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## SHELL ENTRY AND SHELL SELECTION OF HYDROID-COLONIZED SHELLS BY THREE SPECIES OF HERMIT CRABS FROM THE NORTHERN GULF OF MEXICO\*

WILLIAM R. BROOKS† AND RICHARD N. MARISCAL

*Department of Biological Science, Florida State University, Tallahassee, Florida 32306*

### ABSTRACT

The shell entry and shell selection of hydroid-colonized (either *Hydractinia echinata* or *Podocoryne selena*) shells by two populations each of *Pagurus pollicaris*, *P. longicarpus*, and *Clibanarius vittatus* were observed under various conditions. All three species either initially chose or subsequently switched into bare shells, even in the presence of a predator. The population of *P. pollicaris* where *Octopus joubini* was more abundant initially selected hydroid-colonized shells more frequently in one experiment than did the other population of crabs. The general avoidance of hydroid-colonized shells is probably due to the crabs' being stung by nematocysts.

### INTRODUCTION

Because hermit crabs live in empty gastropod shells, their shell selection can be viewed as a form of habitat selection (Conover, 1978). Therefore, just as many other animals selectively choose their habitat (Cox *et al.*, 1976), hermit crabs nonrandomly select their gastropod shells. Most studies involving shell selection by hermit crabs have focused on the significance of various shell characteristics such as species, volume, weight, aperture size, and morphology (Reese, 1962, 1963; Völker, 1967; Markham, 1968; Childress, 1972; Kuris and Brody, 1976; Conover, 1978).

Organisms that colonize the exterior surface of the gastropod shell also affect shell selection (Conover, 1976). In particular, epifaunal hydroid colonies (*e.g.*, *Hydractinia echinata*) typically form encrusting mats on the shells. Certain hermit crabs select hydroid-colonized shells more often than bare shells when given a choice. For example, Jensen (1970) demonstrated that *Pagurus bernhardus* preferred *Hydractinia*-colonized shells to bare shells. Similarly, Grant and Ulmer (1974) showed that *P. acadianus* also preferred *Hydractinia*-colonized shells.

Apparently, however, the preference for hydroid-colonized shells can vary within a species. For example, differences in shell selection have been reported for the hermit crabs *P. pollicaris* and *P. longicarpus*, both of which are common along the North American coasts of the Atlantic Ocean and the Gulf of Mexico. *Pagurus pollicaris* preferred hydroid-colonized shells in studies by Wright (1973), Conover (1976), and Mercado and Lytle (1980). Mills's (1976a) preliminary observations, however, showed that *P. pollicaris* rejects hydroid-colonized shells. Similarly, *P. longicarpus* preferred hydroid-colonized shells in studies by Wright (1973) and Conover (1976), but both Mills (1976a) and Mercado and Lytle (1980) reported that it usually rejected them.

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Although the differences in the above studies may be due, in part, to differing laboratory conditions, the hermit crabs in each study were collected from different localities. Therefore, it is possible that behavioral differences exist between populations of these hermit crabs. Abrams (1978) found that one population of the terrestrial hermit crab *Coenobita compressus* selected larger shells than another population. In addition, Bertness (1982) showed that two populations each of the hermit crabs *Clibanarius albidigitus* and *Calcinus obscurus* differed in shell-species selection. The populations of the two species located where predation pressure was higher selected shells that would minimize predation pressure (Bertness, 1982). Similarly, Scully (1979) found that two populations of *P. longicarpus* chose different sized shells. He also suggested that physical differences between the two collection sites affected the hermit crabs' shell preference.

The preference of some hermit crabs for and the avoidance by others of hydroid-colonized shells may allow coexistence of similar-sized species. Although Grant and Ulmer (1974) found that *P. adrianus* preferred hydroid-colonized shells, the closely related *P. pubescens*, which is sympatric with *P. adrianus*, preferred bare shells. This difference in shell selection could serve to separate resources (shells) and allow these two species to coexist (Grant and Ulmer, 1974). Similarly, Wright (1973) reported that *Clibanarius vittatus*, which is a competitive dominant over *P. pollicaris* (Wright, 1973), prefers shells without hydroids. He found that when *C. vittatus* contacted the hydroid colony it was apparently stung and avoided any further contact with the hydroid. Wright (1973) never observed *P. pollicaris* or *P. longicarpus*, both of which selected shells with hydroids, to behave as if stung. Wright (1973) suggests that because *Pagurus* occasionally eat the hydroid's polyps, this diet might somehow provide *Pagurus* protection from the cnidae, allowing them to inhabit the hydroid-colonized shell.

The avoidance of hydroid-colonized shells may be due, in part, to the effect on the hermit crab of contact with specialized dactylozoid polyps. These coiled polyps, located at the aperture of the shell, have been described as being "defensive" (Schiffjma, 1935; Stokes, 1974a, b). When the colony is mechanically or electrically disturbed, they uncoil and lash down toward the lumen of the shell (Schiffjma, 1935; Stokes, 1974a, b; Mills, 1976a). During shell entry, a hermit crab would no doubt contact these dactylozoid polyps and, if stung, might make no further attempt to enter the shell.

In the present study, the shell entry and shell selection of hydroid-colonized shells by *P. pollicaris*, *P. longicarpus*, and *C. vittatus*, all from the northeastern Gulf of Mexico, were observed under various conditions and compared with previous studies. Tests were performed to determine whether cnidae from the dactylozoid polyps discharge onto the surface of the hermit crabs. In addition, the shell selection of two populations for each of the three species was tested. The results of this work show that all three species of hermit crabs either initially choose or subsequently switch into bare shells, even when a predator (a stone crab or octopus) is present. Some populational differences in shell selection existed with *P. pollicaris*. The rejection of hydroid-colonized shells may be due to the crabs' being stung by cnidae from the dactylozoid polyps, because cnidae do discharge onto the hermit crabs.

#### MATERIALS AND METHODS

All of the animals were kept in closed-system aquaria in the laboratory at the Florida State University, Tallahassee, Florida. The hermit crabs *P. pollicaris* Say, *P. longicarpus* Say, *Clibanarius vittatus* (Bosc), and the stone crab *Menippe mercenaria* (Say) were collected from two intertidal sites in the northeastern Gulf of Mexico at

depths less than 2 meters. One locality was near the Florida State University Marine Laboratory (FSUML), Turkey Point, Florida, about 45 miles south of Tallahassee, Florida. The other locality was in St. Joseph Bay adjacent to Port St. Joe, Florida, and about 50 miles west of the FSUML. These two sites differed in that the salinity at the FSUML fluctuated because of freshwater runoff from a nearby creek. The salinity in St. Joseph Bay remained constant at 30–31‰. *Octopus joubini* Robson was collected from St. Joseph Bay, where it is very common (cf. Mather, 1972; Butterworth, 1982). *Octopus joubini* is uncommon near the FSUML site (pers. obs.; P. Wilber, Florida State University, pers. comm.). Nearly every *P. pollicaris* was a male. The sex ratios of both *P. longicarpus* and *C. vittatus* were approximately 50:50 male:female.

All of the hermit crabs and the stone crabs were fed Tetra Min flaked fish food. *Octopus joubini* was fed fiddler crabs (*Uca* sp.) and small hermit crabs (*Pagurus* sp.). The shell of the gastropod *Polinices duplicatus* (Say) was used in all of the experiments. In addition, the living hydroid (either *Hydractinia echinata* (Fleming) or *Podocoryne selenia* Mills) was used when it was on *P. duplicatus* shells and covered over 90% of the shell's exterior surface. Both species of hydroid commonly occur in the two localities discussed above, but cannot always be clearly distinguished unless in the appropriate reproductive condition (cf. Mills, 1976b).

#### *Shell entry of hydroid-colonized shells*

We removed hermit crabs from their shells by stroking the crab's abdomen with a flexible plastic cord inserted through a hole drilled in the shell. Each naked hermit crab was placed in a finger bowl filled with sea water, and a hydroid-colonized shell was added to the bowl, with its aperture down. The behavior of the hermit crab was observed. The initial contact was observed closely to determine whether the crab responded as if stung. A sting response was defined generally as a rapid movement of a body part or the whole crab away from the hydroid following contact with one or more polyps. For example, if one of the hermit crab's antennae touched the hydroid and the crab quickly jerked the antenna away, this action was considered a sting response.

#### *Cnida discharge tests*

When *P. pollicaris* that live in hydroid-colonized shells retreat into the shells, their eyestalks, antennules, antennae, and chelipeds eventually contact the dactylozoid polyps. Therefore, sensory hairs from the carpus of the cheliped were removed with forceps, stained with 1% Toluidine blue solution, and then examined under a light microscope to determine whether cnidae had discharged onto them (Fig. 1). Ten sensory hairs were removed from each of ten *P. pollicaris*, and the number of cnidae on them was counted. The sensory hairs were chosen rather than the eyestalks, antennules, or antennae because of their small size (which makes them suitable for staining and counting cnidae) and the large number present on the appendages. Furthermore, these sensory hairs are usually associated with sensory endings (Shelton and Laverack, 1970). If the hermit crabs are stung by the hydroids, then the sensory hair is probably at least one of the sites where the stinging stimulus is received and transmitted.

Ten sensory hairs were also removed from each of ten *P. pollicaris* and five *C. vittatus* and rubbed along the fringe of dactylozooids, four times (to ensure contact), from one side of the lip of the shell to the other. These sensory hairs were also stained

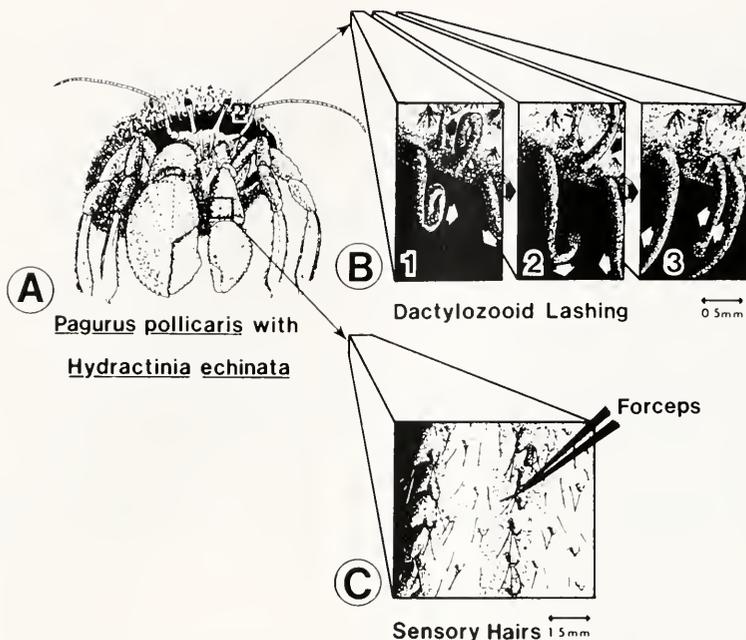


FIGURE 1. The hermit crab *Pagurus pollicaris* inhabiting a *Polinices duplicatus* shell that is colonized by the hydroid *Hydractinia echinata* (A). The dactylozoid polyps of the hydroid uncoil and lash downward when the colony is disturbed (B). Sensory hairs from the carpus of *P. pollicaris* are removed (C) and examined under a light microscope for the dactylozoid's cnidae.

with a 1% Toluidine blue solution and examined under a light microscope to count the cnidae.

In all of the above tests, sensory hairs approximately 3 mm in length were used.

### Shell preference tests

Two populations (one from the FSUML and the other from St. Joseph Bay) of each of the hermit crabs, *P. pollicaris*, *P. longicarpus*, and *C. vittatus*, were used in the following experiments, unless stated otherwise.

*Experiment 1.* A naked hermit crab was placed in a 1-liter aquarium with two *Polinices duplicatus* shells of the same size (same shell aperture  $\pm 1$  mm). One shell, however, was colonized by a hydroid while the other was bare. The shell choice of the hermit crab was recorded at the ends of two observation periods: (1) at five minutes and (2) at eight hours. Two observations were made to determine whether the hermit crabs stay in the shell they choose first.

*Control 1.* This experiment was done to determine whether there were additional factors associated with hydroid-colonized shells that caused the hermit crabs either to select or avoid these shells. Therefore, the hydroid colonies were scraped off of the shells with a knife. The naked hermit crabs were then given a choice between the shells that had had their hydroids scraped off and shells that were originally bare. The two shells were the same size. The shell choice of the hermit crab was recorded at five minutes and eight hours.

*Experiment 2.* Hazlett (1980) has argued that the degree of avoidance of hydroid-colonized shells by hermit crabs may be overestimated using naked crabs. Therefore,

in an experiment designed to determine whether the naked hermit crabs select hydroid-colonized shells differently from hermit crabs in shells, the naked crabs were first allowed to enter *P. duplicatus* shells in which only their abdomen could fit. They were then given a choice between hydroid-colonized shells and bare shells of the same size. After the hermit crab entered the small shell, it was then placed in a 1-liter aquarium, and its shell choice was recorded after five minutes and after eight hours.

*Control 2.* Again, the naked hermit crabs were first allowed to enter small *P. duplicatus* shells. This time, however, the hydroids were scraped off of the shells as was done in Control 1 to determine whether additional factors (other than the hydroid) associated with the hydroid-colonized shells affect the shell choice of the hermit crabs. The hermit crab was then given a choice between a shell with its hydroid colony removed and a shell of the same size that was originally bare. The shell choice of the hermit crab was recorded at five minutes and at eight hours.

*Experiment 3.* The naked hermit crab was placed in a 1-liter aquarium with one hydroid-colonized shell. Observations were made at five minutes and eight hours to determine whether the hermit crab entered the shell.

*Experiment 4.* In an experiment to reveal shell selection behavior in the presence of a crustacean predator, the naked hermit crab was placed in a 4-liter aquarium with the stone crab *M. mercenaria* present. The hermit crab was given a choice between hydroid-colonized shells and bare shells. The two shells were the same size. The shell choice of the hermit crab was recorded at five minutes and at eight hours.

*Experiment 5.* In an experiment designed to compare shell selection behavior in the presence of a cephalopod predator, the naked hermit crab was placed in a 4-liter aquarium with the octopus *O. joubini*. The hermit crab was given a choice between hydroid-colonized shells and bare shells. The two shells were the same size. The shell choice of the hermit crab was recorded after five minutes and after eight hours.

## RESULTS

### *Shell entry of hydroid-colonized shells*

*Pagurus pollicaris* usually used its antennae to make initial contact with the hydroid, but did not respond as if stung (Table I). Subsequently, the crabs frequently climbed onto the shells with their abdomen in full contact with the hydroid's polyps, but again, no evidence of stinging was observed (although some of the polyps did cling to sensory hairs on the abdomen). *Pagurus pollicaris* then lifted the lip of the shell off of the substrate with its chelipeds and placed its abdomen in the aperture of the shell and entered the shell. While the crab was entering the shell, no apparent stinging was observed.

TABLE I

*The responses of naked Pagurus pollicaris, P. longicarpus, and Clibanarius vittatus to initial contact with a hydroid-colonized shell*

Hermit crab	n	Initial contact elicits		Chi-squared test	
		Sting response	No sting response	$\chi^2$	P
<i>P. pollicaris</i>	10	0	10	10.0	<.01
<i>P. longicarpus</i>	10	9	1	6.4	<.05
<i>C. vittatus</i>	14	10	4	2.6	NS

*Pagurus longicarpus* also commonly made initial contact with the hydroid with its antennae, but usually responded as if stung by quickly jumping back several centimeters (Table I). Those crabs subsequently avoided any further contact with the shell.

*Clibanarius vittatus* also initially contacted the hydroid with its antennae, and, while most of the crabs reacted as if stung, some did not (Table I). Three of the crabs that showed no stinging response upon initial antennal contact climbed on top of the shell, as *P. pollicaris* did. At this time, all three of the crabs appeared to be stung and avoided further contact with the shell. Seven of the crabs did enter the shell in a manner similar to that of *P. pollicaris*, including some of the crabs that had apparently been stung upon initial antennal contact.

### *Cnida discharge tests*

The average number of cnidae discharged onto sensory hairs pulled directly off of *P. pollicaris* in hydroid-colonized shells was 2.1 (S.D. = 1.3). The average number of cnidae on the sensory hairs of *P. pollicaris* that had been touched by one of the authors to the dactylozoid polyps was 9.4 (S.D. = 5.9). Significantly more cnidae discharged onto the sensory hairs of the latter group (T-test,  $P < .005$ ) (see Fig. 2). In addition, cnidae also discharged onto the sensory hairs of *C. vittatus* that were touched by one of the authors to the dactylozoid polyps (mean = 8.2, S.D. = 5.6) (see Fig. 3).

### *Shell preference tests*

*Experiment 1.* Table II summarizes the results of the experiment in which the naked hermit crabs were given a choice between hydroid-colonized shells and bare shells.

The shell types occupied by *Pagurus pollicaris* from the FSUML were random after five minutes, but after eight hours bare shells had been chosen more frequently. Eleven shell switches occurred between the two observations. Although eight of the eleven shell switches involved the crab's switching from shells with a hydroid to bare shells, this ratio is not significantly different from random.

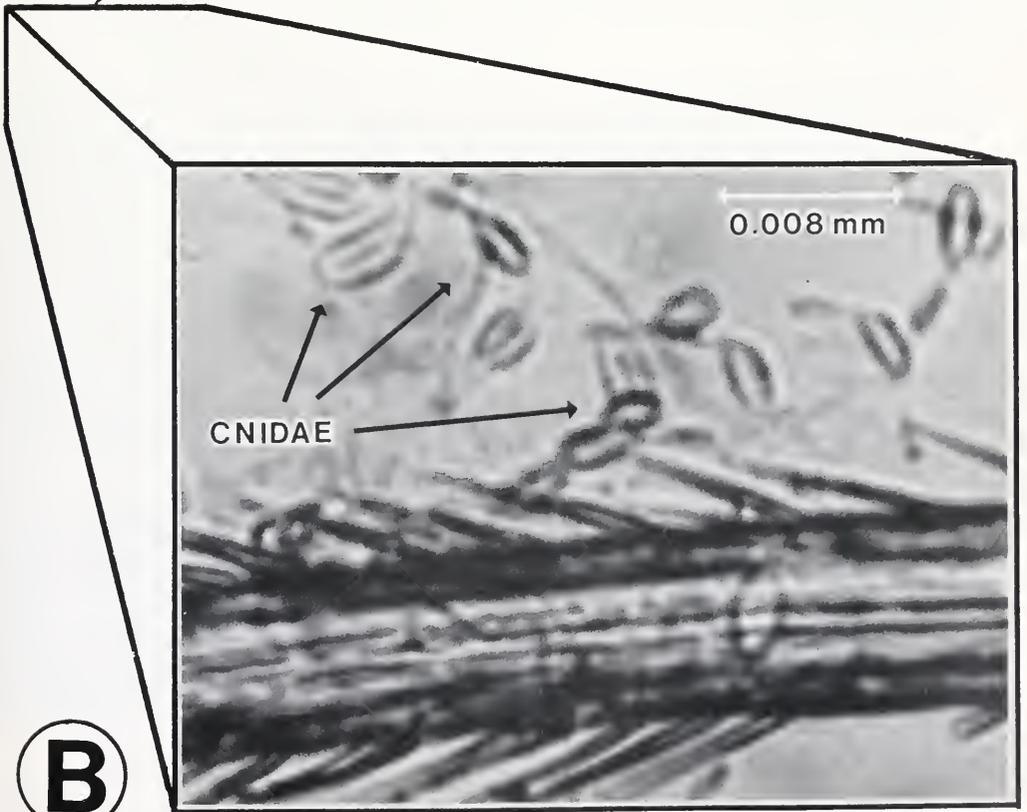
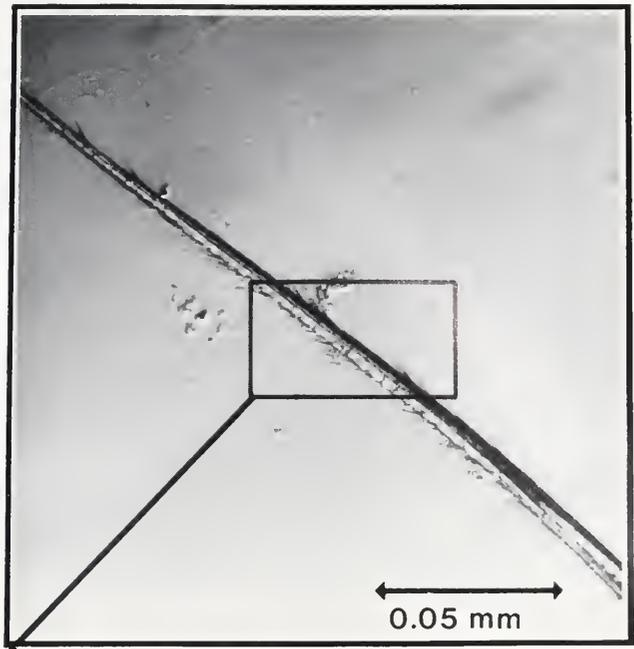
*Pagurus pollicaris* from St. Joseph Bay differed from their FSUML conspecifics in shell choice after both five minutes and eight hours. *Pagurus pollicaris* from St. Joseph Bay had chosen shells with hydroids more frequently after five minutes, but after eight hours the shell types occupied did not differ from random. Thirteen shell switches occurred between the two observations. Ten of the 13 switches ( $P = .06$ ) involved the crabs' switching from a shell with a hydroid to a bare shell.

The shell types occupied by both populations of *P. longicarpus* from the FSUML and St. Joseph Bay were random after five minutes, but bare shells had been chosen much more frequently after eight hours ( $P < .001$ , for the pooled data from both populations). Five switches occurred between the two observations for the crabs from the FSUML. Four of the five switches (not significant) involved the crabs' switching from hydroid-colonized shells to bare shells. Only two switches occurred with the crabs from St. Joseph Bay, both from hydroid-colonized shells to bare shells. Overall,

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FIGURE 2. A sensory hair from the carpus of *Pagurus pollicaris*, shown before (A) and after (B) being rubbed along the fringe of dactylozoid polyps of the hydroid colony. Notice the numerous discharged cnidae on the sensory hair that has been touched to the hydroid.

**A**

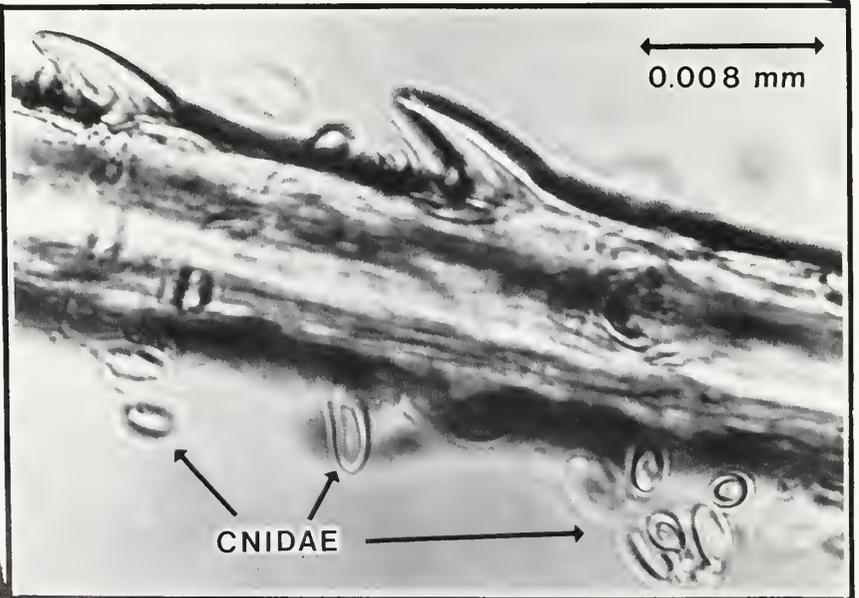


**B**

**A**



**B**



seven switches occurred, six of them involving the crabs' vacating hydroid-colonized shells ( $P = .07$ , for the pooled data of the two populations at both observations).

Both populations of *C. vittatus* had chosen bare shells much more frequently than hydroid-colonized shells after five minutes and after eight hours ( $P < .01$ , for the pooled data of the two populations after both observation periods). No shell switches occurred between the two observations.

*Control 1.* In this experiment, the naked hermit crabs were given a choice between shells that had had their hydroid colony scraped off and bare shells.

The shell types occupied by each population of *P. pollicaris*, *P. longicarpus*, and *C. vittatus* were random after both observation periods. The pooled data for the two populations of *P. pollicaris* shows that after five minutes 26 out of 53 hermit crabs had chosen scraped shells, and after eight hours 29 out of 60 had chosen scraped shells. The pooled data for the two populations of *P. longicarpus* shows that after five minutes 7 out of 19 hermit crabs had chosen scraped shells, and after eight hours 8 out of 19 had chosen scraped shells. The pooled data for the two populations of *C. vittatus* shows that after five minutes 11 out of 18 had chosen scraped shells, and after eight hours 14 out of 27 had chosen scraped shells. No shell switches occurred between the two observations.

*Experiment 2.* Table III summarizes the results of the experiment in which the naked crabs were first allowed to enter shells in which only their abdomens could fit and then given a choice between hydroid-colonized shells and bare shells.

Only 12 of the 20 *P. pollicaris* from the FSUML had chosen a shell after five minutes. Nine of the 12 shells chosen were bare shells (not significant), but after eight hours all 20 crabs had chosen a shell, and 16 of them were bare shells ( $P < .01$ ). No shell switches occurred between the two observations.

Although *P. pollicaris* from St. Joseph Bay occupied shells randomly after both observation periods, it appears that after eight hours there is a trend toward bare shells (14 of 22 shells chosen were bare shells). Only two shell switches occurred, both from shells with a hydroid to bare shells.

The pooled data from the two populations of *P. pollicaris* indicate that after five minutes the shell types occupied were random, but after eight hours bare shells had been chosen more frequently.

Each population of *P. longicarpus* and *C. vittatus* had selected bare shells more frequently at both observations. A total of only three shell switches (all from shells with hydroids to bare shells) occurred between observations for both hermit crab species.

*Control 2.* In this experiment, the naked crabs were first allowed to enter shells in which only their abdomens could fit and then given a choice between shells that had had their hydroid colony scraped off and bare shells.

The shell types occupied by each population of *P. pollicaris*, *P. longicarpus*, and *C. vittatus* were random at both observations. The pooled data for the two populations of *P. pollicaris* shows that after five minutes 14 out of 33 hermit crabs had chosen scraped shells, and after eight hours 17 out of 39 had chosen scraped shells. The pooled data for the two populations of *P. longicarpus* shows that after five minutes 10 out of 19 hermit crabs had chosen scraped shells, and after eight hours 11 out of 20 had chosen scraped shells. The pooled data for the two populations of *C. vittatus*

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FIGURE 3. A sensory hair from the carpus of *Clibanarius vittatus*, shown before (A) and after (B) being rubbed along the fringe of dactylozoid polyps of the hydroid colony. Notice the numerous discharged cnidae on the sensory hair that has been touched to the hydroid.

TABLE II

Results of shell-selection tests of naked *Pagurus pollicaris*, *P. longicarpus*, and *Clibanarius vittatus* from two localities: hydroid-colonized shells versus shells without hydroids\*

Hermit crab	Locality	No. tested	Time of observation	No. selecting shells		$\chi^2$	P	No. switching shells between 5 min and 8 h, from		Chi-squared test	
				With hydroid	Without hydroid			With hydroid to without hydroid	Without hydroid to with hydroid	$\chi^2$	P
<i>P. pollicaris</i>	FSUML	30	5 min	15	14	0.03	NS	8	3	2.27	NS
			8 h	9	21	4.80	<.05				
<i>P. pollicaris</i>	St. Joseph Bay	30	5 min	21	7	7.00	<.01	10	3	3.77	NS
			8 h	15	15	0.00	NS				( <i>p</i> = .06)
<i>P. pollicaris</i>	Pooled	60	†					18	6	6.00	<.05
<i>P. longicarpus</i>	FSUML	10	5 min	5	5	0.00	NS	4	1	1.80	NS
			8 h	1	9	6.40	<.05				
<i>P. longicarpus</i>	St. Joseph Bay	10	5 min	2	4	0.67	NS	2	0	2.00	NS
			8 h	1	9	6.40	<.05				
<i>P. longicarpus</i>	Pooled	20	5 min	7	9	0.25	NS	6	1	3.57	NS
			8 h	2	18	12.80	<.001				( <i>P</i> = .07)
<i>C. vittatus</i>	FSUML	20	5 min	1	9	6.40	<.05	0	0		
			8 h	1	18	15.21	<.001				
<i>C. vittatus</i>	St. Joseph Bay	10	5 min	2	6	2.00	NS	0	0		
			8 h	0	10	10.00	<.01				
<i>C. vittatus</i>	Pooled	30	5 min	3	15	8.00	<.01	0	0		
			8 h	1	28	25.14	<.001				

\* "Pooled" means that the results from the two populations of hermit crabs have been combined.

† Pooling inappropriate because of differences in shell selection between the two populations.

TABLE III

Results of shell-selection tests of *Pagurus pollicaris*, *P. longicarpus*, and *Clibanarius vittatus* (all from two localities) who were first allowed to enter shells in which only the abdomen could fit: hydroid-colonized shells versus shells without hydroids\*

Hermit crab	Locality	No. tested	Time of observation	No. selecting shells		$\chi^2$	P	No. switching shells between 5 min and 8 h. from		Chi-squared test	P
				With hydroid	Without hydroid			With hydroid to without hydroid	Without hydroid to with hydroid		
<i>P. pollicaris</i>	FSUML	20	5 min	3	9	3.00	NS	0	0		
			8 h	4	16	7.20	<.01				
<i>P. pollicaris</i>	St. Joseph Bay	22	5 min	10	11	0.05	NS	2	0	2.00	NS
			8 h	8	14	1.64	NS				
<i>P. pollicaris</i>	Pooled	42	5 min	13	20	1.48	NS	2	0	2.00	NS
			8 h	12	30	7.77	<.01				
<i>P. longicarpus</i>	FSUML	10	5 min	3	5	0.11	NS	1	0	1.00	NS
			8 h	2	8	3.60	NS ( $P = .06$ )				
<i>P. longicarpus</i>	St. Joseph Bay	10	5 min	1	7	4.50	<.05	1	0	1.00	NS
			8 h	1	9	6.40	<.05				
<i>P. longicarpus</i>	Pooled	20	5 min	4	12	4.00	<.05	2	0	2.00	NS
			8 h	3	17	9.80	<.01				
<i>C. vittatus</i>	FSUML	17	5 min	0	14	14.00	<.001	0	0		
			8 h	0	17	17.00	<.001				
<i>C. vittatus</i>	St. Joseph Bay	10	5 min	1	8	5.44	<.05	1	0	1.00	NS
			8 h	0	10	10.00	<.01				
<i>C. vittatus</i>	Pooled	27	5 min	1	22	19.17	<.001	1	0	1.00	NS
			8 h	0	27	27.00	<.001				

\* "Pooled" means that the results from the two populations of hermit crabs have been combined.

show that after five minutes 12 out of 23 hermit crabs had chosen scraped shells, and after eight hours, 14 out of 26 had chosen scraped shells. No shell switches occurred between the two observations.

*Experiment 3.* In this experiment, the naked crabs were given a single hydroid-colonized shell.

The majority of *P. pollicaris* from the FSUML and St. Joseph Bay populations entered the hydroid-colonized shells. The pooled data show that after five minutes 18 of 20 crabs had entered the shell ( $P < .001$ ), and after eight hours all 20 had entered the shell ( $P < .001$ ).

Only some *P. longicarpus* from both populations had entered the hydroid-colonized shell after five minutes (7 of 20, random success-failure rate). The pooled data after eight hours, however, show that 19 of 20 ( $P < .001$ ) *P. longicarpus* entered the shell.

Only 1 of 24 ( $P < .001$ ) *C. vittatus* from both populations had entered the hydroid-colonized shell after five minutes. The pooled data after eight hours, however, show that 10 of 24 (random success-failure rate) *C. vittatus* entered the shell.

*Experiment 4.* Table IV summarizes the results of the experiment in which, in the presence of a stone crab, *M. mercenaria*, the naked hermit crabs were given a choice between hydroid-colonized shells and bare shells.

Sixteen of 24 (not significant) *P. pollicaris* from the FSUML had chosen bare shells after five minutes, and after eight hours 18 of 22 ( $P < .01$ ) had chosen bare shells. Two *P. pollicaris* in bare shells were killed by *M. mercenaria* sometime between the two observations. Four shell switches occurred, all from hydroid-colonized shells to bare shells ( $P < .05$ ).

The shell types occupied by *Pagurus pollicaris* from St. Joseph Bay were random after both observation periods. Again, two *P. pollicaris* in bare shells were killed by the stone crab. No switches occurred between the two observations.

The shell types occupied by both populations of *P. longicarpus* were random after five minutes, but 20 of 24 ( $P < .01$ ) crabs had chosen bare shells after eight hours. Seven *P. longicarpus*, all in bare shells, were killed by the stone crab. Four of the crabs killed were from St. Joseph Bay, and the remainder from the FSUML.

*Clibanarius vittatus* was not tested in this experiment.

*Experiment 5.* Table V summarizes the results of the experiment in which the naked hermit crabs were given a choice between hydroid-colonized shells and bare shells in the presence of the octopus *O. joubini*.

The shell types occupied by *Pagurus pollicaris* from the FSUML were random after five minutes, but after eight hours 17 of 20 ( $P < .01$ ) crabs had chosen bare shells more frequently. One *P. pollicaris* in a bare shell was killed by the octopus. Three shell switches occurred, all from hydroid-colonized shells to bare shells (not significant).

The shell types occupied by *Pagurus pollicaris* from St. Joseph Bay were random after both observation periods. Again, one *P. pollicaris* in a bare shell was killed by the octopus. Three shell switches occurred, all from hydroid-colonized shells to bare shells (not significant).

The shell types occupied by both populations of *P. longicarpus* were random after five minutes, but 32 of 35 ( $P < .001$ ) crabs had chosen bare shells after eight hours. One FSUML *P. longicarpus* in a bare shell was killed by the octopus. Seven shell switches occurred (all from shells with hydroids to bare shells,  $P < .01$ ) between the observations by both hermit crab species. Four of the seven switches occurred by the St. Joseph Bay crabs, and the remainder from the FSUML.

*Clibanarius vittatus* was not tested in this experiment.

TABLE IV  
*Results of shell-selection tests of naked Pagurus pollicaris and P. longicarpus from two localities: hydroid-colonized shells versus shells without hydroids in the presence of Menippe mercenaria\**

Hermit crab	Locality	No. tested	Time of observation	No. selecting shells		$\chi^2$	P	No. switching shells between 5 min and 8 h, from		Chi-squared test	
				With hydroid	Without hydroid			With hydroid to without hydroid	Without hydroid to with hydroid	$\chi^2$	P
<i>P. pollicaris</i>	FSUML	24	5 min	8	16	2.67	NS	4	0	4.00	<.05
			8 h	4	18	8.91	<.01				(2 killed)
<i>P. pollicaris</i>	St. Joseph Bay	18	5 min	6	12	2.00	NS	0	0		(2 killed)
			8 h	6	8	0.29	NS				
<i>P. pollicaris</i>	Pooled	42	5 min	†				4	0	4.00	<.05
			8 h	†							
<i>P. longicarpus</i>	FSUML	17	5 min	5	12	2.88	NS	2	0	2.00	NS
			8 h	2	12	7.14	<.01				(3 killed)
<i>P. longicarpus</i>	St. Joseph Bay	14	5 min	6	8	0.29	NS	1	0	1.00	NS
			8 h	2	8	3.60	NS (P = .06)				(4 killed)
<i>P. longicarpus</i>	Pooled	31	5 min	11	20	2.61	NS	3	0	3.00	NS
			8 h	4	20	10.67	<.01				

\* "Pooled" means that the results from the two populations of hermit crabs have been combined.

† Pooling inappropriate because of differences in shell selection between the two populations.

TABLE V  
*Results of shell-selection tests of naked Pagurus pollicaris and P. longicarpus from two localities: hydroid-colonized shells versus shells without hydroids in the presence of Octopus joubini\**

Hermit crab	Locality	No. tested	Time of observation	No. selecting shells		$\chi^2$	P	No. switching shells between 5 min and 8 h, from		Chi-squared test	
				With hydroid	Without hydroid			With hydroid to without hydroid	Without hydroid to with hydroid	$\chi^2$	P
<i>P. pollicaris</i>	FSUML	21	5 min 8 h	7 3	14 17	2.33 9.80	NS <.01	3	0	3.00 (1 killed)	NS
<i>P. pollicaris</i>	St. Joseph Bay	18	5 min 8 h	10 8	6 9	1.00 0.06	NS NS	3	0	3.00 (1 killed)	NS
<i>P. pollicaris</i>	Pooled	39	5 min 8 h	† †				6	0	6.00	<.05
<i>P. longicarpus</i>	FSUML	15	5 min 8 h	3 0	9 14	3.00 14.00	NS <.001	3	0	3.00 (1 killed)	NS
<i>P. longicarpus</i>	St. Joseph Bay	21	5 min 8 h	8 3	11 18	0.22 10.71	NS <.01	4	0	4.00	<.05
<i>P. longicarpus</i>	Pooled	36	5 min 8 h	7 3	24 32	9.32 24.01	<.01 <.001	7	0	7.00	<.01

\* "Pooled" means that the results from the two populations of hermit crabs have been combined.

† Pooling inappropriate because of differences in shell selection between the two populations.

## DISCUSSION

When an organism's habitat selection is studied, it is important to consider the factors associated with the habitat that will affect the survival of the organism. In the present study, the effect of a hydroid colony on the shell selection behavior of three species of hermit crabs was investigated. But of what value is a hydroid colony to these or other crabs? Schifjsma (1935) suggested that the hydroid constantly enlarges the volume of the shell by growing outward on the lip of the shell. Grant and Pontier (1973) and Brooks and Mariscal (in prep.) have shown that a hydroid-colonized shell protects *Pagurus* from predatory crabs and octopuses. Grant and Pontier (1973) have also shown that *P. acadianus* inhabiting hydroid-colonized shells were dominant over similar-sized crabs in bare shells in 74% of the trials. In addition, Wright (1973) and Grant and Ulmer (1974) have suggested that competition between sympatric species for shells may be lessened because some species reject shells covered with a hydroid.

Regardless of the proposed advantages of inhabiting hydroid-colonized shells, nearly all of the hermit crabs in the present study chose bare shells under all of the experimental conditions. In fact, if the crabs did choose hydroid-colonized shells after five minutes, they frequently vacated these shells for bare shells during the remainder of the trial, even when predatory crabs and octopuses were present. Interestingly, *O. joubini* activates the anemone-transferring behavior of *P. pollicaris* towards its symbiotic sea anemone, *Calliactis tricolor* (Brooks and Mariscal, in prep.), which provides some protection from octopuses (McLean, 1983). Neither the octopus nor the stone crab, however, stimulated the hermit crabs to select hydroid-colonized shells, which also provide protection (Brooks and Mariscal, in prep.).

One population of *P. pollicaris*, from St. Joseph Bay, chose hydroid-colonized shells more frequently in one trial. Seventy-five percent of the *P. pollicaris* from St. Joseph Bay inhabited hydroid-colonized shells after five minutes in Experiment 1, compared with only 52% of *P. pollicaris* from the FSUML in the same experiment. This difference in shell selection between these two populations corroborates the discovery by Brooks and Mariscal (in prep.) that *P. pollicaris* from St. Joseph Bay are more active in acquiring the sea anemone *C. tricolor* than *P. pollicaris* from the FSUML. These populational differences observed in *P. pollicaris* may be due to differences in selective pressures between the two localities. For example, differences in predation pressure (cf., Bertness, 1982) may affect the behavior of hermit crabs toward the hydroid-colonized shells and possibly account for the shell selection discrepancies reported in the literature for both *P. pollicaris* and *P. longicarpus*. The hermit crabs from St. Joseph Bay inhabit areas with more *O. joubini* than those crabs from the FSUML (pers. obs.). Therefore, the St. Joseph Bay crabs may "prefer" hydroid-colonized shells for protection. This observation does not explain, however, why *P. pollicaris* from St. Joseph Bay generally switched from hydroid-colonized shells to bare shells, even in the presence of *O. joubini*.

Apparently, living in a hydroid-colonized shell has some disadvantages. The most likely disadvantage of inhabiting a hydroid-colonized shell is being stung by the hydroid's nematocysts. Of the three species of hermit crab tested in this study, only *P. pollicaris* appeared unaffected by contact with the hydroid. There were, however, discharged nematocysts on the sensory hairs of *P. pollicaris* before and after their being manually touched to the dactylozoid polyps. Although *P. pollicaris* did not react as if stung, it presumably could still be affected by the discharged nematocysts. Perhaps *P. pollicaris* subsequently switch out of hydroid-colonized shells to avoid the lashing dactylozoid polyps, which appear to contact the hermit crab repeatedly. Wright (1973) reported that four *P. pollicaris* switched from hydroid-colonized shells

to bare shells, but two of the crabs soon returned to their former shells. In the studies by Wright (1973), Conover (1976), and Mercado and Lytle (1980), where *P. pollicaris* preferred hydroid-colonized shells, the selective pressures (e.g., predation or competition for shells) on each population may have been too great for the crabs to vacate their shells normally in favor of bare shells.

Although all three hermit crab species in this study preferred bare shells, if given no other choice they would enter hydroid-colonized shells. Wright (1973) reported that only one *C. vittatus* entered a hydroid-colonized shell and soon began to pick off all of the polyps it could reach until the polyps could not reach the crab. A total of 23 *C. vittatus* entered hydroid-colonized shells in the present study, but none was observed picking at the hydroid colony.

The *P. pollicaris*, *P. longicarpus*, and *C. vittatus* collected in the present study are all sympatric. Wright (1973) has suggested that because *C. vittatus*, a competitive dominant over both *P. pollicaris* and *P. longicarpus*, usually avoids hydroid-colonized shells, competition for shells between these three species is reduced. In the present study, both *C. vittatus* and *P. longicarpus* rejected hydroid-colonized shells more frequently than *P. pollicaris*. The effect of this difference on shell competition between these crabs is unclear. *Clibanarius vittatus* from the FSUML were usually found in *Melongena corona* shells (which rarely have hydroids), whereas similar-sized *Pagurus* from the same localities usually inhabit *Polinices duplicatus* shells, which often bear hydroid colonies (pers. obs.). Therefore, if *C. vittatus* prefers different shell species in this area, then its rejection of hydroid-colonized *P. duplicatus* shells probably has little effect on its interactions with *Pagurus*. *Clibanarius vittatus* from St. Joseph Bay, however, were commonly found in *P. duplicatus* shells, as were similar-sized *Pagurus* (pers. obs.). In St. Joseph Bay, competition for shells between *C. vittatus* and *P. pollicaris* may be reduced because of differences in the selection of hydroid-colonized shells.

The occupation of a hydroid-colonized shell can potentially provide hermit crabs with certain benefits. Predation levels can be minimized, fitness increased, and competition with other shell-seeking hermit crabs reduced. Certain hermit crabs, however, still prefer bare shells to hydroid-colonized shells. Apparently, those hermit crabs that select hydroid-colonized shells are either not stung or tolerant of the hydroid's cnidae. Nonetheless, the preference for hydroid-colonized shells implies that the benefits of inhabiting this shell are greater than the harmful consequences.

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## DIEL VERTICAL MIGRATION AND PHOTORESPONSES OF THE CHAETOGNATH *SAGITTA HISPIDA* CONANT

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### ABSTRACT

In estuarine waters at Beaufort, North Carolina, adult *Sagitta hispida* perform a diel vertical migration. By day, few adult chaetognaths are found in the 7 m water column. Shortly after sunset their numbers increase rapidly at all depths. We hypothesized that the evening ascent is dependent on photoresponses elicited by some aspect of the change in light intensity occurring at sunset. In the laboratory, *S. hispida*'s upswimming increases markedly whenever light intensity drops below  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$ . Animals adapted to light intensities below this level, and to darkness, show strong upswimming. This indicates that continuously decreasing light intensity is not required to maintain the upswimming response. In the field, in the afternoon and evening, downward irradiance values of  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$  are found only at sunset. These findings suggest that the daily ascent of *S. hispida* occurs as an all-or-none phenomenon, dependent only on exposure to light intensities below approximately  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$ . This threshold intensity for ascent lies above the threshold for photoreception in this species, suggesting that the ascent begins as a positive phototaxis. However, experiments in which light direction was reversed indicated that the ascent is geotactic or photokinetic, since changes in light direction had little bearing on the orientation of the upswimming response.

### INTRODUCTION

In surveys of the vertical distribution of zooplankton, chaetognaths are often found to perform a diel vertical migration (Alvarino, 1965). This type of migration is characterized by a twilight or nighttime ascent to shallow levels and daytime descent to greater depths (Hutchinson, 1957). For all zooplankton, light is considered the most important environmental factor involved in the control of diel vertical migration (Forward, 1976).

Partly on the basis of observations on chaetognaths, Michael (1911) and later Russell (1927) proposed that diel vertical migration results as zooplankton move up or down in an effort to maintain their position in some optimal or preferred range of light intensities. Support for this proposal (the preferendum hypothesis) came from studies of the movement of the oceanic deep scattering layer, which sometimes remains within a particular narrow range of light intensities during a diel vertical migration (Boden and Kampa, 1967). More recently, Forward *et al.* (1984) have shown that vertically migrating estuarine crab larvae aggregate during daytime near the depth where the irradiance corresponds to their threshold intensity for phototaxis. During

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sunset, the larvae ascend as this particular irradiance value (isolume) is found higher in the water column.

An alternative hypothesis relating vertical migration and photobehavior was proposed by Clarke (1933) and Ringelberg (1964). They suggested that the initiation of vertical migration depends on the rate and direction of change in light intensity at sunset and sunrise. Evidence in support of this hypothesis came from studies of the photoresponses of the cladoceran *Daphnia*, which accelerates its upswimming movements as a function of the rate of decrease in light intensity (Ringelberg, 1964). A similar relationship holds between *Daphnia*'s downswimming and the rate of increase in light intensity (Daan and Ringelberg, 1969). In addition, Buchanan and Haney (1980) observed that vertical migrations of arctic zooplankton are dependent on the rate of change of light intensity.

Recently, Stearns and Forward (1984) studied the photobehaviors responsible for the vertical migration of the copepod *Acartia tonsa*. Light can act to control, initiate, and/or direct vertical migration (Bainbridge, 1961). For *A. tonsa*, photoresponsiveness is controlled by the level of light adaptation. The initiating cues for vertical movement are the direction and rate of change in light intensity. Light is not used as a directional cue for vertical movements.

The work reported here was undertaken to examine the role of photobehavior in the diel vertical migration of the chaetognath *Sagitta hispida* Conant. Details of the timing of the ascent phase of the migration were determined in field studies. To determine how light might control, initiate, or direct the migratory ascent, the field observations were compared to experimentally elicited photoresponses involving upswimming. The results suggest that *S. hispida*'s vertical migration conforms to the preferendum hypothesis, and light acts only to control vertical movement.

## MATERIALS AND METHODS

### *Field studies*

Chaetognaths were collected from a platform attached to the Piver's Island Bridge at Beaufort, North Carolina. Total depth at this location ranged from 5 to 7 m, depending on the state of the tide. Stratified plankton samples were obtained by suspending opening/closing nets in the tidal flow beneath the bridge at 1, 3, and 6–7 m. Net mouth diameter was 0.5 m, and each net was equipped with a calibrated flowmeter (TSK, Yokohama). The nets were made of 0.5 mm mesh Nytex®, and retained animals >6 mm in length. These chaetognaths were of Stage II or III of Russell's (1932) sexual maturation classification scheme (maturing male and female gonads).

For two field studies, sets of stratified plankton collections were made at intervals of approximately 3 h over the course of two to three days. On three other occasions, 5–7 collections were made at intervals of about 40 minutes immediately prior to and after sunset. Samples were preserved in borate-buffered 5% formalin in sea water.

During each field study, water samples were collected at 1, 3, and 6–7 m, and their temperatures and salinities measured using, respectively, a thermometer and refractometer (American Optical). During the two longer field studies, these measurements accompanied each series of plankton samples. During the short term field studies, temperature and salinity were measured at the beginning, midpoint, and end of each study.

During two of the short-term studies, field light intensity was measured with a submersible radiometer (Kahl, model 268 WA 310). This device was fitted with a

cosine collector, and measured downwelling irradiance. The spectral response of the radiometer was restricted to the region 400–620 nm through insertion of a filter (Corning 4-72). *Sagitta hispida* is most sensitive to light in this region of the visible spectrum, with maximum sensitivity at 500 nm (Sweatt and Forward, 1985). The radiometer was calibrated with a laboratory photometer (EG & G model 550), and the field measurements expressed as photons  $\text{m}^{-2} \cdot \text{s}^{-1}$ . On the evening before the field study of 17 September 1982, light intensity was measured at several depths during sunset in order to document the rates of change in light intensity at the depths where the plankton were to be sampled. During subsequent field studies, irradiance was measured only at the surface, and the values at depth determined through calculations based on the previously determined light profile.

### *Photobehavior experiments*

For experimental studies, *Sagitta hispida* were obtained from the field study site at night, when the animals were most abundant in the water column. Conditions of capture and maintenance of the chaetognaths are described by Sweatt and Forward (1985). Sweatt (1983) found no evidence that *S. hispida*'s vertical migration is controlled by endogenous rhythms in activity or phototaxis. Hence the experiments reported here were designed to assess the tendency of chaetognaths to swim upward following a decrease in light intensity within the range of intensities found in the field near sunset. *S. hispida* is negatively buoyant, and its swimming pattern consists of repetitive head-upwards darting motions, each followed by a period of passive descent. In prolonged darkness and under prolonged overhead light (fluorescent room lights), this behavior causes most of the animals to stay near the top of a vessel. Therefore, to be able to observe unequivocal upswimming responses following changes in light intensity, chaetognaths were initially confined near the bottom of a vessel, and released after light intensity was manipulated. The procedure is described in detail below.

Experiments were performed only with animals which had been light adapted for at least 1.5 h prior to testing. Light sources for adaptation and stimulus presentation were slide projectors, equipped as described by Sweatt and Forward (1985). Light adaptation wavelength was 500 nm, obtained with an interference filter (Ditric Optics; half band width 9 nm). Using a mirror, the adaptation light was directed downward onto the chaetognaths, which were held in groups of 13–15 in 50 ml beakers. Before every experiment, light intensities were measured with a laboratory photometer (EG & G Model 550). The photometer probe was placed on the optical axis of the projector-mirror apparatus, at the level of the beakers, and directly faced the light source.

For experimentation, the projector used to present the test stimulus was equipped with a Corning 4-94 glass filter. This filter provided a near natural spectral distribution for the chaetognaths, in that its maximum photon transmission occurs at 560 nm (half band width 80 nm). This wavelength is close to the spectral transmission maximum for water in the Beaufort area (Sweatt, 1983). The stimulus light beam was collimated with several lenses set on an optical bench, and reflected downward onto the test vessel using a large mirror. All optical components except the mirror were situated behind a light-tight partition, out of view of the test vessel.

The test vessel was a transparent Lucite® chamber divided along its vertical axis into five equivalent sections by a set of removable partitions. The partitions were attached to a handle and could be moved in unison (see Fig. 1). With the partitions withdrawn, the portion of the vessel accessible to the animals was 30 cm high, with

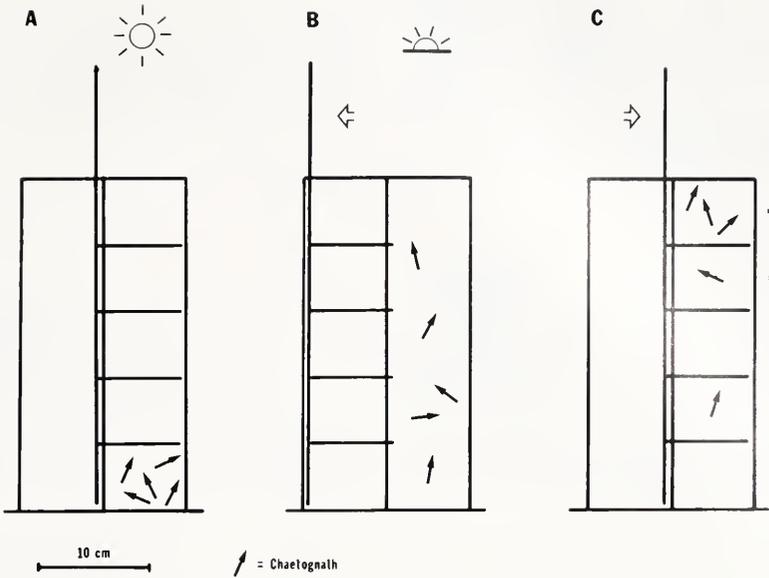


FIGURE 1. Diagram of experimental procedure for tests of upswimming by *S. hispidia*. A. At original adaptation intensity, animals are placed at bottom of chamber, and partitions slid into place. B. Light intensity is manipulated, and, fifteen minutes later, the partitions are withdrawn. C. Three minutes later, partitions are replaced. Those animals found in uppermost two sections of the vessel are considered the percent upswimming.

a cross section  $3 \times 5$  cm. Light intensities were measured as described above except that the probe was placed immediately below the empty chamber.

The basic procedure for the experiments is described here. Details concerning light intensities used in the experiments are given with the results. In performing a test, 13–15 light adapted adult *S. hispidia* were pipetted to the bottom section of the test vessel and the partitions were slid into place. The vessel was then filled with filtered sea water and placed in the stimulus beam, the intensity of which was adjusted to match the original adaptation intensity. Following a one minute pause to allow the animals to recover from the transfer to the test vessel, the intensity of the stimulus beam was reduced by placing a neutral density filter in the light path, or the light source was switched off. In a third test condition, light intensity was not changed from the original adaptation intensity. After a fifteen-minute waiting period at the new light intensity, or in darkness, or at the original adaptation intensity, the partitions of the test vessel were removed in a smooth, gentle motion. Three minutes later, the vertical distribution of the animals was determined (Fig. 1). The proportion of animals swimming in the two uppermost sections of the vessel was recorded as the percent upswimming. The three-minute response period was chosen on the basis of preliminary experiments in which initial upswimming responses tended to be completed within three minutes. For one set of experiments, the configuration of the light source, mirror, and test vessel was altered so that the stimulus was presented from below the animals.

The fifteen-minute waiting period was chosen to ensure that all startle responses were extinguished by the time the partitions were removed. Under highly directional illumination, *S. hispidia* swims very quickly toward the light source in reaction to

mechanical shock or sudden reductions in light intensity. This reaction was first described for *Sagitta crassa* by Goto and Yoshida (1981), who called it target-aiming behavior. It is unlikely that this transient response is related to vertical migration, and it was avoided by pausing between light intensity reduction and withdrawal of the chamber partitions.

## RESULTS

### Field studies

The field studies were planned to control for the possibility that migration is related to tidal phase. Some estuarine zooplankton show a tidal vertical migration pattern (e.g., decapod larvae; Cronin, 1982). Thus in the August 1981 study, the time near sunset (when the animals would likely be migrating) coincided with a rising tide. In the June 1982 study, a falling tide occurred near sunset. During each study temperatures generally fell at night and rose during the day (Fig. 2). The variation in salinity followed the tidal cycle, with lowest salinities occurring at low tide, and highest salinities at high tide (Fig. 2).

Changes in *Sagitta hispida* abundance are shown in Figure 3. Temporal trends in abundance variations were compared between depths by calculating Spearman's rank correlation coefficient (Snedecor and Cochran, 1967). Results showed that there were coherent trends in abundance changes at the three sampling depths in 1981 (correlation coefficients  $\leq 0.850$ ,  $P < .05$ ). In the 1982 study (Fig. 3B), while a general increase in abundance in the water column at night was evident, there were no strongly correlated or statistically significant temporal trends across depth (correlation coef-

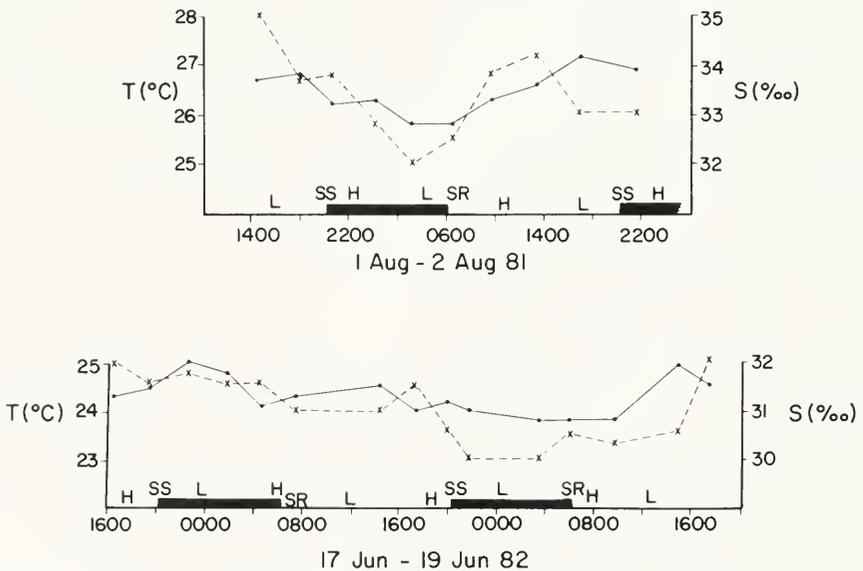


FIGURE 2. Temperature (solid line) and salinity (dashed line) at Piver's Island Bridge, Beaufort, NC, during the studies of diel vertical migration. Values plotted are means of measurements taken at 1, 3, and 6-7 m. There was little variation with depth: T:  $\pm 0.2^{\circ}\text{C}$ ; S:  $\pm 0.4$  ppt. Dark horizontal bar indicates period between sunset (ss) and sunrise (sr). H = high tide; L = low tide.

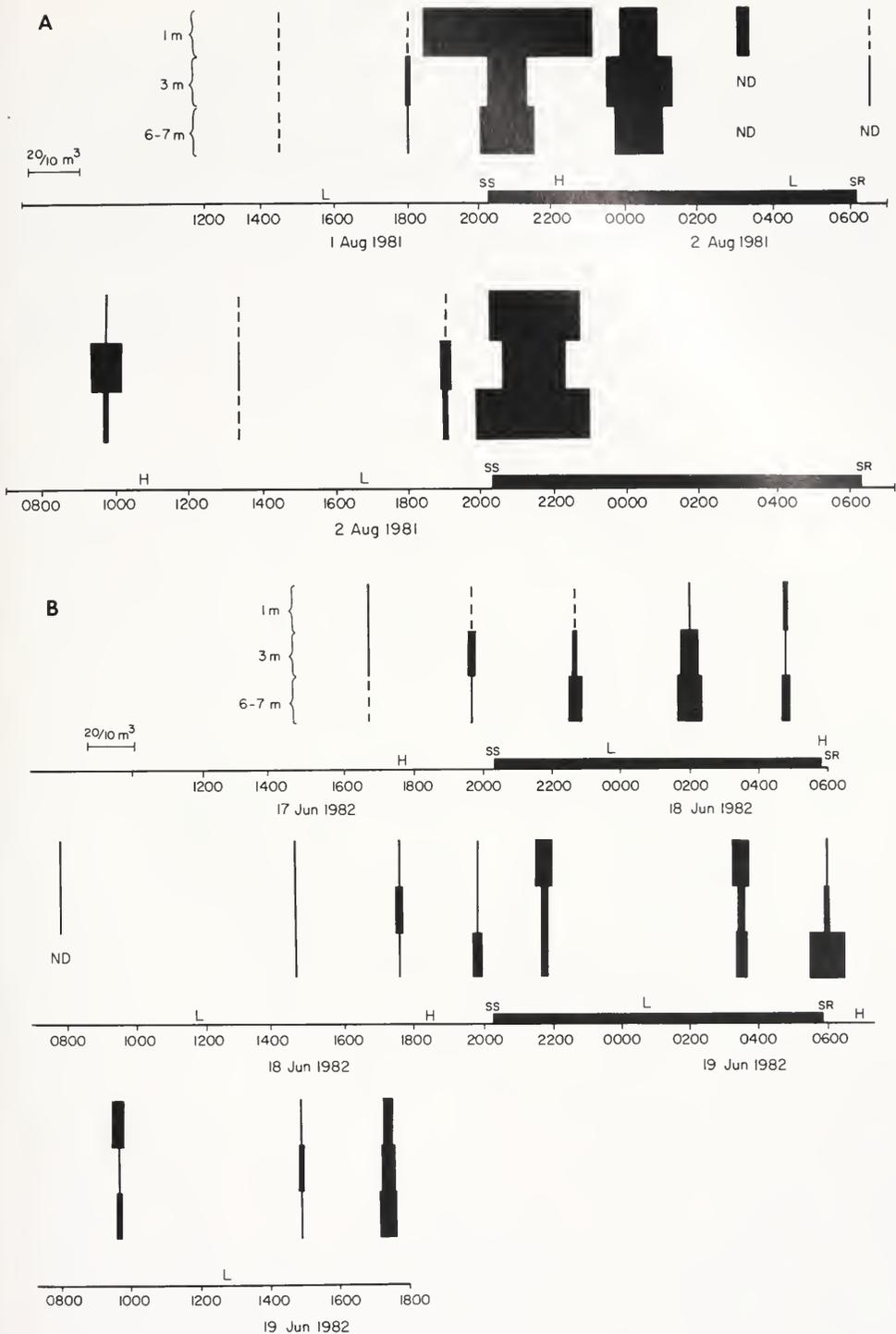


FIGURE 3. Vertical distribution of *Sagitta hispida* at Piver's Island Bridge, Beaufort, NC. A. 1-2 August 1981. B. 17-19 June 1982. For each sampling time, abundance (no./10 m<sup>3</sup>) at 1, 3, and 6-7 m is indicated by rectangles with widths proportional to abundance. Solid vertical lines show abundances < 1/10 m<sup>3</sup>. Dashed vertical lines show abundance = 0. ND = no data. Other symbols as in Figure 2.

ficients  $\leq 0.510$ ). This result may have been due to the fact that, in June 1982, *S. hispida* abundance was generally very low.

The three short-term field studies were also planned so that the chaetognaths were sampled at sunset on both rising and falling tides (Fig. 4). As before, temperature and salinity varied little with depth. Temperatures generally fell after sunset, and salinities rose or fell in correspondence with the phase of the tide. During each study, *S. hispida* abundance increased dramatically at each sampling depth shortly after sunset (Fig. 4).

The daily variations in the abundance of *S. hispida* in the water column indicate that this species performs a vertical migration, and ascends in the evening during both rising and falling tides (Fig. 3). The increase in near-surface abundance at night

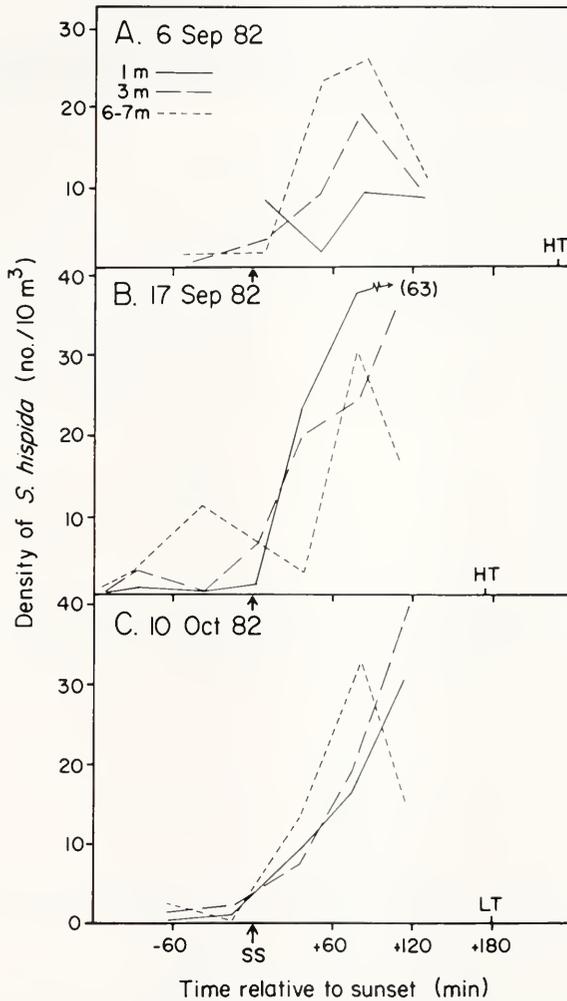


FIGURE 4. Abundance of *Sagitta hispida* at Piver's Island Bridge, Beaufort, NC, during three short term field studies. Values are plotted separately for each sampling depth. ss = time of sunset. HT, LT = times of high and low tide.

is particularly striking in the study of August 1981 (Fig. 3A), and in the short term investigations (Fig. 4).

Figure 5A shows the downward irradiance at several depths during sunset at the study site on the day before the second short term field study (Fig. 4B). Figure 5B shows surface irradiances and extrapolated bottom irradiances at the time of the second and third field studies (Fig. 4B, C). Surface irradiance differs between dates due to variations in cloud cover. On each date, downward irradiance was relatively constant during the late afternoon, and fell nearly simultaneously at all depths during

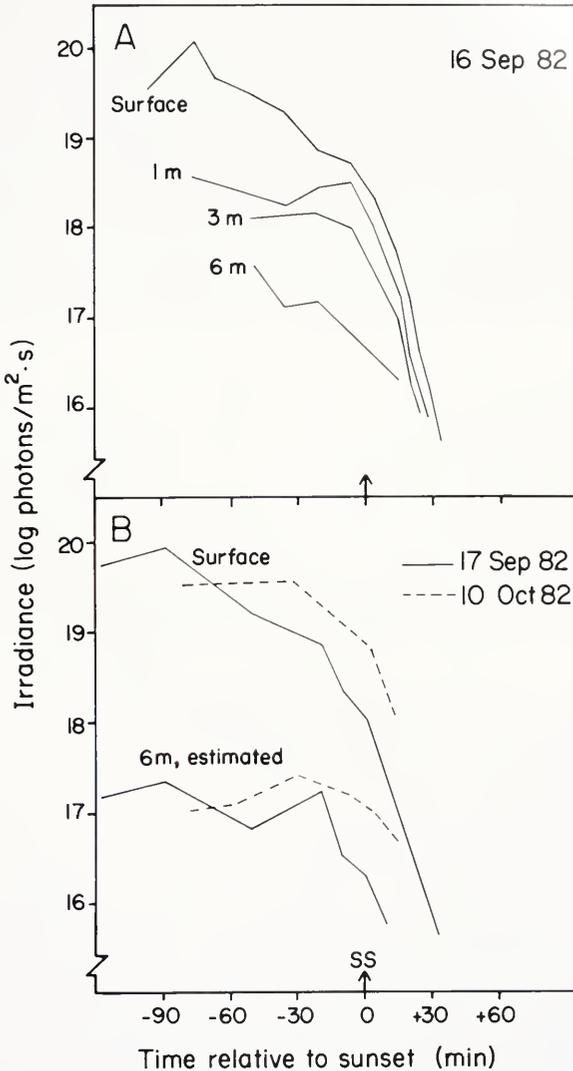


FIGURE 5. Downward irradiance at Piver's Island Bridge, Beaufort, NC. A. Irradiance measured at several depths during sunset, 16 September 1982. B. Irradiance measured at the surface during two of the field studies: 17 September and 10 October 1982. Values plotted for 6–7 m for these data were extrapolated from surface measurements.

sunset. The most rapid decreases in irradiance occurred just after sunset. At sunset, irradiance values over the 7 m water column ranged from  $10^{16}$  to  $10^{19}$  photons  $m^{-2} \cdot s^{-1}$ .

### Photobehavior experiments

In the first series of experiments, upswimming was tested for animals adapted to each of five different light intensities (Fig. 6). Animals were used only once in any experiment. Upswimming was tested at each of the original adaptation intensities (LA), at one log unit below those intensities ( $LA^{-1}$ ), and in darkness (D). Statistical comparisons were made between responses measured under the three test conditions at each adaptation intensity (one-way analysis of variance). In one case, tests were conducted only at the original adaptation intensity and in darkness. In the five sets of tests, upswimming responses were weakest when measured at the original adaptation intensity. Leaving animals in darkness invariably led to a stronger response. Reducing the light intensity to one log unit below the original adaptation intensity had variable effects. At the two highest original adaptation intensities, upswimming following such intensity reductions ( $LA^{-1}$ ) was not significantly different from the response observed with no change in light intensity (LA). However, at two lower adaptation intensities, responses following the one log unit reduction became more like those measured for animals left in darkness (D). This indicates that reducing the intensity by one log unit ( $LA^{-1}$ ) led to marked and significant increases in upswimming (relative to the

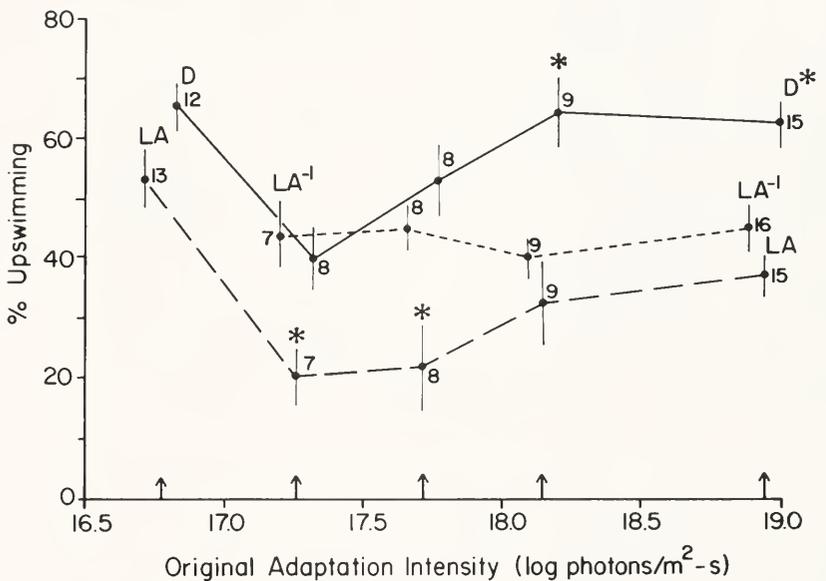


FIGURE 6. Upswimming responses of *Sagitta hispida* adapted to various light intensities. Means and standard errors are shown on the vertical axis. Sample size for each experimental condition is indicated next to mean response level. Ordinate indicates original adaptation intensity. For each set of tests, animals were adapted to the original light intensity for at least 1.5 h prior to being tested under one of three conditions: LA = upswimming measured 15 minutes after animals were placed in test chamber at the original adaptation intensity;  $LA^{-1}$  = after 15 minutes in test chamber at an intensity 1 log unit lower than the original adaptation intensity; D = 15 minutes after being placed in test chamber in darkness. Separate statistical comparisons (ANOVA) were made for data obtained using animals adapted to each of the respective original adaptation intensities. \* Indicates the experimental condition for which upswimming responses were significantly different from the other two conditions ( $P < .05$ ).

LA condition) only when the final light intensity was  $10^{16.72}$  photons  $m^{-2} \cdot s^{-1}$  or lower (Fig. 6). Finally, at the lowest original adaptation intensity ( $10^{16.8}$  photons  $m^{-2} \cdot s^{-1}$ ), upswimming responses in darkness and in light (without intensity reduction) were strong and not significantly different from each other.

In the second series of experiments, upswimming was measured for animals originally adapted at  $10^{17.66}$  photons  $m^{-2} \cdot s^{-1}$ , and irradiated from above or below. Upswimming was tested at the adaptation intensity, and at one log unit below this intensity. This adaptation intensity was chosen because animals had previously shown significantly different responses at a comparable intensity and at one log unit below it (Fig. 6, adaptation intensity  $10^{17.72}$  photons  $m^{-2} \cdot s^{-1}$ ). For comparison with previous experiments, upswimming was also measured for animals kept in darkness.

Results are displayed in Figure 7. As seen in the previous experiment, upswimming increased after light intensity reduction. In darkness, the usual strong upswimming response was observed. No downswimming was observed for animals irradiated from below. The responses of irradiated animals were compared using two-way analysis of variance. Interaction between light intensity and direction was significant at  $P = .046$ . For an interaction with significance at this level, it is still useful to compare main effects. A strong main effect was observed for light intensity alone ( $P < .01$ ) while light direction alone had no significant effect on the upswimming response. Comparison of mean response levels (Bonferroni's multiple comparison) indicated that irradiation at the reduced intensity ( $LA^{-1}$ ) elicited a significantly greater mean response than irradiation at the original adaptation intensity ( $P < .05$ ).

#### DISCUSSION

The nighttime abundance of adult *Sagitta hispida*, coupled with their virtual absence during the day, provides strong evidence that this species performs a diel vertical migration. Animals larger than 6 mm are nearly absent from daytime samples, while their abundance increases markedly at all depths after sunset (Figs. 3, 4). The abundance fluctuations correlate with day-night cycles, and the evening ascent occurs on both rising and falling tides. Figures 2 and 3 show that in the hour after sunset, temperature and salinity change little, while the abundance of *S. hispida* rises sharply. Thus, the post-sunset elevation of chaetognath abundance in the water column is not necessarily associated with movement of a distinct new water mass (with new plankton organisms) into the sampling area.

The short term studies provided further evidence for the close association of sunset with the daily appearance of large numbers of *S. hispida* in the water column (Fig. 4). Migration was again observed on both rising and falling tides. Based on the data collected on 17 September 1982 (Fig. 4B), the upward migration begins no earlier than 10 minutes after sunset (beginning of increases in animals at 3 m). By this time, irradiance near the bottom of the estuary would have fallen below the daytime level of approximately  $10^{17}$  photons  $m^{-2} \cdot s^{-1}$  (Fig. 5).

The most striking aspect of the migration of the larger *S. hispida* is their almost total absence from the water column during the day. Since plankton samples were collected to within 0.5 m of the bottom, and few chaetognaths were found in any daytime samples, it appears that these animals stay very close to the bottom during daylight. Similar observations have been made for *Sagitta elegans*, another chaetognath found in coastal waters (Pearre, 1973; Weinstein, 1973; Sweatt, 1980).

*S. hispida* can attach itself securely to the sides of glass and plastic aquaria (Reeve and Walter, 1972). This species also tolerates prolonged contact with natural substrates, such as sand (pers. obs., A.J.S.). Considering these observations and the vertical distribution data, it seems possible that older *S. hispida* could maintain contact with the bottom of the estuary during the daytime, even in the presence of tidal currents.

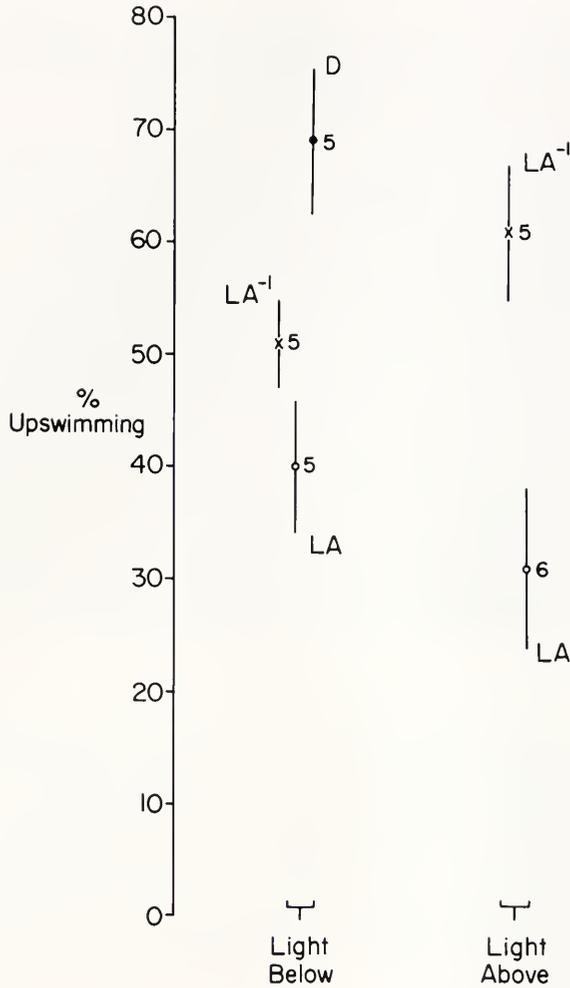


FIGURE 7. Upswimming by *Sagitta hispida* with reversal of light stimulus direction. Means, standard errors, and sample sizes are shown as in Figure 6. Original adaptation intensity was  $10^{17.66}$  photons  $m^{-2} \cdot s^{-1}$  for all tests (see text for rationale). Arbitrary ordinate indicates whether animals were irradiated from above or from below. Other symbols as in Figure 6.

Based on the sampling methods used (horizontal plankton tows) one cannot say whether the continued nighttime appearance of *S. hispida* results from sustained upswimming by individual animals or from rapid fluxes of animals between all depths (Pearre, 1979). Therefore, the behavior which initiates the post-sunset upward movement of *S. hispida* is the only migration-related behavior the direction and timing of which are known with certainty. We hypothesized that this behavior is dependent on photoreception for its initiation.

Light can act to control, initiate, and direct vertical movements of zooplankton (Bainbridge, 1961). Studies have shown that light adaptation level controls responsiveness of zooplankton for migration (Stearns and Forward, 1984), while the actual cue which initiates vertical movement can be a change in light intensity (Ringelberg, 1964; Daan and Ringelberg, 1969; Stearns and Forward, 1984). In addition, overhead

light can act as a directional cue for orienting the animal for swimming upward or downward.

The results of the photobehavior experiments show that reductions of light intensity are followed by increased upswimming by adult *S. hispidus*. Reduction of the light intensity by one log unit elicited enhanced upswimming when the final stimulus intensity was  $10^{16.72}$  photons  $m^{-2} \cdot s^{-1}$  or lower. It is important to note that upswimming was not enhanced following one-log unit reductions which ended at intensities above this level (Fig. 6). For all adaptation intensities, relatively strong upswimming was observed following a decrease in intensity to zero (darkness).

These results support the hypothesis that the ascent phase of the vertical migration of *S. hispidus* involves visual behavior. The type of upswimming response observed following experimental reductions in light intensity could well account for the post-sunset ascent of these animals. In addition, it appears that sustained upswimming by *S. hispidus* does not depend on continuously changing light intensity or on particular rates of change of intensity. This is evident from the experimental results, where upswimming was enhanced even 15 minutes after the light intensity was reduced. Even more salient was the observation of strong upswimming by animals exposed to a constant low light intensity. The lack of a requirement for continuously changing light levels for initiation of ascent was evident in the field data, where the concentration of *S. hispidus* in the water column continues to increase even two hours after sunset (Fig. 4). By this time light intensity at the surface would have reached a constant low value.

At Piver's Island Bridge, adult *S. hispidus* appear to spend the day near the bottom, or at 6–7 m depth. At this depth, light intensities below  $10^{16.72}$  photons  $m^{-2} \cdot s^{-1}$  are reached only at sunset and sunrise (Fig. 5). Based on these field observations and the experimental findings, we proposed that the ascent phase of the vertical migration of *S. hispidus* is controlled by light intensity, and occurs once the intensity falls below a particular level in the evening. This "threshold" light intensity appears to lie near  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$ .

The concept of a threshold for ascent is a variation of the preferendum hypothesis (Michael, 1911; Russell, 1927). It could be predicted that, without depth restrictions, the center of distribution for a population of adult *S. hispidus* should lie near the depth corresponding to the threshold intensity of  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$ . Animals finding themselves much below this depth would tend to ascend, once they were adapted to subthreshold intensity. Those at shallower levels would experience net sinking due to a reduced frequency of upswimming movements upon adaptation to suprathreshold intensities. This prediction should be tested through field studies in deep water.

The threshold effect described for *S. hispidus* is seen in several other animals which exhibit daily behavioral cycles. Dreisig (1980) reported a threshold effect for nocturnally active moths. The beginning of evening flight activity in these animals coincides with the onset of particular low levels of illumination, and is not initiated by the rate of change in light intensity (Dreisig, 1980).

Another example of a threshold effect for evening activity has been shown by Forward *et al.* (1984) for vertically migrating larvae of the crab *Rhithropanopeus harrisi*. Over the course of the day, larvae in the field appear to congregate at depths where the intensity is close to the lower threshold for phototaxis in these animals. Forward *et al.* (1984) also showed that, at suprathreshold intensities, *R. harrisi* larvae sink, while at subthreshold intensities or in darkness, the animals display a negative geotaxis (upswimming).

For the crab larvae, the threshold for ascent coincides with the threshold for phototaxis, or, as determined by Forward *et al.* (1984), the threshold for vision. The

crab larvae may shuttle between depths of perceived light and perceived darkness as they rise and sink near the depth corresponding to their visual threshold. For *S. hispidus*, however, the threshold light intensity for ascent is well above the threshold for dark adapted phototaxis (ascent threshold:  $10^{16.7}$  photons  $m^{-2} \cdot s^{-1}$ ; phototaxis threshold: near  $10^{13.0}$  photons  $m^{-2} \cdot s^{-1}$ ; Sweatt and Forward, 1985). It may be that perception of each threshold is mediated by a different component of the chaetognath visual system. By analogy with the duplex retina of some vertebrates, *S. hispidus* may possess photoreceptors specialized for vision at different light intensities. Morphological evidence for heterogeneity of photoreceptors in *S. hispidus* is presented elsewhere (Sweatt, 1983).

The second set of experiments was designed to test the hypothesis that light direction affects the swimming orientation of *S. hispidus* at intensities below the threshold for ascent. Since *S. hispidus* can perceive light intensities below the threshold for ascent, it may use light as a directional cue during the evening ascent. Thus the ascent might result from positive phototaxis. Alternatively, light direction may be irrelevant to swimming orientation during the ascent. In this case, the ascent could result from negative geotaxis and/or increased activity (photokinesis). Since *S. hispidus*'s normal swimming mode includes active upward movement (followed by passive descent), no attempt was made to experimentally distinguish between geotaxis and photokinesis. However, since *S. hispidus* can attach to surfaces, such as the bottom of the estuary, it is conceivable that the evening ascent could initially involve arousal of animals from immobility. This would be a purely photokinetic effect.

In the relevant experiment, animals were irradiated from above or from below at intensities both greater than and less than the threshold intensity for ascent. Analysis of the results indicated that light intensity and direction may act together to influence the upswimming response, though their statistical interaction is not particularly strong. Ignoring this possible interaction, it was found that light intensity alone has a much greater influence on upswimming than does light direction alone. These results are interpreted to mean that *S. hispidus*'s upswimming response at low light intensities is basically a photokinesis or negative geotaxis, and that a directional light response (phototaxis) is not deeply involved in this behavior. Light intensity apparently serves only to modify the magnitude of the upswimming response, since no downswimming was observed. A similar scheme of light-controlled geotaxis was invoked by Esterly (1919) and Pearre (1973) to explain the migratory ascent of other chaetognaths.

Further evidence against the primary involvement of phototaxis is that *S. hispidus*'s migratory ascent continues into the night in constant light intensities which lie below the visual threshold (Figs. 3, 4). This is consistent with the experimental observation that *S. hispidus* swims upward in darkness. Such behavior was also reported for other chaetognaths by Esterly (1919), Pearre (1973), and Goto and Yoshida (1981). This aspect of chaetognath swimming behavior can only result from orientation with respect to gravity. A likely candidate for gravity perception may be the mechanoreceptive ability of chaetognaths. These animals possess vibration-sensitive hair fans, or setae, distributed over the surface of the body (Feigenbaum, 1978). The hair fans function in prey detection (Feigenbaum and Reeve, 1977), but may also play a part in orienting the body in space. During the sinking phase of *S. hispidus*'s characteristic dart and sink swimming pattern, the mechanoreceptors may be stimulated by the shear field around the animal. This recurrent, predictable stimulus could provide an animal with information as to which way is up, even in the absence of light. Strickler (1982) recently speculated that copepods take advantage of a similar set of circumstances, including a stereotypical swimming pattern and mechanoreception, to determine the direction upward.

## ACKNOWLEDGMENT

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## SPECTRAL SENSITIVITY OF THE CHAETOGNATH *SAGITTA HISPIDA* CONANT

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### ABSTRACT

Using phototaxis as a behavioral measure of photosensitivity, the spectral sensitivity of *Sagitta hispida* Conant (Chaetognatha) was determined. *S. hispida* is most sensitive to blue-green light, with maximum sensitivity at 500 nm. From 400–580 nm, the shape of the action spectrum for phototaxis approximates an absorbance spectrum for a rhodospin-based visual pigment. This suggests that photoreception is mediated largely by a single major pigment. An accessory pigment may play a role in photoreception at longer wavelengths. *S. hispida* is adapted for greatest photosensitivity wherever blue-green light dominates the available spectrum. This finding is consistent with the geographical range of this species, which comprises relatively clear blue-green tropical and subtropical seas.

### INTRODUCTION

At Beaufort, North Carolina, we determined through field studies that the chaetognath *Sagitta hispida* Conant performs a nocturnal diel vertical migration. For adult animals, the migration is characterized by near absence from the 7 m water column by day, and appearance at all depths shortly after sunset (Sweatt and Forward, 1985). It is generally believed that, in performing such vertical migrations, zooplankton are responding to some aspect of changes in light intensity associated with sunset or sunrise (Forward, 1976). It was hypothesized that the vertical migration of *S. hispida* depends on photoreception for its initiation. Accordingly, laboratory studies were undertaken to examine the role that light might play in determining the timing of the ascent phase of the migration.

Initial investigations dealt with the basic photophysiology of *S. hispida*, and we report here our findings concerning the spectral sensitivity of this species. The spectral dependence of variations in phototactic tendency was taken as a measure of photosensitivity. It was found that *S. hispida* is most sensitive to blue-green light. Photoreception appears to be mediated by a rhodopsin-like visual pigment with maximal absorbance near 500 nm. This finding is discussed with reference to the spectral distribution of light in this chaetognath's environment.

### MATERIALS AND METHODS

Chaetognaths were collected in plankton nets suspended in the tidal flow beneath the Piver's Island Bridge at Beaufort, North Carolina. Net mouth diameters ranged from 0.25 to 1.0 m, and all nets were constructed of 0.500 mm Nytex® mesh, which

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retains chaetognaths > 6 mm in length. To minimize damage to the chaetognaths, a large cod end receptacle (4 l capacity) was attached to each net. Collections were made at night, when light sources consisted of distant street lamps and a small red light on the bridge platform. The animals were transported to the laboratory in darkness and not exposed to bright light until the following morning. The day after capture, *S. hispidus* were separated from other zooplankton with the aid of large bore pipettes and a dissection microscope.

In the laboratory, the chaetognaths were kept in aerated 30 l glass aquaria filled with sea water filtered to remove particles larger than 5  $\mu\text{m}$ . Sea water was obtained from the Duke University Marine Laboratory's running sea water system, and its temperature and salinity (19–22°C, 34–36 ppt) were close to the field values at the time of collection (13.5–20.0°C, 33–35 ppt). During experiments, temperature and salinity changes were minimized to avoid eliciting photoresponses or other behaviors which might be related to escape from stressful environments (e.g., positive phototaxis upon exposure to a salinity increase; Forward, 1976).

The aquaria were maintained under a 12L:12D photoperiod (cool white fluorescent lights; light intensity:  $10^{19}$  photons  $\text{m}^{-2} \cdot \text{s}^{-1}$ , measured at the tops of the aquaria). The chaetognaths were supplied daily with food organisms (newly hatched *Artemia salina* nauplii), and placed in new, filtered sea water at least every other day.

Prior to each phototaxis experiment, groups of 20–25 *S. hispidus* were placed in sea water-filled 50 ml beakers. The animals were dark adapted for at least 1.5 h before each experiment. To avoid possible complications in interpretation of results due to endogenous rhythms in activity or photosensitivity, the experiments were performed from 1330 to 1830 h each day.

The experimental light source was a slide projector (Spindler and Sauppe, Model SL-750), equipped with a 300 or 750 W incandescent bulb. Heat was removed from the light with heat filters (Corning, #1-75), and hot mirrors (Baird Atomic, Inc.), while wavelength was controlled by interference filters (6.8–11.5 nm half band width; Ditic Optics, Inc.). Light intensity was regulated with neutral density filters (Ditic Optics, Inc.). The projector was housed in a box such that the projected light exited only from a small aperture.

The test vessel was a horizontal trough, 41  $\times$  8  $\times$  7 cm, constructed of transparent plastic (Lucite®). The long axis of the trough was aligned with the optical axis of the projector. Along its length, the vessel was divided by partitions into five equivalent sections. The partitions were attached to a horizontal cross-piece, and could be moved vertically in unison. Light intensity was measured using a laboratory photometer (EG & G, Model 550). The photometer probe was placed inside the empty vessel, against the end closest to the light source, for measurement.

In performing a test, the vessel was filled with sea water, and the partitions put in place. In darkness, a beaker of dark-adapted *S. hispidus* was then gently immersed in the center section of the trough, rotated to release the chaetognaths, and removed. After pausing for 30 s in darkness to allow the animals to adjust to the chamber, the partitions were gently withdrawn and the light source switched on. Following a three-minute stimulus period, the partitions were replaced, and the distribution of animals among the sections of the vessel was determined. Control experiments were conducted in the same manner, except that the animals were not irradiated. Chaetognaths found in the section of the test chamber closest to the light source were considered positively phototactic, in that they swam at least 8 cm toward the light source. Those in the distal section of the chamber were considered negatively phototactic. For each test performed, the percent of animals exhibiting positive or negative phototaxis was determined. The three-minute stimulus period was chosen so that over the range of

stimulus strengths employed, the animals would exhibit both saturated and control level phototactic responses.

The stimulus-response function for positive phototaxis was determined at 15 wavelengths spaced at 20 nm intervals over the region 400–680 nm. The data were used to determine an action spectrum for phototaxis. Details of the calculation of the action spectrum are given with the results.

## RESULTS

*S. hispida* displayed only positive phototaxis in these experiments. Negative phototaxis rarely exceeded the 10% level, with a mean negative control response of 7.2% (SEM: 0.7). The swimming pattern during phototaxis was the characteristic dart-and-sink motion described for *S. hispida* by Feigenbaum and Reeve (1977), and for *S. crassa* by Goto and Yoshida (1981, 1983). Quick target-aiming behavior, a type of light adapted startle response described by Goto and Yoshida (1981), was not observed in these experiments, but has been seen in tests with light adapted *S. hispida* (Sweatt, 1983).

As data were accumulated, plots of percent phototaxis *versus* stimulus intensity indicated that a roughly hyperbolic stimulus-response relationship held for positive phototaxis. Over the lower part of the range of stimulus intensities employed, responses at each wavelength were generally below 10%. This level of responsiveness was little different from that seen in dark control experiments (Mean: 3.9% SEM: 0.5). As stimulus intensity was increased at each wavelength, responses rose sharply between 10% and 50% phototaxis, and leveled off at about 50%. In order to accurately characterize the relationship between stimulus intensity and response strength, subsequent tests were performed at stimulus intensities in the range which elicited responses lying in the rising portion of the hyperbola (*i.e.*, 10–50% phototaxis). Figure 1 shows, for

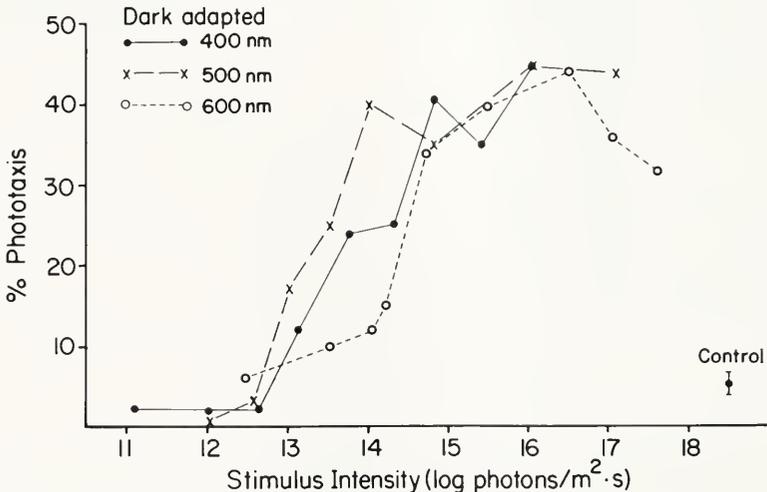


FIGURE 1. Representative stimulus-response functions for dark adapted positive phototaxis by *S. hispida*. % Phototaxis is the proportion of animals swimming a distance of at least 8 cm toward a collimated light source within a three minute period. Stimulus light intensity is expressed in log units. Each point represents the mean of three tests. For clarity, standard errors were omitted from the plot. Control responses were determined by performing phototaxis tests in darkness. Control level and standard error based on 60 tests.

three representative wavelengths, stimulus-response functions for responses lying below 50% phototaxis.

The spectral dependence of the phototactic response can be displayed by an action spectrum. The action spectrum is determined by calculation of the quantal flux necessary at each wavelength to elicit a response of a given magnitude. This method provides a measure of spectral dependence which depends only on the number of quanta absorbed by the system under study, and is not affected by the choice of response (Rodieck, 1973). Thus, it is possible to compare a behaviorally determined action spectrum with, for example, an absorbance spectrum for a photopigment.

To determine the action spectrum for phototaxis by *S. hispidus*, the percent response data were subjected to arcsine transformation (Sokal and Rohlf, 1981) and a linear regression was fitted to the points lying in the steeply rising portion of the stimulus-response function for each test wavelength (*i.e.*, for all responses between 10% and 50% phototaxis). The regression technique provided a method for objectively describing and comparing the stimulus-response functions. Analysis of covariance (Snedecor and Cochran, 1967) revealed that the slopes of the 15 regression lines were not significantly different from each other, indicating that the shape of the stimulus-response function was essentially the same at all test wavelengths. Regression line intercepts differed significantly ( $P < .01$ ), an indication of spectral variation in the sensitivity of the phototactic response. The 30% phototaxis response was chosen as the criterion response for the action spectrum, as this value lies at the midpoint of the response range used to fit each linear regression. For each test wavelength, the stimulus intensity necessary to elicit a 30% phototactic response was estimated from the appropriate regression equation. The reciprocal of this quantity was then plotted, on a relative scale, against wavelength (Fig. 2).

The action spectrum shows that, based on phototactic responsiveness, *S. hispidus* is most sensitive to blue-green light, with maximum sensitivity at 500 nm. Sensitivity at wavelengths above 620 nm was an order of magnitude lower than the lowest sensitivity shown in Figure 2. Included in Figure 2 is an absorbance spectrum for visual pigment having maximum absorbance at 500 nm. This curve was calculated from a nomogram based on the characteristic shapes of absorbance spectra for rhodopsin-based visual pigments (Dartnall, 1953; Ebrey and Honig, 1977). The absorbance spectrum approximates the action spectrum from 400–520 nm, but deviates from it at longer wavelengths.

## DISCUSSION

Positive phototaxis has been reported for chaetognaths by Esterly (1919) and Pearre (1973). Esterly's observations were basically anecdotal, in that no dark control experiments were performed, and the animals (*Sagitta eunertica*; Alvarino, 1965) could initially swim only toward the light sources. In work with *Sagitta elegans*, Pearre (1973) included proper controls, and allowed animals to swim either toward or away from a light source. He reported that 58.9% of the animals swam toward the light, while 44.7% swam away from the light in a horizontal tank. The apparent weakness of the positive phototactic response may have been due to use of a stimulus period of 20 minutes, which may have obscured initially strong phototactic responses. The unequivocal positive phototaxis reported here for *S. hispidus* appears to be comparable to that described for dark adapted *Sagitta crassa* by Goto and Yoshida (1981, 1983). Both of these species were tested using relatively short stimulus periods (3 minutes and less than 10 minutes, respectively).

*S. hispidus*'s response was useful as a measure of photosensitivity, and allowed

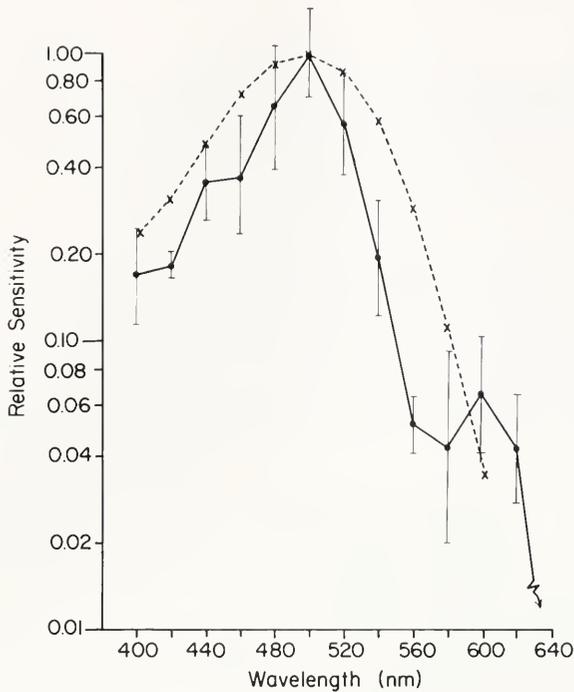


FIGURE 2. Action spectrum for positive phototaxis by *S. hispidia*. For each wavelength the reciprocal of the quantal flux required to elicit 30% phototaxis was calculated. Each value was then divided by the value obtained at 500 nm, where sensitivity was maximal. This quotient is shown as the relative sensitivity. Error bars were calculated similarly, based on the standard error of the 30% phototactic response at each wavelength. Dashed line shows an absorbance spectrum calculated from a nomogram for a visual pigment having maximum absorbance at 500 nm.

the construction of an action spectrum for phototaxis (Fig. 2). The action spectrum shows that *S. hispidia* is most sensitive to blue-green light, with maximum sensitivity at 500 nm. In Figure 2, the shape of the action spectrum corresponds roughly with the absorbance spectrum of a rhodopsin-based visual pigment with maximum absorbance at 500 nm (Ebrey and Honig, 1977). This may be interpreted as evidence that *S. hispidia* possesses a visual pigment which has an absorbance maximum near 500 nm. The small shoulder in the action spectrum at 600–620 nm could indicate the presence of another visual pigment, with an absorbance maximum near 600 nm.

Experimental evidence suggests that chaetognaths are not visual predators (Reeve, 1964; Feigenbaum and Reeve, 1977). It is probable that chaetognath photoreception is mainly concerned with light dependent orientation behaviors, which could include vertical migration. Among zooplankton, light intensity is considered the most important environmental cue involved in vertical migration (Forward, 1976). If chaetognath photoreceptors are primarily used during vertical migration, then it could be expected that their spectral sensitivity should match the spectrum of available light in their underwater environment. Alternatively, a spectral sensitivity maximum which is offset from the environmental spectral transmission maximum may signify an adaptation to enhance contrast sensitivity, which might be useful in object recognition (Lythgoe, 1966; Forward and Cronin, 1979).

In the open ocean, the median of the photon spectral transmission function lies at 470 nm (McFarland and Munz, 1975). Accordingly, open ocean zooplankton which undergo vertical migration frequently have visual pigments with main absorption maxima in the region 460–495 nm (Forward, 1976). Inshore, higher concentrations of phytoplankton, detritus, and complex organic molecules shift the spectral transmission maximum to longer wavelengths (>500 nm). For example, in the estuary where *S. hispidus* was collected, the photon transmission maximum lies at 575 nm (Sweatt, 1983). The absorbance maxima of the visual pigments of many coastal and estuarine zooplankton are in the region 500–600 nm (e.g., Stearns and Forward, 1984). *S. hispidus*, with maximum photosensitivity at 500 nm, could be considered to be better adapted to open ocean spectral environments than to estuaries.

The geographical range of *S. hispidus* comprises the tropical and subtropical eastern Atlantic (Alvarino, 1965). Throughout most of this region, but especially offshore, this species is more likely to encounter clear blue water than the greenish yellow waters characteristic of temperate areas (Smith, 1974). However, the shape of the action spectrum for phototaxis by *S. hispidus* suggests the presence of a second visual pigment, with maximum absorbance near 600 nm. Such an accessory pigment may provide for an increase in photosensitivity in estuarine waters, where much of the available light lies at longer wavelengths.

Thus the spectral sensitivity of *S. hispidus* seems to be adapted to available light. This agreement suggests that vision could be involved in vertical migration. The roles of phototaxis and vertically oriented swimming in the diel vertical migration of *S. hispidus* are addressed in a separate publication (Sweatt and Forward, 1985).

#### ACKNOWLEDGMENT

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INTRA-ORGAN BIOCHEMICAL TRANSFORMATIONS ASSOCIATED WITH OOGENESIS IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS CONCENTRICUS* (SAY), AS INDICATED BY  $^{14}\text{C}$  INCORPORATION

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ABSTRACT

Incorporation of  $^{14}\text{C}$  into lipid, carbohydrate, and protein fractions of digestive gland, adductor muscle, and ovary body components of the bay scallop, *Argopecten irradians concentricus* (Say), varied seasonally in conjunction with oogenesis. Resting stage scallops (June–July) were characterized by net radiocarbon losses in ovary fractions. Nutrient storage during this period was indicated by relatively small radiocarbon losses in digestive gland fractions that were equaled by gains in adductor muscle carbohydrate and protein fractions. During the period of oocyte growth (August–October)  $^{14}\text{C}$  losses in digestive gland and adductor muscle fractions always exceeded progressive gains in the ovary. Increased carbon turnover (catabolism) of digestive gland lipid and adductor muscle carbohydrate fractions accompanied decreased turnover (anabolism) of ovary lipid, indicating the utilization of these reserves for the production of ova. After spawning (November),  $^{14}\text{C}$  was lost from all body component fractions, indicating a state of negative energy balance and generally poor physiological condition. These results directly reinforce the pattern of energy storage and utilization in *A. irradians concentricus* indicated by previous studies on growth, biochemical composition, and substrate catabolism.

INTRODUCTION

Gametogenesis in marine bivalve molluscs is an energetically expensive process. Nucleic acids are required in the male for sperm production, and lipid and protein are accumulated in developing eggs in the female. These energetic demands are met from incoming food, stored reserves, or a combination of both (Gabbott, 1975; Bayne, 1976; Sastry, 1979).

Seasonal changes in body component weights, biochemical compositions, and physiological indexes (O/N, RQ) provide indirect information regarding the ways in which lipid, carbohydrate, and protein fuels are used in gamete production. Periodically monitoring the uptake and distribution of a radiotracer within the body of an animal, however, can provide direct information regarding the storage and utilization of specific nutrient pools in response to gamete development.

The use of radiotracers in the investigation of bivalve reproductive energy metabolism has been limited. The most comprehensive study involved the seasonal distribution of  $^{14}\text{C}$  and  $^{32}\text{P}$  in acid soluble, lipid, and protein fractions of several body components of *Mytilus edulis* (Thompson, 1972). The translocation of radiolabel from digestive gland to gonad in conjunction with reproductive development was demonstrated for the scallops *Argopecten irradians* (Sastry and Blake, 1971) and

*Chlamys hericia* (Vassallo, 1973). Allen (1962, 1970) studied the incorporation and release of  $^{32}\text{P}$  into tissues of several bivalve species.

The bay scallop, *Argopecten irradians concentricus* (Say), is a functional hermaphrodite that in Florida undergoes one complete reproductive cycle in a 12–18 month life span; gametogenesis is initiated in July and spawning commences in October (Barber and Blake, 1983). Investigation into bay scallop reproductive energy metabolism has revealed that energy stored in the digestive gland and adductor muscle prior to the initiation of gametogenesis is subsequently utilized to offset the cost of reproduction (Barber and Blake, 1981, 1983, and unpubl. data). In the present study, incorporation of  $^{14}\text{C}$  into lipid, carbohydrate, and protein fractions of digestive gland, adductor muscle, and ovary body components of *A. irradians concentricus* was monitored monthly to characterize the intra-organ biochemical transformations associated with reproduction.

#### MATERIALS AND METHODS

Scallops of the 1983 year class were hand collected by divers at monthly intervals between June and November from the Anclote Estuary, Tarpon Springs, Florida. Upon return to the laboratory, fouling organisms were removed and scallops were placed in aquaria containing water obtained at the time of collection, adjusted to environmental temperature ( $\pm 1^\circ\text{C}$ ), which ranged from 21.5 to 31.7°C. Six scallops were dissected to determine mean wet weights (WW) and dry weights (DW) of the three body components.

The next day 24 scallops were transferred to a separate feeding tank containing 20 l water adjusted to environmental temperature. *Tetraselmis sp.* culture ( $1.5 \text{ l}$  containing  $1.5 \times 10^6$  cells  $\text{ml}^{-1}$ ) which had been inoculated 24 h previously with 140  $\mu\text{Ci}$  sodium ( $^{14}\text{C}$ ) bicarbonate (Amersham Corp.) was centrifuged and resuspended to remove unincorporated  $^{14}\text{C}$ . This was then added with a peristaltic pump to the feeding tank over a six hour period. Pseudofeces production was negligible, indicating complete ingestion of the radiolabeled cells. After feeding, the scallops were returned to the holding tank where they were fed non-radioactive *Tetraselmis sp.* at the rate of 100 ml animal $^{-1}$  day $^{-1}$  for the duration of the experiment.

Six scallops were sacrificed 1, 4, 7, and 10 days after ingestion of the radiolabel, and 0.20 g (WW) portions of the ovary, digestive gland, and adductor muscle were removed. Each body component piece was then homogenized with a tissue grinder in 5 ml of 2:1 chloroform:methanol in a test tube. NaCl (1 ml 0.9%) was added to each test tube, followed by thorough mixing. After separating overnight in a refrigerator, the lower phase (lipid fraction) was removed with a Pasteur pipette and transferred to a tared scintillation vial. After evaporating the solvent, vials were reweighed to obtain mg (DW) lipid. After lipid phase removal, 1 ml 20% TCA was added to the 3 ml of solution left in each of the test tubes to make a 5% TCA solution. The tubes were then heated in a boiling water bath for 30 min, cooled, and centrifuged. The supernatant (carbohydrate fraction) was poured (along with a distilled water rinse) into a scintillation vial and evaporated to dryness. The precipitate (protein fraction) was rinsed with distilled water into a tared scintillation vial, evaporated to dryness, and reweighed to obtain mg (DW) protein. Carbohydrate (mgDW) was derived from the difference between calculated total dry tissue weight (based on mean DW/WW relationships) and the sum of lipid and protein weights.

Each of the biochemical fractions was solubilized in the scintillation vial with 1 ml tissue solubilizer (NCS, Amersham Corp.). 10 ml organic counting scintillant (OCS, Amersham Corp.) was added to each vial. The radioactivity in each vial was

counted in an Isocap 300 liquid scintillation counter (Nuclear-Chicago Corp.). Corrections for tissue color quenching were applied, and the results were expressed as counts per minute (CPM)  $\text{mgDW}^{-1}$  for each of the body component fractions. Total CPM for a particular body component was obtained by adding its respective lipid, carbohydrate, and protein fraction counts.

Differences in  $^{14}\text{C}$  incorporation (CPM) by the three body components and their respective biochemical fractions as a function of time were analyzed statistically using single factor analysis of variance and the Duncan new multiple range test (Steel and Torrie, 1960).

## RESULTS

Scallop body component CPM  $\text{mgDW}^{-1}$  on Days 1, 4, 7, and 10 are given in Table I. Statistical analysis revealed that digestive gland CPM decreased significantly ( $P < 0.05$ ) between Day 1 and Day 4 in all months. Ovary CPM increased significantly ( $P < 0.05$ ) between Days 1 and 4 in October but decreased significantly ( $P < 0.05$ ) between Days 4 and 7 in all months. Adductor muscle CPM increased significantly ( $P < 0.05$ ) between Day 1 and Day 4 in June and July but decreased significantly ( $P < 0.05$ ) in all months between Days 1 and 4 (September and November) or Days 4 and 7 (June, July, August, and October). Thus,  $^{14}\text{C}$  assimilation and subsequent biochemical transformation took place within 4 days of radiolabel ingestion, and only Day 1 and Day 4 data were considered further.

TABLE I

CPM  $\text{mgDW}^{-1}$  ( $\pm 1$  S.D.) for bay scallop digestive gland, adductor muscle, and ovary body components 1, 4, 7, and 10 days after ingesting radioactive cells

Month	Day 1	Day 4	Day 7	Day 10
Digestive gland				
June	1293 $\pm$ 190	928 $\pm$ 263	423 $\pm$ 187	353 $\pm$ 131
July	457 $\pm$ 44	349 $\pm$ 105	195 $\pm$ 16	95 $\pm$ 26
Aug.	678 $\pm$ 177	210 $\pm$ 75	103 $\pm$ 22	70 $\pm$ 42
Sept.	1197 $\pm$ 431	399 $\pm$ 109	196 $\pm$ 47	133 $\pm$ 27
Oct.	1444 $\pm$ 407	534 $\pm$ 54	253 $\pm$ 60	231 $\pm$ 55
Nov.	1797 $\pm$ 941	527 $\pm$ 155	299 $\pm$ 80	221 $\pm$ 55
Adductor muscle				
June	143 $\pm$ 80	500 $\pm$ 83	220 $\pm$ 88	204 $\pm$ 88
July	52 $\pm$ 22	187 $\pm$ 75	99 $\pm$ 20	62 $\pm$ 35
Aug.	163 $\pm$ 40	120 $\pm$ 26	121 $\pm$ 53	106 $\pm$ 31
Sept.	337 $\pm$ 172	147 $\pm$ 42	247 $\pm$ 49	144 $\pm$ 45
Oct.	419 $\pm$ 49	379 $\pm$ 52	229 $\pm$ 77	251 $\pm$ 52
Nov.	2940 $\pm$ 1911	1223 $\pm$ 840	482 $\pm$ 282	565 $\pm$ 263
Ovary				
June	2220 $\pm$ 654	2099 $\pm$ 637	1327 $\pm$ 710	699 $\pm$ 234
July	1337 $\pm$ 270	1282 $\pm$ 319	679 $\pm$ 293	442 $\pm$ 129
Aug.	929 $\pm$ 270	978 $\pm$ 16	427 $\pm$ 99	313 $\pm$ 87
Sept.	1158 $\pm$ 522	1469 $\pm$ 426	602 $\pm$ 97	455 $\pm$ 128
Oct.	1219 $\pm$ 530	1884 $\pm$ 394	1150 $\pm$ 230	638 $\pm$ 187
Nov.	4682 $\pm$ 3432	3025 $\pm$ 2931	2220 $\pm$ 1077	1301 $\pm$ 302

Comparison of CPM data between months for any body component or biochemical fraction was prevented by apparent differences in overall  $^{14}\text{C}$  uptake efficiency of the food source and/or scallops. Within a month, the digestive gland and/or ovary had significantly ( $P < 0.05$ ) higher Day 1 CPM than the adductor muscle in all months except November, when adductor muscle CPM were almost double those of the digestive gland. In all months except September and October, ovary CPM were higher than digestive gland CPM on Day 1, probably because scallop intestine is intertwined throughout the ovary and was not excluded completely. By Day 4, due to the aforementioned  $^{14}\text{C}$  losses in digestive gland and/or gains in ovary CPM related to reproduction, ovary CPM were significantly ( $P < 0.05$ ) greater than digestive gland CPM in all months. Also on Day 4, adductor muscle CPM were not statistically different from digestive gland CPM.

In order to compare trends in  $^{14}\text{C}$  incorporation within the body components and their respective biochemical fractions over the reproductive cycle, results were expressed as the difference in mean CPM  $\text{mgDW}^{-1}$  between Day 4 and Day 1, divided by 3 (to get the rate of change or slope). A positive slope thus indicated a net gain in radiolabel (relatively low turnover) and a negative slope indicated a net loss of radiolabel (relatively high turnover). A log transformation was performed on the slopes for plotting purposes.

Figure 1 illustrates the dynamics of  $^{14}\text{C}$  incorporation by the three body components over the reproductive cycle. In June and July,  $^{14}\text{C}$  losses between Day 1 and Day 4 in the ovary and digestive gland were offset by gains in the adductor muscle. Turnover started to increase in August as oogenesis commenced. By November, after spawning had occurred,  $^{14}\text{C}$  was being lost from all components very rapidly.

The digestive gland exhibited  $^{14}\text{C}$  loss between Day 1 and Day 4 (*i.e.*, the slope was negative) for every month sampled, as assimilated carbon was transferred to other

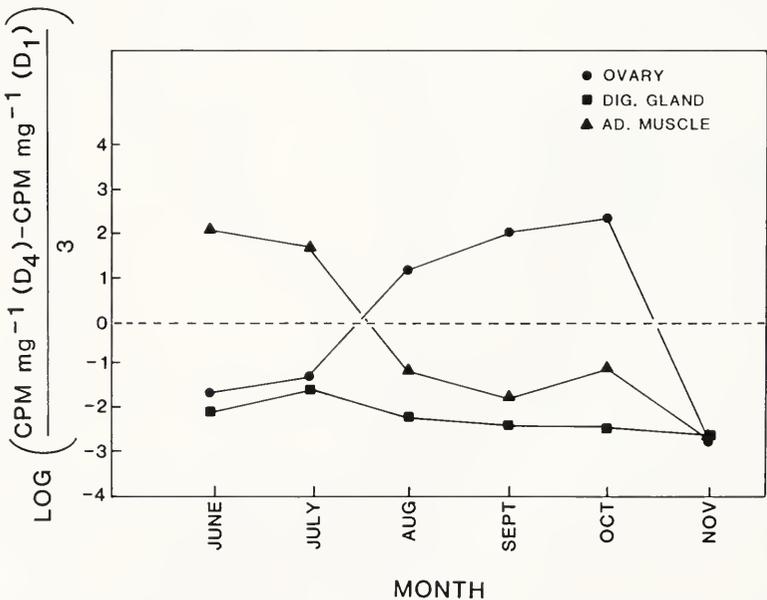


FIGURE 1. Relative seasonal incorporation of  $^{14}\text{C}$  into ovary, digestive gland, and adductor muscle body components of *A. irradians concentricus*.

body components. This loss was less in June and July than it was from July through November.

Adductor muscle gains in  $^{14}\text{C}$  in June and July were inversely correlated to digestive gland and ovary losses. Between July and August, as the ovary slope went from negative to positive, the adductor muscle slope went from positive to negative. The slope remained negative in September and October samples, but became much more negative in November.

The ovary showed a loss of  $^{14}\text{C}$  in June and July, as reflected by negative slopes. By August, a slight gain was found, which increased considerably in September and again in October when a maximum gain was seen. In November, a major loss of  $^{14}\text{C}$  occurred between Day 1 and Day 4.

Lipid, carbohydrate, and protein (CPM  $\text{mgDW}^{-1}$ ) in the three body components on Days 1 and 4 are given in Table II. For the digestive gland, the carbohydrate fraction contained the greatest CPM on both Day 1 and Day 4 in June and July, being significantly ( $P < 0.05$ ) greater than both lipid and protein fractions in all cases except Day 1 of July. The lipid fraction contained the second highest level of radioactivity on Day 1 of June and Days 1 and 4 of July. This trend was reversed from August through November when the lipid fraction contained more CPM than the carbohydrate fraction on both Days 1 and 4. These differences were significant ( $P < 0.05$ ) for all months on Day 1, but only for September and November on Day 4. The digestive gland protein fraction always contained the fewest CPM, and in most cases was significantly ( $P < 0.05$ ) lower in radioactivity than the other two fractions.

TABLE II

CPM  $\text{mgDW}^{-1}$  lipid, carbohydrate, and protein for bay scallop digestive gland (DG), adductor muscle (AM), and ovary (OV) body components 1 and 4 days after ingesting radioactive cells

Month	Day 1			Day 4		
	Lipid	Carbo.	Protein	Lipid	Carbo.	Protein
June						
DG	1002 ± 907	3544 ± 4627	775 ± 225	186 ± 106	5004 ± 1317	750 ± 247
AM	296 ± 140	33 ± 15	96 ± 39	226 ± 139	1459 ± 608	194 ± 82
OV	4376 ± 1621	2076 ± 821	1762 ± 395	2536 ± 1255	3515 ± 1275	1341 ± 551
July						
DG	1071 ± 714	2186 ± 1017	80 ± 54	688 ± 303	1829 ± 715	176 ± 83
AM	182 ± 96	438 ± 284	12 ± 7	194 ± 63	650 ± 342	59 ± 32
OV	1760 ± 910	1928 ± 949	546 ± 299	1488 ± 642	1432 ± 502	833 ± 373
Aug.						
DG	2913 ± 734	1020 ± 319	142 ± 34	570 ± 132	422 ± 143	73 ± 41
AM	196 ± 44	1198 ± 395	26 ± 11	126 ± 51	894 ± 163	35 ± 14
OV	1928 ± 508	1142 ± 384	554 ± 201	2546 ± 661	772 ± 210	486 ± 182
Sept.						
DG	4230 ± 714	1247 ± 431	612 ± 298	1003 ± 277	380 ± 98	261 ± 79
AM	279 ± 117	1881 ± 1027	85 ± 38	139 ± 44	769 ± 28	48 ± 20
OV	2450 ± 1353	1260 ± 595	1198 ± 616	3749 ± 3048	783 ± 426	909 ± 306
Oct.						
DG	4291 ± 1241	2006 ± 569	410 ± 154	1021 ± 87	869 ± 125	315 ± 61
AM	571 ± 211	9043 ± 1766	76 ± 22	468 ± 121	8568 ± 1803	137 ± 40
OV	2840 ± 1233	1678 ± 1481	1972 ± 1549	5507 ± 1328	1080 ± 209	1924 ± 424
Nov.						
DG	6204 ± 1946	2271 ± 1399	317 ± 168	1634 ± 526	556 ± 201	282 ± 74
AM	566 ± 203	32104 ± 18824	339 ± 243	415 ± 192	9878 ± 3471	424 ± 301
OV	7559 ± 1982	17574 ± 6403	2768 ± 1099	2881 ± 1063	2675 ± 187	884 ± 326

The adductor muscle carbohydrate fraction contained significantly ( $P < 0.05$ ) more CPM than the lipid and protein fractions for both Day 1 and Day 4 in all months except June, when Day 1 scallops had significantly ( $P < 0.05$ ) more CPM in the lipid fraction than the carbohydrate fraction. The lipid fraction had the second greatest CPM at all times except for the Day 1 June sample and the November Day 4 sample when protein CPM exceeded the lipid CPM. These differences were significant ( $P < 0.05$ ) only in the June and July Day 1 samples. Otherwise, the protein fraction contained the fewest CPM within the adductor muscle.

For the ovary, CPM were significantly ( $P < 0.05$ ) greater in the lipid fraction than both carbohydrate and protein fractions in all months except July (Day 1) and June, July, and November (Day 4). The carbohydrate fraction contained the most CPM when the lipid fraction did not, although the differences were not statistically significant. The protein fraction contained the fewest CPM at all times except in September (Day 4) and in October (Day 1 and Day 4) when it exceeded, but was not significantly greater than the carbohydrate fraction.

Relative rates of carbon turnover in the biochemical fractions of the three body components over the course of the study are shown in Figures 2, 3, and 4. Digestive gland lipid activity was lost quite rapidly over the whole study period, with the rate of loss increasing after the July sample (Fig. 2). Radioactivity increased in digestive gland carbohydrate in June, but decreased between Day 1 and Day 4 in succeeding months. Protein was the least dynamic of the digestive gland substrates, showing a slight increase in slope in July but minor decreases in all other months.

For the adductor muscle (Fig. 3), the lipid fraction recorded a small gain in radiolabel in July, but losses in all other months. Adductor muscle carbohydrate  $^{14}\text{C}$  incorporation showed a seasonal pattern in which decreasing gains were found in June and July and increasing losses occurred from August through November. Adductor muscle protein was the least variable, with positive slopes in all months but September, when a slight loss was noted.

For the ovary (Fig. 4), the lipid fraction showed a seasonal trend, with radiolabel loss between Day 1 and Day 4 being greatest in June but lower in July. In August, September, and October an increase in gain in radiocarbon lipid was followed by a

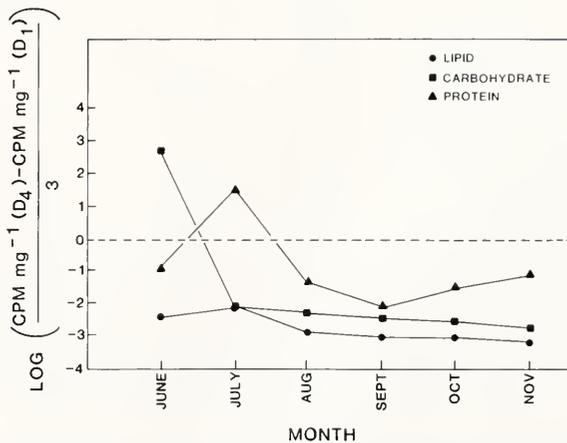


FIGURE 2. Relative seasonal incorporation of  $^{14}\text{C}$  into lipid, carbohydrate, and protein fractions of *A. irradians concentricus* digestive gland.

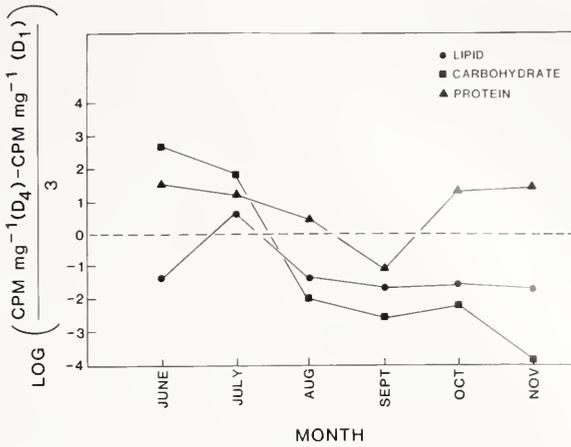


FIGURE 3. Relative seasonal incorporation of  $^{14}\text{C}$  into lipid, carbohydrate, and protein fractions of *A. irradians concentricus* adductor muscle.

drastic loss in November. The carbohydrate fraction showed a gain in radioactivity only in June, with losses occurring in the other months. For the protein fraction, a gain was seen in July, but losses were found in all other months.

#### DISCUSSION

This study provides an account of the intra-organ metabolic transformations occurring in the bay scallop in response to reproductive energetic demands. Labeled food carbon is incorporated into lipid, carbohydrate, and protein components of the digestive gland, adductor muscle, and ovary at rates which vary over the course of the reproductive cycle. Differences in  $\text{CPM mgDW}^{-1}$  between Day 1 and Day 4 indicate the relative rate of turnover of a particular carbon compound within a body component, with a net gain in  $^{14}\text{C}$  (positive slope) indicating anabolism (relatively

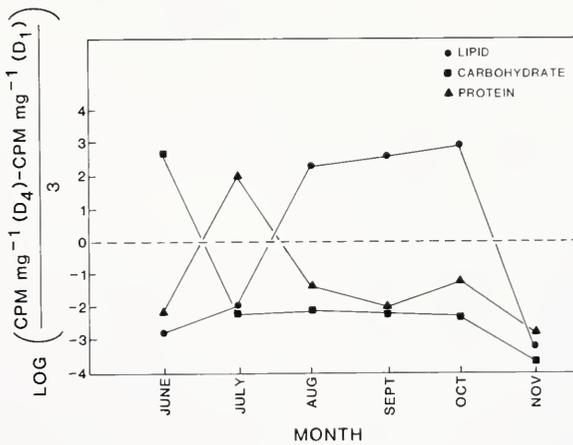


FIGURE 4. Relative seasonal incorporation of  $^{14}\text{C}$  into lipid, carbohydrate, and protein fractions of *A. irradians concentricus* ovary.

low turnover) and a net loss in  $^{14}\text{C}$  (negative slope) indicating catabolism (relatively high turnover). The fact that most of the radiolabel in all fractions is gone after Day 4 (*i.e.*, on Days 7 and 10) suggests that carbon turnover is higher in this species than in *M. edulis*, which retains  $^{14}\text{C}$  for longer periods (Thompson, 1972). This may reflect inter-specific differences in overall rates of carbon assimilation and transformation.

Over the six month study period, body component carbon incorporation in *A. irradians concentricus* is divided into growth (energy storage) and reproductive (energy utilization) periods. June and July represent a period of nutrient storage in that radiocarbon losses from digestive gland lipid and protein fractions are relatively small and are equaled by gains resulting from carbohydrate and protein anabolism in the adductor muscle. Apparent losses from ovary lipid and protein fractions at this time are probably due to unassimilated  $^{14}\text{C}$  in the intestine which is voided by Day 4. (These losses probably occur in subsequent months as well, but are masked by the accumulation of  $^{14}\text{C}$  in developing oocytes.) In August active oogenesis is indicated by a gain in ovary lipid radiolabel in conjunction with an increased rate of  $^{14}\text{C}$  loss from digestive gland lipid and protein fractions. Overall, the adductor muscle loses radiocarbon for the first time during this period, with most of it being in the carbohydrate fraction. In September and October, radiolabeled lipid accumulates at an increased rate in the ovary, suggesting active oogenesis. Digestive gland lipid and adductor muscle carbohydrate fractions both continue to lose radiolabel at increasing rates. The timing and magnitude of radiocarbon losses from these two body component fractions suggest that they are involved in the reproductive process. After spawning occurs in November, carbon is rapidly lost from all body component fractions, indicating that the physiological condition of these animals is poor. Mortality in the Anclote scallop population increases after spawning (Barber and Blake, 1983).

The importance of the digestive gland as the site of carbon assimilation, storage, and transfer in bivalves (Owen, 1966; Weel, 1974) is seen in this study. The digestive gland has initially high activity that is rapidly lost as carbon is transferred to other body components. During the resting stage, loss from the digestive gland is comparatively reduced, with most of this being incorporated into adductor muscle nutrient pools. However, as oogenesis takes place, the rate of loss of  $^{14}\text{C}$  from the digestive gland increases, possibly indicating a carbon transfer between digestive gland and ovary body components. Initiation of gametogenesis in *A. irradians* is associated with the depletion of digestive gland nutrient reserves (Sastry, 1968, 1975), and the transfer of radiocarbon from digestive gland to gonad in association with gametogenesis occurs in the scallops *A. irradians* (Sastry and Blake, 1971) and *Chlamys hericia* (Vassallo, 1973). Digestive gland reserves support vitellogenesis in *M. edulis* (Thompson *et al.*, 1974). Thus the digestive gland of *A. irradians concentricus* functions primarily as a short-term storage organ in that assimilated carbon from recently ingested food can be transferred to storage sites (growth centers) or rapidly turned over to meet increased energetic demands associated with reproduction.

In the digestive gland, lipid is the most important fraction in terms of radiolabel content and metabolic participation, since it is the fraction most rapidly turned over throughout the study. Lipid is a valuable energy substrate due to its high energy yield per unit weight (Giese, 1966). A loss of digestive gland lipid stores in conjunction with gametogenesis occurs in several scallop species, including *Patinopecten yessoensis* (Mori, 1975), *A. irradians concentricus* (Barber and Blake, 1981), and *Placopecten magellanicus* (Robinson *et al.*, 1981). The transfer of radiolabeled lipid from the digestive gland to the gonad of *Chlamys hericia* suggests a mechanism for oocyte yolk synthesis whereby lipid is broken down into fatty acids and glycerol in the digestive gland and transferred to the ovary where triglycerides and hydrocarbons are

synthesized (Vassallo, 1973). The demonstration of increased lipid plasma levels during oogenesis in *P. magellanicus* (Thompson, 1977) and *Crassostrea gigas* (Allen and Conley, 1982) supports this proposed mechanism.

The scallop adductor muscle is an important site of energy storage, and the utilization of its reserves is associated with reproductive development in the species *Chlamys septemradiata* (Ansell, 1974), *Pecten maximus* (Comely, 1974), *C. opercularis* (Taylor and Venn, 1979), *A. irradians concentricus* (Barber and Blake, 1981), and *P. magellanicus* (Robinson *et al.*, 1981). In this study energy storage is represented by a gain in adductor muscle radiocarbon in June and July which appears to be supplied from the digestive gland. Once oogenesis is initiated, however, radiocarbon is initially lost from the adductor muscle. Adductor muscle reserves continue to be catabolized throughout the reproductive period, presumably providing energy for reproduction. In *A. irradians concentricus* the adductor muscle is a long-term storage organ in the sense that nutrients stored in it prior to gametogenesis are used months later to support oocyte synthesis.

Carbohydrate is the most metabolically active fraction in the adductor muscle. During the growth phase it is most rapidly synthesized and during reproduction it is most rapidly utilized. The importance of carbohydrate in bivalve energy metabolism is linked to its more efficient conversion of energy to ATP and its availability to anaerobic metabolism (Gabbott, 1976). A loss of adductor muscle carbohydrate reserves occurs over the reproductive period of the scallop species *Chlamys septemradiata* (Ansell, 1974), *Pecten maximus* (Comely, 1974), *Patinopecten yessoensis* (Mori, 1975), *C. opercularis* (Taylor and Venn, 1979), *Argopecten irradians concentricus* (Barber and Blake, 1981), and *Placopecten magellanicus* (Robinson *et al.*, 1981). This commonly observed synchrony between glycogen utilization and oogenesis suggests that carbohydrate reserves are converted to lipid in developing ova (Gabbott, 1975, 1976).

The rate of incorporation of  $^{14}\text{C}$  into scallop ovary is indicative of reproductive stage. During the nonreproductive period, radiocarbon is lost from the ovary between Day 1 and Day 4. During gametogenesis, the ovary gains radiolabel at a rate that increases as oogenesis continues. The synthesis of gametogenic material occurs in conjunction with the loss of reserves in the adductor muscle and recently assimilated carbon in the digestive gland. However, there is not a 1:1 correlation between  $^{14}\text{C}$  gains and losses over the reproductive period, most likely due to the metabolic "cost" of manufacturing oocytes. The proportion of adductor muscle and digestive gland reserves that actually supports reproduction directly is not known, but it is evident that recently ingested food alone is not sufficient for supporting both maintenance and reproductive metabolisms. After spawning commences, ovary  $^{14}\text{C}$  is rapidly lost, possibly due to a continued release of eggs and/or the resorption of remaining ova.

The ovary lipid fraction essentially parallels the oocyte growth curve for this population of *A. irradians concentricus* (Barber and Blake, 1983). Ovary lipid activity decreases during the resting stage but increases as oogenesis proceeds and fatty yolk accumulates in developing ova. Ovary lipid levels increase in conjunction with seasonal reproductive cycles for a number of scallop species including *Chlamys septemradiata* (Ansell, 1974), *Pecten maximus* (Comely, 1974), *C. opercularis* (Taylor and Venn, 1979), *C. tehuelcha* (Pollero *et al.*, 1979), *A. irradians concentricus* (Barber and Blake, 1981), and *Placopecten magellanicus* (Robinson *et al.*, 1981). Lipid stored in developing ova becomes an important energy source to planktonic larvae (Giese, 1966; Bayne, 1976).

This work reinforces the results of previous studies on seasonal variations in bay scallop body component weights and biochemical compositions and substrate catabolism (Barber and Blake, 1981, 1983, and unpubl. data). Together they provide a

clear picture of the reproductive energy metabolism of this sub-species at this location. The digestive gland functions primarily as the site of carbon assimilation and distribution to other body components and secondarily as a storage organ. During the resting stage some carbon is retained in the digestive gland in the form of lipid, but most of it is shunted to the major energy storage organ, the adductor muscle, where considerable glycogen and protein stores are accumulated. Once gametogenesis begins, the utilization of these pre-stored reserves occurs in a definite sequence. Digestive gland lipid is utilized either as a direct response to the initiation of gametogenesis or because of an increased storage demand in the adductor muscle. At some point, however, digestive gland carbon ceases to be directed to the adductor muscle, being involved instead in the production of gametes. Adductor muscle glycogen utilization follows digestive gland lipid utilization and is continuous over the cytoplasmic growth phase. During vitellogenesis adductor muscle structural protein is broken down as the glycogen supply is depleted. Thus, the digestive gland plays a secondary role to the adductor muscle as an energy storage organ in *A. irradians concentricus* in Florida. This is in contrast to more northerly bay scallop populations (Barber and Blake, 1983) and other marine bivalve species.

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GAMETOGENESIS AND LARVAL PRODUCTION IN A POPULATION  
OF THE INTRODUCED ASIATIC CLAM, *CORBICULA* SP.  
(BIVALVIA:CORBICULIDAE), IN MARYLAND

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ABSTRACT

Histological assessment of gametogenesis and larval production in monthly samples of *Corbicula* sp. collected from December 1981 to October 1983 in the Potomac River, Maryland, revealed that the clams were simultaneous hermaphrodites. Gametes of both sexes were present year-round, including winter, although male reproductive tissue was less common than female tissue. Both male and female tissue were found in the smallest clam examined (9.6 mm long). Eggs and sperm were often produced in the same follicle and occurred together in the gonoducts of a number of specimens. Stereological analysis was used to quantify tissue change during the study. No clear cycles of reproductive tissue volume fractions (developing or ripe gametes) were demonstrated, suggesting that this species may be capable of responding rapidly throughout the year to suitable environmental conditions by spawning. In our samples, larvae were produced over two extended time periods, one in spring and one in fall, with the months involved varying somewhat from year to year. The smallest clam containing larvae was 13.4 mm long.

INTRODUCTION

Asiatic clams of the genus *Corbicula* were apparently introduced to the West Coast of North America prior to 1938 and their descendants have since become widespread in fresh waters of the continent (Britton and Morton, 1982; McMahan, 1982). In some areas they have become so abundant that they foul irrigation canals and industrial plumbing systems, including the water-supply systems of electric power plants (McMahan, 1977; Mattice, 1979), to the point of becoming pests of commercial significance.

In Maryland, Stotts *et al.* (1977) were the first to report on the presence of Asiatic clams, in this case at the mouth of the Susquehanna River. Dresler and Cory (1980) reported that *Corbicula fluminea* had become established in the Potomac River on the Western Shore of Chesapeake Bay, and Counts (1981) recorded this species from the Wicomico River on the Eastern Shore. Since then, we have collected Asiatic clams from Nassawango Creek, which is a tributary of the Pocomoke River in Maryland, and from Butler Mill Creek which flows into Lewis Creek, a short tributary of the Nanticoke River just across the Maryland state line in Delaware.

With these clams thus becoming widely distributed in Maryland and adjacent waters in recent years, we embarked on a study of reproduction in a large population living in the Potomac River. There have been some studies of Asiatic clam reproduction elsewhere in North America, predominantly in the South (see Britton and Morton, 1982 and McMahan, 1983 for reviews) but none reported for the Mid-Atlantic states

or for natural waters (*i.e.*, not industrially heated) this far north. None of the studies used quantitative evaluation of gametogenesis. We employed a recently described quantitative method of stereological analysis (Lowe *et al.*, 1982) to delineate gametogenic patterns in our study population, and we also documented periods of larval production.

#### *Taxonomic status of Asiatic clams*

With regard to the species of Asiatic clam we studied, Britton and Morton (1979) declared that *Corbicula fluminea* was the correct scientific name of North American animals. However, Hillis and Patton (1982) presented electrophoretic evidence that two "species" of *Corbicula* (one with white nacre and one with purple nacre) live in the Brazos River, Texas. We have also found a clam population with purple nacre in Butler Mill Creek, although all other populations in the Bay watershed that we have examined (four) have white nacre. While the Maryland clams studied for this report are similar to those being called *Corbicula fluminea* (white form), we feel that assignment of a species name is not warranted until conclusive taxonomic studies have been performed. We therefore refer to our research animals as *Corbicula sp.* When referring to the reported works of others, we have retained the species' names they used. Voucher specimens of our Whites Ferry study population (USNM 804414) and the Butler Mill Creek population (USNM 836237) have been deposited with the Smithsonian Institution's National Museum of Natural History.

#### MATERIALS AND METHODS

With the aid of a mesh-lined clam rake, Asiatic clams were collected at approximately monthly intervals (except when the river was frozen or during high river flow periods) from shallow inshore water just below Whites Ferry, Maryland (approx. 39°09' N; 77°31' W), on the Potomac River. Collections began in December 1981 and ended in October 1983. Temperatures were measured at time of collection.

Except for early samples, two sets of about 20 animals each were selected to cover a range of lengths (measured to the nearest millimeter along the anterior-posterior axis). These clams were held in reverse-osmosis water for 1–2 days to allow them to cleanse themselves of dirt. They were then shucked whole. Individual dry body tissue weights for one set of clams (size range: 7.5–33.5 mm) were determined by holding the shucked clams at 95°C for 24 h and then weighing them to provide data for log length *versus* log dry weight analysis using least-squares regression. The second set of clams [size range: 9.6–41.9 mm (Table I), with 65% in the 20–30 mm range] was fixed for 24 h in Davidson's Solution at 4 to 8°C, placed in 50% alcohol for at least 2 h at room temperature, and stored in 70% alcohol. Fixed clams were taken through an ascending alcohol series, cleared in xylene, and embedded in 56°C Paraplast. Sections were cut at 6–8  $\mu\text{m}$  thickness and stained in Harris' hematoxylin and counterstained in eosin.

To determine the best orientation for viewing reproductive follicles in body tissue and to ensure that we obtained the most representative section of the tissue, we initially prepared both transverse (cross) and sagittal (longitudinal) sections. We concluded that sagittal sections were more satisfactory for viewing a wider area of gill and body tissue and these sections were thus used exclusively in this study. In preparing sections, when the first section containing substantial follicular tissue was encountered it was retained and two to four additional body sections were cut at 100  $\mu\text{m}$  to 200  $\mu\text{m}$  intervals into the body (depending on clam size). Each of the three to five resulting sections was placed on separate slides for analysis. The sections prepared for each

monthly sample were examined for the presence of larvae in the gills and of eggs and sperm in follicular tissue.

To quantify the process of gametogenesis, stereological analysis (Lowe *et al.*, 1982; Newell *et al.*, 1982) was performed on 10 clams per month (except December 1981 when only 6 were used). A projection microscope was used to project the image of the gonadal tissue in three different sagittal sections per clam onto a sheet of paper marked with 42 points. The coincidence of a point with one of six tissue types, *i.e.*, (1) interfollicular connective tissue; developing (2) male or (3) female gametes; morphologically ripe (4) male or (5) female gametes; and (6) spawning/spent tissue (Fig. 1) was recorded for three randomly selected fields per sagittal section. This provided 9 sets (three fields  $\times$  three sagittal sections) of 42 determinations of the frequency of occurrence of the 6 tissue components within the gonadal region of each specimen. The average value for each set of nine was calculated for each tissue component, resulting in six average values of tissue frequency for each clam. From these values the percentage volume fraction of each of the six tissue types was determined for each clam (Lowe *et al.*, 1982). Following this, an average volume fraction (%) for each tissue type was calculated for each monthly sample of clams. To calculate confidence intervals, these monthly averages were transformed by arcsine transformation (Sokal and Rohlf, 1981) and are presented retransformed with 95% confidence intervals in Figure 2.

During those periods that larvae were being brooded internally, release of brooded material from animals recently collected in the field would take place in holding bowls. In June 1982, this material was examined and length measurements were made on its components (cleavage stages, trochophores, straight-hinge larvae, juveniles).

## RESULTS

Figure 1 presents representative examples of various gametogenic stages, including developing and ripe male and female gametes, spent follicles, and interfollicular connective tissue. The pattern of gametogenesis for Whites Ferry clams is shown in Figure 2 by changes in volume fraction (VF) percentages of six tissue components over time. The preponderant tissue type was interfollicular connective tissue (ICT), the average proportions of which ranged from 74% in August 1982 to 47% in May 1983 and back to 74% in October 1983. Average VF of developing female gametes ranged from 6 to 13% over time; these values were higher than those of contemporaneously developing male gametes (0–6%). Ripe female gametes comprised the second most abundant tissue type (after ICT), ranging from 11 to 28% of all tissues, compared with a range of 0 to 2% for ripe male gametes (the least common tissue type). Finally, spawned or spent tissue ranged in occurrence from 1 to 11% of total tissues examined.

No clear cycles in VF in any of the tissue types are apparent. A peak in proportion of ICT occurred in August 1982 followed by a decline to May 1983 (to a lower point than the previous May). A relatively rapid increase in VF occurred in June and July 1983. Values then declined in August but increased in the following two months. Developing female gametes generally remained within a few percentage points of 10% VF through the sampling period whereas ripe female gametes varied around 20% VF (with a slight tendency for peaks to occur in spring and fall). Both developing and ripe male gametes were very low in VF, with a peak in November 1982 and May–June 1983 for developing male gametes and small peaks in May and September 1982 and June 1983 for ripe male gametes. Spawning or spent tissue was usually less than 10% VF during the year, with peaks in March and June 1982 and May and August 1983.

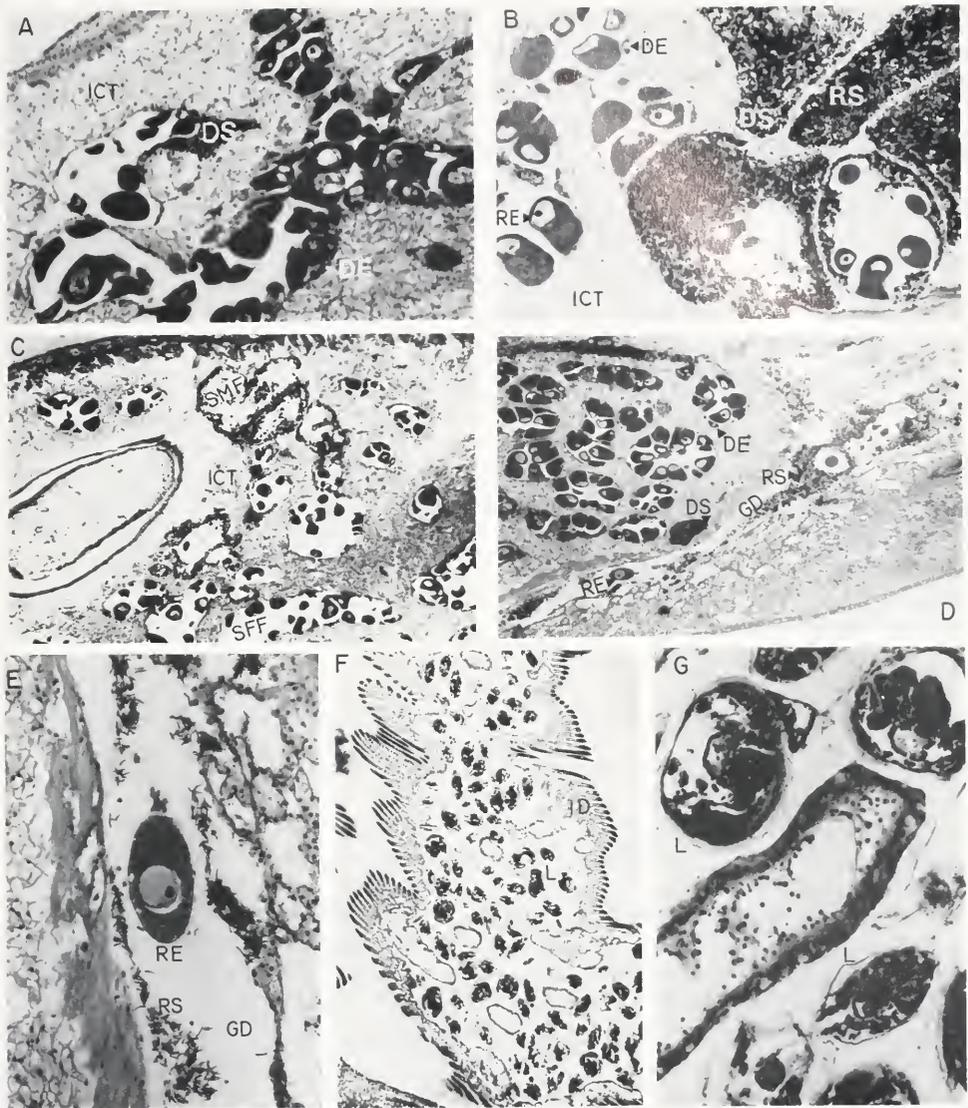


FIGURE 1. Representative examples of gamete development, spawning, gamete intermingling, and larval stages of *Corbicula* sp. A. Follicles containing developing sperm, and developing and ripe eggs; B. As A, later in development, with eggs and sperm co-occurring in some follicles; C. Gonad in advanced spawning/spent state; D. Gonoduct with ripe eggs and ripe sperm intermingling; E. Close-up of D, with egg surrounded by sperm; F. Inner demibranch of gills filled with developing larvae; G. Close-up of larvae in gill. Abbreviations: DE = developing egg; DS = developing sperm; GD = gonoduct; ICT = interfollicular connective tissue; ID = inner demibranch; RE = ripe egg; RS = ripe sperm; SFF = spent female follicle; SMF = spent male follicle. Magnification:  $40\times = C, D, F$ ;  $100\times = A, B$ ;  $400\times = E, G$ .

Table I lists the percentages of Asiatic clams examined that contained female gametes, male gametes, and larvae. Throughout the study, every clam examined contained female gametes (in October 1983 one clam contained extensive male follicles with almost no eggs present). However, the proportion of clams in which male gametes

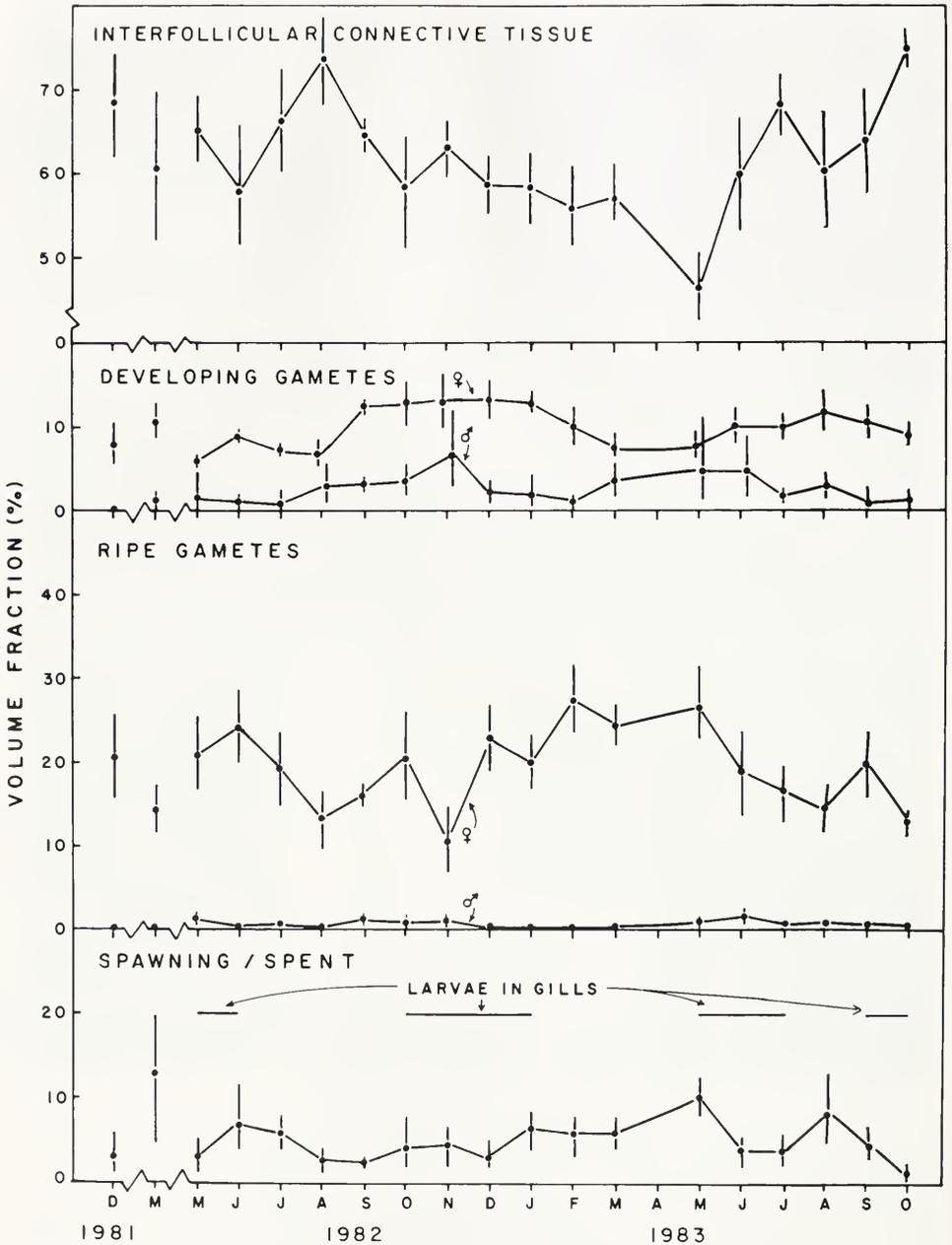


FIGURE 2. Average volume fractions (with 95% confidence intervals) of six tissue components in the gonadal region of *Corbicula* sp. from Whites Ferry, MD.

were noted fluctuated from 43 to 100%, although after March 1982, 85% or more of the monthly samples of clams contained male gametes. The smallest clam we sectioned (9.6 mm) contained both male and female gametes. However, when the

TABLE I

Percentages of Asiatic clams that contained female gametes, male gametes, and larvae (Whites Ferry, Maryland)

Month	Length range (mm)	Female	Male	Larvae
1981 Dec	15.8-24.8	100 (28) <sup>1</sup>	43 (28)	0 (21)
1982 March	18.3-41.9	100 (12)	50 (12)	0 (12)
May	15.6-28.3	100 (16)	88 (16)	67 (15)
June	13.4-23.7	100 (20)	100 (20)	85 (20)
July	14.4-34.7	100 (20)	85 (20)	0 (20)
Aug	18.7-28.9	100 (20)	95 (20)	0 (20)
Sept	21.6-28.1	100 (20)	100 (20)	0 (20)
Oct	13.9-29.0	100 (19)	89 (19)	11 (18)
Nov	9.6-29.0	100 (20)	90 (20)	40 (20)
Dec	11.9-29.2	100 (20)	100 (20)	40 (20)
1983 Jan	19.2-30.1	100 (20)	90 (20)	10 (20)
Feb	23.3-28.8	100 (20)	85 (20)	0 (20)
March	11.6-30.4	100 (20)	100 (20)	0 (19)
May	19.0-29.7	100 (20)	100 (19)	25 (20)
June	21.6-30.9	100 (14)	93 (14)	100 (14)
July	13.1-32.3	100 (20)	90 (20)	45 (20)
Aug	16.7-36.7	100 (20)	90 (20)	0 (20)
Sept	19.8-32.8	100 (20)	95 (20)	80 (20)
Oct	29.7-33.4	100 (20) <sup>3</sup>	90 (20)	75 (20)
Smallest <sup>2</sup>		9.6 mm	9.6 mm	13.4 mm

<sup>1</sup> Number in parentheses is sample size.

<sup>2</sup> Sizes of the smallest animals containing gametes or larvae.

<sup>3</sup> Almost no eggs noted in one animal in which male follicles were widespread.

proportion of clams  $\geq 20$  mm long with male gametes was compared with clams  $< 20$  mm long with male gametes, the former comprised 92% compared with 78% for the latter size class (this difference is significant at the  $P < 0.001$  level as determined by the G-test of independence: Sokal and Rohlf, 1981).

In numerous instances, both male and female gametes were found to be developing within the same follicles (*e.g.*, Fig. 1B). In addition, it was not uncommon during months of spawning to find male and female gametes mingling in gonoducts (*e.g.*, Figs. 1D, E).

Larvae appeared in the inner demibranchs of clams in spring and fall 1982 and in spring-early summer and fall 1983. The smallest clam which contained larvae was 13.4 cm long.

Figure 3 presents information on clam dry weight changes over the study period. Each point represents the predicted dry tissue weight of a representative 20 mm clam as determined from length-weight curves (GM regression; Ricker, 1973) we developed each month (Table II). Coefficients of determination were high and the variance ratios ( $F_s$ ) for each regression were all significant at  $P < 0.001$  (Sokal and Rohlf, 1981). Initially, in December 1981 and March 1982, the predicted dry weight was low (Table II, Fig. 3). It rapidly increased to a peak in May 1982, declined in June, and then increased and reached its highest value for this study in August. Thereafter there was a decline to October 1982 followed by a general increase until January 1983. A decline in February was followed by another peak in March 1983. (Note that the values for December 1982 and March 1983 were higher than they had been

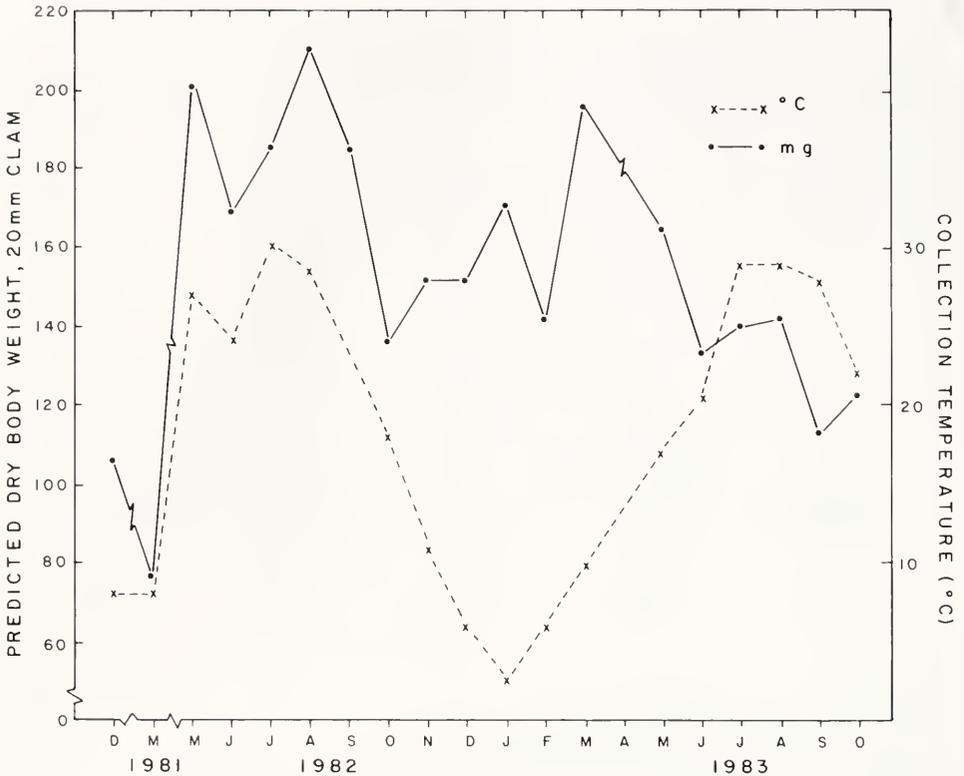


FIGURE 3. Collection temperature and changes in predicted body weight of a representative 20 mm specimen of *Corbicula* sp. from Whites Ferry, MD.

one year earlier.) After March, dry weight declined through June, rose slightly in July and August, then declined again.

Observations of larvae released into bowls by animals recently collected in the field revealed that two kinds of release appeared to occur. In some instances, fully developed juveniles with an active foot were ejected from the adult clam. In other instances, a mixture of material was released; it included juvenile clams, a few younger straight hinge or trochophore larvae, larvae with apical flagellae, and large, ciliated objects that we assume were early cleavage stages or morulae. Samples of this material were measured with an ocular micrometer. The large ciliated objects ranged in size from 160 to 220  $\mu\text{m}$  ( $\bar{x}$  = 183  $\mu\text{m}$ ;  $n$  = 19). Straight hinge larvae were somewhat smaller, ranging from 160 to 190  $\mu\text{m}$  ( $\bar{x}$  = 174  $\mu\text{m}$ ;  $n$  = 10). Newly released juveniles measured at the greatest anterior-posterior distance of their shell were 210–250  $\mu\text{m}$  long ( $\bar{x}$  = 236  $\mu\text{m}$ ;  $n$  = 12). The larvae were not active swimmers. The circular objects and the trochophore-straight hinge stages generally moved little, other than in small circles. The juveniles used their active foot to pull themselves along. However, they did not move very far, tending to remain clumped in the vicinity of the adult which released them rather than spreading throughout the still water of the container.

#### DISCUSSION

The Asiatic clams sampled in this study were found to be simultaneous hermaphrodites, with male and female reproductive tissue present in all months of the

TABLE II

Monthly values for intercept ( $u$ ) and slope ( $v$ ) for allometric equation of  $\log$  dry tissue weight =  $u + v \log$  length, with  $R^2$  = coefficient of determination and  $w$  = dry tissue weight (mg) of standard *Corbicula* sp. of 20 mm shell length

Month	$u$	$v$	$R^2$	$w$
1981 Dec	-2.72	3.65	0.988	105.9
1982 Mar	-1.87	2.88	0.954	74.8
May	-1.38	2.83	0.976	199.1
June	-2.13	3.35	0.980	167.9
July	-1.81	3.14	0.968	187.1
Aug	-1.72	3.11	0.982	210.4
Sept	-1.90	3.20	0.973	182.0
Oct	-2.56	3.61	0.973	135.8
Nov	-2.38	3.51	0.982	152.4
Dec	-2.01	3.22	0.979	150.0
1983 Jan	-1.98	3.24	0.992	170.6
Feb	-2.17	3.32	0.986	140.0
Mar	-1.94	3.26	0.994	198.6
May	-1.91	3.18	0.996	167.5
June	-2.10	3.25	0.993	133.4
July	-2.06	3.24	0.993	141.9
Aug	-2.51	3.59	0.994	143.5
Sept	-2.44	3.46	0.987	114.3
Oct	-2.54	3.56	0.968	122.5

year. However, male reproductive tissue was less common than female tissue (Table I, Fig. 2). This has been the case elsewhere, with Heinsohn (1958) being the first to demonstrate this (in California), followed by Kraemer and Lott (1977) in Arkansas and Britton *et al.* (1979) in Texas.

Male and female gametes were found in the smallest clam we sectioned (9.6 mm). Elsewhere, Kraemer (1978) found both types of gametes in Arkansas specimens  $\geq 8$  mm long and Aldridge and McMahon (1978) found that Texas specimens were sexually mature at 10 mm. In a summary paper, Britton (1982) gave 7–10 mm as the size at which sexual maturity in *C. fluminea* was attained.

Male tissue represented the smallest tissue VF in the gonadal region (Fig. 2), with highest averages being 6% for developing male gametes and 2% for ripe male gametes. Note, however, that a few clams had extensive development of male tissue, both developing and ripe: Nov. 1982, 21.0 mm; May 1983, 24.5 mm; June 1983, 28.9 mm; Oct. 1983, 30.7 mm (the latter was not included in the sample of 10 clams selected haphazardly for the determination of the VF for October). Again, similar low instances in proportions of male tissue have been reported in Arkansas by Kraemer and Lott (1977) who estimated that male tissue occupied no more than 15% of follicular material, and in Texas by Britton and Morton (1979) who provided an estimate of 30% (in neither case was it explained how the estimates were derived). These low figures are not possible evidence for protandric consecutive hermaphroditism (Britton and Morton, 1979) because the proportion of clams with male gametes increased significantly ( $P < 0.001$ ) with shell size (78% of clams  $< 20$  mm long contained male gametes vs. 92% in clams  $\geq 20$  mm long) as noted earlier.

The fact that no clear cycles in VF of any reproductive tissue type were demonstrated (Fig. 2) may indicate that Asiatic clams are not much affected by predation and competition (Suchanek, 1978; 1981). It may also indicate that the species is capable of responding rapidly to suitable environmental conditions by spawning at any time of the year. However, the literature contains no evidence that larval production

in the United States has been occurring other than in spring through fall (Heinsohn, 1958; Aldridge and McMahon, 1978; Britton *et al.*, 1979; Eng, 1979; Dreier and Tranquilli, 1981), usually with peak abundances in spring and in fall. [The presence of "larvae" in monthly plankton samples collected over 14 months in the Altamaha River, GA (Sickel, 1979) is not evidence that larval release was occurring monthly. It could be the result of resuspension of juvenile clams by water movement (Prezant and Chalermwat, 1984).] Similarly, at Whites Ferry, clams with larvae in their gills were found over two extended time periods (Table I, Fig. 2). The spring 1982 pulse occurred with water temperatures of 24° to 27°C (we do not know if larvae were present in April 1982 as extremely high river flows and dangerous river conditions precluded collecting material) whereas for spring 1983, the corresponding water temperatures ranged from 17° to 29°C. The fall 1982 and 1983 pulses occurred while temperatures were dropping (from 18° to 2°C in 1982 and 29° to 24°C in 1983). Larvae were found in December 1982 and not in December 1981, perhaps due to the colder temperatures of the latter month (see below). Elsewhere, Aldridge and McMahon (1978) noted that Texas specimens began "spawning" (larval release) at 19°C, with this behavior inhibited by temperatures > 32°C. Fall veliger release declined sharply below 18°C. In California, Eng (1979) found "spawning" (presence of marsupial larvae) to begin as temperatures exceeded 16°C with a second spawning occurring while temperatures were at summer highs.

Percentages of Whites Ferry clams with marsupial larvae (Table I) were greater in spring 1982 (67–85%) than in fall 1982 (10–40%) and in spring 1983 (100% in June) compared with fall 1983 (75–80%). Similar results have been reported for two other populations. In California, Eng (1979) found all clams (100%) sampled in spring 1974 to contain larvae *versus* 20% in fall 1974. In Texas, Aldridge and McMahon (1978) reported 60–90% of adults to be brooding larvae in spring 1975 compared with 20–70% in fall 1975. However, Britton *et al.* (1979) presented a figure for another Texas population (their Fig. 3) showing that whereas in spring of the sampling year approximately 83% of clams contained larvae, in fall a peak of 80% were brooding. Such differences in peak intensity of larval brooding undoubtedly reflect differences in environmental conditions and are probably less important than extent of the brooding and larval release periods. The fall periods when larvae were present were longer than spring periods in Maryland and Texas, but not California (Table I, see also Aldridge and McMahon, 1978; Britton *et al.*, 1979; Eng, 1979). Further, Aldridge and McMahon (1978) quantified larval releases and found a total fall release of over 750,000 veligers m<sup>-2</sup> compared with spring values of about 390,000 veligers m<sup>-2</sup>.

At Whites Ferry, a possible response to environmental conditions was noted in the predicted dry tissue weight of a 20 mm animal over the study period (Fig. 3). The collection temperatures for December 1981 and 1982 on Figure 3 can be misleading because, as mentioned, the winter of 1981–1982 was colder than that of 1982–1983. Temperature records made daily in the Potomac River by the Washington Suburban Sanitary Commission reveal that temperature from December 1981 to February 1982 ranged from 1.0 to 3.3°C with minima of 0.0 to 1.0°C and maxima of 1.4 to 7.0°C. Temperatures increased from December through February. By contrast, from December 1982 to February 1983, average temperatures were 4.6–8.7°C, with minima of 1.0–5.0°C and maxima of 7.0–15°C (temperatures decreased from December to February). These temperature differences may account for the different predicted dry body weights for the two collection periods (Fig. 3), with weights in December 1981 and March 1982 being up to 60% lower than for comparable periods in 1982–1983. On the other hand, dry weights increased rapidly in early 1982 after the cold winter, reaching levels greater than in comparable months in 1983. Although the colder winter may have depressed body weights until spring, the population which

survived the winter recovered body weight quickly and produced larvae over four different time periods thereafter until our sampling ceased (Fig. 2). Indeed, in September 1983 we estimated clam densities to exceed  $10,000 \text{ m}^{-2}$  at Whites Ferry. This recovery from a very cold winter is significant because Asiatic clams are considered to be intolerant of severe winter conditions, thus being generally limited to areas below  $40^\circ \text{ N}$  latitude (Britton and Morton, 1982). They may be able to escape the cold by burrowing more deeply into the sediment. Our subjective impression is that they lie nearer the surface of the river bed in summer (where they are readily seen) than in winter (when they are much less conspicuous, even in the clear water of that time of year).

In conclusion, Asiatic clams from this more northerly population were broadly similar in their reproductive patterns to those populations that have been studied further south in the United States. Given the year-round presence of eggs and sperm and their occurrence in clams from about 10 mm in size and up, the brooding of larvae for two relatively long periods per year, and the possibility of self-fertilization, it is probable that the recent invasion of this exotic species into Maryland waters will continue and expand.

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CELL-CELL RECOGNITION AND ADHESION DURING  
EMBRYOGENESIS IN THE SEA URCHIN: DEMONSTRATION  
OF SPECIES-SPECIFIC ADHESION AMONG *ARBACIA*  
*PUNCTULATA*, *LYTECHINUS VARIEGATUS*, AND  
*STRONGYLOCENTROTUS PURPURATUS*

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ABSTRACT

The present study investigates species-specific recognition and adhesion between dissociated embryonic cells of hatched blastulae of *Arbacia punctulata*, *Lytechinus variegatus*, and *Strongylocentrotus purpuratus*. The assay used is a modification of one previously developed by McClay and Hausman (1975) and involves collection of labeled single probe cells to unlabeled collecting aggregates. The results indicate that probe cells fixed by glutaraldehyde or formaldehyde or disrupted by sonication do not adhere significantly to their homospecific aggregates. Moreover, fixation of aggregates by glutaraldehyde greatly diminishes binding of labeled probe cells. Thus adhesion, as measured by the present assay, requires both living probe cells and aggregates. In addition, adhesion of probe cells to their homospecific aggregates is found to be significantly greater than adhesion to heterospecific aggregates. The results demonstrate reciprocal species-specific adhesion between *Arbacia punctulata* versus *Lytechinus variegatus*, *Arbacia punctulata* versus *Strongylocentrotus purpuratus*, and *Lytechinus variegatus* versus *Strongylocentrotus purpuratus*. These results extend previous work with other species and suggest that species-specific recognition and adhesion is a universal property of dissociated cells of sea urchin embryos.

INTRODUCTION

The pioneering studies of Herbst (1900) first showed that sea urchin embryos can be dissociated into separate cells in calcium-free sea water, and that these cells will reaggregate into embryo-like structures when returned to normal sea water. Later studies by Giudice (1962), Giudice and Mutolo (1970), Spiegel and Spiegel (1975), and McClay and Hausman (1975) have shown the reaggregation process to be species-specific between *Paracentrotus lividus* and *Arbacia lixula*, *Lytechinus pictus* and *Arbacia punctulata*, and *Tripneustes eschulentus* and *Lytechinus variegatus*, respectively. It is particularly noteworthy that species-specific adhesion is observed not only with dissociated cells from embryos at the hatched blastula through the prism stages (Giudice, 1962; Giudice and Mutolo, 1970; McClay and Hausman, 1975) but also with dissociated cells from blastulae prior to hatching (Giudice and Mutolo, 1970) and from embryos at the 16-cell stage (Spiegel and Spiegel, 1975). Although Giudice and Mutolo (1970) and Spiegel and Spiegel (1975) have reported loose adherence between species followed by "sorting out" when reaggregation occurs in stationary culture, a high

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degree of species-specificity of adhesion is observed by Giudice (1962), McClay and Hausman (1975), and McClay *et al.* (1977) for reaggregation of cells in suspension culture. The apparent nonspecific association initially observed in stationary culture may result from factors influencing motility, chemotaxis, or cell to substratum adhesion, rather than species-specific recognition and adhesion. Giudice (1962), Giudice and Mutolo (1970), Okazaki (1975), Sano (1977), and Spiegel and Spiegel (1980) have shown that embryonic cells do not appear to de-differentiate upon dissociation but rather they appear to retain the same pattern of metabolic activity and embryonic differentiation characteristic of the stage at which they were dissociated. Since Giudice and Mutolo (1970) have shown by autoradiography that dissociated cells of labeled blastulae will reaggregate with dissociated cells of prisms, it would appear that species-specificity rather than stage (or tissue) specificity may predominate initially in this system. Moreover, since Giudice and Mutolo (1970), Spiegel and Spiegel (1975, 1980), McClay and Hausman (1975) and others have demonstrated that reaggregation of dissociated cells can eventually lead to the formation of almost normal embryos, it appears that there is a further sorting out according to tissue after the initial species-specific event. Indeed previous experiments by Spiegel and Spiegel (1978) with isolated, vitally-stained micromeres support this idea. Undoubtedly, reaggregation of dissociated embryonic cells of the sea urchin involves many complex and multiple molecular interactions. However, it would seem quite probable that a recognition event involving species-specific adhesion might indeed be one of the earliest in a series of cell-cell interactions which lead to further sorting out (tissue-specific adhesion) and differentiation. The present work describes species-specific recognition and adhesion among sea urchin species readily available in this country—*Arbacia punctulata*, *Lytechinus variegatus*, and *Strongylocentrotus purpuratus*—by the use of a quantitative reaggregation assay. A portion of this work has appeared previously in abstract form (Schneider, 1983).

