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OF LARVAL PALAEEMONETES (DECAPODA:NAT-
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The Ohio State University, Ph.D., 1962
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THE DEVELOPMENT AND FUNCTION OF NEUROSECRETORY SITES
IN THE EYESTALKS OF LARVAL PALAEEMONETES
(DECAPODA:NATANTIA)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
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by

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The Ohio State University
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Approved by

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INTRODUCTION

Histological evidence of neurosecretion has been shown in numerous organisms throughout the animal kingdom. The original concept of neurosecretion was based upon cytological observations of nerve cells found to behave as gland cells. Neurosecretory cells are often defined as neurons with Nissl bodies, dendrites, axons, and neurofibrils (E. Sharrer, 1954). This definition serves to emphasize that in the minds of comparative endocrinologists, the cells in question are in fact modified nerve cells. A discussion of the idea that such cells have evolved from displaced epithelial secretory cells has been presented by Clark (1956). All concerned, however, have come to recognize that the characteristic property of such cells is the ability to secrete physiologically active substances. Actually, one can no longer clearly distinguish between nervous and endocrine systems.

The secretory products of neurons may be categorized as either neurohumors, that is, substances operating over short distances such as synapses and neuromuscular junctions, or neurohormones, acting on target tissues far removed from the point of release. Acetylcholine and 5-hydroxytryptamine are examples of the former group, and vasopressin, the vertebrate hormone, the latter. In the light of these phenomena, the nervous system has been considered an endocrine complex in which electrical properties are of no greater significance than they are in muscle (Welsh, 1961).

The use of the term neurosecretion, in the histological sense, is usually confined to those cells suspected of producing neurohormones. Neurosecretory cells are not distributed at random but generally compose discrete aggregates located at specific sites in the nervous system. In crustacea, cells displaying secretory activity are usually associated with well-defined systems. Such a complex includes not only the source of active substance but also specialized nerve endings associated with blood channels and commonly known as neurohaemal organs (Carlisle & Knowles, 1953). The first morphological study of invertebrate neurosecretion was by Hanstrom (1931), who described the X-organ complex of crustacea. A vesicular structure associated with the optic ganglia was mentioned by Bellonci (1881) in his work on the head of an isopod. This was probably Hanstrom's X-organ.

For many years, the eyestalks have been recognized as control centers of metabolic and behavioral patterns in crustacea. Pouchet (1876) studied Palaemon and Crangon in addition to several other invertebrates. He observed changes in chromatophores after eyestalk removal but also commented on the peculiar position assumed by Palaemon from which the eyestalks had been removed. At that time, Pouchet associated chromatophore control with the photoreceptive function of the eyes. In fact, he observed that many lower crustaceans lacking eyes also lack chromatophores. Keeble & Gamble (1904) published a detailed study of chromatophore behavior in shrimp. These workers demonstrated some basic differences among the species studied, including opposite chromatophore response following eyestalk removal in Hippolite and Palaemon. The former exhibited pigment contraction after the

operation, while in the latter, pigments were fully expanded. With reference to background response, the larvae of Hippolite and Palaemon reacted as did the adults, although to a lesser degree. Chromatophore response to background was demonstrable at the "stalk eyed" stage but not before. This response was also attributed to direct optic control.

Current interest in the physiological importance of crustacean eyestalks began with the work of Perkins (1928) and Koller (1929). Working independently (on Palaemonetes and Crangon), they showed that injection of eyestalk extracts results in paling an experimentally darkened animal by contraction of chromatophores. In the decade that followed, the notion of endocrine control of many physiological processes in crustacea was firmly established (Abramowitz & Abramowitz, 1940; Brown, 1933, 1934, 1935; Brown, Ederstrom & Scudmore, 1939; Brown & Cunningham, 1929). The entire subject of crustacean endocrinology has been reviewed thoroughly several times since (Brown, 1941, 1944, 1952; Carlisle and Knowles, 1959; Gabe, 1954; Hanstrom, 1939, 1947; Kleinholz, 1942; Knowles and Carlisle, 1956; Panouse, 1947; Welsh, 1961) and need not be reiterated here.

Literature on the subject of neurosecretory systems in crustacea has been concerned mainly with those found in adult animals. Hanstrom's (1931) pioneer work included a description of the X-organ-sinus gland complex in Palaemonetes. The histology of incretory elements in the crab Sesarma was presented in great detail by Enami (1951_b). Bliss and Welsh (1952) published descriptions of the X-organ-sinus gland systems of eleven species of crabs. Carlisle and Passano (1953) defined the anatomical relationships of the cells of the

X-organ to the sinus gland in a number of decapods, noting, especially, basic differences between those found in Brachyurans and Natantians. By then it was evident that neurosecretory systems in crustacea were far more complex than originally had been supposed. In an effort to unify studies in this area, Carlisle and Passano proposed that a distinction be made between the X-organ of Hanstrom, which they called the pars distalis x organi, and the ganglionic X-organs of other workers.

More recently, a number of studies have been published describing neurosecretory cells associated with the central nervous system of decapods (Carlisle, 1953, 1959; Durand, 1956; Matsumoto, 1958; Parameswaran, 1956; Potter, 1954). All were concerned with adult tissues. Our knowledge of neurosecretory systems operating in larval crustaceans is based entirely upon the work of Pyle (1943) on Homarus and Pinotheres. Matsumoto worked on newly hatched crabs; however, the species he studied (Potamon dehaani), like many freshwater decapods, hatches in the form of a small adult, having passed the larval stages of other decapods in the egg. Dahl (1957) described the embryology of the X-organ in Crangon allmanni. His paper, unfortunately, contained no figures and no information on activity of the cells observed. Broch (1960) studied the effects of tissue extracts on isolated chromatophores of Palaemonetes zoeae. Costlow (1961) reported on the effects of Sesarma zoeal eyestalk extracts on adult chromatophores.

