The Veliger Larva of *Trichotropis cancellata* and Comparative Aspects of the Caenogastropod Apical Ganglion

by

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B.Sc., University of Victoria, 1998

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

We accept this thesis as conforming to the required standard

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Abstract

The early life history of the caenogastropod, *Trichotropis cancellata*, was observed and described. The planktotrophic veliger larvae were reared from hatching through to metamorphosis. Specimens were prepared for scanning electron microscopy and histological examination to complement observations of live larvae, and progressive changes in development are described at a number of stages: late embryo, hatching veliger, post-hatching veliger and post-metamorphic juvenile.

The apical ganglion (also called the apical or cephalic sensory organ) is a key structural landmark in a wide range of invertebrate phyla. One of the hallmarks of the apical ganglion (AG) is the presence of serotonin (5-hydroxytryptamine, 5-HT) in the neurons associated with it. The gastropod apical ganglion has been most thoroughly studied in planktotrophic opisthobranch larvae (subgroup of the Heterobranchia). Using light microscopy, immunohistochemical localization of 5-HT antigenicity, and scanning and transmission electron microscopy, the present study provides comparative data on the apical ganglion in five caenogastropod species (*Trichotropis cancellata*, *Euspira [Polinices] lewisii*, *Marsenina stearnsii*, *Lacuna vincta*, and *Amphissa columbiana*). Ultrastructure of the AG is similar amongst these species and the overall layout corresponds to the tri-lobed arrangement described for opisthobranch larvae. The serotonergic neurons of the AG show interspecific variation in these caenogastropods with the number of somata ranging from 3 to 6; quite unlike the characteristic 5 neurons

associated with opisthobranch larvae. The AG is a larval specific structure and completely degenerates within four days of metamorphosis in *T. cancellata.* These and other comparative data suggest that common ancestry is a major determinant of overall AG design within the Gastropoda.

The larval nervous system of *T. cancellata* was further probed with antibodies against FMRFamide. The pattern of immunoreactivity for FMRFamide revealed two apical cells that transiently express the peptide in late embryo and early veliger stages, as well as a series of cells and fibres along the metatroch (post-oral ciliary band) of the velum and in the cerebral and pedal ganglia. An immuncytochemical assay for dopamine ß-hydroxylase and a histochemical assay for nitric oxide synthase yielded negative results.

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Acknowledgements

It is a pleasure to extend my heartfelt thanks to all those who provided encouragement and support during the course of this study.

I would like to thank my supervisor Dr. Louise Page for providing me with the opportunity to undertake this project. Dr. Bruno Pernet and Ms. Erika Iyenegar, both at the Friday Harbor Laboratories, provided me with *Trichotropis cancellata* egg masses. Dr. Robert Burke and Mr. Ross Atkins kindly introduced me to the use of the laser scanning confocal microscope. Dr. Chaman Singla provided many helpful suggestions and good company in the electron microscopy laboratory. Thanks to Carin Bondar for her infectious alacrity. Claire Holeton toiled diligently to maintain algal cultures over the spring and summer of 2000. Ryan Cawker and Bruce Stafford provided indispensable computer assistance and also accompanied me on many sanity-preserving trips to The Dump. My father, Ernest Parries, has been a source of inspiration and has always encouraged me in my academic endeavours. Thanks to Conan and Winnie for making me laugh. Finally, I would like to thank Alexandra Eaves for her advice and assistance in the lab, her infinite patience and unending efforts to bolster my spirits even at the most enervating of times, and simply for being a good friend.

Funding for this research was provided by a Graduate Student Fellowship from the University of Victoria and by an NSERC research grant to L.R. Page.

Introduction

Investigations concerning the early life history of marine organisms, while not as prominent as they have been in the past, have intrinsic merit nevertheless. Very often only the biology of the adult organism is understood to any degree, a situation that can have a number of consequences. A complete understanding of the ecology of an animal, for instance, cannot be achieved without knowledge of the larval and juvenile forms in addition to what is known of mature stages. With the re-emergence of "evolutionary developmental biology" as a full sub-discipline of the life sciences, the importance of studies concerning embryogenesis and morphogenesis cannot be understated (Hall, 1992: Raff, 1996). In attempting to understand the relationship between ontogeny and phylogeny the need for comparative developmental studies is paramount, especially when examining a group of organisms as taxonomically and phylogenetically complex as the gastropod molluscs.

The Gastropoda, containing some 40, 000 described species, are extraordinarily diverse and have long been favourite subjects of embryologists and developmental biologists. However, historical bursts of rapid diversification and convergences associated with shared lifestyles have made the construction of phylogenetic hypotheses very difficult, and indeed such hypotheses are in a constant state of flux. One current hypothesis separates the Gastropoda into two primary groups, the Eogastropoda (the limpets and their ancestors) and the Orthogastropoda (all other gastropods), and recognizes a number of major clades within these two groups (Ponder and Lindberg, 1997). The ensuing discussion

will primarily consider two of these clades, both from the Orthogastropoda: the Caenogastropoda (a consolidation of the Neogastropoda and the paraphyletic Mesogastropoda), and the Heterobranchia (a consolidation of the Opisthobranchia and the Pulmonata). Current phylogenies, constructed with either morphological or molecular characters, view these groups as sister clades (Ponder and Lindberg, 1997; Harasewych et al., 1998; Winnepenninckx, 1998) with a free-swimming, planktotrophic larval stage in the ancestral life history.

Aspects of reproduction, embryogenesis, development, and larval biology in molluscs have been extensively studied and are reviewed by Raven (1958), Verdonk et al. (1983), Fretter and Graham (1994), and Collier (1997) among others. Indirect development is very common, particularly amongst marine species, and in such cases embryogenesis builds a free-swimming, post-embryonic larval form. Pelagic larvae convey dispersal ability to the species, but require extensive morphological differentiation both before and after settlement into a benthic juvenile. Two larval forms occur in the Gastropoda: the trochophore and the veliger. Only in some of the most basal gastropod clades, in which gametes are shed freely into the water column and fertilization is external, does a free-swimming trochophore exist and it quickly develops into a veliger. The veliger larva is characterized by the presence of a velum; a bi-lobed, ciliated structure responsible for locomotion and food capture in planktotrophs. During the course of the larval period, the veliger continues to grow and the differentiation of internal organs also occurs. It can be cautiously said that many of these larval organs are functionally retained post-metamorphically in the adult,

with the notable exception of the velum and the larval heart (Fretter and Graham, 1994). However, in some species, metamorphosis involves abrupt and often drastic changes in morphology. The metamorphic event culminates with the loss of the velum in all species, and makes the transition from pelagic to benthic lifestyles irreversible.

Primary Study Organism

Trichotropis cancellata (Hinds, 1849), in the gastropod superfamily Calyptraeoidea, is one four species in the genus found in the Pacific Northwest (Kozloff, 1996a). It is abundant in shallow subtidal waters, although it is occasionally found in the intertidal zone, and individual snails often reside in close association with one of a number of suspension-feeding polychaete worms. Adults of the species are quite distinctive; they grow to approximately 3cm and the periostracum is elaborated into coarse hairs, similar to the situation in *Fusitriton oregonensis*, a larger and more conspicuous local gastropod (Yonge, 1962; Kozloff, 1996b). Long considered a suspension feeder that uses its ciliated ctenidium to collect and capture suspended particles, it has now been shown that *T. cancellata* is also a facultative kleptoparasite, capable of diverting and ingesting food particles captured by the polychaetes with which it associates (Pernet and Kohn, 1998). Such a feat is accomplished via the insertion of the animal's pseudoproboscis, an elongate, ciliated elaboration of the ventral lip, into the mouth of the host worm. *T. cancellata* is thereby able to take advantage of the elaborate suspension feeding system of its host while reducing its own metabolic costs that would normally be associated with food capture. The biology of the

mature animal is relatively well characterized (Yonge, 1962), but very little is known about the larval and juvenile stages of the life cycle. The present study was undertaken to address some of those unknowns.

Life History of *Trichotropis cancellata*

T. cancellata, as described by Yonge (1962), is a protandric hermaphrodite, a condition in which the animal functions first as a male and later as a female. This is a common condition among members of the Calyptraeoidea, although *T. cancellata* is unusual in that the penis is retained throughout life and is a prominent structure even in fully functional females. During copulation, a smaller snail, acting as a male, positions itself on the anterior right side of a larger female and inserts its penis into the right side of the mantle cavity (personal communication with Dr. B. Pernet). Egg masses consist of a number of individual capsules attached to a solid substrate. Once deposition is finished, the depositing snail remains on its brood for the duration of embryogenesis and if removed it will immediately return (personal communication with Dr. B. Pernet and personal observation). Embryogenesis has not yet been described in detail for this species, but the interval between egg mass deposition and the hatching of veligers is approximately 45-60 days post-deposition, when kept at approximately 10–12 °C in the laboratory (personal communication with Dr. B. Pernet). There is a lengthy pelagic period during which the larvae grow and disperse, before settlement and metamorphosis occurs.

Larval Nervous Systems

Larval forms of marine invertebrates are fascinating organisms. They lead complex, often lengthy, pelagic existences wholly distinct from those they take up after metamorphosing into a benthic adult. They possess a considerable repertoire of behaviours and are able to respond accordingly to the incalculable environmental stimuli they encounter in the plankton (for review see Pawlik, 1992). It has long been recognized that larval behaviours, like those of their adult counterparts, are coordinated by a central nervous system and peripheral sensory receptors. Gastropod larvae have a particularly well-developed nervous system organized around a series of paired ganglia joined across the midline by a commissure. Individual ganglia on each side, the cerebral and the pedal ganglion for instance, are linked by connectives, the cerebropedal connective in this case.

The typical gastropod nervous system includes paired cerebral, pedal, buccal, pleural, and oesophageal or intestinal ganglia, as well as the unpaired visceral, and osphradial ganglia. Generally these ganglia arise by the proliferation and subsequent delamination and/or invagination of cells at specific ectodermal placodes (Jacob, 1984; Bulloch, 1985). Gangliogenesis tends to progress along the anterior-posterior axis, with the cerebral ganglia reportedly developing first, followed by the pedal ganglia, and then the other more posterior ganglia associated with the visceral loop and osphradium (for review see Raven, 1958; Fretter and Graham, 1994; Lin and Leise, 1996a). The cerebrals are generally well developed at hatching and send connectives to the pedal, buccal, and pleural ganglia and innervate anterior sensory structures such as the optic vesicles,

cephalic tentacles, statocysts, and epidermis (Smith, 1935; Crofts, 1938; Raven, 1958; Bullock, 1965; D'Asaro, 1965, 1966, 1969; Bedfod, 1966; Hyman, 1967; Demian and Yousif, 1975; Dorsett, 1986; Fretter and Graham, 1994). The cerebral commissure is also very closely associated with the apical sensory organ as will be discussed below (Lin and Leise, 1996a, b; Marois and Carew, 1997a, Kempf et al., 1997, Page and Parries, 2000).

The pedal ganglia, which are also present in hatching larvae of most species, develop in the base of the foot, and are linked by a well-defined commissure. The pedals are also linked to both the pleural and the cerebral ganglia by connectives. As the larvae approach metamorphic competency, the foot develops an anterior propodium in addition to the more posterior metapodium, giving it a well-defined, planar crawling surface. Large nerves are sent into both these regions of the foot by the pedal ganglia.

The pleural ganglia develop immediately behind the velum, dorsal to the statocysts (Raven, 1958) and may or may not be formed at hatching. The timing of the differentiation of the major ganglia varies from species to species. D'Asaro (1966, 1969) reported the presence of all the major ganglia but the buccals at hatching in the caenogastropods *Thais haemastoma floridana*, *Bursa corrugata*, and *Distorsio clatharta.* In studies of another caenogastropod, however, the queen conch, *Strombus gigas*, only the cerebral and pedal ganglia were distinguishable at hatching (D'Asaro, 1965). Opisthobranchs generally have only a small pair of cerebral ganglia and an even smaller pair of pedal ganglia at hatching (Page, 1992a,b). There is also variation in the degree of fusion with other

ganglia exhibited by the pleurals. In basal gastropod clades, the pleurals are often fused with the pedal ganglia (Crofts, 1938: Raven, 1958). In more derived gastropod clades however, the pleurals tend to lie in closer apposition to the cerebral ganglia (Fretter and Graham, 1994). In addition, the supposed homology of the pleurals between opisthobranchs and caenogastropods is a point of contention (Dorsett, 1983; Page, 1992b; Carrol and Kempf, 1994). The pleurals tend to innervate the lateral body wall, the columellar muscle, and the mantle skirt in caenogastropods (Fretter and Graham, 1994).

The buccal mass, which contains the rather complex odontophoral apparatus, is innervated by the small, paired buccal ganglia. These develop from the wall of the foregut near the radular sac, are connected by a commissure, and are linked to the cerebral ganglia by the cerebrobuccal connectives (Raven, 1958; D'Asaro, 1965; Hyman, 1967; Fretter and Graham, 1994). They provide both sensory and motor innervation to the buccal mass and contain the interneurons of the adult feeding pattern generator (Dorsett, 1986).

The remaining ganglia are strung out along the visceral loop and generally appear somewhat later in the larval phase (D'Asaro, 1965, 1966, 1969; Demian and Yousif, 1975; Fretter and Graham, 1994)). The visceral loop of caenogastropods is streptoneurous, a situation in which the visceral connectives are crossed in a figure eight pattern with one branch passing dorsally over the digestive tract and the other passing ventrally under, due to the effects of ontogenetic torsion. This results in the right, or supraoesophageal (also called the supraintestinal) ganglion, being shifted to the left side of the body, and the left, or

suboesophageal ganglion, being shifted to the right side of the body (Raven, 1958; Fretter and Graham, 1994). Further asymmetries occur and the two oesophageal ganglia can vary widely in terms of position and their apposition or fusion to other ganglia. In a study of *Marisa cornuarietes*, for example, the suboesophageal ganglion shifts to the right and forward to fuse with the right pleural ganglion, while the supraoesophageal persists as a discrete unit (Demian and Yousif, 1975). Regardless, the oesophageal ganglia arise from the ectoderm of the floor of the mantle cavity (Raven, 1958) and tend to innervate the kidney and ctenidium.

The osphradium, an organ located so as to be directly in the incoming current of water that enters the mantle cavity, is a major chemosensory organ (Hyman, 1967; Fretter and Graham, 1994). In many caenogastropods, a single osphradial ganglion innervates the osphradium and it is linked to the supraoesophageal ganglion by the osphradial nerve (Fretter and Graham, 1994).

The unpaired visceral ganglion is located beneath the posterior extremity of the mantle cavity (Raven, 1958; Fretter and Graham, 1994). Connectives from this ganglion run to each of the oesophageals to complete the visceral loop. The visceral ganglion differentiates quite late in the larval phase from an ectodermal placode lining the roof of the mantle cavity at its posterior end (Raven, 1958; D'Asaro, 1965, 1966, 1969). Although generally unpaired, Fretter and Graham (1994) describe the visceral ganglion as bilobed in adult caenogastropods. The visceral ganglion, as its name implies, innervates much of the visceropallium, including: the gut and anus, reproductive organs, the kidney and digestive gland,

the heart, and adjacent areas of the body wall (Bullock, 1965; Dorsett, 1986; Fretter and Graham, 1994).

