Fraser, 1948) have not yielded any local metagenic *Corymorpha,* and the Pacific fauna, in general, includes few metagenic *Corymorpha* species. Uchida (1927) suggested either *C. tomoensis* Ikeda or *C. carnea* (Clark) as the polyp stage of *Euphysora bigelowi.* The morphology of *C. tomoensis* (Ikeda, 1910) is similar to that of *C. bigelowi,* particularly the medusa buds. There are, however, substantial differences in hydranth size, number and morphology of the tentacles, complexity and development of the basal region, and mode of budding. In addition, *C. tomoensis* has not been reported from western North America (Fraser, 1948). *Corymorpha carnea* (Clark, 1876), while reported from North America, has not been adequately described, and its known distribution is restricted to northern Alaska (Torrey, 1902). At present, *C. bigelowi* cannot be positively associated with any other previously described species of *Corymorpha,* although *C. tomoensis* and *C. carnea* cannot be unequivocally eliminated. It is possible that the polyp phase of *C. bigelowi* has not yet been found in the field.

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Summary

1. Five individuals of the corymorphid jellyfish, *Euphysora bigelowi* Maas, were collected in 1973 in Monterey Bay, California, for a range extension of more than 8,000 km across the northeastern Pacific Ocean.

2. Larvae released by these medusae were cultured and the resulting polyps, the first known from this medusa genus, are described.

3. The polyps are a *Corymorpha,* but share some minor characteristics with polyps of the corymorphid genus *Euphysa.*

4. The polyp and medusa are assigned the name *Corymorpha bigelowi* (Maas); the systematic implications of this revision are discussed.

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THE ANATOMY OF THE DECAPOD CRUSTACEAN AUXILIARY HEART

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An auxiliary heart is found in many decapod crustaceans at the anterior end of the dorsal median artery before the artery branches to supply the supraesophageal ganglion and the peripheral oculomotor and visual systems. Although the existence of this auxiliary heart had been noted earlier, when it was named the cor frontale (Baumann, 1917), very little information was provided beyond a description of the muscles involved (for review, see Maynard, 1960). The following is a more thorough account of the anatomy of the cor frontale in several decapods with particular emphasis on the neural elements of the system.

Materials and Methods

Specimens of *Callinectes sapidus,* the American blue crab, and *Panulirus interruptus,* the Californian lobster, were used for the most complete dissections. Specimens of *Scylla serrata,* the Australian mud crab, and two Californian marine crabs, *Cancer productus* and *Cancer antennarius,* were also investigated.

The primary method used to trace the neural elements was *in vivo* methylene blue staining by perfusion through the dorsal medial artery. Fixation of the material so stained was done by the method of Pantin (1969). Light microscopic histological preparations of the heart nerves and tendon ganglia were made using glutaraldehyde fixation, Epon embedding and toluidine blue staining. Electron microscopy of the muscles was done with a 3% glutaraldehyde, 1% paraformaldehyde, collidine buffer, 1178 m osmol fixation and Epon embedding. To trace the course of vessels of the system, liquid latex (Connecticut Valley Biological Supply) was injected into the cerebral vascular system via the dorsal median artery.

Results

The basic anatomy of the cor frontale, which is remarkably similar in all the decapods studied, is illustrated in Figures 1, 2, and 3. The blood flows anteriorly from the main heart through the dorsal median artery to supply the supraesophageal ganglion and the peripheral optic ganglia and oculomotor system (Fig. 1). Before the blood is distributed to these areas, it flows through the auxiliary heart. The anatomy of this heart can most conveniently be described by breaking it down into three elements: the blood vessels, the muscles and tendons, and the associated neural system.

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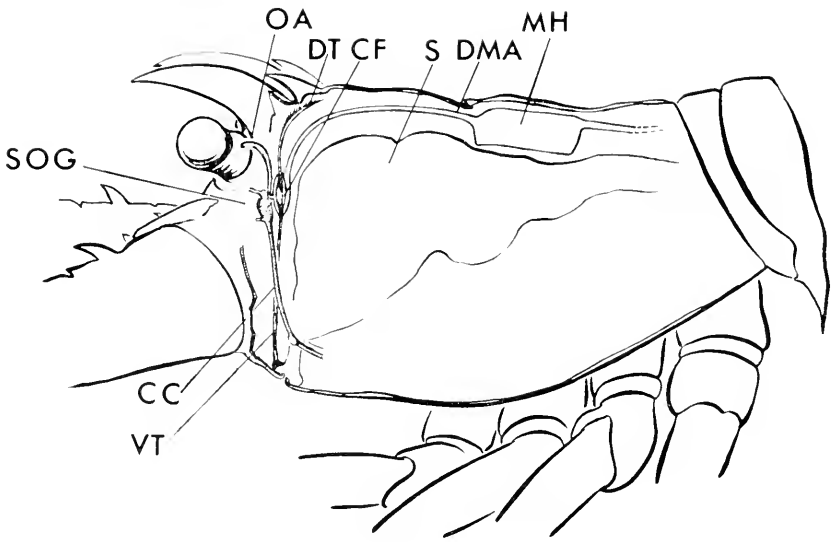

FIGURE 1. Overview of the location of the cor frontale (CF) in relation to other structures in the cephalothorax of the spiny lobster, *Panulirus interruptus*. Blood flows from the main heart (MH) through the dorsal median artery (DMA) over the stomach (S) to the cor frontale (CF) from which it exits to the eyecup via the ophthalmic artery (OA) and to the supraesophageal ganglion (SOG) via the cerebral artery (CA). Other abbreviations are: dorsal tendons of the cor frontale (DT); single ventral tendon (VT); and circumesophageal connectives (CC).

Blood vessels

The wall of the auxiliary heart is formed by the dilated terminal end of the dorsal median artery. Note (Figs. 2 and 3) that no muscle is contained in the wall of the artery itself. Rather, the wall of the cor frontale is composed of the same two layers as that of the dorsal median artery.

The course of the blood vessels from the cor frontale can be seen in the latex injected preparation of *Callinectes* in Figure 4. Blood enters the cor frontale sinus from the dorsal median artery and leaves via the cerebral artery which supplies the supraesophageal ganglion, a few small vessels which supply the nearby eyestalk muscles and the two large ophthalmic arteries which supply the visual and oculomotor system in the eyecup. (The dorsal median artery is sometimes referred to as the ophthalmic artery, a misnomer, since the true ophthalmic arteries, which run to the eyes, receive only part of the supply of the dorsal median artery.)