To comparative endocrinologists, Palaemonetes is a classic experimental animal. Five species inhabit the Eastern United States.

Palaemonetes vulgaris (Say), P. pugio Holthuis, and P. intermedius Holthuis are marine or estuarine forms. Palaemonetes kadiakensis Rathbun and P. paludogus (Gibbes) inhabit freshwater. The larval development of all five species has been described (Broad, 1957; Broad and Hubschman, 1960, 1962_b; Dobkin, 1962; Faxon, 1879). Experimental and histological data concerning the development of neurosecretory sites and functions in the eyestalks of these larval forms are presented in this paper.

METHODS

Rearing of larvae

The procedure for rearing was essentially the same for both freshwater and marine species and followed that outlined by Broad (1957). Adult, male and female Palaemonetes kadiakensis were collected in and around the Sandusky Bay region of Western Lake Erie during the summer of 1960. This portion of the work was carried out at the Franz Theodore Stone Laboratory at Put in Bay, Ohio. The shrimp were kept in aquaria containing Lake Erie water and fed macerated parts of several local fishes, usually whole emerald shiner, Notropis atherinoides. Ovigerous females were examined daily. When embryos reached a late stage of development, the female was isolated in a culture dish until the eggs hatched. Upon hatching, the entire brood was placed in a clean dish and fed day-old Artemia salina nauplii. The female was allowed to molt and then was preserved in 5 per cent formalin. The larvae were fed and the water changed daily until they reached a desired stage of development at which time they were fixed. The larvae of some broods were fixed all at the same time, while from other broods, samples were removed throughout the developmental period.

Several females produced eggs in the laboratory. By using some of these eggs, a series of embryos was fixed for later sectioning.

Eggs were fixed at two-hour intervals during the first day and twelve-hour intervals thereafter until hatching. Individuals of a select group were fixed at six-hour intervals during the last three days of embryonic development.

Late embryos, larvae, and post-larvae of the other freshwater species P. paludosus, fixed in alcoholic Bouin's solution, were supplied by Mr. Sheldon Dobkin of the Marine Laboratory, University of Miami, Florida.

Rearing and experimental work on larval stages of the marine species were carried out at the Duke University Marine Laboratory, Beaufort, North Carolina. Adult P. vulgaris, P. pugio, and P. intermedius were collected in the tidal areas around Beaufort throughout the summer of 1961. The shrimp were maintained in sea water aquaria on a diet of the soft parts of the mud snail, Nassarius obsoletus, the mussel, Modiolus demissus, and the oyster Crassostrea virginica. Females with eggs in late stages of development were isolated in individual bowls of sea water. After hatching, some larvae were reared in mass culture as above, while those used in experiments were placed in individual bowls (3" Carolina stacking culture dishes), and complete molting and developmental histories were kept. All larvae were fed Artemia nauplii. The sea water in which larvae were reared was adjusted to 30 $\frac{1}{10}$ ‰ salinity by the addition of demineralized water, and was changed every day. At the same time, the larvae were examined and a new supply of food was introduced.

Histological methods

All larval stages of P. vulgaris, P. pugio, P. intermedius, P. kadiakensis, and P. paludosus were sectioned. Preliminary to the study of larval tissues, the eyestalks of adult P. vulgaris, P. pugio, P. intermedius and P. kadiakensis were studied, establishing a basis for the interpretation of developmental processes. Five fixing reagents and five multiple staining procedures were used. As a result, serial sections of approximately five hundred individuals required the preparation of approximately 2500 finished slides.

The fixing reagents used were 5 per cent formalin, formalin-alcohol-acetic acid (FAA or AFA), Zenker's fluid, Helly's fluid, and Smith's alcoholic Bouin's solution (Guyer, 1953). Fixation time varied from 8 to 24 hours depending upon the tissue and reagent. Embryos, larvae, and adult tissues were stored in 70 per cent alcohol. Some tissues were stored in AFA or alcoholic Bouin's solution for as long as one year with no undesirable effects.

Embryos and larvae were fixed by being placed directly into the fixing reagent. At first larvae were anesthetized with a few drops of Urethan, but this was found to be unnecessary. Adult shrimp were fixed in several ways. Most were placed directly into the reagent with no preparatory treatment. In some cases, the carapace was slit to insure rapid penetration of the reagent. Separate eyestalks were washed in crustacean saline to remove blood and then immersed in alcoholic Bouin's solution. Freshly dissected eyestalks were studied by using the methylene blue technique suggested by Bliss and Welsh (1952).

Preparatory to dehydration and imbedding, the larval cuticle was broken to insure penetration of the imbedding medium. A tangential cut was made through the ommatidia at the distal end of the eyestalk. Some thoracic appendages and the posterior portion of the abdomen were also removed. A similiar tangential cut was made through the ommatidia of separate adult eyestalks. These operations were performed under a stereoscopic microscope at 40X.

All tissues to be sectioned were dehydrated in alcohol and cleared in xylene. Embedding was done in summer grade rubber-base paraffin (O.S.U. Reagent Control Laboratory formula), melting point 56-58° C. It was found that tearing of the cuticle during sectioning could be minimized if the larvae were cut in an embedding mass that had been held in an oven, in an open beaker, for several weeks at 61° C. This treatment seemed to drive off the more volatile components and resulted in a medium that was harder at any given melting point. This treatment was relied upon rather than attempting to alter the basic formula of the medium. Serial sections were cut at 6 or 8 micra and mounted on glass slides with Myer's albumin adhesive (Guyer, 1953).

Smith's alcoholic Bouin's fixative was found to produce the most satisfactory results. Problems of overfixation were not encountered, and the cuticle was not rendered brittle, as was the case following Zenker or Helly fixation. The later reagents also produce undesirable background coloration with the stains used. Fixation in formalin or AFA did not always provide good differentiation, but this was corrected

by mordanting the sections in Bouin's fluid during the hydration process.

