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SENSORY STRUCTURES AND BEHAVIOR IN OPISTHOBRANCH VELIGER LARVAE

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F.-S. Chia and R. Koss (1991) Sensory structures and behavior in opisthobranch veliger larvae. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 455-484. In opisthobranch molluscan larvae, certain behaviors or changes in behavior, can often be associated with the development of identifiable larval sensory structures. For example, planktotrophic larvae possess the ability to swim, sink, withdraw into the shell and feed upon hatching. All these behaviors most probably involve receptors which are integrated into the nervous system and process sensory information. Swimming and sinking behavior may involve the statocysts, while retraction and selection of food particles during feeding may involve the cephalic sensory organ. Larval defence, *i.e.*, sinking or withdrawal into the shell, can be accomplished by retracting the cephalo-pedal complex into the larval shell and covering the opening with the operculum; this behavior could involve the cephalic sensory organ. Therefore, sensory structures such as the statocysts, cephalic sensoy organ, and foot are obvious, morphologically recognizable, and well-developed before the veliger is liberated from the egg mass. Morphogensis of the eyes precedes the onset of negative phototaxis within the latter part of larval life, enabling downward migration of larvae for locating an appropriate substratum for settlement. Toward the termination of the larval phase, the propodium of most species develops as a structural and functional marker that indicates the larva is capable metamorphosis (metamorphic competence) because it creates the crawling surface of the gastropod foot. Advanced veligers of certain species also settle and metamorphose only in response to chemical(s) within the adult habitat; this capacity may require the histogenesis of specialized sensory organs or tissues which is timed to enable the larva to terminate its pelagic existence. For example, competent larvae of the nudibranchs, *Rostanga pulchra* and *Onchidoris bilamellata*, respond to chemical cues related to the adult prey species. In *Rostanga*, rhinophores develop just prior to metamorphosis, and are probably utilized in substratum selection during settlement since larvae are incapable of metamorphosis before these structures differentiate. The propodium of *Onchidoris* veligers is elaborated into peripheral

ganglia which are suspected to be involved in settlement by perceiving environmental cues, and then triggering the behavioral responses of settlement. Crawling behavior or settlement may involve other sensory receptors located elsewhere in the foot suggesting that the foot generally be considered an sensory structure which universal to all gastropod larvae. Metamorphosis, transforming a larva into a juvenile, involves complex behavior which is likely initiated be a number of different sensory receptors.

Key words: Larva, Chemosensory mechanosenory organs, Behavior, Mollusca, Nudibranchiata.

Larval behavior plays as important role in the success of recruitment of marine benthos and colonization of substrata. This is especially true of invertebrates with life history patterns involving long-term planktonic larval periods. In many species of opisthobranch molluscs, larvae develop within and hatch out of an egg mass as motile pelagic veligers. They may spend lengthy obligatory periods (35 days or more) within the plankton before seettlement, since initially they are neither morphologically nor neurologically capable of undergoing the transformation from a pelagic larva to a benthic juvenile. Veliger larvae must undergo somatic growth and differentiation to be capable of enacting settlement and metamorphosis and assume the benthic life style. The energy for these processes is derived from the plankton and thus larvae must differentiate new or prospective adult structures, designed for a benthic existence, while maintaining those which effect feeding and swimming which are attributed to the larva. The ontogenetic changes in larval behavior of many opisthobranch molluscs are associated with the sequential development of larval sensory structures which are adapted for larval or adult survival. For example, larval swimming may involve the statocysts, while larval defence (sinking, retraction into the shell) and selection of food particles may involve the cephalic sensory organ. Therefore these structures have differentiated before hatching. The appearance of eyes precedes the onset of negative phototaxis, or toward the latter part of larval life, enabling downward migration of larvae for locating an appropriate substratum for settlement. Advanced veligers of certain species settle and metamorphose in response to chemical(s) within the adult habitat.

In the nudibranchs, *Rostanga pulchra* and *Onchidoris bilamellata*, competent larvae respond to chemical cues related to the adult prey. In *Rostanga*, rhinophores develop prior to settlement, and are probably utilized in substratum selection since larvae are incapable of metamorphosis before this event. The propodium in both species develops as a structural marker, indicating metamorphic competence; it creates the crawling surface utilized by the larva after settlement. The propodium of *Onchidoris* veligers contains unique ganglia which are thought to be involved in settlement by perceiving environmental cues, and then triggering the behavioral responses of settlement. Here, settlement will refer to behavioral events which may be reversible, whereas metamorphosis will include those events which are structural and irreversible.

It is thought that the larval nervous system is responsible for the perception of external stimuli, including naturally occurring chemical inducers associated with settlement and metamorphosis (reviewed by Hadfield and Pennington, 1990; Morse, 1990). Although all the structures listed above have been implicated in larval sensory functions, there is little direct evidence of their nervous activity, and the underlying mechanisms remain poorly understood. Diminutive larval size, lack of reliable sensory organ identification, and poor accessibility for electrophysiological study have precluded the elucidation of cellular mechanisms controlling settlement and metamorphosis of marine invertebrate larvae in general. To date, chemoreceptive structures and sensory cells involved in the transduction of settlement cues, have been directly demonstrated for only a single species. The anterolateral ganglia of *Onchidoris bilamellata* have been shown to be functional nervous entities which respond to known settlement cues with low amplitude depolaizations. Recently we have further developed this system whereby the sensory neurons and interneurons of these ganaglia can be isolated, allowing for the possibility of electrophysiological characterization of the receptor cells. The techiques utilized in excising, dissociating, culturing, and identifying these cells will be presented. Presently we are undertaking voltage clamp, second messenger and immunocytochemical experiments to establish the cellular mechanisms

underlying larval settlement. Hopefully, this will contribute a better link between the physiological, developmental and ecologial aspects of larval settlement and metamorphosis.

Metamorphosis, or transforming a larva into a juvenile, also involves complex behavior which is likely initiated be the nervous system and a number of different sensory receptors. This paper affirms the ontogenetic relationship between larval behavior and sensory receptors in opisthobranch molluscs and we will primarily make reference to our own morphological and behavioral studies as they relate to settlement in the nudibranch species, *Rostanga pulchra* and *Onchidoris bilamellata*. New informating regarding the differentiation and involvement of the larval foot in controlling behavioral changes that contribute to metamorphosis will be described and future directions for research on opisthobranch settlement and metamorphosis will be discussed.