The Apical Ganglion

Despite the fact that gastropod molluscs have been intensively studied on many levels over the last century, surprisingly little work has been focused on neurodevelopment. This is especially odd when one considers the wealth of information that has been obtained from neurobiological studies spawned by the relative simplicity and accessibility of the adult gastropod nervous system (e.g. Bulloch and Ridgway, 1995; Satterlie et al., 1995; Byrne and Kandel 1996). Only recently has a concerted effort begun to delve into understanding the structure and function of larval nervous systems. Neuroanatomical studies have been the most common (e.g. Demian and Yousif, 1975; Bonar, 1978; Bickell and Kempf, 1983; Page 1992a,b, 1993; Lin and Leise, 1996a), with a more recent emphasis being placed on neurochemistry (e.g. Kempf et al., 1991, 1992, 1997; Goldberg et al., 1989, 1992; Barlow and Truman, 1992; Marois and Carew, 1997a,b,c; Diefenbach, et al. 1998). However, work on molluscan larvae has been biased in favour of members of the Heterobranchia, the group of gastropods comprising the opisthobranchs and the pulmonates. Recent studies concerning the neurodevelopment of the caenogastropods, the largest and most diverse clade of the extant Gastropoda, have been less common.

An apical organ, generally assumed to be sensory in nature, occurs in larval forms throughout the invertebrate phyla with possible homologues occurring in both the Protostomia and the Deuterostomia. Alternatively referred

to as the "apical sensory organ", the "cephalic sensory organ", the "apical complex", or the "apical ganglion", apical organs have been described in larvae of cnidarians (Chia and Koss, 1979), polychaetes (Lacalli, 1981, 1984; Hay-Schmidt, 1995), brachiopods (Hay-Schmidt, 1992), phoronids (Hay-Schmidt, 1989, 1990a,b), molluscs (Bonar, 1978; Chia and Koss, 1984; Page, 1992; Uthe, 1995; Lin and Leise, 1996a,b; Marois and Carew, 1997a,b,c; Kempf et al. 1997; Page and Parries, 2000), and echinoderms (Bisgrove and Burke, 1986, 1987; Nakajima, 1988) among others. Most often these apical organs are associated with a tuft of elongate, relatively stiff cilia. More recent neurochemical studies have revealed another commonality, the presence of serotonin (5-hydroxytryptamine, 5-HT) in a subset of the neurons making up the organ proper. Conklin (1897) was perhaps the first to suggest that this structure might be homologous across supraphyletic groupings of invertebrates, and other, more recent, authors have made similar proposals (e.g. Bonar, 1978; Nielsen, 1994, 1995; Kempf et al., 1997; Hay-Schmidt, 2000). If such an interpretation is accurate, then the apical organ is an ancient metazoan neuronal structure indeed.

As the ultrastructure and pharmacological profile of the various invertebrate apical organs continues to be explored, it is becoming evident that in many cases they conform to the general definition of an invertebrate ganglion; that is, they consist of a group of neuronal somata surrounding a neuropil. This is certainly the case in the gastropods examined to date and, consequently, the apical organ will be referred to as the "apical ganglion" hereafter.

The apical ganglion (AG) has been most thoroughly studied in gastropod molluscs, particularly amongst the opisthobranchs. In all cases the apical ganglion is situated between the velar lobes at the level of the shell aperture and is just dorsal to the cerebral commissure. The earliest ultrastructural studies of the apical ganglion in the opisthobranchs revealed a number of striking interspecific similarities. Bonar (1978) identified "flask-shaped" sensory neurons in the apical ganglion of the nudibranch *Phestilla sibogae* that extend an apical neck through the overlying epidermis. They are characterized by a deep, central invagination beginning at the apical surface that gives rise to a lumen packed tightly with internalized cilia. Chia and Koss (1984) described similarly specialized cells in the veligers of another nudibranch, *Rostanga pulchra*, and dubbed them "ampullary cells". They also identified two other potential sensory cell types: ciliary tuft cells, characterized by an apical process projecting through the pretrochal epidermis and ending in a distal swelling that gives rise to a number of cilia, and parampullary neurons which have an apical projection similar to that of the ampullary neurons, but lack the invagination and ciliated lumen. The apical processes of all three cell types are gathered together into three distinct bundles that penetrate the overlying epidermis at discrete locations. More recent ultrastructural investigations of the AG in other opisthobranch species have also revealed these three cell types organized in much the same way (Marois and Carew, 1997a; Kempf et al., 1997).

Immunocytochemical assays for serotonin-like antigens within the apical ganglion of opisthobranch larvae have also shown pronounced interspecific

similarity. In all opisthobranch veligers thus far examined, there is a characteristic grouping of five serotonergic somata in the AG: a lateral pair of sensory neurons, a lateral pair of non-sensory neurons, and a medial sensory neuron (Marois and Carew, 1997a,b,c; Kempf et al., 1997). All these cells extend axons into the very prominent neuropil of the AG, a structure that is located immediately dorsal to the cerebral commissure. In addition, serotonergic neurites project anteriorly and then radiate throughout the velum (Marois and Carew, 1997a,c; Kempf et al., 1997). In larvae of *Aplysia californica*, serotonergic velar axons form a single synaptic bouton beneath each of the ciliated cells of the prototroch, the main ciliary band of the velum (Marois and Carew, 1997c).

These velar serotonergic tracts also contact a number of muscle fibres within the velum (Marois and Carew, 1997c; Kempf et al., 1997). Phalloidin is a toxin that binds to filamentous actin (f-actin) and, when conjugated with a fluorophore, is an effective stain of actin filaments (Wulf et al., 1979). Phalloidin has been used to identify myofibrillar actin filaments in the larvae of the gastropod *Haliotis rufescens* (Naganuma and Degnan, 1994) and to investigate the development of the musculature in the limpet, *Patella caerulea* (Wanninger et al., 1999). The present study will use phalloidin to map similar elements in the velum of *T. cancellata.*

Kempf et al. (1997) suggested that the similarity of structure and neurochemistry in the AG, particularly among protostome groups, denotes homology. They further suggested that continued comparative studies of the AG could provide useful data for construction of phylogenetic hypotheses,

particularly among the gastropod molluscs. Current phylogenies for the Gastropoda regard the Heterobranchia (a consolidation of the opisthobranchs and pulmonates) and the Caenogastropoda (a consolidation of the Meso- and Neogastropoda) as sister clades (e.g. Ponder and Lindberg, 1997; Harasewych et al., 1998; Winnepenninckx, 1998). Since the opisthobranch AG has been studied in detail, an appropriate place to collect further comparative ultrastructural and neurochemical data would be among the caenogastropods. Such studies have in fact begun: Uthe (1995) delivered the first ultrastructural study of the AG in a caenogastropod veliger (*Littorina littorea*), Dickinson et al. (1999) described apical serotonergic cells in embryonic *Crepidula fornicata*, and Page and Parries (2000) provided a description of the ultrastructure and serotonergicity of the AG in early veligers of four species of caenogastropods. Again, the morphological similarities are apparent, though the caenogastropods seem to display a greater degree of interspecific variability than do the opisthobranchs. Regardless, Page and Parries (2000) argue that the combination of complexity and similarity within the basic plan of the AG in both the opisthobranchs and caenogastropods indicates a single evolutionary origin for this structure in these two groups.

Previous research also indicates that the AG is a larval-specific structure that rapidly degenerates post-metamorphically (Bonar, 1978b, Lin and Leise, 1996b; Marois and Carew, 1997b). Indeed there has been a long history of speculation that the AG is responsible for detection and neural integration of the cue for metamorphosis (e.g. Bonar, 1978; Chia and Koss, 1984). Hadfield et al. (2000) provided the first experimental data supporting that hypothesis for one

species of opisthobranch, the nudibranch *Phestilla sibogae.* However, observations of post-metamorphic AG degeneration have been made on only a few species, mostly opisthobranchs, and more comparative data are necessary to further assess the ultimate fate of the AG.

Pharmacological Profile of Larval Nervous Systems

Increased interest in the biology of larval nervous systems has led to the discovery that a number of neurotransmitters and neuromodulators are present even in the very earliest stages of the life history of many marine invertebrates. Immunocytochemistry in particular, has become a staple of biological inquiry and by elucidating neurochemical distribution, has proven especially useful in studies of the nervous system (e.g. Bisgrove and Burke, 1986, 1987; Kempf et al. 1991, 1992, 1997; Goldberg and Kater, 1989; Elphick et al., 1989; Diefenbach and Goldberg, 1990; Thorndyke et al., 1992; Hay-Schmidt, 1989, 1990a,b,c, 1992, 1995, 2000; Marois and Carew 1997a,b,c; Diefenbach et al., 1998; Dickinson et al., 1999; Page and Parries, 2000). Other histochemical techniques have proven valuable in this task as well (e.g. Lin and Leise, 1996a,b; Croll et al., 1997; Serfözö et al. 1998), particularly for elucidating the location of neuroactive substances, such as catecholamines or nitric oxide, for which reliable antibodies are not available.

Gastropod embryos and larvae examined to date have a robust pharmacological profile. Neuroactive substances thus far identified include small cardioactive peptides (SCP) (Barlow and Truman, 1992; Kempf et al. 1992), the tetrapeptide Phe-Met-Arg-Phe-amide (FMRFamide) (Kempf et al. 1992; Croll

and Voronezshkaya, 1995,1996; Dickinson, 1999), catecholamines (Croll and Chiason, 1990), serotonin (5-hydroxytryptamine, 5-HT) (Goldberg and Kater, 1989; Kempf et al., 1991, 1997; Goldberg et al., 1994; Marois and Carew, 1997a,b,c; Diefenbach et al., 1998; Page and Parries, 2000) and nitric oxide (Lin and Leise, 1996a.b) among others. The majority of studies concerning these early neuronal elements in the Gastropoda have been conducted with species from the Heterobranchia, and comparative data from the Caenogastropoda would be of use in furthering our understanding of molluscan neurodevelopment. Consequently, a portion of the present study was dedicated to examining the distribution of certain neuroactive substances in the developing nervous system of *T. cancellata* as well as several other caenogastropod species. In particular, assays for serotonin, FMRFamide, dopamine ß-hydroxylase and nitric oxide were carried out.

The present study was undertaken to: 1) provide a description of the larval development of *Trichotropis cancellata*, 2) provide further information on the pharmacological profile of the larvae of *T. cancellata* and caenogastropods in general, 3) examine the relationship between the developing musculature and serotonergic nervous system of *T. cancellata,* 4) provide comparative data on the serotonin immunoreactivity and structure of the caenogastropod AG, and 5) assess the post-metamorphic fate of the AG in *T. cancellata*.

Materials and Methods

Larval Cultures

Adults of *Trichotropis cancellata*, the principle study organism of this investigation, were collected by Dr. Bruno Pernet (University of Washington, 1999) and Ms. Erika Iyenegar (Cornell University, 2000) from waters surrounding San Juan Island, Washington and maintained in the sea water tables at the Friday Harbor Laboratories, University of Washington. Egg masses were deposited in the sea tables in late-winter/early-spring and were subsequently transported to facilities at the University of Victoria. Egg collars of *Euspira lewisii* were hand collected from the intertidal zone of Patricia Bay, near the northwest corner of the Saanich peninsula, Vancouver Island. *Lacuna vincta* egg masses, deposited on fronds of the brown rock kelp *Fucus distichus*, were collected from the lower midlittoral zone on rock outcrops within Margaret's Bay and Arbutus Cove on southeastern Vancouver Island. Egg masses of *Marsenina stearnsii* were collected by Dr. Louise Page, also at Margaret's Bay, and were found associated with the compound ascidian, *Trididemnum opacum*, on which adults of the species feed. Adults of *Amphissa columbiana* were hand collected from the Ogden Point Breakwater in Victoria, British Columbia and were maintained in the recirculating sea water system at the University of Victoria. *A. columbiana* egg capsules were deposited in the sea tables during the spring.

In 1999, natural seawater for the maintenance of egg masses, larvae, and algal cultures was hand collected from the ocean. Ten Mile Point, a coastal headland on southeastern Vancouver Island extending into Haro Strait, is subject

to substantial tidal currents and therefore provided a well-mixed source of seawater. In 2000, improvements in the water quality in the recirculating seawater system at the University of Victoria allowed for its substitution in the maintenance of study organisms.

In the laboratory, egg masses of all species examined were removed from the substratum and placed in vessels containing coarse-filtered (~0.8μm) seawater. The vessels were continuously aerated, maintained at ~10-12 °C, and were observed daily for signs of hatching. The seawater was poured or siphoned off very 1 or 2 days and replaced with freshly filtered seawater.

All veligers studied during the course of this investigation were planktotrophic and needed to be fed unicellular phytoplankton. Consequently, algal cultures were maintained in concert with the larval cultures. During the first year of study, only the golden-brown alga, *Isochrysis galbana*, was cultured as a food source for the larvae. In 1999, algal culturing was carried out in 500mL Erlenmeyer flasks containing approximately 400mL of 0.45μm Millipore filtered seawater (MFSW) enriched with Fritz Algal Media constituents A and B (Fritz Chemical Co., Dallas, Texas). Once inoculated, the cultures were maintained under constant fluorescent illumination at room temperatures (ca 20°C) with constant aeration. Every 5-7 days fresh medium was prepared and inoculated with algae from older cultures. In 2000, separate cultures of another golden-brown alga, *Pavlova lutheri*, and a red alga, *Rhodomonas salina*, were cultured to increase the complexity and quality of the larval diet. Culture methods were virtually identical to those used in the first year, except that laboratory prepared

Provasoli's media was substituted for the Fritz media, and separate stock cultures were maintained in order to reduce the risk of contamination during the inoculation of fresh cultures. All glassware involved in algal culturing was autoclaved prior to use.

Unicellular algae were fed to the larvae at concentrations of 5×10^4 cells mL^{-1} for the first week of growth and at 1×10^5 cells mL^{-1} subsequent to that. A haemocytometer was used to determine the cell density of a given algal culture or mixture and to calculate the volume of algae needed to obtain the appropriate concentration for larval cultures. Prior to introducing the algae to larval cultures, it was centrifuged at a speed of 2300 rpm for 6 minutes in order to remove the algal culturing medium and then re-suspended in fresh, coarse-filtered (~0.8 μm) seawater. Feeding occurred concurrently with the changing of larval culture seawater, a process described below.

Several cultures of the various study animals were reared over the course of this investigation. The length of the larval period varied from species to species and was also dependent on diet; larvae reared with the mixed diet became metamorphically competent earlier than those reared on *I. galbana* alone. As the developmental process unfurled, individual cultures or portions thereof were fixed for subsequent analytical procedures.