#### MATERIALS AND METHODS

For the studies performed in Woods Hole, Massachusetts, *Arbacia punctulata* were supplied by the Marine Biological Laboratory and *Lytechinus variegatus* were purchased from the Center for the Study of Cells, Reproduction, and Development (C.S.C.R.D.), Florida State University, Tallahassee, Florida. Animals were maintained in laboratory sea tables supplied with constantly flowing sea water (20–22°C). For the studies performed in Omaha, *A. punctulata* and *L. variegatus* were purchased from the C.S.C.R.D., Florida State, and *Strongylocentrotus purpuratus* were obtained from the Pacific Biomarine Corporation, Venice, California. The animals were maintained in refrigerated aquaria at 16°, 13°, and 21°C, respectively, in artificial sea water (Instant Ocean, from Aquarium Systems, Inc.; Mentor, Ohio). Gentamicin was obtained from the Schering Corporation. Prosil-28 was purchased from PCR Research Chemicals, Inc.; Gainesville, Florida. Paraformaldehyde was obtained from J. T. Baker; glutaraldehyde and L-leucine were supplied by Sigma. Leucine [L-4,5-<sup>3</sup>H, 56.5 Ci/mmol] was purchased from New England Nuclear.

#### *Fertilization of eggs and culturing of embryos*

Gametes were obtained from the animals by intracoelomic injection with 0.5 M KCl (*S. purpuratus* and *L. variegatus*) or by electrical shock (*A. punctulata*). Eggs at concentrations of 0.5–1 mg of protein per ml (~1% suspension, v/v) were fertilized with a two to three-fold excess of sperm (0.02–0.3  $\mu$ l of dry sperm per ml of eggs)

and washed several times by gravity sedimentation. Embryos were cultured with gentle stirring by a paddle wheel at 0.5–1 mg of protein per ml in artificial sea water (ASW; MBL formula; Harvey, 1956) containing 5 mM Tris-Cl, pH 8.0 and 10  $\mu$ g/ml gentamicin. For studies at Woods Hole, *A. punctulata* and *L. variegatus* were cultured at 20–22°C. For studies in Omaha, *A. punctulata*, *L. variegatus*, and *S. purpuratus* were cultured at 16.5–19.5°C, 19.5°C, and 16.5°C, respectively.

#### *Dissociation of embryos at the hatched blastula stage*

Embryos at the hatched blastula stage (0.5–0.75 ml packed cell volume) were recovered by centrifugation by hand and resuspended at 0°C in 10 ml of calcium and magnesium-free sea water (CMFSW: 0.45 M NaCl, 0.01 M KCl, 0.025 M Na<sub>2</sub>SO<sub>4</sub>, and 0.0025 M NaHCO<sub>3</sub>) containing 5 mM Tris-Cl, pH 8.0, and 1 mM EDTA. Embryos of *A. punctulata* were washed 3–4 times in this medium; those of *L. variegatus* and *S. purpuratus* were washed twice. The embryos were resuspended in 2 ml of CMFSW containing Tris and EDTA and gently dissociated at 0°C by repeated passages through a 5 $\frac{3}{4}$ " Pasteur pipette. When dissociation was complete, as assessed by examination in a phase contrast microscope, the single cells were recovered by centrifugation at 0–5°C for 3 min at 1200 rpm in a Sorvall RC-3 clinical centrifuge.

#### *Preparation of collecting aggregates*

Dissociated cells from hatched blastulae were washed in 3 ml of CMFSW at 0°C, centrifuged at 1200 rpm for 3 min, and resuspended in 6 ml of CMFSW to a concentration of 5–10 mg of protein per ml (about 6–13  $\times 10^7$  cells/ml). These were diluted ten-fold into plastic Petri dishes (Bellco, 100  $\times$  20 mm) containing 16 ml ASW plus gentamicin. Dissociated cells were allowed to reaggregate undisturbed in stationary culture. Cultures of *A. punctulata* and *L. variegatus* cells were incubated at 20–22°C in Woods Hole and at 16.5°–18°C and 18°–20°C, respectively, in Omaha. Cultures of *S. purpuratus* cells were incubated at 16.5°–18°C. Dissociated cells from hatched blastulae exhibited varying degrees of initial adhesion to the plastic substratum, depending upon the species, and after a given time were observed to form clusters or "chains of beads" as previously described by Spiegel and Spiegel (1975). These detached from the plastic substratum in about 1 $\frac{1}{2}$ –2 h for *A. punctulata* and *L. variegatus* at 20–22°C; 4–5 h for *A. punctulata* at 16.5°C, and 6–7 h for *S. purpuratus* at 16.5°C. Aggregates of cells were transferred by Pasteur pipette to conical centrifuge tubes and recovered by gentle centrifugation with a hand-driven centrifuge. The aggregates were washed three times in ASW at 0°C by gravity sedimentation and resuspended to the desired concentrations of 0.1–2.5 mg of protein per ml in ASW containing gentamicin and 1 mM leucine. The average size of the isolated collecting aggregates was equal to that of a normal blastula or somewhat larger. Yields of collecting aggregates from single cells of *A. punctulata* and *L. variegatus* averaged 25–60%; those from *S. purpuratus* averaged 25% in 1982–83 but dropped to 5–10% in 1983–84. These yields represent lower estimates of the percent reaggregation of dissociated cells, since many smaller aggregates were discarded during the gravity sedimentations. Collecting aggregates were distributed to siliconized 25 ml Erlenmeyer flasks and stored at 0°C until ready to be used in the collecting aggregate assay (see below). For preparation of aggregates fixed in glutaraldehyde, collecting aggregates at concentrations of 1–2.5 mg of protein per ml were treated in ASW containing 1% glutaraldehyde at 0°C for 5–10 min, gently centrifuged in a hand-driven centrifuge, and washed three times with ASW before resuspension in ASW containing gentamicin and unlabeled leucine.

*Labeling of embryos with [<sup>3</sup>H]leucine; preparation of labeled probe cells*

Embryos at the hatched blastula stage (0.25–0.3 ml packed volume) were recovered by centrifugation by hand or allowed to settle by gravity sedimentation at 0°C. These were resuspended in 10 ml ASW containing Tris and gentamicin and transferred to a 100 × 20 mm plastic Petri dish. Tritiated leucine (carrier-free; 50 μCi) was added, and the embryos were incubated in stationary culture for 2–4 h at the same temperature as the original 1% culture stirred by the paddle wheel. At the end of the labeling period, the embryos were centrifuged by hand and washed several times with 10 ml ASW at 0°C. Aliquots of the recovered supernatant were counted in a triton and toluene-based scintillation fluid. Aliquots of a suspension of the washed embryos were assessed for tritium content by counting and for protein content by the method of Lowry *et al.* (1951). In addition, the amount of [<sup>3</sup>H]leucine incorporated into protein was assessed in initial experiments by treatment of the washed embryos with ice cold 5% TCA. Under these conditions, it was found that hatched blastulae from all three species took up 70–80% of the labeled amino acid from the medium; moreover, they incorporated 70–90% of the total intracellular label into protein. To ensure that collecting aggregates and labeled probe cells were of the same developmental age, blastulae to be used for probe cells were kept at 10°C while other embryos of the same batch were dissociated and allowed to form collecting aggregates. The cold treatment had no effect on development other than to slow it. In some experiments, portions of the same egg batch were fertilized at an interval of several hours for production of collecting aggregates and probe cells. Both methods gave similar results. To prepare labeled probe cells, 0.25–0.30 ml embryos were washed in 10 ml of ice cold CMFSW containing Tris and EDTA and dissociated as described above. When dissociation was complete, the labeled single cells were recovered by centrifugation at 1200 rpm, washed once in 3 ml of CMFSW, and resuspended in 6–12 ml of CMFSW to a concentration of 1–3 mg of protein per ml (about  $1.3\text{--}3.9 \times 10^7$  cells/ml). Aliquots of this suspension were assessed for tritium and protein content as described above. In addition, aliquots of the supernatants from washing and dissociation of embryos were also counted. By these measurements, it was found that only about 4–12% of the total label in whole embryos was lost during the dissociation procedure for all three species. Moreover, the total label in probe cells ( $4\text{--}6 \times 10^6$  dpm/mg, or about 0.3–0.5 dpm/cell) closely correlated with that found in whole washed embryos. Sonicated probe cells were prepared by treatment of dissociated cells in CMFSW at 0°C in a Branson sonifier equipped with a microtip and set at the maximal setting. Complete breakage (absence of cells) was assessed by microscopic examination and required about four to five bursts of 5 s each. For preparation of probe cells treated in glutaraldehyde or formaldehyde, 3.25 ml of ice cold ASW containing 1.23% of the fixative was added to 0.75 ml of the suspension of probe cells in CMFSW. The cells were treated at 0°C for 10 min, recovered by centrifugation at 1200 rpm, and washed once or twice in 5 ml of ASW before resuspension to 0.75 ml. (Formaldehyde was prepared fresh from paraformaldehyde by boiling in distilled water; a concentrated solution of ASW was added to this to obtain the desired amount in ASW.)

*Collecting aggregate assay for measurement of cell adhesion*

Freshly prepared labeled probe cells (10–50 μl) were added to the Erlenmeyer flasks containing various concentrations of homospecific (or heterospecific) collecting aggregates in a total volume of 2 ml. These were gently shaken on a gyratory shaker (Henkert and Humphreys, 1970) for 2–3 hours at 10–25 rpm. The temperature of

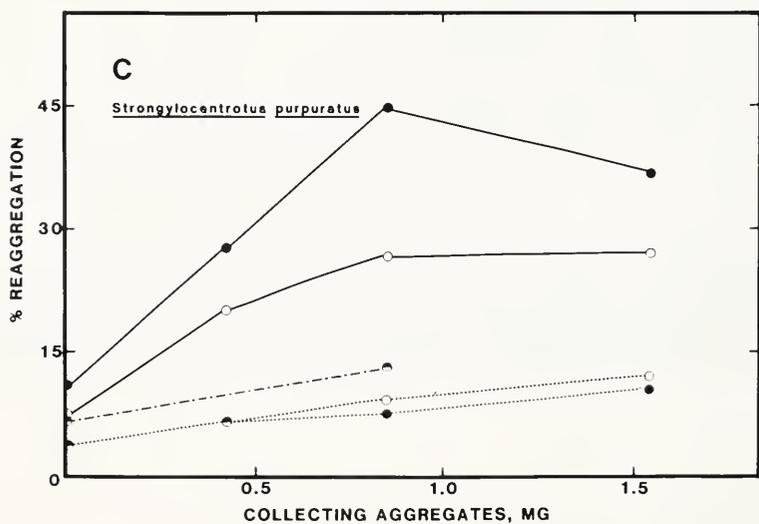
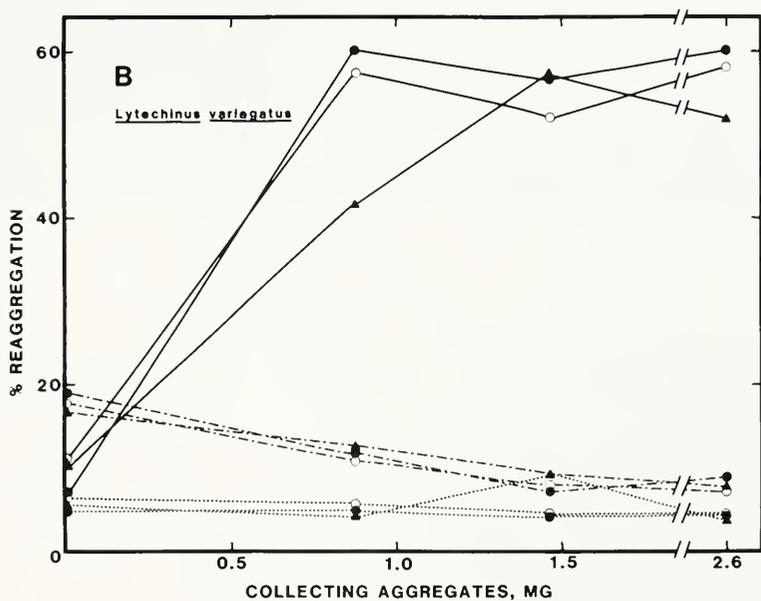
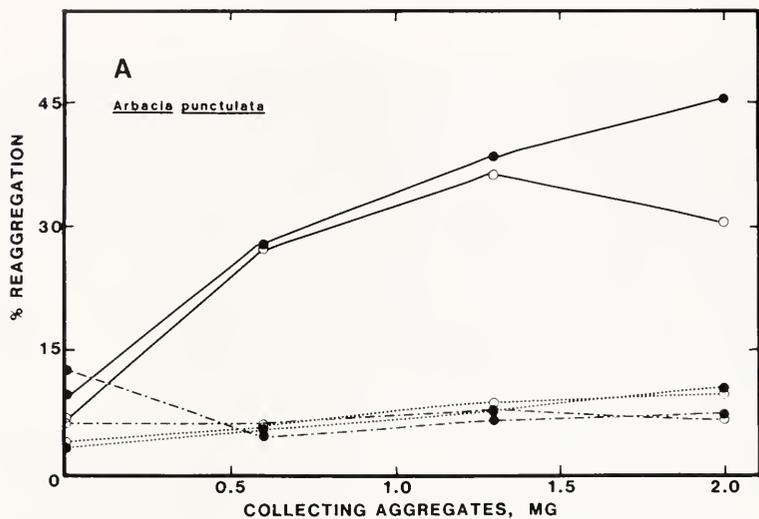
the shaker was controlled in a refrigerated incubator and varied between 16.5°–22°C, depending upon the species used. At the end of the incubation period, the flasks were chilled on ice, and their contents transferred to conical centrifuge tubes. The flasks were washed with 0.5 ml ASW containing 1 mM leucine, and this wash was added to the contents in the centrifuge tubes. Collecting aggregates with adhered probe cells were recovered by hand centrifugation; the aggregates were washed twice with 1 ml of ice cold ASW containing unlabeled leucine. In this manner, three supernatant fractions of 2.5–1.0, and 1.0 ml were obtained; to these were added 10% SDS to a final concentration of 1%. The pelleted aggregates were resuspended in 1 ml of 10% SDS and sonicated. Aliquots of the pellet and supernatant fractions were counted. The percent reaggregation was calculated from the total dpm recovered in the pellet fraction divided by the total dpm recovered ( $\times 100\%$ ). In nearly all cases, 80–100% of the total dpm added to the flasks as probe cells was recovered. The per cent reaggregation was then plotted as a function of the total amount of collecting aggregates (mg of protein) in the assay flask. Although collecting aggregates with adhered probe cells were routinely washed at 0–5°C, trial experiments in which these were washed at the temperature of the adhesion assay yielded similar results. It was decided to routinely wash at the colder temperatures to help prevent “fusion” of aggregates when these were centrifuged into a small packed volume (a problem especially with aggregates of *S. purpuratus*). In early studies the probe cell concentration was varied in addition to that of the collecting aggregates so that the experimental data represented results from single flasks. In later experiments, the probe cell concentration was held constant so that duplicate or triplicate flasks could be assayed for each concentration of aggregates and results could be expressed as the mean plus or minus the standard error.

## RESULTS

### *Adhesion of labeled probe cells to collecting aggregates*

Figure 1 illustrates adhesion of labeled probe cells to their homospecific collecting aggregates for *A. punctulata* (A), *L. variegatus* (B), and *S. purpuratus* (C). A high degree of adhesion is observed for two or three different concentrations of probe cells as a function of the amount of collecting aggregates in the assay, compared to the situations where collecting aggregates were omitted from the assay. In contrast, probe cells which had been sonicated or treated with glutaraldehyde or formaldehyde failed to adhere to the collecting aggregates. Thus, the observed adhesion of probe cells to their homospecific aggregates cannot be due simply to uptake by aggregates of labeled amino acid or proteins from broken or leaky probe cells. In addition, adhesion of probe cells to collecting aggregates appears to require living probe cells. It was of further interest to determine if adhesion in this two-component system also requires living aggregates. Figure 2 illustrates adhesion of *A. punctulata* probe cells to both living and glutaraldehyde-fixed collecting aggregates of *A. punctulata*. Very little adhesion of probe cells to aggregates fixed in glutaraldehyde is observed, in contrast to the living aggregates. Similar data has also been obtained for *L. variegatus* (see Fig. 6) and *S. purpuratus*, although in the latter species, collecting aggregates treated with glutaraldehyde tended to dissociate into single cells, and the recovery of aggregates fixed in glutaraldehyde was sharply reduced (data not shown).

Since adhesion of probe cells to their homospecific aggregates requires both living cells and aggregates, it was important to assess the viability of these in each experiment. As a check on the viability of the probe cells and collecting aggregates, these were routinely returned to Petri dishes containing ASW and gentamicin. The probe cells in all the experiments presented here were observed to reaggregate among themselves



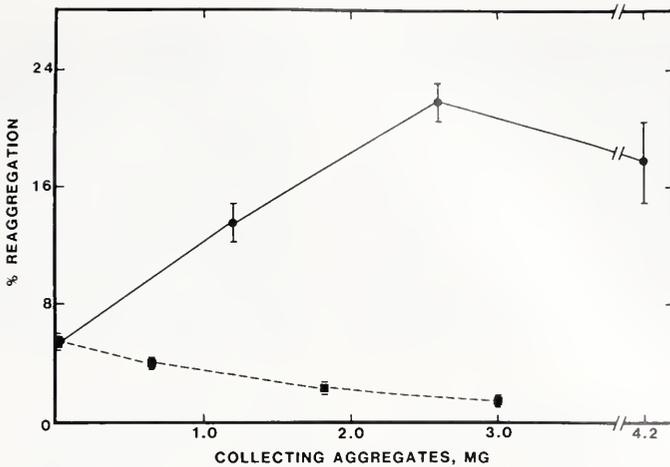


FIGURE 2. Adhesion of labeled probe cells to living and glutaraldehyde-fixed homospesific collecting aggregates. Labeled probe cells from hatched blastulae of *A. punctulata* ( $7.03 \times 10^6$  dpm/mg;  $35 \mu\text{g}$  or  $4.5 \times 10^5$  cells) were incubated at  $16.5^\circ\text{C}$  for 2 h at 10 rpm with the indicated amounts of living (●—●) or glutaraldehyde-treated (■----■) collecting aggregates of *A. punctulata* in a total volume of 2 ml. The data represent results of triplicate assay flasks, plus or minus the standard error of the mean (S.E.M.).

in stationary culture. Moreover, the collecting aggregates were observed to differentiate into embryo-like structures. This process involves transition from a solid mass of adhered cells to blastula-, gastrula-, and finally pluteus-like structures, which are shown for all three species in Figure 3. It can be seen that a very high percentage of these have formed guts, pigment granules, and spicules. Although many of the larger reaggregates contain more than one gut and an abnormal number of arms, a large proportion of the smaller aggregates appear as almost normal plutei. In a few experiments, collecting aggregates without probe cells were shaken for 2–3 h on the gyratory shaker and then returned to stationary culture. Since these were observed also to differentiate into pluteus-like structures, it can be concluded that gentle shaking during the assay does not seriously impair the viability of the collecting aggregates.

FIGURE 1. Adhesion of labeled probe cells to homospesific collecting aggregates. (A) Labeled probe cells ( $4.01 \times 10^6$  dpm/mg) of *A. punctulata* hatched blastulae (●—●,  $169 \mu\text{g}$  or  $21.8 \times 10^5$  cells; ○—○,  $68 \mu\text{g}$  or  $8.75 \times 10^5$  cells) were incubated at  $16.5^\circ\text{C}$  for 2 h at 10 rpm with the indicated amounts of *A. punctulata* collecting aggregates in a total volume of 2 ml. In addition, sonicated probe cells ( $4.29 \times 10^6$  dpm/mg; ●···●,  $157 \mu\text{g}$ ; ○···○,  $63 \mu\text{g}$ ) and probe cells treated with glutaraldehyde ( $3.95 \times 10^6$  dpm/mg; ●- - - - ●,  $82 \mu\text{g}$ ; ○- - - - ○,  $33 \mu\text{g}$ ) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. (B) Labeled probe cells ( $\sim 4.2 \times 10^6$  dpm/mg) of *L. variegatus* hatched blastulae (●—●,  $144 \mu\text{g}$  or  $17.5 \times 10^5$  cells; ○—○,  $58 \mu\text{g}$  or  $7.05 \times 10^5$  cells; ▲—▲,  $29 \mu\text{g}$  or  $3.5 \times 10^5$  cells) were incubated at  $20\text{--}22^\circ\text{C}$  for 2 h at 12 rpm with the indicated amounts of *L. variegatus* collecting aggregates in a total volume of 2 ml. In addition, probe cells treated with formaldehyde ( $\sim 5.1 \times 10^6$  dpm/mg; ●···●,  $94 \mu\text{g}$ ; ○···○,  $38 \mu\text{g}$ ; ▲···▲,  $19 \mu\text{g}$ ) and glutaraldehyde ( $\sim 5.2 \times 10^6$  dpm/mg; ●- - - - ●,  $144 \mu\text{g}$ ; ○- - - - ○,  $58 \mu\text{g}$ ; ▲- - - - ▲,  $28.9 \mu\text{g}$ ) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. (C) Labeled probe cells ( $10.3 \times 10^6$  dpm/mg) of *S. purpuratus* hatched blastulae (●—●,  $99 \mu\text{g}$  or  $13.5 \times 10^5$  cells; ○—○,  $49 \mu\text{g}$ , or  $6.7 \times 10^5$  cells) were incubated at  $16.5^\circ\text{C}$  for 2 h at 20 rpm with the indicated amounts of *S. purpuratus* collecting aggregates in a total volume of 2 ml. In addition, sonicated probe cells ( $11.5 \times 10^6$  dpm/mg; ●···●,  $102 \mu\text{g}$ ; ○···○,  $51 \mu\text{g}$ ) and probe cells treated with glutaraldehyde ( $8.1 \times 10^6$  dpm/mg; ●- - - - ●,  $72 \mu\text{g}$ ; ○- - - - ○,  $36 \mu\text{g}$ ) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. All points in A, B, and C represent data from single assay flasks.

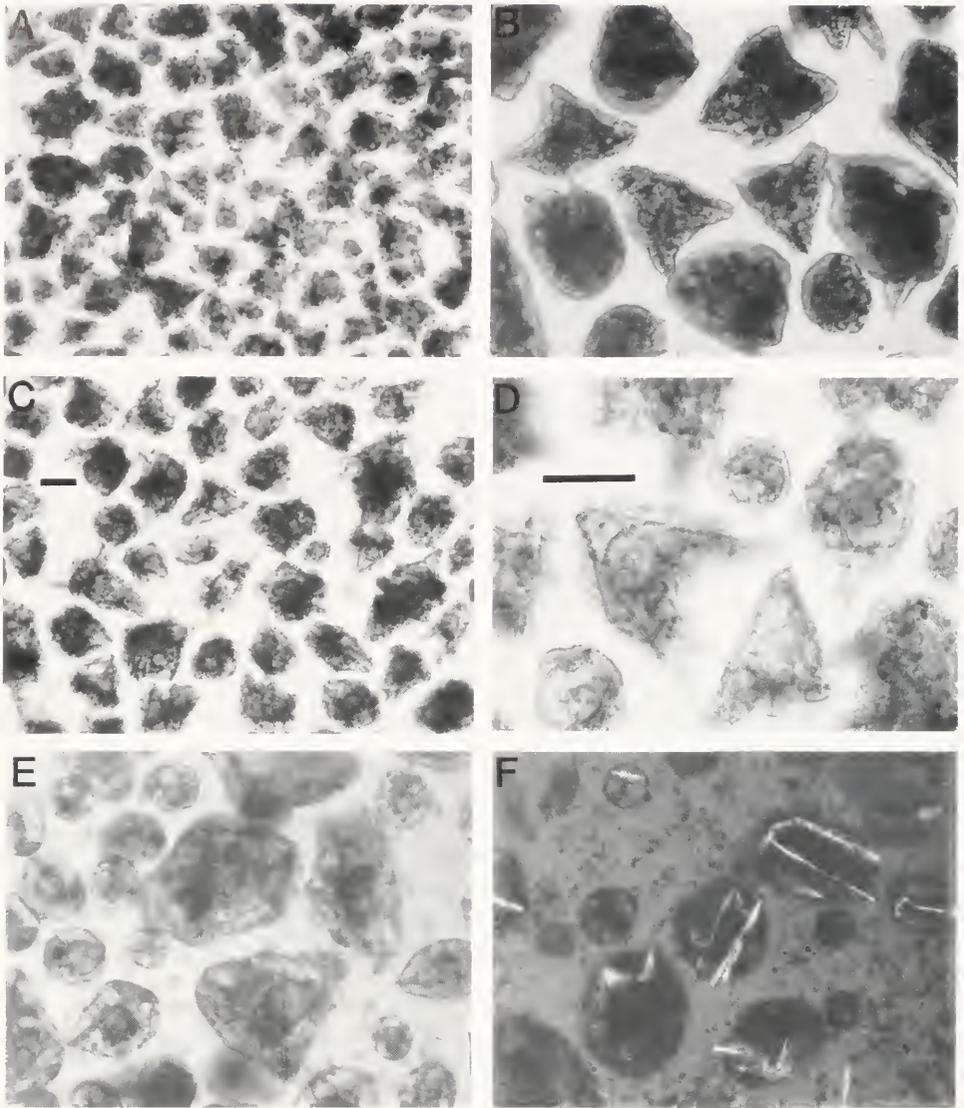


FIGURE 3. Differentiation of collecting aggregates into pluteus-like structures. A and B: *A. punctulata* embryoids after incubation in ASW in stationary culture for two days at 20–22°C. C and D: *L. variegatus* embryoids after incubation in ASW in stationary culture for two days at 20–22°C. E and F: *S. purpuratus* embryoids after incubation in ASW in stationary culture for four days at 16.5°C. All photographs were taken with the aid of a Wild-Heerbrugg phase contrast microscope with a Polaroid camera attachment. A and C: 40× magnification; B, D, E, and F: 100× magnification. The photograph in F was taken with polarized optics to highlight differentiation of the skeleton (spicules). Scale bars = 100  $\mu$ m.

*Species-specificity of adhesion: reaggregation of probe cells to homospecific versus heterospecific collecting aggregates*

Figure 4 illustrates adhesion of labeled probe cells from hatched blastulae of *A. punctulata* to collecting aggregates of *A. punctulata* and *L. variegatus*. A significant

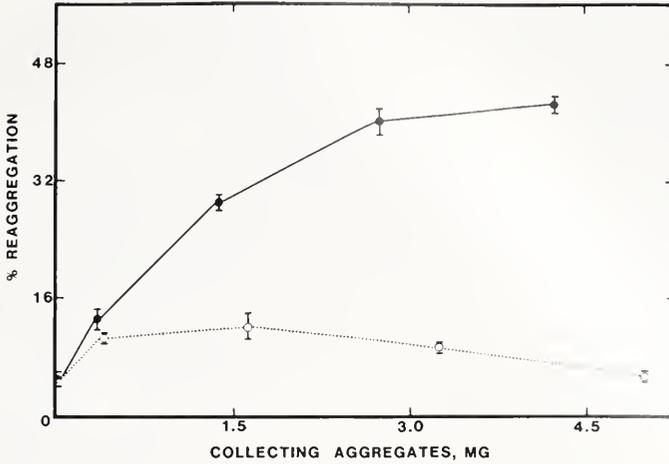


FIGURE 4. Adhesion of labeled probe cells of *A. punctulata* to collecting aggregates of *A. punctulata* and *L. variegatus*. Labeled probe cell from hatched blastulae of *A. punctulata* ( $4.5 \times 10^6$  dpm/mg;  $42 \mu\text{g}$  or  $5.4 \times 10^5$  cells) were incubated at  $18\text{--}20^\circ\text{C}$  for  $2\frac{1}{2}$  h at 12 rpm with the indicated amounts of collecting aggregates of *A. punctulata* (●—●) or *L. variegatus* (○ · · · ○) in a total volume of 2 ml. The data represent results of triplicate assay flasks.

preference for *A. punctulata* collecting aggregates is demonstrated by these probe cells. In a parallel experiment with the same collecting aggregates, probe cells from blastulae of *L. variegatus* demonstrated a preference for collecting aggregates of *L. variegatus* as compared to *A. punctulata* (data not shown).

Figure 5 illustrates adhesion of labeled probe cells from hatched blastulae of *A. punctulata* to collecting aggregates of *A. punctulata* and *S. purpuratus*. A significant

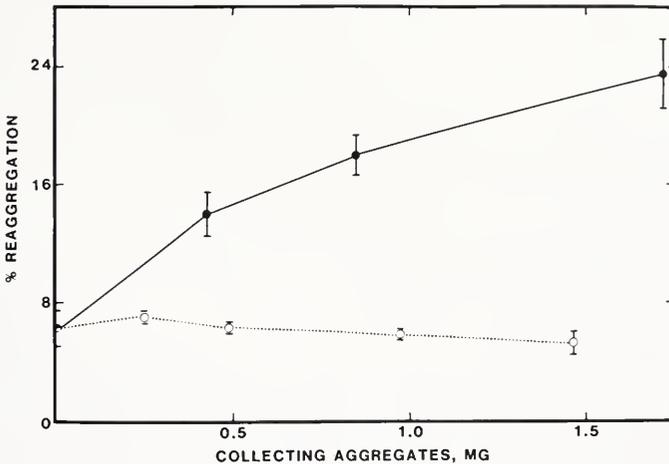


FIGURE 5. Adhesion of labeled probe cells of *A. punctulata* to collecting aggregates of *A. punctulata* and *S. purpuratus*. Labeled probe cells from hatched blastulae of *A. punctulata* ( $10^7$  dpm/mg;  $56 \mu\text{g}$ , or  $7.2 \times 10^5$  cells) were incubated at  $16.5^\circ\text{C}$  for 2 h at 10–12 rpm with the indicated amounts of collecting aggregates of *A. punctulata* (●—●) or *S. purpuratus* (○ · · · ○) in a total volume of 2 ml. The data represent results of duplicate assay flasks.

preference for *A. punctulata* collecting aggregates is again demonstrated by probe cells in this experiment. (It should be noted that the percent reaggregation of probe cells to their homospecific aggregates in this experiment reached a maximum of about 28% at 2.5 mg of aggregates. These data are not shown, since there were not enough *S. purpuratus* aggregates available for a comparison.)

Figure 6 illustrates adhesion of labeled probe cells from hatched blastulae of *L. variegatus* to collecting aggregates of *L. variegatus*, *S. purpuratus*, and *A. punctulata*. In addition, adhesion of probe cells to homospecific aggregates fixed in glutaraldehyde is also investigated. It can be seen that a significant preference for *L. variegatus* aggregates compared to *S. purpuratus* or *A. punctulata* aggregates is demonstrated by these probe cells. Although binding of probe cells to *S. purpuratus* aggregates is somewhat higher than to *A. punctulata* aggregates, the former adhesion is no greater than that seen with homospecific aggregates fixed in glutaraldehyde.

Finally, the species-specificity of adhesion of *S. purpuratus* probes to collecting aggregates was investigated in the experiment shown in Figure 7. Here adhesion of labeled probe cells from gastrulae of *S. purpuratus* to collecting aggregates of *S. purpuratus*, *L. variegatus*, and *A. punctulata* is examined. It can be seen that in this species as well, a significant preference for homospecific compared to heterospecific aggregates is demonstrated. Although the experiment in Figure 7 was done with probes from *S. purpuratus* gastrulae, very similar results have been obtained with probes from *S. purpuratus* blastulae and *S. purpuratus* versus *A. punctulata* collecting aggregates (data not shown).

#### DISCUSSION

The experiments presented here demonstrate reciprocal species-specific adhesion between *A. punctulata* versus *L. variegatus*, *A. punctulata* versus *S. purpuratus*, and *L. variegatus* versus *S. purpuratus*. These results extend those of Giudice (1962) and

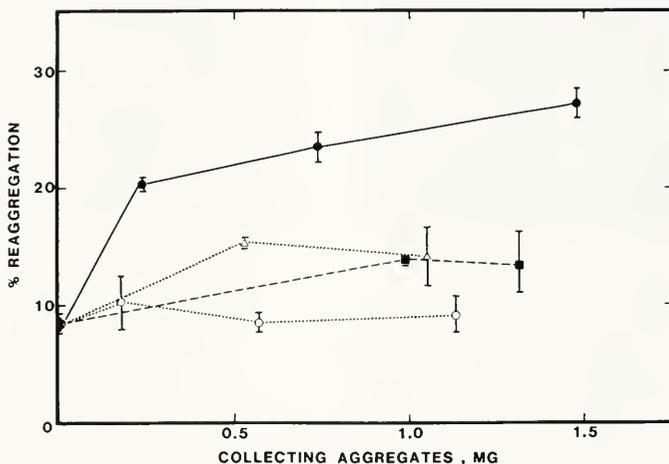


FIGURE 6. Adhesion of labeled probe cells of *L. variegatus* to collecting aggregates of *L. variegatus*, *S. purpuratus*, and *A. punctulata*. Labeled probe cells from hatched blastulae of *L. variegatus* ( $5.1 \times 10^6$  dpm/mg;  $16 \mu\text{g}$  or  $1.9 \times 10^5$  cells) were incubated at  $18^\circ\text{C}$  for 2 h at 20 rpm with the indicated amounts of collecting aggregates of *L. variegatus* (●—●), *S. purpuratus* (△···△), or *A. punctulata* (○···○); and *L. variegatus* collecting aggregates treated with glutaraldehyde (■----■) in a total volume of 2 ml. The data represent results of duplicate assay flasks.

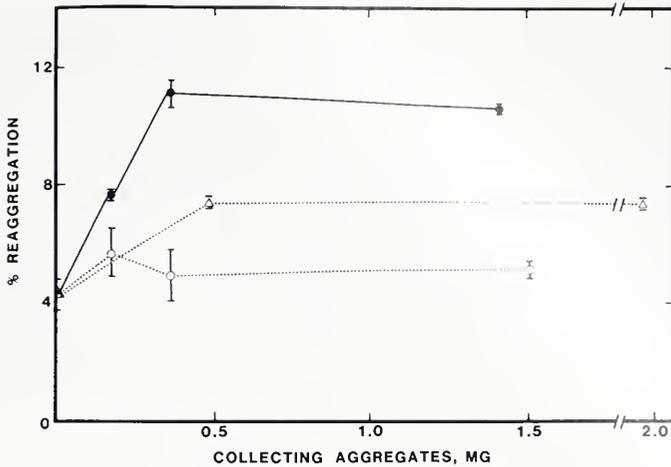


FIGURE 7. Adhesion of labeled probe cells of *S. purpuratus* to collecting aggregates of *S. purpuratus*, *L. variegatus*, and *A. punctulata*. Labeled probe cells from gastrulae of *S. purpuratus* ( $10^7$  dpm/mg; 62  $\mu$ g) were incubated at 18°C for 2 h at 25 rpm with the indicated amounts of collecting aggregates of *S. purpuratus* (●—●), *L. variegatus* ( $\Delta \cdots \Delta$ ), or *A. punctulata* (O  $\cdots$  O) in a total volume of 2 ml. The data represent results of duplicate assay flasks.

Spiegel and Spiegel (1975) who observed species-specific sorting out between *P. lividus* and *A. lixula*, and between *A. punctulata* and *L. pictus*, respectively. Moreover, these results extend those of McClay and Hausman (1975), McClay *et al.* (1977), and McClay (1982) who demonstrated reciprocal species-specific adhesion between *L. variegatus* and *T. eschulentus* using a quantitative collecting aggregate assay similar to the one used in the present studies. The collecting aggregate assay described here differs in several important aspects from the assay used by McClay and co-workers. First, since the separation of collecting aggregates with adhered probe cells from unadhered probe cells in the present study involves a simple centrifugation procedure, the quantities of probe cells and collecting aggregates in these assays are significantly greater (~ten-fold) than the quantities used in the McClay assay. In addition, the collecting aggregates used in these studies are on the average larger than those used in the studies by McClay. Secondly, the shaker speed in the present studies is 10–20 rpm with a 2" radius of gyration, while that in the McClay studies is 70 rpm with a  $\frac{3}{8}$ " radius of gyration (McClay and Baker, 1975). The third important difference in the assay used here compared to that used in the studies by McClay and co-workers is that the present assay measures an end point of adhesion of probe cells to aggregates, not the rate of their adhesion. And finally, McClay and co-workers compared adhesion of different species of probe cells to a single species of collecting aggregates, while in this study adhesion of a single species of probe cell is compared to different species of collecting aggregates.

The present assay offers a few advantages over the assay developed by McClay and co-workers. Since it relies on a simple centrifugation procedure to separate collecting aggregates and probe cells, it eliminates the need for a double filtration procedure and the need to label collecting aggregates with  $^{14}\text{C}$  amino acid in order to correct for aggregates which are lost through the Nitex mesh filter and recovered with unadhered probes. According to McClay and Baker (1975) this loss can be appreciable (up to 75%). An obvious disadvantage of the present assay is that it requires much more

biological material. With regard to differences in shaker speed, as discussed by Henkart and Humphreys (1970), the centrifugal acceleration in a gyratory shaker may be increased by increasing either the shaker speed or the radius of gyration. In the present studies we chose to increase the radius of gyration rather than the shaker speed to minimize the increase of liquid shear forces, which have an adverse effect on cell adhesions. In any event, the results presented in Figure 6 for *L. variegatus* probes and aggregates agree favorably with those of McClay and Hausman (1975) and McClay *et al.* (1977) with regard to maximal extent of adhesion of probe cells to aggregates.

Perhaps the most serious objection which might be raised to the specificity measurements reported here is the possible problem of differences in aggregate size between two species. That is, probe cells may adhere to homospecific collecting aggregates simply because these collecting aggregates are smaller and their relative surface area per mg of protein is larger than for heterospecific aggregates. This problem has been discussed in detail in an early paper by Roth and Weston (1967). These authors have observed that larger aggregates collect even fewer probe cells than the *same number* of smaller aggregates, presumably because the larger aggregates remain in orbits further from the center of the flask while the probe cells gather in the vortex at the center. In answer to these objections, it should be pointed out that when the amount of collecting aggregate is varied in the present assay, the degree of adhesion of probe cells is found to reach a constant maximum level. Hence, observed differences in adhesion due to size differences in aggregates of different species should be minimized by varying the amount of collecting aggregates. In these studies it is found that increasing the amount of heterospecific aggregates in the assay never results in the level of adhesion seen with homospecific aggregates. Secondly, the size distribution of aggregates in all three species is very similar, although aggregates of *S. purpuratus* tend to fuse into macroaggregates during the assay compared to the other two species. In addition, the location of aggregates observed in the flasks during the assay is fairly central rather than peripheral; this is probably due to the large radius of gyration and slow shaker speed. Moreover, as shown by Roth and Weston (1967), even when aggregates of different size are used, isotypic adhesions are found to be significantly greater than heterotypic adhesions. Finally, for every experiment presented here, the reciprocal cross was also done at the same time with probes from the other species and the two types of aggregates. In every case, species-specificity was observed. This is certainly evident in Figures 6 and 7, which were from the same experiment. (It should be noted that the probe cell concentrations of *L. variegatus* and *S. purpuratus* were quite different in this experiment).

Species-specific adhesion as measured by the present assay and that of McClay and Hausman (1975) most probably measures the formation of the most stable adhesions, since the washing procedures used by both assays generate enough shear force to remove all but the most stably adhered probe cells. It is not unreasonable to assume that formation of these adhesions may involve several different steps and that some of these may be more dependent on metabolism (and hence temperature) than others. Evidence in favor of this idea has recently been reported by McClay *et al.* (1981). These workers used neural retina cells in an adhesion assay involving binding of labeled probe cells to stable monolayers in microtiter plates and measured the centrifugal force required to dislodge the probe cells when the plates were sealed and inverted. In this study, these workers identified three types of cellular interactions; the first, an initial binding, occurred at 4°C and exhibited recognition specificity. It is noteworthy that recognition specificity is observed as an initial event by this type of assay. In a presentation at the first symposium for Developmental Biology of the Sea Urchin at the Marine Biological Laboratory in August of 1982, D. R. McClay

presented work on sea urchin adhesion using this new monolayer assay which demonstrated species-specific adhesion between *L. variegatus* and *A. punctulata* at 0–4°C. Preliminary experiments in this laboratory have also demonstrated species-specific adhesion between these species at 0–4°C using the monolayer assay. These observations support the idea suggested earlier that species-specific recognition is an initial event during reaggregation of sea urchin cells and may be followed by later sorting out of cells in the aggregate according to their tissue specificities.

In recent years Noll and co-workers have extracted dissociated sea urchin cells with n-butanol and identified a factor in these extracts which promotes reaggregation of unextracted cells and is essential for reaggregation of extracted cells (Noll *et al.*, 1979, 1981). However, it is not clear what relationship this factor has to species-specific recognition, since the factor itself is not species-specific and will promote reaggregation of both *P. lividus* and *A. lixula* cells. Recently work by McCarthy and Spiegel (1983) has shown that the enhancement of reaggregation of cells by the butanol extract appears to be relatively non-specific, since dissociation supernatant, human plasma fibronectin, and bovine serum albumin all enhance reaggregation in a concentration-dependent manner. Moreover, these workers question the presumed extracellular localization of the factor extracted by butanol. On the other hand, Oppenheimer and Meyer (1982) have identified a component in the dissociation supernatant of sea urchin cells which appears to be both stage and species-specific.

At present it is unclear whether the cell surface molecules assumed to be involved in species-specific recognition in the sea urchin are localized in the hyalin-containing extracellular matrix, the embryonic plasma membrane, or both. Thus far, components identified in the extracellular matrix such as laminin and fibronectin (Spiegel *et al.*, 1983), and collagen (Spiegel and Spiegel, 1979) have not been isolated from sea urchin embryos and tested for possible species-specific effects in a quantitative reaggregation assay. In addition, plasma membranes from embryos or dissociated cells have not been isolated in highly purified form and tested in a quantitative reaggregation assay. Current efforts in this laboratory are aimed at such experiments to localize the species-specific reaggregation factors.

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## THE EFFECTS OF SHELL SIZE AND SHAPE ON GROWTH AND FORM IN THE HERMIT CRAB *PAGURUS LONGICARPUS*

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### ABSTRACT

*Pagurus longicarpus* from two geographic locations were raised in the same environment in three species of gastropod shell. These shell species differed in shape and maximum size. Crabs in small, high-spired shells attained smaller sizes than those in large, low-spired shells. Further, the relative growth rates of male crabs showed differences related to shell differences. Males in small, high-spired shells produced relatively longer claws and greater right/left claw asymmetry than males in large, low-spired shells. These results show the close interaction between hermit crabs and utilized shells and may explain the geographic variation of *P. longicarpus*. Along the Atlantic coast, southern crabs are smaller and have relatively longer claws and greater right/left claw asymmetry than northern crabs. Southern crabs utilize small, high-spired shells almost entirely, whereas northern crabs utilize a high proportion of large, low-spired shells. Size and shape differences between geographic populations of *P. longicarpus* thus may be due to differences in inhabited shells.

### INTRODUCTION

Hermit crabs are anomuran crustaceans which generally inhabit gastropod shells. This shell-living habit has resulted in the modification of many aspects of hermit crab biology (Jackson, 1913; Reese, 1969). Since shells are easily measured and manipulated and since they are a critical resource for hermit crab populations (Provenzano, 1960; Hazlett, 1970; Vance, 1972; Kellogg, 1976; Spight, 1977; Abrams, 1980; Bertness, 1980), research has focused on the hermit crab/shell interaction (see Hazlett, 1981). This interaction has many subtle ramifications. For instance, hermit crab body size and clutch size (and hence fitness) vary depending on the shell inhabited (Markham, 1968; Fotheringham, 1976; Bertness, 1981). This study investigates how the sizes and shapes of *Pagurus longicarpus* hermit crabs vary depending on the sizes and shapes of the inhabited shells.

*P. longicarpus* exhibits geographic variation along the Atlantic coast of North America (e.g., Tables II and VI, Fig. 1). Southern individuals from South and North Carolina tend to be smaller, have relatively longer claws, and exhibit greater right/left claw asymmetry than northern individuals from New Jersey, Long Island Sound, and Massachusetts. The morphological variation of *P. longicarpus* is correlated with differences in the sizes and shapes of the shells used in the different geographic areas. Further, these geographic differences in shell use are due partly to the introduced gastropod *Littorina littorea*. This snail is common in Massachusetts and Long Island Sound, rare in New Jersey, and absent in the Carolinas (Vermeij, 1978, 1982).

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Abbreviations: ASL = anterior shield length, ASW = anterior shield width, RCL = right cheliped length, RPL = right propodus length, RPW = right propodus width, LPL = left propodus length.

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## MATERIALS AND METHODS

*Field sampling*

Samples (Table I) were collected at low tide from the low intertidal zone; to avoid biases every individual within a given area was collected. (Sample area varied depending on the density of the hermit crabs.) Samples were preserved in formalin, and each crab's anterior shield length (ASL) was measured using a dissecting microscope equipped with an ocular micrometer. The ASL is the length of the hard part of the carapace and correlates with total carapace length and body weight (Blackstone, 1984). The length (at maximum parallel to the columellar axis) and the width (at maximum perpendicular to the columellar axis) of each crab's shell was measured using a hand caliper. While factor analysis has been used to characterize the shell resource in hermit crabs (Kuris and Brody, 1976), geographic differences in the shell resource of *P. longicarpus* can be seen by using  $\frac{1}{2}$  (shell length + shell width) to estimate shell size and shell length/shell width to estimate shell shape (*cf.*, Gould, 1977).

*Lab experiments*

In September, 1980, small adult *Pagurus longicarpus* from Beaufort, North Carolina, (n = 67) and Guilford, Connecticut (*i.e.*, Long Island Sound) (n = 98) were collected, isolated in fiberglass mesh aquarium compartments (1 crab per compartment), and raised under constant conditions (temperature = 16°C., photoperiod = 12 hours light/12 hours dark, salinity = 30‰, pH = 8.0) for over three years. The Beaufort sample was taken from an intertidal site; the Guilford sample was taken from an intertidal aggregation of very small crabs.

Each isolated individual was fed every two days during the first year and every three days thereafter. At each feeding each individual was fed to excess with fresh or frozen mussels or occasionally one of several commercial fish foods. Crabs also fed extensively on algae growing on the fiberglass mesh, on detritus which accumulated

TABLE I

*Sites and dates of collections of geographic samples*

Site	Date	n
Nahant, Massachusetts	29 August 1981	136
	31 August 1982	88
Woods Hole, Massachusetts	28 August 1982	122
Guilford, Connecticut	July–September 1980	664
	July–September 1981	299
Cold Spring Harbor, New York	11 September 1982	166
	4 September 1982	146
Barnegat Bay, New Jersey	17 August 1980	148
	20 September 1981	102
Beaufort, North Carolina	7 August 1982	75
	20 September 1980	139
Topsail Inlet, North Carolina	22 August 1982	156
	21 August 1982	114
Carolina Beach, North Carolina	19 August 1982	85
Southport, North Carolina	19 August 1982	136
Little River, South Carolina	18 August 1982	165
Murrell's Inlet, South Carolina	17 August 1982	86
Pawley's Island, South Carolina	16 August 1982	79

in the sandy substrata, and, by climbing the mesh to the surface, on surface zooplankton using the characteristic surface-feeding behavior described by Scully (1978). The isolation of the individuals did not permit courtship and mating.

Each isolated individual was assigned to a shell treatment and given shells accordingly (see below). As individuals molted and grew each molt of each individual was removed, dried, and stored, forming a permanent record of each individual's growth (*cf.*, Fotheringham, 1976). Crabs were sexed from their molts (the female gonopore is at the third pereopod base; sex reversal does not occur). Morphometric data were collected from these molts using a dissecting microscope equipped with an ocular micrometer. Final size was judged by a crab's ASL at the time of its death. (A few individuals died in less than a year and were excluded from the comparisons.) Measurements of the right claw seemed most likely to detect shape differences between crabs of the different shell treatments, since in many hermit crab species this claw exhibits clear "fittedness" to inhabited shells (*e.g.*, Benedict, 1900, Hay and Shore, 1918, Edmondson, 1946). Hence, shape comparisons were made by regressing right cheliped length (RCL; the total length of the major claw) on body size (ASL), by regressing right propodus length (RPL; the length of the last segment or chela of the right cheliped) on body size (ASL), and by regressing right propodus length (RPL) on width (RPW). In males, two additional shape comparisons were made. Right/left symmetry of the chelae was measured by regressing right propodus length (RPL) on left propodus length (LPL). Body shape was measured by regressing anterior shield width (ASW) on length (ASL). These bivariate shape relationships are allometric, and regressions of the natural logarithms of the variables allows comparing relative growth rates.

Three shell treatments were used to test the effects of shells on the 165 isolated individuals. These treatments paralleled the geographic differences in shell use (see Table II and Fig. 1): small, high-spined shells *versus* large, low-spined shells. For the high-spined treatment, crabs collected in high-spined shells in the field (*Nassarius vibex* at Beaufort and *Nassarius trivittatus* at Guilford) were offered several properly fitting shells of *Ilyanassa obsoleta*. At each molt, several larger shells were offered. When the crabs outgrew the maximum size shells of *I. obsoleta*, shells of the similar-shaped but larger *Urosalpinx cinerea* were offered up to its maximum size. (This treatment is hereafter referred to as the *U. cinerea* treatment.)

For the low-spined treatment, Beaufort crabs (necessarily collected in high-spined shells) were offered *Littorina littorea* shells. Those that switched into these shells were confined to them by immediate removal of the vacated original shell. Those that did not switch were used in the high-spined treatment. This procedure was employed because removing hermit crabs from their shells requires heat; this might affect development (Waddington, 1954). This problem did not arise with Guilford crabs, for here low-spined shells (*Littorina* species) were occupied by some of the small crabs collected in the field; these crabs were given shells of *Littorina littorea*. Both the Beaufort and Guilford crabs in this treatment were offered *L. littorea* shells up to the maximum size of this species.

A second low-spined treatment was done with shells of *Polinices duplicatus*. The methodology employed here was similar to that used for the Beaufort *L. littorea* treatment. Crabs were offered *P. duplicatus* shells; if they switched, they were included in this shell treatment. (Few small crabs would switch to *P. duplicatus* shells.)

After two years, shell-switching tests were carried out with the Guilford males used in the above experiments. Males inhabiting maximum-size *U. cinerea* shells were divided into two groups. One group was left in the *U. cinerea* shells, while the other was offered shells of *L. littorea* up to the maximum size. Males inhabiting

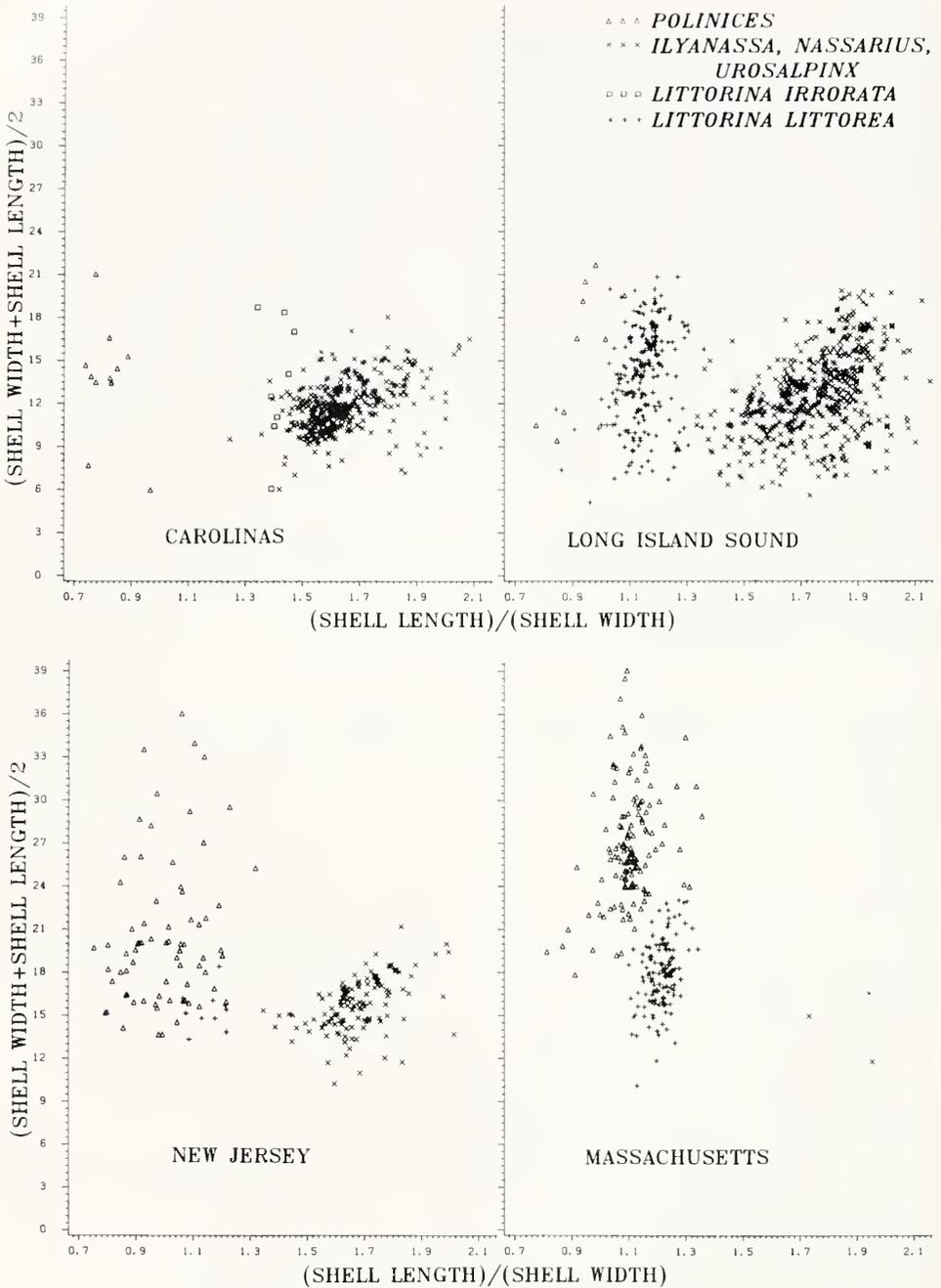


FIGURE 1. For shells inhabited by male *Pagurus longicarpus*,  $\frac{1}{2}$  (shell length + width) approximates shell size and shell length/shell width approximates shell shape; these measures are plotted for shells inhabited in North and South Carolina, Long Island Sound, New Jersey, and Massachusetts. Symbols correspond to the indicated shell species. For easier display, seldom used shell species and the very high-spired shells of *Terebra dislocata* (inhabited in the Carolinas) are excluded. Northern crabs inhabit larger, lower-spired shells. When present, *Littorina littorea* shells bridge the gap in the native shell resource between small, high-spired shells and larger, lower-spired shells.

maximum-size *L. littorea* shells were also divided into two groups. One group was left in the *L. littorea* shells, and the other was offered *P. duplicatus* shells.

For all crabs, maximum shell size was estimated by  $\frac{1}{2}$  (shell length + shell width) of the final occupied shell. Also, measures of the aperture length (at maximum parallel to the aperture lip, excluding the siphonal notch if present), the aperture width (at maximum perpendicular to the aperture length), the length, and the width for a haphazard sample of the shells used in the experiment were taken. Regressing aperture length on aperture width and length on width provided estimates of shell shape. Shells used in the experiments were collected from Guilford, Connecticut, intertidal areas containing many empty shells.

## RESULTS

### Field results

Table II compares the geographic data from South and North Carolina, New Jersey, Long Island Sound, and Massachusetts. Northern crabs are larger and inhabit larger and lower-spired shells than southern crabs. Long Island Sound crabs are stunted as are some other invertebrate taxa in this area (W. D. Hartman, pers. comm.). These data agree with those from other studies (South Carolina, Young, 1979; North Carolina, Mitchell, 1975, Kellogg, 1971, 1976; Rhode Island, Scully, 1979).

TABLE II

*Regional differences in Pagurus longicarpus size, utilized shell size, and utilized shell shape (all measures in mm)*

Sex	Crab size <sup>1</sup> mean ± S.D.	Shell size <sup>2</sup> $\frac{1}{2}$ (length + width) mean ± S.D.	Shell shape <sup>3</sup> length/width mean ± S.D.	n
Southern, South and North Carolina				
Males	2.47 ± 0.58	12.37 ± 2.77	1.88 ± 0.68	408
Females	2.34 ± 0.40	12.04 ± 2.92	1.96 ± 0.75	552
Northern, New Jersey				
Males	4.22 ± 0.84	17.67 ± 4.29	1.41 ± 0.35	208
Females	3.32 ± 0.34	14.85 ± 1.69	1.65 ± 0.11	117
Northern, Long Island Sound				
Males	2.88 ± 0.72	12.95 ± 3.38	1.54 ± 0.33	817
Females	2.32 ± 0.45	10.89 ± 2.58	1.62 ± 0.29	427
Northern, Massachusetts				
Males	5.27 ± 1.27	22.66 ± 6.01	1.16 ± 0.11	268
Females	3.82 ± 0.65	15.69 ± 1.77	1.24 ± 0.14	79

<sup>1</sup> Northern crabs (except Long Island Sound females) are larger than southern crabs ( $P < 0.0001$  Wilcoxon Rank Sum).

<sup>2</sup> Northern shells (except those of Long Island Sound crabs) are larger than southern shells ( $P < 0.0001$  Wilcoxon Rank Sum).

<sup>3</sup> Northern shells are lower spired than southern shells ( $P < 0.0001$  Wilcoxon Rank Sum).

### Lab results

*Size.* There are some differences between the initial sizes of the crabs assigned to the shell treatments (Table III). Beaufort females assigned to *L. littorea* and *P. duplicatus* shells are larger than those assigned to *U. cinerea* shells, while Guilford males assigned to *P. duplicatus* shells are larger than those assigned to the other two shells. These non-random assignments occur in cases where the individuals were able to select their shell treatment. Apparently, smaller crabs have a stronger preference for high-spired shells than larger crabs. This is also shown by shell preference studies (Mitchell, 1975; Blackstone, 1984; Blackstone and Joslyn, 1984).

Because of these differences in initial sizes, both the growth increment data and the final size data should be used to judge the differences between shell treatments. In all cases, the crabs in *L. littorea* shells grew more than those in *U. cinerea* shells. The small sample sizes of crabs in *P. duplicatus* shells hamper comparisons to the other shell treatments, but in the case of the Guilford crabs, those in *P. duplicatus* grew significantly more than those in the other shells. There are no significant differences in the lifespans of crabs grown in the different shells.

The shell-switching experiments provided similar data (Table III). Guilford males raised in *U. cinerea* shells, then offered *L. littorea* shells attained larger sizes than males held in *U. cinerea* shells. Guilford males raised in *L. littorea* shells and offered

TABLE III

*Sizes of Pagurus longicarpus raised in three shell types (all measures in mm)*

Location	Sex	Shell	n	Initial size (mean ASL ± S.D.)	Final size (mean ASL ± S.D.)	Growth <sup>1</sup> (mean ± S.D.)	Days <sup>2</sup> (mean ± S.D.)
Beaufort	F	<i>Urosalpinx</i>	21	2.32 ± 0.25	4.28 ± 0.32	1.96 ± 0.37	877 ± 368
		<i>Littorina</i>	19	2.46 ± 0.19	5.05 ± 0.60	2.58 ± 0.64	1018 ± 393
		<i>Polinices</i>	1	2.90	5.00	2.10	542
		ANOVA <sup>3</sup> P=		0.02	<0.0001	0.002	0.30
Guilford	F	<i>Urosalpinx</i>	26	1.88 ± 0.26	4.55 ± 0.42	2.67 ± 0.57	1174 ± 332
		<i>Littorina</i>	24	1.88 ± 0.28	5.21 ± 0.40	3.34 ± 0.50	1245 ± 219
		<i>Polinices</i>	5	2.02 ± 0.26	6.00 ± 0.19	3.98 ± 0.43	1335 ± 47
		ANOVA P=		0.53	<0.0001	<0.0001	0.40
Beaufort	M	<i>Urosalpinx</i>	10	2.64 ± 0.40	5.61 ± 0.24	2.97 ± 0.48	606 ± 185
		<i>Littorina</i>	11	2.43 ± 0.46	6.10 ± 0.93	3.67 ± 0.92	669 ± 347
		<i>Polinices</i>	4	2.83 ± 0.51	6.45 ± 0.96	3.63 ± 1.10	492 ± 151
		ANOVA P=		0.28	0.13	0.13	0.53
Guilford	M	<i>Urosalpinx</i>	8	1.81 ± 0.18	5.62 ± 0.64	3.81 ± 0.68	1078 ± 369
		<i>Littorina</i>	13	1.75 ± 0.25	6.44 ± 0.55	4.69 ± 0.63	1108 ± 287
		<i>Polinices</i>	7	2.03 ± 0.17	8.27 ± 0.83	6.24 ± 0.87	1005 ± 231
		ANOVA P=		0.04	<0.0001	<0.0001	0.74
Guilford <sup>4</sup>	M	U/U	6	5.33 ± 0.28	5.88 ± 0.50	0.55 ± 0.28	642 ± 142
		U/L	8	5.51 ± 0.36	6.45 ± 0.39	0.94 ± 0.51	606 ± 212
		ANOVA P=		0.34	0.03	0.12	0.73
		L/L	9	6.07 ± 0.42	6.68 ± 0.42	0.61 ± 0.31	585 ± 158
		L/P	7	6.30 ± 0.29	7.51 ± 0.82	1.21 ± 0.57	558 ± 221
	ANOVA P=		0.23	0.02	0.02	0.79	

<sup>1</sup> Total growth increment.

<sup>2</sup> Days from first molt until death.

<sup>3</sup> ANOVA of the three shell treatments; P is the probability that the treatments are the same.

<sup>4</sup> Shell-switching experiments: U/U = *Urosalpinx* to *Urosalpinx* (control), U/L = *Urosalpinx* to *Littorina* (test), L/L = *Littorina* to *Littorina* (control), L/P = *Littorina* to *Polinices* (test).

*P. duplicatus* shells grew more and attained larger sizes than males held in *L. littorea* shells.

For final shell size, Table IV shows that *P. duplicatus* shells are significantly larger than shells of the two other species, but *L. littorea* shells are not significantly larger than *U. cinerea* shells. It may be that shell-type affects crab growth and size (see Bertness, 1981).

Individuals from the same locality and shell treatment varied in final size (see standard deviations in Table III). In the *U. cinerea* and *L. littorea* shell treatments, the crabs outgrew the largest shells available, and there were slight differences among individuals in the sizes of their final shells (see standard deviations in Table IV). But Table V shows that there is no within-shell treatment correlation between final crab size and final shell size in these shell treatments. A correlation is present with the *P. duplicatus* treatments, but this likely results from the preferences of the individuals; in this treatment larger shells were always available.

*Shape.* Table VI shows bivariate regressions of the log-transformed data from the molts of the lab-grown individuals. The slope of each regression represents rate at which the shape relationship between the two variables changes throughout the ontogeny (Huxley, 1932; Gould, 1966). A slope greater than unity indicates that the body part represented by the dependent variable grows relatively faster than the body part represented by the independent variable. In terms of actual shape, the body part represented by the dependent variable would become relatively longer throughout the ontogeny. A slope less than unity indicates that the dependent variable grows relatively slower than the independent variable; thus that body part would become relatively shorter throughout the ontogeny. A slope equal to unity entails no relative growth differences between the parts in question and no shape changes during the ontogeny.

The data of Table VI show a number of general patterns. First, there are regularities of growth which have been found in other hermit crabs (Bush, 1930) and other decapod crustaceans (Huxley, 1932), e.g., the right claw (RCL) grows faster than the body (ASL), and in most cases the chela of the right claw (RPL) grows relatively still faster. Second, there are differences between the sexes, and the males show greater relative growth of the claws (this is also true of other decapods, see Bush, 1930;

TABLE IV

Final shell size (in mm) of male *Pagurus longicarpus* raised in three shell species

Location	Shell	n	Final shell size $\frac{1}{2}(\text{length} + \text{width})$ mean $\pm$ S.D.	ANOVA <sup>1</sup> <i>P</i> =	Pairwise <i>t</i> -tests <sup>2</sup> <i>P</i> =		
					U	L	P
Beaufort	<i>Urosalpinx</i>	10	21.43 $\pm$ 0.46	0.0001	—	0.74	0.0001
	<i>Littorina</i>	11	21.57 $\pm$ 0.85			—	0.0001
	<i>Polinices</i>	4	26.98 $\pm$ 1.74			—	—
Guilford	<i>Urosalpinx</i>	8	21.04 $\pm$ 0.68	0.0001	—	0.23	0.0001
	<i>Littorina</i>	13	21.95 $\pm$ 1.21			—	0.0001
	<i>Polinices</i>	7	28.74 $\pm$ 2.85			—	—

<sup>1</sup> ANOVA of the three shells: *P* is the probability that the shells are the same size.

<sup>2</sup> Paired *t*-tests: U = *Urosalpinx*, L = *Littorina*, P = *Polinices*, *P* is the probability that the pair of means is the same; thus, *P* = 0.74 that the means of the Beaufort *Urosalpinx* and *Littorina* treatments are the same, etc.

TABLE V

Within shell treatment correlation of final shell and crab size in male *Pagurus longicarpus* raised in three shell species

Location	Shell	Regression <sup>1</sup>	n	R <sup>2</sup>	Significance level	
					F	P
Beaufort	<i>Urosalpinx</i>	Y = -0.09X + 7.45	10	0.03	0.22	0.65
	<i>Littorina</i>	Y = 0.27X + 0.31	11	0.07	0.68	0.43
	<i>Polinices</i>	Y = 0.53X - 7.73	4	0.91	19.76	0.05
Guilford	<i>Urosalpinx</i>	Y = 0.31X - 0.80	8	0.10	0.70	0.43
	<i>Littorina</i>	Y = 0.08X + 4.58	13	0.03	0.39	0.54
	<i>Polinices</i>	Y = 0.27X + 0.65	7	0.83	24.39	0.004

<sup>1</sup> Y = crab anterior shield length, X = 1/2(shell length + width).

Huxley, 1932). Third, there are consistent differences between the Beaufort and Guilford crabs, and the latter exhibit reduced relative growth.

There are also differences among the shell treatments. Guilford males show clear trends. Regressing RCL on ASL shows that males in *U. cinerea* shells have a higher rate of right claw growth relative to body growth than do males in *L. littorea* and *P. duplicatus* shells. Males in *L. littorea* shells have an intermediate rate, but this rate is not significantly higher than the rate of males in *P. duplicatus* shells. (As with the size comparisons, shape comparisons of crabs in *P. duplicatus* shells are hampered by small sample sizes.) Regressing RPL on ASL shows that males in *U. cinerea* shells have a higher rate of right chela growth relative to body growth than do males in *L. littorea* and *P. duplicatus* shells. Males in *L. littorea* shells again have an intermediate rate. Regressing RPL on LPL shows that males in *U. cinerea* shells have a higher rate of right chela growth relative to left chela growth than do males in *L. littorea* and *P. duplicatus* shells. Males in *L. littorea* shells have an intermediate rate. Regressing ASW on ASL shows that males in *U. cinerea* shells have a higher rate of body width growth relative to body length growth than do males in *L. littorea* and *P. duplicatus* shells. (Males in *U. cinerea* shells exhibit a slightly positively allometric rate, while those in *L. littorea* and *P. duplicatus* shells exhibit a slightly negatively allometric rate.) Finally, regressing RPL on RPW shows that males in *U. cinerea* shells have the same rate of right chela length growth relative to right chela width growth as do males in *L. littorea* and *P. duplicatus* shells.

These differences in relative growth rate translate into differences in shape. Males in *U. cinerea* shells have longer claws, greater right/left asymmetry of claws, and wider bodies than males in *L. littorea* and *P. duplicatus* shells, while males in *L. littorea* shells are intermediate in shape.

Guilford males used in the shell-switching experiments show shape changes which parallel these between shell treatment differences. Table VII shows the results from crabs raised in *L. littorea* shells and then either held in these shells or offered *P. duplicatus*. Regression of RCL or RPL on ASL show that for the control crabs the rate of right claw growth relative to body growth decreases, but not significantly, while for the test crabs this rate decreases significantly.

Overall, the patterns seen in Guilford males apply to Beaufort males, though some differences exist, possibly because of the smaller sample sizes: (1) while differences in the rate of cheliped growth (RCL regressed on ASL), chela growth (RPL regressed on ASL), and right/left chela growth (RPL regressed on LPL) show the same polarity

as in the Guilford males, these differences are not significant in all comparisons, (2) the rate of body width relative to body length growth (ASW regressed on ASL) shows no significant differences between shell treatments, and (3) the rate of claw length relative to width growth (RPL regressed on RPW) shows slight differences between shell treatments and males in *L. littorea* have a faster rate than males in the other shells.

Females show a different pattern than males. Except for very slight differences in the *P. duplicatus* treatment (which are likely due to sampling biases), Guilford females do not show among shell differences in the rates of shape changes of the variables measured. Beaufort females show insignificant differences in the rates of claw growth (RCL or RPL regressed on ASL) and show significant differences in the rates of claw length relative to width growth (RPL regressed on RPW). As with the Beaufort males, females in *L. littorea* shells have a faster rate than those in *U. cinerea* shells.

Table VIII presents the data on the aperture shape (aperture length regressed on width) and shell shape (length regressed on width) of the sample of shells used in the experiments. Shells used in the high-spined treatment (*I. obsoleta* and *U. cinerea*) are long and narrow with long, narrow apertures. *L. littorea* shells are short and wide with short, wide apertures. *P. duplicatus* shells are very short and wide, but have somewhat long apertures.

## DISCUSSION

Markham (1968) and Fotheringham (1976) show that hermit crabs given larger shells attain larger sizes than those given small shells. Drapkin (1963) makes similar statements, but does not present quantitative data. Bertness (1981) shows that the type of shell inhabited by a hermit crab can influence its growth. The data presented here agree with the results of these workers. These data also show that a hermit crab can continue to grow once its shell has become too small. For instance, the control males in the shell-switching experiments were too large for their shells at the onset of the experiment, but continued to grow (see Table III), albeit more slowly than crabs given shells which favored growth.

These data also provide insight into whether hermit crabs are morphologically molded by their inhabited shells. Goldschmidt (1940) summarized evidence to support this idea. Much of this evidence, however, has been discredited (for instance, see discussion in Wolff, 1961). Further, Thompson (1904) concluded that hermit crabs could not be molded by their inhabited shells. Nevertheless, the molding hypothesis is still suggested by some (e.g., see Elwood *et al.*, 1979).

If the molding hypothesis is correct, the shape of a crab should change with growth to better conform to the shape of its inhabited shell. Both aperture shape and total shell shape would affect the crabs' shape. Long, narrow, highly asymmetric shells with long, narrow apertures present a crab with a living space which is elliptical in circumference, long in length, and oriented asymmetrically. Shorter, wider, more symmetric shells with rounder apertures present a crab with a living space which is more circular in circumference, shorter in length, and oriented more symmetrically. Thus crabs which inhabited *I. obsoleta* and *U. cinerea* shells should develop longer and more asymmetric appendages and flatter, wider bodies than those which inhabited *L. littorea* or *P. duplicatus* shells. Additionally, the right chela, which serves as an operculum, should be molded to fit the aperture shape; crabs in *I. obsoleta* and *U. cinerea* shells should have long, narrow chelae, while those in *L. littorea* and *P. duplicatus* shells should have shorter, wider chelae.

TABLE VI

*Shape regressions of molts of Pagurus longicarpus raised in three shell types*

Variables <sup>1</sup>	Shell <sup>2</sup>	Regression	Molts	R <sup>2</sup>	ANCOVA <sup>3</sup> P =	Orthogonal contrasts <sup>4</sup>	
						<i>Littorina</i> P=	<i>Polinices</i> P=
Beaufort males							
X = log(ASL)	U	Y = 1.48X + 0.98	85	0.98	0.0002	0.03	0.002
Y = log(RCL)	L	Y = 1.39X + 1.07	115	0.98	—	—	0.06
	P	Y = 1.30X + 1.19	30	0.99	—	—	—
X = log(ASL)	U	Y = 1.47X + 0.01	85	0.98	0.001	0.66	0.008
Y = log(RPL)	L	Y = 1.44X + 0.03	115	0.98	—	—	0.008
	P	Y = 1.31X + 0.20	30	0.99	—	—	—
X = log(LPL)	U	Y = 1.38X - 0.06	81	0.98	0.0001	0.0001	0.001
Y = log(RPL)	L	Y = 1.28X + 0.11	114	0.99	—	—	0.88
	P	Y = 1.27X + 1.12	30	0.99	—	—	—
X = log(RPW)	U	Y = 1.27X + 0.60	85	0.97	0.0005	0.04	0.49
Y = log(RPL)	L	Y = 1.34X + 0.51	115	0.98	—	—	0.03
	P	Y = 1.19X + 0.64	30	0.98	—	—	—
X = log(ASL)	U	Y = 1.00X + 0.05	85	0.99	0.08	0.12	0.98
Y = log(ASW)	L	Y = 0.98X + 0.08	114	0.99	—	—	0.23
	P	Y = 1.00X + 0.05	33	0.99	—	—	—
Guilford males							
X = log(ASL)	U	Y = 1.37X + 1.07	189	0.99	0.0001	0.0007	0.0002
Y = log(RCL)	L	Y = 1.31X + 1.12	273	0.99	—	—	0.18
	P	Y = 1.25X + 1.15	99	0.99	—	—	—
X = log(ASL)	U	Y = 1.42X + 0.02	189	0.99	0.0001	0.0001	0.0001
Y = log(RPL)	L	Y = 1.35X + 0.09	273	0.99	—	—	0.005
	P	Y = 1.27X + 0.16	98	0.99	—	—	—
X = log(LPL)	U	Y = 1.25X + 0.10	186	0.99	0.0001	0.002	0.0001
Y = log(RPL)	L	Y = 1.22X + 0.14	269	0.99	—	—	0.03
	P	Y = 1.18X + 0.18	99	0.99	—	—	—
X = log(RPW)	U	Y = 1.14X + 0.69	189	0.98	0.95	0.93	0.39
Y = log(RPL)	L	Y = 1.13X + 0.69	273	0.99	—	—	0.33
	P	Y = 1.13X + 0.67	99	0.99	—	—	—
X = log(ASL)	U	Y = 1.01X + 0.04	187	0.99	0.0001	0.006	0.0002
Y = log(ASW)	L	Y = 0.98X + 0.06	264	0.99	—	—	0.07
	P	Y = 0.97X + 0.08	97	0.99	—	—	—
Beaufort females							
X = log(ASL)	U	Y = 1.19X + 1.19	226	0.97	0.18	0.28	0.57
Y = log(RCL)	L	Y = 1.16X + 1.22	218	0.98	—	—	0.72
	P	Y = 1.12X + 1.27	8	0.99	—	—	—
X = log(ASL)	U	Y = 1.23X + 0.16	226	0.95	0.15	0.17	0.19
Y = log(RPL)	L	Y = 1.20X + 0.20	218	0.98	—	—	0.29
	P	Y = 1.08X + 0.35	8	0.99	—	—	—
X = log(RPW)	U	Y = 1.01X + 0.74	226	0.97	0.0001	0.006	0.45
Y = log(RPL)	L	Y = 1.08X + 0.69	218	0.97	—	—	0.20
	P	Y = 0.96X + 0.81	8	0.99	—	—	—
Guilford females							
X = log(ASL)	U	Y = 1.11X + 1.25	396	0.98	0.77	0.47	0.62
Y = log(RCL)	L	Y = 1.10X + 1.26	364	0.99	—	—	0.93
	P	Y = 1.10X + 1.26	80	0.98	—	—	—

TABLE VI (Continued)

Variables <sup>1</sup>	Shell <sup>2</sup>	Regression	Molts	R <sup>2</sup>	ANCOVA <sup>3</sup> P =	Orthogonal contrasts <sup>4</sup>	
						<i>Littorina</i> P =	<i>Polinices</i> P =
Guilford females							
X = log(ASL)	U	Y = 1.15X + 0.19	396	0.98	0.80	0.15	0.43
Y = log(RPL)	L	Y = 1.15X + 0.22	366	0.98	—	—	0.97
	P	Y = 1.14X + 0.22	81	0.98	—	—	—
X = log(RPW)	U	Y = 1.02X + 0.70	396	0.99	0.57	0.70	0.01
Y = log(RPL)	L	Y = 1.02X + 0.70	367	0.99	—	—	0.007
	P	Y = 1.01X + 0.74	81	0.99	—	—	—

<sup>1</sup> ASL = anterior shield length, LPL = left propodus length, RPW = right propodus width, RCL = right cheliped length, RPL = right propodus length, ASW = anterior shield width.

<sup>2</sup> U = *Urosalpinx*, L = *Littorina*, P = *Polinices*.

<sup>3</sup> ANCOVA tests for homogeneity of slopes between the three shell treatments: P is the probability that the slopes are the same; thus, for log(RCL) regressed on log(ASL) for Beaufort males, P = 0.0002 that the slopes of the *Urosalpinx*, *Littorina*, and *Polinices* regressions are the same, etc.

<sup>4</sup> Orthogonal contrasts between indicated pairs of shell treatments: P is the probability that the treatments are the same; thus, for log(RCL) regressed on log(ASL) for Beaufort males, P = 0.03 that the *Urosalpinx* and *Littorina* treatments are the same; P = 0.002 that the *Urosalpinx* and *Polinices* treatments are the same, etc.

In some ways, the data presented here agree with these predictions. Male crabs raised in *U. cinerea* shells did grow relatively longer chelipeds, longer chelae, and greater right/left asymmetry of chelae than those raised in *L. littorea* or *P. duplicatus* shells. Also, the Guilford males in *U. cinerea* shells grew wider bodies (ASW regressed on ASL) than the other crabs. The growth of the right chela (RPL regressed on RPW), however, does not conform to the predictions of the molding hypothesis. In Guilford males, crabs in *U. cinerea* shells do not grow narrower chelae than crabs in *L. littorea* or *P. duplicatus* shells. In Beaufort males, crabs in *L. littorea* shells grew narrower chelae than crabs in *U. cinerea* shells. This is surprising in view of the tendency for

TABLE VII

Shape regressions of molts of Guilford male *Pagurus longicarpus* used in the shell-switching experiments

Original shell/test shell <sup>1</sup>	Regression	Molts	R <sup>2</sup>	ANCOVA <sup>2</sup>		
				F	P	
X = log(anterior shield length), Y = log(right cheliped length)						
<i>Littorina/Littorina</i>	Before	Y = 1.32X + 1.12	108	0.99	0.75	0.39
	After	Y = 1.22X + 1.27	39	0.66		
<i>Littorina/Polinices</i>	Before	Y = 1.33X + 1.09	77	0.99	9.90	0.002
	After	Y = 1.09X + 1.50	32	0.90		
X = log(anterior shield length), Y = log(right propodus length)						
<i>Littorina/Littorina</i>	Before	Y = 1.36X + 0.09	108	0.99	2.33	0.13
	After	Y = 1.17X + 0.41	39	0.61		
<i>Littorina/Polinices</i>	Before	Y = 1.36X + 0.07	77	0.99	8.48	0.004
	After	Y = 1.12X + 0.48	32	0.87		

<sup>1</sup> For each group, regressions are presented for rate before and after shells were switched.

<sup>2</sup> ANCOVA test for homogeneity of slopes: P is the probability that the before and after slopes are the same.

TABLE VIII

Shape regressions from a sample of the shells used to raise *Pagurus longicarpus*

Shell species <sup>1</sup>	n	Aperture shape <sup>2</sup>		Shell shape <sup>3</sup>	
		Regression	R <sup>2</sup>	Regression	R <sup>2</sup>
<i>Ilyanassa obsoleta</i>	164	$Y_1 = 2.15X_1 + 0.29$	0.93	$Y_2 = 2.14X_2 - 3.33$	0.96
<i>Urosalpinx cinerea</i>	92	$Y_1 = 1.62X_1 + 1.53$	0.64	$Y_2 = 1.59X_2 + 3.55$	0.75
<i>Littorina littorea</i>	229	$Y_1 = 1.20X_1 + 1.35$	0.90	$Y_2 = 1.15X_2 + 0.13$	0.96
<i>Polinices duplicatus</i>	85	$Y_1 = 1.41X_1 + 0.70$	0.99	$Y_2 = 0.96X_2 - 1.36$	0.96

<sup>1</sup> The first two species constituted the high-spined treatment.

<sup>2</sup>  $X_1$  = aperture width,  $Y_1$  = aperture length.

<sup>3</sup>  $X_2$  = shell width,  $Y_2$  = shell length.

globose shell-living hermit crabs to have wide, operculate chelae (e.g., *Pagurus pollicaris*, *Coenobita compressus*, *Calcinus obscurus*, for other examples see Benedict, 1900, Hay and Shore, 1918, Edmondson, 1946).

Further problems with the molding hypothesis are apparent when males in *L. littorea* and *P. duplicatus* shells are compared. The latter shell is somewhat more globose and low-spined than the former but the shape differences are not as dramatic as those between *L. littorea* and *U. cinerea* shells (Table VIII). However, males in *P. duplicatus* shells differ as much in relative cheliped growth (RCL regressed on ASL), relative chela growth (RPL regressed on ASL), and right/left chela growth (RPL regressed on LPL) from males grown in *L. littorea* shells as these males differ from those grown in *U. cinerea* shells (Table VI).

The data from female *P. longicarpus* are inconsistent with the molding hypothesis. In Guilford females, shell shape has no effect on relative cheliped growth (RCL regressed on ASL), on relative chela growth (RPL regressed on ASL), or on the growth of the right chela (RPL regressed on RPW). In Beaufort females, the only significant effect is on the growth of the right chela, and the differences are the opposite of those expected from the molding hypothesis (crabs raised in *L. littorea* shells grow narrower chelae than crabs in *U. cinerea* shells).

The variation between the sexes suggests another explanation for the differences in claw length between males of the different shell treatments. Male hermit crabs fight for mates and large males succeed more than small males (see Hazlett, 1981; this is particularly true of *Pagurus longicarpus*, Thompson, 1904, and pers. obs.). It may be that small-shelled males which are unable to grow large in absolute size, compensate by growing a relatively longer right claw. Since the right claw is the main instrument of combat (e.g., see Reese, 1983), males with a relatively longer claw might be more successful than those with a shorter claw. Selection may have favored a growth program in males in which stunting in size results in increased relative growth of the right claw.

This sexual selection hypothesis, however, does not explain why the Beaufort females should show greater differences in shape between shell treatments than the Guilford females (Table VI). A final hypothesis can explain these differences as well as the differences between the sexes. Size, shape, and developmental timing are interrelated phenomena not only evolutionarily (e.g., Gould, 1977; Bonner and Horn, 1982) but developmentally as well. Vermeij (1980) summarizes the results of field studies which show this interrelationship in gastropods. Generally, slower growth correlates with smaller final size and faster relative growth. A field experiment by

Kemp and Bertness (1984) supports these findings. They grew *Littorina littorea* snails under crowded and less crowded conditions. The crowded snails grew more slowly and exhibited increased negative allometry. (The slopes of the double logarithmic regressions were all less than unity, and the crowded snails had reduced slopes as compared to the normal snails.) These results are similar to those presented here. In both cases, stunting individual's growth rates through unfavorable environmental conditions (crowding in the case of the snails, small shells in the case of the hermit crabs) resulted in slower total growth of the body, but more rapid relative growth (= change in body shape). The hermit crab results would further suggest that the greater the degree of allometry the greater the effects of stunting. Males have greater positive allometry than females (Bush, 1930; Huxley, 1932; Table VI) and hence display greater differences between shell treatments. Beaufort females have greater positive allometry than Guilford females (Table VI) and hence display more similarity to the males.

The results of Ray (1960) are somewhat different. Working with a number of taxa, Ray grew individuals at low and high temperatures. At low temperatures individuals grew more slowly, exhibited reduced positive allometry, and achieved larger final size. The correlation between larger size and reduced allometry is expected and agrees with results presented here. However, the correlation of slower growth with reduced positive allometry differs from results presented here and in Kemp and Bertness (1984). Possibly, there are fundamentally different effects associated with slowed growth caused by slowed metabolic processes as opposed to slowed growth caused by crowding and stunting.

The relationship between body growth rate and relative growth rate needs further clarification. Because the growth of a part is often judged relative to the growth of the body (Huxley, 1932; Gould, 1966; Table VI), a correlation between faster body growth and slower relative growth could be spurious. For instance, regressing RCL on ASL to judge the relative growth of the claw automatically implies that if the growth rate of ASL increases, the relative growth rate of RCL decreases. However, Guilford females in small shells exhibit reduced body growth but no change in relative growth, and male crabs exhibit changes in the growth of the chelae relative to each other (RPL regressed on LPL). The inverse relationship between body growth and relative growth is more than just a simple mathematical tautology; rather, this interaction indicates the existence of underlying developmental mechanisms. Perhaps negative feedback systems controlling growth act to maintain specific relationships between body growth and relative growth (*cf.* Stebbing and Heath, 1984).

Laird *et al.* (1968) and Barton and Laird (1969) propose that allometry is the result of temporal, not spatial, growth gradients. This suggests that hermit crabs in small shells exhibit an accelerated growth program. Perhaps as the small shell causes premature curtailment of normal body growth, homeostatic feedback mechanisms cause premature acceleration of normal appendage growth. Thus a small crab is produced with the shape that would normally be found in a large crab, *i.e.*, longer appendages and greater right/left asymmetry.

The interrelationship of growth rate, relative growth, and final size is of significance to a mechanistic understanding of general processes of growth and form. Further experimental manipulation of organisms' environments would doubtless provide additional insight into how these parameters equilibrate during an organism's development. Because hermit crabs are uniquely dependent on the gastropod shells they occupy and because these shells can be easily measured and manipulated, hermit crabs are ideal subjects for further research in this area.

*Geographic variation in Pagurus longicarpus*

Comparisons of *Pagurus longicarpus* raised in small, high-spined shells and those raised in large, low-spined shells show that the former are smaller than the latter and that males in the former grow relatively longer chelipeds and chelae and greater right/left asymmetry of chelae than males in the latter. These differences may provide insight into the geographic variation of *P. longicarpus*. Southern individuals are smaller and grow relatively longer claws and greater right/left asymmetry of claws than northern individuals (Table II, Table VI). Further, southern individuals inhabit smaller, higher-spined shells than northern individuals (Table II, Fig. 1). It may be that the differences in inhabited shells caused the observed morphological variation in *P. longicarpus*. Three points will be made in this regard:

(1) *Geographic differences are endogenous.* Morphological differences can be induced in individuals of the same geographic population by inhabiting different shell-types. These ecophenotypic differences parallel differences between individuals of the different geographic populations. Nevertheless, individuals from the different geographic populations grown in the same environment in the same shell-type still exhibit differences. Morphological differences between geographic populations thus likely have a genetic basis. If shells were an evolutionary cause for this genetic divergence, mechanisms such as genetic assimilation (Waddington, 1954; Ray, 1960) or selection must be invoked.

(2) *Some geographic differences cannot be induced by shells.* In male *P. longicarpus* chela shape shows clear geographic differences but no induced differences. Further, females show clear geographic differences in cheliped length, chela length, and chela shape, but none of these differences can be induced by shell-type. If shells are the cause of geographic variation, these differences could be explained by pleiotropy (linkage with genes that were assimilated or selected for), additional selection, or genetic drift.

(3) *Littorina littorea: a causal agent?* Raising individuals in large, low-spined shells (versus small, high-spined shells) induces differences which parallel some of the differences between northern and southern *P. longicarpus*. *Littorina littorea* is one of the major large, low-spined shells in Massachusetts and Long Island Sound, but is not found at all in the Carolinas (Fig. 1). This shell is a European species which has become common in Massachusetts and Long Island Sound in the last century and a half (Bequaert, 1943; Vermeij, 1978, 1982). In these northern areas, *L. littorea* shells bridge the gap of size and shape between small native shells (*Nassarius*, *Ilyanassa*, *Urosalpinx*) and large native shells (*Polinices*). It may be that *L. littorea* shells constitute a significant difference in the shell resource between southern and northern areas. Thus the introduction of *L. littorea* and its displacement of native snails (Brenchley and Carlton, 1983) may have changed the perception of the shell resource by *P. longicarpus*. By inhabiting the introduced shells, crabs grew larger and began to use large native shells as well. Size and shape differences were at first ecophenotypic but were genetically assimilated or selected for. Other shape differences possibly occurred through pleiotropy or additional selection. This scenario must be considered in view of the work of Drapkin (1963) who describes the introduction of a large gastropod to the Black Sea followed by an increase in the size of the native hermit crabs. The presence of a large native littorine, *Littorina irrorata*, and the scarcity of *L. littorea* south of Long Island Sound weaken this hypothesis. On the other hand, *L. irrorata* is a higher-spined shell than *L. littorea*, and does not bridge the gap in the native shell resource (Fig. 1). Also, even in New Jersey, where *L. littorea* is rare, its shells occupy a central position in the shell resource (Fig. 1).

In summary, the data presented here show that there is a close interaction between hermit crabs and inhabited shells during ontogeny and that shell size and shape can have profound effects on crab size and shape. These data suggest that the size and shape spectrum of utilized shells be considered when studying morphological differences in hermit crabs. However, all available evidence should be carefully considered before drawing any causal connections between hermit crab morphology and the morphology of inhabited shells. This is especially true for morphological variation in *P. longicarpus*. While it is possible to make hypotheses concerning the effects of shells, particularly the introduced *L. littorea*, at present such hypotheses are only partially supported by available data. Historical records of *P. longicarpus* must be investigated, alternative hypotheses must be considered, and general processes of growth and development must be further explored.

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## MECHANISMS OF SPATIAL COMPETITION OF *DISCINISCA STRIGATA* (INARTICULATA: BRACHIOPODA) IN THE INTERTIDAL OF PANAMA

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### ABSTRACT

The inarticulate brachiopod *Discinisca strigata* uniformly wins competitive interactions for space with other sessile epifauna in the intertidal of the Pacific Panamanian coast. This result is achieved through *D. strigata's* abilities to (1) metamorphose directly on the surface of bryozoan colonies, (2) maintain a pool of particle-depleted water around most of the shell, (3) abrade underlying calcareous epifauna with the shell, eroding them to the level of the substrate, and (4) abrade the tissues of adjacent sponges and bryozoan zooid buds with modified lateral setae. (1) and (2) are allowed by a reversal of the described flow patterns in brachiopods and the possession of a functional siphon formed from modified setae; (3) is possible because the inorganic component of the brachiopod's shell, calcium phosphate, is much harder than calcium carbonate. Three of these mechanisms are not available to articulate brachiopods and the fourth is apparently not exploited, which may explain differences in competitive abilities between the two classes.

### INTRODUCTION

Despite the considerable paleontological importance of brachiopods, their ecology is poorly known; only about 350 species are extant, but on some hard substrates brachiopods are a prominent component of the sessile fauna. All living brachiopods except lingulids and a few articulates (Neall, 1970; Richardson and Watson, 1975a, b) are permanently attached by the pedicle or by cementation of one valve (Thayer, 1981). As immobile epifauna, brachiopods have inhabited hard substrates, presumably competing for space with other epifauna, since the Lower Cambrian. Colonial animals such as sponges and bryozoans are generally superior competitors when competing with solitary animals (Jackson, 1977; but see Greene and Schoener, 1982), and recent studies have clarified the competitive relationships (see Jackson, 1983) and mechanisms (Buss and Jackson, 1979; Buss, 1981) of these and other sessile epifauna, but most studies of brachiopod ecology have concentrated on the demography and population structure of articulates (see Thayer, 1981; Witman and Cooper, 1983). Only Doherty (1979) has addressed how articulate brachiopods fare in competition with other sessile organisms for substrate space; no work has addressed the competitive abilities of inarticulates.

The present study of *Discinisca strigata* grew out of continuing work on brachiopod hydrodynamics (LaBarbera, 1977, 1981), so the samples were not specifically collected to investigate spatial competition. However, description of the competitive relationships and mechanisms implied by evidence from these specimens seems warranted because these results contrast sharply with the reported competitive abilities and mechanisms of solitary animals in other phyla.

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## MATERIALS AND METHODS

Rock fragments were collected from overhangs and crevices in the mid-intertidal at Punta Patilla, Panama. The ecology (Reimer, 1976a, b) and competitive relationships of some epibionts (Buss, 1980, 1981) at this site have been described. Specimens were held briefly in tanks at the Smithsonian Tropical Research Institute, Galeta, Panama, before being hand-carried to Chicago in a styrofoam cooler. On arrival, the fauna were acclimated to the water in the holding tanks over a 3-hour period; all animals survived transport. The animals were fed a mixed culture of diatoms supplemented with commercial bakers yeast when diatom cultures ran low.

Each rock was mapped at 3× using a Wild M5A stereo microscope and camera lucida; all epifauna were outlined and living fauna distinguished from remnants of epibionts. The rocks were also scanned at 6× and 12× to detect overlooked fauna (primarily juvenile *D. strigata*) and to clarify the nature of faunal interfaces observed at 3×. When work on *D. strigata*'s behavior and hydrodynamics was complete, the rocks were submerged in a container of sea water, aliquots of 7% MgCl<sub>2</sub> added until the animals no longer responded to disturbance, and buffered formalin added to yield a 10% solution. The rocks were later resubmerged in sea water and each *D. strigata* removed from its substrate by carefully cutting the pedicle. Any epifauna revealed were recorded on the maps of the rocks.

Areas were measured from the maps using an Apple II+ microcomputer and digitizing pad. All areas reported are the coverage as seen from a viewpoint perpendicular to the plane of the substrate. Where large epibionts were themselves covered by epizoans, the encrusted area was counted twice; such instances represent a minor fraction of the total reported coverage.

## RESULTS

*General*

Rock areas varied from 21 to 116 cm<sup>2</sup>. The fractional area covered by epifauna averaged 49%; free space varied from 32–74%. *D. strigata* covered 2–26% of the surfaces; bryozoans, primarily *Antropora tinctoria*, covered 5–38%. Other major epibionts included serpulids (2.5–29%), spirorbids (0.01–7.3%), and sponges (0.5–8.2%). The most densely encrusted rock is reproduced in Figure 1; coverage data is summarized in Table I. Single individuals of *Isognoman jamus* and *Hippomix panamensis* were also found, but are omitted from this tabulation.

A total of 194 *D. strigata* were seen; 17 were juveniles under 2 mm in diameter. In contrast to the northern Gulf of California (Paine, 1962), the *D. strigata* at Punta Patilla commonly occurred in clusters (animals separated by less than 2 mm) of from two to over a dozen animals; solitary individuals were a minority. One third of the *Disiniscia* (17% of the total valve area) bore epizoans, primarily bryozoans and spirorbids, with occasional *D. strigata*, serpulids, and small sponges.

*Behavior of the living animals*

All *D. strigata* opened within 45 min after transfer to the tanks; all appeared healthy. Details of *D. strigata*'s flow patterns will be described elsewhere (LaBarbera, in prep.), but note that flow directions are the reverse of articulates (LaBarbera, 1981); water enters the shell anteriorly and exits through the lateral gapes (Fig. 2). Paine (1962) noted this pattern, but it has been subsequently overlooked.

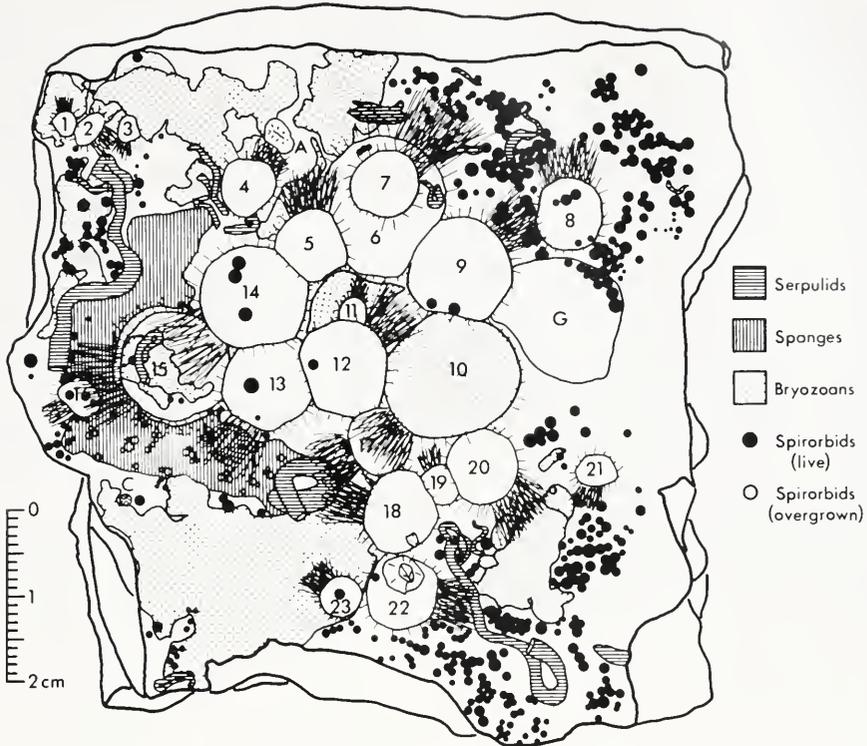


FIGURE 1. Camera lucida drawing of rock 6 (Table 1). All *D. strigata* are numbered. Note the clear zone separating brachiopod 15 from the surrounding sponge and the inhibition of the bryozoan encrusting it by brachiopod 14's anterior setae. Brachiopods 6 and 22 bear a *D. strigata* (number 7) and barnacle, respectively. The interactions occurring in the vicinity of brachiopods 1-4, 15-16, and 17-23 are shown in greater detail in Figure 4. Bryozoan colonies (*Antropora tincta*) are marked by stippling, sponges by vertical hatching, serpulids by horizontal hatching, and spirorbids by circles (solid if exposed, open if overgrown). More complex patterns indicate overgrown. C = solitary coral, A = anemone, G = gastropod (*Crepidula striolata*).

*D. strigata*'s densely packed anterior setae function as a siphon (Fig. 2). These setae are very long (comparable in length to the valves) and bear fine lateral processes (Fig. 3) which mechanically interlock and decrease the mean size of the spaces between the setae. No detectable flow occurs between the anterior setae except near their most distal tips; incurrent water is drawn from well in front of the animal, usually in a plane above the substrate. The lateral setae are about half the length of the anterior setae and much less densely packed; their lateral processes (Fig. 3) are short, stout, and thorn- or hook-like. The posterior setae bear similar ornamentation, but are much shorter than the lateral setae.

When disturbed (and at irregular intervals with no obvious stimulus), the animals rapidly closed their valves and initiated a stereotyped behavior pattern:

(1) With the valves nearly closed, the dorsal valve was rotated clockwise and counterclockwise through a total arc of 60-120°. This movement rubbed the lateral setae of the dorsal mantle over and past the ventral setae. The setal siphon was distorted by this movement but remained patent.

TABLE I

*Epizoans on the rocks collected at Punta Patilla, Panama*

Rock	1	2	3	4	5	6	7	8	9	10	11
Total area (cm <sup>2</sup> )	11.6	20.8	45.0	50.2	51.5	52.9	61.8	66.1	68.7	72.3	116.4
Epizoans (% cover) <sup>1</sup>											
<i>Discinisca</i>	9.8	11.8	3.2	8.7	13.0	26.0	1.8	16.8	19.6	7.7	9.9
<i>Antropora</i>	12.8	1.1	6.9	23.8	14.5	22.5	37.7	1.7	0.6	—	7.3
<i>Onychocella</i>	—	18.5	—	1.6	0.9	—	—	6.6	—	3.3	—
<i>Microporella</i>	—	—	—	2.4	—	—	—	—	—	1.6	—
Sponges	—	—	7.0	1.9	1.2	6.9	6.4	8.2	6.9	0.5	2.1
Serpulids	2.5	4.8	4.2	9.2	9.4	4.4	5.7	10.1	14.1	28.8	3.7
Spirorbids	1.3	3.3	0.01	6.9	5.6	5.2	7.3	3.9	1.2	4.2	5.5
<i>Crepidula</i>	—	—	5.8	—	—	3.0	0.3	—	—	0.2	10.5
Vermetids	—	—	—	—	—	—	—	0.3	—	2.9	3.7
Barnacles	—	—	0.3	0.4	0.2	0.3	—	—	0.4	0.2	0.5
Corals	—	—	—	—	—	0.03	—	—	—	4.4	0.1
Anemones	—	—	—	—	—	0.19	—	—	—	—	—
Oysters	—	—	—	—	—	—	—	—	—	2.6	—
Fragments	—	—	7.6	—	—	—	—	—	—	—	—
Free space	73.6	60.6	65.1	45.3	55.4	31.5	40.8	52.4	57.3	43.7	56.8

<sup>1</sup> Blank entries indicate that no representatives of that group were found. The entries labeled *Discinisca*, *Antropora*, *Onychocella*, *Microporella*, and *Crepidula* are the brachiopod *D. strigata*, the bryozoans *A. tineta*, *O. alula*, and *M. umbracula*, and the gastropod *C. striolata* respectively; the fragments listed are fragments of cemented oyster valves and the bases of the sessile gastropod *Hipponix panamensis*.

Values listed for the epizoans and for free space are percentages of the total projected area of the rock surface.

(2) When the dorsal valve returned to its normal alignment with the ventral valve, the valve margins were clamped together tightly and both valves were rotated as a unit through a total arc of 60–150°.

(3) On return to rest position, the valve margins were clamped tightly to the substrate. After a few seconds to several minutes, the valves returned to a position slightly elevated above the substrate and the animal slowly reopened.

#### *Spatial competition between Discinisca and other epifauna*

Numerous examples of apparent spatial competition between *D. strigata* and the other epifauna, particularly sponges and bryozoans, were noted. Although sponges on these rocks had overgrown serpulids, spirorbids, and bryozoans, only once was there any suspicion of a sponge overgrowing a *D. strigata*; the shell found under this sponge was small (approximately 5 mm diameter), and so badly eroded that it could not be positively identified even to phylum. Most of the sponges were thin, encrusting forms which grew up to the lateral or anterior margins of the brachiopods only when hidden in irregularities in the substrate; where the substrate lacked relief, the brachiopods were surrounded by a clear zone approximately the length of the lateral setae (Figs. 1, 4a). Whenever these sponges grew within 2–3 mm of the posterior margin of a *Discinisca* they exhibited arrested growth and a distinct ridge produced by vertical growth. Four large, thick sponges grew beside or around brachiopods; a distinct clear zone devoid of sponge occurred around the brachiopods except near



FIGURE 2. A live *D. strigata*, actively pumping. The anterior setae are interlocked to form a functional siphon; water is drawn into the animal anteriorly and exits laterally. The catheter tube is filled with a 1:3 mixture of milk and sea water.

the shell anterior. Near the substrate, this zone was equal or slightly smaller in width than the length of the setae, but, a few millimeters above the substrate, the sponges overhung the brachiopods' shells (Fig. 4a). The sponges never actually touched the shell; the two were usually separated by 2–4 mm.

Thirty-six *A. tinctoria* colonies abutted or surrounded brachiopods; in 12 of these, zooids adjacent to the brachiopod had produced a distinct ridge in the colony through frontal budding. Near the anterior or posterior valve margins, this ridge was less than 2 mm from the brachiopod, but near the lateral margins, the ridge occurred at the tips of the lateral setae. In most cases, the location of the bryozoan's growing edge indicated that the brachiopod had been overtaken by a colony expanding its spatial coverage (Fig. 4b). Three other *A. tinctoria* colonies partially encrusted a brachiopod whose posterior half was brushed by the tips of a second brachiopod's anterior setae; where brushed by the setae, these colonies exhibited arrested growth and a ridge 2–3 zooids thick (e.g., Fig. 1, animals 14 and 15).

Undercutting and wear was apparent on epifauna adjacent to or overlapped by brachiopods. Where bryozoans extended beneath brachiopods (23 of the 177 adult brachiopods), zooids near valve margins were visibly worn and some had been bisected (Fig. 5). The 7 spirorbids near the edges of brachiopods (7 cases) were similarly damaged; one fourth to three fourths of each whorl was worn nearly to the substrate (Fig. 4a, c, 6a). Ten serpulids (Fig. 4b), two vermetids, and two corals were also worn, although the damage to these animals was not as dramatic as for bryozoans or spirorbids and the individuals had survived.

When the brachiopods were removed, evidence of past interactions was found beneath the valves (Fig. 4). All spirorbids (24) found under *D. strigata* (13 animals)

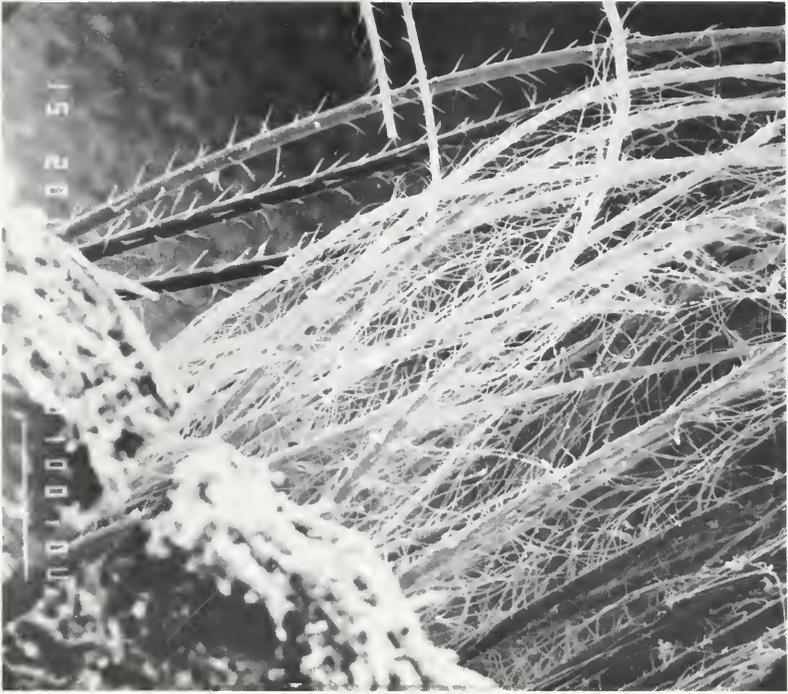


FIGURE 3. SEM micrograph of the anterior (AS) and lateral (LS) setae of *D. strigata*. The anterior setae bear long, thread-like lateral processes that entangle in forming the anterior siphon; the processes of the lateral and posterior (not shown) setae are short, stout, and thorn-like. Scale bar = 100  $\mu\text{m}$ .

were highly worn; most were so eroded that the entire tube was exposed (Fig. 4a, c, 6b). All 18 colonies of sheet-like bryozoans hidden by the brachiopods' valves (Fig. 4a) were extensively worn as were the 9 colonies of "runner" (Jackson, 1979) morphology (Fig. 4c). [Runner-like bryozoans are early colonists but poor competitors (Jackson, 1979); they occurred on the free surface of the rocks only twice.] The 10 fragments of serpulid tubes (under 7 brachiopods) were all highly worn and polished (e.g., Fig. 4c). The undamaged shell of a dead juvenile (1.7 mm diameter) *D. strigata* was found attached beside the pedicle of a second, larger animal. Remains of epifauna were even found underlying the brachiopods' pedicles. Such cases included nine sectioned spirorbids under the pedicles of eight *D. strigata* (Fig. 4c), six worn sheet-like bryozoan colonies (6 brachiopods), four of which underlay the entire pedicle attachment (Fig. 4c), six worn runner-like bryozoans (2 brachiopods), and three worn and polished serpulid tube fragments (2 brachiopods). Three juvenile *D. strigata* (1.2–2.3 mm diameter) were attached to the frontal walls of zooids in the center of living *A. tinctoria* colonies; thus these animals' pedicles were also underlain by bryozoans and metamorphosis must have occurred directly on the living colony.

#### DISCUSSION

The rocks studied were small and may not be a representative sample of the habitat; only 617.3 cm<sup>2</sup> of this habitat was investigated. Static samples are not ideal

for reconstructing competitive relationships, but this approach has been used previously and yields qualitatively valid results (Buss, 1980, 1981; Quinn, 1982; Russ, 1982; Jackson, 1983). Despite these limitations, some aspects of *D. strigata's* ecology seem clear.

*D. strigata* is the spatial dominant on only 3 of the 11 rocks investigated, even though it dominates in competitive interactions. However, only in the case of serpulids on rock 10 does another sessile, solitary animal dominate the space. Before speculating on why *D. strigata* does not dominate to a greater extent, those features that mediate its offensive and defensive functions should be clarified.

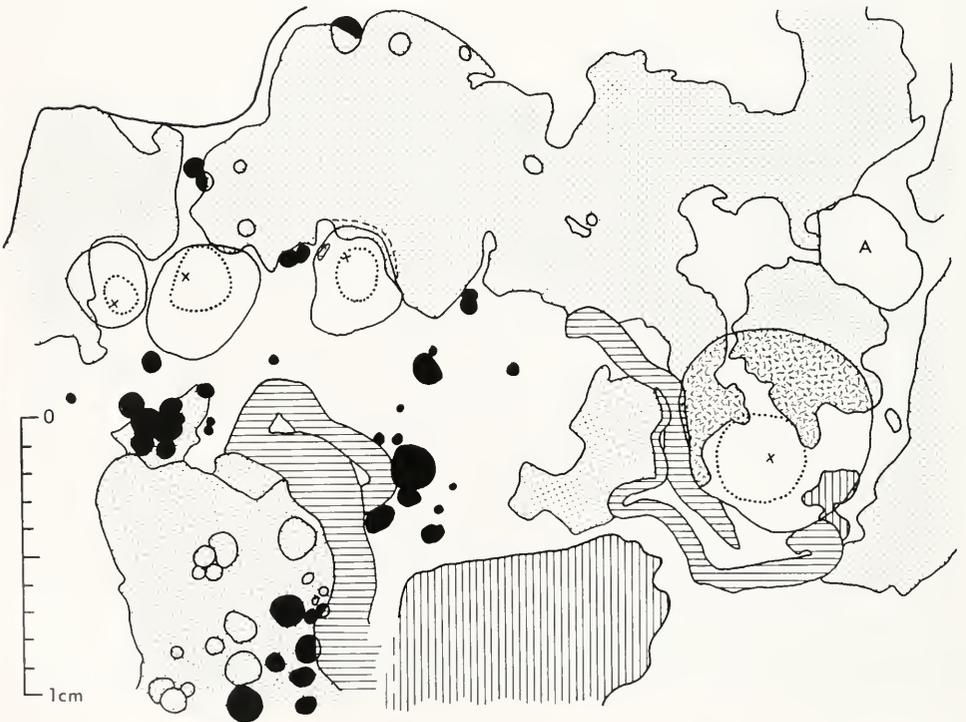
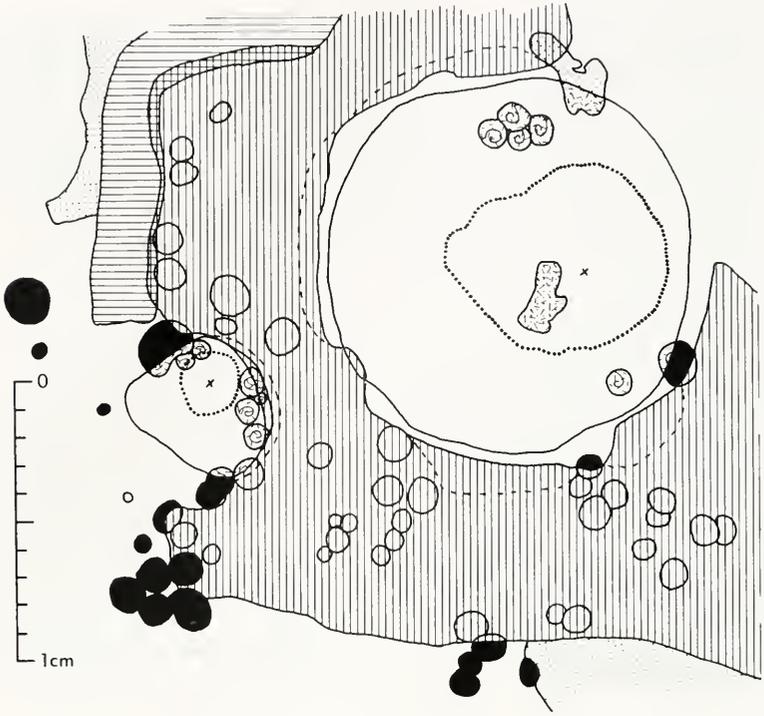
#### *The role of water flow patterns*

Utilization of the anterior setae to form an incurrent siphon may be crucial to *Discinisca's* success in competitive interactions. Since bryozoan zooids expel filtered water towards the substrate, and brachiopods and bryozoans probably exploit the same size fraction of particles (*cf.*, Winston, 1976; Jorgensen *et al.*, 1984), the water near the surface of a bryozoan colony would be devoid of food for a newly-metamorphosed brachiopod; the low local Reynolds number (see Vogel, 1981) implies poor mixing. Three juveniles were seen which had metamorphosed on living bryozoan colonies; if the presence of a single bryozoan colony underlying the entire pedicle is acceptable evidence for a brachiopod's metamorphosis on a living colony, then seven such cases occurred. *D. strigata's* functional siphon allows juveniles to draw water from above the bryozoan's lophophores, permitting feeding and ultimately allowing it to usurp space occupied by the colony. For larger juveniles and adults, the ability to draw water from well above the substrate will minimize the effects of particle depletion (see Buss and Jackson, 1981; Jackson, 1983) by other suspension feeding epifauna.

Since filtered water exits the brachiopods through the lateral shell gapes at low speeds (LaBarbera, in prep.), water on the sides of the brachiopods will be particle-depleted. This nutritionally depleted water might act as a barrier to bryozoan encroachment if colonies grow towards nutritionally favorable microenvironments (Winston, 1976). Since sponges can filter submicron sized particles (Reiswig, 1971) while brachiopods poorly retain particles smaller than 2  $\mu\text{m}$  (Jorgensen *et al.*, 1984), feeding interference by *D. strigata* might seem unlikely. However, particles smaller than 1  $\mu\text{m}$  constitute less than 5% of the diet of sponges (Reiswig, 1971). The growing edge of sponges quickly becomes functionally independent of the main body (Simpson, 1963); if locally available water is depleted of particulates, local growth of the sponge will be repressed. This explanation is consistent with the arching morphology of the larger sponges growing in the brachiopods' vicinity; certainly the brachiopods' other competitive mechanisms (see below) could have no direct effect on portions of the sponges growing more than a few millimeters above the substrate. A similar situation has been described for bryozoans (Buss, 1980, 1981).

#### *Mechanical interference with other epifauna*

The cessation of substrate-level growth of sponges and bryozoans at a distance from the brachiopods equal to the lengths of the lateral and posterior setae implies a direct role of the setae in preventing overgrowth. *D. strigata's* lateral and posterior setae are robust and equipped with stout, thorn-like processes (Fig. 3). *Discinisca's* stereotyped rotation on closure sweeps these setae through an arc around the shell



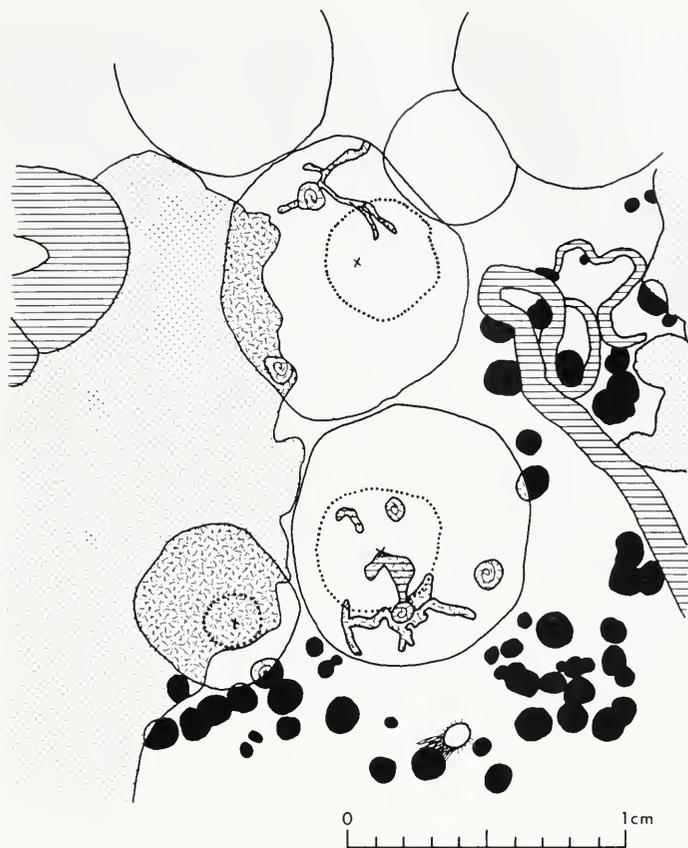


FIGURE 4. Abrasion of epifauna beneath *D. strigata*. The brachiopods' valve margins are outlined, but the valves are drawn as if transparent; setae have been omitted for clarity. Dotted lines outline the pedicle border at its attachment to the substrate; "X" marks the projected position of the brachial valve apex. Epifauna are identified with the conventions of Figure 1; abraded epifauna are indicated by an overlay of short, random hatching.

(a) Encrusting sponge surrounding two *D. strigata* (15 and 16 of Fig. 1). A zone devoid of sponge surrounds each animal near the substrate (dashed line); further from the substrate, the sponge arches over the brachiopods but never touches the shells. Note the abraded bryozoan colony under the pedicle of one brachiopod (right) and abraded spirorbids under both animals; eroded regions end abruptly at the shell margins. The sponge under the anterior margin of the right hand brachiopod lies in a depression in the rock's surface.

(b) Brachiopods 1-4 (left to right) of Figure 1. The anterior and left sides of brachiopod 1 (far left) are elevated due to the slope of the rock beneath the animal; the underlying bryozoans are alive. In contrast, animals 2-4 lie flat on the surface and underlying bryozoans have been abraded to the colony basis. The apparent overgrowth of brachiopod 3 by an *Antopora tinctoria* colony results from the perspective. Near the substrate, the colony margin (dashed line) is located at the tips of the lateral setae; the colony has undergone extensive frontal budding and begun to arch over the shell, but nowhere comes within 1 mm of the brachial valve. As in (a), the sponge under brachiopod 4 (far right) lies in a depression.

(c) Brachiopods 18, 22, and 23 of Figure 1. Abraded bryozoans and spirorbids underly all three animals. The eroded "runner" bryozoans under brachiopods 18 and 22 were not visible elsewhere on the rock, and presumably had been overgrown by other epifauna. The bryozoan on the left underlies brachiopod 23's entire pedicle attachment, implying that this brachiopod metamorphosed directly on the colony's surface. The juvenile *D. strigata* in the lower portion of the drawing was too small to be distinguished in Figure 1.



FIGURE 5. SEM micrograph of a bryozoan colony (*Antropora tincta*) adjacent to a *D. strigata*. The brachiopod's valve margins are indicated by broken lines; note that several zooids adjacent to the brachiopod have been bisected (arrows) and that the colony has been eroded down to its basis beneath the brachiopod. Scale bar = 100  $\mu\text{m}$ .

and mechanically inhibits sponge and bryozoan growth by directly damaging their tissues.

The growing edge of sponges is initially thin (Ayling, 1983) and lacks spicular or fibrous reinforcement (Simpson, 1963). Although the rate of spatial coverage may increase dramatically where the sponge has been disturbed, absolute rates of coverage are maximally 6.98  $\text{mm}^2/\text{cm}$  perimeter/day (Ayling, 1983). Given the frequency with which *D. strigata* sweeps the vicinity with its setae and the vulnerability of sponge tissues, such low expansion rates are easily nullified.

Newly budded bryozoan zooids are weakly calcified (Ryland, 1970); contrary to the usual pattern (Jackson, 1983), here the bryozoan's actively growing edge is more vulnerable than the fully calcified regions where growth is arrested. *Antropora tincta* exhibits frontal budding when growth is blocked (Buss, 1980, 1981; Jackson, 1983); for colonies around *D. strigata*, the only available agent for blockage is the brachiopod's setae. As noted above, frontal budding can be induced in *A. tincta* by the anterior setae of adjacent brachiopods. Since these setae are longer (thus exerting smaller forces at their tips) and lack the spines of the lateral and posterior setae, these bryozoans will be highly vulnerable to the disturbance imposed by the latter.

Numerous eroded epizoans occurred under the brachiopods' ventral valves, although no abrasion of the valve itself was seen. The edge of the ventral valve is the most likely abrasive agent as evidenced by: (1) ground and polished regions on adjacent serpulids, vermetids, and corals, (2) bisected spirorbids and bryozoans where overlapped by a *D. strigata*, (3) a dead but undamaged *D. strigata* juvenile beside the pedicle of

an adult, and (4) the clearance I observed between the central regions of the ventral valve and the substrate. The inorganic component of *D. strigata*'s valves is about 75%  $\text{Ca}_3(\text{PO}_4)_2$  (Jope, 1965), a mineral with a Mohs hardness of 5.0; calcite and aragonite, the inorganic skeletal components of most calcareous epifauna, have hardnesses of 3.0 and 3.5–4.0, respectively. Thus the preferential abrasion of the epifauna arises from the much harder mineral comprising the brachiopod's valves. Whether the minute periostracal spines of *D. strigata* (Williams and Mackay, 1979) play any part in this abrasion is unknown. The characteristically abraded epifauna present beneath adult brachiopods' pedicles implies that this mechanism is effective even in juveniles.

### *Brachiopods as spatial competitors*

Since up to three-fourths of the space on these rocks was unoccupied, it might be argued that discussion of spatial competition is moot. However, from the perspective of sessile epibionts, the only relevant space is that bordering the individual or colony; if this space is contested and lost, the loser will incur a cost in terms of potential growth and thus reproductive potential. The numerous observed overgrowths of epifauna by sponges and bryozoans imply that competition for space does occur; the contests inferred for *D. strigata* also represent local competition for space around individuals.

If *D. strigata* dominates in both direct and indirect (Woodin and Jackson, 1979) competitive interactions, why has it not monopolized the space on these rocks? Although no definitive answer is available, the possibilities appear to be limited to physical disturbance, predation, or failure to secure space as fast as it opens up. This study can offer no insight into the frequency of physical disturbance, and the only evidence of predation was the presence of small (less than 500  $\mu\text{m}$  diameter), straight-sided boreholes in three *D. strigata*, all of which were still alive. No scars on the rocks or fauna indicating removed animals were noted. However, a poor ability of co-opt newly opened space is implied by the determinate growth of adult *D. strigata* (see Jackson, 1979) and the low frequency of juveniles. All juveniles were approximately the same size and thus probably represent a single recruitment episode; if recruitments are annual and all of this magnitude, it would take over ten years (assuming no mortality) to build up the observed adult population. Even if *D. strigata* is the competitive dominant, its domination of space is thus likely to be a protracted exercise. If this argument is valid, this system's dynamics follow Greene and Schoener's (1982) "fixed lottery" model.

*D. strigata* is effective at defending space against encroachment, can co-opt space at metamorphosis that colonial animals previously occupied, and can acquire additional space as it grows – abilities unexpected in sessile, solitary animals (Jackson, 1983). Brachiopods are generally presumed (e.g., Jackson *et al.*, 1971; Thayer, 1981) to be competitively inferior, but the evidence for this view is meager and restricted to articulate brachiopods. Thayer (1981) reports that articulate brachiopods are poor competitors for space when competing with mobile animals such as mussels. In the present study *D. strigata* usually occupied more space than the only mobile epibiont present, *Crepidula striolata* (Table I). Doherty (1979) has documented frequent overgrowth of juveniles of the articulate brachiopod *Terebratella inconspicua* by both bryozoans and sponges. In contrast, of the 17 live juvenile *D. strigata* seen in the present study, none appeared to be in any danger of overgrowth; the only juvenile which had unequivocally lost such an interaction had been smothered by an adult *D. strigata*.

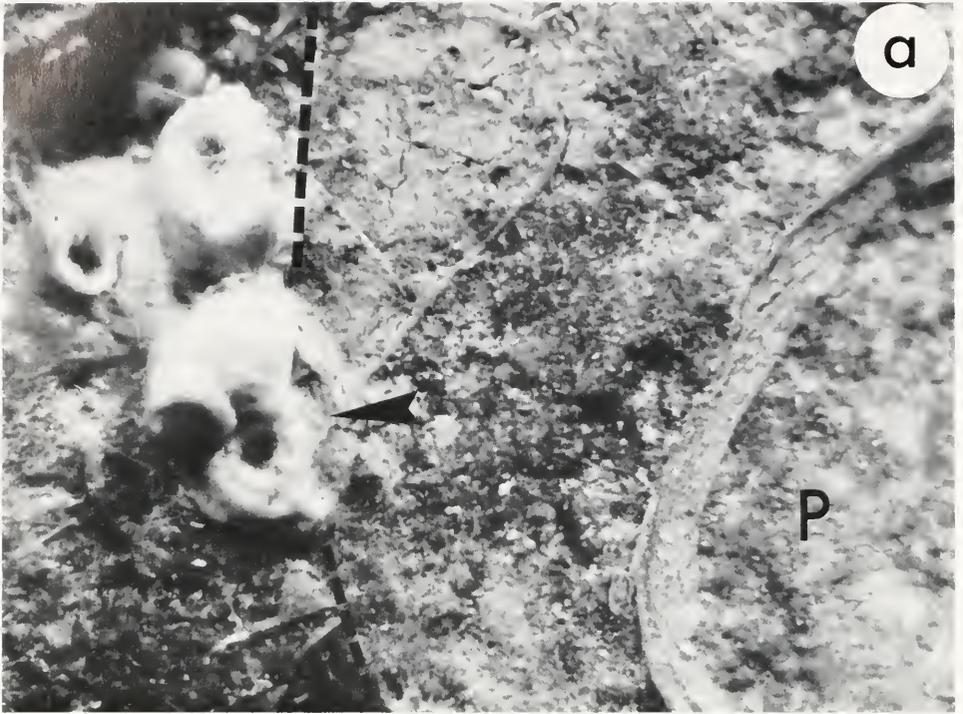


FIGURE 6. Abrasion of spirorbids by the ventral valve of *D. strigata*. Both photographs are of dried specimens; scale bar = 5 mm. (a) A spirorbid, partially overlapped by a *D. strigata*, with a portion of one whorl abraded to the substrate (arrow). The margin of the ventral valve of the brachiopod is indicated by the broken line; a portion of the cuticle covering the pedicle (P) can be seen to the right. (b) A spirorbid

Differences in competitive abilities between the articulates studied by Doherty (1979) and the inarticulates studied here are likely due to a suite of characters. Pedunculate brachiopods' mobility on the pedicle makes them a functionally unstable substrate for overgrowth; both LaBarbera (1977) and Doherty (1979) note that this mobility helps articulate brachiopods avoid overgrowth. In all articulates, however, the pedicle foramen is marginal; the more central foramen in *D. strigata* affords greater protection for the pedicle and insures that the entire shell margin, including regions adjacent to the pedicle, sweep through a sizable arc when the animal rotates, thus inhibiting epifaunal growth at a greater distance from the shell than is possible for articulates.

Rotation of the valves in *D. strigata* mechanically abrades surrounding epibionts through the actions of both the shell and the setae; neither mechanism is well developed in articulate brachiopods. *Discinisca's* distinctively ornamented setae are unique; the setae of both lingulids (Blochmann, 1900; Storch and Welsch, 1972; Orrhage, 1973; Westbroek *et al.*, 1980) and a variety of articulates (Gustus and Cloney, 1972; Orrhage, 1973) are simple straight shafts. Some articulates prune surrounding non-calcified epizoans with the shell during rotation (LaBarbera, 1977), but abrasion of calcified epifauna may not be possible; all articulates possess a calcium carbonate shell and any abrasion of calcareous epifauna would equally abrade the shell.

### General

The anterior incurrent/lateral excurrent flow in *D. strigata* has not been described for any other brachiopod, but I have observed similar patterns in the inarticulate *Crania californica* (LaBarbera, unpubl.). Given the paucity of work on living brachiopods, this pattern may be typical of the seven genera of acrotretid inarticulates.

The strong differentiation in length and ornamentation of the anterior, lateral, and posterior setae appears to be characteristic of the genus; Blochmann (1900) describes and figures similar setal structure and differentiation in *Discinisca lamellosa*. Blochmann (1900) does not mention flow directions through *D. lamellosa* or whether the anterior setae form a siphon, but he worked from preserved specimens where the setal siphon would be difficult to discern and information on flow directions unobtainable. Given the similarities between *D. strigata* and *D. lamellosa*, particularly the differentiation between the anterior and lateral setae and the similar shell compositions, *D. strigata's* mechanisms of competitive interaction are likely to be characteristic of the genus and may have been important in insuring the genus' success since it arose in the lower Jurassic (Rowell, 1965).

It is often possible to reconstruct competitive relationships among fossil epibionts (see, *e.g.*, Jackson, 1983). For fossil *Discinisca* preserved *in situ*, it should be possible to recognize the characteristic shell-generated abrasion of underlying calcareous epibionts, and careful observations on the distribution of epibionts might also produce evidence for setal abrasion.

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which was completely overlapped by a *D. strigata* and abraded to the point where nearly the entire tube has been opened. The brachiopod lay at an angle due to the slope of the underlying substrate; note the smooth bevel of the spiriferid's abraded surface. Conventions as in (a).

## ACKNOWLEDGMENTS

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## EVOLUTION OF THE TELSON NEUROMUSCULATURE IN DECAPOD CRUSTACEA

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### ABSTRACT

The neuromusculature in the telsons of three macrurans (*Pandalus platycernus*, *Procambarus clarkii*, *Upogebia pugettensis*) and three anomurans (*Munida quadripinna*, *Blepharipoda occidentalis*, *Emerita analoga*) are compared to provide a framework for neurophysiological comparisons of their roles in the swimming behaviors of these decapods. The stereotypical arrangement in macruran telsons comprises a group of massive axial muscles and a trio of small appendage muscles (Fig. 1). The various arrangements of telson neuromusculature in the anomurans (Fig. 3) are interpreted in terms of specific modifications of particular macruran features. Homologies among muscles and nerve roots in the telsons of the six decapods are identified (Figs. 1, 3, 4, Table II) and homologies among particular axial and appendage motoneurons in the sixth abdominal ganglia are suggested (Fig. 5, Table III). The appendage neuromusculature in decapod telsons is inferred to be ontophyletically part of the seventh abdominal segment that was present in the ancestors of decapods. These muscles and their motoneurons, like most of the axial neuromusculature in the telson (Dumont and Wine, 1983, in prep.), may be serial homologs of muscles and motoneurons in abdominal segments.

### INTRODUCTION

Phylogenetic histories of neuronal circuits could contribute to neurobiology by revealing what features of neurons and neural circuits respond to selective pressures by evolving new behaviors and what features are conserved through evolution. The neural control of the decapod crustacean tailfan is particularly well suited for such a phylogenetic analysis. This is because the tailfan is an ancestral structure in decapods. It contributes to tailflipping locomotory behaviors that are already subjects of intensive neurobiological investigations in the crayfish, a macruran, and it has been modified in several anomuran families for use in other behaviors.

The tailfan is a tripartite structure comprised of the telson flanked by the paired appendages of the last abdominal segment, the uropods. Paleontological and embryological observations indicate that the tailfan is an ancestral decapod structure that is present during some part of the life cycle in all decapods. The fossil record documents its long history from the Paleozoic Era (Schram, 1982); the fossilized abdomen and tailfan of the "first decapod," *Paleopalaemon newberryi*, is very similar to that of modern reptant macrurans (Schram *et al.*, 1978). Since the Paleozoic, the tailfan has been structurally and functionally modified to perform new behaviors in several

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*Abbreviations.* Muscles: AT, anterior telson; ATU, anterior telson-uropodalis; DR, dorsal rotator; F6, terminal component of fast flexors in segment 6; LTU, lateral telson-uropodalis; PTF, posterior telson flexor; PTU, posterior telson-uropodalis; Re, uropod remotor; RS, uropod return-stroke; STF, slow telson flexor; TU, telson-uropodalis muscles; VR, ventral rotator; VTF, ventral telson flexor.

anomuran families descended from macrurous decapods (see *e.g.*, Chapple, 1966, 1977; Paul, 1981a, b), so that a wealth of comparative material is potentially available to allow verification of conservation of features (among macrurans and unchanged parts of anomurans) against which newly evolved anomuran features can be recognized.

Most investigations of the macruran tailfan have been done on crayfish. They have concerned its role in different forms of tailflipping (Wine and Krasne, 1982), activation of some of the telson muscles in these different behaviors (Kramer *et al.*, 1981a; Kramer and Krasne, 1984), the relationship of telson musculature to the fast flexor neuromusculature of the abdomen (Larimer and Kennedy, 1969a, b; Dumont and Wine, 1983, in prep.), the integration of sensory input (Wilkens and Larimer, 1972; Calabrese, 1976; Wiese, 1976), and the addition of mechanoreceptive hairs and neurons to the tailfan during adult growth (Letourneau, 1976). Recently, some of the motoneurons, local interneurons, and projection interneurons in the terminal abdominal ganglion have been described (Takahata *et al.*, 1981; Reichert *et al.*, 1982; Sigvardt *et al.*, 1982; Dumont and Wine, 1983, in prep.; Nagayama *et al.*, 1983). It has been difficult to integrate neurobiological investigations on the tailfans of other decapods with these crayfish studies because the organizational plan of the telson neuromusculature has not been fully described. Published treatises on the musculature of macrurans (Schmidt, 1915; Berkeley, 1927; Daniel, 1931; Young, 1959) are detailed but predate the modern neurobiologist's functional perspective, and they do not include innervation of individual muscles. Larimer and Kennedy (1969a) began to rectify this for the crayfish from the perspective of differentiation of neuromusculature into phasic and tonic systems. But the arrangements of muscles and nerves in the tailfans of some other decapods appear, at least superficially, very different from crayfish.

We have compared the tailfans in representatives of six families (Table I) to find out whether there is a fundamental plan of organization that describes the musculature in all decapod telsons. We conclude that, even though the telson is not a somite and has no appendages of its own, the telson neuromusculature is divisible into axial and appendage systems, each with entirely separate innervation. And within these two groups, individual muscles and their motoneurons can be recognized and compared among families. Thus, the various arrangements of muscles in the different anomuran telsons can be understood in terms of specific modifications of particular features in the basic macruran plan. Our results provide the necessary ground work for the identification of homologies among the motoneurons serving the tailfan in different decapod groups and for rigorous testing of hypotheses regarding the evolution of neural circuits mediating new behaviors (Paul, 1979, 1981a, b).

#### MATERIALS AND METHODS

*Procambarus clarkii* were obtained from Beachcomber Biological, Oakland, California, and held in continuously flowing freshwater aquaria. Prawns, *Pandalus platycoccus*, squat lobsters, *Munida quadrispina*, and mud shrimps, *Upogebia pugettensis*, were collected locally. Sand crabs, *Blepharipoda occidentalis* and *Emerita analoga*, were collected from Monterey Bay, California. The marine animals were maintained in aquaria in a recycling, 10°C sea water system.

Anatomical investigations were made on freshly dissected specimens. The animals were anaesthetized by chilling before severing the abdomen from the thorax. Most dissections were made from the ventral side, but dorsal and sagittal perspectives of the internal anatomy of segment 6 and the telson were obtained, by the appropriate dissections, to verify the relative positions of muscles and nerves as described from

the ventral approach. Diagrammatic drawings of the features of interest were made with the aid of a camera lucida mounted on a Wild M5 stereomicroscope. Our anatomical descriptions are based on dissections and drawings of a minimum of ten specimens of each species.

Innervation from the terminal (sixth) abdominal ganglion of tailfan neuromusculature and sensory fields was traced by staining freshly dissected tailfans with methylene blue and by electrophysiological methods. We used suction electrodes to stimulate and record from individual nerves to verify that they innervated particular muscles or contained afferents from sensory hairs on the exoskeleton as shown by the staining. Muscle responses were noted by observing contractions under low power magnification of a stereomicroscope, or by recording electrical responses via small suction electrodes or 20 Megohm KCl-filled microelectrodes in muscle fibers. Conventional methods were used to amplify and display the signals on an oscilloscope (Paul, 1971b).

The number of motoneurons in selected nerves, positions of their somata, and the morphology of their principal neurites in the ganglion were revealed by immersing the cut nerve end in 250 mM NiCl<sub>2</sub> at 4°C for 4–18 hours, followed by precipitation of the Ni<sup>2+</sup> with dithiooximide (rubeanic acid; Quicke and Brace, 1979). The stain in some ganglia was intensified with silver (Bacon and Altman, 1977). All ganglia were processed conventionally (Paul, 1981b) and viewed at 160–250× magnification with a Zeiss compound microscope that was equipped with camera lucida. Nerves of interest were backfilled repeatedly (10–20 times) in each species until we were confident, within the limitations of the backfilling technique, that the largest number of motoneurons that we filled (observation repeated in at least three specimens) was the actual number of motoneurons in the nerve.

## RESULTS

The crustacean body terminates in the telson, which thus articulates with the last true segment (abdominal segment 6 in decapods). The telson is flanked by uropods, the paired appendages of segment 6, and together with them forms a tripartite structure called the tailfan. Elsewhere in the body, skeletal muscles are readily subdivided into appendages (arising in the trunk and inserting on an appendage) or axial (arising and inserting along the body trunk), but in the telson of macrurans, such as crayfish, the organization of muscles appears complex, and functional divisions are difficult to understand. We first describe the arrangement in the macruran telson based on examination of members of three families (Table I), and show that a division between axial and appendage muscles also exists. We then consider the organization of the telson neuromusculature in three anomurans (Table I) with modified tailfans. Finally,

TABLE I

*Decapods of the suborder Pleocyemata used in this study (classification to family according to Bowman and Abele, 1982)*

Infraorder	Family	Genus, species
Caridea	Pandalidae	<i>Pandalus platycerus</i> Brandt
Astacidea	Astacidae	<i>Procambarus clarkii</i> Girard
Thalassidea	Upogebidae	<i>Upogebia pugettensis</i> Dana
Anomura	Galatheididae	<i>Munida quadrispina</i> Benedict
	Albuneidae	<i>Blepharipoda occidentalis</i> Randall
	Hippidae	<i>Emerita analoga</i> Stimpson

we interpret the arrangements in these anomuran telsons in terms of specific modifications of particular features of the ancestral macruran plan.

### *Macruran telson*

We use the crayfish as our 'type specimen' for macruran tailfans for several reasons. Precedence: published descriptions of tailfan neuromusculature are more complete for crayfish than for any other macruran (Schmidt, 1915; Larimer and Kennedy, 1969a). Crayfish have been used much more frequently for neurophysiological studies than other macrurans, so that a nomenclature for the nerves and muscles is becoming established (Larimer and Kennedy, 1969a; Kramer *et al.*, 1981a, b; Wine and Krasne, 1982; Dumont and Wine, 1983; Kramer and Krasne, 1984). Finally, we have concluded from our work with crayfish, other macrurans, and anomurans (this study; Paul, 1981b) that the crayfish plan and nomenclature are applicable to the neuromusculature in the telsons of other decapods.

*Axial muscles.* The apparent complexity in functional arrangement of the seven telson muscles in crayfish stems from the fact that four of them insert on one tendon that is continuous with the caudal-most component of the anterior-oblique, fast flexor muscles in segment 6. Near the caudal end of segment 6, this flexor tendon is focally attached to the tendon of the ventral rotator muscle at a point antero-medial to the rotator's insertion on the uropod propodite (Fig. 1A<sub>1</sub>). It is also bound to the partial arthrodistal membrane between segment 6 and telson, through which it passes, so that the three components of the tailfan are mechanically coupled to each other. Three of the telson flexor muscles [the ventral, slow, and posterior telson flexor (ventral head), VTF, STF, and PTF] arise from the ventral cuticular membrane, whereas two muscles (the rest of PTF and the anterior telson muscle, AT) arise from the inner dorsal surface of the telson (Fig. 1A; Fig. 2A). This means that the force vector generated by contraction of any one of the six muscles that share the tendon must depend on the degree of activity in the other muscles. All four of these telson muscles are part of the axial musculature by the criteria of their shared tendon with the fast flexor muscles in segment 6, and by their innervation through root 6 (see below) by motoneurons that, with one exception, are the homologs in G6 of flexor motoneurons in more rostral ganglia (Dumont and Wine, 1983). The VTF, PTF, and AT muscles are active during non-giant mediated tailflips (Kramer and Krasne, 1984); VTF and PTF continue the line of axial flexors down the ventral side of the animal (Figs. 1A<sub>1</sub>, 2) and contribute to flexion of the telson. The AT muscle is the single exception to the clear division between axial and appendage musculature. It inserts on the dorsal side of the flexor tendon (Fig. 2) and not directly on the uropod propodite but, because of its nearly vertical orientation and the tight mechanical linkage between tendons and arthrodistal membranes on the ventral side at the juncture of telson, uropods, and segment 6, this 'axial' muscle pronates the uropod and makes no contribution to flexion of the axis (Dumont and Wine, in prep.). The AT muscle (ATF in Larimer and Kennedy, 1969a) is anomalous in another respect for it has no homolog in the abdominal segments, nor is its single motoneuron homologous to any of the rostral flexor motoneurons (Dumont and Wine, in prep.; see Discussion).

*Appendage muscles.* The remaining muscles in the telson, the telson-uropodalis (TU) muscles (anterior, posterior, and lateral; ATU, PTU, and LTU), arise from a common tendon attached to the dorsal surface of the telson at a point slightly lateral and caudal to the origin of AT (Fig. 1A). They diverge before their insertions on the uropod, PTU and LTU close together on the inner ventral surface of the propodite and ATU on its medial, rostral rim, adjacent to the insertion of the dorsal rotator

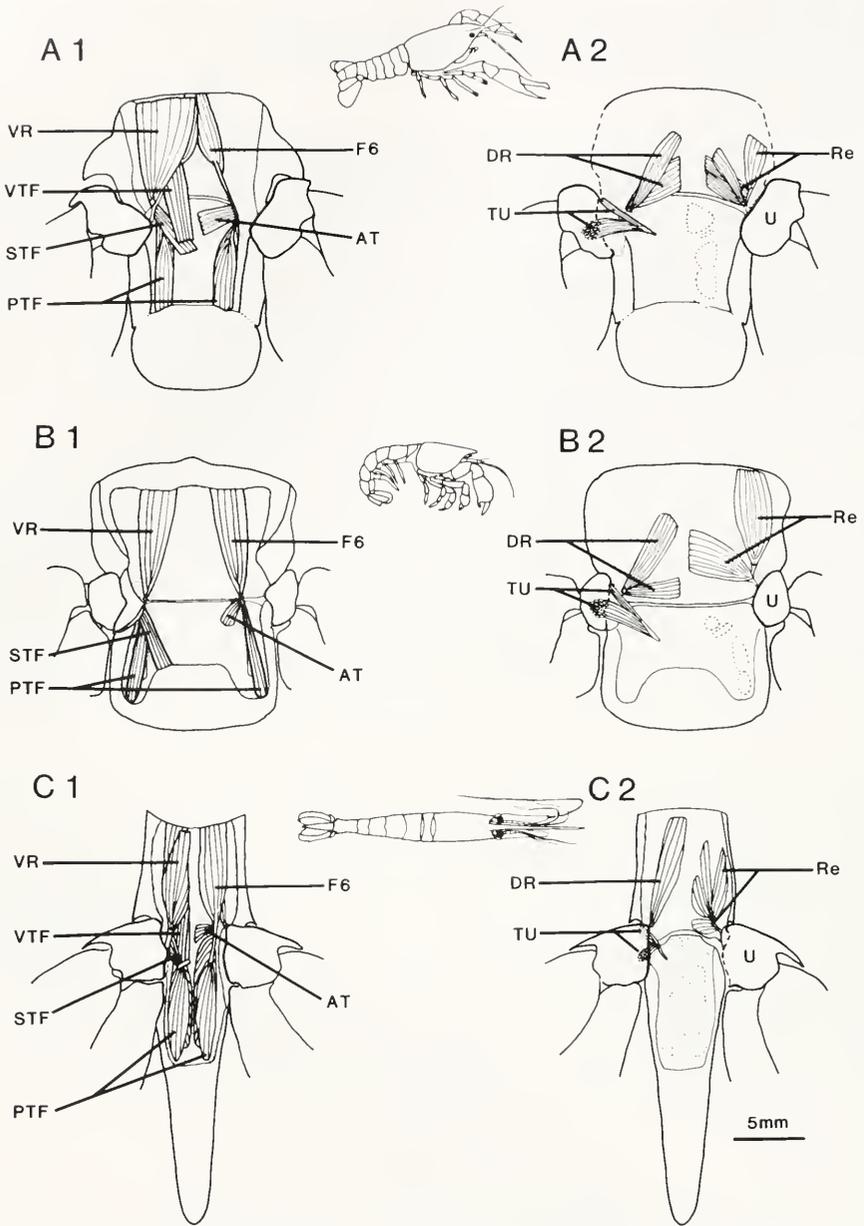


FIGURE 1. Macrurans: ventral aspects of the sixth abdominal segment and tailfan dissected to show all of the telson muscles and those muscles in segment 6 that insert on the uropod. Anterior towards the top. A: *Procambarus*; B: *Upogebia*; C: *Pandalus*. A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>: axial muscles. A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>: appendage muscles that insert ventrally (left side of each figure) and dorsally (right side of each figure) on the uropod propodite (U). Areas enclosed by dotted lines, origins of muscles (axial and appendages) on inner dorsal surface of telson.

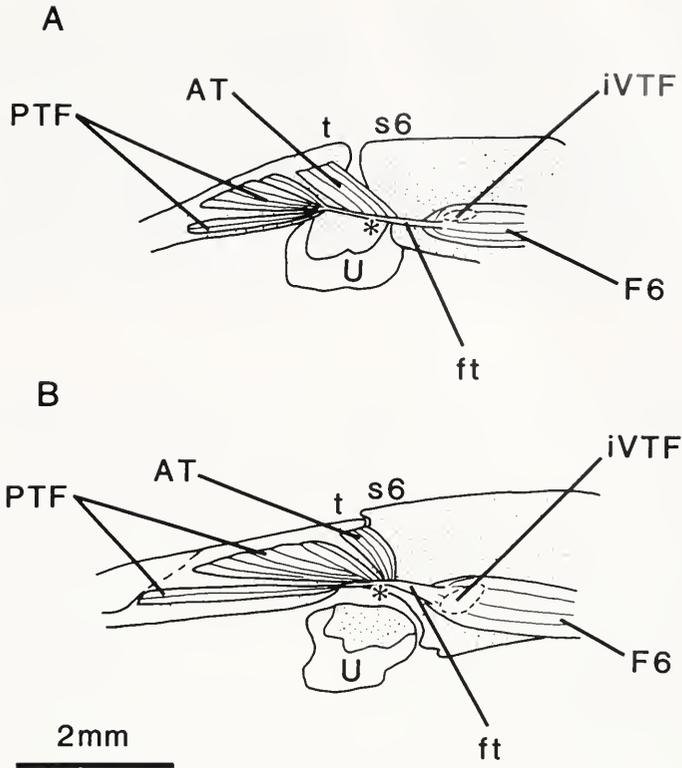


FIGURE 2. Sagittal view of left side of telson (t), segment 6 (s6), and medial edge of left uropod propodite (U) in A, *Procambarus* and B, *Pandalus* to show the flexor tendon (ft) that in macrurans interconnects the axial muscles in the telson and s6. iVTF, insertion of VTF on F6 muscle; \*, approximate location of the interconnection between the ft and the more lateral, perpendicularly oriented TU tendon (see Fig. 1 and text).

muscle. The TU muscles are very small compared to the massive telson flexor muscles. Their actions would appear to be depression of the uropod (see also Larimer and Kennedy, 1969a). The TU tendon is mechanically linked by strong connective tissue to both the arthrodistal membrane in the plane between sixth segment and telson and to the longitudinally oriented flexor tendon (Figs. 1, 2); this would appear to severely limit the TU muscle's independence from the axial musculature.

#### *Is the crayfish plan general for macrurans?*

Macrurans comprise two subgroups, the Reptantia or "crawlers," with abdomen flattened dorso-ventrally, and the Natantia or "swimmers," with abdomen flattened laterally. *Upogebia*, like crayfish, is a reptantian. Its telson muscles are virtually identical to those in crayfish with the exception that VTF is absent (Fig. 1B). The telson of natantians, such as *Pandalus*, is narrower and the uropods more ventral than in Reptantia, but the four axial muscles in the telson are arranged as in crayfish (Figs. 1, 2). The TU muscles are also present and arise from a long, slim tendon that bifurcates to attach to the anterior-dorsal telson, lateral to the origin of AT, and to the posterior-dorsal margin of segment 6, and in mid-course to the arthrodistal membrane and flexor tendon, as in Reptantia. The TU muscles are shorter, however, because

they arise ventral to the PTF muscle; they extend ventrally to insert on the inner surface of the propodite in positions somewhat more lateral than their homologs in crayfish (Fig. 1C<sub>2</sub>).

Although the Reptantia-Natantia division is not taxonomically valid (Schram, 1982), it recognizes the association between two body forms and two modes of living, primarily benthic and primarily pelagic, and is therefore functionally useful. Indeed, the similarity in internal organization despite disparate external form between macruran telsons (compare A and C, Fig. 1) highlights the internal modifications of the ancestral macruran plan in anomuran telsons that externally resemble those of reptant macrurans (see below; compare Fig. 3A, B with Fig. 1A, B; Paul, 1981b).

### *Anomuran telsons*

*Axial muscles.* The number of muscles in the flexor system is smaller in Anomura than in macrurans. Nevertheless, the homology of individual muscles with specific telson flexors in crayfish can be recognized on the basis of relative positions, origin, and innervation by the homolog of crayfish's R6, supplemented by positions and morphologies within the ganglion of the motoneurons innervating each muscle (Fig. 3A-C; see *Innervation*, also Chapple, 1977; Mittenthal and Wine, 1978; Paul, 1981b).

The PTF is the most robust axial muscle in all the decapods. In *Munida* and *Blepharipoda*, it retains its role in telson flexion; but in *Emerita* it inserts directly on the uropod propodite and its sole action is to pronate (and protract) the uropod; it has become functionally an appendage rather than an axial muscle (Fig. 3C, Table II; Paul, 1981b). *Emerita's* PTF (=VM) muscle has been erroneously homologized with the sixth segment ventral rotator muscle in the Galatheid *Galathea strigosa* (Maitland *et al.*, 1982). [This paper also misnamed the dorsal rotator muscle (MR in *Emerita*) that occurs in both macrurans and anomurans (Figs. 1, 3) as the medial remotor; the remotor muscles, as their name implies, insert on the dorsal, not the ventral side of the propodite (Figs. 1, 3).] The STF muscle is reduced in size and innervation in all of the anomurans (Fig. 3; Table III). The VTF is absent, as it is in some macrurans (Fig. 1B), and so is the AT muscle. The loss of AT in the Anomura was probably related to the greater mobility of their uropods: in crayfish the AT muscle contributes to cupping of the uropods (Dumont and Wine, in prep.), a function that has been taken over by a muscle that inserts directly on the propodite, the ATU muscle.

*Appendage muscles.* In contrast to the simplification of the axial muscles, the appendage (TU) muscles in the telson of the anomurans have become enlarged compared to their homologs in macrurans (Figs. 3A-C). TU muscle fibers arise directly from the inner dorsal surface of the telson over rather broad areas (Fig. 1A<sub>2</sub>-C<sub>2</sub>). Three separate heads are recognizable in *Munida* and *Blepharipoda* by their slightly different orientations (origins and insertions) (Fig. 3A<sub>2</sub>, B<sub>2</sub>); they have been called the coxopodite adductor muscle (ATU + PTU) and the accessory coxopodite adductor (LTU) in *Galathea strigosa* (Maitland *et al.*, 1982). In *Emerita* the three TU muscles have become functionally specialized into the dorsomedial, the power-stroke, and the lateral muscles (Paul, 1971b, 1981b) which we think are the respective homologs of ATU, PTU, and LTU in crayfish (Fig. 3C). The ATU muscle in all three anomurans strongly resembles part of the macruran axial musculature, the AT muscle, in its origin (antero-dorso-medial telson), its orientation, and, to a lesser extent, the approximate position of its insertion (respectively, on and close to the medial ventral rim of the propodite): compare ATU in Figure 3A-C with AT in Figure 1A-C. We

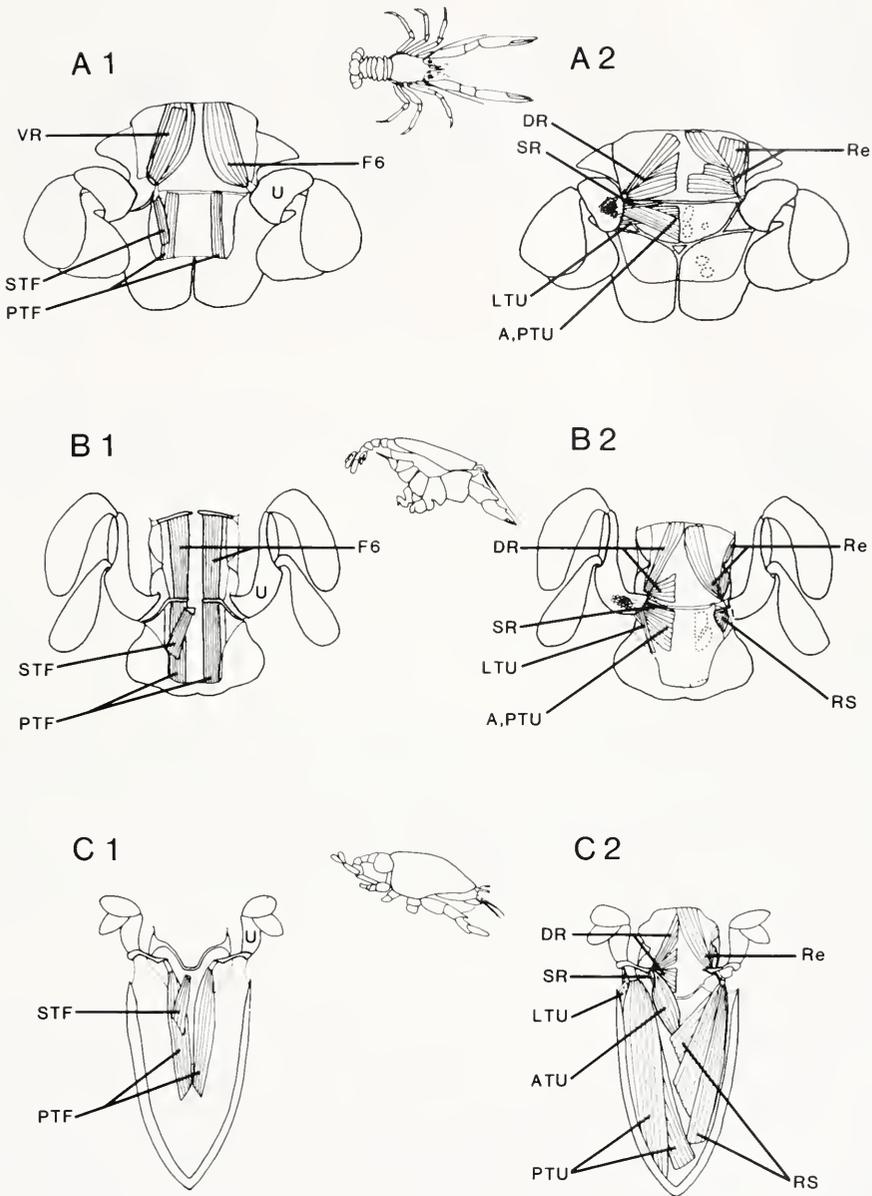


FIGURE 3. Anomurans: ventral aspects of sixth abdominal segment and tailfan to show muscles in telson and segment 6 of A, *Munida*, B, *Blepharipoda*, C, *Emerita*. Layout, scale, and abbreviations the same as for Figure 1. (B, C modified from Paul, 1981b). The dorsal surface of the telson of *Munida* is composed of plates separated by unsclerotized arthrodial membrane. Two heads of the PTF muscle arise from the caudal plate. The stretch receptors (SR) in A and B are shown in their correct position but actually would be partially hidden by ATU in this perspective. Alternate names in galatheids (from Maitland *et al.*, 1982) and in sand crabs (Paul, 1971b, 1981b): ATU: galatheid coxopodite adductor, sand crab dorsomedial muscle (DM); DR: sand crab medial rotator (MR); LTU: galatheid accessory coxopodite adductor, sand crab lateral muscle (LA); PTF: sand crab ventromedial muscle (VM); STF: sand crab medial muscle (ME); VR: in part, galatheid uropod-telson flexor.

TABLE II

*Telson muscles*

	Axial				Appendage		Stretch receptor
	VTF	AT	STF	PTF	TU	RS	
<i>Procambarus</i>	+	+	+	+	+	-	-
<i>Pandalus</i>	+	+	+	+	+	-	-
<i>Upogebia</i>	-	+	+	+	+	-	-
<i>Munida</i>	-	-	+	+	+	-	+
<i>Blepharipoda</i>	-	-	+	+	+	+	+
<i>Emerita</i>	-	-	+	+	+	+	+

were, in fact, misled by this strong anatomical resemblance into considering the ATU in anomurans to be the homolog of the macruran AT muscle until we had investigated their innervations (see *Innervation*; Figs. 4, 5).

*Comparison between telson musculature in macrurans and anomurans*

The muscles present in the telsons of the six animals are summarized in Table II. In the order listed, there is a decrease in number and in size (relative to size of tailfan) of individual axial muscles. The anomurans have done away with the flexor tendon between the axial muscles in the telson and segment 6. *Munida* and *Blepharipoda* have lightly sclerotized and quite flexible telsons, so that their axial muscles both bend the telson and flex it on the abdomen. In *Emerita* the principle axial muscle, PTF, is retained even though the hinge between segment 6 and telson is nearly immobile, but it inserts directly on the uropod and is active during the power stroke of this appendage (Table II; Paul, 1979, 1981b).

The TU muscles in anomurans are much larger than in macrurans. They all arise directly from the dorsal telson, each muscle from a different area (Fig. 3), in contrast to their origin from one tendon in macrurans (Fig. 1). Their insertions on the propodite are in roughly similar relative positions in all six animals.

TABLE III

*Motoneurons in R6 of G6*

<i>Procambarus</i> <sup>1</sup>	<i>Upogebia</i>	<i>Munida</i>	<i>Blepharipoda</i>	<i>Emerita</i>
Muscle:			predicted numbers	
VTF 5(2FF + 2FI + MoG)	0	0	0	0
AT 1	1	0	0	0
STF 4	4	4	4	≤4
PTF 7(4FF + 2FI + MoG)	7	6	6	6
total = 15 <sup>1</sup> Expect:	12	10	10	≤10
Found:	12	~8	8 <sup>3</sup>	6 <sup>2,3</sup>

<sup>1</sup> Physiological identifications of motoneurons in *Procambarus* from Larimer and Kennedy (1969) and Dumont and Wine (in prep.). FF, fast flexor motoneurons; FI, flexor inhibitor—both are shared by VTF and PTF; MoG, motor giant.

<sup>2</sup> Paul (1971b).

<sup>3</sup> Paul (1981b).

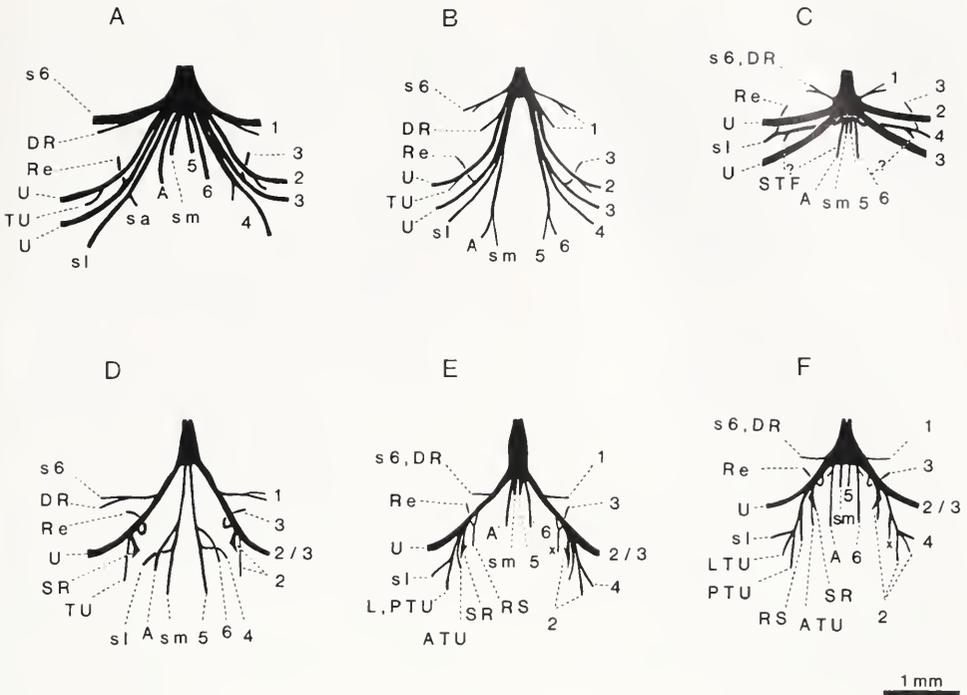


FIGURE 4. The terminal abdominal ganglion, G6, in A, *Procambarus*, B, *Upogebia*, C, *Pandalus*, D, *Munida*, E, *Blepharipoda*, F, *Emerita*. The ganglionic roots on the left side are labelled by the target they innervate, and on the right side by number according to their homology with roots in *Procambarus*: in D-F, roots 2 and 3 actually enter uropod as separate nerves as they do in macrurans. A, axial muscles (see Table II for muscles present in each species); field of sensory roots: s6, lateral and dorsal surfaces of segment 6; sa, sl, sm, anterodorsal, lateral, medioposterior telson, respectively; \* (E, F), RS nerve = ontophylogenically a branch of R3 (see Discussion). Ganglia, not roots, are drawn to the same scale—some of the roots are shown enlarged and spread apart for clarity.

Two additional features are included in Table II. First, to complete the list of telson muscles, is the return-stroke muscle (RS), peculiar to sand crabs (Fig. 3Bii, Cii). This “new” component of the appendage neuromusculature in the telson is the antagonist of the TU muscles, since its action is to elevate (and in *Emerita*, remote) the uropod (Paul, 1971b, 1981b). And finally, in the Anomura, a stretch receptor (SR) spans the basal joint of the uropod from anterior, dorsal telson to ventral, medial rim of the propodite (Fig. 3). It is closely allied with the ATU muscle (this study; Paul, 1971c, 1972) and its few muscle fibers and sensory neurons with central somata may have been derived from this part of the appendage neuromusculature. The receptor in galatheids was first reported in *Galathea strigosa* by Maitland *et al.* (1982), who incorrectly described the position of its dorsal attachment as in the middle of segment 6, rather than at the anterior edge of the telson; their illustration shows it to be in the same location as the SR in *Munida* (Fig. 3A<sub>2</sub>).

#### *Innervation of the telson*

*The sixth abdominal ganglion.* The terminal ganglion in decapods is fused embryonically from primordial abdominal ganglia six and seven, plus a terminal cell

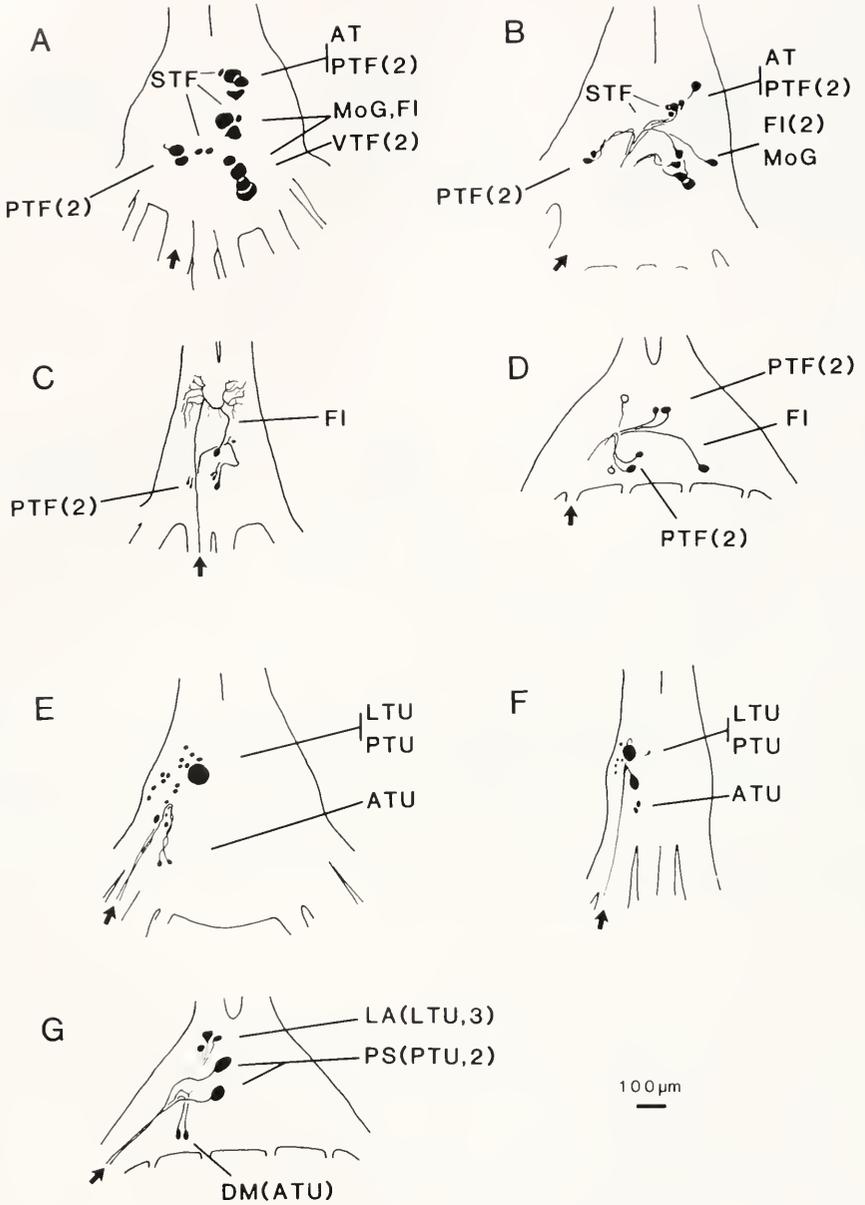


FIGURE 5. Motoneurons in G6 with (A–D) axons in R6 that innervate the axial muscles (see Table III) and (E–G) axons in R2 that innervate the appendage muscles in the telson. Anterior toward top of page. A, E, *Procambarus*; B, *Upogebia*; C, F, *Munida*; D, G, *Emerita*. Identification of fast flexor motoneurons in crayfish from Dumont and Wine (in prep.). Some of the R6 motoneurons in the other species are labelled by the similarities in their soma positions and neurite structures (not shown) with those of the identified crayfish motoneurons, but these identifications have not been confirmed physiologically. The open somas in D show the two alternate positions occupied in different specimens by one motoneuron (probably the STF motoneuron); the positions of the other five are invariant. R2 motoneurons are labelled from backfills of individual nervelets to the three muscles in *Emerita* (G; see Paul, 1981b) and their putative homologs suggested for crayfish (E) and *Munida* (F). The representative backfills shown for crayfish's and *Munida*'s TU branch of R2 may not include all of the smaller neurons. The SR somas are not included in F and G (see Paul, 1972; Maitland *et al.*, 1982).

cluster (Bullock and Horridge, 1965; Dumont and Wine, in prep.). It is the sixth free ganglion (G6) in the abdomen of macrurans. In the three anomurans we have studied, and also in pagurid anomurans (Chapple, 1977), the terminal ganglion is the fifth free ganglion, because abdominal ganglion 1 has fused with the last thoracic ganglion. Further condensation of the anterior abdominal nervous system has occurred in some other Anomura, further reducing the number of free ganglia in the abdomen (Bullock and Horridge, 1965). But, the terminal ganglion in all species is the homolog of the macruran G6 and, to facilitate inter-specific comparisons, we advocate that it and the other abdominal ganglia be numbered according to the number of their homologs in the uncondensed decapod nerve cord (see Paul, 1979).