The most frequently used staining procedure was a modification of an azocarmine-aniline blue-orange G process suggested by Gomori (1939, 1946). This modification has been described elsewhere (Hubschman, 1962) and will be referred to from here on as Azan.

Sections intended for general histological study were also stained in Mallory's triple connective tissue stain or with acid fuchsin followed by the counterstain of the Azan process. Stains that are semi-selective for neurosecretory material have been proposed by a number of workers. Two of these that have been widely used for crustacean tissues were employed here. The first, Gomori's (1941) chromhematoxylin-phloxine, will be referred to hereafter as CHP. The other, Dawson's (1953) modification of Gomori's (1950) aldehyde fuchsin, will be designated PAF.

Photomicrographs of histological sections were made with an A.O. Spencer (Microstar) compound microscope with Orthoilluminator and 35 mm camera attachment. Agfa IFF 13 film was used. Development was in Agfa Rodinal fine grain developer (diluted 1-100) for 13 minutes at 18°C. Line drawings were made with the aid of a camera lucida.

Destalking experiments

Experiments involving eyestalk removal from adult shrimp were primary in the sequence of events that originally established these structures as control centers of metabolic activity. In order to evaluate the role of eyestalks in larval development, a series of

experiments was conducted with zoeal forms of Palaemonetes pugio. A group of sibling larvae (usually an entire brood) was separated into experimental and control groups. Each shrimp was placed in an individual bowl of sea water and assigned a number. Rearing methods were those described in the previous section. Complete molting and developmental records were kept for all larvae. Each day the shrimp were removed with a large-bore pipette and examined under a stereoscopic microscope. Any change in developmental form was recorded and the dish examined for the presence of exuviae.

Eyestalks were removed with a fine blade honed from a number 2 insect pin mounted in a wooden handle. Three or four hours before the operation, the larvae were cooled to 10°C. The eyestalks were then severed at the base and the animals returned to room temperature over a period of two hours. Later, since it was found that the operation performed at room temperature resulted in no higher mortality than was experienced after the cooling process, that step was eliminated. About 75 per cent of the larvae survived the operation.

Two types of control animals were used. The first was unaltered, normal larvae. The second had the antennae removed to allow for possible differences in molting or development resulting from operative stress. Removal of the antennule, while an easy operation, resulted in excessive bleeding due to the relatively large size of the base of that appendage in early zoeal forms. The operated control animals, therefore, had the antennal scale and flagellum removed by cutting through the basal segment (either basipodite or coxopodite). Except for the operation, all larvae were treated in the same manner. The

first zoea hatches from the egg with eyes enclosed in the protozoal carapace and could not be treated as a stalk-eyed form. Experiments were performed on all later larval stages.

Laboratory conditions did not permit precise control over temperature during the experiments. The room used was air-conditioned, but high outside temperatures and equipment operating in other parts of the laboratory placed a burden on the system. Thus during the period of the experiments the mean daily temperature fluctuated between 23.7° and 27.0°C, about an average of 25.5°C. The average daily fluctuation in temperature was 3.47°C, but twice was 6.0°C.

RESULTS

Adult eyestalks

As a basis for comparative study, eyestalks of adult shrimp were examined on living animals, as freshly dissected eyestalks, and in serial histological section. The distribution of secretory cell groups was found to agree closely with that described by previous workers. The eyestalk organs of P. vulgaris were pictured by Hanstrom (1939) in his survey of crustacean central nervous systems. The histology of the eyestalk of Palaemonetes as well as that of Uca was presented in some detail by Milburn (1958). An account of the anatomical aspects of neurosecretory systems in adult Palaemonetes and Uca is apparently in preparation at this time (Milburn, personal communication).

The eyestalk of P. vulgaris, P. pugio, P. intermedius, and P. kadiakensis were found to be essentially alike. Figure 1 is a diagrammatic sketch of the eyestalk of P. kadiakensis. Following the suggestion of Carlisle (1959), the terminology of Russell is used in describing the relationships of the structures discussed here. The sinus gland, shown to be composed of nerve fiber terminations (Bliss & Welsh, 1952), is located on the dorsal, abaxial surface of the ganglionic mass in a crevice between the medulla interna and the medulla externa. It surrounds the junction of the inner and outer

Figure 1. Dorsal view of the right eyestalk of adult Palaemonetes kadiakensis. The muscles, connective tissue, and most ganglionic nuclei have been omitted for clarity. Legend: APS, accessory pigment spot; LG, lamina ganglionaris; ME, medulla externa; MEGX, medulla externa ganglionic X-organ; MI, medulla interna; MIGX, medulla interna ganglionic X-organ; MT, medulla terminalis; MTGX, medulla terminalis ganglionic X-organ; SG, sinus gland; SPX, sensory pore X-organ.

Figure 1

blood sinuses. The X-organ of Hanstrom, the pars distalis or the sensory pore X-organ (referred to hereafter as SPX) occupies a position opposite the sinus gland (Figure 2). It is located between the adaxial, ventral surface of the medulla terminalis and the cuticle. The sensory pore proper is positioned ventrally in the adult eyestalk. There are secretory cell groups associated with each of the ganglionic masses. Beginning at the proximal end of the eyestalk (Figure 1), they are: the group associated with the medulla terminalis usually referred to as the pars ganglionaris or medulla terminalis ganglionic X-organ (MTGX); the group located on the medulla interna, called the sinus gland group or medulla interna ganglionic X-organ (MIGX); the secretory cell mass on the medulla externa, designated the dorsal group or the medulla externa ganglionic X-organ (MEGX).

Two important additional details were observed in fixed and stained preparations. The first of these was the existence of an extremely large, solitary, monopolar secretory neuron. This cell is located on the abaxial surface of the medulla interna and closely associated with, but seemingly not contributory to, the sinus gland tract (Figure 3). Its axon penetrates the fiber mass of the medulla terminalis. This giant neuron has a diameter of approximately 40μ . It has a centrally placed nucleus (diameter 19μ) containing two prominent nucleoli. The cell usually stains blue with Azan and bronze to purple with PAF. This cell is an important feature of the larval eyestalk and will be discussed later.