Upon hatching, a 64 μm or 93 μm Nitex sieve was used to collect the larvae, and these were subsequently pipetted into culture vessels containing 500mL of coarse-filtered (~0.8 μm) seawater and the appropriate amount of algae. The sieves were constructed in the laboratory by removing the bottom of a 150mL

disposable plastic beaker and replacing it with a piece of Nitex screening fixed in place with silicone glue. Initially, larvae were maintained at densities of up to several hundred individuals per culture vessel. This number was reduced quite rapidly over the first several seawater changes so that culture densities ranged from approximately 100-200 larvae per vessel (0.2-0.4 larvae ml^{-1}). Seawater changes were performed by submerging the bottom of a sieve into a seawater-filled Pyrex dish. A given culture could then be carefully poured into the sieve, allowing soiled culture water to be removed while retaining the larvae. Once in the sieve, larvae were examined using a dissecting microscope and pipetted into clean culture vessels containing freshly filtered seawater and an aliquot of algae. Bacterial infestations were controlled by the administration of an antibiotic solution. A stock solution of 0.5 g penicillin G (Sigma Chemical Co., St. Louis, MO) and 0.6 g streptomycin sulphate (Sigma) in 100 mL of MFSW was prepared and frozen. This solution, once thawed, could then be added at a proportion of 1 mL stock to 100 mL culture water. Antibiotics were only used when necessary, as there are some potential teratogenic side effects, particularly for the younger, less-hardy larvae. Culture temperatures varied depending on the species being reared. *T. cancellata* and *A. columbiana* were reared at both 12°C and 16°C, *E. lewisii* was reared at 20-22°C (Pedersen and Page, 2000), and *M. stearnsii* and *L. vincta* were both reared at 12°C only. All species were cultured through metamorphosis to feeding juveniles, indicating that development under laboratory conditions was normal.

Fixation and Experimental Protocols

Anaesthetization

Due to the propensity to withdraw into the larval shell, larvae had to be extensively relaxed prior to fixation. Anaesthesia was accomplished with the use of a high magnesium/low calcium artificial seawater solution (225 mM NaCl, 5 mM KCl, 102 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$). Larvae were placed into 10 mL glass fixation vials containing 1 part anaesthetizing solution to 3 parts natural seawater at 4°C. The proportion of anaesthetizing solution was gradually increased over 2-3 h until the larvae were exposed to neat relaxant. This exposure lasted for approximately 1 h at which point the majority of the relaxant was aspirated off. An ice-cold, saturated solution of chlorobutanol in seawater was then added drop-wise over several minutes to a maximum of 12-15 drops. Observation of the relaxed larvae under a dissecting microscope showed the velum to be fully extended with a disrupted metachronal ciliary beat. Anaesthetized larvae were then exposed to the appropriate primary fixative as described below.

Histological Sections

Primary fixation was carried out over 1-2 h at room temperature using 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.6) and 0.14 M NaCl. Fixed larvae were stored at ~8°C for up to several weeks. Prior to the embedding process, it was necessary to decalcify the fixed larvae with a 1:1 mixture of primary fixative and 10% (by weight) ethylenediaminotetraacetic acid, disodium salt (EDTA). The EDTA in this solution rapidly sequesters calcium

ions from the larval shell and statocysts, making it possible to cut sections through the entire animal. Spent solution was periodically aspirated off and replaced with fresh EDTA mixture. Decalcification required several hours to overnight, depending on the species in question and the age of the veliger or post-larva being examined.

Following decalcification, larvae were rinsed several times (at least three) in 2.5% sodium bicarbonate ($NaHCO_3$) buffer (pH 7.2) and post-fixed for 1 h in 2% osmium tetroxide (OsO_4) in 1.25% $NaHCO_3$ buffer. Specimens were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 95%, 3x 100%) for 12-15 min per wash, rinsed three times in propylene oxide (PO), and infiltrated overnight in a 1:1 mixture of Epon epoxy resin and PO, followed by 8 h in neat Epon epoxy. Larvae were transferred into shallow plastic dishes and the Epon resin was polymerized at 60°C for 24-48 h. Semi-thin (1 μm) histological sections were cut with glass knives using a Reichert U2 ultramicrotome and stained with Richardson's stain (1% methylene blue and 1% azure blue in 1% sodium borax (Richardson *et al.* 1960). Silver-gold sections for transmission electron microscopy (TEM) were cut by Dr. Louise Page with a Diatome diamond knife, also on a Reichert U2 ultramicrotome. Thin sections were stained with 2% aqueous uranyl acetate followed by 0.2% lead citrate and were viewed using an Hitachi 7000 electron microscope.

Some specimens were embedded in JB-4/Immunobed (glycol methacrylate). This slightly softer resin allowed cutting of larger and thicker sections. Fixation and dehydration was identical to that used for Epon, except

that the PO rinses were eliminated as glycol methacrylate is water-soluble. Infiltration was carried out overnight at 4°C on a shaker table. Polymerization was carried out at room temperature in mould-forms that attached directly to the microtome holder. Sections from 1-4 μm in thickness were cut using glass knives on a JB4 microtome. Staining was the same as for Epon epoxy sections.

Scanning Electron Microscopy

Preparation of specimens for scanning electron microscopy was much the same as that for histological sectioning and TEM. Specimens were anaesthetized, fixed, and dehydrated following the same protocol with the following exceptions. In an effort to reduce contamination of specimens by debris, fewer numbers of embryos, larvae or post-larvae (5-50 per vial) were processed at a time, the buffers and anaesthetizing solution were passed through a 0.45 μm membrane filter mounted on a 10 cc syringe, and the ethanol solutions were coarse filtered prior to use. Decalcification was unnecessary and therefore omitted. Following fixation and dehydration, specimens were critical point dried using liquid carbon dioxide as the transitional fluid. Small dabs of nail polish were applied to SEM stubs and allowed to partially dry. Specimens were individually mounted onto the stubs by using a fine sable brush to place them onto the tacky dabs of nail polish. The stubs were sputter-coated with gold and viewed with an Hitachi S3500N scanning electron microscope.

Immunocytochemistry - Fluorescence

Fixations of anaesthetized specimens for immunocytochemical assay were carried out for 2-3 h using a chilled solution of 4% paraformaldehyde in 0.2 M Millonig's phosphate buffer (pH 7.6) and 0.14 M NaCl. Following fixation, specimens were rinsed several times (at least 3) in phosphate buffered saline (PBS; pH 7.3) containing 0.1% (by weight) sodium azide (NaN_3) and were stored in this solution at ~4°C until immunolabelling experiments were begun, a period ranging from overnight to several weeks. The cephalopodium of some larvae, particularly larger and older specimens, was removed from the visceral hump using a pair of fine stainless-steel insect pins mounted onto orange sticks with epoxy. This completely removed the shell and also provided direct access to the neuronal structures of interest, which greatly improved penetration by labelling reagents. In the earliest immunolabelling experiments, specimens were passed through a dehydration-rehydration series of ethanol solutions (30%, 50%, 70%, 90%, 70%, 50%, 30%) at 7 min per rinse in an effort to increase membrane permeability and facilitate subsequent antibody penetration. Later experiments deemed this to be unnecessary and it was therefore omitted. Specimens were rinsed twice in PBS containing 0.1% (by weight) NaN_3 and 0.1% (by volume) Triton-X-100 (PBS+). Regardless of the ensuing assay, all specimens were subjected to a preliminary incubation in 3% heat-inactivated goat serum in PBS+ (GS-PBS+) in order to block non-specific binding sites to the secondary antibody. All antibodies were diluted in GS-PBS+ and all incubations were carried out at 4°C with agitation on a shaker table unless otherwise noted.

Following blocking, specimens were incubated for 12-16 h in either a polyclonal rabbit anti-serotonin primary antibody (Incstar, Stillwater, MN) diluted at 1:900, polyclonal rabbit anti-FMRFamide primary antibody (Chemicon, Temecula, CA) diluted at 1:1000, or polyclonal rabbit anti-dopamine ß-hydroxylase primary antibody (Chemicon) in dilutions ranging from 1:250 to 1:2500. This was followed with at least five rinses in PBS+ over several hours.

The specificity of serotonin antibodies was assessed by preabsorption with a range of concentrations of a serotonin (Sigma) solution. In the control experiments, a 1:900 dilution of the preabsorbed antibodies were used in place of the primary antibodies. Negative controls, in which the primary antibodies were omitted from the immunolabelling procedure, were also carried out for both serotonin and FMRFamide assays.

For fluorescence, specimens were incubated overnight in polyclonal goat anti-rabbit immunoglobulin (IgG) secondary antibody conjugated to the fluorophore Alexa-488 (Molecular Probes, Eugene, OR) diluted 1:400 in GS-PBS+. After several rinses in PBS+, specimens were mounted either in Slow Fade (Molecular Probes) or in a laboratory prepared anti-fading media (1:1.5 PBS to glycerol containing 0.1% NaN_3 and 0.3% n-propyl gallate). Coverslips were often propped upon plasticine supports to minimize damage to wholemount specimens and were also commonly sealed to the slide surface with petroleum jelly to prevent dry-out. Mounted specimens were viewed either on a Zeiss Axioskop with epifluorescence capability or a Zeiss 410 laser scanning confocal

microscope, both operated with excitation and barrier filters appropriate for fluorescein isothyocyanate (FITC).

Immunocytochemistry – Histological sections of anti-5HT labelled specimens

For semi-thin (1 μm) sectioning, specimens were processed through to primary antibody as described above with the addition of a decalcification step following the initial fixation. Decalcification was accomplished by immersing the specimens in 5% EDTA in 0.2 M Millonig's phosphate buffer and 0.14 M NaCl for two to several hours. The majority of sections cut in this instance were through the anterior portion of the animal, therefore only the apertural rim of the shell needed to be completely decalcified. Again, some specimens had the cephalopodium dissected away from the visceral hump as described above.

Once the primary antibody incubation and ensuing rinses were complete, specimens were incubated overnight in biotinylated anti-rabbit IgG secondary antibody (Histostain-SP kit, Zymed, San Francisco, CA) and then rinsed several times in PBS containing 0.1% Triton-X-100. This was followed by an overnight incubation in streptavidin-horseradish peroxidase conjugate (Zymed kit) and then several rinses in PBS + 0.1% Triton-X-100. Specimens were then pre-incubated in a 0.05% diaminobenzidine tetrahydrochlroide (DAB kit, DAKO Diagnostics Canada Inc., Mississauga, Ont.) in PBS for 1 h in the dark at room temperature with agitation. The pre-incubation solution was removed and replaced with a substrate solution of 0.05% DAB and 0.02% H_2O_2 in kit-provided buffer for 5-20 minutes. Several rinses in PBS+ halted the reaction. Dehydration, infiltration,

embedding, and the cutting and staining of sections were carried out as described above for normal histological sections.

Anti-5HT immunofluorescence and Alexa-488 Phalloidin Assay

The relationship between serotonergic axons and larval muscles in *T. cancellata* larvae was investigated using antibodies to serotonin and a marker for filamentous actin. Larvae were fixed and fluorescently labelled for serotonin exactly as described above except that the secondary antibody was conjugated to the fluorophore Alexa-546 rather than Alexa-488, and that the dehydration-rehydration step was omitted because it adversely affected the ensuing assay for f-actin.

After being labelled for serotonin and rinsed several times in PBS+, specimens were immersed in a 1:40 dilution of Alexa-488 phalloidin (Molecular Probes) in PBS+ for 1-2 h. This was followed with a series of rinses in PBS+. Specimens were mounted as described above and viewed on a Zeiss Axioskop equipped with both a rhodamine filter set (Alexa 546) and an FITC filter set (Alexa 488).

NADPH-diaphorase (NADPHd) Histochemistry

NADPHd is identical to nitric oxide synthase (NOS), the enzyme responsible for the production of nitric oxide (NO) (Dawson et al. 1991). The application of the NADPHd histochemical technique causes a blue formazan precipitate to accumulate in neurons containing NOS (Elofsson *et al.*, 1993). An

attempt to assay larvae of *T. cancellata*, *A. columbiana*, and *M. stearnsii* for NOS was carried out during the course of this study.

Larvae were decalcified prior to fixation so that histological sections could be cut once the histochemical reaction was complete. This was accomplished by incubating the larvae overnight in Millipore filtered MBL artificial seawater (Strathmann, 1987) without bicarbonate and buffered to ~pH 7.0 with 10 mM Trizma (Pires and Hadfield 1993). Larvae were relaxed as described in the anaesthetization section above and then fixed for 1 h in chilled 4% paraformaldehyde in 0.2 M Millonig's phosphate buffer (pH7.6) and 0.14 M NaCl. This was followed with 3 rinses in PBS and a subsequent incubation in the histochemical reagents, a freshly prepared solution of: 1 mM ß-NADPH, 0.5 mM Nitroblue tetrazolium (NBT), 0.1 mM dicumoral and 0.1% Triton-X-100 in a 0.5 M tris buffer at pH 8.0 (all reagents from Sigma). This incubation was carried out at room temperature, in the dark, with agitation for 3 h. Control incubation solutions were prepared either by eliminating the ß-NADPH or by replacing it with ß-NADH. Following the incubation, larvae were rinsed three times in tris buffer, and then dehydrated, infiltrated and embedded in EPON epoxy as described earlier. Histological sections (1 µm thickness) were cut on a Reichert U2 ultramicrotome and stained either with Richardson's stain, a 1% thionin solution, or basic fuchsin.

Results

Observations on the Development of *Trichotropis cancellata*

The veligers of *Trichotropis cancellata* are obligate planktotrophs, living and feeding as pelagic larvae for a considerable period of time before settling and metamorphosing into benthic juveniles (Fig. 1). During this period the animals undergo a progressive series of morphological changes, acquiring features necessary for both larval and adult life. The length of the larval period is variable, and consequently, the degree of differentiation exhibited by a given individual is not reliably indicated by age. In 1999, cultures took 42-45 days to reach metamorphic competency, whereas changes to the larval diet in 2000 reduced the larval period to 28-32 days. The following will describe progressive changes in development at a number of stages: late embryo, hatching veliger, post-hatching veliger and post-metamorphic juvenile.

Egg Masses and Embryological Observations

Egg masses consist of a number of individual capsules that are attached to the substrate in the form of an irregular spiral (Fig. 2A). The capsules are ovoid, but their shape is somewhat distorted as they are moulded against neighbouring capsules during egg mass formation. The number of capsules per mass varies, generally ranging from 10-20. The walls of each capsule are approximately 70 μm thick, nearly transparent and slightly striated. Individual capsules are approximately 4-5 mm in width and 3-4 mm in height. The bright yellow developing embryos are originally contained within a membrane inside the capsular wall (Fig. 2A), keeping them in a well-defined mass in the centre of the

Figure 1. Diagrammatic representation of the life history of *Trichotropis cancellata.* Sketches are not drawn to scale.