*Nerve roots of ganglion 6.* The innervation of the axial and the appendage muscles is from two separate roots of G6. Because the number and positions of the nerve trunks leaving the ganglion are different in each species, we have adopted the numbering system for the roots in crayfish to describe the neuroanatomy of the other tailfans in order to facilitate inter-specific comparisons (Paul *et al.*, 1983, Fig. 4). Figure 4A shows the six roots that innervate the skeletal muscles of the crayfish tailfan (the 7th root, to the gut, is omitted); their destinations are given in the figure and legend (see also Larimer and Kennedy, 1969a). In the other macrurans and in anomurans some of the roots emerge from G6 as single trunks, giving the appearance of fewer than six ganglionic nerves. However, the distributions of the various branches to their final destinations (determined by methylene blue staining and electrical stimulation and recording from individual nerve branches; see Materials and Methods) reveal which are the roots corresponding to crayfish's. We have numbered them accordingly in Figure 4B-F.

*Innervation of telson muscles.* The arrangement of musculature in macruran tailfans is sufficiently uniform to present little problem in making interfamilial comparisons (Fig. 1), although their ganglionic roots are rather different (Fig. 4A-C). But anomuran tailfans have diversified so much that gross anatomical observation is inadequate to suggest homologies between individual telson muscles and their counterparts in macrurans (Paul, 1971b). In these cases innervation provided the clue to recognizing the subdivision between axial muscles, innervated by R6, and TU muscles, innervated by R2 (Fig. 4). And within each group, axial and appendage, the motoneurons serving individual muscles can be identified by backfilling with dye their individual nerve branches (Fig. 5). Homologs in the different families can then be suggested based on similarities in position and morphology of the motoneurons (Fig. 5; Paul, 1981b; see also: Chapple, 1977; Mittenthal and Wine, 1978; Sillar and Heitler, 1982). We have used this approach to examine the innervation of the axial muscles in the telsons of five of these decapods (all but the prawn).

The results of unilateral Ni-backfills of R6 in each animal are summarized in Table III (see also Paul *et al.*, 1983). On the left are listed the four muscles in *Procambarus*, followed by the number of motoneurons innervating each; the physiological identifications of motoneurons given in parentheses are from Larimer and Kennedy (1969a) and Dumont and Wine (in prep.). To investigate whether muscles and their motoneurons might be retained or lost together during evolution, we have compared the expected and actual number of motoneurons in R6 for each of the four genera. In *Upogebia* we found three fewer motoneurons than in *Procambarus*, as expected by the loss of the VTF with its two fast flexor motoneurons and one motor giant (both flexor inhibitors are shared with PTF and so would be expected to be retained). In the three anomurans we expected a reduction in numbers of R6 neurons greater than predicted by loss of the VTF and AT muscles (3 excitatory and 1 motor giant motoneurons) because none of these animals has giant interneurons

(Paul, 1971a; Paul and Then, unpub.), and we predicted that none would have the remaining motor giant, reducing PTF's innervation by one motoneuron. There are, in fact, even fewer R6 motoneurons than predicted. We attribute the discrepancy in *Munida* and *Blepharipoda* primarily to reduction in STF innervation concomitant with the reduction in size of this muscle, because this is the case in *Emerita*, where the STF (ME) muscle is innervated by a single motoneuron (Paul, 1971b). Analogous reductions in numbers of motoneurons correlated with hypotrophy of muscles have occurred in the abdomen of pagurids (Chapple, 1977; see also Mittenthal and Wine, 1978), whereas the number has remained constant for the homologous muscles of similar size in macrurans (Kahan, 1971). The PTF (VM) muscle in *Emerita* is innervated by five motoneurons (Paul, 1971b), two fewer than in crayfish; from some positions and neurite branching patterns of the R6 motoneurons it appears that in addition to its motor giant one of the peripheral inhibitory neurons has been lost. We think this may also be the case in *Munida* and *Blepharipoda*. The uncertainty in the latter two animals stems from multiple branching of the nerves to both STF and PTF which reduces the confidence with which we could assign motoneurons backfilled from individual nervelets to particular muscles. In conclusion, Table III should be considered as setting forth a series of specific hypotheses about telson axial neuromusculature that can be tested experimentally by comparing physiological properties of individual motoneurons with those of their homologs in crayfish (Dumont and Wine, 1983, in prep.; Larimer and Kennedy, 1969a; see also Kahan, 1971; Chapple, 1977; Mittenthal and Wine, 1978; Sillar and Heitler, 1982).

#### DISCUSSION

We have described the organization of the neuromusculature in the telsons of decapod Crustacea. We use this plan to suggest homologies between individual muscles, nerve roots, and motoneurons in three macrurans and three anomurans (Table II; Figs. 4, 5). The most significant functional difference in the telson musculature of these anomurans compared to macrurans is the freedom of the appendage (TU) muscles from actions of the axial muscular system. This difference arose by two definitive peripheral changes: elimination of mechanical coupling between flexor and TU muscles and the elimination of the TU tendon, so that the TU muscle fibers in anomurans arise directly from the dorsal telson. Our results also reveal some specific neural changes that accompanied the evolution of the anomuran tailfans but that are not, apparently, correlated with any change in mode of swimming (tailflipping).

#### *Neural correlates of the emancipation of the TU muscles from the axial muscular system*

*Loss of giant interneurons and motor giants.* Macrurans have one or two pairs of giant interneurons (the medial and lateral giant neurons, MG and LG) that mediate in part rapid flexions of the abdomen and tailfan (Bullock and Horridge, 1965; Wine and Krasne, 1982). The pair of giant interneurons in *Callinassa* (and *Upogebia*) is the homolog of the MGs in crayfish (Turner, 1950). The loss of the LGs in these two macrurans was probably secondary to adoption of their burrowing habit. Wine and Krasne (1982) summarize evidence from the cellular organization of crayfish escape behavior that suggests evolution of the giant systems (interneurons, motor giants, and segmental giants) from non-giant neurons. We assume that the giant interneurons were present in the macrurans from whom anomurans evolved. They may have been

present in the earliest decapods, considering that (1) their putative homologs occur in a primitive eumalacostracan (Silvey and Wilson, 1979), (2) the abdomens and tailfans of Devonian fossil and modern macrurans are externally very similar (Schram *et al.*, 1978), and (3) the likelihood that decapods evolved in a near-shore marine habitat (Schram, 1981) in which, presumably, the different forms of rapid, escape tailflips would have been as adaptive as they appear to be today.

Increased autonomy of the enlarged TU muscles in anomurans is correlated with the absence of the giant interneurons (Paul, 1971a; Sillar and Heitler, 1982; Paul and Then, unpub.). Concurrent with the demise of the giant interneurons may have been the loss of the specialized fast flexor motoneurons, the motor giants (MoG) in G6. Our R6 motoneuron counts and morphological data suggest that the one MoG innervating PTF is present in G6 of *Upogebia*, but not in G6 of *Emerita* or either of the tailflipping anomurans, *Munida* or *Blepharipoda* (Table III; Fig. 5). Sillar and Heitler (1982) proposed that an "unspecialized" fast flexor motoneuron in the mid-abdominal ganglia of *Galathea strigosa* (Galatheididae) is homologous to crayfish's MoG. This suggests that members of the serial set of MoGs may have fared differently in the evolution of galatheid anomurans, those in G6 having been lost (one with the demise of VTF, the other reducing PTF's innervation), while those in other abdominal ganglia were retained. Alternatively, an unspecialized MoG may be retained in G6 and one of the fast flexor motoneurons lost. Physiological descriptions of the R6 motoneurons in anomurans to compare with their crayfish homologs (Dumont and Wine, 1983, in prep.) are needed to distinguish between these possibilities.

*Uropod stretch receptor*. The uropod coxal receptor (SR: Fig. 3A<sub>2</sub>-C<sub>2</sub>) has been described previously in *Emerita analoga* (Paul, 1971c, 1972) and in *Galathea strigosa* (Maitland *et al.*, 1982). The latter authors mistook the articulation between telson and segment 6 for an "anterior mid-dorsal hinge in the middle of the 6th abdominal segment" and, therefore, erroneously described the SR's dorsal attachment as being to the middle of segment 6 instead of to the anterior telson.

Serially homologous SRs, innervated by receptor neurons with central somata, occur at the base of segmental appendages in the head (Pasztor, 1969; Pasztor and Bush, 1983), the thorax (Alexandrowicz and Whitear, 1957; Blight and Llinas, 1980), and the abdomen (Heitler, 1982); such sensory structures might have been associated with each of the appendages in primitive crustaceans, including the terminal pair, which probably were more swimmeret- than uropod-like (*cf.*, *e.g.*, modern Nebaleidae). They would then have been lost from the uropods in the subsequent evolution of the eumalacostracan tailfan. Among decapods, uropod SRs occur in the galatheids and albuneids, anomurans that swim by tailflipping in a manner similar to their macruran ancestors (Paul, 1981b, unpub.; Maitland *et al.*, 1982; this study).

The coxal SRs reflexly excite motoneurons in dissected animals, but in most cases little is known about their role in behavior (Blight and Llinas, 1980; Heitler, 1982; Maitland *et al.*, 1982). In *Emerita* they mediate a "complete" resistance reflex that includes reciprocal excitation and inhibition of both excitatory and peripheral inhibitory motoneurons of the PS (PTU) and RS muscles, and they also modulate VM (PTF) motoneuron activity (Paul, 1971c, 1972, unpub.). One function of this reflex is to coordinate uropod power strokes (PTU motoneuron bursts) in the 'treading water' behavior of this sand crab (Paul, 1976). When more is known about the role of the uropod SRs in tailflipping anomurans (Maitland *et al.*, 1982), comparison with *Emerita*'s nonspiking SRs may reveal what specific advantage analog signaling conveys that counterbalances the metabolic cost of developing and maintaining such large neurons (Pearson, 1979; Shepherd, 1981).

*From tailflipping to swimming with the uropods*

This and other studies (Larimer and Kennedy, 1969; Paul, 1971a, b, 1972, 1981a, b; Maitland *et al.*, 1982; Dumont and Wine, 1983, in prep.) provide enough comparable data on the tailfans of different decapods to suggest an evolutionary history of the telson neuromusculature in *Emerita* from the macruran plan. Although the rami of macruran uropods can be flared horizontally by the action of muscles located within the propodite, movement of the appendage relative to the body is quite restricted. Correlated with greater uropod mobility in galatheids and albuneids are reduction of axial flexor muscles, hypertrophy of the TU muscles, and the appearance of the uropod SR. Mechanical linkage between appendage and axial muscles in the telson is gone in both families, but in galatheids the VR muscle in segment 6 is partially fused with the deep-lying fast flexors as it is in macrurans (Fig. 3A). In albuneids, potentially greater independence of the uropods from axial movements is made possible by loss of the VR muscle and addition of a 'new' appendage muscle in the telson (the RS muscle) to elevate the uropod (Fig. 3B; Paul, 1981b). The absence of RS in galatheids, which otherwise albuneids resemble in both telson neuromusculature and tailflipping behavior, would appear to confirm that RS is a 'new' muscle, peculiar to sand crabs (Paul, 1981b). Finally, correlated with the transition from tailflipping to the hippid behavior of swimming with the uropods were three kinds of changes in the telson, all involving muscular and neural components present in the tailflipping, albuneid sand crabs: (1) the insertion of PTF, the principal remaining axial muscle, was moved from axis to uropod; (2) two appendage muscles and their motoneurons experienced tremendous hypertrophy: an ancestral macruran muscle, PTU, to become the uropod power-stroke muscle, and the sand crabs' new RS muscle (Fig. 3; Paul, 1981b); and (3) the physiology of the uropod stretch receptor neurons changed from digital to analog signaling (Paul, 1972, unpub., Maitland *et al.*, 1982).

Paul (1981b) had suggested possible derivation of *Emerita*'s power-stroke (PS = PTU) muscle from the macruran axial muscle, PTF, with a change in exit of the two PS motoneurons from R6 to R2. Having now identified all of the homologs between the telson muscles in *Emerita* and macrurans (Table II) and located their motoneurons in G6 (Fig. 5), we can discard this hypothesis. The resemblance between crayfish's PTF muscle and *Emerita*'s PTU muscle (compare Fig. 1A<sub>1</sub> and Fig. 3C<sub>2</sub>) is clearly an example of convergent evolution, the result of the need for both muscles to be oriented longitudinally because the swimming strokes they mediate are parallel to the long axis of the body—the flexion-extension of the abdomen in the ancestral behavior of tailflipping and the uropod stroke in the hippid mode of swimming with the uropods. The axons exit through the appropriate root to innervate axial and appendage respectively. Thus the conservation of root of exit of motoneurons in mid-body ganglia (Mittenthal and Wine, 1978) appears to be true for the terminal ganglion as well.

The cumulative observations leading to this scenario reinforce the suggestion that swimming with the uropods evolved directly from non-giant mediated tailflipping, and that homologies between elements of the neuronal circuitries mediating each of these two behaviors must exist (Paul, 1971a, 1979, 1981a, b). Both behaviors rely on central generation of the reciprocal pattern of motor activity for power and recovery phases of the swimming stroke (Paul, 1979; Reichert *et al.*, 1981). In *Emerita*, this pattern is expressed by centrally generated bursting of both PTF and PTU motoneurons. Comparable recordings of the motor pattern in crayfish have not been made because it is not spontaneously expressed by the isolated nervous system and a way of eliciting

it has not yet been found. However, neurites of the PTU motoneurons in *Procambarus* (Paul, unpub.) and their homologs, the PS motoneurons, in *Emerita* (Paul, 1981b) project rostrally in G6 to comparable regions of large, dorsal cord axons (DCA) entering from the 5–6 connective. In *Procambarus*, some of these DCAs belong to the non-giant circuitry for tailflipping (Kramer *et al.*, 1981a, b; Wine and Krasne, 1982; Kramer and Krasne, 1984), but nothing is yet known about activity of the TU muscles during this behavior. These comparisons raise two questions that are particularly relevant to the evolution of the hippid swimming behavior: are crayfish PTU motoneurons included in the centrally generated motor pattern for repetitive tailflipping as their central anatomy might suggest? And, are the DCAs in *Emerita* part of the circuit for the uropod central motor program, and, therefore, candidate homologs of specific DCAs in crayfish? If both answers are affirmative, then the connections between DCAs and the PTU (as well as the PTF) motoneurons are potential loci for neural changes incurred during the evolution of the new hippid swimming behavior.

### *General Discussion*

Since the telson is not a somite, with appendages and a ganglion of its own, and, in fact, is little more than a skeletal tailpiece in primitive Crustacea, it is not obvious how in decapods it came to house a neuromusculature nearly as rich and diverse as is present in the body segments. We suggest that the appendage musculature in decapod telsons is ontophyletically neuromusculature of abdominal segment 7. Without development of a seventh abdominal segment in decapods, the terminal ganglion became a fusion of the sixth and seventh abdominal ganglia (plus a terminal cell cluster) (Bullock and Horridge, 1965; Dumont and Wine, in prep.), and muscles of primordial segment 7 that were retained came to be located in the anterior part of the telson.

In a penaeid (suborder Dendrobranchiata), whose morphology is considered to represent a "generalized decapod condition," Young (1959) described protopodite remotor and rotator muscles that appear to be homologs of the TU muscles in the suborder Pleocyemata. The main difference is that their dorsal origin is from connective tissue in the plane of articulation between segment 6 and telson, a position that suggests they could be derivatives of the primordial seventh abdominal segment. They are unlikely to be segment 6 appendage muscles because these are accounted for by the rotator and remotor muscles (Figs. 1, 3). This would make the TU muscles the serial homologs of the dorsal rotator muscles in segment 6.

When the uropods of anomurans acquired greater mobility than in macrurans, additional structures characteristic of segmental appendages were expressed: the SRs with central somata, and, in sand crabs, uropod remotor (=RS) muscles in the telson. In an earlier study Paul (1981b) surmised that the RS muscle had been derived from the remotor muscles (=LR) in the 6th segment on the basis of the innervations, and similarities in positions and morphologies of the motoneurons innervating these two muscles. A reinterpretation of these features is that they reflect serial homology of the RS and the 6th segment remotor neuromusculature, the RS muscle being segment 7 remotor expressed in the telson. Thus, the "new" muscle and its motoneurons in the sand crab telsons, as well as the phylogenetically older TU muscles, would be products of an ontogenetic potential for a 7th abdominal segment the development of which was repressed in decapod evolution. These ideas are highly speculative, and developmental studies of G6 and telson in these species would help clarify the relationships among the neuromuscular components of their tailfans.

Two other components of the tailfan's neural and muscular systems are also thought to be derivatives of both 6th and 7th segments: the fast flexor neuromusculature in the telson (Dumont and Wine, 1983, in prep.), and the uropod SR in *Emerita* which has four nonspiking sensory neurons (Paul, 1972) compared to the two nonspiking neurons in the swimmeret SR (Heitler, 1982). It appears, therefore, that in decapods the only neuromusculature in the telson with no rostral homologs may be the AT muscle and its single excitatory motoneuron (Dumont and Wine, in prep.). This muscle and its motoneuron have been lost in anomurans.

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## TAXONOMIC CORRELATES OF BIOLUMINESCENCE AMONG APPENDICULARIANS (UROCHORDATA: LARVACEA)

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### ABSTRACT

Larvaceans, common members of marine plankton communities, filter-feed with renewable, external, mucous houses. The houses of some species of Oikopleuridae produce endogenous bioluminescent flashes upon mechanical stimulation and may contribute significantly to surface luminescence. To determine which members of the Oikopleuridae are luminescent, we examined several species for stimulable luminescence and for morphological features responsible for or associated with light production. Luminescence is newly reported from house rudiments and from clean, particle-free houses of *Oikopleura rufescens* and *Stegosoma magnum*. In these species, light emanates from previously undescribed fluorescent inclusions in the house rudiment. Neither fluorescence nor luminescence were detected from other parts of the body. Both species also possess oral glands, which apparently are not directly involved in light production but serve as a convenient taxonomic marker of luminescence. All six known luminescent species of larvaceans possess fluorescent and luminescent house rudiment inclusions and oral glands. We predict on these morphological grounds that all twelve species of *Oikopleura (Vexillaria)* plus the oikopleurids *S. magnum* and *Folia gracilis* are luminescent. In two other oikopleurids that lack oral glands, *O. fusiformis* and *Megalocercus huxleyi*, neither fluorescent inclusions nor luminescence were detected in clean houses and animals with house rudiments. However, some field-collected houses of these species produced luminescent flashes, perhaps from dinoflagellates on or in the houses. This report should facilitate assessment of the contribution of larvaceans to surface luminescence on a global scale.

### INTRODUCTION

Larvaceans, important members of marine plankton communities (Alldredge and Madin, 1982), are pelagic tunicates that filter nanoplankton with the aid of an external, renewable, mucous, feeding apparatus, termed a house (Fig. 1) (Fol, 1872; Lohmann, 1899; Alldredge, 1976a). New houses are expanded five to ten times per day (Paffenhöfer, 1973; King, 1981) from mucous rudiments secreted by the animal's trunk during occupation of the previous house (Fol, 1872; Lohmann, 1899; Alldredge, 1976a, c). Evidence that occupied and discarded houses of certain species produce endogenous, luminescent flashes upon mechanical stimulation (Galt, 1978; Galt and Sykes, 1983) indicates that these widespread, often abundant zooplankters may contribute substantially to stimulable, surface luminescence, a view shared by Swift *et al.* (1983).

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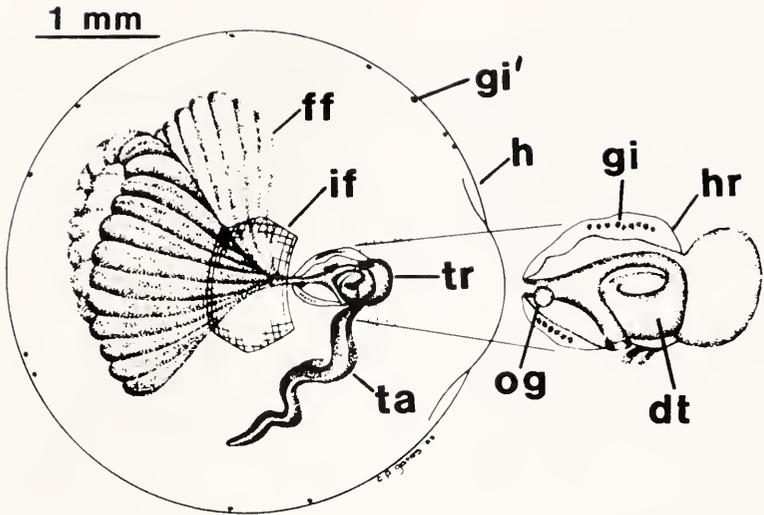


FIGURE 1. Diagram of oikopleurid larvacean within its expanded house (h) with granular inclusions (gi'); enlargement shows house rudiment (hr) with granular inclusions (gi), and oral gland (og). dt: digestive tract, ff: feeding filter, if: incurrent filter, ta: tail, tr: trunk. Drawn from life by Cynthia P. Gates and used with permission of Springer-Verlag, New York.

Until now, only four larvacean species were known to luminesce (Table I): *Oikopleura albicans* (Lohmann, 1899), *O. dioica* and *O. labradoriensis* (Galt, 1978; Galt and Sykes, 1983), and *O. vanhoeffeni* (Tarasov, 1956). These species have in common two taxonomically important morphological features: paired oral glands and a species-specific pattern of inclusions in the house rudiment (Fig. 1) (Bückmann and Kapp, 1975). Oral glands occur in the *Oikopleura* subgenus *Vexillaria* (Lohmann and Bückmann, 1926; Lohmann, 1933) and in *Stegosoma magnum* and *Folia gracilis*. Secretions from the oral glands have been regarded as the source of bioluminescence (Lohmann, 1899, 1933; Fredricksson and Olsson, 1981), but this was not confirmed by Galt and Sykes (1983). On the other hand, house rudiment inclusions are known to be the actual sites of luminescence in two species of *Vexillaria* (Galt and Sykes, 1983), and inclusions are reported for all but one species (*O. rufescens*) of this subgenus (Bückmann and Kapp, 1975). Luminescence has not been reported in any other oikopleurids or in the Fritillaridae or Kowalevskiidae.

Understanding the morphological bases of bioluminescence in larvaceans should help to clarify the mechanisms of light production and permit prediction of luminescence along taxonomic lines. To this end, we examined five species of oikopleurids occupying various taxonomic positions (Table I): *Oikopleura rufescens* and *Stegosoma magnum* possess oral glands, but are reported to lack house rudiment inclusions (Lohmann, 1896, 1933; Lohmann and Bückmann, 1926; Bückmann and Kapp, 1975); *Oikopleura fusiformis* and *Megalocercus huxleyi* lack oral glands and house rudiment inclusions (Lohmann, 1933; Bückmann and Kapp, 1975). We also re-examined *Oikopleura dioica*, which has oral glands and house rudiment inclusions, to confirm our previous observations (Galt and Sykes, 1983).

We report here the first *in situ* observations and photometric records of bioluminescence by animals with house rudiments, freshly collected houses, and particle-free houses in *Oikopleura rufescens* and *Stegosoma magnum*. We also establish house rudiment inclusions as the sites of light production in these two species and, on this

TABLE I

Classification of Larvacea (Fenaux, 1966; Bückmann and Kapp, 1975) indicating species examined in the present study (\*) and, in the right column, species known (§) or predicted (remaining eight species) to possess endogenous bioluminescence

Species without oral glands and rudiment inclusions Non-luminescent	Species with oral glands and rudiment inclusions Luminescent
Oikopleuridae (32 species)	
<i>Oikopleura</i> (Coccaria)	<i>Oikopleura</i> (Vexillaria)
<i>O. cornutogastra</i>	§ <i>O. albicans</i>
* <i>O. fusiformis</i>	<i>O. cophocerca</i>
<i>O. gracilis</i>	§* <i>O. dioica</i>
<i>O. graciloides</i>	<i>O. drygalskii</i>
<i>O. intermedia</i>	<i>O. gaussica</i>
<i>O. longicauda</i>	§ <i>O. labradoriensis</i>
<i>Megalocercus abyssorum</i>	<i>O. mediterranea</i>
* <i>M. huxleyi</i>	<i>O. parva</i>
<i>Bathochordaeus charon</i>	§* <i>O. rufescens</i>
<i>Althoffia tumida</i>	<i>O. valdiviae</i>
<i>Pelagopleura</i> spp. (6)	§ <i>O. vanhoeffeni</i>
<i>Sinisteroffia scrippsi</i>	<i>O. weddelli</i>
<i>Chunopleura microgaster</i>	§* <i>Stegosoma magnum</i>
	<i>Folia gracilis</i>
Fritillariidae	
28 species	
Kowalevskiidae	
2 species	

basis, conclude that endogenous luminescence probably occurs in fourteen species of larvaceans. Additionally, we report the lack of house rudiment inclusions and endogenous luminescence in *O. fusiformis* and *Megalocercus huxleyi*. Finally, we report that discarded houses of even those species of larvaceans incapable of endogenous luminescence may at times produce secondary luminescence, possibly emanating from microorganisms that are associated with the houses.

#### MATERIALS AND METHODS

We conducted this study near Isla El Pardo (110°36.5' W, 24°50.5' N), south of Isla San José in the southern Sea of Cortez, Mexico, during 10–19 August, 1981, aboard the shrimp trawler, B/M MARSEP V, owned by the Secretary of Public Education and operated by Centro de Estudios Tecnológicos del Mar en La Paz, Baja California Sur, Mexico. Surface water and air temperatures were nearly constant at 30°C, thus eliminating the effect of temperature on light production.

We commonly encountered specimens of *Megalocercus huxleyi*, *Oikopleura fusiformis*, *O. rufescens*, *Stegosoma magnum*, and, less commonly, *O. dioica*. We collected animals at night (2000–2400 h, MST) for visual, photometric, and microscopic observations by snorkeling and using a hand-held dive light. The diver could identify most species *in situ* on the basis of behavior and house morphology (Alldredge, 1976c, 1977). The diver enclosed each occupied house in a 50–150 ml wide-mouth jar and passed these to personnel on shipboard. Each animal was identified, removed by gentle prodding from its field-collected house, and placed in a small dish containing 50–100 ml of 0.45 µm filtered sea water, where it built a new, particle-free house. The animal was then removed from its new house, and the field-collected house, the

particle-free house, and the animal with its closely adhering house rudiment were each placed in separate 20-ml scintillation vials with 5–10 ml of particle-free sea water.

We recorded luminescence from these preparations in a light-tight chamber viewed by a side-window photomultiplier tube as described by Galt and Sykes (1983). Each preparation was stimulated twice in succession by forcefully injecting 2 ml of particle-free sea water into the vial. Control injections into water in which the animal had built its house did not elicit luminescence. For visual confirmation of luminescence, we agitated some preparations in a darkened area. All of our observations and recordings were made at night.

Using Nomarski and incident fluorescence microscopy (Galt and Sykes, 1983), we examined animals and their intact house rudiments for fluorescent inclusions, luminescence from the inclusions, and extraneous bioluminescent microorganisms.

## RESULTS

### *Luminescent species*

Of the five species, *Oikopleura rufescens*, *O. dioica* (see also Galt, 1978; Galt and Sykes, 1983), and *Stegosoma magnum* were endogenously luminescent. We consistently recorded flashes upon stimulation of free animals with house rudiments, field-collected houses, and, except in *O. dioica*, new, particle-free houses (Fig. 2, Table II). Visual observations, *in situ* and on shipboard, of blue-green flashes from both

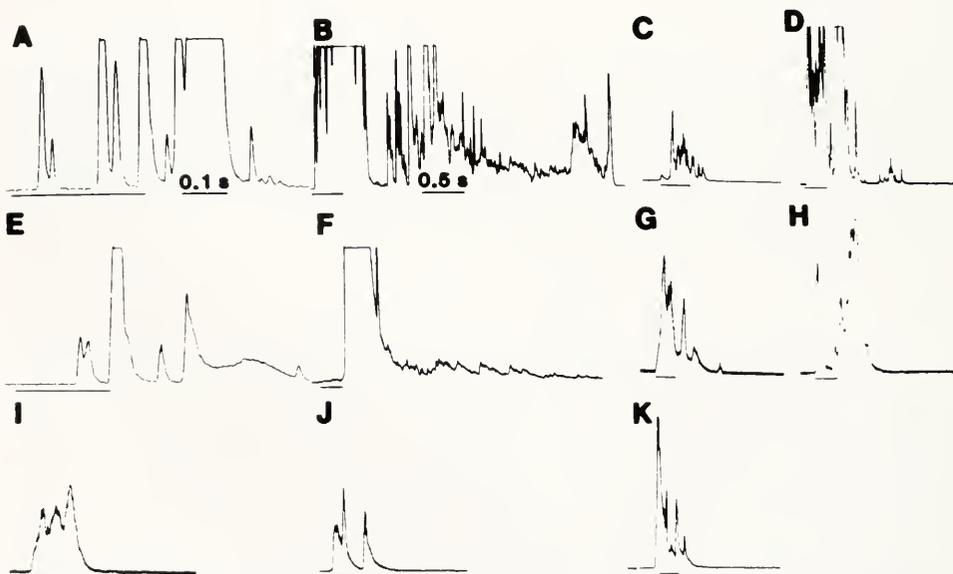


FIGURE 2. Luminescence mechanically elicited from *Stegosoma magnum* (A, B, C, D), *Oikopleura rufescens* (E, F, G), *O. dioica* (H, I), and field-collected houses of *O. fusiformis* (J) and *Megalocercus huxleyi* (K). Recordings were from animal with house rudiment (A, E, H); isolated, particle-free house rudiment (B); unoccupied, particle-free house (C, F); and unoccupied, field-collected house (D, G, I, J, K). Time bar = 0.1 s for A and E and 0.5 s for B–D, F–K. The vertical light intensity scale is arbitrary and is the same for all records. Horizontal bar beneath the start of each trace represents the duration of the stimulus (see text). All flat peaks represent off-scale responses. These flash forms are only examples and do not represent consistent patterns for a given type of preparation.

TABLE II

*Luminescent responses of Sea of Cortez larvaceans to mechanical stimulation*

Species	Response	Field house	Animal with house rudiment	Clean house
<i>Stegosoma magnum</i>	Positive	17	5	8
	None	0	0	0
<i>Oikopleura rufescens</i>	Positive	7	4	4
	None	0	0	0
<i>O. dioica</i>	Positive	4	2	—
	None	0	0	—
<i>Megalocercus huxleyi</i>	Positive	3	0	0
	None	6	5	1
<i>O. fusiformis</i>	Positive	9	0	0
	None	4	6	2

"Positive" indicates multiple, summated flashes in response to one or both stimulations applied to a single preparation. Field houses were captured with animals inside; clean houses were expanded by the animal in particle-free sea water. —Data unavailable.

species upon agitation verified these results. Divers reported that the light produced by these species upon agitation *in situ* appeared as numerous point sources over the surface of the house and sometimes from the animal's trunk, and that the light appeared to account for the majority of stimutable luminescence in the surface water during the study. As in other species (Galt and Sykes, 1983), microscopic examination showed that light from the free animals actually emanated from their house rudiments. In several cases we confirmed microscopically that animals and fresh houses were free of microorganisms.

Our method of stimulation produced turbulence in the vial that elicited bursts of multiple, summated flashes from a single preparation. The resulting photometric records were highly variable and showed no consistent patterns within species or preparations (Fig. 2). From the records for which it was possible, we estimated rise time, half-decay time, and flash duration. Durations ranged between 40 and 240 ms with rise times of 5 to 44 ms (Table III). These values must be regarded as approximations until recordings can be made from individual luminescent sources.

Examination with fluorescence microscopy of the trunks of *O. rufescens* and *S. magnum* revealed greenish yellow, fluorescent inclusions in the house rudiments (Fig. 3). The inclusions fluoresced faintly in some preparations and brightly in others but always faded during the 10 to 20 min of observation. In both species, application of a cover slip or tapping the slide on a darkened microscope stage (Galt and Sykes, 1983) elicited luminescent flashes from the house rudiment inclusions. The pressure of the cover slip caused prolonged emissions of one or more seconds. The luminescent pattern clearly coincided with the pattern of inclusions viewed with dim Nomarski illumination. There was no evidence of luminescence or fluorescence from the oral glands or any other part of the animal, except for red or green fluorescence from food in the gut.

The house rudiment inclusions were difficult to see in both species, perhaps accounting for the failure of earlier authors to report them. Moreover, inclusion patterns in some species become more complex with animal age (Galt, unpubl.). Nonetheless, we have the following preliminary descriptions. In *O. rufescens* (Fig. 3A), the inclusions comprised 0.4–0.8  $\mu\text{m}$  granules scattered over the house rudiment, some arranged into streams or tracks coursing down the side of the rudiment. In *S. magnum* (Fig.

TABLE III

*Kinetics of luminescent flashes of Sea of Cortez larvaceans estimated from chart records*

	Rise time ms	Half-decay time ms	Flash duration ms
<i>Stegosoma magnum</i>			
Animal with rudiment	9 ± 3 (25)	10 ± 4 (25)	56 ± 21 (25)
Particle-free house	8 ± 4 (14)	8 ± 3 (14)	40 ± 16 (13)
Field-collected house	9 ± 7 (20)	13 ± 12 (20)	84 ± 47 (20)
<i>Oikopleura rufescens</i>			
Animal with rudiment	10 ± 5 (13)	20 ± 13 (13)	108 ± 70 (12)
Particle-free house	44 (1)	40 (1)	200 (1)
Field-collected house	5 ± 2 (4)	41 ± 20 (3)	210 ± 82 (4)
<i>O. dioica</i>			
Animal with rudiment	24 (1)	28 (1)	120 (1)
Particle-free house	8 (1)	104 (1)	240 (1)
<i>O. fusiformis</i>			
Field-collected house	11 ± 7 (6)	37 ± 12 (6)	150 ± 85 (6)

Values are mean ± standard deviation of the number of measurements in parentheses.

3B), the pattern on one side comprised looping rows of block-like inclusions that were finely granular (less than 0.5 μm), 5–10 μm wide, and of various lengths. We assume by analogy with other species that the patterns are bilaterally symmetrical.

#### *Non-luminescent species*

The results were less clear-cut for *Megalocercus huxleyi* and *Oikopleura fusiformis*. For both species, neither the free animals invested with house rudiments nor their newly-formed, clean houses luminesced (Table II). However, 1/3–2/3 of their field-collected houses flashed upon stimulation (Fig. 2, Tables II, III). These results suggest the presence of luminescent microorganisms on or in the field-collected houses but not endogenous luminescence by the larvaceans. Microscopic examination of field houses in some cases revealed naked and armored dinoflagellates.

Microscopic examination of the trunks and house rudiments of numerous specimens of *M. huxleyi* and *O. fusiformis* revealed no evidence of fluorescent inclusions in the house rudiments. The only fluorescent structures in these species were the gut contents.

#### DISCUSSION

Our results increase the number of known luminescent larvacean species to six (Table I). Moreover, our discovery of luminescent inclusions in the house rudiments of *Oikopleura rufescens* and *Stegosoma magnum*, contrary to earlier reports (Lohmann, 1896, 1933; Lohmann and Bückmann, 1926; Bückmann and Kapp, 1975), supports the conclusion of Galt and Sykes (1983) that house rudiment inclusions are the sole source of endogenous luminescence in larvaceans. All six known luminescent species also possess oral glands. Therefore we conclude that all fourteen larvacean species with oral glands also possess a species-specific pattern of house rudiment inclusions that are the sites of endogenous luminescence (Table I). The basis for the co-occurrence of oral glands and house rudiment inclusions is unknown, but Fredricksson and Olsson (1981) reported that inclusions derive from oral gland secretions, a view disputed by Galt and Sykes (1983). It seems clear that oral glands are not directly

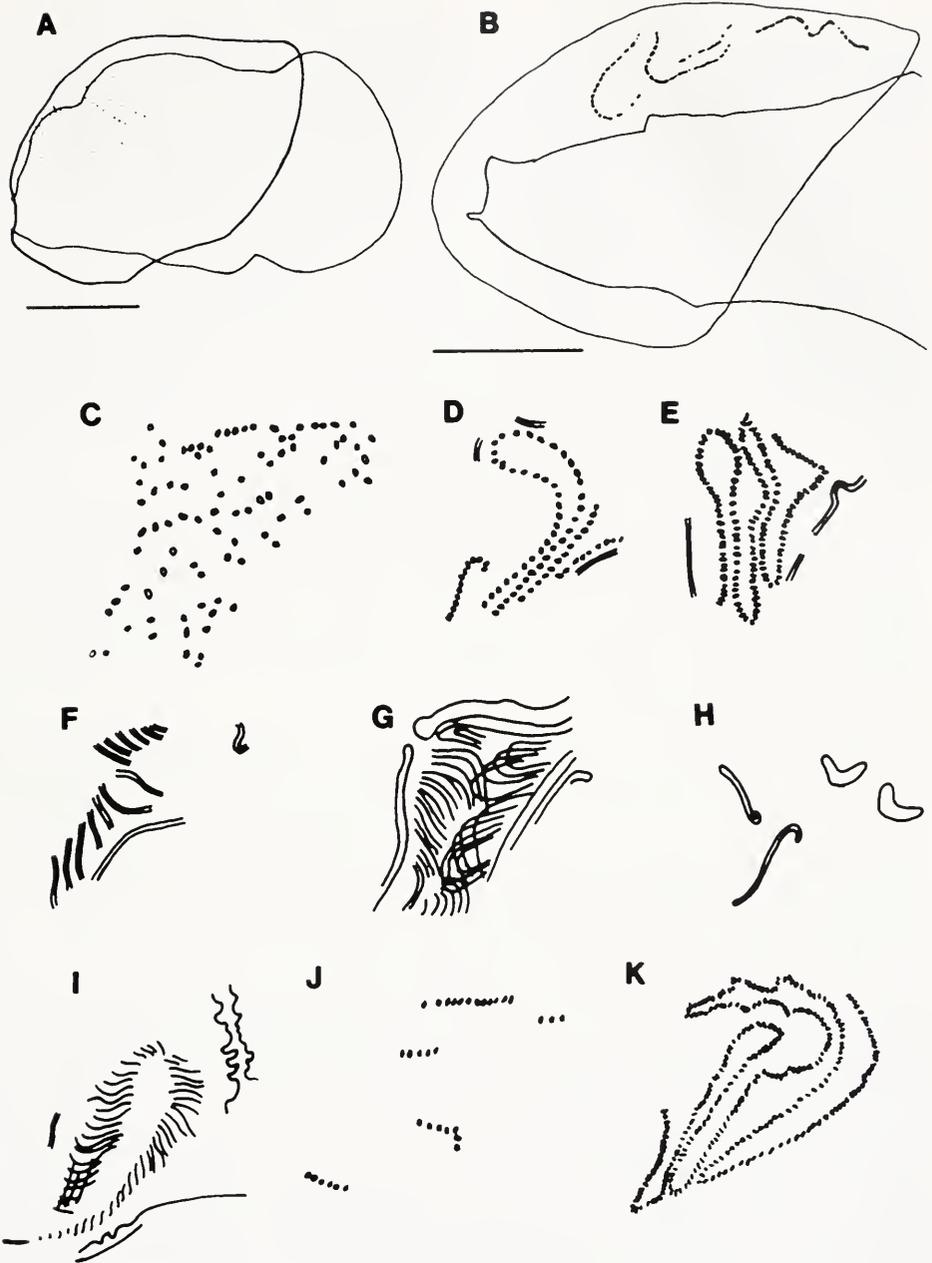


FIGURE 3. Diagrams of inclusion patterns in left side of house rudiment in (A) *Oikopleura rufescens*, (B) *Stegosoma magnum*, (C) *O. vanhoeffeni*, (D) *O. valdiviae*, (E) *O. gausica*, (F) *O. parva*, (G) *O. albicans*, (H) *O. cophocerca*, (I) *O. drygalskii*, (J) *O. dioica*, (K) *O. labradoriensis*. In A, inclusions in house rudiment are shown over outline of animal's trunk. In B, house rudiment is shown over the anterior two thirds of the trunk. Scale bars for A and B, 0.5 mm. Remaining patterns not drawn to scale but each occupies approximately the anterior two thirds of the animal's trunk. A, B this report, all others redrawn from Lohmann and Bückmann (1926), with permission of Walter de Gruyter and Company, Berlin.

involved in light production (see also Galt and Sykes, 1983). Nevertheless, their presence provides a useful indicator of luminescence in larvaceans for field studies, because oral glands are a feature of internal anatomy, whereas the house rudiment with its inclusions may be damaged or lost during capture.

Given our previous conclusion, it follows that larvaceans lacking oral glands, and therefore rudiment inclusions, are not endogenously bioluminescent (Table I). This conclusion is supported by our inability to elicit luminescence from animals and clean houses of *Oikopleura fusiformis* and *Megalocercus huxleyi* (Table II) and *O. longicauda* in southern California (Grober and Galt, unpubl.). However, we recorded luminescence from some field-collected houses of *O. fusiformis* and *M. huxleyi*. We assume these flashes were due to luminescent dinoflagellates, which are responsive to mechanical stimuli and have flash kinetics similar to field-collected houses (Table III; Widder and Case, 1981). Larvacean houses accumulate small phytoplankton in their filters as part of the feeding process (Lohmann, 1899; Alldredge, 1976b), and the meshes of the incurrent filters of *M. huxleyi* (about 54  $\mu\text{m}$ ; Alldredge, 1977) are large enough to admit luminescent species of dinoflagellates. Moreover, larvacean houses, as components of marine snow (Alldredge, 1976b, 1979; Silver and Alldredge, 1981), accumulate various organisms on their surfaces, including dinoflagellates (Silver *et al.*, 1978; Trent *et al.*, 1978; Davoll, 1982, 1984). Davoll (1984) found tens to hundreds of small dinoflagellates per discarded larvacean house in Monterey Bay. Finally, Mackie and Mills (1983) observed stimulated flashes from macroscopic aggregates and discarded larvacean houses *in situ*. Thus, even though some species of larvaceans may be incapable of endogenous luminescence, their discarded houses may be secondarily luminescent.

Comparison of flash kinetics data is difficult because of the variability of flash patterns (see also Galt, 1978; Galt and Sykes, 1983) and the small sample size for some of the estimates. However, the mean rise times and flash durations estimated for *Stegosoma magnum* and *Oikopleura rufescens* animals (Table III) are significantly smaller than those presented by Galt and Sykes (1983) for *O. dioica* and *O. labradoriensis* (Mann-Whitney *U*-tests of pairwise comparisons,  $P < 0.05$ ). The differences, although between different species, are nonetheless consistent with a temperature coefficient ( $Q_{10}$ ) of 2 to 3, since the present recordings were made at about 10°C warmer than the recordings of Galt and Sykes (1983).

Larvaceans are found in the surface layers of all the world's oceans (Lohmann, 1933; Fenaux, 1967). We have surveyed the distributional literature (Galt and Tisdale, 1983) and conclude from more than 80 reports that, world-wide, most coastal and cold-water larvacean assemblages are dominated by species with endogenous luminescence (*Oikopleura dioica*, *O. labradoriensis*, *O. vanhoeffeni*, *O. valdiviae*). Although endogenously luminescent species may be common in warm waters (*O. rufescens*, *Stegosoma magnum*), these areas are usually dominated by forms without endogenous luminescence (*O. longicauda*, *O. fusiformis*). However, the latter species' houses may form secondarily luminescent sites as they are colonized by luminescent dinoflagellates.

Swift *et al.* (1983) concluded from photometric records correlated with plankton samples that zooplankton, perhaps including larvaceans and their houses, make a major contribution to near-surface luminescence in the Sargasso Sea. The ability to predict luminescence capability from easily discerned morphological features, information from distributional studies (Galt and Tisdale, 1983), and quantification of total light emission from larvacean houses (Galt and Grober, 1985), will facilitate estimation of the contribution of larvaceans to stimulable, surface bioluminescence on a global scale.

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## PHYSIOLOGICAL AND MORPHOLOGICAL ADAPTATIONS OF ADULT *UCA SUBCYLINDRICA* TO SEMI-ARID ENVIRONMENTS<sup>1</sup>

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### ABSTRACT

Salinity tolerance, osmotic and ionic regulation abilities, desiccation tolerance, and gill morphometry of *Uca subcylindrica*, which inhabits semi-arid supratidal areas, were compared with more typically intertidal fiddler crabs, especially *U. longisignalis*. Salinity tolerance was less in *U. subcylindrica* (2–90‰) than in *U. longisignalis* (0.08–110‰). Blood osmolality and sodium and chloride concentrations were regulated over a wide range of salinities in both species, but *U. subcylindrica* maintained a smaller gradient against the external medium at the lowest salinity at which it survived and at the higher salinities. The osmoregulatory responses were contrary to predictions based on field distributions, but *U. subcylindrica* generally survived desiccation longer and always tolerated a greater percent body water loss than *U. longisignalis*, *U. rapax*, and *U. panacea*. Differences in gill morphometrics among the four species were consistent with features accompanying increasing terrestriality.

### INTRODUCTION

In the western Gulf of Mexico, several species of the typically intertidal genus *Uca* occur, including *U. longisignalis*, *U. rapax*, and *U. panacea* (Crane, 1975; Barnwell and Thurman, 1984). These crabs inhabit coastal marshes, intertidal areas bordering bays, lagoons and tidal creeks, and the periphery of wind tidal flats. One species, however, *U. subcylindrica*, inhabits areas distinctly different from the others (Rabalais, 1983; Thurman, 1984). This species is restricted to semi-arid habitats from Copano Bay, Texas to Tampico, Mexico. *Uca subcylindrica* lives in supratidal areas removed from permanent bodies of water and also occurs along intermittent stream courses and near ephemeral ponds up to 35 km from tidewater. Other species of *Uca* seldom occur in the habitats of *U. subcylindrica*.

Due to the low rainfall, limited tidal exchange with marine waters, and generally high temperatures and evaporation rates in this region, habitat conditions for *Uca subcylindrica* often include high salinity in the available water (up to 90‰; Rabalais, 1983), lack of standing water for extensive periods, and highly variable and extreme salinities in both standing water and burrow water, due to the periodically heavy rainfall. Salinity conditions for other fiddler crabs are more moderate: e.g., *U. longisignalis* occupies habitats with burrow water or adjacent bay waters ranging from 18 to 34.5‰ (Rabalais, 1983).

Physiological responses to salinity and drying conditions are important in the distributions and differential habitat selection of other decapod crustaceans (e.g., Teal, 1958; Barnes, 1967; Engel, 1977; Felder, 1978; Young, 1978, 1979). The purpose of

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the present investigation was to assess the responses of *Uca subcylindrica* to various stresses associated with their peculiar habitat, to compare these responses with other *Uca* species of the region, and to compare the morphometry of the gills among *Uca* species of varying degrees of terrestriality.

## MATERIALS AND METHODS

### *Collection and maintenance of animals*

*Uca subcylindrica* were collected from two main sites: an intermittent fresh to hypersaline creek (Santa Gertrudis Creek near Kingsville, Texas) and an ephemeral pond near the junction of the Laguna Madre and Baffin Bay, Texas. Individuals from the two areas did not differ in salinity tolerance, percent body water, and blood osmotic and ionic parameters across a salinity range of 2 to 90‰ (Rabalais, 1983), and were combined for comparison with the other species. Specimens of *U. longisignalis*, *U. rapax*, and *U. panacea* were collected from various salt marsh and intertidal habitats of the Corpus Christi, Nueces, and Aransas Bay systems.

Crabs were maintained in large circular tanks with natural sediment and water of about 30‰ salinity available. Feeding was halted 3 days prior to experiments, except in the osmoregulation experiments, in which food was given every 5 days during the 45-day experimental period. Only adult ( $\geq 12$  mm, carapace width) intermolt males and non-ovigerous females were used; most were 15 to 20 mm.

### *Osmotic and ionic regulation*

Crabs were kept in large fingerbowls with 2 cm of water which allowed access to air but kept the animals partly submerged. Animals were maintained at  $22 \pm 1^\circ\text{C}$  under a 14L:10D photoperiod and initially acclimated to 30‰. Other salinities were prepared either by dilution of natural sea water with deionized water or by concentration with artificial sea salts (Instant Ocean). An artificial pond water (0.5 mM NaCl, 0.4 mM  $\text{CaCl}_2$ , 0.2 mM  $\text{NaHCO}_3$ , and 0.05 mM KCl) gave the lowest physiologically meaningful salinity of 0.08‰.

The time required for adjustment to salinity change was determined in a preliminary experiment with three groups of five *Uca subcylindrica*. After 7 days at 30‰, one group was transferred to 10‰, another to 60‰, and the third left at 30‰. Blood osmolality values at 0.5, 1, 2, 3, 5, and 7 days showed that although there were some (non-significant) fluctuations in the first 3 days, there were no significant changes after 5 days. Blood parameters of crabs held 9 days at 2 and 80‰ did not differ significantly from those held 5 days. Thus, an acclimation period of five days was considered sufficient at all salinities and was used in all further salinity acclimation experiments.

The long-term salinity experiments with *Uca subcylindrica* and *U. longisignalis* were conducted as follows: for each species an initial group was acclimated for 5 days at 30‰ after which 5 individuals were sampled. Then half of the group was moved to a higher salinity, and half to a lower, left 5 further days, sampled, and moved again until either the entire range of 0.08 to 120‰ was covered or no more crabs survived. The salinity was monitored daily, readjusted as necessary, and the water changed every two days.

Blood samples were removed with a syringe from the arthrodistal membrane at the base of the fifth pereopod, allowed to clot, centrifuged, and refrigerated until analyzed, usually within 4 to 48 h. Osmolality of blood and water samples was determined on a vapor pressure osmometer (Wescor), chloride concentrations with

an amperometric titrator (Buchler-Cotlove), and sodium by flame photometry (Radiometer).

For field comparison, water samples were aspirated from burrows through flexible plastic tubing. Crabs, blood samples, and water samples were placed on ice until returned to the laboratory for processing as described above.

#### *Desiccation tolerance*

Crabs held for 3 days in water of 30‰ were blotted dry, weighed, and placed in individual ventilated plastic vials in a desiccator containing 250 g of dried CaSO<sub>4</sub>, which produces a relative humidity of 0 to 10% (Jones and Greenwood, 1982). Blood osmolality of a few individuals not desiccated was measured at the beginning as a control. Vials were weighed at 4-h intervals until animals began to die, then hourly observations were made. All crabs were weighed on the same schedule so that manipulations and exposure to room air were consistent. Laboratory conditions were 22 ± 15°C and 24-h dark.

Death was defined as lack of responsiveness to probing of antennae or appendages. At death, the final weight and blood osmolality were determined. The percent water loss was expressed as the loss in weight (assumed to be all water loss) as a percentage of initial weight of body water.

#### *Gill morphometry*

Gills from crabs held 1 week at 30‰ were preserved in either 10% buffered formalin or 0.1 M Na cacodylate buffer. Those used for examination of thick and thin epithelium were post-fixed in 1% OsO<sub>4</sub> for 2 h (Copeland and Fitzjarrell, 1968). Some of the gills were also paraffin embedded and sectioned for measurement of thickness of epithelia.

To avoid size-related differences, similar-sized crabs were used. Because the gills were generally subquadrate, they were cut into three sections—a small distal end, a small proximal end, and a large middle section that was visibly about the same circumference for its length. A representative platelet was cut from each section, the area determined, and the number of platelets per section counted, allowing the calculation of gill area by:

$$\sum_{i=1}^6 [(D_i \cdot a \cdot 2) + (M_i \cdot a \cdot 2) + (P_i \cdot a \cdot 2)] \cdot 2;$$

where, D, M, and P = number of platelets in distal, middle, and proximal sections; a = area of a representative platelet from each; and i = the number of the gill pair. The percentage of thick epithelia was determined from *camera lucida* drawings of representative platelets with an integrating planimeter.

## RESULTS

The highest and lowest tolerated salinities, those in which 50% survived 5 days, were 2 and 90‰ for *Uca subcylindrica* (Fig. 1). The highest tolerated salinity for *U. longisignalis* was 110‰; there was 100% survival in the lowest tested salinity of 0.08‰ (Fig. 1). Increased mortalities at the extremes could be attributed to these conditions, because high survival (>90%) occurred in 30‰ for longer than the 25 days when survival began to decrease in the long-term salinity experiments.

Blood osmolality, as well as the principal ions Na<sup>+</sup> and Cl<sup>-</sup>, were well regulated

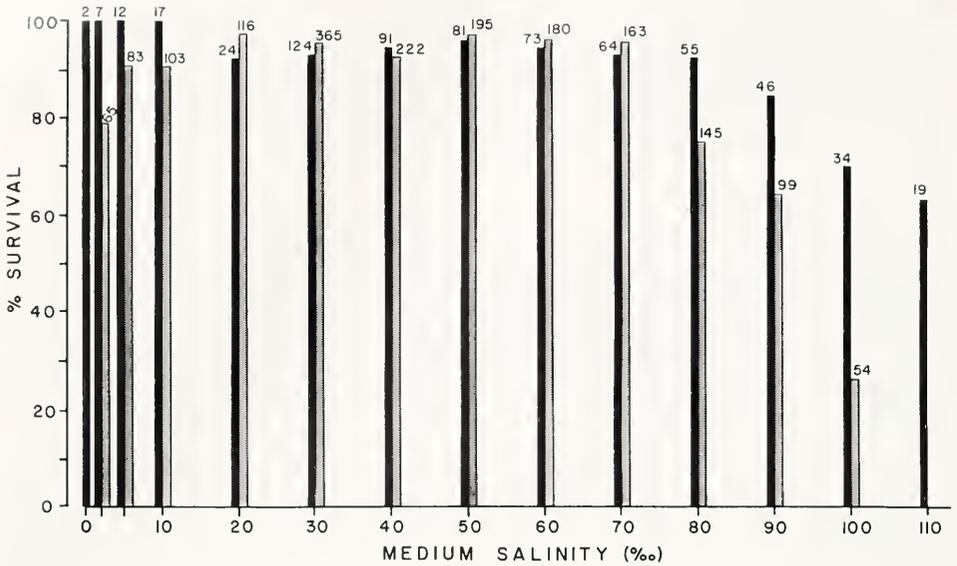


FIGURE 1. Percent survival of *Uca subcylicindrica* in a range of salinities from 2 to 100‰ (shaded histograms) and *U. longisignalis* across a range of salinities from 0.08 to 110‰ (dark histograms). n for each group given above histograms.

over a wide range of salinities in both species (Fig. 2). The isosmotic and isoionic points were higher in *U. subcylicindrica*. *Uca subcylicindrica* maintained less of a gradient against the external media at the lowest salinity (2‰) in which it survived and at the higher salinities (40‰ to the limits of survival) than *U. longisignalis* (*t*-Test,  $P \leq 0.05$ ). Blood parameters for *U. subcylicindrica* were more variable than those for *U. longisignalis*. The average percent body water of *U. subcylicindrica* at all salinities was slightly greater than *U. longisignalis* ( $65.1 \pm 0.4\%$  versus  $63.1 \pm 0.4\%$ ), but values were not consistently greater across the range of salinities and usually not significantly different within a salinity.

In the field, *Uca subcylicindrica* also regulated effectively, with blood osmolality staying within narrow limits in the presence of water of widely varying salinity (Fig. 3). The  $[Na^+]$  and  $[Cl^-]$  data (not shown) reflected a similar pattern with  $[Na^+]$  values generally between 370 and 450 mEq/l and  $[Cl^-]$  values between 280 and 350 mEq/l. Total body water for field-collected animals ranged from 55 to 65%, not significantly different from laboratory-acclimated animals.

#### Desiccation tolerance

Despite an effort to collect similar-sized crabs, the average carapace width differed significantly among the species (*t*-Tests,  $P \leq 0.05$ ) (Fig. 4). Initial weights of *Uca panacea* and *U. subcylicindrica* also differed from each other and from the other two species which were similar. Initial percent body water was not significantly different among species. Initial blood osmolality was similar in all species except *U. longisignalis*. With all values pooled, *U. subcylicindrica* survived longer than *U. longisignalis* and *U. panacea* but not longer than *U. rapax* (Fig. 4). *Uca subcylicindrica* withstood a greater percent body water loss than did the others which is reflected in lower final percent body water and higher final blood osmolality (Fig. 4). Final percent body

water did not differ significantly but final blood osmolality values did. The difference in initial and final blood osmolality was also much greater for *U. subcylindrica* (558 mOsm/kg) than for *U. longisignalis* (295 mOsm/kg), *U. rapax* (395 mOsm/kg), and *U. panacea* (309 mOsm/kg).

There were weak but significant ( $P \leq 0.05$ ) linear relationships between size (as indicated by carapace width and initial body weight) and survival time in *Uca subcylindrica*, *U. longisignalis*, and *U. panacea* but not in *U. rapax*. Percent water loss was not correlated to size for any species. Thus, groups of similar-sized crabs were compared (Fig. 5A, B). In Group A, survival time was longer for *U. subcylindrica* but only significantly longer than *U. longisignalis* (*t*-Tests,  $P \leq 0.05$ ). Within Group A, *U. subcylindrica* withstood a significantly greater percent body water loss. For Group B, *U. subcylindrica* survived significantly longer and withstood a significantly greater percent body water loss. There were no sex-related differences in survival time, but there were in percent water loss in *U. longisignalis* and *U. panacea* (Table I). Comparisons among the same sexes of similar-sized crabs showed that survival time was greater for *U. subcylindrica* but not always significantly and that percent body water loss was always significantly higher for *U. subcylindrica* (Fig. 5C, D).

### Gill morphometry

Gills of *Uca* were morphologically similar to other terrestrial crabs (e.g., *Holthuisana transversa* in Taylor and Greenaway, 1979; *Cardisoma carnifex* in Cameron, 1981) with stiffened platelet margins for structural support, wide interlamellar spacing, and cuticular spines at the base of the efferent blood vessels. There were no obvious morphological differences among the gills of the different species. Gills 1 and 2 contributed little to the total gill surface area with gills 3–6 contributing the bulk (Table II). On the average, 81% of the epithelium was of the thick type (Table II) and averaged 5.2  $\mu\text{m}$  thickness compared to 2.6  $\mu\text{m}$  for the thinner type.

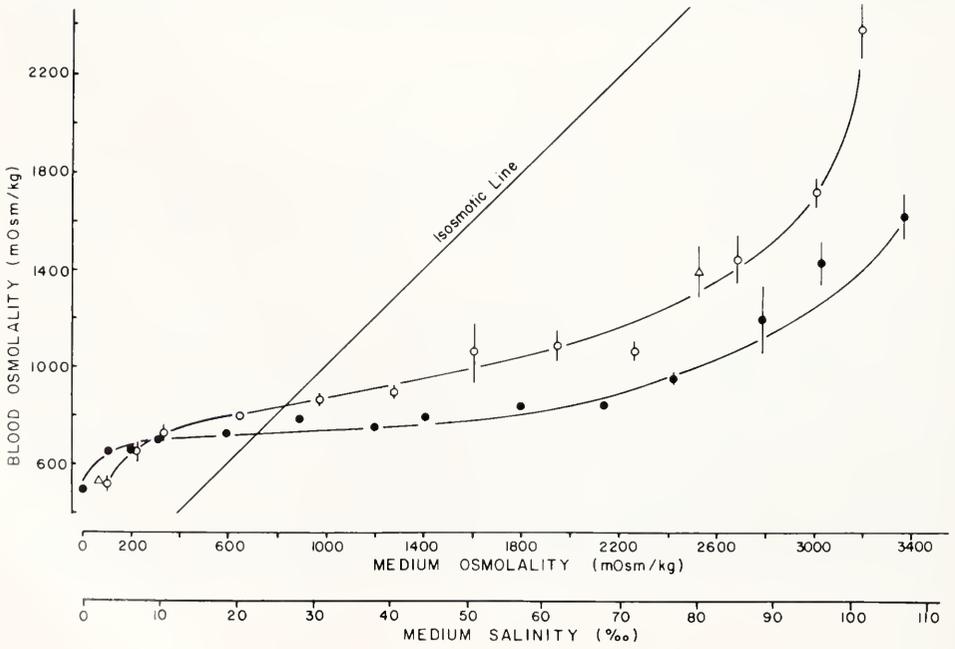
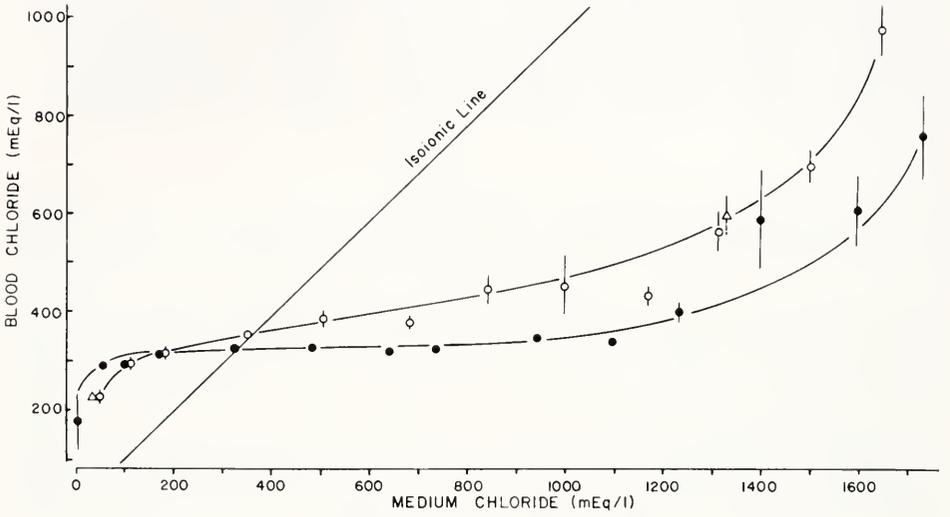
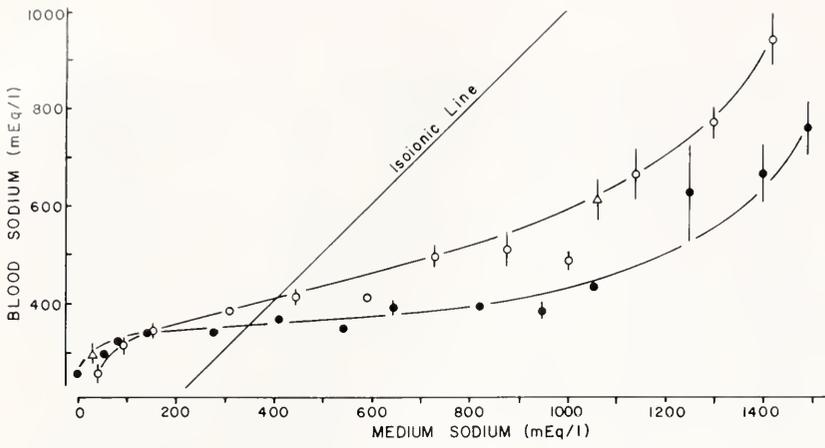
Total platelets, total gill surface area, and gill surface area per wet weight of crab were least in *U. subcylindrica* and varied significantly among the species (Table III). Separation of the data by sex, or expression of area per unit dry weight, ash-free dry weight, or weight minus a major cheliped did not add any significant insights.

## DISCUSSION

### Osmotic and ionic regulation

Several studies have shown *Uca* to accommodate to a wide salinity range by maintaining fairly uniform ionic and osmotic blood levels. Green *et al.* (1959) showed no significant differences in osmoregulation in *U. rapax* and *U. pugilator*, and both species were intermixed in studies by Baldwin and Kirschner (1976a, b). Wright *et al.* (1984) found no major differences in ionic regulation among *U. pugnax*, *U. pugilator*, and *U. minax*. Studies of other decapod crustaceans (Engel, 1977; Felder, 1978; Young, 1979), however, have shown that osmotic and ionic regulatory abilities of closely related species differ and correlate with their distributions in habitats of differing salinities. Based on the more extreme salinities in the habitats of *U. subcylindrica*, we expected to find a greater salinity tolerance and greater osmotic and ionic regulatory ability in these crabs. Even though the osmoregulatory ability of *U. subcylindrica* is considerable, it is not greater than that of *U. longisignalis* in long-term salinity experiments, and, in fact, is less (Figs. 1, 2).

The field data for *Uca subcylindrica* (Fig. 3; Rabalais, 1983) indicate that this species was a nearly perfect regulator in the face of an almost 10-fold variation in



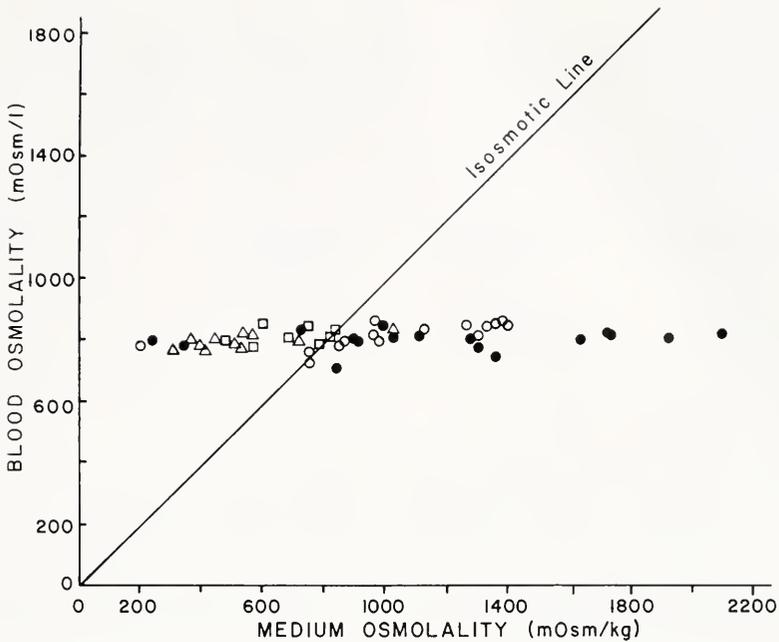


FIGURE 3. Blood osmolality values for *Uca subcylindrica* collected from burrows in the field as a function of the burrow water osmolality. Closed circles represent individuals collected 28 April 1981 from the Laguna Madre area; open circles, 28 May 1981 from the Laguna Madre area; squares, 14 July 1981 from Santa Gertrudis Creek; and triangles, 2 November 1982 from Santa Gertrudis Creek.

the osmolality of available water. This suggests that additional factors, such as behavioral osmoregulation involving selective drinking, may be important. Whether *U. subcylindrica* is more adept in this respect than other fiddler crabs is not known.

#### Desiccation tolerance

Crabs as a group can tolerate significant water loss, varying from 20 to 50% depending on species (reviewed by Jones and Greenwood, 1982). The 29% average for *Uca subcylindrica* reported here is not particularly remarkable, although it is greater than the other *Uca* examined. Young (1978) argued that the percent water loss tolerated was a good unbiased measure of desiccation tolerance, but found more significant interspecies differences based on survival time. In our study, differences in survival time were not as great among the species as the differences in water loss values (Figs. 4, 5).

Given the limited differences in size and initial blood osmolality values but no differences in percent body water and no chance for behavioral modification, the

FIGURE 2. Blood osmolality, chloride, and sodium values for *Uca longisignalis* (closed circles) and for *U. subcylindrica* (open circles) as a function of media osmolality, chloride, and sodium. Values represent the means of 5 determinations  $\pm$  S.E. for *U. longisignalis* and 10 determinations  $\pm$  S.E. for *U. subcylindrica*. Triangles represent groups of *U. subcylindrica* acclimated for nine days as opposed to five days for the others. Approximate salinity values given on a second abscissa below osmolality (lowest values at 0.08‰); data plotted against osmolality and ionic concentrations. Curves fitted by eye.

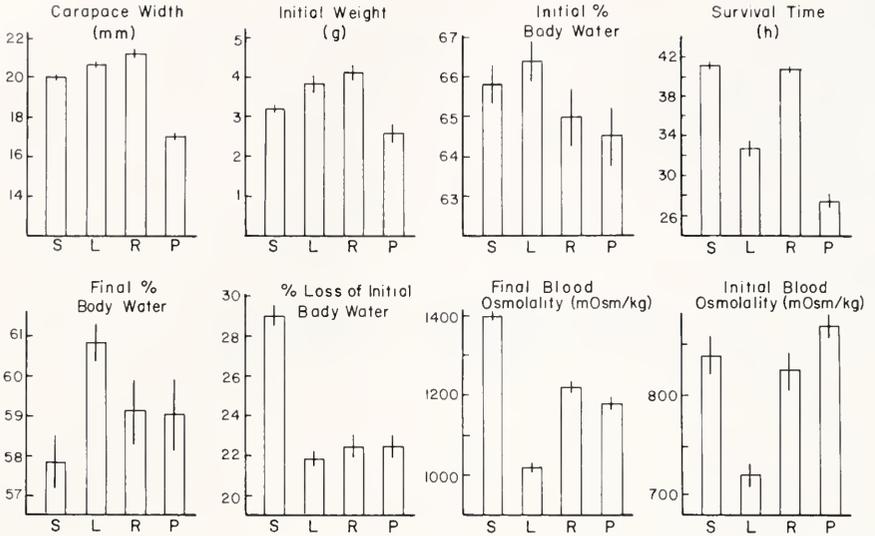


FIGURE 4. Comparison of several parameters in desiccation tolerance experiments for four species of fiddler crabs: S = *Uca subcylindrica*, n = 114; L = *U. longisignalis*, n = 79; R = *U. rapax*, n = 26; and P = *U. panacea*, n = 55. For initial blood osmolality, n = 17 for S, n = 14 for L, n = 5 for R, and n = 13 for P. Vertical lines represent  $\pm$ S.E.

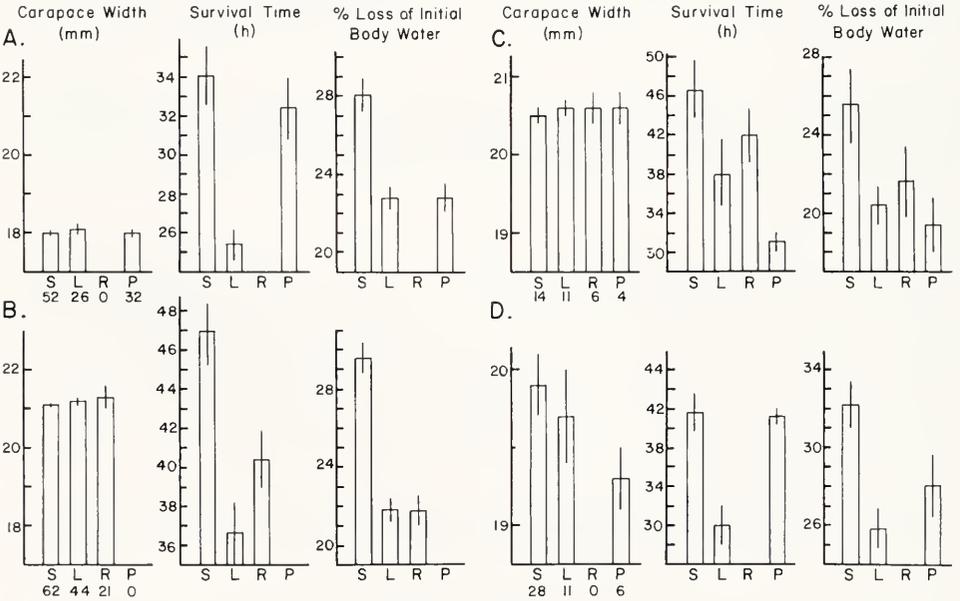


FIGURE 5. Comparison of carapace width, survival time, and percent loss of initial body water in desiccation tolerance experiments for four species of fiddler crabs, grouped by size and sex. Group A is 15.3–19.9 mm, carapace width, males and females; B is 20.0–23.7 mm, carapace width, males and females; C is 20.1–21.3 mm, carapace width, males; D is 18.0–20.1 mm, carapace width, females. S = *Uca subcylindrica*, L = *U. longisignalis*, R = *U. rapax*, and P = *U. panacea*. n for each species in each group given below designation in carapace width data set. Vertical lines represent  $\pm$ S.E.

TABLE I

Comparison of desiccation tolerance data (survival time and percent loss of initial body water) between similar-sized male and female crabs of three species of fiddler crabs.

Species and sex	Carapace width (mm)	Survival time (h)	% Loss of initial body water
<i>Uca subcylindrica</i>			
Males	18.9 ± 0.2	39.6 ± 4.3	24.7 ± 1.1
Females	18.9 ± 0.2	35.1 ± 3.1	29.5 ± 2.6
<i>t</i> -value	-0.8944	0.7080	-1.8380
<i>Uca longisignalis</i>			
Males	19.5 ± 0.3	25.9 ± 1.3	19.6 ± 1.2
Females	19.5 ± 0.3	28.5 ± 1.8	26.2 ± 0.4
<i>t</i> -value	0.7559	-1.3420	-5.6726*
<i>Uca panacea</i>			
Males	18.7 ± 0.3	33.1 ± 2.2	20.0 ± 0.7
Females	18.7 ± 0.3	36.2 ± 2.7	26.5 ± 1.3
<i>t</i> -value	0.1440	-0.9523	-4.3857*

Values of the *t*-distribution marked with an \* are significant ( $P \leq 0.05$ , for  $df = 10$ ).

differences in desiccation tolerance indicate varying physiological abilities among the species and should provide an index of the ability to survive such conditions in the field. In that way, the laboratory data on *Uca subcylindrica* do correlate with the known field distributions and habitat conditions.

The ability of *Uca subcylindrica* to tolerate desiccation better may be related to reduced permeability, reduced surface areas, or an ability to lose sufficient water from the blood or tissues across gill surfaces to maintain a relative humidity in the branchial chamber necessary for respiration. A reduced gill surface area (Table III) would reduce the loss of water by transpiration. The increased blood osmolality was probably not a factor based on known comparative salinity tolerances and osmoregulatory abilities of *U. subcylindrica* and *U. longisignalis* (Figs. 1, 2).

In the field, behavioral mechanisms not possible in laboratory experiments may importantly compensate for desiccating conditions. Activity peaks around dawn in the warmer months (pers. obs.) may be related to the crabs' use of dew which has condensed on sediments and plants. These activity patterns, however, may as likely

TABLE II

Distribution of gill area by individual gill pair and by epithelium type for *Uca subcylindrica* and *U. longisignalis*

Gill pair (anterior to posterior)	<i>Uca subcylindrica</i>		<i>Uca longisignalis</i>	
	% of total area	% thick epithelia	% of total area	% thick epithelia
1	1.9 ± 0.1	87.8	1.7 ± 0	81.6
2	2.5 ± 0.6	80.2	1.0 ± 0.1	88.6
3	17.8 ± 0.8	80.0	19.5 ± 1.6	80.7
4	39.0 ± 1.0	79.4	30.7 ± 0.5	81.7
5	22.0 ± 0.4	77.9	26.1 ± 1.2	79.5
6	16.9 ± 0.7	83.7	20.9 ± 1.2	80.4

Values are means ± S.E.

TABLE III

*Comparison of gill morphometry for four species of Uca*

Species	Carapace width (mm)	Wet weight (g)	Total platelets	Total gill surface (mm <sup>2</sup> )	Gill area/wet weight (mm <sup>2</sup> /g)
<i>Uca longisignalis</i> (6)	21.3 ± 0.7	3.9 ± 0.9	926 ± 17	2237 ± 181	658 ± 78
<i>Uca rapax</i> (1)	21.1	2.9	846	1751	595
<i>Uca panacea</i> (3)	19.6 ± 0.3	2.8 ± 0.1	775 ± 18	1575 ± 142	566 ± 44
<i>Uca subcylindrica</i> (8)	20.7 ± 0.5	3.5 ± 0.2	698 ± 8	1482 ± 78	432 ± 19
F-value, df = 2, 14	1.3207 NS	0.6405 NS	89.7118 ***	10.0912 ***	5.8385 *

Values are means ± S.E.; n for each group given in parentheses following species designation. NS: not significant,  $P > 0.05$ ; \*:  $0.01 < P \leq 0.05$ ; \*\*\*:  $P \leq 0.001$ .

be related to avoidance of hotter temperatures during mid day and predators at night. *Uca subcylindrica* may also utilize water from plants brought into their burrows, e.g., *Batis*, *Sueda*, and *Borrchia* (pers. obs.).

#### Gill morphometry

The reduced gill area in *Uca subcylindrica* is no doubt related to the lesser requirement for gas exchange area in terrestrial crabs (Cameron, 1981) but may also play a role in reducing evaporative water loss in arid environments. Comparisons of gill area have been made for widely disparate groups (e.g., Pearse, 1929; Gray, 1957; Cameron, 1981). The species in this study are of similar size, closely related, do not span the 100- to 650-fold size range of other studies (Greenaway, 1984), and thus provide meaningful comparisons. The activity and terrestriality differences among the *Uca* examined here are more subtle but still distinct. *Uca subcylindrica* lives in the most elevated and arid habitats and most distant from water. *Uca panacea* prefers the periphery of sandy wind tidal flats, and often lacks water in the burrow (Powers, 1975) but is more closely tied to the water than *U. subcylindrica*. The others, *U. longisignalis* and *U. rapax*, are found in the intertidal zone and nearly always have water in their burrows.

It is generally accepted that of the epithelial types in brachyuran crab gills the thinner is the site for respiratory gas exchange while the thicker is the site of active ion transport (Copeland and Fitzjarrell, 1968; Aldridge and Cameron, 1982; Barra *et al.*, 1983). The gills as the site of exchange of gases, ions, and water are of interest to aspects of osmoregulation and desiccation tolerance in fiddler crabs of semi-arid habitats. Reduced gill surface area would be advantageous in reducing the loss of water by transpiration. On the other hand, this reduced area without a concomitant increase in thick epithelia may be a disadvantage to *Uca subcylindrica* from the standpoint of osmotic and ionic regulation. Based on an ecological series of gammaridean amphipods, Moore and Taylor (1984) predicted that an increase in gill area may be promoted in reduced salinities as a means of facilitating ion uptake. The lesser range of salinity tolerance and decreased osmoregulatory ability in *U. subcylindrica* in long-term salinity experiments may be related to the observed differences in gill morphometry.

Part of the respiratory function in terrestrial crabs is taken on by the branchial chamber lining (e.g., Greenaway and Taylor, 1976; Díaz and Rodríguez, 1977). Among

fiddler crabs, *Uca subcylindrica* has the greatest branchial chamber volume, which lends to its distinctive shape and its name. There is no obvious elaboration of its surface area or vascularization, however, so it is not clear whether this is of any great adaptive significance. The egg mass volume of *U. subcylindrica* is twice that of any other *Uca* (Rabalais and Gore, 1985), and the function of the enlarged branchial chamber may be to accommodate this egg mass prior to deposition.

In summary, some of the physiological responses and gill morphometric differences among the four species of *Uca* examined are consistent with their distribution patterns and some are not. *Uca subcylindrica* inhabits by far the driest habitat and the one most subject to salinity extremes, but its osmoregulatory ability was not greater than that of *U. longisignalis*. On the other hand, *U. subcylindrica* was more tolerant of desiccation. This was paralleled by a reduced gill surface area which probably helps to reduce evaporative water loss. The ability to withstand a greater loss of body water would be advantageous to any intertidal organism subjected to periodic exposure. These factors, coupled with a more supratidal or nontidal existence in a semi-arid climate, would be of particular importance to *U. subcylindrica*. That the other species of *Uca* are potentially capable, physiologically, of tolerating many of the conditions that *U. subcylindrica* faces in its peculiar habitat suggests that factors additional to physiological abilities determine their non-overlapping distribution patterns. Primary among these are size, behavior, reproductive biology, life history patterns, dispersal, and characteristics of the early life history stages (Rabalais, 1983; Rabalais and Cameron, 1983; Rabalais and Cameron, 1985).

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## THE EFFECTS OF FACTORS IMPORTANT IN SEMI-ARID ENVIRONMENTS ON THE EARLY DEVELOPMENT OF *UCA SUBCYLINDRICA*<sup>1</sup>

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### ABSTRACT

Lack of food and salinity extremes are conditions encountered by the early life stages of *Uca subcylindrica* in their semi-arid habitats, where nursery areas are temporary rainfall puddles. Several characteristics of the larvae and early postlarvae promote high survivorship in these extreme environments. When starved, yolk reserves in the two zoeal stages lasted through metamorphosis to the megalopal stage and allowed  $\geq 50\%$  survival for 11 days after that. Food was required by the megalopae, however, before molt to crab I occurred. Larvae and early postlarvae survived and developed in a wide range of salinities under laboratory conditions—0.08 to  $\sim 50\%$ . The ability to hyperosmotically regulate the blood was present at hatch. Tolerance to higher salinities increased with successive stages as the ability to hypo-osmotically regulate improved. The early stages of other fiddler crab species are unlikely to survive and develop in the conditions experienced by the zoeae, megalopae, and early crabs of *U. subcylindrica* in their unusual habitats.

### INTRODUCTION

Early development of *Uca subcylindrica* is unique among the Ocypodidae in that larval development is completed with only two brief, maturationally advanced zoeal stages. Metamorphosis occurs within 2 to 3 days of hatch, producing morphologically and behaviorally advanced megalopae. First crabs may appear within 4.5 days of hatch but average 8 days. This abbreviated development is critical since the early stages develop in temporary rainfall puddles which last only a few days (Rabalais and Cameron, 1983). Besides a restricted time for development, the early stages of *U. subcylindrica* also face salinity extremes due to high evaporation and sporadic heavy rainfall in their semi-arid habitats. Larval and early postlarval stages have been collected in the field from water ranging from fresh up to 65‰ salinity (Rabalais, 1983). Finally, suitable food is limited in these temporary puddles.

The effects of salinity and nutrition have been studied in the early life stages of several decapod crustaceans but seldom in species with abbreviated development. Rabalais and Gore (1985) compared closely related species with different developmental sequences and found that those with abbreviated development benefited from higher survival rates than those with more prolonged planktonic existence. Most authors point to degrees of lecithotrophism as the overriding advantage of abbreviated development. Several characteristics, including stored food reserves, appear to be im-

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portant in the high survivorship of early stages of *Uca subcylindrica*. We therefore conducted experiments to test the effects of selected ecological factors on survival and development of larval and early postlarval *U. subcylindrica* and to record the physiological responses of successive stages to salinity.

## MATERIALS AND METHODS

### *Maintenance of ovigerous females and cultures*

Ovigerous females were collected from Santa Gertrudis Creek, Kingsville, Texas. Females with late-stage eggs (Rabalais, 1983) were held in large fingerbowls with 2 cm of 15‰ salinity water and would usually release zoeae within a few days. Females with early- or mid-stage eggs were placed on moist (with 15‰ salinity water) paper toweling in a darkened jar, and hatching occurred within 9 to 24 days. Separate broods were designated by letters.

Only vigorously swimming, healthy-appearing zoeae were used. Larvae and early postlarvae were offered freshly hatched *Artemia salina* nauplii daily, except in starvation experiments. Unless noted otherwise, cultures were maintained under a 14L:10D photoperiod, 26°C, and 15‰ salinity. No antibiotics or fungicides were used. Cultures were counted at 24-h intervals for live individuals of each stage; exuviae and dead individuals were removed and preserved.

Salinities from 2 to 65‰ were prepared by dilution of natural sea water with deionized water or by concentration with artificial sea salts (Instant Ocean). A single exception is noted below. Artificial pond water (0.5 mM NaCl, 0.4 mM CaCl<sub>2</sub>, 0.2 mM NaHCO<sub>3</sub>, and 0.05 mM KCl) gave a salinity of 0.08‰. Salinity was checked daily, readjusted as necessary, and water changed every two days.

### *Treatment of data*

Survivorship and development curves were plotted for each culture as in Figure 1. Data considered "landmarks" were obtained from these graphs and included percent survival of all individuals of all stages, percent survival to the megalopal stage, percent survival to the crab I stage, time required for 50% to reach a particular stage, and intermolt duration, number of days required for 50% of the individuals at a particular stage to attain the next stage. Development times were secondarily derived from the graphs at intersections of the percent and time scales and represented values of  $\pm 0.1$  day. Data are presented as means  $\pm$  S.E. derived from replicated group cultures. Differences in survival and development times were tested by analysis of variance (one way) and analysis of covariance (one way). Differences in blood osmolality values and sizes of individuals in the starvation experiments were tested by *t*-Tests.

### *Variability among broods*

Because the number of larvae per female was relatively small (Rabalais and Cameron, 1983), several broods were used. The variability among broods was studied using 4 different broods reared in 15‰. Five replicates of 20 individuals each from brood B were reared in 250-ml fingerbowls; 2 replicates of 25 individuals from brood C, in 500-ml fingerbowls; 10 replicates of 12 individuals from brood D, in 200-ml containers; and 4 replicates of 15 individuals from brood E, in 250-ml containers. The volume per larva ranged between 12.5 and 20 ml.

### *Effects of starvation*

Five cultures of 20 larvae each were starved. Exuviae and dead individuals were removed immediately so that they would not become food. Cultures were held in 250-ml fingerbowls for 24 days. Brood B (Fig. 1) served as the control culture of fed individuals.

Additional cultures of approximately 75 individuals were maintained along with both the fed and starved replicate cultures. To test for the effect of starvation on size, individuals were removed from these cultures and preserved at recorded intervals. Carapace lengths of zoeae were measured laterally from the base of the rostrum to the most posterior margin. For megalopae, carapace width was measured at the greatest distance across the carapace.

When survivorship began to decrease significantly on day 12 (*t*-Test,  $P \leq 0.05$ ) in replicated starved cultures, the starved mass culture was divided, with feeding resumed for half the individuals and starvation continued for the others ( $n = 24$  individuals per condition), for 12 more days.

### *Effects of salinity*

The effects of salinity on survival and development were assessed in two series of experiments. In the first, 2 replicates of 25 individuals each from brood C were held in 500-ml containers and acclimated in 5‰ steps of 0.5 to 1 h duration to final salinities of 0, 2, 15, 25, 35, 45, and 55‰. In the 45‰ culture for brood C, the salinity rose to 50–51‰ on day 2; thus the development for brood C more closely represented ~50‰, at least initially, and is designated as such. From brood E, a second series of 10 replicates of 12 individuals each in 200-ml containers were acclimated in similar fashion to 2, 5, 15, 25, 35, and 45‰. Five replicates of 20 individuals in 250-ml containers were also acclimated from this brood to 0.08‰.

### *Osmoregulation in the early stages*

For these experiments, water (except 0.08‰) was prepared with artificial sea salts and dechlorinated tap water. Cultures were maintained under a 12L:12D photoperiod and 25°C. Zoeae I were acclimated to 0.08, 2, 5, 30, and 45‰ from 20‰ by 5‰ increments over 0.5 to 1 h intervals, and maintained at the final salinity for 4 h prior to sampling. If the time period was extended much longer, many of the zoeae I would be molting to zoeae II. Results of previous larval studies have shown that the acclimation time for step-wise salinity changes is approximately 1 h, as judged by the period required for body fluids to reach osmotic equilibrium (Foskett, 1977; Young, 1979; Russler and Mangos, 1978). Some zoeae II which survived in 45‰ salinity were acclimated to 55‰ and sampled 1 day later. Because survival was reduced by the megalopal stage at the extremes of salinity, megalopae and crab I individuals reared in 2‰ were acclimated to 0.08‰ and sampled 1 day later. Step-wise acclimations were also made on megalopae and crab I individuals reared at 15‰ up to 45‰ and those reared at 30‰ up to 55 and 65‰ and sampled 1–3 days later. To avoid possible changes in blood osmolality associated with ecdysis as found by Kalber and Costlow (1966), but not by Foskett (1977), samples were taken approximately midway through stages. A few unhatched, late-stage embryos which were dropped from the broods of two females were also sampled.

Blood sampling followed the techniques of Foskett (1977). A larva was removed from the rearing medium, caught in an oil-filled, funnel-shaped glass trap, and blotted

free of excess water. Under a dissecting microscope, micropuncture and blood sampling was accomplished by inserting an oil-filled micropipet through the epithelium between the posterodorsal edge of the carapace and first abdominal somite and into the cardiac area. Postlarvae, which did not conform to the glass traps, were held immobile at the frontal region of the carapace with finely pointed forceps. Approximately 10 nl of the 20 to 80 nl blood sample aspirated was delivered to an oil-filled sample chamber of a direct reading nanoliter osmometer (Clifton Technical Physics). Samples as small as 1 to 10 nl could be analyzed with a reproducibility of  $\pm 10$  mOsm/kg. Blood osmolality was usually determined immediately, but could be determined as long as 20 or 30 min later without change in the freezing point depression. Samples of the medium were taken concurrently and their osmolality ( $\pm 3$  mOsm/kg) determined on a vapor pressure osmometer (Wescor Model 5130) within 0.5 days of sampling. Both osmometers were calibrated with the same osmolality reference standards. Readings between the machines did not vary more than the error of measurement in the nanoliter osmometer.

## RESULTS

### *Variability among broods*

Inter-brood variability (Fig. 1) was statistically significant, but due entirely to brood E, which had a high mortality rate in zoeae II and poorer survival at the megalopal state ( $P \leq 0.05$ ) and at the crab I stage ( $P \leq 0.01$ ). The lower survival of brood E was also apparent in the later salinity series (Fig. 4). Brood E also had shorter intermolt durations in the megalopae ( $P \leq 0.001$ ) and in crab I individuals ( $P \leq 0.01$ ) (Fig. 1). Except for this one brood, there did not appear to be any substantial variation in survival or developmental timing among different broods. There was a statistically significant ( $P \leq 0.05$ ) difference in the intermolt duration for megalopae among broods B ( $5.1 \pm 0.1$  days), C ( $5.0 \pm 0.0$  days), and D ( $4.2 \pm 0.3$  days), but these small differences were probably related to slight differences in the timing of counts and the rapidity of the first zoeal stage. Brood D reached crab I stage significantly faster ( $P \leq 0.001$ ), but the difference disappeared by the crab II stage.

### *Effects of starvation*

Survivorship did not differ between fed and starved larvae until day 14 (*t*-Test,  $P \leq 0.05$ ) (cf. Figs. 1, 2A), although a precipitous decline in survivorship in starved cultures began between day 11 and 12 (*t*-Test,  $P \leq 0.05$ ). In fed cultures, development proceeded into crab IV by day 22 (Fig. 1, Brood B). In starved cultures, development proceeded through zoea I and II and into the megalopal stage, and followed essentially the same timing and pattern as fed cultures through day 6 (Fig. 2A), but molt to crab I did not occur.

Size differences in starved and fed larvae (zoeae II) and megalopae were not evident on days 2, 4, or 6. By day 14, when survivorship fell significantly, starved megalopae were smaller than starved megalopae on day 6 (*t*-Test,  $P \leq 0.05$ ). When feeding was resumed on day 12 in cultures which had been starved, survival leveled off (Fig. 2B). When feeding was resumed, molt to crab I occurred within 3 days and development proceeded to crab III by day 23 (Fig. 2C), or within 12 days of refeeding.

### *Effects of salinity*

Larvae and early postlarvae survived and developed through a wide salinity range—0.08 to  $\sim 50\%$  (Fig. 3). There was 100% mortality within 24 h for larvae in 0‰ and

within 36 h in 55‰. No zoea I molted at these extremes. The effects of salinity on survival for megalopal and crab I stages for broods C and E are summarized in Figure 4. Mean survival to megalopa was significantly lower in 0.08‰ than in 2‰ ( $P \leq 0.05$ ) but still high. Between 0.08‰ and 35‰, survival to megalopa averaged 82% for both series. In the  $\sim 50$ ‰ cultures, survival was significantly lower than in the 45‰ cultures ( $P \leq 0.001$ ) but leveled off when salinities were returned to 45‰. Mean survival to crab I was significantly lower at 0.08 to 2‰ within the E series than at the other salinities ( $P \leq 0.05$ ) (Fig. 4). Still, mean survival to crab I ranged between 55 and 85% for most cultures between 2 and 45‰ and did not fall much below this in 0.08‰ (Fig. 4). Survival to crab I was below 20% in the  $\sim 50$ ‰ cultures and differed from that in the 45‰ cultures ( $P \leq 0.05$ ) for reasons discussed earlier. In most cultures at most salinities (Fig. 3), mortality was usually greater in the second zoeal stage and during metamorphosis to megalopa. After attainment of megalopae, survivorship leveled off, with highest mortality during molting.

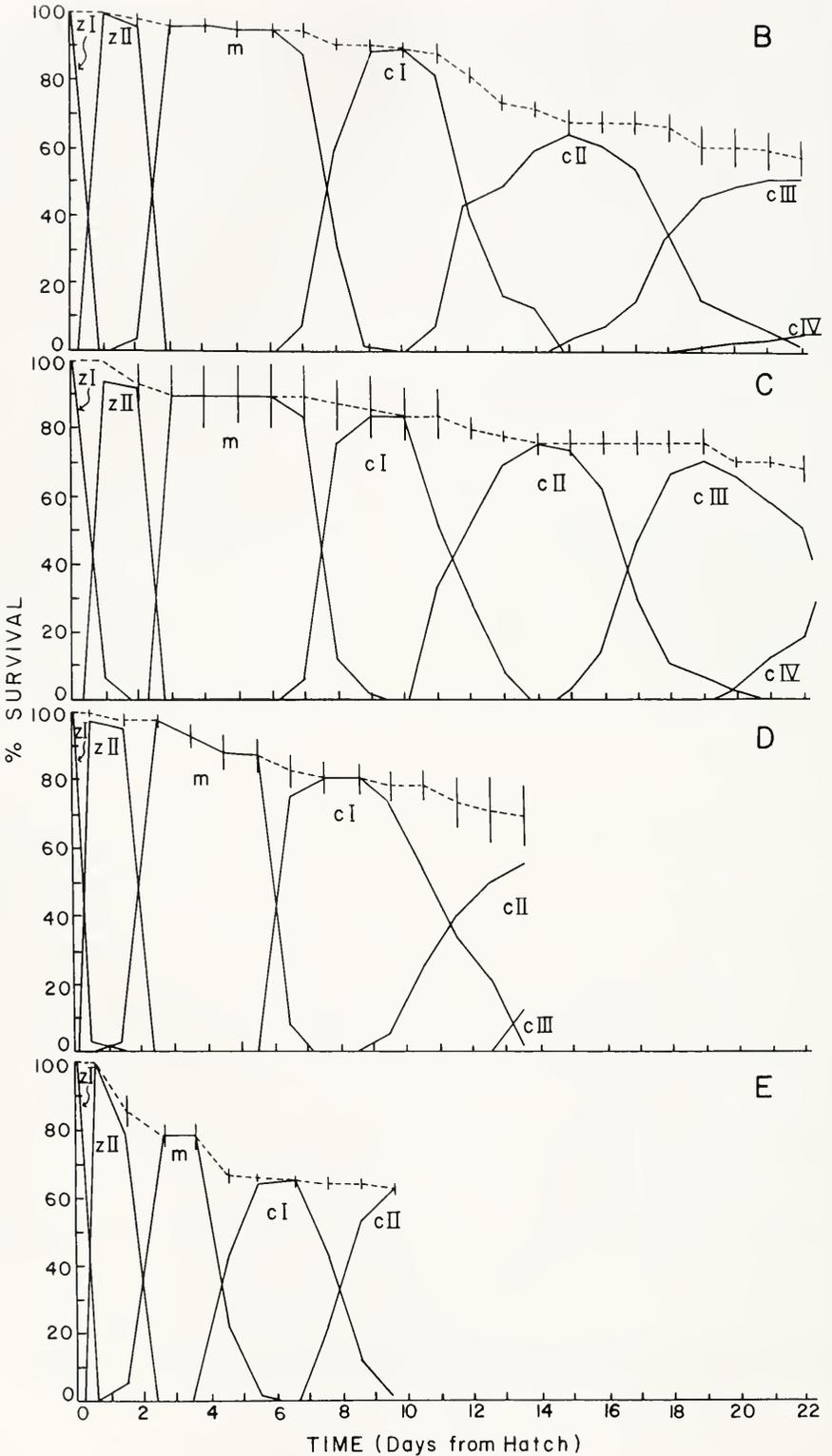
Any effect of salinity on development time was not obvious. Analysis of covariance showed differences between the C and E series across salinities in intermolt duration for zoeae II ( $P \leq 0.001$ ) and in intermolt duration for megalopae ( $P \leq 0.001$ ). Differences in intermolt duration for zoea I were incalculable (no deviation around the means in the E series) and were insignificant in crab I. In another analysis of covariance between the two series, there were differences between C and E in time for 50% of the individuals to attain crab I ( $P \leq 0.01$ ) and crab II ( $P \leq 0.01$ ) but not megalopae ( $P > 0.05$ ). Because of the variability shown between these two series, analyses of variation in development times along a salinity gradient were computed within each series. Although not always significantly longer, there was a tendency for prolongation in development time at the higher and lower salinities, within each series. Optimal development times were seen mostly in the 5, 15, and 20‰ salinity cultures.

### *Osmoregulation in the early stages*

Zoeae I hyperosmotically regulated over 0.08 to 45‰ salinity with the strongest gradient at the lowest salinities (0.08, 2, and 15‰) (Fig. 5). Zoeae II also hyperosmotically regulated over the same salinity range (Fig. 5). Blood osmolality of zoeae II held at 30 and 45‰, however, was only slightly higher than the medium. Zoeae II survived in 55‰ where there was a tendency towards hyporegulation of blood osmolality.

In the megalopae (Fig. 5), values for shorter *versus* longer acclimation times did not differ except for those at 2‰ ( $P \leq 0.05$ ) and at 45‰ ( $P \leq 0.02$ ), but the number of values for these comparisons was low. These differences were also not consistent across salinities or the period of acclimation. The gradient between the blood and 0.08‰ salinity was greater in the megalopae than in zoeae II, as was the reversed gradient at the higher values of 45 and 55‰ (Fig. 5). The isosmotic point was 40‰. Megalopae survived and hyporegulated at 65‰, whereas zoeae II did not survive. Some megalopae in 65‰ were covered with bacteria, and most remained listless on the bottom of culture dishes.

For crab I individuals (Fig. 5), in only one instance did the time of acclimation affect blood osmolality values. At 45‰, mean blood osmolality values for those acclimated 1 day from 15‰ differed from those acclimated 2 days and from those reared in 45‰ ( $P \leq 0.001$ ), but the latter two did not differ significantly. The osmoregulatory pattern for crab I individuals differed from that of megalopae (Fig. 5); there was a larger gradient between the blood and medium at 45‰, and a lower isosmotic point ( $\sim 30$ ‰).



Osmolality values obtained from 4 late-stage embryos which fell from egg masses onto moist paper toweling or into water of 20‰ (630 mOsm/kg) 2 to 4 days prior to hatch of the remainder of the brood averaged  $947 \pm 4$  mOsm/kg.

## DISCUSSION

As a subject for these studies, *Uca subcylindrica* presented both advantages and difficulties. The ovigerous females were difficult to collect because of their seasonal reproductive activity and secretive habits and, thus, were limited in number. They also have relatively few eggs, which imposed a limit on replication. On the other hand, their relatively large and distinctive larval and postlarval stages greatly facilitated the assessment of survival and developmental stages. Maintenance of the early stages was also easy, since yolk reserves sustained them well into the megalopal stage, when they were easily fed. Their lecithotrophic nature also eliminated cannibalism as a problem for mass cultures (Dawirs, 1982). Although the use of mass cultures does not allow exact age determination for each individual, as recommended by Dawirs (1982), there was sufficient synchrony in the replicates (e.g., Figs. 1, 3) to allow accurate determination of developmental "landmarks" for comparison by several statistical techniques.

### *Variability among broods*

With the exception of one anomalous brood, variability among broods in survival was insignificant and in development time was minimal. Within a brood, variability was also minimal (0.5 to 1 day) as shown by consistent development times and intermolt durations seen in the two salinity series at 5 to 25‰. The single anomalous brood where differences approached 2 days in intermolt duration and the minimal differences among the others support the findings of Provenzano *et al.* (1978) and Sandifer and Smith (1979) that genetic or other quality variations among broods of larvae may account for significant variation in larval duration and/or survival. Lack of variability cannot be considered a truism for species with abbreviated development (e.g., Dobkin, 1963; Fielder, 1970), at least for *Uca subcylindrica* (data presented here and in Rabalais and Cameron, 1983), and this variability should be considered when studying effects of laboratory manipulations. Differences, however, were small, and effects outside this range could be attributed to experimental treatments.

### *Effects of starvation*

Zoae of *Uca subcylindrica* are lecithotropic, i.e., they hatch from large, yolky eggs and use stored yolk as energy for growth and metamorphosis rather than relying on planktonic prey. Yolk globules are retained as a mass beneath the carapace in both zoeal stages and the megalopae (Rabalais and Cameron, 1983). These yolk reserves are apparently sufficient for development into the megalopal stage, but molt to crab I will not occur without food (Fig. 2). Accessory setae are reduced or absent on feeding appendages of the zoeal stages (Rabalais and Cameron, 1983). On the

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FIGURE 1. Inter-brood variability in survival and development as shown in four different broods (B-E) reared at similar conditions of temperature (26°C), salinity (15‰), and photoperiod (14L:10D). Values for stages are means of the replicates. Total survival of all individuals  $\pm$ S.E. plotted across the top of the stages with a dashed line. Roman numerals denote stages: z = zoea, m = megalopa, and c = crab stage.

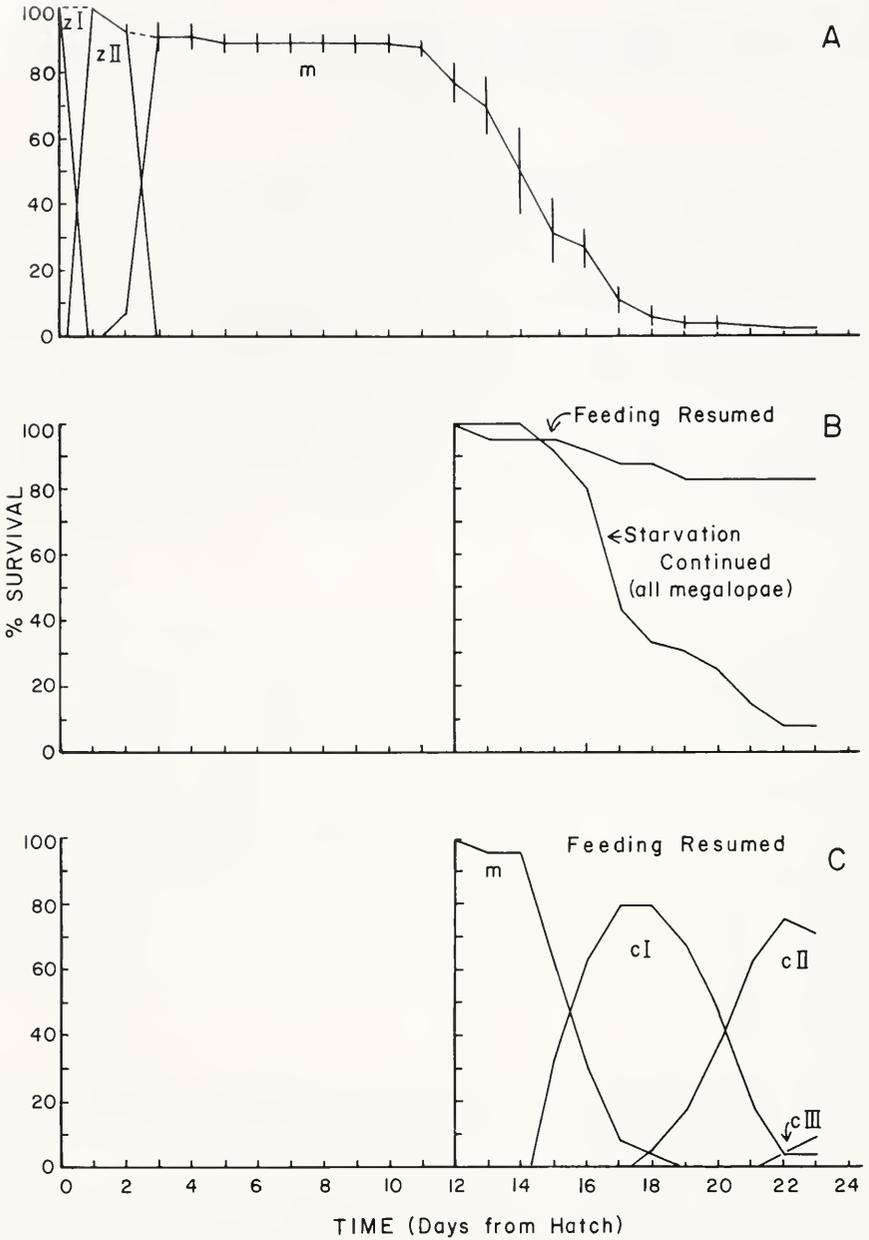


FIGURE 2. A. Percent survival and development for starved cultures at 15‰, 26°C, 14L:10D photoperiod. Values are means of 5 replicates of 20 individuals. Total survival of all individuals  $\pm$ S.E. plotted across the top of the stages with a dashed line. B. Percent survival of all individuals of all stages for the starved culture in which feeding was resumed for half the individuals and starvation was continued for the other half (n = 24 each). C. Percent survival of the individual stages and development for the 24 individuals in which feeding was resumed after 11 days of starvation. Roman numerals denote stages; z = zoea, m = megalopa, c = crab stage.

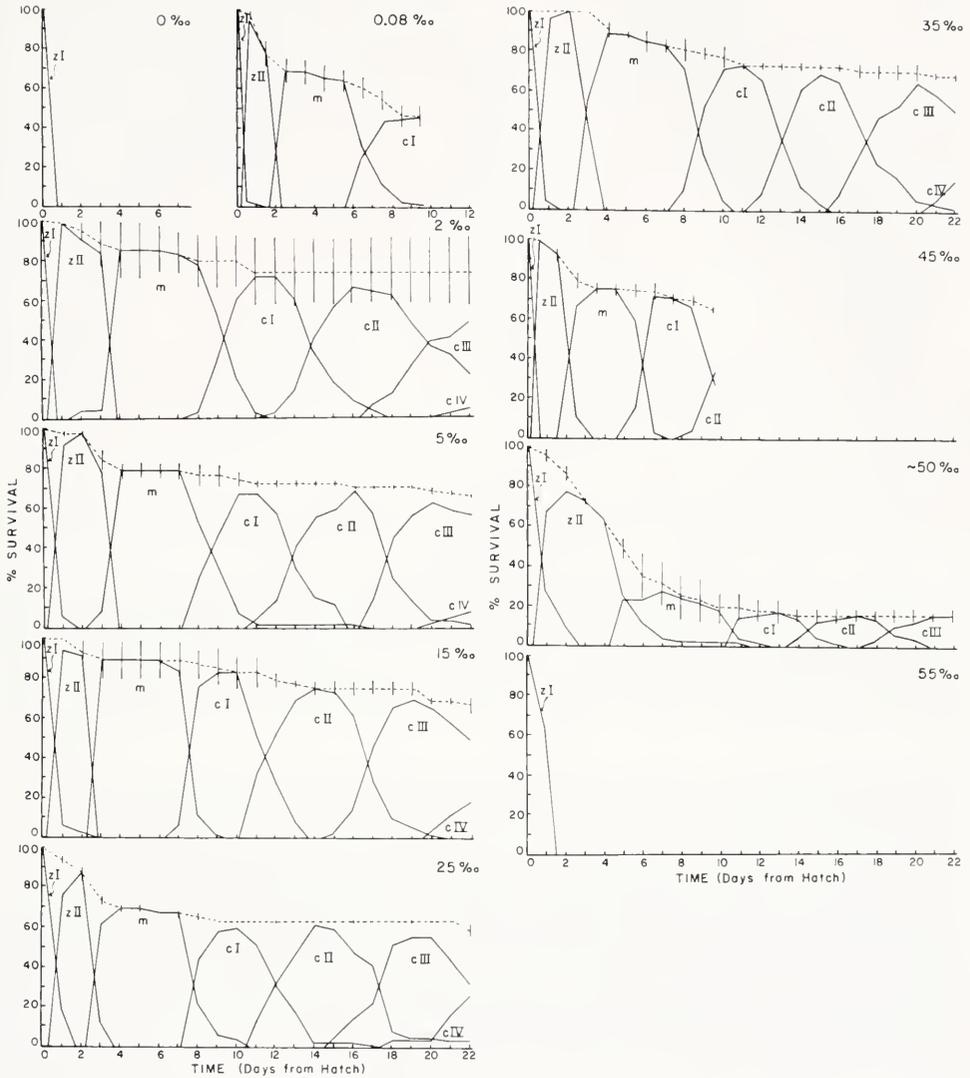


FIGURE 3. Survival and development for larvae and postlarvae reared at various salinities shown at upper right in each panel. Animals at 0.08 and 45‰ from brood E; the rest from brood C. The ~50‰ was 50–51‰ in the initial stages then 45‰. Data presented as in Figure 1.

other hand, feeding appendages of the megalopae are notably more setose with a greater variety of setae than other described *Uca* megalopae, a condition more typical of a first crab. Feeding behavior is consistent with these morphological features. Zoeae would not take food (*Artemia* nauplii, rotifers, and other *Uca* zoeae I) which was offered but the megalopae were voracious feeders and actively swam after, captured, and consumed live *Artemia* nauplii. Early crabs were also active predators. Both megalopae and early crabs scavenged dead organisms.

In brachyuran crabs, energy reserves are usually not sufficient to allow development during starvation, and there is a particularly critical period in the beginning of larval

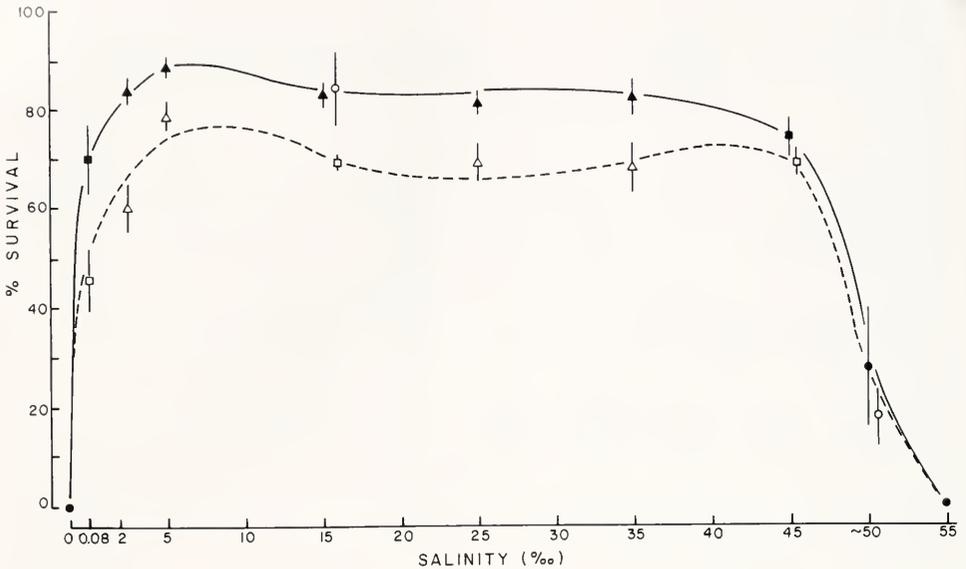


FIGURE 4. Summary of survival data for megalopae (darkened symbols) and crab I individuals (open symbols) from Figure 3. Triangles represent the means of values for broods C and E which were not significantly different. When different, the C series are shown as circles and the E series as squares. Values at 0.08 and 45‰ are brood E (squares) and at ~50‰ are brood C (circles). Salinity axis not to scale at the lower values. Distribution curves (solid line for megalopae and dashed for crab I stages) approximated.

development beyond which starvation may cause irreversible damage to a hormonal or enzymatic system controlling molt (Anger and Dawirs, 1981; Anger *et al.*, 1981). The time when 50% of the starved larvae could not recover when being refeed (Point of No Return,  $PNR_{50}$ ; Anger *et al.*, 1981) was not determined but was obviously longer than the approximately 1 to 2 days for planktotrophic larvae. Megalopae of *Uca subcylindrica* recovered with only 4.2% mortality when feeding was resumed after 12 days of starvation. We estimated that the  $PNR_{50}$  occurs between day 14, when survivorship fell below 50% and size differences were noted, and day 18, when listless behavior of megalopae was observed.

#### *Effects of salinity*

Some euryhaline crabs have a wide range of salinity tolerance during early development: 10 to 40‰ for *Sesarma reticulatum* (Foskett, 1977), 5 to 40‰ (but with poor survival in 5‰) for *Clibanarius vittatus* (Young, 1979), and 2.5 to 40‰ (but with poor survival at the extremes) for *Rhithropanopeus harrisi* (Costlow *et al.*, 1966). Larvae and early postlarvae of *Uca subcylindrica* survived and developed in a greater range of salinities under laboratory conditions—0.08 to ~50‰—than recorded for any other decapod crustacean (Fig. 3). In the osmoregulation salinity series, zoeae II survived in 55‰, and megalopae and crab I individuals survived in 55 and 65‰. This range of tolerance in early stages differs from that of adults, which survived (>50%) 2 to 90‰ in long-term salinity experiments (Rabalais and Cameron, in prep.). The inability of the early stages of other *Uca* to survive in salinities less than 15–20‰ or greater than 40–45‰ (Fig. 6) makes them unlikely to tolerate the salinity ranges in the habitats of *U. subcylindrica*.

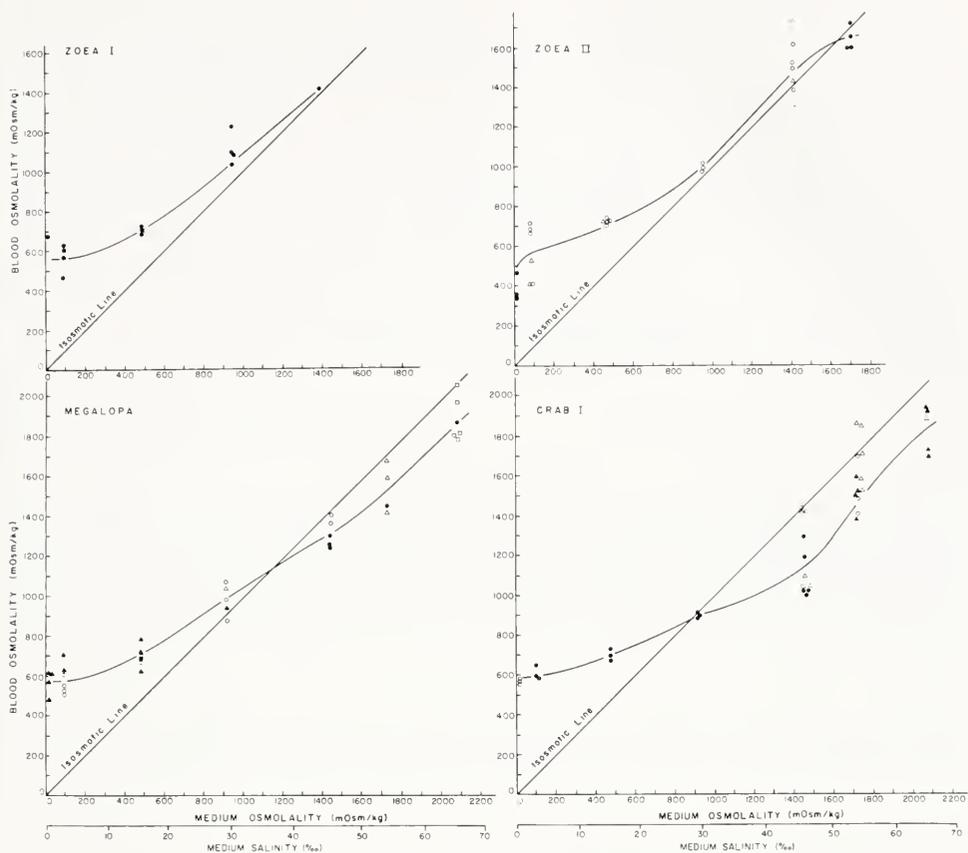


FIGURE 5. Osmoregulation curves for larvae and early postlarvae. Zoeae I from broods G (open circles) and H (closed circles), 6.5–12 h old. Zoeae II from brood H (triangles), brood I acclimated 12 h (open circles), and brood I acclimated 1 day (closed circles). Megalopae from brood G, 7 days old (open circles); brood G, 8 days old (closed circles); from brood G, 9 days old (open triangles); brood H acclimated 1 day to lower salinities (closed triangles); brood I acclimated 1 day to higher salinities (open squares); and brood I acclimated 2 days to higher salinities (closed squares). Crab I individuals from brood F acclimated 10 days (closed circles); brood F acclimated 1 day at the lower and higher salinities (open circles); brood H acclimated 3 days in 55‰ and 1 day in 65‰ (closed triangles); brood G acclimated 2 days at the higher salinities (open triangles). Curves drawn through the mean for each salinity, with the exception of 45‰ in crab I which includes only the lower values (open triangles and closed circles). Approximate salinity values given on second abscissa below medium osmolality.

Tolerance of early stages of *Uca subcylindrica* to a wide range of salinities would be a decided advantage in the habitats in which they are known to develop. To date zoeae I have been found in natural nursery areas with salinities from fresh water to 33‰; zoeae II, in salinities from 2 to 65‰; and megalopae in fresh water to 42‰ (Rabalais, 1983). These values correspond to salinity tolerances in the laboratory (Fig. 3), except for zoeae II in 65‰. The highest value at which zoeae II were found to survive in the laboratory was 55‰, where there was slight hypo-osmotic regulation (Fig. 5), and it is reasonable to expect that survival at 65‰ may occur. Zoeae II which survive in 65‰ may not be able to metamorphose to megalopae at this salinity. Megalopae may only be found in the field at such high salinities if the preceding

SALINITY TOLERANCE OF  
LARVAE & POSTLARVAE

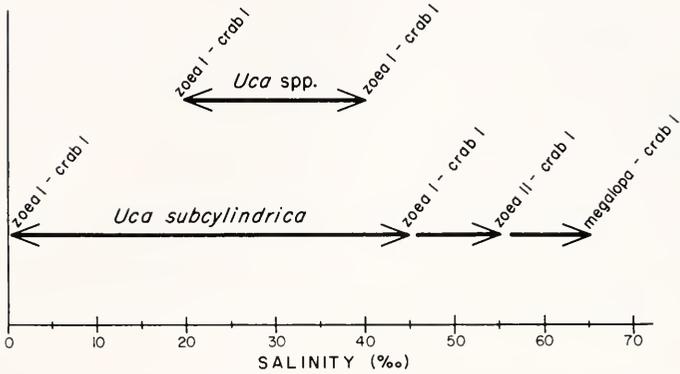


FIGURE 6. Salinity tolerance of larvae and early postlarvae of closely related species—one with abbreviated development, *Uca subcylindrica*, and other *Uca* with longer-developing larvae. Data for *Uca* species from Dietrich (1979) on *U. pugilator*, which did not survive in  $<20\text{‰}$  or  $>40\text{‰}$  and from personal observations for *U. panacea*, *U. longisignalis*, and *U. rapax* zoeae I, which did not survive in  $<15\text{‰}$  or  $>45\text{‰}$  (Rabalais, 1983). Horizontal arrows represent limits for survival and development at various salinities for the stages shown. Lower limit for *U. subcylindrica* was a pond water mixture of  $0.08\text{‰}$ .

zoeal stages molted at a lower salinity before the effects of temperature and evaporation created higher salinities for the subsequent megalopae.

#### Osmoregulation in the early stages

Foskett (1977), who studied *Sesarma reticulatum* and reviewed what was known of larval osmoregulation in brachyuran crabs, found that osmoregulation patterns did not change between successive stages. Young (1979), who studied an anomuran hermit crab, supported this finding. On the other hand, Read (1984) reported noticeable changes in osmotic responses between larval and postlarval stages of a palaemonid shrimp. Foskett (1977) also noted that there was no consistent osmoregulatory pattern across a diversity of brachyuran crab species and that there was no clear trend toward development of adult osmoregulation capabilities at the end of larval life (which included megalopae in his consideration). Osmoregulation patterns of the early stages of *Uca subcylindrica* did differ from stage to stage (Fig. 5) and began to approach those of adults by the megalopal and crab I stages. Hyperosmotic regulation in lower salinities by all early stages was similar to adult crabs (Rabalais and Cameron, 1985). On the other hand, a tendency to hypo-osmoregulate at higher salinities began in zoeae II and developed further in megalopae and crab I individuals. The isosmotic point also approached that of the adults by crab I.

The ontogeny of osmoregulation capabilities in larvae and early postlarvae was consistent with the survivorship curves for these stages. For example, in  $45\text{‰}$ , the greatest mortality was in zoeae II (Fig. 3) where there was hyperosmoconformity (Fig. 5) and leveled off in the megalopal and early crab stages (Fig. 3) when hypo-osmotic regulation began for this salinity (Fig. 5). Mortality was usually greater in larval stages than in early postlarval stages in most salinities. Combined with results from the salinity tolerance experiments, it is obvious that *Uca subcylindrica* larvae and postlarvae

are surviving in a wide range of salinities as a result of regulation of blood osmolality, not just by conforming to and tolerating the external media.

Several characteristics of the early development of *Uca subcylindrica*, especially the wide range of salinity tolerance, prove advantageous in the semi-arid habitats in which these crabs live. Since ecdyses are critical periods in larval life and highest mortality of cultured decapod larvae often occurs then (Knudsen, 1960; Roberts, 1971), reduction in the number of premetamorphic molts may increase larval survivorship (Sandifer, 1973). The mortality among larvae of several species during this critical period may be related to an inability to maintain an osmotic or ionic gradient at the time of molt. A reduction in the number of molts and their associated physiological stress would be advantageous to species which inhabit more dilute media, are exposed to extremes in salinities, or are incapable of appropriate behavioral responses necessary to avoid suboptimal conditions. Early development of *U. subcylindrica* benefits from both a reduced number of molts and stages which can regulate against lower and higher external media.

In summary, several features of the early stages of *Uca subcylindrica* promote high survivorship in a variety of culture conditions and include the lecithotrophic nature of the larvae and early postlarvae, maturationally advanced morphological characteristics and behavior, a reduction in the number of critical molt periods, and the physiological ability to survive and develop through an extremely wide range of salinities. These factors are important since food shortages and extremes of salinity which would tax any organism are found in their nursery areas. The larvae and early postlarvae of other *Uca* are unlikely to survive and develop under such conditions. Combined with differences in the physiology and morphology of adult crabs (Rabalais and Cameron, 1985), knowledge of the characteristics of the early stages contributes to our further understanding of why other fiddler crab species are not usually found in the semi-arid habitats of *U. subcylindrica*.

#### ACKNOWLEDGMENTS

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## FLUID MECHANICS OF THE THALLUS OF AN INTERTIDAL RED ALGA, *HALOSACCION GLANDIFORME*

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### ABSTRACT

The elongate, ellipsoidal thalli of *Halosaccion glandiforme* (Gmel.) Rupr. are alternately exposed and immersed with tidal cycles. These sea water-filled thalli are penetrated, mainly in the distal third, by 5 to 15 pores. During emersion, water is lost by evaporation from the surface of the thallus and by bulk flow through the pores; surface tension at the pores prevents entry of air. During reimmersion, thalli reinflate as a result of the elasticity of their walls and via pressure differences due to flow of ambient water along their surfaces. The drag of thalli is low, and, concomitantly, separation of flow is well downstream; as a result, their relatively weak stipes are adequate to resist hydrodynamic forces.

### INTRODUCTION

*Halosaccion glandiforme* (Gmel.) Rupr. is a common inhabitant of relatively sheltered rocky coastal areas of the north Pacific. Its thallus forms a nearly symmetrical fusiform body of revolution up to 25–30 cm long, 3–4 cm in diameter, distally rounded, and tapering proximally to a thin, short stipe. The thallus is thin-walled (about 200  $\mu\text{m}$ ), filled with sea water (and usually about a cubic centimeter of gas), and punctured by 5 to 15 irregularly shaped (often cruciform) pores up to 200  $\mu\text{m}$  across. A gentle squeeze squirts tiny streams of water from the larger pores. On San Juan Island, Washington, the algae occur mainly in the lower intertidal zone; a receding tide exposes water-filled thalli which then gradually lose varying amounts of water. This loss of water typically results in lateral and then bilateral compression of the thalli without entry of air. Upon reimmersion, the algae reinflate. Just before and immediately after exposure, the algae are subjected to the relatively rapid currents generated as surface waves interact with rocks.

The shape of the thallus suggests some degree of streamlining, an unusual occurrence in a sessile organism exposed to currents which are far from unidirectional. For streamlining to be functional, *Halosaccion* must operate as a weathervane, a behavior more common among planar, flexible forms (Vogel, 1984).

The water within the thallus appears to reduce desiccation or heating of tissue during periods of emersion. Thus DePamphilis (1978) found that three hours of exposure on a sunny summer day killed plants whose internal water had been removed but did not kill intact plants. And Muenscher (1915) noted that the length of exposure needed to dry the algae beyond the point of recovery increased with the amount of water inside the thallus.

The torpedo-like shape and perforation of a sea water-filled thallus and its cyclic deflation and inflation are unique among marine algae (Hansen, pers. commun.). The present study is an inquiry into the identity and relative contribution of the

various physical processes which might be concomitant with this peculiar morphology and behavior. Our focus is on devices which (1) determine the rate of water loss and prevent entry of air when the thallus is emersed, (2) enable replacement of water after reimmersion, and (3) prevent dislodgement of an alga by hydrodynamic forces.

Several agencies might, *a priori*, be involved. (1) Since a water-filled bag is exposed to air, *surface tension* must be considered, either of a cylindrical film of water around the thallus or of spherical interfaces at the pores. (2) Since evaporation occurs on the outside only, *concentration gradients* will develop. (3) Since a thallus has a preferred "resting" shape, inflated rather than flat, *elasticity of the thallus wall* is appreciable. (4) Since water can clearly flow through the pores, the *resistance of the pores to bulk flow* may be important in determining rates of water movement. Since environmental water currents may be substantial, (5) *drag*, (6) the *mechanical strength of the stipe*, and (7) *pressures generated by flow around the thallus* deserve attention. In some concerted fashion, these factors must accomplish the three functions mentioned above.

Clearly, the distribution and abundance in nature of an organism such as *Halosaccion* must be profoundly influenced by its adaptations to the physical conditions of the environment. The present study attempts to elucidate major components of that adaptation. It does not involve direct measurement of the relevant environmental variables nor does it address the precise habits of the organism in nature. These crucial matters are currently the subject of another investigation (Ladd Johnson, pers. comm.).

## MATERIALS AND METHODS

### *Formulas and conventions*

The following formulas will be employed in this paper.

(1) The Reynolds number, a dimensionless index to the character of flow (Vogel, 1981):

$$\text{Re} = \frac{\rho l U}{\mu} \quad (1)$$

(where  $\mu$  is the dynamic viscosity of the medium,  $l$  a characteristic length of the immersed object,  $U$  the relative velocity of fluid with respect to object, and  $\rho$  the density of the medium).

(2) The drag coefficient, a dimensionless drag (Vogel, 1981):

$$C_d = \frac{2D}{\rho S U^2} \quad (2)$$

(where  $D$  is the drag and  $S$  is a specified area characterizing the object).

(3) Flow through a circular aperture (Happel and Brenner, 1965):

$$Q = \frac{a^3 \Delta P}{3\mu} \quad (3)$$

[where  $Q$  is total flow (volume per time),  $a$  the radius of the aperture, and  $\Delta P$  the pressure difference across the aperture]. For sharp-edged apertures of no depth, the equation is trustworthy up to a Reynolds number of 3.2 based on pore diameter and mean velocity through the pore. For pores which are neither sharp-edged, negligibly deep, nor circular in cross-section, this equation is not strictly applicable, but it can be used to calculate (from pressure and flow measurements) "nominal diameters"

for the pores—the diameters of functionally equivalent circular pores of negligible depth.

(4) Pressure difference across (a) spherically or (b) cylindrically curved air-water interfaces (Rouse, 1946):

$$\Delta P = \frac{2\gamma}{a} \quad (4a)$$

$$\Delta P = \frac{\gamma}{a} \quad (4b)$$

(where  $\gamma$  is surface tension and  $a$  here is the radius of curvature of the interface). If  $a$  is approximated by the radius of a pore and if wetting of the thallus is incomplete, then (4a) will overstate the pressure difference necessary to force air through a pore into a water-filled thallus.

(5) Resistance to flow through an aperture or pipe:

$$R = \frac{\Delta P}{Q} \quad (5)$$

The ratio of pressure difference to total flow is obtained from either (3) above or the Hagen-Poiseuille equation; for laminar flow, resistance is independent of speed.

(6) Pressure coefficient (Goldstein, 1938):

$$C_p = \frac{2\Delta P}{\rho U^2} \quad (6)$$

Pressure coefficient is analogous to drag coefficient (and, like the latter, is dimensionless). When shifting media to facilitate the use of models, maintaining constancy of the Reynolds number assures constancy of pressure coefficient, not pressure; and the latter must be recomputed with this formula.

The following values of physical parameters have been used: (1) surface tension of sea water,  $0.074 \text{ N m}^{-1}$ ; (2) density of sea water,  $1.03 \times 10^3 \text{ kg} \cdot \text{m}^{-3}$ ; (3) viscosity of sea water,  $1.36 \times 10^{-3} \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$ ; all for  $12^\circ\text{C}$ ; (4) density of air,  $1.18 \text{ kg} \cdot \text{m}^{-3}$ ; (5) viscosity of air,  $18.3 \times 10^{-6} \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$ ; the latter at  $25^\circ\text{C}$ .

As used here, "imprecision" refers to the standard deviation of a series of measurements whether calculated or estimated without reference to any accepted value; "systematic error" is the maximum difference from some "true" value as a result of calibration artifacts; "uncertainty" combines the two either where systematic error is negligible compared to imprecision or where a distinction is impractical.

Equations (7) through (9) presume S.I. units.

### Material

Specimens of *Halosaccion* ranging in length from 6 to 12 cm were collected from the northern shore of Friday Harbor ("Cantilever Point," Friday Harbor Laboratories) and the southern shore of the entrance to False Bay ("Mar Vista Resort"), both on San Juan Island, Washington, during August of 1981 and 1983. Collection typically involved gently pulling on and breaking the stipe; plants were maintained without attachment in running sea water at about  $12^\circ\text{C}$  at the Friday Harbor Laboratories and, except as noted, were used for experimental purposes within a day following collection. Additional algae were preserved in a solution of formalin in sea water buffered with sodium tetraborate for observations on the distribution of pores.

A brass model of a thallus (Fig. 1A) was constructed on a lathe in order to investigate the relationship between pressure and location on the thallus; the model was life-sized and differed from a normal alga only in being a fully symmetrical body of revolution. Measurements for the model were obtained from a projected lateral photograph of a small but otherwise ordinary specimen tethered by its cut stipe in a flow tank at  $0.33 \text{ m} \cdot \text{s}^{-1}$ ; the "fineness ratio" (length divided by diameter) of the specimen was 3.55. Uncertainty in the dimensions of the model is estimated as 0.2 mm. A small hole penetrated the model axially from the apex almost to the stipe; at the apex this hole was continuous with a brass tube, 2.4 mm in diameter, which functioned as the support ("sting") and pressure-transmitting conduit. A lengthwise row of 15 additional holes of 1.0 mm diameter, each connecting the model's surface with the axial hole, served as pressure taps; in practice all but one of these latter holes were occluded by small pieces of plastic adhesive tape.

#### *Pressure and total flow through the pores*

For manipulations involving pressures in water above 200 Pa ( $\text{N m}^{-2}$ ) the pressure chamber shown in Figure 1B sufficed; it consisted of two plexiglas plates, each with a 25 mm central hole, between which a piece of thallus sandwiched between rubber gaskets could be fit. Each hole communicated with a chamber and the latter with a fitting for the attachment of rubber tubing. Glass plates on the chambers permitted viewing the thallus as pressure changed. Pressures were applied by adjusting the

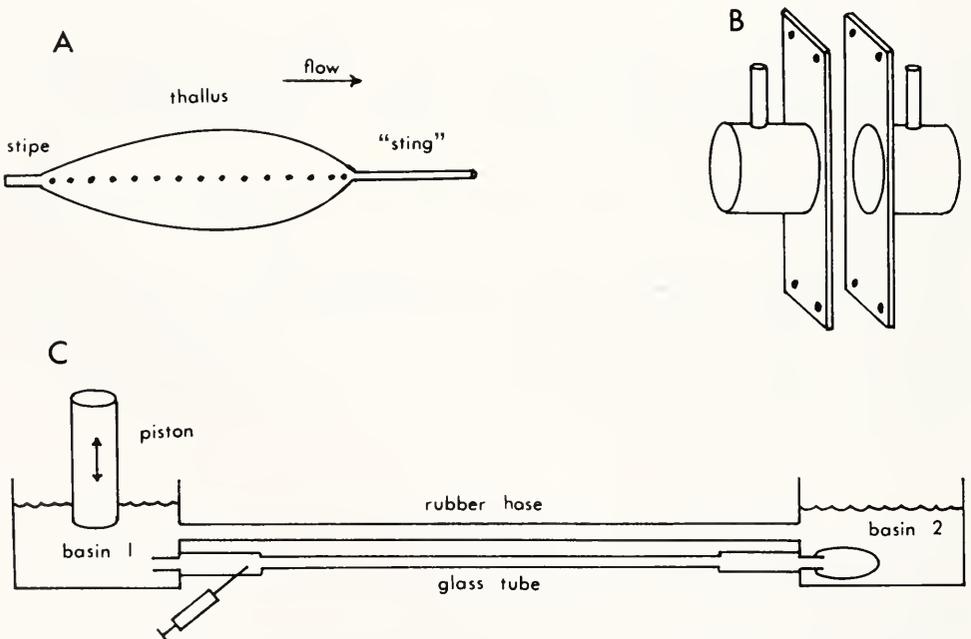


FIGURE 1. (A) Brass model of a *Halosaccion*, showing location of pressure taps; together, thallus and stipe are 62 mm in length. (B) Pressure chamber for use with pieces of thallus wall; rubber gaskets normally seal the piece of thallus between the halves of the chamber. (C) Apparatus for applying pressure to a cannulated thallus or for observing refilling due to wall resiliency. The glass tube attaches to the basins via short pieces of rubber tubing through which dye can be injected; both conduits connecting the basins are equipped with pinch clamps.

relative heights of the water columns on each side of the chamber. Uncertainty in the production of pressure differences was estimated as 10 Pa.

Where pressures in water were below 200 Pa, the apparatus of Figure 1C was used. Two rectangular basins were connected by a straight glass tube and appropriate fittings and by a rubber hose; either connection could be closed. Lowering a cylindrical piston into one basin using a precision worm-drive ("Unislide") raised its water level by a small amount since the piston area ( $62.1 \text{ cm}^2$ ) was much less than that of the air-water interface in the basin ( $403.3 \text{ cm}^2$ ). This interface was large enough to minimize the effects of surface tension. Uncertainty in the resulting pressure differences between the basins was estimated as 0.2 Pa. An entire thallus could be attached to an extension of the glass connection between the basins as shown in Figure 1C: a tapered plastic hose connector was inserted into the hole left after amputation of the stipe, and an annulus of latex tubing was rolled over the cut end of the thallus. Leakage was checked by injecting a sea water solution of fluorescein (uranine) into the nearest rubber tube and applying pressure.

With either pressure-producing apparatus, total flow was measured by injecting a pulse of fluorescein solution and timing the passage of the dye front through a glass tube, taking advantage of the consistent two-fold difference between axial flow speed and average speed for fully developed laminar flow. Minimum detectable total flow was  $5 \text{ mm}^3 \cdot \text{s}^{-1}$ ; uncertainty was estimated as the larger of  $5 \text{ mm}^3 \cdot \text{s}^{-1}$  or 3% of any determination.

Pressures in air were measured with a multiplier manometer constructed and calibrated as previously described (Vogel, 1981; 1983); imprecision was estimated as 0.1 Pa and systematic error as 0.2 Pa. A reference pressure was provided by a hole of 1.6 mm diameter flush with the surface and 28 mm downstream from the leading edge of a thin, flat plate parallel to the airflow.

### *External flows and force measurements*

Measurements of drag and visualization of flow were done in a small flow tank with a working section 10 cm deep and 10 cm wide: the tank was designed as described previously (Vogel, 1981) except for two features. (1) A standpipe downstream from the working section and a tap in the return pipe permitted continuous exchange of sea water. (2) The drive consisted of two sequential propellers separated by a 6 cm long, three-vaned stator: the latter supported the shaft bearings, increased the advance ratio to about 1.1, and improved maximum speed, smoothness of flow, and efficiency. Calibration was done by timing the flow of dye pulses over a 50 cm course (best at low speeds) and by determining the drag of a circular disk oriented normal to flow, 3.73 cm in diameter, of known and constant drag coefficient (most useful at high speeds). Systematic error and imprecision are each estimated as 3% for flows more than 2 cm from either walls or air-water interface. For visualization of flow patterns, fluorescein was injected through the drawn end of a polyethylene catheter tube (tip diameter about 0.1 mm) by a micrometer-equipped syringe.

To measure drag, thalli were attached by their stipes to the end of a cylindrical "sting," 1.83 mm in diameter, which extended vertically out of the flow tank. The sting in turn attached to the end of an aluminum beam,  $60 \text{ mm} \times 12.7 \text{ mm} \times 1.27 \text{ mm}$ , with foil strain gauges centered on each face. The gauges formed two arms of a DC Wheatstone bridge, the imbalance of which was amplified by an Intersil ICL7605CJN instrumentation amplifier in the manufacturer's recommended circuit. Calibration, with an uncertainty of 1%, was done using balance weights with the beam held horizontally. Measurements of the drag of the sting alone were subtracted

from the data; the former figures agreed closely with calculations from flow speed and accepted values of drag coefficient, and thus they provided a check on flow and force calibrations.

The force necessary to pull a thallus from its attachment in nature was determined by connecting a string between a small alligator clip and an Ametek force gauge (T500G-TC), grabbing a stipe with the clip, and noting the highest force as the thallus came loose. Uncertainty was about 5%.

Airflow was produced by a large wind tunnel described by Tucker and Parrott (1970), calibrated with a whirling vane (axial flow) anemometer with an estimated systematic error of less than 5% and an imprecision of less than 2%.

### *Other methods*

Salinity was measured with an American Optical hand-held salinity refractometer.

The location of holes in preserved specimens was determined on castings. Deflated thalli were filled with plaster of Paris through a cut in the stipe end, and the plaster was allowed to set while thalli were hung in water. The thalli filled with plaster were dipped in a basic fuchsin solution for two minutes, and the thalli were then peeled off the casting; purple spots marked hole locations.

## RESULTS

### *Resistance of pores to flow*

For low pressures, volume flow through the pores proved to be directly proportional to pressure. Total flow was determined on a thallus for eight pressures from 19.6 to 114 Pa by forcing water out of a cannulated thallus with the apparatus of Figure 1C. No threshold pressure was necessary to produce a detectable flow, and the resistance (corrected for that of the glass dye-timing tube) averaged  $3.07 \times 10^9 (\pm 0.38 \times 10^9)$   $\text{N} \cdot \text{s} \cdot \text{m}^{-5}$  (equation 5) with little if any systematic variation with changes in applied pressure (see below). Forcing dye through the thallus, an automatic consequence of the measurements, indicated nine open pores in the thallus, so the resistance was equivalent to that of nine identical pores of  $2.76 \times 10^{10} \text{N} \cdot \text{s} \cdot \text{m}^{-5}$  resistance and  $103 \mu\text{m}$  nominal diameter (equation 3). Using this nominal diameter and the average flow through the pores, the Reynolds numbers (equation 1) range from 35 to 250, well above the value of 3.2 given as the maximum for confident use of equation (3). A regression of log-transformed data, though, gave a slope of 1.10 for total flow as a function of pressure, so the assumption of direct proportionality in the equation is not seriously violated (the lack of sharp edges on the pores probably postponed the onset of turbulence to higher Reynolds numbers than otherwise expected):

$$Q = (2.206 \times 10^{-10})\Delta P^{1.10}, \quad r = 0.989 \quad (7)$$

For higher pressures, volume flow was no longer directly proportional to pressure. Total flow was determined for pressures from  $1.56 \times 10^3$  to  $5.97 \times 10^3$  Pa with a piece of thallus containing a single pore using the apparatus of Figure 1B; three measurements were made at each of seven values of pressure. With water on both sides of the tissue, no differences were noted between flows either in or out of the thallus, and again no threshold value of pressure was necessary to produce a flow. The equation determined by linear regression of log-transformed data was:

$$Q = (1.322 \times 10^{-9})\Delta P^{0.631}, \quad r = 0.916 \quad (8)$$

Here the exponent (0.631) was closer to the value of 0.5 expected for Reynolds numbers above 30,000 (Happel and Brenner, 1965) than to that of unity expected below 3.2. Resistance was no longer "ohmic," and nominal diameter could not be calculated without knowing an empirical orifice coefficient. Using a pore diameter of 200  $\mu\text{m}$ , the range of Reynolds numbers was 640 to 1480. These results indicate essentially ordinary behavior for the pores: bidirectionally equal resistances and the expected change in the character of flow between low and high Reynolds numbers.

With the apparatus of Figure 1B, it was possible to establish an air-water interface across a pore and then to force either air or water across the interface. With water passing through the pore, the relationship between pressure and flow did not significantly differ from the results cited above. No threshold pressure was noted, nor were there appreciable differences between flow in the two directions. It appears that the surfaces of the thallus are sufficiently hydrophilic that wetting is essentially complete and surface tension is of no consequence to the passage of water.

By contrast, air did not easily pass through a pore within which an air-water interface was located. By watching the side exposed to water, it was easy to determine threshold pressures at which air bubbles began to cross the piece of thallus. These pressures ranged from 990 to 17,900 Pa, an eighteen-fold variation. Curiously, the threshold pressures increased steadily after collection: the minimum average pressure ( $1125 \pm 116$  Pa) was obtained for a pore tested on the day of collection and the maximum ( $17,200 \pm 581$  Pa) six days after collection. Pores apparently occlude unless thalli are subjected to the normal tidal cycles of exposure and immersion. There was no appreciable difference in the pressures necessary to force air through pores in the two directions.

Using equation (4) for a spherical interface, the minimum pressure of 990 Pa corresponds to a circular pore with a diameter of 300  $\mu\text{m}$ . The same pressure corresponds to a hydrostatic head of 9.8 cm of sea water, about equal to the maximum pressure of water inside one of the present thalli hanging vertically in air. Thus as long as an air-water interface is present to block pores it is quite unlikely that air can be drawn into an intact thallus.

### *Emptying and refilling*

Three turgid thalli with initial masses (including contained water) from 6.5 to 13.0 g were placed on a horizontal piece of transparent plexiglas and exposed to direct sunlight for two hours. Each lost water at a steady rate, averaging  $0.17 \pm 0.035\%$  of its initial weight per minute, a 20.4% loss during the exposure. For these plants, the rate of water loss was more nearly proportional to surface area than to mass or volume. Two other thalli with 1 mm holes at their bases (damage not uncommon in nature) lost water at more rapid rates: 0.28 and 0.31% of initial weight per minute.

An additional thallus was similarly exposed to sunlight for four hours, and a small sample of its internal water was drawn each hour for determination of salinity. During exposure, salinity rose from 30.0 to 41.0‰ while the mass of internal water dropped from 24.1 to 17.5 g. The product of salinity and mass for the five determinations averaged 721.7 ( $\pm 4.7$ ) (g‰), the constancy of these data indicating negligible salt loss during exposure. It appears that under these conditions water loss occurs entirely by evaporation of pure water.

Thalli held vertically in air by their stipes, however, lost water more rapidly, typically losing over half their water during a two-hour exposure. Sea water, in fact, dripped off the thalli. Loss rates were highly variable, though, even among undamaged

thalli. By equation (3) a water loss of 5 ml in two hours with a 400 Pa (4 cm) hydrostatic pressure head implies a single pore of 38  $\mu\text{m}$  diameter or nine identical pores of 18  $\mu\text{m}$ .

When immersed in water, deflated thalli inevitably refilled, although the rates of reinflation were quite variable. Two sets of measurements were made. Five thalli had as much water as possible gently squeezed out through the pores, with an average weight reduction of 87%. In the first ten minutes of reimmersion they regained water at 3.4 ( $\pm 2.2$ )% of their fully inflated weight per minute. Eleven measurements on four other thalli with an average weight reduction of 35% gave refilling rates averaging 1.9 ( $\pm 0.9$ )% per minute.

Spontaneous refilling in still water requires that the pressure within a thallus be below ambient. This pressure difference was measured by cannulating thalli in the apparatus of Figure 1C and then determining the rates at which partially deflated specimens refilled through a fixed resistance with the same water level in the two basins. In practice, the glass dye-timing tube provided the resistance and was calibrated by production of known pressures (using the cylindrical piston) with no thallus attached. Rates of refilling during these measurements were sufficiently higher than those cited above that flow through the pores was negligible. Five measurements were made on each of four thalli; the average pressure difference was 53.6 ( $\pm 26.3$ ) Pa. Variability occurred mainly among the different specimens rather than among measurements on individual thalli: standard deviations for the latter averaged only 14.2 Pa. The variation for individual thalli seemed to depend upon the location of the concavity caused by deflation; functional wall resilience is not constant for a thallus, even at a given degree of deflation.

A refilling rate of 2.5%/min driven by an inflation pressure (due to wall resilience) of 53.6 Pa corresponds, by equation (3) to a single pore of a nominal diameter of 146  $\mu\text{m}$  or to nine 70  $\mu\text{m}$  pores.

### *Ambient flow and drag*

Small surface waves generate substantial currents within about half a meter of the shore or bottom; observations of the movement of dye pulses indicate that flow speeds may routinely reach  $1 \text{ m} \cdot \text{s}^{-1}$ . Shortly before emersion and immediately after reimmersion, *Halosaccion* will be exposed to these flows. Since these elongate thalli with flexible attachments operate as weathervanes, overall flow will never deviate very much from an axial direction from stipe to apex whatever the spatial and temporal complexity of local currents. Consequently it is appropriate to observe the results of ambient flows on either thalli or models oriented parallel to flow in a unidirectional flow tank.

The most immediate and obvious result of water motion on an attached organism is a force directed parallel to flow tending to dislodge it, its drag. For an object of non-negligible frontal area at moderate or high Reynolds numbers, high drag is associated with an anterior point of separation of flow and a wide wake; low drag reflects a delayed, posterior separation and a narrow wake. Dye was injected around and behind each of four thalli in the flow tank at four speeds from 0.12 to 0.33  $\text{m} \cdot \text{s}^{-1}$ , and the algae were photographed in side view (Fig. 2). The "separation point" was taken as the distance along the axis of the thallus from the stipe to where flow separated from the surface divided by the total length of the thallus. It was always well downstream and did not shift appreciably with changes in speed (S.D. < 0.025 for each individual). The separation point did vary among individuals, from 0.795 to 0.914; and the rank order among the four individuals matched that of the "finesness

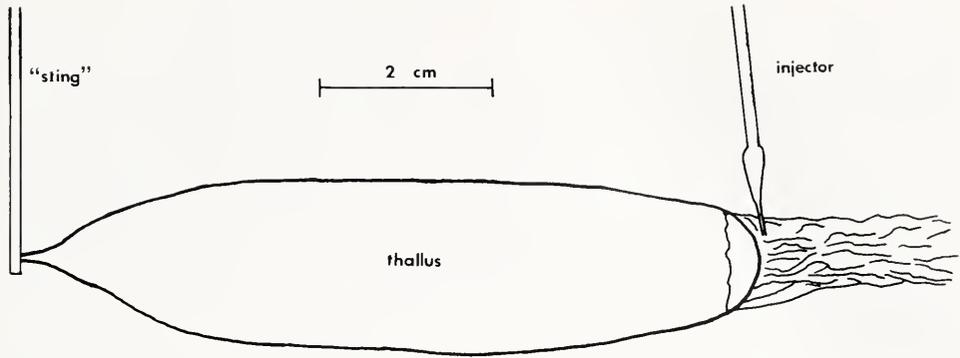


FIGURE 2. Tracing from a photograph taken during injection of dye into the wake of a thallus; dye moves upstream, "peels off" at the separation point, and marks the wake. The sting is idealized. Speed:  $0.33 \text{ m} \cdot \text{s}^{-1}$ ; fineness ratio: 4.09.

ratio" (length, over diameter), the latter ranging from 3.52 to 5.87. In short, separation occurred farther upstream in more rotund specimens.

A variety of measurements of drag were made on thalli of *Halosaccion*, of which the most complete set will be cited here; these were made on a particularly ordinary thallus and seem typical. The thallus had a length of 8.2 cm and a volume (calculated from its mass) of  $8.85 \text{ cm}^3$ . Drag was measured at eleven speeds from  $0.12$  to  $0.71 \text{ m} \cdot \text{s}^{-1}$ , corresponding to Reynolds numbers (based on length) from 7450 to 44,100; data for drag were converted to drag coefficients (equation 2), with volume raised to the two-thirds power used as reference area. No discontinuities in the results were detectable; and, as will be discussed, the figures for drag were impressively low. The equation determined by the linear regression of log-transformed data for drag coefficient as a function of Reynolds number is

$$C_d = 608 \text{ Re}^{-0.86}, \quad r = 0.92 \quad (9)$$

How much force is, in fact, required to detach a thallus? Force to detach was measured for 20 thalli between 8.9 and 11.4 cm in length, all submerged while being pulled upon. Failure uniformly occurred at the stipe rather than the holdfast;  $1.51 \pm 0.48 \text{ N}$  was required. Even the weakest attachment (0.88 N) had a strength about six times the weight of the thallus, so hanging during emersion puts an alga at no hazard of detachment. Nor is the drag measured at the highest speed of the flow tank near values needed for detachment:  $0.0062 \text{ N}$  at  $0.71 \text{ m} \cdot \text{s}^{-1}$  is more than a hundred-fold too low.

#### *Ambient flow and transmural pressures*

In thalli, the concavities resulting from deflation first appear midway between base and apex. The pores, as determined by squeezing in air or, less violently, by observation of fuchsin spots on the castings, are predominantly located in the downstream third. If the pressure difference across the wall is more negative (net outward) near the middle than further downstream, a water current across a thallus could aid reinflation by causing a net inflow through the holes driven by this greater outward pressure on the middle of the thallus. It should be noted that both casual observations and measurements of elastic modulus (unpubl.) indicate that the thallus wall is functionally inextensible at the pressures of present interest.

Results of comparisons of refilling rates in still and in moving ( $0.30 \text{ m} \cdot \text{s}^{-1}$ ) water were statistically equivocal: retrospectively, the speed chosen is likely to have been too low. Given the location of the investigators and the present interest in mechanisms as well as phenomena, recourse was made to the brass model described earlier.

The model was tested in the wind tunnel at speeds corresponding to water flows from  $0.25$  to  $1.0 \text{ m} \cdot \text{s}^{-1}$ . Converting data to pressure coefficients proved to reduce to insignificance variation resulting from the use of several speeds and thus allowed easy averaging of data. Inserting the density of water and the corresponding water velocities into equation (6) permitted calculation of the pressure differences which would obtain in the normal medium. Pooled and averaged data are shown in Figure 3; the graph is similar to one presented for streamlined bodies in Goldstein (1938—Fig. 215) which was obtained at Reynolds numbers nearly two orders of magnitude higher. Differences from published results on streamlined bodies are mainly in data from the downstream portion, near and behind the separation point, as expected for a body distally rounded rather than tapering to a point.

The strongly positive pressures near the upstream (stipe) end are probably of little functional significance since the upstream ends are stiffer, imperforate, and do not deflect observably inward in the flow tank. But the difference between the minimum pressure coefficient and that further downstream is of some interest. A difference of  $0.063$  corresponds to a pressure difference in sea water of  $2.0 \text{ Pa}$  at  $0.25 \text{ m} \cdot \text{s}^{-1}$ ,  $8.0 \text{ Pa}$  at  $0.50 \text{ m} \cdot \text{s}^{-1}$ , and  $32.0 \text{ Pa}$  at  $1.0 \text{ m} \cdot \text{s}^{-1}$ . The latter figure, at least, is not insignificant when compared to the refilling pressure due to wall resiliency of around  $50 \text{ Pa}$  found earlier.

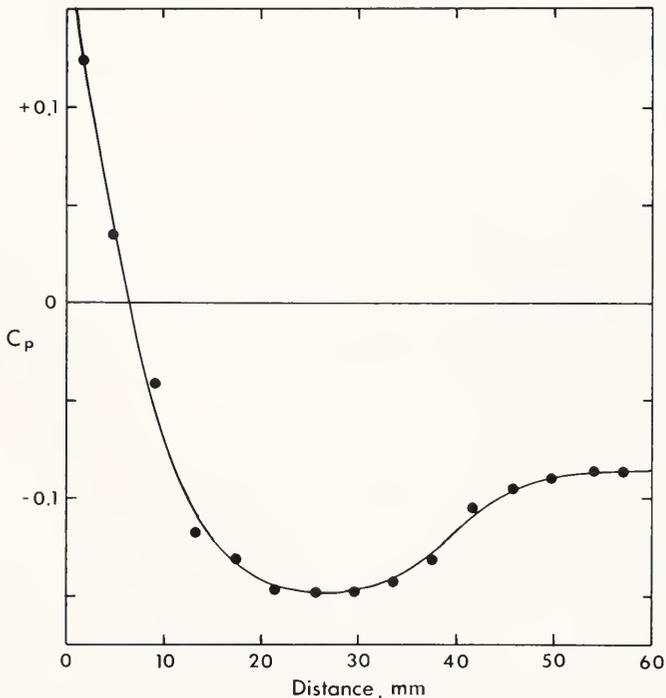


FIGURE 3. Pressure coefficient (equation 6) as a function of distance on the surface of the model thallus (Fig. 1A) from the stipe-thallus junction downstream to the apex, 60 mm in all.

## DISCUSSION

*Shape and drag*

From several viewpoints, a thallus is well-designed to minimize drag. First, in absolute terms drag is low. At  $0.65 \text{ m} \cdot \text{s}^{-1}$  ( $\text{Re} = 40,700$  for the thallus) the drag of the thallus was 5.6 times lower than that of a sphere of the same frontal area (area projecting normal to flow) and 8.7 times lower than that of a sphere of the same overall volume. Indeed it is somewhat awkward to arrange a cylindrical “sting” for mounting a thallus which does not have more drag than the thallus itself. Concomitant with the low drag is the very rearward separation point—an object with a high drag coefficient such as a cylinder normal to flow or a sphere has a separation point at comparable Reynolds numbers about halfway from upstream to downstream extremity. In short, the thalli are impressively well streamlined. Streamlining, of course, works only for a narrow range of orientations of a body to the local flow; here it is facilitated by the flexible stipe and consequent automatic weathervaning.

Moreover, drag does not increase as drastically with increasing flow speed as it does for unstreamlined (bluff) bodies. In the present range of Reynolds numbers (of the order of  $10^4$ ), the drag of a bluff body is proportional to velocity to the power 2.00. For a streamlined body, drag is proportional to velocity to the power 1.50—a less drastic increase. For the present thalli, the exponent is still lower, 1.14; so drag is more nearly proportional to the first than to the second power of flow speed.

It is customary to consider the manner in which the drag coefficient varies with the Reynolds number instead of how drag varies with speed (see Vogel, 1981; 1984). For a given object in a given medium, the drag coefficient is proportional to drag divided by the square of speed and Reynolds number is proportional to speed itself. Thus the exponents relating values of drag coefficient and Reynolds number (as in equation 9) may be obtained by subtracting 2.00 from those above, giving 0.00,  $-0.50$ , and  $-0.86$  respectively.

The functional significance of such low drag is not self-evident. As mentioned, it is far below the force needed to detach a thallus at the speeds considered here. What about higher speeds? The results of extrapolations beyond  $0.71 \text{ m} \cdot \text{s}^{-1}$  depend on the choice of exponent for the relationship between  $C_d$  and  $\text{Re}$ . In the unlikely event that the splendidly low exponent of  $-0.86$  persists at higher speeds, the weakest alga would detach at  $54.8 \text{ m} \cdot \text{s}^{-1}$ , far higher than the maximum speeds ever recorded for water flows in nature. Assuming a streamlined body with an exponent of  $-0.5$  gives detachment at  $19.3 \text{ m} \cdot \text{s}^{-1}$ , comparable to the highest speeds in storms on exposed rocky coasts (Vogel, 1981; Denny, 1982). For a bluff body and an exponent of 0.0, probably an unreasonably conservative assumption, detachment would occur at  $8.5 \text{ m} \cdot \text{s}^{-1}$ , still an heroic velocity. In short, the quality of the streamlining of the *Halosaccion* thallus is far beyond what might be required to resist ordinary hydrodynamic drag.

Two possible explanations of this apparent “overdesign” might be mentioned. (1) The actual drag figures in nature might be higher than those measured here. Thalli frequently bear epiphytes, mainly filamentous algae, which must contribute to their drag. In addition, partially deflated thalli most likely have more drag than fully inflated ones. (2) It is possible that the critical matter is the use of local currents to augment reinflation, as will be discussed below, and that the streamlined shape is, in part, an indirect consequence of design for an appropriate lengthwise pressure distribution.

An additional force tending to detach thalli is the so-called “acceleration reaction,” a force dependent on the acceleration of the fluid relative to a body, the mass of fluid displaced, and a shape-dependent coefficient. For very prolate spheroids, the coefficient is low (Daniel, 1984); and, while water flowing over thalli is constantly changing

speed, the accelerations do not seem to be great. Thus the acceleration reaction is unlikely to be significant in this system.

### *Emptying and refilling*

As anticipated, a set of physical devices, acting in concert, determine the rate at which a thallus empties while it is exposed to air. Two mechanisms promote water loss. *Evaporation* can, as shown here, occur from the thallus wall. It might generate a significant transmural concentration gradient on a sunny, summer day such as that for which DePamphilis (1978) noted that internal water was necessary for survival. *Gravity* will force water out through the pores; it will be most important when a thallus is hanging vertically but will, of course, be largely independent of temperature or illumination. For a thallus 8 cm long with holes mostly about 75% of the way from base to apex, the gravitational pressure will be about 600 Pa.

Another phenomenon, acting at two different points, will oppose water loss. At the pores, *surface tension* will prevent the entry of air through any pore across which the net pressure is inward, that is, where some combination of wall resiliency and other stresses reduce the pressure inside below that outside. Entry of air would, of course, relieve the inward pressure of such mechanical actions and thus permit more rapid egress of water. But the threshold pressure to force air through a pore is greater than any pressure likely to occur naturally. In fact, air-filled thalli are not uncommon during emersion; but they inevitably prove to have been damaged, presumably through the bites of herbivores. Surface tension might also have some effect over the outer surface of a wet thallus in air. Here, though, it is a minor matter, since the radius of curvature is that of the whole thallus and the shape is closer to cylindrical than to spherical. By equation (4b) the inward pressure will only be about 7 Pa.

Refilling, likewise, results from the actions of several physical agencies. Three mechanisms might create an inward movement of water during immersion. *Osmosis* is likely to be minor under most circumstances since the salinity difference across the wall will rarely be very large. However one can imagine occasional cases of occluded pores, substantial rates of evaporation, or gradual increases in salinity through alternating evaporative water loss and bulk sea water replacement in which osmotic refilling might become appreciable. *Wall resilience* appears to be the basic and most consistent mechanism, independent of local flows or the past mechanism of water loss. As determined here, it produces an inward pressure of about 50 Pa, although the variation among individuals is large. Finally there is *flow-induced transmural pressure* resulting from the inverse relationship between speed of flow and pressure along a streamline (Bernoulli's principle). At this point the contribution of the latter is difficult to estimate since it is proportional to the square of flow speed and flow speed is as variable as any parameter of the habitat.

While we believe we have identified the principal mechanisms involved in emptying and refilling, we regard as premature any attempt to draw up a balance sheet reflecting overall pressures, flows, and estimated minimum ratios of immersion to emersion times. Such a balance sheet would, however, permit robust hypotheses concerning the local distribution of the species in the intertidal. Might, for example, more rapid refilling due to faster flows permit survival further above the low water mark?

### *Characteristics of the pores*

Pore morphology will determine the relative importance of different physical processes. Larger pores would allow greater exchange by bulk flow of external sea

water with the water within a thallus when the plant is immersed (and thus more rapid refilling). Small pores will restrict the introduction of air through the pores when the plant is exposed since the pressure difference that an interface can support is inversely related to orifice size (equation 4a). The size and shape of the pores may therefore represent a compromise between keeping air out during emersion and permitting exchange of water during immersion.

Measurements of pressure *versus* flow, of gravity-driven water loss, and of threshold for the entry of air permit calculation of nominal diameters for the pores by equations (3) and (4a) respectively. The results are surprisingly varied. Forcing water through a cannulated thallus leads to an estimate of about 100  $\mu\text{m}$ . The threshold for passage of air implies a diameter of about 300  $\mu\text{m}$ . The rate of water loss for a thallus held vertically in air implies pores of around 20  $\mu\text{m}$ . A ordinarily large pore, according to DePamphilis (1978) is about 100  $\mu\text{m}$  across; our observations agree with his figure. But the pores have a depth greater than their diameter, which will decrease pressure-driven flows; and they are closer to cruciform slits than to circles in cross-section, which will substantially increase the threshold pressure for entry of air as well as decrease pressure-driven flows. In short, pores should have nominal diameters much less than their maximum transverse dimensions, and the figure of 20  $\mu\text{m}$  is probably closer to functional reality than the larger figures. Indeed, by equation (3) a thallus suspended in air with pores of nominal diameter between 100 and 300  $\mu\text{m}$  would deflate with quite unnatural rapidity.

Uncertainties in the measurements, however, are not sufficiently large to account for an order of magnitude overestimate of nominal diameter. A more likely explanation is that the experimental manipulations inadvertently enlarged the pores: even gentle squeezing to expel water, the pressures involved in the cannulations, and those to which the pieces of thallus were subjected functionally enlarged the pores. Only in determining gravitationally driven water loss were the thalli not pressed or squeezed at all. We suggest that in future investigations the absence of visual changes in the pores not be taken as evidence that the pores have not been mechanically altered.

Several phenomena were not encountered. No evidence was found of any mechanical "valving:" curves of pressure *versus* flow were usually linear from the origin in the interesting range of pressures. The resistance of pores to the flow of water was not appreciably different in the two directions. And the threshold for forcing air through pores with water on the other side did not depend on which way the air was forced.

#### ACKNOWLEDGMENTS

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## HEMAGGLUTININ AND HEMOLYSIN LEVELS IN THE COELOMIC FLUID FROM *HOLOTHURIA POLII* (ECHINODERMATA) FOLLOWING SHEEP ERYTHROCYTE INJECTION

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### ABSTRACT

After injection of formalinized sheep erythrocytes into the coelomic cavity of *Holothuria polii* the activity of the naturally occurring hemagglutinins remained constant, while the hemolysin level rose over an eight day period. The kinetics of the response were the same after a further injection, although the hemolytic titers reached higher levels over a longer period. Results obtained using rabbit erythrocytes indicate that this response can be considered a secondary one: higher titers were demonstrated over a 24 h period. Some properties of both naturally occurring and induced hemolysins are discussed.

### INTRODUCTION

Coelomic fluid from the polian vesicles of *Holothuria polii* shows hemagglutinating and hemolytic activity against a variety of erythrocyte types. Studies of anti-rabbit erythrocyte hemagglutinin and hemolysin have shown that they are proteins with differing chemico-physical properties.

Hemagglutinin is relatively heat stable (85°C) and sensitive to low pH and high ionic strength; its activity is independent of divalent cations (Ca<sup>2+</sup> or Mg<sup>2+</sup>) (Parrinello *et al.*, 1976). Hemolysin is a thermolabile molecule which lyses erythrocytes in alkaline medium supplemented with Ca<sup>2+</sup> (Parrinello *et al.*, 1979). Further differences concern molecular weight and subunit organization (Canicattì and Parrinello, 1983).

Since these proteins may be involved in holothurian internal defense, as reported in other invertebrates (McKay *et al.*, 1970; Cooper *et al.*, 1974; Stein *et al.*, 1981, 1982), the possibility of increasing the hemagglutinating and hemolytic activity of the coelomic fluid was investigated by means of erythrocyte injections. Some properties of the hemolysins are also reported.

### MATERIALS AND METHODS

Adult *Holothuria polii* were collected from the Gulf of Trapani and maintained at 15°C in running sea water. The coelomic fluid was obtained by intracoelomic puncture and from the polian vesicles, pooled, and centrifuged at 400 × g for 30 min at 4°C to remove cells. The supernatant was stored at -75°C.

To prevent lysis, sheep erythrocytes were formalinized according to Csizmas's method (1960). After several washings with phosphate buffered saline at pH 7.4 (PBS),

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Abbreviations: SE = sheep erythrocytes; RE = rabbit erythrocytes; HE = human ABO erythrocytes; fSE = formalinized sheep erythrocytes; CF = coelomic fluid from the body cavity; PVCF = coelomic fluid from the polian vesicles; PBS = phosphate buffered saline (0.01 M pH 7.4 phosphate buffer containing 0.15 M NaCl); EDTA = ethylenediaminetetra-acetic acid.

packed formalinized sheep erythrocytes (fSE) were suspended in PBS at a concentration of  $6 \times 10^8$  cells/ml. 0.15 to 0.20 ml of this suspension was injected into the coelomic cavity. Animals injected with 0.15–0.20 ml PBS were used as control. To study the secondary response a second series of injections were given 11 days after the first one.

Hemagglutinin titers and degree of hemolysis were evaluated as previously described (Parrinello *et al.*, 1976, 1979). Injected specimens (5–10) were sacrificed daily, and the coelomic fluids from the body cavity and polian vesicles were separately pooled and tested with sheep (SE), rabbit (RE), and human ABO (HE) erythrocytes. Unless otherwise specified, the coelomic fluid tested was from the body cavity. To absorb the hemolytic activity, packed formalinized RE or SE, washed several times with Tris 0.05 M–NaCl 0.15 M, were added to an equal volume of coelomic fluid previously dialyzed against Tris–NaCl–CaCl<sub>2</sub> 0.02 M. After incubation for 1 h at 37°C followed by 12 h at 4°C the mixture was centrifuged and the supernatant used for hemolytic assays.

The inhibitory effect of carbohydrates was tested by adding coelomic fluid (0.2 ml) to an equal volume of 2-fold serial dilutions of 0.004 M saccharide solution. After incubation for 30 min at 37°C, 0.2 ml of erythrocyte suspension was added. D-galactose, D-glucose, D-fucose, L-fucose, D-xilose, L-xilose, D-glucosamine, D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-mannose, melibiose, cellobiose,  $\alpha$ -lactose, sucrose, and raffinose (Sigma) were used.

The Folin-Ciocalteu method as described by Lowry *et al.* (1970) was used for protein content determination. Bovine serum albumin was the reference standard. Each value was expressed as the mean of three determination  $\pm$  S.E.

Coelomic fluid was fractionated by gel filtration utilizing Bio-gel A5m (Bio-Rad) column (0.9  $\times$  60 cm) equilibrated with PBS. Samples were concentrated by ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, MA) and dialyzed overnight against the starting buffer. The fractions were monitored for UV absorbancy at 280 nm and tested for hemagglutinating and hemolytic activities.

## RESULTS

### *Hemolytic and hemagglutinating activity following primary and secondary sheep erythrocyte injections*

After the injection of the formalinized sheep erythrocytes the anti-SE hemagglutinating activity of the coelomic fluid was the same as that of controls, whereas the hemolysin titer rose, reaching its highest value (1:8), twice that of the controls (1:2), after eight days. The hemolytic activity dropped back near the control level on the tenth day (Fig. 1, CF).

On the first day the undiluted coelomic fluid samples showed a degree of hemolysis (14–43%) lower than that found in the controls (80–90%). This value increased steadily reaching over 80% on the third day. The highest degree of hemolysis (90%) was found on the fourth day (Fig. 2).