Figure 2

Diagrammatic section through A-A' of Figure 1.

GM, nuclei of ganglionic mass; IBS, inner blood sinus; M, muscle; MI, *medulla interna*; MT, *medulla terminalis*;

OBS, outer blood sinus; SG, sinus gland; SP, sensory pore; SPX, sensory pore X-organ.

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Figure 3. Frontal section through eyestalk lobes of *Palaemonetes pugio* (Azan).

Legend for figure 3. ME, *medulla externa*; MI, *medulla interna*; MT, *medulla terminalis*; GN, giant neuron; SGT, sinus gland tract.

The second histological feature that can be observed consistently is the vacuolar nature of the lobes of the SPX (Figure 4). Hanstrom (1939) noted that the vacuolar portions appeared empty (except for secretory droplets) in preparations fixed in Bouin's solution but they were filled with a stainable substance following Zenker fixation. (At times there were large accumulations of droplets in the vacuole regardless of fixation). Hanstrom considered these structures to be secretory and attributed differences in appearance to stages in secretory production. Since then, the so-called onion bodies associated with these vacuoles have been shown to be nerve fiber terminations (Carlisle and Knowles, 1959). It seems reasonable to consider the onion body-vacuole portion of the SPX to be a point of release for secretory products.

Larval eyestalks

The histogenesis of neurohaemal structures was essentially the same in P. kadiakensis, P. pugio, P. vulgaris, and P. intermedius. Palaemonetes paludosus, which has an abbreviated larval development (Dobkin, 1962), passed through the same sequence of changes in a shorter period of time.

The incubation period of P. kadiakensis can be expected to range between 24 and 28 days (Broad & Hubschman, 1962). The topography of the definitive eyestalk is recognizable from about the tenth day. Prior to this, the eyestalk region is composed of undifferentiated neuroblasts. With differentiation, the nerve cells have characteristic brilliant red nuclei (following Azan) and very sparse cytoplasm. The

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Figure 4. Section through Sensory Pore X-organ of adult *Palaemonetes pugio* (Azan).

Legend for figure 4. CT, connective tissue; CU, cuticle; EP, epidermal layer; MT, *medulla terminalis*; OB, onion bodies; V, vacuole with secretory droplets.

Figure 5. Sinus gland of adult Palaemonetes kadiakensis.
Compare with Figure 2 for anatomical relations (Azan).

Figure 6. Sensory pore cells of adult Palaemonetes kadiakensis.
Compare with Figure 2 (Azan).

cytoplasm of these cells is confined almost entirely to the axons stretching far from the cell body. The neuropile appears pale purple. Undifferentiated neuroblasts contain cytoplasm that takes up the orange G component of Azan.

About 5 to 6 days before hatching, the structures contributing to the future SPX can be seen. The most obvious feature of this complex is a single cavity or vacuole closely applied to the outer surface of the eyestalk (Figure 7). This cavity contains a very fine filamentous substance, strands of which are near the limit of resolution of the light microscope. This has the appearance of connective tissue and stains blue with Azan. The thickened wall of the cavity is in direct contact with the epidermal layer at this time. Onion bodies can be seen forming in nests of ganglionic cells. These are always more axial than the vacuole and do not contact the surface of the eyestalk. The onion bodies stain blue, but not as strongly as does the vacuolar portion. The vacuole-onion body complex (SPX) is surrounded by undifferentiated neuroblasts and occupies a position at the dorsal surface of the eyestalk. In some preparations a fine connection can be traced from the medulla terminalis to the SPX. It may be that a nerve fiber migrates some distance from the neuropile and then its terminal portion differentiates under the influence of specific cells. This gives the appearance of the nests mentioned above. It could not be determined whether or not the onion body was actually within the cytoplasm of the nest cell.

Figure 7. Section through eyestalk of *Palaemonetes kadiakensis* embryo (Azan).

Legend for figure 7. EP, epidermal layer; MI, *medulla interna*; MT, *medulla terminalis*; OB, onion bodies; OM, developing ommatidia; V, vacuole of SPX.

Just before hatching the onion bodies become more conspicuous. At this time they stain more deeply with aniline blue, but this may be simply because they are larger and more dense. Nerve cells on the surface of the ganglionic mass have begun to assume the elongate shape of sensory cells (Figure 8). The sheath of the vacuole is now clearly cellular. Several onion bodies appear in the lower (axial) portion of the vacuole. At this time there seem to be three membranes present; one around the vacuole, one around the onion bodies, and an outer one covering both.

At hatching (Form I) the vacuole is approximately 15μ in diameter. Individual onion bodies are up to 6 by 8μ in diameter. Mitotic figures are numerous in the ganglionic mass. A presumptive neurosecretory cell appears in the crevice between the medulla terminalis and the medulla interna (Figure 9). This is the giant cell of the sinus gland group. It seems to differentiate from a ganglionic cell rather than from a typical neuroblast. This giant cell changes in staining reaction during the first larval stage. At hatching it stains orange (as do neuroblasts) but, by the first larval molt, the cell appears blue after Azan and changes from pale to deep purple with PAF.

During the second larval stage (Form II) the onion bodies continue to increase in size and number. The SPX is by now a definite organ. It is no longer in contact with the surface of the eyestalk, but is still dorsal in position (Figure 10). At this time very small secretory droplets can be seen in the vacuole. These droplets are barely visible and appear only as dark specks after all stains.

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Figure 8. Section through dorsal surface of embryonic eyestalk (Azan).

Legend for figure 8. EM, egg membranes; PC, protozoal cuticle; OM, developing ommatidia; SP, ganglionic cells elongating in the formation of the SPX.

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Figure 9. Section through the eyestalk of the first larval stage (Form I) of *Palaemonetes intermedius* (Azon).

