THE
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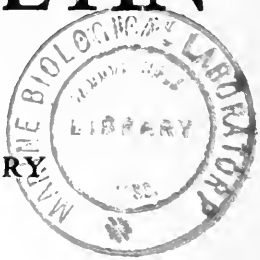

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LARVAL DEVELOPMENT IN THE ASTEROID *ECHINASTER ECHINOPHORUS*

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Echinaster echinophorus (Lamarck), belonging to the order Spinulosida and family Echinasteridae, is a shallow-water asteroid previously reported from the Caribbean (Bahamas) down to Brazil (Kempf, 1966). This species, having a small central disc and five short radiating arms, occurs in 1 to 2 meters of water on *Diplanthera* and *Thalassia* grass beds, submerged rock deposits and oyster banks. When the breeding season begins, the sea stars move out from beneath grass beds and rocks and clump together in groups of two to six. *Echinaster*, spawning from early April to the end of July, produces large yolky eggs (0.8–1.3 mm diameter) in small numbers (50–250) and exhibit lecithotrophic development with radial, holoblastic, equal cleavage (first three cleavages).

The species *E. echinophorus* appears to either contain several morphologically distinct variants or a complex of closely related species. Morphologically dissimilar animals (spine arrangement, coloration pattern and size) have been observed within the same general habitat to spawn two characteristically different egg types (Atwood, unpublished observations). One egg type (Type 1) ranges from 1.0 to 1.3 mm in diameter, is colored brownish-black and floats throughout early development. The modified brachiolaria becomes a free-swimming larva at about 2 days and settles, attaching to the substratum by means of a larval sucker, by the third day. This developmental type has been reported once before in *Echinaster* found in *Diplanthera* grass beds of Brazil (Kempf, 1966).

The second egg type (Type 2) produced by this species is bright orange, somewhat smaller (0.84–0.88 mm diameter) and settles immediately to the substratum after spawning. These eggs are surrounded by a heavy adhesive material which serves to attach them in groups of 2 to 25 to grass blades, rocks or oyster shells. The modified brachiolaria larvae do not pass through a free-swimming stage.

The purpose of this study is to report on the external morphogenesis and larval growth rate of laboratory cultured *Echinaster* eggs of the second type.

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Materials and Methods

Adult specimens of *Echinaster echinophorus* were collected from shallow-water oyster banks in the Anclote estuary off the coast of Tarpon Springs, Florida, and were maintained in the laboratory in recirculating seawater systems (25° C) until spawning was observed. Eggs were collected from spawning females and placed in aquaria containing dilute sperm suspensions. Males were induced to spawn by injections of radial nerve extracts (Chaet, 1966; Kanatani and Ohguri, 1966; Atwood and Simon, 1971). Larvae were relaxed in 6.5% magnesium chloride, or 0.15% propylene phenoxetol (Goldschmidt Chemical Corp., New York; Owen, 1955; Humason, 1967), fixed in Bouin's solution and stored in 70% ethanol. Natural breeding habits were observed in the field throughout the normal spawning season. All developmental stages were photographed following fixation with the aid of a Nikkormat camera and Wild M-5 dissection microscope.

Sea star size was expressed in terms of R (the distance from the center of the mouth to the tip of the ray), r (the distance from the center of the mouth to the middle of the interbrachial arc), R/r (the ratio between R and r) and wet weight. All arms of each specimen were measured with an ocular micrometer and the average R used. One measurement of r (the largest of the five values) was recorded for each specimen.

Wet weights were measured by removing sea stars from water, holding until most of the fluid had dripped from them, blotting with filter paper and weighing together with any liquid that continued to exude.

Observations

Cleavage occurred rapidly at the laboratory culture temperature of 25° C with the 32-cell stage being reached 6½ hours after fertilization. The young gastrula was formed by the end of the first day. By 48 hours the gastrula had elongated into an oblong larva consisting of a large larval body and a smaller preoral lobe (PL), with rudiments of four larval arms (two dorsal—A, B; and two ventral—C, D) and a central sucker (S) (Fig. 1). Three days after fertilization bulges of the five hydrocoel lobes and the first two pairs of tube feet on each ray were evident on the left side of the larval body of the modified brachiolaria (Fig. 2). The larvae had increased in length from 0.86 to 1.48 mm by the fourth day and begun to flatten laterally. The hydropore (H) appeared on the right side of the larval body by the end of the fourth day (Fig. 3).

Larvae of five days (Fig. 4) had reached a length of 1.53 mm. The bulbous arms increased in size as the centrally located sucker became differentiated. The water ring and radial canals appeared more distinct as bulges for the terminal podia (P) became evident. Both pairs of tube feet on each ray showed signs of growth with the proximal pair being better differentiated. Counting rays in a clockwise direction beginning with the ray at the base of the preoral lobe (Fig. 4), the formation of tube feet paralleled that of the hydrocoelic pouches, with those of rays 3 and 4 developing first, followed by rays 2 and 5, and finally number 1.

By 7 days the tube feet were functional as the juvenile sea star began to move about. Terminal podia (P) were differentiated and exceedingly extendable (Fig. 5). Absorption of the preoral lobe at this stage was evident as it began to fold

toward the oral side of the sea star. Larval arms (A, B, C, D) had reached their maximum size and begun to show stress under the folding process (Fig. 5).

Distinction between the left (B) and right (A) dorsal larval arms became difficult at $7\frac{1}{2}$ days as absorption of the preoral lobe continued and the folding proceeded toward completion. Absorption of the right ventral arm (C) preceded

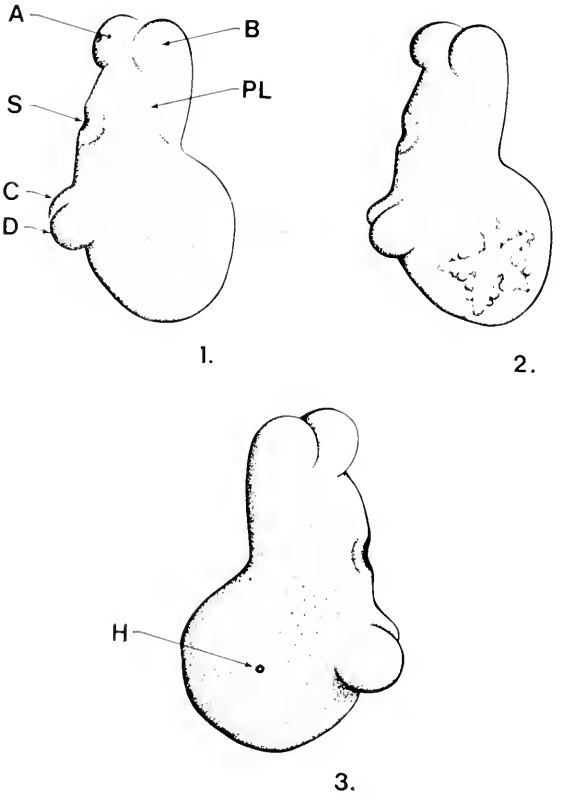


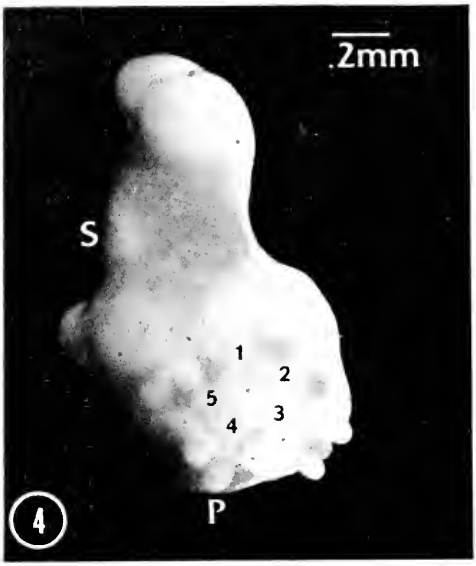
FIGURE 1. Left side (oral) view of a 2-day-old brachiolaria showing the larval right dorsal arm (A), left dorsal arm (B), right ventral arm (C), left ventral arm (D), larval sucker (S) and preoral lobe (PL).

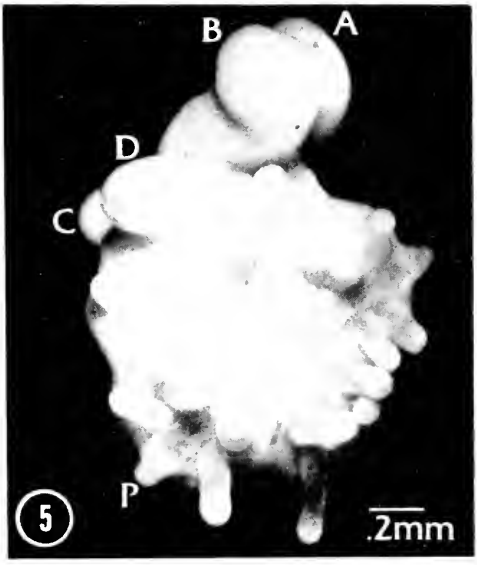
FIGURE 2. Three-day-old brachiolaria showing bulges for the five hydrocoel pouches and first two pairs of tube feet.

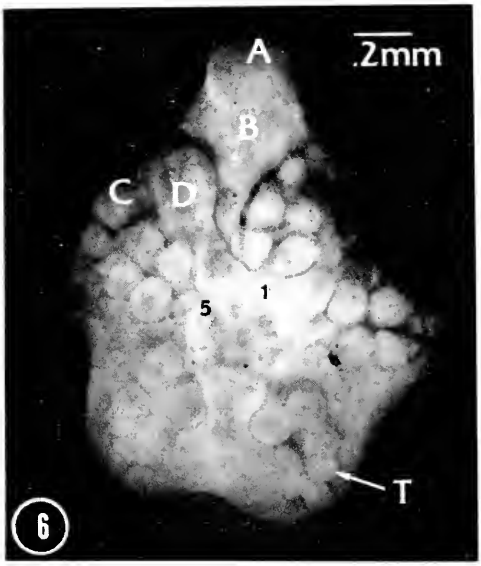
FIGURE 3. Right side (aboral) view of 4-day-old brachiolaria with functional hydropore (H).

that of the left (D). After folding was complete (8 days), the left ventral arm (D) was positioned between the first and fifth rays. The right ventral arm (C) was shifted distally and aboral to the left (Fig. 6). Absorption of the larval sucker appeared to be complete as the third pair of tube feet (T) on each ray appeared on the eighth day (Fig. 6).

The preoral lobe (PL) had been reduced to a small bulge between the first and fifth rays by the tenth day (Fig. 7). A fourth pair of tube feet (T) on each







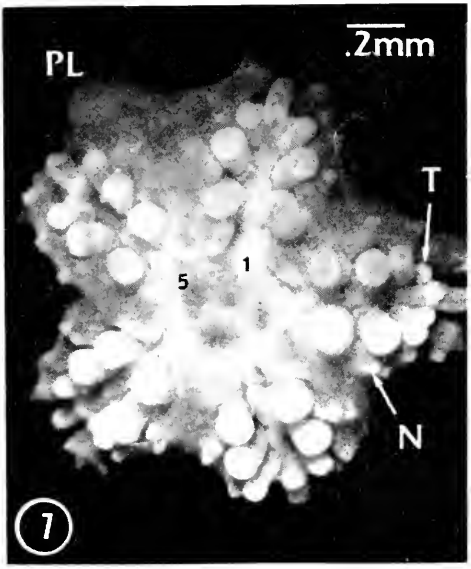


FIGURE 4. Five-day sea star (oral view) with larval sucker (S), bulges for terminal podia (P) and rays 1–5.

FIGURE 5. Preoral lobe folding in 7-day sea star with larval arms (A, B, C, D refer to Fig. 1) and terminal podia (P).

FIGURE 6. Eight-day sea star with larval arms (A, B, C, D refer to Fig. 1), third pair of tube feet (T) and rays 1 and 5.

FIGURE 7. Ten-day-old *Echinaster* juvenile showing remaining preoral lobe (PL), fourth pair of tube feet (T), spines (N) and rays 1 and 5.

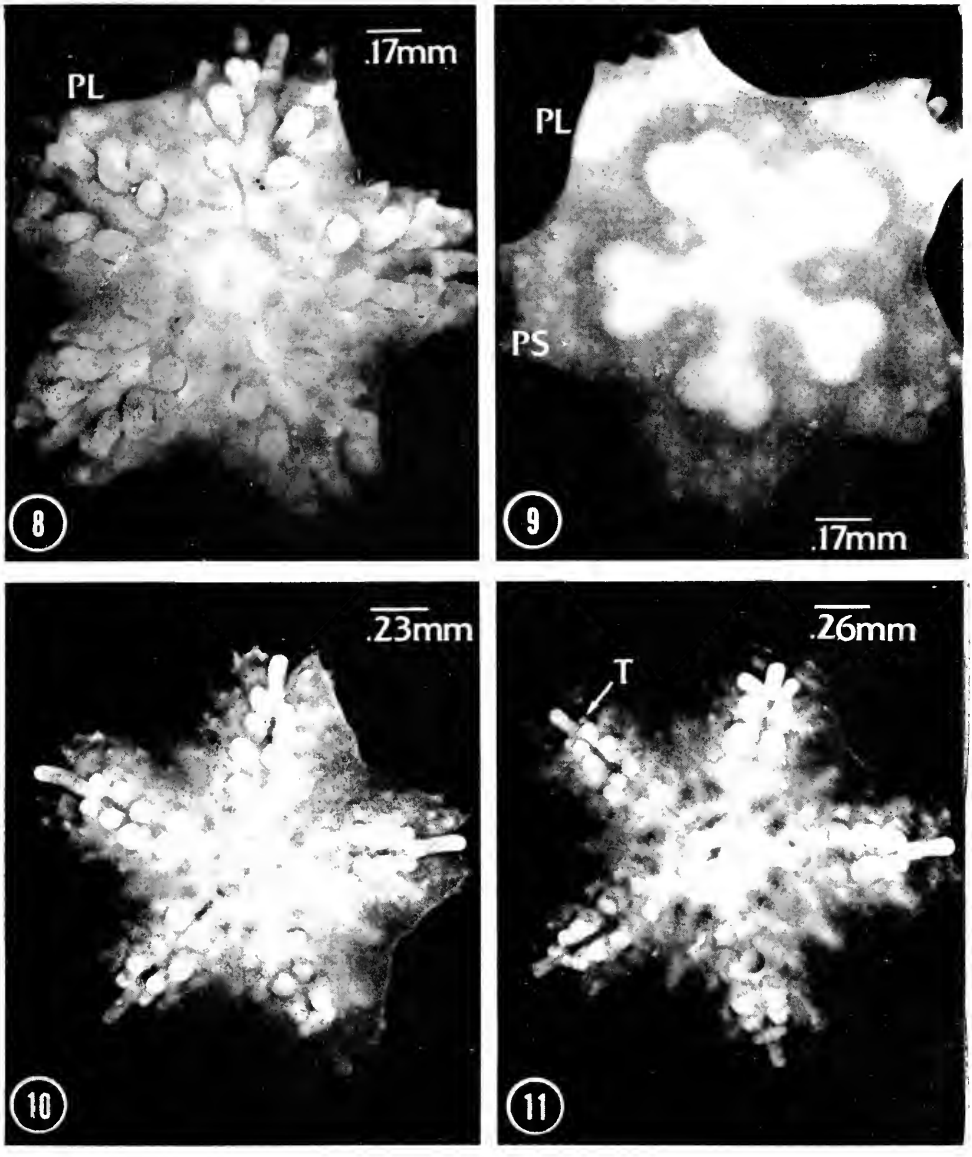


FIGURE 8. *Echinaster* juvenile at 13 days showing small quantity of remaining preoral lobe (PL) tissues.

FIGURE 9. Aboral surface of 13-day sea star with remaining preoral lobe (PL) and light pigment spots (PS).

FIGURE 10. Fourteen-day-old *Echinaster* with newly formed mouth.

FIGURE 11. *Echinaster* at 35 days showing the translucent appearance and the recently developed fifth pair of tube feet (T).

ray formed between the third pair and the terminal podium as spines (N) developed on the oral and aboral surfaces. Spines were larger and more numerous on the oral surface near the terminal portion of each ray (Fig. 7). Thirteen days after fertilization, the preoral lobe (PL) was reduced to a small thickening and light-colored pigment spots (PS) were evident on the oral and aboral surfaces (Figs. 8, 9). The thin membrane sealing the oral region ruptured in 50% of examined juveniles at fourteen days exposing a small functional mouth (Fig. 10). All four pairs of tube feet on each ray were functional at this stage. The anus opened when the larvae were 16 days old. The larval preoral lobe was completely absorbed by this time.

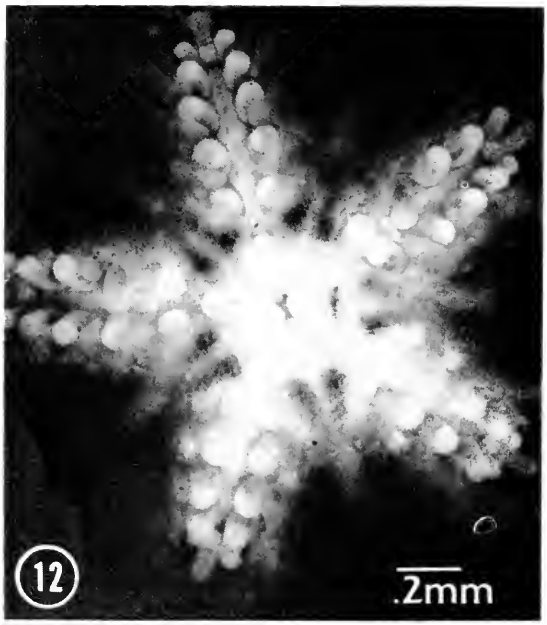


FIGURE 12. Young sea star at 77 days.

Yolk material was completely used up at 35 days and the young sea star appeared translucent. The fifth pair of podia (T) on each ray had developed distal to the fourth pair by this time (Fig. 11). Figure 12 shows the five functional pairs of tube feet and the well developed, muscular mouth of a 77-day-old sea star.

The chronological development as discussed in the preceding text is summarized in Table I.

Echinaster embryos increased in diameter from 0.86 to 0.99 mm within the first 6½ hours after fertilization. By 6 days the larvae reached a length of 1.55 mm (Table II). Growth rates based on ray lengths were employed when larvae attained a sufficient measuring size at 6 days. Juvenile ray lengths showed dramatic increases between 6 and 10 days (0.37 to 0.68 mm) followed by a second burst of growth at 14 days (0.69 to 0.80 mm) (Table III, Fig. 13). Values for the ratio R/r are presented in Table III. Growth rates expressed by wet weights

TABLE I

Chronology of larval development

Time	Developmental events
0 hr	Fertilization
1½ hr	Germinal vesicle breakdown
5 hr	4-cell stage
6½ hr	32-cell stage
8 hr	64-cell stage
10 hr	Morula
23 hr	Blastula; gastrulation
48 hr	Modified brachiolaria larvae with preoral lobe, four small larval arms and sucker
3 days	Five hydrocoel lobes appear; bulges for the first two pairs of tube feet on each ray develop
4 days	Lateral flattening of larval body; hydropore opens to right side of larva
5 days	Differentiation of larval arms and sucker; water ring and radial canals are distinct; bulges of terminal podia appear
6 days	Differentiation of first two pairs of tube feet on each ray
7 days	Tube feet are functional; larval attachment is released; juvenile under own power; terminal podia are differentiated; preoral lobe absorption begins as it folds towards left side of larva
8 days	Formation of third pair of tube feet on each ray; preoral lobe folding completed
10 days	Formation of fourth pair of tube feet on each ray; development of spines on oral and aboral surfaces
14 days	Mouth opens
16 days	Anus opens; preoral lobe appears to be completely absorbed
35 days	Fifth pair of tube feet developed on each ray; yolk materials appear to be used up

were calculated (Table III) and showed a correlation coefficient of 0.85 when compared to growth rates expressed in terms of ray lengths.

DISCUSSION

Fragmentary observations on the external development of *Echinaster echinophorus,* spawning eggs of the buoyant type, were reported by Kempf (1966). Once the embryos of this type completed their free-swimming stage and settled to the substratum, development was comparable to that of the demersal type ob-

TABLE II

Growth rates of early stages of Echinaster echinophorus *expressed as larval lengths (mm) based on a minimum sample size of 10*

Time	Length range	Mean length
1½ hr	0.84–0.88	0.86
5 hr	0.88–1.00	0.91
6½ hr	0.96–1.00	0.99
2 days	0.99–1.26	1.24
3 days	1.20–1.37	1.34
4 days	1.35–1.48	1.48
5 days	1.50–1.54	1.53
6 days	1.48–1.58	1.55

TABLE III

Growth rates of juvenile Echinaster echinophorus *expressed as ray lengths (R, mm) and wet weights (mg) based on a minimum sample size of 10*

Time (days)	R		R : r	Wet weight	
	Range	Mean		Range	Mean
6	0.36–0.40	0.37	1.01	0.1–0.2	0.15
7	0.40–0.56	0.49	1.10	0.1–0.2	0.17
8	0.54–0.60	0.57	1.18	0.1–0.2	0.18
10	0.62–0.72	0.68	1.25	0.2–0.3	0.26
12	0.66–0.70	0.69	1.26	0.3–0.4	0.34
13	0.60–0.72	0.69	1.26	0.3–0.4	0.38
14	0.76–0.84	0.80	1.28	0.3–0.5	0.40
16	0.74–0.84	0.82	1.30	0.3–0.5	0.43
17	0.76–0.84	0.82	1.30	0.3–0.5	0.43
20	0.82–0.92	0.87	1.32	0.3–0.5	0.43
23	0.88–0.90	0.88	1.32	0.5–0.6	0.52
28	0.82–0.98	0.90	1.34	0.5–0.6	0.53
35	0.82–1.00	0.94	1.35	0.5–0.6	0.56
43	0.90–1.00	0.95	1.36	0.5–0.6	0.58
77	0.98–1.10	1.03	1.37	0.8–1.0	0.85

served in the present study. The free-swimming stage which was completed by the end of the third day lasted for 24 hours. The remaining observations of Kempf (1966) are summarized in Table IV.

Kempf's (1966) observations showed the formation of tube feet was much slower (culture temperature at 27° C), with the third pair forming at 10 days compared to 8 days in the present work, and the fourth developing at 23 days, compared to 10 days in the present study. Animals reached a length of 2.2 mm at

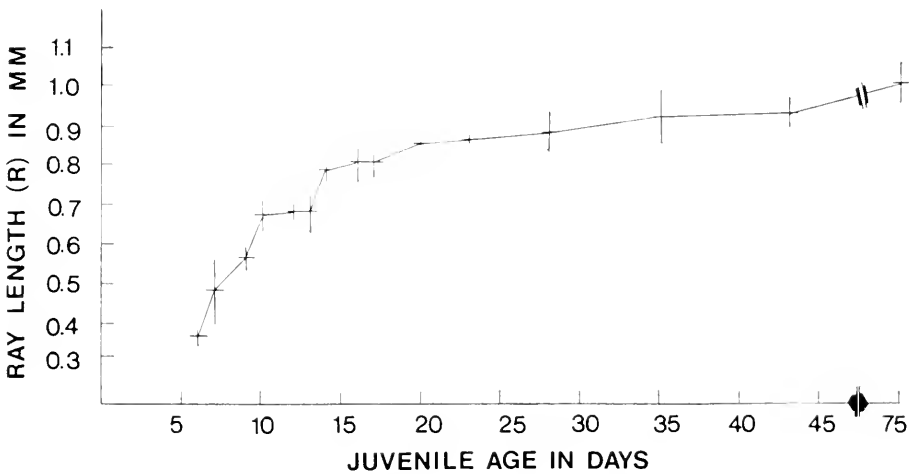


FIGURE 13. Juvenile growth rate of *Echinaster echinophorus*. Vertical lines represent one standard deviation; horizontal lines represent means based on a minimum sample size of 10.

2 days and increased to 3 mm by 4 days. R values enlarged from 0.8 mm to 1.05 mm between 4 and 12 days and reached 1.6 by 100 days. R/r values ranged from 1.26 at 6 days to 1.39 at 100 days (Kempf, 1966). Unfortunately, no comparative data concerning culture conditions, formation of the fifth pair of tube feet, mouth, spines or anus is available.

Löhner (1913) reported that embryos of European *Echinaster sepositus*, measuring 1 mm in diameter when spawned, attained a R of 1.2 mm and a R/r of 1.5 at 20 days of growth. Nachtsheim (1914) observed that eggs of *E. sepositus* were bright red, passed through a free-swimming stage (similar to that described

TABLE IV

Observations on development of Echinaster echinophorus *(Kempf, 1966) and* Echinaster sepositus *(Löhner, 1913; Nachtsheim, 1914)*

Time (days)	Developmental events	
	Echinaster echinophorus (Kempf, 1966)	*Echinaster sepositus* (Löhner, 1913; Nachtsheim, 1914)
3	Growth of larval arms and sucker; larvae settle to substratum	Modified bipinnaria
4	Development of bulges for first two pairs of podia on each ray	
5	Both pairs of podia on each ray are differentiated; water ring and radial canals with primary podia become distinct	Development of four larval arms and sucker; fixation of larvae to substratum
6	Podia become functional; larval attachment is released	Development of bulges for first two pairs of podia and terminal podium on each ray
8–9	Coloration changes from brown to orange	
10	Third pair of podia develop on each ray	Podia are functional; absorption of preoral lobe begins
14		Development of third pair of podia on each ray; absorption of preoral lobe nears completion
23	Fourth pair of podia develop	

by Kempf, 1966) and reached a R of 1.9 mm and a R/r of 1.7 by $7\frac{1}{2}$ months. Development was somewhat slower in the European species which may reflect the influence of lower culture temperatures (Table IV).

Folding of the preoral lobe towards the oral surface of the 7- to 8-day *Echinaster* larva (Figs. 5, 6) has not been observed by previous investigators. Löhner (1913), Nachtsheim (1914) and Kempf (1966) have indicated that absorption of larval tissues took place in an uniform manner disregarding any folding towards either the oral or aboral surface. Soon after larval fixation, the preoral lobe of *Henricia sanguinolenta* (*Cribella oculata*) (order Spinulosida, family Echinasteridae) becomes twisted towards the central disc as the disc rotates in a counterclockwise direction. Through this process the right lateral arm extends backwards towards the right (aboral) larval side as the single dorsal arm twists

with the disc through about 15° (Masterman, 1902). Masterman (1902) viewed this process as a purely mechanical torsion resulting from the folding of the disc towards the oral surface. MacBride (1896) also observed sharp flexure of the disc downwards towards the left larval surface of *Asterina gibbosa* (order Spinulosida, family Asterinidae).

Larval tissues of *Solaster endeca* and *Crossaster papposus* (order Spinulosida, family Solasteridae) undergo a characteristic flexion and torsion at 13 to 19 days of growth (Gemmill, 1912, 1920). A lateral flexion of the preoral lobe towards the oral surface of the embryo and a torsion of the neck of the lobe in a clockwise direction occur simultaneously. Both movements proceed in a gradual manner and are completed by 21 to 22 days after fertilization. The preoral lobe is folded against the left side of the body and is incorporated into the central area of the oral surface through which the mouth will open after another 40 to 50 days. Preoral lobe folding in *Echinaster echinophorus* is not as extensive as that of *Solaster* and *Crossaster* and is restricted to the peripheral tisues between rays number 1 and 5. Neither is there an apparent incorporation of the preoral lobe into the central region of the oral disc of *Echinaster*.

Various invertebrate species have been shown to exhibit different developmental patterns in different regions within their geographic ranges (Thorson, 1950). Thorson (1950) concluded that this phenomenon, poecilogony, is insignificant in the echinoderms. Animals presently classified as *Echinaster echinophorus,* however, shed two characteristically different egg types which undergo different developmental patterns.

The species *echinophorus* in the Florida study area contains at least four morphologically distinct variants. The demersal egg type (Type 2) described in the present study is consistently spawned by the smallest of the variants which measures approximately 6 cm from the tip of a ray to the tip of the opposite ray, occurs in 1–2 meters of water and has a bright red and black coloration pattern. The buoyant egg type (Type 1) has been observed to be spawned by an *echinophorus* which is at least 5 cm larger in diameter, is colored pale orange and normally occurs in slightly deeper water ranging from 3–5 meters in deepth. No spawning has been observed in the remaining two variant types.

Preliminary cross fertilization experiments have shown that mature sperm from either of the two morphological variants will fertilize mature ova of the other. Larval and juvenile development appear to progress normally.

Either this species exhibits differential reproductive behavior in the same locality at the same time, or two or more species are present. This problem can only be solved by completing an extensive taxonomic and developmental study.

I would like to thank Mr. David L. Ballantine for assistance in culture, and Drs. J. M. Lawrence and J. L. Simon of the University of South Florida and Dr. F. S. Chia of the University of Alberta for critically reading the manuscript.

Summary

1. *Echinaster echinophorus* off the west coast of Florida was observed to spawn, from early April to late July, two different egg types: one type is pelagic,

brown to black in color and 1.0–1.3 mm in diameter, while the second is bright orange, ranges from 0.84 to 0.88 mm in diameter and settles immediately to the substratum after spawning.

2. The present study reports on external morphogenesis of *Echinaster* eggs of the second type.

3. Cleavage stages occurred rapidly at the laboratory temperature of 25° C with the gastrula being reached 24 hours after fertilization. By 48 hours a modified brachiolaria develops with 4 larval arms and a central sucker. Folding of the preoral lobe toward the oral surface at 7 days of growth is reported for the first time in *Echinaster*.

4. A chronology of larval and juvenile development is presented.

5. Larval growth rates expressed as lengths (mm) as well as juvenile growth rates expressed as ray lengths (mm) and wet weights (mg) are presented.

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ENDOGENOUS AND PHOTOPERIODIC DIURNAL RHYTHMS OF *IN VIVO* LIGHT ABSORPTION AND SCATTERING IN THE GREEN ALGA *ULVA LACTUCA* L.[1]

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The *Ulva* thallus is two cells in thickness. Each cell contains one large chloroplast covering the outer face of the cell (Taylor, 1937). Held to the light, *Ulva* appears as a translucent, homogenous green sheet. Britz, while at the Chesapeake Biological Laboratory during the summer of 1970, observed the *in vivo* visible light absorption spectra of locally collected *U. lactuca* and noted the absorbance varied considerably over time at all wavelengths in a seemingly rhythmic pattern (unpublished data) (see Fig. 2A). This paper presents a closer examination of these observations under controlled conditions.

Methods and Materials

At the end of November, 1970, free floating specimens of *U. lactuca* were collected along shore near the mouth of the Patuxent River, Solomons, Maryland. These specimens were cultured in the laboratory through the end of April, 1971, at which time fresh specimens were obtained from the same location. Provasoli ASW-8 nutrient medium was used with the substitution of Chesapeake Bay water for sea water in the formula (Provasoli, 1958). Specimens were grown in beakers or flasks covered with clear plastic Petri dish covers in a water bath at 24 ± 1° C. Overhead illumination was provided by two 20W cool white fluorescent and two 40W incandescent lights. Light intensity at the water bath surface was approximately 200 ft-c. A light-dark cycle of 16 hours of light and 8 hours of darkness was employed, the cycle being synchronized to a natural photoperiod with D + 8 at 0600 EST. Considering the possibility that epiphytic bacteria could be involved in normal thallus morphogenesis (Provasoli, 1958) no attempt was made to culture *Ulva* axenically. Epiflora were assumed to be removed by careful washing of the thalli. Bacterial growth in the nutrient medium was held down by changing the containers and medium weekly. At the same time thalli were cleaned and washed by hand.

Initial measures of light absorption were obtained from spectra of live *Ulva* thalli recorded against a bleached thallus reference using a Bausch and Lomb Spectronic 505. Due to light scattering and reflection, it should be emphasized that the optical density values recorded cannot be construed as absolute measures of light absorption. For a more detailed discussion of the problems and methodol-

[1] These investigations were supported by U.S.A.E.C. Contract No. AT(11-1)3278.

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ogy of *in vivo* spectroscopy the reader would be advised to consult Butler (1964). More specifically, Mestre (1935) related the problems involved in using a bleached thallus reference for absorbance measurements in *Ulva*. For the purpose of this work, however, it was considered sufficient to use relative values of light absorption, since a rhythmic phenomenon provides its own internal standard. It was assumed, furthermore, that the thick cell walls of the *Ulva* thallus would diffuse effectively the transmitted and scattered light, acting to some extent like opal glass in providing an internal correction for scattered light effects (Butler, 1964). Recognizing the above distinctions, the recorded optical density values are referred to as Relative Optical Density (ROD) values.

ROD measurements were made on 13 mm diameter *Ulva* thallus circles cut with a cork borer and placed between two square glass sample holders cut from microscope slides. Caution was exercised to avoid wrinkles and air bubbles in the light path. The sample holder was placed in the "optical bench trough" resting flush against the cuvette holder on the phototube side. Eight measurements of ROD were obtained for each thallus by rotating the sample holder 90° for each face. An average ROD was then calculated. In general, one thallus circle would be monitored throughout a particular experiment. This technique served to eliminate minor variations in ROD from thallus to thallus. Relative standard deviations of the mean ROD were of the order of a few per cent.

For all ROD measurements a reference cell was used consisting of a thallus circle bleached 24 hours in dimethylsulfoxide (DMSO). The bleached thallus was soaked for several minutes in distilled water, rinsed twice, and placed between two glass plates of a sample holder which was then sealed with clear nail polish. The ROD spectrum of the reference thallus versus a water blank revealed a smooth, monotonically increasing curve with decreasing wavelength. This was interpreted as being due to light scattering. At 350 and 800 nm ROD values were 0.85 and 0.51, respectively. Between 560 and 760 nm the curve was approximately linear.

Extractable chlorophyll *a* and *b* concentrations were obtained by a modification of the method of Reger and Krauss (1970). Thallus circles 13 mm in diameter were cut with a cork borer, placed in 5 ml of prechilled 20% DMSO in methanol, and left to extract in the dark at 6° C. Extraction of pigments was complete within two hours and the pigments were stable for at least 24 hours under the same conditions as extraction. Using a separatory funnel, the pigments were transferred to prechilled anhydrous ethyl ether. The ether solution was adjusted to 5 ml total volume and dried for several minutes over anhydrous Na_2SO_4. After the extract had warmed to room temperature, optical densities at 642 and 662 nm were recorded on a Bausch and Lomb Spectronic 505 and the values applied to the simultaneous equations of Smith and Benitez (1955). All pigment extraction work was done in either very dim white or green light.

To measure a possible photoperiodic change in optical dichroism, the differential absorption of vertically and horizontally polarized light was measured in a Beckman DK-2 Spectrophotometer. Substantial horizontal polarization of light by the optics of the Bausch and Lomb Spectronic 505 made this instrument unsuitable. A cradle for the glass sample holder was designed to fit a Beckman DK-2 light path. Polaroid *J* filters were inserted in the light beam to produce either horizontal or vertical polarization. A bleached *Ulva* reference cell was used. ROD

values were calculated from the relative transmittance at 682 nm using a correction factor derived to account for the differences in transmittance between horizontal and vertical polarizing orientations of the filter.

Results

Figure 1 shows a typical light absorption spectrum for an *Ulva* thallus at mid-photophase. At all wavelengths ROD values varied rhythmically with periods

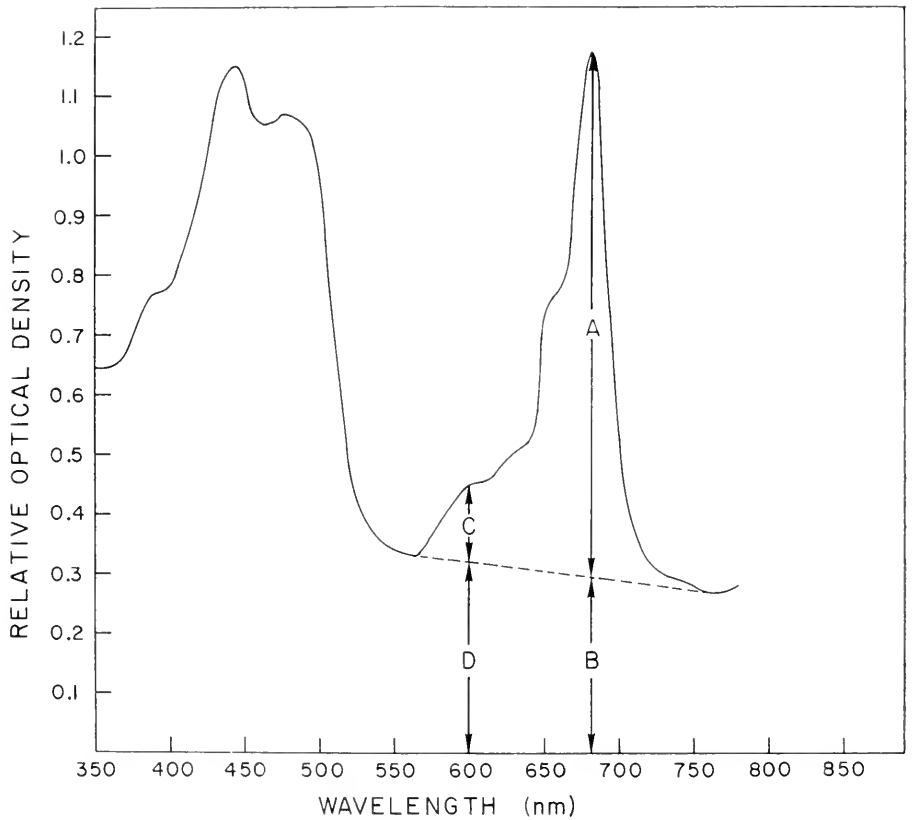

Figure 1. Typical *in vivo* absorption spectrum of mid-photophase *Ulva lactuca* L. recorded with a Bausch and Lomb Spectronic 505. See explanation under Results.

of 24 hours. However, the time of maximum ROD varied with wavelength. For example, ROD maxima at 432, 470, 493, 560, 600, 654, 682, and 760 nm occurred at L + 9, L + 9, L + 9, L + 14, L + 14, L + 12, L + 9, and D + 2, respectively. Values of ROD at 760 nm, where pigment absorption should be negligible, are defined to represent relative light scattering. The above observations were then interpreted as resulting from the addition of two different rhythms—light absorption by the photosynthetic pigments and light scattering. The light absorption rhythm seemed to have a maximum around mid-photophase, while light scatter-

ing was maximal during early scotophase. At a particular wavelength, the time of the observed ROD maximum would depend on the relative amplitude of the two rhythmic components.

On the basis of measurements conducted over three days, mid-photophase ROD values at 432, 470, and 493 nm were, respectively, 2.15, 1.97, and 1.89 times the scotophase minimum values. Although these comparisons are complicated by overlapping pigment absorption bands and light scattering, the constancy of change at three wavelengths where first chlorophyll *a*, then chlorophyll *b*, and finally the carotenoids, respectively, would be expected to be major absorbers suggests the light absorption rhythm involves both chlorophylls *a* and *b* and perhaps carotenoids.

High ROD values at 560 and 760 nm (see Fig. 1) suggested the bleached thallus reference was not correcting entirely for light scattering. Thus, in the portion of the ROD spectrum between 560 and 760 nm a further correction was made by connecting these points graphically with a straight line (dashed line in Fig. 1) designated the Light Scattering Baseline (LSB). This assumed a linear light scattering curve, such as seen for the ROD spectrum of the bleached thallus reference, and neglible pigment absorption at 560 nm. The latter assumption, however, is probably not correct, since the ROD_{560} rhythm is not in phase with the ROD_{760} rhythm—indicating the presence of substantial pigment absorption. The portion above the LSB (A and C in Fig. 1) was designated the Pigment Absorption Component (PAC), while the portion below the LSB was designated the Light Scattering Component (LSC). PAC values are defined to be relative measures of light absorption corrected for light scattering. It was noted that PAC rhythms at 600, 654, and 682 nm were in phase and had maxima at L + 8. The ability of the LSB correction to resolve a single light absorption rhythm at different wavelengths (where the ROD rhythms were out of phase) was taken as evidence for the validity of the procedure, at least at wavelengths where the amplitude of the PAC rhythm was large with respect to the amplitude of the LSC rhythm.

In Figure 2B typical values of relative light absorption, represented by PAC_{682}, and light scattering, given by ROD_{760} are plotted over three normal light-dark cycles and 57 hours of continuous darkness following the last scotophase.

The rhythm of PAC_{682}, while dampening, continued clearly for at least three cycles of continuous conditions with a periodicity that seemed to remain close to 24 hours. The ROD_{760} rhythm also appeared to continue, albeit rather erratically toward the end. It did not appear to change its phase relation to the PAC_{682} rhythm. At the conclusion of the period of continuous darkness the thallus was transferred to a normal light-dark cycle where over a period of a week its regained a normal rhythm pattern.

In an attempt to elucidate the basis of the light absorption rhythm, chlorophyll *a* and *b* concentrations were determined three times over a 24 hour period. Five 13 mm diameter thallus circles were extracted at each time, and the average content per thallus of chlorophyll *a* and *b*, respectively, was as follows: 21.5 ± 0.7 and 9.2 ± 0.4 μg at D + 7.7, 22.5 ± 0.2 and 9.5 ± 0.2 μg at L + 8.2, and 22.8 ± 1.0 and 9.3 ± 0.3 μg at L + 15.4. Cholorphyll *b* concentrations did not seem to change, but cholorophyll *a* concentrations did appear to increase gradually about 6% over the 16 hour light period. However, comparison of the maximum and

minimum chlorophyll *a* values by t-test showed no significant difference to approximately 85% certainty. Even if significant, the chlorophyll *a* concentration increase was neither large enough to account for the amplitude of the light absorption rhythm nor was the change in chlorophyll *a* concentration in phase with the rhythm.

Alternatively, it was considered that the light absorption rhythm might be brought about by a rhythm of cholorphyllide *a* and *b* incorporation as chlorophyll *a* and *b* by the thylakoid membranes. Ethyl chlorophyllides *a* and *b* have absorb-

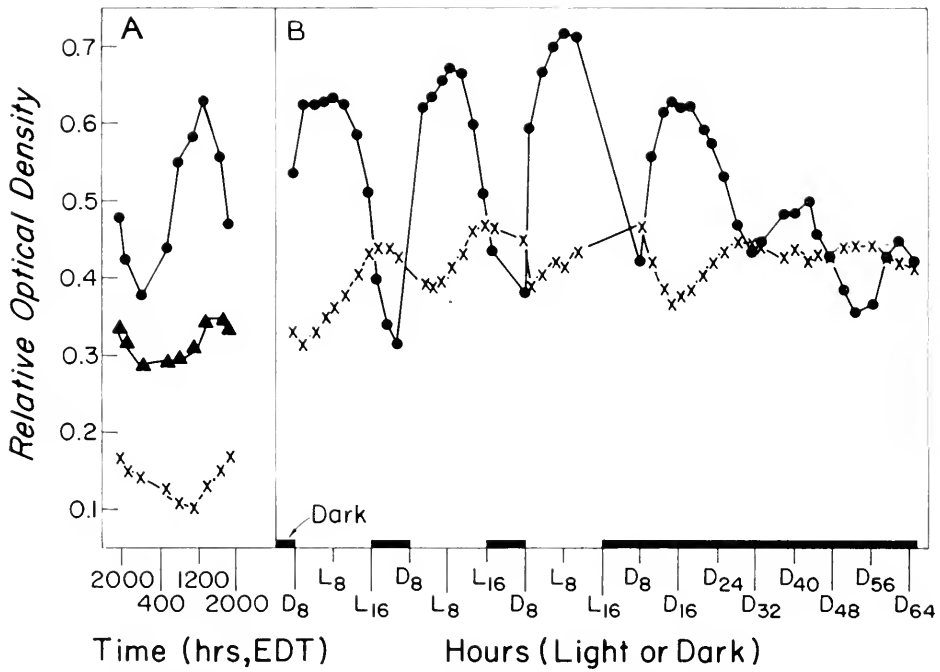

FIGURE 2. (A.) The change in ROD over July 13–14, 1970 (See Introduction) at 445 (●—●), 673 (▲—▲), and 615 nm (x—x). Measurements were made on a Coleman Model 6-A Spectrophotometer. *U. lactuca* was grown in glass-wool filtered, running bay water with a natural photoperiod from window light illumination. Light intensity at 1430 EDT was 5×10^3 erg/cm^2/sec; (B.) PAC_{672} (●—●) and ROD_{700} (x—x).

ance spectra very close to chlorophyll *a* and *b*, respectively (Holt and Jacobs, 1954). Electron microscopy of *U. mutabilis* F. (Lövlie and Bråten, 1968) showed the thylakoids were oriented largely in the plane of the thallus. If a similar situation existed in *U. lactuca*, then the potential for a detectable change in linear dichroism would exist. If a large proportion of chlorophyll *a*, for example, dissociated to chlorophyllide *a* during scotophase, then the average orientation of the porphyrin dipole moment should change from being approximately in the plane of the thallus to being randomly oriented, and the absorbance should decrease. For a light beam oriented perpendicular to the plane of the thallus, there should be no difference in the absorption of vertically and horizontally polarized light whether

the orientation of the dipole moment of the absorber is in the plane of the thallus or randomly oriented. If the light beam were incident at 45°, then the electric vector of the horizontally polarized light would have only one component with half the intensity in the plane of the thallus. If the dipole moment of the absorber were largely in the plane of the thallus, one might expect up to twice as much absorption of vertically polarized light as horizontally polarized light relative to random orientation.

It was found, however, that at both D + 7.5 and L + 8.5 the ratio of 682 nm vertically polarized light absorbed to horizontally polarized light absorbed was equal to one for both 90° and 45° angles of incidence of the light beam. This indicated the rhythm of light absorption at that wavelength could not be accounted for in terms of an incorporation of oriented chlorophyll *a* into thylakoids from previously randomly oriented cholorphyllide *a*. It was assumed that similar results would be found for the case of chlorophyll *b* and cholorophyllide *b*.

Chloroplast movement is a well known phenomenon for many different plants (for a review, see Zurzycki, 1962). In *Ulva* the appropriate shifting of the chloroplast might decrease light absorption through a shading effect. However, *in vivo* light microscopy observations did not reveal any obvious change in chloroplast morphology between scotophase and photophase.

Discussion

Under constant conditions, the persistence of the light absorption and scattering rhythms and the maintenance of periods approximately 24 hours imply that these are endogenous circadian rhythms. Any postulated mechanism must account for what seems to be the participation of both chlorophyll *a* and *b* and perhaps carotenoids. The light absorption rhythm was shown not to be due to changes in pigment concentration or in the overall change of absorber dipole moment orientation. It is unlikely that changes in the pigment environment drastic enough to alter the extinction coefficient by factors of two or three would occur, since no changes in wavelengths of maximum absorption were observed. Although no obvious changes in chloroplast position were noted, the small size of the cells (about 10 μm in diameter), their bilayer arrangement, and the large chloroplast made it difficult to visualize the chloroplast position.

Løvlie and Bråten (1968) reported qualitatively what seemed to be a rhythm of cell division in *U. mutabilis*. Most division occurred shortly after the beginning of the dark period and involved the chloroplast shifting to the side of the cell during cleavage. It does not seem likely that this type of chloroplast movement would be responsible for the light absorption rhythm reported here where the decrease in absorbance begins soon after mid-photophase. In addition, for our *Ulva* cultures only about one-quarter of the cells would be dividing per day (on the basis of an observed doubling time of about four days). Even if the dividing cells absorbed no light at all after chloroplast movement, the decrease in absorbance would only be about one-fourth, instead of the one-half to two-thirds actually seen. It does seem possible, however, that the light scattering rhythm reported here could be related to the cell division of Løvlie and Bråten, since the two rhythms seem to be in phase. Further light microscopy is indicated to resolve the issue.

Murakami and Packer (1970a) described light induced reduction of thylakoid membrane thickness, increased ordering and flattening of the thylakoid discs, and chloroplast volume changes in the dark adapted thalli of *Ulva* and *Porphyra* sp. These changes were correlated with decreases in the 180° transmittance and increases in the 90° light scattering at 540 nm. Studies with isolated spinach chloroplasts revealed similar results (Murakami and Packer, 1970b). Though these changes are not endogenous rhythms, they do suggest that alteration of the thylakoid membrane structure can produce changes in light transmittance and scattering. The possibility that such changes could be involved in the rhythms reported here should be subject to investigation by electron microscopy. While Murakami and Packer do not report the presence of light absorption or scattering rhythms in their *Ulva* specimens, the possibility of such rhythms should be considered as a potentially influential factor in light scattering investigations of light induced chloroplast structural changes.

The adaptive significance of light absorption and scattering rhythms in *Ulva* remains to be investigated. It will be of interest to determine whether the rhythms are related to rates of or capacity for photosynthesis.

Summary

Techniques of *in vivo* spectroscopy were employed to demonstrate the presence of rhythms of light absorption and scattering in the green thalloid alga *Ulva lactuca* L. maintained in artificial nutrient medium under constant photoperiod. The absorbance during photophase at 682 nm, the chlorophyll *a in vivo* absorption maximum, was typically two to three times that during scotophase. Prephased endogenous rhythms (in continued darkness) were observed for a time period equal to three photoperiods. The absorbance rhythm did not correlate with changes in extractable chlorophyll *a* or *b* concentration. No changes in linear dichroism were observed, indicating the rhythm of light absorption could not be explained on the basis of orientation of individual absorber molecules. *In vivo* light microscopy did not reveal differences in chloroplast orientation. Alternative mechanisms are discussed.

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BIOLUMINESCENT BEHAVIOR IN *RENILLA*. I. COLONIAL RESPONSES [1]

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The colonial soft coral (alcyonarian) *Renilla*, or "sea pansy," consists of a flat, bilobed, nearly circular platform or rachis, which normally rests on the ocean bottom, and a central holdfast or peduncle which extends down into the sand from the under surface. From the upper surface of the rachis project several thousand zooids, which are of two kinds: larger retractile feeding polyps or autozooids, which occur singly, and much smaller, modified individuals called siphonozooids, which occur in clusters and function as intake pores for the sea water which circulates through the spongy tissue of the rachis and peduncle. The colony undergoes peristaltic muscular movements and periodic deflations and expansions (Parker, 1920a; Hyman, 1940).

The luminescence of *Renilla*, which, in the words of Agassiz (1850, page 209), "shines at night with a golden green light of a most wonderful softness," has long stimulated the curiosity of zoologists. The colony does not ordinarily luminesce spontaneously under laboratory conditions: rather, the light is manifested as a wave or series of waves radiating over the rachis from any point of stimulation. The polydirectional and non-decremental spread of luminescence have been assumed to mean that an unpolarized nerve (neuroid) net is present in the rachis.

Using mainly mechanical stimulation, Parker (1920b) defined many of the basic properties of the luminous response in *Renilla*. More than thirty years later his observations were extended by me (Buck, 1953, 1955) and particularly by Nicol (1955a, 1955b), using electrical stimulation. The present paper gives some new anatomical details and amplifies such of my visual behavioral observations as were not superceded by Nicol's photometric recordings.

Material and Methods

Renilla köllikeri (miscalled *R. amethystina* by Parker) from two Californian sources was used. For the bulk of the visual work colonies were dredged weekly from a depth of 75 feet in the mouth of Newport Harbor in June and July and stored in running sea water at 19–24° C in aquaria exposed to diffuse daylight. Supplementary observations were made on colonies brought up by divers from Los Angeles Harbor in March and stored in refrigerated aquaria. As will appear, and as noted also by Nicol (1955a), the behavior of "summer" and "winter" pansies differed strikingly in certain respects. Since all colonies contributed to understanding zooid and colony behavior, both populations were accepted as normal

[1] Dedicated to Professor Curt Stern of the University of California for his 70th birthday.

without attempting to ascertain the cause of the differences or whether they are in fact consistently seasonal or geographically distinct.

For visual observation colonies were studied at magnifications up to 100 × in four-inch finger bowls of standing sea water. Since it is known that ambient light can inhibit luminescence, colonies were dark-adapted for at least a half hour before testing.

Colonies were stimulated *via* condenser shocks of 100 msec duration and nominal strengths up to 80 volts. Click-stop control knobs enabled stimulus parameters to be set in total darkness. The electrode consisted of two strands of no. 24 enameled

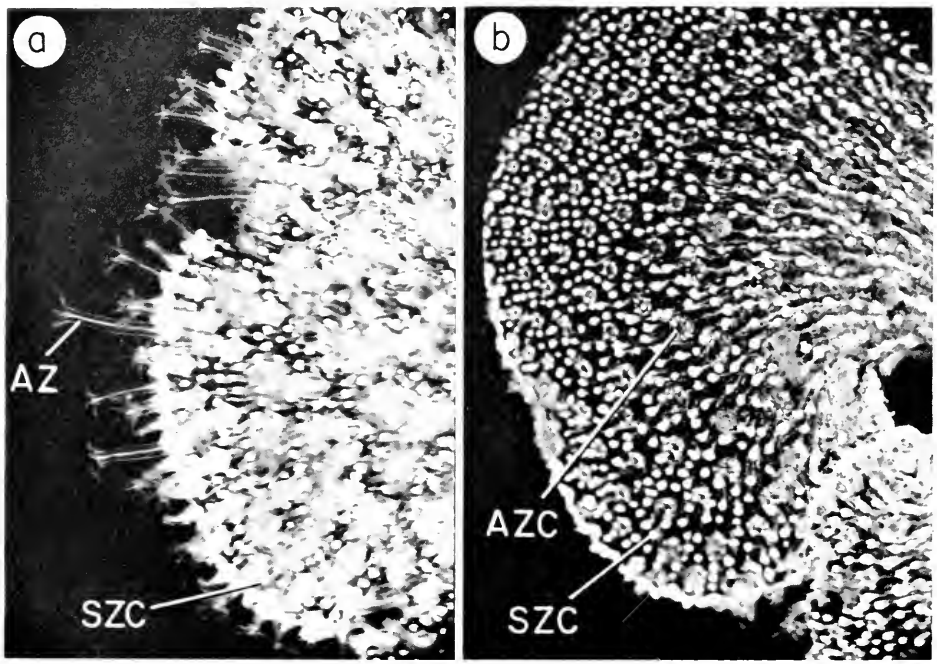

FIGURE 1. Portions of living *Renilla* rachis in sea water, × 3; (a), expanded colony; (b), contracted colony; SZC, siphonozooid cluster; AZ, expanded autozooid polyp; AZC, calyx or base of autozooid.

copper wire sealed in a glass tube except for the tips, 3 mm apart. The bare metal was cleaned periodically with a carborundum stick to reduce fouling by mucus. The electrode was carried on a sliding rack so that it could be lowered to the rachis in total darkness by feel. The electrode was usually positioned about a third of the distance in from the margin of the rachis to the center and was pressed firmly into the surface to prevent shifts in position due to peristalsis or deflation. Voice descriptions of behavior were tape-recorded concurrently with the stimulus signals. Water temperatures varied between 20° and 25°.

Since the absolute intensity of *Renilla* luminescence is low, care was taken to dark-adapt the eyes for at least 20 minutes before beginning observations.

Attempts were made to detect local responses by recording through a small hole in an opaque screen covering the rachis and by using a 1.5 mm diameter light guide. Technical details of the oscilloscopic records and image intensifier photographs presented will appear in a following paper.

Results

Morphology

The colony is quite muscular and a large rachis can vary in diameter between 10 cm when fully distended with water to as little as 5 cm when maximally contracted. Independently of the contractility of the rachidial tissue itself, the autozooid polyps (AZ, Figs. 1, 2) are able, by shortening the tentacles and trunk and turning the trunk inside out, to retract completely into the interior of the rachis so that only the star-shaped, saucer-like calyx remains above the rachidial surface (Figs. 1b, 2b). The siphonozooid clusters, however, do not change shape appreciably even on exposure to fixing solutions (Figs. 1, 2). The clusters often lie along radii reflecting the mode of colony growth (Wilson, 1884). The autozooids are more closely spaced near the outer margin than in the interior of the rachis (Fig. 1b). In a large rachis there may be 400–500 autozooids and 1500–2000 siphonozooid clusters comprising perhaps 15,000 individual polyps. Areal distributions of the 442 autozooids and 1456 siphonozooid clusters on a preserved rachis 55 × 65 mm are given in Figure 4. In this specimen the average number of siphonozooids per cluster was about 6.

The autozooids have eight pinnate tentacles (Fig. 2a). These are of equal size except in autozooids at the extreme margin of the rachis, where two are typically larger than the others. The calyx has five lobes, representing preferential development of interradial regions of the basic octomerous plan (Fig. 2b). The siphonozooid clusters consist of a single, somewhat larger "principal siphonozooid" with two vestigial tentacles (VT, Figs. 2a, 3) surrounded by 4–15 smaller ovoid individuals (Fig. 3). The autozooid calyx lobe tissue contains granular material that is white by reflected light. Similar refractile material occurs in two parallel narrow strips in each autozooid tentacle and in the siphonozooids.

Particularly when the mucus feeding canopy (MacGinitie and MacGinitie, 1968) is stripped off the rachis it can be seen that the colony surface is thickly strewn with minute aciculate spicules, usually deep blue but occasionally amber or even colorless (SP, Fig. 2b, 3). Spicules are also banked up around the bases of the autozooids and the spihonozooid groups, like tartar around a molar tooth. The minority of colonies in which the spicules are amber rather than blue have, when expanded, a predominantly pink overall rachidial color as compared with the uniform purplish color of specimens with only blue spicules, and more prominent radii. No difference in luminosity between the two types of pansy was observed.

Sites of luminescence

Luminescence in *Renilla* is not as simply localized as might be expected. In Newport specimens the first touch usually caused an immediate retraction of the autozooids which lasted at least half an hour even without further stimulation.

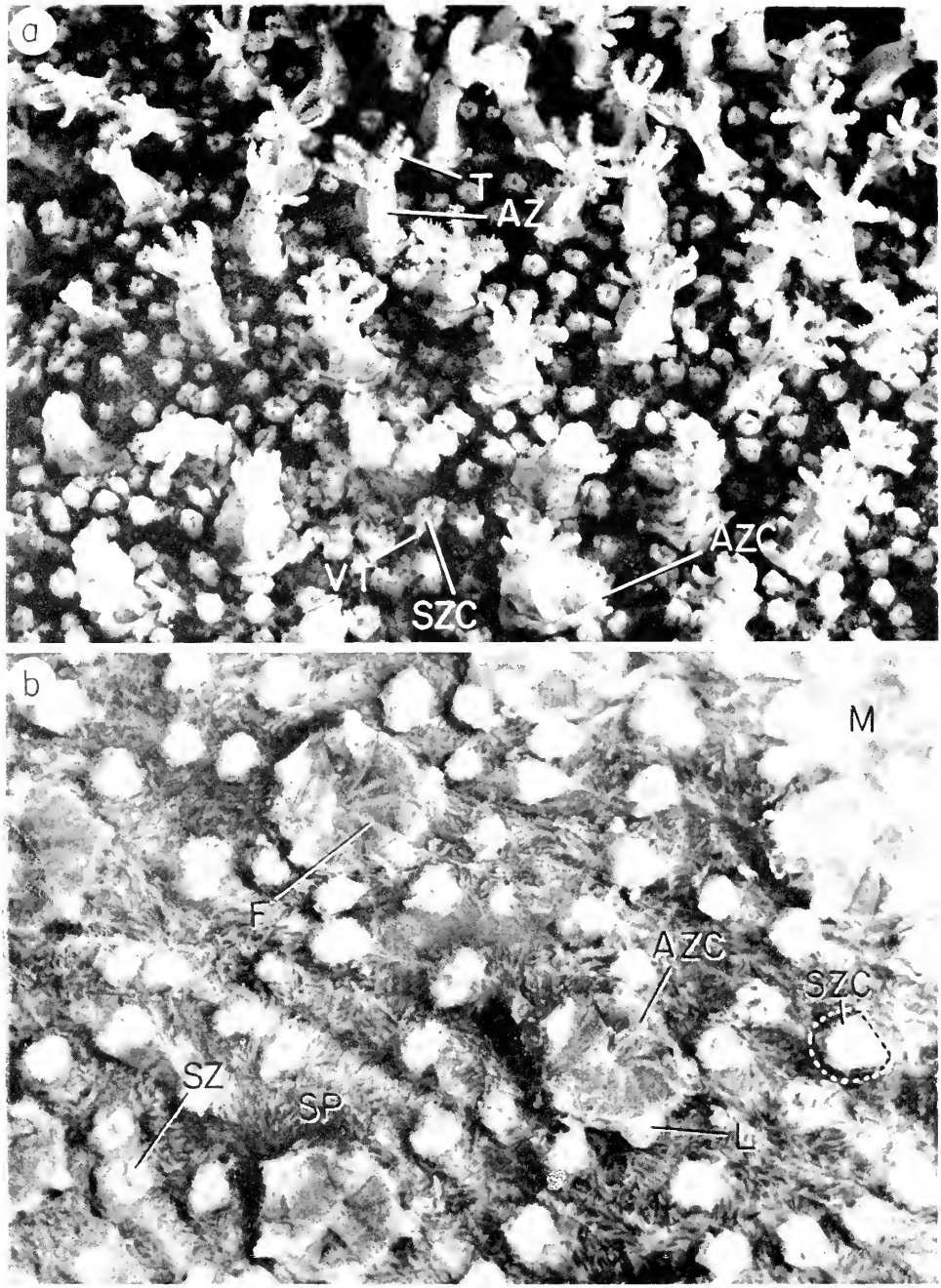

FIGURE 2. Surface views of rachides fixed by sudden flooding with hot formalin, × 10; (a), partly expanded colony; (b) contracted colony; AZ, autozooid with eight pinnate tentacles (T); SZ, one of several siphonozooids of a cluster (SZC); SP, spicules. In a, one

When such a summer rachis was stimulated, small, circular, dark islands were seen among the multitude of spots that lit up briefly as the response wave swept across the colony (Fig. 5). By using very dim incident light during the response it was seen that each luminous spot was a siphonozooid cluster whereas each dark area contained a retracted autozooid.

Since the autozooids retracted upon touch in summer specimens it was not known whether they participated in the luminous waves that occur with moderate and infrequent stimulation but at least the calices were non-luminous. In summer pansies in long-stagnant water, in which the autozooids remained extended after stimulation of the rachis, the autozooid trunks and tentacles were not luminous

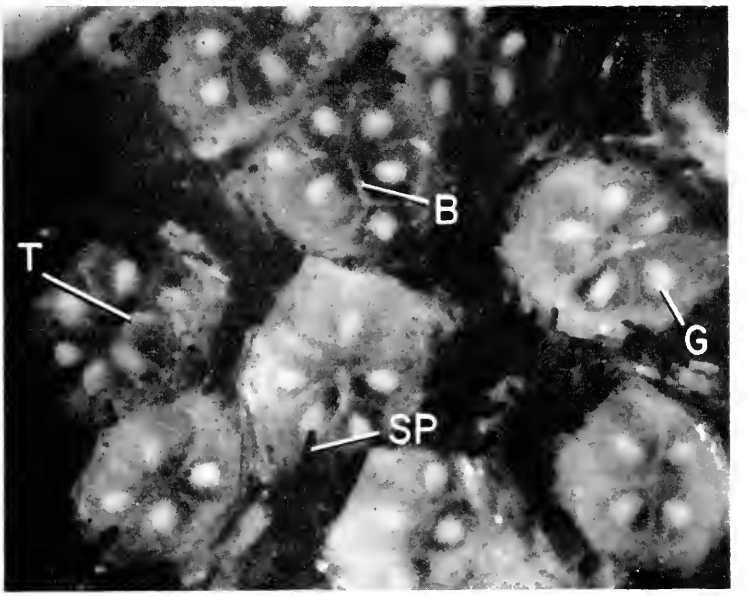

FIGURE 3. Nine siphonozooid clusters of fixed, contracted rachis, × 40; B, polygonal boundaries between individual siphonozooids; G, shrunken gastroderm; T, vestigial tentacle; SP, spicule.

though the siphonozooids still responded. However, in normal pansies undergoing strong repetitive stimulation the autozooid calices did begin to produce light. This light was considerably dimmer and much longer lasting than that from siphonozooid clusters and seemed concentrated near one or more of the five lobes of the calyx.

Winter pansies from Los Angeles differed from Newport summer animals in (a) not immediately withdrawing their autozooids upon stimulation, (b) often being poised in a hyperexcitable state such that a single touch led to a brief and spectacular general luminescence after which the colony was almost inexcitable for

cluster shows the two vestigial tentacles (VT) of its principal siphonozooid. In b the mucus feeding canopy (M) has been stripped from most of the surface. Calices of involuted autozooids (AZC) have five fleshy lobes (L) and enclose folds (F) corresponding to the eight internal mesenteries of the column.

a long period, and (c) having autozooids which were not only luminous but brilliantly so. Due to simultaneous siphonozooid activity it was difficult to be sure that the stalks or columns of these extended autozooids were entirely non-luminous, though they appeared to be so, but there was no doubt that each crown of tentacles gave flashes fully as sharp and bright as those of a siphonozooid cluster. In fact, as seen in a localized region under the microscope, the autozooids gave the luminous wave a striking bimodal character. The first peak represented the typical siphonozooid activity on the rachidial surface, and was followed by a relatively dark period of a quarter to half second, presumably representing the

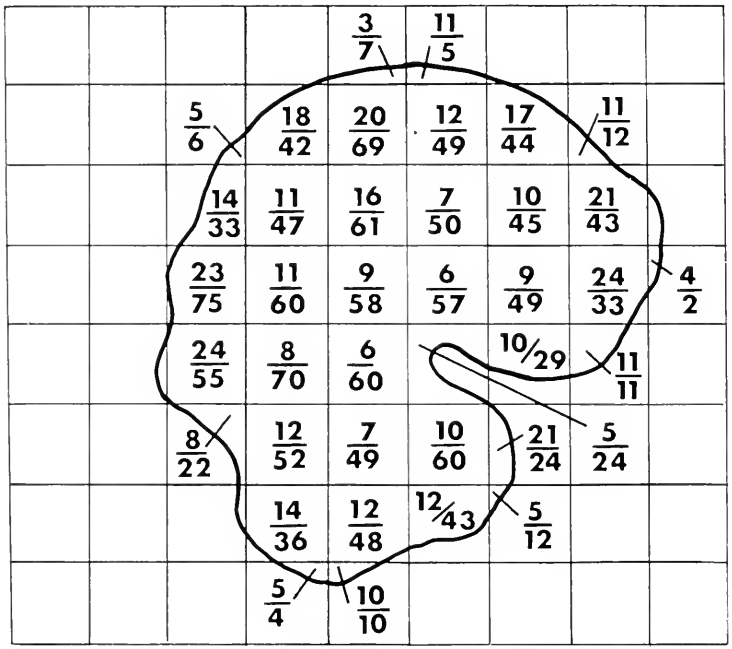

FIGURE 4. Relative distributions of 442 autozooids and 1456 siphonozooid clusters over fixed 55 × 65 mm largely contracted rachis. Note AZ/SZC ratios varying from up to 1 : 1 at the margin to 1 : 10 in the center.

time required (at the 10–15° temperature of the sea water in which the colonies were originally examined) for the excitation to climb the centimeter-long, apparently non-luminous autozooid columns, after which a second peak occurred as light fairly blazed up in the tentacles and ran to the tips.

The response characteristics of the winter animals persisted during 24–36 hours' exposure to water of 20–25°, hence seemed not to be a temporary response to low temperature.

At 20 to 30 diameters magnification the light of *Renilla* seemed to come principally from the neighborhood of the whitish refractile substance within the siphonozooids, the lobes of the autozooid calyx and the strips along each autozooid tentacle. By analogy with other bioluminescent organisms it would be expected

that the actual photogenic tissue is translucent and that its apparent association with the refractile material is simply due to enhancement of the luminescence by reflection. Luminous material was never found in the ambient water or on utensils or hands, even after considerable handling of the rachis. One could easily be deceived on this point, however, if employing injurious stimulation such as stabbing

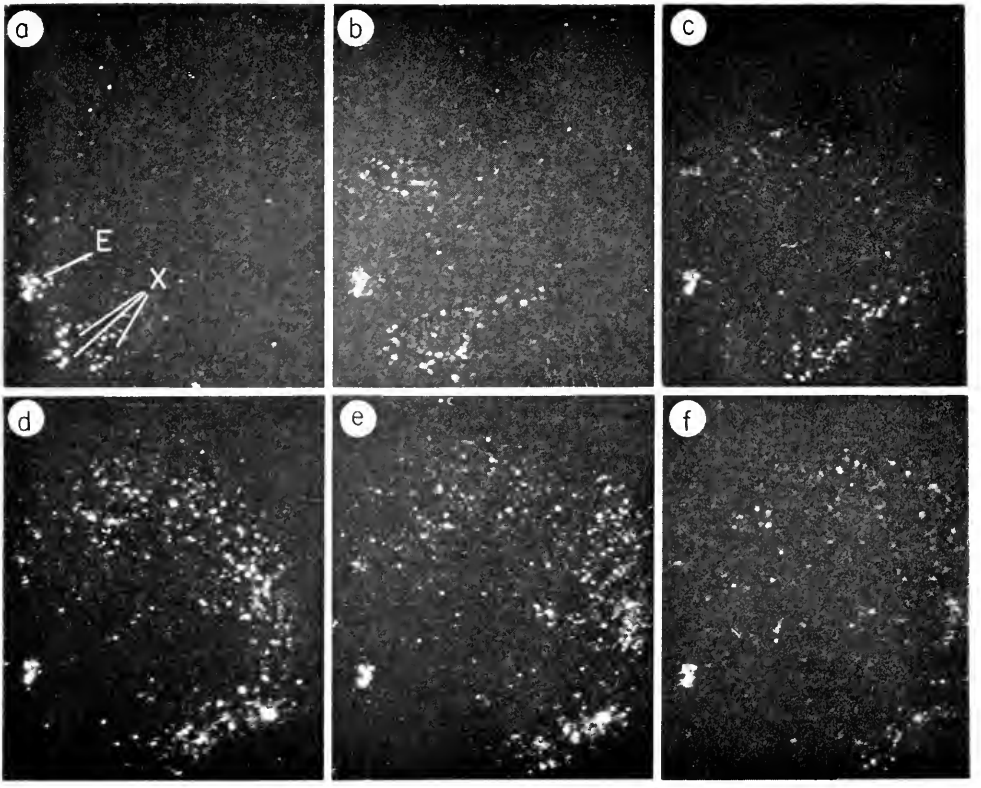

FIGURE 5. Six stages in passage of a luminous wave from the point of electrical stimulation (E) across a *Renilla* rachis from left to right. Panels are, respectively, the 1st, 9th, 17th, 25th, 33d and 41st frames of a 16 mm cinema film exposed at 16 frames/sec in photographing the phosphor of an image intensifier tube, × 3. The "X"s in panel a indicate presumed sites of non-luminous autozooids amid circles of luminous siphonozooid clusters. Several such dark circular islands are visible also in the 11 to 1 o'clock and 4 to 6 o'clock sectors of panel c and in the 11 to 2 o'clock sector of panel d.

with a needle, since small bits of luminous tissue readily become lodged in the communal mucus feeding canopy. Spawning male colonies could also be deceptive since the sperm-filled surrounding water assumes a milky luminosity when illuminated by flash or glow. The ability to flash for long periods without significant fatigue (see below) is an additional indication of intracellular luminescence.

Effects of ambient light and of stagnation on excitability

Working on summer pansies I confirmed reports (Parker, 1920b; Nicol, 1955a; Kreiss and Cormier, 1967) that ambient daylight inhibits luminescence and found also that light adaptation raises the threshold for stimulation. However, in contrast to the inhibitory effect of diffuse daylight, pansies exposed for 5–30 minutes to direct sunlight, though drastically contracted, showed low threshold and bright luminescence upon stimulation and even a tendency to become autoexcitable. The temperature rise in the water did not exceed 2° during such exposure.

Stagnation of water in the finger bowls in the dark had no effect up to 8 hours, but thereafter increasing numbers of colonies showed inflated rachides, permanently extended autozooids, induced spawning, higher thresholds and dim luminescence, the first three effects showing also in colonies kept in the light. However, *Renilla* usually survives 24–36 hours in a finger bowl at 20–22°.

Types of luminous response

As will appear, *Renilla* has a large repertory of luminous emissions which vary with both physiological state of the colony and intensity of stimulation. The simplest of these is the single wave evoked by weak electrical stimulus, which I will call the "normal" wave (Fig. 5). With the equipment used in this study the threshold stimulus, using 100 msec shocks and fresh, dark-adapted summer pansies, was about 4 V.

The normal wave. I confirmed previous descriptions of response waves as spreading radially, non-decrementally and with the leading edge of the wave brightest. I also found that the breadth of a wave—that is, the number of ranks of zooids participating at any instant—differed widely in different specimens and under different conditions. Curiously, the luminescence did not usually appear to originate at the point of electrode contact; rather, the electrode seemed surrounded by a circular dark zone 5–10 mm in diameter out of which the waves became visible.

The velocity of excitation spread across the rachis was in the 4–7 cm sec range at 20–25°. Though waves usually moved with uniform velocity the impression was occasionally gained of local variations in conduction velocity, particularly in rachidial strips (Parker, 1920b) in which the total path for passage of a wave can be increased from about 7 to 30 cm (Figs. 6a, 6b). A rare phenomenon seen also in such strips was an apparent "fraying out" of the originally single wave into two or more by the time it reached the end of its travel.

As observed also by others, it is the almost invariable rule that a luminescent wave, once initiated, sweeps across the entire rachis with no decrement in velocity or intensity. This is true of waves induced by barely adequate stimulation as well as those induced by intense shocks, and of dim waves as well as the most brilliant. In fact, rather than dying out, very dim waves sometimes first become noticeable at points well removed from the electrode and seemed to brighten a little during their passage. Out of thousands of waves observed I have seen only a half dozen failures to affect the whole rachis and most of those involved dimly luminous pansies, long resident in darkness, in which secondarily non-luminous but still conducting regions might have been involved. In a single pansy that had been ex-

posed to direct sunlight, however, decremental conduction seemed to be shown clearly. In this specimen, which, contrary to the usual effect of sunlight, had a high threshold and was consequently being stimulated with high voltage and at a

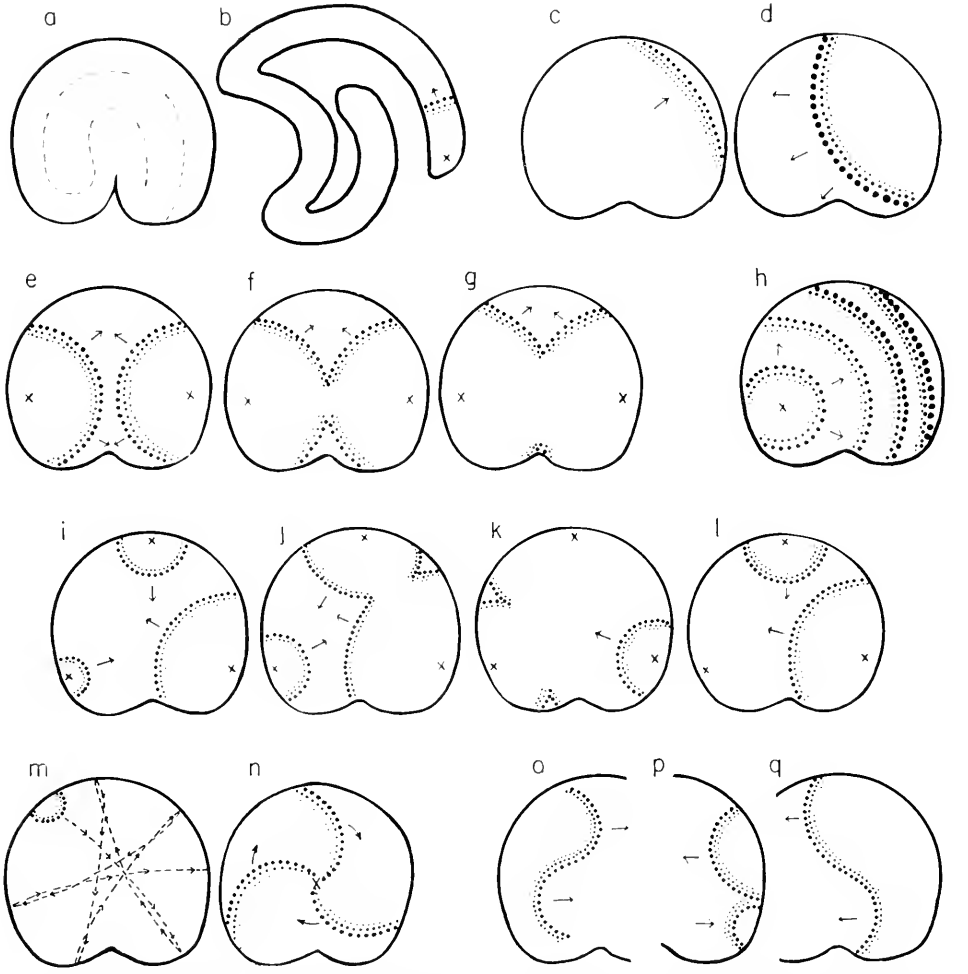

FIGURE 6. Diagrams showing various luminosity phenomena of *Renilla* rachis: a, b, method of cutting rachis to yield a strip with approximately quadrupled conduction path; c, right-moving luminous wave about to die out at rachidial margin in 2 o'clock sector; d, left-moving wave which originated spontaneously at about point where wave diagrammed in Figure 6c died out; e, f, g, three stages in collision and mutual cancellation of two luminous waves originating simultaneously from two electrodes (X) (see text); h, volley or family of luminous waves induced by mechanical stimulation at X (see text); i, j, k, l, four successive stages in a type of frenzy with several excitation centers, giving the impression of "boiling" luminescence (see text); m, simple type of frenzy in which an apparently single luminous wave bounces back and forth across the rachis (see text); n, type of frenzy giving the impression of a rotating luminescent "propeller"; o, p, q, type of frenzy in which a wave may have a front combining both convex-forward and concave-forward segments (o) which then reverse upon "reflection" (q).

frequency of 1/sec, the response began locally with the 6th shock, increasing in brightness and spreading farther out until at the 10th shock the whole rachis was involved.

Conduction rate in relation to possible tissue path was compared in broad and narrow strips. For example, a strip 12 mm wide and 90 mm long was split nearly to one end into strips 2 and 10 mm wide: A stimulus at the junction point elicited waves that were conducted at the same rate (4 cm/sec) in both strips. This suggests that if there are individual connections within the rachidial tissue these are numerous enough that a very direct conduction path can be utilized between any two points.

Facilitation and adaptation. Typically *Renilla* did not respond to the first few of a series of near-threshold stimuli repeated once every two seconds, and usually, after beginning to respond, produced several successively brighter waves until a plateau of wave intensity was reached. If stimulation was continued, responses usually began to be skipped, at first occasionally and then more and more frequently until the colony ceased to respond. A modest increase in stimulus strength then ordinarily reinstated response for another period, after which adaptation again supervened. The existence of effector facilitation is also suggested by the succession of progressively less frequent and less bright spontaneous waves which often followed the cessation of intense stimulation ("defacilitation").

Neuroeffector facilitation in autozooid calices is to be inferred from the brightening of the coarse-grained background glow that often paralleled the increase in intensity of the fine-grained siphonozooid flashes during serial stimulation, but even among winter pansies the autozooids did not remain extended during stimulation consistently enough to permit conclusions about possible facilitation of luminescence in tentacles.

A single mechanical stimulus was usually much more effective than electrical stimulation in inducing intense luminescence (*cf.* "flare," below) and quick effector facilitation.

The spatial uniformity of facilitation in the rachis was investigated in winter pansies, using two electrode pairs 22 mm apart. With repeated near-threshold stimuli delivered alternately at two second intervals, starting with electrode pair A, the first response most frequently occurred at the second shock—*i.e.*, at electrode B. Thereafter wave intensity built up stepwise for the first 6 to 8 responses just as if the successive stimuli were all being applied at one site. It was also possible to facilitate the rachis by repeated stimulation through one electrode and then, maintaining the same cadence, to obtain a response to the first shock delivered through the other electrode pair, 22 mm distant, rather than on the 2d to 4th, as is usual with non-facilitated rachides. Many such tests on a number of colonies are summarized in the first data line of Table I. The evidence that the rachis has been facilitated uniformly (92 responses on the first trial after switching, out of 107) is very strong, particularly in view of the possibility that some of the failures to respond (last column) were due to inadequate contact of the second electrode pair with the rachidial surface. However, in 5 of the 92 instances in which response to electrode B was immediate, luminescence only continued for a few additional cycles, as if net-wide sensory adaptation had already been nearly reached during stimulation via electrode A.

In 44 series stimulation through one electrode was continued until the rachis had ceased to respond (second data line of Table I), then the current was switched to the other electrode pair. In 8 of the 28 experiments of this group in which there was a response it occurred after the first shock. This result suggests that sensory adaptation in such instances was local. The average interval between the last response at electrode A and the time of switching electrodes was 21 seconds, the longest being 36 seconds.

In several hundred records of serial stimulation of summer pansies the number of the shock inducing the first visible response varied between 2 and 24. The number was seemingly less related to frequency or strength of stimulation than to "physiological state" of the colony. Similarly the number of stimuli required to reach fully facilitated luminescence varied over a wide range, some colonies reaching the plateau level in as few as 3 to 8 cycles whereas others might still be increasing in brightness after 20 successive stimuli (Fig. 7a) and still others might show hardly any increase in brilliance during long stimulation series.

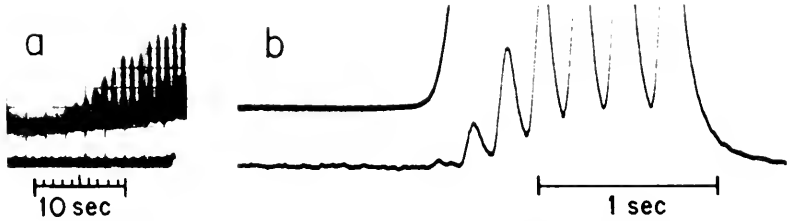

FIGURE 7. Oscillographic records of responses of *Renilla* to electrical stimulation. (a) Eighteen (?) successive responses to serial stimuli delivered at 1/sec; light trace above (left to right), stimuli below; sweep 2 secs/cm division; first response apparent after third shock; (b.) Response to stimuli delivered at 5/sec; upper trace, light detected from large area of rachis; lower trace, light detected from small area through 1–$\frac{1}{2}$ mm light guide.

The duration of the facilitated state was variable also. The most unequivocal measure would appear to be the length of time during which a particular mode of repetitive stimulation can be suspended before the rachis fails to respond at once when stimulation is resumed. Nearly a hundred such tests were made on fully facilitated winter pansies, with the result that 3 shocks at a frequency of one every two seconds, and 6 at a frequency of one per second, were the largest numbers that could be skipped with confidence that the colony would respond to the first subsequent shock. The time seemed little influenced by either the intensity of the shock or by the brightness of the luminescence prior to the skipping. Facilitation induced by moderate stimulation thus appeared to persist for approximately 8 seconds, or possibly as long as 10 if we accept the infrequent responses after 4 and 7 skips, respectively, at the two frequencies. This estimate is thus only a half to a third that obtained in the eight local adaptation experiments (Table I).

The effects of repetitive stimulation in relation to facilitation and adaptation will be considered below in further detail, but it is appropriate here to mention response limits. The highest frequency at which a rachis could usually be driven for more than a few cycles was about 3/second. Beyond this level the colony might become autoexcitable (q.v.), or refractory or appear to give light con-

timonsly for a short time ("flare"). In this last connection, photometric recordings through small holes showed, as expected, that the luminescence cycles of limited regions of the rachis were shorter than the integrated record of the whole rachis wave. This point is also well illustrated in a two channel recording in which the light emitted by a small area of a rachis being stimulated 5 times per second was detected through a light guide (Fig. 7b). This recording shows that although the rachis appeared to the eye, and in the integrative recording, to be tetanized, some of the effector tissue, at least, was responding 1 : 1 and showing facilitation in addition to some apparent summation.

Colliding waves. If the rachis was stimulated at the same time at opposite points on the margin, two simultaneous waves were induced which swept across the rachis and canceled each other upon meeting. This resulted in a curious "quadrille" effect in which two normal convex-forward waves advancing centripetally along one axis (Fig. 6e) transformed into two concave-forward waves traveling centrifugally at right angles to the original direction (Figs. 6f, 6g). Corresponding cancellations were observed when three or more wave-generating centers were active (v.i.).

TABLE 1

Experiments on spatial extent of facilitation. Colony first stimulated to plateau brilliance through electrode pair on one side of the rachis (usually at 30 shocks per minute), then by a pair on the opposite side, maintaining the cadence of stimulation. (Summary of tests on many colonies)

	Response at B to first shock after switch from A	Response to 2d or subsequent shock after switch	No response after switch
Responding 1:1 before switch (107)	92	6	9
Refractory before switch (44)	8	20	16

Extra and skipped waves. Vigorous pansies frequently showed another type of response, particularly during long-continued rhythmic stimulation at somewhat above threshold strength. In this response a wave originated at the electrode at the usual interval after stimulus, but was followed, before the next stimulus, by a second wave, also originating at the electrode, but much brighter. When the next stimulus occurred it might also elicit a brighter-than-normal response wave, or none at all. One may have, then, in the midst of a regular response series, either a "repeat" wave involving two waves abnormally bright and abnormally close together in time and space, the second occurring at normal response time and originating at the same site, or a single, brighter "extra" wave not at the normal response time, with the next normal response being skipped. In pansies that had been responding serially for a long time, waves were also skipped without warning, the wave immediately preceding such a skipped response seeming to be normally bright. Sometimes, however, such a skip was preceded by one or more waves of less than average brightness.

Reverse wave. In vigorous, low-threshold pansies stimulated on one side of the rachis every two seconds at somewhat above threshold intensity the wave of luminescence sometimes spread across the rachis and disappeared as usual,

(Fig. 6c) but was then followed, after a very brief delay, and before the next stimulus, by a brighter wave traveling in the opposite direction (Fig. 6d). This "reverse" wave originated at or near the point where the luminescence of the normal wave had died out, *i.e.*, usually at the point most distant from the original point of stimulation, and had the appearance of having been reflected back. Like the extra waves discussed above, a reverse wave might be followed, at the next stimulus, by either a brighter-than-normal wave from the electrode site or by a skipped wave.

Since excitation is known to traverse nonluminous parts of the colony such as the peduncle it seemed possible that the reverse wave represents a response to excitation that has spread from the electrode in the opposite direction from that in which the wave moves, passing around via the under surface of the rachis to the opposite edge, where the re-excitation of siphonozooids occurs. This possibility

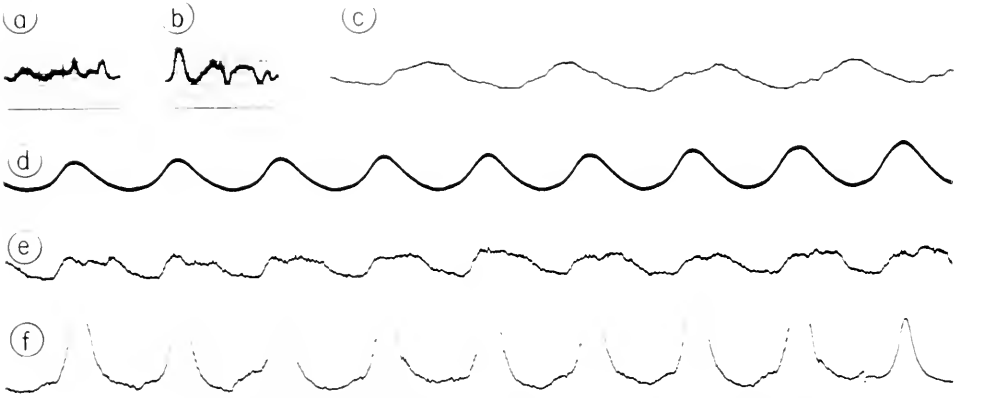

FIGURE 8. Oscillographs of *Renilla* luminosity during frenzy; a, b, slow sweep integrative records from large rachidial area 500 milliseconds between vertical grid lines; c, d, e, f, waveforms of colony luminescence in different stages of frenzy; wave frequency $\frac{3}{4}$ seconds in c; 1/second in d, e, f. Gain reduced for last wave of Figure 8f. See text.

was investigated by cutting a 3 mm strip off the margin of the rachis for half its circumference, presumably thus interfering with the continuity between possible subepidermal nerve nets on top and bottom surfaces of the rachis. However, when such rachides were stimulated at points opposite to the cut margin, typical reverse waves still occurred.

Volley or familial waves. With a single mechanical stimulation such as the initial contact with the electrode, a volley or family of waves was often induced rather than a single wave. Such an episode began with very brilliant waves in such quick succession that several were on the rachis simultaneously (Fig. 6h) but "ran down" or defacilitated in the course of a few seconds, the individual waves decreasing rapidly in brilliance and the inter-wave intervals increasing progressively. The contrast between the brilliant response to mechanical stimulation and that elicited by moderate electrical stimulation was especially striking in colonies that were fatigued or had acquired the high threshold and characteristically dim luminescence induced by long sojourn in stale water. Occasionally such

familial volleys were followed, at intervals of 10 seconds or more, by one or more additional waves, bright, and sometimes in the reverse direction. A similar phenomenon was often seen at the end of a long series of strong shocks at frequencies above the limit of 1 : 1 response. Here, after stimulation ceased, there originated from the electrode contact point a half dozen or more flashes of progressively decreasing frequency and brilliance. Rarely, a wave even occurred a minute or so after stimulation and luminescence had ceased, raising the question of whether it represented a long-delayed response to the original excitation or was truly spontaneous.

Frenzy. The most extraordinary type of light emission in *Renilla* is one which I shall call "frenzy" and whose appearance is suggested by terms in my tape-recorded descriptions: "sputtering," "rippling," "circling," "oscillating," "boiling," "pinwheel," "propeller." The common features of these bewildering manifestations were long-continued autoexcitation and a constantly shifting point or points of wave origin. Not only was the activity spontaneous but, once well started, it seemed relatively independent of environmental influences. If the electrode was left in position (with the stimulator turned off) the contact point sometimes appeared to be a point of origin of some of the spontaneous waves, but excitation continued equally well with nothing touching the rachis.

A simple type of frenzy, with two alternating excitation centers was that diagrammed in Figure 6m, where the wave rocked back and forth across the rachis, the excitation centers progressing around the margin. In "boiling," waves spread out radially from two or more excitation centers active either synchronously or asynchronously, canceling on impingement (Figs. 6i–6l). Occasionally the luminous waves became phased for a short time so as to give a continuously rotating effect like a two or three bladed propeller (Fig. 6n). Figures 6o–6q, illustrate how multiple centers and wave "reflection" combined to create concave-forward waves and a whiplashing impression. In all these behaviors there was an accompanying lasting glow of autozooid calices which tended to fill in the intensity troughs between the waves and make photometry difficult, though the eye was able to sort out the siphonozooid activity.

With the rachis constantly alight at one point or another it was difficult to make accurate comparisons with normal waves, but the propagation rate of the fragmented waves during frenzy appeared normal and did not change with time. By concentrating attention upon a single small area of the rachis it was seen that although the region was occasionally dark for several cycles, a fairly regular rhythmicity underlay the apparently highly irregular activity. The frequency of this spontaneous auto-excitation was consistently about 37/min at 21°. Figures 8a and 8b illustrate the irregular fluctuations in rate of total light output from the rachis that often prevailed, whereas Figures 8c–8f show the rather regular rhythmicity that obtained at other times.

Frenzy might continue for only a few minutes or persist for more than an hour. It ordinarily involved considerably brighter waves than are usual, for example, with one electrical stimulus every two seconds. However, particularly in winter pansies that had (it seems) exhausted themselves in an initial burst of activity due to mechanical contact of the electrode, the luminescence might be very dim. In general the average luminous intensity decreased slowly with time, but in several instances in which cessation of frenzy was actually observed the luminescence was still moderately intense at that time. In numerous pansies in

which a bout of frenzy lasted only a few minutes it was readily re-excited and proceeded again at full intensity. On the other hand, a pansy that had gone through a long frenzy was often not re-excitable until it had had a rest period.

The conditions which induce frenzy are discussed in more detail below, but for present purposes it can be said that the phenomenon was more characteristic of vigorous colonies than of those with high thresholds or dim luminescence and that it could usually be induced by strong, long-continued repetitive electrical stimulation at frequencies of 30–45 per minute or higher.

Flare. A phenomenon not infrequently seen with initial mechanical or strong electrical stimulation of summer pansies, and commonly seen in winter pansies, was a brilliant even glow which lasted for a brief period at apparently constant intensity and then died away over as long as 30 seconds. Though such flares usually showed no trace of waves the light was often not absolutely uniform but gave the impression that the individual luminous points over the rachidial surface (siphonophore clusters) were pulsing or scintillating at high frequency. In a few instances a flare resolved into familial waves.

An interesting feature of glows and flares was that by repetition of the precipitating stimulus the light could be "pumped up" repeatedly to peak brilliance.

Autozooid glow. The long-lasting autozooid glow which often developed during serial stimulation could become quite bright but was always dimmer than the siphonozooid waves that occurred at each stimulus and was coarser-grained. The relation between persistent interwave glow and the flash is not simple. Glow was observed most often in bright, vigorous pansies but occurred also in stale colonies. Similarly, both low-threshold, dark-adapted pansies and light-adapted pansies sometimes glowed, and glows were seen together with either narrow waves or broad. The speed of development of glow also varied, some pansies still showing complete extinction of luminescence between waves after 50 or even 100 successive responses whereas in others a glow might develop with fewer than 10 successive stimuli. Glows were most frequent with stimulation well above threshold strength and with frequencies higher than the standard 30 min, but some pansies developed autozooid glow at threshold strength and low frequency.

Glows occurred after familial waves and during frenzy, indicating that spontaneous excitation as well as applied stimuli was effective. One consistent generalization is that the threshold for glowing was at least as high as for flashing: Glows never developed at stimulus strengths below that required to induce waves and as soon as stimulus strength was reduced below the threshold for waves any glow that might have developed began to fade.

The glow was generally uniform over the rachis. Occasionally it seemed more pronounced at the margins, an effect that could be due either to the greater concentration of zooids peripherally or to the more tangential view along the down-curving surface of the margin. Persistence of glow was quite variable, being usually of the order of 20 seconds after cessation of stimulation but not uncommonly differing from this by a factor of 2 in either direction.

Interrelations of behavior types

Though some of the different types of light emission in *Renilla* seemed to depend on particular modes of stimulation, their interrelations were not immediately

obvious. In order to clarify this matter, stimulus intensity, frequency and number were explored systematically. Ability to follow 1 : 1 through a series of 10–15 consecutive stimuli was tested at each of several intensity-frequency combinations and if possible each series was repeated several times with each pansy. Also whenever possible the effects of stimulus strength and frequency were tested separately by facilitating the colony at one set of stimulus parameters then changing either the intensity or frequency. Long term behavior was also explored. Development of frenzy was an obstacle to repetition in many tests, particularly with high stimulus frequencies and strengths.

TABLE II

Relation between strength of stimulus and ability to give 1:1 response to a given frequency of stimulation. The categories of response ("grouped," "failing," etc.) are defined in the text. The number following the category name is the number of series of at least 10 successive stimuli in which the indicated type of response was observed

Nominal strength (V)	30 per minute	60 per minute		120 per minute		180 per minute	
4	response	response	6	grouped	4	single	4
		failing	3	alternating	1	failing	1
		grouped	6				
		alternating	1				
16	response	response	16	response	18	failing	14
		failing	8	failing	20	grouped	3
		grouped	1	alternating	4	alternating	1
				frenzy	3	frenzy	2
72	response	response	7	response	16	response	10
				failing	11	failing	14
				frenzy	2	alternating	2

Table II summarizes several hundred tests with 12 combinations of stimulus strengths and frequencies. All pansies that responded to stimulation at 30 per minute were able to maintain response for 10–15 cycles at all voltages tested. At higher frequencies there were also successful series, particularly with the higher voltages, but there was an increasing percentage of breakdowns of several kinds. In "grouped" responses a few consecutive skips alternated with a few consecutive responses. In "alternating" behavior the colony skipped every other response, or sometimes two out of three potential responses. In the "failing" category the pansy started out apparently responding in perfect correspondence with the stimulation frequency but gradually fell behind and by the 10th consecutive wave or so was out of phase by a third or half cycle. If stimulation was then continued the responses became "grouped" and then more and more irregular until they ceased altogether.

There was, of course, considerable difference between individuals, but a number of definite trends were observed. First, ability to follow a given frequency was definitely greater the greater the stimulation intensity. This is well shown by the series made with 3 shocks per second, in which at threshold strength the almost invariable

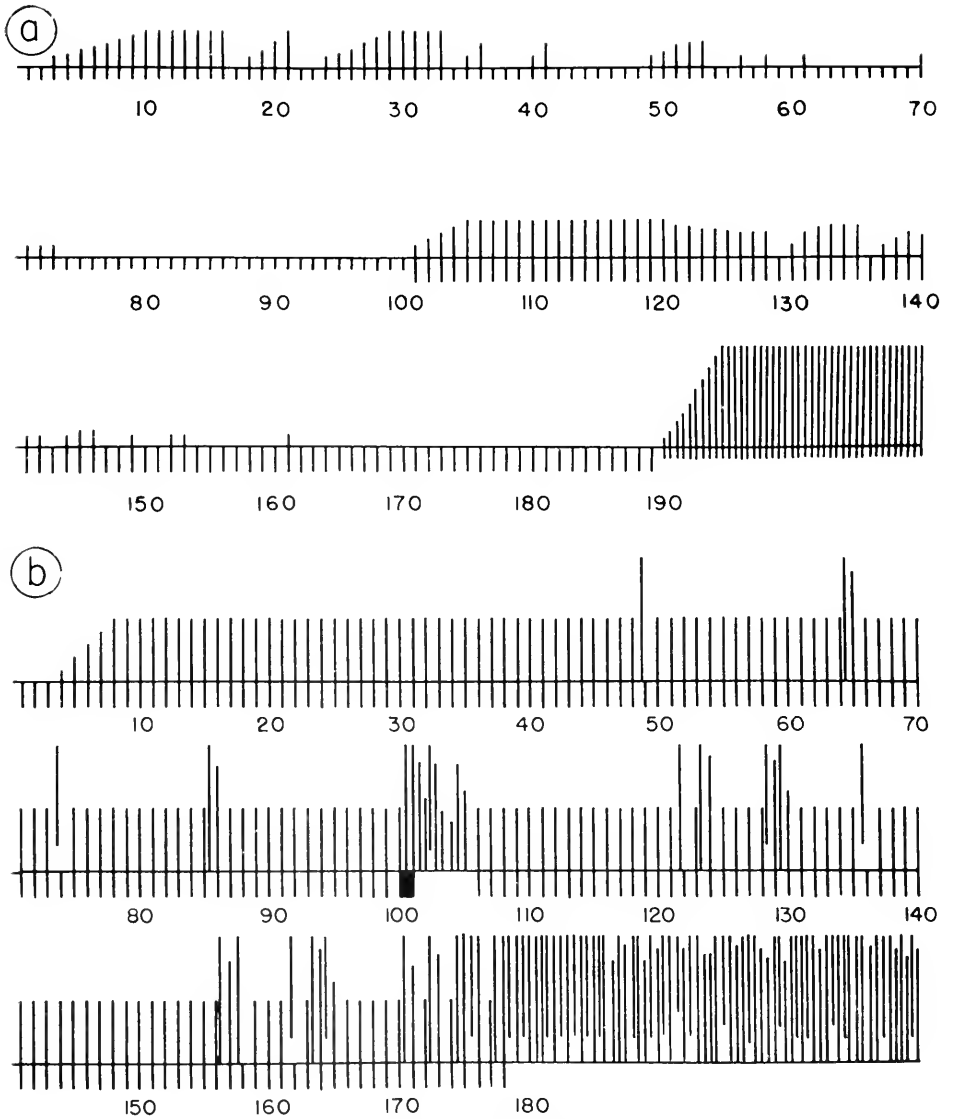

FIGURE 9. Diagrams of long records of response of *Renilla* colonies to serial electrical stimulation. Wave intensities indicated by lengths of vertical lines above horizontal axis. Relative intensities and frequencies of stimulation indicated by lines below horizontal axis. No attempt is made to show latencies. "Reverse" waves suggested by vertical lines not reaching horizontal axis. (a.) One hundred successive stimulations at threshold strength (4 V) and frequency of 30/min are followed by 89 stimuli at increased voltage but same frequency, and then by 41 stimuli at 4 V and 1/sec. Facilitation indicated at episodes 3–10, 24–33, *etc.*; skips at 17, 21, 22, *etc.* (b.) One hundred successive stimulations at 16 V and frequency of 30/min are followed by mechanical stimulation (eliciting "extra" and "reverse" waves), then resumption of original stimulations; leading to autoexcitation (frenzy) by about episode 178. Brighter extra waves indicated just after responses 64, 85, *etc.* followed by facilitated waves; brighter waves just after responses 48, 121, *etc.*, causing skipping; reverse waves at episodes 73, 136, *etc.*

response was a single flash, whereas at a strength of 72 V, or nearly twenty times the threshold stimulus strength, 1:1 responses were obtained in 10 of 26 trials. Secondly, performance tended to deteriorate with repetition. For example, a pansy might follow perfectly for 10 shocks on the first test at 60 per minute and threshold strength, but fall behind in successive trials in spite of intervening periods at 30 min (which was always followed without difficulty). Thirdly, the "grouped" type of response seemed characteristic of the lower frequencies and strengths of stimulation, whereas failure to maintain response at higher frequency-strength

TABLE III

Five representative stimulation series at 30 stimuli per minute which terminated in frenzy. Numbers in all but first three columns are stimulus sequence numbers at which indicated event occurred

Exp. No.	Stim. Str. (V)	First Response Start Shock	Extra, with brighter normal	Extra, with skip	Reverse with brighter normal	Reverse with skip	Frenzy	Remarks
1	16	3			51*, 143*, 182, 200, 229, 235, 238	117	258	225, dimmer; 258, two centers active
2	40	3	8, 57**	37			165	100, dimmer
3	16	2	70*	277‡		175†, 256†	280	83, two simultaneous waves from different points
4	16	4	78, 95**, 240*		154, 164, 323, 345	288	377	100–150, dimmer
5	16	2		300		102, 261, 271, 279	375	332, skipped; 100–180, brightest; 338–350, dim

* Not much facilitation of next normal wave.
** Extra wave of normal brightness.
† At proper time for normal wave.
‡ From second center.

combinations more usually took the form either of complete refractoriness or alternation, and it was of course also in this region that the response was most likely to pass over into frenzy.

Within limits, intensity of luminescence increased with increasing frequency of stimulation. Stepwise increase in brilliance as the pansy responded to the new frequency indicated that facilitation was involved, and a reverse or "defacilitatory" process was seen when stimulation frequency was suddenly reduced. If stimulus strength was suddenly increased, even as much as 10-fold, during the plateau phase of a response series, no increase in luminescence was observed. The effect of stimulus strength in maintaining response in long serial stimulation (overcoming adaptation) has already been mentioned.

Long-continued stimulation usually induced progressive changes in luminous behavior leading either to failure of response or to autoexcitation. Failing response, which was common with mild stimulation, is considered due to "adaptation" rather than to junctional or effector fatigue both because, in some series with higher voltages, many hundred successive responses could be induced and because very prolonged periods of spontaneous flashing occurred during frenzy. Adapting series might terminate without warning but ordinarily involved a few sporadic and short-lived resumptions of response after the initial failure. Frenzy, on the other hand, developed via a variety of the spontaneous behaviors already discussed. Table III summarizes five actual series, with some indication of intensity fluctuations as well as my interpretation of successive episodes. Composite diagrams to illustrate typical development of adaptation and of frenzy, as recorded from visual observation, are given in Figures 9a and 9b.

Siphonozooid activity during rachidial responses

Though records of luminescent waves delimit roughly the activity cycle of the siphonozooid clusters they give only a statistical view of the frequency of participation of the individual clusters and the temporal coordination of their activities. Visual observations of such phenomena must always be somewhat questionable because of the difficulty of maintaining fixation between flashes, but from microscopic viewing of many hundred waves during both electrical stimulation and frenzy, the following tentative conclusions were drawn: (a) a given cluster participated in each successive wave, though not always at the same brightness, (b) different clusters could be of quite different peak brilliance, (c) the increase in intensity of light emitted during facilitation was due to increase in the activity of the individual clusters, not to activation of additional clusters interspersed among those originally responding, (d) not every individual siphonozooid in a cluster necessarily produced light at every stimulus, (e) not all the individual siphonozooids of a cluster were of equal brightness, (f) the brilliance of a given individual siphonozooid could vary from wave to wave. From the fact that each wave was brightest at its leading edge it was deduced (g) that the ultimate unit response is asymmetrical—*i.e.*, has a relatively more rapid rise phase than decay phase.

Though *Renilla* colonies characteristically responded as a whole, and excitation is thought to affect all parts of the rachis equally, sometimes a few points of light were seen in response to the first one or two shocks of a strength which proved to be below threshold for the rachis as a whole, thus suggesting that the thresholds of the individual zooids or siphonozooid clusters differed somewhat. Similarly, when a rachis had become adapted and no longer responded as a whole, or had become fatigued by frenzy, a few zooids sometimes continued to light up in apparent response to the passage of a wave of excitation to which the rest of the zooids were refractory. Also, individual clusters occassionally flashed spontaneously in pansies that had not been stimulated for several minutes, perhaps indicating the attainment of local autoexcitatory states.

Mechanical injury sometimes elicited local luminescence, such as shown at the electrode site in Figure 5, which persisted steadily for many minutes. An interesting and unexplained phenomenon is that a single siphonozooid of a cluster, or a single lobe of an autozooid calyx, occasionally remained lit up steadily through

several to many seconds, apparently independent of injury, general rachidial waves or stimulation, and with a brilliance at least equal to that of any point on the rachis during passage of a normal luminous wave.

One further type of luminescence, sometimes seen briefly after mechanical stimulation, gives the impression of scintillation—*i.e.*, as if many of the siphonozooid clusters were sparkling rapidly and out of phase with each other and independently of the usual wave luminescence or indeed of any rachis-wide coordinated excitation.

Discussion

Assuming that the spread of luminescence over the *Renilla* colony mirrors the paths of excitation in a nerve net, the types of behavior described above might permit an analysis of net behavior hardly attainable from any other material. Some conclusions, particularly in relation to autoexcitability, are already apparent and are discussed below. Other very suggestive indications would be strengthened by a more critical discrimination between conductional, junctional and effector roles, and by better knowledge of the excitation cycles of the individual polyps and their parts, such as could be obtained by local stimulation. Preliminary results from such experiments (Buck and Hanson, 1967) were so promising—for example, in disclosing the existence of non-propagated responses and differing latency classes—that most of the discussion of nerve net physiology and theory, and the relation of the work on *Renilla* to the extensive literature derived from work on other coelenterates, have been deferred to a subsequent paper (Hanson and Buck, in preparation).

The data for polyp population (Fig. 4) and gross conduction velocity permit estimation of the minimal extent of the putative nerve net. The facts that the waves spread evenly, with the leading edge a smooth arc and with no sign of preferred direction relative to rachidial geometry, require siphonozooid interconnections in at least six directions. Assuming isolateral triangular connectivity and equal distance between clusters, and considering the rachis a hexagon of equivalent area, the number of siphonozooid clusters and the lengths of their interconnections are related by the formula $N = 3\ d(d + 1) + 1$, where N is the total population of siphonozooid clusters and d is the number of unit distances (cluster-to-cluster) along one edge of the rachidial hexagon. Substituting 1500 for N and solving for d, the corresponding hexagonal rachis works out to be about 22 connectivity units on an edge, or 44 units for a diameter (vertex to para position vertex). Using 6.6 cm as a reasonable actual diameter, the unit cluster-to-cluster distance is therefore about 1.5 mm. Assuming that each siphonozooid cluster transmits excitation to its three nearest centrifugal neighbors, and neglecting all autozooid connections, the total length of net involved during passage of a wave over a 6.6 cm colony would be not less than $1500 \times 3 \times 0.0015$, or about 6.7 m.

The differences between "summer" and "winter" colonies, whatever their cause and generality, are interesting and valuable from several aspects. If summer pansies had been used exclusively I would certainly have concluded that rapid autozooid withdrawal and rachidial wave luminescence are coupled responses. I was not able to tell whether the startle reaction can occur without luminescence but the fact that autozooids in winter pansies sometimes did not withdraw after excita-

tion strong enough to elicit luminescence from the whole rachis (including the autozooid tentacles) shows that muscular and bioluminescent responses are not in obligatory linkage. This does not mean that the two systems necessarily require separate conduction pathways, since differing requirements for facilitation could divorce the responses. Neither do my observations prove that rapid withdrawal, when it does occur, is triggered by the same excitation that induces flashing, though the two events often occur simultaneously. Parker (1919, 1920a, 1920b) considered the rapid withdrawal to be mediated by a different system from the slow rachidial peristalsis.

A second deduction from winter pansies is that at least the tentacles of the autozooids are connected to the general rachidial net that transmits excitation to the siphonozooid cluster. My inclination to believe that the trunk or column of the autozooid is non-luminous, or at least far dimmer than the tentacles, accords with Parker's conclusion from crushed autozooids, but since his treatment did not elicit light from the tentacles either, the matter must be left in abeyance. The variability observed in the laboratory reinforces the need for behavioral observations in the native habitat, particularly in connection with the questions of whether the colonies luminesce spontaneously, whether the autozooid tentacles participate regularly in rachidial luminescence along with the siphonozooids, whether there is autozooid withdrawal whenever a wave is generated, and whether autoexcitation occurs in nature.

By poking the white "granules" around the autozooid (the calyx lobes) Parker (1920b) discovered that the calyx is luminous but did not realize that the tissue also luminesces in response to distant stimulation of the rachis. Actually, the behavior of the calyx stands in curious contrast to that of both the autozooid tentacles and of the siphonozooids. The fact that no autozooid luminescence is usually seen in summer pansies during rachidial waves evoked by low frequency electrical stimulation that is near the threshold for siphonozooid responses could be attributed to the calices having a systematically higher threshold than the siphonozooids, or lower brilliance, or both, but the much greater sluggishness of the luminescence, when it does occur, suggests the possibility of a different effector tissue type or differing mode of excitation. On the other hand, the facts that calyx luminescence does augment in parallel with siphonozooid luminescence during vigorous repetitive stimulation, and that it does appear during frenzy, argue for excitation *via* the primary net.

The indication that luminescence in *Renilla* is intracellular is somewhat surprising, for although this is usual in coelenterates, secretion of a luminous slime was seen in the closely related sea pen *Cavernularia* (Harvey, 1917).

Cancellation of light emission when waves collide (Fig. 6e–6g) was observed by Panceri (1872) and Moore (1926) in *Pennatula*. The phenomenon was ascribed by Moore to a refractory state in the nerve net behind the advancing wave of luminescence that persists long enough to block conduction in the opposite direction. This seems a reasonable explanation and seems also compatible with the phenomenon of skipped waves during serial stimulation and with the origin of the "repeat" category of spontaneous waves from the site of electrode contact, the region of the rachis that has had the longest time to recover. However, Moore's

hypothesis is difficult to reconcile with "reverse" waves (Fig. 6c, 6d), which appear to originate from the region of the rachis most recently excited.

The virtual absence of decrement in the spread of luminescence in *Renilla* argues against the presence of facilitation-requiring synapses in the hypothetical nerve net itself, as does the fact that luminescence spreads evenly and apparently with equal facility in any direction. The presence of well-marked stepwise augmentation of luminescence, however, would argue strongly for the presence of neuroeffector junctions if it could be proven that this phenomenon is not due primarily to recruitment of additional luminous units. Insofar as I can judge visually, recruitment is not a major factor—hence my use of "facilitation"—but the necessity for periodic interruptions of view make it impossible to be sure. Ability to produce stepwise augmentation of luminescence by stimulating at two sites alternately, and to obtain a response to the first shock applied at one point after repetitive stimulation at another, provide additional evidence for net-wide facilitation, as does the persistence of a ready state for up to perhaps 30 seconds.

There is no reason to suppose that frenzy, a consequence of violent or repetitive stimulation, is a normal response for *Renilla* in its native habitat. Nonetheless, analysis in terms of combinations of autoexcitatory (spontaneous) behaviors such as repeat and reverse waves, with collision cancellation and facilitation, brings some order to an otherwise bewildering phenomenon. The existence of multiple and shifting autoexcitatory centers is a secondary complication to the primary problem, which is the persistence of spontaneity long past the time when any hyperfacilitation or after-discharge would be expected to have died out. The possibility of continuing excitation by a trapped circuit wave of the sort seen in the scyphozoan sub-umbrellar nerve (Bullock, 1943) is attractive, particularly during frenzies in which the luminescence actually does circle the perimeter of the rachis. Conceivably the constantly shifting pattern of spread and the appearance of luminescence in the most recently active regions (reverse waves) will turn out to reflect excitation that is circling the rachis vertically, so to speak, rather than horizontally, via the non-luminous under surface or via some internal pathway. The rather constant natural frequency of about one 'sec at 25° for frenzied flashing is reasonably close to the conduction delay expected for circuits of that length, although my experiments involving trimming the rachidial margin did not support the idea of net connections via non-luminous parts.

My visual observations and Nicol's (1955a, 1955b) recordings confirm and complement each other in most of the aspects of *Renilla* luminescence that we both studied. In certain matters where there appears to be some disagreement—for example, in our respective estimates of the persistence of facilitation (Nicol, 10 minutes; Buck, 30 seconds), in the extent of sensory adaptation over the rachis (Nicol, uniform; Buck, sometimes local), in the involvement of recruitment in the augmentation of luminescence (Nicol, postulated; Buck, not visible)—it is possible that differences in techniques may be involved. Ordinary photometric recordings from any considerable portion of the rachis (*e.g.*, Figs. 8a–8f) can be unreliable indicators of siphonozooid kinetics during the passage of a wave, even in regard to overall waveform (Buck, 1955), and even when intensity changes with time are not further obscured by sustained glowing of autozooid calices. Recordings made through a light guide (Fig. 7b) indeed show that small areas may

have a much shorter cycle than the mass wave though they do not identify the sources anatomically. What is needed here, as in other connections discussed earlier, are the data derived from individual stimulation of single zooids (Hanson and Buck, in preparation).

Most of this work was done at the Kerckhoff Marine Laboratory of the California Institute of Technology during tenure of a Visiting Professorship and in the laboratory of Professor T. H. Bullock at the University of California, Los Angeles. Special thanks are also due George and Nettie MacGinitie, George Beadle, T. D. Coyle and J. F. Case for valuable assistance. Numerous colleagues have ministered to the manuscript during its long gestation and Dr. Nicol is particularly thanked for his generous acknowledgment of its content. The photographs for Figure 1 were taken by Professor MacGinitie. The oscilloscope records used for Figures 7 and 8 were made at the National Institutes of Health in collaboration with Dr. Frank Hanson, and the image intensifier photographs of Figure 5 were made at Princeton University by Dr. Hanson in collaboration with Professor George Reynolds. Figure 4 was prepared by Edith Bidwell.

Summary

1. Some details are given of the external morphology of the autozooids and siphonozooids and of their distribution in the colony. It is estimated that a minimum of over 6 meters of nerve net would be required to conduct excitation across an average-size colony during the passage of a wave of luminescence.

2. An account is given of the localization of luminescence in the two types of polyp and of their apparently differing behaviors in colonies collected in summer and winter.

3. In summer colonies the sharp luminous waves induced by electrical stimulation are entirely due to siphonozooids. Under strong stimulation the autozooid calices produce a long lasting glow.

4. Neuroeffector facilitation takes place uniformly throughout the colonial conduction system. Decay of facilitation requires 10–36 seconds, by different tests. There are indications that sensory adaptation in the (hypothetical) net can be local.

5. Local recording shows that the response cycle in small areas of the colony is much shorter, and its frequency response much higher, than indicated by integrative recordings of the wave response as a whole.

6. Individual siphonozooid clusters can flash repetitively in successive waves, fail to participate in every wave and vary in intensity from wave to wave. The increase in light intensity during successive facilitating waves seems due to increase in the activity of individual clusters, not to recruitment of additional clusters. There were indications of individual differences in threshold, adaptation and autoexcitation between clusters.

7. During strong repetitive electrical stimulation there may arise extra siphonozooid waves of augmented brightness, running in the same direction as the "normal" waves (*i.e.*, centrifugally from the electrode) or in the reverse direction.

colony may then enter an autoexcitatory state ("frenzy"), independent of external stimulation and often involving development of several excitation centers, in which waves of irregular and constantly changing form course over the rachidial surface for up to an hour.

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Note added in proof: Professor Reynolds has just published several interesting image intensifier photographs of *Renilla* luminescence (Reynolds, George T., 1972. Image intensification applied to biological problems. *Quart. Rev. Biophysics*, 5: 295–347.)

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DISTRIBUTION AND FUNCTIONS OF ELASTIC FIBERS IN THE INVERTEBRATES

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Histochemical and functional anatomical evidence has been presented in Elder and Owen (1967) and in Elder (1973) for the existence of both collagen fibers and elastic fibers in the polychaete *Polyphysia crassa* and in several other invertebrates. In view of the unique fibrous architecture of the body wall connective tissues of *Polyphysia* and of the unusual locomotory mechanism employed in that species, and upon which most of the work cited above has been based, it was of relevance to discover how widespread are the elastic fibers amongst the invertebrates. While evidence from x-ray diffraction, physical properties, biochemical analyses and electron microscopy has established the existence of collagen fibers in all metazoan groups examined, both vertebrate and invertebrate (Marks, Bear and Blake, 1949; Piez and Gross, 1959), no correspondingly unequivocal demonstration of elastic fibers has been given amongst the invertebrates and even amongst mammalian tissues elastic fibers are extremely difficult to define biochemically (Piez, 1968; Partridge, 1970; Bodley and Wood, 1972). Moreover, both histochemically and ultrastructurally there are clear differences between the invertebrate elastic fibers and those of vertebrates (Elder and Owen, 1967 and Elder, 1972). The protein "elastin," characteristic of vertebrates (Ross and Bornstein, 1969) appears to be absent from invertebrates (Piez, 1968). The only definitive test for an invertebrate elastic fiber would be the physical demonstration of the "property of rubber-like extension and elastic snap on recoil" characteristic of vertebrate elastin (Partridge, 1970, page 593); preliminary tests on the radial fibers in *Polyphysia* body wall were reported in Elder and Owen (1967) and the physical extensibility has been shown from histological sections of the body wall fixed in different configurations (Elder, in preparation).

Staining techniques, such as the spirit blue (Owen, 1959; Elder and Owen, 1967) and aldehyde fuchsin (Gomori, 1950; Gabe, 1953) methods, readily distinguish invertebrate collagenous fibers, which do not take these dyes, from other fibers which stain readily. Amongst the latter, elastic fibers may be found. As a first step, however, it was considered essential to determine how widely distributed amongst the invertebrates the spirit blue positive fibers were and in what anatomical locations they occurred.

Materials and Methods

Most of the specimens were collected in the Woods Hole region, Massachusetts. Other specimens were collected in the Cobscook Bay area of Northern Maine or were supplied by the Scottish Marine Biological Association, Millport, Isle of

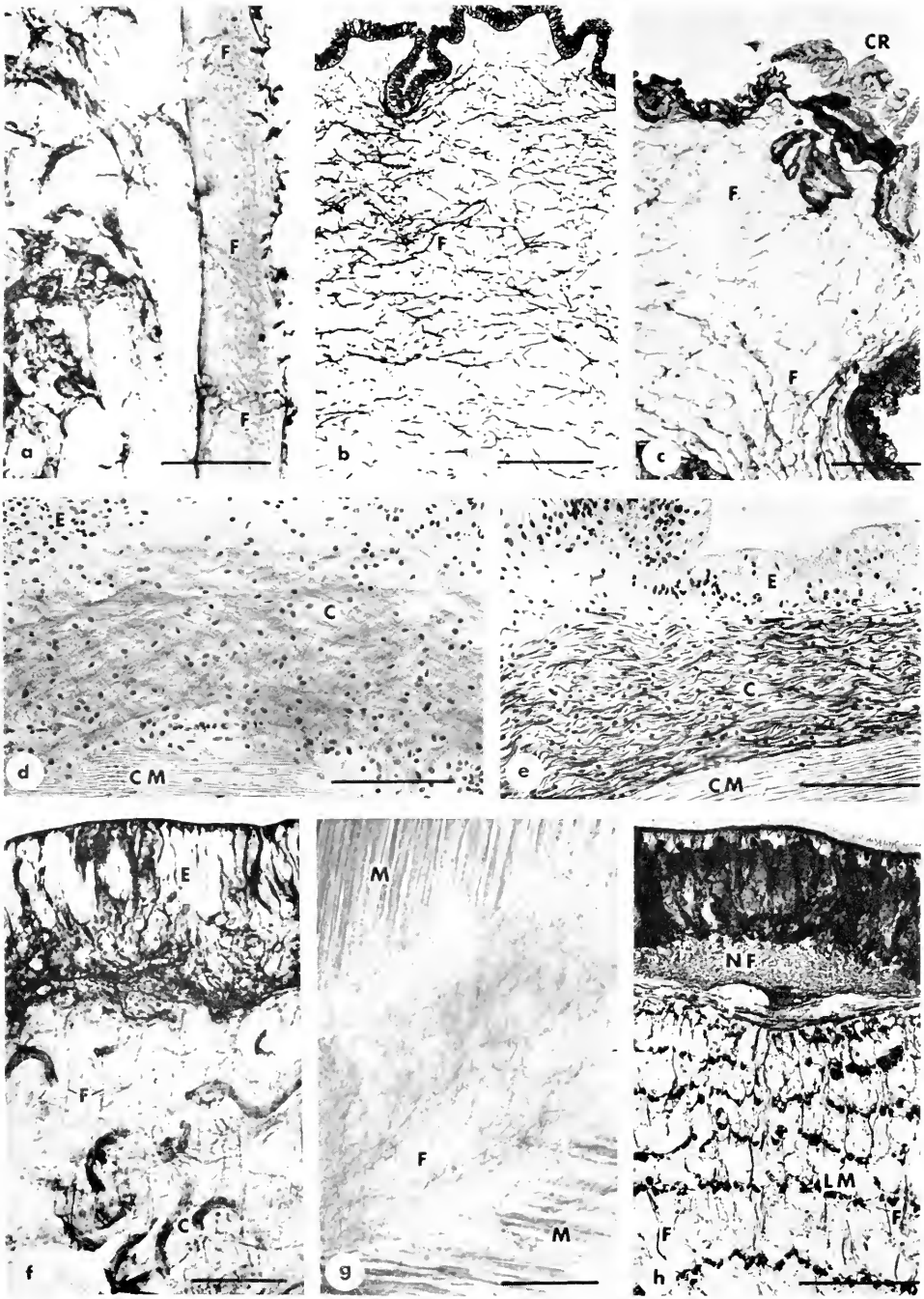

FIGURE 1. (a) Micrograph of the mesoglea of the hydroid polyp *Corymorpha pendula*, stained with spirit blue and van Gieson. The mesogleal matrix stains as for collagen but a

Cumbrae. Much of the histology was done in the Marine Biological Laboratory, Woods Hole, Massachusetts. Specimens were fixed in Bouin's fluid and embedded in paraffin or ester wax (B.D.H. Ltd.) and cut at 2–8 μm. The routine stains used were Masson's trichrome, aldehyde fuchsin (Gabe, 1953) and fast green, and spirit blue and van Gieson (Elder and Owen, 1967).

For electron microscopy specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 with 3% sucrose, post fixed in 1% osmium tetroxide in the same buffer and embedded in araldite. Sections were cut on L.K.B. I or III ultramicrotomes, stained in uranyl acetate and lead citrate and examined on A.E.I. EM6 and EM6B electron microscopes of the Departments of Zoology and Physiology of the University of Glasgow. Light micrographs were taken on a Leitz Orthoplan.

Observations on the burrowing behavior of animals were carried out at the MBL, Woods Hole. Animals were observed while burrowing into the surface of the substratum in open dishes or down the sides of a glass tank. Electronic flash photographs were made for examination and analysis.

Results

Some forty selected species from twelve invertebrate Phyla were examined histologically for the presence of spirit blue positive fibers. A detailed description of all the results is not appropriate to the present work but the major findings are summarized in Table I and preliminary accounts have been given in Elder (1966a

number of radially oriented, spirit blue positive fibers (F) are seen traversing the thickness of the mesoglea; 50 μm bar. (b) The field shows part of the exumbrellar region of the bell of the scyphozoan *Dactylometra quinquecirrha*. The epithelium is folded due to the significant shrinkage during processing. The extensive network of spirit blue positive fibers (F), organized approximately tangentially to the exumbrellar surface, is apparent; spirit blue and van Gieson stained; 200 μm bar. (c) A sector of the collenchyme and epidermis of the ctenophore *Mnemiopsis leidyi* is shown; spirit blue and van Gieson stained. Again considerable distortion has occurred during preparation but the network of spirit blue positive fibers (F) is apparent throughout the collenchyme; CR, ctene row; 200 μm bar. (d) Tangential section through the body wall of the holothurian *Leptosynapta tenuis;* Masson trichrome stain. The long axis of the body is from top to bottom of the micrograph and it can be seen that collagen (C) fibers occur predominantly in right and left handed helices along the long axis of the body; CM, circular muscles; E, epidermis; 100 μm bar. (e) Transverse section through the body wall of *Leptosynapta tenuis;* Masson trichrome stain. The radial integration of the thick dermal collagen layer, achieved by the wavy course adopted by the individual collagen fibers, is apparent. Immediately beneath the epidermis and above the collagen is a pale layer which contains the ossicles and does not stain with this method. Lettering is as in previous figures; 100 μm bar. (f) Transverse section through the outer layer of the dermis in *Leptosynapta;* stained with spirit blue and van Gieson. This layer, unstained in 8e above, stains intensely by this method. A number of stout, wavy collagen fibers link the epidermis to the underlying dense collagen layer (not in the field). A number of radially oriented, spirit blue positive fibers are seen traversing the deeply stained matrix of this outer dermal layer. Lettering is as above; 50 μm bar. (g) Tangential section through the gizzard of the polychaete *Aphrodite aculeata;* spirit blue and van Gieson stained. In the connective tissue between muscle layers an extensive network of spirit blue positive fibers (F) is apparent; M, muscle; 50 μm bar. (h) Transverse section through the proboscis of the hemichordate *Saccoglossus kowalewskii;* spirit blue and van Gieson stained. Densely stained spirit blue positive fibers are seen radially oriented, running within a meshwork of fine collagen fibers and linking the concentric layers of longitudinal muscle fibers, (LM); NF, nerve fiber layer underlying the ciliated epidermis; 100 μm bar.

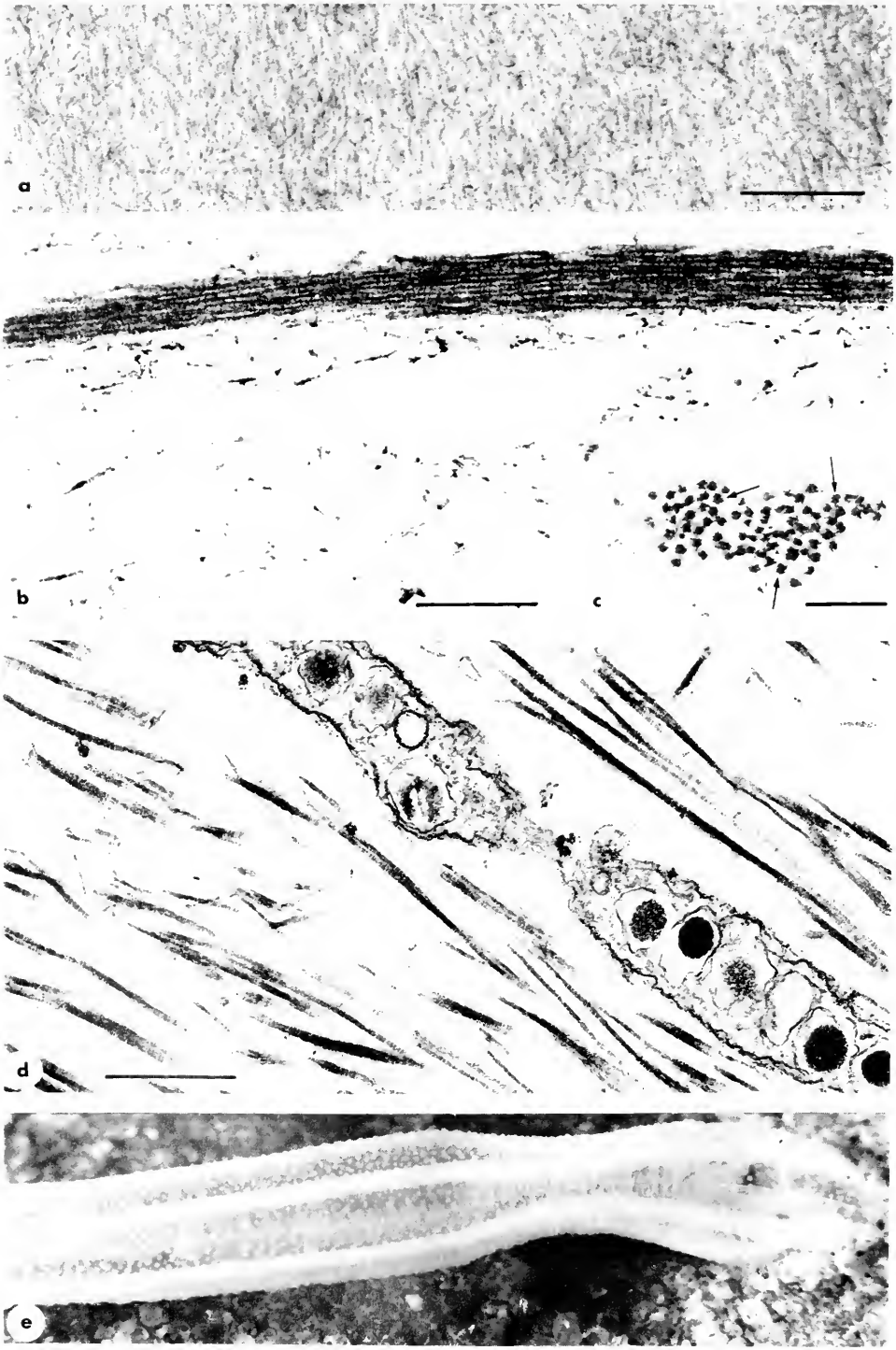

FIGURE 2.

and 1966b). While an investigation of this type can be regarded as no more than a preliminary survey, several interesting facts and general conclusions do emerge together with a number of pointers for future work. Spirit blue positive fibers were found in most of the species examined; the only groups in which none were found were the Anthozoa (two species) and the Platyhelminthes (three species).

Cnidarian mesoglea

Examination of sections of the hydrozoan polyps *Hydra* and *Tubularia* failed to reveal any fibrous structure in the very thin mesoglea, though an extensive fibrous architecture has been described from whole preparations of *Hydra* mesoglea by Hausman and Burnett (1969). Staining by aldehyde fuchsin (Gabe, 1953) failed to demonstrate in these polyps the affinity for the dye reported by Bouillon (1957) in *Limnocnida* polyps. The giant hydroid *Corymorpha pendula* was examined in the hope that its much thicker mesoglea might be more easily investigated microscopically. Some evidence for the presence of radially oriented fibers in an otherwise amorphous mesoglea was found (Fig. 1a), particularly in sections from the neck region where the mesoglea is thickest. The staining reactions were equivocal and it was not clear whether the radially oriented structures represent true fibrous elements traversing the amorphous mesoglea or simply condensations of the amorphous matrix. Electron micrographs reveal a fairly dense and apparently randomly oriented newtork of fine filaments some 10 nm in diameter (Fig. 2a) but so far have failed to reveal any of the radially oriented fibers.

In contrast to the anthozoa and hydrozoan polyps the medusae of those species of scyphozoa and hydrozoa examined have abundant mesogleal fibers which stain strongly with spirit blue and aldehyde fuchsin. The disposition of these fibers corresponds to that given by previous workers. The principle fibrous organization consists of a network of fibers running in all directions in a plane tangential to the exumbrellar surface (Fig. 1b), and stout, radially oriented, arborescent fibers which traverse the mesoglea (Chapman, 1953a, 1959; Bouillon and Vandermeerssche, 1956). The organization of similar fibers traversing the mesoglea of the stauromedusae has been described in Elder and Owen (1967). The electron microscopical appearance of the stout fibers traversing the mesoglea of *Haliclystus auricula* is shown in Figure 2b and c. Although the fibers show an indistinct periodicity by virtue of the beaded nature of the component filaments, the appear-

FIGURE 2. (a) Electron micrograph of the fine fibers, apparently randomly oriented in the mesoglea of the giant hydroid polyp *Corymorpha pendula;* U and Pb stained; 0.5 μm bar. (b) One of the "spirit blue positive" fibers from the mesoglea of *Haliclystus auricula* is cut in longitudinal section in this electron micrograph. The fiber is seen to be comprised of finer, beaded fibrils. Associated with the fiber are finer filaments which also form a loose meshwork throughout the mesoglea; U and Pb stained; 0.5 μm bar. (c) One of the spirit blue positive fibers of *Haliclystus* is seen in transverse section in this electron micrograph. The component fibrils are clearly seen and each appears to be comprised of finer filaments (arrows) some 6 nm in diameter; U and Pb stained; 0.25 μm bar. (d) Section through the dermis of *Leptosynapta tenuis* shows collagen filaments with the characteristic cross-striation pattern (approximately 50 nm periodicity). A number of finer, unstriated filaments (some 8 nm in diameter) are found associated with the collagen. Part of a cell (probably a fibrocyte) with a number of membrane-bound, electron dense inclusions is also seen; U and Pb stained; 0.5 μm bar. (e) Flash photograph of the mid trunk region of a specimen of *Leptosynapta tenuis* burrowing into the surface of the sand; the head is buried and to the right. A direct peristaltic wave involving simultaneous longitudinal and circular muscle contraction, is seen passing along the body from left to right.

ance is quite distinct from that of collagenous fibers. In their staining properties under the light microscope and in their electron microscopical appearance these fibers resemble those described by Bouillon and Vandermeerssche (1956), though only low power micrographs were presented by these authors. The ultrastructure of the medusan fibers differs from that of the spirit blue fibers described from *Polyphysia*, *Peripatus* and molluscan tissues (Elder and Owen, 1967) in being composed of aggregates of filaments associated together apparently by cross bridges (Elder, 1966b).

A loose network of fine fibers, some 10 nm in diameter, is also present throughout the mesoglea (Fig. 2b). The filaments resemble those found in the mesoglea of *Corymorpha* above. Though not so dense as in the latter species, these filaments are probably responsible for the diffuse collagen-like staining reactions of the medusan mesogleal matrix. In the stauromedusan *Craterolophus convolutus* they form a coarse network visible with the light microscope (Smith and Elder, 1967).

In the Phylum Ctenophora the collenchyme was found to contain a network of spirit blue staining fibers (Fig. 1c) in *Mnemiopsis leidyi*, the only species examined.

Dermal connective tissues

In view of the important role which the elastic fibers of the body wall have in the locomotion of the polychaete *Polyphysia* (Elder, 1972; and 1973), it was considered worthwhile investigating the dermal connective tissue organization and composition in a variety of burrowing invertebrates. Radially oriented spirit blue positive fibers were encountered in the body wall connective tissues of at least some members of the Nemertea, Annelida, Sipunculida, Aschelminthes (Priapulida), Onycophora, Echinodermata (Holothuria) and Hemichordata.

(*a*). *Apodous holothurian body wall.* It was of great interest to find a thick dermal connective tissue layer comprising both collagenous and spirit blue positive fibers in the burrowing apodous holothurian *Leptosynapta tennis*. The collagen fibers are organized in a three dimensional lattice which would allow radial, circumferential or longitudinal extension. Tangential sections (Fig. 1d) show that the fibers are organized in right and left handed helices around the long axis of the body while transverse (Fig. 1e) or longitudinal sections reveal that the fibers pass from one level to another, integrating the system radially. In the superficial levels of the dermis the collagen fibers are sparse but stout bundles are always found traversing the connective tissue to attach to the epidermal basement membrane (Fig. 1f). Most of the volume of the superficial dermal layer is occupied by a "felt-like" network of fine fibers which stain with the spirit blue and aldehyde fuchsin techniques (Fig. 1f). Radially oriented spirit blue positive fibers are also readily demonstrated traversing this layer (Fig. 1f). It is not clear whether the latter are discrete fibers or simply condensations of the felt-like network. While the spirit blue positive "felt" and the radial fibers are most easily seen in the superficial dermal layer, the radial fibers can be traced inwards through the collagen lattice to the muscle layer and the fine fiber network forms an envelope investing the collagen bundles. Under the electron microscope the fine "felt" and the collagen fibers have been found (Fig. 2d) but the radially oriented fibers have not yet been located.

Staining with alcian blue 8GX and Hale's dialyzed iron methods revealed a distribution of acid mucopolysaccharides similar to that of the fine fibrous network. While the radially oriented fibers are stained they do not appear to take these dyes more strongly than the surrounding felt-work, as they might be expected to do if they are simply condensations of that network. Fullmer (1960) has shown that aldehyde fuchsin stains some acid mucopolysaccharides after peracetic oxidation and it may be that the affinity of the fine network fibers for spirit blue or aldehyde fuchsin after permanganate oxidation is also due to the presence of acid mucopolysaccharides.

The bases of the epithelial cells are separated from each other by spaces, apparently fluid filled, which frequently extend through most of the height of the epithelium. Tangential sections through the epithelium, stained as above for glycosaminoglycans, show each cell outlined by this space, strongly stained for acid mucopolysaccharides. Electron micrographs reveal that the fine felt-work fibers penetrate radially through the space to attach apically by hemi-desmosomes.

(*b*). *Burrowing mechanism in* Leptosynapta. In an attempt to determine the significance of the complex dermal connective tissue construction, observations were made on the burrowing mechanism of *Leptosynapta*. Electronic flash photographs were taken of specimens moving over the substratum and burrowing into it and of animals burrowing in sand sandwiched between glass plates. Coelomic pressure recordings using a Statham pressure transducer and pen recorder have also been made (Hunter and Elder, 1967). These studies will be reported fully elsewhere. For present purposes it is sufficient to note that, with reference to *Leptosynapta*, previous accounts of the burrowing mechanism in holothurians (Gerould, 1897; Clark, 1901; Yamanouchi, 1926, 1929) summarized in Trueman and Ansell (1969) are inadequate or erroneous. Burrow excavation is performed by the lateral scraping of the ring of circum oral tentacles, and body progression is achieved by means of direct peristaltic waves involving simultaneous longitudinal and circular muscle contraction. At no phase of the burrowing activity is a "terminal anchor" formed and the periodic contractions of the longitudinal musculature are associated with burrow consolidation (Hunter and Elder, 1967), not with pulling the body up to a terminal anchor (Trueman and Ansell, 1969). Figure 2e shows a peristaltic constriction travelling anteriorily along the body of *Leptosynapta* during forward locomotion. This type of locomotory and burrowing mechanism has been described before only for the polychaete *Polyphysia crassa* (Elder, 1973).

During the passage of the peristaltic constriction the radial dimension of the body wall probably increases significantly. The construction of the three dimensional collagen lattice allows radial increase when circular and longitudinal muscle are contracted simultaneously and, as in *Polyphysia*, the radially oriented spirit blue fibers are probably extended in this configuration of the body wall.

(*c*). *Pedate holothurian body wall.* Sections of the pedate holothurian *Thyone briareus* revealed a thick dermal layer, the staining reactions of which suggest that it is composed principally of dense collagenous layers. A suggestion of spirit blue staining material was present but no discrete fibers were distinguishable.

(*d*). *Sipunculid burrowing and body wall structure.* *Golfingia gouldi* may be taken as representative of burrowers of the terminal anchorage type. Regions of the trunk form a penetration anchor during forceful protrusion of the proboscis

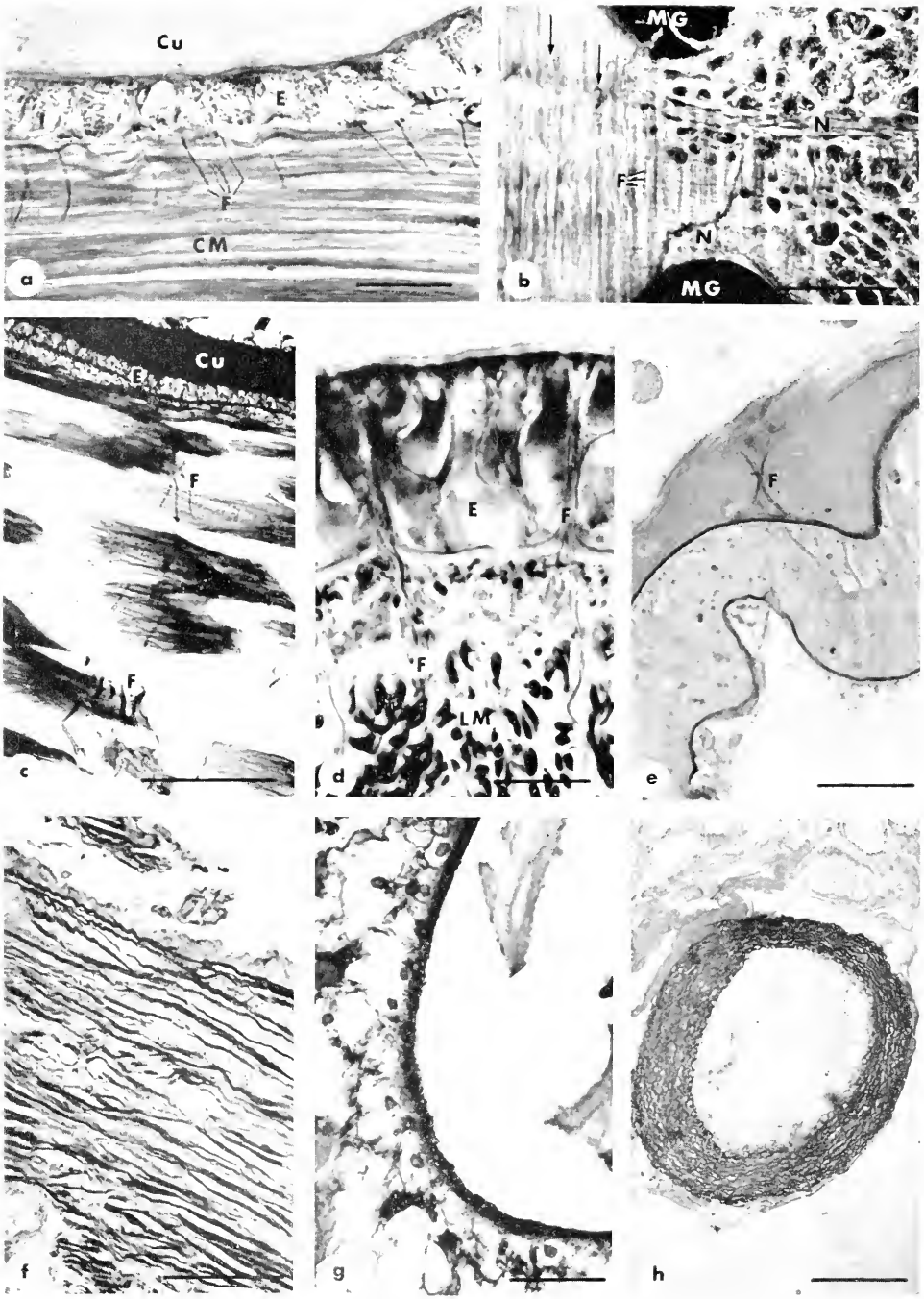

FIGURE 3. (a) A transverse section through the outer layers of the body wall of the sipunculid *Golfingia gouldi* in the trunk region; spirit blue and van Gieson stained. Part of

into the substratum. The everted proboscis then dilates to form a terminal anchor while the longitudinal muscles contract to pull the body forward (Clark, 1964; Trueman and Ansell, 1969). While coelomic pressures of only a few cm of water are employed during burrowing in *Leptosynapta*, with peaks of around 20 cm during burrow consolidation, *Golfingia* was found to utilize pressures of up to 250 cm during rapid burrowing (Hunter and Elder, 1967). A further contrast with burrowers of the lateral scraping type lies in the structure of the body wall. The trunk region of *Golfingia gouldi* possesses a very thick collagenous cuticle and a very thin dermal connective tissue layer (Hyman, 1959 and present observations, Fig. 3a). Radially oriented spirit blue positive fibers are, however, found traversing the thick circular muscle layer (Fig. 3a and Elder and Owen, 1967). At their outer extremities these fibers attach to longitudinally oriented spirit blue positive fibers of the dermal layer (Fig. 3b) and at their inner extremities to the connective tissue around the innermost circular muscle fibers. It is probable that these spirit blue positive fibers are elastic for they follow a straight course in sections from narcotised animals. However, the thickness of the circular muscle layer must increase significantly on contraction and at the same time the length of the longitudinal muscles and longitudinally oriented spirit blue fibers must also increase. The function of the radial fibers is probably to retain the position of the longitudinal muscle blocks relative to the epidermis and cuticle, irrespective of circular muscle contraction.

the stout, lamellated cuticle (Cu), which stains like collagen, overlies the cuboidal epithelium (E). From the thin dermis radially oriented spirit blue positive fibers (F) extend through the circular muscle layer (CM); 20 μm bar. (b) Tangential section through the outer layers of the body wall of *Golfingia* in the trunk region; Aldehyde fuchsin and fast green stained. The bases of the epithelial cells with granular cytoplasm are seen to the right and the circular muscle layer to the left. The margins of mucous glands (MG) are seen top and bottom, with associated nerve fibers (N). The system of fine, longitudinally oriented, "spirit blue positive" fibers is seen running within the dermis. These longitudinal fibers link at intervals of every three or four muscle fibers with rows of radially oriented, spirit blue positive fibers (arrows). Other lettering is as above; 50 μm bar. (c) Transverse section through the outer layers of the body wall of the aschelminth *Priapulus caudatus* in the trunk region; spirit blue and van Gieson stained. Unlike the cuticle of *Golfingia* above, the stout cuticle of *Priapulus* stains deeply with spirit blue. Radially oriented, spirit blue positive fibers are seen linking the connective tissue sheaths around the circular muscle fibers. Lettering is as in the previous plates; 50 μm bar. (d) Transverse section through the outer layers of the body of the heteronemertean *Micrura leidyi;* spirit blue and van Gieson stained. Radially oriented, spirit blue positive fibers traversing the outer longitudinal muscle layer (LM) aggregate into bundles which traverse the narrow dermis and penetrate into periodic clefts amongst the ciliated epithelial cells. Lettering is as in previous plates; 20 μm bar. (e) Transverse section through the intestinal wall of the polychaete *Polyphysia crassa;* spirit blue and van Gieson stained. The gut lumen is to the bottom of the field. Spirit blue positive fibers form networks in the walls of the peri-intestinal haemal sinus and periodic columns of radially oriented fibers (F) traverse the sinus lumen; 50 μm bar. (f) Transverse section through the wall of the posterior aortic bulb in the bivalve mollusc *Mya arenaria;* spirit blue and van Gieson stained. Spirit blue positive fibers form concentric lamellae interspersed with muscle fibers and collagen. The lumen is to the top of the field; 100 μm bar. (g) Transverse section of the wall of the dorsal abdominal artery of the crustacean *Nephrops norvegicus;* spirit blue and van Gieson stained. A single, stout, spirit blue positive lamella is present, surrounded by a cuboidal epithelium; 50 μm bar. (h) Transverse section of the anterior dorsal artery in the xiphosuran *Limulus polyphemus;* stained with spirit blue and van Gieson. Concentric layers of spirit blue positive fibers are interspersed with muscle fibers; 200 μm bar.

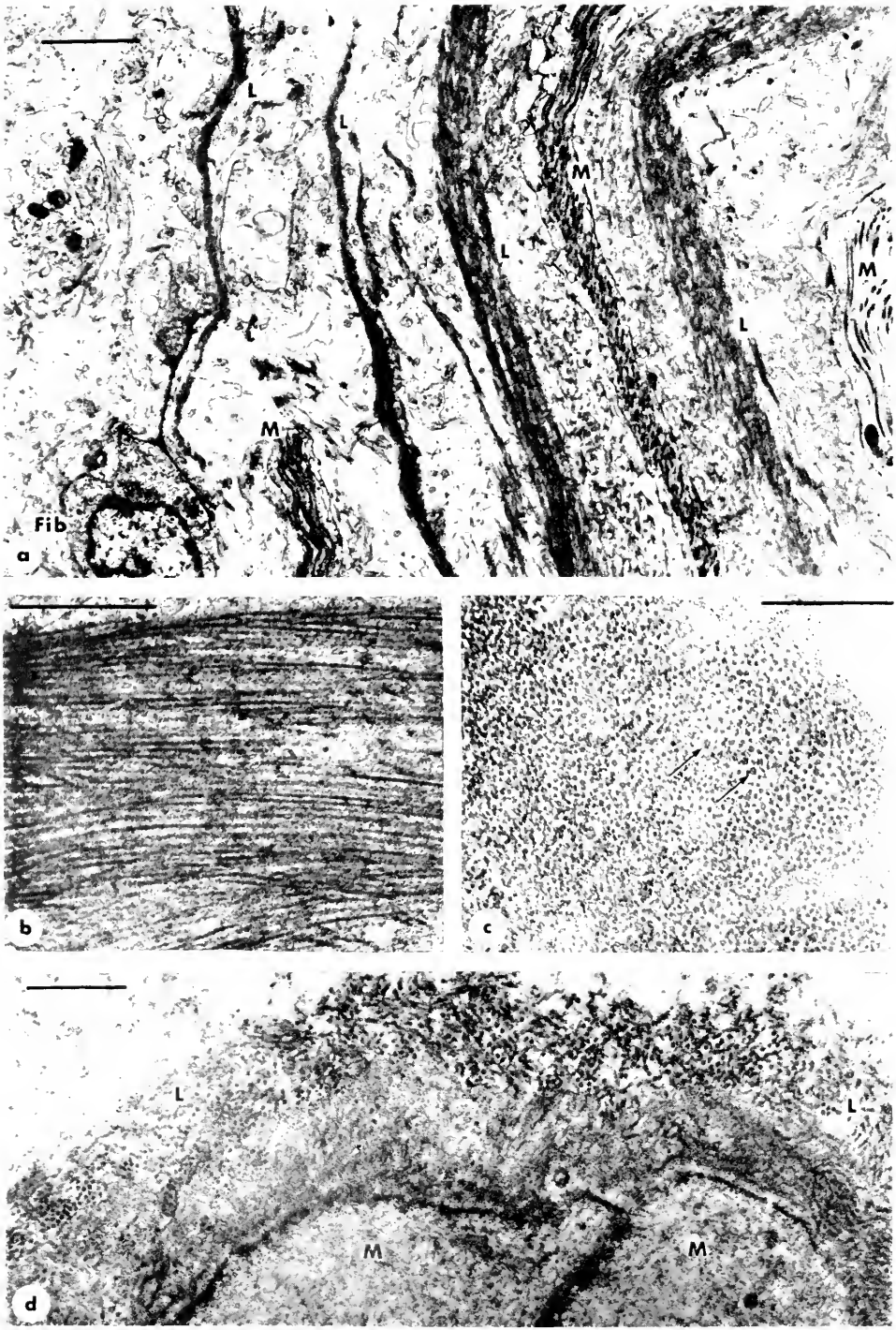

FIGURE 4.

(*e*). *Nemertean parenchymal fibers.* A similar case can be made for the properties and functions of spirit blue positive fibers which follow direct radial courses through the muscle layers of the body of *Micrura leidyi* and other nemertean worms (Fig. 3d). The fibers do not attach to the base of the epithelium but penetrate between the cells in spaces, apparently fluid filled, which periodically separate the bases of the epithelial cells (Fig. 3d), and extend at least half the height of the epithelium.

(*f*). *Connective tissues of the hemichordate proboscis.* Amongst the Hemichordata, a complex system of radially oriented spirit blue positive fibers is found associated with collagen fibers linking the nerve fiber layer of the neurectoderm and the concentrically arranged longitudinal muscle fiber layers in the proboscis of *Saccoglossus kowalewskii* (Fig. 1h).

(*g*). *Priapulid body wall.* Spirit blue positive fibers are found radially oriented amongst the circular muscle fibers of the Aschelminth *Priapulus* (Fig. 3c). Careful inspection of sections, however, suggests that these fibers are simply condensations of material which forms a sheath around each muscle fiber. Since, at least in the middle of each segment where the majority of these radially oriented condensations are found, the muscle fibers are well spaced contraction may not result in stretching of the radial elements. Unlike the cuticle of annelids and *Golfingia* which stain like collagen, the thick cuticle of *Priapulus* is strongly spirit blue positive. There is, however, no evidence to suggest that either the cuticle or the perimuscular sheaths have elastic properties. It is possible that the spirit blue affinity of these elements in *Priapulus* may, as in the case of the network of fine filaments in the dermis of *Leptosynapta,* be due to the presence of acid mucopolysaccharides.

(*h*). *Annelid body wall fibers.* Amongst the annelids the distribution of spirit blue fibers in the dermis of *Polyphysia* has been described by Elder (1972) and Owen (1959) figures spirit blue positive fibers in the body wall of *Lumbricus.* Similar fibers are present also in the Hirudinea (Elder and Owen, 1967). Several polychaete species were examined in the present series and spirit blue positive fibers were found to be of widespread occurrence in the group. Figure 1g shows a network of fibers between muscle layers in the gizzard in the errant polychaete *Aphrodite.*

Blood vessels

The blood vessels of a number of invertebrate groups were examined as a possible location in which elastic fibers might be found; spirit blue positive fibers

FIGURE 4. (a) Low power electron micrograph of part of the wall of the posterior aortic bulb in the bivalve *Mya arenaria.* The lumen lies out of the field to the right. Concentric "spirit blue positive" lamellae (L) are interspersed with muscle cells (M), collagen (C) and fibrocytes (Fib), U and Pb stained, 2 μm bar. (b) In this electron micrograph the fibrils (some 21 nm in diameter) which comprise the single lamella in the dorsal abdominal artery of *Nephrops norvegicus* are seen in longitudinal section. A faint periodicity is apparent in the densely staining fibrils; U and Pb stained; 0.5 μm bar. (c) The fibrils which comprise the lamella of the *Nephrops* artery are seen in transverse section in this electron micrograph and appear to be comprised of aggregates of finer filaments. The number of component filaments may be variable (arrows); U and Pb stained; 0.5 μm bar. (d) Part of one of the "spirit blue positive" lamellae (L) of the anterior dorsal artery of *Limulus polyphemus* is seen in this electron micrograph. As in the crustacean *Nephrops* above, the lamella is composed of electron dense fibrils which may be aggregates of finer sub units. A layer of amorphous material lies between the lamella and the periphral sarcoplasm of two obliquely sectioned muscle cells (M); 0.5 μm bar.

were found in all species examined. In the peri-intestinal haemal sinuses of the polychaete *Polyphysia* spirit blue positive fibers are located, radially oriented, traversing, the sinus between the intestinal and mesothelial surfaces (Fig. 4d). Blood pressure in this region of the circulatory system is probably negligible but it has been suggested (Elder, 1973) that movements of the body wall may play an important role in compressing the sinuses and propelling the blood. At any particular locus distension of the sinus would tend to extend the spirit blue positive fibers. Radially oriented spirit blue positive fibers were similarly found traversing the lumen of the peri-intestinal haemal sinus in *Thyone briareus*.

In the Mollusca spirit blue positive fibers were found disposed in concentric layers in the cephalic artery of the cephalopods *Loligo pealii* and *Eledone cirrhosa* and in the aortic bulb of the bivalve *Mya arenaria*, (Fig. 3f). They have also been found amongst the viscera of the Pacific polyplacophoran *Cryptochiton* (Elder and Owen, 1967). Ultrastructurally the concentric lamellae of the spirit blue staining fibers in *Mya* artery (Fig. 4a) closely resemble those of the radial fibers from the dermis of *Polyphysia* (Elder, 1966b; Elder and Owen, 1967). The fine filaments of some 3 nm diameter which constitute the lamellae appear to be disposed in a predominantly circumferential orientation. Distension of these vessels would tend to extend the muscle fibers and to stress the concentric fiber layers circumferentially.

In the crustacean *Nephrops norvegicus* the structure of the dorsal abdominal artery is strikingly different from that of the molluscan vessels described above. Columnar cells lining the haemocoel surround a single stout spirit blue positive lamella which is lined by squamous endothelial cells (Fig. 3g). Electron micrographs reveal that the thick lamella is made up of fibers oriented largely along the long axis of the vessel. Moreover, the fibers appear to be compound, comprising from 3–7 filaments each some 5 nm in diameter (Figs 4b and 4c). In this respect they resemble the compound fiber type found in scyphozoan mesoglea.

The xiphosuran *Limulus polyphemus* has a more extensive vascular system than that of other arthropods. The complex structure of the anterior dorsal artery in which concentric spirit blue positive lamellae are interspersed with muscle fibers is shown in Figure 3h. Ultrastructurally these lamellae are composed of fibers longitudinally oriented, as in *Nephrops* (Elder, 1966b), and are found adjacent to extensive areas of amorphous, electron dense material initimately associated with the muscle fibers (Fig. 4d).

Discussion

The remarkable range of body form of which *Metridium* at least is capable amongst Anthozoa has been described by Batham and Pantin (1950) and a three dimensional collagen lattice was described in *Calliactis* and *Metridium* by Chapman (1953a) who also drew attention to the visco-elastic properties of the mesoglea. These properties were also studied by Alexander (1962) who described a long period reversible deformation which could not be ascribed to the known properties of the collagen rich mesoglea. Recently Gosline (1971a and 1971b) has carried out extensive tests on *Metridium* mesoglea which show that the properties of extensibility and elasticity can be accounted for by the presence of a high molecular weight amorphous polymer network in the mesogleal matrix, the collagen being

mainly a "reinforcing filler" which provides short term rigidity (Gosline, 1971b). The physical properties of *Metridium* mesoglea seem admirably adapted to the slow postural changes and very variable enterocoelic volume in this animal. Further studies may reveal instructive differences in the mesogleal structure and properties in other species with less extensible bodies, such as *Calliactis,* or more active habits, such as the burrowing anemone *Peachia* (Ansell and Trueman, 1968) or the swimming anemone, *Stomphia* (Robson, 1966). Thus although the anthozoan mesoglea displays elastic properties, the absence of any fibrous elements with such properties need not be surprising.

TABLE I

Phyletic and anatomical distribution of spirit blue positive fibers in invertebrate tissues examined

Phylum and sub phylum	Class and sub class	Species	Anatomical location of spirit blue fibers
CNIDARIA	HYDROZOA	Hydra pseudoligactis	—
		Tubularia larynx	—
		Corymorpha pendula	? polyp mesoglea
		Gonionemus murbachii	medusan mesoglea
	SCYPHOZOA	Dactylometra quinquecirrha	medusan mesoglea
		Lucernaria quadricornis	mesoglea
		Halicystus auricula	"
		Craterolophus convolutus	"
	ANTHOZOA	Actinia equina	—
		Haloclava producta	—
CTENOPHORA	TENTACULATA	Mnemiopsis leidyi	collenchyme
PLATYHELMINTHES	TURBELLARIA	Bdelloura candida	—
		Stylochus zebra	—
	CESTODA	Schistocephalus solidus	—
NEMERTEA	ANOPLA	Micrura leidyi	radial in muscles & mesenchyme
		Cerebratulus lacteus	radial in muscles & mesenchyme
ANNELIDA	POLYCHAETA	Arenicola cristata	body wall
		Polyphysia crassa	body wall & blood vessels
		Scalibregma inflatum	body wall
		Nereis virens	body wall
		Aphrodite aculeata	gizzard
	OLIGOCHAETA	Lumbricus terrestris	body wall
	HIRUDINEA	Hirudo medicinalis	body wall
SIPUNCULIDA		Golfingia gouldi	body wall
ASCHELMINTHES	PRIAPULIDA	Priapulus caudatus	? body wall
MOLLUSCA	POLYPLACOPHORA	Cryptochiton species	visceral connective tissues
	GASTROPODA	Helix aspersa	artery
	BIVALVIA	Mya arenaria	aortic bulb
	CEPHALOPODA	Loligo pealei	cephalic artery
		Eledone cirrhosa	" "
ARTHROPODA	CRUSTACEA	Nephrops norvegicus	dorsal abdominal artery
	MEROSTOMATA	Limulus polyphemus	cephalic artery
ONYCOPHORA		Peripatoides novae-zelandiae	body wall
ECHINODERMATA	HOLOTHURIA	Leptosynapta tenuis	body wall
		Thyone briareus	intestinal haemal sinus
HEMICHODATA	ENTEROPNEUSTA	Saccoglossus kowaleswkii	proboscis

Amongst the hydrozoan polyps a system of crossed fibers parallel to and perpendicular to the oral-aboral axis in whole preparations of isolated mesoglea of *Hydra* has been described by Hausman and Burnett (1969). Their physical and histochemical study revealed the presence of elastin-like and collagen-like proteins and they concluded that the normal movements of *Hydra* could be accounted for by the presence of the elastin-like protein of the fibers. Electron micrographs revealed a fine fibrous network of 6–8 nm fibrils but, as in the present study with *Corymorpha,* the stouter fibers seen in the light micrographs were not found; Hausman and Burnett (1969) suggest that the latter are aggregations of the fine fibrils into fibers some 0.3 μm in diameter.

The divergent views of Bouillon and Vandermeerssche (1956), who suggested that the fibers of the medusan mesoglea were elastic, and those of Chapman (1953a, 1953b, 1959, 1966), who has presented convincing evidence of the presence of collagen in the mesoglea have been discussed by Elder and Owen (1967) who concluded that two distinct types of fiber were present, collagen and a type of invertebrate elastic fiber.

In previous theoretical considerations of the role of the scyphozoan mesoglea it has been assumed that the elasticity of the mesoglea serves as the antagonist for the sub-umbrellar circumferential muscles and that the radial connective tissue fibers transversing the mesoglea are stretched during contraction of the bell (Chapman, 1958, 1966). In a recent study of the swimming of medusae Gladfelter (1970) found that in many species the mesoglea does not form a mechanically homogeneous structure but has structural joints at which the bulk of bell deformation occurs. He also found that in a given medusa the amount of stretch in a region of the bell wall during contraction is inversely proportional to the number of fibers per unit area there and considers that the major role of mesogleal fibers is that of "maintaining the radial integrity of the bell during deformation." Therefore in the light of Gosline's (1971a and 1971b) work showing that the elastic properties of an anthozoan mesoglea reside in the matrix rather than in the fibrous connective tissue components, the question of whether the undoubted elasticity which the bell mesoglea possesses resides in the stout fibers or in the mesogleal matrix remains an open one.

Hyman (1940) noted the presence of a network of connective tissue fibers in the ctenophoran collenchyme and in volume V (Hyman, 1959) she supported the view of the origin of ctenophores from the trachyline hydrozoa. It is possible therefore that the spirit blue fibers observed in *Mnemiopsis* are similar to, and derived from, the type of fibers found in the hydromedusan mesoglea.

Attention has been drawn to the convergent development in *Peripatus* and *Polyphysia* of a thick collagenous dermal layer in the body wall with radially oriented elastic fibers (Elder and Owen, 1967). The extension which the latter undergo in *Polyphysia* when simultaneous circular and longitudinal muscle contraction occurs during the locomotor cycle has been estimated (Elder, in preparation). It is probably of significance therefore that, in its behavior, *Peripatus* is adapted to penetrating crevices. Manton (1961) has found that *Peripatus* can pass through a hole only one ninth the normal diameter of the body and describes how the animal passes the body through the aperture by sequential constriction of the segments. It seems probable that the elaborate dermal connective tissue will prove to have a function in freeing the underlying musculature from the restricting influence of the thick integument in a manner analagous to that in *Polyphysia*.

An even more significant convergence of dermal structure and burrowing behavior is found in the comparison of *Polyphysia* and *Leptosynapta*. In both, burrowing excavation is achieved primarily by the lateral scraping action of tentacles at the anterior end, the trunk forming a *point d'appui* which allows the head to press continuously forwards. Trunk progression is achieved by means of direct peristaltic waves with constrictions involving simultaneous circular and longitudinal muscle contraction; no terminal anchor is involved. A very flexible body wall structure appears to be a prerequisite of this type of locomtion involving the

simultaneous contraction of both muscle layers and is achieved in both animals by an elaborate three dimensional collagen lattice and a system of radially oriented fibers, probably elastic in properties. This method of burrowing, examples of which have not been described before, utilizes, and indeed would demand (Mettam, 1969), very low coelomic pressures at least in *Leptosynapta* (Hunter and Elder, 1967) and probably also in *Polyphysia* (Elder, 1973). In *Polyphysia* both the neuromuscular activity patterns and the connective tissue fiber architecture have probably evloved from sand burrowing ancestors as adaptations to burrowing in the soft mud habitat (Elder, unpublished). It is probably significant that, while most burrowing animals are closely adapted to a particular type of substratum (Yonge, 1949), *Leptosynapta tenuis* (= *Synapta inhaerens* see Smith, 1964) lives and burrows in sand, muddy sand or even in pure mud (Clark, 1901). It is suggested that this degree of freedom in choice of habitat is conferred by the unique burrowing mechanism. The degree of development of the dermal connective tissues and coelomic pressures employed by these lateral scraping excavators contrasts strongly with the slight development of the dermis and relatively high coelomic pressure recorded in terminal anchorage burrowers such as *Golfingia* and *Priapulus*.

The presence of radially and longitudinally oriented spirit blue staining fibers which are most probably elastic has been noted above in the body wall of *Golfingia gouldi*. Although spirit blue staining elements are present, radially oriented in the circular muscle layer of the body wall of *Priapulus* the case for their being elastic is much weaker. In another group of the aschelminthes, the Acanthocephala, the presence of radially oriented spirit blue positive fibers in the body wall of the trunk region has been noted by Hammond (1966) although no comment was offered as to their possible function. Nicholas and Mercer (1965) have presented electron micrographs of the radially oriented fibers in the trunk wall of an acanthocephalan, which reveal a strong resemblance to the ultrastructure of the radially oriented fibers in the dermis of *Polyphysia* (Elder and Owen, 1967 and Elder, 1972).

Robson (1957) described the existence of fluid filled spaces between the bases of the epithelial elements of the musculo-epithelial cells of *Metridium*. She stated that the function of this sub-epithelial fluid compartment was to enable the epithelium to follow rapid muscular contractions without delay owing to the hydrostatic action of the fluid in thrusting it outwards. Similar fluid filled spaces have been noted between the bases of the epithelial cells of *Leptosynapta* and *Microura*. It is probably of significance that, in common with *Metridium*, these animals have highly deformable body walls. Even in *Polyphysia*, in which the deformability of the body wall is probably greater than in most other cuticularized animals, subepithelial fluid spaces of this type are absent and the epithelium and cuticle conform to body wall deformation by folding rather than by increase in thickness (Elder 1972, 1973).

The finding of a thick and predominantly collagen-staining dermis in the holothurian *Thyone* agree with the results of Piez and Gross (1959) who examined the dermal connective tissue of *Thyone* by light and electron microscopy and performed amino acid analyses. In addition to the abundant collagen fibrils of 15–200 nm diameter and 65 nm periodicity these authors also figured and noted a second, unidentified type of "very thin 'beaded' fibril" (Piez and Gross, 1959,

page 27). It remains to be shown if aggregates of the beaded fibrils are responsible for the suggestion of spirit blue staining noted above and what properties and functions this second type of fiber have.

On the basis of staining characteristics and anatomical distribution elastic fibers have been described in molluscan tissues for more than a century (Leydig, 1854). On the basis of negative result with orcein Argaud (1908, 1909) and Jullien, Cardot, Ripplinger and Claudey (1956) concluded that elastic fibers were absent from the skin of cephalopods. But using Gomori's (1950) aldehyde fuchsin Jullien, Cardot and Ripplinger (1957, 1958) have reinvestigated previous descriptions and reversed their own previous conclusion. They particularly noted the similarity between the structure of cephalopod arteries, with respect to the concentric distribution of (presumed) elastic lamellae, which they figure, and the structure of vertebrate elastic arteries. Systolic pressures of up to some 50 mm Hg have been recorded in the cephalic artery of *Octopus* during activity (Johansen and Martin, 1962). Using Weigert's resorcin and orcein, Wetekamp (1915) described elastic fibers in a variety of tissues, including the blood vascular system of the large bivalve *Anodonta cellensis*. Ventricular systolic pressures of 3 mm Hg at the normal resting level and up to almost 8 mm Hg during active pedal probing have recently been recorded from *Anodonta* (Brand, 1972). In bivalves, such as *Mya*, with well developed posterior aortic bulbs periodic pressures greater than these may occur if, as Brand (1972) suggests, blood is forced back into the bulb during siphon retraction.

Amongst the arthropods literature contains occasional reference to the identification of elastic fibers, usually on the basis of staining techniques. Thus Boissezon (1930) records elastic fibers associated with the anterior intestine of insect *Culex* and Nutting (1951) figures elastic fibers in the dorsal diaphragm of orthopterans. Mabillot (1954, 1955) reports the presence of elastic fibers in the digestive tract of the crustacean *Gammarus* and Barnes and Gonor (1958) found a loose meshwork of elastic fibers associated with the nerve cord of the cirripede *Pollicipes*. Lacombe (1970) believes that elastic fibers are involved in cement extrusion from the cement glands of some large balanid barnacles. And in the Xiphosura elastic ligaments suspending the heart and elastic fibers in the anterior pericardial wall have been described by Krumbach (1935). In the present investigation arteries in the Norway lobster *Nephrops norvegicus* and the king crab *Limulus polyphemus* were found to have spirit blue staining fibers. Systolic pressures of 9–20 mm Hg have been recorded from the posterior dorsal aorta of *Homarus americanus* (Scheer, 1963). It seems possible that the spirit blue staining lamellae have elastic properties but further studies on the circulatory dynamics of these invertebrates are required.

Amongst mammalian tissue fiber types it is clear that, on the basis of the staining properties described above and in section 1, the invertebrate elastic fibers most clearly resemble the pre-elastic and oxytalan fibers (Fullmer and Lillie, 1958; Fullmer, 1960). Argaud (1908, 1909) had previously reached a similar conclusion. He noted that the molluscan fibers had the physical characteristics of vertebrate elastic fibers but would not take elastin stains and in these respects resembled the first formed fibers in young human embryos. Ultrastructural studies have revealed further similarities between the invertebrate fibers and the oxytalan fiber

(Carmichael and Fullmer, 1966) and "elastin" precursors (Ross and Bornstein, 1969, 1970, 1971). All three fiber types are comprised of beaded filaments which stain densely with the cationic stains, uranyl acetate and lead citrate. In these respects they differ markedly from the "amorphous component" seen in ultra-thin sections of mature mammalian elastic fibers which stains with anionic stains such as phosphotungstic acid but remains unstained after application of the lead and uranium salts. There appears to be variation in the dimensions of the beaded filamentous elements of both the invertebrate fibers (Elder, 1966b) and the mammalian elastic fiber precursors (Ross and Bornstein, 1969).

The mechanism of the staining reaction between spirit blue and the invertebrate fibers remains unknown. Of a variety of stains tested (Elder and Owen, 1967) it most closely resembles aldehyde fuchsin in its staining properties. Fullmer (1965) comments that mammalian elastic fibers have a remarkable affinity for phenols, napthols and ferric salts and it is significant that all the common elastic stains (except aldehyde fuchsin) contain phenols. Aldehyde fuchsin, formed when paraldehyde reacts with basic fuchsin (Gomori, 1950) probably contains aromatic amines which may react in a way similar to the phenols of other elastic stains (Fullmer, 1965). The fact that alcohol soluble aniline blue (spirit blue) also contains aromatic amine groups (Gurr, 1962) is probably therefore of significance in the staining reactions of the spirit blue technique.

While the above may be of value in attempting to explain the ability of spirit blue to act as a selective stain for vertebrate elastic fibers it does not explain the requirement of the invertebrate fibers for oxidation before a positive reaction can be obtained with spirit blue or aldehyde fuchsin. The oxytalan fibers of the human periodontal membrane will similarly give positive staining with aldehyde fuchsin only after oxidation (Fullmer and Lillie, 1958), and there is strong evidence that these staining properties of the oxytalan fibers are due to conjugated mucopolysaccharide (Fullmer, 1965).

As discussed above some of the invertebrate connective tissue elements which stain strongly by the spirit blue method may have no physical elasticity or chemical affinity with elastic proteins and may stain only by virtue of associated glycosaminoglycans. Even amongst those fibers for which there is some indirect evidence of physical elasticity from anatomical considerations there appear to be several morphological types. Thus the annelids and molluscs have fibers composed of bundles of fine beaded filaments while in the coelenterates and arthropods the fibers are composed of compound fibrils each of which consists of aggregates of fine filaments apparently crosslinked. These structural differences may reflect differences in chemical composition and mechanical properties.

The mechanical properties of the vertebrate elastin precursor fiber do not appear to have been studied in detail. Amino acid analysis of purified samples have shown that, in contrast to the mature "amorphous" elastin, the fibrillar component is a glycoprotein rich in polar amino acids (Ross and Bornstein, 1969, 1970). Therefore the molecular organization and elastic properties of the precursor filaments (if indeed they are elastic) cannot be due to the type of two phase model involving interlinked hydrophobic globules in an aqueous phase, proposed by Partridge (1968, 1970) or the "liquid drop elastomer" of Weis-Fogh and Andersen (1970). An amino acid analysis of purified spirit blue staining fibers would be

of great interest and relevance although the presence of numerous polar groups need not deny rubber-like properties, as Bailey and Weis-Fogh (1961) and Weis-Fogh (1965) have shown for the protein resilin from arthropod cuticle. In ultra-thin sections vertebrate elastin usually appears amorphous (Cox and Little, 1961; Ross and Bornstein, 1969). Partridge (1968) has recently presented micrographs which reveal a network of globular particles in a three dimensional array and considers that the beaded fibrillar apparance of some negatively stained elastin preparations (Gotte, Meneghelli and Castellani, 1965; Gotte, Mammi and Pezzin, 1968) has relevance only to a super stretched condition. Weis-Fogh and Andersen (1970; page 721) however point out that "one advantage of a liquid drop elastomer is that one can imagine the existence of elastic fibrils of much smaller diameter than is possible for rubberlike elastomers, because a single linear row of interlinked globular molecules would show an elastic behavior which is similar to that of a larger aggregate of globules." Further mechanical, ultrastructural and chemical studies of the invertebrate fibers described above are clearly required before many of the questions raised above can be answered.

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Summary

1. A microscopical survey of some forty species from twelve invertebrate phyla confirmed the presence of collagen in all groups.

2. A second group of connective tissue fibers, distinguishable from collagen by permanganate/spirit blue staining and which includes invertebrate elastic fibers was found in all groups except the anthozoan coelenterates and the turbellarians.

3. Spirit blue positive fibers were variously found in the dermis, around nerves, amongst muscles, in blood vessels and epithelial basement membranes and traversing the mesoglea of several coelenterate types. It is probable that further work will confirm the physical elasticity of many of these fibers.

4. Anatomically the "spirit blue fibers" are oriented to antagonize muscles in the medusae, accommodate fluid pressures in vascular systems and oppose tissue deformation in many soft-bodied animals.

5. Specifically, in the latter category, radially oriented dermal "spirit blue fibers" oppose the radial distension of the body wall during the simultaneous circular and longitudinal muscle contraction which occurs in the passage of direct peristaltic waves in the burrowing holothurian *Leptosynapta tenuis*.

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CLONE SPECIFIC SEGREGATION IN THE SEA ANEMONE *ANTHOPLEURA ELEGANTISSIMA*

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The west coast sea anemone *Anthopleura elegantissima* commonly lives intertidally in dense beds. These beds are frequently divided into two or more separate aggregations by anemone-free zones (Fig. 1). These zones may be irregular and rather unremarkable areas occupied by limpets, chitons, snails, barnacles and algae. However, under crowded conditions on inclined surfaces that face toward the sea, these zones are often conspicuous, long, narrow, relatively bare strips anywhere from a fraction of a centimeter to about 5 cm wide (Fig. 1). I have also observed that the position of these zones is relatively constant over periods of up to four years. Whether they are wide or narrow, regular or irregular, these zones separating adjacent aggregations of anemones are apparent to the careful observer because the distance across the zones is obviously greater than the distance between anemones in the midst of the aggregations.

There are also short anemone-free pockets penetrating single aggregations of the anemones. Some of these clearings may be made by the chiton *Mopalia muscosa*, which Field has found to be capable of maintaining and extending artificially created clearings within aggregations of the anemones (Field, Department of Biology, University of California, Santa Barbara, personal communication in 1969), or by the turban snail *Tegula funebralis* which I have observed living in such pockets.

The subject of this paper and associated work (Francis, 1973) is the nature of the relatively permanent anemone-free zones separating adjacent aggregations of the sea anemone *Anthopleura elegantissima* and the relation of these zones to the distribution and behavior of the anemone.

Materials and Methods

Animals and collecting methods

The anemones used in the experiments were all individuals of the aggregating form of *Anthopleura elegantissima* collected intertidally within ten miles of the Hopkins Marine Station at Pacific Grove, California. I removed them from the very large rocks on which they were found by gently working a thin spatula under the edges of the pedal discs.

Laboratory holding conditions

At Hopkins Marine Station the anemones were kept in glass finger-bowls supplied with flowing sea water at about 13° C. The anemones were fed intermittently except during experiments, when food was withheld. Anemones to be used in be-

havioral experiments were always kept in the laboratory for a few weeks to allow them time to recover from minor damage inflicted during collecting, and to shed the debris that was usually pulled loose from the rocks by the clinging pedal discs.

Only animals that appeared healthy and that attached to the holding bowls by their pedal discs were subsequently used in behavioral experiments. To remove anemones from a holding bowl, I hit the bowl firmly and repeatedly against a hard surface until the anemones released their footholds. Anemones treated in this way appeared to suffer no damage, and they settled in the experimental chambers much more quickly than did anemones that had been forcefully pried loose.

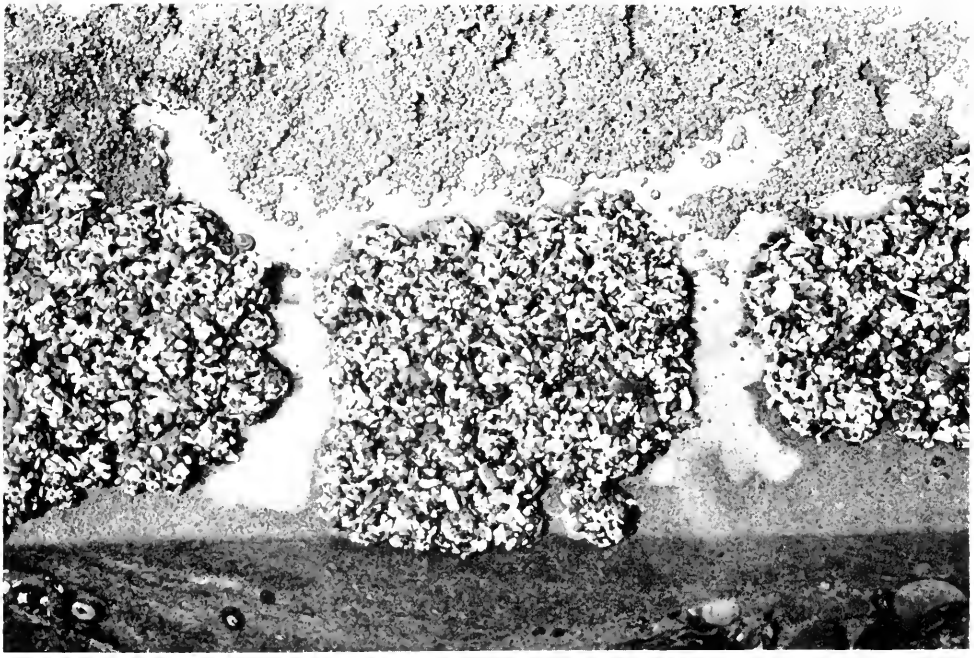

FIGURE 1. A conspicuous anemone-free strip separating adjacent groups (clones) of anemones (intertidal rocks, Arroho Honda, near Baviota, California, December 1970).

Sex determination

The freshly collected anemones were cut in half lengthwise or in the case of larger specimens, in quarters; and the mesenteries were examined for the presence of gonads. During the summer months when the gonads are ripe (Ford, 1964), female gonads are obvious as brownish-pink bodies. Because the yellowish-white male gonads are less easily identifiable, several mesenteries from each non-female anemone were examined microscopically for the presence of spermatocytes. Occasional checks were also made for the presence of oocytes in the smaller female gonads. Anemones having at least one identifiable gonad containing oocytes or spermatocytes were scored as reproductively mature.

Dry weight determination

After sex determination was accomplished, the anemones were blotted using a piece of tissue paper and weighed to ± 0.01 g. The animals were then individually dried to constant weight in a drying oven at between 75° and 91° C (18 to 24 hours were usually required).

Procedures and Results

Clonal nature of the aggregations

The anemones living in aggregations separated by anemone-free strips were examined closely in order to determine whether those living on opposite sides of the strips differed from each other in any discernible way.

Anthopleura elegantissima shows a variety of color patterning which allows for considerable variation among individuals. For example, a pink pigment is present in the tentacle tips of some but not nearly all individuals. Further, the mesenterial insertion line on the oral disc may be marked with pigment ranging from red-brown to purple in color, and the oral disc may or may not be marked with a radiating white or gold pattern. All of the anemones belonging to a continuous aggregation were observed to have the same color patterning. If more than one color pattern was observed in a bed of anemones on a single rock, the bed was always found to be divided by anemone-free spaces into aggregations of anemones that were segregated by color pattern.

The sexes are separate in this anemone, and samples were taken July 13, 1968 to determine the distribution of the sexes with respect to the anemone-free zones. Samples of at least nine anemones were collected from each of ten contiguous aggregations, each of which was separated from at least one adjacent aggregation by an anemone-free zone. Care was taken in collecting the anemones to select animals from widely separated parts of each aggregation and to include animals from the edges as well as animals from the middle of each aggregation. The results (Table I) show no mixing of the sexes within a single contiguous aggregation. In the single case in which male and female anemones were found living on the same rock, they were found in two unisexual groups separated by an anemone-free strip. In the experiment described below, 99 anemones (a significant fraction of the aggregation) from a single aggregation were collected and examined for the presence of gonads. Of these 21 had female gonads and 78 had no gonads. There were none with male gonads.

Since it is known that these anemones reproduce asexually by longitudinal fission (Hand, 1955), it is apparent that each contiguous aggregation of the sea anemone *Anthopleura elegantissima* is a single clone and that the anemone-free zones described above separate adjacent clones of the anemones. No other hypothesis can simply explain the observed segregation into unisexual groups in which all the group members have the same color patterning.

Size and sexual maturity

It may be noted that some anemones from each clonal group lacked gonads at what is usually the peak period for the gonad index (Ford, 1964).

TABLE I

Distribution of males and females on rocks bearing adjacent aggregations of anemones separated by anemone-free zones

Identifying no. for each aggregation	No. of developed males	No. with sex undeveloped	No. of developed females	Comparison of sex between adjacent aggregations
100	—	8	2	♀/♀
101	—	8	2	
102	—	6	4	♀/♀
103	—	9	1	
104	—	6	3	♀/♀
105	—	8	2	
106	—	2	8	♀/♀
107	7	2	—	♀/♂
108	10	8	—	♂/♂
109	8	2	—	

Both Ford's work and my own casual observation suggested a correlation with the size of the animals. To test this idea, I collected all the anemones (99 anemones) from one section of a female clone on July 29, 1968, and after assaying the reproductive state, I determined the dry weights for each anemone. The results (Table II) show that within this clone, animals smaller than 0.6 g dry weight were not reproductive. The pooled data from smaller samples from 12 different clones, both male and female, collected between July 13 and August 18, 1968, show that none of the individuals below 0.2 g dry weight were reproductive (Table II).

From this it would appear that reporductive state is related to size in these anemones, even among genetically identical individuals, the smallest individuals

TABLE II

Gonad maturity and dry body weight

Whole body dry weight (g)	99 anemones from a single female clone		94 anemones from 12 separate clones both male and female	
	No. of individuals with developed gonads	No. of individuals without developed gonads	No. of individuals with developed gonads	No. of individuals without developed gonads
0.0–0.2	0	16	0	10
0.2–0.4	0	18	4	19
0.4–0.6	0	20	3	10
0.6–0.8	3	10	12	7
0.8–1.0	2	7	4	0
1.0–1.2	8	1	8	2
1.2–1.4	2	2	5	0
1.4–1.6	1	2	5	0
1.6–1.8	1	1	0	0
1.8+	4	1	5	0

being non-reproductive while some proportion of those over 0.2 g dry weight are reproductively mature.

Clonal segregation and separation in the laboratory

Reaggregation into uniclonal groups. An experiment was conducted in order to determine whether or not the observed segregation into clonal groups is a result of an active process carried on by the anemones.