To study the secondary response, a further dose of formalinized sheep erythrocytes was injected 11 days after the first one. No differences were found in the kinetics of the response when compared to the primary one, although the hemolytic titers reached higher levels over a longer period (Fig. 1).

In assays of the undiluted samples after the secondary injection, the degree of hemolysis reached the same levels (80–90%) as the controls throughout the period (Fig. 2).

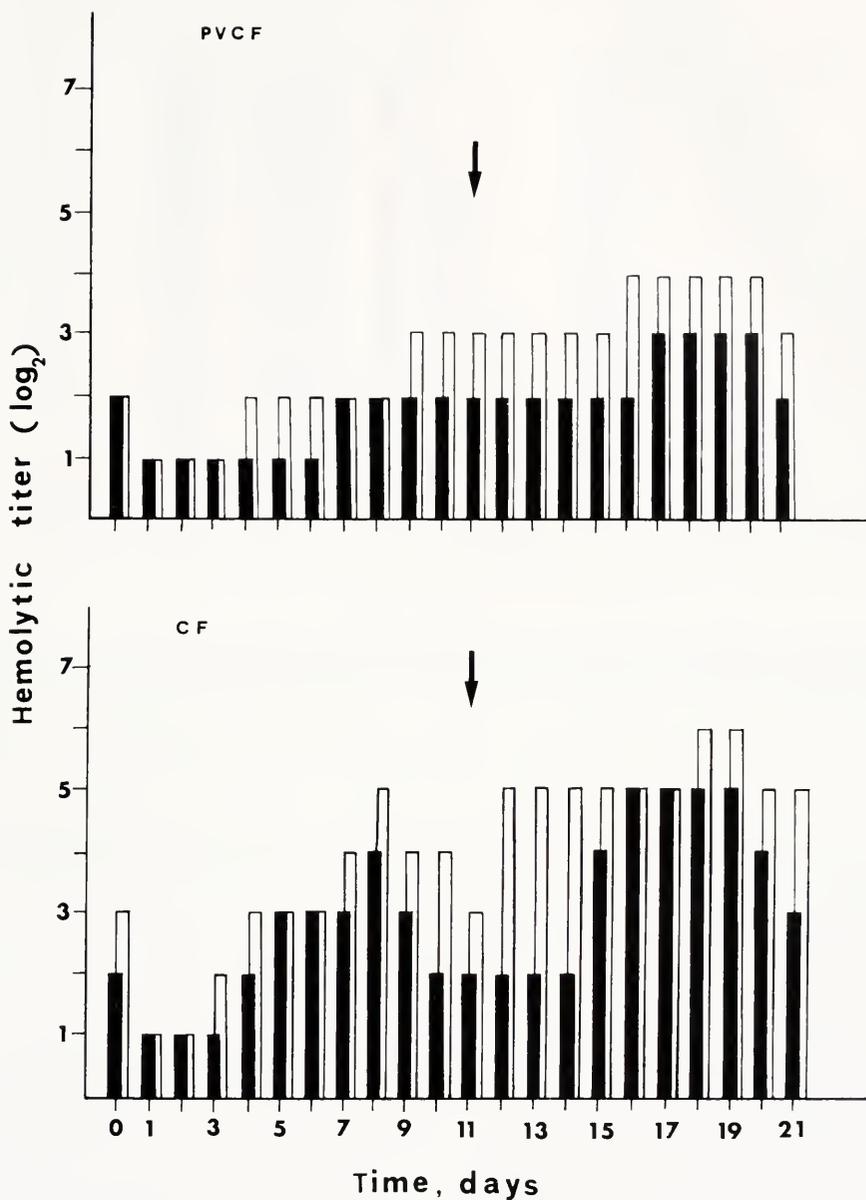


FIGURE 1. Hemolysin levels in the polian vesicle coelomic fluid (PVCF) and body cavity coelomic fluid (CF) of *Holothuria polii* following two injections of 0.15–0.20 ml of  $6 \times 10^8$ /ml formalinized sheep erythrocytes. The diagrams show titers measured with sheep ■ and rabbit □ erythrocytes. The end titer was the reciprocal of the highest dilution revealing hemolysis (at least 10%). The arrows indicate the second injection point.

The hemolysin levels in the coelomic fluid from the polian vesicles were different. Titters increased slightly after the primary and secondary challenges, and in comparison with the coelomic fluid from the body cavity, lower titers were observed over a longer period (Fig. 1, PVCF).

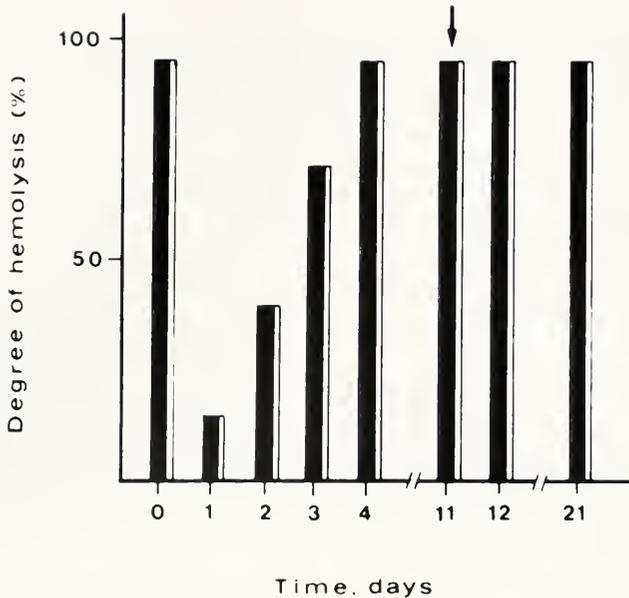


FIGURE 2. Degree of hemolysis of undiluted coelomic fluid following primary and secondary injection of formalinized sheep erythrocytes. ■ = sheep erythrocytes; □ = rabbit erythrocytes. Mean values. S.E. < 1.46, n = 3.

#### *Specificity of the hemolytic response and saccharide inhibition experiments*

Each immune sample was assayed with rabbit erythrocytes. The samples obtained seven days after the primary and secondary injection were also tested with ABO human erythrocytes. Increased hemolytic titers were obtained in both cases.

The anti-RE activity varied in the same way as the anti-SE in the period following the first injection; it increased sharply 24 hours after the second fSE injection (Fig. 1, CF).

The titer of hemolysin against ABO-HE was 1:4 in the control coelomic fluid and increased to 1:16 in the immune samples.

Absorption experiments were carried out by mixing (v/v) coelomic fluid with formalinized sheep or rabbit erythrocyte suspensions. The supernatants obtained by centrifuging the reaction mixtures did not show anti-SE, anti-RE, or anti-HE activity.

In attempts to identify the membrane components which react with hemolysin, inhibition experiments with saccharides were performed. None of the sugars used exerted inhibitory activity on hemolysin of immune or control samples. The same results were obtained when the reaction mixtures containing sugar were tested for hemagglutinating activity.

#### *Gel filtration of the immune coelomic fluid*

The coelomic fluid obtained after the first and second fSE injections were separately pooled and compared with control coelomic fluid by chromatographic separation. The elution patterns were similar although the hemolysin fraction (third peak) from the immune coelomic fluid showed the greatest UV absorbances, corresponding to increased anti-RE hemolytic titers (Fig. 3). Anti-SE activity in the third peak was

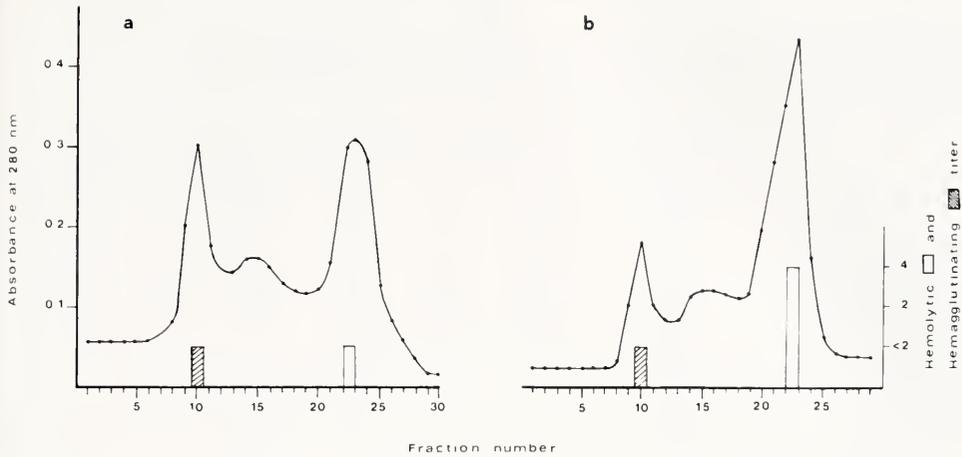


FIGURE 3. Bio-gel A5m elution patterns of *Holothuria polii* coelomic fluid from animals injected with phosphate buffered saline (a) or formalinized sheep erythrocytes (b). Bars indicate the distribution of hemolytic  $\square$  and hemagglutinating  $\text{▨}$  activities against rabbit erythrocytes. The end titer was the reciprocal of the last dilution revealing a clear agglutination, or hemolysis (at least 10%).

found only when the pooled fractions were concentrated by ultrafiltration to a fifth of their volume. The titer was higher (1:8) in the immune than (1:2) in the non-immune coelomic fluid; quantification also showed higher protein content ( $0.314 \pm 0.5$  mg/ml) of the pooled active fractions in comparison with the corresponding peak of the non-immune samples ( $0.110 \pm 0.5$  mg/ml).

#### Temperature treatments

The anti-RE hemolytic activity of the non-immune coelomic fluid disappeared after heating at  $56^{\circ}\text{C}$ , while a residual activity was maintained against SE until  $100^{\circ}\text{C}$ . The same treatments were carried out on samples from specimens immunized with a second fSE injection. As shown in Table I, the heat stable fraction increased after this injection and also reacted with RE.

In the coelomic fluid from polian vesicles the heat stable fraction was evident only when immune-samples were tested; however, a low degree of hemolysis was also found (Table I).

#### Role of divalent cations

While the hemolytic activity of the non-immune coelomic fluid was reduced following EDTA (8 mM, pH 8) dialysis, a residual activity (40%) was found in the immune samples.

#### Characteristics of the hemolytic reaction

Some properties of the immune coelomic fluid obtained following a secondary injection were compared to those of the control samples.

To evaluate the reaction time, fixed amounts of coelomic fluid were incubated with a constant RE suspension ( $6 \times 10^8$  cells/ml) and the degree of hemolysis estimated at different times. The reaction of the naturally occurring and induced hemolysin

TABLE I

Effect of temperature treatments on naturally occurring and induced hemolytic activity of *Holothuria polii* coelomic fluid

Samples	Erythrocytes used for the hemolytic reaction	Degree of hemolysis (%) after temperature treatments <sup>1</sup>					
		37°C	56°C	70°C	80°C	90°C	100°C
CF <sup>2</sup> from control animals	RE	87	16	14	14	14	10
	SE	84	51	46	40	40	40
CF from injected animals <sup>3</sup>	RE	94	55	53	50	42	36
	SE	94	84	84	84	80	68
PVCF <sup>4</sup> from control animals	RE	18	2				
	SE	2					
PVCF from injected animals <sup>3</sup>	RE	75	2				
	SE	25	25	23	ND		

<sup>1</sup> Mean values, S.E. < 1.7, n = 3.

<sup>2</sup> CF = coelomic fluid from the body cavity.

<sup>3</sup> The samples were obtained after a second stimulation with formalized sheep erythrocytes.

<sup>4</sup> PVCF = coelomic fluid from the polian vesicles.

RE = rabbit erythrocytes; SE = sheep erythrocytes.

was very fast; after 5 minutes the degree of hemolysis rose to 60% and its highest value (94%) was reached after 20 minutes.

To determine the amount of the immune coelomic fluid required to lyse a constant number ( $6 \times 10^8$  cells/ml) of RE, sample scalar dilutions were incubated with erythrocytes. The curve presented in Figure 4 shows that from 15 to 80% there is a linear relationship between degree of hemolysis and amount of coelomic fluid while, as the degree of hemolysis approaches 0 or 100%, large increases in coelomic fluid result in smaller increases in the degree of hemolysis. Similar behavior and curve shape were found when HE ( $6 \times 10^8$  cells/ml) were used.

## DISCUSSION

The results show that the hemolytic system of *Holothuria polii*, when examined by hemolysin titration, responded in three phases: an initial decrease, probably due to the involvement of the hemolysins in the clearance of foreign materials; an increase, which could depend on stimulation and differentiation of the producer cells; and a final decrease to the level of the controls. This stimulation of the hemolytic system is also indicated by the gradual increase, after an initial reduction, in the degree of hemolysis in the undiluted samples collected over the whole response period reaching maximum before the titer values increase.

A further injection of the same erythrocytes in the third phase of the primary response produced a similar pattern, so far as the interval between injection and titer increase are concerned, although its magnitude changed. There was no first phase decrease in titers and hemolysin levels, the former subsequently reaching values which were significantly higher than those of both the controls and the primary response.

The induction of the coelomic fluid hemolytic fraction was also shown by the quantification of the third peak in the elution chromatographic pattern of the immune sample.

The hemolytic system of *H. polii* therefore seems to be primed after the first injection, and the response to a further injection can also be considered a secondary



FIGURE 4. Relationship between concentrations of coelomic fluid from animals injected with formalinized sheep erythrocytes, and degree of hemolysis of a constant number ( $6 \times 10^8$  cells/ml) of rabbit erythrocytes. Mean values, S.E. < 2.5,  $n = 3$ .

one on the basis of the results obtained using rabbit erythrocytes as a test system. These red cells are more sensitive to hemolysis by *H. polii* coelomic fluid (Parrinello *et al.*, 1979) and, as shown by absorption studies, their receptors are recognized as cross-reactive by anti-SE hemolysin. Thus the secondary response appears faster than the primary one, and high titers are achieved after 24 hours. We do not know if this is a specific response because the induced hemolysin also reacts with the other erythrocytes used in this study (HE) probably due to its wide specificity range.

The hemolytic reaction is very fast. As indicated by the dose-response curve, several factors could be involved in the hemolytic activity of the coelomic fluid. The sigmoidal shape of the curve could be explained by a model requiring various components acting together to lyse the erythrocyte. The possibility that the multiple binding of a component is required for lysis cannot be excluded.

The coelomic fluid contains heat-sensitive and heat-stable hemolysins, of which the latter lyses SE, and, as suggested by absorption experiments, it is able to link up with the RE surface. The heat-stable hemolysin has been induced by fSE injections; it is also able to lyse RE. As shown by EDTA treatments, the activity of the induced fraction could be independent from divalent cations. Further investigation is needed to clarify the differences between these two components of the *H. polii* hemolytic system, and their relationships with complement factors should be studied.

The starting mechanism of the lytic reaction seems to exclude membrane receptors characterized by sugars, which in this study are used for competitive inhibition experiments.

The production of hemagglutinins in the coelomic fluid of *H. polii* was not stimulated by the formalinized erythrocyte injections. However, the dose and/or formalinization of the erythrocytes could influence the strength of the holothurian response.

#### ACKNOWLEDGMENT

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## HISTOCOMPATIBILITY RESPONSES IN *VERONGIA* SPECIES (DEMOSPONGIAE): IMPLICATIONS OF IMMUNOLOGICAL STUDIES

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### ABSTRACT

The results of immunological analyses are correlated to those of *in situ* grafting experiments in the marine demosponge, *Verongia longissima*. The use of agglutination and cross-absorption techniques substantiate the existence of self recognition and perhaps strain-specificity in this sponge species. This specificity is consistent with previously documented clonal or strain-related patterns identified in grafting experiments.

### INTRODUCTION

Since the illustrious studies of Wilson (1907) on the phenomenon of species specific aggregation in the lower Metazoa, interest in sponges has focused on cell reaggregation (Galstoff, 1925; Curtis, 1962; MacLennan, 1974). Van de Vyver (1970) was the first to record intraspecific incompatibility in the Porifera. She concluded that local populations of the freshwater sponge, *Ephydatia fluviatilis*, and the marine sponge, *Crambe crambe*, consist of a number of strain types. Each strain was defined by tissue contact incompatibility with other members of the same species in contact zones, creating a discrete border or zone of non-coalescence separating the allogeneic individuals while members of the same strain fused compatibly.

Until the 1970's the possession of an immune system was considered to be a vertebrate trait; invertebrate defense systems were considered to rely on phagocytosis and show only a crude specificity (Manning and Turner, 1976). More recently, tissue transplantation studies on the Porifera have focused on the immunorecognition and adaptive immune responses present at this lower phylogenetic level (Evans *et al.*, 1980; Hildemann *et al.*, 1980; Kaye and Ortiz, 1981; Bigger *et al.*, 1982; Curtis *et al.*, 1982; Buscema and Van de Vyver, 1983, 1984; Johnston and Hildemann, 1983; Van de Vyver, 1983; Van de Vyver and Barbieux, 1983; Neigel and Schmahl, 1984). Variability in the results of these tissue grafting studies and the diversity of histoincompatibility behavior they have demonstrated, indicate the present impracticality of formulating predictions concerning immune reactions in still uninvestigated sponge species.

The existence of strain-specificity in the marine sponge, *Verongia longissima*, has been suggested (Kaye and Ortiz, 1981) although clonal relationships for this population (Neigel and Avise, 1983) cannot be discounted. The present study investigates the possibility of employing immunological techniques such as agglutination, cross-absorption, and immunofluorescence in substantiation of apparent allogeneic incompatibility in this sponge.

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## MATERIALS AND METHODS

All field work and some preliminary experiments were conducted at the Bellairs Research Institute of McGill University, Barbados. Further experiments and final analyses were performed at McGill University, Montréal.

The marine demosponge, *Verongia* (= *Aplysina*) *longissima* (Carter, 1882), was the primary species studied in this investigation, and to a lesser extent the closely related species, *V. cauliformis* (Carter, 1882). *V. fistularis* (Pallas, 1766) was also observed and used in several of the experiments.

The collection area selected for this study was the same site used for previous grafting experiments (Kaye and Ortiz, 1981). Eighteen tissue samples were collected for immunological analyses. Branches of test specimens were cut from the donor and transferred to plastic bags underwater and immediately returned to the laboratory.

*Preparation of antigens*

Specimens were dissociated according to the procedure of Humphreys *et al.*, 1960. Cell counts of suspensions were taken with a Neubauer haemocytometer. Merthiolate was added to each suspension to retard bacterial growth. Six of the suspensions were divided into 0.1 ml aliquots for immunization, and 10 ml aliquots for agglutination and cross-absorption tests. The remaining 12 suspensions were used only for the tests.

*Immunization*

Six young adult rabbits (4 to 6 kg) were injected subcutaneously with 0.1 ml of the antigen emulsified with 0.2 ml of Freund's complete adjuvant on days 1, 3, 5, 7, 9, 17, 19, 21, and 23. Blood (100 ml) was collected via cardiac puncture on day 31. Sera were frozen in 5 ml aliquots for use in the agglutination and cross-absorption tests.

*Agglutination tests*

Reaction wells of microtitre plates contained 25 $\lambda$  of diluted antisera (serially diluted by doubling dilutions of calcium-magnesium-free sea water (CMF-SW) and antiserum) and 25 $\lambda$  of diluted antigen (1:1 with CMF-SW). Control wells contained only 25 $\lambda$  of diluted antigen and 25 $\lambda$  of CMF-SW. Plates were incubated at room temperature for 8 hours and then macroscopic and microscopic observations were recorded.

*Cross-absorption tests*

These tests were performed on two of the antisera to confirm the agglutination tests. Varying cell numbers of each of several antigens were spun down at 7000 rpm. The pellets were washed once in CMF-SW, resuspended, and spun down again. The pellets were then mixed with 0.3 ml of the antiserum, incubated at room temperature for 1 hour, and spun down. Agglutination tests were performed as before testing the supernatant (absorbed antiserum) against its homologous antigen. Immunofluorescence tests were confounded by autofluorescence of the sponge cells.

## RESULTS

*Agglutination tests*

A positive reaction (agglutination) was scored if the wells containing antiserum and antigen were cloudy and showed no pellet formation such as occurred in control

wells containing antigen only. For microscopic analyses a positive reaction was scored if sponge cells had formed aggregates which had not been disturbed during gentle mixing, and were not observed in the generally even distribution of cells in the controls.

Table I presents the results of quantitative analyses to determine agglutinating antibody content of the antisera. The antisera titres represent the highest dilution of antisera that showed agglutination. These results indicate that antisera 2 and 12 reacted with antigens 2, 11, and 12 (high titre values in antisera columns). These two antisera are therefore likely to have antibodies of similar specificity as are antisera 71 and 73 which both reacted with antigens 71, 73, 75, and 23. Antiserum 31 reacted with antigens 31, 33, and 35; and antiserum 89 reacted with only its homologous antigen, 89.

Previous work on grafting (Kaye and Ortiz, 1981) found that sponges 2, 11, and 12 were tissue-compatible and thus of the same strain of *V. longissima*; sponges 71, 73, 75, and 23 were all of the same strain of *V. longissima*; sponges 31, 33, and 35 were of the same strain, all members of *V. cauliformis*; and sponge 89 was the only sponge of strain type 15 of *V. longissima*. Thus agglutination results conform to the pattern of recognition derived from grafting studies, supporting the concept of specificity at population, strain, or individual genotype levels.

TABLE I

Comparison of agglutination results in three species of *Verongia*

Antigen	Antigen cell counts ( $\times 10^6/\text{ml}$ )	Antisera titers					
		<i>V. longissima</i>					<i>V. cauliformis</i> 31
		2	12	71	73	89	
<i>V. longissima</i>							
2	259.6	32	32	1	1	1	1
11	253.4	32	32	1	1	1	1
12	203.2	32	32	1	1	1	1
23	147.4	2	2	32	32	1	1
71	160.0	2	2	32	32	1	1
73	177.8	2	2	32	32	1	1
75	162.4	2	2	32	32	1	1
89	210.6	1	1	0	0	64	0
19	164.4	1	1	1	1	0	1
20	168.2	2	2	0	0	0	2
44	161.2	1	1	1	1	1	1
67	149.8	1	1	0	0	0	0
77	213.2	0	0	0	0	0	0
87	215.4	2	2	2	2	2	2
<i>V. cauliformis</i>							
31	194.4	1	1	0	0	0	32
33	177.0	1	1	0	0	0	32
35	190.8	1	1	0	0	0	32
<i>V. fistularis</i>							
TA	281.8	0	0	0	0	0	1

### Cross-absorption tests

These tests were performed on antisera 71 and 12, and the results are represented in Figures 1A and 1B. These tests involved absorbing-out an antiserum by incubating various cell numbers of an antigen with the antiserum. When the antigen cell concentrations were plotted against the antiserum titres an absorption curve was obtained for the homologous antigen (Fig. 1A, #71; and Fig. 1B, #12). These curves were used as controls to compare the efficiency of the other antigens to absorb the antibodies.

The results of the cross-absorption tests for sponge antiserum 71 (Fig. 1A) dem-

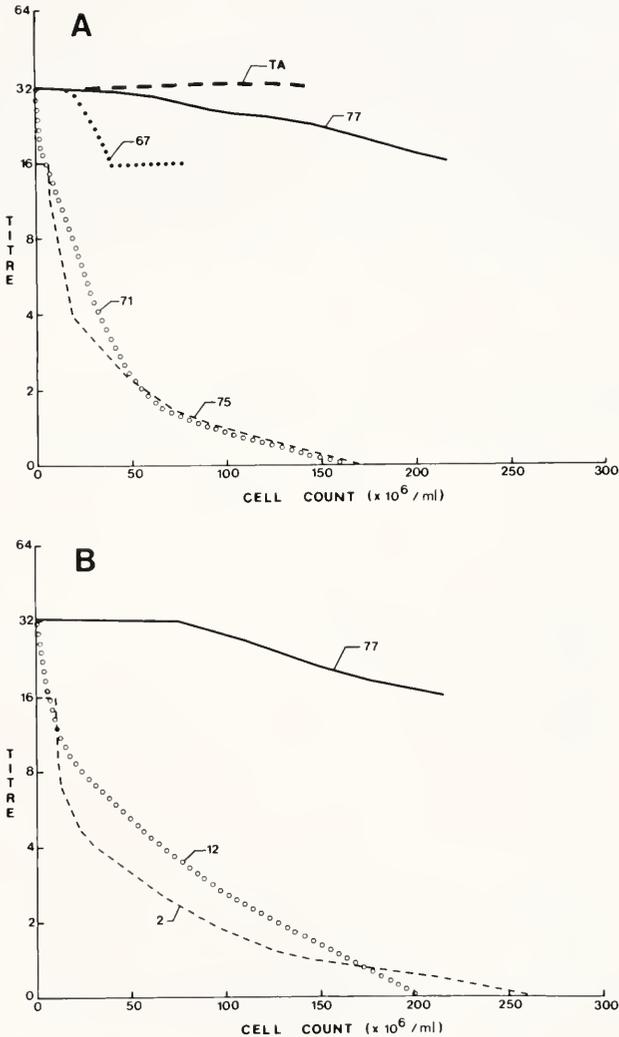


FIGURE 1. A. Results of agglutination test after absorption of  $\alpha$  71 by various sponge cell suspensions. The reciprocal of the dilution (TITRE axis) and concentration of sponge cell suspension (CELL COUNT axis) causing an agglutination reaction to occur are shown. Specimen TA is *Verongia fistularis*, 67 and 77 are *V. longissima*, and 71 and 75 are the same strain (type 3) of *V. longissima*.

B. Results of agglutination test after absorption of  $\alpha$  12 by various sponge cell suspensions. The reciprocal of the dilution (TITRE axis) and concentration of sponge cell suspension (CELL COUNT axis) causing an agglutination reaction to occur are shown. Specimen 77 is *V. longissima*, and 2 and 12 are the same strain (type 2) of *V. longissima*.

onstrate that sponges 71 and 75 are similar and for sponge antiserum 12 (Fig. 1B) demonstrate that sponges 12 and 2 are also similar.

## DISCUSSION

Grafting experiments have provided evidence for the occurrence of strain-specificity in some sponge populations (Van de Vyver, 1970; Curtis, 1979; Evans *et al.*, 1980; Kaye and Ortiz, 1981). The allogeneic incompatibility demonstrated in these experiments is the expression of defense mechanisms that ultimately preserve the genetic integrity of the individual.

In the present study the results of the agglutination and cross-absorption tests exhibit antigenic specificities and these specificities are directly related to strain designations based upon earlier grafting experiments (Kaye and Ortiz, 1981), although clonal identity by fragmentation cannot be ruled out (Neigel and Avise, 1983). The low agglutination titres often observed with heterologous antigens from different sponge strains could be explained by: (1) the sponge cells showing antigenic cross-reactions, (2) the cells having surface components common for all strains but each strain having different levels of each of these components, and (3) the antiserum having antibodies against surface components for species and some for strain.

The agglutination test is somewhat insensitive, and a more detailed analysis is required to elucidate the type of recognition that is occurring at the low agglutination titres using other immunological techniques. Therefore, the cross-absorption test was employed. This test demonstrates the ability of same strain antigens to completely absorb the antiserum, same species antigens to absorb very little antiserum, and different species antigens to absorb none of the antiserum. It is apparent from these cross-absorption tests that the antigens have qualitative differences. In other words, the type of recognition that is occurring at the low agglutination titres is more likely to be due to a species recognition and not the results of cross reactivity or quantitative differences in the surface components of the sponge cells. However, the fact that a small amount of antibody was absorbed by heterologous sponge cells may indicate that some antibodies were formed against components common to the species. Nevertheless, it appears that surface components on the cells of *V. longissima* responsible for strain recognition in this sponge play the dominant role in eliciting antibody formation in rabbits.

In light of reaggregation and grafting studies that have demonstrated species and presumed strain-specific recognition in sponges, it seems that allogeneic effects may have been missed by other workers in reaggregation studies (Spiegel, 1954; MacLennan and Dodd, 1967) because factors were isolated from bulk sponge material, possibly derived from multiple allogeneic individuals. This problem may require the reinterpretation of these results, and should certainly be considered in future studies.

The limited supply of antigens and antisera restricted the extent to which the agglutination and cross-absorption tests could be carried out. To allow for immunological results to be employed as conclusive evidence regarding histocompatibility among specimens of *V. longissima* the tests should be carried out for all the sponges employed in the grafting experiments (Kaye and Ortiz, 1981). However, sufficient data has been presented to demonstrate the possibilities of employing immunological methods to show the occurrence of compatibility between individual specimens of *V. longissima*, whether of strain type or clonal basis. Once the results of these techniques are extensively compared to grafting results, and if the results consistently agree, these immunological methods can be employed to gain conclusive evidence regarding strain specificity in other sponges.

This study offers another technique that could be extended to future research in this area which might provide insight into the mechanisms involved in histocom-

patibility reactions occurring in allogeneic rejections, and in the exquisite immune system that these lower invertebrates possess.

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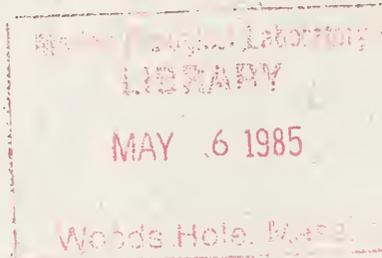
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## ERRATUM

THE BIOLOGICAL BULLETIN, Volume 167, Number 3, Page 663

The following correction should be made in the paper by R. Vitturi *et al.* entitled, Chromosome polymorphism in *Gobius paganellus*, Linneo 1758 (Pisces, Gobiidae) (1984, *Biol. Bull.*, **167**: 658–668): the entry found two lines up from the bottom (specimen 25) in the final column of Table III should read 1.37 instead of 0.37.



## SELECTED ASPECTS OF LENS DIFFERENTIATION

SEYMOUR ZIGMAN

*Department of Ophthalmology (Box 314) and Department of Biochemistry, University of Rochester  
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### ABSTRACT

Recent reviews and papers regarding lens differentiation have been considered in this brief review intended for a general biological readership. Structural and biochemical bases for the initial formation and the continuing growth of the lens are discussed as embryological and maturational processes. Cell division, conversion of epithelial to fiber cells, elongation and invagination, and positional differences in individual lenses are discussed. Emphasis on protein aggregation and chemical changes in fiber cell membranes leads to the conclusion that cataracts not due specifically to toxic, environmental, or genetic factors are merely the result of terminal lens differentiation in which the lens nucleus scatters light excessively.

### INTRODUCTION

The ocular lens has often been used as a model in studies of cell differentiation, and many articles and reviews have been published on this subject. It is not the purpose of this review to summarize all of the previous literature, but it refers to recent selected papers. The material presented is that portion of the information with which the author is most familiar. Since lens cells continue to differentiate throughout the life of the individual, the following major differentiation processes are going on throughout life.

Lens epithelial cells grow and divide, and become fiber cells as they invaginate and elongate. Older fiber cells are relocated centrally due to the encapsulated nature of the lens. Post-synthetic modification of the lens proteins (*i.e.*, crystallins) leads to aggregation and to altered fiber cell membranes especially toward the oldest central portion of the lens, a collection of events that eventually leads to light-scattering in the lens nucleus.

Ocular lens biochemistry and structure have been remarkably preserved throughout the vertebrate phylum. The lens usually is of a spherical or spheroid shape, sometimes having a more flattened anterior surface. Teleosts, elasmobranchs, and many rodents have nearly spherical lenses, while those of humans, cows, rabbits, and squirrels are spheroids with anterior surface flattened. Generally, spheroid lenses accommodate passively by being stretched thin (for far vision) or relaxed thick (for near vision). However, the non-elastic spherical fish lenses (*i.e.*, teleost and elasmobranch) must also perform the refractive function of the cornea, since the refractive index of sea water is nearly the same as that of the cornea. Lenses of sharks thus may be moved forward and back to allow for accommodation (Gilbert, 1984).

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Reprint requests: Dr. Seymour Zigman, Ophthalmic Biochemistry Laboratory (Box 314), University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642.

The lens develops very early in embryogenesis from ectodermal anlagen. In most animals, the lens is fully developed at birth, and it grows throughout life, although the rate of growth plateaus in late adulthood. There is a progression of cellular changes that converts the epithelial cells from a monolayer over the anterior aspect of the lens (just beneath the connective tissue capsule) into elongated cortical fiber cells that are relocated internally. Further changes that accompany internalization are a gradual loss both of fiber cell nuclei and other subcellular particles (*i.e.*, mitochondria and ribosomes). Cortical fiber cells are then relocated into the lens interior (*i.e.*, the lens nucleus). As this process continues, much of the cytoplasm is lost, and the fiber cells become a concentrated collection of concentric cell membranes in the nucleus with much aggregated (formerly soluble) protein associated by both covalent and noncovalent bonds.

As a result of the process outlined above, the nuclear region of the lens becomes a depository for the oldest and most differentiated cells. An increase in aggregated proteins and relative losses of cytoplasm and decrease in water content (*i.e.*, dehydration) contribute to the enhancement of light-scattering in the nucleus, and the ability to maintain transparency there is diminished. This is clearly observed by slit-lamp examination of the lens in most species. Figure 1 illustrates such nuclear light-scattering in human and elasmobranch lenses.

Lens differentiation can be considered in two different phases: embryological and maturational. Relatively speaking, embryological lens differentiation takes a very short time to be completed (*i.e.*, several months). This phase of differentiation establishes the tissue as having outer anterior epithelial cells and interior elongated fiber cells. The maturational process continues for the life of the individual (*i.e.*, as long as a century in man), and it merely adds new layers of fiber cells over the old. Figures 2a and b illustrate embryological development both schematically and with a chick eye photomicrograph. Figure 3 schematically illustrates maturational differentiation.

#### EMBRYOLOGICAL DIFFERENTIATION

Embryogenesis is the first stage of lens differentiation. Ectodermal cells become epithelial cells at the lens placode stage. It is well-known that a number of factors stimulate lens induction at this time, but the number and types have not been totally elucidated. Agreement has been reached among researchers (Simonneau *et al.*, 1983) that the neural retina provides growth factors that stimulate further lens development from this point on. Recently, *in vitro* studies have shown that these neural factors, substances in serum, and specific hormonal factors can stimulate lens epithelial cell division in cultures and that differentiation into fiber cells and even lentoid bodies occurs readily (Harding *et al.*, 1971).

The second stage of embryological differentiation is elongation. Neural factors stimulate ectodermal cell division and lead to invagination and elongation of these epithelial cells into primary lens fiber cells of the cortex (Arruti and Courtois, 1978). In embryogenesis, lens ectodermal cells elongate so as to form a vesicle which separates the posterior of the tissue from the retina. Ectodermal cells convert anteriorly into an epithelium under the connective tissue capsule. A solid tissue forms as the vesicle is filled with internalized fiber cells that have increased in number due to epithelial cell division, migration, elongation, and invagination. The process of epithelial cell division at the equatorial or bow region of the lens,

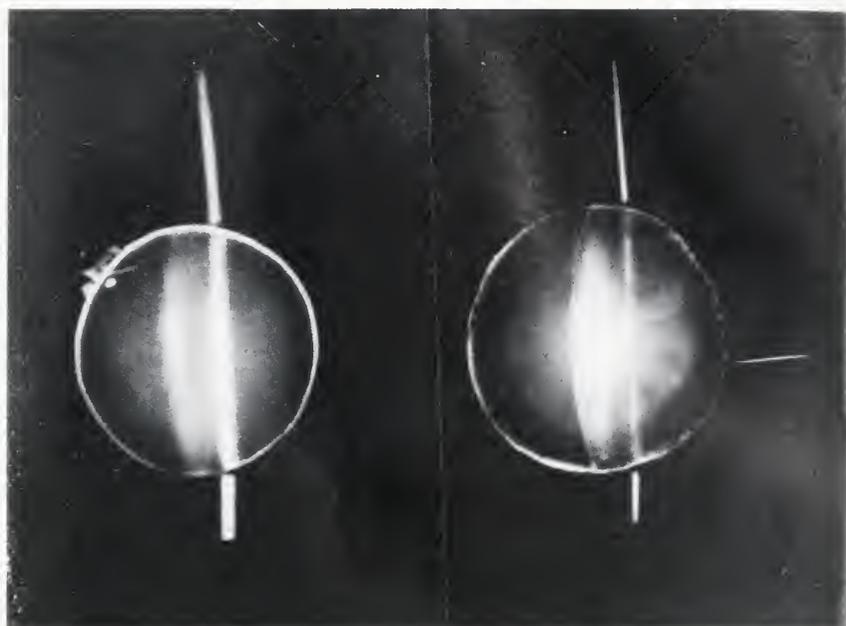
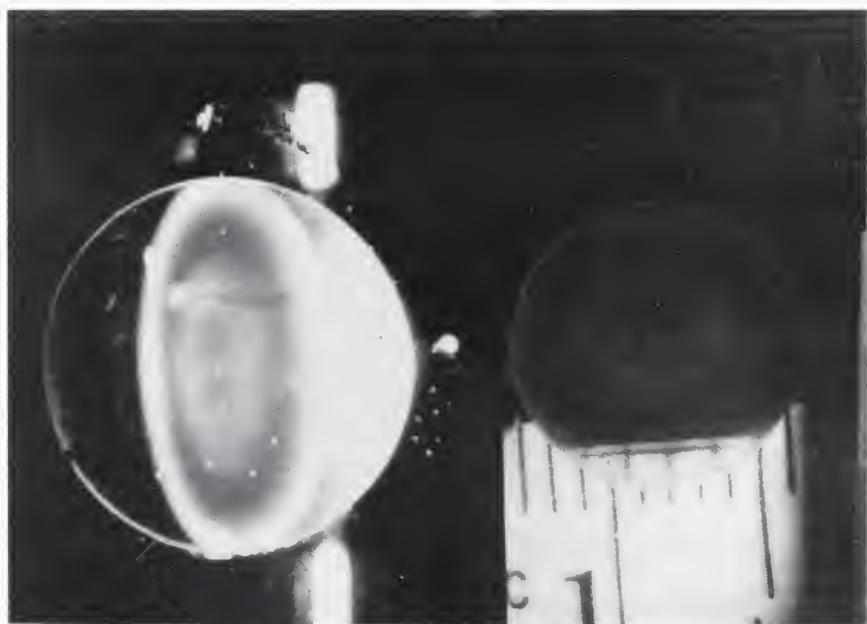
**A****B****C****D**

FIGURE 1. Photographs of human and elasmobranch lenses made with slit lamp illumination (A, B, C) so as to exaggerate light-scattering areas. A. Human normal lens, 55-yr-old; B. Nuclear cataractous lens; C. Adult smooth dogfish lens; D. Adult skate lens (anterior view photograph).

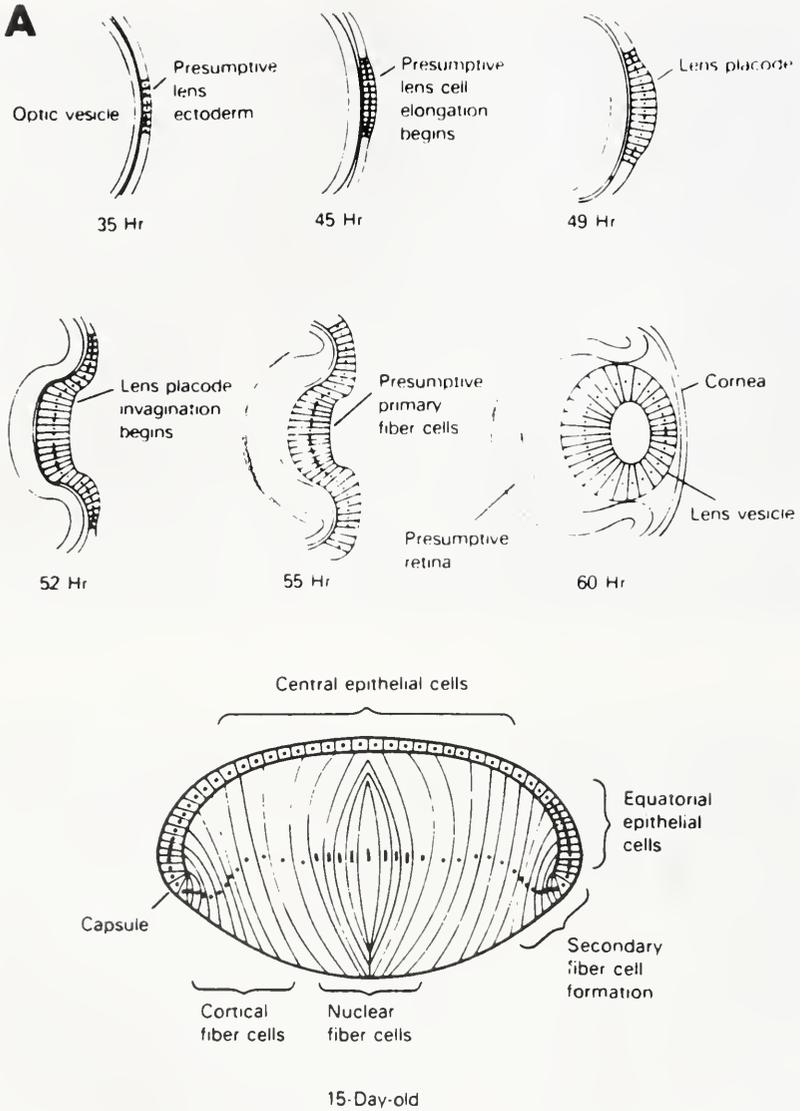


FIGURE 2. A. Embryogenesis of the chick lens from 35 h to 15 days of age. B. Photomicrograph of a cross section of a 4-day-old chick eye that illustrates the differentiation process in the lens (after Piatigorsky, 1981).

invagination, and elongation continues throughout the life of the individual, but at a constantly declining rate.

Chemical factors that are present in neural retina extracts stimulate elongation of lens epithelial cells and crystallin synthesis in rats (Simonneau *et al.*, 1983). Lentropin, a 60,000 dalton heat-labile glycoprotein from the vitreous humor, also stimulates delayed crystallin synthesis in embryonic chick lens epithelium (Piatigorsky, 1981).



FIGURE 2. (Continued)

#### MOLECULAR EVENTS

Molecular events in differentiation lead finally to the accumulation of crystallins with the distribution found in the mature lens. While lens maturation in adult

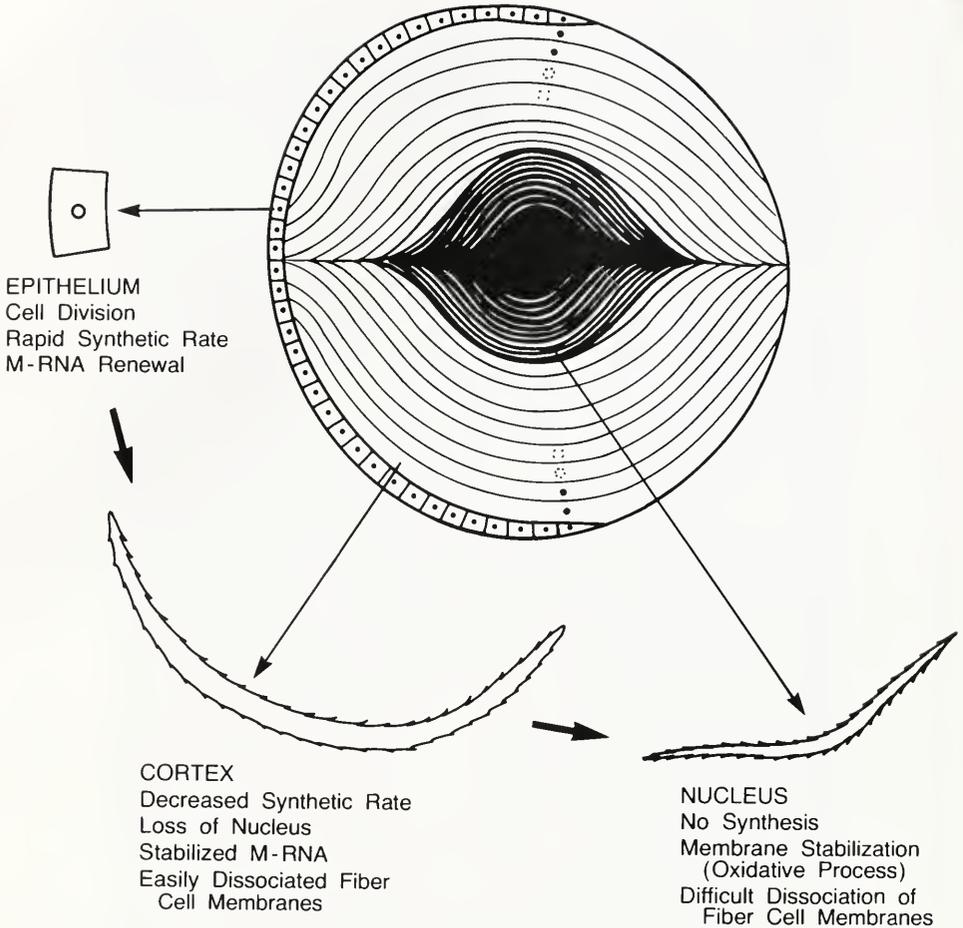


FIGURE 3. Illustration of the maturational differentiation of a schematic lens. Origin of the cells form and growth characteristics of the three major forms of lens cells.

animals results in a distribution of crystallins that will be detailed later in this review, nucleic acid modification also takes place to control this distribution.

Papaconstantinou (1967) has provided a summary of these events that is shown in Figure 4. A controversial concept is that mRNA is stabilized during conversion of epithelial into fiber cells. This conclusion was based upon the use of actinomycin D to prevent mRNA synthesis. However, more recently (Piatigorsky, 1981) has shown that actinomycin D is cytotoxic to adult chicken lens epithelial cells, so that their mRNA instability could be due to toxicity of another sort than mRNA synthesis inhibition. How mRNA stabilization occurs is not known, but hypotheses have been proposed that a ribonuclease inhibitor in the lens is involved (Ortwerth and Byrnes, 1971; Delcour and Piessens, 1980). Another point is that mRNA state is related to polyribosomal conformation.

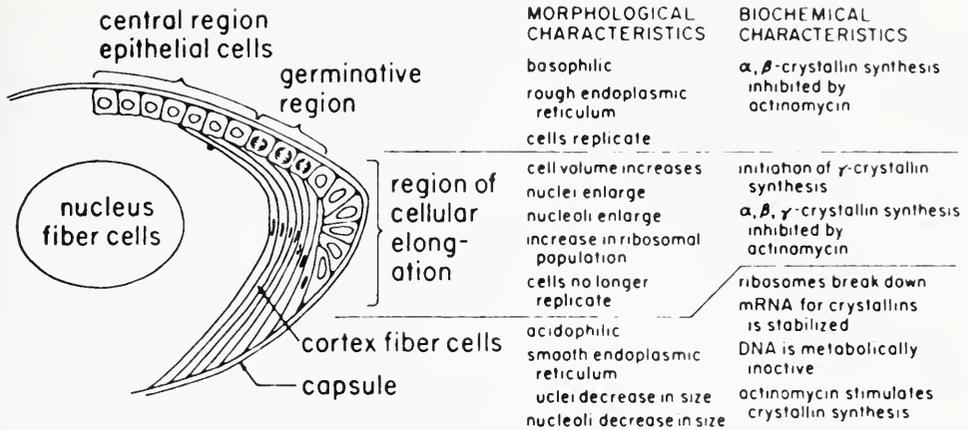


FIGURE 4. Morphology and biochemistry of lens differentiation (after Papaconstantinou, 1967).

One interesting feature of lens differentiation is the loss of cellular organelles and DNA with aging, as defined by position within the lens. As epithelial cells become cortical fiber cells and then nuclear fiber cells, the cell nuclei become pyknotic and the DNA is totally depleted. The cell nucleus first rounds up and then fragments prior to DNA degradation. DNA is thought to become degraded initially by single strand breaks and then is cut into smaller units as determined by alkaline sucrose density gradient studies. Inability to repair DNA may be the manner in which DNA is degraded, but reduction of DNA polymerase does not seem to occur. It is not known how the other organelles are broken down as fiber cells become aged by virtue of their more central positioning in the lens (Modak and Perdue, 1970).

Regions of the lens synthesize RNAs at different rates during the differentiation process. In chick lens, RNA synthesis is most rapid at the equator of the lens where epithelial cells are converted into fiber cells (Modak and Persons, 1971).

As genetic defects are expressed during embryogenesis, they strongly influence the normalcy of the differentiated lens product. These defects eventually lead to disturbances in the transparency of the lens, and results in lenses useless for vision. Many animal models of genetic lens defects based upon specific biochemical lesions have been described. An example is the Nakano mouse model, in which an excess of  $\text{Na}^+/\text{K}^+$  ATPase inhibitor causes opacity due to osmotic imbalances and swelling (Kinoshita *et al.*, 1974). Another is the galactosemia of infants, in which the enzyme U.D.P.G. galactosyl-transferase is lacking and sugar alcohol accumulation leads to osmotic cataract (Chylack, 1981). Other genetic defects that interfere with lens differentiation have been discovered in chickens and mice (Piatigorsky, 1981).

#### MATURATIONAL DIFFERENTIATION

Two major biochemical processes are the most important events in the maturational differentiation of the lens. The first is the change in the synthesis and accumulation of the lens crystallins, and the second is protein aggregation either as a separate process or one that involves the association of the protein with the membranes of the fiber cells.

Figures 5 through 8 illustrate some of the well-known features of the three major lens crystallins. Figure 5 demonstrates the sedimentation rate of an aqueous extract of a dogfish lens. Figure 6 shows both the undenatured (shark) and denatured (human) polyacrylamide gel electrophoretic profiles of aqueous buffer homogenate supernatants, and Figures 7 and 8 show the Sephadex G200 resolvable proteins of the bovine lens, and their profiles by electrophoresis. Three similar structural soluble protein classes are found in the lenses of nearly all vertebrates, with the exception of the birds and the reptiles. These have been termed alpha ( $\alpha$ ), beta ( $\beta$ ) and gamma ( $\gamma$ ) crystallins, but in birds and reptiles,  $\gamma$ -crystallins are lacking and another protein named delta ( $\delta$ ) crystallin is present.  $\alpha$ -crystallin is the largest of the crystallins, with molecular weights ranging from 700,000 to 900,000 daltons, but they are composed of multiples of subunits in the 20,000 to 23,000 dalton range. This protein is composed of two acidic and two basic types of subunits.  $\beta$ -crystallins have heterogeneous sizes ranging from 30,000 to 250,000 daltons. The  $\gamma$ -crystallins are a group of single-chain multi-isomeric proteins with a molecular weight of approximately 20,000 daltons, and they differ greatly in isoelectric points and thus in their electrophoretic mobilities (*i.e.*,  $\alpha$  is the most electronegative and  $\gamma$  is the least). Due to a high tryptophan content,  $\gamma$ -crystallins are the most UV-absorbing and fluorescent lens proteins, and they exhibit the greatest molar absorptivity. They are also the most reactive since they contain the greatest -SH content of all the crystallins. Delta ( $\delta$ ) crystallins of birds and reptiles are distinctly (immunologically) different from mammalian crystallins and they are composed of four subunits of approximately 50,000 daltons each, which all differ from each other immunologically, in isoelectric points, and in amino acid compositions (Bloemendal, 1981). When the various crystallins are denatured in SDS, the bands in Figure 7 are observed by polyacrylamide gel electrophoresis.

In the several stages of differentiation, questions arise about the order of appearance and the location in the lens of these crystallins. The distribution of the crystallins varies in different species. For example,  $\alpha$ -crystallins appear anteriorly first in rats and mice in the central anterior lens at the stage of ectodermal invagination, while  $\beta$ - and  $\gamma$ -crystallin appearance follows. In newts,  $\beta$ -crystallins appears first, when the primary fiber cells begin to form the lens vesicle;  $\gamma$ - and  $\alpha$ -crystallins appear subsequently. However, in the chicken (Delta) crystallin is the first to appear at the lens placode stage, and is followed by the appearance of  $\beta$ - and then  $\alpha$ -crystallin. There appears to be a relationship between the formation of the crystallins and the process of cell division, in that while  $\alpha$ -crystallin is synthesized in all metabolically active lens cell,  $\beta$ - and  $\gamma$ -crystallins are not readily synthesized in lens cells that are actively dividing (Piatigorsky, 1981). But even though the lens epithelium contains dividing cells, small amounts of  $\beta$ - and  $\gamma$ -crystallins are still found to be present (McAvoy, 1981).

Generally speaking, there is a species difference in the relative levels of the three crystallins. While in bovine and human lenses  $\alpha$ -crystallins are present at the highest levels, amphibians and elamobranh lenses contain mainly  $\gamma$ -crystallin. In terms of distribution within the lens, it appears that  $\alpha$ -crystallins predominate in the epithelium and outer cortex whereas  $\gamma$ -crystallins predominate in the inner nucleus (McAvoy, 1981).

#### LENS MEMBRANES

Due to lens growth processes and the conservative nature of the fiber cells, the nucleus grows larger with increasing age, as shown in the dogfish (Fig. 9). Changes



FIGURE 5. Analytical ultracentrifugation (left to right) of the total water-soluble extract of a dogfish lens illustrating the three lens crystallins  $\alpha$ - (the heaviest and furthest migrating),  $\beta$ - (intermediate in molecular weight), and  $\gamma$ - (the lightest and slowest migrating). Note also the fringes (to the left) representing high molecular weight colloidal protein aggregates.

in the thickness and in the interdigitating knob structures of the fiber cells of the skate are shown in Figure 10.

Lens nuclei contain some of the longest-lived cell membranes in biological systems. Two views of the structure and extrinsic protein binding to lens fiber cell

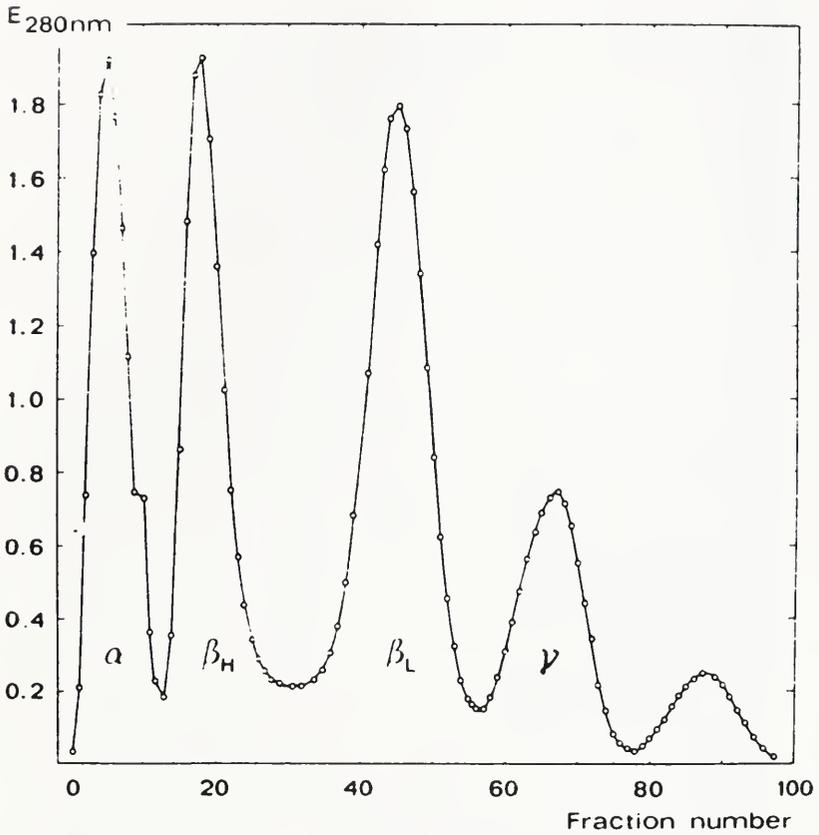


FIGURE 6. Separation of the bovine lens crystallins using Sephadex G 200 (after Bloemendal, 1981). H = heavy, L = light.



FIGURE 7. Polyacrylamide gel electrophoresis of lens water-soluble extracts. A. Tris glycine buffer solvent; shark lens extract. B. SDS-phosphate buffer solvent; human lens extract.

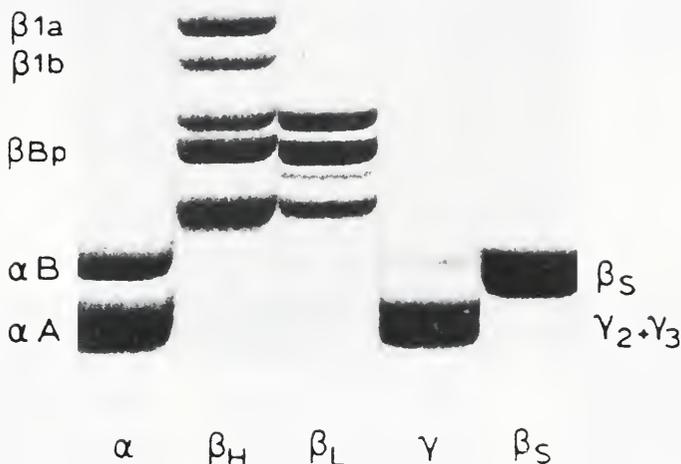


FIGURE 8. SDS polyacrylamide gel electrophoresis of Sephadex G 200 separated bovine lens crystallin subunits (after Bloemendal, 1981).  $\beta_5$  is a low molecular weight beta (27,000 daltons) that resembles gamma components 2 and 3.

membranes are illustrated in Figure 11 (Spector *et al.*, 1979; Broekhuysse, 1981). Not only do the cytoplasmic elements differentiate as lens epithelial cells become fiber cells, but the cell membranes also change substantially in this way. It is clear that with the loss of cytoplasm and organelles as fiber cells are relocated toward the lens core, the fiber cell membranes collect much bound non-membrane proteins. This increases the protein-to-lipid ratio so as to produce an abnormal membrane environment. There is also a dehydration in the nuclear core as compared with the outer portions of the lens.

Another important influence on fiber cell membranes relative to differentiation is the accumulation of formerly water-soluble proteins in an aggregated form and an unfolding of these proteins so as to expose reactive side groups and to stimulate unnatural protein:protein interactions. The exposure of SH groups is an example of this process, and it has been shown by many investigators (Roy and Spector, 1976; Bloemendal, 1981). Spector *et al.* (1979) have shown that a protein of 23,000 dalton molecular weight has a special affinity for membrane surface proteins in human

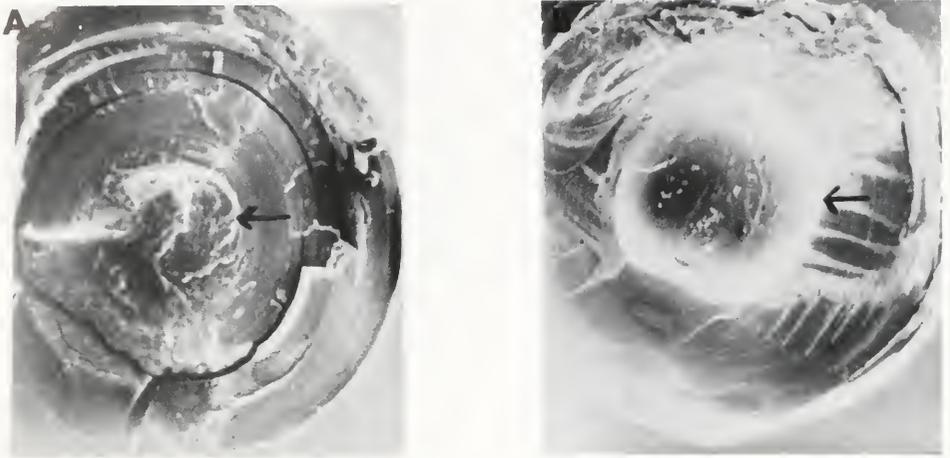


FIGURE 9. Growth of the lens nucleus in the dogfish as shown by scanning electron microscopy of an animal (A) 10 inches in length; and (B) the lens of an animal 45 inches in length. The arrows represent the outer limits of the nuclear region of each lens. Reduction: A, 1 cm = 0.09 cm; B, 1 cm = 0.25 cm.

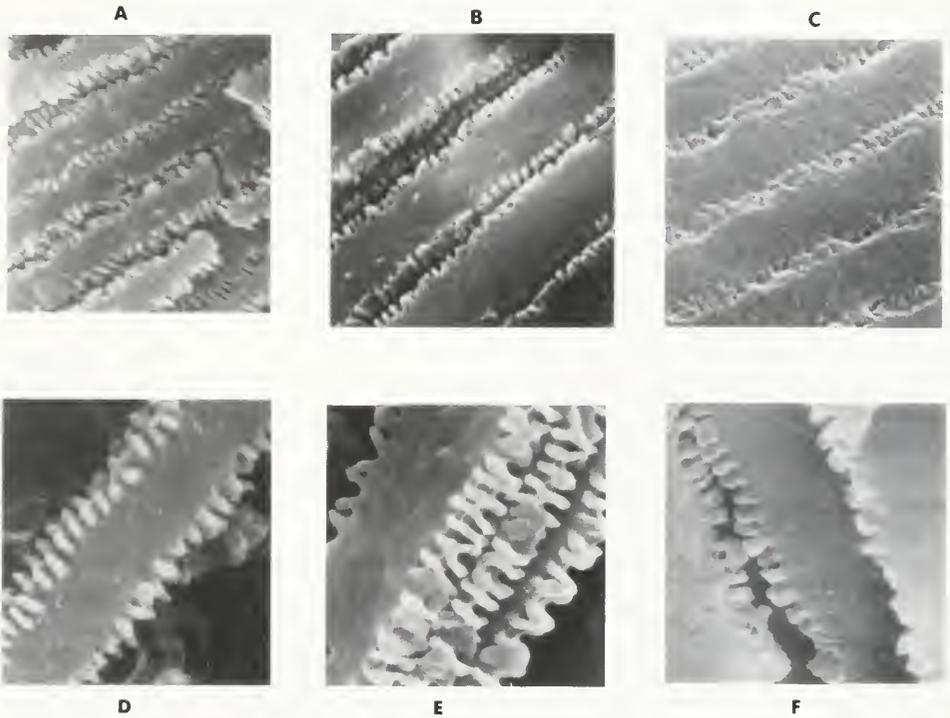
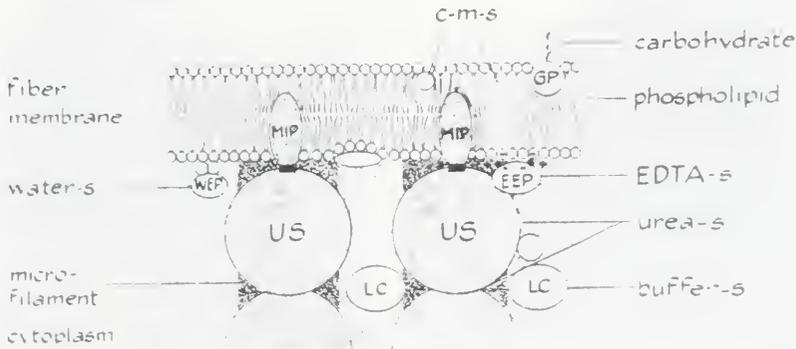


FIGURE 10. Scanning electron microscopic view of the alterations in the morphology of dogfish lens fiber cells relative to age and position in the lens. A, B, C at 2000 $\times$ ; D, E, F at 5000 $\times$ . A, D. Nuclear fiber cells of a 15-inch-long animal; B, E. Cortical fiber cells of 36-inch-long animal; C, F. Nuclear fiber cells of the 36-inch-long animal.

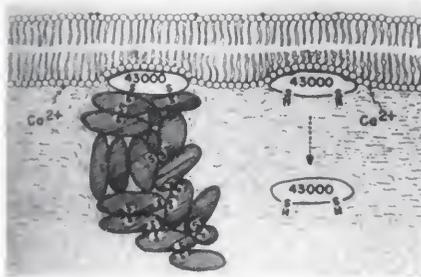
cataracts via -SH to -SS reactions, and that other lens crystallins such as  $\gamma$ -crystallin also react with 23,000 dalton proteins. In this way, areas of large scale aggregation result along the inner fiber cell membranes. Other forms of covalent bonding between formerly soluble proteins and membrane proteins occur. Figure 11 illustrates the above (Horwitz, unpub. data).

Another aspect of lens fiber membrane protein changes with differentiation involves the intrinsic membrane protein of 26 to 27,000 daltons that represents the major protein of the gap junction (see Fig. 12). With aging, maturation, and position in the lens, the relative amount of the main intrinsic protein (MIP) to the other membrane proteins increases (Horwitz *et al.*, 1979; Zigman *et al.*, 1982).

Extraction of fiber cells consecutively with aqueous buffer, 8 M urea, 1% SDS, and then 1% SDS plus 50 mM DTT indicates the stability of the membrane to dissolution (see Fig. 13 for the dogfish lens). Table I indicates further how separated skate lens nuclei and cortices respond to such consecutive extractions. It is surprising that SDS alone still leaves much fiber membrane undissolved. DTT added to the



A



B

FIGURE 11. Schematics of lens fiber cell membranes. A. Association of water-insoluble but urea-soluble proteins with the fiber cell membrane, and summary of other associated molecular species (after Broekhuysse, 1981). B. Association of the 43,000 dalton water-soluble protein with the lens fiber cell membrane, and aggregation of other soluble crystallins to the membrane via -SS bonding (after Spector *et al.*, 1979).

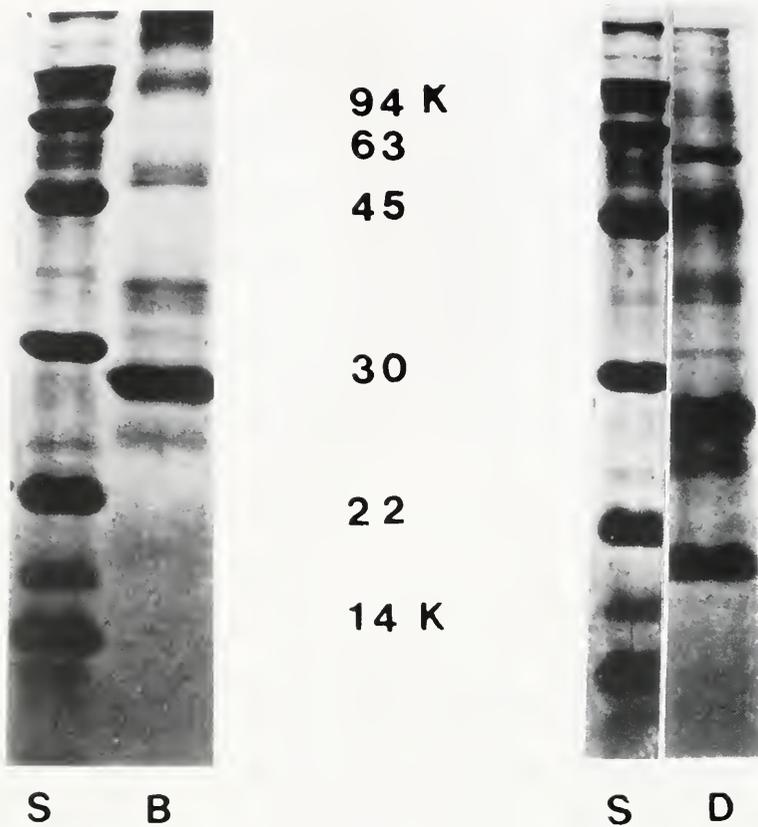


FIGURE 12. SDS-polyacrylamide gel electrophoresis of the urea-insoluble membrane fraction of bovine (B) and dogfish (D) lens homogenates. Note the presence of the 26,000 to 27,000 dalton major bands in both lenses, but other bands that differ in protein and concentration.

SDS apparently does not totally disperse the membrane components (see Zigman *et al.*, 1982).

The major gross change in the nucleus of the lens that represents terminal differentiation is the enhanced light-scattering that is observed in a variety of animals, including elasmobranchs and humans. Such nuclear scattering is the end result of the aggregation due to membrane and soluble protein interactions that occur naturally with aging as a result of lens differentiation. One can thus equate the formation of nuclear opacity (or cataract) to maturation and aging changes that lead to this extreme of the differentiation process (Table I).

#### CYTOSKELETAL ELEMENTS

An interesting feature of the ocular lens relates to its cytoskeleton and the architecture of the fiber cells. These features change with altered fiber cell shape as they are internalized to the lens nucleus. Figure 14 summarizes the process by illustrating the cytoskeletal elements in the fiber cells. In the cortex the intermediate filaments appear distinct and clearly defined with good connections to the plasma membranes, whereas, the filaments are absent in the nuclear fiber cells and there is clumping of the protein chains to the plasma membranes (see Maisel *et al.*,

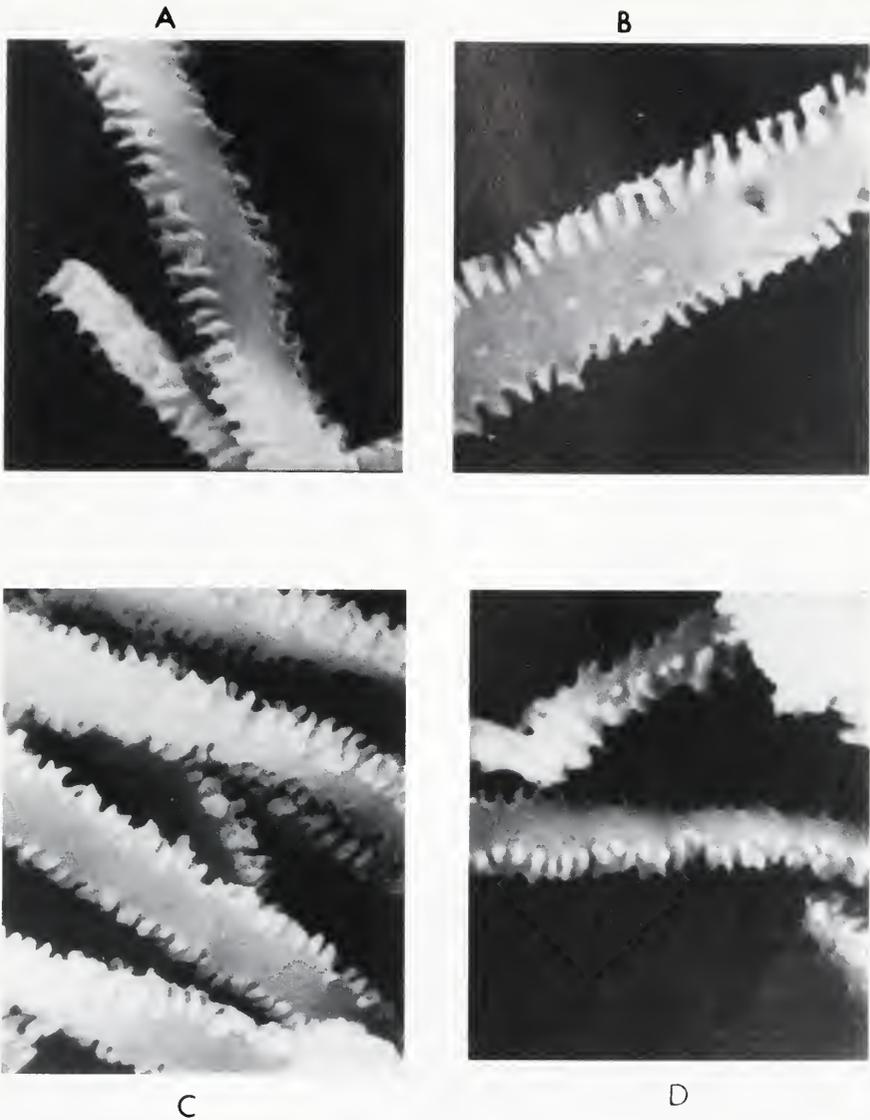


FIGURE 13. Stability of dogfish lens nuclear fiber cells after extraction with tris buffer (A), 8 *M* urea (B), 1% SDS (C), and 1% SDS plus 50 *mM* DTT. (D) 1% SDS plus 50 *mM* DTT for 24 h. The fibers are thinner and appear to disintegrate with time. Only SDS plus DTT was capable of totally disintegrating the fiber membranes.

1981). These structural alterations parallel the diminished elasticity of the nuclear fiber cells.

On a chemical basis, one finds that there are two major elements of the cytoskeleton: vimentin and actin. An SDS polyacrylamide gel of the lens fiber membrane-cytoskeletal complex reveals peptides at 55,000 and at 45,000 daltons, which represent vimentin (55,000) and actin (45,000). Figure 15 illustrates the SDS-polyacrylamide gel profile of skeletal and other membrane elements of cow lenses (Benedetti *et al.*, 1981). The loss of intermediate filaments in the nucleus is

TABLE I

*Distribution of insoluble proteins in the skate lens†*

a) Total insoluble protein as percent of total protein				
Young (Average* lens weight of 22 mg)	Cortex 20%		Nucleus 28%	
Adult (Average* lens weight of 98 mg)	Outer cortex 38%	Inner cortex 34%	Outer nucleus 23%	Inner nucleus 50%
b) Solubility of the insoluble proteins				
Young	Cortex		Nucleus	
1) Extracted with 8 M urea and then 1% SDS	67%		49%	
2) Extracted with 1% SDS plus 50 mM DTT	33%		51%	
Adult	Outer cortex	Inner cortex	Outer nucleus	Inner nucleus
1) Extracted with 8 M urea and then 1% SDS	66%	66%	65%	55%
2) Extracted with 1% SDS plus 50 mM DTT	34%	35%	35%	45%

\* Average of 3 determinations.

† *Raja eglanteria*.

accompanied by the loss of vimentin in the nucleus. Thus, with maturation as judged by position within the lens, the basic protein chemistry and structural elements of the cytoskeleton show great changes. Loss of nuclear zone elasticity with maturation may be the result of these changes.

#### POSITIONAL MATURATION OF LENS PROTEINS

Lens differentiation continues to take place throughout the lifetime of an individual. Post-translational changes occur in the distribution and interaction of the lens crystallins, as well as in their chemical features. When adult lenses of many species were separated into concentric layers, the soluble crystallin distribution varied similarly in all. While heavier proteins predominated in the total water-soluble fraction of the outer layers, the lower molecular weight species were more abundant toward the lens center or nucleus. In absolute terms as well, the content of the heavier crystallins was greatly diminished in the nuclear core (see Figs. 16–19).

In parallel with the losses of heavier crystallins with internalization, there is an increase in the water-insoluble aggregated fractions. This is true for the water-insoluble protein that can be solubilized in 8 M urea and in 1% SDS. But in the water insoluble but SDS plus DTT soluble fraction (*i.e.*, the fiber cell membranes themselves), the insoluble protein remains nearly constant in all layers. The latter fractions would represent the intrinsic lens fiber cell membrane proteins.

It is thought by many lens researchers that the lower molecular weight gamma crystallins have the greatest potential for aggregation and covalent attachment to the fiber cell membranes. This is due to their abundant cysteine and tryptophan

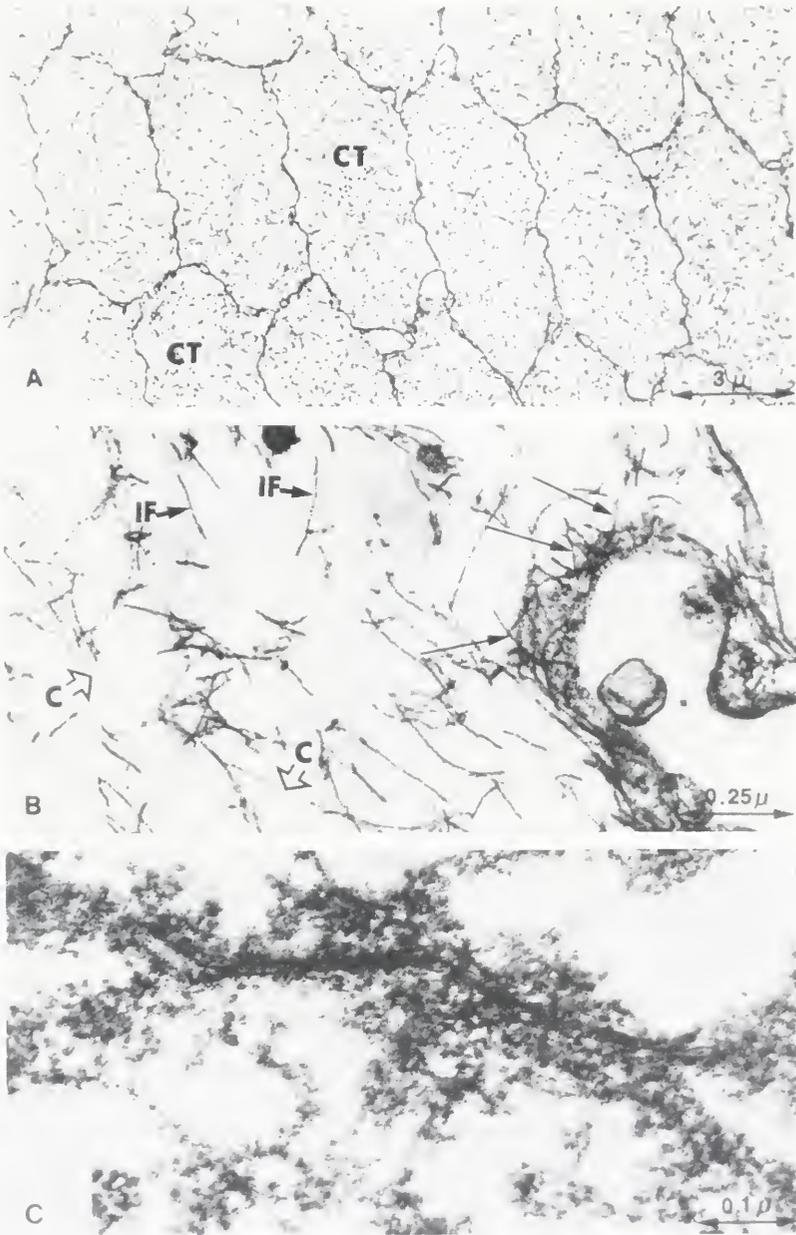


FIGURE 14. Transmission electron microscopic morphology of glycerol extracted fiber cells of the cow lens, prepared so as to illustrate the cytoskeletal elements. A. Presence of cytoskeletal elements in fiber cells cross sections to show relationship between membranes and cytoskeleton; B. cortical intermediate filaments; and C. lack of intermediate filaments in the nuclear preparation and clumping onto the cell membrane (after Maisel *et al.*, 1981).

contents relative to the other crystallins. Both of these amino acids have easily oxidizable side-chains that enhance the reactivity of crystallins due to oxidation reactions. Free -SH groups of  $\gamma$ -crystallins are known to become oxidized, resulting

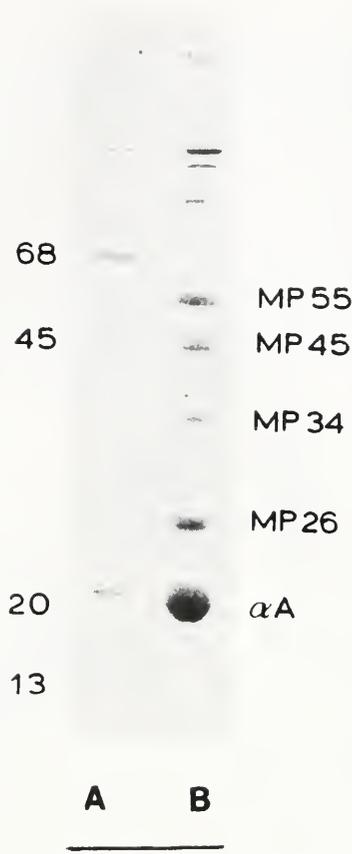


FIGURE 15. SDS polyacrylamide gel electrophoretic profiles of the membrane-associated water-insoluble proteins of the cow lens, showing vimentin (MP 55) and actin (MP 45) of the cytoskeleton, the membrane intrinsic proteins (MP 26, 34), and the A chain of alpha crystallin. A = standards; B = membrane-cytoskeleton extract (after Benedetti *et al.*, 1981).

in the formation of intra- and inter-molecular crosslinks via -SS bond formation (Takemoto and Azari, 1977). Again, due to the conservative growth process of the lens, these post-translational changes in protein side-chains eventually lead to aggregates and light-scattering in the nucleus.

The indole rings of protein tryptophan can also be oxidized to form kynurenine and other products (*i.e.*, beta carbolines, anthranilic acid) that can serve as fluorescent crosslinks between protein molecules (Dillon, 1976). N-formylkynurenine has been isolated from the lens and identified by Pirie (1971) and 3-OH-kynurenine glucoside has been identified in the lens by Van Heyningen (1973) and Bando (1983). These tryptophan products are known photosensitizers. Such photosensitization reactions lead to enhanced protein photo-oxidation that is ultimately found in the nuclear fiber cells. The presence of these aggregated molecules in the nucleus of the lens due to metabolic and radiant energy-induced oxidation has been well-documented, and is most likely responsible for much of the high degree of nuclear light-scattering.

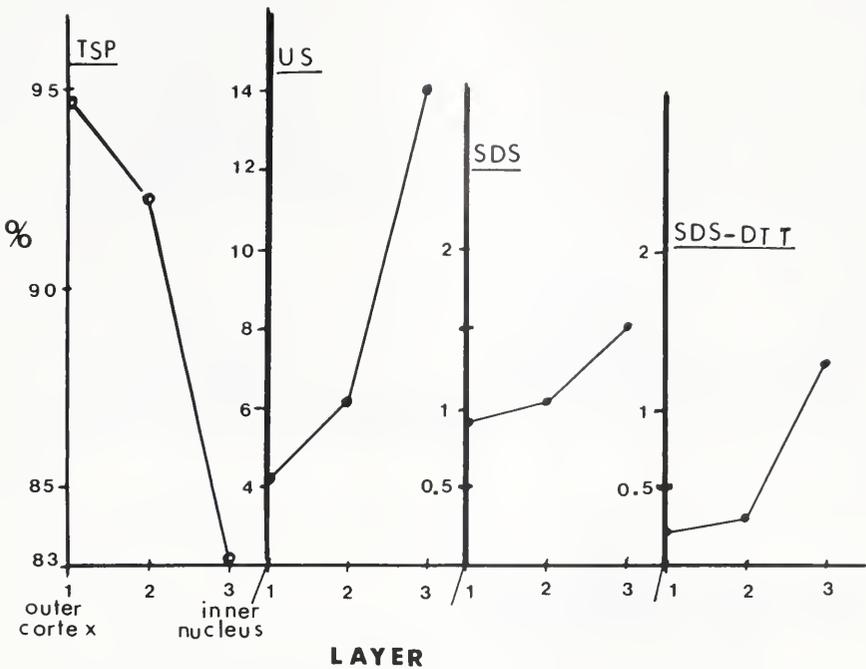


FIGURE 16. Changes in both water-soluble and water-insoluble protein content in concentric layers of the squirrel lens. TSP = water-insoluble; US = urea-soluble; SDS = sodium dodecyl sulphate-soluble; SDS + DTT = SDS plus dithiothreitol soluble proteins.

Positional differences are seen not only with regard to structural proteins, but they also apply to many enzymes of great importance to cell function (see Hockwin and Ohrloff, 1981). For example, the ATPase activity is greatest in the outer layers of the lens, and tend to diminish toward the nucleus (see Table II). With regard to the  $\text{Na}^+/\text{K}^+$  enzyme, diminished activity in the nucleus leads to light-scattering stimulation due to disturbances in the salt and water balance in this region.

Positional differences between outer less mature and inner more mature regions of the lens apply to several other features of the lens. In one case, there are stable free radicals in the lens whose concentration diminishes toward the nucleus (Fig. 20). It has been hypothesized that the chemical entities that represent the free radicals are bound to proteins and in fact serve as cross-linking agents. Due to the conservative nature of the lens, such cross-linking agents react with proteins that are finally found in the nucleus, and therefore become quenched with regard to free radical properties (Zigman, 1981).

Another lens component that changes in properties and levels from the outer to inner portions of the lens is the yellow pigment that is present nearly exclusively in diurnally active animals, such as squirrels, monkeys, and humans. The pigments that are present in the lens at birth both in humans and in squirrels, are water-soluble, low molecular weight entities (see Fig. 21). Tentatively speaking, the human pigment is 3-OH kynurenine-glucoside (Bando, 1983; Van Heyningen, 1973), while the squirrel pigment is thought to be n-acetyl,3-OH kynurenine (Van Heyningen, 1973). Both of these are metabolic products of tryptophan via a tryptophan

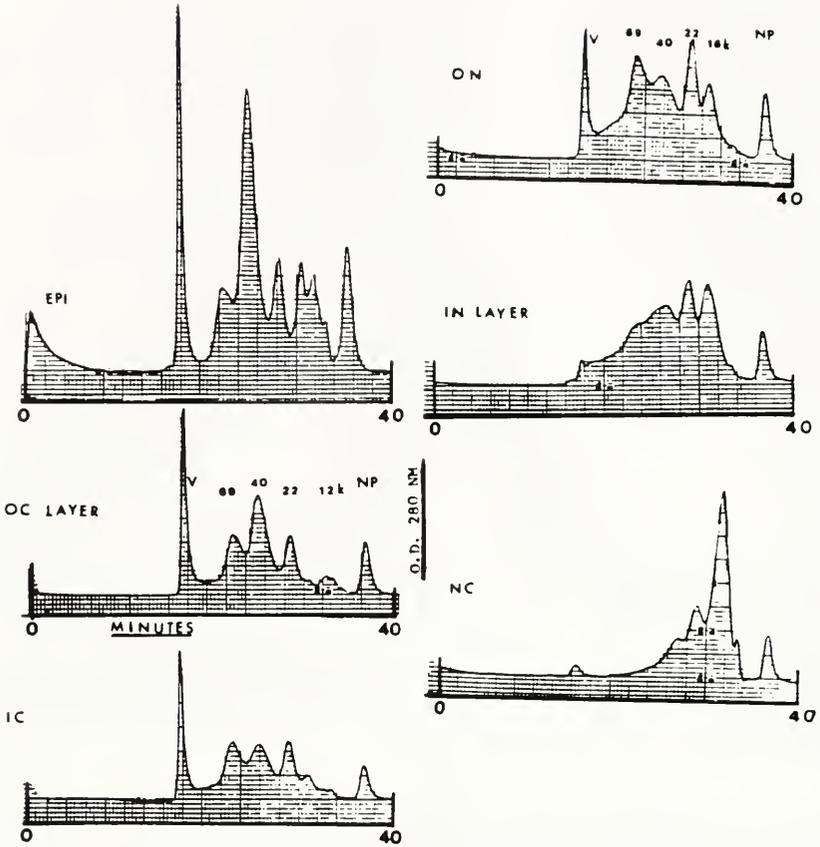


FIGURE 17. High performance liquid chromatography of the water-soluble proteins in the epithelium and five concentric layers of the lens of a squirrel. Note loss of heavy and increase in lighter proteins going from the outer to the inner portion of the lens.

oxygenase enzyme (Van Heyningen, 1973). While in the human, this type of low molecular weight water-soluble lens pigment diminishes with maturation and aging, it appears to increase in concentration as the squirrel matures. In the squirrel lens, the balance of oxidation-reduction of this pigment appears to be maintained with maturation, while in the human lens there is a buildup of new tryptophan oxidation

TABLE II

*Distribution of ATPase activity in bovine lens*

ATPase of:	Total ATPase	Mg <sup>++</sup> -ATPase	Na <sup>+</sup> K <sup>+</sup> -ATPase
Bovine	Micromoles per mg per hour		
Lens epithelium	0.15	0.08	0.07
Lens cortex	0.03	0.02	0.01
Lens nucleus	0.005	0.004	0.001

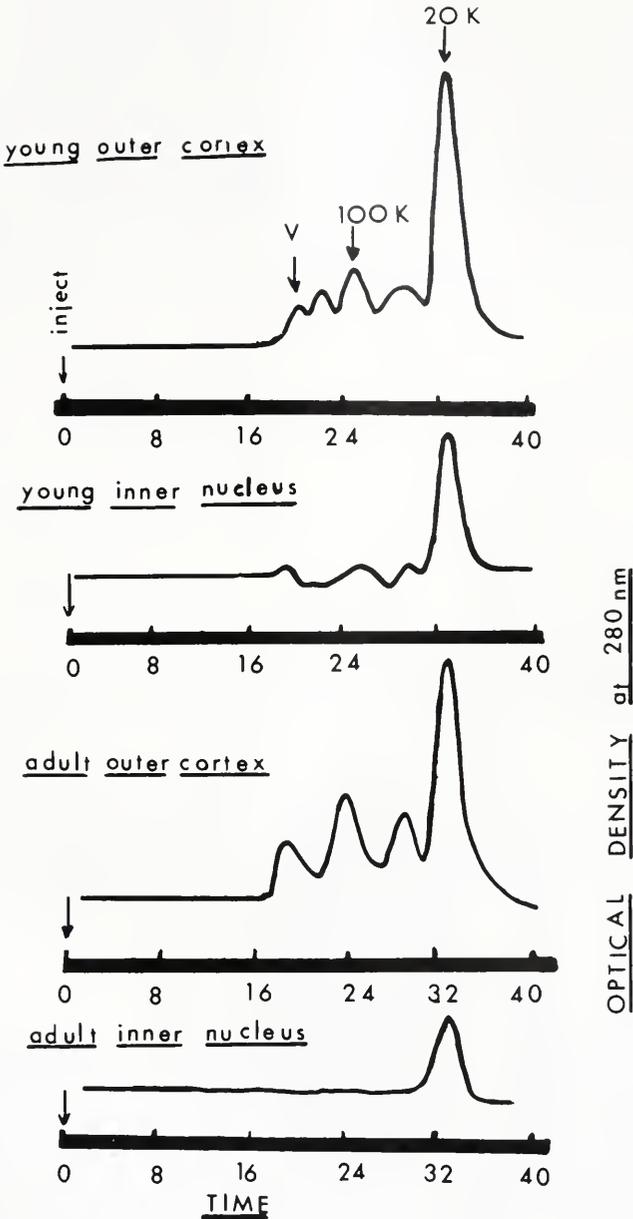


FIGURE 18. HPLC profiles of the outer and inner cortices and nuclei of young (15 inches long) and adult (33 inches long) skates. Note loss of heavy and increase of light protein bands going toward the central core of the lens.

products with aging (*i.e.*, over 30 years of age). For example *n*-formylkynurenine and B-carbolines become covalently associated with the structural proteins, and via fluorescent cross-links, they enhance aggregation and light-scattering (Dillon *et al.*, 1976). This process of enhanced concentration of protein-bound pigment has only been observed in human lens thus far, perhaps due to the relative longevity of the

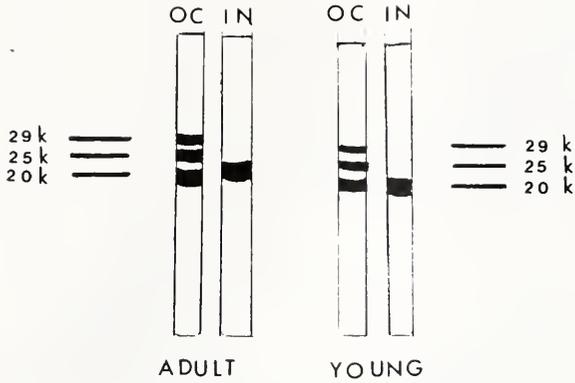


FIGURE 19. SDS-polyacrylamide gel electrophoresis of skate lens proteins. OC = outer cortex; IN = inner nucleus.

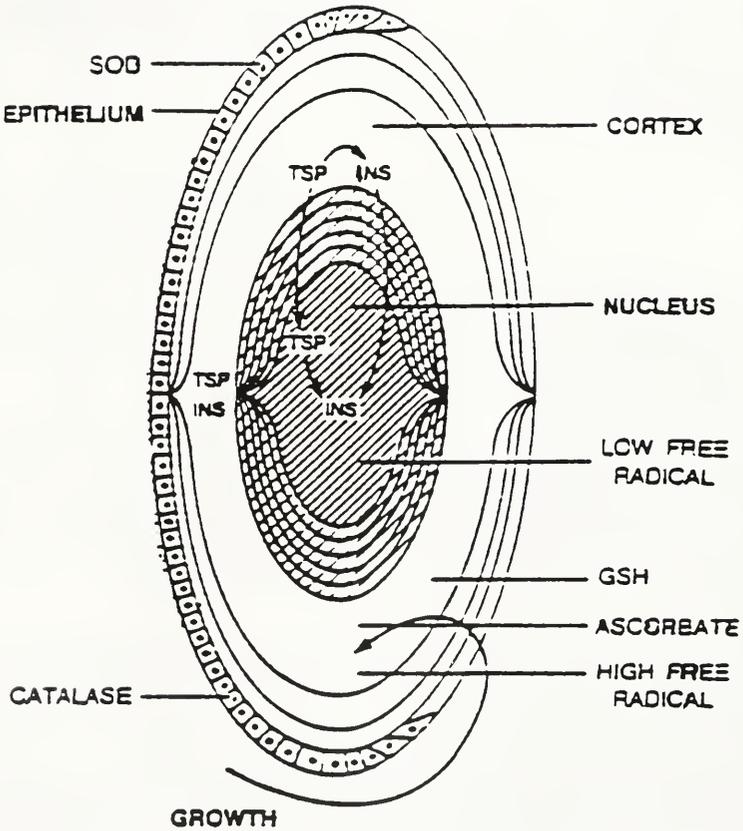


FIGURE 20. Scheme of distribution of stable free radicals, reducing agents, and anti-oxidant enzymes in the ocular lens.

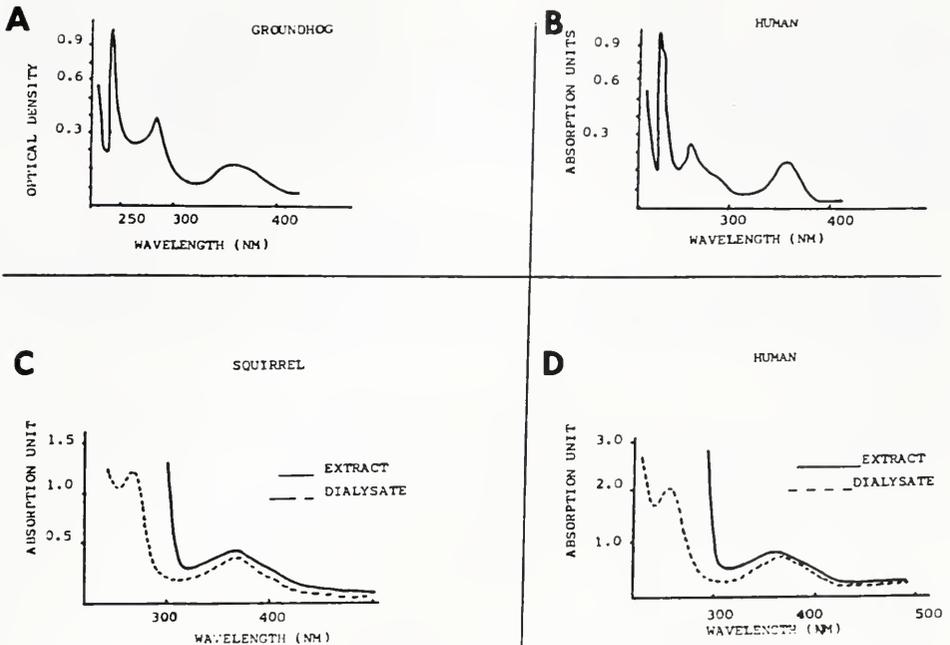


FIGURE 21. Spectral properties of the near-UV and blue-visible absorbing pigments of groundhog, human, and squirrel lenses. A, B are pressure dialyzed non-protein extracts; C, D show both dialyzed and non-dialyzed extracts.

human species. The pigment density becomes quite high only in the nucleus and not in the cortex with an occasional exception. This phenomenon has been ascribed to a paucity of reducing agents (*i.e.*, glutathione, ascorbic acid, etc.) in the nucleus to counteract metabolic oxidant buildup and photosensitized radiant energy absorption effects.

### CONCLUSION

This brief review supports the concept that cataract (*i.e.*, excessive extinction of light) is actually a case of terminal differentiation of the lens as influenced by genetic, nutritional, internal biochemical, and environmental factors. With regard to the biochemical influence on cataract formation, the major contributing processes appear to be protein aggregation and association of protein species with the fiber cell membranes leading to light-scattering in the nucleus of the lens. These processes are stimulated by oxidation reactions, and oxidants formed as the result of metabolism play a role in the loss of transparency. Little is yet known about the nutritional aspects of cataract, except that trace elements that function as antioxidants and enzyme cofactors must be maintained to prevent cataract formation. Thus far the genetic influences on cataract formation seem to be mainly related to the lack of enzyme activities which maintain the exchange of substances between the lens and the aqueous humor. Radiant energy seems to be the most influential in cataract formation, in that it has been shown experimentally that free radical formation, fluorescent cross-link stimulation in proteins, and enhanced pigment darkening are stimulated by excessive exposure to the long wavelength light present in the sunlight.

While some of these factors apply more or less to the non-human species, naturally occurring cataract is relatively rare in them. However, the problem of cataract is most acute in humans, in whom all of these factors apply. Longevity is an additional factor that does not apply to most other species. Details of the current studies on human cataract can be found in several recent books (Bloemendal, 1981; Duncan, 1981; Maisel, 1985).

#### ACKNOWLEDGMENTS

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## LONG-LASTING SUBSTRATE MARKING IN THE COLLECTIVE HOMING OF THE GASTROPOD *NERITA TEXTILIS*

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### ABSTRACT

Field observations and experiments were conducted on the intertidal gastropod *Nerita textilis* Gmelin along the Somalian coast to determine if its rhythmical mass-homing includes the detection of durable substrate marking as well as short-term trail-following. The snails' first response to displacement is a zonal orientation compensating for the vertical component of experimental shifting. The homing performance of symmetrically transferred animals supports the hypothesis that a marked area is present in and below the aggregation site, detectable by the homer snails 24 hours after its deposition by spontaneously moving conspecifics. No specific marking of different collective homes resulted from these experiments.

### INTRODUCTION

Experimental evidence shows the importance of trail-following in the orientation of molluscs toward goals of different ecological significance, including rest sites (Newell, 1979; Underwood, 1979). The Indo-Pacific intertidal gastropod *Nerita textilis* Gmelin performs looped feeding excursions whose homeward branch partly overlaps its outward path. Moreover, arena tests show inter-individual short-term retracing as well (Chelazzi *et al.*, 1983). Under high population density this species shows markedly rhythmical clustering during all high tides and low tides occurring between about midnight and noon (Vannini and Chelazzi, 1978). This rhythmical aggregation is controlled by external (tidal) factors and spatial interactions between members of the population (Chelazzi *et al.*, 1984).

Collective homing of *N. textilis* and other gregarious intertidal gastropods (Moulton, 1962; Magnus and Haacker, 1968; Willoughby, 1973) would seem to require not only a capacity to follow freshly deposited mucous trails but also the ability to detect long-lasting chemical cues, including a durable system of trails connecting feeding and resting places, as well as the marking of collective homes. The capacity to follow durable mucous trails and the use of stable chemical labeling of the rest site are both present in some solitary-homer intertidal gastropods (Funke, 1968; Cook, 1969).

The displacement experiments reported in this paper were designed to verify if *N. textilis* homes not only through the use of short-term trail-following, but also by detection of long-lasting chemical cues.

### MATERIALS AND METHODS

Experiments and observations were conducted at different sites along the Benadir coast (Somalia), whose morphology and intertidal ecology have been described elsewhere (Chelazzi and Vannini, 1980a). Observations on natural behavior were performed using photography at set intervals.

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Tides are semidiurnal along the Somalian coast and during the test period (around spring tide) low tides occurred at about 11:00 and 23:00 h. Displacement tests were performed by collecting all snails resting in each cluster during the morning (08:00–11:00), marking them individually by a number-color code, and transferring them immediately to the release point. Their position was recorded 24 h later at the following diurnal low tide. Since *N. textilis* moves only once a day (for about 7 h) during afternoon and night low tides (Vannini and Chelazzi, 1978), the time between displacement and recording comprised only one activity phase.

For the *single-cluster* tests a total of 140 snails resting in three aggregations were divided into four groups of equal size ( $n = 35$ ) and released from points 150 cm above, below, to the left, and to the right of their original cluster. Their position with respect to the release site was recorded 24 h later. Moreover, an additional 403 snails were transferred as above from 6 aggregations; the number of those returned to their original cluster was recorded 24 h later.

The *crossing* tests consisted of collecting all snails resting in twin clusters 160–200 cm apart at the same shore level. Members of left and right clusters were divided into two lots of almost equal size and transferred below each original cluster or obliquely, below the side cluster. The number of displaced snails returned to each cluster was recorded 24 h later. Three replicates of the experiment were performed on different cluster couples, for a total of 133 vertically and 138 obliquely transferred snails.

Circular distributions of recovery directions (*single-cluster* test) were analyzed according to current circular statistics (Batschelet, 1981).

## RESULTS

### *Field observations*

Photographs taken during clustering and disaggregation show evidence of interaction between moving snails. During their return from the feeding zone the snails either form pre-aggregation clumps which move compactly to the sheltered areas (fast return under strong wave movement) or sparsely follow a web of trails (slow return under moderate wave action). Mucous trails are evident under the latter circumstances and the web progressively clumps into a few major trails leading to the cluster site (Fig. 1). Snails follow the trail system singly or in small groups. Moreover, queuing is commonly observed during the early downward migration following disaggregation (Fig. 2).

Besides the web of trails traced on the shore during migration, rest places also show distinct long-lasting features: where clusters usually form, the rocky wall differs in color from the neighboring zones. Moreover, cluster sites are constant not only day after day but also after their periodic vacancy during the synodic month (Chelazzi *et al.*, 1984). The long-lasting marking of rest areas is also suggested by the return rate of snails to temporarily abandoned clusters. In most cases this phenomenon appears suddenly: at the first rest phase of occupancy the mean number of aggregating snails is about 72% of the saturation size of each cluster.

### *Experimental displacement*

Inspection of the shore during the 24 h period between displacement and position-recording confirmed that throughout the test period transferred snails moved only during the nocturnal low tide. The angular distributions obtained from the *single-cluster* tests show that the snails adjusted zonally to displacement. When

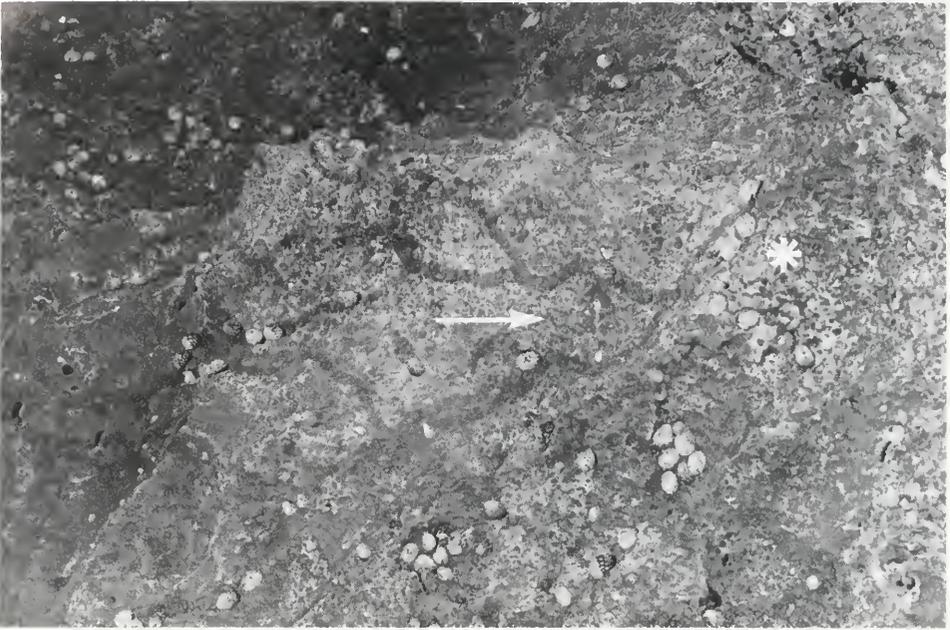


FIGURE 1. Night photograph of the shore during early cluster formation, showing a web of mucous trails leading to a cluster area (asterisk). Snails returning from their feeding excursions following trails are visible (arrow: direction of movement). Sea is at the left.



FIGURE 2. Afternoon photograph of the same stretch of shore as in Figure 1, during early downward movement (arrow) from the cluster area (asterisk).

released above (Fig. 3A) and below (Fig. 3B) their original site they headed respectively downwards and upwards (V test,  $P < 0.01$  for both distributions). The two distributions differ statistically from each other according to Watson's  $U^2$  test ( $U^2_{25,31} = 1.06$ ;  $P < 0.01$ ). The lateral displacements (Fig. 3C-D) were followed by bimodal heading distributions (Rao's test,  $P < 0.01$ ) since the snails moved in both horizontal directions after release. The two distributions are not statistically different ( $U^2_{29,34} = 0.09$ ;  $P > 0.10$ ), but the cumulative distribution of headings after lateral displacements differs statistically from that obtained after upwards ( $U^2_{25,63} = 0.65$ ;  $P < 0.01$ ) and downwards ( $U^2_{31,63} = 0.96$ ;  $P < 0.01$ ) releases.

The number of snails which returned to their original cluster site differs between the various release sites (Fig. 4). Homing performance was significantly higher following downward rather than upward ( $\chi^2 = 23.99$ ;  $P < 0.01$ ) or horizontal displacement ( $\chi^2 = 25.91$ ;  $P < 0.01$ ). The return from release sites above the cluster area was slightly lower than from the lateral places, but the difference was not statistically significant in the present sample size ( $\chi^2 = 0.91$ ;  $P > 0.05$ ).

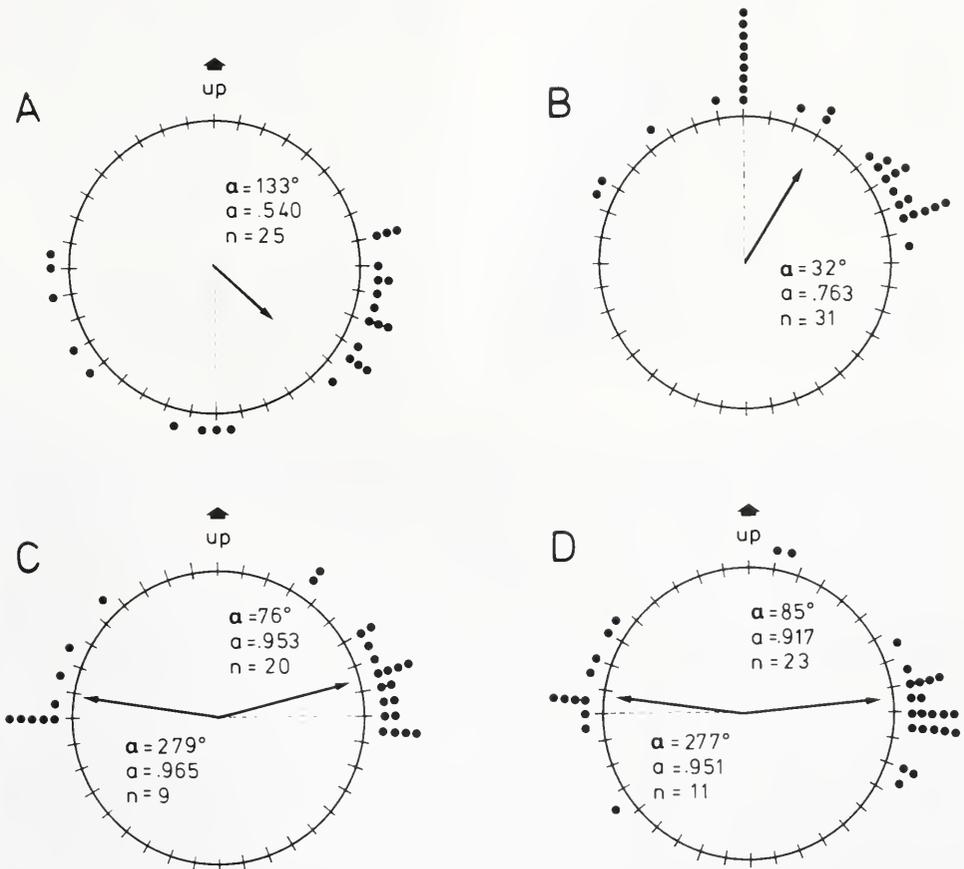


FIGURE 3. Headings of snails after displacement above (A), below (B), to the left (C), and to the right (D) of their cluster site. Inner dashed line: original home direction; inner arrow: mean vector. The polar coordinates and sample size of each distribution are also shown. C and D distributions were analyzed as bimodal (rightward and leftward) distributions.

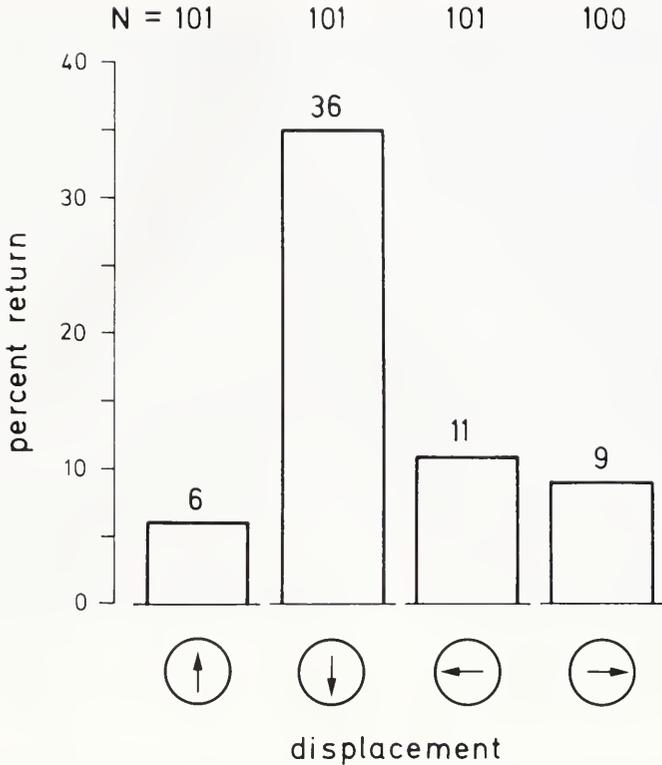


FIGURE 4. Homing performance relative to the *single-cluster* test, expressed as percent of the number displaced in each direction (N). Number of animals recovered in their original cluster after each displacement is shown above each histogram.

The majority of the non-homed snails were either lost—probably dislodged by waves during high tide (about 15%)—or remained scattered, while a fraction was observed in adjacent clusters. *Crossing* tests were performed in order to quantify the change of cluster after displacement and to verify if snails significantly preferred their original aggregation. Out of a total of 144 snails recovered in both test clusters, 68 were found in the cluster above the release site (47.2%) and 76 to the side ( $\chi^2 = 0.57$ ;  $P > 0.05$ ). Figure 5 shows no evidence for a preference of original *versus* adjacent aggregation ( $\chi^2 = 0.37$ ;  $P > 0.05$ ).

#### DISCUSSION

The first response of *Nerita textilis* specimens, after being transferred from their cluster sites, is a compensation for the vertical component of experimental displacement. Long-distance (10–50 m) seaward displacement of this species, and the congeneric *N. plicata*, has revealed this compensative zonal orientation (Chelazzi and Vannini, 1976; 1980b) which has also been demonstrated in *Littorina irrorata* (Hamilton, 1978), *L. littorea* (Gowanloch and Hayes, 1926; Gendron, 1977), and *L. punctata* (Evans, 1961). But the present study revealed a very precise and fast zonal adjustment.

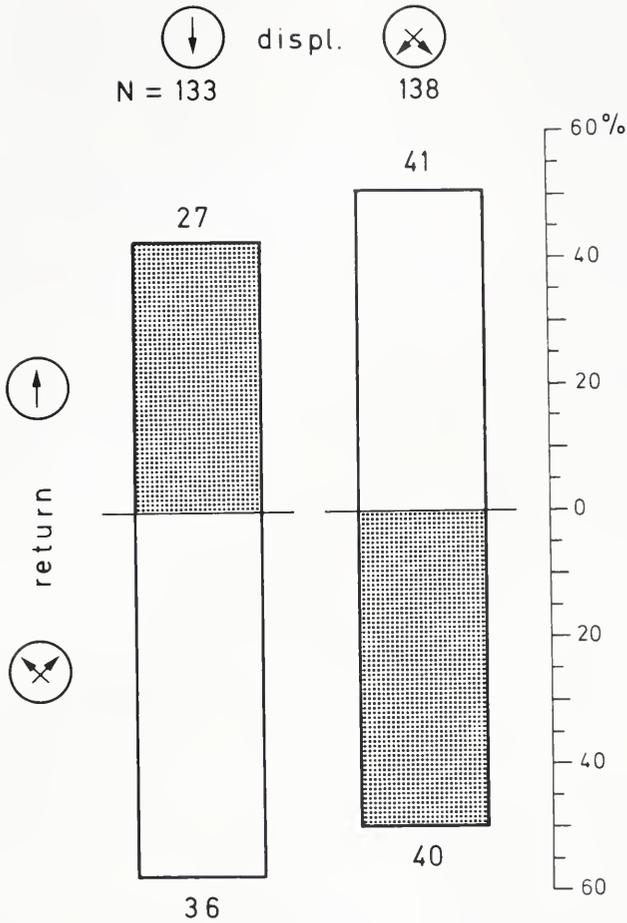


FIGURE 5. Homing performance relative to the *crossing* tests: upward (above) and diagonal (below) return, expressed as percent of snails recovered after downward (left) and oblique (right) displacement. Total numbers of displaced snails are shown above the histograms (N). Shaded histograms: snails returned to the original cluster.

Among the probable factors informing the animals about their vertical shift are the variation of exposure to waves during high tide and physical substrate conditions (hydration and temperature) during low tide. These cues could trigger a directional orientation such as geotaxis, following the complex integration between releasing and orienting mechanisms frequently involved in the zonal orientation of intertidal gastropods (Fraenkel, 1927; Kristensen, 1965; Bock and Johnson, 1967; Bingham, 1972; Underwood, 1972a, b; Chelazzi and Focardi, 1982).

However, alone this precisely tuned zonal behavior cannot guarantee the relocation of a spatially definite goal such as the aggregation area, 10–40 cm in diameter. An additional stopping effect on moving animals based on long-lasting marking of the cluster area could explain some aspects of the natural mass-homing of *N. textilis*, including the sudden occupancy of aggregation sites and their long-term spatial stability. Nonetheless, the difference in the homing performance

recorded from four symmetrical release sites supports the hypothesis that the marked area extends downward from the aggregation site and is probably arranged in a roughly triangular shape with its vertex in the cluster area and the base downward. This could explain the significantly higher homing performance after downward displacement with respect to upward and lateral transfer.

A channeling effect due to shore morphology (crevices, etc.) can be ruled out in these experiments as the rock surface around the test clusters showed no special features. *N. textilis* generally congregates either in tide-pools of various shapes or on flat areas (Chelazzi *et al.*, 1984). While collective homing could be facilitated by drainage channels spreading down from the pools, the frequent clustering on flat surfaces must be based on factors not related to cliff morphology. Moreover, even when clustering in tide-pools, snails do not randomly use every site suitable for resting but congregate in a few areas which greatly resemble the unfrequented sites.

Chemical marking is evidently produced by the repeated release of mucus as the snails migrate downward and up the cliff during their natural feeding excursions (Chelazzi *et al.*, 1983). The experimental procedure, involving the complete destruction of original clusters during a rest phase and the control of homing 24 h later, excluded the possibility that homing of displaced animals was based on freshly lain trails; the marked area had an age of at least one day under present test conditions.

These conclusions do not contradict the available laboratory information on the survival of orienting cues in the trails of other gastropods. While in the freshwater snails *Biomphalaria glabrata* (Townsend, 1974; Bousfield *et al.*, 1981) and *Physa acuta* (Wells and Buckley, 1972) or periwinkles *Littorina planaxis* (Raftery, 1983) and *L. littorea* (Gilly and Swenson, 1978) the directional information contained in the trail seems to be significantly retained only shortly after deposition (10–30 min), other species produce long-lasting trails whose detectability by conspecifics ranges from 4 h in the mud snail *Ilyanassa obsoleta* (= *Nassarius obsoletus*) (Trott and Dimock, 1978) to one or two days in the pulmonate limpets *Siphonaria normalis* and *S. alternata* (Cook, 1969, 1971). Longer trail survival was found in the terrestrial slug *Limax grossus* (= *L. pseudoflavus*) (Cook, 1976).

Laboratory tests show that *N. textilis* recognizes the direction of freshly lain trails (Chelazzi *et al.*, 1983), but its clustering in the field does not necessarily require intrinsic trail-polarization since collective homing is performed by this species on vertical rocky cliffs where the correct (homeward) following could be based on such external cues as gravity.

Finally, no evidence emerged from our tests about the informational difference between trail-webs spreading out from various clusters, which agree with the usually observed inter-cluster turnover in the collective homing of *N. textilis* (Chelazzi *et al.*, 1984) and other gregarious gastropods (Moulton, 1962; Willoughby, 1973).

#### ACKNOWLEDGMENTS

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## INDIVIDUAL VARIATION IN ASSOCIATIVE LEARNING OF THE NUDIBRANCH MOLLUSC *HERMISSENDA CRASSICORNIS*

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### ABSTRACT

Retention of learned suppression of positive phototaxis in the nudibranch mollusc *Hermissenda crassicornis*, induced by exposure to trials of paired light and rotation, was determined for individuals within groups trained in two, three, four, and six daily sessions of 100 trials each. Significant increases in latency to light (acquisition) were measured within all paired treatment groups when these were tested before treatment and 24 hours after the last session. No significant differences in latency were found within four unpaired and one random control group. Next, retention of phototactic suppression (increased latency to respond to light) for each individual was assessed by comparing its post-treatment suppression ratio (SR) scores to a population median score derived from the frequency distribution of scores from a naive group of animals repeatedly tested over a 31-day period. Retention, defined as the consecutive number of days post-treatment on which an animal's SR scores were suppressed below the population median score, was significantly longer in groups trained four and six days than in the two- and three-day paired treatment groups. When retention day score distributions from paired groups were compared to those from the unpaired and random control groups, a significant increase in phototactic suppression was found only for groups trained four and six days. Maximum retention, or resistance to extinction, was measured at 17–18 days (one animal) after 6 sessions. All paired treatments contained animals which did not acquire the association. Retention increased with experience (number of sessions) and the number of animals per group which showed no acquisition decreased.

Investigations on the neural correlates of this behavioral change in *Hermissenda* are currently in progress; an understanding of the relationship between the degree of phototactic suppression in a sample of animals and the number of training sessions will aid in design and interpretation of experiments in which biophysical and biochemical data are correlated with behavioral measures.

### INTRODUCTION

Interest in the learning abilities of gastropod molluscs has been stimulated by the discovery that these relatively simple animals provide useful models for studies on the neuronal basis of learning. Associative learning has now been studied in five gastropod species (Mpitsof and Davis, 1973; Alkon, 1974; Gelperin, 1975; Crow and Alkon, 1978; Walters *et al.*, 1979; Audesirk *et al.*, 1982). Behavioral acts modified by conditioning procedures include: (1) feeding behavior—*Limax maximus*

(Gelperin, 1975; Sahley *et al.*, 1981), *Pleurobranchaea californica* (Mpitsos and Davis, 1973; Mpitsos and Collins, 1975; Davis *et al.*, 1980), *Lymnaea stagnalis* (Alexander *et al.*, 1982, 1984; Audesirk *et al.*, 1982); (2) escape-withdrawal locomotion—*Aplysia californica* *Pleurobranchaea* (Mpitsos and Collins, 1975; Walters *et al.*, 1979; Carew *et al.*, 1981, 1983); and (3) positive phototaxis—*Hermisenda crassicornis* (Alkon, 1974; Crow and Alkon, 1978; Crow and Harrigan, 1989; Farley and Alkon, 1980, 1982; Crow, 1983; Crow and Offenbach, 1983).

Retention is generally defined as the period of time post-treatment over which statistically significant differences are detected between experimental and control groups or within experimental groups relative to a pre-treatment response level. Duration of retention varied from 3 to 4 days for conditioned suppression of phototaxis in *Hermisenda* (Crow and Alkon, 1978) to at least 14 to 19 days for modification of feeding behavior in *Pleurobranchaea* (Mpitsos and Davis, 1973) and *Lymnaea* (Alexander *et al.*, 1978). Data on individual differences in acquisition and retention are available for food-aversion learning in *Limax*. Of a sample of 12 animals, 33% retained the aversion for 9 to 26 days after one or two trials; the remaining animals required 3 to 6 trials before retention reached significance (Gelperin, 1975). Mean retention of two non-associative forms of learning, habituation and sensitization of the siphon- and gill-withdrawal reflexes in *Aplysia*, approximated 21 days (Carew *et al.*, 1972; Pinsker *et al.*, 1973). However, because the relationship between training procedures and persistence of the learned response has not been systematically explored in any of these species, these retention periods should be regarded as approximate.

Significant increases in latency, defined as the time an individual *Hermisenda* takes to respond to light, have previously been shown to be specific to temporal pairing of light and rotational stimuli, and to be specifically restricted to locomotion in a light gradient (Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983). Because exposure to paired stimulation results in a decrease in an animal's responsiveness to light, we refer to this learned behavioral change as 'associatively suppressed phototaxis.' Here we report the results of experiments designed to define the range of variation in acquisition and retention of associatively suppressed phototaxis between individual specimens of *Hermisenda*, and the effect of increasing numbers of treatment sessions on retention.

We are interested in describing individual variation in acquisition and retention of this behavioral change for the following reasons. First, the small numbers of neurons in the sensory structures (eye and statocyst) which transduce light and gravitational stimuli, and in the interconnecting sensory pathways, have permitted cellular processes associated with this behavioral change to be analyzed in single neurons (see review by Alkon, 1980; also, Alkon, 1982–1983). To adequately measure changes, often small in magnitude, in membrane currents (Alkon *et al.*, 1982; Farley *et al.*, 1984; Forman *et al.*, 1984) and protein phosphorylation (Neary *et al.*, 1981) that are specific to treatment with paired stimulation, it is important to optimize treatment procedures to induce maximum expression of the learned behavior. Second, variation in retention may itself be correlated with measurable biophysical and biochemical changes. Such correlation, if found, would aid in relating specific cellular processes to behavioral features of learning. Third, since laboratory-reared animals are capable of acquiring the association (Crow and Harrigan, 1979), selective cultivation of strains of animals with long or short retention capacity, as defined by the range in retention measured in the laboratory, could also provide material for study of the behavioral, biochemical, and biophysical components of associative learning.

## MATERIALS AND METHODS

Specimens of *Hermisenda* were obtained weekly, year-round, from Sea Life Supply, Sand City, California, and maintained at 12–14°C in a refrigerated aquarium (Dayno Mfg. Co.). Two fluorescent bright sticks (Sylvania Corp.) provided illumination on a cycle of 12 hours light:12 hours dark (on at 0600) at an intensity of  $3.6 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  (Radiometer Model 65A, Yellow Springs Instrument Co.). Animals were stored in the aquarium in individually numbered clear plastic slotted containers. All animals were acclimated in the laboratory 4–5 days before the start of an experiment.

Responsiveness to a light gradient was markedly affected by food consumption. Well-fed animals tended to be less responsive to light than semi-starved animals. To ensure survival of animals for long-term (one month) experiments and to control for the effects of food intake on positive phototaxis, it was necessary to standardize feeding so that each animal continued to grow but was not satiated at the time of testing. The feeding schedule selected, by trial-and-error, was 0.10 cm<sup>3</sup> of tunicate viscera (*Ciona intestinalis*) per animal per day, fed at the end of each day's session. This maintenance diet was doubled on two out of seven days for animals larger than 5 cm body length.

Body length of each animal was measured when the animal was fully extended and moving forward. All animals measured 1.50–3.70 cm at the start of an experiment. Sizes were remeasured after 31 days for the group of test-only animals.

Experimental procedures and apparatus have been described by Crow and Alkon (1978) and Tyndale and Crow (1979). Behavioral procedures were divided into two modes, testing and treatment. In the testing mode an animal was secured by a clear plastic gate at one end of a sea water filled clear lucite tube measuring 230 mm by 13 mm (inside diameter) (Fig. 1). Ten tubes were attached to a horizontal turntable, animals at the periphery, in an incubator at 12–14°C. Animals were dark-adapted 10 minutes. The gates were then removed in a darkened room and a light above the turntable center turned on. The latency, or time taken by each animal to move from the dim periphery to the brighter central area was recorded. The turntable did not rotate during testing. In the treatment mode animals were exposed to programmed sequences of 30 seconds of light and 30 seconds of rotation. The rotational stimulus was generated by spinning the turntable. Light and rotation stimulus presentations were either completely paired, unpaired, or randomized (Table 1). In the treatment mode animals remained confined at the turntable periphery, where they were exposed to a gravitational force of  $g = 2.24$  during rotation. Latency scores obtained during testing, which preceded and followed treatment, were analyzed to determine the degree to which paired, unpaired, or random light and rotation stimulus presentations affected the animals' responsiveness to the light gradient.

For the present series of experiments illumination conditions were standardized as follows. Light was provided by a series of 150-watt tungsten-halogen lamps (Sylvania Corp. No. EKE) housed outside the incubator (Dolan-Jenner Fiberlite, Model 180). Output from the lamps was combined in branching fiber light pipes of 6.5 mm diameter (Dolan-Jenner Industries) and filtered to  $500 \pm 25 \text{ nm}$  (green) through a 25.8 cm<sup>2</sup> glass filter (Oriol Corp. No. 5756). The filtered light source was mounted 49.5 cm normal to the turntable center. Peak transmittance of this filter is near peak sensitivity of the photoreceptors as determined by intracellular recordings (510 nm, Alkon and Fuortes, unpub. obs.). In the testing mode, animals experienced a maximum illumination of  $2.5 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  at the center, decreasing to approximately  $2 \times 10^2 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  at the periphery. In the treatment mode light

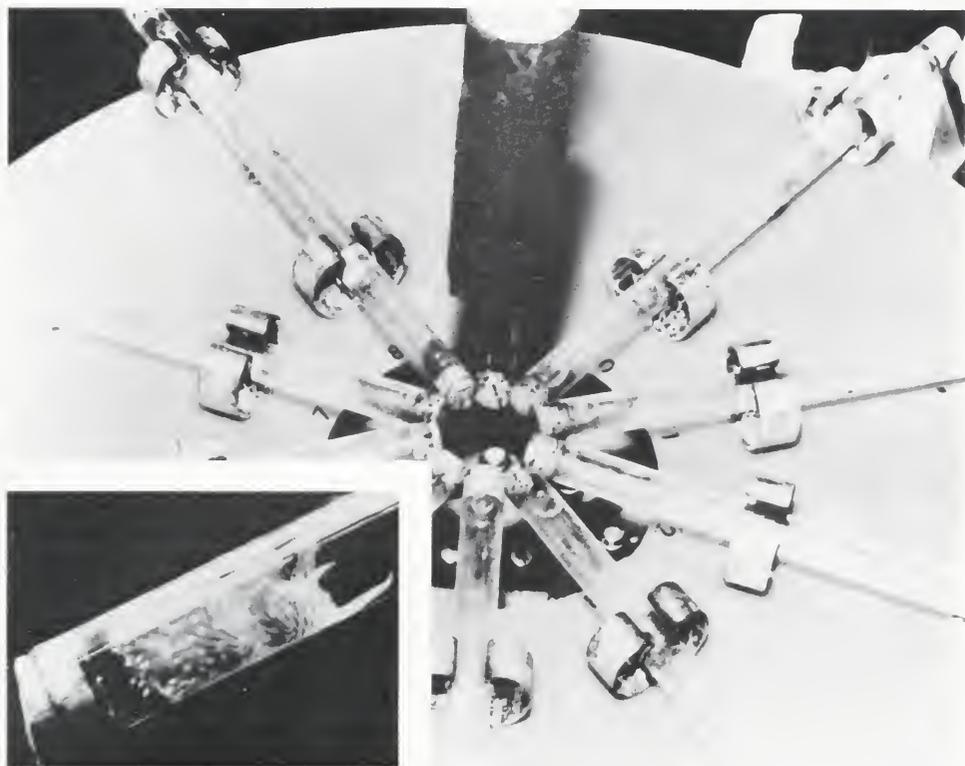


FIGURE 1. Apparatus used for measuring animals' latencies to respond to light (testing), and for treatment with paired, unpaired, or random light and rotation stimulus configurations. Light source is normal to the turntable center; inset shows an animal in the starting position (from Crow and Alkon, 1978).

intensity was increased through the same filter so that animals received a maximum of  $2.5 \times 10^3 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  at the periphery. That is, treatment light intensity equalled test light intensity at the turntable center. Light intensity within the  $500 \pm 25 \text{ nm}$  band emitted by the aquarium maintenance lights was less than  $10 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ .

### *Experimental protocol*

Only undamaged animals which fed in the laboratory and responded to light within 30 minutes on the initial, or baseline test, were included in experiments. Within each sample of ten animals tested, only three or fewer typically failed to respond. All tests subsequent to baseline response measurement were cut off at 60 minutes. Latencies were recorded by manually activating event recorder pens wired to switches mounted outside the incubator. The response criterion was that the anterior end of the animal, initially one or both tentacles, make physical contact with the plate covering the central end of the tube. Because animals were clearly visible in the experimental green light, minimal uncertainty was involved in this decision. To check possible bias, latency measurements of a sample of ten animals were taken simultaneously by two different experimenters. Recorded latencies were

TABLE I

*Experimental design: twenty animals per treatment group were subjected to each of the three stimulus configurations listed (paired, unpaired, or randomized) for 2, 3, 4, and 6 days for each configuration*

Treatment	Stimulus configuration	No. of treatment days (100 trials/day)	No. of animals per treatment
Paired	30 s LR on-90 s off	2	20
L and R	(1 trial = 120 s)	3	20
		4	20
		6	20
Unpaired	30 s L on-30 s off	2	20
L and R	30 s R on-30 s off	3	20
	(1 trial = 120 s)	4	20
		6	20
Randomized	30 s L on-0 to 240 s off	4	20
L and R	30 s R on-0 to 240 s off		
	(1 trial = variable to 270 s for each stimulus)		
Test-only	—	—	20
			Total animals = 200

In the random treatment, stimulus intervals were independently randomized, resulting in partial stimulus overlaps of 20–25%, with about 5 complete pairings occurring per 100 trials.

L = light; R = rotation.

within 0.1 minute per animal. This check was occasionally repeated with a second set of experimenters with the same result.

Within 30–45 minutes after baseline testing, animals were transferred for the first session of light-rotation trials to identical tubes containing clean sea water, dark-adapted for 10 minutes, then subjected to one of the treatments listed in Table I. Because we were interested in maximizing suppression of phototaxis, all sessions were 100 trials each, double the number originally shown to produce significant suppression (Crow and Alkon, 1978). A 60-minute latency test preceded each treatment session. Latencies were also measured in all groups 24-hours post-treatment (the first post-treatment test) on each of the next four days, and once per two days thereafter until each individual's latency recovered to the population median pre-treatment level. All daily latency tests were conducted at approximate 24-hour intervals. Latencies for individual animals were therefore recorded from a baseline value across all treatment days, then until response recovery was observed.

Because the extensive testing and treatment schedule precluded running control and experimental groups simultaneously, effects of variation between shipments and seasonal effects were controlled for by alternating paired and control treatments and by insuring that each treatment group contained animals from at least three different shipments.

In order to exclude possible effects of habituation and sensitization of phototaxis that might arise over several weeks of repeated testing of the same animal, it was first necessary to assess stability of latencies to light in naive animals over a one month period. An initial group of 10 animals was tested daily for 11 days, simulating 6 treatment days and 5 retention days, then once per 2 days until day 31, simulating a maximum retention of phototactic suppression of 25 days. Choice of an estimated maximum retention period of 25 days was based on results from other gastropods

(see Introduction). During the study similar data were obtained from ten additional animals. These 20 animals formed the test-only group (Table I).

A repeatedly tested group of animals sometimes included individuals which became unresponsive to light within the first 14 days and died within a month thereafter. Because the learned behavior is expressed as a decreased or absent response (to light), care was taken to exclude animals that may have become slow to respond due to a disease process. Therefore, data is reported only from test-only animals which survived in the laboratory at least two weeks after the test period ended.

Because cut-off scores were included in the data, statistical tests were primarily non-parametric (Siegel, 1956; Hollander and Wolfe, 1973). Two forms of latency scores were analyzed. First, all within-group differences and correlations were tested using raw scores in minutes. For the test-only group and for assessment of retention in individuals, raw scores were converted to suppression ratio (SR) scores of the form  $A/(A + B)$ , where  $A$  = baseline latency,  $B$  = latency on any subsequent test. A score of 0.50 indicates that baseline and subsequent test latencies were equal; lower scores mean that test latencies have slowed relative to baseline latencies.

### *Definitions*

We define the terms 'acquisition' and 'retention' as they apply to our description of the results as follows.

*Acquisition:* a statistically significant trend of increasing latencies to light as a function of two, three, four, or six consecutive treatment sessions. For each treatment group, latencies (in minutes) were arrayed from baseline values across latencies from the daily tests preceding each treatment session to the results of the first post-treatment test. This data was analyzed using Page's L-statistic for ordered alternatives, a non-parametric test useful for detecting trends in treatment effects. A value of L was calculated from latency scores ranked within each paired treatment group and control group and its level of significance determined.

*Retention:* retention of suppressed phototaxis in an individual is defined as the number of consecutive days post-treatment, beginning with the first post-treatment test, on which an animal's suppression ratio (SR) score was less than a median latency score derived from the frequency distribution of SR scores from the test-only group. Retention day scores were determined in this manner for both paired and control group animals.

## RESULTS

### *Stability of phototaxis in the test-only group*

Although within-individual latencies varied considerably on successive days, daily median SR scores calculated for the sample were stable over the 31-day test period. When tested against a time trend (days), these scores seemed to decrease with time, but not significantly (Theil test,  $C^* = 1.46$ ,  $P = 0.07$ , one-tailed). Median scores for individuals over the test period were all  $\geq 0.40$ .

The median score from the frequency distribution of all SR scores combined from this group, with 95% confidence limits, was  $SR = 0.44$  (0.41–0.45). Distribution of scores from the first one-third of the test period (days 2–11, consecutive daily tests) was not significantly different from that obtained over the second two-thirds (days 13–31, tests once/2 days) (Chi square = 6.95,  $df = 8$ ,  $P \leq 0.46$ , Fig. 2). Because we did not detect any significant changes in latency to light in this group

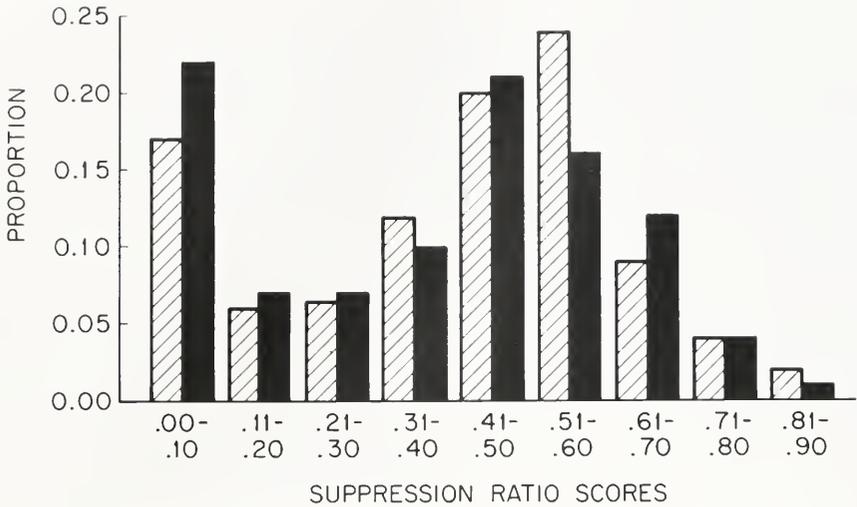


FIGURE 2. Frequency distributions of suppression ratio (SR) scores from the test-only group across days 2-11 (striped bars, 10 tests,  $n = 200$  scores), and across days 13-31 (shaded bars, 10 tests,  $n = 200$  scores). Median scores and 95% confidence intervals for each distribution are  $SR = 0.45$  (0.42-0.48) for days 2-11, and  $SR = 0.42$  (0.40-0.45) for days 13-31. The frequency class 0-0.10 consists entirely of cut-off scores.

over 31 days, we defined the lower 95% confidence limit of the frequency distribution of all scores combined,  $SR = 0.41$ , as a conservative score representing the average response obtained from a naive animal tested repeatedly over 31 days. This score is also the entering score for the modal class in the combined distribution, the 0.41-0.50 class. The number of consecutive tests in which an animal's SR score remained below this expected median value could, therefore, represent either a spontaneous run of increased latencies in a control treatment animal, or retention of associatively suppressed phototaxis in a paired treatment animal. Runs of increased latencies to light were considered to be extinguished whenever an individual achieved a test score  $\geq 0.41$ .

Next, we summarized the distribution of runs of increased latencies (scores  $< 0.41$ ) in the test-only individuals (Fig. 3). The most active animal scored  $< 0.41$  on 2/20 test days; at the other extreme one animal had a seven-day run of increased latencies. Seventy-five percent of the runs of suppressed phototaxis in this group were one or two test days in length (Fig. 3). The probability of a spontaneous run of scores  $< 0.41$  for as long as seven days was 1/20 animals, or  $P = 0.05$ .

There was no significant correlation between baseline latencies in minutes (18/20 animals responded in less than seven minutes), and the total number of days on which each animal scored  $< 0.41$  ( $r = -0.201$ ,  $df = 18$ ,  $P > 0.05$ ). Animals slower to respond to light in the baseline test were no more likely than initially faster animals to score  $< 0.41$  on repeated tests.

Because the trend (not significant) toward decreasing median latencies across days could have been a function of growth, we compared body lengths measured at the start and end of the 31-day test period. Increase in body length was significant, from  $2.32 \pm 0.59$  cm to  $3.54 \pm 0.71$  cm ( $t_{38} = 2.1617$ ,  $P < 0.05$ ). However, size was not correlated with latency measured on the same day either on day 1 ( $r = 0.267$ ,  $df = 18$ ,  $P > 0.05$ ) or on day 31 ( $r = 0.351$ ,  $df = 18$ ,  $P > 0.05$ ) of testing,

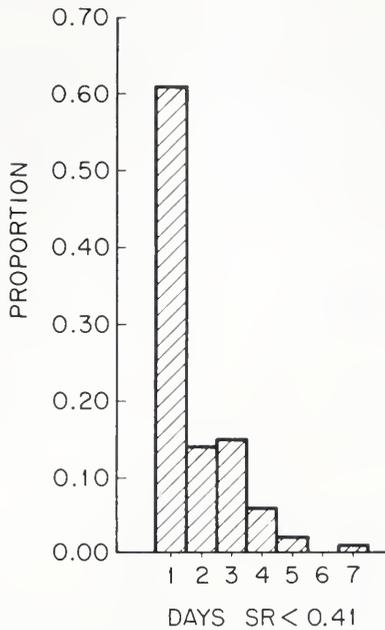


FIGURE 3. Frequency distribution of runs of spontaneously suppressed phototaxis in the test-only group. All animals scored  $<0.41$  at least once in 20 tests over 31 days.

indicating that larger animals in the sample were not consistently slower or faster than the smaller ones. Factors influencing latencies in naive animals across time were not identified in this study.

We conclude that positive phototaxis in *Hermisenda* does not significantly habituate or sensitize over one month of testing and that, within the limits reported, latencies are not a significant function of body size. Prolonged periods of reduced responsiveness to light in animals treated with paired stimulation may, when compared statistically to spontaneously occurring runs of suppressed phototaxis in control treatment animals, be assigned to long-term retention of associatively suppressed phototaxis.

### Acquisition

Ninety percent of all experimental animals responded to light within ten minutes in the baseline test. When baseline latencies were compared between all groups no significant difference was found (Kruskal-Wallis one-way ANOVA,  $H' = 13.578$ ,  $df = 9$ ,  $P \sim 0.14$ ).

Although results from the test-only group showed that latencies fluctuate on a daily basis, exposure of animals to paired stimulation should, if acquisition increases with experience, result in a trend towards increasing latencies with number of sessions in the paired but not the unpaired and random groups (Table II, Fig 4).

Arrays of within-group latencies, measured daily from baseline scores across treatment days to the first post-treatment test, were analyzed with Page's L-test for ordered alternatives. All groups (two, three, four, six days) exposed to paired light and rotation showed significant ordered increases in latency across treatment sessions ( $P \leq 0.05$ ). Page's L-statistic did not reach significance (at the 0.05 level) in any

TABLE II

Median latencies in minutes, corresponding median SR scores, and number of animals per group with cut-off scores listed for all treatments from baseline test to first post-treatment test

Paired treatment groups:				Unpaired and random treatment groups:			
	Median latency (min)	Median SR	No. cut- off scores		Median latency (min)	Median SR	No. cut- off scores
<i>2 days</i>				<i>2 days</i>			
Baseline	3.8	—	0	Baseline	4.2	—	0
Day 2	4.3	0.47	4	Day 2	3.6	0.54	7
Day 3	5.1	0.43	5	Day 3	3.2	0.57	1
<i>3 days</i>				<i>3 days</i>			
Baseline	4.0	—	0	Baseline	2.4	—	0
Day 2	6.4	0.38	2	Day 2	3.4	0.41	3
Day 3	5.7	0.41	6	Day 3	2.6	0.48	1
Day 4	15.2	0.21	6	Day 4	3.4	0.41	2
<i>4 days</i>				<i>4 days</i>			
Baseline	2.6	—	0	Baseline	3.3	—	0
Day 2	5.4	0.32	4	Day 2	2.4	0.58	5
Day 3	5.1	0.34	4	Day 3	7.4	0.31	4
Day 4	60+	0.04	11	Day 4	3.4	0.49	3
Day 5	60+	0.04	12	Day 5	4.1	0.45	1
<i>6 days</i>				<i>6 days</i>			
Baseline	2.9	—	0	Baseline	3.2	—	0
Day 2	3.8	0.43	6	Day 2	3.2	0.50	3
Day 3	9.9	0.23	8	Day 3	2.4	0.57	2
Day 4	60+	0.05	13	Day 4	3.4	0.48	3
Day 5	60+	0.05	10	Day 5	3.2	0.50	3
Day 6	60+	0.05	11	Day 6	3.7	0.46	3
Day 7	60+	0.05	14	Day 7	3.0	0.52	1
				<i>Random:</i>			
				<i>4 days</i>			
				Baseline	3.0	—	0
				Day 2	7.4	0.26	9
				Day 3	4.7	0.39	3
				Day 4	12.8	0.19	8
				Day 5	5.5	0.35	3
				<i>Unpaired*</i>			
				<i>1 day</i>			
<i>1 day*</i>				Baseline	3.0	—	0
Baseline	3.4	—	0	Day 2	3.0	0.50	17
Day 2	4.3	0.44	16				

\* *1 day* scores are from the 2 to 6 day treatment groups combined ( $n = 80$ ). Other values are for independent  $n = 20$  treatment groups.

group subjected to unpaired or random stimulation. Within all paired treatment groups, animals responded significantly more slowly to light on the first post-treatment test than during baseline testing (Wilcoxon matched-pairs signed-ranks test,  $P \leq 0.05$ , one-tailed). Unpaired and random groups did not show a significantly slower response to light when their first post-treatment test scores were compared to baseline latencies (Wilcoxon matched-pairs signed-ranks test,  $P > 0.05$ , one-tailed).

Although a separate group exposed to a single treatment session of 100 trials was not run, latencies measured 24 hours after the first session were available from

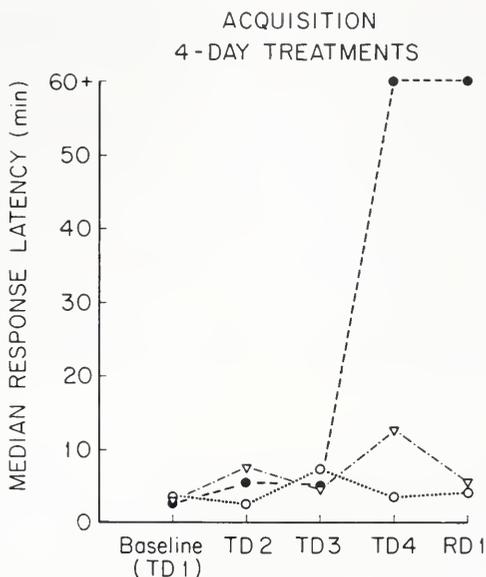


FIGURE 4. Relationship between median response latency in minutes and number of treatment sessions during acquisition in the 4-day groups. Animals were tested before each 100-trial session on treatment days (TD) 1-4. Retention day 1 (RD1) is the first post-treatment test. Paired treatment ●—●; unpaired treatment ○---○; random treatment ▽-·-▽. Note rapid increase in median response latency of the paired group after three treatment sessions.

all groups. Latencies were significantly slower than baseline values 24-hours after one paired treatment session (all paired treatment groups combined, Wilcoxon matched-pairs signed-ranks test,  $n = 77$ ,  $P = 0.002$ , one-tailed). No significant increases in latency were detected between latencies measured before and 24-hours after any control treatment (all unpaired groups combined, Wilcoxon matched-pairs, signed ranks test,  $n = 78$ ,  $P = 0.32$ , one-tailed; one random group, Wilcoxon test,  $n = 20$ ,  $P > 0.05$ ).

These results demonstrate that acquisition of the behavioral change, or increase in latency to respond to light, may be detected 24 hours after exposure to at least 100 trials with paired light and rotation. However, the increase in within-group median latency measured after one 100-trial session is small, approximately one minute (Table II), and large sample sizes of  $n \geq 80$  may be necessary in order to consistently measure a 24-hour associative effect. Latency increases within paired groups were consistently significant with sample sizes of  $n = 20$  when training sessions spanned two or more days. Trends in latency scores measured 24 hours after each unpaired or random treatment session were not significant even after six consecutive sessions.

Inspection of latency scores from the first post-treatment tests from all paired treatment groups indicated that the magnitude of the change in latency increased with the number of sessions. As the number of paired treatment sessions increased from two to six, the number of animals scoring  $<0.41$  on the first post-treatment test increased. Also, a discontinuity was observed in the latency score distributions that separated results of two and three treatment sessions from those of four and six sessions (Table II). Numbers of non-responding animals, that is, those that did not traverse the light gradient within 60 minutes and were given cut-off scores,

counted within each group were five and six on the first post-treatment test for animals trained two and three days, increasing to 12 and 14 for animals trained four and six days. Within unpaired and random groups, on the first post-treatment test, numbers of non-responding animals did not increase across sessions (Table II).

Next, within each paired-treatment group, latencies for those animals that responded to light before the 60-minute cut-off were compared with their baseline latencies. These distributions were not significantly different within the two or three day groups, but reached significance within the four and six day groups combined [Wilcoxon matched-pairs, signed-ranks test, one-tailed:  $n = 15$ ,  $P > 0.05$  (two days);  $n = 13$ ,  $P > 0.05$  (three days);  $n = 12$ ,  $P = 0.025$  (four and six days)]. These results suggest that individuals differ in sensitivity to the learning paradigm, with less sensitive animals showing significant behavioral suppression only after four or six paired treatment sessions.

### Retention

The discontinuity on the first post-treatment test between numbers of non-responding animals (within the 60-minute test) from paired groups treated two and three days *versus* four and six days is reflected in the retention day score distributions (post-treatment runs of SR scores  $< 0.41$ ). Animals subjected to paired light and rotation for four or six days had a significantly broader distribution of retention day scores than did the two and three day groups (Table III, Fig. 5A, B). Retention day scores were bimodally distributed in the four-day paired group; 14/20 animals scored 0–4 retention days and 6/20 scored 11–14 days. The distribution was smoother after six treatment days (Fig. 5A). Maximum retention was measured in the six-day group at 17–18 days (one animal). As the number of sessions increased both the number of animals scoring at least one retention day and the maximum number of retention days increased relative to the distribution of similarly determined runs of suppressed phototaxis in the unpaired and random groups (Fig. 5A, B).

TABLE III

*Tests of significance between retention day distributions*

Comparison	Statistic
Unpaired groups on 2, 3, 4, 6 treatment days and 4-day random group	$H' = 2.522$ , $df = 4$ (NS)
Paired groups on 2, 3, 4, 6 treatment days	$H' = 22.643$ , $df = 3$ , $P \leq 0.01$ (by Miller's multiple treatment comparisons: days 4, 6, sig. dif. ( $P \leq 0.05$ ) from days 2, 3)
2 days: paired <i>versus</i> unpaired	$D_{\max} = 3$ (NS)
3 days: paired <i>versus</i> unpaired	$D_{\max} = 6$ (NS)
4 days: paired <i>versus</i> unpaired	$D_{\max} = 8$ ( $P \leq 0.05$ )
4 days: paired <i>versus</i> random	$D_{\max} = 9$ ( $P \leq 0.05$ )
6 days: paired <i>versus</i> unpaired	$D_{\max} = 12$ ( $P \leq 0.01$ )

$H'$  = Kruskal-Wallis statistic (one-way ANOVA by ranks).

$D_{\max}$  = Kolmogorov-Smirnov statistic (test for broad alternatives).

All tests are one-sided.

NS = not significant at  $P \leq 0.05$ .

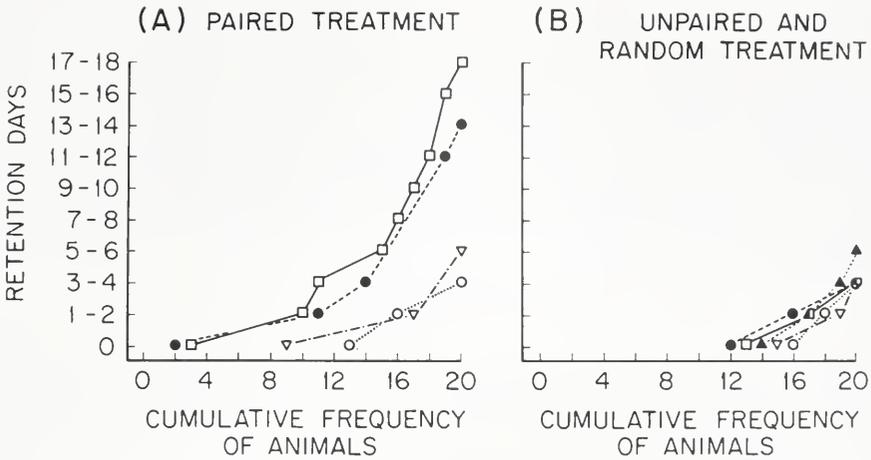


FIGURE 5. Relationship between retention day score distributions and number of treatment sessions, expressed as cumulative frequency of animals across retention day score classes. (A) Effect of paired treatments. Note the discontinuous distribution of retention days between the 2- and 3-day paired groups and the 4- and 6-day groups. (B) Effect of unpaired and random treatments. Distributions of post-treatment runs of spontaneously suppressed phototaxis for these groups. Note that more than half of each control group consisted of animals scoring zero retention days. Two days ○ --- ○; 3 days ▽ — · — ▽; 4 days ● --- ●; 6 days □ — □; 4-day random group ▲ --- ▲.

Significant increases in retention in paired relative to control groups were detected only for the four and six day groups (Table III). There was no significant correlation between baseline latencies of animals in the four and six day paired groups and their retention day scores ( $r = -0.0563$ ,  $df = 38$ ,  $P > 0.05$ ).

If animals scoring more than seven post-treatment retention days, scores unique to the paired treatment (Fig. 5A) and likely to occur with a probability of less than 0.05 (see results from the test-only group), are defined as 'long-retainers,' then 11/40 (27.5%) of the four plus six day paired groups may estimate that fraction of the laboratory population capable of long-term retention of associatively suppressed phototaxis.

*Can first post-treatment test scores predict retention day scores?*

Because the criterion for recovery of responsiveness to light to an average pre-treatment level was expressed in the form of an SR score ( $SR \geq 0.41$ ), latencies were analyzed for their predictive value in this form. SR scores  $< 0.41$  on the first post-treatment test were combined for all paired groups and arranged into four samples of scores for animals that subsequently scored 1, 2, 3-6, and 7+ retention days (medians = 0.28, 0.04, 0.08, and 0.05, respectively). Overall, these scores were significantly different (Kruskal-Wallis one-way layout,  $H' = 14.955$ ,  $df = 3$ ,  $P \leq 0.005$ ). The sample of animals that scored one retention day had significantly higher SR scores on the first post-treatment test than did samples of animals scoring more than one retention day (Dunn's multiple comparison method,  $P \leq 0.05$ ). From inspection of the data, 75% of animals with one retention day ( $n = 14$ ) had SR scores greater than 0.20, whereas among animals scoring more than one retention day ( $n = 39$ ) 75% scored less than  $SR = 0.20$ .

A similar analysis of SR scores from the unpaired groups resulted in no significant differences detected between SR scores arranged into three samples of 1,

2, and 3–4 retention day scores ( $H' = 0.669$ ,  $df = 2$ ,  $P \sim 0.70$ ). Median SR scores for these groups were 0.18, 0.15, and 0.16, respectively.

Discontinuities in behavioral responses between subgroups of paired treatment animals, such as differences in retention measured between animals scoring above or below  $SR = 0.20$  on the first post-treatment test or the bimodal distribution of retention days seen in the four-day paired treatment group, suggest that data on cellular events taken from animals within different behavioral subgroups be compared to see if the behavioral differences are reflected in biophysical and/or biochemical differences.

## DISCUSSION

Our data suggest that the extensive variation in retention observed among animals exposed to paired stimulation with light and rotation for four and six daily sessions may represent differences in individual capacities for associative learning. A factor that may contribute significantly to this variability is the level of food intake of the animals. Latency measurements obtained while determining an optimum feeding level for the experimental animals indicate that animals fed to excess (more than they would consume) tended to respond more slowly to light and with greater variability than did semi-starved animals.

Even greater variation between individuals may have been detected if experimental animals had not been selected for uniformly fast responsiveness to the light gradient before treatment. Most animals tested (70–100% of each sample of ten) were likely to have baseline latencies less than 30 minutes. Those animals with initially longer latencies (30–60+ minutes; not included in present study), when retested on a later day, either responded faster, equally slowly, or were unresponsive to the test light over ten or more test days (J. Harrigan, pers. obs.). Results reported here were obtained only from animals capable of a strong photopositive response during baseline testing, and may not be applicable to the small proportion of the population less responsive to light gradients.

Selection of initially fast animals, 90% with latencies less than ten minutes, also reduced the possibility of including in experiments animals whose latencies may have been suppressed by recent experience with paired light and gravitational stimulation in the ocean, or animals which did not recover from collection and shipment. Elapsed time between field collection and the start of an experiment was approximately 9–11 days. Our results indicate that only 27% of experimental animals (the four plus six day paired treatments) exhibited suppressed phototaxis for seven or more days, and that increased latencies during this period were usually in excess of 60 minutes (cut-off scores). However, when working with animals from wild populations the influence of prior experience and the effect of behavioral 'savings' (Crow and Alkon, 1978) on subsequent experiments on learning cannot be completely ruled out.

In previous investigations, animals were trained with light and rotation on a schedule consisting of 50 trials per day for three days. Routinely, statistically significant latency increases were found using either within-group tests or comparisons with the latencies of control groups. Previously published values for mean and median latency scores for paired groups, expressed as minutes or SR scores, are similar to values reported here for three days of training with 100 trials per day. Crow and Alkon (1978) report a median SR score of 0.30 for a paired treatment group 48 hours post-treatment with 150 trials over three days (present study = 0.21

SR; at 24 hours post-treatment, Table II). Farley and Alkon (1982) present two figures (Figs. 1, 2, Farley and Alkon, 1982) comparing mean latencies in minutes and SR scores for groups of paired treatment animals in a horizontal light gradient. They report mean baseline latencies of approximately 9–13 minutes (present study = 2.6–4.2 minutes, Table II), and 24 hours post-treatment latencies of about 24–28 minutes, or SR about 0.30–0.35 (present study = 15.2 minutes, SR = 0.21, Table II). Mean increase in latency between the baseline and first post-treatment tests was estimated at 15 minutes in Farley and Alkon's experiment, and 11 minutes in the present study. Longer latencies in the baseline test in Farley and Alkon's experiment may reflect differences in maintenance and in experimental lighting conditions; however, the mean latency increase after 150 trials was nearly the same as that reported here for 300 trials over three days.

These results suggest an interaction between treatment days and trial density during acquisition. Latency measurements for all paired treatment groups in our study show an average increase of 1.3–7.0 min between baseline and day three tests (after two training days), increasing to 11.2–60+ minutes after three training days (Table II). We found consistently large increases in mean latency (to 60+ minutes) mainly in the groups trained four and six days; shorter training schedules were associated with increased variability between groups (Table II). Although significant acquisition was measured in all paired-treatment groups, a significant increase in the number of days that the association was retained was measured only in groups trained more than three days (Table III).

Retention may also be affected by trial density. Longer retention of associative learning after spaced rather than massed trials has been demonstrated in *Lymnaea* (Alexander *et al.*, 1982), which is also capable of significant acquisition after one trial (Alexander *et al.*, 1984). Retention of habituation and sensitization of the gill-and siphon-withdrawal reflex in *Aplysia*, two non-associative forms of learning, were also significantly enhanced by spaced rather than massed trials (Carew *et al.*, 1972; Pinsker *et al.*, 1973). Influence of trial density on retention in *Hermisenda* has not yet been quantified.

Each post-treatment test can also be considered a measure of resistance to extinction. Retention of suppressed phototaxis was surprisingly persistent considering the frequency with which latencies were measured. Longer periods of suppression may possibly have been measured with a less frequent testing schedule. Also, because the presence of learning in *Hermisenda* was expressed as a reduction in responsiveness (to light), an animal could be assigned a retention day score only when its latency recovered to the average score for the naive population,  $SR \geq 0.41$ . It is possible that some animals were 'permanently' trained, and that retention exceeding 18 days was missed because animals died before recovering to  $SR > 0.41$  and were therefore excluded from the data.

Associatively suppressed phototaxis has been demonstrated in laboratory-reared *Hermisenda*. Three consecutive generations of animals reared from wild parents (the  $F_1$ ,  $F_2$ , and  $F_3$  generations) acquired the behavioral change after three days of training with 50 trials per day of paired light and rotation (Crow and Harrigan, 1979). These cultured populations showed significantly less variation in their latencies to respond to light than did animals from wild populations (Crow and Harrigan, 1979). It is not known if retention day scores would also be less variable in laboratory-reared animals. Along with possible heritable components of learning, the ability of an individual *Hermisenda* to acquire this associative task is also influenced by environmentally induced alterations in sensory system morphology

that occur during larval and juvenile development (Crow and Harrigan, 1979; Harrigan, Crow, Kuzirian, and Alkon, in prep.).

In a review of definitions of learning as they might apply to *Pleurobranchaea*, Mpitsof *et al.* (1978) concluded that the effects of different controls on neural functioning must be understood before the one most appropriate for the particular feature of learning under investigation can be selected. Choice of controls for conditioning procedures, especially for initial demonstrations of associative learning, have generally been some combination of naive, unpaired, random, and single stimulus presentations tailored to demonstrate specificity of the particular association under study to the temporal pairing of stimuli. In recent studies on *Hermisenda* combining behavioral and cellular analyses, the control treatment selected has been either random or unpaired. In the random control, partial stimulus overlaps were obtained with separately randomized light and rotation plus a small number (fewer than 5) of complete pairings, providing a conservative control against which cellular events induced by paired stimulation can be assessed. In the unpaired control, it is assumed that no association between stimuli is formed (for example, Crow and Alkon, 1982).

In the present study and other studies on *Hermisenda* in which acquisition was measured 24 hours post-treatment, no significant behavioral differences were found within or between unpaired, random, or single stimulus treatment groups; these controls were behaviorally identical. Because our study included only behavioral data, adequate controls were considered to be unpaired groups corresponding to each paired treatment, and a single random (four-day) control treatment as a check for the presence of any non-associative effects that might have accrued from increasing the number of trials and sessions over those previously used. The small number of complete pairings that occurred during the random treatment (15) had no measurable effect on post-treatment relative to baseline latencies.

In a series of conditioning experiments on *Hermisenda*, Crow (1983) found significant non-associative effects, affecting paired and random treatments equally, 30 minutes after exposure to 50 paired trials with light and rotation. These non-associative effects decremented by 45 minutes post-training when paired and random groups became significantly different, reflecting the appearance of longer-term associative effects and did not accumulate over multiple training sessions (Crow, 1983). Non-associative effects, therefore, were not detectable with the 24-hour interval employed in the present study.

The behavioral change is pairing-specific and affects phototaxis in both vertical and horizontal planes (Crow and Alkon, 1978; Farley and Alkon, 1982). Animals have been observed, after training, to move around in non-gradient illumination and feed normally (J. Harrigan, pers. obs.), indicating that only orientation components have been affected. Because strong positive phototaxis appears primarily in semi-starved animals (Alkon *et al.*, 1978; present study), Alkon (1980) has suggested that phototactic suppression may enhance survival in the natural habitat by inhibiting migration from depleted food supplies into brightly lit surface waters during strong surge, which the rotational stimulus mimics.

Hypotheses regarding possible adaptive advantages of conditioned phototactic suppression and its behavioral variability will have to be tested in the field rather than the laboratory. Differences in patterns of light and gravitational stimulation between the laboratory and the animals' natural habitat, as well as differences in the stimulus parameters themselves, especially the rotational stimulus employed in the laboratory, preclude any generalizations from behavioral results obtained in the laboratory to naturally occurring behavior patterns.

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## HISTOSPECIFIC ACETYLCHOLINESTERASE DEVELOPMENT IN QUARTER ASCIDIAN EMBRYOS DERIVED FROM EACH BLASTOMERE PAIR OF THE EIGHT-CELL STAGE

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### ABSTRACT

Recent cell lineage studies of ascidian embryos have shown that muscle cells of the larval tail are derived not only from the B4.1-cell pair of 8-cell embryos, as was formerly believed, but also from the b4.2- and A4.1-cell pairs. Therefore, we re-examined the developmental autonomy of blastomere pairs in 8-cell ascidian embryos. The four blastomere-pairs (a4.2, b4.2, A4.1, and B4.1) were isolated from the 8-cell embryos of *Ciona intestinalis* and *Halocynthia roretzi* and allowed to develop into quarter embryos. More than 80% of the B4.1 quarter embryos of both species produced histochemically detectable, putative muscle-specific acetylcholinesterase (AChE). About 10% of the *Ciona* b4.2 quarter embryos and 1% of the *Halocynthia* b4.2 quarter embryos showed AChE activity. About 2% of the *Halocynthia* A4.1 quarter embryos developed AChE activity, but none of the *Ciona* A4.1 quarter embryos showed AChE activity. Although the frequency of the b4.2 or A4.1 quarter embryos with AChE activity was relatively low, these results indicate that not only isolated B4.1 blastomeres but also isolated b4.2 or A4.1 blastomeres could produce AChE independently from the interaction with progeny cells of the other pairs. In addition, about 3–4% of the a4.2 quarter embryos of both species produced AChE. This activity, found in cells thought not to contribute to the muscle cell lineage, may be due to the expression of AChE activity in the larval brain of *Ciona* and the larval brain and pharynx of *Halocynthia*.

### INTRODUCTION

Descriptive and experimental studies have demonstrated that the selection of different developmental pathways in ascidian embryos is not mediated by a stable intrinsic nuclear lineage but by cytoplasmic determinants localized in predetermined regions of the egg (Conklin, 1905a, b; Reverberi and Minganti, 1946; Whittaker, 1973, 1980, 1982; Tung *et al.*, 1977; Deno and Satoh, 1984; Deno *et al.*, 1984). The cytoplasmic determinants are thought to be segregated by cleavage into certain lineage cells, where they bring about the activation of genes responsible for tissue-specific enzyme development (see Davidson, 1976; Whittaker, 1979; Jeffery *et al.*, 1984 for reviews).

One line of evidence for the segregated cytoplasmic determinants is the capacity of isolated blastomeres to differentiate autonomously according to the developmental fates predicted by their cell lineages (see Reverberi, 1971; Whittaker, 1979 for reviews). According to the cell lineages devised by Conklin (1905a) and Ortolani (1955), muscle cells of the larval tail originate from the B4.1 pair of blastomeres (the posterior vegetal blastomeres) of the 8-cell stage (Fig 1). Autonomous develop-

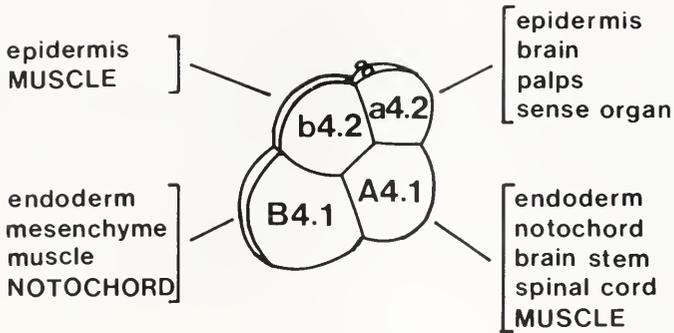


FIGURE 1. A diagram illustrating the nomenclature of blastomeres of the 8-cell-stage ascidian embryos according to Conklin (1905a), and also listing the derivatives from each blastomere pair. The derivatives shown with small letters are based on previous studies (Conklin, 1905a; Ortolani, 1955) while those with capitals are those obtained in our recent study (Nishida and Satoh, 1983).

ment of a putative muscle-specific enzyme, acetylcholinesterase (AChE), and of myofibrils has been shown in partial ascidian embryos derived from isolated B4.1 pairs (Whittaker *et al.*, 1977; Crowther and Whittaker, 1983). Recent analyses of cell lineages in ascidian embryos, however, have demonstrated that muscle cells are derived not only from the B4.1-cell pair, as was formerly believed, but also from both the A4.1- (the anterior vegetal blastomeres) and b4.2-cell pairs (the posterior animal blastomeres) (Fig. 1; Nishida and Satoh, 1983; Zaloker and Sardet, 1984). In a previous study, we isolated the B4.1-cell pairs from 8-cell *Ciona* embryos and showed that AChE development as well as myofibril differentiation took place in partial embryos originating not only from isolated B4.1 pairs, but also from 8-cell embryos lacking B4.1 progeny cells (Deno *et al.*, 1984). The goal of the present study was to determine the ability of quarter embryos derived from the isolated B4.1, A4.1, b4.2, and a4.2 pairs to express AChE activity.

## MATERIALS AND METHODS

### *Animals, gametes, and embryos*

Eggs of the ascidians *Ciona intestinalis* (L.) and *Halocynthia roretzi* (Drashe) were used in this study. *C. intestinalis* adults were collected at Takahama, Wakasa Bay, Japan and maintained in temperature-controlled aquaria (18°C) under constant light to induce oocyte maturation. Eggs were removed surgically from the gonoducts and fertilized with a dilute suspension of sperm of other individuals. Fertilized eggs were reared in filtered sea water at 18°C. Under these conditions they reached the 8-cell stage about 2 h after fertilization and hatched at about 17 h of development. *H. roretzi* adults were collected in Mutsu Bay, Aomori, Japan and maintained in aquaria of the Marine Biological Station of Asamushi. Naturally spawned eggs were fertilized by mixing sperm suspensions of different animals, and reared in filtered sea water at 13°C. At this temperature, they developed to the 8-cell stage about 3.5 h after fertilization and hatched at about 34 h of development.

### *Blastomere isolations*

Each experiment was carried out in a room in which the temperature was 18°C for *Ciona* embryos and 13°C for *Halocynthia* embryos. Fertilized eggs of both species

were dechorionated with sharpened tungsten needles between 10 and 30 min after fertilization. Dechorionated eggs were cultured to the 8-cell stage in 0.9% agar coated Falcon petri dishes. Only 8-cell embryos of normal appearance were used for the experiments. The four cell-pairs of the 8-cell embryos (*i.e.*, a4.2 + a4.2, b4.2 + b4.2, A4.1 + A4.1, and B4.1 + B4.1) were separated with a glass needle under a dissecting microscope (Fig. 2). The location of polar bodies, the configurations of the blastomeres, and the distribution of pigments were used as landmarks for orientation of the embryos. Isolated blastomeres were cultured separately in 0.9% agar coated Falcon 24-well multiwells. For culture of dechorionated embryos and isolated blastomeres, Millipore-filtered (pore size, 0.2  $\mu\text{m}$ ) sea water containing 50  $\mu\text{g}/\text{ml}$  streptomycin sulfate was used. Dechorionated control embryos and isolates were reared until the appropriate developmental stages, and then fixed for histochemical examinations.