Legend for figure 9. GN, giant neuron in its primary (ventral) position;
 LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna;
 MT, medulla terminalis.

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Figure 10. Section through the anterior end of the first larval stage (Form I) of *Palaemonetes kadiakensis* (Azan).

Legend for figure 10. LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; SPX, sensory pore X-organ, (vacuole appears in eyestalk at left, onion body clusters can be seen in both eyestalks).

By Form III, the onion bodies become collectively larger than the vacuole. Individual bodies may range up to 11 by 16 μ in size, while the whole organ may be 45 μ in diameter. Neuroblasts around the SPX continue to divide. The vacuole is approximately 18 μ in diameter and contains more droplets. These droplets stain orange with Azan and deep purple with PAF. A layer of ganglionic nuclei has now formed between the SPX and the surface of the eyestalk. Slightly elongate epidermal sensory cells project in a cone to the pore at the surface. The pore is approximately 5 μ wide and 7 μ deep (Figure 11).

The SPX continues to be displaced, by differential growth, from its peripheral location to a site deeper in the ganglionic mass. Throughout larval life, the onion bodies increase in number while a single cavity remains. Eventually, the onion bodies come to lie between the vacuole and the surface of the eyestalk as they do in the adult. The accumulation of secretory droplets becomes continually greater. There is, however, no evidence of secretory activity by cells immediately surrounding the SPX.

By the fifth larval stage, but not earlier, the sinus gland can be recognized. While not a functional complex as yet, an accumulation of nerve fiber endings can be seen at the site of the future junction of the external and internal blood sinuses (Figure 13). The nerve fibers, mostly from the medulla terminalis, form a delicate fan extending over the sinus area. The giant cell is in its definitive position and a number of presumptive neurosecretory cells occupy the site of the future MIGX. This area is ventrally located in the larval eyestalk.

Figure 11. Diagram of Sensory Pore X-organ in the third larval form of Palaemonetes. EP, epidermal cell; MT, medulla terminalis; N₁, nucleus of sensory pore cell; N₂ nucleus of ganglion cell; N₃, nucleus of cell contributing a process to the SPX; OB, onion body; NB, neuroblast; SN, nucleus of sheath cell; SP, sensory pore; V, vacuole of SPX.

Figure 12. Diagram of Sensory Pore X-organ of post-larval Palaemonetes. MT, medulla terminalis; N₁, nucleus of sensory pore cell; N₂, nucleus of ganglionic cell; OB, onion body; SD, secretory droplet; SN, nucleus of sheath cell; V, vacuole.

Figure 11

Figure 12

Figure 13. Sinus Gland forming in fifth larval stage (Form V) of *Palaemonetes pugio* (PAF).

Legend for figure 13. C, cuticle; BS, blood sinus; EP, epidermal-connective tissue layer; ME, *medulla externa*; MI, *medulla interna*; MT, *medulla terminalis*; SG, sinus gland; SGT, sinus gland tract.

The vacuole is a constant feature of the SPX complex from the fifth day before hatching to the definitive adult organ. The adult conformation of the SPX can be recognized in the early post-larval shrimp (Figure 12). At metamorphosis, the eyestalk begins to rotate on its longitudinal axis. The result is, that by the third post-larval molt, the SPX is ventral. This is just the opposite to the embryonic and larval position on the dorsal surface of the ganglionic mass. This rotation in effect results in the maintenance of the orientation of the sensory pore with the substrate, while the rest of the animal assumes the characteristic dorsal side up position of the adult (Figure 15). In effect the shrimp rotates around the fixed eyestalk.

At metamorphosis, the sinus gland develops its definitive shape (Figure 14). No accumulation of secretory material could be detected during larval life. With the onset of postlarval existence (3rd or 4th postlarval molt), however, the sinus gland assumes functional importance. Examination of sections of destalked larvae revealed no build-up of stainable droplets in the brain stubs as shown in adult crabs by Matsumoto (1958). Apparently the migration of neurosecretory substances from the brain and thoracic ganglia does not begin until postlarval life. The ganglionic X-organs of the adult eyestalk are not functional during larval development. Only after metamorphosis, can the cells of the MTGX, MIGX, and the MEGX be recognized histologically. With the procedures used, the secretory activity in these cells can be demonstrated for the first time in shrimp that have completed several postlarval molts.

Figure 14. Compact Sinus Gland of Form VII (first postlarva) of *Palaemonetes pugio* (PAF).

Legend for figure 14. BS, blood sinus; GN, giant neuron; ME, *medulla externa*;
 *MI, *medulla interna*; MT, *medulla terminalis*; SG, sinus gland.

Figure 15

Figure 15 shows the attitude of larval (A) and postlarval (B) shrimp with reference to the relative position of the Sensory Pore X-organ.

Destalking experiment -

In spite of the wealth of information available on decapod larval development, nothing is known of the mechanism of control of crustacean metamorphosis. Passano (1961) has suggested that an unknown hormonal control may exist and called our attention to the need for experiments involving interference with the normal process to support his hypotheses.

Broad (1957) has described seven regular zoeal forms of P. pugio. The sequence of these seven forms, while not invariable, is usually from I to VII with none of the intermediate forms omitted or repeated. Thus, each number represents the form a larva assumes during a given intermolt. (i.e. A third zoea has molted twice and resembles other third zoea. After the next molt, the animal will become a fourth zoea.) The sequence of developmental stages or zoeal forms was identical in destalked animals, those with antennae removed, and unaltered controls. The duration of repeated molt cycles (ecdysis to ecdysis) of 187 larval P. pugio is given in the body of Table 1. These data are derived from daily observations of 133 destalked larvae, 36 from which the antenna had been removed and 18 intact controls. The mean intermolt period varies from 2.3 to 2.5 days, but the maximum duration of the intermolt does not exceed 5 days.