Muscles and tendons

The cor frontale muscles are two distinct strips of striated muscle originating from tendons outside the dorsal median artery. In the crab, the tendons begin as multiple insertions on the dorsal carapace just behind the middle cylinder of the eyestalk. This origin can be seen as two indentations on both the underside and on the external surface of the dorsal carapace. In the lobster, which has no

eyestalk middle cylinder, the tendons originate in an equivalent position on the dorsal carapace between the two large spines of the rostrum. Each of these two tendons is joined by an orthogonal lateral tendon before the tendons pass through the wall of the dorsal median artery. At this point, the cor frontale sinus begins (Fig. 2). As the tendons pass into the sinus they give rise to the two muscles

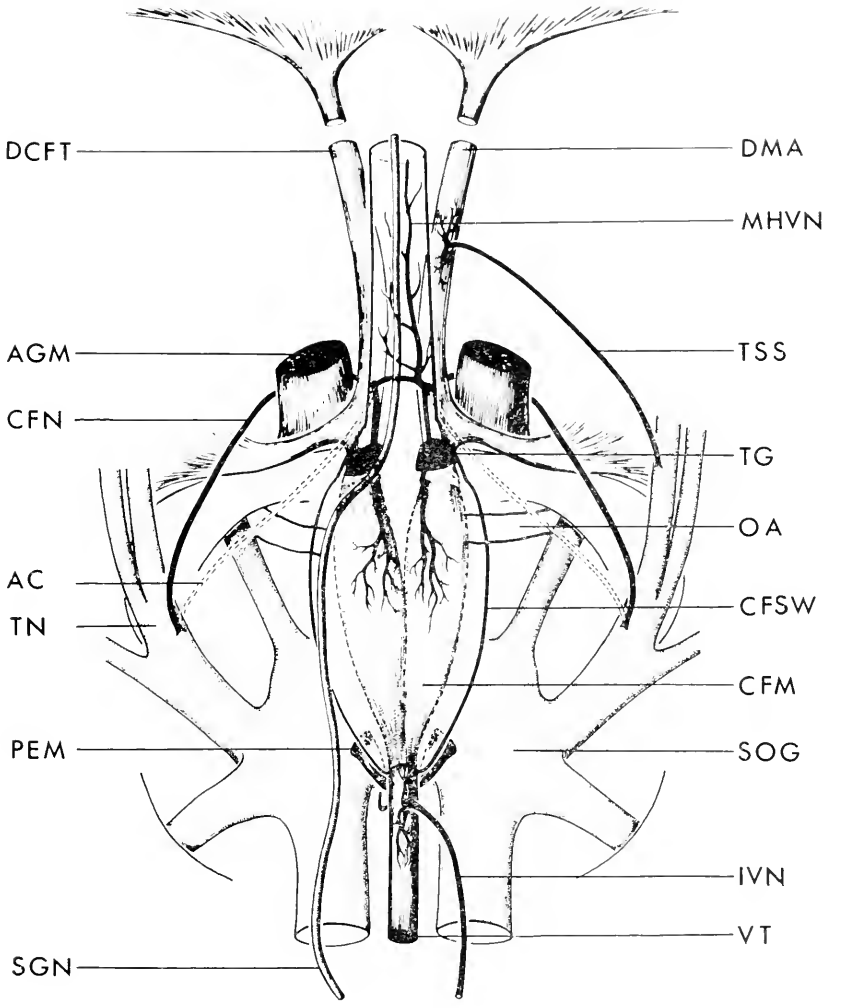

FIGURE 2. Transverse view of the cor frontale of *Panulirus interruptus*. Abbreviations are: dorsal cor frontale tendons (DCFT); anterior gastric muscles (AGM); cor frontale nerve (CFN); alternate course of the cor frontale nerve (AC); tegumentary nerve (TN); stomatogastric nerve (SGN); ventral tendon (VT); inferior ventricular nerve (IVN); posterior eyestalk muscles (PEM); supraesophageal ganglion (SOG); cor frontale muscles (CFM); cor frontale sinus wall (CFSW); ophthalmic artery (OA); tendon ganglion (TG); occasional separate tendon sensory supply (TSS); and nerve to main heart valve (MHVN); dorsal median artery (DMA).

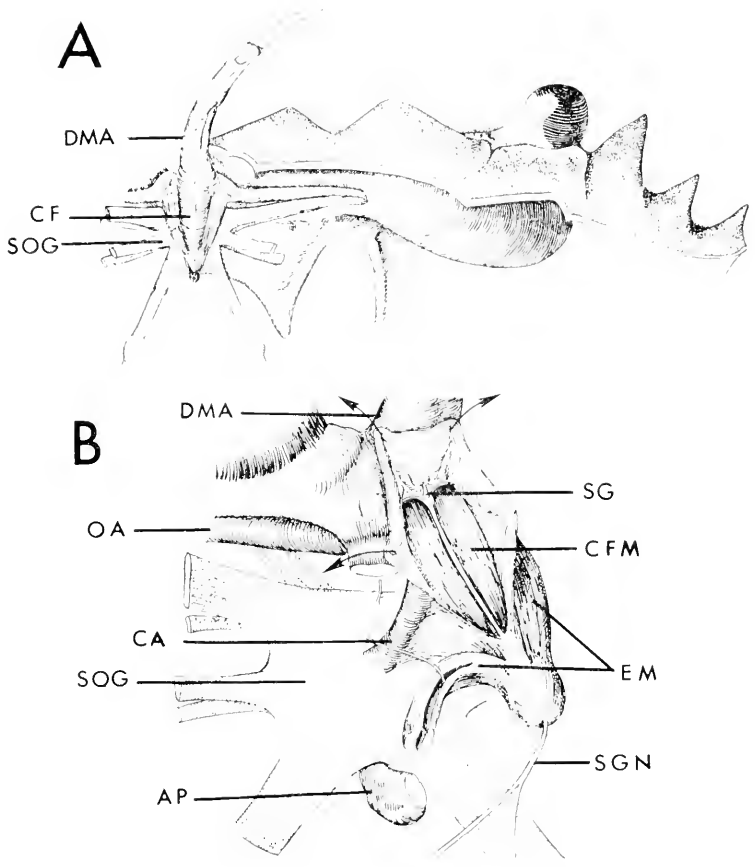

FIGURE 3. Transverse view of the cor frontale in cephalothorax of the *Callinectes sapidus*. A shows relation of cor frontale (CF) to cephalic structures. DMA indicates dorsal median artery entering the cor frontale. Muscles bordering cor frontale (EM) are the eyestalk muscles. The supraesophageal ganglion (SOG) lies under the cor frontale and receives its blood supply via the cerebral artery (CA). B shows enlarged view of center of (A) showing the cor frontale sinus walls opened at arrows to expose the enclosed cor frontale muscles (CFM) and stomatogastric ganglion (SG). The stomatogastric ganglion (SG) lies inside the cor frontale. SGN is the stomatogastric nerve exiting the cor frontale at the point where the tendon has been detached by dissection from its apodeme (AP).

of the cor frontale. These muscles extend the length of the sinus and exit at the ventral posterior end as a single tendon attached to an apodeme. This apodeme (Fig. 3), which arises from an epistome above the mouth, is a common attachment for the cor frontale muscles, the dorsal eyestalk muscles and several esophageal muscles.