### Enzyme histochemistry

AChE is thought to be a tissue-specific enzyme of muscle cells in the tail of developing ascidian embryos (Durante, 1956; Meedel and Whittaker, 1979). The enzyme activity was detected histochemically in dechorionated whole embryos as well as in partial embryos by the "direct-coloring" thiocholine method of Karnovsky and Roots (1964) using acetylthiocholine iodide as a substrate. In the case of *C. intestinalis* the embryos were first cooled at 4°C then fixed with cold (4°C) 80% ethanol for only 30 s as described previously (Deno *et al.*, 1984), whereas *Halocynthia* embryos were fixed with cold (4°C) 5% formalin sea water for 30 min. It has been revealed that the enzyme activity detected by the present histochemical technique is attributable to the presence of AChE and not to pseudocholinesterase (Fromson and Whittaker, 1970; Meedel and Whittaker, 1979; Satoh, 1979). Histochemically stained embryos were dehydrated in a graded series of ethanol solutions, cleared in xylene, and mounted in balsam for microscopic examination and photomicrography.

## RESULTS

### Acetylcholinesterase development in control embryos

Dechorionated eggs were allowed to develop in microwells for the same length of time as that of partial embryos. These control embryos of both species, however, did not always develop to the normal tailbud stage. Although the reason is obscure, a similar inclination was noticed in previous studies (Whittaker, 1982; Deno *et al.*,

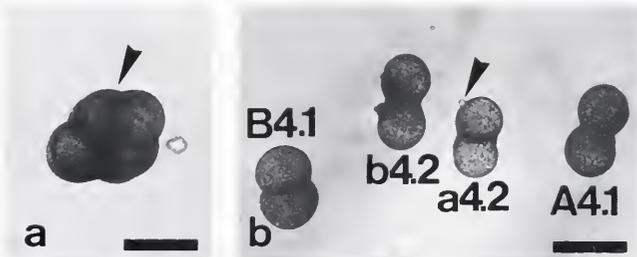


FIGURE 2. Photomicrographs demonstrating blastomere isolation. (a) Dechorionated 8-cell-stage *Ciona* embryo viewed from the left side. (b) Isolated blastomere pairs from the 8-cell-stage embryo. Arrowheads indicate the polar bodies, which mark the animal pole of the egg. Scale bar, 50  $\mu\text{m}$ .

1984). In this study, 64% (234/264) of the dechorionated 8-cell-stage embryos of *C. intestinalis* formed morphologically normal tailbud stages. However, almost all of these tailbud stages which had developed for more than 8 h produced AChE activity in tail muscle cells (Fig. 3a). In addition to the enzyme activity localized in muscle cells, some control embryos examined at 13.5 h of development showed the enzyme activity in a strand-like structure in the dorsal region of the head, as did almost all of the control embryos which had developed for more than 15 h (Fig. 3b). Furthermore, a spot of AChE activity appeared in a region posterior to melanocytes of all 17-h embryos examined (Fig. 3c). This spot appeared to be attached to the strand-like structure (Fig. 3c). From its location, the spot of AChE activity seems to be a part of the central nervous system, which may presumably be the "adult brain," as has been pointed out by Meedel and Whittaker (1979).

More than half of dechorionated 8-cell embryos of *H. roretzi* failed to complete neural-tube formation, resulting in morphologically abnormal embryos. However, these abnormal embryos, as well as morphologically normal tailbud stages which had developed for more than 14 h, showed AChE activity in tail muscle cells (Fig. 4a), suggesting a normal production of AChE in spite of the failure of normal

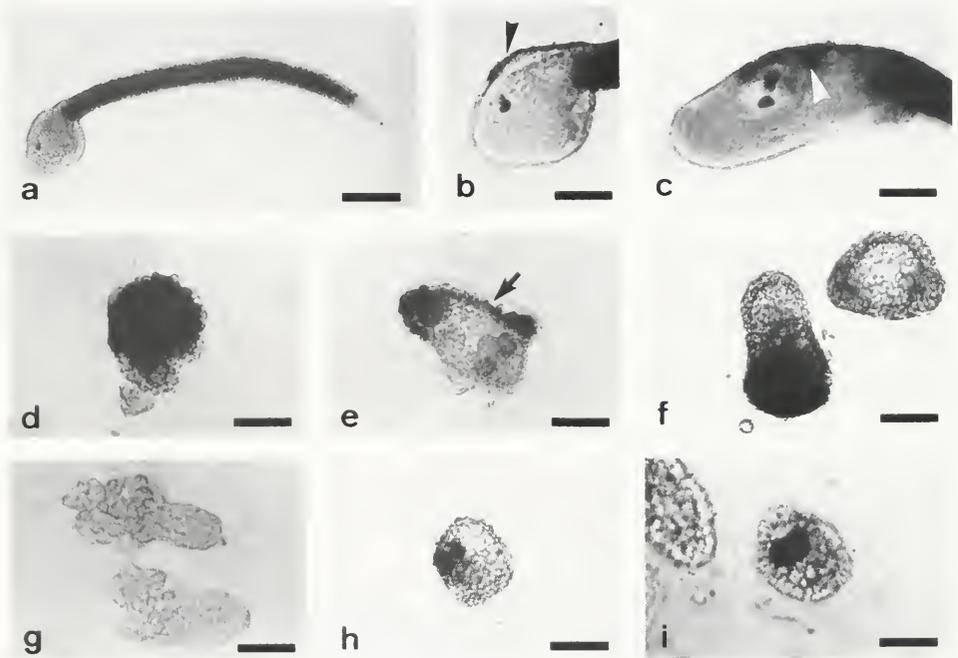


FIGURE 3. Histochemical localization of AChE activity in *C. intestinalis* whole and quarter embryos of various ages. (a) Tailbud-stage embryo developed from dechorionated whole egg (13 h of development). AChE activity developed only in the muscle cells of the tail. (b, c) Head regions of dechorionated whole embryos after 13.5 h (b) and 19 h (c) of development, respectively. Arrowhead in (b) indicates a strand-like structure with AChE activity and arrowhead in (c) indicates "adult brain" with the enzyme activity. (d) B4.1 quarter embryo showing AChE activity (13.5 h of development). (e, f) B4.2 quarter embryos with AChE activity; (e) 13.5 h, (f) 19 h. A right quarter embryo in (f) does not show the enzyme activity. Arrow indicates a strand-like ectodermal structure with the enzyme activity. (g) A4.1 quarter embryos (13.5 h of development). They did not show AChE activity. (h, i) A4.2 quarter embryos at 19 h (h) and 22 h (i) of development, respectively. A patch of cells clearly show the enzyme activity. Scale bars, 50  $\mu$ m in (a) and 25  $\mu$ m in (b-i).

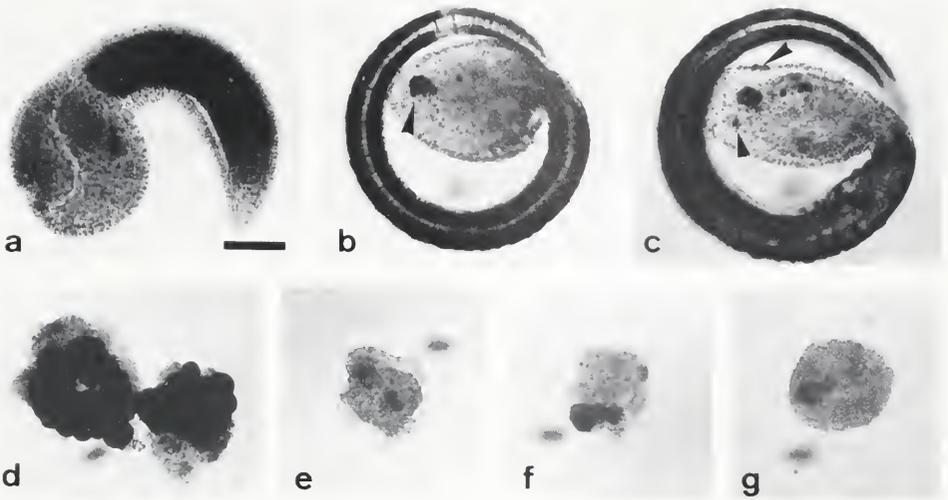


FIGURE 4. Histochemical localization of AChE activity in *H. roretzi* whole and quarter embryo of various ages. (a, b, c) Dechorionated whole embryos after 22 h (a), 28 h (b), and 32 h (c) of development, respectively. In (a) muscle cells of the caudal tip region do not produce AChE, but in (b) and (c) they show the enzyme activity. Arrowhead in (b) indicates the primordial pharynx with AChE activity and arrowheads in (c) indicate small spots with the enzyme activity. (d) B4.1 quarter embryos with AChE activity (30 h of development). (e) b4.2 quarter embryo at 30 h of development. A few cells show the enzyme activity. (f) A4.1 quarter embryo with the enzyme activity (30 h of development). (g) a4.2 quarter embryos developing AChE activity in a few cells (46 h of development). Scale bar, 100  $\mu$ m.

morphogenesis. At this time, however, muscle cells of the caudal tip of the tail did not show the enzyme activity; AChE activity in this region first appeared in 24-h embryos (Fig. 4b). Like *Ciona* embryos, normal *Halocynthia* embryos also produced AChE in tissues other than the tail muscle cells. AChE activity was noticed in the region between the sensory vesicle and the papillae in 24-h embryos (Fig. 4b). This structure is in the region of the primordial pharynx. In addition, 32-h embryos produced AChE in several other small spots near both dorsal and ventral sides of the primordial pharynx region (Fig. 4c).

#### *Acetylcholinesterase development in quarter embryos*

In this report, partial embryos derived from isolated a4.2-, b4.2-, A4.1-, and B4.1-blastomere pairs are designated for convenience as a4.2, b4.2, A4.1, and B4.1 quarter embryos, respectively. AChE activity in these quarter embryos is summarized in Table I (*C. intestinalis*) and in Table II (*H. roretzi*).

*The B4.1 quarter embryos.* According to recent cell lineage study (Nishida and Satoh, 1983), among the 36 (*C. intestinalis*) or 42 (*H. roretzi*) muscle cells of the larval tail, 28 cells located in the anterior and middle parts of the tail originate from the B4.1 pair of blastomeres. In *C. intestinalis* isolated B4.1-pairs usually developed to raspberry-shaped partial embryos (Fig. 3d). B4.1 quarter embryos which developed for more than 12 h (beyond the normal time of AChE synthesis) produced AChE activity (Fig. 3d) at a high frequency (92% on an average; Table I). The B4.1 quarter embryos of *H. roretzi* which had developed for more than 22 h also became raspberry-like cell aggregates (Fig. 4d). They also frequently developed AChE activity (81% on an average; Table II; Fig. 4d).

TABLE I  
*Development of histochemically detectable acetylcholinesterase activity in quarter Ciona intestinalis embryos*

Origin of quarter embryos	Time (h) of development No. of batches	No. of embryos with acetylcholinesterase activity							Total	
		12	13.5	15	17	19	22	10		
B4.1-cell pair	3									
	$\frac{53}{59}$ (90%)	$\frac{76}{78}$ (97%)	$\frac{42}{48}$ (88%)	$\frac{20}{23}$ (87%)	$\frac{14}{14}$ (100%)	$\frac{29}{32}$ (91%)	$\frac{234}{254}$ (92%)			
b4.2-cell pair	3									
	$\frac{0}{82}$ (0%)	$\frac{3}{51}$ (6%)	$\frac{6}{44}$ (14%)	$\frac{5}{42}$ (12%)	$\frac{3}{23}$ (13%)	$\frac{4}{37}$ (11%)	$\frac{21}{279}$ (8%)			
A4.1-cell pair	3									
	$\frac{0}{78}$ (0%)	$\frac{0}{56}$ (0%)	$\frac{0}{38}$ (0%)	$\frac{0}{30}$ (0%)	$\frac{0}{14}$ (0%)	$\frac{0}{33}$ (0%)	$\frac{0}{249}$ (0%)			
a4.2-cell pair	3									
	$\frac{0}{82}$ (0%)	$\frac{0}{69}$ (0%)	$\frac{2}{45}$ (4%)	$\frac{1}{30}$ (3%)	$\frac{2}{23}$ (9%)	$\frac{4}{38}$ (11%)	$\frac{9}{287}$ (3%)			

TABLE II

*Development of histochemically detectable acetylcholinesterase activity in quarter Halocynthia roretzi embryos*

Origin of quarter embryos	Time (h) of development No. of batches	No. of embryos with acetylcholinesterase activity					Total
		22	26	30	46		
		4	3	5	5	17	
B4.1-cell pair		$\frac{147}{242}$ (61%)	$\frac{307}{357}$ (86%)	$\frac{200}{208}$ (96%)	$\frac{317}{389}$ (82%)	$\frac{971}{1196}$ (81%)	
b4.2-cell pair		$\frac{0}{252}$ (0%)	$\frac{2}{361}$ (1%)	$\frac{2}{187}$ (1%)	$\frac{10}{313}$ (3%)	$\frac{14}{1113}$ (1%)	
A4.1-cell pair		$\frac{2}{215}$ (1%)	$\frac{3}{314}$ (1%)	$\frac{2}{218}$ (1%)	$\frac{13}{352}$ (4%)	$\frac{20}{1099}$ (2%)	
a4.2-cell pair		$\frac{0}{268}$ (0%)	$\frac{5}{338}$ (2%)	$\frac{3}{201}$ (2%)	$\frac{35}{327}$ (11%)	$\frac{43}{1134}$ (4%)	

*The b4.2 quarter embryos.* A recent cell lineage study has reported that 4 (*C. intestinalis*) or 10 (*H. roretzi*) muscle cells of the caudal tip region are derived from the b4.2-cell pair (Nishida and Satoh, 1983). The b4.2 quarter embryos of *C. intestinalis* developed into a blastula-like structure with a cell mass in the cavity (Figs. 3e, f). AChE activity was not found in the b4.2 quarter embryos examined at 12 h of development (0/82; Table I). However, localized enzyme activity began to appear in some of the b4.2 quarter embryos at 13.5 h (3/51, 6%; Table I), and an average of 12% of the b4.2 quarter embryos which had developed for more than 15 h showed AChE activity (Figs. 3e, f; Table I). Most of the b4.2 quarter embryos that developed AChE activity showed it only in a small number of cells in the interior portion of the blastula-like structure (Fig. 3f), but some embryos developed the enzyme activity not only in these cells but also in the cell of a strand-like ectodermal structure (Fig. 3e).

The b4.2 quarter embryos of *H. roretzi* also developed into blastula-like structures with a cell-mass in the cavity (Fig. 4e). No AChE activity was found in the b4.2 quarter embryos examined at 22 h of development (0/252; Table II). The b4.2 quarter embryos which had developed for more than 26 h, however, produced localized enzyme activity, although the frequency was very low (14/861, 1.6%; Table II; Fig. 4e). In contrast to *Ciona* b4.2 quarter embryos, a strand-like structure with the enzyme activity did not appear in *Halocynthia* b4.2 quarter embryos.

*The A4.1 quarter embryos.* Four muscle cells of the posterior part of the larval tail are the descendants of the A4.1-cell pair. Isolated A4.1-cell pairs of *C. intestinalis* gave rise to gourd-shaped partial embryos. However, no AChE activity was detected in the A4.1 quarter embryos examined at times between 12 and 22 h after fertilization (0/249; Table I; Fig. 3g). The A4.1 quarter embryos of *H. roretzi* became raspberry-shaped structures similar to those produced by the B4.1 quarter embryos. Although the percentage of the positive embryos was low (2% on the average; Table II), the A4.1 quarter embryos did develop enzyme activity in some of their cells (Fig. 4f).

*The a4.2 quarter embryos.* According to all the current ascidian cell lineage studies the a4.2-cell pair gives rise to cells of the epidermis, brain, and sensory organs, but not to tail muscle cells (Fig. 1). The a4.2 quarter embryos of *C. intestinalis*, similar to the b4.2 quarter embryos, developed into blastula-like structures with clumps of tissue in the cavity (Figs. 3h, i). Unexpectedly, some of the a4.2 quarter embryos also developed AChE. The enzyme activity first appeared at 15 h of development (4%; Table I), and the proportion of quarter embryos with enzyme activity increased to about 10% by 19 h of development (Table I). The distribution of AChE activity in the a4.2 quarter embryos was not spread but localized in particular cells (Figs. 3h, i).

Blastula-like a4.2 quarter embryos of *H. roretzi* also produced AChE activity, which first appeared after 26 h of development (Fig. 4g). About 11% of the 46-h, a4.2 quarter embryos also showed the enzyme activity (Table II).

## DISCUSSION

More than two-thirds of muscle cells in the larval tail are descendants of the B4.1-cell pair at the 8-cell stage. As clearly shown in this study, if the B4.1 blastomeres are isolated and allowed to develop into quarter embryos, these partial embryos produce AChE at a high frequency (more than 90% in *Ciona* and about 80% in *Halocynthia*). This result confirms previous studies (Whittaker *et al.*, 1977; Whittaker, 1982; Deno *et al.*, 1984).

The b4.2 quarter embryos of both species as well as *Halocynthia* A4.1 quarter embryos also showed the enzyme activity. In *Ciona* b4.2 quarter embryos AChE activity was found in a strand-like epidermal structure in addition to some cells in the interior of the blastula-like structures that formed. According to the most recent cell lineage study (Nishida and Satoh, 1983) a strand of epidermal cells at the dorsal head region of tailbud embryos is derived from the b4.2-cell pair, and as shown in this study, this structure can develop AChE. Therefore, the strand-like structure exhibiting AChE activity in the *Ciona* b4.2 quarter embryos may be correlated with the epidermal structure of normal embryos.

The frequency of AChE development in b4.2 or A4.1 quarter embryos was very low, particularly in A4.1 quarter embryos. The reason for this is obscure, but blastomere isolation could disturb the normal division pattern of the isolated cells, causing abnormal segregation of the cytoplasmic determinants responsible for AChE development. However, the possibility that induction phenomena between blastomeres are involved in AChE development in b4.2 or A4.1 quarter embryos cannot be ruled out (Pucci-Minafra and Ortolani, 1968). So far, several blastomere isolation experiments in ascidian embryos have demonstrated muscle cell development in partial embryos lacking the progeny cells of B4.1 blastomeres (Von Ubisch, 1939; Reverberi and Minganti, 1946, 1947). In the previous study, for instance, we showed that more than 85% of *Ciona* a4.2 + b4.2 + A4.1 partial embryos developed AChE (Deno *et al.*, 1984). However, we know of no report of muscle development in the b4.2 or A4.1 quarter embryos. If cleavage-stage ascidian embryos are permanently arrested with cytochalasin B, an inhibitor of cytokinesis, the arrested embryos produced AChE in muscle lineage blastomeres (Whittaker, 1973a; Satoh, 1979). However, the blastomeres which developed enzyme activity were only the B4.1-line muscle lineage cells; the b4.2- and A4.1-line muscle lineage cells did not produce the enzyme. This phenomenon may be related to the low frequency of b4.2 or A4.1 quarter embryos that show the enzyme activity. In any event the present study clearly shows that, in addition to the B4.1 quarter embryos, the b4.2 or A4.1 quarter

embryos also produced AChE, although the frequency of the embryos with the enzyme activity is low. The results also support the recent cell lineage study suggesting that muscle lineage cells of the ascidian embryos arise from more than one line of founder cells.

Unexpectedly, about 3–4% of the a4.2 quarter embryos of both species also developed AChE. As shown in a previous study (Meedel and Whittaker, 1979) as well as in this study, *Ciona* embryos and larvae produce AChE in the brain, whereas *Halocynthia* embryos produce AChE in the brain and/or the pharynx region, in addition to the tail muscle cells. The brain and pharynx originate from the a4.2-cell pair at the 8-cell stage (Nishida and Satoh, 1983). In each species the time of the first appearance of AChE activity in the a4.2 quarter embryos almost coincided with that at which normal embryos differentiated the enzyme activity in these tissues. Therefore, it is unlikely that AChE development in the a4.2 quarter embryos is related to muscle differentiation. Instead it is probably associated with the differentiation of the brain cells (*Ciona* and *Halocynthia* embryos) or with pharynx cells (*Halocynthia* embryos).

This study suggests a way to improve the biological assay system for identifying and purifying the cytoplasmic determinant(s) responsible for AChE development. Recently, we have transplanted cytoplasm from B4.1 blastomeres of 8-cell *Halocynthia* embryos into the A4.1 blastomeres of another embryo by microinjection (Deno and Satoh, 1984). When the host 8-cell embryos were then arrested with cytochalasin B, a few of them developed AChE activity in the A4.1 cells in addition to the B4.1 cells. At the time these experiments were being done this result suggested a possible assay system for isolation and characterization of cytoplasmic determinants. The recent cell lineage study, however, revealed that some of the tail muscle cells are derived from the A4.1 blastomeres, and suggest that a better assay system might be developed by transplanting cytoplasm from the B4.1 cells into the a4.2 cells (Nishida and Satoh, 1983). However, as clearly shown in this study, a finite number of the a4.2 blastomere progeny also produce AChE without differentiating into muscle. Therefore, the a4.2 cells are not necessarily the most desirable hosts for the assay system. Instead, cytoplasm from B4.1 cells or from isolated yellow crescents (Jeffery *et al.*, 1984) should be injected into an isolated A4.1-cell pair and be allowed to develop into a quarter embryo. If five or more progeny cells of the transplanted A4.1-cell pair showed the enzyme activity, this could be used as a potential biological assay for cytoplasmic determinants.

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## HOLOTHURIAN OOCYTE MATURATION INDUCED BY RADIAL NERVE<sup>1</sup>

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### ABSTRACT

Endogeneous substances responsible for maturation of holothurian oocytes were examined. Water-extracts of radial nerves from five species of sea cucumbers induced oocyte maturation. Cross-experiments on the maturation-inducing activity of radial nerve extracts indicated that the radial nerve extracts cross-react effectively among the sea cucumbers examined. From its heat-stability, protease-sensitivity, dialyzability, and elution-patterns through 'Sephadex' G-15 and G-50 columns, the active factor appears to be a heat-stable peptide of several thousands' daltons. The active factor, termed radial nerve factor, acted on oocytes in isolated ovaries or isolated oocytes having follicle cells to induce maturation. It was ineffective on oocytes deprived of follicle cells. Isolated ovaries or testes incubated with radial nerve factor produced a secondary factor which directly induces maturation of the follicle cell-free oocyte. Follicle cells isolated from follicle-oocyte complexes also produced the secondary factor in the presence of radial nerve factor. These results show that the radial nerve factor stimulates the follicle cells to produce a secondary factor, and the latter, in turn, directly induces oocyte maturation.

### INTRODUCTION

The meiotic resumption is thought to be triggered by hormonal substances specific to the animal groups. In the sea cucumber (Holothuroidea; Echinodermata), the full-grown oocyte is arrested in the prophase-I stage of meiosis, and meiotic resumption seems to occur just before spawning. Although a number of means have been reported for induction of spawning or oocyte maturation in sea cucumbers (Ohshima, 1925; Inaba, 1937; Colwin, 1948; Strathmann and Sato, 1969; Ishida, 1979, Maruyama, 1980), the endocrine substances responsible for oocyte maturation and spawning are not yet known.

In starfish (Asteroidea; Echinodermata), a gonad-stimulating substance (GSS), a peptide, of the radial nerve stimulates the gonads to induce oocyte maturation and spawning (Chaet and McConnaughy, 1959; Chaet, 1967; Kanatani, 1964, 1973; Kanatani *et al.*, 1971). GSS acts on the follicle cell in the ovary (Hirai and Kanatani, 1971; Cloud and Schuetz, 1973; Hirai *et al.*, 1973) to produce a secondary substance (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967), identified as 1-MeAde (Kanatani *et al.*, 1969). One-MeAde acts on a receptor site on the oocyte membrane to trigger the meiotic resumption (Kanatani and Hiramoto, 1970; Dorée and Guerrier, 1975; Morisawa and Kanatani, 1978; Ikadai and Kanatani, 1982).

The radial nerve factor, a polypeptide, which induces sperm-shedding of isolated testis fragments, was also found in radial nerves of *Strongylocentrotus purpuratus*

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Abbreviations: GSS, gonad-stimulating substance; GVBD, germinal vesicle breakdown; 1-MeAde, 1-methyladenine; RNE, radial nerve extract; RNH, radial nerve homogenate.

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(Echinoidea; Echinodermata) by Cochran and Engelmann (1972, 1976). They showed that the radial nerve factor stimulates ovaries to produce an ovarian factor. This factor also induces gamete-shedding from testicular fragments. In sea urchins, 1-MeAde is also reported to play an important role in oocyte maturation and spawning (Kanatani, 1974).

Attempts to demonstrate maturation-inducing activity from the radial nerve of sea cucumbers have been unsuccessful (Noumura and Kanatani, 1962). Several investigators have examined effects of 1-MeAde and starfish GSS on sea cucumber oocyte maturation (Strathmann and Sato, 1969; Stevens, 1970; Hufty and Schroeder, 1974; Ikegami *et al.*, 1976; Kishimoto and Kanatani, 1980; Maruyama, 1980). However, the endocrine substances directly concerned with maturation of sea cucumber oocytes remain unknown, and accumulating evidence suggests that sea cucumber oocyte maturation is controlled by substances other than 1-MeAde. On the other hand, presence of a common mechanism in oocyte maturation is suggested by successful induction of oocyte maturation with such disulfide-reducing agents as dithiothreitol in both sea cucumbers (Maruyama, 1980; Kishimoto and Kanatani, 1980) and starfishes (Kishimoto and Kanatani, 1973). It was also shown that injection of cytoplasm from maturing starfish oocytes, containing maturation-promoting factor, into immature sea cucumber oocytes induced germinal vesicle breakdown (Kishimoto *et al.*, 1982).

The present study reinvestigated effects of the radial nerve of sea cucumbers on oocyte maturation. Water-extracts of radial nerves of sea cucumbers were used. Maturation-inducing activity was assayed with ovarian oocytes in a piece of isolated ovaries, isolated oocytes with the follicle cells, and isolated oocytes deprived of the follicle cells. The presence of a maturation-inducing factor in the radial nerve, its action site, and presence of a secondary factor are examined in this paper.

## MATERIALS AND METHODS

Sea cucumbers (*Holothuria leucospilota*, *Holothuria pervicax*, *Holothuria moebi*, *Holothuria pardalis*, and *Stichopus japonicus*) were collected from June through August (1982 and 1983) near the Seto Marine Biological Laboratory. Except for *S. japonicus*, their gonads were fully developed. Experiments were made in sea water at 27–29°C, the approximate average summer sea water temperature. All tests were intraspecific, *e.g.*, radial nerve preparations and oocytes from the same species, unless otherwise stated.

### *Preparations of radial nerve homogenate or extract*

To obtain the radial nerve of the sea cucumber, the body wall was cut longitudinally with scissors. The radial nerve, located between a pair of bands of the longitudinal muscles, was isolated using a razor and a forceps. This radial nerve preparation contained the radial nerve and its overlying epithelia of water-vascular and coelomic canals. The radial nerves were blotted with filter paper and then weighed. They were used immediately or stored at –20°C until use. Radial nerves were cut into small pieces with scissors, and homogenized, with a glass homogenizer, in de-ionized water for 20 min at 27–29°C. The homogenate was mixed with an equal volume of double strength artificial sea water (modified Herbst's sea water), to make the ionic condition close to the physiological state. This original radial nerve homogenate, usually containing 50 mg wet weight of radial nerves per ml, was used after serial sea water dilution. In some instances, the radial nerve

homogenate (RNH) was centrifuged ( $26,000 \times g$ , 30 min,  $4^{\circ}\text{C}$ ). The clear supernatant is designated radial nerve extract (RNE). The 'concentration' of radial nerve component in the extract was defined tentatively by the concentration (mg/ml) of radial nerves in the original homogenate. The radial nerve homogenate or its supernatant were used immediately or stored at  $-20^{\circ}\text{C}$ . Storage of the radial nerves at  $-20^{\circ}\text{C}$  before or after homogenization did not decrease their activities for up to three months.

The above procedures were also used to obtain homogenates of the other tissues, close to the radial nerve, of the body wall; *i.e.*, longitudinal muscle at the ambulacral zone, and circular muscle and dermis at a middle portion of the interambulacral zone.

### *Sea water and chemicals*

Modified Herbst's sea water (NaCl, 2.6%; KCl, 0.07%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2%;  $\text{CaCl}_2$ , 0.11%;  $\text{NaHCO}_3$ , 0.045%) (Motomura, 1938) was used. Ca-free or Ca·Mg-free sea water were prepared, following this formula, with addition of appropriate amounts of NaCl. In some experiments, sodium bicarbonate was replaced by boric acid (0.31%) and pH was adjusted to 8.2–8.3 with NaOH. Pronase (50,000 units, Calbiochem-Behring) was dissolved at 0.1% in sea water. Pronase treatments of RNE were made by incubating RNE (12.5 mg/ml, final concentration) with 0.01% (final) pronase for about 3 hours at  $29^{\circ}\text{C}$ . After heating ( $85^{\circ}\text{C}$ , 15 min) to inactivate pronase, the mixture was assayed. In controls, sea water was substituted for the pronase solution. Trypsin (Bovine Pancreas Type III-S, Sigma) was dissolved at 0.1% in sea water. Trypsin treatments were made by incubating RNE (90 mg/ml, final concentration) with 0.01% (final) trypsin at  $37^{\circ}\text{C}$  for 60 min. After addition of soybean trypsin inhibitor (Type I-S, Sigma, 0.06% final conc.), the mixture was assayed. As a control for trypsin treatments, RNE was treated with both 0.01% (final) trypsin and 0.06% (final) soybean trypsin inhibitor at  $37^{\circ}\text{C}$  for 60 min, and then assayed. Dialysis was performed with cellophane tubing (8/32 inch cellophane tubing-seamless, Union Carbide). An aliquot (0.5 ml) of RNE (100 mg/ml) was dialyzed against 0.5 ml of sea water for 17 hours at  $4^{\circ}\text{C}$ , and then assayed. As a control, an aliquot (0.5 ml) of sea water was 'dialyzed' against the same volume of sea water for 17 hours at  $4^{\circ}\text{C}$ , and the 'dialyzate' was assayed. Sephadex G-15 and G-50 (Pharmacia fine chemicals) were swollen in distilled water for 3 hours at  $27-28^{\circ}\text{C}$ , and transferred into columns. The columns were equilibrated with sea water (pH 8.2–8.3).

### *Bioassay materials*

Sea cucumber ovaries with many fully grown oocytes were isolated, immediately washed several times with natural sea water, and then placed in sea water for immediate use.

*Ovarian oocytes.* Isolated ovaries were cut into pieces, 1 cm long, with fine scissors just before use. After several sea water rinses, the ovarian fragment was transferred into a test solution with forceps. Intact oocytes in such an ovarian fragment are designated 'ovarian oocytes.' In some experiments, an isolated ovary was torn open with two forceps from its cut-ends, longitudinally, and then cut into pieces, 0.5 cm long, with scissors. This 'torn out' ovarian fragment was also used as assay material.

*Isolated oocytes with intact follicle cells.* Oocytes were squeezed out from freshly isolated ovaries with two forceps. They were immediately washed ten times with

ten volumes of sea water by a hand-centrifuge to avoid sporadic occurrence of the oocyte maturation. The washed oocytes were stored in ten volumes of sea water. Among these washed oocytes, those with intact follicle cell-coats were isolated individually with a micropipette and used as assay materials.

*Oocytes deprived of follicle cells.* The washed oocytes were treated with ten volumes of Ca-free or Ca·Mg-free sea water two or three times, each for 10–20 min. Follicle cell-free oocytes were isolated individually with a micropipette and used as assay materials.

To determine maturation-inducing activity, about 180  $\mu$ l of a test solution was placed in a plastic dish. To this drop, one piece of an ovarian fragment, about 20 oocytes with the follicle cells, or about 20 oocytes deprived of the follicle cells, were transferred. Percentage of germinal vesicle breakdown was scored one hour later. In typical experiments using the ovarian fragment, two groups of oocytes (those remaining within the fragment and those extruded from it) were observed separately; the former was observed soon after being squeezed out from the fragment with two forceps. The maturation-inducing activity of the radial nerve of a sea cucumber was assayed by using oocytes of the same species unless otherwise stated.

#### *Preparation of the follicle cell suspension*

About  $10^6$  oocytes were squeezed out of freshly isolated ovaries of *H. leucospilota*, with two forceps, into 40 ml of sea water or Ca·Mg-free sea water. The follicle-oocyte complexes were pipetted several times to enhance detachment of the follicle cells. About 20 min later, the number of oocytes and the percentage of follicle cell-free oocytes were determined. Usually 70–80% of the oocytes were follicle cell-free. Oocytes in the suspension were removed by using a hand-centrifuge twice. The supernatant, virtually free of oocytes or ovarian fragment contamination, was centrifuged at  $1500 \times g$  for 15 min to collect pellets of follicle cells. The follicle cells were used after serial sea water dilutions. The density of follicle cells in the suspension was expressed in terms of the number of follicle cell-coats per unit volume, calculated on the basis of the frequency of follicle cell-free oocytes in the original suspension. This calculation may involve a certain degree of over-estimation, since some oocytes were follicle cell-free when they were squeezed from the ovaries.

## RESULTS

#### *Presence of maturation-inducing factor in radial nerves*

Tissue-homogenates containing radial nerves successfully induced oocyte maturation and 'spawning' in isolated ovarian fragments of *Holothuria leucospilota* and *Holothuria pervicax* (Figs. 1B, C). In *H. leucospilota*, ovarian fragments incubated with radial nerve homogenates (10 mg/ml) began to extrude follicle cell-free oocytes from their cut-ends after a rather constant time-lag of 14 min ( $n = 4$ ) at 28–29°C. These extruded oocytes matured subsequently; the germinal vesicle began to migrate to the region of the micropyle process at 15 min (after the start of RNH treatment), and after attaching to the region germinal vesicle breakdown (GVBD) occurred at 18–20 min (Maruyama, 1980, 1981). Polar bodies formed from the micropyle process. On insemination, the oocytes began normal development (Fig. 1F). By contrast, control ovarian fragments in sea water did not show such an extrusion of oocytes (Fig. 1A), and the oocytes remaining in the ovary did not mature even after several hours in sea water. These events with a similar time-course were also observed in *H. pervicax* when the ovarian fragments were incubated with the radial nerve homogenate.

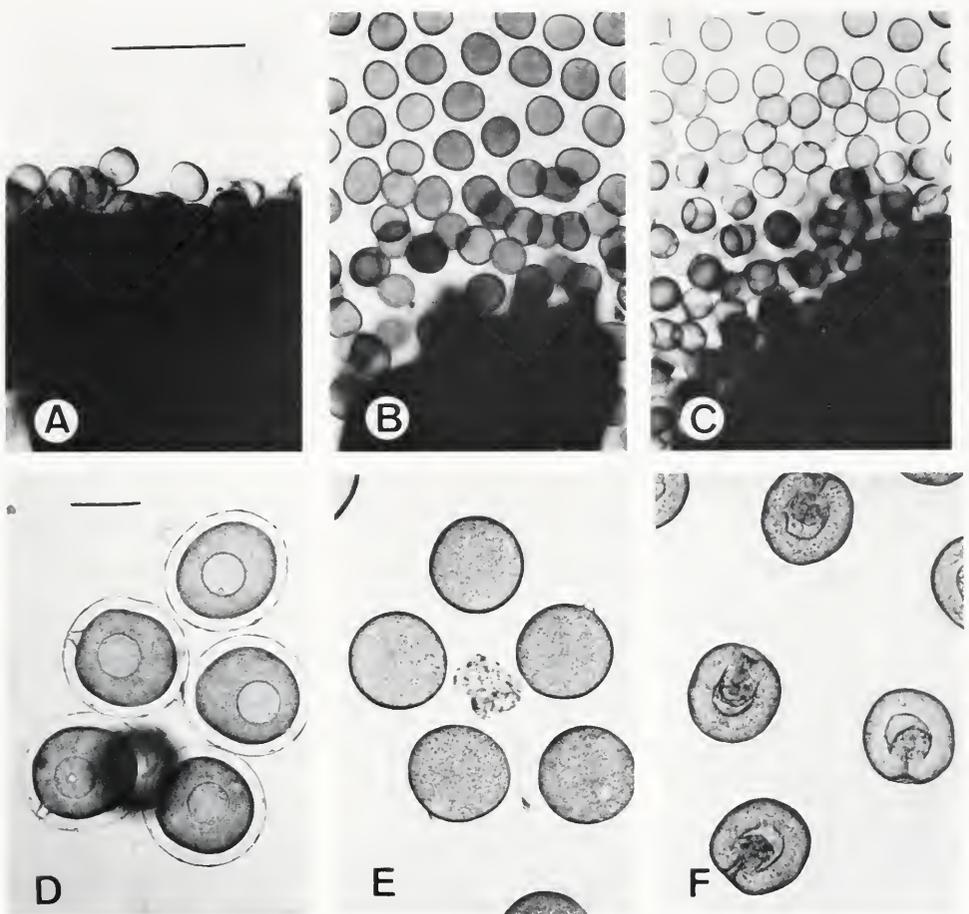


FIGURE 1. Effects of radial nerve homogenates on ovarian fragments and on oocytes with follicle cells. A: control ovarian fragments after 50 min in sea water. *H. leucospilota*. The bar (500  $\mu$ m) is common to A, B, and C. B: mature oocytes extruded from a cut-end of an ovarian fragment incubated with RNH (10 mg/ml) for 30 min. *H. leucospilota*. C: same as B. *H. pervicax*. D: isolated oocytes with follicle cells in sea water (control). *H. pervicax*. The bar (100  $\mu$ m) is common to D, E, and F. E: isolated oocytes with follicle cells, incubated with RNE (14 mg/ml) for 40 min. *H. pervicax*. The germinal vesicle is broken down after migrating to the micropyle process. Follicle cells are detached from the oocytes to form a large cell mass (center). F: early gastrulae with invaginating archenteron (16 hours post-fertilization) from oocytes which have been induced to mature by RNH and inseminated 70 min later. The largest optical sections through the main axes of embryos (not compressed) were photographed. *H. leucospilota*.

It is reasonable to regard such an extrusion of oocytes as 'spawning' by an ovarian fragment, because it occurs only after a time-lag following stimulation and most (usually 100%) of the extruded oocytes mature. However, many oocytes still remained in the ovarian fragment after one hour of incubation. Therefore, in experiments using ovarian fragments as assay materials, both oocytes extruded from the fragment and those remaining within the fragment were observed separately, if necessary.

Figure 2 shows GVBD response of ovarian oocytes incubated with various concentrations of radial nerve homogenates in *H. leucospilota*. Threshold concentrations of radial nerve homogenates for inducing spawning lie at 0.5–1.0 mg/ml.

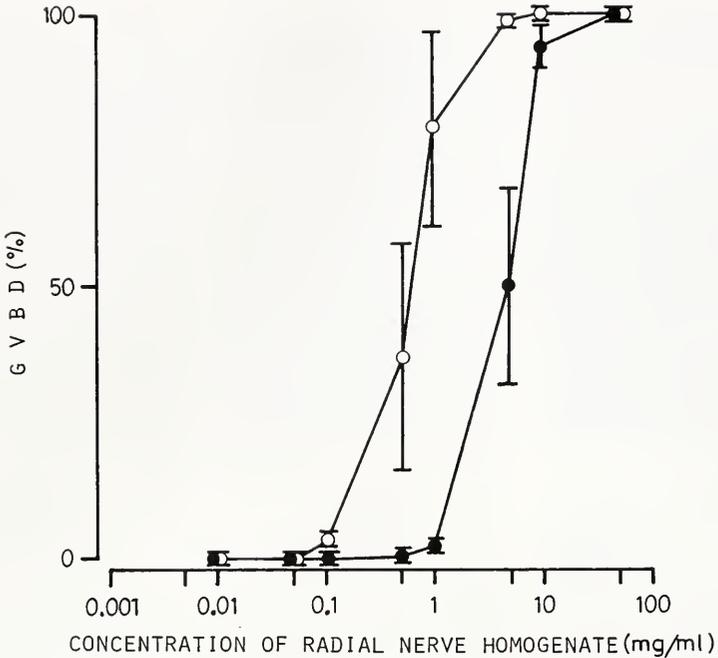


FIGURE 2. Effects of radial nerve homogenate on induction of oocyte maturation in ovarian fragments of *H. leucospilota*. Oocytes extruded from the ovarian fragment (open circles) and those remaining within the ovarian fragment (closed circles) were observed simultaneously. Each point represents mean  $\pm$  SE of five experiments.

In subthreshold concentrations, a low percentage of oocytes located at the cut-ends of the fragment showed GVBD. There was a range (1–5 mg/ml) of concentrations of radial nerve homogenates where spawning (and subsequent maturation of the spawned oocytes) occurs but GVBD rates of oocytes remaining in the fragment are very low (Fig. 2). The curve indicates that a 10-fold higher concentration is required for GVBD in unextruded oocytes. Nearly all the oocytes remaining in ovarian fragments were induced to mature at a concentration of 5–10 mg/ml. Similar results were observed in other sea cucumbers (Fig. 3A, Table II). A very high concentration (400 mg/ml) of radial nerve homogenate from *H. leucospilota* also successfully induced maturation of all oocytes and spawning in ovarian fragments of *H. leucospilota* and *H. pardalis*.

These results show that the radial nerve has a factor(s) responsible for spawning and oocyte maturation.

Radial nerve homogenates (or extracts) were then applied to isolated oocytes with or without follicle cells (see Figs. 1D, 5A). In *H. leucospilota*, most ( $83 \pm 8\%$ ,  $n = 3$ ) of the isolated oocytes with follicle cells were induced to mature by 1 or 5 mg/ml of radial nerve homogenate. By contrast, none of follicle cell-free oocytes were induced to mature by the homogenate at a wide range of concentration (0.01–50 mg/ml) ( $n = 3$ ) (*c.f.*, Fig. 5D). In control sea water, no GVBD was observed in oocytes with or without follicle cells. In *H. pervicax* also, radial nerve homogenates at 5 mg/ml induced maturation in most ( $99 \pm 2\%$ ,  $n = 2$ ) isolated oocytes with follicle cells (Figs. 1D, E), but ineffective to follicle cell-free oocytes. In control sea water, no GVBD was observed in oocytes with or without follicle cells. Figure 3B

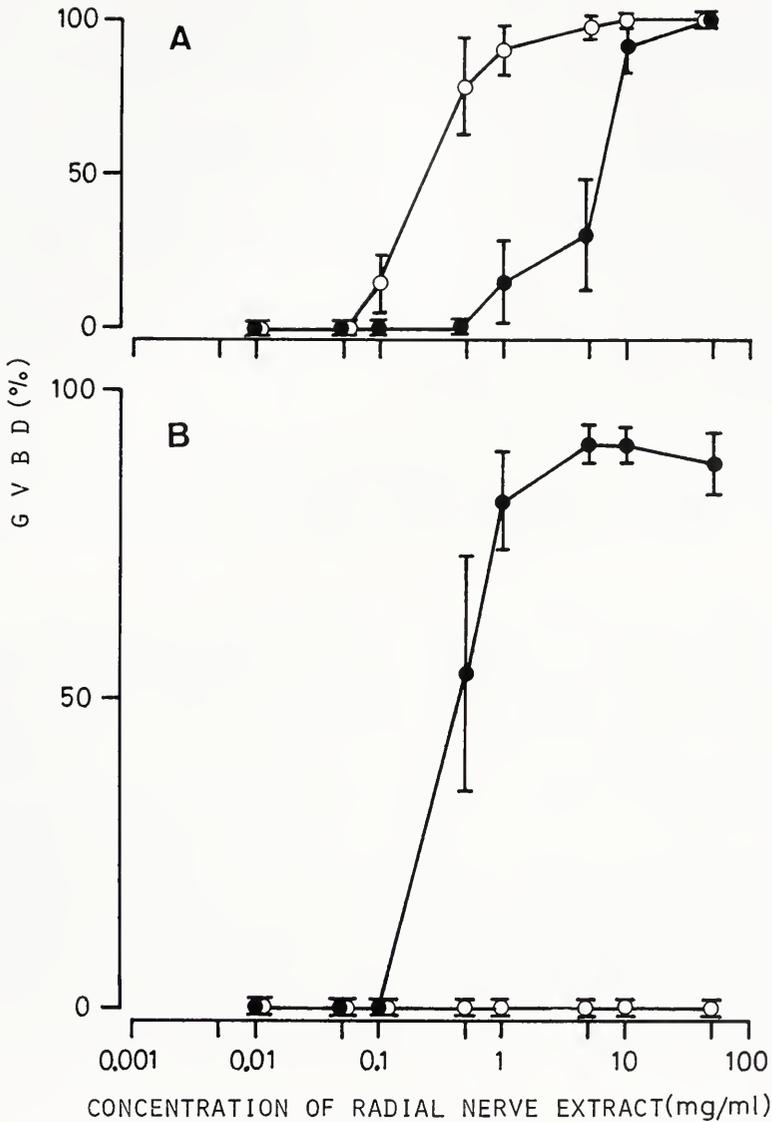


FIGURE 3. Effects of radial nerve extract on induction of maturation in *H. pervicax*. (A) GVBD in oocytes in ovarian fragments. Open circles: oocytes extruded from ovarian fragments. Closed circles: oocytes remaining within ovarian fragments. Each point is mean  $\pm$  SE of seven experiments. (B) GVBD in isolated oocytes with follicle cells (closed circles) or without follicle cells (open circles). Each point is mean  $\pm$  SE of three experiments.

shows dose-dependence of GVBD response in isolated oocytes of *H. pervicax*. Sensitivity of isolated oocytes with follicle cells (closed circles in Fig. 3B) is nearly equal to that of the ovarian fragment assessed by extruded oocytes (open circles in Fig. 3A). Also, the threshold concentration of radial nerve extract for inducing maturation of oocytes with follicle cells, attaching to a piece of 'torn-out' ovarian walls, was nearly equal to that in isolated oocytes with follicle cells (data not shown).

These results in *H. leucospilota* and *H. pervicax* show that a maturation-inducing factor in the radial nerve acts on the isolated oocyte with follicle cells, but does not act directly on the follicle cell-free oocyte.

*Maturation-inducing activity in tissues adjacent to radial nerves*

To examine for maturation-inducing activity in body wall tissues other than the radial nerve, tissue fragments of the interambulacral zone, muscle bands of the ambulacral zone, and radial nerve tissues were separately homogenized from three *H. leucospilota* individuals, and applied to ovarian fragments at a concentration of 5 mg/ml. The frequencies of maturation in oocytes are shown in Table I. Only the radial nerve homogenate exhibited high activity for inducing oocyte maturation and spawning; other tissue homogenates showed low spawning-inducing and maturation-inducing activity even at a higher concentration (50 mg/ml).

These results exclude the possibility of uniform distribution of the maturation-inducing activity all over the body wall, and suggest that the radial nerve is the predominant source of the maturation-inducing activity. Therefore the factor responsible for the activity may be called 'radial nerve factor.' It is possible that the oocyte maturation *in vivo* is due to the factor in the radial nerve.

*Common occurrence of radial nerve factor in sea cucumbers*

Species-specificity of the radial nerve factor in five species of the order Aspidochirotida was examined by applying radial nerve homogenates at a wide range (0.01–50 mg/ml) of concentrations to ovarian fragments. One hour later the ovarian fragments were observed for spawning and the frequencies of matured oocytes either spawned or remaining in mid-portions of the fragments. In all combinations examined, spawning and oocyte maturation were induced. Table II shows minimal concentrations of radial nerve homogenates required for induction of spawning and maturation. There were no consistent differences in effectiveness between homotypic and heterotypic combinations. By contrast, none of the follicle cell-free oocytes were induced to mature by a wide range (0.01–50 mg/ml) of concentrations of radial nerve homogenates in all combinations examined among *H. leucospilota*, *H. pervicax*, *H. pardalis*, and *H. moebi*.

These results indicate that an active factor in the radial nerve is common among five sea cucumber species.

TABLE I

*Maturation-inducing activity in tissues adjacent to radial nerves in H. leucospilota*

Source of tissue homogenate	Percentage of GVBD <sup>1</sup>	
	5 mg/ml	50 mg/ml
Tissue at interambulacra	0 ± 0 <sup>2</sup>	36 ± 30
Longitudinal muscle	0 ± 0	27 ± 12
Radial nerve	100 ± 0	100 ± 0

<sup>1</sup> Percentage of GVBD in oocytes extruded from cut-ends of ovarian fragments at the end of 60 min incubation.

<sup>2</sup> Mean ± SE of three experiments. Neither spawning nor maturation of oocytes was observed in control ovarian fragments in sea water.

TABLE II

*Cross-effects of radial nerve homogenate on oocyte maturation among sea cucumbers*

Source of ovarian fragments	Source of radial nerve homogenate				
	<i>H. leucospilota</i>	<i>H. pervicax</i>	<i>H. moebi</i>	<i>H. pardalis</i>	<i>S. japonicus</i>
<i>H. leucospilota</i>	+++ (++) <sup>1</sup>	++ (+)	+ (+)	++ (+)	+ (+)
<i>H. pervicax</i>	+++ (++)	+++ (+++)	+++ (++)	+++ (++)	+ (+)
<i>H. moebi</i>	+++ (++)	+++ (+++)	++ (+)	+++ (++)	?
<i>H. pardalis</i>	++ (+)	++ (+)	++ (+)	++ (+)	?

<sup>1</sup> Symbols indicate minimal concentrations of radial nerve homogenates effective for either spawning (and subsequent maturation) or maturation of oocytes unextruded from the ovarian fragment. The latter is shown in parentheses. +++, 0.1–1 mg/ml; ++, 1–10 mg/ml; +, 10–50 mg/ml; ?, no test.

In *H. leucospilota* and *H. pervicax* radial nerve homogenates from both female and male individuals were similarly effective for inducing the spawning and oocyte maturation, suggesting no sexual differences of the radial nerve factor.

#### *Chemical nature of the radial nerve factor*

The chemical nature was examined primarily using radial nerve extract from *H. leucospilota* (Table III). Heating (92°C for 15 min) did not inactivate the maturation-inducing activity of the radial nerve extract. When radial nerve extract was dialyzed against an aliquot of sea water through cellophane tubing, the activity was found in the dialyzate. Pronase (0.01%) and trypsin (0.01%) inactivated the maturation-inducing activity. Similar results were obtained in radial nerve extracts of *H. pervicax*. Such heat-stable, dialyzable, and protease-sensitive properties suggest that the radial nerve factor is a peptide.

The radial nerve factor was separated by gel-filtration. Radial nerve extract of *H. leucospilota* was fractionated on Sephadex G-15 and G-50 columns (Fig. 4). Its elution patterns were simultaneously monitored with 280 nm absorption. An aliquot (180 µl) of each fraction was assayed with an ovarian fragment. In a Sephadex G-15 column, the activity was eluted at the void volume of the column. In a Sephadex G-50 column, the activity was retarded but eluted apparently as a single peak (fraction No. 11, 12, 13, and 14) just before the column volume. Similar results

TABLE III

*Effects of heating, dialysis, or protease-treatments of radial nerve extract in H. leucospilota*

Treatments	%GVBD <sup>1</sup>	
	Experiments <sup>2</sup>	Controls <sup>2</sup>
Heating (92°C for 15 min)	98%	100%
Dialysis through cellophane tubing	94%	8%
0.01% pronase	0%	95%
0.01% trypsin	0%	100%

<sup>1</sup> Ovarian fragments were used for assay. The GVBD rate was obtained from all the oocytes of an ovarian fragment incubated with a test solution.

<sup>2</sup> See Materials and Methods.

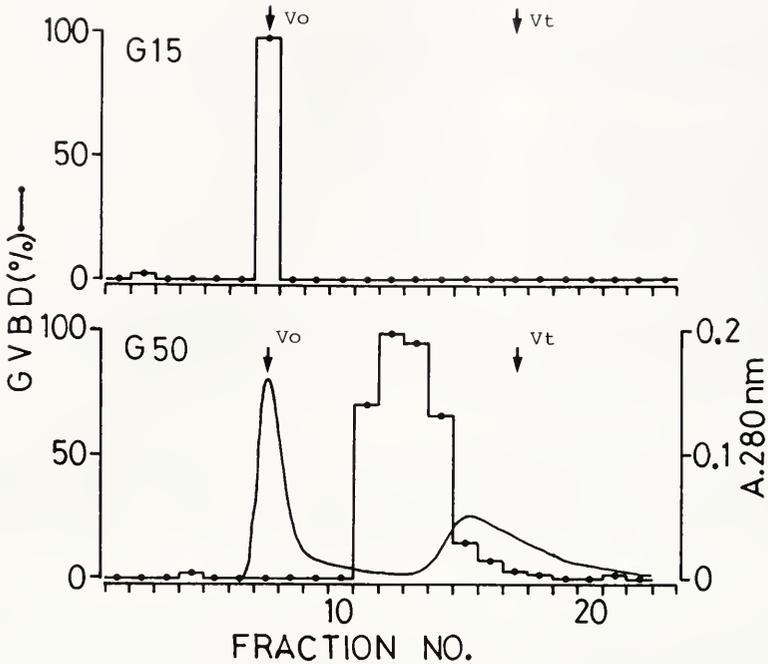


FIGURE 4. Gel-filtration of radial nerve extracts from *H. leucospilota*. Two or three ml of RNE (100 mg per 1 ml of sea water) were placed in Sephadex G-15 columns (1.6 × 42 cm) and Sephadex G-50 columns (1.6 × 42 cm), respectively. Sea water (pH 8.2–8.3) was used as eluant (30 ml/h), and fraction size was 5 ml. Each fraction was assayed with an ovarian fragment, and the GVD rate was obtained from all the oocytes of the fragment. The elution pattern was simultaneously monitored with 280 nm absorption, and its elution pattern through the G-50 column was shown in a curved-line. The scale of 280 nm absorption is shown in the ordinate at the right side of the figure. Vo, void volume. Vt, column volume or total volume of the packed bed volume.

were obtained in radial nerve extracts of *H. pervicax*. Fractionation range in molecular weight of Sephadex G-50 is 1500–30,000 d ('Sephadex gel-filtration in theory and practice' from Pharmacia Fine Chemicals). The extrusion limit in Sephadex G-15 is reported as 1500 d. Therefore, the molecular weight of the radial nerve factor appears to be larger than 1500 but considerably smaller than 30,000. This implies that the radial nerve factor is a peptide with molecular weight of several thousands' daltons.

#### *Presence of a secondary factor for oocyte maturation*

Ovaries or testes (2–3 g, wet weight) of *H. leucospilota* were separately incubated with 1 ml of radial nerve homogenate (50 or 100 mg/ml) for 2 or 3 hours. After removing gonads and spawned oocytes (or sperm in males) by low-speed centrifugation, the incubation mixtures were centrifuged at  $26,000 \times g$  or  $10,000 \times g$  for 30 min at 4°C to remove cells or debris, and the resulting supernatants were assayed with follicle cell-free oocytes. The supernatant induced the maturation of follicle cell-free oocytes (Fig. 5). As shown in Table IV, ovaries or testes, in the presence of radial nerves, produce a factor which induces the maturation of the follicle cell-free oocyte. Identical results were obtained by using *H. pervicax*. The controls did not show the maturation-inducing activity (Table IV, Fig. 5D).

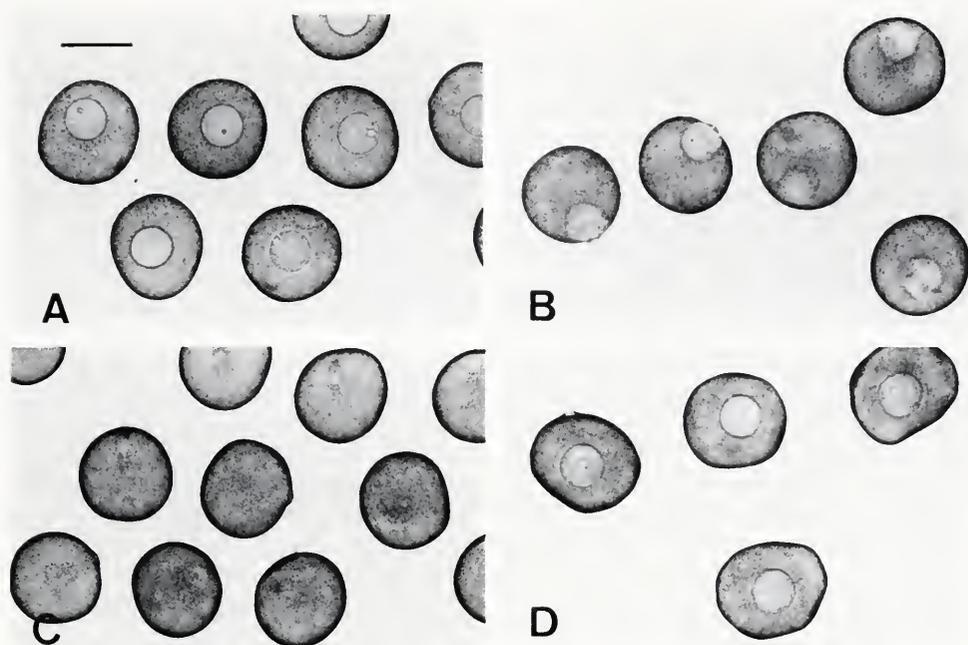


FIGURE 5. Effects on the follicle cell-free oocytes of incubation mixtures of ovaries and radial nerve homogenate in *H. leucospilota*. Ovaries (2–3 g) were incubated with 1 ml of RNE (100 mg/ml) for 2 or 3 hours. The supernatant of the mixture was applied to follicle cell-free oocytes. Water temperature was 28°C. Bar: 100  $\mu$ m. A: 0 min. B: 20 min after incubation with the supernatant. GVBD just occurs at the micropyle process. C: 110 min. Two polar bodies formed. D: follicle cell-free oocytes incubated with RNH (50 mg/ml) for 60 min.

These results show that a secondary factor which induces maturation in the follicle cell-free oocyte is produced by ovaries or testes incubated with the radial nerve factor.

TABLE IV

Production of a secondary factor by ovaries or testes incubated with radial nerve homogenate (RNH) or extract (RNE)

	No. of experiments	%GVBD of oocytes in serially diluted media				
		$1/1$	$1/2$	$1/4$	$1/8$	$1/16$
<i>H. leucospilota</i>						
Ovaries + RNH (50 mg/ml) <sup>1</sup>	4	88 $\pm$ 13 <sup>2</sup>	24 $\pm$ 41	0 $\pm$ 0	–	–
Ovaries + RNH (100 mg/ml)	3	98 $\pm$ 2	55 $\pm$ 34	21 $\pm$ 30	0 $\pm$ 0	0 $\pm$ 0
Ovaries + sea water	3	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
RNH (100 mg/ml)	3	2 $\pm$ 2	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Testes + RNH (100 mg/ml)	2	95 $\pm$ 0	50 $\pm$ 21	3 $\pm$ 3	–	–
Testes + sea water	2	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	–	–
RNH (100 mg/ml)	2	8 $\pm$ 2	3 $\pm$ 2	3 $\pm$ 2	–	–
<i>H. pervicax</i>						
Ovaries + RNE (50 mg/ml)	4	96 $\pm$ 3	63 $\pm$ 37	12 $\pm$ 8	2 $\pm$ 3	0 $\pm$ 0

<sup>1</sup> The supernatant of the incubation mixture was serially diluted with sea water and assayed with the follicle cell-free oocyte from the same species.

<sup>2</sup> Mean  $\pm$  SD of GVBD oocytes observed 1 hour later.

*Production of a secondary factor by follicle cells*

Results in the foregoing sections suggest that follicle cells are the action site of the radial nerve factor, as in the case of starfishes. Follicle cell suspensions were prepared from oocyte-follicle complexes squeezed out from freshly isolated ovaries in *H. leucospilota* (see Materials and Methods). Various densities of follicle cell suspensions were prepared after serial sea water dilutions, and to each suspension the radial nerve homogenate was added at the final concentration of 17 mg/ml. Two or three hours later, an aliquot (180  $\mu$ l) of each suspension was withdrawn and examined for maturation-inducing activity on follicle cell-free oocytes of *H. leucospilota*. The follicle cell-free oocytes matured with increasing frequency as the density of the follicle cells increased (Fig. 6). Nearly 100% GVBD was obtained with suspensions of  $10^{5-6}$  follicle cell-coats per ml. In control incubations (no follicle cell or radial nerve), the maturation-inducing activity was rarely detected. Both sea water and Ca·Mg-free sea water isolated follicle cells produce maturation-inducing activity (Fig. 6).

These results show that the radial nerve factor acts on the follicle cell to produce a secondary factor, which directly induces maturation of the oocyte.

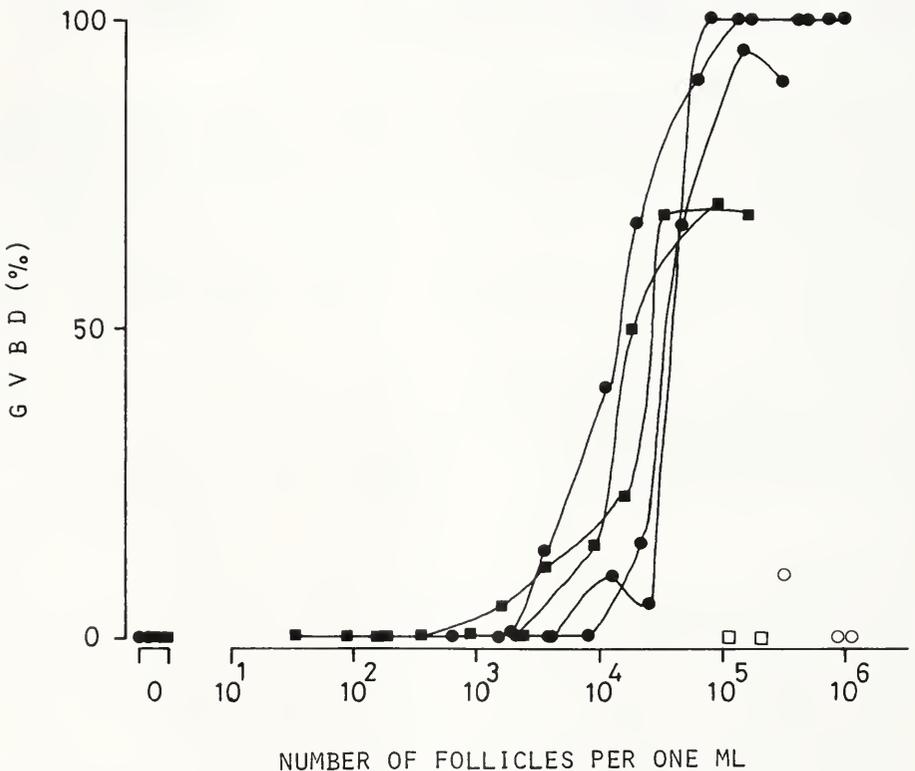


FIGURE 6. Production of a secondary factor by isolated follicle cells incubated with radial nerve homogenate in *H. leucospilota*. The assay was made by the follicle cell-free oocyte. Closed symbols: follicle cell suspensions incubated with RNH (17 mg/ml at the final concentration). Open symbols: original follicle cell suspensions without RNH. Two different media for follicle cell isolation, sea water (squares), and Ca·Mg-free sea water (circles), gave similar results.

## DISCUSSION

This study demonstrates a radial nerve factor in five sea cucumber species of the order Aspidochirotrida. The factor is apparently a peptide of a low molecular weight (several thousands' daltons) and is common to sea cucumbers. The radial nerve factor acts on oocytes with follicle cells, but does not act directly on an oocyte. This radial nerve factor of the sea cucumber has characteristics similar to radial nerve factor, gonad-stimulating substance (GSS), of starfishes (Kanatani, 1973) and sea urchins (Cochran and Engelmann, 1972, 1976), suggesting that these substances are closely related molecules. It remains unclear what types of cells in the radial nerve tissue contain or secrete the radial nerve factor and whether the factor is produced in tissues other than the radial nerve. Further studies are necessary for identification of the secretory cells and transport route of the radial nerve factor.

This study also demonstrated the presence of a secondary factor which is produced by follicle cells and directly acts on oocyte to mature. These results show that the oocyte maturation of the sea cucumber is regulated via follicle cells. In starfishes, such a secondary factor was identified as 1-MeAde (Kanatani *et al.*, 1969), which is a non-species specific maturation-inducing substance in starfishes (Kanatani, 1973; Kanatani and Nagahama, 1983). However, 1-MeAde has been shown to be ineffective for oocyte maturation of the sea cucumber *H. leucospitota* (Maruyama, 1980, unpub. data). Ineffectiveness of 1-MeAde for sea cucumber oocyte maturation was also reported by Stevens (1970), Ikegami *et al.* (1976), and Kishimoto and Kanatani (1980). The secondary factor of the sea cucumber should be a substance other than 1-MeAde. Purification and identification of the secondary factor in sea cucumbers remains to be done.

The ovarian fragments incubated with the radial nerve homogenate retained a considerable number of immature oocytes even after spawning many oocytes, and a higher concentration of the radial nerve homogenate was required for the maturation of the remaining oocytes. This might suggest the presence of some inhibitors within ovaries against the action of the radial nerve factor. Spawning inhibitors have been reported in starfish ovaries (Ikegami *et al.*, 1967; Ikegami, 1976).

The accumulated evidence shows the neurosecretory mechanism of oocyte maturation or gamete-shedding in three out of five classes of Echinodermata. In addition, a regulatory (inhibitory) system to such a control is now found in sea cucumbers as well as starfishes. Demonstration of similar neurosecretory mechanism in the other classes of Echinodermata is awaited.

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BEHAVIORAL RESPONSES OF OCEANIC ZOOPLANKTON  
TO SIMULATED BIOLUMINESCENCE

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## ABSTRACT

A defensive function often has been suggested for the bioluminescence of dinoflagellates and copepods, but there is only limited experimental evidence. Using closed circuit television equipment and infrared illumination we have recorded the behavioral responses of planktonic copepods, ostracods, polychaetes, chaetognaths, and euphausiids to simulated bioluminescent flashes. The swimming patterns of these organisms were then quantified using a video-computer system for motion analysis (the Bugwatcher). The photophobic response exhibited by certain copepod species in response to simulated dinoflagellate flashes, as well as the lack of response by several potential predators on copepods to their simulated bioluminescence, provide new insight into the roles of bioluminescence in plankton ecology. Comparison of the responses of the non-bioluminescent copepod *Calanus finmarchicus* and the bioluminescent copepod *Metridia longa* to simulated copepod bioluminescence show that *Metridia* is much more responsive than *Calanus*. This suggests that bioluminescence in *Metridia* may be recognized as a warning signal by conspecifics in addition to serving as a defense against predation.

## INTRODUCTION

Most of the bioluminescence observed in the epipelagic zone is attributed to dinoflagellates and planktonic crustaceans such as copepods, ostracods, and euphausiids (Tett and Kelly, 1973; Swift *et al.*, 1983). Although the physical characteristics of the bioluminescence of these plankters has been carefully studied in several instances (*e.g.*, Harvey *et al.*, 1957; Eckert, 1967; Biggley *et al.*, 1969; Swift *et al.*, 1973; Widder *et al.*, 1983) there has been relatively little experimental work investigating the adaptive value of bioluminescence to planktonic organisms. Dinoflagellate bioluminescence has received the most attention, and the results of several studies provide evidence to support the hypothesis that dinoflagellates bioluminescence functions as a defense against nocturnal grazers such as copepods (Esaias and Curl, 1972; White, 1979; Buskey and Swift, 1983; Buskey *et al.*, 1983). Experimental studies also have suggested a defensive role for copepod bioluminescence (David and Conover, 1961).

One problem with studies of the behavior of planktonic bioluminescent organisms is that their bioluminescence is often photoinhibited at even low ambient light levels, making direct observation of behavioral interactions difficult or impossible. Another problem is that the behavioral interactions among zooplankters that lead to bioluminescent displays (*e.g.*, predator-prey interactions) are often low-frequency events and thus only rarely observed. We have overcome these problems by using infrared illumination to record on videotape the behavior of various planktonic organisms in darkness and by using an artificial light source to simulate the

bioluminescent emissions of dinoflagellates and copepods. The responses of a variety of zooplankton species to bioluminescent flashes were observed and quantified using this technique, and this information was used to provide new evidence for the proposed roles of bioluminescence in zooplankton ecology.

#### MATERIALS AND METHODS

Live zooplankton samples were taken in the vicinity of Iceland aboard the R/V Endeavor during cruise EN-103 in July 1983. Oblique tows were taken with 333 or 202  $\mu\text{m}$  mesh plankton nets towed between the surface and *ca.* 100 m depth. A ship speed of  $<1$  knot was maintained during these tows to reduce injury to the zooplankters. Upon recovery the contents of the cod ends of the nets were immediately diluted into one gallon glass jars with sea water at ambient temperature. From this container individuals of the plankton species chosen for study were then captured with a large bore pipette and transferred to 11 cm diameter Carolina culture dishes containing filtered sea water. These organisms were then observed under a dissecting microscope to check species identifications and to inspect for injury. Specimens showing signs of injury (*e.g.*, broken setae) were not used. Specimens were then held in incubators at ambient temperature for 12–24 hours before experimentation.

To test the effects of simulated bioluminescent flashes on the swimming behavior of the various zooplankton species collected, bioluminescent flashes were simulated using a diffuse horizontal light beam from a high intensity tungsten lamp passed through a 480 nm narrow band interference filter (10 nm half band width). Light intensity was adjusted using neutral density filters and by controlling lamp current (Oriel Model 6329 controller). Flash duration was adjusted by passing the light beam through a shutter with a Uniblitz model 310 controller, and photon flux was measured with a LICOR model 158A light sensor with quantum probe.

A flash of 480 nm blue light for 60 ms at an intensity of *ca.*  $2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  was used to simulate dinoflagellate bioluminescence (Buskey and Swift, 1983). This flash approximates the light flux per unit area through the surface of a bioluminescent dinoflagellate (Seliger *et al.*, in prep.) and thus represents the maximum light dose that would be received by direct contact of a flashing dinoflagellate with a copepod eye. Copepod bioluminescent flashes are composed of light of similar spectral composition and intensity as in dinoflagellates (David and Conover, 1961; Herring, 1983; Widder *et al.*, 1983) but their light emission lasts considerably longer. The duration of bioluminescent displays by copepods are reported to range from 0.1 s to one minute or more (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972) and seem to be highly dependent on the method of stimulation. When stimulated either by electrical shock (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972) or by placing the animals on filter paper and removing the water (Clarke *et al.*, 1962), copepods produce considerably longer bioluminescent emissions than those produced by mechanical stimulation via a stirring rod (Barnes and Case, 1972; Swift *et al.*, in prep). Mechanical stimulation is most similar to the stimuli which normally induce bioluminescence in nature (*e.g.*, attempted capture by predators). Copepod bioluminescence is characterized by a rapid rise in intensity, a slower decay and a dim afterglow occasionally lingering for a period of up to a minute, but with  $>90$  percent of light production in less than 1 s. Since this intensity pattern cannot be duplicated with our electronic shutter, a constant intensity flash of 600 ms duration was used to simulate copepod bioluminescence.

All experiments were performed on board ship in a darkened room. A closed

circuit television system was used to monitor and record swimming behavior of the zooplankters. Darkfield substage illumination, passed through an infrared transmitting filter (Kodak Safelight Filter No. 11), provided light for a Cohu 4400 television camera with a macro lens. Movement was monitored from above in the horizontal plane.

Four hours before video recording each experiment, organisms were transferred to  $10 \times 10 \times 5$  cm lucite chambers. From 1 to 5 individuals were placed in each chamber, depending on their size and activity level. These chambers had silicon rubber gaskets on their lids which allowed the chambers to be completely filled with sea water and sealed shut. The absence of air in the cuvette almost completely eliminated passive movement of the animals within the chamber caused by movements of the ship. Reported respiration rates for copepods (Vidal, 1980) and euphausiids (Mauchline, 1980) indicate that oxygen concentrations within the cuvettes should be depleted by less than five percent over the course of the experiments. The sealed chambers were placed in incubators at ambient temperatures (*ca.* 2–6°C) in complete darkness. Just prior to video recording, the experimental chambers were removed from the incubator and placed in a water bath to reduce changes in water temperature during the *ca.* 5 min video recording session. To ensure that the organisms were isolated from extraneous light produced by the experimental equipment, samples were placed in an opaque enclosure, with openings for the video camera, substage illumination, and horizontal light source.

In a typical experiment, the swimming behavior of the organisms was videotaped for a period of two minutes in the absence of simulated bioluminescence, and then for two minutes with light flashes introduced through the side of the chamber at five-second intervals. This experimental design allowed paired comparisons using Student's *t*-test of the swimming behavior of the same group of organisms. The paired comparison design is extremely useful for investigations of behavioral parameters since measured values can vary considerably even between individuals from the same population. To avoid recording interactions of the zooplankton with the side walls of the cuvette, only the central area (*ca.*  $8 \times 8$  cm) was included in the field of view of the video camera.

After the cruise, videotapes of copepod swimming behavior were played back through a video-to-digital processor, the "Bugwatcher" (Wilson and Greaves, 1979), and the location of the digitized outline of each organism in the video field was input to a Data General Eclipse S120 computer at a rate of  $10 \text{ frames} \cdot \text{s}^{-1}$  for organisms with slow or consistent swimming speeds (euphausiids, polychaetes and chaetognaths) or at a rate of  $15 \text{ frames} \cdot \text{s}^{-1}$  for organisms with more rapid or variable swimming behavior (copepods and ostracods). The mean swimming speed was computed from the digitized paths of all organisms. The number of swimming speed bursts was determined by counting bursts that exceeded a threshold of  $15 \text{ mm} \cdot \text{s}^{-1}$ . Since frame by frame observation of videotapes revealed that these swimming speed bursts sometimes occurred in less than a single video frame ( $60 \text{ frames} \cdot \text{s}^{-1}$ ), the measured speed of these bursts based on a sampling rate of  $15 \text{ frames} \cdot \text{s}^{-1}$  is at most  $\frac{1}{4}$  of the true speed. In some cases, swimming speed bursts were so dramatic that the organisms jumped out of the field of view of the video camera (which included *ca.* 65% of the cuvette). Since speed bursts could not be measured by the computer in these cases, videotapes were also visually monitored to count the number of speed bursts when responses were too extreme to be quantified by computer.

The turning behavior of the organisms in the horizontal plane of observation were quantified as rate of change of direction and net to gross displacement ratio.

Rate of change of direction is simply the turning rate measured in degrees per second. The tendency of organisms to remain within an area by changing their turning behavior is indicated by the net to gross displacement ratios (NGDR) of their paths of travel. This measure is the ratio of the linear distance between starting point and ending point of the path (net displacement) to the total distance traveled for each path (gross displacement). Thus an increase in NGDR indicates a more linear swimming path, and a decrease in NGDR indicates a less linear, more circuitous swimming path. Direction of travel measures the angle between each segment of the path (for each 1/10 or 1/15 s) and the light source ( $0^\circ$ ). Distributions of direction of travel are calculated as percent distribution within each of twelve  $30^\circ$  arcs. These distributions of direction of travel are compared, using a Chi-square test, with a theoretical uniform distribution of direction of travel (Batschelet, 1965).

### RESULTS

The most commonly observed behavioral response of copepods to simulated dinoflagellate flashes are characterized by a sharp increase in swimming speed a few ms after the beginning of the flash (Fig. 1). Sometimes swimming speed bursts are preceded by turning behavior. We refer to all the responses to rapid changes in light intensity which elicit a transient alteration in the activity of the organism (e.g., a burst of swimming speed) as photophobic responses (*sensu* Diehn *et al.*, 1977). The responses of the copepods tested in this study are similar to those previously observed in the estuarine copepod *Acartia hudsonica* exposed to both natural and simulated dinoflagellate bioluminescence (Buskey and Swift, 1983; Buskey *et al.*, 1983). The major difference between present and previous results was that the photophobic responses observed on this cruise were more intense than any previously recorded.

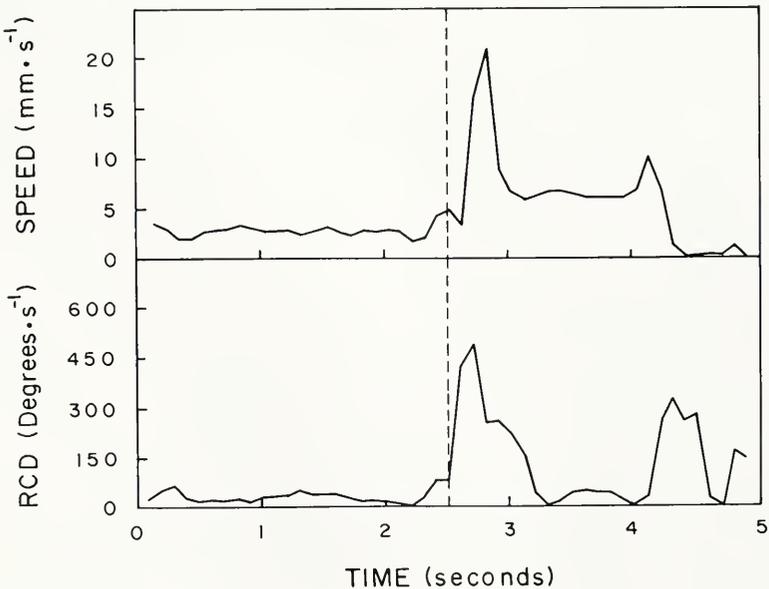


FIGURE 1. Record of swimming speed and the rate of change of direction (RCD) over time for a single *Calanus finmarchicus* exposed to a simulated bioluminescent flash (475 nm blue light for 60 ms duration at an intensity of  $2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ); the dashed line indicates the time of the flash.

The effects of simulated bioluminescence on the swimming behavior of a variety of copepod species (Table I) indicated strong photophobic responses and increased average swimming speeds for four of the six copepod species tested (*Calanus finmarchicus*, *Metridia longa*, *Metridia lucens*, *Temora longicornis*). Three of these

TABLE I

Responses of oceanic zooplankton to simulated bioluminescent flashes (475 nm peak emission, 60 ms duration,  $2.0 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity)<sup>a</sup>

Species	Percent response <sup>b</sup>	Mean speed (mm · s <sup>-1</sup> )	Bursts/min <sup>c</sup>	NGDR <sup>d</sup>
<i>Calanus finmarchicus</i>	C	2.02	2.1	0.41
	80	(0.80)*	(2.6)*	(0.04)*
	E	4.98	10.3	0.71
<i>Calanus hyperboreus</i>	C	1.14	1.3	0.32
	16	(0.18)	(0.9)	(0.05)
	E	1.31	1.8	0.36
<i>Euchaeta norvegica</i>	C	1.29	0.6	0.34
	0	(0.18)	(0.8)	(0.03)
	E	1.12	0.2	0.39
<i>Metridia longa</i>	C	5.65	0.7	0.53
	58	(0.48)*	(4.1)*	(0.03)*
	E	7.02	18.3	0.81
<i>Metridia lucens</i>	C	4.13	2.7	0.45
	64	(1.09)*	(3.6)*	(0.06)*
	E	8.47	14.3	0.69
<i>Temora longicornis</i>	C	2.68	0.5	0.79
	86	(0.83)*	(2.7)*	(0.03)
	E	6.05	15.9	0.75
<i>Meganyctiphanes norvegica</i>	C	4.71	0	0.41
	0	(1.61)		(0.07)
	E	4.11	0	0.35
<i>Conchoecia borealis</i>	C	20.6	1.6	0.84
	0	(2.4)*	(2.1)	(0.04)*
	E	29.9	3.9	0.42
<i>Eukrohnia hamata</i>	C	0.95	0	0.31
	0	(0.61)		(0.11)
	E	0.88	0	0.42
<i>Tomopteris septendrialis</i>	C	12.7	0	0.61
	43	(3.5)	(2.1)*	(0.08)
	E	18.3	4.9	0.54

<sup>a</sup> Grand means for 10 groups of 1 to 5 organisms exposed to no light flash (C) and to flashes of blue light at 5 s intervals (E). The estimated standard error of the mean difference is given in parentheses. Significant differences are designated with an asterisk (Student's *t*-test with paired comparison design,  $\alpha = 0.05$ ).

<sup>b</sup> The proportion of zooplankton responding to simulated bioluminescent flashes (Percent response) is based on visual monitoring of videotaped experiments.

<sup>c</sup> The number of swimming speed bursts for each experimental trial (Bursts) was normalized by dividing by the total number of minutes that zooplankton tracks were observed during the 2 minute video recording. Speed bursts had peaks  $>15 \text{ mm} \cdot \text{s}^{-1}$  for copepods,  $>30 \text{ mm} \cdot \text{s}^{-1}$  for *Tomopteris*, and  $>50 \text{ mm} \cdot \text{s}^{-1}$  for *Conchoecia*.

<sup>d</sup> Net to Gross Displacement Ratio.

four species (except *T. longicornis*) also showed a significant tendency to swim in straighter paths (increased NGDR), although *M. longa* and *M. lucens* exhibited rapid spiralling behavior while swimming in what was otherwise an essentially straight path. *Calanus hyperboreus* exhibited an occasional weak photophobic response but showed no significant changes in average swimming speed in the presence of simulated bioluminescence. *Euchaeta norvegica* showed no evidence of a photophobic response or other change in swimming speed. Despite the absence of strong photophobic responses, both *C. hyperboreus* and *E. norvegica* were occasionally observed to make "grasping" motions with their feeding appendages immediately after simulated bioluminescent flashes.

The paths of copepods exhibiting a photophobic response to the first light flash were pooled and analyzed separately from the paths of copepods not responding to the light for each species (see Table I for percent of animals responding). No significant difference was found between direction of travel distributions during the 1 s intervals before and after the flash (Chi-square test,  $\alpha = 0.05$ ). This lack of difference suggests that the orientation of copepods with respect to the light source prior to the flash does not influence the frequency of response, nor does there seem to be a tendency for copepods responding to the flash to move preferentially toward or away from the light source immediately after the flash. Since bioluminescence in both dinoflagellates and copepods is stimulated by mechanical disturbances, potential predators and their prey should often be in direct contact when a bioluminescent flash is stimulated. Any subsequent photophobic response would tend to separate the predator and its prey, regardless of their direction of travel.

The responses of several other zooplankton species to simulated bioluminescence were also tested. Neither the euphausiid *Meganycitiphanes norvegica* nor the chaetognath *Eukrohnia hamata* showed any behavioral responses to simulated bioluminescence (Table I). The ostracod *Conchoecia borealis* exhibited no distinct photophobic response, but showed a general increase in swimming speed in the presence of simulated bioluminescence (Table I, Fig. 2) and a significant decrease in NGDR (Table I). These changes in behavior were the result of a rapid looping swimming pattern in the presence of simulated bioluminescence. In contrast, the planktonic polychaete *Tomopteris septendrionalis* showed a photophobic response consisting of a sharp turn followed by a rapid increase in swimming speed (Fig. 3) that was quite similar to the response in copepods (see Fig. 1).

More extensive tests, including the effects of changing flash color, intensity, and duration on photic responses were made with two common copepod species, *Calanus finmarchicus* (which is not bioluminescent) and *Metridia longa* (which is bioluminescent). The wavelength of light was varied to determine the wavelengths of greatest sensitivity for the photophobic responses of *Calanus finmarchicus* and *Metridia longa* (60 ms duration,  $0.2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity). Both species showed strong photophobic responses over a range of wavelengths between approximately 460–560 nm (Fig. 4). At this intensity (which represents *ca.* 10% of that given off by a bioluminescent dinoflagellate) the reactions of the copepods were so strong that it was impossible to define a narrower range of maximum sensitivity based on this photophobic response.

Varying the intensity of blue 60 ms flashes indicated that a strong photophobic response still occurs for copepods exposed to light intensities as low as  $0.002 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (Table II). Lower light intensities were not used since our light sensor was not sensitive enough to measure light in this intensity range. At all four intensities tested there was a significant increase in average swimming speed, number of bursts in swimming speed, and NGDR for copepods exposed to simulated

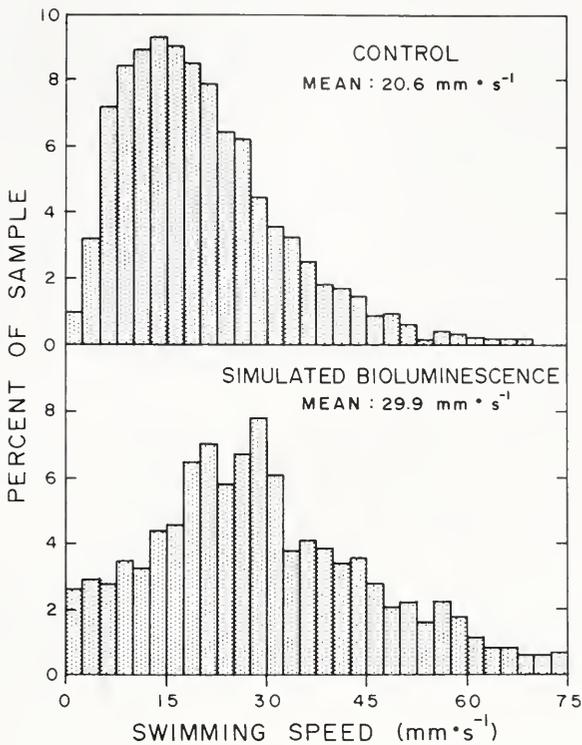


FIGURE 2. Distribution of swimming speeds for the ostracod *Conchoecia borealis* in complete darkness (top) and with a simulated bioluminescent flash (475 nm, 60 ms duration,  $2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity) presented every 5 s (bottom). These distributions are based on the pooled results from trials on 10 groups of ostracods with *ca.* 5 ostracods per trial. The mean is based on the total number of measurements of swimming speed made as the ostracods swam through the field of observation.

bioluminescent flashes compared to those under control conditions ( $\alpha = 0.05$ , Student's *t*-test with paired comparison design).

No effect of flash duration was found for the response of *Calanus finmarchicus* when the copepod was exposed to either 60 or 600 ms flashes. These responses were most easily compared as percent of copepods responding to the light flash (Table III). In contrast, *Metridia longa* showed a significantly greater percent response to 600 ms flashes than to 60 ms flashes at all intensities tested. By comparing the response of copepods to longer flashes at a given intensity *versus* shorter flashes at a higher intensity, it is also apparent that the difference in response to short and long flashes was not simply a function of total light dose. A 600 ms flash of  $0.02 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity delivers the same total light dose as a 60 ms flash of  $0.2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity, yet the 600 ms flash still resulted in a greater percent response by *Metridia longa*.

#### DISCUSSION

One of the most commonly suggested functions of the bioluminescence of dinoflagellates and copepods is as a deterrent against nocturnal predation (Tett and Kelly, 1973; Buck, 1978; Porter and Porter, 1979; Morin, 1983; Young, 1983). All

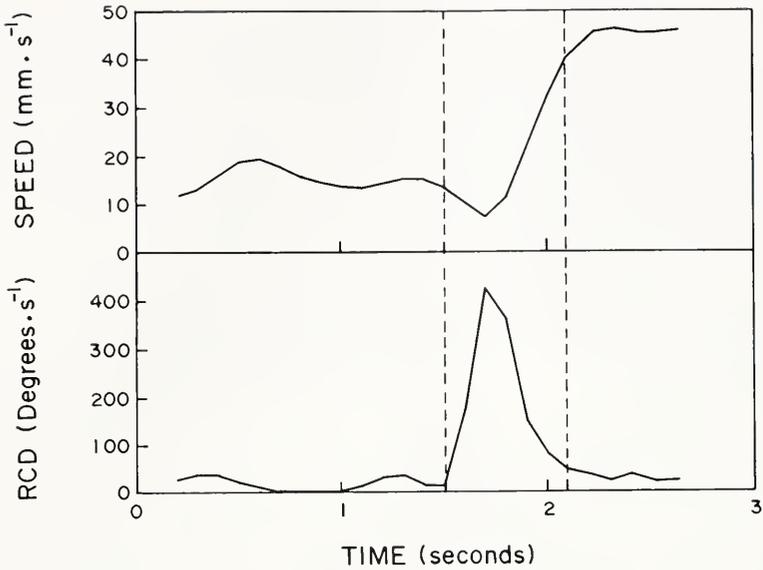


FIGURE 3. Record of swimming speed and rate of change of direction for a single *Tomopteris septentrionalis* exposed to a simulated bioluminescent flash (475 nm blue light for 60 ms duration at an intensity of  $2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ); the dashed lines indicate the beginning and end of the flash.

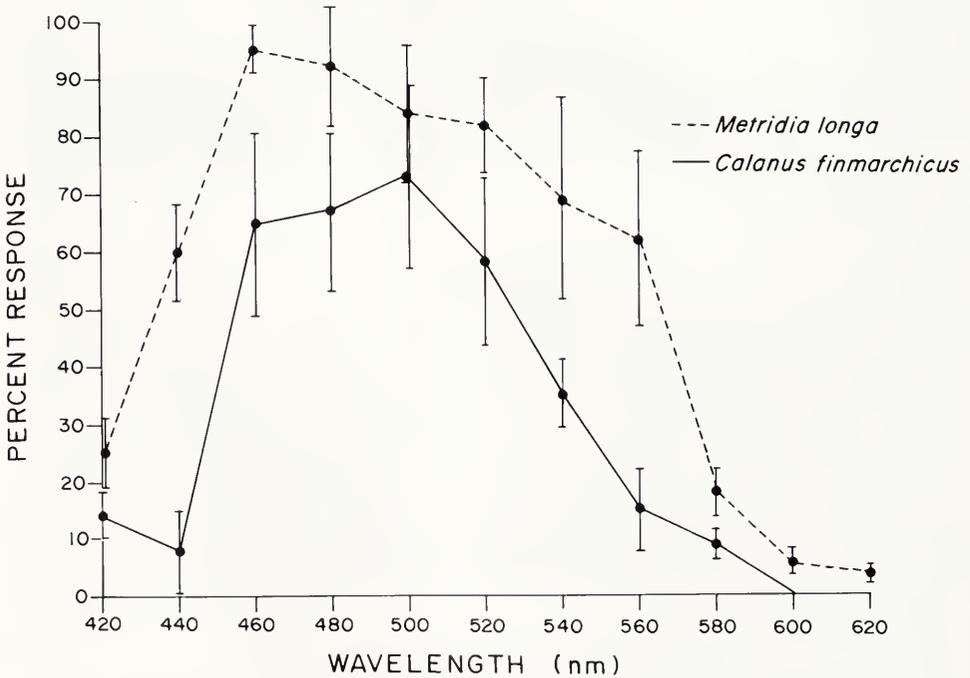


FIGURE 4. Effects of varying light color on the proportion of *Calanus finmarchicus* (solid line) and *Metridia longa* (dashed line) responding to simulated bioluminescent flashes (60 ms duration,  $0.2 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  intensity) with a burst of swimming speed. Vertical bars indicate the standard error of the mean value, based on 5 trials at each intensity with ca. 5 copepods per trial.

TABLE II

Parameters describing swimming behavior for *Calanus finmarchicus* and *Metridia longa* exposed to simulated dinoflagellate flashes at different intensities<sup>a</sup>

Intensity ( $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ )		Mean speed $\text{mm} \cdot \text{s}^{-1}$	Bursts/ min	NGDR
<i>Calanus finmarchicus</i>				
2.0	C	2.02 (0.80)*	2.1 (2.6)*	0.41 (0.04)*
	E	4.98	10.3	0.71
0.2	C	1.98 (0.53)*	3.6 (1.7)*	0.34 (0.09)*
	E	3.68	8.5	0.62
0.02	C	2.45 (0.56)*	6.3 (1.1)*	0.39 (0.06)*
	E	5.81	13.7	0.64
0.002	C	1.83 (0.88)*	2.8 (1.4)*	0.37 (0.07)*
	E	4.35	9.7	0.68
<i>Metridia longa</i>				
2.0	C	5.65 (0.48)*	0.7 (4.1)*	0.53 (0.03)*
	E	7.02	18.3	0.81
0.2	C	4.51 (1.03)*	1.5 (3.8)*	0.47 (0.04)*
	E	8.12	13.9	0.64
0.02	C	5.40 (0.87)*	2.1 (2.7)*	0.66 (0.06)*
	E	9.14	14.2	0.85
0.002	C	4.21 (0.39)*	1.7 (2.8)*	0.52 (0.04)*
	E	7.45	11.9	0.78

<sup>a</sup> Numbers are grand means for 10 groups of 5 copepods exposed to no light flash (C) and to flashes of blue light (E) (wavelength at peak emission 475 nm, duration 60 ms). The estimated standard error of the mean difference is given in parentheses. Significant differences are designated with an asterisk (Student's *t*-test with paired sample design,  $\alpha = 0.05$ ).

the calanoid copepods we tested that were potential grazers on dinoflagellates responded to simulated dinoflagellate flashes with a photophobic response (Table I). These include species considered to be mainly herbivorous such as *Calanus finmarchicus* (Conover, 1960; Anraku and Omori, 1963; Gauld, 1966) and other copepod species considered to be omnivorous such as *Calanus hyperboreus* (Conover, 1966), *Temora longicornis* (Gauld, 1966), *Metridia lucens* (Haq, 1967; Harding, 1974), and *Metridia longa* (Haq, 1967). Our results provide further support for the hypothesis that dinoflagellate bioluminescence acts to deter predation by nocturnal grazers such as copepods. The bioluminescent flash stimulated by contact between a copepod and a bioluminescent dinoflagellate should elicit a photophobic response in the copepod. This burst of swimming speed by the copepod will interrupt the

TABLE III

Effect of flash duration on percent startle response for the non-bioluminescent copepod *Calanus finmarchicus* and the bioluminescent copepod *Metridia longa*<sup>a</sup>

Intensity ( $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ )	<i>Calanus finmarchicus</i>		<i>Metridia longa</i>	
	60 ms	600 ms	60 ms	600 ms
2.0	80	65	58	96
		ns	*	*
0.2	76	69	69	97
		ns	*	*
0.02	63	72	62	98
		ns	*	*
0.002	50	67	45	92
		ns	*	*

<sup>a</sup> Responses of copepods to 60 and 600 ms flashes at each intensity, and the responses of copepods to 60 ms flashes at one intensity and 600 ms flashes at lower intensity (same total light dose) were compared using the Chi-square test for two independent samples. An asterisk indicates a significant difference at  $\alpha = 0.05$ . Sample size range: 35–54 copepods per intensity-duration treatment.

feeding behavior of the copepod (Rosenburg, 1980) and physically separate the copepod and dinoflagellate by a distance of several centimeters. *Calanus hyperboreus* only exhibited occasional photophobic responses to simulated dinoflagellate bioluminescence (Table I). It is unclear whether this reduced response compared to other omnivorous copepods was due to the physiological state of the copepods (e.g., trauma associated with their capture or handling) or if the limited response indicates that *C. hyperboreus* is truly less susceptible to the bioluminescent defenses of dinoflagellates.

There have been several detailed studies of bioluminescent copepods (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972) but only the study of David and Conover (1961) investigated the role of bioluminescence in copepod ecology experimentally. In their experiments the euphausiid *Meganyctiphanes norvegica* and ca. 10 bioluminescent copepods (*Metridia lucens*) were placed in a beaker in front of a photomultiplier tube in darkness. The number of multiple flash sequences corresponded well to the number of copepods consumed, and these multiple flashes were assumed to represent bioluminescence stimulated during capture and consumption of copepods. Since flashes were rarely recorded when *Metridia* was held separately or in the presence of non-predatory euphausiids or amphipods, single flashes were assumed to represent attempted captures and successful escapes. The results of David and Conover do not provide evidence that bioluminescence aided the escape of the *Metridia lucens* from *Meganyctiphanes norvegica*, however. Since bioluminescence is stimulated mechanically in copepods, bioluminescence should be produced during any attempted capture and subsequent handling of the copepods. Additional experimental evidence is needed to determine if the bioluminescent flash increases the probability of escape. The results of our study show no apparent change in the behavior of *M. norvegica* when exposed to simulated copepod bioluminescence (Table I). This lack of response by *M. norvegica* could be due to a number of experimental conditions (i.e., confinement, shock from capture, etc.) so these results do not rule out a defensive role for bioluminescence in *M. norvegica*-*M. lucens* predator-prey interactions. However, the lack of response to simulated bioluminescence by three potential invertebrate predators on *Metridia*

(*Meganyctiphanes*, *Eukrohnia*, and *Euchaeta*) suggests that the proposed defensive function of bioluminescence in *Metridia* may have evolved instead for defense against visual predators such as planktivorous fish, or for some purpose other than defense. We have not yet tested the responses of fish to simulated bioluminescence.

Of the potential predators on copepods we tested, only *Tomopteris septendriionalis* responded to a simulated copepod flash with a sharp increase in swimming speed (Fig. 3, Table I). Thus bioluminescence of *Metridia* and other bioluminescent organisms could potentially serve as a deterrent to predation by *Tomopteris*, although the extent to which this predation occurs in nature is unknown. Dales (1971) has suggested that bioluminescence in *Tomopteris* might serve as a mating signal. It seems unlikely that the photophobic response to a diffuse blue light flash that we observed for *Tomopteris* would represent an adaptation for mate location. The bioluminescent display produced by *Tomopteris* is quite different than that produced by *Metridia*, however, and a specific photic signal might be required to elicit mating behavior in *Tomopteris*. The peak wavelengths of light emission is between 560–580 nm for *Tomopteris septendriionalis* (Terio, 1960 cited in Dales, 1971) compared to a peak of ca. 480 nm for *Metridia lucens* (David and Conover, 1961). The occurrence and distribution of the light-producing rosette organs in the parapodia of *Tomopteris* varies between different *Tomopteris* species and thus could produce species specific patterns that might act as recognition signals for mating (Dales, 1971).

Ostracods of the genus *Conchoecia* are reported to feed mainly on dead crustaceans and small masses of detritus (Lochhead, 1968; Angel, 1970) and are probably not potential predators on either dinoflagellates or copepods. Therefore lack of a photophobic or “startle” response by *Conchoecia borealis* to simulated dinoflagellate and copepod bioluminescence observed in this study neither supports nor refutes a hypothetical defensive function of bioluminescence in these groups. The response of *C. borealis* to simulated bioluminescence was a general increase in swimming speed and a increased curvature of swimming paths (Table I). The adaptive value of this photokinetic response is not obvious, but perhaps these changes in behavior represent an avoidance response elicited when in the presence of high concentrations of bioluminescent dinoflagellates, whose luminescence might reveal the location of the ostracods to visual predators (Burkenroad, 1943). *C. borealis* is itself bioluminescent, and although the biochemistry of luminescence is well known in the Ostracoda, little is known about the ecology of their bioluminescence (Tett and Kelly, 1973). The secretion of bioluminescent clouds by ostracods in response to mechanical stimulation (Angel, 1968) suggests at least a defensive role for ostracod bioluminescence.

In this study a bioluminescent copepod (*Metridia longa*) was found to be more responsive to simulated copepod bioluminescence than to simulated dinoflagellate bioluminescence (Table III). No similar difference in responsiveness was found in either the non-bioluminescent copepod tested during this study (*Calanus finmarchicus*) or in the non-bioluminescent estuarine copepod tested in a previous study (*Acartia hudsonica*, Buskey and Swift, 1983). This difference suggests that the behavioral response of *Metridia longa* may have evolved by increasing its sensitivity to longer duration flashes such as those produced by conspecifics. This type of response could have potential adaptive value since long duration (>600 ms) bioluminescent signals from conspecifics could indicate an attack by a predator such as a euphausiid, since bioluminescence in copepods is stimulated primarily by interaction with predators (David and Conover, 1961).

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## LIPID DECLINE IN STRESSED CORALS AND THEIR CRUSTACEAN SYMBIONTS

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### ABSTRACT

Total lipid levels, determined by the phosphosulphovanillin colorimetric method, declined significantly in ramose scleractinian corals and their xanthid crab symbionts during the 1983 El Niño warming event on the Pacific coast of Panamá. This decline was observed in a controlled laboratory experiment, employing host corals (*Pocillopora damicornis*) and obligate crab symbionts (*Trapezia corallina* and *Trapezia ferruginea*), concurrently with morbidity and mortality observed on coral reefs in the field. Lipid levels decreased from 0.59% (dry weight) to 0.34% in normal *versus* affected and dead corals, and from 4.54% (dry weight) to 1.20% in normal *versus* affected and dead crabs in a two-week period. Lipid depletion in corals accompanied the loss of zooxanthellae and increased morbidity and death; in crabs, a decrease in the number of egg-carrying females, a high emigration rate, a slight increase in mortality, and a decline in defensive behavior occurred. These findings suggest that symbiotic crabs were deprived of food from their coral hosts who initially lost zooxanthellae, an event correlated with the prolonged El Niño sea warming.

### INTRODUCTION

Studies on the feeding biology of obligate crustacean symbionts (crabs and shrimp) inhabiting corals have indicated a strong trophic dependency, with crustaceans using coral host mucus and entrapped organic matter (Knudsen, 1967; Patton, 1974, 1976; Castro, 1976). The high lipid content of mucus, an important energy source for the crustaceans (Benson and Muscatine, 1974), is believed to be derived from the symbiotic zooxanthellae present in coral tissue (Crossland *et al.*, 1980; Davies, 1984). Reef-building corals harboring crustacean symbionts were stressed—presumably due to a prolonged El Niño warming spell—and experienced massive zooxanthellae loss (bleaching) and widespread mortality in tropical eastern Pacific waters in 1983 (Glynn, 1983a, 1984). Observations in Panamá demonstrated that mucus release declined significantly in affected (bleached) corals and that the number of crustacean symbionts per colony and agonistic (defensive) behavior of the crabs also declined during this period (Glynn, in prep.).

Advantage was taken of this large-scale warming disturbance to determine the concentrations of lipid stores in the affected corals and in their crustacean symbionts. If the lipid reserves of affected crustacean symbionts could be shown to decline simultaneously with the deterioration of their principal food source, then this would suggest the influence of food deprivation in disrupting the coral-crustacean symbiotic

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bond during the 1983 EL Niño event. It is also possible that the symbiotic crustaceans were adversely affected by temperature alone, but no other reef organisms, including numerous crustacean species, were apparently stressed during the warming period.

In this study we examine lipid levels in coral hosts and crab symbionts affected by the 1983 El Niño sea warming episode in Panamá. Major emphasis is given to the coral-crab mutualism in outdoor aquaria in the upwelling environment of the Gulf of Panamá. These observations are supplemented with data obtained from field populations in the non-upwelling waters of the Gulf of Chiriquí.

#### MATERIALS AND METHODS

The scleractinian coral host *Pocillopora damicornis* (Linnaeus), and its xanthid crab symbionts, *Trapezia corallina* Gerstaecker and *Trapezia ferruginea* Latreille, were examined. All animals were collected at 3–8 meters depth on a small, pocilloporid patch reef at Urabá Island (8°47'03"N; 79°32'22"W), Taboga Islands, Gulf of Panamá. Three collections were made over the period 1 June to 25 July 1983 to carry out observations during the unusual warming event experienced in 1983 in the Gulf of Panamá. Coral populations in the Gulf of Chiriquí (northwestern Pacific coast of Panamá) were affected in February–March, and corals in the Gulf of Panamá were affected later, beginning in June 1983, after the upwelling season (Glynn, 1983a, 1984). Nearly all corals in the first two collections lost their zooxanthellae and died within a week of collection. By the third collection (25 July), the disturbance had stabilized somewhat, *i.e.*, normal and affected corals remained visibly the same, retaining zooxanthellae and bleached tissues respectively, for 3–4 weeks. This last collection provided the main material for the laboratory observations in this study.

Eight coral colonies of each of four conditions—*normal* (N), usual brown color uniformly present; *partially bleached* (PB), loss of some color, especially on upper branches; *bleached* (B), colony nearly uniformly white; *dead* (D), live coral tissues absent, colony covered with thin growth of pioneering filamentous algae—were selected and assigned to each treatment. These corals were 10–14 cm in maximum colony diameter and contained all live branches, or recently dead branches in the case of the dead group. Crab symbionts were also collected from normal *Pocillopora damicornis* corals obtained on 25 July 1983 at Urabá Island, and one naturally paired male and female was added to each colony in the four treatments. Twenty-one pairs of *Trapezia corallina* and 11 pairs of *Trapezia ferruginea* were introduced onto the experimental corals. Crab carapace widths ranged from 8 to 14 mm ( $\bar{x}$  = 10.9, S.D. = 1.73) and dry weights from 137.9 to 758.5 mg ( $\bar{x}$  = 326.8, S.D. = 183.4).

Each coral colony with its pair of crab symbionts was maintained in a 2.6 l glass bowl. Plastic netting was attached to the rim of each bowl to prevent the escape of crabs in the overflow. Continuously flowing sea water, filtered through medium coarse #20 silica sand, was supplied at a rate of about 1 l/min. Colonies were placed on partially shaded, outdoor tables receiving 50–70% natural lighting. Colony locations were determined by strict random assignment and they were not moved during the experiment (28 July–11 August, 1983).

At the end of the experiment, branch tips (2–3 cm long) of corals and whole crabs were rinsed gently with distilled water to remove salts and dried to constant weight *in vacuo* over silica gel for seven days. Gravid female crabs with eggs were analyzed *in toto*. After drying, the samples, including tissues and skeleton, were reduced to a fine powder by grinding with a mortar and pestle. Total lipid contents

were determined by the phosphosulphovanillin, colorimetric method of Barnes and Blackstock (1973).

Field observations on the condition of host corals and crab symbionts were also made during the warm water periods March to June, 1983, at Uva Island (7°48'46"N; 81°45'35"W), Gulf of Chiriquí, and during June to October, 1983, at Urabá Island, and the northern Pearl Islands (Saboga Island, 8°37'29"N; 79°03'23"W), Gulf of Panamá.

## RESULTS

Median lipid levels (% dry weight) in the host coral *Pocillopora damicornis* differed significantly among the four conditions examined ( $P \ll 0.001$ , Kruskal-Wallis test). The highest levels found in normal corals (median = 0.59%) were significantly higher than those of the affected (medians = 0.28–0.37%) and dead corals which had statistically similar levels (Kruskal-Wallis multiple comparison procedure, Daniel, 1978, Table I). The lipids found in dead corals (median = 0.36%) were probably present in the algae and other organisms colonizing the skeletal surface.

*Trapezia* spp. crabs inhabiting normal coral hosts had significantly higher lipid levels, with median = 4.5% lipid, than crabs present in affected and dead corals, with median lipid levels that ranged from 0.80 (in dead coral) to 1.48% (in partially bleached coral) ( $P \ll 0.001$ , Kruskal-Wallis test). *A posteriori* multiple comparison testing (Daniel, 1978) indicated that the low lipid levels of crabs present in partially bleached and bleached corals were similar, as were the low lipid levels detected in crabs from bleached and dead corals (Table II). A comparison of lipid levels between the two crab species (*Trapezia corallina* and *Trapezia ferruginea*) within each of the four coral conditions failed to reveal any significant differences between species (Table III).

No significant differences in lipid levels between crab sexes were evident (Table III). This result for crabs present in normal corals was unexpected because 50% of the females were carrying large numbers of eggs. Body lipid levels generally increase in female crustaceans in preparation for and during breeding periods (Du Preez and McLachlan, 1983; Tessier *et al.*, 1983). Only one female in eight (12.5%) was gravid in each of the affected and dead groups of coral. The frequency of gravid female

TABLE I

Total lipid levels (% dry weight) in normal (N), affected (PB, B), and dead (D) branch tips of *Pocillopora damicornis*

	Condition of coral host			
	N	PB	B	D
Median	0.59	0.37	0.28	0.36
MCP <sup>1</sup>				
Range	0.24–0.85	0.11–0.45	0.09–0.48	0.14–0.64
0.95 conf. lim. <sup>2</sup>	0.44–0.66	0.25–0.41	0.14–0.45	0.24–0.51
Number	8	8	8	8

<sup>1</sup> *A posteriori* multiple comparison procedure (Kruskal-Wallis test, Daniel, 1978),  $\alpha = 0.20$ , line joins statistically equal median values.

<sup>2</sup> Confidence limits of median calculated as  $K = 0.5(n + 1) - (n)^{1/2}$  of the range, where K is the number of units from each end of the distribution toward the median.

TABLE II

Total lipid levels (% dry weight) of *Trapezia* spp. crabs inhabiting normal (N), affected (PB, B), and dead (D) pocilloporid corals

	Condition of coral host			
	N	PB	B	D
Median MCP <sup>1</sup>	4.54	1.48	1.32	0.80
Range	2.47-12.15	0.95-4.74	0.85-3.05	0.57-1.70
0.95 conf. lim. <sup>2</sup>	3.76-5.25	1.24-1.85	1.04-1.61	0.66-1.05
Number <sup>3</sup>	12(4)	10(6)	10(6)	10(6)

<sup>1</sup> *A posteriori* multiple comparison procedure (Kruskal-Wallis test, Daniel, 1978),  $\alpha = 0.20$ , lines join statistically equal median values.

<sup>2</sup> Confidence limits of median calculated as  $K = 0.5(n + 1) - (n)^{1/2}$  of the range, where K is the number of units from each end of the distribution toward the median.

<sup>3</sup> First entry denotes number of *Trapezia corallina*, entry in parentheses denotes number of *Trapezia ferruginea*. Results from the two crab species were pooled because no species differences were evident (see Table III).

crabs was significantly higher ( $P = 0.042$ , Fisher exact test) in normal than in affected and dead coral hosts combined. Since the reproductive condition of crabs was noted only at the end of the experiment, it is not known if the frequency of

TABLE III

Comparisons of total lipid levels (% dry weight) in two crab species (*T. corallina* and *T. ferruginea*) and in female and male crabs

	Condition of coral host							
	Normal		Partially bleached		Bleached		Dead	
	<i>Trapezia corallina</i>	<i>Trapezia ferruginea</i>						
Crab species differences								
Median	4.04	6.66	1.56	1.33	1.40	1.28	0.80	0.89
Probability <sup>1</sup>	>0.05		>0.05		>0.05		>0.05	
Range	2.47-12.15	4.55-12.02	1.01-4.74	0.95-2.15	0.85-3.05	1.04-1.61	0.57-1.57	0.62-1.70
0.95 c.l. <sup>2</sup>	3.76-4.85	4.55-12.02	1.36-1.85	1.16-1.78	0.95-1.69	1.17-1.39	0.67-0.96	0.66-1.06
Number	12	4	10	6	10	6	10	6
Crab sex differences								
Median <sup>3</sup>	♀ 4.70	♂ 4.04	♀ 1.40	♂ 1.56	♀ 1.46	♂ 1.30	♀ 0.80	♂ 0.85
Probability <sup>1</sup>	~0.29		>0.36		~0.25		>0.48	
Range	3.50-12.02	2.47-12.15	0.95-4.74	1.01-3.53	0.90-3.05	0.85-1.69	0.57-1.06	0.61-1.70
0.95 c.l. <sup>2</sup>	3.76-5.25	3.75-8.62	1.24-1.85	1.16-2.15	1.04-2.60	0.95-1.45	0.67-1.05	0.62-1.57
Number <sup>4</sup>	8(4)	8	8(1)	8	8(1)	8	8(1)	8

<sup>1</sup> Associated with Mann-Whitney U test.

<sup>2</sup> Confidence limits of median calculated as  $K = 0.5(n + 1) - (n)^{1/2}$  of the range, where K is the number of units from each end of the distribution toward the median.

<sup>3</sup> Crabs of each sex were pooled since no significant species differences were evident.

<sup>4</sup> Number of gravid crabs in parentheses; eggs included in analysis.

gravid females declined in the stressed coral groups, if more crabs released eggs in the normal group, or if their numbers remained unchanged over the two-week period (see field results below).

*Trapezia* spp. showed a higher rate of emigration from bleached and dead coral hosts than from normal corals (Table IV). The data demonstrating this trend are from two experiments initiated in July and discontinued because of high coral mortality and unrestricted movements or loss of some crabs. In these first experiments the bowls were not rimmed with netting to confine the crabs to their respective coral hosts. Median emigration rates were significantly different among the coral hosts of varying condition in both the 5 July and 20 July experiments ( $0.01 > P > 0.001$  in both cases, Kruskal-Wallis test). From 5 to 6 July, 2 crabs/colony/day emigrated from bleached and dead corals, whereas normal and partially bleached colonies lost only 1 crab/day each (Table IV). From 20 to 21 July, no crabs left their normal hosts, but median emigration rates of 0.5 to 1 crab/colony/day were observed in partially bleached, bleached, and dead corals.

All crabs that died in laboratory experiments were associated with either partially bleached, bleached, or dead corals (Table V). Overall crab death in the two experiments ranged from 4.0% in dead corals to 15.4% in bleached corals. Although these results suggest that crab mortality was highest in affected (partially bleached and bleached) and dead corals, statistical testing of the pooled data—normal ( $n = 32$ ) versus affected ( $n = 81$ )—indicate a nonsignificant difference ( $X_3^2 = 6.46$ ,  $0.10 > P > 0.05$ ).

Although no attempt was made to quantify the defensive behavior of the crabs—which typically involves threat displays and attacks directed toward intruding competitors and predators (Glynn, 1983b; Abele, 1984)—it was clear that by the end of the 28 July experiment the crabs associated with normal corals were more alert and forceful in their movements than those on bleached and dead corals.

TABLE IV

*Number of Trapezia spp. crab symbionts emigrating per day from individual coral colonies of varying condition in the laboratory, July 1983*

	Condition of coral host			
	N	PB	B	D
<i>5-6 July</i>				
Median <sup>1</sup>	1	1	2	2
MCP <sup>2</sup>				
Range	0-2	0-2	1-2	2-2
0.95 conf. lim. <sup>3</sup>	0-2	0-1	1-2	2-2
<i>20-21 July</i>				
Median <sup>1</sup>	0	0.5	1	1
MCP <sup>2</sup>				
Range	0-0	0-2	0-2	0-2
0.95 conf. lim. <sup>3</sup>	0-0	0-1	0-2	1-1

<sup>1</sup> Based on eight coral colonies, each with one pair of crabs.

<sup>2</sup> *A posteriori* multiple comparison procedure (Kruskal-Wallis test, Daniel, 1978).  $\alpha = 0.20$ , lines join statistically equal median values.

<sup>3</sup> Confidence limits of median calculated at  $K = 0.5(n + 1) - (n)^{1/2}$  of the range, where K is the number of units from each end of the distribution toward the median.

TABLE V

Number of *Trapezia* spp. crab symbionts that died in coral colonies of varying condition in the laboratory, July–August 1983

	Condition of coral host			
	N	PB	B	D
<i>5–20 July</i>				
Number dead	0	3	2	1
Percent dead <sup>1</sup>	0	21.4	20.0	11.1
Total number crabs	16	14	10	9
<i>28 July–11 August</i>				
Number dead	0	1	2	0
Percent dead	0	6.2	12.5	0
Total number crabs	16	16	16	16
Overall percent dead	0	13.3	15.4	4.0

<sup>1</sup> Crabs lost from bowls are omitted from calculations of percent dead.

Threat displays could be easily elicited from crabs in normal corals by probing with forceps, whereas crabs in bleached and dead corals moved away from the probe, remained motionless, or responded only weakly. The median defensive behavior of *Trapezia* spp. in normal coral hosts in the field (Uva Island reef, Gulf of Chiriquí, 27–28 April 1983) was 10 responses per colony per 3 min; this declined to 3 responses in partially bleached corals and the crustacean guards in fully bleached and dead colonies were virtually unresponsive, each exhibiting median responses of 0 (Glynn, in prep.).

As in the laboratory results, a higher proportion of female *Trapezia* spp. were gravid in normal than in affected or dead corals in the field (Table VI). In two collections, the overall frequency of gravid crabs in normal corals was 43.9%, whereas this ranged from 17.9% (partially bleached) to 26.7% (bleached) in affected corals. However, this difference is not statistically significant ( $X_3^2 = 5.71$ ,  $0.20 > P > 0.10$ ). The frequency of gravid crabs also declined in each of the four host coral conditions from April to June (Table VI). This decrease in egg-carrying crabs was

TABLE VI

Number of gravid *Trapezia* spp. in normal, affected, and dead colonies of *Pocillopora damicornis* at Uva Island reef in April and June, 1983

	Condition of coral host			
	N	PB	B	D
<i>28 April</i>				
Number gravid	9	2	2	2
Percent	75.0	50.0	33.3	40.0
Number ♀♀ sampled	12	4	6	5
<i>24 June</i>				
Number gravid	9	3	2	0
Percent	31.0	12.5	22.2	0
Number ♀♀ sampled	29	24	9	4
Overall percent gravid	43.9	17.9	26.7	22.2

statistically significant in normal corals ( $P = 0.011$ , Fisher exact test), but not so in partially bleached ( $P = 0.124$ ), bleached ( $P = 0.396$ ), or dead corals ( $P = 0.277$ ).

#### DISCUSSION

Large amounts of lipid are often found in the mucus of healthy corals and commonly range from about 20 to 90% dry weight (Benson and Muscatine, 1974; Ducklow and Mitchel, 1979; Daumas *et al.*, 1982). These high lipid levels seem to be derived from zooxanthellar lipogenesis and translocation to coral tissues (Crossland *et al.*, 1980; Davies, 1984). Even if the pure mucus of some coral species contains little lipid (3–4% reported by Krupp, 1982), contamination by nematocysts, bacteria, organic debris, and especially zooxanthellae will usually increase lipid levels considerably. Such high lipid levels probably represent an important energy source for the crustacean symbionts that feed on coral mucus. This nutrient-rich pathway may have served as a basis for selection favoring the formation of coral-crustacean mutualisms, as suggested by Thompson (1982) in the evolution of the mutualistic symbiosis of coral and zooxanthellae. The coral-crustacean energy shunt is one step removed from the usual host-symbiont interactions in nutrient-poor environments, *e.g.*, lichens (algae and fungi) and ant-plant mutualisms. In these mutualisms the algae and ants supply low nutritional inputs to their hosts. In the coral-crustacean partnership the trophic input is opposite in direction with the coral host providing nutrition to the crustacean symbionts via its plant symbionts. While it is possible that the waste products of crustacean symbionts provide nutrients (*e.g.*, nitrogen) for host zooxanthellae, this kind of interaction has not yet been demonstrated (Patton, 1976).

With the approximately 50% decline in lipids in coral branches and 40% decline in mucus release among stressed corals (unpub. data), it is highly likely that crustacean symbionts were deprived of a significant proportion of their food resource. Only symbiotic crustaceans on stressed corals showed a debilitated state (increased emigration, apparent decline in reproductive activity, and reduced defensive behavior), and had a marked reduction in lipid content, to about 30% of values observed in healthy coral hosts. Castro (1978) observed movement in *Trapezia* spp. and concluded that one of the reasons for increased emigration was an insufficient supply of coral mucus. Thus, it appears that the decline in energy-rich mucus of host corals was the main factor leading to the disturbances observed in the symbiotic crabs.

This study provides evidence that lipid reserves can be drawn down rapidly in both corals and crustacean symbionts during periods of stress. In just 14 days lipid levels were reduced by 50% in corals and by 78% in crabs. A rapid decline in lipids, 60% loss in 15 days, has also been found in *Pocillopora damicornis* subjected to reduced light levels in Hawaii (Stimpson, pers. comm.; see Clayton and Lasker, 1982, and Szmant-Froelich and Pilson, 1980 for additional examples of rapid weight loss and lipid decline in corals).

The principal organisms affected during the 1982–83 El Niño warming period were coelenterates containing endosymbiotic zooxanthellae (Glynn, 1984; Suharsono and Kiswara, 1984; Lasker *et al.*, in press). This severe and prolonged sea warming event probably disrupted the coral-dinoflagellate mutualism, resulting in the expulsion or emigration of zooxanthellae. Numerous bleached corals, especially ramose species with high growth rates, died in 2–5 weeks following the loss of zooxanthellae. Repeated observations and quantitative sampling of reef organisms other than reef-building corals (polychaete worms, gastropods, crustaceans, echinoderms, and fishes),

during and after the warming episode in both upwelling and non-upwelling environments, failed to reveal evidence of stress or mortality in non-zooxanthellate bearing species. Even the ahermatypic (non-zooxanthellate) coral *Tubastraea coccinea* Lesson was unaffected by this disturbance. Thus, it seems unlikely that whatever conditions (probably prolonged high temperatures) stressed and killed hermatypic corals also independently affected only the coral crustacean symbionts.

Some evidence suggests that the crustacean symbionts of *Pocillopora* are generally more sensitive to environmental extremes than are their coral hosts. Upwelling and attendant extreme conditions (Abele, 1976, 1979), as well as oxygen depletion (Glynn, unpub. obs. in Panamá and Guam) can differentially kill crab and shrimp symbionts. Such stressful conditions sometimes cause partial coral mortality, but usually the crustacean symbionts are more sensitive and succumb to these disturbances, thus leaving only the surviving coral hosts. It should be noted, however, that extreme low tidal exposures occasionally kill shallow, reef flat corals and allow a high initial survival of crustacean symbionts because of their ability to emigrate to slightly deeper reef habitats (Glynn, 1976). In this study the mass mortality of normally hardy coral hosts caused a secondary and severe mortality of the more sensitive obligate crustacean symbionts. This mortality event is similar to Futuyma's (1973) prediction that the elimination of inflexible or hardy species among groups of species in usually constant environments may lead to the elimination of a suite of interdependent species.

Abele (1976) has demonstrated that crustaceans associated with pocilloporid corals in Panamá show a higher species richness in fluctuating (upwelling) than in constant (non-upwelling) environments. He hypothesized that the periodic disturbances caused by upwelling prevent specialist species from monopolizing the limiting resources of host corals. Since coral host and crustacean symbiont mortalities were similar in the upwelling and more constant non-upwelling environments, it is not likely that this disturbance had a selective and diversifying effect on coral-associated crustaceans in the seasonally fluctuating upwelling environment.

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DEVELOPMENT AND METAMORPHOSIS OF THE BRITTLE STAR  
*OPHIOCOMA PUMILA*: EVOLUTIONARY AND  
ECOLOGICAL IMPLICATIONS<sup>1</sup>

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ABSTRACT

The development to metamorphosis of the shallow-water tropical-Atlantic ophiuroid *Ophiocoma pumila* is described for the first time. This species possesses an eight-armed planktotrophic ophiopluteus larva that has a potential pelagic existence of three months (26–27.5°C), considerably longer than other brittle star species so far studied. This likely accounts for the amphi-Atlantic distribution of *O. pumila*. During metamorphosis, all of the larval arms are resorbed into the developing ophiuroid rudiment although the right antero-lateral arm disappears last. The metamorphosing larva transforms into a highly mobile vitellaria possessing transverse ciliary bands and tube feet. Ophiuroid vitellariae have hitherto been described only for species with abbreviated lecithotrophic development. The vitellaria of *O. pumila* can delay settlement for at least a week and probably functions as a pre-settling exploratory stage. The discovery of both an ophiopluteus and a vitellaria in the ontogeny of a brittle star strongly supports the proposal that ophiuroid vitellariae are related to ophioplutei and are not a divergent larval series. Abbreviated development in brittle stars with vitellariae may be the outcome of heterochrony caused by an acceleration of metamorphosis. Little is known about ophiuroid metamorphosis so it is possible that the ophioplutei of many other species pass through a vitellaria stage towards the end of their pelagic existence.

INTRODUCTION

The fact that in several forms, especially those which keep one of the anterolateral arms intact during metamorphosis, the ciliated band is broken up in pieces, so as to recall the ciliated rings in the Auricularian pupa, may merely be hinted at here. The discussion of its meaning must be left for another occasion.

Mortensen, 1921, p. 125

Many ophiuroid echinoderms have a feeding (planktotrophic) larva, the ophiopluteus, which usually possesses four pairs of arms supported by skeletal spicules and bordered externally by a ciliated band. Ophioplutei generally have a planktonic existence of about one month or less (Hendler, 1975) followed by a relatively gradual metamorphosis that commences prior to settlement (Chia and Burke, 1978; Strathmann, 1978). Two basic patterns of metamorphosis are presently known (Mortensen, 1921, 1931). In the first (here called Type 1) as exemplified by *Ophiothrix* spp., *Ophiomaza cacaotica*, and *Ophiopholis aculeata* (MacBride, 1907; Mortensen, 1937, 1938; Olsen, 1942; Mladenov, 1979), three pairs of shorter, inner

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arms are simultaneously resorbed into the developing ophiuroid rudiment while an outer pair of longer postero-lateral arms remains intact. The rudiment quickly develops tube feet and becomes suspended, oral surface downwards, from the postero-lateral arms. This form probably functions as a pre-settling exploratory stage with the ciliated postero-lateral arms used for swimming and maneuvering and the tube-feet for substrate testing and attachment.

A second type of ophiuroid metamorphosis (here called Type II) has been described only superficially for ophioplutei of *Amphiura filiformis* and *Ophiura albida* (Mortensen, 1931), and for several unidentified ophioplutei collected from the plankton during metamorphosis (Mortensen, 1921). In these, all four pairs of larval arms are resorbed into the rudiment, although the right antero-lateral arm apparently disappears last. Mortensen (1921) made the interesting but little known observation that during the metamorphosis of some of these larvae the ciliated band associated with the shrinking arms appears to become discontinuous, forming rings around the developing rudiment. The rudiment then resembles, in Mortensen's words, an auricularian pupa (= holothurian doliolaria; see Mortensen, 1927, p. 355). No one has yet verified this observation nor speculated on its possible evolutionary and functional significance.

Strathmann (1978) recognized that the timing of metamorphosis relative to settlement in brittle stars with Type II metamorphosis is a conundrum which requires further study. Settlement could occur before the arms, with their band of cilia, are completely resorbed. If so, the larvae would retain some maneuverability at settlement (important, presumably, for site selection) but attachment structures (*i.e.*, tube feet) would not be fully developed. Alternatively, metamorphosis could be completed in the plankton with juveniles settling and attaching to the bottom. However, juveniles, because they lack a ciliated band, would have limited site selection capacity.

This paper reports that the ophiopluteus of the brittle star *Ophiocoma pumila* Lütken is potentially teleplanic and passes through a vitellaria (= doliolaria) stage during Type II metamorphosis. The evolutionary and ecological implications of these findings are considered.

#### MATERIALS AND METHODS

*Ophiocoma pumila* were collected at a depth of 7 m from Columbus Park Reef (located on the west side of Discovery Bay, Jamaica) on the afternoon of 12 July 1982. At this site, *O. pumila* is abundant on and beneath the coralline red alga, *Amphiroa tribulis* (Ellis and Solander) Lamouroux. The brittle stars were taken to the nearby Discovery Bay Marine Laboratory and placed in fingerbowls containing sea water. Males and females spawned spontaneously at 1835 h on the same day. The fertilized eggs were rinsed several times with Millipore-filtered sea water and cultured in polystyrene dishes at 26–27.5°C (approximate temperature at the collecting site) in stirred Millipore-filtered sea water containing antibiotics (Mladenov, 1979). The resulting larvae were fed a mixture of the following algae: *Amphidinium carterae*, *Isochrysis galbana*, *Dunaliella salina*, *Phaeodactylum tricorutum*, and a Tahitian strain of *Isochrysis galbana*. The algal culturing procedures are outlined in Mladenov (1984).

On 30 July 1982 about 200 larvae were placed in a thermos with about 2 l of culture medium and transported to the Biology Department, Mount Allison University, Canada. Total transit time was 28 h. At Mount Allison the larvae were reared at 27°C as described above; the cultures were not stirred. The culture

medium was prepared from Discovery Bay sea water which was also transported to Mount Allison.

Photographs were taken in transmitted light with a Zeiss compound microscope equipped with an Olympus OM-2n camera.

## RESULTS

### *Description of gametes*

The spawned fertilized eggs of *Ophiocoma pumila* are approximately 73  $\mu\text{m}$  in diameter ( $n = 7$ ) and pink-red in color due to the presence of red pigment granules scattered throughout the cream colored ooplasm. Each egg is surrounded by a smooth fertilization envelope (Devaney, 1970). A single, clear polar body was usually visible beneath the fertilization envelope indicating that eggs are spawned and fertilized as secondary oocytes. Spermatozoa have spherical heads approximately 2.5  $\mu\text{m}$  in diameter and tails approximately 40  $\mu\text{m}$  in length ( $n = 5$ ).

### *Larval development*

A chronology of larval development for *O. pumila* is presented in Table I and a brief supplemental description is provided here.

Hatching from the fertilization envelope takes place at the early gastrula stage. Gastrulae are uniformly ciliated and possess a glycocalyx (= cuticle) which is easily resolved with the light microscope and which persists throughout larval development. The gastrulae swim just beneath the surface of the culture water; postero-lateral arm buds are evident two days after fertilization and by five days (Fig. 1A) the gastrulae have developed into feeding ophioplutei with postero-lateral arms and incipient antero-lateral arms. During the next eight weeks the ophioplutei slowly assume their final form, acquiring post-oral and postero-dorsal arms, and growing much larger in overall size. The fully formed ophiopluteus of *O. pumila* is a singularly beautiful larva characterized by a large pre-oral region; short, broad antero-lateral arms; and a pair of epaulettes. These are specialized regions of the ciliated band having longer cilia than the rest of the band and located at the base of the arms (Fig. 1B). The distance between the tips of the postero-lateral arms is approximately 1.1 mm. The skeleton is somewhat unusual because the body rods, which normally join in the

TABLE I

*Chronology of larval development of Ophiocoma pumila (26.0–27.5°C)*

Time	Developmental events
0	Spawning and fertilization.
3.5 h	Four-cell stage.
21 h	Gastrulae hatched from fertilization envelope.
1.5 days	Skeletal spicules are evident in gastrulae.
2.0 days	Postero-lateral arms begin to develop.
2.5 days	Digestive tract fully formed and feeding has begun as shown by the accumulation of phytoplankton cells in the stomach.
5.0 days	Antero-lateral arms begin to develop.
9.0 days	Post-oral arms begin to develop.
35 days	Postero-dorsal arms are evident.
61 days	Fully formed ophiopluteus larva with four pairs of arms and a set of epaulettes.
79 days	First signs of metamorphosis: developing hydrocoel evident and arm resorption begins. Some ophioplutei delayed commencement of metamorphosis for at least 12 days.

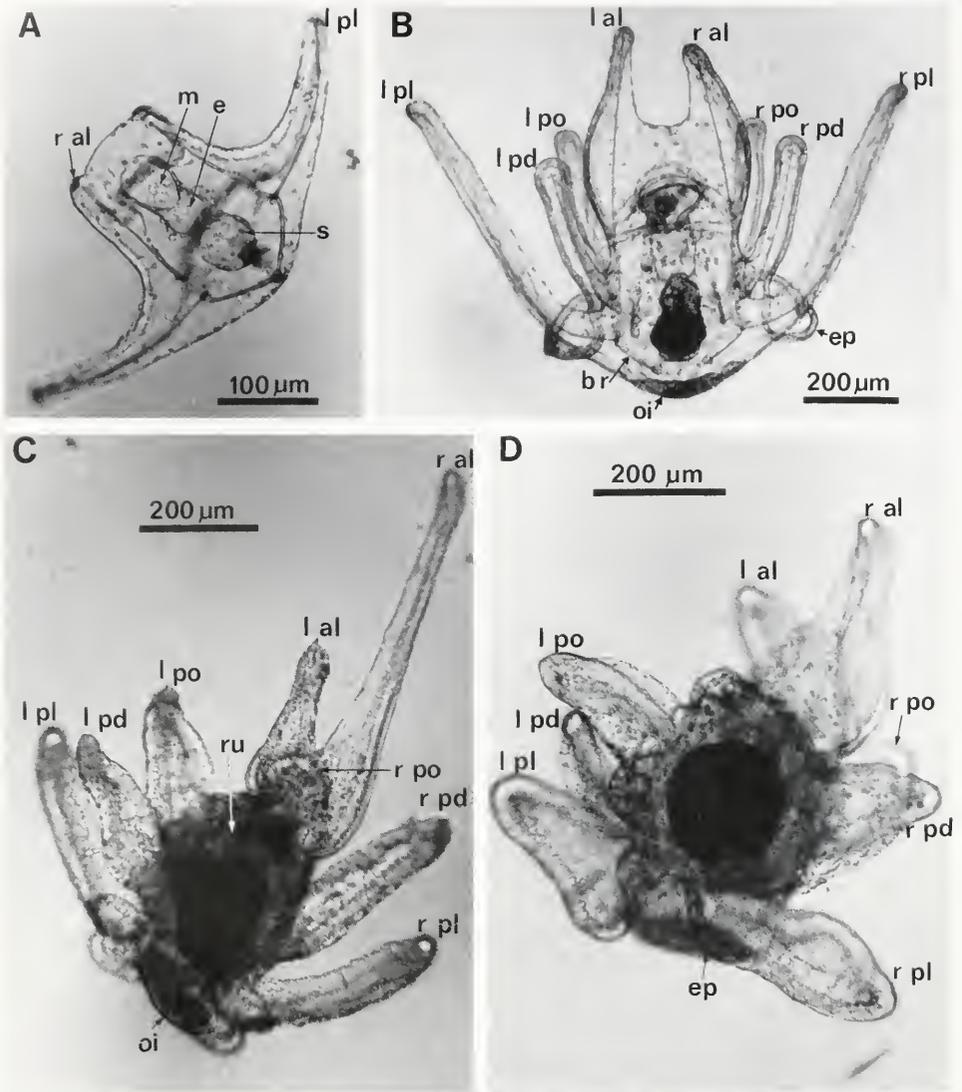


FIGURE 1. Larval and early metamorphic stages of *Ophiocoma pumila*. All photographs are of living material. A. Ventral view of a five-day-old ophiopluteus larva with well-developed postero-lateral arms, incipient antero-lateral arms, and a functional digestive tract. B. Dorsal view of a fully developed, 61-day-old ophiopluteus larva; note the epaulettes at the base of the postero-lateral arms, the rudimentary body rods, and the oily accumulation in the posterior end. C. Dorsal view of a 79-day-old larva in the early stages of metamorphosis; all arms, except for the right antero-lateral arm, are being resorbed into the ophiuroid rudiment; the right post-oral arm (which is visible beneath the antero-lateral arm) is bent across the ventral surface of the rudiment. D. A slightly later stage of metamorphosis (approximately 24 h after the commencement of arm resorption); note that the right antero-lateral arm has begun to be resorbed; the dark structure in the middle of the rudiment is the stomach. br, body rod; e, esophagus; ep, epaulette; l al, left antero-lateral arm; l pd, left postero-dorsal arm; l pl, left postero-lateral arm; l po, left post-oral arm; m, mouth; oi, oily accumulation; r al, right antero-lateral arm; r pd, right postero-dorsal arm; r pl, right postero-lateral arm; r po, right post-oral arm; ru, ophiuroid rudiment; s, stomach.

posterior end of the body of ophioplutei, become separated from one another by a gap during development (Fig. 1B). Furthermore, end and transverse rods, prominent at the posterior tip of each body rod in most ophioplutei, are very reduced in *O. pumila*. An unknown substance with an oily appearance accumulates within the posterior tip of the ophiopluteus during its development (Fig. 1B). Overall, the larva has a faint yellowish tint due to the presence of yellow-colored cells which are scattered throughout the epithelium but which are particularly noticeable in the region beneath the ciliary band.

### Metamorphosis

The first indication of metamorphosis in *O. pumila* was the appearance of a 5-lobed hydrocoel on the left side of the oesophagus starting about 79 days after fertilization. Shortly afterwards, all of the larval arms, except for the right antero-lateral, begin to be resorbed slowly into the developing ophiuroid rudiment (Fig. 1C) (Table II). All arms are resorbed in place except for the right post-oral arm which is bent ventrally across the rudiment during resorption. The resorption of the right antero-lateral arm begins before the other arms are completely resorbed (Fig. 1D). The basal portion of this arm is still visible when the other arms have nearly disappeared (Fig. 2A). As each arm is resorbed, the internal skeletal rod appears to dissolve in proximal-distal fashion at about the same rate as the arm itself shortens. Two days after the commencement of arm resorption, the metamorphosing larva looks like a typical ophiuroid vitellaria (Fig. 2B). Vestiges of all the resorbed larval arms, except for the left antero-lateral arm, contribute to specific structures in the vitellaria: (1) the remains of the right antero-lateral arm form an anterior preoral lobe which has a ciliated band at its tip. (2) The right post-oral arm, which was bent ventrally during resorption, becomes a ciliated ridge positioned ventrally at the base of the preoral lobe. (3) A part of the ciliated band that was formerly associated with the right postero-dorsal arm becomes a ciliated band which is present interradially on the right ventral aspect of the vitellaria. (4) Similarly, the left post-oral and left postero-dorsal arms contribute to two bands of cilia, present in interradiial positions on the left ventral surface of the vitellaria. (5) Tracts of cilia derived from the left and right postero-lateral arms form a ciliated band at the right posterior corner of the vitellaria. The oily accumulation within the posterior part of the ophiopluteus becomes incorporated into the posterior of the vitellaria. The ciliated epaulettes do not appear to contribute to any structures in the vitellaria. Note that clear vesicle-like structures develop at the tips of the remnants of the resorbed larval arms (see Fig. 2B). The significance of these structures is not known,

TABLE II

*Chronology of metamorphosis of Ophiocoma pumila (26.0–27.5°C)*

Time	Events
0	Arm resorption begins.
2 days	Arm resorption completed. There is now a vitellaria-type larva, with developing tube feet.
3 days	Tube feet are functional.
4 days	Vitellariae begin to settle. The ciliated bands are retained.
11 days	Preoral lobe has been resorbed and ciliated bands begin to disappear. The vitellaria has become a benthic juvenile.
25 days	Juvenile with five tiny arm buds.

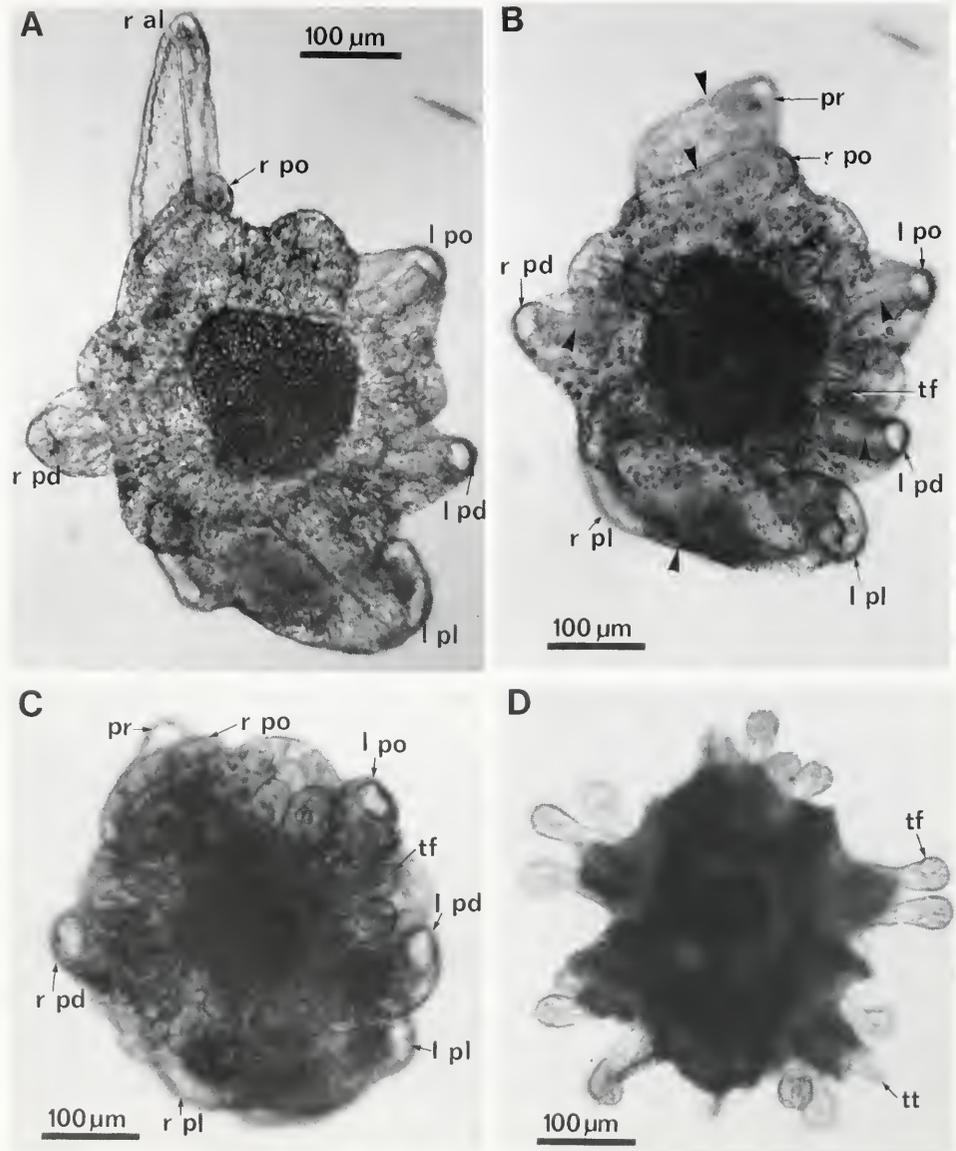


FIGURE 2. Late metamorphic, vitellaria, and juvenile stages of *Ophiocoma pumila*. All photographs are of living material. A. Ventral view of a late metamorphic stage (approximately 36 h after the commencement of arm resorption); note that all arms are nearly completely resorbed except for the right antero-lateral arm. B. Vitellaria (48 h after the commencement of arm resorption) showing the ophiuroid rudiment with developing tube feet, and the remains of the resorbed larval arms; the remnants of the right antero-lateral arm form the preoral lobe; the arrowheads indicate the position of ciliated bands. Note the clear vesicles at the tips of the larval arm remnants. C. An eight-day-old vitellaria; the ciliated bands are still present but the preoral lobe has shrunk considerably. D. Juvenile brittle star approximately three weeks after settlement; note the well-developed tube feet and the terminal tentacle evident at the tip of one of the developing arms. l pd, left postero-dorsal arm; l pl, left postero-lateral arm; l po, left post-oral arm; pr, preoral lobe; r al, right antero-lateral arm; r pd, right postero-dorsal arm; r po, right post-oral arm; tf, tube foot; tt, terminal tentacle.

but they may represent a by-product of arm resorption. The vitellaria possesses five pairs of tube feet, one pair per radius.

Although some ophioplutei in culture began to metamorphose 79 days after fertilization, some still had not begun metamorphosis at 91 days. This suggests that under natural circumstances delay of metamorphosis may occur.

The vitellariae of *O. pumila* are fast and maneuverable swimmers. In culture, they begin to settle four days after the commencement of metamorphosis, using the tube feet for attachment. However, vitellariae retain their ciliated bands for at least a week, during which time they can detach repeatedly from the substrate, swim about for a variable period of time, and then reattach and walk. The preoral lobe is gradually reabsorbed during this period (Fig. 2C). Then, the ciliated bands gradually disappear and the vitellaria becomes a benthic juvenile. The juvenile can move quickly over the bottom using the tube feet. If disturbed, it securely attaches itself to the bottom and is difficult to dislodge with a jet of water from a pipette. By roughly 25 days following the commencement of metamorphosis, the juvenile has 5 tiny arm buds, each with a small terminal tentacle at its tip (Fig. 2D).

## DISCUSSION

### *Comments on development within the genus Ophiocoma*

In general, *Ophiocoma* ophioplutei are characterized by a large pre-oral region, short, broad antero-lateral arms, and well-developed epaulettes. However, discernible species-specific differences in shape, size, and coloration as well as in the structure and ontogeny of the body skeleton do exist (Grave, 1898; Mortensen, 1931, 1937). The *O. pumila* ophiopluteus is most similar in shape and size to that of *O. pica* from the Indo-Pacific. Furthermore, both have rudimentary body skeletons, the body rods being separated by a gap. In *O. pumila*, however, the body rods are joined at first (Fig. 1A) and then gradually separate as the ophiopluteus matures (Fig. 1B). In *O. pica*, on the other hand, the body rods are, apparently, never joined (Mortensen, 1937, p. 52). Devaney (1970) reported that in *O. pumila* the body rods were joined in this species. He did not rear the larvae long enough, though, to record the eventual separation of the body rods in the fully developed ophiopluteus. An oily accumulation, as observed in the larva of *O. pumila*, has not been reported or figured in other ophiocomid larvae.

Devaney (1970) separated *Ophiocoma* species into four groups on the basis of adult characters, with *O. pumila* and *O. pica* being placed into the PUMILA and PICA groups, respectively. The differences in the ontogeny of the body skeletons described here would seem to provide additional support for the separation of these two species. As Devaney (1970) suggested, more detailed studies of other premetamorphic characters might aid in the separation of *Ophiocoma* species into valid groups.

### *Evolutionary implications*

At present, seven species of Ophiuroidea are known to possess a vitellaria larva: *Ophioderma brevispinum* (Brooks and Grave, 1899), *Ophionereis squamulosa* (Mortensen, 1921), *Ophiolepis cincta* (Mortensen, 1938), *Ophioderma longicaudum* (Fenaux, 1969), *Ophiolepis elegans* (Stancyk, 1973), *Ophioderma cinereum* (Hendler, 1979, p. 153) and *Ophionereis annulata* (Hendler, 1982). In all of these, development is abbreviated and the gastrula transforms directly into a vitellaria without passing through a pluteus stage.

The ophiuroid vitellaria would appear, at first analysis, to be fundamentally different from an ophiopluteus larva. The vitellaria is an armless, barrel-shaped, non-feeding larva with multiple transverse ciliary bands that are used solely for locomotion. The ophiopluteus, on the other hand, is an armed feeding larva with a single ciliated band which is used for both feeding and locomotion. It is not surprising then that several investigators (Hamann, 1901; Fell, 1945; Williams and Anderson, 1975) concluded that the vitellaria is evolutionarily distinct from the ophiopluteus. Mortensen (1921, p. 176), however, contended that the vitellaria was actually a reduced ophiopluteus. His argument was strengthened by his discovery of small calcareous structures in the vitellaria of *Ophiolepis cincta* (Mortensen, 1938) which he interpreted to be vestiges of an ophiopluteus skeleton. Still, this proposal remained questionable until Hendler (1982) presented persuasive evidence that certain skeletal spicules present in the vitellaria of *Ophionereis annulata* were indeed remnants of an ophiopluteus skeleton. Mortensen (1921) and later Hendler (1982) suggested that some ophioplutei have undergone a progressive reduction in number of arms and complexity of the skeleton thereby becoming vitellariae. The vitellaria is thus regarded as having evolved from an ophiopluteus form.

As shown in this paper, a vitellaria is the terminal stage in the larval ontogeny of *Ophiocoma pumila*. To be more precise, the vitellaria consists of the ophiuroid rudiment together with structural remnants of the ophiopluteus. There is great structural similarity between the *O. pumila* vitellaria and the vitellariae that have been described for species with abbreviated lecithotrophic development (Brooks and Grave, 1899; Mortensen, 1921, 1938; Fenaux, 1969; Stancyk, 1973; Hendler, 1982). Apart from the fact that all have a preoral lobe, similarities in pattern of ciliation are striking. Generally, lecithotrophic vitellariae possess a tuft of cilia at the tip of the preoral lobe, a band of cilia at the base of this lobe, three interradiat tracts of cilia on the ventral aspect of the rudiment (two on the left side and one on the right side), and a band of cilia at the right posterior corner of the rudiment. The pattern of ciliation on the *O. pumila* vitellaria is identical. This strongly suggests that there is more than a superficial resemblance, and that the *O. pumila* vitellaria is homologous to the lecithotrophic vitellaria.

The discovery of both an ophiopluteus and a vitellaria in the ontogeny of a single species of brittle star implies an evolutionary affinity between brittle stars with an ophiopluteus larva and those with abbreviated development and a vitellaria larva. It also allows comment on the evolutionary mechanism linking the two forms. If the ontogeny of *O. pumila* is considered to be of an ancestral type, then the vitellaria could not have evolved from an ophiopluteus by reduction, since both ophiopluteus and vitellaria stages occur in the same life cycle. An alternative explanation is that the ophiopluteus has been lost from the life cycle whilst the vitellaria persists. This outcome might be the result of heterochrony, caused by an acceleration (see Gould, 1977) of metamorphosis such that it begins earlier and earlier in the ontogeny of descendants, thereby eclipsing the ophiopluteus stage altogether (Fig. 3). A similar argument was originally invoked by Fell (1945) (he labelled the process "recession of metamorphosis") to explain the abbreviated ophiopluteus larvae which form a continuous reduction series with fewer and fewer arms than normal. Fell's scheme is useful so long as it is recognized that the vitellaria is probably not the ultimate outcome of this reduction series but is, rather, a distinct stage which may have persisted through phylogeny despite reduction of the ophiopluteus itself.

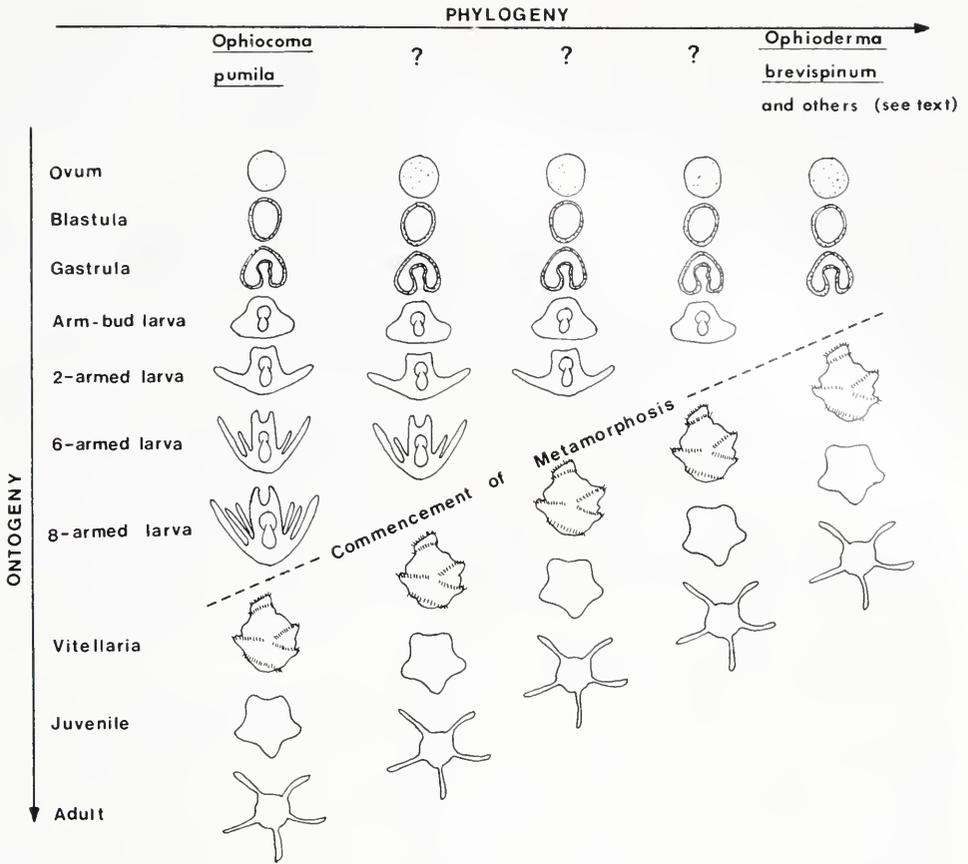


FIGURE 3. Diagram illustrating the outcome of a gradual acceleration of metamorphosis in a line of brittle stars with Type II metamorphosis. See text for further explanation.

The *O. pumila* life cycle is likely not unique among ophiuroids. It must be remembered that developmental information is available for only about 4% of the 2000 living ophiuroids and much of this is fragmentary, especially the details of metamorphosis (Hendler, 1975; pers. notes). Indeed, it is possible that a vitellaria exists in many or, perhaps, all species with a Type II metamorphosis.

The relationship between brittle stars with Type I and Type II metamorphosis is obscure. It is possible that brittle stars with Type I metamorphosis never possessed a vitellaria stage in their ontogeny and that their "suspended rudiment" stage serves the same function as the vitellaria (*i.e.*, pre-settlement exploration of the substrate; see next section). If so, Type I and Type II metamorphosis may be indicative of separate lines of larval evolution within the non-euryalinid brittle stars. Alternatively, it is possible that the vitellaria stage has been secondarily lost in this group and has been replaced by the "suspended rudiment" stage.

If acceleration of metamorphosis is a gradual process, as envisioned by Fell (1945), then further study should yield ophiuroids which have a reduced ophiopluteus (less than 8 arms) along with a Type II metamorphosis resulting in a vitellaria (Fig.

3). A vestigial ophiopluteus skeleton in some vitellariae, but not in others (Mortensen, 1921, 1938; Hendler, 1982) is likely a consequence of the extent of acceleration of metamorphosis *i.e.*, in some the skeleton starts to develop before the onset of metamorphosis; in others, metamorphosis takes place before the skeleton begins to develop.

#### *Ecological implications*

It is generally thought that ophioplutei have a relatively short pelagic existence (a month or less) and are incapable of long-distance transport in ocean currents (see Hendler, 1975). Although rate of development in laboratory culture is not necessarily indicative of what occurs in the ocean, it can be argued that *O. pumila* has the ability to survive in the plankton for up to three months and then metamorphose and settle normally. The larva of this species is thus potentially teleplanic and capable of long-distance dispersal. This may explain the wide geographic distribution of *O. pumila*. It has been recorded from insular and mainland regions of the Caribbean north to Bermuda and south to Brazil, and also from the west coast of Africa including the Azores, Gulf of Guinea, and Cape Verde Islands (Devaney, 1970). In fact, *O. pumila* is one of the few shallow-water ophiuroids with a tropical amphi-Atlantic distribution (Hyman, 1955). From Scheltema's (1977, Table 3) calculations, *O. pumila* larvae could be easily carried from the Gulf of Guinea to Brazil in the South Equatorial Current (60–154 days required) and occasional transport from Brazil to the Gulf of Guinea in the Equatorial Undercurrent may also take place (approximately 96 days required).

The *O. pumila* vitellaria, with its ciliated bands, which confer maneuverability, and its tube feet, which provide both temporary and final attachment, appear admirably adapted for pre-settlement exploration of the substrate. The ciliated bands remain for about a week providing the vitellaria with an extended opportunity to encounter a substrate suitable for the survival of the juvenile. The slowly shrinking preoral lobe, and the oily accumulation, probably sustain the vitellaria during this exploratory phase. Incidentally, the vitellariae resulting from abbreviated development in *Ophionereis squamulosa* and *Ophiolepis elegans* are also active swimmers and appear to "test" the substrate (Mortensen, 1921; Stancyk, 1973).

The question raised by Strathmann (1978) concerning the actual timing of settlement in brittle stars with Type II metamorphosis is resolved, at least for *O. pumila*. This species settles as neither an ophiopluteus nor a juvenile, but as a vitellaria. The vitellaria functions as the necessary intermediary between the pelagic and benthic phases of the life cycle. As mentioned previously, other species with Type II metamorphosis likely pass through a vitellaria phase as well.

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## MORPHOLOGICAL ADAPTATIONS TO BURROWING IN *CHIRIDOTEA COECA* (CRUSTACEA, ISOPODA)

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### ABSTRACT

The burrowing isopod *Chiridotea coeca* lives in medium to coarse sand. It preferentially burrows into sands of particle size ranges which most closely resemble its native substrate (particle sizes 250–1000  $\mu\text{m}$ ), and burrowing is fastest in these substrates. Pereiopods and pleopods are fouled in substrates with particles smaller than 100  $\mu\text{m}$ .

Locomotory rhythms above and below the substrate are similar. Phase differences between adjacent legs are 30% with metachronal waves moving forward. Power stroke for all pereiopods is by extension at the coxo-basal and basi-ischial articulations. Directions of leg thrust vary from almost perpendicular to the longitudinal body axis for gnathopods, to directly posterior for the last ambulatory leg.

The body is dorso-ventrally flattened. Power and recovery stroke occur in very nearly the same plane and limb planes approach vertical posteriorly. Pereiopods lack the ventrally directed carpo-propodal flexure found in most free living isopods. Setae provide a conspicuous morphological adaptation to burrowing. Stout, posteriorly directed serrulate setae on the pereiopods increase the area of contact with sand grains, and provide an expanded tip around the dactyl. On the second and third gnathopods and all locomotory legs, a fringe of posteriorly directed plumose setae provides a shelter into which the leg behind can swing during recovery stroke. These setae increase in length and number with body size but the intersetal distance remains sufficiently close to exclude virtually all of the native sand grains from the spaces between the legs. Other morphological adaptations are discussed and the species is compared with other burrowing Peracarida.

### INTRODUCTION

Isopods are a common faunal constituent of marine sand beaches. Dahl (1952) proposed a scheme of beach faunal zonation in which members of the family Cirolanidae comprise a distinct and regular horizontal zone in many regions of the world. This phenomenon does not seem to apply to the east coast of North America, where there is little or no evidence of a "cirolanid belt." One intertidal species which frequently occurs, however, is *Chiridotea coeca* (Valvifera: Idoteidae). This species ranges from Nova Scotia to Florida (Richardson, 1905), and is typically intertidal (Tait, 1927; Wigley, 1960), but may extend into the subtidal zone (Shealy *et al.*, 1975).

The distributions of many infaunal Peracaridia are limited by substrate particle size (Crawford, 1937; Meadows, 1964; Jones, 1970; Morgan, 1970; Rees, 1975; Oakden, 1984, and others). This also appears to be the case with *C. coeca*, which occurs in coarse sand (Tait, 1927). Few attempts have been made to relate morphology of burrowing peracaridans to substrate particle size preference. The functional morphologies of interstitial forms have been described (Pearse *et al.*,

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1942; Swedmark, 1964), but larger crustaceans have been, for the most part, neglected. These forms often bear extensive setal armature, but very rarely has setation in beach-dwellers been described functionally. An understanding of setal functions greatly increases the understanding of the habits and general lifestyle of a species. The work of Manton (1952; 1977) and Hessler (1981; 1982) dealt with surface walking forms bearing few, inconspicuous setae. Species descriptions commonly do not include any details on setation. Menzies (1957) and Fish (1972) described in detail the setation of *Limnoria* sp. and *Eurydice pulchra* respectively, but did not provide a functional explanation for them. Few isopodan species are as setose as *C. coeca*, so workers on other forms have not felt obliged to examine setal function. In *Chiridotea*, a study of burrowing would not be complete without consideration of the role of setal armature.

Locomotory mechanisms for surface-walking arthropods have been thoroughly studied (Manton, 1952; Lochhead, 1961; Hessler, 1982), as have burrowing mechanisms for true burrowers such as *Leptocheirus* (Goodhard, 1939). The locomotory mechanisms of forms such as *Chiridotea*, which plow forward beneath the substrate, are known only vaguely (Lochhead, 1961).

*Chiridotea* and other members of the subfamily Glyptonotinae show marked morphological divergence from the more primitive Idoteidae. In this study, the morphology of *C. coeca* will be discussed in terms of functional adaptation to the burrowing habit.

#### MATERIALS AND METHODS

Specimens of *C. coeca* were collected from among sand ripples in shallow puddles in the intertidal zone of New River Beach, Bay of Fundy, New Brunswick in August, 1982 and 1984. They were kept in lots of 100 animals in shallow plastic trays (inside dimensions 24 by 36 cm) with 2 cm of native beach sand and 4 cm of sea water, at 10°C, and were fed scraps of earthworms and fish. The water was changed every two days.

Five large sand samples were gathered at New River Beach from the top 1 cm in areas where *Chiridotea* trails were found. The sand was oven dried at 80°C and passed through a series of mechanically shaken, graded sieves and weighed. Mineral particle size fractions were expressed as percent of total weight. A further sample of several kilograms was similarly fractionated for substrate preference tests.

To determine the organic content of the native substrate of *C. coeca*, five 0.5 l samples of surface sand from areas of the beach where isopods were found were oven dried and weighed before and after ashing in a muffle furnace at 550°C for 1 hour. *C. coeca* does not occur on muddy substrates. Five substrate samples from a mudflat close to New River Beach were dried and ashed for comparison with the habitat of *C. coeca*.

A substrate choice experiment was set up in order to determine if *C. coeca* preferentially burrows into substrates of certain particle size ranges. A plastic tray with inside dimensions 24 by 36 cm was subdivided into 5 equal areas using 2 cm deep plastic dividers. Each area was filled to the level of the dividers with a fraction of New River Beach sand of one of the following particle size ranges: <250  $\mu\text{m}$ , 251–500  $\mu\text{m}$ , 501–1000  $\mu\text{m}$ , 1001–2000  $\mu\text{m}$ , 2001–4000  $\mu\text{m}$ . The tray was then filled with sea water, care being taken to prevent mixing of the substrates. Fifty isopods were placed in the tray, 10 in each area. After six hours, the five substrate areas were removed, one at a time, and the number of individuals per substrate was recorded. Five trials were performed, with random rotation of substrate positions between trials.

The ability of *Chiridotea* to burrow in different substrates was tested. Surface sand gathered randomly at New River Beach was passed through the same set of sieves. Six particle size ranges were obtained (from 126–500  $\mu\text{m}$  up to 4001–8000  $\mu\text{m}$ ). Five cm of each sand substrate were placed in a 10 cm diameter finger bowl. An additional bowl was filled 5 cm deep with mud. The finger bowls were topped with sea water. Groups of five isopods, 9 to 10 mm in length, were allowed to burrow in each substrate. Mean time taken for each individual to disappear completely beneath the substrate was recorded from 5 to 10 separate trials. In the two coarsest substrates, *Chiridotea* could not burrow, so behavior was noted.

To examine the effects of lowered water content of substrate, as at low tide, surface water was removed from each substrate. Isopods were reintroduced to the finger bowls and burrowing time was recorded as before. Buried animals were gently prodded with a glass rod to determine ability to move.

Specimens were fixed in 10% formalin or in 2% glutaraldehyde for 24 hours for light microscopy and SEM respectively. Storage was in 3% formalin. Gross morphology and pereopod orientation were examined using a stereoscopic dissecting microscope. For SEM, dissected appendages were cleaned and rinsed in distilled water, freeze-dried, and sputter-coated with gold. In each leg segment, setae were counted and classified according to the scheme of Pohle and Telford (1981). Because this study is concerned with functional morphology related to burrowing, only the larger types of macrosetae (*sensu* Fish, 1972) will be dealt with in detail. Ten individuals ranging from 4.0 to 14.2 mm were used. Detailed setal descriptions are based on an individual 8.9 mm in length but many more individuals were used to confirm the observations and to determine the extent of variation. Intersetal spacing of plumose setae on the posterior margins of the pereopods was determined from SEM micrographs and by direct measurement under a compound microscope with an eyepiece micrometer.

Preliminary observations of burrowing indicates that burrowing motions seem to be a continuation of the walking mechanism during and after submergence in the sand. Films of walking individuals were used to determine leg movements and locomotory rhythms. Walking animals were filmed from above and below, in a five gallon aquarium containing about 5 cm of sea water. A JVC video camera fitted with a macro lens was used. Animals were allowed to roam freely, and were cooled by frequent changes of water. Walking was analyzed frame by frame by tracing images from the video monitor directly onto acetate sheets. The sequences, gait patterns, and phase differences were determined for all pereopods. This was done by recording the displacement of each leg over time, by measuring the change in angle at the midline between the tip of the dactyl and a transverse line through the body at the level of the coxae.

Burrowing was also filmed, from above and below the substrate surface, using a 16 mm motion picture camera at 36 frames per second. To permit viewing of burrowing from underneath, less than 1 cm of sand was placed in a glass-bottomed aquarium, covered with approximately 3 cm of sea water. In sand this deep, animals could bury themselves entirely, but became visible from below. Contact with the glass surface of the aquarium did not significantly affect locomotory behavior so far as we could see. These films were viewed frame by frame and in slow motion. Leg movements were charted frame by frame on to paper from the projected image.

## RESULTS

### *Substrate and behavior*

The percentages of the different size classes of substrate particles in New River Beach sand are summarized in Table I. More than 90% of the substrate (by weight)

TABLE I

*Percentages of size classes of particles in New River Beach sand*

Particle size class ( $\mu\text{m}$ )	Percentage in substrate (by weight)	Standard deviation
<250	2.4	0.57
251-500	61.3	9.18
501-1000	29.4	9.98
1001-2000	5.1	2.05
2001-4000	1.4	0.53
>4000	0.4	0.32

was made up of particles between 251 and 1000  $\mu\text{m}$  in diameter, with the greatest portion (61.3%) between 251 and 500  $\mu\text{m}$ .

New River Beach sand has a low percentage of organic content. In five samples, the mean organic content was  $1.2\% \pm 0.3$  S.D. The extreme values were 0.8% and 1.6%. Mud samples from an adjacent beach contained  $6.7\% \pm 1.4$  S.D. organic content.

In five substrate selection tests, most animals (mean 44.7%) chose the particle size range 251-500  $\mu\text{m}$ , which was found by sieving to be the dominant fraction of sand (Table I). The next most frequently chosen particle size range was 501-1000  $\mu\text{m}$  (18.2%). These fractions together comprise about 90% of the natural sand by weight. A chi-square test (Sokal and Rolf, 1981) showed that this distribution differed significantly from random ( $P < 0.005$ ) which would be expected if no selection occurred.

Analysis of variance (Sokal and Rohlf, 1981) showed that burrowing times (Fig. 1) differ significantly ( $P < 0.0001$ ) among different substrates. An *a posteriori* test,

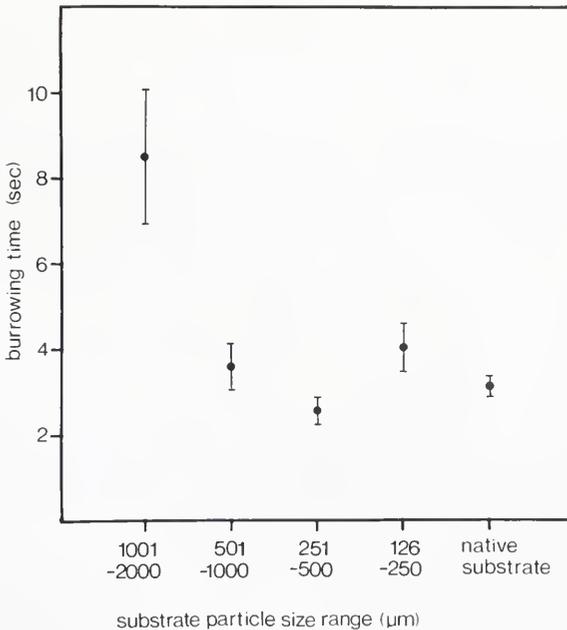


FIGURE 1. Time required for complete burial in various substrates. Vertical bars represent 95% confidence intervals.

the Newman-Keuls procedure (Snedecor and Cochran, 1967), at the 0.05 level separated the mean values into three homogeneous subsets. The first contained the value for the 1001–2000  $\mu\text{m}$  size range. In this substrate burrowing was slowest. The second group contained burrowing times from substrate particle size ranges 126–250  $\mu\text{m}$ , 501–1000  $\mu\text{m}$ , and the native substrate. The third group contained the lowest burrowing time which was found in the substrate of particle sizes 251–500  $\mu\text{m}$ , the dominant fraction of New River Beach sand (Table I). No burrowing time was recorded for the two coarsest substrates because the isopods could only rarely manage to bury themselves. In most instances they merely wedged themselves between pebbles. Burrowing in mud was as fast as, or faster than in the sand particle size range with the lowest burrowing time (251–500  $\mu\text{m}$ ).

Reduced water content, achieved by pouring off all free water, hampered locomotory abilities to some degree in all substrates. Burrowing time increased, and once buried, little horizontal movement was found in any substrate. In coarser substrates, most individuals worked their way down deeper into the sediment to a level of greater interstitial water content. After six hours, the experimental animals were recovered from the substrate samples. Those in sand were readily responsive to stimuli and capable of burrowing. Those in mud were unresponsive, and required 1–2 hours in fresh sea water before recovering. The pereopods and pleopods of individuals which had been in mud for even a short period of time were befoiled, but this did not occur in the coarser substrates.