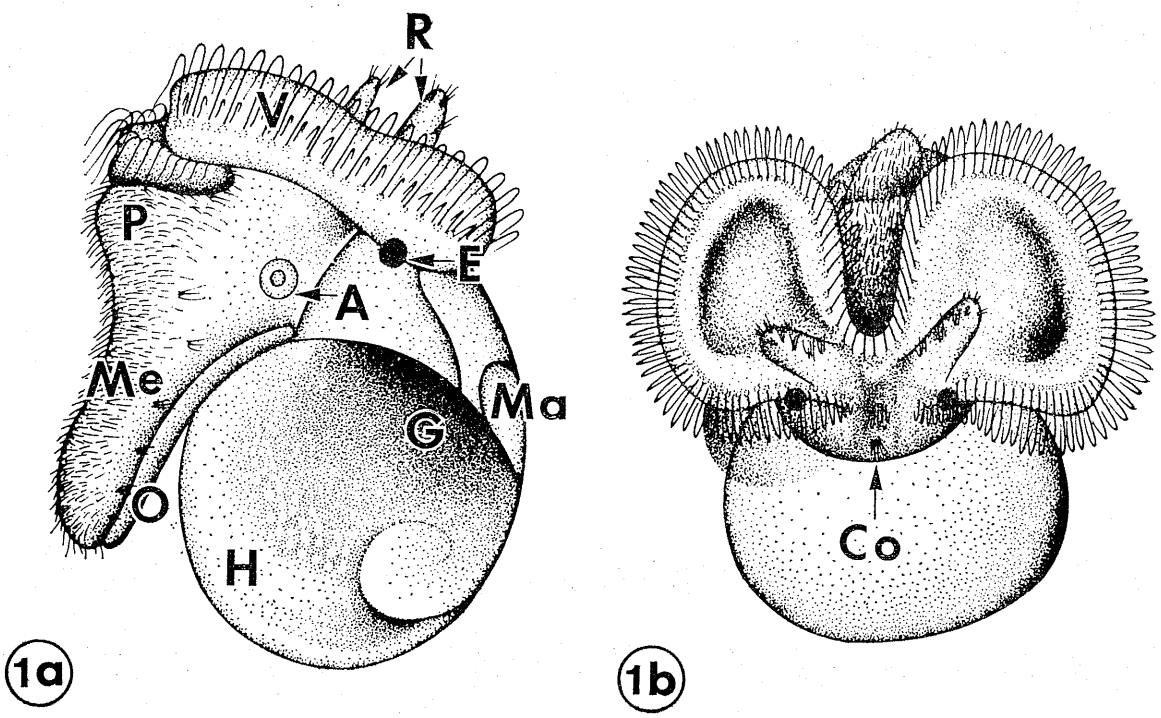
MORPHOGENETIC CHANGES DURING PLANKTOTROPHIC DEVELOPMENT

The life cycles of many temperate opisthobranchs include a long-term planktotrophic veliger larval stage. Typically, in *R. pulchra* and *O. bilamellata*, embryonic development occurs within an egg capsule encased by a gelatinous matrix which is housed in a larger spiral egg mass. Both species of prehatching veligers are formed at one to three weeks before being liberated from the egg mass. Both prehatching and posthatching larvae possess a protective shell which is coiled (refer to Hurst, 1967) and possesses an articulating operculum. The veliger is into two tissue groups; the cephalo-pedal and visceropallial masses. The cephalopedal mass contains the bilobed velum which is composed of two rows of cilia: the postoral band (large multiciliated cells) which provides the propulsive locomotory force for swimming, and a subvelar or preoral band which is involved in feeding (Bonar, 1978). At this stage the foot is recognizable as a metapodium, a small flattened triangular structure with several small ciliary tufts located along its lateral margins. The cephalo-pedal mass can be withdrawn into the shell by contraction of the retractor muscle attached to the left side of the head-foot and the operculum; the operculum seals the opening of

the shell upon retraction. Throughout the duration of larval development, the larva is capable of retracting into the shell. The visceropallial mass is composed of the digestive tract, the mantle, the perivisceral membrane and the retractor muscle. The veliger's digestive system is composed of a mouth located centrally between the velar lobes, an esophagus, a stomach, a right digestive gland (left digestive gland is present but reduced), an intestine and an anus. The digestive tract loops within the shell and exits at the right side of the larval foot.

Development of newly hatched veligers proceeds in a progressive sequence according to a predictable pattern (reviewed by Hadfield and Switzer-Dunlap, 1984). The larval period stops when the herbivorous larva assumes a carnivorous diet within the adult habitat through metamorphosis. The duration of larval life is species specfic and can be modified by water temperature, food availability, and availability of a suitable substratum for settlement. The following description is a brief outline on the obvious structural changes occurring during development, although we will not review the histological or ultrastructural changes of the gonadal rudiments, kidney complex, mantle fold, muscle systems, or alimentary tract (for a detailed description refer to Bickell and Chia, 1979). Under laboratory conditions, the pelagic period for reaching metamorphic competence for both *R. pulchra*, is approximately 30-40 days at 10-12°C (Chia and Koss, 1978, 1988), and shell growth doubles from 150 μm to 300 or 320 μm. The right digestive gland is transparent at hatching, but grows considerably larger and becomes darkly pigmented with age; accumulations of lipid droplets represent the food reservoir of the larva to be utilized during or after metamorphosis. In *R. pulchra* and *O. bilamellata* the eyespots become faintly visible after about two weeks, and become progressively more obvious and pigmented after three weeks.

A flap of tissue, representing the larval heart differentiates on the dorsal side of the cephalic region, inside the mantle cavity. The heart undulates back and forth in the mantle cavity. Four weeks after hatching the mantle thickens and retracts from the dorsal rim of the shell; this marks the end of shell deposition and growth. At the same time the proximal portion of the foot (metapodium) thickens into a



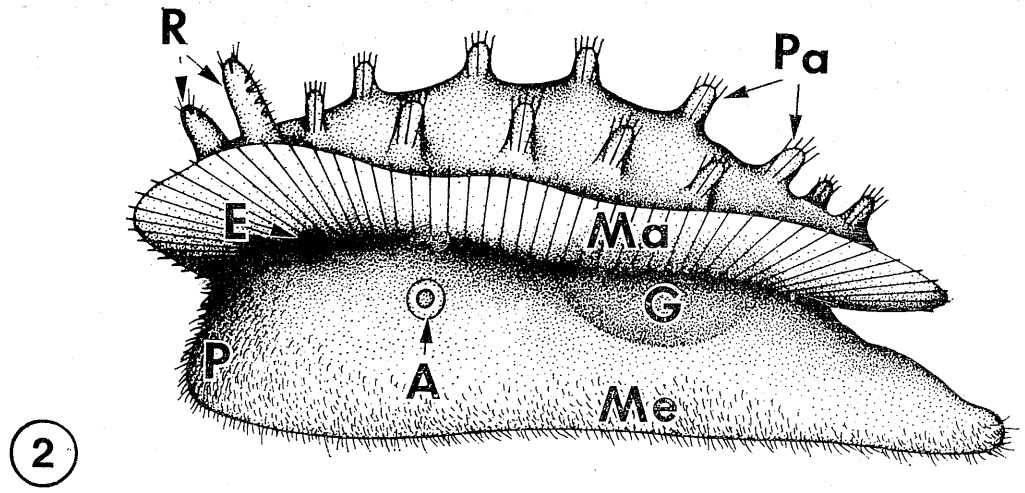


Fig. 1. a. Diagram of competent veliger of *Rostanga* indicating the propodium (P), metapodium (Me), statocyst (A), eye (E), velum (V), operculum (O), shell (H) and digestive gland (G). b. Dorsal view showing cephalic sensory organ (Co).

Fig. 2. Diagram of a *Rostanga* juvenile, 24 h after metamorphosis, showing the incorporation of the digestive gland (G) and the relocation of the rhinophores (R). The mantle (Ma), papillae (Pa) on the notum, eye (E), statocyst (A), propodium (P) and metapodium (Me) are also noted.

bump, representing the propodium. The propodium provides a convenient indicator in discerning that the larva now has the ability to settle and metamorphose; it is referred to as being metamorphically competent (Fig. 1). The development of the propodium establishes the crawling surface for the larva, the juvenile and the adult. Settlement and metamorphosis can not proceed without the differentiation of this structure. Concomitantly, specialized structures may differentiate and dictate whether the larva is capable of metamorphosis. Such structures may be as unique as the rhinophores in *R. pulchra*, or the propodial ganglia in *O. bilamellata* (Fig. 7).

Settlement and metamorphosis of nudibranchs often involves the selection of a site in the adult habitat, and is associated with the food of the adult. For example, the larvae of *R. pulchra* can metamorphose only in the presence of the sponge, *Ophilitaspongia pennata* (Chia and Koss, 1978), and the larvae of *O. bilamellata* can settle in the presence of living barnacles, but metamorphosis can only take place upon contact with the barnacle (Todd, 1981; Chia and Koss, 1988). Metamorphosis involves the loss of the velar cilia and the reabsorption of the velar lobes. The shell is cast off by separating from the retractor muscle, and the mantle is reflected back upon exiting the shell. The operculum is shed and the visceral mass composed of the digestive gland and the stomach, is eventally incorporated into the posterodorsal portion of the foot (Fig. 2). The mantle grows posteriorly to cover the digestive gland. Detorsion occurs at the time of shell exit.

LARVAL BEHAVIOR

Prehatched veligers are capable of swimming through velar activity, and spin around within the egg capsule, toward the latter phase of intracapsular development. Encapsulated veligers were observed to be capable of stopping ciliary activity and withdrawing the velum and cephalo-pedal mass into the shell. Because of the organization of the larval body, the velum of hatched veligers faces upward while the visceral mass and shell are located below. When the velum and foot are protracted out of the shell, activity of the preoral cilia naturally produces upward swimming, although by adjusting the relative positions of the velar lobes,

larvae are also capable of swimming horizontally and turning. The position of the velar lobes appears to be controlled by subepidermal muscle fibers.

Because the density of the larva is greater than that of seawater, veligers sink when the preoral cilia are arrested. The sinking response is not synonymous with withdrawal into the shell, as larvae can determine a particular level in the water column by sinking or swimming. Sinking involves either folding the velar lobes upward onto themselves or shrinking the parimeter of entire velum through muscle constriction; postoral cilia are arrested during these bouts. Both sinking and retraction into the shell may be avoidance behavior or a form of larval defence. Sinking can be triggered by light, or at metamorphic competence, by a chemical inducer; withdrawal into the shell occurs after mechanical disturbance.