A. Adult snail perched near the aperture of the calcareous tube of a polychaete host, *Serpula vermicularis.*

B. Benthic egg mass attached to substrate.

C. Newly hatched free-swimming veliger

D. Metamorphically competent free-swimming veliger. Note the echinospira shell.

E. Newly settled and metamorphosed juvenile snail in the process of migrating to a polychaete host.

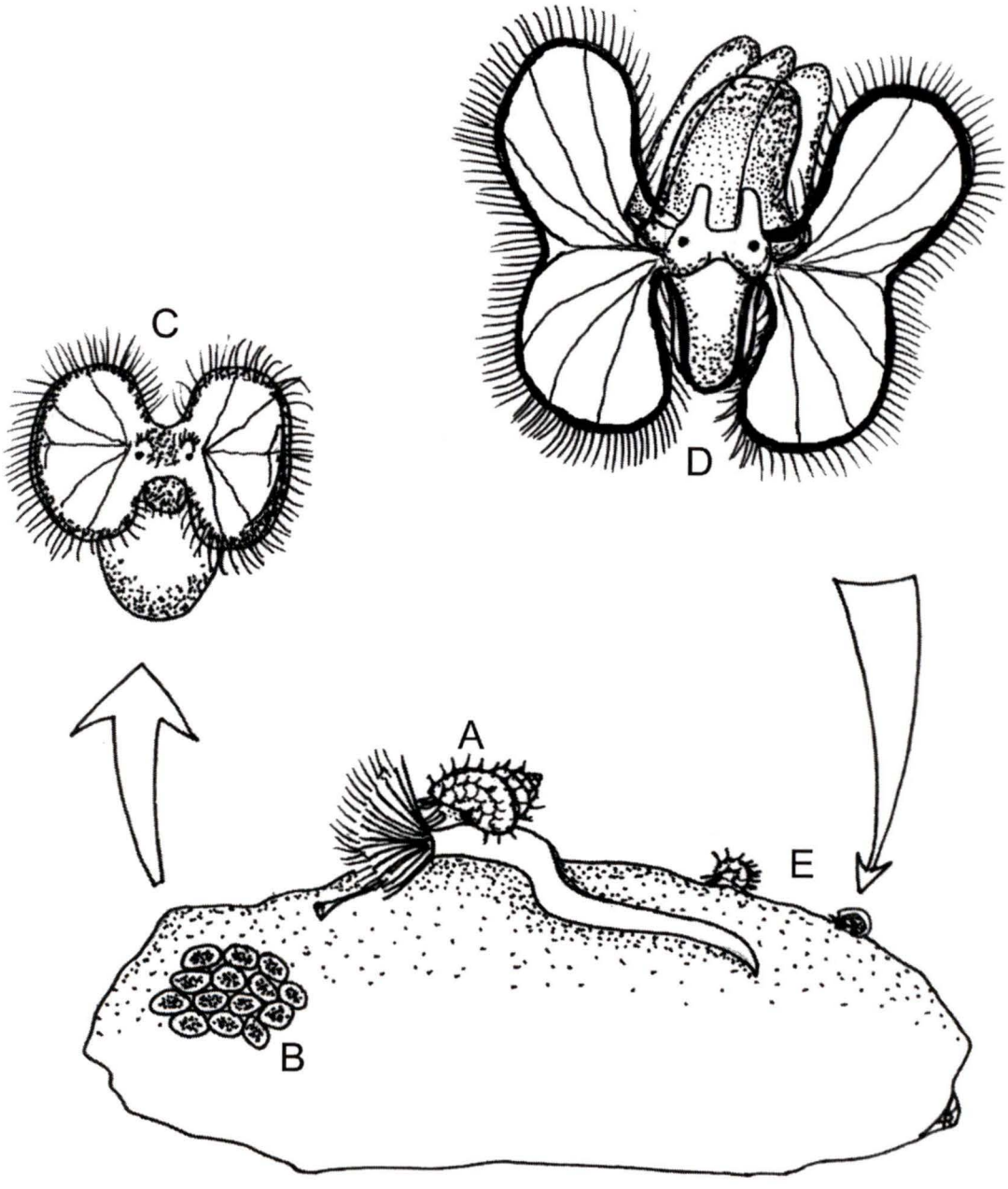
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Figure 2. Egg mass and embryonic stages of *Trichotropis cancellata*.

A. Egg mass of *T. cancellata* consisting of 15 individual capsules. Early embryos are maintained within a membrane inside of each capsule keeping them in a well-defined, centralized mass. Scale Bar = 3mm.

B. Apical view of *T. cancellata* embryo at approximately 30% embryonic development. The rudiments of the foot, prototroch and larval kidneys are visible. Scale Bar = 40μm.

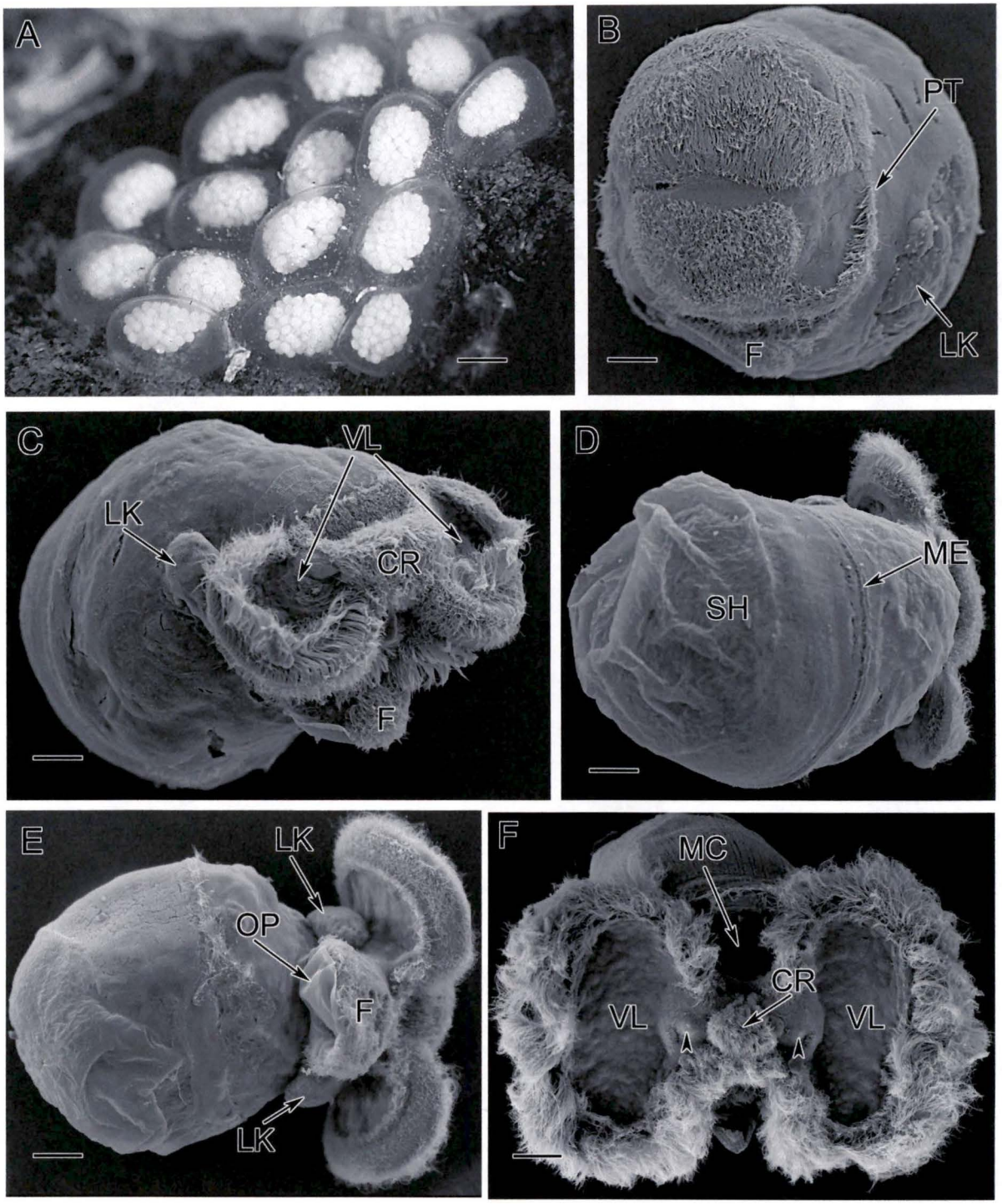
C. Oblique anterior view of *T. cancellata* embryo at approximately 50% embryonic development. The expanding velar lobes, apical ciliary crown, foot, and right larval kidney are visible. Scale Bar = 40μm.

D. Dorsal view of *T. cancellata* embryo at approximately 50% embryonic development showing the developing shell and the ciliated mantle edge. Scale Bar = 40μm.

E. Ventral view of *T. cancellata* embryo at approximately 65% embryonic development showing the foot, operculum, and larval kidneys. Scale Bar = 50μm.

F. Anterior view of *T. cancellata* embryo at approximately 90% embryonic development. Ontogenetic torsion is complete and the mantle cavity is apparent. Arrowheads on either side of the ciliary crown indicate the tentacular rudiments. Scale Bar = 50μm.

Lettering
CR = ciliary crown
F = foot
LK = larval kidney
MC = mantle cavity
ME = mantle edge
PT = prototroch
OP = operculum
SH = shell
VL = velar lobe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capsule. Attempts at counting embryo numbers per capsule were foiled by the often simultaneous hatching of several capsules, but is estimated at 100-150. At what I estimate to be one third of the way through the embryonic phase, the ovoid embryos, approximately 325 by 200 μm in length and width (Fig. 2B), begin rotating slowly within the inner membrane. The inner membrane disappears shortly thereafter, allowing the ciliated embryos to move about within the entire interior of the capsule. Over the next 7 days, the velum begins to develop (Fig. 2C), as do the eyespots. An apical crown of elongate cilia arises between the velar lobes (Fig. 2C,F). The shell also becomes visible, initially resembling a small, convex disk covering the posterior portion of the animal (Fig. 2D,E). The velum continues to enlarge for the remainder of the embryonic phase, some 15-20 days, and red pigment accumulates along its periphery. At hatching, the velum is approximately 450 by 600 μm (Fig. 2F). Near the time of hatching, individual capsules are packed very tightly with embryos and the pigment in the velum changes the overall cast of the egg mass from yellow to red. All embryos in a given capsule develop synchronously and there is no evidence of adelphophagy. Escape holes open at the apex of each capsule, through which the larvae emerge.

The Veliger Larva of *Trichotropis cancellata*

Emergent veligers of *T. cancellata* are torted, have a pair of velar lobes rimmed with red pigment, a pair of eyespots, a small, ciliated foot with a delicate operculum, and a functional digestive tract. Two small protuberances where the cephalic tentacles eventually arise (Fig. 3A,C) are located on either side of the

Figure 3. Early veliger larvae of *Trichotropis cancellata.*

A. Anterior view of a newly hatched veliger of *T. cancellata* showing the velar lobes, ciliary crown, foot and operculum, and tentacular rudiments. Scale Bar = 60μm.

B. Frontal view of a newly hatched veliger of *T. cancellata*. Note the presence of both a pre-oral prototrochal ciliary band and post-oral metatrochal ciliary band. The unelaborated shell, foot and operculum are also visible. Scale Bar = 60 μm.

C. Anterior view of *T. cancellata* veliger at hatching showing the ciliated crown between the velar lobes. Scale Bar = 30μm.

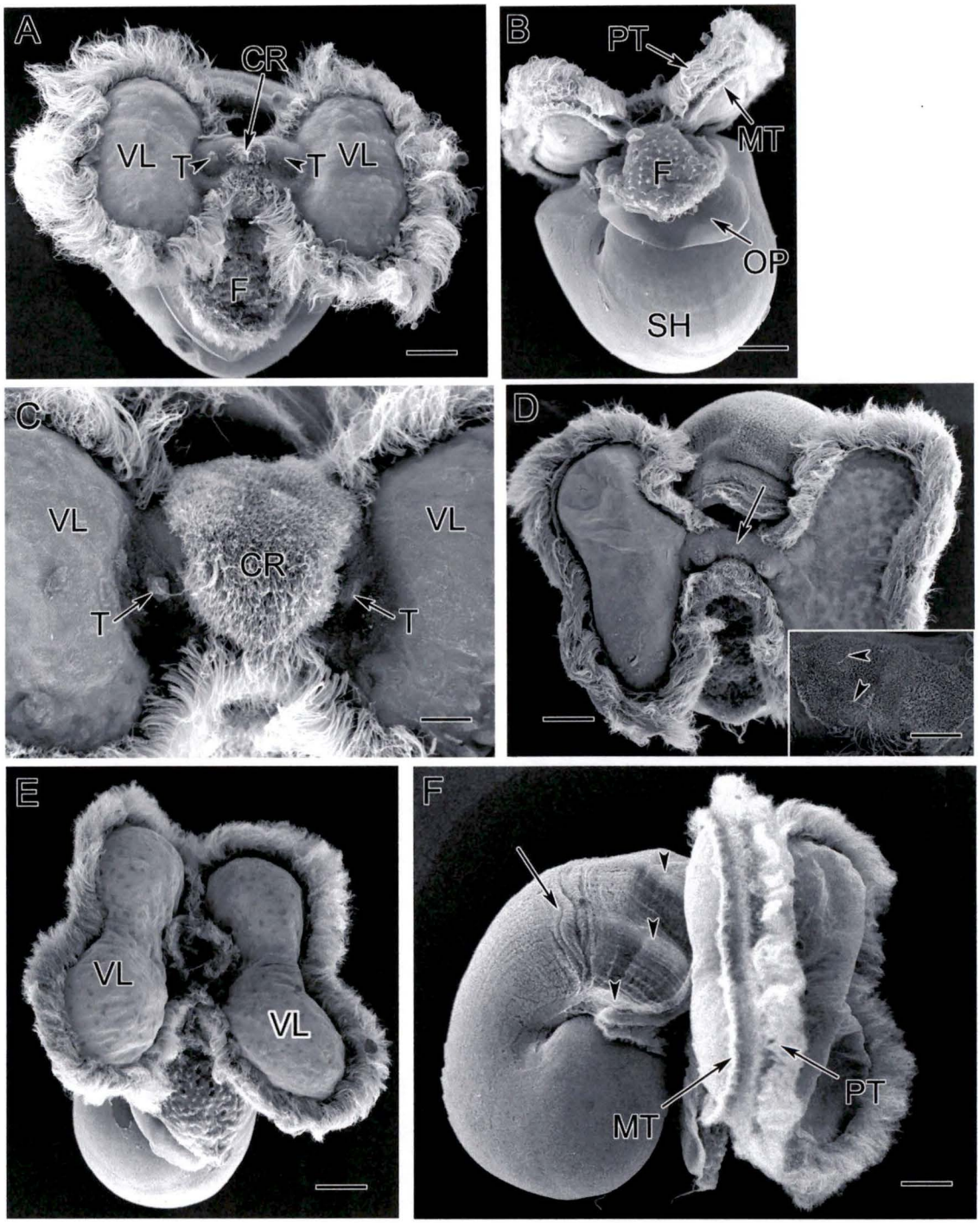
D. Anterior view of *T. cancellata* veliger at hatching in which the ciliary crown is no longer present. Arrow indicates a patch of elongate microvilli revealed once the ciliary crown is lost. Scale Bar = 75μm

Inset shows an enlargement of the microvillar patch. Arrowheads indicate occasional short cilia protruding from the patch of microvilli. Scale Bar = 15μm.