### *General morphology*

In *Chiridotea*, the middle pereonites (3rd and 4th) are the widest (Fig. 2). The body is flattened and the head is depressed and shield-like (Fig. 3). The abdomen is elongate and tapers unevenly to a point. The first three pairs of pereopods (Fig. 4A, C), hereafter referred to as gnathopods, are stout and subchelate with a compact merus, carpus, and propodus, characteristic of the idoteid subfamily Glyptonotinae. The posterior four pereopods, hereafter referred to as ambulatory legs, are robust, elongate, and posteriorly directed. They possess simple, peg-like dactyls. The major axes of all segments of each pereopod lie within a single plane, known as the limb plane. Limb planes of successive pereopods differ with respect to a hypothetical vertical plane passing through the major axis of the body. The limb planes of the gnathopods are angled forward relative to this plane, those of the first ambulatory legs are almost perpendicular to it, and those of the third to seventh ambulatory legs are directed posteriorly, becoming serially closer to parallel with it. Limb planes also differ successively with respect to a hypothetical vertical plane passing through the points of insertion (coxo-basal articulations) of each pair of legs. For all pereopods, the anterior surface of the limb plane faces upwards, towards the body. The angle each limb plane makes with its corresponding vertical plane decreases gradually from the first gnathopods, whose planes are almost horizontal, to the fourth ambulatory legs, whose planes approach vertical. The regions of the limb planes which contain locomotory motions are stacked one on top of another, each lying beneath its adjacent anterior neighbor (Fig. 5). Because the bases of the last pair of legs have the greatest degree of vertical direction, and bases of all legs are of similar length, the animal has a forward-leaning stance (Fig. 3.)

The abdominal appendages are arranged, for the most part, in the typical idoteid fashion, as described by Naylor (1955). There are six pairs of abdominal limbs, five pairs of pleopods and one pair of uropods. Each pleopod is a biramous lamellar structure which projects posteriorly beneath the abdomen. The uropods are large,

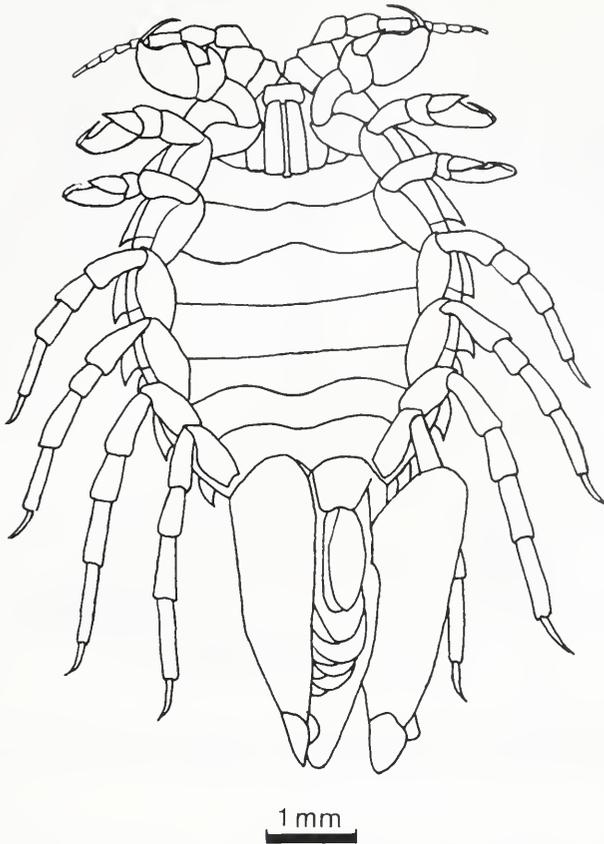


FIGURE 2. Ventral view of *C. coeca* showing arrangement of thoracic and abdominal appendages. Setae omitted.

flattened, and laterally hinged to the abdomen so that they are able to fold under the abdomen and cover the pleopods. They bear small endopods which are attached to the postero-lateral margin and lie ventral to the apex of the telson when the uropods are closed (Fig. 2).

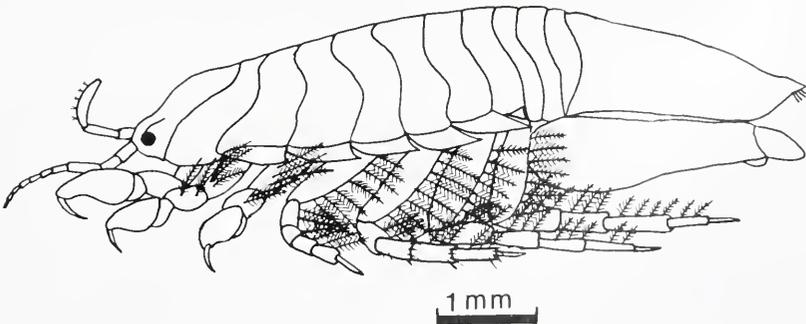


FIGURE 3. Lateral view of *C. coeca* showing pereopod orientation and distribution of plumose setae.

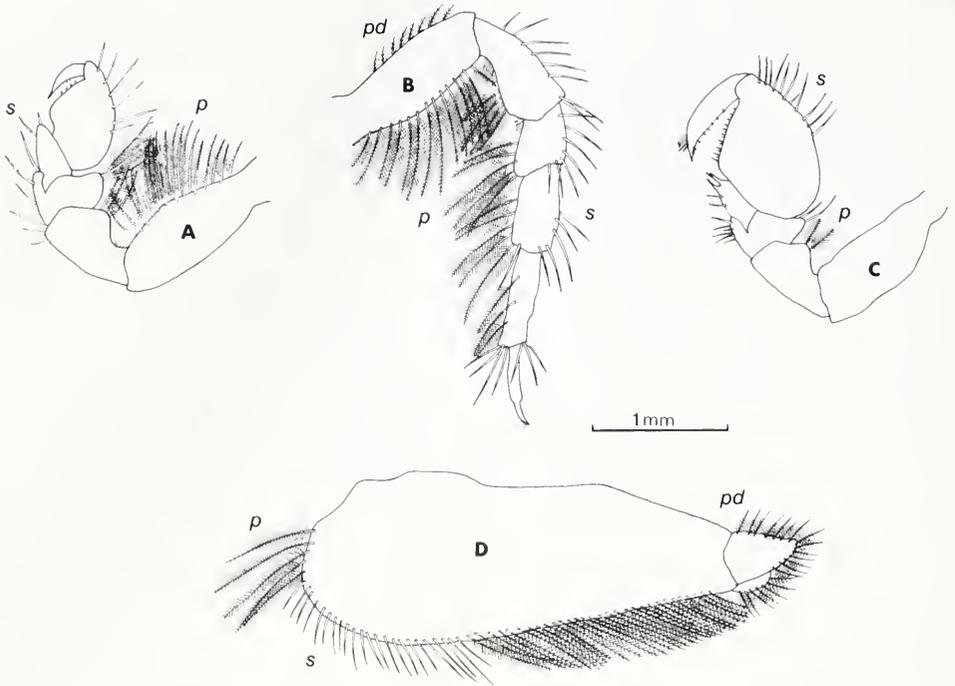


FIGURE 4. Setation of A, left second gnathopod; B, left first ambulatory leg; C, left first gnathopod; D, left uropod, showing placement of p, plumose setae; pd, plumodenticulate setae; and s, serrulate setae.

### *Leg articulation*

There are two major flexures of the pereopods in resting *Chiridotea* (Fig. 4). The first is between the coxa and basis: the basis projects ventro-medially in the gnathopods and ventro-laterally in the ambulatory legs. The second articulation is between the basis and ischium: from this articulation, the leg extends as a relatively straight limb, antero-laterally in the gnathopods, almost laterally in the first ambulatories, and posterolaterally in the remaining pairs (Fig. 2). There is no ventral flexure between carpus and propodus (Fig. 4A–C). A third major flexure is found in the gnathopods; the dactyls are articulated against the propodi to form food grasping organs. The dactyls of the ambulatory legs are peg-like and ventrally directed.

### *Setation of pereopods*

*Plumose setae* (Fig. 6A). These are the very conspicuous (400–1000  $\mu\text{m}$ ) feathery setae on the posterior margins of the basis, ischium, merus, carpus, and propodus of the ambulatory legs and the basis, ischium, and merus of the second and third pairs of gnathopods (Figs. 4A, 4B). They lie within the limb plane and are perpendicular to the long axis of each segment (Fig. 6B). The setules are 50–150  $\mu\text{m}$  long, and 10–30  $\mu\text{m}$  apart. They occur in two opposite rows (Fig. 6A). Plumose setae are arranged in single, evenly spaced rows, except on the basal segments where they occur in two adjacent rows. The first gnathopods may bear a few plumose setae (Fig. 4C), but often have none.

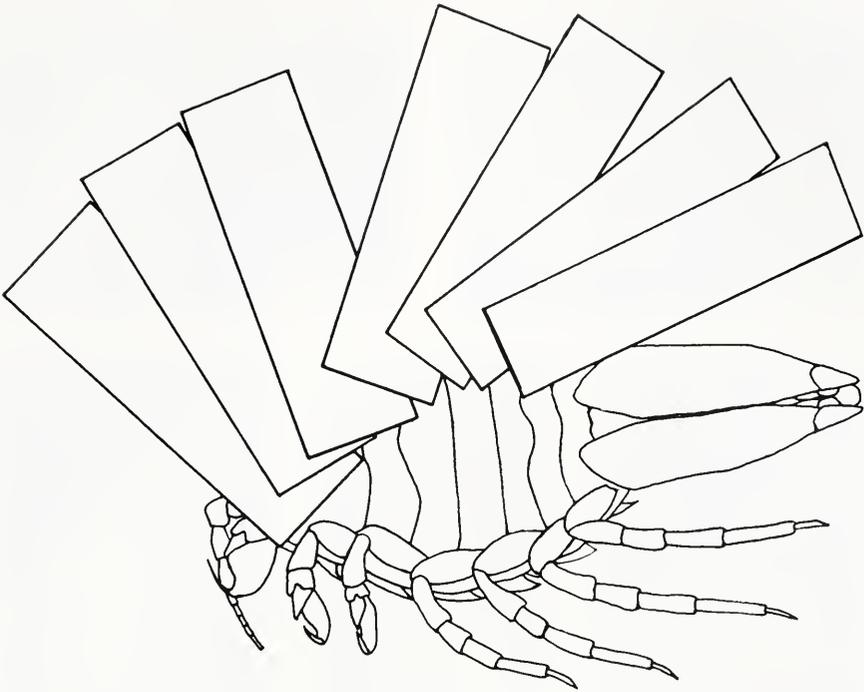


FIGURE 5. Limb planes of pereiopods of *C. coeca*. Quadrilaterals represent planes of equal dimensions oriented within the limb plane of each pereiopod, identical in position relative to a vertical plane through the coxae of each pereiopod pair, and a vertical plane through the major axis of the body.

*Plumodenticulate setae* (Fig. 6C). These are 200–250  $\mu\text{m}$  long, with a basal diameter of 10  $\mu\text{m}$ . The setules are 80–120  $\mu\text{m}$  long, with pointed, tooth-like setulettes. Two opposite rows of pointed denticules, 1–4  $\mu\text{m}$  long, occur post-annularly. These become narrower and more closely applied to the shaft near the pointed tip. There is no terminal pore (Fig. 6D). Plumodenticulate setae occur in a single row of 5–10 on the anterior margin of the basis of each ambulatory leg (Fig. 4B).

*Serrulate setae* (Fig. 6E). These setae, 200–400  $\mu\text{m}$  long, have a basal diameter of 10–15  $\mu\text{m}$ . The shaft is smooth until approximately one-tenth the length from the tip, where, on one side only, the surface is raised in a dense concentration of 3  $\mu\text{m}$ -long fingerlike denticules. The tip is tapered, emerging from underneath the denticulate area. There is a crease-like subterminal pore (Fig. 6E). These are the stout, distally pointing setae on the anterior and distal margins of the basis, ischium, merus, carpus, and propodus of the ambulatory legs and the merus, carpus, and propodus of the first and second gnathopods (Fig. 4A–C). A tuft of these setae emerges from the posterior side of the tip of the propodus on the ambulatory legs. The dactyl emerges adjacent to the setal tuft, forming a “splayed foot.” A single row of serrulate setae is found on the posterior margin of the propodus of each gnathopod.

*Serrate setae* (Fig. 6F). These are 400–500  $\mu\text{m}$  long. The basal diameter is about 15  $\mu\text{m}$ . There are two rows of large, V-shaped denticules which end approximately 10  $\mu\text{m}$  from the roughened, expanded tip. There is a cup-like terminal depression, but no pore. One or two setae of this type may occur on the distal margin of the

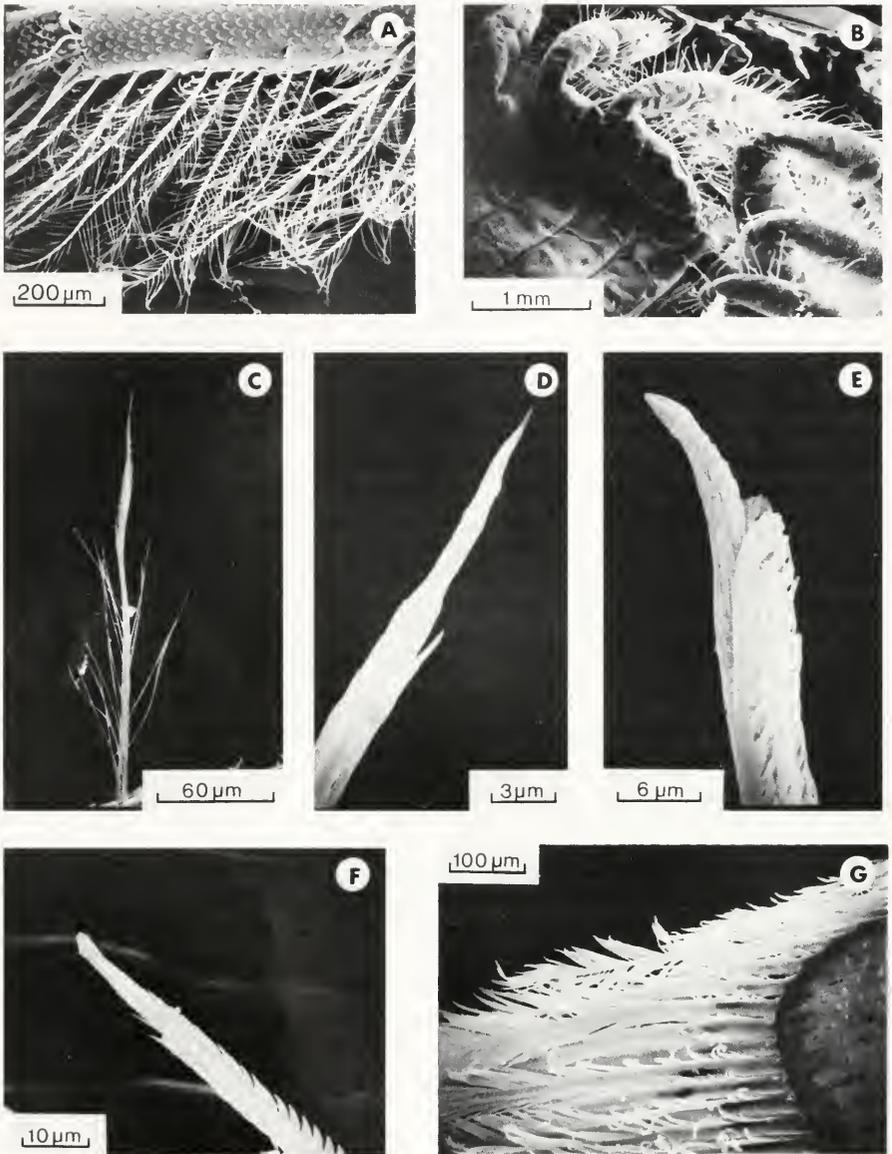


FIGURE 6. SEM of *C. coeca* setae. A, pallisade of plumose setae from basis of first ambulatory leg; B, ventral view of several consecutive pereiopods, showing continuous overlap of setae between legs; C, plumodenticulate seta; D, tip of plumodenticulate seta; E, tip of serrulate seta; F, tip of serrate seta; G, plumose setae on first pleopod.

merus on the ventral faces of the ambulatory legs, and the second and third gnathopods (not shown in Fig. 4).

#### *Setation of abdominal appendages*

There is a single dense row of plumose setae on the margins of both rami of the first two pleopods. The third pleopod bears plumose setae only on the margin of

the outer ramus. Pleopodal plumose setae are longer (800–1200  $\mu\text{m}$ ), thicker (basal diameter 20–30  $\mu\text{m}$ ), and more densely packed than pereopod setae. The setules are 40–50  $\mu\text{m}$  long, and intersetal spaces are approximately 5–8  $\mu\text{m}$  (Fig. 6G). Plumose setae (500–950  $\mu\text{m}$ ) occur on the anterior margin of the exopodite of the uropods. Shorter plumose setae (300–500  $\mu\text{m}$ ), line most of the lateral margin, except for the antero-lateral region, which bears a row of widely spaced serrulate setae (Fig. 4D). The endopods bear plumodenticulate setae of the sort found on the bases of ambulatory legs (Fig. 4D).

### *Setation and growth*

The length and number of plumose setae on each pereopod segment increases with the length of the animal (Fig. 7A). However, the average spacing of setae on a given segment does not increase with size (Fig. 7B). For animals of all size classes, plumose setae on each thoracic leg are sufficiently long to overlap the anterior margins of the two or three most proximal segments (basis, ischium, merus) of the adjacent posterior legs. Thus there is a continuous layer of plumose setae which lies dorsal to the pereopods and lateral to the body.

### *Locomotory rhythms*

*Chiridotea coeca* has three main locomotory patterns. It swims, usually venter up, at or below the surface of the water using only the pleopods. It does this in the pools of water left on the beach at low tide. It burrows into and tunnels through submerged sandy substrate using thoracic and pleonal appendages. It walks on the surface of the substrate and may assist the walking motions of the thoracic appendages with swimming motions of the pleopods. The term "walking" will be used here to connote action of the pereopods only.

Videotapes of walking individuals show that the first pair of gnathopods are not locomotory. They are held immobile beneath the head, while the second and third pairs are employed in the same fashion as the ambulatory legs. For all pereopods, the power stroke involves almost simultaneous extension of the coxo-basal and baso-ischial articulations. In the recovery stroke, the reverse process takes place, with baso-ischial flexion occurring slightly before coxo-basal flexion. There is a slight rotation of limb plane at the coxo-basal articulation in the gnathopods but it appears that in the ambulatory legs, all locomotory movements are in the same plane. The point of contact with the substrate for all pereopods is the dactyl, which is extended in the gnathopods during locomotion. Legs thrust outward, within the limb plane. They do not push in a direction parallel to the body axis. Thus, a force combining both forward and lateral components is delivered to the body by each leg.

In walking and burrowing, metachronal waves pass from posterior to anterior, with each leg about 0.3 cycles ahead of the next anterior to it. Therefore when leg "n + 1" is undergoing a recovery stroke, it swings forward beneath the palisade of plumose setae on leg "n," which is in a more posteriorly directed posture, in an earlier stage of recovery stroke.

Opposite appendages of each pair are 0.5 cycle out of phase. The leg on one side serves as a pivot for the opposite leg to work against so that there is a slight wobble in the gait. Unaided by thrust from the pleopods, *Chiridotea* walks on sandy substrates at speeds usually between 0.3 and 1.0 cm per second. In this sort of locomotion, the ratio of the durations of the forestroke and backstroke is approximately 1:1, Manton's "middle gear" (Manton, 1952). An individual traveling in a

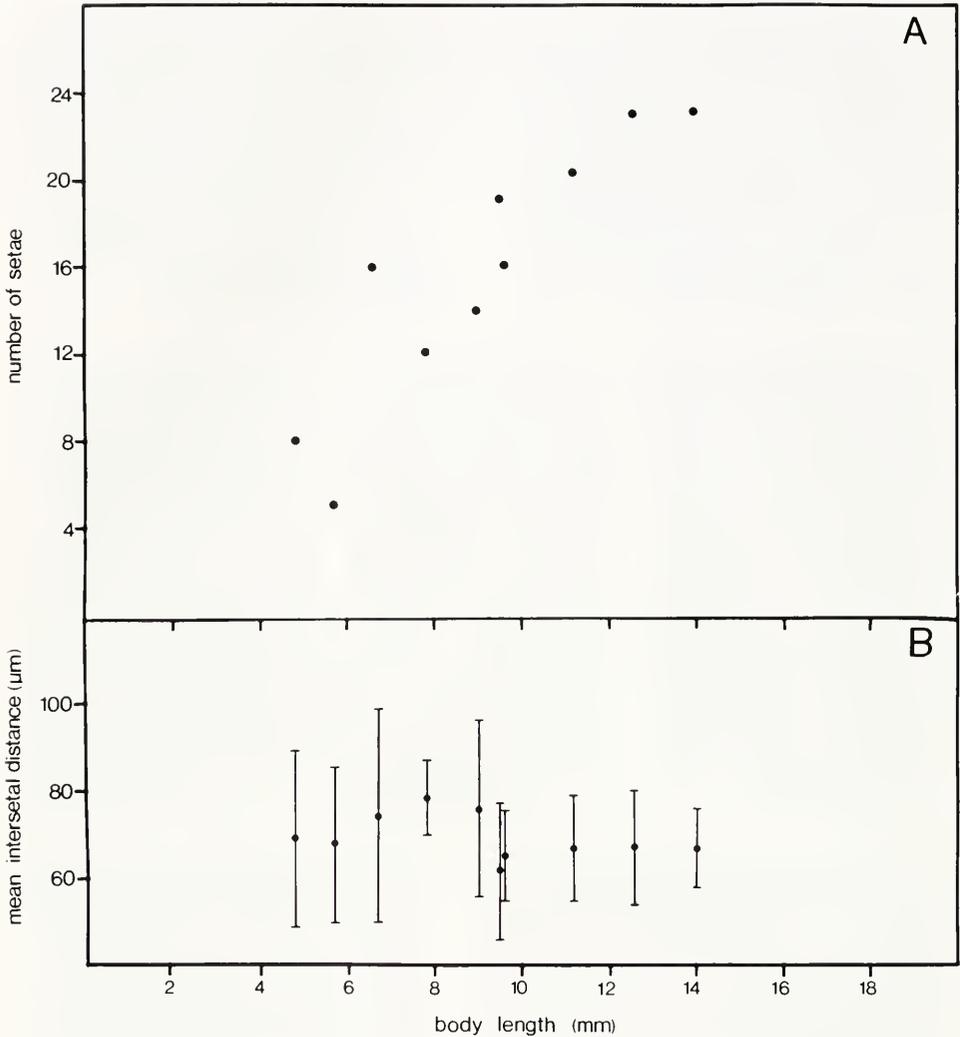


FIGURE 7. A, number of setae on a single leg segment (basis of first ambulatory leg) plotted against body length (anterior margin of head to tip of telson). B, average intersetal distance for a single leg segment (basis of first ambulatory leg) plotted against body length. Vertical bars represent one standard deviation.

straight line changes speed by altering the period of the leg cycle. A medium-sized individual traveling at a speed of 0.5 cm per second undergoes between 2 and 3 leg cycles per second. The length of stride and angle of swing of the legs do not change with speed in animals walking in straight lines, but do change during turning. Turning may be enhanced by usage of legs on one side of the body only for one or more metachronal cycles.

Burrowing may be initiated from either reptant locomotion or resting posture. As burrowing commences, the abdomen is tilted upwards and the head is lowered against the substrate. The pleopods beat rapidly, which forces a current of water downwards and backwards and further adds to the forward tilt of the body by

forcing the pleon upwards and forward. The gnathopods thrust into the sand, and force particles to the side. The current created by the pleopods draws sand out from under the body, and adds forward thrust during the early stages of burrowing, forcing the body down into the substrate. The animal works its way down into the substrate, moving the pereopods in the usual metachronal rhythm. Little interruption occurs between walking and burrowing, especially if the animal burrows into the side of a sand ripple or other substrate irregularity. After the head and forward half of the thorax are submerged, the uropods close under the abdomen and the animal continues to tunnel forward horizontally using the pereopods alone. The pleopods continue to beat, but only enough to maintain a respiratory current. After total submergence, the animal commences to tunnel forward, usually less than 1 cm below the sand surface. In *Chiridotea*, the locomotory mechanism used in tunneling is only slightly altered from that of walking: forward speed during tunneling is slower than that of walking and the forestroke:backstroke ratio decreases to approximately 1:2, Manton's "low gear."

The sand-water mixture through which *Chiridotea* tunnels is a very unstable medium. Each foothold slips backward as force is applied. As a result, there is horizontal displacement of sand particles. Sand is also displaced vertically, being forced up and over the convex surface of the body as the animal moves forward. These movements have been mapped by tracing the paths of individual sand grains in video and film sequences, from above and beneath the animals. *Chiridotea* is considered a tunneler rather than a true burrower because an open burrow is not left behind as it moves forward. The surrounding sand settles, loosely packed, at the posterior of the animal as it passes, and that which is carried over the dorsal surface of the body settles on top of this sand, forming a raised trail in the substrate.

#### DISCUSSION

The surface sand at New River Beach is moderately coarse (36% of particles by weight greater than 500  $\mu\text{m}$  in diameter) and contains a certain amount of pebbles and shell fragments (represented as particles greater than 4000  $\mu\text{m}$  in diameter). This substrate is similar to, or perhaps somewhat finer than that described by Tait (1927) who said that this species occurs in sand with occasional fragments of shell and stone, the diameters of particles being between 500 and 1500  $\mu\text{m}$ . Ashing showed that New River Beach sand has an organic content of less than 1.2%. This is much lower than that of the nearby mudflat (6.7%). The physical effects of substrate on psammobionts at New River Beach are almost entirely due to the movement of sand particles within a water matrix and are not complicated by the presence of interstitial organic material.

When offered a choice, *C. coeca* preferentially burrowed into substrates which comprise a large portion of its native substrate. Individuals were unable to burrow in particles greater than 2000  $\mu\text{m}$ . Burrowing speed increased inversely with substrate coarseness between substrates 1001–2000  $\mu\text{m}$  and 251–500  $\mu\text{m}$ . This, no doubt, is due to the ease with which the smaller particles can be moved by the combined action of pereopods and pleopods. The porosity of packed particles of uniform shape is independent of particle size (Allen, 1982; Leeder, 1982; Beard and Weyl, 1973). However, the permeability decreases as the particles become smaller (Beard and Weyl, 1973). In the finest sand substrate (<250  $\mu\text{m}$ ), burrowing was slower than in the native substrate, or in the two substrates which comprise more than 90% of it (Fig. 1). The finest substrate comprises very little of the native substrate (<3% by weight). *C. coeca* apparently has difficulty penetrating sands which are significantly finer than its native substrate.

Maintenance of a fresh respiratory flow probably becomes increasingly difficult as the mean sediment particle size decreases. Very fine particles in clays and organically rich muds are held together by cohesive electrostatic forces (Allen, 1982) and by adhesiveness of organic molecules. *Chiridotea coeca* became mired very rapidly in mud. The porosity of well sorted natural sands is 40–45% (Beard and Weyl, 1973). In poorly sorted sediments, porosity can be as low as 5–10% (Allen, 1982). *Chiridotea* species have only been reported in well-sorted substrates consisting principally of medium-coarse sand grains (Tait, 1927; Wigley, 1960; Harper, 1974). This marked preference is probably due to ease of locomotion and maintenance of respiratory flow.

Burrowing is a behavior common to beach macrofauna of widely divergent taxa. It provides protection against desiccation and reduces the occurrence of dislocation due to wave action. At high water, burrowing provides protection from predation by fish, which may be an important biotic pressure on intertidal isopods (Wallerstein and Brusca, 1982). At low water, predation from terrestrial animals such as shorebirds (Myers *et al.*, 1980) is counteracted by burrowing.

The walking mechanism of *Chiridotea* is different from that of the primitive asellotan, oniscoidean, valviferan, and phreaticoidean forms as described by Hessler (1982). In those forms, the dactyl of an anterior pereopod (*e.g.*, pereopod II) “is lifted from the substrate, stretched forward, primarily through extension at co.-ba., ba.-i. and c.-p., but also at i.-m. and p.-d., and set down again so that the dactyl again makes contact. The limb is then flexed, primarily at the same articulations, and this pulls the animal forward. Flexion is the propulsive mechanism, and extension is used in recovery. With posteriorly directed appendages, such as pereopod VII, flexion and extension are also the dominant motions, but their roles are reversed” (Hessler, 1982: p. 259). In contrast, frame by frame analysis of video and motion picture film of *C. coeca* shows that the power stroke for all pereopods (except gnathopod I, which does not participate in locomotion) involves extension at the coxo-basal and basi-ischial articulations, and the recovery stroke involves flexion at these articulations. There is a substantial amount of lateral thrust in the power stroke of each leg, which varies over the pereopodal series. The movement of the gnathopods provides very little forward propulsion. These legs push laterally and sweep through a small angle, so that the greatest direction of thrust is almost perpendicular to the direction of body motion. The gnathopods thus push sand aside, allowing the widest part of the body to move forward unimpeded. Isopods which utilize “extensible strut” (Hessler, 1982) or the swinging of legs beneath the body as in *Ligia* (Manton, 1952) involve vertical displacement of legs. These sorts of mechanisms would be inefficient and impractical for moving beneath an unstable substrate, where vertical motions encounter resistance, and a small anterior surface area is desirable. *Chiridotea* does not lift pereopods, and the anteriorly tilted stance and flattened body provide a small anterior surface area.

For many crustaceans which plow through mud or sand, locomotory rhythms in the substrate are the same as those used in walking (Lochhead, 1961). This is mostly true in *Chiridotea*, where leg motions above and below the substrate surface are identical, except for a decreased forestroke:backstroke ratio. This decrease is probably due to the greater resistance to backward thrust encountered by legs during tunneling. The metachronal rhythm remains the same, as does the 0.5 cycle phase difference between opposite legs. This difference in phase cycle is not unique to *Chiridotea*, but in combination with the lateral component of pereopod movement may be an asset to tunneling, for it produces the wobble of the body which may exert a shearing force on the surrounding substrate, reducing resistance to forward motion.

*Chiridotea* is morphologically very different from the Idoteinae (*sensu* Tait, 1927). Dorso-ventral flattening of the head is extreme, and lateral expansion of the middle pereonites is much greater than in most idoteine species. The pereiopods of *Chiridotea* are robust. There is a marked dimorphism between the strongly subchelate gnathopods and the elongate ambulatory legs, reflecting the importance of the feeding role and different locomotory role of the former. In *Idotea* and related species, pereiopods are weakly subchelate, adapted for grasping onto fronds of marine grasses (Hessler, 1982). The pereiopods of *Chiridotea* lack the carpo-propodal flexure found in *Idotea* and the other groups described by Hessler (1982). The presence of this flexure causes the body to have a half-hanging stance (Lochhead, 1961), which is adaptive for clinging. The absence of this flexure in *Chiridotea* reflects the function of these pereiopods, which act as poles which push against the substrate. Any flexure below the basi-ischial articulation would reduce the rigidity of the leg, and reduce the force delivered to the dactyl.

In the primitive isopodan configuration, the angle of the limb plane with vertical shifts for successive limbs (Hessler, 1982). In anterior pereiopods, the anterior surface of the plane is tilted upwards. In posterior pereiopods it is tilted downwards. The plane of the fourth pereiopod is almost vertical. In *Chiridotea*, the anterior surfaces of all limb planes are tilted upwards. The regions of the limb planes within which the legs move are stacked one on top of another, each lying beneath its anterior neighbor (Fig. 5). The anterior slope of all limb planes ensures a flattened form. A more extreme case of anterior slope of all limb planes is found in *Serolis* (Flabellifera: Serolidae). This is an extremely flattened form which also plows beneath the substrate (Moreira, 1974).

Setae are a very conspicuous element of the morphology of *Chiridotea*. Tait (1927) and Pearse *et al.* (1942) discussed morphological adaptations of *Chiridotea* to burrowing, but did not describe the function of the pereiopod plumose setae. Because pereiopods extend laterally, they are not sheltered by the body. In the absence of setae, there would be a tendency for sand to fall between the legs and beneath the body, which would hinder or block the anterior swing of pereiopods during recovery stroke. Metachronal waves move anteriorly, therefore during recovery stroke, each pereiopod swings forward beneath the palisade of setae on the adjacent anterior leg, which is in a posterior position, in an earlier stage of recovery stroke.

The spacing of plumose setae suggests that they are very effective in screening out the sand grains of the native substrate. The vast majority of the substrate particles were too large to pass through the intersetal spaces. During growth, intersetal spacing does not change significantly (Fig. 7B). Number of setae per segment increases (Fig. 7A), as does setal length, but intersetal distance does not. The spacing is therefore thought to be important to the animal, most likely for the screening mechanism described.

The possession of palisades of plumose setae is relatively rare in sand-dwelling Peracarida. Members of the dominant sand-dwelling isopod family, the Cirolanidae, may bear short plumose setae on the posterior margin of the telson (Richardson, 1905; Menzies and Frankenberg, 1966; Jones, 1974; Jones, 1976; Fish, 1972) but the pereiopods typically bear stout spine-like setae. Beach-dwelling cirolanids are usually smaller than *Chiridotea* species, and have relatively smaller limbs which are ventrally directed beneath the body in a more typical isopodan fashion. Haustoriid amphipods are commonly found on exposed sand beaches (Dahl, 1952; Bousfield, 1973) and typically have a lot of plumose setae. Watkin (1940) described the burrowing method of the haustoriid *Urothoe marina*. Burrowing locomotion in this species is dependent on the maintenance of a water current which is drawn through a trough

beneath the body. The walls of the trough are formed by coxal plates and the plumose setae on the pereopods. These setae form a screen which prevents sand from falling into the ventral trough. The plumose setae of *Chiridotea* are analagous to those of haustoriid amphipods. The coxal plates of *Chiridotea* are moderately setose and probably also perform a sand-blocking role. In the flabelliferan isopod *Serolis* the coxal epimeres are expanded to such a degree that the legs are entirely covered. The pereopods of *Serolis* bear no plumose setae, the coxal epimeres performing the same function as the plumose setae in *Chiridotea*.

The plumose setae on the pleopods of *Chiridotea* (Fig. 6G) differ from those on the pereopods and gnathopods. They are longer, thicker, more closely packed, and much more densely setulose. They increase the propulsive area of the swimming appendages without increasing their inertia.

Serrulate setae increase the area of contact between pereopods and substrate during the power stroke. The direction of thrust of each pereopod is described by the motion of the tip of the leg. These setae are located on the lateral surfaces of the ischium, merus, carpus, and the lateral and medial surface of the propodus of the pereopods. Because they are distally directed, each exerts a force on the substrate through its tip, parallel to the force exerted by the dactyl. They therefore increase the thrust delivered to the unstable surrounding substrate.

*Chiridotea* and its glyptonotine allies have diverged significantly from the primitive idoteid stock. The typical members of the family (*Idotea* and similar forms) have retained a stance, general morphology, and locomotory pattern which are shared with members of other suborders. *Chiridotea* and closely related species are seen as highly modified, having a unique posture, general morphology, setal complement and locomotory mechanism, which are seen as adaptations to a fossorial existence in a sandy substrate.

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## FLEXIBILITY: A MECHANISM FOR CONTROL OF LOCAL VELOCITIES IN HYDROID COLONIES

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### ABSTRACT

A design conflict exists in passive suspension feeding colonies between maximizing surface area for feeding and minimizing drag-related forces on the colony. The importance of colony flexibility as a homeostatic mechanism was demonstrated experimentally on the scale of both the entire colony and the polyp. On the colony scale, flexibility reduces the relationship between drag and water velocity from a square to a first power dependence. This finding is consistent with the discovery that flexion in trees also reduces drag to a linear function of velocity. On the polyp scale, colony flexibility strongly damps flow velocity changes at the polyp over at least an order of magnitude change in ambient velocity. This previously unappreciated consequence of flexibility may be an important selective force affecting the evolution of colony form. The separate consequences of flexion at the polyp and whole colony level are considered in a simple conceptual model incorporating polyp feeding success and colony detachment probability over a range of flow velocities. Inspection of the model reveals that the lower velocity limit at which a colony can survive is likely to be constrained by polyp feeding success, while the upper velocity limit may be constrained by either polyp feeding success or the probability of colony detachment.

### INTRODUCTION

Suspension feeding is a common trophic mode among colonial marine invertebrates. For a broad range of invertebrate taxa in the Cnidaria (Gorgonacea; Hydroidea: Plumulariidae, Sertulariidae) (Warner, 1977) and Bryozoa (Cheilostomata; Cyclostomata), colonies that live in habitats with uni- or bi-directional currents maximize their feeding area with a planar morphology perpendicularly oriented to the prevailing current direction. However, a design constraint arises from the conflicting demands to increase surface area to maximize food intake and to decrease surface area to minimize drag forces. Most of these colonies appear to solve the design dilemma of increased drag by being flexible, thus bending rather than breaking as current-induced drag forces increase (Murdoch, 1976; Wainwright and Koehl, 1976; Patterson, 1983).

Although drag on a rigid object is proportional to the square of ambient velocity, flexibility can reduce the exponent relating velocity to drag. For example, Fraser (1963) reported that drag on trees is directly proportional to velocity rather than to velocity squared; this drag reduction is attributable to a bending of the branches and consequent streamlining of the tree, yielding changes in both the cross sectional area exposed to the flow and the drag coefficient. Koehl (1977) and Vogel (1984) have also demonstrated that drag is reduced in sea anemones and trees respectively, by such profile changes.

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An analysis of the effects of current-induced drag is complicated in colonial forms because hydrodynamic forces act on two spatial and functional scales: on the architecture and stature of the entire colony, and on the stature and behavior of individual polyps. Drag forces can dislodge colonies (Birkeland, 1974; Patterson, 1983) and disrupt the success in particle capture of polyps (Leversee, 1976; Okamura, in press). It is useful to discriminate between these scales because the consequences of increasing colony size and high drag forces differ at the two levels. For example, drag-induced dislodgement becomes an increasing source of mortality in larger colonies (Birkeland, 1974), while the drag forces on individual polyps are more disruptive to the feeding of small colonies (Okamura, in press). Although drag reduction on the whole colony is a frequently cited consequence of flexibility, an important and seldom discussed consequence of colony flexion is the resulting change in flow regime around the feeding units. As a colony flexes and streamlines, ambient flow will increasingly tend to pass around a colony rather than through it. We postulate that colony flexion is an important homeostatic mechanism operating to buffer changes in the hydrodynamic forces both for the individual polyp and the entire colony.

To test our hypotheses about colony design and hydrodynamics, we investigated the behavior of colonies of the hydroid *Abietenaria rigida* to variable currents in a flow tank. *Abietenaria rigida* is a planar branched hydroid whose colonies consist of one or more erect stipes bearing alternating, symmetrical side branches (Fig. 1). This morphology is a common architectural arrangement in hydroids from high current habitats. *A. rigida* is locally abundant in Puget Sound at 15–30 m depth in high current areas; at the locale where our colonies were collected, maximum average current speeds measured at 0.10 m above the substrate range from 0.2 to 0.4 m/s (Schopf *et al.*, 1980). At these sites colonies are oriented perpendicular to the predominant current direction in a stable, but maximum, drag configuration (Wainwright and Dillon, 1969). To interpret the consequences of flexion for *A. rigida*, we experimentally altered the stiffness of colonies and measured (1) how drag on stiff or flexible colonies varied with velocity and (2) how flow velocity at the polyp location varied with mainstream velocity for stiff or flexible colonies.

#### MATERIALS AND METHODS

For rigid objects at high flow velocities (high Reynolds number) drag is given by:

$$D = \frac{1}{2} C_d \rho S U^2$$

Over a limited range of Reynolds number the coefficient of drag ( $C_d$ ) can be considered a constant; drag is thus proportional to the surface area ( $S$ ) perpendicular to the flow and the flow velocity ( $U$ ) squared (see Vogel, 1981). The density of water ( $\rho$ ) is assumed constant.

Drag on the colonies was measured in a 15 by 15 cm square cross section flow tank (Vogel and LaBarbera, 1978). A foil strain gage bonded to a 0.130-cm thick aluminum beam was attached to the colony by a lever arm. The signal from the strain gage was amplified through an Intersil ICL7605 integrated circuit using the manufacturer's recommended circuit and recorded on a Brush 2200 chart recorder. The gage was calibrated by hanging known masses off the lever arm. Net sensitivity was approximately  $10^{-4}$  N. Water velocities were calculated from the measured drag on a 3.7 cm diameter disc attached to a second strain gage beam. This provided an accurate measure of velocity at high speeds because the force measured (drag) is



FIGURE 1. Colony architecture and polyp placement on *Abietenaria rigida*. P, polyp; scale, 1 cm.

proportional to velocity squared and the coefficient of drag for a disc is constant over a broad range of Reynolds numbers (Vogel, 1981). At low velocities, water velocity was measured by timing the passage of a dye cloud over a known distance.

Stiffened colonies were prepared by soaking selected fresh colonies in distilled water for two hours and drying them overnight at 60°C. A steel wire (0.030 cm diameter) was carefully threaded up the central axis of the colony, the colony was impregnated with cyanoacrylate cement, and then sprayed with five coats of an aerosol acrylic plastic. To quantify the difference between natural and stiffened colonies, the flexural stiffness EI (see Wainwright *et al.*, 1976) was determined by measuring the force necessary to deflect the specimens through known distances and applying the standard formula for deflection of a cantilever beam. The mean value of EI for fresh *A. rigida* colonies was  $6.84 \times 10^{-6} \text{ Nm}^2$ ; the mean EI for stiffened colonies was  $1.02 \times 10^{-4} \text{ Nm}^2$ .

Detachment strength, the force required to detach a colony from the substrate, was measured on 21 colonies ranging from 2.8–7.0 cm high. Force was applied

parallel to the colony axis with a Schaevitz model FTD-G-1K force transducer. The signal was recorded on a Brush 2200 chart recorder; accuracy was  $\pm 0.05$  N.

Local current speeds around colonies were measured with a thermistor flowmeter modified after the design of LaBarbera and Vogel (1976). Spatial resolution was 0.5 mm; precision and accuracy were  $\pm 5\%$ . Measurements at the polyp locations were made by positioning the probe 1 mm above branches where the polyps are normally positioned. The probe was positioned about 1/2 way down the stipe, and at the midpoint of a side branch.

## RESULTS

The drag on flexible *A. rigida* colonies is directly proportional to mainstream current velocities (Fig. 2), but the drag on artificially stiffened colonies is proportional to the square of mainstream velocity. The decreased drag on naturally flexible colonies relative to artificially stiffened colonies is a function of colony profile changes due to flexion; as a colony flexes, more water can move around or over the colony rather than through it and a reduced surface area is presented to the current. At velocities of 20–30 cm/s, flexible colonies experience an order of magnitude lower drag forces than artificially stiffened colonies of similar size.

Detachment strength, the force required to dislodge an *A. rigida* colony from the substrate, ranged from 1.4–6.0 N and was independent of colony size. In 95% of the colonies tested, failure occurred at the interface between the holdfast and the substrate, not within the colony itself. A linear regression of detachment force measured in newtons *versus* colony height measured in centimeters yielded the equation: detachment force = 0.222 (height) + 1.95 (n = 21; r = 0.284). This poor fit to a linear model suggests that there is little correspondence between colony size measured as height and detachment force. Extrapolating the data in Figure 2, a

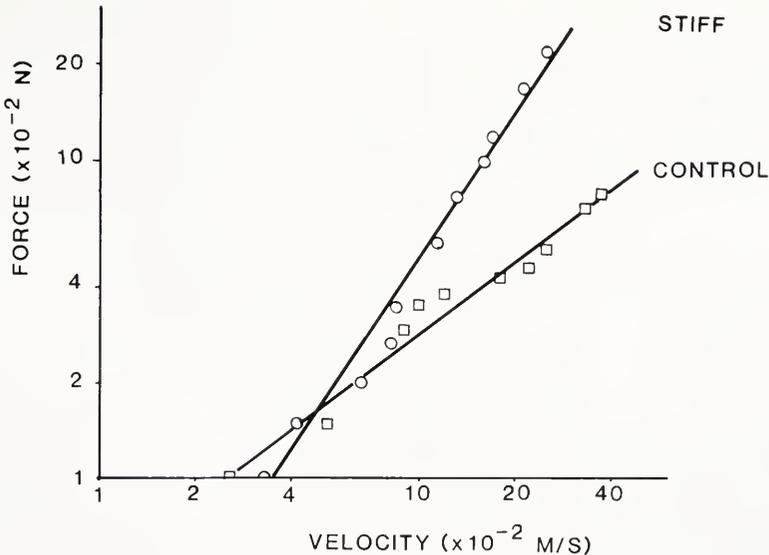


FIGURE 2. Drag force as a function of flow velocity for experimentally stiffened (O) and normal flexible colonies (□). The least squares linear regressions fit to these log plot data are: log force (N) = 1.91 U (m/s) - 0.46 (stiffened) and log force (N) = 0.94 (m/s) - 1.65 (controls).

stiffened colony would be dislodged by drag at a mainstream velocity of about  $5 \times 10^2$  cm/s; a flexible colony would only be dislodged by currents in excess of  $1.5 \times 10^4$  cm/s. These values far exceed the velocities that would ever be experienced by subtidal populations. Thus colonies are far more flexible than they need to be to avoid dislodgement. From this, we conclude that reduction of whole colony drag forces is not the primary selective force favoring colony flexion.

The local flow velocity around different portions of *A. rigida* colonies varies considerably, from values nearly equivalent to mainstream velocities to essentially still water. The high degree of spatial variation in flow speeds provides an opportunity for polyps to behaviorally mediate their flow environment. At low velocities, polyp tentacles are rigid and outstretched, creating a complete mesh between the branches through which water flows. As mainstream velocity increases and the colony flexes, the polyps themselves bend and the tentacles move behind the branch to areas of lower velocity, perhaps capturing food particles in eddy currents (Leversee, 1976; Patterson, 1983). The local flow velocity in regions where the polyps normally are located remains approximately constant (2–3 cm/s) over a 17 cm/s range of mainstream velocities (Fig. 3). For artificially stiffened colonies, the variation in velocity at the polyp locations with increases in mainstream velocity is much greater. In addition, although velocity at the polyp on both stiffened and flexible colonies increases in almost direct proportion to increases in mainstream flow, the slope of the increase in flexible colonies is considerably less than that for stiffened colonies (Fig. 3). Thus, polyps on normal, flexible colonies experience a damped flow environment with reduced variability in flow speeds relative to stiffened colonies.

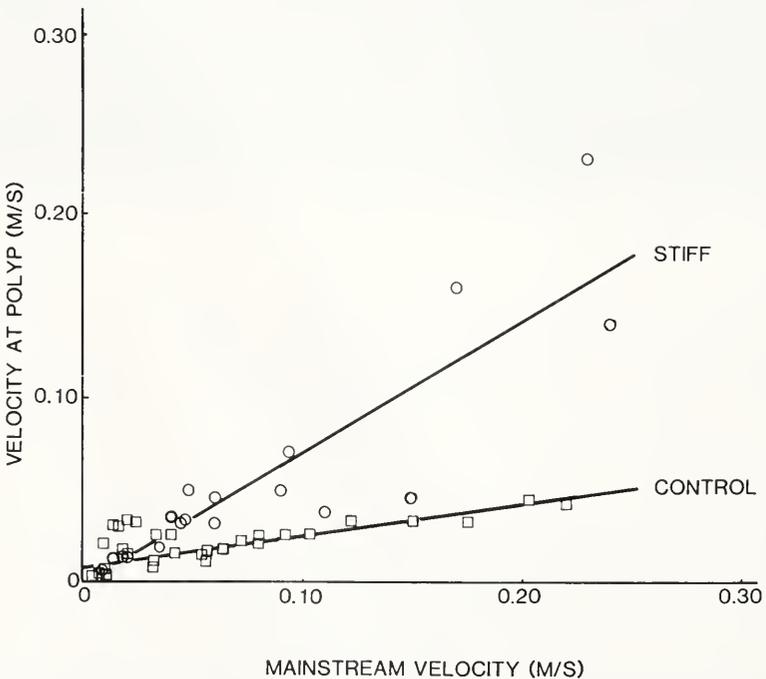


FIGURE 3. Velocity at the polyp as a function of mainstream velocity for experimentally stiffened (○) and normal, flexible control colonies (□).

## DISCUSSION

The design of passive suspension feeding colonies appears to be constrained by the conflicting requirements of minimizing total drag and maximizing the flux of capturable particles through the feeding structures. Colony flexion resolves this conflict and allows simultaneous maximization of colony surface area and minimization of flow variability at the feeding units. The striking constancy of flow velocity at the polyp over a wide range of mainstream flow speeds is a previously unappreciated consequence of flexibility.

LaBarbera (1984) has argued that the hydrodynamics of small particles near surfaces implies that suspension feeders may capture particles with maximal efficiency only over a narrow range of flow velocities. Okamura (in press) reports reduced feeding of zooids from small, upright bryozoan colonies at faster velocities. Larger colonies are less sensitive to increases in flow velocity. We predict that several mechanisms exist to minimize flow variability at the polyp in variable mainstream flows, ranging from colony flexion and reorientation to polyp behavioral changes. Passive suspension feeders can be categorized on the basis of the mechanism of particle capture (LaBarbera, 1984). *A. rigida* appears to use a combination of aerosol particle capture mechanisms, depending upon the mainstream water velocity and the density of particle. The benefits of decreased flow variability may be greater with some feeding mechanisms than others. For example direct interception, which is likely to be the predominant particle capture mechanism in *A. rigida*, may be strongly dependent upon polyp posture and local flow velocities. For all mechanisms, success in feeding is highly dependent upon surface area exposed to flow, which is in turn affected by flow speed. As flow speed changes, colony and polyp postures change, and at the highest flow speeds, hydroid polyps retract and thus do not feed at all. A simple mechanism to circumvent (or at least reduce) the requirement for frequent changes in polyp posture is colony flexion. The cost of changing postures is probably a decreased gross efficiency in particle capture, although the rate of particle capture may actually increase.

It has been shown that some hydroid colonies can change stiffness with current regime; colonies grown under higher flow speeds were less stiff than their clonemates grown in lower flows (Hughes, 1978). This developmental control of stiffness supports the notion that colony stiffness is of great selective importance. However, the major variable dictating the appropriate stiffness of a colony may be the optimal flow range for polyp feeding rather than drag reduction as previously suggested (Wainwright *et al.*, 1976; Vogel, 1981).

Flexion appears to be a general, interphyletic mechanism for dealing with variability in hydro- and aerodynamic forces, but it should be noted that the specific implications of flexibility will depend on the biology of the organism involved. In marine passive suspension feeders, design trade-offs between feeding units and whole colonies may vary as a function of colony size. For example, mortality due to drag-induced dislodgement accounts for 80% of the mortality of Caribbean sea fans (Birkeland, 1974). For these sea fans, as with hydroids in this study, failure often occurred in the substrate rather than the colony. The strength of the substrate or colony-substrate interface sets an absolute upper limit to colony stiffness and size, and is more likely to be a problem in large, high surface area colonies than small colonies (Denny *et al.*, 1985). In small colonies, such as *A. rigida*, dislodgement due to drag forces does not appear to be a large source of mortality. We have shown that the force required to dislodge a colony is far in excess of the drag normally encountered. In general, we postulate that the evolutionary effects of drag on colony

design will be dependent on colony size: selection will act on large colonies to minimize drag, while for small colonies, constancy of flow at the polyp may be of greater selective importance.

A simple conceptual model of the relative constraints imposed by the sometimes conflicting requirements of drag minimization *versus* particle capture maximization is presented in Figure 4. A successful colony is likely to maximize the combined probabilities of surviving dislodgement and particle capture over some range of flow velocities. Viewing the probabilities of these events separately and then in combination allows us to determine which level constrains performance at any given velocity. The probability of surviving dislodgement over a range of flow velocities is plotted in Figure 4a as one minus the probability of dislodgement. This probability is one at low velocities and declines as some function, here shown as a sigmoid, of increasing flow. At some extreme high velocity, the colony will eventually detach, either due to substrate or colony failure; at this point the probability of survival is zero. Similar functions and the rationale for a sigmoid curve are presented in Denny *et al.* (1985). Because there is no single good measure of the feeding success of a colony and because the true shape of the curve is not known, we will plot some function of the probability of polyp capture success over a range of flow velocities in Figure 4b. We can assume the curve is peaked with low probabilities at extreme low and high velocities. Viewing the combined probability of surviving dislodgement and polyp capture success (Fig. 4c) allows us to determine which level is constraining

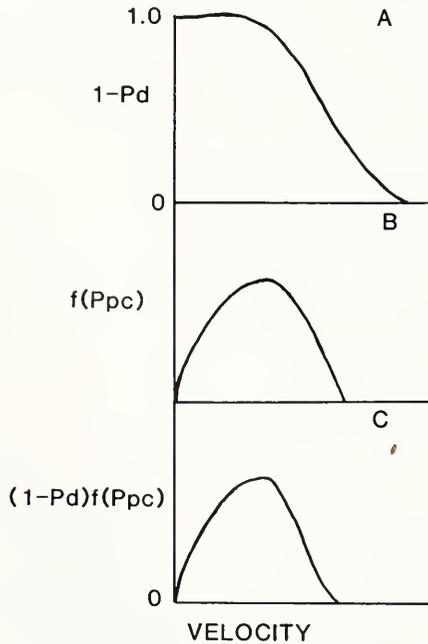


FIGURE 4. Model incorporating the probability of surviving colony detachment and polyp particle capture success as a function of velocity. A. The probability of surviving detachment. B. A function of the probability of polyp capture success. C. The combined probability of surviving detachment and polyp particle capture success. Pd—Probability of detachment. Ppc—Probability of polyp particle capture.

performance. In the example shown, the probability of particle capture (Fig. 4b) drops to zero at a lower velocity than the probability of surviving dislodgement (Fig. 4a). This is analagous to the situation measured for *A. rigida* and represents a situation where the requirements of particle capture set the upper velocity at which a colony can succeed. In the model, the zero probability of polyp capture success forces the resultant probability depicted in Figure 4c to be zero at the same velocity. The upper velocity at which a colony survives can be determined by either low polyp feeding success or a low probability of surviving dislodgement. From the model, the lower velocity limit is set by polyp capture success since the probability of surviving detachment is always one at low velocities. The measurements made on *A. rigida* provide an example of a colony where the upper velocity limit is set by particle capture success. An example where the upper limit may be set by dislodgement is that already discussed for tropical gorgonians. In general, we expect that the maximum velocities tolerated by large colonies are more likely to be limited by whole-colony drag or the risk of dislodgement than by polyp capture success.

For *A. rigida*, an abundant hydroid frequenting high flow environments, flexion acts to reduce drag and minimize variability in local flow velocities. Mechanisms promoting constancy in local flow may prove to be important adaptations possessed by many colonies living in high flow environments.

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## FORM AND FUNCTION OF THE ASYMMETRIC CHELAE IN BLUE CRABS WITH NORMAL AND REVERSED HANDEDNESS

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### ABSTRACT

The form and function of the paired asymmetric chelae were examined in blue crabs with the normal (right crusher, left cutter) and reverse (left crusher, right cutter) chela laterality or handedness. The force generated by the crusher chela in intact blue crabs was significantly greater than that of its counterpart cutter chela. The relative strength of the crusher is due to its greater mechanical advantage and larger muscle volume. The closer muscles of both chelae were capable of exerting similar forces. For a comparable frequency of stimulation, the fast excitor axon was more effective in the cutter than in the crusher chela. The paired closer muscles were composed of fibers with long sarcomere lengths ( $>6 \mu\text{m}$ ) characteristic of slow fibers with no significant difference in their mean value or in their distribution between crusher and cutter chelae. This was corroborated by finding that the myofibrillar ATPase activity and oxidative capacity was uniformly similar over most of the muscle in both chelae except for some differentiation in the most proximal and distal regions of the muscle. Such homogeneity in sarcomere lengths and enzymatic properties of the paired closer muscle occurred in blue crabs with the normal and reverse chela laterality. Thus functional differences brought about by motoneuron activation between the asymmetric chelae are probably due to muscle fiber properties other than their sarcomere lengths and enzymatic profiles, such as their cable and contractile properties as well as to neuronal and neuromuscular properties.

### INTRODUCTION

A prominent feature of many crustaceans is the bilateral asymmetry of the paired chelae consisting of a major (crusher) chela which is usually larger and more elaborate in form than the minor (cutter) chela. Striking examples of such chela dimorphism are found among male fiddler crabs, snapping shrimp, and lobsters, in all of which the paired chelae behave differently as well. In the male fiddler crab *Uca*, the major chela is used for territorial defense and courtship display while the minor is used for feeding and grooming (Crane, 1975). Similarly in the snapping shrimp, *Alpheus*, the major chela functions in agonistic behavior and the minor in manipulating objects. In the lobster *Homarus*, the crusher chela, which is slow-acting and powerful, is used for crushing the shells of mussels, etc. (Herrick, 1895), and the cutter chela, which is fast-acting but weaker, has been observed to capture swimming fish (Lang *et al.*, 1978). The basis for these different behaviors in the lobster *Homarus americanus* has been attributed to chela morphology including the fiber composition of the closer muscle. Crusher chelae have greater mechanical advantage and closer muscle volume than similarly sized cutter chelae (Elnor and

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Campbell, 1981). The crusher closer muscle is composed of all slow fibers while the cutter closer muscle has a majority of fast fibers and few slow fibers (Jahromi and Atwood, 1971; Lang *et al.*, 1977; Ogonowski *et al.*, 1980).

The blue crab, *Callinectes sapidus* Rathbun, also has dimorphic crusher and cutter chelae which have different functions in feeding. The crusher, which is capable of generating much more force than the cutter, is used in crushing mollusc prey, while the cutter manipulates the prey item and tears at the exposed flesh (Blundon and Kennedy, 1982). However, there is little available data on the functional and structural differentiation of the dimorphic chelae or on the composition of their closer muscles. The present study addressed these points.

Another reason for studying blue crabs is the apparent reversal of chela laterality that they display. The normal chela laterality is right crusher and left cutter (Przibram, 1931). However, the reverse configuration of left crusher and right cutter which occurs in a small percentage (14.4%) of the population and among large crabs only, suggested to Hamilton *et al.* (1976) that this configuration could arise when the crusher is lost and in its place a cutter regenerates. The existing intact cutter transforms into a crusher. Reversal of chela laterality found in the snapping shrimp *Alpheus*, involves a transformation in the fiber composition of the closer muscles (Stephens and Mellon, 1979; Mellon and Stephens, 1980). It was therefore of interest to determine the fiber composition of the closer muscle in blue crabs with the normal and reversed chela laterality.

#### MATERIALS AND METHODS

##### *Chela performance in vivo*

Blue crabs weighing between 150 g and 260 g were caught with crab pots in the Choptank River, Chesapeake Bay, in June 1984. They were placed in water tables (at Horn Point Environmental Laboratory, Cambridge, Maryland) supplied with running estuarine water (25°C) for 24 hours before testing. Crabs were induced to grip two steel bars of a force transducer (Fig. 1). One bar was connected to a

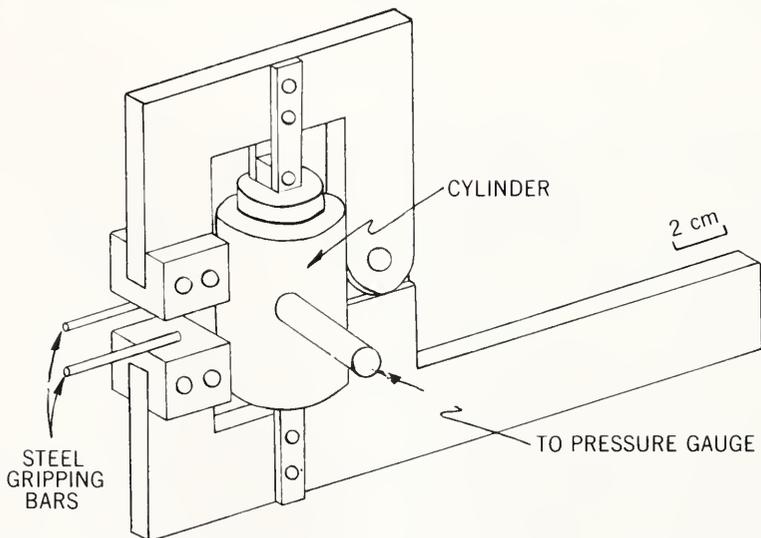


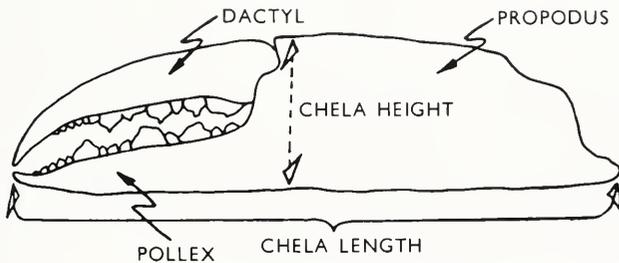
FIGURE 1. Diagram of the force transducer used to measure the force exerted by the chelae in intact blue crabs. Scale mark = 2 cm.

stainless steel cylinder, the other bar was attached to a piston which inserted into the cylinder. The cylinder was filled with vacuum pump oil. Squeezing the bars together raised the pressure inside the cylinder which was shown on a pressure gauge. The transducer was calibrated by hanging a weight on the proximal and distal ends of the steel bars and recording the deflection on the pressure gauge. Force recordings were taken from both crusher and cutter chelae of the crabs. The points of force application along the bars of the transducer and along the dactyls were measured with dial calipers. The force applied to the transducer by the dactyl ( $F_{\text{bar}}$ ) was used to calculate the force applied by the closer muscle to the dactyl at the point of apodeme insertion ( $F_1$ ). Assuming the dactyl pivot is frictionless, then

$$F_1 = (F_{\text{bar}})(L_{\text{bar}})/L_1$$

where  $L_1$  is the distance from the dactyl pivot to the point of apodeme insertion onto the dactyl, and  $L_{\text{bar}}$  is the distance from the dactyl pivot to the point of force application along the dactyl (Fig. 2). The product of ( $F_1$ ) ( $L_1$ ) divided by the distance from the dactyl pivot to any point  $x$  along the dactyl equals the dactyl force at point  $x$ . Other measurements included carapace width, chela length, chela height, chela thickness, mechanical advantage ( $L_1$  divided by the distance from the dactyl pivot to the dactyl tip), angle of muscle fiber pinnation, and surface area of both sides of the apodeme. After removing the dorsal surface of the propodus and the chela opener muscle, the dactyl was fixed open at  $30^\circ$  (to simulate its crushing position) and viewed under a dissecting microscope equipped with a camera lucida drawing tube. The apodeme and several muscle fibers on both sides of the apodeme were traced; the angles of muscle fiber attachment onto the apodeme (angles of pinnation) were then measured with a protractor. The apodeme was subsequently

### CRUSHER



### CUTTER

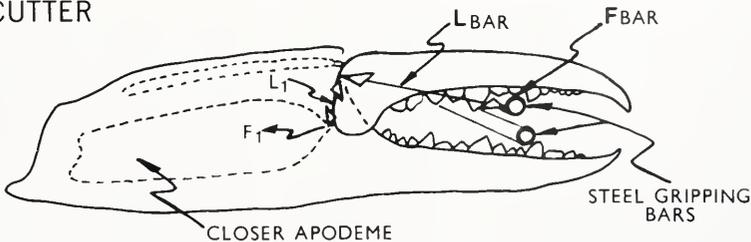


FIGURE 2. Crusher and cutter chelae of *Callinectes sapidus* showing the mechanical and morphological parameters used to characterize the chelae.

dissected away from the chela. Its area was measured with a meter conventionally used by botanists to measure leaf surface area.

The amount of stress (S) per unit of muscle fiber cross-sectional area was determined by

$$S = F_1/A \sin 2\theta$$

where  $F_1$  is the force applied to the dactyl by the closer muscle,  $A$  is the area of one side of the apodeme (the formula uses total apodeme area  $2A$ ) and  $\theta$  is the mean angle of pinnation (Alexander, 1969).

The means of all strength and morphological measurements were compared among right and left crushers and cutters using a one-way analysis of variance. If this test rejected the null hypothesis that the measurements were similar, a Student-Newman-Keuls multiple comparison test was then used to determine which means were significantly different ( $\alpha = 0.05$ ) (Sokal and Rohlf, 1969).

### *Chela performance in situ*

*Callinectes sapidus* weighing between 150 and 180 g were purchased from local suppliers and held in artificial sea water (at University of Toronto, Scarborough, Ontario) at approximately 10°C for a few days before being used. All experiments were performed on crabs with fully differentiated crusher and cutter chelae and not on regenerating chelae which were recognized by their relatively smaller size. The majority of animals used had right crusher and left cutter chelae while a few had the opposite configuration of right cutter and left crusher and even fewer had both chelae of the cutter type. We have not as yet encountered any blue crabs with paired crusher chelae.

In order to determine the closing behavior of a chela, it was removed from the animal by inducing autotomy. Next the nerve to the chela closer muscle was exposed in the carpus where it was stimulated via platinum wire electrodes. The resulting axon spike was recorded extracellularly via a suction electrode placed more distal to the point of stimulation. The preparation was bathed in 10°C marine saline (470 mM NaCl, 8 mM KCl, 10 mM MgCl<sub>2</sub>, 15 mM CaCl<sub>2</sub>, 33 mM glucose, 10 mM HEPES at pH 7.4). The fast and slow axons in the closer nerve were selectively stimulated with 0.1 ms pulses either by varying the stimulus intensity or the placement of the nerve on the electrodes. Resulting contractions of the dactyl were monitored by having its distal end attached to a probe fitted to the moveable anode pin of an RCA 5734 mechano-electric transducer.

### *Muscle composition*

The fiber composition of the closer muscle was assessed in two ways: by measuring the average sarcomere length (SL) of histologically fixed fibers or by staining for myofibrillar ATPase and NADH dehydrogenase activity in frozen cross sections of the muscle. In the former method, the closer muscle was exposed on its dorsal surface by removing the overlying opener muscle and injected with alcoholic Bouins fixative in which it was allowed to saturate for 24 h (Lang *et al.*, 1977). Care was taken to ensure that the fibers were fixed in a relaxed position by holding the dactyl partly open. Subsequently, the closer muscle was exposed and divided into nine sections along its inner face in order to sample all areas (Lang *et al.*, 1977). The samples were then stored in 75% ethanol. Measurements of SL were made in teased wet mount preparations using a compound microscope equipped

with a micrometer. Five consecutive sarcomeres were measured at 3 separate regions of a myofibril and this total of 15 sarcomeres gave an average SL for a fiber. Usually 10 fibers were measured in each of the 9 samples, for a total of 90 fibers for each muscle. Three pairs of closer muscles were analyzed with the above technique for the normal chela laterality of right crusher and left cutter; one pair for the reverse configuration of right cutter and left crusher and one pair for the paired cutter configuration.

For the histochemical study, the cuticle of the chela was ground down to almost the hypodermis with a high speed hobby drill. The entire propodus was mounted on a chuck, coated with embedding material and frozen in isopentane chilled with liquid nitrogen. Serial thick (16  $\mu\text{m}$ ) cross-sections were obtained by methods described elsewhere (Ogonowski and Lang, 1979). Histochemical techniques for detecting myofibrillar ATPase activity were modified from a method by Padykula and Herman (1955) and these modifications are described elsewhere (Tse *et al.*, 1983). Staining methods for NADH dehydrogenase was first described by Nachlas *et al.* (1958). Four pairs of chelae were analyzed histochemically for crabs with the normal laterality of right crusher and left cutter; two pairs for the reversed configuration of left crusher and right cutter, and one pair for the paired cutter configuration.

## RESULTS

### *Chela performance in vivo*

In blue crabs with the normal laterality of right crusher and left cutter the dactyl of right crushers exerts greater force than the dactyl of left cutters (Table I). However, there was no significant difference in stress exerted by crusher or cutter

TABLE I

*Mechanical and morphological measurements (mean  $\pm$  SD) of the paired crusher and cutter chela of blue crabs, Callinectes sapidus*

	Normal laterality		Reversed laterality	
	Right crusher (n = 18)	Left cutter (n = 18)	Left crusher (n = 9)	Right cutter (n = 9)
Force at dactyl tip (newtons)	42.8 $\pm$ 13.8 <sup>a</sup>	24.6 $\pm$ 7.3 <sup>b</sup>	31.2 $\pm$ 8.9 <sup>b</sup>	19.9 $\pm$ 8.0 <sup>b</sup>
Muscle stress (newtons/cm <sup>2</sup> )	63.8 $\pm$ 17.8 <sup>a</sup>	51.4 $\pm$ 14.3 <sup>ab</sup>	60.2 $\pm$ 18.8 <sup>ab</sup>	45.2 $\pm$ 12.1 <sup>b</sup>
Mechanical advantage	0.216 $\pm$ 0.012 <sup>a</sup>	0.171 $\pm$ 0.016 <sup>b</sup>	0.188 $\pm$ 0.011 <sup>c</sup>	0.176 $\pm$ 0.011 <sup>bc</sup>
Apodeme area/ chela length	0.0429 $\pm$ 0.0044 <sup>a</sup>	0.0374 $\pm$ 0.0035 <sup>bc</sup>	0.0397 $\pm$ 0.0057 <sup>ab</sup>	0.0344 $\pm$ 0.0057 <sup>c</sup>
Chela height/ chela length	0.273 $\pm$ 0.012 <sup>a</sup>	0.253 $\pm$ 0.010 <sup>b</sup>	0.263 $\pm$ 0.006 <sup>c</sup>	0.252 $\pm$ 0.012 <sup>b</sup>
Chela thickness/ chela length	0.221 $\pm$ 0.011 <sup>a</sup>	0.211 $\pm$ 0.011 <sup>b</sup>	0.218 $\pm$ 0.008 <sup>ab</sup>	0.207 $\pm$ 0.009 <sup>b</sup>
Chela length/ carapace width	0.543 $\pm$ 0.019 <sup>a</sup>	0.542 $\pm$ 0.019 <sup>a</sup>	0.539 $\pm$ 0.021 <sup>a</sup>	0.534 $\pm$ 0.026 <sup>a</sup>

Means with at least one superscript letter in common are not statistically different ( $\alpha = 0.05$ ).

Apodeme area measured in cm<sup>2</sup>; chela height, length, and thickness measured in mm.

closer muscles. Right crushers possessed a significantly greater mechanical advantage, apodeme size, chela height, and chela thickness, than their counterpart left cutters. The chela length, however, was similar between the dimorphic claws.

Angles of pinnation along the distal-proximal axis of the dorsal surface of the crusher and cutter closer muscles were similar except for the most proximal 10% of the muscle, where the angles became more acute. A similar pattern of fiber arrangement has been found in the shore crab *Carcinus maenas* (Warner *et al.*, 1982). Mean angles of pinnation between right and left crushers and between right and left cutters were not significantly different, although cutters had a significantly greater angle of pinnation than crushers (33° and 31° respectively). Angles of pinnation were not measured for fibers deeper within the muscle, although Warner *et al.* (1982) found such fibers of *C. maenas* to have similar angles relative to the dorsal fibers.

In animals with the reversed laterality of left crusher and right cutter, the crushers were not able to exert greater forces than the cutters (Table I). Left crushers had greater apodeme size and chela height than right cutters, but had similar values of mechanical advantage, chela thickness, and chela length. When comparing similarly sized right and left crushers from two different crabs, left crushers were weaker and had a smaller mechanical advantage.

These measurements were taken from male crabs. A few measurements from female crabs revealed differences between the dimorphic chelae which were similar to those for the male. However, female crusher and cutter chelae were approximately 30% and 23% shorter than their male counterparts for crabs with similar carapace widths.

#### *Chelae performance in situ*

The fast axon to the chela closer muscle was readily distinguished from the slow axon because it usually had a lower threshold for firing and a higher conduction velocity. Both these features are shown in the extracellular recording of their spikes from the closer nerve (Fig. 3A). It was therefore possible by the appropriate placement of the electrodes to fire each axon by itself and to record the closing behavior it evoked.

In the cutter chela (Fig. 3B) stimulation of the fast axon at 1 Hz evoked small twitches only when twin pulses (6–8 ms apart) were used. The individual twitches were visible after a couple of stimuli and they summated markedly with each succeeding stimuli thereafter. At a higher frequency of contraction such as 20 Hz, a smooth contraction resulted which increased in speed as the frequency was raised. Stimulation of the slow axon to this same muscle evoked a barely perceptible contraction which increased in speed and strength as the frequency was increased.

In the crusher chela (Fig. 3C) no twitch contractions were seen even with twin pulses at 5 Hz at which frequency, however, a small tonic contraction was evident. Increasing the stimulating frequency to 20 Hz dramatically increased the closing behavior of the chela. The slow axon to this muscle evoked a small contraction at about 50 Hz stimulation. Doubling the rate of stimulation correspondingly increased the speed of chela closing.

The above results highlight two important points concerning chela closing behavior in blue crabs *viz.* (1) that within each chela the fast axon is effective at a much lower frequency of firing than the slow axon and, (2) that the axons to the cutter chela are effective at a lower frequency of firing than their homologs in the crusher chela.

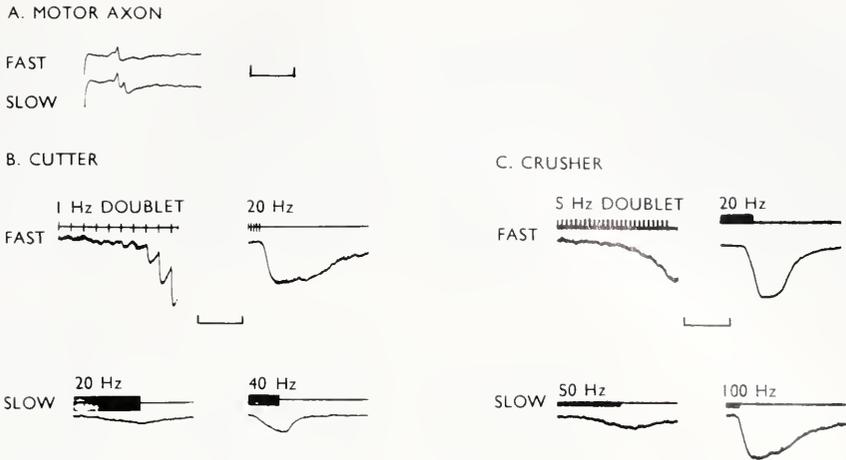


FIGURE 3. A. Extracellular recording from the closer nerve to the crusher chela showing a fast axon spike by itself (upper trace) which is followed at a higher stimulating voltage by a slow axon spike which has a slower conduction velocity (lower trace). B, C. Closing behavior of the paired cutter and crusher chelae with stimulation of fast and slow axons to the closer muscle. The upper trace monitors the stimuli and the lower trace records the closing movement of the dactyl. Calibration: A, 5 ms, B, fast contractions 0.2 s; slow contractions 0.5 s; C, fast contractions 0.5 s; slow contractions 0.2 s.

*Muscle composition*

The fiber composition of the paired cutter and crusher muscles based on sarcomere length (SL) was essentially similar (Fig. 4). Both have fibers with only long (>6  $\mu\text{m}$ ) SL which are typical of slow fibers. The range of SL extended between 6 to 15  $\mu\text{m}$  in all five blue crabs examined. The mean SL calculated for each

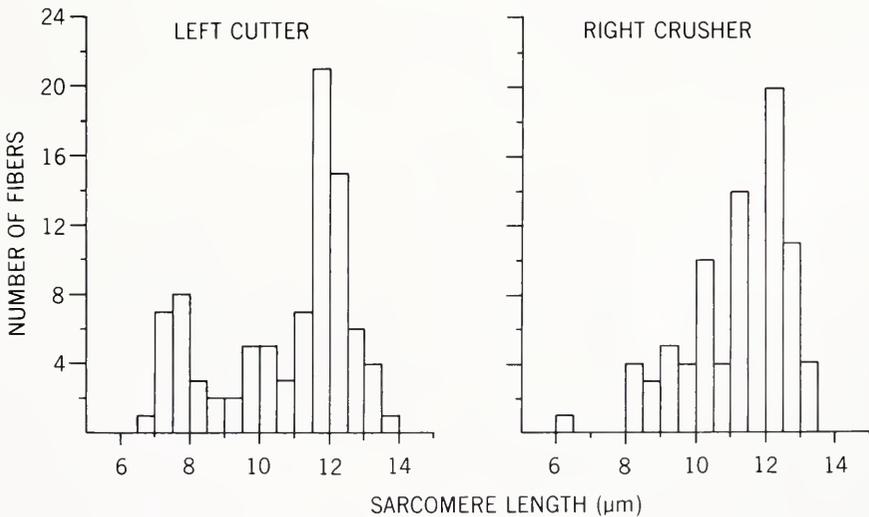


FIGURE 4. Frequency histogram of muscle fibers with characteristic sarcomere lengths from paired closer muscles of a blue crab with the chela laterality of right crusher and left cutter. Ninety fibers were sampled in each muscle.

chela was not significantly different from that of its counterpart (Table II). Furthermore, muscle fiber populations were compared using the Kolmogorov-Smirnoff two-sample test and showed no significant differences in all five blue crabs listed in Table II. These results demonstrate that the paired chela closer muscles are essentially similar in their fiber composition (consisting of all slow fibers) in any three of the chela configurations examined in this study.

The paired closer muscles were also examined by cutting cross-sections of the entire chelae and staining for myofibrillar ATPase and NADH diaphorase activity in several animals. In both claws the muscles stained with a uniform intensity over most of its length (Fig. 5B, F) suggesting a uniform population of fiber types in keeping with the SL measurements. However, some differentiation within this slow fiber population was seen especially in the proximal and distal regions of the muscle. In the distal region a small group of central fibers close to the exoskeleton showed a lower ATPase and higher diaphorase activity than the remaining fibers (Fig. 5A, E). In the proximal region, a central band of fibers extending between apodeme and exoskeleton displayed a much higher ATPase and diaphorase activity than the remainder (Fig. 5C, G). In some cases this proximal region was even further differentiated showing at least three intensities of staining for ATPase activity (Fig. 5D).

#### DISCUSSION

The fiber composition of the closer muscle in the paired crusher and cutter chelae is similar, consisting of all long SL ( $>6 \mu\text{m}$ ), slow fibers. Within this category of slow fibers there is some further differentiation into subtypes especially in the proximal region of the muscle where several intensities of myofibrillar ATPase staining were seen. Such differentiation within slow fibers has been seen in lobster muscle as well (Kent and Govind, 1981; Costello and Govind, 1983), and underscores the fact that there may well be a continuum of sub-types within the broad category of slow muscle. The paired muscles are also similar in the amount of stress they exert per unit cross-sectional area. Despite these similarities there are some functional differences between the asymmetric chelipeds in their closing behavior. Thus the force during claw closure developed *in vivo* was significantly greater for the crusher than the cutter claw. This is attributable in part to the larger muscle volume and

TABLE II

*Mean sarcomere length ( $\mu\text{m}$ ) of paired chela closer muscles from Callinectes sapidus with various chela lateralities*

	Left cutter			Right crusher		
	Body wt (g)	Mean $\pm$ SD		Mean $\pm$ SD		<i>P</i>
#1	170	10.51 $\pm$ 1.38	90	10.97 $\pm$ 0.47	90	ns
#2	161	10.61 $\pm$ 1.23	90	11.52 $\pm$ 0.82	90	ns
#3	142	10.42 $\pm$ 1.67	60	11.17 $\pm$ 0.89	60	ns
	Left crusher			Right cutter		
#4	137	10.51 $\pm$ 1.29	90	10.42 $\pm$ 2.05	90	ns
	Left cutter			Right cutter		
#5	154	12.13 $\pm$ 1.53	90	11.66 $\pm$ 1.53	90	ns

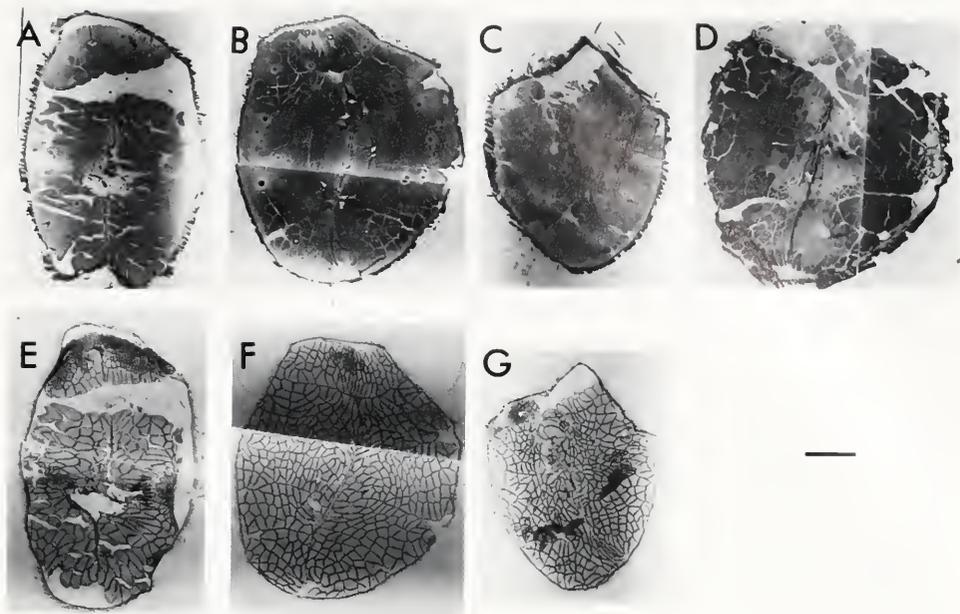


FIGURE 5. Frozen cross-sections taken through the distal (A, E), middle (B, F) and proximal (C, D, G) regions of a crusher chela and stained for myofibrillar ATPase (upper row) and NADH diaphorase (lower row) activity. The closer muscle occupies most of the cross-sectional area of the propodus with the opener muscle occupying a small area in the dorsal part of the chela. Adjacent sections for ATPase and NADH diaphorase are shown for each region from one chela and a single proximal section (D) from another crusher chela is shown to emphasize the variation in ATPase activity in this region. Scale mark = 1 mm.

greater mechanical advantage of the crusher compared to the cutter and it may also be attributable to motor firing patterns. In the latter respect the experiments on isolated chelae showed clear differences between cutter and crusher types. The cutter chela gives twitch contractions to twin pulses whereas the crusher does not twitch. Presumably some of the slow fibers in the cutter closer muscle are of the fast-follower type in their contractile characteristics as has been demonstrated among slow fibers in the lobster closer muscle by Jahromi and Atwood (1971) and more recently by Costello and Govind (1983). These fast-follower types have relatively short rise times when depolarized and this would account for the twitch-type contraction. The crusher closer muscle in the blue crab judging from its inability to twitch, would presumably have no fast-follower types among its slow fibers. Further functional differentiation between cutter and crusher chelae in blue crabs is seen in the fact that for a comparable stimulus frequency the cutter closer muscle contracts more effectively than its crusher counterpart. This taken together with the finding that the crusher chela generates greater force than the cutter would make the paired chelae suited to perform different roles in feeding if not in other behaviors as well.

Chela asymmetry in the blue crab, when compared with other crustaceans in which asymmetry has been studied, shows the least degree of specialization. The present study uncovered a functional and structural asymmetry between major and minor chelae in blue crabs with no corresponding asymmetry in the composition or strength of the closer muscles. Furthermore we would predict that there are no asymmetries in the size of motoneurons or the number of sensory axons to the

chela based on the fact that they are not greatly different in size. A somewhat greater degree of specialization between the paired chela is seen in the lobster *Homarus americanus*. Not only do the lobster chela show functional asymmetry (Govind and Lang, 1974) but there is also a corresponding asymmetry in the fiber composition of the paired closer muscles (reviewed by Govind, 1981). Forces measured at the dactyl of lobster chela can be five to ten times greater in the crusher than the cutter. The crusher has greater mechanical advantage and muscle volume than the cutter, but even after these differences have been taken into consideration, the crusher closer muscle exerts two to three times the stress displayed by the cutter chela (Blundon, unpub.). There is, however, no asymmetry between homologous motoneurons to the closer muscle (Hill and Govind, 1983) nor in the size or number of sensory axons to the paired chela (Govind and Pearce, 1985). The greatest degree of specialization between the paired chela occurs in the snapping shrimp *Alpheus* (reviewed by Mellon, 1981). In this genus, the functional asymmetry (Przibram, 1931; Wilson, 1903) is complemented by asymmetries in the fiber composition of the closer muscle (Stephens and Mellon, 1979; Mellon and Stephens, 1980) in the size of the motoneuron somata to the closer muscle (Mellon *et al.*, 1981), and in the number of sensory axons to the chela (Govind and Pearce, 1985).

Finally, with regard to the reversal of chela asymmetry proposed by Hamilton *et al.* (1976) for blue crabs, the present report shows that such reversal would not involve changes in the sarcomere length, myosin ATPase activity, or oxidative capacity of the closer muscles. They may, however, involve changeover of the contractile properties from fast-follower type fibers to slow-follower type in the transforming cutter-to-crusher chela. They certainly involve changes in the external form of the chela; the cutter acquires a heavier dentition and possibly a slightly larger muscle in its transformation into a crusher. The mechanical advantage and muscle volume of the newly formed left crusher, although being superior to the newly regenerated right cutter, is not as great as the original crusher. More importantly, the essential similarity in SL and enzyme properties between the closer muscle of the cutter and crusher claws demonstrates that these muscle properties are not responsible for any functional differences between the paired claws brought about by firing of their motoneurons. Rather these differences are in part due to properties of their motor axons, such as conduction velocities, and their neuromuscular synapses, such as the amount of transmitter released and the degree of facilitation. Part of the functional differentiation may also be due to properties of the muscle fibers themselves such as differences in cable properties and in the threshold for excitation-contraction coupling.

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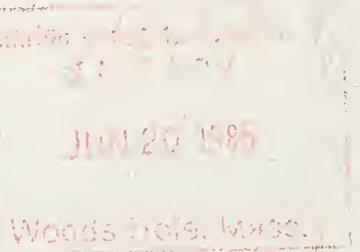
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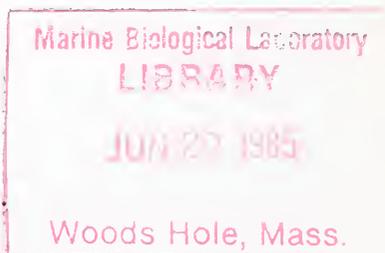
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## RETINAL CIRCUITRY AND CLINICAL OPHTHALMOLOGY\*

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### ABSTRACT

Six different neurotransmitters are now comparatively well established in the retina, and for several we know both which cells they are in and what contacts these cells make with other neurons. We also know there must be many more neurotransmitters, although they have not yet been clearly identified.

Psychoactive drugs can be expected to affect not only neurons in the brain but also in the retina. Dopaminergic neurons, GABA neurons, and indoleamine neurons are among the ones likely to be affected. Drugs which influence these systems are already in widespread use, and new and more powerful ones are constantly being developed. However, comparatively little is known about what the drugs do to vision. Very likely, this is so because clinical ophthalmological methods are usually not sophisticated enough to detect the effects of the drugs on vision. There is a great need for better clinical diagnostic methods, based on experimental knowledge of what different types of neurons may do in the retina and what different signal systems there are.

There may be as many different specialized tasks for the retina as there are neurotransmitters. It is important that these tasks are duly identified in the laboratory and that the results are brought to the ophthalmologists so that sensitive and selective clinical testing procedures can be developed.

### INTRODUCTION

The eye is, in many respects, similar to a camera, and light induces some special chemical reactions both in the photographic film and in the retina at the back of the eye. However, unlike the film, the retina cannot be taken out to be developed and therefore it has to transform the chemical reactions into nerve signals that can be sent into the brain so we can see. This is done by means of various interactions between the many and different nerve cells that it contains, producing a coded signal that can be efficiently transmitted to the brain.

Ordinary microscope sections of the retina show a number of nerve cells, but not much of the different connections they make. With the more than a century-old silver staining methods, the form of individual nerve cells can be studied in great detail, and with the procedure, very complex network diagrams of different cell types have been produced (Polyak, 1941). However, as intricate and detailed as the diagrams are, they have so far been of only limited value. The reason for this is that some very important information is missing. The diagrams give no details about what the different nerve cells actually do, or how they do it.

Modern neurobiology is changing this. We are now able to say a little about the actions of the different components in the retina and some examples will be

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presented here on how the cells in the retina send signals to each other. Some special attention will be given to systems which seem particularly relevant to clinical ophthalmology.

It was once maintained by some that the signal transfer between different neurons was rather trivial. All that was needed, it was said, was something similar to the binary system of a computer: an excitatory signal (or plus signal if you wish) and an inhibitory (or minus) signal. As will be seen, this is far from what the situation is like in the retina or, for that matter, in any other part of the nervous system.

### THE SIMPLIFIED RETINA

It is useful to simplify the structure of the retina as in Figure 1, where one cell of each of the main types is shown.

The pigment epithelium cells serve to reduce reflections in the eye and thus to prevent glare. However, more importantly, they are positioned in between the blood vessels of the choroid and the photoreceptor cells, and, as will be further discussed, they therefore interact with them as a kind of helper cell.

The photoreceptor cells are highly specialized for capturing light. As indicated in Figure 1, light passes through most of the retina before it reaches these cells and excites them. They will in turn affect the next neuron, the bipolar cell. From the structure of the junction between the two types of cells we know that it is a chemical synapse, that is, a neurotransmitter is released from the photoreceptor cells to influence the next cell in line. There is also a different kind of synapse in the retina, gap junctions, which couple cells together electrically (Yamada and Ishikawa, 1965; Witkovsky and Dowling, 1969; Kolb and Nelson, 1983, 1984; Witkovsky *et al.*, 1983). But our knowledge about them is still not sufficient to make it possible to discuss how they might affect vision, however interesting they may be.

The bipolar cell passes the signal received from the photoreceptor cell on to the ganglion cell in a second chemical synapse. The ganglion cell has a long axon with which it sends the extensively coded information to the brain.

There are also cells that modify the transmission of information through the retina. Horizontal cells are present at the first synapse between the photoreceptor cells and the bipolar cells. Amacrine cells affect the signal transfer at the second synapse, *e.g.*, the synapse between the bipolar cells and the ganglion cells. They also form important links in multineuron signal chains. For instance, in the cat retina, certain amacrine cells are intercalated between rod bipolar cells and ganglion cells so that, in effect, a four-neuron path is produced from the photoreceptor cells to the brain (Kolb and Nelson, 1983). In the ground squirrel, some ganglion cells get their information exclusively from amacrine cells, and also in these cases a four-neuron path must be assumed (West and Dowling, 1972).

A recently discovered, slightly different cell type, the interplexiform cell, appears to send information backwards from the second to the first synapse, in what electronic engineers would call a feedback loop. This arrangement was originally recognized as the result of studies on a special neurotransmitter in fish retinas, dopamine (Dowling and Ehinger, 1975; Dowling *et al.*, 1976), but the cells have more recently also been observed with other methods (Ehinger, 1982, 1983a).

A special type of ganglion cell has recently been described (Mariani, 1983; Zrenner *et al.*, 1983). It contacts the photoreceptor cells directly without any intermediary cells, but has so far been seen only in cynomolgus monkeys.

The image on the retina is thus in most cases sent from the photoreceptor cells

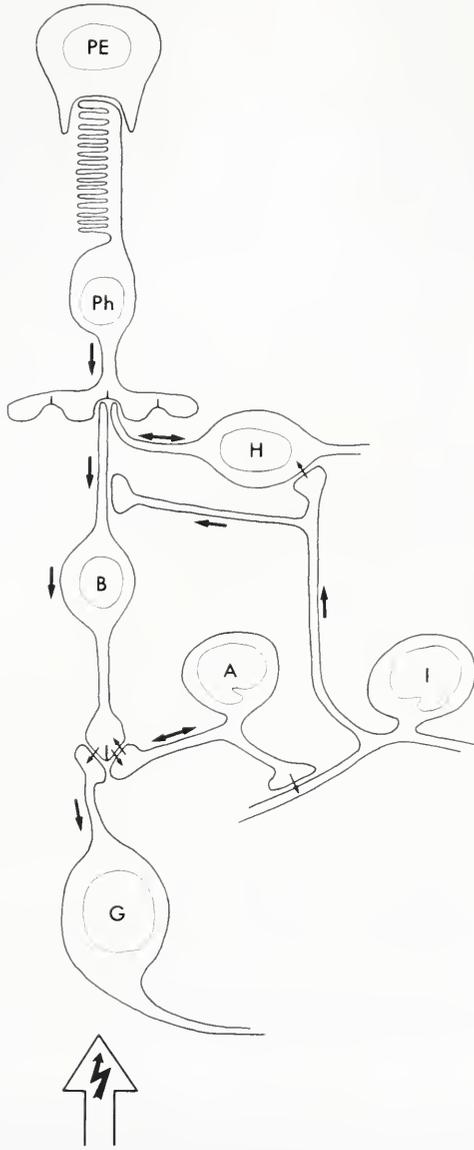


FIGURE 1. Simplified diagram of the retinal neurons. Light enters from below and passes through the various cells until it reaches the photoreceptors (Ph), where it is converted to an electrical signal. PE is the pigment epithelium. The signal is transmitted by means of a network of cells to the ganglion cell (G) which sends it to the brain. The network consists of horizontal cells (H), bipolar cells (B), amacrine cells (A), and interplexiform cells (I). The arrows indicate the possible flow of information in the different parts.

to the brain through a chain of at least three neurons, the photoreceptor cell, the bipolar cell, and the ganglion cell. The signals will be modified when they pass from one cell to the next at the synapses and therefore it is important to understand how they are being transformed when passing through this chain of neurons.

### THE NEUROTRANSMITTERS

Since most of the neurons operate with the aid of neurotransmitters, it is of interest to know what these chemicals are and how they act. This is a field where substantial progress has been made the last few years. Six substances are relatively well documented as retinal neurotransmitters. They are, in approximate descending order of documentation: dopamine, acetylcholine, glycine, GABA, 5-hydroxytryptamine (in non-mammalian vertebrates), and glutamic acid. Also more than a dozen other substances are suspected to be neurotransmitters for different reasons. These include many neuropeptides, but the evidence is in these cases less complete. Most likely, there are also many neurotransmitters in the retina which we have not recognized.

The three main criteria for classifying a substance as a neurotransmitter are: (1) that the substance is present or can be rapidly synthesized in neurons, (2) that it can be released by nerve activity, and (3) that selective receptors are present. In addition, some other criteria are often used: (4) there should be some inactivation mechanism for the transmitter. (5) Synthesizing enzymes should be demonstrable. (6) Some protected storage mechanism should be present. These criteria open up many ways to identify a neurotransmitter: by chemical analysis, by physiological or pharmacological experiments, or by morphological work. All approaches have been used in different studies, and various examples will follow, although some emphasis will be given to morphological results.

### DOPAMINERGIC NEURONS

Many of the drugs used today by psychiatrists affect the function of, among others, dopaminergic neurons, and such cells have been demonstrated in the retina. Figure 2 is a fluorescence micrograph of the dopaminergic neurons in the cynomolgus monkey retina. Human retinas are very similar. Dopamine has been turned into a fluorescent compound in the tissue section by reacting it with formaldehyde with the method of Falck and Hillarp (see Björklund *et al.*, 1972). Fluorescence is seen in amacrine cells, indicating they contain dopamine.

Dopaminergic neurons vary in intriguing ways between different species, but nevertheless occur in all vertebrates investigated so far (Fig. 3). Evidently, then, they must be important for vision. However, simple clinical observations tell us that powerful as they are, the neuroleptics used by the psychiatrists do not seem to affect vision in any readily discernible way even though they are well known to interfere



FIGURE 2. Formaldehyde induced fluorescence in a baboon retina, demonstrating a dopaminergic cell body (arrow) and dopaminergic processes forming a narrow sublayer in sublamina 1 at the border between the inner plexiform layer and the inner nuclear layer (arrowheads). Fluorescence micrograph, 110 $\times$ .

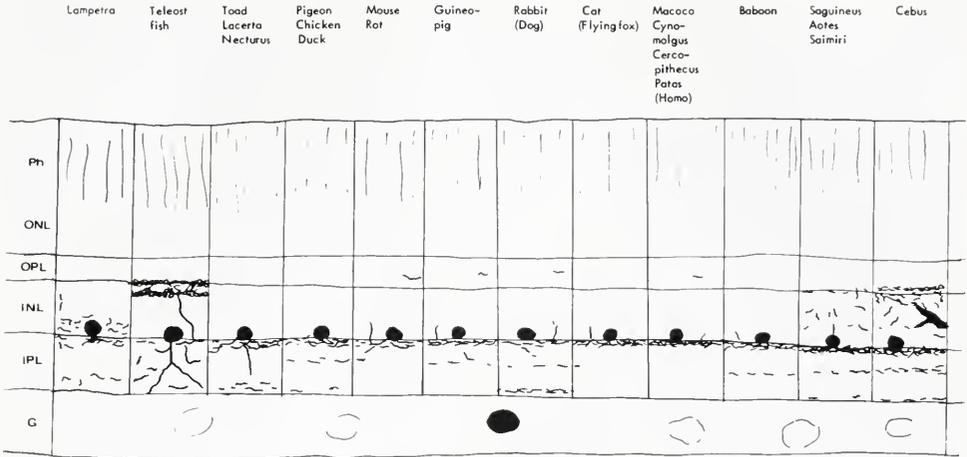


FIGURE 3. Scheme of dopaminergic neurons demonstrable with formaldehyde induced fluorescence in different species. Note that dopaminergic amacrine cells occur in all species but that the distribution of their processes varies widely.

with dopaminergic systems similar to the one found in the retina. This is of course good and well for the psychiatric patient but may seem somewhat surprising, and perhaps also a little disappointing from the neurobiologist's viewpoint. However, something of an explanation can be found if one goes a little more into the details of how these cells connect to other cells.

Dopaminergic neurons very actively accumulate their transmitter and certain similar substances. 5,6-dihydroxytryptamine is such a dopamine-like substance, and it makes the membranes and the cytoplasm appear darker in the electron microscope, and also induces some swelling of the processes and organelles of the dopaminergic neurons. Therefore these neurons can be identified at the ultrastructural level (Fig. 4; Dowling and Ehinger, 1975, 1978). Indoleamine accumulating neurons also are labeled by the treatment, but they can be removed so that they will not interfere with the analysis (Ehinger and Florén, 1978).

In favorable sections, one can identify the pre- and postsynaptic members of the synapse and thus determine which contacts the dopaminergic cell makes. The analysis shows that they contact only other amacrine cells (Fig. 5; Dowling and Ehinger, 1976, 1978; review: Ehinger, 1983b).

The main and most direct signal pathway from the photoreceptor cells to the brain is through the bipolar and ganglion cells. Dopaminergic neurons are not coupled directly to either of these cells (Ehinger, 1983a). Consequently, it seems reasonable to guess that the dopaminergic neurons have some general but specialized governing function rather than an influence on details in the visual field. Actually, the overall morphology of the dopaminergic neurons suggests the same: the cells are relatively sparse, but branch widely so that a single cell can affect many others. Such a general but special function may be difficult to observe clinically because the methods routinely available do not test any special function of vision. This might be the explanation why psychoactive drugs have not been observed to influence human vision.

If dopaminergic drugs do not affect vision in a readily detectable way because dopaminergic neurons are not directly connected to the neurons which form the

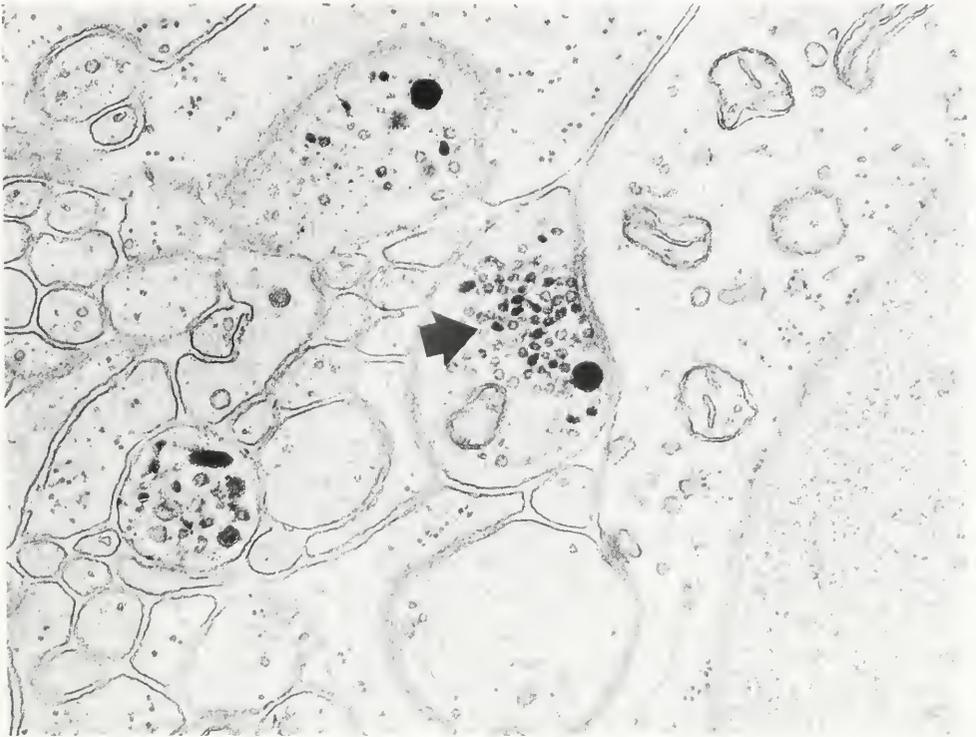


FIGURE 4. Electron micrograph from rabbit retina, showing three dopaminergic processes, indicated by their dark vesicles. They were labeled with 5,6-dihydroxytryptamine. One of the processes (arrow) makes a synapse with an amacrine cell body. Indoleamine accumulating processes had been removed in order not to interfere with the analysis. 40,000 $\times$ .

most direct link between photoreceptors and the brain, then other cells which do contact this link should affect vision significantly. As the following examples will show, this is perhaps the case, although only too few studies directly address this point.

#### CHOLINERGIC NEURONS

There is now very good evidence that cholinergic amacrine neurons occur in the retina. Acetylcholine is present in the tissue, as is its synthesizing and degrading enzymes, and specific receptors have also been demonstrated with several different methods (reviews: Neal, 1976, 1983). Moreover, there is a calcium-dependent and light-driven release system for acetylcholine (Masland and Livingstone, 1976; Baughman and Bader, 1977; Baughman, 1980; Neal and Massey, 1980; Neal, 1982, 1983). By autoradiography and other morphological methods, the cholinergic cells have been identified as amacrine cells, most likely of the special, so-called starburst type (Masland and Mills, 1979; Masland, 1980; Famiglietti, 1983a, b). Both physiological and pharmacological experiments suggest that these neurons contact the bipolar cells and presumably also the ganglion cells (Fig. 6). In birds, autoradiography suggests that certain bipolar cells perhaps are cholinergic (Baughman and

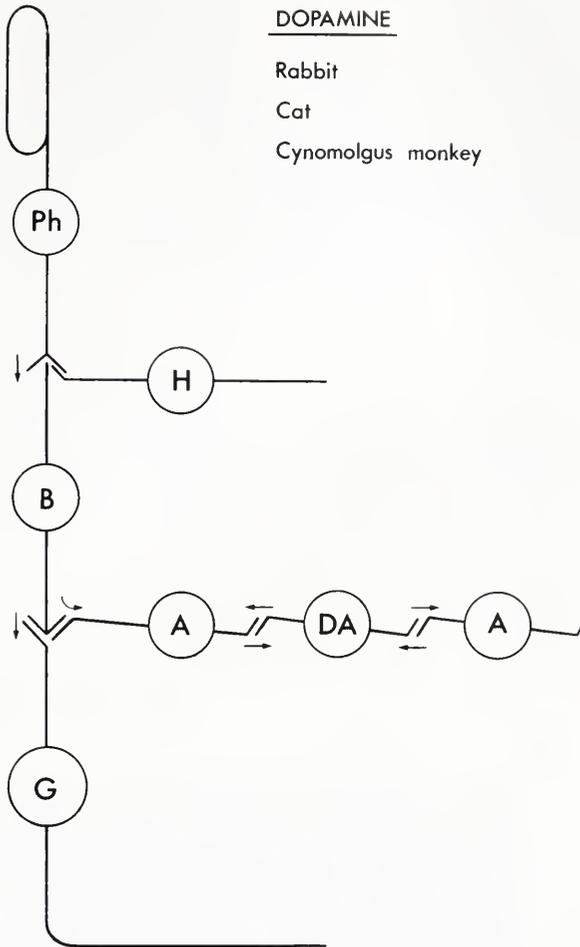


FIGURE 5. Schematic diagram of dopaminergic neurons (DA) in the rabbit retina. These cells are a special subgroup of amacrine cells that contact only other amacrine cells, *i.e.*, they are interamacrine neurons. The diagram is based on electron microscopical studies with autoradiograms and labeling with 5,6-dihydroxytryptamine. Ph, photoreceptor; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell.

Bader, 1977) and in toads and goldfish there is evidence that a small percentage of the ganglion cells may be cholinergic (Oswald and Freeman, 1980).

In clinical ophthalmology, cholinomimetic drugs such as pilocarpine or various cholinesterase inhibitors have long been used as topical eye drops for the treatment of glaucoma. These drugs are likely to reach the retina in significant concentrations, especially in patients which have had their lenses removed because of cataract. However, neither textbooks nor the scientific literature usually suggest that the treatment has any effect on the retina. Nevertheless, all patients on pilocarpine or similar eye drops complain about blurred and obscured vision, at least initially. However, they still usually perform well on ordinary visual testing, so doctors tend not to pay very much attention to these complaints. The standard explanation the doctor gives to the patient is that his or her vision gets obscured because the pupil

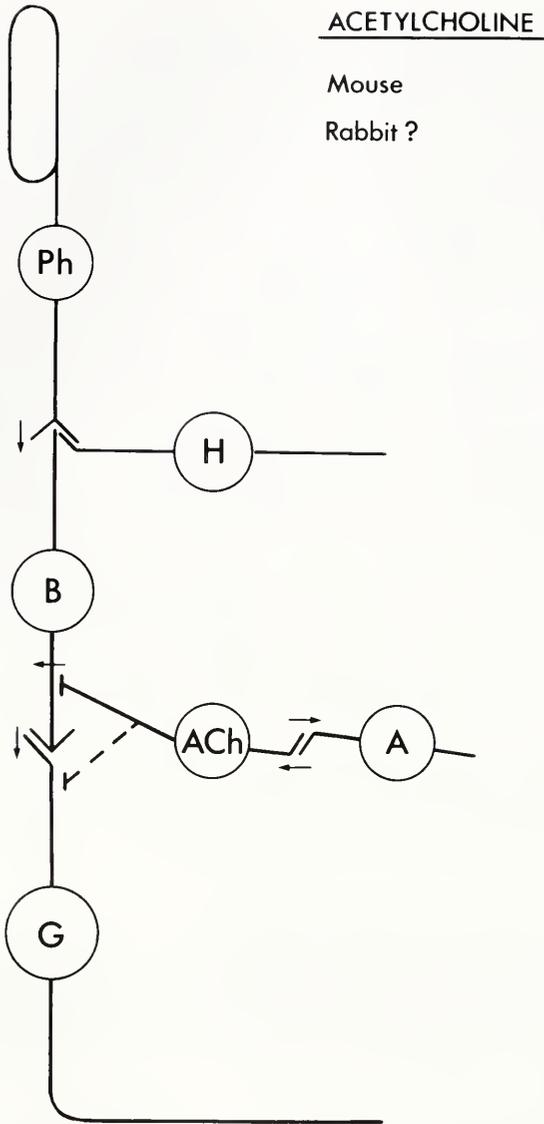


FIGURE 6. Cholinergic neurons (ACh) in the mouse and rabbit retina seem to contact both amacrine cells (A), bipolar cells (B) and, perhaps, ganglion cells (G). The diagram is somewhat tentative and based on receptor binding studies with alpha-bungarotoxin, autoradiography, electron microscopy, and certain electrophysiological experiments. Ph, photoreceptor; H, horizontal cell.

has been made so small by the drug. This effect is easily seen by the doctor, and the patient (or at least some relative) can readily confirm the observation, so the statement is usually accepted without any further discussion. The doctor would ask the patient to put up with the problem or lose vision. The choice seems easy.

However, the retina is able to change its sensitivity about a hundred thousand fold by adjusting the gain in its amplifier system. Constriction of the pupil by a

drug can diminish the light falling on the retina only about fifteen-fold, provided that no complicating factor is present, such as opacification of the lens by a cataract. Clearly, the doctor's simple standard explanation must be in doubt in many cases. One can reasonably suspect a pharmacological action of the cholinergic drugs on the retina in addition to the change in pupil size. However, currently our standard methods for clinical investigations are too crude to tell easily what is actually happening. Clinicians will be able to design effective clinical testing methods only when they can be guided by results from experimental neurobiology, showing what functions the cholinergic neurons may have.

### GABA NEURONS

There is a similar story for another neurotransmitter, *gamma*-amino butyric acid, usually abbreviated GABA.

There are good reasons to believe that GABA is a neurotransmitter in certain amacrine cells in the mammalian retina. It is present in high concentration in the same region of the retina as the amacrine cells, where the synthesizing enzyme can also be found (Kuriyama *et al.*, 1968; Graham, 1972; Voaden, 1978; Brandon *et al.*, 1979; Lam *et al.*, 1979; Brandon *et al.*, 1980; Famiglietti and Vaughn, 1981; Vaughn *et al.*, 1981; Wu *et al.*, 1981; Zucker *et al.*, 1984). There is also a highly selective and very active uptake mechanism for GABA in certain amacrine cells which can be illustrated by autoradiography (Fig. 7). Moreover, tritiated GABA can be released from the retina by light flashes. Finally, different types of experiments in several laboratories have shown GABA receptors to be present in the retina (reviews: Ehinger, 1982, 1983b).

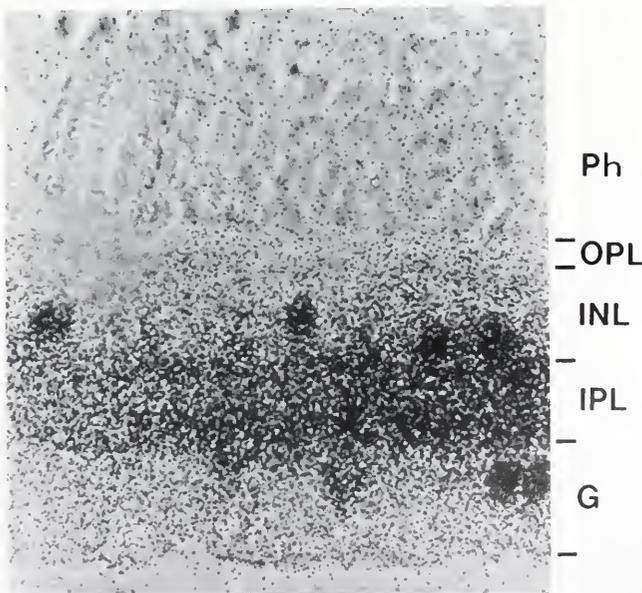


FIGURE 7. Autoradiogram of a rabbit retina 4 hours after the intravitreal injection of ( $^3\text{H}$ )-GABA. Amacrine cells, some ganglion cells (G), and the entire inner plexiform layer (IPL) have become labeled. Ph, photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Phase contrast micrograph, 400 $\times$ .

Immunohistochemical analyses of the localization of the enzyme that synthesizes GABA have suggested that the GABA neurons connect directly to bipolar cells and, possibly, ganglion cells (Brandon *et al.*, 1980; Famiglietti and Vaughn, 1981; Vaughn *et al.*, 1981; Zucker *et al.*, 1984). The postulated organization is shown in Figure 8.

When driving an automobile, it is extremely important that you are able to detect moving objects in the periphery of your field of vision, like a running child emerging from the sidewalk, for instance. It has been shown that GABA receptor blockers degrade the function of cells in the rabbit retina which detect movements (Wyatt and Daw, 1976; Caldwell *et al.*, 1978). Similar results have been seen more recently in frogs (Bonaventure *et al.*, 1983). Certain benzodiazepine tranquilizers like Valium (R) and Librium (R) are known to affect the GABA neurons and it would seem that one of the reasons why benzodiazepine drugs are unfit for drivers is that they make it difficult to detect the child running in from the side. Regrettably,

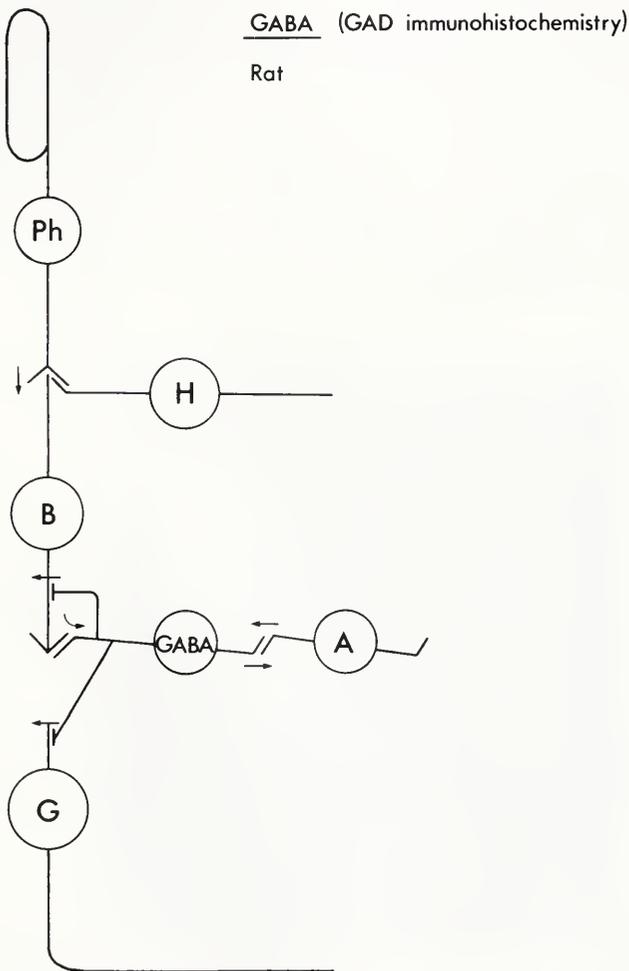


FIGURE 8. GABA neurons probably contact bipolar cells, ganglion cells, and amacrine cells. They also make feedback contacts onto bipolar cells. The diagram is based mainly on GAD immunohistochemistry. Ph, photoreceptor; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell.

this has not been properly tested, presumably because there are no suitable and well-developed clinical test systems. Therefore we do not know if some benzodiazepines are more detrimental than others. However, one report suggests that this should be testable. The ability of humans to track moving stripes after having been dazzled by a strong light decreased with increasing concentrations of a benzodiazepine in their blood (Bergman *et al.*, 1979). The authors' interpretation of their results did not include any discussion of the GABA receptors in the retina, and any inference about an effect on the retinal GABA neurons must await appropriate control experiments. However, the results do show that with properly refined clinical methods one is likely to be able to make significant observations on the function of neurotransmitters, but the tests will need a theoretical background that only animal experimentation can provide.

#### INDOLEAMINE ACCUMULATING NEURONS

There are other neurotransmitters in the retina which should be of some future concern to both ophthalmologists and neurobiologists. In cold-blooded vertebrates, 5-hydroxytryptamine has been shown to be present in amacrine cells (Fig. 9) and it seems likely that it is a neurotransmitter. The substance is present in presynaptic terminals, it can be inactivated by re-uptake into neurons, receptors have been demonstrated for it, and there appears to be stores for it (reviews: Ehinger, 1982; Osborne, 1984; Redburn, 1984). Furthermore, tritiated hydroxytryptamine can be released by depolarizing stimuli and by light (Thomas and Redburn, 1979; Osborne, 1980, 1982a, b; Redburn, 1984; Ehinger, Tornqvist, and Waga, unpubl.). There are similar neurons in mammals, but it is not certain that 5-hydroxytryptamine is their transmitter because their content of the substance is at least 50 times less than in neurons known to be 5-hydroxytryptaminergic (Ehinger, 1982; Redburn, 1984). However, collectively these neurons can be called indoleamine accumulating neurons, and their similarities warrant that they be discussed together.

The indoleamine accumulating neurons can be identified using the electron microscope because they can take up 5,6-dihydroxytryptamine. This drug induces morphological changes similar to the ones seen in Figure 4 (Dowling *et al.*, 1980; Ehinger, 1982; Holmgren-Taylor, 1982a-c) and makes it possible to trace their connections with the electron microscope. Since the dopaminergic neurons also accumulate the substance, these neurons must first be eliminated so that they do not interfere with the analysis. This can be done with the aid of the selective neurotoxin, 6-hydroxydopamine (Ehinger and Nordenfelt, 1977).

When the indoleamine-accumulating neurons are studied in the electron microscope, they are seen to contact bipolar cells (Fig. 10). Bipolar cells form part of the main signal pathway from the photoreceptor cells to the brain, and the indoleamine-accumulating neurons can thus be suspected to influence vision directly; but almost nothing is known about their function. Therefore it is not possible to predict what interfering with the indoleamine accumulating neurons will do to vision. Currently, however, many pharmaceutical companies are very actively testing new psychotropic drugs which act on the 5-hydroxytryptamine neurons, and it is likely that they will affect the function of the retina. Since there is at present no way of knowing what type of side effects to expect, it is not very likely that they will be discovered in the initial drug testing phases, but only when the drugs have come into extensive use. Again, we do not have good enough clinical methods available to analyze routinely the different components of vision.

As mentioned above, indoleamine accumulating neurons are somewhat complicated. It is now quite clear that there are two types, one that contains significant

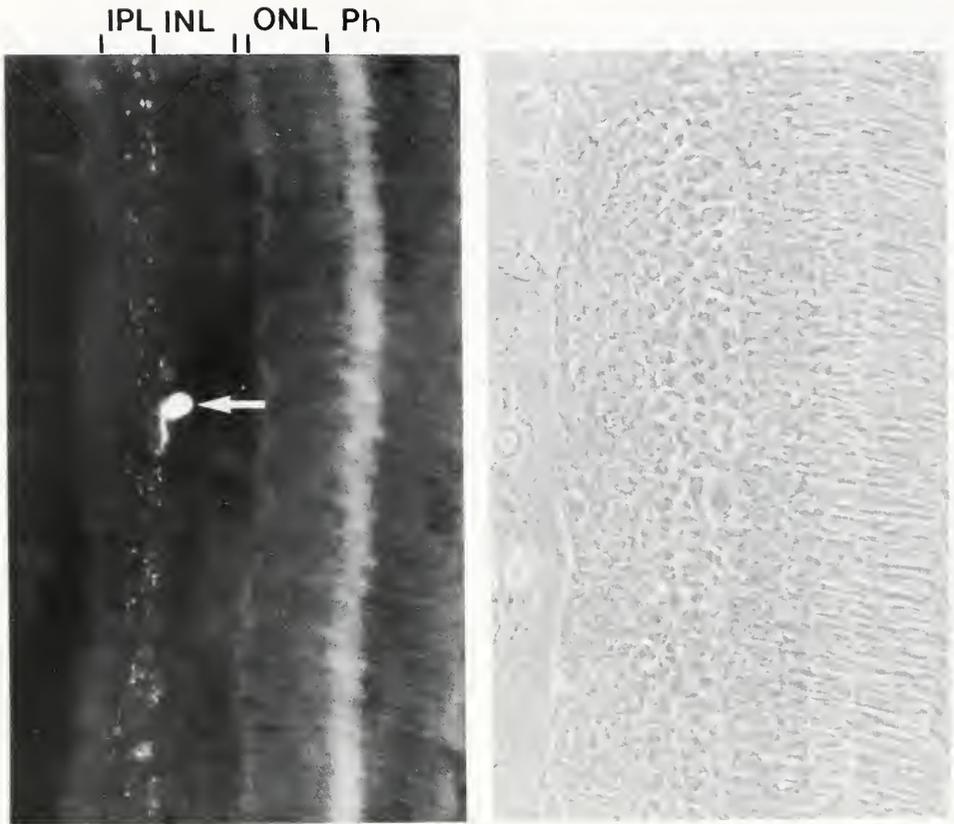


FIGURE 9. Left, immunohistochemical demonstration of 5-hydroxytryptamine in the skate retina. There is an immunoreactive amacrine cell body (arrow) and there are numerous immunoreactive processes in the outer half of the inner plexiform layer. Right, phase contrast micrograph of the same region. Ph, photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; 280X.

amounts of 5-hydroxytryptamine and one that does not (Bruun *et al.*, 1984). Mammalian retinas contain the type that does not contain very much 5-hydroxytryptamine. However, the pharmaceutical companies direct their work towards the ones which do contain 5-hydroxytryptamine and which are found in the mammalian brain. Some drugs that will be developed are likely to affect only the type containing much 5-hydroxytryptamine, others will presumably affect both types, and yet others may perhaps even affect only the type with little 5-hydroxytryptamine. This will cause confusion, and will make it even more difficult to detect whatever side effects on vision drugs acting on the indoleamine systems may have.

#### NEUROPEPTIDES

Neuropeptides presumably act as neurotransmitters, and many have now been found in the retina, most of them in amacrine cells (Table I). Much of the work has been done with immunohistochemistry (Tornqvist, 1983; Brecha *et al.*, 1984),

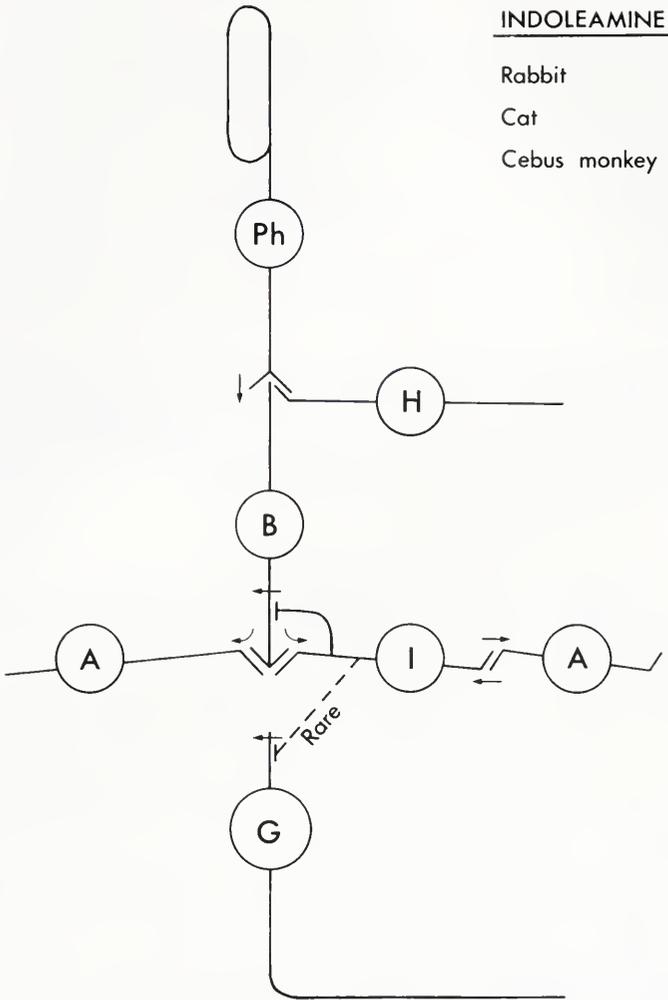


FIGURE 10. In mammals, indoleamine accumulating neurons (I) make contacts with bipolar cells and amacrine cells. They also form reciprocal synapses with bipolar cells at the dyads. Contacts with ganglion cells are rare. The diagram is based on studies with electron microscopy after labeling with 5,6-dihydroxytryptamine. Ph, photoreceptor; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell.

and exact identification is available only for a few of the substances and in a limited number of animal species. Also, no retina has been shown to have all of them. The number of known neuropeptides is growing rapidly, and many will no doubt be added to the list.

Little is known about the function of the neuropeptides in the retina, so it is more than premature to speculate about their possible clinical implications. However, it is important to note that they are present, because drugs acting as either agonists or antagonists are now very actively being developed in many laboratories. Substance P may be taken as an example. A substance P antagonists [Spantid (R)] has been shown to have anti-inflammatory effects in rabbits (Holmdahl *et al.*, 1981; Bynke,

TABLE I

*An alphabetical list of some neuropeptides shown or presumed to be present in the retina of different animals*

Bombesin	NPY
Cholecystokinin (CCK)	PHI
Endorphins	Somatostatin
Enkephalins	Substance P
Glucagon	TRH (no immunohistochemistry)
LHRH	VIP
Neurotensin	

1984) and it seems possible that it may find use in ophthalmology as an anti-inflammatory drug. Others are likely to follow. Therefore there are good reasons to investigate what the drugs affecting the function of neuropeptides might do to vision, first by finding out where the neuropeptides are in the retina and then to continue with physiological and pharmacological work.

#### PIGMENT EPITHELIUM

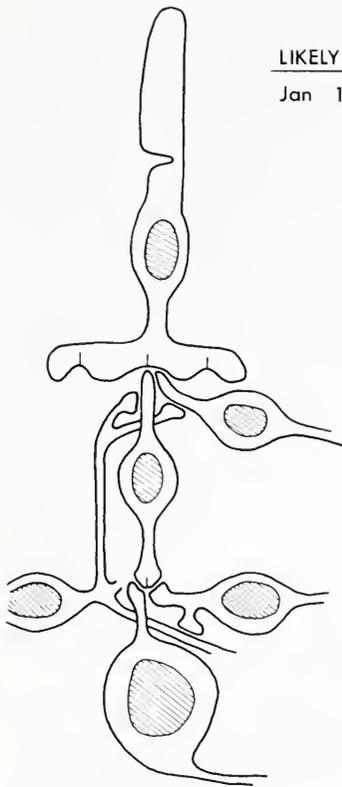
The examples above all show how signals are being sent between nerve cells in the retina. There are several other very important signal systems in it, and one is of particular significance to ophthalmologists.

It has been known for some time that normal functioning of the photoreceptor cells is dependent on the pigmented epithelium. Figure 1 shows how the pigment epithelium sits just next to the photoreceptor cells. In some very elegant studies only little more than a decade ago Young (1967) showed that photoreceptor cells grow continuously and shed their tips in order to keep their length constant. The pigment epithelium phagocytizes the discarded photoreceptor tips (Hollyfield and Basinger, 1978).

Since the phagocytosis of photoreceptor tips has been found to take place only during a few hours and only once daily for a given class of cells, there must be a very precise system to tell the pigment epithelium when it is time to consume a little of the photoreceptor cells, and which ones. We are just beginning to suspect that certain chemicals, present in the retina, may turn this system on or off, although the picture is still very far from clear (Ogino *et al.*, 1983; Besharse *et al.*, 1984). Different disturbances in the turnover system of the photoreceptor cells are well known in various laboratory animals. In humans, there may be similar situations in a special group of diseases, collectively called retinitis pigmentosa. These disorders are relatively common, stealing the eyesight from some 50,000 or perhaps more Americans. In most cases the patients are young, and otherwise the disease does not effect them so that they live an ordinary life except that they are blind. It is tantalizing that it is realistic to hope for a treatment in these cases. However, there is much work to be done before any treatment will be available. Therefore, it seems extremely important to point out that studies on the interactions between the retinal pigment epithelium and the photoreceptor cells carry the potential of helping to retain the eyesight of tens of thousands of people in the U. S. alone and many many more in other countries. High priority should be given to such work.

#### CONCLUSIONS

We now know a number of neurotransmitters in the retina (Fig. 11) and for several, the circuits of the cells they are in have been traced. This knowledge is



LIKELY NEUROTRANSMITTER:

Jan 1985

PHOTORECEPTORS: Aspartate?  
Glutamate?

HORIZONTAL CELLS: ? (GABA in goldfish)

BIPOLAR CELLS: ? (Indoleamine in skate)

INTERPLEXIFORM CELLS: Dopamine (teleost fish, New World monkeys)  
(GABA in cats?)  
(Glycine in goldfish?)

AMACRINES: Dopamine	Enkephalins?	TRH ?
Glycine	Substance P?	NPY ?
GABA	Glucagon?	PHI ?
Indoleamine (5-HT)	Neurotensin?	FMRFamid?
Acetylcholine	Bombesin?	GRP ??
VIP ?	Cholecystokinin?	
Samatostatin?	LHRH ?	

GANGLION CELLS: Glutamate? (5-10%)  
Acetylcholine (frags)

FIGURE 11. Summary diagram of the localization of neurotransmitters in the retina as presently known. The identification of most of the peptides is only tentative and the evidence that they are neurotransmitters is only suggestive. Parentheses indicate that there are important restrictions for the substance.

potentially useful in two ways: (1) it can help us pinpoint the cause of certain diseases so that remedies can constructively be sought for and (2) it can help us predict the effects of new drugs, favorable or unfavorable.

At the same time it seems evident that with this multitude of different transmitters, there are perhaps as many different tasks that the retina performs. There is a shortage of adequate methods for testing possible functions in the clinical setting. This is the task for the neurobiologists: find the functions of the different neurotransmitters and their circuits and tell the clinicians how to search for the function also in patients. No doubt such work will have to start on cold blooded vertebrates which are comparatively easy to work with and therefore suitable as models for more complicated systems.

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NEMATOCYST DISCHARGE, HISTOINCOMPATIBILITY, AND THE  
FORMATION OF SWEEPER TENTACLES IN THE CORAL  
*GALAXEA FASCICULARIS*

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ABSTRACT

The roles of nematocyst discharge of ordinary tentacles and histoincompatibility responses in the formation of sweeper tentacles in the coral *Galaxea fascicularis* were examined. Colonies of *G. fascicularis* could be divided into several groups according to whether their ordinary tentacles discharge nematocysts against each other. Polyps isolated from colonies of the same or different groups were set about 5 mm apart with tentacles touching and maintained for about five months in this position. When two polyps isolated from colonies of different groups were paired, they developed sweeper tentacles. Usually, one of the paired polyps was damaged, but in some combinations both polyps survived without damage. Polyps that did not discharge nematocysts against each other did not compete through sweeper tentacle formation. In some combinations, paired polyps showed histoincompatibility and developed tentacles at intermediate stages between ordinary and sweeper tentacles. In other combinations, paired polyps fused and developed no or only a few intermediate tentacles. The present results show that the nematocyst discharge response has a major role in eliciting formation of sweeper tentacles, but that the histoincompatibility response may also stimulate transformation of ordinary tentacles into sweeper tentacles.

INTRODUCTION

Corals compete for space in several ways. Interspecific competition through extracoelenteric digestion with mesenterial filaments (Lang, 1973), overgrowth (Porter, 1974; Connell, 1976), and sweeper tentacle formation (den Hartog, 1977; Richardson *et al.*, 1979; Wellington, 1980; Bak *et al.*, 1982; Chornesky, 1983) are well known. Although corals generally do not use mesenterial filaments in intraspecific competition for space, they compete with other conspecific colonies through cytotoxic histoincompatibility (Hildemann *et al.*, 1975, 1977a, b) and direct overgrowth (Potts, 1976, 1978; Rinkevich and Loya, 1983). *Galaxea fascicularis* uses sweeper tentacles in intraspecific competition (Hidaka and Yamazato, 1984). Sweeper tentacles are formed when corals come into contact with other colonies (Hidaka and Yamazato, 1984) or with other corals and some anthozoans (Chornesky, 1983). Sweeper tentacles have a different complement of nematocysts than ordinary tentacles (den Hartog, 1977; Hidaka and Yamazato, 1984). But there are tentacles at intermediate stages between ordinary and sweeper tentacles both in terms of nematocyst composition and external appearance (Hidaka and Yamazato, 1984). Hidaka and Yamazato (1984) suggested that ordinary tentacles transform into sweeper tentacles and that changes in nematocyst composition accompany this

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transition of tentacles. This is also the case with catch tentacles, which are similar to sweeper tentacles, and used in aggression by some sea anemone species (Purcell, 1977; Watson and Mariscal, 1983).

It is, however, not yet understood whether nematocyst discharge of ordinary feeding tentacles plays some role in intraspecific interactions. It also remains to be elucidated what kind of stimulus induces development of sweeper tentacles. Recently, Chornesky (1983) found that artificial damage or tactile stimuli cannot induce sweeper tentacle formation, though damage caused by extruded mesenterial filaments of other corals elicits sweeper tentacle formation in *Agaricia agaricites*.

The present paper first examines the roles of (1) nematocyst discharge of ordinary tentacles and (2) the histoincompatibility response in self and non-self recognition. Second, it examines whether the nematocyst discharge of ordinary tentacles or histoincompatibility response is necessary for inducing formation of sweeper tentacles. Finally, it shows that the nematocyst discharge response of ordinary tentacles plays a major role in eliciting formation of sweeper tentacles, though the histoincompatibility response may also stimulate formation of sweeper tentacles. The results also show that the histoincompatibility response is more sensitive in recognition of self and non-self than is the nematocyst discharge response.

#### MATERIALS AND METHODS

Colonies of *Galaxea fascicularis* were collected from the reef at Sesoko Marine Science Center, University of the Ryukyus, in Okinawa. They were maintained in tanks supplied with running sea water until used. Colonies used in this experiment could be divided into several morphs according to polyp coloration: morph 1 (BG), brown with greenish oral disk; morph 2 (Gt), brown but lateral tentacles are light green; morph 3 (Gs), brown but tentacles on the major septa are light green; morph 4 (B), entire polyp is pale brown; and morph 5 (Wt), brown with white fluorescent lateral tentacles.

#### *Nematocyst discharge responses*

Polyps with their corallites were isolated from a colony by pulling with forceps. The isolated polyps were placed in a holding tank for at least 6 h or sometimes for about 2 months to recover from the possible damage caused during the isolation process. The subject polyp was then placed in a dish filled with sea water and allowed to adapt to the experimental condition until its tentacles were extended. Lateral wall tissue of the stimulus polyp was applied to the tentacle tip (acrosphere) of the subject polyp for about one second. Then the stimulus polyp was gently removed from the tentacle tip to examine whether the tentacle tip adhered to the target tissue. When the tentacle tip adhered to the target tissue, it was assumed that it discharged nematocysts against the target tissue (as assumed in Lubbock, 1980; Ertman and Davenport, 1981). This test was repeated three times for each tentacle until the tentacle tip adhered to the target tissue, although adhesion usually occurred at the first trial. At least three tentacles of a polyp were examined for each combination of colonies. For the colony pairs used in contact experiments, the test was performed on at least nine tentacles of three polyps. The nematocyst discharge responses between nine colonies were examined and two series of such experiments were performed.

### *Contact experiments*

Colonies of *G. fascicularis* were divided into several groups according to whether they discharged nematocysts against each other (see Results). In this experiment, polyps isolated from colonies belonging to the same group or to different groups were paired. Seven replicated pairs of polyps were made for each combination of colonies. The paired polyps were kept at a distance of about 5 mm so that the ordinary tentacles of the two polyps touched each other. Isolated polyps were held on a polyvinylchloride stage by inserting the basal skeletal portion of the polyp into a hole in the stage. The paired polyps were placed in a tank supplied with running sea water with a distance between pairs of about 10 cm. They were observed at intervals of about two weeks for up to five months. It was recorded whether the polyps had developed sweeper tentacles, whether they suffered damage from the neighboring polyp, and whether the tissues of both polyps had fused.

At the end of the contact experiment, the paired polyps were fixed in 10% formalin in sea water. Their tentacles were cut off and their nematocyst composition was examined under a microscope at a magnification of 200–400 $\times$ . The tentacle tips (acrospheres) of ordinary and sweeper tentacles have characteristically different types of nematocysts, microbasic p-mastigophores, and large microbasic b-mastigophores, respectively (Hidaka and Yamazato, 1984). The acrosphere region of the squashed tentacle was scanned under a microscope and the number of each type of nematocyst was counted. When the acrosphere of a tentacle contained numerous microbasic p-mastigophores and no more than 20 large microbasic b-mastigophores, the tentacle was identified as ordinary tentacle. When the acrosphere of a tentacle contained numerous large microbasic b-mastigophores and no more than 20 microbasic p-mastigophores, the tentacle was classified as sweeper tentacle. When more than 20 of both microbasic p-mastigophores and large microbasic b-mastigophores were present in the acrosphere of a tentacle, the tentacle was classified as intermediate tentacle. In this experiment, the longest 20 tentacles that extended laterally were examined, though polyps usually have 30–50 tentacles. When the first 12 tentacles were revealed to be ordinary tentacles (this occurred only in histoincompatible pairs), the nematocyst composition of the remaining tentacles were not examined but they were assumed to be ordinary by external appearance alone.

After the examination of nematocyst composition, five pairs out of seven replicates were immersed in a 1:1 mixture of 10% formalin in sea water and 10% acetic acid to decalcify. After the completion of decalcification, the specimen was prodded lightly with forceps to examine whether the tissues of both polyps were continuous. The two remaining pairs were immersed in 5% commercial sodium hypochlorite solution to remove the soft tissue. The exposed skeletons were examined under a stereomicroscope to examine whether the skeletons were continuous or separated by a fine gap.

## RESULTS

### *Nematocyst discharge responses*

Colonies of *Galaxea fascicularis* were divided into several groups according to whether their ordinary tentacles discharged nematocysts against each other (Table I). Polyps did not discharge nematocysts against polyps of other colonies with the same or similar color. Polyps of a Wt or B colony did not discharge nematocysts against polyps of another Wt or B colony, respectively. There was an exception (Wt11–Wt12), although Wt11 is slightly different from other Wt colonies in that its

TABLE I

*Nematocyst discharge responses between colonies of Galaxea fascicularis*

A

Response	Stimulus								
	Wt11	Wt12	B13	B14	BG15	Gs16	Gs17	Gs18	Gs19
Wt11		+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)
Wt12	+(3)		+(9)	+(9)	+(3)	+(3)	+(3)	+(3)	+(3)
B13	+(3)	+(6)	-(9)	-(3)	+(9)	+(3)	+(3)	+(3)	+(3)
B14	+(3)	+(6)	-(3)		+(3)	+(9)	+(3)	+(3)	+(3)
BG15	+(3)	+(3)	+(9)	+(3)		-(9)	-(3)	-(3)	-(3)
Gs16	+(3)	+(3)	+(3)	+(9)	-(9)		-(3)	-(3)	-(3)
Gs17	+(3)	+(3)	+(3)	+(3)	-(3)	-(3)		-(9)	-(3)
Gs18	+(3)	+(3)	+(3)	+(3)	-(3)	-(3)	-(9)		-(3)
Gs19	+(3)	+(3)	+(3)	+(3)	-(3)	-(3)	-(3)	-(3)	

B

Response	Stimulus								
	Wt21	Wt22	B23	B24	B25	BG26	BG27	Gs28	Gt29
Wt21		-(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)	+(3)
Wt22	-(3)		+(9)	+(18)	+(3)	+(9)	+(3)	+(3)	+(3)
B23	-(3)	+(3)		-(9)	-(3)	+(9)	+(3)	+(3)	+(3)
B24	-(3)	+(1)	-(9)		-(3)	+(3)	+(3)	+(3)	+(3)
B25	-(3)	-(3)	-(3)	-(3)		+(3)	+(3)	+(3)	+(3)
BG26	+(3)	+(9)	+(9)	+(3)	+(3)		-(13)	-(13)	-(19)
BG27	+(3)	+(3)	+(3)	+(3)	+(3)	-(13)		-(13)	-(3)
Gs28	+(3)	+(3)	+(3)	+(3)	+(3)	-(13)	-(13)		-(14)
Gt29	+(3)	+(3)	+(3)	+(3)	+(3)	-(19)	-(8)	-(14)	

A and B show results of two series of experiments. +, Adhesion. -, Non-adhesion. Letters represent color morphs and numbers following the letters represent individual colonies. Tentacles of B morph colonies adhered only slightly to polyps of Wt morph colonies when it adhered. Number in parentheses indicates the number of tentacles examined.

tentacles on the major septa are whitish. Polyps of BG, Gs, and Gt colonies did not discharge nematocysts against each other, forming one group according to the present examination of the nematocyst discharge response. Colonies belonging to different groups discharged nematocysts against each other. In one combination of colonies (Wt-B), however, the nematocyst discharge appeared to be unidirectional. Polyps of B colonies failed to adhere or sometimes only weakly adhered to the target tissue of Wt colonies, while tentacles of Wt colonies strongly discharged nematocysts against the tissue of B colonies. Polyps of the same B colony discharged nematocysts normally against polyps of morphs other than Wt.

#### Contact experiments

When polyps isolated from colonies of different groups were paired, both of the paired polyps developed many sweeper tentacles (Table III). In some combinations,

TABLE II

*Outcomes of the contact experiments between isolated polyps of Galaxea fascicularis*

Source of the paired polyps	Nematocyst* discharge	Contact responses			
		Fusion	Filling	Aggression**	No contact and no response
Allogeneic					
BG15-B13	+(9) +(9)	0	1	6 (BG15 > B13)	0
Wt22-BG26	+(9) +(9)	0	0	7 (Wt22 > BG26)	0
B14-Gs16	+(9) +(9)	0	7	0	0
B23-BG26	+(9) +(9)	0	3	0	4
Wt12-B13	+(9) +(6)/-(3)	0	0	7 (Wt12 > B13)	0
Wt12-B14	+(9) +(6)/-(3)	0	0	7 (Wt12 > B14)	0
Wt22-B23	+(9) +(3)/-(6)	0	0	5 (Wt22 > B23)	2
Wt22-B24	+(18) +(1)/-(17)	0	0	7 (Wt22 $\cong$ B24)	0
Gt29-BG26	-(19) -(19)	0	6	0	1
Gt29-Gs28	-(14) -(14)	0	7	0	0
B23-B24	-(9) -(9)	0	6	0	1
Gs16-BG15	-(9) -(9)	7	0	0	0
Gs17-Gs18	-(9) -(9)	7	0	0	0
Syngeneic					
B13-B13	-(9)	7	0	0	0

Seven replicated pairs were made in each combination of colonies and the number of pairs which fell into each category are shown. Letters indicate color morphs and numbers following the letters indicate individual colonies.

\* +, Tentacle tips adhered to the opponent polyp. -, No adhesion was observed. Number in parentheses indicates the number of tentacles examined. Adhesion of tentacles of B morph colonies to polyps of Wt morph colonies was very weak when it occurred. The data are same as those shown in Table I.

\*\* Aggression means that one of the paired polyps was damaged; the dominant polyps are on the left side of the inequality sign. Polyps of the Wt22 colony were dominant in four pairs and died in the other three pairs.

one of the paired polyps was damaged (aggression) (Table II; BG15-B13, Wt22-BG26). In other combinations, both polyps survived, without suffering any damage from the neighboring polyp, for five months (Table II; B14-Gs16, B23-BG26). Tissue fusion did not occur and a more or less vertical ridge was formed at the interface as a result of interfacial cementation (filling).

When polyps isolated from B and Wt colonies were paired, polyps of B colonies usually suffered damage from polyps of Wt colonies (Table II), though both of the paired polyps developed many sweeper tentacles (Table III). This indicates that polyps which discharge nematocysts against the opponent polyp are dominant over the polyps which do not, since it appeared that only polyps of a Wt colony discharged nematocysts against polyps of a B colony. In some cases, polyps from Wt colonies died in the early stage of the experiment (Table II; Wt22-B24). But this may be due to the fact that polyps of Wt colonies have shorter column wall tissue than polyps of other morphs and seem to be more sensitive to overgrowth by algae when isolated.

When polyps isolated from different colonies belonging to the same group were paired, filling or fusion occurred (Table II). In the former case, both polyps survived without suffering any damage from the neighboring polyp, but tissues of the paired

TABLE III

*The number of ordinary, intermediate, and sweeper tentacles in the paired polyps of Galaxea fascicularis at the end of the contact experiment*

Source of the paired polyps	Nematocyst* discharge	Number of tentacles (mean $\pm$ S.D.)		
		Ordinary	Intermediate	Sweeper
Allogeneic aggression				
BG15 > B13				
BG15	+	4.4 $\pm$ 1.1 (7)	2.7 $\pm$ 1.0 (7)	12.9 $\pm$ 1.7 (7)
B13	+	0.5 $\pm$ 0.8 (6)	1.2 $\pm$ 1.6 (6)	13.8 $\pm$ 3.9 (6)
Wt12 > B13				
Wt12	+	4.6 $\pm$ 2.9 (7)	8.7 $\pm$ 2.6 (7)	6.7 $\pm$ 2.1 (7)
B13	+/-	1.7 $\pm$ 2.1 (7)	2.7 $\pm$ 1.5 (7)	13.1 $\pm$ 3.2 (7)
Wt12 > B14				
Wt12	+	3.3 $\pm$ 3.0 (7)	7.0 $\pm$ 3.9 (7)	9.7 $\pm$ 5.3 (7)
B14	+/-	6.2 $\pm$ 2.8 (6)	2.2 $\pm$ 1.2 (6)	10.5 $\pm$ 3.3 (6)
filling				
B14-Gs16				
B14	+	3.0 $\pm$ 2.8 (7)	1.1 $\pm$ 1.2 (7)	13.6 $\pm$ 3.8 (7)
Gs16	+	3.0 $\pm$ 2.8 (7)	2.6 $\pm$ 1.3 (7)	14.4 $\pm$ 3.5 (7)
Gt29-Gs28				
Gt29	-	2.1 $\pm$ 2.4 (7)	17.7 $\pm$ 2.6 (7)	0.1 $\pm$ 0.4 (7)
Gs28	-	9.4 $\pm$ 7.2 (7)	10.4 $\pm$ 7.4 (7)	0.1 $\pm$ 0.4 (7)
Gt29-BG26				
Gt29	-	2.3 $\pm$ 2.1 (7)	17.7 $\pm$ 2.1 (7)	0 (7)
BG26	-	6.9 $\pm$ 4.5 (7)	11.9 $\pm$ 5.3 (7)	1.3 $\pm$ 2.6 (7)
B23-B24				
B23	-	4.7 $\pm$ 2.8 (7)	15.3 $\pm$ 2.8 (7)	0 (7)
B24	-	18.3 $\pm$ 1.3 (7)	1.6 $\pm$ 1.1 (7)	0.1 $\pm$ 0.4 (7)
fusion				
Gs16 = BG15				
Gs1	-	20 (7)	0 (7)	0 (7)
BG1	-	19.4 $\pm$ 1.5 (7)	0.6 $\pm$ 1.5 (7)	0 (7)
Gs17 = Gs18				
Gs17	-	20 (7)	0 (7)	0 (7)
Gs18	-	18.7 $\pm$ 2.4 (7)	1.3 $\pm$ 2.4 (7)	0 (7)
Syngeneic fusion				
B13-B13				
B13	-	20 (14)	0 (14)	0 (14)

The number of the polyps whose tentacles were examined is shown in parentheses. Letters indicate color morphs and numbers following the letters indicate individual colonies. Inequality signs represent aggression; the dominant polyps are on the left side of the sign. Minus signs represent filling, and equality signs represent fusion.

\* +, Tentacle tips adhered to the opponent polyp. -, No adhesion was observed. +/-, Weak adhesion was sometimes observed.

polyps did not fuse (Table II; Gt29-BG26, Gt29-Gs28, B23-B24). In this case a considerable number of intermediate tentacles were developed, though either polyp developed no or only a few sweeper tentacles (Table III; Gt29-BG26, Gt29-Gs28, B23-B24). In the latter case, tissues of the paired polyps fused with each other (Table II; Gs16-BG15, Gs17-Gs18). In this case both polyps possessed no sweeper tentacles and no or only a few intermediate tentacles (Table III; Gs16-BG15, Gs17-Gs18). Decalcified specimens did not separate into two pieces when prodded lightly,

indicating that the soft tissues are continuous. Examination of the skeletons also proved that the interface of the skeletons is smooth without an apparent gap between them.

When syngeneic polyps were paired, they fused and developed no sweeper or intermediate tentacles (Tables II and III) as previously reported (Hidaka and Yamazato, 1984).

#### DISCUSSION

The present results show that ordinary tentacles of *Galaxea fascicularis* play some role in self and non-self recognition. Colonies of *G. fascicularis* can be divided into groups according to whether their ordinary tentacles discharge nematocysts against each other. This is consistent with the previous study in which specimens of *G. fascicularis*, collected from another site, were used (Hidaka and Miyazaki, 1984). These groups corresponded to previously described color morphs, although color morphs Gs, Gt, and BG formed a group according to the present examination of nematocyst discharge response. Ordinary feeding tentacles must play some role in self and non-self recognition in sea anemones, since contact of feeding tentacles with nonclonemates can initiate aggression with acrorhagi or catch tentacles (Francis, 1973; Bigger, 1976; 1980; Lubbock, 1980; Purcell, 1977).

When two polyps that discharged nematocysts against each other were paired, both polyps developed many sweeper tentacles. When two polyps that did not discharge nematocysts against each other were paired, neither polyp developed sweeper tentacles. This indicates that the nematocyst discharge response of ordinary tentacles is important in eliciting formation of sweeper tentacles. Chornesky (1983) reported that, in *Agaricia agaricites*, sweeper tentacle formation can be elicited also by contact with tentacle tips of other corals. This may also indicate that nematocyst discharge plays an important role in eliciting sweeper tentacle formation. However, incompatible polyps developed many intermediate tentacles when kept in contact, even if they did not discharge nematocysts against each other. This suggests that the histoincompatibility response may also stimulate formation of sweeper tentacles though at a slower rate.

An alternative interpretation is that both nematocyst discharge and sweeper tentacle formation are elicited when the degree of histoincompatibility exceeds a certain limit and that nematocyst discharge is not necessarily involved in eliciting sweeper tentacle formation. A further study might be necessary to exclude this possibility.

When two polyps that do not discharge nematocysts against each other but which were isolated from different colonies were paired, the tissues of the paired polyps sometimes fused and at other times did not fuse. This indicates that polyps sometimes recognize polyps from other colonies as non-self at the level of histoincompatibility response, even when the ordinary tentacles do not discharge nematocysts against them. This indicates that self and non-self is more finely recognized in the histoincompatibility response than in the nematocyst discharge response, and is consistent with the previous results with *Pocillopora damicornis* (Hidaka, in prep.). It is not clear whether fused polyps were derived from syngeneic colonies produced by asexual reproduction such as fragmentation. It was suggested that colonies of *G. fascicularis* may reproduce asexually by fragmentation in addition to sexual reproduction (Hidaka and Yamazato, 1982). It is also possible that allogeneic polyps can fuse with each other when genetic difference is very small, contrary to the "uniqueness of the individual principle" proposed by Hildemann *et al.*, (1977a,

1980). Recently, Willis and Ayre (1985) observed one fusion of electrophoretically different colonies of *Pavona cactus*.

It is interesting that some pairs survived for five months without suffering any damage from the neighboring polyp even though both polyps had developed many sweeper tentacles. It is probable that some process of habituation occurs and allows allogeneic polyps to survive side by side without suffering any damage. Such a habituation process may occur in the sea anemones *Metridium senile* (Purcell and Kitting, 1982) and *Anthopleura xanthogrammica* (Sebens, 1984). Purcell and Kitting (1982) suggested that habituation is a major factor that allows different clones of sea anemones to live intermingled with each other without interclonal aggression.

The present results strongly suggest that the nematocyst discharge response has an important role in eliciting formation of sweeper tentacles and that self and non-self are more finely recognized in the histoincompatibility reaction than in the nematocyst discharge response.

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NON-RANDOM, SEASONAL OSCILLATIONS IN THE ORIENTATION  
AND LOCOMOTOR ACTIVITY OF SEA CATFISH (*ARIUS FELIS*)  
IN A MULTIPLE-CHOICE SITUATION

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ABSTRACT

A quantitative analysis of angular orientation and locomotor activity of 80 sea catfish (*Arius felis*) over 12 consecutive months, under controlled conditions of photoperiod, temperature, and water quality, revealed non-random oscillations in the monthly mean orientation vectors and monthly mean activity of the experimental population. Marquardt modeling of monthly mean activity and of the sine and cosine components of monthly mean orientation indicated significant annual, bimodal cycles for all three variables. These cycles correlated with the observed, seasonal inshore-offshore migrations of *A. felis*, as documented in the literature, and had significant periods of 11.40, 5.11, and 6.64 months for the sine and cosine components of orientation and for activity, respectively. Photoperiod alone apparently acted as the exogenous cue triggering these cyclic changes in orientation and activity.

INTRODUCTION

Seasonal changes in activity patterns have been documented for a number of fishes, e.g., brook trout, *Salvelinus fontinalis* (Eriksson, 1972), the sculpins *Cottus gobio* and *C. poecilopus* (Andreasson, 1973), and burbot, *Lota lota* (Müller, 1973). Brook trout are day-active all year, except for a “desynchronized” interval in summer. The sculpins and burbot are day-active in winter and night-active in summer. Andreasson (1973) suggested that two separate oscillators, one “light-active” and one “dark-active,” controlled activity rhythms in these fishes, with seasonal changes in the phase angle between the two proposed oscillators (entrained by exogenous factors) triggering the observed changes in activity patterns. Although these investigators discounted any temperature effect on seasonal activity patterns, Byrne (1968) found that a photoperiod-temperature interaction produced changes in activity patterns of juvenile sockeye salmon, *Oncorhynchus nerka*. In addition, Goodlad *et al.* (1974) demonstrated that, for their study area (four lakes in the Fraser River system), the entrance times of salmon fry into nursery areas were related to seasonal patterns of water temperature (and other factors).

Similarly, the patterns of migration and movements of fishes have been investigated by numerous individuals, several of whom have suggested mechanisms directing the observed behavior. For example, it has been suggested that orientation during migration is associated with various physical, chemical, and biological environmental “clues” (as defined by Harden Jones, 1984a), e.g., direction-resolvable acceleration from ocean swell (Cook, 1984); direction-resolvable Langmuir circulations (Barstow, 1983; Leibovich, 1983); geomagnetism (Quinn, 1982; Walker, 1984); enviroregulation (Neill, 1984); tidal and lunar cycles (Arnold and Cook, 1984;

Pfeiler, 1984); temperature gradients (Dodson and Dohse, 1984; McCleave and Kleckner, in press); and, see the review by Hasler and Scholz (1983).

Beverton and Holt (1957) suggested the theoretical concepts of diffusion and oriented dispersion to explain observed migrations and movements, but these concepts have been applied to very few field results (Saila, 1961). In the Beverton and Holt concept, such "diasporic migration" [a term originally used by Wilkinson (1952) to describe the reassembly of birds dispersed over a large territory back to a breeding zone] is considered satisfactory to explain most examples of fish migration. Beverton and Holt (1957) further postulated that current direction is an orientation clue for migration by marine fishes. Saila (1961) further developed the concept of diasporic migration to explain the annual inshore-offshore migrations of winter flounder, *Pleuronectes americanus*. He did not include a "guiding factor" for direction in this model, and considered the observed "open-water" migration a result of "random search," insofar as fish relocating the coast was concerned. The "homing" phase to specific spawning sites was considered to occur by other means. Saila and Shappy (1963) then formalized this concept of random search into a mathematical model for describing the "open-ocean" phase of salmon migration in returning fish to coastal areas. This random search model (a numerical probability model based on Monte Carlo simulation techniques) was combined with a "low degree" of orientation to an (unspecified) outside stimulus source to produce a "small bias" in the directional orientation of the fish toward their natal coast. The assumptions of the model are oversimplified, and no mechanism for producing the preferred bias in orientation is suggested. However, the random walk model formulated by Saila and Shappy (1963) resulted in a greater return probability of salmon (using only random movement combined with a "small" preferred directional bias) in its simulations than is observed in tag returns. These results indicate that neither the capability for bicoordinate (or, celestial) navigation, nor an extremely precise directional orientation are necessary to insure "successful" migration by fishes. This hypothesis has been supported by Able (1980), Harden Jones (1984a), Leggett (1984), and McCleave and Kleckner (in press).

However, Quinn and Groot (1984a) questioned the correctness of this hypothesis in their criticism of the model formulated by Saila and Shappy (1963). They present convincing evidence that at least three assumptions underlying the model's formulation are incorrect (*i.e.*, salmon swimming speed, duration of migration, and return success). These errors result in a significant under-estimation of the intensity of homeward orientation in migrating salmon. Recent evidence suggests a strong degree of directed orientation in the open-ocean migration of these fishes (Quinn, 1982, 1984; Quinn and Groot, 1984a). Field studies by Hasler *et al.* (1958, 1969) on the open-water orientation and homing of white bass, *Roccus chrysops*, provided earlier support for claims of precise, non-random orientation by fish. White bass were shown to make prolonged, oriented movements in open waters, although the exact mechanism underlying the orientation could not be determined. However, experimental studies on lake-migrating juvenile sockeye salmon have documented mechanisms of solar and magnetic compass orientation in this species (Brannon, 1972; Quinn, 1980; Quinn and Brannon, 1982).

Because a wide variety of animals display compass orientation or directional preferences in artificial testing arenas (Matis *et al.*, 1974; Quinn and Groot, 1984b), it is possible to investigate the mechanisms triggering (*i.e.*, "cues," as defined by Harden Jones, 1984a) and directing (*i.e.*, clues) biases in orientation of some species in the laboratory. For example, Hasler *et al.* (1958) conducted laboratory studies which demonstrated a sun-compass mechanism of orientation in white bass, bluegill

sunfish (*Lepomis macrochirus*), and pumpkinseed sunfish (*L. gibbosus*). Fish were first trained to take food or find shelter in a specific compass direction, using the sun as the only reference clue. Subsequent testing, at a different time of day, showed that the fish were able to compensate for the sun's movement, and orient in the trained direction.

Quantitative investigations of non-random changes in angular orientation of fish have been conducted with goldfish, *Carassius auratus*, by Matis *et al.* (1974, 1977). Their analyses of the monthly mean orientation vectors of 129 fish over a 41-month period (not entirely consecutive) revealed both long-term (monthly) and short-term (hourly) non-random oscillatory changes in the angular orientation of these fish. In addition, the population exhibited a significant long-term cycle (33.6 months) in the sine component of their orientation. However, these authors were unable to relate their findings to natural populations and attempts to provide a mechanism for cueing the observed "seasonal" changes in orientation were inconclusive. Although Matis *et al.* (1974) conjectured that periods of inactivity in the goldfish were related to oscillations in orientation, no quantitative analysis was attempted.

The present study was undertaken to examine quantitatively seasonal changes in both angular orientation and activity in a migratory species. The hardhead sea catfish, *Arius felis*, was selected as the experimental species for several reasons. Firstly, it has been utilized extensively in this laboratory as a bioassay model for examining the behavioral toxicology of copper to marine fishes (*e.g.*, Scarfe *et al.*, 1982; Steele, 1983). All such experiments consist of a control recording of the locomotion of a single, naive sea catfish for 24 h following their placement into a behavioral testing arena in which the locomotion was monitored prior to experimental manipulation with copper. Fish size, treatment prior to monitoring, and experimental conditions during monitoring were identical for all control periods of experiments covering a span of 12 consecutive months. Thus, the data, stored on computer disks, are suitable for a quantitative analysis of seasonal changes in orientation and activity of these fish.

Secondly, sea catfish are dark-active (nocturnal) year-round, although light-to-dark transitions apparently synchronize their diel activity patterns (Steele, 1984, 1985). Thus, a consideration of seasonal changes in diel activity as noted for brook trout (*S. fontinalis*), sculpins (*C. gobio* and *C. poecilopus*), and burbot (*L. lota*) is unnecessary (see Eriksson, 1972; Andreasson, 1973; Müller, 1973). Although photoperiod does produce short-term alterations in orientation patterns in sea catfish, the minimum day-length needed to trigger these alterations in angular orientation (19.2 h) is not considered to be frequently encountered in the fish's natural habitats, and certainly not along the Texas coast (Steele, 1984).

Finally, the natural history of *A. felis* is well-documented (*e.g.*, Henshall, 1895; Hubbs, 1936; Lee, 1937; Gunter, 1947; Ward, 1957) which facilitates relating observed changes in seasonal activity and orientation in the laboratory to the behavior of natural populations. Generally, sea catfish decrease in abundance in the bays and estuaries along the northern coast of the Gulf of Mexico during winter, except in southern Florida, and move to deeper waters offshore or to deep-water "holes" within an estuary system (Gunter, 1947; Gunter and Hall, 1965). This annual offshore migration occurs from approximately October through December in the Northern Gulf of Mexico, along the Texas coast (*e.g.*, Gunter, 1947; Ward, 1957; pers. obs.). According to Franks *et al.* (1972) this species becomes widely scattered during winter months after returning to the Gulf. Although present in small numbers year-round within estuary systems, sea catfish become quite "rare" over winter, but regain abundance during spring and summer as fish return to the

estuaries to spawn (Gunter, 1947; Ward, 1957; Franks *et al.*, 1972; Morgan, 1974; pers. obs.). Although fish begin returning to the estuaries as early as March (Gunter, 1947; Ward, 1957), the spawning season of sea catfish along the northern Gulf coast usually occurs from about the first week in May to as late as the first week in August (Ward, 1957). Observed changes in activity and angular orientation in the laboratory may be correlated with these observed annual inshore-offshore migrations and spawning periods of sea catfish.

## MATERIALS AND METHODS

### *Experimental procedure*

Eighty hardhead sea catfish, *Arius felis* (average total length  $274 \pm 13$  mm), were obtained either by hook and line or from a commercial supplier in Port Aransas, Texas, between July and September 1977. They were maintained for at least three months prior to experimentation in slate holding tanks containing filtered, recirculating artificial sea water (30‰ S; 21–24°C) and fed every third day with fresh-frozen shrimp, except during behavioral monitoring.

Fish were monitored individually for 24 h, each, in the behavioral monitoring system. All monitoring was conducted in constant darkness to reduce visual clues in the behavioral arena. The monitoring system used for data collection has been described previously in detail (Kleerekoper, 1977). Briefly, it consists of a cylindrical steel rosette tank (210 cm diameter) with double walls forming a peripheral channel. The periphery of the interior is incompletely partitioned into 16 radial compartments and an open central area (110 cm diameter) by hollow wooden dividers. The system operates on the principle of multiple choice in which there is equal access to every compartment. Alternate dividers contain either light sources ( $705 \pm 35$  nm) or photodiode arrays, which together form photoelectric "gates" at the entrance to each compartment. Data are collected during an experiment when a fish, on entering or leaving a compartment, activates the photoelectric gate. This "event" is then recorded (as time and compartment identity) via a logic interface onto a high-speed paper-tape punch. Locomotor activity is then quantified on a computer by the total number of events per experiment.

Additionally, the orientation vector (turning angle) of each successive movement between compartments, defined as the angle between the compartments of origin and of destination, is also determined. Each compartment covers an arc of  $22.5^\circ$ , therefore orientation vectors range from  $22.5^\circ$  to  $360^\circ$  at  $22.5^\circ$  intervals, with  $180^\circ$  indicating a straight path across the monitor tank and  $360^\circ$  indicating a return to the compartment immediately vacated. Computer analysis thus produces a time series of absolute orientation vectors of a fish's movements.

### *Treatment of data*

For each fish a frequency distribution of the orientation vectors over an entire 24-h monitoring period was constructed. Since angular resolution is limited to  $22.5^\circ$  intervals, due to tank design, orientation vectors were grouped into  $22.5^\circ$  categories. Table I lists such data for a representative fish.

Prior to analytical modeling of the monthly time series of orientation vectors, it must first be determined whether the series is non-random (*i.e.*, oscillatory). The determination of non-randomness is of prime importance here. Data was first analyzed for each fish to determine the monthly mean orientation vector ( $\bar{\theta}$ ) and the intensity of this mean vector ( $r_v$ ). Individual data was then pooled for all fish

TABLE I

Frequency distribution of orientation angles for a representative sea catfish, *Arius felis* (Expt. No. 938, June 1978)\*

Interval midpoint	Frequency	Interval midpoint	Frequency
0 (360)°	88	180°	633
22.5°	84	202.5°	699
45.0°	134	225.0°	516
67.5°	157	247.5°	506
90.0°	133	270.0°	286
112.5°	145	292.5°	131
135.0°	271	315.0°	124
157.5°	351	337.5°	49

\* Mean vector:  $\bar{\theta} = 201^{\circ}27'$ ; Vector intensity:  $r_v = 0.4753$ .

monitored in the same month, and a group monthly mean  $\bar{\theta}$  and  $r_v$  were calculated. Because orientation data is circular in nature, simple arithmetic means could not be used to determine monthly mean vectors. Analyses were conducted according to the procedures of Batschelet (1965, 1972, 1978, 1981) for handling orientation data. Monthly mean vectors were calculated using the equation,

$$\bar{\theta} = \tan^{-1}(\bar{Y}/\bar{X}) \quad (1)$$

where  $\bar{X}$  and  $\bar{Y}$  are the set of mean rectangular coordinates of orientation for a data set. These are also termed the (weighted) sines and cosines since,

$$\bar{X} = r \cos \theta \quad (2)$$

and,

$$\bar{Y} = r \sin \theta \quad (3)$$

Operationally,  $\bar{X}$  and  $\bar{Y}$  are determined using equations (4) and (5),

$$\bar{X} = (\sum w_i x_i) / \sum w_i \quad (4)$$

$$\bar{Y} = (\sum w_i y_i) / \sum w_i \quad (5)$$

where  $x_i$  and  $y_i$  are the cosines and sines, respectively, of each orientation vector category. The "weights" for each sine and cosine,  $w_i$ , are simply the frequency of occurrence of each orientation vector category within a data set (see Table I). The intensity ( $r_v$ ) of each monthly mean vector was then calculated using,

$$r_v = (\bar{X}^2 + \bar{Y}^2)^{1/2} \quad (6)$$

Second-order statistical analysis of the directional data thus determined for each fish was performed using Hotelling's one-sample test (Hotelling, 1931), as detailed by Batschelet (1978). Rejection of the null hypothesis indicates a concentration of the individual vectors around a mean direction (vector). In this instance, Hotelling's test was performed on each monthly set of individual orientation vectors. Calculation of each monthly test statistic ( $F_{2,n-2}$ ) was performed without the use of a confidence ellipse (see Batschelet, 1978).

Two tests have been found to be statistically powerful for detecting non-randomness in orientation data (Matis *et al.*, 1974) and will be described. The Spearman rank correlation test (Siegel, 1956; Mendenhall, 1975) determines a rank correlation between time (*i.e.*, the month) and the magnitude of the  $\bar{X}$  and  $\bar{Y}$

components of each monthly mean orientation vector ( $\bar{\theta}$ ). The null hypothesis proposes that time and magnitude are linearly uncorrelated, while the alternative hypothesis indicates either an increasing or decreasing linear correlation. The correlation coefficient,  $r_s$ , of a series is determined and its significance tested by exact theory.

Randomness in a series is tested by the Wald and Wolfowitz serial correlation test (Wald and Wolfowitz, 1943). The serial correlation coefficient of lag 1,  $R_1$ , measures dependency between consecutive observations and is tested for significance by approximate theory. The null hypothesis states that successive observations are independent. The alternative hypothesis indicates regularity (predictability) in observed fluctuations in the data. These same two statistical tests were also used to search for non-randomness in the monthly mean activity of all fish monitored in a given month.

Together, the Spearman rank correlation test and the Wald and Wolfowitz serial correlation test classify a time series as follows. If the Spearman rank correlation test does not reject the null hypothesis, the significance or non-significance of the serial correlation test indicates either oscillation or random fluctuations in the data, respectively. If, however, linear correlation is indicated, the strength of this correlation is indicated by the serial correlation test ( $H_0$ : weak linear correlation). Figure 1 is a decision-making flow chart which summarizes the above procedures.