Upon liberation from the egg mass, many species of opisthobranch larvae swim upwards. This movement is generally considered negative geotaxis (Hadfield and Switzer-Dunlap, 1984). Eyes do not appear until about two or three weeks post-hatching in veligers of *R. pulchra* and *O. bilamellata*. Within this time frame, and throughout the remainder of the larval period, most laboratory-cultured veligers accumulate and remain close to the bottom of the culture vessels where swimming and feeding continues. Such changes may be the result of a reversal of phototactic or geotactic behavior. This overall shift in behavior would ultimately have an impact on larval swimming patterns, probably bringing them closer to acceptable substrata for settlement and metamorphosis. In both *R. pulchra* and *O. bilamellata* the environmental inducing cues are located within intertidal to subtidal regions, with reduced light levels,

Full development of the larval foot, which involves differentiation of the propodium, provides the advanced larvae with the ability to crawl. Crawling behavior is assumed at the onset of settlement and involves creeping with the pedal sole cilia. In *O. bilamellata*, crawling also can occur when induced by the brushing action of water when individual larvae were pipetted from one vessel to another. This observation suggests that water turbulence may contribute to the expression of crawling behavior.

In *O. bilamellata*, upon recognition of a chemical cue associated

with barnacles, competent larvae were observed to cease swimming and settle to the bottom of culture vessels. Automatically the foot begins rhythmic contortions and crawling commences; crawling is in an anterior direction and the velum remains folded and partially withdrawn into the shell. During bouts of crawling, the position of the velum alternates from retraction to protraction. Crawling continues if a barnacle shell is contacted, with metamorphosis following. If a barnacle is not encountered, crawling stops and the larva will again resume swimming in the water column, or lying on its side on the bottom of the culture vessel.

LARVAL SENSORY STRUCTURES

In larval opisthobranchs, the inferred involvement of the nervous system in behavior, especially in settlement and metamorphosis, has received considerable attention (reviewed by Pawlik and Hadfield, 1990). The nervous system develops sequentially along with the structures which it innervates (Kriegstein, 1977; Bickell and Chia, 1979). Therefore, it is not unreasonable to suggest that the development of sensory structures can therefore be correlated to the important changes in larval behavior. Many behaviors in adult opisthobranchs have been show to be directed by the peripheral nervous system which involve sensory structures (Alkon, 1984); most of these structures are present in the larva form and there is no reason to believe they are not functional at this level in the life cycle.

In the planktonic larvae of *R. pulchra* and *O. bilamellata*, a pair of cerebral ganglia is present at hatching. The cerebral ganglia are presumably capable of directing larval behaviors such as swimming and feeding, capacities which young larvae possess. Both of these fundamental behavioral processes are controlled by the action of the velum. Velar activity is under nervous control (Arkett *et al.*, 1987; Barlow, 1990), and has been shown to be under cerebral ganglionic in other gastropod veliger larvae (Mackie *et al.*, 1976). Concomitantly the associated sensory structures present at hatching are a pair of statocysts and the cephalic sensory organ; both of which are innervated by the cerebral ganglia (Chia *et al.*, 1981; Chia and Koss, 1984). The statocysts have been shown to be

responsive to gravity in adults, and in larvae are probably associated with initial negative geotactic response of newly veligers. The cephalic sensory organ may be involved in feeding or in larval defence.

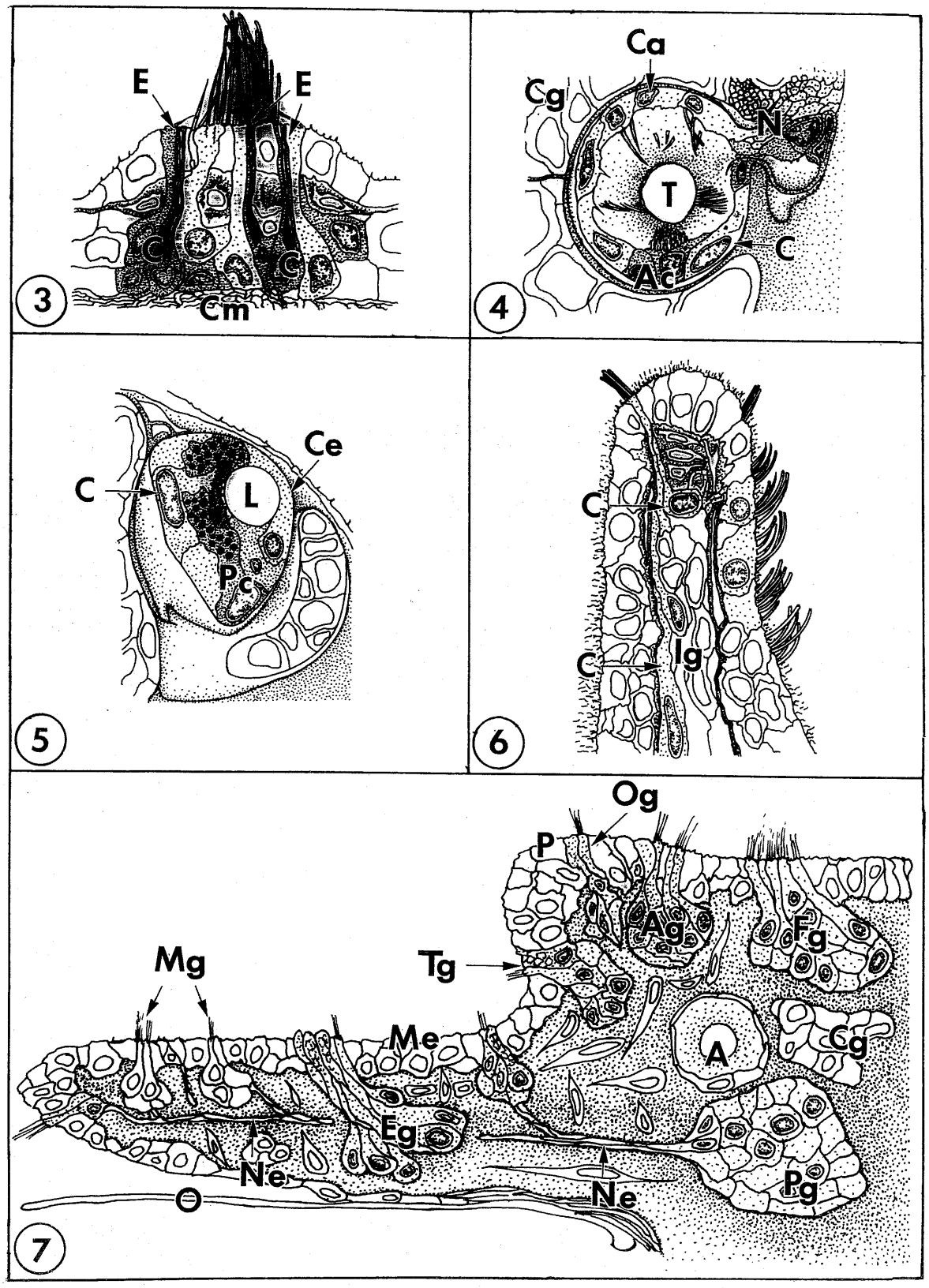
The statocysts of *R. pulchra* are hollow fluid-filled capsules, 15 to 20 μm in diameter, located on opposite sides at the base of the larval foot (Fig. 4). They remain unchanged throughout larval life. Each statocyst contains a single large concretion and several smaller ones which roll around internally to provide the directional stimulation enabling gravity detection. The statocyst capsule is composed of hair cells or sensory cells and accessory cells. Sensory cells are of two types and may respond to different types of stimuli. The first type is characterized by cilia being positioned randomly on the roof of the statocyst and may be sensitive to multidirectional stimuli. The second type is confined to the base of the statocyst and contains cilia arranged at regular intervals in a curved row which may be receptive to only bi-directional stimuli. The distribution of weight indicates that throughout larval life, the heavy larval shell orients the veliger in a normal swimming position with the velum pointing upward. Swimming would then be monitored by the polarized sensory cells encompassing the base of the statocyst and may be sensitive to tilting of the body or upward motion. However, because there are only a few receptor cells, detection of small displacements may not occur and position sense is probably crude. Sinking may be controlled by cilia on the roof of the statocyst. The sinking orientation approximates the swimming orientation, although locomotory cilia of the velar lobes are arrested. When sinking, the velum and foot are folded into a retracted state and the statolith would be forced onto the cilia of cells on the statocyst roof. These same cells may indicate turbulence to the larva and initiate or maintain the retracted condition.