Anemones from two clones (Clone 1 and Clone 2) living adjacent to each other in the field were collected and brought into the laboratory where they were kept with their clonemates in bowls supplied with running sea water.

Ten small to medium sized anemones (from about 0.5 to about 2.3 cm across the expanded oral disc) from each of the two clones were pinned with insect pins to a foam plastic ball about 5 cm in diameter. They were crowded together as closely as possible in five rows of four animals, the individuals from the two clones being arranged alternately in both the horizontal and vertical rows to cover more than half the surface of the ball. This arrangement maximized contact between anemones and allowed contact between both clonemates and non-clonemates. The buoyant ball was fastened to a lead weight using a piece of monofilament line and placed in an aquarium where the ball was held below the surface of the water by the weight. Running sea water at 13° C was continuously supplied to the aquarium. In three days when all the anemones seemed to be adhering to the ball, the pins were removed to allow the anemones to move freely.

After 13 days of free movement, four anemones had fallen off the ball and the remaining 16 anemones had rearranged themselves into two segregated clonal groups. There was at that time no contact between the tentacles of non-clonemates. The anemones continued to move about after 13 days and some further reassortment occurred, but it remained apparent that contact was maintained only between clonemates.

It is apparent from this that clonal segregation within aggregations can be actively accomplished by the anemones.

Formation of an anemone-free trail in the laboratory. The following experiment was designed to determine whether or not the anemones will form anemone-free zones between adjacent clonal groups in the absence of naturally associated species and various environmental factors such as tidal cycle.

The anemones used in the experiment were collected from two different but not adjacent clones (Clone 3 and Clone 4). These animals were maintained in the laboratory for periods varying from several months to a year prior to this experiment. A pyrex baking dish approximately 36 cm by 18 cm and 5 cm deep was lined with a sheet of foam plastic glued to the bottom. One end of the sheet was entirely covered by Clone 3 anemones pinned side by side with insect pins, and the other end was covered with Clone 4 anemones. Where the two clones met at an uneven interface, a barrier of microscope slides fastened together with tape was set on edge between the two clones. The uneveness of the interface served to maximize the area of contact between the clones. Running sea water was introduced into the dish at one end; and because the anemones tend to move upstream (Buchsbaum, 1968), the position of the water inlet was changed periodically to prevent a consistent unidirectional migration in response to current. After a few

days when the pedal discs of the anemones had become attached to the substratum, the pins attaching the anemones to the foam plastic sheet were removed as was the barrier between the clones. The animals that released their footholds on the substratum during the rest of the experiment were removed from the dish on the assumption that such animals would have been removed by wave action under field conditions.

Photographs were taken from a fixed position above the dish once a day. The position of the anemones at the beginning of the experiment just after the barrier

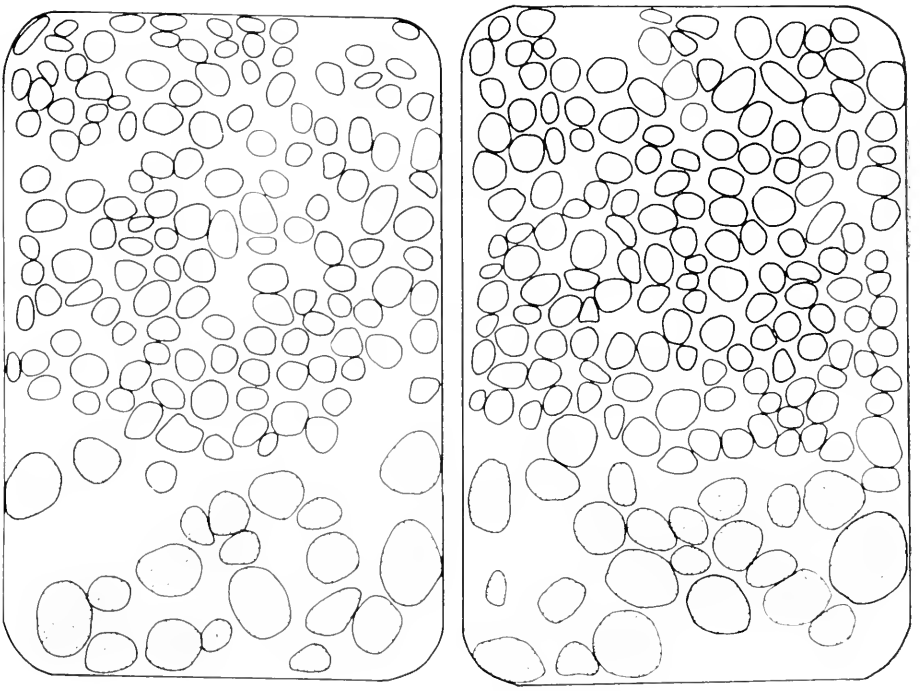

FIGURE 2. The position of anemones from Clone 3 and Clone 4 just after the barrier between the two groups was removed (right) and 14 days after the barrier was removed (left), (see text for details).

was removed and their position 16 days later are shown in Figure 2. The outlines of the oral discs exclusive of tentacles are shown here, and the outlines of the anemones from Clone 3 are shown stippled.

Under these conditions an anemone-free zone was formed between the two clonal groups within three weeks (Fig. 2, left). During this time numerous aggressive episodes (Francis, 1973) were observed at the border between the two groups. (This was not observed during the previous experiment, which was conducted before I discovered the aggressive behavior of this animal.) Apparently then, clonal isolation as well as clonal segregation be actively accomplished by the anemones.

Discussion

The anemones in a single continuous aggregation of the sea anemone *Anthopleura elegantissima* are observed to resemble each other in two obvious ways: (1) all have the same color pattern, and (2) all individuals having developed gonads are of the same sex. Furthermore, while sustained contact between individuals within an aggregation is very common, contact between individuals from different aggregations has been found to initiate an intraspecific aggressive response resulting in the separation of these individuals (Francis, 1973). These animals are known to reproduce asexually by longitudinal fission, and is therefore concluded that the aggregations must each be comprised of anemones from a single clone. It seems to me improbable that anything other than genetic identity could account for aggregations of up to thousands of individuals of the same sex, having the same color patterns and showing mutual tolerance for contact with each other and for no other members of the species.

What might be the advantage to the anemones of living in segregated aggregations? There are a number of possible advantages to a small anemone in living adjacent to other members of the species rather than living alone. (1) Contact between anemones decreases their effective surface areas. This reduces water loss during low tides (Roberts, 1941) and minimizes the area exposed to the pulling and battering effects of the waves and the abrasive effects of suspended matter. (2) An area of rock closely covered by a sheet of anemones provides no place for the settlement of other sessile organisms (such as the larger algae) which may compete with the anemones for space. (3) A single small anemone would have little chance of catching and holding large organisms against the force of the waves; however, I have often seen several anemones in an aggregation together holding and ingesting a large jellyfish or squid.

Anemones of the solitary form of this species are usually larger than individuals of the clonal form. These animals live in more protected circumstances than individuals of the clonal form, usually in nooks and crevices, and often with their bases attached to rocks below the sand level (Hand, 1955). That these larger animals should be found in a more protected habitat than their smaller conspecifics fits well with the preceding analysis. Increased size would make these anemones more vulnerable to the pulling and battering of the waves and therefore less able to occupy flat exposed rock surfaces. At the same time, decrease in the surface to volume ratio of the solitary as compared with the clonal form decreases water loss by evaporation, thus reducing this problem of the solitary living habit. I have observed (as the name of the form itself suggests) that individuals of the solitary form of *Anthopleura elegantissima* also remain isolated from adjacent members of the species.

The advantage to the anemones of segregation into strictly uniclonal groups and separation from adjacent clonal groups is not immediately apparent (see Francis, 1973). However, segregation among genetically different animals of the same species is not peculiar to the species or to the phylum. The growth patterns reported for some colonial coelenterates, tunicates and bryozoans seem to be of the same type.

Schijfsma (1939) reports that young colonies of the hydroid *Hydractinia echinata* seem to fuse completely but that, "When they meet at an older (perhaps

different) age a remarkable zone of demarcation is formed," (p. 102). He also notes that when the borders of the same colony meet, for example in growing around a shell, no such line of demarcation is formed.

Theodor (1966) reports a similar phenomenon for the gorgonian *Eunicella stricta*. Two specimens were found growing very close together; and although the bases of the animals were in close contact, the tissues remained unfused. In the laboratory he found that homografts (grafts between genetically different individuals of the same species) always failed to show tissue fusion. Autografts (grafts involving tissue from only one individual) were always successful; and the fused tissues in the graft area appeared normal histologically.

Workers have also observed complete fusion at the interface between separate growing edges of the same colony both in the encrusting ascidian *Botrylus* and in a variety of bryozoa. Knight-Jones and Moyse (1961), referring to these observations, report that, "if two clonies of the same species (of encrusting ascidian or bryozoan) meet, each seems to respect the other's well-marked frontier and spreads only in other directions," (page 88).

What ties these phenomena together with the clone specific segregation and separation reported here for *Anthopleura elegantissima* is the contrast between the intimacy of association among genetically identical "individuals" in colonies or clonal groups, and the relative isolation between genetically different individuals of the same species. All of these animals apparently respond differently to contact with a genetically identical conspecific than to contact with other conspecifics.

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Summary

The anemones in a single continuous aggregation of the sea anemone *Anthopleura elegantissima* are observed to resemble each other in two obvious ways: (1) all have the same color pattern, and (2) all individuals having developed gonads are of the same sex. Furthermore, while sustained contact between individuals within an aggregation is very common, contact between individuals from different aggregations has been found to initiate an intraspecific aggressive response resulting in the separation of these individuals (Francis, 1973). These animals are known to reproduce asexually by longitudinal fission, and it is therefore concluded that the aggregations must each be comprised of anemones from a single clone. It seems to me improbable that anything other than genetic identity could account for aggregations of up to thousands of individuals of the same sex, having the same color patterns and showing mutual tolerance for contact with each other and for no other members of the species.

Even among anemones within a single clone, the presence of gonads containing gametes is shown to be related to size. Anemones smaller than 0.2 g dry wt consistently lack developed gonads, while some proportion of those over 0.2 g have gonads with gametes.

In the field, adjacent clonal groups are observed to remain separated from each other. In the laboratory a mixed group of anemones will reaggregate into isolated uniclonal groups, implying that clone specific segregation is actively accomplished by the anemones.

Living in aggregations has a number of potential advantages for the anemones such as reducing their effective surface area and thereby reducing water loss and the battering effects of wave action, excluding interspecific competition for space by promoting effective blanketing of an area, and allowing for cooperation in the capture and holding of larger prey. The function for the anemones of clone specific segregation and separation is not yet clear (see Francis, 1973), however the phenomenon is not without parallel since some other coelenterates as well as some bryozoans have also been observed to respond differently to contact with a genetically identical conspecific than to contact with other conspecifics.

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INTRASPECIFIC AGGRESSION AND ITS EFFECT ON THE DISTRIBUTION OF *ANTHOPLEURA ELEGANTISSIMA* AND SOME RELATED SEA ANEMONES

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Contiguous aggregations of the west coast sea anemone *Anthopleura elegantissima* are composed of individuals from a single clone, the products of asexual reproduction. In the field, adjacent clones of anemones are observed to be isolated from each other by anemone-free spaces; and in the laboratory a group of anemones of mixed clonal origins will reaggregate into isolated uniclonal groups. (Francis, 1973) . In the field I have also observed that individuals of the large solitary form of *Anthopleura elegantissima* also remain isolated from adjacent members of the species. Contacts between genetically different individuals of this species initiates in one or both individuals an elaborate behavior pattern that usually results in damage to one or both animals. This is aggressive behavior according to the definition of Carthy and Ebling (1964), "An animal acts aggressively when it inflicts, attempts to inflict, or threatens to inflict damage on another animal. The act is accompanied by recognizable behavioral symptoms and definable physiological changes" (page 1). This behavior has never before been reported for this species, and my purpose here is to describe this specialized aggressive behavior and to investigate the relationship between the aggressive behavior of this and related species and the observed field distributions.

Materials and Methods

Animals and collecting methods

In synthesizing a description of the aggressive response, I observed individuals of both the solitary and the aggregating form of the anemone *Anthopleura elegantissima* (most recently described by Hand, 1955). The animals were observed and/or collected intertidally on the California coast between Pacific Grove and Santa Barbara, California.

The specimens of *Anthopleura elegantissima* used in the experiments were small to large sized individuals (from 1 to 5 cm across the expanded oral disc) of the aggregating form and large individuals (from about 5 to about 8 cm across the oral disc) of the solitary form of the anemone. These animals were collected intertidally within ten miles of the Hopkins Marine Station at Pacific Grove, California and between Gaviota and Santa Barbara, California. The very small sub-adult anemones used in one set of experiments were collected at Arroyo Hondo, an area just south of Gaviota. These animals were collected in October of 1971 in a mussel bed where they were attached to mussels and interspersed pebbles. The specimens of *Anthopleura artemisia* and *Anthopleura xanthogrammica* were also collected intertidally near Hopkins Marine Station. Specimens of *Actinea equina* used in

experiments were collected near the Gatty Marine Laboratory, St. Andrews, Scotland and near the Plymouth Laboratory, Plymouth, England. These anemones were removed from the rocks on which they were found by gently working a thin spatula under the edges of the pedal discs.

Laboratory holding conditions

At the four marine laboratories at which this work was done (Hopkins, Santa Barbara, the Gatty, and Plymouth) the anemones were kept in bowls supplied with flowing sea water. The anemones were fed intermittently except during experiments, when food was withheld.

Conditions under which aggression was observed

Contact between two genetically different anemones, not mere proximity, appears to be the condition necessary for the initiation of the aggressive response.

In my early experiments anemones collected from different clones were brought into contact in the laboratory by first allowing some of the animals to attach to pieces of glass or stone, and then moving these portable animals to within contact distance of other anemones. More recently the anemones were stimulated to initiate the aggressive response by repeatedly touching the experimental animal on the tip of a tentacle with a tentacle excised from a non-clonemate. This was done in a manner designed to simulate natural contacts between anemones. Contacts between anemones and excised tentacles were as brief as possible (lasting less than a second), and sufficient time (2–3 minutes) was allowed for the retracted tentacles of the experimental animal to re-extend between successive contacts. A single set of repeated contacts was always restricted to the tentacles of one quarter section of the anemone. Stimulation was discontinued when the acrorhagi of the anemone began to inflate rapidly, signaling the beginning of the inflation stage of the aggressive behavior. If the behavior did not then proceed to completion, additional stimulations were given. The total number of stimulations needed to elicit a complete aggressive response (including the movement of application) is designated the aggressive threshold. Stimulation was discontinued if the reaction was not elicited within a preset number of contacts.

An excised tentacle was used for up to ten successive contacts with the same anemone, and the tentacle was kept in cool sea water between contacts. (I found for *Anthopleura elegantissima* that tentacles used for many more than ten intraspecific contacts and even those rubbed repeatedly and with considerable force among the tentacles and against the column of a non-clonemate were still completely effective at eliciting aggression upon contact with any non-clonemate. This was true as long as the surface of the tentacles was kept free of any superficial clinging material.)

Procedures, Observations and Results

Anthopleura elegantissima

Description of the aggressive behavior. Although, as a matter of convenience, tentacles excised from another anemone were used to stimulate anemones during

most laboratory experiments, it was found that contact with any of the external surfaces of a non-clonemate including the column, uninflated acrorhagi, or the intact surface of the pedal disc elicited aggression in laboratory maintained anemones.

I have also observed the behavior occurring naturally in tidepools during daytime low tides, especially as the tide begins to rise, bringing cool aerated water into the stagnant pools and submerging anemones previously exposed to the air. Aggression is common among these expanded anemones as they are jostled together by the movement of the water.

The following description of the aggressive response of *Anthopleura elegantissima* is, then, a composite description based on my numerous observations of the behavior rather than a description of a single episode.

For convenience I have separated the aggressive behavior of *Anthopleura elegantissima* into five stages: (1) stimulation (or initiation), during which the tentacles of the two anemones repeatedly come into contact and withdraw from contact; (2) inflation, during which the acrorhagi commence to become turgid; and (3) the movement of application, during which the inflated acrorhagi are pushed toward the source of stimulation; (4) application of ectoderm, during which damage is inflicted on another anemone; and (5) recovery, during which the anemone returns to its normal posture. The various stages, which correspond roughly with those described by Bonnin (1964) for the aggressive behavior of *Actinea equina*, are illustrated in Figures 1 through 7 and are described in full below.

When an expanded anemone is moved toward and first makes contact with another expanded anemone, the tentacle tips are usually the first parts to make contact. Typically, if two clonemates meet in this way their tentacles will at first withdraw from the contact and will then re-extend. This may happen several times in succession until finally the tentacles cease withdrawing and remain more or less in contact. If the anemones then move toward each other, their tentacles interlace without apparent further interaction.

If anemones from different clones come within contact range of each other, the reaction is very different. Tentacle contact between anemones from different clones is followed by rapid withdrawal of those tentacles involved and often of some nearby tentacles as well. The retracted tentacles then slowly re-extend until contact is again made between the two anemones. The tentacles withdraw again from contact, and the process of contact and withdrawal is repeated until the next stage of the behavior begins. During the period of repeated tentacle contact the acrorhagi (also called marginal spherules) closest to those tentacles involved in the contacts often become visible as white (or rarely pinkish) spherules beneath the outer cycle of tentacles (Fig. 1c). In unaroused anemones the acrorhagi, which are more or less numerous depending on the individual, are often difficult to see as they are usually quite small and lie hidden beneath the tentacles and in the fosse (a channel between the collar of the column and the tentacles).

The inflation stage of the aggressive behavior begins as the tentacles of the aggressor withdraw from the area of stimulation after repeated contact with a non-clonemate. As the tentacles begin to re-extend after the last contact, the anemone often assumes a more upright posture (Fig. 3). This may be accomplished by contraction of the circular muscles of the column and/or relaxation of

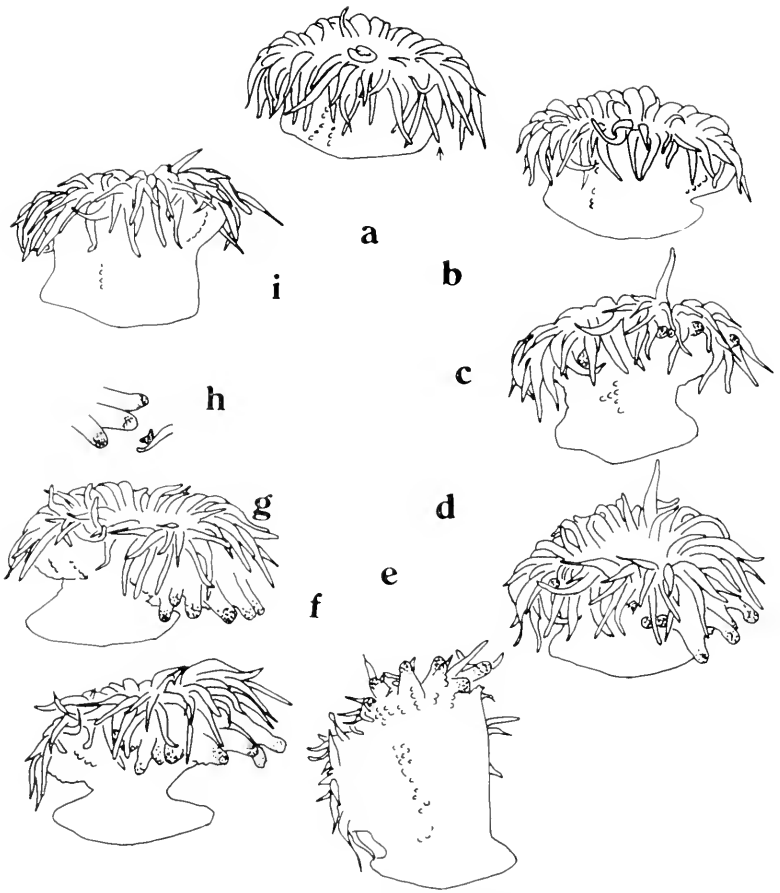

FIGURE 1. The positions of a specimen of *Anthopleura elegantissima* (the aggregating form) were traced from photographs taken during one aggressive episode. (a) The anemone is shown at rest (before contact with the non-clonemate); the arrow indicates the region that will come into contact with the non-clonemate. (b) Initiation: the anemone is shown just after contact with the non-clonemate; the relative retraction of the tentacles and a slight swelling of the capitulum (to the right) may be noted. (c) Inflation: the white tips (stippled) of the inflating acrorhagi have become visible in the area adjacent to the tentacles receiving the stimulation; the shape of the column is notably different from that shown in Figure 1a. (d) Inflation: the acrorhagi in the area of stimulation are strongly inflated, appearing white at the tips (stippled) and transparent at the bases. (e) Movement of application: the inflated acrorhagi are drawn upward and back away from the source of the stimulus. (f) Movement of application: the acrorhagi are sweeping downward and may, as in this case, continue downward to the position shown in Figure 1g, or the movement may stop in the position shown here with the anemone leaning over the adjacent non-clonemate. (g) Movement of application: the acrorhagi have reached the bottom of their downward sweep and are being wiped against the body of the victim. (h) Release of ectoderm: acrorhagial ectoderm has been released from one of the acrorhagi which is now transparent (unstippled) at the tip where the ectoderm is missing; the ectoderm is shown clinging to a tentacle of the victim. (i) Recovery: the acrorhagi have deflated but the anemone has not yet fully recovered its preaggression posture; the column is still somewhat elongated and the capitulum is still swollen and somewhat elevated in the area proximal to the area of stimulation.

the longitudinal muscles. There is usually also some swelling of the capitulum at the base of the stimulated tentacles, perhaps the result of relaxation of the circular muscles just below the stimulated tentacles and contraction of those muscles below the remaining tentacles. At this time the acrorhagi associated with the stimulated tentacles become distended, changing in appearance from shriveled white spherules to transparent rounded cones with white tips (Fig. 1d). The gastrovascular cavity of these anemones extends into the hollow tentacles and acrorhagi, and it is possible that the contractions of the circular muscles described above serve to force water from the columnar portion of the gastrovascular cavity into these acrorhagi, causing them to become distended.

Sometimes there are, in addition to the above movements, peristaltic contractions that begin at the base of the elongated anemone and travel up the column. These muscular contractions (probably of the circular muscles) may serve to force water into the expanding acrorhagi.

Along with an increase in the turgidity of the involved acrorhagi during the inflation period, there is usually a progressive increase in the number of acrorhagi that are inflated. Recruitment progresses in both directions around the capitulum so that more and more acrorhagi on either side of the originally stimulated tentacles become involved. Typically four to twelve acrorhagi become fully turgid during a single aggressive episode.

Acrorhagial inflation has never before been reported for this species; and except in rare, unexplained cases full inflation of the acrorhagi seems to occur only during the aggressive response.

The movement of application is a rather rapid movement for an anemone, requiring between 30 and 120 seconds for the complete movement.

As the expanding acrorhagi begin to distend, the swollen edge of the capitulum is extended upward drawing the turgid acrorhagi upward and away from the original site of contact with the other anemone (Fig. 4). This movement probably involves elongation of the longitudinal columnar muscles adjacent to the area of acrorhagial inflation. The anemone may remain in this position for a few seconds. Then the area of the oral disc between the mouth and the stimulated tentacles begins to elongate. This might be effected by relaxation of radially arranged muscles of the oral disc in this region and contraction of other muscles of the column and oral disc which would cause additional gastrovascular fluid to be forced into the area. As the oral disc changes shape the swollen edge of the capitulum moves downward, and the expanded acrorhagi point outward or somewhat downward (Fig. 5). This motion, which is perceived as a smooth downward sweep of the expanded acrorhagi, sometimes stops as the acrorhagi reach the position shown in Figure 1f (the aggressor is leaning over the spot where the victim normally should be). More often, the downsweeping movement continues until the acrorhagi are on a level with the pedal disc (Fig. 6) or actually touching the substratum. During this time inflation of the acrorhagi often continues, and more acrorhagi at both edges of the inflated area continue to be recruited.

The full movement of application sequence may be repeated one or more times, or the anemone may slowly recover its normal posture after only one movement of application.

If during the downward motion of the movement of application the white tip of one or more of the aggressor's fully inflated acrorhagi (*i.e.*, acrorhagi so turgid

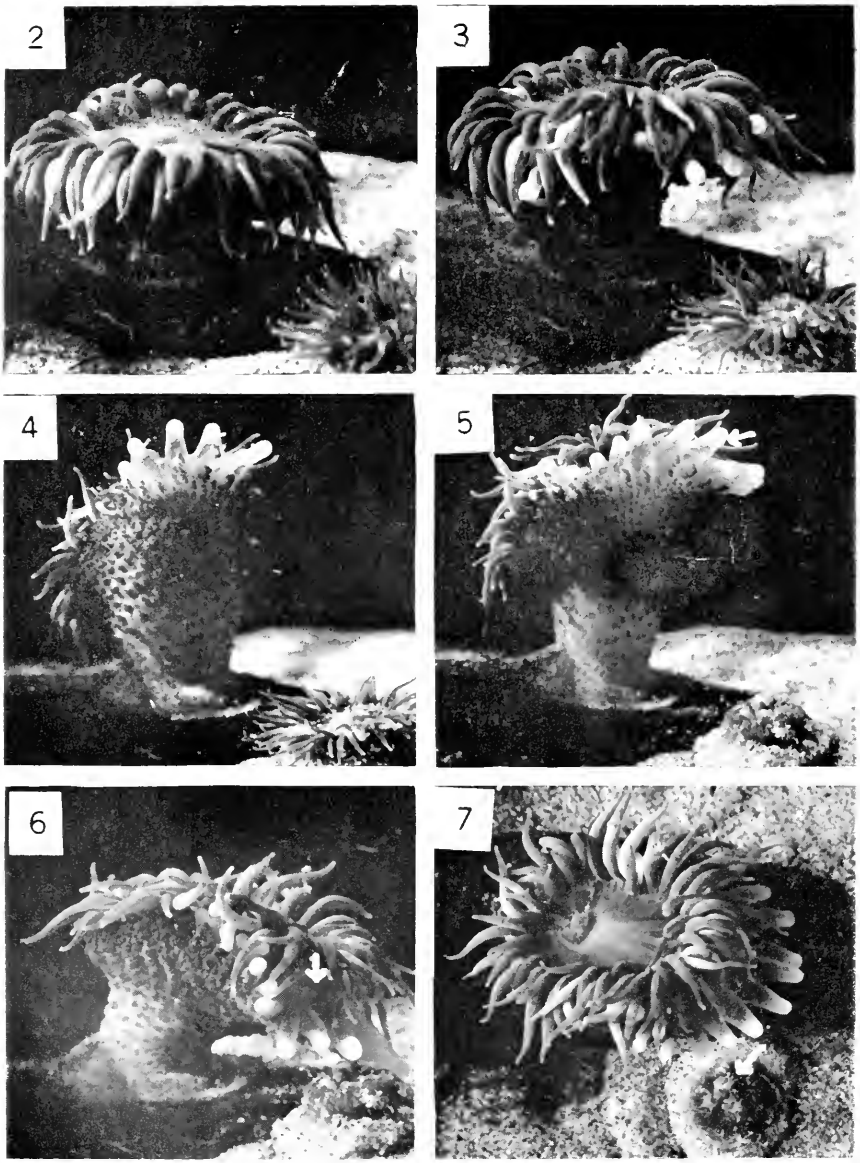

FIGURE 2. The anemone is shown at rest.

FIGURE 3. Inflation: the white tips of the inflating acrorhagi have become visible; the column has become more elongated.

FIGURE 4. Movement of application: the fully inflated acrorhagi are drawn upward and back and are just about to begin moving downward toward the victim.

FIGURE 5. Movement of application: a second movement of application is underway and more fully inflated acrorhagi have been recruited (*cf.* Fig. 4); transparent areas are obvious at the tips of several acrorhagi (⟶) where acrorhagial ectoderm was released from the acrorhagi and applied to the victim during the first movement of application.

that their bases appear transparent, see Fig. 7) comes into contact with a non-clonemate, the white surface layer can be observed to pull loose from the acrorhagus and adhere to the body of the non-clonemate (Fig. 1h and 7).

Fully expanded acrorhagi that had released part of their white surfaces as described above were sectioned using freeze sectioning and standard paraffin embedding techniques. In the region from which the white material was released the entire ectoderm layer was absent, and only the endoderm and mesogleal layers remained. *In vivo*, the tips of such acrorhagi appear transparent where the ectoderm is missing (Fig. 5).

During the slow return to a normal posture following the aggressive response, the anemone first rises from the arched over position (Fig. 1g) to an upright posture. The acrorhagi frequently lose their turgidity as the anemone returns to the upright position (Fig. 1i). If the anemone does not again come into tentacle contact with a non-clonemate, the acrorhagi eventually deflate and within a matter of hours the animal slowly returns to its more usual posture.

The anemone to which acrorhagial ectoderm has been applied (the victim of an aggressive episode) may respond by returning the aggression; more frequently, however, it will at first retract its tentacles, shorten its column, and contract the large sphincter at the top of the column. The damaged anemone may then move away from the aggressor; or sometime, after re-extending the tentacles and column and relaxing the sphincter, it may lean away, thus avoiding contact that would trigger another attack by the aggressor.

In other cases, apparently after having had insufficient time to attach its foot to the substratum or after having received severe damage as a result of repeated attacks, the victim may release its hold on the substratum. In the field, such an animal would certainly be carried away by the waves and might conceivably resettle elsewhere. In the laboratory, animals that have released their foothold after being attacked and damaged by a non-clonemate have subsequently resettled and recovered when they were removed to a separate bowl.

The tissue of the victim in the area of applied acrorhagial ectoderm shows obvious signs of damage and deterioration within about a day. This is especially evident for the tentacles, which contract and remain contracted as soon as acrorhagial ectoderm is applied. Within a few days, the obviously necrotic tissue in the damaged area is sloughed off along with the whitish patches of foreign acrorhagial ectoderm.

Tentacles to which acrorhagial ectoderm was applied were fixed, embedded in paraffin, sectioned, cleared, and stained (Masson's Trichrome dyes). In these sections it was possible to see the threads of the large atrich nematocysts (in this species, peculiar to the acrorhagi) penetrating the tentacle tissue.

As the following experiment demonstrates, an anemone that is repeatedly attacked and that can neither move away nor be washed away from the vicinity of an aggressor will eventually be killed.

FIGURE 6. Movement of application: the acrorhagi have reached the bottom of their downward sweep and are being wiped against the body of the victim; swelling of the fosse area between the inflated acrorhagi and the tentacles is apparent here (→).

FIGURE 7. Movement of application: seen from overhead as the acrorhagi reach the level of the victim, elongation of the oral disc in the area proximal to the inflated acrorhagi is apparent; scraps of applied acrorhagial ectoderm are visible on the body of the victim (→).

A single anemone from one clone (Clone 2) was first allowed to settle on a small pebble and then placed in a bowl so that it was close to and completely surrounded by anemones from another clone (Clone 1). The Clone 2 anemone was repeatedly attacked and damaged. Its pedal disc quickly became detached from the substratum (see Response of the victim, above); and being unable to escape the continued attack, it was killed within ten days. This experiment was repeated seven times, and each time the single Clone 2 animal was eventually killed.

As a control, one of two Clone 2 anemones placed in a bowl of Clone 1 anemones was protected from contact by a four-layered cylinder of plastic screening; the pebble on which the anemone was settled was the bottom of the cylinder. The tentacles of the protected Clone 2 anemone touched the inside layer of the screen, and the tentacles of the adjacent Clone 1 anemones touched the outside layer a fraction of a centimeter away. As a result, there was no aggression directed toward the protected Clone 2 anemone, and it remained undamaged and healthy in appearance for the several weeks that it remained in the dish. Within the same period the unprotected Clone 2 anemone was attacked, damaged, and killed.

Specificity of the aggressive response. In order to determine the range of circumstances in which *Anthopleura elegantissima* employs the aggressive response, the anemones were brought into contact with various animals that they might or might not encounter in their natural habitat. Both casual contact and repeated stimulation (described above under methods) were always used in attempting to elicit a response from the anemones.

Some of the invertebrates used were those that I have observed in the laboratory or in the field either feeding on *A. elegantissima* ("predator species") or being eaten by these anemones ("prey species"). Included among the predator species were the nudibranchs *Hermissenda crassicornis* and *Aeolidia papillosa*, the snails *Calliostoma annulatum* and *Epitonium* sp., and the omnivorous batstar *Patiria miniata*. The two prey species used were young mussels *Mytilus* sp., and the surface-living coelenterate *Velella velella*. The anemones did not aggress after repeated contact with any of the predator or prey species, nor would the fully expanded acrorhagi of anemones in full aggression release ectoderm upon contact with any of these species.

The tentacles of numerous coelenterates and a chain of eudoxids from a calycophoran were also used in attempts to elicit aggression in *A. elegantissima*. These results were not always consistent. I observed a tendency for the tentacles of the two animals to stick together. Afterwards, the tentacles of *A. elegantissima* involved in the contact often looked shriveled and failed to react in the usual way to contact with the tentacles of a non-clonemate. Tentacles recently involved in intra-specific contacts only never appeared to be damaged. If, as seems apparent, damage is involved in at least some inter-specific contacts, this could cause some interference with the initiation of aggression. For this reason, each of the species of coelenterates tested was used for two to four series of stimulations and was recorded as eliciting aggression if even one series of contacts elicited the full aggressive behavior. None of the three Hydrazoans tested (the unidentified calycophoran, *Velella velella* and *Corymorpha palma*) was capable either of eliciting aggression or of triggering the release of acrorhagial ectoderm upon contact with the fully expanded acrorhagi of an aggressing anemone. All eight species of Anthozoans tested (*Anthopleura xanthogrammica*, *Anthopleura artemesia*, *Tealia* sp., *Cnidopus ritteri*,

Epiactis prolifera, Cerianthis sp., *Corynactis californica, Balanophyllia elegans* and *Paracyanthis stearnsii*) elicited aggression in *Anthopleura elegantissima* as well as triggering the release of acrorhagial ectoderm.

Inert objects were also used in an attempt to elicit the aggressive response. Aggression was not elicited by repeated tentacle contact with inert objects such as a clean glass rod or metal probe. Mechanical stimulation such as pinching the tentacles with a pair of forceps was also ineffective as was repeated electrical stimulation of the tentacles.

The following experiment was designed to determine whether the anemones aggress against all non-clonemate members of their own species or whether there might be clones that are mutually compatible.

Anemones from twenty-five different clones of the aggregating form of *A. elegantissima* and a large individual of the solitary form were collected from several different areas for use in this experiment. Several anemones from each of three clones (Clones 1, 2, and 5) were used as test animals in this experiment. Because it might be possible for an anemone to wander some distance from its clonemates and form a separate but genetically identical clone, care was taken when collecting in the vicinity of Clones 1, 2, and 5 to select only clones having color patterns observably different from those of the three test clones.

In the laboratory, securely settled anemones from each of the three clones were stimulated in the usual way with tentacles from each of the twenty-six genetically different anemones mentioned above. This included one set of forty stimulations using, as a control, tentacles from a clonemate not recently in contact with the experimental animal.

Because the members of the three clones used in this experiment responded aggressively to contact with the tentacles of all twenty-five non-clonemates but not to contact with a clonemate's tentacle I think it likely that aggression is triggered by contact with any and all non-clonemates.

The following experiment demonstrates that these anemones, while reacting aggressively to contact with the tentacles of any non-clonemate, quite reliably fail to aggress upon contact with the tentacle of a clonemate.

(1) An anemone was stimulated as usual using the excised tentacles of a non-clonemate. Stimulation was discontinued if the reaction was not elicited within ten contacts, and those anemones not reacting within ten contacts were eliminated from the experiment at this point. (2) After a five minute rest period, the anemone was stimulated in exactly the same manner with the tentacle of a clonemate (one kept in a separate bowl to eliminate the possibility of acquired tolerance involving specific individuals). Stimulation was discontinued if the aggressive response was not elicited within twenty contacts. (3) After a five minute recovery period, the anemone was again stimulated with the tentacles of a non-clonemate, and the threshold was recorded.

This experiment was repeated thriteen times using anemones from four different clones. Two animals were eliminated from the experiment after step 1 because they had thresholds greater than ten. Of the remaining eleven animals (Table I) all but one (K) failed to aggress after repeated contact with a clonemate's tentacle. The behavior pattern of anemone K was peculiar in that the agressive behavior was directed ninety degrees away from the area of stimulation, while normally aggression is directed toward the area of stimulation. In this case, aggression was di-

TABLE I

Inter-clonal specificity in the initiation of the aggressive response. The symbol 7+ indicates that seven contacts with the excised tentacle initiated the aggressive response in the experimental animal, while the symbol 20-0 indicates that twenty contacts with an excised tentacle did not initiate the aggressive response

Individual anemone	Number of stimulations—response		
	Non-clonemate's tentacles	Clonemate's tentacles	Non-clonemate's tentacles
A	7+	20-0	5+
B	1+	20-0	1+
C	8+	20-0	1+
D	6+	20-0	2+
E	3+	20-0	2+
F	1+	20-0	1+
H	8+	20-0	3+
I	5+	20-0	1+
K	3+	16+	1+
L	5+	20-0	7+
M	1+	20-0	2+

rected toward the source of stimulation during step 1. (It should also be mentioned here that there is considerable variation in the behavior of these animals as there is in the behavior of anemones in general. From time to time and for no apparent reason a member of this species has failed to react to stimulation that at another time was quite adequate to elicit a reaction. Sometimes a given anemone has responded less quickly to stimulation than usual. I have also observed delayed movements of application following a few minutes after a usual aggression, and occasional examples of apparently spontaneous aggression. The response of anemone K during step 2 of the experiment may have been an example of a delayed movement of application, since it was directed toward the area of stimulation during step 1.) In step 2 of the experiment, all eleven anemones proceeded into full aggression after less than ten contacts with the tentacle of a non-clonemate, showing that the animals were still capable of responding aggressively to appropriate contact.

Apparently, then, the aggressive response of *A. elegantissima* is elicited by contact with non-clonemates or with any of a number of Anthozoans, but not by contact with clonemates, with hydrozoans, with predator or prey species, or with inert objects.

Aggression-related damage at interclonal borders. Examination of adjacent clonal borders in the field has frequently shown aggression and aggression related damage (white patches of acrorhagial ectoderm on tentacles and column, each spot surrounded by a ring of necrotic tissue) among the animals at or near these borders. Aggression is rarely observed among anemones in the midst of a clonal group.

During low tide anemones were collected from the adjacent borders and from the middles of two clones whose common interclonal boundary had been observed to be relatively stable over a one year period. These animals were retracted when

collected and no aggression-related damage was evident; however damage was evident when the anemones expanded after they were placed in sea water in the laboratory. Of the seven anemones collected from the border (3 from one clone and 4 from the other), all showed aggression related damage. Of the nine anemones collected from mid-clone (4 from one clone and 5 from the other) none showed aggression related damage.

Here is evidence that in the field aggression is occurring at the boundaries between adjacent clonal groups and not within clonal groups.

In the laboratory, two adjacent clonal groups that were forced into close proximity eventually developed an anemone-free zone between the two groups (Francis, 1973). During this time numerous aggressive episodes between nonclonemates were observed at the border between the two groups suggesting that aggression was involved in the formation of the anemone-free space between the clones.

Reaction of young anemones to intraspecific contact. Small specimens of *A. elegantissima* were found in a mussel bed at Arroyo Hondo (an area just south of Gaviota, Calif.) in October of 1971. Careful examination showed that the smallest of these (those having 30 or fewer tentacles) had not yet developed acrorhagi. Larger specimens having 30 to 40 tentacles were developing acrorhagi on the adoral side of the verucae closest to the oral disc. It seems reasonable to assume (a) that these incompletely developed anemones were relatively recent products of sexual reproduction, and (b) that they were too young to have undergone asexual binary fission and so were each genetically distinct individuals (non-clonemates).

During the experiments described below I observed interactions between several young anemones without developed acrorhagi. When these animals came into tentacle contact with each other their tentacles twitched, writhed, and retracted from the contact. Often the postures of the animals also changed noticeably after such an encounter, the column becoming longer and thinner. Occasionally I observed movements similar to those of the aggressive response; there were frequently bending and writhing movements of the column, and the edge of the oral disc in the tentacle contact area was sometimes lifted up and back as happens in the aggressive response early in the movement of application (Fig. 4). However, none of these anemones showed the full set of movements in the sequence displayed during the adult aggressive response (*i.e.*, tentacle contact and retraction, column elongation, proximal edge of the oral disc drawn up and back, proximal edge of the oral disc extended out and down). During the 4 such encounters that I watched, one of the anemones (the one that had moved into contact range of the other) moved out of contact range within an hour. Obviously these juveniles lacking acrorhagi were unable to damage each other by the application of acrorhagial ectoderm, and their distribution and response to contact with each other is therefore of interest in ascertaining the relation between the damage inflicted as a result of the aggressive behavior and the clone specific segregation observed for the adults of this species.

Both on the mussels and pebbles on which they were collected and on the glass bowls in which they were kept in the laboratory, these young anemones were usually solitary, that is, out of contact range of each other. When within contact distance they were never observed in passive tentacle to tentacle contact as is common within the clonal groups of this species, but one or both of any proximal

pair was always retracted. Groups of 5 to 9 of the young anemones lacking acrorhagi were placed close together in containers of various sorts in order to make it easy for them to settle close to an another, but these animals always moved apart in from 24 to 48 hours. Apparently then, young specimens of the anemone *A. elegantissima* tend to move in such a way as to eliminate contact with genetically different members of the species, even in the absence of the aggressive response.

Actinea equina, variety *mesembryanthemum*

I observed the north Atlantic intertidal sea anemone *Actinea equina* (var. *mesembryanthemum*) in its natural habitat along the northwestern and southeastern coasts of Scotland and along the southern coast of England. There I found the anemones to be solitary, living out of contact distance with each other. Stephenson, 1935, reports asexual reproduction in the species as rare.

Bonnin (1964) described an aggressive response for this variety of *Actinea equina* (color types green and red) and did some preliminary studies on the specificity of the response which indicated that these anemones more often aggress against animals of the opposite color type than against those of their own color type. In studies that I conducted at the Gatty Marine Laboratory, St. Andrews, Scotland, and the Plymouth Laboratory, Plymouth, England, my findings were entirely to the contrary.

Because many of the anemones that I saw could not clearly be classified as either color type red or color type green, each individual is referred to by a number beside

TABLE II

Specificity of the aggressive response of Actinea equina

Source of the excised tentacles used for stimulation*	Response of 6 different individuals to contact with tentacles excised from 12 different individuals†					
	5R,R′	11r,R	390R,r	220,OG	350G,*g*	29G,G
5R,R′	O	+	+	+	+	/
11r,R	+	O	+	+	+	+
12r,R	+	+	+	±	+	/
20r,R	+	+	+	+	+	/
19r,r	+	±	+	+	+	+
390R,r	+	+	O	+	+	/
220,OG	+	+	+	O	+	±
350G,*g*	+	+	+	+	O	+
32g,g*	+	+	±	+	+	·
29G,G	+	+	+	+	+	·
10G,G	+	+	+	+	+	·
41G,G	+	+	+	+	+	·

* The numbers each designate a different individual. The first letter(s) following each number indicates the tentacle color of the individual (R = red, r = light red, OR = orangish red, O = orange, OG = orangish green, g = light green, G = green) and the letter(s) following the comma indicates the color of the column (R′ = dark red, *g* = light green with darker green spots).

† The symbol + indicates that stimulation of the anemone elicited an aggressive response within 20 contacts while ± indicates that the anemone failed to respond aggressively to one or two sets of stimulations and responded positively to another set. The symbol O indicates that no aggressive response was elicited. The symbol / indicates that there is no information.

which are letters indicating tentacle color and column color (Table II). Six of the anemones covering the color range from green to red were tested in the usual way by repeated contact with tentacles excised from twelve different animals of various colors; this included 20 contacts with one of their own tentacles. Stimulation was discontinued if an anemone did not respond after 20 stimulations, but the results were not reported as negative unless the animal failed to respond to the stimulation on three different occasions. Tests in which the anemone failed on at least one occasion to respond aggressively to twenty contacts with an excised tentacle, but subsequently responded positively are indicated in Table II, by an underscored +. The results are shown in Table II where each individual is indicated by a number beside which the tentacle color and column color are indicated. The specificity of the response in *Actinea equina* seems to be the same as that in *Anthopleura elegantissima*; the anemones aggress in response to contact with tissue from any genetically distinct individual regardless of color type but not upon contact with tissue from a genetically identical individual (in this case their own tentacle). There was no apparent correlation between color type combinations and the ease with which aggression was elicited.

Anthopleura artemisia

Anthopleura artemisia is found living intertidally along the west coast of America from Alaska to southern California, where it lives attached to rocks and buried in mud, sand, or gravel so that usually only the tentacular crown is extended above the surface (Hand, 1955). I have generally found this species to be solitary, although I have occasionally observed close contact between two animals having similar colors and patterning on the oral disc and tentacles. Since this species is known to reproduce asexually by longitudinal fission (Hand, 1955), such pairs are probably clonemates.

The coloring and patterns of this species are highly variable, and I have never observed close contact between two specimens having distinguishably different color of patterning.

Anthopleura artemisia was observed in the laboratory to aggress in response to contact with non-clonemate members of the same species and in response to contact with its congenerics *A. elegantissima* and *A. xanthogrammica*. The aggressive behavior of *A. artemisia* was identical at least superficially to that described for *A. elegantissima*.

Anthopleura xanthogrammica

According to Hand (1955) *Anthopleura xanthogrammica* "is known from Japan (?) to southern California" (page 53), where the adults are commonly found in pools and crevices just below the mussel beds. In the intertidal near Pacific Grove, California where I have observed and collected these animals I have frequently observed very close contact within groups that could only be non-clonemates since this species does not reproduce asexually.

Anthopleura xanthogrammica has never been observed to display an aggressive response, even when provoked by repeated contact with members of the same species or with its congenerics.

Discussion

The aggressive response of some members of the family Actiniidae is another example of a surprisingly specialized and complex behavior pattern displayed by sea anemones, animals having "primitive" systems for nervous integration and often assumed, therefore, to display a very limited behavioral repertoire. I would like to compare this behavior with some other complex behaviors described for anemones and other coelenterates in order to point out the common as well as the distinctive features of the aggressive behavior.

The aggressive response of *Anthopleura elegantissima, Actinea equina* (Bonnin, 1964), and *Anthopleura artemisia* is like both the raptorial feeding of coelenterates and the shell transfer behavior of some anemones (Ross, 1967) in being, a series of movements directed towards a goal. In addition the conspicuous movements are very similar in the three different kinds of behavior patterns in that they are grossly asymmetrical and directed with respect to the source of the stimulus. The aggressive response includes both symmetrical postural changes that involve the whole animal (see Fig. 3, the elongated, upright posture assumed during aggression) and localized, coordinated movements that are radially asymmetrical and oriented with respect to the source of contact stimulation (*e.g.*, the movement of application, Fig 7). Raptorial feeding in coelenterates could involve symmertical changes in the condition of the whole animal (*e.g.*, postural changes or changes in the threshold of the nematocysts involved in feeding), however the conspicuous movements are asymmetrical (*e.g.*, movement of the mouth and tentacles) and directed with respect to the area of contact with the prey. Aggression is also comparable, in this regard, to the transfer behavior (movement of the anemones from glass or another surface to a shell) shown by *Calliactis parasitica* (Ross and Sutton, 1961; Ross, 1967), *Actinostola* (Ross, 1967) and *Stomphia coccinea* (Ross, 1967) in response to contact with a shell. The transfer behavior appears to involve symmetrical postural changes in the anemones as well as asymmetrical movements that are oriented with respect to the source of stimulation (*e.g.*, in *Stomphia*, swelling of one side of the pedal disc and movement toward a shell with which the tentacles are in contact).

Aggression might also be compared with the swimming response of coelenterates. In the jellyfish swimming involves rhythmically repetitive, radially symmetrical movements. Directedness is normally achieved not by gross asymmetry in the movements themselves but by subtle and temporary asymmetry in the movements which then leads to changes in the orientation of the whole animal. The same is apparently true for the swimming behavior of the Actinians *Gonactinia, Boloceroides, Bolocera*ctis and *Bunodiopsis medusoides,* all of which use their tentacles in swimming (Robson, 1966). The case is less clear for the swimming of the anemones *Actinostola* and *Stomphia* (Yentsch and Pierce, 1955; Ross and Sutton, 1967). The behavior here initially involves movements that seem relatively symmetrical and that lead to elongation of the column, expansion of the crown, and finally detachment from the substratum. The swimming movement itself, while apparently producing no effective orientation in the net movement of the anemone, could nonetheless be described as asymmetrically oriented with respect to the source of the stimulus since it begins as alternate bending of the column away from and toward the source of stimulation. However, the orientation of the rhyth-

mic bending then slowly shifts around the column (Robson, 1966), making this swimming movement a difficult one to classify with regard to symmetry and directedness.

It does seem apparent that in some important ways the aggressive behavior is more nearly like raptorial feeding and transfer behavior than it is like swimming; aggression is a directed behavior involving asymmetrical movements that are not repeated rhythmically. However, in some other respects the aggressive response does resemble the swimming behavior of *Stomphia* and *Actinostola*. Both of these behavior patterns involve a specific sequence of movements that, once initiated, continues to completion in the absence of additional or continuing stimulation. In both cases the behavior is initiated by contact with a limited number of species (Ross and Sutton, 1967; and Bonnin, 1964), implying that the initiating stimuli are very specific and presumably chemical in nature.

Several characteristics of the aggressive response are distinctive within the repertoire of anemone behavior. One of these is the peculiar manner in which the response is initiated. For *Anthopleura elegantissima* the initiating stimulus involves repeated contact of the tentacles with some part of a non-clonemate conspecific or some other anthozoan. In describing the aggressive response of *Actinea equina* Bonnin (1964) has noted that a definite time lapse between successive contacts during stimulation seems important in reaching the threshold for the response. Too rapid or massive stimulation usually results in complete retraction of the anemone without eliciting an aggressive response. The same seems to be true for *Antholeura elegantissima*.

The aggressive behavior of these anemones is also remarkable in being a very specialized behavior that functions under very particular and limited circumstances (see also Bonnin, 1964) and that involves the use of specialized movements and equipment. Contact and retraction of the tentacles during initiation of the behavior does not appear to be particularly specialized (the animals respond in a similar way to mild mechanical or electrical stimulation of the tentacles); however some of the movements during the inflation stage of the behavior (*e.g.*, the puffing up of one edge of the capitulum and the associated acrorhagi) and during the movement of application (*e.g.*, the asymmetrical elongation of the oral disc and the downsweeping movement of the inflated acrorhagi) are quite distinctive movements of the aggressive behavior. The acrorhagi are specialized "organs" which have been observed to function only during aggression (Abel, 1954; Bonnin, 1964) and apparently the acrorhagial atrich nematocysts are also used only during aggression.

From the evidence presented on the specificity of the aggressive response of *Anthopleura elegantissima*, it is apparent that the behavior is not directly involved either in defense against predators or capture of prey. Aggression is elicited only by contact with conspecifics and other Anthozoans. Bonnin (1964) found the same to be true for *Actinea equina*.

For *Anthopleura elegantissima* the aggressive response seems to have a clear function in intraspecific interactions. Briefly summarized, the pertinent evidence, presented in this and a previous paper (Francis, 1973) is as follows: (1) Within clonal groups of the aggregating form of *Anthopleura elegantissima* the individuals are in close contact with their neighbors and the tentacles of the adjacent animals

are interlaced. In the field, adjacent clones of these anemones are isolated from each other by anemone-free spaces; and in the laboratory a group of anemones of mixed clonal origins will reaggregate into isolated uniclonal groups (Francis, 1973). (2) The specificity of the aggressive response is directly related to that shown in the segregation and separation phenomena seen both in the laboratory and in the field. Contact between clonemates does not elicit aggression and is commonly seen both in the laboratory and in the field. Contact between non-clonemates elicits aggression and is not maintained between these anemones either in the laboratory or in the field. (3) The response of the victim of an aggressive episode is such as to affect its separation from the non-clonemate aggressor. The damaged anemone may move away from the aggressor, or sometimes it may merely lean away. In the laboratory, anemones that were severely damaged as a result of repeated aggressive episodes have been observed to release their hold on the substratum; in the field, such animals would certainly be swept away in the surf. All of these responses tend to remove the victim from contact with the attacking non-clonemate. (4) The evidence also shows that aggression does occur at the boundary between two clonal groups. In the field, anemones collected from an interclonal border showed aggression-related damage while their clonemates that were collected from the center of the clonal groups showed no such damage. In the laboratory numerous aggressive episodes were observed over a three week period as two clonal groups that had been brought into artificially close proximity interacted at their common boundary (Francis, 1973). This suggests that aggression is important in the formation and maintenance of anemone-free zones between adjacent non-clonemates in this species.

It is possible that the activities of some associated animals such as the chiton *Mopalia muscosa* and the turban snail *Tegula funebralis* are involved in the maintenance, especially of very wide trail-like anemone-free spaces between adjacent clonal aggregations. Field (Biology Department, University of California, Santa Barbara, personal communication in 1969) has found that *Mopalia* is capable of maintaining and extending artificially made clearings in aggregations of *A. elegantissima;* and I have observed the appearance of short anemone-free pockets occupied by *Mopalia* or *Tegula* in previously uninterrupted borders of clonal aggregations.

In addition, the observations and experiments with the pre-aggression juveniles of *A. elegantissima* indicate that the aggressive behavior may not be a necessary condition for clone specific segregation and separation in this species. Contact between genetically different individuals was avoided even in the absence of the aggressive response.

However, the direct effects of the aggressive behavior of *A. elegantissima* would be sufficient to account for clone specific segregation and at least minimal separation between clones; and although other factors may be involved, the aggressive response undoubtedly functions as part of an intraspecific territorial behavior in this species.

Assuming that the aggressive response of acrorhagus-bearing members of the family Actiniidae is monophyletic in orgin, and knowing that no function outside of aggression is known for the acrorhagi (Abel, 1954), we may speculate on the evolutionary advantages and history of this highly specialized behavior.

I have shown that aggression is effective in intraspecific interactions for *Anthopleura elegantissima*. In *Actinea equina* also the specificity of the aggressive response correlates with the intraspecific associations apparent in the field; these anemones are solitary and they respond aggressively to contact with any other conspecific. For *Anthopleura artemisia* the data are less complete since no extensive work has been done on the intraspecific specificity of aggression for this species. Nonetheless these anemones do respond aggressively to contact with conspecifics, and they have not been found living in contact with genetically different conspecifics. *Anthopleura xanthogrammica* apparently lacks an aggressive response while having the specialized equipment (acrorhagi bearing atrich nematocysts) used in this behavior. However, the relationship between distribution and behavior is also apparent for *A. xanthogrammica* since genetically different members of this species are frequently found living in close contact, with the tentacles of adjacent non-clonemates passively interlaced. Taken together, this information suggests that the aggressive response of acrorhagus-bearing anemones is presently effective in intraspecific interactions.

This suggests the possibility that aggression arose in this group of animals through intraspecific interactions. If this were true, what might be the origin of and selective advantage(s) for intraspecific territoriality involving mutal repulsion among genetically unlike members of a species? I would suggest that an anemone that repulses all members of the species except clonemates may gain space on the substratum for its genotype. This could be a decided advantage to the genotype possessing this characteristic, especially if there were competition among members of the species for space itself or for the available food in a given area. As long as the adverse effects of this characteristic, such as the inability to form inter-clonal aggregations and thereby reduce water loss (Roberts, 1941) and the energy loss involved in the repulsion of non-clonemates, were not too great any anemone that developed some form of genetically determined interclonal "repulsiveness" would have a competitive advantage over its inoffensive neighbors. This genotype and the post-larval progeny inheriting the characteristic would be assured of adequate "living space" and would therefore have an increased probability of survival and reproductive success. It may have been in this way that the evolutionary precursor of the aggressive response (perhaps a simple tendency to avoid contact, as in the case of the pre-aggression young of *Anthopleura elegantissima*) became fixed in the common ancestor of these anemones.

It seems apparent to me that an aggressive behavior that arose through intraspecific competition would be of value only to the individual anemones that display the behavior and not to the species as a whole. (Of course a characteristic theoretically need not be of advantage to the species in order to be selected for, because selection is believed to operate at the level of the individual.) Once aggression and armament are adopted and convey their temporary advantage to the aggressor, an evolutionary trend toward more potent weapons and more effective ways of using them seems very likely. Among the acrorhagus-bearing anemones the specialized nematocysts carried on specialized protuberances (the acrorhagi) and the relatively effective behavior pattern that brings these weapons into play may have evolved in this way.

It is also possible that the aggressive response evolved through interspecific

interactions with other anthozoans and served to counter direct competition for space and food by other large sessile coelenterates. This hypothesis is appealing in that it provides a selective advantage to the species as well as to the individual in the origin and development of an aggressive response. However, while intraspecific contact is effective at eliciting aggression in all three species for which the response has been described, interspecific contact is a less reliable elicitor than intraspecific contact for one of the two species tested in this regard. Both interspecific and intraspecific contact are reported to be completely effective at eliciting aggression in *Actinea equina* (Bonnin, 1964), while intraspecific contact is much more reliable than interspecific contact at eliciting aggression in *Anthopleura elegantissima*. For *Anthopleura xanthogrammica* both the interspecific and the intraspecific components of the response have apparently been lost (presumably in recent time since the supposedly vestigal acrorhagi do not appear to be different in form or number from the functional ones of *A. elegantissima*).

Before this question of evolution can be dealt with further, we clearly need more information, especially on the interspecific aspects of the aggressive response of acrorhagus-bearing Actiniids.

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Summary

Tentacle contact between genetically different individuals of the west coast sea anemone *Anthopleura elegantissima* initiates in one or both individuals an elaborate behavior pattern that usually results in damage to one or both animals. This aggressive behavior may be described as progressing through five stages: (1) stimulation (or initiation) during which the tentacles of the two anemones repeatedly come into contact and then withdraw from contact; (2) inflation, during which the acrorhagi commence to become turgid; (3) the movement of application, during which the inflated acrorhagi are pushed toward the source of stimulation; (4) application of ectoderm, during which damage is inflicted on the other anemone by the application of acrorhagial ectoderm to the body of the victim; and (5) recovery, during which the anemone returns to its normal posture.

The tissue of the victim to which acrorhagial ectoderm has been applied by an aggressor becomes necrotic and sloughs off within a few days. Sections of such tissue that were fixed and sectioned immediately after the application of ectoderm showed penetration of the tissue of the victim by the large atrich nematocysts in the aggressor's acrorhagial ectoderm.

The aggressive response of *Anthopleura elegantissima* is like raptorial feeding and the shell transfer behavior of some anemones in that it is a directed behavior involving asymmetrical movements that are not repeated rhythmically. Aggression resembles the swimming behavior of *Stomphia* and *Actinostola* in that the specific

sequence of movements, once initiated, continues to completion in the absence of additional or continuing stimulation. The deployment of specialized effector "organs" (the acrorhagi) bearing specialized nematocysts (the large acrorhagial atrichs), the necessity for repeated contact in initiating the response, and the functioning of aggression in intraspecific territoriality are interesting characteristics of this behavior that are unique in the repertoire of anemone behavior.

For *Anthopleura elegantissima* the aggressive response is not directly involved either in defense against predators or capture of prey. Evidence presented here suggests that for this species the aggressive response is involved in intraspecific interaction. Pertinent evidence, presented in this and a previous paper (Francis, 1973) is as follows: (1) Within clonal groups of the aggregating form of *Anthopleura elegantissima* the individuals are in close contact with their neighbors and the tentacles of the adjacent animals are interlaced. In the field, adjacent clones of these anemones are isolated from each other by anemone-free spaces; and in the laboratory a group of anemones of mixed clonal origins will reaggregate into isolated uniclonal groups (Francis, 1973a). (2) The specificity of the aggressive response is directly related to that shown in the segregation and separation phenomena seen both in the laboratory and in the field. Contact between clonemates does not elicit aggression and is commonly seen both in the laboratory and in the field. Contact between non-clonemates elicits aggression and is not maintained between these anemones either in the laboratory or in the field. (3) The response of the victim of an aggressive episode is such as to affect its separation from the non-clonemate aggressor. The damaged anemone may move away from the aggressor, or sometimes it may merely lean away. In the laboratory, anemones that were severely damaged as a result of repeated aggressive episodes have been observed to relase their hold on the substratum; in the field, such animals would certainly be swept away in the surf. (4) The evidence also shows that aggression does occur at the boundary between two clonal groups. In the field, anemones collected from an interclonal border showed aggression-related damage while their clonemates that were collected from the center of the clonal groups showed no such damage. In the laboratory numerous aggressive episodes were observed over a three week period as two clonal groups that had been brought into artificially close proximity interacted at their common boundary (Francis, 1973a). This suggests that aggression is important in the formation and maintenance of anemone-free zones between adjacent non-clonemates in this species.

The direct effects of the aggressive behavior of *A. elegantissima* would be sufficient to account for clone specific segregation and at least minimal separation between clones; and although other factors, such as the activities of associated animals and the tendency of the young anemones to move apart even in the absence of the aggressive response, may be involved, the aggressive response undoubtedly functions as part of an intraspecific territorial behavior in this species.

In *Actinea equina* also the specificity of the aggressive response correlates with the intraspecific associations apparent in the field; these anemones are solitary and they respond aggressively to contact with any other conspecific. No work has been done on the specificity of the aggressive response in *Anthopleura artemisia*, however these anemones do respond aggressively to contact with conspecifics, and they have not been found living in contact with genetically different conspecifics.

Anthopleura xanthogrammica apparently lacks an aggressive response while having the specialized equipment (acrorhagi bearing atrich nematocysts) used in this behavior. However, the relationship between distribution and behavior is also apparent for *A. xanthogrammica* since genetically different members of this species are frequently found living in close contact, with the tentacles of adjacent non-clonemates passively interlaced. Taken together, this information suggests that the aggressive response of acrorhagus-bearing anemones is presently effective in intraspecific interactions.

It is suggested that the evolutionary origin of the aggressive response among acrorhagus-bearing members of the family Actiniidae is monophyletic and that the behavior may have arisen in connection with competition for space either within the species of with other anthozoan species.

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THE ECOLOGY AND REPRODUCTION OF A MARINE BIVALVE, *MYSELLA PLANULATA* (ERYCINACEA)[1]

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Mysella planulata (Stimpson) is a small erycinacean bivalve which occurs primarily in muddy sands of the *Zostera marina* community. The recent distribution of *M. planulata* extends from Nova Scotia to Cape Hatteras on the American Atlantic coast and includes the Texas coast of the Gulf of Mexico. The species also occurs in Pliocene and Pleistocene deposits of Florida and Maine (Dall, 1900). While *M. planulata* is not a conspicuous member of the benthos, it may be an abundant and at times even a dominant species. Yet, as with so many other marine invertebrates of no apparent economic importance, virtually nothing is known of its biology. This report presents data on the density, distribution, growth, age-structure and reproduction of a population of *M. planulata* from Beebe Cove, at the mouth of the Mystic River, eastern Long Island Sound, Connecticut.

Beebe Cove is a small bay, approximately 1500 m long by 305 m wide. The habitat is sublittoral, but water depth does not exceed 2 m; salinity varies between 29 and 31‰. The sediment is a mixture of fine sand, silt, and clays, the latter components comprising more than 70%. Beds of *Zostera marina* cover the bottom and contribute large quantities of organic detritus to the benthic ecosystem. Temperature ranges from −1 to 26° C and an ice cover may be present in winter. Beebe Cove is isolated from the Mystic River by a railroad trestle embankment and exchange occurs only through two narrow channels. Consequently, through most of the cove currents are restricted and sluggish and siltation rates are high. Except for a thin layer at the mud/water interface, the benthic environment is highly reducing.

Sampling was restricted to a single station near the center of the cove, marked with a buoy which remained in place throughout the experimental period. Most of the data in this report are based on replicate samples taken at this single station between April 1970 and November 1971, although some information is presented for the same station from sampling dates in 1969.

Replicate samples were collected using a 0.07 m^2 Peterson grab operated by hand from a small skiff. Samples were washed through a 1 mm mesh stainless steel screen with seawater.

For the studies on larval development, animals from the field collections were transferred to finger bowls of seawater. Frequently the act of screening and handling the adults seems to stimulate release of larvae, but a constant supply of newly released veligers was provided by adults maintained in finger bowls containing sediment from Beebe Cove. Larvae were collected every other day and

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reared at a concentration of 5–20 larvae per ml, at a constant temperature of 25° C, and were fed *Isochrysis galbana* at a concentration of 100 cells/ml. Cultures were changed daily or every other day.

To determine the stage of gametogenesis, smears of live animals were prepared by removing the animals from their shells under the dissecting microscope. Soft parts were examined under a coverglass with a compound microscope, and the presence or absence of eggs, veligers and active sperm was easily determined.

Results

Density and distribution

The areal density of *Mysella* at a series of sampling dates for 1970 and 1971 is shown in Table I. The variance/mean ratio (coefficient of dispersal) is greater than unity on all dates, indicating a degree of contagion or parchiness.

Table I

Sampling statistics for Mysella planulata, *April 1970–November 1971 based on replicate Peterson Grab samples from a single station*

Sample No.	Date	$\bar{x}$ per Grab	S^2	Number of replicate grabs	Moment estimate of k*	1/k**
1	4/7/70	18.4	76.3	5	5.857	0.179
2	5/25	14.6	54.0	5	5.410	0.195
3	6/29	6.2	32.7	5	1.450	0.831
4	7/14	5.2	15.2	5	2.704	0.417
5	7/28	8.4	22.8	5	4.900	0.218
6	8/25	4.2	13.7	5	1.857	0.637
7	12/3	30.6	358.3	5	2.857	0.379
8	4/13/71	12.2	54.2	5	3.544	0.304
9	5/25	4.8	22.1	5	1.332	0.929
10	6/8	22.0	338.6	5	1.529	0.760
11	7/7	20.5	112.4	5	4.573	0.231
12	7/27	21.3	123.2	5	4.452	0.237
13	8/17	8.4	67.2	7	1.200	0.965
14	11/30	4.3	10.9	10	2.801	0.379

* $k = \bar{x}^2/Y'$ (see text).
** $1/k = Y'/X'$ (see text).

The negative binomial distribution is considered to be the most flexible of several mathematical frequency distributions in which the variance exceeds the mean (Anscombe, 1949; Bliss and Fisher, 1953). The parameters of this distribution are the arithmetic mean, and the exponent k. The latter is related to the spatial dispersion of organisms and its reciprocal is a measure of the degree of aggregation or clumping of the individuals in a population (Elliott, 1971). As $1/k$ approaches 0, the distribution approaches the Poisson series in which organisms are randomly distributed ($S^2 = \bar{x}$). In this study, the values of k (Table I) were calculated by the moment estimate in which $k = \bar{x}/(S^2 - \bar{x})$.

As seen in Table I, moment estimates of k ranged from 1.200 to 5.857. This would suggest a fair degree of stability over a two year period. When plotted, there does not appear to be a relationship between $\bar{x}$ and $1/k$, *i.e.*, the degree of

clumping as indicated by $(1/k)$ is not correlated with the sample mean. A rough estimate of a common k-value (k_c) has been calculated using the statistics X′ and Y′ where $X' = \bar{x}^2 - (S^2/n)$ and $Y' = S^2 - \bar{x}$; $1/k = Y'/X'$ (Elliott, 1971). The common k (k_c) is calculated: $1/k_c = Y'/X'$. Calculated in this way (Table I), a k_c of 2.68 is obtained.

At the single location studied, changes in density with time vary somewhat during 1971. The reasons for these differences will become clear following the discussion of age structure (see below). A feature common to both years is the decline in density which occurs during the winter and early spring. Between December 1970 and May 1971, mean density declined from 30.6 animals per grab to 4.8 per grab. After the low point in numbers is reached in late summer (Table I), no increases occur until the addition of the newly recruited year class appears

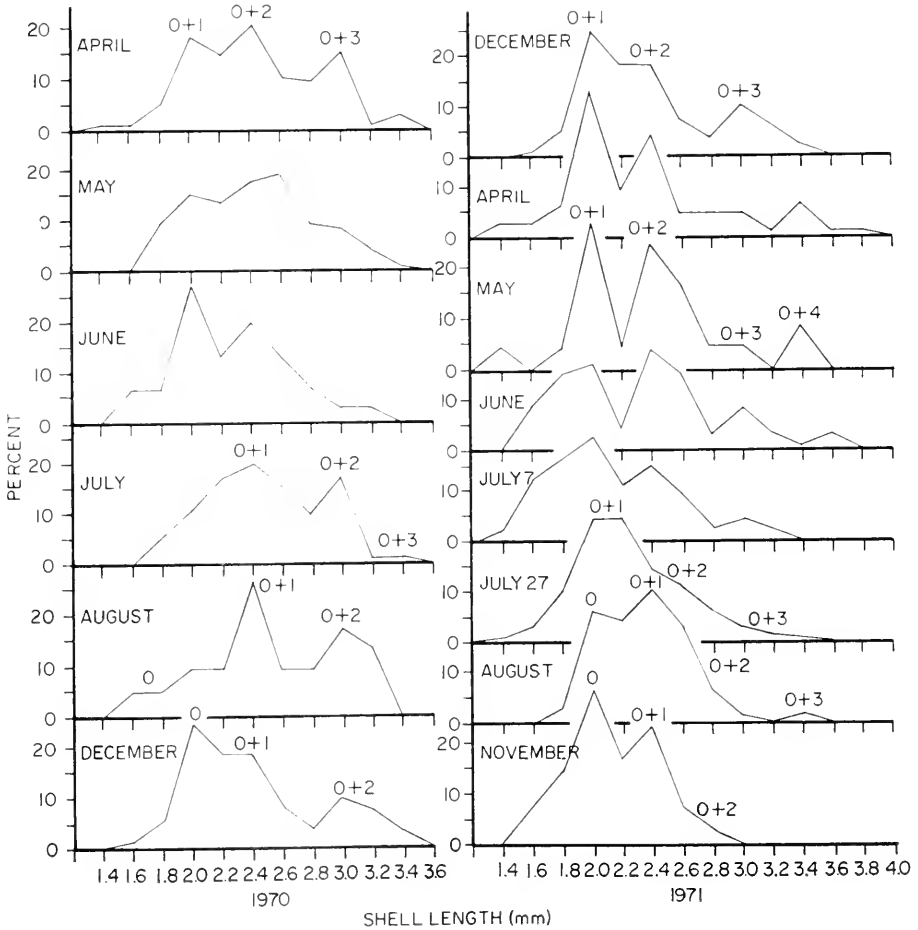

FIGURE 1. Size distribution of *Mysella planulata* populations from Beebe Cove, April 1970 through November, 1971. Numbers above the distributions indicate the age classes.

(December sample, 1970). In 1971, however, an increase in density occurred between May and June. As will be shown below, these differences are explainable on the basis of age-specific mortality, and of differences in the age composition of the population between the two years.

Population age structure and growth

Information on population age structure and size for 1970 and 1971 (Fig. 1) is supplemented by data from collections of September, 1969 (not plotted), from larval settlement collectors (Fig. 2) and from analysis of growth cessation marks (Fig. 3). The pattern revealed in the size-frequency histograms (Fig. 1) is consistent for both years. At the beginning of the growth season, the population comprises 3 fairly well-defined modal groups plus a less well-defined group at the upper end of the histogram. These peaks are considered to correspond to 3 year classes, with the remnants of possibly one or more older classes at the right. Between April and June there is an increase of very small animals in the youngest year class, particularly evident between April and May of 1970 and between May

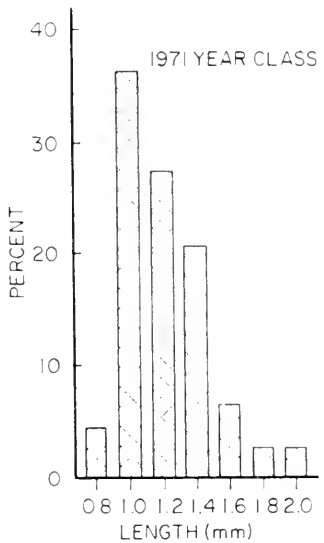

FIGURE 2. Size distribution of zero class animals from benthic collectors in Beebe Cove. This collection was retrieved on 30 August, 1971 and the animals are a maximum of 2 months old.

and June of 1971. This is accounted for by growth of very small animals which, prior to this, were below the size retained on the 1.0 mm screen. By late July, the 0 + 1 class (clams in their second growth season) is fully represented in the histograms. The new zero class makes its first appearance in August. By the end of the season, December (1970) and November (1971), the zero class is numerically dominant. The 0 + 3 class (clams in their fourth season) fades completely from the distributions by August of both years.

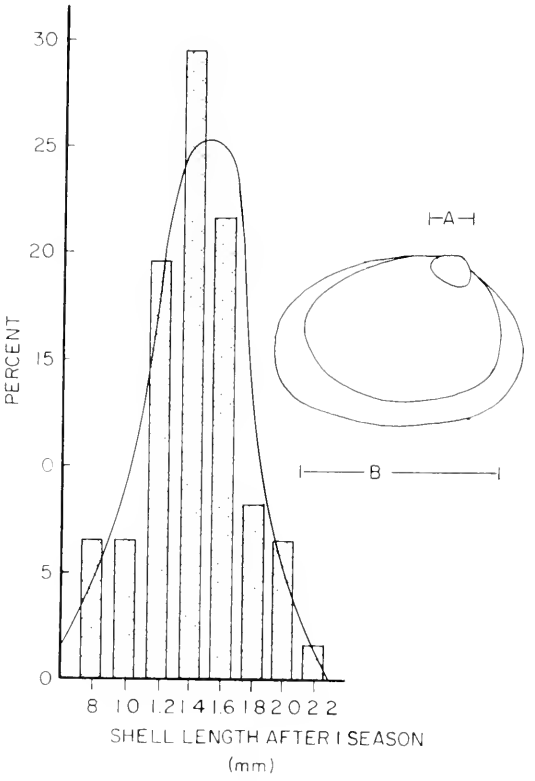

FIGURE 3. Size distribution of clams at the end of their first growing season based on length B, the maximum length at the time growth stopped. Length A represents the length of the prodissoconch (larval shell) at time of metamorphosis. The smoothed curve was fitted using normal probability paper.

The difference in density pattern between 1970 and 1971 (Table I) is largely accounted for by internal changes in the relative abundance of age groups. During both years, increments in the number of young animals occur in May and June. In 1971, this increment is reflected by the increase in density in June; in 1970, however, increases attributable to the addition of small animals were offset by mortality of older animals, the 1966 and 1967 year classes. The following year, the analogous year classes were less numerous to begin with.

Another obvious difference between the two years is evident in November 1971 where the 0 + 2 class seems to disappear completely, a trend already evident in the immediately previous sample. This is probably correlated with the drastic overall decline in the population density of *Mysella* in Beebe Cove over this period.

Figure 2 shows the length-frequency distribution of a collection of *Mysella* from Beebe Cove sediment collectors. The collectors, one-quart wide mouth glass jars (80 mm diameter) in a wood frame anchored on the bottom with several cinder blocks, were placed in the cove on 1 July and retrieved on 30 August.

Although the wooden frame rested on the bottom, the jars themselves were raised above the bottom making it unlikely that juvenile clams crawled into the collectors. The maximum age of the animals in Figure 2 is therefore 2 months. Although most of the clams in the collectors are in the 1.0 mm size class, the largest and presumably oldest have attained 2.0 mm. Figure 3 is a similar distribution of shell length (B) at the time of growth cessation. Assuming that the growth interruption mark indicates the shell length at the beginning of the new growth season (which is likely but not certain) animals range from less than 0.8 to about 2.3 mm at that time. If specimens with growth interruption marks accurately reflect the total population, the modal size at the end of the first season would be 1.4 mm (Fig. 3). This is significantly smaller than the modal size of the zero class in the size-frequency distributions for December 1970 and November 1971 in which the mode would appear to be close to 2.0 mm. However, growth cessation marks are not observable on all animals. If, for example, they are obliterated by erosion on the older animals, the distribution in Figure 3 would represent only the younger members of the zero class. While it is not possible from the present data to assign precise boundaries to the zero class, it is clear that in their first growth season these clams attain a maximum size between 2.2 and 2.5 mm with a mode between 1.4 and 2.0. By late summer (July or August) of the following year, this class attains a modal size of about 2.4 mm; and one year later, about 3.0 mm. By the end of the fourth season, the modal size may be close to 3.4 mm. While the calculations are imprecise, the overall picture is probably a reasonable approximation.

Reproductive biology

Information on gametogenesis, based on the examination of whole animal smears, is shown in Table II. In the period from April through July, active sperm were observed in almost all animals. However, developing ova were not seen until May, and the percentage of animals with developing eggs increased to a high of 67 per cent in June. By July, oogenesis had proceeded to the point where fertilized ova were seen being brooded within the mantle cavity.

The range in size of animals with sperm and ova is shown in Figure 4. Active sperm were observed in animals from 1.33 to 3.82 mm, and all individuals with developing ova were also producing active sperm. Animals with sperm but no ova ranged up to about 2.4 mm. In early spring, only individuals larger than 2.3 mm were seen to have ova, but by late May ova were seen in some smaller animals also.

TABLE II

Gametogenesis—1971

Date	No. animals examined	No. with active sperm	%	No. with ova	%
13 April	20	19	95	0	0
4 May	9	9	90	2	22
25 May	24	24	100	13	54
9 June	12	12	100	8	67
5 July	12	11	91	6	50

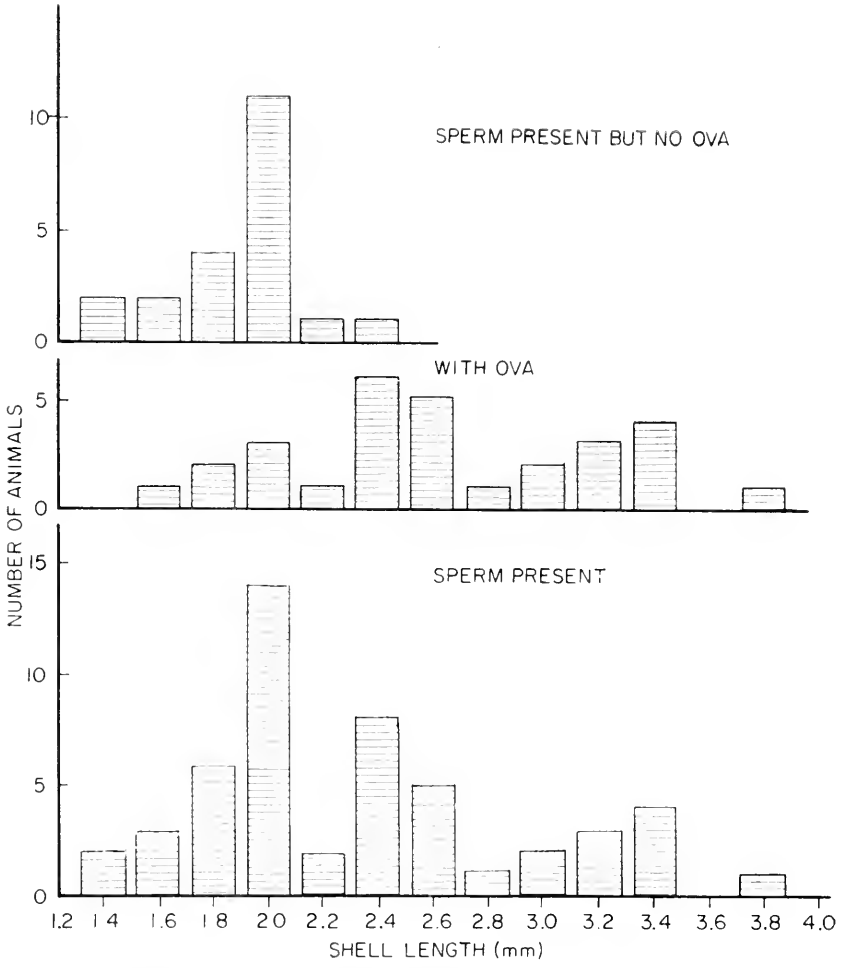

FIGURE 4. The relationship between gametogenesis and shell length based on smears of living animals.

The information in Figure 4 and Table II shows that *Mysella planulata* is a simultaneous hermaphrodite. Spermatogenesis precedes oogenesis so that by the time the latter process begins, most of the sperm are already matured and ready for use. However, sperm are always present, even when oogenesis is maximal.

Although hermaphroditism is widespread in the Erycinacea, and occurs in all Montacutidae, the existence of self-fertilization has never been proven. In order to test this possibility in *M. planulata,* a group of 10 individuals were isolated and maintained separately in standing seawater while another group of 10 similar-sized animals was kept together to facilitate cross-fertilization. This experiment was initiated on 4 May and terminated on 23 June. During this period, the containers were undisturbed and the water was not changed. At the end of the experimental

period, veliger larvae were observed in the grouped control. But four of the ten isolated animals had also produced veliger larvae. Since the animals were isolated before ova had matured and entered the suprabranchial cavity, the fertilization of the ova leading to the release of the veligers by the isolated specimens must have been effected by self-fertilization. This experiment does not eliminate the possibility that cross-fertilization may also occur. It is significant, however, that no spontaneous release of sperm has ever been observed.

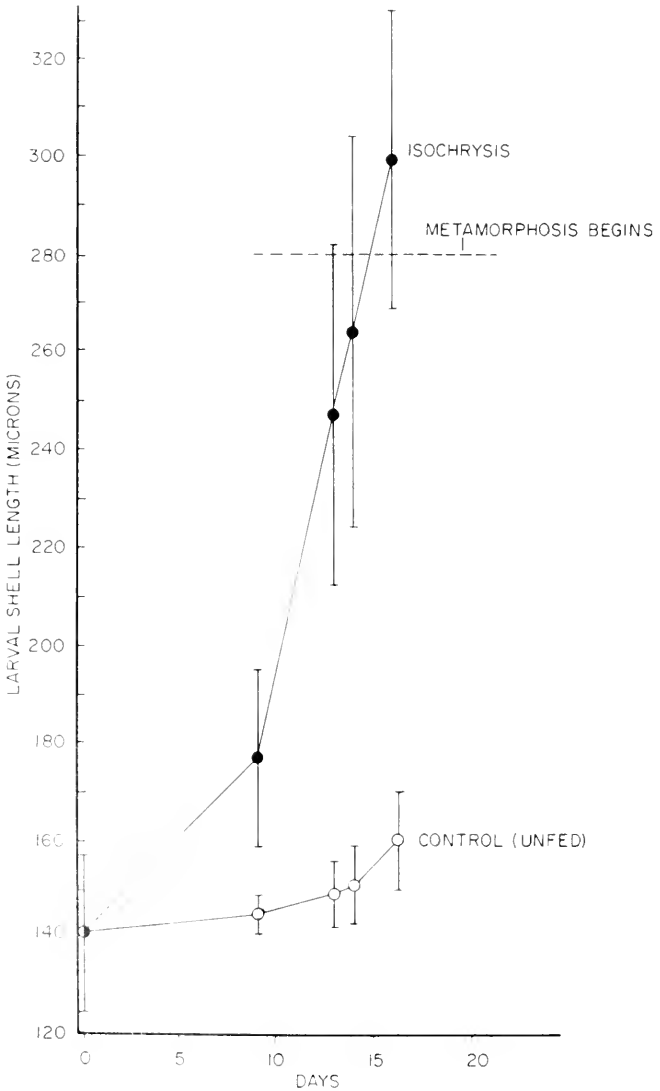

FIGURE 5. Cumulative growth curves of *Mysella* larvae reared in the laboratory. Solid circles, fed *Isochrysis galbana;* clear circles, starved. Lines indicate standard errors of the means. The horizontal dashed line shows the approximate size at which metamorphosis begins.

Development

Fertilized ova in cleavage stages were observed in the suprabranchial cavity toward the end of May. By early June, development had proceeded to the veliger stage. Most veligers appear to be released in June but a small proportion of animals continued to release larvae into July or even August. At emergence, the veliger shell length ranged from 126 to 150 microns ($\bar{x} = 141 \pm 16$ microns) and larvae are at the straight-hinge stage. A cumulative growth curve for larvae reared in the laboratory is shown in Figure 5. Although there is significant growth in the starved control group, these never exceed 160 microns and do not metamorphose. It is clear that the larvae of *M. planulata* are planktotrophic: the veligers must feed on the plankton and grow in order for metamorphosis to occur. Variability in size of the fed larvae is high. Some individuals grow rapidly, others very slowly. Metamorphosis begins in cultures at about 15 days and pediveligers, at metamorphosis, range from 225 to 355 microns.

Figure 6 shows the prodissoconch length-frequency distribution for a series of laboratory grown animals as compared with field-collected juveniles. The means of 299 and 286 microns (laboratory-reared and field-collected specimens, respectively) are not significantly different. However, the variance of the field-collected population is significantly lower than the variance of the laboratory-reared group.

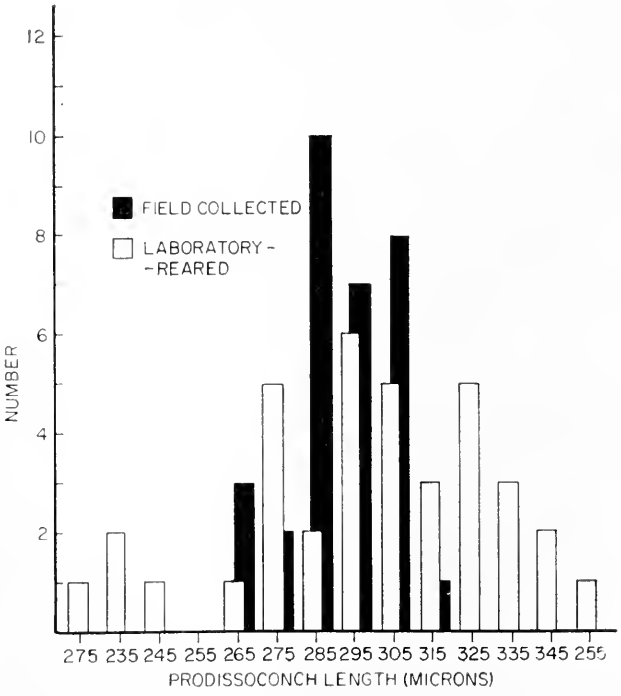

FIGURE 6. Prodissoconch length-frequency distributions for laboratory-reared and field-collected *Mysella*.

Discussion

It is evident from Table I that *Mysella planulata* exhibits a fairly constant high degree of clumping. Values of k in the same range ($0 < k < 5$) were reported by Lie (1968) for the related species *Mysella tumida* in Puget Sound. Lie suggested that the high degree of patchiness shown by *M. tumida* is correlated with brood protection. However, the present study shows that although larvae are retained for a period of time, they nevertheless spend a significant period of time in the plankton and are therefore not restricted to settling in close proximity to adult parent clams.

Possibly late stage larvae are attracted by the presence of adults in the substrate, resulting in a non-random distribution. Alternately, a gregarious response may occur following metamorphosis. In view of the capacity of *Mysella* for self-fertilization, gregariousness would have the beneficial effect of increasing heterozygosity in the gene pools of local aggregations.

A high degree of contagion might be expected in symbiotic species, which include many—perhaps the majority—of erycinacean bivalves, assuming that the host species are themselves contagiously distributed. However, this study provides no evidence for an obligative symbiosis with any invertebrate species, and even a facultative or temporary symbiosis seems unlikely. Small specimens and dead shells are sometimes found adhering to the podia of *Thyone briareus,* a burrowing holothurian. But this is of doubtful significance because the podia of *Thyone* tend to adhere to a variety of detritus. In any case, *M. planulata* gives every indication of being free-living in Beebe Cove, where it lives in the upper oxygenated layer of sediment. Full understanding of its distribution may require a better knowledge of how *Mysella* functions in this microhabitat. The eel grass itself is not distributed evenly over the bottom. Even within a dense *Zostera* stand, patches of bare or almost bare substrate occur. The microenvironment within a *Zostera* meadow has not been studied but it is possible that this is the key to the non-random distribution of *M. planulata.*

In common with other Erycinacea, *Mysella planulata,* exhibits larviparity (the mode of development in which the eggs develop within the confines of the maternal body and are then released as larvae) and partial brood protection. *M. planulata* is also a simultaneous hermaphrodite. As noted by Fretter and Graham (1964), simultaneous hermaphroditism in marine bivalves appears to be linked with brood protection, particularly in the form of ovoviviparity and viviparity. These forms of brood protection, whatever their specific selective advantage, reinforce non-random dispersal since juveniles occur in close proximity to their parents. The loss of a larval dispersal phase may be compensated by the advantage of access to a suitable substrate immediately upon release from the female, particularly in bivalves of restricted habitats. Sellmer (1967) noted that brood protection is most prevalent among very small bivalves and may be related to the efficiency of egg production. Very small animals may simply lack the energy resources to produce enough planktotrophic eggs to guarantee survival. The larviparity of *Mysella planulata* confers the benefits of both brood protection and planktonic larvae. Retention of the early larval stage provides protection during a critical period but the subsequent release of planktotrophic veligers allows the species to

exploit the phytoplanktonic food source as well as providing a mechanism of dispersal.

Mysella varies seasonally in abundance. Jørgensen (1946) observed that the larvae of *M. bidentata* constitute an important part of the plankton of the Øresund at certain times of the year. In the Beebe Cove benthos, population densities exceeding 5000 per m^2 have been observed in late summer and fall, mostly newly-settled juveniles. At other times, populations of adults rarely exceed 600 per m^2 in this area, and may be much less. Thus, such a diminutive and inconspicuous species may assume an unexpectedly significant part in the function of this ecosystem by virtue of its role as a secondary producer in the plankton, and the subsequent transfer of energy into the benthos where it is available for exploitation by upper trophic levels of the benthic food chain.

In Beebe Cove, larvae metamorphose at a size between 265 and 315 microns with a mean of 299 microns. The greater range among laboratory-reared individuals suggests that in nature environmental factors—most likely predation—eliminate individuals which either delay metamorphosis or develop too slowly.

The data presented above show that *Mysella planulata* is a small (<4.0 mm) bivalve, a simultaneous hermaphrodite with larviparous development. For most individuals sexual maturity occurs in the third season. Mature animals produce up to 1000 eggs per season. Fertilized eggs are retained in the suprabranchial chamber until the larvae attain the straight-hinge stage and are released as planktotrophic veligers. Larvae settle after about two weeks in the plankton (Fig. 5). Animals continue to grow and reproduce and live a maximum of about 4 years.

The picture which emerges is of a species which, despite its minute size, exhibits a degree of iteroparity, *i.e.*, the capacity to reproduce at least twice per lifetime. The significance of this is apparent when considered in the light of the fecundity of the species. Since egg production is limited to animals larger than 1.7 mm, probably not more than 25 per cent of the 0 + 1 class contributes to the annual egg production. Estimates of the potential reproductive contribution of the various age classes in the population at the beginning of the reproductive season are shown in Figure 8. The estimates were made with the aid of the regression (Fig. 7), relating total eggs produced as a function of shell length. The numbers of individuals attributable to the several age classes were determined by visually assigning limits to the modal size groups. The potential egg production for each size class was calculated by multiplying the numbers of individuals in that class by the total number of larvae released per adult (Fig. 7). The production of all the size classes within each age class was summed and the proportion of the total egg production attribute to each age class determined (Fig. 8). Where age class limits overlap, potential egg production was divided evenly between adjacent classes.

The estimates assume that only a single batch of larvae is released per adult clam. It is possible that a second brood of fertilized eggs passes into the suprabranchial chamber of the clams following the release of the initial batch of veligers. However, dissections of brooding animals indicate very few maturing ova retained in the ovaries. The scatter in Figure 7 probably arises from abnormally low counts which could be explained by the occurrence of secondary broods. In any case, it is probable that the regression in Figure 7 under estimates egg production

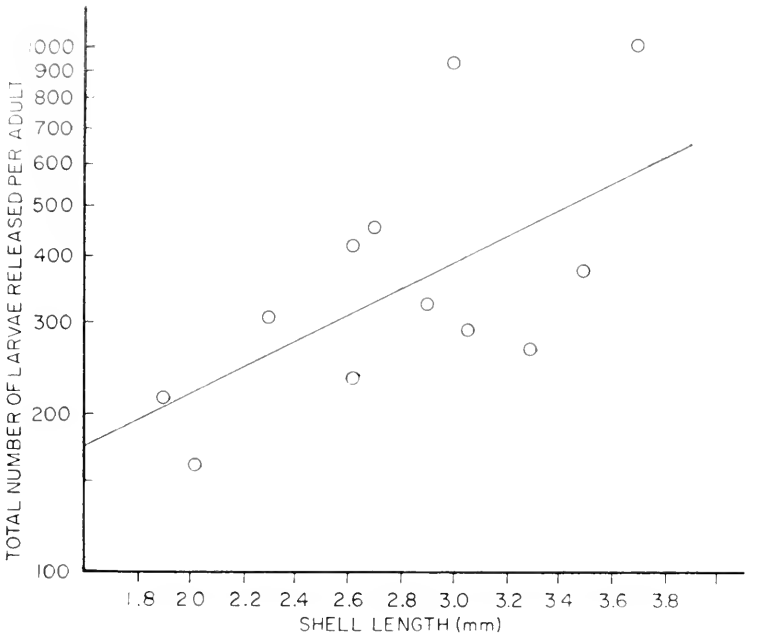

FIGURE 7. Relationship between shell length and the total number of larvae released per adult. The regression line is fitted by the method of least squares: $\text{Log}_{10}Y = 1.860 + 0.243X$ where X is shell length and Y is the number of larvae.

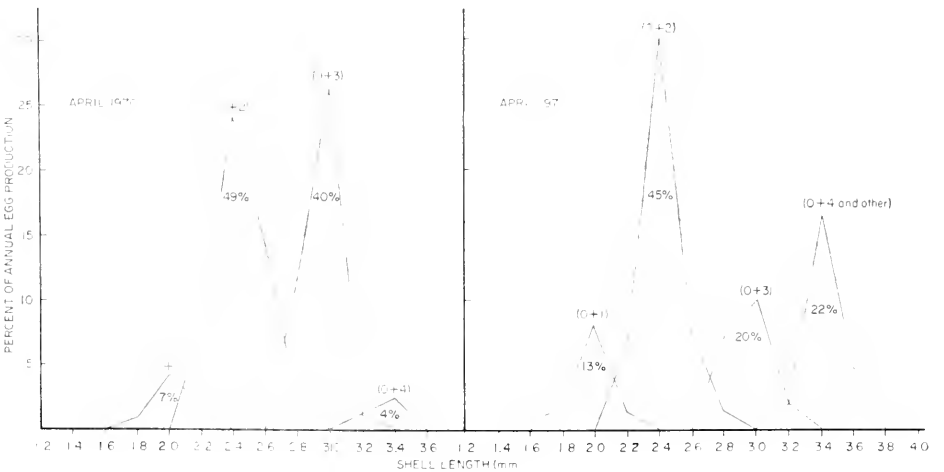

FIGURE 8. Potential reproductive contribution of age classes at the beginning of the reproductive seasons, April 1970 and 1971. Numbers in parenthesis above the distributions indicate the age classes; numbers under the peaks indicate the percentage contribution of the age groups.

of the larger individuals and probably overestimates production by very small individuals.

It is evident from Figure 8 that although the major reproductive burden is carried by the 0 + 2 class (clams in their 3rd growing season), the remaining year classes, and particularly the older classes, do contribute significantly to the annual production. This pattern is adaptive since it provides a margin of safety against the loss of one or more entire year classes. Between 1970 and 1971, (Fig. 8) the potential reproductive contribution of the 0 + 3 class dropped from about 40% in 1970 to 20% in 1971. This was caused by high mortality in the 1968 year class, which is the 0 + 2 class in 1970 and becomes the 0 + 3 class in 1971.

Murphy (1968) provides evidence that iteroparity has selective advantages in species in which there is uncertainty in survival from zygote to first maturity. In the case of *Mysella*, as with other planktotrophic temperate species, variability in the survival of the zero class is normal. This variability may result from variation in the level of predation pressure on larvae and juveniles, and in density-independent environmental factors such as unfavorable currents, unusually severe reducing conditions in the sediment, *etc*. Where environmental instability may cause fluctuations in population density, the adaptive value of distributing the reproductive responsibility over a wider range of age groups is obvious. The 0 + 2 class, which evidently bears the major reproductive burden, represents the best possible compromise between maximum numbers of individuals and maximum biomass per individual.

Iteroparity is not the only reproductive option open to small marine bivalves. The venerid clam *Gemma gemma* is an ovoviviparous species which appears to reproduce only once (Sellmer, 1967; Green and Hobson, 1970). Populations of *Gemma* are known to suffer severe predation so that there may be an adaptive advantage for this species to mature and reproduce rapidly. Ovoviviparity in *Gemma* could be interpreted primiarily as a mechanism for enhancing survival of offspring, thus increasing biotic potential. In *Mysella*, however, the occurrence of a planktonic dispersal phase puts a constraint on the development toward complete brood protection. The adjustment toward iteroparity may thus be interpreted as an alternate option for increasing biotic potential.

The feeding mechanism of *M. planulata* was not studied. However, from the posture of the living animal *in situ*, and its normal location at the sediment/water interface, either detrital organic matter or suspended material is potentially available for food. Unlike the situation in most bivalves, except the Protobranchia and certain primitive eulamellibranch genera, the pathway of water through the mantle cavity of *Mysella* and related genera is anterior to posterior. There are no siphons; gill filaments are few in number and, in *M. planulata*, the outer demibranchs are reduced in size. Ciliary currents are present but weak. The palps are also quite small but their appressed surfaces are grooved and ciliated, suggesting the presence of a sorting mechanism. A distal food groove is present on the ctenidia. All of these morphological characters suggest suspension feeding. The suspended matter is quite likely very small aggregates of particulate organic matter, some or all of which may be resuspended by the movement of the animal as it moves through the sediment. Nevertheless, a role for dissolved organic material in this species is not ruled out.

Summary

1. In Beebe Cove, a shallow sublittoral bay, the bivalve *Mysella planulata* is dispersed contagiously throughout the year. This dispersion is not associated with the mode of development of the species but may be correlated with non-random variability in the microhabitat. There is no evidence that *M. planulata* in Beebe Cove is symbiotically associated with any invertebrate species.

2. *Mysella planulata* has a life span encompassing four growing seasons and a maximum size of about 4.0 mm. About 50% of maximum length is attained by the end of the first growing season.

3. *Mysella planulata* is a simultaneous hermaphrodite. Spermatogenesis precedes oogenesis. The species is capable of self-fertilization and although no animals were ever observed to release sperm, the possibility of cross-fertilization is not ruled out. Egg production is limited to animals in excess of 1.7 mm.

4. Larvae are retained in the suprabranchial chamber to the straight-hinge stage of development. Upon release, the larvae are planktotrophic; starved larvae in culture grow slightly but will not metamorphose. Metamorphosis in culture occurs in about two weeks at 25° C. In the laboratory, larvae metamorphose over a wider range of size than in nature, probably reflecting predation pressure in nature.

5. Estimates of the potential reproductive contribution of the various age classes show that although the major burden is carried by animals in their 3rd season (0 + 2 class), older classes contribute significantly. This pattern is adaptive in unstable environments where high mortality of the 0-year class is probable.

6. On morphological grounds, *M. planulata* is believed to be a suspension feeder; probably feeding on very fine particulate organic matter.

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THE SEARCH DYNAMICS OF RECRUITED HONEY BEES, *APIS MELLIFERA LIGUSTICA* SPINOLA

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The foraging efficiency of a honey bee colony is maintained by workers experienced at collecting from various crops surrounding the hive. Because these experienced foragers associate the distinctive odors of flowers with the nectar and pollen rewards (Wenner and Johnson, 1966), they may be stimulated to leave the hive merely by perceiving these odors clinging to the bodies of other returning foragers or when artificially injected into the hive (Steinhoff, 1948; Johnson and Wenner, 1966). Using landmarks learned from previous flights (Wolf, 1926, 1927), these experienced bees can quickly return to those sites which they associate with the odor stimuli.

Yet, there are always some bees which lack sufficient experience to be recruited to the field by this method. Bees which have not previously collected food are continually added to the foraging population, a new crop may appear which is not in the experience of some of the bees, or a group of foragers may become idle with the depletion of a food source. Not being attached to a particular crop, these bees collectively form the potential recruit pool. By attending the dances of active foragers, potential recruits are thought to be stimulated to leave the hive in search of currently productive areas (von Frisch, 1946).

Dance attendants may be exposed to information about the feeding site in three modes: (1) tactile signals (including sounds) generated by the maneuvers of the dance, (2) gustatory signals from the offered food, and (3) olfactory signals from the odors clinging to the body of the dancing bee (reviewed in von Frisch, 1967a). However, which of this information recruits use in searching for the signalled feeding site is presently the center of controversy (von Frisch, 1967b; Wenner and Johnson, 1967; Wenner, 1971).

Von Frisch (1946) proposed that recruits rely on the quantitative information contained in the tactile signals of the figure-8 dance, while the olfactory signals merely indicate the "kind of flower frequented" by the dancer. In his summary volume (von Frisch, 1967a, page 57) he states: "When the goal is 100 m or farther from the hive the round dance is replaced by the tail-wagging dance. Like the round dance, it announces in the first place the existence of a profitable source of food, secondly the kind of flower frequented (by means of the floral odor clinging to the dancer), and thirdly the potential productivity of the food source (by means of the liveliness of the dances); and here too the scent organ is set into action on the return to the feeding place and the newcomers' locating this correctly is thereby facilitated. But beyond this the tail-wagging dance makes known the distance and compass direction to the goal, the first by the tempo of the dance, the latter through the direction of the straight segment of the run in the dance

pattern. This description of the location enables the newcomers to fly rapidly and with certainty to the indicated flowers, even when these are kilometers away—an accomplishment on the part of the bees that is without parallel elsewhere in the entire animal kingdom."

More recently Wenner has indicated that the original experiments of von Frisch lacked necessary controls. He and co-workers have proposed that, although the dance and food may be required to initiate search behavior, it is the odor associated with the dancing forager which guides successful recruits to the proper site. (See Wenner, 1971, for a discussion of this point of view.)

Conversely, other investigators have challenged Wenner and co-workers and have concluded that their data support the dance language hypothesis (Mautz, 1971; Esch and Bastian, 1970; Gould, Henery and MacLeod, 1970). But, the communication efficiencies (*i.e.*, the percentage of dance attendants successful at locating the feeding site—Mautz, 1971) obtained in these studies were only 32% (Mautz) and 13% (Gould *et al.*). Esch and Bastian reported on only those dance attendants which left the hive and found that 41% of these were successful. Additionally, for distances which should require only 30 seconds flying time, the average search times of these successful recruits were 3.2 min for 200 m (Mautz), 8.5 min for 200 m (Esch and Bastian) and approximately 13 min (but with a median value of 8 min) for 120 m (Gould *et al.*). These data indicate quite clearly that recruits did not leave the hive and fly directly to the food source. Instead, the communication efficiences and the extended search times of recruits necessitate the accumulation of searchers in the field.

Investigators have necessarily formulated their interpretations while little was known of the flight paths or search dynamics of recruits. By assuming that recruits fly a direct path to the food source, investigators have paid little attention to the behavior of these bees. In light of the current controversy, I conducted a series of experiments between 1967 and 1970 which was designed to contrast and test the predictions of the two major hypotheses (dance language and odor dependence) while providing information on the search dynamics of recruits. More specifically, several questions were posed the answers to which would allow an approach to the primary question: What determines the flight paths and success of newly recruited honey bees?

The variables selected for testing the hypotheses were as follows: Is recruit success dependent on (1) the presence of odor in the food, (2) the number of foragers visiting the food, (3) the distance and direction to the food relative to the wind, and/or (4) the velocity of the wind within the normal flight activity ranges? If recruits can rely solely on the dance's distance and direction information between the hive and feeding site, they should be independent of the above variables; but if using only odors associated with foragers, they should be highly dependent on each of the above.

Materials and Methods

This investigation of recruit search dynamics was conducted using capture techniques to sample the densities of searching bees at strategic locations. The technique used in the past has consisted of the placement of an array of dishes containing scented sucrose solution in the field while a group of foragers collected a

similar solution at a feeding station. These early experiments were designed to test the accuracy of site location by searching recruits. If, however, the searching recruits use odor as a guide, the addition of an array of odor sources would itself alter the system and dramatically influence the distribution to be measured (Wenner, 1971). Placing only a single scented station into the field after a searching population has been established should diminish this redistribution. If left for a brief period of time, a repeated use of such a monitoring station at various locations may yield a closer approximation of the distribution of searching bees. The distributions thus obtained under different experimental conditions could be compared to yield an average search pattern for a population of recruits and some of the variables which determine this pattern.

Under this assumption experiments were performed on the Lagoon Island Reserve of the University of California's Santa Barbara campus (UCSB) and at Santa Barbara Shores (SBS), 10 km west of the UCSB campus. Both sites provided large, level areas of land receiving predictable winds from the Pacific Ocean. A single hive of Italian strain honey bees (*Apis mellifera ligustica* Spinola) was used at UCSB, while the designs of experiments run at the SBS site necessitated the incorporation of a second hive containing cordovan bees (Italian strain, cordovan gene, *cd*, for body color). The cordovan bees, being of lighter color, could easily be distinguished from the darker wild type but displayed no discernible differences in behavior. In all cases two-story hives containing 50,000–60,000 bees were used.

During each experiment a single feeding station was provided for a specific number of foragers from the hive(s) involved. These foragers were trained to visit the feeding site in a manner similar to that described by Wenner (1961) and individually marked with enamel paint. All unmarked bees arriving at a feeding site were either killed by placing each in 50% ethanol with a pair of forceps or were marked as replacements of regular foragers.

Sucrose solution (1.0 or 1.5 M) was provided on vinyl-covered platforms during training and experimentation. For scented stations, an essential oil was added to the food solution. Foragers feeding on this scented solution remained lightly scented (to the human observers) even after scent was removed from the feeding station. Thus, following scented periods, unscented feeding sites remained "functionally scented" with low levels of odor provided by foragers at the station, along the flight path, and when dancing in the hive.

Glass dishes containing fresh sugar solution were placed on clean filter paper on the tabletop at regular intervals, not exceeding thirty minutes. Soiled filter paper and dishes with remaining sugar solution were doubly sealed in plastic bags and deposited in containers odor tight to humans. If an unscented period followed a scented period, the containers were removed from the area and the platforms were wiped with damp sponges.

The monitoring station used to determine the density of searching recruits did not have a fixed location but sampled various sites at different times. The locations of such monitoring sites are described separately for each experiment. This station was always scented but without regular foragers, and all bees arriving at a monitoring station were killed and tallied.

Observation periods for each experiment were restricted to morning hours be-

tween 0800 and 1230 DST, and only during these times was food provided. A variety of data was collected at the experimental (feeding) site: time, temperature, wind speed and direction, number of captured recruits, number of regular foragers, number of visitations made by these foragers, and in some experiments recording the arrival and departure times of regular foragers and the capture times of recruits was necessary. Except as related to regular foragers, the same data were collected at the monitoring station.

Results and Discussion

The influence of foragers on recruit success

When altering the number of foragers which visited a dish, the amount of time that dish was occupied by a given number of bees was disproportionate to the change in forager numbers (Wenner and Friesen, unpublished observations). With ten bees visiting a feeding site 210 m from the hive, the food dish was occupied by at least three bees 63% of the time; while with five bees, three or more foragers were present only 18.5% of the time. In addition, with ten bees a vacant dish was a rare event, *i.e.*, less than 1% of the time; while with only five bees the dish was unoccupied 15% of the time.

If, as Kalmus (1954) claimed, the presence of foragers at a dish makes that site more attractive to new arrivals, then the change in the numbers of foragers visiting a site should change the efficiency of searching recruits. Thus, the recruit success rate (number of recruit arrivals/forager round trip) as a measure of this efficiency should not remain constant, but should decrease in the case of a reduction in the numbers of foragers flying between the feeding station and the hive.

To test this, the number of foragers visiting a single feeding site 360 m from the hive was varied and recruit arrivals measured. Ten bees gathered scented sugar solution (0.30 ml oil of cinnamon/liter 1.5 M sucrose) on two days. Then, the number of foragers was reduced to five, and these were allowed to forage on four days. Finally, the number of foragers again was increased to ten, and recruit arrivals and forager round trips were recorded on two more days.

These data (Table I) show that halving the number of foragers does not simply halve, but diminishes to less than one quarter, the number of recruits successful at locating the feeding site. Thus, the recruit success rate, rather than remaining constant, shows a 58% decrease indicating recruit efficiency was positively correlated with, but disaproprotionate to, the number of foragers visiting the feeding site.

To test whether forager presence in the field (rather than dance frequency in the hive) was sufficient to account for the above correlation, another experiment was conducted using two hives at SBS. A feeding station midway between the cordovan and Italian strain hives (Fig. 11, site III) provided 1.0 M sucrose solution scented with clove oil (0.05 ml/liter) for three hours on each day of the experiment. For three days ten bees from each hive foraged at the feeding site. Then, after a reduction to five bees from the cordovan strain hive, the station was run an additional six days. The results of this experiment are contained in Table II.

Although the number of foragers from the Italian strain hive remained constant, the recruit arrivals from that hive dropped dramatically with the reduction of

TABLE I

The influence of the number of foragers on recruit success rates

Date	Number of foragers	Time (hrs)	Total round trips (RT)	Recruit arrivals (r)	Recruit success rates (r/RT)
11 Aug. 68	10	2¼	204	41	
12 Aug. 68	10	2¼	206	44	
20 Aug. 68	10	2¼	204	45	0.19
21 Aug. 68	10	2¼	199	25	
14 Aug. 68	5	2	105	3	
15 Aug. 68	5	2	96	9	0.08
16 Aug. 68	5	2	125	13	
17 Aug. 68	5	2	129	10	

foragers from the other hive. With twenty bees providing feeding activity at the dish, the recruit arrival rate for the Italian strain hive was 0.46; whereas after the reduction to fifteen foragers this rate fell to 0.29. (Using the Mann-Whitney U test on the original data, these values were found to differ significantly with $P = 0.048$.) In addition, search times of recruits with twenty foragers were less than with fifteen. With twenty foragers the average arrival time of the fifteenth recruit from the Italian strain hive was 55 min and from the cordovan strain hive, 63 min. However, with only fifteen foragers these averages increased to 77 min for the Italian strain hive and 82 min for the cordovan bees. Again, the data suggest that the disproportionate recruit success with different numbers of foragers visiting a feeding site may be attributed to the density of these foragers in the field.

The influence of odor on recruit success

The magnitude of the odor dependence of searching bees was first suggested in a preliminary experiment testing the effect of odor withdrawal from two feeding sites each visited by ten regular foragers. With the stations scented, large numbers of recruits arrived; and when this scent was removed on subsequent days recruit

TABLE II

The influence of the number of foragers from one hive on the recruit success rates of bees from a second hive. The lack of a change in the recruit success rate for the cordovan strain bees may be explained by the downwind position of their hive (see text)

Number of days	Number of foragers	Recruit arrivals/ day (r)	Forager round trips/day (RT)	Recruit success rates (r/RT)
Upwind hive (Italian)				
3	10	189.7	408.7	0.46
6	10	118.3	406.8	0.29
Downwind hive (Cordovan)				
3	10	438.7	329.0	1.33
6	5	150.7	116.3	1.30

arrivals diminished, but not to zero. By reducing the number of foragers to five at each station and maintaining the unscented condition, the recruit success rate fell to less than half (from 0.89 with ten to 0.35 with five foragers) as could be expected from the above experiments. More interesting, with fewer foragers visiting the stations, searching bees began diligently investigating the data pads and other paper near the stations! Only when foragers were at or near the dishes did these searchers approach the stations. These successful recruits paid little attention to the food on arrival, but investigated the paper towelling upon which the food dishes sat!

The paper towelling apparently had a distinctive odor to bees; and this odor, previously unnoticed by the observers at the stations, seemed to be a common factor to several types of paper. This unexpected observation emphasized the importance of location odor and resulted in later experiments being run with HCl-processed filter paper which had no demonstrable odor to bees.

Better techniques for controlling odor were initiated after the experiences of this early experiment, and the resulting data are worthy of mention. A feeding site (E_1, Fig. 2) located 360 m from a hive provided scented food (0.15 ml oil of cinnamon/liter 1.5 M sucrose) to ten foragers for two hours. The two-hour scented period was followed by two and one-quarter hours during which only unscented food was provided.

Figure 1 demonstrates the recruits' dependence on odor. With scent removal recruit arrivals virtually ceased. The occasional recruits arriving after the two-hour scented period can be explained by the low level of honey bee and location odors occurring at any site visited by bees. As evidenced by the slight decline in the forager visitation rate, regular foragers orienting to the experimental site

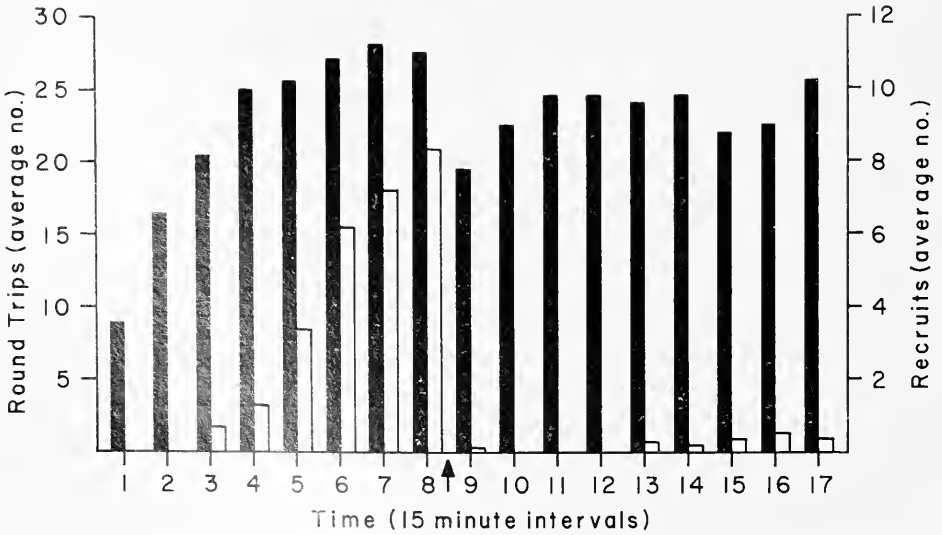

FIGURE 1. The influence of scent removal (arrow) on recruit arrivals (white bars) at a feeding site (E_1, Fig. 2) to which ten foragers were making round trips (black bars).

primarily by landmarks were influenced by odor differences as well (see also Ribbands, 1955).

Wenner, Wells, and Johnson (1969, page 84) later obtained data consistent with this result while working with unscented food in a low scent area. They reported: "That bees locate a food source by olfaction is especially possible in view of the extremely low recruitment rate of regular foragers collecting unscented sucrose at an unscented site. On 25 July 1968, for instance, in the absence of a major nectar source for the colony, we received only five recruits from a hive of approximately 60,000 bees after ten bees had foraged at each of four stations for a total of 1374 round trips during a three-hour period." (*Recruitment rate* referred to in the above is equivalent to *recruit success rate* as used in this paper.)

Since foragers dance more frequently for an unscented site (Wells and Wenner, 1971) and presumably recruit more bees to the field, the reduction in recruit arrivals after scent removal appears to be a powerful indicator of the dependence of recruits on odor for success. The recruit success rates cannot be correlated with dance frequency but support the concept of a recruit search behavior based on the odor characteristics of the feeding site. The further dependence on the number of foragers visiting a site may simply be an expression of this odor dependence in terms of bee and bee-carried odors at the feeding site and distributed by these foragers between that site and the hive.

The spatial and temporal distributions of searching bees

The spatial and temporal distributions of searching recruits were determined with Italian strain honey bees searching for one of two potential feeding sites (E_1 or E_2) at a distance of 360 m from their hive (see Fig. 2). During the course of experimentation, the wind velocity was less than 5 m/sec. The flight line flown by ten regular foragers to E_1 was crossed by a southwest wind, while in a later experiment a western wind was nearly parallel to the flight line between the hive and E_2.

As mentioned earlier the addition of a single scented station within the system of searching bees should attract nearby recruits, giving a density value for that particular location. If left for a short period of time or if the earliest intervals of the monitoring periods are examined, a close approximation of the distribution of searching bees may be formed on the basis of the data obtained from several such monitoring locations.

Eleven sites separated by angles of 22.5° surrounded the hive at a distance of 150 m and were used to determine the radial distribution of recruits. For the determination of the longitudinal and temporal distributions of searching bees, eleven monitoring sites at thirty meter intervals directly beneath the flight path were observed (Fig. 3).

The experimental periods lasted four and one-quarter hours—a two-hour build-up period followed by two one-hour monitoring periods separated by a fifteen minute interval. During the first two hours, the system of searching bees was allowed to build up with the use of scented sugar solution (0.15 ml oil of cinnamon/liter 1.5 M sucrose) at the feeding site. Unscented sugar solution was provided at that station during the last two and one-quarter hours, while a scented

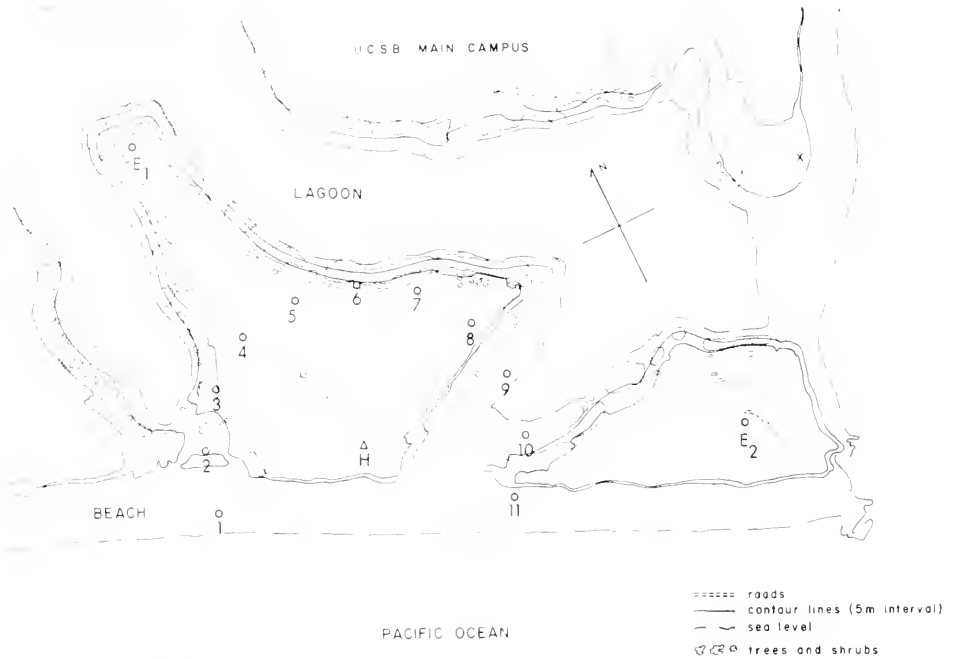

FIGURE 2. Map of the UCSB Lagoon Reserve indicating the locations of the eleven radial distribution monitoring sites (1–11), the feeding sites (E_1 and E_2), and the hive (H). The distance from the hive to the monitoring stations was 150 m and to the feeding sites 360 m.

monitoring station sampled the density of searching bees at point sources in the field. The monitoring station, located at one of the eleven selected sites during the first hour of the experimental period, was moved to a second location during the fifteen minute interval and run for a second hour. The sequence of locations thus run was determined by lot. All bees captured at the monitoring sites were killed and tallied.

As a control against the possibility that bees captured at the monitoring sites had found the unscented feeding station but were returning to the hive because of a lack of odor, the first radial distribution experiment was performed a second time with an odor plate at E_1. The odor plate consisted of a dish (like the feeding dish but screened to prevent forager contact) containing a filter paper disk kept moist with the same scented sugar solution used during the previous scented period. This dish was placed at the feeding site with the unscented dish and provided odor for the searching recruits. The conformity of the data run with and without the odor plate (see Fig. 4) allowed this control to be eliminated from other experiments.

The radial distributions obtained when using E_1 and E_2 as feeding sites both contained peaks lying on the flight path of regular foragers (Figs. 4 and 5). With the wind nearly parallel to the flight line between the hive and E_2, few recruits were captured at areas other than near this path (Fig. 5). However, when using E_1 as the feeding site the wind was approximately perpendicular to the

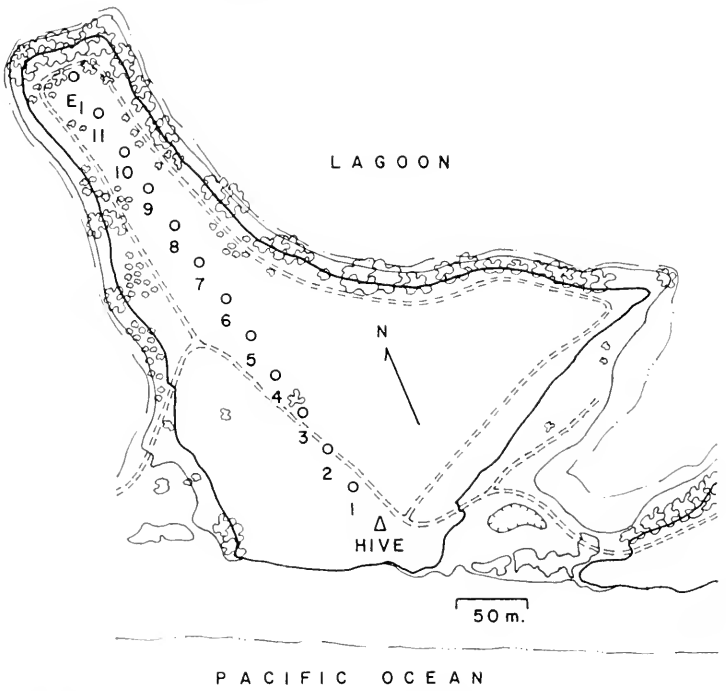

FIGURE 3. Map of the UCSB Lagoon Reserve indicating the locations of the eleven longitudinal distribution monitoring sites (1–11) and the feeding site (E_1).

flight path of regular foragers, and significant numbers of bees were captured at monitoring sites upwind and downwind of this line (Fig. 4).

Apparently, searching recruits were distributed along the flight paths of regular

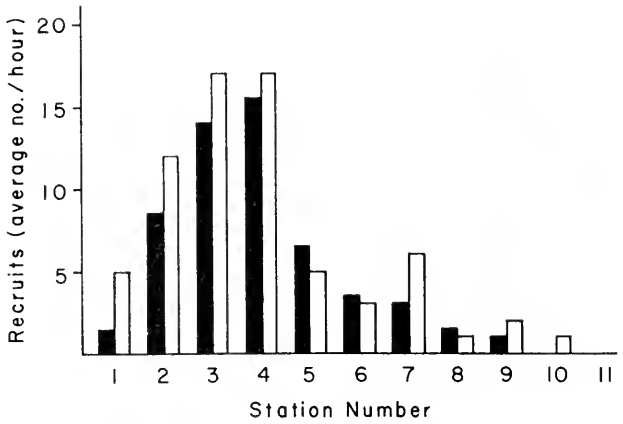

FIGURE 4. Recruit arrivals at eleven scented monitoring sites (see Fig. 2) with a forager flight path crosswind to a scented (black bars) and an unscented (white bars) feeding site (E_1).

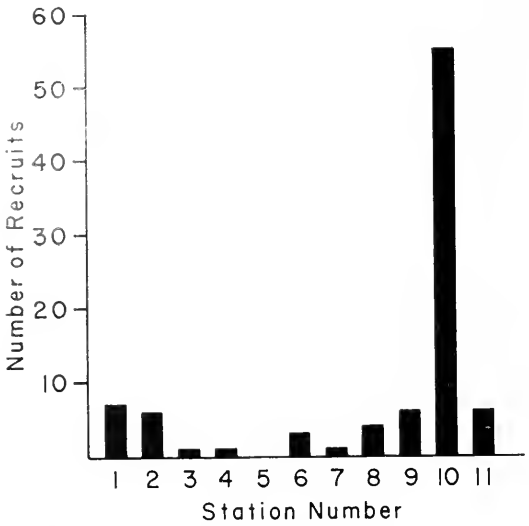

FIGURE 5. Recruit arrivals at eleven scented monitoring sites (see Fig. 2) with a wind parallel to the forager flight path between the hive and a scented feeding station (E_2).

foragers and responded positively by flying upwind (positive anemotaxis) to the odors which they associated with these regular foragers. This behavior has been described for several insects which rely on air transmission of odor signals for site location (for a discussion of olfactory guidance, see Butler, 1967). Bossert and Wilson (1963) calculated that such behavior was necessary for efficient odor following because the extremely small concentration gradient at the downwind limit of threshold response could not be used for orientation.

Recruits arrived at upwind stations in numbers proportional to each station's distance from the flight line. Apparently, the odors from the upwind monitoring sites were windborne to the flight path and attracted recruits to these sites. Thus, the data from the experiment using E_1 as the feeding site (Fig. 4) may be interpreted as an indication of the relative attractiveness of an odor source at various upwind distances (stations 1–4). When using E_2, only sites 1 and 2 were upwind of the flight path, and the number of recruit arrivals at these stations reflects their distance upwind (Fig. 5).

Of greater interest were the recruits which arrived at stations downwind of the flight line (Fig. 4). Recruits could not have been attracted from the flight line to downwind sites by odor. However, the arrival of recruits at these downwind sites may be explained if, while searching along the flight line, these bees periodically lost the odor to which they were orienting and flew downwind (negative anemotaxis) where that odor might more easily be contacted.

That this interpretation is valid may be supported by observations of recruits rapidly flying downwind after they had lost the odor of a station by drifting too far laterally or as a consequence of having flown beyond the source of odor. After such downwind flights, recruits normally reapproached from downwind on a decreasing zigzag path, presumably with the odors from the station and other bees as

cues (see also von Frisch, 1967a). This behavior occurred at the feeding site and at all scented monitoring sites in the field which received recruits. Thus, the recruit arrivals at stations 5–8 (Fig. 4) may represent the frequency distribution of distances downwind through which searchers dropped before capturing the lost odor.

These factors influencing the radial distribution of recruits are graphed in Figure 6. The distributions of points upwind and downwind of the flight line show different relationships, as might be expected from data resulting from different behavior patterns (positive and negative anemotaxis).

If odors were the foundation of recruit search behavior, the concentration of recruits along the flight line with a parallel wind should not imply communication efficiency but should merely reflect the wind direction and the resulting distribution of odors. The apparent accuracy seen in the direction of search by recruits to E_2 (Fig. 5) would predict a high success rate on the basis of the dance language hypothesis; yet, the downwind position of this feeding station would predict a low success rate according to the odor dependence hypothesis, since the wind would have carried the odor from this site away from the hive and the population of searching bees. In fact, far fewer recruits arrived at E_2 than at E_1, even though the data from the latter displayed a more diffuse distribution of recruits. These data for the two-hour scented periods at the two feeding sites are graphed in Figure 7.

The eleven sites used to determine the distribution of recruits between the hive

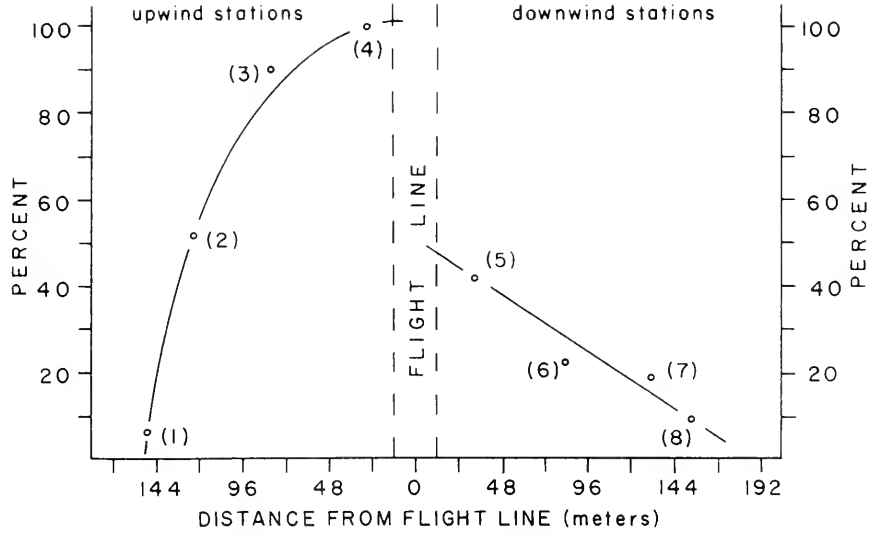

FIGURE 6. Recruit arrivals at scented monitoring stations various distances upwind and downwind of the forager flight line provided information about the influence of odor and distance on upwind flights (positive anemotaxis) and the distances downwind flown by recruits (negative anemotaxis). Each value was plotted as a per cent of the number of recruits arriving at the upwind station nearest the flight line (4). Wind velocity was always less than 5 m/sec.

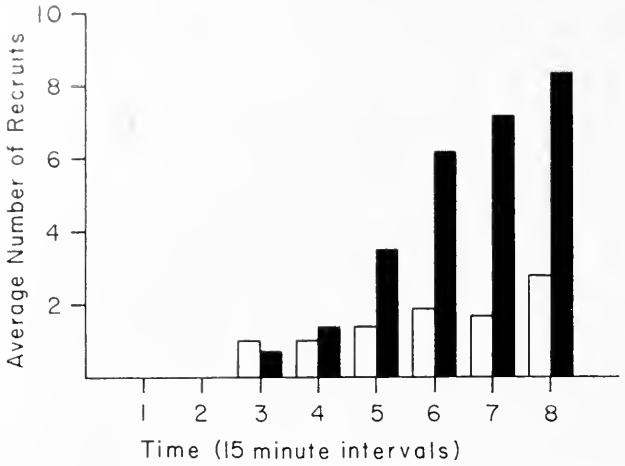

FIGURE 7. Recruit arrivals (average no./15 min) at crosswind (E_1, black bars) and downwind (E_2, white bars) scented feeding sites (see Fig. 2) without scented monitoring stations in the field.

and E_1 were monitored as two groups—the even and later the odd-numbered sites (see Fig. 3). Again, a two-hour build-up period with the feeding site scented preceded the monitoring periods with the feeding site unscented. In addition to the experimental days, the feeding site was periodically monitored while remaining scented and without other stations in the field.

Because the total data for the second hour of the two-hour monitoring period conformed to that of the first for the even-numbered sites, the odd-numbered sites were run only once—either during the first or second monitoring period. This loss of data for the first hour for some of the odd-numbered monitoring sites was unfortunate, since a later analysis revealed that the data obtained during the first half hour of the monitoring period was perhaps the more important for the determination of the distribution of searching recruits.

The data of the first hour, when partitioned into half hour intervals, show an initial distribution (Fig. 8A and C) quite different from that obtained during the second half hour (Fig. 8B and D). Apparently, the data from this first half hour more closely approximated the established distribution of searching recruits, while the second half hour reflects the build-up of recruits with the change to unscented sucrose at E_1.

In the second half hour, all sites beyond 5 received nearly equal numbers of recruits when these searchers were not drawn from this area to the scented feeding station. The area of the peak in the first half hour received substantial numbers of recruits immediately, while all other stations did not attract recruits until after 10 min. Newly accumulated searchers in the area of this peak appear to have been beyond the influence of the feeding site. That this high density was formed by searchers accumulating in the field is suggested by the decreased recruit arrival rates in this area during the second half hour (Fig. 8, sites 6 and 7), even though under normal conditions recruitment increases with time (see Fig. 1).

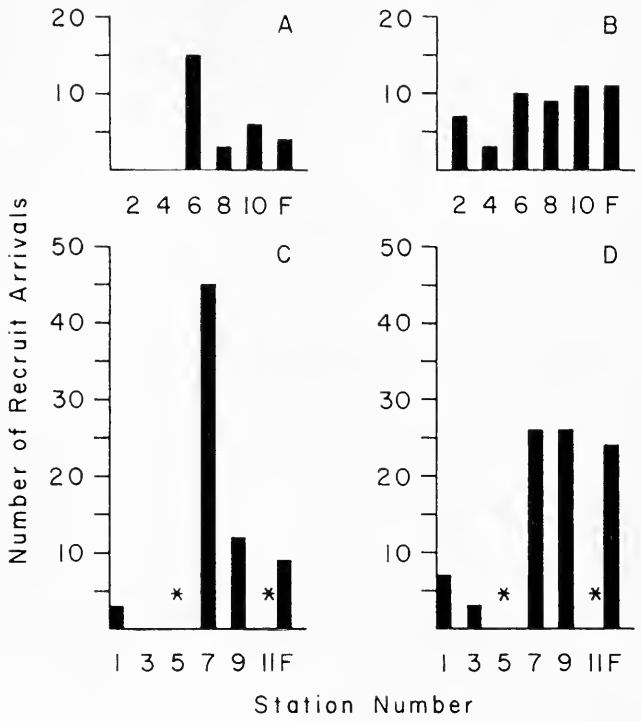

FIGURE 8. Recruit arrivals at singly-run, scented monitoring stations (see Fig. 3) compared to the feeding site (F) during the initial half hour (A and C) and the second half hour (B and D) following a two-hour build-up period. Asterisk indicates no data taken.

The distribution of the first half hour may be explained by considering that a population of searching recruits existed between site 6 and the feeding station. The removal of these bees from the more distant areas of the searching population left the highest density of searchers at sites 6 and 7. Thus, the stations, when monitored immediately after this distribution had been established, reflected the feeding station's influence. When unscented sugar solution replaced the scented solution, the feeding site no longer removed bees from the searching population and the continued presence of the monitoring station through the second half hour demonstrated the equality of attraction of new searchers to the various sites (Fig. 8B and D).

Of course under natural conditions, recruits would not be killed at the feeding place; and the searcher density would increase exponentially as these recruits recruited still others to the field. This would result in a positive feedback system with increased density leading to increased recruit success.

Even without allowing recruits to return to the hive to recruit other bees, the recruit arrival rate increases at a feeding site visited by a constant number of foragers (see Fig. 1). Wenner, Wells, and Johnson (1969) suggested that this increase, occurring at times when an increase in dancing was not exhibited in the hive, could be caused by an odor accumulation in the hive. An alternative now

would be that the increasing recruit arrival rate may be due to an increasing number of recruits accumulating along the flight line. The extended search times of unsuccessful, as well as successful, recruits (Esch and Bastian, 1970) and the data from stations 6 and 7 substantiate this field accumulation of searching bees.

With the same arrangement of monitoring sites used in the last experiment (see Fig. 3), arrival times of the first recruits were recorded at sites 3, 6, 9, and the feeding site (E_1). Unlike the last experiment one of the intermediate stations was opened with the feeding station, both were scented, and both remained open from 0800 until fifteen recruits were captured at each site. All recruits captured were killed and their arrival times recorded. Ten bees collected scented sugar solution (0.13 ml oil of cinnamon/liter 1.0 M sucrose) at E_1; the same solution was offered at the monitoring station. E_1 was run alone one day as a standard.

Because the scented feeding and monitoring stations were placed in the field together, recruit arrivals do not reflect the distributions of established populations, but reflect the alterations of such distributions as they grew about the two odor foci. The delays of recruitment at E_1 and the times of recruit arrivals at the various sites may be compared with each other and the more natural distribution seen in Figure 8. The recruit arrival times for each of the monitoring stations and the feeding site when run alone are displayed in Figure 9. The recruit arrival times for the feeding site when run alone and with each monitoring site are compared in Figure 10.

Most interesting are the large time differences for the arrivals of the first recruits at the various stations (Fig. 9). Using the feeding site when run alone as a standard, station 6 received its fifteenth recruit at about the same time as E_1;

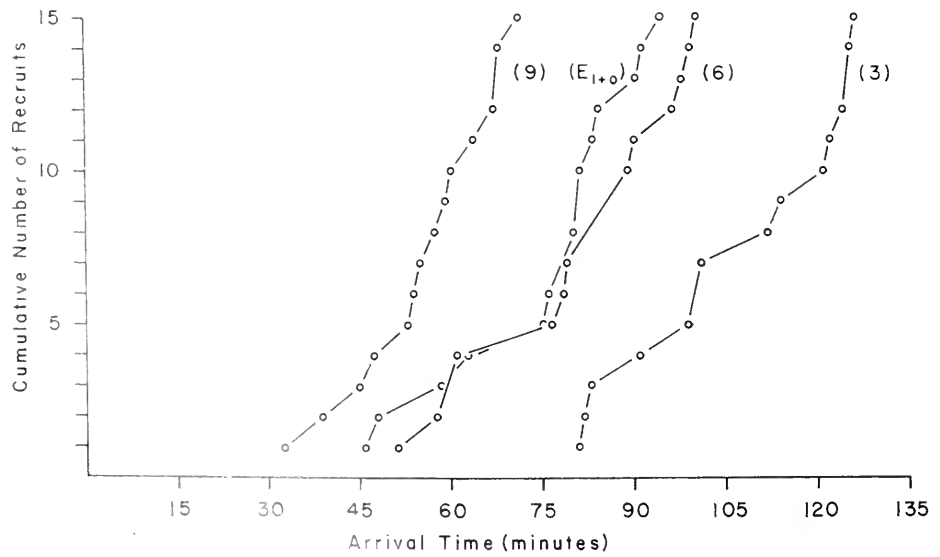

FIGURE 9. Temporal distributions of the first fifteen recruit arrivals at three monitoring sites (3, 6, and 9) compared with that at the feeding site ($E_1 + 0$) when run alone (see Fig. 3).

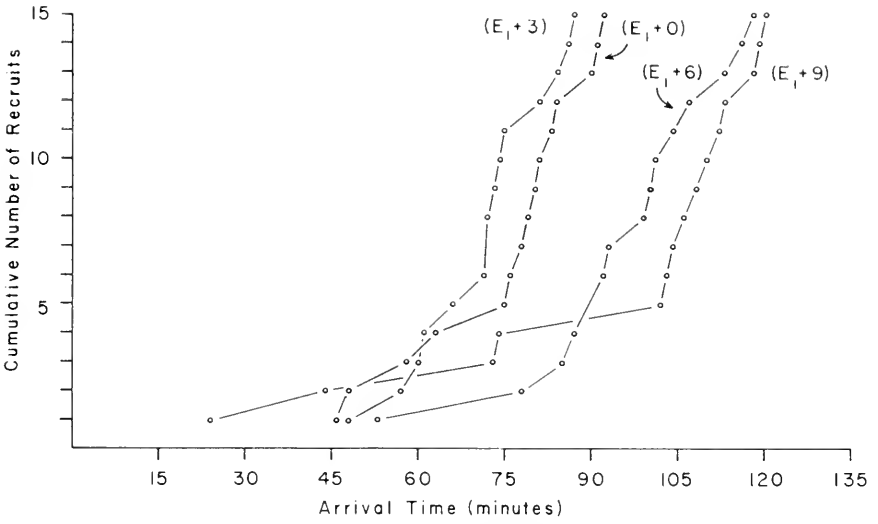

FIGURE 10. Temporal distributions of the first fifteen recruit arrivals at the feeding site (E_1, Fig. 3) when run alone ($E_1 + 0$) or with one of the monitoring sites ($E_1 + 3$, +6, or +9).

while station 9 received fifteen recruits approximately 20 minutes before E_1 and station 3 over 30 minutes after. Because of these extremely long intervals between the onset of recruitment at areas only a short distance apart along the flight path, one cannot assume that recruits were distracted from a direct flight to the goal. The feeding site run without intermediate "distractions" received recruits 20 minutes later than 9 and was only 90 m farther along the forager flight path. In addition, station 6, 90 m nearer the hive than 9, received recruits 20 minutes later also.

The later onset of recruitment at E_1 with the accumulation of bees from the searching population at sites 6 and 9 (Fig. 10) and the long intervals between the onset of recruit arrivals along the flight path may well support the concepts of the bee density dependence of recruit arrivals and the growth and expansion of the searching population of recruits. Earlier experiments demonstrated that recruit success was dependent on the field density of foragers (Table I), even though these bees may have been from different hives (Table II). These data and the earlier arrival of recruits at sites of high bee density suggest that searchers in the field are reinforced in their efforts by the presence of other bees.

An hypothesis consistent with the data from the previous experiments suggests that a population of searching bees accumulates within an area under the influence of bee and bee-carried odors. A monitoring station placed in this population of searching bees will receive recruits in numbers and times dependent on the odor of the station and the density of bees searching in that area for the same odor. For example, station 9 placed in the center of such a distribution received the first recruits; while the feeding site and station 6 at the extremes of the distribution and at equal distances from its center received recruits later and with approxi-

mately the same delay. Station 3, outside the early distribution received recruits nearly one hour later than station 9.

Although the data suggest that it is not the dance information which guides recruits to the proper distance, the data do not suggest why recruits reject the stations closer to the hive. Perhaps a study of the scenting behavior of foragers as they approach a feeding site or of the search behavior of new recruits as they leave the hive will provide an answer.

Recruit success as a function of wind

The effects of wind were minimized in the foregoing experiments by considering data from only those days which received wind from a relatively constant direction and with a speed of less than 5 m/sec. Certainly, wind speed and direction are factors which must be considered when dealing with flying insects, especially if windborne odors may be contributing to the dynamics of their search behavior. By assuming that recruits possess preflight directions, investigators have considered wind as a complicating factor and have used "adverse wind conditions" as an explanation of data anomalous to the dance language hypothesis (*e.g.*, von Frisch, 1967a). However, if the success of searching bees is mediated by odors, then wind should not be studied as a complicating factor but as a necessary condition for recruit success. For this reason the following experiments were designed to measure the effects of wind on the success of searching bees.

Five potential feeding sites were chosen along a line between a cordovan strain hive and a hive containing the darker Italian strain bees (Fig. 11). The hives were placed so that the line of sites lay in an approximate east-west direction and, therefore, at an angle to the prevailing southeast and southwest winds. The odor from a scented (0.05 ml oil of clove/liter 1.0 M sucrose solution) station placed at one of the feeding sites and visited by a group of foragers from each hive would be carried more toward one hive than the other. In this way the two hives served as reciprocal controls.

Wind direction was determined at five-minute intervals using a wind vane; and wind force was monitored continuously with an anemometer and recorded as revolutions/min. All other data were collected as usual.

Between 18 July and 8 August 1969 the wind was consistently from the southwest, toward the cordovan strain hive. A feeding station visited by ten bees from each hive monitored the recruit arrivals at each site on four different days during two complete runs up and down the line of sites. The station was first placed at site IV and on successive days was placed at adjacent sites along the line, except at the end sites which were run two consecutive days before reversing direction. The combined data for each site are displayed separately for the two hives in Figures 12 and 13.

It is clear that wind was a limiting factor for recruit arrivals at various distances from the hives. The slight distance dependence exhibited by the cordovan strain bees from the downwind hive (Fig. 12) contrasts sharply with that of the Italian strain bees from the upwind hive (Fig. 13). Bees which searched for a feeding site upwind of the hive were more successful than those bees which searched for a site downwind of the hive. These data are consistent with those ob-

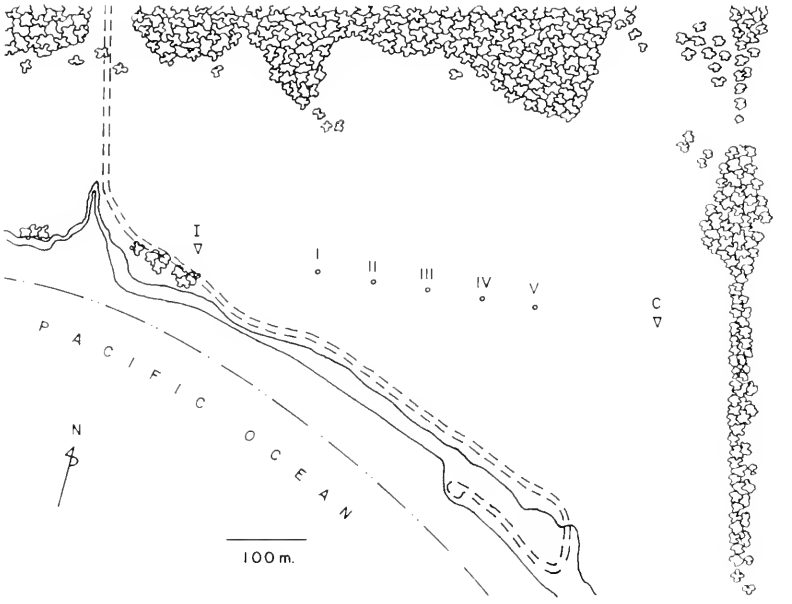

FIGURE 11. Map of the Santa Barbara Shores area indicating the locations of the five feeding sites (I–V) and the Italian (I) and cordovan (C) strain hives. The distance between feeding sites was 75 m and between hives, 600 m.

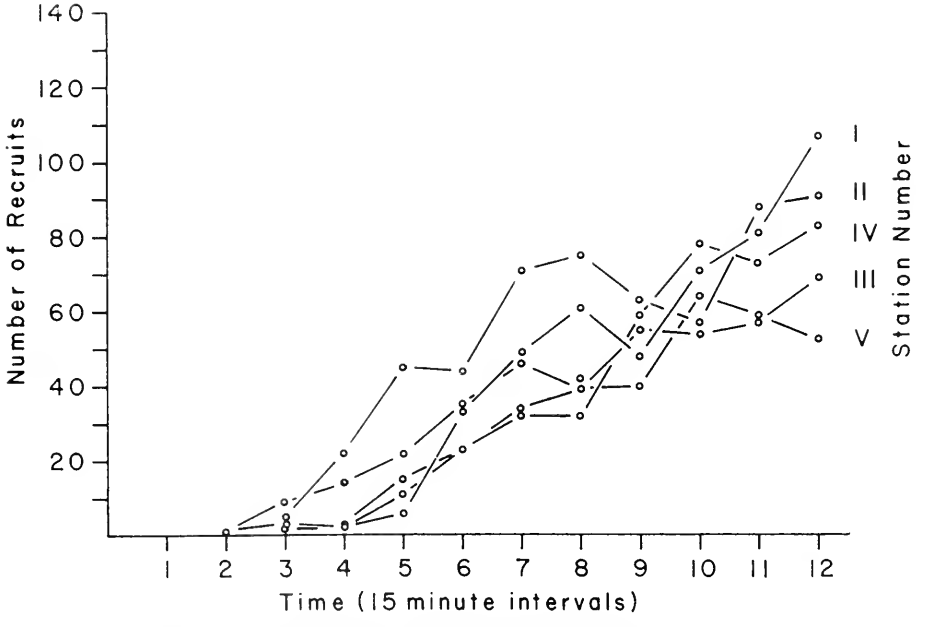

FIGURE 12. Recruit arrivals from the downwind cordovan colony at each of five feeding sites (I–V, Fig. 11) various distances from the hive.

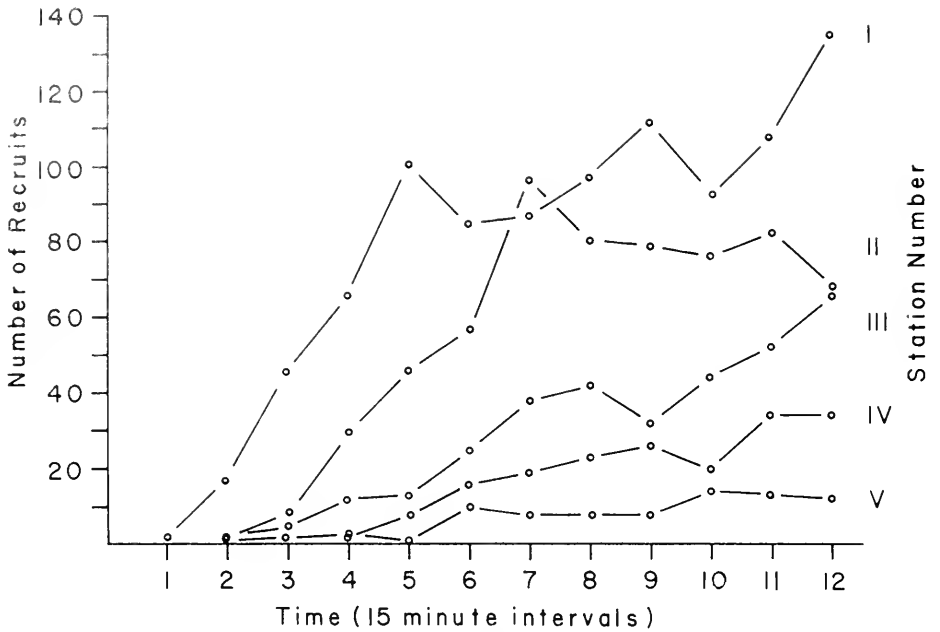

FIGURE 13. Recruit arrivals from the upwind Italian colony at each of five feeding sites (I–V, Fig. 11) various distances from the hive. Compare with the downwind hive data (Fig. 12).

tained during experiments using a crosswind and a downwind feeding station (Fig. 7) and suggest that wind direction was only indirectly important to the success of recruits, since flying against the wind aided, not hindered, their progress. Apparently, at the velocities considered wind direction was a factor only as it affected the distribution of odor and, thus, the search pattern of recruits.

The higher number of recruits which arrived at feeding sites upwind of the hive may have been a consequence of reduced search times of these successful bees. This possibility was checked indirectly by another experiment. Ten bees from each hive were allowed to forage at site III, midway between the hives, until the first recruits were captured. The temporary capture of one group of foragers then stopped the outflow of recruits from that hive; and a maximum search time for successful recruits was estimated from the arrival time of the last recruit to find the station. The later release of these same foragers allowed a minimum search time to be estimated from the subsequent arrival of the first recruit successful at locating the station. The foragers from the other hive provided feeding activity at the station and on other days served as the experimental group.

Several factors limit the reliability of these estimates. The time required to capture all foragers was as great as 3 min 30 sec; and the lack of forager flight between the hive and the feeding site after this capture created an unnatural condition for searchers remaining in the field. After the release of caged foragers, the times of the first dances were unknown. Finally, there can be no estimate of the search

times of unsuccessful recruits. (Unsuccessful recruits' flights have been given as an average of approximately 7 minutes by Mautz, 1971.)

Although the absolute value of the maximum and minimum search times may not be obtained by this method, their estimates may be compared for data collected during different wind conditions. Therefore, wind direction could be examined as a factor influencing recruit search times.

As mentioned above, feeding site III served as the experimental station at which ten bees from each hive foraged. After attaining a level of ten recruit arrivals per fifteen minute interval from the experimental hive, the regular foragers from that hive were captured and retained in a ventilated, wax-lined box. The arrival times of subsequent recruits from that hive were recorded. Following the fifteen minute interval during which no additional recruits arrived, the captured foragers were released, and the arrival times of recruits were again recorded for thirty minutes.

The data were partitioned into that group which came from a hive upwind of the feeding station and that group which came from downwind of this station. Arrivals after the capture and release of regular foragers are displayed in Figures 14 and 15, respectively. Maximum and minimum search time estimates for successful recruits are shown in Table III.

Both maximum and minimum search time estimates were shorter when the station was upwind of the experimental hive, and show a much narrower range (6:04–12:30) than did those for the station when downwind of the hive (11:20–36:10). Additionally, the arrival pattern after the release of foragers showed a more rapid acceleration in recruit arrivals to the upwind station. These data again agree with earlier observations that wind direction affects recruit success and

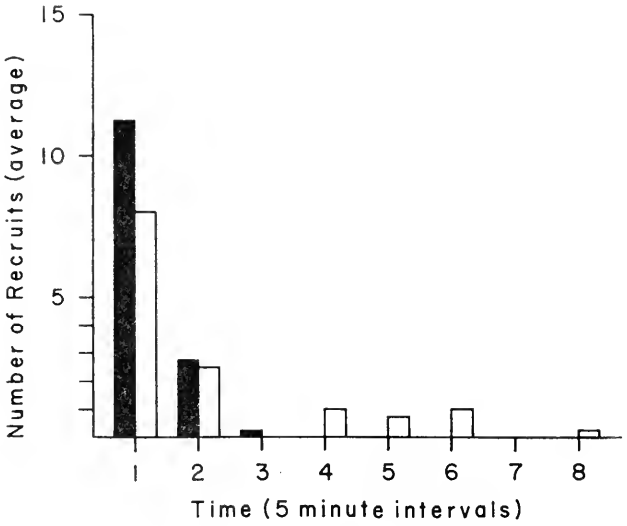

FIGURE 14. Recruit arrivals (average no./5 min) at feeding site III (Fig. 11) from the downwind hive (black bars) and the upwind hive (white bars) after the capture of their respective foragers.

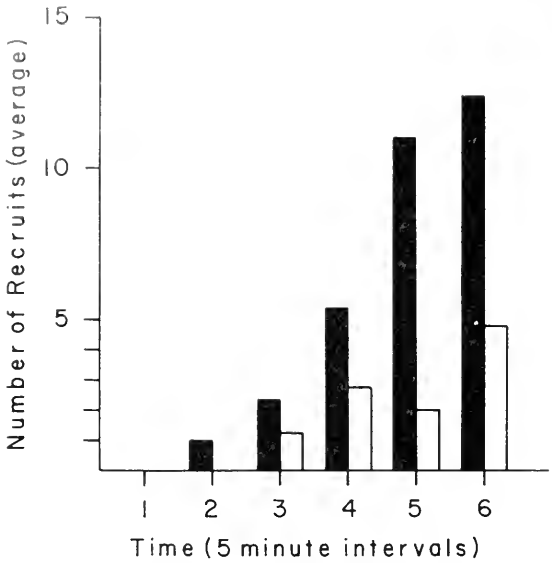

FIGURE 15. Recruits arrivals (average no./5 min) at feeding site III (Fig. 11) from a downwind hive (black bars) and an upwind hive (white bars) after the release of their respective foragers.

provides that, at least in part, this influence was due to the rapidity with which a recruit was able to locate the scented feeding site at its direction from the hive relative to the wind.

TABLE III

Arrival times of the last recruit after forager capture and the first recruit after forager release at site III (Fig. 11) partitioned into those groups arriving from the downwind and upwind hives

Date	Last recruit captured	First recruit captured
	Downwind hive	
12 Aug. 69	6 min 4 sec	10 min 45 sec
12 Aug. 69	8 min 10 sec	6 min 45 sec
19 Aug. 69	9 min 40 sec	10 min 10 sec
21 Aug. 69	11 min 20 sec	12 min 30 sec
	Average 8.8 min	Average 10 min
	Upwind hive	
14 Aug. 69	26 min 30 sec	11 min 20 sec
15 Aug. 69	36 min 10 sec	17 min 30 sec
16 Aug. 69	5 min 30 sec	12 min 10 sec
20 Aug. 69	29 min 00 sec	13 min 00 sec
	Average 24.3 min	Average 13.5 min

After observing that recruit success was dependent on the directional component of wind, the other vectorial component, wind speed, was examined as a possible factor influencing recruit success. Often during the course of experimentation, recruits appeared to arrive in clusters at times of increased wind speed. This subjective observation led to tests conducted at SBS between 23 August and 6 September 1969.

Again, site III located midway between the hives was used (Fig. 11). Ten bees from the Italian strain hive were allowed to forage at the station. During the days of the first half of this experiment, ten bees from the cordovan strain hive also foraged at this station; yet, during the last half of the experiment, only five cordovan bees were allowed to forage with the ten Italian strain bees as part of a concurrent experiment (see Table II). No other manipulation of bees except the usual killing of recruits and replacement of foragers was performed.

The data collected over a three-hour period were partitioned as a one-hour build-up period followed by a two-hour experimental period. The numbers of recruits arriving during the first and second hours of this two-hour experimental period were compared to changes in wind speed during those times. As stated, wind speed was continuously monitored using a wind speed anemometer and was recorded as revolutions per minute. The wind was always from the southwest. Data for the two hives appear in Table IV; and that for the upwind Italian strain hive is graphed in Figure 16.

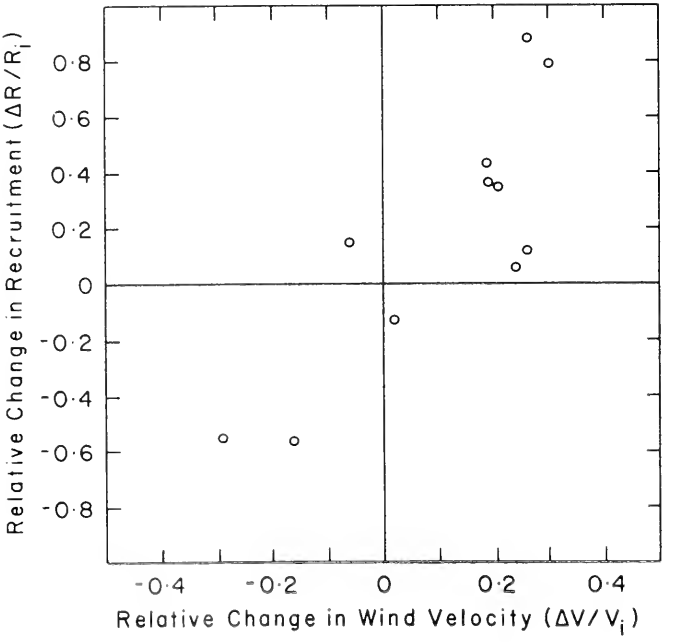

FIGURE 16. The relative change in wind velocity versus the relative change in the number of recruit arrivals to a feeding site (III, Fig. 11) from the upwind Italian strain hive (see Table IV and the text for further details).

TABLE IV

Changes in wind velocity (V), measured as anemometer revolutions/hour, compared with changes in the levels of recruit arrivals from the downwind cordovan (C) and upwind Italian (I) strain hives at feeding site III (Fig. 11) over a two hour period

Date	No. of recruit arrivals				No. of anemometer revolutions		R_1-R_2/R_1		V_1-V_2/V_1
	1st hour (R_1)		2nd hour (R_2)		1st hour (V_1)	2nd hour (V_2)			
	(I)	(C)	(I)	(C)			(I)	(C)	
23 Aug. 69	31	105	42	66	174.5	211.5	0.35	−0.37	0.21
24 Aug. 69	41	91	18	56	237.5	199.5	−0.56	−0.38	−0.16
25 Aug. 69	39	133	45	102	278.5	262.5	0.15	−0.23	−0.06
27 Aug. 69	87	67	39	79	282.0	203.0	−0.55	0.18	−0.28
28 Aug. 69	40	33	35	39	124.0	127.0	−0.13	0.18	0.02
31 Aug. 69	32	84	34	102	303.0	376.0	0.06	0.21	0.24
1 Sep. 69	14	32	19	61	328.0	389.0	0.36	0.91	0.19
3 Sep. 69	14	26	20	47	222.0	264.0	0.43	0.81	0.19
4 Sep. 69	28	16	50	33	188.0	245.5	0.79	1.06	0.30
5 Sep. 69	26	42	49	37	309.0	389.5	0.88	−0.14	0.26
6 Sep. 69	34	51	38	63	220.0	274.0	0.12	0.24	0.26

The cordovan strain hive data showed no obvious correlation between changes in the level of recruit arrivals and increases and decreases in wind speed. This would be expected on the basis of the upwind position of the feeding site from this hive. Since bees are able to compensate for changes in wind velocity and maintain a relatively constant ground speed (Wenner, 1963), searchers downwind of an odor source need only exhibit positive anemotaxis upon perceiving the proper combination of odors to quickly reach the goal. The successful recruits from the cordovan strain hive, having flown upwind to the odor source, display this anticipated lack of wind speed influence.

The Italian strain recruits, on the other hand, travelled downwind to the odor source and had their progress influenced by wind speed changes. These recruits were less successful than cordovan strain recruits; but within this group, bees searching during higher wind speeds were the most successful (Fig. 16). The exhibition of negative anemotaxis by searchers after odor loss offers an explanation for this wind speed dependence.

The bee and bee-carried odors from the flight path of foragers would be carried more toward the feeding site than the hive. Recruits searching downwind of the flight path would perceive the odors from foragers flying toward the feeding site as pulses of odor, since these odors would be trailed as a front at an angle to the wind's direction. On the other hand, recruits following odors from foragers flying toward the hive would be led back to the hive on a continuous trail.

Outward flights of foragers would be responsible for the recruits' progress toward the feeding site. With the perception of each odor pulse, searchers would immediately orient upwind and lose the scent. Thus, the subsequent downwind flights after each odor loss would bring these bees nearer the goal. Higher wind speeds would allow longer downwind flights, and these longer flights would be responsible for the shorter search times exhibited.

Search dynamics: odor, dance, and the scent gland

There is now a substantial body of evidence which demonstrates complex interrelationships among the variables of the recruitment process. Those variables seen to have the greatest importance to recruit success are the initial dance, food odor, forager density in the field, and the vectorial components of wind. Wenner and Wells (in press), working with foragers performing disoriented dances, have shown that at least the direction information of the dance is not necessary for recruit success. Odor, however, is apparently crucial to recruit sucess. The further dependencies of recruit success and distribution on the field density of foragers carrying this odor and the vectorial components of wind distributing this odor suggest a recruitment system based on windborne odor trails between the feeding site and the hive.

The apparent accuracy in the direction and distance of search by recruits now seems to be due, not to preflight information enabling a direct flight to the food source, but to the association of recruits with the odors from the forager flight path and the feeding site itself. The extended search times of flights repeatedly taken by unsuccessful, as well as successful, recruits demonstrates the accumulation of these bees within a searching population outside the hive. As a consequence these data have failed to support the dance language hypothesis.

Apparently positive feedback systems operating on the accumulated searching population enhance the ultimate success of recruits searching in the field. Certainly, the cumulative attractiveness of an area with increasing bee density is an example of such a system. The disproportionate increase in recruit success with increased bee density at the feeding site and the earlier arrival times of recruits at stations placed within a high searcher density suggest some communication among bees in the field as the foundation of this positive feedback system.

Because recruits frequently display Nasanov gland scenting as they approach monitoring stations and orient to the exposed Nasanov glands of both foragers and recruits near these stations and the feeding site, it may well be that the odors from this gland serve as a link among foragers and recruits in the field. Although not necessarily an attractant (Wells and Wenner, 1971), Nasanov gland scent may orient and reinforce recruits searching in the presence of odors to which they have been conditioned. Thus, the distribution of the searching population would initially be dependent on the odors from foragers and the feeding site while the growth of this population would allow later recruits to orient to these other searchers—a positive feedback system enhanced by the cumulative influence of new recruits.

Wells and Wenner (1971) have reported evidence suggesting that both forager dancing in the hive and Nasanov gland exposure in the field are dependent on the size of the potential recruit pool. Their interpretation (page 207) was: "unsuccessful recruits not only contribute to Nasanov gland exposure through their contact with oriented foragers in the field, but these unsuccessful recruits also return to the hive and contribute to an enlarged recruit pool. Presumably, contact with numerous available recruits induces successful foragers to dance, leading to the correlations we have observed." Thus, two positive feedback systems coordinating the enlargement of the searching population with increased scenting along the flight

path and near the feeding site could be controlled by and lead to the success of the searching population.

Although speculative this interpretation compares favorably with the observations of temporal distributions of recruit arrivals along the forager flight path. Conceivably, the concentrations of both forager and food scent near the feeding site accounted for the higher density and earlier arrival of recruits at areas of the outer half of the forager flight path. Apparently, the growth and expansion of the searching population with its growing influence on foragers and new recruits was responsible for the progression of recruit arrivals in both directions along the flight path from the area of earliest recruitment.

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Summary

Some variables in the recruitment process of honey bees were studied as they affected the distribution and success of the searching population in the field. The dance language and odor dependence hypotheses were contrasted and their predictions compared with the following observations.

1. Recruits were attracted to the odors from the food which were carried by foragers and were dependent on these odors for success.

2. A monitoring of recruit densities in the field demonstrated an association of searchers with the forager flight path.

3. The degree of correspondence between the distribution of recruits and the direction of the flight path to the feeding site was correlated with wind direction, not search efficiency.

4. Feeding stations upwind of the hive provided the highest recruit success rates, shortest search times, and the least dependence on wind speed. Downwind stations provided the lowest recruit success rates, the longest search times, and the greatest dependence on wind speed.

5. A disproportionate increase in recruit success with an increase in the number of foragers visiting a feeding site was correlated with the density of the foragers in the field.

6. Increased bee densities at the feeding site, even with bees from different hives, increased recruit success and shortened search times.

7. The progression of and the extremely long intervals between the onset of recruit arrivals at areas along the forager flight path suggested communication among bees in the field and a dependence of recruit success on the density and growth of the searching population.

These observations are compatible with an odor dependent search behavior and together fail to support the predictions of the dance language hypothesis. Dance attendants appeared to have been conditioned to the odors associated with re-

turning foragers and, after leaving the hive, entered a searching population dependent on these odors for success. The dependence of recruit success on food odor at the feeding station, the density of foragers between this station and the hive, and the direction of the wind indicates that the integrity of the forager flight path was extremely important to this success. The distributions and extended search times of recruits indicated a search behavior based on positive anemotaxis during the perception of the proper combination of odors and negative anemotaxis after the loss of this stimulation.

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PACHYCHELES MONILIFER (DANA, 1852): THE DEVELOPMENT IN THE LABORATORY OF LARVAE FROM AN ATLANTIC SPECIMEN WITH A DISCUSSION OF SOME LARVAL CHARACTERS IN THE GENUS (CRUSTACEA: DECAPODA; ANOMURA

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The porcellanid crab genus *Pachycheles* which is essentially tropical and worldwide in distribution consists of 14 Atlantic, 18 Eastern Pacific and 11 Indo-Pacific species (see Haig, 1956; 1960; 1966b). The larval development is known completely or in part for only six species (Table I), two from each of the mentioned oceans. In the western hemisphere two species of the genus are known to occur on both sides of the Panamanian isthmus, *Pachycheles chacei* Haig, 1956 and *P. monilifer* (Dana, 1852, 1855). The eastern Pacific record of the latter species, however, is based on a single juvenile female collected at La Libertad, Ecuador. Thus, it may not be *P. monilifer* but a new, although very closely related, species (see Haig, 1960, page 162). In the Atlantic *P. monilifer* is known to occur from Sebastian Inlet, Florida (a northern extension of range hereby noted in this study) to Ilha da Victoria, Brazil (Haig, 1966a).

Previous studies on larvae from other trans-Panamanian porcellanid crabs have shown that Atlantic larvae differ from their eastern Pacific counterparts in many respects (Gore, 1970, 1971, 1972a). Hence, the larval development of *Pachycheles monilifer* is of interest for additional reasons besides that of of identifying such larvae in the plankton. If *P. monilifer* does indeed have an eastern Pacific population then it is of interest to compare the larvae of the Atlantic population with that of the eastern Pacific to determine what differences, if any, exist between the two. Secondly, should the Pacific specimen recorded by Haig (1960, page 160) ultimately prove to belong to a specifically distinct population then comparison may still be made between the larvae of both species as a possible means of providing further grounds for their separation (see *e.g.*, Gore and Abele, in press). In addition, continued studies on larvae reared from known adults may allow elucidation of relationships in the genus as well as providing identification of such larvae on a worldwide basis.

In this paper the complete larval development of an Atlantic specimen of *Pachycheles monilifer* is described and illustrated. The larvae are compared with those known from other species in the genus and some tentative features which may characterize the genus on a worldwide basis are suggested.

Materials and Methods

Two ovigerous females were collected from the Indian River region of the central Florida east coast. One was taken at Rio Mar Reef, Vero Beach, the other

at Sebastian Inlet, on April 12 and 13, 1972, respectively. Both inhabited the interstices of phragmatopomid worm reefs common in the areas. Each female occurred with a male. Prior to this report *Pachycheles monilifer* was known only as far north as Hillsboro Reef, in south Florida (Haig, 1956, page 14).

The females were returned to the Harbor Branch Foundation Laboratory, Ft. Pierce, Florida (HBFL) placed in 19 cm diameter glass bowls filled with non-flowing seawater (34‰) until hatching occurred on 13 April. A series of 48 zoeae were placed, one each, in individual compartments of 24-compartmented rearing trays. No controlled temperature units were used. Instead, all stages were exposed to ambient light, and were held at room temperature which was adjusted via central reverse-cycle air conditioning to maintain a water temperature of about 28° C (± 2 C°, mean = 27.8° C). Water was changed and *Artemia* nauplii were provided as food every day. Salinity of ocean water stored in a 13-gallon container and used throughout the study was 34‰. Measurements of zoeae and megalopae were made with a Wild slide micrometer and are expressed as the arithmetic average of the specimens examined. For further details involving rearing, measuring and describing of larvae consult previous studies by Gore (*e.g.*, 1970, 1972a).

A spent female and a complete series of larval stages are deposited in the National Museum of Natural History, USNM 141323, 141324; a second series will be deposited in the British Museum of Natural History.

Results

Rearing experiment

Pachycheles monilifer hatches as a pre-zoea, remaining thus for as little as five minutes or up to one hour before molting to the first zoeal stage. This stage is not described.

At temperatures of 26.5–28.5° C the first zoeal stage lasts four to six days, but usually five days before molting to stage II. At temperatures of 28.5 to 30° C the second zoeal stages lasts five, usually six, and rarely as long as nine days. At temperatures of 26.5–28.5° C the megalopal stage lasts from six to 10 days, with

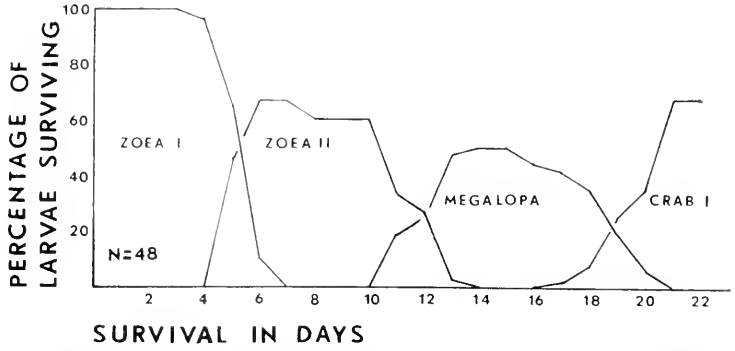

FIGURE 1. Percentage and duration of survival at room temperature of larvae of *Pachycheles monilifer* (Dana). N reflects the number of larvae reared in the series.

TABLE I

Comparison of some zoeal features in six species of Pachycheles*

ZOEA I	*P. monilifer*	*P. haigae*	*P. pubescens*	*P. rudis*	*P. stevensii*	*P. natalense*
Antenna						
Exo::Endo	1.5×	2×	1.75-2×	2×	*ca.* 2×	¼×
Exopod	3 spines	4 spines	3–4 spines	3 spines	3 spines	3 spines
Mandible						
Palp	Absent	Absent	Absent	Absent	Absent	Absent
Maxillule						
Endopod	4 setae	4 setae	3–4 setae	4 setae	4 setae	4 setae
Endites	9 basal	9 basal	8–9 **basal	9 basal	7 basal**	9 basal**
	8 coxal	8 coxal	8*–9 coxal	8–9 coxal	8 coxal**	8 coxal**
Maxilla						
Endopod	4,2,3 setae	9(?4,2,3)**	3–4,2,3 setae	4,2,3 setae	4,2,3 setae	9(?4,2,3)**
Scaphognth.	4–5 + 1 setae	7 + 1 setae	6–7 + 1 setae	6–7 + 1 setae	6 + 1 setae	7 + 1 setae
Maxilliped 1						
Coxopod	2 setae	No data	2 setae	2 setae	No data	No data
Basipod	2,2,2,3	2,2,3,3	2,2,2–3,3	2,2,2,3	?,2,2,3**	2,2,3,3
Endopod	3,3,2+3,8–9+1	2,4,3+4,10+1	2–3,3,1–2+3, 8–9+1	3,2,2+3–4,7–9+1	3,3,2+3,6+1**	"2,4,6,8" = 2,4, 2+4,8+1?**
Maxilliped 2						
Coxopod	Naked	No data	Naked	Naked	No data	No data
Basipod	1,2	1,1	1,2	1,2	1,2†	1,2
Endopod	2,2,1+2,5+1	3,3,1+1,7+1	2,2,1+2,5–6+1	2,2,1+2,5+1	2,2,1+2,4+1†	2,2,1+2,5+1
Abdomen somites						
Lateral spines	3,4,5	5	4,5	4,5	3**,4**,5	5
Dorsal teeth	Present	No data	Present	No data	Present	No data
Telson setae						
Distal Hooks	1,2	1,2	1,2	1,2	1,2	1,2,3,4,5
Carapace margin	Serrate	Smooth**	Smooth**	Smooth**	Serrate	Smooth**
ZOEA II						
Antennule						
Aesthetascs	4,5,3,3,2+3–4	"8"+"a series"	4,5,3,3,2+3–4††	4,5,3,3,2+3–4	No data	No second stage
Protopod	No long seta	No long seta	1 long seta	1 long seta	No data	data available

TABLE I—(*Continued*)

ZOEA II	*P. monilifer*	*P. haigae*	*P. pubescens*	*P. rudis*	*P. stevensii*	*P. natalensis*
Antenna						
Exo::Endo.	$\frac{2}{3}$	$\frac{2}{3}$**	$\frac{2}{3}$	$\frac{2}{3}$–$\frac{3}{4}$	$\frac{1}{2}$	
Exopod	3 hairs	Naked	Naked	Rudimentary spines or naked	Naked**	
Mandible						
Palp	Present	Absent	Present	Present	Present	
Maxillule						
Endopod	4 setae	4 setae	4 setae	4 setae	4 setae†	
Endites	10 basal	11–12 basal	10 basal	10 basal	No data	
	10 coxal	10 coxal	10–11 coxal	10–11 coxal	No data	
Maxilla						
Endopod	4,2,3 setae	6,2 setae	4,2,3 setae	4,2,3 setae	No data	
Scaphognth.	25 setae	24 setae	21–22 setae	20–22 setae	No data	
Maxilliped 1						
Coxopod	2 setae	No data	2 setae	2 setae	No data	
Basipod	2,2,2,3	2,2,3,3	2,2,2,3	2,2,2–3,3	?,2,2,3**	
Endopod	3+1,3+1,2+3+1, 9–10+1	2+1,4+1,3+4 +1,10+1**	3+1,3+1,1+3+1 6–10+1	3,2+1,1–2+3–4 +1,8–9+1	3+1,3+1,2+3+1, 7–8+1**	
Maxilliped 2						
Basipod	1–2,2	1,2**	1,2	1,2	1,2**	
Endopod	2+1,2+1,1+2+1, 5+1	3+1,3+1, 1+2+1,7+1	2+1,2+1,1+2+1 5–6+1	2–3,2+1,1+2+1, 5+1	2+1,2+1,1+2+1, 4(?5)+1	
Maxilliped 3	2 setae	2 setae	4–6 setae	2 setae	1–3 setae	
Telson setae						
Distal Hooks	1,2	1,2	1,2	1,2	1,2	
Carapace margin	Serrate postero-laterally	Smooth**	Smooth**	Smooth**	No data	

* Species data from authors as follows: *P. haigae*, Boschi, Scelzo and Goldstein, 1967; *P. pubescens*, Gonor, 1970, MacMillan, 1972; *P. rudis*, Gonor, 1970, Knight, 1966; *P. stevensii*, Kurata, 1964; *P. natalensis*, Sankolli, 1967.

** No specific description, data interpreted from illustration.

† Probable situation based on stage II illustration.

†† Most commonly occurring situation.

most molting to crab 1 around day seven or eight. The temperatures just cited reflect those prevailing during the stages discussed. The duration of these stages and the percentage of larval survival throughout the study is expressed in Figure 1.

Sea water temperatures monitored in the Indian River adjacent to HBFL were generally 1–2.5° C lower than laboratory temperatures. For this reason the duration of larval development in the laboratory is considered to be close, although perhaps not identical, to that which occurs in nature at this time of year. If anything, because of slightly higher temperatures, the laboratory-reared specimens might have attained each developmental stage slightly faster than their counterparts in the plankton, although there is no way to determine if this was so. At any rate, *Pachycheles monilifer* is capable of completing its larval development under laboratory conditions in as little as 15 days, although the usual time is close to three weeks according to data obtained from this study.

Description of the Larvae

Zoea I

Carapace length: 1.47 mm
Number of specimens examined: 10

Carapace: (Fig. 2, A). Typically porcellanid, with elongate rostral and posterior carapace spines; former heavily armed overall with spinules to its tip and up to six times carapace length (CL); latter from 2.6–3.0 × CL. Lower margin of carapace from just posterior of eye to origin of posterior spine distinctly armed with numerous small spinules, becoming large recurved spines along ventral margin of posterior carapace spines; remainder of posterior spines covered with small nubs dorsally, and to a lesser extent, on interior margin. Three pair of setae dorsally on carapace, two pair anterior to eyes, third pair above midgut. Eyes sessile.

Antennule: (Fig. 3, A). An elongate slightly swollen rod; three aesthetascs, three setae, as shown.

Antenna: (Fig. 3, B). Endopodite fused to protopodite, a single thin hair at tip next to small, distinct spine; exopodite half again as long as endopodite, three distinct curved spines along upper margin, as shown.

Mandibles: (Fig. 3, C). Asymmetrical, heavily dentate processes; left with about 10 small teeth in semi-circle when viewed from interior; right with large molar process.

Maxillule: (Fig. 3, D). Endopodite unsegmented, four terminal setae, basal and coxal endites with 6, 3, and 5, 3, spines and setae, respectively.

Maxilla: (Fig. 3, E). Endopodite unsegmented, setae progressing terminally 4, 2, 3; lobes of basal endite, distally and proximally, with 3, 5, and 3, 4, spines and setae, respectively; those of coxal endite 2, 1, and 4, 3; scaphognathite with four (occasionally five) setae around outer margin, plus one long apical seta.

Maxilliped 1: (Fig. 3, F). Coxopodite with two setae posteriorly; basipodite setate progressing distally 2, 2, 2, 3 (occasionally 2, 3, 2, 3); endopodite setae 3, 3, 2 + 3, 8 (rarely 9) ventrally plus one (= I) plumose seta dorsally; dorsal setules on segments as shown. Exopodite indistinctly two-segmented, four natatory setae.

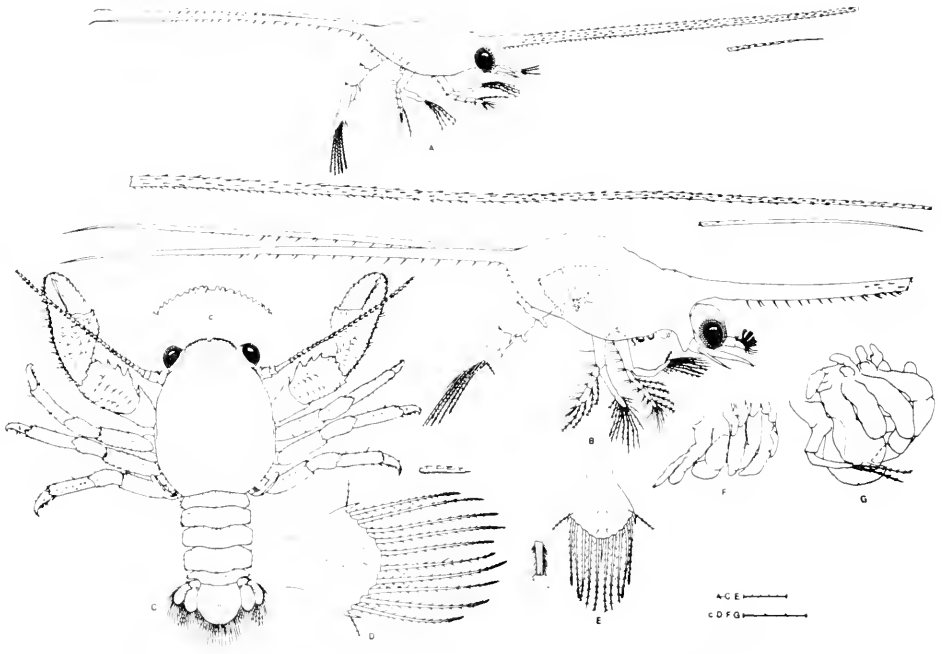

FIGURE 2. The zoeal and megalopal stages of *Pachycheles monilifer* (Dana); A, First zoea; B, Second zoea; C, Megalopa; c, Detail, megalopal frontal region; D, Telson, first zoea; E, Telson, second zoea; F, Maxilliped 3 and pereiopods, late stage first zoea; G, Same, late stage second zoea. Scale lines equal a total of 0.3 and 0.5 mm according to divisions.

Maxilliped 2: (Fig. 3, G). Coxopodite naked; basipodite setae 1, 2; endopodite setae 2, 2, 1 + 2, 5 + 1; setules dorsally on segments as shown; exopodite as above.

Maxilliped 3 and pereiopods: (Fig. 3, H). Small, amorphous buds; gill buds present in late stage; bud of future maxilliped endopodite elongate but without segmentation; no setae present during stage but may be extruded just prior to subsequent molt (Fig. 2, F).

Abdomen: (Fig. 2, A). Lateral spines present on somites 3, 4, 5, increasing in size posteriorly; dorsal posterior margin of above somites armed with series of minute teeth or spinules; pleopod buds visible only as primordia in late stage.

Telson: (Fig. 2, D). Fifth pair of plumose setae on central prominence of telson; setae 1 and 2 armed with distinct hook-like spinules at tips (detail), remaining setae appear serrate but without spines; large lateral spines of telson smooth; anal spine present.

Color: Rostrum and posterior carapace spines diffusely orange, all somites of abdomen with at least one red-orange chromatophore dorsally and ventrally, two ventrally on somites 1 and 5; when chromatophores are expanded entire abdomen

FIGURE 3. First zoeal appendages of *Pachycheles monilifer* (Dana); A, Antennule; B, Antenna; C, Mandibles; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2; H, Maxilliped 3 and pereiopods, early stage. Scale lines equal 0.3 mm.

appears ringed with red-orange bands; same color extends ventrally along underside of carapace between maxillipeds and over entire mouthpart region; telson with three large orange chromatophores, two laterally toward abdomen, one mesially near central prominence; eyes dull green with black eyespots.

Zoea II

Carapace length: 2.35 mm
Number of specimens examined: 10

Carapace: (Fig. 2, B). Now more inflated; armature of rostral and posterior spines most developed ventrally, in two to four rows; rostral spine up to five times CL; posterior spines nearly twice CL; ventral margin of carapace now unarmed except for postero-lateral margin where from six to 10 small spinules appear; dorsal setae on midline of carapace not easily seen and may be absent in some specimens; eyes now mobile.

Antennule: (Fig. 4, A). Endopodite fused to protopodite, foreshortened, approximately $\frac{1}{2}$ exopodite length; latter with five rows of aesthetascs progressing distally as 4, 5, 3, 3, 2, plus three or occasionally four terminally and other setae as shown. Additional setae on protopodite as illustrated.

Antenna: (Fig. 4, B). Exopodite now without spinules distally but with three extremely fine hairs in their place, visible only under 40 × objective magnification. Exopodite approximately $\frac{2}{3}$ endopodite length; other setae as shown.

Mandibles: (Fig. 4, C). More heavily dentate than stage I, left with about seven large teeth in curved row along interior margin, right with massive molar process as illustrated; palp buds now present.

Maxillule: (Fig. 4, D). Endopodite segmented, retaining stage I setation; spines and setae on basal and coxal lobes: 7, 3, and 6, 4, respectively.

Maxilla: (Fig. 4, E). Endopodite segmented, retaining stage I setae of 4, 2, 3; spines and setae on endites as follows: basal distal, 3, 7, + 1 stub, proximal 3, 6, + 1 stub; coxal distal, 2, 3, + 1 strong seta, proximal 3, 5 encircling lobe, plus 1–2, scaphognathite with about 25 (± 2) around margin.

Maxilliped 1: (Fig. 4, F). Coxopodite and Basipodite setae unchanged; each endopodite segment with a dorsal plumose seta (I), formula of stage I retained (*viz.* 3 + I, 3 + I, 2 + 3 + I) except terminal segment which carries nine or very rarely 10 setae, plus one dorsally. Fine setules absent; exopodite distinctly two-segmented, 14 setae of type and position as illustrated.

Maxilliped 2: (Fig. 4, G). Basipodite may rarely have 2, 2, instead of 1, 2, setae ventrally; endopodite setae 2 + I, 2 + I, 1 + 2 + I, 5 + I; exopodite as above. The only variation seen on these appendages was in the third segment of maxilliped 1 in one specimen, with two long dorsal setae instead of one, and in the other setae of both maxilliped 1 and 2 noted above.

Maxilliped 3: (Fig. 2, F, G). Future endopodite bud much elongate and swollen but only faintly segmented at early stage; exopodite distinctly two-segmented with two long setae terminally, these setae first appearing during molt to stage II (Fig. 2, F).

Perciopods: (Fig. 2, G). Now well formed but mostly unsegmented buds in early stage; segmentation and gill bud enlargement proceeds throughout stage,

FIGURE 4. Second zoeal appendages of *Pachycheles monilifer* (Dana); A, Antennule; B, Antenna; C, Mandibles; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2. Scale lines equal 0.3 mm.

entire mass hanging from underneath carapace shortly before ensuing megalopal molt.