The cor frontale of the lobster differs from that of the crab primarily in the extent of the development of the tendons. The dorsal tendons of the lobster are much larger and the single ventral tendon is greatly elongated. These differences are consistent with both the larger size and the dorso-ventral elongation

of the lobster cephalothorax (Fig. 1). The size of the tendons is particularly striking in view of the relatively small size of the cor frontale muscles. Associated with these tendons is a well developed sensory innervation.

There are around one hundred individual muscle fibers in a cross section of the muscle (Fig. 5). At two points on the muscle perimeter are areas which contain much connective tissue, large motor axons and some fibers which contain dense granules also seen in the tendon ganglia. The muscles of the cor frontale are striking in their compactness and white hue, being distinctly whiter and more dense than the fastest portions of the eyestalk muscles which border them. The preliminary electron microscopy which was done shows only a few small mitochondria which may account for the whiteness of the muscle. The banding pattern of the sarcomeres is not well defined. The Z band is moderately dense and appears to be continuous across the sarcomere. The sarcoplasmic reticulum is scarce and connections with the well-developed T tubular system are rare. The appearance is that of a crustacean somatic, rather than heart, muscle; and, in fact, it has been suggested that the cor frontale muscles are somatic muscles secondarily adapted for cardiac function (Maynard, 1960).

Neural anatomy

Cor frontale nerve. This nerve, which is the neural connection between the auxiliary heart and the supraesophegeal ganglion, exits from the supraesophageal ganglion with the tegumentary nerve and splits off as a small diameter branch to curve back and up to the dorsal aspect of the cor frontale. The nerve passes under the cor frontale tendon to enter the dorsal median artery near the entry of the tendon.

In the lobster, the cor frontale nerve either leaves the tegumentary nerve close to the ganglion and takes a direct route to the heart (dashed line in Fig. 2), or more commonly, it continues with the tegumentary nerve up to the anterior gastric muscle where it leaves the tegumentary nerve and passes around the gastric muscle to enter the dorsal aspect of the artery. In the crab, the course of the nerve through the cephalothorax is invariant but quite long and difficult to trace. It leaves an anterior branch of the tegumentary nerve laterally and curves back to the cor frontale as a fine nerve embedded in the dorsal hypodermis.

Cross section of the cor frontale nerve shows seven fibers. After methylene blue staining one may observe two large axons which can be traced to the cor frontale muscles, one large axon which runs out the dorsal median artery to the valve of the main heart (see below) and several small axons whose origin and termination could not be determined because of their poor staining. The motor neuron somata of the fibers supplying the cor frontale muscles and main heart valve originate in the supraesophageal ganglion. This was shown by electrophysiological recording and by methylene blue staining in which the two fibers can be traced clearly from the ganglion to innervate the muscles. Attempts to back fill the somata with cobalt or procion yellow have, to date, been unsuccessful.

In the lobster and crab, motor axons to the heart muscle split into two branches as the nerve enters the artery. One branch supplies the ipsilateral muscle and the other passes across the artery to join with the axons of the contralateral cor

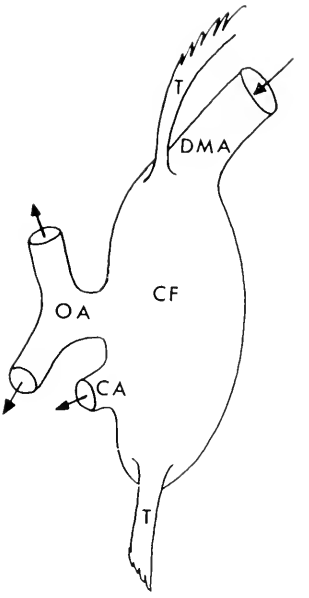

FIGURE 4. Sketch of a liquid latex injected cast of *Callinectes sapidus* cor frontale. Blood flows via the dorsal median artery (DMA) into the cor frontale (CF) and out via the cerebral artery (CA) and the ophthalmic artery (OA). Dorsal and ventral tendons (T) of the cor frontale muscles are shown exiting the cor frontale sinus.

frontale nerve to supply the contralateral muscle. Since the same motor axon splitting occurs on both sides, the result is that each muscle is innervated by four axons, two from each half of the supraesophageal ganglion. In the lobster, this crossing of the motor axons forms a distinct central neural cross bar structure which is embedded in the dorsal wall of the artery near the tendons. In the crab, when the two cor frontale nerves enter the artery, they do not form the distinct neural cross bar structure seen in the lobster. The same splitting and crossing over of the motor axons occurs, but at lower level closer to the muscles.

Main heart valve nerve. In the lobster and crab, a single axon from each cor frontale nerve joins a contralateral partner near the right dorsal tendon to form a nerve which turns away from the cor frontale and, embedded in the arterial wall, travels the entire length of the dorsal median artery. The two axons innervate the valve of the artery as the latter leaves the main heart. Histological sections of the nerve in the lobster show two large axons and three much smaller axons. The two large axons stain darkly with methylene blue and clearly go directly to and end in the arterial valve. The others stain poorly with methylene blue and branch repeatedly in the arterial wall. The three small fibers may be a sensory system which supplies the arterial wall since the wall contains no muscle.

The valve of the dorsal median artery at the main heart has two components. One, a well developed semilunar valve with cusps opening to the arterial side which appear to operate passively to prevent backflow and the other, a circular ring of muscle fibers between the valve and the heart muscle proper. It is this

circular muscle which the two axons innervate. As the two axons approach the valve, each axon splits first into two and then into four branches. Two branches of each axon charactistically cross the midline of the valve so that each half of the circular muscle ring is supplied by axons from both halves of the supraesophageal ganglion. The axons terminate in an extensive plexus among circular muscle fibers of the valve.

In a transilluminated methylene blue stained preparation, these muscle fibers are quite distinct from those of the main heart muscle and appear similar to those described by Alexandrowicz (1932) in several crustacean heart valves. From their termination on the circular muscle of the valve and the lack of any other nerve supply to these muscles, it is assumed that the axons to the valve are motor.