The cephalic sensory organ was discovered in the larva of the nudibranch *Phestilla sibogae* by Bonar (1978), who suggested it was involved in chemoreception at settlement and metamorphosis. However, in *R. pulchra* and *O. bilamellata* this organ is present at hatching, possibly indicating that this structure may function in ways other than substratum selection, as larvae are

incapable of these events at this stage. This structure was observed in other nudibranch species at hatching as well (Bickell and Kempf, 1983). In *R. pulchra* and *O. bilamellata*, the cephalic sensory organ can be recognized externally as a tuft of cilia positioned dorsally between the velar lobes (Fig. 1). It is innervated by the cerebral ganglia, and is composed of three morphologically different sensory receptors with cell bodies located in the cerebral commissure (Fig. 3). Their dendrites which passed through the epidermis to terminate externally (Chia and Koss, 1984). The cephalic sensory organ may also be utilized in feeding (Chia and Koss, 1984) as it is in direct line with the ciliary band on the velum which carries food particles to the mouth (Thompson, 1959). Water currents initiated through velar activity, may bring food items in contact with the organ. Here choices regarding acceptance or rejection of food particles can be made on the basis of taste, size, weight, etc.

Throughout the larval period, veligers are capable of defensive behavior by retracting into their shell and closing the aperture with the operculum (Garstang, 1928). An escape response, in the form of sinking, may also involve the cephalic sensory organ, in that it may provide information regarding the degree of folding of the velum. The lobes of the velum fold upward and inward, toward the midline when the larva is disturbed. Disturbances, such a tapping the culture vessel with a glass rod, do not always produce withdrawal into the shell. The velar lobes, when folded in this manner, would be pressing against the cilia of cephalic sensory organ. The cephalic sensory organ may provide the veliger with information initiating retraction. It may also involve monitoring the animal's position inside the shell and this can be accomplished by brushing against the shell aperture with the sensory cilia. Foot receptors, located along the edge of the foot or just under the apex, may also be involved in defensive behavior.

At approximately two weeks after hatching, the optic ganglia and eyes begin differentiating (Fig. 5). Presumably, progressive development of the negative phototaxis, or a ligh-induced positive geotactic response, in *R. pulchra* is associated with differentiation of the larval eyes (Chia and Koss, 1978, 1983). The eyes are clearly visible as darkly pigmented spots located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at the base of the velar lobes. They are structured as simple cups composed of sensory cells and pigmented cells (Chia and Koss, 1983). Microvilli, or the receptive surface, project into and fill the space behind the lens. A lens of heterogeneous consistency occupies the opening to the cup end a single corneal cell covers its dorsal surface. Because the eyes are small and there is virtually no cavity behind the lens, it is doubtful that they are capable of focusing or image formatting. Instead veliger eyes are probably responsive to light intensity or possess a sensitivity to certain wavelengths. This perception can initiate the behavior that brings the veligers close to the substratum at settlement.

When comparing the advanced larvae of different opisthobranch species, especially those with a lengthy obligatory planktotrophic phase, it becomes apparent that certain species develop unique structures which can be considered ontogenetic preparation for the expected changes at settlement and metamorphosis (Bickell and Kempf, 1983). The attainment of metamorphic competence may revolve around the development of the nervous system, and more specifically, the differentiation of sensory structures and their respective ganglia.

The importance of the larval nervous system in the perception of

Fig. 3. Drawing of the cephalic sensory organ of *Rostanga* showing sensory cells (C) with cell bodies located within the cerebral commissure (Cm), and dendrites (E) emerging ciliated and nonciliated clusters on the external surface.

Fig. 4. Drawing of the left statocyst of *Rostanga* showing two types of sensory cells (C and Ca), accessory cells (Ac), statolith (T), and static nerve (N) joining into the cerebral ganglion (Cg).

Fig. 5. Drawing of the left eye of *Rostanga* with sensory cells (C), pigment cells (Pc), corneal cell (Ce) and lens (L).

Fig. 6. Drawing of the rhinophore of *Rostanga* showing the rhinophoral ganglion (Ig) with sensory cells (C) extending ciliated dendritic endings through its tip.

Fig. 7. Drawing of the left foot region, in sagittal view, of a competent *Onchidoris* veliger showing the propodium (P), metapodium (Me), statocyst (A), operculum (O), frontal ganglion (Fg), anterolateral ganglion (Ag), pedal ganglion (Pg) and cerebral ganglion (Cg). The pedal nerve (N) originating from the pedal ganglion, innervates the accessory metapodial glands (Mg), the large propodial (Og), pedal groove (Eg) and metapodial (Tg) glands. All the subepidermal multicellular glands contain a complement of secretory and sensory cells.

the chemical factors which induce settlement and metamorphosis has received considerable attention, especially in abalone larvae (Morse *et al.*, 1980; Baloun and Morse, 1984; Trapido-Rosenthal and Morse, 1986; Barlow, 1990; Morse, 1990), and nudibranchs (Hadfield, 1978; Hirata and Hadfield, 1986; Yool *et al.*, 1986; Pennington and Hadfield, 1989; Hadfield and Pennington, 1990). It has been suggested that the attainment of larval competence was the result of the readiness of sensory receptors or nervous pathways in responding to stimulation (Hadfield, 1978; Barlow 1990). The involvement of the nervous system in larval settlement is supported by several studies. Excess external potassium can apparently generate nervous depolarization in molluscan larvae leading to metamorphosis (Baloun and Morse, 1984; Yool *et al.*, 1986). Neuroactive compounds which impact the nervous system have been found to be related to metamorphosis (reviewed by Morse, 1990; Hadfield and Pennington, 1990; Pawlik and Hadfield, 1990). However the identification and location of the receptor cell(s) remain unclear. In larval molluscs, at the primary level, the process of perceiving natural or artificial inductive substances may be associated with an external epidermal sensory cell(s) or neuroreceptor (Baloun and Morse, 1984; Trapedo-Rosenthal and Morse, 1986; Yool *et al.*, 1986). Unfortunately, none of these studies have shown that an external neuroreceptor is involved in the transduction of settlement and metamorphic cues because the physical location of the external neuroreceptor remains elusive (Barlow, 1990).

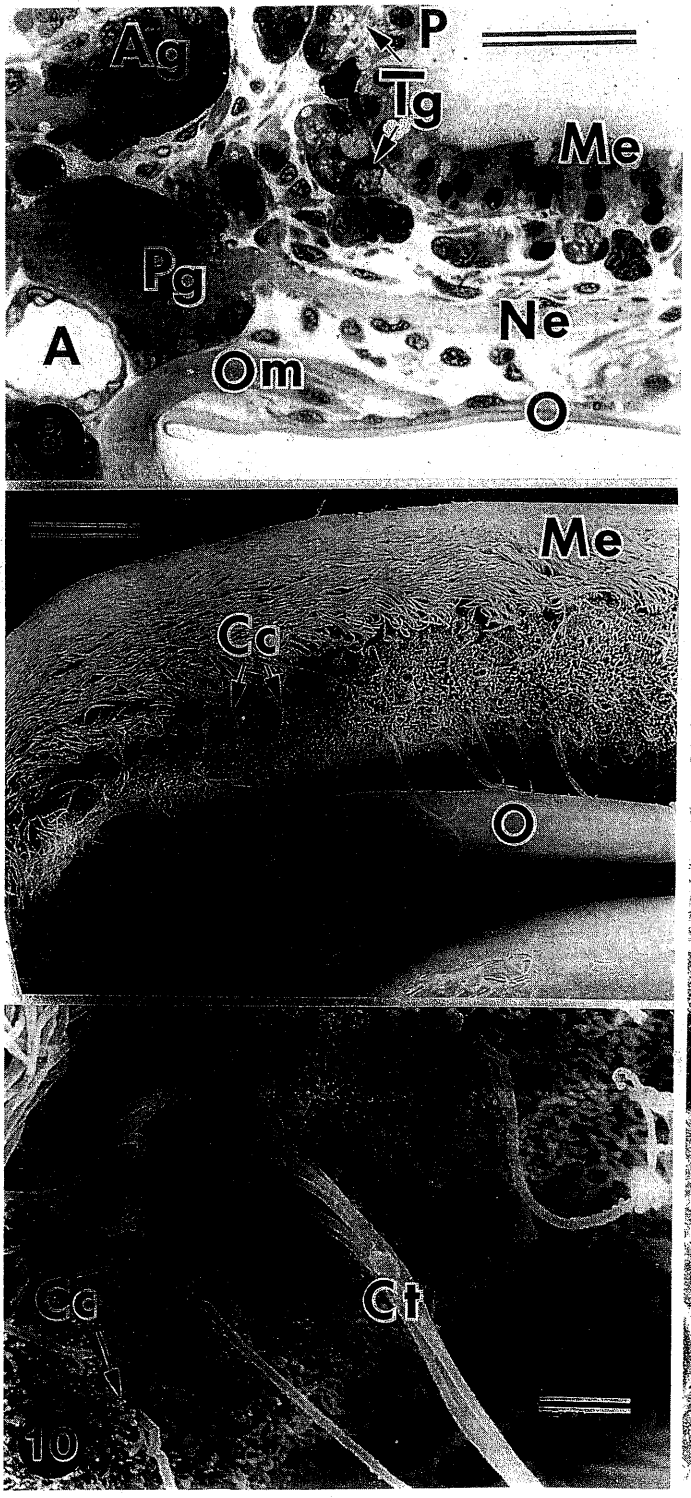
A few putative external receptor-cell locations that could potentially be involved in settlement and metamorphosis, have been structurally identified to be the cephalic sensory organ (Bonar, 1978; Morse *et al.*, 1980; Chia and Koss, 1984), the rhinophores (Chia and Koss, 1982) and the foot (Chia and Koss, 1989).