Abdomen: (Fig. 2, B). Lateral spines except for somite five reduced; small spinules on postero-dorsal margin of somites 3, 4, 5, retained. Pleopod buds present on somites 2, 3, 4, 5, enlarging as stage progresses.

Telson: (Fig. 2, E). Fifth pair of plumose setae retained on central prominence but a median spine now also present; distal tips of plumose setae 1 and 2 still with hook-like spinules. Mesially along the shaft of each plumose seta are a series of small spinules visible only under high magnification (40 × objective; see detail).

Color: As in stage I but appearing more intense due to larger size; pereiopods become tinged with blue as they develop; expanded orange-red chromatophores on abdomen cover nearly entire somite; rostrum and posterior spines nearly completely diffused with orange; because of this any color change in these spines signalling forthcoming molt is masked.

Megalopa

Carapace length × width: 1.7× 1.4 mm.
Number of specimens examined: 8

Carapace: (Fig. 2, C). Suboval, inflated, smooth, regions fairly well delimited; frontal region produced, not expanded, projecting just beyond eyes, armed on anterior margin with a series of 12 to 14 small, sharp spinules (detail) on each side, separated in middle by distinct U-shaped notch; dorsal surface with many short stiff hairs; epibranchial angle to outer orbital angle armed with about six to eight tiny spinules, outer orbital angle otherwise little developed; epimera separated but without small pieces posteriorly which characterize the genus.

Antennule: (Fig. 5, A). Peduncle three-segmented; basal segment distinctly spinous, with additional setae as illustrated. Lower ramus three-segmented with thirteen extremely long setae, seven dorso-laterally and six latero-ventrally, placed as illustrated on proximal segment. Ventral setae may be confused with three other setae which appear on ventral surface of distal segment of peduncle (Fig. 5, A detail) but former are nearly twice as long as latter. Upper ramus five or indistinctly six-segmented; aesthetascs on segments two through five usually in the following sequence of rows and numbers; 2 rows (7, 5–6, + 1 setae), 2 rows (5, 4, + 1 seta), 2 rows (2–3, + 1 seta, 3–2), 1 row (3–4), plus additional long setae at tip of ramus. Other smaller scattered setae as illustrated on both rami.

Antenna: (Fig. 5, B). Peduncle three-segmented; basal and following movable segments spinous, and with setae as illustrated. Flagellum with 25–26 segments, number differing on either flagellum in same animal; setae around distal edges of each segment as shown.

Mandibles: (Fig. 5, C). Calcified scoop-shaped processes open on interior side; each with three-segmented palp; palp with two setae basally and about 15 short spinous seta terminally.

Maxillule: (Fig. 5, D). Endopodite unsegmented, naked except for single subterminal spine; basal endite with 17 spines, 9 adjacent setae, five strong setae anterior to spines and three additional setae down side, interiorly (latter may be easily overlooked; coxal endite terminally with eight spines and one strong seta,

FIGURE 5. Megalopal sensory and feeding appendages of *Pachycheles monilifer* (Dana); A, Antennule; detail of third peduncular segment on right; B, Antenna; C, Mandible; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2; g, spine position on penultimate segment; H, Maxilliped 3; h, spine position on last three segments. Not all aesthetascs nor elongate setae are completely figured. Scale lines equal 0.3 mm.

on either side of which are six setae plus eight additionally adjacent to these; other setae lower down as illustrated. Endite prolonged into thin lobe fringed with fine hair and with two long setae basally.

Maxilla: (Fig. 5, E). Endites so heavily crowded with setae that numbers and position difficult to ascertain, but approximately as follows: basal endite, distal lobe, 36 or more terminally, proximal lobe, about 20; coxal endite distal lobe, 11 terminally and subterminally, plus eight or nine down the side, proximal lobe with 13 spines terminally, four behind these, plus 23 strong setae in ring around lobe; endopodite indistinctly two-segmented, probably only one segment, with six (less often four) long setae as illustrated; scaphognathite with about 60 to 72 setae around outer margin; other shorter setae and spinules on appendage endites as illustrated.

Maxilliped 1: (Fig. 5, F). Exopodite distinctly segmented at base, endopodite indistinctly so, setae on both as illustrated; protopodite divided into two lobes, setae on anterior lobe about 37, plus two posteriorly, and ten additional shorter setae behind and subterminally; on posterior lobe two rows, 10 terminally and seven subterminally, plus others as illustrated.

Maxilliped 2: (Fig. 5, G, g). Exopodite two-segmented, endopodite four-segmented, setae as illustrated. Those on terminal segments of endopodite crowded and much elongate, about 18–20; three additional large spines on penultimate segment as illustrated (detail).

Maxilliped 3: (Fig. 5, H, h). Coxopodite and basipodite as illustrated, extremely setose, with spine on former; exopodite two-segmented, four terminal setae plus others as illustrated; endopodite five-segmented, ischium, merus and carpus with lateral edges developed into dentate lamellae; a spine interiorly on ischium; remaining segments with numerous long setae in approximate numbers as follows: merus, 16; carpus; 16 propodus, 10 exteriorly, 13 interiorly; dactylus, 10 exteriorly, 7 interiorly. Dagger-like spines on last three segments as illustrated in detail (Fig. 5, h).

Pereiopods: (Fig. 6, A, B). All walking legs setose, with elongate spines ventrally on propodus and dactyl as illustrated. Pereiopod 5 chelate, with six long scythe-like setae laterally and ventrally, and others as illustrated. Surfaces of chelipeds, and walkings legs to a lesser extent, with distinct granulate rugae transversely in longitudinal rows; several small setae project from each ruga on chela, but not walking legs. Rugae are miniature version of rounded granules that adults exhibit. Outer margin of hand and fingers, dorsally and ventrally sculptured into single, bifid, or occasionally trifid, spinules. Anterior margin of carpus of chelipeds developed into four large, serrate, spine-like teeth.

Pleopods: (Fig. 6, C, D). Four pairs, simple biramous processes. Setae number inconsistent in the same specimen, but usually appearing as 13 + 1, 13 + 2, 14 + 2, 12 + 2, on exopodite and endopodite, respectively, progressing toward telson. Endopodites also with 4–5 hooks which function as *appendix interna.*

Tail Fan: (Fig. 6, E). Telson with eight or nine elongate marginal setae on either side of midline, plus smaller setae interspersed between these, number inconsistent in same specimen. Uropodites with setae on exopodites, 15–16, endopodites, 10–12, also inconsistent in same specimen. Ventral surface of telson with four, long distinct setae adjacent to cloaca, plus additional pairs dorsally and ventrally on midline, and elsewhere, as illustrated.

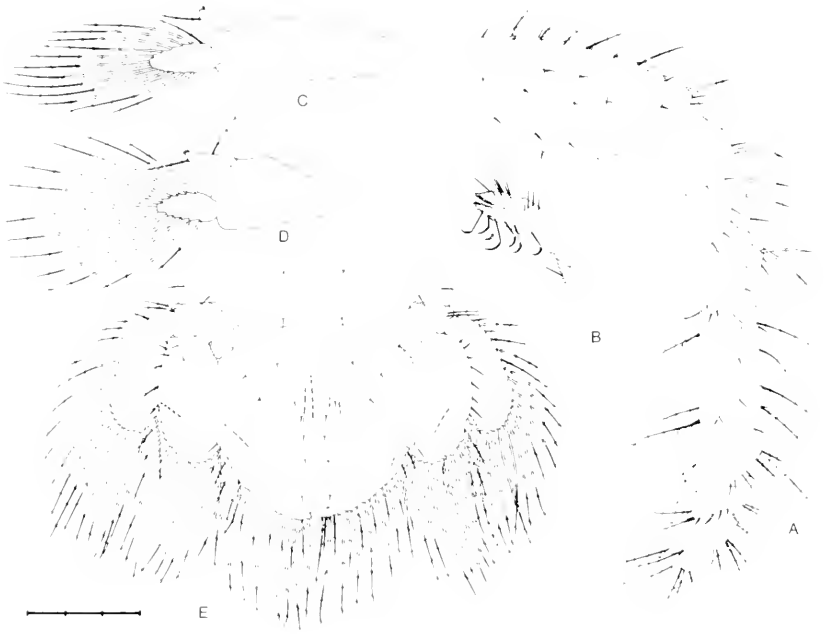

FIGURE 6. Megalopal locomotory appendages and tail fan of *Pachycheles monilifer* (Dana); A, Pereiopod 1; B, Pereiopod 5; C, Pleopod 1; D, Pleopod 4; E, Tail fan (dorsal view). Scale line equals 0.3 mm.

Color: Carapace a deep transparent blue throughout, chelipeds and pereiopods more transparent but of same blue color; entire megalopa peppered overall with numerous scattered red chromatophores, most prevalent as follows: on manus of cheliped forming a distinct red band; scattered dorsally on carpus of same, less so ventrally; walking legs banded with red anteriorly and posteriorly; additional scattered red chromatophores on epimera, and between walking legs 1 and 2 ventrally; on posterior margin of abdominal somites in interrupted line; dorsally on surface of telson in three large expanded groups; in addition, numerous white spidery or "snowflake" chromatophores on frontal region; eyes dark brown.

For comparative purposes the data on zoeal and megalopal stages in other species of *Pachycheles* are still quite sketchy, and even where complete development is known many inconsistencies appear in the same species (see *e.g.,* Table I and Discussion below). However, a provisional synopsis of zoeal features using the known data that seem most reliable may be of aid in identifying at least some of the porcellanid larvae in the plankton to the genus *Pachycheles*.

SYNOPSIS OF LARVAL CHARACTERS IN THE GENUS *PACHYCHELES*

Carapace

Rostral spine elongate in both zoeal stages, generally straight, not upswept, heavily spinose overall or armed only laterally and ventrally in some; lower mar-

gin of carapace serrate, dentate, with small curved hooks, on entire; postero-lateral margins may have distinct spinule.

Abdomen

Postero-dorsal margin of abdominal somites three through five with or without row of small teeth; postero-lateral spines present at least on somites four and five; pleopods on somites 2, 3, 4, 5, in stage II.*

Antenna

Stage I exopodite longer than endopodite; three to four spines distally in a row. Stage II exopodite shorter than endopodite, armature reduced or lacking.

Mandible

Without palp in stage I; palp usually present in stage II.*

Maxillule

Endopodite with four setae both stages.

Maxilla

Endopodite with nine setae both stages, usually placed 4, 3, 2.

Maxillipeds

Setae on terminal endopodal segments different depending on species; maxilliped 1 penultimate segment usually with 2 medial, 3–4 distal setae;* maxilliped 2 formulae usually 2, 2, 1 + 2, 5 + 1 in stage I, with one additional dorsal seta (= I) added to segments 1–3, both maxillipeds, in stage II;* (dorsal seta on segment 1 may be lacking in some species).

Telson

First two elongate plumose setae armed distally with hook-like spinules, remainder not armed in most species; fifth pair plumose setae on central prominence in stage I, a median spine added here in stage II.*

These features marked thus* also occur in larvae of *Petrolisthes* and *Megalobrachium* to some extent. Such "suprageneric" characters have been used to classify larvae of these two genera along with *Pachycheles* in the *Petrolisthes*-group of larvae based on an original grouping by Lebour (1943). Other larvae differing in some or all of these features are currently assigned to either the *Porcellana*-group or the *Petrolisthes platymerus*-group (see Gore, 1971, 1972b, for a more complete discussion).

Although even less data are available for megalopal stages in the genus some features appear to be consistent. The megalopa, in general, seems to resemble the adult to some degree, the margin of the frontal region is often serrate or with distinct setae dorsally, spinules may be present along the epibranchial margin of

the carapace, the third antennular peduncle segment may have two groups of elongate setae dorsally and ventrally, the lamellar plates of the ischium, merus and carpus of maxilliped 3 are often dentate, and two pairs of elongate setae may appear on the ventral surface of the telson adjacent to the cloaca.

Discussion

The zoeal stages of *Pachycheles monilifer* may be identified in the plankton by several features. Most notable is the color of the live zoeae which appear even to the unaided eye to be banded with orange on the abdomen, and diffused with orange on the rostral and posterior carapace spines. Other features in the first zoeal stage include the distinctly spinulose lower margin of the carapace, the large recurved spinules on the posterior carapace spines, the small teeth dorsally on the posterior margin of abdominal somites three to five, the three small spines in a row on the antennal exopodite and the large hook-like spinules on the distal tips of plumose telson setae 1 and 2.

In the second stage *P. monilifer* possesses carapace spinulation only on the postero-lateral margin, but retains the enlarged spinules proximally on the posterior carapace spines, the small teeth on the postero-dorsal margin of the abdomen and the hook-like spines on the distal tips of telson setae 1 and 2. Three fine hairs replace the row of spines on the antennal exopodite. In addition, second stage *P. monilifer* possesses a median spine on the telson which places it in Lebour's (1943) category of the *Petrolisthes*-group of larvae, the group to which other known members of the genus belong (see Knight, 1966; Boschi, Scelzo and Goldstein, 1967, MacMillan, 1972).

The megalopal stage of *P. monilifer* has a noticeably spinulose frontal margin, dentate epibranchial angles, and heavily spined chelipeds covered dorsally with distinct longitudinal rows of rugae or enlarged granules. Other easily observed features include the dentate lamellar segments of maxilliped 3, the numerous short hairs on the frontal region, the elongate setae groups on the dorsal and ventral margins of the terminal segment of the antennule, and the two pairs of elongate setae ventrally on the telson adjacent to the cloaca.

In the megalopae the sidewalls of the carapace are not yet divided into the additional plates which characterize adults of the genus. However, sutures appear postero-laterally in this region and division of the epimera probably occurs during early crab stages. The telson also is not yet divided into five plates, nor is there any way to distinguish male pleopods at this stage, much less to be able to forecast their presence or absence at sexual maturity. Except for these features, the megalopae resemble the adult in many respects, most notably in the ornamentation of the dorsal and anterior surfaces of the chelipeds.

The blue color of the carapace and chelipeds and the numerous scattered red chromatophores, especially those which form bands of red on the manus and the walking legs, may also aid in identification of this species at this stage of development.

All of these colors are lost upon molt to first crab stage whereupon the animal assumes a mottled greenish-brown and white color which becomes more intense and less muddy in later crab stages as the animal progresses through the juvenile state.

Pachycheles haigae is the only other species of the genus in the western Atlantic for which the complete development is known (Boschi, Scelzo and Goldstein, 1967). Although *P. haigae* has not been recorded north of Pernambuco, Brazil (7°–10° S; in Boschi, 1963; also Coelho, 1963), *P. monilifer,* on the other hand, has been reported as far south as the state of Sao Paulo, Brazil (about 25° S; in Haig, 1966a). The larvae of these two species, therefore, overlap between these two areas and might possibly be confused. However, the study by Boschi *et al.* (1967) is quite thorough and by referring to that work the larvae of the two species should be easily distinguished. Briefly, *P. haigae* differs from *P. monilifer* in having the rostral spine unarmed dorsally, the posterior margin of the carapace unarmed except for a single spinule, in having four (not three) spines in a row on the antennal exopodite in stage I and being unarmed in stage II, possessing a different number (*i.e.* formula) of setae on both maxillipeds, and, most exceptionally, in lacking a palp on the mandible in stage II. Both zoeal stages apparently lack the small teeth on the postero-dorsal margin of the abdominal somites which are present in *P. monilifer.*

The megalopal stage of *P. haigae* may be distinguished by its rosy-red color, by lack of spination on the frontal and epibranchial regions (according to the illustrations examined), by the reduced carpal spines on the cheliped, and by the presence of only five elongate dorsal and lateral setae on the third or terminal segment of the antennular peduncle.

I have also a very limited series of laboratory-cultured first zoeae of *Pachycheles serratus,* another western Atlantic species. In these zoeae the following differences appear: the entire lower margin of the carapace is armed with small, rounded, anteriorly-directed hook-like spinules, the antennal exopodite has two or three very small, hair-like spines, and the setae number and position on the first two maxillipeds is 3, 3, 2 + 4, 10 + I, and 2, 2, I + 2, 5 + I, respectively. The first zoea of *P. serratus,* unlike those of *P. monilifer,* has only a few minute teeth on the postero-dorsal margin of the abdominal somites. The first two elongate plumose setae on the telson, however, do possess distinct hook-like spinules distally.

In the eastern Pacific Ocean, larvae are known for *Pachycheles rudis* and *P. pubescens,* both of which are not known to occur farther south than the Baja California peninsula. Comparison of *P. monilifer* with *P. rudis* and *P. pubescens* shows that the larvae of all three are quite similar (Table I). In the first zoeal stage of these species the chief differences appear in the length ratio of the antennal endopodite and exopodite, in the spinulation on the postero-dorsal margins of the abdominal somites, and whether the lower margin of the carapace is armed. The only difference in maxilliped setation which does not appear to show variation is that of the ventral setae on the second endopodal segment (3 in *monilifer* and *pubescens,* 2 in *rudis*). This last feature must therefore be used with care at the species level.

In stage II the species differ in the presence or absence of a single long seta on the antennular protopodite next to the junction with the endopodite, in the armature of the antennal exopodite, the number of setae on the exopodite of maxilliped three, and in the abdominal-somite and carapace-margin features mentioned previously. *P. rudis* lacks a dorsal seta on the first endopodal segment of both maxilliped 1 and 2, and this basipodal and endopodal ventral setation may allow

the species to be distinguished from *P. monilifer* and *P. pubescens*. Apparently, variation in setae formulae does not occur as often in stage II as in stage I. The number of setae around the margin of the scaphognathite might also prove to be a valuable character at the species level.

At the megalopal stage little difficulty should be encountered in live material since, as far as is known, only *P. monilifer* is colored blue with scattered red chromatophores. In preserved specimens the armature of the anterior margin of the carpus of the cheliped should be distinctive (4 spines in *monilifer*, 2 in *pubescens*, 1 in *rudis*).

An examination of Table I shows that several features are recurrent and may thus be indicative at the generic level. These include the presence of spines or spinules on the antennal exopodites in stage I, plus four setae on the endopodite of the maxillule, and the hook-like spinules on the distal tips of the first two elongate plumose telson setae in both zoeal stages (except in *P. natalensis*). The setal formula of the maxillary endopodite may also have value since the number is usually 9 setae, and the positioning appears to be 4, 2, 3, in most cases, based on either descriptive text or good illustrations. This last features must also be used with caution since the determination as to whether setae are terminal or subterminal is, at times, quite subjective. In the known species of *Petrolisthes*, for example, some also have 9 setae on the maxillary endopodite, but these are placed 3, 3, 3; thus, this feature is of limited value at present when used by itself at the generic level. Other features of somewhat limited value are the previously noted spinules on the postero-dorsal margins of the abdominal somites and the armature of the lower carapace margin. Both features occur inconsistently in some species or are lacking entirely. Both features, because of their small size, required higher objective magnification (20× – 40×) to be seen, hence they might have been inadvertently overlooked in those species in which they are described as absent.

MacMillan (1972) noted that both *P. rudis* and *P. pubescens* lacked setules on the dorsal margins of the maxillipedal endopodites, and suggested this as a possible generic character for *Pachycheles* larvae. However, setules are present in both *P. monilifer* and *P. serratus* and are apparently indicated in *P. natalensis* (see Sankolli, 1967, page 748, Fig. 2,g). Thus, this feature becomes less useful as a generic character, especially in larvae from two of the three known Atlantic species, although it presently may still be used to distinguish these from the two known eastern Pacific species.

It is entirely possible, of course, that some of the features noted in the discussion above and in the earlier synopsis may ultimately prove to be unreliable or too inconsistent to be safely used at the generic level. This would not be unexpected in such a large and widespread genus as *Pachycheles*. Nor would it be surprising to find some of the above noted features also occurring in larvae of the closely related genus *Neopisosoma*. Future studies, carefully done, may not only clarify such relationships but provide additional or more stable criteria for determining planktonic porcellanid larvae at the generic level.

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Summary

The complete larval development from hatching through megalopal stage is described and illustrated for *Pachycheles monilifer*, a shallow water porcellanid crab. The development at room temperature (27.8° C) under laboratory conditions consisted of a prezoeal stage of approximately one hour duration, two zoeal stages of approximately five and six days duration, respectively, and a megalopal stage of about eight days duration. The entire larval/postlarval portion of the life cycle is completed under the conditions described herein in approximately three weeks.

The zoeal and megalopal stages of *P. monilifer* were compared with larvae known from two other western Atlantic species, and two species each from the eastern Pacific and Indo-Pacific oceans. As might be expected, larvae of the genus are quite similar in many respects, and difficulty may be encountered in separating them at the specific level. However, certain morphological features are recurrent in the zoeal stages and may be indicative at the generic level; among these are the presence of three spines on the antennal exopodite, four setae on the maxilliulary endopodite, and the hook-like spinules on the first two elongate plumose setae of the telson. A provisional synopsis is provided utilizing the most salient features occurring in all known *Pachycheles* larvae as an aid in recognizing such larvae, at least at the generic level, in the plankton.

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THE DIFFERENTIAL RADIOSENSITIVITY OF OOGONIA AND OOCYTES AT DIFFERENT DEVELOPMENTAL STAGES OF THE BRINE SHRIMP, *ARTEMIA SALINA*

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The radiosensitivity of germ cells and oocytes varies between different species and is dependent on the age of the animal at the time of irradiation.

Artemia is well suited for genetic or radiobiological studies because it is easily cultured in the laboratory and has a generation time of 25 to 30 days. The development of female germ cells is synchronous as each brood and the stages of their maturation are easily recognizable from external features.

The effects of acute irradiation on adult life span and reproductive performance of *Artemia* were reported by Grosch and Erdman (1955), Metalli and Ballardin (1962, 1972), Cervini and Giavelli (1965) and Squire (1970). Cervini and Giavelli (1965) studied the sensitivity of *Artemia* oocytes at different meiotic stages using hatchability as the criterion of radiation damage. They found that oocytes were more sensitive in prophase I than in metaphase I. However, histological or cytological observations of oogonia and oocytes in ovaries of irradiated *Artemia* have not been reported. The present study was initiated to obtain this information.

Materials and Methods

Dry eggs of the California strain (diploid amphigonic) of *Artemia* obtained from the Aquarium Society of San Francisco, Hayward, California, were hatched in artificial sea water and reared to maturity. The culture methods employed were similar to those described previously (Iwasaki, 1970). In the present experiment, three developmental stages of the brine shrimp were used for irradiation; dry eggs, immature animals (6–8 mm in body length) and adults.

For irradiation with ^{60}Co γ-rays, eggs were placed in a small lucite chamber under dry conditions. Young or adult shrimps were put into a cylindrical lucite vessel, 2 cm in diameter, containing artificial sea water about 0.5 cm in depth. Dry eggs were irradiated with 2, 5, 10, 25, 50, 100, 200 and 300 krads of γ-rays at a dose-rate of 10 krads/min. Young and adult shrimps were irradiated with 2, 5, 10 and 20 krads, at a dose-rate of 2 krads/min. After irradiation, eggs were hatched in artificial sea water and were cultured to maturity; irradiated shrimps were kept in culture medium.

To observe histological changes in the gonads after γ-irradiation, shrimps at each dose level were fixed in Bouin's fluid at desired intervals. Serial longitudinal sections, cut in paraffin at a thickness of 5 μ, were stained with Mayer's hemalum and eosin. There were at least 6 animals in each group.

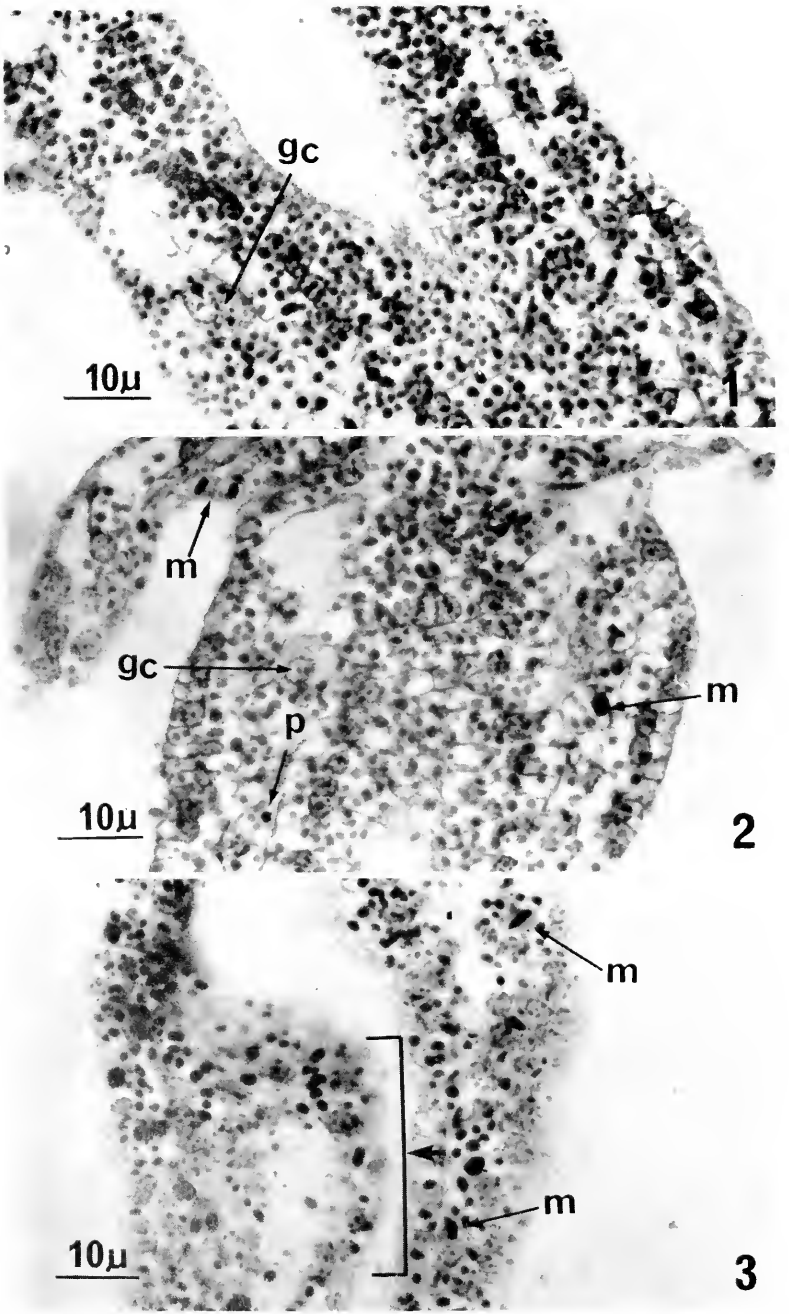

FIGURE 1. Longitudinal section of a 1-day-old nauplius of *Artemia* hatched from an egg irradiated with 5 krads of γ-rays. The cells appear normal. The germ cell group (gc) consists of 4 to 8 cells, × 1200.

Results

1. *Dry eggs*

Encysted dry eggs temporarily cease development at the gastrula stage and produce thick shells. Their radiosensitivity is extremely low, 50% hatchability requiring a dose of several hundreds krads (Iwasaki, 1964, 1965).

In this experiment, only a dose of 300 krads prevented hatching of dry eggs, but the time required to reach 50% hatching increased considerably as the dose increased from 2 to 200 krads. Nauplii derived from dry eggs irradiated with 50 krads or less reached the mature stage as well as the unirradiated control. γ-ray exposure of 100 krads greatly reduced the viability of the nauplii which hatched from these eggs, and it allowed some individuals to mature but malformations, for example twisted tails, were observed in some animals. In the groups receiving more than 150 krads, essentially all of the animals died within one week.

Microscopic observations indicated that nuclei in the cells of nauplii hatched from eggs irradiated with less than 25 krads were intact and normal in appearance (Fig. 1), but some pycnotic cells were found in nauplii of eggs irradiated with 25 krads or more (Fig. 2). In nauplii hatched from eggs receiving 150 krads, normal mitoses were still found, however, the cells were loosened in appearance (Fig. 3).

No changes were observed in the ovaries of adults hatched from eggs receiving 10 krads or less (Figs. 4a, 4b). When the dose to the eggs was increased to 100 krads, normal growing oocytes were observed in the ovaries of mature survivors, but some pycnotic oocytes were found as well as shown in Figures 5a and 5b.

2. *Immature animals*

The ovary of *Artemia* in this stage, consists of a large population of mitotically active oogonia. Changes in the ovary were apparent at all radiation levels employed, although progressively more damage was present with increasing dose.

In young shrimps receiving a γ-ray dose of 2 krads, some oogonia immediately underwent mitosis, but pycnotic cells also appeared within one day after irradiation as shown in Figure 6. During the first 4 days after irradiation, oogonia degenerated, and the ovary became greatly depleted (Fig. 7). At 10 days to 2 weeks, however, some surviving oogonia began to enter mitosis, and a small number of healthy-appearing oogonia were found (Figs. 8a, 8b); the ovary had increased in size by this time.

The effects of 5, 10 or 20 krads on the cells of the ovary were similar to, but much more pronounced than, those observed at 2 krads. However, following these

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Figure 2. Longitudinal section of a 1-day-old nauplius hatched from an egg irradiated with 25 krads. Many mitotic cells (m) and a few cells with pycnotic nuclei (p) are found throughout the body, × 1200.

Figure 3. Longitudinal section of a 3-day-old nauplius hatched from an egg irradiated with 200 krads. Arrow indicates an abnormality in the gut lumen. The cells lose their rigidity and many of their structural details. Mitotic cells are still found, × 1200.

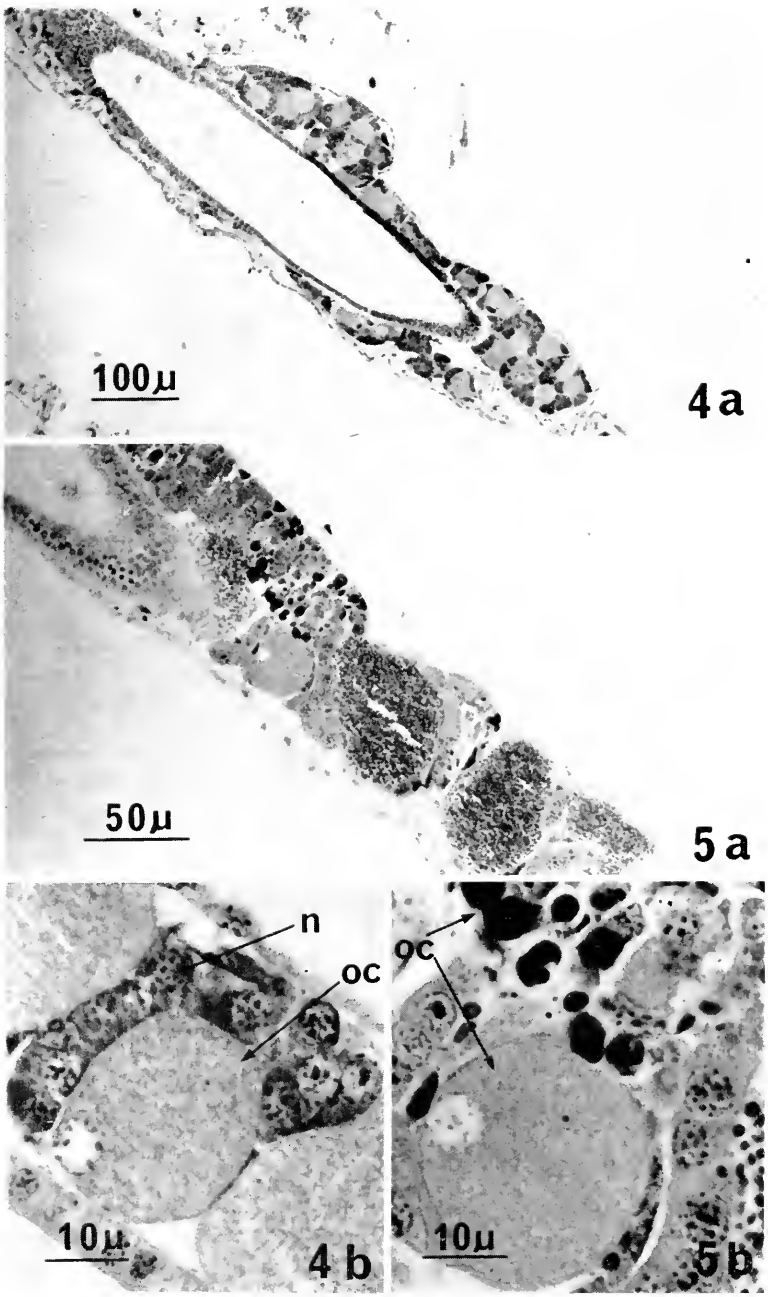
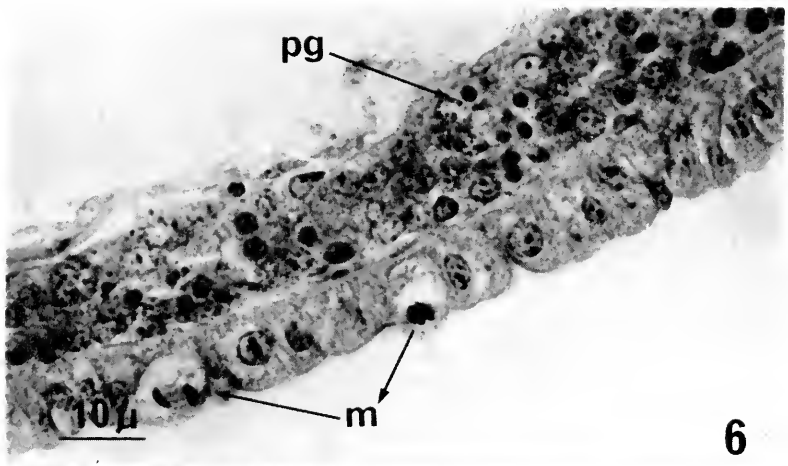


FIGURE 4. Longitudinal section of an ovary of an adult *Artemia* derived from an egg irradiated with 5 krads. Many normal growing oocytes (oc) surrounded by nutritive cells (n) arranged in rows are shown; a: × 120, b: × 1200.

FIGURE 5. Longitudinal section of an ovary of an adult *Artemia* derived from an egg irradiated with 100 krads. The ovary contains degenerated oocytes, nutritive and follicle cells, and normal growing oocytes; a: × 300, b: × 1200.



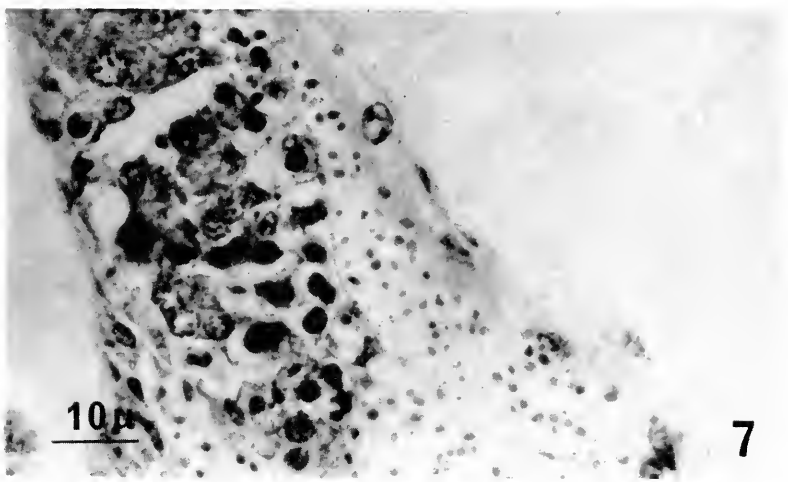


FIGURE 6. Ovary of a young *Artemia* 1 day after irradiation with 2 krads. Some pycnotic oogonia (pg) are shown. Two dividing cells (m) in the gut lumen are also evident, × 1200.

FIGURE 7. Degenerating ovary of a young *Artemia* 4 days after irradiation with 2 krads. There has been a progressive loss of cells, but a few pycnotic and normal cells are still found, × 1200.

doses, animals were in generally poor condition and usually died before recovery of ovarian damage reached completion.

3. *Adults*

In the normal ovary of an adult shrimp, all prophase oocytes are located along the oviduct. When they are pushed ahead to form two lateral masses in the uterine cavity, the chromosomes are on the equatorial plate of the maturation division. After the second polar body is expelled in the central pouch of the uterus, embryonic development takes place in the uterine cavity as far as the gastrula stage (oviparous) or nauplius stage (viviparous) (Metalle and Ballardin, 1962).

The effect of γ-rays on the ovary of adult shrimps largely depends on the

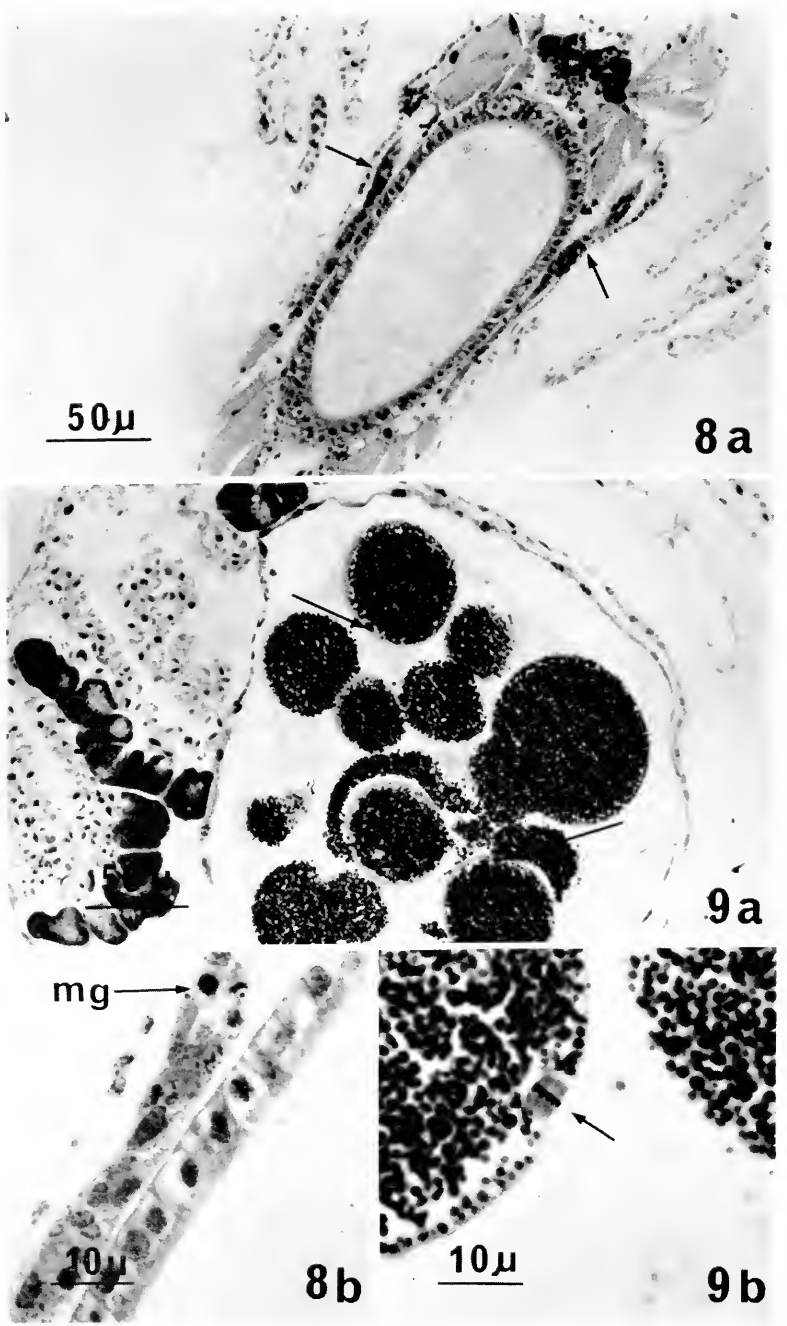


FIGURE 8. Regenerating ovary of a young *Artemia* 2 weeks after irradiation with 2 krads. Mitotic oogonia (mg) and newly proliferated oogonia are found; a: × 300, b: × 1200.

FIGURE 9. Oocytes in the uterus of an adult *Artemia* 2 weeks after irradiation with 2 krads. Arrows show normal metaphase of maturation division. The cells were in an early meiotic stage at the time of irradiation; a: × 300, b: × 1200.

stage of development of the oocytes at the time of irradiation. In *Artemia* receiving 2 krads of γ-rays, oocytes in various stages (pre-meiotic, meiotic prophase and the first maturation division) and eggs undergoing early cleavage in the uterus remained apparently intact during the first day following irradiation. Two weeks after irradiation, most growing oocytes present at the time of irradiation had reached the mature stage, and they subsequently underwent normal maturation division (Figs. 9a, 9b).

In contrast to this, oocytes in adults receiving 5 krads were damaged to various degrees depending on their stage of development (Figs. 10, 11). Generally, oocytes at the pre-meiotic stage were severely damaged, and their nuclei became

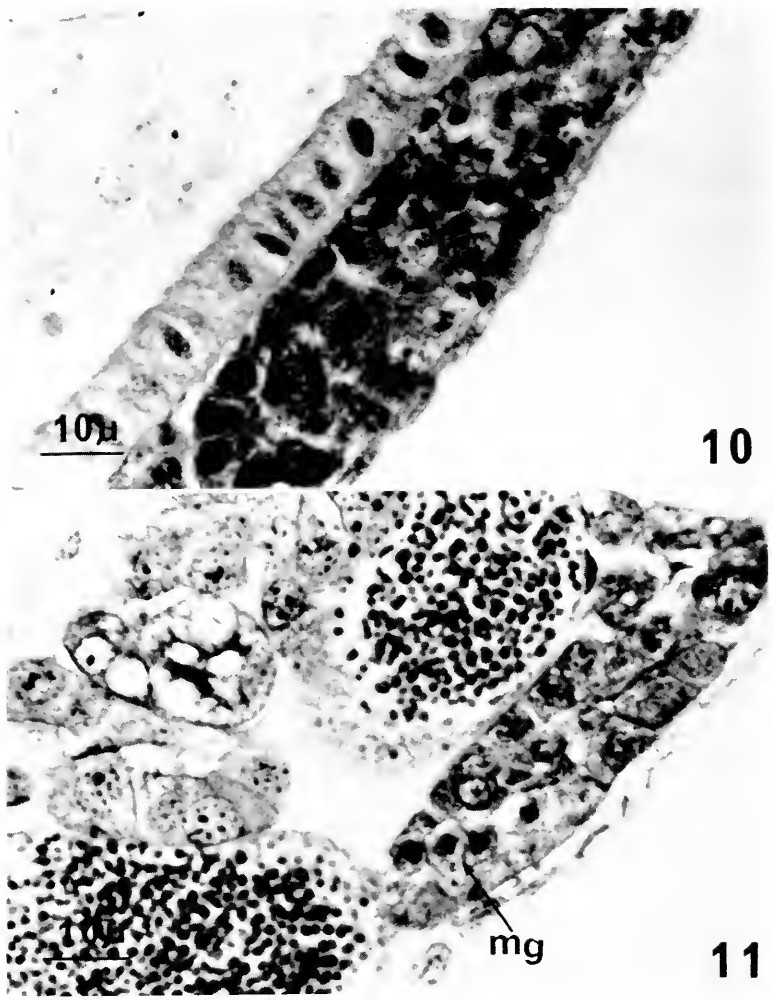


FIGURE 10. A portion of an ovary of an adult *Artemia* 1 day after irradiation with 5 krads. Pre-meiotic oocytes are pycnotic, × 1200.

FIGURE 11. A portion of an ovary of an adult *Artemia* 1 day after irradiation with 5 krads. Four metaphasic oogonia (mg) are shown in the neighborhood of growing oocytes. Figures 10 and 11 are different areas of an ovary in the same individual, × 1200.

pycnotic within one day after irradiation (Fig. 10). On the other hand, growing oocytes did not show any immediate effects of radiation (Fig. 11), and the maturation division and the nuclei of eggs undergoing cleavage were generally normal in appearance although some individuals having eggs with abnormal nuclei were occasionally found.

It should be pointed out that in adults as well as young shrimps, repopulation of the surviving oogonia was observed. That is, some dividing oogonia as well as oocytes were found at various times after irradiation in the ovary of adult shrimps receiving 2 or 5 krads (Figs. 11, 12a, 12b). In the ovaries of unirradiated adult shrimps, this situation is rarely observed. However, whether or not these

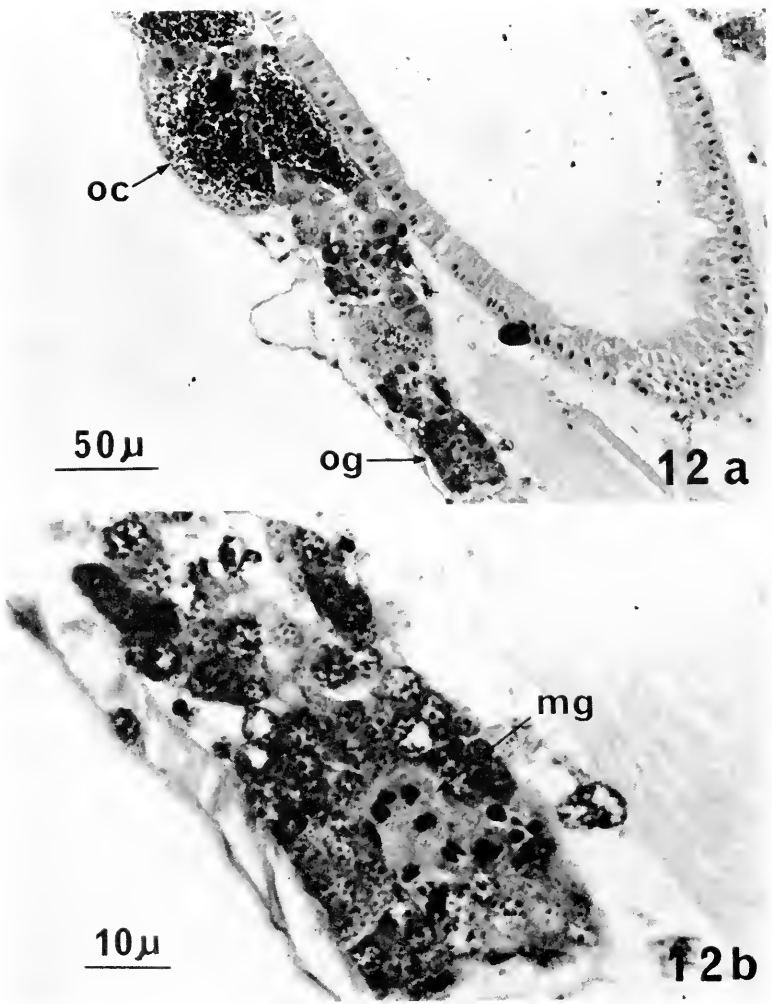


FIGURE 12. Ovary of an adult *Artemia* 2 weeks after irradiation with 2 krads showing some mitotic oogonia (mg); a: × 300, b: × 1200.

newly formed oogonia become functional oocytes has not been determined in these experiments.

Discussion

In a previous paper, the author reported that if radiosensitivity was determined on the basis of hatchability, it was very low, the 50% hatching dose being about 400 krads (Iwasaki, 1964, 1965), and that although 200 krads of γ-rays had no significant effect upon the hatchability of the eggs, it greatly reduced the viability of nauplii derived from them (Iwasaki, Maruyama, Kumamoto and Kato, 1971). The author also reported that in squashed preparations of 1-day-old nauplii derived from eggs receiving 200 krads or less of γ-rays the number of mitotic cells did not decrease below control levels, but that pycnotic cells were found at doses as low as 20 krads (Iwasaki *et al.*, 1971). These cytological data obtained by squashed preparations are in agreement with the histological observations in the present experiments, that is, many normal mitoses were found in nauplii hatching from eggs irradiated with 150 krads although pycnotic cells were observed in nauplii derived from eggs irradiated with as low a dose as 25 krads.

According to Bowen (1963), a dose of 50 kR administered to encysted dry eggs did not kill during embryonic development but instead killed during later developmental stages (through the eighth instar). In our experiment, some nauplii derived from the dry eggs irradiated with 50 to 100 krads did reach maturity, but their ovaries contained pycnotic oocytes in addition to oocytes with normal nuclei It may be expected on the basis of these histological observations that these doses would result in reduced fecundity of *Artemia*, although studies of fecundity and fertility were not performed in the present experiment.

Compared to dry eggs, young shrimps are very sensitive to radiation; even a dose of 2 krads results in severe damage to the ovary and the eventual life-shortening of the shrimp. This radiosensitivity may be correlated with high mitotic activity of the oogonia. Because, it is in this stage that oogonia undergo repeated mitotic divisions and rapidly increase in number. The results are similar to those found in the rat by Beaumont and Mandl (1962) and Beaumont (1965), who concluded that oogonia are intrinsically more radiosensitive than oocytes based on a comparison of the numbers of germ cells recorded 24 hours after irradiation.

Studies with a number of organisms using a variety of endpoints have shown that the radiation sensitivity of the germ cells depends upon their stage in meiosis at the time of exposure. In rats, the last pre-meiotic division is highly sensitive to radiation whereas meiotic prophase is relatively less sensitive (Beaumont and Mandle, 1962). In a recent review, Baker (1971) states that in mammals, sensitivity increases during the final pre-meiotic division, and the oocyte subsequently becomes increasingly resistant to radiation as it progresses from leptotene to pachytene. In *Habrobracon*, metaphase of meiosis is 20 times more sensitive to x-rays than prophase I for the induction of dominant and recessive lethal mutations (Whiting, 1945). Similarly, in a parthenogenetic stick insect, oocytes in the first metaphase are about 8 times more sensitive at the LD_{50} level than those in the first prophase (Pijnacker, 1971). In the silkworm, germ cells in the first meiotic division are more resistant to lethal damage than those in the second meiotic division (Murakami, 1971). Metalli and Ballardin (1962, 1972) and Cervini and Giavelli

(1965) have investigated the relationship between meiotic stage sensitivity and dominant lethality as the basis of hatching inhibition in *Artemia;* hatchability was lowest in the earliest prophase stage scored and increased througout prophase reaching a maximum at metaphase. That is, oocytes were more sensitive in prophase than in metaphase. Based on histological evidence, the present experiments indicate that pre-meiotic oocytes are much more sensitive to γ-rays than growing oocytes or oocytes at maturation division (metaphase I). It may be significant that the former stage is characterized by a high rate of DNA synthesis but not the latter one (Iwasaki, 1970). Since no correlation was observed between genetic damage and cell killing, our results are not directly comparable to those of Metalli and Cervini. However, the pattern of radiosensitivity for cell killing during meiosis of *Artemia* oocytes was the same as that observed for the production of dominant lethals.

Squire (1970) has proposed that the *Artemia* ovary normally contains a permanent oogonial stem-cell component which contributes to each successive brood. In the ovary of adult *Artemia* irradiated with 2 or 5 krads, we observed some oogonia entering mitosis after irradiation, resulting in an increase in the number of germ cells. Therefore, some oogonia having the potential to undergo mitosis persist in the ovary of adult shrimp, lending support to Squire's proposal.

Grosch and Erdman (1955) reported that *Artemia* females die within several days after irradiation with 150 kR of x-rays and that the sterility dose for the female is 2 to 3 kR. Recently, Squire (1970) has also reported on female reproductive performance. According to him, sterility was almost complete after 5 kR of γ-rays, and the number of broods was significantly decreased at doses of 2 kR and above. Judging from our histological observations, it may be suggested that at doses of 5 krads or less the first brood derived from oocytes which were in postmeiotic or early cleavage stages at the time of irradiation may be laid normally. The subsequent brood, however, may not be produced or may be reduced in number, even though partial regeneration of oogonia has occurred.

I am indebted to Dr. H. Matsudaira for his continued interest and encouragement in this research. I also thank Dr. J. D. Wilson for his critical reading of the manuscript.

Summary

Histological changes in the gonads of γ-irradiated *Artemia* were studied. Three different developmental stages were irradiated; dry eggs, immature young and adults. Normal growing oocytes and some pycnotic oocytes were found in the ovaries of quite a few surviving adult animals derived from dry eggs irradiated with 100 krads of γ-rays. Oogonia and primary oocytes in ovaries of immature *Artemia* irradiated with 5 krads of γ-rays became pycnotic within one day after irradiation, and became greatly reduced in number in 4 days. However, 10 to 14 days after irradiation, some surviving oogonia began to undergo mitosis. In adult animals, although pre-meiotic oocytes were damaged growing oocytes did not show any immediate damage after dose of 5 krads of γ-rays. Seven days after irradiation, they reached the mature stage, and normal maturation division occurred.

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THE SWITCHOVER FROM VIRGIN TO MATED BEHAVIOR IN FEMALE CECROPIA MOTHS: THE ROLE OF THE BURSA COPULATRIX

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In their reproductive behavior the non-feeding giant silkmoths are well adapted to a short life span of 7 to 10 days. The female emerges with almost a full complement of mature eggs (Telfer and Rutberg, 1960) and soon thereafter begins to release sex pheromone ("call") in response to specific environmental cues (Riddiford and Williams, 1971). As a virgin the female lays no eggs for at least the first 3 days after emergence. Beyond this time eggs are laid at a low rate of approximately 7% of her mature eggs per day (Truman and Riddiford, 1971). Mating causes an abrupt switch in the behavior of the female: she ceases to "call" and begins to lay eggs at a greatly increased rate. Mated females of *Hyalophora cecropia* oviposit an average of 36% of their mature eggs on the first night and thereafter lay approximately 35% of their remaining eggs each succeeding night (Truman and Riddiford, 1971).

Intrinsic neurosecretory cells of the corpora cardiaca were found to control both the "calling" and the oviposition behavior of these moths (Riddiford and Williams, 1971; Truman and Riddiford, 1971). Yet the question remained as to how mating stimulated the corpora cardiaca to release the presumed hormone which caused the change to mated behavior. The failure of matings to castrated males to trigger the increase in oviposition rate (Truman and Riddiford, 1971) indicated that sperm were necessary for the switchover in behavior. The present study was done to determine the type of link between the presence of sperm in the genital tract of the female and the neuroendocrine system.

Materials and Methods

Experimental animals

Pupae of *Hyalophora cecropia* were purchased from dealers or reared outdoors on netted trees (Telfer, 1967). These pupae were stored at 5° C for at least 12 weeks prior to use.

Castrations

The pupal testes were removed from chilled male diapausing pupae as described by Truman and Riddiford (1971). These castrated pupae formed apparently normal moths which mated and produced sterile but well-formed spermatophores.

Organ implantations

The bursa copulatrix was excised just proximal to the chitinized ductus bursae and placed into Ringer's (Ephrussi and Beadle, 1936). The adhering fat body and, in the case of mated females, the spermatophore were removed. The empty bursa was then rinsed in fresh Ringer's and implanted into a lightly anesthetized (10 minutes under CO_2) two-day-old virgin Cecropia female through a slit in the dorsal part of the second abdominal segment. A few crystals of a 1 : 1 mixture of phenylthiourea and streptomycin (Williams, 1959) were placed in the wound, which was then closed by a hemostat. One to 2 hours later, the hemostat was removed and melted paraffin was used to cover the wound site.

The spermatheca including the lagena was removed to Ringer's. Most of the sperm were removed from those from mated females, although it was later found that the presence of sperm did not alter the results obtained. Implants were performed as with the bursa.

Hemolymph injections

The blood was expressed from a middorsal slit in the sixth abdominal segment by pressing down steadily on the first abdominal segment. The blood was removed with a Pasteur pipet to a microscope slide. A few crystals of a 1 : 1 mixture of phenylthiourea and streptomycin were added to prevent darkening. The blood was then taken up into an 100 μl Hamilton syringe with a fixed 30-gauge needle. Fifty μl were injected into each recipient two-day-old female between the first and second ventral abdominal segment just below the spiracle. The wound was sealed with melted paraffin.

Collection of eggs

After organ implantation or blood injection, the female moth was placed in a large paper bag. The number of eggs laid each day was then counted. After death, the female was dissected under Ringer's solution and the number of mature chorionated eggs remaining in the ovarioles counted. Thus, the total number of mature eggs was determined and used as a base for the cumulative percentage laid per day.

Only host females which lived for at least four days after the experiment were included in the data. Females that died in two to three days tended to produce a sudden burst of oviposition the day of their death, irrespective of the treatment. Similarly, females which laid no eggs after treatment were discarded, although some lived as long as two weeks.

H^3-inulin

Ten μl of water containing 0.36 μg inulin-methoxy-H^3 (New England Nuclear, 139 μCi/mg) were injected into the dorsal thorax of two-day-old virgin female Cecropia moths. Twenty-two hours later the females were bled and 10 μl of blood was counted in a Tracerlab liquid scintillation counter.

Results

Implantation of spermathecae

Since matings to castrate males did not result in a mated oviposition response (Truman and Riddiford, 1971), it seemed likely that the presence of sperm in the

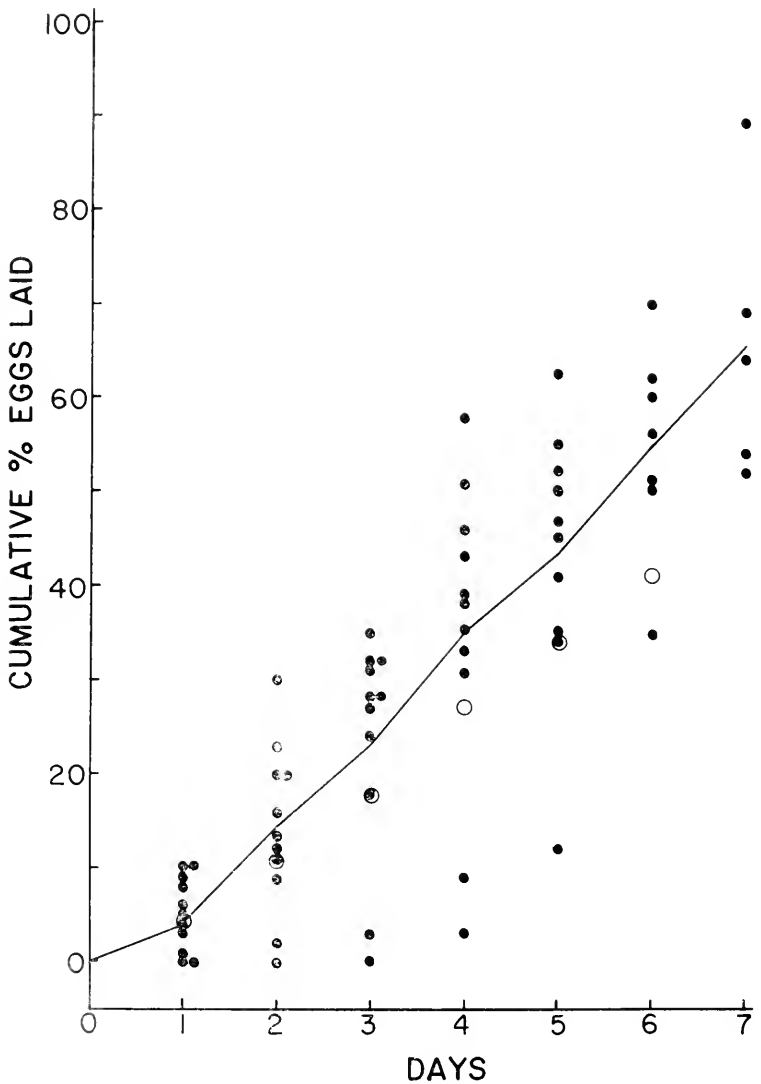

Figure 1. The oviposition pattern of virgin Cecropia females, each of which has received an implant of a spermatheca from a mated female. The abscissa refers to days after implantation. The line is drawn through the average of the closed circles. The open circles represent the average cumulative percentage of eggs laid by normal, intact virgin females, beginning on day 3 of life (Truman and Riddiford, 1971).

spermatheca was the trigger for the switchover from a virgin to a mated oviposition rate. Thus, 12 females were mated. The spermatheca was removed the morning after mating was completed and was implanted into a virgin female. Figure 1 shows that these females laid eggs in a pattern which was essentially identical to that displayed by virgin females.

Implants of spermathecae from 4 two-day-old virgin females also gave the same pattern.

Implantation of the bursa copulatrix from mated females

Cecropia mate in the early morning and remain *in copulo* throughout the day until the onset of darkness at which time the female begins laying eggs. Figure 2A shows that implantation of the empty bursa copulatrix from a mated female into a virgin female caused the switchover from the virgin to the mated oviposition pattern. Five of the 17 bursae were removed immediately after the termination of mating before the female began laying eggs; 10 on the next morning; and 2 were removed on the following day. No detectable difference in the oviposition rate

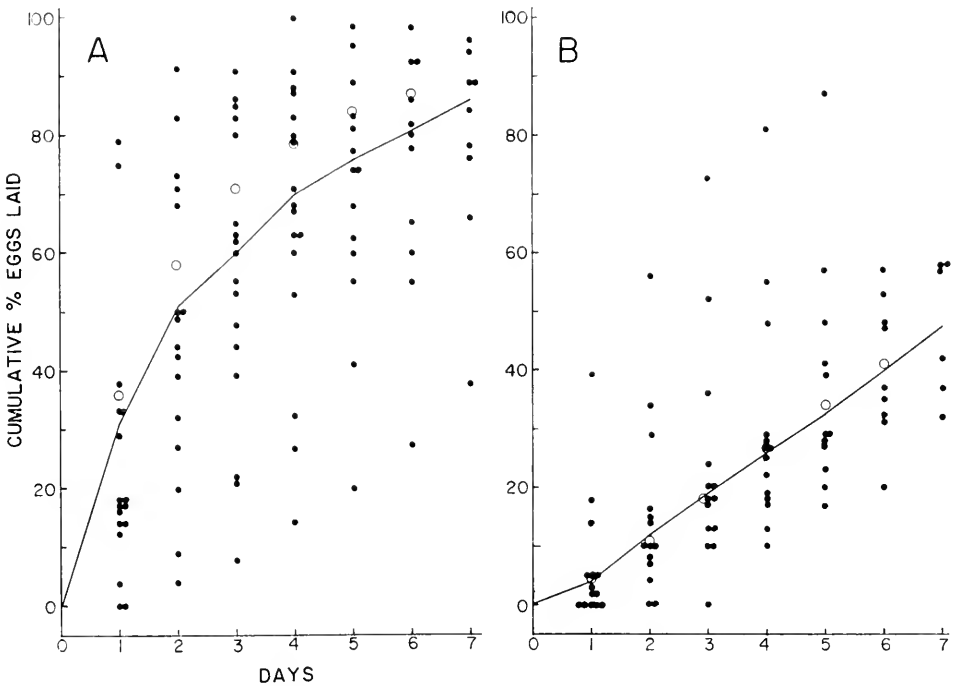

FIGURE 2. The oviposition patterns of virgin female Cecropia, each of which has received an implant of a bursa copulatrix. The abscissa refers to days after implantation. The lines are drawn through the average of the closed circles excluding the lowest set in 2A and the highest set in 2B: (A). mated bursa implanted; (B.) virgin bursa implanted. The open circles represent the average cumulative percentage of eggs laid by normal, intact mated and virgin females respectively (Truman and Riddiford, 1971).

was noted among these groups. All females except two laid eggs in the mated oviposition pattern.

Figure 2B indicates that the trauma involved in the implantation of the large bursa was not responsible for the increased oviposition rate. When bursae were removed from 14 two-day-old virgin Cecropia and each implanted into a virgin female, the oviposition pattern obtained was, with the exception of one female, always that of a virgin female. Consequently, one may conclude that bursae from mated females emit a blood-borne factor which effects the switchover in oviposition behavior after mating.

Implantation of the bursa copulatrix from females mated to castrate males

Eleven females were mated to castrate males. On the morning following completion of mating the bursa was excised and the spermatophore removed. These bursae when implanted into virgin females failed to cause a switchover in oviposition pattern (Figure 3). The per cent of eggs laid was only slightly increased over that seen when virgin bursae were implanted. Therefore, the oviposition pattern remained essentially that of a virgin female. Since these bursae had contained a well-formed but sterile spermatophore, apparently an interaction of the bursa with the sperm is necessary to trigger the release of the bursa factor.

Injection of hemolymph from mated females

Since both the corpora cardiaca (Truman and Riddiford, 1971) and the bursa copulatrix of mated females presumably release hormones into the blood, the hemolymph from these females should be able to stimulate oviposition. Injected hemolymph proved somewhat toxic such that death often occurred in 12–48 hours. All females which lived at least 3 days after the injection were included. Since many died soon thereafter, the data for only the first 4 days are included in Figure 4.

Figure 4A indicates that injections of 50 μl of blood from two-day-old virgin females did not stimulate oviposition. Similarly, injections of 50 μl of Ephrussi-Beadle Ringer's solution did not change the oviposition pattern.

Blood was drawn from females on the morning after mating was completed. Of 11 females injected with this "mated blood," nine began laying eggs at an increased rate as indicated in Figure 4B. By the third day the oviposition rate had returned to the virgin level (Table I). Surprisingly, injection of blood from females immediately after mating (about 12 hours after the beginning of mating) did not cause the switchover in behavior (Table I). Apparently, the circulating hormone(s) titer must increase with time after mating.

The blood volume of two two-day-old Cecropia females was measured by injecting 0.36 μg of inulin-methoxy-H^3. Inulin is neither excreted nor taken up by the cells of these insects (Cherbas and Cherbas, 1970). Equilibration appears to be complete in 4–6 hours in pupae (Cherbas and Cherbas, 1970); but to be certain, blood samples were taken 22 hours after the inulin injection. The blood volume of the 4.1 g and the 4.25 g females was found to be 1.1 and 1.4 ml $\pm$ 10%, respectively. Thus, the 50 μl hemolymph injections were about 4% of the total hemolymph volume.

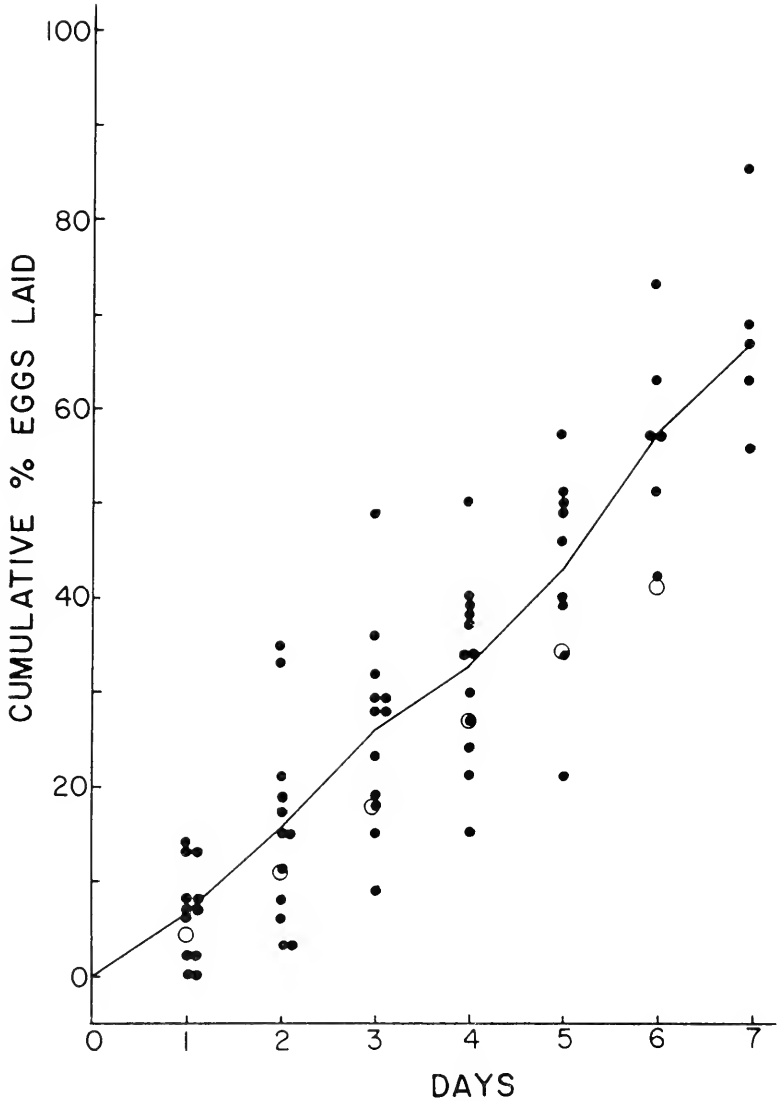

FIGURE 3. The oviposition pattern of virgin Cecropia females, each of which has received an implant of a bursa copulatrix from a female mated to a castrate male. The abscissa refers to days after implantation. The line is drawn through the average of the closed circles. The open circles represent the average cumulative percentage of eggs laid by females after mating to castrate males (Truman and Riddiford, 1971).

DISCUSSION

In the Lepidoptera, mating has long been known to stimulate oogenesis and increase the rate of oviposition (Klatt, 1920; Norris, 1933; Mokia, 1941; Benz, 1969; Truman and Riddiford, 1971). In all these cases the presence of sperm in

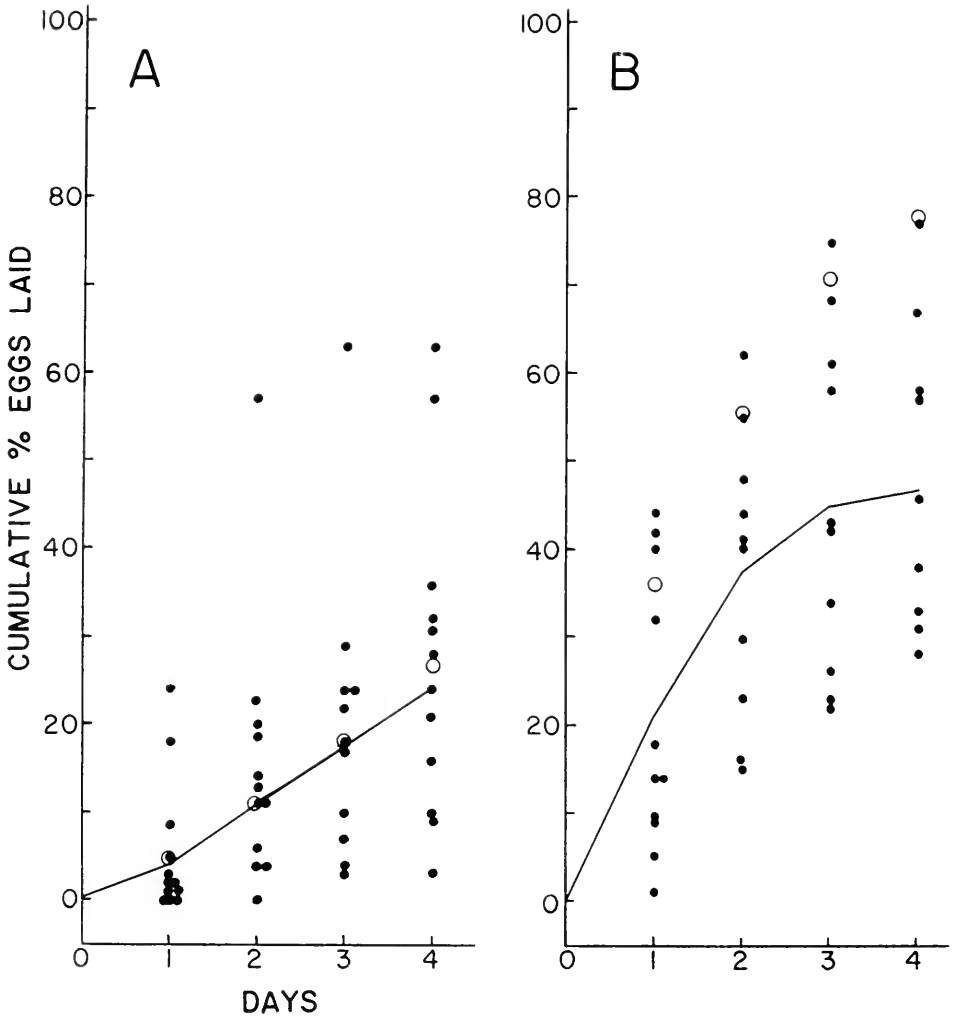

FIGURE 4. The oviposition pattern of virgin Cecropia females, each of which has received 50 μl of hemolymph. The abscissa refer to days after implantation. The line is drawn through the average of the closed circles excluding the highest set in Figure 4A: (A.) virgin hemolymph; (B.) mated hemolymph. The open circles represent the average cumulative percentage oviposition by intact virgin and mated females, respectively (Truman and Riddiford, 1971).

the female reproductive tract appears to be necessary for this switchover. It has been assumed that the presence of sperm in the spermatheca (Norris, 1933; Mokia, 1941; Benz, 1969) is the trigger just as has been found in the hemipteran *Rhodnius prolixus* (Davey, 1965). But the results reported above show that, at least in the non-feeding saturniid moth, *H. cecropia,* the important step is probably an interaction between the sperm and the bursa copulatrix. The male accessory gland

TABLE 1

Average daily oviposition of virgin female Cecropia moths after hemolymph injections

Treatment	Number	Average % eggs laid per day						
		Day after Treatment						
		1*	2	3	4	5	6	7
Untreated virgin females**	12	5	7	7	9	7	7	—
Untreated mated females**	24	36	22	13	7	6	3	—
Virgin females injected with blood from virgin females	12	4	10	6	6	10	11	8
Virgin females injected with blood from mated females 12 hours after the beginning of mating	3	1	4	3	5	10	16	16
Virgin females injected with blood from mated females the morning after mating	11	21	16	12	5	6	5	14

* Day 1 refers to day 3 of life. Day 3 is the day when most normal virgin females begin to lay a few eggs and is also the day following injection.

** From data of Truman and Riddiford (1971).

secretions which comprise the spermatophore do not trigger this response as indicated by the fact that implanted bursae of females mated to castrate males do not cause a change in oviposition rate. We have not ruled out, however, the possibility that, besides producing sperm, the testis also makes another substance which is responsible for activating the bursa.

After a fertile mating the bursa secretes a substance into the blood which causes a switchover from virgin to mated behavior. Secretory cells have been observed in the distal bursa copulatrix of several female Lepidoptera (Klatt, 1920; Weidner, 1934). The function of these cells is unknown, although Weidner (1934) proposes that the secretion may prevent the re-entry of the sperm into the bursa after they have emigrated to the spermatheca. Omura (1938) observed secretory cells in the anterior portion of the ductus bursae in the silkmoth *Bombyx mori,* but ascribes no function to them. Possibly one of these secretions is responsible for triggering the mated response.

This bursa factor does not stimulate directly an increase in oviposition, since mated cardiaectomized-allatectomized females lay eggs in the virgin pattern (Truman and Riddiford, 1971). Instead, this substance must act either directly or indirectly on the intrinsic cells of the corpora cardiaca to shut off the release of the calling hormone and to turn on the release of the oviposition stimulating hormone (Truman and Riddiford, 1971). Additional support for this role of the bursa factor is afforded by the observation that virgin females receiving bursa implants from mated females displayed other behaviors which were typical of mated females—they never "called" and they showed enhanced flight activity (Truman, Lounibos, and Riddiford, unpublished observations). Since intact nervous con-

nections between the brain and corpora cardiaca are necessary for this release (Truman and Riddiford, 1971), it seems probable that the bursa factor acts indirectly on the corpora cardiaca. The exact mode of action is presently unknown.

Moika (1941) showed that injection of blood from mated *B. mori* females induced oviposition in virgin females. Similiarly, in Cecropia mated hemolymph provoked an increase in oviposition rate over that of the normal virgin. But it should be noted that the response was transient and did not reach the mated level. This lack of a complete response is not surprising since the volume of hemolymph injected was only about 4% of the total blood volume. The absence of a continuing source of hormone as is present in the hosts receiving bursa implants from mated females is likely responsible for the swift decay back to the virgin level.

The bursa factor does not act directly to stimulate oviposition, but rather causes the corpora cardiaca to secrete an oviposition-stimulating hormone. Thus, hemolymph obtained from mated females may contain either or both the factor from the bursa and the hormone from the corpora cardiaca.

In Cecropia the spermatophore is completely formed in the bursa copulatrix by 3 hours and sperm transfer to the spermatheca occurs for the next 6 to 10 hours (J. A. Shepherd, Harvard University, personal communication). Thus, any crucial interaction of the sperm with the bursa must occur by 13 hours. The fact that the bursa is fully competent by 12 hours after the onset of mating to cause the change to the mated oviposition behavior is consistent with this hypothesis. As noted above, Weidner (1934) reported that during sperm transfer a secretion appears in the bursa. Possibly the release of the bursa factor into the blood also begins at this time.

Yet the blood taken from females 12 hours after the onset of mating lacks oviposition-stimulating activity. Normally, a mated Cecropia female under our 17L:7D photoperiod conditions begins oviposition about 17 to 18 hours after the initiation of mating (*i.e.* at or soon after the lights-off signal). Consequently, the corpora cardiaca probably release their oviposition-stimulating hormone about this time. Thus, 6 hours earlier, the oviposition-stimulating hormone was probably absent from the blood and the titer of the bursa factor was apparently not high enough to stimulate the corpora cardiaca of an injected host. By the following morning a sufficient titer of one or both hormones was present to stimulate oviposition.

The female Cecropia moth has a finely tuned hormonal control over her reproductive behavior. As a virgin female she releases her sex pheromone at a specific time of day determined by photoperiod (Riddiford and Williams, 1971). Once mated, pheromone release ceases and eggs are laid at an increased rate (Truman and Riddiford, 1971). The cue for this switchover is an interaction of sperm or substances from the testes with the bursa copulatrix. This interaction somehow causes the secretion of a factor from the bursa which in turn stimulates the corpora cardiaca to secrete the oviposition-stimulating hormone.

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Summary

1. Mating greatly increases the oviposition rate of female Cecropia silkmoths.

2. Implantation of the spermatheca from a mated female (either with or without sperm) into a virgin female did not alter the typical virgin oviposition pattern.

3. After implantation of the bursa copulatrix (minus the spermatophore) from a mated female, the virgin female oviposited eggs in the typical mated pattern.

4. Similar implantations of bursae from virgin females or from females which had mated with castrate males did not alter the virgin oviposition pattern.

5. Injections of hemolymph from mated females into virgin females caused an increase in oviposition rate. Blood from virgin females had no effect on oviposition.

6. Thus, the change in oviposition upon mating is due to a blood-borne factor which is secreted by the bursa copulatrix after contact with sperm or other substance from the testis. This bursa factor most probably acts to trigger the release of the oviposition-stimulating hormone from the intrinsic cells of the corpora cardiaca.

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EFFECTS OF SALINITY AND STARVATION ON THE UPTAKE AND UTILIZATION OF DISSOLVED GLYCINE BY *AURELIA AURITA* POLYPS

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The active uptake of amino acids from dilute solution by marine invertebrates of a number of phyla is a well documented phenomenon that has been adequately reviewed elsewhere (*e.g.*, Anderson and Stephens, 1969; Johannes, Coward and Webb, 1969; Southward and Southward, 1970; Stephens, 1972). Webb, Schimpf and Olmon (1972) have characterized the free amino acid (FAA) pools of recently fed *Aurelia aurita, Chrysaora quinquecirrha* and *Cyanea capillata fulva* scyphistomae, and Raum (1970) has made similar observations on *Aurelia* polyps that had been starved for one month. Although the uptake of dissolved amino acids by cnidarians has been documented among the Anthozoa (Stephens, 1962) and the Hydrozoa (Stephens and Schinske, 1961), such reports are lacking for the Scyphozoa.

A major controversy has concerned the possible role of dissolved amino acids as an energy source for marine invertebrates, with (in recent years) Stephens (1967, 1968), Little and Gupta (1968), Southward and Southward (1968, 1970, 1972) and others maintaining that this is the case. Other workers have observed a net loss of FAA by marine invertebrates; Johannes *et al.* (1969) review and discuss much of this literature, stating that the net loss of these substances precludes their utilization as a significant source of nutrition.

Johannes *et al.* (1969), using C^{14}-labeled amino acids and ion exchange chromatography, monitored the total flux of FAA in the turbellarian *Bdelloura candida;* the latter technique was also used in determining the rates of release of FAA by starved turbellarians at different salinities (Webb, Johannes and Coward 1971). Stephens (1962, 1963, 1964) and Stephens and Virkar (1966) have examined the role of dissolved labeled amino acids in the energy budgets of a variety of marine invertebrates, emphasizing salinity induced effects. In the investigation of the significance of dissolved FAA as a supplemental energy source, an important extension of the above experiments is to examine the effects of starvation on the uptake, subsequent distribution and utilization of labeled amino acids over a range of salinity. Since glycine is the major constituent of the FAA pools of *Aurelia* polyps (Raum, 1970; Webb *et al.*, 1972), such experiments were undertaken using ecologically meaningful concentrations of glycine in seawater.

Materials and Methods

Polyps of *Aurelia aurita* were obtained from the Virginia Institute of Marine Science, Gloucester Point; the animals were from a culture isolated by Spangenberg (1964) at Corpus Christi, Texas. Groups of scyphistomae were acclimated

to salinities of 10, 20, 30 and 40‰ (artificial seawater prepared according to the formula of Spangenberg, 1965) at room temperature (22°–24° C) for two months prior to the experiments. Iodide was deliberately withheld, lest effects of iodine induced metamorphosis (Spangenberg, 1967) complicate the experimental results. Polyps were fed *Artemia salina* nauplii twice weekly, and the water in the culture dishes was changed approximately six hours after feeding.

Preliminary experiments in which polyps were preincubated for 21 hours in streptomycin sulfate (200 mg/l seawater) indicated that bacterial contamination altered neither total glycine uptake nor the distribution of radioactivity in the various fractions. Therefore, to minimize external variables, streptomycin was not used in the main body of experiments, and the following decontamination procedures were utilized. Polyps were removed from the culture dishes either 48 or 288 hours after feeding, adhering debris was removed with a pipet, and the animals were washed four times with Millipore filtered (0.45 μ pore size) artificial seawater of the appropriate salinity.

Groups of 10 polyps of uniform size were transferred to sterile test tubes in 0.5 ml of sterile seawater of the acclimation salinity. To each tube was added 1.0 ml of sterile seawater of the acclimation salinity with a C^{14} glycine (U.L.) concentration of 1.27 μ moles/l (0.1 μCi/ml), giving a final glycine concentration of approximately 0.85 μ moles/l in the exposure medium. This concentration was felt to be ecologically realistic (*cf.* Stephens, 1963; Webb and Wood, 1967).

Scyphistomae were exposed to the labeled medium in sealed test tubes for one hour, quickly washed in 4 changes of sterile, unlabeled seawater, and incubated for an additional 23 hours in sterile, unlabeled seawater at room temperature. CO_2 was collected throughout the exposure and incubation periods in 10% KOH on ground glass rods imbedded in the stoppers sealing the individual test tubes. The KOH was rinsed into individual counting vials at the end of the incubation period and counted in Aquasol Universal L.C.S. Scintillator on a Beckman LS-200B liquid scintillation system. The polyps were again washed in unlabeled seawater and finally placed in 1.5 ml of 80% ethanol for 24 hours. Aliquots of the EtOH extracts (FAA) were then added to scintillation cocktail (6 g PPO/l toluene, added 2:1 to Triton X-100). The polyps were rinsed four times in clean ethanol, air dried briefly, placed in counting vials with 0.5 ml NCS Solubilizer and digested for 24 hours. Scintillation cocktail was then added and the pH adjusted to approximately 6 with glacial acetic acid. All samples were allowed to stand for 24 hours before counting, and were corrected for background and for quenching.

Experimental procedure prevented weighing the experimental animals, although mean dry weights for recently fed (48 h) and starved (288 h) polyps were determined using two replicates of three uniformly sized individuals from every experimental group. Polyps were removed from the culture dishes, rinsed briefly in glass distilled water, placed in groups of three on tared foil strips, dried for 24 hours at 55° C and weighed to the nearest 0.01 mg.

Results

General

Polyps increased in size and reproduced asexually at all experimental salinities, with the highest growth and reproductive rates occurring at 30‰, and the lowest

TABLE I

C^{14} glycine distribution and utilization (cpm μg dry wt. ± S.D) by groups of 10 Aurelia polyps at various salinities and times since feeding. Mean dry weight (μg/polyp) for each experimental group is given in parentheses

Fraction	10‰		20‰		30‰		40‰	
	48 h (29)	288 h (23)	48 h (42)	288 h (39)	48 h (43)	288 h (27)	48 h (33)	288 h (30)
	n = 3	n = 4	n = 3	n = 4	n = 3	n = 4	n = 4	n = 4
CO_2	15.9 ± 1.5	45.7 ± 8.9	8.3 ± 1.1	19.7 ± 7.4	5.2 ± 0.7	15.4 ± 3.6	13.8 ± 2.0	16.5 ± 1.5
FAA	10.2 ± 2.3	10.0 ± 3.6	61.5 ± 10.3	67.0 ± 5.6	105.1 ± 14.8	77.6 ± 22.8	65.5 ± 24.3	73.6 ± 22.7
Ethanol insoluble	122.2 ± 8.7	116.0 ± 19.3	165.8 ± 3.4	106.2 ± 17.0	127.8 ± 1.8	138.8 ± 29.4	113.5 ± 16.7	118.2 ± 15.5
Total uptake	148.3	171.7	235.6	192.9	238.1	231.8	192.8	208.3

rates at 40‰. Rates at 10‰ approached the low levels observed for the animals at 40‰, while those at 20‰ were only slightly less than those of the scyphistomae at 30‰.

Mean dry weights (μg/polyp) are given in Table I. Absolute values (expressed as cpm/μg dry weight) for $C^{14}O_2$, labeled FAA, ethanol insoluble radioactivity and total radioactive glycine uptake are given in Table I. When corrected for machine efficiency, 100 cpm are equivalent to approximately 0.6 pico Moles of C^{14} glycine. Values for the various fractions expressed as per cents of the total uptake at a given salinity and time since feeding are given in Figure 1.

Recently fed (48 h) polyps

Both the total glycine uptake and the size of the labeled FAA pools of recently fed scyphistomae increased (on absolute and relative bases) with salinity from 10 to 30‰ (Table I; Fig. 1); this relationship appears to be widespread among

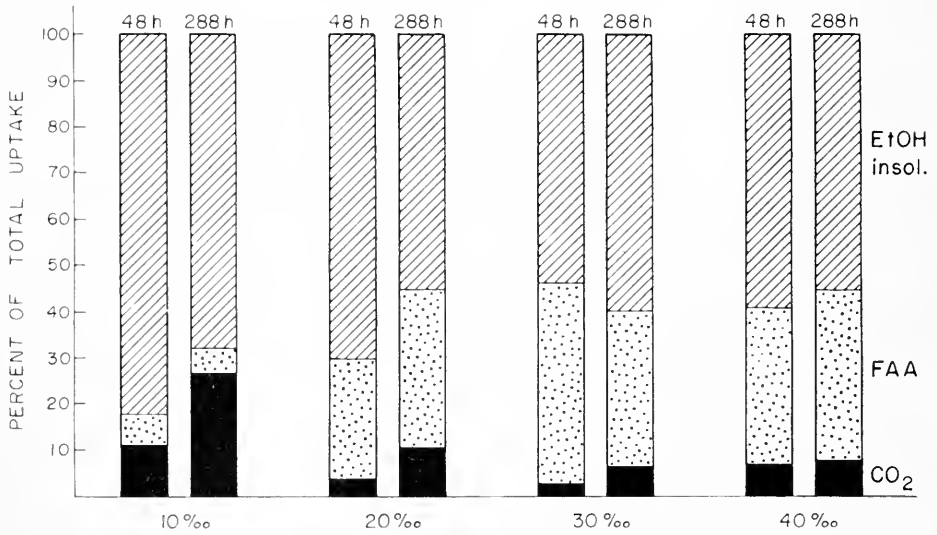

FIGURE 1. Distribution and utilization of C^{14} glycine by *Aurelia* polyps, expressed as per cents of total uptake at various salinities and times since feeding.

euryhaline marine invertebrates. The sixfold increase in radioactivity present as FAA in Corpus Christi polyps at 20‰ when compared to animals at 10‰ closely parallels the change in glycine levels of the total FAA pools of Chesapeake Bay polyps 48 hours after feeding reported by Webb *et al.*, (1972). Similarly, the reduced size of the free glycine pool at 40‰ (Table I) is again paralleled by the decline of glycine in the pools of Chesapeake Bay polyps above 30‰; the decrease probably reflects, as do the depressed growth and reproductive rates of the Corpus Christi polyps, the effects of physiological stress at elevated salinities.

The relative amounts of label present as ethanol insoluble material showed an inverse relationship with salinity between 10 and 30‰ (Fig. 1). This phenomenon has been noted in a variety of marine and estuarine organisms (Stephens, 1964;

Stephens and Virkar, 1966; J. W. Anderson, personal communication), and probably serves to reduce the amount of osmotically active small molecules in the tissues at lower salinities.

The high levels of $C^{14}O_2$ production at 10 and 40‰ (Table I; Fig. 1) may again indicate stress at salinity extremes, although detailed metabolic studies would certainly be necessary to clarify this point; both groups of polyps exhibited depressed growth and reproductive rates. It may be significant with respect to this hypothesis that the lowest $C^{14}O_2$ production was observed at 30‰, the optimum salinity for growth and reproduction in the present study.

Starved (288 h) polyps

In comparisons with 48 h polyps, Student's t tests revealed no significant differences ($P > 0.05$) in total C^{14}glycine uptake after starvation at any experimental salinity. The production of $C^{14}O_2$ by polyps 288 hours since feeding is from two to three times greater than that of the more recently fed animals at 10, 20, and 30‰ (Table I; Fig. 1). Production of $C^{14}O_2$ increased (on absolute and relative bases) in starved polyps at 10‰, while free C^{14}glycine remained at the same low level (Fig. 1). At 20‰, $C^{14}O_2$ produced by starved animals increased, while free labeled glycine again remained essentially the same as in the 48 h polyps. The increase in labeled CO_2 at 30‰ occurred with a concomitant decrease in the amount of free labeled glycine (Table I; Fig. 1). Total glycine uptake by starved polyps at 40‰ matched that of recently fed scyphistomae (Table I), and it is noteworthy that the relative levels of all fractions also remained constant (Fig. 1). The apparent lack of starvation effects at 40‰ may indicate a minimum critical level of glycine uptake and an optimum distribution of the amino acid among catabolic pathways, FAA pools and synthesis of larger molecules at this stressful salinity that remain constant even during the added stress of 288 hours of food deprivation.

Discussion

At this point, explanations of the above observations must be largely speculative. Webb *et al.* (1971) have demonstrated that starved turbellarians decrease the amount of FAA released into the medium, although these authors present no data concerning the rate of uptake under similar conditions. Unfortunately, the total amino acid flux in scyphozoan polyps, either recently fed or starved, has not been determined, although the results of the present study indicate that the rate of glycine uptake is essentially unaffected by 288 hours of food deprivation. Thus, the often observed net outward flow of FAA may be reversed to some degree during starvation.

The low level of free C^{14} glycine in 48 h and 288 h polyps at 10‰ is suggestive of an optimum and necessarily low concentration of an osmotically active amino acid at reduced salinity that remains constant even during starvation. The absolute values and percentages of free glycine in both 48 h and 288 h polyps at 20 and 40‰, and in 288 h polyps at 30‰, are very similar (Table I; Fig. 1;) this similarity over such a range of salinity perhaps indicates that the pools serve as

sources for a metabolic pathway or pathways, rather than having a strictly osmotic function. The larger free glycine pool in 48 h polyps at 30‰ may be correlated with the observed maximum growth and reproductive rates (and hence, presumed maximum metabolic efficiency) at this salinity; these polyps therefore accumulate large amounts of this amino acid into FAA pools, channeling much of the pool into catabolic pathways during food deprivation, a supposition that is supported by the work of Raum (1970). Further, the glycine that is taken up from solution during starvation at this salinity is broken down fairly rapidly, rather than remaining in the large pools observed in the recently fed animals.

The two-to-threefold increases in $C^{14}O_2$ production by starved polyps when compared to recently fed animals are probably explained by the larger percentage of labeled glycine relative to unlabeled glycine in the FAA pools of the starved animals. However, since the amount of incorporation of labeled glycine into ethanol insoluble material either decreases or remains essentially unchanged after starvation, the possibility of an enhanced rate of glycine oxidation by starved polyps exposed to dissolved glycine cannot be discounted. Whichever is the case, there is proportionately greater participation of exogenous glycine in the catabolic pathways of the starved scyphistomae. The estimation of the significance of this increase must await the determination of the total glycine flux in these animals. It must also be pointed out that the $C^{14}O_2$ produced is not necessarily respiratory in origin, for Greenberg (1961) has indicated that the catabolism of glycine via serine and pyruvic acid and subsequent entry into the tricarboxylic acid cycle is of little quantitative significance in vertebrate liver. A similar situation seems to exist in scyphozoan polyps; more than 99% of the radioactivity recovered from *Chrysaora quinquecirrha* polyps exposed to C^{14} glycine was still present as glycine, while the radioactivity recovered from polyps exposed to labeled serine was determined to be 73.8% in serine and 20.7% in glycine (Dr. K. L. Webb, personal communication).

Previous investigations of a nutritive role of dissolved organic compounds have been performed using well fed animals having presumably high levels of metabolic substrates. The present investigation has demonstrated that the oxidation of glycine taken up from solution assumes increased importance when substrates derived from prey or other solid food may be limiting.

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Summary

1. The size of the labeled free glycine pools in recently fed *Aurelia aurita* polyps exposed to dissolved C^{14} glycine increased with salinity from 10 to 30‰; the decline at 40‰ is probably a reflection of stress on the animals.

2. The percentage of radioactivity present as ethanol insoluble material was inversely related to salinity between 10 and 30‰.

3. The rate of glycine uptake was unaltered after 288 hours of food deprivation at 10, 20, 30 and 40‰.

4. The oxidation of radioactive glycine taken up from solution, as measured by the collection of $C^{14}O_2$, increased two- to threefold in starved polyps at 10, 20 and 30‰.

5. It is suggested that experiments utilizing starved animals, in which substrates derived from solid food are low, are of importance in elucidating the role of dissolved organic compounds as supplemental energy sources for marine invertebrates.

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DEMONSTRATION OF AN ACTION OF ACETYLCHOLINE ON THE CENTRAL NERVOUS SYSTEM OF A CRAB

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A question which has remained unsettled for some time concerns the function of the acetylcholine (ACh) found in crustacean nervous systems. The most likely role for this compound is that of a synaptic transmitter agent in the central nervous system but of those criteria listed by Paton (1958) for evaluation of suspected transmitter agents, one has resisted experimental verification: administration of ACh to the central nervous system has not consistently produced effects which mimic the action of a natural transmitter. Although Bonnet (1938) reported the demonstration of such an effect of ACh on the crayfish, neither an extensive study by Prosser (1940) nor more recent attempts (Welsh, 1961) confirmed the earlier study.

The purpose of this report is to show that small doses of ACh, when added to a crab's central nervous system, do indeed have definite effects and moreover these effects are influenced by eserine and atropine. The present results support the contention that ACh is a synaptic transmitter agent in the central nervous system of crabs.

Materials and Methods

The crab *Carcinus maenas* was used for these experiments. The physiological saline solution (Fatt and Katz, 1953) contained (mM) NaCl (513), KCl (13), $CaCl_2$ (12), and $MgCl_2$ (24). The saline was buffered at pH 7.4 by 5 mM Tris-Maleate. The drugs (acetylcholine chloride, atropine sulfate, and eserine salicylate) were obtained from Sigma Chemical Company. Stock solutions were made up in distilled water and stored in the refrigerator until use. These stocks were made up frequently. All experiments were done at room temperature, 22–25° C.

Two types of experiments were performed. The first type involved perfusion of the thoracic ganglion through the sternal artery and recording the mechanical activity of the legs in response to ACh. The administration of drugs to the central nervous system *via* the arterial system was necessary for the success of the experiments. Although crustaceans have open circulatory systems, there is a well-defined vascular system in the thoracic and supra-esophageal ganglia (Sandeman, 1967). The second type involved adding the drug directly to the ganglion by electroosmotic injection from a glass micropipette and recording the induced electrical activity in the neuropile by means of a second, adjacent, micropipette.

For the experiments which required the recording of mechanical activity the crab was tied, with rubber bands, to a small Plexiglas platform. The crab's legs hung over the edge of the platform and thus could move freely. The crab was held

in air over a large dish which was used to catch the perfusate dripping out of the animal. The carapace over the heart was removed and, with the aid of a stereo microscope, the dorsum of the heart was removed and the blood rinsed out with saline. A small polyethylene cannula was then inserted into the orifice of the sternal artery in the ventral part of the heart; the artery was tied to the cannula with silk suture, and a slow perfusion of saline from a Mariotte flask was started. ACh was injected in aliquots of one ml while eserine and atropine were perfused through continuously from separate flasks. In this preparation, mechanical activity of the legs was monitored on a Grass Model 7 Polygraph. This was achieved by attaching one or more legs with thread at convenient places, to a Grass FT.03 strain gauge.

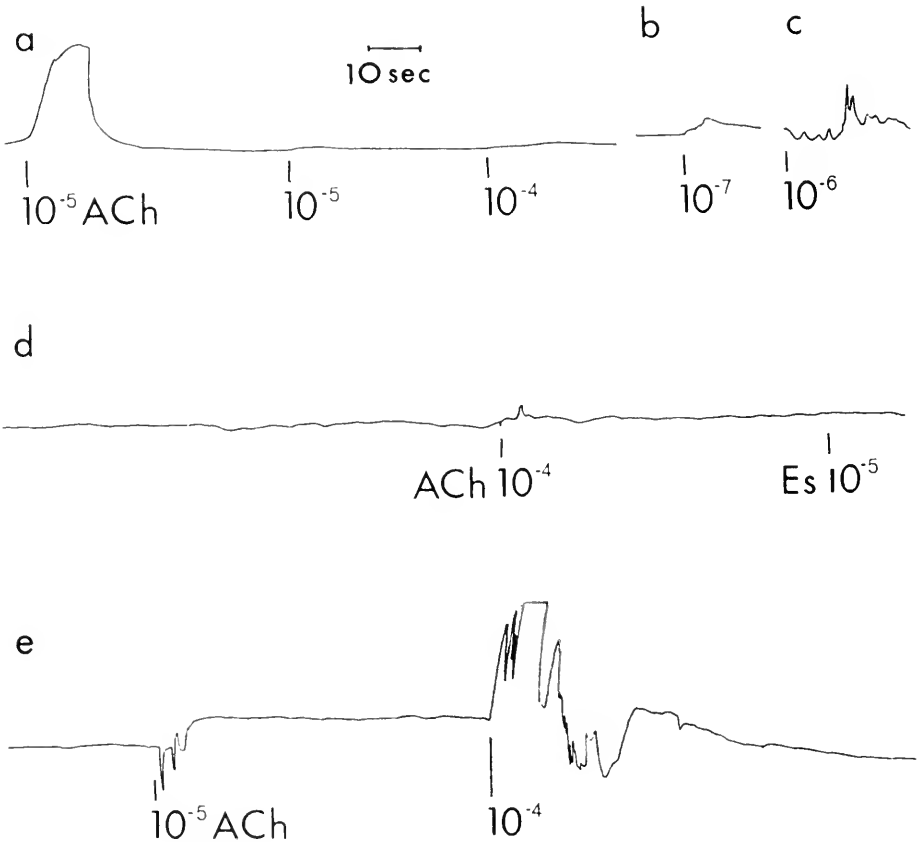

FIGURE 1. The effect of acetylcholine and eserine on leg movements of *Carcinus*. In (A) the injection of one ml of ACh-saline (10^{-5} g/ml) into the perfusion line elicited a transient leg movement. Subsequent application of the drug at the same and at a greater concentration showed no notable effect. In (B) and (C) the responses of two different crabs to lower concentrations of acetylcholine are shown. In (D) the response of a fourth crab to an injection of acetylcholine (10^{-4} g/ml) is displayed and in (E) the responses of the same crab at a lower and at the same concentration as before but both in the presence of eserine (10^{-5} g/ml).

For the second type of experiment, the crab's legs were removed at the point of autotomy, and the crab was tied to the platform ventral side up. The tail, ventral exoskeleton, and underlying musculature were then removed in conjunction with copious rinsing in seawater. The thoracic ganglion thus exposed was kept moist by topical application of the saline solution. Micropipettes with a tip diameter of less than 0.5 microns were drawn from Pyrex tubing using the Industrial Sciences puller. One micropipette, used to inject ACh, contained only ACh (1–10 mM) in distilled water. The second pipette, used to record electrical changes in the neuropile, was filled with 3M KCl and had a resistance of 5–15 megohms. A Ag: AgCl electrode in the fluid next to the ganglion completed the circuits. The mode of recording was single-ended, and the amplifier used was a Bioelectric Instruments NFI. The neuropile potentials were observed on one channel of a Tektronix 561 oscilloscope and were photographed with a Grass Kymograph camera on a "slave" oscilloscope. The pipette used to deliver the ACh was connected to a Tektronix 161 pulse generator through an operational amplifier circuit designed to insure constant currents, despite large changes which might be encountered in the resistance of the micropipettes. A description of this current-clamp device is being prepared for publication (G. Katz, in preparation). The second channel of the oscilloscope was used to monitor the current pulse during the electroosmotic injection. The method of electroosmotic injection (Krnjevic and Whittaker, 1965; Chiarandini, Reuben, Brandt and Grundfest, 1970) is attractive because of its ease of use and simplicity; for quantitative studies however, iontophoretic application would be preferable.

The pipettes were held by the same micromanipulator, a Narishige MD-2, which allowed independent positioning of the tips to within 0.2 mm. The spacing was within 0.2 mm at the start but after insertion it was probable that the spacing changed. By selective probing of the ventral surface of the ganglion, areas in the neuropile were found which responded to ACh with electrogenesis. Many insertions showed no response. No intracellular recordings were made and no responses were recorded from points near the surface. No systematic study was made of the frequency or location of responsive sites within the neuropile because the intent was qualitative; that is, to determine whether or not direct injection of ACh would produce an effect.

Results

The effect of acetylcholine

Figure 1 depicts the result obtained upon addition of one ml of saline containing ACh to the perfusion line. Three different preparations with different response sensitivities are shown in Figure 1A, B, and C. I found that the sensitivity of the crab to ACh diminished with time; if the application of ACh was made within a few minutes after cannulation, concentrations as low as 10^{-8} g/ml injected into the perfusion line could cause leg movement. After an hour of perfusion and with no other treatments, 10^{-6} g/ml or more were required. In no instance was a concentration greater than 10^{-4} g/ml necessary.

I also found that repeated application of ACh resulted in diminished sensitivity. An example of this is shown in Figure 1A where a second application of 10^{-5} g/ml

was ineffective and even 10^{-4} g/ml showed little effect. This decrease in sensitivity was also observed with the electroosmotic injection method and it will be discussed later.

Finally, as noted by many previous investigators, topical application of ACh in concentrations as high as 10^{-3} g/ml was ineffective. This method of application involved dripping the solution directly on the ganglion.

The effect of eserine and atropine

Eserine by itself caused leg movements of low magnitude but it was easily shown that the effect of ACh were enhanced during eserine treatment. Figure 1D and E shows this potentiation. While there was relatively little control response to 10^{-4} g/ml ACh, upon the addition of eserine, even a ten-fold reduction in dosage elicited an easily-recorded response and a repeat of the control dose caused vigorous and prolonged leg movements. Discontinuation of perfusion with eserine resulted in a loss of this heightened responsiveness to ACh. In connection with these observations, spontaneous increases in sensitivity to ACh were never observed. Instead, the common observation was, as noted above, a decrease in sensitivity. Thus, the result to be emphasized is the increase in sensitivity during eserine treatment rather than the decrease in sensitivity after eserine perfusion was discontinued.

Eserine also caused an increased responsiveness to mechanical stimulation. Normally there were no spontaneous movements; the crab, after several minutes perfusion, remained limp and unresponsive to moderate tapping on the rostrum. In contrast, the eserine-treated animal responded to tapping on its rostrum with extensive leg movements. Figure 2A depicts this effect.

Atropine reduced the response to ACh. Figure 2B and C displays an example of this effect. After the addition of atropine, the response to ACh was recorded and then, after extensive perfusion with normal saline, another response was recorded and was found to be considerably greater. The effect of atropine was difficult to reverse and in this case, 45 minutes perfusion was necessary. Diminished sensitivity to repeated applications of ACh made it difficult to establish a control response before the addition of this drug, which also attenuated the response. For this reason, in this instance, I chose not to test the animal before the addition of atropine in order to work with as fresh a preparation as possible. Since I have never observed a spontaneous increase in sensitivity to ACh, it seems reasonable to ascribe the larger response in Figure 2C to the absence of atropine.

Electroosmotic injection of acetylcholine

Since the sternal artery passes through the thoracic ganglion and branches to form an artery for each leg, it is conceivable that the effect of ACh obtained by perfusion is initiated peripherally and not in the central nervous system. A direct test of this hypothesis is obviously desirable. The change in electrical activity following electroosmotic injection of ACh into the neuropile is shown in Figures 3 and 4.

In Figure 3, records taken from four different insertions (two animals) are shown. The voltage record (top trace) before the pulse is stable but upon initiation of electroosmotic flow there is considerable electrical activity. The interpretation

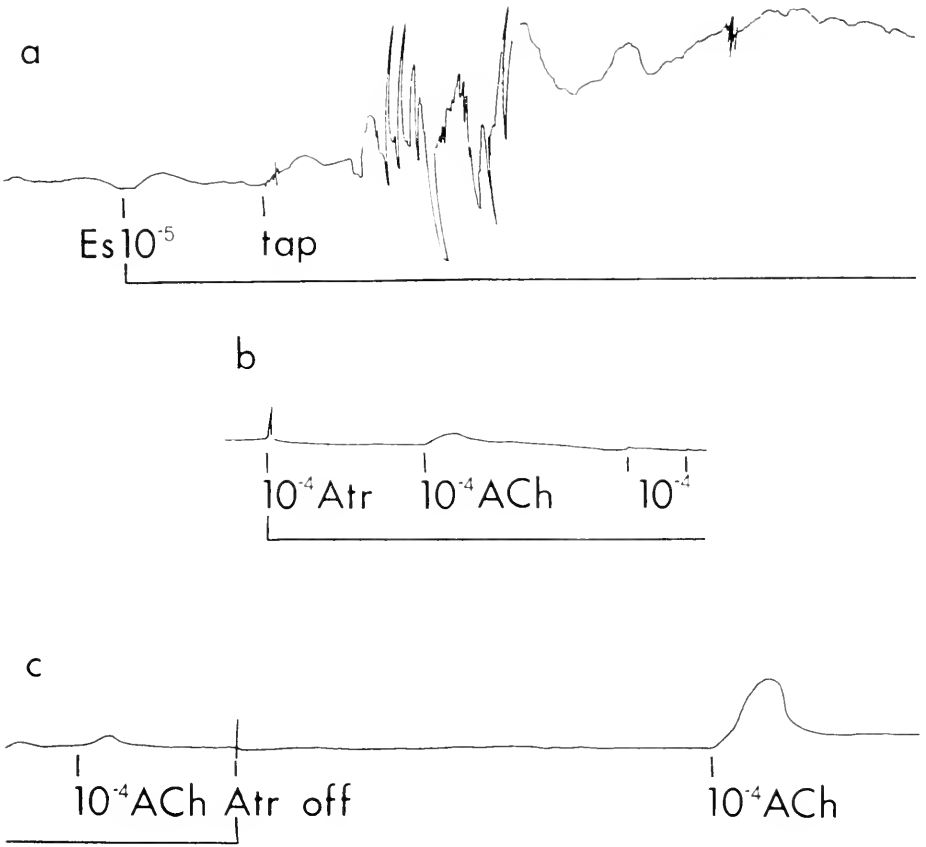

FIGURE 2. The influence of eserine on mechanical stimulation and of atropine on the acetylcholine effect. In (A) the leg movements in response to a sharp rap on the rostral carapace after the perfusion of eserine. Normally the crabs are flaccid and do not respond to such stimulation. In (B) atropine (10^{-4} g/ml) was added before the acetylcholine and a small response was noted. In (C) the response was depressed until after removal of the atropine by extensive perfusion.

of these potential changes is that they represent current flow through the resistance formed by the extracellular fluid lying between different neuropile elements; it is probable that both synaptic and axonal responses contribute, the latter in response to the former. It is unlikely that the current flow from the delivery pipette itself caused the observed effects because the majority of attempts resulted in no electrical response at all. Figure 3 also shows that the electrical activity subsided toward the end of the pulse even though the current pulse remained steady. This can probably be attributed to desensitization of the postsynaptic element. Another fact which suggests that the primary effect is on synaptic rather than electrically excitable membrane is that the effect of ACh was graded; the size of the response grew with increase in electroosmotic current.

Repeated application of ACh resulted in diminished responses. Figure 4A shows again that the acitivity subsided with time during one pulse. Figure 4B, C, and D all show that repetition of the pulse at short intervals could attenuate or even abolish the response. The repeated pulses are superimposed. If the interval between pulses was on the order of a few seconds, this diminished response was not observed; this can be another manifestation of desensitization. The presence of graded activity and of desensitization argue further against the possibility that the current flow itself could cause these neuropile potentials, since synaptic membrane is electrically inexcitable (Grundfest, 1957).

I would like to emphasize that the important point of this section is that the effect of ACh can be localized to the thoracic ganglion. Even though the possibility of an effect of ACh on peripheral elements of the nervous system was not

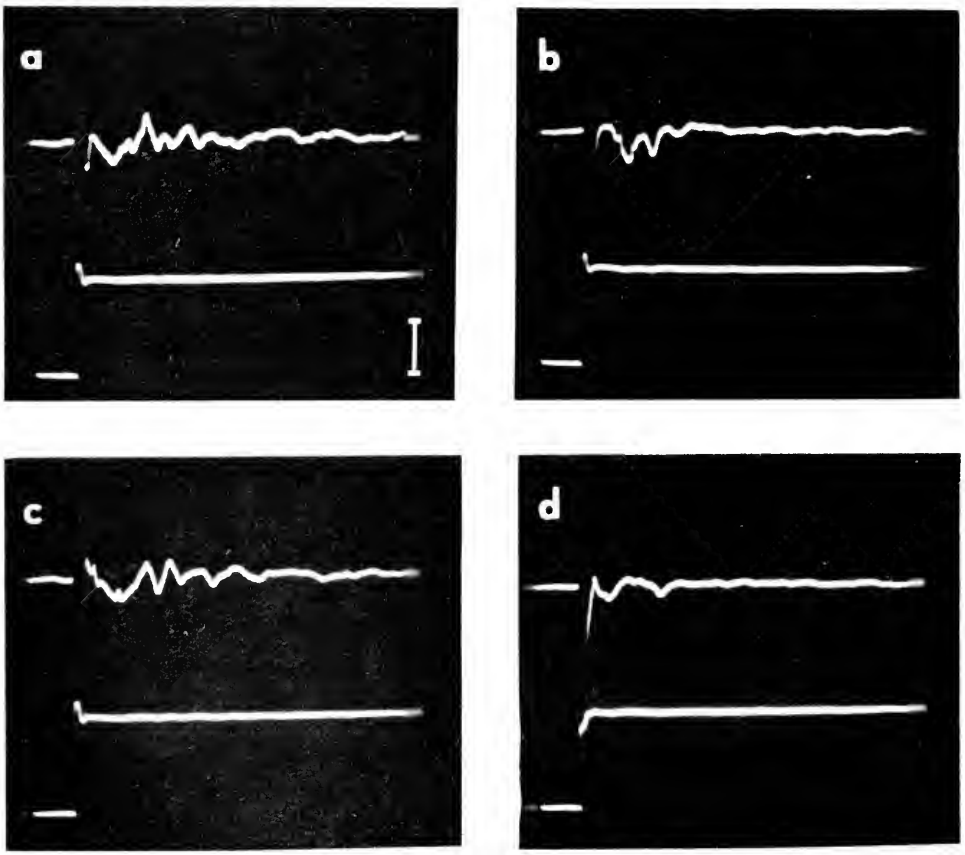

FIGURE 3. The effect of electroosmotic injection of acetylcholine on the electrical activity of the crab neuropile. Sections (A-D) represent the responses of two crabs to injection in four different areas. The top traces are the extracellularly-recorded neuropile potentials. The vertical bar represents 5 millivolts. The bottom trace shows the current record in response to a 100 volt driving pulse of 100 milliseconds duration. The pulse stopped at the end of the sweep.

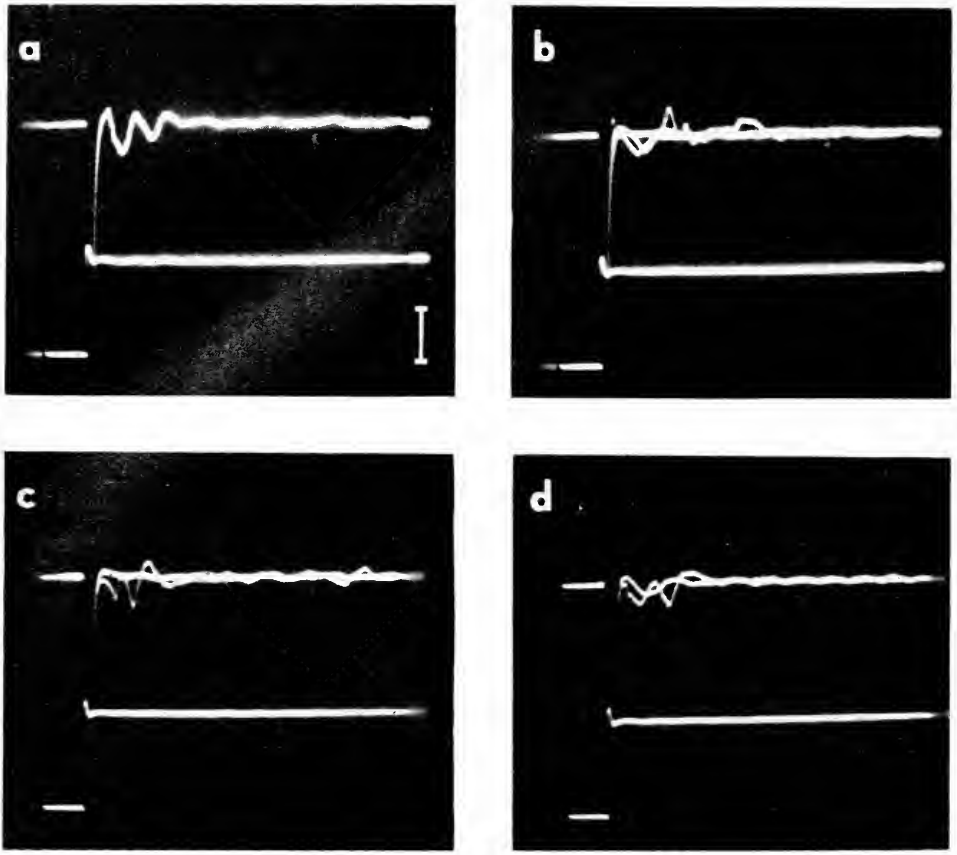

FIGURE 4. The effect of continued and repetitive injections of ACh by the electroosmotic method. In (A) the transient nature of the response, in spite of continuous injection, is shown. (B) shows the effects of three separate pulses with a repetition interval of 0.75 seconds and (C) displays two pulses. In (D) the interval was increased to 1.5 seconds.

directly ruled out, the results of this section made it unnecessary to postulate such actions.

DISCUSSION

Acetylcholine, adminstered in low concentrations by arterial perfusion to the thoracic ganglion, caused leg movements in crabs. Direct injections into the neuropile show that the effect of ACh can be attributed to its action on neural tissue. Furthermore the actions of eserine in potentiating this action of ACh, of atropine in blocking it, and the presence of desensitization and gradedness, all strongly support the contention that the ACh is acting on synaptic membrane. It might be argued that ACh is, in some manner, inducing a release of a transmitter rather than acting on synaptic membrane directly. Until there is evidence suggesting that the situation is more complex, it seems advisable to retain the simplest possible explanation.

A discussion of the experiments of earlier investigators is hampered by the diversity of preparations used and of the experimental conditions employed. Only Bonnet (1938) has reported a positive effect of ACh in low concentrations. He found that there was a short-lived enhancement of spontaneous electrical activity of the isolated ventral nerve cord of crayfish, following application of ACh in concentrations ranging from 10^{-9} to 10^{-7} g/ml. This action was followed by a depression of nervous activity. Prosser (1940) was unable to confirm Bonnet's work, finding that concentrations of ACh as large as 10^{-3} g/ml were necessary to obtain an effect.

A succession of investigations dealing with shrimp, lobsters, and crayfish also produced negative results. ACh, applied topically to the ventral nerve cord, either caused no change in spontaneous activity or, if changes were observed, the concentrations necessary to produce an effect were considered to be too large to be physiological (Ellis, Thienes and Wiersma, 1942; Turner, Hagins and Moore, 1950; Wiersma, 1952; Hichar, 1960; Welsh, 1961). I too, was unable to produce an effect in the present experiments by applying ACh topically on the thoracic ganglion. Similar difficulties have been encountered during the study of insect ganglia (Treherne, 1966).

Other investigations (Turner *et al.*, 1950; Wiersma, 1952) have employed preparations which were later shown to transmit electrically rather than by release of chemicals. The cause for a failure to find an effect of ACh in these cases is thus apparent.

The failure of ACh to act in low concentrations can be ascribed to the action of cholinesterase. In both insects and crustaceans, there is an active cholinesterase and a sizable connective tissue sheath surrounding the central nervous system (Treherne, 1966). Direct application by injection into the neuropile of crabs in the present experiments and of insects (Kerkut, Pitman and Walker, 1969; Pitman and Kerkut, 1970) has been successful in circumventing these barriers.

As I previously mentioned, in addition to the desensitization (Figure 4), the crab thoracic ganglion appears to lose sensitivity to ACh over a period of time; This phenomenon may also be responsible, at least in part, for some of the earlier failures to find a convincing effect of ACh. The mechanism of loss of sensitivity is unknown. One explanation is that perfusion may remove a substance necessary for maintenance of normal excitability. An alternative explanation is that these ganglia are sensitive to low oxygen levels and to the lack of nutrients in the perfusion fluid. Prosser and Buehl (1939)have demonstrated the importance of oxidative metabolism for electrical activity in ganglia taken from crayfish. Future experiments such as the present study must include better control of oxygenation. It is unlikely that this loss of sensitivity is a desensitization since it appears to happen without previous application of ACh.

The presence of ACh in crustacean central nervous systems has been reported by many investigators (Welsh, 1938; Jullien and Vincent, 1938; Welsh, 1939; Smith, 1939; Schallek, 1945; Tobias, Kollros and Savit, 1946; Easton, 1950; Walop, 1950; Hichar, 1960; Florey and Biederman, 1960). Furthermore both the synthesizing and degrading enzymes have been shown to be present. Choline acetylase has been found in ganglia of crayfish by Easton (1950) and of crabs by Walop (1950). The presence of cholinesterase was first shown by Marnay

and Nachmansohn (1937) for lobsters; Walop and Boot (1950) demonstrated the presence of the enzymes in *Carcinus* thoracic ganglia. E. Maynard (1964, 1971) has studied the localization of cholinesterase in lobster ventral ganglia by histochemical techniques.

A great many pharmacological studies have been performed on crustaceans using drugs known to affect cholinergic systems in other phyla. Curare injections are known to cause paralysis, depression of reflex activity, and lethargy in crustaceans (Katz, 1936; Ellis *et al.*, 1942; Wright, 1949). Atropine and eserine have also been studied. Although Prosser (1940) found no activity of atropine on the crayfish ventral nerve cord preparation, Turner *et al.*, (1950) found that it caused increased spontaneous activity and Ellis *et al.*, (1942) reported that it blocked nerve conduction. The concentrations of atropine employed were rather large. On the other hand, eserine in low concentrations (10^{-5} g/ml) has been reported to cause increased spontaneous activity (Prosser, 1940; Turner *et al.*, 1950; Hichar, 1960) and a hypersensitivity of reflex action (Wright, 1949; Florey, 1967). Easton (1957) perfused the ventral nerve cord of crayfish *via* the heart and although he was unable to demonstrate an effect of ACh alone, he found that eserine permitted actions of ACh to be seen. He observed an attenuation of reflex transmission from the legs to the abdominal muscles concerned with the tail flip. He further observed the production of slow rhythmical abdominal flexions in response to the presence of ACh in the eserine-treated preparation. However, eserine, like atropine, can cause direct effects on nerve (Ellis *et al.*, 1942) and Knowlton (1942) has found actions of high concentrations of eserine on the neuromuscular junction of a crab leg even though this is not a cholinergic system (Katz, 1936). Therefore, although the pharmacological studies involving direct application of drugs are suggestive, it is only when they are studied in conjunction with positive effects of ACh application, as in the present experiments, that they are persuasive.

A final consideration is the demonstration of a release of ACh during nervous activity. Many investigators (Welsh, 1939; Schallek, 1945; Walop, 1951) have shown that ACh is released from nervous tissue taken from crustaceans. Since there is a concomitant spontaneous electrical activity it can be said that ACh is released during nervous activity. It remains to be seen, however, if this release is correlated with the electrical activity or is merely coincidental. Walop (1950) for instance was unable to demonstrate that the release of ACh from the thoracic ganglion of *Carcinus* was augumented by electrical stimulation of the ganglion. Florey (1967) however has recently reported that perfusates of crab central nervous systems contained ACh-like activity during sensory-induced mechanical activity. His report removes an important obstacle in satisfying the criteria for ACh as a transmitter in crabs.

In summary, since ACh is present, along with enzymes for its synthesis and inactivation, and since ACh has now been shown to have an effect on the central nervous system with the attendant and expected effects of eserine and atropine, and finally, since ACh is released from crustacean nerve and ganglia in response to sensory input, it would appear safe to conclude that ACh may function as a chemical transmitter in the central nervous system of crustaceans.

Florey and Biederman (1961) suggested that ACh may be the transmitter of sensory neurons in crabs because they found little or no ACh in either the excita-

tory or inhibitory axons. ACh has long been known to be present in crustacean nerve as well as in the ganglia. Davis and Nachmansohn (1964) found that lobster leg nerve *sans* its large efferent axons, and therefore presumably entirely composed of sensory axons, was capable of synthesizing ACh. Synthesis of ACh by abdominal stretch receptors of lobsters has recently been reported by Hildebrand, Barker, Herbert and Kravitz (1971). These observations and those of Wright (1949) and of Easton (1957) which showed increased reflex responsiveness following administration of eserine, all indicate that ACh may be a transmitter in sensory neurons. However, although ACh appears to function as a chemical transmitter in sensory axons, it should be pointed out that ACh need not be the transmitter in all crustacean sensory systems nor need ACh be restricted to sensory systems. Indeed, careful experiments by Prosser (1940) showed that reflex transmission elicited by movements of caudal sensory hairs in crayfish was influenced by neither ACh nor eserine. It may also be that ACh is a transmitter of one or more interneurons.

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Summary

1. The administration of acetylcholine to the thoracic ganglion of *Carcinus* by means of the sternal artery resulted in leg movements. Low concentrations of acetylcholine were effective and the action of acetylcholine was potentiated by eserine and attenuated by atropine.

2. Injection of acetylcholine into the thoracic ganglion by means of electro-osmotic injection caused electrical activity in the ganglion and desensitization was found to accompany this activity.

3. The present results and those of other investigators indicate that the classical pharmacological criteria for identification of ACh as a chemical transmitter appear to be fulfilled.

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THE EFFECTS OF ACUTE GAMMA IRRADIATION ON THE BRINE SHRIMP, *ARTEMIA*. III. MALE F_1 REPRODUCTIVE PERFORMANCE FOLLOWING PATERNAL IRRADIATION OF MATURE SPERM[1]

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It was reported in the first paper of this series (Squire and Grosch, 1970) that the F_1 sons of irradiated males are much more sterile than are the irradiated males themselves. However, data were not available for quantitative analysis at that time.

Population studies have indicated two major patterns of response following acute irradiation of mature gametes. The majority of the species studied to date demonstrate a greater degree of sterility in the irradiated generation than in subsequent descendants. The irradiated generation may thus serve as an immediate assay for reproductive damage. In the second pattern of radiation response, the descendants of irradiated animals are much more sterile than is the irradiated generation itself. This second pattern of response has been only recently explored, and is correlated with chromosome structure (see La Chance *et al.*, 1970). Instead of having a single region of centromeric activity per chromosome (monokinetic chromosome structure), these latter organisms have many regions of centromeric activity per chromosome (diffuse centromeres, holokinetic or polycentric chromosomes). The holokinetic nature of the chromosome is expected to reduce acentric fragment loss following irradiation, and hence reduce the degree of induced P_1 sterility associated with such induced genetic dominant lethal events. The surviving offspring, however, may contain many simple and complex chromosomal rearrangements. After meiosis, this is expected to result in significantly increased F_1 sterility due to the production of genetically unbalanced gametes which cause lethality in the F_2. The excellent paper by LaChance, Degrugillier and Leverich (1970) discusses the cytogenetic aspects in much greater detail and provides a review of the earlier papers dealing with holokinetic chromosome structure. The vast majority of these radiation studies in which increased F_1 sterility was found utilized lepidopteran, homopteran or hemipteran insects.

Stefani (1963c) reported that *Artemia* has polycentric chromosomes and indicated their possible interest to radiation biologists. Stefani's studies were often based upon early blastomere divisions, but he has examined meiotic stages as well (Stefani, 1961, 1963a, 1963b, 1964, 1967). In their extensive review, Metalli and Ballardin (1970–72) question Stefani's interpretation, stating that they have found clear primary constrictions in a metacentric position when studying larval stages, oocytes and male meiosis. Based on electron microscope examinations,

[1] Financial support was provided by LIU Grants In Aid for 1969–70 and 1970–71.

Fautrez-Firlefyn and Roels (1968) consider *Artemia* chromosomes to have a diffuse kinetochore.

The present study therefore provides indirect evidence supporting the holokinetic nature of the *Artemia* chromosome. The small numerous chromosomes ($2n = 42$) of the of the *Artemia* complement make cytogenetic analysis of induced aberrations rather difficult, but such a study has begun.

Materials and Methods

Culture techniques were essentially the same as reported earlier (Squire and Grosch, 1970). Male brine shrimp were hatched from commercially obtained California cysts, and were approximately 5–6 weeks old at the time of exposure. Doses were 0, 1, 2, 3.5, 5, or 10 kR acute gamma radiation using a Gammator-50 (507 R/min, 137-Cs).

Irradiated and control males were individually pair mated to homozygous white eyed females (Xw/Yw). This use of a recessive genetic marker from the female, and a dominant allele from the male proves the paternity of the offspring and removes the necessity of using unmated females. (There is no sperm storage in this species and each brood is produced by a separate mating.) In general, the F_1 were raised to maturity and scored for survival to adulthood and sex ratio. Several F_1 sons (3 if available) were selected from each P_1 male and individually pair mated to comparable females which lacked any radiation in their ancestry. (Thus the control "F_1" females became the mates for the F_1 males of control and of irradiated ancestry.) Each F_1 female was either unmated or virgin when used. Pedigree records of each mating were maintained. Care was exercised to avoid any brother-sister matings, since inbreeding depression is extremely pronounced in this species (Squire, 1969), and would be confounded with induced sterility. It is important to note that the terms F_1 and F_2 as used in this paper refer to outcrossed rather than inbred generations.

These experiments were conducted over a period of $2\frac{1}{2}$ years and hence encompassed some variation in environmental conditions. Temperatures ranged from 70° to 85° F, but were usually maintained at 78° to 82° F by use of a room airconditioner. These variations are of little importance since treated animals were always concurrent with their controls. Repeated tests at 0, 5 and 10 kR gave essentially identical results. Although full P_1 and F_1 reproductive data were obtained at some selected dose levels, the magnitude of data precluded such an analysis at others. The hatchability of cysts obtained from P_1 and F_1 animals was usually low, whether or not there was a history of irradiation. This low hatchability limited the kinds of comparisons which could be made. Survival to adulthood and sex ratio data were obtained by placing 50 (if available) freshly produced nauplii from a single viviparous brood in a quart jar of brine. The sample was never smaller than 25 nauplii, and was restricted to a maximum of 50 nauplii in order to minimize the selective effects of crowding. Different samples represented the products of different pairs.

Data were restricted to young which were produced during the first 11 days (8 days for 1 kR) after paternal irradiation in order to further ensure the probability that they were derived from cells which were spermatozoa at the time of irradiation (Squire and Grosch, 1970).

TABLE I

Survival to adulthood and sex ratios of F_1 Artemia after a paternal dose of 5 or 10 kR

Dose (kR)	No. of young produced	Survival to adulthood				Sex ratio % males
		Males	Females	Total	%	
0	754	292	288	580	76.9	50.3
5	593	196	140	336	56.7	58.3
10	539	176	121	297	55.1	59.3
X^2 comparison				88.1 $P < 0.005$		9.24 $P < 0.01$

It should be noted that the *Artemia* utilized in this study had different genetic backgrounds than did the animals used earlier (Squire and Grosch, 1970; Squire, 1970). Some of these genetic differences were probably reflected in differences in life span and fertility data which become apparent when comparing experiments. However, the similarities in response are much more pronounced than are the differences.

OBSERVATIONS

5 and 10 kR

On the basis of 1886 F_1 shrimp, there is a significant 27 per cent reduction in F_1 survival to adulthood after a paternal dose of either 5 or 10 kR. A significant increase in male sex ratio was also observed (Table I). Two replicates were run and the data were pooled since no differences were found between replicates. All Chi-square contingency tests were conducted according to Steel and Torrie (1960, pages 370–372).

Three F_1 males were selected from each brood when available, and individually pair mated to untreated females. Each pair mating was scored throughout the entire life span of the F_1 male. F_1 males were almost completely sterile after a paternal dose of either 5 or 10 kR. The paternal dose, number of F_1 pairs, number of fertile F_1 males, total number of viviparously produced F_2 progeny, and average F_1 male adult life span are indicated in Table II. No F_2 progeny were produced ovoviviparously after paternal doses of 5 or 10 kR.

TABLE II

F_1 male sterility and average adult life span after a paternal dose of 5 or 10 kR

Dose (kR)	No. of F_1 pairs	No. of fertile F_1 males	Total no. of F_2 progeny	F_1 male adult life span (days)	
				$\bar{X} \pm$ S.E.	t
0	12	12	15,006	88.4 ± 16.5	
5	12	4	51	40.4 ± 4.9	2.79*
10	14	2	9	66.6 ± 10.6	1.11NS

* Significant at 0.02.

TABLE III

F_1 male reproductive data (scored for 35 days) after a paternal dose of 1 kR

Criterion	0 kR	1 kR
Fraction of pairs surviving 35 days	20/30	19/27
Average number of viviparous broods per surviving pair	4.30	3.00
Average number of ovoviviparous broods per surviving pair	2.15	3.10
Number of sterile males in surviving pairs	0	1
Average number of young per viviparous brood from surviving pairs	96.20	80.23
Average per cent cyst hatchability on a per pair basis	11.7	15.9

1 kR

Since 5 and 10 kR were sufficient to induce almost complete F_1 male sterility, a lower dose of 1 kR was next investigated. Survival to adulthood was 93.8 per cent in each group and there was no effect on sex ratio. F_1 male life span and reproductive data were kept for 35 days and failed to detect any effect of P_1 irradiation (Table III).

2 kR

A new experiment was therefore initiated using a paternal dose of 2 kR. A preliminary analysis failed to detect any effect of paternal irradiation on F_1 male reproductive performance, and this experiment was abandoned.

3.5 kR

A final experiment was conducted using a paternal dose of 3.5 kR. This dose resulted in an 11 per cent reduction in survival to adulthood. The change in sex ratio was not significant with the sample size used (Table IV). This dose resulted in a 44 per cent increase in the number of sterile pairs (Table V). Several of the fetrile males were characterized by broods which repeatedly contained incompletely developed embryos in addition to normal-appearing nauplii. Pairs were scored until (a) they had produced several viviparous broods, (b) they had produced at least three ovoviviparous broods, or (c) the male died. It was initially

TABLE IV

Survival to adulthood and sex ratios of F_1 Artemia *after a paternal dose of 3.5 kR*

Dose (kR)	No. of young produced	Survival to adulthood				Sex ratio % males
		Males	Females	Total	%	
0	266	107	109	216	81.2	49.5
3.5	647	261	204	465	71.9	56.1
X^2 comparison				8.18 $P < 0.005$		2.32NS $P > 0.10$

TABLE V

Number of sterile pairs after a paternal dose of 3.5 kR

Dose (kR)	Fertile pairs		Sterile pairs		Total
	Number	%	Number	%	
0	16	69.6	7	30.4	23
3.5	14	30.4	32	69.6	46
Total	30	43.5	39	56.5	69

$X^2 = 8.03$, $P < 0.001$.

assumed that cyst analysis would give a reliable estimate of fertility in the absence of viviparous reproduction. However, cyst hatchability was too low in this experiment to use as a criterion of damage, and probably gave an inflated estimate of the actual number of sterile males in both the controls and the descendants of irradiated males. Nevertheless, the per cent reduction in the number of fertile males (treated *vs.* control) is probably a meaningful estimate. The low cyst hatchability observed here is believed due to a genetic deterioration of the white eye unirradiated stock which resulted in improper cyst formation. Many females consistently produced translucent cysts or unencysted embryos which subsequently failed to develop.

DISCUSSION

The current survival to adulthood and sex ratio data after a paternal dose of 5 or 10 kR are based on larger samples than those of Squire and Grosch (1970). In our earlier paper, we noted a non-significant trend toward increased male sex ratio. This trend reached statistical significance in the present study. The shift in sex ratio could be due to reduced female viability. The female is the heterogametic sex in *Artemia*, and perhaps the X chromosome is partially hemizygous for viability genes. Sufficient genetic data is lacking for evaluating this hypothesis at the present time and alternative hypotheses could be evoked.

A paternal dose of 5 or 10 kR resulted in a 27 per cent reduction in F_1 survival to adulthood. No difference was observed between the 5 and 10 kR treatments, and this failure cannot be explained at the present time. Survival to adulthood was reduced by 11 per cent after 3.5 kR, but was not affected at lower doses.

The F_1 male sterility and average adult life span data confirm and extend the qualitative observations of Squire and Grosch (1970). A paternal dose of 5 or 10 kR results in virtually complete sterility of surviving F_1 males. A paternal dose of 1 kR had no effect on F_1 survival to adulthood, sex ratio or male reproductive performance. Incomplete data indicated that the same holds true for a paternal dose of 2 kR. A paternal dose of 3.5 kR resulted in a 44 per cent reduction in the number of fertile males, and also reduced the degree of fertility of at least some of the reproducing males (*i.e.*, partial sterility).

An unexpected finding in these experiments is the relatively narrow dose range sufficient to span the gap from no demonstrable F_1 male sterility (2 kR or more?)

on the one hand, to virtually complete F_1 male sterility (5 kR or less) on the other. It should be emphasized that even this higher dose of 5 kR was without obvious effect on the initial fertility of the irradiated males themselves, although it did reduce F_1 post natal viability. F_1 survival to adulthood measures viability during development of the free-swimming nauplius into an adult, and is thus a measure of lethal events which occur relatively late in development. Early embryonic deaths would be manifest as aborted embryos which failed to develop into free-swimming nauplii. Such embryos were found in the F_2 after 3.5 kR, but not in the F_1. Delayed F_1 mortality and the greater degree of F_1 male sterility (as compared to their fathers) are characteristic of organisms with holokinetic chromosomes, but diametrically opposed to results obtained with organisms which have monokinetic chromosomes.

In organisms which contain holokinetic chromosomes it has been repeatedly demonstrated that the F_1 sterility is associated with genetically unbalanced gametes which result from improper meiotic segregation of heterozygous translocation complexes. An additional factor may be secondary chromosome fragmentation and fragment loss (Brown and Wiegmann, 1969; LaChance *et al.*, 1970; Nordenskiold, 1963; North and Holt, 1968). Although such species are able to transmit small fragments through many mitotic divisions, there is increasing evidence supporting the view that many fragments which are retained in successive mitotic divisions are nevertheless eliminated during meiosis in the mealy bug, *Planococcus* (Brown and Wiegmann, 1969), and the silk worm, *Bombyx* (Inagaki and Nakao, 1970). However at least some such fragments are retained over successive generations in the large milkweed bug, *Oncopeltus* (LaChance *et al.*, 1970), and the wood rush, *Luzula* (Nordenskiold, 1963). It is not yet clear whether such a preferential elimination is in some way caused by a difference in the way in which the spindle fibers attach to the chromosomes during meiosis as compared to mitosis (Braselton, 1971; Buck, 1967; Comings and Okada, 1972).

In the current study, no attempt was made to distinguish between chromosomal sterility and possible abnormal histological, physiological or behavioral factors which might also be involved in F_1 sterility.

It is important to note that in organisms with holokinetic chromosomes, the effects of radiation exposure may be much more pronounced in the F_1 than in the irradiated generation itself. It is therefore quite possible for a natural population to receive a radiation dose which will result in population collapse of the subsequent generation. The usual methods of sampling field populations might fail to detect such a change before irreversible damage occured. This implication has become well known to insect-control researchers, but has remained largely unnoticed by many other investigators. Animals with holokinetic chromosomes include several groups of insects (Lepidoptera, Hemiptera, Homoptera, Odonata), brine shrimp (*Artemia*), and the Brazilian scorpion (*Tityus*). Many of these species are of considerable ecological and economic importance.

Summary

Male brine shrimp (*Artemia*) were hatched from commercially obtained Californian cysts and irradiated as adults with acute doses of 0, 1, 2, 3.5, 5 or 10 kR

gamma rays. Each male was individually pair mated to a white eye female. The F_1 were raised to maturity and scored for survival to adulthood, sex ratio, and F_1 male reproductive performance. There was a significant decrease in F_1 survival to adulthood after paternal doses of 3.5, 5 and 10 kR, but not at lower doses. A significant increase in male sex ratio was observed after 5 and 10 kR, and these males were almost completely sterile. A paternal dose of 3.5 kR resulted in a 44 per cent decrease in the number of fertile males and some fertile males were semi-sterile. No apparent effect was observed after 1 or 2 kR.

These data support the proposed holokinetic nature of the *Artemia* chromosome. They also demonstrate that the observable effects of radiation may be much more extreme in the F_1 animals than in the irradiated generation itself. This observation has important implications when assaying the effects of radiation on natural populations.

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PHYSIOLOGY OF INSECT ECDYSIS. II. THE ASSAY AND OCCURRENCE OF THE ECLOSION HORMONE IN THE CHINESE OAK, SILKMOTH, *ANTHERAEA PERNYI*

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The transformation of the silkmoth pupa into the moth culminates with the emergence, or eclosion, of the adult from the old pupal skin. This event marks the "turning-on" of adult behavior and is accomplished through a series of distinct behavioral acts (Truman, 1971a; Truman and Sokolove, 1972). Early experiments which involved brain extirpation and transplantation suggested that eclosion was triggered by a neurosecretory hormone which was released by the brain (Truman and Riddiford, 1970). This inference was then confirmed by the demonstration of eclosion stimulating activity in homogenates of the silkmoth brain and corpora cardiaca (Truman and Riddiford, 1970; Truman, 1971a). The present study extends these preliminary observations. It describes in detail the assay for the eclosion hormone and also its appearance and distribution in the nervous system of the Chinese oak silkmoth, *Antheraea pernyi*.

Materials and Methods

1. *Experimental animals*

Diapausing pupae of *Antheraea pernyi* were obtained from Japanese dealers and stored at 5° C. When needed, pupae were brought to 26° C. Typically, adult development was initiated within a week, and adult emergence followed about 3 weeks later. Some experiments also utilized the tobacco hornworm, *Manduca sexta*. The origin and handling of these insects was as described in Truman (1972).

2. *The biological assay for the eclosion hormone*

In the Pernyi moth, the initiation of adult development is signalled by the retraction of the wing epidermis from the overlying pupal cuticle. Once initiated formation of the adult then requires 19 days at 26° C (Truman, 1970 as modified from Williams and Adkisson, 1964). At the end of this period one finds a "pharate moth"—a moth which has completed all overt signs of adult development but which is still encased in the pupal skin.

The eclosion hormone assay utilized pharate Pernyi moths. For at least one week prior to their use the developing moths were exposed to a defined photoperiod. Routinely, this was a 17L : 7D regimen (photophase from 07 : 00 to 24 :00). Under these conditions pharate specimens of Pernyi eclose only during a "gate" in the late evening which occurs between 18 : 30 and 23 : 30 (Truman, 1971b).

Tissues to be assayed for hormone activity were homogenized with a ground glass homogenizer in a small volume (usually 20 μl) of insect Ringer's (Ephrussi and Beadle, 1936). Homogenates were either assayed immediately or frozen until tested. Each sample was injected by means of a 50 μl Hamilton syringe into the mesothoracic tergum of an assay animal. Pharate Pernyi moths were injected approximately 10 hours before their anticipated eclosion gate. At this stage the surrounding pupal cuticle shows complete digestion of the endocuticle and the ecdysial seams rupture when gentle pressure is applied with the fingers.

The presence of eclosion hormone activity was indicated by eclosion 1.5 to 4.5 hours after injection. If the moth did not emerge until its normal gate that same evening, the assay was scored negative. Occasionally a moth would not emerge on the day of injection but, rather, during its gate on the following day. In such cases it was assumed that the moth was not competent to respond at the time of injection and the assay was discarded.

Results

1. Quantitative aspects of the Pernyi assay

During the early stages of this study it was noted that extracts presumed to be rich in hormone (*i.e.*, from pharate moths) provoked eclosion more rapidly than did extracts thought to be poor in hormone (from newly emerged adults). Thus, it appeared that the latent period between injection and eclosion could provide a semiquantitative estimate of the amount of hormone injected.

As seen below, the corpora cardiaca (CC) from early day-19 Pernyi were very potent sources of the ecolsion hormone. Therefore, these organs were removed from pharate moths at this stage, homogenized, diluted to various concentrations and injected into weighed test insects. The reciprocal of the latent period (the time between injection and eclosion) was taken as the score of the positive responses. Negative responses were scored as zero.

Figure 1 shows the results of 63 assays performed on corpora cardiaca-corpora allata (CC-CA) complexes obtained from day-19 pharate moths. The amount of hormone injected is expressed in corpora cardiaca equivalents per gram of assay animal (CC equiv/g). As seen in Figure 1, a dose of 0.15 CC equiv/g caused one-half of the assay animals to emerge. Beyond 0.30 CC equiv/g responses were almost exclusively positive.

The scores computed for the positive assays showed considerable scatter, but a clear dependence of score upon dose was observed. Table I gives the average scores computed for each 0.1 CC equiv/g increment in dosage. From concentrations of 0.05 to 0.60 CC equiv/g, the average score increased in a linear fashion to a value of 0.60. Concentrations greater than 0.60 CC equiv/g failed to produce higher average scores (Fig. 1).

The substantial scatter was probably due to individual variation in both the assay animals and the CC-CA complexes tested. The variation in the latter was underscored by a series of 17 assays performed on the pooled material from 9 CC-CA complexes (Fig. 1, open circles). The scores obtained with the pooled homogenate showed considerably less scatter than did the remainder of the assays which were each performed on single pairs of glands.

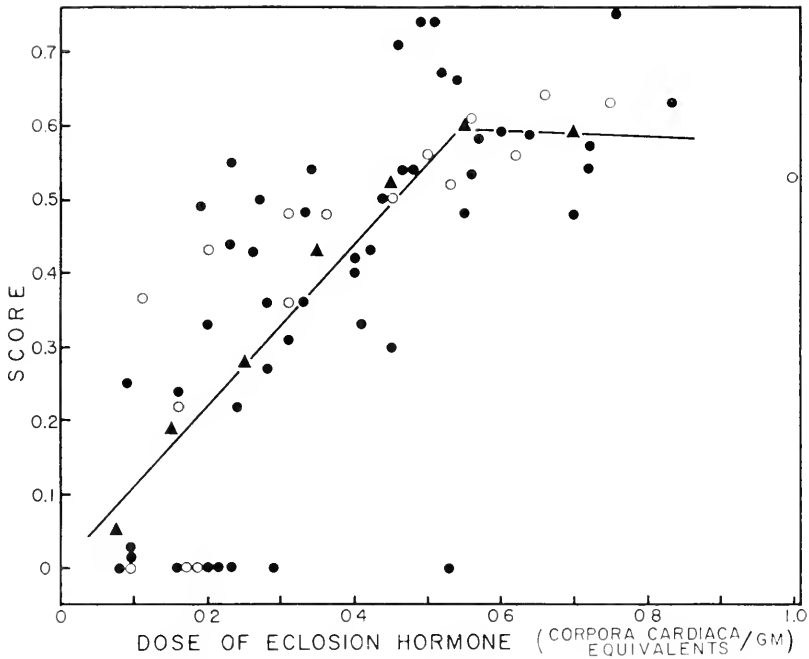

FIGURE 1. A dose-response curve for the eclosion hormone using the Peryni assay. The score of positive assays was computed as the reciprocal of the latent period, the time between injection of the hormone and emergence of the moth. Filled circles were tests performed on single CC-CA complexes. The open circles were tests performed on the pooled material from 9 complexes. The filled triangles are average scores obtained from Table I. The lines were obtained by the least squares method for the data from 0 to 0.6 and from 0.5 to 1.0 CC equiv/g respectively. The zero score at 0.53 CC equiv/g was omitted.

A score of 0.60 represents a latent period of approximately 1.7 hr. Of this time, the last 1.25 to 1.5 hr is required for the performance of the pre-eclosion behavior—a stereotyped piece of behavior which immediately precedes eclosion (Truman, 1971a). Therefore, the hormone injection triggers a change in overt

TABLE I

The effect of various doses of eclosion hormone containing homogenates in the Pernyi assay

Dosage (CC equiv/g)	No. of assays	Scores	Average score
0.05–0.10	5	0,0,0,0,0.25	0.05
0.11–0.20	9	0,0,0,0,0.22,0.24,0.33,0.43,0.49	0.19
0.21–0.30	10	0,0,0,0.22, 0.27, 0.36, 0.43, 0.44, 0.50, 0.55	0.28
0.31–0.40	9	0.31,0.36,0.36,0.40,0.42,0.48,0.48,0.48,0.54	0.43
0.41–0.50	10	0.30,0.33,0.43,0.50,0.50,0.54,0.54,0.56,0.71,0.74	0.52
0.51–0.60	10	0,0.48,0.56,0.53,0.58,0.59,0.61,0.66,0.67,0.74	0.60*
>0.60	10	0.48,0.53,0.54,0.56,0.57,0.58,0.63,0.63,0.64,0.74	0.59

* Zero score was considered to be a false negative assay and therefore was not included in the computation of the average score.

TABLE II

Distribution of eclosion hormone in the nervous system of day-19, pharate, Pernyi moths

Tissue	No. of assays	% Positive responses
Abdominal ganglia	5	0
Thoracic ganglia	5	0
Brain	10	60
Corpora allata-corpora cardiaca complex	9	100
Corpora allata	8	0
Corpora cardiaca	7	100
Body of CC	11	91
CC nerve leading to heart	10	60

behavior within 10 to 20 minutes. Presumably the time between hormone release and the onset of the pre-eclosion behavior in the intact animal is similar.

A pair of CC injected into a 3 g test animal is equivalent to a dose of 0.067 CC equiv/g or approximately 4.5 times the hormone required to give a 50% positive score. Thus, a day-19 pharate moth has stored 2 to 3 times the hormone necessary to trigger eclosion.

2. Distribution of eclosion hormone in the moth nervous system

Specimens of day-19 Pernyi were dissected. Various portions of the nervous system were excised and tested in the Pernyi assay. Table II shows that eclosion hormone activity was confined exclusively to the cephalic portion of the nervous system—homogenates prepared from the thoracic or abdominal ganglia consistently failed to cause eclosion.

In the head considerable activity was obtained from the brain—positive scores were obtained in 6 of 10 trials. But as seen in Table II, the highest activity was recovered from the CC-CA complex. In order to identify the source of activity in the complex, the CA were carefully dissected from the CC and the two glands assayed separately. In all assays the CC produced positive responses, whereas the CA had no effect.

The CC of silkmoths is a complicated structure which receives nerves from the brain and also sends a number of roots to the periphery. The largest nerve leading from the CC extends the short distance to the heart and ends in the heart wall. In 11 CC-CA complexes, the nerves leading to the heart were severed as close to the body of the CC as possible. The CC and the severed nerve including the section of heart into which it entered were then each tested. The CC consistently showed activity. Also, in many cases the nerves leading to the heart had substantial activity. Therefore, eclosion hormone may be transported from the CC to the heart wall in preparation for release. This arrangement would ensure the most rapid distribution of hormone through the moth.

3. Source of the eclosion hormone

When the corpora cardiaca are removed from Cecropia pupae, the neurohaemal portions of these structures rapidly regenerate, but the intrinsic functions of the

CC are lost (Stumm-Zollinger, 1957). The moths which result from such pupae, however, show a perfectly normal timing of eclosion (Truman and Riddiford, 1970), indicating a release of eclosion hormone at the time of adult emergence. Therefore, the high titers of activity observed in the CC undoubtedly arise from hormone which is produced in the brain and then is transported to the CC for storage and eventual release. In the moth brain the neurosecretory cells are found primarily in median and lateral cell clusters. Also, a few are located in a small

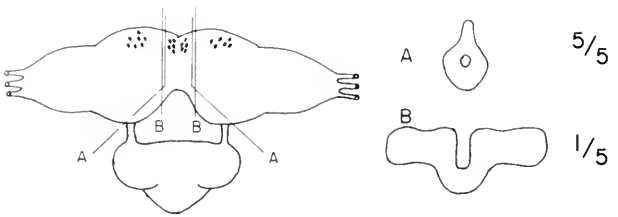

FIGURE 2. Left: schematic representation of the pupal brain showing the cuts made to isolate the median (A) or lateral (B) neurosecretory cells. In both cases the piece to be implanted was left attached to the subesophageal ganglion; Right: brain fragments after metamorphosis: (A) piece containing median neurosecretory cells; (B) piece containing lateral cells. The fraction shows the number of assays which were positive for the eclosion hormone.

posterior group (Herman and Gilbert, 1965). In order to determine which of the two major groups might be responsible for hormone production, brains from *Manduca* pupae were divided as shown in Figure 2. Hornworms were chosen for this experiment because both the median and lateral clusters of neurosecretory cells are clearly visible in the living brain when viewed against a black background under a dissecting microscope. The brain fragments were implanted into brainless hornworm pupae which were then caused to develop by injection of β-ecdysone. On the next to last day of adult development, the moths were sacrificed and the fragments recovered. As noted in Figure 2, pieces containing the median neurosecretory cell cluster had activity in 5 of 5 cases, whereas the lateral group gave a positive response in 1 of 5 assays. The positive response obtained in this latter group was presumably due to a few median neurosecretory cells being retained in a lateral piece. These data, therefore, indicate that the cells in the median neurosecretory cluster are probably responsible for production of the eclosion hormone.

4. Eclosion hormone activity in the brain and corpora cardiaca

Larval brains and corpora cardiaca. In tobacco hornworms, the fourth instar requires 2 to 3 days. The larvae then release prothoracicotropic hormone and, 50 hrs later, ecdyse to the 5th instar (Truman, 1972). Brains with attached CC were removed from pairs of *Manduca* larvae which were within 12 hr of the time of ecdysis to the 5th instar. As seen in Table III, no eclosion hormone activity was recovered from larvae at this stage.

During the feeding portion of the fifth instar, which lasts 4 to 5 days, tests on the brain and CC of individual larvae revealed little activity. Tissue removed during the first half of this period gave positive responses in only 1 of 7 assays.

Similarly, late 5th instar larvae did not yield any positive scores. This marginal level of activity continued through the cessation of feeding and the voiding of the gut (Table III).

Prepupal brains and corpora cardiaca. The onset of the prepupal period is signalled by retraction of the ocellar pigment and occurs approximately 1.5 days after the voiding of the gut. The brain and corpora cardiaca from the early prepupae showed only a very low level of activity. Even late prepupae, which had begun tanning of the underlying pupal cuticle, failed to yield substantial activity.

TABLE III

Amount of eclosion hormone activity found in brain and corpora cardiaca of tobacco hornworms at various stages in their life history

Stage	No. of assays	No. of positive scores	Average score
Pharate fifth instar larva	4*	0	0
Early fifth instar	7†	1	0.04
Late fifth instar	11†	0	0
Day of voiding gut	11†	1	0.02
Early prepupa	12†	2	0.04
Late prepupa	12†	1	0.02
Fresh pupa	13†	2	0.04
Pharate adult	12†	11	0.37

* Each assay utilized the brain and corpora cardiaca from two larvae.
† Each assay was performed on the brain and corpora cardiaca from a single donor.

Pupal brains and corpora cardiaca. Hornworm pupae were sacrificed within 24 hours after pupation and the brain and CC removed. As seen in Table III, hormonal activity in these structures was minimal.

Pharate adult brains and corpora cardiaca. Larvae of *Manduca* which had been reared under a 17L:7D photoperiod initiate adult development on the day following the pupal ecdysis. Seventeen days later development is complete and the moth emerges during that evening (M. M. Nijhout, Harvard University, unpublished). As is evident from Table III, pharate moths which were in early day 17 showed high concentrations of hormone in the brain and CC. This result will be considered in detail in the following section.

5. *The titer of eclosion hormone during adult development of Pernyi moths*

Between the initiation of adult development and eclosion, the developmental age of the Pernyi silkmoth can be estimated by features of the developing moth which can be seen through the pupal cuticle (Williams and Adkisson, 1964, as modified by Truman, 1970). Assays were performed separately on the brain and the CC from individuals at various stages in this development. Individual variation in both the donor and assay animals produced considerable scatter in the data, but some clear trends were evident.

Activity from the developing adult brain. A total of 79 assays were performed using homogenates prepared from brains of Pernyi pupae, developing adults, and adults. Figure 3A shows that activity could not be detected in single brains from

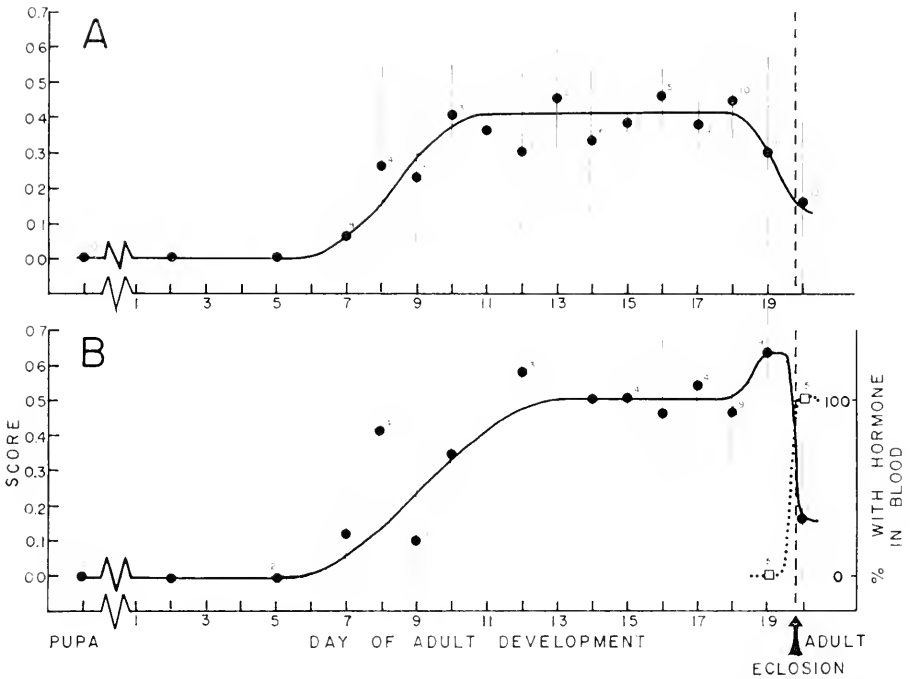

FIGURE 3. Changes in the eclosion hormone titer in the Pernyi silkworm as a function of the developmental age of the donor; (A) brain; (B) corpora cardiaca (●—●) and blood (□...□). The mean and standard deviation for each day are given. The number of animals assayed on each day is indicated.

Pernyi pupae or from developing adults up to day 7. On day 7, one of 4 brains tested showed low activity (score of 0.30). The average scores then increased through the next three days. Between day-10 and day-18, the brain titer appeared to remain relatively constant. There was a certain scatter in the data but the majority of the assays (69%) fell in the range between 0.30 and 0.50.

In a 17L:7D photoperiod, Pernyi emerge during the late evening of day-19. Therefore, it was surprising to find that on the morning of day-19, almost 12 hours before the time of eclosion, hormonal activity was disappearing from the moth brain (Fig. 3A). Of the 10 pharate moths which were sacrificed at this time, four gave negative assays.

This decline in activity was further evident in the adult. The brain of the newly emerged moth contained a level of hormone activity which was lower than any time during the preceding two weeks. Furthermore, 8 of 12 brains failed to show eclosion hormone activity.

Activity from the developing adult corpora cardiaca. During the pupal stage and the first week of adult development, the hormone titers in the CC mimicked those seen in the brain (Fig. 3B). Activity was first detected on day 7. The hormone concentration then increased until approximately day-12.

As with the brain, the amount of hormone in the CC appeared to remain rela-

tively constant between day-12 and day-18. During this period the average activity observed in the CC was consistently higher than that obtained from the brains from Pernyi moths of similar age.

As noted above, the hormone concentration in the brain begins to decline early on day-19. It is therefore of interest that the CC at this time reach their highest activity. Apparently, at this point in development, the brain is rapidly transporting the remaining hormone to the CC in preparation for its release that evening.

During the evening of day-19, the CC show a precipitous drop in hormone titer. These glands, excised from freshly emerged adults, produced an average score of only 0.17. Moreover, 6 of the 11 CC tested at this stage gave negative assays.

Activity from the haemolymph. Abdomens were severed from pharate or newly emerged Pernyi moths. Gentle pressure was applied to the fragments to express the haemolymph into a small vial which contained a few crystals of phenylthiourea (Williams, 1952). Approximately 200 to 500 μl of haemolymph was obtained from each animal in this manner. Samples which were contaminated with gut contents were immediately discarded. The blood from each moth was lyophilized and the resulting powder resuspended, as well as possible, in 50 μl of Ringer's. This syrupy mixture was then assayed as described under Methods.

Haemolymph collected from each of five pharate moths on the morning of day-19 was tested for eclosion hormone activity. All samples gave negative results (Fig. 3B). By contrast, blood collected form Pernyi moths during the performance of the pre-eclosion behavior or just after emergence that evening (prior to the initiation of wing inflation) provoked eclosion for all five samples tested. Therefore, the precipitous drop in hormone activity in the CC is mirrored by its appearance in the blood.

6. Test for activity in non-Saturniids

As shown in Table III, homogenates prepared from the brains and CC of pharate tobacco hornworms has marked activity in the Pernyi assay. Brains and CC from two hemimetabolous insects were also tested for the presence of the eclosion hormone. The tissues were dissected from pharate adults of either the cockroach, *Leucophaea maderae,* or the linden bug, *Pyrrhocoris apterus,* and tested in the usual manner. As seen in Table IV, no activity was recovered from either of these insects.

TABLE IV

Tests of brains and corpora cardiaca from non-lepidopteran species in the Pernyi eclosion hormone assay

Donor	No. of assays	No. of brains and CC-CA complexes per assay	% positive responses
Leucophaea maderae	1	1	0
	3	2	0
	1	3	0
Pyrrhocoris apterus	1	60	0

TABLE V

Tests of juvenile hormone, ecdysone and bursicon in the Pernyi eclosion hormone assay

Material tested	Dosage	No. of assays	% positive responses
C18 Cecropia juvenile hormone	0.2 μg	10	0
β-ecdysone	8 μg	10	0
Bursicon (abdominal nerve cord from *Manduca*)	1	10	0

7. Non-activity of other insect hormones in the Pernyi assay

The effects of C-18 Cecropia juvenile hormone and of β-ecdysone in the Pernyi assay are recorded in Table V. The former was injected in 10 μl of olive oil and the latter in 10% isopropanol solution. Both hormones were injected in physiological doses (Meyer, Hanzmann, Schneiderman, Gilbert, and Boyette, 1970; Williams, 1968) and both proved to be completely inactive.

Since release of the tanning hormone, bursicon, is associated with the act of eclosion (Cottrell, 1962; Fraenkel and Hsiao, 1962), it was of interest to test this hormone in the Pernyi assay. The abdominal nerve cord of pharate adult *Manduca sexta* is a potent source of bursicon (Truman, in preparation). Table V shows that homogenates prepared from pharate nerve cords were without activity.

DISCUSSION

Two neurosecretory hormones are associated with insect molting: the prothoracicotropic hormone (PTTH), which promotes the secretion of ecdysone by the prothoracic glands (Williams, 1952), and bursicon, which is responsible for the cuticular tanning of freshly emerged insects (Cottrell, 1962; Fraenkel and Hsiao, 1962, 1965). The data in Table V clearly show that the eclosion hormone is distinct from bursicon. Portions of the moth nervous system which contain bursicon show no eclosion hormone activity. Similarly, homogenates of the brain and CC of pharate adult hornworms will not provoke tanning (Truman, unpublished).

At present there is not conclusive proof that the eclosion hormone is distinct from PTTH. However, the failure to find significant quantities of eclosion hormone during larval life—a time when PTTH is known to be released—casts doubt on the hypothesis that the two hormones are identical. Therefore, from the available evidence, I suggest that the triggering of eclosion is the function of a previously unknown insect hormone rather than a new function for a described hormone.

The suggestion of the name "neurotropic ecdysis hormone" in our initial report of the hormonal control of emergence (Truman and Riddiford, 1970) suggested that this mechanism might be generally applicable to the control of all insect ecdyses. However, a recent study of larval molting in *Manduca* has pointed out a major difference in the control of larval ecdysis *versus* adult eclosion (Truman, 1972). Adult eclosion is a gated event which is triggered by a biological clock located in the moth brain (Truman and Riddiford, 1970). By contrast, larval ecdysis is

not gated but is developmentally triggered. At a given temperature it occurs a constant time after the initiation of the molting process by PTTH and ecdysone (Truman, 1972). Furthermore, the timing of larval ecdysis remains unaltered, even when all influence of the head is abolished by neck ligation (Truman, 1972).

The data presented here also argue against the hypothesis that the eclosion hormone triggers the larval-larval and/or the larval-pupal ecdysis. No individual from the developing fifth instar larva through to the pupal stage contained significant amounts of the eclosion hormone (average scores of 0 to 0.04). Even prior to an ecdysis, a time at which one would logically expect a build-up of titer in the CC, the hormone is practically absent.

From the dose-response curve given in Figure 1, one can estimate that in *Manduca* the background levels of hormonal activity seen during the larval and pupal stages (scores of 0 to 0.04) represent, at best, one-tenth of the material found in the pharate hornworm moth (score of 0.37). Similarly, the failure to find activity in single brains or CC of Pernyi pupae indicates that a similar relationship holds for this species. Thus, in both species, synthesis of the eclosion hormone occurs primarily during adult development.

Since in moths the eclosion hormone functions only during adult emergence and not during any of the preceding ecdyses, the failure to find activity in cockroaches or bugs is not surprising. The latter are hemimetabolous insects which do not have a pupal stage interposed between the feeding larva and the adult. Thus, in these insects the adult ecdysis differs little from the larval ecdyses. Therefore, one is presented with the intriguing possibility that the eclosion hormone may be confined only to the holometabolous orders of insects. The validity of this interpretation awaits further study.

The restriction of the appearance of the eclosion hormones to a specific portion of the moth life history allows for a preliminary identification of periods of synthesis, transport, and release of hormonal material. The study of Scharrer (1962) on the cockroach, *Leucophaea maderae*, clearly showed that neurosecretory material is transported down the neurosecretory axons from the brain to the CC. Similarly, in the moth, activity recovered from the brain presumably represents material either in the cell body or in transit down axons leading from the median neurosecretory cluster. Activity in the CC would then be due to the accumulation of the hormone in the axon endings.

In Pernyi, hormone synthesis by the median neurosecretory cells begins approximately on day-7. At this early stage hormone transport is also occurring as manifest in the recovery of activity in the CC. Hormone titers continue to increase in the brain until day-10, at which time a plateau is attained. This constant level of hormone activity could be due either to the rate of transport becoming equal to the rate of synthesis or to the stopping of both synthesis and transport. The continuing increase of activity in the CC would favor the former alternative through day-12. But the subsequent attainment of a plateau in the CC indicates that both hormone synthesis and hormone transport cease after day-12. This interpretation, of course, assumes that there is no breakdown of hormone in the axon terminals and that release only occurs after the completion of adult development. With the onset of day-19, hormone transport resumes and the brain essentially empties of activity. Presumably at this stage synthesis remains shut-off.

During the evening of day-19, the pharate moth secretes the eclosion hormone into the haemolymph. At this time approximately 75% of the hormonal material stored in the CC is released. The fact that hormone can be recovered from nerves which lead directly from the CC into the heart suggests that this may be the final pathway of hormone into the blood.

I thank Prof. L. M. Riddiford for her suggestions during this study and for a critical reading of the manuscript. The advice of Prof. C. M. Williams is also gratefully acknowledged. The β-ecdysone was kindly supplied by Prof. K. Nakanishi and the juvenile hormone by Zoecon Corporation. This study was supported by a NSF predoctoral fellowship, the Harvard University Society of Fellows and grants from the William F. Milton Fund and the Rockefeller Foundation.

Summary

1. A semi-quantitative biological assay for the eclosion hormone is described.

2. In the pharate moth hormonal activity is confined to the brain and the corpora cardiaca (CC).

3. During the life history of the moth, activity is practically absent from the brain and CC of larval and pupal stages and appears only in preparation for adult emergence.

4. The titer of eclosion hormone in the brain and CC of Pernyi moths was followed through adult development. In both structures activity first appeared on about day 7. Titers then increased for the next 3 to 5 days followed by a plateau. On the morning of the day of emergence, the titer in the brain fell and that in the CC correspondingly reached its highest level. At the time of eclosion in the evening, the CC titer then dropped and hormone appeared in the blood.

5. Tests on brains and CC from cockroaches and bugs failed to show activity.

6. It was concluded that the eclosion hormone is used only for the pupal-adult ecdysis and, thus, is probably restricted to the holometabolous orders of insects.

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THE REPRODUCTIVE CYCLE OF *SPHAERIUM SIMILE* [1]

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Reproduction in the fresh water family Sphaeriidae shows a number of consistent and specialized characteristics. Virtually all members of the family are hermaphroditic, and the developing young are retained in special brood pouches known as marsupial sacs, located in the inner gill of the adult, until fully prepared for a free living existence. The general structure, histology and development of the marsupial sacs, particularly in the genus *Sphaerium*, have been extensively studied (Poyarkoff, 1910; Groenewegen, 1926; Okada, 1935b). Other investigators have made observations on a number of different aspects of reproduction in the sphaeriids. Drew (1896) described the general anatomy of the genital system of *Sphaerium sulcatum* (= *simile;* Herrington, 1965). Gilmore (1917) gave a brief outline of some of the main points of reproduction in *Sphaerium simile*. Woods (1931) described the development of the genital system in *S. striatinum* from the time of fertilization through the attainment of sexual maturity in the individual. Foster (1932), by analyzing seasonal changes in the relative abundance of individuals of different sizes in the population, determined the life cycle of *S. solidulum* (= *striatinum;* Herrington, 1962). Okada undertook a very thorough investigation of virtually every aspect of reproduction in *S. japonicum* (formerly identified as *Musculium heterodon*). In a series of 4 papers (1935a, 1935b, 1935c, and 1936, he described the genital system, gametogenesis, the structure, development and function of the marsupial sacs, the reproductive cycle, and the early embryology of the species.

More recently, Thomas (1959, 1963, 1965) studied the growth and life history of a population of *Musculium partumeium* living in a temporary pond. It is evident from these studies that the life cycle of some members of the family Sphaeriidae may be particularly suited to meet unusual environmental conditions. Heard (1965) compared the life cycles of a number of species of the genus *Pisidium* found in North America. Another recent work involved a general examination by Mitropol'skii (1969) of the life cycle of *S. corneum,* a species previously studied by Thiel from 1924 to 1930 (cited in Okada, 1935b). Finally, Avolizi (1971) has investigated growth rate, mortality, fecundity and biomass turnover in populations of *S. simile* and *S. striatinum* located in New York State.

It would appear from the number and scope of the investigations already undertaken that virtually no stone has been left unturned in the examination of reproduction in the Sphaeriidae. Heard (1965), however, noted that there are marked differences in reproductive cycles among the several genera of this family. These differences include the relative life span of the species, the size and number of

[1] This paper represents an abridgement of a thesis submitted to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science.

litters produced each year, and the exact seasons in which such processes as gametogenesis are most active. Furthermore, he noted that differences also occur among different species of the same genus and occasionally among members of the same species found in different environments.

Heard's observations have made it quite evident that many aspects of reproduction in the genus *Pisidium* are highly variable. A review of the literature in the field clearly indicates that as in *Pisidium,* seasonal variations in the time of gametogenesis, fertilization and birth occur among species of *Sphaerium* as well, and many significant details of reproductive biology remain to be elucidated, particularly in species such as *Sphaerium simile* which have not been exhaustively studied.

Although Drew (1896) described the genital system of *Sphaerium simile* and Gilmore (1917) studied some of the major aspects of its reproductive morphology and physiology, no one has undertaken a detailed investigation of the reproductive cycle of this species. The purpose of the present study, therefore, was to elucidate the annual reproductive cycle, for comparative purposes, in a species in which it has not been examined.

H. B. Herrington, an authority on the sphaeriids, has made a number of revisions in the taxonomy of this group which will be used in this paper (Herrington, 1962 and 1965). He indicates that the name *Sphaerium striatinum* should replace *Sphaerium solidulum* (Herrington, 1962), and the species identified as *Sphaerium sulcatum* should now be referred to as *Sphaerium simile* (Herrington, 1965). In addition, the clam identified by Thomas (1959, 1963, and 1965) as *Sphaerium partumeium* is properly termed *Musculium partumeium* (Herrington, 1965).

Materials and Methods

A large population of *Sphaerium simile* occupies the soft, flocculent, silty bottom of the stream draining the large pond in the Lloyd-Cornell Reservation, locally referred to as McLean Bog, located off Route 13 between Dryden and Cortland, New York. This population is such a conspicuous feature that the stream is known as Sphaerium Brook (Sibley and Leffingwell, 1926). Information concerning the environmental characteristics of this brook and their probable relationship to the reproductive cycle of this population of *S. simile* will be cited in the discussion.

Collections were made in the brook on about the twentieth of each month from November, 1969 through October, 1970. Because of inclement weather in February, 1970, it was impossible to obtain a collection for this month until March 2; the normal March collection was taken on March 21. Specimens were obtained by scooping up the substratum several times with an ordinary 6 to 8 inch kitchen sieve, mesh size about $\frac{1}{16}$ of an inch, with the objective of securing a random sample consisting of varying numbers of individuals. The number of specimens collected each month ranged generally between 100 and 300. The material was suspended in water from the brook for transportation to the laboratory.

At the time of each collection a record was made of the water temperature in the brook, which was found to range from 2° C in December, 1969 to 26° C in August, 1970

At the laboratory the clams were immediately sorted from the substratum and placed in large fingerbowls containing aerated brook water, set in a water bath maintained at about 15° C. From each collection, 15 individuals ranging in size from 8.5 or 9 mm to over 16 mm were selected for histological examination. The same numbers of specimens of exactly the same sizes were not chosen each month, but an overall effort was made to select approximately equal numbers of clams of various sizes. Only those animals that had their feet and siphons extended and otherwise appeared to be in good condition were chosen. These individuals were measured with vernier calipers or a millimeter scale placed under a dissecting microscope. They were then thoroughly relaxed in approximately 300 ml of brook water containing several drops of Propylene Phenoxetol (Owen, 1955). This compound was obtained from Goldschmidt Chemical Corp., New York, New York.

When the valves were gaping widely, usually after about an hour in the relaxing solution, each clam was fixed in warm Bouin's fluid. It was allowed to remain in the fixative for several weeks, until the shells of the embryos it contained were completely decalcified. At this time the remains of the adult valves were removed with fine forceps. The soft tissues were then soaked for a few days in several changes of 70% ethyl alcohol, dehydrated, and embedded in paraffin in a vacuum oven. Serial cross sections of the entire area between the anterior and posterior adductor muscles were cut at 8 μ to 10 μ and mounted on albuminized slides. The sections were stained in Delafield's or Ehrlich's hematoxylin and counterstained with a 0.1% solution of eosin Y in 95% alcohol.

After the specimens selected for histological preparation had been fixed, the lengths of all the remaining individuals in the sample were measured with vernier calipers or a millimeter scale and the number of clams in each size category (taken at 1 mm intervals) recorded. These data were used to calculate the percentage of "newborn" young in each monthly sample.

Each month from January through October 1970, approximately 20 specimens, ranging in length from 13.5 mm to over 16 mm, were dissected, and the embryos of maximum size that they contained were removed and measured. Data recorded included the length of the parent and the length of the largest embryo in each of its inner gills. Despite the fact that only the maximum size embryos in the parents were measured, these young showed considerable variations in length each month, sometimes ranging from less than 1 mm to approximately 7 mm long. These measurements were made, at the latest, on the day after the collection had been made. Only a few such measurements were recorded for specimens collected in November and December, 1969.

Observations

Seasonal changes in the testes

As previously described by Drew (1896) and Gilmore (1917), the male reproductive system of *Sphaerium simile*, which is located behind the stomach and below the pericardial cavity, consists of paired gonads, each composed of a number of testicular follicles grouped around a common sperm duct. Although the 2 gonads are at first separated by the intestine, in the more posterior regions of

the animal this barrier disappears and the groups of follicles come to lie close together above the foot, a condition that frequently obscures the paired nature of the gonads at this point. It is at this level that the gonadal mass reaches its greatest width, and data to be cited later refer to the breadth of the organ at this point.

The overall condition of the male gonads in terms of their size and activity varies both with the seasons and with the size of the animal, and these changes will now be described. The exact size at which *Sphaerium simile* reaches sexual maturity is not known, since in even the smallest individuals examined (*i.e.*, approximately 8.5 mm) the testes contain spermatozoa.

In animals collected from September through April the male gonads show many consistent characteristics. In specimens less than 12 mm in length, the entire gonadal mass usually ranges from 0.2 mm to 0.45 mm in greatest width, although in some cases this measurement may exceed 0.5 mm. In the majority of larger individuals (*i.e.*, exceeding 12 mm in length), the entire gonadal area varies from 0.3 mm to 0.7 mm in greatest width, but usually it is approximately 0.5 mm to 0.6 mm wide.

During this period specimens ranging from 8.5 mm to just below 12 mm in length appear to be undergoing moderate amounts of spermatogenic activity (Figs. 1 and 3). Spermatogonia and to a lesser extent spermatocytes are readily visible, particularly in the early spring (especially April) when they frequently fill the entire follicular cavity, obscuring any evidence of a central lumen (Fig. 3). In many follicles, however, particularly in those found in smaller individuals collected before April, cells in more advanced spermatogenic stages are often located in the interior regions of the follicle. Spermatids can often be seen in the fall, although they are considerably less common from December through April. In many testicular follicles there is a distinct central lumen which contains spermatozoa, although this phenomenon is not as common in April as it is in previous months. Eosinophilic material (to be described in more detail below), although occasionally visible in specimens over 10 mm in length, is mostly found in small amorphous aggregations, usually confined to the periphery of the follicle.

In the majority of specimens over 12 mm in length collected between September and April, the testicular follicles show evidence of a decided reduction in spermatogenesis. The gonads may contain one or a few follicles which have retained spermatogenic activity, but the majority of follicles contain a mixture of spermatogenic cells and eosinophilic material (Fig. 2). The latter substance, although occurring in a variety of shapes, is often arranged in prominent spheres, many of which exceed 20 μ–25 μ in diameter. With the stains employed, its color varies from deep pink to purple. When the eosinophilic bodies are the predominant material in the follicles, as frequently happens, the spermatogenic cells are fewer in number, show no evidence of the organization of cell types that is normally characteristic of spermatogenesis and are considerably reduced in variety. In particular, spermatids and spermatozoa are rare, especially from December until April. In extreme cases the eosinophilic bodies are the only material found in the follicle.

Eosinophilic material is never found to any great extent in follicles in which active spermatogenesis is occurring. Although blood cells have not been seen in the process of phagocytosis, they are sometimes found adhering to the outer wall of a testicular follicle or, more rarely, actually within a follicle or sperm duct.

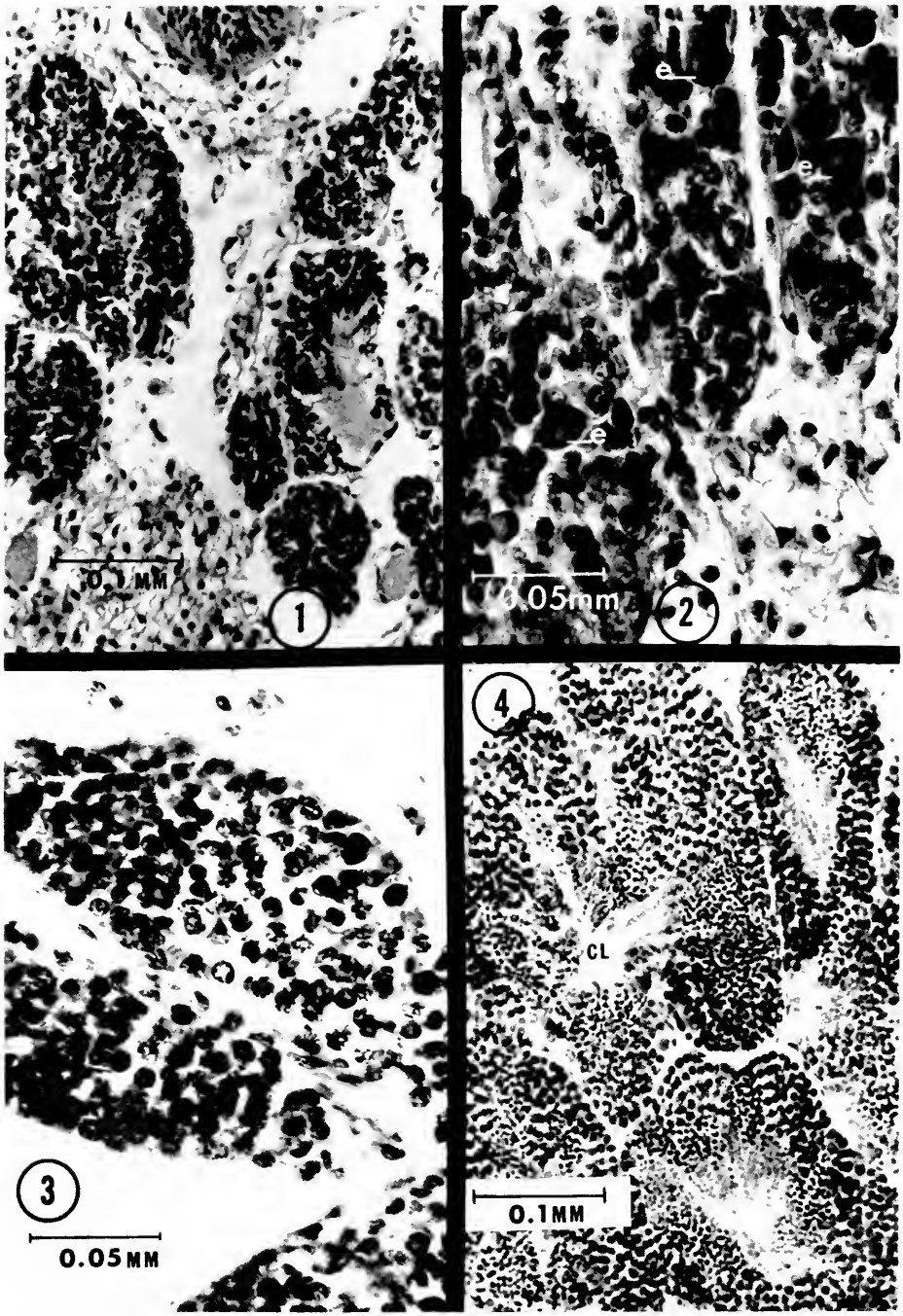

FIGURE 1. Testicular follicles of specimen 10 mm in length collected in December. In December (as in the other months from September to April) specimens under 12 mm in

Occasionally rounded masses of sperm heads can be seen which are apparently surrounded by an eosinophilic substance. In general, the evidence suggests that the eosinophilic material may represent the results of the phagocytosis, by blood cells, of debris and degenerating follicular contents.

With few exceptions, as indicated above, by April the testes of the average specimen show very few spermatozoa. This situation results from the fact that while the smaller specimens have gonads with little sign of degeneration, the follicles contain chiefly immature spermatogenic cells. The larger specimens, on the other hand, are showing extensive testicular degeneration and decreasing spermatogenic activity.

In May specimens over 12 mm in length may contain at least a few follicles whose poor development, obvious shrinkage and large quantities of eosinophilic material are reminiscent of conditions found in preceding months. Nevertheless, from May to July the great majority of individuals examined show a marked increase both in the overall size of the gonads and in the amount of spermatogenic activity taking place. The contrast between the gonads of smaller and larger individuals is much less pronounced than during the period from September through April.

From May through July, specimens under 12 mm in length have testes ranging from 0.5 mm to 0.75 or 0.8 mm wide. In specimens longer than 12 mm the overall range in maximum width of the gonad is from 0.5 mm to well over 1.0 mm, but most commonly the gonadal mass in these individuals is approximately 0.7 mm to 0.8 mm wide.

The testicular follicles contain abundant quantities of cells in all stages of spermatogenesis (Fig. 4). Spermatids show a particularly marked increase from previous months. They often occupy much of the interior of the follicle, and especially during June and July, frequently extend out to the periphery as well. Although spermatozoa are visible in May, they are far less numerous than other cell types. In June and July, on the other hand, spermatozoa are usually readily visible in varying numbers, frequently filling a central lumen which may be quite prominent (Fig. 4). Even in June and July, however, the younger stages still greatly outnumber the spermatozoa, indicating an obviously great potential for further production of male gametes.

It has already been indicated that in May specimens over 12 mm in length

length show evidence of moderate spermatogenic activity and little sign of testicular degeneration.

FIGURE 2. Testicular follicles of specimen 13 mm in length collected in December. Testicular follicles such as those illustrated in this figure are characteristic of specimens longer than 12 mm collected from September through April. They are also commonly found in adults greater than 12 mm in length collected in August. Note the prominent eosinophilic masses mingled among the spermatogenic cells; e, eosinophilic mass.

FIGURE 3. Testicular follicles of specimen 11.3 mm in length collected in April. Particularly in April, the testicular follicles of clams less than 12 mm long often have spermatogonia and spermatocytes extending throughout the entire follicular cavity, obscuring any evidence of a central lumen.

FIGURE 4. Testicular follicles of specimen 12.7 mm in length collected in June. In June the testicular follicles of most specimens, regardless of size, are quite massive and show evidence of very active spermatogenesis. The prominent central lumen is lined with abundant spermatozoa; CL, central lumen.