Cor frontale tendon ganglia. Tightly adhering to the upper tendon of each cor frontale muscle as it passes through the arterial walls is an ill-defined aggregation of neural tissue here termed the tendon ganglia (Fig. 2). The motor axons to the muscles pass directly through this ganglion and cannot be easily separated from it. In light microscopic toluidine blue stained sections of the ganglion, several distinct types of somata are found. Two of these somata types contain numerous either large or small darkly stained granules suggestive of neurosecretory vesicles. A third class of somata exhibits a clear cytoplasm and distinct nucleus. Numerous neural processes are also seen in the ganglia, some of which contain the same granules seen in the somata. These processes do not appear to form a distinct neuropile, although there is a somewhat circular course of the fibers within the ganglion. The density of the ganglion excludes its function as a neurohaemal organ. Several types of fibers enter or leave the tendon ganglia. There are many small fibers which can be traced from the ganglia to extensive ramifications in the arterial wall. The arterial and cor frontale sinus walls are innervated by fibers which appear to terminate in the tendon ganglia. Also associated with the tendon ganglia are fibers which, when stained with methylene blue, can be seen to branch extensively over the upper tendons of the cor frontale muscles. These fibers are presumed to be sensory, since they are never found extending beyond the tendon to the muscle. All these above fibers are very fine and their termination difficult to follow. In the lobster, the tendon sensory fibers sometimes go directly to the supraesophageal ganglion via a separate sensory nerve which joins the tegumentary nerve above the origin of the cor frontale motor nerve (Fig. 2).

Finally, connections can sometimes be traced between the stomatogastric nerve and the tendon ganglia. In the lobster the stomatogastric ganglion is located inside the dorsal median artery where this artery passes over the stomach (a few centimeters posterior to the cor frontale). The nerve then exits from the artery but adheres to it, giving off many small branches, one of which can sometimes be traced to the tendon ganglia or to the nerve carrying the axon to the main heart valve. In the crab, the stomatogastric ganglion is enclosed within the sinus of the cor frontale directly between the two muscles (Fig. 3). Two lateral nerves from this ganglion leave the sinus to supply the anterior gastric muscles. These gastric nerves leave the sinus near the cor frontale tendons and give off fine branches to the tendon ganglia. The stomatogastric ganglion inside the cor frontale appears to be the same ganglion referred to as the ventricular ganglion (Baumann, 1917; Maynard, 1960).

Ventral tendon sensory units. On the single long ventral tendon of the lobster a distinct sensory innervation is found which extends down the tendon. The fibers from this nerve enter the inferior ventricular nerve. The inferior ventricular nerve, after leaving its origin in the supraesophageal ganglion, passes through an opening in the cor frontale tendon. The sensory units from the tendon join the nerve and travel toward the inferior esophageal ganglion. In the crab, the ventral tendon is very short (Fig. 3), and the inferior ventricular nerve does not pass through the tendon. No sensory fibers comparable to those of the lobster ventral tendon have been found in the crab.

Auxiliary heart in the eyecup. When the oculomotor muscles in the eyecups are exposed by dissection (while the ophthalmic arteries are inflated by saline perfusion or by liquid latex injection), muscle number 21 (nomenclature of Cochran, 1935) is found to lie within the arterial lumen and shows anatomical features similar to the cor frontale. This is most obvious with the latex injection when the latex is found within the lumen of the artery completely surrounding the muscle. The muscle divides easily into two sections. Part of the muscle has the appearance of the other eyecup muscles and the rest has the white dense appearance of the cor frontale muscles.

The presence of this muscle in the lumen of the blood vessel, its physical resemblance to the cor frontale muscle and its location within the arterial lumen immediately before the artery enters the neuropile of the eyecup indicate that it may be another auxiliary heart. The optic neuropile, like the supraesophageal ganglion, but unlike most crustacean ganglia, requires a constant blood supply and fails soon after this flow is interrupted. This muscle has been noted before to be "heavily vascularized" (Sandeman, 1967), but its presence inside the vessel lumen and possible auxiliary heart function were not noted. In the shrimp, *Palaemon*, an eyecup muscle inserted in the ophthalmic artery has been described and a blood pumping function ascribed to it (Debaisieux, 1944; Demal, 1953).

Discussion

The term auxiliary heart, rather than accessory heart (Maynard, 1960), has been used here for the cor frontale, since its fine structure and electrophysiological reflex response (Steinacker, 1978) suggest a phasic function which is recruited only when the main heart activity is insufficient for the circulatory requirements of cerebral nervous system. The anatomy of this heart reveals a complex organ whose function appears to be controlled by and integrated with several other systems. From anatomical and electrophysiological evidence (Steinacker, 1978), the main integrative center appears to be in the supraesophageal ganglion where the motoneurons are located. The tendon ganglia may be a second, local integrative center, with perhaps a neurosecretory function whose control could be exerted at two sites; as a direct action of neurosecretory products on the muscle and/or by a central effect on the neurons in the supraesophageal ganglion. Since blood flows past the tendon ganglia to the supraesophageal ganglion, neurosecretory products will be carried directly to a central integrative system in the supraesophageal ganglion. In addition, afferent or interneuronal fibers from the tendon ganglia may travel in the cor frontale nerves to or from the tendon ganglia and the

supraesophageal ganglion. There are at least four fibers in the cor frontale nerves which are not motor and which may arise all or in part from the tendon ganglia.

The involvement of the stomatogastric ganglion with an auxiliary heart deserves mention. In a decapod with an open venous system and inflexible carapace, variations in volume of a highly distensible stomach will have a considerable influence on blood pressure. In addition to the passive influence of stomach volume on blood pressure, active uptake of salt and water by the gut in crustaceans has been demonstrated (Weisman, 1874; Fox, 1952; Croghan, 1958). The stomatogastric system may be involved in hemodynamics through the passive effect of the stomach volume on blood pressure and through active control of salt and water uptake. The stomatogastric system and cor frontale also have a possible neural communication via the supraesophageal ganglion and lower control centers. Evidence is building for a common control center (or centers) for the gills, main heart, auxiliary heart and stomatogastric system. Excitatory and inhibitory fibers, which have been found in the circumesophageal connectives, govern these systems (Wiersma and Novitski, 1942; Mendelson, 1971; Field and Larimer, 1975; Wilkens, Wilkens, and McMahon, 1974; Steinacker, 1978). Command fibers for the stomatogastric system are thought to originate in the supraesophageal ganglion (Dando and Selverston, 1972) and neurosecretory cells connecting the supraesophageal and lower neural centers exist (Goldstone and Cook, 1971).