The larval rhinophores are obvious dorsal structures located at the base of the velar lobes (Chia and Koss, 1982). In *R. pulchra*, rhinophores develop prior to metamorphosis, and are thought to be involved in the chemical perception of the preferred sponge substratum. Each is cylindrical but tapers apically; a row of ciliary tufts is visible externally along the lateral margins and four or more shorter bristle-like tufts were observed on the terminals (Fig. 6). The rhinophoral epidermis

houses an cylindrical extension of the rhinophoral ganglion. The rhinophoral ganglia are joined to the cerebral ganglia by thick connectives. Sensory cells with cell bodies located at the tip of each ganglion, send dendritic endings that traverse the rhinophoral epidermis to form ciliary tufts on the external tip of the rhinophore. The intravelar location of the rhinophores enables the larva to sample water currents created by the velum; they would also be in close proximity to the sponge during crawling (Chia and Koss, 1978).

The veliger foot participates in a number of larval activities. The foot of the newly hatched veliger possesses a ciliary groove running along the length of its midline upon which food particles are rejected. During ontogeny, the foot of a pelagic opisthobranch veliger larva undergoes morphogenetic changes which contribute to the onset of metamorphic competence, and enable larval settlement and metamorphosis (refer to Bonar, 1978; Bickell and Chia, 1979; Bickell and Kempf, 1983). A large swelling, the propodium, appears on the proximal ventral face of the foot, and provides a suitable shape and surface area for crawling by settling larvae. It has been suggested that the foot of advanced veligers facilitates crawling (Bickell and Chia, 1979), and shell-loss at metamorphosis (Bonar and Hadfield, 1974; Bonar, 1976) through the concomitant development of, and secretion by, pedal glands. The different types of glands and their ontogeny within the larval foot have been extensively described (Thompson, 1958; Bonar, 1976; Bickell and Chia, 1979). However, in addition to these functions, the foot of some species of opisthobranch larvae may also serve a sensory function during settlement. Overall, the foot may the largest sensory structure of the larval body, and may contribute the most sensory information to the animal especially at settlemant because it is in close contact to the substratum. The propodium in *O. bilamellata* has been shown to be largely devoted to a sensory function (Chia and Koss, 1988, 1989; Arkett *et al.*, 1989). The metapodium, in contrast, has received little attention.

In *O. bilamellata*, preliminary observations indicated that the distal portion of the foot contains an infrastructure of elaborations of the nervous system. The foot contains central nervous elements such as the pedal ganglia and peripheral nervous tissues which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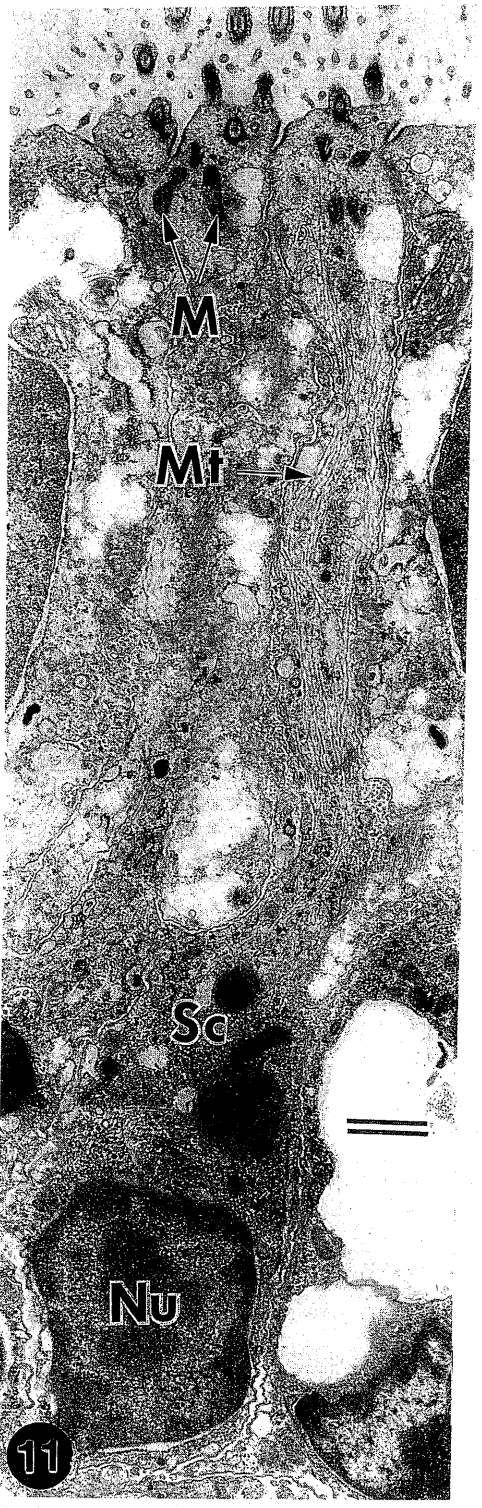
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include the propodial ganglia, many smaller ganglia composed of clusters of sensory cells (Figs. 7-12), and a basiepidermal nerve plexus with individual indigenous nerve cells (Fig. 9).

The pedal ganglia differentiate about two weeks after hatching. In metamorphically competent larvae of *O. bilamellata*, each pedal ganglion is located at the base of the propodium, adjacent to the operculum. They possess thick pedal nerves which travel toward the up of the foot (Figs. 7 and 8). Each pedal nerve bifurcates to be partitioned into two main groups of nerves; one group passes into the epidermis to be associated with clusters of intraepithelial ganglia composed of sensory cells that give rise to the ciliary tufts or single ciliary cells. The second group is reduced to smaller subepidermal ganglia that innervate the mulitcellular glands of the foot. Intraepithelial sensory cells are found in clusters of 5 to 10 cells, that are situated just under the apex and at paired locations along the sides of the foot. Each group of cells is recognizable on the external surface of the metapodium as an isolated ciliary tuft, measuring 15 to 20 μm in length, and composed of 10 to 15 cilia (Figs. 9 and 10). The tuft underlying the tip of the foot possesses approximately 30 cilia, and a greater number of cells. Sensory cells are generally flask-shaped and lightly staining. Free ribosomes are distributed throughout the cell. The dendritic portion of the cell contains numerous microtubules, mitochondria, dense-cored vesicles

Fig. 8. Sagittal section (1 μm thickness) of an advanced veliger of *Onchidoris* showing the propodium (P) and metapodium (Me) composing the foot, pedal ganglion (Pg), pedal nerve (Ne), anterolateral ganglion (Ag), and metapodial glands (Tg). The operculum (O), statocyst (A) and opercular muscle (Om) are also shown. Scale bar=25 μm.

Fig. 9. Scanning electron micrograph (SEM) of an *Onchidoris* veliger showing the metapodium (Me) with dense pedal sole ciliation extending to the tip of the foot. Sensory cells with single cilia (Cc) and tufts (Ct) are shown to be located along the margin and tip of the foot. The operculum is also shown. Scale bar=10 μm.

Fig. 10. Higher magnification SEM shown single cilia (Cc) of sensory cells interspersed between ciliary tufts (Ct) emanating from sensory cell-clusters. Scale bar=1 μm.

Fig. 11. Section through the tip of the foot showing a cluster of sensory cells (Sc) located within the epidermis. The nucleus (N), mitochondria (M) and microtubles (Mt) of a sensory cell are shown. Scale bar=1 μm.