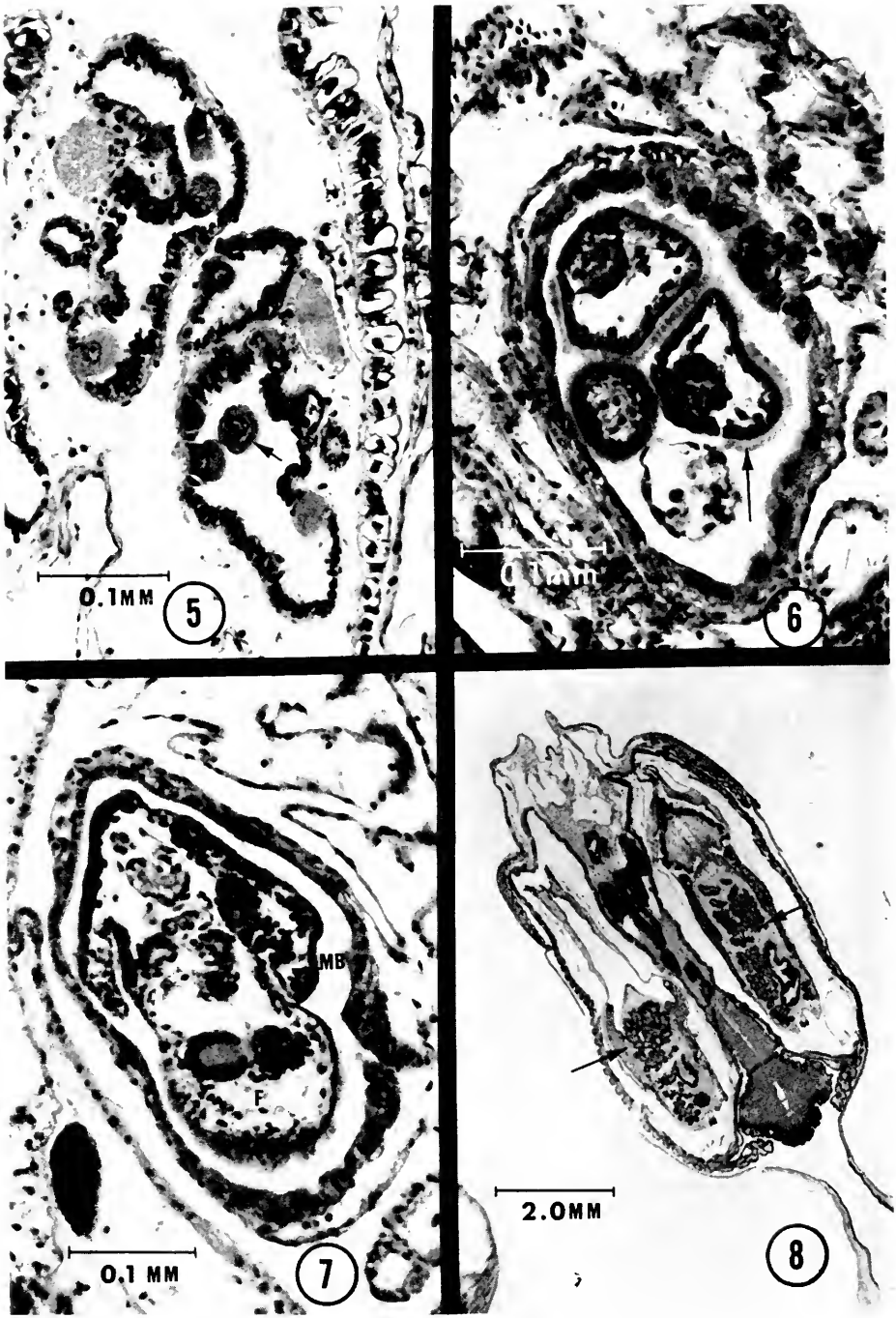

FIGURE 5. Ovaries. Note the characteristic manner in which developing oögonia bulge into the ovarian lumen. The spherical cell completely free in the lumen (arrow) is a primary oöcyte.

often contain at least a few testicular follicles with large quantities of eosinophilic material. In general, however, from May to July this substance, although occasionally visible, is not common in the testes of the majority of individuals examined, regardless of size.

There is evidence of declining spermatogenic activity in some of the specimens longer than 15 mm found in July as well as in the majority of specimens greater than 12 mm in length collected in August. In general the testes are similar in their overall size and follicular contents to those of clams over 12 mm long examined from September to April. The testes of these specimens often contain much more eosinophilic material at the expense of spermatogenic cells, particularly spermatozoa. Nevertheless, cells in all stages of spermatogenesis are still readily visible and are definitely in the majority in most of the follicles examined.

In August, the testes of specimens less than 12 mm in length range from approximately 0.2 mm to 0.45 mm in greatest width. Despite this decrease in size, however, the testes show evidence of active spermatogenesis, and all cell types can be found in varying proportions in the testicular follicles. In addition, eosinophilic material is generally not common in individuals less than 12 mm long.

Seasonal changes in the ovaries

In *Sphaerium similc* as in other members of the genus *Sphaerium*, the paired ovaries are located behind the testes and above the foot. Each ovary tapers posteriorly and merges with a hermaphroditic duct which also receives the products of the sperm duct.

The general process by which mature female gametes are produced in *S. similc* is virtually identical to the detailed and precise account of oögenesis given by Okada (1935a) for *S. japonicum*. In both species cells of the ovarian epithelium develop into oögonia, gradually accumulate cytoplasm and bulge into the ovarian lumen until their bases of attachment to the ovarian wall become extremely tenuous. Eventually the cells are released into the lumen as primary oöcytes. Cells in various stages of oögenesis are illustrated in Figure 5. In *S. japonicum* the maturation divisions of the egg follow soon after sperm penetration. In the present study these events have not been observed, but it seems safe to assume that they occur in a similar manner in *S. similc*.

Oögenesis occurs throughout the year in all clams examined, although there are variations in the extent of this activity among individuals which are not firmly correlated with seasonal differences or with the size of the specimen. Within a single individual, in addition to very early oögonial stages, older oögonia of varying sizes can be found clearly bulging into the ovarian lumen (Fig. 5). Most commonly these developing cells extend to a distance of 25 μ to 46 μ into the cavity, but in those adults in which oögenesis is at a low level, most of the prominent oögonia

FIGURE 6. Several very early fetal larvae in a marsupial sac (one of the embryos is indicated by an arrow). Note that these embryos are primitive in development and bear no resemblance to typical adult clams.

FIGURE 7. Embryo in the earliest stage of "intermediate" development. In this section, mantle buds and a definite foot are readily visible, giving the embryo a more "clam-like" appearance than that of previous embryonic stages; MB, mantle bud; F, foot.

FIGURE 8. Very large embryos (indicated by arrows) lying in each inner gill of the parent clam. These young are over 5 mm in length, well developed, and nearly ready to be born.

are approximately 25 μ to 35 μ in height. In cases where the level of activity is greater, a phenomenon most commonly found in specimens collected from June through August, many of the cells are larger, extending 35 μ to 46 μ, and sometimes as much as 60 μ into the lumen. In addition, at least some of these cells show evidence of a decidedly narrowing base of attachment to the ovarian wall. Normally each adult has from 10 to well over 30 oögonia clearly extending into the ovarian lumen. Although smaller individuals tend to contain fewer such cells than larger adults, there is no definite relationship between the size of the specimen and the number of oögonia it contains.

Primary oöcytes (*i.e.*, cells ranging from approximately 35 $\mu \times 28$ μ to 58 $\mu \times$ 46 μ lying free in the ovarian lumen) are considerably less numerous than the earlier stages of oögenesis. Generally, at least one adult in every monthly collection contains one or a few, and these cells are more readily found in specimens collected during the summer. Primary oöcytes are particularly common in June, when over 40% of the individuals examined have ovaries containing well developed cells unmistakably free in the ovarian cavity. Throughout the year, some individuals can also be found containing prominent cells which seem to be lying against the wall of the ovary with no obvious attachment to it. These cells do not clearly appear to be free in the lumen, but even if they have not yet been released, their appearance and relation to the ovarian epithelium make it seem obvious that they will soon be primary oöcytes.

The general pattern suggested by these observations shows a continuous low level of egg production throughout the year, with a recognizable peak occurring in June. In addition, it appears that oögenesis is generally more active during the summer than in other seasons. The fact that oögonia tend to be larger at this time, and the fact that primary oöcytes are somewhat more common, lend support to this view.

Sphaerium simile must be at least 9 mm long and more often over 10 mm in length before it begins to brood young. The younger adults in the population undergo several periods of fertilization while they themselves are increasing in length. By this process they gradually accumulate young in several stages of development. By the time they are 15 mm in length, adults are usually maintaining the maximum number of young customarily found in a member of this species. Clams of this size usually contain at least 6 to 8 marsupial sacs, and the young within the parent are in 3 or 4 different phases of development. Normally a marsupial sac contains only one embryo, but 3 or 4 young may be enclosed in one brood pouch if they are in very early developmental stages (*i.e.*, zygotes to early "fetal larvae"). Only individuals at least 13.5 mm in length contain embryos ready to be born, and even in animals of this size the phenomenon is not common. Embryos very near birth are most frequently found in adults from 15 mm to 17 mm in length.

The number of embryos in a parent of specified length shows little seasonal variation, but the developmental stages of young that are present may differ in consistent ways depending on the time of the year. These variations will now be examined more closely in sections which will deal successively with (a) fertilizations occurring each month, (b) the embryos of intermediate development, and (c) the embryos of maximum size found in the parent at different times of the year.

Fertilizations occurring each month

In the present study, embryos ranging from zygotes to the earlier phases of "fetal larval" development are considered to represent the products of fertilizations most likely to have occurred during the month since the last previous collection. Okada (1936) uses the term "fetal larva" to describe the embryonic stage that commences immediately after gastrulation in *Sphaerium japonicum*. Young in similar stages of development can be found in *Sphaerium simile* (Fig. 6). In this species a comparatively early fetal larva is spherical, pear-shaped or top-shaped, and its overall outline bears no resemblance to that of a typical clam. Cilia can be seen on some of the external surfaces of the embryo. Internally, although various cell masses represent the primordia of future organs, the only clearly recognizable structure is a simple digestive tube, ciliated in part. Unfortunately, because no information is available concerning the rate of embryonic development in *Sphaerium*, it is impossible to determine exactly when the fertilizations occur that produce the earliest developmental stages found each month, especially the early "fetal larvae". Nevertheless, these embryos are still very small and primitive in structure, representing a stage that begins to develop just after gastrulation; it seems likely, therefore, that these young are less than 1 month old.

Approximately 50% of the specimens (of all sizes) examined histologically in September and about 40% of the clams studied in this manner each month from October to April contain stages that are either early fetal larvae or, much less commonly, younger embryos, resulting from even more recent fertilizations (*i.e.*, blastula or gastrula stages). Zygotes and cleavage stages are never seen. Specimens examined in May show no evidence of any stages earlier than early fetal larvae and only one clam contains these. Beginning in June and continuing throughout the summer months, there is considerable evidence of fertilization. Stages of development ranging from zygotes to the earlier phases of fetal larval development, which are almost non-existent in May, are found in 67% of the June specimens, 80% of the July specimens and over 70% of the August specimens examined. These are the only months of the year when zygotes and cleavage stages can be found. Embryos in the blastula and gastrula stages, which were very infrequently encountered in previous months, are commonly seen throughout the summer.

In general it appears that fertilization continues to some extent throughout the year. This activity is at a particularly low level in May, but it shows a marked increase during the summer months.

Intermediate embryos

Although not all the adults examined each month contain embryos less than 1 month old or young almost ready to be born, practically all specimens over 12 mm long are always maintaining embryos in various stages of development between these 2 extremes. For the purposes of the present discussion, such young will be termed "intermediate" embryos. Embryos included in this category range in development from embryos approximately 0.3 mm long to young which are slightly over 3 mm in length. The smallest intermediate embryos have a distinct "clam-like" outline due to the presence of a definite foot and small mantle and gill buds

TABLE I

Seasonal variations in numbers of intermediate young in clams over 14 mm in length

Number of intermediate young	Winter (Dec.–Mar. 2)	Spring (Mar. 21–May)	Summer (June 22–Aug.)	Fall (Sept.–Nov.)
2	0.0%	0.0%	17.6%	0.0%
3	6.7%	0.0%	17.6%	25.0%
4	33.3%	35.3%	52.8%	16.3%
5	20.0%	23.5%	6.0%	16.3%
6	40.0%	41.2%	6.0%	42.4%
Number of adults examined	15	17	17	12

(Fig. 7). Since they appear to be considerably more advanced in both internal and external development than early fetal larvae, it is quite probable that these more well developed embryos are greater than 1 month old. Only specimens exceeding 14 mm in length show definite seasonal variations in the number of intermediate young they contain; therefore, the intermediate embryos of only those adults longer than 14 mm will be considered in the section that follows.

The figures in Table I show that during the winter and spring months most of the large specimens contain between 4 and 6 intermediate embryos. These represent the results of at least 2 or 3 distinct fertilization periods. It is interesting to note that although an active birth period occurs during the winter, while fewer young are released in the spring, the variations in the number of intermediate embryos found in individuals collected during the 2 seasons are surprisingly small. Apparently, fertilizations occurring throughout the winter and early spring supply new young to replace those released, and these embryos grow and develop during this period, gradually entering successively more advanced phases of intermediate development.

As shown in Table I, during the summer months the majority of adults over 14 mm in length contain between 2 and 4 intermediate embryos. There are several factors which could account for this phenomenon. Since fertilization appears to occur at a very low rate between late April and late May, relatively few embryos would be produced which might attain intermediate status by June. Indeed, 4 out of 6 of the adults over 14 mm in length examined at this time are devoid of the smallest intermediate embryos, although they are commonly found in adults of this size in other months of the year. Furthermore, during the summer, when birth is intense, many of the more advanced intermediate embryos continue their growth and development until they are ready for release into the outside environment. Finally, the adults themselves may be undergoing a particularly rapid burst of growth during the summer, and it is possible that smaller adults could enter a larger size category without a concomitant increase in the number of intermediate young they possess.

The continuation of fertilization activity throughout the summer and fall and the declining rate of birth in the autumn enable the majority of specimens longer than 14 mm to accumulate between 4 and 6 intermediate young during the fall months.

Embryos of maximum size in the parent and the percentage of "newborn" young in the population

As is true of the other embryos in a single monthly collection, the oldest young may vary considerably in size and development even among parents of approximately the same length. Nevertheless, both the relative size of the largest embryos in the parent and the percentage of newly released young in the population do show seasonal variations, and together these provide a good indication of when birth most commonly occurs in *Sphaerium simile*. Both of these factors will now be examined. Since clams less than 13.5 mm in length rarely, if ever, contain young which are ready for release, only the embryos of those adults larger than 13.5 mm will be considered.

The data pertinent to this section are summarized in Table II and Figure 9. It should be emphasized that in collecting the data for Table II, only the largest embryo being brooded in each inner gill of the parent was considered; even if smaller young were present, information concerning their sizes was *not* included in this table. In most collections some adults could be found containing, as their young of maximum size, embryos less than 1 mm in length. It was very difficult to obtain accurate measurements of these young with the methods available; therefore, although the percentage of adults containing such embryos is indicated in Table II, young less than 1 mm in length are not included in the data concerning the average size of the largest young. With regard to Table II it should also be noted that histological examination shows that embryos greater than 3.5 mm in length are fully developed. They resemble the adult in every respect other than size and the fact that the gonads are not yet producing gametes (Fig. 8). From these observations, it is concluded that embryos of this size are very large young near birth.

Clams ranging from 5 mm to just under 8 mm in length represent the smallest individuals occurring in the population. Since embryos of 6 mm or 7 mm in length

TABLE II

Embryos of maximum size in adults over 13.5 mm in length

	Percentage of adults whose embryos of maximum size are:			Average size of largest embryos over 1 mm long	Total number of adults examined
	Less than 1 mm long	1 mm–3.5 mm long	over 3.5 mm long		
Nov.	0.0%	57.2%	42.8%	3.5 mm	7
Dec.	no specimens examined				0
Jan.	6.3%	87.4%	6.3%	2.0 mm	16
Feb.	41.7%	41.7%	16.6%	2.5 mm	12
Mar.	5.6%	72.2%	22.2%	2.5 mm	18
Apr.	0.0%	71.4%	28.6%	2.8 mm	22
May	0.0%	40.0%	60.0%	3.9 mm	20
June	25.0%	37.5%	37.5%	3.7 mm	24
July	9.1%	72.7%	18.2%	2.4 mm	22
Aug.	41.7%	25.0%	33.3%	3.6 mm	24
Sept.	22.7%	63.6%	13.7%	2.4 mm	22
Oct.	9.1%	68.2%	22.7%	2.7 mm	22

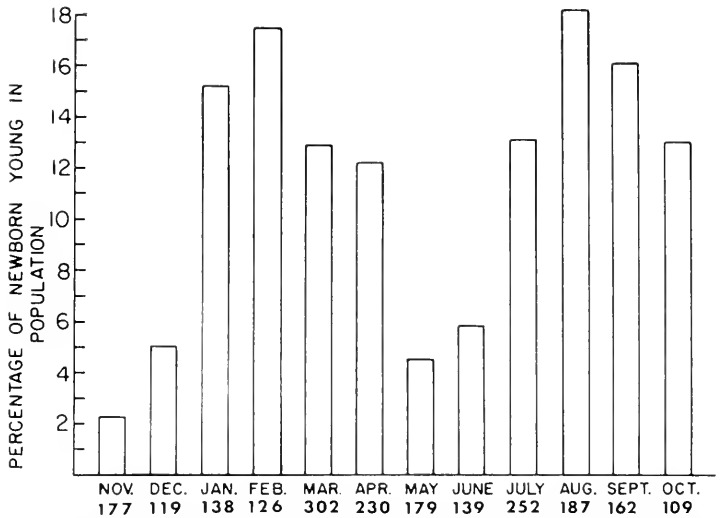

FIGURE 9. Monthly variations in the percentage of "newborn" young in the population. The number under each month indicates the total number of individuals (ranging in length from 5 mm to over 18 or 19 mm) collected that month.

are found inside adults, it is assumed that clams of comparable length in the outside environment represent "newborn" young. Figure 9 shows the percentage of such newborn young, 5 mm to just under 8 mm long in each of the monthly samples.

Figure 9 indicates that although newborn young can be found in the population throughout the year, the 2 major periods of birth for *S. simile* appear to occur during the winter and summer months. Between December and January, the percentage of newly released young in the sampled population shows a threefold increase, and there is a further increase during the following month. The second great burst of birth activity begins to occur between June and July and reaches its peak in August, although a comparatively large percentage of newborn young can still be found in the sample population collected in September. During the spring and fall months, on the other hand, the percentage of young between 5 mm and just under 8 mm long in the sampled populations steadily decreases. It thus appears that these 2 seasons are periods of declining birth activity.

The size of the largest embryos being brooded by the parents also shows seasonal variations, although these are not as clearly defined as the patterns of birth. In both the spring and fall months, when fewer young are released, the average size of the largest embryos in the parent gradually increases in preparation for the next major period of birth. It should also be noted that in those months when the percentage of newly released young in the sampled population is at a particularly low level (*i.e.*, November and December, May and June), the average size of the largest embryos in the parent is, for the most part, considerably greater than it is at other times of the year (Table II). In addition, adults maintaining embryos over 3.5 mm long are also very common at these times. Although the largest embryos of only 2 specimens were examined in December, in both cases the young are between 3 mm and 5 mm in length. In addition, over four-fifths of the adults

studied histologically in November and December contain embryos nearly ready to be born. In summary, both the histological observations made in November and December as well as the data in Table II indicate that a large percentage of the adults examined in the months immediately preceding a period of high birth activity contain embryos nearly ready to be born.

In general, during the months when many newborn young are found in the outside population, the largest embryos being brooded by the adults are considerably smaller than they are when birth is at a low level. There is a sharp drop in the average size of the largest embryos in the parents between December and January, June and July, and August and September. It appears that the average size of these embryos rises somewhat between January and "February" (*i.e.*, March 2) and is very high in August, despite the fact that a large percentage of newborn young are found in the outside population in both these months. Nevertheless, it must also be noted that in both "February" and August over 40% of the adults examined contain, as their young of maximum size, embryos less than 1 mm long, whose measurements are not included in the average sizes listed in Table II. Apparently during these months, and especially in August, a number of adults still contain young nearly ready to be born, while many others have released young very recently, and the embryos remaining inside them must undergo considerably more growth and development before they will be ready for birth.

Discussion

The successive phases of reproduction including the production of gametes, fertilization of eggs, maintenance of embryos and birth of young occur to a limited degree at all times. Nevertheless, each activity varies considerably in extent depending on the month or season being considered, and it is these seasonal variations that produce the annual reproductive cycle characteristic of *Sphaerium simile*.

Both gametogenesis and fertilization occur throughout the year. The fact that mature gametes are not present in large numbers from September to April probably accounts for the fact that less than half the individuals examined in each of these collections contain embryos resulting from very recent fertilizations. The testes of all specimens examined in both April and May generally contain very few spermatozoa, and this fact helps to explain the particularly low level of fertilization observed in May. During the period from June to August, when mature gametes of both sexes are more common than they are in other months, fertilization shows a marked increase.

Young in several stages of development can be found in parent clams throughout the year. During both the spring and the fall months the largest embryos of adults over 13.5 mm in length gradually increase in size, and these young are born mainly during the winter and summer months. In general, individuals of specified length show surprisingly minor variation in the number of young they contain, regardless of the season. It thus appears that a continuous succession of young is maintained in parent clams. When the oldest embryos are released, the other groups of young in the parent continue their development, and ultimately a new group is ready for release. Meanwhile the replacement cycle continues as new groups of eggs undergo fertilization.

In many species of the genera *Sphaerium* and *Musculium* each phase of reproduction occurs in several seasons of the year. Although Drew (1896) and Gilmore (1917) did not make any attempt to study seasonal changes in the gonads of *S. simile,* both investigators observed mature gametes throughout the year in this species. A similar phenomenon is observed in *S. striatinum* (Woods, 1931). Okada (1935b) noted that in *S. japonicum* the presence of mature gametes and zygotes is limited to the spring and fall.

With regard to the release of young, Okada (1935b) noted that in *S. japonicum* birth occurs most commonly during the spring and autumn. Investigations on the life cycle of *S. corneum* carried out by Thiel (cited in Okada, 1936b) and more recently by Mitropol'skii (1969) indicate that in this species birth is most frequent in the summer and fall, although Mitropol'skii found no sharp division between these 2 birth periods. Foster (1932), working with *S. striatinum,* reported that in this species birth occurs mainly during the summer and winter, and the present study shows that the situation is similar in *S. simile.* The main difference between the times of birth in these 2 species is that in *S. striatinum* these periods are more sharply defined than they are in *S. simile.* In *S. striatinum* many young are released in the winter, and then birth is minimal until July when births increase until August. In *S. simile* birth continues at a high rate throughout the winter and summer months and gradually declines during the spring and fall. The differences in birth cycles in these 2 species may be partly explained on the basis of the numbers of embryos that each is capable of maintaining. Although Avolizi (1971) observed that *S. striatinum* may contain as many as 12 embryos, Foster (1932) reported that most of the adults in the population of *S. striatinum* that he observed contain 2 to 4 developing young. In the population of *S. simile* investigated in the present study there are normally 6 to 8 developing young. It may be that it simply takes longer for the larger number of young produced in *S. simile* to be released into the general population, and this may lead to the larger overall birth periods, tapering off gradually, observed in this species.

With regard to litter size in *Sphaerium simile,* Gilmore (1917) reported that no more than 2 to 4 embryos are found in the inner gill of this species. The discrepancy between Gilmore's observations and those of the present study may be explained by the fact that Gilmore based his conclusions concerning brood size primarily on data obtained from a number of gross dissections of clams examined in July 1917. With the technique used he was unable to observe embryos less than 0.5 mm in length, and smaller young were therefore not included in the results of this part of his study. In the present investigation, it was observed that in July many of the largest young are released from the parent by birth, and in addition, many new eggs are fertilized. In the majority of specimens examined, at least half the embryos they contain are considerably less than 0.5 mm long and are often not visible without the aid of a microscope. Thus it seems probable that the methods Gilmore employed were not adequate to determine the total number of young being brooded by *S. simile.*

In some sphaeriid populations reproductive activities are limited to those seasons of the year when environmental conditions are most favorable. Thomas (1963) studied a population of *Musculium partumeium* living in a temporary pond which freezes in the winter and dries up during the summer. Both growth of the

individuals and the release of young are confined to the spring months when the amount of water in the pond and its temperature provide suitable conditions for these activities. The habitat of the population of *S. simile* investigated in the present study does not undergo extreme environmental changes. Sphaerium Brook never dries up, and a steady moderate current is present throughout the year. This flow of water prevents the brook from freezing; even during the intensely cold weather in the winter of 1969–1970 water temperatures of 2° C to 3° C were measured, and ice formed only along the banks. In late summer, although water temperatures as high as 26° C were noted, conditions favorable for reproduction are maintained through the steady movement of oxygenated water from the pond. This comparatively mild environment permits reproductive activities to continue throughout the year in *S. simile*.

Both Foster (1932) and Thomas (1965) reported that the total life span of the species they investigated was approximately 1 year. Herrington (1948), on the other hand, working with *S. occidentale*, noted from marking experiments that this species can survive longer than 1 year. The populations of *S. striatinum* investigated by Avolizi (1971) have a life span of 18 to 24 months. Avolizi's study also indicates that *S. simile* survives approximately 26 months and has a steady but low rate of mortality.

The present study has indicated the annual reproductive cycle of *Sphaerium simile*. It is hoped that this investigation together with Avolizi's work concerning the growth rate and longevity of this species will provide a more comprehensive understanding of the entire life cycle of *Sphaerium simile*.

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Summary

1. Reproduction occurs throughout the year in *S. simile* although definite variations can be observed in different reproductive activities at different times.

2. Gametogenesis and mature gametes are visible to some extent during the fall, winter and early spring (particularly in specimens less than 12 mm long); but the production of gametes appears to be most intense in all adults, regardless of size, in the late spring and summer (especially June and July).

3. Fertilization seems to be most active during the summer, although parents with young resulting from very recent fertilizations can be found throughout the year.

4. Embryos in one or more stages of development are present in the great majority of individuals over 10 mm in length at all times.

5. The birth rate is highest during the winter and summer months. There is a gradual decline in the number of births throughout the spring and fall. During these seasons, however, the embryos inside the parent continue their growth and

development; the size of the largest embryos in the adults continues to increase in preparation for the next major period of birth.

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THE PHYSIOLOGICAL RESPONSE OF THE ESTUARINE CLAM, *RANGIA CUNEATA* (GRAY), TO SALINITY. II. UPTAKE OF GLYCINE [1]

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The literature concerning the uptake and utilization of dissolved organics by estuarine and marine invertebrates has been reviewed by Stephens (1972). Stephens (1964) reported that the capacity of two polychaetous annelids, *Nereis limnicola* and *N. succinea* to take up C^{14}-labeled glycine was related to the chloride concentration of the acclimation and exposure media. For both species, the uptake was nearly zero at chlorosities of 150 meq Cl^-/l (10‰ S) or less, and a significant increase in glycine removal occurred at 200 meq (about 13‰). From the work of Smith (1959) and the data of Oglesby (1965), it appears that these polychaetes begin chloride regulation at approximately 210–225 meq (14‰ S).

Stephens and Virkar (1966) studied the uptake of glycine and other amino acids by the brittle star, *Ophiactis arenosa*. Since these animals are not truly euryhaline, they were only tested over a salinity range of 50 to 100% sea water (16.5–33.0‰). Post-incubation after exposure to C^{14}-glycine showed a decrease in salinity was correlated with a decrease in ninhydrin positive material (NPM), and an increase in the radioactivity associated with the alcohol insoluble fraction of the animals. There was a corresponding decrease in the alcohol soluble radioactivity as salinity decreased from 70 to 50% sea water.

The literature regarding the utilization of free amino acids (FAA) in the osmoregulation of lamellibranch mollusks and other estuarine and marine invertebrates was reviewed by Virkar and Webb (1970). Gilles (1972) has shown that amino acids played a part in the cellular osmoregulation process in the intertidal bivalves studied. Allen (1961) found that *Rangia cuneata* taken from different salinities contained quantities of alanine, aspartic acid, glutamic acid and glycine, which increased as the salinity increased from 3 to 17‰. As the environmental

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salinity increased from 17 to 25‰ there was a slight decrease in the levels of these compounds. Alanine was found to be the most prominent amino acid in *Rangia,* while in *Mytilus edulis* taurine is retained in high concentration (Allen and Awapara, 1960).

The estuarine clam, *Rangia cuneata,* is extremely tolerant to wide salinity fluctuations (Hopkins and Andrews, 1970) and is of potential economic importance (Hopkins, 1970). Unusual among the bivalves which have been studied (Robertson 1964; Pierce, 1970), *Rangia* have been shown to be capable of osmoregulation in low salinity water (Bedford and Anderson, 1972). The blood osmotic concentration becomes hyperosmotic to the environment at salinities less than 10‰. From 5‰ to 1‰ salinity (S) an osmotic differential of approximately 55 to 65 milliosmoles per liter above the environment was maintained by the blood.

Since the osmoregulartory activity of *Rangia* had been established and since these clams were tolerant to salinity extremes, it was felt that they would be excellent subjects for study on the relationship between salinity and the uptake of amino acids from solution.

Methods

The specimens of *Rangia* were collected from McCollum Park, Trinity Bay, Chambers County, Texas. The salinity of this region fluctuates widely, but is generally between 5 and 15‰ S. The substrate was a very uniform hard sandy mud, and the water depth ranged between 45 cm at shore to 95 cm approximately 300 meters from shore. The density of the clam population at this site was between 45 and 49 individuals per square meter.

The animals were maintained in the laboratory in 15 gallon aerated aquaria containing water of the same salinity as that of the collection site. Water used for holding and experimental work was prepared with distilled water and Instant Ocean (Aquarium Systems, Inc.). After two days of maintenance at the collection salinity, they were acclimated in steps of 5‰ per 2 days. This procedure resulted in a survival rate of 98 per cent or greater. Animals were held at the final experimental salinity for at least 7 days, and no longer than 24 days before use. In every case all clams utilized in a given experiment were collected on the same date. There was no attempt to feed the animals, since preliminary studies have shown that they survive for over two months in the laboratory without food. Allen (1959) has reported that these animals survive long periods of starvation and no appreciable changes occur in the amino acid composition after 21 days without food. Maintenance and experimentation were in an air conditioned laboratory at 22 ± 1° C.

Per cent body water and per cent ash

After acclimation for 10 days at salinities from 1 to 32‰, clams were shucked, blotted, and the wet weight was taken. The tissue was dried to a constant weight (approximately 72 hours) at 95° C, and then ashed in a muffle furnace at 550° C to a constant weight (24 hours). These data were used to prepare curves for per cent body water and per cent ash-free dry weight at each salinity. Where direct determinations of ash-free dry weight were not possible in a given experiment, values were corrected using the above curves. To determine the rate at

which volume regulation occurred, clams were transfered from 15‰ to 5 or 25‰ S and analyzed for per cent water at various time intervals.

Glycine uptake

Animals utilized in uptake experiments were carefully selected for uniform size (55 ± 3 mm) to reduce variation due to weight. A random selection of 44 clams within this size range gave a mean ash-free dry weight of 1.31 grams and a standard deviation (SD) of 0.27 grams.

Whole animals were tested for their ability to remove C^{14}-labeled glycine at various salinities by exposing them for different time intervals to mixtures of C^{14} and C^{12}-glycine. After exposure they were shucked and the meat rinsed about 5 seconds in tap water, blotted, weighed and placed in 80% ethanol (ETOH). During a period of approximately 48 hours the ETOH extracts and tissue were agitated several times. Two 1 ml aliquots of each extract were analyzed for radioactivity with a Beckman 200-LS liquid scintillation counter and the C^{14}-activity expressed as counts per minute per mg ash-free weight (cpm/mg). Initial and final samples of media were also counted in all experiments.

In one instance a flowing system was utilized to expose *Rangia* to C^{14}-glycine over a 16-hour period. Water of 15‰ S was filtered through a 0.45 μ Millipore filter before the addition of glycine. A peristaltic pump (1 ml/min) was used to pull water through individual containers with *Rangia* and the effluent was collected in a fraction collector. The source medium and the chambers were constantly mixed by use of air-powered magnetic stirrers. Effluents and ETOH extracts of the animals were counted by liquid scintillation.

To obtain data on short term (less than one hour) uptake, the gills (demibranchs) were removed from acclimated animals and utilized in an uptake experiment. After dissection, gills were maintained in water of the appropriate salinity and then transferred after blotting to smaller containers with the exposure media. Groups of gills were removed from the C^{14}-glycine at intervals between 10 and 90 minutes, rinsed in three changes of water, and treated as described for whole animals.

A series of experiments were conducted to determine the kinetics of glycine uptake by *Rangia* at several salinities. When all animals were actively siphoning, a small volume of solution was added to each container, such that all clams were exposed to the same amount of C^{14}-glycine, but the total concentration of C^{14} plus C^{12} was between 10^{-3} and 10^{-5} molar glycine. After one hour of exposure in these concentration gradient studies, the usual extraction and counting procedures were conducted, and the velocity of uptake calculated as follows:

$$\frac{\text{cpm/g/hour (animals)}}{\text{cpm/ml (initial medium)}} \times \text{medium concentration (moles/ml)} = \text{Velocity of Uptake (moles/g/hr)}$$

These data were used to prepare a Lineweaver-Burke curve, where the reciprocal of the velocity is plotted against the reciprocal of the substrate concentration. As noted by other workers (Stephens, 1967), the use of this approach is not a suggestion that uptake is an enzyme-dependent process.

Glycine release

To examine the use of glycine in the osmoregulatory activity of the *Rangia*, animals were first exposed to C^{14}-glycine for four hours at a salinity of 22‰. After exposure, three clams were extracted as usual, while the remainder were placed in separate containers at salinities between 2 and 22‰ for 24 hours. Samples of medium from each container were taken at several time intervals, and the counts were expressed as radioactivity released in cpm/mg ash-free dry weight.

Fate of glycine

Animals which had been acclimated to various salinities were exposed to C^{14}-glycine for 2 hours and washed free of the radioactivity by six successive changes of the appropriate salinity water. They were then maintained in glycine-free

TABLE I.

Flow sheet for biochemical separation of the major tissue components

1. Extract 0.8 g wet weight of homogenized tissue in 10 ml of 80% ETOH for 24 hours.
2. Centrifuge* and pour off supernatant, repeat with 5 ml wash of 80% ETOH.
3. Extract lipids from pellet with 10 ml of ether-70% ETOH (3:1 v/v) for 18 hours with frequent mixing, centrifuge, repeat with 5 ml of ether-ETOH for 2 hours.

Combined supernatants (lipids)
1. Evaporate in vacuum desiccator (insert a small beaker of paraffin oil in desiccator).
2. Weight when dry.

Pellet
1. Add 5 ml TCA (trichloroacetic acid) and mix well.
2. Heat to 80° C for 30 minutes, then hold at 50° C for 2 hours.
3. Cool and centrifuge, repeat with 5 ml of cold TCA.

Combined supernatants (glycogen and nucleic acids)
1. Make the supernate 52% ETOH.
2. Allow to stand at 0° C for 18 hours.
3. Centrifuge.

Pellet (proteins)
1. Wash twice with 10 ml of absolute ETOH.
2. Dry to constant weight at 95° C.

Supernatant (nucleic acids)
1. Remove TCA by extraction with 30 ml of ether in separatory funnel. Repeat 4 times.
2. Evaporate to dryness at 95° C.

Precipitate (glycogen)
1. Wash twice with 5 ml of absolute ETOH.
2. Dry to constant weight at 95° C.

* All centrifugation at 3000 rpm for ten minutes.

water of the appropriate salinity for periods up to 99 hours. At various intermediate intervals samples from each salinity were extracted in 80% ETOH as described above, and the remaining alcohol insoluble (incorporated) components were determined as follows: tissue was washed with alcohol, blotted and homogenized in a micro-Waring blender with enough 80% ETOH to bring the volume to 50 ml. Three 1 ml samples of each homogenate were solubilized with 1 ml of Protosol (New England Nuclear), neutralized with glacial acetic acid and counted by liquid scintillation. The alcohol soluble and insoluble radioactivities were corrected by use of quenching curves, and the data were expressed as per cent of

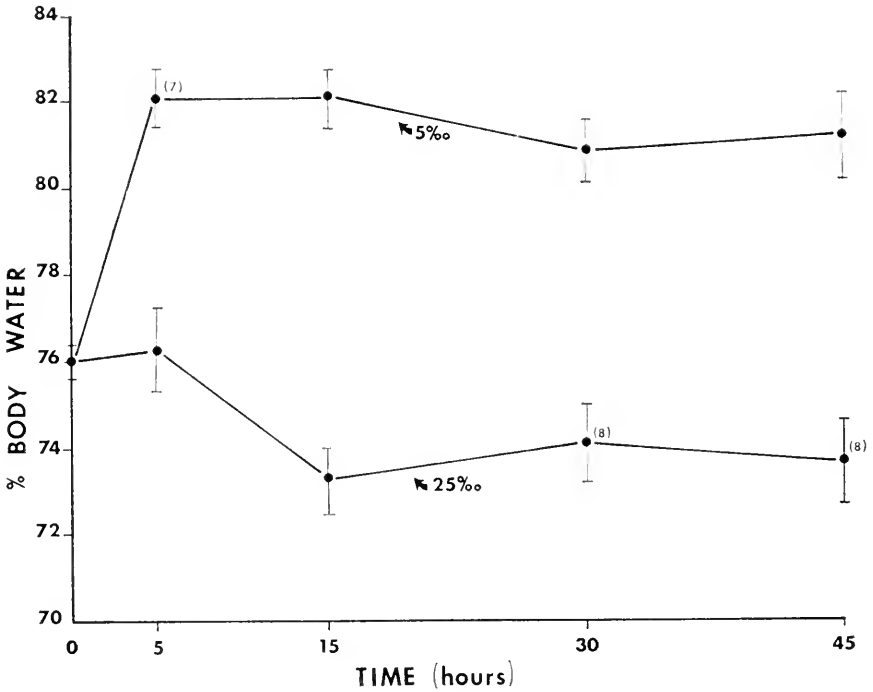

FIGURE 1. Volume regulation as a function of time. Vertical bars indicate standard deviations (SD), where n = 10 (except where noted).

the total activity. This method facilitates comparisons between salinities and compensates for individual differences in total activity.

Additional samples of the homogenates were quantitatively separated into protein, nucleic acids, lipids and polysaccharides (glycogen). The basic methodology (outlined in Table I) used was that of Shibko, Koivistoinen, Tratnyek, Newall, and Friedman (1967) with modifications as suggested by Graff (1970). After their qualitive separation, the four tissue fractions were prepared for counting as follows: first, the dried components were put into solution with 5 ml of 10% KOH, using heat where necessary. Then 1 ml of each tissue fraction was neutralized with glacial acetic acid and counted using the Beckman LS-200B

liquid scintillation system with an Aquasol cocktail. This uniform preparation for counting greatly reduced any differences in the quenching properties of the different fractions.

Results

Per cent body water and per cent ash

The percentage of tissue water increased by approximately 8 per cent (73 to 80.8%) as the acclimation salinity decreased from 32 to 1‰ S. The inorganic

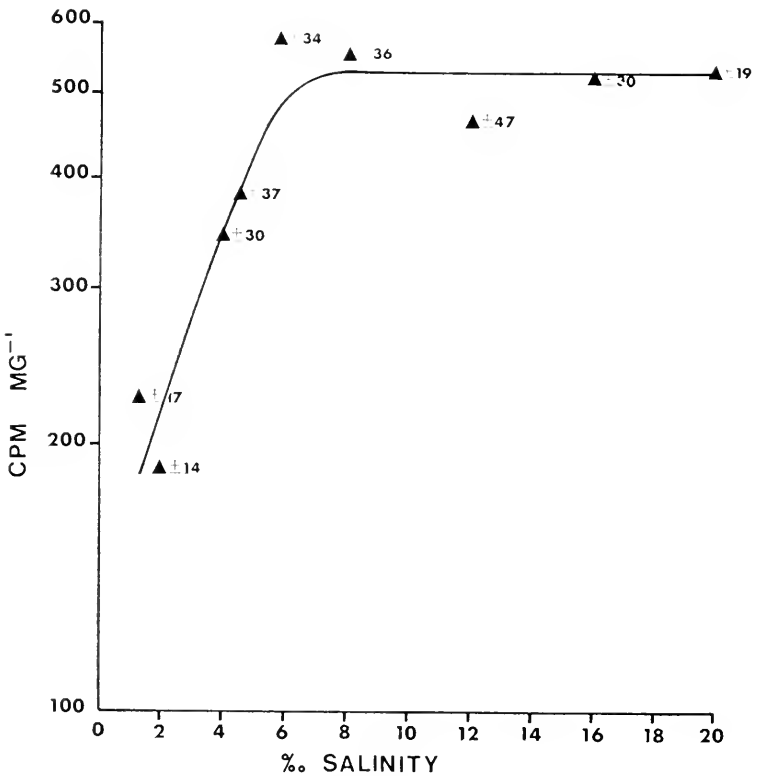

Figure 2. Ethanol soluble radioactivity of whole *Rangia* as a function of salinity; exposed to 1×10^{-5} molar glycine for 5 hours. SD shown as ±, where n = 6.

constituents of the tissue (ash) increased from 2.5% at 1‰ S to 7.6% at 32‰ S. The data exhibited good linear fit in both cases, with correlation coefficients of 0.964 and 0.995, respectively. In a few instances where it was not practical to determine ash-free dry weight directly, curves prepared from the above data were used to convert the values.

When specimens of *Rangia*, acclimated to 15‰ S, were immediately transferred to 5 or 25‰ S, the rate of change in per cent body water was measured (Fig. 1). It should be noted that those animals exposed to a decrease in salinity (5‰ S)

had already reached their approximately new steady state (82%) by the 5 hour interval. Clams transferred to 25‰ S presumably had not begun active siphoning by the 5 hour interval, since relatively little change had occurred in the per cent of body water. By the 15 hour interval both groups of clams had reached a level approximating their final steady state.

Uptake of glycine

Uptake of glycine by whole animals, as a function of salinity, is shown in Figure 2. The values plotted on the logarithmic scale represent the ethanol soluble activity after 5 hours of exposure in a 1×10^{-5} molar solution of $C^{14} + C^{12}$-

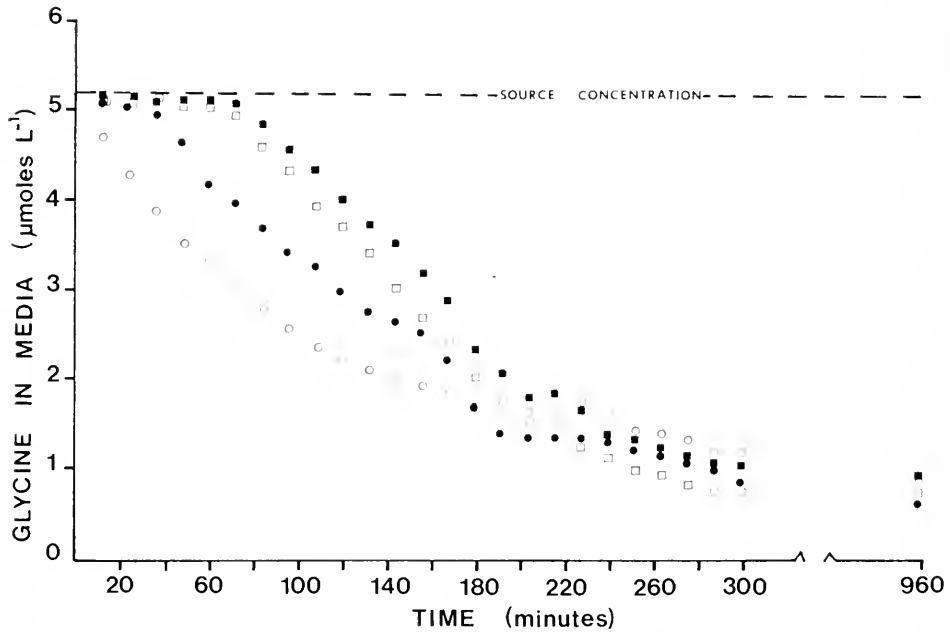

FIGURE 3. Glycine uptake by 4 individuals in separate flow-through chambers.

glycine (700 cpm = 1×10^{-3} micromoles). Resulting activities remained relatively constant (450–550 cpm/mg or about 0.7 μ moles/g) at salinities from 20 to 6‰ but decreased rapidly at lower salinities. The uptake at 6‰ S was shown to be 3 times that at 2‰ S. The uptake levels at the higher salinities (6–20‰ S) represent a 75 fold accumulation of glycine over that in the ambient medium.

The nature of the uptake process over time is shown in Figure 3. From these data it appears that the process is a continuous one, at least at the concentrations and time intervals tested. This experiment was conducted at 15‰ S with the glycine being introduced via a flow-through system. Uptake of glycine during the first 300 minutes was apparently dependent on the activity of each of the four clams, since the rate at which a constant differential was reached varied. From 300 minutes to 960 the removal of glycine was very stable. The 4.4 μ mole differential,

between source and outflow, presented on the graph represents a rate of glycine uptake equal to 0.264 μ moles per hour (calculated using the flow rate of 0.06 l/hr). Using the average ash-free dry weight of the clam tissue (1.58 g) the uptake was 0.161 μ moles/g/hr, for a period of approximately 11 hours. At a flow rate of 1 ml per minute there was considerable reduction in the concentration of dissolved oxygen present in the chambers after 16 hours of exposure. The concentrations were in the range of from 1 to 2 ml O_2 liter at termination and unpublished data indicate that respiration at these levels is approximately 0.5 ml O_2/g ash-free dry weight/hr. Since constant dissolved oxygen (D.O.) readings were not taken, it is not possible to relate D.O. to uptake, but the constant rate of glycine removal from

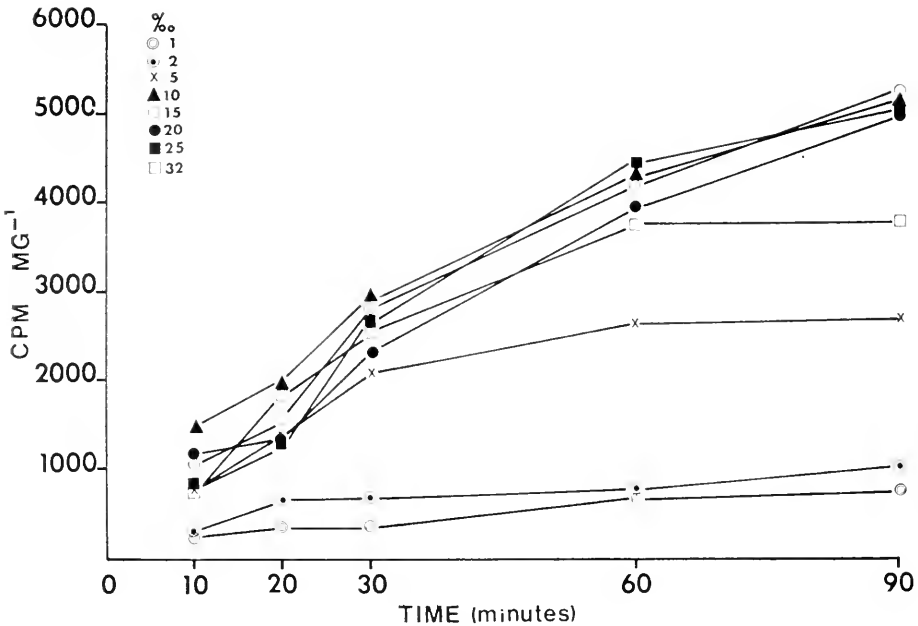

FIGURE 4. Ethanol soluble radioactivity of gill tissue as a function of salinity and time. SD values were approximately 7% of the plotted points, where n = 4.

3 to 16 hours, while D.O. was decreasing, indicates that uptake was independent of D.O. concentration.

The uptake by *Rangia* gills at various salinities from a 3.8×10^{-6} molar solution of C^{14} plus C^{12}-glycine is shown in Figure 4. These gills were removed immediately before exposure from clams that had been acclimated to each of the test salinities. Uptake was quite uniform and linear for gills at 10 through 25‰ S. The radioactivity associated with the gills at 1 and 2‰ S was relatively small and increased only slightly from 10 to 90 minutes. Uptake of glycine was intermediate for gills tested at 5 and 32‰ S, with the uptake rate sharply decreasing at 30 and 60 minutes, respectively. The data may be converted to μ moles of glycine by using a ratio of 1000 cpm/mg = 0.74 μ moles/g. After 90 minutes of exposure the gills at 10, 15, 20 and 25‰ had removed approximately 3.84 μ moles/g, while those at

1 and 2‰ had only accumulated 0.56 and 0.75 μ moles/g, respectively. The intermediate uptake at 5‰ S was expected from data on whole animals (Fig. 2), but the reduced uptake by gills at 32‰ after 90 minutes was not anticipated and will be discussed later. It should be noted that at no time was the glycine concentration in the exposure media reduced below 1×10^{-6} molar, and was therefore not limiting.

The relationship between the ambient glycine concentration and the rate of uptake by whole *Rangia* is shown in Table II. A Lineweaver-Burke plot of these data produced regression lines for each salinity with correlation coeffcients (Table II) which indicate excellent fit of the data to the curves. The standard estimate of error for each curve was less than 0.2, with the exception of one 2‰ S curve,

TABLE II

The relationship between the ambient glycine concentration (S) and the rate of uptake (V) at different salinities. Each V value represents the mean of 5 individuals. Kt represents that concentration of glycine at which uptake is half maximal (V_{max}).

Salinity (‰)	S ($\times 10^{-4}$ Molar)	V (μ moles/g/hr)	Slope of 1/V *versus* 1/S	V_{max} (μ moles/g/hr)	Kt ($\times 10^{-4}$ Molar)	Correlation coefficient
2	10.0	0.900	0.491	0.851	0.418	0.993
	5.0	1.200				
	1.0	0.457				
	0.5	0.445				
	0.1	0.166				
2	10.0	1.253	0.464	1.025	0.476	0.987
	5.0	0.819				
	1.0	0.633				
	0.5	0.545				
	0.1	0.178				
4	10.0	2.638	0.342	2.849	0.974	0.989
	5.0	1.905				
	1.0	1.478				
	0.5	1.078				
	0.1	0.264				
6	10.0	2.859	0.159	2.079	0.331	0.987
	5.0	2.398				
	1.0	1.289				
	0.5	1.054				
	0.1	0.492				
10	10.0	—	0.172	2.703	0.462	0.992
	5.0	2.839				
	1.0	1.468				
	0.5	1.608				
	0.1	0.478				
11	10.0	2.710	0.137	2.288	0.314	0.986
	5.0	2.263				
	1.0	1.772				
	0.5	1.171				
	0.1	0.561				

which was 0.4. When 95% confidence limits were prepared for each curve, the limits for 6, 10 and 11‰ S overlapped, while those for 4‰ were significantly different from the latter and also those enclosing both 2‰ S curves. Since the intercepts of the 4, 6, 10 and 11‰ S curves were nearly equal, the confidence limits did overlap as they approached the Y-axis (at higher concentrations). The slopes of the curves for 2 and 4‰ S are approximately the same, and those for 6, 10 and 11 are relatively close to each other. The data in Table II appear to represent three distinctive patterns of uptake. First, the data from the two separate experiments at 2‰ S are quite similar and indicate a slow rate of uptake, characterized by a low V_{max} (0.851–1.025). Secondly, the values for slope, V_{max} and Kt are relatively uniform for uptake of glycine at 6, 10 and 11‰ S. Finally, up-

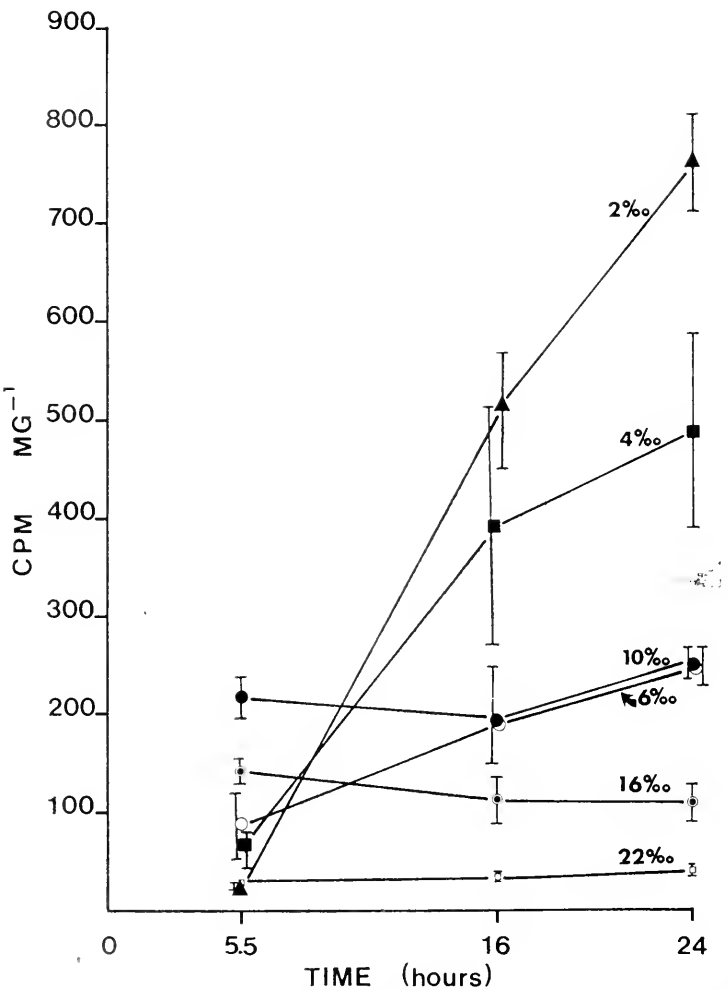

FIGURE 5. Release of C^{14}-activity as a function of salinity and time; *Rangia* previously exposed for 5 hours to C^{14}-glycine at 22‰ S. Vertical bars are standard errors, where n = 3.

take at 4‰ S appears to exhibit characteristics intermediate between the latter two groups, since the slope was high (as at 2‰ S) and the Kt was very large (0.974) with the resulting V_{max} similar to those of 6, 10 and 11‰ S.

When specimens of *Rangia* were first exposed to C^{14}-labeled glycine at 22‰ S and later moved to salinities of 16, 10, 6, 4 and 2‰, the accumulated radioactivity was released as shown in Figure 5. With decreasing salinity there was a corresponding increase in the radioactivity released into the media. The greatest release in radioactivity, which occurred after 24 hours, was at 2 and 4‰ S (762 and 440 cpm/mg, respectively). The release at 6‰ S was similar to that at 10‰ S (249 and 255 cpm/mg) and only slight amounts of radioactivity appeared at the higher salinity of 16‰. The 42 cpm/mg obtained at 22‰ was due to "Hot" exposure

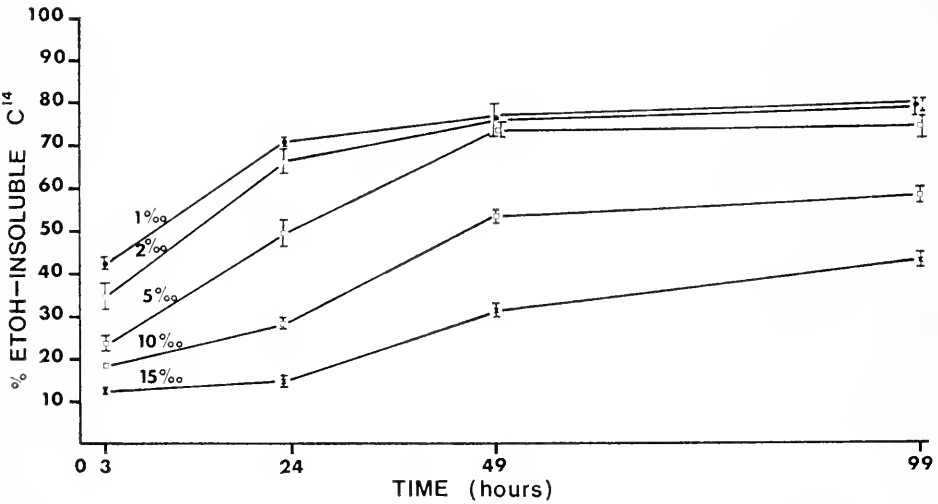

FIGURE 6. Percentage of total activity in the ETOH-insoluble fraction as a function of salinity and time. Vertical bars indicate SD, where n = 4.

media carried over when the clams were transferred to fresh media. The low levels of released activity at the 5.5 hour interval for the clams at 2 and 4‰ S was in all probability due to the extreme salinity shock inhibiting the siphoning activity of the clams.

Fate of glycine

The first aspect of a study to determine the fate of the glycine after uptake was to examine the percentage of activity that remains as free pool compounds and the percentage that was incorporated into the tissue components . These percentages are plotted versus time for salinities 1 through 15‰ S (Fig. 6). The components that are soluble in 80% ethanol have been defined as free pool components and the insoluble portion as incorporated compounds. Only the incorporated or ETOH insoluble fraction is plotted, as the pool or ETOH soluble fraction is the reciprocal of Figure 6. The most rapid and greatest incorporation occurred at the lower salinities of 1, 2, and 5‰ S, resulting in approximately 75% of the total activity

being converted to ETOH-insoluble components 49 hours after exposure to the labeled glycine. During the same time interval the clams in 10 and 15‰ S had only incorporated 54 and 32%, respectively, of the total accumulated glycine. At 99 hours the figures for the *Rangia* at 1, 2 and 5‰ S had not changed appreciably. However, incorporated radioactivity had increased for the animals at 10 and 15‰ S (58 and 44%, respectively). The total (free pool and incorporated) activity, for the 99 hour interval, is plotted against salinity in Figure 7. The total C^{14}-activity due to accumulation of labeled glycine was 3.5 times greater at 10 and 15‰ S than at 1‰ S. The total activity present in FAA pools and tissues of the animals de-

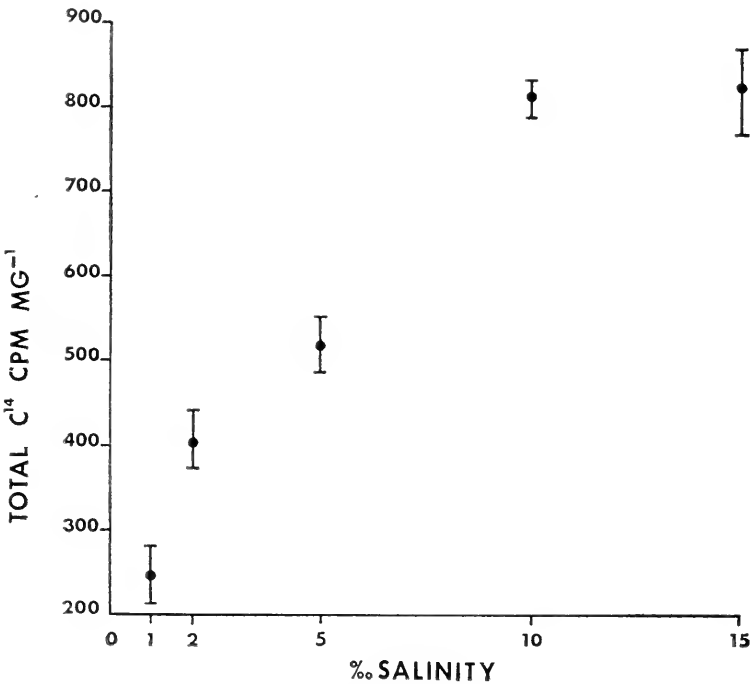

FIGURE 7. Total C^{14}-activity 99 hours after exposure to glycine at various salinities; vertical bars = SD, where n = 4.

creases sharply below 10‰ S. A comparison was made between the total activity present in the clams at 24 hours after exposure to that at 99 hours. The results of the comparison are presented in Table III as the per cent decrease in the total tissue activity and as the equivalent in micromoles of glycine per gram of tissue. It appears that with increasing salinity there was an increase in the utilization as well as uptake of glycine.

In Figures 8 and 9 the percentages of the incorporated activity which were found in the protein, nucleic acid, glycogen, and lipid fractions are presented for the 3 and 99 hour intervals, respectively, after exposure to C^{14}-labeled glycine. These fractions made up the following percentages by weight of the total tissue components: protein, 59.4 ± 4.9; nucleic acid, 15.8 ± 1.3; glycogen, 19.0 ± 5.3; and

lipid, 5.7 ± 1.8. The ± refers to the standard deviation, where n = 39. These percentages total very close to 100% which was expected, since the free pool compounds were extracted before analysis and the inorganic matter was subtracted from the total weight. There was no significant difference in the percentages of

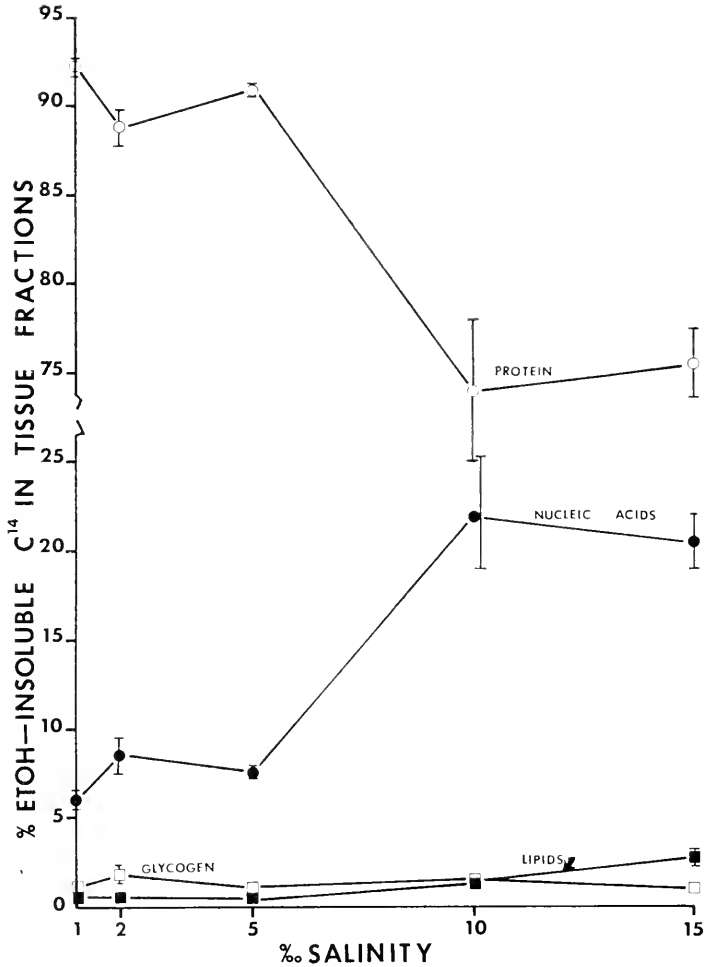

FIGURE 8. Percentage of ETOH-insoluble activity in the various tissue fractions 3 hours after exposure to C^{14}-glycine, vertical bars = SD, where n = 4.

the tissue components at the different salinities. There was, however, a significant difference in the fate of the labeled glycine with regard to both salinity and time. After three hours of exposure to C^{14}-glycine those clams at 1, 2 and 5‰ S had used approximately 90% of the incorporated glycine in protein synthesis, while the clams at 10 and 15‰ S had used only 75%. This difference was accounted for by a higher percentage of labeled nucleic acids in animals from the 10 and 15‰

salinities. After 99 hours the percentage of C^{14} in the protein of the low salinity clams had dropped to 87% and the percentage in the clams at 10 and 15‰ S had increased to 93%. The nucleic acid fractions at 10 and 15‰ S had decreased to a level where there was no significant difference with salinity at 99 hours. The percentage of C^{14} activity in the glycogen fractions had increased during the 96 hours from 1 or 2% to 5 or 6% at the low salinities. The C^{14}-activity associated with lipids increased slightly with salinity at the 3 hour interval (Fig. 8), but did not show any variation with salinity at the 99 hour period (Fig. 9). As pointed out in Table III the loss in the total tissue activity after 75 hours ranged from 9.2 to 69.2%, depending on salinity. This loss of activity was not accounted for by an increase in the media activity, thus the C^{14} must have been expelled as $C^{14}O_2$, indicating metabolism of labeled compounds.

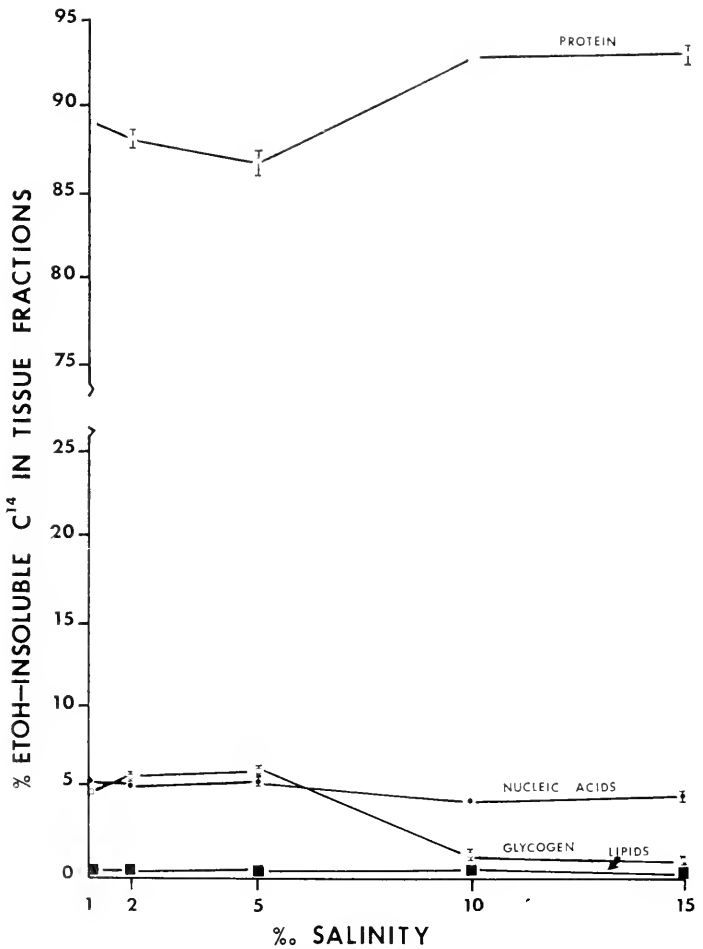

FIGURE 9. Percentage of ETOH-insoluble activity in the various tissue fractions 99 hours after exposure to C^{14}-glycine; vertical bars = SD, where n = 4.

Discussion

When relating the physiological response of estuarine organisms to salinity it is extremely important to quantitate the data in a manner which is independent of salinity. Over the salinity range tested (1 to 32‰ S) the per cent body water was found to decrease from approximately 81 to 73%. Since clams acclimated to 32‰ S contain significantly higher levels of inorganic materials than those at 1‰ S, the use of dry weight is not as accurate as ash-free dry weight in the quantitation of metabolic processes. The inorganic constituents (ash) were shown to increase from 2.5% at 1‰ S to 7.6% at 32‰ S. The data for per cent body water and per cent ash compare quite closely to those of Allen (1961), with the exception that no decrease in the percentage dry weight or ash was noted at 25‰ in this study. All uptake data were calculated in terms of radioactivity or micromoles per unit ash-free dry weight.

As might be expected, the results of studies on the rate of volume regulation

Table III

The percentage decrease in radioactivity and μ moles of glycine in Rangia *tissue at various salinities. The decrease occurred between 24 and 99 hours after exposure*

Salinity (‰)	Per cent decrease in total radioactivity	Decrease in glycine (μ moles/g tissue)
1	9.2	0.015
2	21.3	0.067
5	45.5	0.260
10	53.8	0.419
15	69.2	1.108

compare closely with the earlier data on osmoregulation (Bedford and Anderson, 1972). In both instances, a new steady state was reached by the *Rangia* in approximately 24 hours, after transfer to media 10‰ hypo- or hyperosmotic to the acclimation medium. Again, the clams exhibited a more rapid rate of acclimation to the new environment under conditions of dilution (Bedford and Anderson, 1972). Inhibition of siphoning activity for at least 5 hours was apparent (Fig. 1) when animals were transfered from 15 to 25‰ S.

At the concentrations examined, *Rangia* was found to remove glycine from solution at all salinities tested. In the curves relating uptake to salinity there was a breaking point at approximately 6 to 10‰ S. At salinities of 6‰ or greater the uptake of glycine proceeded quite rapidly and at a rate which was independent of salinity. The single exception in the data was the suppression of uptake by gill tissue acclimated to 32‰ S. It should be noted that although specimens of *Rangia* survive well as adults in the laboratory at 32‰ S, they do not occur in habitats which attain salinities greater than 25‰ S for any significant amount of time. These clams as well as their larvae prefer salinities of 15‰ or less (Bedford and Anderson, unpublished data).

By various means, it has been shown that the uptake of glycine by whole *Rangia* and by their gill tissue declines sharply at salinities of 5‰ or less. After one hour of exposure to the same concentration of C^{14}-glycine, gill tissue from

clams acclimated to 10‰ S contained nearly an order of magnitude greater alcohol soluble radioactivity than gills from 1‰ S animals (Fig. 4). It should be noted that uptake of glycine by gill tissue occurred at a rate which was much more rapid and greater levels were obtained when compared to whole animals. Of course this was to be expected, since on a per unit weight basis gill tissue is certainly the most metabolically active tissue of bivalves. In addition to being the site for respiratory exchange, water propulsion, food sorting and ionic regulation, the gill has been shown to be responsible for a major portion of the glycine removal.

It would appear that some stress is exerted on the whole animals at salinities of 5‰ and less and also on the gill tissue at 32‰ S. As it has been shown (Bedford and Anderson, 1972) that *Rangia* maintain their blood at about 60 milliosmoles per liter above the environment at salinities below 5‰, presumably this osmotic work could be considered a stress. Stephens (1964) found that the uptake by *Nereis limnicola* decreased sharply at approximately the point at which chloride regulation began (14‰ S). He stated in a later publication (Stephens, 1967 page 371), "It may be that the processes that underlie osmotic regulation are incompatible with the rapid accumulation of amino acids from the ambient medium." Whether we consider the reduction of uptake at low salinity due to stress, competition for energy or competition for transport sites on the membrane is perhaps academic at this time. Much more information is required regarding transport mechanisms and their energy demands.

In addition to the possibility that the total osmotic concentration of the medium affects the uptake system, individual inorganic ions may influence the permeability and/or transport systems of the membrane. Although this subject has not been investigated at length, our unpublished results and those of Preston and Stephens (1969) and Stephens (1964) indicate that reductions in levels of sodium or chloride are not directly responsible for suppression of uptake at low salinity.

Stephens (1968) and Chien, Stephens and Healey (1972) have demonstrated long-term removal of amino acids by polychaetes at a constant rate of accumulation. The results of this investigation have shown that not only was the short-term accumulation of glycine by gill tissue relatively linear for 90 minutes at salinities between 10 and 25‰, but also long-term removal by whole animals at 15‰ was constant over a period of approximately 11 hours. Using a flowing system, the clams were found to remove 0.16 μ moles of glycine/g/hour when supplied with a concentration of 5.2 μ moles of glycine per liter (Fig. 3).

The release of C^{14}-labeled material by *Rangia*, which had previously been exposed to C^{14}-glycine and then transfered to various dilutions, was, as expected, dependent on the degree of dilution. Although no attempt was made to identify the material released, it was assumed to be glycine, since Stephens (1964) found the majority of alcohol soluble radioactivity of *Nereis* in the form of glycine, several hours after exposure. Since CO_2 was not driven off before counting and since certain polychaetes have been shown to rapidly interconvert amino acids in the FAA pool (Wong and Stephens, 1970), the character of the radioactivity remains uncertain. The use of free amino acids in the volume regulation and osmoregulation of lamellibranch mollusks is well documented (Virkar and Webb, 1970; Gilles, 1972; Allen, 1961). It is interesting to note that the release of radioactivity took place quite rapidly for *Rangia* subjected to slight reductions in salinity (6, 10 and

16‰), while those exposed to extreme dilution (2 and 4‰ S) had not released a significant amount until the 16 hour interval. To obtain better quantitative data relative to time, and to avoid the possibility of uptake of released material, such research should utilize a flow-through system.

From the results presented, it appears that the incorporation of the accumulated glycine by *Rangia* in response to salinity dilution follows much the same pattern as that of nereid polychaetes (Stephens, 1964). At all time intervals tested the per cent of ETOH insoluble C^{14}-activity (relative to the total activity) increased as the salinity decreased. In addition, the rate of incorporation, during at least the first 24 hours, increased as salinity decreased (Fig. 6). Further evidence of rapid incorporation of accumulated glycine by animals at low salinity is the high level of labeled protein in clams 3 hours after exposure (Fig. 8). It seems that nucleic acid synthesis was still the major activity of higher salinity *Rangia* at the 3 hour interval, but they had reached the same level of protein synthesis by the 99 hour analysis (Fig. 9). This type of response by organisms acclimated to low salinities would be a functional advantage, allowing them to rapidly transfer the glycine from the osmotically active FAA pool and yet utilize the compounds in synthetic pathways.

Although *Rangia* at salinities of 5‰ or less transferred the glycine from the FAA pool to protein and other large compounds at a rapid rate, the turnover rate of the total radioactivity was relatively slow. The total C^{14}-activity associated with the clams decreased over the 75 hour period at rates which increased as salinity increased (Table III). Apparently, *Rangia* maintained at salinities of 10‰ or greater utilize the accumulated glycine at a higher rate by conversion to secretory, excretory and respiratory products. It might be assumed that differences in the respiratory rate of the clams at various salinities would account for the variations in glycine utilization. However, a considerable amount of data (Bedford and Anderson, in preparation) demonstrate that the rate of oxygen consumption by *Rangia* at salinities between 1 and 32‰ is quite uniform after acclimation.

Finally, the aspect of nutrition, which has been discussed at length in several studies (Stephens, 1963; Stephens, 1967; Stephens, 1968; Johannes, Coward and Webb, 1969; Stephens, 1972) should be examined. At a glycine concentration of 4.6×10^{-5} moles/liter (Kt) the rate of uptake by whole animals was 101 μg of glycine/g/hr at 10‰. Since 2.2 mg of glycine requires roughly 1 ml of oxygen for complete oxidation (Stephens and Virkar, 1966), and since at 10‰ and 22° C the clams require 1.2 ml O_2/g ash-free dry weight/hr, the accululated glycine represents approximately 3.8% of the total oxygen requirement of *Rangia*. At 2‰ S the glycine taken up was equivalent to only 1.5% of the energy necessary for respiration. The concentration of 4.6×10^{-5} is perhaps high for a single amino acid even in an estuary, but Stephens (1963) has reported values between 6×10^{-5} and 1×10^{-4} moles/liter for total free amino acids in interstitial water of mud flats. Even if one assumes higher levels of glycine are present in the habitat of *Rangia* or that they are simultaneously removing other amino acids, it would appear that these mollusks can not support as large a portion of their metabolic energy needs as other invertebrates investigated. Ecological studies (Darnell, 1958) have shown that in addition to feeding on phytoplankton, *Rangia* utilize detritus, which is generally in abundance in the upper reaches of the estuary.

We wish to acknowledge our laboratory assistant, Miss Susan Baldwin, for the many helpful duties which she performed. We are also grateful for the funds furnished by the U. S. Army Corps of Engineers under contract, DAW 39–71-C-007, which provided supplies and personnel.

Summary

1. The effect of salinity on the uptake of dissolved C^{14}-labeled glycine by *Rangia cuneata* and isolated gill tissue was studied.

2. The percentage water in *Rangia* tissue ranged from 81% at 1‰ S to 73% at 32‰ S, while the ash-free dry weights at these same salinities were 2.5% and 7.6%, respectively. The per cent water and per cent ash values were linear throughout this salinity range.

3. The uptake of glycine was relatively uniform at salinities between 6 and 32‰ for whole animals and between 10 and 25‰ for gill tissue.

4. At salinities below 6‰ uptake decreased sharply and in a linear fashion to a low at 1‰ S. Suppression of uptake was also exhibited when gill tissue was exposed for 90 minutes at 32‰ S.

5. With increasing salinity the percentage utilization of accumulated glycine increased, but the rate of glycine incorporation into the alcohol insoluble fraction decreased.

6. Removal of glycine by gill tissue from salinities of 10 to 25‰ occurred at a relatively linear rate up to 90 minutes and the levels obtained were considerably higher than those for whole animals when compared on a basis of cpm per mg ash-free dry weight.

7. In a constantly flowing system *Rangia* at 15‰ S removed 0.16 μ moles of glycine/g/hour for a period of 11 hours, when supplied with 5.2 μ moles of glycine per liter.

8. The significance of the relationship between salinity and glycine uptake, and possible explanations for this relationship are discussed.

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REGENERATION IN THE AFRICAN LUNGFISH, *PROTOPTERUS*. III. REGENERATION DURING FASTING AND ESTIVATION

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Problems related to growth are among the most interesting in contemporary biology. Of unique interest are those special systems which can be isolated from, or juxtaposed with, normal somatic growth and maintenance. Limb regeneration in lower vertebrates is such a system, providing the opportunity to observe the reorganization of a replacement structure whose growth rate and degree of differentiation temporarily differ from those of the body as a whole.

The African lungfish, *Protopterus,* is especially suited to such experiments because growth and regression are already a part of its annual life cycle. Found in many of the river systems and lakes of tropical Africa, lungfish lead an aquatic life in flooded swamplands during the rainy season but survive the dry season in a state of metabolic torpor known as estivation. For five to seven months they remain buried in drying earth, unable to eat or excrete, protected from desiccation by a cocoon of dried mucus, and released only when the rains come again.

Estivation effects significant loss of body weight and shrinkage of trunk and appendages. Yet when not estivating, these fish are capable of prodigious growth and of full limb and tail regeneration (Conant, 1970). The experiments reported here counterpose these capabilities of regression and growth. Estivation has been induced in regenerating animals in order to investigate what influence, if any, the depressive effects of estivation may have on regeneration. Initial studies (Conant, 1970) have shown that very little regeneration occurred if estivation was induced prior to blastema formation. This paper will report on the effects of estivation begun during the growth phase of regeneration and will compare those results with observations on regeneration during periods of starvation.

Material and Methods

The fish used were 9 individuals of *Protopterus annectens* Owen and 2 of *P. aethiopicus* Heckel, ranging in weight from 51 to 382 grams after amputation. Each was maintained in a separate aquarium at 21° C ± 3° C and fed beef strips twice a week. Portions of pectoral and pelvic limbs and up to 8 cm of tail were removed during anesthesia with 1:900 ethyl m-aminobenzoate methane-sulfonate: water, and regeneration was followed for up to 12 weeks in the water environment before estivation was induced. Over the course of three years, 15 experiments were completed in which the fish were induced to estivate while in the process of limb and tail regeneration. They were kept in the mud 3 months or more, a time period adequate for complete regeneration in the non-estivating, well-fed controls. The stage of the regenerating structures at the beginning of estivation varied with

different fish from 7 days to more than 2 months after amputation. At the end of an average time of 129 days (92–147 days), the fish were excavated, weighed and measured, and returned to water where growth resumed under conditions of adequate food and normal day-night illumination. Two additional fish died in my attempt to force estivation, and two died in the mud.

Estivation was induced by introducing the fish into a deep bucket of water and mud and gradually withdrawing the water over a period of 6–8 days. Grass seed

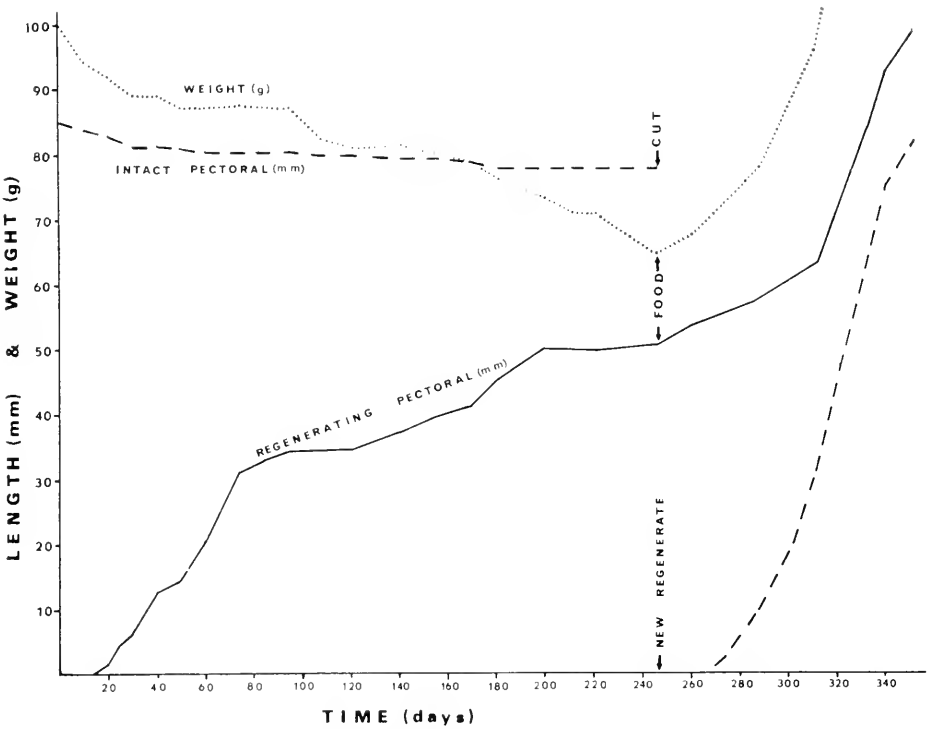

FIGURE 1. Changes in limb length (in mm) and body weight (in g) of fish #18, *Protopterus aethiopicus*, fasted for 246 days and then fed. Growth of regenerating pectoral limb measured under conditions of both fasting and feeding. Shortening of intact contralateral pectoral limb measured during fasting; then that limb was removed, feeding was resumed, and measurements were taken of the new regenerate.

planted near the respiratory channel enhanced dehydration and helped prevent localized cracking as the mud dried.

Three fish were starved for 212, 246, and 308 days, respectively, following the amputation of one pectoral limb 3 mm from the body. Regular records were kept of weight, snout-tail length, and the length of the two pectoral limbs. In the case of fish #12, the pectoral limb left intact had itself regenerated 8 months prior to the beginning of the experiment. The control limb on the other two fish (#18 and #28) had not regenerated in at least the three previous years. At the end of the fasting period, the control limb was removed and feeding resumed.

Fish #28 developed fungus infection and died, but growth was followed in the other two.

Observations

Regeneration during fasting

A regenerating appendage in a well-fed lungfish kept in water passes through a period of wound healing (1–2 days), a latent period of 2–3 weeks, and then periods of first accelerating and then decreasing growth, with replacement complete after 3–4 months (Conant, 1970). The regenerating pectoral limb in the three fasting fish showed healing and latent periods roughly similar to those of fish that were being fed, but elongation during the growth phase was considerably attenuated.

Figure 1 summarizes changes in limb length and body weight observed in fish #18 during and after a starvation period of 246 days. Data from the other two fasted fish closely resembled these data from #18. At the end of 3 months, by

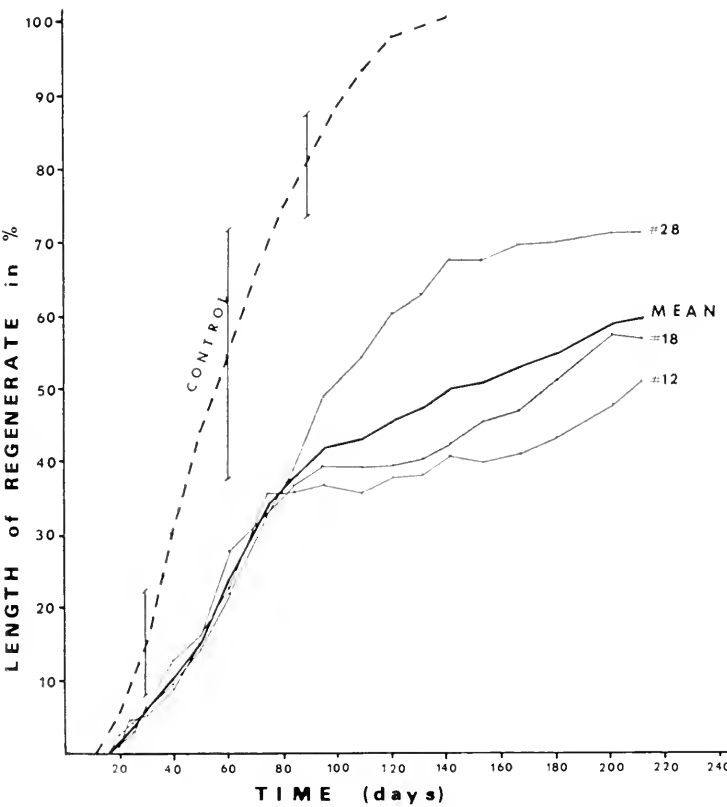

Figure 2. Growth curves of the regenerating pectoral limb of three fasting fish and their mean, compared to a curve representing the mean pectoral regeneration in 12 control fish kept in water with adequate food. All curves are expressed in terms of per cent replacement of the amputated segment over time. Vertical lines intersecting the control curve at days 30, 60, and 90 represent standard error.

which time a pectoral regenerate would have been totally restored under well-fed circumstances, the regenerating pectoral of the fasting fish attaind a length of 30–35 mm, barely a third of the 87 mm removed. By 8 months, the new limb measured approximately 50 mm, or about 60% of its original length. Upon resumption of feeding, substantial weight gain accompanied accelerating growth in the original regenerate, and 3½ months later the limb was 100% restored.

Figure 2 depicts the growth curves of regenerating pectoral limbs in all three fasting fish compared to the mean rate of pectoral regeneration in 12 control fish kept in water and fed regularly. Statistical comparisons of these growth rates were made by two methods. A comparison of the extent of limb replacement at 10-day time intervals showed significant differences between the fasting and control fish with probabilities of identity being less than 0.05 from day 40 onward, less than

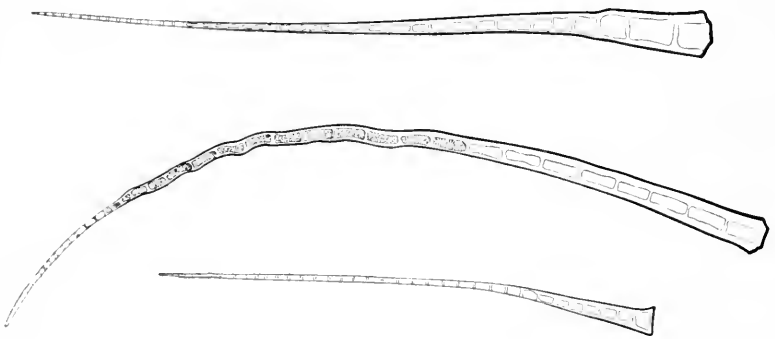

FIGURE 3. Tracing of three pectoral limbs taken from fish #28 before and after 212 days' fast. The uppermost limb is the left pectoral, recently regenerated, removed on day 1 of the experiment. The middle limb is the right pectoral, removed on day 212, terminating fast. Note its deformation in the course of atrophy, the axial segments most involved having been stippled. The lowermost limb is the left pectoral regenerate, grown during the fasting period and removed on day 215 when the animal died of fungus infection. Note the straight axis of both the new regenerate and the limb removed as the experiment began. Note also that the terminal segments are less distinct in the lower two limbs.

0.001 from day 70 onward. The second approach involved examination of the slope of the growth curves. Using the arc tangent of the increment of growth added during each successive 10-day time period, the curves were expressed in degrees of slope. Comparison of the slope angles for the first 130 days shows significant differences between the experimental and the control rates with P values of 0.05 or less from day 30 to day 50 and from day 90 to day 120.

The loss of body mass during starvation was manifest in several ways. Weights decreased steadily: the three fish lost 40%, 36%, and 26% of their initial weights in the 7–10 months' fast, the fish showing the greatest loss (fish #28) dying of fungus infection shortly after feeding was resumed. At the end of these prolonged fasts, the atrophy of the head musculature was clearly visible in fish #18 and #28, and the skin seemed to "fit loosely." Axial shrinkage amounted to 8% or less of the original body length; girth measurements might have been more revealing.

The intact pectoral limb also shortened during the fasting period. In fish #18 and #28, this "control" limb was 7 mm shorter after 7–8 months, a loss of

about 10%. In fish #12, the intact limb had itself recently regenerated; it shortened during fasting by 19 mm, about 20% of its length at the beginning of the experiment. In each case, most of the loss in length occurred in the early weeks, and after several months the straight axis of the shortened limb was deformed by wavy irregularities (Fig. 3).

In order to investigate whether or not the prolonged fast exerted a lasting depressant effect on growth, the intact pectoral limb was removed at the end of the fasting period and the regrowth of its replacement was observed under conditions of feeding (Fig. 1). Statistical comparisons of both the extent of growth and the degree of slope of the new post-fast regenerate *vs.* the control curve (Fig. 2) showed no significant differences.

TABLE I

Summary of regeneration/estivation results, ranked in order of per cent replacement at the start of estivation

Fish # (weight)	Induced to estivate on day # after amputation	Length of regenerate at beginning of estivation in % replacement:			Mean %	Mean in mm	Total days in estiv. (weight at end)	Length of regenerate at end of estivation in % replacement:			Mean %	Mean in mm
		Pectoral	Pelvic	Tail				Pectoral	Pelvic	Tail		
#28,a (202 g)	7	0	0	0	—	—	129 (150 g)	4.7	2.8	0	*2.5*	*1.5*
#29,a (132 g)	7	0	0	0	—	—	129 (100 g)	3.6	5.7	0	*3.1*	*1.3*
#14 (286 g)	19	2	0	0	*0.7*	*0.5*	146 (264 g)	5.4	12.2	0	*5.9*	*3.3*
#07 (110 g)	18	1.7	3.3	0	*1.7*	*0.8*	147 (100 g)	6.9	11.1	12.3	*10.1*	*5.7*
#12 (382 g)	21	1	2.8	1.1	*2.6*	*1.3*	136 (358 g)	23.3	17.1	32.6	*24.3*	*15.8*
#27,a (203 g)	18	5.7	12.5	6.3	*8.2*	*3.0*	92 (194 g)	11.4	32.5	18.8	*20.9*	*7.3*
#18 (100 g)	18	12.5	16.7	1.1	*10.1*	*3.7*	92 (98 g)	17.5	22.2	20.7	*20.1*	*7.5*
#28,b (169 g)	34	19.0	18.2	3.9	*13.7*	*8.2*	140 (135 g)	22.2	20.9	9.2	*17.4*	*10.5*
#29,b (102 g)	29	19.5	15.3	10.0	*14.9*	*5.8*	140 (93 g)	21.9	27.8	40.0	*29.9*	*11.7*
#21,b (235 g)	39	22.5	33.3	7.1	*20.9*	*12.7*	136 (180 g)	20.0	38.1	21.2	*26.4*	*16.7*
#21,a (177 g)	42	40.5	40.0	10.0	*30.2*	*12.3*	130 (158 g)	29.9	30.0	16.7	*25.5*	*10.3*
#16 (51 g)	72	{38.5 {28.5	36.6	33.3	*34.2*	*13.2*	129 (49 g)	{35.9 {14.3	36.6	44.4	*32.8*	*14.0*
#22 (130 g)	88	40.0	72.4	21.3	*44.6*	*26.3*	129 (124 g)	30.0	55.2	24.0	*36.4*	*19.3*
#27,b (166 g)	87	90.0	60.0	37.7	*62.6*	*37.7*	133 (138 g)	73.3	40.0	35.0	*49.4*	*30.3*
#19 (143 g)	71	96.6	79.4	47.4	*74.5*	*42.7*	133 (130 g)	75.9	48.9	41.0	*55.3*	*32.5*

Regeneration during estivation

The data from the regeneration/estivation experiments are presented in Table I. The data are ranked in terms of the average amount of regeneration in the three appendages compared to the average amount removed ("% regrowth") at the time estivation was induced. The mean length of the regenerates in millimeters is also recorded, as is the length of the estivating period and the weight before and after estivation. It should be remembered that a well-fed control fish in water would show virtually 100% replacement of parts removed during the 3–5 months time period of the estivation experiments.

These data indicate that estivation sharply curtailed but did not stop regeneration when the fish appendages were in the latent or early growth phase of regeneration at the time of entry into the mud. Estivation, however, not only inhibited further growth of longer regenerates but actually effected a loss in length.

Fish induced to estivate at the end of week one of the latent phase showed growth of only a few millimeters (2–3%) at the end of 129 days in the mud, a point which

would have been reached within 14 days had the fish remained in water. Regenerates which were somewhat farther along at the time of estivation increased an additional 5–15% in length during the 3–5 months in the mud, although there was such individual variability that no direct linear relationship was found between length before and after estivation. When the regenerating structures averaged more than about 1/3 replacement at the outset, however, estivation exerted still greater limitation such that, in a series of increasingly mature regenerates, first the pectoral, then the pelvic, and finally the tail regenerates shortened in length while in the mud. All these regenerates, no matter what their stage and whether

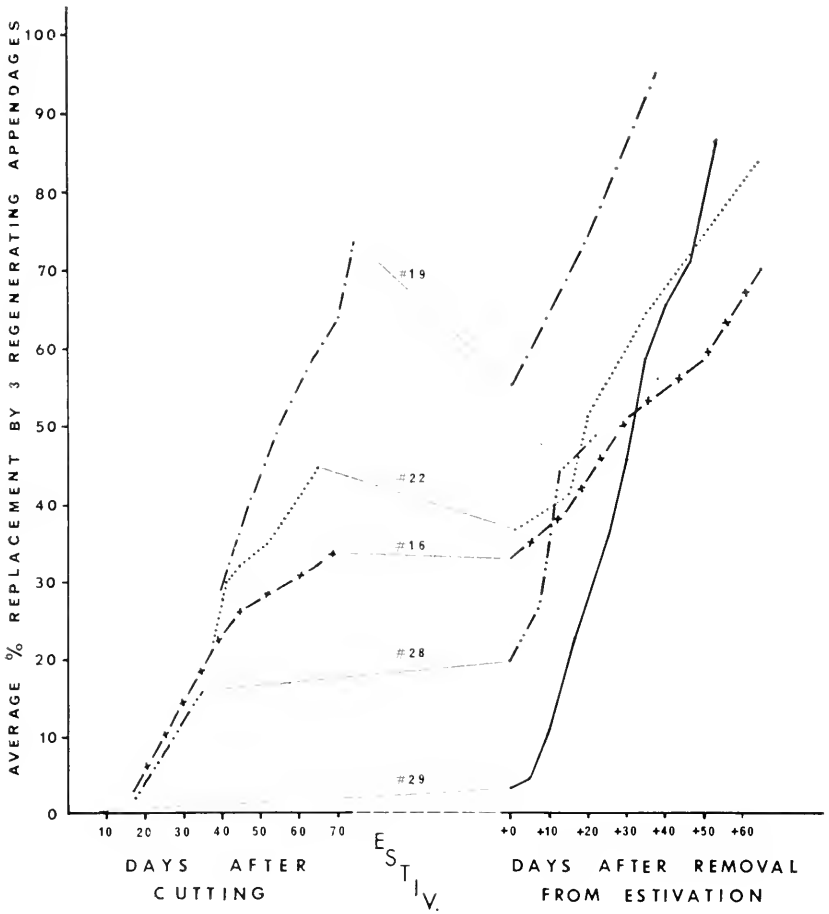

FIGURE 4. Representative growth curves of five lungfish before and after estivation. The curves at the left are segments of the growth curves following amputation, drawn to show successively longer regenerates. The dashed lines in the middle represent 3–5 months in estivation, not drawn to scale. The curves at the right represent growth data after the fish were returned again to water. Note the limited growth during estivation in the earlier stages of regeneration, the loss of length during estivation by limbs in the later stages of regeneration, and the resumption of growth after estivation was terminated.

they had grown or shrunk during estivation, resumed growth when the fish were returned to water and feeding was reëstablished (Fig. 4).

There were characteristic differences in growth patterns among the two limbs and tail. Figure 5 expresses growth before and after estivation in absolute length (mm) in bar-graph form, ranking each experiment in terms of average length of the three regenerates at the time of induced estivation. This graph, and the

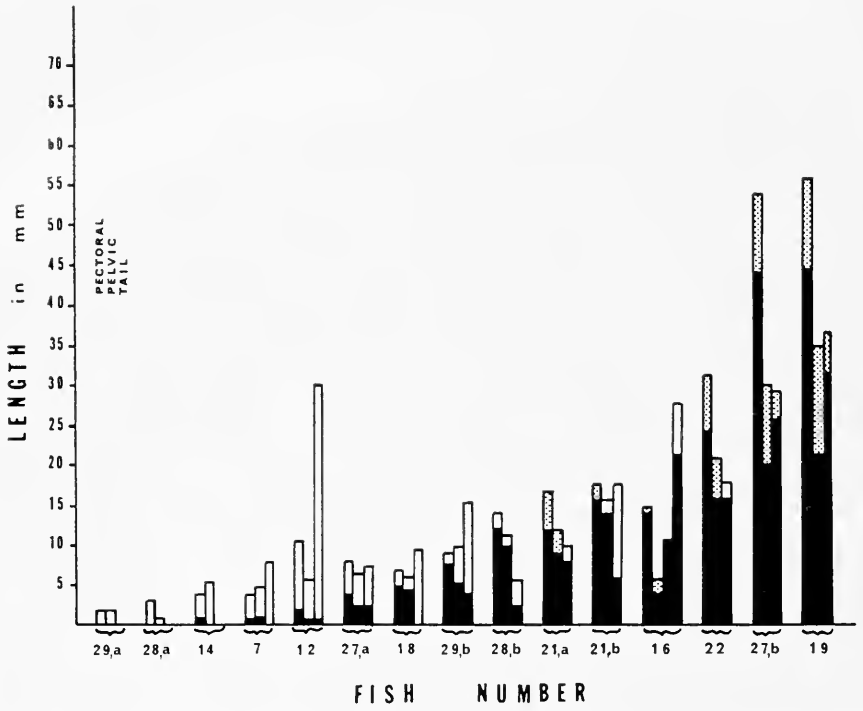

FIGURE 5. Regeneration/estivation data from 15 experiments, using measurements in absolute length (mm) and ranked in order of increasing length at the time of estivation. Each trio of bars represents measurements of, from left to right, the pectoral limb, the pelvic limb, and the tail. Data from fish #16 includes a second pectoral limb at the left. Dark area represents extent of growth at the beginning of estivation; white area represents increment of additional growth during estivation; stippled area represents the extent of shrinkage during estivation, *i.e.*, the highest point of the stippled bar denotes the length when estivation was induced, the stippled area indicates decrement lost during estivation, and the height of the blackened section defines the length of the appendage when estivation was terminated.

comparable information in Table I, shows that the three appendages responded somewhat differently to amputation and estivation. Typically, the pectoral limb breaks the lag phase first, grows the fastest, and completes regeneration before the others, consistent with previous observations that rapid growth compensates extensive limb amputations (Conant, 1970). In absolute measurement, the pelvic limb responds to amputation a little more slowly, and, until later in the growth process, the tail grows at the slowest rate of all. Hence, in Figure 5, the blackened

bars representing the extent of regeneration when estivation was induced usually show that the greatest length was attained by the pectoral limb.

This characteristic "lead" of the pectoral regenerate in the water environment was not maintained during estivation. In Figure 5, the white bars represent growth during estivation. In the majority of cases, the incremental growth of the pectoral limb while in the mud was less than that of the pelvic limb or tail, and of all structures, the tail seemed the least affected by estivation. The regenerating tail, for example, frequently added length while the limbs showed gain or even loss, and in one case (fish #12) where cocoon formation was incomplete, the tail regenerate added nearly 3 cm in 136 days in the mud. The pectoral limb, on the

TABLE II

Regeneration during short-term estivation

Fish #:	Fish #21 (1971	Fish #26	Fish #21 (1972)	Fish #22	Fish #12
Pre-estivation:					
% regrowth, pectoral	16.7%	41.3%	59.5%	43.1%	86.7%
% regrowth, pectoral				53.8%	54.5%
% regrowth, pelvic	16.7%	27.3%	7.4%	36.7%	41.2%
% regrowth, pelvic				32.4%	
% regrowth, tail	2.5%	34.3%	37.7%		64.5%
Mean of all regenerates in % regrowth	*11.9%*	*34.3%*	*34.9%*	*41.5%*	*61.7%*
Mean of all regenerates in mm	*4.7 mm*	*21.7 mm*	*20.3 mm*	*19.8 mm*	*41.3 mm*
Days in estivation:	21	21	27	27	27
Condition of cocoon:	unformed	unformed	dry	dry	dry
Post-estivation:					
% regrowth, pectoral	23.8%	43.8%	43.2%	34.5%	70.7%
% regrowth, pectoral				41.0%	48.1%
% regrowth, pelvic	30.0%	30.9%	18.5%	33.3%	38.2%
% regrowth, pelvic				26.0%	
% regrowth, tail	5.0%	36.0%	41.3%		77.8%
Mean of all regenerates in % regrowth	*19.6%*	*36.9%*	*34.3%*	*33.7%*	*58.7%*
Mean of all regenerates in mm	*7.7 mm*	*23.3 mm*	*18.0 mm*	*16.5 mm*	*37.3 mm*

other hand, appeared to be the most labile, the first to start growth, but the first to show inhibition. In Figure 5, the stippled sections of the bars represent the extent of loss of length during estivation, and it can be seen that the pectoral limb was the first, the tail last, to undergo shrinkage.

One experiment tested the effects of estivation on an induced pectoral branch. Accessory structures can be elicited in lungfish by removing from the limb a wedge of tissue which includes some axial endoskeleton (Conant, 1972). Fish #14 had been operated on in this fashion, and by the time of estivation (19 days), the cut had filled in and a 1 mm projection had formed in its place. After 146 days in the mud, the projection had neither grown nor shrunk, but it sprouted rapidly once the fish had been returned to water and to feeding. This was a case in which a new structure was held in abeyance by estivation but was not inhibited when conditions improved.

Table II lists the data from five fish (17 appendages, total) induced to estivate when the appendages were in varying states of regeneration and which were excavated after 3–4 weeks instead of 3–5 months. Because of differences in the number of appendages cut, the format of Table II is reversed from that used in Table I. It can be seen that fish in earlier stages of regeneration and those with less complete cocoon formation were less affected by estivation than those induced to estivate at a later stage or whose cocoons were fully formed.

Discussion

Many lower vertebrates can grow and/or regenerate despite adverse conditions. As early as 1909 Ellis showed that fasting tadpoles could regenerate at the same rate and to the same degree as well-fed counterparts, and as recently as 1970 Hui and Smith reported that *Ambystoma* larvae unfed for 10 weeks regenerated hind limbs fully as rapidly as controls despite a 50% loss of body weight.

Lungfish would seem to be exceptions to this general rule. They routinely encounter environmental adversities, and in the course of evolution have developed numerous adaptations to minimize the effects. Sluggish as well as poikilothermic, they are capable of prolonged fasting and may experience 6 months without food in estivation as an annual event. Smith (1930, 1935a), in work later expanded by Janssens (1964), examined the metabolism of two specimens of *P. aethiopicus* starved until death and found several metabolic adjustments. Levels of body carbohydrate and enzyme titers were found to differ significantly in control, starving, and estivating animals. Smith further reported a persistent decrease in O_2 consumption roughly following a logarithmic curve. Using his figures, the fish described here would be consuming only about 20% as much O_2 after 7–8 months' fast as normally. Since protein degradation, an inefficient energy source, accounts for 50% or more of the metabolic energy, the fasting or estivating lungfish obtains only about 1.6 cal for every gram of body weight lost (Smith, 1930). At the end of 250 days, the two fish in Smith's experiments had lost approximately 9% and 18% of their original body weight, smaller figures than those reported here in my specimens. His experiments were run at 20° C. One final observation supporting the notion of the conservation of body energy during fasting concerns the animal's activity. Normal well-fed lungfish show limited nocturnal movement in the form of occasional episodes of swimming alternating with quiet periods. These movements can be detected by electrodes in the water and noted on a chart recorder. After 2 months of fasting, however, all such movements had been abolished, and, except for respiration, the fish remained apparently motionless for days at a time.

In the light of this inactivity during fasting and the enforced immobility of estivation, one wonders if "disuse atrophy" might play a role in the loss of body mass and in the retardation of regeneration found under both conditions. Inasmuch as structural proteins are used as an energy source, it would be difficult to differentiate what proportion of limb or tail regression could be attributed to metabolic degradation to counteract caloric deficiency and what proportion to simple disuse. Denervation experiments in combination with fasting or estivation might provide some useful clues.

In any case, considering these multiple responses to starvation, it may not be

surprising that the lungfish limb regenerated at a significantly lower rate when food was withheld. When feeding was resumed, the growth rate of this regenerating limb increased, consistent with Smith's observation (1935b) that feeding-metabolism levels of O_2 consumption were rapidly reinstated once the fast was broken. Unlike Buchanan's report on anurans (1938), there was no evidence of compensatory increase in growth rate after the adverse conditions were relieved. This might be explained by Smith's further observation (1935b) that a maximum level of O_2 consumption was reached in lungfish beyond which ever-increasing amounts of food had no effect.

The regression of the intact pectoral limb during fasting took place at the same time that the new regenerate had begun to grow. Over the total period without food, the intact control limbs of fish #18 and #28 lost 10.6% and 7.5% of their original length and the intact but recently regenerated control limb of fish #12 lost 20% in length. This atrophy of the unamputated limb paralleled shrinkage of the axial snout-tail length by about 8% and losses of body weight in the 3 fish of 26–40% such that the regeneration of the amputated pectoral, albeit limited, could be seen as net growth taking place despite the overall tendency towards tissue reduction. The same can be said for those tissues which added mass during estivation despite a general loss of body mass in the time in the mud.

Estivation imposes even greater stringencies on the animal than fasting. Although cessation of feeding is an aspect of estivation, additional elements are involved such as immobility, cocoon formation, reduced heart rate and O_2 consumption, numerous biochemical and enzymatic adjustments (Smith, 1930; Janssens, 1964), virtual cessation of erythrocytopoiesis (Jordan and Speidel, 1931), changes in endocrine activity (Godet, 1959, 1962; Godet, Michel, and Dupé, 1964; Leloup, 1958), and, according to Swan, Jenkins and Knox (1969), the possible production of a neurohumoral antimetabolite.

Reminiscent of the gross results of fasting, these internal changes of the lungfish in the mud are reflected in gross changes, most clearly in overall weight loss and in modification of body form. Estivation of only a few months in drying conditions results in the loss of up to 27% of the body weight (Smith, 1930; Lüling, 1961; Table I here). Further, during estivation, the tail tip becomes noticeably blunter as the axis shortens and all limbs lose some of their length. Blanc, d'Aubenton and Plessis (1956) reported that after 28 weeks of estivation, one fish had lost 57 g out of 345 g originally, was 35 mm shorter than the original 400 mm snout-tail length, had pectoral limbs only two-thirds their initial length, and pelvic limbs shortened by over 10%. In field observations in the Congo, Poll (1938) observed that estivating individuals of *Protopterus* excavated near the Lualaba had limbs reduced to as little as a third of the length of those belonging to active fish taken from open water. My own measurements of non-regenerating limbs show reductions in length of 6–11% in the pelvic and 19–35% in the pectoral limbs resulting from estivation.

From the experiments reported here, appendages which had regenerated about two-fifths or more of the amputated portion responded to estivation like whole limbs in that their lengths were decreased rather than increased during estivation. In light of the greater vulnerability of the recently regenerated limb to reduction during fasting referred to earlier, it would be interesting to know whether limb

shortening during estivation is more pronounced in regenerates than in those limbs which had never regenerated. Not having reared any of my experimental fish from larvae, I do not know their full history and cannot make this comparison.

In these experiments, early stages of regeneration were observed under four different conditions, and the resumption of growth by older regenerates was observed under two. New regenerates were seen developing (1.) under control conditions of water and food, (2.) during fasting, (3.) after the fast had been broken, and (4.) after release from estivation. Regeneration in later stages of growth was followed after the termination of fasting and estivation.

Comparison of the growth curves of the early stages of new regenerates reveals basic similarities in lag phase, time of blastema formation, and early growth. With food, whether after fasting or after estivation, development of the new structure virtually paralleled that of the controls; during fasting, development was slower than in the controls but much closer to the control rate for the first 11 weeks than later (Fig. 2). This suggests that the initiation of growth and its early stages are not profoundly affected by existing or recently terminated adverse conditions.

Older regenerates, on the other hand, showed a two month lag in the resumption of growth after 8–10 months' fast, a condition in marked contrast with the normal growth of the newly-amputated regenerate. Likewise, there is some indication in Figure 4 that the older regenerates did not resume the normal growth rate as speedily after estivation as did those whose growth had been interrupted in earlier stages.

In short, the state of the regenerate, so important in the growth/regression response to estivation, appears to affect the recovery process as well. Comparative studies of vascularization, mitotic index, enzyme levels and innervation might help define the degree of this state-dependency.

It is tempting to consider estivation in relation to hibernation, estivation resembling hibernation in metabolic depression but not in response to lowered temperatures. Can experiments in estivation help separate out the simpler effects of temperature from those of metabolic austerity and immobility? Invertebrate regeneration during hibernation has been studied with segmented worms, although they defy generalization in that some groups can regenerate during diapause while others cannot (Saussay, 1966a, 1966b). Among higher vertebrates, Lyman and others have examined changes in cold-acclimated and hibernating rodents. During hibernation, cell division (Mayer and Bernick, 1958) and DNA synthesis (Manasek, Adelstein and Lyman, 1965) were sharply curtailed, hematopoietic organs were inactive (Lyman, Weiss, O'Brien and Barbeau, 1957), and even neoplastic transplants in the hamster cheek pouch did not grow (Lyman and Fawcett, 1954). Among Chiroptera, there have been observations on changes in osteocytic activity (Whalen, Krook and Nunez, 1972) and on the lack of healing during hibernation (R. J. Goss, Department of Biology, Brown University, personal communication).

These experiments with *Protopterus* suggest an equally profound depressant effect under estivating conditions, this despite temperatures of about 23° C in the laboratory and 10° C or more higher in field measurements of dry mud in East Africa (Swan and Hall, 1966). The only growth noted during estivation was of appendages in early stages of regeneration and even that was sharply curtailed.

The process of estivation involves several stages: progressive penetration to the

deeper layers of the mud and the consequent formation of the respiratory channel, assumption of the final estivating position, production of heavy mucus, and the drying of mucus in final cocoon formation. The dryness of the environment determines in large measure the pace of these steps, and in some natural settings such as parts of the Congo, at least one species, *P. dolloi*, remains active in moist mud all during the dry season with no cocoon formation whatsoever (Brien, 1958). Observations based on the mud and containers used in this laboratory established that by 3 weeks the animal had taken up the final deep position and was covered with heavy mucus and that by 4 weeks with daily deep withdrawal of water, the cocoon was complete.

It is important to know what fraction of the growth recorded after 3–5 months in the mud had in fact taken place in the first 3–4 weeks. Accordingly, 5 fish with appendages in varying stages of regeneration were induced to estivate as usual but were retrieved after 3–4 weeks (Table II). The results mirror those seen after extended estivation. The fish that showed the greatest growth in the series was the one whose regenerating appendages were in their earliest stages, averaging 11.9% or 4.7 mm, and whose cocoon was not yet fully formed. This animal showed an average additional increment of regeneration during the 3 weeks of 3 mm, or 7.7%. Another fish (#26) whose regeneration was farther along at the time of estivation (34.3%, or 21.7 mm average) and who was excavated at 3 weeks before the cocoon was complete, showed additional growth of but 1.6 mm on the average (2.6%). Still less growth, or even shrinkage, was found in fish whose regenerates were still more extensive and/or whose dry cocoon had fully formed.

When do the depressant effects of estivation become operative? There are several clues to suggest that the relevant metabolic and behavioral adaptations take effect almost immediately. The regeneration figures themselves are the first clue (Tables I and II; Fig. 1). If 3 weeks in the mud preceding cocoon formation were as metabolically "normal" as 3 weeks of fasting in water, then one might expect growth figures to be comparable. Yet the increments added during the process of estivation are even less than those added during fasting, and indeed may even be decrements, suggesting that the conservative influences of even pending estivation are felt from the beginning. Elements of lungfish behavior likewise support this view. Within hours of being introduced into the mud, the fish will assume the tail-over-eyes estivating position even though this immobility is periodically interrupted by episodes of burrowing and position change. The respiratory behavior also changes in most fish within the first day, from the semi-regular lung-filling associated with the aquatic environment to clusters of breaths separated by periods of 20–50 minutes below the surface.

Nevertheless, the growth in the first few weeks of the short-term experiments does constitute a significant fraction of changes noted after longer estivation periods in other fish. Indeed, it may be that some of the growth of the pre-cocoon estivating stages would later have been nullified by limb shortening during extended periods in the final estivating state. For this reason, the results support the notion that maximum metabolic depression accompanies full cocoon development.

In conclusion, the dual capacity of the lungfish both to resorb and regenerate, both to estivate and grow, provides the opportunity to study growth under special conditions. It is just one more example of the many interesting features found in the Dipnoi.

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Summary

1. Limb and tail regeneration of *Protopterus annectens* and *P. aethiopicus* was observed under conditions of fasting and induced estivation.

2. Three fish were fasted for 212, 246, and 308 days, respectively. During this period, weight losses of 26–40% were recorded. A regenerating pectoral limb on each fish showed healing and latent phases comparable to well-fed controls, but growth itself was considerably slower and averaged only 50–60% replacement after several hundred days. The contralateral intact pectoral shrunk 7–20% during the same period and axial length shortened by about 8%. After feeding resumed, weight was rapidly regained, the old regenerating limb increased its growth rate, and a new pectoral regenerate grew normally.

3. Fifteen fish were induced to estivate after they had regenerated varying amounts of limb and tail tissue. Estivation sharply limited but did not halt further growth if the regenerate was in the latent or early growth stages, but it did stop growth or even cause shrinkage of more mature regenerates. The pectoral regenerate, first to grow after amputation, was also the first to be inhibited by estivation; the tail was the last to be affected.

4. Short-term estivation experiments revealed that the bulk of the growth took place in the 3–4 weeks preceding dry cocoon formation. It is concluded that deep estivation has an inhibitory effect on regeneration.

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CORRELATION OF *IN SITU* FLUORESCENCE AND BIOLUMINESCENCE WITH BIOTA IN THE NEW YORK BIGHT

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Two aspects of optical oceanography appear to offer the possibility for *in situ* optical observations that may be correlated with concurrently existing specific biota. One technique is *in situ* measurement of stimulated bioluminescence, and the other is *in situ* measurement of visible fluorescence from ultraviolet excitation. These methods are of great interest because their use permits detailed vertical profiling with immediate information access. Synoptic information may be acquired by deploying unattended instrumentation at a number of locations.

Modern *in situ* photometric measurements of bioluminescence began with the use of a bathyphotometer by Clarke and Wertheim (1956), Boden and Kampa (1957), and Hardy and Kay (1964), who describe observations in Lock Fyne in 1957. The bathyphotometer designed by Clarke and Wertheim (1956) was used by Clarke and Breslau (1959, 1960) and Clarke and Backus (1956) to show that large scale bioluminescence exists at all depths of the sea and is an important factor in the ecology of marine life. Clarke and Kelly (1964) were able to estimate the distance over which one subsurface organism is able to communicate with another by measuring the intensity of the bioluminescent flashing and the transparency of the Indian Ocean. Clarke and Kelly (1965) also developed an *in situ* photometer usable day and night in contrast to previous units that could be used at night or only at great depths. This new photometer was light-tight and permitted water to be pumped through and to be subjected to controlled physical agitation to produce bioluminescence. Egan (1969) described an *in situ* photometer used aboard the submersible *Ben Franklin* during its 30-day drift mission, that was capable not only of measuring bioluminescence, but fluorescence as well.

Boden, Kampa and Snodgrass (1960) describe instrumentation similar to that of Clarke and Wertheim (1956) that was used by Boden and Kampa (1957). They found layers of strong luminescence associated with the sonic scattering layer.

These instruments used for bioluminescence measurements differ in detail in the electrical and optical characteristics. The device described by Boden, Kampa and Snodgrass (1960) was carefully calibrated and used a 931-A (response between 0.36 and 0.54 μm) photomultiplier, and 5 interference filters with peaks presumably at 434, 460, 491, and 514 nm. Switching time between filters was 10 s, and the system time of response to bioluminescent flashes was essentially limited by the recorder to about 50 cycles per minute (Kampa and Boden 1956). There was a serious question as to whether the measurement was of spontaneous bioluminescence or stimulated bioluminescence caused by motion of the photometer

in the water. In answer to this criticism, Boden, Kampa and Snodgrass (1965) developed a coincidence sensing technique using two photometers viewing a common volume included in the intersection of two 10 degree cones viewed by the photometers at a distance of one meter. The sensor was a type 6472 photomultiplier (response between 0.36 and 0.54 μm). The system time of response to bioluminescent flashes is about 20 milliseconds. The experiment is still open to question because sympathetic flashing may occur in biota in the sensed area due to undetected flashes near the sensor caused by physical agitation of the water from sensor motion.

The photometer of Hardy and Kay (1964) used a 931A photomultiplier, but in association with a 200 cps rotating shutter and an ac amplifier for fast response. No optical filters were used, and the system was calibrated at one level of illumination to check the gain stability. The system time of response was limited by the Fielden Servograph to about 1 cps for bioluminescent flashes.

Clark and Kelly (1965) used an end window type 5819 photomultiplier (response between 0.34 and 0.55 μm) with no optical filtration in their *in situ* measurements of stimulated bioluminescence from a motor driven vane. Graphical records of radiance from bioluminescent flashing are presented with the impeller on and off. The system time of response appears to be of the order of a fraction of a second.

All of the foregoing instrumentation responds to bioluminescent flashes in a way that is limited by the time of response of the system, and the flashes are recorded as pulses. Another approach to the sensing of bioluminescence is that of recording the time average of the radiance from bioluminescent organisms. This time average is proportional to the energy output of the bioluminescent organisms which should be related to the number and species present. By including stimulated bioluminescence, some causes of natural variations in light emission are eliminated and a more realistic representation is obtained. An integrating type *in situ* sensor of this type was used to sense bioluminescence in the Gulf Stream (Egan, 1969), and in the New York Bight (Egan, Cassin and Hair, 1972). However, the correlation of the *in situ* bioluminescence with biota remains to be presented.

Fluorescence in sea water from ultraviolet excitation is another optical phenomena that can be used to advantage for *in situ* measurements of chlorophyll and Gelbstoff. Long wavelength ultraviolet red fluorescence from chloropigments is related to total chlorophyll and chlorophyll *a* (Yentsch and Menzel, 1963). Since certain species of phytoplankton contain chlorophyll, and if the distribution of these species is known, the amount of long wavelength fluorescence of chlorophyll *a* is proportional to the biomass of these species. Another aspect of fluorescence is that short wavelength ultraviolet fluorescence produces a blue-green radiation in luminescent organisms (Kelly, 1968). Another source of fluorescence in sea water in Gelbstoff (Kalle, 1966). Gelbstoff is a collective term that includes humic acids and melanoidines that are formed whenever decomposition of living cells occurs. The decomposition releases carbohydrates and amino acids (Kalle, 1966). Gelbstoff is found in areas where coastal waters from rivers and estuaries flow into the sea. Representative areas are the North Sea and the Baltic Sea (Jerlov, 1955; Kalle, 1966) and the Dutch Wadden Sea (Otto, 1966; Postma and Rommets, 1970). The New York Bight, similarly, is typical of coastal waters fed by highly

fluorescent fresh water (Egan, Cassin and Hair, 1972). A blue fluorescence occurs from organic substances present during the formation of the melanoidines during ultraviolet radiation (Kalle, 1966). This fluorescence is then proportional to the quantity of these substances present, and for a given area, related to the Gelbstoff present.

In order to make *in situ* investigations of bioluminescence and fluorescence in estuaries and deep sea environments, the photometric equipment used in the Gulf Stream drift experiment (Egan, 1969) was assembled in a geometrical configuration identical to that on the submersible used in the Gulf Stream. Subsequently additional instrumentation was added (Fig. 1). The other instruments on the assembly (Fig. 1) were used to gather information for a previous paper (Egan, Cassin and Hair, 1972). Thus, k is a flow type pH electrode, and b is its preamplifier; e is a lamp used to measure transmission in the red spectral region with sensor c; j is a dissolved oxygen probe; h is a Beckman conductivity probe for salinity measurements in association with the temperature sensing thermistor l; a is a thin walled stainless steel pivoted tube containing a thin film type water velocity probe (a fin for aligning the tube in the direction of maximum water velocity is not shown); and m is the hoist cable. The multiconductor cable from the instrumentation to the surface is not shown to emphasize clarity. Of the many instruments in this assembly, only certain units are relevant to this paper.

These were the photometers c and d, the ultraviolet lamps f and g, and the electric motor driven propeller, i. A very detailed description of the optical properties of the ultraviolet lamps and the photometers will be presented in a subsequent paper (Egan, in preparation). However, briefly, photometer d is a wideband sensor for bioluminescence using a 6199 photomultiplier with optical filtration to produce a response between 0.46 and 0.55 μm, sensing blue-green bioluminescence expected from dinoflagellates (Kelly, 1968). The optical band-pass is limited by interference filters. This same photometer measures Gelbstoff fluorescence within the acceptance band resulting from short wavelength ultraviolet radiation. This is provided by an S4T5 short wavelength ultraviolet lamp (peak radiation at 254 nm), with a Corning CS7-54 filter to attenuate the visible radiation. The measurement sequence consists of a background dc measurement of the photomultiplier output; then a measurement of the output with the motor alone operated; then a measurement with the short wavelength lamp alone operated. After the subtraction of the background dc measurement, and using photometric calibrations against an Eppley thermopile with neutral density filters to attenuate the radiance sensed by the photomultipliers, a radiance level is determined. This is then proportional to the bioluminescence or Gelbstoff depending upon the measurement.

For chlorophyll *a* measurements by fluorescence, the upper sensor is used in a similar way. It is a type 6199 photomultiplier with a Corning Glass CS2–60 filter to sense red radiance (0.62 to 0.65 μm) as a result of long wavelength fluorescent radiation from an S4T5 ultraviolet lamp, g. The UV lamp has a radiation peak at 370 nm, achieved with a fluorescent coating and a Corning CS7-60 glass filter to attenuate the visual and red spectral regions. Chlorophyll calibrations are based on samples acquired during the measurement sequence and analyzed at the Adelphi Institute of Marine Science (Strickland and Parsons, 1968). Both ultraviolet lamps, f and g, were protected from the sea by pressure resisting housings with UV transmitting quartz windows capable of withstanding depths to 600

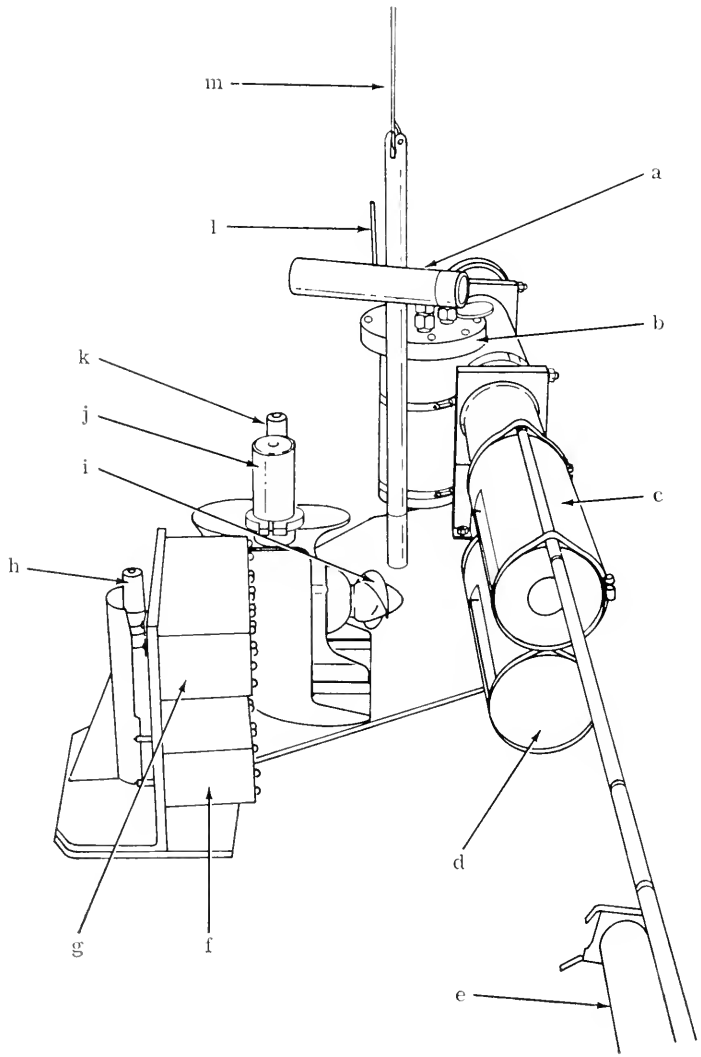

FIGURE 1. Sensor assembly; a, thin film anemometer unit; b, pH sensor preamplifier; c, red photometric sensor unit; d, broadband photometric sensor unit; e, transmissometer light source; f, 254 nm ultraviolet source; g, 370 nm ultraviolet source; h, conductivity cell; i, propeller agitator; j, dissolved O_2 sensor; k, flow type pH sensor; l, thermistor; m, hoist cable.

m. The photometers are of an extremely simple design with high voltage batteries within the housings, and gain variable by a set of relays also within the housings. Signals are transmitted to the surface through a multiconductor cable with vulcanized connections.

The utility of this *in situ* photometric instrumentation lies in the fact that bioluminescence and fluorescence measurements made *in situ* are not restricted by collection problems and are not handicapped by time lapse between sample collec-

tions and analyses. The instrumentation is well suited for unattended long term monitoring of estuarine areas.

To determine the applicability of this *in situ*, photometric instrumentation to estuarine research, a series of observations were made in the New York Bight.

By comparison of bioluminescence, short-wavelength ultraviolet-produced fluorescence, and long-wavelength ultraviolet-produced fluorescence with the laboratory determined biota distribution, the causative organisms may be inferred. The inferences are drawn on the basis of previous work on bioluminescent organisms, specifically dinoflagellates, and the known existence of chlorophyll *a* in certain phytoplankton.

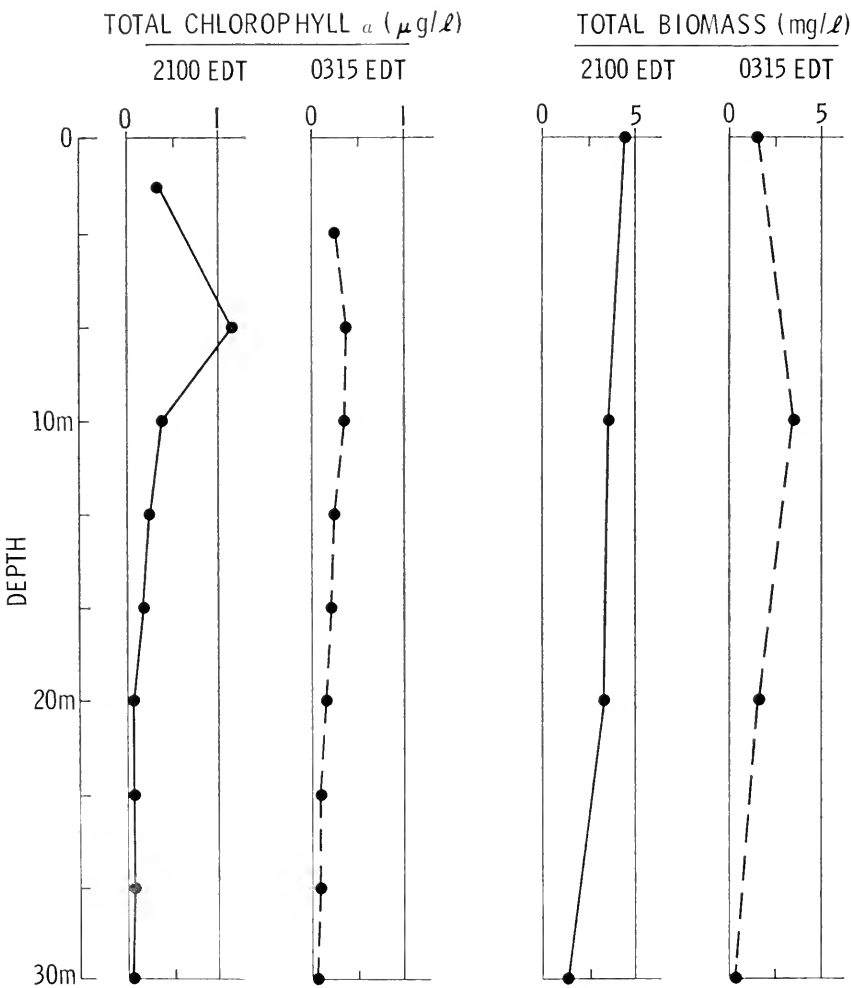

FIGURE 2. Comparison of *in situ* chlorophyll *a* measurements with total biomass at different depths and times.

Materials and Methods

There were two aspects to the experimental procedure: the first involves the *in situ* measurements, the second involves the laboratory methods for phytoplankton analysis. Both aspects of the experimental procedure were carried out concurrently.

A measurement program was conducted at night on October 19–20, 1970, one-half mile north of Ambrose Tower. *In situ* profiles were made using the assembly previously described. Profiles started at the surface with depth increments generally of 3 m. A background level was recorded on each photometer, and then each ultraviolet lamp was activated, and the photometer output and ultraviolet lamp input recorded. Photometer sensitivities were capable of remote incremental variation. For bioluminescence observations, input to the propeller agitator was adjusted to 250 w, and output of appropriate photometers recorded (with the

Table I

Standing crop from laboratory analyses

Date	Time EDT	Depth m	Zooplankton (total/l) $\times 10^3$	Phytoplankton (total/l) $\times 10^3$	Total biomass (mg/l)
19–10–70	2140	Surface	0	0.83	4.4
		10	60	3.15	3.53
		20	10	0.774	3.19
		30	50	0.64	1.2
20–10–70	0315	Surface	10	1.53	1.47
		10	60 (tintinnids)	1.63	3.34
		20	0	0.56	1.53
		30	110 (polychete larvae)	0.15	0.015

ultraviolet lamps off). The readings of the sensors, after the background level is subtracted, is then proportional to the fluorescence of total chlorophyll, the fluorescence of bioluminescent organisms, Gelbstoff or dissolved minerals, or bioluminescence.

Samples for laboratory analyses were collected concurrently with the *in situ* measurements at 2100 hours on October 19 and at 0315 hours on October 20, 1970. For phytoplankton, liter samples were collected in two 500 ml polyethylene bottles from Nansen bottles submerged to depths of 1, 10, 20, and 30 m. Samples were concentrated to a final volume of 10 ml, using continuous centrifugation (American Public Health Association, 1965; Kimball and Wood, 1965). After microscopic examination to identify forms that would be damaged by preservation, concentrate was fixed in 3 percent neutral formalin. Counts were performed in a Palmer nannoplankton cell. Raw counts were adjusted to cells per liter using appropriate dilution and efficiency factors (Cassin and McLaughlin, 1973).

Numbers of zooplankton were recorded with no attempt to classify by genus or species. Phytoplankton standing crop was recorded, and organisms identified to genus or when possible to species. Dimensions were measured by calibrated Whipple disc, and volumes (in cubic μm), derived by assigning one of three geometrical forms: rectangle, cylinder, or sphere. Estimated biomass (mg/1) was

derived from calculated cell volumes (Strickland, 1966; Willin, 1959). Paasche (1960) noted a 0.62 correlation between cell volume and productivity.

Results

Results of *in situ* fluorescence and bioluminescence measurements are shown in Figures 2 and 3. Laboratory analyses for standing crops are presented in Table I. *In situ* measurements indicate that total chlorophyll is high at the surface and decreases with increased depth, as expected. This correlates with laboratory determinations of total biomass. Total chlorophyll pigments are related (in a variable way) to total biomass, and would be expected to correlate with it. Laboratory

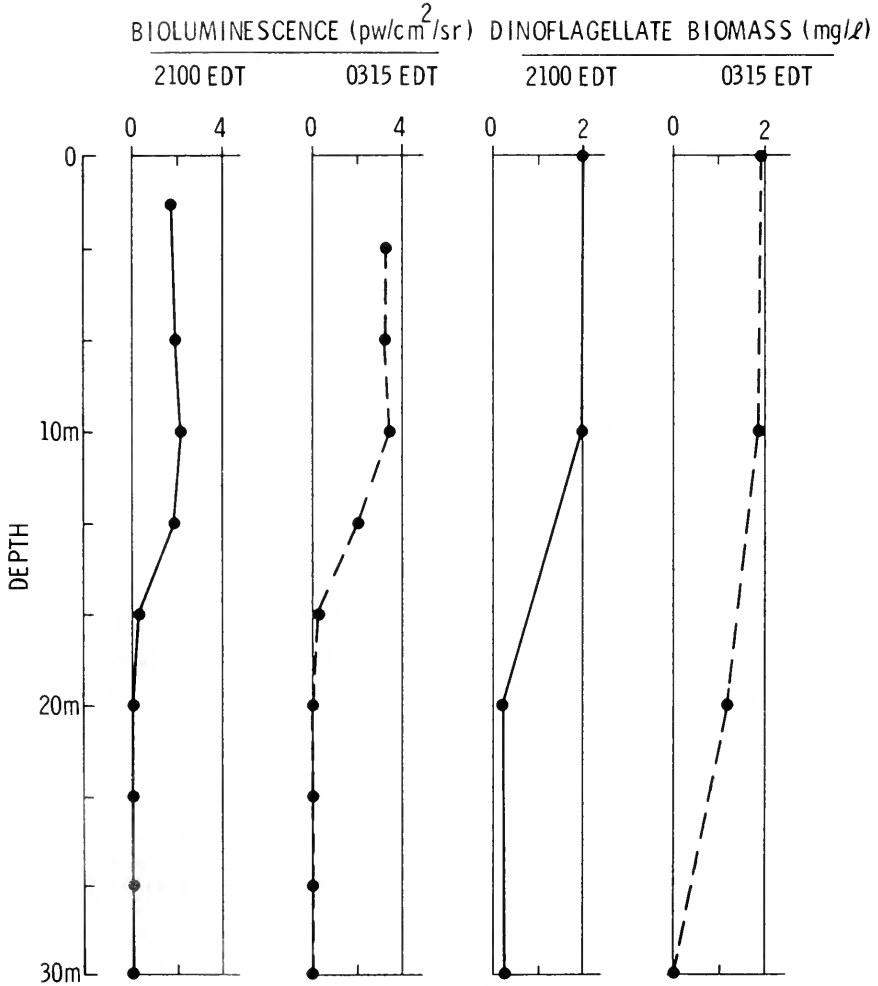

Figure 3. Comparison of *in situ* bioluminescence measurements with dinoflagellate biomass at different depths and times.

TABLE II

Phytoplanktonts occurring in regions of high long wavelength (370 nm) ultraviolet fluorescence; depths: A-surface, B-10 m, C-20 m, D-30 m

Species	Date - Time		Chlorophyll *a*
	19-10-2140	20-10-0315	
Cyclotella caspia Grunow		C	yes
Skeletonema costatum (Greville) Cleve	A, B, C	A, B, C	yes
Thalassiosira condensata Cleve		B	yes
T. gravida Cleve	A, B, C		yes
T. nordenskioeldi Cleve	A	C	yes
Actinocyclus sp.		C	yes
Chaetoceros decipiens Cleve	A		yes
Biddulphia pulchella Gray	B		yes
Thallassiothrix frauenfeldi Grunow		B, C	yes
Cylindrotheca clostridium (Ehrenberg) Reimann and Lewis	A, C, D	B	yes
Nitzschia seriata Cleve	A, D		yes
N. affinis Grunow	B, D	A, C	yes
Exuviella marina Cienowski	A		yes†
Prorocentrum micans Ehrenberg	A, C	B	yes†
P. scutellum Schroeder	A, B, C, D	A, B, C	—
Gymnodinium simplex (Lohmann) Kofoid and Swezy	B		yes*
Goniaulax diegensis Kofoid	A, D		—
G. polygramma Stein	A, D		—
G. spinifera (Claparede and Lackmann) Diesing		A	yes*
Peridinium depressum Bailey	A, B, C	B, C	no*
P. minusculum Pavillard	A	A	no*
P. ovatum (Bouchet) Scheutt		C	yes†
P. trochoideum Lemmerman	A, B	A, B, C	yes*
Dinophysis ovum Schuett	B	A, B	yes*
Glenodinium sp.	A		—
Ceratium lineatum Ehrenberg	B		—
C. arcticum Ehrenberg	B	A, B	—
Olisthodiscus luteus Carter		A, B	yes
Eutreptiella marina de Cunha	A		yes
Cryptomonas salina Wisloch	B		yes

† Raymont, 1967.
* Lebour, 1925.
—undetermined

analyses of the phytoplankton (Table II) indicate the major portion of the biomass to be made up of Bacillariophyceae and Dinophyceae. Also listed in Table II are the phytoplankton occurring in the regions of high long-wavelength fluorescence (surface and 10 m at 2100 hours, the surface and 10 and 20 m at 0315 hours), and the concurrent depth locations where they were found; there is a slight amount of overlap at the greater depths. Also indicated in Table II are those organisms that contain chlorophyll *a* and thus would be expected to fluoresce in long-wavelength ultraviolet light, permitting detection by this technique. In Figure 2, at 2100 hours at 7 m there is a chlorophyll increase not picked up in the bottle sampling.

The *in situ* observations of bioluminescence correlate with the dinoflagellate biomass (Fig. 3), as would be expected from the work of Kelly (1968). Haxo and Sweeney (1955) and Hastings and Sweeney (1958) have demonstrated an endogenous diurnal rhythm in laboratory cultures of *Gonyaulax polyedra*. An anomalously high value of dinoflagellate biomass occurs in the bottle sampling at 0315 hours at a depth of 20 m; this appears to be the result of local turbulence occurring at about 18 m depth since it does not agree with the *in situ* measurement. Turbulence in the water was observed with the thin film anemometer located on the platform (Fig. 1, sensor a). Note that the bioluminescence at 0315 hours is 1/3 higher than at 2100 hours, even though the dinoflagellate biomass is the same near the surface; this is presumably the result of the continuing increase subsequent to normal daylight inhibition of the luminescence of dinoflagellates, or an endogenous diurnal rhythm reported by Kelly and Katona (1966).

Correlation of the strong *in situ* bioluminescence occurring down to the thermocline (at 18 m) with laboratory determinations of dinoflagellates in the same region is presented in Table III. Known luminescent dinoflagellates are indicated and those with unknown characteristics denoted by a dash. It can be seen from the distribution of phytoplankton and known bioluminescent organisms that the bioluminescence observed is the result of the Dinophyceae: Goniaulaceae, and Peridiniaceae; many of the species recovered by us enhance and confirm the work of Kelly (1968) and Tett (1971), but we had certain dinoflagellates (Prorocentraceae) not reported as being bioluminescent, but observed within the population.

It appears that the zooplankton do not contribute to the total bioluminescence.

Augmenting factors that affect the efficiency of a luciferin-luciferase bioluminescence are water salinity, pH, pressure, and temperature, all of which varied with depth, and thus could cause some effect. The temperature of the water dropped from 16.4° C to 9.8° C below the thermocline, which would tend to decrease the bioluminescence.

There was no correlation of biomass, or dinoflagellate biomass, with the short wavelength ultraviolet fluorescence, indicating the dominance of Gelbstoff and/or dissolved minerals over biomass.

Discussion

Much of our present knowledge of the phytoplankton physiology arises from indirect sources such as laboratory experiments on marine cultures, or studies of general plankton ecology emphasizing primary production. However, it is most desirable for the environmental physiologist to conduct experiments at sea utilizing natural populations. However, in natural populations the investigator generally must work with low biota concentrations and techniques that the insensitive for experimentation. We have approached this problem with our *in situ* instrumentation, whereby a large volume of water is subjected to the experimental investigation to achieve a higher sensitivity. The importance of *in situ* measurements in this regard has been emphasized by Yentsch (1962) and Kelly (1968). This situation existed at the time of our study. Our observations were made during a transition time when the typical temperate diatom population was taking dominance over the typical summer flora, as evidenced by the appearance of Thallasiosira species and other diatoms (Table II).

Our technique for the measurement of bioluminescence differs from that of Kelly (1968) in that our index of bioluminescence is a photometric measurement of total integrated energy during agitation of the media. Kelly (1968) measured a flashing rate with his undersea photometer during agitation. The flashing rate does not measure the energy unless a time-intensity integration is performed on the luminescent flash record. The technique used by Kelly is applicable for areas

TABLE III

Dinoflagellates occurring in region of high bioluminescence; depths of samples: A-surface, B-10 m, C-20 m, D-30 m

Species	19–10–2140	20–10–0315	Luminescent ?
Prorocentraceae:			
Exuviella marina Cienowski	A		—
Prorocentrum minimum var. triangulatum (Martin) Hulbert	A, C	B	no*
P. scutellum Schroeder	A, B, C, D	A, B, C	—
Gymnodiniaceae:			
Gymnodinium simplex Kofoid and Swezy	B		—
Goniaulaceae:			
Goniaulax diegensis Kofoid	A, D		probable*
G. polygramma Stein	A, D		yes*
G. spinifera (Claparede and Lackmann) Diesing		A	yes*
Peridiniaceae:			
Peridinium depressum Bailey	A, B, C	B, C	yes*
P. minusculum Pavillard	A	A	—
P. trochoidium Lemmerman	A, B	A, B, C	no*
Dinophysiaceae:			
Dinophysis ovum Schuett	B	A, B	no*
Glenodiniaceae:			
Glenodinium sp.	A		no*
Ceratiaceae:			
Ceratium lineatum Ehrenberg	B		no*
C. arcticum Ehrenberg	B	A, B	no*

* Kelly (1968, Ph.D. thesis, Harvard University) and Kelly, 1969; Tett, 1971.

where the bioluminescence is weak, whereas when strong bioluminescence or a wide range of bioluminescence occurs, our technique, responding to the integrated light intensity, appears to be the best solution. Flashing rate measurements require a pulse counting technique, which is more complicated than the integrated light energy approach. The circuitry must be of the fast response, low time constant type, in order to respond to light pulses occurring at rapid rates, and to distinguish between nearly coincident pulses. Further, the interpretation of physiochemical reactions requires energy balance considerations, and the integrated light measurement furnishes the pertinent information directly. It is important to note

that this technique requires that there be an adequate supply of luminescent organisms and the necessary oxygen (McElroy and Seliger, 1963) which would not generally exist in a laboratory environment. A further consideration in the measurement of bioluminescence is that the spottiness noted by Kelly (1968) was also observed in our *in situ* measurements. This requires an averaging be made over a period of time that is long compared to the variations.

If measurements of bioluminescence are made without intentional agitation, either by virtue of a motor-driven impeller or by the motion of the photometer through the water, there is an uncertainty as to the physical cause of the bioluminescence (Tett, 1971).

Our fluorescence measurements of chlorophyll *a*, bioluminescent organisms, Gelbstoff or dissolved minerals similarly are made *in situ*, in a large volume of water. This has a limitation of application in highly turbid, estuarine water, because the ultraviolet radiation from the source lamps will be scattered and absorbed very strongly, as will be the fluorescent radiation. In an application of this sort, an alternative would be a flow through a Turner fluorometer because it has shorter light path lengths (Strickland and Parsons, 1968). However, the sensitivity would generally be severely decreased because of the smaller volume of turbid water with its increased absorption both of the ultraviolet and fluorescent radiation.

A criticism of the present instrumentation is that the configuration (Fig. 1) has the highest sensitivity when the background light level is lowest. This is no great problem for our measurements because the bioluminescence is greatest at night, and chlorophyll or Gelbstoff fluorescence measurements may be made over a night tidal cycle. However, if a 24 hour operational cycle is desired, a light tight shroud can be placed around the system and a recalibration made.

The ultimate use of *in situ* measurements is for mathematical modelling of a specific area. One application of *in situ* measurements for mathematical modelling has been made for Bahia Fosforescente by Seliger, Carpenter, Loftus, Biggley and McElroy (1971), although not without extensive associated laboratory investigations. They proposed three conditions as sufficient for modelling this bioluminescent bay, with two parameters to define the equilibrium state of the bay (the concentrations of *Pyrodinium bahamense* Margalef and *Ceratium furca hircus* Margalef). Effects are attributed to salinity, tidal flow, wind conditions, rain runoff, and geology. Nutrient concentrations are treated in a gross manner, since the detailed physiology of dinoflagellates and their relation to nutrients is as yet poorly determined (Kelly, 1968).

It is of interest to note that *Olisthodiscus luteus* Carter and *Eutreptiella marina* Cunha (Table II) appeared in high concentrations, although not indicated in the tabular data. Typical concentrations were 5000/liter at 0315 hrs at 30 m depth for *Olisthodiscus* and 1000 to 50,000/liter between 1735 and 2100 hrs at various depths. The appearance of these organisms has been noted by one of us as appearing at Bridgeport Harbor, Hempstead Harbor, and other small eutrophic embayments of Long Island. Palmer (1969) has indicated the Euglenoid group as a prime indicator of nutrient rich waters. As shown in Table II, both of these organisms fluoresce when irradiated with long wavelength ultraviolet radiation. Hence an *in situ* fluorescence technique offers possibilities for the monitoring of water quality.

The use of fluorescence as an indicator of chlorophyll, Gelbstoff, or dissolved

minerals for mathematical modelling is not yet tractable. It appears that an approach utilizing selective wavelength excitation and sensing would be advantageous to determine the form of chlorophyll *a in situ* (Goedheer, 1964). Further, any fluorescent dissolved minerals would have excitation and emission bands determined by their atomic and molecular bonds, which would characterize them. However, spectral resolutions necessary would be of the order of a nanometer, which requires a monochromator, with associated higher instrumental complexity. Nevertheless, for ecological monitoring of estuarine areas in terms of primary production and algal blooms, *in situ* fluorescence instrumentation permits rapid evaluation and subsequent monitoring of estuary conditions.

The authors thank Dr. M. E. Hair for his spectrophotometric chlorophyll determinations and the Grumman Aerospace Corporation and the Adelphi Institute of Marine Sciences for their support of this work.

Summary

We have described a series of measurements emphasizing the *in situ* aspects of bioluminescence and fluorescence photometric observations. The fundamental question of isolating a particular organism and determining whether it fluoresces or biolumineses has not been answered; however, we have observed a phenomenon and sought to deduce the probable causative organisms. It appears that the causative organisms may be inferred by a comparison of the *in situ* bioluminescence, short-wavelength ultraviolet-produced fluorescence, and long-wavelength ultraviolet-produced fluorescence with the laboratory determined biota distribution.

Using this approach, we find that strong bioluminescence occurs above the thermo/halicline and appears to be caused mainly by Peridiniaceae and Gymnodiniaceae.

The long-wavelength ultraviolet fluorescence is correlated with the total chlorophyll and consequently with the total biomass.

It is evident that *in situ* bioluminescence and fluorescence instrumentation is not restricted by collection problems, and is not handicapped by the time lapse between sample collections and analyses; it is well suited to long term monitoring of estuarine areas.

To understand the energetics of a bioluminescent reaction, it is necessary to express the transformation in terms of an average light energy output rather than a flash rate.

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COMPARISON OF THE PIGMENTARY EFFECTOR TROPINS IN THE EYESTALKS AND ABDOMINAL NERVE CORD OF THE PRAWN *PALAEMONETES VULGARIS* [1]

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In 1935 Brown concluded that each of the chromatophoric pigments in the prawn *Palaemonetes vulgaris* was under separate hormonal control. This conclusion was based on his observation that each pigment was capable of movements which were completely independent of those of the other pigments. Perkins (1928) had found a red pigment-concentrating hormone in the eyestalks of this prawn. Later it was shown that the eyestalks also contain a substance that causes dispersion of the melanin in crab melanophores (Brown, 1940; Brown and Scudamore, 1940). *Palaemonetes* itself lacks melanophores. Still later, Brown, Webb and Sandeen (1952) found that this prawn also produces a red pigment-dispersing hormone. The ratio of red pigment-dispersing hormone to red pigment-concentrating hormone appeared to be highest in the abdominal nerve cord. The abdominal nerve cord of *Palaemonetes* also possesses the substance that evokes melanin dispersion in crabs (Fingerman and Couch, 1967). Not only are the erythrophores of *Palaemonetes* controlled by pigment-dispersing and pigment-concentrating substances but also the leucophores (Fingerman, 1970). In the meantime, Kleinholz (1936) found that eyestalk extracts from *Palaemonetes*, in addition to their chromatophorotropic activities, evoked light adaptation of this prawn's distal retinal pigment.

The data of Fingerman and Couch (1967) suggested that the substance in *Palaemonetes* that caused red pigment dispersion in the prawn was not the same substance as that which had been found in the prawn and caused melanin dispersion in the fiddler crab. The portions of the central nervous system which were assayed differed with respect to the ratio of the amounts of melanin dispersion and red pigment dispersion they evoked. In these earlier experiments, however, crude extracts were used and, therefore, no definite conclusion could be drawn concerning that possible identity or non-identity of these substances. The changes in this ratio of red pigment-dispersing activity to melanin-dispersing activity were due to either differences in the relative quantities of a pigment-dispersing substance which acted on both types of chromatophores and antagonistically-acting pigment-concentrating substances among the tissues assayed or, alternatively, the fact that melanin dispersion and red pigment dispersion were caused by different substances.

The main object of the experiments described below is to examine further the

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question of whether red pigment dispersion in the prawn and melanin dispersion in the fiddler crab are caused by the same or different substances from the prawn. The technique of gel chromatography on Bio-Gel P-6 which has been used to separate pigment-dispersing and pigment-concentrating substances of crustaceans from each other was used herein. For example, this technique has allowed the separation of red pigment-dispersing and melanin-dispersing activities from the corresponding pigment-concentrating activities in extracts of eyestalks from the fiddler crab *Uca pugilator* (Fingerman, Bartell and Krasnow, 1971; Fingerman and Fingerman, 1972). Use of this technique would provide us pigment-dispersing material without interference from pigment concentrators and thereby allow resolution of the problem. Furthermore, Fingerman, Krasnow and Fingerman (1971) have suggested that if the melanin-dispersing substance found in *Palaemonetes* is not the same substance as the red pigment-dispersing substance it may be serving as the distal retinal pigment light-adapting hormone of the prawn inasmuch as the prawn which has a high titer of this melanin-dispersing substance in its eyestalks lacks melanophores. In the present experiments this possibility is explored further also.

Materials and Methods

The specimens of the prawn *Palaemonetes vulgaris* were collected in the vicinity of Woods Hole, Massachusetts, by members of the Supply Department of the Marine Biological Laboratory. The fiddler crabs, *Uca pugilator*, were provided by the Gulf Specimen Company of Panacea, Florida.

The system of Hogben and Slome (1931) was used to stage the chromatophores. According to their scheme stage 1 represents maximal pigment concentration, stage 5 maximal dispersion and stages 2, 3 and 4 the intermediate conditions. The chromatophores were staged at the time of injection of the extract being tested, 5, 15 and 30 minutes thereafter and subsequently at 30 minute intervals until the chromatophores had returned to their original stage. The recorded stage values were then used to calculate Standard Integrated Responses (SIR) which are a measure that includes both the amplitude and duration of the response (Fingerman, Rao and Bartell, 1967). With the prawn, the erythrophores and leucophores in the epidermis adhering to the portion of the carapace dorsal to the heart were staged; with the fiddler crab, the melanophores seen through the exoskeleton on the anteroventral surface of the second walking leg on the right side of the animal were staged. Eyestalkless crabs with maximally concentrated melanin were used to assay for the melanin-dispersing substance. Intact crabs in black pans with maximally dispersed melanin were used to assay for a melanin-concentrating substance. Eyestalkless prawns with their white pigment in an intermediate state of dispersion (Fingerman, 1970) were used to assay for white pigment-dispersing and pigment-concentrating substances. These eyestalkless prawns were also used in the assays for the red pigment-concentrating substance because their red pigment became maximally dispersed after their eyestalks had been removed. Prawns having one eyestalk were placed in white containers until their red pigment maximally concentrated and then served in the assays for the red pigment-dispersing substance. Prawns with one eyestalk are more sensitive to this substance than are intact prawns and, therefore, provide a better assay system for it (Brown, Webb

and Sandeen, 1952). The injected dose throughout these experiments was 0.05 ml for both species.

Assays for distal retinal pigment light-adapting activity were performed using the method of Sandeen and Brown (1952). Their technique consists essentially of placing a prawn on the stage of a dissecting microscope and measuring with the aid of an ocular micrometer and transmitted light: (A) the width of the transparent area of the distal portion of the eye in the direction of the long axis of the eyestalk and (B) the distance from the corneal surface to the proximal edge of the pigment spot at the base of the eye on the dorsal surface of the eyestalk. The ratio of A/B is known as the Distal Pigment Index (DPI). Each morning prawns to be used in an assay for the distal retinal pigment light-adapting substance were taken from the stock aquaria and placed in black pans in a darkroom until their distal retinal pigment had become fully dark-adapted. The extracts were then injected with the aid of a dim red lamp while the prawns were still in the photographic darkroom. The prawns were then kept in darkness for one additional hour at the end of which the DPI of each prawn was determined. Control prawns received saline alone.

Gel chromatography of extracts of eyestalks and abdominal nerve cords from the prawn was performed in essentially the same manner as described by Fingerman and Fingerman (1972) with eyestalks of other crustaceans. After either 100 eyestalks or 50 abdominal nerve cords had been extracted in 0.3 ml of 0.065 M sodium chloride, the extract was centrifuged for five minutes at 1815 g and 23–25° C. The supernatant was then applied to the top of a 0.8×31.0 cm column of Bio-Gel P-6, 100–200 mesh (Bio-Rad), prepared with 0.065 M sodium chloride. The void volume of this column was 6.5 ml and its flow rate was 0.5 ml per minute. The active material was eluted with the 0.065 M sodium chloride solution. One ml fractions were collected. To each fraction was added 0.29 ml of 400% crustacean saline (Pantin, 1934) which made the resulting solution so close to the osmotic concentrations of the blood of the prawn and the crab, that coupled with the dilution of the injected material in the blood of the recipients, control specimens showed no significant response in the absence of pigmentary effector activators. Twice as many eyestalks were used as abdominal cords because each prawn had two eyestalks but only one abdominal nerve cord. The prawns of the size used in these experiments had an average weight of 0.57 g. One of their eyestalks averaged 3.0 mg and their abdominal nerve cord averaged 1.3 mg.

In order better to compare the quantities of pigmentary effector tropins in the fractions obtained from the columns, dosage-response curves were obtained by assaying dilutions of the fractions which were most active in evoking melanin dispersion, red pigment dispersion and light adaptation of the distal retinal pigment. Then by use of these curves in which relative concentration was plotted *versus* SIR or DPI we could determine the relative difference in concentration of active material present in different fractions after they had been used in an assay. For example, when fractions evoked different red pigment-dispersing SIR's, examination of the appropriate dosage-response curve would reveal the relative concentration of the red pigment-dispersing substance in each fraction. These dosage-response curves provided a way to compare the quantity of a pigmentary effector tropin in each of these partially purified fractions. With such partially purified fractions one does not have to contend with the complications that occur when

dealing with a mixture of antagonistically-acting substances. Simply comparing SIR's or DPI's does not reveal relative concentrations. For example, an SIR of "4" does not necessarily mean there is twice as much hormone present in one fraction as in a second which evoked an SIR of "2."

Each of these experiments was performed twice. Assays for chromatophore activators were performed on three animals each, those for distal retinal pigment

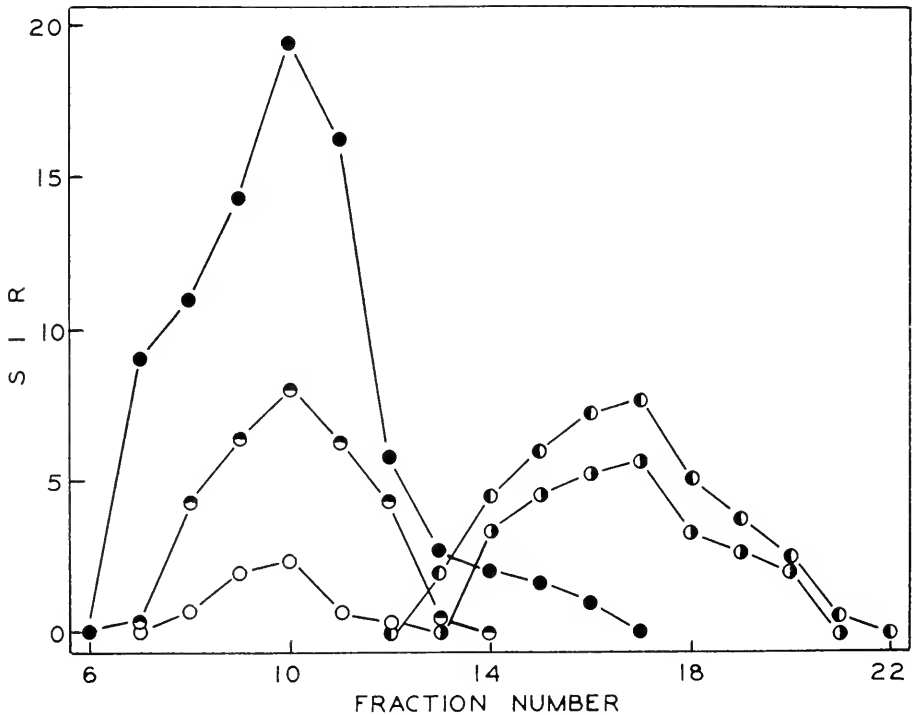

FIGURE 1. The Standard Integrated Responses (SIR) of the melanophores of fiddler crabs and the erythrophores and leucophores of prawns evoked by the fractions obtained by passing extracts of prawn eyestalks through a column of Bio-Gel P-6; dots, melanin-dispersing responses; circles, red pigment-dispersing responses; circles half-filled on top, white pigment-dispersing responses; circles half-filled on left, red pigment-concentrating responses; circles half-filled on right, white pigment-concentrating responses.

light adaptation on seven prawns each. Therefore, the averaged results in the following figures for chromatophoric responses represent the mean for six specimens and for distal retinal pigment light adaptation a mean of 14 specimens.

EXPERIMENTS AND RESULTS

The object of the first set of experiments was to determine the elution profiles of chromatophorotropins from prawn eyestalks and abdominal nerve cords which had been passed through a column of Bio-Gel P-6 and to compare the responses obtained from assays of these partially purified substances with the chromatophoric

responses obtained from assays of crude extracts of these organs. The mean results of the gel chromatographic analysis of the eyestalk extracts are presented in Figure 1. It can be seen there that the largest SIR's were obtained from the fractions that had been assayed for melanin-dispersing activity in eyestalkless fiddler crabs. In addition, red pigment-dispersing, white pigment-dispersing, red pigment-concentrating and white pigment-concentrating activities in the prawn were found. No black pigment-concentrating activity in the fiddler crab was found. All of the pigment-dispersing activities were eluted with an R_f of 0.65 whereas the two pigment-concentrating activities were eluted from the column later and both had an R_f of 0.38; R_f = void volume/elution volume of maximal activity.

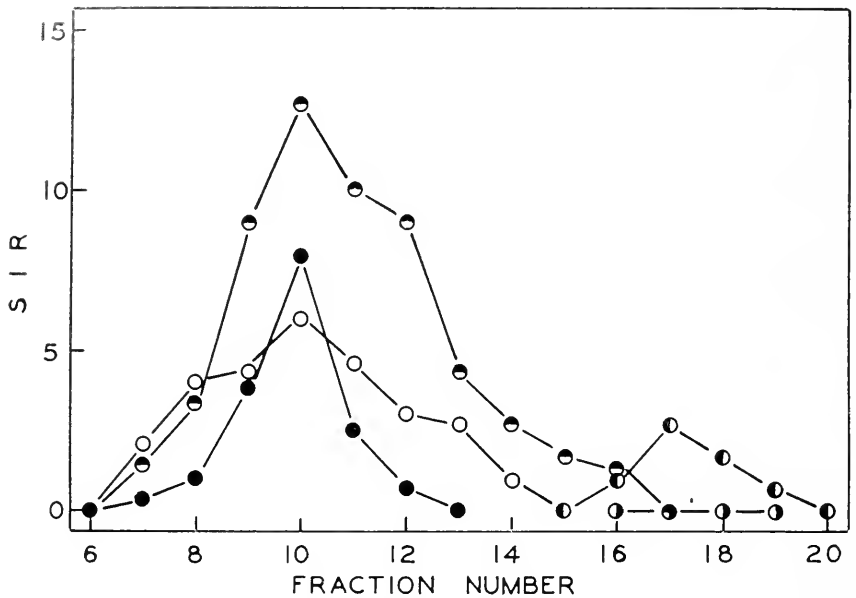

FIGURE 2. The Standard Integrated Responses (SIR) of the melanophores of fiddler crabs and the erythrophores and leucophores of prawns evoked by the fractions obtained by passing extracts of prawn abdominal nerve cords through a column of Bio-Gel P-6. See Figure 1 for key to symbols.

The mean results for the gel chromatographic analysis of the abdominal nerve cord extracts from the prawn are presented in Figure 2. Peaks of red and white pigment-dispersing activities in the prawn and melanin-dispersing activity in the fiddler crab, each having the same elution value (R_f 0.65) as the three pigment-dispersing activities from the eyestalks, were present here also. However, although a small red pigment-concentrating activity was noted (R_f 0.38), separated from the pigment-dispersing activities, neither white pigment concentration in the prawn nor melanin concentration in the fiddler crab was detected with these fractions of the abdominal nerve cords. If this prawn has melanin-concentrating activity in its eyestalks or abdominal nerve cord and white pigment-concentrating activity in its abdominal nerve cord, these activities must be present in very low concentrations

for these assays to have failed to detect them. Comparison of Figures 1 and 2 reveals that there was a considerable difference among the melanin-dispersing and red pigment-dispersing potencies of the eyestalks and abdominal nerve cords. The abdominal nerve cords contained more red pigment-dispersing activity but less melanin-dispersing activity than did the eyestalks. In contrast the relationship of the white and red pigment-dispersing activities to each other was the same in both the eyestalks and abdominal nerve cords, there having been consistently more of the former than the latter.

The crude extracts of the prawn abdominal nerve cord were prepared in a concentration of 1/12 equivalent per dose, prawn eyestalk extracts in both 1/6 and 1/12 equivalent per dose. The SIR's evoked by these extracts are presented in Table I. It can be seen there that injection of the crude eyestalk extracts resulted in a strong melanin-dispersing response in the crab and a strong red-pigment concentrating response in the prawn but no red pigment dispersion occurred in the prawn. In contrast, the abdominal nerve cord extracts evoked very similar SIR's for both melanin dispersion in the crab and red pigment dispersion in the prawn

TABLE I

Standard Integrated Responses (SIR) to crude extracts of eyestalks and abdominal nerve cords of the prawn

Organs extracted	Concentration (organ equivalents per dose)	SIR for melanin dispersion in the fiddler crab	SIR for red pigment dispersion in the prawn	SIR for red pigment concentration in the prawn
Eyestalks	1/6	17.1	0.0	15.2
Eyestalks	1/12	12.5	0.0	11.6
Abdominal nerve cords	1/12	4.9	5.3	0.0

but failed to evoke concentration of the red pigment in the prawn. The lack, for example, of a red pigment-dispersing response with the crude eyestalk extracts but its appearance when the partially purified eyestalk extracts were assayed (Fig. 1) is an example of the sort of antagonism which can occur between pigment-dispersing and pigment-concentrating chromatophorotropins. In the eyestalks there is sufficient red pigment-concentrating hormone to mask completely the red pigment-dispersing substance.

In order to gain further information concerning the relative quantities of the melanin-dispersing and red pigment-dispersing activities in the eyestalks and abdominal nerve cords, fraction 10 of the eyestalks (Fig. 1) which evoked the largest melanin-dispersing response and fraction 10 of the abdominal nerve cords (Fig. 2) which evoked the largest red pigment-dispersing response were diluted with 100% crustacean saline to concentrations of 1/3, 1/9, 1/27 and 1/81 of the original. The dilutions of the eyestalk fraction were then assayed to determine the melanin-dispersing SIR's they evoked and the dilutions of the abdominal nerve cord fraction were similarly assayed to determine their red pigment-dispersing SIR's. The averaged results of these assays were used in the preparation of the dosage-response curves of Figure 3. By use of the data of Figure 3, as described above, we find that the red-pigment dispersing SIR evoked by fraction 10 of the

abdominal nerve cords (Fig. 2) was due to 3.3 times as much active material as was responsible for the red pigment-dispersing SIR obtained with fraction 10 of the eyestalks (Fig. 1). A similar analysis reveals that the melanin-dispersing SIR of fraction 10 of the abdominal nerve cords was due to 0.3 as much melanin-dispersing material as caused the melanin-dispersing SIR of fraction 10 of the eyestalks.

The following experiment was designed to examine the possibility that the hormonal control of the erythrophores is not the same as that of the distal retinal pigment of *Palaemonetes*. These particular chromatophores were chosen for study

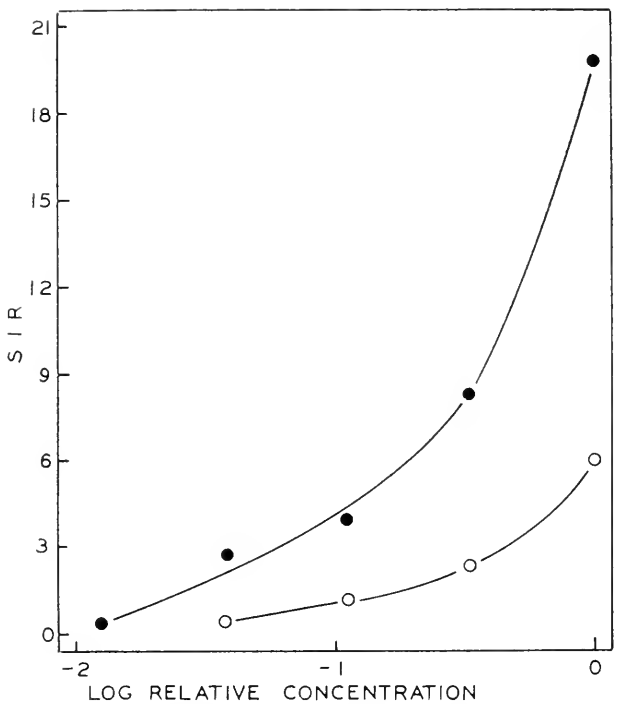

FIGURE 3. Relationships between the Standard Integrated Response (SIR) and the logarithm of the relative concentration of fraction 10 of the prawn eyestalks with respect to melanin dispersion in the fiddler crab (dots) and fraction 10 of the prawn abdominal nerve cords with respect to red pigment dispersion in the prawn (circles).

because they are the predominant type in determing the coloration of this prawn. Twenty intact prawns were evenly divided among two black and two white pans. The prawns in one black and one white pan were then exposed to an illumination of 0.52 lux while the remaining prawns were exposed to an illumination of 1400 lux. One hour later the stages of the erythrophores and the DPI of these prawns were determined. The experiment was repeated and the data averaged. The data revealed that under both light intensities the means stage of the erythrophores of the prawns in the white pans was 1.0 (maximally concentrated) and of those in the black containers 4.8 (nearly maximally dispersed). The mean DPI of the prawns

in the white pans exposed to 0.52 lux was 0.172; in the black pans at 0.52 lux, 0.093; in the white pans at 1400 lux, 0.261; in the black pans at 1400 lux, 0.208. We see from these data that the DPI and erythrophore stages are independent of each other. For example, the DPI of prawns in black pans under an illumination of 1400 lux was larger than the DPI of the prawns in white pans under an illumination of 0.52 lux but smaller than the DPI of the prawns in white pans under an illumination of 1400 lux, yet the red chromatophoric pigment of the prawns in the black pans was nearly maximally dispersed while this pigment was always maximally concentrated in the prawns in the white pans. On the basis of these data, it is valid to conclude that the hormonal control cannot be completely the same for both of these pigmentary effectors.

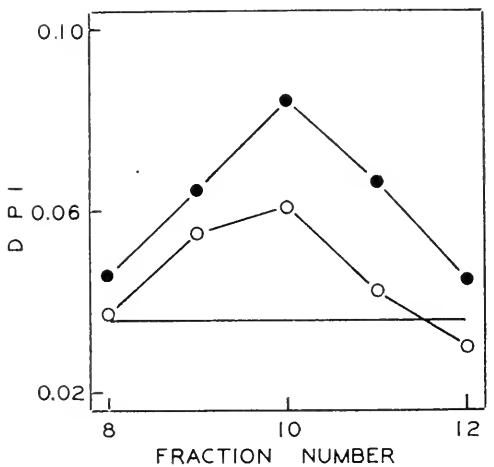

FIGURE 4. The Distal Pigment Indexes (DPI) of prawns which had received injections of fractions of prawn eyestalks (dots) or abdominal nerve cords (circles). The horizontal line at DPI = 0.036 is the mean value of the controls.

The aim of the next experiment was to compare the distal retinal pigment light-adapting potencies of crude extracts of eyestalks and abdominal nerve cords. Prawns that received the equivalent of one-half abdominal nerve cord per dose had a DPI of 0.074 one hour after the extract had been injected whereas prawns that received eyestalk extracts prepared in a concentration of either one-half or one eyestalk equivalent per dose had DPI's one hour after the extracts were injected of 0.168 and 0.219, respectively. The mean value for the controls was 0.036. The crude extracts of the abdominal nerve cords evoked less light adaptation of the distal retinal pigment than did the crude extracts of the eyestalks.

Figure 4 presents the mean results obtained when fractions of the eyestalks and abdominal nerve cords obtained from the Bio-Gel P-6 column were assayed to determine their light-adapting potencies. With each set of fractions, the maximal distal retinal pigment light-adapting activity was obtained with fraction 10 (R_f = 0.65) just as occurred when these fractions were assayed for pigment-dispersing activities on the chromatophores (Figs. 1 and 2). The overall light-adapting re-

sponse to the eyestalk fractions was greater than that evoked by the fractions of the abdominal nerve cords, which is in agreement with the results of the previous experiment where with the crude extracts the eyestalks were more effective than the abdominal nerve cords in evoking a light-adapting response.

The diluted preparations of fraction 10 of the eyestalks which had been used to obtain the melanin-dispersing dosage-response curve of Figure 3 were then assayed to provide data for a dosage-response curve showing the relationship between their distal retinal pigment light-adapting activities (DPI) and relative concentrations (Fig. 5). Using Figure 5 in the same manner as was done to calcu-

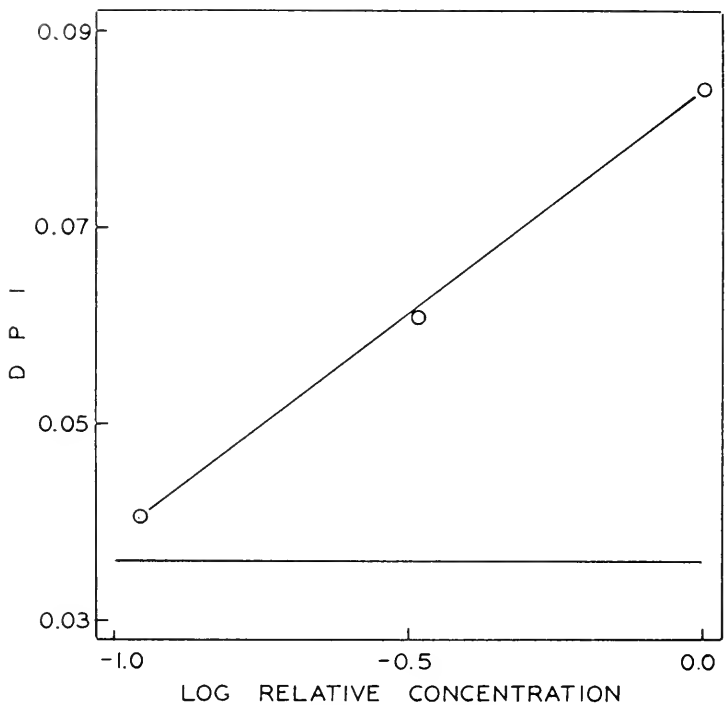

FIGURE 5. Relationship between the Distal Pigment Index (DPI) and the logarithm of the relative concentration of fraction 10 of the prawn eyestalks which was injected. The horizontal line at DPI = 0.036 is the mean value of the controls.

late the relative concentrations of pigment-dispersing material in the fractions of the eyestalks and abdominal nerve cords, we found that fraction 10 of the abdominal nerve cords contained 0.3 as much distal retinal pigment light-adapting substance as did fraction 10 of the eyestalks.

DISCUSSION

These experiments have revealed that the substance in the prawn *Palaemonetes vulgaris* which causes red pigment dispersion and perhaps white pigment dispersion as well is different from the substance also found in this prawn that evokes melanin

dispersion in the fiddler crab *Uca pugilator*. Kleinholz (1970) had chromatographed extracts of eyestalks alone from the prawn *Pandalus jordani* and because the elution profiles of all the pigment-dispersing activities were the same he suggested that (a) a single substance activates more than one chromatophore type or (b) different but closely related molecules control each chromatophore type. However, when the pigment-dispersing activities of fractions of abdominal nerve cords are compared with those of eyestalk extracts as was done herein (Figs. 1 and 2) it becomes clear that at least two substances are involved. The eyestalk fractions evoked larger melanin-dispersing SIR's (particularly fractions 7–11) in the fiddler crab than red or white pigment-dispersing SIR's in the prawn (Fig. 1), but when the fractions of the abdominal nerve cords were assayed (Fig. 2) all of the fractions containing pigment-dispersing activities showed a greater effect on the prawn's leucophores than on the crab's melanophores and only one fraction evoked a larger SIR in the crab than it did with the erythrophores of the prawn. These differences among the relative overall melanin-dispersing and red and white pigment-dispersing potencies of the several fractions from the eyestalks and abdominal nerve cords would not have occurred if all these pigment-dispersing responses were due to a single substance. On the basis of these results it is clear that the eyestalks contain more of the melanin-dispersing substance and less of the red and white pigment-dispersing activities than do the abdominal nerve cords. The data with crude extracts (Table I) support the results shown in Figures 1 and 2 with respect to the relative quantities of the melanin-dispersing and red pigment-dispersing substances in the eyestalks and abdominal nerve cords. However, it was possible that there was an antagonism between the substances controlling dispersion and concentration of each pigment which could result in a much smaller response, or no response at all, than would occur if each substance was assayed in the absence of its antagonist. Therefore, it was necessary to filter these extracts through a gel known to separate pigment-dispersing and pigment-concentrating substances from each other (Fingerman, Bartell and Krasnow, 1971; Fingerman and Fingerman, 1972) before a definitive conclusion could be arrived at concerning the identity or non-identity of these pigment-dispersing substances.

The large amount of melanin-dispersing substance in the eyestalks of the prawn, which lacks melanophores, is enigmatic unless, of course, this substance has a different role in the prawn itself. The data presented above support the hypothesis that the role of this melanin-dispersing substance in the prawn is that of the hormone that causes light-adaptation of the distal retinal pigment for the following reasons. The elution patterns of the melanin-dispersing and distal retinal pigment light-adapting activities from both the eyestalks and abdominal nerve cords are the same (Figs. 1, 2, and 4). This similarity was also noted with extracts of eyestalks from the prawn *Pandalus jordani* (Kleinholz, 1970) and the fiddler crab *Uca pugilator* (Fielder, Rao and Fingerman, 1971). Furthermore, with both crude extracts and these partially purified fractions the eyestalks were more potent than the abdominal nerve cords with respect to both the melanin-dispersing and distal retinal pigment light-adapting activities. These fractions with distal retinal pigment light-adapting activity would be devoid of the dark-adapting hormone. Fingerman, Krasnow and Fingerman (1971) found that the latter was eluted much later than the former from Bio-Gel P-6, just as the pigment-concentrating material was

separated from the pigment-dispersing activities by this gel. Finally, the relative amounts of both the melanin-dispersing and distal retinal pigment light-adapting activities in the eyestalks and abdominal nerve cords were the same. Analysis of the data presented above by use of the dosage-response curves of Figures 3 and 5 showed that fraction 10 of the abdominal nerve cords contained not only 0.3 as much of the melanin-dispersing substance as did the eyestalks, but also only 0.3 as much distal retinal pigment light-adapting material as well.

The conclusion of Brown (1935) that the hormonal control of the erythrophores in this prawn has to be different from that of the leucophores was referred to above. However, the present experiments have not allowed us to conclude whether the difference in their control is due to the hormones which regulate dispersion of the red and white pigments, their concentration, or perhaps both processes. By the same logic which Brown (1935) used to arrive at his conclusion about the control of the chromatophores in *Palaemonetes,* a similar conclusion can be drawn that the hormonal control of the erythrophores and distal retinal pigment of this prawn cannot be the same because the pigment migrations which occur within one of these effectors are independent of migrations going on in the other. As seen above, when prawns are on a white background their red chromatophoric pigment is maximally concentrated and when they are on a black background this pigment is nearly maximally dispersed, but the distal retinal pigment may occupy a position that approaches the dark-adapted one or the light-adapted position regardless of whether the red chromatophoric pigment is dispersed or concentrated. Furthermore, on the basis of the present experiments we can conclude that one of the differences between the hormonal controls of each of these pigmentary effectors is that dispersion of the red and white chromatophoric pigments of *Palaemonetes* cannot be due to the substance which causes light adaptation of its distal retinal pigment. Whereas the eyestalk fractions were overall more potent than those of the abdominal nerve cords in evoking light adaptation of the distal retinal pigment (Fig. 4), the situation was reversed in the assays for chromatophoric pigment-dispersing activities in the prawn (Figs. 1 and 2).

Sandeen and Brown (1952) found that the distal retinal pigment of *Palaemonetes* responds to the brightness of the visual field (not an albedo response) whereas the responses of its chromatophores to black and to white backgrounds are a true albedo response. The observation herein that at each of the two light intensities used the mean DPI of the prawns in the black pans was lower than the mean DPI of the prawns in the corresponding white containers is in conformity with the results of Sandeen and Brown. Fingerman, Krasnow and Fingerman (1971) had suggested that this difference in response to illumination (albedo *versus* brightness of the visual field) by the chromatophores and distal retinal pigment of *Palaemonetes* respectively might be due to an underlying difference in their hormonal controls. The experiments described above revealed that such a difference does indeed exist.

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Summary

1. Extracts of the eyestalks and abdominal nerve cords of the prawn *Palaemonetes vulgaris* were chromatographed on Bio-Gel P-6. The fractions of both extracts revealed melanin-dispersing activity in the fiddler crab *Uca pugilator* and red and white pigment-dispersing activities and distal retinal pigment light-adapting activity in the prawn. In addition, the eyestalk fractions contained red and white pigment-concentrating activities whereas only the red pigment-concentrating activity was found in the fractions of the abdominal nerve cords.

2. The pigment-dispersing and distal retinal pigment light-adapting activities were eluted from the column ahead of the pigment-concentrating activities and were consequently separated from them. However, the pigment-dispersing and distal retinal pigment light-adapting activities did not separate from each other. The pigment-concentrating activities likewise did not separate from each other.

3. The substance from the prawn which evokes melanin dispersion in the fiddler crab is not the substance that causes red pigment dispersion and perhaps white pigment dispersion as well in the prawn itself.

4. Furthermore, dispersion of the red and white chromatophoric pigments in the prawn is not caused by the substance that evokes light adaptation of its distal retinal pigment.

5. Evidence is presented to support the hypothesis that light-adaptation of the distal retinal pigment in *Palaemonetes* is caused by the substance found in the prawn that causes melanin dispersion in the fiddler crab. *Palaemonetes* itself lacks melanophores.

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SOLUTE ADJUSTMENTS IN THE COELOMIC FLUID AND MUSCLE FIBERS OF A EURYHALINE POLYCHAETE, *NEANTHES SUCCINEA*, ADAPTED TO VARIOUS SALINITIES [1]

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Neanthes (*Nereis*) *succinea* (Frey and Leukart) is a nereid polychaete found in both hypersaline (Carpelan and Linsley, 1961) and brackish waters (Oglesby, 1965). Studies on the survival of *N. succinea*, and other euryhaline nereids, in sea water below 30% have been well reviewed by Oglesby (1969). Survival is achieved at the organismal level by the hyper-regulation of sodium chloride in the coelomic fluid (Oglesby, 1965), together with compensatory adjustments in the body wall permeability to water (Smith, 1964) and salts (Smith, 1963). At salinities greater than 30% SW the coelomic fluid of these worms conforms osmotically with the environment. Since any changes in the coelomic fluid osmotic activity, either in the regulating or conforming range, are presumed to be accompanied by equivalent osmotic adjustments in the intracellular compartment, the means by which this cellular equilibration is accomplished is most important.

Osmotic regulation in polychaetes has not heretofore been examined at the cellular level, and at the tissue level only with respect to the suggested osmoregulatory role of the large free amino acid pool consistently found in the tissues of these organisms (Clark, 1968a, 1968b). In euryhaline polychaetes large decreases in intracellular osmotic activity presumably are achieved by the reduction of a considerable portion of this nitrogenous solute pool, together with the removal of water (Jeuniaux, Duchâteau-Bosson, and Florkin, 1961; Clark, 1968b). The responses of other tissue solutes of marine annelids to osmotic stress have not been examined, save for one brief account of *Arenicola* muscle tissue at two salinities (C. B. Cowey and J. Shaw, unpublished, cited in Potts and Parry, 1964).

It is apparent that the distribution of intracellular solutes in marine annelids is poorly understood relative to other aspects of the osmotic physiology of these organisms. This paper presents the results of our studies on the partitioning of the major organic and inorganic solutes and water in both extra- and intracellular compartments of *Neanthes succinea* adapted to various salinities.

[1] This study represents portions of two theses presented to the faculty of California State University at San Diego by the first two authors in partial fulfillment of the requirements for M.S. degrees. Part of this work was supported by a California State University at San Diego Foundation Grant (#261134) to Mary E. Clark.

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Materials and Methods

Collection and maintenance

Specimens of *Neanthes succinea* were collected in Colorado Lagoon, Alamitos Bay, Long Beach, California. Although not a true estuarine environment, the salinity of the lagoon varied from 97% SW to 24% SW (100% SW = 560 mM Cl) over the period of January 1970 to January 1972. Worms were kept at 15 to 18° C without feeding in filtered artificial sea water of the following composition (or dilutions thereof): Na, 487.2 mM; K, 9.0 mM; Ca, 9.1 mM, Mg, 48.4 mM; SO_4 25.5 mM; Cl, 556.1 mM; HCO_3, 2.1 mM. Experimental specimens were always moved through salinity gradients in a stepwise fashion, remaining several days at each dilution. Only worms kept at the final acclimation salinity for five or more days were used experimentally.

Sample preparations

No particular distinctions were made regarding either the sex or size of experimental animals. Regenerating worms were frequently used, but only when sufficiently advanced in caudal development. Specimens were also utilized in a variety of reproductive states; however extremely ripe individuals and heteronereids were discarded.

Coelomic fluid was removed from unanesthetized specimens with a fine tipped, hand drawn capillary. Samples were taken only from the anterior half of the worm with as few punctures as possible (generally three). The supernatant of the centrifuged coelomic fluid was stored at 4° C prior to analysis.

A uniform tissue type was obtained by excising only the anterior ventral musculature. Care was taken to remove the parapodia, gut, and large blood vessels. This procedure resulted in a nearly homogeneous tissue sample, since only nephridial fragments and the thin epidermis and external cuticle remain attached to the muscle. Except where sample pooling was necessary tissue solutes and water content were determined on the same piece of tissue.

Inorganic tissue solutes were extracted in glass distilled water at 4° C for at least 36 hours. Justifications for using this procedure have been given by Webber and Dehnel (1968). This method was preferred here to avoid interferences associated with acid extracts during the subsequent chemical analyses.

The water contents of whole animals and tissue pieces were obtained by drying the samples to constant weight at 60° C. Weights were recorded with a Mettler Type H-16 analytical balance to the nearest hundredth milligram.

Analytical methods

Sodium, potassium, and calcium were determined with a Beckman DU flame photometer equipped with a photomultiplier. In the estimation of K and Ca, sodium was added to the standards to adjust for spectral interferences attributable to this ion. Total calcium plus magnesium was measured by an ethylenediaminetetraacetic acid titration using Eriochrome Black T as an indicator (Walser, 1960). The magnesium concentration was then obtained by difference.

Chloride was determined by microtitration following the method of Schales and Schales (1941). Phosphate levels were analyzed by the technique of Parvin and

Smith (1969). Identification of the various phosphate fractions (inorganic orthophosphate, phosphagen phosphate, and adenosine triphosphate) was accomplished by inorganic phosphate analysis following varying periods of hydrolysis in 1 N HCl of the trichloroacetic acid extracts of pooled, freshly excised tissues (slightly modified from Shaw, 1958a).

Total ninhydrin positive nitrogen (Nin +N) was determined on alcoholic extracts of pooled fresh tissues following the method of Clark (1964).

The osmotic activity of coelomic fluid samples was estimated by the comparative melting point method of Gross (1954). Results of these analyses are expressed in milliosmoles/l by assuming 0.50 M NaCl is equivalent to 1000 milliosmoles/l.

All analyses of solutes from individual worms were performed in duplicate. Triplicate analyses were made on pooled samples of approximately equal weights of tissue or volumes of coelomic fluid from at least six worms.

Extracellular space

The extracellular space (ECS) of the muscle tissue was determined by two separate methods using ^{14}C-inulin (New England Nuclear, lot #606–204). In the first method an *in vivo* technique was used (Clark, 1972). Specimens, partially anesthetized in a 0.1% MS 222 sea water solution, were slowly injected with 0.25 μCi of ^{14}C-inulin in 10 μl of a sea water carrier by means of a fine tipped glass needle attached to an Agla micrometer syringe. After a predetermined equilibration time coelomic fluid samples were withdrawn from the anterior portion of the coelom. Duplicate 5μl aliquots of the centrifuged coelomic fluid supernatant were placed in 10 ml of a scintillation cocktail (2 parts of a toluene solution, containing 8 g Omnifluor (New England Nuclear) per liter, and one part Triton X-100) and counted on a Packard TriCarb Liquid Scintillation Spectrometer.

Excised muscle tissues from the same region of each specimen were weighed and then dissolved in 1 ml of 88% formic acid in a scintillation vial. After digestion, 10 ml of the scintillation cocktail were added and the mixture counted.