It seems obvious that the measurement of duration of molt cycle should have been made in some unit smaller than days. Palaemonetes larvae, however, molt only at night (usually not before 8:00 or 9:00 P.M.) and observations more frequent than daily ones could add nothing

Table 1

Duration of Intermolt Period in Days of Palaemonetes pugio Larvae Having
Eyestalks Removed, Antenna Removed and Left Intact

Form	Brood	DESTALKED					ANTENNAE REMOVED					INTACT				
		Duration of Molt Cycle in Days					Duration of Molt Cycle in Days					Duration of Molt Cycle in Days				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
III	322	-	26	6	-	-	-	5	12	-	-	-	14	6	-	-
III	325	-	29	14	-	-	-	14	12	-	-	-	-	-	-	-
II	328	-	10	5	-	-	1	6	12	-	-	-	-	-	-	-
V	330	1	13	6	-	-	-	9	6	-	-	-	3	5	-	-
III	333	5	181	77	4	2	-	56	37	2	2	-	46	7	4	2
IV	339	6	87	39	5	-	-	-	-	-	-	-	-	-	-	-
Totals		12	346	147	9	2	1	90	79	2	2	-	63	18	4	2
Means				2.30					2.50					2.48		

of value to the data. The variate, duration of molt cycle, is actually a discontinuous one and properly may be measured in days. Molting data are presented graphically in Figure 16. No significant difference in duration of molt cycle could be observed between experimental and control animals.

Larvae from which eyestalks had been removed metamorphosed between 18 and 30 days after hatching (mean, 20.79 days). Operated control larvae metamorphosed between 20 and 25 days (mean, 22.88 days) and unaltered control metamorphosed between 18 and 22 days (mean 20.33 days). These data are presented graphically in Figure 17. Analysis of variance fails to reveal that these differences are significant.

Growth may properly be separated from development. It was convenient however, to consider the influence of eyestalk ablation on growth of the same groups of larvae studied above. As shown by experiments on adult Cambarus (Smith, 1940), Uca (Abramowitz & Abramowitz, 1940) and Carcinus (Carlisle, 1955), destalking results in increased size. Destalked larvae at metamorphosis had an average over-all length (tip of rostrum to end of telson) of 10.1 mm. Control animals were approximately 7.1 mm at the same stage of development. No differences in average length could be observed between altered and intact control animals.

Adult shrimp differ from their brachyuran relatives in the chromatophore response following eyestalk removal. Crabs exhibit an over-all paling while shrimp darken following the operation. In adult Palaemonetes there is complete dispersal of red pigments following

Frequency distribution of intermolt period of destalked and control larvae of *Palaemonetes pugio*.

Figure 17

Frequency distribution of metamorphoses of destalked and control
larvae of *Palaemonetes pugio*.

removal of the eyestalks (Perkins, 1928). While quantitative aspects of these phenomena were not studied here, it should be noted that eyestalk removal resulted in complete paling of the larval shrimp. The pale condition persisted through subsequent molts and developmental phases through metamorphosis. The immediate condition seemed to result from contraction of chromatophores but possibly was accompanied by loss of pigment in later stages.

It has been observed previously, that removal of eyestalks proximal to the optic ganglia will result in regeneration of an antenna in place of the missing eyestalk in some adult decapods (Bliss, 1960). Regeneration of a short, setose appendage was commonly observed among the larvae destalked in these experiments (Figure 18). No external eyestalk or portion thereof was ever observed to regenerate. Larvae having antenna removed were found to replace this structure quite rapidly. Often the full antenna was present by the time of metamorphosis. In several instances the nerve supply to the regenerated antennule (in eyestalk position) arose from both sides of the brain. Such a crossing-over is shown in Figure 19.

Fingerman and Aoto (1961) have demonstrated in Cambarellus that, after severance of eyestalks, an accumulation of secretory products can be seen in the terminal portion of the remaining stub. This supports the findings of Matsumoto (1958) mentioned earlier. Adult P. kadiakensis were destalked to see if this build-up could be observed in shrimp. The animals were held for six days, then fixed, sectioned, and stained with Azan. An accumulation of red droplets

Figure 18

Destalked postlarva bearing a regenerated antennule of three segments.

29 • NNR •

Figure 19. Section through the anterior end of *Palaemonetes pugio* from which eyestalks had been removed during larval development (Azan).

Legend for Figure 19. B, brain; C, cuticle; EP, epidermal layer; N, nerve fiber tract crossing over and merging with nerve supply of regenerated appendage; R, rostrum; RA, regenerated appendage.

was found in the nerve stubs of destalked shrimp. The optic nerves of normal adults were free of droplets. It should be recalled that no accumulation of stainable material could be detected in the brain stubs of destalked larvae.

29 • NHP •

Figure 20. Sinus Gland of juvenile *Palaemonetes pugio* with accumulation of secretory material (Azan).

Legend for Figure 20. IBS, inner blood sinus; OBS, outer blood sinus; M, muscle; MI, *medulla interna*; SD, secretory droplets; SG, sinus gland.

DISCUSSION

Dahl (1957) in his account of the embryology of the X-organs in Crangon allmanni, states that the first onion body lies directly beneath the integument and is surrounded by cells of the SPX. This is not the case in Palaemonetes. The most prominent feature of the SPX in this shrimp is the sac-like vacuolar portion, which, indeed, does contact the epidermal layer in early development (Figure 7). The onion bodies are always more axial (i.e., proximal to the neuropile) in position and never contact the integument. During larval development, however, these do change in relative position when the onion bodies by-pass the vacuole due to differential growth. By the time the adult stage is reached the vacuole lies between the onion body cluster and the neuropile (Figure 4). While the vacuole may not be present in Crangon, this difference could be explained on the basis of the similarity of staining properties of these structures. The developing vacuole and associated onion bodies both appear blue after Azan, purple after PAF, and gray with CHP. A tangential section of the vacuole near the surface of the eyestalk could be misinterpreted as an onion body.