The sensory innervation of the tendons of the cor frontale introduces the possibility of either feedback control or coordination of cor frontale function with the other cardioregulatory systems. In the case of the ventral tendon, this infor-

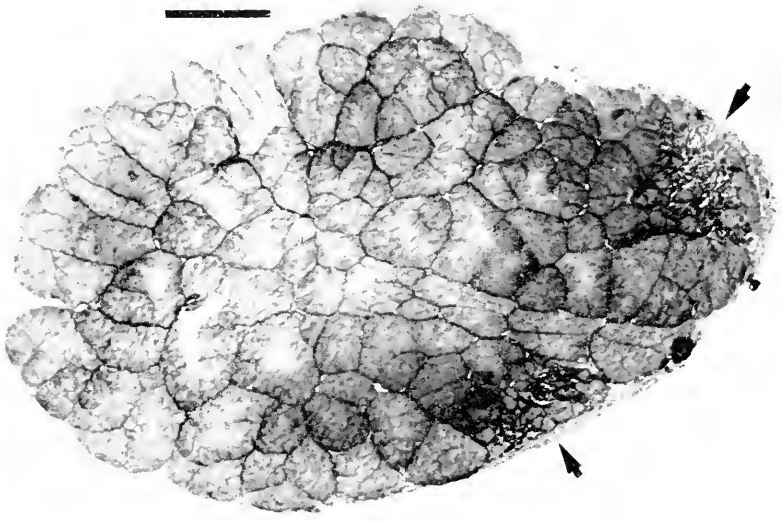

FIGURE 5. Light micrograph of transverse section of a single cor frontale muscle illustrating somatic nature of the muscle. Note small number of homogeneous fiber types with the exception of two areas (at arrows) where small muscle fibers, nerve fibers and connective tissue stroma is found. Scale equals 100 microns.

mation appears to be feeding into the esophageal and/or stomatogastric system since the sensory fibers travel away from the supraesophageal ganglion. In the dorsal tendons, either the tendon ganglia and/or the supraesophageal ganglion receive the sensory input from the tendons. In addition, the innervation of the walls of the artery and the sinus wall of the cor frontale may provide direct information on blood pressure levels which could be used by either the supraesophageal ganglion or the tendon ganglia.

The nerve which carries the two axons to the main heart valve from the supraesophageal ganglion appears to be the often cited nerve of Lemoine (Lemoine, 1868) or nervus cardiacus anterior (Police, 1908; Alexandrowicz, 1932; and Health, 1941), which was believed by them to originate in the stomatogastric ganglion. However, in all the decapods examined in the present study, these two axons, stained darkly by methylene blue, could be followed clearly from the supraesophageal ganglion in the cor frontale nerve down the length of the dorsal median artery to the main heart valve where they provide the sole innervation of the valve. The wall of the dorsal median artery along its entire length is a meshwork for fine nerve fibers, some of which can be traced to the stomatogastric nerve and others to the nerve in which the two axons run to the heart valve. In some cases, distinct connections could be found between the stomatogastric nerve and the nerve from the cor frontale carrying the two axons to the heart valve. These connections may be the source of error as to the origin of the heart valve axons in the earlier literature.

It may appear strange that such a well developed system as the cor frontale has previously escaped detailed attention, particularly in view of the wide interest in crustacean neurophysiology. The muscles are fairly conspicious, although they had been previously been confused with the eyestalk muscles judging from their inclusion in the eyestalk numbering system and the name, musculi oculi basilis posterior, applied to them (Cochran, 1935). However, the small size and circuitous route of the nerves from the supraesophageal ganglion to the cor frontale and the diffuseness of the system (in comparison to the simplicity of the main heart) may also explain the neglect. In addition, electrophysiological work on the supraesophageal ganglion in an isolated preparation has been hampered by the lack of proper perfusion techniques (Steinacker, 1975) and so (with the exception of recording from intact animals) the cephalic portion of the decapods has been relatively unexplored in comparison to the extensive work on more peripheral crustacean ganglia.

I thank Dr. Donald Kennedy for his interest and encouragement, Teppy Williams for illustrating Figures 1 and 2 and Jim Brodal for Figure 3. This work was supported by NIH postdoctoral fellowship 1FO 2 EY-55-012-01 and 1F 32 EY-05-055-01.

Summary

The anatomy of an auxiliary heart found in many decapod crustaceans is described. This heart is found at the anterior end of the dorsal median artery

before the artery divides to supply the cerebral nervous system. The heart is essentially two strips of modified somatic muscle located inside a sinus in the dorsal median artery. These muscles are innervated by four motoneurons located in the supraesophageal ganglion. Sensory innervation and possible neurosecretory elements are also described.

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RECTAL GLAND OF FRESHWATER STINGRAYS, *POTAMOTRYGON* SPP. (CHONDRICHTHYES: POTAMOTRYGONIDAE)

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The rectal salt gland of elasmobranchs (also known in the English literature as the caecal, cloacal, anal, superanal, rectal, vermiform and digitiform gland, process or appendage) has been amply treated in the older literature (Hoskins, 1917; Crofts, 1925). The gland has been likened to the ink sac of cephalopods, various intestinal diverticula found in other vertebrates and a urinary bladder, and has been assigned digestive, reproductive, secretory and blood-cleansing functions. More than sixty years before the discovery of its true function, Crawford (1899, p. 60) stated, "The rich blood supply, the character of the secreting cells, resembling so closely as they do the cells of the kidney, and the occurrence of urea in considerable amount in the secretion, all point to the structure having an excretory function, and playing the part of a supplementary kidney." Crawford has not been given appropriate credit for his prescience, which was essentially confirmed by Burger and Hess (1960) when they demonstrated that the rectal gland of *Squalus acanthias* secretes sodium chloride in a concentration approximately twice that of the plasma.

Perhaps because of the technical difficulties in collecting the rectal gland fluid, except for *S. acanthias,* it has been collected and analyzed only from the lip shark, *Hemiscyllium plagiosum* (Chan, Phillips and Chester Jones, 1967) and the stingray, *Dasyatis sabina* (Burger, 1972; Beitz, 1977). It can nevertheless be reasonably assumed that the salt secreting function of the rectal gland is universal among marine elasmobranchs.