The second method for determining the ECS was an *in vitro* technique. Weighed pieces of body wall musculature were incubated in the appropriate sea water solution containing approximately 0.1 microcurie ^{14}C-inulin per milliliter. Following a predetermined incubation time the tissues were rinsed, blotted on a non-absorbent surface, and digested as before. Aliquots of the incubation medium were treated and counted as the coelomic fluid samples in the *in vivo* method. Water content was determined on a parallel sample of tissue.

Counting error was less than two per cent, and counts of all samples were quench corrected.

Results

The solute composition of the adaptational salinity, coelomic fluid, and muscle tissue of *Neanthes succinea* is presented in Table I. Tissue values are presented here for comparative purposes, since much of the published data concerning marine invertebrate muscle solutes have not been corrected for extracellular space.

TABLE I

Solute composition of the coelomic fluid and muscle of N. succinea *at various salinities. Concentrations are given in millimoles per l or per kg tissue H_2O ± 1 S.E. (N)*

Solute	100% SW			50% SW		
	Medium	Coelomic fluid	Tissue	Medium	Coelomic fluid	Tissue
Na	478.6 ±4.7 (5)	483.3 ±2.5 (9)	125.1 ±4.7 (8)	233.9 ±3.6 (5)	240.5 ±4.7 (7)	58.4 ±3.1 (9)
K	8.9 ±0.1 (5)	13.7 ±0.5 (8)	194.9 ±9.0 (9)	4.7 ±0.1 (2)	8.4 ±0.4 (8)	153.3 ±6.6 (9)
Ca	12.9 ±0.2 (6)	12.5 ±0.5 (8)	13.8 ±0.8 (6)	4.4 ±0.1 (7)	4.1 ±0.1 (8)	8.2 ±0.5 (8)
Mg	36.7 ±1.9 (7)	44.1 ±0.9 (7)	21.6 ±0.3 (7)	21.3 ±0.6 (5)	21.3 ±0.4 (7)	16.0 ±0.7 (7)
Cl	568.8 ±7.4 (5)	544.8 ±6.6 (9)	123.8 ±4.0 (8)	278.6 ±6.7 (6)	280.0 ±5.6 (8)	56.7 ±2.0 (7)
PO_4	—	—	34.7	—	—	29.6
Phosphagen	—	—	11.6	—	—	8.9
ATP	—	—	8.8	—	—	2.5
Nin + N	—	9.3	412.1	—	7.3	154.6
Total solute	1105.9	1107.7	—	542.9	561.6	—
Measured milliosmolarity	1129.0 ±28.7 (4)	1107.5 ±33.2 (5)	—	545.0 ±16.9 (3)	565.0 ±16.6 (5)	—

Solute	35% SW			20% SW		
	Medium	Coelomic fluid	Tissue	Medium	Coelomic fluid	Tissue
Na	178.1 ±1.9 (5)	183.6 ±2.6 (8)	37.7 ±0.8 (8)	96.2 ±0.5 (5)	124.3 ±0.6 (8)	26.9 ±2.2 (8)
K	3.1 ±0.1 (5)	6.9 ±0.5 (5)	119.8 ±2.6 (9)	1.7 ±0.1 (5)	4.4 +0.2 (8)	119.4 ±10.0 (9)
Ca	3.1 ±0.1 (5)	3.0 ±0.1 (9)	7.9 ±0.3 (8)	1.9 ±0.0 (5)	2.6 ±0.1 (7)	6.5 ±0.3 (8)
Mg	16.9 ±0.3 (3)	16.9 ±0.1 (8)	13.1 ±0.6 (8)	9.9 ±0.3 (5)	12.2 ±0.7 (8)	9.2 ±0.4 (7)
Cl	200.4 ±3.8 (5)	201.4 ±2.6 (8)	38.7 ±1.4 (8)	116.7 ±0.6 (5)	130.8 ±1.7 (8)	26.7 ±1.4 (7)
PO_4	—	—	—	—	—	—
Phosphagen	—	—	—	—	—	—
ATP	—	—	—	—	—	—
Nin + N	—	7.3	136.5	—	5.1	76.6
Total solute	401.6	419.1	—	226.4	279.4	—
Measured milliosmolarity	396.0*	409.0*	—	226.0*	270.0*	—

Solute	10% SW		
	Medium	Coelomic fluid	Tissue
Na	55.3 ±0.2 (4)	76.2 ±1.7 (8)	22.3 ±0.8 (8)
K	1.0 ±0.0 (4)	3.5 ±0.2 (8)	110.8 ±3.1 (8)
Ca	1.0 ±0.0 (5)	1.9 ±0.1 (7)	5.5 ±0.2 (8)
Mg	4.5 ±0.1 (4)	8.9 ±0.5 (5)	9.8 ±0.4 (5)
Cl	62.5 ±2.1 (4)	91.6 ±2.1 (8)	18.4 ±1.1 (8)
PO_4	—	—	23.1
Phosphagen	—	—	11.0
ATP	—	—	3.0
Nin + N	—	2.5	67.6
Total solute	123.4	184.6	—
Measured milliosmolarity	113.0 ±5.6 (5)	200.0 ±14.9 (6)	—

* Values interpolated from a plot of the osmotic activity of the coelomic fluid *vs* the medium.

The coelomic fluid

With the exception of K, the osmotic and ionic composition of the coelomic fluid of *N. succinea* conforms with adaptational salinities as low as 35% SW. Potassium exists in a hyperionic state at all salinities examined, yet it contributes little to the total osmotic activity of the coelomic fluid. The ratio K_{cfl}/K_{sw} is not constant however, varying from 1.5 to 3.5 in 100% and 10% SW, respectively. Below 35% SW the coelomic fluid is hypersomotically regulated. This hyperosmotic plateau results primarily from the retention of NaCl, with minor contributions

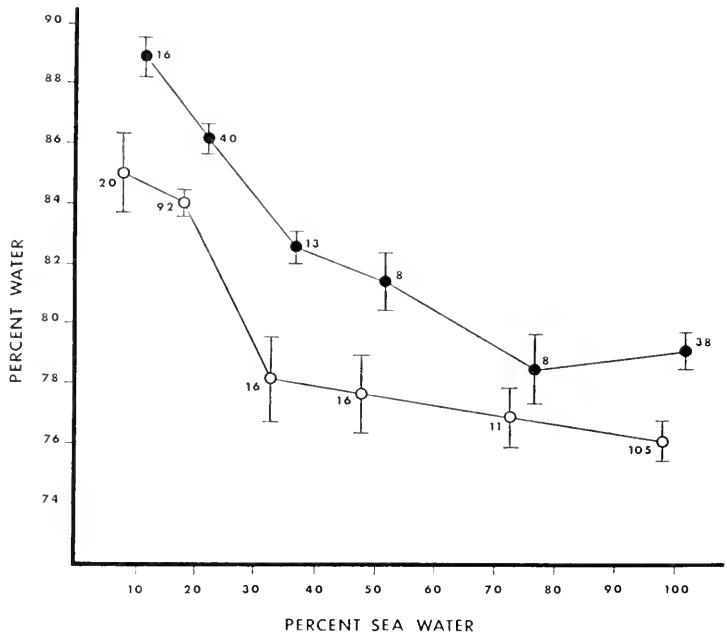

FIGURE 1. The per cent water content of whole animals (closed circles) and body wall pieces (open circles) of worms adapted to 10, 20, 35, 50, 75 and 100 per cent sea water. The vertical bars represent ±2 S.E. and the sample size is indicated beside each point. For clarity the points have been slightly displaced to each side of the appropriate per cent sea water.

by Ca and Mg. Although the survival limits of this population were not determined here, it was observed that coelomic chloride remains isoionic with media as concentrated as 160% SW, while it continues to be hyper-regulated at salinities as low as 7% SW.

It is reasonable to assume that all of the coelomic fluid solutes have been accounted for, since the differences between the summed solute levels and that measured by freezing point depression are negligible. Addition of sulfate and bicarbonate (which were not directly measured) to the summed sea water totals would only increase them by 2%. The contribution of these ions to the total solute levels in the coelomic fluid is also probably small.

The extracellular space

As the salinity is reduced there are increases in the hydration of both the whole animal and the muscle tissue (Fig. 1). An approximation of how this water gain is apportioned between the intra- and extracellular spaces is given by the chloride space of the tissue:

$$\text{Cl space} = \frac{[\text{Cl}]\ \text{tissue mmoles / kg tissue } H_2O}{[\text{Cl}]\ \text{ctl mM}}.$$

The major assumption here is that all of the chloride measured in the muscle tissue is restricted to the ECS. Since this is likely to be false, the value obtained represents a maximum size for this compartment.

TABLE II

Extracellular space estimates of muscle tissues excised from worms adapted to various salinities

% SW	% Cl space	% Inulin space $\bar{X} \pm 2$ S.E. (N)	
		In vitro	*In vivo*
100	22.7	15.6 ± 1.1 (63)	17.7 ± 1.0 (130)
50	20.2	—	—
35	19.2	—	—
20	20.2	15.3 ± 1.2 (53)	17.5 ± 1.1 (58)
10	20.1	—	—

The inulin space, derived in the same manner as the Cl space, more accurately represents the ECS, since inulin is excluded from the fiber interior. Inulin and chloride space values from worms adapted to the various salinities are presented in Table II. Three interesting features emerge from these data. First, the inulin space is consistently less than the chloride space. Therefore, some chloride must be located within the fiber interior. Secondly, the mean inulin space values indicate a larger ECS in the *in vivo* technique. Hydrostatic pressure within the coelomic compartment might force more fluid into the extracellular spaces, thereby expanding the inulin space. With the *in vitro* method no hydrostatic pressure gradient surrounds the tissues, thus a slightly smaller ECS value might be expected.

Of greatest interest is the fact that the steady state ECS, as measured by both the Cl and inulin spaces, is not significantly altered as the salinity of the medium is lowered. Since the water content of the tissues increases (Fig. 1), the constancy of the extracellular space must be interpreted to mean that the volume of the muscle fibers, relative to the extracellular fluid volume, is constant. That is, the water gain is proportionally distributed between the two compartments.

Intracellular solute levels

The apparent intracellular concentration ([I], mmoles/kg cell water) of a given solute was calculated as follows:

$$[I] = \frac{[T] - ([C] \cdot (ECS))}{1 - (ECS)}$$

In this equation [T] is the mean tissue concentration of a given solute in mmoles/kg tissue water; [C] is the mean coelomic fluid solute concentration, mM; ECS is the extracellular space as estimated by the *in vivo* inulin space. It should be noted that this equation incorporates two assumptions of questionable validity. It is assumed that all of the analyzable water is acting as solvent, and furthermore, that all of the solute measured is osmotically active. Because of the limitations imposed

TABLE III

Apparent intracellular solute concentrations in the muscle fibers of worms adapted to various salinities (all values expressed as mmoles per kg cell water; $\bar{X} \pm 2$ S.E.)

Solute	100% SW	50% SW	35% SW	20% SW	10% SW
Na	48.1 ± 5.3	19.5 ± 3.1	6.6 ± 2.4	6.3 ± 1.6	10.3 ± 2.2
K	233.9 ± 2.7	184.2 ± 2.4	143.9 ± 1.9	143.8 ± 1.9	133.5 ± 1.8
Ca	14.1 ± 0.0	9.1 ± 0.1	10.0 ± 0.1	7.3 ± 0.1	6.3 ± 0.1
Mg	16.8 ± 0.3	13.5 ± 1.5	12.3 ± 0.1	8.6 ± 0.1	10.0 ± 0.0
Cl	33.3 ± 6.2	9.0 ± 3.5	4.0 ± 2.7	4.6 ± 1.8	2.9 ± 1.2
Phosphate fractions*	66.9 ± 0.8	49.3 ± 0.7	49.3† ± 0.3	47.2† ± 0.4	46.0 ± 0.6
Nin + N	494.9 ± 2.6	186.1 ± 2.5	161.3 ± 0.6	91.8 ± 1.2	81.4 ± 1.1
% of total coelomic osmotic activity	81.9	83.3	94.7	114.6	145.2

* Phosphate fraction: free PO_4, phosphagen PO_4, ATP.

† Interpolated estimates.

by these assumptions (see discussion) the values in Table III are described as *apparent* intracellular solute concentrations.

The greatest source of variance in estimating intracellular solute concentrations was the measurement of ECS. Thus, the ranges of the values shown in Table III have been calculated using the mean value of ECS ± 2 S.E.'s. Although inulin space estimates were made at only 100% and 20% SW, the fact that neither this parameter nor the Cl space (which was measured at all salinities) changes appreciably as the salinity is reduced has encouraged us to estimate intracellular solute levels at the other salinities by using the same ECS values.

In muscle cells from worms adapted to full strength sea water the major osmotic constituents, Nin + N and K, make up 54% and 26%, respectively, of the solutes measured. In muscle fibers from worms adapted to 10% SW, Nin + N and K are again the major components, but in this diluted condition their respective contributions are 28% and 46%. In other words, as the salinity decreases, the steady state osmotic roles of free amino acids and potassium are reversed. This reversal of importance is indicative of the large decrease (some 414 mmoles/kg cell water) in intracellular Nin + N upon dilution from 100% to 10% SW.

It is not known if all of the intracellular osmolytes present in *Neanthes* muscle have been accounted for in this study. In Table III the summed concentrations of the individual solutes have been expressed as a fraction of the measured coelomic fluid osmotic activity. In muscle fibers from worms adapted to 100% SW only 82% of the extracellular activity was accounted for. When expressed as a fraction of the coelomic fluid activity the intracellular solute totals increase with decreasing salinity. Thus, in muscle fibers from 10% SW adapted worms the summed intracellular solute level is 145% of the coelomic fluid activity. This observation is considered in more detail in the discussion.

It is evident that all intracellular solutes decrease in concentration with decreasing salinity. The extent to which these solute reductions result from increments in cell hydration is of major interest. Solute concentrations ($[I]_{predicted}$)

TABLE IV

The ratios of the observed (O) to predicted (P) apparent intracellular concentrations of the various muscle solutes from worms adapted to several dilutions

Solute	50% SW O/P	35% SW O/P	20% SW O/P	10% SW O/P
K	1.06	0.97	1.03	1.14
Ca	0.87	1.11	0.87	0.87
Mg	1.08	1.15	0.84	1.18
Phosphate fractions*	0.96	1.11	1.13	1.32
Na	0.55	0.21	0.22	0.42
Cl	0.37	0.19	0.23	0.17
Nin + N	0.49	0.49	0.31	0.36

* Phosphate fractions: free PO_4, phosphagen PO_4, ATP.

which would result solely from cell hydration changes may be estimated by the following relation (modified from Clark, 1968b):

$$[I]_{predicted} = \frac{[I] \cdot (\%H_2O_{100}) \cdot (1 - ECS_{100}) \cdot (\%DW_{exp})}{(\%H_2O_{exp}) \cdot (1 - ECS_{exp}) \cdot (\%DW_{100})}.$$

Previously undefined terms used in this equation are: $\%H_2O$, the per cent tissue water; $\%DW$, the per cent dry weight of the tissue. The subscripts, 100 and exp, refer to the 100% and experimental salinities, respectively. The assumptions previously presented for the calculation of apparent intracellular concentrations are also inherent in this relation.

The predicted concentrations, resulting from changes in tissue hydration alone, have been computed for each of the muscle solutes. The results are presented in Table IV by comparing the observed (O) to the predicted (P) concentrations as an O/P ratio. At least two major responses may be noted. The first group of solutes have predicted concentrations similar to the observed levels. Cellular potassium is most consistent in this respect, having O/P ratios very close to one at all salinities. Calcium, magnesium, and the summed phosphate fractions also appear to fall within this category, but with less certainty. The second group includes intracellular sodium, chloride, and the Nin + N fraction. These solutes exist in

the diluted fibers at apparent concentrations much less than would be predicted on the basis of increases in cell water. That is, the influx of water does not wholly account for the observed reductions in concentration of these solutes.

Discussion

The coelomic fluid of this population of *Neanthes succinea* is isosmotic with the environment to about 35% SW, below which hyperosmotic regulation occurs. Other populations have been shown to respond to dilution in a qualitatively similar fashion (Oglesby, 1965). The salinity at which hyperregulation begins appears to be similar for all osmoregulating nereids, while major differences are often observed with respect to their capacity and extent of regulation. Thus, specimens of *N. succinea* from Alamitos Bay are much weaker hyperosmotic regulators than the same species from other localities. For example, the San Francisco population in 10% SW has a coelomic fluid osmotic activity some 70 milliosmoles greater than the worms studied here (Oglesby, 1965). These discrepancies between populations of the same species are considered as being indicative of the physiological, and perhaps genetic, plasticity of nereid polychaetes. Such physiological variability has been noted previously in populations of *N. diversicolor* from different parts of its geographical range with respect to osmoregulation (Ellis, 1937) and chloride regulation (Smith, 1955). Intraspecific variation in the amino acid composition of isolated populations of *N. succinea* from Alamitos Bay and the Salton Sea have been described (Mearns and Reish, 1969), further supporting the idea that these organisms are physiologically diverse.

The maintenance of hyperosmotic body fluids, achieved mainly by the retention of NaCl, at salinities less than 35% SW in *N. succinea* and *N. diversicolor* (Oglesby, 1970) suggests an active process. Two avenues for the active regulation of solutes have been demonstrated in nereid polychaetes. First, in *N. diversicolor* the urine concentration in the conforming range is isosmotic with the coelomic fluid, while in the regulating range a progressively more hyposmotic urine is elaborated with decreasing salinity (Smith, 1970b). It is possible that solute retention by means of hyposmotic urine production is also important in *N. succinea*. Secondly, it has been recently demonstrated that a sodium translocating system exists in the body wall of *N. succinea* (Doneen, 1971). This system is present in worms acclimated to salinities lower than 35% SW, but appears to be inactive in animals living in the conforming range. Smith (1970a) has also demonstrated active chloride uptake in *N. diversicolor* at salinities where regulation occurs.

In the present study coelomic fluid potassium was shown to be hyperionic to the medium over the entire range of salinities. Both Oglesby (1970) and Fletcher (1970) obtained similar results with *N. diversicolor*. Coelomic fluid ultrafiltrates prepared by Fletcher indicated that the elevated K is not attributable to binding by coelomic fluid proteins. Oglesby (1970) proposed that the hyperionic state of K measured in the coelomic fluid of *N. diversicolor* and other polychaetes (Oglesby, 1969) is an artifact of the sampling procedure, contending that repeated penetration of the body wall causes sufficient tissue leakage to increase the coelomic fluid K levels. If, however, intracellular leakage were a major factor, other solutes such as Nin + N might also be expected to contribute similarly to the coelomic fluid Nin + N levels. Thus, if Nin + N leakage were proportional to the presumed K

leakage in worms adapted to 100% SW, then an amount of Nin + N greater than actually measured would have come from this source.

When *N. succinea* is acclimated to low salinities, Ca and Mg are concentrated in the coelomic fluid in addition to NaCl. A qualitatively similar finding was reported by Fletcher (1970) for *N. diversicolor*. In this species Fletcher demonstrated that about 20% of the Ca and 14% of the Mg are bound to coelomic fluid proteins. This amount of binding, however, was not sufficient to explain the observed concentration of these solutes in the coelomic fluid at low salinities. Fletcher proposed that a major portion of this solute accumulation results from the passive diffusion of Ca and Mg across the body wall into the coelomic fluid in response to the inside negative electrical potential generated across the body wall of *N. diversicolor* at low salinities. Calcium and magnesium retention via the production of a hypotonic urine was not ruled out however. The inside negative potential generated by *N. succinea* at low salinities is slight (not exceeding −3.5 mV in 20% SW, Doneen, 1971), and could account for only three-fourths of the observed divalent solute levels. Since the extent of binding of these solutes to coelomic fluid proteins in *N. succinea* is not known, it is difficult to state with certainty whether an active or passive process (or both) is involved.

It is of interest to note that the ability to control body volume in *N. diversicolor* is in part governed by the presence of Ca in the environmental medium (Ellis, 1937). When 20% SW acclimated worms are transferred to an isotonic, Ca-free sea water there is a large and rapid influx of water. The observed accumulation of Ca at extreme dilutions may be essential for the establishment or maintenance of reduced body wall permeabilities required for survival. In this sense Ca concentrating mechanisms may be as important as the Na accumulating systems at low salinities.

Tissue inorganic solute levels have been reported for several marine worms from stenohaline habitats. Whole muscle solute concentrations, particularly K, in *Phascolosoma* (Steinbach, 1940), *Eunice* (Dejorge, Petersen, Ditadi, and Sawaya, 1966), and *Sipunculus* (Dejorge, Petersen, and Ditadi, 1970) are lower than those observed in body wall musculature of *N. succinea* (present report) or *Arenicola* (Cowey and Shaw, unpublished, in Potts and Parry, 1964) adapted to 100% SW. Reasons for such variations are not apparent. They are unlikely to result from variations in the ECS, since potassium, primarily an intracellular solute, is up to three times more concentrated in *Neanthes* and *Arenicola* muscle than in the other worms.

The major osmotic component in the muscle tissue of *Neanthes* is the Nin + N fraction. This is in agreement with the observations of Clark (1968a) on a variety of polychaetes, including the closely related *N. vexillosa*. The tissue amino acid concentrations of *Arenicola* are similar to those of *N. succinea* (Jeuniaux *et al.*, 1961; Cowey and Shaw, unpublished, cited in Potts and Parry, 1964), and result primarily from two amino acids, alanine and glycine. Although no qualitative analyses of the amino acids in *N. succinea* muscle tissue were made in this study, a disproportionality in the levels of individual amino acids has been previously reported. In specimens of *N. succinea* from Alamitos Bay it was noted that up to 50% of the total alcohol extractable amino acids from whole worms are alanine and glycine (Mearns and Reish, 1969).

Intracellular solute concentrations have never been determined for any polychaete muscle tissue prior to the present report. Gilbert and Shaw (1969), however, measured the cation concentrations in the extruded axoplasm of the single median giant axon of the sabellid *Myxicola*. This fluid was found to be isosmotic to the bathing medium. In the following considerations, the assumption is made that the osmotically active water of the muscle fiber interior is in osmotic equilibrium with the extracellular *milieu*. This assumption is supported by direct observations, such as the freezing point depression of intact fibers, on several different muscle tissues (Potts, 1952; Shaw, 1958b; Dick, 1971).

Evidence supporting the inhomogeneous nature of the cell interior is accumulating. The assumptions inherent in the method of calculation of intracellular concentrations are most likely invalid on two accounts. First, much evidence can be adduced to support the conclusion that a significant fraction of the total cell water behaves as though it were osmotically inactive. In *Callinectes*, for example, 33% of the muscle fiber water is osmotically inactive (Lang and Gainer, 1969a). Using seven independent estimates Hinke (1970) concluded that 25% of the analyzable water in *Balanus* fibers does not act as solvent for myoplasmic solutes. Furthermore, this non-solvent space was shown to have a volume equal to the osmotically inactive space of these fibers.

This concentrative effect of reduced solvent volume may be offset by reductions in intracellular osmotic activity. Various experimental approaches have indicated that a significant portion of inorganic ions are compartmentalized or bound in invertebrate muscle. In *Homarus*, immobile or non-exchangeable components for both Na and Cl have been observed (Dunham and Gainer, 1968). McLaughlin and Hinke (1966) measured Na and K activities and the solvent space of the myoplasm of *Balanus* fibers and concluded that 92% of the intracellular Na and 38% of the K are bound. Robertson (1961) found that the fluid mechanically expressed from *Nephrops* muscle tissue differs in ion composition from intact muscle. In this case 82% of the Na and 26% of the myoplasmic K were considered bound.

One of the salient features of the data presented here is the fact that the apparent total intracellular solute concentration in *Neanthes* muscle fibers is not proportional to the measured coelomic fluid osmotic activity over the range of salinities studied. That is, in fibers from animals adapted to 100% SW the total measured solute concentration is only 82% of that in the coelomic fluid, while in fibers from worms adapted to 20% SW the summed solute level is 115% of the coelomic concentration. Although confidence can be placed only on the data from animals adapted to 100% and 20% SW, since those at the other salinities were calculated using estimated ECS values, Table III suggests that the apparent total intracellular solute concentration, relative to that in the coelomic fluid, progressively increases as the salinity is reduced.

The most plausible explanation for this sort of result arises from the questionable validity of assuming isosmoticity between the total cell water and the coelomic fluid. That is, the volume of the solvent space or the extent of solute binding may not be constant over the range of coelomic fluid osmotic pressures to which the muscle fibers are exposed. For example, as the salinity is reduced, particularly from 50% to 10% SW, either there may be increases in the osmotically active volume (solvent space) relative to the total cell water, or there may be increments

in the proportion of solute bound to intracellular protein. Additionally, it is possible that both phenomena could occur together to produce the observed result, namely an apparent increase in the osmotic activity in diluted fibers relative to that in the coelomic fluid.

Our observations indicate that the changes in concentration of certain intracellular solutes of *N. succinea* (K, Ca, Mg, PO_4 fractions) could be explained merely by changes in the total intracellular water content, while others (Na, Cl, Nin + N) can not. This agrees well with the results presented by Shaw (1955) for single muscle fibers of *Carcinus* and the data of Cowey and Shaw (in Potts and Parry, 1964) for whole muscle solutes of *Arenicola*. Shaw's tracer studies on *Carcinus* muscle (1958a) indicate that inorganic phosphate is merely diluted by increases in cell hydration because the muscle fibers are impermeable to this solute. The dilution of potassium, however, appears to result from equal inward and outward fluxes, rather than to its being impermeant. Due to the similarity of muscle solute responses to dilution between *Neanthes* and *Carcinus* it would not be surprising to find that these solutes are distributed in *Neanthes* muscles because of similar membrane properties.

The reductions in intracellular amino acid concentration below the level expected after hydration is a commonly observed phenomenon in marine polychaetes (Jeuniaux *et al.*, 1961; Cowey and Shaw, in Potts and Parry, 1964; Virkar, 1966; and Clark, 1968b) and euryhaline marine invertebrates in general (Florkin and Schoffeniels, 1969). It is not necessary that these nitrogenous solutes be actually removed from the myoplasm, but only that they be rendered osmotically inactive. Whether intracellular amino acids in *N. succinea* are extruded from muscle fibers as in *Callinectes* (Lang and Gainer, 1969b) and *Eriocheir* (Vincent-Marique and Gilles, 1970), or sequestered from the osmolyte pool by incorporation into protein as in *Melanopsis* (Bedford, 1971) and *Ophiactis* (Stephens and Virkar, 1966) remains to be demonstrated.

Osmotic inactivation of nitrogenous solutes, regardless of the means by which it occurs, is believed to be an important part of the osmotic adjustments of the cell interior during adaptation to reduced salinities. Cell volume regulation in response to hypotonic salines has been described in single muscle fibers of *Callinectes* and inferred from the minimal tissue hydration changes in many other euryhaline marine invertebrates. Isolated muscle tissues of *N. succinea* also show a definite volume regulatory response to moderately hypotonic salines (Freel, unpublished observations). Despite the dynamic role of free amino acids in intracellular osmoregulation this is probably not their major function in the tissues of marine invertebrates (Shaw, 1958b). Strong support for this contention has been provided by Clark (1968a) who noted that tissue Nin + N levels in several stenohaline polychaetes are even greater than the levels reported for *N. succinea*. It is, then, the mechanism of osmotic inactivation that defines the capacity for intracellular osmotic regulation, rather than the high concentrations of free amino acids *per se*. Due to the ubiquitous distribution of high levels of amino acids in the tissues of marine invertebrates a more fundamental hypothesis regarding the steady state function of these nitrogenous solutes is required.

Summary

1. The solute composition of the coelomic fluid and ventral musculature of a euryhaline polychaete, *Neanthes succinea,* acclimated to various salinities was examined.

2. The coelomic fluid of these worms conforms osmotically and ionically (with the exception of potassium) with salinities as low as 35% SW. Below this point hyperosmotic regulation of the coelomic fluid occurs as a result of the retention of NaCl. Calcium and magnesium are also slightly concentrated in the regulating range but they do not contribute significantly to the hyperosmotic plateau. The means of solute retention in the coelomic fluid are considered.

3. The extracellular space of the muscle tissue remains unchanged over the salinity range, although the tissue does become more hydrated. Thus, the water gain upon adaptation to dilute conditions must be proportionally distributed between the intra- and extracellular spaces.

4. The sum of the apparent concentrations of the intracellular solutes measured was not a constant proportion of the coelomic fluid osmotic activity, possibly indicating that either the solvent volume or the extent of solute binding varies with the external osmotic activity.

5. Intracellular solute concentrations fall with decreasing salinity. The reduction in concentration of K, PO_4 fractions, Ca, and Mg may be explained merely on the basis of increases in cell hydration, while the lower concentrations of Na, Cl, and Nin + N are not wholly accounted for by the influx of water. The removal of a large fraction of the free amino acid pool is the major osmotic adjustment observed in the muscle fibers of this worm.

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SERUM COMPOSITION OF FRESHWATER STINGRAYS (POTAMOTRYGONIDAE) ADAPTED TO FRESH AND DILUTE SEA WATER

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The osmotic adaptation of elasmobranch fishes to marine environments involves the retention of organic molecules, particularly urea, to maintain the osmotic pressure of body fluids at, or slightly in excess of, environmental levels. Recent discoveries that urea retention is lost in freshwater stingrays from South America (Thorson, Cowan and Watson, 1967; Junqueira, Hoxter and Zago, 1968) and is present in the coelacanth (Pickford and Grant, 1967; Lutz and Robertson, 1971), however, indicate that the mechanism is neither ubiquitous in nor restricted to elasmobranchs. The observations that serum urea levels are markedly reduced when marine or euryhaline elasmobranchs are subjected to dilutions in environmental salinity (Smith, 1931; Price and Creaser, 1967; Urist, 1962; Thorson, 1967) and that renal urea loss is increased (Smith, 1931; 1936) suggest, conversely, that freshwater stingrays might decrease urea loss when subjected to increased salinity and utilize this molecule in osmoregulation. Preliminary investigations of this possibility by Thorson (1970) failed to demonstrate elevated urea levels in the blood of fish adapted for short periods of time to increasing salinities. Clearly further investigations, employing more gradual adaptation, are needed.

From the point of view of inorganic electrolytes and osmolarity the freshwater stingrays have levels lower than marine elasmobranchs and comparable with those of teleosts (Thorson *et al.*, 1967; Junqueira *et al.*, 1968). Whether the mechanisms of ionic and osmotic regulation are also comparable is not known. While other elasmobranchs are known to enter fresh water (Smith, 1931) only the South American stingrays are permanent residents in this medium. In fact the group has probably lived in fresh water for millions of years. Larrazet (1886) described fossil stingrays from Tertiary deposits in the Rio Parana basin which Garman (1913) placed in his genus *Potamotrygon*. This long adaptation to fresh water of a predominantly marine group makes the river rays of considerable interest in terms of physiological evolution.

The purpose of the present investigation is twofold: to survey the major inorganic and organic serum constituents of freshwater stingrays in an attempt to

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elucidate the factors permitting such rays to live permanently in fresh water in contradistinction to all other known elasmobranchs, and to ascertain whether gradually-increased salinity affects these parameters. In addition, some data on electrolyte composition in other body fluids (pericardial and perivisceral) are presented.

Materials and Methods

The fish used in the present studies, reputedly from the Amazon basin, were obtained through a local tropical fish retailer (Connecticut Aquarium; East Haven, Connecticut) in late April, 1970. A total of 20 fish were acquired; twelve of these succumbed to infections prior to the initiation of experiments and data on eight fish are reported here. All were juveniles of small size, ranging in weight from 61 to 190 grams.

The stingrays were initially adapted to running, dechlorinated New Haven tap water at 25° C. Fish were maintained in separate 20 gallon aquaria and were fed a diet of live tubifex worms. The fish were kept under laboratory conditions for one and one half months before the initiation of experiments. At this time the surviving stingrays (13) were divided into two groups: 6 freshwater controls and 7 fish subjected to increased salinity. Salinity was raised by permitting Long Island Sound water (salinity 29‰) to slowly drip into the aquaria. Increases of 0.7‰ per day were accomplished in this manner. A preliminary test on a single fish indicated that osmoregulatory failure occurred at 20.6‰, and it was concluded that a salinity of 14.5‰, intermediate between this value and one isosmotic with serum (based on the data of Thorson *et al.*, 1967), would be ideal for our purposes. Of the six remaining experimental fish, three succumbed to infections during the 20 days that salinity was being increased (at 7.0, 8.7 and 10.6‰); two of the control fish died also. At the time of autopsy there remained four freshwater controls and three experimentals at a salinity of 14.5‰. All fish save one of the experimentals appeared to be in excellent condition.

At autopsy the fish were anesthetized in MS 222 (one part to 1000 parts of water from the tank), sponged with distilled water, blotted dry, weighed, and the pericardical cavity was exposed. Pericardial fluid was collected in microhematocrit tubes, the pericardium was blotted dry, and the heart was punctured with fine scissors. Whole blood was drawn immediately for pH measurements and blood was taken for hematological studies. Blood was then collected in microhematocrit tubes, permitted to clot, and centrifuged. The abdomen was opened, perivisceral fluid was taken, and the livers removed, weighed, and frozen for shipment to Dr. Leon Goldstein, Brown University. Aliquots of serum were taken for total CO_2, $Na+/K+$ and chloride, and the remainder was frozen in tightly-corked vials at −20° C for subsequent analyses.

The methods and procedures used in the analysis of serum and other fluids were essentially identical to those developed for studies on *Fundulus heteroclitus* and described in detail in Pickford, Grant and Umminger (1969). Only microliter samples were required in most cases and all analyses were completed within one month of autopsy. Procedures for hematological studies were as described in Pickford, Srivastava, Slicher and Pang (1971).

Some difficulties of systematics

It was observed that our stingrays included a diverse assemblage of different forms in respect to external morphology. As it seemed possible that such morphological variability might be associated with physiological differences and as an aid in comparison of our data with other studies, an attempt was made to establish a sound nomenclatural designation for our fish. Our attempts to relegate specific names to the stingrays studied in the present report proved less than successful for several reasons: (1) uncertainty as to the applicability and status of the generic names within the family Potamotrygonidae; (2) presence within our sample of forms not readily assignable to any described species of river ray; (3) uncertainty as to the status of and relationships between any of the putative species of Potamotrygonidae; and (4) ignorance regarding the precise region(s) within the Amazon drainage from which our rays were collected. In view of the current interest in these fishes and, perhaps, as a spur to systematists to settle some of the taxonomic ambiguities in the river rays, some further discussion appears pertinent.

The first point to be made concerns the generic appellation *Potamotrygon* (Garman, 1877) frequently applied to the river rays. Of the five nominal genera in the family Potamotrygonidae three (*Elipesurus* (Schomburgk, 1843), *Paratrygon* (Dumereil, 1865) and *Disceus* (Garman, 1877)) have priority over *Potamotrygon*. Recently Castex (1968), after concluding that the type species of *Elipesurus*, *E. spinicaudata,* was identical to *Trygon brachyurus* Günther (a species generally placed in the genus *Potamotrygon*), proposed for reasons of simplicity that the genus *Elipesurus* be suppressed under the plenary powers of the International Commission on Zoological Nomenclature (I.C.Z.N.) in favor of *Potamotrygon*. Castex (1968) avoided use of the nominal genus *Paratrygon* used by Fowler (1948) on the grounds that identification of the type species (the "aiereba" of Marggrave) was not possible. In a reply to Castex, Bailey (1969) agreed with Castex's treatment of *Paratrygon* but suggested that *Elipesurus spinicaudata* was identical to *Disceus thayeri* Garman. Bailey thought it unwise to suppress *Elipesurus* and suggested that it should replace *Disceus*. Although both authors have stressed the fact that the type species of *Paratrygon* is unidentifiable we might note that Dumereil's description of the genus was based explicitly on a specimen (in the Munich museum) rather than Marggrave's figure of the "aiereba." Subject to rulings of the I.C.Z.N., identification of the Munich specimen could establish the identity of the type species of *Paratrygon* and make the genus available for use. While authors have consistently regarded *Disceus* and *Potamotrygon* (or their equivalents) as distinct from one another, one of the forms studied in the present report is intermediate in several respects between *Disceus* and *Potamotrygon* as defined by Garman (1913). In view of the nomenclatural uncertainty we might suggest, pending revisionary studies of the group, that future physiological studies on the river rays avoid indiscriminate use of generic names. Already Bailey (1969) has used the existence of the name *Potamotrygon* in the physiological literature (apparently based solely on Thorson *et al.* (1967) and Mathews (1966); Smith (1931) referred only to *Elipesurus* and Thorson *et al.* (1967) also referred to *Elipesurus* and *Disceus*) as an argument for its retention.

A comparison of our specimens with published descriptions of river rays (compiled in Garman, 1913) demonstrated that one form agrees with '*Potamotrygon*'

motoro and a second with *'P.' reticulatus*. A third form was the aforementioned intermediate between *'Potamotrygon'* and *'Disceus'* and three are apparently undescribed types of *'Potamotrygon.'* As little is known of geographic variation or species relationships in river rays and as we do not know the localities from which our fish were collected further systematic treatment is not possible. The physiological data presented here involves only three forms: three specimens of *'Potamotrygon' motoro* (one in fresh water and two in dilute sea water); four of an unidentified *'Potamotrygon'* (two specimens in fresh water, one in dilute sea water and one failing at 20.6‰); and one specimen of the intermediate in fresh water. Fortunately, we observed no consistent variability in the physiological parameters studied which could be correlated with morphological differences or sex and we feel

TABLE I

Hematology and collateral data on rays adapted to fresh or dilute sea water

	Fresh water	One half sea water	
		With sick fish	Sick fish omitted
Number	4	3	2
Hepatosomatic index	2.08 ± 0.16	1.56 ± 0.34	1.87
RBC (10^6/mm^3)	0.296 ± 0.032	0.253 ± 0.057	0.298
RBC length (μ)	17.65 ± 0.87	17.97 ± 0.69	17.30
RBC width (μ)	11.90 ± 0.60	11.60 ± 0.68	11.10
RBC nuclear length (μ)	7.66 ± 0.24	7.60 ± 0.21	7.40
RBC nuclear width (μ)	5.69 ± 0.57	5.10 ± 0.10	5.00
Thrombocytes (10^3/mm^3)	33.8 ± 1.3	35.0 ± 0.47	30.0
Leucocytes (10^3/mm^3)	5.12 ± 1.49	3.70 ± 1.26	4.95
Sm. lymphocytes (% WBC)	65.3 ± 6.4	70.0 ± 10.0	80.0
Lg. lymphocytes (% WBC)	8.5 ± 2.9	10.7 ± 4.7	13.5
Monocytes (% WBC)	13.8 ± 9.0	9.3 ± 7.9	1.5
Neutrophils (% WBC)	7.5 ± 4.8	5.0 ± 2.0	2.5
Eosinophils (% WBC)	5.0 ± 1.7	5.0 ± 2.9	2.5

justified in lumping forms for statistical comparison. The carcasses of all stingrays studied in the present investigation have been preserved and deposited in the fish collections of the Peabody Museum of Natural History, Yale University (Bingham Oceanographic Collection catalogue number 6995).

RESULTS

Hematology and collateral data

Data on the hematology and on collateral parameters of freshwater stingrays are presented in Table I. No significant effect of salinity was found on any hematological parameter studied nor was the hepatosomatic index or liver color affected by the salinity of the medium. There is an apparent correlation of hepatosomatic index and erythrocyte and leucocyte counts with health; the fish which had the lowest values for these parameters was markedly sluggish at autopsy and had very low levels of serum organic constituents (glucose, total carbohydrates, cholesterol, urea and proteins). As the possibility exists that the aberrant values of this fish

for collateral data and serum organic constituents might have been induced by high salinity rather than health, we have presented the data on these parameters both with the sick fish included and omitted (Tables I and III). Blood smears from most of our specimens revealed the presence of a bacterium similar to that responsible for hemorrhagic disease in *Fundulus heteroclitus* (*Aeromonas* sp.). There was no relationship between severity of infection and health; the sick fish had only a moderate infection while several heavily infected fish seemed to be in perfect health.

A comparison of our data on liver size with values on marine elasmobranchs reviewed by Olivereau and Leloup (1950) showed that our values were relatively low. Many factors including maturity, sex, diet and buoyancy functions affect the hepatosomatic index in elasmobranchs, however, and it is difficult to interpret our values in respect to those of marine species.

Red cell counts in river rays were in the range reported for marine elasmobranchs by Malassez (1872) and Saunders (1966); these levels are much lower than those of teleosts. Erythrocyte size averaged $17.8 \times 11.8\ \mu$; a value comparable with those reported for many marine elasmobranchs by Saunders (1966) but somewhat smaller than values reported for other batoids by Malassez (1872), Kisch (1951) and Saunders (1966). Our data on white cell counts and differential counts are in essential agreement with those of Saunders (1966) on marine elasmobranchs. We observed no basophils in our fish.

Survival in hyperosmotic media

Our preliminary data, based largely on the failure of a single healthy fish, indicated that juvenile stingrays are unable to survive at salinities in excess of 20.6‰. Earlier failures (at 7.0, 8.7 and 10.6‰) of experimental fish were associated with bacterial or fungal infections and control fish showed equivalent mortality. Three specimens reached a salinity of 14.5‰, and of these, two were still healthy.

Our data, such as they are, appear to conflict with those of Thorson (1970) who adapted several fish to salinities in excess of 20‰, one of which reached a final salinity of 32.3‰. Thorson did not report that any of his fish adapted to high salinities were at or near failure. Several factors could be responsible for the apparent discrepancy: size differences, differences in rate salinity increase and possible inter- or intraspecific differences. While Thorson's fish averaged 2.24 kilograms, ours were under 200 grams. It is possible that osmoregulatory failure proceeds more rapidly in small fish. Although we are not certain of the actual rate of salinity increase in Thorson's study (as he only gave the final salinity and days elapsed since the beginning of salinity increase), it is apparent that our fish were acclimated more gradually (*ca.* 0.7‰ per day *vs.* 5.1 to 21.6‰ per day). The possibility of large inter- or intraspecific differences in salinity tolerance exists. Both phenonena are evident in species of the teleost genus *Fundulus* that are restricted to fresh water (Griffith, 1972). Additional studies are necessary before the factors affecting salinity tolerance in river rays are fully understood.

Inorganic cations ($Na+$, $K+$, $Ca++$, $Mg++$)

Significant increases in serum sodium (21%), calcium (48%) and magnesium (51%) were observed in fish adapted to one half sea water when compared with

freshwater controls (Table II). Serum potassium was also somewhat higher in the experimental fish (21%) although the increase was not significant. It is of interest that serum sodium in the saline-adapted fish is iso-ionic with the environment (198.3 *vs.* 197.7 mEq/l; Tables II and V). Determinations of serum sodium on one failing fish at a salinity of 20.6‰ (Table IV) showed levels only slightly less than calculated environmental sodium concentrations (262 *vs.* 280 mEq/l). Although serum calcium and magnesium are elevated, both electrolytes are maintained at levels below the environment in dilute sea water.

Our data on serum cation levels in fresh water and in dilute sea water show minor differences from those reported previously (Thorson, 1970; Junqueira *et al.*,

TABLE II

Serum inorganic electrolytes and blood pH in rays (Potamotrygonidae) adapted to fresh or to one half sea water (salinity, 14.5‰)

	Fresh water	Half sea water	Per cent change
Number	4	3	
Na+ (mEq/l)	164.0 ± 5.6	198.3 ± 2.7	+20.9**
K+ (mEq/l)	4.45 ± 0.25	5.37 ± 0.38	+20.7
Ca++ (mEq/l)	3.04 ± 0.41	4.50 ± 0.12	+48.0*
Mg++ (mEq/l)	2.31 ± 0.17	3.49 ± 0.22	+51.1**
Cl− (mEq/l)	151.7 ± 5.0	183.1 ± 2.0	+20.7**
Total CO_2 (mM/l, equilibrated with 7% CO_2)	9.75 ± 0.79	11.35 ± 1.75	+16.4
HCO_3− (mEq/l, estimated)	7.95 ± 0.79	9.55 ± 1.75	+20.1
Total P (mM/l)	1.96 ± 0.67	2.59 ± 0.35	+32.1
Inorganic P (mM/l)	1.29 ± 0.24	1.74 ± 0.05	+34.9
Inorganic P (mEq/l at pH 7.3)	2.28 ± 0.42	3.07 ± 0.08	+34.6
pH (whole blood)	7.296 ± 0.025	7.323 ± 0.100	
Total cations mEq/l)	173.8 ± 5.1	211.7 ± 3.0	+21.8**
Total anions (mEq/l)	161.9 ± 5.3	195.7 ± 3.7	+20.9**
Cation excess (mEq/l)	11.9 ± 1.9	16.0 ± 1.9	+34.5
Total inorganic ions (mM/l)	332.1 ± 10.6	402.1 ± 6.5	+21.1**

* Significantly different from freshwater controls; $P < 0.05$.
** Significantly different from freshwater controls; $P < 0.01$.

1968). Serum sodium is somewhat higher in freshwater controls while potassium, calcium and magnesium are somewhat lower. These discrepancies might be due to differences between unstressed, laboratory-acclimated fish and wild rays subjected to recent capture. Thorson (1970) reported increases in serum sodium and magnesium at elevated salinities but these changes were not consistently related to the final salinity of the medium. Effects of salinity on calcium and potassium were equivocal in Thorson's study.

In general, our data support the statement of Thorson *et al.* (1967) that inorganic electrolyte levels are comparable to those of teleosts rather than marine elasmobranchs. Our data also suggest that the river rays are unable to actively excrete sodium. This would fit with the reported absence of the rectal gland in potamotrygonids (Goldstein and Forster, 1971), an organ implicated in active sodium excretion in marine elasmobranchs (Burger and Hess, 1960). Our rays possessed an organ in the anatomical position of the rectal gland, but histological

TABLE III

Serum organic constituents and osmolarity of stingrays adapted to fresh or one half sea water

	Fresh water	One half sea water	
		With sick fish	Sick fish omitted
Number	4	3	2
Organic P (mM/l)	0.59 ± 0.44	0.80 ± 0.35	1.07
Total cholesterol (mM/l)	2.80 ± 0.76	2.77 ± 0.87	3.64
Total free carbohydrates (as mM/l glucose)	4.91 ± 0.32	4.33 ± 1.12	4.72
Glucose (mM/l)	1.22 ± 0.17	0.68 ± 0.42	1.02
Non-glucose carbohydrates (as mM/l glucose)	3.72 ± 0.30	3.32 ± 0.55	4.21
Urea (mM/l)	1.08 ± 0.13	2.31 ± 0.77	2.95
Total protein (mg%)	829 ± 218	819 ± 290	1071
Fraction I (mg%)	64 ± 22	67 ± 29	85
Fraction II (mg%)	289 ± 123	239 ± 126	324
Fraction III (mg%)	207 ± 30	283 ± 78	361
Fraction IV (mg%)	118 ± 31	116 ± 47	157
Fraction V (mg%)	152 ± 40	110 ± 26	136
Percent albumin (V/total)	19.2 ± 3.7	15.1 ± 2.6	12.7
Calculated osmolarity†	338.1 ± 10.3	408.7 ± 6.0*	
Measured osmolarity (milliosmoles/liter	282.0 ± 16.8	477.7 ± 49.5*	
Calculated-measured osmolarity	+56.1	−69.0	

† Calculated osmolarity includes all inorganic ions, urea and total carbohydrates.
* Significantly different from freshwater controls; $P < 0.01$.

examination (Leon Goldstein, Roy Forster and William Doyle, personal communication) revealed that it was structurally unlike the rectal gland of marine elasmobranchs and was probably non-functional in salt secretion. While calcium and magnesium seem to be regulated somewhat, excretion of these divalent cations is urinary (Burger, 1967; Hickman and Trump, 1969). Freshwater stingrays are capable of active sodium uptake (Pang, Griffith and Kahn, 1972), although this uptake is inefficient at low environmental sodium levels. It appears that the pattern of sodium balance as well as serum levels of this electrolyte resembles that of freshwater teleosts (*cf.* Maetz, 1970).

Inorganic anions (Cl−, total CO_2, inorganic P)

We observed a significant increase in serum chloride (21%) and similar, but on account of high variance, non-significant increases in total CO_2 (16%) and inorganic phosphorus (35%) in fish adapted to dilute sea water (Table II). A further increase in serum chloride was observed in the fish failing at 20.6‰ (Table IV). The changes in chloride were similar to those in sodium in terms of percentage change and milliequivalents per liter. As chloride was maintained at levels lower than those of sodium, and as chloride in sea water is higher than sodium, serum chloride levels were not iso-ionic with the environment at elevated salinities.

A comparison of our data with that of Thorson (1970) showed similar levels of chloride in freshwater sera although inorganic phosphorus was lower in our

study. Thorson (1970) reported elevated chloride in saline media, in substantial agreement with our findings. Serum chloride levels in river rays are intermediate between those of freshwater teleosts and marine elasmobranchs (Holmes and Donaldson, 1969). Bicarbonate [estimated from total CO_2 and the known equilibration pressure of CO_2 using the nomograph of McLean (1938)] is higher than the levels found in marine elasmobranchs but is comparable to that found in *Fundulus heteroclitus* (Pickford *et al.*, 1969). Our levels of inorganic phosphorus are similar to those in marine elasmobranchs and are lower than typically found in teleosts (Holmes and Donaldson, 1969).

Serum urea

Although there was a strong trend towards an increase of serum urea (114%) in dilute sea water, low values for the sick fish prevented the difference from being significant (Table III). Were only healthy fish considered, small numbers would preclude demonstration of the significance of the increase (173%). The levels of urea in control fish (1.1 ± 0.1 mM/l) are in close agreement with the results of Thorson *et al.* (1967) (0.7 to 1.8 mM/l), and are but slightly lower than those of Junqueira *et al.* (1968) (1.9 ± 0.3 mM/l), thus reaffirming the unique hypouremia of the river rays among elasmobranchs. Marine and euryhaline elasmobranchs have urea concentrations ranging from 80 to 450 mM/l (Holmes and Donaldson, 1969). Our data correspond to the observation of Thorson (1970) that high salinities elicit small and irregular increases in serum urea.

While urea levels in river rays are very low, the fish possess the necessary ornithine-urea cycle enzymes for urea biosynthesis, albeit at activities much lower than in marine elasmobranchs (Goldstein and Forster, 1971). Assays of our fish livers for carbamyl phosphate synthetase, the rate limiting enzyme in the urea biosynthesis pathway in marine elasmobranchs, failed to demonstrate consistent effects of salinity on activity or correlations between serum urea and enzyme activity (Leon Goldstein, personal communication). The data clearly demonstrate that the river rays are unable to accumulate urea to levels comparable to those

TABLE IV

Distribution of sodium, potassium, and chloride in serum, pericardial fluid and perivisceral fluid of rays in fresh or dilute sea water (values in mEq/l)

Group	Ion	Serum	Pericardial fluid	Perivisceral fluid
Fresh water (N = 4)	Na+	164.0 ± 5.6	112.9 ± 14.4*	115.7 ± 14.4*
	K+	4.45 ± 0.25	5.48 ± 0.95	6.65 ± 1.27
	Cl−	151.7 ± 5.0	157.0 ± 6.0	159.7 ± 6.9
Half sea water (N = 3)	Na+	198.3 ± 2.7	147.8 ± 0.8**	156.3 ± 8.8**
	K+	5.37 ± 0.38	6.23 ± 2.14	6.90 ± 0.67
	Cl−	183.1 ± 2.0	199.7 ± 7.8	189.1 ± 2.1
Failing at 20.6‰ (N = 1)	Na+	261.8	218.0	223.4
	K+	12.6	13.5	11.5
	Cl−	222.4	267.1	286.4

* Significantly different from serum values; $P < 0.02$.
** Significantly different from serum values; $P < 0.001$.

in marine elasmobranchs, even when subjected to high salinities. Whether this inability is due solely to a failure to increase enzyme activities in response to salinity or reflects, as well, a failure of the kidney to actively resorb urea at high salinities is uncertain. Both factors appear to contribute to the low urea levels in fresh water (Goldstein and Forster, 1971).

Serum carbohydrates

We failed to detect significant changes in serum glucose, total carbohydrates, or non-glucose carbohydrates in stingrays adapted to dilute sea water (Table III). Our levels of glucose are lower than typical elasmobranch values (Kiermeir, 1939) although this could be related to activity patterns, feeding, handling, or differences in method (Kisch, 1929; Kiermeir, 1939). A correlation with health is apparent in the sick fish which had no detectable glucose. Our data show a striking discrepency between glucose (1.22 mM/l) and total carbohydrate concentrations (4.91 mM/l as glucose) in control fish, suggesting that the predominant carbohydrates in stingray sera are not glucose. Similar relationships have been reported in the shark, *Scyliorhinus caniculus*, by Florkin (1936), Bocquet (1967) and Pérès and Rigal (1969). Parallel determinations of glucose and total reducing substances in the elasmobranchs *Raia erinacea* (Grant, 1964) and *Squalus acanthias* and the holocephalian *Hydrolagus collici* (Patent, 1970), however, failed to detect differences suggestive of significant levels of non-glucose carbohydrates. Although glucose appears to be the most abundant sugar in *Scyliorhinus caniculus* blood, Bocquet (1967) detected significant amounts of arabinose, fucose, xylose and rhamnose. Nixon (1965) found levels of meso-inositol in the related *S. stellaris* of 2.3 to 6.8 mg%. The identification of the non-glucose carbohydrates in the blood of river rays is a problem worthy of investigation.

Cholesterol and organic phosphorus

We observed no marked effect of salinity on measured serum cholesterol or total phosphorus, nor on derived values for organic phosphorus (Table III). Our cholesterol values are comparable to those reported in marine elasmobranchs by Mayer and Schaeffer (1913), Morris (1959), Sulya, Box and Gunter (1960), Urist and Van de Putte (1967) and Lauter, Brandenberger-Brown and Tram (1967). Our values for organic phosphorus were extremely variable (0.04 to 1.91 mM/l in freshwater controls) and parameters which might be derived from these values (*e.g.* phospholipid and the ratio of phospholipid to cholesterol) were correspondingly variable and are omitted. A highly significant correlation ($r = 0.933$; $P < 0.005$) exists between organic phosphorus and one of the protein fractions. The relationship appears to be a linear function with origin at 0; suggesting that most of the organic phosphorus in stingray sera is protein bound.

Serum proteins

An interesting finding of our study is the presence of significant levels of serum albumin in freshwater stingrays. While some of the early literature (*e.g.* Nolf, 1907; Roche, Derrien and Fontaine, 1940; Cordier, Barnaud and Brandon, 1958) reported the presence of "albumin" in elasmobranchs, it is now apparent that marine elasmobranchs lack significant amounts of a serum component with the electrophoretic mobility of mammalian albumin (Deutsch and McShan, 1949;

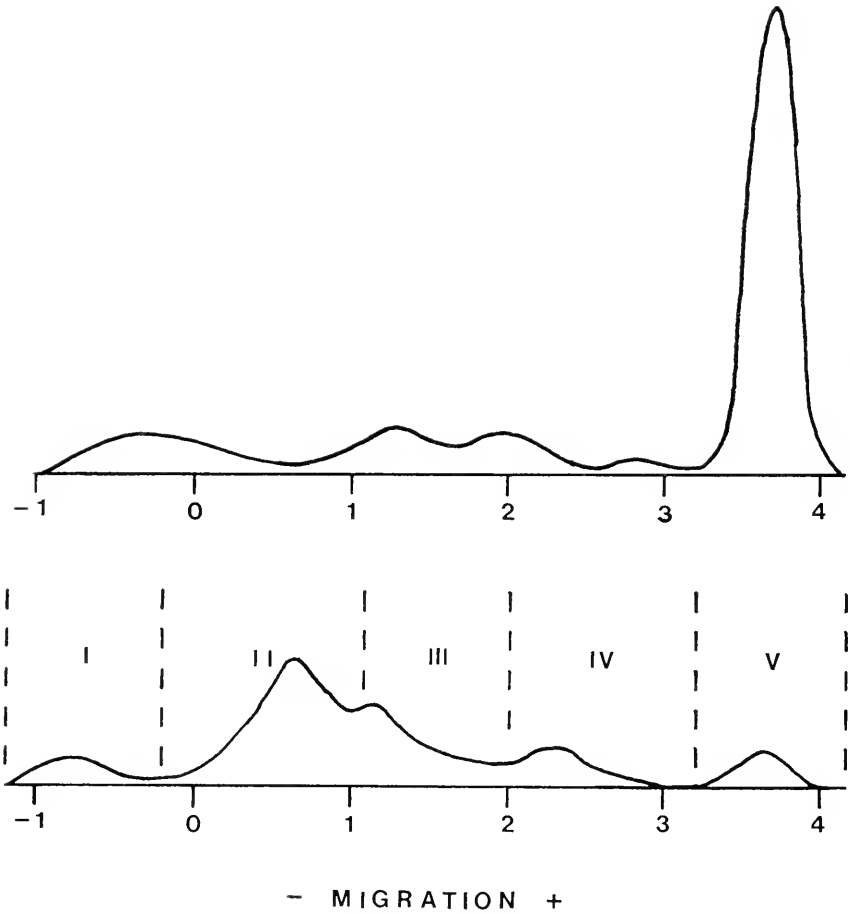

FIGURE 1. Comparison of densitometric tracings from protidograms of human (top) and freshwater stingray (bottom). Human pattern is from single application of standard Versatol (lot 0287050) and stingray pattern is from triple application of a freshwater control. Division of stingray pattern into five fractions is illustrated.

Irisawa and Irisawa, 1954; Sulya, Box and Gunter, 1961; Urist, 1961; Rasmussen and Rasmussen, 1967) or its physical properties (Urist, 1961). Urist and Van de Putte (1967) have used the absence of albumin as one of the distinguishing features of elasmobranchs, although a high mobility serum protein fraction is lacking in many teleosts as well (Pickford *et al.*, 1969). A statistical comparison of the positions of human albumin and the most mobile stingray fraction showed that the two were electrophoretically indistinguishable (Fig. 1). This comparison was between 18 stingray samples (including duplicates and several failing fish) and human standard sera (Versatol) run concomitantly, with the electropherograms positioned carefully on the densitometer so as to be directly comparable. Although the level of albumin was not high (58 to 231 mg%) it constituted a substantial fraction (10 to 26%) of the total protein.

Total protein levels (314 to 1430 mg%; Table III) are below those reported previously in river rays by Thorson *et al.* (1967) (1100 to 2300 mg%) but are similar to those found by Junqueira *et al.* (1968) (700 to 1300 mg%). Junqueira *et al.* (1968), using electrophoretic techniques, found five major protein zones. Our results are similar (Fig. 1). We found no differences in the position or distribution of the protein fractions which could be related to morphological type. A close correlation of one of the fractions (fraction II, Fig. 1) with organic phosphorus suggests that this component is a phosphorus-containing protein. We failed to detect any effect of salinity on total protein, on any individual fraction, or on the ratio of albumin to total protein. Nevertheless, it is worth speculating that the presence of albumin is one of the factor permitting the river rays to inhabit dilute media.

Serum osmolarity and total osmoticallly-active substances

Significant increases in both measured serum osmolarity (69%) and osmolarity calculated from the sum of the measured osmotically-active substances (21%) were observed in fish adapted to dilute sea water in comparison to those in fresh water (Table III). A rather large discrepancy between measured and calculated osmolarities was observed; the latter being 56.1 milliosmoles greater than the former in freshwater fish. Karhausen (1962) has pointed out that such discrepancies can occur as a consequence of ion-complexing substances in the blood or factors affecting salt dissociation such as temperature, pH or ionic concentrations.

In the present study it was found that the difference between measured and calculated osmolarities was reversed in dilute sea water; measured osmolarity being 69.0 milliosmoles greater than calculated values compared to 56.1 milliosmoles less in fresh water. This may be attributed to large increases in unmeasured serum components in the fish at the high salinity. The possibility that trimethylamine oxide, which like urea is retained by marine elasmobranchs at high levels, accounts for much of the unexplained increase in osmolarity would seem to be discounted by Thorson (1970) who found negligible amounts of this compound in saline-adapted river rays.

Thorson *et al.* (1967) give values for serum osmolarity (301 to 320 milliosmoles) which agree with our freshwater data in terms of levels (247 to 317), but differ in that they agree closely with calculated osmolarities. Junqueira *et al.* (1968) give somewhat higher osmolarities (350 ± 32 milliosmoles), but an insufficient number of parameters were determined to estimate total osmotically active substances. We believe that our observed difference between measured and calculated osmolarities in freshwater control fish is due to a physico-chemical property of stingray sera. Using identical techniques on *Fundulus heteroclitus* this laboratory has consistently found that measured osmolarity slightly exceeds calculated values based on a comparable number of serum parameters (Pickford *et al.*, 1969; Srivastava and Pickford, 1972; Umminger, 1969).

Blood pH and cation excess

Environmental salinity was without effect on either blood pH (which ranged from 7.145 to 7.481) or on the difference between the sums of the measured cations and anions (which ranged from 7.5 to 19.0 mEq/1). Neither parameter

was correlated with total CO_2, inorganic phosphorus, total serum proteins or albumin; serum constituents potentially involved in acid-base balance. We found a barely significant negative correlation ($r = 0.832$; $P < 0.05$) between blood pH and serum cation excess. The possibility that the two are correlated in this way through differences in blood lactic acid concentrations may be inferred from the studies of Piiper and Baumgarten (1969) who suggested that increases in cations would be necessary to maintain electrical neutrality in the blood of acidotic *Scyliorhinus stellaris* with low blood pH and high blood lactic acid. Our values for blood pH are similar to those reported for marine elasmobranchs by Heinemann and Hodler (1953), Green and Hoffman (1953) and Murdaugh and Robin (1967). Our values for cation excess are somewhat lower than those observed in the teleost *Fundulus heteroclitus* by Pickford *et al.* (1969).

TABLE V

Electrolyte concentrations in the Amazon River and in experimental tanks

Parameter	Amazon River†		Fresh water	Half sea water††
	Mean	Range		
Salinity (ppm)	44.75	37–59	100	14500
Sodium (mM/l)	0.047	0.030–0.068	0.150	197.7
Potassium (mM/l)	0.034	0.026–0.048	0.016	4.20
Calcium (mM/l)	0.196	0.136–0.312	0.167	4.30
Magnesium (mM/l)	0.048	0.021–0.073	—	22.5
Chloride (mM/l)	0.072	0.062–0.088	0.241	230.5

† Data after Clarke (1924); based on four analyses at different parts of river basin.
†† Values for other parameters estimated from salinity.

Perivisceral and pericardial fluids

Data are presented in Table IV for sodium, potassium and chloride of serum, perivisceral fluid and pericardial fluid for stingrays in fresh water, dilute sea water and failing at a salinity of 20.6‰. It is apparent that sodium and chloride of the three fluids are elevated in dilute sea water. This rise is not evident in potassium although levels of this ion were high in the failing fish. Of interest is the fact that both pericardial and perivisceral fluids are markedly hyponatremic, yet iso- or slightly hyperchloremic to serum in all media. These data are in agreement with Thorson *et al.* (1967), who also noted that the other cations measured (K+, Mg++, Ca++) in these fluids were at lower levels than in the serum. These data suggest that both pericardial and perivisceral fluids have a large cation deficit in contrast to the serum where there is a cation excess; a finding similar to observations on marine elasobranchs by Bernard, Wynn and Wynn (1966), Rodnan, Robin and Andrus (1962) and Murdaugh and Robin (1967).

DISCUSSION

In adapting to fresh water, the river rays of the family Potamotrygonidae have developed several physiological characteristics which set them apart from marine elasmobranchs. Most apparent is the elimination of urea retention as an osmoregu-

latory mechanism. Marine elasmobranchs have serum urea levels ranging from 209 to 453 mM/l (Holmes and Donaldson, 1969). Euryhaline species sampled in fresh water have concentrations ranging from 81 to 180 mM/l (Smith, 1931; Urist, 1962; Thorson, 1967). In striking contrast, the river rays have urea levels of 1 to 2 mM/l; concentrations which are, at most, tripled by adaptation to moderately high salinities. It would appear that urea retention in elasmobranchs is a specific adaptation to marine environments, subject to modification in response to dilute media through physiological mechanisms to only a limited extent, but labile in an evolutionary sense. The probably independent acquisition in the coelacanth (Pickford and Grant, 1967; Lutz and Robertson, 1971) and the certainly independent acquisition of the urea retention mechanism in the frog *Rana cancrivora* (Gordon, Schmidt-Nielsen and Kelly, 1961) suggest that urea retention is an "obvious" way for aquatic vertebrates to cope with the osmoregulatory problems inherent in maintaining a moderately low serum specific ion content in a medium with much higher electrolyte concentrations.

The question might be asked as to why the teleost fishes did not adopt the urea retention habitus while while in sea water, particularly in light of recent demonstrations that all of the enzymes of the ornithine-urea cycle are present in teleosts (Huggins, Skutch and Baldwin, 1969; Read, 1971). The answer may lie in an advantage inherent in the teleost mechanism of osmoregulation in sea water (*i.e.*, drinking sea water and excreting salts extrarenally; *cf.* Smith, 1930) in adapting to increases and decreases in environmental salinity. An as yet untested corollary of this hypothesis is the possibility, assuming that urea retention is metabolically inexpensive relative to drinking sea water and excreting ions, and assuming that urea tolerance is not a sufficient deterrent, that marine teleosts which have not been subjected to changes in environmental salinity for geologically long periods of time (*e.g.* bathybenthic and bathypelagic groups) may retain urea for osmoregulatory purposes. Of particular interest would be species which are ovoviviparous, viviparous, or have encapsulated eggs, developments which might be interpreted as consequences of urea retention (Smith, 1936; 1953; Price and Daiber, 1967).

A second feature making the river rays distinctive is the regulation of serum inorganic electrolytes at reduced levels compared to marine elasmobranchs. An analogous difference in normal levels of serum electrolytes may be found between marine and freshwater teleosts although the difference is not as marked in this group. Euryhaline teleosts maintain their serum electrolytes within narrow limits irrespective of the adaptation medium (Pickford *et al.*, 1969). In both elasmobranchs and teleosts it seems likely that the final serum electrolyte levels are the result of a balance between the expense of maintaining ion gradients which, unchecked, would result in salt loss in fresh water and salt gain in sea water, and the ability of tissues to tolerate changes in their ionic milieu. In both taxonomic groups the balance points differ between freshwater and marine species.

A third unique characteristic of the Potamotrygonidae among elasmobranchs is the presence of serum albumin. It is possible that the binding of ions by this protein is a mechanism of decreasing the osmotic gradient between the fish and its environment while maintaining the specific ion content of the serum within reasonable values. A function of serum albumin in adaptation to fresh water has

also been suggested for the teleosts (Roche, Derrien and Chouaiech, 1939; Drilhon and Fine, 1957), although it is clear that many marine teleosts have substantial amounts of an albumin-like protein (Sulya *et al.*, 1961; Morris, 1959) and that albumin plays no role in the adaptation of *Fundulus* species to fresh water (Pickford *et al.*, 1969; Griffith, 1972).

Finally, the adaptation of river rays to fresh water has proceeded to such an extent that they are no longer able to tolerate sea water. Failure to withstand high salinities is apparently due to an inability to excrete monovalent salts (sodium and chloride) which accumulate to high levels in fish living in dilute sea water. The loss of a functional rectal gland which would be of no use in a salt deficient environment possibly accounts for the inability of the river rays to excrete monovalent salts. Their failure to accumulate and/or retain urea in saline media is probably of secondary importance in preventing adaptation to high salinities.

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Summary

1. Juvenile freshwater stingrays of the family Potamotrygonidae are unable to survive in salinities in excess of 20.6‰ when gradually acclimated.

2. No differences were observed in blood pH or hematological parameters when fish adapted to a salinity of 14.5‰ were compared with freshwater controls.

3. Significant increases were found in serum sodium (21%), chloride (21%), calcium (48%), and magnesium (51%). Increases in total CO_2 (16%), potassium (21%), and inorganic phosphorus (35%) were not significant on account of high variance. Serum osmolarity increased 69%.

4. There was no apparent effect of salinity on serum total cholesterol, organically bound phosphorus, or total carbohydrates. Glucose contributed only 25% of the latter.

5. Serum urea was low (1.1 mM/l) as previously reported, and the trend to increase in a saline environment was not osmotically significant.

6. Freshwater stingrays are unique among elasmobranchs in possessing significant amounts of a protein with the electrophoretic mobility of human serum albumin. There was no significant change in this fraction or in serum total protein in fish adapted to a saline medium.

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THE UTILIZATION OF FOODSTUFFS AND UREA PRODUCTION BY A LAND SNAIL DURING ESTIVATION

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Inhabitants of arid regions are periodically subjected to lengthy periods of drought. During times of low or even no rainfall considerable physiological demands are placed on a land snail. For instance, snails must have ample food reserves to assure survival because normal feeding may not resume for months. In addition the snails must endure high temperatures and conserve water. To escape desiccation terrestrial snails go into dormancy (estivation).

One aspect of the physiology of estivation that is of especial interest is the metabolic role of the accumulation of urea during dormancy (Horne, 1971). Why do some and not other snails buildup their urea levels? And what is the physiological importance of the urea when it is stored? Information on the metabolism of estivation in molluscs is generally scarce. Most of the studies have been conducted on the aquatic pulmonates (von Brand, 1931; von Brand, Nolan and Mann, 1948; von Brand, Baernstein, and Mehlman, 1950; von Brand, McMahon and Nolan, 1957; Coles, 1969) and on the prosobranchs (Meenakshi, 1958 and 1964).

To ascertain how one terrestrial pulmonate snail, *Bulimulus dealbatus,* has coped with the problems of water loss and starvation during estivation, the current study was initiated. The crux of the study was to answer the question of the importance of urea to the snail, and to determine if the urea production was related to the foodstuffs used during starvation or to osmotic stress.

Methods

Pulmonate snails, *Bulimulus dealbatus mooreanus* (Pfeiffer), were collected locally (Hays County, Texas) and were maintained in the laboratory at 22 ± 2.0° C and at a relative humidity ranging from 45–75%. Photoperiod was not controlled. Active snails were fed lettuce *ad libitum*. *Bulimulus* was identified by Dr. Joseph Rosewater, Curator, Division of Mollusks, United States National Museum.

Over an 80 day estivation period snails were weighed every 10 to 14 days. Periodically specimens were sacrificed and the wet weights and the dry weights determined to show the rate that tissue was metabolized. To get an accurate wet weight the whole snail was weighed; then by carefully removing and drying the shell to constant weight at 65° C, it was possible to obtain a good estimate of the wet weight. By drying the soft body at 65° C the dry weight was acquired.

In as much as the concentration of foodstuffs were changing in relation to each other, it was essential that the concentration be expressed in terms of an internal standard that was not affected by starvation (such as DNA). Thus an average DNA concentration was established for the active feeding snails. If, for instance, in an estivating snail the DNA increased by 25%, then a decrease in dry weight

of 25% would have had to have occurred to account for the higher DNA values. Therefore, it was possible to adjust the data on estivation to the initial concentrations of active feeding snails. This manipulation was indispensable to the expression of the foodstuff concentrations.

Total reducing polysaccharide in whole snails was prepared for analysis by the procedure presented by Oser (1965). The method of Nelson (1944) and Somogyi (1945) was used for color development and quantitative analysis. No attempt was made to distinguish between glycogen and galactogen.

Lipid in whole snails was homogenized in 10 ml methanol and then diluted with 20 ml chloroform (1 : 2) and separated according to the procedure of Sperry (1955). The lipid fraction was air dried in a weighing bottle and weighed to the nearest 0.05 mg.

TABLE I

Rate of weight loss of estivating Bulimulus dealbatus *in the laboratory**

Estivation in days	Wet wt. loss mg/g snail ± S.D. (N)	Dry wt. loss mg/g snail (10% of wet wt)	Dry wt. loss mg/g snail (calculated from DNA)
0	—	—	0.0 679 ± 103(5)**
17	99.7 ± 28.2 (12)	9.9***	11.0 789 ± 134(6)
26	127.7 ± 32.7 (12)	12.7	—
60	215.6 ± 39.1 (18)	21.5	20.1 880 ± 225(6)
80	312.0 ± 64.6 (11)	31.2	29.9 978± 218(5)

* Relative humidity = 45 to 78%; temperature = 22.0 ± 2.0° C.
** Actual DNA values [Mg DNA ± S.D. (number)].
*** Dry weight equals 10% of wet weight.

Total protein was precipitated with 10% trichloroacetic acid, centrifuged at 2000 × *g* for 10 minutes, the supernatant discarded and the precipitate redissolved in 1 N NaOH. For quantitative color development the method for Lowry, Rosebrough, Farr and Randall (1951) was used.

Lactic acid determinations were carried out by the Barker and Summerson method (1941) as presented in Oser (1965).

Urea was estimated by the colorimetric method of Archibald (1945) or the urease method of Conway (1957).

Measurement of aerobic respiratory rates was done with a conventional Warburg respirometer (Precision Scientific Co., Chicago) at 22.0 ± 1.0° C. It was unnecessary to shake the flasks since there was no fluid phase, except that of the 0.2 ml of 20% KOH. Thus, the snails were not distrubed by shaking (Cole,

1969). Active non-crawling snails were those individuals with the foot either extended or retracted, but without an epiphragm. Individual readings were made at 30 minute intervals and over a period of 2 to 3 hours.

Carbon dioxide and respiratory quotients were measured by the direct manometric method (Umbreit, Burris, and Schauffer, 1959).

Constant humidities were maintained with saturated salt solutions (Winston and Bates, 1960) in large vacuum desiccators. To assure adequate gas exchange with air the desiccators were left open. The published relative humidity values for the salt solutions employed here were checked with a Bacharach Instrument Co. (Pittsburg) humidity meter. The measured humidities were always within 1½% of the value reported by Winston and Bates (1960).

Results

During an estivation period of 80 days (relative himidity = 45–75%; temperature = 22.0 ± 2.0%) approximately 31% of the initial dry weight was consumed (Table I). Throughout estivation the ratio of dry weight to wet weight was approximately 1 : 10 (Table II), thus somewhat simplifying estimation of dry weight from the wet weight. Also by using DNA as an internal standard, it was possible to relate back to the initial conditions. For example, if the DNA content increased by one third on a gram basis, then a decrease in one third of the other tissue constituents would have had to occur. There was a little difference between the dry weight values calculated from percentage wet weight and DNA. The relatively consistent water content illustrated that evaporative water loss was replaced by metabolic water and that desiccation was not the most pressing physiological problem of estivating *Bulimulus*. In fact, the water content may have increased slightly. Active crawling snails had 87.0 ± 2.3% (15) water, whereas the estivating snails had about 89 to 91% water (Table II).

Table II

Influence of relative humidity on the water content and on the number of epiphragms formed by Bulimulus *after 58 days of estivation*

	KCl (85%)	$Ca(NO_3)_2$ (55%)	$MgCl_2$ (33%)	LiCl (14%)
Water				
$\bar{x} \pm$ S.D.	91.2 ± 2.0	88.5 ± 2.6	89.3 ± 1.8	89.2 ± 2.7
	(6)	(6)	(6)	(6)
Epiphragms				
$\bar{x}$	2.0	3.5	2.7	3.7
(range)	(1–4)	(1–7)	(1–6)	(1–7)
(N)	(10)	(16)	(12)	(13)

A clear relationship between humidity and the number of epiphragms formed was not noted (Table II). Nevertheless, the snails in the highest humidity had fewer epiphragms than those in the lower humidities.

The rate of disappearance of foodstuffs during estivation is shown in Table III. Because carbohydrate, lipid and protein were changing in relation to one another

TABLE III

Utilization of foodstuffs during estivation

Foodstuffs (mg/mg DNA)	Feeding snail	Estivating snail (60 days)	Amount consumed in 60 days
Protein	86.1 ± 3.8 (20)*	67.8 ± 12.0 (21)	18.1 (12.3)**
Carbohydrate	13.2 ± 5.3 (10)	2.0 ± 1.6 (5)	11.2 (7.6)**
Lipid	13.3 ± 2.4 (12)	15.5 ± 4.1 (11)	—

* Mean ± standard deviation (number).
** MG consumed g wet weight.

as they were utilized, their respective concentrations were expressed in terms of the internal standard DNA. By using DNA values to adjust the foodstuff concentrations of estivating snails to values comparable to those of active snails, it was possible to estimate the amount of foodstuff metabolized on a wet weight basis during dormancy.

Surprisingly little lipid was used, whereas carbohydrate and protein accounted for almost all of the dry weight loss. Protein made up 57% and carbohydrate 35% of the dry weight consumed for energy. Complete depletion of polysaccharide deposits (glycogen and galactogen) occurred in about 70 days (Fig. 1). It is not yet known if both lipid and protein or only protein are used once all of the

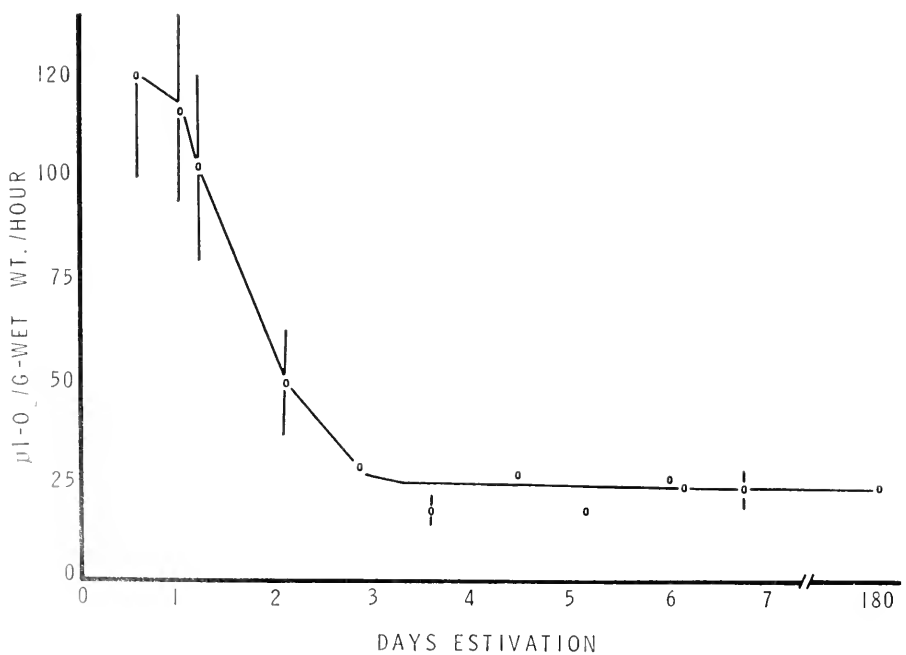

FIGURE 1. Rate of disappearance of reserve polysaccharide with estivation; Mean ± S.D. (Number ≥ 5)

carbohydrate reserve is gone. The average respiratory quotient for snails that have estivated for 120 days was 0.82.

The rate of oxygen consumption dropped sharply as the snails entered estivation, and more or less stabilized after three days at about 16% of the resting level (not crawling) (Fig. 2). The consumption of an average of 20 μl-0_2/g/hr by an estivating snail suggested that the snails were burning about 450 to 550 μg-foodstuff/g/day (one μl-0_2 = approximately one μg-protein; Cantarow and Schepartz, 1967). That some snails used as little as 2 μl-O_2/g/hr indicated that a few *Bulimulus* could withstand extended periods of dormancy. *Bulimulus* apparently did not seem to rely on anaerobic respiration since lactic acid values of only 170 ± 60 μg/g (10) were found in snails that had estivated for six months.

Upon being exposed to a relative humidity of 85%, snails that had been in estivation for six months would become active within one to three hours. Such a quick response to a moist situation assures that *Bulimulus* can capitalize on such environmentally favorable conditions. In Central Texas the snails are out feeding for short periods about twice a month in the spring and fall, but during the hot summer they may remain dormant for 3 to 4 months, depending on the pattern of rainfall. *Bulimulus* is also dormant during the cool winter months of December, January and February. Since the snails are active only during or shortly after a rain, they rarely have the opportunity to feed for more than 24 to 36 hours. During short periods of dormancy, the snails probably depend on carbohydrate for energy.

In the laboratory *Bulimulus* that have fed for several weeks store only 8.8 ± 3.5 mg-polysaccharide/g wet weight (10). The largest value recorded was 14 mg/g wet weight. It is doubtful that such deposits would be attained outside of the laboratory.

Humidity had an unexpected effect on the accumulation of urea during dormancy in as much as the response to humidity did not seem to be related to

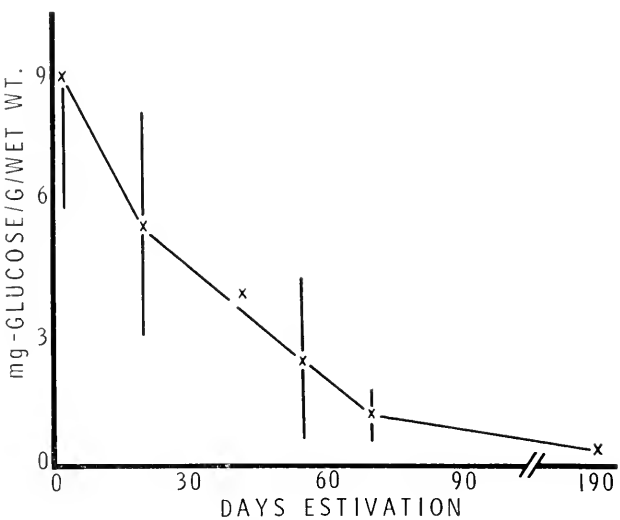

FIGURE 2. Changes in aerobic respiratory rates with estivation, Mean ± S.D. (N = 5)

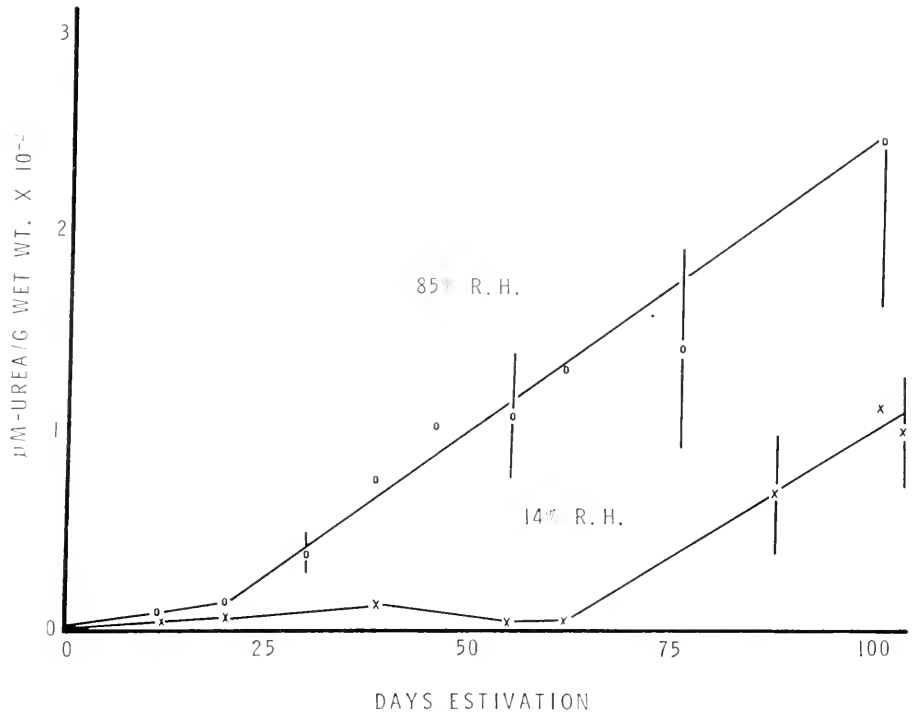

FIGURE 3. The effects of estivating at different relative humidities on urea accumulation. Mean ± S.D. (N = 5) The regression lines were calculated by least squares.

water loss, but rather to the activity of the animal (Fig. 3). In an atmosphere over saturated KCl (85% RH) *Bulimulus* remained active for two weeks before going into estivation. Yet even after two weeks, sixty-four of seventy-five snails were inactive and without epiphragms, while 11 had formed epiphragms. In an atmosphere over saturated LiCl (14% RH) all of the snails were estivating within nine hours. Since the buildup of tissue urea was greater at the higher humidities, the elevated urea values could not have been due to a rsponse to osmotic stress, but seemed only to further illustrate the metabolic demand on protein catabolism during starvation.

That relative humidity has little effect on dehydration of *Bilimulus* is illustrated in Table II. At all of the humidities studied the ratio of dry weight to wet weight remained constant. Hydration of the tissues was maintained by metabolic water.

DISCUSSION

Since protein declined the most during estivation and the estivating respiratory quotient was 0.82, the accumulation of urea with dormancy may be due in part to the enhanced catabolism of tissue protein. Apparently, some protein is used at about the same time that polysaccharide is mobilized. The carbohydrate reserves of snails fed *ad libitum* for at least 10 days, however, last only for about 70 days at

22° C. In Southwest Texas it would be unlikely that *Bulimulus* would ever have the opportunity to store so much carbohydrate.

During starvation and desiccation the aquatic pulmonate, *Australorbis*, depleted its polysaccharide and lipid stores, and seemed to depend on protein during extended periods of dormancy (von Brand, McMahon and Nolan, 1957). In their study the most pronounced reduction in carbohydrate and lipid occurred in the first ten days. On an annual basis the lipid content of *Helix pomatia* fluctuated only slightly while glycogen was stored prior to estivation and then consumed thereafter (von Brand, 1931). In other examples of the utilization of food stores, the aquatic snail, *Planorbis*, consumed mostly carbohydrate, while the desert pulmonate, *Sphincterochila*, appeared to use mainly carbohydrate and protein (Emerson,, 1967; Schmidt-Nielsen, Taylor and Shkolnik, 1971). At high humidities *Bulimulus* appeared to start utilizing a lot of protein by the fourteenth day, and therefore was somewhat like *Australorbis*.

That aerobic respiration persisted throughout estivation and that no lactic acid accumulated in the tissues was not especially surprising for a terrestrial snail. Both of the helicid snails, *Helix* and *Sphincterochila*, seemed to respire aerobically during estivation (Fischer, 1931; Schmidt-Nielsen, Taylor and Shkolnik, 1971). Even the aquatic pulmonates studied by von Brand *et al.* (1948) that have a limited capacity for anaerobiosis are also primarily aerobic.

The decrease in respiratory rate to about 16% of the normal resting rate demonstrated that the snails had entered a resting state. However, this state could be interrupted easily by placing the snails in an atmosphere with a relative humidity of 85%. At such a humidity all of the snails would be actively crawling within one to three hours. Both Fischer (1931) and Meyer and Thibaudet (1937) reported that *Helix pomatia* became inactive at any time of the year if food and water were removed, while activity resumed if food and water were provided. Following the onset of dormancy *Australorbis*, *Bulinus* and *Sphincterochila* reduced their respiratory rates between 9 and 30% of the initial rate (von Brand, McMahon and Nolan, 1957; Coles, 1969; Schmidt-Nielsen, Taylor and Shkolnik, 1971). *Australorbis* also showed a reduction in heart rate. A reduction in respiratory rate would be essential if such snails as *Bulimulus* are to survive many months in estivation. With an average oxygen consumption rate of 20 μl/g/hr, a one gram snail (130 mg dry weight) would use about 450–550 μg-tissue/d (one μl-O_2 = approximately one μg protein). At 22° C this means that *Bulimulus* would metabolize 50% of its dry weight within 4–6 months. Those individuals with respiratory rates lower than the average could undoubtedly estivate for several months longer. The desert snail, *Sphincterochila*, can estivate for longer periods than *Bulimulus* and seems to metabolize all tissue components (Schmidt-Nielsen, Taylor and Shkolnik, 1971) as does *Bulimulus*. At a relative humidity of 96% and at a temperature of 27° C *Australorbis* consumed 50–60% of its dry wt within 128 days (von Brand, McMahon and Nolan, 1957).

The catabolism of protein for energy is not surprising when the activity patterns of *Bulimulus* are considered. For example, *Bulimulus* is out actively feeding only during or shortly after a rain. At most, the snails feed for about 24 to 36 hours and are starving the rest of the time. In other words, *Bulimulus* spends most of its time in estivation in semi-arid Central Texas, U.S.A. With such short periods

of feeding it is doubtful that *Bulimulus* can ingest enough food to form large polysaccharide deposits. Even in the laboratory where lettuce is fed *ad libitum* the concentrations of carbohydrate was extremely variable. The highest concentration was 14 mg/g snail. Polysaccharide values in other species are generally much higher. Glycogen values in *Pila* ranged from 20.2–24.5 mg/g (Meenakshi, 1958), and for four aquatic pulmonates, von Brand, Baernstein and Mehlman (1950) reported values of 10 to 35 mg/g snail. These data show that *Bulimulus* deposits much less polysaccharide than many other snails. The high individual variation in reserve carbohydrate may explain the differences in the amounts of urea that accumulate during estivation. That is, snails with a high food reserve would catabolize less protein and thus need to detoxify less ammonia than a snail with a low polysaccharide reserve.

That low humidity does not enhance the production of urea clearly illustrates that osmotic stress does not effect the control of urea biosynthesis in snails as it may in some animals (McBean and Goldstein, 1971). In a relative humidity of 14%, where the threat of water loss by evaporation was greatest, the production of urea was least. The much more rapid buildup of urea in the environment of 85% R.H. was perhaps due to a more rapid mobilization and breakdown of protein. During estivation the end-product of nitrogen catabolism seems to be urea, not ammonia or uric acid (Horne, 1971). At the high humidity, starving *Bulimulus* remained actively crawling for about two weeks before estivating, while at the lower humidity the snails were all estivating within nine hours. Carbohydrate reserves would be metabolized much sooner in the active crawling snails.

High urea levels could conceivably be of some importance in reducing evaporative water loss in a snail buried in the soil. Urea concentrations of 300 μmoles/g would increase the osmotic pressure of a snail by about 6 atmospheres. However, since the water content of the snails that had estivated 58 days in humidities ranging from 14 to 85% did not differ, it is unlikely that the role of urea is related to water conservation. Apparently, the epiphragms in *Bulimulus* are what really reduceds water loss, even though the number of epiphragms formed did not seem to be affected by humidity. The numerous chambers formed by the epiphragms may reduce evaporation by decreasing air circulation in the spaces between epiphragm. Machin (1968, 1972) discussed the permeability of helicid epiphragms to water and has emphasized the importance of the mantle in reducing evaporative water loss, whereas the operculum of *Pila* was found by Meenakshi (1964) to be critical in decreasing water loss in this species.

Water loss is not the most critical problem encountered by estivating *Bulimulus*. But rather, it is the depletion of food reserves that determines how long a snail may remain dormant. The reduction in respiratory rate allows for prolonged periods of dormancy. The physiological implications of the elevated urea levels are uncertain, except that they seem to be related to protein catabolism, ammonia detoxification and the fact that the snails are not voiding urine during inactivity.

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Summary

1. During a sixty day estivation period, the land pulmonate, *Bulimulus dealbatus*, metabolized 21.5 mg-tissue/g dry wt. Of the tissue consumed, protein and polysaccharide made up 57% and 35% of the loss.

2. By the third day after onset of estivation aerobic respiratory rates declined to 16% of the resting level.

3. Snails maintained in a 85% R.H. were active longer and accumulated urea faster than those snails in 14% R.H. The high concentrations of urea were probably related to an enhanced catabolism of protein and not to osmotic stress.

4. The water content of *Bulimulus* and the number of epiphragms formed was not correlated with the estivation humidity.

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OOGENESIS IN *DROSOPHILA VIRILIS*. I. INTERACTIONS BETWEEN THE RING CANAL RIMS AND THE NUCLEUS OF THE OOCYTE

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The polytrophic, meroistic ovaries of flies belonging to the genus *Drosophila* are made up of clusters of egg tubes. Each ovariole is composed of an anterior, sausage-shaped germarium and a vitellarium which contains a single-file array of individual egg chambers that are attached to one another by stalks of follicle cells. In the germarium a single, apical cystoblast gives rise to 16 cells by a series of four consecutive mitotic divisions. These 16 cystocytes are connected by intercellular channels that are arranged in a specific pattern: 2 cells have 4 canals each, 2 have 3, 4 have 2, and 8 have 1 canal each. In the posterior region of the germarium individual clusters are surrounded by a single layer of follicle cells. These enveloped 16 cell clusters then enter the vitellarium (King, 1970).

The ring-shaped canals that connect the 16 sister cells of a cluster are of interest because of the invariable relationship that exists between the number of canals and the developmental fate of the cystocytes. While the 16 cells are all descendants of a single cell and presumably contain identical genomes, two cells (the pro-oocytes) enter meiotic prophase, and the others do not. Each of the two pro-oocytes always has four canals. This paper will give an account of oogenesis in *Drosophila virilis*, with specific emphasis on ring canal structure and function.

Materials and Methods

Females of *Drosophila virilis* from the Pasadena wild type strain were reared at room temperature on David's medium (David, 1962) during a natural cycle of light and darkness. All work was done on mated flies that were between 5 and 8 days old. The ovaries were dissected from etherized flies immersed in *Drosophila* saline solution (see King, Rubinson and Smith, 1956, for the recipe). Whole mount preparations were used to determine the ovariole number, stage distribution, chamber dimensions, and the state of the chromatin in the follicle cells, nurse cells, and oocytes during oogenesis. The DNA was visualized using Feulgen staining (see King, Burnett, and Staley, 1957, page 242 for the procedure).

Ovaries that were to be sectioned were dissected from females immersed in the fixative, which contained paraformaldehyde and glutaraldehyde, both at a final concentration of 4% in a 0.2 M cacodylate buffer (Karnovsky, 1965). The ovaries were fixed for two hours at room temperature, passed through several washes of 0.2 M cacodylate buffer containing 10% sucrose to approximate the osmolality of the fixative, and then post-fixed for two hours in aqueous OsO_4 and $K_2Cr_2O_7$ (both at final concentrations of 1%). Maser, Powell and Philpott (1967) have determined osmolality values for various fixative, buffer, salt, and sugar solutions, and

we employed their data to obtain fixatives and rinsing fluids of similar osmolalities. The ovaries were rapidly dehydrated through an ethanol series and transferred to propylene oxide. The ovaries were infiltrated over-night in a 1:1 propylene oxide: plastic mixture, and then they were transferred to pure Epon-Araldite (Mollenhauer, 1964). Polymerization took 72 hours at 60° C.

Serial sections 0.5 to 2 μ thick were cut using an LKB Ultrotome III fitted with a glass knife. Sections were picked-up with hair loops and transferred to water drops on glass slides. A hot plate was used for rapid evaporation and attachment of selections to the slides. Egg chambers in each of the 14 developmental stages described by King, Rubinson and Smith (1956) were serially sectioned (1 μ) and stained with Richardson's technique (Richardson, Jarett and Finke, 1960). These sections were used to characterize intranuclear structures. Sections for general morphological work were cut 2 μ thick and stained with azure B at a variety of pH values (4, for differentiation of DNA and RNA, and 7 or 9, for non-specific, but more intense staining). In addition, selected 1 μ sections were stained with fast green (pH 2) for detection of proteins and by the periodic acid-Schiff (PAS) method for polysaccharides (see King, 1960, for the rationale and techniques). All light sections were studied using a Wild-Heerbrugg M20 research microscope fitted with a drawing tube. Using the drawing tube, the magnified images of chambers in various stages were traced on Kodak diffusion sheets. These tracings were then stacked on top of one another in proper sequence to gain information concerning three-dimensional interrelations of various structures.

Tracings of this sort were also useful for volume estimates. By placing a tracing over a ruled grid and counting the squares enclosed by a given outlined structure, an estimate of the area of the sectioned object could be obtained. Since the thickness of the sections and the image magnification factors were known, we could calculate the volume of each section, and by adding together the volumes of all the sections through the object, its volume was determined. The data presented in Figure 7 were obtained by these methods.

Tissues used for electron microscopy were fixed and embedded in the same manner as that described for light microscopy. Silver to grey sections were cut with glass knives using the LKB Ultrotome III and picked-up with an LKB section collector. Two types of copper grids were used: slot grids for serial work, and mesh grids for general work. Carbon-coated Formvar specimen support films were prepared according to the procedure of Meek (1970). The tissue was first stained with saturated, aqueous, uranyl acetate followed by lead citrate (Reynolds, 1963). Sections were viewed and photographed with either a Hitachi HS 8 or HU 11A electron microscope. Tracings were made of selected serial electron micrographs and stacked in proper order to obtain 3-dimensional reconstructions of certain organelles.

Observations and Results

The morphology of canal rims

In *Drosophila virilis* the pattern of interconnections among the 16 sister cystocytes (see inset, Fig. 7) is the same as that first described in *Drosophila melanogaster* (Brown and King, 1964). Only two cells in a cluster have four canals, and it is these cells that differentiate into pro-oocytes in the germarium. One of

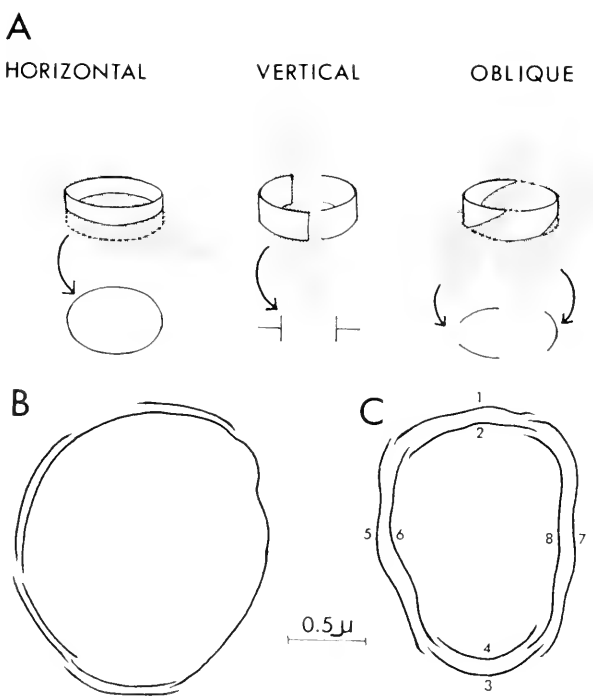


FIGURE 1. (A.) Ring canals as they appear when sectioned in each of three different planes; (B.) a ring canal reconstructed from horizontal sections through a stage 3 oocyte of *Drosophila virilis;* (C.) a ring canal in a germarial cystocyte of *Habrobracon juglandis* (from Cassidy and King, 1969).

the pro-oocytes invariably differentiates into the oocyte, while the other enters the developmental pathway followed by the other 14 nurse cells.

The intercellular connections have been called ring canals because their rims are ring-shaped. A ring has varied appearances when sectioned in different planes, and Figure 1A illustrates the terminology we will use throughout the paper to describe the planes of section and the appearances of the sectioned canals. Ring canals are frequently sectioned in oblique (Figs. 2A, 4D and 5A) and vertical planes (Figs. 2A, 3ABC, and 4A) and rarely in the horizontal plane (Fig. 4C).

When consecutive horizontal sections are photographed, traced, and reconstructed, we find that the canal rim of *Drosophila virilis* is composed of several overlapping leaves (Fig. 1B). The canal rim leaves resemble those described in germarial cystocytes of *Habrobracon juglandis.* In this wasp each rim is composed of 8 leaves arranged in 4 pairs. The opposite leaf pairs have similar dimensions: leaves 1, 2 and 3, 4 are 1.1 μ long and 0.7 μ high; while leaves 5, 6 and 7, 8 are 2.0 μ long and 0.4 μ high (Fig. 1C). The leaves have equal widths. In the *Habrobracon* electron micrographs the space between the paired leaves is wider than in *Drosophila* (compare Figs. 1B and 1C). Unfortunately we do not have all horizontal sections through a *virilis* canal rim and therefore cannot estimate the length and height of each of the leaves. However, we have seen some horizontal

sections that demonstrate that leaves corresponding to 1 and 2 are taller than leaves corresponding to 5 and 6.

In horizontal sections, *Drosophila* canal rims appear to be composed of vertically oriented tubules, each with a diameter of 20 mμ, which are separated from their

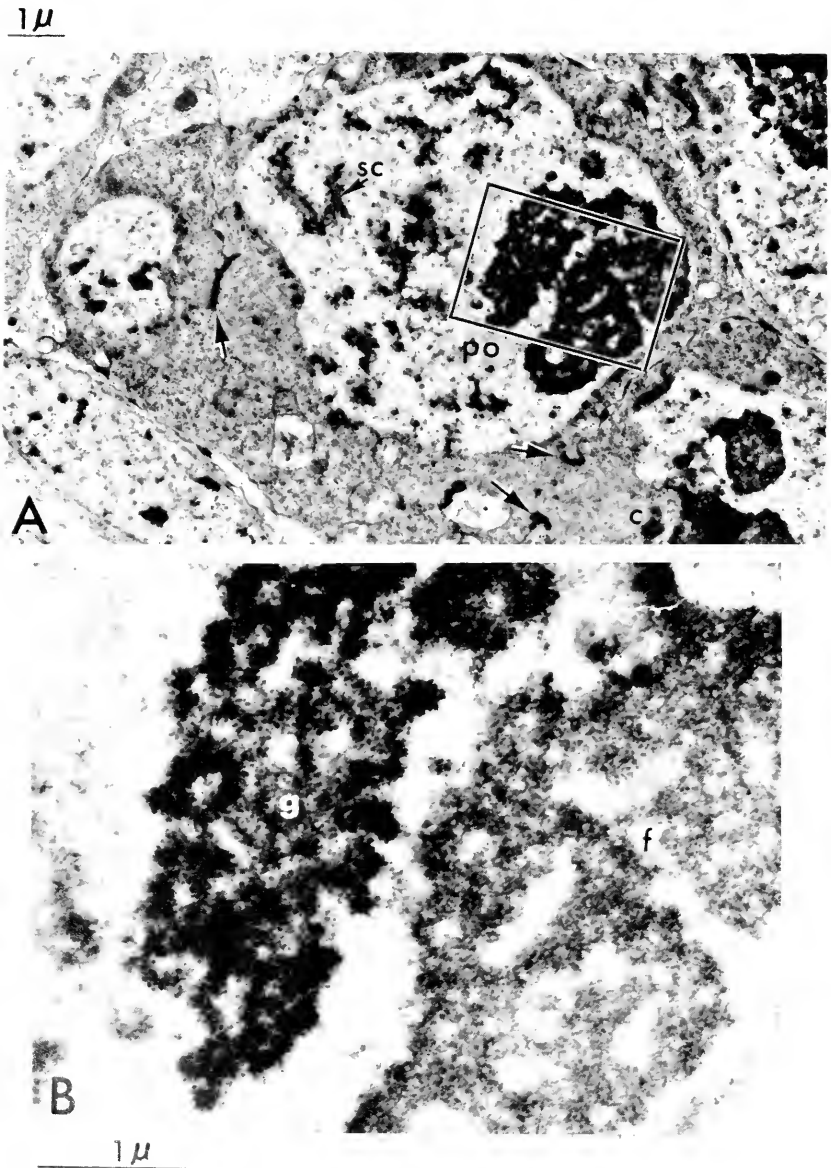


FIGURE 2. (A.) An electron micrograph of a pro-oocyte in the germarium. Its nucleus contains synaptonemal complexes (sc). Arrows point to ring canal rims; one in oblique section, the other in vertical section. A centriole (c) is seen lying near a nurse nucleus; (B.) an enlargement of the nucleolus in A showing both granular (g) and fibrillar (f) elements.

nearest neighbors by approximately 12 mμ. Each canal rim has short pieces of membrane attached to it that radiate out into the cytoplasm. In *Habrobracon*, the dimensions, spacing and orientation of the tubules are similar, and so are the membrane attachments.

Pro-oocytes of *Drosophila virilis* are characterized by nuclei that contain synaptonemal complexes (Fig. 2). The ring canals of pro-oocytes can be classified into four generation groups on the basis of their morphologies and positions. The oldest or first generation canal is always the largest, the most morphologically complex, and the one which connects the pro-oocytes. The diameters of canals from all four generations increase through stage 10B. For example, the first generation canal has a diameter of 1.4 μ in S1 and of 6.8 μ in S10B. The height of the rim also increases with development through at least S8 (from 0.5 μ in S1 to 2.8 μ in S8).

The material located in or adjacent to the canals changes during development. In the most anterior 16 cell clusters, a canal contains flocculent material which assumes an hour glass shape with the waist through the canal. Such areas are free of ribosomes and contain short segments of microtubules, and we assume that these areas represent the remnants of mitotic spindles. By S2 there is a marked decrease in the flocculent material in and adjacent to canals (Fig. 3A). However, short microtubules remain in some of the canals. The younger, fourth generation canal (Fig. 3B) has more tubules and a larger spindle remnant area than the older canal (Fig. 3C). The S2 canals also contain both ribosomes and mitochondria. By S3 no obvious spindle remnants remain in any of the canals (Figs. 4C and 5A). Mitochondria and ribosomes account for the majority of the material seen within canals until the onset of vitellogenesis.

The rims of canals in the youngest 16 cell clusters, found in the anterior half of the germarium (Fig. 2A), are uncoated. However, by stage 1 a coating can be resolved. The first generation canal has the thickest coating, and the fourth generation canal the thinnest. By stage 2, the thickness of the coating increases, but differences are still seen in the development of canals formed at different generations (Fig. 3). The volume of the coating material increases through S6, and then begins to decrease. The thickness of the coating, however, begins to decrease by S5, probably as the result of stretching as the canal expands.

The material coating canal rims has been shown to be a polysaccharide-free protein by cytochemical tests. When the canal is sectioned vertically (Figs. 3B and C), the coating appears to be made up of tightly packed fine particles. However, horizontal sections reveal that the coating contains many microfibrils whose orientation is parallel to the circumference of the canal rim (Figs. 4C and D, 5B). The average diameter of a microfibril is about 65 Å, and the center to center interval between adjacent microfibrils is roughly 160 Å.

Rim material in the cytoplasm

Large clusters of microfibrils are frequently seen free in the ooplasm and occasionally in nurse cytoplasm. The one shown in Figure 5A and 5C is 0.7 μ wide and 3 μ long. Four of these masses have been serially sectioned; the largest one was 0.8 × 6 μ; the smallest, 0.4 × 2.3 μ. They contain microfibrils with diameters in the 65–75 Å range and with center-to-center intervals of about 200 Å. In all masses observed, the microfibrils are oriented parallel to the long axis and

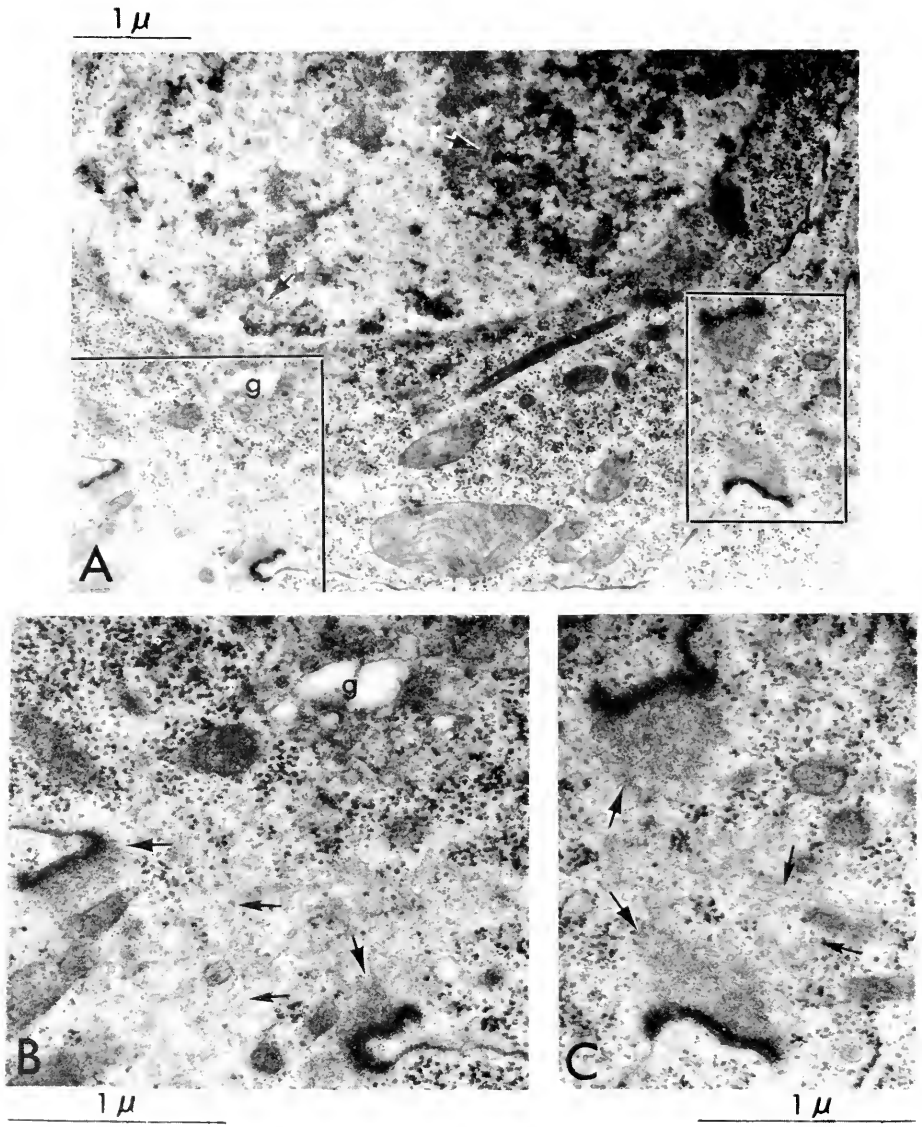


FIGURE 3. (A.) An electron micrograph of a portion of a stage 2 chamber. The nucleus is from a pro-oocyte that has turned to the nurse cell developmental pathway. Note the remnants (r) of old synaptonemal complexes. A well developed golgi apparatus (g) lies next to the nucleus; (B.) an enlargement of the left outlined area in A showing a 4th generation ring canal in vertical section. Large arrows point to the microfibrils coating the rim. The small arrows point to microtubules of the spindle remnant; (C.) an enlargement of the right rectangle in A, showing a 2nd generation ring canal cut in vertical section. Note the increase in the thickness of rim coating and the decrease of spindle remnants relative to B.

perpendicular to the short axis, and they are not enclosed by a membrane. The earliest stage in which these masses have been observed is S3. However, since these clusters are relatively small and are randomly distributed, we may have missed

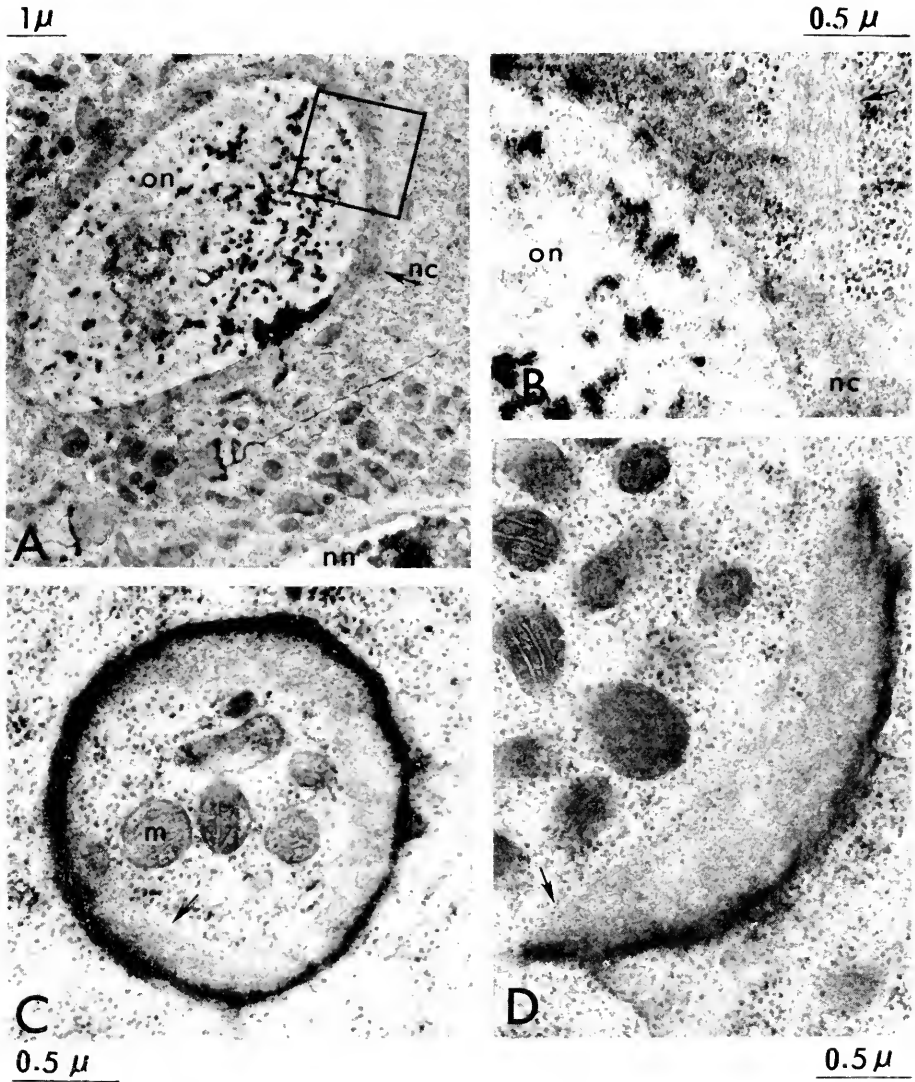


FIGURE 4. (A.) A stage 4 oocyte nucleus (on) and its nuclear coating (nc). The nurse nucleus (nn) has no coating; (B.) an enlargement of the square in A, showing a microfibrillar mass (arrow in contact with the nuclear coat (nc); (C.) a horizontal section through a stage 3 ring canal. The arrow points to microfibrils which are oriented circumferentially about the ring canal rim. The canals contain mitochondria (m) and ribosomes; (D.) an oblique section through a S4 ring canal. The arrow points to microfibrils in the rim coating.

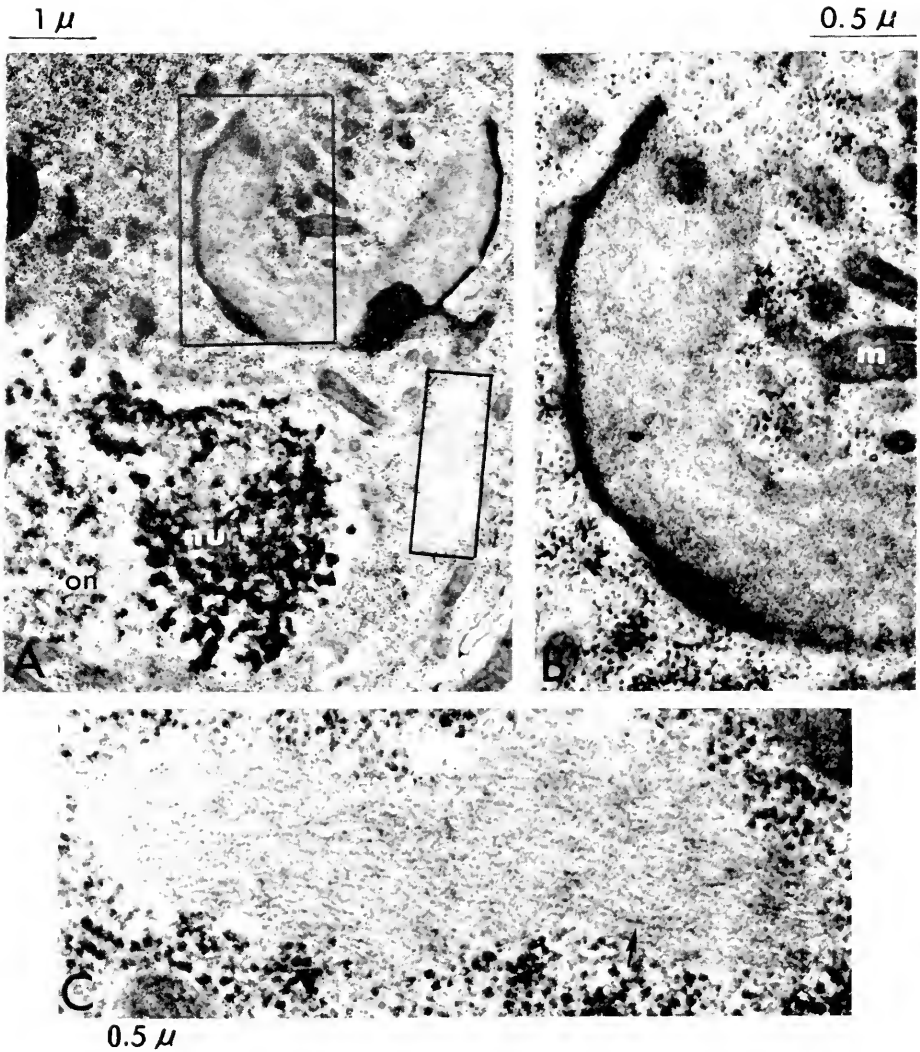


FIGURE 5. (A.) A stage 3 oocyte nucleus (on) containing a fragmenting nucleolus (nu); (B.) an enlargement of the large rectangle in A, showing the rim coating in an obliquely sectioned ring canal; (C.) an enlargement of the small rectangle in A, which shows the fine structure of a cytoplasmic mass of microfibrils. The arrow points to several microfibrils which are oriented parallel to the long axis of the mass.

them in younger cystocytes. Two observations point to canal rims as a source of the clusters of microfibrils seen in the cytoplasm. First, there is similarity of microfibril size and spacing. Secondly, the microfibrils are seen first on canal rims (S1) and later (S3) in cytoplasmic clusters. By S7 the quantity of material on canal rims begins to decrease, and this may be due to the coating material detaching to form the cytoplasmic masses.

The coating of the oocyte nucleus

The fate of these masses is even more intriguing. In one series of electron micrographs, a mass of microfibrils ($0.4 \times 2.5\ \mu$) was seen attached to a coating which is forming about the oocyte nucleus (Figs. 4A and B). From a static picture it is obviously impossible to determine whether the mass is leaving or entering the coating. However, we favor the idea that the microfibrils detach from the rim and attach subsequently to the oocyte nucleus because as is shown in Figure 5A, masses of microfibrils are first seen in the cytoplasm before there is appreciable coating on the oocyte nuclear envelope. The microfibrils around the rims and in the cytoplasm are oriented in parallel while the fibrils in the "amorphous" nuclear coating show no consistent orientation.

The coating or "halo" begins to be formed about the oocyte nucleus in stage 3 (see S3 of Fig. 6). Initially this coating is incomplete, with gaps free from deposits and periodic clumps of the material. By S4 this coating is almost continuous (Fig. 4A). From S5 through S9 the halo is complete, and it continues to accumulate until it reaches a thickness of about 1 μ with a calculated maximum volume of 400 μ^3 (see Fig. 7). We calculated the volume of the halo by subtracting nuclear volume from the volume determined on the basis of nuclear radius plus average halo thickness. After the S9 peak, the character of the coating begins to change. By S10A small gaps reappear (see Fig. 6) with a concurrent decrease in the thickness of the coating. The changes in the character and thickness of the coating may indicate that the addition of new halo material has not kept pace with the rapid growth of the oocyte nucleus. Stage 11 is the last stage before the breakdown of the nuclear envelope in preparation for the 1st meiotic division. The material coating the S11 nucleus is less dense than at the previous stages. In a stage 8 oocyte (Figs. 8A and 8B) the density of the coating is such that mitochondria and ribosomes are excluded from the halo region. However, in stage 11 this exclusion is no longer complete.

One datum worth stressing is that the coating is restricted to the oocyte nucleus (see Fig. 4A). We found no coatings on the nuclei of sister nurse cells, although many were photographed or traced (Table 1) and many more observed. The nurse cell derived from a pro-oocyte also lacks the coating.

The halos are fast green-positive, PA/S negative, Feulgen-negative, and stain green with azure B (pH 4). We conclude that the coating contains protein and lacks appreciable amounts of polysaccharides and nucleic acids. The amorphous appearance of the nuclear coating may indicate that the protein fibers coil up prior to their deposition or that uncoiled microfibrils precipitate on the nucleus in a "brush heap" configuration. During stages S3 through S6 the coating resembles that seen when ring canals are sectioned vertically, exposing the rim coating microfibrils only in cross and tangential sections (Fig. 4A). Later some compacting or clumping of halo material occurs (Figs. 8A and 8B).

Behavior of oocyte nucleus

The cytological changes which characterize the growing oocyte nucleus are diagrammed in Figure 6. Meiotic prophase must begin in the pro-oocytes immediately following the final cystocyte mitosis. The most anterior pro-oocytes we observed were always in a stage where synaptonemal complexes were forming

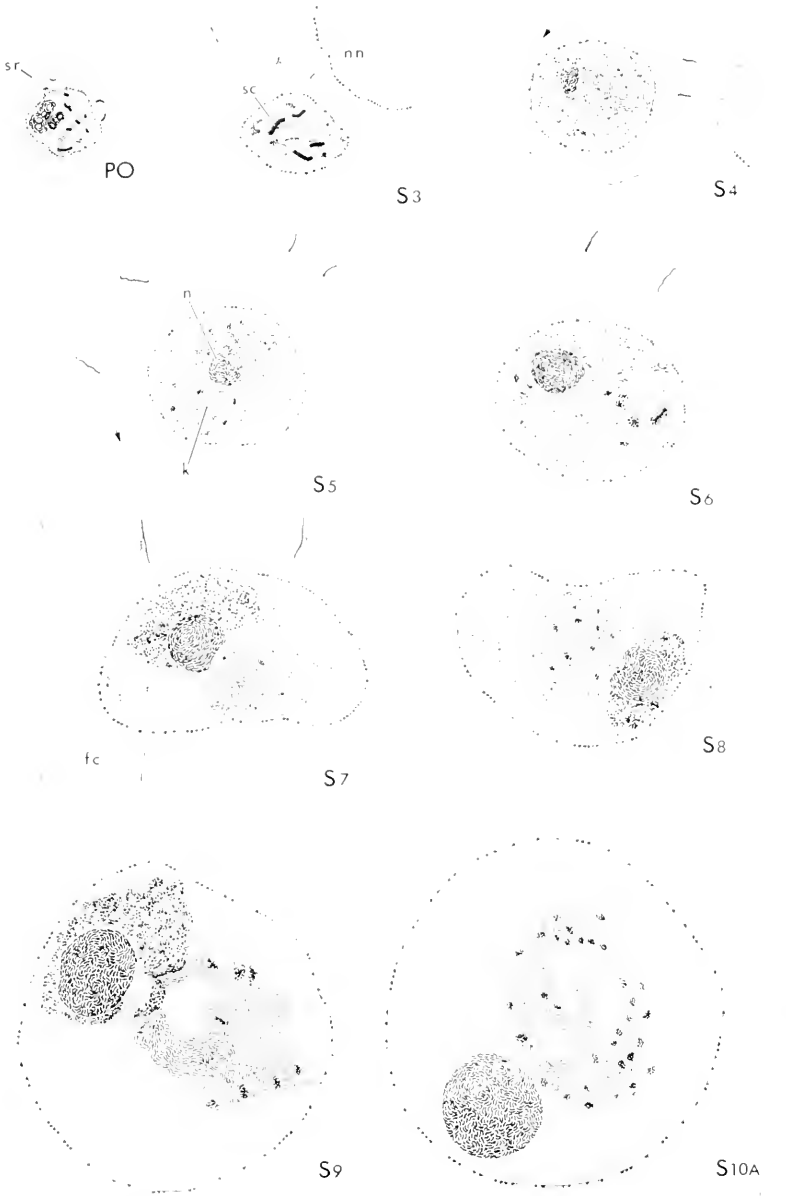
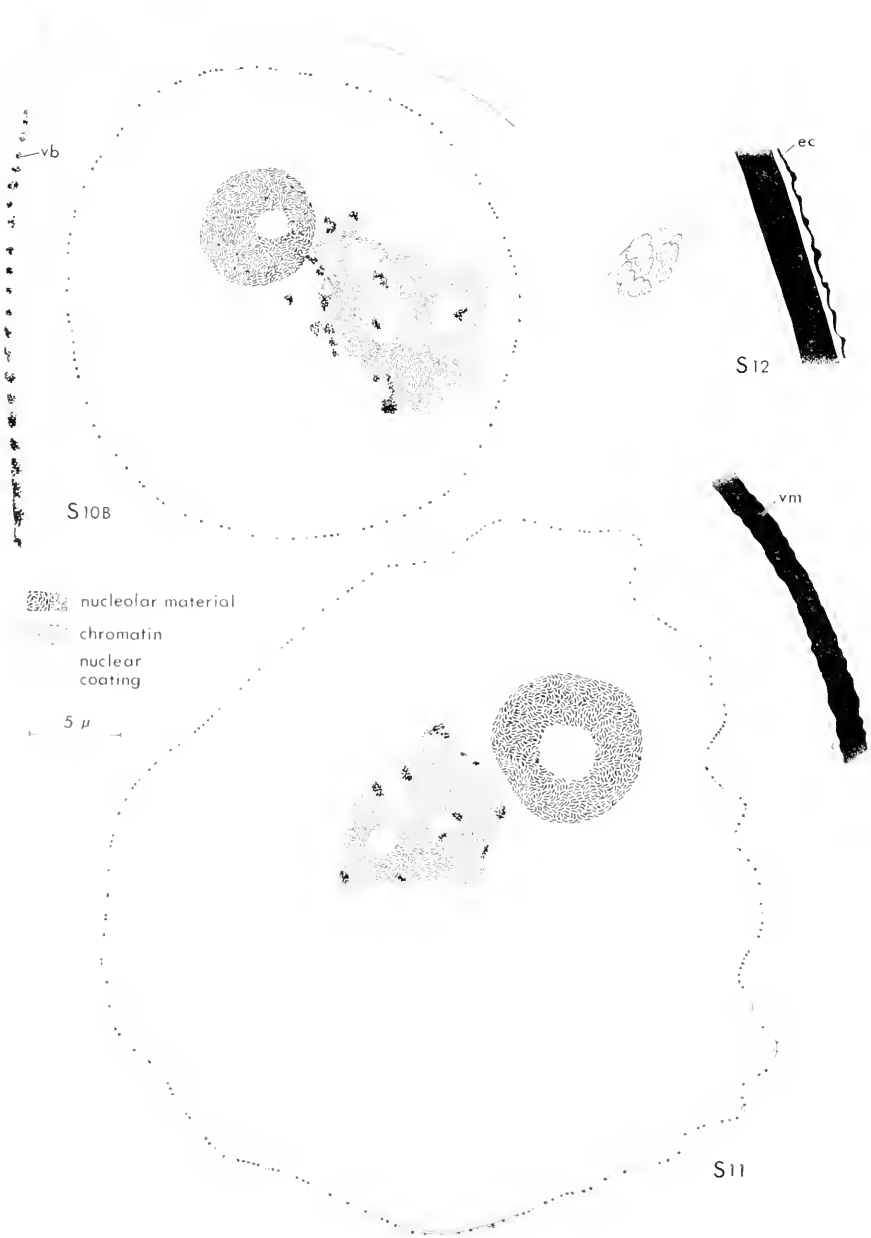


FIGURE 6. Diagrams of stages in the development of the oocyte nucleus from the pro-oocyte (PO) through stage 12. Synaptonemal complexes (sc) occur in the pro-oocyte (PO) and stage 3 (S3) nuclei. Spindle remnants (sr) lie near young ring canals. In oocytes later than S8 ring canals were not included in those sections that contained the nucleus. An amorphous halo forms about the oocyte nucleus in S3 through S9. During S10 and S11 the coatings become thinner and discontinuous. Arrows point to cytoplasmic masses of microfibrils. In S5 a spherical nucleolar structure (n) appears, and it continues to grow through



S11. By S5 the chromatin has aggregated into a karyosome (k) which expands through S10B. In S5, small packets of material (depicted as heavy dots) are seen near the nucleolus, and they persist through S9. Small clumps of granules are associated with the karyosome from S6 through S11. In S10B, vitelline bodies (vb) are deposited, and these coalesce to form a vitelline membrane (vm) by S11. The endochorion (ec), is laid down during S12. The S12 oocyte has distinct chromosomes associated with spindle microtubules. No nuclear envelope or centrioles are present.

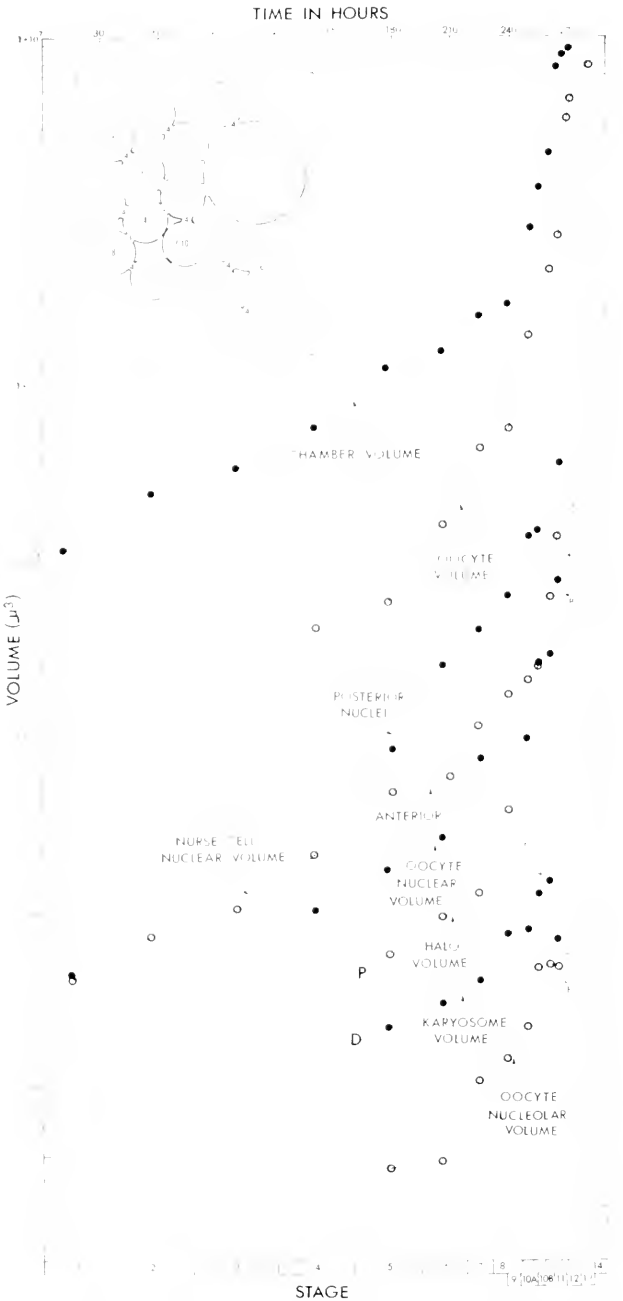


FIGURE 7. The volumes of various ovarian structures as a function of developmental stage; B, breakdown of nuclear envelope; C, condensation of karyosomes into individual bivalents; D, degeneration of nurse cell nuclei; F, fragmentation of the oocyte halo and nucleolus. Inset: the pattern of interconnected fourth generation ovarian cystocytes seen in *Drosophila virilis*.

(zygonema). Leptonema must be very rapid, since it was not observed. In the two germaria we sectioned there were eight 16-cell clusters which were considered newly formed, since they were adjacent to clusters containing 4 or 8 cells. Synaptonemal complexes were never seen in 8-cell clusters; whereas in the adjacent 16-cell clusters the two cells with 4 canals contained synaptonemal complexes. In *D. melanogaster* complexes have been reported to occur in 75% of the newly formed 16 cell clusters (Koch, Smith, and King, 1967).

By the time the oocyte enters the vitellarium it is in the pachytene stage, and synapsis of homologues is complete. Meanwhile, the other pro-oocyte turns to the nurse cell developmental pathway, and the synaptonemal complexes disappear. The true oocyte contains these complexes through S3. In S4 complexes are missing, and chromatin masses are seen distributed throughout the nucleus. By S5 the chromatin is incorporated into a karyosome which expands until S11, when it condenses (see Fig. 7). During S12 the nuclear envelope breaks down, short spindle microtubules appear, and the condensed chromosomes take up positions characteristic of prometaphase. In S13 and 14 the chromosomes are arranged on the metaphase plate, and the spindle microtubules lengthen. We have seen no evidence of either centrioles or asters in our electron micrographs of S12 and S13 meiotic spindles.

In *Drosophila virilis,* from the time the 16 cell cluster forms through S2 or S3, the oocyte nucleolus has granular and fibrillar elements quite similar to those seen in the nucleoli of nurse cells (Figs. 2A and 2B). The nucleolus then begins to fragment. The granular elements are lost, and the fibrillar portions of the old nucleolus begin to reaggregate forming a spherical structure, the "endobody" (Figs. 8A and 8B; Fig. 6, S5–S11). The endobody increases in diameter, from 1.8 μ in S5 to 4.8 μ in S11. It disappears during S12. From stages 5 through 9 the endobody is a densely packed fibrillar sphere with rough edges (Fig. 8B). However, by S10A the border of the endobody becomes smooth. The S10B endobody develops a cavity (*ca.* 5 μ^3) which approximately doubles in volume by S11 (see Fig. 6, S11). The endobody volume gradually increases from S5 until a maximum is reached by S10A (see Fig. 7). The volume then remains essentially constant through S10B and S11. Therefore, the increases in outer diameter seen in the S10B and S11 endobody are the result of the growth of the internal cavity rather than an increase in endobody material. From S5 through early S11 the endobody is in contact with the karyosome, but in late S11 this association is lost. The endobody stains with azure B in the characteristic manner of nucleolar material.

Small "packets" (*ca.* 600 Å diameter) of fibrillar material are seen in the oocyte nucleus from S4 through S9 (Fig. 6, S5-S9). At first this material is widely distributed, but as development proceeds these small clumps congregate in the area adjacent to the endobody. After S9 only a very small amount of the packet material is seen, and it is widely distributed throughout the nucleus. Figures 8A and 8B show the relationship between the endobody and packets in the S8 oocyte. The similar appearance of the material of the endobody and the packets and the

Cell 1 is the oocyte and cells 2–16 are nurse cells. The number in each canal specifies the division at which it was formed. The cystocytes are numbered so that only cells 1 and 2 possess a canal formed at the first division; cells 3 and 4 possess canals formed at the second and following divisions; cells 5–8 possess canals formed at the third and fourth divisions, and cells 9–16 possess canals formed only at the last division.

observed rough surface of the endobody suggests that there is movement of the packets either into or out of the endobody. By S10A the surface is smooth, and packets are no longer seen in the vicinity. We favor the movement of packets into

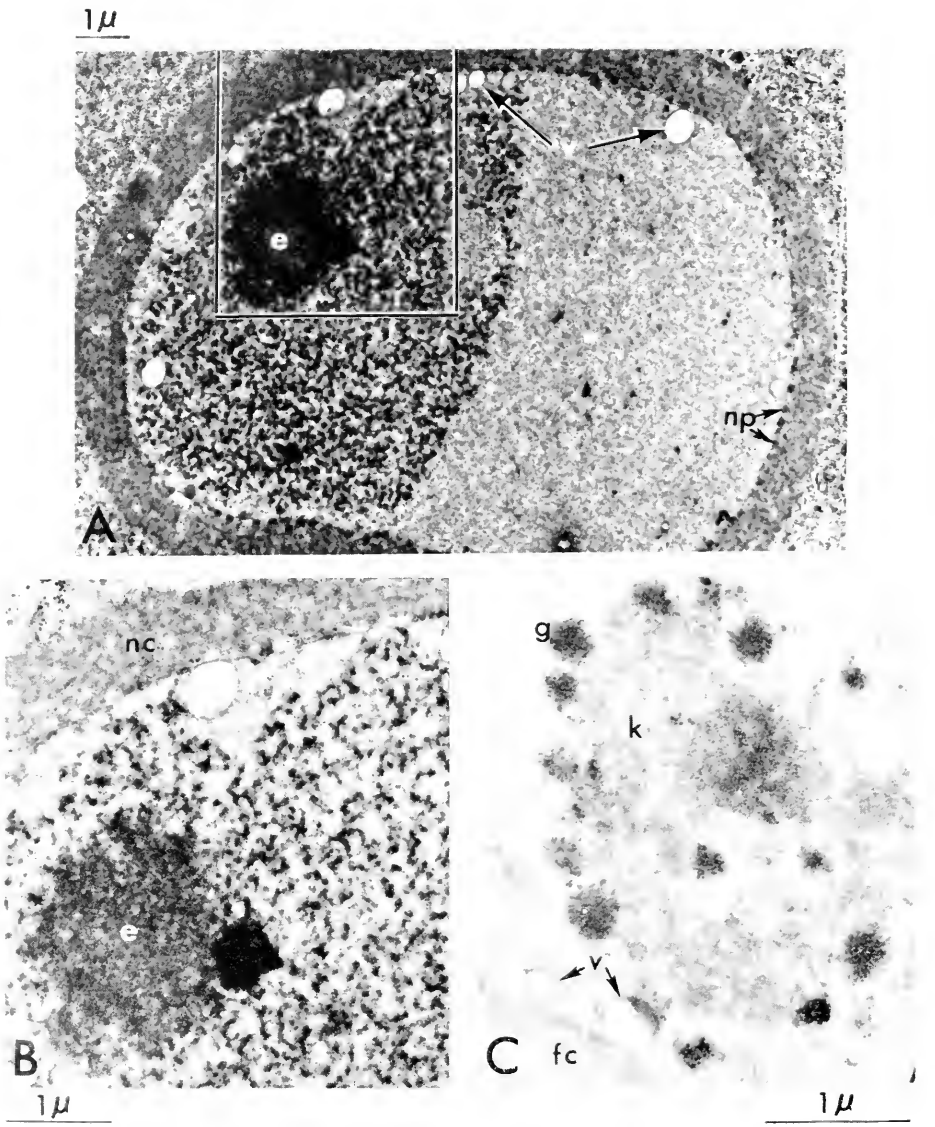


FIGURE 8. (A.) A section through a S8 oocyte which does not include the karyosome. A dense endobody (e) is seen surrounded by packets of nucleolar material. Vesicles (v) are present inside the oocyte nucleus, and nuclear pores (np) are evident; (B.) an enlargement of A, showing the rough border of the endobody and the 600 Å packets. The nuclear coat (nc) contains numerous dense areas; (C.) the karyosome (k) of another S8 oocyte contains clumps of granular (g) material.

the endobody because (1) as development proceeds, the packets get closer together and are found nearer the endobody, and (2) the endobody seems to grow at the expense of the packets, since a maximum endobody volume is seen at S10A, just

TABLE I

The number of oocyte and nurse nuclei traced at the light level and the nuclei photographed at the EM level. The "near" nurse nuclei are those in cells that are directly connected to the oocyte by ring canals, while the "distant" nurse nuclei are in cells separated from the oocyte by at least one other nurse cell. "N," the number of nuclei sectioned; "S," the average number of sections through each nucleus; (−) no nuclear halo visible; (+) to (+++++) indicate the relative amounts of nuclear coating visible

		oocyte nuclei		near nurse nuclei		distant nurse nuclei	
		N	S	N	S	N	S
stage 1	lt.	1 (−)	4	4 (−)	4	11 (−)	4
	em.	1 (−)	6	4 (−)	6	11 (−)	5
stage 2	lt.	2 (−)	5	12 (−)	5	32 (−)	5
	em.	1 (−)	3	2 (−)	10	8 (−)	7
stage 3	lt.	2 (−)	6	9 (−)	6	23 (−)	6
	em.	3 (+)	2	5 (−)	2	1 (−)	1
stage 4	lt.	3 (−)	6	18 (−)	6	38 (−)	6
	em.	2 (++)	5	3 (−)	3	5 (−)	3
stage 5	lt.	7 (+)	5	28 (−)	7	79 (−)	6
	em.	2 (+++)	4	4 (−)	2	7 (−)	4
stage 6	lt.	4 (++)	5	15 (−)	9	38 (−)	7
	em.	3 (++++)	8	3 (−)	2	6 (−)	6
stage 7	lt.	2 (+++)	7	10 (−)	12	22 (−)	10
	em.	1 (+++++)	12				
stage 8	lt.	5 (++++)	8	12 (−)	14	31 (−)	12
	em.	2 (+++++)	8				
stage 9	lt.	2 (+++++)	12	8 (−)	26	22 (−)	17
	em.						
stage 10A	lt.	1 (++++)	13	4 (−)	29	11 (−)	18
	em.	1 (++++)	3	1 (−)	1		
stage 10B	lt.	1 (+++)	18	4 (−)	33	11 (−)	24
	em.						
stage 11	lt.	2 (++)	26	8 (−)	35	22 (−)	30
	em.	1 (++)	5				
stage 12	lt.	1 (−)	6	Degenerate			
	em.	1 (−)	4				

after packets disappear; and (3) the volume of the endobody remains constant, once the packets are gone.

Small granules (about 400 Å in diameter) are associated with the karyosome of *Drosophila virilis*. These granules appear in aggregates of various sizes up to 0.3 μ in diameter. Although the amounts of this material are difficult to quantitate, it is very evident that there is an increase with time, and the maximum is reached at S10A. Cytochemical test with azure B (pH 4) indicate that these granules contain RNA.

Discussion

The general features of oogenesis in *Drosophila virilis* are very similar to those of *Drosophila melanogaster*. For example, the stage designations which were developed for *D. melanogaster* by King, Rubinson and Smith (1956) are applicable to *D. virilis* with little or no modification. As we will show in a subsequent paper, the relative times spent in events such as vitellogenesis, the follicle cell migrations, and the deposition of protective membranes are quite similar in the two species. In addition, the general developmental behavior of the nurse cell chromatin and nucleolar material is almost identical in the two species (see Cummings and King, 1969; King 1970 for *D. melanogaster*).

Koch, Smith and King (1967) demonstrated in *Drosophila melanogaster* that both cystocytes with four ring canals formed synaptonemal complexes, while the other 14 sister cystocytes did not. They also showed that, as it enters the vitellarium, one of the pro-oocytes switches to the developmental pathway being followed by the 14 nurse cells. The same developmental sequence was found in *D. virilis*. The synaptonemal complexes disappear from the oocyte nucleus by S4, and, as in *D. melanogaster* (Smith and King, 1968), by S5 the chromatin is incorporated into a compact karyosome. The small granules (about 400 Å in diameter) associated with the karyosome in *D. virilis* have also been seen in *D. melanogaster* and *D. immigrans* (Mahowald and Tiefert, 1970). We have determined that the number of granules reaches a maximum in S10A, a stage in which Mahowald and Tiefert have demonstrated RNA synthesis in the karyosome of *D. melanogaster*.

The behaviors of the oocyte nucleoli of *D. virilis*, *D. melanogaster*, and *D. immigrans* are quite similar during previtellogenic stages. In all three species the granular elements of the nucleoli are lost by S3 (see Mahowald and Tiefert, 1970, for *D. melanogaster* and *D. immigrans*). The fibrillar elements then seem to reaggregate forming a spherical endobody. Bier, Kunz and Ribbert (1967) first described this structure in the nuclei of oocytes from a variety of insect species characterized by both panoistic and meroistic ovarioles (the genera *Blatella*, *Gryllus*, *Carabus*, *Musca*, *Calliphora*, *Pterostichus* and *Dytiscus* were represented). They found that the structure does not take up H^3-uridine in short term experiments, but does take up H^3-amino acids. Differences were seen in the size, shape and longevity of the endobody depending on the species. Even in the genus *Drosophila*, variations are evident. While the endobody in *D. melanogaster* and *D. immigrans* disappears by the onset of vitellogenesis (Mahowald and Tiefert, 1970), the *D. virilis* endobody remains until the beginning of metaphase I in S12. Both *D. melanogaster* and *D. immigrans* have the 600 Å packets of densely-staining material

which we have described and followed in *D. virilis*. Our evidence suggests that most of the packets are incorporated into the *D. virilis* endobody by S10A.

Sonnenblick (1950) made a light microscopic investigation of the meiotic divisions in recently laid eggs of *Drosophila melanogaster*. According to his account no centrioles or asters were present in the maturation division figures. *Drosophila virilis* oocytes appear to complete meiotic prophase earlier than do *D. melanogaster* oocytes. Meiotic metaphase takes place during S13 and S14 in *D. virilis*, also in the absence of centrioles and asters! The polar areas of the spindles contain diffuse filamentous material and some accumulations of membranes, much like those described in the meiotic divisions of mammalian oocytes (Zamboni, 1970; Szollosi, 1972; Calarco, Donahue and Szollosi, 1972). Centrioles are found within germarial cystocytes and within nurse cells and oocytes of egg chambers in the vitellarium (Koch and King, 1969; Mahowald and Strassheim, 1970). As development proceeds the number of centrioles per oocyte increases and so does the number of nurse cells without centrioles. These findings suggest that after the last cystocyte division the centrioles detach from the cell membrane, that those within nurse cells are carried through the canals in the protoplasmic stream, and that eventually almost all of these centrioles are transferred to the oocyte. The population of centrioles presumably degenerates, since they do not seem to be required for the meiotic divisions.

Ring canals have been observed during the gametogenesis of organisms as diverse as annelids and mammals (see Cassidy and King, 1969; Ruby, Dyer, Gasser, and Skalko, 1970; Dym and Fawcett, 1971; King and Akai, 1971a; Moens and Go, 1972). The canals are found between sister gametocytes during both spermatogenesis and oogenesis. In those insects possessing polytrophic meroistic ovarioles, all but one of the sister germ cells subsequently function as nurse cells, but in many higher mammals the morphological distinction between nurse cells and oocytes is not obvious. However, Davidson (1968), noting that the majority of early oocytes degenerate in mammals, has proposed that some of the interconnected germ cells may function as trophocytes.

There is a general structural similarity in ring canals despite the wide phylogenetic separation of the organisms in which they occur. In all cases, the canal is seen to be round or oval in horizontal section, to be attached to the plasmalemma, and to have a thickened, densely staining rim. The specific features of canal morphology have been studied in detail in only a few organisms, but there is reason to believe that several fine structural aspects may be common at least among some insect orders. The great similarity between the canals of a hymenopteran, *Habrobracon juglandis* and a dipteran, *Drosophila virilis*, is seen when comparisons are made of leaves, vertical microtubules, and membrane connections. These two species are separated enough phylogenetically so that we anticipate finding these same fine structural features in other insect species.

During oogenesis the ring canals allow passage of materials from the nurse cells to the oocyte. Many of the long-lived mRNAs, and ribosomes needed during embryogenesis are provided at an earlier stage by the endopolyploid nurse cells. After fertilization, chromosomes are engaged in rapid mitoses and are inactive as far as transcription is concerned (Fan and Penman, 1970). King and Burnett (1959), Zalokar (1960), and Bier (1963) have demonstrated both the synthesis of

TABLE II

The final number of cystocytes per cluster during gametogenesis

Species	Sex	N	Reference
Drosophila hydei	Male	8	Hess and Meyer, 1968
Drosophila melanogaster	Male	16	Hess and Meyer, 1968
	Female	16	King, Rubinson and Smith, 1956
Habrobracon juglandis	Female	32	Cassidy and King, 1972
Bombyx mori	Male	64	King and Akai, 1971b
	Female	8	King and Akai, 1971b

RNA by nurse cells and its transport into the oocyte. The nurse cells are also major suppliers of mitochondria, lipid droplets, and low molecular weight materials (King, 1960; Cummings and King, 1970).

In the germaria of *Drosophila melanogaster* mitotic figures are found to be grouped into clusters of 2, 4, or 8 (Bucher, 1957; Grell, 1967; Johnson and King, 1972), and this observation demonstrates that interconnected cystocytes divide simultaneously. In *Xenopus laevis* somatic cell nuclei can be induced to undergo DNA replication by exposing them to egg cytoplasm (Graham, Arms, and Gurdon, 1966). This finding demonstrates that the test cytoplasm contains factors that stimulate mitosis. Perhaps in *Drosophila* the system of canals between cystocytes allows passage of soluble "mitogens" which initiate division in all cells in a given cluster. Thus, sister cystocytes should show an "all or none" type of mitotic activity, and their number (N) after their final division should be given by $N = 2^M$, where M equals the number of consecutive mitoses preceding meiosis. The values of N observed in a number of higher insects (see Table II) follow the 2^M rule, and these observations support the hypothesis that the ring canal system insures the mitotic synchrony of sister cystocytes during both oogenesis and spermatogenesis.

Dividing cystocytes are rarely observed in fixed *Drosophila* germaria. The data of Johnson and King (1972) indicate that only one germarium in 25 contains such cells. However, in two instances dividing cystocytes have been encountered in electron microscopic studies. In the cytokinesis illustrated by Koch and King (1969, their Figs. 2 and 3) vesicles and small tubules are aligned along the division plane separating two sister cystocytes. The canal rim is embedded in the center of this plaque. In the division illustrated by Mahowald (1971, his Figs. 1 and 2) paired, convoluted membranes extend to the canal. The tissues were fixed differently in the two cases. Koch and King (1969) used chromate-OsO_4 and Mahowald, glutaraldehyde. Taken together these results provide some useful information concerning cystocyte cytokineses. We now interpret the vesiculated areas to represent recently synthesized portions of the plasmalemma (see Figs. 9B and 9C). Presumably these are stabilized by glutaraldehyde, but tend to fragment when exposed to the harsher fixative. However, the portion of the plasmalemma to which the contractile ring microfibrils are attached is stable enough to resist the chromate-OsO_4 fixation and is therefore retained in both cases. The concept that new membrane is synthesized immediately beyond the contractile ring during cleavage formation is supported by the studies of Selman and Perry (1970) and Bluemink (1971) on cleaving amphibian zygotes.

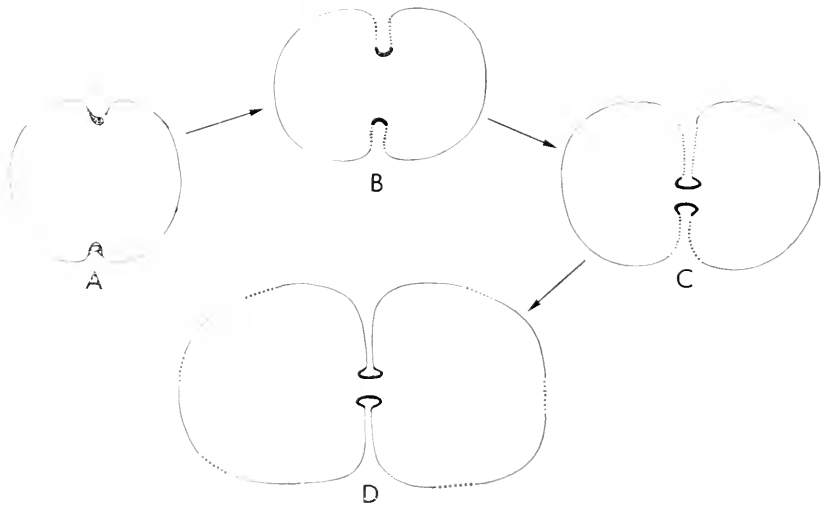


FIGURE 9. A diagrammatic representation of the proposed synthesis of new plasma membrane during the cleavage and subsequent growth of cystocytes. The contractile ring is represented by a thick solid line, old membrane by a thin solid line, and newly synthesized membrane by a stippled line. See text for further discussion.

Koch and King (1969) have shown in *Drosophila melanogaster* that, while the canal rims undergo morphogenetic changes, the average distance between ring canals remains relatively the same as the cystocytes grow. Therefore the plasmalemmal regions that bind microfibrils are not disturbed as the surfaces of the cystocytes expand. The above observations suggest that once cytokinesis is complete and fourth generation cystocytes start to grow, new plasma membrane is added to regions relatively distant from the canal rims (see Fig. 9D).

In the *Drosophila* male all cystocytes develop in an identical fashion, once divisions cease. In the female, however, the nurse cells and the oocyte follow different developmental pathways, although they are interconnected (see King, 1970 for details). Studies on the *female sterile* mutation provide clues to the mechanisms terminating cystocyte mitosis and initiating differentiation. Females homozygous for *fes* are sterile and produce "tumorous" ovarian chambers (King, Burnett and Staley, 1957, King, Koch and Cassens, 1961, Koch and King, 1964, King, 1969a). The mechanism which stops fourth generation cystocytes from further division in wild type *D. melanogaster* does not operate in the majority of *fes* chambers. In the mutant Johnson and King (1972) have shown that the normal arrested cleavage of cystocytes is disturbed so that divisions are often complete. They conclude that the product of the fes^{+} gene is required for the formation of a stable canal system, and they suggest that the product of the mutant gene is defective in this regard. In the abnormal cystocyte clusters found in *fes* an oocyte is produced only in those cells containing four canals (King, 1969b). If fewer canals are present, oocyte differentiation does not occur, regardless of the total number of cystocytes in the cluster. Johnson and King (1972) suggest that the signal that normally stops fourth generation cystocytes from further mitoses is the differentiation of pro-oocytes, and that these cells receive their cue to differentiate from the four canal rims that they alone possess.

We have presented cytological evidence suggesting that protein microfibrils continue to be deposited on the canal rims and that later masses of clustered microfibrils break off of each rim and are carried away in the cytoplasmic stream. The sizes of masses vary, but as one would expect the long and short axes of the masses are always parallel to the long and short axes of the constituent microfibrils. The masses seem to bind specifically to the oocyte nucleus and to form a coating that eventually reaches a thickness of a micron or more and is dense enough to exclude perinuclear mitochondria and ribosomes.

An interesting comparison can be made between the microfibrils associated with ring canals and the microfibrils making up the contractile rings found in cleaving animal cells (see Selman and Perry, 1970; Szollosi, 1970). Many investigators (see reviews by Rappaport, 1971; Tucker, 1971) believe that sliding interactions between the overlapping microfibrils generate the constrictive forces required for cleavage. Contractile ring microfibrils have a diameter of 30–70 Å and are always oriented circumferentially in the plane of the constriction. The microfibrils we see on the canal rims are of similar diameters and also have a circumferential orientation. We suggest that the ring canal rim is composed of old equatorial plasmalemma that retains an affinity for contractile ring microfibrils. However, we have no evidence for or against the argument that the microfibrils that attach subsequent to the arrested cleavage represent a heterogeneous assemblage of protein molecules that bind both to canal rims and to the surface of the oocyte nucleus only because these structures carry the appropriate receptors. For example, spindle microtubules are composed of fibrous protein subunits (see review by Adelman, Borisy, Shelanski, Weisenberg, and Taylor, 1968). These "tubulin" molecules, which have diameters ranging between 40 and 50 Å, should be abundant near canals and might contribute to the coatings. Regardless of the composition of the coatings, it is clear that the ring canal rims and the outer surface of the oocyte nucleus both tend to bind fibrous protein molecules and that this binding ability is shown by the rims before the nucleus. Perhaps the receptor molecules necessary for the subsequent binding of microfibrils pass from the rims to the oocyte nucleus; but this is but one of many plausible interpretations.

Studies on the mechanism of action of steroid hormones in birds and mammals (O'Malley, Sherman, and Toft, 1972; Steggles, Spelsberg, Glasser, and O'Malley, 1971) indicate that the cells of target tissues contain specific receptor proteins that bind to the hormones. The receptor protein-hormone complex then enters the nucleus, binds to the chromatin, and stimulates the transcription of mRNAs for the proteins whose synthesis is known to be induced by the hormones. In the *Drosophila* egg chamber the nucleus of the oocyte is much less active in terms of transcription than are those of the sister nurse cells. We assume that the nuclear coating prevents the transfer of high molecular weight materials to and from the oocyte nucleoplasm and thus helps to insulate the oocyte chromosomes from the influence of those compounds that stimulate transcription in the sister nurse cells.

The agent responsible for ring canal formation is probably a long-lived spindle apparatus (Fawcett, Ito and Slautterback, 1959; Brown and King, 1964) which arrests the advancing cleavage furrow and thus prevents separation of the sister cells. The portion of the spindle called the midbody remains in the bridge connecting the sister cells. Within the midbody, microtubules extending from the spindle poles overlap and are embedded in a dense matrix (Byers and Abramson,

1968). Buch and Tisdale (1962) report that the longevity of the midbody varies according to the species and the cell type in which it is found. Although the exact nature of ring canal formation is not understood, there is evidence that the advancing edge of the furrow may actually interact with the tubules making up the periphery of the midbody. This view is based on the structure of *Habrobracon* and *Drosophila* canal rims where the tubules found embedded in the leaves have the same orientation as the midbody microtubules (Cassidy and King, 1969).

Studies on ciliate cleavage (Tucker, 1971) suggest that the process includes two phases; the first rapid, the second slow. The rapid phase effectively separates the dividing cells except for a small neck. In a much slower phase, the last small interconnection is severed. Tucker suggests that the final nipping off of the sister cells is due to an additional or different mechanism from that involved in ring constriction. The ring canals of cystocytes could result from failure of this final cleavage mechanism.

Two patterns of cell division have been studied by electron microscopists. The first, which is commonly observed in animal tissues, requires the formation of a plasmalemmal furrow (Fawcett, Ito, and Slautterback, 1959). The second, which is commonly observed in higher plants, involves the laying down of a plaque of interconnected vesicles and tubules along the plane of division and later the coalescence of nearby tubules to form continuous sheets of membrane which segregate the cytoplasm of the sister cells (Porter and Machado, 1960). Centrioles and asters are associated with the mitotic apparatus in the first type of cytokinesis (Stubblefield and Brinkley, 1967). Centrioles and asters are missing from the mitotic apparatus in the second type of cytokinesis (Pickett-Heaps, 1971). The *Drosophila* oocyte is generated by the division of a third generation cystocyte, and a conventional mitotic apparatus with centrioles and asters is employed. The subsequent meiotic division figure lacks centrioles and asters, and the cell does not cleave. Taken together, the above facts suggest that centrioles are not necessary for the formation of spindles and for the sister chromosomes to be separated in an orderly fashion. This function is performed by much less conspicuous organelles. Pickett-Heaps (1971) has referred to these as microtubule-organizing-centers (MTOCs). Rappaport (1971) has reviewed the evidence which demonstrates that the establishment of a cleavage furrow requires two asters working in concert. Perhaps the centrioles generate asters, and in the zone where microtubules from the two asters are confluent an equatorial ring of plasma membrane is modified in some way that facilitates the assembly of contractile ring microfibrils. In *Drosophila melanogaster* according to Sonnenblick (1950) the polar body nuclei are not extruded from the egg but remain peripherally disposed in an anterodorsal island of ooplasm where they eventually disintegrate. It follows that in the case of the oocyte, centrioles can be dispensed with, since furrowing of a conventional sort does not occur during the meiotic divisions.

The fertilizing sperm brings in a centriole (Sonnenblick, 1950), and this is the progenitor of those seen during embryogenesis. After fertilization twelve synchronous mitoses occur, and the nuclei generated form a blastema. At the next mitosis cytokinesis takes place, and a cellular blastoderm is formed. In their study of this event in *Drosophila montana* Fullilove and Jacobson (1971) concluded that the oolemma starts to form furrows at those regions where the microtubules of adjacent asters overlap (see their Fig. 16).

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Summary

In females of *Drosophila virilis*, germ cells, characterized by conventional division, differentiate into "cystocytes" that engage in a specific number of mitoses, each followed by incomplete cleavage. The result is a cluster of 16 cells joined by canals. The canal rims are made up of overlapping leaves similar to those already described for *Habrobracon juglandis*. The *virilis* canal rims become coated with protein microfibrils. Masses of these fibrils detach from the rims and are carried in a stream of cytoplasm to the oocyte. Clusters of microfibrils adhere to the oocyte nucleus which eventually develops a coating of amorphous material 1 μ thick. The nuclei of the 15 sister nurse cells never develop such a coating. We suggest that the nuclear coating prevents the transfer of large molecules to the oocyte nucleoplasm and so insulates oocyte chromosomes from the influence of those compounds that stimulate transcription in the sister nurse cells. Centrioles and asters take part in cystocyte divisions, but are absent from the meiotic division figures. We conclude that centrioles function to generate asters. These in turn facilitate the attachment to localized regions of the plasmalemma of contractile microfibrils that enable furrowing to take place. Less conspicuous "microtubule-organizing centers" organize the spindle which functions to distribute the chromosomes during mitosis.

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MUSCULAR ACTIVITY UNDERLYING VENTILATION AND SWIMMING IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* (LINNAEUS)[1]

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Ventilation and swimming in *Limulus polyphemus* (Linnaeus) are two behaviorally distinct, rhythmic movements involving coordinated metachronal activation of the opisthosomal appendages. Normally, ventilation consists of slow, rhythmic movements of all five gill plate pairs causing circulation of water through the subjacent gill books. The ventilatory cycle ranges from a low frequency, low amplitude movement of only the terminal gill plates to a vigorous rhythm (hyperventilation) involving the genital operculum as well as all gill plates. Vigorous ventilatory movements, however, do not develop significant propulsive force. Swimming consists of rapid gill plate and opercular movements which are qualitatively similar to those of ventilation but which differ substantially in amplitude and various temporal characteristics. Frequently the prosomal walking legs participate in swimming behavior, moving in coordination with the opisthosomal appendages.

Hyde (1893) found that *Limulus* opisthosomal ganglia, isolated from the rest of the CNS by nerve sectioning, are capable of generating ventilatory movements in their corresponding gill plates. More recently Fourtner, Drewes and Pax (1971) demonstrated that the motor output is sustained in the absence of peripheral feedback and therefore probably is centrally programmed. Intracellular recordings and stimulation revealed that motoneurons participating in the ventilatory bursts do not interact (Wyse, 1971). These results suggest that the origin of the oscillatory motor output which controls ventilation involves premotor interneurons within each opisthosomal ganglion. The overt similarities between swimming and ventilatory movements, and the fact that reciprocal excitation of the same antagonistic flexor and extensor muscles are responsible for the gill plate movements of both behaviors, suggest a common central control mechanism of rhythmic output. This investigation utilizes gill plate muscle activity recorded during periods of swimming and ventilation to predict characteristics of the underlying motoneuron bursts and to analyze various aspects of their coordinating mechanisms.

Material and Methods

Horseshoe crabs, *Limulus polyphemus,* were collected by the supply department of the Marine Biological Laboratory (Woods Hole, Massachusetts) and held

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until use in tanks of running sea water at room temperature either in Woods Hole or in Santa Barbara. A Nikon Super 8 movie camera (film speed 24 frames per second) was used for detailed observation of appendage movements during *Limulus* swimming and ventilation. The films were analyzed by frame-by-frame projection and tracing of the position of each participating appendage as it underwent metachronal movements. The paths described by the basal segment of each leg and the distal articulation of the opisthosomal appendages were plotted as a function of time.

Ventilation was monitored while the animal rested submerged in a container of sea water. Swimming studies were conducted with the animal suspended by a clamp on the prosomal carapace, which allowed free movement of the opisthosoma and all appendages. Specimens supported in this manner usually began swimming spontaneously, although in some cases gentle tactile stimulation was required to elicit this behavior.

Electromyograms were recorded from the prosomal tergo-coxal muscles, which retract the ambulatory appendages, and the opisthosomal abductor and external branchial muscles, which extend and flex respectively the gill plates and the gential operculum (for terminology of the musculature see Lankerster, Benham, and Beck, 1885). Monopolar electrodes of insulated silver or copper wire were implanted through small holes bored in the prosomal or opisthosomal carapace at the tergal insertion of the appropriate muscles. The electrodes were secured to the carapace with Eastman 9–10 adhesive and plastic tape. Muscle potentials were amplified with Tektronix Type 122 low-level preamplifiers and displayed on a Tektronix 564 oscilloscope or an Offner Type RB dynograph. A one second time constant was used for integrated records of muscle activity.

Since variations in the degree of electrical contact made by individual electrodes bias the recorded muscle activity amplitudes and durations, only burst interval data are quantitatively comparable between electrodes. Comparisons between electromyogram amplitude and duration are valid only if the data were recorded from the same electrode implantation. Thus, burst amplitude is here computed as the percentage of maximum amplitude recorded during rapid swimming movements, and intersegmental delay is defined as the measured time between the peaks of integrated burst activity from sequential extensor or flexor muscles.

Results

Ventilation

Ventilation consists of repetitive extensions and flexions of the opisthosomal gill plates beginning with the terminal pair and proceeding sequentially forward. Each gill plate extends slowly, actively bent at its distal articulation by muscles in the outer lobe, and sweeps out an arc from approximately 3–5 degrees during slow ventilation to 65–70 degrees during periods of hyperventilation. Extension of the gill plate forces the branchial leaflets apart, allowing for the irrigation of the subjacent gill books. Gill plate extensions are followed by a metachronal wave of more rapid gill plate flexions, during which time the outer lobe muscles of each plate momentarily relax. Each gill plate is extended in response to a burst of activity in its corresponding abductor muscle (Fig. 1a, 1b). The burst is almost symmetrical in shape with the amplitude peak toward the center of the burst. Gill plate flexion results from a similarly symmetrical muscle burst in the external

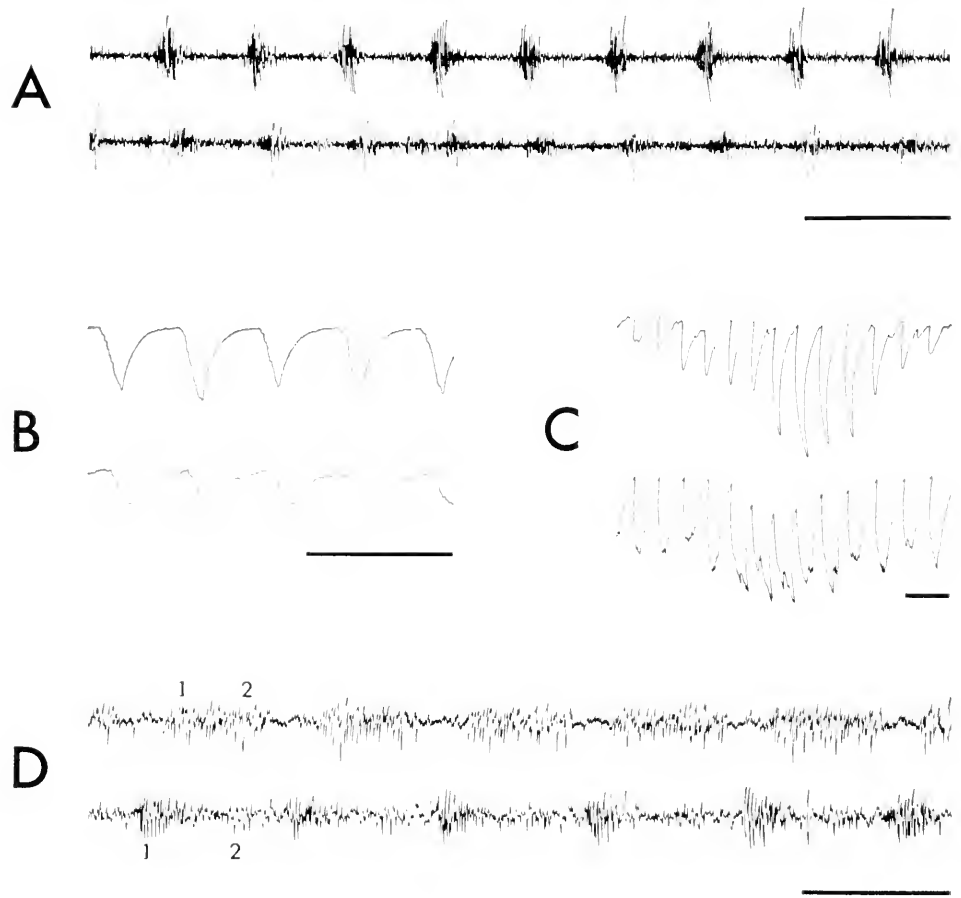

FIGURE 1. Flexor and extensor bursts during ventilation and hyperventilation. (A) exemplifies the reciprocal activity in flexor (top) and extensor (bottom) muscles during ventilation as recorded from the musculature of the first gill plate. (B) is an integrated record of (A) showing the symmetrical shape of the flexor and extensor muscle bursts (a downward deflection indicates activity). In (C) an integrated record of flexor (top) and extensor (bottom) bursts during gradual transitions from ventilation to hyperventilation and back to ventilation demonstrate the concomitant transformations of the flexor burst shape from symmetrical to saw-tooth, and of the extensor burst shape from symmetrical to double peaked. (D) shows double peaked extensor bursts in the first (top) and fourth (bottom) gill plates during hyperventilation. The first activity peak (1) in both muscles is consistantly strong, whereas the second activity peak (2) is considerably weaker in the more caudal muscle. Bar indicates 5 sec.

branchial muscle (Fig. 1b). These muscle burst shapes correlate with the symmetrical motoneuron firing patterns in the external branchial nerve and medial branchial nerve as recorded by Fourtner *et al.* (1971).

Electromyograms from extensor and flexor muscles indicate that the following elements of the ventilatory burst cycle are variable: (1) the number of participating appendages; (2) the muscle burst interval; (3) the intersegmental delay of muscle

TABLE I

Recorded ranges of the variable elements in flexor muscle burst during ventilation, hyperventilation and swimming (Values taken from data on sixteen different animals). Abbreviations are: gp, gill plate; go, genital operculum; wl, walking leg.

	Appendages involved		Burst interval (sec)		Burst duration (sec)		Burst amplitude (per cent max.)		Intersegmental delay*** (msec)	
	Slow*	Fast**	Slow	Fast	Slow	Fast	Slow	Fast	Slow	Fast
Ventilation	gp 1	gp 1–5	20.0	1.0	1.80	0.28	<5%	40%	500	30
Hyperventilation	gp 1–5 go	gp 1–5 go	4.0	0.6	1.00	0.25	30%	85%	70	10
Swimming	gp 1–5 go	gp 1–5 go wl 1–5	1.8	0.4	1.10	0.20	78%	100%	30	<10

* slow limits were set by larger animals (16 to 18 cm across the prosomal carapace).
** fast limits were set by smaller animals (8 to 10 cm across the prosomal carapace).
*** the measured time between peaks of integrated burst activity from sequential flexor muscles.

activation; (4) the amplitude of integrated muscle activity; and (5) the duration of the muscle activity. The recorded range for each element of the flexor burst cycle is presented in Table I. Occasionally, during periods of extremely slow ventilation, only the caudal gill plate pair or pairs are active; these movements are slight, with correspondingly small muscle potentials and large intersegmental delays, up to 500 msec in some cases. If the animal receives gentle tactile stimutation, the more rostral gill plates and the genital operculum will be recruited into the rhythmic movement with a concomitant increase in both cycle frequency and muscle activity, and a decrease in the intersegmental delay. Figure 2 suggests that the above variables are linearly related to burst interval.

Hyperventilation

Hyperventilation is behaviorally distinguishable from ventilation by the active participation of the genital operculum in the rhythmic metachronal cycle. Since there are no gill books associated with the operculum, this movement appears only to enhance the circulation of water for the more caudal gill plates and their corresponding gill books. The transition from ventilation to hyperventilation may be gradual, with no obvious change in the metachronal movements other than incorporation of the genital operculum, or it may be abrupt, in which case there will be observed a pronounced increase in amplitude of the gill plate movements (Fig. 3). As hyperventilation becomes more vigorous, a transition in the extensor and flexor muscle burst shapes takes place (Fig. 1c). The extensor burst assumes a double peak conformation in which the first peak is larger and more consistent, while the second is somewhat erratic, being almost imperceptible in the most caudal gill plates, and becoming larger in the more rostral gill plates (Fig. 1d). The burst shape in the flexor muscle changes from symmetrical, during slow to moderate hyperventilation, to a saw-tooth shape during rapid hyperventilation (Fig. 1c). This transition may occur gradually, in which case the peak of muscle activity appears to migrate slowly toward the beginning of the burst. This change in

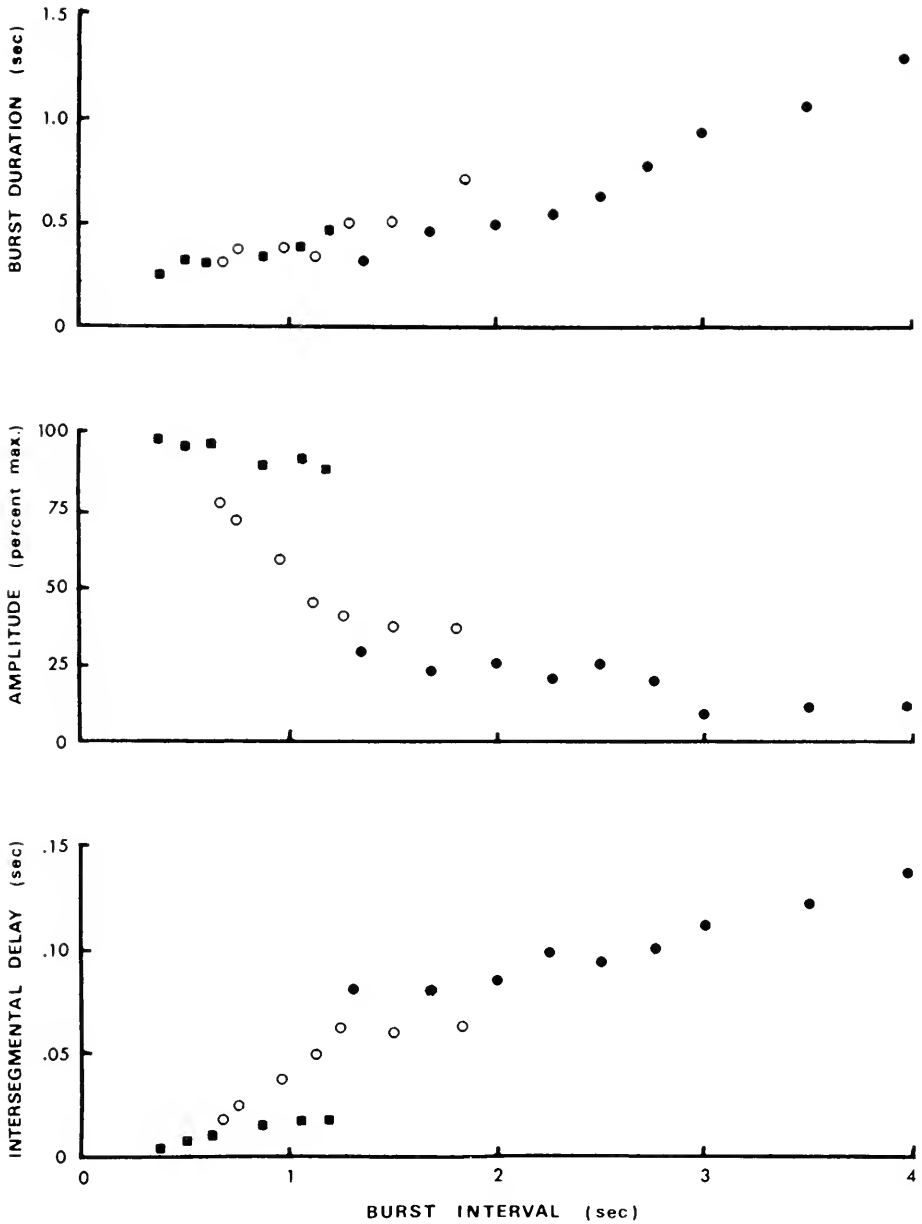

FIGURE 2. Change in flexor burst duration, amplitude and intersegmental delay as a function of muscle burst interval for ventilation (closed circles), hyperventilation (open circles), and swimming (closed squares). The data was taken from the flexor muscles in the first and second gill plates of 12 cm (across prosoma) males (N = 10). Similar relationships are found in the extensor muscle activity.

shape reflects a similar alteration in the causative motoneuron burst from one which is symmetrical to one which is biased toward burst onset.

Measurements of hyperventilatory intersegmental delay and muscle potential amplitude vary from those recorded during ventilatory movements (Fig. 2). Flexor and extensor muscles undergo substantially greater increases in muscle amplitude and decreases in intersegmental delay with decreasing burst interval. Also, the lower limit of the burst interval greatly decreases during hyperventilation (Table I), which allows for more rapid movements of the gill plates than occurs during ventilation.

Swimming

Swimming behavior varies from the previously described activities of ventilation and hyperventilation in that prosomal appendages are incorporated into the ongoing metachronal process. The onset of swimming is signaled by a slight retraction of the fifth pair of walking legs, which usually does not affect the anterior appendages. This retraction is immediately followed by repetitive waves of coordinated appendage protractions and retractions beginning at the terminal gill plates and proceeding rostrally to the first pair of walking legs (Fig. 4). The gill plates move powerfully following the basic ventilatory movement pattern, but sweep out a considerably greater arc and bend to a greater extent at their distal articulation during extension than is the case for ventilation. The prosomal walking legs, on the other hand, describe a more elliptical path (as viewed from the side) (Fig. 5), protracting slowly under the protection of the carapace and then suddenly extending and rapidly retracting, beginning with the fifth leg. Movement of the fifth leg is followed by a simultaneous retraction of the four anterior legs. The chelae of the first four walking legs, and the spatulate spines of the fifth walking leg spread simultaneously with extension which probably serves to increase thrust. Following retraction, the chelae and tarsal spines close and the legs are again brough up under the carapace.

All the aforementioned appendages continue in their stereotyped movements as long as the specimen swims unobstructedly. If, however, an obstacle is contacted,

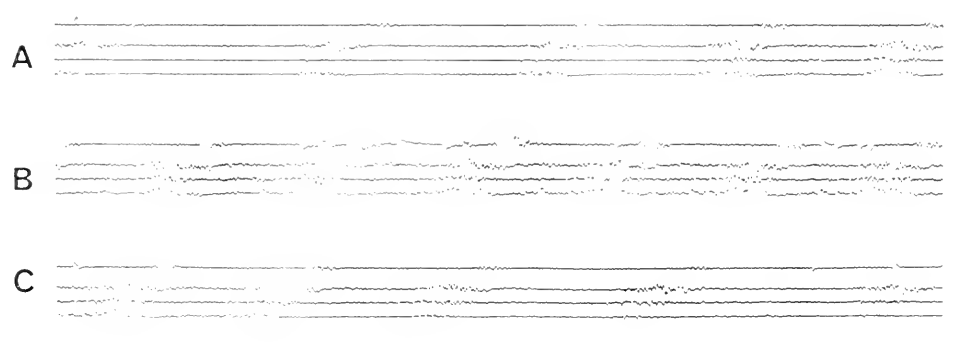

FIGURE 3. Activation and deactivation of the flexor muscle to the genital operculum during a transition from ventilation (A) to hyperventilation (B) and back to ventilation (C). Shown are consecutive recordings from the flexor muscles of the genital operculum and first, second and third gill plates (top to bottom). Bar indicates 0.5 sec.

the animal can navigate around it by maintaining swimming movements in certain legs while others remain at rest. Thus all the legs have been observed operating independently of the others and in various combinations with ipsilateral as well as contralateral legs, and independently of the gill plates and the operculum. The opisthosomal appendages, however, do not exhibit this degree of freedom. Every wave initiated by the fifth gill plate during swimming carries through to the operculum, which is not the case for ventilation where occasionally only the posterior two or three gill plate pairs will be affected by a single wave of excitation.

It is possible to identify swimming behavior from electromyograms since activity in the gill plate muscles is correlated with activity in the leg muscles. Analogous to the transition from ventilation to hyperventilation, the transition from ventilation to swimming may take place gradually, accompanied by a progressive increase in muscle burst amplitude and frequency (Fig. 6a), or suddenly, in which case burst amplitude and frequency increase abruptly (Fig. 6b). The extensor and flexor muscle bursts consist of uniform, high amplitude muscle potentials (Fig. 6a) which probably result from extremely intense short duration motor nerve activity. The temporal characteristics of the flexor and extensor bursts change little with decreasing burst interval during swimming, as opposed to the cases of ventilation and hyperventilation (Fig. 2). Although a discernible increase in burst amplitude and decrease in burst duration and intersegmental delay occurs with decreasing burst interval, the rates of change are less than those noted for hyperventilation and ventilation.

Lesion experiments

Lesion experiments similar to those by Hyde (1893) were performed in this study to segregate those opisthosomal movements which require contact with the brain from those which are within the repertoire of the isolated opisthosomal nervous system. A cut in the ventral nerve cord anywhere posterior to the brain leaves appendages caudal to the cut unable to carry out swimming movements, while appendages rostral to the cut perform apparently unaltered swimming motions. Appendages caudal to the cut will, however, participate in coordinated ventilation, which proceeds continually unless the gill plates are physically restrained, and hyperventilation, which may be provoked for short periods through tactile stimulation of the opisthosoma. Thus, the opisthosomal neural circuitry is capable of ventilatory and hyperventilatory movements in isolation, while swimming seems to require descending information from the brain.

Coordination in the ventral nerve cord

Tactile stimulation applied to the anterior lamella of any gill plate causes its immediate flexion and a general cessation of ventilatory movements, when the nervous system is intact. If, however, the ventral cord has been severed between the brain and first opisthosomal ganglion, this gill plate reflex is localized to the stimulated gill plate, which immediately resumes its ventilatory movements after flexing. To determine the extent to which such peripheral sensory input influences temporal aspects of the rhythmic activities, serial transections of the ventral nerve cord were made to isolate the fused fifth/fourth, the third, the second, and the first opisthosomal ganglia forcing them to coordinate their outputs through sensory

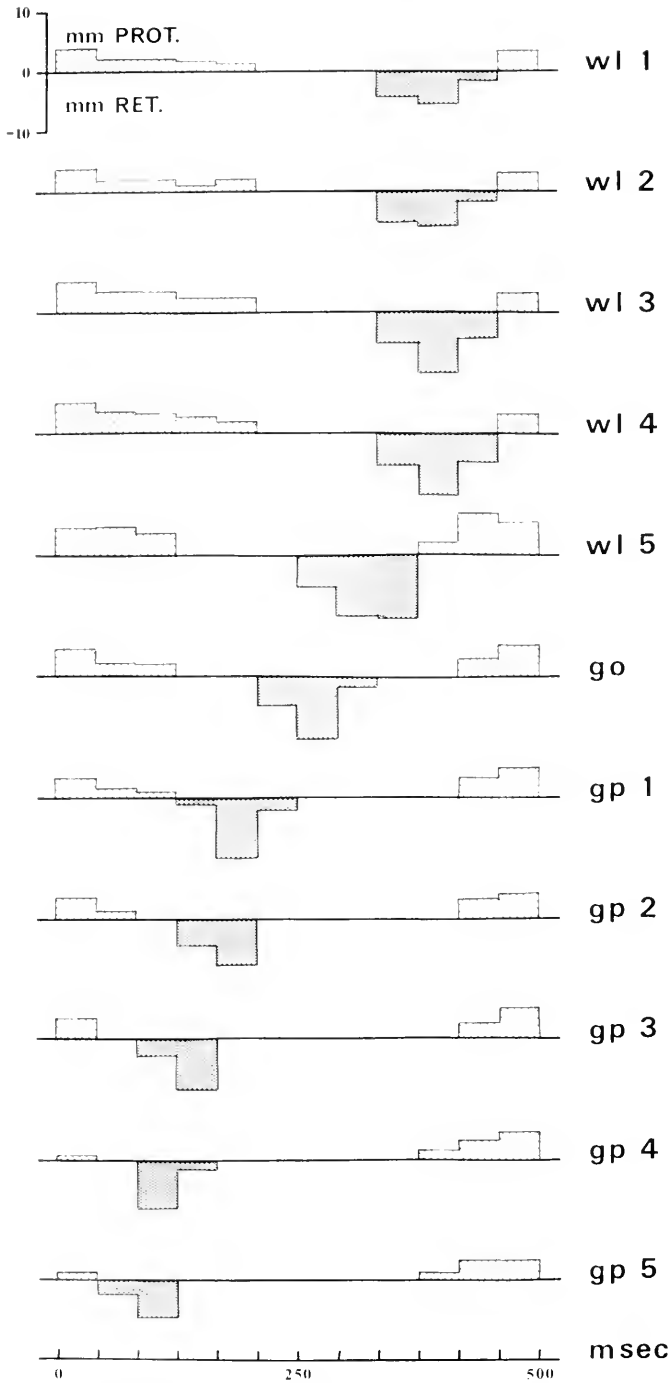

FIGURE 4. One swimming cycle of appendage protractions (positive) and retractions (negative) derived from frame by frame analysis of a freely swimming, 13 cm (prosomal

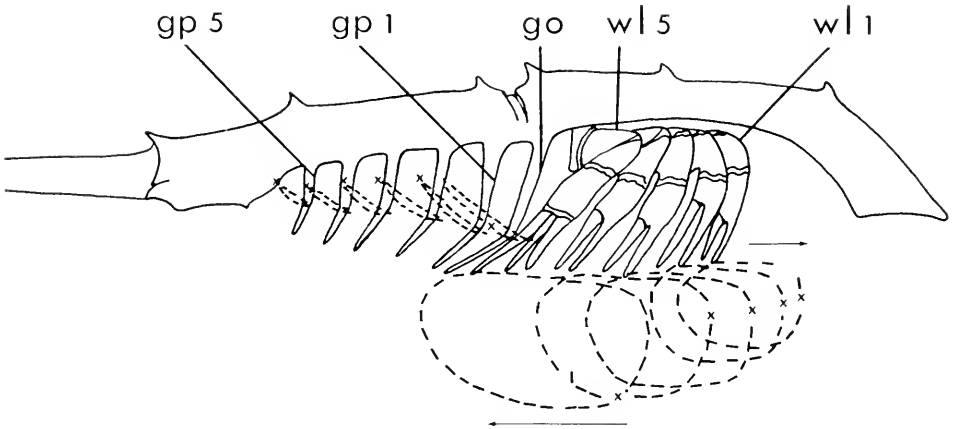

FIGURE 5. Cut-away view of *Limulus* showing the paths described by the various appendages during swimming as derived from consecutive frame tracings. The figure is drawn such that the appendage positions correspond to time 0 in Figure 4, and the "X's" denote the position of each appendage at 250 msec. Abbreviations are: go, genital operculum; gp, gill plate; wl, walking leg.

input alone. Muscle electrodes monitored activity in each external branchial muscle. Figure 7 shows the results of ganglionic isolation.