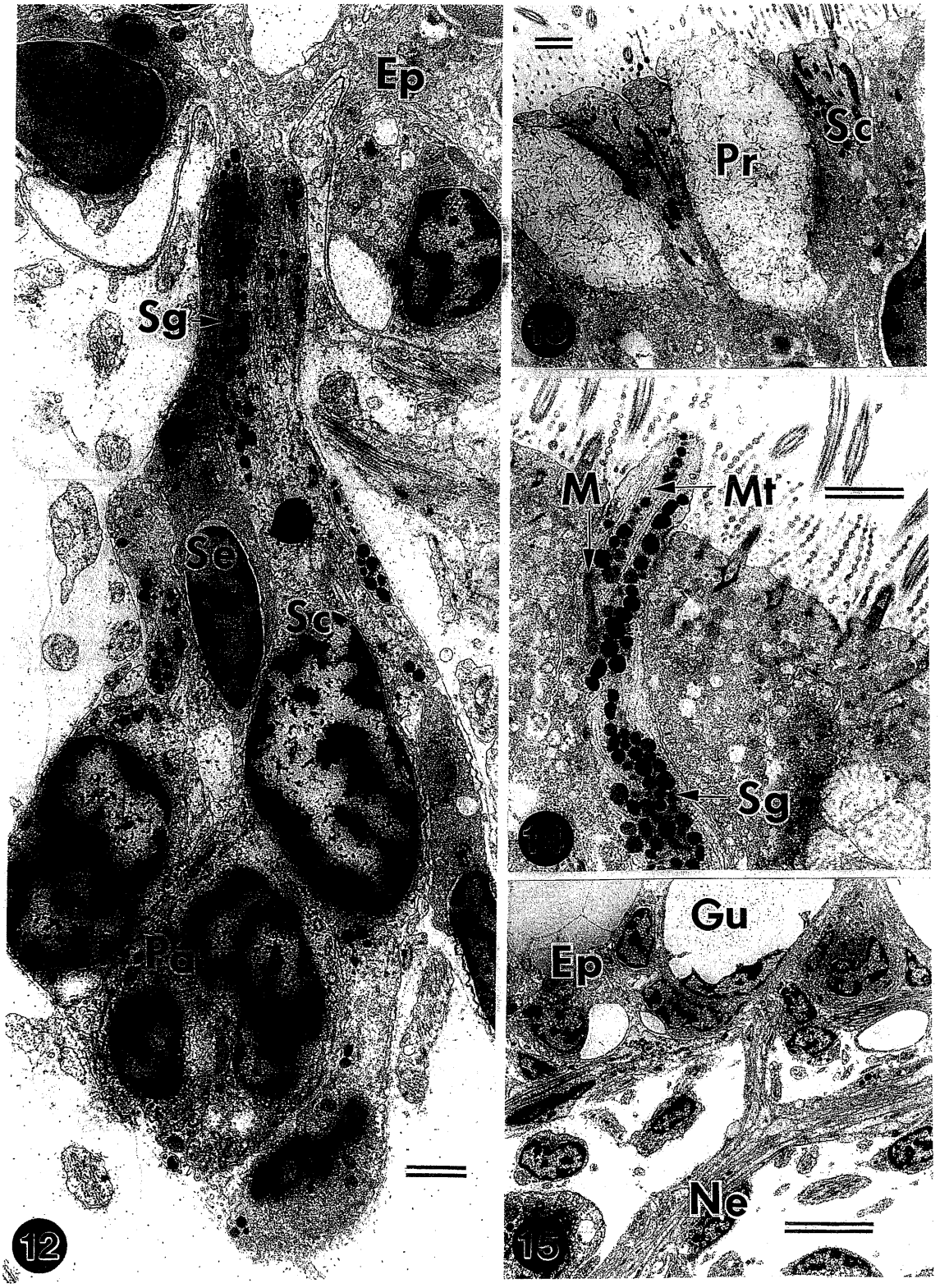
averaging 0.08 μm in diameter, and occasionally vacuoles of 0.3 μm in diameter; 3 to 4 cilia together with microvilli arise from the distal surface (Fig. 11). A Golgi body is located above a basally positioned nucleus. A few electron dense granules measuring about 0.5 μm in diameter and cisternae of RER are also situated in this nuclear zone (Fig. 11). A basally derived axon passes into the underlying nerve plexus. These processes contain microtubules, electron translucent vesicles averaging 0.06 μm in diameter, and dense-cored vesicles of 0.08 μm in diameter. The foot may be sensitive to mechanical disturbances through the stimulation of ciliary tufts along its margins, resulting in retraction of the larva into the shell (Bonar, 1978; Bickell and Chia, 1979). These ciliary tufts are unique larval structures which disappear after metamorphosis. In *O. bilamellata*, these tufts may also be important in contact perception of a suitable substratum for metamorphosis as these sensory-cell clusters are innervated by the pedal ganglia.

The secretory cells constituting the various glands all originate subepidermally, but terminate apically in localized groupings on the epidermal surface. An accompanying collection of sensory receptor cells arise and terminate among these groupings (Fig. 13). Subepidermally, aggregations of sensory cells interconnect the different types of multicellular glands, although being situated immediately outside and between secretory cell groupings (Fig. 12). Tiny branches of the pedal nerve innervates each group of receptors as well as the multicellular pedal glands. Subepidermal sensory cells are flask-shaped and appear to be of two types (Fig. 12). In the first, the cytoplasm is relatively clear and lightly staining. An elliptical nucleus occupies the base of the cell. A Golgi body and electron opaque granules measuring 0.2 μm in diameter are situated in juxtanuclear positions. Microtubules together with some RER occupy the neck region of the cell. More distally, numerous mitochondria are interspersed among oval vacuoles measuring about 0.1 μm in diameter. Three or four cilia together with microvilli emerge from the apical surface (Fig. 13). The second type of subepidermal sensory cell is more numerous, nonciliated, stains densely, and terminates beyond the level of the general ciliated epidermis (Figs.

12 and 14). Free ribosomes are distributed throughout the cell. The portion of the cell, beginning above the nucleus, that penetrates the epidermis is filled with small characteristic membrane-bounded electron opaque granules averaging 0.08 μm in diameter (Figs. 12 and 14). Microtubules and a few mitochondria are found among these inclusions. Aggregations of Golgi bodies, mitochondria, and RER are situated around an elliptical, electron dense nucleus that possesses a nucleolus. These sensory neurons are likely involved in substratum recognition and pedal integrating pedal gland secretion.

Although the receptive capacities of most of the above structures and organs have been inferred from morphological characteristics and whole larval behavioral correlations, there is little direct evidence to suggest that any these structures are functional, and predisposed to perceiving settlement or metamorphic cues. To date, the *Onchidoris* larval foot, or specifically the anterolateral propodial ganglia, represents the only system where morphologically identified chemosensory receptor cells have been shown to electrophysiologically respond to a known settlement cue (Arkett *et al.*, 1989). The propodium of the advanced veliger larva of *O. bilamellata*, contains the anterolateral and frontal ganglia (Chia and Koss, 1989). These structures were thought to be involved in the perception of chemical cues from barnacles, which induce settlement and metamorphosis (Chia and Koss, 1988). Both sets of propodial ganglia are connected into the cerebral ganglia. The ganalia are located subepidermally, but send dendritic processes through the epidermis as terminate as ciliary or microvillar endings (Fig. 7). Both sets of ganglia are represented as sensory fields externally which are discernible from the surface by central regions devoid of ciliation (Fig. 17).

These regions can be exposed electrophysiological characterization by immobilizing larvae in seawater with high Mg^{++} and low Ca^{++} concentrations, and then tethering them with cactus spines (Fig. 17) so that this region points upward. The results of our recent work showed that the receptor cells of the anterolateral ganglia can respond to the settlement cue with slow, low-amplitude depolarizations when measured *in situ* intracellular recording methods (Fig. 16; Arkett *et al.*, 1989). However, the activity of the


Ep
Sg
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M
Mt
Sg
Gu
Ep
Ne
12
13

activity of the sensory cells is variable in terms of duration and amplitude. Such variability may be caused by an undetermined amount of crude stimulus being administered, the developmental status of the receptors or the degree of damage caused by the electrode upon entering the cell. It is also unclear at what depth within the ganglion the electrode was placed or if the epidermal tissues overlying these subepidermal structures altered the response of receptor cells.