Since the rectal gland functions to rid the body of excess salt, it is reasonable to expect that secretion would stop in a euryhaline species when it enters fresh water. Although this has not yet been conclusively demonstrated, Oguri (1964) and Gerzeli, Gervaso and De Stefano (1969) have noted that the rectal glands of *Carcharhinus leucas,* the highly euryhaline bull shark, taken from a freshwater environment, are smaller than in the same species taken from marine water. Furthermore, by histological examination, they noted concomitant regressive changes in the secretory tubules of the freshwater specimens.

Carcharhinus leucas moves back and forth between fresh water and the sea (Thorson, 1971) and can readily tolerate both media (Thorson and Gerst, 1972). Presumably, when movement takes place between salt and fresh water the rectal gland alternates between activity and inactivity. However, the family Potamotrygonidae, freshwater stingrays of South American river systems, live permanently in fresh water and have apparently been limited to fresh water for a very long time. They no longer retain high concentrations of urea, so uni-

[1] Deceased October 26, 1975.

versally employed as an osmoregulatory agent by marine and euryhaline elasmobranchs (Thorson, Cowan and Watson, 1967; Junqueira, Hoxter and Zago, 1968); nor do they build up their urea content when transferred to varying dilutions of sea water (Thorson, 1970; Griffith, Pang, Srivastava and Pickford, 1973; Gerst and Thorson, 1977). The urea retaining ability is apparently of no further survival value to elasmobranchs in a freshwater environment. Since salts are in extremely short supply in the fresh water of tropical South American rivers, an interesting question is posed concerning the fate of the strictly freshwater stingray's rectal gland, for whose salt-secreting function there is likewise no further use.

This paper presents findings concerning the morphological aspects of this question, as well as some of their physiological connotations.

Materials and Methods

Freshwater stingrays of the genus *Potamotrygon* were procured at Leticia, Colombia, from the Amazon River drainage in Brazil and from aquarium suppliers in the United States. The latter specimens were imported from dealers on the Amazon River and were clearly rays of the subject genus, although identification to species was not always possible.

Sections were made of portions of appropriate tissues from numerous rays, but the illustrations and discussion are based primarily on two: specimen A, a juvenile female (*Potamotrygon motoro*) of 160 mm disc width, purchased from a Nebraska supplier; and specimen B, a female approaching sexual maturity (*P. circularis*), 413 mm disc width, taken by a local dealer from the Itacoai River, an Amazon tributary in extreme western Brazil, near Leticia, Colombia.

From the rays selected for study, a section of the lower end of the alimentary canal, with associated structures, was immersed either directly in Bouin's fluid or in 10% formalin and later transferred to Bouin's. Specimens were transferred through several changes of 70% alcohol to remove the excess Picric acid. All the tissues were passed through successive increasing strengths of alcohol up to absolute, to insure thorough dehydration. The last change of 100% alcohol was replaced with xylene to which dryrite had been added. After clearing, they were placed in molten paraffin and subsequently embedded in wax. Sections were cut at 8 to 10 micra and the ribbons affixed to slides with Meyer's albumin fluid. The sections were stained with Ehrlich's acid hematoxylin and Eosin as tinctorial agents, and mounted in balsam under glass cover slips.

Micrographs were taken with a Zeiss photo-microscope II on Ilford Pan F film.

Results

The rectal gland and associated tissues of specimen A (a juvenile female *Potamotrygon motoro*) are shown in Figure 1. The gland is a short, slender structure, directed anteriorly from the dorsal side of the post-valvular intestine. It is closely associated with three ovoid masses of white tissue. Both the gland and the three white lobes are covered with peritoneum. The same structures and arrangement were found in specimen B, a female *P. circularis* approaching sexual

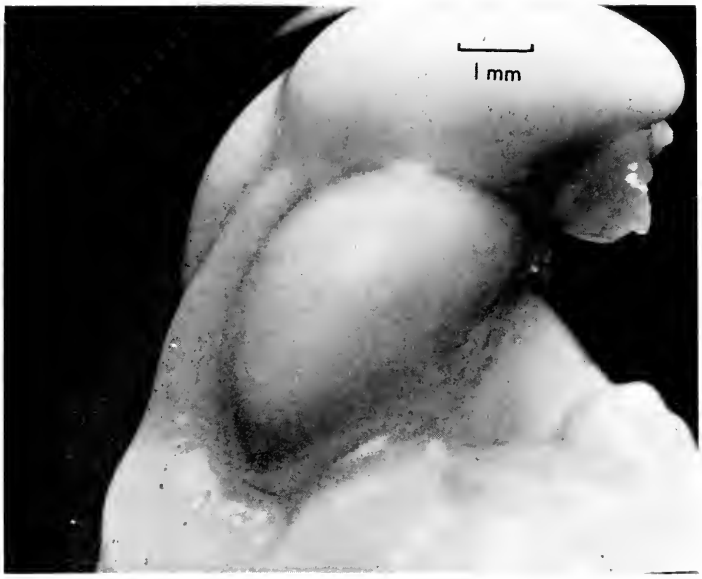

FIGURE 1. Rectal gland and associated myeloid lobes of a juvenile *Potamotrygon motoro* (160 mm disc width). The gland is the slender structure at left.

maturity (Fig. 2). In this larger animal the white masses have become further lobed and irregular in shape.

A representative cross section of the rectal gland from specimen A is shown in Figure 3 and an enlarged area of the section in Figure 4. The glandular portion, surrounding a central lumen (A), occupies approximately the central half of the gland's diameter. It includes a series of tubules (B) which are composed of simple cuboidal cells and drain into the central lumen, as at C. Surrounding the central glandular portion and forming most of the remainder of the gland, is a broad band of connective tissue containing blood vessels (D) and sinuses. The free surface of the gland is covered by a stratified columnar epithelium (E). The central lumen is lined with a simple squamous epithelium which becomes stratified as it comes closer to and enters the rectum.

The gland, although in close connection with the associated lobes (Fig. 1), is clearly and completely independent, being separated from them by a broad layer of connective tissue (Fig. 3).

Examination of the lobes associated with the rectal gland discloses an external epithelial layer continuous with that of the gland. The epithelium covers a thin connective tissue stratum and inside is a heavy concentration of leucocytes of various kinds and stages, including some with mitotic figures.