With each transection the following occurs: (1) there is a prolongation and sporadic desynchronization of the bursts from the isolated ganglion; (2) the pacemaker function is assumed by the most caudal intact ganglion; and (3) the burst duration and intersegmental delay of the intact ganglia become increasingly variable (Fig. 7b). All of the ganglia are capable of pacemaker activity and all maintain their rhythmic output after isolation. When all ganglia are isolated, the burst pattern of each remains loosely coordinated with activity in neighboring ganglia, although the order of burst initiation may vary (Fig. 7c). This loose coordination probably results from tactile stimulation by adjacent gill plates, whose movements trigger activity in other ganglia.

The pacemaker function, usually exercised by the fifth opisthosomal ganglion during normal ventilation, can be exercised by any of the first five ganglia under conditions of differential ganglionic stimulation if the nerve cord remains intact. Figure 8a represents the normal metachronal progression of the ventilatory rhythm. Figures 8b and 8c show the results of differential stimulation of the second and first ganglia by severing their gill plates. A distinct reversal in firing order is observed, with the stimulated ganglion assuming the pacemaker function for the system. Whichever ganglion functions as pacemaker, the coordinating circuitry is organized so that the wave of excitation propagates from the pacemaker, sequentially exciting adjacent ganglia both anteriorly and posteriorly.

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width) female. Each bar represents the distance traveled, in a rostral or caudal direction, by the corresponding appendage between consecutive frames (41.7 msec). Abbreviations are: go, genital operculum; gp, gill plate; wl, walking leg (compare with Figure 5).

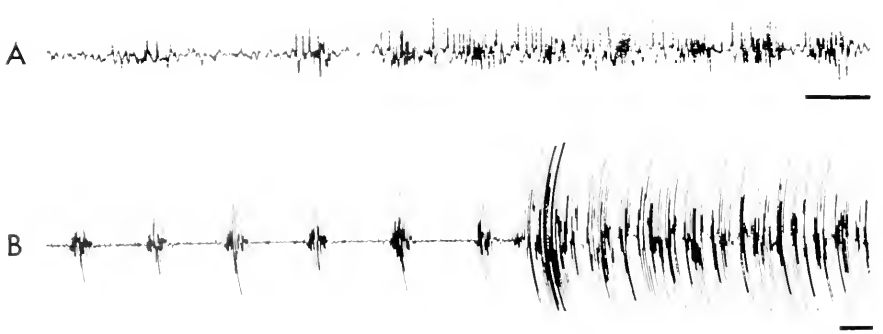

FIGURE 6. Flexor burst records during gradual (A) and abrupt (B) transitions from ventilation to swimming. Both recordings are from the first gill plate flexor, but (A) is at a lower gain than (B). Bar indicates 1 sec.

DISCUSSION

Interpretation of the muscle activity graphs in Figure 2 gives some insight as to the underlying motoneuron activity corresponding to ventilation, hyperventilation, and swimming. If each ventilatory motor burst consists of a nearly equal number of motoneuron discharges, then the observed decrease in burst duration would entail a progressively smaller interspike interval. Assuming that the opisthosomal flexor and extensor muscles undergo summation and facilitation, such a reduction in interspike interval would explain the observed increase in muscle potential amplitude. The amplitude change in the muscle activity with the onset of hyperventilation may result from recruitment of motoneurons causing additional muscular summation and facilitation. The burst shape transition from symmetrical to saw-tooth (Fig. 1c), noted during periods of hyperventilation, may represent

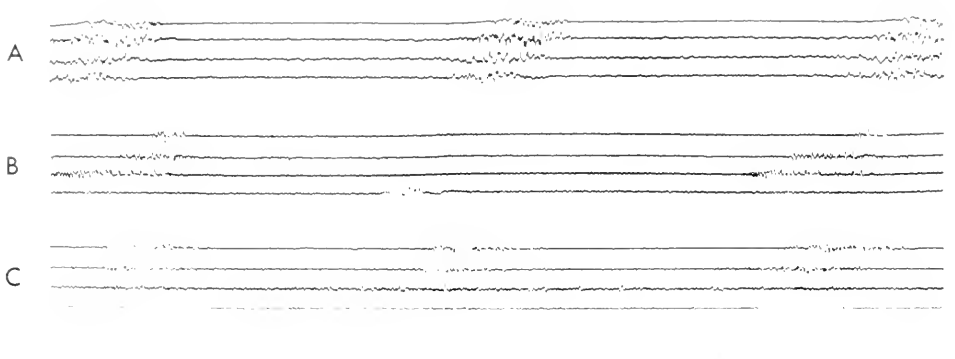

FIGURE 7. The effect of progressive isolation of the opisthosomal ganglia upon ventilatory activity in the flexor muscles of gill plates one through four (in order from top to bottom). The rhythmic, metachronal muscle potentials in (A) are typical of normal ventilatory activity. Desynchromized activity of the fourth gill plate flexor muscles, shown in (B), occured after the connectives between the third and fourth ganglia were severed. Muscle bursts following the isolation of all opisthosomal ganglia, (C), demonstrate some loose coordination of activity. Bar indicates 1 sec.

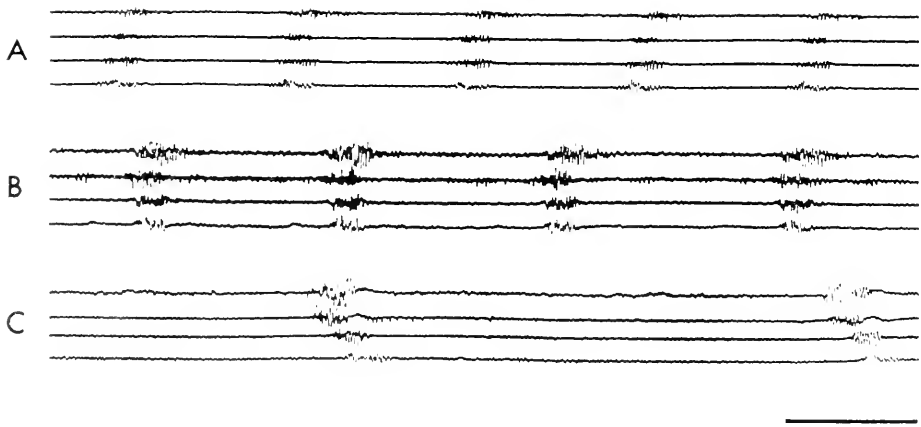

FIGURE 8. Shift in pacemaker location in the intact nervous system following differential stimulation of the leading ganglion as reflected by flexor muscle activity in the first four gill plates (top to bottom). (A) shows the normal metachronal progression of flexor activation. (B) and (C) show the second and first ganglia exercising the pacemaker function. Bar indicates 1 sec.

the migration of these newly recruited motoneuron discharges from the middle to the beginning of the flexor burst. The substantially greater muscle potential amplitude in the flexors and extensors during the performance of swimming movements suggest still further recruitment of motoneurons to the respective motoneuron bursts. The similarities of the burst amplitude - burst interval plots (Fig. 2) and the intersegmental delay - burst interval plots suggest that amplitude and delay are governed by the same principle.

The variability of the pacemaker in the intact nervous system indicates that although the coordinating mechanism for the metachronal rhythm is certainly inherent to the CNS (Fourtner *et al.* 1971), the directionality of the mechanism is not so stable that it may be considered polarized, as is the case in the crayfish swimmeret control system (Ikeda and Wiersma, 1964). The observation that selective stimulation of any one ganglion triggers ventilatory output in that ganglion and that this activity in turn excites the more remote ganglia both caudally and rostrally in a sequential manner (Fig. 8) intimates that the triggering of these opisthosomal ganglionic oscillators is a threshold phenomenon based on intergrated peripheral and central input and that the firing of an oscillator activates interneurons which synapse, either directly or indirectly, upon the more caudal and more rostral oscillators. Although correlated bursting of isolated opisthosomal ganglia may be sustained through sensory input triggering alone (Fig. 7c), burst duration and delay seem to be critically modulated by such interganglionic neural activity possibly in the form of phasic excitation and inhibition transmitted *via* coordinating interneurons analogous to those found in the crayfish swimmeret control system (Stein, 1971). That transection of the connectives to the most caudal ganglion interferes substantially with the control of interganglionic delay and burst duration in the remaining, intact ganglia (Fig. 7b) implies that the

coordinating information emanating from the single ganglion influences the burst generating mechanism in each of the other ganglia.

The initial proposition of a common central control mechanism which mediates both ventilatory and swimming outputs is still not clearly resolved. Judging from the graphs in Figure 2, hyperventilation is most probably an extension of ventilatory activity. The aberration of muscle burst amplitudes and intersegmental delay during swimming suggest the influence of a second control system. Nerve lesion experiments, in which the opisthosomal nervous system is isolated from the brain, seem to confirm this interpretation. However, frequency dependent command fiber tracts in *Limulus* have been reported by Wyse (1971), thus, the apparent loss of swimming movement capabilities in the isolated appendages may be attributed to the elimination of descending command information which coded for a swimming output from the single control system. The swimming and ventilatory coordinating mechanisms are similar, if not one and the same, in that each opisthosomal ganglion is capable of functioning as the pacemaker for both behaviors after lesion of its caudal connectives. Furthermore, the transition from ventilation to swimming can be a gradual progression in terms of muscle activity (Fig. 6a), devoid of observable interaction of two control systems. Since recorded muscle activity is many times removed from the pattern generating event, conclusive evidence in support or repudiation of a single central control mechanism must come from direct central and motor nerve recordings.

The guidance of Drs. James Case and Allan Gelperin is gratefully acknowledged; and I am indebted to Drs. Anthony Barnes, James Parmentier, and Gordon Wyse for critical readings of the manuscript.

Summary

1. Ventilatory and swimming movements in *Limulus polyphemus* are described and the underlying muscular activity is analyzed.

2. Ventilatory and swimming muscle activity in gill plates consists of repetitive, metachronal bursts in both extensor and flexor muscles beginning in the caudal gill plate muscles and proceeding sequentially forward.

3. Ventilatory muscle bursts are approximately symmetrical, being of relatively long duration, small amplitude and long intersegmental delay, affecting exclusively the gill plate musculature.

4. Hyperventilation involves the active participation of the genital operculum in the rhythmic cycle. Rapidly decreasing intersegmental delay, increasing muscle burst amplitude and transition in the muscle burst shapes accompany decreasing interburst intervals.

5. Swimming movements consist of comparatively high frequency, large amplitude excursions of the gill plates, genital operculum and, to various degrees, the walking legs. Metachronous, high amplitude, square shaped muscle bursts, which proceed rostrally with a short intersegmental delay, are responsible for these propulsive movements.

6. Swimming movements of the gill plates require descending information from the brain, whereas the neural circuitry required for ventilation and hyperventilation is endogenous to the opisthosomal ganglia.

7. The pacemaker function for swimming and ventilatory movements may be served by any of the five involved opisthosomal ganglia if its caudal connectives are severed.

8. The ventilatory coordinating mechanism is not polarized; whichever ganglion functions as the pacemaker, the wave of excitation propagates both rostrally and caudally, sequentially exciting neighboring ganglia.

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EFFECTS OF JUVENILE HORMONE ANALOGUES ON THE METAMORPHOSIS OF BEETLES *TROGODERMA GRANARIUM* (DERMESTIDAE) AND *CARYEDON GONAGRA* (BRUCHIDAE)

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Several authors—for example Bowers and Thompson (1963) in a paper on *Tenebrio molitor* (Tenebrionidae), Sláma and associates in a study on *Dermestes vulpinus* (Dermestidae), and De Wilde (1971) in a note concerning *Leptinotarsa decemlineata* (Chrysomellidae)—demonstrated that application of juvenile hormone analogues (JHa) to freshly molted pupae of beetles inhibits imaginal differentiation. Detailed description of the effects elicited, however, is available only for the flour beetle, *Tenebrio molitor* (Rose, Westermann, Trautmann, Schmralek and Klauske 1969). This species is also the only beetle in which the effects of JHa on the larvae were studied (Schmialek, 1963). Therefore, in the present study we have examined in detail the effects of JHa on both the larval-pupal and pupal-adult transformation of representatives of the coleopteran families Dermestidae and Bruchidae.

The dermestids and bruchids differ from one another in many features, and it was intriguing to compare their responses to JHa. Both families include serious pests. The khapra beetle, *Trogoderma granarium*, is polyphagous and infests stored grains, malt, seeds, flower, dry milk products, and woolen cloths in many parts of the world, particularly in hot dry regions (Hadaway, 1956). The hairy larvae undergo at least 4 larval molts in males and 5 in females but the number of larval instars may more than double. The larvae may diapause for more than a year at a temperature around 20° C. The last larval instar of nondiapausing insects lasts 13 days (including 2 days of the prepupal stage) and the pupal instar lasts 7–8 days. The pupae, which are also covered with hairs, remain in the last larval exuvia until completion of adult development.

By contrast, the groundnut beetle, *Caryedon gonagra*, attacks only, or at least primarily, the stores of peanuts in West Africa (Davey, 1959). After hatching, its grub-like larvae bore into the seeds and remain inside until the fourth larval instar. The larvae leave seeds 13–14 days after ecdysis into the fourth instar to spin cocoons. The spinning continues for about 1 day; then the larvae rest inside the cocoons for 2–3 days before ecdysing as pupae. The pupal instar lasts 7–8 days. The adults escape from the cocoons a few days after imaginal ecdysis.

Material and Methods

(1.) *The juvenile hormone analogues*

Twenty-seven analogues were selected to include compounds with diverse chemical structures (Fig. 1). The substances were prepared and kindly provided

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by Drs. M. Romaňuk (Nos. IV, IX, X, XI, XVI, XVII, XVIII); Václav Jarolím (Nos. III, VII, VIII, XIII, XIV, XV); K. Hejno (Nos. I, II); Z. Arnold (Nos. XX, XXI, XXII, XXIII); P. Beran (Nos. XXV, XXVI); and J. Kahovcová (No. XXVII)—all of the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences—and by Dr. J. B. Siddall of the Zoecon Corporation, Palo Alto, California (Nos. V, VI, XII, XIX, XXIV). In most experiments the compounds were dissolved in acetone (grade *pro chromatography*, product of Lachema, Brno) in the ratio 1 : 10, 1 : 100, 1 : 1000 etc.; the solutions were stored at —10° C for up to six months.

(2.) *Biological tests*

Both species of beetles were reared on roasted peanuts in constant darkness at 27° C and 60–70% R.H. The last instar larvae or freshly ecdysed pupae were collected daily from the stock culture and kept in groups of 10 specimens in Petri dishes under the same regime. Fresh food was provided every second week. The insects selected for the expriments (females were used in most cases) were treated with 0.5 μl (*Caryedon*) or 1 μl (*Trogoderma*) of a solution of JHa on the dorsal body side. No anesthesia was used. The solutions were dispensed from a microsyringe driven by means of a screw. In the case of prepupae and pupae of *Caryedon*, the syringe-needle was inserted through the cocoon. Since the pupae of both species were sensitive to oily materials (10% medicinal olive oil in acetone killed 50% of pupae) the highest concentration of analogues used in most experiments was 1%. Application of 1% olive oil in acetone to the controls had no effect.

In a few experiments, the compounds were administered in the form of vapors. To this end a measured amount of analogue was dissolved in 0.5 ml acetone and soaked into a disk of filter paper (8 cm in diameter); the solvent was evaporated and the impregnated paper put in the bottom of a glass Petri dish (diameter 9 cm). Larvae or pupae of *Trogoderma* were placed in an uncovered dish (diameter 4 cm) and the latter was put upon the filter paper. The Petri dish was covered with a lid and the entire assembly was placed in another covered Petri dish (diameter 14 cm).

The treated larvae were periodically observed until they either died or developed into adults. The number of ecdyses was recorded and all ecdysed insects were examined for morphological abnormalities; it was often necessary to remove old exuvia with forceps. Effects on pupae were evaluated two days after the controls had emerged as adults; ordinarily the affected insects produced a new cuticle but could not escape from the old exuvia. The specimens that died prior to the deposition of a new cuticle were disregarded in evaluating results.

Results

(1.) *Action of the juvenile hormone analogues on pupae*

Pupae treated with JHa often molted into various intermediate forms (Fig. 2) that were classified with the aid of a scoring system (Table I) based on the ratio of pupal and adult characters. The maximum effect, *i.e.*, formation of a perfect second pupa, was never observed in either species. Even the most affected specimens of *Trogoderma* displayed slightly pigmented adult eyes, outlines of the segmentation of appendages, and, most important, lacked the pupal hairs. The maximally affected individuals of *Caryedon* resembled normal pupae but their eyes

and appendages also showed adult differentiation. In the less influenced insects of either species, the adult characteristics gradually spread from the head and thorax while the pupal features simultaneously disappeared; the distribution of

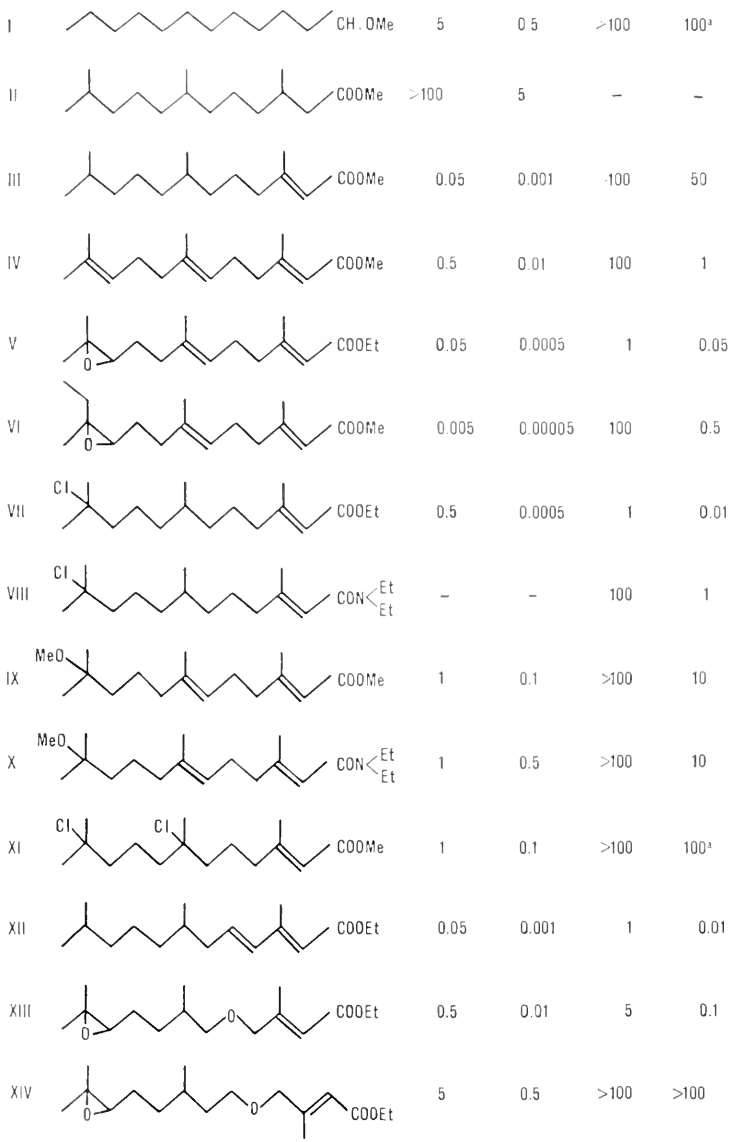
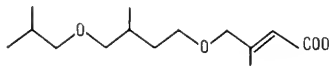
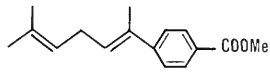
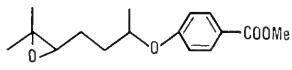
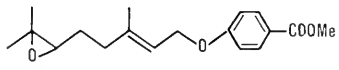
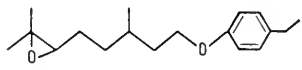
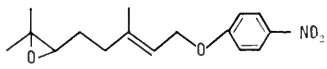
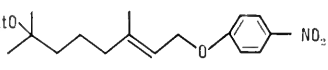
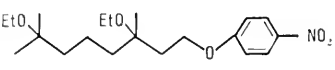
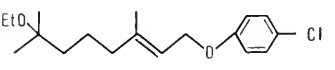
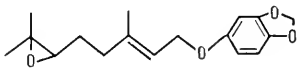
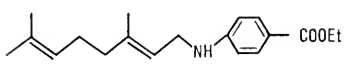
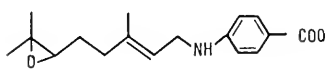
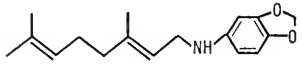


FIGURE 1. Activities of the juvenile hormone analogues in pupal assays. The compounds (all racemic mixture except VI which was *trans, trans, cis* isomer; the aliphatic substances contained mostly two thirds of the 2-*trans* isomers) were applied in 0.5 μl (*Carycdon*) and 1 μl (*Trogoderma*) of acetone on the surface of freshly ecdysed pupae. The elicited effects were scored as described in Table I and are expressed here in ID_{50} and LD_{50}—amount of the com-

pupal and adult characteristics always followed a precise spatial pattern. The least affected animals appeared as normal adults except that their wings were crumpled and, in the case of *Trogoderma*, the cuticle on the dorsal side of abdomen

XV		>100	10	>100	>100[a]
XVI		50	5	>100	100
XVII		10	0.5	100	50
XVIII		10	0.5	100	10
XIX		10	0.1	–	–
XX		5	0.1	50	5
XXI		>100	5	>100	5
XXII		>100	5	>100	100
XXIII		>100	10	>100	10
XXIV		0.001	0.000005	0.1	0.01
XXV		–	–	>100	100[a]
XXVI		>100	10	>100	50
XXVII		5	0.1	>100	100

pound (in μg) provoking in the average the effect of score 2; LD_{50}—amount of the compound (in μg) causing small [scores (1) and 1] but nevertheless lethal effects in 50% of treated insects. The doses marked (a) were toxic. A pupa of *Trogoderma* weighed about 3.5 mg and that of *Caryedon* about 18 mg. Ten pupae, 0–24 hrs after ecdysis, were used in each assay.

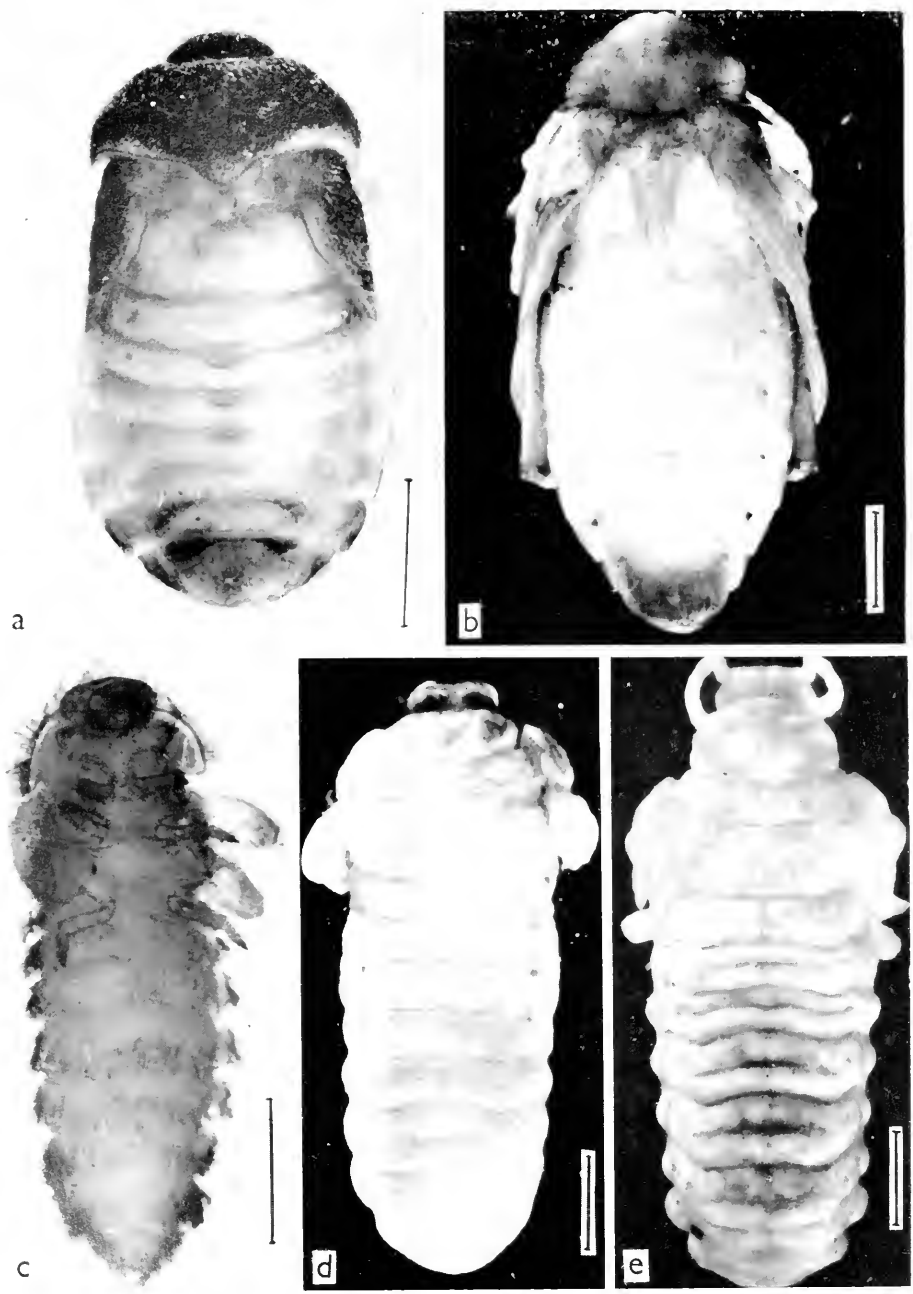


FIGURE 2. The pupal-adult (top) and larval-pupal (bottom) intermediates produced by JHa in *Trogoderma* (pictures a and c) and *Caryedon* (pictures b, d, and e). The pupal-adult intermediates were classified with score 2, the larval-pupal ones with score 4 (pictures c and d) and 2 (picture e). The lines indicate length of 1 mm.

was not completely pigmented. These insects were probably affected internally because they behaved like insects with external juvenile hormone effects and died without escaping from the old exuvia.

Some of the pupal-adult intermediates showed a deep depression in the integument on either side of the prothorax. These depressions were probably caused by contractions of muscles attached to the corresponding parts of the integument at the time when the newly formed cuticle had not yet hardened. Treatments with high concentrations of the analogues occasionally inhibited sclerotization of the emerging insects. This effect was also observed after treatments with olive oil and its cause was obscure. A rare effect of JHa was prolongation of the pupal instar; in this case the treated insects ecdysed as adults up to three times later than the controls.

TABLE I

Scoring system for assessing juvenile effects in pupal assays

Score	Characteristic features	
	Trogoderma granarium	*Caryedon gonagra*
0	Normal adult	Normal adult.
(1)	Adult lacking the cuticular tanning in small areas of abdominal tergites or having crumpled wings.	Adult with crumpled wings or adult failing to extract some body parts from the pupal exuvia.
1	Virtually normal adult remaining in the pupal exuvia. Wings point downwards as in pupa.	Virtually normal adult remaining in the pupal exuvia; wings point downwards as in pupa.
2	Head and thorax are nearly of adult form but large portions of abdominal tergites remain untanned.	The cuticular tanning is lighter than in adults and the number of adult hairs is reduced; wings often appear swollen.
3	Head and thorax are predominantly of adult form but most of the abdomen is covered with a pupal-like cuticle lacking pupal hairs.	Head and thorax are of nearly adult form but the eyes are more remote from one another than in the adults and their pigment is incompletely developed; the abdomen remains white as in pupae; wings are often swollen.
4	Head and thorax are intermediate between pupa and adult; appendages are virtually adult; wings are slightly tanned; the abdomen is pupal-like except for the lack of pupal hairs.	Nearly perfect second pupa but appendages are differentiated and the tips of mandibles sclerotized; wings often stretch at right angles from the body.
5	Perfect second pupa.	Perfect second pupa.

The morphological effects were most pronounced when the compounds were applied immediately after the pupal ecdysis. Pupae 0–24 hrs old were therefore used for assaying activities of different JHa. Since the effects produced by some treatments varied as much as over 3 grades of our scoring system (10 insects were used in each assay), the average effect was calculated for each concentration tested. The activity of each analogue was expressed in terms of the dose provoking an average effect of grade 2 (ID_{50}; *cf.* Sláma,, 1971) and also in terms of the critical dose causing small but lethal developmental derangements [grades 1 and (1)] in 50% of treated insects (LD_{50}).

Figure 1, which summarizes the results, shows that the compounds differ considerably in their activities. In the case of *Trogoderma* pupae, LD_{50} of the best compounds ranged from several picograms to a few nanograms; in the case of *Caryedon* pupae ten or more nanograms were required. With each of the most active materials the range of effects from score 1 to score 4 was realized within 10^4–10^7 increase in dose. Less active compounds produced only small effects even when applied in the highest amounts and a few others had no effect on the development of *Caryedon*.

(2.) *Action of JHa on* Trogoderma *larvae*

Larvae treated within the first nine days of the last instar (the total length of the instar was 13 days) underwent up to six extra larval molts (Table II) and

TABLE II

Development of Trogoderma *larvae treated with 3,4-methylendioxyphenyl 6,7-epoxygeranyl ether (compound No. XXIV)*+

Dose (μg)	Time of treatment*	Number of extra larval molts	Number of dead larvae	Number pupating	Number of larval-pupal intermediates	Time of ecdysis into pupae or intermediates†
0	1	0–2	0	10	0	1–7
0	5	0–2	0	10	0	1–4
0.00001	1	0–4	0	10	0	5–17
0.00001	5	0–4	0	10	0	11–19
0.00001	9	0–4	0	9	1	5–16
10	1	2–5	0	10	0	6–28
10	3	1–5	3	6	1	10–27
10	5	2–6	2	8	0	8–20
10	7	2–6	1	9	0	7–19
10	9	1–5	0	9	1	7–27
10	11	0–3	0	2	8	2–8
10	12 (prepupa)	0	0	10	0	1

+ Ten insects were used in each assay.
* Time of application in days after the final larval ecdysis.
† Time of ecdysis is given in weeks after the application.

some of them lived more than four months longer than the controls (the whole life cycle normally lasts two and a half months). The first ecdysis following application of JHa was significantly delayed. The extra larval instars appeared as normal larvae although some of them seemed to have fewer hairs. Prolongation of larval life was not accompanied by an increase in size despite the continued feeding of the larvae (the amount of consumed food was not established). Their body weight periodically fluctuated around 3.5 mg in relation to the molting cycles: it was at its lowest just after the ecdysis but increased slowly throughout the following instar, reaching a maximum shortly before the succeeding ecdysis.

Most superlarvae eventually either died or pupated. Death occurred mostly in the intermolt period and its immediate cause was unknown. Some insects died at ecdysis because they could not free themselves from the old exuvia. Superlarvae that managed to pupate usually produced morphologically perfect adults. A very few molted into pupal-adult intermediates.

Intermediates between larva and pupa were rarely observed except when the larvae were treated at the very end of the instar (11 days after the last larval ecdysis). Even in this case two individuals molted into superlarvae which eventually pupated (Table II). When JHa was administered to older animals (prepupae, 12 days after the last larval ecdysis), normal-looking pupae resulted, but these pupae developed into the pupal-adult intermediates. These intermediates resembled those produced by applying the compounds to freshly molted pupae but many of them possessed some well developed pupal hairs. Formation of a second pupa perfect in every respect, however, was also never observed.

The larval-pupal intermediates from different experiments could be arranged into a continuous series of transitions between the larval and pupal appearance. To facilitate their description, they were divided into several categories (Table III).

TABLE III

Scoring system for assessing juvenile effects in larval assays

Score	Characteristic features	
	Trogoderma granarium	*Caryedon gonagra*
0	Normal pupa.	Normal pupa.
1	Pupa with incompletely developed appendages; tarsi are larval; antennae shorter and simpler than in pupae; mouth-parts intermediate between larva and pupa.	Pupa with unusually long prothorax and larval-like tip of abdomen maintaining the larval motility.
2	Pupal-like intermediate with larval legs and atypical head bearing both larval and pupal features	Pupal-like intermediate with nearly perfect pupal wings and appendages but with a larval-like abdomen.
3	Rather pupal-like body shape, long wings, intermediate hair pattern on the dorsal body side, and nearly larval head and appendages.	Pupal-like thorax, considerably differentiated wings and appendages, and larval abdomen.
4	Larval-like intermediate with everted wings reaching $\frac{1}{3}$ of their pupal length and slightly rounded shape of the body.	Larval-like intermediate with small everted wings, slightly differentiated appendages and thoracic segments; head not retracted as much as in a normal larva.
5	Perfect superlarva but no increase in body size.	Perfect superlarva never obtained.

The larval-like intermediates shed the old exuvia while the pupal-like ones did not. The intermediates of all categories, however, usually developed further into creatures displaying a combination of larval, pupal, and adult features. The amount of imaginal differentiation seemed to depend on the type of the intermediate and, probably, on the concentration of JHa left in the body. Some intermediates, in particular those of scores 1–2, maintained their original appearance. The epidermis produced the pupal-like cuticle with hairs and the appendages kept the larval-like form. The only organs undergoing imaginal differentiation were the eyes; these remained small but formed pigmented ommatidia.

The majority of larval-pupal intermediates, particularly those that developed from the superlarvae, attained a more adult appearance during subsequent development. Some parts of the epidermis secreted adult cuticle and the number of pupal hairs was greatly reduced in other areas where a pupal type cuticle had formed.

The elytra were often sclerotized as in adults and the compound eyes attained adult shape and color. The appendages and wings remained small and undifferentiated and did not change their position. Consequently, some larval-pupal intermediates of score 1 developed into nearly normal adults except for the presence of larval appendages.

(3.) *Action of JHa on* Caryedon *larvae*

Table IV records the responses of last instar larvae of *Caryedon* to one of the most active analogues (compound No. XXIV): (1) Some larvae reduced their feeding, survived up to a fortnight after the treatment, but eventually died; (2) The larvae molted into the larval-pupal intermediates; (3) The larval life was prolonged but the insects eventually pupated; (4) The pupation was delayed only slightly or not at all, whereas the pupae developed into the pupal-adult intermediates.

Larval death or prolongation of larval life occurred when the compounds were administered at any time before the larvae began spinning. The prolongation of larval life was never accompanied by extra larval molts or any noticeable increase in the body size. The larval-pupal intermediates (Fig. 2d and 2e,) were mostly produced by applying the substances within the second half of the last larval instar, preferably to larvae spinning cocoons. On the other hand, applications to prepupae (pharate pupae) did not often affect the larval-pupal transformation but caused derangements in the following pupal-adult transformation, *i.e.*, the insects molted into pupae that developed into pupal-adult intermediates similar to those obtained by treatment of freshly ecdysed pupae (Table I). One of the prepupae developed

TABLE IV

Development of Caryedon *larvae treated with 3,4-methylendioxyphenyl 6,7-epoxygeranyl ether (compound No. XXIV)*[+]

Dose (μg)	Age of larvae*	Number of dead larvae	Number of larval-pupal intermediates	Number of pupae	Number of pupal-adult intermediates†
0.1	young	0	0	10	0
0.1	old	0	2	8	0
0.1	spinning	0	3	7	0
0.1	prepupae	0	1	9	1
1	young	6	1	2	0
1	old	0	2	8	1
1	spinning	0	4	6	1
1	prepupae	0	6	2	1
50	young	6	0	3	0
50	old	0	6	4	0
50	spinning	0	8	2	0
50	prepupae	0	6	4	3

[+] Nine to ten larvae were tested in each assay.

* The last instar larvae were divided into the following categories: young—first third of the instar (about 6 days); old—second third of the instar; spinning—period encompassing two days after the start of spinning; prepupae—insects in cocoons (0–3 days before the pupal ecdysis). The total length of the last larval instar was 17 days.

† The difference between number of pupae and that of pupal-adult intermediates indicates how many pupae molted into normal adults.

into a nearly perfect second pupa. It differed from a normal pupa by having small and pigmented adult eyes.

The larval-pupal intermediates were scored as in the case of *Trogoderma* (Table III). In contrast to the latter, only the most pupal-like intermediates (score 1) succeeded in escaping from the larval exuvia. Many intermediates developed further into creatures possessing combinations of larval, pupal, and adult features. The most larval-like intermediates ecdysed into larval-like creatures with adult cuticle restricted to the head and tiny regions on the thorax and with small but otherwise perfect adult eyes. The most pupal-like intermediates formed rather adultoid creatures possessing an unusually long prothorax, large patches of a pupal-like cuticle on the abdomen, and occasionally undifferentiated tips of appendages, particularly the antennae. The abdomen apparently maintained the larval musculature and muscle innervation because it often was as movable as in a normal larva.

(4.) *Administration of analogues as vapors*

Amounts of 0.1 to 10 mg of analogues Nos. VII and XXIV were allowed to evaporate in Petri dishes of 15 ml volume. The insects (Khapra beetle was used in these experiments) were exposed to the vapors for 6 weeks. The results are summarized in Table V.

All of the last instar larvae underwent 1 to 4 extra larval molts and eventually molted either into the larval-pupal intermediates or into normal pupae. Some pupae produced the pupal-adult intermediates, others developed into morphologically normal adults. Their fecundity was not examined.

Insects exposed to the vapors only from the start of the pupal instar mostly developed into normal adults, presumably because they passed the sensitive period

TABLE V

Effects of the vapors of JHa on the larvae and pupae of Trogoderma granarium[+]

Compound (No.)	Dose* (mg)	Number of extra larval molts	Number of larval-pupal intermediates	Number of pupal-adult intermediates	Number of adults
Exposure of larvae beginning midway the last instar					
VII	10	2–4	4	6	0
VII	1	2–3	4	5	1
VII	0.1	1–3	0	9	1
XXIV	1	1–2	0	0	10
Exposure beginning just after the pupal ecdysis					
VII	10	—	—	4	6
VII	1	—	—	2	8
VII	0.1	—	—	0	10
XXIV	1	—	—	0	10

[+] Ten insects were tested in each assay.

* The indicated amount of JHa was impregnated into filter paper and the latter placed in a covered Petri dish of 15 ml volume. Exposure of the insects began 2 hrs later.

before there could be sufficient uptake of analogue from the vapor phase. Their fertility, however, was considerably lower than in normal insects. For example, the adults which had developed in the presence of 10 mg of compound VII deposited only 4 eggs per female compared with 34 eggs in the control. The hatchability of the eggs removed from further contact with the vapors was approximately 25%, so that each female produced only one offspring. Furthermore, the hatched larvae mostly died within the first two or three larval instars.

The filter paper soaked with JHa seemed to produce effective concentrations of the vapor for a considerable length of time. Thus, insects which were placed in the dishes as late as 6 weeks after the administration of the compound were affected almost to the same degree as in the experiments just described.

Discussion

The larval-pupal transformation of both *Trogoderma* and *Caryedon* was prevented or deranged by the application of JHa during a considerable part of the last larval instar. High doses of JHa were effective even if administered to prepupae shortly before the secretion of the pupal cuticle. Late application did not impede the formation of pupae but often caused the latter to form pupal-adult intermediates. The pupal-adult intermediates were also produced by administering the compounds within the first third of the pupal instar.

Similar periods of sensitivity also have been found in other beetles. Thus the larval-pupal transformation of *Tenebrio molitor* is deranged by implanting active corpora allata into mature, last instar larvae (Radtke, 1942). So also the pupal-adult transformation is affected by administering JHa at the beginning of the pupal instar of *Tenebrio molitor* (Bowers and Thompson, 1963; Schmialek, 1963; Socha and Sehnal, 1972), *Dermestes vulpinus* (Sláma, Hejno, Jarolim and Sorm, 1970), *Tribolium confusum* (Mori, 1971), and other beetles.

A typical characteristic of both larval-pupal and pupal-adult intermediates is the predictable pattern in which the new features appear and spread over the body while the old ones simultaneously regress. The distribution of "metamorphosed" and "non-metamorphosed" tissues obviously depends on the progress of determination of metamorphosis which is known to be dependent upon determinative cell divisions (Hinton, 1963; Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Sehnal and Novák, 1969; *etc.*). The sensitivity of tissues to JHa is lost after the determinative cell divisions have been completed.

The first organs failing to respond to JHa administered to the larvae investigated in the present study were the eyes and wings and in the case of *Caryedon* also the legs. The legs of *Caryedon* and the wings and epidermis of either species lost sensitivity to JHa over a prolonged period of time. For example, certain regions of the epidermis failed to respond to an early application of the hormone while other regions were still affected by administrations to prepupae. The appendages of *Trogoderma* and the tip of the abdomen of *Caryedon* maintained their sensitivity longer than any other body part.

Comparing the larval-pupal intermediates of *Trogoderma* and *Caryedon* we find that the pattern of determination of the metamorphic changes is related to the differences between larva and pupa. For example the larval-pupal transformation of the head and appendages in *Trogoderma* seems to be relatively simple and is both

determined and accomplished within a short period of time at the end of the last larval instar (application of JHa at this time provoked development of "pupae with larval legs"). On the other hand, the larval legs of *Caryedon* seem to degenerate while the pupal legs develop from the imaginal discs. The determination of the disc development encompasses a long period of time; the successive steps of the leg differentiation appear to be determined consecutively (all various larval-pupal intermediates possessed legs with both larval and pupal features).

During the pupal-adult transformation, the organs differentiate in their fine structure and develop musculature and innervation. In both species, the loss of capacity to secrete pupal cuticle and, simultaneously, the attainment of ability to secrete adult cuticle followed a similar pattern as has been established in *Tenebrio molitor* (Rose *et al.*, 1968; Socha and Sehnal, 1972). The determination was accomplished within the first third of the pupal instar. Some changes, such as the development of adult eyes, segmentation of appendages, and in *Trogoderma* also the loss of pupal hairs, seemed to be determined prior to the pupal ecdysis. None of these changes were prevented by administering JHa after the pupal ecdysis.

The responses of larvae to JHa suggest that different insects possess diverse mechanisms for preventing lethal developmental derangements that could occur in normal development if the control of JH secretion by the corpora allata failed. One can tentatively distinguish the following types of these mechanisms: (1) The mature larvae undergo "stationary" larval molts with no increase in the body weight as long as the titer of JH remains high. When the titer decreases, the insects pupate. This is the case of *Trogoderma granarium*. (2) The mature larvae undergo extra larval molts accompanied by an increase in the body size that partly compensates for the delay in pupation. This is the case of *Tenebrio molitor* (Radtke, 1942; Schmialek, 1963) and *Galleria mellonella* (Sehnal, 1971). (3) The mature larvae do not molt as long as the JH titer remains high. In some instances they might continue to grow but only within narrow limits. This is the case of *Caryedon gonagra*.

The action of juvenile hormone has been called the "*status quo*" effect (Williams, 1961). The term is pertinent, because both JH and JHa preserve the existing stage of development. The larval-pupal intermediates, which are produced by impeding further progress of metamorphosis in the middle of the larval-pupal transformation, maintain the capacity to molt and are thus capable of further development. In the absence of further JHa they should theoretically produce pupal-adult intermediates; the original larval tissues would metamorphose into pupal ones and the original pupal tissues into the adult ones. If the titer of JHa remains high, the larval-pupal intermediates should maintain the *status quo* and appear after ecdysis the same as before.

The intermediates without further supply of JHa indeed developed towards the adult stage. Some body parts, however, always metamorphosed more than the others. This suggests that the sensitivity of different tissues to JHa diversified; differentiation of some tissues was hindered by the remnants of JHa in the body whereas other tissues differentiated despite the presence of JHa.

The results of our tests confirm that certain changes in the chemical structure of analogues significantly alter the biological activity. The comparison of these data with the information on *Tenebrio molitor* (reviews Williams, 1970; Sláma, 1971), *Dermestes vulpinus* (Sláma *et al.*, 1970) and *Tribolium confusum* (Bowers,

1971; Mori, 1971) indicates the relationships between the chemical structure and the activity on different coleopteran families. For example, the activity of the farnesane-type compounds seems to depend on the following parts of the molecule:

(1) The number of double bonds—the beetles respond most readily to substances having 2, 3 and 6, 7 double bonds (Wakabayashi, Sonnet and Law., 1969; Sláma *et al.*, 1970). The present results demonstrate that the shift of the 6, 7 double bond to the 4, 5 position does not considerably alter the activity.

(2) Hydrochlorination or epoxydation of the 10, 11 double bond generally increases the activity (Bowers, Thompson and Uebel, 1965; *et al.*, 1970; Mori 1971). *Trogoderma* seems to be particularly sensitive to the 11-chlorine derivative. Compounds with the methoxy group on C-11 appear to be less active.

(3) Prolongation of the side chain on C-11 also increases the activity (Röller and Dahm, 1968; Wakabayashi *et al.*, 1969). Substances with two ethyl groups on C-11 were reported to be even more active on *Tenebrio* and *Tribolium* (Mori, 1971).

(4) Additional substitution on C-7 generally decreases the activity (Sláma, Romaňuk and Sorm 1969). The diethylamid of 6, 10-dihydro-7, 11-dichlorofarnesoate (not tested in the present study), however, proved to be very active on *Tenebrio* (Cruickshank, 1971).

Similar relations between chemical structure and biological activity may be found in the group of aromatic JHa. The most active of all compounds tested on *Trogoderma* and *Caryedon* appears to be, 3,4-methylendioxphenyl 6,7-epoxygeranyl ether. The compounds of this type are also very active on *Tenebrio* (Bowers, 1969) and *Tribolium* (Bowers, 1971). Some aromatic substances may act rather specifically only on some families of Coleoptera. For example, 4-nitrophenyl 7-ethoxygeranyl ether (XXI) is very active on *Tenebrio* (Sláma, 1971) but nearly inactive on *Trogoderma* and *Caryedon*.

Beetles of different families differ one from another by their responsiveness to certain types of analogues as well as by their sensitivity to JHa in general. For example, *Tenebrio* is generally more sensitive than *Caryedon* but, in regard to certain compounds, it is less sensitive than *Trogoderma*. Consequently, results of assays on one species cannot be extended to all Coleoptera.

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Summary

1. The metamorphosis of *Trogoderma granarium* and *Caryedon gonagra* may be deranged with as little as 0.000005 μg and, respectively, 0.05 μg of certain juvenile hormone analogues.

2. Under the prolonged influence of analogues the larvae of *Trogoderma* undergo several "stationary" extra larval molts with no increase in the body size.

The larvae of *Carycdon* do not molt but their pupation is considerably delayed. In extreme cases the larvae die.

3. Exposure of *Trogoderma* and *Carycdon* to the analogues during the last third of the last larval instar often induces development of larval-pupal intermediates. The species differ in the distribution of larval and pupal features in these intermediates.

4. The larval-pupal intermediates develop into creatures composed of larval, pupal, and adult tissues.

5. Treating the insects shortly before or after the pupal ecdysis results in formation of pupal-adult intermediates with a similar distribution of pupal and adult tissues in both species.

6. Pupal assays revealed that the investigated species differ from one another as well as from other beetles in their responsiveness to certain analogues and also in their responsiveness to the analogues in general.

7. *Trogoderma* is affected by the vapors of analogues.

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LOCALIZATION OF *LIMULUS POLYPHEMUS* OXYGEN SENSITIVITY

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Rhythmic metachronal movements of the five book gill-bearing opisthosomal appendages provide respiratory ventilation in the horseshoe crab, *Limulus polyphemus* (Hyde, 1893; Waterman and Travis, 1953). Two book gills are situated on the lateral posterior surfaces of each pair of fused gill appendages. The first pair of opisthosomal appendages, the genital operculum, moves in concert with the first through fifth pairs of gill appendages. Ventilation results from the alternate abduction and adduction of each paired gill appendage. Each wave of abduction begins in the fifth gill appendage and sweeps anteriorly; abduction is closely followed by an anterior sweeping wave of adduction.

The rate and amplitude of the opisthosomal ventilatory movements are dependent upon the oxygen concentration in the sea water environment (Hyde, 1906; Waterman and Travis, 1953). Under anoxic conditions ventilation ceases. When oxygen is introduced into anaerobic sea water, there is an immediate resumption of ventilation suggesting that external oxygen receptors are present (Waterman and Travis, 1953). Previous attempts to identify these external receptors have been unsuccessful (Waterman and Travis, 1953; Schlein and Barber, 1971).

This report describes experiments in which the external sites of oxygen sensitivity were identified by examining the effects which sectioning nerves and otherwise inactivating possible sensory structures had upon the oxygen dependent ventilatory reflexes. The results demonstrate that oxygen responsiveness of the *Limulus* ventilatory system is dependent upon the integrity of the opisthosomal book gills (Fig. 1B) and the prosomal intercoxal sensory cuticle (Fig. 1C). Generation of the ventilatory rhythm in intact animals is dependent upon afferent input from these structures.

All appendages and sensory structures to which reference is made in the text of this report are labeled in Figure 1.

Methods

Adult and immature (prosoma widths of 6–8 and 3–4 inches respectively) specimens of *Limulus polyphemus* (L.) were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Upon receipt the telsons were removed. Animals were fed pieces of beef liver and maintained for 1–3 months in a circulating sea water tank (at the MBL) or in a 120 gallon aquarium containing Dayno synthetic sea water (at Ohio).

Animals were placed in a sea water-filled plexiglass chamber and secured in an extended position, ventral side up, with four hooks inserted through the margins of the prosomal and opisthosomal carapace (Fig. 1A). Air, nitrogen and oxygen

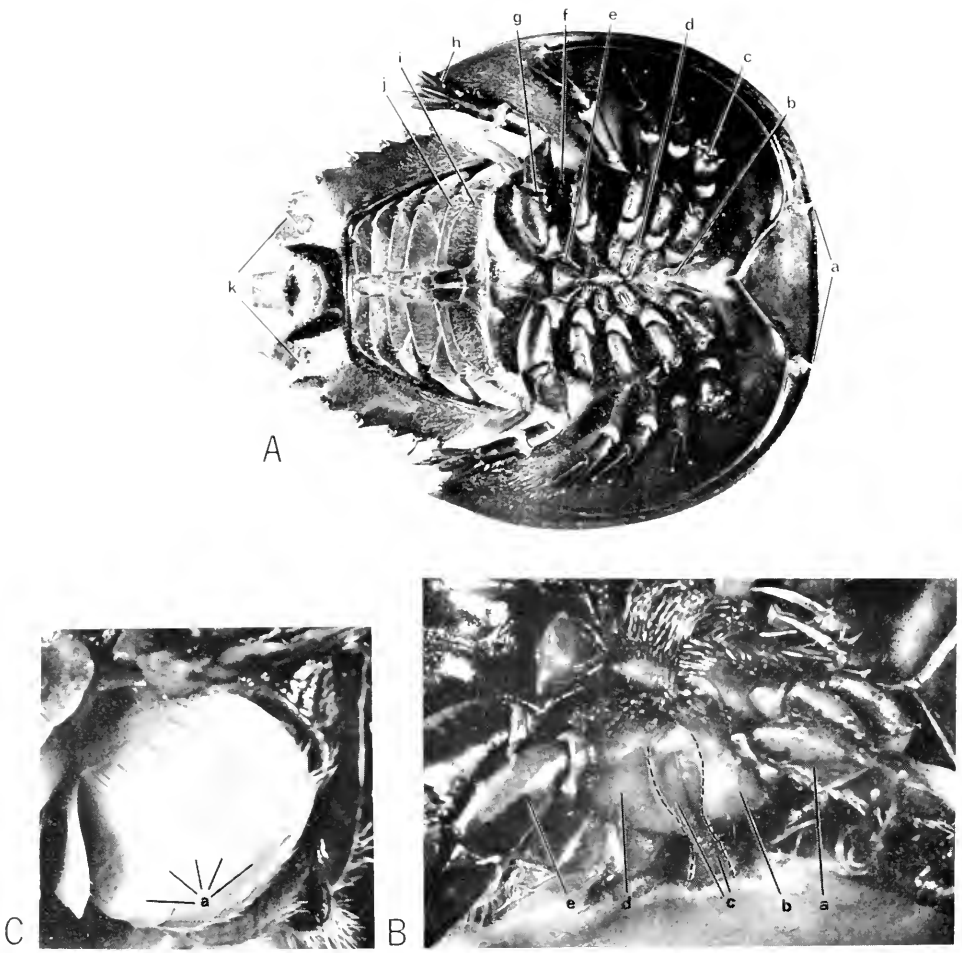

FIGURE 1. Appendages and regions of oxygen sensitivity in an adult *Limulus*: (*A*) Ventral view. Symbols used are: a = hooks securing prosoma; b = ventral eye; c = 1st walking leg (w.l.); d = gnathobase spines of 2nd w. l.; e = chilaria; f = flabellum of 5th w. l.; g = basipodite of 5th w. l.; h = spatulate podite appendages of 5th w. l.; i = operculum; j = 1st gill appendage; k = hooks securing opisthosoma. (B) is a view of intercoxal sensory cuticle. Symbols used are a = basipodite of 3rd w. l.; b = coxa of 3rd w. l.; c = intercoxal cuticular membranes (enclosed by dashed line); d = coxa of 4th w. l.; e = basipodite of 4th w. l. (C) is a view of book gill on posterior surface of 1st gill appendage; a = lamellae of book gill.

gases were introduced into the sea water through air stones placed in the chamber corner adjacent to the anterior left edge of the carapace. The experimental procedure was to seal the chamber with a sheet of Parafilm, displace the oxygen from the air-saturated sea water by bubbling nitrogen into the chamber until the sea water was sufficiently anaerobic (usually less than 2×10^{-4}% O_2), turn the nitrogen off and on several times to control for the possibility of the animal responding to

changes in the flow of gas bubbles from the air stones and then introducing oxygen while recording the response of the animal.

Oxygen levels of the sea water were monitored with an oxygen electrode (Yellow Springs Instrument Co. #5418) located in the posterior right chamber corner. All oxygen measurements were in parts per thousand (‰) corrected for temperature and salinity with the #51A oxygen meter. The temperature range was 19°–22° C.

Movements of the gill appendages were monitored by differential recording between two insulated 100 μm copper wires—one affixed to the side of the experimental chamber and the other attached to the exopodite of the first gill appendage. For recording muscle activity, bipolar electrodes—made by cementing together two 100 μm insulated copper wires—were inserted into the extensor muscle of the basipodite of the fifth walking leg and into either the abductor or adductor muscles of the first gill appendage. With these electrodes movement artifacts were minimized.

All potentials were differentially amplified with a Grass P15 preamplifier, fed into a Physiograph DC amplifier and recorded with a Physiograph pen recorder.

Prosomal and opisthosomal components of the ventilatory reflex were differentiated in immature animals whose opercular nerves and ventral nerve cords were sectioned immediately posterior to the chilaria. The opercular nerves and ventral nerve cord exit from the nerve ring and run posteriorly to the operculum and more posterior opisthosomal segments respectively (Patten and Redenbaugh, 1899). The surgical procedure was to make an incision in the cuticle between the chilaria and operculum exposing the opercular nerves and ventral nerve cord and then to section them. In addition, in several animals the first dorsal nerves, which arise from the anterior ventro-lateral aspect of the nerve ring and run posterior to innervate the ventral cuticle in the opisthosoma (Patten and Redenbaugh, 1899) were exposed through an incision in the dorsal prosomal cuticle slightly medial to the lateral eye and sectioned.

Animals whose opercular nerves and ventral nerve cords had been sectioned were allowed a 3 week recovery period before their ventilatory reflexes were examined. Although prosomal-opisthosomal coordination was absent, the activity levels and responsiveness of these animals appeared normal. After completion of the experiment surgical interruption of the sectioned nerves was checked by dissection.

A series of experimental procedures were employed to localize those sensory structures which are concerned with oxygen ventilatory reflex responsiveness. These structures were either surgically removed, destroyed by cauterization or inactivated by covering them with a layer of low melting point wax (Tackiwax, Cenco). The question of the involvement of book gill receptors in the oxygen ventilatory reflex was examined by monitoring the effects of each of the following experimental procedures upon the oxygen ventilatory reflex: (i) covering the book gill lamellae with wax; (ii) sectioning the gill nerve just proximal to the point at which it innervates the book gill lamellae (Patten and Redenbaugh, 1899); (iii) careful surgical removal of all book gill lamellae which can be accomplished with only slight blood loss—perhaps the dorsal abdominal flexors close off the afferent branchial arteries under these conditions (Lochhead, 1950).

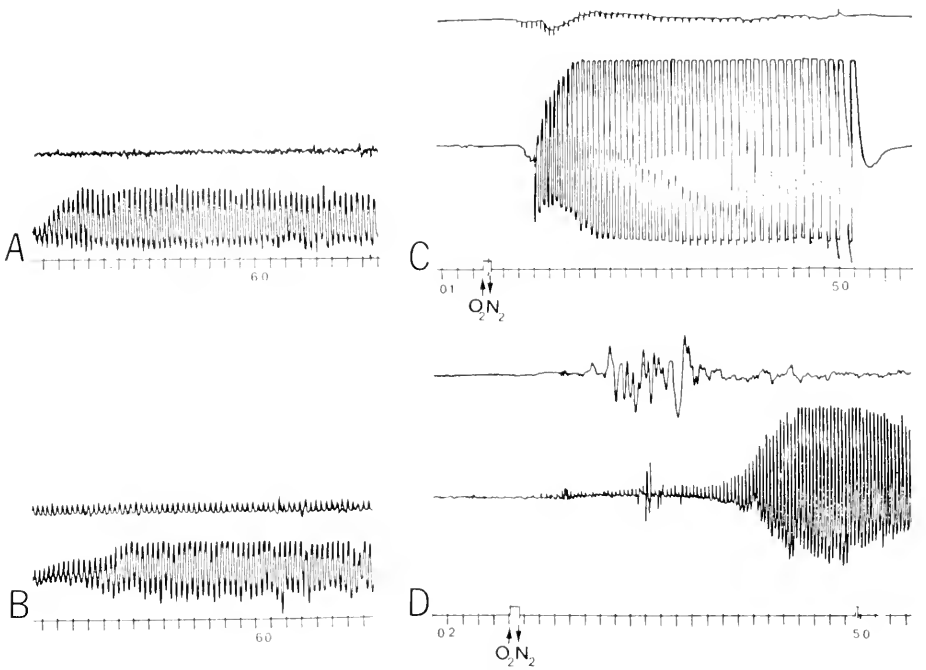

FIGURE 2. *Limulus* ventilatory reflexes. (A) Respiratory movements of gill appendages. Note absence of walking leg movement. (B) Coupled swimming movements of walking legs and gill appendages. (C) Walking leg and gill appendage responses to the introduction of oxygen into the anaerobic chamber. (D) Oxygen responsiveness of walking legs and gill appendages in an animal whose opercular nerves and ventral nerve cord were previously sectioned between the prosoma and opisthosoma; upper trace: recording of movement (A and B) or basipodite muscle responses (C and D) of 5th walking leg; middle trace: recording of movement (A and B) or adductor muscle responses (C and D) of 1st gill appendage; lower trace: 5 sec time marks; all measurements of oxygen are in $\% \times 10^{-3}$; animals were immature. These records have been retouched for photographic reproduction.

RESULTS

Low amplitude metachronal respiratory movements of the opisthosomal appendages (operculum and 5 pairs of gill appendages) usually served to ventilate the book gills of adults secured in the experimental chamber (Fig. 2A). However on occasion, intermittent bouts of swimming—metachronal movements of the opisthosomal appendages coupled with rhythmic movements of the prosomal walking legs—were observed (Fig. 2B). Although swimming in adults was intermittent, periods of sustained swimming were often observed in immature animals.

The respiratory rate was proportional to the logarithm of the oxygen concentration from 1×10^{-4} to 2×10^{-3} % O_2 (Fig. 3). Observed respiratory rates ranged from 5 per min (below 3×10^{-4} % O_2) to 40 per min (above 2×10^{-3} % O_2). Respiratory movement amplitude also decreased as the oxygen level was lowered to 5×10^{-4} % O_2.

With sufficient exposure to anaerobic conditions ventilatory movements (res-

piration and swimming) always ceased. In agreement with Waterman and Travis (1953) when oxygen was introduced into the anaerobic sea water, ventilation would resume in 5 to 60 seconds. Rhythmic metachronal movements of the opisthosomal appendages, evoked by the introduction of oxygen, were usually accompanied by movements of the prosomal walking legs (Fig. 2C). Occasionally these leg movements were nonrhythmic; at other times they were rhythmic and coupled with opisthosomal appendage beating, resembling normal swimming movements. Resumption of ventilation was in response to the appearance of oxygen; not to physical vibration of the water resulting from turning the oxygen inflow on. Turning nitrogen inflow off and on again usually elicited no walking leg or gill appendage responses (Fig. 4C) although on occasion a short duration bout of low frequency movements was observed (see also Waterman and Travis, 1953).

Prosomal walking leg and opisthosomal appendage responsiveness to the introduction of oxygen into the anaerobic chamber could be differentiated in animals whose opercular nerves and ventral nerve cords had been sectioned between the prosomal chilaria and the opisthosomal operculum. Five to 60 seconds following the introduction of oxygen into the anaerobic chamber rhythmic movements of the gill appendages and nonrhythmic movements of the prosomal walking legs were invariably initiated (Fig. 2D).

Oxygen responsiveness of the gill appendages in animals with sectioned opercular nerves and ventral nerve cords depends upon the integrity of the book

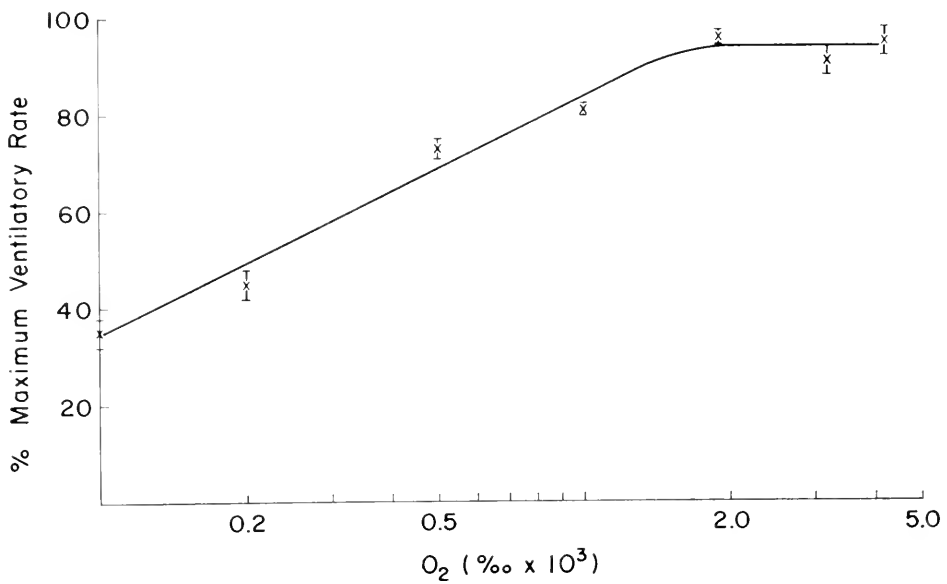

FIGURE 3. Dependence of *Limulus* ventilatory rate on the oxygen concentration of the environmental sea water. Data were obtained for four mature animals. Per cent changes in ventilatory rate were calculated for each animal. Mean values for the four animals are plotted with standard errors. Measurements were made from records obtained during four typical experimental runs in which nitrogen slowly displaced the oxygen present in air saturated sea water.

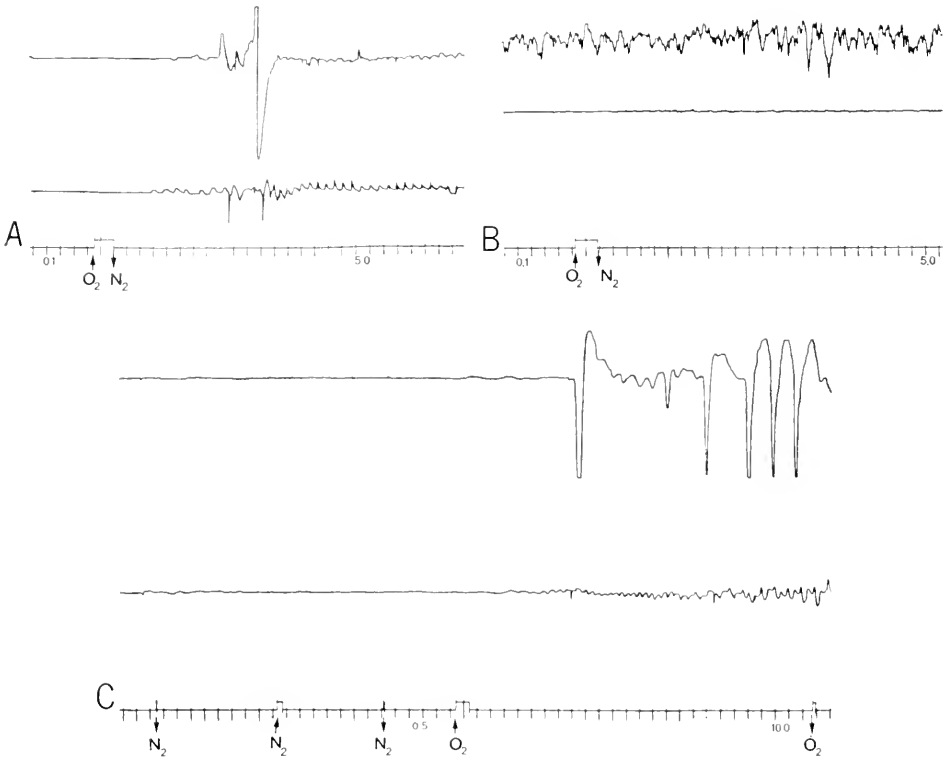

FIGURE 4. The effects of eliminating oxygen receptive structures upon *Limulus* ventilatory reflexes; (A) oxygen responsiveness of walking legs and gill appendages following removal of all book gills. Note rhythmicity of response; (B) oxygen responsiveness of animal in A (all book gills removed) following cauterization of all intercoxal sensory cuticle. Note the absence of a response to oxygen introduction; (C) oxygen responsiveness of walking legs and gill appendages following sectioning of all branchial blood sinuses. The opercular nerves and ventral nerve cord of this immature animal were previously sectioned. Note the absence of a response to turning nitrogen off and on again; upper trace: basipodite muscle responses of 5th walking leg; middle trace: adductor muscle responses in 1st gill appendage; lower trace: 5 sec time mark. All measurements of oxygen are in $\% \times 10^{-3}$. Records A and B were obtained from a mature animal with intact nerve cord. These records have been retouched for photographic reproduction.

gills. Gill appendage responses to the introduction of oxygen into the anaerobic sea water (but not to tactile stimulation) disappeared following any of these procedures: (i) section of all gill nerves which innervate the book gills; (ii) covering all book gill lamellae with wax; (iii) careful surgical removal of all book gill lamellae. The surgery usually resulted in only slight blood loss (see Methods). In contrast animals in which all branchial blood sinuses were cut open, with consequent massive blood loss, continued to initiate rhythmic respiratory movements when oxygen was introduced into the anaerobic sea water environment (Fig. 4C). Control manipulations including removal of all gill appendage endopodites and exopodites as well as covering the opisthosomal carapace with wax had no effect on oxygen responsiveness.

Prosomal responses to oxygen (but not to tactile stimulation) could be eliminated in animals with sectioned opercular nerves and ventral nerve cords by either waxing or cauterizing the membraneous cuticle between the coxa of the 5 pairs of walking legs. Removal of the coxal gnathobase spines, the flabellum or the spatulate propodite appendages of the fifth walking leg had no effect on walking leg responses to oxygen introduction. Nor was prosomal oxygen responsiveness affected by (i) cauterizing the sensory membraneous area anterior to the ventral eye; (ii) covering the ventral surface of the prosomal carapace with wax or (iii) sectioning the first dorsal nerves of the prosoma which innervate the ventral surface of the opisthosoma (Pattern and Redenbaugh, 1899).

In three animals with intact ventral nerve cords rhythmic responses of the gill appendages to the introduction of oxygen into the anaerobic chamber were reduced after waxing all book gill lamellae (Fig. 4A) and eliminated following cauterization of all prosomal intercoxal sensory cuticular areas (Fig. 4B). After removal of the wax covering the book gills rhythmic responses to oxygen introduction were restored.

Discussion

There are two morphologically different oxygen receptive systems concerned with *Limulus* ventilatory reflexes. Each provides sufficient sensory input to the central neural ventilatory pacemaking system to generate a rhythmic motor output to the gill appendages.

Oxygen sensitivity is dependent upon the integrity of the prosomal intercoxal sensory cuticle and the opisthosomal book gills. Unfortunately, consistent quantitative measurements of the behavioral effects of eliminating these structures are very difficult to obtain since: (i) the ventral nerve cord and the nerves which arise from it run within blood vessels—section of a nerve or connective produces considerable blood loss; (ii) extirpation or waxing the surfaces of the book gills drastically reduces the cuticular surface area available for respiratory gas exchange thereby leading to anoxia. However the observation of Hyde (1906) that after removal of the heart and massive blood loss—and therefore during presumed anoxia—rhythmic gill appendage movements were maintained, and the present observation (Fig. 4C) that oxygen responsiveness was not appreciably affected by cutting open all branchial blood sinuses, suggest that neither anoxia nor blood loss plays a decisive role in depressing the oxygen responsiveness of the ventilatory system.

Since neither rhythmic respiratory nor rhythmic swimming movements were ever observed after complete blockage of the prosomal and opisthosomal oxygen sensitive structures, in the intact animal afferent input from the oxygen receptive areas must be important in the maintenance of the excitation level in the central ventilatory neural network. In isolated opisthosomal nerve cord preparations Wyse (1971) has on occasion observed rhythmic output in the apparent absence of any neural input to the ventilatory rhythm generating neural network. However in the isolated single ganglion preparation, neural input is required via either the gill appendage sensory innervation (Hyde, 1906) or electrical stimulation of the nerve cord connectives (Fourtner, Drewes and Pax, 1971; Wyse, 1971).

The opisthosomal book gills are extensively innervated by the sensory gill nerve (Patten and Redenbaugh, 1899). Sensory buds and free nerve endings have been described in the book gill cuticle (Patten, 1912). However there is no

information available concerning which of these sensory elements may be the oxygen receptors, nor am I aware of any descriptions of sensory elements in the intercoxal cuticle. Information on the detailed morphology and physiology of sensory structures in the book gills as well as morphological and physiological descriptions of oxygen sensitive structures in the intercoxal sensory cuticle is presently being obtained.

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Summary

1. The rate of *Limulus* opisthosomal gill appendage respiratory movements is proportional to the logarithm of the environmental oxygen concentration. Respiratory movements cease in anaerobic sea water.

2. Oxygen introduction into anaerobic sea water elicits movement of the prosomal walking legs as well as the rhythmic gill appendage movements described by Waterman and Travis (1953).

3. The prosoma and opisthosoma each contain oxygen receptive structures. Prosomal oxygen responsiveness depends upon the sensory cuticular membranes located between the walking leg coxa. Opisthosomal oxygen sensitivity depends upon book gill integrity.

4. Sensory input from either the prosomal intercoxal cuticle and/or the opisthosomal book gills is required to generate rhythmic gill appendage movements in response to oxygen introduction into anaerobic sea water.

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CONSTITUENTS OF UNIONID EXTRAPALLIAL FLUID. I. ELECTROPHORETIC AND IMMUNOLOGICAL STUDIES OF PROTEIN COMPONENTS

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The mechanism of shell formation in mollusks is not well understood (Wilbur and Yonge, 1964). Bivalve shell formation has been shown to occur in a compartment bounded by the shell and the mantle (Bevelander and Nakahara, 1969). The fluid of this compartment, the extrapallial fluid, contains the proteins necessary for the synthesis of an organic matrix able to bind Ca^{++} and $CO_3^{=}$ ions in a characteristic crystalline configuration (Wilbur and Watabe, 1960). Due to difficulty in working with the extrapallial fluid, the number and nature of the proteins of this fluid is not yet understood. Kobayashi (1964a, 1964b) reported a partial separation of these components from a number of molluscan species using paper and acetate strip electrophoresis. We report a more definitive separation using acrylamide gel electrophoresis. Using extrapallial fluid from several species representing three subfamilies within the family Unionidae, the electrophoretically produced patterns of protein bands and immunological studies reveal a surprising uniformity in the soluble protein composition.

Methods and Materials

Mussels

Live mussels were used as the source of extrapallial fluid. Specimens of *Quadrula pustulosa* (Lea), *Quadrula quadrula* (Rafinesque), *Amblema costata* (Rafinesque), *Pleurobema cordatum* (Conrad), *Obliquaria reflexa* (Rafinesque), *Lampsilis siliquoidea* (Barnes), *L. ventricosa* (Barnes), *Actinonaias carinata* (Barnes), and *Anodonta grandis* (Say), were collected from the Muskingum River at the Beverly, Ohio, bed (Bates, 1970). They were transported to Ypsilanti packed in wet rags and were thereafter maintained in well aereated, circulating tap water at room temperature.

Preparation of samples

Access to the extrapallial fluid was achieved by prying the shell open 2–3 mm, and cutting the adductor muscles. The mantle was pierced near the pallial attachment with an 18 gauge 2-inch needle; then gentle suction was applied to remove the fluid.

Each clam provided enough fluid for a single electrophoresis gel. Normal extrapallial pH for 200 individuals of three species tested was 8.15. Two parts

[1] James Pietrzak died of burns incurred in a house fire on April 28, 1972. Mr Pietrzak was a graduate student at Eastern Michigan University at the time this study was performed, and was employed at Grand Valley State College at the time of his death.

fluid were mixed with one part 60% sucrose in a 2.5 ml syringe; then 0.3 ml of this mixture was immediately applied to a gel. Samples were run within five minutes of extraction. Between extractions the syringe was rinsed three times with distilled water to prevent cross contamination.

For injection into rabbits fluid from 3–6 individuals of a single species was spun in a clinical centrifuge to remove the suspended flocculent material, and the supernate was sterilized using a bacterial filter.

Samples of extrapallial fluid to serve as antigen in the agar-gel diffusion studies were pooled from 3–6 individuals and placed directly into the antigen well of the agar plate.

Electrophoresis

A Heathkit IPW-17 constant voltage power supply was used in conjunction with a Buchler 12-gel capacity water-jacketed electrophoresis chamber. Cold tap water (15° C) was circulated through the water jacket during the operation.

Acrylamide; N, N'-Methylenebiscrylamide (BIS); 2-Amino-2-(hydroxymethyl)-1, 3-propanediol (TRIS); N, N, N', N'-Tetramethylethylenediamine (TEMED); Naphthol Blue Black (Amido Schwartz 10B); Glycine (ammonia free); and Photo-Flo were all obtained from Eastman Kodak and used as supplied. Sucrose (analytical grade) and Ammonium Persulfate (analytical grade) were obtained from the Mallinkrodt Co. and also used as supplied.

Electrophoresis buffer: 28.8 g glycine, and 6.00 g TRIS was diluted to one liter with water. If necessary the solution was adjusted to pH 8.3; Fixative-stain solution: one gram Amido Schwartz per 100 ml 7% (v/v) aqueous acetic acid; Photo-Flo solution: 0.5% (v/v) aqueous Kodak Photo-Flo.

Preparation of acrylamide gels

The procedure of Davis (1964) was followed with the modifications indicated as follows: *Solution A:* 48 ml 1N HCl, 36.6 g TRIS, 0.23 ml TEMED, diluted to 100 ml with water; *Solution B:* 48 ml 1N HCl, 5.98 g TRIS, 0.46 ml TEMED, diluted to 100 ml with water and adjusted with 1N HCl to pH 6.7; *Solution C:* 30.0 g acrylamide, 0.80 g BIS, diluted to 100 ml with water; *Solution D:* 10.0 g acrylamide, 2.5 g BIS, diluted to 100 ml with water; *Solution E:* 40% (w/v) aqueous sucrose; *Solution F:* 0.14% (W/v) aqueous ammonium persulfate; *Solution G:* 0.004% (w/v) aqueous riboflavin.

Separatory gel (small pore)

A mixture of solutions A, C, and F (1, 2, and 4 volumes, respectively) was drawn into a syringe and injected into clean 8.5 cm gel tubes to fill each tube to a height of 4.5 cm. With a 0.5 ml syringe and 26 gauge needle a 2 mm layer of water was placed over the gel. The tubes were allowed to stand 20 minutes as polymerization took place.

Spacer gel (large pore)

The water layer was removed from the surface of the separatory gel, and a mixture was made of solutions B, D, G, and E (1, 2, 1 and 4 volumes respectively), and was injected from a syringe to a height of 6 mm above the separatory gel. A 2 mm layer of water was placed carefully over the spacer gel. The tubes were

placed within one inch of a fluorescent tube for 20 minutes to initiate photopolymerization.

Separation and detection procedures

With the gels in the apparatus and the buffer compartments filled with fresh buffer, the protein-sucrose solutions were layered directly onto the spacer gels. A blank gel was included in each set. All blanks indicated no contamination of upper buffer by exteraneous protein. Electrophoresis was performed at 2 mAmp/tube for 15 minutes, then the voltage was turned to the maximum setting, normally producing a current of 6 mAmp/tube, for 50–55 min. The gels were removed from the tubes by squirting water with an 18 gauge needle between the gel and the tube. Each gel was stained overnight in fixative-stain solution and destained electrophoretically in 7% (v/v) aqueous acetic acid at 10 milliAmp per tube for one hour. Gels were then scanned by means of a photometric scanner attached to an Aminco Bowman SPF-125 Spectrophotofluorimeter (Scott and Pietrzak, 1971).

Injection of rabbits for antibody production

Fourteen female virgin New Zealand White Rabbits were injected for antibody production. Injections were every three days as follows: 1 and 2—0.5 ml intravenous, 3—2.0 ml subcutaneous, 4 through 14—0.2 ml subcutaneous plus 0.5 ml Freund Complete Adjuvant (Difco), final—2.0 ml intraperitoneal.

Immunodiffusion

Difco Nobel-Agar was obtained from the Difco Co. and was used as received. Boric acid, sodium tetraborate decahydrate, and sodium chloride were all Baker reagent grade. Merthiolate was obtained from the Lilly Co. and was used as supplied.

Large quantities of whole blood were obtained by cardiac puncture one week after termination of the immunization program. The blood was stored at 4° C for 24 hours to allow clotting. Serum was drawn off with a syringe, made 0.01% in merthiolate to inhibit bacterial growth (Ouchterlony, 1968), and stored frozen at −20° C until use.

Just prior to use, serum was thawed and incubated in a water bath at 56° C for 30 minutes to deactivate the serum complement. Diffusion agar was prepared according to the recommendations of Carpenter (1956). Plastic petri-dishes 5 cm in diameter and 10 mm deep were half filled (6 ml) with agar. Four peripheral wells 7 mm in diameter were punched equidistant from a central well (16 mm center to center). For each cross test 0.1 ml of extrapallial fluid was placed in each peripheral well, and 0.1 ml of antiserum was placed in the center well.

Patterns were allowed to develop in an incubator at 10° C until maximal precipitate arcs formed, (approximately 21 days). In addition, control diffusion-plates using sera from non-innoculated rabbits were run simultaneously with each immuno-system. No immunological response occurred in any control. All tests were run in triplicate, all plates giving identical results within a group.

Results

Table I summarizes the results of electrophoretic separations of soluble extrapallial fluid proteins on acrylamide gels. The A band because of its dependability

TABLE I

Electrophoretic results

Species*	Number of specimens	Type of Data†	Bands observed											
			A	B	C	D	E	F	G	H	I	α1	α2	α3
Qq	25	M	1.00	0.70	0.59	0.50	0.40	0.35	0.30	–	0.14	1.40	1.63	2.15
		SD	—	±0.013	±0.012	±0.013	±0.012		±0.009	–	±0.013	±0.000	±0.050	±0.050
		P	100	88	96	100	36	4	96	0	72	40	24	17
Qp	20	M	1.00	0.70	0.059	0.49	0.38	0.35	0.29	–	0.13	1.40	–	–
		SD	—	±0.012	±0.010	±0.017	±0.011	±0.012	±0.010	–	±0.019			
		P	100	95	15	85	24	56	60	0	60	4	0	0
Ac	30	M	1.00	0.66	0.59	0.50	0.39	0.35	0.30	0.26	0.15	–	–	–
		SD	—	±0.012	±0.013	±0.011	±0.010	±0.010	±0.011	±0.013	±0.016			
			100	87	42	94	42	13	74	49	87	0	0	0
Pc	4	M	1.00	0.80	0.60	0.50	0.39	0.34	0.30	0.25	0.16	–	–	–
		P	100	75	75	100	100	50	75	50	100	0	0	0
Or	3	M	1.00	0.72	–	0.49	0.40	0.35	0.32	0.26	0.14	–	–	–
		P	100	67	0	100	33	67	33	100	100	0	0	0
Ls	4	M	1.00	0.80	–	–	–	0.38	–	0.30	–	–	–	–
		P	100	25	0	0	0	50	0	75	0	0	0	0

* Qq = *Quadrula quadrula*, Qp = *Quadrula pustulosa*, Ac = *Ambelma costata*, Pc = *Pleurobema cordatum*, Or = *Obliquaria reflexa*, LS = *Lampsilis siliquoidea*.

† M = mobility referenced to A = 1.00, SD = Standard deviation of mobility, P = Percentage of samples displaying the band.

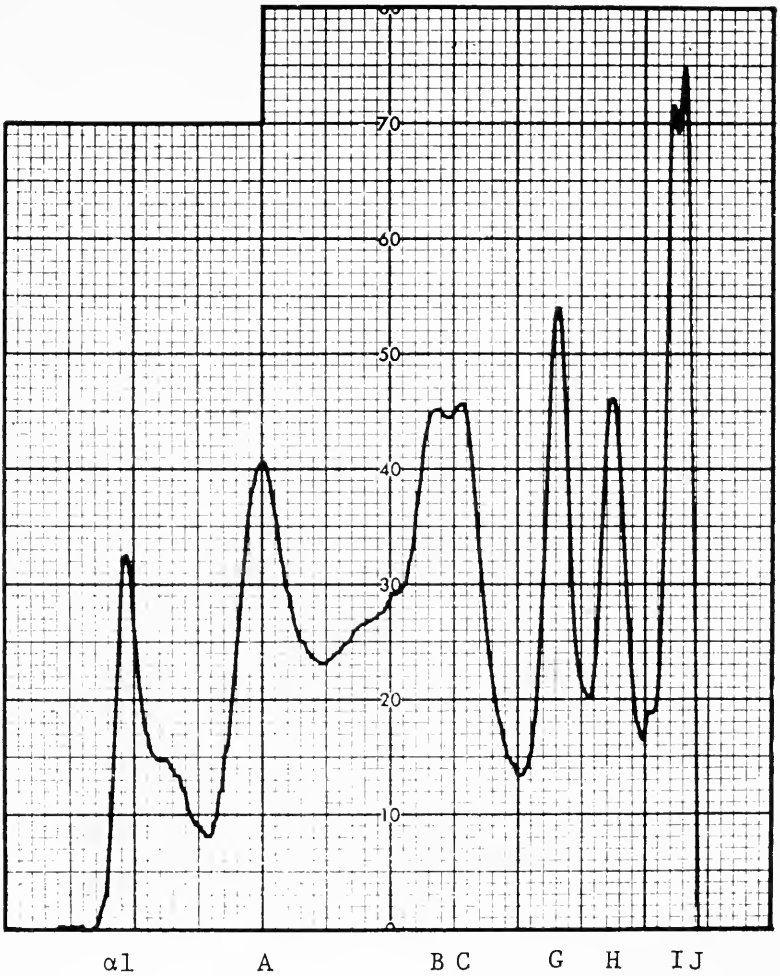

FIGURE 1. A typical densitometer scan of an acrylamide gel electrophoretic separation of extrapallial fluid.

of occurrence, was assigned a relative mobility of 1.00, and all other bands are reported as fractions or multiples of this value.

All gels were scanned by a densitometer, a typical result being shown in Figure 1. This technique proved superior to visual inspection for the detection of individual bands and facilitated accurate calculations of mobilities. Due to background coloration and overlapping of bands, no attempt was made to integrate the areas under the peaks as a measure of total protein in each band. It is obvious from inspection of the scans, however, that bands A, E, F, and G represent proteins present in relatively high concentration. Bands B, C, D, and I represent compounds of only moderate concentration, while the α bands are too diffuse to allow a reasonable estimation of concentration to be made. When present the H band could be either

intense or weak. The J band was too slow moving to permit a measurement of its mobility. In fact, considering its position at the end of the gel it could well be an artifact.

The frequency of occurrence of each band was considered. The A band was particularly significant being present in good concentration in every organism studied. Bands B, D, G, and I were generally present, while bands C, F, and H were less dependable and showed considerable species variation. For example, band C was virtually always present in *Quadrula quadrula* but seldom present in *Quadrula pustulosa*. There was only one individual occurrence of an α band outside of *Quadrula quadrula*, and even in that species they appeared in less than half of the samples assayed.

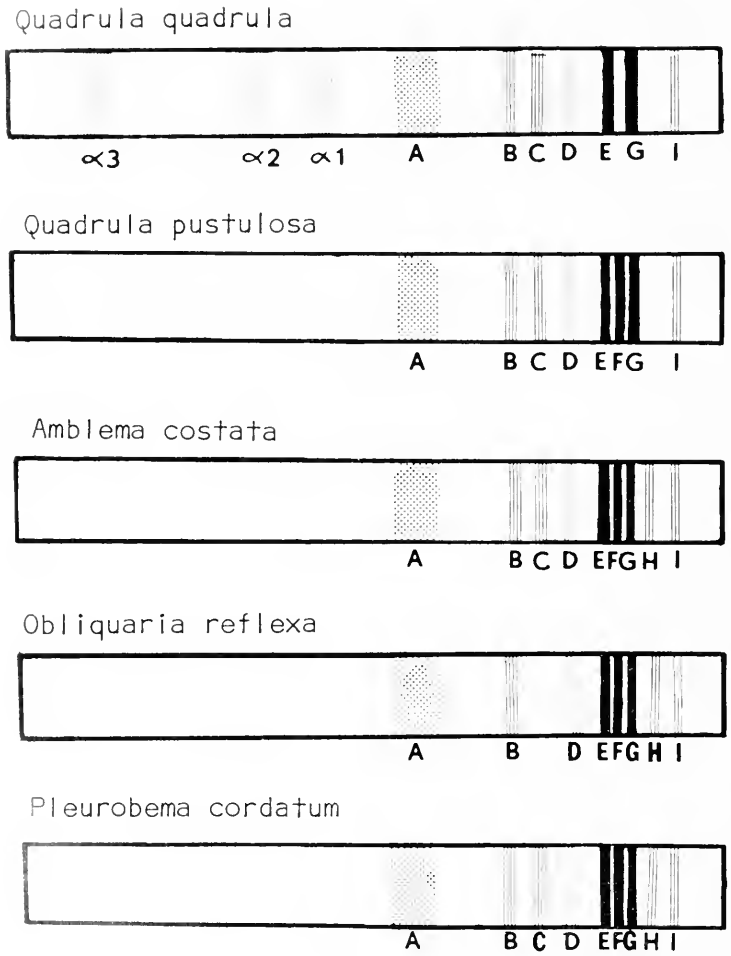

FIGURE 2. Patterns for five species showing the relative positions of frequently occurring bonds.

The size and age of each specimen was recorded and correlated with the assay of its fluid. Examination of this data failed to reveal any relationship between age and the frequency of appearance of any particular band.

It is noteworthy that band B showed a much greater variation in mobility from one species to another than any of the other bands. Where large samplings were taken, it showed a very reproducible value for the particular species. Typical band patterns for five species are presented for comparison in Figure 2.

On the basis of the interpretation of the acrylamide gel data, immunological studies to ascertain if the similar patterns in fact represented identical protein species were undertaken.

An "F" reaction is defined as a cross reaction between homologous and heterogenous systems with deviation and complete fusion of the immuno-precipitate arc as in an Ouchterlony "Identity" reaction (Ouchterlony, 1968). A "C" reaction is defined as a cross reaction between homologous and heterogenous systems in which the immuno-precipitate arc was of insufficient density or extension to absolutely establish an "F" type reaction. The "C" reaction was attributed to lower antigen or antibody concentration in the complex systems tested. Table II indicates the results of test crosses between homologous and heterogenous immuno-systems. Cross reactions occurred without exception in all test crosses of heterogenous antigen and homologous systems among species from all three subfamilies. The cross reactions were often characterized by Ouchterlony "Identity" reactions-deviation and complete fusion of precipitate arcs. The generally accepted interpretation of this reaction is that the antigens have a factor in common and are, due to this determinant, precipitated by the corresponding antibody component to this

TABLE II

Results of test crosses between homologous and heterogenous extrapallial fluid immuno-systems. Symbols are: *A-Qq, A-Qp, A-Ac, A-Xc, A-Ls, A-Ag are the antisera and Qq, Qp, Ac, Xc, Ls, Ag are the antigens corresponding to* Quadrula quadrula, Quadrula pustolosa, Amblema costata, Actinonaias carinata, Lampsilis siliquoidea, *and* Anodonta grandis. *Lv is the antigen corresponding to* Lampsilis ventricosa. *F and C refer to F type and C type immuno-reactions, while the number in brackets indicates the number of bands of the particular reaction. N.R. indicates that the combination was not tried.*

Cross test antigen	Homologous antibody—antigen					
	1	2	3	4	5	6
	A-Qq + Qq	A-Qp + Qp	A-Ac + Ac	A-Xc + Xc	A-Ls + Ls	A-Ag + Ag
Qq	F(1)C(2)	F(4)	F(1)C(1)	F(2)	F(1)	C(2)
Qp	F(1)C(1)	F(4)	F(1)C(1)	F(1)C(1)	C(2)	C(2)
Ac	C(2)	F(1)C(1)	F(1)C(1)	C(1)	C(2)	C(2)
Xc	N.R.	N.R.	N.R.	F(2)	N.R.	N.R.
Ls	C(3)	C(2)	F(1)	F(1)	F(1)C(1)	N.R.
Lv	N.R.	N.R.	N.R.	N.R.	F(1)C(1)	C(2)

common determinant. These "F" type reactions were evident in test crosses between heterogenous antigen in 12 of 21 crosses.

Discussion

The shell matrix has been established to be protein in nature. Gregoire, Duchateau, and Florkin (1955) reported the presence of a polypeptide and two proteins, nacrine and nacrosclerotine, in this material. X-ray diffraction patterns indicated an α or β keratin structure (Wilbur and Watabe, 1963). Within the $CaCO_3$ crystal both water soluble and insoluble components were found (Gregoire, 1955). The non-dialyzable soluble fraction was found to precipitate with trichloroacetic acid, inferring a protein substance. This was found in each of thirty-two species of gastropod tested and accounted for nearly half of the total matrix protein in some of these species (Florkin and Stotz, 1968).

A recent elegant study (Bevelander and Nakahara, 1969) proposed a mechanism for shell formation based on shell electron micrographs. A compartment was described as forming near the surface of the shell, attaching to the shell and trapping a modified extrapallial fluid. The compartment fills with fibers and granules, this being followed by the formation of carbonate crystals. The envelope described is presumably protein in nature, probably what has been described elsewhere as the shell matrix. The protein precursors thus incorporated and the enzymes necessary for these transformations must have originated in the extrapallial fluid.

Previous studies with marine species (Gregoire, 1957, 1960) involving electron micrographs of shell matrix revealed that patterns and sizes of perforations are differentially characteristic of Gastropoda, Pelecypoda, and Cephalopoda. Hare and Abelson (1965) demonstrated that the amino acid compositions of matrix proteins are strikingly similar among species within a single family of Pelecypoda, but that differences are significant between families.

Gel electrophoresis provided resolution of the protein components of the extrapallial fluid superior to that previously reported with paper or cellulose acetate strips (Kobayashi, 1964). Evidence presented in this report supports the concept that within a single species there are some proteins which appear in all or virtually all samples of extrapallial fluid. Other proteins occur in a more variable manner from one individual to the next. This is not inconsistent with the concept of a functioning system maintaining a constant supply of some components and generating others as necessary. Conceivably some enzymes and a supply of the basic unit of shell matrix protein might be present as stable components while other enzymes or matrix intermediates vary according to the current rate or stage in shell formation by the individual. The similarity of the patterns from one species to another supports the concept that the mechanism of shell formation may well be very similar or identical among those species.

In terms of the immunological evidence, it is not possible to determine the nature of the proteins or conjugated proteins found in the fluid. Further, it is not intended to reveal the absolute number of components involved since the acrylamide data provides superior resolution in this regard. It is possible to state that definite close antigenic relationships do exist among some components of extrapallial fluid of both closely and distantly related species of the family Unionidae. These similarities manifest themselves as extensive cross reactions between heterogenous immunosystems and are characterized by frequent "F" type interactions.

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Summary

Components of extrapallial fluid of several species within the three sub-families of the family Unionidae have been comparatively analyzed. It has been found that the proteins involved in shell formation for these species are strikingly similar, electrophoretically and immunologically. These data infer that the same protein components may well be involved in the formation of shell within species of the family Unionidae.

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OPERCULAR REGULATION IN THE POLYCHAETE *HYDROIDES DIANTHUS* (VERRILL, 1873). I. OPERCULAR ONTOGENY, DISTRIBUTION AND FLUX[1]

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The cosmopolitan polychaete *Hydroides* is usually found subtidally at moderate depths, but may be seen occasionally on rocks along the shoreline. The animals spend their post-larval life in calcareous tubes attached to substrates such as rocks, mollusc shells, pilings and ship bottoms.

In this genus the head consists of a prostomium bearing two hemicirclets of ciliated and pinnuled branchiae as the only appendages. Normally these are the sole exposed portions of the body and they serve as respiratory, feeding and sensory organs. Two dissimilar opercula attached to the dorsal base of these structures are modified branchiae. The larger operculum functions as a tube plug. In its mature form it is modified for defensive purposes by having a non-pinnuled, thickened stalk and an enlarged tip consisting of two cup-shaped structures, one arising from within the other (Fig. 1a). On the side opposite the functional operculum is a small, rudimentary operculum with a swollen but otherwise undifferentiated tip. The functional operculum may be associated with either the left or the right branchial half-circlet, hence the designation of animals as "left-handed" or "right-handed."

Zeleny (1905, 1911), in his classical account of the ontogeny of *Hydroides dianthus* larvae, established that the initial operculum developed from the middle member of the original three branchiae on the left. These branchiae were provided with a number of elongated and ciliated respiratory pinnules. The pinnules on the opercular branchia soon dropped off or were resorbed, leaving a simple primary functional operculum on the now naked stalk. Shortly thereafter the middle of the three branchiae on the right dropped off and a primary rudimentary operculum, consisting of a short, undifferentiated stalk, developed at the site of autotomy. Two unchanged branchiae then remained on each side. One or two new branchiae were added ventrally, but no other changes occurred until the animal was about six months of age, when the primary functional operculum on the left was autotomized. The primary rudimentary operculum on the right simultaneously enlarged to become a secondary functional operculum which had a mature, two-tiered structure. The residual stump on the left formed a secondary, mature type of rudimentary operculum. Therefore all animals should be right handed at this stage. No further observations were made by Zeleny.

[1] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island.

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While working at Woods Hole, it became apparent that the development of many *Hydroides dianthus* larvae could not be reconciled with this account. Observations suggested that only the initial appearance of the functional operculum is programmed and that thereafter the animals follow a variety of pathways.

That the position of the adult functional operculum can be reversed was first shown by Zeleny (1902, 1905). After amputation of the functional operculum the rudimentary operculum differentiated into a functional one, while the stump of the former functional operculum regenerated a rudimentary structure. Zeleny proposed that natural physiological reversals might occur, although he had no direct evidence to support this suggestion. Ichikawa and Takagaki (1942) found that, during several months of maintenance, four of 17 asymmetric juveniles of *Hydroides ezoensis* reversed. They postulated that this was due to physiological aging of the functional operculum, which would eventually be discarded.

The existence of occasional individuals with two functional opercula has not been satisfactorily explained. Rioja (1919) regarded them as throw-backs to an ancestral bi-operculate type. La Greca (1949, 1950), finding that double-operculate animals in his laboratory population of *H. norvegica* were regenerating branchiae, proposed that involution of, or injury to the branchiae could allow the rudimentary operculum to develop. During starvation or other suboptimal conditions, natural involution of branchiae can occur but double functional opercula rarely form during subsequent regeneration (Greuet, 1962). Bi-operculate animals can be experimentally produced by the simultaneous removal of both opercula (Zeleny, 1905; Okada, 1933; Abeloos, 1952a), by a transverse thoracic section (Zeleny, 1902, 1905; Okada, 1933; Abeloos, 1952a; Cresp, 1964), or by the excision of at least one-half of the branchiae (La Greca, 1950). It is dubious whether any of these conditions would arise frequently enough to account for the presence of substantial numbers of these animals in a natural population.

This study investigates opercular development, opercular dynamism, and population structure in specimens of *Hydroides dianthus* at Woods Hole.

Methods

Specimens of *Hydroides dianthus* of various ages were dredged from a depth of 60 feet in Buzzard's Bay by the Supply Department of the Marine Biology Laboratory, Woods Hole, Massachusetts. During the breeding months (June through September) tiny juvenile worms may be found interspersed among the adult tubes on mollusc shells and rocks. These immature animals with their tubes were carefully removed from the substrate with fine forceps. Only individuals less than 3 mm in body length were utilized. In some cases the fragile tube shattered during the process, but generally enough remained to enable the animal to repair and extend it (*cf.* Neff, 1969). The animals were placed in small vials filled with 20 ml of sea water, which was changed every few days and kept at ambient temperatures (20–23° C). As the animals did not attach to the bottoms of the vials, they could be easily removed with a Pasteur pipett. For experimental manipulations, specimens were placed in a drop of sea water on a glass slide, and opercular and branchial amputations were performed with a No. 5 forceps and a microdissecting needle.

After complete or partial removal from their tubes, adult animals were maintained in fingers bowls covered with cheesecloth. The bowls were submerged in a

sea water table, and were cleaned frequently to remove algal deposits. The most expeditious method of removing adults from their tubes was by cracking and removing the posterior end of the tube. The worm was then pushed out through this opening with a piece of fine polyethylene tubing.

For field population studies wooden frame boxes were enclosed with nylon screening. An opening was made in one side of the screening to permit small predators to enter. Intact specimens on shells were placed inside the boxes and were submerged in Vineyard Sound in approximately 20 feet of water. The bottom in the chosen location was silty, rather than rocky, as is the usual habitat of these animals. The remaining animals in these studies were maintained in sea water tables in the laboratory, as described above. Observations on both groups of animals were made at one-week intervals.

Experimental groups were compared using the chi-square distribution. Chi-square 2×2 contingency tables were used in these calculations and manipulations were performed on a Hewlett Packard 9100A calculator.

Observations

Ontogeny of the branchiae and opercula

The early development of *Hydroides* embryos is typical of other annelids, and the trochophore is indistinguishable from that of other Serpulid genera. The cleavage and larval stages of Serpulids have been described in detail by Conn (1884), Soulier (1898), Shearer (1911), Segrove (1941) and Sentz-Braconnet (1964). At metamorphosis the larvae settle on the substratum and begin to secrete calcareous tubes. According to Zeleny (1905) and Wisely (1958), just prior to this time the protuberant head of the free-swimming larva differentiates into a more flattened structure with dorso-lateral lobes. Three or four branchial rudiments appear simultaneously from each of these lobes (Fig. 1b). These stumpy, ciliated rudiments elongate and develop minute protuberances which indicate the future position of respiratory pinnules. These grow rapidly as the branchial filaments enlarge. At the stage when the primary operculum first appears (about four weeks after fertilization) the worm possesses three symmetrical, pinnuled sets of branchiae (for details of branchial development, see Zeleny, 1905, 1911).

The first indication of an operculum is a swelling at the apex of the left median branchia (Branchia II). The orientation of this operculum appears to be invariable in the larvae of most species of *Hydroides* examined but Ichikawa and Takagaki (1942) found that in 5 of 77 *H. ezoensis* juveniles the initial operculum appeared on the right side. The terminal swelling of the branchial filament enlarges into a small, funnel-shaped opercular cup perched on top of an otherwise unexceptional pinnuled branchia. This structure will be henceforth referred to as the branchial operculum (Fig. 1c). The opercular branchia has two long pinnules in *H. dianthus,* although eight were reported by Zeleny in the same species (1905).

The initial sample consisted of 55 pre-opercular juveniles. The number of animals in the groups below is small because of death or lack of opercular development by many larvae under laboratory conditions.

In three animals the branchial operculum originated as described above and within three to 14 days the pinnules on the opercular branchia were lost. The

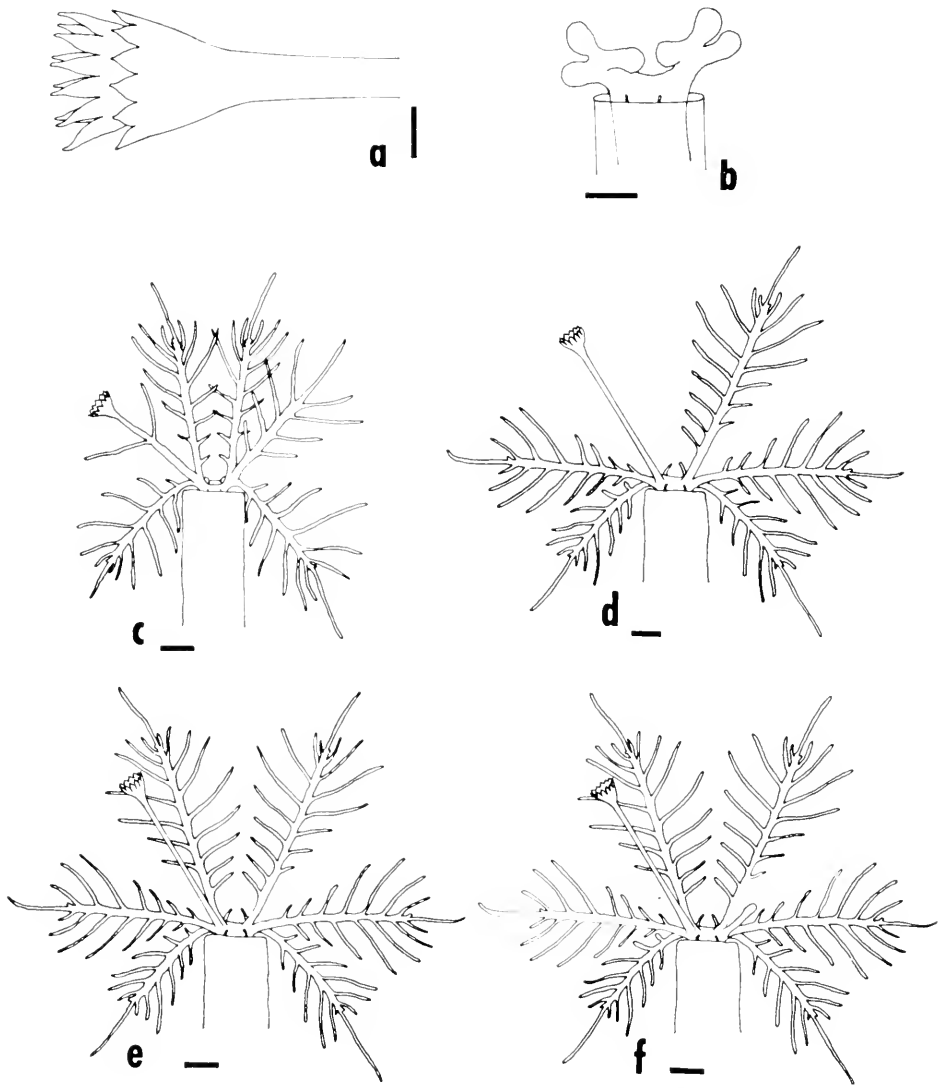

FIGURE 1 (a) A mature functional operculum of *Hydroides dianthus*, with a characteristic double-cupped structure and thickened spines, marker length = 1 mm; (b) head of a post-metamorphic *H. dianthus* juvenile, demonstrating the branchial rudiments. The animal at this time is approximately 1 mm in length, marker length = 0.1 mm; (c) dorsal view of a 1 mm juvenile with a branchial operculum on the left median branchia. The most anterior branchiae may or may not be pinnuled at this time. The animal is shown projecting from its tube, marker length = 0.1 mm; (d) dorsal view of a juvenile with a left primary functional operculum and asymmetric distribution of branchiae, marker length = 0.1 mm; (e) dorsal view of a juvenile with a left primary functional operculum and symmetric branchiae (modified from Zeleny, 1905), marker length = 0.1 mm; (f) a juvenile with a left primary-type functional operculum and a primary rudimentary operculum on the right (modified from Zeleny, 1905), marker length = 0.1 mm.

corresponding right branchia was not autotomized at any time to make space for a primary rudimentary operculum, as Zeleny (1905, 1911) and Wisely (1958) noted. Therefore these animals had three pinnate branchiae on the right side and two on the left side, in addition to the non-pinnuled operculum, which may now be called the primary functional operculum (Fig. 1d). The majority of the juveniles which survived through the developmental period, however, did not exhibit this branchial asymmetry. Each of these retained three pairs of pinnuled branchiae throughout early development. In six individuals the opercular cup disappeared from the tip of the left Branchia II, and a non-pinnuled primary functional operculum appeared on the left, in addition to the three intact branchial pairs (Fig. 1e). These animals had three sets of pinnuled branchiae but were rendered asymmetric by the presence of the primary functional operculum on the left side. In four other larvae, the operculum formed was a primary rudimentary type, which ceased to develop further. Three remaining larvae lost the branchial operculum without any ensuing opercular formation.

The numerical imbalance between individuals showing branchial symmetry and asymmetry was sustained in older animals. Six of seven with a left primary-type functional operculum but no rudimentary one had symmetrical branchiae. This is more difficult to explain than the cases in which the primary operculum is derived from the second branchia by a simple loss of pinnules from the branchial stalk (Zeleny 1905, 1911). One possible explanation for this phenomenon assumes the formation of a new branchia on the left to replace the one which has lost its pinnules and become an operculum. However, regenerating branchiae are never observed under these circumstances. A more plausible alternative is that the left median branchia does not lose its pinnules, but that the opercular cup at the apex of this branchia is autotomized and the primary functional operculum formed *de novo* from the branchial base. In such an eventuality there will be a developmental difference between the transient branchial operculum which appears on the branchial apex and the primary functional operculum which arises independently on the same side. It should be emphasized that, although the branchial filaments and the opercular stalk are almost identical in morphology and in histological structure, none of the opercula subsequent to the branchial one is derived directly from the branchiae; rather they develop from the basal portion of the branchial crown. The tissue of the branchial base has the capability of producing new opercular structures at all stages of development.

The probability of a non-branchial origin of the primary functional operculum is supported by the following observation. Over a period of five weeks the branchial filament supporting the opercular cup in one animal became progressively more attenuated at the terminal end until at last the cup was autotomized, leaving a normal-appearing branchia. Consequently this worm had three pairs of branchiae but no operculum. Subsequently a rudimentary type of operculum appeared on the left side, and the animal did not change thereafter. While this phenomenon was observed only once, loss of the branchial operculum normally occurs soon after its formation, and the chances of detecting its loss would be slight. This particular animal was unusual in having retained the branchial operculum for almost five weeks. In addition, several larvae were found with symmetrical branchiae and an operculum developing on the left side. This also suggests a *de novo* formation of the primary operculum.

However the primary functional operculum originates, it is dropped within a few weeks and a rudimentary operculum may develop on the right side. Eight specimens, after losing the primary functional operculum, replaced it with a primary rudimentary one which subsequently developed into another primary-type functional operculum. In three of these animals there was a contemporaneous rudimentary operculum (Fig. 1f); nevertheless, the primary functional operculum was preferentially replaced by another functional one on the same (left) side and no opercular reversal occurred at this time. In three additional animals, however, the loss of the primary functional operculum signaled the primary rudimentary operculum present on the right to differentiate into a functional operculum. These proved to be very transient and were rapidly replaced by rudimentary structures again. In other animals the primary rudimentary operculum which developed on the left after the loss of the primary functional one did not differentiate into a functional one, and another rudimentary operculum was usually formed on the right. In most cases (22 out of 33) these animals retained the two rudimentary opercula for a lengthy period of time. They may represent a quiescent stage, such as the six-month hiatus reported by Zeleny (1905) between the appearance of the primary opercula and the first reversal. In a few individuals, one or the other of the rudimentary opercula would appear enlarged or swollen, only to revert to normal size. It was as though the presence of a second rudimentary operculum inhibited the first from developing into a functional type.

In addition to the juveniles collected at a pre-opercular or primary opercular stage, there were 65 specimens with a functional operculum already present on the right side and a rudimentary one on the left. These animals were more mature than a large majority of the left-handed specimens, as indicated by their size, the opacity of the body and tube, the mature form of their opercula and the appearance of pigmented stripes on the functional operculum. Eleven of these animals autotomized the functional operculum and regenerated a rudimentary operculum in its place without reversal. The remainder died or did not change during the observation period.

Population composition

In order to determine whether opercular changes occur naturally in *Hydroides dianthus,* it was necessary to ascertain the normal population composition. Few quantitative studies of Serpulid populations exist and, except for the genus *Hydroides*, conclusions about opercular distribution have been made on the basis of small numbers of animals. Zeleny (1902, 1905) found a slight predominance of right-handed specimens in *H. dianthus*, *H. uncinata*, and *H. pectinata* collections. Ichikawa and Takagaki's (1942) survey of an enormous *H. czocnsis* population from Japan gave similar results. No statistical tests were used by these investigators, but they all assumed a preponderance of right-handed animals. Application of chi-square tests to their data indicated that none of these populations differed significantly in "handedness" from a 50–50 distribution, thereby corroborating the observations of Ludwig and Ludwig (1954) who worked with the European species, *H. norvegica.*

Since opercular distribution in a population might vary with age and sexual maturity, the worms used in this study were separated into the following categories: Adult—large animals with heavily-calcified tubes and approximately 30 pigmented

branchiae; Young—smaller animals with thin delicate tubes and fewer, lightly-pigmented branchiae; and Juvenile—very young post-metamorphic animals, 3 mm or less in total length, with extremely thin translucent tubes.

According to Grave (1933) and Hill (1967), under optimal conditions individuals of the genus *Hydroides* become sexually mature six to eight weeks after metamorphosis. Therefore the first two samples should both be of reproductive age.

One thousand, one hundred and twelve adult, 952 young and 209 juvenile specimens of *Hydroides dianthus* were tabulated. Table I presents the distribution of left- and right-handed individuals in this population. Neither adult nor young animals differed from a 50–50 distribution of left- and right-handed animals. In contrast, the juveniles taken as a group were heavily skewed in favor of left-handed specimens and, consequently, the proportions of the juvenile population differed significantly from that of both adult ($P < 0.005$) and young ($P < 0.005$) populations. Ichikawa and Takagaki (1942), Ludwig and Ludwig (1954) and H. W. Ludwig (1957) also reported that juvenile specimens were predominantly left-handed.

The juvenile group was subdivided according to size into three categories. Group A was 1.5 mm or less in length; Group B, 1.5 to 2 mm; and Group C, 2 to 3 mm. Of these only Group A, the youngest animals, did not demonstrate an equal

TABLE I

Distribution of left-handed, right-handed and bi-operculate animals in a population of Hydroides dianthus. *Abbreviations are: LH, left-handed specimens; RH, right-handed specimens; B, bi-operculate specimens*

	N	%	Chi-square value (Variation from 50–50 distribution excluding bi-operculate animals	*P*
Adult	LH 531	(43.8)		
	RH 528	(43.6)	0.00	$P > 0.975$
	B 153	(12.6)		
Young	LH 389	(40.9)		
	RH 408	(42.8)	0.23	$0.9 > P > 0.5$
	B 155	(16.3)		
Juvenile groups				
A (<1.5 mm)	LH 56	(8.84)		
	RH 8	(12.1)	20.94	$P < 0.005$
	B 2	(3.0)		
B (1.5–2.0 mm)	LH 36	(45.6)		
	RH 31	(39.2)	0.19	$0.9 > P > 0.5$
	B 12	(15.2)		
C (2–3 mm)	LH 23	(35.9)		
	RH 26	(40.4)	0.09	$0.9 > P > 0.5$
	B 15	(23.4)		
Combined	LH 115	(55.0)		
	RH 65	(31.1)	7.60	$0.01 > P > 0.005$
	B 29	(13.9)		

distribution of left- and right-handed individuals (Table I). It therefore appeared that the difference between the older worms and juvenile worms was attributable entirely to Group A, as neither Group B nor Group C was statistically different from the adult ($0.9 > P > 0.5$) or young ($0.5 > P > 0.1$) samples.

The proportion of animals with two functional opercula in the three populations was more variable than the left-right ratios, ranging from 3 to 23% of the population in question (Table I). Comparisons of these values indicate that, whereas adult and young populations did not differ in left-right ratios, the young group had a significantly higher proportion of bi-operculate specimens ($0.25 > P > 0.01$). Neither older sample varied from the juvenile group as a whole in this regard. However, the proportion of bi-operculate animals in the adult population was significantly higher than that of Group A and lower than that of C (in both cases, $0.025 > P > 0.01$). The young animals had significantly more bi-operculate forms than A ($P < 0.005$), but were not different from B and C. Groups B and C did not differ from each other. Thus the older portions of the juvenile population resembled the young rather than the adult group in the distribution of bi-operculate individuals. The animals of juvenile group A had only reached the earlier stages of opercular development and many still had only the primary functional operculum, whereas the adults showed a marked decline in bi-operculate specimens from the proportions in the younger age groups.

In the course of examining approximately 5000 specimens of *Hydroides dianthus*, I encountered two animals with supernumerary opercula. Each had two rudimentary and one functional operculum. In one, both rudimentary opercula branched from the same peduncle; in the other, the functional operculum and one of the rudimentary ones were joined at the base. These individuals were apparently the result of developmental aberrations although the opercula themselves were morphologically normal. Zeleny (1905) and La Greca (1950) also reported finding an animal with three opercula.

Opercular Flux

The ability of these serpulids to replace a missing functional operculum by the development of the rudimentary one enhances the probability that reversal might be a natural event. Collected specimens were occasionally found with a developing functional operculum on one side and a new rudimentary operculum regenerating from the opercular stump on the opposite side. Whether this reversal was provoked by accident or by an intrinsic physiological process has not been determined.

To study possible natural variations in *Hydroides* populations, a simple format was used in which left-handed, right-handed and bi-operculate animals were maintained separately for observation. The following groups were observed at intervals of one week for a six-week period: *Group 1:* Adult, left- and right-handed animals kept in the laboratory; *Group 2:* Adult, bi-operculate animals kept in the laboratory; *Group 3:* Adult animals of all types, kept in the field; *Group 4:* Young left- and right-handed animals kept in the laboratory; *Group 5: Young* bi-operculate animals kept in the laboratory. Because of the excellent survival rate of the young animals, it was possible to follow them for 6–8 weeks. Animals of Groups 4 and 5 which had undergone some change were segregated into the following four

categories. All were maintained in the laboratory. *Group 6:* Young left- and right-handed animals from Group 4, which had reversed during the course of observation; *Group 7:* Young left- and right-handed animals from Groups 4 and 5 which had previously been bi-operculate; *Group 8:* Young animals which had lost their functional opercula, from all groups of young animals; *Group 9:* Young animals from Groups 4 and 7 which became bi-operculate during the course of the experiment, but which were not so at the beginning.

Group 1: (N = 77) adult left- and right-handed animals. The majority of these animals did not change during any one week. Those which did had either autotomized the functional operculum, in which case the rudimentary operculum had enlarged (reversal) or, alternatively, the rudimentary operculum had developed although the functional one remained intact. The latter process resulted in a bi-operculate animal. In a few of these cases, the functional operculum was obviously degenerating.

A constant rate of change was observed from one week to the next in this group except for the last two of the six weeks. By this time the animals may have been in a precarious physiological state. Data are given in Table II, Column 1.

Group 2: (N = 71) bi-operculate adult animals. The only alteration observed in bi-operculate animals was the autotomy of one of the opercula and its subsequent replacement by a rudimentary operculum. Thirty-seven of the original 71 animals underwent this change, 21 died without change, and only seven remained bi-operculate after three weeks. This is an average change of 37.7% per week. Note that this rate of alteration is much greater than changes in asymmetric animals (Group 1), and thus bi-operculate worms would be rapidly converted to left- and right-handed individuals under these circumstances.

In most bi-operculate individuals one operculum is more mature than the other, and animals can be categorized on this basis. Those in which the left operculum was older were denoted as L/B (N = 40); those in which the right one was older, R/B (N = 31). In general the animals lost the more mature operculum. Seventeen of the L/B individuals became right-handed, and only two autotomized the younger right operculum to become left-handed. Of the R/B individuals, 16 lost the right operculum, and two lost the less-developed left operculum. The remainder in both groups died. Therefore almost 90% of the surviving animals autotomized the older operculum, a process which results in reversal of the prior asymmetry.

Group 3: (N = 127) adult animals maintained in Vineyard Sound. As it proved impossible to distinguish left-handed and right-handed animals in their tubes with an accuracy of more than 70 to 80%, they were classified as to whether they had mature, immature, double or aberrant functional opercula.

This group was initially composed of 112 asymmetric individuals, 10 bi-operculate ones, two without one of the branchial half-circlets, one without any visible opercula, and one which had lost both head and upper thorax. Observations were unfortunately curtailed the third week of observation by heavy silting of the habitat and consequent mortality.

The changes occurring in originally normal, asymmetric animals consisted of (a) loss of the mature functional operculum and its replacement by a young, developing one; (b) loss of the mature functional operculum without any observable replacement; (c) doubling, *i.e.* formation of bi-operculate individuals from left- or right-handed animals, (d) loss of the opercular cup, and (e) loss of branchiae

TABLE II

Comparison of changes in left-handed and right-handed individuals of groups 1, 3, 4, 6, and 7

Initial sample size	*Group 1* Laboratory maintained adults N = 77	*Group 3* Field adults N = 112	*Group 4* Young animals N = 349	*Group 6* Young LH&RH animals changed N = 50	*Group 7* Young LH&RH animals formerly bi-operculate N = 30
First week	N = 67	N = 105	N = 160	N = 46	N = 22
Reversal	1 (1.5%)	10 (9.5%)	23 (14.4%)	6 (13.0%)	2 (9.1%)
Bi-operculate	2 (3.0%)	1 (1.0%)	10 (6.3%)	—	3 (13.6%)
No visible operculum	—	19 (18.1%)	4 (2.5%)	5 (10.9%)	—
Other change	—	2 (1.9%)	—	—	—
Total change	3 (4.5%)	32 (30.5%)	37 (23.1%)	11 (23.9%)	5 (23.7%)
Second week	N = 47	N = 61	N = 129	N = 26	N = 15
Reversal	2 (4.3%)	4 (6.6%)	16 (12.4%)	1 (3.8%)	2 (13.3%)
Bi-operculate	1 (2.1%)	1 (1.6%)	2 (1.6%)	—	—
No visible operculum	—	11 (18.0%)	2 (1.6%)	—	1 (6.7%)
Other change	—	6 (9.8%)	3 (2.3%)	—	—
Total change	3 (6.4%)	22 (36.1%)	23 (17.8%)	1 (3.8%)	3 (20.0%)
Third week	N = 30	N = 0	N = 95	N = 21	N = 10
Reversal	1 (3.3%)		6 (6.3%)	2 (9.5%)	—
Bi-operculate	1 (3.3%)		3 (3.2%)	—	—
No visible operculum	—		7 (7.4%)	—	—
Other change	—		—	—	—
Total change	2 (6.7%)		16 (16.8%)	2 (9.5%)	—
Fourth week	N = 18		N = 74	N = 16	N = 8
Reversal	—		7 (9.5%)	3 (18.8%)	—
Bi-operculate	1 (5.6%)		2 (2.7%)	1 (6.2%)	1 (12.5%)
No visible operculum	—		2 (2.7%)	—	—
Other change	—		3 (4.1%)	—	—
Total change	1 (5.6%)		14 (18.9%)	4 (25.0%)	1 (12.5%)
Fifth week	N = 7		N = 59	N = 11	N = 4
Reversal	—		7 (11.9%)	3 (27.3%)	—
Bi-operculate	—		1 (1.7%)	1 (9.1%)	—
No visible operculum	—		2 (3.4%)	—	—
Other change	—		—	—	—
Total change	—		10 (16.9%)	4 (36.4%)	—
Sixth week	N = 2		N = 44		N = 1
Reversal	—		2 (4.5%)		—
Bi-operculate	—		—		—
No visible operculum	—		—		—
Other change	—		—		—
Total change	—		2 (4.5%)		—
Sum	N = 171	N = 166	N = 561	N = 120	N = 60
Reversal	4 (2.3%)	14 (8.4%)	61 (10.9%)	15 (12.5%)	4 (6.7%)
Bioperculate	5 (3.0%)	2 (1.2%)	18 (3.2%)	2 (1.7%)	4 (6.7%)
No visible operculum	0	30 (18.1%)	17 (3.0%)	5 (4.2%)	1 (1.7%)
Other change	0	8 (4.8%)	6 (1.1%)	0	0
Total change	9 (5.3%)	54 (32.5%)	102 (18.2%)	22 (18.3%)	9 (15%)

and/or some thoracic segments. Data from this sample are found in Table II, Column 2.

Originally bi-operculate animals did not remain bi-operculate, and either one or both of the functional opercula was rapidly autotomized. Loss of both opercula was not observed in bi-operculate specimens of Group 2, above.

Other animals with missing parts usually regenerated these structures. Severe injury resulted in the subsequent disappearance of the animal.

Opercular modifications in this population were considerably augmented in comparison with the adult group maintained in the laboratory. During each of the week-long periods, more than one-third of the animals varied from their previous state. At the end of the first week, 36.4% of the worms had altered; by the second, a further 39.8% of the unchanged animals had done so. Only 34.8% of the 112 original right- and left-handed animals remained unchanged for the two periods, and only one of the bi-operculate individuals sustained both opercula for more than one week.

Animals with a young functional operculum and at least some of those with no visible operculum were apparently in the process of reversing. If one assumes that all of these animals were doing so, then by the end of the first period, almost 30% (29 of 105) of the surviving normal worms were reversing, and almost 25% (15 of 61) of the asymmetric animals surviving to the second week did likewise. A comparable figure for the laboratory-maintained adults (Group 1) would be approximately 4%.

The incidence of animals with missing branchiae, segments, opercula, or parts thereof presumably reflects the activity of predators. The incidence of predation cannot be exactly determined because the animals which disappeared from their tubes during the course of the experiment may have succumbed to other environmental factors.

Group 4: ($N = 349$) young left- and right-handed animals. Many of these animals died during the early portion of the experiment, but subsequently survival rates were excellent. Each animal was observed for six weeks, or until the termination of the experiment.

The changes observed in these young animals were similar to those found in the comparable older specimens (Group 1); *i.e.*, opercular reversal, doubling, and occasional aberrations such as opercular or branchial loss. Total variation was considerably greater than that in Group 1, but much lower than that of the adults kept in the field (Group 3). The data from the group appear in Table II, Column 3.

Group 5: ($N = 50$) young bi-operculate animals. After one week, 11 of the 12 worms observed had autotomized one of the opercula. Four of these had become left-handed and seven, right-handed. The remaining animal had lost head and thorax and was discarded. One sample of 11 animals was not observed until two weeks after the beginning of the experiment, and of these, five had become left-handed and six, right-handed.

Young bi-operculate specimens appeared to be highly labile, maintaining this state for a short time only. All of the individuals then reverted to an asymmetric opercular pattern, and they did so much more rapidly than the older animals (Group 2). Adult bi-operculate animals in the field (Group 3) however, demonstrated as rapid a turnover as these younger animals.

The tendency of altered animals to repeat these changes was tested by segregating healthy specimens of Groups 4 and 5 into Groups 6 through 9.

Group 6: ($N = 50$) *young asymmetric animals which had reversed.* While seven animals did not undergo any subsequent changes for five consecutive weeks, a few others changed several times. The average rate of change proved to be almost the same as that of the original asymmetric population (Group 4). See Table II, Column 4.

As these animals were not all kept for a full six periods because of the termination date of the experiment in the autumn, the rate of variation given here may be somewhat low.

Group 7: ($N = 30$) *previously bi-operculate young animals, now asymmetric.* The rate of variation in such animals (*i.e.*, becoming bi-operculate or reversing) was similar to the corresponding rates of young asymmetric individuals (Group 4) or those which had previously reversed (Group 6). See Table II, Column 5.

Group 8: ($N = 8$) *young animals without functional opercula.* Four specimens had previously been right-handed, and four, left-handed, but one had been bi-operculate. They eventually regenerated new functional opercula, but half of them did not reverse, and regenerated a new functional operculum on the same side as the old one. No subsequent opercular changes were noted.

Group 9: ($N = 17$) *young animals which became bi-operculate.* Only two of the 14 survivors in this group were still bi-operculate after one week. One of these retained both functional opercula for two, and the other for three weeks before reverting to an asymmetric state. The rapid turnover is quite comparable to that in the animals of Group 5. In every case, the worm retained the younger operculum, resulting in a reversal of the initial asymmetry.

The animals of Group 9 were kept from one to five weeks longer, and during that time none of them demonstrated any further changes. The prior history of these animals is interesting, however. Six of the 17 had been bi-operculate at an earlier time during the experiment; five within the two previous weeks and one, within the five previous weeks.

Comparisons between groups

Groups 1, 3, 4, 6 and 7 (asymmetric animals) were compared with respect to two criteria: the incidence of reversals and the incidence of bi-operculate animals. Chi-square tests between the paired groups indicated that most groups had similar rates of reversal, with the exception of the laboratory-maintained adults, Group 1. The animals in this sample had a significantly lower proportion of individuals undergoing reversal than any of the other groups except Group 7; (for Group 3, $0.025 > P > 0.01$; for Groups 4 and 5, $P < 0.005$). Group 7 did not differ significantly from any of the other groups.

Most groups were approximately equivalent in the rate of production of bi-operculate animals. Less than 7% of the specimens became bi-operculate during any period in any group, and ordinarily the percentage was much lower. The highest production of bi-operculate specimens was found in Group 7, in which the incidence was significantly higher than among field adults of Group 3 ($0.025 > P > 0.01$), but not higher than any of the other groups.

The laboratory-maintained adults, then, did not reverse the positions of the opercula as frequently as did the young animals. Adults in the field, under more

stressful conditions than prevail in the laboratory, tended to lose opercula with high frequency, which suggests that many reversals in nature may occur as a result of trauma, rather than unprovoked autotomy. Young animals had an elevated frequency of opercular loss even in the laboratory, and therefore in the field they might have had a rate comparable to or exceeding that of the field-maintained adults of Group 3.

Opercular flux in juveniles

The deviation of both left- and right-handed juveniles with rudimentary opercula from the normal adult reversal pattern (see above) could be attributed to several things: nutrient deprivation (although there were algal growths and small plankton in the vials), a natural quiescent stage, or an undeveloped reversal mechanism in these young animals. Some insight into this problem is given by the observations described below.

A number of juveniles collected had two functional opercula, and in older samples as many as one-third were bi-operculate, the proportion increasing with augmented size and age of the animals. These bi-operculate juveniles are interesting with regard to the origin of the first mature type of functional operculum, which can be distinguished from a primary functional operculum by the presence of a secondary cup arising from the center of the proximal one. According to Zeleny (1905, 1911) each individual produced only one primary functional operculum. The animals then proceeded, upon the loss of this operculum, to develop a mature functional operculum by growth and differentiation of the rudimentary operculum which lies on the right side. In the present sample, no such regularity was observed in the time of appearance of this mature operculum, as most animals recapitulated the primary-type functional operculum more than once. This apparent randomness was emphasized by the behavior of the bi-operculate animals.

Fourteen bi-operculate juveniles were considered. Six of these had one mature and one primary-type functional operculum; the latter were present on either side. In five other individuals both opercula were of the mature type, and the remaining three had two primary-type functional opercula.

Of the four surviving animals of the first sample, three autotomized the primary-type operculum. Two of these had this operculum on the right; one, on the left. The preferential loss of the primary-type operculum supports the idea that at a particular point in ontogeny the primary operculum is succeeded by a mature operculum. The fact that the primary operculum may be located on either side indicates that prior reversals have occurred in at least some animals. The fourth animal in this group autotomized the mature-type operculum, and was left with the primary one. This suggests that there may be a transitional period during which the mature opercular form is not yet definitively established, and during which it may be supplanted by an immature structure. The animals in the second group were puzzling, as two of the three survivors autotomized the younger, developing mature operculum. Later the older operculum was also lost, to be replaced by a rudimentary operculum. The two surviving animals of the last sample both lost the left primary operculum. It seems that the bi-operculate state is a transient one, but the result is not immediate reversal of the prior opercular positions as in adult animals.

Experiments on pre-opercular animals

The period of experimental observation was two months, or until the death of the specimen. The animals in this group were all at stages prior to the appearance of the opercular cup. Either three or four pairs of branchiae were present. Only long-term survivors are enumerated. In the first group of 10 animals, the entire left or right branchial circlet was extirpated. The branchiae removed were rapidly regenerated and a highly accelerated growth rate soon re-established the parity between the branchiae of the two sides. After one to two weeks the branchiae on the amputated side could not be distinguished in size or development from those on the opposite side. In eight of the ten survivors, no further development occurred within the observation period, but the remaining two developed a single rudimentary operculum. In one animal it appeared on the left; in the other, on the right.

The control animals were left to develop undisturbed. Of the four survivors, two formed an opercular cup within one week; the other two showed no opercular development. The difference between the control animals and the experimental animals indicates that removal of the branchiae retards opercular development even though normal branchial morphology is restored. The formation of primary rudimentary, rather than branchial opercula in two of the experimental animals suggests that these animals associated surgical removal of branchiae with loss of the operculum, and so the normal developmental pattern was disrupted by the trauma. In these cases the branchial opercular stage was by-passed.

Experiments on animals with branchial opercula

Basal excision of the left Branchia II with its associated branchial operculum yielded contradictory results. The development of one animal was so disturbed that only branchial regeneration occurred. A second worm produced two primary-type rudimentary opercula, and a third developed a primary functional operculum. This last response was also typical of two specimens in which the entire left branchial circlet was removed together with the opercular branchia. The operculum was thereafter lost and replaced by a rudimentary one. A mature operculum developed on the right side in one specimen and the last surviving animal in this group regenerated the entire pinnuled branchial operculum.

Zeleny (1911), in somewhat analogous experiments, removed the upper stalk of the opercular branchia, or the opercular cup alone, but did not amputate the entire stalk. Either level of amputation resulted in the regeneration of the opercular cup from the severed end of the branchial filament. A peculiar feature in two of the animals was the appearance of a fleeting pre-opercular swelling on the tip of the right Branchia II, a feature which I found also in two undisturbed pre-opercular animals, but not in any of the experimental animals. In all cases the swelling was soon resorbed. Only one of the animals in the present experiment responded to total extirpation of the opercular branchia by regeneration of the original pinnuled form, in contrast to the results when only the upper half of the stalk is severed (Zeleny, 1911).

The four surviving control animals which were untreated retained their primary functional opercula for several weeks before replacing them with rudimentary ones, so that their development corresponded closely to that of the experimental animals.

Experiments on animals with primary functional opercula on the left

These animals had a primary functional operculum without pinnules on the left and no rudimentary operculum. The right branchial circlet was severed at the base. The three surviving animals reproduced the missing branchiae and could not thereafter be distinguished from the control group.

In a second group of juveniles, the primary functional operculum was removed, with or without concomitant amputation of the left branchial apparatus. The primary functional operculum was replaced by a rudimentary one in four cases, and another primary-type rudimentary operculum formed later on the right side in two of them. The remaining two formed no opercula at all. At the termination of the experiment none of the rudimentary opercula had differentiated into functional ones. Two of the six surviving control animals retained the primary operculum throughout the observation; the other four replaced it with a rudimentary one.

Experiments on animals with both primary functional and rudimentary opercula

Sixteen juveniles had a rudimentary operculum in addition to a primary functional operculum, but the removal of the latter did not induce differentiation in the rudimentary one except in two individuals. In these two the rudimentary operculum enlarged into a mature type of functional operculum, and the animals became right-handed. One other individual formed a second primary-type functional operculum in place of the amputated one, but the remainder of the animals regenerated only rudimentary opercula (10) or no opercula at all (3) on the site of amputation.

Of the five surviving control animals, one retained the primary functional operculum during the entire experimental period. The others autotomized it and developed a rudimentary operculum in its place..

Experiments on animals with functional opercula on the right side

These were among the larger specimens, and they invariably had rudimentary opercula on the left side. Both opercula were of the mature type. When the functional operculum was cut off at the base, in eight of the ten specimens the rudimentary operculum enlarged into a functional one and the amputated side regenerated a rudimentary operculum. In the other two animals there was only replacement of the functional operculum by a rudimentary one. When the entire branchial crown, including the opercula, was removed, the entire appartus regenerated. In two animals the functioned operculum appeared on the left side; the other two produced rudimentary opercula on both sides. Opercular growth was retarded in these animals. Six of the eight surviving control animals lost the functional operculum and replaced it with a rudimentary one; the other remained unchanged. No reversals occurred in this group.

Discussion

Asymmetry of external structure is not confined to Serpulids. In species of Crustacea in which the adults have asymmetric chelae the juveniles sometimes have two equal-sized claws. They are usually both small, as in the lobster, *Homarus*, and in the fiddler crab, *Uca*. In other genera, such as the Alpheids (snapping shrimp), the two chelae differentiate directly into two distinct types. In the lobster and the alpheids, the alteration of one claw into a different form is a normal

ontogenetic step (Herrick, 1895, 1907; Emmel, 1908; Przibram, 1905, 1931). Morgan (1923, 1924), however, felt that the loss of one of the claws of a juvenile fiddler crab *Uca* fixed the symmetry of the animal. Vernberg and Costlow (1966) on the other hand, did not observe any effect of cheliped removal on laboratory-raised male *Uca* juveniles. In species where the asymmetry is determined by hereditary pattern it is relatively easy to account for the distribution of left- and right-handed members of the population. Even in organisms such as *Alpheus*, the pistol shrimp, in which reversal of chela types can be induced, the population balance is hereditary. Approximately half of the larvae will develop directly into left-handed adults and half, into right-handed ones.

In the Serpulid *Hydroides*, however, the position of the functional operculum does not seem to genetically determined. The great majority of specimens in the adult population of *H. dianthus* sampled were asymmetric with regard to the two opercula, although bioperculate animals appeared in every collection. The first operculum to appear in juvenile animals invariably formed on the left (Zeleny, 1905; 1911; Ichikawa and Takagaki, 1942; Wisely, 1958), but both the population distribution of juvenile groups and the observations made of them demonstrated that they made a rapid transition from an entirely left-handed population at the earliest stages of opercular development to an equilibrium population comprising equal numbers of left-handed and right-handed individuals. Zeleny (1905, 1911) and Wisely (1958) found that one reversal occurred early in juvenile life. This eliminated the primary left operculum and, by growth of the right rudimentary operculum, the animals became right-handed. However, they had no information on how this theoretically right-handed juvenile population became transformed into an asymmetric adult one. In the present study it was found that *Hydroides* juveniles have an ontogenetic pattern which appears to be precisely programmed only through the early post-metamorphic stages. A branchial operculum invariably developed on the left side and the juvenile animal became asymmetric for the first time. The development of this operculum at the apex of Branchia II on the left side was followed either by a loss of pinnules from this branchia or, apparently, by the autotomy of the opercular cup from the tip of the branchial stalk. In the first instance, the branchial operculum was converted directly into a primary functional operculum; but in the latter event, this structure had to be developed *de novo* from the dorsal region of the branchial base. Support for this hypothesis was also provided by the observation that juveniles were frequently found with a small differentiating functional operculum on the left side. The branchial operculum persisted for a variable period of time—several days ordinarily and five weeks in the most extreme case. Zeleny (1911) showed that excision of a portion of the branchial operculum can result in regeneration of the absent part rather than in further developmental changes in opercular structure. In the present work juveniles proceeded to later developmental stages after the amputation of the entire branchial operculum.

Prior to the formation of the primary operculum the juvenile may follow either of the two above pathways, but there were a number of possibilities thereafter. The irregularity of opercular development after the loss of the primary functional operculum implied that environmental circumstances are involved with later opercular events, as is true in adult animals. Ludwig and Ludwig (1954) found that juveniles of *H. norvegicus*, if undisturbed, followed the pattern described by Zeleny (1905, 1911) for specimens of *H. dianthus*. However, if the primary

operculum were amputated, another primary-type operculum would develop on the opposite side. Repeated ablation of these opercula during a period of time equivalent to the normal life-span of the primary operculum (approximately 6 months, according to Zeleny, 1905) resulted in continual reversals involving only primary-type opercula, but at the end of this period a mature functional operculum was formed (Ludwig and Ludwig, 1954). The present work indicates that such repetitive development of a primary-type operculum also occurs frequently under normal conditions in *H. dianthus* juveniles. Rarely was the initial operculum the only primary functional operculum which the juvenile produced during the course of its maturation, as Zeleny (1905, 1911) supposed. In the animals he observed, the primary operculum was supplanted by a mature functional operculum which developed from the rudimentary operculum present on the right side. This pattern cannot explain the appearance of primary-type functional opercula on the right side or even on both sides, as in some bi-operculate juvenile animals. An individual often lost and regenerated a primary type functional operculum one or more times, frequently on the same side. In contrast to Ludwig and Ludwig's (1954) results I found that extirpation of the primary operculum did not lead to its regeneration, but instead, to a static condition with one or two rudimentary opercula, or with no opercula at all. It therefore appeared that in specimens of the Woods Hole region there is no fixed "life-span" for the primary functional operculum. After some time, one of the rudimentary opercula developed into a mature functional operculum and often no more primary type functional opercula were formed in later reversals. There were enough exceptions to the above order of events to indicate the existence of an indeterminate stage in opercular development. During this flexible period the animal might develop, interchangeably, either primary or mature-type opercula. The mechanism for the final maturation or determination of the adult opercular type is unknown. It is not, apparently, rigidly time-determined but it does occur by the time an animal approximates a certain size (2–3 mm) and when the branchiae have become pigmented. By then the capacity of the branchial base tissue to form a single-cupped operculum has been lost.

Most heterochelous crustaceans differ from these polychaetes in that asymmetry is essentially fixed before the attainment of maturity and there is no natural oscillation between asymmetric chelae. The ability to autotomize an operculum or claw under duress and to regenerate it is a useful adaptive mechanism, but the crustacean claw is slowly regenerated. To attain full size it is necessary for several molts to intervene and meanwhile the animal is at a competitive disadvantage. In *Hydroides* adults a missing operculum is readily replaced by the growth of the rudimentary operculum which becomes a functional defensive device within a few days. In juveniles, however, there is little such regularity. In only 12.5% of these animals did the rudimentary operculum enlarge in response to the excision of the primary one. In slightly older animals, such as those with a mature operculum on the right side, 80% reversed under these conditions. These results demonstrate a correlation between the ability of the young animal to reverse and the establishment of a mature functional operculum. Apparently the development both of the mature operculum and of the opercular response system are dependent upon the differentiation and maturation of the juvenile animal. It may be that tissue response is deficient at early ages, or perhaps both processes are dependent upon the maturation of some extra-opercular control system (nervous, hormonal, etc.).

Observations made on specimens of *Hydroides dianthus* of various ages showed that physiological transposition of opercula is a normal pattern in the animals' lives. The result was a series of opercular "oscillations" although no definite periodicity was detected. As soon as a juvenile animal had a rudimentary operculum as well as a functional one, it was theoretically possible for a natural reversal to occur. This did happen, particularly among the older specimens of the juvenile population. Alternatively, at these early stages, the functional operculum (especially if it were a primary-type one) might simply be reproduced on the same side as the autotomized one without any concomitant differentiation of the rudimentary operculum.

There were two patterns by which reversal could be accomplished, in adults or in juveniles. The functional operculum could be cast off first, followed by the differentiation and growth of the rudimentary one. As an alternative mechanism, the rudimentary operculum developed into a functional one even though the old functional operculum was still intact. The older operculum was thereafter autotomized and supplanted by a rudimentary one. The two patterns differ primarily in the timing of the autotomy of the original functional operculum. Bi-operculate animals thus are not aberrent or atavistic specimens, nor do they represent a third stable opercular form. As juveniles became older, right-handed and bi-operculate individuals appeared concomitantly. By the time the animals were 2 to 3 mm long they had established a 1 : 1 left-right opercular ratio, but there were elevated proportions of bi-operculate specimens. Paired functional opercula occur when by some means the rudimentary operculum overcomes the "inhibition" of the functional operculum and becomes capable of enlargement and development. The two opercula are unable to co-exist for long in this balanced state, and one of them, generally the older, is autotomized and replaced by a rudimentary operculum. Therefore bi-operculate animals can be regarded as indicators of reversal. The elevated frequency of bi-operculate animals in the young sample paralleled the enhanced reversal rate found in this group whereas the adult samples showed both fewer bi-operculate members and a lower reversal rate.

There was a slight tendency in young animals to autotomize the functional operculum and to replace it with a new functional operculum on the same side, although this was much less marked than in juvenile specimens. In older animals loss of an operculum was almost always followed by reversal. However, as the worm aged, the tendency to reversal became increasingly retarded, which might be a concomitant of a general metabolic slowdown. The rudimentary operculum enlarged more slowly, and if the animal became bi-operculate both opercula could be maintained for several weeks. A similar phenomenon was found in such species as *Alpheus*, in which the rate of claw inversion and the ability to reverse are inversely proportional to the size (*i.e.*, age) of the animal (Przibram, 1908, 1931; Huxley, 1932). In older animals transposition was virtually precluded (Huxley, 1932). This is related to alterations in the growth rates of the heterogonic chelae; the relative rates of growth have changed so that reversal can no longer be induced (Dawes, 1934; Huxley, 1932). In *Hydroides*, in contrast, natural inversion continues throughout life, albeit at a decreasing frequency. In *Hydroides* juveniles the functional operculum is enormously exaggerated with respect to the tiny body, so the operculum must, like the cheliped, slow its initial growth rate. As the animal elongates the operculum becomes increasingly shorter in proportion to body length. Whether negative allometry is intimately related to the phenomena

controlling reversal or whether they are merely congruent phenomena has not been determined.

How can opercular reversal be accounted for? Opercular aging is one postulate to explain the physiological reversals found in specimens of *Hydroides*. Ichikawa and Takagaki (1942) proposed that there might be an aging of the functional operculum since they found that in bi-operculate individuals one of the opercula always appeared older and less active than the other. Wrinkled, necrotic opercula were occasionally observed in both normal and bi-operculate adult individuals in the present population as well. W. Ludwig (1941) and Ludwig and Ludwig (1954) claimed they could distinguish aging opercula by vital staining and thus predict reversal events. Aging, they postulated, was correlated with the decline in production of some specific chemical agent which inhibits the rudimentary operculum and maintains the vitality of the functional one.

Opercula of dissimilar ages may respond differently to experimental manipulation, also suggesting a natural cycle for the operculum. Abeloos (1952b) noted that amputation of an old functional operculum resulted in a conventional reversal, but that when the same operation was performed on a newly-developed one, it was replaced by another functional one on the same side. This phenomenon has also been noted in young animals and juveniles (see above) and it may be characteristic of organisms and structures in a still-differentiating state. Ludwig and Ludwig (1954) removed the upper cup of one functional operculum in bi-operculate animals and found that if the functional operculum were the older structure it was autotomized; if younger, it remained until it became senescent. If both cups were removed the older of the two opercula was autotomized and replaced by a new one, whereupon the second of the two opercula dropped off.

Arguing against a regular aging process as a determinant of natural reversal are the comparisons of reversal rates in the various groups kept in the laboratory. These indicated that opercular changes were random, in that animals which had recently reversed did not subsequently show enhanced or depressed rates of reversal. Each event was apparently independent. The substantially enhanced rates of change in field-maintained animals demonstrated that many alterations can be accredited to predation and other environmental factors. The major predators of *Hydroides* are probably sea urchins, crabs and other small Crustacea, and various molluscs. Branchial and opercular cup losses noted in the field group were obviously traumatic events and not degenerative ones, as the animals rapidly regenerated new branchiae and opercula. Alterations in most of this population were therefore not attributable to abnormalities, although there might have been greater physiological activity in the sea than in sub-optimal laboratory conditions. In addition, laboratory animals could have been hindered by removal from the tube.

Once a left-right equilibrium of opercula had been established in the *Hydroides* population, it was maintained in a dynamic state by a combination of physiologically-controlled oscillation and environmental stress. Both seemed requisite to account for the differences in reversal rate between the laboratory population and those animals kept in a natural situation. In the field experimens, the loss of opercula to predators was superimposed on the intrinsic reversal pattern of the individuals in the population to create a high rate of flux. At present it is difficult to determine the intrinsic factors which promote reversal, and discussion of chemical agents is purely speculative.

I wish to thank my advisor, Professor Richard J. Goss, for his valuable guidance during the course of this project, and in addition to acknowledge the advice of Dr. Louis Homer on the statistical handling of the data.

Summary

Although any population of *Hydroides dianthus* maintains an equilibrium of equal proportions of right- and left-handed animals, individual members are continually modifying their condition. They may spontaneously autotomize the functional operculum, permitting the rudimentary one to enlarge. Alternatively, the rudimentary operculum may develop independently to yield an unstable situation in which the animal bears two large opercula. This may persist for several weeks; then the older of the two opercula autotomizes. Either mechanism results in a reversal of the original opercular asymmetry. The rate of variation in left- and right-handed individuals is equal, so that population balance does not shift even though its component organisms undergo continual flux.

Each animal undergoes several reversals during a normal life span, beginning with the larval stages. The rate of change declines gradually so that the older the animals, the less frequently it will reverse. Animals maintained in the ocean subject to predators show a high rate of reversal and traumatic aberrations.

In larval ontogeny the origin of an opercular cup on the second left branchial rudiment is followed by (a) loss of pinnules on the opercular stalk to form the primary operculum or (b) severance of the opercular cup from the branchia, perhaps by progressive attenuation of the terminal end of the filament. In the latter case, a primary operculum must be developed on the left. This seems to be accomplished by the formation of a left rudimentary operculum, developing later into a primary one. This operculum, no matter how it originates, may either be lost and replaced by a similar structure, or reversal may occur. These processes may be repeated until this simple operculum is permanently replaced by an adult double-cupped operculum. The time at which this occurs is not fixed, and there is a transitional period during which either type of operculum can appear. After further maturation, only the adult type of functional operculum can be formed. This coincides with the development of the ability to carry out opercular regulation.

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CHEMICAL CONTROL OF THE EVISCERATION PROCESS IN *THYONE BRIAREUS* [1]

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Evisceration, in Holothuroidea, is a complex form of autotomy, involving expulsion of the viscera through a rupture in the body wall. It occurs in several species of sea cucumbers in response to a variety of noxious stimuli. Among more or less natural stimuli, fouled aquaria, high temperatures, and rough treatment are effective. Evisceration can be produced effectively in the laboratory by injection of strychnine or methylene blue (Pearse, 1909), injection of distilled water (Domantay, 1931; Dawbin, 1949), electrical stimulation, or immersion in a dilute solution of NH_4OH in sea water (Kille, 1931, 1935). In *Thyone briareus*, evisceration occurs through the anterior end and results in the loss of the aquapharyngeal bulb (or lantern) and its associated oral structures, as well as the viscera (Pearse, 1909; Scott, 1914; Kille, 1935). Generally, the lantern, the gut, and possibly the gonad are discarded together, neatly packaged in the weakened and stretched introvert portion of the body wall (Kille, 1935). For this loss to occur, the introvert must be ruptured, and the viscera and lantern freed from the internal support of mesenteries and retractor muscles.

The lantern is firmly attached to the body wall by the five pharyngeal retractor muscles. The anterior end of each muscle inserts on the anterior portion of one of the calcareous plates composing the lantern; the posterior end inserts at a distinct point on the superior surface of the longitudinal body wall muscle (LBWM). During evisceration, each lantern retractor muscle undergoes autotomy at its posterior terminus—the junction with the LBWM (Kille, 1935). Although the site of muscle breakage never changes, the timing of autotomy in the sequence of events comprising evisceration varies. All of the muscles may break even before swelling of the introvert begins, or they may break only after the process of evisceration is nearly complete (Kille, 1935). In one animal observed by Kille, two muscles were still attached after the initial swelling of the introvert, while the three other muscles were already autotomized. Thus, while the rupture of the introvert and autotomy of the PRMs are mandatory events in evisceration, they are partially independent.

In this report, the mechanical stresses involved in muscle autotomy are determined. Then, isolated pharyngeal retractor muscles are used to test for endogenous,

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autotomy inducing factors released by the evisceration stimulus. A preliminary report describing the assay system and the initial demonstration of such an endogenous factor was presented to the American Society of Zoologists (Smith, Greenberg, and Hill, 1971). We now describe the source and isolation of an endogenous factor capable of initiating the sequence of events leading to autotomy and evisceration.

Materials and Methods

Animals

Specimens of *Thyone briareus* were obtained from the supply department of the Marine Biological Laboratory at Woods Hole, Massachusetts. For our experiments at the Marine Biological Laboratory, the animals were maintained in trays of running sea water. In Toronto, specimens of *Thyone* were maintained in 25 gallon Instant Ocean culture systems at 16° C. Animals were always used within two weeks of delivery.

Medium sized specimens of *Mercenaria mercenaria*, also obtained from Woods Hole, were used for the clam heart bio-assay.

Anesthesia

Animals dissected without anesthetic always eviscerate. Even though the coelomic pressure necessary to complete evisceration is relieved by an incision through the body wall, the introvert portion of the wall loses tensile strength and becomes a soft gel (G. N. Smith, Jr., unpublished data). Moreover, the pharyngeal retractor muscles autotomize when such a cut is made. Fortunately, all the events of evisceration may be completely blocked by an appropriate anesthetic.

The anesthetic of choice is a 0.1% solution of chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol; "Chloretone") in sea water. *Thyone* can be safely dissected after an immersion of only 7–8 min in this solution. Sea water, saturated with propylene phenoxytol (Hill, 1966), is also effective. Treatment with this substance for 15 to 20 min produces adequate anesthesia, and animals recover after immersion for as long as an hour. Isotonic (0.5 M) $MgSO_4$ is an effective relaxant, but we no longer use it. This anesthetic is difficult to wash out. Furthermore, both the responsiveness of the muscles, and the condition of the connective tissue structures, may be modified by long exposure (30 min) to Mg^{++} ion.

In any case, exposure to the anesthetic should be as brief as possible to reduce the washout time and to ensure recovery of full muscle reactivity. Moreover, since effective exposure times are highly variable, the experimenter must follow the state of relaxation by palpating the animal with his fingertips. If an unrelaxed animal is gently pressed it will respond by contracting and stiffening to the extent that the calcareous lantern can no longer be felt through the body wall. A *Thyone* is relaxed enough for dissection when two successive attempts to feel the lantern through the body wall are successful.

Preparation of everted animals and of isolated pharyngeal retractor muscles

Thyone can be everted if it is first relaxed with a suitable anesthetic. The operation is performed by cutting off the cloacal end of the body, and pushing the lantern and viscera through the resulting opening with a finger or a glass rod.

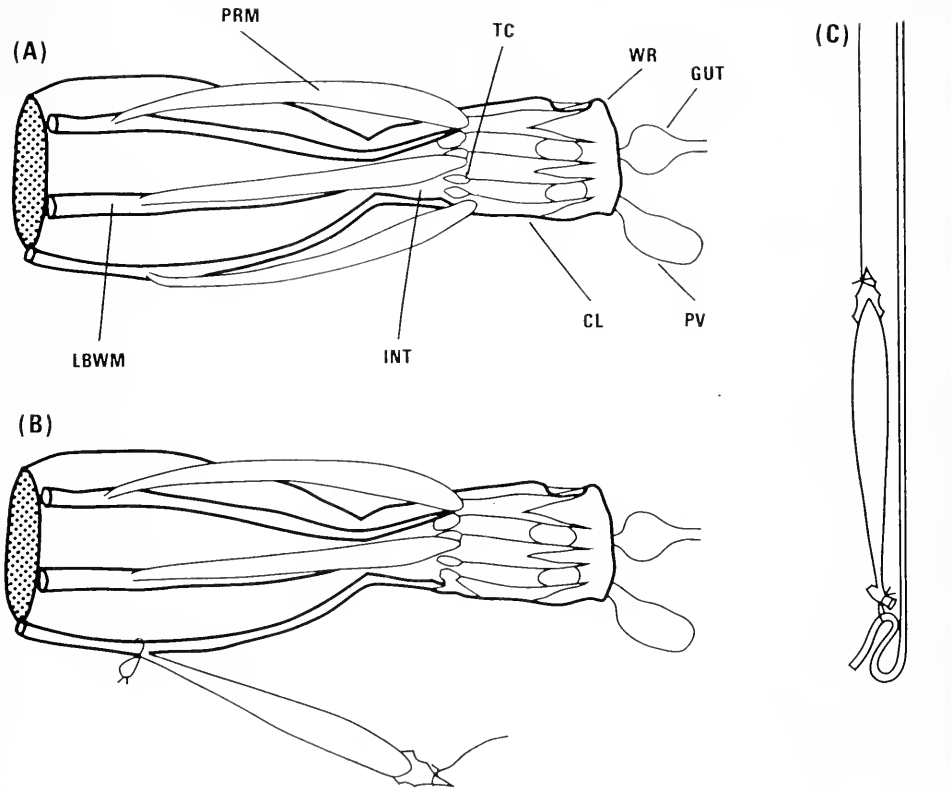

FIGURE 1. Preparation of isolated pharyngeal retractor muscles (PRM) from everted *Thyone;* (a) The relationship between the PRM and the longitudinal body wall muscle (LBWM). Note the calcareous lantern (CL), and the relationship between the tentacular canals (TC) and the anterior insertion of the PRM. Other abbreviations are PV, polian vesicle; WR, water ring; INT, introvert. (b) The anterior end of the PRM is freed by inserting a scissors in the tentacular canals and cutting a pie-shaped piece from the ossicle. Threads are tied near each attachment of the PRM: one on the ossicle and one on the LBWM; (c) The isolated PRM, tied to a stainless steel hook, and immersed in the testing bath (not shown). The upper thread is attached to a force-displacement transducer.

After the bulk of the viscera have been removed, the lantern, retractor muscles, and the body wall are washed with sea water. After sufficient washing, the animal will recover without undergoing either autotomy of the pharyngeal retractor muscles (PRMs) or softening of the introvert wall.

If the isolated PRMs are to be prepared, the freshly everted animal is washed with anesthetic solution instead of sea water. The lantern attachment of the PRM can then be freed by inserting the sharp point of a fine scissors in the exposed tentacular canal on either side of the anterior insertion of the muscle and cutting a wedge shaped piece from the calcareous plate of the lantern. Once the piece of lantern has been cut away from the introvert, the anterior end of the PRM is detached (Fig. 1b). A thread is attached to the lantern fragment.

Next, a fine forceps is forced under the longitudinal body wall muscle (LBWM) posterior to its junction with the PRM. A thread is pulled under the LBWM, and tied firmly around the muscle. A small loop is tied in this thread to facilitate attachment of the muscle to a hook in the bottom of a muscle bath (Fig. 1b). The junction of the PRM and the LBWM is freed by carefully cutting through the LBWM anterior to the junction, then cutting the LBWM away from the body wall with fine scissors, and finally cutting through the LBWM again, posterior to the thread. Now, the muscle is free, and both insertions are intact and undamaged. The entire preparation can be suspended between a stainless steel hook and a force-displacement transducer in an aerated, temperature-regulated organ bath. The bath fluid used was either natural sea water (at Woods Hole) or artificial sea water (Harvey, 1945).

Many PRMs are subdivided into 2–8 distinct bundles posterior to their anterior insertion on the lantern (see also Pople and Ewer, 1954; *Cucumaria sykion*). An animal with multiple bundles in one PRM usually has the same number of bundles in all of its five muscles. Although each bundle has its own insertion on the longitudinal body wall muscle, the individual junctions are tightly grouped. PRMs with multiple bundles were dissected and treated as single muscles.

Bioassay of acetylcholine

Isolated ventricles of *Mercenaria mercenaria* were used to assay acetylcholine in tissue extracts. The classic preparation of Welsh and Taub (1948) was employed. Benzoquinonium chloride (Mytolon) (Sterling-Winthrop) was used as a specific antagonist of the acetylcholine response.

Isolated PRMs were also used as ACh assay objects. The effect of ACh—a contracture—develops rapidly, is graded according to dose, and can be washed out quickly at any time during the response. Threshold is about 10^{-9} M, without eserine treatment, and the responses are repeatable over a long period of time.

Preparation of extracts

Hot distilled water was added to tissue in a glass homogenizer (1 ml per gram fresh tissue; 1 ml per 0.01 gram lyophilized tissue) and the mixture heated in a boiling water bath for three minutes. After vigorous homogenization with a motor driven Teflon pestle, the extract was heated for three minutes more.

The homogenized, boiled samples were cooled and centrifuged at 4° C, for 20 minutes at 27,000 × *g* (Sorval Model RC-2B). The supernatant fluid was retained. Those extracts not assayed immediately were frozen and stored at −20° C. Preparations stored 3 months were still active. Some extracts were lyophilized to concentrate the activity.

Gel filtration

Active extracts were fractionated by column chromatography (Sephadex G-15) with a 0.025 M phosphate buffer (pH 7.0) as the elution fluid. The separation was carried out in two steps. The initial fractionation was on a short column (2.6 cm × 30 cm). The resulting active fractions were combined, lyophilized, and rechromatographed on a long column (1.6 cm × 88 cm). All chromatography was carried out in a cold room at 4° C. The volume of sample applied was 1.5 ml on the short column, and 0.5 ml on the long column.

Five milliliter fractions were collected automatically and assayed on the PRM and the clam heart. The buffer affected neither assay object.

Recording

Responses of the clam heart and the PRM to contracture inducing agents were recorded on either a Grass polygraph (Grass Instrument) or a Physiograph Four (Narco BioSystems). The Grass recorder produces curvilinear recordings; the Physiograph, rectilinear recordings. Either system gave easily recognizable, unique response patterns for the agents assayed.

Results

The stimulus for evisceration and autotomy in normal and everted animals

Evisceration of intact *Thyone* could be induced by either of the two classical techniques of Kille (1935): immersion in ammonia water (1 ml of 7 N NH_4OH in 800 ml of sea water) or electrical stimulation. Electrical stimuli (50 V; 6 msec; 4/sec) were delivered for 30–60 sec to the external surface of the body with a square wave stimulator (Grass Model SD9) equipped with Ag-AgCl electrodes. Some variation in threshold was seen, but long duration and low frequency were important characteristics of successful stimuli. If, when electrical stimulation failed, it was followed immediately by gentle manipulation, evisceration rapidly ensued.

On the hypothesis that the mechanism of electrically induced evisceration is massive stimulation of the nervous system, KCl was introduced into the coelom as a possible evisceration stimulus. In fact, intraperitoneal injection of 0.5–2 ml of isotonic (0.54 M) KCl solution always produced rapid evisceration, followed by quick recovery and apparently normal regeneration of the treated animals.

If evisceration is caused by nervous stimulation, then anesthetics should block the response. Accordingly Mg^{++} ion, chlorobutanol and propylene phenoxytol were tested. All three substances were effective antagonists of evisceration either by electrical stimulation or by KCl injection.

Everted animals, stimulated either electrically or with KCl, demonstrate the evisceration response (G. N. Smith, Jr., unpublished data). Not only introvert softening, but also PRM autotomy, can be observed more clearly in such preparations. To test the specificity of KCl as an evisceration stimulus, solutions of common sea water ions, distilled water, and acetylcholine were tested. The ionic solutions, KCl, NaCl, $MgSO_4$, $CaCl_2$, NH_4Cl, and Ca^{++}, Mg^{++}— free sea water, were isosomotic with sea water. One tenth of the animal's volume was injected into intact *Thyone,* while 1/10 of the total volume was added to the bathing fluid containing everted preparations.

Of all the solutions tested, only KCl was effective as a stimulus of both intact and everted sea cucumbers. Ammonia water seldom elicits evisceration when applied intraperitoneally, and has no action when applied directly to everted preparations. Thus, ammonia applied to the external surface may act by stimulating cutaneous sensory structures. Distilled water, an occasionally effective eviscerant when injected, presumably acts by osmotically stimulating nervous elements.

Acetylcholine, whether injected or directly applied, causes strong contractions of the body wall musculature, but never evisceration. Doses as high as 10^{-4} M were tested.

The site of PRM autotomy in normal and everted animals

During evisceration, autotomy always occurs at the junction of the pharyngeal retractor muscle (PRM) with the longitudinal body wall muscle (LBWM). Careful examination of twelve animals eviscerated by immersion in ammonia water showed 60 clean breaks; all were at the junctions. During the course of this study, and others, hundreds of animals have been stimulated to evisceration by electrical shock, distilled water injection, rough treatment, polluted aquaria, immersion in dilute NH_4OH, and KCl injection. In no case did breakage ever occur at any site but the junction.

Since only the weight of the lantern opposes PRM shortening in everted preparations, the break at the junction must occur at very low tensions. However, in spite of these low break tensions, and although the junction appears to be a predetermined fracture plane, it is not a site of mechanical weakness. Three relaxed animals were everted, but the $MgSO_4$ solution was not washed out. The anterior end of the passive muscles, together with their attached ossicles, were freed and pulled by hand until they broke. The 15 muscles treated in this manner never broke at the junctional fracture plane. Thirteen tore near the mid-point of the muscle, and two failed at their insertions on the calcareous plate.

Autotomy in isolated retractor muscles

An isolated PRM, exposed to 100 mM KCl in the bathing solution, contracts and then autotomizes (Fig. 2). A similar response may be obtained by electrical stimulation. Autotomy can be blocked by anesthetising the muscle before stimulating it. The break always occurs at the junction of the PRM with the LBWM (*e.g.*, Table I).

Acetylcholine, in doses as high as 10^{-4} M in the bath, contracts the muscle but does not produce autotomy. Two such PRMs remained in ACh contracture for 6 hr 28 min, and 7 hr 43 min respectively; finally they tore, but in the middle of the muscle.

Therefore, insofar as they respond to the same stimuli, the autotomy of isolated PRMs is analogous to that in whole or everted *Thyone*.

Comparison of passive and autotomy breakage tensions

Breakage tension, during both passive stretch and autotomy, was also measured on unanesthetized, responsive, isolated retractor muscles.

Passive tension was developed by a spring loaded device which broke the muscle by stretching it 5 cm in 0.5 sec. Fifteen muscles (including those in Table I) were passively stretched. The mean breakage tension was 110.0 ± 17.8 grams ($\pm$ standard deviation). Tension was approximately proportional to muscle size. All but three of the breaks were at sites other than junctions.

Autotomy breakage tension was measured by allowing KCl treated muscles to contract isometrically until they failed. In each of four animals, one pair of muscles was treated with KCl, while two others were passively stretched. The resulting breakage tensions and sites of breakage, for each muscle, are displayed in Table I. The bundles comprising the PRM usually break in concert. In some instances, however, bundles broke separately (Table I, muscle 1E; Figs. 2 and 7).

TABLE I

Breakage tensions of pharyngeal retractor muscles during KCl-induced autotomy and under passive stretch

Animal	Muscle*	Weight of muscle (mg)	No. of bundles	Treatment	Breakage tension (g)	Breakage site
1	C	57	2	KCl	8	Junction
	A	46	2	KCl	3.5	Junction
	E	60	2	Stretch	90†	PRM
					35	Junction
	B	58	2	Stretch	100	PRM
2	B	15	1	KCl	<0.1	Junction
	A	25	1	KCl	<0.1	Junction
	E	24	1	Stretch	30	Junction
	C	29	1	Stretch	50	Ossicle
3	B	34	3	KCl	2.75	Junction
	D	53.4	3	KCl	5	Junction
	A	24.4	3	Stretch	180	Ossicle
	E	31	2	Stretch	300	PRM
4	B	56	3	KCl	4	Junction
	A	37.9	3	KCl	5.5	Junction
	D	62.1	3	Stretch	100	LBWM
	E	73.7	2	Stretch	120	Ossicle

* Muscle nomenclature after Carpenter, 1884 (in Hyman, 1955).
† Two bundles broke separately.

Ten muscles treated with KCl (including the pairs in Table I) developed tensions isometrically and broke in 6–10 sec. The mean breakage tension of autotomy was 3.9 ± 1.04 grams. However, muscles contracted by KCl actually lose tension before breakage occurs. The breakage shown in Figure 2, at high chart speed, would be recorded as having occurred at 5 g tension, although the muscle actually tore at about 1 g. Therefore, the measurements used to calculate the mean breaking tension (*e.g.*, Table I) tend to be high.

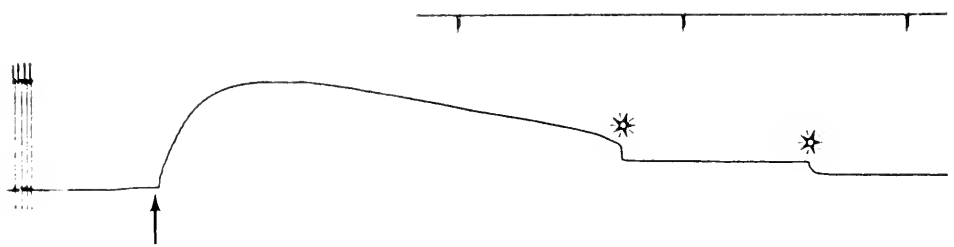

FIGURE 2. KCl induced autotomy of an isolated two-stranded pharyngeal retractor muscle of *Thyone*. KCl concentration of the bath increased to 100 mM, at the arrow. Stars indicate autotomy of the individuals muscle bundles; tension calibration: 5 g; time mark: 5 sec; rectilinear recording.

Muscles treated with KCl also break under isotonic conditions. PRMs loaded with 250–750 mg tear in 60–180 sec.

The evisceration factor

As the introvert of an eviscerating *Thyone* ruptures, quantities of coelomic fluids are expelled. A sufficiently large amount of this material, injected into a whole animal, can stimulate evisceration of the recipient. In contrast, the coelomic fluid of normal, intact *Thyone* never evokes the evisceration response. These results suggest that an endogenous evisceration factor (EF) is present in the coelomic fluid of eviscerating animals.

Coelomic fluid from eviscerating animals causes slow, rhythmical contractions in isolated PRMs; if the EF activity of the fluid is sufficiently high, autotomy occurs. The contraction produced by EF-containing fluid develops slowly, in contrast with ACh contractures (Fig. 3). Furthermore, once maximum tension has developed,

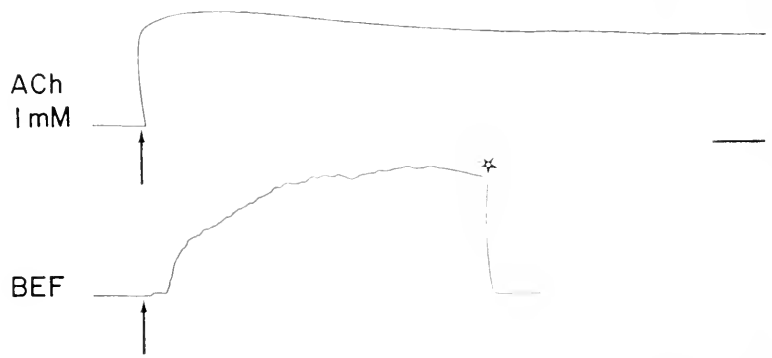

FIGURE 3. Responses of isolated pharyngeal retractor muscles of *Thyone;* (a) Effect of acetylcholine (ACh); (b) Effect of boiled evisceration factor (BEF). Substances were added at the arrows. Star indicates autotomy of the muscle, time mark: 10 sec; curvilinear recording.

the muscle relaxes along a time course similar to its contraction. The EF induced contraction also differs from that of ACh in its washout characteristics. ACh washes out much more rapidly than EF. Furthermore, if EF is washed during contraction, the contraction continues about to the level which would have been reached in the continued presence of EF. If the bath fluid is not changed, the muscle will enter successive cycles of contraction and relaxation, and will continue to show this slow, rhythmic activity until autotomy supervenes. The moment of autotomy for an isolated PRM depends on the EF activity administered. High concentrations of the factor produce breakage during the first contraction (Fig. 3). Less active preparations induce autotomy later in the series of contractions. If very low concentrations are given, autotomy may not occur at all. One preparation underwent 22 contractions over a period of 8 hr 9 min without breaking.

Autotomy need not occur at the maximum tension developed by the muscle. For example, since the waves of contraction diminish, with time, in both amplitude and duration, breakage may occur at a tension less than the maximum developed during the previous cycle. Furthermore, autotomy may occur on the relaxation as well as on the contraction phase of a cycle.

Autotomy inducing activity can be detected in the coelomic fluid extruded into the swollen introvert immediately on evisceration, but much higher activities develop if the eviscerated organs are allowed to steep in the fluid. Thirty minutes after evisceration, activity in the coelomic fluid is always high. If, however, active coelomic fluid, with the viscera removed, is allowed to stand at room temperature, the activity decays. This decay is not prevented by centrifugation at $27{,}000 \times g$ to remove coelomocytes, but is prevented by heating the coelomic fluid in a boiling water bath for a minimum of three minutes. Longer boiling (10 to 15 min) diminishes activity.

The relative heat stability of the activity in coelomic fluid suggests that the active substance is a small molecule. Dialysis of 25 ml of active coelomic fluid against 100 ml of distilled water at 4° C for 24 hours resulted in activity in both compartments. Further dialysis against 6 liters of distilled water completely eliminated the activity of the material in the dialysis bag. A sample kept in the cold room as a control and tested before and after the dialysis period retained its activity. Lyophilized material from the dialysate was tested in high concentration (*i.e.,* 50 ml of external dialysis medium were lyophilized and redissolved in 5 ml) and caused slow contraction and autotomy of isolated PRM.

These results suggest that evisceration activity is produced by one or more small molecules, and that these are inactivated by a soluble enzyme system.

Assay of the evisceration factor

The evisceration of whole *Thyone* is an ungraded, all-or-none phenomenon. Thus, many animals would be required to assay the activity of even one tissue extract. On the other hand, the effects of evisceration factor on PRMs are graded. Furthermore, one cucumber could provide up to 5 assay objects. Therefore, these muscles were conveniently used to assay the EF activity in the coelomic fluid of eviscerated animals and in tissue extracts.

Since autotomy of the muscle would terminate its usefulness, test solutions were sufficiently diluted so that they would not induce breakage on the first contraction. Furthermore, the muscles were washed out either before, or just at, the peak of the initial contraction. Thus, only the effectiveness of a particular extract as a contracture agent was measured.

In developing the PRM assay, we took advantage of two dose-dependent characteristics of the EF effect. First, the rate of contraction increases with activity; secondly, the delay between the administration of an active extract and the onset of contraction decreases as EF concentration increases. Some muscles may show only the rate or only the delay dose dependency (compare Figs. 4a and 4b).

The quantitative effect of the same dose of active material on the same muscle are not precisely repeatable. In view of this lack of precision, a set of responses of half-log-unit dilutions of haemal system extract was taken as the quantal responses of each assay muscle (Fig. 4). The effects of all other extracts on that muscle, were compared with these quantal responses. By means, the values shown in Table II were determined.

The source of the evisceration factor

Since activity develops slowly in evisceration fluid only if fragments of the viscera are present, extracts of ejected organs were tested for EF activity.

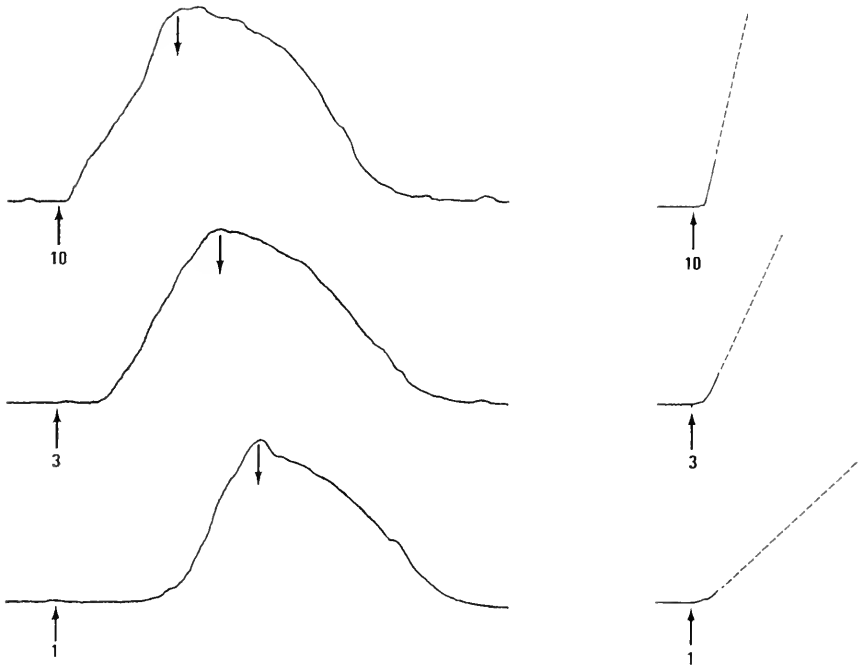

FIGURE 4. Standardization of evisceration factor bioassay on two isolated pharyngeal retractor muscles (PRM) of *Thyone*. Quantal responses are related to half-log-unit dilutions of haemal system extracts; quantal response of undiluted extract defined as 10. Doses (0.4 ml) administered to both PRMs: top records—undiluted haemal system extract (HS); middle records—3-fold dilution of HS (HS/3); bottom records—10-fold dilution of HS (HS/10). Note two types of responses: (A) delay between dose and contraction increases as dose decreases; (B) rate of tension development increases with dose. Extract added to the bath at upward-pointing arrows; washout at downward-pointing arrows; rectilinear recordings.

TABLE II

Relative activity of evisceration factor (EF), and concentration of acetylcholine (ACh), in tissue extracts from uneviscerated Thyone

Tissue	EF (relative activity*)	ACh (moles/liter)
Haemal system	10	10^{-12}–10^{-10}
Respiratory tree	1	10^{-9} –10^{-8}
Mesentery	1	10^{-9} –10^{-8}
Gut	0.3	10^{-9} –10^{-8}
Gonad	0.01	10^{-12}–10^{-10}
PRM	0.01	10^{-8} –10^{-7}

* Undiluted haemal system extract (10 mg/ml) is the standard preparation with a defined relative activity: 10. The relative activities of other undiluted extracts are given by the amount of haemal system extract necessary to match their effects. Thus, the activity of 10 mg respiratory tree equals that of 1 mg haemal system extract (see Fig. 4).

Ejected viscera were divided, immediately after evisceration, into four components: introvert, "lantern," "gut," and gonad. "Lantern" included both the PRM and the tentacles; "gut" included both mesentery and haemal system. These tissues were allowed to stand in 10 ml of sea water, at room temperature, for 30 minutes and then centrifuged. The supernatants were assayed but activity was detected only in the fluid in which gut was steeped. Extracts of the pellets were made in boiling sea water. Again, the "gut" extract was active, although low EF activity also appeared in the gonad pellet. ACh was detected in all tissues. Thus, of the ejected viscera, gut and its associated structures was the major source of extractable EF activity.

We next determined the distribution of the active component in intact organisms. Tissues from relaxed *Thyone* were prepared: extracts of gut, including mesentery and haemal system; isolated mesentery; isolated haemal system; respiratory trees; gonads; and pharyngeal retractor muscles were assayed on the PRM. Haemal system was the most active tissue (Table II) with respiratory tree, mesentery, and gut, showing mixed ACh and EF responses (Fig. 5). Haemal sys-

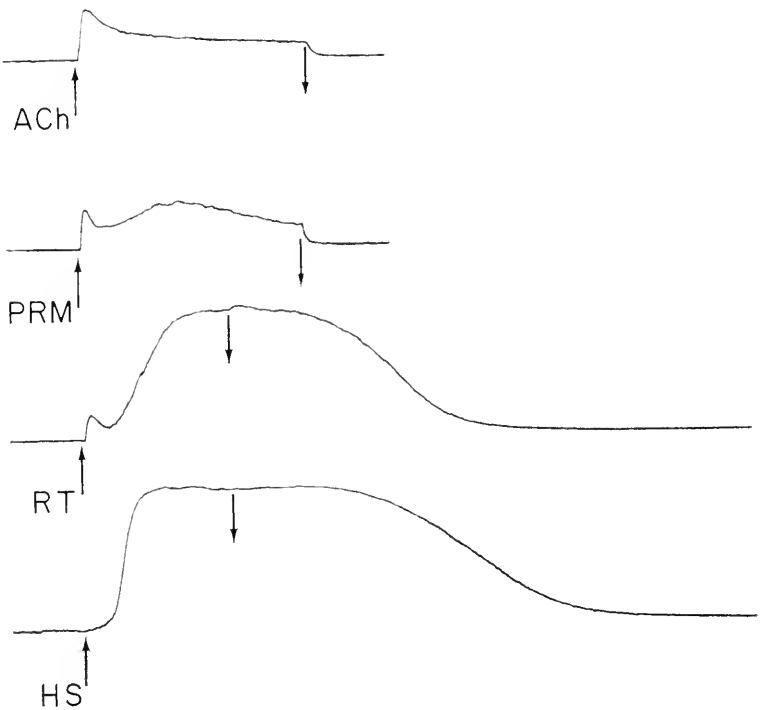

FIGURE 5. The effects of acetylcholine (ACh 10^{-7} M), and of evisceration factor (EF) from various tissues, on an isolated pharyngeal retractor muscle of *Thyone*. Extracts: pharngeal retractor muscle (PRM)—10 mg lyophilized tissue in 1.0 ml distilled water; respiratory tree (RT)—10 mg/ml; haemal system (HS)—1 mg/ml; doses of extract: 0.6 ml in 10 ml bath. Note that responses to PRM and RT contain both ACh and EF effects. Substances added at upward pointing arrows; washout at downward pointing arrows; time from beginning of record to dose: 100 sec; rectilinear recording.

tem sometimes has low ACh activity, detectable on the clam heart, but not on the PRM. The PRM extracts have low EF activity barely detectable in the presence of the high levels of ACh present.

Fractionation

Active evisceration fluid, and extracts of haemal system, respiratory tree, gut, and PRM were chromatographed on Sephadex G-15, with 0.025 M phosphate buffer as the eluent fluid. Only haemal system extract was placed on the long column directly. All other materials were first chromatographed on a short column; the active fractions containing both ACh and EF were lyophilized and resuspended in about 1 ml of water, then applied to the long column. All of the preparations contained an active factor which was identified as EF by its response on the PRM. The material eluted at a relative elution volume (Ve/Vo; where Ve = elution volume, Vo = void volume) of 1.58. The K_{av} was 0.36. (K_{av} is a partition coefficient defined as Ve-Vo/Vt-Vo, where Vt = total volume of the column.) The ACh eluted at a relative elution volume of 1.39 ($K_{av} = 0.225$). The peaks were close, but clearly separated (Fig. 6). ACh was identified by its characteristic effects both on isolated clam hearts and PRMs. Although 8 void volumes were tested, no other peaks active on the pharyngeal retractor muscle were found.

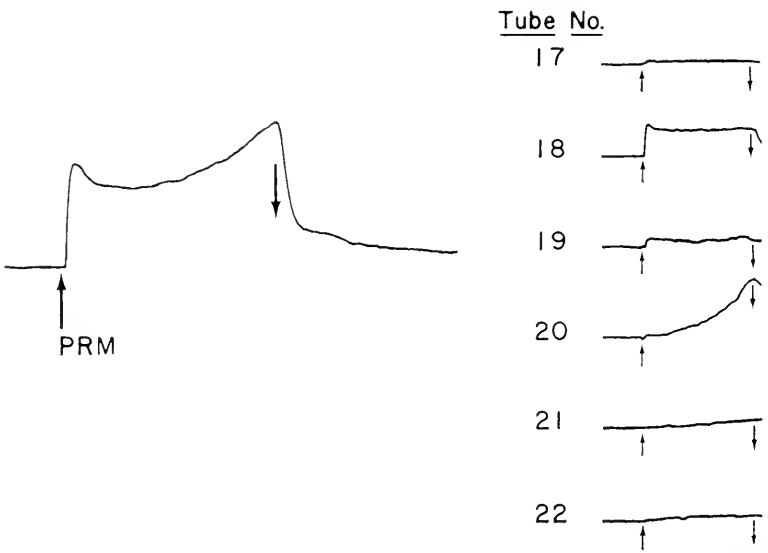

FIGURE 6. Fractionation of an extract of pharyngeal retractor muscle (PRM). Initial extract of lyophilized PRM in water passed through short column. Combined active fractions assayed (left-hand record); dose: 0.4 ml. Note both acetylcholine (ACh) and evisceration factor (EF) effects in the response. Combined active fractions passed through long column and assayed (right-hand records); dose: 1 ml of 5 ml fractions from long column. Tube 18 contains most of ACh; tube 20 contains EF. All assays performed on the same isolated PRM; tension amplification unvarying. Doses added at upward pointing arrows; washout at downward pointing arrows; rectilinear recording.

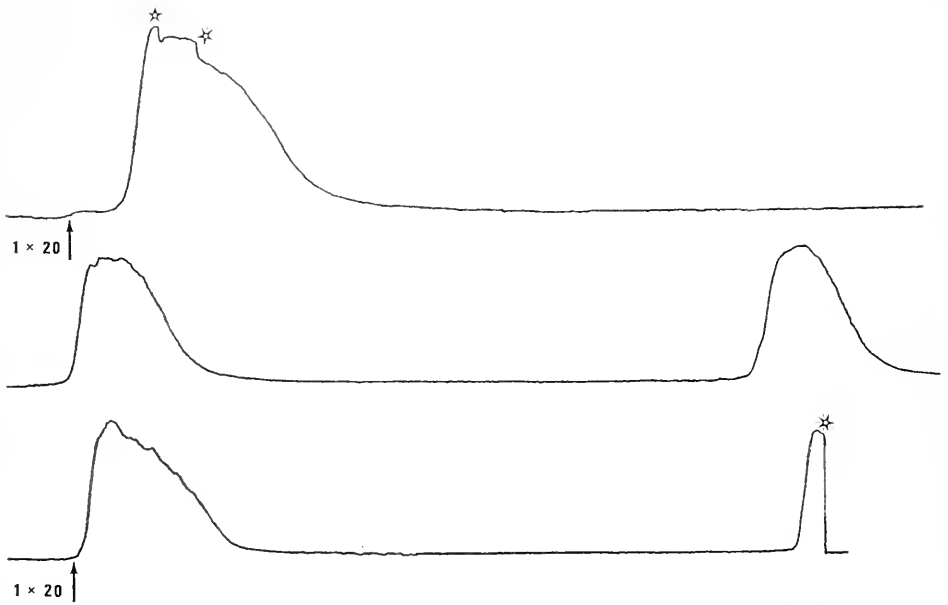

FIGURE 7. Autotomy of a three-stranded pharyngeal retractor muscle (PRM) induced by purified evisceration factor (EF). EF obtained from tube 20 of long column fractionation of haemal system extract (see Fig. 6); doses: 1 ml of the 5 ml fraction. Stars indicate autotomy of the individual muscle bundles; the third autotomy occurs only after a second dose of EF; tension at 1st peak is 5 g and tension at 3rd peak is 3 g; continuous record; total elapsed time: 2 hr, 18 min; rectilinear recording.

All tissue extracts producing the slow contraction waves in PRMs characteristic of EF yielded, on fractionation, the same component with activity identical to the whole extract. Large doses of this purified component induce autotomy (Fig. 7) and, after concentration by lyophilization, cause evisceration on injection into intact organisms. Probably, therefore, the EF activity of evisceration fluid is produced by a single molecule.

DISCUSSION

A factor inducing both autotomy of isolated pharyngeal retractor muscles and evisceration of whole animals appears in the coelomic fluid of *Thyone* upon evisceration. We have isolated this substance from the coelomic fluid as well as from tissue extracts. We propose that the many diverse conditions and compounds inducing evisceration all act through the common agency of this factor. Presumably the various stimuli release evisceration factor (EF) from intracellular stores, and the sequence of events comprising evisceration then follows perforce.