E. Anterior view of *T. cancellata* veliger at 5 days post-hatching showing the definitive butterfly shape of the velum. Scale Bar = 75μm.

F. Right lateral view of *T. cancellata* veliger at 6 days post-hatching revealing the onset of shell elaboration into spiral ridges. The arrow marks the transition from protoconch I (embryonic shell) to protoconch II (larval shell) and the arrowheads indicate individual spiral ridges. Also note the two distinct ciliary bands of the velum. Scale Bar = 60μm.

Lettering
CR = ciliary crown
F = foot
MT = metatroch
OP = operculum
PT = prototroch
SH = shell
T = cephalic tentacle
VL = velar lobe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apical crown of cilia that develops prior to hatching. The crown itself may or may not be present at hatching (compare Fig. 3C and 3D), but it is absent in all larvae by the first few days post-hatching. Once the crown has been lost, an ovoid patch of elongate microvilli with occasional short cilia can be seen between the developing cephalic tentacles (Fig. 3D). The protoconch I is smooth and planorbiform with a relatively thick, unsculptured periostracum. It has approximately 1 whorl, and is 300-350μm in length. Observations of live larvae show the larval heart, a vesicular apparatus, pumping steadily in the floor of the mantle cavity, although contractions are interrupted when animals retract into their shell. The velum has both a pre-oral and post-oral ciliary band extending about the periphery of the velar lobes (Fig. 3B,F). Feeding begins immediately.

Within a few days of hatching, the velum acquires a characteristic butterfly shape, with four broad, blunt lobes, that persists until metamorphosis (Fig. 3E). The red pigment along the periphery of the velum remains obvious for a variable amount of time, so that in some older veligers it remains, where in others it does not. The cephalic tentacles, each distally ciliated, lengthen considerably as development unfolds and are very prominent in competent specimens.

At approximately one quarter of the way through the obligate larval phase, the adult heart becomes evident. It is located posterodorsally, just beneath the floor of the mantle cavity. The adult and larval hearts are distinguishable because their beats are asynchronous. The larval heart persists for the duration of the larval phase, although its activity becomes more sporadic as the animal

approaches competency. The adult heart continues to elaborate during the larval phase and has a strong beat throughout.

When the velum takes on its definitive butterfly shape, the periostracum of the shell elaborates into 3-5 spiral ridges (Fig. 3F, 4A,B). These grow such that the larvae can be considered to be of the echinospira type; that is, the larvae effectively have two shells. The outer shell, or scaphoconch, is colourless and seems almost gelatinous in consistency (Fig. 4A, C-E). Sections through the scaphoconch show the ridges to be riddled throughout with vacuoles. There is also a thin membranous layer lying over the top of the spiral ridges that creates a space within the scaphoconch itself (Fig. 4D,E). The calcified inner shell, the "true shell", remains unsculptured and transparent, and grows in a helicoid spiral.

At approximately halfway through the larval phase, a small pit appears in the apical epidermis, in the middle of the microvillar patch between the cephalic tentacles (Fig. 5A-C). Histological sections show the pit to have an almost dumb-bell like shape, with two larger lobes connected by a short, curved channel (Fig. 5D,E). Microvilli from the patch are carried into the pit as the epidermis invaginates and occasional short cilia protrude from the microvillar patch lining the pit (Fig. 5C). Also at this time, gill lamellae appear along the roof of the mantle cavity (Fig. 5F,G). Beating of gill cilia can be easily observed in whole-mounts of live larvae.

The ventral lip of the mouth elongates during the course of larval development. At hatching, the mouth is merely a ciliated opening at the position where the velar lobes and foot come together. As development proceeds however,

Figure 4. The echinospira shell of *Trichotropis cancellata.*

A. Oblique anterior view of *T. cancellata* veliger at 13 days post-hatching. Note the transparent scaphoconch (accessory shell). Scale Bar = 40μm.

B. Oblique lateral view of *T. cancellata* veliger at 17 days post-hatching. Arrowheads indicate the spiral ridges of the scaphoconch. Also note the transition from protoconch I to protoconch II indicated (arrow). Scale Bar = 125 μm.

C. A sketch of the echinospira shell of *T. cancellata* to show the relationship between the outer scaphoconch and the inner calcified shell. Modified from Pilkington, 1976.

D. Cross section through a metamorphically competent (32 days post-hatching) *T. cancellata* veliger showing the structure of the echinospira shell. The vacuolated scaphoconch ridges are indicated by arrow-heads and the innermost calcified layer is labeled. Also note the membranous layer loosely appressed to the scaphoconch ridges to create a large (presumably fluid-filled) space. Scale Bar = 100μm.

E. Magnified view of the shell shown in D showing the calcified inner shell layer lying in close apposition to the scaphoconch. Scale Bar = 20μm.

Lettering
CSH = calcified shell
E = eyespot
SC = scaphoconch
T = cephalic tentacle

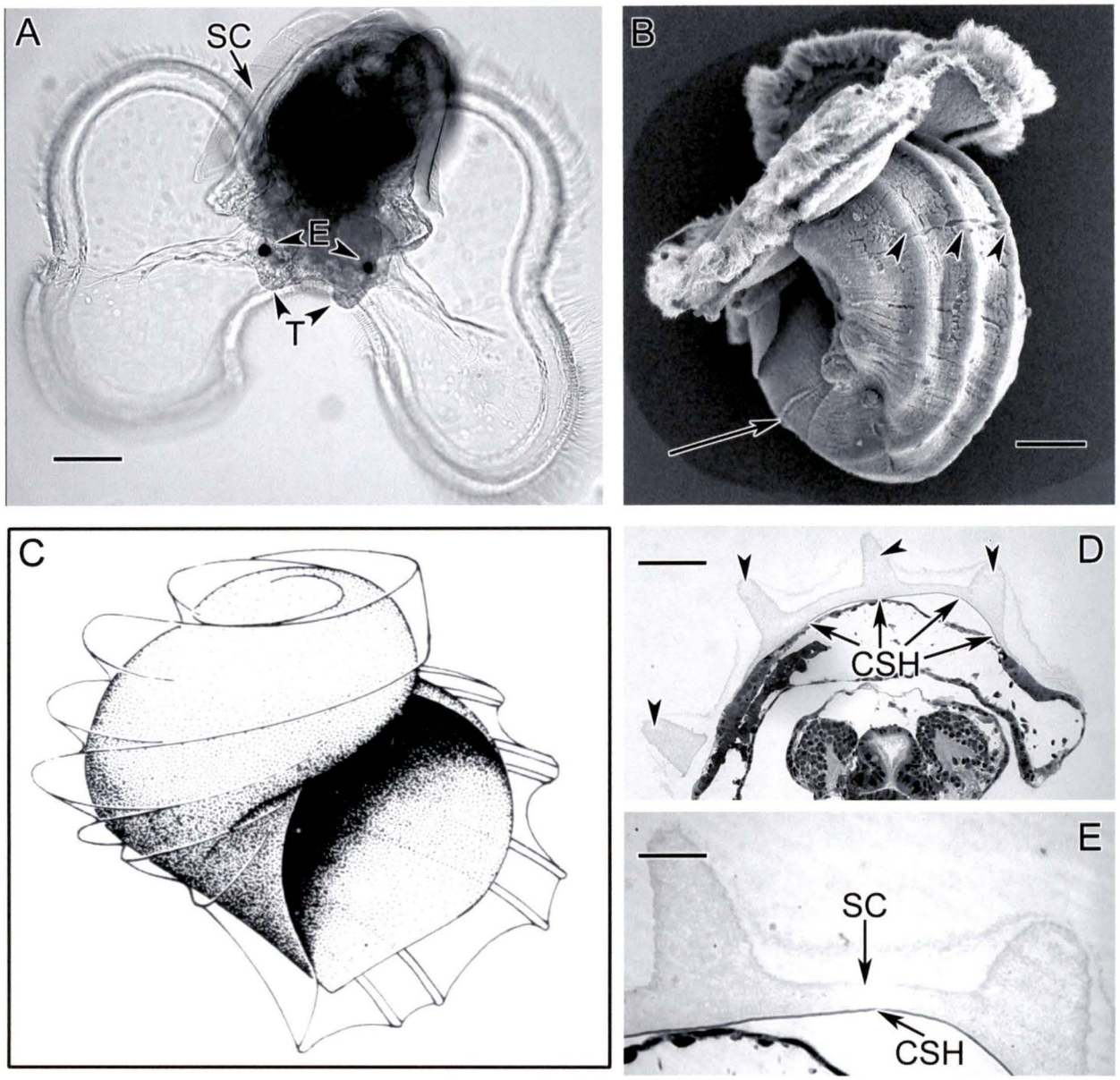
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Figure 5. The apical pit and ctenidial filaments of *Trichotropis cancellata* veliger larvae.

A. Anterior view of *T. cancellata* veliger at 15 days post-hatching. Arrow indicates the location of the apical pit that forms approximately halfway through the larval phase. The apical ganglion lies directly below the epidermis at that location. Scale Bar = 75μm.

B. Anterior view of *T. cancellata* veliger at 15 days post-hatching showing the apical pit between the two cephalic tentacles in the centre of a patch of elongate microvilli. Scale bar = 20μm.

C. Enlargement of the apical pit shown in B. Note that the pit consists of two lateral lobes connected by a narrow gully. Arrowheads indicate occasional short cilia protruding out of the main microvillar patch. Scale Bar = 5μm.

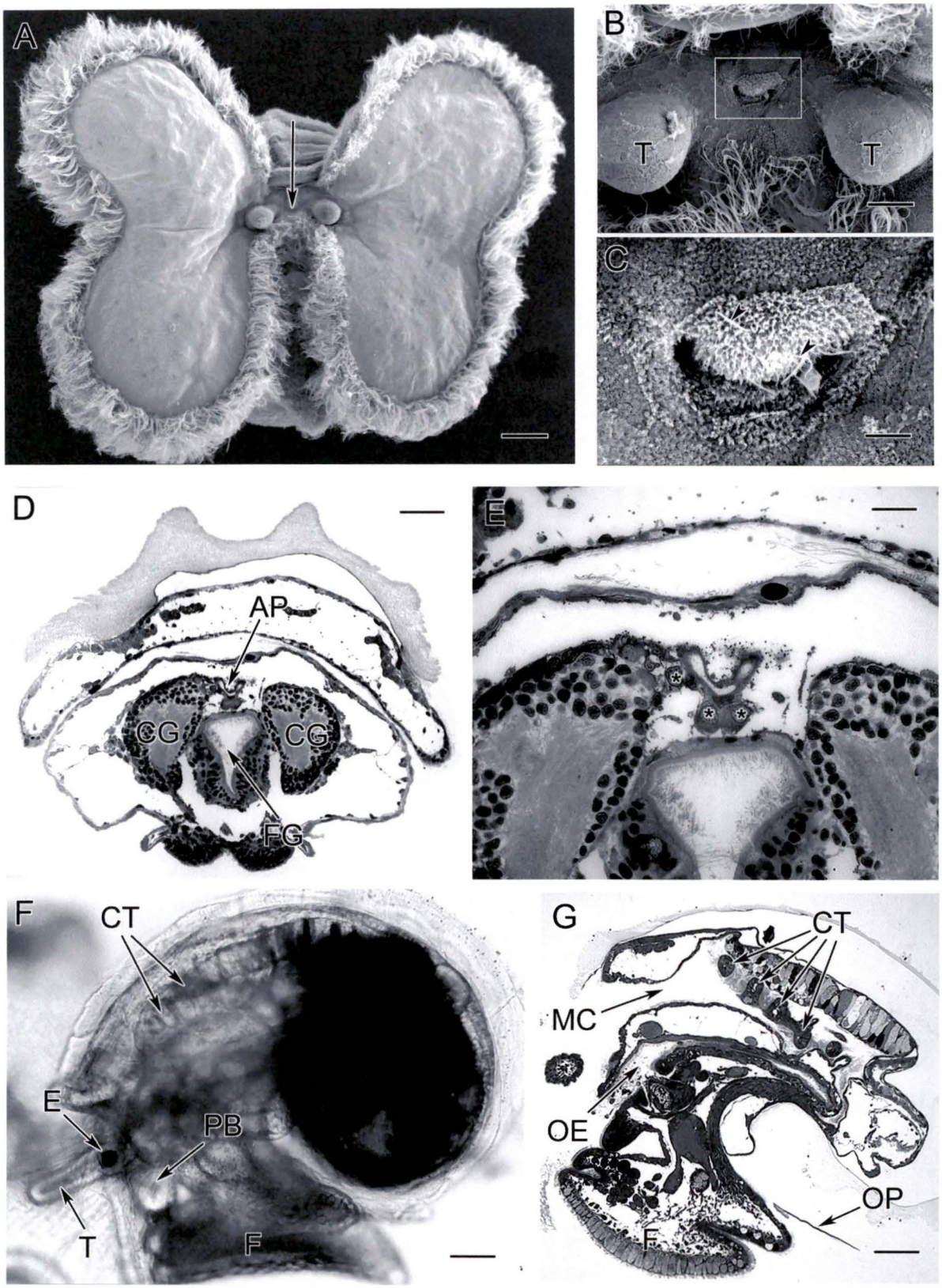
D. Anterior cross section through a late-stage (38 days post-hatching, 1999 culture) veliger larva of *T. cancellata* showing the location of the apical pit between the two cerebral ganglia. Scale Bar = 15μm.

E. Enlargement of the apical pit shown in D. Asterisks indicate cells of the apical ganglion. Scale Bar = 7μm.

F. Left lateral view of late-stage veliger larva of *T. cancellata* showing the elongate cephalic tentacles, eyespots, foot, pseudoproboscis, and ctenidial filaments. Scale bar = 75μm.

G. Median sagittal section of a late stage veliger larva of *T. cancellata* showing the larval oesophagus, foot and operculum, mantle cavity and ctenidial filaments. Scale Bar = 75μm.

Lettering
AP = apical pit
CG = cerebral ganglion
CT = ctenidium
F = foot
FG = foregut
MC = mantle cavity
OE = larval oesophagus
OP = operculum
PB = pseudoproboscis
T = cephalic tentacle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the ventral lip begins to protrude (Fig. 6A). At competency, the ventral lip is somewhat spade-shaped and held flat by its association with the velar lobes (Fig. 6B). It extends from the mouth and often comes into contact with the propodium (Fig. 6C). A strip of cilia runs from the mouth down the midline of the lip (Fig. B). Post-metamorphically, this structure is released from its association with the velum and rolls up transversely to become the pseudoproboscis (Fig. 6D-F). The pseudoproboscis is very extensible, but it did not seem to be withdrawn into the animal at any time. Instead it folds backward on itself and is tucked under the ventral portion of the head when not in use.