This sac-like SPX in the larval eyestalk appears more like the X-organ of the Mysidacea than it does the definitive form of adult Palaemonetes. In the Mysids, Eucopia and Boreomysis, the X-organ is

sac-like and situated close to the eye papilli (Hanstrom, 1939). In one (Boreomysis), the large vesicle is filled with intensely stained, winding threads which call to mind the filamentous inclusions of the larval SPX in Palaemonetes. The resemblance of many decapod larvae to the Mysidacea is superficial and probably reflects the pelagic environment (Snodgrass, 1956), but it is interesting that there is internal similarity, at least in one detail.

Other aspects of the developing SPX in Palaemonetes agree more closely with that in Crangon. Cells of the SPX as well as the ganglionic X-organs develop from neuroblasts of the ganglionic layer. As is the case in Crangon, these neuroblasts first assume the appearance of regular nerve cells then mature into the definitive types. Many of the cells of the SPX differentiate during embryonic development and those of the ganglionic X-organs become recognizable only after metamorphosis. The first onion body appears 5 or 6 days before hatching. As Dahl (ibid.) observed, this coincides with the appearance of eye pigments. There is no known significance of this occurrence, other than that it furnishes an outward clue to the state of development. This could be of aid to the experimental embryologist.

Carlisle (1953) has shown that onion bodies in Lysmata are expanded endings of nerve fibers emerging from the medulla terminalis. This appears to be the case in Palaemonetes. A fine connection can be traced from the medulla terminalis to the developing onion body. The end of this nerve fiber appears to differentiate into an onion body while in close association with cells of the ganglionic mass. The developing body is surrounded by nuclei that seem to participate

in the maturation process. This gives the appearance of the rosette or nest mentioned earlier. Since the nerve tract connecting the onion body cluster with the neuropile is not visible until the onion bodies form, the direction of growth cannot be stated with certainty. There is no reason, however, to believe that the nest cells send out the processes in the direction of the medulla terminalis.

The remainder of the SPX, including the terminal sensory pore portion, is derived from cells of the nerve ganglion. There is no evidence to support the view that the sensory pore complex is derived from epidermal cells. Late in embryonic development, the sensory portion develops from ganglion cells that elongate within the ganglionic mass (Figure 8).

Throughout embryonic development and larval life, the cells of the future ganglionic X-organs fail to show any activity with CHP or PAF. The single exception to this is the giant cell located peripheral to the MIGX, close to the sinus gland tract. This cell apparently becomes active early in larval life. During the first phase of free-living existence (Form I), this cell stains deeply with aniline blue and appears purple with PAF (Figure 9). Since this is the only cell that seems to be active during larval life, and its activity slightly precedes the accumulation of droplets in the SPX, it may well be the source of this stainable material. Admittedly, the CHP and PAF techniques are selective rather than specific for neurosecretion (Bliss, Durand, & Welsh, 1954), but previous work has shown physiological activity to be associated with the products of cells demonstrated with these processes (Passano, 1951_a, 1951_b, 1954). Carlisle (1954) has

reported the movement of droplets from the ganglionic X-organs to the SPX. He suggests that the SPX is a point of storage and release for neurosecretory products. This is probably true of Palaemonetes. Since there was no accumulation of stainable material at the stubs of the brain in destalked larvae, the droplets found in the vacuole of the SPX probably were produced within the eyestalk. In adult eyestalks (Figure 3), the axon of the giant cell can be seen to enter the neuropile of the medulla terminalis and is not directly incorporated into the sinus gland tract. The fibers could not be traced out of the neuropile.

Pyle (1943) described the position of the X-organ (SPX) in embryos of Pinnotheres and Homarus, as that which will become the median ventral side of the eyestalk in the first zoea. This is very different from what I found in Palaemonetes. The SPX develops embryologically in that portion of the eyestalk that becomes dorsal in the free-swimming larva. It remains dorsal throughout larval life, becoming ventrally located during the changes associated with metamorphosis. At metamorphosis the eyestalk rotates on its longitudinal axis, the dorsal surface becomes ventral and the ventral surface becomes dorsal. All internal structures retain a constant relation to each other. It is interesting that shrimp zoea swim upside down and backwards during larval life. At metamorphosis, the animal assumes the dorsal side up position of the adult. The sensory pore remains oriented toward the substrate throughout this change in attitude (Figure 15). A functional difference between dorsal and ventral surfaces of the eyestalk was hinted at by Keeble and Gamble (1904), but their experiments involving changes in direction of illumination failed to support this idea.

From the structural complexity of the SPX, especially in adult shrimp, it is difficult to believe that the so-called sensory pore does not have functional importance (Figures 4 and 6).

The sinus gland cannot be seen in Palaemonetes until the fifth larval stage (Form V). Between this and the Form VI larva (final), the nerve fiber endings comprising the sinus gland become increasingly compact. Staining reactions indicate that the structure does not become functional until post larval life. Pyle (ibid.) found that the sinus gland was not recognizable until the third stage after hatching in Homarus americanus. By the fourth stage the sinus gland had increased in size but still failed to show the brilliant stain of the adult structure. Morphologically the fourth stage in Homarus is a post-larva in spite of its continued pelagic existence (Herrick, 1895). The activity of the sinus gland (i.e. storage and release of neurosecretory products), therefore, seems to be limited to postlarval life. After metamorphosis, internal development can no longer be related to external morphology. In Palaemonetes the sinus gland appears to be functional after the second or third postlarval molt (Figure 20). Because of rearing difficulties, Pyle was not able to study the development of Pinnotherea larvae. He does state, however, that the sinus gland was not evident in late embryos or in the first zoeal stage.

Broch (1960) reported that all larval stages of P. vulgaris, from hatching through the "8th zoeal stage", responded to background coloration with changes in chromatophores. He found that the chromatophores completely contracted over a white background and were fully

expanded upon the addition of abdominal nerve cord extract, and contracted when eyestalk extract was added. It was not stated that the tissue extracts were of adult origin. Broch concluded that "endocrine control of chromatophores in the zoea of P. vulgaris is comparable to that of the adult."