If oscillation was found in a data set, the  $\bar{X}$  and  $\bar{Y}$  components (the rectangular coordinates) of each monthly mean orientation vector were plotted for each fish for each month. Both the  $\bar{X}$  and  $\bar{Y}$  components were then analyzed for periodicity over time according to the cyclical models,

$$\bar{Y}_t = a_1 + A_1 \cos(c_1 t + \Omega_1) \quad (7)$$

$$\bar{X}_t = a_2 + A_2 \cos(c_2 t + \Omega_2) \quad (8)$$

where  $t$  is time (in months) and the parameters  $a_i$ ,  $A_i$ ,  $c_i$  and  $\Omega_i$  represent the mean level, the semi-amplitude, the frequency and the phase angle parameters, respectively (Bliss, 1970; Matis *et al.*, 1977). A similar model was derived to analyze monthly mean activity and is given by,

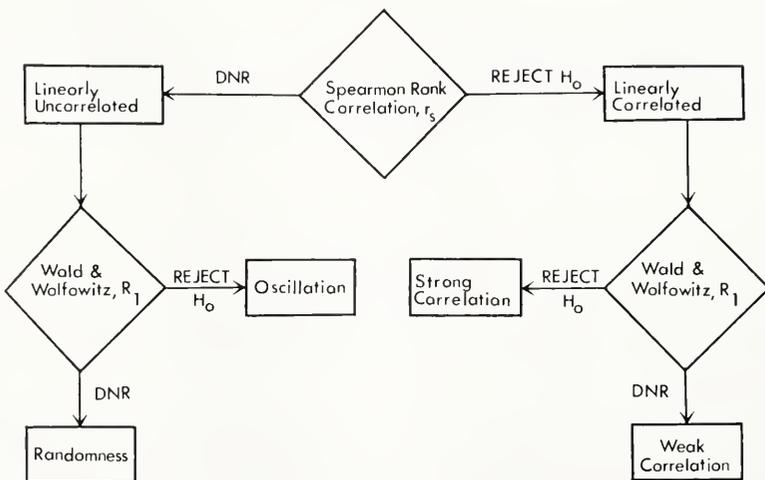


FIGURE 1. Decision-making flow chart used in determining if non-randomness exists in the data. See text for discussion of the statistical tests.

$$\bar{Y}_{\text{act}} = a_3 + A_3 \cos(c_3t + \Omega_3) \quad (9)$$

where  $\bar{Y}_{\text{act}}$  is used as conventional regression notation and does not indicate a component of rectangular coordinates.

These models were fitted to the data by the Marquardt technique of nonlinear least squares (Marquardt, 1963; and see Draper and Smith, 1981; Matis *et al.*, 1977), which generates combinations of the parameters ( $a_i$ ,  $A_i$ ,  $c_i$  and  $\Omega_i$ ) to minimize the error sum of squares (SSE). Although the theory of nonlinear least squares does not guarantee that a given set of parameters which produces the smallest local minima on the SSE hypersurface represents the absolute minimum of the SSE, those parameter sets which do will be considered the "best-fitting" cycle for each model, respectively.

## RESULTS

The monthly mean orientation vectors ( $\bar{\theta}$ ) and their intensities ( $r_v$ ) are given in Table II and illustrated in Figure 2 (which also contains the individual data). The

TABLE II

*Composite monthly mean vector, vector intensity, and monthly mean activity for sea catfish, Arius felis*

Month <sup>a</sup>	Monthly code (m)	N	Total no. observations in mean vector	Total no. hours of recording	Angle ( $\bar{\theta}$ ) of mean vector	Intensity ( $r_v$ ) of mean vector	Monthly mean activity <sup>b</sup> ( $\pm$ S.D.)
Jan	0	6	28,688	144	189°57'	0.4222	4778 (648.55)
Feb	1	7	45,269	168	195°55'	0.3346	6467 (753.91)
Mar	2	7	52,080	168	266°22'	0.2952	7440 (749.43)
Apr	3	6	37,590	144	257°05'	0.0709	6265 (531.77)
May	4	6	29,544	144	215°15'	0.0580	4924 (708.15)
June	5	8	33,440	192	181°10'	0.3387	4180 (567.65)
July	6	6	26,268	144	149°25'	0.0284	4378 (636.82)
Aug	7	6	34,392	144	119°14'	0.2302	5732 (766.14)
Sep	8	7	56,679	168	94°22'	0.1837	8097 (1713.91)
Oct	9	8	57,344	192	88°14'	0.1996	7168 (694.95)
Nov	10	6	39,882	144	179°49'	0.3229	6647 (850.51)
Dec	11	7	35,021	168	183°55'	0.3304	5003 (772.91)

<sup>a</sup> All months in 1978.

<sup>b</sup> Activity quantified as the number of entries into compartments in the behavioral monitoring arena per 24 h.

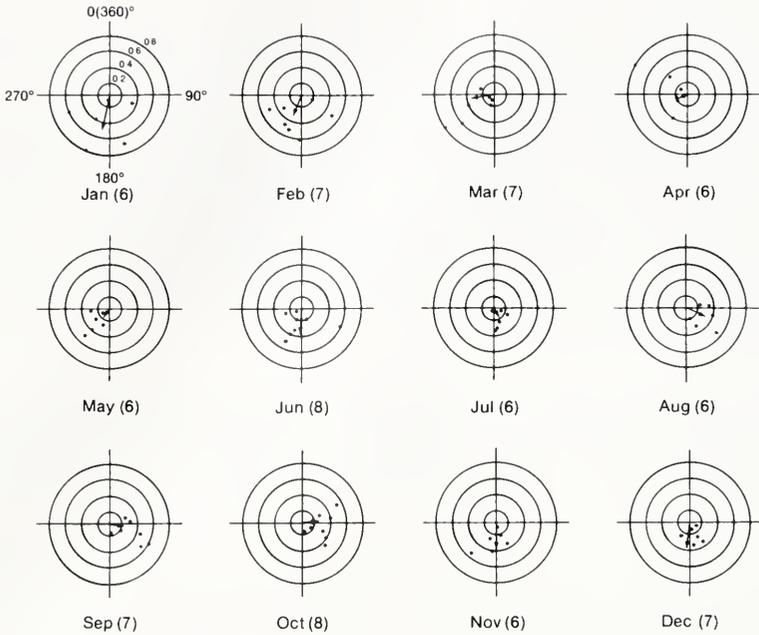


FIGURE 2. Monthly mean orientation vectors from January 1978 to December 1978 for 80 sea catfish (*Arius felis*). Number of fish in parentheses; vectors of individual fish are plotted in each month.

weighted sines ( $\bar{Y}_t$ ) and cosines ( $\bar{X}_t$ ) of the monthly mean vectors are plotted in Figures 3 and 4, respectively. Monthly mean activities are also listed in Table II and are plotted in Figure 5. Table III contains the results of Hotelling's test on the individual orientation data for each month. The individual vectors are significantly concentrated around the monthly mean vector for each month except January.

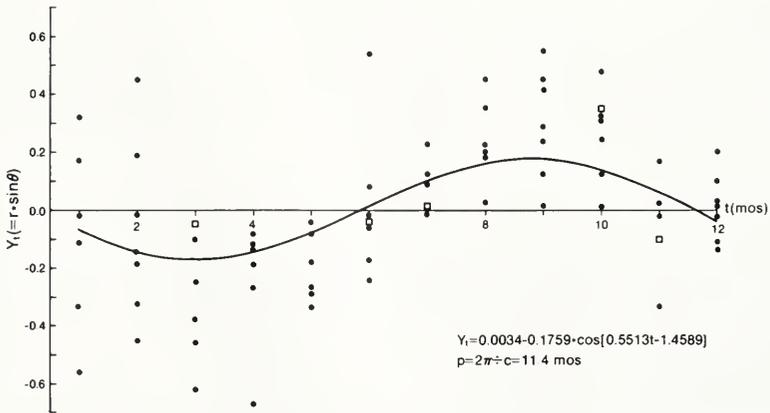


FIGURE 3. Observed and fitted values of sine,  $Y_t$ , of monthly mean orientation vectors for 80 sea catfish (*Arius felis*) plotted against month,  $t$ , of observation. Parameters of the cyclical model used to fit the data (mean level, semi-amplitude, frequency, and phase angle, respectively) and period of oscillation are also given. Open squares indicate more than one data point.

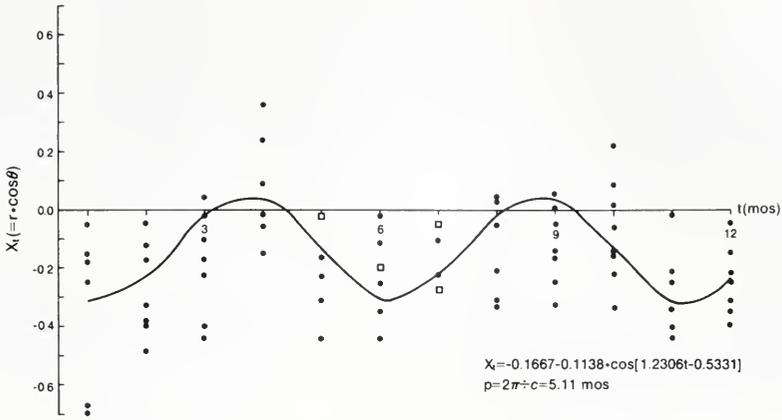


FIGURE 4. Observed and fitted values of cosine,  $X_t$ , of monthly mean orientation vectors for 80 sea catfish (*Arius felis*) plotted against month,  $t$ , of observation. Parameters of the cyclical model used to fit the data (mean level, semi-amplitude, frequency, and phase angle, respectively) and period of oscillation are also given. Open squares indicate more than one data point.

The apparent tendency of the monthly mean vectors of sea catfish to oscillate non-randomly with time (Fig. 2) was confirmed by the results of the Spearman rank correlation and the Wald and Wolfowitz serial correlation tests performed on the rectangular coordinates (*i.e.*, the weighted sines and cosines) of the vectors. Neither the time series of sines ( $r_s = -0.478$ ) nor cosines ( $r_s = 0.098$ ) reject the null hypothesis, indicating nonlinearity in the data ( $r_{s12,0.05} = \pm 0.591$ ). The subsequent serial correlation tests indicate significant oscillation ( $R_{I12,0.01} = \pm 0.780$ ), with  $R_I = 0.865$  and  $R_I = 0.830$  for  $\bar{Y}_1$  and  $\bar{X}_1$ , respectively. Similar statistical results confirm non-random oscillation in the observed monthly mean activity of the fish with  $r_s = 0.196$  and  $R_I = 0.889$ .

Table IV lists certain parameter combinations which yield minimal SSE for the cyclical models of the  $\bar{Y}_1$  and  $\bar{X}_1$  components of the monthly mean orientation vectors and for the cyclical model of monthly mean activity of sea catfish. Although

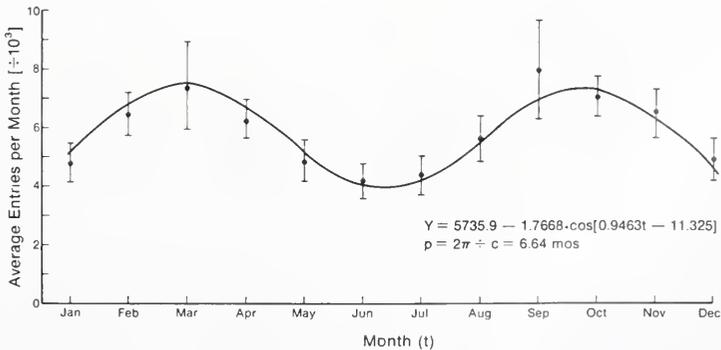


FIGURE 5. Observed and fitted values of monthly mean activity,  $Y_{act}$ , of 80 sea catfish (*Arius felis*) plotted against month of observation,  $t$ . Parameters of the cyclical model used to fit the data (mean level, semi-amplitude, frequency, and phase angle, respectively) and period of oscillation are also given. Numbers of fish observed each month are given in Table II.

TABLE III

Results of Hotelling's second-order test on the individual orientation vectors per month for sea catfish, *Arius felis*

Month	N	F <sub>2,n-2</sub>	Month	N	F <sub>2,n-2</sub>
Jan	6	3.12 <sup>NS</sup>	July	6	12.27 <sup>a</sup>
Feb	7	9.01 <sup>a</sup>	Aug	6	7.92 <sup>a</sup>
Mar	7	5.94 <sup>a</sup>	Sep	7	9.39 <sup>a</sup>
Apr	6	6.95 <sup>a</sup>	Oct	8	10.52 <sup>a</sup>
May	6	7.21 <sup>a</sup>	Nov	6	7.83 <sup>a</sup>
June	8	10.70 <sup>a</sup>	Dec	7	15.08 <sup>b</sup>

NS = Not Significant; <sup>a</sup>P ≤ 0.05; <sup>b</sup>P ≤ 0.01. See Mendenhall (1975) for critical F-values for each value of F<sub>2,n-2</sub> (or any comparable statistics text).

three sets of parameters are listed for each model for comparison, following the decision criteria described, the parameter sets producing the smallest residual SSE for each model were designated "best-fitting," and are the only ones plotted in Figures 3-5 and further discussed. The total (corrected) sums of squares for  $\bar{Y}_t$ ,  $\bar{X}_t$ , and  $\bar{Y}_{act}$  are 5.70599, 2.84663, and 4.18387, respectively. Thus, the "best" model for  $\bar{Y}_t$  (frequency = 0.5513) is significant (P ≤ 0.001) with F<sub>4,76</sub> = 14.98; that for  $\bar{X}_t$  (frequency = 1.2306) is also highly significant (P ≤ 0.001) with F<sub>4,76</sub> = 26.04;

TABLE IV

Parameter combinations (± asymptotic standard error for the "best-fitting" model) yielding local minima on the SSE hypersurface

A. Sine component of orientation, Y <sub>t</sub>					
a <sub>1</sub>	A <sub>1</sub>	c <sub>1</sub>	Ω <sub>1</sub>	SSE	F-value <sup>a</sup>
0.00340	-0.17588	0.55132	-1.14589	3.19402	14.98
(±0.02170)	(±0.03130)	(±0.04690)	(±0.32594)		
-0.00261	-0.15026	0.54390	-1.02940	3.53778	
-0.02176	-0.09426	0.43086	-0.37075	4.20367	
B. Cosine component of orientation, X <sub>t</sub>					
a <sub>2</sub>	A <sub>2</sub>	c <sub>2</sub>	Ω <sub>2</sub>	SSE	F-value <sup>a</sup>
-0.16673	-0.11378	1.23058	-0.53312	2.31088	26.04
(±0.02023)	(±0.02745)	(±0.08022)	(±0.50694)		
-0.16362	-0.11076	1.27339	-0.84907	2.33175	
-0.16891	-0.10535	1.17558	-0.13387	2.33984	
C. Activity, Y <sub>act</sub>					
a <sub>3</sub>	A <sub>3</sub>	c <sub>3</sub>	Ω <sub>3</sub>	SSE	F-value <sup>b</sup>
5.73587	-1.76680	0.94632	-11.32450	1.75769	4.97
(±0.14791)	(±0.20497)	(±0.03027)	(±0.19310)		
5.74040	-1.70508	0.94323	-11.33090	1.79394	
5.73078	-1.61667	0.98327	-11.30412	3.03329	

<sup>a</sup> Significant at P ≤ 0.001; <sup>b</sup>significant at P ≤ 0.005.

and, the "best" model for  $\bar{Y}_{act}$  (frequency = 0.9463) is highly significant ( $P \leq 0.005$ ) with  $F_{4,76} = 4.08$ . The period of oscillation for each "best-fitting" model is determined using the frequency,  $c_i$ , such that  $2\pi/c_i =$  the period. The periods of oscillation, along with their asymptotic 95% confidence intervals, are (in months) 11.40 (9.53, 14.17); 5.11 (4.52, 5.86); and 6.64 (6.18, 7.17) for  $\bar{Y}_t$ ,  $\bar{X}_t$ , and  $\bar{Y}_{act}$ , respectively. The asymptotic standard errors for the frequencies are 0.04690 ( $\bar{Y}_t$ ), 0.08022 ( $\bar{X}_t$ ) and 0.03027 ( $\bar{Y}_{act}$ ).

## DISCUSSION

Outstanding among these results is the unambiguous existence of non-random, seasonal oscillations in both the sine and cosine components of monthly mean angular orientation and the monthly mean activity of sea catfish, *Arius felis*. These cycles had significant periods of oscillation of 11.40, 5.11, and 6.64 months, respectively. These significant oscillations were exhibited by fish exposed only to the natural, local photoperiod, but otherwise maintained and monitored in a homogeneous laboratory environment. The existence of such a (seasonal) time-compensated sun compass [or, sun-orientation rhythm (Hasler and Schwassman, 1960)] is indicative of a circadian neural mechanism influencing behavior (Meirer and Fivizzani, 1980). Time-compensating sun orientation has been demonstrated for many fishes (see Quinn, 1984).

In addition, since all behavioral monitoring was conducted in constant darkness, these observed oscillations in angular orientation and activity indicate the existence of an endogenous oscillator(s) (presumably entrained by the exogenous photoperiod) which triggers non-random cycles of directional biases in monthly mean angular orientation and non-random cycles in monthly mean activity of these fish which persist for at least 24 h without reference to the entraining photoperiod. This would be of importance to the fish during migration should they swim below the photic zone, at night, or on overcast days. The distributions of orientation angles and activity of sea catfish monitored for 24 h in constant darkness have previously been shown to be not statistically different from fish similarly monitored under an LD 12:12 photoperiod approximating the local photoperiod (Steele, 1984). This persistence of the seasonal activity and orientation rhythms following removal of the environmental cue (*i.e.*, the sun) is a recognized characteristic of endogenous rhythms (*e.g.*, Schwassman, 1960).

For all three cyclical models the oscillations had annual, bimodal distributions which can be roughly correlated with the observed return migration of sea catfish to estuaries for spawning [from as early as March to as late as August (Gunter, 1947; Ward, 1957)] and their subsequent migration to offshore waters from approximately October through December (Gunter, 1947; Ward, 1957; pers. obs.). The sine ( $\bar{Y}_t$ ) component of monthly mean orientation had a relative minima in its cycle during March and a relative maxima during September (Fig. 3). The cycle for the cosine component ( $\bar{X}_t$ ) exhibited relative maxima during March-April and August-September. Relative minima occurred during June and November (Fig. 4). Monthly mean activity (Fig. 5) peaked during March and September-October, with minimal activity occurring during June-July (when the fish would be, presumably, in the estuaries) and December-January (when the offshore migration has presumably ended). March has been documented as the earliest month of the inshore spawning migration of sea catfish, with October through December approximately encompassing the period of offshore migration (*e.g.*, Gunter, 1947; Ward, 1957). More precise

correlation of the behavior of the experimental population with field data is not possible for a number of reasons.

Given the wide range in dates of observed migration, and the extended period during which fish were collected (July through September) it is possible that sea catfish from a number of "subgroups" making the annual inshore-offshore migration at different times during the season are represented. Such a situation may account, at least in part, for the lack of significant correlation between monthly mean activity and monthly mean vector intensity ( $r_s = -0.1468$ ). Intuitively, these parameters should have a significant positive correlation, especially if they are related to migration. Also, the presence of fish from different spawning groups may explain the sometimes "wide" range seen in monthly individual orientation data (Figs. 2-4). This may also be compounded by the relatively small monthly sample sizes.

Additionally, the absence in this laboratory environment of other environmental cues for triggering the migratory response and clues for its subsequent direction which the fish may normally use could also interfere with precise correlation of the observed laboratory behavior with field data. Other laboratory factors which may also inhibit the complete expression of "natural" migratory orientation and activity include the relatively confined spaces of the behavioral monitoring tank and holding tanks (thus severely limiting the fishes' progression in a "preferred" direction) and the lack of social facilitation during behavioral monitoring. Although the fish were maintained in community holding tanks, the behavioral data were collected from individuals because of design limitations of the data acquisition apparatus. Sea catfish migrate in schools (*e.g.*, Gunter, 1947; Ward, 1957). Lack of social facilitation, as well as the absence of other environmental cues and clues which may normally be used, may be especially important if the fish collected were repeat migrants, and if learning is involved in the migratory process (*e.g.*, Bitterman, 1984).

Finally, the fish were transported approximately 300 miles north of their capture site (Port Aransas, lat.  $27^{\circ}50.0'$  N, long.  $97^{\circ}3.5'$  W; College Station, approximate lat.  $30^{\circ}19.6'$  N, approximate long.  $96^{\circ}16.5'$  W). Although changes in latitude (and, therefore, the sun's altitude) alter sun-orientation responses in fishes, the displacement distances reported are considerably greater than in this study (see Hasler and Schwassman, 1960; Schwassman, 1960; Schwassman and Hasler, 1964). The fish were maintained a minimum of three months in the laboratory prior to experimentation and, therefore, exposed to the local daily photoperiod for at least this time (longer if a fish was caught at the beginning of the collecting period and/or monitored later in the following year; no individual data is available on time of capture *versus* time of monitoring). How the change in location may have affected monthly mean orientation, or whether the experience with the local photoperiod was of sufficient duration to ameliorate any such effects could not be evaluated.

However, as seen in Figure 2, the individual monthly mean orientation vectors observed in the laboratory are appropriate for fish entering and leaving the estuary system in the vicinity of Port Aransas (roughly west and east, respectively, with undoubtedly some north-south component) at the corresponding times reported for the inshore-offshore migrations (*e.g.*, Gunter, 1947; Ward, 1957). Results of Hotelling's second-order test (Hotelling, 1931; Batschelet, 1978; Table III) indicate significant clumping of the individual vectors around each monthly mean vector, except during January [Franks *et al.* (1972) have reported that sea catfish become "widely dispersed" during the winter months.] Such maintenance of appropriate seasonal orientation (and activity) by the experimental population, especially when considering the above discussion of factors which may have adversely affected an appropriate response, is considered evidence that the seasonal migrations of sea catfish must be

strongly oriented and directed in the field, and do not proceed by a simple random-walk process.

Why some fishes possess endogenous rhythmicity is not yet completely understood (Goudie *et al.*, 1983). Numerous exogenous mechanisms have been implicated in cueing both activity and orientation of fishes, including photoperiod and light intensity (*e.g.*, Richardson and McCleave, 1972; Goudie *et al.*, 1983; Steele, 1984), temperature (*e.g.*, Byrne, 1968; Goodlad *et al.*, 1974; Neill, 1984), feeding and social behavior (Goudie *et al.*, 1983), tidal and lunar cycles, and currents (*e.g.*, Sails, 1961; Arnold and Cook, 1984; Pfeiler, 1984), etc. However, the results presented here suggest that photoperiod alone can act as an exogenous mechanism for entraining an endogenous oscillator(s) in cueing (*i.e.*, triggering) seasonal oscillations in angular orientation and activity of sea catfish. Indeed, previous work (Steele, 1984) indicates the existence of at least two distinct control mechanisms governing angular orientation and locomotion of sea catfish that are light regulated (directly or indirectly) either by the length of the photoperiod (angular orientation) or by the transition from light to darkness (diel locomotor activity). This is not to say, however, that sea catfish (and other fishes) do not use additional exogenous cues in their natural habitats to "fine tune" their migratory behavior. Additionally, orientation during migration need not be confined to a single sensory modality or system of clues (Adler, 1970; Leggett, 1977). Observed migration patterns in fishes are undoubtedly the result of complex summations of many environmental forces (*e.g.*, see Clark, 1925; Walker, 1952; Gerking, 1959; Goodlad *et al.*, 1974; Matis *et al.*, 1974, 1977; Meier and Fivizzani, 1980; Barstow, 1983; Arnold and Cook, 1984; Cook, 1984; Neill, 1984). However, photoperiod alone apparently triggered non-random, seasonal oscillations in angular orientation and activity in this experimental population of sea catfish. These oscillations seen in the laboratory correspond roughly to observed migratory patterns in the species, and could be caused by an entrained endogenous mechanism controlling the cueing of migration. Several studies have demonstrated that Pacific salmon (*Oncorhynchus* spp.) also select appropriate directional headings in the absence of rheotactic, thermal, olfactory, or salinity cues (see review by Quinn, 1984). Quinn (1982) proposed that the assessment of photoperiod (daylength, or rate of change in daylength) combines with an endogenous circannual rhythm in these salmon to provide a "calendar sense." Such a mechanism could also be operating in sea catfish.

The exact nature of this (hypothesized) endogenous cueing of angular orientation and activity in migrations of sea catfish (and other fishes) remains unknown but must have an underlying physiological basis. Physiological involvement of the pineal organ in seasonal reproduction (depending on season, photoperiod and/or temperature) has been demonstrated in some fishes (*e.g.*, Fenwick, 1970; De Vlaming, 1975; De Vlaming and Vodcicnik, 1978). Although relatively few studies examining seasonal changes in the pineal gland of fish have been reported (see Vivien-Roels *et al.*, 1979), there are indications of seasonal functional variations in the biochemical activity of the pineal (see Vivien-Roels, 1981), and of pineal involvement in regulating seasonal rhythms in fishes (*e.g.*, Morita, 1966; Eriksson, 1972; Kavaliers, 1980). Melatonin synthesis by the teleostean pineal gland is a well-documented nocturnal event (Gern *et al.*, 1981). It has been hypothesized that melatonin may provide a "template" to provide timing information for maintenance of entrained diel, circadian and circannual cycles (Gern *et al.*, 1981; Steele, 1984). Melatonin may act either directly or indirectly to influence biological rhythms, perhaps through the actions of serotonin, a well-known neurotransmitter, which is an intermediate in the synthesis of melatonin (Martin, 1978; Norris, 1980) and for which

non-random, seasonal oscillations in content in the pineal complex and lateral eye of lamprey, *Lampetra planeri*, has been demonstrated (Vivien-Roels and Meiniel, 1983).

From the results of this study, however, it is not possible to assert a physiological role for the pineal complex in cueing the observed non-random, seasonal oscillations in angular orientation and locomotor activity of sea catfish, nor their migratory behavior in the field. However, the results presented demonstrate that exposure of the experimental population to daily fluctuations in the natural photoperiod alone is sufficient to trigger seasonal cycles in the orientation and activity of the fish, which, combined with information in the literature (*e.g.*, Fenwick, 1970; De Vlaming, 1975; De Vlaming and Vodcicnik, 1978; Gern *et al.*, 1981; Goudie *et al.*, 1983) supports such an hypothesis. Additional experimental data on the relative roles of photoperiod and other environmental factors on pineal activity and its interactions with hormones involved in the reproductive endocrinology of fishes in general (*e.g.*, Donaldson, 1973) and sea catfish in particular are needed before a complete explanation of the observations is possible.

To state that the observed maintenance of seasonally appropriate compass headings by sea catfish monitored in constant darkness is characteristic of an endogenous rhythm is not particularly instructive in understanding the mechanism involved in directing this orientation. However, the guidance mechanism of oceanic, or open-water, orientation and navigation during migration is still not understood, and a matter of considerable controversy (Leggett, 1977, 1984; Quinn, 1984; Quinn and Groot, 1984a). Many hypothetical mechanisms have been proposed, some of which are applicable to this study, while others are not. For example, the random walk hypothesis (Saila and Shappy, 1963; Leggett, 1984) appears insufficient (as discussed above) to explain the observed seasonal cycles in orientation, as are the hypothesized guidance mechanisms of bicoordinate navigation and magnetic-, electroreception (see Quinn, 1982, 1984). The confines of the behavioral arena limit the movement of fish to 210 cm in any direction. This distance is certainly insufficient for bicoordinate navigation to be operating, or to cross the necessary lines of declination required if magnetic guidance was being used (see Quinn, 1982; Leggett, 1984). Also, the double-walled steel of the monitoring tank would certainly disrupt any "normal" electromagnetic patterns. The effects of other environmental clues potentially useful in directing movement (*e.g.*, thermal, salinity, or olfactory gradients, currents, wave swell, etc.) and the influence of prior learning during migration could not be evaluated.

The underlying mechanism being used by the fish for directing the observed cycles in orientation (at least in this laboratory situation, *i.e.*, in constant darkness and the absence of environmental clues) may be time-compensating sun-compass orientation and/or inertial guidance. A number of fish species have been shown to possess a sun-compass mechanism for orientation (*e.g.*, see Hasler *et al.*, 1958, 1969; Quinn, 1984). The results of this study indicate that photoperiod entrains an endogenous oscillator to cue the observed seasonal cycles in orientation and activity. The fish may also be using the sun to maintain a preferred direction once selected. If so, the 24 h in constant darkness was insufficient to uncouple the "internal clock" of the circadian rhythm by which a sun-compass mechanism operates (Schwassman, 1960; Adler, 1970). Thus, time-compensated movements relative to the "learned" rate of change of daylength (and, therefore, the sun's position) could be used to maintain the appropriate compass heading. Inertial guidance (*i.e.*, angle compensation), for which there is some evidence in fishes, cannot, however, be ignored. Kleerekoper *et al.* (1969, 1970) provided evidence of an inertial guidance system in

the orientation of goldfish (*Carassius auratus*) monitored in a laboratory situation. Recently, Harden Jones (1984a, b) presented field data suggesting the use of inertial clues by migrating plaice (*Pleuronectes platessa*). Harden Jones hypothesized that the labyrinth could provide the basis for an inertial guidance system, especially if the selected heading could be continually corrected by reference to external clues. However, Kleerekoper *et al.* (1969, 1970) maintained that a direction of progression (orientation), once established, could be maintained by continuous compensation of turning angles in the absence of directional clues, as is the case here.

Whether either, or both, of these proposed systems of directional guidance are actually used by sea catfish to maintain a selected compass heading in this laboratory environment (or, in the field) cannot, of course, be asserted without reservation. Too little is known about the clues used for orientation and concomitant movement by this species under natural conditions. It is also unknown whether sun-compass orientation and/or inertial guidance could adequately direct orientation in sea catfish for longer than 24 h without reference to external clues. However, given the earlier discussion of proposed directing mechanisms, and the results of this study, time-compensating sun-compass orientation and/or a system of inertial guidance (angle compensation) appear to be the only appropriate mechanisms which could be directing the orientation of the fish in this situation.

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## MULTIPLE SPAWNING AND MOLT SYNCHRONY IN A FREE SPAWNING SHRIMP (*SICYONIA INGENTIS*: PENAEOIDEA)

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### ABSTRACT

Field sampling was conducted to examine patterns of molt and reproductive activity in populations of the free spawning, penaeoid prawn, *Sicyonia ingentis*; some of these patterns were also verified by laboratory studies. Multiple spawning in the field was inferred from the high frequency of prominent oocyte features which are termed cortical specializations. In a prior laboratory study (Anderson *et al.*, 1984), the time from cortical specialization stage to spawning was determined to be less than one week.

Molt studies showed that females began a synchronous molt cycle in June which was not completed until late October or early November, after the spawning season was over. Males exhibited similar synchrony, but the period was shorter, and there was more variability. Data indicate that not only the absolute duration of the molt cycle varied with season, but the relative duration of molt cycle stages also varied. December and January were the only months of the year in which the relative frequency of molt cycle stages approximated literature values.

Potential multiple spawning throughout the summer, coupled with almost no molting in the summer, suggest that multiple spawning occurred without intervening molt or mating in field populations. This was substantiated by laboratory studies which directly demonstrated multiple spawning by these prawns within a single, prolonged molt cycle.

### INTRODUCTION

The relationships between molt and reproduction in the Crustacea have been studied for decades (Adiyodi and Adiyodi, 1970). Both proximate and ultimate factors which may potentially determine molt and reproductive patterns have been considered. The proximate endocrine factors have received the most attention (Adiyodi and Adiyodi, 1970; Kleinholz and Keller, 1979, for reviews). However, the roles of ultimate ecological forces and environmental variables have also been examined (*e.g.*, Aiken, 1969; Cheung, 1969; Reaka, 1976; Steel, 1980; Webster, 1982; Nelson *et al.*, 1983; Quackenbush and Herrnkind, 1983).

Much research has been done on molt and reproduction in the Crustacea, but most of this work has been conducted on brooding species. Remarkably, relationships between molt and reproduction in free spawning crustaceans have not been well characterized. The most frequently studied free spawners are the prawns of the families Penaeidae and Sicyoniidae, although all members of the superfamily Penaeoidea (suborder Dendrobranchiata) exhibit this characteristic (for recent tax-

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Abbreviations: GSI, gonadosomatic index; CL, carapace length; CS, Cortical specializations.

onomic revisions see Bowman and Abele, 1982). In the Penaeoidea, sperm are transferred by deposition of either an internal or external spermatophore and fertilization occurs at spawning.

The few studies (Emmerson, 1980; Penn, 1980; Crocos and Kerr, 1983) which have addressed the interrelationships of molt and reproduction in Penaeoidea have left two interesting questions unanswered. First, is multiple spawning within a single molt cycle possible in field populations, and second, what is the relative duration of molt cycle stages in field populations? Emmerson (1980) provides the only evidence that multiple spawning of both eyestalk-ablated and intact prawns can occur within a single molt cycle. Emmerson (1980) examined spawning induction in the laboratory but did not include an analysis of multiple spawning with respect to molt activity in the local field population. Crocos and Kerr (1983) state that there is no evidence that multiple spawning can occur within a single molt cycle in the field.

Knowledge of the timing of oocyte development is used in this work to study multiple spawning in field populations of the ridgeback prawn (*Sicyonia ingentis*: Sicyoniidae), an abundant benthic organism in the Southern California Bight. Anderson *et al.* (1984) employed an ovarian biopsy technique in the laboratory to determine the relative timing of postvitellogenic ovarian stages for *S. ingentis*. They found that after vitellogenesis, approximately four days are required to pass through the most advanced oocyte stages; that is from the time when the cortical specializations (CS) are fully formed until spawning.

To our knowledge, no one has followed the pattern of molt cycles and reproductive stages through time in any field population for the Penaeoidea. Emmerson (1980), however, provided information on the coincidence of the various molt stages with given ovarian stages in laboratory populations of *Penaeus indicus*, and Crocos and Kerr (1983) provided similar information for field populations of *P. merguensis*. This then, is the first study to examine the relative duration of molt cycle stages in the field for any of the Penaeoidea. Furthermore, these field observations on molt cycles and multiple spawning are verified in laboratory studies.

## MATERIALS AND METHODS

### *Animal collection and field sampling regime*

Field observations on reproduction and molting were made monthly for two years (15 November 1979 to 13 November 1981) and on an irregular basis during the spring and summer of 1982. The monthly sampling was conducted at one fixed station in the Santa Barbara channel off of Santa Barbara, California (Fig. 1). Commercial shrimp semiballoon trawl gear was used. Nets were approximately 30 m long and 7 m wide at the mouth. The mesh at the cod end was approximately 11.5 cm on the diagonal. The fixed station was located (Fig. 1) in 145 m of water approximately 25 km offshore. Random trawls were also made on some of the same dates as trawls at the fixed station (Fig. 1).

Monthly samples included two groups of animals. First, approximately 50 live prawns were selected randomly from the trawl. Second, a group of approximately 100 animals was taken from the same tow each month and frozen. Both live and dead animals were selected in this second group to prevent underrepresentation of postmolt animals due to death in the trawl. In the first group (the live sample), body wet weight, ovary wet weight, carapace length, molt stage, and presence or absence of CS were determined. In the second group (the frozen sample), sex,

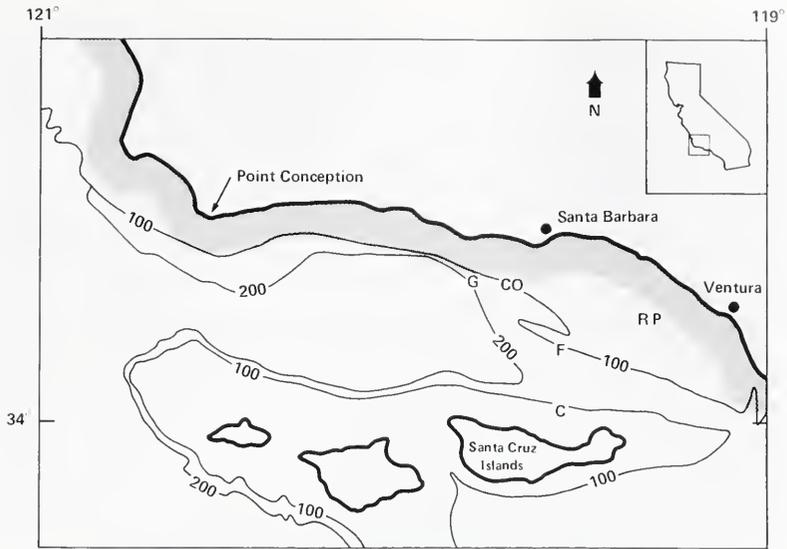


FIGURE 1. Study area in the Santa Barbara Channel. Insert shows the location of the Santa Barbara Channel along the coast of California. Shaded area denotes the three mile (4.8 km) limit. Depths in meters. F = fixed station (145 m), R = Rincon Pt. (60 m), P = Pitas Pt. (80 m), G = Gaviota area (220 m), CO = Coal Oil Pt. (150–250 m), C = China Cove (180 m).

carapace length, gross softness (indicating molt activity), and gross ovarian development were observed. Further details are given below.

### Reproduction

The spawning season was delineated in two ways. First, gonadosomatic index (GSI) was determined for 20–30 live females monthly. GSI is defined as follows (Giese, 1958):

$$\frac{\text{ovarian wet weight}}{\text{body wet weight}} \times 100 = \text{GSI}$$

Animals were dried with paper towels until no water drained from the branchial cavity. Body and ovarian wet weights were recorded. Second, a gross estimate of reproductive activity was obtained by counting the number of ripe females in the frozen sample of approximately 100 animals. Shrimp with a green ovary extending from the base of the eyestalks to the base of the telson were recorded as ripe. The ovary is initially cream-colored. As oocyte development progresses, it gradually becomes green without any intermediate coloration. Therefore, animals scored as ripe are advanced vitellogenic or postvitellogenic. This corresponds to an "R" (ripe) stage ovary as described by King (1948). This gross estimate of ripeness was also determined on samples taken at random trawl sites.

The periods of spawning (1981 and 1982) were determined by microscopically observing ovarian fragments (from ovaries used for GSI determinations) for the presence of cortical specializations (CS); these are prominent oocyte features which form late in oocyte development (Duronslet *et al.*, 1975; Anderson *et al.*, 1984). Figure 2 shows a postvitellogenic oocyte which has not yet developed CS. Figure 3

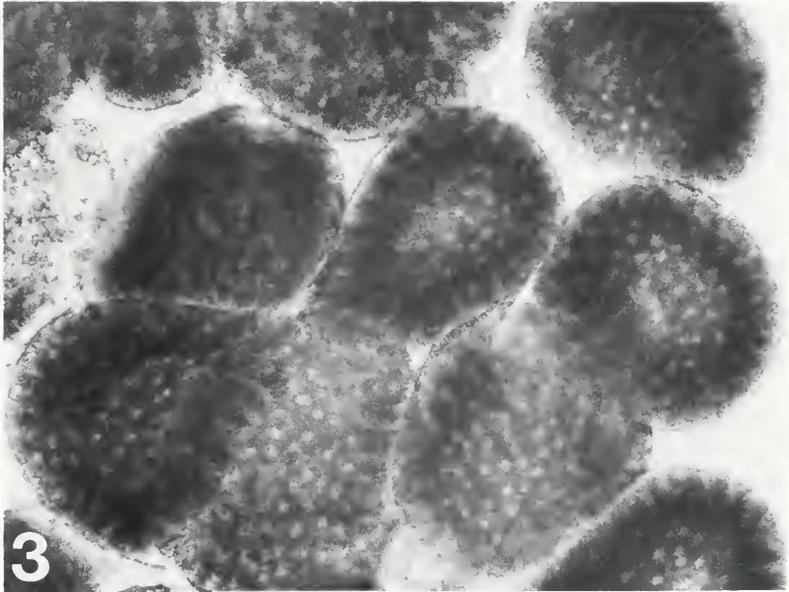
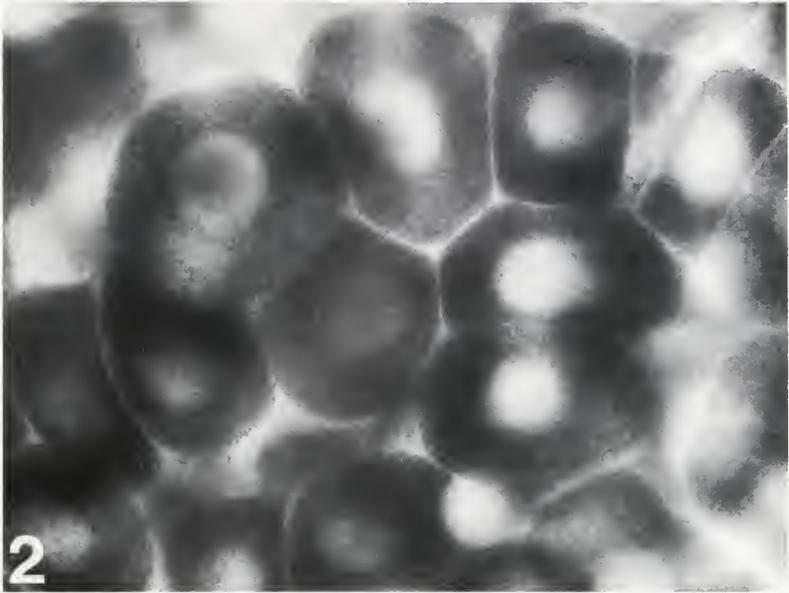


FIGURE 2. Postvitellogenic oocytes which have not yet developed cortical specializations (CS). 100 $\times$ .

FIGURE 3. Cortical specialization (CS) phase oocytes. CS in these oocytes are the discrete light areas. 100 $\times$ .

shows an oocyte with prominent CS which appear as discrete light areas. Presence of CS at the oocyte cortex indicates that spawning will frequently occur in less than one week (Anderson *et al.*, 1984). Consequently, CS serve as an index for spawning activity.

### *Molting*

Molt activity was assessed in two ways. First, microscopic molt staging was performed on approximately 20 live females monthly (same animals used for GSI determinations) for two years and on approximately 20 live males monthly for one year. One antennal scale and one uropod were examined on each animal. Molt stage criteria are summarized in Table I. These criteria are modified from those of Drach (1939), Schafer (1968), Stevenson (1972), and Aiken (1973) specifically for application to *S. ingentis*, and the duration of the soft condition was determined by us in the laboratory. Molt stages were grouped into five categories (Figs. 4–7, Table I).

The second procedure for estimating molt activity, which was conducted at both the fixed and random stations (Fig. 1), was a determination of the percent of soft animals present in the sample of approximately 100 frozen animals. Soft animals are in stage A and part of stage B (Table I). This second means of estimating molt activity served as a comparison for determinations of the percent of animals in early postmolt taken from the live animal sample. These determinations were important because animals in early postmolt are very soft and are less likely to survive collection compared to their hardened cohorts. They are, therefore, less likely to be represented proportionately to their field abundance in a sample of live animals.

### *Morphological characteristics*

Carapace length (CL) was measured from the posterior margin of the orbit to the posterior border of the carapace. Males were distinguished from females by the presence of the petasma (copulatory structure) in the former.

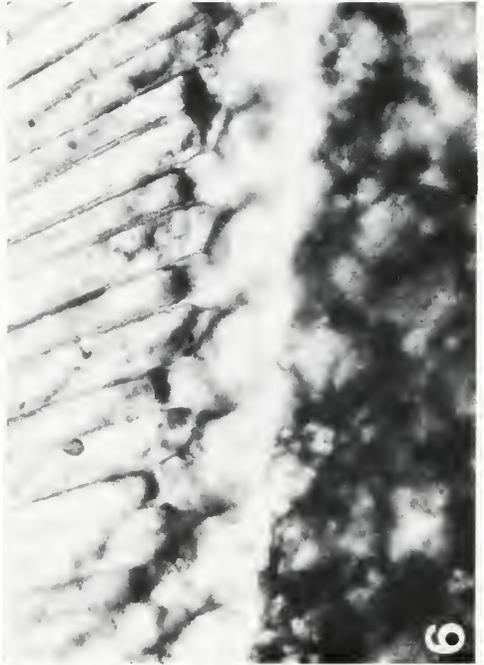
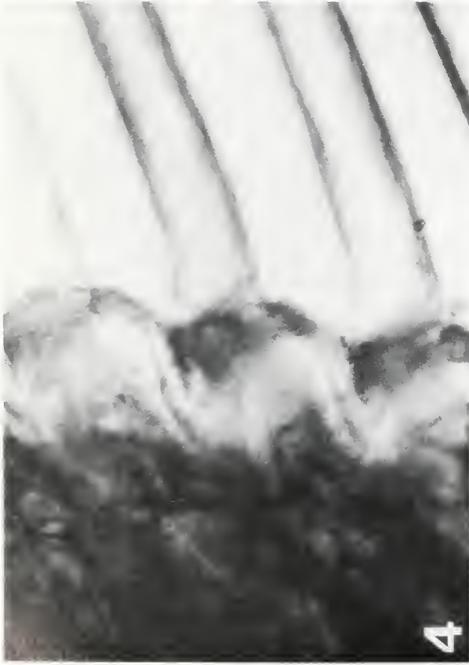
### *Multiple spawning and molt activity in laboratory populations*

Two different laboratory approaches were taken to investigate the potential for multiple spawning occurrences without intervening molt or mating. One approach was to determine if sperm were retained in females after spawning and if so, whether these sperm represented an adequate amount to fertilize a clutch of eggs. To

TABLE I

#### *Criteria for molt stage determination*

Molt stage	Criteria
A-B	cones at base of setae not formed or just organizing carapace may be extremely soft (A) or only barely yield to pressure (B) soft condition lasts 24–48 h
C	cones at base of setae are being completed or are complete pigmented epidermis has not retracted from the cuticle
D <sub>0</sub>	pigmented epidermis has retracted from the cuticle, retraction may be partial or epidermis may form straight line at base of setae or slightly below base of setae
D <sub>1</sub> -D <sub>2</sub>	epidermis slightly scalloped in appearance and/or new setae barely visible progressive stages of setagenesis occur
D <sub>3</sub> -D <sub>4</sub>	carapace loose or slightly loose and may be peeled off new setae are fully formed



accomplish this, four animals were killed immediately after spawning, and thelyca (sperm storage structure) were examined for the presence of retained sperm. Thelyca were dissected and homogenized lightly in 1 ml of cold crustacean Ringers (Pantin, 1934) with a Dounce homogenizer (B pestle). The homogenate was then vortexed, and sperm were counted using a hemacytometer. Additionally, an estimate of the number of eggs produced in a spawn was made to determine whether retained sperm represented an adequate quantity to fertilize a subsequent spawn. All of the eggs from six spawns were siphoned into formalin. Duplicate 5 ml aliquots of each spawn were counted and averaged.

The second approach used was to select animals just after spawning in the laboratory, to hold them in individual compartments (13 cm  $\times$  13 cm  $\times$  20 cm deep) of troughs supplied with running sea water (12–18°C, 33–34 ppt). These animals were monitored for egg development, spawning, and molt activity. The compartments were covered with clear plexiglass so that dim fluorescent light reached the tanks 13–16 h per day. Animals were fed *Tubifex* and brine shrimp to excess. Deaths, spawns, and molts were recorded. Signs of ovarian development and resorption that were visible through the carapace were also recorded.

Embryos were examined approximately 18 h after spawning. Two samples of 30 embryos each were counted and averaged. The number of embryos which had passed through the 8-cell stage was recorded.

Statistical methods used to compare seasonal tendency to molt in the laboratory were the Bonferroni t procedure and the Scheffé's S test (Kirk, 1982).

## RESULTS

### *Reproduction*

The spawning season was delineated by determining the frequency of ripe animals ( $n =$  approximately 100) in frozen samples and the mean gonadosomatic index ( $n = 20$ –30) on live animals taken in monthly trawls. Use of the gonadosomatic index to determine reproductive condition assumes that animals in the body size range studied have the same ratio of gonad size to body size, within a known limited range of variation in body size, at a given time of year, in a given locality (Gonor, 1972). To determine whether these data met such a condition, regression analyses were performed to determine whether GSI is dependent on body size. Log transformed GSI data were used because diagnostic plots of mean GSI *versus* variance showed that the variance increased with the mean. Log transformation effectively removed this dependence of variance on the mean. February and June of 1980 and 1981 were studied in order to compare periods of uniform high and uniform low ovarian bulk.

The relationship between GSI and body size was examined and in only one case out of four (June 1980), was a "t" test for the slope of the regression line of GSI

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FIGURE 4. Molt stage A-B. Note the absence of setal cones. 50 $\times$ .

FIGURE 5. Molt stage C. Note the organization of the setal cones and the retraction of the epidermis. 50 $\times$ .

FIGURE 6. Molt stage D<sub>0</sub>. Note the retraction of the epidermis. 50 $\times$ .

FIGURE 7. Molt stage D<sub>1</sub>-D<sub>2</sub>. Note the slight scalloping of the epidermis and presence of setal shafts. 50 $\times$ .

versus CL significant ( $P < 0.05$ , Table II). Correlation coefficients,  $r$ , were not extremely low. These data suggest that any dependence of GSI on CL was weak or nonexistent. Regression of GSI on carapace length for all observations in the experiment ( $n = 613$ ) was also performed. The slope was significantly different from zero ( $P < 0.01$ ). However, the correlation coefficient was low ( $r = 0.105$ ) and the number of observations was very large. Furthermore, the size range of animals in the area studied was narrow (22–45 mm CL).

The reproductive season for *S. ingentis* in the Santa Barbara Channel during 1980 and 1981 is outlined in Figure 8. Frequency of ripe animals closely paralleled the profile of log mean GSI. A period of low GSI was evident in the winter months from November until the end of April. Occasionally, animals with developed ovaries were seen at this time of the year, but they were not as well developed as during the peak season. GSI began to increase in late April, and this continued through May and into early June. This was the period when the first vitellogenesis of the season occurred. During late June, July, August, and September, high mean GSI was evident. The ovary accounted for 10–15% of the body weight. In these months, 75–95% of the population was ripe. A large drop in the number of ripe animals and ovarian bulk occurred in October and November, and the final spawning of the season occurred at this time. Similar results were obtained from samples taken at random stations.

Several lines of evidence raised questions as to whether these shrimp hold their eggs throughout the summer, have multiple spawns, or migrate into and out of spawning grounds. High mean GSI was maintained from late June through September, but the range in GSI values was very large. Moreover, 5–25% of the population was scored "not ripe" during these months. Examination of oocytes for CS showed a high incidence of this advanced stage throughout the summer, with maximum values in late July through early September (Table III). These data indicated a strong potential for multiple spawning during a single annual reproductive season.

TABLE II

*Regression analyses examining the relationship between Log Gonadosomatic Index (GSI) and Carapace Length (CL) (for four individual months and for the overall study of two years of monthly observations)*

Time period (n)	Regression equation Log GSI = a + b (CL)	r
Feb 1980 (n = 20)	a = 0.958 ± 0.766 b = -0.015 ± 0.020	0.176
Feb 1981 (n = 30)	a = -0.083 ± 0.492 b = 0.027 ± 0.013	0.359
June 1980 (n = 30)	a = 0.056 ± 0.109 b = 0.006 ± 0.003	0.366*
June 1981 (n = 30)	a = 0.079 ± 0.3600 b = 0.020 ± 0.010	0.342
Overall (n = 615) 1980–1981	a = 0.149 ± 0.172 b = 0.013 ± 0.005	0.105**

\* Significant at  $P < 0.05$ .\*\* Significant at  $P < 0.01$ .

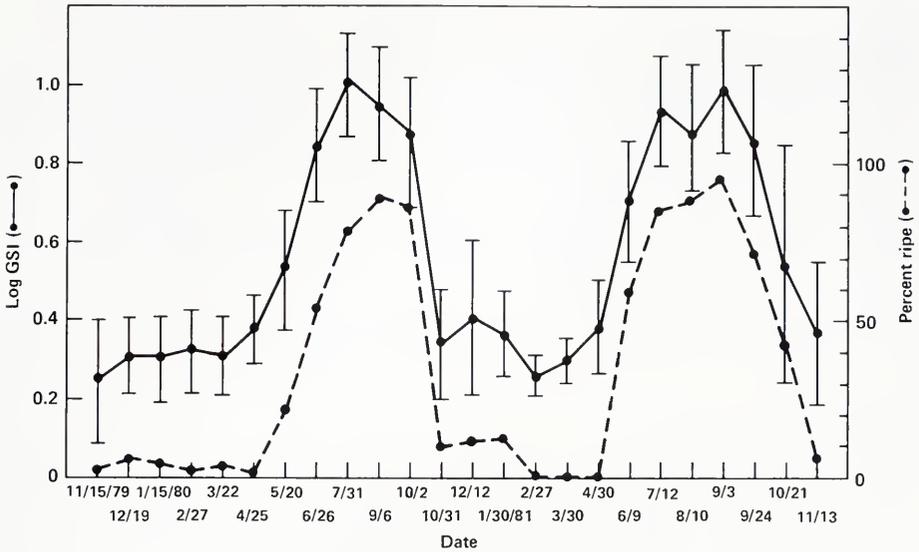


FIGURE 8. The reproductive season for *S. ingentis* in the Santa Barbara Channel (1980-81). Solid line is mean and standard deviation of log transformed monthly GSI values. Broken line represents mean percent ripe as judged by the presence of a green ovary in each month.

To determine whether frequency of occurrence of CS was uniform between size classes, the hypothesis that animals with CS differed in size from the mean size of the entire sample was tested using a "t" test. The mean carapace length of all animals in a given sample was tested against the mean carapace length of those with CS. In four out of six samples, the means were not significantly different (Table IV). Although the data remain inconclusive, there is apparently no strong association between incidence of CS and size, within the range tested.

TABLE III

*Incidence of Cortical Specializations (CS)*

1981*		1982*	
Date	% with CS	Date	% with CS
6/9	0	7/12	30
7/13	3	7/26	57
8/11	25	8/25	48
9/4	50		
9/25	25		
10/22	20		
11/14	0		

\* Data for 1981 were collected at one fixed station and were taken from live animals. Data for 1982 were supplementary to the main study. Samples were taken from random stations, and ovarian fragments were formalin fixed. Results between the two years are not directly comparable.

TABLE IV

*Size specificity of the frequency of Cortical Specializations (CS)*

Date	Carapace length for the entire sample Means in mm (n)	Carapace length for animals with CS Means in mm (n)
9/4/81	37.0 (20)	36.6 (10)
9/25/81	34.7 (20)	36.0 (5)
10/22/81	35.0 (20)	33.2 (4)
7/9/82	36.7 (26)	40.3 (8)
7/26/82	35.5 (29)	37.7 (15)
8/25/82	35.6 (29)	35.8 (14)

\*  $P < 0.01$ .*Molting*

The two year profile of male and female molt activity based on the frequency of soft animals is given in Figure 9. For females, a high and broad peak of molt activity occurred both years between late February and early May. The exact frequencies of soft animals, however, varied between the two years. Between late October and early November of each year, another smaller peak in molt activity was observed; a slightly elevated frequency of soft animals was also observed in December. Throughout the summer, no soft females were observed. For males, no discernible peaks in molt activity were detected. However, soft animals were rarely (1980) or never (1981) seen during the summer. Data taken at random stations generally agreed with the data taken at the fixed stations.

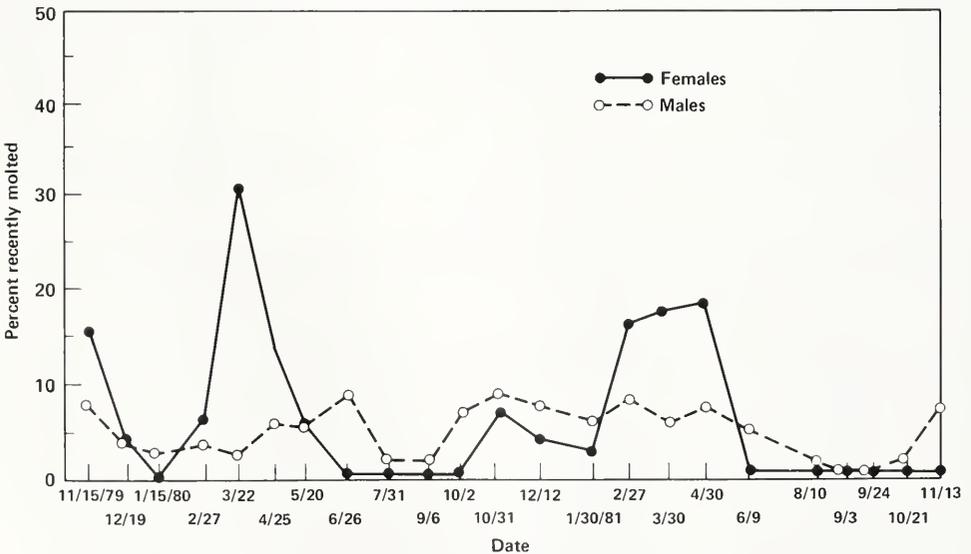


FIGURE 9. Molt activity (based on the frequency of soft animals) for *S. ingentis* in the Santa Barbara Channel. Solid and broken lines indicate data taken on females and males respectively at one fixed station.

Monthly determinations of molt stages were made microscopically for two years on females and for one year on males (Fig. 10). During November 1979, the period of the fall molt, 80% of the animals observed were in D<sub>3</sub>-D<sub>4</sub>. From December through January, 50-80% of the animals were in D<sub>0</sub> with lesser representation of other stages. The pattern for December and January roughly coincided with the expected relative duration of molt cycle stages as described in other shrimp species (Drach, 1944; Scheer, 1960). In March and April, no single molt stage predominated, and the frequency of the low probability stage, A-B, was relatively high. This indicates a very high frequency of molting, and it also indicates variability in length of the molt cycle. The high frequency of molting is corroborated by the data in Figure 9.

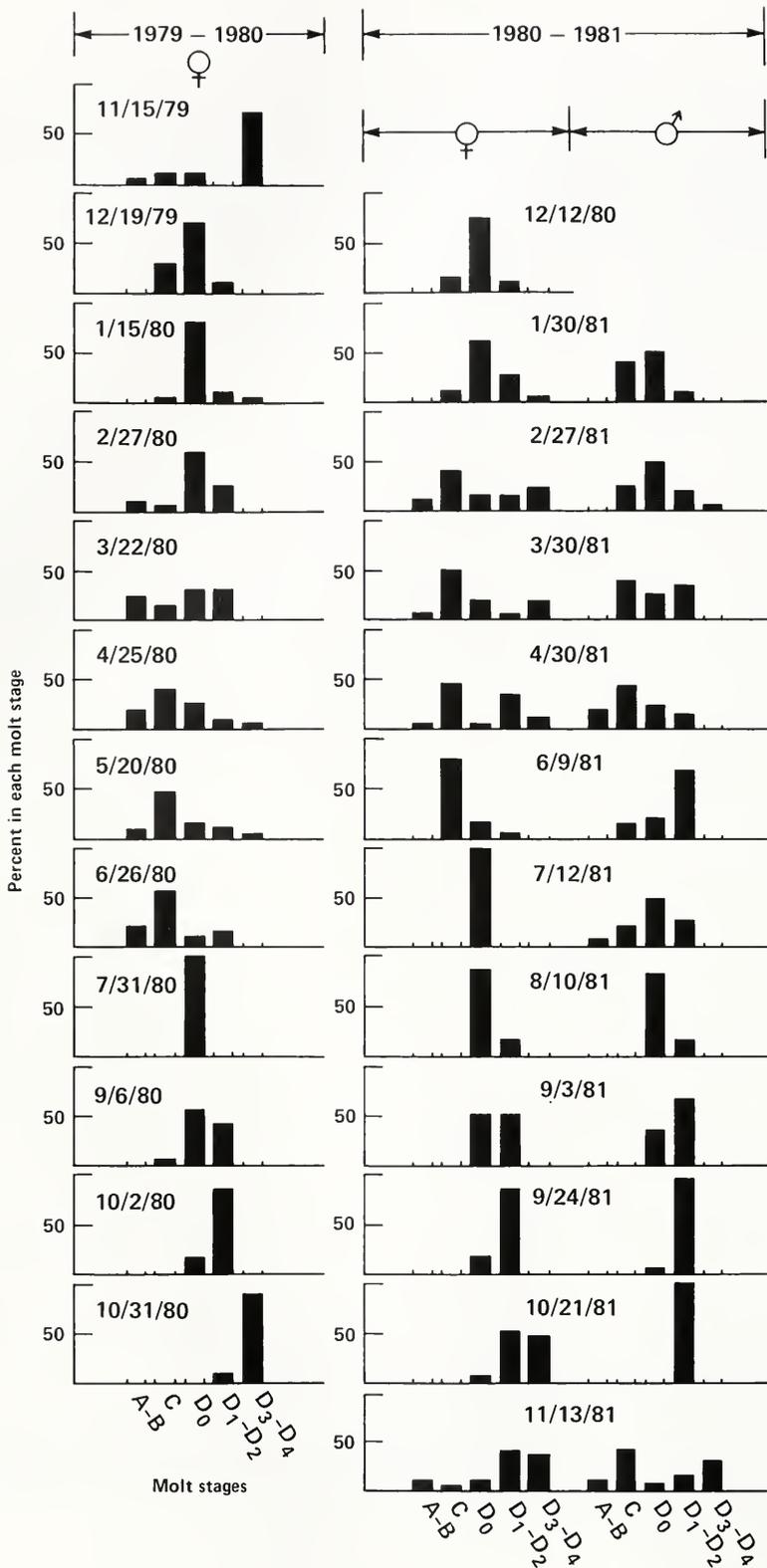
After the animals became vitellogenic in May, the range of observed molt cycle stages declined. In June, 50% were in stage C; by the end of July, 100% were in D<sub>0</sub>. In September and October, animals were increasingly observed in later premolt stages. By late October (as with the previous November), nearly all animals were in D<sub>3</sub>-D<sub>4</sub>. These data indicate that a synchronous progression through a single prolonged molt cycle occurred. These data further indicate variability not only in length of the molt cycle but also in the relative duration of molt cycle stages. Data for females in 1980-1981 were nearly identical to 1979-1980 data already described, indicating that the observed patterns were reproducible.

Molt patterns for males from January through June (Fig. 9) indicated asynchronous molt activity. This is generally corroborated in the microscopic molt stage data; however, it is not known why a higher representation of molt stage D<sub>0</sub> or even C was not observed. In August, when the frequency of soft males declined to zero (Fig. 9), a synchronous progression through postmolt was initiated. This is similar to the data for females, except that the duration of the long premolt period was one month shorter.

For both males and females, microscopic molt staging indicated that the fall molt occurred between 21 October 1981 and 13 November 1981 (Fig. 10). This molt was never detected in an increased frequency of soft animals at the fixed station (Fig. 9). This indicates that the fall molt may have occurred very rapidly and could have been missed when only the frequency of soft animals was recorded.

Comparison of the two indices (Figs. 9 and 10) shows that Stage A-B animals were underrepresented in some of the live animal (microscopic staging) samples. However, this does not have much effect on the overall interpretation of the molt stage data. For the females during the period of anecysis, there were zero molts so there is zero error. On 22 March 1980, 30% soft animals was recorded in the molt frequency sample, and 25% Stage A-B was reported in the live animal (microscopic staging) sample. Whereas in February, March, and April of 1981, 16-19% soft was reported and only 5-10% of the animals were in molt Stage A-B. Stage A-B was under-represented but the molt frequency (percent soft) data provide insight to this problem. During the months of December and January, when molt frequency was low and there was a spread of molt stages present, only the molt frequency (percent soft) sample with the high number of observations included the postmolt animals. In males, where synchrony was lower, there was approximately 5% underrepresentation of Stage A-B during the entire winter and spring.

Indication of a strong potential for multiple spawning throughout the summer, coupled with the observation of near-zero molt in the summer, indicated that multiple spawning occurred without intervening molt or mating. This possibility is further addressed in the next section.



*Multiple spawning potential and molt activity in laboratory populations*

The purpose of the sperm enumeration work was to determine whether *S. ingentis* could potentially spawn multiple batches of fertile embryos without an intervening molt or mating. The number of sperm in four freshly spawned females ranged from  $3 \times 10^6$  to  $40 \times 10^6$  with a mean of  $23 \times 10^6$ . The number of embryos produced in 6 spawns ranged from 47,000 to 131,000 with a mean of 86,000. The size of animals also varied (25.4–35.7 mm CL). In any case, the lowest number of sperm present in a spawned female was more than an order of magnitude greater than the number of embryos in the largest spawn. These initial results provided a strong indication that multiple spawning without an intervening molt or mating was possible.

The second approach to the analysis of potential multiple spawning without an intervening molt involved laboratory observation of spawning and molt activity. The first group (6 August 1982), consisted of 14 females which were isolated after spawning in the laboratory. Fifty percent of these females spawned again (Table V), despite the fact that no special conditioning regimes were employed. The time between observed spawns was  $19 \pm 2$  days (mean  $\pm$  S.D.). No prawns molted before spawning, and the time to molt for the 11 animals which survived to molting was  $52 \pm 14$  days (mean  $\pm$  S.D.). The embryo quality data showed that these animals spawned viable ova which were successfully fertilized and developed well. Embryo quality values were comparable to those reported for recently field collected animals (Anderson *et al.*, 1984). For the 20 August and 22 September groups, multiple spawning was observed again; and in no case, did an animal molt before it spawned (Table V).

Field results indicated that prawns began to molt in the fall as the spawning season drew to a close (Figs. 9, 10). This result was corroborated in laboratory results (Table V). Animals collected in early August did not molt in the laboratory for nearly two months, whereas those collected in late September molted within one month. Moreover, females collected in September showed a decreased tendency to spawn. Most laboratory molting, then, occurred throughout the month of October after the spawning season was over. Similarly, peak molting in the field occurred in late October and early November when spawning was completed. These seasonal changes in time to molt and tendency to spawn were statistically analyzed.

When the hypothesis that mean time to molt was the same for the three groups was tested by one-way ANOVA (Table V), this hypothesis was rejected ( $P < 0.0001$ ). Subsequently, pairs of means were compared in Bonferroni trials with the following results: 6 August *versus* 26 August,  $P = 0.0143$ ; 6 August *versus* 22 September,  $P = 0.0001$ ; 26 August *versus* 22 September,  $P = 0.0014$ . A Scheffé contrast was used to determine whether mean time to molt decreased with time ( $\mu_1 > \mu_2 > \mu_3$ ,  $P < 0.01$ ). These results demonstrate that mean time to molt decreased significantly as the spawning season came to an end.

When the hypothesis that the ratio of spawns was the same for the three groups was tested by a  $2 \times 3$  contingency table (Table V), the null hypothesis could not be rejected ( $0.05 < P < 0.10$ ). However, the percent of animals which exhibited repeated spawning was 50, 42, and 10 for the three groups. Although the result was not statistically significant at the  $P < 0.05$  level, it is believed that this is primarily due to the biologically significant fact that few spawns occur late in the season, making

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FIGURE 10. Microscopic molt stage patterns for *S. ingentis* in the Santa Barbara Channel. The frequency of molt stages is given for females (1979–1981) and males (1980–1981).

TABLE V

*Incidence of multiple spawning without an intervening molt in laboratory populations of S. ingentis*

	Collection date		
	8/6/82	8/26/82	9/22/82
Number observed <sup>a</sup>	14	12	10
Number that spawned <sup>b</sup>	7 <sup>c</sup>	5 <sup>d</sup>	1 <sup>e</sup>
Time to spawn (days): Mean $\pm$ S.D.	19 $\pm$ 2	19 $\pm$ 8	14
Number that molted	11	9	7
Time to molt (days): <sup>b</sup> Mean $\pm$ S.D.	52 $\pm$ 13	39 $\pm$ 6	28 $\pm$ 5

<sup>a</sup> Prawns observed for spawn and molt activity all spawned once in the laboratory after field collection. They were then placed in the holding facilities used for this experiment.

<sup>b</sup> The hypothesis that the ratio of spawns was the same for the three groups was tested in a  $2 \times 3$  contingency table ( $\chi_{\text{calc}}^2 = 4.807$ ,  $0.5 < P < 0.1$ ). The hypothesis that the time to molt was the same for the three groups was tested in one-way ANOVA ( $P < 0.0001$ ). Subsequently, the hypothesis that  $\mu_1 > \mu_2 > \mu_3$  was tested using the Scheffé's multiple comparison procedure ( $P < 0.01$ ).

<sup>c</sup> Six of these prawns survived to molt. None molted before spawning.

<sup>d</sup> Three of these prawns survived to molt. None molted before spawning.

<sup>e</sup> This prawn survived to molt. It spawned before molting.

it difficult to detect statistically significant effects without a very high number of observations.

#### *Molt stage and reproductive activity*

Individual records on field collected and laboratory maintained animals revealed that vitellogenesis occurred in molt stages late B through D<sub>1</sub>-D<sub>2</sub>, and spawning occurred in stages C through D<sub>1</sub>-D<sub>2</sub>. As the animals progressed into D<sub>3</sub>-D<sub>4</sub>, ovaries were either spent or resorptive.

## DISCUSSION

### *Spawning season*

The spawning season of *Sicyonia ingentis* in the Santa Barbara Channel is from June through October with possible multiple spawning occurring throughout the summer (Fig. 8; Tables III, V). Although regression analyses examining the relationship between GSI and body size (Table II) were somewhat contradictory, we have no reservations about the use of GSI to delineate the reproductive season. This is based upon the observation that frequency of ripe animals gave the same results as did the GSI data (Fig. 8). Random station data were in agreement with data taken at the fixed station. Therefore, it seems valid to generalize the spawning season for the majority of the Santa Barbara Channel region.

The spawning period of *S. ingentis* is more highly seasonal than that of other grooved Penaeoidea on the Atlantic and Gulf Coasts of the United States. The spawning period is restricted to 4 months, and there is a very high incidence (75–95%) of ripe animals at any time within the season. Kennedy *et al.* (1977) studied rock shrimp populations (*S. brevisrostris*) off the eastern coast of Florida. They found that spawning occurred year round with peaks in January through March. The maximum frequency of animals with developed ovary in any month was approximately 75%. The minimum was approximately 10% with 6–7 months of the year in the 20–30% range. Cobb *et al.* (1973) studied *S. brevisrostris* on the western coast

of Florida. They also found year-round spawning. Peaks occurred in October through February. The maximum percent ripe (by adding their percent developing and percent ripe) was 65%. Numerous investigations on prawns of the genus *Penaeus* in Gulf and Atlantic coast waters also indicate year-round spawning for several species. This includes, but is not limited to, *Penaeus duorarum* (Cummings, 1961; Kennedy and Barber, 1981) and *Penaeus aztecus* (Renfro and Brusher, 1963).

### *Multiple spawning*

The frequency of CS stage oocytes in ovaries of *S. ingentis* was used as an index of spawning activity in the field. Laboratory determinations (Anderson *et al.*, 1984) revealed that the time from the onset of CS phase to spawning ranges from 1 to 9 days with a mean of 4 days at ambient fluctuating temperatures of 12–18°C. It is not known how environmental parameters affect this rate. Nevertheless, in interpreting these data, it may be concluded that the majority of animals observed to be in CS phase will probably spawn within a week.

Assuming animals with CS will spawn within a week, then for every month in which at least 25% of the population was observed to be in CS phase, we can predict approximately one spawn per month for each animal. This criterion was met or exceeded in three months during 1981 (Table III). Attempts to examine any size specificity in the occurrence of CS were inconclusive (Table IV); therefore, we are not certain whether the frequency of repeated spawning is the same in all size classes.

Direct documentation of multiple spawning within a single molt cycle has been provided by Emmerson (1980) in laboratory studies on *P. indicus*. It was found that unablated, recently wild caught females could spawn up to three times without a molt. However, no field studies were conducted. Our laboratory studies were initiated halfway through the spawning season, and two spawns within one molt cycle were noted for 13 animals. It was also demonstrated that mean time to molt in the laboratory decreased as the spawning season in the field ended. The prolonged molt cycle which occurs in the summer made it possible for us to also infer multiple spawning within a single molt cycle in field populations.

An alternative explanation to the possibility of repeated spawning within a single prolonged molt cycle is that the selected sampling station is a spawning ground through which ripe prawns (possibly in a favored molt stage) move during the summer. This possibility cannot be entirely refuted; however, four lines of evidence argue against this possibility. First, the size distribution of both males and females at the sampling station did not change over two years (Anderson *et al.*, 1985). Second, although the sex ratio at the sampling site varied, no systematic pattern was detected (Anderson *et al.*, 1985). Third, data collected on molt and reproductive activity at random stations were in agreement with fixed station data. Fourth, laboratory data demonstrated the potential for repeated spawning within a single molt cycle (Table V), and animals collected in early August delayed their molt in the laboratory until October, when the spawning season in the field was drawing to a close (Table V).

### *Molting*

Monthly analyses of two indices of molt activity for *S. ingentis* populations revealed patterns of molt activity which vary dramatically with changes in reproductive status of the prawns and with season (Figs. 9, 10). Molt frequency was highest in

the winter and spring months when ripe animals were rarely found. Molt frequency was especially high in the late spring, prior to the onset of the spawning season. Ovarian bulk rose rapidly in May. At this time, the majority of the females began to progress synchronously through a single molt cycle which was not completed until late October or early November, after the spawning season was over. Males exhibited similar synchrony, but the synchronous period was shorter and there was more variability. To our knowledge, this is the first documentation of such dramatic molt synchrony in a population of free spawners.

Although molt stage data (Fig. 10) indicate that a synchronous molt occurred in November after the reproductive season was over, this was not always reflected in a peak percentage of soft animals (Fig. 9). This peak was narrow, and the period of maximum molting can be missed. This means that if a synchronous molt occurs over a short time period, sampling only for soft animals may not be sufficient to routinely detect this transient peak. It is also possible that significant mortality occurs after the fall molt and this affects the height of the peak.

Other interesting findings of this study are, first, that during the reproductive anecdyosis of this free spawner, all molt stages from  $D_0$  through  $D_3$ - $D_4$  were prolonged, not just  $D_0$ . In contrast, investigators working on brooding species (Hiatt, 1948; Aiken, 1973) have noted that  $D_0$  or  $C_4$  are the stages which are prolonged when anecdyosis occurs. Second, it was shown that not only the absolute duration but also the relative duration of molt cycle stages may vary with sex and season.

Drach (1944) and Scheer (1960) have used the proportion of field collected animals in a given molt stage as an indication of the relative duration of molt cycle stages in brooding prawns. We caution against the use of this method when collections are not numerous. Furthermore, it is important to note that different molt cycle stages imply behavioral changes which may affect results. The latter source of error could not be addressed in our study because of the great depth at which these prawns are found.

### *Interactions between reproduction and molting*

Our observations on the co-occurrence of molt stages with selected reproductive conditions agree with those of Emmerson (1980) who found ripe *Penaeus indicus* in molt stages B- $D_2$ . It was also noted that  $D_2$ - $D_3$  ovaries resorb to an undeveloped state. Aquacop (1975) also noted that molting animals were never ripe. The relationships described above only apply to free spawning species, and they should not be generalized to brooding crustaceans. For example, the brooding prawn *Macrobrachium nobilii* undergoes a molt immediately prior to extrusion (Pandian and Balasundaram, 1982).

Our data do not permit final conclusions on the ultimate ecological significance of the long period of molt synchrony in *S. ingentis* populations, but we would like to discuss five pertinent hypotheses. First, there is no cannibalism in this species, so the argument proposed by Reaka (1976) for stomatopods, that molt synchrony provides a temporal refuge from cannibalism, is unlikely in this case. Second, Klapow (1972) demonstrated semi-lunar rhythms of molting and reproduction of the isopod *Excirologa chiltoni*. He believed these rhythms optimized success of soft adults and hatchlings with respect to tidal exposure. The environment that *S. ingentis* inhabits poses no such problems. Third, it is possible that fall molt synchrony provides an opportunity for heightened mating success. However, this is not likely because the prawns will molt again, thus losing their sperm packet, before they become ripe the next summer. Fourth, molt synchrony may cause a predator

satiation effect. We feel this is an interesting hypothesis, possibly implying selection at the population level. However, this is a difficult hypothesis to test. Finally, it is possible that proximate factors, such as the physiological demands of egg production are of overwhelming importance in the determination of this molt synchrony phenomenon. This rationale for molt inhibition is not new (Adiyodi and Adiyodi, 1970), but it has not been effectively tested. The physiological demands of egg production are dictated by the reproductive strategy of the organism. In the case of *S. ingentis*, multiple spawning occurs throughout a single synchronous molt cycle. We suggest that the dramatic molt synchrony which occurred in this *S. ingentis* population during the spawning season may be related to their highly seasonal spawning activity and may not occur in populations of penaeoids with protracted spawning seasons and more rapid growth rates. It would be possible to test this hypothesis by determining whether both molt synchrony and multiple spawning occur only in penaeoids with restricted spawning seasons.

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FEMALES INHIBIT MALES' PROPENSITY TO DEVELOP INTO  
SIMULTANEOUS HERMAPHRODITES IN *CAPITELLA*  
SPECIES I (POLYCHAETA)

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ABSTRACT

The propensity for a male of *Capitella* species I to develop into a simultaneous hermaphrodite is shown to be inhibited by the presence of females. However, even when females are rare, males which develop into hermaphrodites do so nearly as quickly as males held in all male cultures or in isolation. When held in isolation males which do not switch are smaller than their male siblings which do. The maternal parent has no effect on the propensity of switching; no differences are found between male offspring which are derived from either females or hermaphrodites. It has been suggested that hermaphroditism in *Capitella* is an adaptation to low density. Yet females do not become hermaphroditic, hermaphrodites do not self-fertilize, and hermaphrodites function primarily as females. It seems much more likely that hermaphroditism in *Capitella* is an adaptation to living in small local populations which experience local mate competition.

INTRODUCTION

The polychaete species *Capitella capitata* is known to actually be comprised of a large group of unnamed sibling species (Grassle and Grassle, 1976; Grassle, 1980) which exhibit a great deal of diversity in life history and sexuality. In one species, designated *Capitella* sp. I (Grassle and Grassle, 1976), natural populations are a mixture of males, females, and hermaphrodites which arise from males that have secondarily developed eggs. Hermaphrodites can function as either sex and offspring are the result of sexual reproduction (Holbrook, 1982). Since *Capitella* sp. I rapidly colonizes a disturbed habitat (Sanders *et al.*, 1980), the occurrence of hermaphroditism in the species has been linked to its opportunistic life history (Grassle and Grassle, 1976; Grassle, 1980; Holbrook, 1982; Holbrook and Grassle, 1984).

Recently, it has been shown that sex determination in *Capitella* sp. I is a simple two-factor system (Petraitis, 1985). Under careful rearing of progeny from single crosses with less than 20% mortality, females produce a 1:1 sex ratio while hermaphrodites which function as females produce nearly all male offspring. All female offspring from hermaphrodites are protogynous hermaphrodites and secondarily develop male copulatory setae. The simplest explanation is that males (*i.e.*, hermaphrodites) are the homogametic sex.

Holbrook and Grassle (1984) found that male offspring from broods with either "high male" or "low male" sex ratios show no difference in their propensity to develop into hermaphrodites. The first objective of this research was to examine for differences between male offspring from females and hermaphrodites as maternal parents.

A second objective was to explore alternative explanations for the adaptive significance of hermaphroditism in *Capitella* sp. I. Holbrook and Grassle (1984) call the development of hermaphroditism an "emergency adaption" to low density. Holbrook (1982) suggests that the presence of females or a female pheromone suppresses ovum development in males. The low density model (Tomlinson, 1966; Ghiselin, 1969; Charnov, 1982) predicts that individuals which can develop into simultaneous hermaphrodites when mates are rare will be at a selective advantage. The observations that *Capitella* sp. I females do not become simultaneous hermaphrodites, that hermaphrodites cannot self-fertilize, and that older males will become hermaphrodites even at high density are not explained by the low density model.

As an alternative explanation, I propose that hermaphroditism in *Capitella* may be the result of mate competition. Since *Capitella* often colonizes areas over a short time period, I hypothesize that mating may be highly synchronized as most females reach maturity at the same time. As females are mated and begin to brood eggs, the ratio of receptive females to males would decline. At this point a male which could develop eggs and function as a female would be favored. If colonizers of a patch are closely related, competition among siblings for mates would have the same effect (Taylor, 1981). Thus one would predict some males should develop eggs when females are rare, regardless of the density. The second objective was to test this prediction.

#### MATERIALS AND METHODS

All worms are from a stock culture started in the fall of 1980 with offspring from *Capitella* sp. I females from Woods Hole (Petraitis, 1984). For the last two years, stock cultures have been maintained at 15°C. Procedures for maintenance are identical to methods given in Petraitis (1984) except that worms older than three weeks are fed finely chopped frozen spinach in a bed of fine (less than 300  $\mu\text{m}$ ) sand. All experiments were done between March 1983 and February 1984.

In order to control for differences among families, full sibling offspring from a single cross were treated as experimental blocks. Offspring from each cross were raised in a single 4 inch culture bowl until they were used for an experiment. Sibships were then subdivided and used in each treatment.

A few broods which were directly drawn from the stock culture were used. The maternal parent was always female in these cases. Female and hermaphroditic offspring from the original broods were raised in isolation and then crossed with males from stock culture. Male offspring from the female X male crosses were used in experiments as "female-derived" males. Male offspring from the hermaphrodite X male matings provided "hermaphrodite-derived" males.

I examined the propensity of sex change in male offspring from hermaphrodites and females in an isolation experiment similar to the experiment described by Holbrook and Grassle (1984). From each of 15 sibships from hermaphrodite X male matings and 11 from female X male matings, 20 males were randomly chosen, and each worm was singly placed into a small petri dish (0.05 worms/cm<sup>2</sup>). Worms ranged from 42 to 60 days in age. Water and food was changed twice a week. At the end of three weeks the number of males which had developed eggs were tallied. The length of all worms was measured with an ocular micrometer. As a check on length as a measure of body size, 100 worms were randomly drawn from the stock culture, measured, and then dried and weighed. The correlation between dry weight and length is +0.94.

Observations were also taken on cohorts of worms from the same sibships which were used in the isolation experiments. A group of 25 larvae from each sibship was placed into a 4 inch culture bowl. Bowls were checked every week for females and hermaphrodites with brood tubes. Tubes were removed, offspring were counted, and mothers returned to bowls. Every three weeks the bowls were censused and 10 worms were measured. Data were collected for life table analysis which will not be discussed here, however information on the occurrence of hermaphrodites is relevant and is discussed. Twenty broods from hermaphrodite X male crosses and 19 broods from female X male crosses were used.

Two experiments were conducted to test if the initiation of ovum development in males was affected by the presence of females. For the first experiment male offspring from a brood were randomly assigned to four treatments: a pure male treatment of 6 males per petri dish ( $0.29 \text{ worms/cm}^2$ ), a 50:50 sex ratio treatment of 3 males and 3 females per dish, a biased sex ratio treatment of 1 male and 5 females per dish, and a control of 1 male per dish ( $0.05 \text{ worms/cm}^2$ ). I set up two or three controls per sibship and one was randomly chosen for analysis. Progeny of a single brood thus formed a "block" and data were analyzed with Friedman's test (Sokal and Rohlf, 1981). If worms died, that block was not used. The sibships ranged from 35 to 69 days old with an average of 48 days at the start of the experiment. Dishes were maintained for 56 days or until at least one male in each treatment dish had developed ova. Dishes were checked at least twice a week. Thirteen blocks were successfully run. The second experiment was simply a continuation of the first except that an additional treatment of 5 males and 1 female per dish was added. This experiment used progeny from seven different broods.

## RESULTS

When held in isolation, males from hermaphrodite X male and female X male matings show no significant difference in the development of ova. All 20 male siblings developed ova in 8 of the 15 hermaphrodite-derived sibships and in 3 of the 11 female-derived sibships. The average percentage is 91% for hermaphrodite-derived males and 79% for female-derived males. These means are based on untransformed data; a *t*-test for difference between means, which were calculated with data transformed by the arcsine of the square root, is not significant ( $t = 1.57$ , d.f. = 24). Transformed means and standard deviations are  $78.71 \pm 15.51$  for hermaphrodite-derived males and  $69.93 \pm 19.44$  for female-derived males. Survivorship is quite good in both groups with 453 of the 520 males surviving. Mean survivorship is 17.9 out of 20 for hermaphrodite-derived males and 16.8 out of 20 for female-derived males.

Isolated males which develop ova are slightly larger than their male siblings which do not (Table I). When results of both types of sibships are combined, males which develop ova average 13.97 mm in length while their siblings are 11.38 mm. Since the observations are blocked by sibships, a paired *t*-test was used, and the difference is significant. Offspring from both types of sibships show the same trend, although the difference in hermaphrodite-derived families is not significant. This is probably due to small sample size.

The cohort observations are quite different. Hermaphrodites develop in 17 of the 20 cohorts from hermaphrodite X male matings and in 3 of the 19 cohorts from female X male matings. Hermaphrodites first appear in the bowls between 6 and 21 weeks; the average is 13 weeks. Males which develop into hermaphrodites are no bigger than their male siblings (Table I). Males from the cohort bowls are

TABLE I

Mean lengths of males which developed into hermaphrodites and of their male siblings which did not

	Length of siblings which are		Difference between pairs of observations
	herm.	male	
<i>Isolation observations</i>			
hermaphrodite-derived sibships (6)			
mean	11.36	9.06	2.31
S.D.	0.87	2.67	2.21
female-derived sibships (8)			
mean	9.86	8.18	1.69*
S.D.	0.68	1.65	1.37
combined (14)			
mean	10.51	8.56	1.95*
S.D.	1.07	2.10	1.73
<i>Cohort observations</i>			
hermaphrodite-derived sibships (17)			
mean	12.74	12.23	0.51
S.D.	3.32	2.56	1.98
female-derived sibships (3)			
mean	15.00	12.87	2.13
S.D.	3.00	1.63	1.63
combined (20)			
mean	13.08	12.32	0.75
S.D.	3.30	2.42	1.99

Means and standard deviations (S.D.) are given in units from the ocular micrometer scale. One unit = 1.33 mm. Numbers in parentheses are the number of sibships used in each case. Since males and hermaphrodites are paired by sibship, the mean difference is given. Significant paired *t*-test at the 5% level is denoted by asterisk.

larger than their male siblings held in isolation, however this is not surprising since males held in isolation were measured when they were about 10 weeks old while males held in cohorts were measured when they were usually older and thus larger.

Sex ratio, when density is held constant, has a strong effect on the initiation of ovum development (Table II). Males held in pure male dishes develop ova as frequently as males held in isolation. Indeed it appears that males held in pure male dishes develop ova more quickly. However note the means in Table II are calculated without taking block (*i.e.*, progeny from a single cross) effects into consideration.

For ten of the blocks in experiments 1 and 2, hermaphrodites appeared in both the pure male treatment and the control. When the difference between the control and treatment for these families is taken, males in pure male dishes take an average of 2.8 days longer to develop into hermaphrodites (S.D. = 6.2 days). The difference is not significant.

Since the difference between the treatments and the control may reflect density effects, treatments with females should be compared against the pure male treatment. When the pure male treatment value for each block is subtracted from other treatment values, it is clear that the presence of females depresses the initiation of

TABLE II

Mean and standard deviation of the number of days required for the first male to develop ova under different sex ratios

	Males/females per dish				
	6/0	5/1	3/3	1/5	1/0
Expt. 1.					
mean	18.6		29.6	38.7	21.6
S.D.	5.8		8.7	4.9	7.3
percent	84.6		46.2	15.4	100.0
rank	1.5		3.4	3.5	1.7
Expt. 2.					
mean	27.0	24.8	35.0	29.0	17.0
S.D.	12.7	2.5	7.1	10.5	4.9
percent	71.4	42.9	28.6	42.9	100.0
rank	2.3	3.4	4.1	3.8	1.1

Row labelled "percent" gives the percentage of sibships in which at least one male did switch. "Rank" row is mean rank which is used in Friedman's test. Test is significant for both experiments. For experiment 1.,  $H = 26.75$ ,  $df = 3$ ,  $P \leq 0.001$  and for experiment 2.,  $H = 11.74$ ,  $df = 4$ ,  $P \leq 0.05$ .

ovum development in males (Table III). One female in the presence of five males however is not sufficient to affect the rate.

There are no differences between hermaphrodite-derived and female-derived males in either likelihood or speed of sex change. When the observation of all control dishes are analyzed as a nested analysis of variance (Sokal and Rohlf, 1981), there is no significant difference between origin of male or among siblings within type of cross (Table IV). The likelihood of switching is also nearly identical. Under the pure male treatment 83% of the broods from hermaphrodite X male matings and 78% of the broods from female X male matings show the development of at least one hermaphrodite per dish.

#### DISCUSSION

Holbrook and Grassle (1984) found that males held in isolation required an average of 22 days to develop ova (their Table II) and suggest that not only the

TABLE III

The difference in days of each treatment minus the "pure male" (i.e. 6/0) treatment

	Males/females per dish		
	5/1	3/3	1/5
mean	-2.3	10.7*	14.8*
S.D.	10.7	6.2	14.3
n	3	7	5

Results are combined for both experiments shown in Table II, and the variate used to calculate the mean and standard deviation is the difference between treatments within sibships. The sample size,  $n$ , is the number of sibships in which at least one male changed sex in both the pure male treatment and the treatment under consideration. Paired  $t$ -tests which are significant at the 5% level are denoted by an asterisk.

TABLE IV

*Nested analysis of variance of the number of days required for a male to develop into a hermaphrodite when held in isolation*

Type of mother	Statistics for each sibship used in analysis					
Female						
mean number of days	27.3	21.0	19.5	16.5	13.5	19.5
standard deviation	2.9	0.0	5.0	3.5	6.4	7.8
sample size	3	2	2	2	2	2
Hermaphrodite						
mean number of days	20.3	21.0	19.0	18.5	15.0	
standard deviation	6.8	0.0	0.0	7.8	0.0	
sample size	3	3	3	2	2	
Analysis of variance						
Source of variation	df	MS	F			
Between type of mother	1	7.54				
Among sibships within type	9	35.86	1.75			
Among progeny within sibships	15	20.52				

Data are control dishes (*i.e.*, 1/0 treatment) from experiments shown in Table II. Analysis of variance is not significant.

absence of females but also an excess of food may be required for development of ova by males. My result for the rate of development is quite similar; males develop ova in 20 days (Table IV). While my isolation experiments show that males which develop ova are larger than their brothers, this difference is not seen in males held in groups (Table I). It should be noted that larger size of the sex changers in the isolation experiment may have little to do with food availability. It is just as plausible that smaller males should be expected to be sex changers if they are shunting available energy into egg production rather than growth. At this point the biological significance of the difference in size is not clear.

Presence of females clearly inhibits the initiation of ovum development in males. In the presence of a single female, males developed into hermaphrodites in only 3 of the 7 experimental blocks (Table II), while combined results of experiments 1 and 2 show 16 of the 20 pure male treatment dishes produce at least one hermaphrodite. Since density and age are controlled, I infer the difference is due to the presence of a female. Data in Tables II and III suggest the presence of females affects the likelihood of switching more strongly than the number of days.

Holbrook and Grassle (1984) found no difference between males from broods with "high male" and "low male" sex ratios in the proportion of males which develop eggs (their Fig. 2). I found no difference between hermaphrodite-derived and female-derived males. I suspect their high male broods are in fact derived from hermaphrodites and their low male broods from females. They report 3 high male broods with a total of 161 males, 12 females, 25 unsexed juveniles, and 52 deaths before sexing. Their 3 low male broods have 96 males, 117 females, 21 unsexed juveniles and 96 deaths before sexing. Assuming no sex-specific mortality, high male broods are 93% male and low male families are 45% male. These ratios are quite similar to the values I found in 8 female and 13 hermaphrodite-derived sibships in which all individuals were sexed and mortality was kept to a minimum (Petraitis,

1985). Broods from hermaphrodite X male matings are 98% male and from female X male matings are 47% male. The six "females" out of the 297 offspring from hermaphroditic mothers all secondarily developed male copulatory setae. The simplest explanation for these sex ratios is that sex determination in *Capitella* sp. I is a two-factor system in which the females are the heterogametic sex (Petraitis, 1985).

Since the development of hermaphroditism is clearly inhibited by females, it is possible hermaphroditism in *Capitella* is an adaptation to mate competition. *Capitella* quickly colonizes newly disturbed habitats (e.g., Sanders *et al.*, 1980), and it is likely small local populations may be highly synchronized. *Capitella* sp. I reduces sexual maturity in 4 to 6 weeks at 15°C and females require 10 to 14 days to brood eggs to larvae. Thus in synchronized patches the ratio of receptive females to males may decline quite rapidly and remain low for several weeks. Males that can develop eggs and function as females would be at an advantage. In larger populations the probability of synchrony would be lower and thus the occurrence of hermaphrodites much rarer.

In fact, hermaphrodites function more as females than males. Holbrook (1982) examined mating success of hermaphrodites under different total densities and proportions of hermaphrodites. She claims that a hermaphrodite's ability to mate as a male and a female depends on total density and frequency of hermaphrodites (Holbrook, 1982; her Fig. 21). Unfortunately she did not take into account the number of matings that would be expected if all worms within a replicate bowl mated at random. When corrected for this expectation there is no effect of density or frequency, however hermaphrodites avoid mating as males (i.e., there are fewer matings by hermaphrodites as males than would be expected under random mating).

Because the original colonists of a patch may be related and since a patch can persist for several generations, local mate competition may play a role. It is very likely that some patches are founded by siblings since when a mother deserts a brood tube with newly emerging larvae, some larvae remain trapped in the tube. If the tube is carried intact to a new location, then the founders would be siblings.

In this situation, the sex ratio will be biased in favor of the sex which has the least amount of competition among kin (Bulmer and Taylor, 1980a, b; Taylor, 1981). Even when the original colonists are unrelated, the bias depends on the number of original colonists and the number of generations the subpopulation is isolated (Bulmer and Taylor, 1980b).

Two observations suggest that male *Capitella* within a patch may be related. First, patches may be founded by a few individuals and persist for long periods (Sanders *et al.*, 1980). Second, founders may be related if intact brood tubes provide a major method of dispersal.

Yet, *Capitella* does not have a female biased sex ratio as predicted by local mate competition (Petraitis, 1985). The lack of bias is based, however, on the assumption *Capitella* is a species with only females and males. In fact data on sex determination in *Capitella* suggest there are only two sexual genotypes i.e., female (ZW) and male or hermaphrodite (ZZ). Under this sexual system the sex ratio should be strongly biased in favor of hermaphrodites (Lewis, 1941; Charnov, 1982). Since *Capitella* shows nearly a 50:50 sex ratio, this must be interpreted as a female biased sex ratio as predicted by local mate competition.

The two mechanisms, synchronized mating with a decline in receptive females and local mate competition among kin, are not mutually exclusive. Both could be important and both predict that hermaphrodites should function primarily as females. Since female *Capitella* do not develop into hermaphrodites when population

levels are low and since hermaphrodites do not self-fertilize (Grassle, 1980), it seems unlikely that hermaphroditism in *Capitella* is an adaptation for periodic declines in population or for ease of colonization of new habitat. Rather, hermaphroditism in *Capitella* appears to be an adaptation to small local populations which are highly age-structured and in which individuals are related.

#### ACKNOWLEDGMENTS

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## BIOLOGY OF HYDRACTINIID HYDROIDS. 4. ULTRASTRUCTURE OF THE PLANULA OF *HYDRACTINIA ECHINATA*

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### ABSTRACT

The fine structure of the ectodermal surface of the planula larva of *Hydractinia echinata* is described and the spatial distribution of cell types quantified. The larval ectoderm contains five cell types: nematocytes, gland cells, epitheliomuscular cells, neurosensory cells, and nerve cells. Nematocytes (atrichous isorhizas and desmonemes) and neurosensory cells are clustered at the tapered posterior end of the larvae. Mucous cells occur over the surface of the planula, but are particularly common at the anterior end. The larva is similar to others described from marine hydroids, except that (a) gland cells display a ring of microvilli emerging from a cavity encircling the cilium and (b) nerve cells appear to lack a cilium.

### INTRODUCTION

A number of sessile marine invertebrates settle preferentially in locations which are buffered from certain physical and biotic stresses (Meadows and Campbell, 1972; Scheltema, 1974; Buss, 1979). Certain sessile species are found in symbiosis with mobile organisms: such associations act to simultaneously limit access of predators and competitors which are unable to locate these surfaces (Jackson, 1977). Among the most specific of these relationships is the symbiosis of sessile species with hermit crabs of the genus *Pagarus* in coastal waters and scallops of the genus *Acopecten* in deeper waters. The relatively low diversity of sessile species occupying these habitats and the apparent specialization of various hydroid (*e.g.*, *Hydractinia* and *Podocoryne* spp.), bryozoan (*e.g.*, *Acyonidium* spp.), and gastropod (*e.g.*, *Crepidula* spp.) species on them, suggests that specialized mechanisms have developed which allow the location of these refugia and the discrimination of these substrata from others.

*Hydractinia echinata* is found primarily on hermit crab shells, although anecdotal accounts of *H. echinata* on other substrata are known (*e.g.*, *Limulus* and other crab carapaces, pilings, and rock substrata). Planulae are capable of sensing movement of nearby objects and are reported to discharge nematocysts to latch onto passing substrata (Chia and Bickell, 1978). Studies of microfloral induction of metamorphosis have demonstrated that certain bacterial species release a metabolite which initiates *Hydractinia* settlement, an effect which can be mimicked by certain monovalent cations (Muller, 1969, 1973; Spindler and Muller, 1972). These same gram-negative bacterial species are found in association with hermit crabs (Muller, 1969).

While the planulae of Anthozoa, Hydrozoa, and to a lesser extent Scyphozoa display considerable cellular differentiation of the ectoderm (see review by Chia and Bickell, 1978), the relationship between planula behavior and planula fine structure is incompletely known. In several species, gland cells appear in the planulae which

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are not expressed in the adult, suggesting that glandular secretions play a functional role in attachment (Chia and Crawford, 1977; Vandermeulen, 1974; Martin and Thomas, 1977; Martin *et al.*, 1983). In addition, several planulae possess specialized ciliary structures, putatively sensory in function, which are postulated to play a role in habitat choice (Lyons, 1973; Vandermeulen, 1974; Mariscal, 1974a; Martin and Thomas, 1977; Chia and Koss, 1979; Martin *et al.*, 1983). Nerves and neurosensory cells are present in most species studied and are known, in some species, to play an important role in settlement (Martin and Thomas, 1977, 1981a, b; 1983). Although the fine structure of several planulae have been described, the planulae of *Hydractinia*, whose behavior is well known, have no such description. As a prelude to experimental analysis of structure—function relationships in *H. echinata* planulae, we describe the ultrastructure of competent planulae, with emphasis on the nature of ectodermal cells.

#### MATERIALS AND METHODS

*Hydractinia echinata* is a dioecious hydroid, whose spawning is initiated in response to light (Bunting, 1894; Ballard, 1942). Laboratory cultivated colonies (Ivker, 1972) were placed in aerated dishes overnight and fertilized eggs were collected the following morning. Fertilized eggs develop into competent planulae within 18 h at room temperature. Planulae used for ultrastructural observations varied in age from 24 to 96 h post-fertilization. Planulae were fixed for 2 h at room temperature in 4% glutaraldehyde in 0.1 M sodium cacodylate buffered, double-strength, filtered sea water at pH 7.0. Planulae were rinsed in buffer for three successive 10 minute washes and then post-fixed in 2% OsO<sub>4</sub> in 0.1 M sodium cacodylate in distilled water at pH 7.0 for 1 h at room temperature. After a second series of three ten-minute washes in buffered sea water, specimens for transmission electron microscopy (TEM) were then infiltrated with propylene oxide and embedded with a Spurr's epoxy mixture of 70°C overnight. Blocks were sectioned on a Huxley ultramicrotome, stained with uranyl acetate and Reynold's lead citrate, and viewed with a Philips 300 series transmission electron microscope. Sections ranged in thickness from 700–2200 Å (silver to blue). Thick sections (purple and blue) were made in order to frequently capture the cilia of the mucous cell and relatively rare neurosensory cell. Specimens for scanning electron microscopy (SEM) were fixed as described above, dehydrated in an ethanol solvent, critical point dried in a Balser critical point dryer with CO<sub>2</sub>, mounted on stubs, sputter coated with palladium and gold, and viewed with an ISI-40 scanning electron microscope. Several hundred sections were observed and complete serial sections made for gland, epitheliomuscular, and nerve cells. The relative frequency of epitheliomuscular cells, nematocytes, sensory, and mucous cells was quantified from scanning electron micrographs. At each of four locations on the surface of the planula, the number of each cell type present was counted for a total of ten planulae. For each planula, the total area sampled per location was chosen so that the number of each cell type per unit area was constant.

#### RESULTS

The planula of *Hydractinia echinata* is cone-shaped, 300–500 μm long and 100–120 μm wide, with a blunt anterior end and a tapered posterior tip. At the blunt end, the planula contains a distinct dimple (Fig. 1). The planula is uniformly ciliated, with ciliary structures extending from each ectodermal cell. The ectodermal surface of the planula harbors five cell types: nematocytes, epitheliomuscular cells

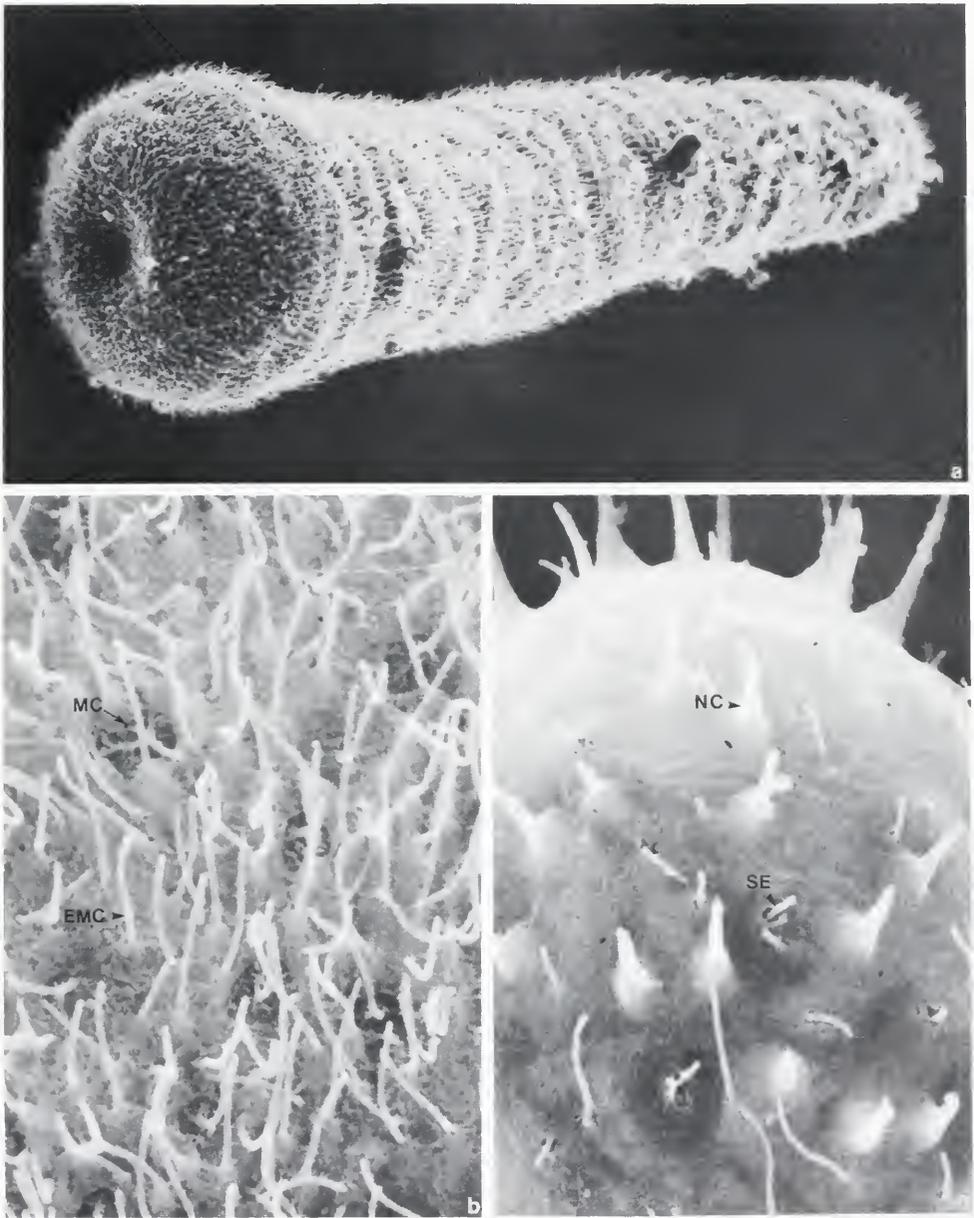


FIGURE 1. *H. echinata* planula. (A) Low magnification scanning micrograph of a planula (232 $\times$ ). (B) Scanning micrograph of the surface of a planula near the blunt end. Note uniform ciliation (2240 $\times$ ). (C) Scanning micrograph of the surface at the tapered tip (2240 $\times$ ). EMC = cilium of an EMC, NC = cnidocil emerging from a nematocyte, MC = cilium and associated microvilli emerging from a mucous cell, SE = cilium emerging from a concavity in a neurosensory cell.

(hereafter EMC), gland cells, neurosensory cells, and nerve cells. No interstitial cells were found in the ectoderm. The fine structure and relative frequency of each cell type is described below.

*Nematocyte*

Nematocytes (Figs. 3, 4) occur along the entire length of the planula, but are exceedingly dense at the tapered end (Fig. 2). Two types of nematocysts occur in the planula: atrichous isorhizas (Fig. 4A) and desmonemes (Fig. 4B) (*sensu* Mariscal, 1974b). The cnidoblast originates from interstitial cells in the endoderm. The external tube extends through the cytoplasm in the early stages of development, but invaginates completely before the cell's migration to the ectoderm. The cnidocil and its associated rods and stereocilia develop while the cell moves through the endoderm and ectoderm (Fig. 4C). At this stage, the nucleus is positioned under the basal end of the mature capsule (Fig. 4C). Mitochondria with well developed cristae are prominent. Slightly to the side of the operculum are two centrioles

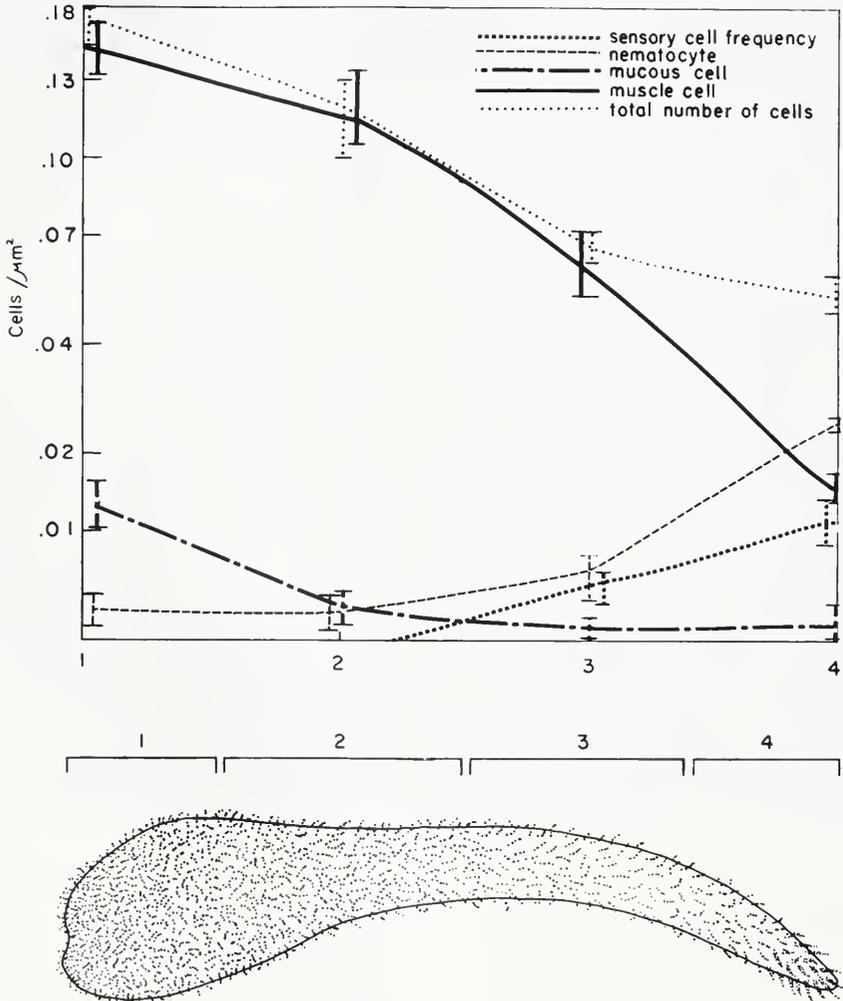


FIGURE 2. Frequency of ectodermal cell types. Numerals refer to location on the surface of the planula as indicated.

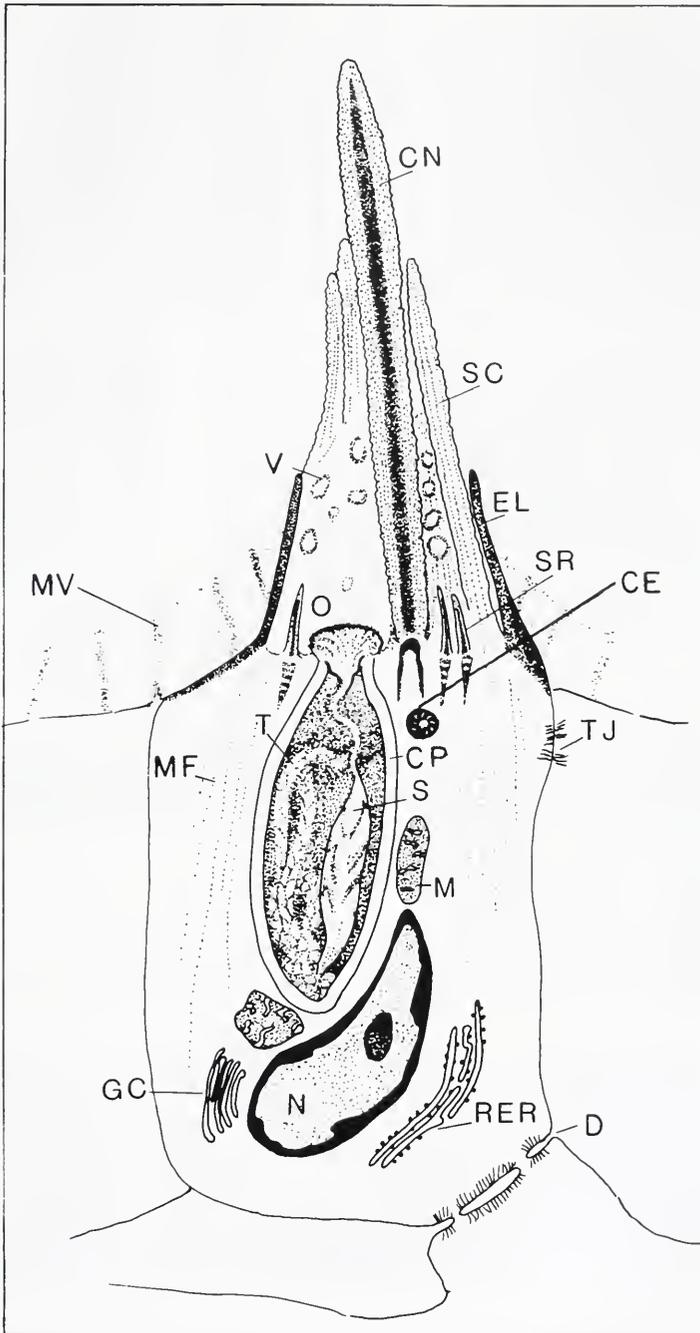


FIGURE 3. Longitudinal reconstruction of the nematocyte extending outer ectodermal surface to the mesoglea. CE = centriole, CP = capsule, CN = cnidocil, D = desmosome, EL = epithelial lip, GC = Golgi Complex, M = mitochondrion, MF = microfilaments, MV = microvillus, N = nucleus, O = operculum, RER = rough endoplasmic reticulum, S = stylet, SC = stereocilla, SR = stiff rod, T = thread, TJ = tight junction, V = irregularly shaped vesicles.

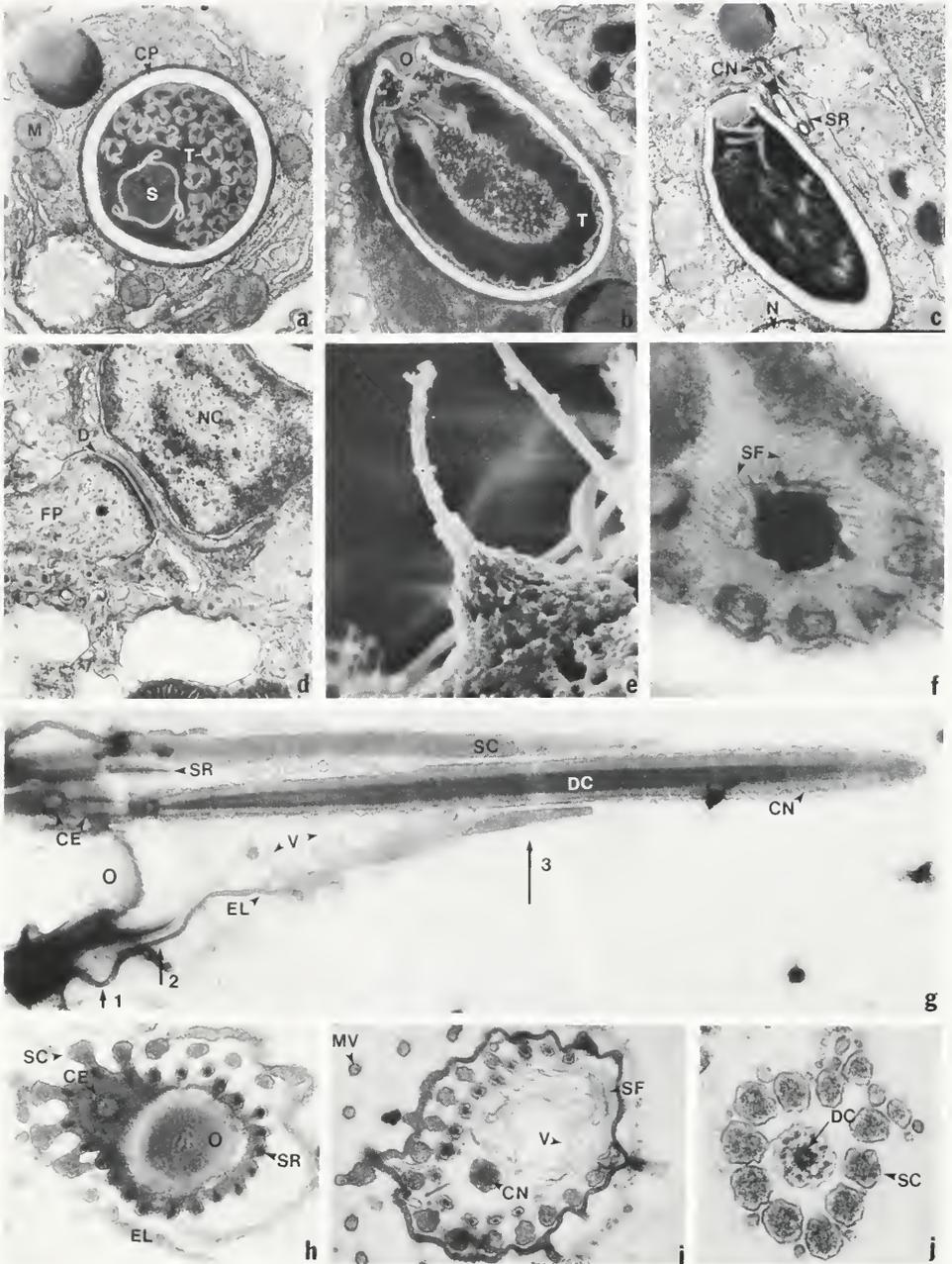


FIGURE 4. Nematocyte. (A) Cross-section of an atrichous isorhiza. Note the stylet and small thread (16,620 $\times$ ). (B) Longitudinal section of a desmoneme. Note the single large thread (16,620 $\times$ ). (C) Cnidoblast in the endoderm (10,234 $\times$ ). (D) Desmosome between the base of a nematocyte and a foot process of an EMC (16,620 $\times$ ). (E) A scanning micrograph of a cnidocil on the surface of a planula (8,867 $\times$ ). (F) Cross-section of a cnidocil and cnidocil-associated apparatus. Note the fine filamentous sheath can be seen surrounding the cnidocil and stereocilia (61,344 $\times$ ). (G) Longitudinal section of a cnidocil, cnidocil-associated apparatus, and operculum (16,080 $\times$ ). (H) Cross-section of centriole, cnidocil, and cnidocil-associated apparatus, and operculum of position of arrow #1 in (G) (28,080 $\times$ ). (I) Cross-

oriented at a  $90^\circ$  angle to each other, above which arises the partially formed cnidocil (Fig. 4C). The striated rootlet of a stiff rod lies to the right of the centrioles.

The mature nematocyte,  $5\text{--}7\ \mu\text{m}$  in length, extends from the ectodermal surface to the neuro-muscular layer except at the extreme posterior and anterior ends of the planula. Here the cell appears to be embedded in EMC's. A desmosome,  $0.5\ \mu\text{m}$  across, lies between the nematocyte and the foot process of an EMC near the base of the cell (Fig. 4D). The capsule of atrichous isorhizas,  $1.5\ \mu\text{m}$  in diameter and  $4.5\ \mu\text{m}$  in length, is composed of a dark-staining outer wall surrounding a light inner wall and houses the projectile apparatus of thread and stylet (Figs. 4A, C). The projectile apparatus of the desmoneme lacks a stylet, but contains a thickened thread (Fig. 4B). An operculum,  $0.63\ \mu\text{m}$  in diameter, caps the nematocyst (Figs. 4B, C, G). A large nucleus, approximately  $3\ \mu\text{m}$  long, is wrapped around the base of the capsule (Fig. 4C). The cytoplasm of the nematocyte contains rough endoplasmic reticulum (RER) (Fig. 4C), mitochondria, a dense stream of microfilaments, and a Golgi Complex (not shown in section) located near the nucleus.

The cnidocil (Figs. 4E, G–J), a modified cilium,  $6.5\ \mu\text{m}$  long and  $0.27\ \mu\text{m}$  in diameter, is characterized by nine doublets of microtubules around the edge of a dark staining inner core,  $90\ \text{nm}$  in diameter, apparently composed of many unpaired microtubules embedded in a dense unpackaged filamentous material (Figs. 4G, J). The inner core narrows at the top as the cnidocil tapers to a tip (Fig. 4G). Two centrioles comprise the base of the cnidocil (Figs. 4C, G). Twenty to twenty-four short, stiff rods,  $0.62\ \mu\text{m}$  long and  $70\text{--}90\ \text{nm}$  in diameter, with striated rootlets,  $0.45\ \mu\text{m}$  long, surround the base of the cnidocil and operculum (Figs. 4C, G–I). The striated rootlets closely resemble those of cilia of the EMC. The stiff rods are partially surrounded by 9–14 stereocilia (Figs. 4H, I),  $3.8\text{--}4.7\ \mu\text{m}$  long and  $90\text{--}140\ \text{nm}$  in diameter, containing a dense array of microfilaments (Figs. 4H–J) that extend down to anchor in the cytoplasm. These large and long spatula-shaped projections encircle the cnidocil above the point where the stiff rods end (Fig. 4J). Also present in the cnidocil-associated apparatus are numerous thin-membraned, irregularly shaped vesicles of unknown content and function, ranging from  $40\text{--}130\ \text{nm}$  in diameter. These may be lined up between a stereocilium and the cnidocil (Fig. 4G) or may be scattered throughout the space above the operculum (Fig. 4I). The rods, stereocilia, and cnidocil are connected by, and embedded in, a matrix of fine filamentous sheets, spread approximately  $30\ \text{nm}$  apart (Fig. 4F).

### *Gland cell*

The gland (or mucous) cell (Figs. 5, 6) is characterized by large, tightly packed, electron-lucent granules, a dense staining basal nucleus, one or more large dark staining bodies at the base, and a single short cilium with a short striated rootlet and associated centrioles. Gland cells are particularly large ( $30\ \mu\text{m}$  long) and common at the dimpled end of the planula (Fig. 5A) and are less common along the edges and at the tip of the planula ( $6.6\ \mu\text{m}$  long) (Fig. 5D), largely because the ectoderm itself narrows in this region (Fig. 2). The cilium is located in a depression,  $1.2\text{--}1.7\ \mu\text{m}$  in diameter,  $1.8\text{--}2.4\ \mu\text{m}$  deep, and  $4.0\text{--}5.3\ \mu\text{m}$  in circumference, and

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section of cnidocil and associated apparatus at position of arrow #2 in (G) ( $22,133\times$ ). (J) Cross-section of cnidocil and stereocilia at position of arrow #3 in (G). The nine doublets and dense inner core can clearly be seen as can the very fine array of microfilaments in the stereocilia ( $32,947\times$ ). DC = dense inner core, FP = foot process of EMC, NC = nematocyte, SF = sheet of fine filaments. Other labels as described in previous figures.

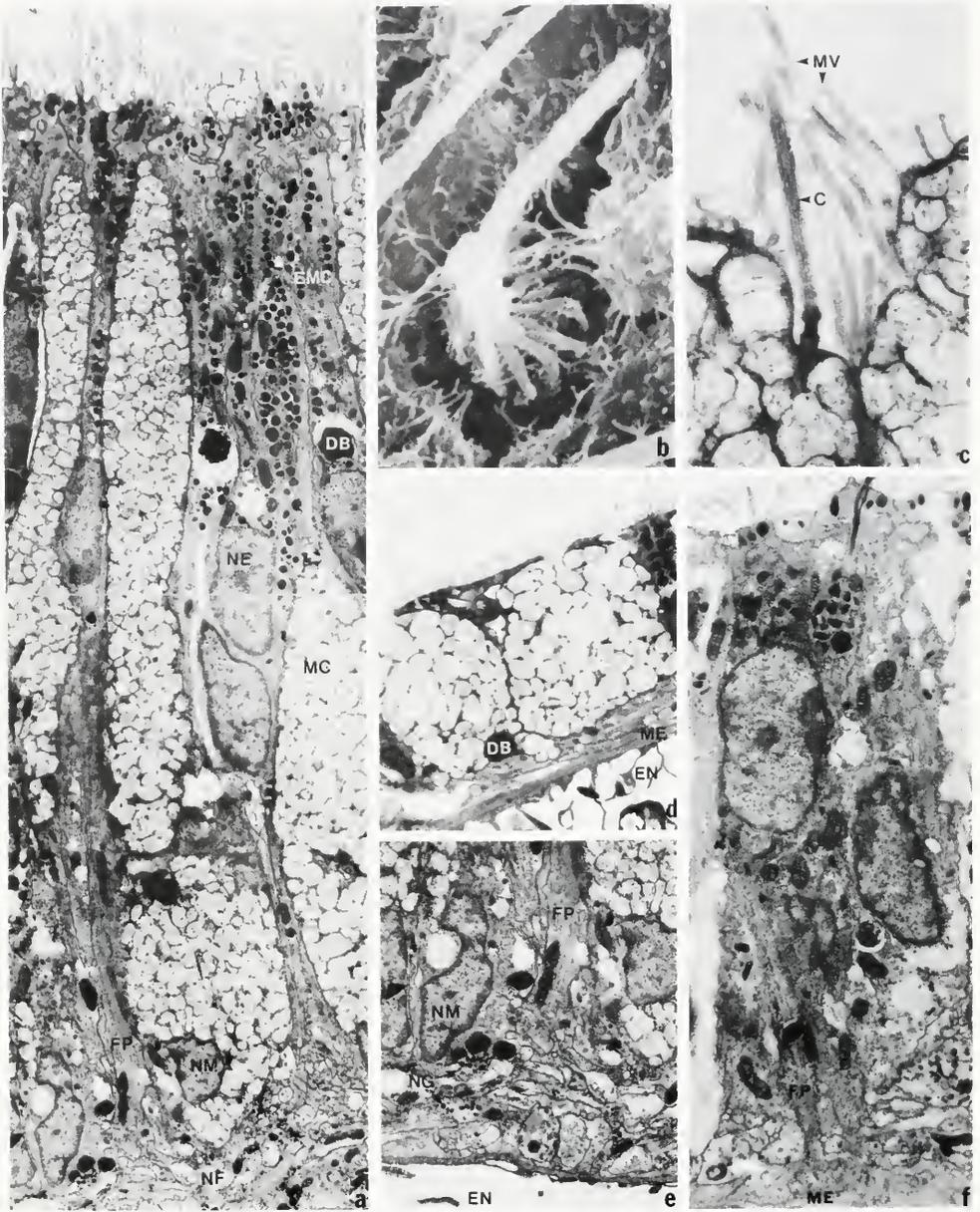


FIGURE 5. Gland cell. (A) Longitudinal section through the ectoderm of a planula at the blunt end (4462 $\times$ ). (B) Scanning micrograph of the cilium of a mucous cell on the surface of a planula. Note the basket of microvilli surrounding cilium, emerging from a concavity (17,113 $\times$ ). (C) Longitudinal section of a cilium and associated microvilli extending from depression in a mucous cell. Microvilli appear to emerge in groups from within crevices between mucous granules in the cytoplasm (11,198 $\times$ ). (D) Longitudinal section of two mucous cells near the tapered tip. Note that the concavities are rich in microvilli, that there is a large dense staining body at the base of one cell, and that both cells lie above a thin nerve layer and mesoglea (3631 $\times$ ). (E) A higher magnification of the basal ectoderm in (A). The mucous cells appear to be in close association with axons of nerve cells, both with and without neurosecretory granules. Microtubular foot processes of EMC's can also be seen extending down to the

is surrounded by a complex basket of elongated microvilli (Figs. 5B, C). This ring of microvilli extends well beyond the opening of the concavity and appears to emerge from between individual mucous granules (Figs. 5C). The light-staining mucoprotein granules, 0.7–1.3  $\mu\text{m}$  in diameter, are wrapped in a thin matrix of cytoplasm and fill the entire cell, except for a region at the base of the cell containing RER, several mitochondria, and dark-staining bodies (0.8–1.4  $\mu\text{m}$  in diameter) (Figs. 5A, G). The large, dark-staining, basal nucleus, 2.3–3.0  $\mu\text{m}$  long, is found in close association with various neurosecretory granules and microtubular extensions of the nerve layer (Figs. 5A, E).

### *Epitheliomuscular cell*

Epitheliomuscular cells (Fig. 5), the most common cells of the ectoderm (Fig. 2), are characterized by large membrane-bound, dense staining granules, a large medially located nucleus, a Golgi complex, numerous large mitochondria, and a single cilium with a 9 + 2 arrangement of microtubules, striated rootlet, and centrioles. The dense granules are concentrated in the apical cytoplasm. These may, however, vary considerably in size (mean: 0.6  $\mu\text{m}$ , range: 0.18–0.75  $\mu\text{m}$  in diameter), number (range: 15–48), and location. Large dark-staining bodies, often containing large white crystals, are occasionally found located near the nuclei of EMC's. Muscular feet at the base of the cell, rich in microtubules, spread out and interdigitate with nematocytes, nerve cells, and other EMC's (Fig. 5F). The cilia of EMC's are either in a concavity, approximately 0.7–1.0  $\mu\text{m}$  in diameter and 1.5  $\mu\text{m}$  deep, surrounded by a collar of cytoplasm or found emerging from a flat or slightly raised cell surface (Fig. 5F). Cilia in concavities are most common at the blunt end of the planula, whereas the flat or raised form is found distributed over the rest of the surface. No other morphological differences have been observed in EMC's displaying the two arrangements of cilia.

### *Neurosensory cell*

The sensory cell (Fig. 7) is the rarest cell type in the ectoderm, reaching its highest concentrations of the posterior (tapered) tip. The cell is characterized by an abundance of dense-cored neurosecretory vesicles approximately 70–100 nm in diameter, one or more prominent Golgi Complexes, a medially located nucleus, and a short cilium that emerges from a concavity in the cytoplasm (Figs. 7A, B). Neurosecretory vesicles are typically concentrated at the base of the cell (Fig. 7C). The cilium lacks any associated microvilli. Some ciliary rootlets are extremely long, extending in one case 9.8  $\mu\text{m}$  from the ectodermal surface to the top of the nucleus. The neurosensory cells at the tapered tip contain large dark-staining granules, approximately 0.2–0.45  $\mu\text{m}$  in diameter, in the aboral cytoplasm (Fig. 7B). Sensory cells along the lateral surfaces of the planula lack these granules and contain an electron-lucent cytoplasm (Fig. 7C). No other morphological distinctions have been observed between neurosensory cells with and without dark-staining granules.

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basal ectoderm (5348 $\times$ ). (F) Longitudinal section of an EMC along the lateral edge of the planula. Note that the cilium emerges from a slightly raised surface (6405 $\times$ ). C = cilium, DB = dark staining body, EN = endoderm, MC = mucous cell, ME = mesoglea, NE = nucleus of EMC, NF = nerve and foot process layer, NG = neurosecretory granules. NM = nucleus of mucous cell. Other labels as in previous figures.

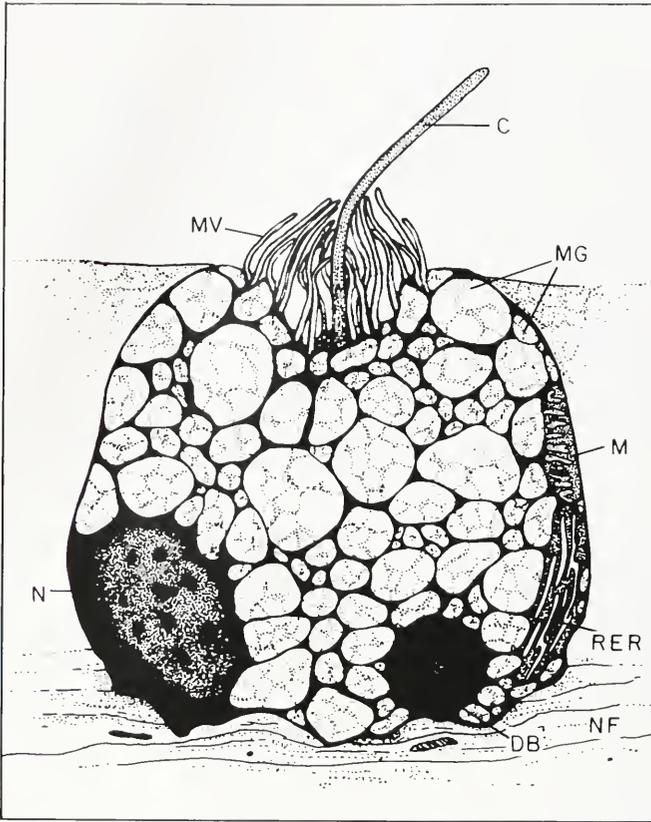


FIGURE 6. Longitudinal reconstruction of mucous cell extending from the outer ectodermal layer to the nerve layer. The mitochondrion and RER are characteristically located at the outer edge of the cytoplasm. MG = mucous granule. Other labels as in previous figures.

### *Nerve cell*

The nerve layer ranges in thickness from 1  $\mu\text{m}$  on the side of the planula to 6  $\mu\text{m}$  at the blunt end, where numerous EMC's and mucous cells are found. The nerve cell (Figs. 7, 8) is the only cell type in the ectoderm, besides the cnidoblast, that is oriented parallel to the mesoglea. The cell is characterized by an irregularly shaped nucleus surrounded by perikaryon with neurites extending along the mesoglea 0.3–0.5  $\mu\text{m}$  wide (Fig. 7D). A prominent Golgi Complex lies above the nucleus. Mitochondria vary in shape, from round in the perikaryon to extremely long in the neurites (Fig. 7D). Some neurites contain neurosecretory granules, approximately 70–100 nm in diameter, while others have only microtubules. It is not clear as to whether these different kinds of neurites occur in a single cell or whether they are derived from different cells. The dense inner core of the vesicles, found in both sensory and nerve cells, vary in both size and shape. Some have thin rectangular inner cores which are distinctly detached from their surrounding membrane, while others are entirely dense with no space between the membrane and the contents. Still others appear to have little or no contents. It is unknown whether this variation represents an artifact of fixation or significant differences in morphology, function,

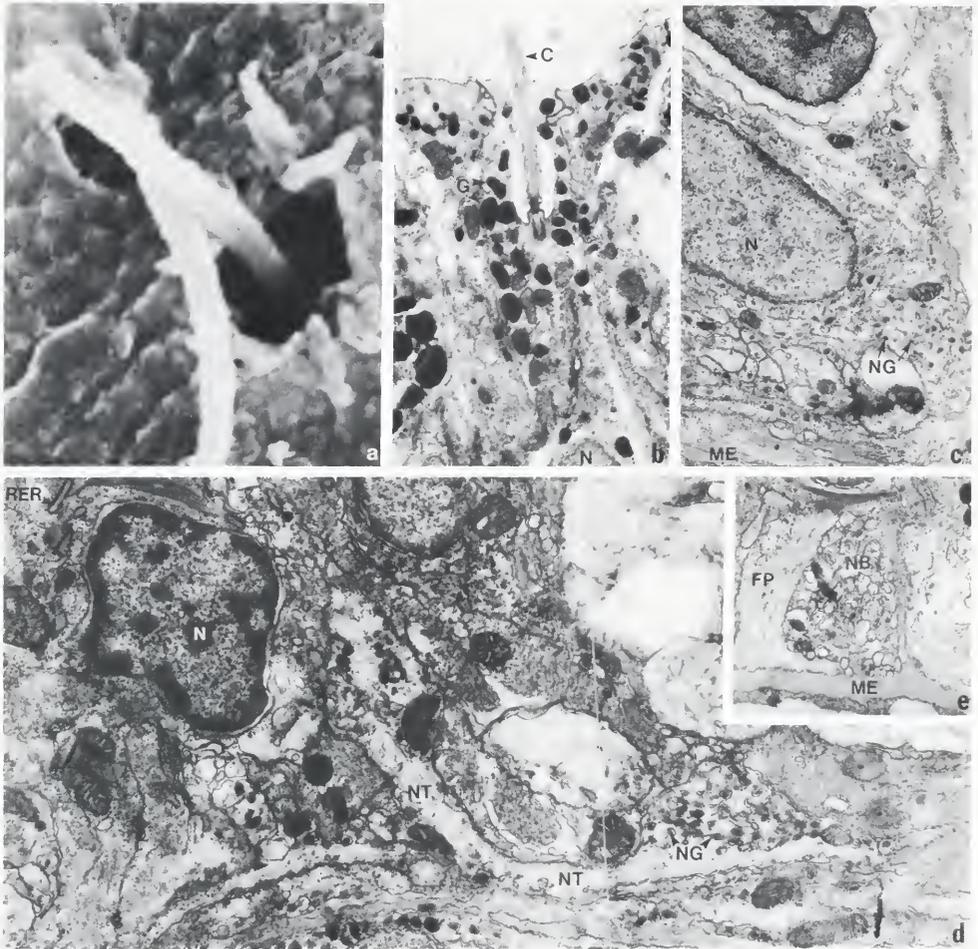


FIGURE 7. Sensory and nerve cells. (A) A scanning micrograph of a sensory cilium. Note its short length and that it emerges from a concavity (15,600 $\times$ ). (B) Longitudinal section of the apical end of a sensory cell at the tapered tip. Note the dark-staining granules in the apical cytoplasm (9521 $\times$ ). (C) Longitudinal section of the basal end of a sensory cell. The basal cytoplasm is rich in neurosecretory granules and can be seen interdigitating with neurites (9071 $\times$ ). (D) A nerve cell in the basal ectoderm. A neurite containing microtubules and neurosecretory granules extends into the nerve layer (15,350 $\times$ ). (E) Low magnification of a nerve bundle in cross-section in the basal ectoderm. The nerve bundle is surrounded by microtubular foot processes of EMC's (9720 $\times$ ). G = dark-staining granules, NB = nerve bundle, NT = neurite. Other labels as in previous figures.

or development. Neurites, often found packaged in large bundles (Fig. 7E), were observed in both longitudinal and cross-sectional sections. No cilium was found in serial sections of nerve cells.

#### DISCUSSION

While the ectoderm of the *Hydractinia echinata* planula possesses cells similar to those described for other cnidarian planulae, each cell type in *H. echinata* differs subtly from related cells in other species. A summary of the ectodermal cell types

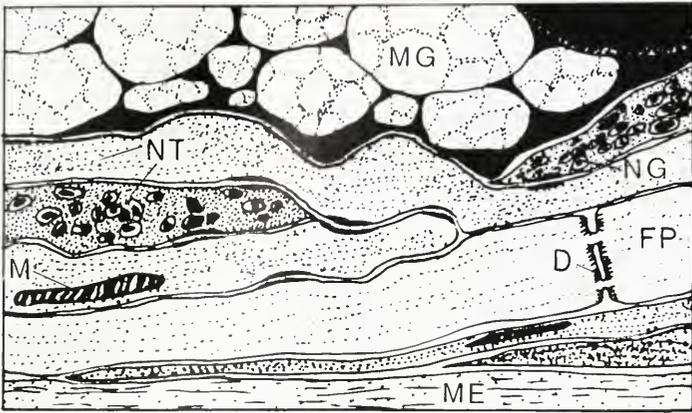


FIGURE 8. Reconstruction of nerve layer below a mucous cell. Neurites extend in both directions. Some contain microtubules, while others contain neurosecretory granules. Labels as in previous figures.

in cnidarian planulae, modified from Martin and Thomas (1977), is presented in Table I. Nematocytes, not studied in depth in many planulae, appear to have the same general cell structure as those described in adult hydroids (e.g., Chapman and Tilney, 1959; Slatteback and Fawcett, 1959; Lentz, 1966, and many others), but with different nematocysts. The supportive cell or EMC of *H. echinata* very closely resembles that of the hydroids *Pennaria tiarella* (Martin and Thomas, 1977) and *Mitrocomella polydiademata* (Martin *et al.*, 1983) in both cell structure and content. All three extend from the mesoglea to the ectoderm and contain locomotory cilia. The EMC's of *P. tiarella* planulae, as in *H. echinata*, contain dense, membrane-bound granules at their apex, whereas *M. polydiademata* lacks apical granules and possesses large basal ones. *Mitrocomella polydiademata*, however, does possess a cell containing dense staining granules which may also be a supportive cell. In contrast to these hydroids, the supportive cells of the four described anthozoan planulae all lack dark-staining granules. The cilia of supportive cells in *Balanophyllia regia* (Lyons, 1973), *Pocillopora damicornis* (Vandermeulen, 1974), and *Anthopleura elegantissima* (Chia and Koss, 1979) all contain collar cells consisting of an ordered, symmetrical arrangement of microvilli around the cilium and complex bands of microfilaments associated with the striated rootlet of the cilium. No such apparatus is found in *H. echinata* or other described hydroid planulae.

The mucous cell of *H. echinata* is also very similar to mucous cells found in *P. tiarella* and *M. polydiademata*, except that these hydroids lack the concavity for the cilium and the associated microvilli found in *H. echinata*. All four anthozoan species possess several gland cells, including an electron-lucent mucous cell. Those of *Ptilosarcus gurneyi* and *P. damicornis* lack cilia and microvilli, but contain granules similar in size and concentration to those of *H. echinata*. The four different gland cells of the anthozoan *B. regia* are ciliated and possess "randomly arrayed microvilli" (Lyons, 1973), but the microvilli do not appear to be arranged in as ordered a fashion as those in *H. echinata*.

The sensory cell of *H. echinata* planulae lacking apical granules has counterparts in the hydroids *M. polydiademata* and *P. tiarella*. Comparable sensory cells occur in only one of the four anthozoan species. Chia and Koss (1979) describe two sensory cells in the ectoderm of the anemone *A. elegantissima* that resemble the sensory cell in *H. echinata*. Of the two cells, one possesses an electron lucent

TABLE I

Distribution of ectodermal cell types\*

Taxa	Reference	Total # cell types	Number of ectodermal cell types							Uniformly ciliated
			Nemato-cysts	Supportive cells	Gland cells	Sensory cells	Nerve cells	Other cells		
<b>ANTHOZOA</b>										
<i>Anthopleura elegantissima</i>	Chia and Koss, 1979, 1983	11	3	1	3	2	1	1	1	yes
<i>Ptilosarcus garneyi</i>	Chia and Crawford, 1977	7	1	2	3	0	1	1	0	yes
<i>Balanophyllia regia</i>	Lyons, 1983	9	3	1	4	0	1	1	0	yes
<i>Pocillopora damicornis</i>	Vandermeulen, 1974	10	3	2	4	0	1	1	0	yes
<b>HYDROZOA</b>										
<i>Mitrocomella polydiademata</i>	Martin, 1983	6	1	1	2	1	1	1	0	yes
<i>Pennaria tiarella</i>	Martin and Thomas, 1977, 1981a,b, 1983	5	1	1	1	1	1	1	0	yes
<i>Hydractinia echinata</i>	This study	6	2	1	1	1	1	1	0	yes
<b>SCYPHOZOA</b>										
<i>Halichystus salpinx</i>	Otto, 1978	3	1	1	0	0	0	0	1	no
<i>Cassiopeia xamachana</i>	Martin and Chia, 1982	2	1	1	0	0	0	0	0	yes

\* Modified from Martin and Thomas, 1977.

cytoplasm without vesicles and the other contains large dense vesicles in the basal portion of the cell. These cells, however, are concentrated at the aboral end of the planula and are associated with the apical organ. The apical organ, with its associated cells and cilia, is far more sophisticated than any sensory structure in *H. echinata*. Nerve cells of *H. echinata* planulae closely resemble the sensory-motor-interneurons described by Westfall and associates (Westfall, 1966, 1970, 1973a, b, 1980; Westfall *et al.*, 1971; Westfall and Kinnamon, 1978; Kinnamon and Westfall, 1982), except that the cell apparently lacks a cilium. Tsuneki and Kobayashi (1977) describe two nerve cells in *Hydractinia epiconcha*, as had earlier workers with *Hydra* (e.g., Lentz, 1966, 1968). The nerve cell in *H. echinata* resembles a combination of the traits described for the two cells of *H. epiconcha*.

The spatial distribution of the various cell types suggests a strong functional specialization of different regions along the surface of the planula. The competent planula is held in an upright posture with the blunt anterior end attached to sand grains and the tapered posterior end suspended above the sediment-water interface (pers. obs.). The anterior end is characterized by a distinct dimple, surrounded by numerous EMC's and mucous cells. The dimple, similar to that described for the *Cassiopeia xamachana* and *P. gurneyi* but lacking in many other free-swimming planulae, likely functions as a suction cup allowing sedentary planulae to temporarily adhere to stable substrata. The tapered end of the planula, which must first interact with approaching hermit crabs, is especially rich in neurosensory cells. These neurosensory cells are adjacent to high concentrations of nematocytes. The spatial association of neurosensory cells and nematocytes is particularly significant, given Spencer and Arkett's (1984) observation that patterns of intercellular electrical communication may be effectively localized by non-overlapping distribution of cell types. Nematocysts have been implicated in the process of securing the planula to the shell (Lyons, 1973; Muller, 1973; Chia and Bickell, 1978). These suggestions are given further support by the common laboratory observation that planulae will adhere to moving objects (Chia and Bickell, 1978; pers. obs.). Surely the desmonemes with their thick, sticky thread might aid in contacting a shell, while isorhizas with their long barbed thread and stylet might aid in securing the initial attachment. Once on a shell, the planula must permanently attach and metamorphose. Martin and Thomas (1977) describe granules in EMC cells of *P. tiarella* similar to those found in *H. echinata* and note that these are absent in the adult, suggesting that apical granules are utilized in metamorphosis. The mucous cell of *Hydractina* are also concentrated at the blunt anterior end of the planula, where permanent adherence to a surface takes place. These cells possess a microvillar net, observed to control the release of mucous during metamorphosis by Crawford and Chia (1974), further implicating the mucous cell in attachment. The presence of dense staining granules of the EMC's in addition to mucous cells suggest the possibility of a "duo-gland adhesive system" as described by Hermans (1983).

While analysis of fine structure alone cannot insure accurate prediction of function, analysis of the fine structure and spatial distribution of ectodermal elements of the *H. echinata* planula suggest the following hypotheses regarding settlement: (1) the dimple of EMC's and mucous cells at the anterior end acts as a suction cup holding the planula upright on sand grains, (2) neurosensory cells sense the activities of nearby hermit crabs signaling a release of the suction, (3) the planula is drawn into contact with the crab by its feeding currents, initiating the discharge of nematocysts, and (4) the nematocysts adhere to the crab's shell facilitating the temporary attachment to the shell prior to eventual secretion by EMC's and/or mucous cells of substances used in permanent attachment.

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## LATITUDINAL DIFFERENTIATION IN EMBRYONIC DURATION, EGG SIZE, AND NEWBORN SURVIVAL IN A HARPACTICOID COPEPOD

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## ABSTRACT

We demonstrate significant genetically based differentiation in embryonic duration (h), egg size ( $\mu\text{m}^3$ ), and newborn survival (number/h) in the harpacticoid copepod, *Scottolana canadensis* (Crustacea), taken from a broad range of latitudes ( $^{\circ}\text{N}$ ) and reared in the laboratory for several generations under the same conditions. Egg development times of the northern-derived (ME) individuals were significantly longer at all test temperatures, and thus did not demonstrate compensation at low temperature. Maine development times may be due to the larger egg size.

## INTRODUCTION

Compensatory responses to cold temperature are common in poikilotherms (Bullock, 1955, 1957). Compensation is manifested in cold-adapted animals by an elevated physiological rate compared to those that are warm-adapted (yet see Barlow, 1961; Pickens, 1965). Adaptation to low temperature, involving metabolic acceleration, results in substantially increased energetic costs at high temperatures, which may result in little energy available for growth or reproduction (Levinton, 1983). This may lead to stabilizing selection for latitudinally fixed physiological rates and thus geographic variation in gene frequencies, or local evolution.

In marine invertebrate traits such as metabolic rate, thermal limits, and egg development, reversible and irreversible environmental effects may both be responsible for latitudinal (Schneider, 1967) or seasonal variation whereas genetic effects may be very minor (Vernberg, 1972; Landry, 1975). In contrast, other studies have suggested that phenotypic differences may reflect local genetic adaptation (Gonzalez, 1974; Bradley, 1975, 1978; Levinton and Monahan, 1983; Lonsdale and Levinton, in press). The sources of physiological and morphological differences between and within populations can be estimated from progeny reared in the laboratory under uniform environmental conditions. This method eliminates both the reversible and irreversible components of physiological adaptation. If a difference is then found among progeny reared under identical laboratory conditions it would suggest that the difference is genetical (Battaglia, 1957; Schneider, 1967; Antonovics *et al.*, 1971).

In this paper we present data that demonstrates genetically based differences in embryonic duration and egg size among latitudinally separated individuals of *Scottolana canadensis* (Willey), a harpacticoid copepod. Our results do not demonstrate compensation for temperature in egg development but we suggest that it may be masked by differentiation in egg size.

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## MATERIALS AND METHODS

*Field collections*

*Scottolana canadensis* is a brackish-water species (Coull, 1972), whose peak population growth is restricted mainly to late winter through early summer along the Atlantic coast (Willey, 1923; Lonsdale, 1981a; B. Coull, pers. comm.). Planktonic nauplii and epibenthic adults were obtained at five sites on the east coast of North America; pertinent collection information is listed in Table I. Separate collections consisted of 250 or more nauplii and a few adults except those for FL-1981 which consisted of 30–40 nauplii. Approximately 50–75% survived transport and reached reproductive age. All five collections appear to contain *S. canadensis* on the basis of morphology (B. Coull, pers. comm.) and reproductive compatibility, although the latter diminishes with distance between the locales (Lonsdale and Levinton, unpubl.).

*Culture methods*

In the laboratory, wild-caught copepods were cultured through one generation in 1000- and 2000-ml Erlenmeyer flasks containing Millipore-filtered ( $0.45 \mu\text{m}$ ) ambient estuarine water. Subsequently, water obtained from Stony Brook Harbor, New York, was used for culture after being glass-fiber filtered, adjusted to 15‰ with distilled water, and autoclaved. Algae were cultured at 20°C and 15‰ with a 14:10 hour light-dark cycle in f/2 nutrient medium (Guillard, 1975) and maintained in approximately a log phase of growth by harvesting and media addition five times weekly. Algae cultures used both in copepod culturing and experiments ranged in age from 5 to 14 days. Assessments of cell densities were made using a hemocytometer or Coulter counter.

A mixture of three algal species, *Isochrysis galbana* (ISO), *Pseudoisochrysis* sp. (VA12), and *Thalassiosira pseudonana* (3H), was added to each copepod culture flask four times weekly in 1981 and 1982; twice to obtain a minimum density of  $1.0 \times 10^5$  cells/ml and alternatively to achieve  $2.5 \times 10^5$  cells/ml. Previous experience

TABLE I

*Location and physical characteristics of collection sites for Scottolana canadensis*

Collection site	Latitude (°N)	Date	Temperature (°C)	Salinity (‰)
Tampa, FL	27	May 1981	27	18
		April 1982	24	18
		March 1983	19	18
Georgetown, SC	33	May 1981	24	15
Lusby, MD	38	May 1981	24	10
		May 1982	20	10
		May 1983	20	10
Mattapoisett, MA	41	June 1981	21	10
		June 1983	23	10
Biddeford, ME	43	June 1981	15	15
		May 1982	13	10
		July 1983	19	10

had shown that high rates of copepod mortality occurred when *Scottolana* were fed greater rations. Presumably, this was from the detrimental effects of substantial settling of algal cells. In 1983, the feeding routine was altered so that each culture reached a minimum density of  $2.5 \times 10^5$  cells/ml three times weekly.

Harvesting of the copepod cultures was begun when wild-caught females began to reproduce. The vast majority of nauplii (nauplius stages I–VI) were found in the upper water column and adults occurred on the bottom of the culture flask. In 1981 and 1982, nauplii were removed weekly by pouring off 25–30% of the culture water. Once a month adult densities were substantially reduced; 50–75% of the adults were removed but a major proportion of nauplii, those that passed through a sieve or were collected with the surviving adults, were retained. Retained nauplii, in addition to subsequently hatched nauplii, were not harvested until the second week following this procedure. At this time, 50% of the copepod culture water was replaced. Initially, some harvested adults were used to start replicate cultures or ones at other temperatures differing from ambient (15, 20, 25, or 28°C). This procedure allows an approximate calculation of the number of generations since a population was removed from the field. All cultures at a given temperature received equal feeding and harvesting treatments with the exception of the FL-1981 culture; nauplii of the first generation (f1) were not harvested and those from the second generation (f2) were used to start other cultures.

Because of coinciding experiments in 1983 that required the frequent (at least once a month) removal of a majority of gravid females in all cultures, it was not necessary to routinely split them as was done in previous years.

### *Experimental procedures*

*Egg development times.* One hundred and fifty to two hundred f2 nauplii (1981) were removed from each representative culture ( $n = 15$ ) and reared in 1000-ml beakers containing autoclaved, glass fiber-filtered sea water (15‰). They were fed an algal suspension of *I. galbana* and *T. pseudonana* in equal cell densities and adjusted to  $2.5 \times 10^5$  cells/ml four times weekly. From these cultures 50 mating pairs were removed and maintained under these same conditions until females began producing eggs. To determine egg hatching times, females were then placed individually in separate wells (1-ml volume) of a multi-depression dish placed in an airtight opaque plastic box. Distilled water in the bottom of the box reduced evaporation from the wells. The culture media, as above, was replaced daily. The location of boxes within an incubator was randomized with respect to locality. Females were monitored every four hours for the appearance of an egg sac and until the time of egg hatching. Approximately ten observations were made at 15 and 20°C while 20 were made at 25 and 28°C. Only three observations were made at both 25 and 28°C for the FL-1981 samples because females had little reproductive success. Also, FL-1981 hatching times were not determined at 15 and 20°C nor were SC-1981 at 15°C because these cultures were not established at the time.

The procedures were repeated for all populations in 1983, (excluding SC) using nauplii from cultures that had been in the laboratory for four to six months. Since in most cases there were not 50 mating pairs, the second step was eliminated; reproductive females for experimental monitoring were removed from the initial experimental culture and placed directly in the depression plates. Sufficient numbers of FL-1983 females reproduced during this series of experiments except at 15°C.

*Egg size.* To test for among-locale differences in copepod egg size, experiments were conducted in 1982 and 1983; the first was a preliminary study at 20°C, and

the second involved a range of temperatures (15–28°C). In the preliminary experiment, 150 to 200 f2 nauplii from three locales (ME-, MD-, and FL-1982) were removed from 20°C cultures and reared under the same conditions as nauplii used in the egg development studies. When females produced egg sacs, they were isolated in 50-ml Stendor dishes containing 20 ml of the same media and observed daily for newly hatched nauplii. Approximately 15 to 20 f3 nauplii from 10 females were removed and again reared in the same manner as the previous generation. Once mating was observed, the beakers were checked daily for the presence of egg sacs on females which were then removed and preserved in 5% buffered formalin. A time-limitation is necessary in order to minimize egg size increases from water uptake (Wittman, 1981). In the second series of experiments, females used in the above described 1983 egg development experiments also were preserved within 24 hours of extruding a second clutch of eggs.

The egg sacs were dissected and, when possible, egg dimensions ( $\mu\text{m}$ ) were determined for four to six eggs for each of 10 females from each locale and test condition. Egg volume was calculated after Allan (1984) by the formula: volume ( $\mu\text{m}^3$ ) =  $4/3\pi r_1 r_2^2$  where  $r_1$  and  $r_2$  are the long and short axis, respectively.

*Newborn survivorship.* One hundred and fifty to two hundred f4 nauplii from each 1981 locale (ME, MA, MD, SC, FL) were reared at 25°C using the same procedure as that for f2 nauplii used in the egg development studies. From these cultures gravid females were isolated and observed every 12 ( $\pm 2$ ) hours until the eggs hatched. The nauplii were individually placed in separate wells of a multi-depression dish as already described. From each of six families, six siblings were equally split among two food levels,  $2.5 \times 10^4$  and  $5.0 \times 10^5$  cells/ml;  $n = 18$  for each locale and ration. Media in the wells was completely replaced daily for nauplii and copepodites. When Copepodite I was reached, an additional 50% was replaced 12 hours later. The location of individuals in an incubator was randomized with respect to family, locale, and food density. Observations on stage of development (6 nauplius, 5 copepodite, and the last adult molt stage) and mortality were made every 12 ( $\pm 2$ ) hours.

## RESULTS

### *Egg development times*

Differences in embryonic duration (h) were not found within localities between years (1981 and 1983) for FL, MD, and MA eggs. (A Two-way ANOVA for year and temperature effects was computed for each locale.) But in 1983, ME eggs on the average took 10% longer to develop ( $P < 0.001$ ) at each temperature than they had in 1981. As there was no temperature-year interaction, we pooled development data for both years for ME as well as for the remaining locales. For all locales, including SC-1981 (One-way ANOVA), temperature was a highly significant factor ( $P < 0.0001$ ). The relationship between development time and temperature was best described by a linear regression for both ME and MA populations ( $r^2 = 0.83$  and  $0.85$ ; Fig. 1) and a log-log regression for MD, FL, and SC ( $r^2 = 0.86, 0.90,$  and  $0.71$ , respectively). For SC, the log-log regression was only marginally better, since  $r^2$  increased by less than 1%, because of the absence of 15°C data). We used a Two-way ANOVA for locale (excluding SC because of the absence of 15°C data) and temperature effects (15–25°C) to explore the possibility of genetically based latitudinal differentiation in egg development rate. Both variables and their interaction term were highly significant ( $P < 0.0001$ ). This analysis indicates both genetic differentiation of copepods among locales and a locale by temperature source of variance.

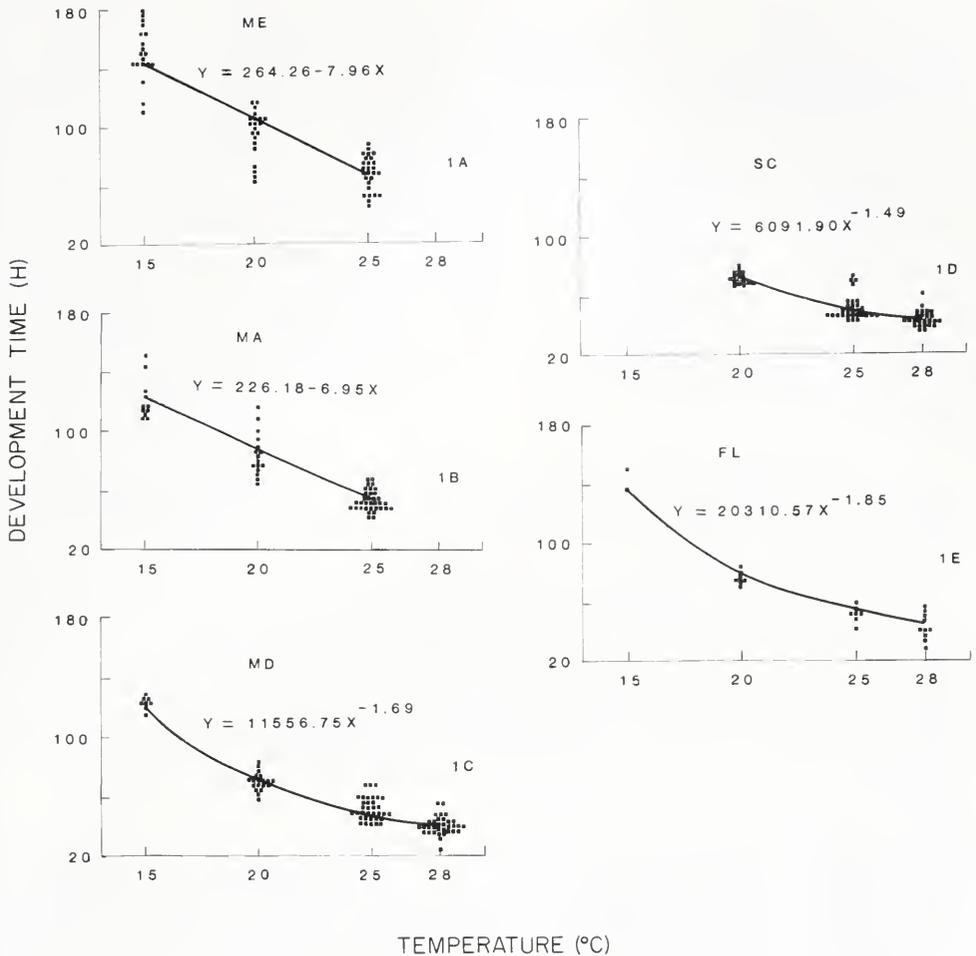


FIGURE 1. Egg development times (h) of *Scotollana canadensis* collected from five locales (ME, MA, MD, SC, FL) and tested at four temperatures (15, 20, 25, 28°C).

Differences in egg development time (h) between some locales were found at each temperature despite culturing under identical conditions (Table II; One-way ANOVA followed by a Studentized Range test; Snedecor and Cochran, 1967). Most striking was the slower egg development rate of the ME copepods at all temperatures tested (15, 20, and 25°C). We were not able to establish ME or MA cultures at 28°C. In contrast, MD, SC, and FL times are similar between 20 and 28°C. At 15°C, the mean hatching time of FL eggs is significantly greater than MD and SC, but because of the small sample size,  $n = 2$ , we are not confident in this result.

From these data, acceleration of egg development, as found for growth (Lonsdale and Levinton, in press), at low temperature is not readily apparent in high-latitude *Scotollana*.

#### Egg size

In the preliminary 1982 egg size study conducted on f3 females at 20°C, significant differences were found among the three test locales (ME, MD, FL), as

TABLE II

Mean egg development times (h) of *Scottolana canadensis* collected from five locales and reared at four temperatures ( $^{\circ}\text{C}$ )

Locale	Temperature ( $^{\circ}\text{C}$ )			
	15	20	25	28
ME	149.2 <sup>a</sup>	97.3 <sup>a</sup>	67.7 <sup>a</sup>	—
MA	123.1 <sup>b</sup>	85.8 <sup>b</sup>	52.7 <sup>b</sup>	—
MD	122.5 <sup>b</sup>	70.4 <sup>c</sup>	52.4 <sup>b</sup>	40.7 <sup>a</sup>
SC	—	71.9 <sup>c</sup>	51.0 <sup>b</sup>	43.9 <sup>a</sup>
FL	143.6 <sup>a</sup>	76.7 <sup>b,c</sup>	51.7 <sup>b</sup>	44.1 <sup>a</sup>

Means with identical superscripts (a, b, or c) are not significantly different (.05 level) at a given temperature.

well as among females within a locale ( $P < 0.005$ ; Nested ANOVA). Eggs of ME females were the largest, averaging  $1.56 \pm .12 \times 10^5 \mu\text{m}^3$  ( $\pm 95\%$  confidence), MD were intermediate,  $1.27 \pm .04 \times 10^5 \mu\text{m}^3$ , and FL were the smallest,  $1.07 \pm .07 \times 10^5 \mu\text{m}^3$ .

In 1983, both the latitude from which the copepod originated and the rearing temperature were significant variables affecting the mean egg volume of a female *Scottolana* ( $P < 0.001$  and  $P < 0.0002$ , respectively; multiple regression). As in the previous year, egg volumes of ME females were the largest, whereas there was no difference between MD and FL eggs (Fig. 2). The influence of temperature is only apparent between 15 and  $20^{\circ}\text{C}$ ; egg volumes are substantially reduced at  $20^{\circ}\text{C}$  in all populations. There is no further reduction in volume between 20 and  $28^{\circ}\text{C}$ .

#### Newborn survivorship

Newborn survivorship curves constructed from the copepod growth rate experiment conducted at  $25^{\circ}\text{C}$  and  $2.5 \times 10^4$  cells/ml are shown in Figure 3. At this ration, survivorship was poor for all five populations. At the termination of the

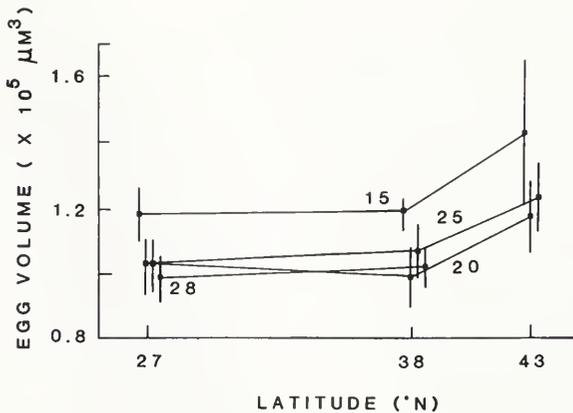


FIGURE 2. Mean egg volumes ( $\times 10^5 \mu\text{m}^3$ ;  $\pm 95\%$  confidence) of *Scottolana canadensis* collected from three locales (ME, MD, FL) and tested at four temperatures (15, 20, 25,  $28^{\circ}\text{C}$ ).

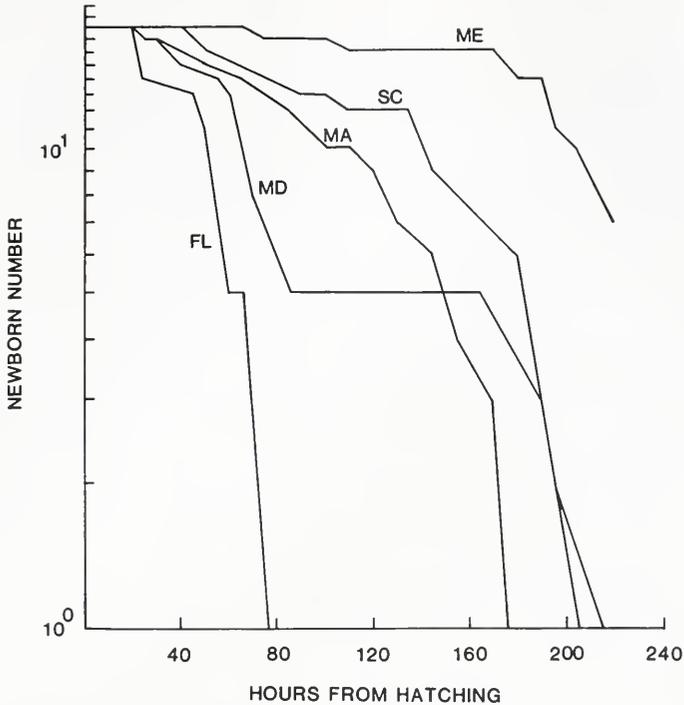


FIGURE 3. Newborn survival (number/h) of *Scottolana canadensis* collected from five locales (ME, MA, MD, SC, FL) and reared at 25°C and  $2.5 \times 10^4$  cells/ml.

experiment, 220 hours, no nauplii had reached Copepodite I, (ME nauplii had developed the fastest, reaching Nauplius V), and most nauplii were dead. Insufficient food levels were the cause of these results since sibs maintained at 25°C and  $5.0 \times 10^5$  cells/ml grew faster, many copepods had reached adult by 200 hours, and had substantially higher rates of survival. After 120 hours at the high ration, all newborns had survivorship rates of 94% or better and at 220 hours, all were greater than 70%; MD nauplii had the lowest rate of survival, 75%, followed by ME, FL, MA, and SC (78, 83, 94, and 100%, respectively). There is no latitudinal differentiation in survivorship at the high ration ( $P > 0.145$ ; Gehan-Wilcoxin test; SAS). In contrast, at the reduced ration, significant differences among survivorship curves emerge ( $P < 0.0001$ ). Maine nauplii had a higher survivorship rate compared to all other populations; 36% were alive after 220 hours whereas 100% mortality of FL nauplii occurred by 120 hours. Massachusetts, MD, and SC survivorship curves were similar among one another, and intermediate between those from ME and FL.

#### DISCUSSION

We show genetically based variation in three reproductive traits of latitudinally separated *Scottolana canadensis*: embryonic duration (h), egg size ( $\mu\text{m}^3$ ), and newborn survival (number/h). High-latitude copepods from ME produce eggs that take longer to develop and that are the largest when compared to all other east

coast copepods. Their nauplii also have the highest rates of survival when food is limiting.

Our data do not demonstrate acceleration in embryonic development for animals from low-temperature locales. Rather, the opposite is true because high-latitude eggs take a significantly longer time to develop. These results are in direct contrast to those reported for inter- and intraspecific embryonic development times in cold- and warm-adapted rotifers and copepods (Herzig, 1983a, b; yet see Hart and McLaren, 1978). Although there are many biological factors that can influence embryonic duration, such as DNA content of the dividing cells or cytoplasmic clocks (Horner and MacGregor, 1983; Hara *et al.*, 1980; respectively), we feel that the latitudinal differentiation in egg development times demonstrated in our study is a consequence of variation in egg size. Maine females produce the largest eggs, which in turn have the longest development times. It has often been observed that larger eggs have longer development times (McLaren, 1966; Corkett, 1972; Bottrell *et al.*, 1976; Steele, 1977; Hart and McLaren, 1978; Woodward and White, 1981; Clarke, 1982.). Several phenomena could explain this correlation but a common explanation is that the rate of carbon dioxide or oxygen diffusion in large eggs may be slower when compared to that for smaller eggs (Berrill, 1935; cited by McLaren, 1966; Clarke, 1982). Diffusion rates would then necessitate a lower metabolic rate (Corkett, 1972). This fact may explain why egg survival is most drastically reduced in species with large eggs when temperatures are elevated (Wear, 1974); diffusion rates may not be sufficient to meet the increased metabolic requirements.

The question then arises as to why ME females produce larger eggs. A positive correlation between egg size and body size is known in some copepods (Hart and McLaren, 1978). Thus ME females may produce eggs that are larger because of their larger body size at all test temperatures compared to FL females (Lonsdale and Levinton, in press). But body size alone cannot explain egg size differences between ME and MD females because their body lengths are similar. Variation in egg yolk content also can contribute to egg size differences. Our hypothesis is that ME *Scottolana* females produce eggs that contain more yolk than those from other locales (MA, MD, SC, and FL), resulting in eggs which are larger and which take longer to develop. At least four possible processes, listed below, may be operating to explain this pattern.

- (1) Differential dietary requirements among *Scottolana* populations; experimental algal diets may not be equal in nutritional value and insufficient maternal diets can reduce energy reserves in eggs.