The foot enlarges a great deal during the larval phase. At hatching the foot is small and simple, possessing only the metapodial rudiment and having an oval operculum on its dorsal surface (Fig. 7A). The metapodial lobes appear as extensions from the side of the foot that begin forming in the first quarter of larval development (Fig. 7B). The propodium becomes evident in the third quarter of the larval phase. Once the larvae begin using it to crawl about in the culture vessels, they are approaching metamorphic competence (Fig. 7B-D). The sole of the foot is marked by a distinctive stripe of brownish pigment granules down its centre, and the propodium becomes marked by a horizontal notch at its anterior end (Fig. 7E,F). The large transparent operculum is present on the back of the foot throughout.

Competent larvae range in shell length from 750-850 μm, and the velar height and width are 1000 μm and 1200 μm (Fig. 8A). A well-developed radula is also apparent. Metamorphosis can be induced by placing the animals on rocks

Figure 6. The pseudoproboscis of *Trichotropis cancellata.*

A. Anterior view of a metamorphically competent (32 days post-hatching) *T. cancellata* veliger. The outlined area indicates the location of the elongate, spade-shaped ventral lip that ultimately becomes the pseudoproboscis. Scale Bar = 175μm.

B. Enlargement of the area outlined in A to show details of the ventral lip. Note the strip of elongate cilia running down the structure's midline. Scale Bar = 20μm.

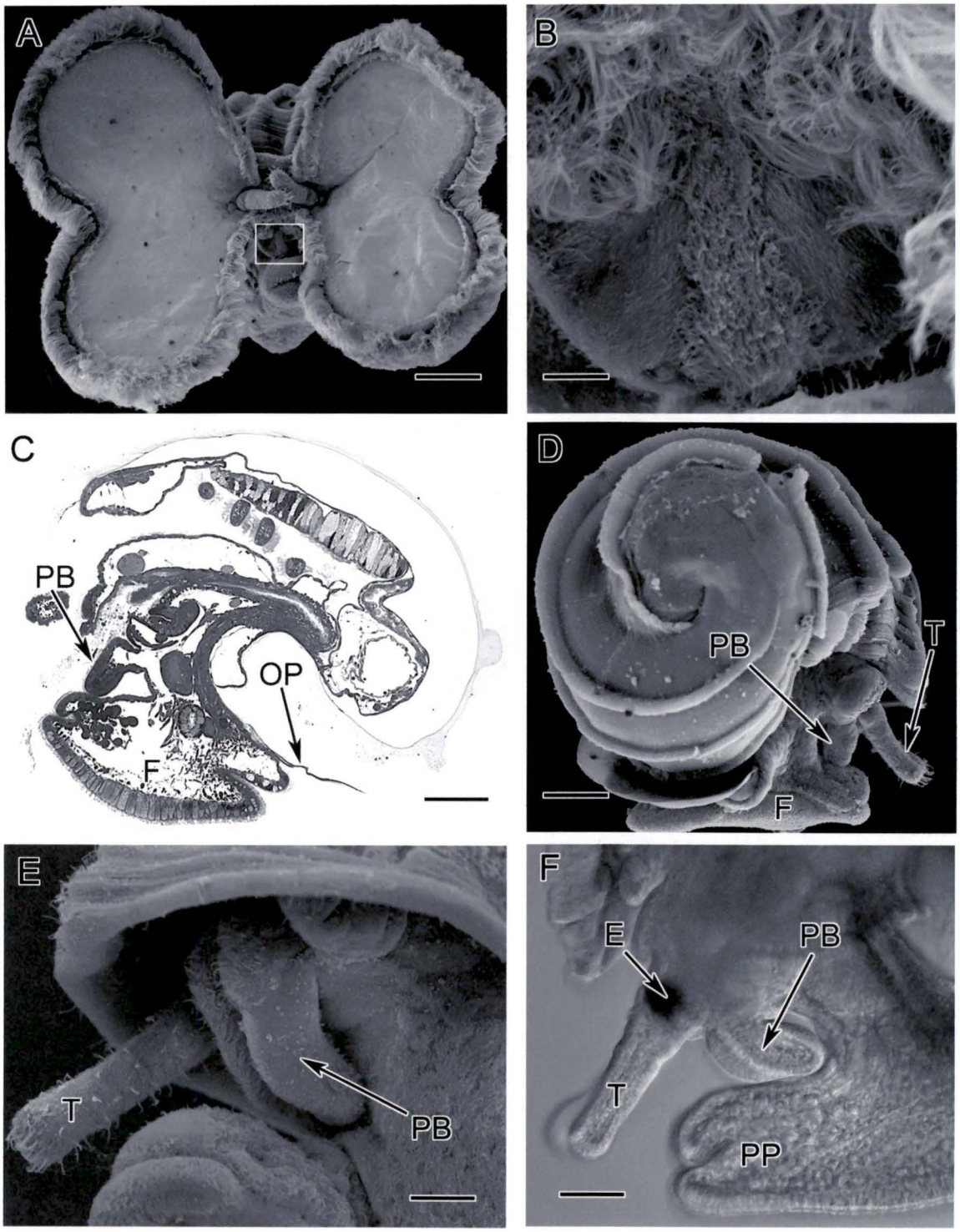
C. Median sagittal section through a late stage veliger larva of *T. cancellata* showing the ventral lip/pseudoproboscis protruding at the base of the mouth. Scale Bar = 75μm.

D. Right lateral view of a *T. cancellata* juvenile 24 hours post-metamorphosis showing the pseudoproboscis extending ventrally form the head. The elongate cephalic tentacles, foot, and shell are also visible. Scale Bar = 200μm.

E. Oblique left lateral view of a partially retracted *T. cancellata* juvenile 3 days post-metamorphosis. The left cephalic tentacle is tucked into the mantle cavity revealing the pseudoproboscis. Note the ciliated central groove that runs down the midline of the pseudoproboscis. Scale Bar = 40μm.

F. DIC/Nomarski image of a left lateral view of the cephalopodium of a *T. cancellata* juvenile 24 hours post-metamorphosis. The pseudoproboscis can be seen jutting out to come into contact with the propodium. Also note the horizontal notch across the anterior end of the propodium. Scale Bar = 40μm.

Lettering
E = eyespot
F = foot
PB = pseudoproboscis
PP = propodium
OP = operculum
T = cephalic tentacle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PB
F
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PB
T
PP

Figure 7. Development of the foot of *Trichotropis cancellata.*

A. Frontal view of emergent veliger of *T. cancellata* showing the foot and operculum. At this stage the foot consists of a metapodial rudiment only. Scale Bar = 75μm.

B. Oblique anterior view of *T. cancellata* veliger 15 days post-hatching (slightly more than half-way through the larval phase) showing the metapodial lobes and developing propodium. Scale Bar = 75μm.

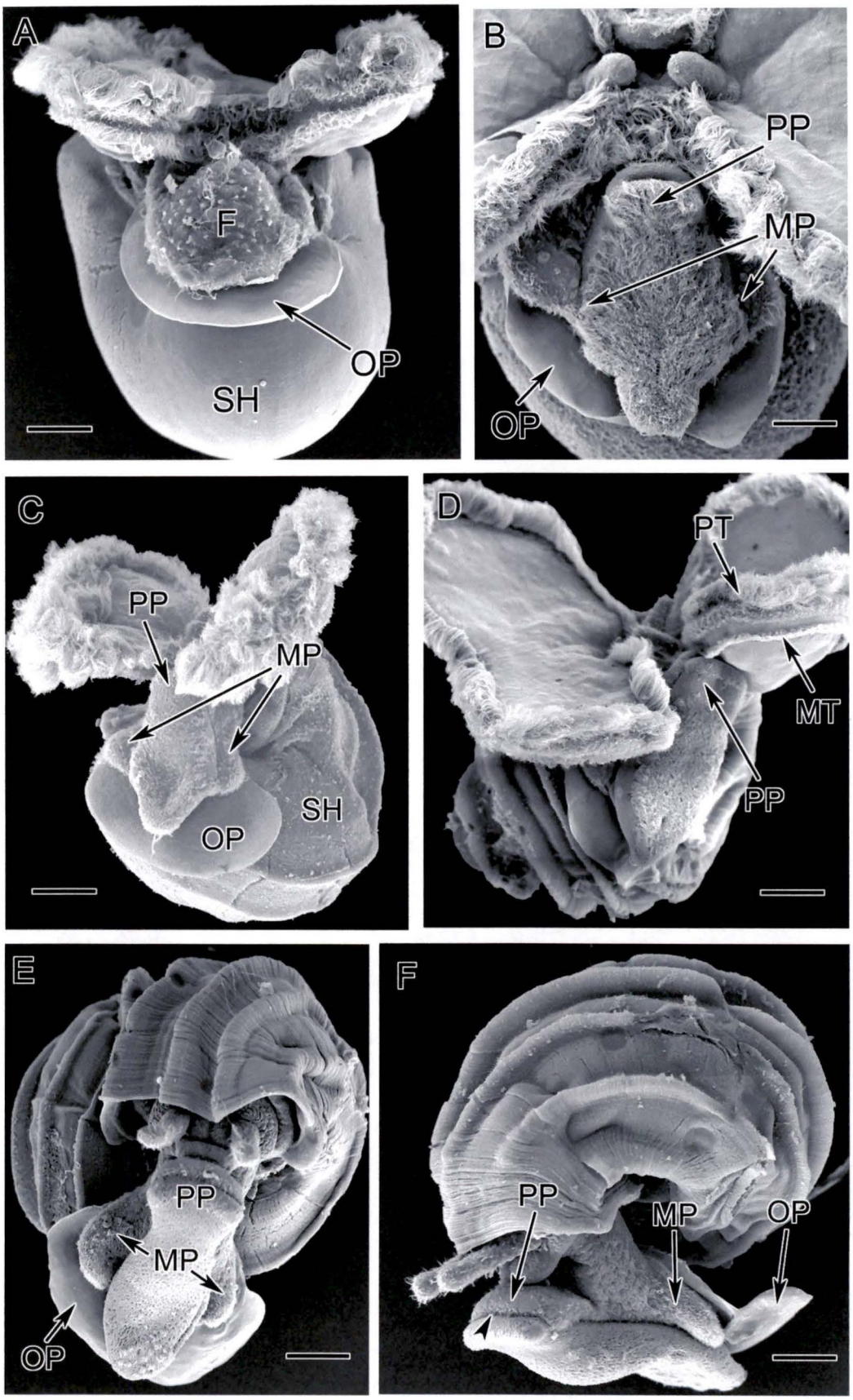
C. Oblique frontal view of a late stage *T. cancellata* veliger. Note the large metapodial side-lobes. The propodium is partially obscured by the left velar lobe. Scale Bar = 150μm.

D. Frontal view of a metamorphically competent *T. cancellata* veliger showing the propodium and metapodial lobes of the foot. At this stage the foot is used to actively crawl on the substrate. Also note the preoral (prototroch) and postoral (metatroch) ciliary bands visible on the left velar lobe. Scale Bar = 175μm

E. Anterior view of a *T. cancellata* juvenile 5 hours post-metamorphosis showing the large propodium and metapodial lobes. Also note the large operculum on the back of the foot. Scale Bar = 175μm.

F. Left lateral view of a *T. cancellata* juvenile 5 hours post-metamorphosis showing features of the foot. Arrowhead indicates the horizontal notch across the anterior edge of the propodium. Scale Bar = 175μm.

Lettering
F = foot
MP = metapodial lobe
MT = metatroch
OP = operculum
PP = propodium
PT = prototroch
SH = shell


A
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MP
OP

Figure 8. Metamorphically competent veligers and juveniles of *Trichotropis cancellata.*

A. Light micrograph of a metamorphically competent veliger larva of *T. cancellata* showing the large, butterfly-shaped velum. Arrow indicates brown pigment granules accumulated along the sole of the foot. Scale Bar = 200μm.

B. Metamorphosing *T. cancellata* veliger. Arrowheads indicate the discarded ciliary bands of the velum. Scale Bar = 250μm.

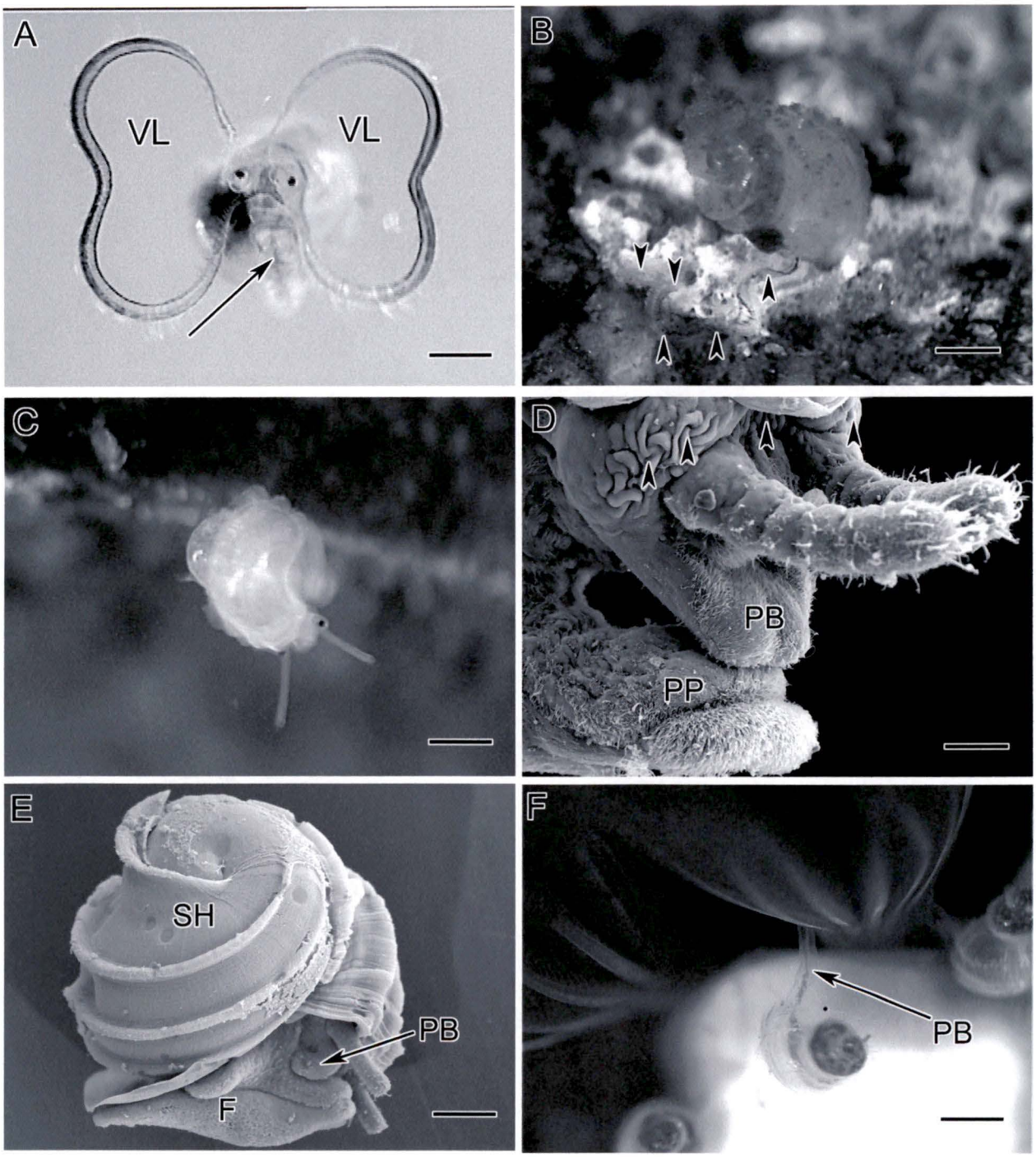
C. Light micrograph of a newly metamorphosed *T. cancellata* juvenile. Note the elongate cephalic tentacles and prominent eyespots. Scale Bar = 300μm.