Perkins (1928) showed that eyestalk removal resulted in dispersal of red chromatophores in adult Palaemonetes. Preliminary experiments with P. pugio larvae indicate that the response to eyestalk removal in larvae is opposite to that in the adult. Destalked larvae become pale following the operation, and the pale condition persisted through metamorphosis. This reaction, opposite or otherwise, indicates that there is some influence exerted on the chromatophore system by larval eyestalks.

Costlow (1961) has reported finding a Uca black dispersing substance in the eyestalk extracts obtained from zoea of Sesarma reticulatum. The chromatophore dispersing substance fluctuates in total activity during the molt cycle. Recognizing the possibility of different hormones controlling color change and molting, Costlow suggests the following hypotheses:

- 1) The Uca black-dispersing substance located within the eyestalks of the larvae fluctuates because of changes in titre associated with moulting but is not directly responsible for, nor involved in, the actual moulting process, or 2) if the endocrine organs within the eyestalks of the larval stages are responsible for production or storage of several hormones, including the moult-inhibiting hormone, the reduction of the titre of the Uca black-dispersing substance may reflect the reduction in titre of the moult-inhibiting hormone also.

Results of the destalking experiment described above suggest that chromatophores and molting are under separate control in the larvae of Palaemonetes. Eyestalk removal definitely affected the chromatophores (contracted) of the larvae, while the operation seemed to have no influence on the frequency of molting.

The molt-inhibiting hormone has been related to the X-organ-sinus gland complex in all decapods studied (Carlisle & Knowles, 1959). Considering the histological evidence for lack of activity in the ganglionic X-organs and the absence of a functional sinus gland during larval life, the failure of eyestalk loss to interfere with molting frequency does not seem unreasonable. If a molt-inhibiting hormone is present in larval shrimp it is probably produced somewhere other than in the eyestalks. Possibly the molt cycle in the larva is uninhibited. The rapid cycle (2 day intermolt) may go on continually as a function of the production of molting hormone by a larval molting gland, if one exists. The duration of proecdysis (Drach's stage D) in adult natantians represents 60% per cent of the molt cycle (Passano, 1960). Morphological details of new spine formation suggest that the proecdysal period may be almost continuous, or at least longer relative duration, during larval development (Broad & Hubschman, 1962_a).

The accumulation of droplets in the larval SPX may represent the storage and/or release of chromatophorotropic hormone. The deprivation of such a substance (or one component) results in the over-all paling in larval shrimp. A similiar material in the eyestalks of Sesarma larvae may be the Uca black-dispersing substance demonstrated by

Costlow. It is noteworthy that Sesarma is unique among crabs since it darkens in response to eyestalk removal (Enami, 1951_a, Prosser & Brown, 1961). This behavior is like that of adult Palaemonetes and opposite to the reaction in all other brachyurans. Carlisle (1955) has shown that molt inhibition and control of water balance are effected by separate hormones in Carcinus. If the relatively large size of destalked larvae is a result of increased water uptake, the control of these processes would seem to be separate in Palaemonetes larvae as well. The destalked larvae at metamorphosis were approximately 50 per cent larger than the control animals. There was no difference in the duration of the intermolt periods between these groups. This suggests another possible factor attributable to the secretory products in the SPX.

SUMMARY

1. The histogenesis of eyestalk organs in Palaemonetes vulgaris, P. pugio, P. intermedius, and P. kadiakensis is essentially the same. Palaemonetes paludosus, having an abbreviated larval development, follows the same sequence of events in a shorter period of time.

2. The developing eyestalk of Palaemonetes has two consistent, characteristic features: a.) a single, large, monopolar secretory neuron that becomes active during the first larval stage, and b.) a sac-like vacuole in the sensory pore X-organ, that appears to be the site of storage and release of secretory products, from the first larval stage through adult life.

3. The sensory pore X-organ develops from neuroblasts of the eyestalk ganglia. It appears five or six days before hatching. It is located dorsally and remains in that position throughout larval development. Secretory droplets appear in the SPX vacuole during the first larval stage. The accumulation of droplets increases throughout larval life.

4. The sinus gland is not recognizable until the fifth larval stage. It apparently does not function as a neurohaemal organ until after metamorphosis.

5. At metamorphosis, the eyestalk rotates on its longitudinal axis. The dorsal surface becomes ventral and the ventral surface

becomes dorsal. Since the animal turns over at this time, the orientation of the sensory pore remains constant with relation to the substrate.

6. Eyestalk removal has no effect on the larval molt cycle.

7. Eyestalk removal has no effect on metamorphosis.

8. Eyestalk removal results in complete paling of larval Palaemonetes. The pale condition persists throughout larval development and metamorphosis.

9. There is no accumulation of secretory material in the brain stubs of destalked larvae. It is concluded that the droplets found in the SPX originate within the eyestalk.

10. It is suggested that the giant neuron is the source of stainable droplets found in the larval SPX.

11. The ganglionic X-organs are not functional during larval development.

12. The endocrine systems operating in Palaemonetes larvae differ in several respects from those of the adult.

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AUTOBIOGRAPHY

I, Jerry Henry Hubschman, was born in Great Neck, New York, on February 4, 1929. I received my elementary and secondary school education in the public schools of Great Neck. In 1956, I graduated from The State University Agricultural and Technical Institute at Farmingdale, New York, with a major in animal husbandry. I received undergraduate training at The Ohio State University which granted me the Bachelor of Science in Agriculture degree in March of 1959. At that time I entered the Graduate School of The Ohio State University as a student in the Department of Zoology and Entomology. I held a graduate assistantship in this department from 1959 through 1961. I was awarded a National Science Foundation Teaching Assistant Fellowship for the summers of 1960 and 1961. During the year 1961-62, I was a National Science Foundation Cooperative Fellow in this department.

I have accepted a position as Research Biologist with the United States Public Health Service at the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.