The factor would appear to be localized in nervous tissue since both KCl and electrical stimulation induce evisceration, while anesthetics block the effect. Although neither radial nerve cord nor the nerve ring is a very rich source of EF, additional nerve fibers are widely distributed in the organism. In particular, nerves have been demonstrated in the pharyngeal retractor muscles of *Thyone* by Margaret Hill in our laboratory at Woods Hole, and have been reported as well in

Thyonella gemmata (Nace, 1971) and in *Cucumaria sykion* (Pople and Ewer, 1954). Since, in addition, EF has been demonstrated in PRM extracts, the autotomy of isolated retractors following KCl or electrical stimulation is explainable on the mechanism proposed above.

Notwithstanding the evidence favoring EF localization in nerve, haemal system extracts were the richest source of the factor. Electron microscopy of the haemal system in *Cucumaria frondosa* revealed numerous bundles of axons in the vessel walls (Doyle, 1967). These neurons appeared to terminate adjacent to muscle cells, sometimes in deep invaginations in the fibers; but no membrane specializations were observed at the terminals. Large, membrane-bound, dense-core vesicles of unknown content occur in the nerve endings and Doyle (1967) suggested that these might be neurosecretory products. Assuming that the haemal system of *Thyone* is similar to that of *Cucumaria,* the high activity of EF in this tissue could be due to the high density of innervation or to the release of a specific neurosecretory substance. In any case, extracts of *Cucumaria frondosa* haemal system have yielded high levels of EF activity in preliminary experiments. The extracts were tested on *Thyone* PRMs and gave a typical EF response. The activity was recovered from the gel column at the expected elution volume.

Only the one substance, EF, is required to initiate the entire evisceration process including, not only autotomy of the pharyngeal retractor muscles, but also softening of the introvert wall. Exactly what the mechanism of EF action might be remains unclear. One hypothesis explaining the loss of tensile strength in an apparently collagenous structure would be the activation of a collagenolytic enzyme by the factor. However, no evidence that such an enzyme occurs in the introvert wall has been found (Smith, unpublished data). The wide tissue distribution of the evisceration factor, and its occurrence is a genus (*Cucumaria*) not normally exhibiting the evisceration response, suggests a further, more general role for the factor. In fact, evisceration may be only an incidental, if not accidential, effect of EF.

Low concentrations of EF induce cycles of slow contractions. Similar contractions have been described for the PRM of *Cucumaria sykion* (Pople and Ewer, 1958). These contractions are under nervous control, and the *Thyone* factor might be the neuromuscular transmitter by which they are effected. The effect of EF on other muscle preparations from *Thyone* is under investigation. The decay of activity at room temperature, and the preservation of activity by short boiling, suggests an enzyme system for removal of such a transmitter.

The electrical events associated with slow contractions are not known, but two types of membrane potentials have been observed in *Thyone* PRM in response to electrical stimulus (Prosser, Curtis, and Travis, 1951). A spike, apparently associated with twitch responses, can be measured. This response fades with increasing frequency of stimulation. In addition, a slow wave of potential may be recorded. Prosser *et al.* (1951) made no mechanical records corresponding to the electrical events, due to excessive fragility of the preparation. But, isolated PRMs from *Thyone* give vigorous contractions (>5 grams tension) on stimulation with ACh. The observed *in situ* fragility was probably due to the autotomy reaction, elicited by the electrical stimulus. In the same study, similar potentials were mea-

sured in *Phascolosoma* (=*Golfingia*) *gouldii*, a sipunculid. In this organism, slow waves were correlated with slow contractions.

The identity of the factor is unknown, but a number of relevant data are available. Neither ACh, 5HT, epinephrine, dopamine, L-dopa, norepinephrine, glutamic acid, 1-methyl adenine, nor histamine produce similar contractions or evisceration. No appreciable ninhydrin positive material occurs in the active fractions, and the absorption spectrum in the ultraviolet reveals no peaks. In particular, at 260 and 280 nm, the absorption is quite low. The material has either no effect, or a slightly excitatory effect (at high concentrations), on the clam heart. It elutes off the Sephadex column after ACh (MW = 181.6) but before leucine (MW = 131).

Evisceration in *Thyone* has been demonstrated in the course in Invertebrate Zoology at the Marine Biological Laboratory at Woods Hole for many years. Kille (1931; 1935) became interested in the problem during his association, as a student, with the laboratory. Our own work, although begun in December, 1970, was given impetus by our association with the Experimental Invertebrate Zoology course during the summer of 1971.

For their technical assistance and contributions to the development of the assay system, we thank John B. Dickson and Karl Brot (project students at Scarborough College, University of Toronto), as well as Margaret C. Hill and Gary Johnson (students in Experimental Invertebrate Zoology at the Marine Biological Laboratory, Woods Hole).

Summary

1. The pharyngeal retractor muscles of *Thyone briareus* autotomize during evisceration at the junction of the PRM and LBWM. Autotomy involves a loss of tensile strength at the connection between the PRM and the LBWM.

2. An evisceration factor (EF) is present in the coelomic fluid expelled by eviscerating *Thyone*. This factor induces autotomy of isolated PRMs.

3. Most tissues have some EF activity, but haemal system is the richest source of the factor.

4. Purified EF causes evisceration on injection into intact animals.

5. The factor is a small molecule (~150 MW), of unknown character. It probably has other roles in muscle and connective tissue physiology.

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INTERORGANISMIC AND ENVIRONMENTAL INFLUENCES THROUGH EXTREMELY WEAK ELECTROMAGNETIC FIELDS[1]

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A large amount of evidence has accrued over the past decade that organisms are not only sensitive to electromagnetic fields as weak as the earth's natural ones (Dijkgraaf and Kalmijn, 1963; Brown, Park, and Zeno, 1966; Lindauer and Martin, 1968; Keeton, 1971; Brown, 1971; Wiltschko and Wiltschko, 1972; Rommel and McCleave, 1972; and many others) but that their rates of metabolism or of "spontaneous" activity may reflect natural fluctuations in electromagnetic fields of the atmosphere. These fluctuations may be correlated with weather system movements (Truchan and Boyer, 1972) and times within the major geophysical cycles (Brown, 1962a, 1962b, 1963, 1965, 1968; Stutz, 1971). Indeed, Cumming (1967) has reported correlations between seed germination and solar radio flux. Others have reported annual variations in seed germination in presumed constant conditions (Bünning and Müssle, 1951; Bünning and Bauer, 1952).

In the course of search for the consequently expected differences in biological phenomena resulting from conducting observations at different sites in a modern laboratory with its greatly and diversely disturbed ambient weak electromagnetic fields, and concurrently at two geographic sites 1000 miles apart, such differences soon became clearly apparent. However, an additional and quite unexpected discovery was made that electromagnetic contributions to the environment by some organisms themselves can lead to an altered behavior of others nearby. The presence of this latter phenomenon and the gradual disclosure of some of its remarkable characteristics as the investigation progressed compelled on a number of occasions diversion from initially planned experimental objectives.

[1] This research was supported by Grant #GB-31040 from the National Science Foundation.

Methods, Experiments and Results

Preliminary studies

To assay biological differences at positions with obviously different electromagnetic fields the rate of water uptake by certified pinto beans *Phaseolus vulgaris*, (University of Idaho #111) first was chosen. From a 50 lb bag beans were used without any selection other than discarding broken ones or the very occasional, odd, extremely small specimens. This seemed to offer opportunity for simple, precise measurement of a reaction rate from a fixed constant starting point. The mean rate of water uptake during an accurately timed 4-hour period following submergence of 10- or 20-bean samples in shallow 6×6 cm square aluminum-screen trays in water could be readily determined.

Water uptake was determined as the difference between initial and final wet weights and then expressed as the percentage of initial dry weight. The flat, low trays with their single bean layer were designed expressly for quick, uniform blotting on absorbent tissue followed by fast weighing on torsion balances. The trays weighed 115 ± 1 centigrams. The first few hundred initial wet weights indicated that 9 to 22 centigrams became added when the beans were wet. These added values showed what appeared to be essentially a normal frequency distribution about a mode close to 15 centigrams. Subsequently, for the wet weight 15 centigrams was routinely added to dry weight of beans and tray to obtain the initial wet-weight value. The errors of measurement were hence all relatively small in relation to the 4-hour, 150–700 centigram water uptake by the beans and contained no systematic error.

The dry weight of the samples characteristically fell at a value between 7.5 and 9 grams and was recorded to the nearest centigram. The water uptake in four hours at room temperature during studies between February 22 and December 1, 1972 ranged from about 18% to more than 80% for 20-bean samples despite the air-conditioned, temperature regulated ($22 \pm 1.5°$ C) laboratory environment. With two exceptions which will be indicated later, all experiments were performed in a groundfloor laboratory suite of Hogan Biological Laboratory, Evanston, Illinois.

In an initial experiment trays of 20 beans were placed in 10 cm glass finger bowls at two sites in the laboratory. Two were juxtaposed on a wooden table top near the center of a room, a position discovered to be as undisturbed in its magnetic-field strength and orientation as any to be found in the laboratory. Another two finger bowls were juxtaposed on a stone chemical bench about 25 cm from a steel utility column at the surface of which a magnetic field of about $1\frac{1}{2}$ oersteds was measurable. Observations were made at these sites generally both morning and afternoon five days a week from February 22, through April 14, 1972.

Although an expected positive correlation ($r = +0.323$, $t = 2.97$, $n = 78$, $P < 0.005$) was found between the concurrent mean values obtained day by day from the two sites located in different rooms and separated by about 50 ft in the laboratory suite (Fig. 1) it was surprising to discover that no significant correlation existed between the two parallel bean samples closely side by side at each site, where it had been anticipated that the correlation would have been highest.

In an attempt to account for this latter absence of correlation, the "structure" of the relationship between the members of the pairs for the pooled data from the

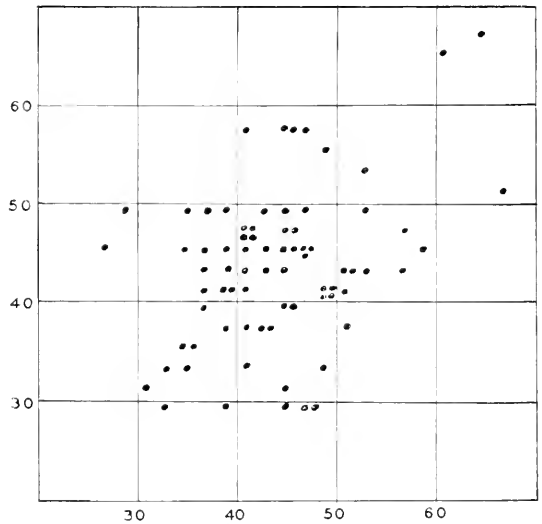

FIGURE 1. Scatterplot of the relationship between the mean water uptake for pairs of 20 bean samples with one pair of juxtaposed vessels located at a magnetically "disturbed" laboratory site (abscissa), and a second, similarly closely apposed, pair about 50 feet away at a "quiet" site (ordinate).

two sites was examined (Fig. 2A). For the scatterplots, with the 20-bean samples the data were grouped into 2% classes, and for the 10-bean ones treated below, into 3% classes.

There was a clear suggestion from inspection of Figure 2A that the values did not comprise a simple homogeneous population; there appeared to be a mixture of two populations, one with smaller variance and a negative correlation and the other with greater variance and displaying a positive correlation. To understand what could be the basis of such a mixture we must consider the significances of the correlations themselves. Being related are the varying values obtained from one sample of beans (abscissa) and the values simultaneously being derived from another (ordinate). Were these two samples fluctuating fully randomly and independently from one sampling period to another, a random distribution of points in the scatterplots would be evident, there would be no statistically significant correlation, and one might expect each, the ordinate and abscissa values, to display a normal distribution.

The hypothesis underlying the present investigation was that the varying values from one sampling period to another were not fully randomly distributed, and the differences reflected to a statistically significant degree the responses of the beans to concurrent variations in subtle, pervasive factors of the atmospheric environment which were postulated to be electromagnetic. Since all atmospheric parameters, whether subtle or obvious, are well-known not to exhibit a random day to day fluctuation or even to possess a normal distribution of their values (*e.g.*, atmospheric temperature or pressure), any variation of the bean water uptake would, to the extent it constituted an environmental response, comparably deviate from normality.

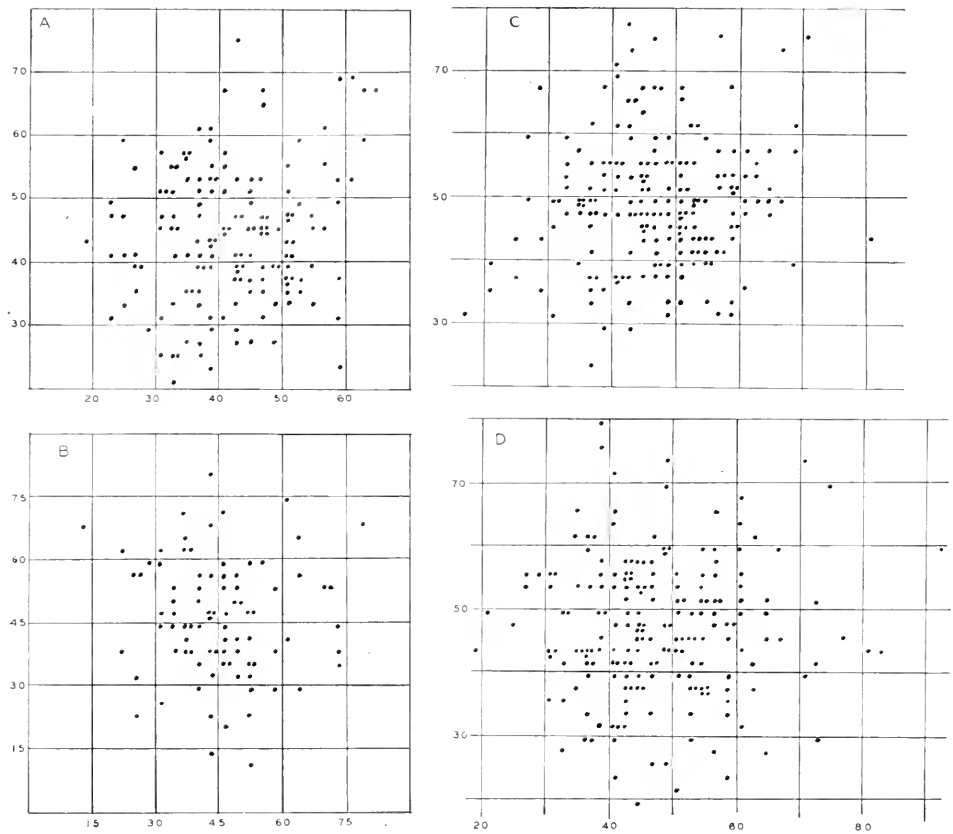

FIGURE 2. Scatterplots between rates of water uptake in paired samples of beans; (A) between the two members of the pairs of Figure 1; (B) between members of closely apposed vessels each containing 10 beans; (C) between 20-bean samples in trays about 35 cm apart (see text); (D) between members of paired 20-bean samples in the weak field of a slowly (2 rpm) clockwise rotating horizontal bar magnet.

The hypothesis that the beans continue to exhibit responses to ambient environmental variations even under conditions of controlled constancy of all obvious ones would, most simply, predict the beans to display a positive correlation between two independent samples investigated concurrently in the same environment. This would result from parallel environmental influences. If this were the case, a statistically significant positive correlation would be expected and the scatterplots should reveal upon inspection evidence for a homogeneity of such character that not only the total mass of points but any and every substantial assemblage of points arbitrarily delimited by boundaries parallel to the ordinate or abscissa coordinates should reflect the positive character of the relationship.

There was an alternative possibility which our hypothesis included, and which had been suggested in many data obtained over many years, namely that the correlations of a biological system with subtle ambient field-variations could be either

positive or negative. Environmentally dependent temporal patterns of variation of two independent biological samples held under different environmental conditions, could conceivably mirror-image one another and a negative correlation exist between them. In the beans investigated concurrently and under the same experimental conditions there could theoretically be for two independent samples day to day parallel fluctuations in sign between positive and negative. Under these circumstances there would be expected only a reinforced positive correlation between two concurrent samples but no expected inhomogeneity in scatterplots. The occurrence of such inversions parallelly for both samples would hence probably not be readily identifiable.

Another possibility existed, namely that little or no correlation would exist between two concurrently studied samples as a consequence of a random and independent distribution of correlating signs of response of the two biological samples to their common environmental fluctuations. In this case the correlation coefficient could conceivably be even zero while two populations of values might be conspicuously evident upon inspection of the scatterplots as an X-formed distribution of values. One population of values could be clearly contributing a negative correlation, the other a positive one.

Our hypothesis, however, had not taken into account the additional possibility that was clearly suggested by the relationship between the two samples illustrated in Figure 2A, namely two populations of points in which a negatively correlating one was not counterbalanced over the same range of values by an equal and opposite positively correlating population of values. The only possibility for the explanation of such a relationship was the existence of a mutual interaction between the two samples that had been hitherto presumed to be behaving independently of one another. The relatively large range of values over which the uncompensated negative correlation occurred suggested that the influence of the interaction was substantial. The simplest hypothesis for the nature of the interactional influence seemed to be that the two samples in some manner biased one another to adopt opposite signs for their correlated responses to the varying subtle geophysical parameters which were effecting the day to day fluctuations in water uptake.

The specific character of the two populations of Figure 2A suggested an *ad hoc* means for confirming existence of its peculiar population admixture. This was qualitatively accomplished through learning that for those correlated values that lay within the range of 32% up to 58% the coefficient of correlation, r, was -0.32, $n = 99$, and for all those values that lay beyond this range $r = +0.33$, $n = 57$. Correlations of this magnitude and with such sign differences are definitely not expected in homogeneous populations of truly random, or even correlated, data which are extracted from scatterplots in this specific manner. These correlations certainly suggest trends but are perhaps questionably accessible to rigorous determination of probabilities.

In view of the uniformity of the beans used, the positive component of Figure 2A was expected from commonly reported general correlation between rates of biological activities and fluctuating subtle parameters, including electromagnetic, of the atmosphere. The negative correlation, spanning from about 70% to 126% of mean rate of water-uptake for that period was, on the contrary, quite unexpected.

Comparing the extent of the negative correlation for the two sites separately, the "undisturbed" location yielded a value of $r = -0.42$, $n = 51$, while the "disturbed" area gave also a negative, but much smaller $r = -0.18$, $n = 48$.

Another series of "paired" experiments was also being conducted both mornings and afternoons commencing on February 22, similarly on the stone-topped chemical bench, but no closer than 75 cm to the service column. In this series the bean samples included only 10 seeds. Again, two pairs of vessels were used; these were separated by only about 50 cm. There were two initial objectives for this series, (a) to learn whether 10-bean samples would provide results quite comparable to those of 20-bean ones and (b) to determine the magnitude of an expected correlation between two pairs only 50 cm apart when they were, therefore, in essentially the same specific laboratory environment. The variances observed among the smaller sized samples were found to be greater than for 20-bean ones. Correlation between the two members of the pairs was also statistically insignificant (Fig. 2B), but as for the 20-bean series a population inhomogeneity was evident upon inspection of the scatterplot; over an intermediate range of values of 28% to 62% a negative value of $r = -0.27$, $n = 59$, was obtained.

Another experiment was performed. This one was initially designed to determine whether differences in strength of ionizing radiation would effect differences in rate of water uptake and if so, whether the influence might differ between when the field was administered parallel and at right angles to the earth's horizontal magnetic vector as had earlier been disclosed for an influence on light response for *Dugesia* (Brown, 1963, 1971).

Between March 6 and April 14, 1972, at a relatively "undisturbed" position in the laboratory, five trays of 20 beans each were placed in a large 33-cm long rectangular plastic vessel and aligned close together in a row toward magnetic north from a weak (24 μCi) gamma source yielding a gradient along the five samples ranging from 17 × background for the closest to 2.5 × for the most distant. A completely comparable series of 20-bean trays was arranged in exactly the same manner in a second large plastic vessel, but directed from the same gamma source toward "magnetic west." For both series, the closest tray was numbered 1, and the most distant, 5. From center to center of the closest bean groups between the two vessels was 15 cm. Correlating percentage water uptake between samples in the two series, N and W, relating them in the reversed order in the gamma radiation gradient (*i.e.*, $1^N \times 5^W$, $2^N \times 4^W$, *etc.*), in order to minimize any possible existent contributions by the gamma-field gradient itself, yielded r as a positive but statistically insignificant $+0.146$, $t = 1.82$, $n = 150$. However, a comparable correlation in a sequence paralleling the gradient (*i.e.*, $1^N \times 1^W$, $2^N \times 2^W$, *etc.*) gave r as a statistically significant $+0.2845 \pm 0.075$, $n = 150$, $P < 0.001$.

Examining the structure of the correlation scatterplot for the reverse-related samples it was noted, now for the third time, that an inhomogeneity in the population existed. This too appeared to result from a mixture of two populations. One sizable population of values which exhibited a negative correlation was responsible in good measure for the relatively small value of r that had been obtained. The substantial magnitude of this negative contribution was indicated by finding that over the range of values from 38% to 56% (Fig. 2C), values spanning the mean, $r = -0.41$, $n = 78$. The negatively correlating population of values was apparently

slightly more restricted in its range under these experimental conditions than under the conditions for the two previous cases.

In the direct serial order correlation this negatively correlating population of values seemed reduced and largely overridden by a relatively strong positively correlating relationship between 4N and 4W, and 5N and 5W, which were the groups most distant from one another, about 43 and 53 cm, respectively. In the "reverse-correlated" study the interdistances ranged around, and close to, 35 cm between all correlated samples.

To learn whether a fluctuating, weak magnetic field might modify rate of water uptake another experimental series was conducted concurrently with the gamma-field one, trays of 20-bean samples were placed in two tandemly arranged 33 cm long plastic containers, each with three trays with all six trays lined up about one tray-width apart to "magnetic east" from a rotating horizontal 18 cm bar magnet, turning clockwise (viewed from above) 2 rpm. The magnet provided at about 46 cm a horizontal field strength equal to the ambient horizontal one at that site in the laboratory. Correlating the first tray of beans in the first container with the first in the second, the second with the second, *etc.*, r was very close to zero. A repetition of the experiment was performed with eight trays of beans in a line of four pairs of 10 cm finger bowls with one juxtaposed pair as close to the rotating magnet as feasible and the remaining pairs at 74 cm intervals away. No significant correlation existed when #1 was related to #3, 2 to 4, 5 to 7, and 6 to 8. Nor was any correlation evident when #1 was related to #2, #3 to #4, etc. The pooled results of these two rotating magnet series gave $r = -0.03$. However, an examination of the structure of the relationship between the samples suggested, from both kinds of series separately, that we were probably here, too, not dealing with randomly varying data. A "figure X" tendency in the scatterplots between the correlated samples suggested that we had, instead, biological systems that were correlating both + and −, with close to equal frequency.

A third study, in the field of the rotating magnet, with a geometric and geographic arrangement exactly as for the gamma study, was also performed. Correlating in "reverse sequence" to minimize any graded contribution resulting from effect of magnetic field strength itself, an "X" tendency in the scatterplot was again suggested, and r was again very close to zero. The pooled results of all three rotating-magnet series are illustrated in Figure 2D. Although here again, two populations of values were suggested, they seemed about equally divided between + and − correlating groups.

With the initial intention of assuring that both members of paired samples of beans would be occupying the same space at essentially the same time and hence any negatively correlating relationship such as those noted earlier could not be ascribed to subtle differences between two fixed locations, another kind of observation was made. Two pairs of trays of 10 beans each, in 10 cm finger bowls, were placed on a counterclockwise (from above) rotating platform, 6 rpm. Two bowls were placed in contact with one another on each side of the axis of rotation. The bean trays in the paired bowls averaged about 10 cm from center to center. The distance of the second pair from the first averaged about 19 cm with the distance between centers of the diagonally opposing bean-trays being about 24 cm. Observations were made of water uptake over 21 half days spanning about two weeks

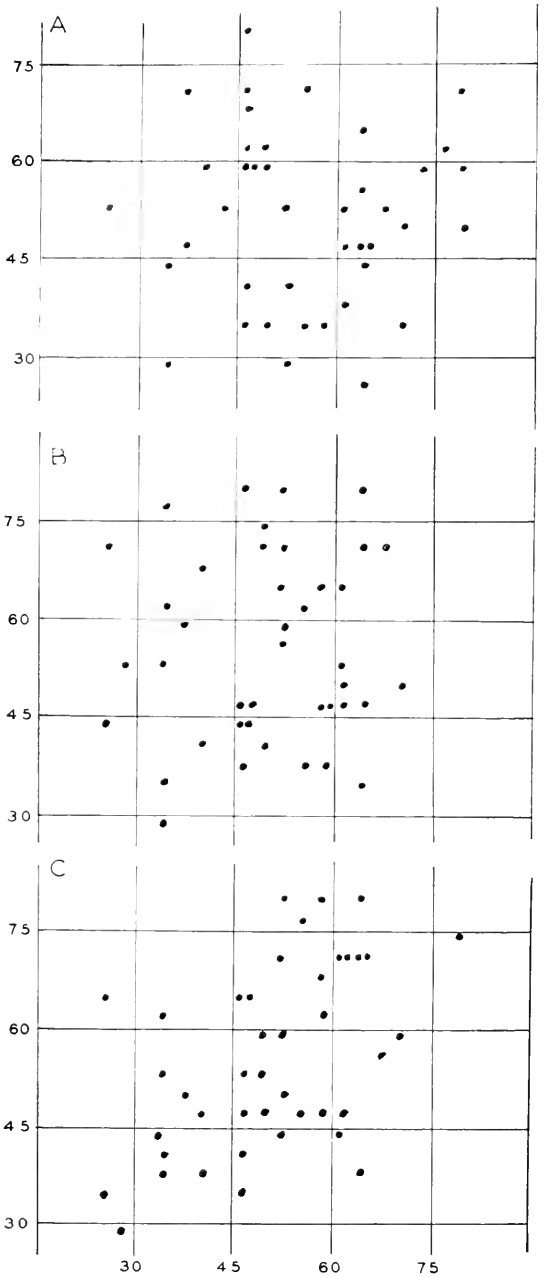

FIGURE 3. Scatterplots between 10-bean samples in four separate vessels on a 6 rpm, counterclockwise rotating platform with two pairs symmetrically paralleling one another 9.5 cm on opposite sides of the center of rotation; (A) between members of pairs; (B) between directly opposite counterparts; (C) between diagonally opposite counterparts.

(April 10–24, 1972). No significant correlations were present either between the two members of the paired samples (Fig. 3A), or between the corresponding tray of the opposite pair (Fig. 3B). However, strikingly, a good, highly significant positive correlation was discovered between the diagonally opposite samples (Fig. 3C), $r = +0.483$, $t = 3.49$, $n = 42$, $P < 0.002$.

A final preliminary study was commenced to learn whether perhaps the negatively correlating element that had been noted between the two members of closely apposed paired groups perhaps depended upon sample proximity itself and would disappear if members of the correlated pairs were separated by more than the 43 to 53 cm distance that the gamma series suggested no longer to be contributing to the negatively correlating central population within the scatterplot. Twelve 10 cm finger bowls, each with a tray of 20 beans, were arranged as three groups of four, each group on a separate table, in the following manner in a relatively "undisturbed" area of the laboratory. In each group were two closely apposed to one another to comprise a "pair." The other two in each group were separated by a distance of 70 cm from the "pair" and more than this from one another, or from beans of any other group. On the basis of a possible influence of sample proximity either no correlation, or a low positive one, over the central range where bean "interactions" appeared to have been displayed was predicted when the two "singles" were correlated. A low positive one could result from a parallel response to their common physical environment. In short, the sign of each sample might be, independently, + or − without being biased by any adjacent bean sample. For the paired samples, on the other hand, a negative correlation would be expected for this central range as one group biased toward opposite sign the adoption of the sign by the other.

The results from 20 half-day experiments from April 24 through May 5, 1972, disclosed that within the same central range of values, 38% up to 58% (Fig. 4B), the paired samples yielded, as predicted if biasing existed, a negative correlation, $r = -0.361$, $n = 31$. For the corresponding "singles" for each group (Fig. 4A), on the other hand, a small positive correlation was present, $r = +0.149$, $n = 30$. Each "single" thus appeared to have its sign, whether positive or negative, determined without influence of the other, with a weak suggestion of operation of a varying component in the physical environment influencing them in a parallel manner.

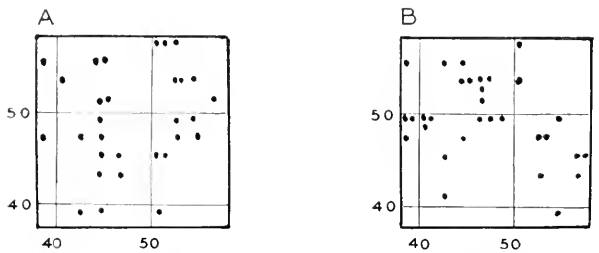

FIGURE 4. Scatterplots of relationships within the range of 38% to 58% of water uptake for (A) two samples separated by more than 70 cm from one another and (B) two samples closely juxtaposed in separate vessels.

"Pairs" vs. "singles"

The precedings "pairs" vs "singles" experiment was continued through August 18, 1972. Assays of water uptake were made both morning and afternoon through June 2, after which only single daily assays were performed. The 10 cm glass finger bowls were replaced by cylindrical, transparent plastic vessels, 9 cm in diameter and 4.5 cm deep. The original certified bean strain was replaced by uncertified pinto beans obtained from a large commercial supplier in Nebraska. The beans were thereafter submerged mornings and their water uptake was determined four hours later, in the afternoons. Figure 5A illustrates the day by day variation in the mean percentage water uptakes, only the afternoon values from May 19 through June 2, and then single daily ones until the end of the study on August 18, 1972, for the "pairs" and "singles" separately. The concurrent two mean values during the whole period of study exhibited a high correlation with one another, $r = +0.806$, $n = 111$. In the same illustration, Figure 5B, are seen the concurrent mean water uptake percentages from June 5 onward for nine 20-bean samples arranged as three triplets of linearly juxtaposed vessels. These last, being investigated in an adjoining room within the laboratory suite to learn in what manner an extra bean sample might influence pair interaction, are noted to be comparably highly correlated in their systematic variation in mean rates with the "singles" and "pairs" in the other series.

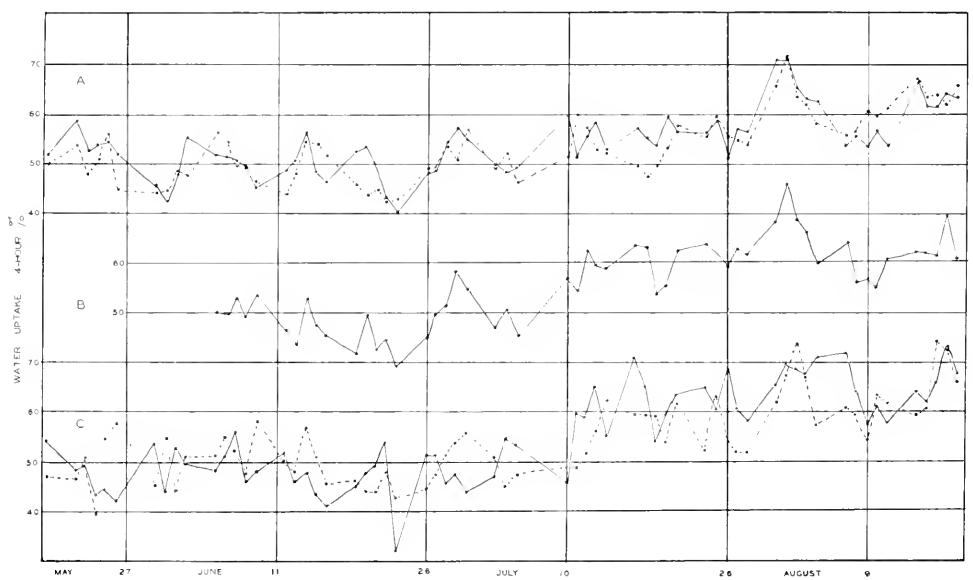

FIGURE 5. Variation from day to day in mean rate of water uptake for (A) (solid line) six 20-bean samples separated by 70 cm from one another, and (broken line) six 20-bean samples arranged on the same three laboratory tables but as closely apposed pairs; (B) nine 20-bean samples arranged as three triplet groups at a second laboratory site bout 40 feet away; (C) (solid line) four 20-bean samples, arranged as two pairs, on a 6 rpm, clockwise rotating platform and (broken line) on a counterclockwise rotating one a short distance away in the same room but a different room from A and B.

In order to abolish the high positive correlation which was a consequence of the conspicuous parallel variation in rate of water uptake in the means for all these bean samples the rate of water uptake in each of the twelve "singles" and "pairs" samples was expressed each day as a deviation from the mean value for all twelve for the concurrent interval. It was recognized that upon simple grounds of probability, a very weak negative correlation would be the null expectation from correlating pairs of these values and therefore statistical significances would depend upon significant differences from appropriate "controls" employing the same population of deviations.

Examination of the correlation scatterplots of the 333 values relating deviations from the daily means for the members of the juxtaposed trays on the one hand, and the pairs of separated trays on the other, revealed a gross difference between them. For the "singles" there was suggested a "figure X" relationship, a relationship not similarly suggested for the closely apposed pairs of trays. The difference is described and quantified in Figure 6. In this figure the variance for one member of a "pair" is plotted against deviation from the mean for the second member for $\pm 4\%$ intervals (the open circles), and the variances of the second member are comparably shown in relation to the deviations of the first (solid circles). The numbers contributing to each point are indicated. For the "singles" (Fig. 6A) it is evident that variance of one member systematically increases with deviation of the other from the mean, a relationship consistent with the apparent "figure X"

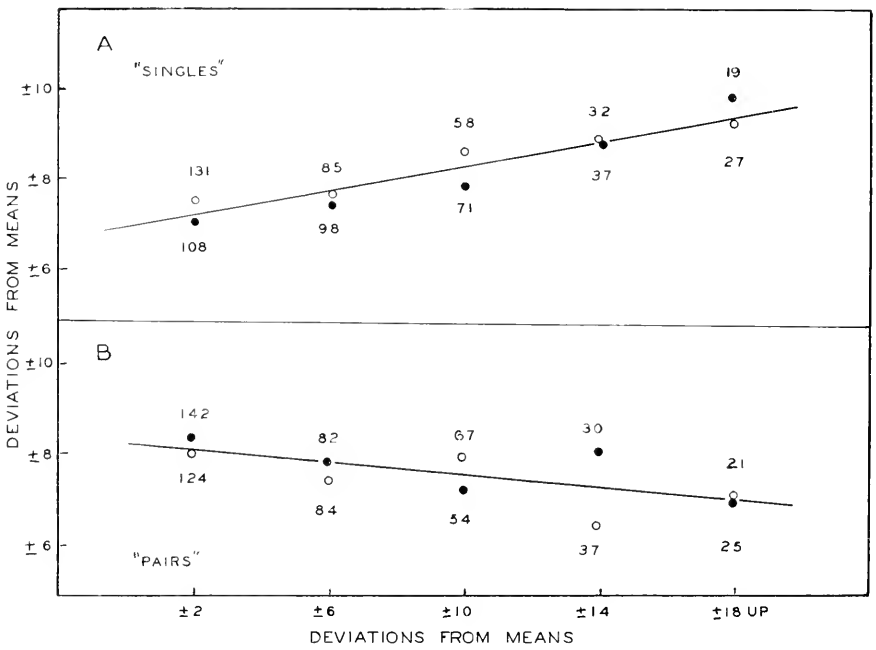

FIGURE 6. The relationship between (A) (circles) the deviation from the mean, without sign, for one of the two correlated "singles" and the concurrent comparable deviation for the second, and (dots) vice versa. (B) The same for the two correlated "pairs."

relationship in which both positively and negatively correlating relationships between the samples appeared to occur with about equal frequency.

When, on the other hand, the members of the closely apposed paired samples were analyzed in an entirely comparable manner the relationship seen in Figure 6B was obtained. Now the *greatest* variance in one is noted for the *smallest* mean deviations of the other, the variances of one *decreasing* systematically as the mean deviations of the other *increase*. In other words, when the value of one member of the pair is near the mean value for that day, the other member is biased away from that mean, and when one member, instead, deviates farther from the mean, the other member is permitted to approach more closely the mean for the day. Indeed, even more, when one member of a pair deviates far from the mean, the other member of that pair appears to be prevented from deviating far, whether in the same or opposite direction.

The preliminary observations of the presence near their means of a negative correlation between members of closely apposed bean samples is, therefore, supported by these additional results. Separation of the members of the pairs by about 70 cm appears to have prevented this interaction thereby permitting each member of the pair to become, independently of the other, positively or negatively correlated with whatever is the effective subtle geophysical factor of factors.

Rotating platforms

The preliminary experiment involving the two pairs of 10-bean samples on a rotating platform at 6 rpm CCW (counterclockwise) was continued, but altered to 20-bean samples and amplified by conducting simultaneously, and nearby in the same small laboratory room, an entirely parallel series differing only in that the rotation of the platform was CW (clockwise). The experiment commenced April 25 and terminated on August 18, 1972. The series were run both mornings and afternoons through June 2 and thereafter only once each day, over the noon period. Plastic vessels were substituted for the glass ones.

The day to day variations in mean water uptake for the two series are shown independently in Figure 5C only for afternoons through June 2 and thereafter daily through August 18. Inspection of this illustration clearly indicates that for the gross trends there is a positive correlation between the means of the four samples on the CW and the CCW rotating platforms. Suggested, however, is the common occurrence of apparent inversions between them for a single days or over a series of several days.

When the nature of any correlations between the individuals of pairs and between diagonally related trays for the two platforms were investigated it became quickly evident that the CW and CCW rotations were clearly not equivalent. Because of the longer-term parallel trends, statistically significant positive correlations were found between members of the pairs and of the diagonals for both rotational directions. However, some statistically highly significant differences were noted among them.

The lowest correlation $r = +0.195 \pm 0.066$ was found between CCW pairs with a higher one, $r = +0.262 \pm 0.064$, for the diagonals. Next in degree of correlations were the diagonals for the CW rotating samples with $r = +0.401 \pm 0.058$. Highest was the correlation between members of the CW pairs, with

$r = +0.477 \pm 0.053$. For each one $N = 220$; the statistical significances are high. Converting r to z the above values became, respectively $+0.195 \pm 0.068$, $+0.266 \pm 0.068$, $+0.420 \pm 0.068$, and $+0.520 \pm 0.068$. The difference between the above CW and CCW pairs was 3.40 times its error ($P < 0.001$). The difference between CW pairs and CCW diagonals was 2.68 times ($P < 0.01$), and between the CW diagonals and CCW pairs, 2.35 times its error ($P < 0.02$). The mean difference between both CW correlations and both CCW ones was 3.36 times its error ($P < 0.001$).

Both inspection of the scatterplots and the particular order of the degrees of correlations suggested that for the CCW rotation, as noted in the preliminary studies, the "pairs" contained a greater negatively correlating element than did the "diagonals." On the other hand, for the CW rotation the diagonals appeared to include more negatively correlating elements than did the pairs. At the same time, CW rotation, relative to CCW rotation, appeared to reduce the degree of that interaction between the bean trays which resulted in the varying degrees of reduction in a fundamentally positive correlation.

Of some interest was whether, perchance, the rotational consequences that were being disclosed might be related in any manner to the effects of extremely slow rotation (1 revolution per day) on plant growth, turgidity, and twining described by Jones (1960). Jones had reported that relative to concurrent plant-growth-rate on stationary platforms the slow CW rotation depressed rate and CCW rotation accelerated it. A comparable influence by very slowly rotating (2 revolutions per day) weak magnets on plant growth rate was reported by Edmiston (1972).

To determine whether the bean water-uptake rate might also be influenced by such slow rotation rates, pairs of vessels containing 20-bean samples were placed on duplicate platforms, arranged side by side, in a light-controlled room at the Marine Biological Laboratory, Woods Hole, Massachusetts. The two pairs were placed on opposite sides, and 30 cm from, the platform center. One platform rotated CW at the rate of 1 rpd, the other was kept stationary. The pairs of bean trays on the rotating platform turned, obviously, through an angle of only 60° in the course of a four-hour run. The experiment continued from June 22 through August 18. In view of the great change in rate of water uptake that was occurring over the course of this 2-month period in all experimental samples (see Fig. 9A) the data were converted daily to deviations from the mean for all of 28 bean samples that were involved in the several experiments proceeding concurrently in the same room.

Correlating members of pairs on the CW rotating platform (1 rpd), $r = -0.129$; $n = 84$, and on the static one, $r = +0.230$; $n = 84$. Transforming r to z the difference between those two was statistically significant, $P < 0.03$.

Lead covers and electrical shielding

Other experimental series led to further interesting observations and conclusions. The possible influences on water uptake by (1) attenuation of the ambient electrostatic field, especially any vertical component, and (2) augmentation of any vertical component of background radiation through the "Rossi effect"

of production of cosmic ray showers through the super-imposition of lead plates, were investigated.

Four pairs of 12-cm square glass vessels were arranged on a large wooden table as 4 juxtaposed pairs (12 cm, center to center). Two of the pairs, separated by 24 cm, were to be involved in the electrical shielding experiment. The other two pairs, separated by 34 cm, were for the lead-cover one.

The specific environment of the laboratory site where these experimental setups were located was very clearly one of the more electromagnetically "disturbed" areas of the laboratory. There was virtually no horizontal vector of magnetism over portions of the table area and over the remainder the needle of a very sensitive surveyor's compass often drifted slowly and erratically to various geographic directions. This highly disturbed state of the ambient magnetic field at this specific location appeared due to the presence of heavy electrical building-service equipment in the basement directly below.

The electrical field was attenuated for one of the pairs of bean-samples by sandwiching the pair of vessels between two horizontal copper plates, $25 \times 47 \times 0.056$ cm, electrically connected with one another, but not grounded. The other pair served as the concurrent control and possessed only a cardboard cover. To control for any influence of the different specific sites of the pairs on the table, the application of the copper plates was alternated on successive days between the two pairs of vessels. Any effects of electrical shielding measured as differences from the concurrent control would, therefore, not include consequences of site difference.

Comparably, for the other two pairs of vessels on the table, comprising experimentals and controls for the altered background radiation series, the lead cover was moved on alternate days from one pair to the other. The lead plate comprised a sheet $30 \times 56 \times 2$ cm. The controls received only a cardboard cover.

The lead-plate and electrical-shielding series were continued from June 5 through September 8, 1972.

The effects of the experimental conditions were first recorded day by day as the *difference* in water uptakes between experimentals and their concurrent controls. The mean rates for the two members of each pair were used for this purpose. A review of these differences disclosed that there was an underlying component possessing a good positive correlation between the day by day "responses" to the lead and electric-field environment for those days that the response values did not exceed about $\pm 14\%$ for either one. This is evident from inspection of the scatter-plot of the relationship between the two conditions (Fig. 7). In Figure 7, sixteen out of a total of 67 values are seen to fall beyond the limits. With these values removed $r = +0.57$; $n = 51$.

In view of what appeared to be the existence of a basic positive correlation between the "responses" over a substantial range of values to the two differing experimental treatments, it was arbitrarily decided to consider any day on which the apparent response to either experimental condition equalled or exceeded 20% as the difference between experimental and control as an *aberrant* day. There were eight of these among the 67. It is evident from Figure 7 that these high values were essentially randomly distributed as far as contributing any given relationship between the two experimental series.

It soon became evident that the aberrant days were significantly different from

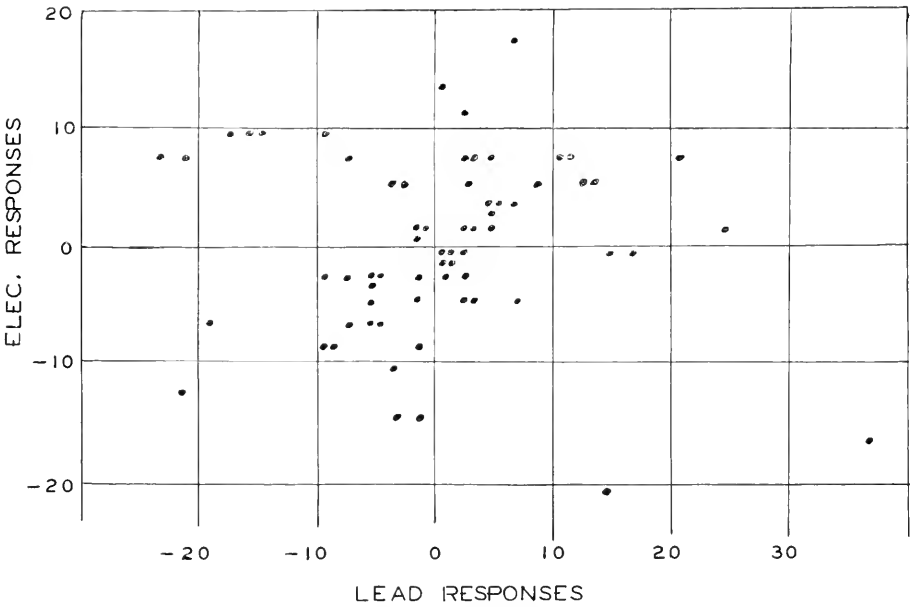

FIGURE 7. Scatterplot of the relationship between concurrent responses (difference between experimentals and controls) to "electrical shielding" and altered background radiation by covering with 2 cm thick lead plates.

the *non-aberrant* ones in also another respect. This was the nature of the interaction between the individual members of the experimental and control pairs when deviations from the daily means of all four pairs on the table were employed. For the aberrant days the coefficient of correlation between members of the pairs (Fig. 8C) was positive, $r = +0.288 \pm 0.10$, $t = 2.7$ ($P < 0.01$) while that between individual members of an experimental pair and an individual member of the control pair (Fig. 8A) was negative, $r = -0.390 \pm 0.093$, $t = 3.8$ ($P < 0.001$) with $N = 84$. On the contrary, for the residual non-aberrant days, the value between members of two different pairs (Fig. 8B) was positive, $r = +0.202 \pm 0.071$, $t = 2.78$ ($P < 0.01$), and that between members of the same pair (Fig. 8D) was negative, $r = -0.348 \pm 0.065$, $t = 5.03$ ($P < 0.001$), with $N = 184$.

These results provide evidence for another apparent property of the phenomenon of interactions between the organisms, namely, an interaction between closely apposed members within pairs may involve not only a biasing of the second of the pair to adopt an *opposite* sign of its correlation with whatever the effective, varying subtle environmental parameter or parameters, but that some environmental conditions may obtain wherein the *intrapair* biasing may be in the direction of the two members adopting the *same* sign.

Geographic and laboratory similarities and differences

Further information concerning characteristics of the environmental variations responsible for the fluctuations in water-uptake and modification of their influences

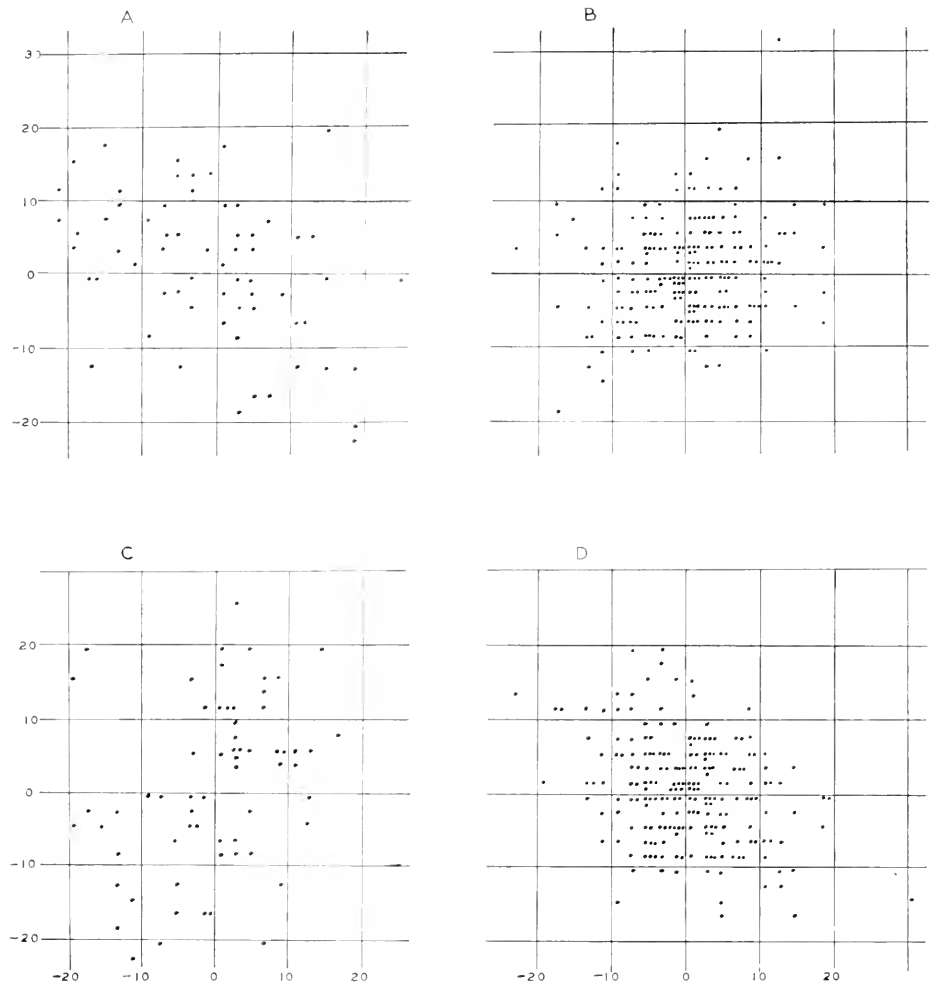

FIGURE 8. Scatterplots of the concurrent relationships between deviations from the daily means for the four electrical shielding and four lead-plate experimental and control pairs of the same day. *Aberrant* days (when response to either shielding or lead cover, exceeded 20) are treated separately from the remaining non-aberrant days. (A) is the relationship for aberrant days between a sample for one member of the experimental with one of the control. (B) is the comparable relationship for the residual non-aberrant days. (C) is, for aberrant days, the relationship between the two members of pairs, and (D) is the same for the non-aberrant days.

by subtle environmental factors came from additional kinds of observations. These involved concurrent studies at widely different geographic points and at different specific sites within the laboratory, including a walk-in, insulated and metal-sheathed, controlled-temperature cabinet ($6\frac{1}{2} \times 7\frac{1}{2} \times 7\frac{1}{2}$ ft).

Figure 9A describes the variation over the period, June 22 through August 4, 1972, of mean water uptake in three rooms in a laboratory suite at the Marine

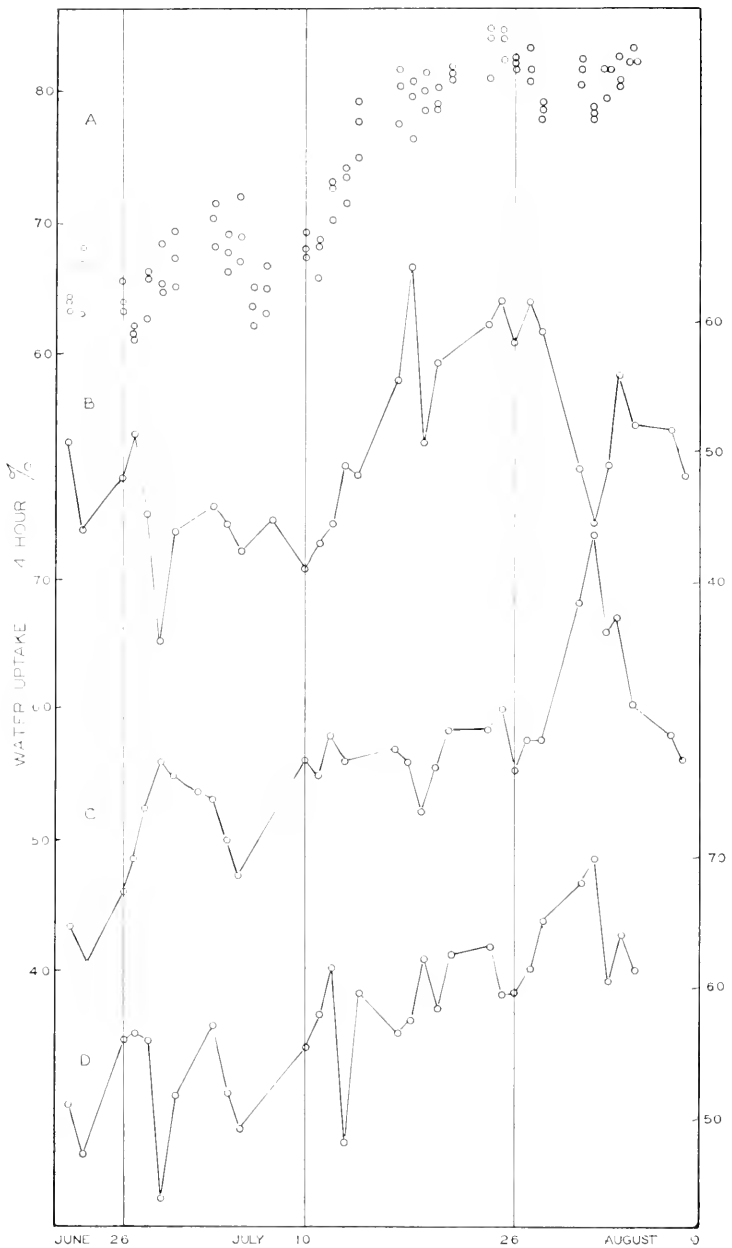

FIGURE 9. Concurrent day by day variation in water-uptake rate (A) in each of three rooms at Woods Hole, Masachusetts, (B) in a walk-in constant temperature cabinet in Evanston, Illinois, (C) on tables in the Evanston laboratory just outside the temperature-controlled cabinet, and (D) in an electromagnetically "disturbed" laboratory site (the electric shielding and lead cover experimental series).

Biological Laboratory, Woods Hole, Massachusetts. The points describe the means for 28 20-bean samples in one room, 32 in a second and 14 in a third for each working day over the period. Figure 9B describes values concurrently obtained by Mr. Jack S. Pierce of Northwestern University as the mean of four samples in the accurately temperature-controlled room in darkness at 23° C in Evanston, Illinois. Figure 9C compares the mean variation for the same period of 21 20-bean samples on wooden table tops in the open laboratory in Evanston in relatively "undisturbed" areas with respect to ambient electromagnetic fields. Figure 9D shows the mean day to day variation for the 8 samples involved in the lead-plate and electrical-shielding experiments on the wooden table in the obviously greatly "disturbed" electromagnetic environment.

It had been noted in the Massachusetts laboratory that a rather abrupt increase in water-uptake rate commenced about July 11, an increase for which there appeared to be no evident explanation in terms of alteration in any obvious environment factor. The level of the mean rate rose from 66% as the average for the interval from June 22 through July 11, to 82% for the period July 17 through August 4. There had thus been a relatively abrupt increase from one general rate level to another, 24% higher.

Essentially the same pattern of rate change had occurred concurrently in Evanston, Illinois, in the temperature-controlled room where, coincidentally, the increase between the same two intervals was also 24%.

For the day to day concurrent variation in the means of the 21 samples involved in the "pairs" and "singles" series described earlier, and in the "triplet" series of experiments referred to earlier (see Fig. 5B) there was, between the same two periods a 22% increase. However, a comparison of the pattern (Fig. 9C) for the total period from June 22 through August 4 suggested that this was quite different from that one for the samples in the controlled temperature room (Fig. 9B) despite the fact that the "singles" and "pairs" were no more than 12 feet away and the "triplet" series no more than 40 feet away in the large laboratory suite just outside the controlled cabinet. Before the July 11 date, the lowest value in the accurately controlled temperature environment (45.7%)occurred on the same day as the highest (55.8%) for those beans outside the controlled room. The variation, both throughout the period of the abrupt rise and after its completion, seemed to possess a relatively strong mirror-imaging relationship. This latter reached its most spectacular proportions when on August 1 one of the two had increased to a peak value of 73% while the other had at the same time dropped to a sharp minimum value of 44.5%.

Meanwhile the eight samples in the electromagnetically disturbed area of the lead and copper shielding series had also exhibited a rise in mean rates between the two intervals from 53.5% to 61.8%, but an increase of only 15.5% (Fig. 9D). An examination of the pattern of water-uptake variation over the whole 44-day period suggested that for about the first two weeks the pattern in the disturbed area paralleled that of the beans in the temperature-controlled room (Fig. 9B), but thereafter appeared to resemble more that of the other, or open laboratory, sample (Fig. 9C).

The apparent general widespread presence and character of the July 12–16 abrupt increase in water uptake rate is again supported by the patterns

revealed for the two rotating-platform (6 rpm, CW and CCW) experiments performed in the separate small room about 30 feet away from all other concurrent experimental series (Fig. 5C). In these the July 12–16 rise also occurred. This rise was from 49.0% to 63.6% in the CW series, and from 46.7% to 59.2% for the CCW one. These amounted to rises of 29.8% and 26.8%, respectively.

These observations lend further support for the presumption that any given sample or group of samples of beans are capable of correlating *either positively or negatively* with at least one of the normally uncontrolled but highly influential parameters of the subtle geophysical environment. Furthermore, it is apparent that differences in sign of response may alone account for differences in a fundamentally important biological process, amounting to more than 60% between two concurrent samples at the same local geographic site under what has usually been deemed constant conditions for them. The mean temperature of the air-conditioned, temperature-regulated laboratory was about 22.0° C, that of the accurately temperature-controlled room, 23.0° C.

Finally a comparison was made of the concurrent day by day fluctuations in mean water uptake for eight 20-bean samples being investigated in light and in darkness by Mr. Jack Pierce in the controlled-temperature room at 25° C for the period from September 28 through November 17 (Fig. 10A) with the mean for

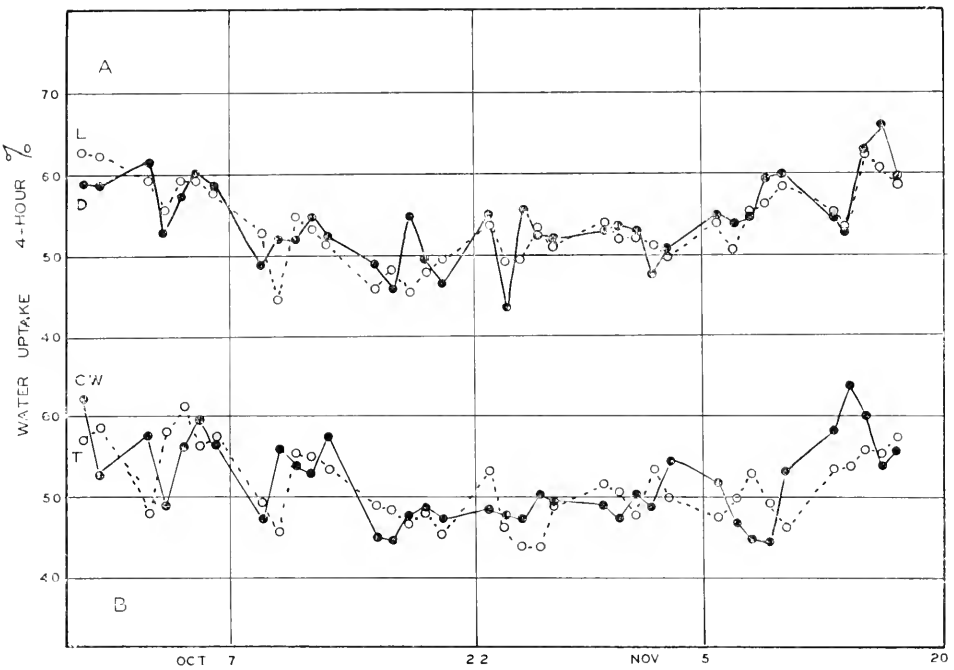

FIGURE 10. Day by day water-uptake variations for (A) (solid line) eight 20-bean samples in a temperature-controlled cabinet at 25° C and in darkness, and (broken line) eight similar samples in the same cabinet but in light. (B) the concurrent variation in the laboratory just outside the cabinet (solid line) for twelve 20-bean samples on a clockwise rotating platform, 2 rpm, and (broken line) nine 20-bean samples arranged as 3 triplet groups.

21 concurrent 20-bean samples in light on a CW rotating (2 rpm) and a stationary platform in the open laboratory (Fig. 10B). Similarities are notable even in relatively minor fluctuations indicating a continuing day by day paralleling influence of subtle pervasive parameters of the atmosphere in inducing fluctuations in such an elementary phenomenon as water uptake by dry seeds. This comparison confirmed also that a mirror-imaging capacity and tendency also commonly occurs as is particularly evident for the last few November values.

Discussion

A high degree of responsiveness to subtle parameters of the geophysical environment has been repeatedly described over the past century but especially over the past two decades for many kinds of organisms. Substantial variations in such phenomena as spontaneous activity, rates of oxidative metabolism in animals and plants, and response to light have been reported to be correlated with solar and lunar related periods even when the organisms are held in constant conditions (Lang, 1965; Brown, Hastings and Palmer, 1970). This indicates that these creatures are able to reflect relatively minute differences in pervasive correlates of these overt environmental fluctuations, correlates that are able to penetrate into experimental environments in which every obvious factor is held in an unvarying state. The current studies confirm and emphasize this state of affairs and indicate a surprisingly large magnitude of their potential effects.

The relevant variations in the external environment appear to be geoelectromagnetic fluctuations. These latter would be expected to be of the same general order of magnitude as those electromagnetic fields generated by the organisms themselves and in whose mutual fields organisms placed in close proximity to one another are found. The field of the organisms might be expected to effect a modification of the natural ambient field comparable in degree to weather-associated and geophysical-period-related alterations.

The biological fluctuations induced by subtle environmental changes, in view of their relatively large ranges, would be predicted to elicit compensatory reactions through the servomechanisms normally operating in homeostatic regulation (Brown, 1972). The activities of these mechanisms would superimpose their own modification upon any immediate responses of the biological systems to the physical factors.

The most plausible and consistent hypothesis to account for the remarkable positive and negative correlations as well as the absences of correlations that have been described in the current study seems to be that any bean sample is potentially able to adopt either a positively or a negatively correlating relationship with whatever the effective, steadily varying subtle geophysical environmental parameter which is involved. Abrupt, inexplicable apparent sign changes with time have been noted and found very frustrating over the years by many investigators who have attempted to discover correlations of diverse biological phenomena in controlled, presumably constant, conditions with geophysical parameters including barometric pressure, atmospheric temperature, background radiation, and primary cosmic radiation (Brown, 1959, 1960, 1968; Brown, Webb and Bennett, 1958). The last one was probably a consequence of response to geomagnetic variation. Sign-

changing correlation of rat activity with sunspot number has also been noted (unpublished records from earlier studies by one of us, F. A. B)

If this sign-changing capacity exists for an organism and, in this instance, for a small cluster of beans then it would appear from the present observations that each of the members of the paired samples of beans, even when present in separate glass or plastic containers, can somehow be influenced by a very weak electromagnetic field produced by the other. By some means, the adoption of a "positive" state in one member of a pair must under some circumstances bias the other member of the pair within their mutual field to adopt the "negative" state. Correlating with opposite sign with the still uncontrolled effective parameter or parameters of the fluctuating pervasive physical environment, the negative correlations over substantial ranges of rates would be thereby effected. Variations in the physical environment calling forth greater organismic response than within this range, would appear often to overwhelm the influence from the mutual association between the members of the pair and under normal conditions lead to a superimposed larger-range positive correlation.

In the instance of the beans on the rotating platform in the preliminary study, such a hypothesis would account for the results if the beans in one bowl biased the sign of the beans in the two closer other bowls toward possessing a sign opposite from itself. Thereupon the beans of the diagonally-opposite bowls would be expected to be, in turn, biased to the opposite sign of its two closest bowl partners. The members of one diagonal couple would be predicted to be mutually "positive," and the other mutually "negative," and a good positive correlation could be under these circumstances the product of relating the diagonal pairs, just as was observed. Under other environmental circumstances, with biasing of the two members of closely apposed pairs to the same sign, the members of a second pair more distant but still within effective range might be biased jointly to the opposite sign as in the lead plate and electrical field shielding series.

It seemed less plausible that the two members of a pair of bean trays located within 7 to 35 cm of one another would be subjected to sufficiently different fields derived from their physical environment to tip the two to oppositely correlating signs as consistently as has been observed. And too, in the rotated groups of beans, all four trays on a given table in effect, over time, occupied the same detailed laboratory site.

It is also of interest in the light of the totality of the results to reexamine, for instance, Figure 1, Figure 3C and Figure 4B and others. In these, the correlations, positive or negative, have been significantly diminished by presence of values which seemed to suggest simultaneous but weaker tendency for correlation with the opposite sign.

The rotating magnetic field is postulated to interfere completely with whatever the mechanism of interaction among the bean samples and yield a state of affairs similar to that observed when bean samples are separated from one another by distances of half a meter or more. Comparable low frequency, relatively weak, rotating magnetic fields have been reported to alter behavioral development in rats (Persinger, 1969; Persinger and Foster, 1970).

One of the interesting aspects of these findings pertains to the current views as to general nature of processes involved during the first few hours of water absorp-

tion by a seed. Only physical processes have been considered to be significant over this period. The results of this investigation, on the other hand, indicate that biological factors probably play a substantial role. Interaction between organisms and organisms and their ambient environment through electromagnetic fields have both been postulated by Presman (1970) to occur. While it is admittedly difficult to credit that biological interactions of this nature can occur, it is perhaps even more difficult to imagine how such negative correlations could be generated in terms solely of interactions between two isolated simple physical systems. *Positive* correlations could, obviously, be readily explicable and indeed have been predicted, but not *negative* ones. It seems reasonable to postulate, therefore, that the embryo within the seed possesses the capacity to regulate within limits the rate of water uptake within that seed, and even in adjacent seeds, other factors equal. It is significant in this connection that a circadian rhythm has recently (Bryant, 1972) been reported for gas uptake in onion seeds.

It is suggestive that a maximum in rate of water absorption during the nine months of this study occurred in late July and August (Fig. 5), and a broad minimum seemed to be occurring in November (Fig. 10). This phenomenon, therefore, appears to involve subtle environmental parameters in common with those responsible for other reported persistent annual variations (see Brown, Hastings and Palmer, 1970), which similarly pass through their maximum and minimum at these same times of year.

It seems probable that the general phenomenon of organismic interaction by very weak electromagnetic fields that has been disclosed in this study is widespread among living things, if not universal. If this is true it carries great implications for all of regulatory biology. It is evident that precise reproducibility of results in time, at least when one is dealing at levels of integrated and functional biological systems will be unlikely. The quantitative, and often even qualitative, character of results may be in part determined by uncontrolled factors even as subtle as the proximity of other individuals of the same, or possibly even different, species as well as by time within the now widely acknowledged relatively predictable, solar and lunar circadian cycles, and monthly and annual ones. Less predictable variations associated with movements of weather systems, and fluctuations in solar activity may also be expected to impose *significant* influences.

And not least, the existence of the phenomenon indicates that we are operating within the range of a biological "uncertainty principle." There is now clear reason to presume that the uses of modern methods, facilities and equipment for making precise measurement of diverse parameters in living systems exert of themselves an influence upon the system being measured, an influence effected by the invariable and characteristic weak accompanying alterations in electromagnetic fields produced by these. Biological processes will reflect in their measured values the methods and conditions under which the measurements are made, and the differences may be substantial.

The relatively large range in variation which appears to be associated with the sign of biological correlation with subtle geophysical factors suggests potential practical roles in the health sciences. Biometeorology with its medical aspects includes a significant influence of weather correlates on living systems. If these in-

fluences may be in either of two directions, as evidence now indicates, and based upon positive and negative correlating relationships, one of these signs can be expected for any given time and circumstances to be deleterious relative to the other. Increased understanding of the factors determining correlation sign together with discovery of the means to regulate living systems at will in this respect could quite conceivably provide a basis by which clinical crisis intervals might be alleviated, or bypassed with decreased stress.

Indeed, in this last connection one can speculate that the reversible correlating sign and its modifications by interaction between organisms or groups is biologically adaptive. Viewing the need for maintenance of a species as transcending survival of single individuals or single groups, the species is steadily provided with two possibilities for survival, + and − states, in its response to the natural fluctuations in its geoelectromagnetic environment.

Summary

1. The mean rate of water uptake by beans during the first four hours of their submergence in water varies substantially from day to day, even when in presumed "constant" conditions.

2. The variations in rate in independent samples at different laboratory sites and even at widely different geographic ones may show a strong positive correlation not explicable in terms of variations in any obvious factor.

3. Superimposed on a major positively correlating state, is a secondary, shorter-period fluctuation wherein the correlation between different groups of samples may exhibit either a positive or a negative correlation.

4. Groups of organisms in closely juxtaposed vessels may bias one another to adopt *opposite* sign of correlation under some conditions and the *same* sign under others.

5. This interorganismic biasing may be prevented by having the paired vessels in the very weak field of a very slowly rotating (2 rpm, CW) horizontal bar magnet, or by separating the vessels by 70 cm or more. In either case, the separate vessels then appear to correlate positively and negatively with about equal frequency, indicating independence of one another.

6. Pairs of vessels upon rotating tables at 6 rpm are modified in their interactions in manners dependent upon direction of rotation. Other factors equal, CW rotation appears to favor positive correlation between members of pairs, CCW rotation favors negative correlation.

7. Platform rotation even at the very slow rate of one revolution a day (CW) appears to effect an alteration in the character of the interaction between closely apposed bean samples.

8. Partial electrical shielding by copper plates and experimental alteration of the ambient background radiation by a lead plate modify the rate of water uptake by beans.

9. The interactions between organisms and organisms and their subtle physical environment as they determine positively and negatively correlating states are able to yield biological differences, even on the order of a 2-fold range, concurrently and under the same conditions of all obvious environmental factors.

10. Parallel and concurrent variations in bean samples as widely separated as Woods Hole, Massachusetts and Evanston, Illinois, suggest wide geographic scope of at least one of the major effective subtle parameters.

11. The nature of the phenomenon for beans is of such character that it appears probable that the living embryo within the dried seed possesses the capacity to regulate to a substantial degree the rate of water absorption by the seed upon its submergence.

12. On the presumption that the phenomenon that has been treated herein is a universal biological one, the implications of these findings are great. They relate to (a) reproducibility of experimental results, (b) biological influence of the weak fields of diverse facilities and equipment employed for measurement of biological phenomena, (c) additional and subtle means through which man's alteration of his environment may influence living creatures and (d) potential practical applications of knowledge of the subject for the health sciences.

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EFFECTS OF SPERM CONCENTRATION, SPERM AGING, AND OTHER VARIABLES ON FERTILIZATION IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.

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Although the horseshoe crab, *Limulus polyphemus* L. and related species have been extensively used in reference to embryology (Lockwood, 1870; Packard, 1885; Kingsley, 1892; Iwanoff, 1932; Roonwal, 1944; and Sekiguchi, 1970), only recently has this species been used for significant fertilization studies. Such studies have involved fine structure of the initial sperm-egg interactions (André, 1963; Shoger and Brown, 1970), immunological approaches to these interactions (Cooper and Brown, 1972; Mowbray and Brown, 1973), and stereoscan observations (Brown and Humphreys, 1971). In examining fertilization in *Limulus* one must be impressed by the large number of spermatozoa (10^5 to 10^6) which can simultaneously attach to the egg surface and still allow normal development to occur. Since this phenomenon can be readily demonstrated, the present study is a quantitative approach to examine the effect of sperm concentration on fertilization and development.

Although in some animal species (*e.g. Arbacia*) a single sperm can approach, attach, penetrate and fertilize an egg (Lillie, 1919; Colm, 1918), in most species the normal number of spermatozoa attaching to or in the immediate vicinity of the egg to be fertilized is usually greater than one (*cf.* Cohen, 1971; Austin, 1969a). Also, as demonstrated in *Arbacia* and other species too many spermatozoa contacting the egg simultaneously can cause pathological polyspermy resulting in abnormal development (*cf.* Austin, 1969b). Thus, we might examine the roles of several spermatozoa during fertilization. As readily demonstrated in several species of crustacean decapods and in *Limulus* large numbers of spermatozoa normally attach to the egg surface (Binford, 1913; Brown, 1966; Hinsch, 1971; Shoger and Brown, 1970). In these species each sperm not only attaches but also undergoes an acrosome reaction, penetrating the egg envelope, but not necessarily the egg plasma membrane. Better known are the conditions in various species of mammals, where many reacted spermatozoa are found associated with the cumulus and corona cells and the zona pellucida (Overstreet, 1970; Bedford, 1970; Franklin, Barros, and Fussell, 1970; Menge, 1971). Thus, in conclusion, during fertilization the phenomenon of several or many spermatozoa contacting and penetrating the egg envelope is common in species of diverse phylogeny. In many of these species, the initial contact and close associations with the egg envelope can be referred to as polyspermic attachment.

Since in *Limulus* polyspermic attachment occurs and each sperm undergoes the acrosome reaction simultaneously, the ultimate question concerns the control of sperm penetration into the egg proper. This study therefore involves in *Limulus*

an initial approach to determine the apparent necessity of a high sperm concentration in facilitating fertilization and some elucidation of the mechanisms involved. Following are observations demonstrating the unique relationship of sperm concentrations and normal development.

Materials and Methods

Specimens of the horseshoe crab, *Limulus polyphemus* L., were collected in the initial stages of this study from breeding grounds on Shackleton Banks near Beaufort, North Carolina, during the summer of 1969. In later experiments animals were either obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, or from the Florida Marine Biological Specimen Company, Panama City, Florida. Specimens were kept in running sea water tables at the Duke University Marine Laboratory (summer, 1969) and in "Instant Ocean" Culture Aquaria (15° C) at Iowa State University. Including all experiments approximately 50 male and 25 female horseshoe crabs were used.

In performing the studies described in this paper, a standard experimental procedure for fertilizing *Limulus* eggs was only established after numerous trials and errors. Following is a description of this standard procedure which quite obviously includes some variables referred to in the next section. Gametes were usually obtained by electrical stimulation after procedures previously described (Shrank, Schechtman, Shoger, and Bishop, 1967). The semen was obtained "dry," routinely diluted in sea water to a 10% sperm concentration, and stored at 4 to 5° C for 72 hours. Before using in experiments, the spermatozoa were checked for viability by placing one drop on a microscope slide containing egg sections (Mowbray and Brown, 1973). In most cases, except where variables were being tested, 1 ml of a 10% sperm concentration was mixed with approximately 100 eggs in a plastic centrifuge tube (the eggs adhere to glass). The eggs in all cases were collected in oviductual fluid ("dry") and immediately mixed with the appropriately prepared sperm concentration. These sperm-egg suspensions were allowed to sit with an occasional swirling for approximately 5 to 10 minutes or 25 to 30 minutes, washed several times, placed in parafilm-lined syracuse dishes with streptomycin-penicillin sea water (Perkins and Menzel, 1964) and observed for development. The per cent of development was based on the number of inseminated eggs to develop to the limb bud stage or into swimming larvae. Unfortunately, it usually took 8 to 10 days (room temperature, 22–25° C) before definitive development was observed. The variables tested in methodology were: (1) different types of egg collecting, (2) effects of seminal fluid, and (3) duration of sperm-egg mixing. After standards were obtained in these methods, experimentation involved the effects of different sperm concentrations and aging of spermatozoa on development.

Observations

Methods of collecting eggs

To observe if the per cent of development was related to the method of collecting, eggs were collected by three different methods: electrical stimulation, dis-

section, and oviduct caressing. The electrical stimulation method was referred to in the previous section. The dissection method involved killing the animal by exsanguination followed by immersion in warm water, dissecting away the dorsal region of the prosoma, and removing eggs directly from the long oviducts. The oviduct caressing method consisted of pressing the oviduct externally with the index finger. By moving the finger ventrally towards the genital opening, eggs could be forced out. In all cases, the collected eggs were immediately mixed with spermatozoa. In electrical stimulation 77.6% development resulted, with dissection 72.5% and with caressing method 57.0%. Since electrical stimulation was the most convenient, it was used in all following experiments.

Effects of seminal fluid on development

In order to determine the effects of seminal fluid on development, 5 ml of freshly prepared 10% sperm concentration was washed thrice by mild centrifugation with 10 ml of sea water and stored for 72 hours at 5° C before mixing with eggs. For comparison, another 5 ml of a 10% sperm concentration was treated similarly except it was left unwashed. In comparing washed spermatozoa (free of seminal fluid) and unwashed spermatozoa, a high per cent of development was obtained in both cases, 70.0% and 64.1% respectively. Since the difference in development between both types of sperm concentrations was insignificant, unwashed sperm concentrations were used in all experiments.

Duration of sperm-egg mixing

In order to determine an optimal time for sperm-egg mixing, approximately 500 eggs were mixed with 5 ml of a 10% sperm concentration. Immediately after mixing and at the following intervals: 5, 15, 30, and 60 minutes, samples of approximately 100 inseminated eggs were removed and washed. In addition, after each time period the sperm-egg mixture was examined for sperm motility. As noted in Table I, the per cent of development for each sample was reasonably high with the exception of the 15 minute sample. As a result either 5 to 10 minutes or 25 to 30 minutes were used as a standard time of mixing. As was noted when observing sea water samples from each sperm-egg mixture, sperm motility generally involved more than 90% of the total number of spermatozoa. Although the greatest decrease of sperm motility occurred after 4 to 5 minutes, a few motile spermatozoa were observed 10 to 15 minutes after mixing.

TABLE I

Duration of sperm-egg mixing

Time of mixing (min)	Number of experiments performed	Number of different females used	Number of eggs	Number of eggs developing	Per cent development
1	3	2	242	224	92.6
5	3	2	319	206	64.6
15	3	2	309	128	41.4
30	3	2	278	195	70.2
60	3	2	264	197	74.6

TABLE II

Aging of spermatozoa

Hours of aging	Number of experiments performed	Number of different females used	Number of eggs	Number of eggs developing	Per cent development
0–1	3	2	331	143	43.2
24	3	2	393	203	51.7
48	3	2	383	262	68.4
72	3	2	501	418	83.5
96	3	2	371	282	76.1

Aging of spermatozoa

For determining the effects of aging on sperm viability, 5 ml of a 10% sperm concentration was stored at 4 to 5° C for 96 hours. At each of the following intervals: 0, 24, 48, 72, and 96 hours, 1 ml of this sperm concentration was removed and mixed with approximately 100 eggs and the percent of development determined. As noted in Table II, the older sperm concentrations (24 to 96 hours) gave higher development than freshly collected spermatozoa. There was no testing beyond 96 hours. Since 72 hours of aging gave the best result (83.5%), this period of aging was generally used for all 10% sperm concentrations before they were used in experimentation.

Sperm concentration and development

In testing the effects of sperm concentration on development, a 10% sperm concentration (stored for 72 hours at 4 to 5° C) was serially diluted so that 1 ml of each of the following sperm concentrations was obtained: 10%, 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001%. In addition 1 ml of a 100% sperm concentration was used. Since previous studies with extensive sperm dilutions in sea urchins showed a rapid loss of fertility during aging (Cohn, 1918), each of these sperm concentrations was immediately mixed with eggs. This was only a precautionary step since recent experiments in our laboratory have demonstrated that the lower sperm concentrations maintain the same fertility level at least 1 hour, a time well within the confines of this experiment.

The effects of serially diluted sperm concentrations on development are tabulated in Table III. Although some development was observed with all per cents of sperm concentration used, the higher average per cents: 68.3, 66.3, and 76.8, occurred with 1%, 10%, and 100% sperm concentrations, respectively. A noticeable decrease in development occurred with 0.1% sperm concentration (33.3%) and became more obvious at 0.01% (17.6%) and 0.0001% (2%) sperm concentrations.

Because of the recent emphasis on a quantitative approach to fertilization (Cohen, 1971), the number of spermatozoa for each sperm concentration was determined with a hemocytometer. From this the approximate number of spermatozoa per egg for each sperm concentration was determined (Table III). Finally, in order to double check the viability of spermatozoa and to determine an

TABLE III

Sperm concentration and per cent of development

Sperm concentration in per cent	Number of experiments performed	Number of different females used	Approximate number of spermatozoa/ml*	Approximate number of spermatozoa egg*	Calculated number of attaching spermatozoa/egg	Number of eggs	Number of eggs developing	Per cent development
100	2	2	10^{10}	10^{8}	—	150	115	76.7
10	11	10	10^{9}	10^{7}	626,000	925	613	66.3
1.0	11	10	10^{8}	10^{6}	118,000	972	664	68.3
0.1	11	10	10^{7}	10^{5}	23,000	936	312	33.3
0.01	10	9	10^{6}	10^{4}	7,400	830	146	17.6
0.001	7	6	10^{5}	10^{3}	750	570	44	7.7
0.0001	5	4	10^{4}	10^{2}	130	499	10	2.0

* In all experiments, approximately 100 eggs were mixed with 1 ml of each sperm concentration.

approximate number of spermatozoa actually attaching to each egg, egg sections were employed. The methods for using *Limulus* egg sections are described in a separate paper (Mowbray and Brown, 1973). Basically, they involve freezing of whole eggs, frozen sectioning at 10 to 12 μ, mixing of 0.1 ml of sperm concentration, and the counting of spermatozoa attaching to a known egg area. From these sperm counts the approximate number of spermatozoa attaching to the whole egg could be determined with each sperm concentration by multiplying the sperm counts per egg section area (0.0056 mm^2) by the surface area of the whole egg (9.42 mm^2). For example, with an 1.0% sperm concentration the sperm count was 70.3, thus allowing a calculated 118,000 spermatozoa attaching to the whole egg (Table III). For each sperm concentration, the ratio of the total number of spermatozoa to egg surface area was approximately the same, regardless of whether using whole eggs or egg sections.

DISCUSSION

Although the emphasis in this study is placed on sperm concentration and sperm aging and their effects on fertilization, the usage of a new research animal, *Limulus polyphemus*, in fertilization studies is also demonstrated. In addition, these studies with this species gametes are particularly unique because of *Limulus* phylogenetic relationship and the study of arthropod fertilization, which is unquestionably difficult due primarily to the evolutionary adaptation of this group to internal fertilization. In reference to the methods for this study the following variables are discussed: methods of collecting eggs, effects of seminal fluids, and duration of sperm-egg interactions.

As demonstrated in this study, fertilization and larval development are readily performed in the laboratory. The availability of *Limulus* and the ease in obtaining an abundance of viable gametes year around certainly enhances the usefulness of this species in fertilization studies. In reference to obtaining gametes, the electrical stimulation method has proven quite satisfactory, although unfertilized eggs, unfortunately, frequently became activated shortly (usually 5 to 20 minutes) after collecting and appeared to be developing normally for as long as

four days before they deteriorated. Other methods of collecting did not significantly prevent this egg activation. The presence or absence of seminal particles or fluids does not effect fertilization, although they probably play a very important role during sperm storage and sperm inactivity in the male animal. An adequate duration for mixture of the gametes is 5 minutes (Table I). Interestingly, this time is directly related to the duration of sperm motility which is 4 to 5 minutes from initial mixing. This implies that the sperm functional time is quite short unless attachment to the egg occurs. One side effect of mixing times is the low per cent of development occurring when eggs are washed 10 to 15 minutes after the mixing of gametes. Presumably a disturbance of a sensitive sperm penetration event during this time causes a malfunctioning in fertilization.

The aging of a 10% sperm suspension at 4 to 5° C for three days increases the per cent of development (Table II). Although no adequate explanation presently exists for this phenomenon, it is interesting that an increase in fertilizability of stored semen has also been observed with bull semen (Salisburg and Hart, 1970) and with chicken semen (Lodge, Fechheimere, and Jaap, 1971), although the latter case was *in vivo*. In their study, Salisburg and Hart interpreted this improvement as the selective death of abnormal spermatozoa. Stored *Arbacia* semen did not produce this effect (Goldfarb, 1918).

In *Limulus* a high sperm concentration per egg definitely enhances a higher per cent of development. As shown in Table III a 1% sperm suspension (1,000,000 spermatozoa/egg) resulted in 68.3% development of eggs into swimming larvae. In comparison, a 0.01% sperm suspension (10,000 spermatozoa/egg) and a 0.0001% sperm suspension (100 spermatozoa/egg) resulted in 17.6% and 2% respectively, a rapid decrease in development even though many spermatozoa were present. Although not numerically the same, there is an interesting comparison between *Limulus* and rabbit fertilization. Overstreet (1970) has observed that a high number of capacitated spermatozoa (10,000+) injected into the oviducts of ovulated rabbits ensures a higher per cent (90% or better) of fertilization and as the sperm number is reduced to 500–1500, a considerably lower per cent (30–50%) of development takes place.

In *Limulus*, many spermatozoa (approximately 10^6) can actually attach to the egg surface and normal development ensues (Brown and Humphreys, 1971). After attachment each sperm undergoes the acrosome reaction and forms an acrosomal filament which penetrates the tough egg envelope (Shoger and Brown, 1970). Interestingly enough, polyspermic attachment (or similar association) has been observed to occur normally in other species, for example, decapods (Binford, 1913; Brown, 1966; Hinsch, 1970), insects (Huettner, 1927), and some mammals (rabbits, Bedford, 1970). Studies by Bedford (1970) and Overstreet (1970) show that many spermatozoa (several hundred) come into contact with the rabbit egg cumulus and many have actually been observed to penetrate into the perivitelline space surrounding each egg. Mammal spermatozoa in contact with the egg cumulus and zona pellucida release the enzymes, acrosomal hyalauronidase and acrosomal proteinases, which aid sperm penetration (Yanagimachi and Teichman, 1972). Supposedly, a greater chance of sperm penetration occurs with numerous spermatozoa releasing these enzymes (*cf.* Austin, 1969a). Since sperm penetration studies in *Limulus* are not complete, whether a similar role could exist

can only be speculated. However, the existence of an acrosomal proteinase has been determined (Bennett and Brown, in preparation). Perhaps, in *Limulus,* a lytic or chemical reaction could result from the large number of reacted spermatozoa, forming canals or causing chemical changes in the egg envelope thus facilitating the movement of one or a few sperm nuclei towards the egg plasma membrane. An alternative would be that the process of penetrating the egg envelope (approximately 45 microns thick) may be selective enough to allow only certain genetically determined spermatozoa or those on specific penetrable sites to pass through the envelope. Quite possibly there may be a combination of these processes. Unfortunately, at present, whether or not polyspermy penetration occurs in *Limulus* is unknown. Since this phenomenon is common in many arthropods (insects, Huettner, 1927; Piko, 1961), the possibility does exist.

In considering all the aspects suggested above and in a superficial answer to the pleas of Cohen (1971) one cannot help but to reflect on the evolutionary significance of a large number of spermatozoa attaching to an egg. This reflection is enhanced by the fact that other arthropods (insects, decapods) share this phenomena, although not to the same degree. Judging from this study, apparently the answer is not merely a large number of reacting spermatozoa chemically facilitating the penetration of one or several spermatozoa, since even a low sperm concentration can occasionally fertilize an egg. One must speculate on why so many spermatozoa attach to *Limulus* eggs whereas other species have evolved various ways (micropyles, sperm dilution, complex reproductive systems, *etc.*) to reduce the sperm number reaching the egg. Apparently in *Limulus* selection for sperm number reduction has taken place within the sperm envelope, so that the selective mechanism does not affect spermatozoa simultaneously binding to the egg surface, but operates within the egg envelope allowing only a minimal number of sperm nuclei to reach the egg plasma membrane. Thus, it is possible that this envelope selective mechanism is variable enough so that while normally large numbers of spermatozoa are necessary for fertilization, occasionally small numbers can also successfully fertilize the egg. This latter would account for the observed development occurring with very low sperm concentrations.

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Summary

The horseshoe crab, *Limulus polyphemus,* is an excellent species for fertilization studies since viable gametes can be easily obtained on a year around basis.

In reference to methodology for *Limulus* fertilization studies, the following prove to be adequate: collection of gametes by electrical stimulation, unwashed or washed spermatozoa (free of seminal fluid), and 5 to 10 minutes duration for sperm-egg mixing.

The aging of a 10% sperm concentration at 5° C for three or four days increases the per cent of embryonic development. This increase may be due to the death of abnormal spermatozoa.

Higher sperm concentrations definitely enhance higher per cent of development. With the dilution of sperm concentration, the per cent of development was drastically reduced even though several hundred spermatozoa per egg were present. The significance of the high number of spermatozoa is speculated as a necessity for (1) the chance penetration of a special site, (2) the release of sperm enzymes causing a chemical lysis in the egg envelope, or, more favorably, (3) a selection mechanism determined by the egg envelope for special genetical spermatozoa.

The number of spermatozoa per egg is calculated for each sperm concentration. In addition, a method for calculating the number of spermatozoa actually attaching to each egg is formulated.

Finally, some speculations on the evolution of such a system as found in *Limulus* are presented.

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DAILY RHYTHMS IN CONCENTRATION OF PLASMA CORTISOL IN MALE AND FEMALE GULF KILLIFISH, *FUNDULUS GRANDIS* [1]

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Daily rhythms of adrenal corticoids have been implicated recently in the regulation of fat stores in vertebrates (review, Meier, 1972). In intact *Fundulus grandis* maintained in continuous light, injections of cortisol entrain a 24-hour rhythm of fattening responses to mammalian prolactin (Meier, Trobec, Joseph and John, 1971). Daily injections of prolactin given 18 or 24 hours after daily injections of cortisol promote increases in fat stores whereas injections of prolactin given 6 or 12 hours after cortisol injections cause decreases in body fat.

Although considerable information regarding the daily rhythm of concentration of plasma adrenal corticoids is available for mammals, the possible existence of such rhythms in fish has received little attention. Daily rhythms of plasma adrenal corticoids have been reported in the male channel catfish, *Ictalurus punctatus* (Boehlke, Church, Tiemeri and Eleftheriou, 1966), and a daily variation in plasma cortisol has been reported in the male *Fundulus grandis* investigated during an 8-hour portion of the day (Srivastava and Meier, 1972). Both studies of fish and most of the studies of mammals and birds were carried out with males only under carefully controlled conditions in the laboratory. Because various stresses may be expected to stimulate increases in plasma concentrations of adrenal corticoids in vertebrates, including fish (Donaldson and McBridge, 1967), the possibility that the rhythms of adrenal corticoids have important physiological roles depends on whether the rhythms exist under natural conditions. This study was performed with *Fundulus grandis* to determine the concentrations of plasma cortisol throughout the day in male and female fish taken from a brackish lake.

Materials and Methods

Blood, gonads and intact carcasses of male and female specimens of *Fundulus grandis* were collected during June and August, 1971. The study site is a deep thirty-six acre brackish lake about 80 miles south of Baton Rouge, Louisiana. During both months of the study the salinity was 115 milliosmoles. Surface temperatures ranged from a low of 29° C at dawn to a high of 31° C nine hours after dawn in June and in August. The temperature on the bottom near the shore at a depth of 0.5 meters was a constant 29° C in both months. Sunrise and sunset on June 6, the first collection date, were at 0502 and 1905 Central Standard Time, respectively. On August 8, sunrise and sunset were at 0527 and 1853 C.S.T.

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Daylength on June 6 is longer by 37 minutes than on August 8. The moon was in the full phase in both months of the study.

Specimens of *Fundulus grandis* weighing 8–20 grams were collected by seining five days prior to the date of exsanguination and distributed equally by number and sex among six hardware cloth holding cages. These cages were positioned in the shallow (depth 0.5 m) water near the shore where the fish are generally found. Aquatic vegetation of the type frequented by *Fundulus grandis* was placed in each cage to provide a refuge for the subordinate individuals. The cages were watched continuously and were not disturbed during the waiting period. During the days prior to exsanguination the fish in the cages exhibited behavior similar to the uncaged fish in the area, including feeding, schooling, and (in males) aggressive display.

Collection of blood was conducted at one hour after dawn (30 minutes after sunrise) and at every fourth hour thereafter for the next twenty hours. At each time of collection, a single cage was removed from the water and the fish were placed in an anesthetizing solution of tricaine methane sulfonate (Sigma) having a concentration of one gram per liter. The fish were immobilized within thirty seconds. Blood was collected directly from the heart with a heparinized capillary tube. Three workers were able to complete the exsanguination of a group of up to fifteen fish well within fifteen minutes. Blood from each fish was labelled according to the fish's order in the sampling sequence, so that a possible change in circulating levels of cortisol initiated by the disturbance could be determined. A single fish usually yielded sufficient blood for three 10 μl samples of plasma. Following the collection, the blood was temporarily stored on ice at the study site. There were 27 blood samples in June and 39 in August that were taken from males, and 30 samples in June and 44 in August that were taken from females. Except for the samples taken from the males in June, the numbers of samples were approximately equal for each of the 6 daily sampling times. The blood specimens taken at 0200 from males in June were accidentally lost.

Upon arrival at the laboratory, the blood was centrifuged and the plasma was separated from the packed cells and stored by freezing. Plasma adrenal steroids are stable for many months if kept frozen (Guillemin, Clayton, Lipscomb, and Smith, 1959). Plasma concentrations of cortisol, the major corticosteroid in poeciliid fishes (Chester Jones, Chan, Henderson and Ball, 1969) were determined using the competitive protein-binding radio-assay of Murphy (1967) using human plasma as the source of CBG and Florisil as adsorbent. For each assay, one sample from each time group was taken in triplicate. Tritium counting was done with a Beckman Liquid System using toluene scintillation solution containing 2,5-diphenyloxazole (0.3% w/v), 1,4-bis-2(5 phenyloxalyl)-benzene (0.01% w/v) and Triton-X100 (2:1 by volume). To test whether other plasma steroids in *Fundulus grandis* might interfere prohibitively, plasma cortisol was measured directly and after separation by thin layer chromatography. In male specimens of *Fundulus grandis* maintained indoors on a 12-hour photoperiod, the results were comparable for blood collected at 1, 5, and 9 hours after the onset of light.

The gonadal weights and fat stores were determined in 20 randomly selected fish in June and again in August. The gonads were preserved immediately in ethanol and weighed after several days. The gonadal weight is expressed as a

percentage of the fresh weights of the fish (gonadosomatic index). The total body lipid was determined by Soxhlet extraction with ether and is expressed as a percentage of the dry body weight (dry lipid index).

Results

Gonadal regression similar to that observed by Matthews (1939) in *Fundulus heteroclitus* occurred in both sexes of *Fundulus grandis* between June and August (Fig. 1). The gonadosomatic index in males was 0.36 ± 0.03 in June and 0.24 ± 0.03 in August. In females, the gonadosomatic index was 4.31 ± 0.23 in June and 1.43 ± 0.36 in August.

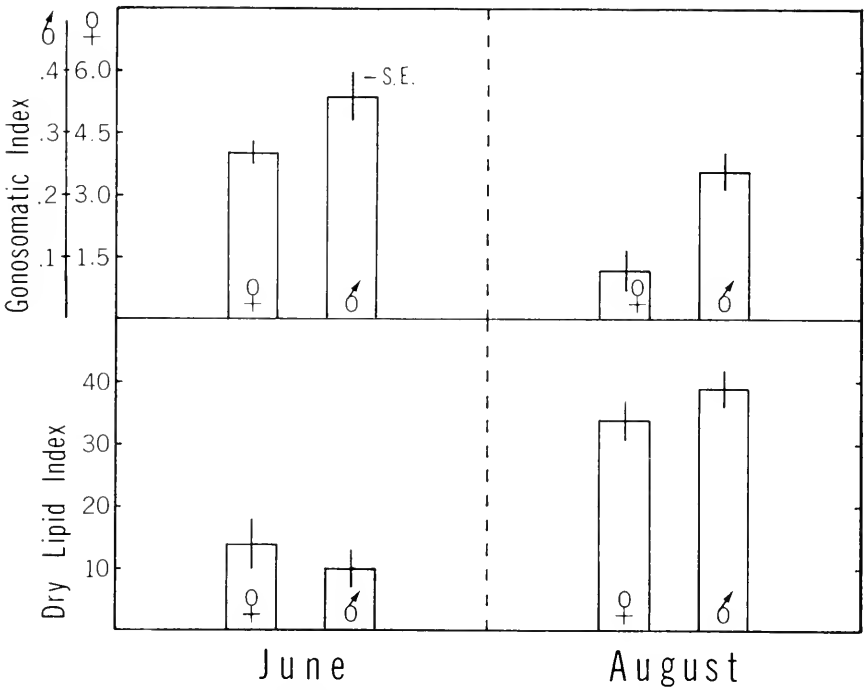

Figure 1. Gonadosomatic and dry lipid indexes in June and July.

The regression of the reproductive system between June and August is accompanied in both sexes by an increase in fat stores (Fig. 1). The dry lipid index rises from 12.10 ± 0.39 in June to 34.75 ± 4.0 in August. There is no significant difference in fat content between the sexes in either month, and there is no correlation between dry lipid index and whole body weight in either month.

The plasma cortisol content (p.c.c.) of all the fish averaged 21.9 μg% in June and 23.2 μg% in August. These overall seasonal means do not differ significantly. The overall means of the sexes are the same (males: 22.5 ± 1.3; females: 22.5 ± 1.1). However, there are marked variations in the p.c.c. of both male and female *Fundulus grandis* during a twenty-four hour period (Figs. 2 and 3). According

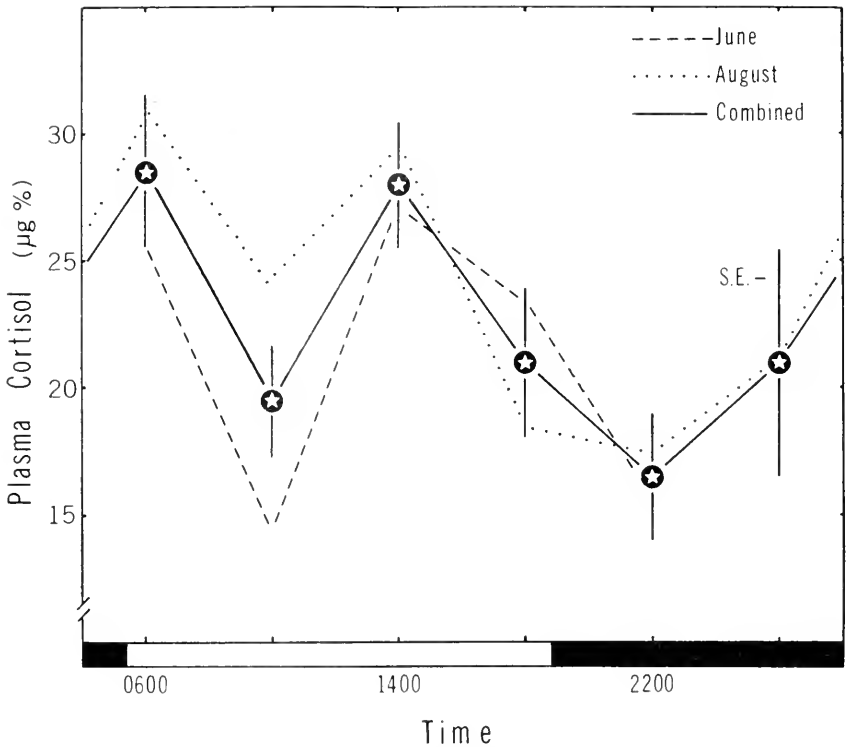

FIGURE 2. Daily rhythms in concentration of plasma cortisol in male specimens of *Fundulus grandis* in June and August.

to a least-squares analysis of variance (Snedecor and Cochran, 1967), the daily variations of the combined data of June and August are significant ($P < 0.05$) for each sex. In addition, the rhythm of each sex is distinct.

According to orthogonal comparisons (Snedecor and Cochran, 1967), the daily rhythm of cortisol in males has linear ($P < 0.05$) and quartic ($P < 0.01$) relationships, indicating that although there is a general downward trend in the cortisol levels in the twenty hours following dawn (linear trend), the rapid rise observed nine hours after dawn at 1400 is also significant (quartic trend). Thus, the daily cortisol rhythm in males in both June and August is bimodal, with peak values occurring near dawn (0600) and eight hours later.

The daily rhythm of plasma cortisol in female *Fundulus grandis* for June and August combined differs from that in the males in that it bears cubic relationships ($P < 0.05$), indicating the presence within each day of a single alternation between periods of high and low concentrations. The smaller sample size in June is unfortunate in that it may have prevented statistical verification of a possible seasonal change in the time of the daily rise of p.c.c. in female fish. During both months, the peak of plasma cortisol occurs at dawn, followed by a rapid fall within four hours, similar to that occurring in males. In the females, however, there is only one daily

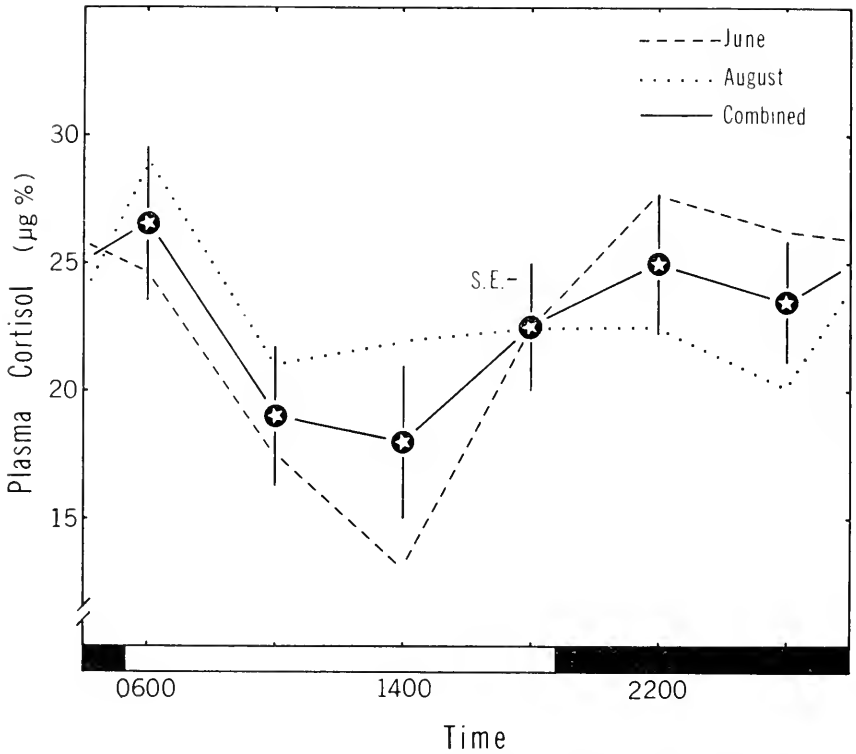

FIGURE 3. Daily rhythms in concentration of plasma cortisol in female specimens of *Fundulus grandis* in June and August.

rise in p.c.c. occurring between 1400 and dusk in June and between 0200 and 0600 in August. High platean levels of cortisol were maintained throughout the night in June.

The disturbance associated with the sampling procedure caused a significant rise in cortisol content within a period of less than fifteen minutes. For the entire group of fish employed in the study, the average increment in p.c.c. for each unit increase in sample sequence number was 0.72 μg%. The regression coefficient (0.72) is significantly different from zero according to analysis of variance of linear regression. The rise in p.c.c. by the end of a fifteen minute series of exsanguinations was about 7 μg%. The data do not permit any conclusions concerning possible changes in p.c.c. occurring in the time interval (30–60 seconds) between the initial disturbance and the first exsanguination.

DISCUSSION

The existence of daily rhythms of plasma cortisol in *Fundulus grandis* is not surprising in view of the widespread occurrence of daily rhythms of adrenocortical hormones in the higher vertebrates. The presence of these rhythms under natural conditions in a fish strengthens the idea that they have important functions.

Temporal relationships between the daily rhythms of prolactin and cortisol have been implicated in the control of fat stores. Prolactin stimulates fat deposition in *Fundulus chrysotus* (Lee and Meier, 1967), *Fundulus kansae* (Mehrle and Fleming, 1970), *Fundulus grandis* (Joseph and Meier, 1971), *Cyprinodon variegatus* and *Fundulus similis* (de Vlaming and Sage, 1972). In all of these investigations, however, the time of injection with respect to the photoperiod proved to be of fundamental importance in determining whether prolactin elicited gains or losses in fat stores. These variations in response to prolactin appear to be the result of daily rhythms of cortisol. In specimens of *Fundulus grandis* maintained in continuous light, daily injections of cortisol entrain daily rhythms of fattening responses to prolactin (Meier, Trobec, Joseph, and John, 1971). That is, daily injections of prolactin given at 6 or 12 hours after injections of cortisol cause losses of fat stores, whereas prolactin injections administered at 18 or 24 hours after cortisol cause increases in fat.

One can derive some interesting temporal correlations by comparing our findings of the daily rhythms of concentration of plasma cortisol with previous studies of *Fundulus grandis* which involve daily rhythms of fattening responses to prolactin entrained by daily photoperiods (Joseph and Meier, 1971) and by daily injections of cortisol (Meier, Trobec, Joseph, and John, 1971). The fattening response to prolactin occurs at 4 to 8 hours after dawn in fish maintained on 8-, 12-, or 16-hour daily photoperiods, and at 18 to 24 hours after daily injections of cortisol in fish maintained in continuous light. In order for cortisol to account for the fattening response to prolactin at 4 to 8 hours after dawn, one would predict on the basis of these findings that the daily rise of plasma concentrations of cortisol should occur about 6 to 12 hours after dawn. This prediction agrees well with our findings in that the first daily increase in plasma cortisol in males occurred about 9 hours after dawn and the daily increase in females occurred about 12 hours after dawn. Inasmuch as the cortisol rhythm is bimodal in the male with a second increase in concentration at 20 to 24 hours after dawn, one would anticipate that another period of fattening response to prolactin might be found in males during the dark, 20 to 22 hours after dawn. Injections at that time of day have not as yet been done in *Fundulus grandis*. For a graphic description of the temporal relations, see Figure 4.

The central role of cortisol in mediating the photoperiodic entrainment of the daily fattening response to prolactin in *Fundulus grandis* is similar to the roles ascribed to adrenal corticoids in the photoperiodic entrainment of other daily responses to prolactin: fattening responses in lizards, birds and mammals; cropsac responses in pigeons; red eft water drive responses in the spotted newt; reproductive photosensitivity and photorefractory responses in sparrows; and migratory restlessness and orientation responses in a migratory bird (reviews, Meier and MacGregor, 1972; Meier, 1972). The close correlation of many of the results obtained by injection of hormones with assays of the hormones (similar to that described in Fig. 4) provides strong evidence that the interrenal system has an important role in photoperiodic entrainment of daily rhythms.

So far as we can ascertain, a sexual difference in the daily rhythm of adrenal corticoids has not been reported in any other vertebrate. It should be noted, though, that the possibility has seldom been taken into account. A comparison of

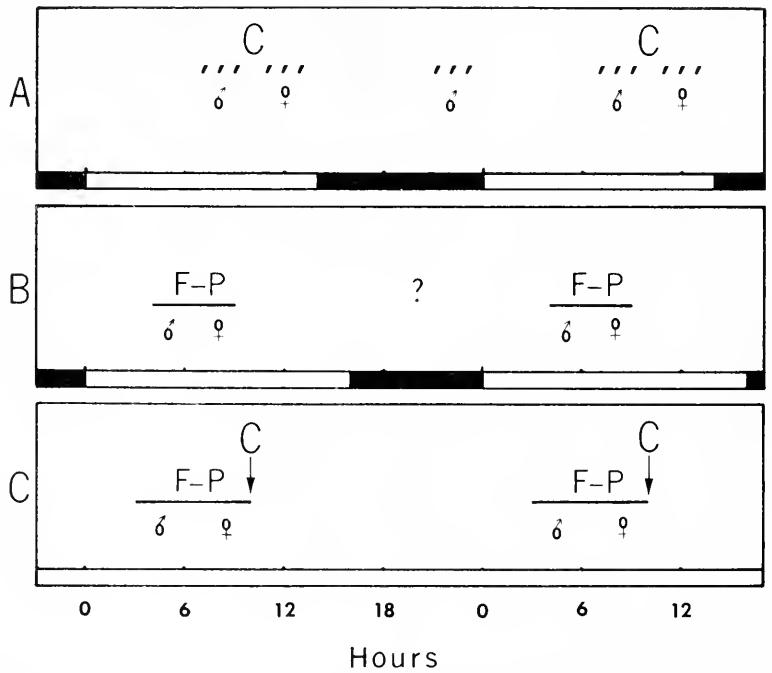

FIGURE 4. Temporal relations of the daily rhythm of cortisol in the photoperiodic entrainment of the daily fattening response to prolactin; (A.) the times of the daily increases in concentrations of plasma cortisol (C) in *F. grandis* outdoor (reported herein); (B.) the time of the daily fattening response to prolactin (F-P) in *F. grandis* maintained on a 16-hour daily photoperiod. Tests were not made during the hours of darkness (Joseph and Meier, 1971); (C.) the temporal relation between daily injections of cortisol and the fattening responses to prolactin in *F. grandis* maintained in continuous light. (Meier, Trobec, Joseph, and John, 1971).

the daily rhythms of the sexes (Figs. 1 and 2) suggests that some factor is influencing the daily rhythm in one sex and not in the other. The most conspicuous difference is the sharp rise in plasma cortisol at 9 hours after dawn followed by a sharp drop at 13 hours after dawn in the male, but not in the female. The possibility that the midday peak in males is a result of interference from gonadal steroids (Murphy, 1967) has been eliminated because this peak is also present after separation of cortisol by thin layer chromatography (see Materials and Methods). In addition, the peak is also present in hypophysectomized *Fundulus grandis* in which the gonadal weights are very small (Srivastava and Meier, 1972). The physiological basis and significance of the sexual difference in the daily rhythm of cortisol remains to be determined.

Because the interrenal gland of all vertebrates is stimulated by adrenocorticotropic hormone (ACTH), it has seemed reasonable to assume that a daily rhythm in concentration of plasma corticoids would be a direct result of a daily rhythm of release of pituitary ACTH. However, when this assumption was tested in hypophysectomized male *Fundulus grandis,* it was discovered that the daily rhythm of plasma cortisol was essentially the same as in intact fish (Srivastava

and Meier, 1972). In addition, the phase of the rhythm may be inverted in both hypophysectomized and intact fish by inverting the 12-hour daily photoperiod. Thus, the existence of the daily rhythm of plasma cortisol as well as the photoperiodic entrainment of the rhythm does not depend on a daily rhythm of pituitary ACTH. It might be expected that the discovery of the mechanism involved in regulating the daily rhythm of plasma cortisol will considerably increase our understanding of endocrinology and biological rhythms. Apparently, cholinergic systems are involved (Meier and Srivastava, unpublished).

The increase in concentration of plasma cortisol after immobilization with tricaine methane sulfonate is in keeping with what is expected in animals after an applied stress. The increase of 7 μg% in fifteen minutes corresponds with an increase of 5.3 μg% (from 8.1 to 13.4 μg%) in *Salmo gairdneri* following half an hour of forced activity in very shallow water (Donaldson and McBride, 1967). An explosive increase in concentration of plasma cortisol (from 6.2 μg% to 11 μg%) has been reported to occur in *Carassius auratus* within 15 seconds of an osmotic challenge with 0.1% NaCl (Singley and Chavin, 1971). However, a similar sharp increase in plasma cortisol following handling disturbances does not occur in *Carassius auratus* (R. E. Spieler, Louisiana State University, unpublished), nor in *Fundulus grandis* (Srivastava and Meier, 1972). The general pattern of change in concentration of plasma adrenal corticoids following a single applied stress in most animals investigated involves a gradual increase in concentration reaching a maximum in 15–30 minutes followed by a decline to normal levels usually within one to two hours.

The increase in plasma concentrations of cortisol following stress might be expected to interfere with any functions controlled by a daily rhythm of plasma cortisol. Recent studies in our laboratory indicate that such is the case in a number of vertebrate species including *Fundulus chrysotus* (Meier, Trobec, Haymaker, MacGregor and Russo, 1973). Disturbances of handling at specific times of day repeated daily for 10 days caused substantial increases or decreases in fat stores depending on the time of day when the disturbances were made. The reproductive system was also stimulated or inhibited depending on the time of disturbance. Under the conditions of the present study which approach natural conditions, whatever disturbances that occur do not obscure the daily rhythm of cortisol. However, appropriate stresses of sufficient intensity and/or duration may be expected to interfere with the daily rhythm of cortisol and with the functions or conditions that are controlled by the rhythm.

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Summary

Daily rhythms of plasma cortisol were found in male and female *Fundulus grandis* examined in a brackish lake in June and August. The rhythm differed in

the two sexes. The rhythm of the males was bimodal; the peaks occurred at one hour and at nine hours after sunrise. The rhythm of the females was unimodal with high concentrations late in the day and at night until one hour after sunrise.

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OBSERVATIONS ON THE SEXUAL BEHAVIOR OF FREE-FLYING *AEDES AEGYPTI* MOSQUITOES[1]

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The first description of copulation of mosquitoes was given by Godeheu de Riville (1760). At the present time the most detailed observations on sexual behavior in mosquitoes are those of Roth (1948), Spielman (1964), and Jones (1973), who worked primarily with free-flying *Aedes aegypti*, and those of Jones and Wheeler (1965) who used mostly the forced-copulation technique. Roth (1948, page 270) concluded that *A. aegypti* mosquitoes ". . . copulate most frequently after the female comes to rest, while copulation partly in flight and at rest is common and copulation exclusively in flight is the least common." He never observed copulation when the male was "astride" the female.

Since Godeheu de Riville (1760), it has been known that *A. aegypti* copulates belly-to-belly or face-to-face, and that the angle formed between the copulating pair varies from 45° to 90°. Once the male is in position he uses mainly his front tarsi to hold the female's hind femora or her other legs. He is also said to use his metathoracic legs to push the female's abdomen or her hind legs just prior to genital contact. After terminalial contact, the male's mesothoracic legs may push on the female's tibiae or tarsi to keep them raised during coitus. Sometimes the claws of the male's metathoracic legs are said to be hooked over the margins of the female's wings (Roth, 1948).

Roth's work (1948) gives the strong impression that copulation occurs almost reflexly, whenever flight is induced. Thus, he states ". . . disturbance induces them to fly and the males immediately pursue the females and seize them" (page 272), and he further wrote that males copulate ". . . at any time of the day, until death, whenever stimulated by flying females" (page 346).

Materials and Methods

All of the observations were made using adults of either the Bangkok or the U. S. Naval Medical strains of *Aedes* (*Stegomyia*) *aegypti* (Linnaeus). The behavior of the mosquitoes was examined while they were confined in either 600 ml or 1000 ml glass beakers with mosquito netting tops or within 1 cu ft cages having one or more clear plastic sides. The insectary was held at 27° ± 2° C and 80% ± 2% R.H. The constant air circulation within the insectary was associated with a rather loud continuous background noise. The insectary was illuminated from 0630 to 1900 hours daily. The observations were made only under well-lighted conditions between 0800 and 1800 hours.

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Observations

Recognition and chasing

Resting males as a rule are not activated to flight by the mere presence of flying females in a 1 cu ft cage. Further, resting males often remain stationary even when females fly within 2 inches of them. In colony cages, males often remain standing despite flights closer than one inch by females, providing a female does not touch him with her trailing legs. Often a female skimming across the surface of a wall may contact a male with her legs and he is then induced to fly. In colony cages, we have seen stationary males which did not fly when females landed very close to them or even when such females happened to touch the males so that the pair were in tarsal contact. In cages containing only one male, if the females come within about 1 inch or less of him, he almost always takes flight very quickly.

If a male is already in flight, the presence of one or many flying females does not necessarily elicit any directed response by a given male. The presence of several males in a cage (some of which may be flying at any given moment) appears to increase the extent of flight by the males as a group so that they can chase and capture more females than solitary males. Males which have been flying in a group for some time seem to be less alert in locating females than those previously stationary males which have just been jostled or otherwise induced to fly either by another male or a female landing on their backs in a colony cage. Females have been seen to fly around or close by stationary or flying males or to cross flight paths with a male and still not be pursued. This was especially noticed with seminally depleted males, but could also be observed even with 3 to 4 day old virgin males which had rotated their terminalia normally and which had extended hairs on their antennae.

Often a flying male was seen to change a presumably undirected flight pattern suddenly, and then rapidly chase a flying female. A pursuing male can cease chasing a given female for no discernible reason and may continue to fly or else land. Occasionally we have observed females whose power of flight and/or maneuverability considerably exceeded those of the males, so that they were able repeatedly to elude any chasing individual from a group. While a very few females successfully avoid capture for extended periods, eventually most of these will be caught. Females can also avoid capture by frequently landing (being air-borne for only a short period at a time), by standing for long periods, or by skimming along the ceiling, floor, or walls at varying heights. Males frequently capture females without a conspicuous or prolonged chase; but, if they do so, males may suddenly fly much faster than normal when chasing some females and often appear to make a series of short zigzags as they get near to one of them.

Capture

During the pursuit the male may fly in one or more loops around a given female. After such a look-over, a male may either attempt to make tarsal contact with a flying female or may cease the chase and generally quickly land or may begin to pursue another female. When the mating status of a female was known, it was observed that males could readily chase, capture, and assume a copulatory

position with both virgin and previously mated females. Further, the duration of genital contacts were within the same range for both types of female.

Once a male has captured a female, grasping her with his prothoracic and perhaps mesothoracic legs, he generally quickly orients himself so that he is ventral to her. Prior to genital contact in flight, the male's abdomen generally hangs slightly downward, parallel to that of the female. At this moment, a number of possible actions may occur, depending upon the activities of the male or female partner. A broad category of distinction may be drawn between activities which occur if the female remains flying with the attached male or if the pair land.

Mid-air copulations

If a female continues to fly with the male holding on in a ventral position, the male may arch his abdomen towards the female and attempt to clasp her cerci. If cercal contact occurs, their flight suddenly and recognizably changes so that the previous rapid spiral and zigzag motions of the pair slow down perceptibly to become smooth, circular or spiral patterns. During this time of slowed flight, it can often be seen that the male's wings are not beating and it can also be determined that the male is indeed in firm genital contact with the female.

If genital union has been made by a flying couple, copulations of variable duration may occur exclusively while air-borne. Because the individuals are moving so rapidly, sometimes in the midst of others, it is frequently not possible to record air-borne coital time with high accuracy. Termination of in-flight coitus can be noted by the sudden lowering of the male's abdomen, and this action is generally rapidly followed by the departure of the flying male. Some air-borne couples which have already achieved firm genital union may land with the female in a position above the male without coitus being interrupted. While most couples land on a side of the cage, some may land on the floor.

Many copulations begin during flight and are broken up on landing. This separation appears to depend to a large degree on the stability of the landing pair and the character of the substrate. Thus, if a copulating couple land on a cloth sleeve or mosquito netting, their union tends to remain firm, but if a couple land on the metal floor or on the edge of the cage, the genital contacts are often broken. For example, a female may land on a ledge so that the male is suddenly violently struck on the head or pronotum by the ledge at the moment of landing. Sometimes a joined couple may land apparently off-balance and before the female can come to a complete halt, the male will have been abruptly dislodged on impact with the substrate. A number of mid-air copulations of long duration (which were apparently nearing their end) were terminated almost immediately, but some couples continue to copulate for a short time after landing.

Where numerous males are present in a cage, the copulation of an individual couple may be broken up in mid-air by another male attempting to seize the same female. Occasionally a pair which may or may not have achieved genital union, while still air-borne, may attract more than one flying male and this may result either in the rapid separation of the entire group or else the intruding males will make tarsal contacts with the pair, causing highly irregular and rapid flight. Most often, due to the added weight, the group will land on the floor. The female and

her accompanying males may then exhibit tumbling, as the males vie to achieve cercal contact. This tumbling may result in cercal clasping by one male while the others remain tarsally in contact with the copulating pair. The other males may become catatonic or make repeated abdominal archings towards the joined pair. Sometimes, these attempts to clasp by intruders will dislodge the first copulating male, resulting either in departure in flight by the female or successful genital contact by one of the intruders. Sometimes after a female has departed, a pair of males may be seen on the floor, flexing their abdomens and attempting to clasp each other.

Copulation following landing

In most cases couples land before the male has made terminalial contact. If the female lands while the male is still attempting to clasp her cerci, the couple will stay tarsally united. The success of cercal clasping depends greatly on the final position of the couple after landing. Thus, a male which has maintained coital alignment will be far enough forward relative to the body of the female so that when he arches his abdomen, the terminalia of the two can meet. Often during landing the male may end up too far posteriorly relative to the female, and thus cannot attain genital contact by abdominal flexing. The male may, nonetheless, continue to make clasping movements. Slightly misaligned males may later gain a more favorable coital position and succeed in making a firm genital contact.

During cercal clasping and copulation in a standing position, the hind legs of the female are generally raised unless the couple's position is unstable, necessitating further bracing by the female's lowered metathoracic legs. When the hind legs of the female are elevated, this may be due either to her own action or to the male's pushing against them with either his mesothoracic or metathoracic legs, with the result that the female's legs are spread out of the way. We have never seen the claws of the male's metathoracic legs hooked over the female's wings. The male's metathoracic legs may be extended from his body against the substrate to form a stabilizing base from which abdominal arching and copulation can occur. The actual clasping of a female's terminalium and subsequent copulation generally result in a slight downward pull of the tip of the female's abdomen by the male. We have seen the hind legs of some seminally depleted males quivering towards the end of a non-inseminating copulation. On many occasions, the slow lowering of the female's hind legs, on her own or due to their release by the supporting legs of the male, occurs just prior to the breaking of genital union and can be a clue to an impending termination of copulation. On a few occasions, in attempting to leave the female after a long copulation, a male may become twisted into a position 180° from that of the female and remain briefly terminally joined to her. A further struggle generally breaks the connection. Shortly after a couple concludes copulation, a male may quickly copulate again with the same female. This subsequent copulation is usually significantly shorter than the first. Very rarely, the female may fly off with a terminally-attached male and the two separate in flight.

Some couples were occasionally observed to land on the mosquito netting on the roof of the cage and these generally then copulated, the pair being suspended only by the prothoracic leg or legs of the female. The duration of such copulations were no different from seemingly more stable positions. Just prior to breaking

genital contact, the male releases his tarsal hold on the female and may swing downward before departing. On several occasions, genital contact was not broken at this time so that the male continued his downward swing through a wide arc, often as much as 180° before the union was severed. Occasionally a couple flew off together from the roof, sometimes still *in copula*, but more often after termination of copulation, so that the partners separated from each other in flight.

Sometimes when couples land on the floor, the struggling pair exhibit violent tumbling and kicking, especially if a firm genital contact had not been achieved in mid-air or had been broken upon landing. On quite rare occasions the male of a couple that has not yet attained a copulatory position in flight may end up in a position superior to the female upon landing. He may be able to clasp her cerci momentarily before being dislodged by her struggles. On only one occasion was the duration of the terminalial union barely sufficient to be classified as a possible copulation (he did not inseminate her).

Males have been seen to chase and capture females and although seemingly perfectly aligned in a normal copulatory pose only lightly touch their terminalia to those of the females once or many times in rapid succession. They sometimes succeed in lightly clasping the cerci or terminal sterna of the females but do not establish firm genital unions. Seminally depleted males reach a stage when they will chase and capture females but no longer copulate with them. Such males were often seen to be correctly aligned and often made repeated light terminalial touches.

Grossly misaligned males

A grossly misaligned male may continue flexing his abdomen even though his terminalium does not contact the body of a female. Such males may be diagonally or laterally positioned so that only minimal tarsal contact with a female is maintained at this time. Repeated kicking and clasping attempts by such males may position his head under the female's terminalium.

A female which is being chased by a male may quickly land and the male may as suddenly land very close to or on the back of the female. Generally, as soon as the male contacts the body of the female, she rapidly takes flight and is then pursued again by the male and is often captured. If the female remains stationary, however, with the male on her back, several things can happen. Males which have landed on the backs of females are quite active and appear to walk over the female but do not attempt to clasp and never achieve genital contact of any kind from a location on top of her back. Males which have landed close beside a recently landed female may begin to flex their abdomen toward the female but are so misaligned that firm genital contact cannot be made. Often during this activity the female may kick the male with one or both metathoracic legs and thus drive him away. It should be noted here that when a female lands upon the back of another female or otherwise comes in contact with one, both females tend to remain standing quietly without kicking. When a normal male fails to make cercal contact due to his being severely misaligned, it is the female which flies away, as he tries to clasp either her thorax or abdomen with his claspers. Nevertheless, many females have been seen to remain standing despite being vigorously and frequently

jostled by misaligned males. An exception to this behavior is that seminally depleted males are nearly always the ones to leave the female. Such males were often misaligned and they generally flew away after a brief attempt to become realigned. Even when the depleted male is being carried by the flying female, he may suddenly lose tarsal contact and drop abruptly downward. On one unequivocal occasion a male landed on the side of the cage close beside a long-standing female (with raised metathoracic legs) and, passing in from the side, was seen to position himself very rapidly underneath her and succeeded in clasping her terminalium and copulated.

Successful copulatory positions

The positions of the male relative to the female that may result in firm genital unions vary quite remarkably. For example, a male may copulate when he is located slightly, and very rarely far, to one side of the female or when he is positioned so far to the rear of her that he is balanced on the front of his pronotum with the result that his abdomen must be strongly upcurved to achieve genital union with a coital angle approaching 90°. A rarely seen variation on this position occurs when a female attracts 2 males simultaneously: upon landing she appears to fall forward which elevates her terminalium so that firm genital contact by one of the males and clasping attempts by the other result in a tripod-like configuration.

If the initial clasping attempts of the male are not successful, both partners may become motionless for variable (often for quite long) periods. It should be noted that during such catatonic states the male remains in firm tarsal contact with the female. Clasping attempts may then resume which may or may not result in cercal contact, depending upon the male's alignment. At no time during this clasping activity (of either constantly active males or those resuming after a catatonic period) was the female ever observed to change her stance to facilitate terminalial union (nor was the male ever observed to elevate the female's abdomen to gain a more favorable coital position). Sometimes when a couple had not achieved terminalial union after several or many clasping attempts by the male, one or both partners (but generally the female) would terminate the activities by flight, leaving the other partner. Sometimes males would hang onto the female and be carried by her to another location where copulatory acts might be resumed.

Termination of copulation

As previously noted, the termination of copulation is often signalled when the female's metathoracic legs descend. The instant of the breaking of firm genital unions is marked by a sudden springing apart of their terminalia. If genital separation does not occur during lowering of the female's hind legs or immediately thereafter, she usually quickly raises them again and brings them abruptly down towards the male, thus kicking his abdomen or at his terminalium. This action may have to be repeated several times before the male will depart. Another technique which may be used separately or in conjunction with the above is the crossing of her hind legs during lowering so that they are placed in the space

between their abdomens. Extension of her crossed legs strikes the joined terminalia and usually without fail leads to his separation. After copulation for 6 secs. or longer, a female may suddenly lift her body upon her greatly extended legs and this action alone may dislodge some males. Sometimes a female may use all or any combination of the above techniques to break genital union. Soon after the female is alone, either having flown off by herself or after the male has left, she may be seen to scrub her metathoracic legs, often rubbing them over her terminalium. However, the occurrence of this action may not necessarily indicate a recently copulated female. We have seen seminally depleted males repeatedly scrubbing their terminalia in a similar manner.

Discussion

Contrary to a widely-held belief gained from Roth (1948), males are not instantly or regularly induced to fly and chase flying females under uncrowded conditions. In fact, most of a male's time is spent either in standing or in apparently non-directed flights. It is only when colony cages containing large numbers of mosquitoes of both sexes are observed that one obtains the impression that sexual behavior is occurring relatively constantly. However, close observations on individual males even in such cases will reveal that only during a small percentage of a male's time is he actually chasing a female or engaging in copulation.

While it can be said that males locate flying females by means of their antennae which are acting to discern the sounds of a female's wing beats (Roth, 1948), the present work indicates that this sensory mechanism of the male may not always activate him to flight or else may not itself be activated by the sound of the female wingbeats. Since males do not always respond to the sound of a flying female, it might be that the response to imputs from this mechanism occurs at will or else is triggered at varying thresholds. Roth (1948) concluded that an adaptation occurred among males which had been exposed for varying periods to sounds simulating female wingbeat frequencies. It may be that the periods during which a male does not respond to the sound of a flying female may represent periods of adaptation to the general sound of females flying in a cage. Activation to flight may then be interpreted as a response to a sound of slightly different but distinguishable frequency from the background noise to which he has adapted, or else to a sound that exceeds, due to the proximity of the female, the level of adaptation.

The performance of an average male in detecting and capturing a female seems truly remarkable considering the inherent difficulties of locating rapidly flying females which are constantly changing flight directions due to the confines of the cage. Furthermore, this ability to distinguish the flight tone of a female as opposed to sound reflexions from either curved glass or smooth plastic surfaces seems all the more striking. Where numerous females are flying in a cage, the ability of a male to locate and track a single female in the midst of interfering noises from other flying females (even allowing for slight variations in flight tone due to age or weight) would indicate truly formidable powers.

It has not been previously noted before this that sometimes despite vigorous chases by males, some females can elude capture for long periods by various tactics.

Once a male has captured a female and begins to copulate with her, the coital angles are essentially the same irrespective of the site of copulation. In agreement with Roth (1948), the present observations indicate that copulations most frequently occur on the landing of a pair which had previously been partially joined in flight. The second most common site of copulation is partially in flight with termination shortly after landing. Less frequently, copulations occur entirely in the air and are terminated while the pair are still in flight. The least common method of copulation was the one case where a male succeeded in aligning himself under a long-standing female and copulating with her.

The effects of changing flight tone to one which is presumably more noticeable to sound-adapted males is demonstrated by the attraction of other males to a couple which have just initiated a steady slow flight pattern.

The importance of the male gaining a firm hold on the female in a position from which his terminalium can touch that of the female prior to her landing is shown by the relative lack of success of misaligned males to achieve copulation.

Adult males will not copulate with freshly inseminated females or with those which have been inseminated for a long time if their terminalia are forcibly rubbed together under a microscope (Jones and Wheeler, 1965). The males make no attempt or only feeble attempts to clasp the cerci of females which are manually presented to them. Furthermore, the males under such artificial conditions will not attempt to grasp the cerci even when the terminal segments of the female are distended so that the cerci are readily available (Jones, unpublished). It is very evident from the present study however that this phenomenon does *not* occur with free-flying mosquitoes. Free-flying males apparently cannot recognize virgin from inseminated females since they readily clasped and maintained a copulatory position with both, usually for a similar period of time.

Termination of copulation may be due to actions of either the male or the female. The female certainly appears to terminate most genital unions by actively dislodging the male, primarily by striking him with her metathoracic legs or by standing on her tarsal tips. It is possible that she cues the male to withdraw and that the lowering of her hind legs is sometimes due to his activities. In order to terminate copulation, the male must reverse the copulatory acts, as follows. He must withdraw his aedeagus from the vagina, then retract his apical paraprocts from her cloacal hollow, and finally must release her cerci.

We wish to acknowledge our thanks to Patricia A. Pilitt for her help with this manuscript.

Summary

1. The sexual behavior of 2 strains of free-flying *Aedes aegypti* in 1 cu ft cages is described.

2. Groups of resting males are not necessarily activated to flight by flying females under uncrowded conditions, even though the females may fly within 2

inches of them. Individual males tend to be activated to flight if females fly within one inch of them or actually touch them.

3. Flying females do not necessarily elicit directed responses by flying males. When males pursue females, they may fly in one or more loops around them before establishing tarsal contact. Only a small percentage of a male's time is spent in chasing or copulating with females.

4. Copulations taking place in mid-air can be recognized by a sudden change in the flight pattern of the couple from a rapid zigzag to a slower spiralling path. Most couples land before the male makes genital contact with the female. On many occasions males were seen to land beside females and to be in tarsal contact with them. Although some of these males made attempts to clasp stationary females from the side, on only one occasion was a male actually seen to crawl beneath a long-standing female and to copulate with her.

5. Females were never seen to change their stance to facilitate copulatory acts of the males. Although males and/or females may terminate copulation, the lowering of the female's metathoracic legs generally signals an impending end to the act. Females may additionally have to cross their hind legs, stand on their tarsal tips or fly away to dislodge some males.

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ORGANIZATION OF PRIMITIVE NERVOUS SYSTEMS. NEUROMUSCULAR PHYSIOLOGY OF *GYROCOTYLE URNA*, A PARASITIC FLATWORM

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The free-living flatworms are usually considered to occupy a strategic position at the base of the metazoan phylogenetic tree. They are the first bilaterally symmetrical animals to possess a brain concommitant with the concentration of neural tissue into discrete nerves. The nervous system itself is of interest because it probably reflects various aspects of the primitive conditions which existed during the evolution of centralization. In a number of species, for example, the anatomical arrangement of nerve cells appears to be intermediate in organization between the diffuse nerve-nets of many coelenterates and the more highly concentrated systems of higher protostomes (Koopowitz, in preparation). Neuromuscular preparations among the free-living flatworms have only been reported from polyclad turbellarians (Gruber and Ewer, 1962; Koopowitz and Ewer, 1970). This scarcity of information reflects the general unsuitability of these animals as neurophysiological preparations. Not only does their acoelous nature preclude easy exposure of nerves but their fragility makes it almost impossible to restrain them for any but the shortest lengths of time. The latter problem can be avoided by using parasitic flatworms which have a tough outer cuticle. However, except for some early mechanical recordings of drug effects on *Fasciola*, the liver fluke (Chance and Mansour, 1949; Mansour, 1957), no neurophysiological investigations appear to have been reported for either trematode or cestode parasites.

The report that follows is a preliminary investigation on the neuromuscular properties and capabilities of preparations made from a cestodarian flatworm, *Gyrocotyle urna*. Cestodarian flatworms are an unusual group of unsegmented animals which are classically placed in a sub-class of the Cestoda, the parasitic tapeworms (Barnes, 1968). Modern workers now consider them an aberrant group, not closely related to present day tapeworms but rather remnants of a stock closer to the original primitive parasitic platyhelminths (Burt, 1970; Wardel and McLeod, 1952). These unique animals have a nervous system reduced to a pair of longitudinal cords with commissures at each end and a posterior nerve ring. The neuronal anatomy appears much simpler than that found in either the polyclads or the freshwater planarians. Besides shedding light on the nervous organization of primitive flatworms, these animals should reflect those adaptations produced to meet an endoparasitic existence. Specific neuromuscular adaptations to parasitic modes of life have so far received little attention.

Methods and Materials

Gyrocotyle urna is found close to the spiral valve in the stomach of *Hydrolagus colliei*, a chimerid fish abundant in the waters of Puget Sound. The fish were

kept in large tanks of circulating sea water at the Friday Harbor Laboratories and flatworms were dissected free as needed. Virtually every fish harbors at least one of these parasites. The animals used ranged from 2 to 6 cm in length and up to 3 cm in width. Isolated flatworms will live for three to four days in cold sea water but the animals were usually used within 24 hours of extraction.

A number of different preparations were made. Most frequently the animal was sliced down its mid-line, any eggs washed out of the uterus and both anterior and posterior ends cut off. The latter operations effectively removed commissures and ganglia. This was verified by inspection. Tiny hooks made from minute pins were inserted into both ends of the preparation. One of these was fastened to the bottom of the container while the other was connected by a length of nylon floss to a mechanoelectrical transducer. Either of two transducers were used: a Brush metripak angular position transducer was utilized for isotonic contractions while a Statham "Gold Cell" was available for isometric measurements. The output from the transducers was displayed on either a Bausch & Lomb VOM6 single-channel chart recorder or on a Clevite Brush Mark 220 two-channel chart recorder.

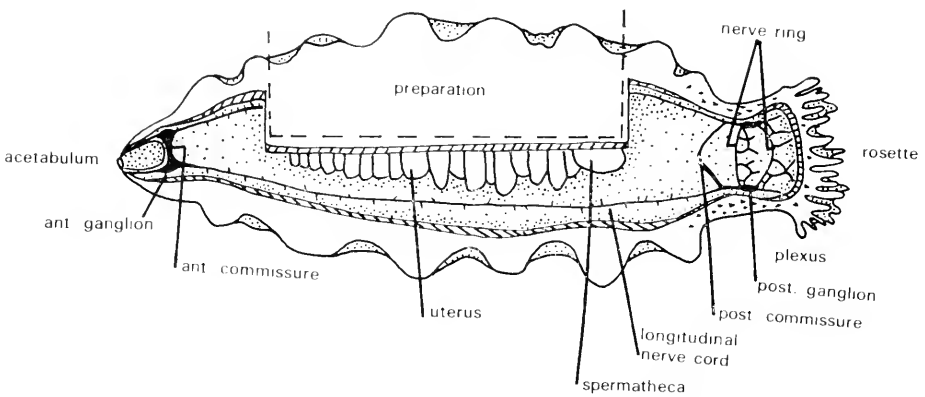

FIGURE 1. The major parts of the nervous system of *Gyrocotyle urna* as seen by dissection. The part used as the preparation was that bounded by the dashed line. This specimen was 3 cm long.

Stimuli were delivered by a pair of silver electrodes. These were insulated in polyethylene tubing sleeves except for their tips which were chlorided and flattened. The electrodes were pushd into the tissue, one electrode on each side of the longitudinal nerve trunk. A Grass S48 stimulator provided positive going square wave pulses. The preparation was suspended in 15–17° C aerated sea water which was changed every half hour.

RESULTS

Anatomy

Figure 1 shows the major nerves; these can be dissected fairly easily. There are two main nerve strands running along the length of the animal. At the anterior end they are joined by a commissure behind the muscular acetabulum. Slight swellings on either side of the commissure have been called ganglia (Watson,

1911). Along their length the major cords give off small nerves which sink into the musculature. In the central two-thirds of the animal, most of this musculature appears to be orientated longitudinally. At the posterior end there is a nerve ring which sends off branches into the funnel shaped sucker. These branches anastomose to form a small plexus. At the level of the ring the longitudinal cords form another pair of ganglionic swellings. Anterior to the ring can also be found a commissure joining the two rings. Giant cells (Watson, 1911) have been reported but, with vital methylene blue staining, they could not be found. Vital staining also showed very few somata in the ganglia. Methylene blue staining, however, is notoriously erratic. Monopolar cells were found in the longitudinal cords. A peripheral nervous system has been described (Watson, 1911) but this was not visible with a dissecting microscope.

Neuromuscular responses

A single stimulus pulse, of above threshold amplitude, results in a smooth contraction followed by a series of secondary contractions. The initial response tends to be quite slow and usually requires more than one second to reach peak tension. Even if secondary activity is not generated the time taken to relax back to the original level of tone is very long, typically more than 40 seconds. The rate of tension generation is dependent on both the intensity (Fig. 2a) and duration (Fig. 2b) of the electrical stimulus. Likewise, the total shortening of the preparation is also dependent on both intensity (Fig. 2c) and duration (Fig. 2d) of the stimulus. These responses do not bear a simple relationship to the amount of current in the stimulus. In Figure 2d the response to a 10 V stimulus of 30 msec duration is less than that to a 20 V, 10 msec shock, although the former stimulus should have greater amperage. When the total amount of current is kept constant (intensity $\times$ duration = constant) increasing the duration does not lead to larger response amplitudes but increasing the stimulus intensity does (Figs. 2e and 2f).

The threshold voltages often appear to be quite high, *e.g.*, 20 volts and 2 msec duration. This is also true of the polyclad, *Planocera* (personal observations). To see if these high thresholds could be due to some artifact, I tested the experimental situation by substituting a fragment of the nemertean *Paranemertes peregina* for the *Gytrocotyle* preparation and found threshold values for this animal closer to the expected range, *i.e.*, 4 volts and 0.3 msec duration. High threshold values may, therefore, be characteristic of platyhelminth preparations.

Inhibitory responses

In many cases, it was noticed that stimuli of intensities slightly below the threshold values for contractions caused very small decreases in the tone of the preparation. Much larger decreases in tone could be obtained if the preparation was already in a state of contraction (Fig. 3a). This case much resembles the "direct inhibition" previously described in *Planocera* (Koopowitz and Ewer, 1970). If a train of stimuli are delivered, then the initial contraction is often followed by a drop in the level of tone and a depression of spontaneous activity (Fig. 3b). After stimulation the depression is followed by a rebound in tone, as well as increases in frequency and amplitude of the spontaneous contractions. In another preparation

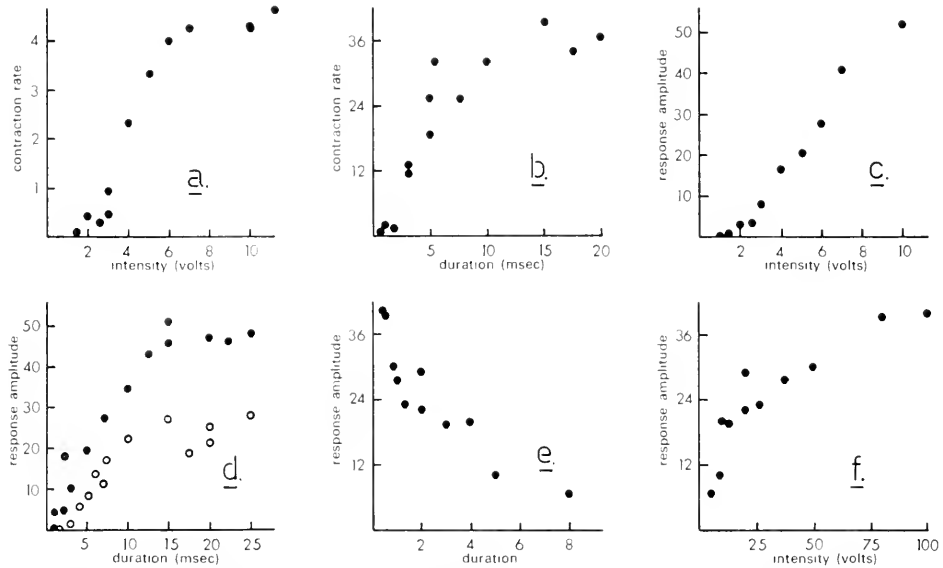

FIGURE 2. (a) Effects of stimulus intensity on rate of contraction; the axis measures the relative rate of contraction as the slope of the rising part of the trace. The abcissa measures intensity in volts. The stimulus duration was kept constant at 1.0 msec. In this, as in all other graphs, each point represents a single reading. For any graph the points are all from a single preparation. (b) Effect of stimulus duration on the rate of contraction; the axis is relative rate of contraction while the abscissa reads stimulus duration in msec. Stimulus intensity was 20 V. (c) Amount of shortening and intensity of the stimulus; axis is the relative height of the response and the abcissa the stimulus intensity in volts. Stimulus duration was held at 1 msec. (d) Amount of contraction with different stimulus durations; axis is the relative amount of shortening and the abscissa the stimulus duration in msec. Solid circles were obtained with an intensity of 20 V while the rings were obtained with an intensity of 10 V. Both were from the same preparation. (e) Amplitude of the response with changing intensity and current kept constant ($v \times d = 40$); axis is relative amount of shortening and abscissa is msec duration. (f) Amplitude of response with changing stimulus duration, current kept constant ($v \times d = 40$); abscissa intensity in volts. Same experiment as in Figure 2e.

(Fig. 3c) which was not spontaneously active, the lowered level of tone was held for 20 sec after the stimulus and the rebound contained two contractions which resembled spontaneous contractions. When the frequency and number of stimuli was increased (Fig. 3d) even more of these contractions followed. It should be noted that multiple stimuli are not necessary to elicit both contraction and relaxation, for a single stimulus can also do this (Fig. 3e) in a favorable preparation. Although cessation of spontaneous activity most likely involves an active inhibitory system, the measured "loss" in tone could also be due to contractions evoked in antagonistic muscle layers. However, the rebound following loss of tone argues against this explanation.

The amount of tension generated is also dependent upon the previous stimulation history of the preparation. Generally, the smaller the interstimulus interval the less the amount of tension generated for identical stimuli. This generalization will be qualified shortly. Figure 4a shows the response by a preparation following

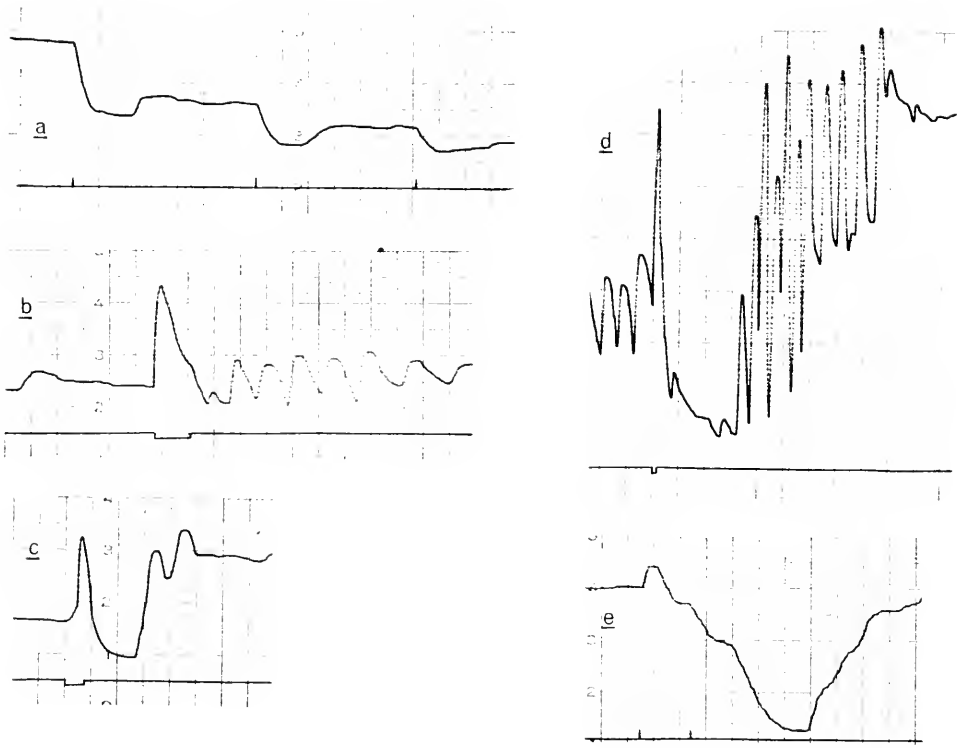

FIGURE 3. Inhibitory effects of stimuli on the level of tone; (a) the effects of three single stimuli each 40 V and 0.5 msec duration; (b) the same preparation as Figure 3c, where the stimulus train was 20 sec long with a frequency of 20 per sec. Intensity was 40 V with 1 msec duration. (c) The effects of a stimulus train 10 sec long; each stimulus was 40 V and of 1 msec duration. Frequency was 5 per sec. (d) Response to a train of stimuli 10 sec; (e) response to two stimuli four sec apart, intensity was 20 V and duration 1 msec.

three different interstimulus intervals. The recovery time for this depression varies from preparation to preparation. It usually lasts for no more than ten to fifteen minutes, although in some cases it was present for a considerably longer period. Response amplitude is plotted against interstimulus interval in Figure 4b. This decreased responsiveness could be due to fatigue in the muscle, failure of the nerve or neuromuscular junction, or some "active" process such as a long lasting inhibitory effect. It is unlikely that fatigue can account for the changes measured as they are evident in very fresh preparations. Depression can also be recorded from preparations using weak stimuli, before increasing the stimulus strength and eliciting very large responses. It is also unlikely that failure or fatigue in the nervous part of the preparation can be responsible because stimuli delivered with interstimulus intervals less than four seconds apart show facilitation.

Facilitatory responses

The contraction recorded from a second stimulus, administered within one or two seconds of the first, often results in an increase in response over that from the

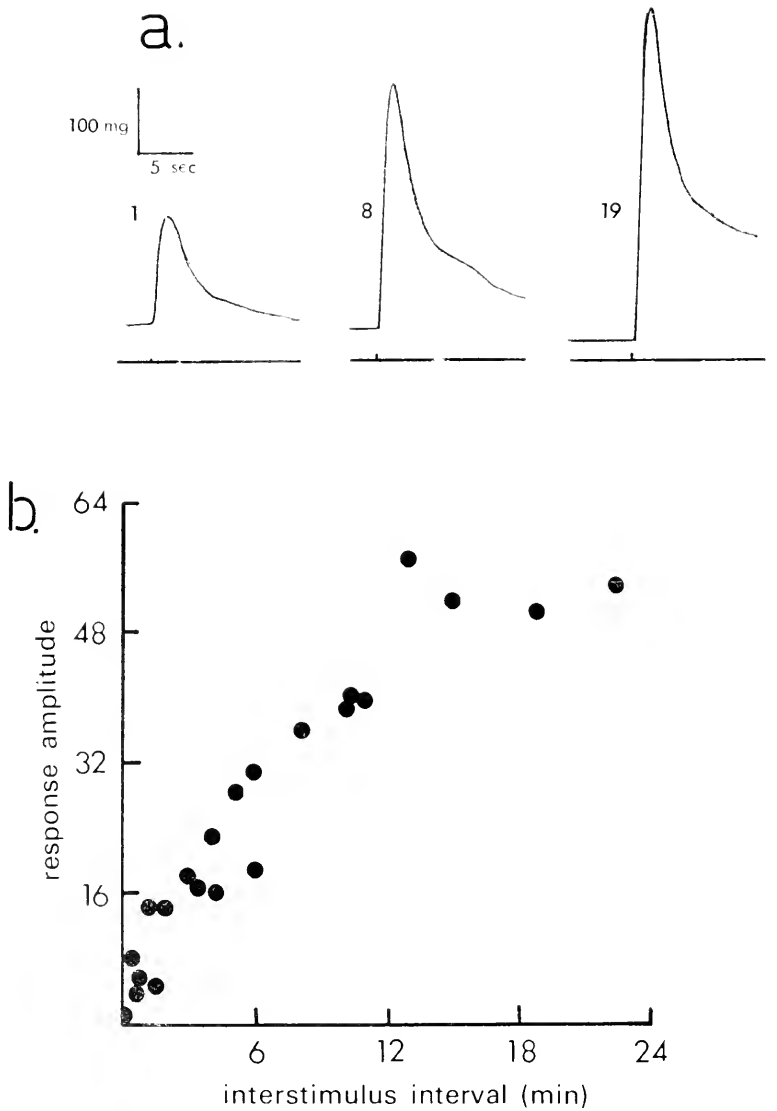

FIGURE 4. (a) Effects of interstimulus interval on the size of the response; stimuli were 30 V and 0.3 msec duration. The number next to each response was the length of the interstimulus interval in minutes. (b) Effects of interstimulus interval on the amplitude of the response; axis is the relative amount of shortening and abscissa the interstimulus interval in minutes. All stimuli were 30 V and 0.1 msec duration.

initial stimulus. Because of the slow nature of the response the facilitation is displayed as increased "treppe" step size. Response amplitude to three single stimuli of 10 msec duration is greater than the response to a 30 msec stimulus of the same intensity and the response to the second or third stimulus is greater than

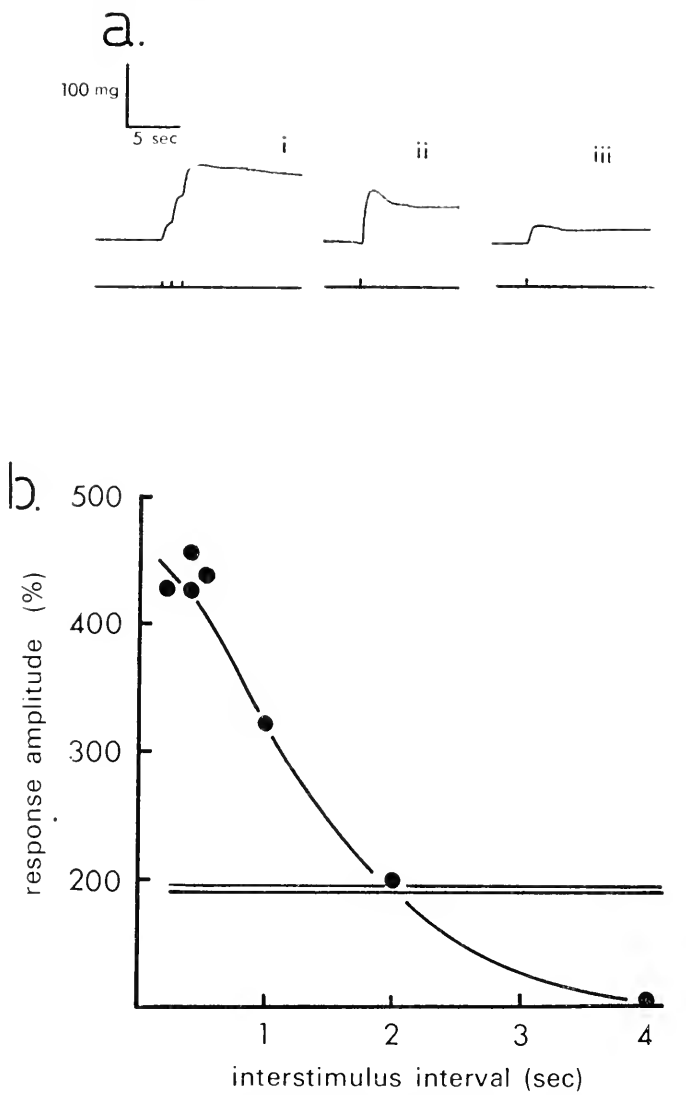

FIGURE 5. (a) Facilitatory effects. Each stimulus had an amplitude of 10 V. In i 3 stimuli of 1 msec duration were delivered at 1 sec intervals, and ii shows the response to a single shock of 3 msec duration while iii is the response to a single stimulus of 1 msec. (b) Changes in amplitude of the response with changing intervals between two stimuli; the axis is the amplitude of the response to the second stimulus expressed as a percentage of the first stimulus amplitude, while the abscissa represents the interstimulus interval in seconds. All stimuli had an intensity of 10 V and a duration of 1 msec. The double line is the response amplitude to a single shock of 10 V and 2 msec duration.

the first (Fig. 5a). In some preparations it was possible to confuse the second stimulus response with secondary activity elicited by the first stimulus especially if these were superimposed on each other. Special care was taken to avoid using

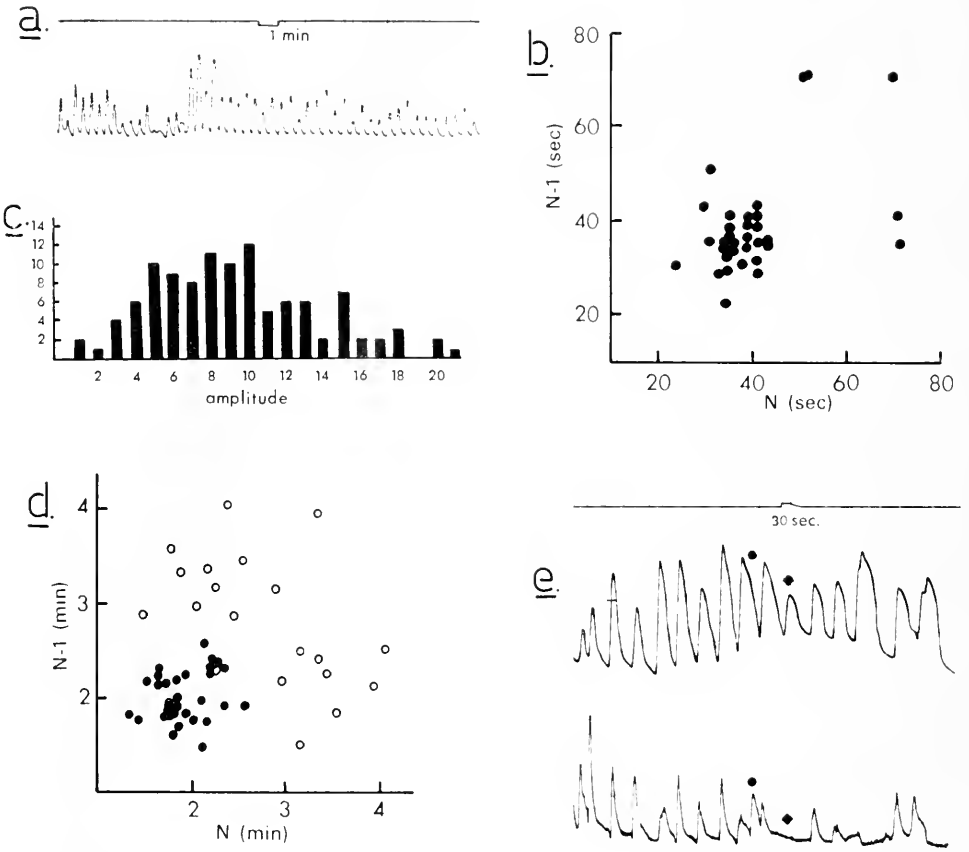

FIGURE 6. (a) Spontaneous activity from a *Gyrocotyle* preparation; (b) a joint interval histogram of the spontaneous activity. The axis is the time interval preceding a spontaneous contraction and the abscissa is the interval following the activity. Intervals are expressed in seconds. (c) Distribution of spontaneous contraction amplitudes; the frequency of occurrence is on the axis and the abscissa represents contraction amplitude or tension. This is from the same preparation in Figure 6(a) but collected for a 65-minute period. (d) Joint interval histogram for the two sides of a hemisected animal; this animal had the posterior commissure intact and the open and closed circles represent the different sides of the preparation. Intervals are expressed in minutes.

this kind of data which might be ambiguous. The time course for facilitation in one preparation is shown in Figure 5b. Here the amplitude of the second response reached a maximum when the pulses were less than 1/2 seconds apart. By the time the interstimulus interval reached 4 seconds, the responses were of equal size. It should be noted that the maximum facilitated response was more than twice the size of the response elicited by a single stimulus of the same amplitude but with twice the duration, suggesting that neuromuscular facilitation does take place. This is the first demonstration of this phenomenon in the flatworms.

Spontaneous activity

About half of the preparations set up show spontaneous contractions (Fig. 6a). The mean interval between contractions varies with the preparation and ranges from less than 20 seconds to more than 4 minutes. Figure 6b displays one preparation for which the intercontraction intervals are plotted against the preceding interval to form a joint interval histogram. The tight clustering of points indicates a fairly constant rhythm though there is some scatter and irregularity. Although the contractions tend to be more-or-less rhythmical, amplitude of contraction is quite variable (Fig. 6c). The site of origin for this activity could be either the nervous system or the muscles. Special preparations were made by removing the anterior commissure and splitting the animal halfway down the midline towards the posterior end; thus, muscular activity could be recorded from both halves of the animal simultaneously. The joint interval histogram from one such preparation (Fig. 6d) indicates that each side contains its own pacemaker system as the rhythms of the two sides are quite different. One is regular while the other tends to have short intervals followed by long. Recordings from the two sides were attempted when the anterior commissure was intact but the posterior one had been removed. Spontaneous activity was only recorded in one such preparation (Fig. 6e). Here the spontaneous activity in both sides of the animal was sychronized and matched except for two contractions. Unfortunately the activity died away before the anterior commissure could be severed. Nevertheless, data presented in the next section suggest that the anterior and not the posterior commissures are involved with coordination of longitudinal muscle activity between the two sides of the animal; thus the synchronization between the two sides displayed in Figure 6e may be of some significance.

Coordination between the two sides

When, as above, partially hemisected preparations are set up, with the anterior commissure intact, then stimulation of one side will set up contractions in both halves. If the commissure is severed, however, then only the directly stimulated side of the preparation will contract. A similar set of experiments was conducted to determine whether transmission also took place across the posterior commissure or ring. In only one preparation out of six did I find transmission through the posterior portion of the animal. The transmitted response was quite different from that usually obtained with anterior conduction. The latency ranged from 2 to 7 seconds compared with less than one second for transmission involving the acetabular commissure; the transmitted response in the posterior conducting system also appears to lack the rapid component seen with anterior transmission. Without more data, however, it is difficult to decide whether or not posterior transmission is of any significance.

Discussion

A neuromuscular explanation for the reported observations is desirable, however, without intracellular records from the nerves and muscle cells themselves, any hypotheses must remain rather tenuous. This system has rather slow contraction rates and even longer times for return to resting tension levels. Both short term

facilitation and a longer lasting inhibition have also been described and spontaneous activity in the animal tends to be in the form of more-or-less rythmical contractions. At first glance *Gyrocotyle* appears to share many of the properties of coelenterate anthozoan preparations. This should not be taken as an indication of phylogenetic closeness as the free-living polyclad *Planocera*, investigated by Gruber and Ewer (1963), shows few similarities to coelenterate neuromuscular systems. Also, the extent to which the *Gyrocotyle* neuromuscular system reflects the parasitic nature of the organism is not clear but there are some surprizing similarities with the unrelated nematode *Ascaris*.

Comparing neuromuscular properties of *Gyrocotyle* with those of *Planocera*, a polyclad flatworm (Gruber and Ewer, 1962; Koopowitz and Ewer, 1970), reveals a number of both similarities and differences. The major difference concerns neuromuscular facilitation. Facilitation, a common property of many invertebrate neuromuscular preparations, cannot be clearly demonstrated in the free-living worms. *Gyrocotyle*, however, does show facilitation. The absence of the phenomenon in polyclads may, therefore, be a peculiarity of that species rather than indicative of a condition in the entire phylum. Nevertheless, even in *Gyrocotyle*, facilitation is difficult to evince unless one has a particularly favorable preparation. In most cases the muscular response to a second stimulus is markedly less than the response to a first stimulus. This antifacilitation occurs even when contractions are elicited by small amplitude stimuli which result in responses that are a tiny fraction of the possible tetanus tension. Similar stimuli are needed to demonstrate facilitation in favorable preparations. The facilitatory effects of a stimulus are short and usually last on the order of seconds (Fig. 5b) in contrast to the depression which may last for a considerable time (Fig. 4b), usually tens of minutes.

The time course for facilitation in *Calliactis* (Pantin, 1935) and *Cerianthus* (Horridge, 1958), both sea-anemones, peaks between 0.1 and 0.2 sec and then rapidly falls away within a few seconds. Maximum facilitation in *Metridium* occurs at stimulus intervals of 0.5 sec (Robson and Josephson, 1969). In a tropical species of *Calliactis* Josephson (1966) found that facilitation only lasted for 0.6 seconds. On the other hand Arai (1965) recorded facilitatory effects that lasted as long as 8 min in *Pachycerianthus*. Except for the latter case these values are similar to those obtained with *Gyrocotyle*. Comparatively little is known of inhibitory systems in coelenterates but evidence suggests that in *Calliactis* inhibition may be prolonged (Ewer, 1960) and much longer lasting than the facilitatory events. Intracellular recording from *Ascaris* (del Castillo *et al.*, 1967) suggests that in the somatic musculature facilitatory events are ephemeral compared to inhibitory. The later event being more easy to evoke. They found, however, that records of "synaptic" transients showed similar time courses for both depolarizations and hyperpolarizations. Multiple stimuli, however, lead to prolonged periods of hyperpolarization. Whether these longer inhibitory effects are due merely to the amount of transmitter released or active post-junctional membrane changes has not been ascertained.

Another difference between *Planocera* and *Gyrocotyle* resides in the inhibitory systems. The inhibitory thresholds in the polyclad are much higher than the excitatory. In *Gyrocotyle*, however, direct muscle relaxation can often be obtained

by stimuli of intensities below those used to make the preparation contract. Other properties of the depression systems appear to be quite similar. Both preparations have similar time courses for the long-term inhibitory decay following stimulation and in both animals inhibitory phenomena can be produced by multiple stimuli where a single stimulus is ineffective. This suggests that some sort of inhibitory facilitation may exist. The data displayed in Figure 3b and Figure 3c are of interest as they show that a period of inhibition can be followed by a period of rhythmical contractions and that the number of contractions may be related to the amount of inhibition. The longer the stimulus train the greater the number of contractions. If, as it appears, this activity involves some sort of a rebound from an inhibitory state then it more likely involves short term rather than long term inhibitory systems. Long term depression normally lasts for many minutes; during these experiments, the tone of the preparation was depressed for a much shorter time period than would have been expected.

With comparable stimuli, both *Gyrocotyle* and *Planocera* (Koopowitz, unpublished observations), require approximately the same length of time to reach peak tension during a twitch. This is quite a slow process and usually takes between 0.5 and 1.0 seconds. Polyclad muscle, however, relaxes in less than 10 seconds, whereas the cestodarian usually requires at least 40 seconds. The longer time course for *Gyrocotyle* appears to involve an "active" component because the tension measured during relaxation is similar whether isotonic or isometric transducers are used. If the slow relaxation was due merely to inelastic properties resisting stretch, one might expect different time courses for relaxation with the different transducers. Further investigation is needed to discover exactly what physiological properties of the system determine the speed of relaxation.

In *Ascaris,* esophageal muscle cell tension appears to be maintained as long as the muscle cell remains depolarized (del Castillo and Morales, 1967) and in fact relaxation can be elicited more rapidly with hyperpolarizing potentials. Whether this is the case in *Gyrocotyle* is not known. Relaxation times for sea-anemones are much slower than the worms but direct comparisons are difficult as multiple stimuli are usually used to evoke activity in anemone preparations. Both *Cerianthus* (Horridge, 1958) and *Pachycerianthus* (Arai, 1965) will produce twitches to single stimuli and here relaxation is much longer than in *Gyrocotyle.* Muscle action potentials measured from *Calliactis* appear to be transient and muscle cell depolarization cannot account for the maintained tonus (Josephson, 1966).

In anemones, two kinds of muscular activity have been reported, twitches and slow contractions. The latter have been difficult to investigate and their nature is unclear (Robson and Josephson, 1969; Ross, 1957). Slow responses were often obtained following evoked twitches and in this way resemble the secondary activity recorded from *Gyrocotyle.* The complex nature of secondary activity and the repetition of the contractions suggest some kind of reverberating activity might exist. Josephson (1966) found that single stimuli could evoke multiple firing in *Calliactis* and this effect, but on a larger scale, could be involved with flatworm secondary activity.

So little is known of the behavioral repertoire and adaptations to an endoparasitic existence that it would be profitable to discuss some aspects of behavior which have been observed with *Gyrocotyle.* Interactions between adjacent animals

occur. On one occasion when a host was opened and two worms were found, one of the animals had attached quite firmly to the other with its acetabulum. Animals lying next to one another in a dish of sea water have been observed to grip each other with their acetabulum. This behavior has also been observed in some free-living polyclads, *e.g., Enchiridium punctatum* (Koopowitz, unpublished observation). It is not clear whether this is part of a prelude to copulation. The acetubulum of *Gyrocotyle* tends to be used in an exploratory manner; when an animal is first introduced into a container of sea water this structure is extended and moved in a number of directions. There is the impression of a sensory structure being used to sample the environment. In another set of chance observations, a young worm was seen to emerge from the genital aperture of a large adult. Most of the young worm's activity during the "birth" seemed to involve its own acetabulum. When the small worm was free, it started to undulate in the nearest approach to locomotory activity that has been seen. A number of waves of dorsoventral flexions passed down the animal's body, rather reminiscent of polyclad swimming, and the animal moved with the acetabulum leading. These observations suggest that the acetabular tip is the anterior end of the animal.

There was an old controversy about the scolex in tapeworms and whether this represented the anterior or the posterior end of the animal. Considerable evidence has been accumulated that the scolex is the anterior end (Wardle and McLeod, 1952) and the problem now appears to be settled. However, the rosette holdfast of *Gyrocotyle* is usually considered to be homologous with the scolex of the *Eucestoda* (Wardle and McLeod, 1952). This paradox is most easily resolved if one considers the *Gyrocotyle* holdfast to be only functionally analogous to the scolex. This may well be the case if, in fact, the two kinds of animals are not closely related.

One of the outstanding features of *Gyrocotyle* is the apparent simplicity of the nervous system and the ease with which it may be exposed for experimental manipulation. Experiments reported here, while not expressly designed to investigate purely neuronal interactions, do give some insight into the organization of the nervous system. It is probable that the pacemakers for spontaneous longitudinal contractions occur in the main longitudinal nerve trunks as this was the main nervous tissue in the preparations. Each side has at least one pacemaker system and synchronization takes place across the anterior commissure. Stimuli from one side of the body are also able to cross to the other side via this commissure. The acetabular commissure then possesses properties similar to those demonstrated in polyclad brains (Gruber and Ewer, 1962). It is tempting to consider the commissure and its "ganglia" as homologues of the early flatworm brain. More intriguing is the possibility that we are dealing with a brain that is still both at a very primitive stage and involved with the initial functions for which brains were evolved. This flatworm is now accepted to be derived from the stock which originally gave rise to the other parasitic platyhelminths (Burt, 1970). The association of primitive parasites with primitive hosts is often exemplified by *Gyrocotyle* and its chimerid host. The ganglia and commissure could, on the other hand, represent a secondarily reduced brain. The animal's parasitic habit, which presumably has resulted in a reduced need for it to interact with its environment, may have left it with only the essential neuronal circuitry necessary to deal

with these limited needs. A careful inventory of the functions and physiology of this "brain" might be expected to throw considerable light on the factors underlying brain evolution.

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Summary

1. The nervous system consists of two longitudinal nerve cords with commissures at each end. Ganglia are reduced to small swellings in the cords adjacent to the commissures. At the posterior end there is a nerve ring with a reduced nerve plexus in the rosette.

2. The responses to electrical stimulation are described. Preparations have high thresholds and relatively slow twitch contractions.

3. Direct stimulation often causes loss of muscular tone. This and decreased responsiveness to repeated stimulation are thought to be due to the presence of inhibitory neurones.

4. Facilitation of the response to electrical stimulation is short lasting. This effect has usually decayed within a few seconds of stimulation.

5. Rhythmical spontaneous contractions occur. Pacemakers for this activity are probably in the longitudinal nerve trunks.

6. The anterior acetabular commissure is used to synchronize the spontaneous activity of the two sides. Electrical stimuli applied to one side will cause the other to contract if the anterior commissure is intact.

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STUDIES ON THE TUBE-BUILDING AMPHIPOD *COROPHIUM TRIAENONYX* STEBBING FROM VISAKHAPATNAM HARBOR: EFFECT OF SALINITY AND TEMPERATURE

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Factors such as salinity and temperature that control the distribution of fouling organisms are of considerable interest. Seasonal variations in salinity are of great importance in the distribution of such organisms because at some places the salinity may be sufficiently high for their survival only during certain months. It would appear that relatively few studies have been made to determine the effect of wide ranges of salinity. Beadle and Cragg (1940) carried out some experiments on the salinity tolerance of *Gammarus* and more extensive work was completed by Kinne (1952, 1953a, 1953b, 1956, 1959 and 1960) on the tolerance of *Gammarus duebeni* and *Gammarus salinus*.

The importance of temperature to stenothermal organisms is well known. Some observations have been made here on the influence of temperature on *Corophium triaenonyx*. Kinne (1952, 1953a, 1953b, 1954, 1956, 1959 and 1970) dealt with the heat tolerance of the amphipod *Gammarus duebeni*. Resistance of the amphipods *Hyalella azteca*, *Gammarus fasciatus* and *Gammarus pseudolimnaeus* to lethal temperatures was studied by Sprague (1963). Every individual has an optimum temperature or upper biokinetic limit above which activity may suddenly cease in what is known as heat coma. Heat coma and death occur in sequence and may not be simultaneous. Low temperatures can effect animals in a similar way.

A study was undertaken to find out the effects of salinity and temperature on the tube building amphipod *Corophium triaenonyx* at Visakhapatnam. Such fouling organisms are known to withstand wide ranges of salinity and temperature. Investigations were therefore undertaken to determine the maximal and minimal salinity and temperature values at which the adults and juveniles of *C. triaenonyx* survive, to study their reactions to gradual changes in salinity and temperature and to observe the tolerance of animals *in situ*.

During the period of study the overall salinity level of the harbor waters showed wide fluctuations. The highest salinity values recorded were 34.67‰ and 34.62‰ and 34.62‰ in the years 1961, 1962 and 1963, respectively. The lowest salinity values for these years are 14.15‰, 14.66‰ and 8.66‰. The maximum temperatures of the water were 30.80° C, 30.80° C and 30.84° C in the years 1961, 1962 and 1963, respectively.

Materials and Methods

For all experiments on salinity tolerance, normal seawater was diluted with glass distilled water as desired. A series of solutions covering a range 8‰ (lowest

recorded in the field) 7‰, 6‰, 5‰, 4‰, 3‰, 2‰, 1‰ down to zero were prepared by dilution. For temperature experiments, finger-bowls containing 200 ml of seawater of salinity 29.86‰ were kept in thermostats at desired temperatures and aerated at intervals. Several experiments were conducted at a time using different thermostats with temperatures ranging from 30° C–40° C.

Twenty animals of both sexes and approximately of the same size groups were used in each experiment and each experiment was repeated at least six times. Animals carefully removed from their tubes were directly transferred from seawater of salinity 29.86‰ and temperature 30.5° C to each of the fingerbowls with experimental media and experimental temperatures. At regular intervals the water was aerated and the animals were observed under a binocular microscope. Dead animals were removed. Emphasis was placed on actual survival rather than on their ability to carry on normal activity. The salinity and temperature at which 50% of the animals failed to recover in a 12-hour period as judged by their inability to respond to needle prickings was considered lethal (Evans 1948). For all experiments controls were maintained. For all salinity experiments a constant temperature of 30.5° C was used and for all temperature experiments a salinity of 29.86‰ was maintained.

Results

Decreased salinity

An initial experiment was designed to find out the maximum salinity at which *C. triaenonyx* survives and also to determine how well they might recover after exposure to abnormally low salinities. In distilled water they die in one hour and forty-five minutes. Animals subjected to salinities between 4‰ and 10‰ died after 6 days and those between 10‰ and 40‰ survived for more than 8 days. The lower lethal salinity lies below 4‰. The survival period in the control bowls was 12–14 days. Another set of experiments was conducted with concentrations from 4‰ down to distilled water. Animals survived for 2 days in 1‰ concentration but they were found to be inactive. So it may be concluded that the minimum salinity for survival activity is 4‰. A series of experiments consisting of concentrations from 1‰ to 0.5‰ was prepared to fix the lower lethal salinity. The results are shown in Figure 1. Death occurred at 0.6‰ concentration. Thus it becomes clear that the lower lethal salinity of *C. triaenonyx* is 0.6‰. At 4‰ and above they remain very active. Of course such low salinities may seldom occur in nature. In the present experiments the fall in salinity is sudden, and more rapid than could possibly occur in the harbor.

Increased salinity

The annual maxima of salinity as observed in the harbor were 34.67‰, 34.62‰ and 34.62‰ for the three years. Animals were placed in solutions of seawater of increased strength from 40‰ to 55‰ adopting the methods described in the foregoing experiments. The different concentrations were prepared by addition of appropriate quantities of sodium chloride. They were able to survive in 55‰ concentration for three days. A second set of solutions was prepared with concentrations ranging from 55‰ to 58‰. In 58‰ they lived for 24 hours. A third

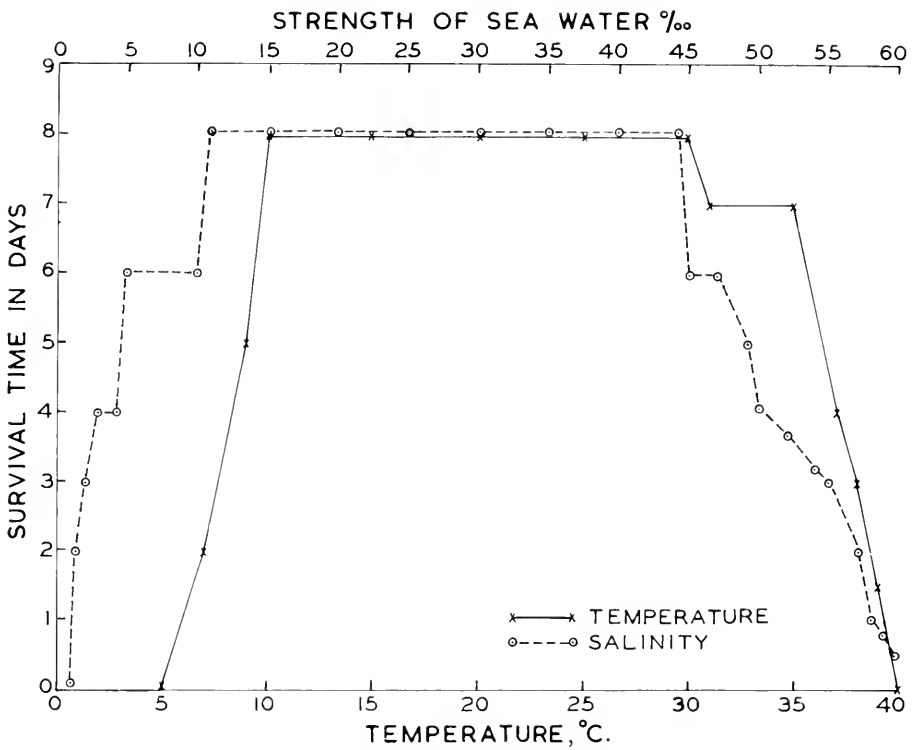

FIGURE 1. Tolerance of *C. triaenonyx* to increased and decreased salinities and temperatures.

set of experiments with solutions consisting of closely graded concentrations ranging from 58‰ to 60‰ at 1‰ intervals. The results are presented graphically in Figure 1. The upper lethal salinity has been found to be 59.8‰, in which concentration immediate death occurred. In media up to 55‰ animals moved about actively and of this concentration and below the animals live quite normally. From all these results it is clear that *C. triaenonyx* is a very hardy form tolerating a wide range of salinity from 0.6‰ to 59.8‰.

Reactions to gradual changes

Some experiments were conducted to assess any differences in tolerance to gradual increase or decrease in salinities. The animals were kept in seawater of salinity 29.86‰ for 24 hours and then they were transferred to the desired decreased or increased concentrations. Procedures described in the prevoius experiments were followed in preparing higher or lower concentrations and the same precautions were taken for aerating the water and removing dead animals as in the previous case. It was found that the survival time is not increased by gradually raising or lowering the salt content of the sea water.

Some experiments were conducted with animals in their tubes. No differences could be found in survival times in the salinity gradients.

Extreme limits of tolerance to temperatures

In all experiments the animals were transferred directly from 30.5° C to the experimental temperatures. At 37° C they lived quite normally for four days and then death ensued. The animals could survive for 10–12 hours at 39° C. At 40° C all the animals succumbed in 2 hours. Thus it could be established that *C. triaenonyx* tolerates wide ranges of temperature up to 40° C but sudden change to unnatural temperature has some effect.

Reactions to gradual changes

In the second set of experiments the conditions and precautions were the same as in the previous experiments. The specimens were placed in a finger bowl consisting of 100 ml of sea water and placed in a bath adjusted to 31° C which is the maximum temperature recorded in the field. Then the temperature was increased by 1° C every day. The temperature of the water in the bowl and the outside air were regularly noted. It was found that with gradual increase in temperature the animals can remain active up to 42.5° C. Beyond this point activity ceased, and at 46° C they were all dead. However with immediate gradual cooling from 42.5° C recovery was possible and the animals once more were observed to be active. Irreversible heat coma apparently sets in from 42.5° C and above (Fig. 1).

A third experiment was conducted on the same lines with a minor change. The temperature was increased by 1° C every hour instead of at 24-hour intervals. In this case the animals become comatose at 42.5° C and at 44° C they appeared dead. It would appear that higher lethal temperature for *C. triaenonyx* lies at 44° C, when the temperature is raised by 1° C every one hour. Some of the animals were removed from the thermostat bath at 42.5° C and brought to room temperature. After 18 hours of cooling some of them recovered. Animals which were held at 44° C or above could not recover.

Some experiments were also conducted with animals in tubes and the following results were obtained. With sudden increase they survived up to 43° C and with gradual increase of 1° C every day up to 48° C and 1° C every hour up to 46° C.

It is clear that the lethal temperatures lie at slightly higher level when the animals are inside the tubes.

Although experiments at lower temperatures were not completed, it is suggested that the lower lethal temperature of *C. triaenonyx* lies at 5° C. Acclimation experiments in this direction could not be conducted.

Effects of salinity and temperature combinations

Kinne (1952, 1953a, 1954 and 1964) made an extensive study of salinity and temperature combinations on *Gammarus duebeni*.

In the present study 6 temperature and 9 salinity combinations were chosen. Twenty specimens were used in each experiment. The salinities and temperatures tested have been 1‰, 5‰, 15‰, 20, 25‰, 30‰, 35‰ and 40‰, and 8° C, 15° C, 22° C, 30° C, 35° C and 39° C respectively. Other conditions were the same as in previous experiments and dead animals were promptly removed in the course of the experiments. The results are given graphically in Figure 2.

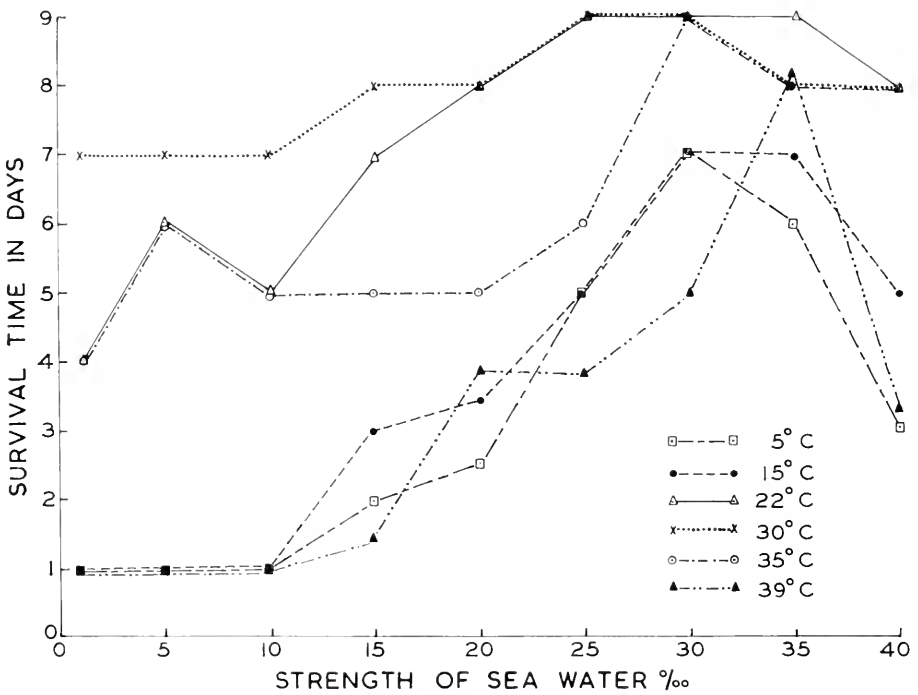

FIGURE 2. Effect of salinity and temperature combinations on *C. triaenonyx*.

It thus becomes (Fig. 2) that *C. triaenonyx* tolerates lower salinities better at higher temperatures, and also the animals are able to tolerate the lower temperatures well when the salinities are nearer normal values.

Effects of size and sex

Size and sex do not seem to have any influence on tolerance. Young ones could be equated to the adults in respect of their tolerance to temperature and salinity. No clearcut correlation between sex and resistance could be made out. However according to Kinne (1953a and 1953b) males are less sensitive to higher temperatures. In the present experiments young females and males appeared to be more resistant than the adults.

DISCUSSION

It is well known that many marine animals cannot tolerate wide ranges of salinities in their environments. However, gradual increase or decrease in salinity may pass unnoticed. *Corophium triaenonyx* is a hardy form and has been found to tolerate wide ranges in salinity. It is able to survive in distilled water for nearly two hours although a flatworm like *Monocelis fusca* dies immediately. (Rees 1941). The present observations demonstrate that this amphipod has a greater resistance to fluctuations. Its lower lethal salinity is 0.6‰ and the upper lethal is 59.8‰.

Under natural conditions, it can survive a rise of 27‰ (from 8‰ to 35‰). The flatworm *Monocelis fusca* can also tolerate wide ranges of salinity, although its behavior in increased salinity is somewhat different from that at lower values (see Rees, 1941). In Kinne's experiments on *Gammarus duebeni* (Kinne, 1959) it was found that all specimens remained motionless when salinity rose to 85‰, but that they regain activity upon transfer to normal conditions. However, they could not remain alive in 85‰ for more than 24 hours.

Gradual acclimation to higher temperature, with a resulting increase in the upper temperature tolerance for the species, has been demonstrated by McLeese (1956) with the lobster *Homarus americanus*. Acclimation to higher temperature also increases the lethal temperature levels for two intertidal crabs, *Hemigrapsus nudus* and *Hemigrapsus oregonensis* (Todd and Dehnel 1960).

Different species have different capacities for temperature acclimation. There is clear proof that when the temperature is increased gradually by 1° C each day, survival at higher temperatures is greatly increased in *C. triaenonyx*. Heat tolerance could be increased in lobster *Homarus americanus* when transferred from 14.5° C to 23° C with about 22 days for total acclimation (McLeese, 1956). Acclimation at high temperatures raised the lethal levels of temperature in *Hemigrapsus nudus* and *Hemigrapsus oregonensis* (Todd and Dehnel, 1960). Acclimation at 20° C increased the lethal level for *Gammarus fasciatus* and *Gammarus pseudolimnaeus* (Sprague, 1963). There was no change in the heat tolerance of *Corophium triaenonyx* during different periods of the year. Edwards and Irving (1943) found a 10° C difference between summer and winter conditions in the death point of the sand crab *Emerita talpoida*.