D. Oblique anterior view of the head of a *T. cancellata* juvenile 9 hours post-metamorphosis. Arrowheads indicate scarring on the lateral sides of the head at the previous site of velum attachment. The elongate, distally ciliated tentacles, propodium and pseudoproboscis are also visible. Scale Bar = 50μm.

E. Right lateral view of a *T. cancellata* juvenile 24 hours post-metamorphosis. The shell consists of 2½ whorls. Scale Bar = 200μm.

F. Light micrograph of newly metamorphosed *T. cancellata* juveniles that have located a polychaete host and are engaging in the kleptoparasitic habit. The ciliated central grove of the pseudoproboscis in the labelled specimen is full of food particles diverted from the polychaete. Scale Bar = 800μm.

Lettering
F = foot
PB = pseudoproboscis
PP = propodium
SH = shell
VL = velar lobe


A
VL
VL
B
C
D
PB
PP
E
SH
PB
F
F
PB

encrusted with either spirorbid or serpulid polychaetes. Larvae crawl about and explore the substratum and, if it is suitable, metamorphose. During the course of metamorphosis, the larvae rock back and forth and twist about on their foot. The periphery of the velum, including the ciliary bands, is discarded as a strip and the rest of the velum is lost in chunks (Fig. 8B). Newly metamorphosed juveniles (Fig. 8C) show tissue scars along the lateral sides of their head, where the velum was attached (Fig. 8D). Metamorphosed individuals have a shell of 2½ whorls and the scaphoconch is retained and becomes incorporated into the adult shell. The cephalic tentacles are elongate and distally ciliated (Fig. 8D). The foot is well developed (Fig. 7E,F and 8E) and individuals immediately migrate to a host polychaete and take up the kleptoparasitic habit, using their pseudoproboscis to steal food from the worms (Fig. 8F). Once a suitable host is found, the snails tend not to move and become essentially sessile; individuals maintained in the laboratory for 5 months did not move from their initial polychaete host.

The Larval Nervous System of *T. cancellata*

I. Serotonin

The development of serotonin-like immunoreactivity was examined in embryonic, larval, and post-larval stages of *T. cancellata*. Pre-absorption of primary antibodies or primary antibody omission controls resulted in a lack of labelling in all cases.

A) Observations on Embryos

The earliest reliable detection of serotonergic immunoreactivity in this study occurred embryonically, in the early post-torsional veliger stage (Fig. 9A-D). The velar lobes have differentiated and begun to grow at this point, as has the foot. The eyespots and larval kidneys are also evident. Two, bilaterally symmetrical, relatively large cells appear between the eyespots, in the region corresponding to the eventual position of the apical ganglion of the larva (Fig. 9A). Neurites project from these cells into a commissure or neuropil-like structure, effectively linking them together (Fig. 9A,B). A third cell with an anterior dendritic projection appears shortly thereafter near the centre of this neuropil, completing the serotonergic compliment of the AG in *T. cancellata* (Fig. 9B). A second pair of bilaterally symmetrical cells also appears, positioned just laterally to each of the eyespots, beneath the site where the cephalic tentacles will eventually differentiate. These cells send projections behind the eyespots into the putative developing cerebral commissure (Fig. 9A).

Neurites originating in the neuropil progress into the expanding velar lobes (Fig. 9C). A main tract extends radially across each velar lobe to the periphery where it bifurcates and joins with a tract that encircles the velar margin below the ciliated cells of the prototroch. A serotonergic tract running dorsally over the developing oesophagus completes the connection between the velar lobes (Fig. 9D). Other neurites run from the neuropil posteriorly along the visceral loop (Fig. 9A), which has a figure-eight configuration due to the effects of torsion, and also into either side of the foot.

Figure 9. Distribution of anti-serotonin immunoreactive cells in post-torsional *Trichotropis cancellata* embryos. All specimens in this sequence were taken from a single egg capsule and fixed simultaneously.

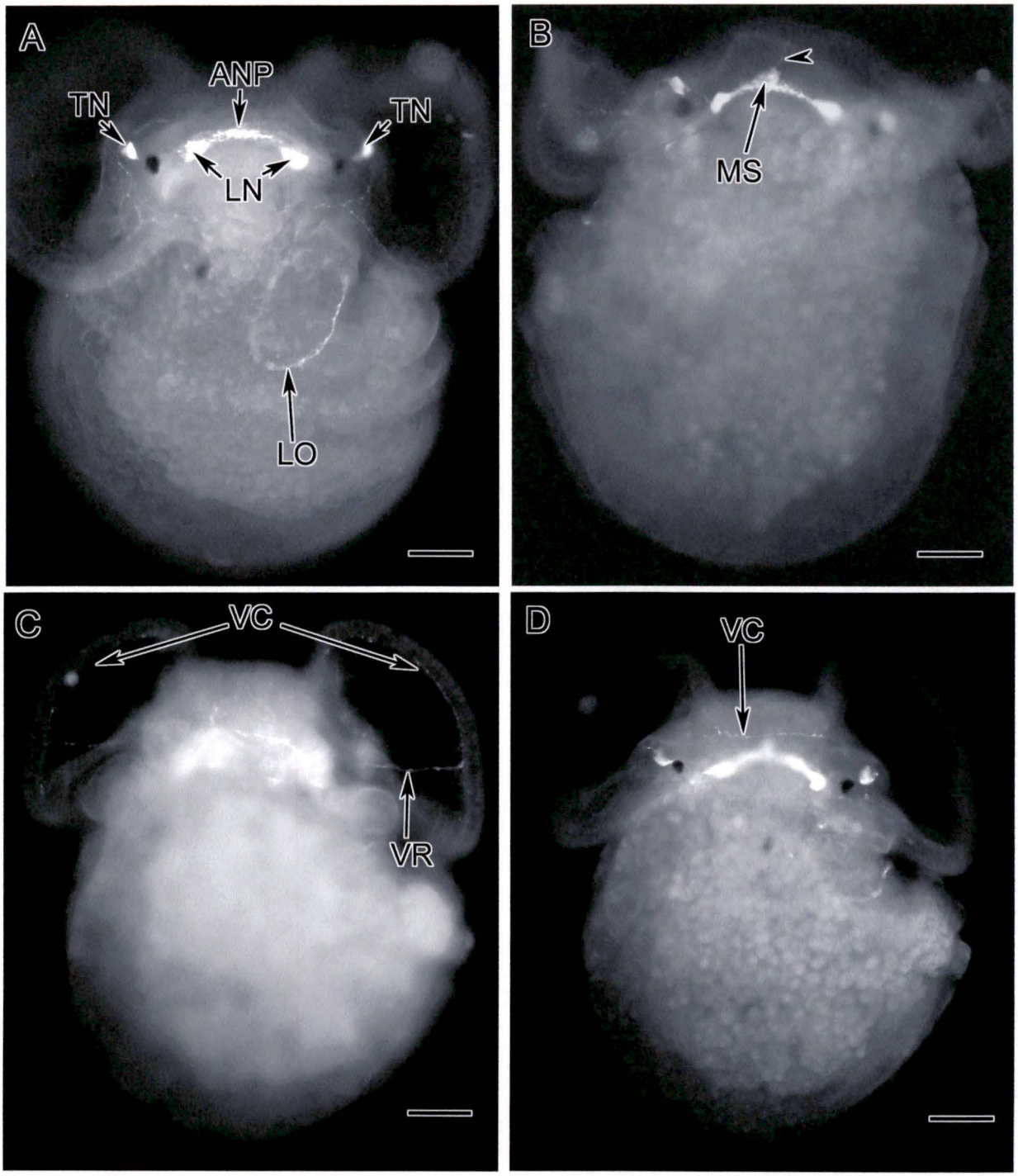
A. Oblique anterior view showing the lateral cells and neuropil of the apical ganglion, as well as cells positioned just laterally to each of the eyespots. Immunoreactive tracts are also visible in the visceral loop, velar lobes and cerebral commissure. Scale Bar = 40μm.

B. Oblique anterior view of a specimen slightly more advanced than that shown in A. The medial cell of the apical ganglion is immunoreactive to serotonin in addition to the two lateral cells. Arrowhead indicates the apical dendritic projection of the medial cell. Scale Bar = 40μm.

C. Oblique anterior view showing the circumferential and radial serotonin immunoreactive axonal tracts in the velar lobes. Scale Bar = 40μm.

D. Same specimen as in C viewed at a different focal plane showing the portion of the circumferential axonal tract that runs dorsally over the larval oesophagus. Scale bar = 40μm.

Lettering
LN = lateral non-sensory neuron of the apical ganglion
LO = visceral loop connective
MS = medial sensory neuron of the apical ganglion
TN = tentacular neuron
VC = circumferential velar tract
VR = radial velar tract


A
ANP
TN
TN
LN
LO
B
MS
C
VC
VR
D
VC

B) Observations on Larvae

The serotonergic nervous system in *T. cancellata* is quite elaborate at hatching. The serotonergic complex of the apical ganglion is fully formed at hatching, consisting of a pair of lateral, non-sensory neurons, and a medial sensory neuron, each of which sends an axonal projection into a pronounced neuropil (Fig. 10A). A few other, less conspicuous tracts join the major radial tracts in the velum and the circumferential velar tract is now very prominent (Fig. 10B). The circumferential tract consists of several fibres with varicosities along their length (Fig. 10A,B). The second pair of lateral serotonin immunoreactive neurons that appeared close to the eyespots in embryos is still evident in hatching larvae (Fig. 10A). Histological sections of specimens labelled with DAB reaction product show that this second pair of neurons are in the developing tentacular ganglia and each sends a dendritic projection through the overlying epithelium (Fig. 10C inset). Cells within the cerebral ganglia of hatching larvae often appear weakly immunoreactive to serotonin antibodies (Fig. 10A,C), and the cerebropedal connective contains serotonergic fibres (Fig. 10D). A pair of labelled neurons in the pedal ganglia is present in most hatching larvae, and tracts exit each pedal ganglion to branch into the foot itself (Fig. 10D). The visceral loop connective shows several labelled axonal projections and a cell within the supraoesophageal ganglion shows serotonin immunoreactivity as well (Fig. 10A,D).

By the midpoint of the larval phase, the velum has taken on its blunt, four-lobed appearance, the cephalic tentacles have lengthened considerably and the

Figure 10. Anti-serotonin immunoreactivity in newly hatched *Trichotropis cancellata* veliger larvae.

A. Wholemount dorsal view. The serotonergic complex of the apical ganglion is visible, as are circumferential and radial axon tracts in the velum, and axons within the visceral loop connective. Asterisks indicate lateral clusters of serotonin immunoreactive cells located outside of the apical ganglion. Arrowhead indicates an immunoreactive cell in the supraoesophageal ganglion. Scale Bar = 100μm.

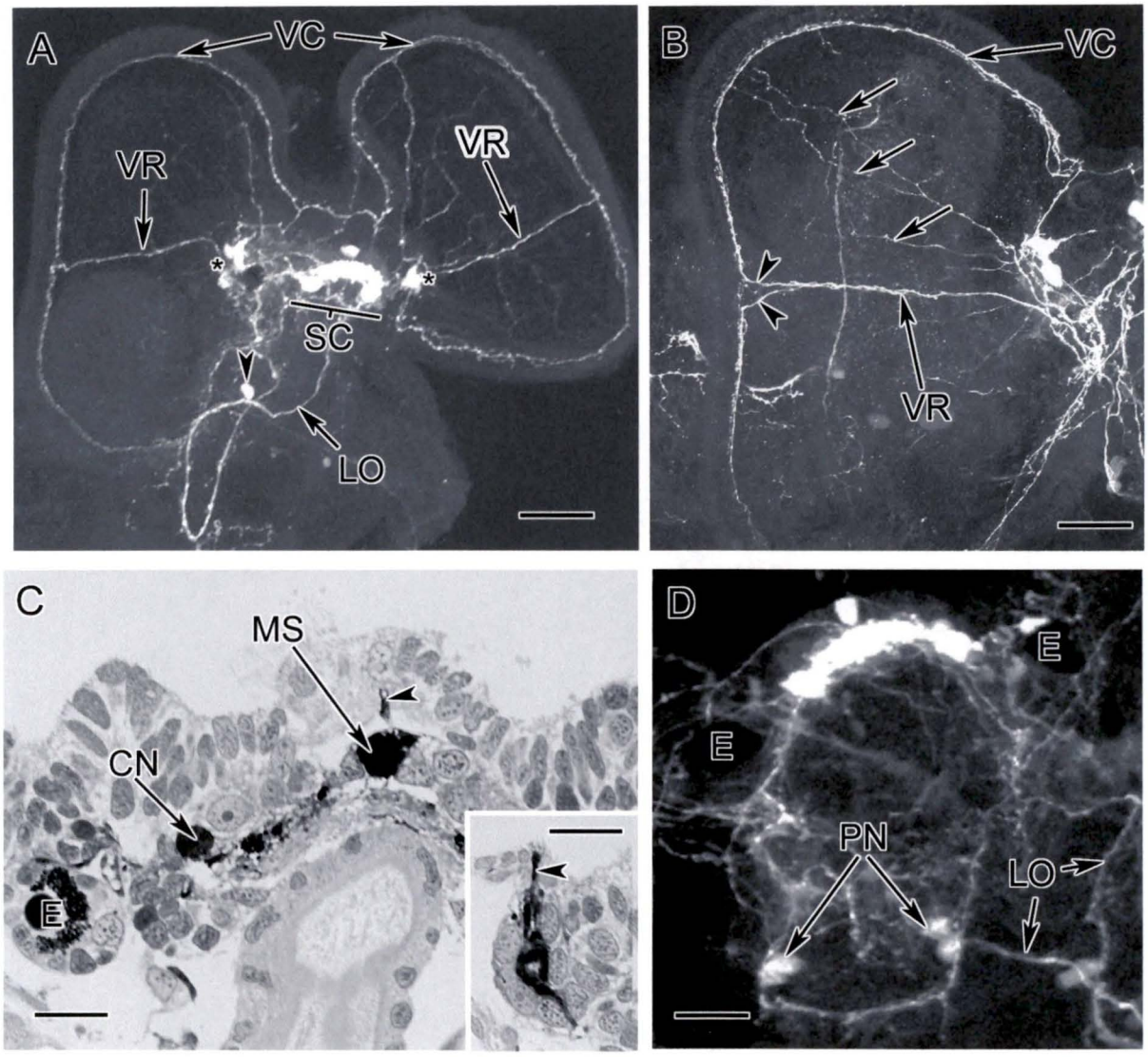
B. Enlargement of the right velar lobe of a newly hatched veliger. The main radial and circumferential axonal tracts are visible. Arrows indicate a number of less prominent radial axonal tracts. Arrowheads indicate the bifurcation of the main radial axonal tract to join with the circumferential axons. Scale Bar = 40μm.