DISCUSSION

The white lobes associated with the rectal gland undoubtedly represent the "lymphoid tissue" described and figured in several earlier accounts of the gross structure of the rectal gland (*e.g.,* Hoskins, 1917). Their histology indicates

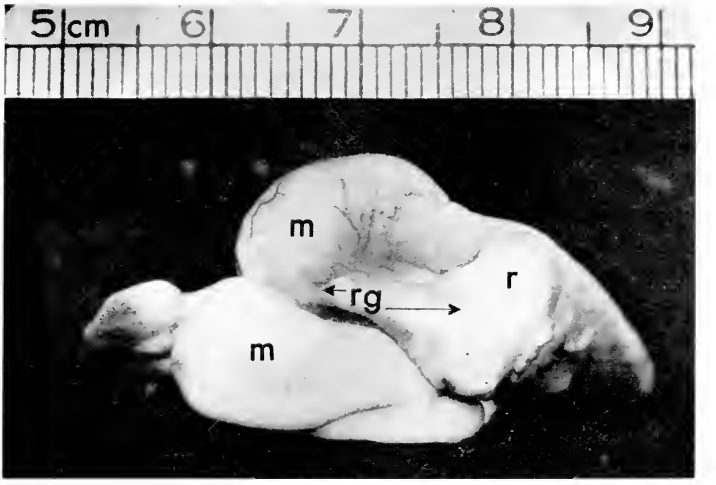

FIGURE 2. Rectal gland (rg) with portion of rectum (r) and associated myeloid lobes (m) of a female *Potamotrygon circularis* nearing sexual maturity (413 mm disc width).

that they are a part of the lymphomyeloid system of cartilaginous fishes recently discussed by Fänge (1977). This system is active in haemopoiesis and in the immune responses. Components of the system mentioned by Fänge include the spleen and thymus; the epigonal organs (associated with the gonads); Leydig's organ in the esophagus; extensive tissue in the cranium (in holocephalans); and aggregations of leucocytes in the connective tissue of the kidneys and the intestine (spiral valve).

The prominence and distinctness of the organs discussed here and their close

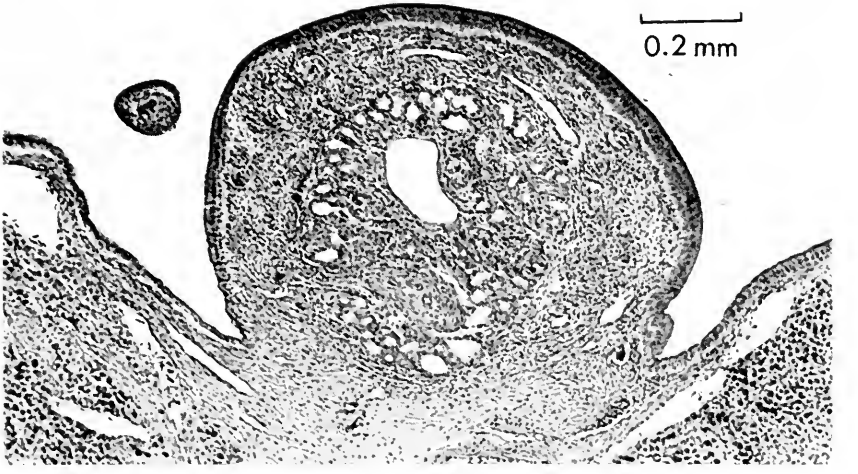

FIGURE 3. Cross section of rectal gland shown in Figure 1.

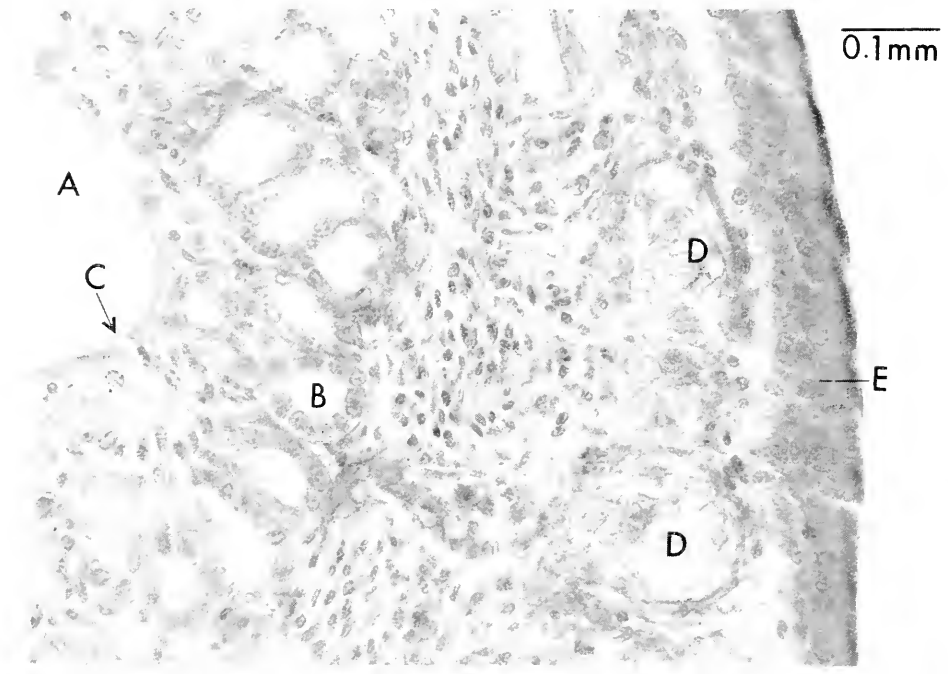

FIGURE 4. Enlarged area of rectal gland section (from Fig. 3): a central lumen (A), lined with simple, squamous epithelium, is surrounded by scattered tubules (B) which empty into lumen, as at C. A wide band of connective tissue, with blood vessels (D) occupies the outer portion of the section, and the gland is covered with a stratified, columnar epithelium (E).

association with the rectal gland and post-valvular gut justify their designation as rectomyeloid bodies.

Goldstein and Forster (1971) were unable to find a rectal gland in *Potamotrygon* sp. Griffith *et al.* (1973) reported that rays studied by them (*Potamotrygon* spp.) had an organ in the anatomical position of the rectal gland, but histological investigation showed that it was structurally unlike the rectal gland of marine elasmobranchs. Gerzeli *et al.* (1969) and Gerzeli, De Stefano, Bolognani, Koenig, Gervaso and Omodeo-Salé (1976) reported a rectal gland in a stingray identified as *Potamotrygon brachyurus* and noted that it was large, with a gland weight/body weight ratio of *ca.* 1×10^{-3}. Gerst and Thorson (1977) reported the presence of a structure in *Potamotrygon* spp. with the location and histological characteristics of the elasmobranch rectal gland, but of reduced proportions.

The present study conclusively establishes the presence of a rectal gland in the Potamotrygonidae, with the location and histological features of the gland found in marine elasmobranchs. The confusion and conflicting reports cited above may be explained by the fact that the gland is small and inconspicuous and may easily be obscured by, or even mistaken for, a part of the lobed myeloid tissue spatially associated with it.