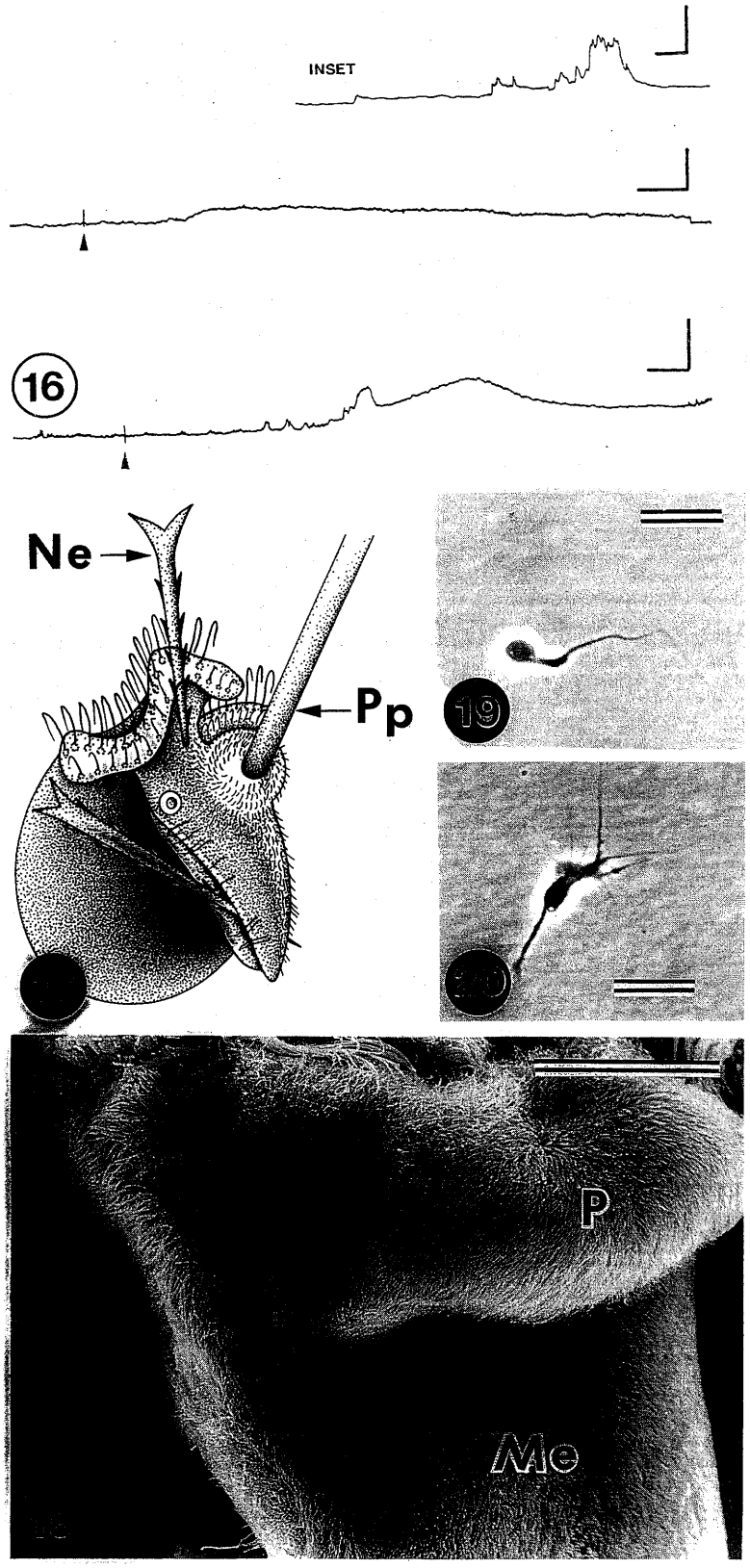
To avoid these problems, we have now developed a technique for excising, dissociating and culturing the lateral propodial region of the veliger foot of *O. bilamellata* (refer to Fig. 21 for a summary; F.-S. Chia, R. Koss, S. Stevens and J. I. Goldberg, in preparation). Individual sensory cells (neurons) and interneurons can be obtained from the anterolateral ganglia, which can survive for several days in culture (Figs. 19 and 20). Larvae were initially relaxed in 2.5 ml of high Mg^{++}, low Ca^{++} seawater mixture (12-15°C) consisting of natural seawater, isotonic (0.33 M) $MgCl_2$, and Ca^{++}-seawater in a volume ratio of 2:1:4.5. Veligers were immobilized by impaling them with a cactus spines in such a way to secure an upward orientation of one side of the propodium and its incumbent receptor field (Fig. 17). Prior to excising a ganglion, pinned larvae were incubated for five minutes in 0.2% trypsin in a modified *Aplysia* (11) defined medium (mADM: 50% Liebowitz L-15 (Gibco special order); 0.26 M NaCl; 9.7 mM $CaCl_2$; 4.6 mM KCl; 26 mM $MgSO_4$; 26 mM $MgCl_2$; 2 mM $NaHCO_3$; 33 mM Dextrose; 10 mM Hepes; 0.015% L-glutamine; 50 μg per ml gentamicin; pH 7.8) which was subsequently replaced with mADM. The tip of

Fig. 12. Oblique section through a cluster of subepidermal sensory cells (Pa). Note sensory cells are either darkly staining (Se) with granules (Sg), or lightly staining (Sc) with ciliated dendritic endings that eventually terminate at the level of the epidermis (Ep). Scale bar=1 μm.

Fig. 13. Terminals of the multicellular propodial gland showing composite cell-types including secretory cells (Pr) and sensory cells (Sc). Scale bar=1 μm.

Fig. 14. Terminal of a subepidermal sensory cell containing granules (Sg), mitochondria (M), and microtubules (Mt). Scale bar=0.5 μm.

Fig. 15. Section through the foot showing part of the pedal nerve (Ne) that splits and passes into the epidermis (Ep) at the base of unicellular gland cell (Gu). Scale bar=10 μm.


INSET
16
Ne
Pp
19
P
Me

a silicon-coated (Sigmacoat) micropipette, with a bore diameter of 20-30 μm, was then placed directly on top of the field representing the anterolateral ganglion (Fig. 17). The anterolateral ganglion was excised by first applying mechanical force with the mircropipette to this region, and then alternating negative and positive pressure within the micropipette through a microsuction device. The excised ganglionic tissue along with portions of the propodial epidermis, were triturated in the micropipette and the dissociated cells were plated into high molecular weight poly-L-lysine coated plastic 35 mm tissue culture plates in cold mMDA. LM identification of living cell-types was corroborated by transmission electron microscopy (TEM). The anterolateral ganglia are composed of sensory receptor cells, interneurons and sheath cells. The majority of interneurons plated from the anteriolateral ganglion were unipolar of flask-shaped (Fig. 19). The flask-shape was represented as a rounded base extending into a long, thin neck. Neuronal cells were occasionally bipolar in profile and sometimes found collectively with sensory cells. Neurites contained microtubules and vesicles ranging from 0.005 to 0.008 μm in diameter. All of the above features correspond identically to the neurons found in the anterolateral ganglia of the veliger (Chia and Koss, 1989). It should be noted that sheath cells at times remained attached to the neurons *in vitro*, further lending support that this technique involving

Fig. 16. Intracellular electrode recordings from receptor cells of *Onchidoris* showing a response to barnacle water (arrow). Horizontal scale=3s (inset, 1s); vertical scale=15 mV. Reproduced with permission from *The Biological Bulletin*, 176: 155-160 (April, 1989).

Fig. 17. Schematic drawing of the *Onchidoris* veliger preparation showing how veliger was pinned with cactus spines (Ne) and the placement of the micropipette (Mp) onto the receptor field of anterolateral ganglion. Scale bar=50 μm.

Fig. 18. SEM of the *Onchidoris* foot, showing propodium (P) with sensory field of the right anterolateral ganglion (arrow) and metapodium (Me). Scale bar=20 μm.

Fig. 19. Photomicrograph (interneuron) of an interneuron plated after dissociation and excision of *Onchidoris* anterolateral ganglion. Scale bar=10 μm.

Fig. 20. Photomicrograph (phase contrast) of a sensory neuron plated after dissociation and excision of *Onchidoris* anterolateral ganglion. Scale bar=10 μm.