- (2) Energy budget limitations imposed by local temperature conditions; higher environmental temperatures, as in FL, may result in less energy available for reproduction thereby resulting in females producing both fewer as well as less costly eggs.

- (3) Variability in primary productivity during the nauplius planktonic stage; restrictions in the more northerly latitudes may necessitate some degree of independence from environmental food resources.

- (4) Limited nauplius development time imposed by cold coastal temperatures; yolker eggs may shorten the planktonic phase and thereby decrease the probability of *Scottolana* being carried into the Gulf of Maine.

#### *Differential dietary requirements*

The diet used in both the egg development and egg size experiments has been shown to maximize growth and reproduction in MD *Scottolana* (Harris, 1977) but

it is equally good for ME, MA, and SC copepods. For example, in this study, the diet resulted in 88, 79, 75, and 87% successful hatching rates of clutches at 20°C for the four populations, respectively. We also have data (unpubl.) which show that the numbers of eggs produced by MD and ME females, corrected for body weight, are not significantly different at any test temperature (15–25°C) and greater than 80% of all adult females produce clutches when food is not limiting. (We did not test either MA or SC females for egg production.) It is apparent from these data that food inequities in the laboratory cannot explain the substantial differentiation in embryonic duration, egg size, and newborn survival, which are presumed to result from greater egg yolk reserves of the ME population relative to the remaining three (MA, MD, and SC) east coast *Scottolana* populations.

In contrast, FL females may have different dietary requirements. Florida females also are very fecund at all test temperatures (15–25°C), since greater than 80% of all adult females reproduce (Levinton and Lonsdale, unpubl.), but in this study at 20°C only 38% of the clutches produced resulted in viable nauplii. The experimental diet may be inferior if assimilation efficiencies are reduced in FL females but our data (unpubl.) show no significant difference in carbon assimilation efficiencies between ME and FL females feeding on *I. galbana* at 20°C. Diet also can affect reproduction if specific vitamin or sterol (lipid) requirements are not met. In many insects, particularly *Drosophila*, proteinaceous materials are necessary for yolk formation, whereas the metabolic pathways associated with this process depend on vitamin, sterol, and salt availability (Engelmann, 1970). Cholesterol has been shown not to affect the number of eggs laid by *Drosophila*, but its deficiency results in low hatching success (Sang and King, 1961; cited by Engelmann, 1970). Egg hatching success in our laboratory-reared FL populations has been inferior to all other populations throughout our three years of study. This does not necessarily mean that yolk content is reduced in the FL eggs, however. First, inviability could be due to male sterility from vitamin deficiencies (Geer, 1966; cited in Engelmann, 1970). But male sterility is most likely not important since our breeding experiments (unpubl.) have shown that FL females are no more fertile with males from other populations despite the fact that other populations have successful hatches more than 80% of the time. Second, if egg maturation is controlled by hormones, nutritional deficiencies may alter only the endocrine system and not yolk formation (Engelmann, 1970).

#### *Energy budget limitations*

At higher temperatures, proportionately less energy may be available for reproduction (Sebens, 1982; Barber and Blake, 1983; Page, 1983; yet see Glebe and Leggett, 1981). Increased temperatures may impose additional metabolic demands not met by increased feeding rates (Sebens, 1982; Levinton, 1983). Thus, reproductive energy constraints, mediated by the local thermal regime, may select for egg size and/or its yolk content in addition to egg number. But lecithotrophic reproduction does not necessarily require greater reproductive effort. In some cases related species show no significant difference in reproductive effort between reproductive modes and in others, planktotrophic effort may exceed that of lecithotrophic effort (DeFreese and Clark, 1983; Todd, 1979; respectively. In both papers, "effort" is defined as egg mass calories per adult calorie.) Given that higher temperatures can restrict the amount of energy available for reproduction, these findings show that it does not necessarily follow that individual eggs will have less allocated energy and thus temperature is not the ultimate factor operating to explain the patterns of differentiation found in this study among latitudinally separated *Scottolana*.

### *Variability in primary productivity*

Variability in primary productivity among locales also may explain our results. Thorson (1950) noted that Arctic marine benthic invertebrates produce large, yolky eggs whose larvae are without a planktonic phase while most temperate species produce eggs with very little yolk and subsequently, the larvae are planktotrophic. Although energetically expensive, yolky eggs have presumably evolved because primary productivity is seasonally restricted in high latitudes and developmental periods are longer, making successful planktotrophic feeding unpredictable if not impossible for many species. Todd (1979), studying two sympatric species of nudibranchs, also has suggested that lecithotrophy may have evolved "in order to offset the unpredictability of energy available for reproduction" (p. 57). In the laboratory, the reproductive strategy of *S. canadensis* is characterized by a long reproductive lifespan (>60 days at 20°C; Lonsdale, 1981b) and in the field gravid females have been found in late summer although planktonic nauplii are rare (Lonsdale, 1981a). Thus, ME nauplii from the first few clutches may complete development within the spring bloom period, but the later ones must contend with declining concentration and changing composition of algae as well as possible increased competition from other dominant copepods such as *Acartia* (Townsend, 1984; B. McAlice, pers. commun.; Lonsdale, pers. obs.).

The variable primary productivity hypothesis is supported by our data which show a correspondance between *Scottolana* embryonic development time, egg size, and the ability to survive as nauplii when food is severely limiting. Planktonic feeding must be a necessity for FL Nauplius II–III as they did not survive past these stages when reared at 25°C and  $2.5 \times 10^4$  cells/ml. In contrast, 36% of ME nauplii had reached Nauplius V at the termination of the experiment (220 h). Excess nutrient reserves sequestered in the cells or gut of newly hatched ME nauplii could explain these very different rates of survival. Other studies also have shown that egg size and larval survival are directly correlated in marine bivalves and yolk content is presumed to determine this relationship (Bayne *et al.*, 1975; Kraeuter *et al.*, 1982). It also is possible that the increased rates of survival in high-latitude forms under low food stress could result if their nauplii were more efficient filter-feeders but had metabolic costs which were similar to low-latitude nauplii. This alternative is not as reasonable given the larger body size of ME nauplii (Lonsdale and Levinton, unpubl.) and the observation that the optimal body size for maximum scope for growth decreases as food supply declines (Elliott, 1975; Vidal, 1980; Sebens, 1982).

### *Nauplius development time restrictions*

The Saco River, from which the ME *Scottolana* were collected, is characterized by extremely heavy freshwater flow as compared to other estuaries along the Maine coast (B. McAlice, pers. comm.) and it is highly likely that nauplii would be lost once carried into the Gulf of Maine due to rapidly declining temperatures (McAlice, 1981). Yolkier eggs may enhance the rate of nauplius development to Copepodite I, at which stage they migrate to the bottom, thereby increasing the probability of *Scottolana* remaining within the estuary. Our data reported in this paper indicate that when food is extremely limiting, ( $2.5 \times 10^4$  cells/ml), ME nauplii have the developmental advantage at 25°C. We also have data (unpubl.) which show a similar result at a less restrictive ration and lower temperature. We have found that at 15°C there is no significant ration effect ( $5.0$  versus  $1.0 \times 10^5$  cells/ml;  $P > 0.05$ ; One-way ANOVA) on total time from hatching to Copepodite I for either FL or

ME nauplii. However, at 20°C, the time is significantly increased at the lower ration for nauplii from FL but not from ME. At 25°C, the development time of nauplii from both locales is significantly affected but ME less so than FL (82 *versus* 98 h at 5.0 and  $1.0 \times 10^5$  cells/ml, respectively, for ME nauplii as compared to 85 *versus* 126 h for FL). Although ME nauplii are not at a developmental advantage at the high ration, since there were no significant between-locale differences at 15, 20, or 25°C ( $P > 0.05$ ; One-Way ANOVAs), we cannot rule out the possibility that ME females produce yolkier eggs which enhance nauplius development rates in order to minimize loss of offspring from the estuary. It is difficult to assess how well our laboratory food levels match that which copepods experience in estuaries.

We feel that either variability in planktonic productivity and/or nauplius developmental time restrictions may be operating to explain the patterns of differentiation in embryonic duration, egg size, and newborn survival among latitudinally separated *Scottolana canadensis*. All three traits can be affected by egg yolk content, and it is this latter trait which is being selected upon in response to environmental factors. Further inquiries into the extent of differentiation in egg yolk content among *Scottolana* populations is warranted.

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## THE NEUROBIOLOGY OF THE ECTONEURAL/HYPONEURAL SYNAPTIC CONNECTION IN AN ECHINODERM

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### ABSTRACT

The nervous system of echinoderms consists of two parts, the ectoneural and the hyponeural. The latter is purely motor in function and of mesodermal origin. It is separated from the main ectoneural nervous tissue by a true basement membrane of collagenous connective tissue. Previous anatomical work has suggested that a chemical synapse occurs across the basement membrane with the hyponeural neurons being post synaptic. The brittlestar *Ophiura ophiura* contains very large ectoneural interneurons and hyponeural motor neurons. This report describes for the first time the use of intracellular recording electrodes to dye-fill the cells and monitor action potentials and synaptic potentials. The synaptic morphology of both ectoneural and hyponeural neurons is described from dye-filled cells and shows that the large axons break up into a fine varicose plexus at the terminal regions. Preliminary recordings have been made from ectoneural neurons. Further work is required before function at the cellular level in this nervous system is understood. The report describes in detail, using simultaneous recording from two electrodes, how activity within the ectoneural system drives the hyponeural motor system and thus produces muscular contractions in the intervertebral muscles.

### INTRODUCTION

The major structures of the nervous system of eleutherozoan echinoderms consist of the radial nerve cords and the circumoral nerve ring. There are two distinct parts, separated by a basement membrane: the oral ectoneural region and the aboral hyponeural region (Hyman, 1955).

Previous accounts (see Smith, 1966) have described the circumoral ring as a C.N.S. which contains behavior-coordinating centers, but there is no physiological evidence for this and recent anatomical evidence does not support it (Cobb and Stubbs, 1982). A different organization based on anatomical descriptions and recent extracellular neurophysiological work has been proposed (Cobb, 1982). In this scheme it is suggested that whole animal behavior is coordinated by patterns of activity which may be generated peripherally in any one of the radial nerve cords and which activity is propagated virtually unchanged throughout the nervous system (see Stubbs, 1982; Moore and Cobb, 1985). The circumoral nerve ring thus acts as a relay between the arms and not as a centralized 'brain.'

The small size of echinoderm neurons has not previously allowed repeatable extracellular unitary potentials to be recorded and there are no previous reports of intracellular techniques being used. Recently, however, a system of giant fibers has been described in a species of brittlestar (*Ophiura ophiura*) with axons up to 10  $\mu$  in diameter. This is at least an order of magnitude larger than axons in other classes of echinoderm where they are very small ( $>1 \mu$ ) (Cobb, 1970; Cobb and Stubbs,

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1981; Stubbs and Cobb, 1981; Cobb and Stubbs, 1982). Although these cells are giant by echinoderm standards, they are still small relative to many in other invertebrate groups. These large neurons have allowed consistent extracellular recordings to be made of unitary activity, and thus make possible investigations into the mechanisms of behavior coordination and the sensory abilities of echinoderms (Brehm, 1977; Stubbs, 1982; Moore, in press, Moore and Cobb, 1985).

Each arm in an ophiuroid brittlestar contains a radial nerve cord with a swelling in each segment in the form of a complex ganglion (Fig. 1a). There are three major features to each ganglion: predictably located areas of neuropile, large axons running transversely, and large axons running longitudinally (for illustrations see Cobb and Stubbs, 1981). The neuropile is characteristic of echinoderms (see Cobb, 1970; Cobb and Pentreath, 1978) and consists of small ( $1\ \mu\text{m}$  or less) varicose axon endings filled with vesicles. There are no specialized synapses reported in echinoderms (Cobb and Pentreath, 1977). Previous anatomical studies of the 'giant' fibers failed to indicate either electrical or chemical synapses between them despite extensive serial section searches (Cobb and Stubbs, 1981). Each ganglionic swelling is joined to that of the next segment by a narrower region of the nerve cord containing only longitudinally orientated axons. The circumoral nerve ring contains giant axons which run circumferentially, connecting the radial nerve cords; it does not show the structure indicative of a complex ganglion (Cobb and Stubbs, 1982).

Associated with each ganglion swelling of the ectoneural radial nerve cord is the hyponeural tissue. This lies aborally and consists of two discrete groups of about 50 giant motor neurons either side of the midline. The hyponeural tissue of a particular segment is not connected to adjacent groups of neurons in hyponeural tissue in segments on either side. The hyponeural system is entirely motor in its function and its presence in the various classes of echinoderms is correlated with major skeletal muscle systems. It is unique in that it's mesodermal; it does not connect directly with the ectoneural nervous system. The two nervous systems are separated by a continuous layer of connective tissue which varies in width between several microns and about 40 nm. This is a true basement in the sense that it occurs between ectoderm and mesoderm cell layers. In regions where the width is above 40 nm there are substantial amounts of collagen fibrils with a basal lamina on either side. Where the basement membrane is thinnest at 40 nm, only the basal lamina is present. Figures 1a and b summarize these anatomical details.

The ectoneural nervous system is unusual in that it does not form neuromuscular junctions directly with most classes of muscle cells. The two main movement-producing systems in echinoderms which are muscle-operated are the tube feet of the water vascular system, and the skeletal system associated with articulating calcite ossicles. Anatomical evidence suggests that the muscles of the tube feet are controlled by a transmitter released across a connective tissue basement membrane from endings of nerves of the ectoneural system (Cobb, 1970; Florey and Cahill, 1977). The skeletal muscles are directly innervated by hyponeural motor nerves. Previous intracellular studies have shown that these skeletal muscles are innervated by the hyponeural motor system at one end, and that they propagate typical action potentials which are triggered by a typical junction potential (Cobb, 1968; Pentreath and Cobb, 1972). Degeneration and general morphological studies on the present species have shown that each giant motor neuron passes to the muscles to be innervated (the intervertebral muscles) and then divides to innervate one end of large numbers of small ( $5\text{--}10\ \mu\text{m}$  diameter) smooth muscle cells (Stubbs and Cobb, 1981). The motor endings are varicose and substantially smaller in diameter than the main axons; they do not form specialized neuromuscular junctions (see Cobb

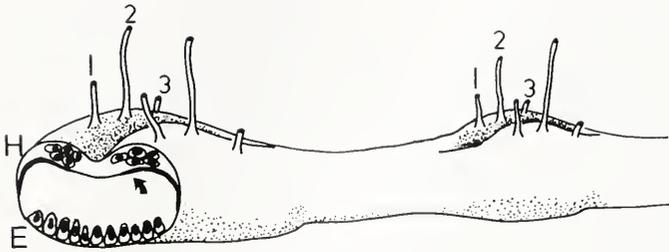


FIGURE 1a. The nervous system of a brittlestar consists, in part, of a circumoral nerve ring and five segmentally ganglionated nerve cords. Two such segmental ganglia are illustrated diagrammatically with one cut in transverse section. The cell bodies of the ectoneural nervous system (E) occur in a layer on the oral surface. The hyponeural motor neurons (H) have cell bodies in aboral swellings either side of the midline. The two nervous systems are separated by a connective tissue basement membrane of varying thickness (arrow). There are three main hyponeural motor axon branches (1, 2, and 3) arising from the swelling on either side of the mid line. These branches ascend aborally to innervate the large intervertebral muscles and to innervate connective tissue (see Wilkie, 1984). The detailed anatomy of this system is illustrated in Stubbs and Cobb (1981).

and Laverack, 1967; Pentreath and Cobb, 1972). Cobb and Pentreath (1976) described the fine structure of the chemical synapse across the basement membrane between ectoneural nerves and the hyponeural motor nerves. They showed that the basement membrane consists, over wide areas, of a thin basal lamina approximately 40 nm thick. The immediately adjacent ectoneural tissue is composed of a continuous layer of axon varicosities filled with vesicles. The hyponeural tissue adjacent is composed of small diameter nerve processes. Cobb and Pentreath proposed that a transmitter was released across the basement membrane by the accepted process of pre-synaptic vesicle exocytosis.

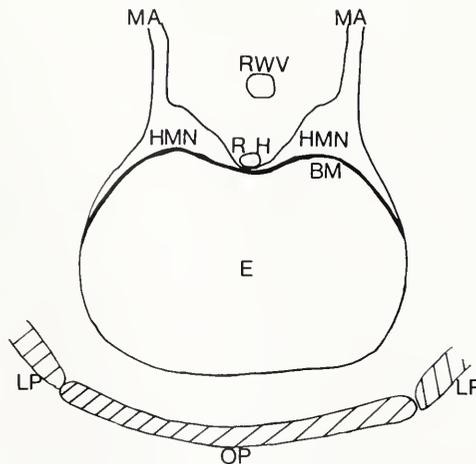


FIGURE 1b. Diagram of a transverse section through the oral part of the arm of a brittlestar. The ectoneural (E) radial nerve cord is covered by the epineural sinus which is enclosed by the oral plate (OP) and part of the lateral plates (LP). A connective tissue basement membrane (BM) separates the hyponeural motor neurons (HMN) from the ectoneural tissue (E). Two motor axon (MA) bundles lead to the radial hemal sinus (RH) and the radial water vascular canal (RWV).

The system of giant fibers in both the ectoneural and hyponeural systems of an ophiuroid has enabled intracellular recordings to be made. This report describes the way motor responses are produced to propagated patterns of activity by interaction between the ectoneural and hyponeural nervous systems.

#### MATERIALS AND METHODS

Large specimens of *Ophiura ophiura* were purchased from Millport Marine Station, Isle of Cumbrae. A single arm preparation was used and dissected from the oral surface to expose the ectoneural nerve cord or from the aboral surface for the hyponeural motor nerves.

Extracellular recordings were made using conventional polythene tipped suction electrodes drawn to a tip diameter of 200–500  $\mu$ . The two connective tissue sheaths, which in effect form the epineural sinus, were removed but otherwise the nerve cords were left *in situ*. Impalements were made with electrodes prepared from 1 mm diameter thin-walled glass tubing and pulled on a Campden 753 electrode puller. For intracellular dye injection, electrodes were filled with a 5% solution of Lucifer Yellow CH in 3 M LiCl and iontophoresed using 500 ms duration, 10 n amp, hyperpolarizing pulses applied at 1 Hz. Most preparations were examined fresh but some were fixed in 5% formol saline for 10 minutes and subsequently dehydrated and cleared in methyl salicylate. Physiological records were obtained using standard apparatus.

Three different methods of stimulation were used to produce propagated activity within the ectoneural tissue of the radial nerve cords:

(1) Photic stimulation was achieved by extinguishing a spot of light directed at a few peripheral segments on the arm. This action typically produces a burst of spikes which is conducted through the ectoneural nervous system (Stubbs, 1982; Moore and Cobb, 1985).

(2) Chemical stimulation was produced using solutions of the amino acids L-leucine or L-lysine at concentrations of  $10^{-10}$  applied with a dropper to the tip of the arm. This produced conducted activity within the radial nerve cords (see Moore, *in press*).

(3) Electrical stimulation: a suction electrode containing two insulated silver chloride wires was applied to an exposed peripheral part of the nerve cord. This stimulation produced unpredictable but large bursts of activity conducted within the ectoneural system. This stimulation, although non-specific, clearly was useful in that it produced substantial numbers of synaptic potentials within the hyponeural motor neurons.

#### *Interpretation of intracellular recordings*

Intracellular recordings from echinoderm neurons have not been reported previously. It is therefore necessary to establish the criteria for judging success with intracellular recordings and iontophoretic dye fills.

The main criterion for a successful cell impalement is a stable and appreciable negative resting potential. With these cells, resting potentials of at least  $-25$  mV, and on occasion as much as  $-60$  mV were recorded. All data reported here were obtained from 40 mV or better impalements. Lower resting potentials are usually associated with injury discharge, which is characterized by a continuous, high-frequency train of small, positive-going potentials. Larger resting potentials indicating better impalements show spikes up to 70 mV in amplitude, which, with a resting

potential of  $-60$  mV, implies a  $10$  mV over-shoot of zero. Such cells can be caused to spike using injected depolarizing current and, on rebound from hyperpolarizing current, they will also sometimes show spikes on stimulation of the peripheral radial nerve cord. These spikes are always of constant amplitude and timecourse. Spontaneous fluctuations in membrane potential of varying amplitude with a slower risetime than spikes were interpreted as synaptic potentials.

Lucifer Yellow was injected by iontophoresis into many neurons in which penetrations were deemed successful by the criteria outlined above. The period of dye injection varied considerably from cell to cell, and in some cases the cells were apparently only partially filled with dye. It is difficult to be certain that a fill is complete but fills were deemed partial if the dye faded in intensity to the point of invisibility while the neuron process remained relatively large in diameter. In contrast, fills were deemed complete if the Lucifer remained at high intensity while the neuron process itself diminished in diameter to the point of invisibility.

## RESULTS

### *Morphology of the ectoneural neurons*

Forty ectoneural cells were partially or completely filled with dye. All but three of these fills were of neurons that run longitudinally and complete fills showed that some of these neurons passed through two complete segments with a terminal plexus of varicosities at each end. The cell body is found at one end of the axon close to the varicose plexus (Fig. 2b.). Many of the fills, however, were partial with the cell body and varicosities visible at one end but the axon fading before reaching the other terminal region. In some fills the cell body was on the opposite side of the midline to the longitudinal axon. The remaining three cells were completely filled and showed a transverse orientation, with the main axon running across the ganglion and showing terminal varicosities either side of the midline (Fig. 2a.). The region of terminal varicosities in both types of neuron covers a relatively large area of each ganglion. This finding explains the failure to find synapses of any sort between the giant fibers themselves in the previous anatomical study and may well be of significance in understanding how integration takes place in the nervous system. The finding of longitudinal and transverse axons also fits the previous anatomical data. The varicose endings in some preparations were in a two dimensional layer at the depth of the basement membrane between the two nervous systems and this again fits the previous anatomical evidence of a complete layer of small varicose vesicle filled profiles in this region.

### *Physiology of the ectoneural neurons*

The ectoneural cells are loosely packed and are difficult to impale successfully for long periods. Impalements were made in both nerve cell bodies and axons (as the cell bodies are invariably on the surface, and the axons deeper, they are distinguishable). A  $-60$  mV impalement produces a spike of approximately  $70$  mV amplitude, *i.e.*, it overshoots zero by about  $10$  mV (Fig. 3c.). Some penetrations (about 1 in 50) are silent with a resting potential of  $-25$  mV or greater. These cells show no signs of synaptic potentials or injury potentials but will spike when depolarizing current is injected and also on rebound from hyperpolarizing current. Some will also spike when the nerve cord is stimulated peripherally (Fig. 3b.) and this is concomitant with extracellularly recorded spikes using a suction electrode. A

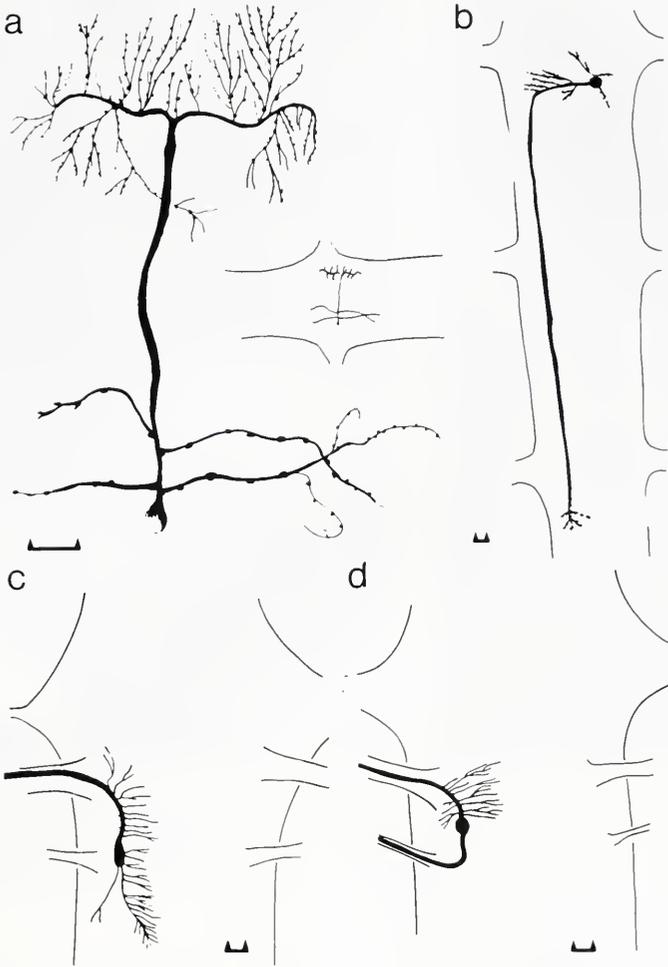


FIGURE 2a. Camera lucida drawing of a neuron filled with Lucifer Yellow in the ectoneural system, lying transversely across the radial nerve cord. Note the varicose terminals of branches. (Inset) The relationship of the neurone to a segment of the nerve cord. Scale =  $20\ \mu$ . b. Ectoneural neuron showing varicose terminals lying longitudinally within three segments of the radial nerve cord. Scale =  $20\ \mu$ . c. Hyponeural neuron showing non varicose fine branches that are post-synaptic dendrites. A single axon lies in the largest motor nerve branch to the muscles of the vertebral ossicles. The outline of a single segment is shown. Scale =  $20\ \mu$ . d. Hyponeural neuron similar to 2c, but with axons in both motor branches to the vertebral ossicles. The outline of a single segment is shown. Scale =  $20\ \mu$ .

small number of cells showed synaptic potentials and did not spike either to injected current or spontaneously (Fig. 3a.).

It is not possible with the small number of fills achieved at present to correlate structure with function, beyond the observation that the longitudinal axons show spike potentials and few synaptic potentials.

#### *Anatomy of the hyponeural neurons*

Usually, the fine dendritic processes arise directly from the cell body with the minimum of branching but generally cover quite a large area of the ganglion. One

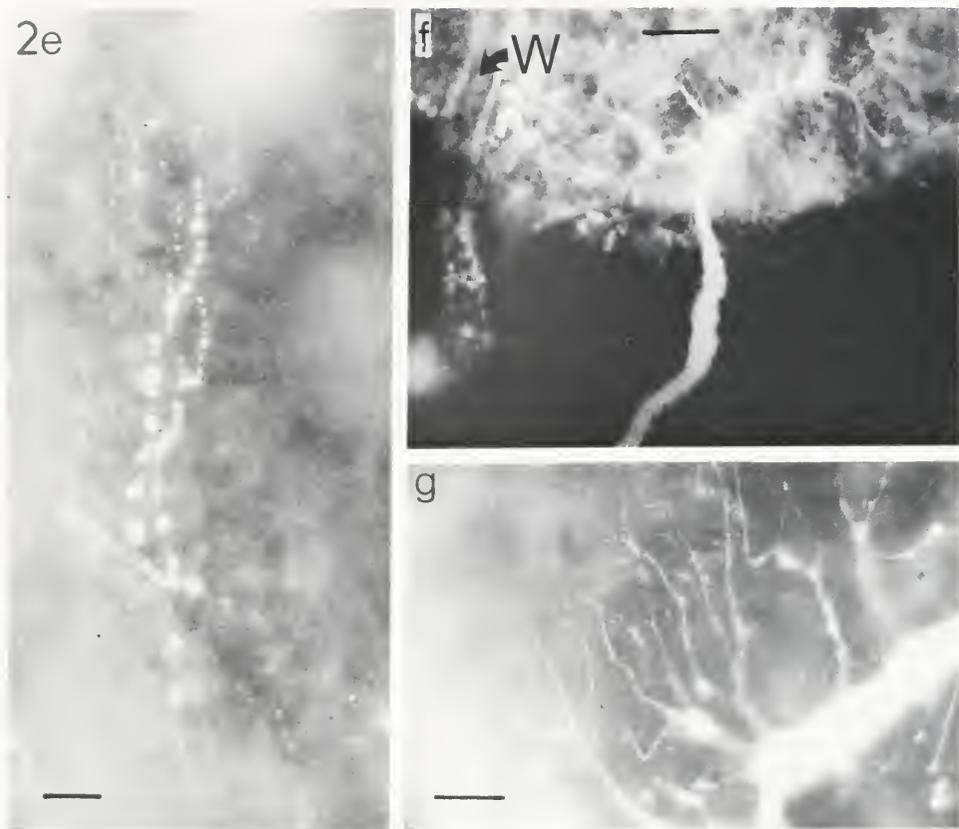


FIGURE 2e. Varicose endings in part of the Lucifer Yellow filled ectoneural neuron illustrated in 2a. Scale =  $10\ \mu$ . f. Dye-filled hyoneural neuron showing axon dissected free from ossicles. The non-varicose endings cover about 1/3 of the ganglionic swelling on one side of the midline. The segmental branch of the water vascular system is shown (W arrow). This cell is similar to that illustrated in 2c. Scale =  $40\ \mu$ . g. Detail of non varicose processes from a dye-filled hyoneural neuron. Note the fine branches leave the main body of the neuron directly. Scale =  $20\ \mu$ .

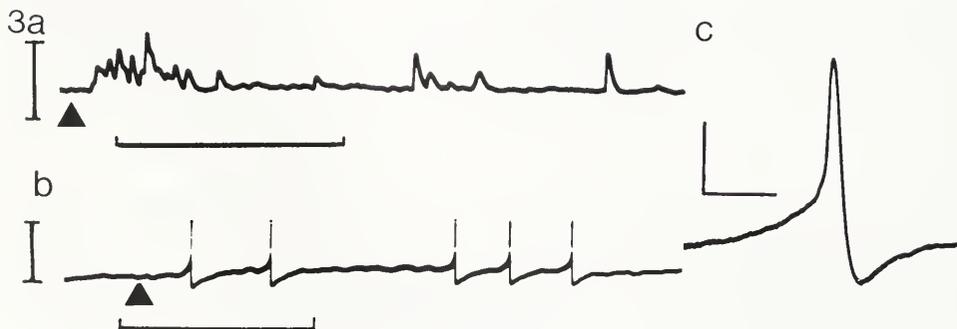


FIGURE 3a. Intracellular impalement of an ectoneural neuron showing post synaptic potentials to a photic stimulus to the arm tip (arrow). This cell did not show spike potentials when depolarising current was injected. Very few impalements of this type of non spiking cell in the ectoneural nervous system have so far been made. b. Longitudinal neuron penetration showing typical spike potentials to a photic stimulus (arrow) to the arm tip. Time scale = 1 s, vertical scale: a = 10 mV, b = 20 mV. c. Intracellular spike potential to photic stimulation filmed at a higher sweep speed to illustrate basic shape. Time scale = 20 ms, vertical scale = 20 mV.

hundred cells were filled with Lucifer Yellow and in no preparation were even the finest processes found to cross the midline or pass to the two adjacent segmental ganglia along the nerve cord. The cells appear either monopolar or bipolar in the sense that they give off either one or two large axons. These axons pass up one or two of the three motor trunks (see Fig. 1a). It is not possible to dissect the entire axon of a motor neuron away from the ossicle to show the region of the neuromuscular junctions, and any sectioning technique requires lengthy decalcification. However, the details of the neuromuscular junction have been worked out using other techniques (Stubbs and Cobb, 1981). The substantial dendritic branching of the hyponeural neurons immediately adjacent to the cell bodies are not typical of invertebrate motor neurons and more closely resemble some type of neuron from vertebrate C.N.S.

Lucifer Yellow was successfully injected into more than 100 hyponeural motor cells. This was deemed sufficient, because of the consistency of results, to identify the general morphology of these cells.

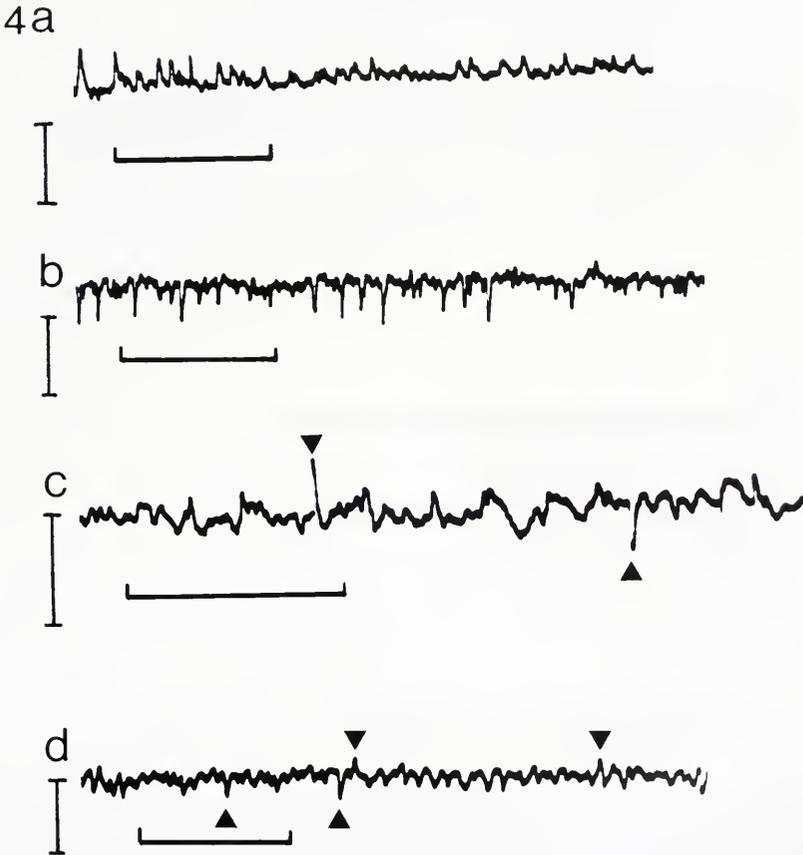


FIGURE 4a-d. Intracellular recordings from single hyponeural neurons showing the characteristic synaptic potentials present in all of many hundreds of impalements. The size and frequency of such events varied with time and from preparation to preparation. All cells impaled in a particular preparation at a particular time tended to show mainly either excitatory (4a) or inhibitory (4b) potentials. Long-term observation of such impalements occasionally show the opposite inhibitory or excitatory synaptic potentials (4c, d arrows). These events are caused by variable pre-synaptic activity in an unidentified class of ectoneural interneuron. Time scale = 1 s, vertical scale = 10 mV.

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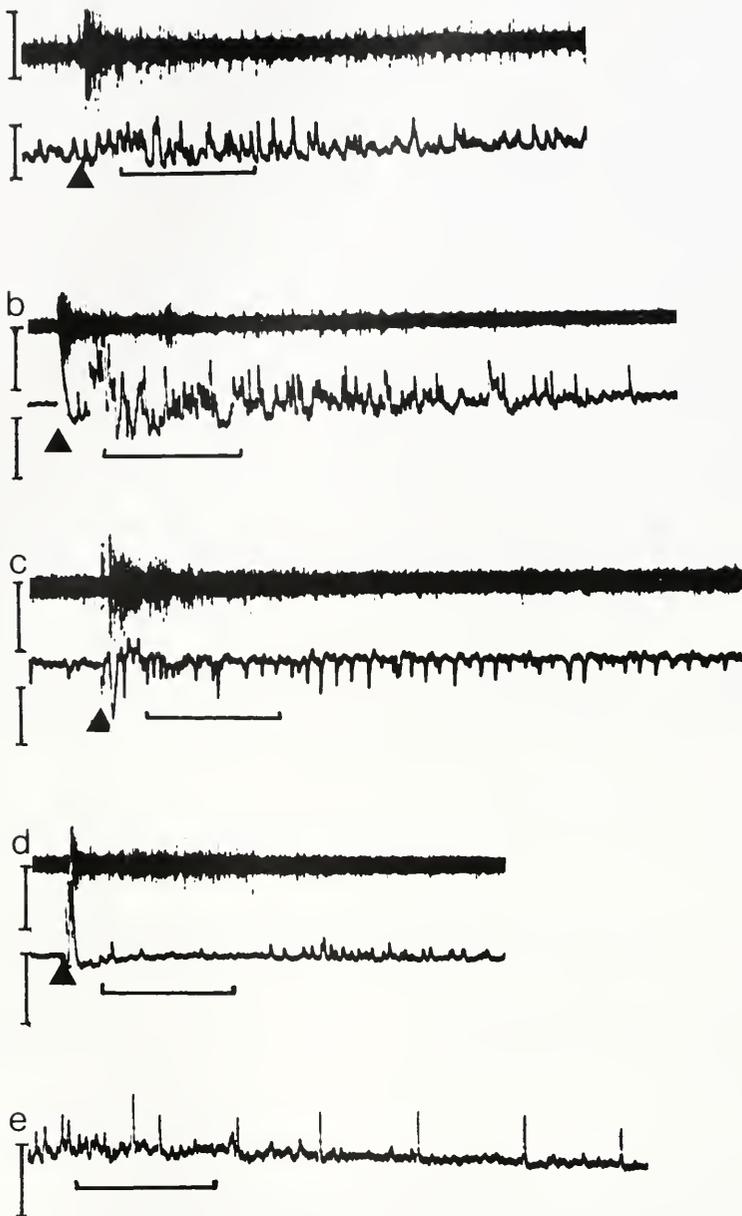


FIGURE 5a-e. Upper trace, extracellular record from ectoneural radial nerve cord showing the propagated burst of spike activity to photic or electrical stimulation (arrow) of the nerve cord six segments distal but which shows a similar pattern where ever it is recorded; lower trace, intracellular record of post synaptic potentials within a hyoneural nerve cell body. a. Excitatory post synaptic potentials to photic stimulation, note summation of potentials cause slight depolarization. b. E.p.s.ps to electrical stimulation, note more substantial summation causing depolarization. (There is an initial stimulus artifact when electrical stimulation is used.) c. Mainly i.p.s.ps.; note some initial summed hyperpolarization, then small e.p.s.ps. and finally a much increased rate of i.p.s.ps., (electrical stimulation). d. Summed e.p.s.ps. followed

*Physiology of the hyponeural neurons*

The hyponeural cells are easier to impale compared to ectoneural neurons. The point where the motor axons leave the ganglion can be impaled with careful dissection. When the region of the cell bodies is impaled injury potentials are never observed but the resting potential ( $-30$  to  $-60$  mV) always shows some small continuous fluctuation. These are present even in behaviorally quiescent preparations but in many cases are clearly inhibitory and excitatory synaptic potentials (Figs. 4a, b.). These cell bodies do not spike to injected depolarizing current or show rebound spikes to hyperpolarizing current. These hyponeural cell bodies do show a burst of synaptic potentials coincident with the burst of spike activity in the ectoneural tissue in response to stimulus. The synaptic potentials may be excitatory or inhibitory and can summate to give potentials up to 15 mVs (Figs. 5a-c.). Impalement of the motor axons often causes injury spike potentials and these axons can also be induced to spike by stimulation of the ectoneural system.

There is a tendency for a particular cell at a particular time to show either excitatory or inhibitory potentials but sometimes they show both (Figs. 4a-d.). Many hundreds of impalements on different preparations, which could be kept alive for 10-12 hours and stimulated using the three different parameters, show that the presence of excitatory or inhibitory synaptic potentials varies with preparation, time, and stimulus and is unequivocally a consequence of presynaptic ectoneural activity. Thus there is only one class of hyponeural motor neuron. The cell body region which is normally impaled is non-excitabile and spikes are initiated in an axon hillock region. The motor axons can also be impaled (Fig. 6a.). Some penetrations of cell bodies showed large rapid depolarizations which may be tonically conducted axon spikes which penetrate the cell soma but there is no absolute criteria to distinguish them from synaptic potentials (Fig. 5e.).

Each hyponeural motor neuron therefore receives both excitatory and inhibitory synaptic input across the basement membrane from ectoneural neurons. This synaptic input can, however, never be correlated directly with units in the propagated spike potentials that can be recorded extracellularly in the ectoneural systems (Fig. 5d.). The extracellular electrodes cover most of the nerve cord and it is likely that unitary potentials would be recorded from most large, longitudinally conducting, ectoneural axons. This lack of correlation implies that another class of interneuron within the ectoneural system is interposed between the longitudinally conducting neurons and the hyponeural motor neurons. The extracellular electrode is placed one segment peripheral to the ganglion impaled but the pattern of spikes recorded is similar in each segment (see Stubbs, 1982). The failure to record activity from this other class of ectoneural interneuron may be due to a smaller size of axon or even that these neurons do not spike.

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by a lowered level of activity, then increased e.p.s.ps. The ectoneural activity recorded extracellularly is in general coincident with synaptic activity but there is no correlation with individual spikes. This is an extreme example since the main burst of synaptic activity is coincident with ectoneural activity only marginally above the non-stimulated state. This implies at least one other class of ectoneural interneuron is interposed between them. Time scale = 1 s, vertical scale =  $40 \mu\text{V}$  (extracellular), 10 mV (intracellular). e. Activity from a stimulated preparation showing synaptic activity. The single large units may be attenuated soma spikes propagated tonically from the spike initiating site. Such potentials are common, especially to electrical stimulation, but there is no absolute criteria to distinguish them from large e.p.s.ps. Time scale = 1 s, vertical scale = 10 mV.

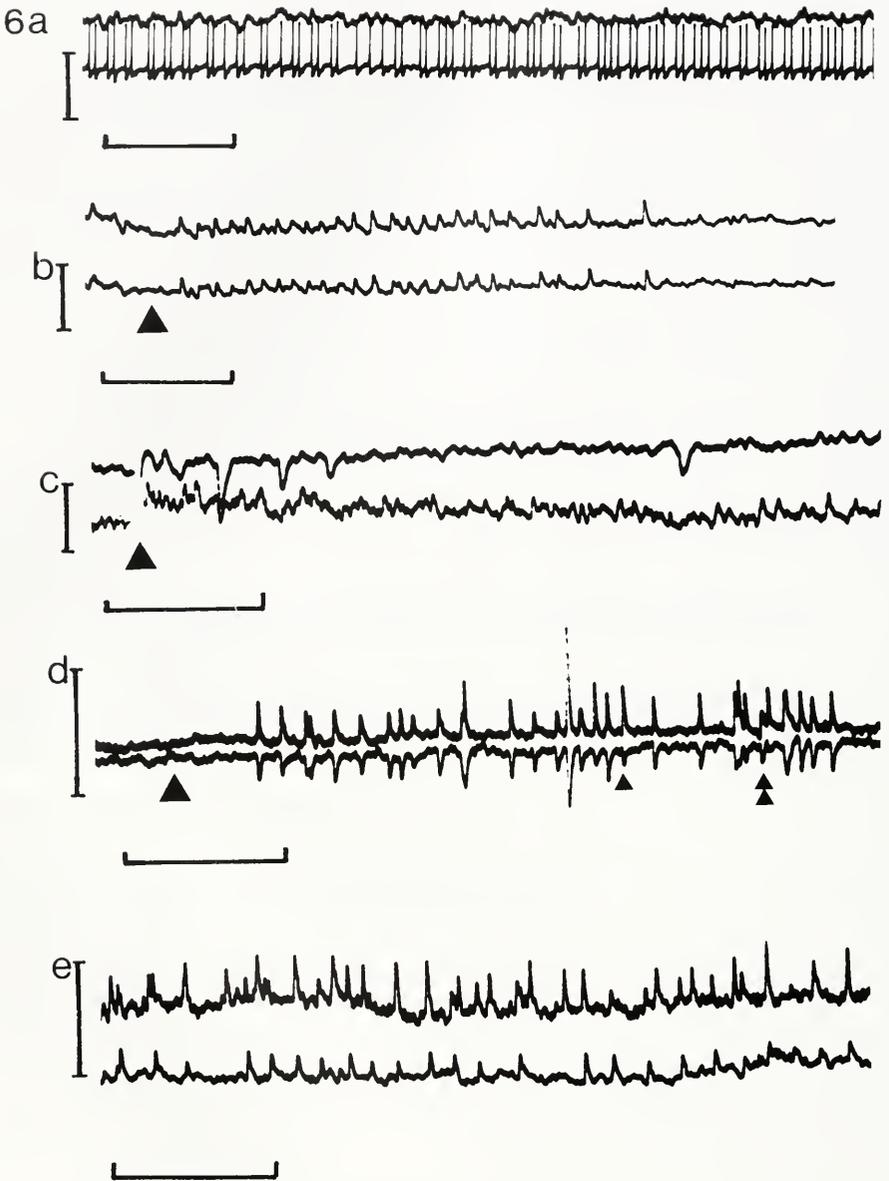


FIGURE 6a. Responses recorded intracellularly in two separate hyoneural motor neurons. Upper trace, small irregular post synaptic potentials from cell body; (bar = 5 mV); lower trace, spontaneous spike potentials from close to axon hillock region (bar = 40 mV). Spikes were never recorded from the cell body region either spontaneously or to injected current. b. Two cells impaled close together in the same segmental ganglion ipsilaterally (photic stimulation arrow). Injected current showed that these were not recorded from a single cell and repeated impalements of other cells in same area of such hyoneural preparations showed a strong correlation between recorded synaptic activity implying many of these cells received input from the same presynaptic units. c. Similar to (b) but cells impaled at opposite ends of a single ganglion ipsilaterally. In general the closer cells are together the greater the correlation in pattern of p.s.ps. (electrical stimulation arrow). d. Two cells impaled contralaterally in the same ganglion both with 60 mV resting potentials. One cell shows e.p.s.ps. and the other i.p.s.ps. Although the correlation is striking it is not an exact mirror image; analysis of responses over a number of seconds shows occasional

Experiments were initiated using two intracellular recording electrodes to compare responses of hyponeural motor neurons in different anatomical positions. Two cells were impaled simultaneously at varying distances apart within the same ganglion including the electrodes being on opposite sides of the midline. Cells in adjacent segmental ganglia were also impaled simultaneously, and in all cases responses to the same stimulus were recorded. Initially all cells impaled in dual electrode experiments were tested with 10 n amps of both depolarizing and hyperpolarizing direct current. In none of these experiments was there any sign that the same cell was impaled twice nor was there any indication that cells were electrically or chemically coupled. In these experiments, the cells of the pair where current was not injected showed no short or long-term changes in resting potential or change in the rate, polarity, or size of synaptic potentials already present. Previous anatomical studies of this region (Cobb, 1970; Cobb and Pentreath, 1976; Stubbs and Cobb, 1981) have not provided any evidence for such synapses at the ultrastructural level.

Experiments with two microelectrodes show that the closer the neurons are together, ipsilaterally in one segment, the greater the correlation in the pattern of junction potentials to stimulus (Figs. 6b, c.). If cells on opposite sides of the midline in the same ganglion are impaled simultaneously, then one cell may show purely e.p.s.ps. and the other i.p.s.ps. (Fig. 6d.). Cells impaled in sequential ganglia ipsilaterally show a looser correlation in the pattern of junction potentials (Fig. 6e.).

#### DISCUSSION

This study is the first stage in developing an understanding of function in the echinoderm nervous system at the cellular level. It has demonstrated the basic morphology of some echinoderm neurons. It has shown that there are fine plexuses of varicose endings at the terminal region of the large ectoneural interneurons. Lucifer fills have also confirmed the electron microscopical evidence for longitudinally and transversely orientated neurons. More work is required to show if these are the only major classes of neurons and to what extent they are divided structurally and functionally into further subclasses.

The manner in which echinoderm neurons make contact synaptically has not been well documented. The unusual contact between ectoneural and hyponeural neurons across the basement membrane shows no membrane specialization associated with it (Cobb and Pentreath, 1976). There are also no membrane specializations at any other chemical synapse between neurons or at neuromuscular junctions (see Pentreath and Cobb, 1972). There have been no published reports of electrical synapses that show the characteristics of gap junctions. Previous anatomical work on the giant fiber system (Cobb and Stubbs, 1981; Stubbs and Cobb, 1981) showed, using serial section analysis and electron microscope evaluation of critical sections, that the individual giant fibers could not be traced for long distances. They failed to show any structure which might be interpreted as either a chemical or an electrical synapse onto another neuron. By comparison with other invertebrate giant

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differences in amplitude (single arrow) and pattern of events (double arrow), electrical stimulation arrow. There is no evidence of any hyponeural axon crossing over between contralateral sides of the ganglia from Lucifer fills; these are undoubtedly separate cells. e. Two cells impaled in sequential segmental ganglia showing some correlation in patterns of synaptic activity as a burst of propagated activity caused by a remote previous chemical stimulus is conducted from ganglion to ganglion through the ectoneural nervous system. Time scale = 1 s, Vertical scale = 10 mV.

fiber systems, large electrical synapses might have been anticipated. The present study has, however, shown that the terminal regions at both ends of ectoneural and hyponeural neurons consist of a fine plexus of neuronal processes. In the case of the hyponeural motor neurons the present evidence shows that these fine processes represent a functional connection with ectoneural neurons.

It seems likely that many impalements with low resting potentials are poor quality with current leakage as confirmed by predictably smaller but undoubted spike potentials. Takahashi (1964) reported extracellular unitary potentials using metal-filled glass microelectrodes in the small fibered nervous system of an echinoid. This work has never been repeated despite very substantial efforts by the author and others using a wide range of refined extracellular electrodes. Suction electrodes, only, have been used successfully by Brehm (1977) and in this laboratory (Stubbs, 1982; Moore, in press; Moore and Cobb, 1985) to record unitary potentials extracellularly, and then only from the giant fibers of ophiuroids. It is therefore very unlikely from this evidence, and from the characteristics of impalements described in an earlier section, that extracellular field potentials from distant spike potentials could account for any of the synaptic activity described in the present paper. Even using small suction electrodes, the largest spikes ever recorded were a few tens of microvolts, and synaptic potentials recorded intracellularly from cells with a resting potential of 30 mV or more were several millivolts.

Function in the echinoderm nervous system can be simplistically divided into three parts. First is the neural mechanism that produces the pattern of behavior coordinating activity due to the local sensory input into a single ganglion. Next is the neuronal function that transmits coordinating activity from ganglion to ganglion. The final step is the production of motor responses within the hyponeural system at each segment in response to the through conducted patterns of activity. This report provides a preliminary description of the anatomy and physiology of this last part of these three general levels of integration. Figure 7 is a summary diagram of the structure of the neurons involved.

The hyponeural motor neurons are either monopolar or bipolar with fine non-varicose dendritic branching and the cell bodies are non-excitabile. The connection between the two nervous systems has been unequivocally demonstrated as a pre-synaptic plexus of small ectoneural endings adjacent across the basement membrane to a post-synaptic plexus of small hyponeural dendrites. This fits the previously somewhat puzzling findings of electron microscopical studies. Physiological connection between the two nervous systems has been demonstrated for the first time and shows that coordinated inhibition and excitation of the hyponeural motor nerves could, for example, produce the rapid arm flexures associated with escape behavior. This situation of a chemical synapse across a basement membrane is similar to that proposed to account for the innervation of the muscles of the ampullae and tube feet. There is anatomical evidence for this (Cobb, 1970; Florey and Cahill, 1977), and recently Florey and Cahill (1980) carried out a mathematical analysis of the various factors involved and showed that although the connective tissue in the tube foot can be as thin as 4–5  $\mu$ , transmission is feasible across as much as 25  $\mu$  onto the muscle cells. In the present situation only a 40 nm basal lamina is present over much of the ectoneural/hyponeural boundary, but in some regions the basement membrane can be several microns thick and is composed of collagenous connective tissue. The reason why a separate mesodermal nervous system has evolved and why the ectodermal nervous system never penetrates any mesodermal tissue is enigmatic. This poses a fascinating developmental question as to how the pre- and post-synaptic elements achieve the correct positional geometry separated by an uninter-

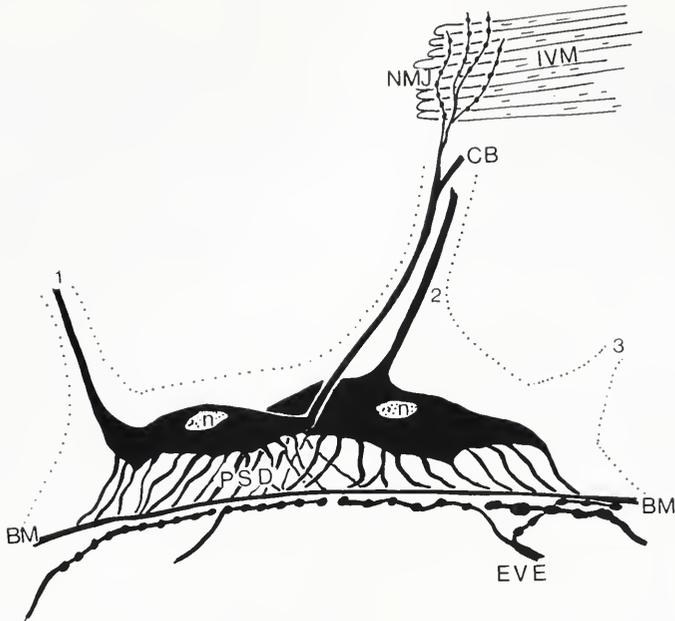


FIGURE 7. Summary diagram of structure of hyponeural motor neurons. Small varicose endings (EVE), form unspecialized synapses against a continuous connective tissue basement membrane (BM). Hyponeural motor cell bodies ( $n$  = nucleus) have a large plexus of post synaptic dendritic (PSD) processes which abut against the basement membrane. The motor cells are either unipolar or bipolar sending one or two branches up the three (1, 2, and 3) axon trunks to the intervertebral muscles (IVM). The motor axons branch, and also innervate the connective (CB) tissue via the juxtaligamentous ganglion. It is not known whether there is a separate population of motoneurons involved in this innervation or whether, as illustrated, the same neurons branch and reduce in size to form single varicose ending type of neuromuscular junction (NMJ). Not to scale.

rupted basement membrane. It has also been shown that whatever the reason for two totally separate nervous systems, the hyponeural is excited or inhibited in a relatively straight-forward way by the ectoneural system. That there is no direct correlation between individual units in bursts of ectoneural spikes that are conducted on through the nervous system and synaptic potentials in the hyponeural neurons implies that there is, at least, a second class of interneurons interposed between these two systems. These unknown interneurons can both excite or inhibit all the hyponeural motor axons in each ganglion depending on levels of excitation present. The records using two electrodes in the hyponeural cells in different positions show these ectoneural interneurons to have a fascinating and perhaps unique mechanism of function and overlapping fields of synaptic activity. It is not, however, worthwhile to speculate as to how coordinated motor output is achieved until more is known about these ectoneural interneurons. It is clear that the key to understanding the unique non-centralized nervous system of echinoderms lies in defining interneuron function within the ectoneural system.

#### ACKNOWLEDGMENTS

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## SARCOPLASMIC RETICULUM IN THE ADDUCTOR MUSCLES OF A BERMUDA SCALLOP: COMPARISON OF SMOOTH *VERSUS* CROSS-STRIATED PORTIONS

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### ABSTRACT

The adductor muscle of the Bermuda scallop, *Pecten ziczac*, is composed of two different types of muscle: cross-striated and smooth. The major portion consists of ribbon-shaped cross-striated muscle cells averaging about ten  $\mu\text{m}$  by 1.5  $\mu\text{m}$  in cross section. Each cell contains only one myofibril. In some of the wider cells, an extra sarcomere sometimes is inserted in the lateral part of the myofibril creating a vernier. The individual striated muscle cells do not span the entire length of the adductor but are connected at their ends via junctions similar to intercalated discs. The minor portion of the adductor muscle consists of smooth muscle cells which are fusiform averaging 6  $\mu\text{m}$  in diameter. There are no specialized cell surface invaginations in either muscle type that correspond to a T-tubule or caveolae system. The sarcoplasmic reticulum in both muscles is confined to the area just beneath the cell surface. In both muscle types, the sarcoplasmic reticulum systems have distended vesicles connected to the cell membrane via surface couplings. These vesicles are interconnected by one to seven tubular elements of the sarcoplasmic reticulum to form a closed and continuous system. The tubular elements of the sarcoplasmic reticulum in the cross-striated muscle are fairly uniform in bore, 40 nm in diameter, as compared to the irregular bore in smooth muscle, 15–40 nm. The striated muscle has twice as much of its surface covered with sarcoplasmic reticulum as does the smooth muscle. Moreover, the striated muscle cells have about 4% of their cross-sectional area devoted to sarcoplasmic reticulum while the smooth muscle cells have only 0.5%. This eight-fold difference in the amount of sarcoplasmic reticulum in the striated muscle is consistent with its reported 50-fold faster contraction rate and 128-fold faster relaxation time over that of the scallop adductor smooth muscle.

### INTRODUCTION

The adductor muscle bundle in the scallop consists of two different types of muscle. The larger part (about 90%) is translucent, is composed of cross-striated muscle cells (Marceau, 1909), and is responsible for the swimming action of the scallop (von Buddenbrock, 1911). The smaller, white part of the adductor consists of smooth muscle cells responsible for keeping the shells closed for long periods of time with low consumption of energy (von Buddenbrock, 1911) and has been termed a "catch" muscle (von Üxküll, 1912; Bayliss *et al.*, 1930). There is as yet no satisfactory explanation as to how the catch mechanism works (Johnson *et al.*, 1959; Lowy and Millman, 1963; Rüegg, 1971; Twarog, 1975). The scallop cross-

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striated adductor contracts at a rate that is fifty times that of the smooth adductor and relaxes after a contraction at a rate that is 128 times faster than the smooth part of the adductor (Prosser, 1973). The present study examines the structure of the sarcoplasmic reticulum in the cross-striated and catch muscles and provides an analysis of the amount and distribution of the sarcoplasmic reticulum in the two parts of the adductor. The results provide a morphological basis for the different contraction and relaxation rates of the two muscle types.

#### MATERIALS AND METHODS

Bermuda scallops, *Pecten ziczac*, were collected from Harrington Sound and kept in aquaria at the Biological Station until used. The scallops were taken out of the water and gently shaken to remove the sea water inside the shell and then placed in sea water (22°C) containing 4% glutaraldehyde (Polysciences, Inc., Warrington, PA). The pH of the glutaraldehyde-sea water was 7.5 and was not adjusted. After 4 to 24 hours the solution was changed and fresh fixative was added.

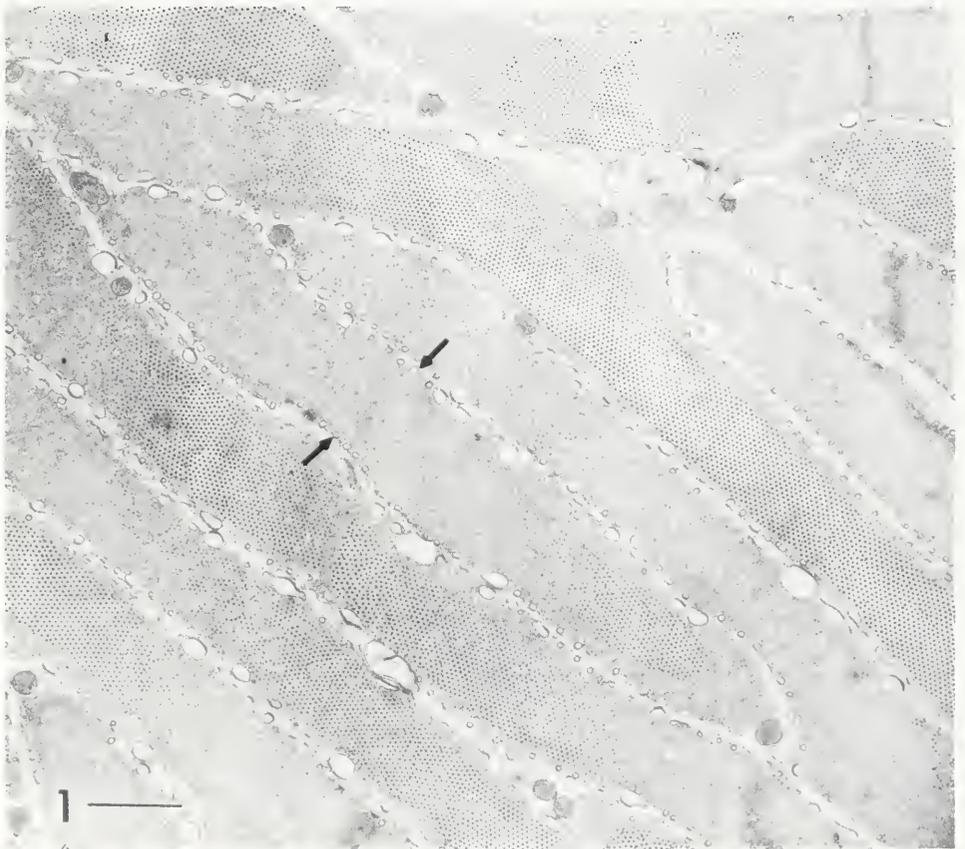


FIGURE 1. Cross-section of striated muscle reveals ribbon shape of myofibrils. Sarcoplasmic reticulum (arrows) are localized just under the cell surface. Scale = 1  $\mu$ m.

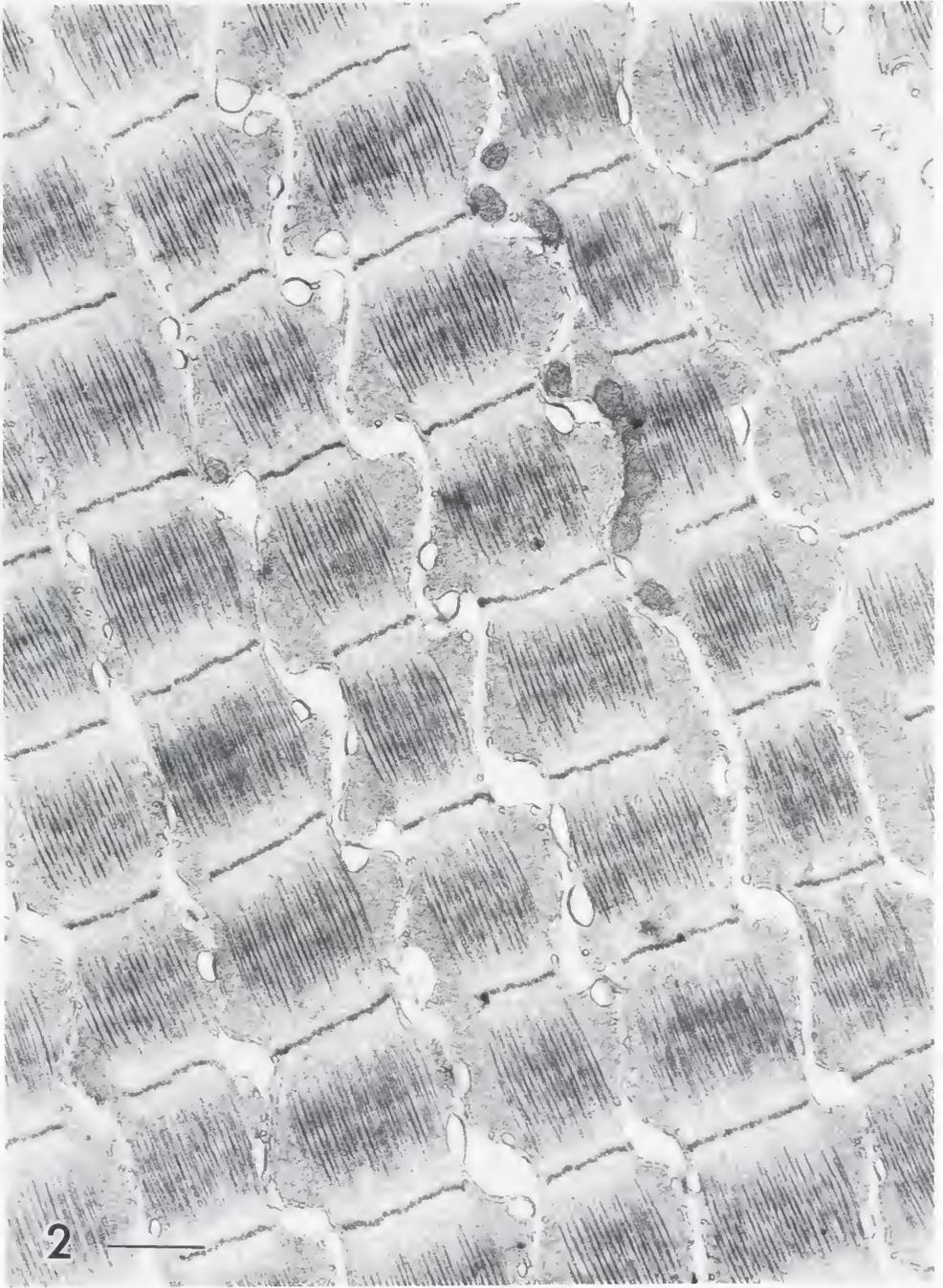


FIGURE 2. Longitudinal section of striated muscle illustrating the narrow part of the ribbon shaped cells. Each cell contains one myofibril. Scale = 1  $\mu$ m.

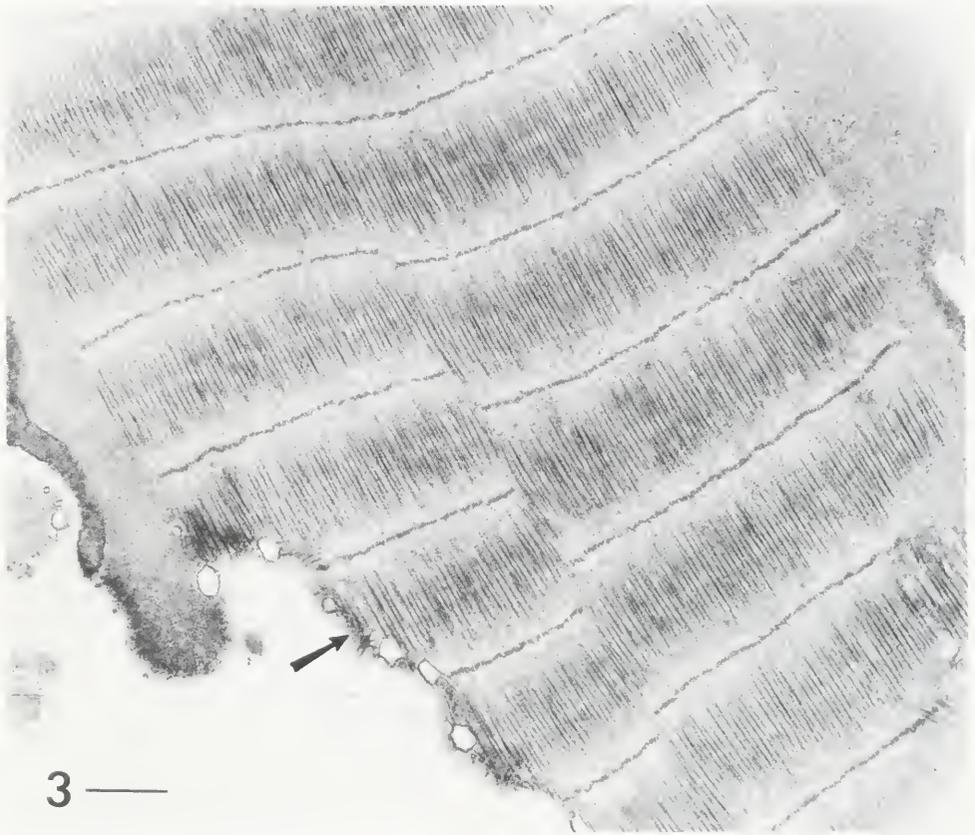


FIGURE 3. Demonstration of a vernier in which an extra sarcomere (arrow) is inserted on the left side of the myofibril. This longitudinal section illustrates the wide part of the ribbon-shaped cell. Scale = 1  $\mu$ m.

The total time in glutaraldehyde fixative was one to two days. The shell was removed from the muscle and the two parts of the adductor, smooth and cross-striated, were separated from one another. Small pieces were cut from each muscle bundle and washed several times over a four to six hour period with filtered sea water and then fixed in osmium tetroxide (1% in 0.1 M phosphate buffer, pH 6.0), in the cold (4°C). The muscle was rinsed at room temperature many times over a one hour period with distilled water, stained *en bloc* with an aqueous solution of uranyl acetate (0.25%) for two to four hours and then rinsed several times with distilled water before being dehydrated in an ethyl alcohol series and embedded in Epon (Luft, 1961). Thin sections were stained with freshly filtered 4% aqueous uranyl acetate solution, followed by lead citrate (Reynolds, 1963) and examined with a Philips EM 201 electron microscope (provided on loan to the Bermuda Biological Station courtesy of Philips Inc. and Lico, Inc.).

Measurement of the amount of sarcoplasmic reticulum was calculated from electron micrographs of cross-sections of the smooth and striated muscle cells. Thirty micrographs, each a cross-section through a different cell (15 smooth and 15 striated), were printed at the same magnification on 11"  $\times$  14" photographic paper. Each cell in the cross-section was then cut out of the paper along the outer cell

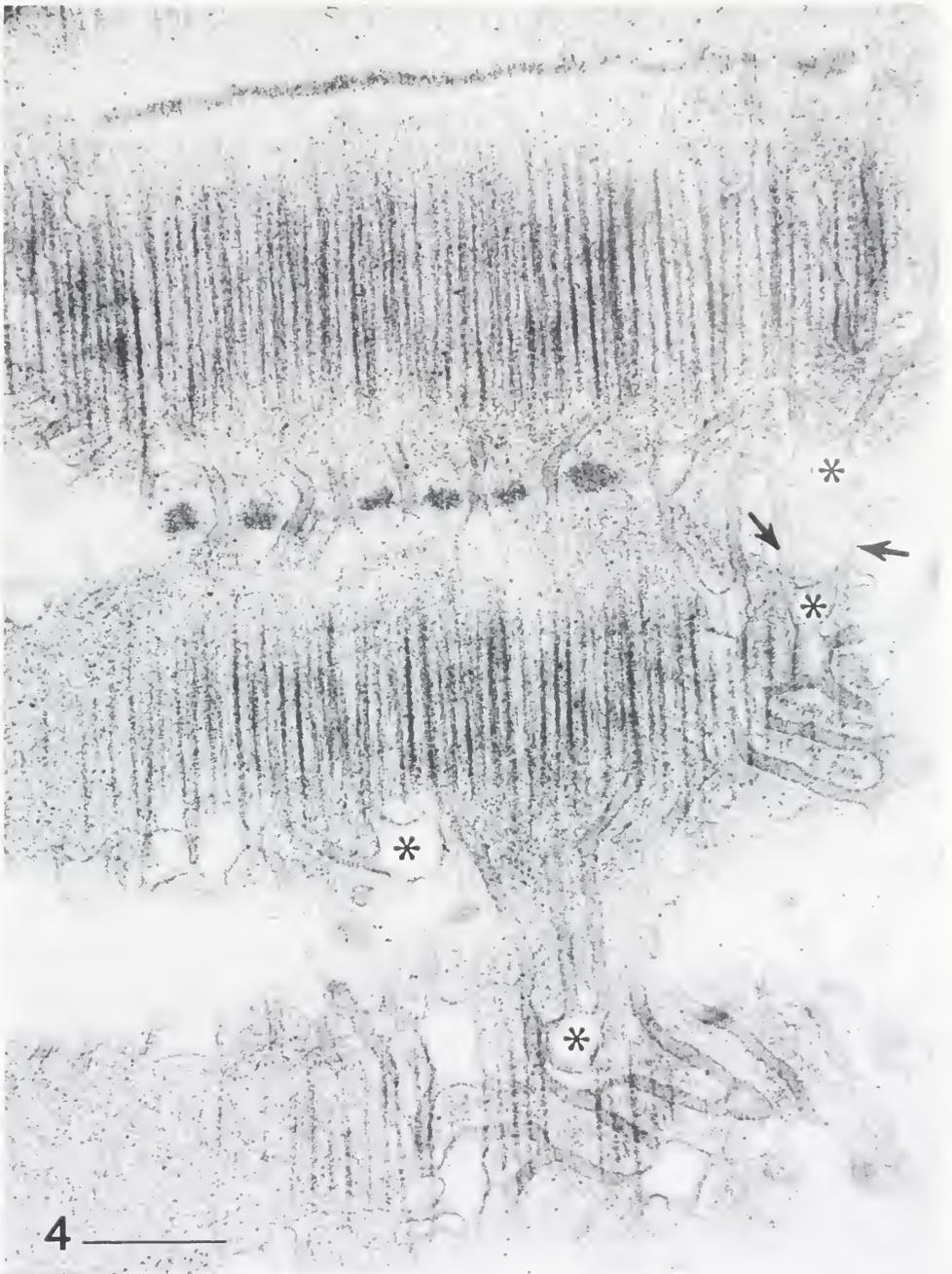


FIGURE 4. The terminal cisternae (asterisks) of the sarcoplasmic reticulum are interconnected by a series of tubular elements (arrows). Scale = 0.5  $\mu$ m.

membrane and the cell profile weighed. An Exacto knife was then used to cut out the elements of the sarcoplasmic reticulum from beneath the outer membrane of the cell profile, and the sarcoplasmic reticulum profiles were then weighed. The

ratio of sarcoplasmic reticulum weight to cell weight was considered to represent the percent of cross-sectional areas of the cell occupied by sarcoplasmic reticulum.

## RESULTS

### *Cross-striated adductor*

The cross-striated muscle cells of the Bermuda scallop, *Pecten ziczac*, are ribbon-shaped, ranging from 0.9 to 2.0  $\mu\text{m}$  (ave. = 1.5  $\mu\text{m}$ ) in thickness and from 3 to 20  $\mu\text{m}$  (ave. = 10  $\mu\text{m}$ ) in width (Figs. 1, 2). They do not run the entire length of the adductor but are connected via junctions between the terminal Z-bands. The junctions are staggered along the length and width of a cell to form interdigitating boundaries similar to those found in the intercalated discs in cardiac muscles. Each cell consists of one myofibril and in some cells an extra sarcomere is inserted laterally creating a vernier effect (Fig. 3). There seems to be only one elongated nucleus per cell and it is located at the periphery of the cell.

The sarcoplasmic reticulum is confined to the area just beneath the cell surface and can be observed most clearly in tangential sections cut just below the plasmalemma (Figs. 4, 5). There are no transverse tubules or sarcoplasmic reticulum in the interior of the cell. The sarcoplasmic reticulum consists of two units: flattened vesicles and tubes linking the vesicles to form a closed system. The vesicles are about 0.16  $\mu\text{m}$  in width and up to 0.28  $\mu\text{m}$  in length and are interlinked by 2-7 tubular elements, all of which have a uniform bore of about 40 nm. Infrequently, one tube terminates in another tube and in a few cases vesicles fuse to each other directly. The sarcoplasmic reticulum vesicles and tubes are scattered all along the surface of the sarcomere with no periodic arrangement at a particular band of the sarcomere. In cross-sections, surface couplings between the vesicles and the cell surface were observed (Fig. 5), but only occasionally were couplings seen between the smooth tubes and the cell surface. Both vesicles and tubes had fine granular material associated with their luminal wall membrane. Together, the vesicles and tubes comprising the sarcoplasmic reticulum accounted for about 4% (average of 15 cells) of the non-nuclear cross-sectional area of the cross-striated adductor cell.

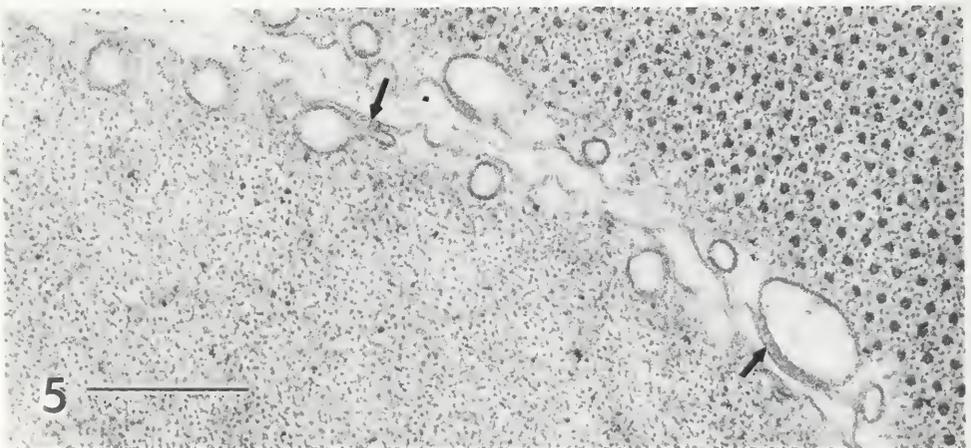


FIGURE 5. Cross-section of striated muscle cells showing the surface couplings (arrows) of the element of the sarcoplasmic reticulum to the cell surface. Scale = 0.5  $\mu\text{m}$ .

*Smooth muscle adductor*

The fusiform cells of the smooth muscle adductor are about  $10\ \mu\text{m}$  at their widest diameter and contain one elongated nucleus per cell located in a peripheral position at the widest point. There are no indentations of the cell surface to form a T-system (Fig. 6). The sarcoplasmic reticulum consists of vesicles and irregularly shaped tubes or channels that connect the vesicles forming a complicated network just under the cell surface (Figs. 7–9). Fewer channels fuse with each vesicle than is the case in the striated muscle and the vesicles are slightly larger and more irregular in outline than those observed in the striated muscle. They range in width from  $0.12$  to  $0.2\ \mu\text{m}$  and in length up to  $0.5\ \mu\text{m}$  with ribosomes often attached. In transverse sections, surface couplings can be seen between the walls of the vesicles and the cell surface (Fig. 9). The tubes in the smooth muscle are uneven in bore, ranging from  $15$  to  $40\ \text{nm}$ . A filamentous coating is associated with the luminal wall of both the

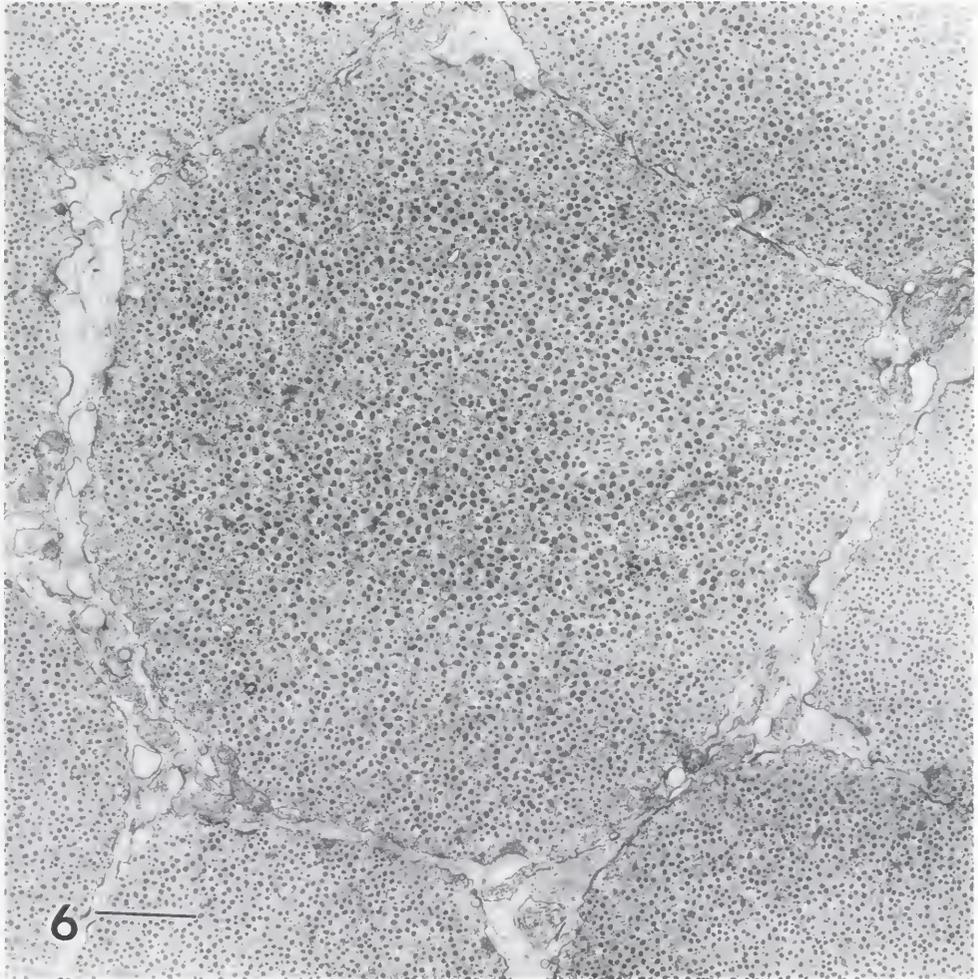


FIGURE 6. Transverse section of the smooth muscle adductor. Scale =  $1\ \mu\text{m}$ .

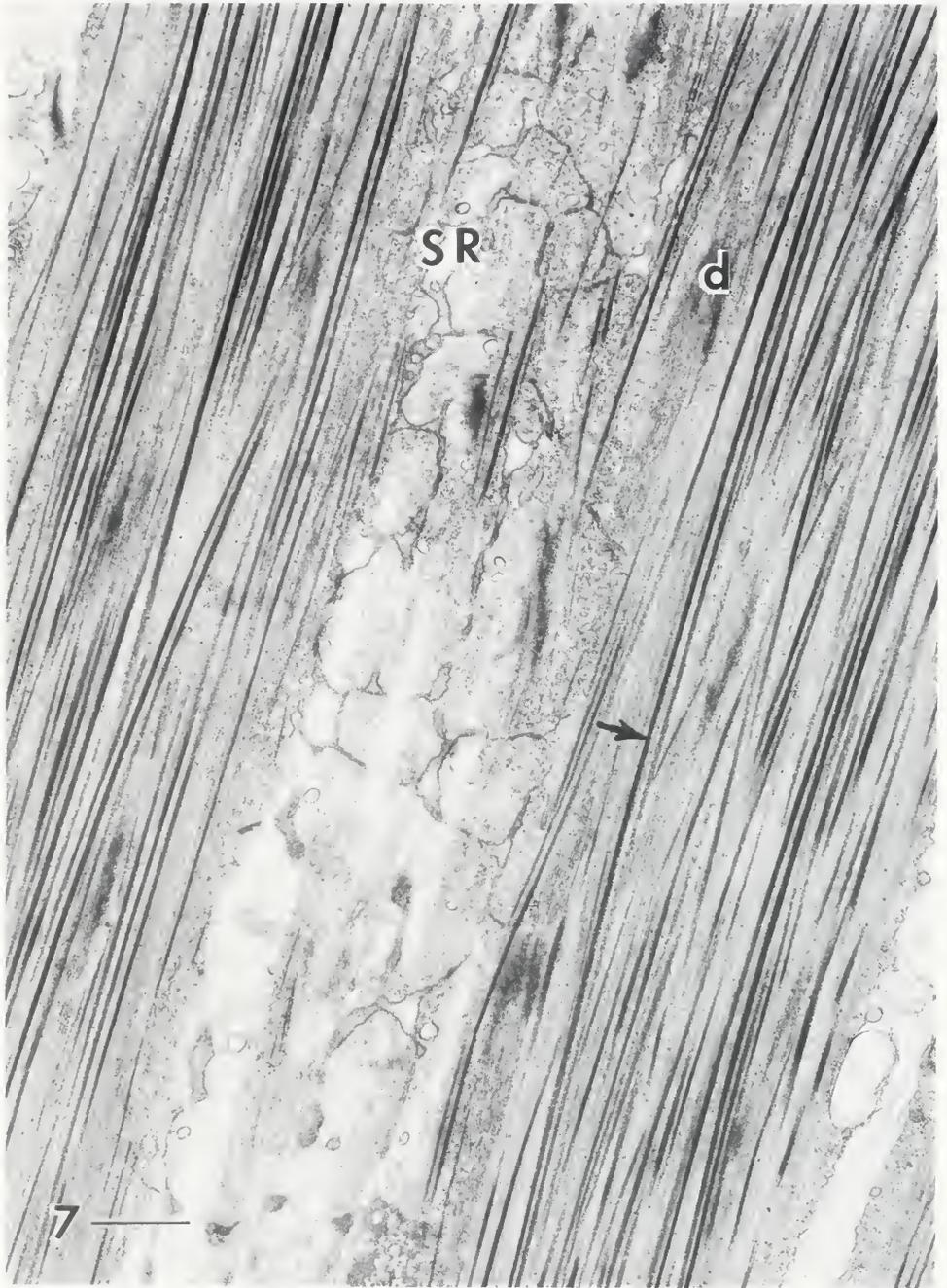


FIGURE 7. Longitudinal section of the smooth adductor illustrating dense bodies (d), thick filaments (arrow), and elements of the sarcoplasmic reticulum (SR). Scale =  $1 \mu\text{m}$ .

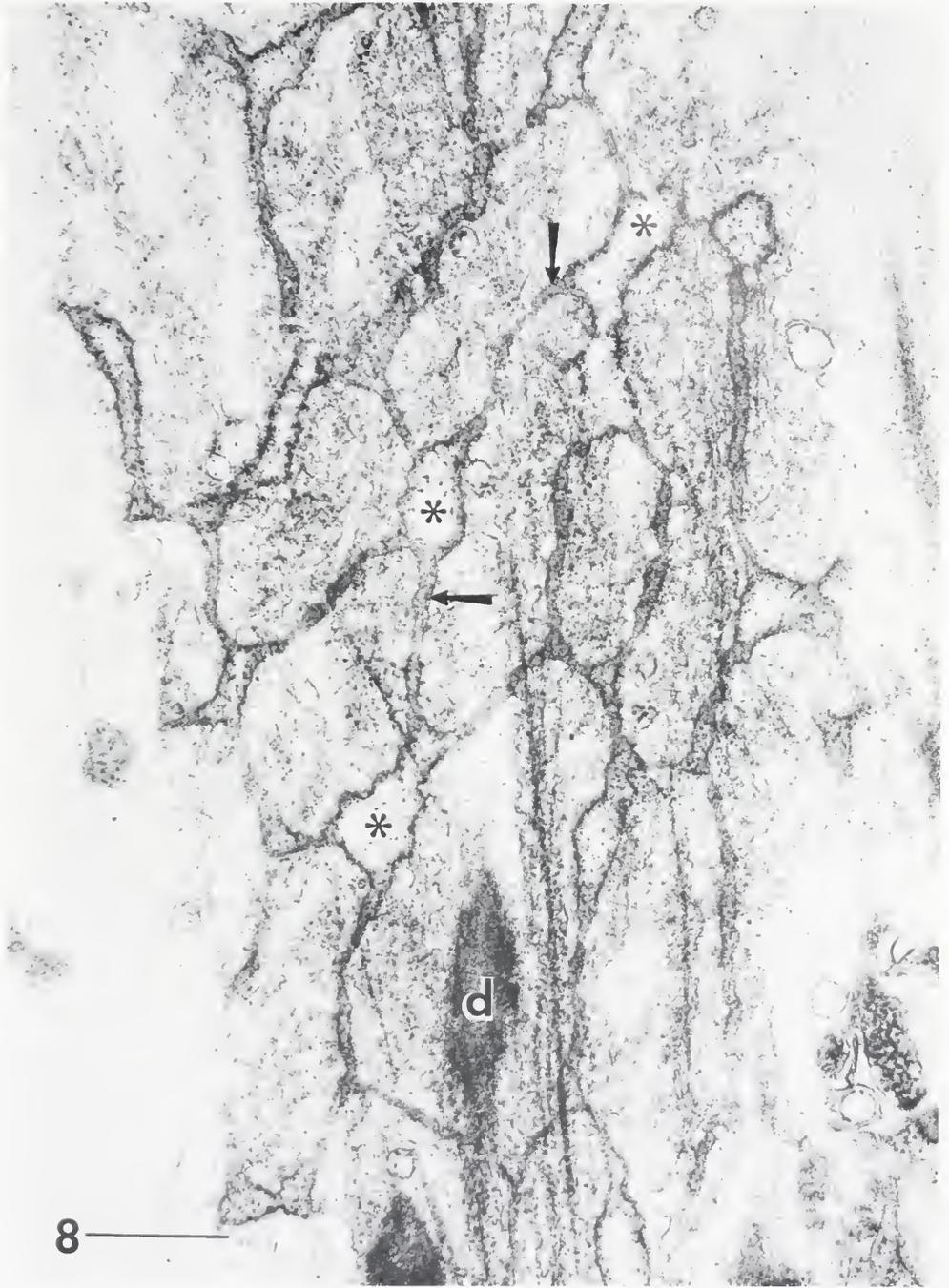


FIGURE 8. Tangential section of the wall of a smooth muscle cell demonstrating dense bodies (d) and the interconnections of the terminal cisternae (asterisks) via irregular tubes (arrows). Scale = 0.5  $\mu$ m.

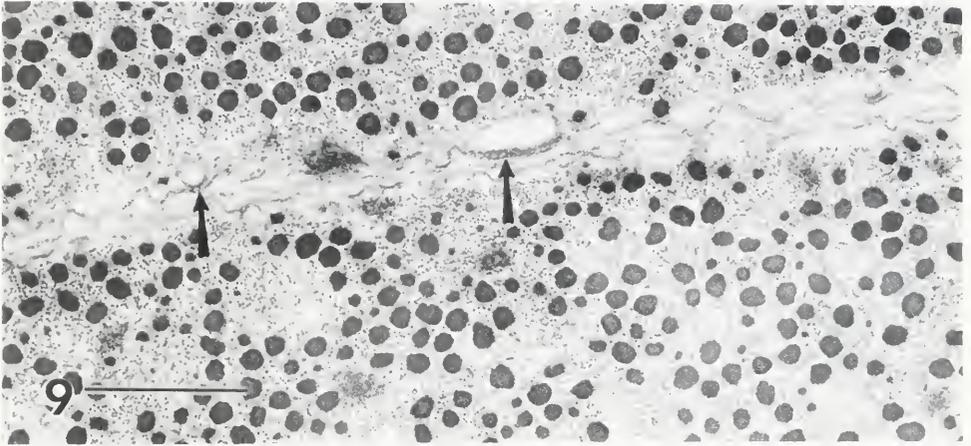


FIGURE 9. Cross-section of the smooth adductor with surface coupling between vesicles of the sarcoplasmic reticulum (arrows) and the cell surface. Scale =  $0.5 \mu\text{m}$ .

vesicles and tubes of the sarcoplasmic reticulum. The area occupied by both elements of the sarcoplasmic reticulum in smooth muscle cells was about  $\frac{1}{2}\%$  of the non-nuclear cross-sectional area of the smooth muscle adductor cell (average of 15 measurements).

#### DISCUSSION

In scallops, both cross-striated and smooth adductor muscles possess a lacy network of sarcoplasmic reticulum localized just under the cell surface (*cf.* Figs. 4 and 8). Both systems have flattened vesicles coupled to the surface cell membrane via short structures, *i.e.*, surface couplings (Franzini-Armstrong, 1972). In the two muscles the vesicles are interconnected via tubules. The uniform diameter of the cross-striated sarcoplasmic reticulum tubules is in contrast to the non-uniform bore of the smooth muscle sarcoplasmic reticulum. The alignment of the tubes is along the long axis of the myofilaments and cell fiber.

A comparison of the two muscles in cross-sections (*cf.* Figs. 1 and 6) and in tangential sections (*cf.* Figs. 4, 10 and 8, 11) reveals at first glance that there is much more sarcoplasmic reticulum in the cross-striated muscle than in the smooth muscle. Our measurements of the sarcoplasmic reticulum demonstrated that 4% of the cross-sectional area of the cross-striated muscle is occupied by sarcoplasmic reticulum while in smooth muscle it is only 0.5%. The average cell size of the scallop striated muscle cell has a width of  $10 \mu\text{m}$  and a thickness of  $1.5 \mu\text{m}$  (area =  $15 \mu\text{m}^2$ ) while the average diameter of a smooth muscle cell was  $6 \mu\text{m}$  (area =  $28 \mu\text{m}^2$ ). Thus the striated cells have about half the cross-sectional area of a smooth muscle cell, but eight times as much sarcoplasmic reticulum, resulting in a sixteen-fold advantage for the cross-striated muscle over that of the smooth muscle. The abundance of sarcoplasmic reticulum in the cross-striated muscle provides a morphological basis for its fifty-fold faster contraction rate and 128-fold faster relaxation time (Prosser, 1973). This sixteen-fold factor may be even greater when the relative rates of biochemical pumping of calcium ions (ATPase) is eventually compared in these two muscles: striated *versus* smooth.

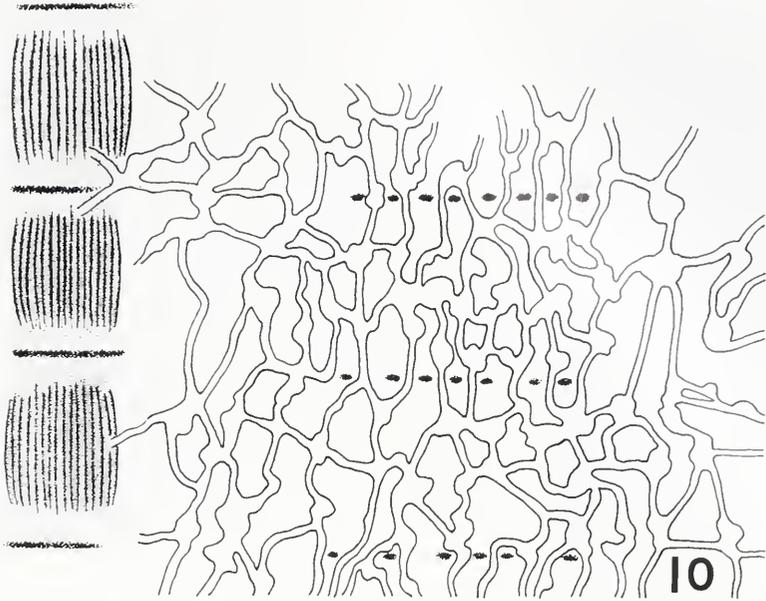


FIGURE 10. Diagram of a tangential section of a cross-striated adductor muscle cell.

The striated part of the adductor muscle is used for swimming and therefore, it is not surprising that it has a great deal more sarcoplasmic reticulum than the smooth muscle which is responsible for keeping the shell closed. The large amount of sarcoplasmic reticulum in the cross-striated muscle as compared to the smooth muscle of the scallop adductor corresponds to the general relationship observed among vertebrate muscles where the faster contracting muscles have a more elaborate and greater quantity of sarcoplasmic reticulum than slower contracting muscles (Franzini-Armstrong, 1972).

In vertebrates, mononuclear presumptive myoblasts fuse with one another to form a large multinucleated cell (reviewed by Fischman, 1972). In the striated scallop adductor, fusion of myoblasts evidently does not occur as evidenced by the absence of multinucleated cells and the presence of junctions connecting the ends of the muscle cells. These junctions resemble the intercalated discs of vertebrate and invertebrate cardiac muscle (reviewed by Sanger, 1979). Similar junctions were also observed in the striated adductor muscle cells of the scallop, *Aequipecten irradians* (Sanger, 1979; Nunzi and Franzini-Armstrong, 1981). In contrast to vertebrate cross striated muscles, there are no invaginations of the cell surface of the scallop cross-striated muscle surface to form a transverse tubular system. The scallop adductor muscle cells are small enough that diffusion of calcium from the cell surface to the sarcoplasmic reticulum can occur without the need for a transverse tubular system. In effect, a single scallop cross-striated muscle cell corresponds to one myofibril in vertebrate skeletal muscle (Sanger, 1971).

Vertebrate smooth muscle has much less sarcoplasmic reticulum than vertebrate striated muscle (Devine *et al.*, 1973) and does not have a transverse tubular system. There are a group of surface invaginations about  $0.3 \mu\text{m}$  deep (caveolae) which are closely associated with the loose network of sarcoplasmic reticulum. Surface couplings have been observed between the cell surface and peripheral elements of the

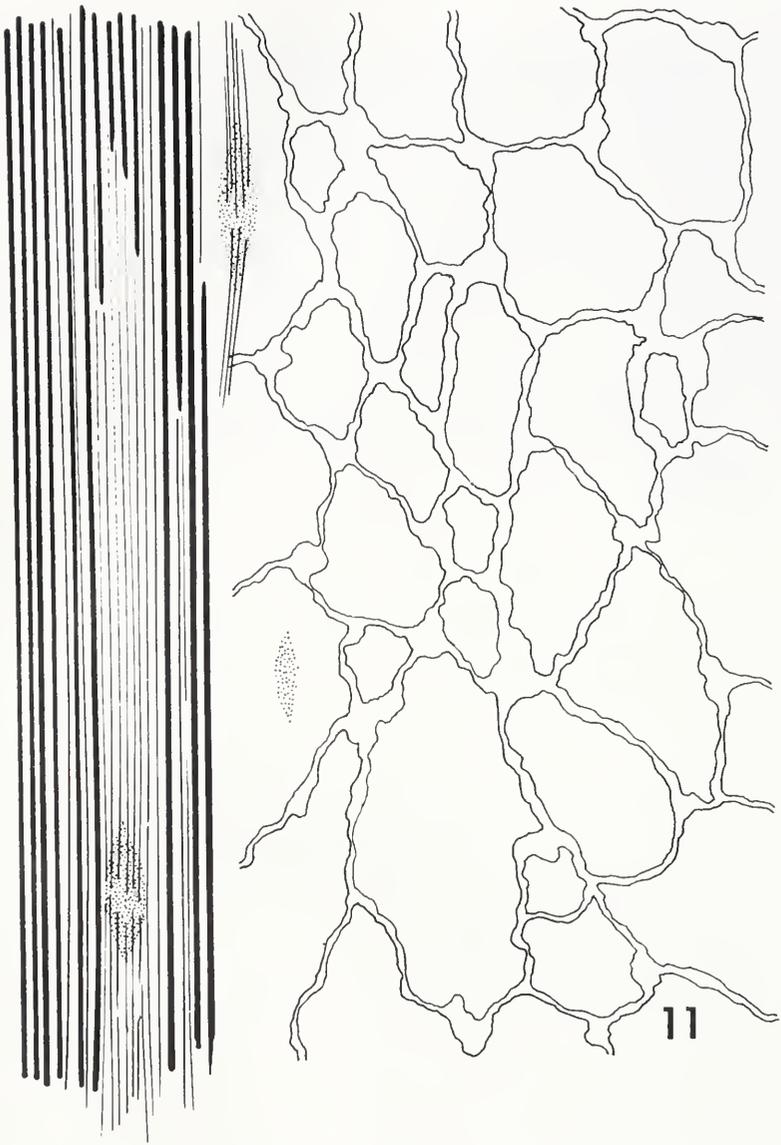


FIGURE 11. Diagram of a tangential section of a smooth adductor muscle cell.

sarcoplasmic reticulum but not between the sarcoplasmic reticulum and the caveolae. In some vertebrate smooth muscles, the sarcoplasmic reticulum consists of flattened vesicles interconnected with tubules of irregular diameter (Fig. 2 of Devine *et al.*, 1973). The pattern is similar to that observed in the scallop smooth muscle.

The scallop smooth adductor muscle like its cross-striated counterpart lacks any surface invaginations. These smooth muscle cells, while larger than the striated muscle cells, contract much more slowly than the striated cells (Prosser, 1973) and thus do not really need surface invaginations. Almost all invertebrate smooth muscle cells lack any surface indentations corresponding to vertebrate smooth muscle

caveolae. Sarcolemmic invaginations in smooth muscle were observed in a few molluscan cells (Sanger and Hill, 1972; Prescott and Brightman, 1976). The best documented case is the radula protractor muscle of the whelk, *Busycon canaliculatum* (Sanger and Hill, 1972, 1973). This muscle exhibited an extensive system of tubular invaginations of the cell surface which were termed sarcolemmic tubules. The tubules, 60 nm in diameter and 0.5  $\mu\text{m}$  in length, were limited to just under the cell surface. Moreover two other smooth muscles (blood vessel and epineural smooth muscle) from the same animal also possessed these surface invaginations (Sanger, 1973). No surface couplings were observed between the sarcolemmic tubules and the sarcoplasmic reticulum (Sanger and Hill, 1972). The significance of these surface specializations with regard to the functions of the smooth muscle is unknown.

Whether the sarcoplasmic reticulum plays a role in the catch properties of the scallop smooth muscle is not known. There does not appear to be any unusual morphological entity present in the sarcoplasmic reticulum of the smooth muscle other than the uneven nature of the bore of the tubular elements in the catch muscle. However until some biochemical analysis is done on the two different sarcoplasmic reticulum systems it is difficult to see what effect this could have on the physiological behavior of the muscles.

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THE USE OF THE URN CELL COMPLEXES OF *SIPUNCULUS NUDUS*  
FOR THE DETECTION OF THE PRESENCE OF MUCUS  
STIMULATING SUBSTANCES IN THE SERUM OF  
RABBITS WITH MUCOID ENTERITIS\*

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ABSTRACT

Urn cell complexes (UCC) from the marine coelomate *Sipunculus nudus* were used to measure a progressive change in mucus stimulating substances (MSS) in the serum of rabbits with mucoid enteritis. The serum, but not plasma, of rabbits with mucoid enteritis induced significant hypersecretion in UCC. Neither diluted nor undiluted serum or plasma from control animals induced hypersecretion. The length of the mucus tail induced in the urn cells by serum of affected rabbits was directly related to the severity of the disease. When the MSS was partially purified by cold precipitation and gel filtration, its molecular weight was determined to be between 10,000 and 13,500 daltons. These studies establish the urn cell assay as a system in which abnormal secretion of mucus can be monitored and followed with non-invasive *in vitro* techniques.

INTRODUCTION

Although aberrations in the secretion of mucus play important roles in many disease processes of animals and humans, the mechanisms that regulate the secretion of mucus in different physiological and pathological states is poorly understood. Factors found in body fluids such as serum have been implicated in the pathogenesis of several diseases in which mucus hypersecretion is a feature (Franklin and Bang, 1980; Kurlandsky *et al.*, 1980; Bang *et al.*, 1983). Serum factors were first suggested to be a possible stimulus to mucus secretion by Johnson (1935) who showed that a measurable increase in the volume of tracheal secretions could be obtained by running a film of blood over the mucous membrane of cat trachea. Hall *et al.* (1978) demonstrated that this increased tracheal secretion came from both submucosal glands and from the goblet cells, the two sources of mucus secretion in the trachea. They suggested that since serum exudates are often found in sputum or bronchial washings of patients suffering a wide range of respiratory diseases, it is likely that there are stimulants of augmented mucus secretion in the serum of these patients.

Bang and Bang (1979) have developed an *in vitro* assay system using the urn cell complexes (UCC) of the marine coelomate, *Sipunculus nudus*, to search for factors which regulate mucus secretion including factors found in serum in different

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\* The work described in this manuscript was begun under the leadership of Dr. Frederik B. Bang. After the untimely death of both Drs. Bang and Talamo, Drs. Silverman and Berninger have completed this work.

pathological states. Urn cell complexes, complexes of ciliated and mucus secreting cells, are a normal component of the coelomic fluid of *S. nudus*. Cantacuzène (1922) observed that bacterial infections of *S. nudus* induced its UCC to hypersecrete mucus. Using the UCC assay, mucus stimulating substances (MSS) have been demonstrated in normal human serum heated to 85°C for 4 minutes (Bang and Bang, 1971), in human lacrimal fluids (Franklin and Bang, 1980) and in other body fluids (Bang and Bang, 1979). Titers of MSS in serum (Kurlandsky *et al.*, 1980; Bang *et al.*, 1983) and lacrimal fluids (Franklin and Bang, 1980) have also been found to be altered in certain disease states.

The present study was designed (1) to determine if there are MSS in the blood of rabbits with mucoïd enteritis (ME), a naturally occurring disease of rabbits in which mucus plugs of the small intestine is pathognomonic (Fig. 1); (2) to determine if these factors have a relationship with the disease; and (3) to isolate, purify, and partially characterize any such factors.

### MATERIALS AND METHODS

#### *Buffer*

PBS (phosphate buffered saline: 0.010 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) was prepared fresh in deionized water with monobasic and dibasic sodium phosphate and sodium chloride (Biological grade, Fisher Scientific, Silver Spring, MD).



FIGURE 1. Colon of rabbit with mucoïd enteritis showing mucus impaction.

### *Animals*

The collection and maintenance of *S. nudus* have been previously described (Bang and Bang, 1972). In brief, *Sipunculus nudus* were dug from the sand at the lowest monthly ebb tide in their natural habitat at Loquemeau, Finistere, Station Biologique, Roscoff, France. They were maintained in France in large tanks of running sea water with beds of sand from their native area. *S. nudus* were flown to the United States packed in thermos bottles and were maintained in Instant Ocean (Aquaria Aquarium Systems, Inc., Eastlake, Ohio) in artificial sea water. They have been maintained in this fashion for up to one year while still yielding viable urn cells. New Zealand white (NZW) rabbits from our colony, with clinically diagnosed mucoid enteritis, and normal aged matched NZW rabbits were used in these studies.

### *Blood collection*

Rabbits were bled from the marginal ear vein and the blood was placed in either 15 ml or 50 ml polypropylene centrifuge tubes (Falcon Plastic, Oxnard, CA). The blood was allowed to clot at 4°C for 4 hours. The blood was then centrifuged at  $200 \times g$  for twenty minutes at room temperature and the serum removed and placed into 15 ml polypropylene tubes, and stored at -70°C. Blood was also heparinized and plasma collected in a similar fashion. In one experiment, plasma was also obtained by placing blood into 3 ml glass tubes containing EDTA (4 mg) (BD, Rutherford, NJ), sodium heparin (BD), or potassium oxalate (6.0 µg potassium oxalate with 7.5 mg sodium fluoride) (BD).

### *Urn cell complexes*

Stocks of UCC for *in vitro* tests were obtained by withdrawing 3 ml of coelomic fluid from *S. nudus* with a 20 gauge needle and putting aliquots into polypropylene 400 µl microcentrifuge tubes (Falcon Plastic). When the heavy blood cells settled, the clear supernatant contained thousands of freely swimming urn cells. There were 6-20 urns/µl of clear supernatant. The preparations remained viable for 3-4 weeks when stored at 4°C.

### *Urn cell complex assay*

Urn cell complexes were used in an assay to detect MSS in the serum and plasma of rabbits. Serum and plasma were usually diluted 1:40 in boiled filtered sea water (BFSW) to make them isotonic with *S. nudus* coelomic fluid and were used unheated. In titration experiments, serum and plasma were used either undiluted or in serial two-fold dilutions. In testing for MSS, 20 µl of the test fluid were added to 20 µl of urn fluid in a well of a glass depression slide. The length of the mucus tail induced in the urn cell by a given time was measured by an eyepiece micrometer in a light microscope and was expressed in multiples of the average diameter of the vesicle cell. Twenty to 30 UCC were counted and a mean tail length was determined. All test substances were coded and all tests were conducted without knowledge as to the source of the test material. A known MSS, heated human serum diluted 1:40 in sea water, was used as a positive control. Boiled filtered sea water (BFSW) and unheated human serum were used as negative controls. All tests were read 15 minutes after the stimulus was added.

### *Isolation of MSS*

Blood was collected from rabbits with mucoid enteritis and from control rabbits as described above and placed into polypropylene test tubes. The blood was allowed to clot and serum was removed and placed in a polypropylene test tube which was subjected to a high speed centrifugation ( $91,000 \times g$ ) at  $4^{\circ}\text{C}$  for 3 hours yielding a pellet which contained the mucus stimulating activity. This pellet was resuspended in 10 ml of PBS (pH 7.4) and allowed to stand at  $4^{\circ}\text{C}$  overnight. A slow speed spin ( $200 \times g$ ) at  $4^{\circ}\text{C}$  for 20 min yielded the mucus stimulating activity in the pellet. This pellet was resuspended at 7.5 ml of PBS and was gel filtered on Sephacryl S-200 Superfine (Lot No. 4119, Pharmacia Fine Chemicals, Uppsala, Sweden). The glass column (Pharmacia Fine Chemicals, Uppsala, Sweden) was siliconized (Prosil-28, PCR Research Chemical Inc.). The column ( $1.6 \times 92.5$  cm) was equilibrated with PBS (pH 7.4) at room temperature. The flow rate of the column was 16 ml/h and 2.3 ml fractions were collected in polypropylene tubes using a LKB 7000 Fraction collector and a duostaltic pump (Buchler Instruments, Fort Lee, NJ). The column was standardized with beef liver catalase (MW 240,000, Boehringer-Mannheim, New York, NY), transferrin (MW 82,000, Sigma Chemical Co., St. Louis, MO), egg albumin (MW 45,000, Boehringer-Mannheim, New York, NY), soybean trypsin inhibitor (MW 21,500, Worthington Biochemical, Freehold, NJ), cytochrome C (MW 12,500, Boehringer-Mannheim, New York, NY), and  $\text{K}_2\text{CrO}_4$  (MW 194.2, Scientific Products, Columbia, MD). Each collected fraction was tested for absorbance at 206, 220 and 280 nm, on a Beckman model 25 spectrophotometer. Fractions were also tested in the urn cell assay.

### *Dialysis of MSS*

Samples of collected fractions were transferred to cellulose dialysis tubing with an exclusion limit of 1000 daltons ( $43 \text{ mm} \times 10 \text{ mm}$  in 1% NaBenzoate, Spectrum Medical Industries, Inc., Los Angeles, CA). The sample was dialyzed against 1000 ml of 1 mM  $\text{NH}_4\text{HCO}_3$  at  $4^{\circ}\text{C}$  for 36 hours with two solution changes, one every 12 hours. The dialysis solution was stirred continuously throughout the process. Samples were rapidly frozen in a dry ice/acetone bath and then lyophilized.

### *Protein determination*

Column fractions were subjected to protein determination by the Bio-Rad Protein Assay. Gammaglobulin (Bio-Rad Laboratories) was used as a standard.

### *Trypsin treatment of MSS*

Column fractions containing MSS activity were subjected to a 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) solution (final concentration) for 15 minutes at room temperature. At the end of 15 minutes, Soybean Trypsin Inhibitor (SBTI) (Worthington Biochem Corp., Freehold, NJ) was added to the mixture, 1 mg SBTI for each 1.53 mg of trypsin, and allowed to stand for 15 minutes at room temperature. The resulting mixture was then tested in the UCC assay as described above. Trypsin and SBTI were also allowed to react in PBS and the resulting complex was mixed with equal volumes of fractions containing MSS activity and was used in the UCC assay. The effect of 0.1% trypsin on preformed UCC tails was also examined.

### *Stability of MSS*

Fractions from the Sephacryl S-200 column were heated to 85°C for 10 minutes to test their heat stability. Sephacryl S-200 fractions with MSS activity were also subjected to three rapid freeze/thaw cycles.

## RESULTS

### *Urn cell response to serum or plasma collected during the natural outbreak of mucoid enteritis*

Blood was collected and prepared as described above. Serum, but not heparinized plasma, from rabbits with mucoid enteritis induced significant hypersecretion in the urn cells (Fig. 2). Undiluted and diluted serum or plasma from control animals failed to induce mucus tails when used in the UCC assay (Table I). Control serum heated to 85°C for 5 minutes also failed to induce mucus tails. The length of the mucus tails induced by the serum of the affected animals correlated well with the clinical grading of the disease (Table II).

### *Plasma and serum*

To address the question of whether the absence of MSS in the plasma of rabbits with mucoid enteritis was due to an artifact of using heparin as an anticoagulant, plasma was prepared from the blood of rabbits with mucoid enteritis using sodium citrate and EDTA, as well as sodium heparin. Sera were also recovered from these animals. Plasma from rabbits with MSS activity in their serum was negative for MSS activity regardless of the anticoagulant used. The plasma, undiluted or diluted 1:20 or 1:40 in BFSW was also heated to 85°C for 5 minutes without generation of MSS activity.

### *Partial characterization of MSS*

*Sephacryl S-200 fractionation.* Sera from 10 ME positive rabbits with MSS activity were pooled and again tested for UCC activity. The pooled sera induced mucus tails that were three times the diameter of the vesicle cell (graded +3). The pooled sera was centrifuged as previously described. MSS activity was found only in the resuspended pellet. The resuspended pellet was then subjected to gel filtration on Sephacryl S-200 at room temperature. The sera of control animals were treated in the same manner (Fig. 3). Upon gel filtration, the resuspended cold precipitate from experimental sera yielded two minor peaks, each with a small amount of absorbance at 280 nm and molecular weights of 97,000 and 25,000. Both peaks, however, lacked MSS activity in the UCC assay. Mucus stimulating activity was detected in the MW range of 11,000–13,000. All active fractions lacked absorbance at 206, 220, or 280 nm. The percentage of MSS activity placed on the column and recovered was 82.0%. All fractions of control sera lacked MSS activity.

When the entire procedure was repeated twice with sera from additional animals with ME, MSS activity was found in fractions having a MW range of 10,750 to 13,500 and 10,000 to 11,000. The percentages MSS recovered in the experiments were only 42.8% and 47.5%, respectively. Control sera continued to yield no MSS activity in any fraction.

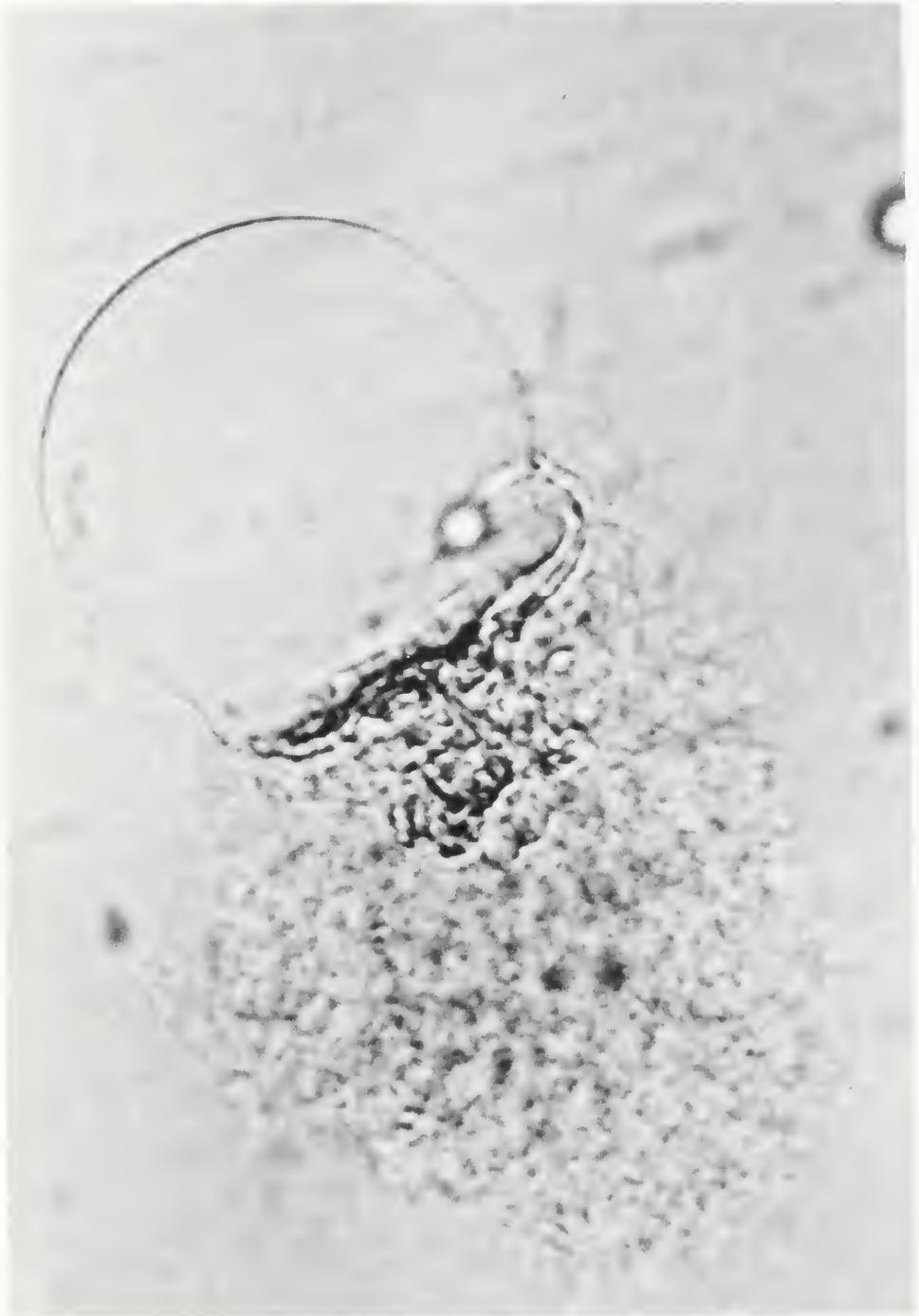


FIGURE 2. Cell complex of *Sipunculus nudus* stimulated to hypersecrete mucus by incubation with serum of rabbits with mucoid enteritis. The serum was diluted 1/40 in BFSW and mixed with an equal volume of coelomic fluid containing approximately 10 UCC/ml. After 10 minutes of incubation, 20 to 30 UCC's were counted and a mean tail length was determined.

TABLE I

*Urn cell response to blood from rabbits with mucooid enteritis*

Group	Serum	Plasma
Controls <sup>a</sup>	0/30	0/30
Mucooid enteritis <sup>b</sup>	13/13	0/13

<sup>a</sup> Controls included 20 apparently healthy rabbits, 5 rabbits with enteric disease as evidenced by diarrhea but not mucooid enteritis, and 5 rabbits with *Pasteurella* infections of the upper respiratory tract.

<sup>b</sup> Rabbits were clinically diagnosed as having mucooid enteritis. The diagnosis was based on the passage of copious amounts of mucus, anorexia, and polydipsia, symptoms diagnostic for the disease.

*Physical properties of MSS*

Aliquot of Sephacryl S-200 fractions containing MSS activity (MW 12,000–13,000) were combined, dialyzed, lyophilized, and reconstituted in PBS with no loss of MSS activity. Aliquots of MW 11,000 which also contained MSS activity were heated at 37°C, 56°C, or 85°C for 10 minutes with little or no change in MSS activity. Sera heated in a similar fashion also maintained its MSS activity. Sera and purified MSS heated to 100°C for 10 minutes, however, lost activity. The purified MSS and MSS containing sera withstood three rapid freeze/thaw cycles with no loss of activity. Purified MSS remained stable for at least 7 days at –70°C and for 3 days at 4°C. Attempts to reprecipitate purified MSS by standing in PBS at 4°C for 12 hours failed. However, when inactive MW 97,000 fraction was mixed with purified MSS (MW 12,000–13,000) and allowed to stand at 4°C for 12 hours, a

TABLE II

*Urn cell response and clinical grading of mucooid enteritis*

Rabbit number	Urn cell <sup>a</sup> response	Clinical <sup>b</sup> grading
320	+	1 <sup>c</sup>
326	+	1
342	+	1
325	++	2
322	++	3
324	++	3
336	++	3
340	++	3
315	+++	4
337	+++	4
338	+++	4
341	+++	4
345	+++	4

<sup>a</sup> Response to a 1:40 dilution of sera from rabbits with mucooid enteritis.

<sup>b</sup> Rabbits passing small amounts of mucus and showing no other clinical signs were graded 1. Rabbits passing small amounts of mucus as well as showing signs of anorexia and polydipsia were graded 2. Rabbits passing copious amounts of mucus and showing signs of polydipsia and anorexia were graded 3. Rabbits which were no longer passing mucus after 2–3 days of passing copious amounts and whose abdomens were distended with impacted intestines were graded 4.

<sup>c</sup> The length of the mucus tail induced in the urn cell was measured by an eyepiece micrometer and was expressed in multiples of the average diameter of the vesicle cell (1 = 50 μm).

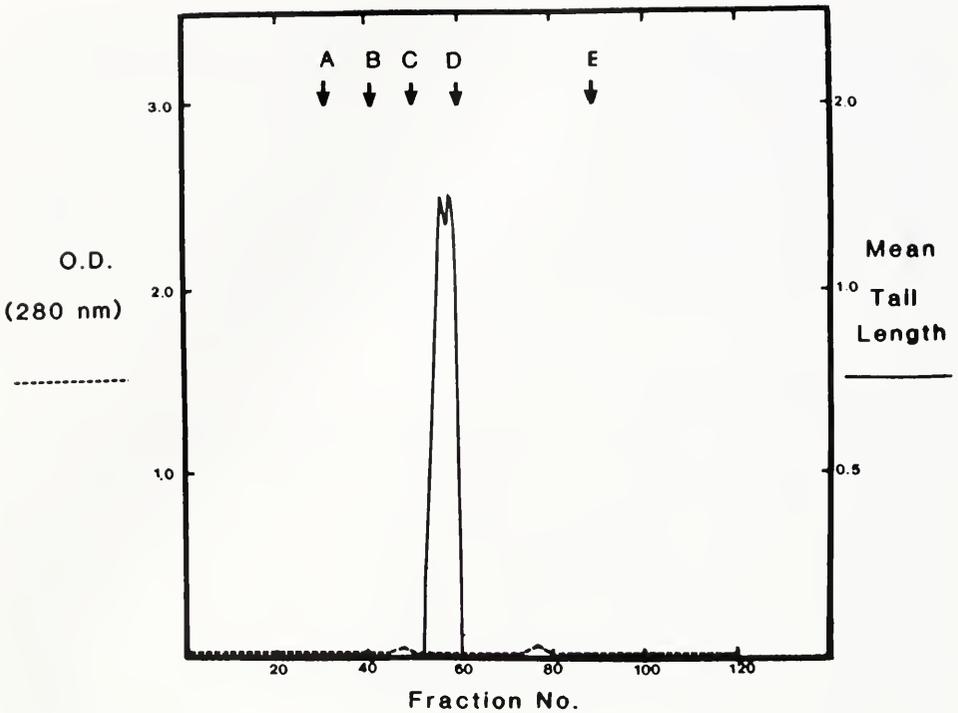


FIGURE 3. Blood was collected from rabbits with mucoid enteritis and allowed to clot. Serum was collected and subjected to a centrifugation at  $91,000 \times g$  at  $4^{\circ}\text{C}$  for 3 hours. The pellet was resuspended and allowed to stand at  $4^{\circ}\text{C}$  overnight. The cold precipitate formed at 280 nm and presence of MSS activity was determined for each fraction. A = beef liver catalase (MW 240,000); B = transferrin (MW 82,000); C = egg albumin (MW 45,000); D = soybean trypsin inhibitor (MW 21,500); E =  $\text{K}_2\text{CrO}_4$  (MW 194.2).

cold precipitate was formed with MSS activity found in the resuspended pellet following centrifugation. Purified MSS activity could be neutralized by treatment with 0.1% trypsin for 30 minutes at room temperature. When trypsin and SBTI were allowed to complex and then added to the MSS, there was no inhibition of MSS activity. Preformed mucus tails were not cleaved by 0.1% trypsin.

*Bio-Rad assay for protein in the Sephacryl S-200 fractions from rabbits with mucoid enteritis*

The Bio-Rad assay was used as a method of determination of the concentration of protein in the fractions of sera from rabbits with mucoid enteritis. Small amounts of protein (2.0–3.2  $\text{ng}/\mu\text{l}$ ) were present in fractions containing MSS activity. Similar amounts (2.0–4.0  $\text{ng}/\mu\text{l}$ ) of these proteins, however, were found in comparable fractions of serum from control rabbits.

DISCUSSION

These studies of MSS in sera of rabbits with mucoid enteritis represent the first attempt to study the progressive changes in MSS titer during the course of an acute

disease which causes excess mucus secretion in experimental animals. The very close correlation between MSS in the serum and the disease as measured by (1) the 100% presence of the factor in the serum of animals with mucoid enteritis and the absence in all controls and (2) the correlation of severity of the disease and the intensity of the UCC response makes it extremely likely that the UCC does, indeed, measure a substance of significance in the pathogenesis of this disease characterized by excessive intestinal mucus secretion.

MSS purified from the sera of rabbits with mucoid enteritis has a low molecular weight (10,000–13,500). The purest rabbit MSS active material obtained by ultracentrifugation, cold precipitation at 4°C, and gel filtration on Sephacryl S-200 yield low concentrations of protein as determined by absorbance at 280 nm and by the Bio-Rad assay for proteins (2–3 µg/µl). The apparent ability to inhibit MSS activity with trypsin, however, suggests that MSS is in fact a protein.

The MSS appears to be very stable. Heating either the whole active serum or active Sephacryl S-200 fractions at 85°C for 10 minutes did not affect the MSS activity, although heating MSS containing sera or purified MSS at 100°C for 10 minutes did abrogate the MSS activity. The MSS active sera and purified MSS were frozen and thawed three times without loss of activity. MSS fractions from the Sephacryl S-200 column could also be stored at 4°C for at least 72 hours without loss of activity. Purified MSS was dialyzed, lyophilized, and reconstituted with little apparent loss in activity.

The finding that MSS appears in the serum and not the plasma of animals with mucoid enteritis suggests that some change occurs during the clotting process, such as secretion from platelets, that is essential in the generation of MSS. Attempts to prepare plasma with a variety of anticoagulants failed to produce MSS in plasma of rabbits with mucoid enteritis. Attempts to isolate MSS from disrupted platelet suspensions of both diseased and control rabbits have been unsuccessful (results not shown). A possible role for platelets in the production or release of MSS remains, however, since platelet aggregation may be necessary for the release of MSS. Aggregation is accomplished experimentally by adding a substrate to the platelet suspension. This was not done in these studies and should be attempted in future studies. The interaction of platelets and the other cells of the clot also may be important in the generation of MSS. Alternatively, MSS may be generated by leukocytes during the clotting process. Finally, the interaction of the clotting process and the production of kinins as well as sequences in the complement cascade may be important in the generation of MSS.

Mucus stimulating substances have been demonstrated in heated normal human serum (Bang and Bang, 1980), unheated human tears (Franklin and Bang, 1980), and unheated filtrates of human cholera stools (Bang and Bang, 1979). It has also been found in unheated sea water dilutions of *Lotus tetragonolbus* lectin (Nicosia, 1979) and in unheated suspensions of sonicated human lymphoblastoid cells (Kulemann-Kloene *et al.*, 1982). Alterations in MSS profiles have been demonstrated in cystic fibrosis patients who demonstrate abnormal mucus secretion (Kurlandsky *et al.*, 1980; Bang *et al.*, 1983). The present studies suggest the use of the urn cell assay as a system in which such abnormal secretion of mucus can be monitored and followed with relatively non-invasive *in vitro* techniques.

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## CRYOPRESERVATION OF SPERMATOPHORE OF THE FRESH WATER SHRIMP, *MACROBRACHIUM ROSENBERGII*

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### ABSTRACT

Spermatophores were removed from the sternum of a female *Macrobrachium rosenbergii* immediately after mating, and equilibrated in either fresh water or physiological saline, both containing 10% glycerol. After 10 to 60 minutes equilibration, these spermatophores were pre-frozen in liquid nitrogen (LN<sub>2</sub>) vapor and submerged in LN<sub>2</sub>. The spermatophores were then thawed by directly putting them into warm water, and attached to the sternum of other females using a glue. Successful fertilization was observed in cases where the spermatophores were equilibrated in fresh water for 15–30 minutes. At 10 minutes equilibration, most of the spawned eggs were not fertilized. Furthermore, fertilized eggs were not obtained without pre-freezing, or where spermatophores were equilibrated in physiological saline.

### INTRODUCTION

Although cryopreservation of gamete or embryo has been attempted and actually applied in mammals (Leverage *et al.*, 1972; Whittingham *et al.*, 1972), teleosts (Horton and Ott, 1976), and invertebrates (Dunn and McLachlin, 1973; Hughes, 1973; Asahina and Takahashi, 1978; Zell *et al.*, 1979), no report has been made on decapod crustacea. Attempts to preserve the spermatophore of the fresh water shrimp *Macrobrachium rosenbergii* have been made by Sandifer and Lynn (1980) and Chow (1982), but fertility only lasted for 24 h in the former while in the latter, the preservation period was extended to 9 days.

This paper presents results on the first attempt at cryopreservation of spermatophore of the fresh water shrimp *Macrobrachium rosenbergii* in liquid nitrogen (–196°C) and observations on successful fertilization using preserved spermatophore.

### MATERIALS AND METHODS

#### *Collection of spermatophore*

Adult males and females of *M. rosenbergii* were maintained individually in 40-liter fresh water aquaria at 25–29°C. The mature female is distinguished by the fully developed ovary observed through the translucent exoskeleton. She also undergoes a pre-spawning molt. Mating followed after transferring the female, which had recently experienced a pre-spawning molt, to an aquarium containing a male. The spermatophore was deposited on the female sternum, and was carefully stripped immediately after the mating.

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*Treatment and cryopreservation of spermatophore*

Fresh water, prepared by filtering tap water through activated carbon, or physiological saline were used as basic media for equilibration of the stripped spermatophores. These media contained 10% (V/V) glycerol as cryoprotectant. The composition of physiological saline was adopted from Nagamine *et al.* (1980) and shown in Table I. The swelling condition of spermatophores, exposed to three kinds of media (fresh water, fresh water containing 10% glycerol, and physiological salines containing 10% glycerol) was observed. After 0, 10, 15, 30, 60, 120, and 180 minutes of swelling the spermatophores were carefully wiped with cloth or cotton and their weights were determined for all media used.

For cryopreservation, swollen spermatophores exposed to fresh water for 10, 15, and 30 minutes or to physiological saline for 30 and 60 minutes at room temperature (25–30°C) were removed from these media and transferred to 1 cm diameter dry glass test tubes. The test tubes were then pre-frozen in liquid nitrogen (LN<sub>2</sub>) vapor for 0, 5, and 10 minutes before submerging them into LN<sub>2</sub>. Spermatophores were thawed in tap water at 30°C. Artificial insemination techniques have been previously described (Chow, 1982).

## RESULTS

*Morphological observations of spermatophore and spermatozoa during equilibration in various media and after preservation*

The extent of swelling of the spermatophores in various media was determined by their weight increase at 0, 10, 15, 30, 60, 120, and 180 minutes (see Fig. 1). As expected, considerable swelling was observed in fresh water, followed by fresh water containing 10% glycerol, then physiological saline containing 10% glycerol. Maximum swelling of spermatophore was observed after 2 h in all media, where weight increases were about 10× in fresh water, 6× in fresh water containing 10% glycerol, and 2.5× in physiological saline containing 10% glycerol.

Remarkable visible changes, namely, the softening of the protective matrix and the slight hardening of the adhesive matrix, were observed in spermatophores equilibrated in fresh water but not in physiological saline.

Light microscopic observation showed no visible deformities of the spermatozoa during equilibrations in these media and even after freezing and thawing.

*Fertility of cryopreserved spermatozoa*

Among 15 trials of inseminations using frozen-thawed spermatophores, fertilized eggs were observed in 8 trials (Table II). In two cases where the spermatophores

TABLE I

*Macrobrachium rosenbergii* physiological saline

NaCl	11.00 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	1.91
KCl	0.52
MgSO <sub>4</sub> · 7H <sub>2</sub> O	2.47
NaHCO <sub>3</sub>	0.17

The salts were dissolved in distilled water and brought up to 1 liter. The pH was adjusted to 7.6 with 1 N NaOH. (From Nagamine *et al.*, 1980).

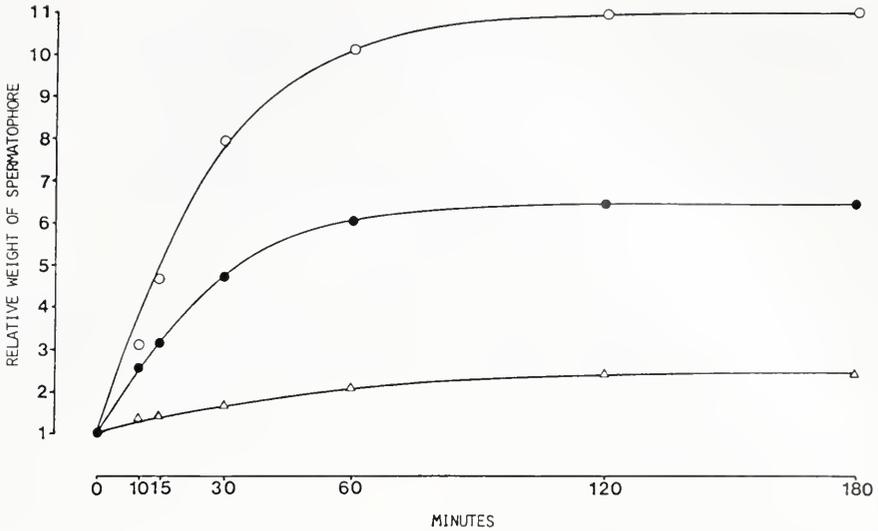


FIGURE 1. Fluctuation of spermatophore weight with the time elapsed after exposure to three kinds of media; fresh water (O), fresh water containing 10% glycerol (●), and physiological saline containing 10% glycerol (Δ).

were equilibrated for 10 minutes in fresh water containing 10% glycerol and pre-frozen for 5 minutes, most of the spawned eggs were not fertilized, and a few fertilized eggs had fallen off within a week together with the unfertilized eggs before growing to eyed-stage. In 7 combinations of 15 and 30 minutes equilibration in

TABLE II

Results of artificial insemination using shrimp spermatophore preserved in LN<sub>2</sub>

Basic medium	Equilibration time (min)	Pre-freezing time in vapor LN <sub>2</sub> (min)	Preservation period in LN <sub>2</sub> (day)	Fertilization	Hatching
Fresh water	10	5	9	fertilized*	not hatched
	10	5	31	fertilized*	not hatched
	15	0	0.5	not fertilized	—
	15	0	26	not fertilized	—
	15	5	0.2	fertilized	hatched
	15	5	30	fertilized	hatched
	15	10	0.08	fertilized	hatched
	15	10	8	not fertilized	—
	30	5	0.13	fertilized	hatched
	30	10	0.17	fertilized	hatched
Physiological saline	30	10	20	fertilized	hatched
	30	10	0.04	not fertilized	—
	30	10	2	not fertilized	—
	60	10	1	not fertilized	—
60	10	1	not fertilized	—	

\* A small number of fertilized and cleaved eggs were observed, and they had fallen off within a week.

fresh water with 5 and 10 minutes pre-freezing, 6 cases provided successful fertilization and hatching, while 2 cases of 15 minutes equilibration in this same medium with no pre-freezing were not fertilized. Furthermore, no fertilized eggs were observed in cases where the spermatophores were equilibrated in physiological saline for 30 and 60 minutes.

Under rearing conditions at 28°C, more than 70% of hatched larvae developed normally and metamorphosed into post larvae after a month.

#### DISCUSSION

Components of the basic media used in this study are simple when compared with the diluent for cryogenic preservation of spermatozoa in other animals studied. In contrast to their spermatozoa which are suspended in seminal plasma, the shrimp sperm are enveloped by the gel matrix (Berry, 1970; Bauer, 1976; Sandifer and Lynn, 1980; Chow *et al.*, 1982). The permeability of the gel matrix to fluids was demonstrated by the swelling of the spermatophore in the three media used. Successful fertilization occurred in cases where spermatophore was equilibrated in fresh water containing 10% glycerol, but not in physiological saline containing the cryoprotectant. This undoubtedly indicates that glycerol has a protective effect against freezing damage and that fresh water as basic medium was more effective in the transport of glycerol into the gel matrix. In physiological saline, the movement of glycerol into the gel matrix was not possible because the difference in osmotic pressure was minimal. This was shown by the slight swelling observed in physiological saline (Fig. 1).

While optimum cooling velocities are different in various tissues, rapid cooling above optimum rate generally causes intracellular freezing or recrystallization during thawing and results in death (Mazur, 1970). Although the cooling and thawing velocities were not extensively examined in this study, the results show that, in the two trials where pre-freezing was not done, fertilization did not occur; light microscopy did not detect any deformities of the spermatozoa. This indicates that rapid cooling may affect the spermatozoa.

#### ACKNOWLEDGMENTS

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## AN INVESTIGATION OF EXTRACELLULAR ELECTRICAL CURRENTS AROUND CYANOBACTERIAL FILAMENTS

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### ABSTRACT

We have searched for currents through gliding filaments of the giant cyanobacterium, *Oscillatoria princeps*, as well as through two species of *Anabaena* and found none. Current loops associated with gliding (and which would therefore have dimensions of the order of a filament length) should have been detected if they had surface densities of 0.03 to 0.1  $\mu\text{A}/\text{cm}^2$  or more; while current loops through *Anabaena* heterocysts should have been detected if they had surface densities of the order of 1 to 3  $\mu\text{A}/\text{cm}^2$  or more. The relationship of these negative findings to earlier reports of large voltages along *Phormidium* filaments is discussed.

### INTRODUCTION

Using a vibrating probe to measure extracellular electrical fields, it has been shown that many growing eukaryotes drive steady electrical currents of the order of 1 to 100  $\mu\text{A}/\text{cm}^2$  through themselves (Jaffe, 1982). However, the only direct indicator of such currents through prokaryotes lies in several reports of extracellular voltages along gliding filaments of the cyanobacterium, *Phormidium uncinatum*. In one setup, Häder (1978) allowed a single *Phormidium* filament (in distilled water) to glide across a tight constriction formed by nearly fusing the end of a glass capillary. Unilateral illumination then induced voltages of up to 10 millivolts across the constriction. Later, Murvanidze and Glagolev (1982) placed a bundle of about 20 filaments, also in distilled water, along a fine groove formed by scratching a piece of Plexiglas. Illumination of one end of the bundle—in some experiments followed by a regime of turning uniform illumination on and then off—yielded voltages of up to 20 millivolts across the groove.

Such voltages could have been generated by the photoinduction of currents through the *Phormidium* filaments. If they were, then the fields so produced might well be measurable with a vibrating probe, without constriction of these currents by a capillary or a groove, since voltages a million times smaller, *i.e.*, of the order of 10 nanovolts or more, can be reliably measured with a vibrating probe system (Jaffe and Nuccitelli, 1974).

Currents through gliding filaments would be relevant not only to the study of the photophobic responses of cyanobacteria (Häder, 1978); but also in the possible mechanism of gliding (Jaffe, 1984). We have looked for such currents in a species of *Oscillatoria* that has exceptionally wide—actually 35  $\mu\text{m}$  wide—filaments and exhibits rapid gliding motility. We also investigated two species of *Anabaena*, one motile and one not. In *Anabaena* we also looked for currents which might be associated with heterocysts, peculiar nitrogen-fixing cells which differentiate from vegetative cells at regular intervals along a filament (Fay *et al.*, 1968). In various

eukaryotes, extracellular currents play a role in differentiation; moreover, heterocysts lack photosystem II (Tel-or and Stewart, 1977), and it seemed possible that they might show differences in photosynthetically driven proton flow through their plasma membranes from those found in vegetative cells—differences that in turn would generate detectible extracellular currents.

#### MATERIALS AND METHODS

*Anabaena flos-aquae* strain 1304/13f from the Cambridge Collection of Algae and Protozoa (CCAP) and *Anabaena cylindrica* strain CCAP 1304/2a were cultured as described by Armstrong *et al.* (1983) at 20°C under an incident light (approximately 1000 lux). Current density measurements were made with filaments suspended in the same medium diluted 50% with distilled water to give a resistivity of 35 K  $\Omega$ cm. *Oscillatoria princeps* strain ID-9-Op (from Dr. R. W. Castenholz, University of Oregon, Eugene) was grown in medium D (Castenholz, 1981). Measurements of current density were made with filaments suspended in this medium at full strength, in this same medium diluted ten times with distilled water, and also in 1  $\mu$ M CaCl<sub>2</sub>, a medium providing higher resistivity (450 K  $\Omega$ cm).

Current-generated fields around filaments were investigated using the vibrating probe with the filaments placed in 35-mm diameter Petri dishes on 1-mm deep layers of 1% (w/v) Difco agar jelly covered by 3 mm of the appropriate medium and a 2-mm layer of an inert paraffin oil. The agar was prepared in the same medium as the overlay. To facilitate adhesion to the agar, filaments were first allowed to stick to the agar jelly without any fluid overlay.

The vibrating probe system was a modified version of that described by Jaffe and Nuccitelli (1974). Among the modifications were the following: (1) the vibrating electrodes were made by electrochemically depositing gold and then platinum black onto the tips of parylene-insulated stainless steel electrodes made primarily for brain recording (from Microprobe Inc., Clarksburg, Maryland). (2) The reference electrode was a non-vibrating platinized platinum wire immersed in the medium about a centimeter away from the vibrating one. (3) No meniscus setter was used. Where necessary, meniscal noise was avoided by covering the aqueous medium with oil. (4) The outputs of the vibrating and reference electrodes went to a differential preamplifier. The bath was kept near ground potential using a second platinized platinum wire and a virtual ground circuit. (5) For measurements of the field components perpendicular to a filament, the electrode was vibrated along its shaft instead of across it. This arrangement allowed a closer approach of the probe tip to the bacterial filaments than the usual lateral vibration, since the insulation did not intervene between the platinum black and the living cells. A close approach during such radial vibrations was also favored by the use of an electrode with a relatively small platinum black tip—one only 6  $\mu$ m wide by 8  $\mu$ m long.

Unless otherwise stated, measurements were made with a system time constant  $T$  of 5 s, *i.e.*, the system output after a step change in input rose to half of its final value in 5 s.

Figure 1 shows a probe near the end of an *Oscillatoria princeps* filament.

The filaments and probe were observed with a Zeiss inverted microscope. The most critical observations were made with a 40 $\times$  objective. Most of the observations on *O. princeps* were made at a lamp voltage that gave an irradiance of 36 Wm<sup>-2</sup> on the specimen. This is equivalent to a light intensity of about 9 klux (see conversion table of Van Liere and Walsby, 1982) and is similar to the light intensity used by Häder (1978). Some of the observations on *Oscillatoria* and on *Anabaena*

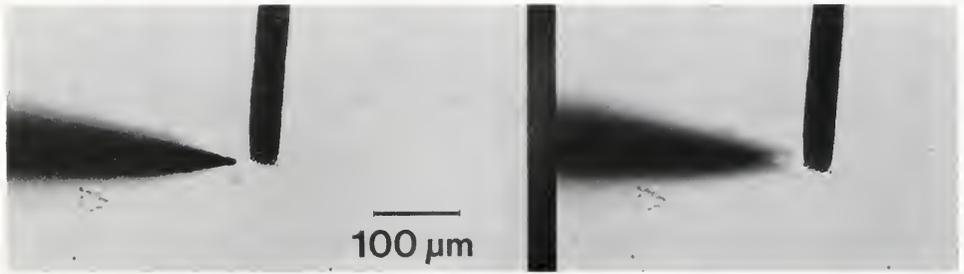


FIGURE 1. Photographs of a probe near the end of an *Oscillatoria princeps* filament. Left: probe static. Right: probe vibrating.

*cylindrica* and all measurements made with the photosensitive *A. flos-aquae* were made with a green filter in the lamp condenser which reduced the irradiance to 19% of the unfiltered value.

## RESULTS

Measurements were made on two cyanobacterial species with typical widths: *Anabaena cylindrica* with filaments about 4  $\mu\text{m}$  wide and *A. flos-aquae* with filaments 6  $\mu\text{m}$  wide. They were also made on the exceptional filaments (36  $\mu\text{m}$  wide) of *Oscillatoria princeps*. Since the measurements on the latter should be most reliable, they are described first.

### *Oscillatoria*

A search for measurable currents was made on at least six different gliding filaments of *O. princeps*. The filaments generally glided at rates of about 1 to 5  $\mu\text{m}/\text{s}$  during measurements with frequent reversals of direction. This search included measurements with the probe tip vibrating in the following directions and positions: (1) parallel to the filament and placed to one side of it with a minimal probe-to-filament gap of about 15  $\mu\text{m}$ . (2) Parallel to and above the filament with a gap of about 40  $\mu\text{m}$ . (3) Perpendicular and to the side with a 15- $\mu\text{m}$  gap. (4) Oblique and above with a gap of about 50  $\mu\text{m}$ . It likewise included measurements in various positions along the filament. In most cases we explored the vicinity of an entire filament (including its necridia) by letting it glide past a vibrating probe kept in one position except for slight adjustments to keep the probe-to-filament gap constant. The search also included measurement before, during, and after spontaneous reversals of the direction of gliding; measurements with the microscope light on or off and measurements during a shift from light on to off, or *vice versa*; as well as some measurements on filaments which were partially illuminated with the aid of the microscope light and the condenser diaphragm. The search likewise included measurements on filaments in medium D, in tenth strength D, as well as in a minimally conductive medium (1  $\mu\text{M}$   $\text{CaCl}_2$  added to glass distilled water) which nevertheless supported continued gliding. These last measurements were done with a half time constant of 1.5 s instead of the usual 5 s.

In no case were currents detected. At the point of measurement we would estimate that the instrumental limits of detectability generally lay between 30 and 60 (or in a few cases 100)  $\text{nA}/\text{cm}^2$ , *i.e.*, 0.03 to 0.1  $\mu\text{A}/\text{cm}^2$ . Tangential or parallel current densities associated with gliding would presumably fall off over dimensions

comparable to the lengths of the measured filaments. Since these filaments were the order of a millimeter or more in length, the tangential current densities should not have fallen off significantly from a filament's surface to the point of measurement; so tangential surface current densities of more than about 30 to 60 nA/cm<sup>2</sup> should therefore have been detected. Perpendicular density components of a 'gliding current,' on the other hand, would be expected to fall off inversely to the distance from a filament's mid line. Hence, perpendicular surface densities should have been 2 to 3 fold higher than at the measurement point, and ones more than about 100 nA/cm<sup>2</sup> should have been detected.

Figure 2 shows two sections of the original records, which illustrate these negative results with *Oscillatoria princeps*.

### *Anabaena*

About ten filaments of *A. cylindrica* were explored in a similar way with similar negative results. However, they glided more slowly than *O. princeps* (0.2–0.3 μm/s instead of 1–5 μm/s). As a result, it was possible to vibrate the probe closer to the filaments. Probe-to-filament gaps as small as about 5 μm were often attained. Furthermore, *A. cylindrica* has heterocysts at about 100 μm intervals. Therefore, it was possible to search for special heterocyst currents in this species. These results

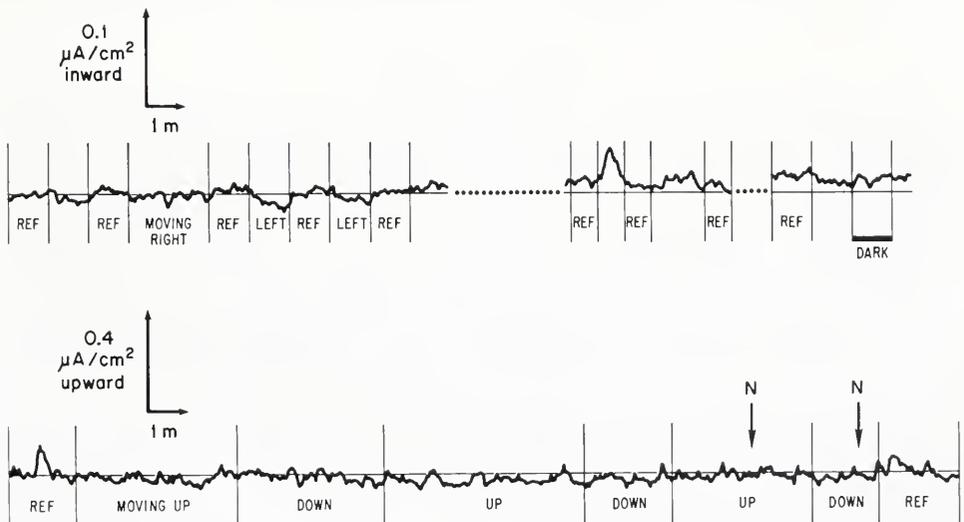


FIGURE 2. Illustrative records of the unsuccessful search for currents near gliding *Oscillatoria* filaments, in full strength medium D.

*Top:* probe vibrating *perpendicular* to filament 2 on 16 September 1984. The probe was to the side of the filament, about 5–10 μm away in its closest test positions (and 200 μm away in the reference positions marked 'REF'). The filament was observed to be gliding rightward or leftward during periods marked right or left; it was also moving at most other times, although the direction was not noted. The microscope light was on except where the chart is marked 'DARK.' During deleted parts of the record, the probe was not vibrating or had touched the filament.

*Bottom:* probe vibrating *parallel* to filament 3 on 17 September 1984. The probe was about 35 μm directly above the filament in its closest test positions and at this same height but 300 μm to the right in its reference positions. The filament was gliding horizontally 'upwards,' *i.e.*, away from the observer, or 'downwards' at about 4 μm/s in the periods marked up or down, respectively. N marks a point at which a necridium passed the probe.

were negative too. That is to say, currents of about  $100 \text{ nA/cm}^2$  or more would have been detected in the region of measurement. Considered as a source or sink of current, each heterocyst is a roughly equi-dimensional object with a  $3\text{--}4 \mu\text{m}$  radius. Currents emanating from a heterocyst should fall off roughly as the square of the distance from the cell's center. Considering the probe tip size of  $6 \times 8 \mu\text{m}$ , the minimum gap of  $5 \mu\text{m}$ , etc., we estimated that perpendicular surface current densities as small as one to a few  $\mu\text{A/cm}^2$  would have given detectable signals in the region of measurement. Since none were detected, we conclude that if there are special heterocyst currents, then their surface densities are less than 1 to 3  $\mu\text{A/cm}^2$ .

Finally, we report some measurement made on the non-motile,  $5\text{--}6 \mu\text{m}$  wide filaments of *A. flos-aquae*. At first we regularly observed apparent outward currents of a few hundred  $\text{nA/cm}^2$  near (*i.e.*, about  $5 \mu\text{m}$  away from) the filaments of this organism, but no corresponding inward currents could be found. Then we observed that these apparent currents were only generated by regions of a filament which were so loose that they visibly vibrated when the probe approached. No such signals were generated by well-stuck regions of a filament which did not visibly vibrate when the probe approached. Evidently, these apparent small outward currents are artifacts somehow produced by vibrating the filament. Perhaps these curious artifacts originate in electrokinetic effects produced by mechanically shearing a double layer at the filaments' outer surfaces. In any case, they are a warning against searching for currents in objects so light and so loosely tethered that the vibrating probe itself can vibrate them.

#### DISCUSSION

The absence of detectable current-generated electrical fields around gliding filaments of *Oscillatoria* and of *Anabaena* raises the question of whether: (1) these filaments are fundamentally different from *Phormidium* filaments, (2) the voltages measured across *Phormidium* filaments were generated by extracellular currents which were (a) too transient or (b) too small to be detected by us, or (3) these voltages were not generated by electrical currents driven through the medium by the filaments, but in some other fundamentally different way.

The first possibility seems unlikely in view of the apparent similarity between different gliding cyanobacterial filaments. Moreover, a preliminary effort to measure current-generated fields near gliding *Phormidium* filaments using a vibrating probe system has also yielded negative results (Häder, pers. comm.).

Half times of the transient voltages recorded by Murvanidze and Glagolev were of the order of 10 to 30 s, so our system half time constant of 5 s should have allowed observations of comparable transients. Moreover, a crude calculation suggests that if the voltages which they recorded had been generated by currents through the filaments, then they would have been large enough to be detected by us. Suppose that their groove had a cross-sectional area of about  $0.01 \text{ mm}^2$ —as their reports suggest—and suppose that the medium in the groove had a resistivity of about  $1 \times 10^5 \Omega\text{cm}$ . Then the resistance of their groove would have been about 1 megohm and the current per filament needed to generate 20 mV would have been about 1 nanoampere. This would have required a surface density of the order of  $10 \mu\text{A/cm}^2$  as it entered or left a filament section  $0.5 \text{ mm long} \times 10 \mu\text{m wide}$ . There are many uncertainties in this calculation! Nevertheless, the figure is two orders of magnitude higher than our limits of detectability. This, in turn, suggests that the third possibility must be seriously considered.

How *could* the voltages recorded along *Phormidium* filaments have been

generated except by current flow through the filaments? One possibility is that they were diffusion potentials generated at liquid junctions outside of the filaments' plasma membranes. This seems particularly plausible when one considers that these voltages were recorded in "distilled water." In such a medium, the ionic strength and conductivity of the medium within the constrictions used—Häder's capillary or Murvanidze and Glagolev's groove—could well have been substantially raised by ions coming out of the filaments themselves. Under such circumstances, the liquid junction potentials within the constrictions may have been reduced relative to those at the ends of these constrictions, so that different liquid junctions at the ends would have generated substantial net voltages. In short, the extracellular *Phormidium* voltages recorded in the literature may indicate transient extracellular concentration gradients—as of pH, pCA, or sulfated polysaccharides in the slime—rather than electrical current flow through the filaments.

Altogether, our negative findings may be taken as evidence against an electrophoretic theory of gliding (Jaffe, 1984) and thus—by elimination—as being in favor of a sliding filament model (Castenholz, 1982).

#### ACKNOWLEDGMENTS

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## TRADE-OFF BETWEEN MALE REPRODUCTION (AMPLEXUS) AND GROWTH IN THE AMPHIPOD *GAMMARUS LAWRENCIANUS*

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### ABSTRACT

A trade-off is found between growth and the length of time male amphipods (*Gammarus lawrencianus*) spend in amplexus. Males spending the majority of time in amplexus showed 45% less growth than unamplexed males. The inability of males to use their gnathopods to feed while in amplexus appears to cause this reduced growth. Growth rates of females appear unaffected by amplexus.

Since male size is correlated with male reproductive success in *G. lawrencianus*, a 45% decrease in size increment at the next molt would represent a similar loss in the incremental male reproductive success. Male mating decisions are therefore based not only on immediate past male investment in amplexus, male size, and population characteristics, but also on the trade-off between present reproduction and future size.

### INTRODUCTION

Reproduction in the marine amphipod *Gammarus lawrencianus* (Bousfield) follows a period of amplexus in which the male and female remain attached together. This precopulatory attachment is a form of "mate guarding," and is usually treated as an investment in time by the male (Parker, 1974).

Guarding of females has been examined in several species. Manning (1975) and Ridley and Thompson (1979) have investigated precopulatory guarding in isopods, Parker (1978) in the dung fly, Hartnoll and Smith (1978, 1980), Birkhead and Clarkson (1980), Wildish (1982), and Hunte *et al.* (in press) have all studied amplexus in amphipods, and Davies and Halliday (1977, 1979) in toads. In these cases, guarding of a female will be advantageous when the expected rate of gain in reproductive success due to amplexus is potentially greater than the sum of (1) the loss due to withdrawal for further searching (Parker, 1974, 1978; Hunte *et al.*, in press), (2) the increased risk of predation due to being a larger more visible target (Strong, 1973; VanDolah, 1978; Ridley and Thompson, 1979; Wildish, 1982), and (3) the physiological expense of amplexing owing to increased energy expenditure (Manning, 1975; Calow, 1979) or to a post-copulatory refractory period (Hunte *et al.*, in press).

Amphipod amplexus occurs when a sexually mature male comes into contact with a female and attempts to take hold. If successful, the male holds her close to his ventral surface by inserting his gnathopods between the segments along her anterior dorsal surface, then turning her around and carrying her longitudinally beneath him.

*G. lawrencianus* is a macrophageous feeder, feeding on coarse solid food which is grasped and manipulated by the gnathopods which are also used to hold the female during amplexus. Females appear to be able to exercise some choice of males

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during the early stages of amplexus, in that females large relative to the male can escape, providing a possible means of sexual selection for larger males. Doyle and Hunte (1981b), and Doyle and Myers (1982) have shown that female fecundity varies directly with size in *G. lawrencianus*.

This study had two aims: (1) to test for an effect of amplexus on the rate of food intake by both sexes, and (2) if a difference in feeding rate exists, to determine how much a reduction in growth may result from that difference. Since female fecundity is correlated with female size and hence with male size, a decrease in male growth rate represents a loss of future male reproductive success. Such a physiological cost to reproduction would cause a trade-off between present and future reproductive success which must be added to the time-budget considerations of Hunte *et al.* (in press).

## MATERIALS AND METHODS

### *Free and amplexed feeding rates*

Amphipods in all experiments were randomly collected from a laboratory-reared population (Doyle and Hunte, 1981a, b). Individual and amplexed amphipods were isolated without food in 25.0 ml petri dishes filled with sea water at 22°C, and having a fecal pellet trap composed of .75 mm plastic screening. After a 24 hour starvation period, the sea water was replaced and a weighed dry sample (2.000–3.500 mg) of 1200 dpm/mg <sup>14</sup>C-labeled cellulose suspended in coagulated egg white was added. The remaining food and fecal pellets were collected and the sea water replaced after 24 hours. A second 24-hour starvation period followed feeding.

Live amphipods were weighed on an electrobalance after blotting with absorbent paper. Each animal was then dissected and the gut with caecae, the body without head, tail, or gut, and the fecal pellets were placed in separate scintillation vials and dissolved in NCS Tissue Solubilizer.

Twenty-seven individual and 33 amplexed males as well as 27 individual and 29 amplexed females were so tested. Animals discarded because they molted, separated, or died are not included in the results. Ten individual males, 10 females, and 11 amplex pairs served as controls, fed with non-radioactive food.

### *Growth rates of free and amplexed males*

Two treatments were prepared, the first composed of "free" male amphipods and the second of male and female amphipods in a one-to-two ratio. The sex ratio in treatment two caused the males to remain in amplexus over much of the experimental period.

In each of 4 free male replicates, 21 males between 25.0 and 35.0 mg were collected, and their live weights determined. Five amplexed male replicates were prepared consisting of 7 males in the same weight range as above plus 14 females (smaller than the males).

Live weights of all males were determined on days 0, 9, 29, and 43 of the experiment. The experiment ended on the 44th day due to mortalities.

### *Growth rate of males without gnathopods*

Gnathopods were cut distal to the basis. Five replicates of this treatment and five uncut controls were prepared. Each of the control replicates contained 16 males between 10.0 and 20.0 mg. Two treatment replicates contained 17 males and the

others 16. All treatment replicates contained five females as well, which if found in amplexus indicated males with regenerated gnathopods. Checked daily, amplexed males had their gnathopods recut. Live weights of all male amphipods were determined every 9 days for 27 days. Examined under a microscope after weighing, gnathopods were recut if necessary.

## RESULTS

### *Free and amplexed feeding rates*

The results of the consumption of both free and amplexed female amphipods is given in Figure 1. The (Gut + Body) dpm data when linearly regressed against female weight shows a low, positive correlation in both cases. The equation describing the free female feeding rates has a slope  $U_f = 7.37$  (S.D. = 2.42,  $R^2 = .27$ ) while that of amplexed females is  $U_a = 7.6$  (S.D. = 3.05,  $R^2 = .19$ ). Although statistically significant, the  $R^2$  is low indicating only slight size dependency of feeding within the range of sizes studied. The low slopes and broadly overlapping size ranges of the two groups make adjustment for weight as a covariate unnecessary, and so a Kruskal-Wallis test (Sokal and Rohlf, 1981a) was used to test for a difference in (Gut + Body) dpm between amplexed and free females. There is no evidence to suggest that the activity measured in the two groups differed ( $\chi^2(1) = .620$ ,  $P > .05$ ). Female feeding rate appears to be independent of amplexus status and nearly independent of wet weight in this experiment.

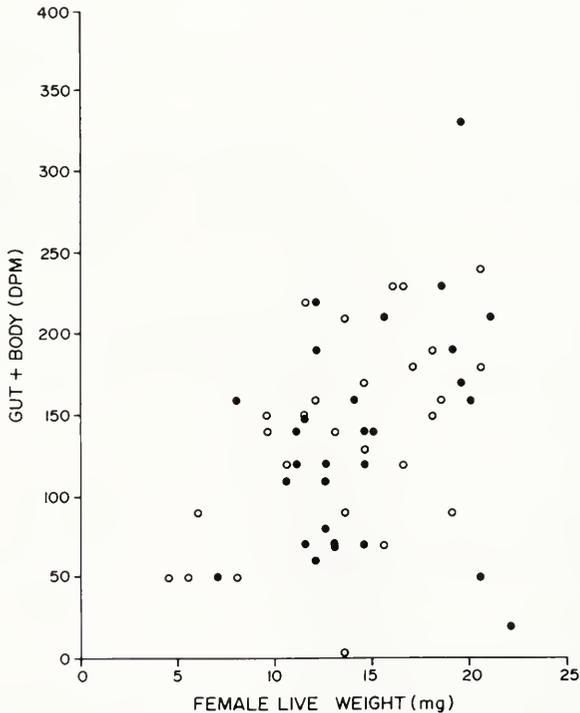


FIGURE 1. Food consumption over 24 h by free (open circles) and amplexed (closed circles) female amphipods.

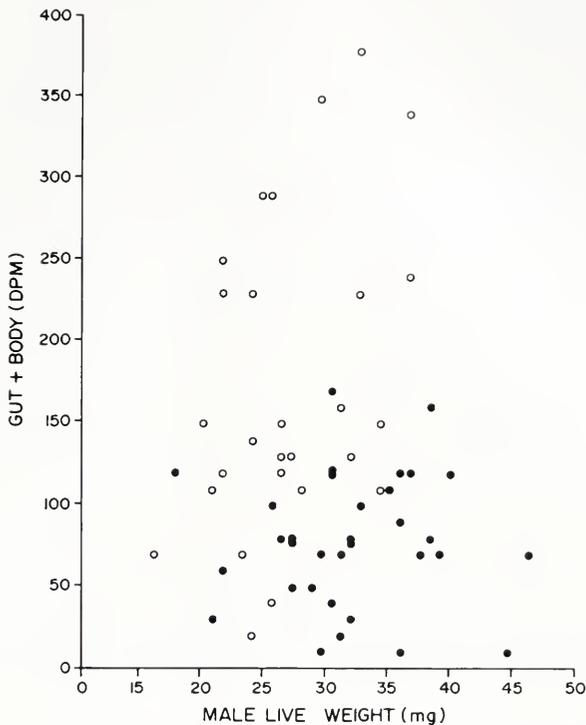


FIGURE 2. Food consumption over 24 h by free (open circles) and amplexed (closed circles) male amphipods.

Free and amplexed male feeding rates are shown in Figure 2. The slope of the linear equation of (Gut + Body) dpm regressed against male weight is  $U_f = 6.84$  (S.D. = 3.29,  $R^2 = .14$ ) and  $U_a = .0795$  (S.D. = 1.19,  $R^2 = 0$ ) for free and amplexed males respectively. Thus free male amphipod feeding rates are not significantly size-dependent. No correlation was shown between the calculated residuals of amplexed male consumption and either the female weight or the ratio of male to female weight, indicating that the feeding rate of amplexed males is independent of the size of the female carried. A Kruskal-Wallis analysis of this data indicates that male guarding of a female reduced male feeding rate ( $\chi^2(1) = 22.5$ ,  $P < .005$ ) by 53%.

#### *Growth rates of free and amplexed males*

The divergence of the mean weights of the two treatments is shown in Figure 3. A two-way analysis of variance (factors; amplexed state by replicate and period, Sokal and Rohlf, 1981b) of the increment of weight increase over the first 9 day period, second 20 day period, and final 14 day period is given in Table I.

Amplexed males in this experiment grew at a rate 45% less than that of free males. All factors, male state, period of growth, and the interaction between the two are significant, although the last less so than the first two.

#### *Growth rate of males without gnathopods*

The change in mean weight of these two treatments over 27 days is shown in Figure 4. The results of a two-way ANOVA (with three 9-day periods) are given in

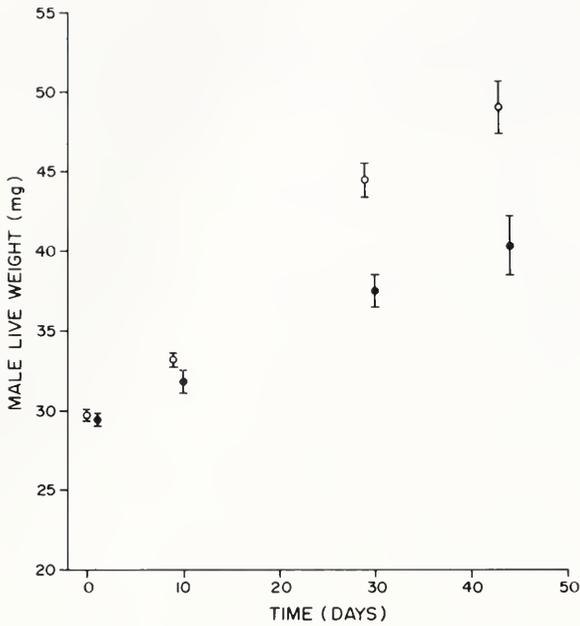


FIGURE 3. Growth of free (open circles) and amplexed (closed circles) male amphipods over 43 days.

Table II. They indicate, with a high degree of significance, that males with gnathopods grew 44% faster than those who had had them removed.

#### DISCUSSION

Mate guarding is advantageous to a male when the cost of amplexus is less than the return in reproductive success to that male (Parker, 1974). The results (Fig. 3) indicate that a major physiological cost incurred by amplexing males is reduced growth. The close correspondence between the growth lost in amplexus and the decrease following gnathopod removal indicates that amplexus in *G. lawrenciamus* has a cost not so much in energy expended to carry a female, but in reduced food consumption by the male.

TABLE I

ANOVA table of growth of free and amplexed males over three periods

Source of variation	df	ss	F <sub>s</sub>
Subgroups	5	232.9	12.7**
A (Time period)	2	145.6	19.9**
B (Amplexus status)	1	59.1	16.1**
A × B (Interaction)	2	28.2	3.84*
Within subgroup error	18	66.0	
Total	23	298.8	

\* 0.01 < P < 0.05.

\*\* P < 0.001.

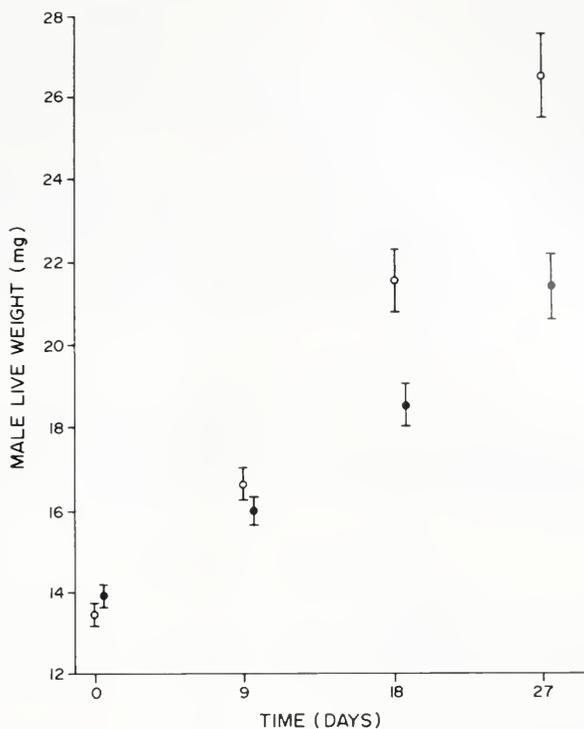


FIGURE 4. Growth of male amphipods with (open circles) and without (closed circles) gnathopods over 27 days.

Size-assortive mating is caused by (1) larger female amphipods selecting for larger mates by escaping smaller males more often (Ridley and Thompson, 1979), and (2) larger males preferentially mating with larger females (Manning, 1975; Birkhead and Clarkson, 1980; Hunte *et al.*, in press). Since larger females are more fecund (Doyle and Hunte, 1981b; Doyle and Myers, 1982), larger male amphipods on average will produce more offspring than smaller males in any one successful mating.

TABLE II

*ANOVA table of growth of males with and without gnathopods over three 9-day periods*

Source of variation	df	ss	F <sub>s</sub>
Subgroups	5	38.6	5.52*
A (Time period)	2	6.07	2.17
B (Gnathopod status)	1	31.1	22.2**
A × B (Interaction)	2	1.44	0.514
Within subgroup error	24	33.6	
Total	29	72.2	

\*  $0.005 < P < 0.001$ .

\*\*  $P > 0.001$ .

The optimal duration of amplexus depends on population characteristics such as mortality rate, sex ratios, and male and female size distribution (Hunte *et al.*, in press). Since the relations between male and female size and female fecundity are reasonably linear, a 45% decrease in size increment at the next molt would represent a similar loss in the incremental male reproductive success (measured as the number of eggs fertilized).

In their examination of *G. lawrencianus*, Hunte *et al.* (in press) determined that immediate past male investment in amplexus may influence mating decisions through change in physiological state (*e.g.*, a post-amplexus refractory period). We now observe that the situation is complicated by yet another factor, a trade-off between present reproduction and future size, as modulated by feeding activity during amplexus.

#### ACKNOWLEDGMENTS

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