Temperature stress in conjunction with salinity has been studied less extensively in amphipods. In the present investigation, *Corophium triaenonyx* tolerates lower salinities better at higher temperatures and lower temperatures at more normal salinities. Broekema (1941) investigated temperature and salinity effects on the shrimp *Crangon crangon*. This shrimp was shown to endure lower salinities better at higher temperatures. Its optimal salinity at 4° C is 33‰ and between 20° C and 22° C it is 28–29‰. McLeese (1956) has demonstrated that the lobster *Homarus americanus* has a better survival when both temperature and salinity were high. Temperature can change the salinity range, and salinity can change the temperature range of a species. Todd and Dehnel (1960) have shown that higher temperatures together with higher salinity were most favorable for *Hemigrapsus nudus* and *Hemigrapsus oregonensis*. A temperature-salinity relationship was shown to exist in the crab *Rithropanopeus harrisii* (Kinne and Rottbauwe, 1952).

Size and sex do not seem to have any influence on the tolerance of *Corophium triaenonyx*. The effect of size and sex on tolerance to heat does vary in other animals. Edwards and Irving (1943) found no difference in tolerance in *Emerita talpoida* between males and females but larger animals seemed more resistant than the smaller ones. McLeese (1956) observed that, in the size-groups of lobster studied (21–28 cm), there is identical response to upper lethal temperatures. (Kinne 1959) showed a lower tolerance in *female* amphipods (*Gammarus duebeni*) to higher temperatures, and higher tolerance in smaller individuals of both sexes. Sprague (1963) observed that resistance to higher temperatures de-

creased with size in *Gammarus fasciatus*, and that female gammarids were more resistant than the males.

It is clear from the experimental results reported here that *Corophium triaenonyx* is hardy both from salinity and temperature points of view. Gradual acclimations over long intervals of time gave a better survival values. For instance when the temperature was increased by 1° C every day they were able to tolerate 46° C. Field data for its natural environment show fluctuations for salinity (8.66‰–34.67‰) and for temperature (26.4° C–30.84° C). The experimental results demonstrate that *C. triaenonyx* in the field lives well within the ranges of tolerance both for salinity and for temperature. However, in an area like Visakhpatnam harbor, factors other than salinity and temperature may also have to be considered. Visakhapatnam harbor receives sewage materials and effluents from industries like the Caltex Oil Refinery and Coramandel Fertilizers, and various other categories of pollution depending upon the nature of such cargo vessels as are berthed there from time to time.

I am indebted to Professor K. Hanumantha Rao, head of the Department of Zoology, Andhra University, for his valuable suggestions and helpful criticism.

Summary

1. Investigations were undertaken to determine the maximal and minimal salinity and temperature values at which the adults and juveniles of *Corophium triaenonyx* survive, to study the effect of gradual changes, and also to observe the temperature and salinity ranges of natural populations in the environment.

2. This amphipod tolerates wide ranges of salinity from 0.6‰ to 59.8‰, which are the lower and upper lethal salinities respectively. Between 4‰ and 55‰ they live quite normally. Gradual increase or decrease in the salt content does not seem to have any effect on survival time. Animals *in situ* behaved in a similar way to those out of the tubes.

3. *C. triaenonyx* can tolerate temperature ranges from 5° C to 40° C. Survival times increase when the animals are subjected to move gradual changes. When the temperature is raised by 1° C every 24 hours they could survive up to 46° C. When the temperature is raised by 1° C every one hour they died at 44° C. Animals within their tubes seem to be more tolerant.

4. These amphipods tolerate lower salinities better at higher temperature, and lower temperatures better when the salinities are nearer normal values. Size and sex do not seem to have any influence on the survival time.

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Reference: *Biol. Bull.*, **144**: 511–524. (June, 1973)

THE REPRODUCTIVE PHYSIOLOGY OF THE INTERTIDAL PROSOBRANCH *THAIS LAMELLOSA* (GMELIN). I. SEASONAL CHANGES IN THE RATE OF OXYGEN CONSUMPTION AND BODY COMPONENT INDEXES[1]

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There have been many methods used to determine the course of the annual reproductive cycle of marine invertebrates (Giese, 1959). Spawning, numbers of larvae, the appearance of ripe gametes in gonads, the brooding of eggs, and the relative size of gonads and other body components have been used by different investigators to define the reproductive season. Giese (1969) has reviewed the available literature on the use of the body component index approach to studying seasonal changes in the relative size of the body components and on the biochemical composition of each component of molluscs.

The only papers which have been published on seasonal changes in body component indexes of gastropods have dealt with the broadcast fertilizing abalones. Boolootian, Farmanfarmaian and Giese (1962) used cross sectional area to follow seasonal changes in the gonad and hepatic indexes of two species of abalone, *Haliotis cracherodii* and *H. rufescens*. They found an inverse relationship between seasonal changes in the size of the gonad and the hepatic gland. Webber and Giese (1969) determined seasonal index changes in the gonad weight of *H. cracherodii* and found a seasonal periodicity in its size. Maximal gonad growth occurred during the summer months just prior to spawning in early fall. Webber (1970) extended the body component analysis of this species to include the digestive tissue and foot and found two periods of rapid gonadal growth, one prior to spawning and a second just after it. The size of the foot decreased during the first (larger) period of gonadal growth, but increased during the second (smaller) period. He found the size of the digestive gland to change little during the reproductive cycle.

Giese (1969) presented body component index data from single collections of three species: the black abalone *Haliotis cracherodii*, the keyhole limpet *Megathura crenulata*, and the moon snail *Polinices lewisii*. He found a wide difference in the shell indexes, ranging from 7.4% for *M. crenulata*, to 37% for *H. cracherodii*, to 61.6% for *P. lewisii*. The foot was the dominant soft part in all three species.

This paper reports the results of an investigation of *Thais lamellosa* (Gmelin) which is a carnivorous neogastropod snail of the west coast of North America. Its diet usually consists of the barnacles *Balanus cariosus* and *B. glandula* (Connell, 1960, 1970; Emlen, 1966). The species can also feed on the mussels *Mytilus*

[1] Adapted from portions of a thesis submitted to University of Saskatchewan, Regina Campus in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

edulis and *M. californianus*. Male and female *T. lamellosa* aggregate to breed between October and June and aggregation is completed in one to three months in the Puget Sound area (Spight, 1972). Fertilized eggs are enclosed in capsules which are attached to rocks. The number of eggs and capsules produced per female is proportional to its size (Emlen, 1966; Spight, 1972). One population which Spight studied was located in the same vicinity as the one investigated by this author.

Nutrient deposition or depletion is dependent upon the balance between anabolism and catabolism. One measure of metabolic activity often used is the rate of oxygen consumption which is affected by factors that fluctuate seasonally such as food availability and water temperature. Changes in the metabolic rate of a species affect metabolite accumulation or depletion.

Methods and Materials

Adult (> 32 mm long) specimens of *T. lamellosa* were collected either intertidally or with scuba gear at approximately monthly intervals from Turn Island, Washington and immediately taken to the Friday Harbor Laboratories. Snails were placed in an aquarium of flowing sea water along with specimens of *Balanus cariosus* which served as food. Surface water temperature was measured at the time of each collection.

Freshly laid egg capsules were collected at Turn Island on January 19, 1969. Capsules were dried to constant weight by lyophilization and weighed individually.

Respiratory rates

Standard Warburg techniques at 10° C and 30‰ salinity with the modifications of Duerr (1967), were used to determine the rate of oxygen consumption for a 24 hour period as soon as possible after the animals had been collected. After removal from the respirometer, animals were dried, numbered, frozen, and stored for further analyses.

Component indexes

Animals were removed from the freezer, the shell and foot wiped dry, and the animals were weighed. This weight was the animal's entire weight. The shell and operculum were removed, the animals were sexed, and then dissected into foot and visceral mass. The plane of dissection was from just in front of the tentacles to the posterior edge of the opercular scar. Excess water was wiped from the foot and visceral mass, and the components were immediately frozen between two cakes of dry ice. The frozen components were then weighed and immediately lyophilized. The foot and visceral mass were dried to constant weight, weighed separately, and stored over calcium chloride or in a freezer until chemical analyses could be made. Each animal was thus divided into four components: shell, body water, visceral mass, and foot.

Giese's definition of the body component index (1959, 1969) has been modified in this paper so that each body component index, except that of body water, is given as dry weight of the component ($\times 100$) divided by the entire weight. Giese has determined all indexes on the basis of the components wet weight.

Complex component indexes

The visceral mass of animals collected during November 1968; February, April, May, June, July, August, September, November, and December, 1969; and January, February, and March, 1970, were more completely dissected and more complex indexes were calculated. The female visceral mass was dissected into three distinct components: the ovary-digestive gland complex, the capsule-albumin gland complex, and the remaining visceral mass. The male visceral mass was dissected into two components: the testis-digestive gland complex and the remaining visceral mass. These components were stored in a similar manner as the visceral mass and foot until chemical analyses could be made.

Shell length–shell weight ratio

The ratio of shell length to shell weight was determined for each time that the population was sampled and a regression line constructed.

Statistical analyses

Body component indexes which were calculated each time the population was sampled and the egg capsule dry weights were analyzed as the mean plus and minus the confidence interval at the 95% level of significance. When a positive correlation (95% confidence level) existed between the natural logarithms of oxygen consumption, and snail dry weight, Bartlett's Best-Fit method was used to calculate the slope of the regression line, Y intercept, and 95% confidence limits of the slope (Simpson, Row and Lewontin, 1960). When no such correlation existed between the natural logarithms of oxygen consumption and dry weight, oxygen consumption was calculated on a per gram dry weight basis.

Orthogonal polynomials were fitted to the shell length–shell weight data by the least squares regression cubed method (Snedecor, 1956). The data was examined for a possible linear or curvilinear correlation of changes in the shell length-weight relationship with the reproductive cycle.

Results

Field observations

Specimens of *T. lamellosa* were collected from among the barnacles, *Balanus cariosus*, except for the time when they were aggregated in breeding clumps. Most breeding clumps were more than five feet from the nearest barnacles and the animals apparently ceased feeding when aggregated.

Aggregation occurred after November 18 and copulation was observed between the aggregated animals on December 8, 1968. The same population was aggregated and copulating December 12, 1969. Spawning began within the aggregation between January 7 and 19, 1969 and around January 10, 1970. Animals had completed spawning and migrated to the barnacles by March 27, 1969 and by March 30, 1970.

The abundance of intertidal *Balanus cariosus* varied seasonally on Turn Island Barnacles were abundant from September to November 1968 but barnacle density

was low from December 1968 to July 1969 with most of the barnacles being large and located high in the intertidal zone. A new barnacle settlement was first observed on July 14, 1969 and the young barnacles had grown considerably by August 17 when the last observation was made.

Respiratory rates

Oxygen consumption data for both sexes is given in Table I. There were times during the year when there was no correlation between the natural logarithms of oxygen consumption and snail dry weight. Both the absolute values of the

TABLE I

Seasonal changes in oxygen consumption of Thais lamellosa

Month	Correlation coefficient	95% signif.	Number of animals	Slope ± 95% conf. interval	$\mu l\ O_2 \cdot g^{-1} \cdot hr^{-1}$
Males					
Oct. 68	0.715	S	12	0.65 ± 0.53	—
Nov. 68	0.517	S	16	0.75 ± 0.71	—
Dec. 68	0.376	NS	17	—	173 ± 36
Jan. 69	0.158	NS	37	—	139 ± 18
Feb. 69	0.361	S	36	0.19 ± 0.29	—
Mar. 69	0.776	S	26	1.02 ± 0.39	—
Apr. 69	0.189	NS	24	—	130 ± 20
May 69	0.327	NS	23	—	154 ± 24
June 69	0.457	S	28	0.48 ± 0.37	—
July 69	0.565	S	20	0.53 ± 0.36	—
Aug. 69	0.634	S	21	0.58 ± 0.37	—
Females					
Oct. 68	0.092	NS	17	—	155 ± 24
Nov. 68	0.572	S	25	0.42 ± 0.33	—
Dec. 68	0.227	NS	17	—	110 ± 23
Jan. 69	0.470	S	21	0.44 ± 0.35	—
Feb. 69	0.643	S	14	0.98 ± 0.75	—
Mar. 69	0.057	NS	24	—	85 ± 20
Apr. 69	0.710	S	24	0.71 ± 0.30	—
May 69	0.287	NS	27	—	124 ± 22
June 69	0.590	S	20	0.63 ± 0.42	—
July 69	0.654	S	31	0.47 ± 0.21	—
Aug. 69	0.558	S	20	0.63 ± 0.46	—

slopes and the confidence intervals about the mean values were quite variable.

In order to compare oxygen consumption data for all months, the oxygen consumption rate of standard animals with a dry tissue weight of 0.5 grams was determined for both sexes each month (Fig. 1). The oxygen consumption rate of males was highest during October, December, and March. It is interesting to note that males were normally actively copulating at the time of the December collection and had just begun feeding when collected in March. The oxygen consumption rate of females appeared to be more closely related to changes in water temperature than was the oxygen consumption rate of males.

Component indexes

Male body component indexes are given in Figure 2. There did not appear to be any seasonal periodicity in the size of the foot index. The male visceral mass index was largest in the fall and declined at a relatively constant rate throughout the period of aggregation. The visceral mass index declined from a December, 1968 value of 3.62 to 2.42 in March, 1969, and from 3.45 to 2.10 during the same time period of the 1969–1970 aggregation. This decline during aggregation was the result of two factors, starvation and copulation. The visceral mass index was smallest during the spring and summer months and was correlated with a lack of obtainable barnacles as prey. The visceral mass index increased 156% from an

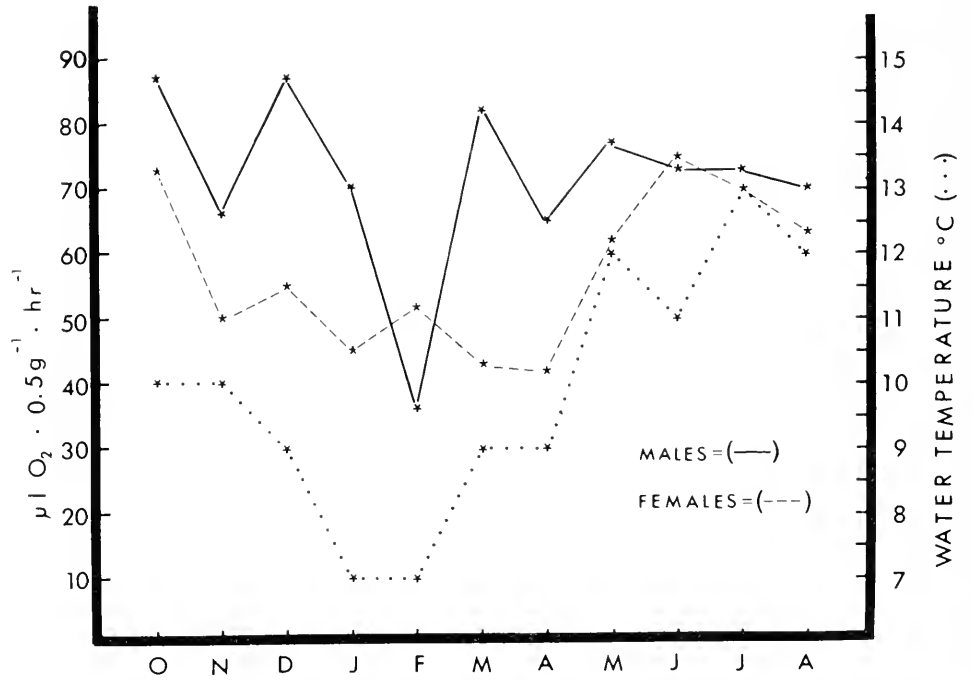

FIGURE 1. The average oxygen consumption rate of 0.5 gram tissue dry-weight male and female standard animals is given in microliters consumed per hour along the left Y axis. Ambient surface seawater temperature is given along the right Y axis.

August value of 2.74 to 4.27 in September, 1969. The body water index cycled in a manner similar to that of the visceral mass and the shell index cycled in an inverse relationship to changes in the visceral mass and body water indexes. The average index of each body component exhibited the following seasonal range: foot, 0.50–0.61; visceral mass, 1.96–5.01; body water, 9.64–12.98; and shell, 81.94–87.74.

Female body component indexes are given in Figure 3. The foot index did not appear to exhibit any seasonal periodicity. The female visceral mass index was

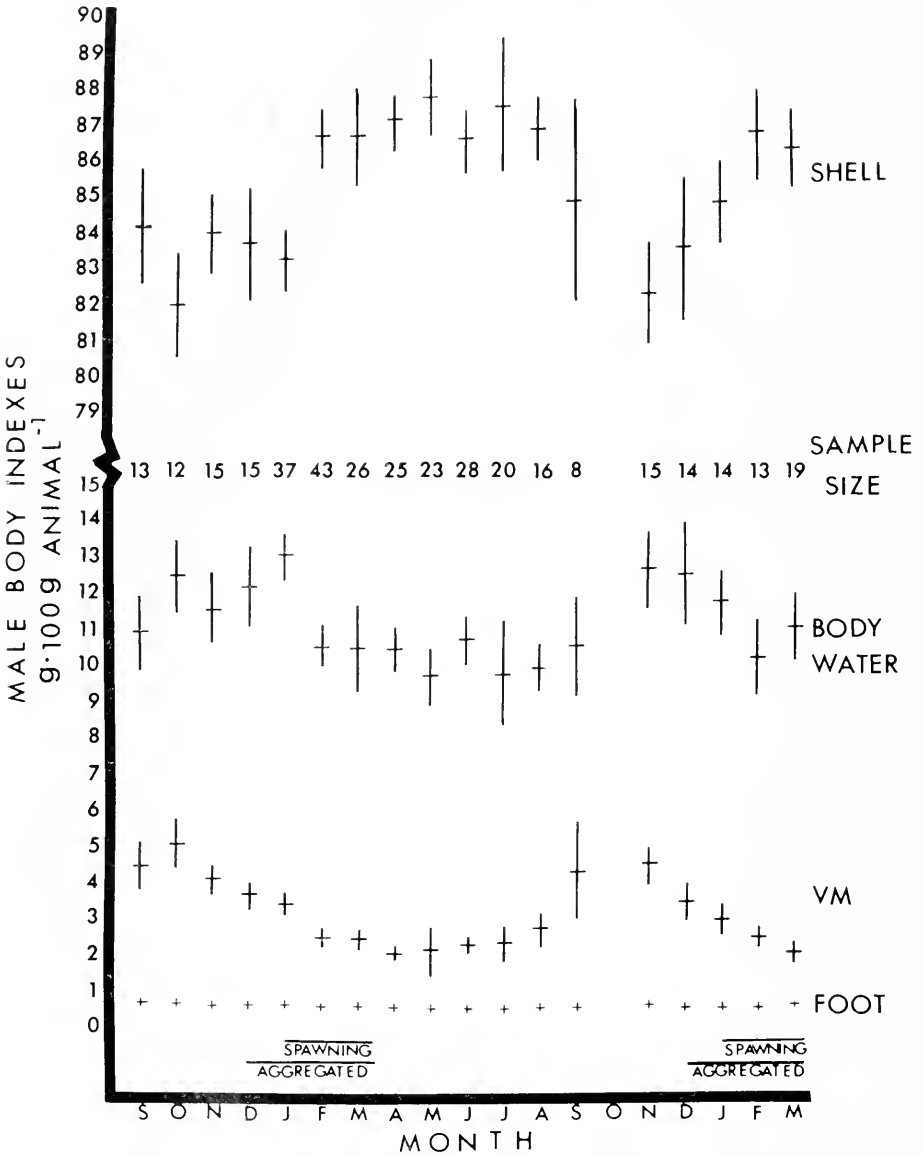

FIGURE 2. Male body component indexes are each represented by a cross. The horizontal line represents the mean and the vertical line the 95% confidence range about the mean.

largest during the fall and early winter with little difference in size occurring during these months. The visceral mass index declined from 4.84 to 3.01 between January and March, 1969 and from 4.53 to 2.04 between December, 1969 and March, 1970. Visceral mass material was lost during aggregation as a result of starvation and spawning by females. The visceral mass index continued to decline

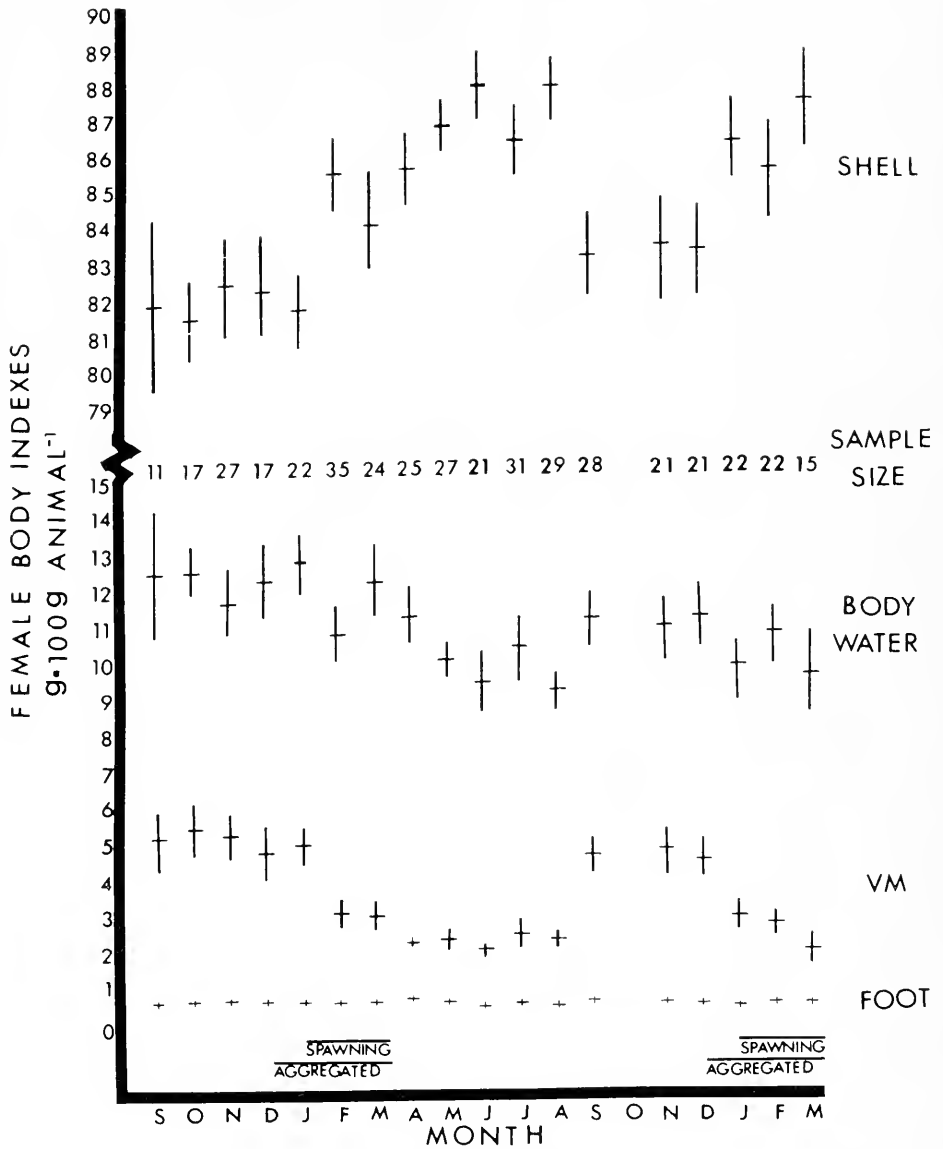

FIGURE 3. Female body component indexes are each represented by a cross. The horizontal line represents the mean and the vertical line the 95% confidence range about the mean.

from March to June, increased slightly in July and August, and doubled from 2.33 to 4.66 between August and September. The body water index cycled seasonally in a similar manner to that of the visceral mass index and the shell index cycled inversely. The average body component indexes of females exhibited the following seasonal range: foot, 0.50–0.66; visceral mass, 2.04–5.39; body water, 9.22–12.43; and shell, 81.49–88.01.

Complex component indexes

Data for the complex visceral mass component indexes of both sexes are given in Table II. There was little seasonal variation in the remaining visceral mass index of males. The testis-digestive gland index cycled seasonally in the same manner as the entire male visceral mass. Therefore, changes in the index of this complex component were responsible for the seasonal cycling of the male visceral mass. The average male complex visceral mass component indexes exhibited the following seasonal ranges: testis-digestive gland, 0.56–2.51; and remaining visceral mass, 1.29–1.98.

All three complex component indexes of the female visceral mass exhibited the same seasonal pattern, largest just prior to spawning and smallest during the late spring and summer. Seasonal changes in the remaining visceral mass index were not pronounced but the ovary-digestive gland complex and the capsule-

TABLE II

Seasonal changes in the complex body component indexes of Thais lamellosa ($g \cdot 100g\ animal^{-1}$)

	Males			*Females*			
Month	Testes-DG complex	Remaining VM	Sample size	Ovary-DG complex	Cap.-Alb.G. complex	Remaining VM	Sample size
Nov.	2.46 ± 0.81*	1.85 ± 0.40	7	2.91 ± 0.77	0.94 ± 0.20	1.63 ± 0.24	12
Feb.	0.99 ± 0.50	1.45 ± 0.32	10	1.11 ± 0.45	0.54 ± 0.31	1.12 ± 0.16	9
April	0.56 ± 0.17	1.36 ± 0.11	12	0.76 ± 0.15	0.28 ± 0.06	1.37 ± 0.19	10
May	0.75 ± 0.22	1.42 ± 0.24	13	0.81 ± 0.24	0.39 ± 0.18	1.31 ± 0.19	10
June	0.66 ± 0.12	1.66 ± 0.12	10	0.65 ± 0.21	0.25 ± 0.07	1.14 ± 0.27	8
July	0.94 ± 0.48	1.62 ± 0.41	9	0.74 ± 0.27	0.20 ± 0.03	1.21 ± 0.25	11
Aug.	1.28 ± 0.25	1.53 ± 0.19	13	0.90 ± 0.20	0.23 ± 0.06	1.15 ± 0.14	12
Sept.	2.50 ± 0.97	1.77 ± 0.38	8	2.47 ± 0.34	0.61 ± 0.08	1.66 ± 0.13	28
Nov.	2.51 ± 0.36	1.98 ± 0.18	15	2.35 ± 0.43	0.87 ± 0.13	1.57 ± 0.17	21
Dec. 69	1.76 ± 0.36	1.77 ± 0.25	12	2.05 ± 0.28	1.04 ± 0.19	1.44 ± 0.12	21
Jan.	1.37 ± 0.16	1.47 ± 0.12	14	1.24 ± 0.26	0.60 ± 0.16	1.16 ± 0.10	22
Feb.	1.14 ± 0.18	1.40 ± 0.14	13	1.13 ± 0.26	0.51 ± 0.11	1.17 ± 0.10	23
March	0.73 ± 0.20	1.29 ± 0.18	19	0.68 ± 0.16	0.28 ± 0.14	1.08 ± 0.20	19

* Mean ± 95% confidence interval.

albumin gland complex indexes exhibited a distinct seasonal cycle. The average female complex visceral mass component indexes exhibited the following seasonal ranges: ovary-digestive gland, 0.65–2.91; capsule-albumin gland, 0.20–1.04; and remaining visceral mass, 1.08–1.66.

Biomass lost per female during spawning

One can estimate the percentage of visceral mass size lost by females during spawning which was deposited as egg capsules. The average dry weight of 78 freshly laid egg capsules was 4.6 ± 0.6 mg. Spight's (1972) estimates of 36.2 capsules laid per female during the 1969 spawning period and 48.3 capsules laid during 1970 were multiplied by the average capsule dry weight to determine the

total capsule weight lost per female during spawning. The average entire weight of the 420 females examined during this study was 11.3208 grams (range—3.9090 to 30.3442 g) and the average length was 40.9 mm (range — 32.0 to 56.0 mm). Visceral mass biomass lost during spawning was adjusted to the amount lost by an 11.3208 gram female from January to March 1969 and from December 1969 to March 1970. The difference between the total visceral mass biomass lost during the spawning period and the amount deposited as egg capsules can be attributed to female respiration. These calculations are presented in Table III.

More biomass was lost from the female visceral mass during 1970 than in 1969. Spight (1972) reported a higher capsule production per female in 1970 than in 1969. Most of the biomass lost from the female visceral mass during both of the spawning periods was deposited as capsular material. The 1969 spawning period was estimated to be 82 days and the 1970 period 78 days.

Shell length–shell weight relationship

Because the shell comprises a large percentage of the body of *Thais lamellosa*, the possibility of seasonal changes in the shell length–weight ratio was investigated.

TABLE III

Biomass lost per gram Thais lamellosa *female during 78–82 day spawning period*

Capsules laid per female*	Dry weight per capsule (mg)	Dry weight lost as spawn (mg)	Dry weight lost from the VM** (mg)	Per cent VM dry weight lost as spawn	Per cent VM metabolized as tissue
1968–69					
36.2	4.6	167	207	81	19
1969–70					
48.3	4.6	218	282	77	23

* From Spight (1972).

** Visceral mass size lost during the spawning period in a 100 gram female was reduced to the amount lost by an 11.3208 gram female and expressed in milligrams.

Significant seasonal changes in the relationship between shell length and weight would be indicative of a variable pattern of shell deposition and such information would be valuable to the explanation of seasonal changes in the other body component indexes.

There was no linear or curvilinear correlation between the shell length–shell weight relationship and the monthly sampling dates for the population. Therefore, it does not appear likely that seasonal changes in the rate of shell deposition are responsible for the reproductive cycle periodicity of the body component indexes. The seasonal changes which occur in the shell index of both sexes are simply a reflection of changes occurring in the magnitude of the other indexes.

DISCUSSION

Thais lamellosa differs in several important ways from the other prosobranchs for which body component indexes are available. *Haliotis cracherodii* and *M.*

crenulata are herbivores whereas *P. lewisii* and *T. lamellosa* are carnivores. *Haliotis cracherodii* and *M. crenulata* are broadcast fertilizers (Webber and Giese, 1969; Morton, 1958). *P. lewisii* produces a sand collar composed of sand mingled with a gelatinous secretion and containing spaces occupied by eggs (Hyman, 1967), and *T. lamellosa* deposits fertilized eggs in a well developed egg capsule.

The foot comprises the predominant body component of *H. cracherodii*, *M. crenulata*, and *P. lewisii* but is a minor component of *T. lamellosa*. Relative size differences of the foot can be best explained by considering the ecological niche of each species. The foot of *H. cracherodii* and *M. crenulata* is modified into a broad flat sole used for creeping over the rocks from which the snails rasp a diatom film. The pedal lobes of the foot of *P. lewisii* can be expanded to completely cover its shell by the animal pumping seawater into the foot's intersititial spaces. Expansion of the pedal lobes presumably protects the shell from the abrasive action of the sand or mud through which the species crawls (Hyman, 1967). In contrast to the other three species, the foot of *T. lamellosa* is unspecialized and is used to attach the species to rocks and prey.

The shell index of *T. lamellosa* is much larger than that of the other three species. Its index ranges from 81.49 to 88.01 seasonally as compared to indexes of 61.6 for *P. lewisii*, 37 for *H. cracherodii*, and 7.4 for *M. crenulata*.

There is much inter-population variability in the shell morphology of *T. lamellosa* (Kincaid, 1957). Much of the variability is undoubtedly genetic in that no planktonic larvae is produced by the species and genetic exchange between populations is rare. In addition, Malone and Dodd (1967) have shown the calcification rate of *Mytilus edulis* to vary directly with salinity and temperature. The shell indexes of a population of *T. lamellosa* from Saint Therese, Alaska ranged from 72.56 to 83.13 (Stickle, unpublished data) as compared to the 81.49 to 88.01 range from the Turn Island, Washington population. The Saint Therese population is faced with colder winters and less saline water during the summer months than the Turn Island population. Environmental conditions are therefore more favorable for shell calcification at Turn Island, Washington than at Saint Therese, Alaska.

Reproductive tract complexity correlates well with the evolutionary status of the species within the subclass Prosobranchiata. Morton (1958) states that archaeogastropods and mesogastropods deposit less extraembryonic material around their gametes than neogastropods. The oviducts of the archaeogastropods, *H. cracherodii* and *M. crenulata* is undifferentiated, that of the mesogastropod *P. lewisii* is somewhat developed, and that of *T. lamellosa* is very well developed.

The reproductive tract morphology of *T. lamellosa* has not been described in the literature but it appears to be similar to that of *T.* (= *Nucella*) *lapillus* (Fretter, 1941; Fretter and Graham, 1962), *T. emarginata*, and *T. canaliculata* (Houston, 1971). The prostate gland is the only major accessory structure of the male and was included in the remaining visceral mass component of this study. The female capsule and albumin glands produce all of the extraembryonic material secreted around *T. lamellosa's* eggs. The albumin gland coats the eggs with a layer of albumin and the capsule gland produces the egg capsule.

It is metabolically expensive for animals to secrete extraembryonic material around gametes. The metabolic expense of producing extraembryonic material is balanced by the production of fewer eggs than occurs in broadcast fertilizers. The female capsule-albumin gland complex declined from 17% to 12% of the visceral mass size during spawning in 1969 and from 26% to 14% in 1970. If body components are adjusted to an 11.3208 gram animal, biomass lost from the capsule-albumin gland complex during spawning would account for 45% of the visceral mass biomass deposited as spawn in 1969 and 39% in 1970. It is also assumed that no biomass was transferred to the capsule-albumin gland complex from other body components. Lawrence, Lawrence, and Giese (1965), however, found a reciprocal relationship to exist between the digestive gland and glandular oviduct indexes of the amphineuran *Katherina tunicata*. An inverse relationship of such nature suggests nutrient transfer from the digestive gland to the oviduct during gametogenesis.

The gonad-digestive gland complex was probably the energy source for the production of gametes and maintenance of body functions during aggregation and spawning. Unfortunately it was impossible to separate the gonad and digestive gland tissue of *T. lamellosa*. Webber (1970) found no seasonal change in the digestive gland index of *H. cracherodii*.

The remaining visceral mass index of *T. lamellosa* does not appear to exhibit a seasonal cycle. However, this is not surprising since it contains such organs as the heart, kidney, gills, mantle, salivary glands, *etc.*, which have never been shown to be nutrient depots.

Seasonal differences in food availability can effect a species' pattern of nutrient deposition. If prey were abundant year around, less food would have to be stored prior to the initiation of gametogenesis.

Webber (1970) suggested that seasonal differences in the feeding rate of *H. cracherodii* may have been responsible for a different relationship between the foot and gonad indexes during two periods of gonad growth. Foot size decreased during the first (larger) period of gonad growth but increased during the second (smaller) period which occurred just after spawning. The abalone population was observed to be feeding more during the postspawning period of gonad growth than at any other time of the reproductive cycle. Webber felt that there was sufficient energy intake during the postspawning period of gonad growth to satisfy energy demands for abalone maintenance, growth, and gonad maturation but that insufficient energy was ingested to satisfy all three energetic demands during the prespawning gonad growth phase.

Barnacle density and intertidal position probably affected changes in *T. lamellosa's* body component indexes. The intertidal barnacles remaining at the conclusion of the snails' 1969 spawning period were very large and located near the high tide line. Both barnacle size and intertidal position would make it difficult for *T. lamellosa* to prey upon them. Emlen (1966) has shown *T. lamellosa* to most efficiently eat barnacles of much smaller size than those found at Turn Island in March 1969. In addition, the higher a barnacle is in the intertidal zone, the less time it is sumberged and *T. lamellosa* only drills barnacles while submerged.

A new barnacle settlement was not observed until July 14, 1969. This lack of prey could explain the rather constant visceral mass index between March and August. The young barnacles had grown considerably by August 17, 1969. The tremendous increase in the visceral mass indexes of both snail sexes between August and September can probably be explained by the fact that the young barnacles had grown to a size where they could be efficiently preyed upon by adult *T. lamellosa*.

A successful barnacle settlement is probably reflected the following year by an increased capsule production by female *T. lamellosa*. Spight (1972) reported the 1969 barnacle settlement to be the most successful in years and reported a higher capsule production by female snails in 1969–1970 than had occurred in 1969.

Aggregation was extremely demanding on adults of *T. lamellosa* which were starving and depositing 77–81% of their prespawning visceral mass size as spawn during that three to four month period. Pearce and Thorson (1967) indicated that females of *Neptunea antiqua* became exhausted following spawning and starving. Eight of nine females observed during spawning in the laboratory died within three months after spawning. Stickle (1971) has shown that adult specimens of *T. lamellosa* could be starved for an additional 91 days after the completion of spawning before 50% of the snails died. Specimens of *T. lamellosa* were capable of an extremely protracted period of starvation at ambient sea water temperature (Stickle and Duerr, 1970; Stickle, 1971). Resistance to starvation allows the species to survive periods of time when food is scarce.

There appeared to be no compensation in *T. lamellosa's* oxygen consumption rate. Less metabolic substrate was therefore utilized by females for body maintenance during aggregation in the winter months than would have occurred if thermal acclimation existed. However, the oxygen consumption rate of males increased significantly at the beginning and conclusion of aggregation. The increased oxygen consumption rate at the beginning of aggregation can be explained by the fact that male copulatory activity is most pronounced during early aggregation and declines with its duration (Spight, 1972). Metabolic conversions involved in sperm production and copulatory activity were probably quite high at the beginning of the aggregation. The increased oxygen consumption rate at the conclusion of aggregation may have been due to an increased rate of lipogenesis. The lipid level and content of males increased from February to March although the increase was not statistically significant (Stickle, unpublished data). However, male snails collected at Turn Island on February 25, 1969 and starved for 91 days at ambient water temperatures synthesized significant amounts of lipid.

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Summary

1. Body component indexes of *Thais lamellosa* were determined over a period of 19 months. Snails were separated into shell, body water, foot, and the visceral mass including its more complex components consisting of the gonad-digestive gland, remaining visceral mass and the female capsule-albumin gland. Indexes were expressed as grams of the component × grams of the entire animal^{-1} × 100. Spawn was collected and weighed. The rate of oxygen consumption was determined for the first year of the study.

2. The body water, visceral mass, gonad-digestive gland, capsule-albumin gland, and female remaining visceral mass indexes exhibited a seasonal cycle of being largest just prior to population aggregation in November, declining during aggregation and remaining low during the summer months. These indexes greatly increased between August and September and remained high until the next population aggregation. The shell index cycled in an inverse pattern to that of the others, merely reflecting changes in the magnitude of the sum total of the other indexes.

3. Aggregation is demanding upon *T. lamellosa* because it is accompanied by spawning and starvation. Spawn accounted for 77–81% of the size of the visceral mass lost during aggregation with the remainder being lost through respiration. Capsule-albumin gland material accounted for 39–45% of the biomass lost from the visceral mass as spawn. The production of extraembryonic material is metabolically demanding of adult snails.

4. The availability of barnacles of optimum size and distribution for predation by *Thais* is integrally related to the snail's pattern of nutrient deposition. Barnacles were unavailable for efficient predation from November-August. The increase in the visceral mass, gonad-digestive gland, and the capsule-albumin gland indexes between August and September was probably related to the presence of barnacles of optimum size for predation.

5. The rate of oxygen consumption of both sexes was directly related to water temperature with the male rate being higher than expected from the water temperature at the beginning and end of aggregation.

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STUDIES ON LARVAE OF STRIGEOID TREMATODES FROM THE WOODS HOLE, MASSACHUSETTS REGION[1]

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The superfamily Strigeoidea Railliet, 1919 was erected to contain the trematodes formerly included in the families Holostomidae E. Blanchard, 1847 and Hemistomidae Brandes, 1888. *Holostomum* Nitzsch, 1819 and *Hemistomum* Diesing, 1850 were suppressed as synonyms, respectively, of *Strigea* Abildgaard, 1790 and *Alaria* Schrank, 1788. These worms were known to Goeze and Rudolphi and are common parasites of birds and mammals. Adults live in the intestine of turtles, crocodiles, birds and mammals. The first intermediate hosts are snails and the cercariae are produced in slender sporocysts. The cercariae have forked tails, penetrate their next hosts and occur as metacercariae in snails, leeches, fish, amphibians and rarely in snakes, birds, and mammals. In members of the genera *Strigea* and *Alaria*, an additional stage, the mesocercaria, may be interposed between the cercaria and the metacercaria and these species have a four host life cycle.

Knowledge concerning the life-cycles of these trematodes was retarded for fifty years by the erroneous belief that they differed essentially from other groups. Von Linstow (1877) embryonated eggs of *Holostomum cornucopia* Molin, 1859 [= *Strigea strigis* (Schrank, 1788) Abildgaard, 1790]. He reported that the "embryo" which emerged, transformed, without sporocyst or cercarial generations, into the metacercaria. This organism had been described by Steenstrup (1842) and was designated as Tetracotyle by de Filippi (1854). The metacercariae of the strigeids have characteristic forms which were regarded as genera by the early investigators and designated Tetracotyle, Tylodelphys, Diplostomum, Conocephalus, *et al.* Leuckart (1889) accepted the idea of von Linstow and designated the type of development as "metastatic," *i.e.*, intermediate between the monogenetic and digenetic life cycles of other trematodes. The error was dispelled when Lutz (1921) demonstrated that strigeid metacercariae developed from forked-tailed cercariae. The discovery was quickly confirmed; Ruszkowski (1922), Mathias (1922) and Szidat (1924) showed that other strigeids have furcocercous cercariae. Szidat (1929) described larval development in the strigeids and showed that the "holdfast" or tribocytic organ is a new structure, peculiar to the group.

In a monograph of the Strigeida, Dubois (1938) recognized two superfamilies: Strigeides Dubois, 1936 and Cyathocotylides Dubois, 1936. The first contained three families: Strigeidae Railliet, 1919; Diplostomidae Poirier, 1886, and Proterodiplostomidae Dubois, 1936. The Strigeidae included two subfamilies, Strigeinae Railliet, 1919, parasites of birds, and Duboisiellinae Baer, 1938, with a single genus *Duboisiella* Baer, 1938, parasite of mammals. The Diplostomidae contained two subfamilies: Diplostominae Monticelli, 1888, parasites of birds and Alariinae

[1] Supported by NSF GB 30662.

Hall and Wigdor, 1918, parasites of mammals. The family Proterodiplostomidae contained three subfamilies: all parasites of reptiles. The superfamily Cyathocotylides contained two families: Cyathocotylidae Poche, 1925, with parasites of both birds and mammals, and Brauninidae Bosma, 1931, parasites of mammals. Dubois (1968, 1970) published a revised "Synopsis des Strigeidae et des Diplostomidae."

LaRue (1957) reviewed the life-cycles and developmental stages of the digenetic trematodes. Basing his determination primarily on homologies in the formation of the excretory system, he proposed a new system of classification in which a new order, Strigeatoidea, included the strigeids, the schistosomes, clinostomes, azygiids, cyclocoelids, brachylaemids, fellodistomids, bucephalids, and renicolids. In all of these families the cercariae lack stylets, have simple membranous excretory vesicles, forked tails, and primary excretory pores located on the tails. Discovery of the life-cycle of *Renicola thaidus* by Stunkard (1964) removed the family Renicolidae from the Strigeatoidea.

Hoffman (1960) compiled a synopsis of Strigeoidea (Trematoda) of fishes and their life-cycles. He recognized four larval groups: Tetracotyle, Diplostomulum, Neascus, and Prohemistomulum. The Tetracotyles are larvae of species in the family Strigeidae and the adults occur in birds; Diplostomulum and Neascus are metacercariae of members of the Diplostomidae which occur in both birds and mammals, while the Prohemistomula are larvae of cyathocotylids that occur in both birds and mammals. The Tetracotyle have lateral pseudosuckers (cotylae) and the tribocytic organ is bilobed. The diplostomes have small, marginal anterior suckers and the tribocytic organ is circular to oval with a median longitudinal vent. Neascus and Prohemistomulum larvae lack accessory suckers. Tetracotyles produce cysts of parasitic origin but the larvae of *Apatemon burti* in leeches do not encyst for five or six weeks and are not infective until encysted (Stunkard, Willey and Rabinowitz, 1941). In the diplostomes the cercariae do not have cystogenous glands and there are no cysts of parasitic origin.

Strigeid trematodes are common parasites of shore birds and Linton (1928) described three species from gulls taken at Woods Hole. One, described as *Proalaria indistincta* (Guberlet) was based on three specimens, two from *Larus argentatus* and one from *Larus atricilla*. A single, damaged specimen from *L. argentatus* was described as *Alaria* species. Three specimens from *L. argentatus*, one from *L. atricilla*, and three from *L. delawarensis* were described as *Strigea bursigerum* (Brandes) Lühe. The first species was identified by Dubois (1970) as *Diplostomum spathaceum indistinctum* (Guberlet, 1923) Hughes, 1929 and the second as *Diplostomum gavium* (Guberlet, 1922) Hughes, 1929. The third species was assigned by Dubois (1968) to *Cardiocephalus mediconiger* Dubois and Vigueras, 1949 [= *Cardiocephaloides mediconiger* (Dubois and Vigueras, 1949) Dubois, 1970].

Although adult specimens of strigeid trematodes are common in shore birds, the larval stages are virtually unknown in marine snails. Cable (1956) described two cyathocotylid species: *Cercaria caribbea L.* and *Cercaria caribbea LI.* from species of *Cerithium* in Puerto Rico. Hutton and Sogandares-Bernal (1959, 1960) described cyathocotylid larvae from *Cerithium muscarum* which encysted in the muscles of mullets, *Mugil* spp., and became sexually mature in the intestine of

the brown pelican, *Pelicanus occidentalis,* the black crowned night heron, *Nycticorax nycticorax,* and the opossum, *Didelphis virginianus.* The adults were identified as *Mesotephanus appendiculatoides* (Price, 1934) Lutz, 1935. A field caught gull, *L. delawarensis,* and a raccoon, *Procyon lotor,* yielded specimens of *M. appendiculatoides* when fed infected mullet flesh. Another life-cycle was reported by Martin (1961) who found sporocysts and cercariae in *Cerithidea californica,* encysted metacercariae in the muscles of *Fundulus parvipinnis* and *Gillichthys mirabilis,* and adults were obtained experimentally in chicks. The worms were identified as *Mesostephanus appendiculatus* (Ciurea, 1916) Lutz, 1935. The species was described originally from the intestine of dogs and cats that had been fed metacercariae encysted in the muscles of various fishes, *Tinca tinca, Aspius aspius, Blicca bjorkna,* and *Carassius carassius,* taken from the Danube River in Roumania. Dubois (1953, page 108) stated that the natural hosts are pelicans and "*Mesostephanus appendiculatus* Ciur. et *M. longisaccus* Chdl. sont des parasites secondaries ou erratiques du chien." Leonov (1958) reported *M. appendiculatus* from *L. argentatus* in Russia and Yamaguti (1971) from *L. delawarensis* in the United States.

With the exceptions of the cyathocotylids mentioned, the only other strigeid larva reported from a marine snail is *Cercaria nassa* Martin, 1945, from *Ilyanassa obsoleta* (syn. *Nassa obsoleta*) taken in the Woods Hole region. The incidence of infection was very low, averaging 0.1 per cent. The cercariae developed in long slender sporocysts, and both sporocysts and cercariae were figured. In addition to the morphology of the cercariae, Martin (1945) reported on their behavior and swimming activity. The cercariae are not responsive to light, are uniformly distributed in the water, and swim with the tail in advance. They manifest alternate periods of activity and rest. At rest the cercaria is suspended in the water, tail uppermost with the furcae spread at an angle of approximately 90 degrees. It slowly sinks until it suddenly darts upward in a spiral course, caused by a sculling movement of the tail, which ends with the body in a horizontal position. As the larva sinks, the body turns downward and after it reaches a vertical position the next upward dash is started. Martin (1945) measured the length of the swimming and inactive periods; the average of 40 observations gave the following data; resting, 7.87 seconds, swimming, 2.08 seconds. As the cercariae grow older the swimming period becomes shorter and the resting period is lengthened. Consequently, the cercariae tend to lie deeper in the water. Martin recognized the cercaria as a strigeid species and compared it with *Cercaria flexicorpa* Collins, 1935. Attempts were made to determine the second intermediate host and common fishes were exposed to the cercariae. The larvae attached by their anterior organs to *Fundulus* spp. and *Paralichthys dentatus* and immediately shed their tails. They remained attached to *Fundulus* for several hours but seemed unable to penetrate and eventually dropped off. They penetrated the thin web of the fin of *P. dentatus* but disintegrated after a few hours.

Hunter and Vernberg (1960) at the Duke University marine laboratory, Beaufort, North Carolina, reported the finding of strigeids in laboratory reared birds, *Rhynchops nigra, Sterna hirundo,* and *Sterna albifrons,* that had been fed small unidentified fishes. Dissection disclosed large numbers of metacercariae, some encysted, in the ventricles and frequently in the eyes of *Menidia menidia* and

Mugil cephalus. Feeding experiments conducted in 1956 yielded worms of different ages and degrees of development when young *M. cephalus* were fed to *R. nigra*. Feeding experiments were continued in 1959. Metacercariae from the brain of *M. menidia* were fed to two *R. nigra* and one *S. hirundo*. The results were negative and the authors concluded that the metacercariae were not infective before encystment. Whole brains were fed to four, one-day old, chicks. Three received 20 and the other 30 brains. Five worms were recovered from one chick after 72 hours and eleven worms from another after seven days. The other two chicks were negative on examination. Worms were submitted to Dr. Dubois and identified as *Cardiocephalus medioconiger* [= *Cardiocephaloides medioconiger* (Dubois and Vigueras, 1949) Dubois, 1970]. Dubois (1970, page 722) reported, "*Cardiocephalus* Szidat, 1928 tombe comme homonyme de *Cardiocephalus* Broili, 1904 (Amphibia: Lepospondyli: Microsauria), type *sternbergi (Permien*, Texas)."

The authors noted that *Cercaria nassa* occurs frequently in *Nassarius obsoleta* in the Beaufort, North Carolina area. They recalled that Martin (1945) had recognized *C. nassa* as a strigeid but had failed to obtain infection in species of *Fundulus* and *Paralichthys dentatus*. They confirmed the results with *Fundulus* spp. However, they reported penetration into small *M. cephalus* and recovery of larvae at intervals of ten hours and 18 days, although the locations were not given. Attempts at penetration of the general body surface of fishes were unsuccessful, but penetration through the roof of the mouth was observed and entrance through the gills was suspected. Encysted metacercariae were found only in the brain, but there was no assurance that the worms resulted from experimental exposure. There is no evidence that *C. nassa* is the larval stage of *C. medioconiger* and such uncritical speculation may lead to unfortunate errors like the one made by Hunter and Vernberg (1953) where the cercaria of *Zoogonus lasius* (Leidy, 1891) was identified as the larval stage of *Gynaecotyla adunca* (Linton, 1905).

An observation by Abbott (1968) has impelled renewed study of *C. nassa*. He reported metacercariae of an unidentified trematode on the brain of *Fundulus heteroclitus*, representing 100% infection of a sample of fishes obtained from the Chesapeake Biological Laboratory, Solomons, Maryland. Although the author was concerned with the function of the pineal gland of the fish, the description and figures of the parasite identify it as a strigeid. Since *C. nassa* is the only described strigeid cercaria from marine snails on the Atlantic coast and is sympatric with *F. heteroclitus*, the possibility appeared that it might be a stage in the life cycle of the metacercariae in the brain of that species. This possibility has been under investigation during the past three summers and a preliminary report was presented at the annual meeting of the American Society of Parasitologists in August, 1971, at Los Angeles, California.

Materials and Methods

During the past three years over 10,000 specimens of *I. obsoleta* have been collected and examined for infection with *C. nassa*. They came from various locations on the seashore near Woods Hole, Massachusetts and from beaches on adjacent Martha's Vineyard, Elizabethan and Weepecket Islands. For ease in computation, they were isolated ten in a bowl and the water was changed daily,

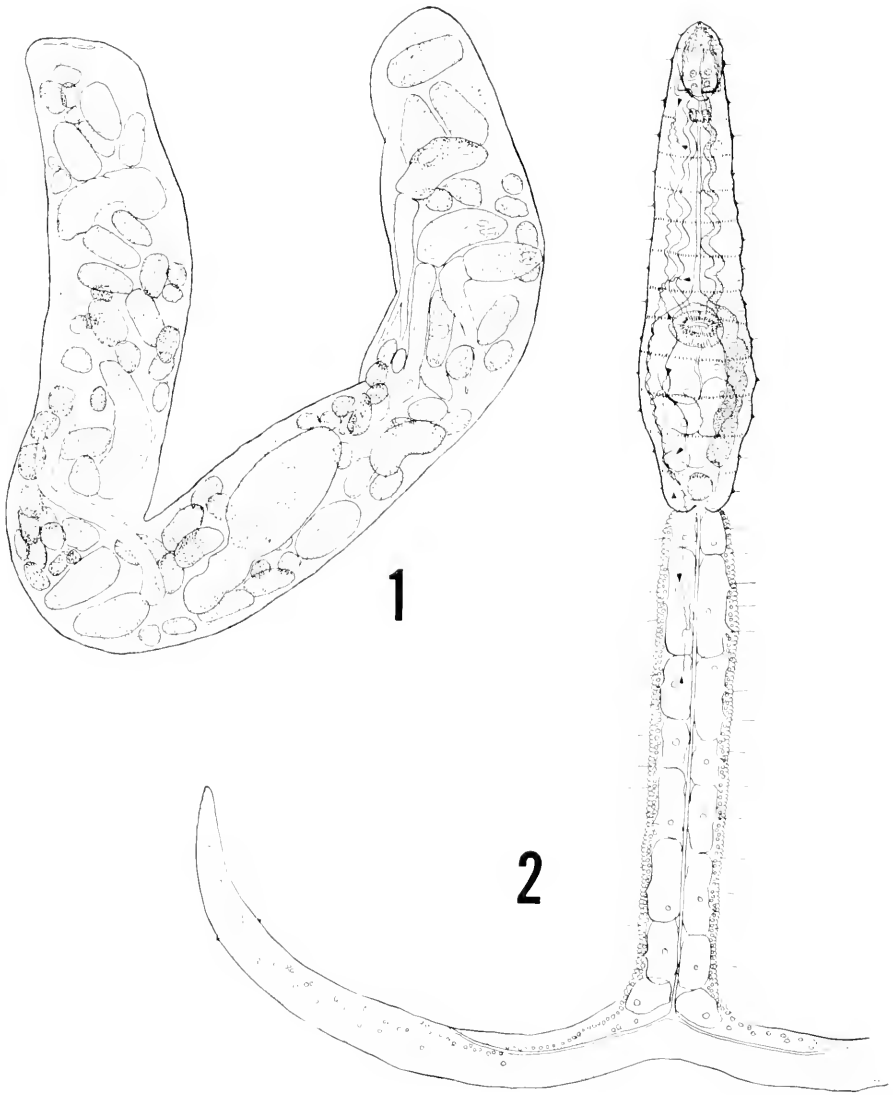

FIGURE 1. Sporocyst of *C. nassa;* specimen 2.20 mm long, 0.20 mm wide. For clarity, less than one-half of the germ balls and cercariae are represented.

FIGURE 2. *Cercaria nassa;* a composite drawing made from pencil sketches of living specimens and study of fixed and stained ones.

sometimes in the morning and sometimes in the late afternoon to check the time of cercarial emergence. When the snails were transferred to a clean bowl, the water from which they were taken was examined for cercariae. Other species, *e.g.*, cercariae of *Zoogonus lasius, Himasthla quissetensis, Lepocreadium setiferoides, Stephanostomum tenue* and *Stephanostomum dentatum* were common while those of *Gynaecotyla nassicola* and *Microbilharzia variglandis* were rare.

After one week to ten days, if no *Cercaria nassa* appeared, sample snails were crushed for examination and others were discarded. As a rule, when snails are brought into the laboratory, with a somewhat higher temperature, they shed readily and they will continue to shed if well fed, but starved snails soon fail to liberate cercariae although the infection persists and shedding can sometimes be induced by feeding the snails. It is clear the reproduction of the parasite is inhibited when the host is unnourished. Shedding of *C. nassa* is unpredictable; some days scores of cercariae emerged followed by several days with few or no cercariae and then a renewal of shedding. The larvae emerged during both day and night but most were shed in the morning hours. Whether or not cercariae are infective immediately after release or only after a period of acclimatization, and for how long a time, are unknown. The cercariae lose vitality after 24 hours and may no longer be infective. When fishes are left in bowls with shedding snails, the number of cercariae shed is unknown, but larvae of different ages and different degrees of infectivity are present.

During the summer of 1969, 2,740 specimens of *I. obsoleta* were isolated and no infections with *C. nassa* were found. In 1970, 3,980 snails were isolated and a collection of 630 snails, taken July 8, in Squiteague Bay, near North Falmouth, yielded three infections, the only ones discovered during the summer. In 1971, 3,590 snails were isolated with negative results, but a single infected specimen was provided by Dr. Paul Krupa who found it in a collection made on Penzance in Great Harbor, Woods Hole. In the past three years, only four of more than 10,000 snails shed *C. nassa*. The incidence of infection in the Woods Hole region is less than that found by Martin some thirty years ago. The snails are as numerous, so the final hosts must be more rare.

On July 17, 1969, Dr. Langley Wood brought about 100 specimens of *Fundulus heteroclitus* taken near the Chesapeake Biological Laboratory, Solomons, Maryland and one week later sent 200 specimens of *Nassarius vibex* from the same area. The fishes were dissected but no strigeid metacercariae were found. Dr. Wood reported that *I. obsoleta* is rare in Chesapeake Bay and is replaced there by *N. vibex*. One of the *N. vibex* shed *C. nassa*. The snail continued to shed cercariae during the summer, sometimes at intervals of several days and was crushed on September 15th. There had been no cercariae for almost three weeks, but the haemocoele was filled with active sporocysts of all sizes. On July 21, 1970, Dr. Victor Sprague sent 200 specimens of *N. vibex* from the Chesapeake Laboratory, but there were no infections with *C. nassa*.

Strigeid trematodes are predominantly parasites of birds and it seemed likely that the adult stage of *C. nassa* would be found in birds that feed on *Fundulus* spp. Accordingly, when an infection by *C. nassa* was found in 1970, Dr. Norman Sinclair and Mr. Peter Oldham collected two nestling snowy egrets, *Egretta thula*, and one black-crowned night heron, *Nycticorax nycticorax*, from the rookery on Martha's Vineyard Island and three cormorants, *Phalacrocorax auritus*, and three gulls, *Larus argentatus*, from the rookery on the Weepeckets. Repeated fecal examinations showed no previous infection by trematodes and the birds were maintained on commercial food to assure against accidental infection. One cormorant and one gull were lost, but the other birds survived and were autopsied in in September.

Experiments and Results

In 1969, when the specimen of *N. vibex* was found to be liberating *C. nassa*, experiments were begun to determine whether or not the metacercariae would attack and develop in *Fundulus* spp. Fishes, provided by the Supply Department of the Marine Biological Laboratory, were placed in bowls with swimming cercariae and although penetration was not observed, the presence of discarded tails on the bottom of the bowls indicated that the bodies of the cercariae had entered the tissues of the fish. Dissection of fishes that had been exposed yielded metacercariae on the surface of the brain. The larger worms were active, not encysted, and fell off the brain-surface when the cranial cavity was opened under sea water. They were not found in the gills, eyes, or optic nerves. The smallest specimen was found on August 5, in a fish that had been exposed on August 1. This specimen was lost. Two other fishes, exposed on August 1, were dissected on August 16. Four metacercariae were recovered; a fixed and mounted specimen (Fig. 3) measures 0.325 mm long and 0.24 mm wide. A fish dissected on September 8, yielded a metacercaria that measured 0.44 by 0.315 mm. Two fishes dissected on September 9 yielded three metacercariae; the largest fixed and mounted, (Fig. 6) measured 0.70 by 0.63 mm. It was very active, with the edges of the body extending and retracting constantly. In it the reticular reserve excretory system was filled with concretions. The purpose of the study was to obtain adult specimens rather than to trace development in the fish, so few fishes were dissected. It was hoped to feed the metacercariae to baby chicks at the end of the summer, but baby chicks are not available in September in the Woods Hole area. Eighteen fishes that had been exposed were decapitated, the brains were removed and fed to a pigeon on September 12. The bird was autopsied on September 18, but the results were negative.

When snails infected with *C. nassa* were found on July 8, 1970, experimental infection of *Fundulus* spp. was renewed and efforts were made to complete the life-cycle by the use of fish-eating birds. The rookeries on Martha's Vineyard and the Weepeckets provided nestlings as noted earlier.

On July 12, two fishes were placed in a bowl with hundreds of cercariae that had been liberated by the three snails. One of the fishes died the next day and the other on the following day. Whether or not these deaths were the result of the massive exposure is uncertain, but large numbers of cercariae must have penetrated the tissues of the fishes because there were hundreds of detached and discarded tails on the bottom of the bowl. In later exposures, the fishes were not subjected to heavy infection; rather, repeated exposures were made with fewer cercariae. After repeated exposures, the fishes were maintained in larger aquaria. For exposure, usually two fishes were placed in a bowl with the cercariae that had emerged during the previous 24 hours, some 20 to 50 larvae. As fishes that had been exposed died during the summer, they were dissected to follow the development of the metacercariae. Exposures were terminated about the middle of August and thereafter the cercariae were studied for morphological details. On August 25, one snail was crushed to obtain sporocysts and developing cercariae. The digestive gland was heavily infected with active sporocysts although for the past two weeks the number of cercariae liberated had been small. A second infected

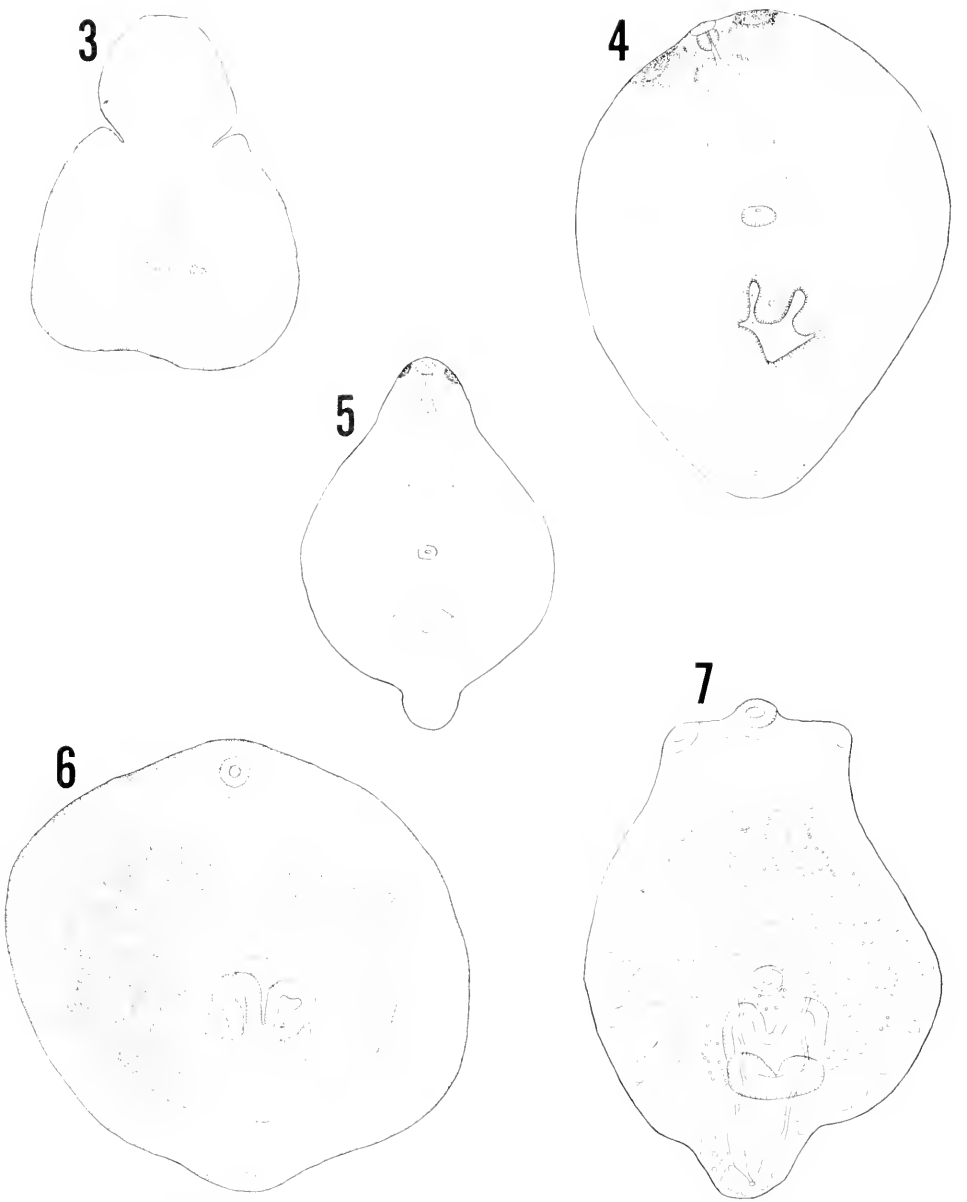

FIGURE 3. Metacercaria from brain of *F. heteroclitus;* taken 11 August, 1969. Fixed and stained, it is 0.325 mm long, 0.24 mm wide and shows cellular aggregates of metamorphosis.

FIGURE 4. Metacercaria taken 15 September, 1970; fixed and stained, is 1.25 mm long, shows outlines of future organs, bilobed tribocytic organ, and beginning of hindbody.

FIGURE 5. Metacercaria taken 15 September, 1970; fixed and stained, is 1.30 mm long with distinct hindbody.

snail was crushed on August 28 and the third on September 3. Both harbored heavy infections.

On September 13, six fishes, exposed repeatedly since mid-July, were fed to each of the experimental birds. On September 15, a lone remaining fish was dissected and seven metacercariae (Figs. 4, 5) were recovered from the surface of the brain. None of the metacercariae was encysted. The two white egrets and the black-crowned night heron were autopsied on September 19, the two cormorants on September 20, and the two gulls on September 21. No trematodes were found in any of the birds. The larvae taken from the fish on September 15 were active, but worms at that stage of development may not be sufficiently mature to survive in the digestive tracts of piscivorous birds.

The single infected snail found by Dr. Paul Krupa on July 21, 1971 liberated cercariae during the summer. Specimens of *Fundulus heteroclitus* were placed in bowls with swimming cercariae as in previous seasons and 28 fishes were exposed. One fish was dissected on August 18, and a single unencysted strigeid metacercaria was found on the brain. It was hoped to feed the metacercariae to day-old chicks and ducks at the end of the summer, but again baby birds were not available. Accordingly, the fishes were held in aquaria of the U.S. Bureau of Fisheries through the courtesy of Mr. Charles Wheeler and in the Marine Biological Laboratory through the courtesy of Mr. John Valois. Six fishes survived in the M.B.L. Day-old chicks were provided by the M.B.L. on June 29, 1972. The fishes were dissected on June 29 and June 30. One fish had two encysted larvae on the brain and another had a single encysted metacercaria between the brain and the cranial wall. The other fishes were negative. The cysts were oval with firm, rather thick walls. The fishes had been isolated over the winter with no opportunity for reinfection, so the parasites were unquestionably carried over from the previous summer. The finding of encysted larvae shows that the tetracotyles ultimately encyst. The encysted larvae were fed to a chick but no worms were found when the bird was autopsied six days later. The failure to obtain infection in the chick is not surprising since only occasionally, or rarely do strigeids persist in chicks.

Experiments conducted over a period of three years have shown that *C. nassa* is not the larval stage of the metacercariae on the brain of *Fundulus* spp., but have not disclosed the final host and adult stage of those metacercariae.

Observations

The metacercariae from the brain of *Fundulus* spp. are obviously members of a single species. They grow and develop on the surface of the brain but encysted specimens have been observed only after a long interval. The smallest were flattened, circular to oval, and about 0.2 mm in diameter. They were immobile, unattached, and floated free when the meniges were removed by dissections in sea water. In these specimens, the cercarial structures were completely obliterated and the larvae consisted of closely packed nuclei, with little or no cytoplasm and no

Figure 6. Metacercaria taken 9 September 1969; fixed and stained, is 0.70 by 0.63 mm. It was very active when alive; the figure shows the suckers, developing pseudosuckers, bilobed tribocytic organ, excretory pore and outline of the excretory tubules.

Figure 7. Drawing made from pencil sketches of living metacercaria to show the concretions in the excretory tubules and the spaces of the reserve bladder.

distinct cell boundaries. The first recognizable features of development were aggregations of nuclei at the locations of the future suckers, the tribocytic organ, and the reproductive organs. The development of the metacercariae involves a complete metamorphosis, comparable to that described by Szidat (1929) in the life-cycle of *Cotylurus cornutus* (Rudolphi, 1808), (syn. *Tetracotyle typica* Diesing, 1858). He compared the metamorphosis of strigeid trematodes with that of certain insects, "Entwicklung mit Umwaldung" and stated p. 668, "Es ist dies Verhalten der Larven der klare Ausdruck einer Metamorphose, und zwar, nach den bestehenden Begriffen, einer holometabolen Metamorphose der Cercarien, wie wir sie bisher bei Trematoden nicht gekannt haben." In a specimen taken August 16, 1969, stained and mounted, (Fig. 3), the oral sucker, acetabulum and reproductive complex are represented by cellular aggregates and the tribocytic organ is forecast by a shallow transverse depression. A more developed specimen taken September 9, 1969, somewhat flattened (Fig. 6) shows the suckers, the cellular condensations that form the lateral pseudosuckers, the bilobed tribocytic organ and the outline of the network formed by the excretory system. Fixed, stained and mounted it is 0.70 mm long and 0.63 mm wide. Figures 4 and 5 were made from two of seven larvae taken September 15, 1970; they measure 1.25 and 1.30 mm respectively in length, and show the dorso-posterior protrusion that becomes the hindbody of the adult. Figure 7 is from pencil sketches of a larva to show the reticular pattern of the excretory system as outlined by rows of concretions and by the spaces of the reserve system at the periphery of the body.

The description of *Cercaria nassa* by Martin (1945) is brief and not entirely correct. He described six penetration glands, but there are only two pairs. A more complete account is presented.

The haemal sinuses of the digestive gland of an infected snail may be filled by hundreds of tangled sporocysts. The snails harbored natural infections and accordingly, only daughter sporocysts were present. They vary in size from small, cylindrical to fusiform specimens, 0.25 mm in length and 0.06 mm in width with a few small germ-balls to large gravid individuals, 3 mm in length and 0.10 to 0.20 mm in width, with hundreds of germ-balls and developing cercariae, (Fig. 1). The increase in length is relatively greater than the increase in width. In general, the length is 10 to 15 times the width of a sporocyst. The ends may be conical, extended and tapering, blunt and flattened, or slightly concave. The ends, especially the anterior end, are more muscular than the rest of the body and have a concentration of nuclei in their walls. Often one or both ends may be rounded and knob-like, separated from the remainder of the body by a constricted, neck-like region. The body wall contains circular, longitudinal and diagonal muscle fibers and young sporocysts are very active. They may proceed with either end in advance. They may be nematoform, or contractions of circular muscles may produce constrictions with accompanying protuberances and bizarre shapes. With increase in size and number of progeny, mobility is reduced and the larger sporocysts are relatively inert. The body wall is covered by a very thin, transparent membrane which is often raised in fixed and stained specimens.

The germ balls are almost spherical until they attain a diameter of 0.03 to 0.04 mm when they become oval and continue to increase in the long axis. At a length of approximately 0.05 mm, the constriction that denotes the tail appears, and as

length increases the bifid character of the tail becomes more pronounced. Further development leads to the fully formed cercariae which leave the sporocyst through a birth pore, situated near the anterior end. The pore is not recognizable in fixed and stained specimens. The increase in number of sporocysts, and the presence of large numbers of small individuals in snails that have been isolated for several months and have continued to liberate thousands of cercariae, strongly suggests more than one generation of sporocysts. The recognition of a daughter sporocyst within a mother sporocyst is impossible, since there are no distinguishing features to discriminate between a daughter sporocyst and a cercaria at that stage of development.

The cercaria

In living specimens under slight coverglass pressure the body measures 0.06 to 0.26 mm in length and 0.03 to 0.05 mm in width. The tail-stem is about as long and as wide as the body; the furci somewhat shorter but very extensile. Each region is capable of independent extension and contraction. The acetabulum, situated in the posterior half of the body, is about 0.02 mm in diameter; the "anterior organ" is oval, 0.033 to 0.040 mm in length and 0.018 to 0.022 mm in width; the pharynx measures 0.008 to 0.009 mm in diameter and is separated from the anterior organ by about the same length. The body and tail bear papillae, each surmounted by a single seta. On the body they alternate with the rows of cuticular spines; on the tail-stem and furci the number and position apparently are not constant. In specimens killed in hot water, fixed in Duboscq-Brasil solution, stained and mounted (Fig. 2), average measurements are body-length, 0.165 mm; width, 0.044 mm, greatest in the posterior half of the body. The tail-stem has almost parallel sides, averages 0.18 mm long and 0.036 mm wide; the furci 0.135 mm long. The surface of the body but not of the tail-stem, bears cuticular spines, largely disposed in annular rows, eight in the preacetabular and four in the postacetabular portion of the body. Contractions of circular muscles at the level of the cuticular spines may give the body a crenate appearance. There are small spines along the edges of the furci. There are two rows of alternating spines around the opening of the acetabulum.

The structure called the "oral sucker" or "anterior organ" is a conspicuous feature of strigeid and schistosome cercariae. Hoffman and Hundley (1957) referred to it as the "penetration organ." It is not an oral sucker although after metamorphosis, its remains are reorganized to form the definitive oral sucker of the adult. The term "anterior organ" is a meaningless and unsuitable designation for this organ, which by its structure and function is adapted to penetrate the tissues of the next host and transmit the secretions of the penetration glands into the resulting wound. For it I propose the name "penetrantorium." The name "perforatorium" would be equally appropriate but since the term has been applied to the acrosome of the spermatozoan, its use would not be approved by semantic purists like the late Dr. Libbie Hyman who maintained that only homologous structures should bear the same appellation. An example of incongruity is the term, acetabulum, which denotes very different structures in digenetic trematodes and vertebrates.

In *C. nassa* the penetrantorium is oval, protrusible and retractile, working with a piston-like movement. It is retracted by fibers that originate more posteriorly from the body-wall and are inserted on the posterolateral aspects of the organ, and on retraction the anterior tip of the body becomes cupuliform. The penetrantorium is driven forward explosively by rapid, successive contractions of circular muscles from the acetabulum to the anterior end of the body. It contains four large secretory cells, two on each side, one dorsal the other ventral. The nuclei, 0.004 to 0.005 mm in diameter, are located in the posterior portions of the cells, the nuclei of the ventral cells posterior to those of the dorsal cells. The function of these cells is obscure; the secretion may contain enzymes of use in penetration, or it may serve to maintain rigidity of the organ and make the forward thrust more effective. When protruded, the anterior tip bears a battery of forward directed spines, typically arranged in five alternating rows, with each spine below and between the two above it. The arrangement varies with muscular contractions of the tip; the most common one presents a dorsal row with 2 spines, the next 3 spines, the next 4 spines, the next 5 spines, and the lowest row above the mouth has 4 spines. In other specimens the number may vary to a condition where the rows have 4, 5, 6, 5, and 4 spines in successive rows. Surrounding the anterior terminal spines, there is a small glabrous circular area and the body wall is then encircled by a band of closely set, alternating spines, typically arranged in eight rows. There is a zone of scattered cuticular spines between this zone and the previously described first annular ring of cuticular spines. The prepharygeal portion of the digestive tract passes forward in the center of the penetrantorium and the ducts of the penetration glands enter on the posterolateral borders, two on each side, extend through the organ, and open on the anterior face, on either side of the mouth.

There are four penetration glands, situated in the intercecal area between the acetabulum and the excretory bladder. Their ducts, two on each side, pass anteriad along the dorsolateral faces of the acetabulum, dorsal to the anterior ends of the digestive ceca and enter the posterolateral faces of the penetrantorium. In their initial portion, they contain particulate matter but in the terminal portion the contents are fluid and stain with vital dyes. If the penetrantorium and anterior end of the body are retracted, the ducts frequently buckle and become dilated before their entrance into the penetrantorium.

The digestive system is well developed. The mouth is situated on the anterior aspect of the penetrantorium and the canal passes through this structure to emerge as a prepharygeal section, about as long as the pharynx before opening into that organ. The esophagus is long, bifurcating anterior to the acetabulum. There are short lateral extensions, lined with cuticula, which open into the digestive ceca. The ceca are lined with a layer of epithelial cells and terminate at the level of the excretory vesicle.

The flame-cell formula of the excretory system is $2[(2 + 2) + (2 + 2) + (2)]$. There is no transverse commissure between the collecting tubules of the two sides. The lateral collecting tubules contain long cilia; they fuse to form the excretory vesicle, a trefoil shaped sac with a larger, median posterior lobe. They separate in the base of the tail and surround the "island of Cort" before they unite again to form the central canal in the tail. At the distal end of the tail-stem, they separate again and enter the furci, opening on the anterior faces about one-fourth of the

distance to the furcal tips. In the body the most anterior flame-cell is ventral in position, the next one is dorsal and they alternate posteriorly. Of the flame cells in the tail, the anterior pair is dorsal and lateral, the posterior pair is ventral and median.

The wall of the tail consists of external circular, median longitudinal and inner diagonal fibers and the core is filled with the "caudal bodies" or "glycogen cells," usually arranged in eight pairs, which are attached to the central excretory canal. These cells become filled as the cercaria matures in the haemal sinuses of the snail; they are depleted and shrink as the cercaria swims by lashing of the tail. According to Ginetsinskaya and Dobrovol'skii (1962; English translation 1968, page 7), "The number and form of the caudal bodies vary in cercariae of the same species and depend upon age. As the glycogen in them is expended, the large caudal bodies take on an irregular, stellate form and then become inconspicuous."

The future gonads and ducts are represented by a cluster of deeply staining cells situated at the level of the excretory bladder.

Discussion

Cercaria nassa closely resembles *Cercaria scudderi* described by Olivier (1941) from the pulmonate snail, *Lymnaea palustris elodes* Say, taken in Cheboygan County, Michigan. The two are almost identical in size, have the same number and distribution of penetration glands and flame-cells, but differ in details of spination. Dubois (1966) predicated that *C. scudderi* is identical with the larva of a species described by Hoffman and Hundley (1957) as *Diplostomum baeri eucaliae*. These authors found metacercariae in the brain of the stickleback, *Eucalia inconstans*, taken from a stream at Grand Forks, North Dakota. Fed to chicks, they obtained adults in four to six days. Eggs were embryonated, miracidia emerged and penetrated laboratory-reared snails, identified as *Stagnicola palustris elodes*, and produced sporocysts and cercariae. Infection of sticklebacks completed the life-cycle. The adults were identified as *Diplostomum baeri* Dubois, 1937, but were designated as a new subspecies, *D. baeri eucaliae*. Dubois (1966) recognized it as a distinct species, *Diplostomum scudderi* (Olivier, 1941). The general morphology of *C. nassa*, and especially the absence of commissures in the excretory system, identify it as a species of *Diplostomum* and it is designated as *Diplostomum nassa* (Martin, 1945). Larvae of the genus *Diplostomum* were included in a larval group, Diplostomulum, by Brandes (1892). These larvae occur in the musculature, eyes, brain, and spinal cord of fishes and tadpoles of frogs and toads. Indeed, the genus *Diplostomum* was erected by von Nordmann (1832) to contain *D. volvens*, a metacercaria from the eyes of freshwater fishes that proved to be the larval stage of *Diplostomum spathaceum* (Rudolphi, 1819). Since diplostome metacercariae occur frequently in the brain and eyes of fishes, a possible connection between *C. nassa* and the metacercariae on the brain of *Fundulus* spp. was a logical presumption. Experiments, however, have dispelled the idea.

Study of metacercariae from the brain of *Fundulus* spp. shows that the tribocytic organ is bilobed, and that the openings of the pseudo-suckers (cotylae) are directed medially, not anterior and marginal as in the diplostomes. The development of these larvae establishes their identity as tetracotyles, the characteristic larvae of the Strigeidae. Tetracotyles of species in the genera *Apatemon* and

Cotylurus encyst in mollusks and leeches with adults in aquatic birds. Tetracotyles of these and other species also encyst in vertebrates. They occur in the body cavities, peritoneum, muscles, liver and eyes, but so far have not been reported from the brain.

The larval groups appear to be characteristic of families but specificity of hosts is not closely restricted and mature worms ordinarily do not persist in their host for a long time. Considering this subject, Baer and Joyeux (1961) reported, page 639 "On observe, chez les Strigeida, une corrélation très étroite entre le biotope de l'hôte définitif et celui du deuxième hôte intermédiare, qui assure un degré élevé de spécificité écologique. Cette constation est d'autant plus frappante qu'il est possible d'infester expérimentalement des hôtes très différents en leur faisant ingérer des métacercaires. Par example, des Chiens et des Chats peuvent être infestés par des Trématodes vivant normalement chez des Oiseaux rapaces et des Pigeons, Caille, Poulet, par des Vers d'Oiseaux aquatiques."

Grateful acknowledgments are made to the previously mentioned individuals for the kind and generous provision of specimens, including snails, fishes, and birds used in the investigation, and to Charles Wheeler and John Valois for maintenance of exposed fishes over winter.

Summary

Metacercarial stages of a strigeid trematode were reported from the brain and eyes of *Menidia menidia* and *Mugil cephalus* at Beaufort, North Carolina by Hunter and Vernberg (1960) and from the brain of *Fundulus heteroclitus* taken in Chesapeake Bay by Abbott (1968). Strigeid metacercariae occur also on the brain of species of *Fundulus* in the Woods Hole, Massachusetts region. The only strigeid cercaria described from the mid-Atlantic coast is *Cercaria nassa* Martin, 1945. Attempts to infect *Fundulus* spp. with *C. nassa* were futile and attempts to infect avian species, egrets, herons, cormorants, and gulls with metacercariae from *Fundulus heteroclitus* gave only negative results. The cercaria and metacercaria are described. *Cercaria nassa* belongs to the larval group, Diplostomulum, and is named *Diplostomum nassa* (Martin, 1945), family Diplostomidae. The metacercaria from the brain of *F. heteroclitus* belongs to the larval group, Tetracotyle, family Strigeidae. Accordingly, the two larvae are members of entirely different groups.

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ULTRASTRUCTURAL STUDIES ON THE FORM AND FUNCTION OF THE GASTRODERMIS OF PROTOPOLYSTOMA XENOPI (MONOGENOIDEA: POLYOPISTHOCOTYLEA)

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Studies of intestinal histology and digestive physiology have been carried out on a number of monogeneans, and the available information has been reviewed by Jennings (1968). There is a fundamental difference in the nutrition of the two sub-groups of the Monogenoidea. Representatives of the Monopisthocotylea feed on the epidermis and associated mucoid secretions of the host while the Polyopisthocotylea feed almost exclusively on the host's blood. Differences in the diet and digestive processes of these groups are reflected in the cellular organization of the gastrodermis. Much of digestive breakdown in the Monopisthocotylea is believed to be extracellular and the soluble products are absorbed by a continuous cuboidal and columnar epithelium. In the Polyopisthocotylea, on the other hand, hemoglobin degradation largely occurs intracellularly and results in the accumulation of hematin within the gastrodermal cells. This insoluble product is thought to be eliminated by the disintegration or shedding of the cell. It has been considered that the digestive process involves the constant degeneration and renewal of the so-called deciduous gastrodermis, and this gives rise to the apparently discontinuous nature of the cecal epithelium. Jennings (1968) has concluded that the consequent wastage of cellular materials during the polyopisthocotylean digestive process represents an incomplete adaptation to the blood-feeding habit.

The ultrastructure of the cecal epithelium has been investigated in only one species of polyopisthocotylean. Halton, Dermott and Morris (1968) reported that in *Diclidophora merlangi* the cecal epithelium is in fact a continuous structure composed of two distinct cell types, with pigmented "hematin cells" alternating with flat, extensive "connecting cells." The latter cover all areas of the cecal wall between the hematin cells and are so thin in section that they cannot be resolved by light microscopy.

The present paper describes the ultrastructural organization of the cecal epithelium of *Protopolystoma xenopi* (Price, 1943) Bychowsky, 1957, a blood-feeding parasite of the African clawed toad, *Xenopus laevis* (Daudin). Observations are made on the growth of the gastrodermis during the parasite's development and on the structural changes accompanying digestion; an interpretation of the functional morphology of the epithelial components is presented.

Materials and Methods

Ultrastructural observations were made on *Protopolystoma xenopi* over a range of developmental stages. Adult worms were obtained from the urinary bladder of naturally-infected *Xenopus laevis* imported from South Africa. Hosts were also

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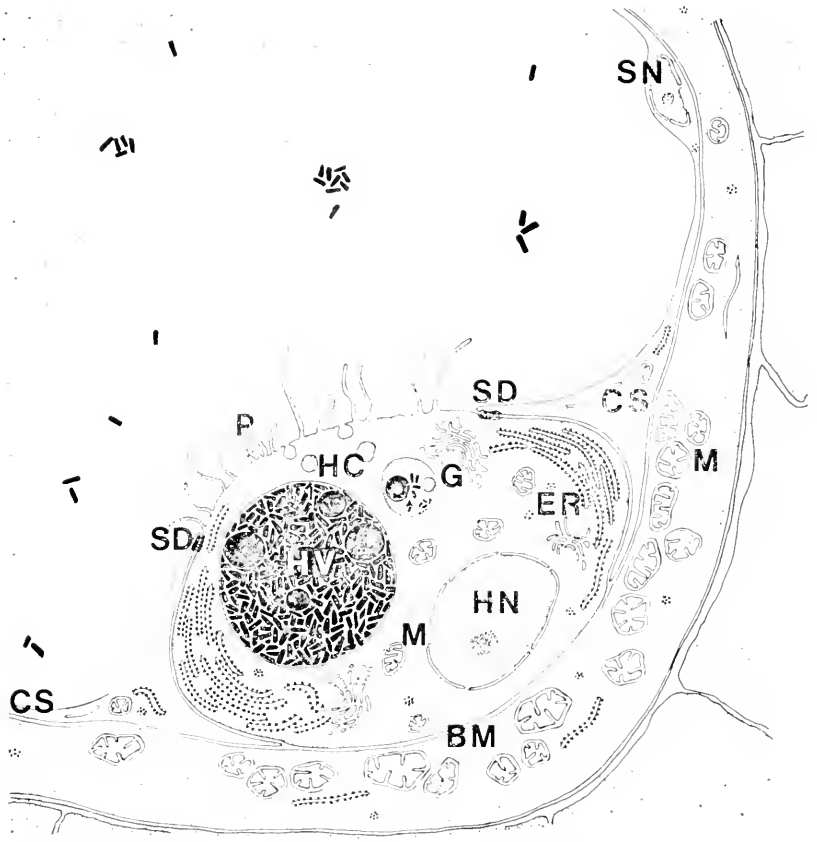


FIGURE 1. *Protopolystoma xenopi*; diagrammatic representation of the structure of the cecal epithelium. The hematin cell (HC) bears cytoplasmic processes (P) on its exposed distal border, the nucleus (HN) is basal and rough endoplasmic reticulum (ER) and associated golgi bodies (G) are situated laterally. Hemoglobin breakdown takes place within large membrane-bound vacuoles (HV) and leads to the appearance of hematin and lipid-like droplets. Mitochondria (M) are scattered in the hematin cell and densely aggregated beneath the basement membrane (BM). The sheet-like connecting syncytium (CS) surrounds the hematin cell laterally and a septate desmosome (SD) occurs at the point of maximum overlap. The nuclei (SN) and organelles of the syncytium are relatively scattered.

infected experimentally in the laboratory (Tinsley and Owen, in preparation); larval parasites were recovered from the kidneys two weeks after infection, juvenile parasites from the bladder after eight to ten weeks, and adults from the bladder after sixteen weeks.

Adult parasites were fixed at progressive intervals after feeding, specimens with bright red gut contents being regarded as very recently fed. These were fixed either immediately or after being maintained for 12, 24 and 48 hours in 33% normal strength *Xenopus* ringer.

All specimens were fixed for two hours in ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. The material was rinsed for 15 minutes in 0.1 M

cacodylate buffer in 1 M sucrose and post-fixed for one hour in 1% osmium tetroxide held at pH 7.3 with 3.0×10^{-2} M veronal buffer containing invertebrate salt solution. A 15 minute rinse in veronal buffer was followed by dehydration in ice-cold graded ethanols, impregnation with propylene oxide at room temperature and embedding in Shell Epikote resin (epon 812). Silver to grey sections were cut with glass knives on a Cambridge (Huxley) ultramicrotome and mounted on formvar-coated or uncoated grids. These sections were double-stained with uranyl acetate and lead citrate and examined with an AEI EM6B electron microscope. Sections cut at 0.5 μ were mounted on glass slides, stained in Azur II in borax (Jeon, 1965) and examined with the light microscope.

The presence in some gastrodermal cells of large quantities of hematin often made the cutting of satisfactory thin sections difficult. The pieric alcohol test described by Halton *et al.* (1968) was employed to identify the hematin.

Observations

The structure of the gastrodermis

The intestinal system of *Protopolystoma xenopi* is composed of two lateral ceca arising immediately behind the pharynx; these extend to the posterior of the worm and bear parallel diverticula on both medial and lateral surfaces. The majority of diverticula are blind-ending pouches but up to six of the medial branches extend across the mid-line and join the right and left ceca.

Light microscope examination revealed that the intestinal system is bordered by discontinuously arranged cells which have an irregular shape, possess basal nuclei and contain varying amounts of hematin enclosed in spheroidal vacuoles. The intervening cecal walls are apparently devoid of cells (Fig. 2).

With the electron microscope the cecal epithelium was found to consist of two components, the larger "hematin cells" referred to above, and a thin cytoplasmic layer composed of "connecting cells" (Halton *et al.*, 1968) covering the basement membrane between adjacent hematin cells. The structure and arrangement of the two components was most clearly observed in juvenile parasites and a relatively constant pattern was recorded (Fig. 1). The hematin cell protrudes from the cecal wall into the gut lumen and a portion of its lateral border is overlapped by an extension of the connecting cell system. The exposed apical plasma membrane bears numerous cytoplasmic processes usually up to 2 μ but occasionally 4 μ in length, and circular in cross section (diameter about 0.05 μ) (Figs. 4 and 5). The processes have an uneven distribution; in some areas they are regularly arranged, about 0.06 μ apart, whilst in others they are relatively scattered at intervals of 0.3–1.0 μ. The hematin cell contains a large basal nucleus with a prominent nucleolus, and lateral to this, in a region overlapped by connecting cell, is an endoplasmic reticulum and associated golgi complex (Figs. 5, 7 and 8). The reticulum forms an arc of up to eleven concentric cisternae which are interconnected laterally and studded with numerous ribosomes. Two golgi bodies are often present, one situated at each end of the endoplasmic reticulum. Small vesicles are associated with each complex and the contents of these and of the golgi cisternae are moderately electron dense. Small mitochondria are scattered throughout the cell whilst larger more numerous mitochondria are aggregated beneath the basement membrane

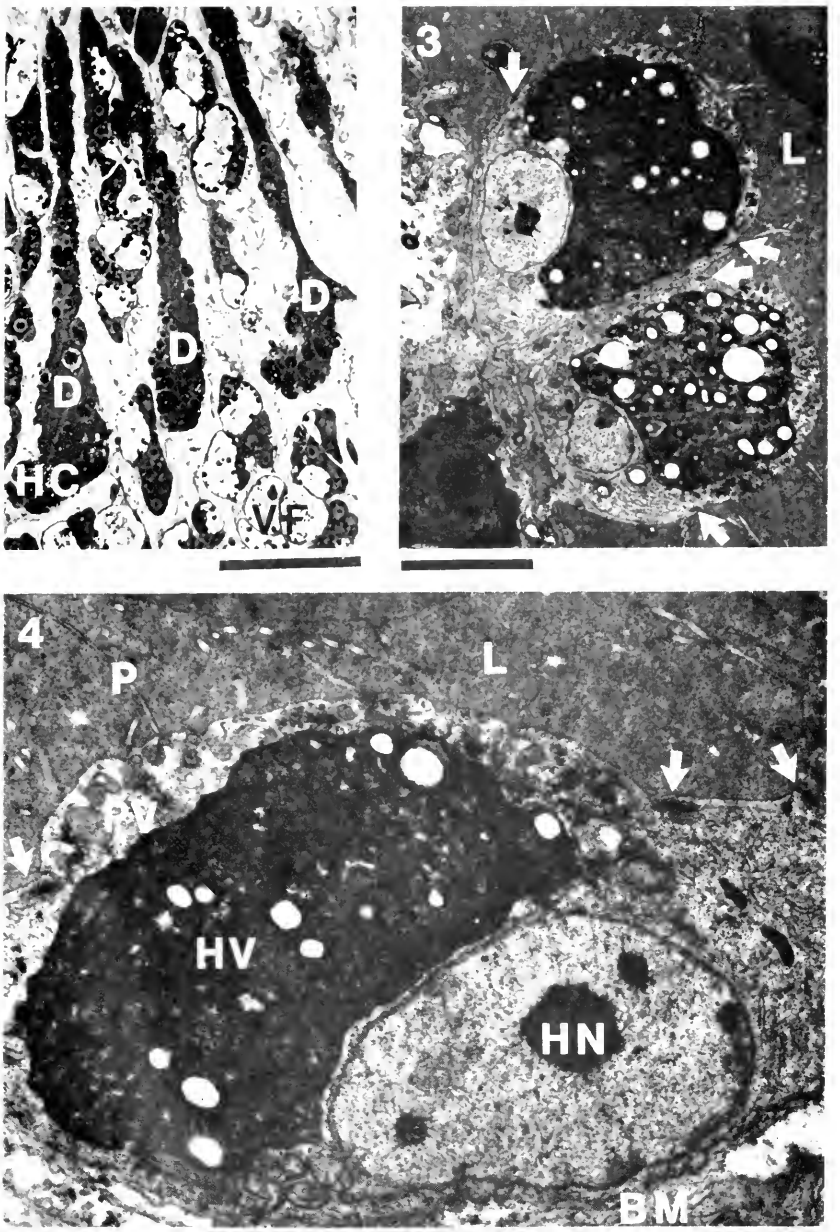


FIGURE 2. *P. xenopi*; light micrograph showing transverse section through cecal diverticula (D). Discontinuously-arranged hematin cells (HC) are interspersed with areas apparently devoid of cells; vitelline follicles (VF) are distributed in the inter-cecal parenchyma; scale bar, 50 μ.

FIGURE 3. *P. xenopi*; part of the gastrodermis showing two hematin cells protruding into the gut lumen (L). The very thin connecting syncytium covers the adjacent cecal wall and

of the gastrodermis (Fig. 8). One or more large membrane-bound vacuoles occur in a distal position in the cell, these usually measure about 12 μ in diameter but may reach 20 μ. They contain varying amounts of dense granular pigment, identified by the picric alcohol solubility test (Llewellyn, 1954; Halton *et al.*, 1968) as hematin, together with homogeneous lipid-like droplets. The hematin is often composed of splinter-like fragments which may also be observed scattered in the intestinal lumen (Fig. 13). Pinocytotic vesicles containing material almost indistinguishable from that in the gut lumen often occur in the distal cytoplasm between the free plasma membrane and the hematin vacuole. Fine tubules, approximately 0.06 μ in diameter, may also ramify through this region.

The connecting cell system is very thin, usually measuring up to 0.3 μ in section. It invariably intervenes between adjacent hematin cells and even where the latter are in close proximity they are separated by a thin cytoplasmic layer (Fig. 7). Where the hematin cells are well separated the connecting system forms a flat sheet overlying the basement membrane. Extensions of this system overlap a variable portion of the lateral border of each hematin cell and a septate desmosome is present at the point of maximum overlap (Figs. 5 and 6). Extensions also pass a short distance beneath the margin of the cell. The free plasma membrane of the connecting system does not form lamellae or other cytoplasmic processes and shows no evidence of pinocytotic activity. The cytoplasm is not as dense as that of the hematin cell and contains relatively scattered organelles. The connecting system intervening between hematin cells may contain small mitochondria, rough endoplasmic reticulum and golgi apparatus, but the extensions overlapping the hematin cells are generally very thin and devoid of inclusions. Pigment-containing vacuoles similar to those characteristic of the hematin cells do not occur in the connecting system. The nuclei are accommodated within swellings of the sheet-like cytoplasm, up to 3 μ in thickness, but they have been observed infrequently in the large number of sections examined. No cell junctions have been recorded, and the cytoplasm of the connecting system is apparently continuous.

Structural changes accompanying digestion

Few intact erythrocytes have been observed in worms fixed during feeding and it is probable that hemolysis occurs immediately after ingestion. The erythrocyte nuclei, however, persist unchanged for several hours. In a recently-fed *P. xenopi* many hematin cells contain vacuoles of homogeneous, moderately electron-dense material almost indistinguishable from that in the gut lumen (Fig. 11). This appears to enter the cells principally by pinocytosis. Numerous pinocytotic vesicles occur at the surface of the hematin cell and these may be observed to pass through the distal cytoplasm and fuse with the large central vacuoles (Figs. 10 and 11). In

surrounds the lower half of each hematin cell; septate desmosomes (arrowed) mark the extent of the overlap. The hematin cell nuclei are basal and the distal region of each cell is occupied by a large vacuole whose contents are in the course of digestive breakdown; scale bar, 5 μ.

FIGURE 4. *P. xenopi*; the organization of the hematin cell. The nucleus (HN) lies close to the basement membrane (BM); cytoplasmic processes (P) project from the free distal border and numerous pinocytotic vesicles (PV) occur containing material indistinguishable from that in the gut lumen (L). The hematin vacuole (HV) contains fragments of hematin and lipid-like droplets. The lateral regions of the hematin cell are overlapped by the connecting syncytium, with septate desmosomes arrowed; scale bar, 1 μ.

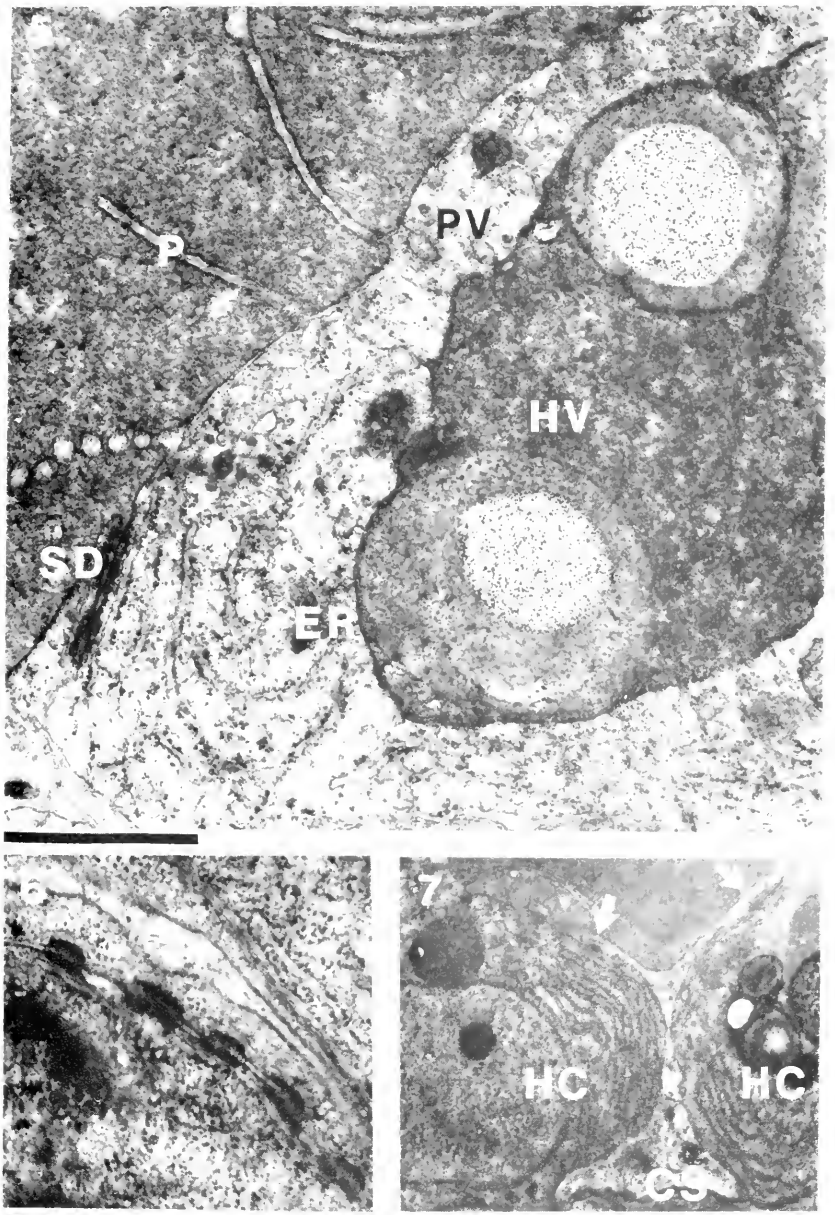


FIGURE 5. *P. xenopi*, hematin cell with vacuole (HV) in the initial phase of digestion, contents slightly denser than those of gut lumen with appearance of ferretin-like granules. Note rough endoplasmic reticulum with interconnected cisternae (ER), cecal cytoplasmic processes (P), probable pinocytotic vesicles (PV), portion of connecting syncytium with septate desmosome (SD); scale bar, 1 μ.

FIGURE 6. *P. xenopi*; detail of septate desmosomes between hematin and connecting cells; scale bar, 0.5 μ.

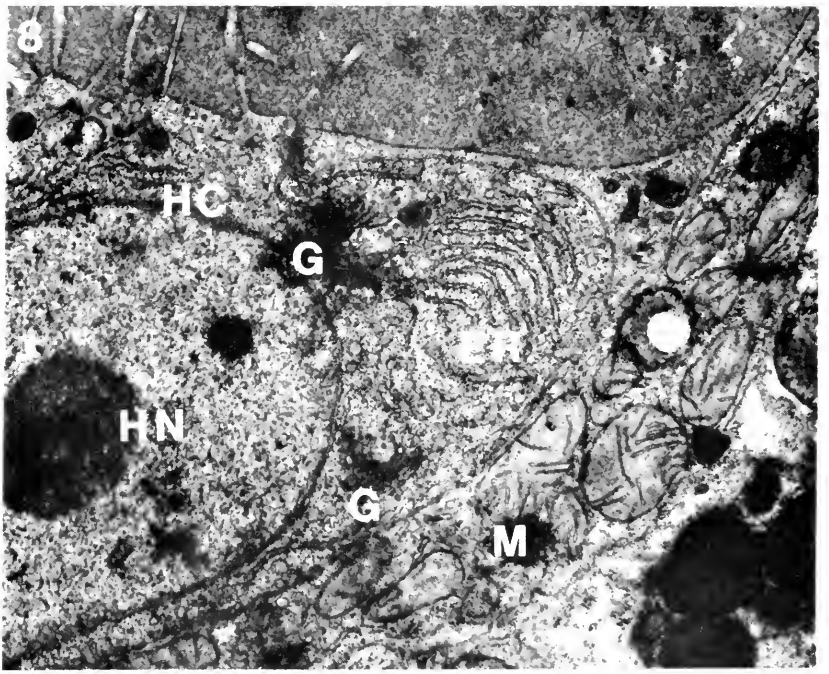
cells apparently taking part in the process of uptake for the first time a large volume of material may be absorbed very rapidly. A single vacuole forms within the cell which may become so swollen that only an extremely thin layer of cytoplasm (sometimes as little as 0.2 μ deep) encloses the vacuole distally. This condition may be achieved before any changes occur distinguishing the contents from those in the gut lumen. An additional means of hemoglobin uptake may be provided by the numerous fine tubules which are often present in the apical cytoplasm of the hematin cell. These apparently communicate between the free plasma membrane and the vacuole and may be observed to contain material similar to that in the gut lumen (Fig. 9).

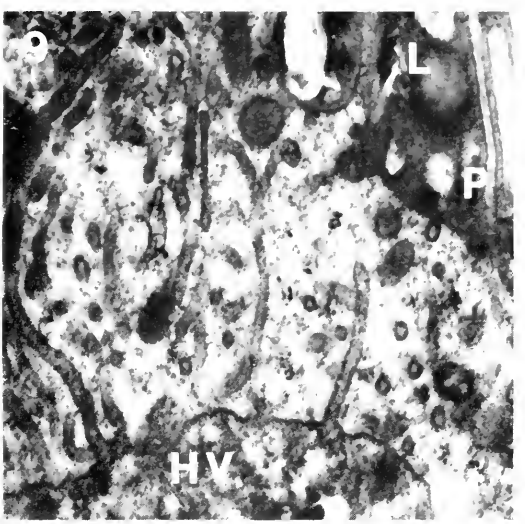
During the course of digestion the contents of the vacuole become more electron-dense, presumably as water is withdrawn, and lipid-like droplets and fragments of hematin form initially around the periphery of the vacuole (Figs. 3 and 5). Twenty-four hours after feeding the contents of the majority of hematin cells are in an advanced stage of digestion. One or more vacuoles occupy the distal region of each cell; the vacuoles are packed with highly electron-dense fragments of hematin together with lipid-like droplets and stacks of myeloid fibrils. The high lipid content of the vacuole may be derived from the breakdown of the phospholipid envelopes of the erythrocytes. Hemoglobin uptake can apparently continue for a considerable period as cells heavily laden with hematin may show signs of active pinocytotic absorption.

The enzymes of intracellular digestion are probably derived from the cisternae of the endoplasmic reticulum. Small lysosome-like vesicles are produced by the extensive network of cisternae and these have been observed to fuse with the membrane of the adjacent hematin vacuole.

Hematin accumulates within the cells over a relatively long period and in living adult worms the major part of the intestinal system is an intense black-brown. Hematin discharge occurs continuously, but even in worms starved for four days a considerable amount of intracellular pigment remains. The membrane-bound hematin vacuoles are discharged intact and have been observed lying free in the gut lumen. In living worms the pigment spheres circulate with the gut contents, they eventually disintegrate and fine fragments of hematin become scattered throughout the gut. The mode of discharge of the hematin vacuole has proved difficult to interpret; the attachment of protruding hematin cells to the cecal wall is frequently very tenuous both in young cells in the initial stages of absorption (Fig. 3) and in older hematin-laden cells (Fig. 12). Hematin vacuoles surrounded by a thin layer of cytoplasm have been observed apparently in the process of detachment from the gastrodermis (Fig. 13); however, in many cases it is probable that the cell was sectioned apically and the vacuole was, in life, attached to the cecal wall at a higher or lower plane. At the ultrastructural level no areas of discontinuity or disruption in the cecal epithelium caused by the possible shedding or disintegration of the hematin cells have been observed, and no detached cecal cells have been found in the gut lumen.

FIGURE 7. *P. xenopi*; view of adjacent hematin cells (HC) showing position of intervening connecting syncytium (CS). Hematin cells with prominent collateral cisternae of endoplasmic reticulum, part of digestive vacuole visible in the cell on the right and the nucleus in the cell on the left. The cytoplasm of the connecting syncytium is less dense, septate desmosomes arrowed; scale bar, 1 μ.





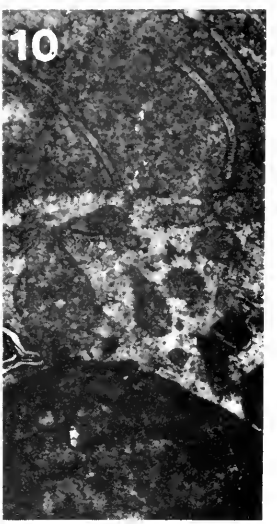


FIGURE 8. *P. xenopi*; lateral region of a hematin cell (HC) overlapped by the thin sheet-like connecting syncytium without cecal processes. Lateral to the hematin cell nucleus (HN) is a well-developed endoplasmic reticulum (ER) with two golgi bodies (G). Large mitochondria (M) are aggregated beneath the basement membrane; scale bar, 1 μ.

FIGURE 9. *P. xenopi*; distal cytoplasm of hematin cell showing intracellular tubules between hematin vacuole (HV) and free distal membrane, the latter bears cytoplasmic processes (P) extending into the gut lumen (L); scale bar, 0.5 μ.

In most pigment-laden cells the hematin vacuole is situated in the distal region and the nucleus and other organelles are confined basally. Discharge of the vacuole may be achieved simply by its extrusion through the free plasma membrane without affecting the rest of the cell. On the other hand, portions of cells distended with hematin which protrude into the gut lumen as in Fig. 12 may become detached more or less accidently due to body movements. Such detachment during the violent contractions accompanying fixation may account for previous observations (Jennings, 1959; Halton and Jennings, 1965) that some hematin-laden cells may break down or be shed intact from the cecal wall. Certain other products of digestion, including isolated lipid-like droplets and membrane-bound myelin figures may be extruded separately through the free plasma membrane of the hematin cell.

Development

The arrangement of alternate hematin and connecting cells observed in adult parasites was found to be established in the youngest larvae of *P. xenopi* examined, those fixed two weeks after experimental infection of the host. The growth and development of the parasite is accompanied by certain changes in the morphology of the gastrodermal components. In juvenile parasites, fixed two months after infection, the hematin cells are scattered over the cecal wall, they usually contain a single hematin vacuole and conform to the relatively constant pattern described above (Fig. 2). In an adult *P. xenopi*, fixed over four months after infection, the hematin cells are numerous and closely packed, each occupies a relatively larger area of the cecal wall and contains several spheroidal hematin vacuoles. There is also variation in the appearance of the connecting system during the course of development. In juvenile parasites, the syncytium is thin and sheet-like, with extensions which partially overlap the well-separated hematin cells (Fig. 14A). The latter may sometimes be more or less circular in section, attached to the basal tissues by a relatively narrow neck. In these circumstances the connecting system encloses the lower half of the cell, supporting it in a cup-like sheath (Figs. 3 and 14B). In older parasites where the more numerous hematin cells are usually closely-packed and columnar the connecting system forms narrow strips sandwiched between adjacent cells. In section the syncytium is relatively deep, extending from the basement membrane to the cecal border of the hematin cells (Fig. 14C).

In view of the gradual increase in the number of hematin cells during the course of the parasite's development, sections have been examined for the occurrence of hematin cell primordia in or below the cecal epithelium. Discrete cells have been observed beneath the syncytium; in section these are largely occupied by the nucleus, surrounded by a thin layer of dense cytoplasm containing ribosomes and mitochondria. These may represent initials which give rise to emergent hematin cells but the process of development has not been recorded.

Discussion

There is a close similarity in the cellular arrangement of the gastrodermis of *Protopolystoma xenopi*, revealed by the present study, and that of *Diclidophora*

Figure 10. *P. xenopi*; distal cytoplasm of hematin cell showing pinocytotic entry of material from the gut lumen to the hematin vacuole; scale bar, 0.5 μ.

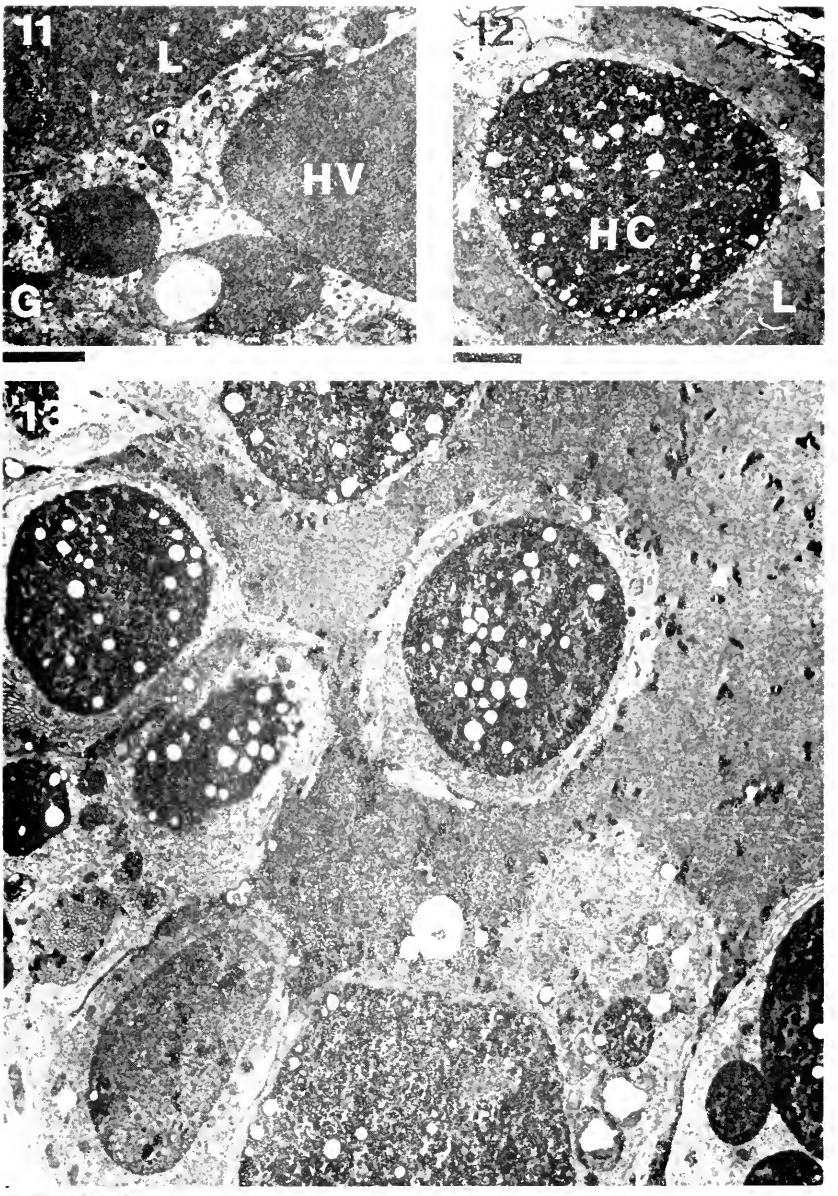


FIGURE 11. *P. xenopi*; hematin cell showing passage of food material from the gut lumen (L) to the digestive vacuole (HV). Break-down is already initiated in one of the incoming vesicles; note the adjacent golgi complex (G); scale bar, 1 μ.

FIGURE 12. *P. xenopi*; part of hematin-laden cell (HC) protruding into gut lumen (L) and attached to the cecal wall by a narrow neck. The connecting syncytium extends around the lower half of the cell (maximum overlap arrowed); scale bar, 4 μ.

FIGURE 13. *P. xenopi*; part of cecal epithelium showing apparent detachment of hematin-laden vacuole, scattered fragments of hematin occur in the gut lumen. The disposition of the

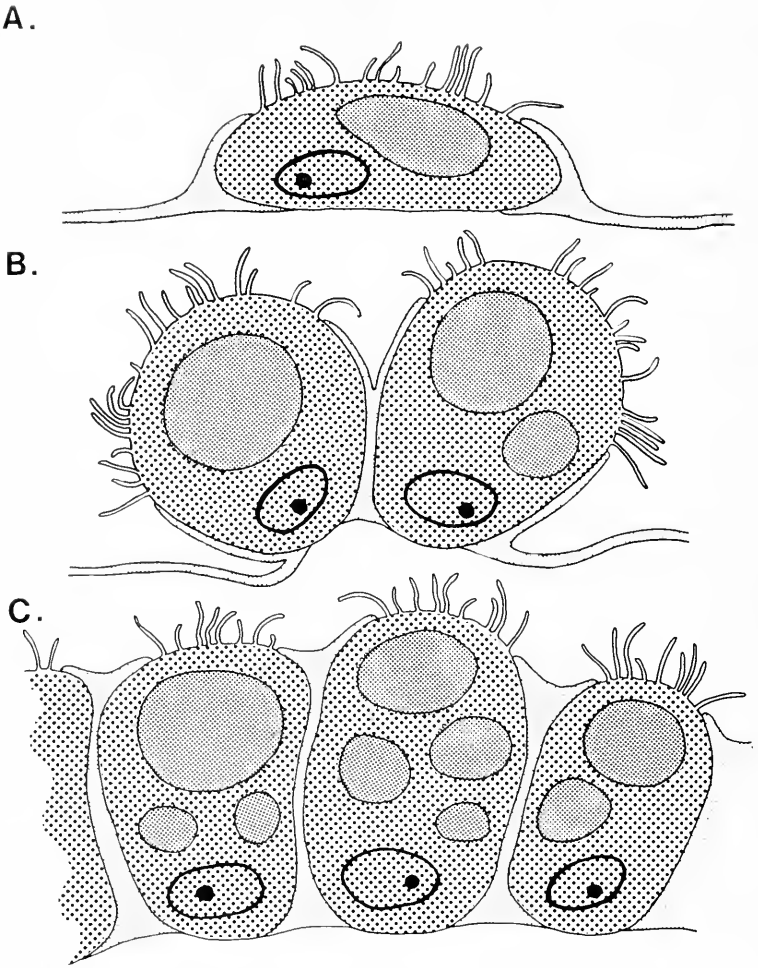


FIGURE 14. *P. xenopi*; variation in the arrangement of the components of the cecal epithelium. Hematin cells with hematin vacuoles and basal nuclei, densely stippled; connecting syncytium, lightly stippled. For explanation see text.

merlangi reported by Halton *et al.* (1968). In both monogeneans the cecal epithelium is composed of two distinct cell types; hematin cells alternate with a previously unrecognized connecting cell system which covers all areas of the cecal wall between adajacent hematin cells. No inter-cellular junctions have been observed within the flat, sheet-like connecting system, and the view of Halton *et al.* (1968) that this exists as a syncytium is supported.

There are certain differences in the ultrastructure of the gastrodermal components in the two species. Halton *et al.* (1968) recorded that in *D. merlangi* protoplasmic projections occur on the free surface of both types of cecal cell. In

epithelium suggests, however, that this plane of sectioning does not represent the actual process of detachment; scale bar, 4 μ.

P. xenopi, on the other hand, these are borne only by the hematin cells and the free plasma membrane of the connecting cell is smooth. The cytoplasmic processes of *D. merlangi* are in the form of lamella which differ in size on the hematin and connecting cells. However, the hematin cell processes of *P. xenopi* are circular in section and considerably longer than the equivalent structures of *D. merlangi* (2.0–4.0 μ and 0.6 μ respectively). The appearance of the cells loaded with hematin differs in *D. merlangi* and *P. xenopi*. In the former, numerous small pigment vacuoles are dispersed in the cytoplasm; in the latter, a few relatively large spheres occur in the mid-distal region of the cell.

A gradual increase in the number of hematin cells occurs during the growth of *P. xenopi* and this may, perhaps, be correlated with the increasing needs of the parasite. The alternate arrangement of the two epithelial components could be derived from the development of hematin cells from initials arising beneath the connecting system. The eruption of these through the syncytium to expose the apical region would result in the observed cellular relationship. However whilst possible primordia have been recorded, the process of eruption has not been observed.

Much of the course of digestion in *Protopolystoma xenopi* revealed by electron microscopy accords with that determined by Jennings (1959) from histochemical and light microscope studies of the closely related *Polystoma integerrimum*. The hematin cell is involved in the visible processes of digestion. The products of extracellular hemolysis of ingested erythrocytes are taken into the cell by pinocytosis and digestion proceeds within large membrane-bound vacuoles. There are apparently no previous records of intracellular microtubules communicating between the distal border and the digestive vacuole. These commonly occur in the actively absorbing cells of *P. xenopi* and it seems probable that they assist in the entry of hemoglobin. Their occurrence may be linked with the observation that the initial absorption of the blood meal is very rapid. The distribution of the connecting syncytium which partially overlaps the distal margins of the hematin cells involves a reduction in the absorptive surface area: it is possible that the system of microtubules compensates for this limitation. The cecal cytoplasmic processes may be concerned with the absorption of low molecular weight compounds. However, they may perhaps facilitate extracellular "contact digestion" as postulated for the similar structures of other animal groups by Ugolev (1960) and Jennings (1968, 1969). This purely catalytic function might explain their occurrence on only one cell type, the digestive cell, in *P. xenopi* but on both cell types in *D. merlangi*. The much greater length of the processes in the former species might compensate for their restricted distribution.

The degradation of hemoglobin leads to the accumulation of an insoluble pigment, probably largely hematin, within the vacuoles and these are shed intact from the cell, apparently by simple extrusion. As suggested by Jennings (1959, 1968), the eventual breakdown of the spent hematin vacuoles could result in the release of intracellular digestive enzymes and these may initiate partial extracellular breakdown of the next blood meal.

Ultrastructural studies indicate the need to revise earlier interpretations of both the form and function of the polyopisthocotylean gastrodermis. The formation of insoluble residues of hemoglobin breakdown within the digestive cells creates problems unique to this parasite group. Previous light microscope studies suggested

that elimination is achieved by the partial breakdown or shedding of the hematin-laden cell. This interpretation, involving the cyclical loss and replacement of the digestive cells, gained support from the apparently discontinuous nature of the gastrodermis. However, electron microscope observations show that a second cell system is interposed between all adjacent hematin cells, and the gastrodermis remains an organized and continuous structure throughout life. Hematin elimination is achieved by the extrusion of intact vacuoles; there is no evidence from ultrastructural studies for the breakdown of the entire cell, and previous light microscope observations may have been influenced by the effects of fixation. The view that the digestive process involves considerable wastage of cellular materials and represents an incomplete adaptation to the blood-feeding habit is considered to be no longer tenable.

The function of the connecting system is obscure. The syncytium apparently plays no obvious role in the digestive processes. There is no evidence of pinocytotic uptake nor of secretory activity; the relatively scattered organelles and the infrequent occurrence of nuclei suggest that an active physiological role is unlikely. Other characteristics indicate the possibility of a physical role. The cecal epithelium is regularly subjected to vigorous deformation by the contractions of the body muscles. The hematin cells protrude from this epithelium and contain relatively dense structures, the hematin vacuoles, in their distal regions. In surface view, the connecting syncytium forms a flat sheet of cytoplasm overlying the whole of the cecal wall and perforated by a system of pores—the tips of the hematin cells. This organization suggests that the syncytium may perform a skeletal function, providing both support and protection for the hematin-laden digestive cells and for the relatively delicate underlying tissues.

The ultrastructural differences between *D. merlangi* and *P. xenopi* concerning the form and occurrence of the cecal cytoplasmic processes may reflect the systematic separation of the two monogeneans. The organization of the two cell type gastrodermis, on the other hand, may be a common feature of all Polyopisthocotylea. Jennings (1968) has noted the constant association of the blood-feeding habit, the intracellular production of hematin and the apparently discontinuous gastrodermis in all the polyopisthocotyleans so far examined. Alternative digestive pathways have been determined in other blood-feeding flatworms. In certain digenean species hemoglobin degradation involves the splitting of the heme group extracellularly and hematin production is confined to the gut lumen, whilst in others digestion results in the breakdown of hemoglobin to completely soluble compounds (Halton, 1967). In these forms the problems associated with the accumulation and elimination of intracellular hematin do not arise. Whilst the digestive pathways of the Polyopisthocotylea probably do not involve the cyclical degeneration and wastage of cellular materials suggested by previous writers, other important functional considerations emerge. The gut of acoelomate animals is highly susceptible to the stresses of body movement and the effects are potentially most harmful where digestive cells contain protruding masses of dense iron-containing pigment. The support and protection of these tissues may have been an important factor in the early development of the blood-feeding habit amongst monogeneans. In accordance with views of monogenean evolution expressed recently by Llewellyn (1963) and Halton and Jennings (1965), the Monopisthocotylea probably occupy the ancestral

habitat and retain the primitive feeding methods. The Polyopisthocotylea, on the other hand, have apparently invaded secondary sites of infection and have become blood-feeders. The present studies throw further light on the form and function of the gastrodermis and suggest that the evolution of the skeletal connecting syncytium was a necessary concomitant of the specific digestive pathways developed by the Polyopisthocotylea.

I am grateful to Dr. J. B. Jennings for advice during the preparation of the manuscript. This study was carried out during the tenure of a Science Research Council Studentship.

Summary

1. Ultrastructural studies reveal that the cecal epithelium of the monogenean *Protopolystoma xenopi* is composed of two cell types. Hematin-containing cells are discontinuously arranged and alternate with a thin cytoplasmic layer, the connecting syncytium.

2. The connecting syncytium forms a flat sheet overlying the cecal wall, it surrounds the individual hematin cells and is perforated only by their distal tips. The ultrastructure of the syncytium suggests that an active physiological role is unlikely.

3. The hematin cells are involved in the visible processes of digestion. After initial extracellular hemolysis the blood meal is absorbed by pinocytosis; intracellular breakdown continues within large membrane-bound vacuoles and leads to the accumulation of hematin; this insoluble product is eventually eliminated by the discharge of intact vacuoles, apparently by simple extrusion.

4. There is no evidence for the detachment or partial disintegration of the hematin-laden cells, and the cecal epithelium remains at all times a continuous structure.

5. Since the hematin cells are partially overlapped by the connecting syncytium, active absorption is limited to a relatively small area of the hematin cell surface. A system of microtubules communicating between the free cell border and the hematin vacuole may participate in hemoglobin uptake and compensate for the reduced absorptive area. The exposed hematin cell border bears numerous cytoplasmic processes and these may facilitate contact digestion.

6. The structural and physiological organization of the polyopisthocotylean gastrodermis is characterised by the formation of dense iron-containing pigment within the relatively vulnerable digestive cells. It is considered that the connecting syncytium performs a skeletal role, giving support and protection to the hematin cells and the underlying tissues.

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LARVA RELEASE IN RESPONSE TO LIGHT BY THE COMPOUND ASCIDIANS *DISTAPLIA OCCIDENTALIS* AND *METANDROCARPA TAYLORI*

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Light has been implicated as a trigger to gamete release by several solitary ascidians (see Lambert and Brandt, 1967, for review). Most solitary ascidians are oviparous with large numbers of relatively small eggs which develop rapidly into a simplified tadpole larva which swims for a short time before selecting a suitable substrate and metamorphosing (Berrill, 1950). Compound ascidians, on the other hand, generally produce only a few large eggs which develop ovoviviparously into a highly differentiated tadpole larva that is only released when development of the swimming larva is complete. Many observations of larva release by compound ascidians have been reported, which include members of both orders in which brooding is common (Abbott, 1955; Costello, Davidson, Eggers, Fox and Henley, 1957; Grave, 1936, 1937; Grave and Woodridge, 1924; Oka, 1943; Scott, 1954). *Aplidium* (= *Amaroucium*) *constellatum* (Scott, 1954; Costello, Davidson, Eggers, Fox and Henley, 1957), *Perophora viridis* (Costello *et al.*, 1957), *Polyandrocarpa tincta* (Grave, 1936) and *Botryllus schlosseri* (Grave and Woodbridge, 1924; Grave, 1937) seem to release tadpoles during the morning when

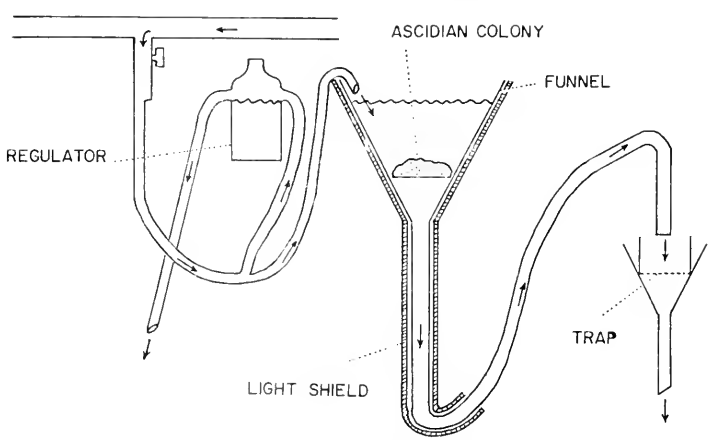

FIGURE 1. Diagram of the tunicate tadpole collector. The water level is maintained by the heights of the regulator and second outflow u tube. The larvae are collected on 370μ Nytex mesh in the trap.

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subjected to natural illumination while *Polycitor mutabilis* (Oka, 1943), *Metandrocarpa taylori* (Abbott, 1955) and *Symplegma viride* (Grave, 1937) have been reported to release larvae throughout a normal day-night cycle. In most of these studies the primary interest was embryological or behavioral or concerned with metamorphosis; larva release was an incidental observation.

In the present study we have examined in some detail larva release by two compound ascidians, *Distaplia occidentalis* (Aplousobranchiata) and *Metandrocarpa taylori* (Stolidobranchiata), in two light regimes. Larva release under natural illumination has been studied in detail over long periods of time throughout the 24 hour cycle. Experimental light-dark cycles have allowed us to clarify the role of light in the natural release cycle of these two ascidians. A portion of the results reported here were presented in a preliminary form elsewhere (Watanabe and Lambert, 1971). The observations and experiments were undertaken at the Friday Harbor Laboratories during 1968 and 1969.

Materials and Methods

Experimental animals

Large colonies of *Distaplia occidentalis* were collected from logs in Jakle's Lagoon on the east side of San Juan Island, San Juan County, Washington. Colonies of *Metandrocarpa taylori* were obtained by dredging ascidian-encrusted cobbles from Peavine Pass between San Juan and Orcas Islands. The colonies of *Metandrocarpa* are broadly attached to the substratum so that it was necessary to leave the colonies attached to their rocks or shells during all laboratory observations and manipulations. Colonies of *Distaplia* are rather pedunculate, making it possible to remove entire colonies without apparent damage. The animals were maintained on running sea water tables until use (generally 2–3 days). Because the Friday Harbor Laboratories sea water system is non-filtered and non-recirculating, filter feeders such as ascidians flourish and grow for long periods of time.

Experimental apparatus and methods

Larva release under various conditions of illumination was examined by means of the larva collector shown in Figure 1. Essentially this is an inverted version of the tunicate egg collector used by Huus (1939). Running sea water constantly swirls through the 254 cm diameter funnel, the water level being regulated by the input flow rate and the heights of the outflow u-tube and regulator. Immediately after release the larvae are washed through the outflow tube to the filter trap which is constructed of a 9 cm diameter circle of 370 μ pore size Nytex cloth cemented to a short Plexiglas cylinder. The 370 μ mesh size filter was chosen because it is large enough to pass diatoms, small algae, *etc.*, without becoming clogged, but small enough to retain the larvae of these ascidians. The larva collector was covered with black plastic and aluminum foil to exclude light except from above. For studies on larva release in the dark the top of the funnel was also light proofed. The illumination for laboratory studies on larva release in the light was furnished by the general fluorescent lighting of the laboratory with an additional 40 watt incandescent lamp 30 cm above the funnel. Experiments involving natural illumination were undertaken with the larva collector installed outside the laboratory away

from all extraneous artificial illumination. The water temperature varied from 11°C to 15°C during the course of these studies. For any experimental series, however, the water temperature was constant within 1°C.

Results

Distaplia occidentalis

The breeding season of *Distaplia* extends from early April to late August with a maximum from May to July. Most of our observations on this ascidian took place between mid-May and mid-June. Larva release by several colonies was examined with similar results so we will present the data obtained from a single representative colony. This colony was 6.5 cm long, 6.0 cm wide and 3 cm in

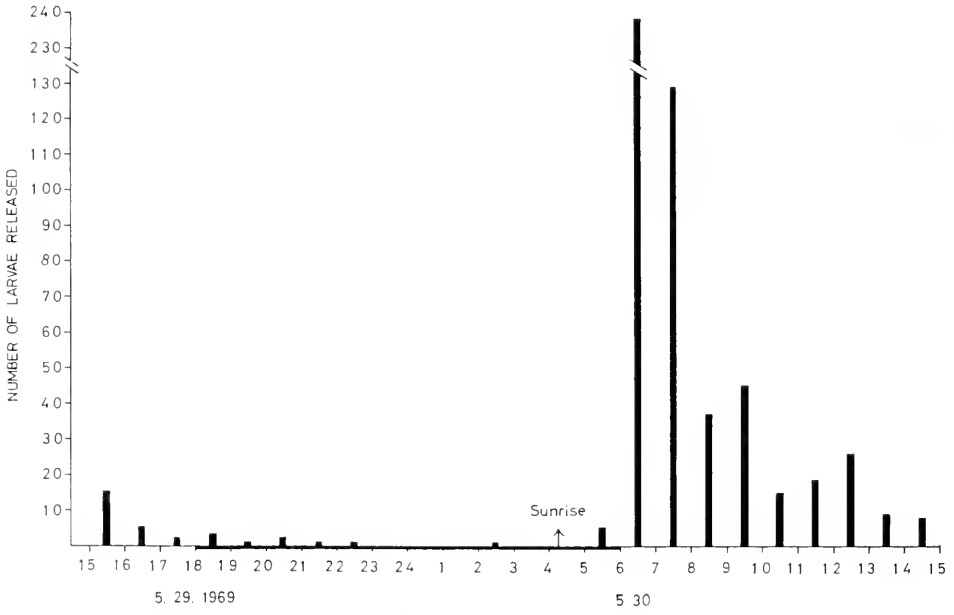

Figure 2. Larva release under conditions of natural illumination by *Distaplia occidentalis*.

height. The colony contained about 150 systems, each composed of 12–15 adult zooids.

The first series of observations examined larva release under natural illumination. During 24 hours of natural illumination a total of 563 larvae were released. As shown in Figure 2, most of the larvae were liberated during the morning hours with 483 (85.8%) being released before noon, 65 (11.5%) released between 1200–1800 and only 15 (2.7%) released during the night (1800–0600). A longer term experiment is shown in Figure 3. Here we have followed larva release during the night, morning and afternoon of three successive days with the same morning peak of larva release being evident. Seventy-five per cent of the 1838 larvae were

released during the morning hours. All of these larvae were fully mature; *Distaplia* does not release eggs or partially developed embryos.

Larva release under natural illumination suggests that light following a dark period elicits the release of larvae. To investigate further this possibility a series of experiments were conducted in which we artificially controlled the timing and duration of the dark and light periods. Figure 4 shows larva release during 44 hours of darkness followed by 3 hours of light, after which the colony was again darkened. During the initial dark period only 20 larvae were released or 5 per hour (Z/h = .5) on the average. Immediately after return to light, larva release began with a maximum within one hour. During the 3 hours of illumination 390 larvae were released (Z/h = 130). Upon returning the colony to darkness for an additional 15 hours, only 7 larvae (Z/h = .6) were released. Under our conditions at least 15 minutes of illumination are required for larva release even though

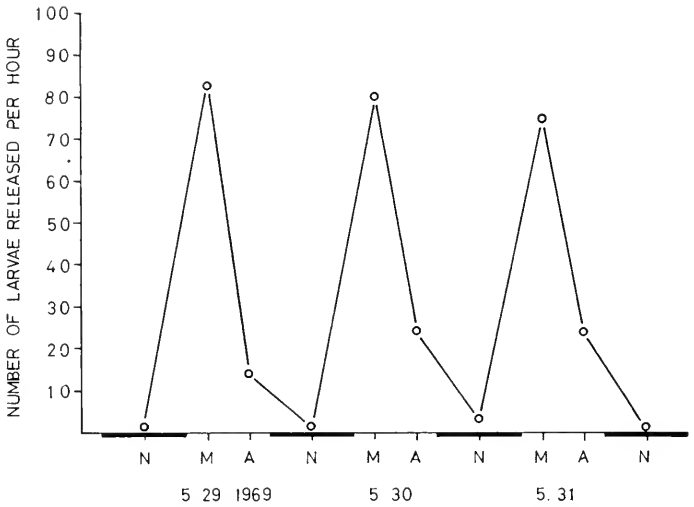

FIGURE 3. Larva release during 3 successive days of natural illumination by *Distaplia occidentalis*. The abbreviations indicate: U = 1800–0600, M = 0600–1200, A = 1200–1800

the larvae were not released until after return to darkness. We then examined the relationship between the duration of darkness and the number of larvae released upon return to light. Table I shows that there is a clear tendency for more larvae to be released after a longer period of darkness than a shorter one regardless of the time of day.

During long periods of continuous darkness very few larvae are released (Table II). We would have predicted that under conditions of continuous illumination, after the initial swarm of larvae were released the rate of release would fall to that of the colony under continuous darkness if a dark period is absolutely requisite for release. That this is not the case is shown in Table II. Here it can be seen that after the initial large release the level of release remains higher than the dark-adapted colony. It should be noted, however, that the initial rate of release during

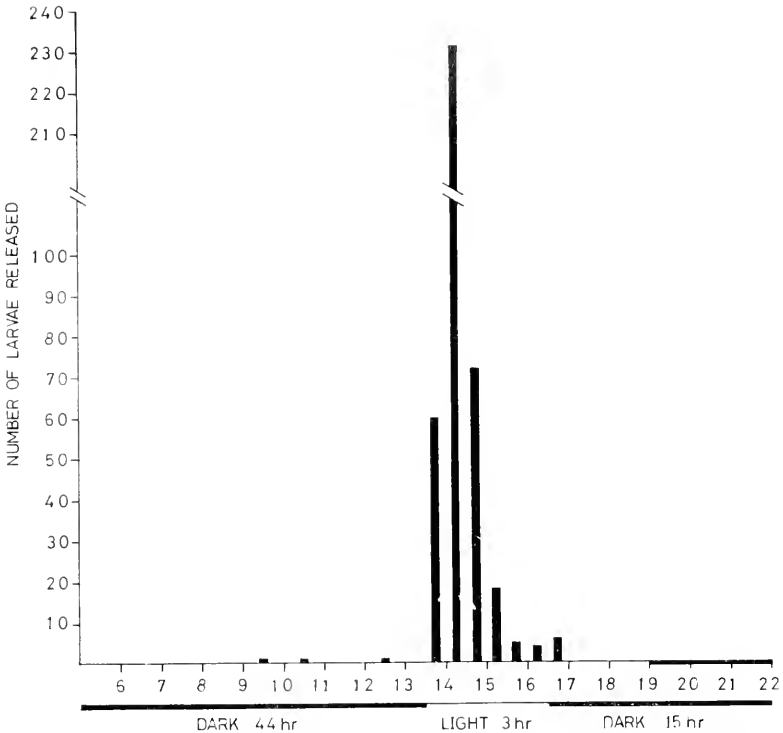

FIGURE 4. The effect of light on larva release by *Distaplia occidentalis*.

the first 6 hours following darkness was 203.5 Z/h as compared to the long term average rate of 46.1 Z/h.

Metandrocarpa taylori

Metandrocarpa breeds throughout the year in Washington waters, which is consistant with its breeding cycle in California (Haven, 1971) and the reproduction

TABLE 1

The relationship between the duration of the dark period and number of larvae released on return to light by Distaplia occidentalis

Duration of dark period	Duration of light period	No. of larvae released	z h
15 hr →	3 hr (19:00–22:00)	146	48.7
20.5 hr	3 hr (14:30–17:30)	173	57.7
42.5 hr	0.5 hr (13:30–14:00)	144	57.6
44 hr	3 hr (13:30–16:30)	390	130.0
48 hr	4 hr (15:00–19:00)	1131	188.5
63 hr	5 hr (08:30–13:30)	520	104.0
84.5 hr	6 hr (08:00–14:00)	1554	310.8

TABLE II

Summary of larva release by Distaplia occidentalis *under all experimental light regimes. D → L refers to larva release occurring up to six hours after the onset of illumination*

Illumination	Duration	No. of larvae released	z/h
Dark	350 hr	332	0.9
Light	238.75 hr	11008	46.1
D → L	30.75 hr	6258	203.5
	Total		
	588.75 hr	11340	

of *Metandrocarpa uedai* in Japanese waters (Watanabe and Tokioka, 1972). Our representative colony of *Metandrocarpa* consisted of 580 blastozooids above 4.0 mm along the antero-posterior axis.

Larva release under a natural light regime roughly parallels the results with *Distaplia*. During 24 hours of hourly observations (Fig. 5), 82 larvae were released. The number of larvae released began to increase around 0700 hours, reached a peak between 0900 hours and 1000 hours and declined afterwards; only a few were randomly released during the rest of the day. The same colony was observed during 7 successive days of natural illumination (Fig. 6). Here we see repeated the early morning mass release of larvae that was observed during the single diurnal cycle. Table III summarizes the long-term observations. Unlike *Distaplia*, *Metandrocarpa* often released a few immature tadpoles and unhatched embryos along with the actively swimming tadpoles. The proportion of immature stages released is the lowest during the peak morning swarms with the afternoon and night being about equal.

The observations under the normal day-night regime again suggested photically controlled larva release; therefore we turned our attention to experimental modifications of the duration and timing of illumination. These experiments were undertaken with several large colonies of *Metandrocarpa* with perfectly uniform results. Here we report on larva release by a representative colony which contained 350 adult zooids above 4.0 mm along the antero-posterior axis. Figure 7 shows the

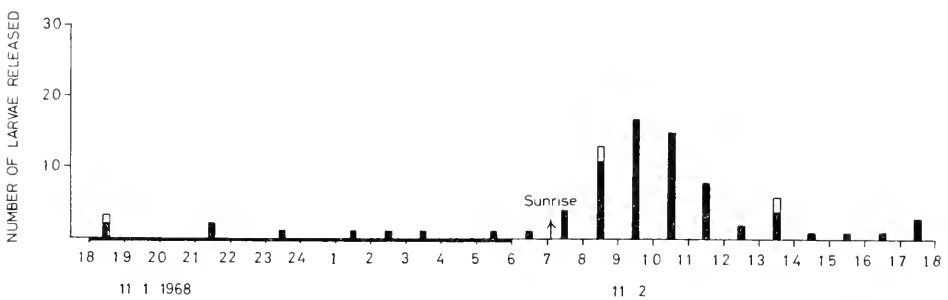

FIGURE 5. Release of larvae under natural illumination by *Metandrocarpa taylori*. The blackened bars indicate the number of mature larvae expelled, the white portions indicate the number of immature stages.

TABLE III

Larva release by Metandrocarpa taylori *under natural illumination. The immature tadpole category includes all developmental stages incapable of effective locomotion*

Illumination	Duration	Mature tadpoles	Immature tadpoles	Total	z, h
Night	84 hr	34 (76.6%)	11 (24.4%)	45	0.5
Day (07:00–12:00)	35 hr	247 (93.6%)	17 (6.4%)	264	7.5
Day (12:00–18:00)	49 hr	39 (70.9%)	16 (29.1%)	55	1.1
	Total 168 hr	320 (87.9%)	44 (12.1%)	364	2.2

results of holding the colony under continuous darkness for 43 hours, then exposing it to continuous light. Again, we see the massive larva release upon returning the colony to light. The number of larvae released on return to light seems to be related to the duration of the dark period (Table IV) with a clear tendency to release fewer immature stages after the longest dark periods. We then determined the minimal duration of exposure to light that would elicit larva release after a suitable dark period. Again, 15 minutes of exposure to light seems necessary for release even though some of the tadpoles were released after return to darkness. Larva release during long, continuous exposure to light or darkness is shown in Table V. Small numbers of larvae were irregularly released during both treatments. Again more larvae were released under illumination than darkness. More immature tadpoles were released during continuous light or dark than under natural or experimental dark-light cycles.

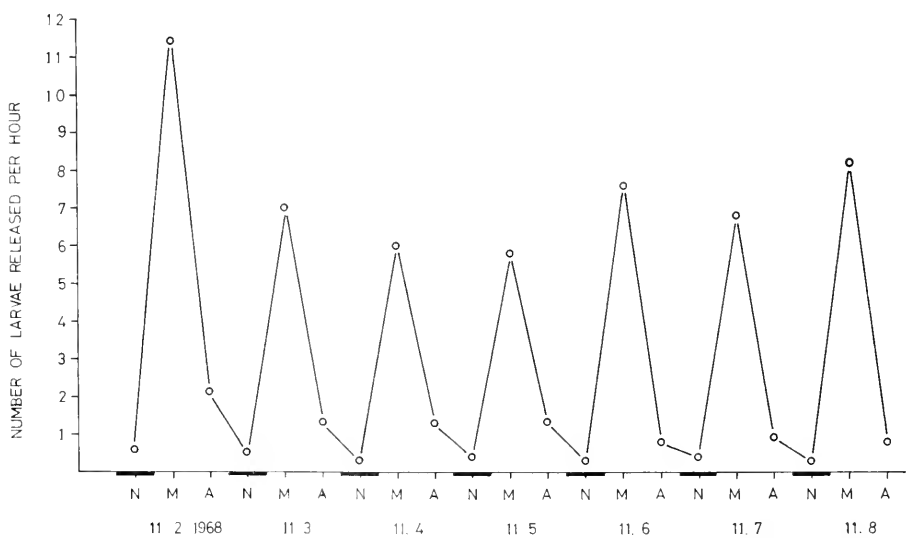

FIGURE 6. Larva release during 7 successive days of natural illumination by *Metandrocarpa taylori*. The abbreviations are the same as in Figure 3.

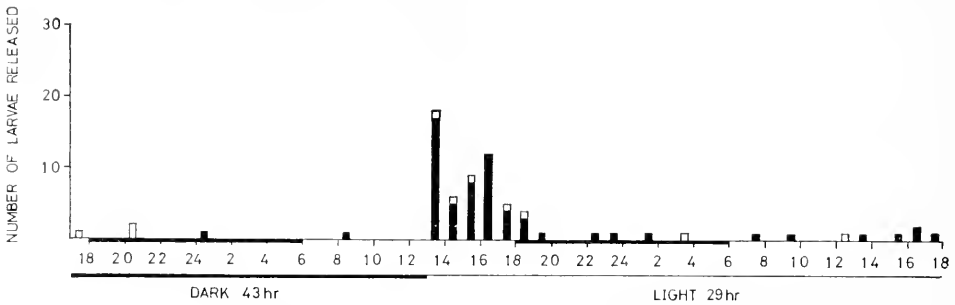

FIGURE 7. The effect of light on larva release by *Metandrocarpa taylori*.

DISCUSSION

Our observations clearly indicate that both *Metandrocarpa taylori* and *Distaplia occidentalis* preferentially release their tadpole larvae upon exposure to light after a period of darkness. This generalization holds for both larva release under a natural day-night cycle and under experimentally altered periods of darkness and illumination. These findings are similar to most of the reports on larva release by compound ascidians. *Botryllus schlosseri* (Grave and Woodridge, 1924; Grave, 1937), *Polyandrocarpa tincta* (Grave, 1936) and *Perophora viridis* (Costello *et al.*, 1957) all release their larvae during the morning in the laboratory when the laboratory was darkened at night. *Aplidium* (= *Amaroucium*) *constellatum* (Scott, 1954; Costello *et al.*, 1957) normally releases larvae 20 minutes after dawn, but can be induced to release 20 minutes after the onset of illumination if maintained in the dark until later in the day. Two other ascidians, *Polycitor mutabilis* (Oka, 1943) and *Symplegma viride* (Grave, 1937) apparently release larvae sporadically throughout a diurnal light cycle. Earlier work with *Metandrocarpa taylori* (Abbott, 1955) also suggested that larva release by this ascidian might not be under photic control. Abbott (1955) observed sporadic release of larvae throughout a day-night cycle by colonies held in finger bowls of still sea water, in the presence of very dim light during the night. It is apparent that Abbott's observations are not directly comparable with ours, because of the different conditions under which release was studied. It would be of considerable interest to examine larva release

TABLE IV

The relationship between duration of the dark period and the number of larvae released on return to light by Metandrocarpa taylori

Duration of dark period	Duration of light period	Mature tadpoles	Immature tadpoles	Total	z/h
13 hr →	5 hr (07:00–12:00)	4	1	5	1.0
20 hr	4 hr (14:00–18:00)	16	3	19	4.8
40 hr	5 hr (07:00–12:00)	22	3	25	5.0
43 hr	5 hr (13:00–18:00)	46	4	50	10.0
70 hr	3 hr (19:00–22:00)	42	0	42	14.0
90 hr	5 hr (09:00–14:00)	74	0	74	14.8
96 hr	4 hr (15:00–19:00)	100	0	100	25.0

by *Symplegma viride* and *Polycitor mutabilis* for long periods in our larva collector to see if these ascidians would also exhibit photic control in running sea water.

Although we did not specifically study the mechanism of larva release, certain aspects of the morphological basis of brooding in *Metandrocarpa* (Abbott, 1955) and *Distaplia* (Berrill, 1948) have been described which are important in any consideration of the mechanism of larva release. The development of *Distaplia* embryos occurs within a brood pouch which is essentially the distal portion of the oviduct (Berrill, 1948). One finds a complete series of developmental stages within the sac with fully formed tadpoles at the distal and early cleavage stages at the proximal end of the sac. As the parental zooid regresses the brood pouch can become completely isolated from the parental zooid leaving the intact brood sac as an independent structure with its contained embryos as the structure from which the larvae are released. Thus larva release is not likely to be under control of the parental nervous system in this species. Reese (1967) has shown that the isolated gonoducts of *Ciona intestinalis* can be induced to spawn by exposure to light which opens the possibility that the brood sac itself may be responding to light in *Distaplia*. Light induced larval activity is probably not a factor in larva release by *Distaplia* as the

TABLE V

The effect of continuous darkness and continuous illumination on larva release by Metandrocarpa taylori

Duration of dark or light period	Mature tadpoles	Immature tadpoles	Total	z/h
136 hr (dark)	13 (68.4%)	6 (31.6%)	19	0.1
115 hr (light)	94 (77.7%)	27 (22.3%)	121	1.1

larvae are not active at the time of actual escape from the colony (Ritter and Forsyth, 1917). In contrast to *Distaplia*, *Metandrocarpa* broods its embryos in the peribranchial cavity, about 15–20 embryos of various stages being found in a large individual. Our findings that 12% of the young of *Metandrocarpa* are released in an immature state are in agreement with those of Abbott (1955), who suggested that the immature larvae and embryos might be accidentally released along with the release of faeces. That the proportion of immature embryos was at a minimum during the morning when the greatest number of larvae are released under natural light conditions (Table III) suggests that the release of mature larvae and immature embryos are under fundamentally different controls. Perhaps the mature, swimming larvae are triggered into swimming activity by light following darkness, while the immature stages are released by mild random contractions of the parent. This hypothesis gains support from the finding that roughly similar numbers of immature tadpoles were released under all conditions of illumination (Table III) while the number of mature tadpoles released showed a nearly tenfold increase following exposure to light. *Metandrocarpa uedai* (Watanabe and Tokioka, 1972), in contrast to *M. taylori*, releases only mature larvae, which suggests that this species has evolved a mechanism for the retention of immature stages that is lacking in *M. taylori*.

The ecological significance of ascidians releasing the majority of their larvae during the few hours following dawn is problematical. Possibly the hour of release is not crucial, the important factor being a large number of larvae released at about the same time. It is difficult to see how synchronous larva release would make any adaptive difference whatsoever in the reproductive success of these ascidians (see Millar, 1972 for review of reproductive strategy). A more plausible explanation, we feel, is related to the function of the ascidian tadpole: habitat selection (Berrill, 1950). Differential orientations to light are important in habitat selection by ascidian larvae. Initially the larvae are photo-positive; later they become photo-negative. Only during the photo-negative period do the larva metamorphose, chiefly in shaded portions of rocks, logs or algae. Thus, it is important that each larva should have a maximum period for seeking a suitable substratum during the day of release. Presumably those larvae which do not find an adequately shaded spot before nightfall would tend to stand a poor chance for colony development and reproductive success.

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Summary

1. Larva release under natural and artificially determined light regimes was studied in the colonial ascidians *Distaplia occidentalis* and *Metandrocarpa taylori*.

2. Under natural illumination, both species show a clear tendency to release their larvae during the morning hours. Sunrise marks the beginning of larva release.

3. Under experimental light conditions, larva release can be initiated at any time of the day or night by exposing suitably dark conditioned colonies to light. Transfer from light to darkness has no effect on larva release.

4. The number of larvae released upon exposure to light is related to the duration of the dark period. A greater number of larvae are released after a long dark period than a short one.

5. The minimum duration of exposure to light that will elicit larva release by dark adapted colonies is 15 minutes. Larva release continues after return to darkness, providing the duration of illumination is adequate.

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THE NEUROMUSCULAR BASIS OF COXAL FEEDING AND LOCOMOTORY MOVEMENTS IN *LIMULUS* [1]

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The control of movements by central and reflex integrative mechanisms has been studied in a number of arthropods (for reviews see Kandel and Kupfermann, 1970, and Evoy and Cohen, 1971). Until recently, little work has been done on control of movements in *Limulus*, despite their widespread use in other areas such as visual physiology. *Limulus polyphemus*, the horseshoe crab, is large, hardy, and tolerant of experimental manipulation. It also possesses several stereotyped behavior patterns that are amenable to analysis of their neural control, including rhythmic gill ventilation (Fourtner, Drews, and Pax, 1971; Wyse, 1972) and tail spine rotation (Silvey, 1971; Eagles, 1971, 1973). The patterns considered in this study are the rhythmic coxal movements associated with feeding and locomotion.

Limulus feeds by rhythmic transverse abduction and adduction of the segments of the walking legs. Each coxa, which is elongated dorsoventrally, moves in an arc about a dorsolateral pivot. Gnathobase spines, directed inward, serve to shred particulate food and push it into the mouth, which is in the middle of the legs. Opposite legs move in phase and adjacent legs move out of phase, so that both first and both third legs move inward while both second and both fourth legs move outward, and vice versa. Both these chewing movements and the anatomy of the coxal muscles mediating them have been described (Manton, 1964). The present study shows that for an individual leg, both the movements and the underlying muscle actions are more complex than indicated by Manton, and describes the patterns of motor output controlling different types of rhythmic feeding movements.

Materials and Methods

Specimens of *Limulus polyphemus* (L.) were obtained from the Marine Biological Laboratory, Woods Hole and were maintained in a 150 gal recirculating sea water system. Animals were usually placed ventral side up in air for observation and recording of feeding activity. They were fed small pieces of frozen ocean perch (*ca.* 0.5–1 g, thawed to room temperature) to elicit feeding movements.

Muscle electrical activity was recorded chronically from intact, restrained animals with insulated 40-gauge stainless steel wires from which the insulation had been removed from the terminal 1–2 mm. These electrodes were inserted through fine holes in the cuticle into the underlying muscles and held in place with Eastman 910 adhesive and vinyl adhesive tape. The origins of the tergocoxal muscles (25–29 in Fig. 1) are clearly visible on the dorsal prosoma of most animals, allowing electrode placement at the origins of these muscles. For the four ventral

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plastrocoxal muscles, each electrode was inserted through the articular membrane between the legs, into the muscle near its coxal insertion. Indifferent electrodes were placed in the prosoma near its lateral margin. In all cases the electrode positions were confirmed by dissection after the experiment.

To clarify patterns of coxal chewing movements, unrestrained *Limulus* were fed, ventral side up in air, and their activity was photographed with a Bolex Rex 16 mm cine camera at 18–24 frames/sec. Frame-by-frame analysis of individual chewing cycles was performed with a time-motion study projector. Coxal movements were also recorded along with muscle activity, by coupling the gnathobase through a weak spring to a Grass strain gauge.

Results

Chewing patterns

The rhythmic chewing movements of a coxa sometimes consist of simple alternate abduction and adduction about the dorsolateral pleurocoxal pivot, as described by Manton (1964). However, we noted that similar in-and-out coxal movements were used to egest food such as fish that was several days old, a finding which argued for greater complexity of the coxal movements. Further observation showed that in most normal chewing, the coxa did not trace a simple arc about its pivot, but instead took an oval path of: depression-adduction-elevation-abduction (Fig. 1). This coxal movement pattern of *ingestive chewing* then resembled a plot of a hysteresis loop rather than a simple arc. *Egestive chewing* resulted from the reversed sequence of: elevation-adduction-depression-abduction.

This "hysteresis" in the chewing cycle requires movement of the pleurocoxal pivot. The coxa articulates with a Y-shaped pleurite, which in turn is set in pliable pleural cuticle. The pleurite serves as a rather firm pivot against anterior-posterior movement, but is relatively free to move dorsolaterally. Such movements of the pleurocoxal pivot are observed during both ingestive and egestive chewing, but the inaccessibility of the pivot and movements of distal leg segments make them difficult to quantify.

Anatomy of coxal muscles

The coxal muscles have previously been described (Lankester, Benham and Beck, 1885; Manton, 1964). Although their actual arrangement is more complicated than previously indicated, only a brief outline of the anatomy will be given here. Each of the first four walking legs has nine coxal muscles (the fifth leg is only tonically active during chewing, and was not examined). The third walking legs were selected for detailed study, but there is little difference in muscle anatomy among the first four legs. Five of the nine coxal muscles originate dorsally on the carapace (tergocoxals), and four (plastrocoxals) originate on the endosternite, a central cartilage-like mass derived from fused muscle tendons (*en* in Fig. 1.). The position of the endosternite can be considered fixed over the course of several coxal chewing cycles; since adjacent legs move out of phase with each other, any movement of the endosternite cannot contribute to a coxal movement cycle.

The muscles are numbered according to Lankester *et al.* (1885). In their description, serially homologous tergocoxal muscles bear the same number, but

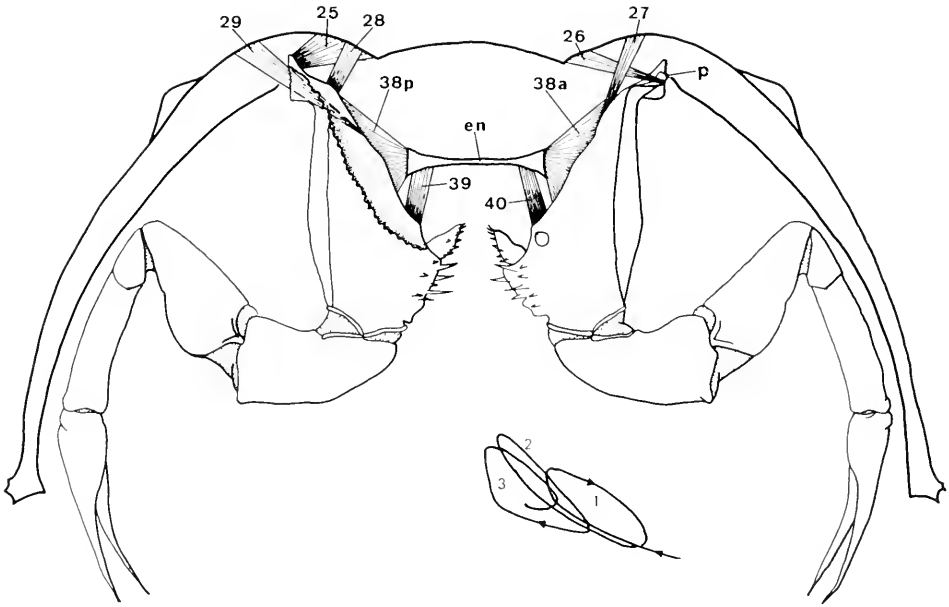

FIGURE 1. Anterior view of a *Limulus* at the level of the third walking legs. On the right are shown the muscles inserting on the anterior face of the coxa of the third leg. On the left, the anterior face and muscles have been cut away, exposing the muscles inserting on the posterior face. Muscles are numbered according to Lankester, Benham, and Beck (1885). Abbreviations are: en = endosternite, p = pleurocoxal pivot. The diagram under the legs shows three consecutive cycles of chewing (labeled 1–3) of a leg. The path of a coxal gnathobase spine was traced from frames of a cine film. Note that the paths are oval rather than simple arcs. The net inward movement of the coxa over several cycles resulted in part from decreased resistance to adduction as the food, a piece of fish, was swallowed. The scale of the path is larger than that of the diagram of the animal above.

serially homologous plastrocoxals bear different numbers for each pair of legs. The numbers used here are for the third legs. The tergocoxals 25, 28, and 29 and the plastrocoxals 38p and 39 insert on the posterior side of the dorsomedial coxal margin (Fig. 1). The tergocoxals 26 and 27 and the plastrocoxals 38a and 40 insert on the anterior side of the margin. Several of the muscles have an anterior or posterior component to their action, not visible in the transverse view of Figure 1. As indicated in Figure 2, 25, 38p and 38a originate anterior to the leg on which they insert, while 26, 39, and 40 originate posterior to their insertions.

Muscle activity during normal ingestive chewing

Activity was recorded from 4–6 muscles at a time, usually from third walking legs, in over twenty intact specimens of *Limulus*. The degree of "hysteresis" in normal chewing was not quantified in recording, but was frequently noted to be present in slight or moderate degree. Representative records of muscle activity during normal chewing are shown in Figure 3. Most of the muscles were active during instroke (adduction, represented in all records by upward deflection of the monitor).

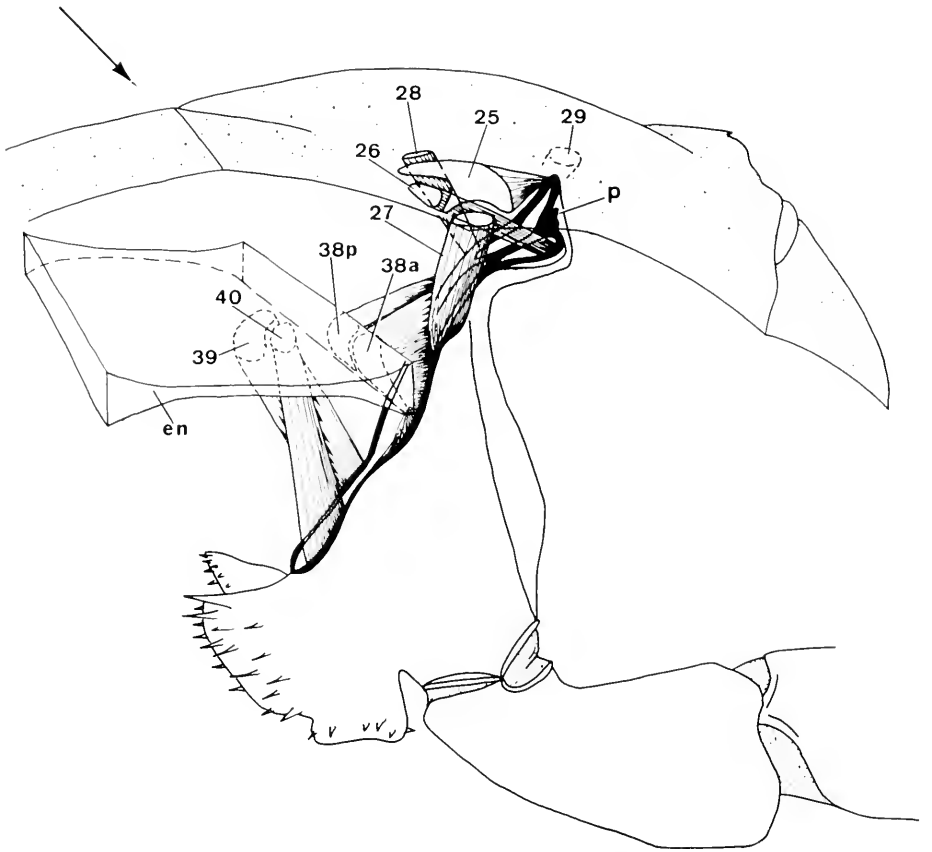

FIGURE 2. Perspective view of the third left coxa, showing muscle attachments. The arrow indicates anterior direction. Note that the directions of muscle action are oblique rather than simply transverse. Abbreviations are: en = endosternite, p = pleurocoxal pivot.

Muscles 39, 40, 38p and 38a (the four ventral plastrocoxal muscles) were always active during instroke (Fig. 3A), although the amplitude in 38p varied. Muscle 40 frequently terminated activity before the end of instroke. Activity of all four usually started 0.1–0.2 sec before the onset of observable inward movement. In some preparations a second period of synchronous activity was present in all four plastrocoxal muscles during outstroke (Figs. 3A, 4). Figure 4 shows the two phases of plastrocoxal activity at a faster time base. Activity during instroke was biphasic negative-then-positive, and was not synchronous between the four muscles. In contrast, the activity during outstroke was highly synchronous for all four muscles, and was biphasic positive-then-negative. Since the adjacent legs move out of phase with the recorded leg, this activity might have resulted from electrode pickup from their adjacent muscles. However, 38a would pick up from the second leg and 38p from the fourth leg, so that synchronous activity would be highly unlikely. The synchronous activity during outstroke did not correlate with

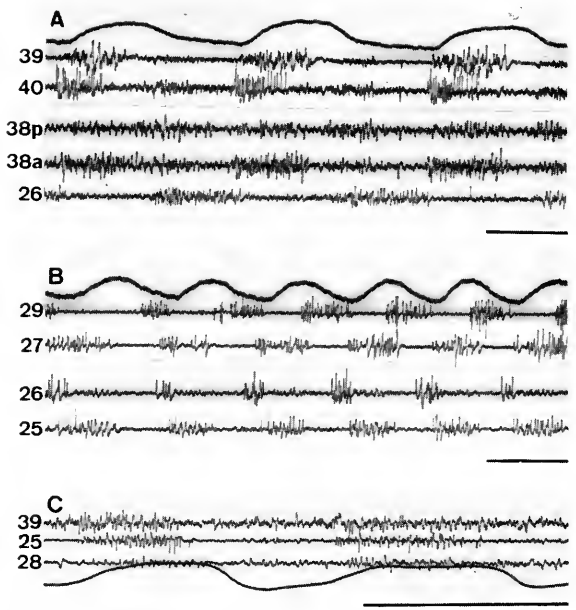

FIGURE 3. Electromyograms of coxal muscles during normal chewing. Upward displacement of the monitor indicates inward coxal movement (top trace in A and B; bottom trace in C); further explanation in the text. All time marks 2 sec.

tension development in the muscles, an observation confirmed by recording tension development in individual muscles, the insertion of which had been dissociated from the coxa. Such muscles developed tension during instroke only and relaxed during outstroke-phase synchronous activity. It seems most likely that this synchronous activity represents the action of a peripheral inhibitor axon common to all four plastocoxal muscles. Although similar evidence for peripheral inhibition has been reported in lobster swimmeret muscles (Davis, 1969), intracellular recording from the plastrocoxal muscles will be required to test the hypothesis of a common inhibitor.

The activity of the five tergocoxal muscles is more diverse (Fig. 3A-C). Muscles 25 and 28 were only active during instroke, although 25 could tail over into the beginning of outstroke. The only muscle with activity consistently during outstroke was 26. Its activity was usually confined to the latter part of outstroke; the record in Figure 3B is more typical than that in 3A in this respect. During weak chewing, there was little or no activity in 25 and 26; they were recruited during stronger cycles.

The two muscles directed nearly radially to the axis of coxal movement had the most variable activity. Muscle 27 was active during instroke, sometimes continuing through early outstroke. Muscle 29 was extremely variable and could act during instroke, outstroke, or both (Figs. 3B, 5). Three patterns of activity were observed most frequently: (1) during instroke, (2) during both late outstroke and late instroke (in two discrete phases), and (3) through late instroke and early

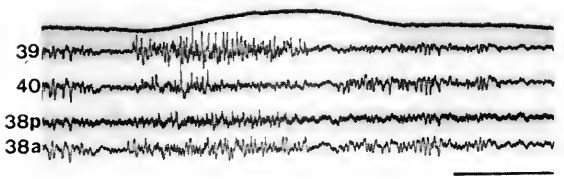

FIGURE 4. Electromyograms of plastrocoxal muscles during normal chewing, showing asynchronous activity during instroke (up) and synchronous activity during outstroke (down); further explanation in the text. Negative is up for all electrodes; time mark 1 sec.

outstroke, lagging 90° or more in phase behind the instroke. During normal chewing, 29 never appears active during the beginning of instroke. Figure 6 summarizes the characteristic patterns of activity of coxal muscles during ingestive chewing.

Muscle activity during egestive chewing

When experimental animals were fed fish that was several days old, the pattern of egestive chewing often resulted, in which the coxal gnathobases moved in the sequence medial-ventral-lateral-dorsal. Representative records of coxal muscle activity recorded during such a pattern are shown in Figure 7, and a summary of the activity patterns in all such cases is shown in Figure 8. Most of the muscles had patterns of activity nearly indistinguishable from those during normal chewing. The only muscles whose pattern changed markedly were the radially directed tergocoxals, 27 and 29. Muscle 29 was active during late outstroke, often with a secondary burst early in instroke. Activity in 27 started in mid-outstroke and continued until late instroke. Taken together, activity in the radially directed muscles 29 and 27 phase-led instroke during egestive chewing (Fig. 8). In contrast, activity in these two muscles tended to phase-lag behind instroke during normal ingestive chewing (Fig. 6). The significance of these changes in phase is discussed below.

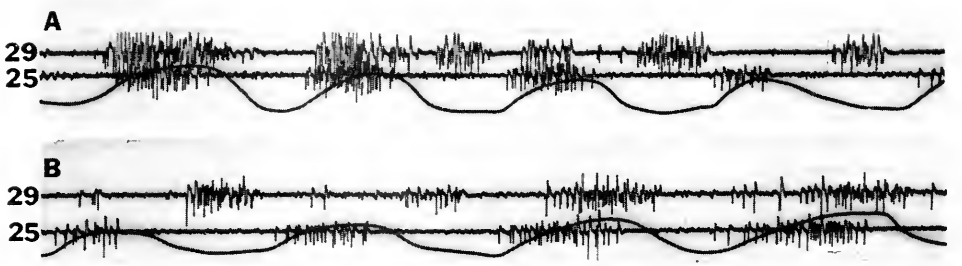

FIGURE 5. Electromyograms of tergocoxal muscles 29 and 25, showing extreme variation in the activity of 29. The two records are in sequence, with two cycles omitted between them. Muscle 29 acted during instroke at the beginning and end of the records, but acted during both phases or during outstroke in intervening cycles; time mark 2 sec.

Coxal promotor and remotor movements

The activity of muscles during the coxal promotor-remotor swing (Manton, 1964) was examined for comparison to activity during chewing. The animals were allowed to walk over a glass plate in air. In contrast to transverse rotation about the dorsolateral pivot in chewing, the anterior-posterior coxal swing must involve two functional pivots. In addition to the fixed dorsolateral pivot, a second indistinct point near the gnathobase at the ventromedial coxal margin is also

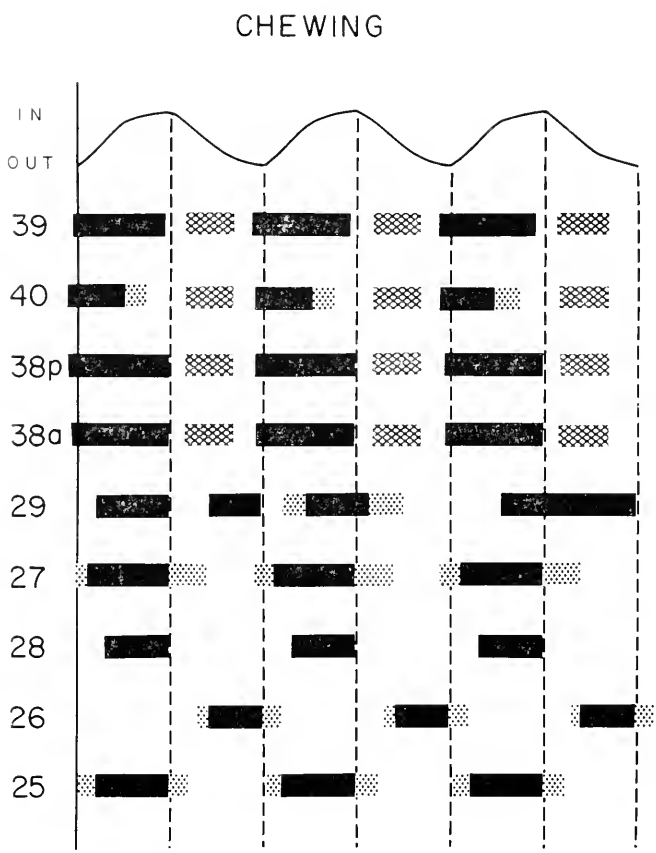

FIGURE 6. Summary of muscle activities of all preparations during normal ingestive chewing. Black bars indicate the period during which a muscle was usually or always active. Dotted areas indicate periods when muscle was sometimes active. Crosshatched areas indicate periods of synchronous, positive-going activity (inhibition?) in 38a, 38p, 39, and 40. For 29, the three common activity patterns are shown.

stationary. The coxa then swings like a door around two hinges. The general pattern of muscle activity during this swing was as follows (see Figs. 9 and 10): muscles 26, 27, and 38a, which attach to the anterior face of the coxa, were active during forward movement (promotion). Muscles 25, 38p, and 39, which attach

to the posterior coxal face, were active during backward movement (remotion). Muscle 29 (recorded once) was inactive or weakly active during remotion; activity in 28 during coxal swing was not recorded. Muscle 40, although inserted on the anterior coxal face, was active during remotion. Secondary activity was sometimes seen in 38a, 26, and 27 during remotion. The secondary activity in 26 and 27 sometimes appeared synchronous and could represent either peripheral inhibition or spread of activity from the large adjacent origin of muscle 25. It seems clear, however, that promotion results from inward pull on the anterior coxal margin and remotion results from pulling in on the posterior margin.

Discussion

In the muscular generation of normal coxal chewing movements, the ventral plastrocoxal muscles 38a, 38p, 39, and 40 must serve as the main adductors. They

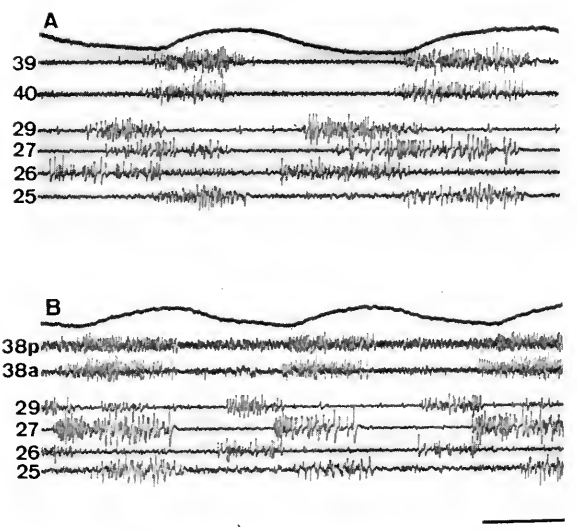

Figure 7. Electromyograms of coxal muscles during egestive chewing; upward deflection of monitor = inward movement; time mark 2 sec.

are the muscles with the greatest leverage around the pleurocoxal pivot (Fig. 1), and their combined activity appears to be an invariant correlate of adduction.

Manton (1964) in her anatomical study of coxal muscles, concluded that the tergocoxal muscle 25 actively abducted the coxa. She felt that 25 inserted dorsal as well as posterior to the pleurocoxal pivot, and hence could abduct. She rejected the possibility of abduction by passive elasticity, feeling that the arthroidial cuticle was not sufficiently elastic. Our results clearly show that 25 is active during adduction only, never during abduction. The only muscle consistently active during abduction was 26. It is anatomically unlikely that 26 could abduct. It is the main promotor during coxal swinging (see below) and is probably of only secondary importance in chewing, being recruited only with stronger cycles. Direct stimula-

tion of muscle 26 through the recording electrode always produced simple promotion of the coxa, with no abduction. Conjoint stimulation of 26 and 29 (since 29 is sometimes also active during abduction) likewise produced simple promotion. It seems clear that none of the coxal muscles are abductors. Abduction must then result from passive forces, perhaps by cuticular elasticity aided by active inhibition of adductors. Lateral displacement of the coxa by adduction of adjacent coxae in the restricted space around the mouth could also aid in passive abduction.

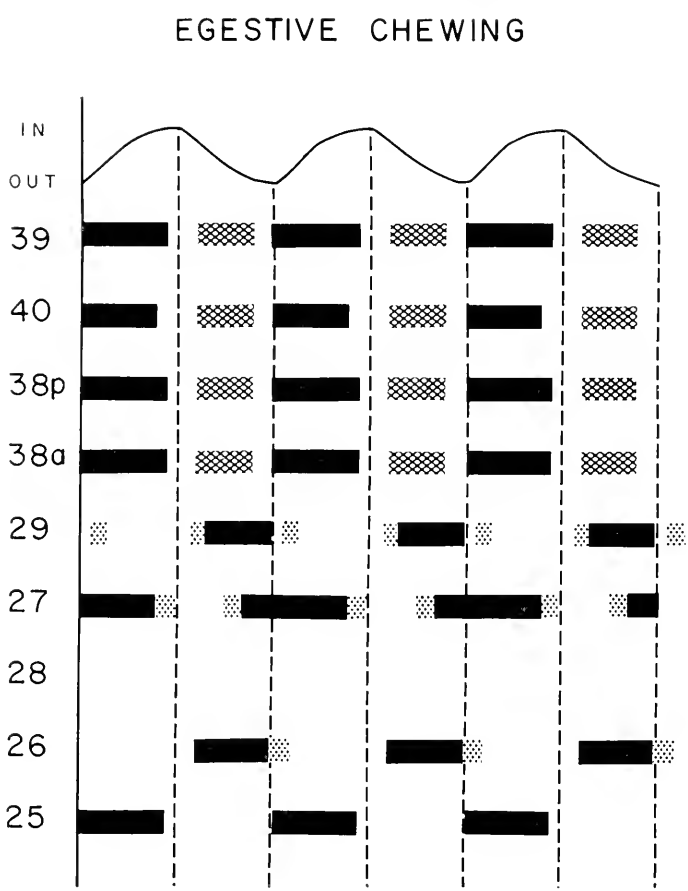

FIGURE 8. Summary of muscle activities during egestive chewing. Symbols are the same as in Figure 6. Activity patterns are similar to those in normal chewing (Fig. 6), except that 29 and 27 tend to phase-lead instroke.

Snodgrass (1950) hypothesized that arthropod jaws evolved from leg coxae, with a primitive condition of direct transverse jaw movements worked by adductor muscles. Abductor muscles were considered to be absent, with passive abduction resulting from cuticular elasticity. Manton (1964) opposed this view, arguing that Snodgrass' hypothetical ancestor did not resemble any living arthropod, and that passive abduction was undemonstrated and unlikely. Manton (1964) concluded

that various arthropod jaw mechanisms had evolved independently, and considered her study to support a hypothesis of polyphyletic origin of arthropods (see Tiegs and Manton, 1958). We find no evidence for a functional abductor muscle in the transverse chewing movements of *Limulus*. Therefore one of Manton's objections to Snodgrass' hypothesis is not verified, but implications concerning the evolution of arthropod groups remain unclear.

In the muscular generation of the promotor-remotor coxal swing of walking, the dorsal tergocoxal muscles must predominate. In agreement with Manton (1964) we conclude that muscle 26 is probably the main promotor, while muscle 25 is probably the main remotor. This conclusion is based in part on anatomical arrangement of the muscles, which allows maximum leverage anterior and posterior to the pleurocoxal pivot. Furthermore, on direct electrical stimulation, 25 always produced simple coxal remotion, while 26 always produced simple promotion. Stimulation of 27 and 28 produced variable mixtures of weak promotion or remotion

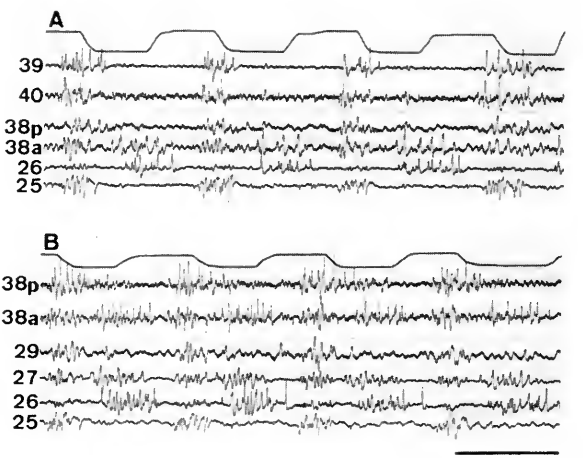

FIGURE 9. Electromyograms of coxal muscles during the coxal swing of promotion and remotion. The movement monitor (top traces, forward promotion is up) was hand-controlled, and gives only an approximation of the time of coxal movement; time mark 1 sec.

and adduction. The plastrocoxal muscles may contribute to promotion and remotion, but their major role is probably stabilization of the ventromedial coxal margin. During walking in aquaria the gnathobases are held rather laterally. Thus the activity of the plastrocoxal adductors must not generate a great degree of shortening of the muscles.

The larger remotor muscle 25 pulls the posterior dorsolateral corner of the coxa both dorsally and medially (see Figs. 1 and 2). The medial component of this force would displace the whole ventral portion of the coxa forward as well as remoting. The combined action of muscles 39 and 40, both of which pull the ventral part of the coxa back as well as in, and both of which act during remotion, would tend to oppose the forward displacement of the coxa and stabilize remotion.

The circularity or "hysteresis" of coxal movements during ingestive and egestive chewing is considered to result from action of muscles 29 and 27. The presence

of any circularity in the arc of transverse movement about the dorsolateral pivot requires that the pivot move on an axis radial to the arc. Muscles 27 and especially 29 are radically directed (Fig. 1) and can act as *pivot shifters*. Of the three relatively stable patterns of action of muscle 29, the one likely to produce the greatest degree of "hysteresis" is with 29 activity phase-lagging 90° or more behind adduction. The coxa would be adducted inward, and then pulled dorsolaterally during late

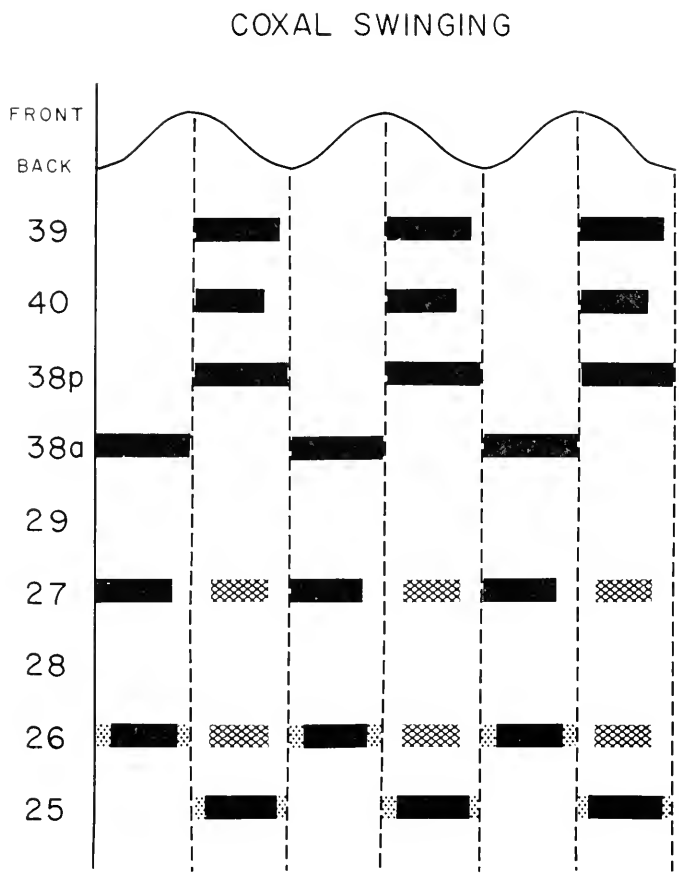

FIGURE 10. Summary of muscle activities during promotor-remotor coxal swinging. Symbols are the same as in Figure 6. Activity of 28 was not recorded during swinging; activity in 29 was recorded in only one preparation and thus could not be characterized reliably.

instroke and much of outstroke. Therefore, the outstroke path would be dorsolateral to the instroke path, producing the sequence medial-dorsal-lateral-ventral.

By the same argument, the pattern of activity of muscles 29 and 27 during egestive chewing is sufficient to produce circularity in the opposite direction. Taken together, the radial muscles phase-lead instroke by about 90°, producing a dorsolateral force vector during late outstroke and early instroke. The resulting movement sequence is the medial-ventral-lateral-dorsal pattern of egestion.

The difference between chewing resulting in ingestion and chewing resulting in egestion is thus a result of a phase shift in the action of two muscles within an otherwise rather stable pattern of muscle activities. The sensory information underlying this phase shift presumably comes from the same gnathobase chemoreceptors (Barber, 1956; Barber and Hayes, 1963) that trigger all chewing sequences. The central integrative mechanisms underlying the chemosensory control of this phase shift are interesting subjects for further research.

Summary

1. Transverse feeding movements of a *Limulus* leg coxa can trace a simple repeating arc of abduction and adduction around a dorsolateral pivot, or may take an oval path of depression, adduction, elevation, and abduction. This ingestive chewing path and the reverse sequence mediating egestion both require movement of the pivot.

2. The actions of the nine coxal muscles were determined by chronic electromyogram and movement recordings in intact animals. In all feeding patterns most muscles act during adduction, the four ventral plastrocoxals being the main adductors. None of the muscles actively abduct the coxa.

3. In the promotor-remotor coxal swing of locomotion the dorsal tergocoxal muscles predominate, although the plastrocoxal muscles are also active. Muscles attached to the posterior margin of the coxa are active during remotion, and those on the anterior margin are active during promotion (except muscle 40, which is active during remotion).

4. The oval paths characteristic of ingestive and egestive chewing result from action of muscles 29 and 27. These muscles act radially to the arc of adduction-abduction and displace the pivot of that arc dorsolaterally. When activity in these muscles phase-lags adduction, the path of ingestive chewing results. When their activity phase-leads adduction, the reverse sequence of egestive chewing is produced. Thus a major behavioral alteration results from phase-shifting the action of two muscles in an otherwise stable pattern of motor output.

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