Marine and brackishwater elasmobranchs have relatively large glands (Table I). The fully euryhaline shark (*Carcharhinus leucas*) that completely tolerates both

TABLE I

Rectal gland/body weight ratio related to habitat.

Reference	Species	Rectal gland wt./body wt. ratio (units per million)
Marine and brackish water species		
Burger (1972)	*Squalus acanthias*	600
	Dasyatis sabina	240
Fänge and Fugelli (1963)	*Selache maxima*	200
Chan and Phillips (1967)	*Hemiscyllium plagiosum*	120
Bonting (1966)	*Squalus acanthias*	444
	Carcharius littoralis	185
	Carcharhinus falciformis	190
	Mustelus canis	214
	Raja eglanteria	202
	Myliobatus freminvillei	164
	Pteroplatea altavela	86
	Squatina squatina	99
Fully euryhaline species		
Gerzeli *et al.* (1969)	*Carcharhinus leucas* (marine)	60
	Carcharhinus leucas (fresh water)	20
Thorson (unpublished)	*Carcharhinus leucas* (fresh water)	30
Freshwater species		
Thorson (unpublished)	*Potamotrygon circularis*	15
Gerzeli *et al.* (1969)	*Potamotrygon brachyurus*	1000*

* See text.

fresh and salt water has a gland of somewhat reduced relative size; the gland appears to be larger when this shark is in sea water than when it is in fresh water (Oguri, 1964; Gerzeli *et al.*, 1969). The completely freshwater rays (Potamotrygonidae) examined in this study have rectal glands of still more reduced size. Furthermore, the number of tubules is considerably reduced and their distribution within the gland is relatively restricted (Fig. 3). The rectal gland weight/body weight ratio of 1×10^{-3} given by Gerzeli *et al.* (1969) is greater than that of any marine elasmobranch listed in Table I. The figure must either be in error or the specimen studied may have included myeloid or other tissue in addition to the gland itself.

Atrophy of the gland might reasonably be expected in rays that have been completely limited to fresh water for a long, although undetermined, period of time. Just as they have abandoned urea retention, the freshwater rays apparently have also abandoned supplementary salt excretion. Both would be counterproductive in a freshwater environment.

The highly euryhaline *Carcharhinus leucas* is able to increase and decrease the urea content of its body fluids in response to changes in environmental salinity. The findings of Oguri (1964), and Gerzeli *et al.* (1969; 1976) suggest that secretory activity of the rectal gland of *C. leucas* also responds to changes in environ-

mental salinity. In *Potamotrygon* spp., on the other hand, transfer to saline environment does not elicit an increase in urea concentration in body fluids, and the loss of urea retention appears to be irreversible (Thorson, 1970; Griffith *et al.,* 1973; Gerst and Thorson, 1977). This fact suggests that the apparent loss of salt secretory activity of the atrophied rectal gland in *Potamotrygon* may also be irreversible. This view is supported by the observation that, in potamotrygonids transferred to dilute sea water, regulation of inorganic ions breaks down. Sodium and chloride concentrations in particular almost double in seawater-acclimated rays. The greatest concentration they can tolerate for any length of time is approximately 40% sea water (Thorson *et al.,* 1967; Thorson, 1970; Griffith *et al.,* 1973; Gerst and Thorson, 1977).

No function, other than salt secretion, has been demonstrated for the fully active rectal gland of marine elasmobranchs. What residual function the atrophied potamotrygonid rectal gland might have, if any, is unknown. Gerzeli *et al.,* (1976, p. 619) reported that the rectal gland of *Potamotrygon brachyurus* "appears very peculiar, showing secretory activity histologically, but lacking any cytochemical evidence related to salt secretion." Otherwise, nothing has been established experimentally or histochemically concerning any specific function for the potamotrygonid rectal gland.

The Chondrichthyes made their appearance in the geological record during the Devonian. Although their presumed ancestors, the placoderms, may have inhabited inland fresh waters, the Devonian Chondrichthyes appear to have been marine since their first appearance (Romer, 1966). This study does little to elucidate the continuing discussion of whether urea retention developed in chondrichthians in response to the invasion of salt water or existed earlier and provided a pre-adaptation for marine life. However, it provides evidence bearing on a related question concerning the potamotrygonid stingrays: does the near absence of urea in the freshwater rays represent a genetic deletion of their ancestral ability to concentrate urea, or were they descended from ancestors that had never left fresh water and had never developed urea retention? The latter possibility was considered remote by Thorson *et al.* (1967) and less plausible than the former by Forster and Goldstein (1969). However, evidence concerning urea retention is not preserved in fossils, and there is in any case no fossil record of the family Potamotrygonidae. Stingrays of the closely related marine family Dasyatidae are known from freshwater assemblages of the Tertiary (Eocene), but reports of fossil Potamotrygonidae in South America (Garman, 1913) and in Africa (Arambourg, 1947) are probably also of dasyatids (Thorson and Watson, 1975).

In the absence of fossil evidence of the history of this group, evidence must be sought from extant rays. Such evidence is now provided by the rectal gland, which is likewise not preserved in fossils. A functional rectal gland can only be viewed as a marine adaptation and its presence, albeit in much reduced form, with no known function, can only indicate a marine ancestry for the freshwater stingrays. The chronology of the gland's earliest history cannot at present be firmly established, but at the time of the first appearance of the stingrays, in the Cretaceous (Romer, 1966), they were almost certainly already marine, as were the other Chondrichthyes, and possessed the functional rectal gland so universally found in the other cartilaginous fishes.

Both the absence of urea retention and the atrophy of the rectal gland bespeak a long history in fresh water for the Potamotrygonidae. The salinity tolerance, urea retaining ability and the size and condition of the rectal gland, studied in a variety of stingray species representing the full spectrum of environmental salinities, are potentially rich sources of evidence regarding the evolution of freshwater adaptation in stingrays as well as elasmobranchs in general.

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Summary

1. Contrary to some reports, a rectal gland is present in strictly freshwater stingrays of South American rivers (*Potamotrygon* spp.).

2. The gland has the location and histological features of the salt-secreting rectal gland of marine elasmobranchs, but is much reduced in size and number of tubules.

3. Its residual function, if any, is unknown.

4. The rectal gland is associated with prominent myeloid lobes, here designated as rectomyeloid bodies.

5. In the absence of potamotrygonid fossils, the atrophied rectal gland is strong evidence of marine ancestry for the freshwater rays.

6. Both the reduced gland and the loss of urea retention in potamotrygonids are indicative of a long history of freshwater adaptation.

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