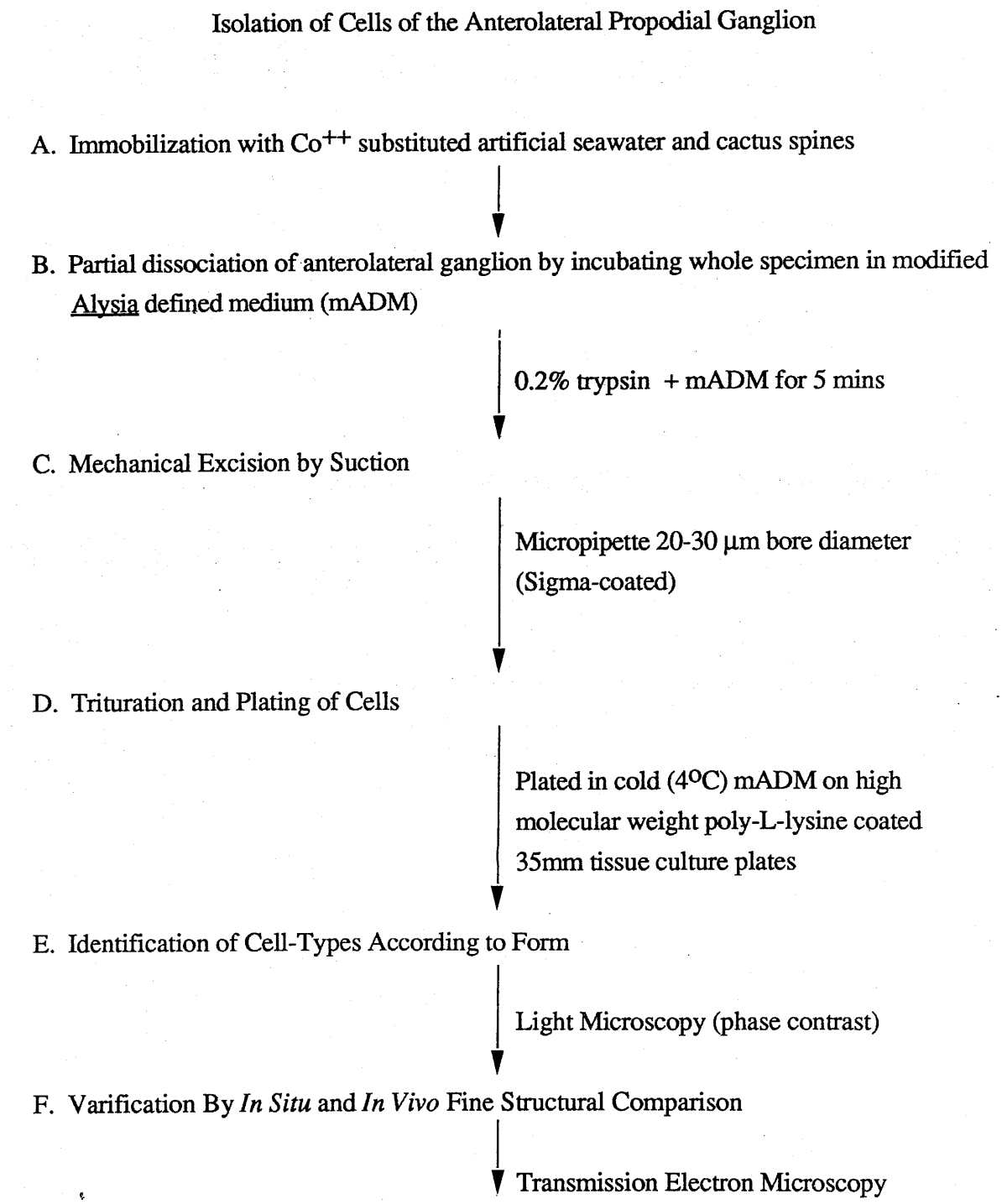


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Fig. 21. Diagrammatic presentation of the method utilized to isolate, culture and identify cells of *Onchidoris* anteolateral ganglion.

micropipette placement and ganglionic excision is accurate. Sheath cells could be identified by a densely staining nucleus, cytoplasm and mitochondria. These features are comparable to those cells found in a larva preseved in its natural state.

Sensory cells were recognized at the level of light microscopy through a bipolar shape with two main processes radiating out from each soma (Fig. 20). One of these processes was invariably thicker in comparison to the opposite neurite. At times the thickened end was observed to be compressed, possibly due to mechanical disturbance during excision, and the nucleus occupied a central location within the cell. The overall form of these cells, and the interneurons, appeared to be conserved from *in situ* to *in vitro* conditions. The spindle-shape of sensory cells is retained through dissociation and plating of the individuil cells in the culture dishes. At the fine structural level, the integrity of the sensory cells also remains unchanged. These cells characteristically contain a relatively clear cytoplasm which stains lightly and contains free ribosomes and an elliptical to spherical nucleus. The nucleus is relatively lightly staining and the axon and dendritic processes can be recognized. The dendrite possesses a single cilium and mitochondria, is generally wider than the axon and lacks vesicles. The cilium lacks a ciliary rootlet which is characteristic of the general ciliated epidermal cells.

We are confident that this culture system will provide an opportunity to investigate the mechanisms by which neurons and sensory cells respond to settlement or metamorphic cues in *O. bilamellata*. The technique developed here also provides a broader basis for studying the mechanisms controlling settlement and metamorphosis in larvae of many different marine invertebrate phyla. Cellular mechanisms of recognizable tissues that putatively control settlement and metamorphic processes can be studied in groups such as polychaetes (Amieva *et al.*, 1987), cnidarians (Chia and Koss, 1979), or echinoderms (Chia *et al.*, 1986). Individual larval sensory cells neurons are uniquely amenable to electrophysiological, neurotransmitter, second messenger, and immunocytological study. This system circumvents the problem of interpreting the actions of a specific substance by correlating whole-larval responses to cellular activity or mechanisms. Assays which utilize

whole-organism responses may be useful in identification of compounds which induce larval settlement and metamorphosis. They provide little information, however, regarding the cellular mechanisms which they trigger, or the structural site at which their effects are imposed. For example, a compound may have multiple effects on the same cells or tissues, multiple effects on different cells and tissues, or a simple effect on a single cell type or tissue that activates a hierarchy of responses along a neurophysiological pathway(s). Therefore, techniques enabling the study of the precise actions of settlement and metamorphic inducers must be developed.

CONCLUDING REMARKS

Veliger behaviors, as described in this paper, include swimming, feeding, defence, settlement, crawling and metamorphosis. Swimming and feeding are acquired before hatching and are continuous until metamorphosis. Defence behavior in veligers is passive and feeble; it is not likely to be very effective against predators and filter feeders (Cowden *et al.*, 1984). Settlement, involving sinking or swimming from the surface water into the adult habitat, is the initiation of metamorphosis but does not always lead to metamorphosis. Settlement behavior can be temportary and reversible or permanent and irreversible. Settled larvae can crawl or rest on the substratum or swim short distances in the boundary layer along a hard substratum. Metamorphosis is not a behavior but a structural event and once committed, cannot be reversed; it involves the loss of the larval shell, the operculum, the velum, and the reabsorption of the visceral mass into the foot.

The development of the larval nervous system and the sensory receptors is closely associated with the ontogeny of larval behavior. The fine structure of veliger receptors and nervous system has been described in several species of veligers, and their correlation to larval behavior has been suggested. For example larval feeding (sorting, ingestion, rejection of food particles) is thought to be associated with the cephalic sensory organ and the foot, swimming with the statocysts, defence with the cephalic sensory organ and the foot, settlement with the eyes, crawling and metamorphosis with the rhinophores and the

foot receptors, and metamorphosis with the rhinophores (*Rostanga*) and the foot receptors. However, electrophysiological data indicating a causal relationship between a sensory receptor and a clearly defined behavior can be found in only one report (Arkett *et al.*, 1989). It is hoped that our recent success in isolating and culturing sensory neurons, can lead to further electrophysiological studies of settlement and metamorphosis. It is acknowledged that morphological studies fall short of determining the direct function of a particular tissue or organ. However, they are invaluable in facilitating the identification of physical nervous locations targeted by different stimuli, and provide a stereotypic atlas for future molecular, biochemical or electrophysiological examination. As pointed out in this report, many approaches fall short of determining the location of sensory neurons because morphological baseline studies are lacking.

The foregoing accounts of the behavivor, morphology and physiology of molluscan larvae may appear to be theoretical, anecdotal and unrelated to the practices or goals of aquaculture. However, a basic understanding of the biology of the animal being reared, especially its life history, is imperative. For economically important food species of invertebrates such as molluscs, many scientific studies have impacted rearing practices by facilitating their cultivation, especially within the larval stage. The larval stage is the most vulnerable phase in the life cycle and most work has focused on increasing the numbers of larvae reaching the juvenile stage through enriched food regimes or by enchancing settlement. More recently, the cultivation of organisms as a source for bioassays has received focus. These bioassays may be utilized in determining toxicity levels of chemicals and their environmental impact or may contribute to an eradication program for bio-fouling species. For example, a viable alternative eradication program, which targets the vulnerable larval stage, may be possible for the problematic zebra mussel. However, little is known about its life history traits, and more specifically the behaviors, settlement and dietary requirements of the larvae. In North America, aquaculture of this species, for the purpose of bioassay, has been relatively unsuccessful because of the lack of

background knowledge about the biology of the animal.

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