C. Frontal section showing DAB label in the medial sensory neuron of the apical ganglion and its apical dendrite. A smaller neuron within the right cerebral ganglion also appears. Scale Bar = 25μm.

   Inset shows a DAB labelled sensory neuron within the developing right tentacular ganglion. Arrowhead indicates the apical dendritic projection of this neuron penetrating the overlying epidermis. Scale Bar = 10μm.

D. Oblique frontal view. Note the presence of labelled neurons within each of the pedal ganglia. Scale Bar = 25μm.

   Lettering
   CN = cerebral ganglion neuron
   E = eyespot
   LO = visceral loop connective
   MS = medial sensory neuron of the apical ganglion
   PN = pedal ganglion neuron
   SC = serotonergic complex of the apical ganglion
   VC = circumferential velar tract
   VR = radial velar tract


A
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CN
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E
PN
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metapodial flaps are obvious (Fig. 11A). The pattern of serotonin immunolabelling within the apical ganglion remains unchanged (Fig. 11B), but the rest of the serotonergic nervous system continues to be elaborated. Several cells, somewhat smaller than those of the apical ganglion, are now evident in the cerebral ganglia (Fig. 11B). The number in each ganglion is variable, but generally ranges from 8-12. The cephalic tentacles show a network of serotonergic fibres along their length that interact with the tentacular and cerebral ganglia (Fig. 11C). The circumferential velar tract has an increased number of fibres (Fig. 11A). Many more fibres in the cerebropedal connectives are evident, as are several cells within the pedal ganglia (Fig. 11D). These pedal neurons are of a similar size to those labelled in the cerebral ganglia, and they are also variable in number, ranging from 10-15 per ganglion. The pedal commissure shows intense labelling (Fig. 11D). Major nerves now extend from each pedal ganglion into the metapodium and the developing propodium, and these show much branching along the ciliated sole of the foot (Fig. 11E). Older larvae are much more robust than their newly hatched counterparts, making antibody penetration to the visceral mass difficult. The size and shape of the shell also made it difficult to get the animal in an orientation that would produce useful whole-mount data. Therefore, the cephalopodium was dissected away from the visceropallial mass in many cases, allowing antibody penetration to the cephalic areas of interest and increasing the ease with which useful mounts could be prepared. Consequently, observations concerning the continuing development of

Figure 11. Anti-serotonin immunoreactivity in mid- and late-stage *Trichotropis cancellata* veliger larvae.

A. Oblique anterior view of the cephalopodium of a 19 day old (approximately two-thirds through the larval phase) veliger. Scale Bar = 150μm.

B. The serotonergic complex of the apical ganglion in a 19 day old veliger. Also note the appearance of a number of smaller cells immunoreactive to serotonin located outside of the apical ganglion. Scale Bar = 50μm.

C. The cephalic tentacles of a late-stage veliger. Scale Bar = 30μm.

D. Frontal view of the foot in a 19 day old veliger. Several cells in each pedal ganglion are immunoreactive to serotonin. Scale Bar = 50μm.

E. Right lateral view of the foot in a 19 day old veliger. Major nerves can be seen extending from the pedal ganglia into the metapodial and propodial regions of the foot. Scale Bar = 50μm.

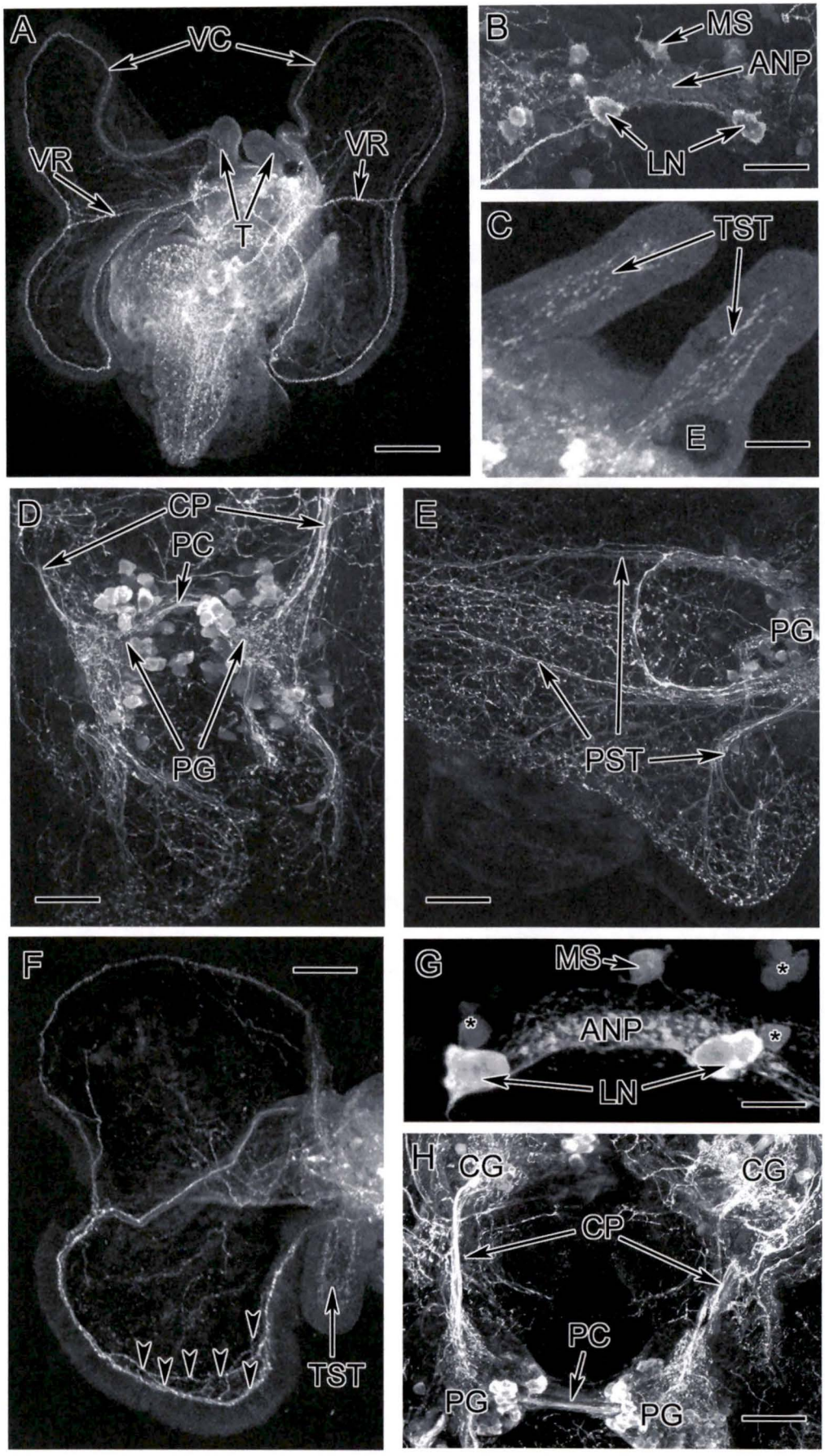
F. The right velar lobe of a metamorphically competent veliger. Serotonergic tracts in the elongate right cephalic tentacle are visible. Arrowheads indicate several individual serotonergic tracts in the circumferential velar lobe. Scale Bar = 100μm.

G. The serotonergic complex of the apical ganglion in a metamorphically competent veliger. The apical neuropil is less distinct than in earlier stages, as it has flattened over the top the cerebral commissure. Asterisks indicate serotonin immunoreactive cells outside of the apical ganglion. Scale Bar = 20μm.

H. Serotonin immunoreactivity in the pedal ganglia of a metamorphically competent veliger. Note the large pedal commissure and cerebropedal connectives. Scale Bar = 20μm.

Lettering

ANP = apical neuropil
CG = cerebral ganglion
CN = cerebral ganglion neuron
CP = cerebropedal connectives
E = eyespot
LO = visceral loop connective
LS = lateral non-sensory neuron of the apical ganglion
MS = medial sensory neuron of the apical ganglion
PC = pedal commissure
PG = pedal ganglion
PN = pedal ganglion neuron
PST = pedal serotonergic tract
SC = serotonergic complex of the apical ganglion
T = cephalic tentacle
TST = tentacular serotonergic tract
VC = circumferential velar tract
VR = radial velar tract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serotonergicity of the visceral loop connective and its associated ganglia were not made.

Metamorphically competent larvae show all of the features already mentioned with few further elaborations. The velar tracts are composed of many individual neurites and continue to show associations with the prototroch as well as with velar muscles (Fig. 11F). The apical ganglion remains the same as it did at hatching, except that the neuropil is slightly less distinct as it flattens somewhat over the top of the now much larger cerebral commissure (Fig. 11G). The long cephalic tentacles are well supplied with serotonergic neurites. Further cells have been added to the cerebral and pedal ganglia, and their associated commissures and connectives are more robust (Fig. 11H). Numbers of labelled axons extending into the foot has increased and these show a greater degree of branching along the entire length of the foot.

C) Observations on Post-Larvae

The metamorphic event results in a number of changes to the serotonergic components of the nervous system in *T. cancellata.* Most notably, the serotonergic cells and neuropil of the apical ganglion disappear within 5 hours of velar loss (Fig. 12A,B). The cerebral ganglia and commissure continue to show strong labelling and are now the most obvious serotonergic structures in the head of the animal (Fig. 12A,B). The pseudoproboscis is supplied with many highly branched serotonergic projections (Fig. 12C). The foot is innervated by many highly branched serotonergic neurites (Fig. 12C,D) and the pedal ganglia contain several serotonin immunoreactive neurons (Fig. 12D).

Figure 12. Anti-serotonin immunoreactivity in the early juvenile (24 hours post-metamorphosis) of *Trichotropis cancellata.*

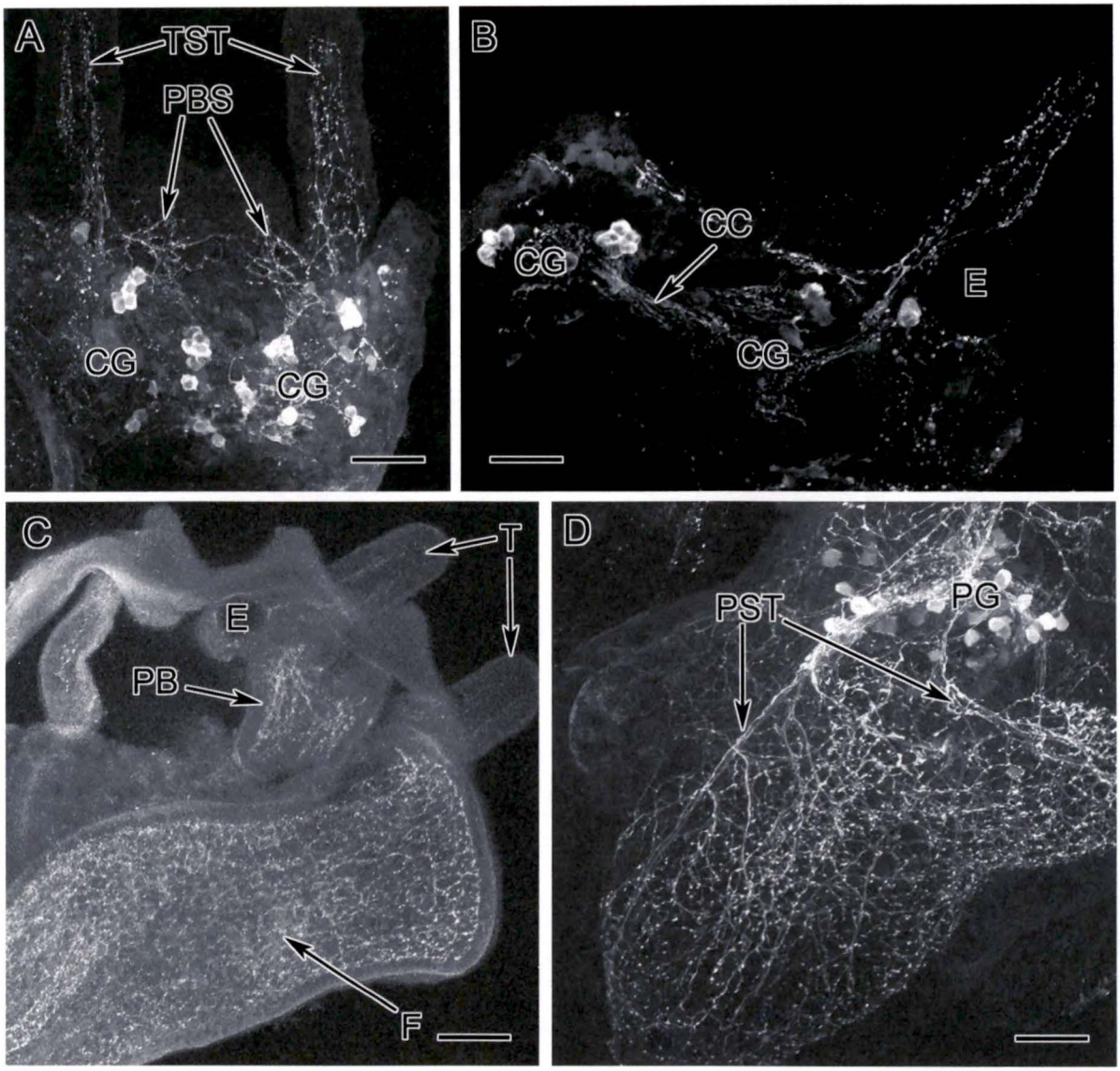
A. Dorsal view of the head. Several cells in the cerebral ganglia show immunoreactivity to serotonin, and serotonergic tracts extend into the pseudoproboscis and cephalic tentacles. The serotonergic complex of the apical ganglion is no longer evident. Scale Bar = 25μm.

B. Dorsal view of the head showing labelled neurites in the cerebral commissure. Scale Bar = 25μm.

C. Oblique ventral view showing a network of labelled neurites within the sole of the foot, in the pseudoproboscis and in the cephalic tentacles. Scale Bar = 50μm.

D. Oblique dorsal view of the foot. Several neurons are labelled within the pedal ganglion, as are the highly branched pedal tracts. Scale Bar = 25μm.

Lettering
CC = cerebral commissure
CG = cerebral ganglion
E = eyespot
F = foot
PB = pseudoproboscis
PG = pedal ganglion
PBS = pseudoproboscis serotonergic tracts
PST = pedal serotonergic tracts
T = cephalic tentacles
TST = tentacular serotonergic tracts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II. FMRFamide

The development of FMRFamide-like immunoreactivity was examined in larval and early post-larval stages of *T. cancellata*. Primary antibody omission controls eliminated labelling in all cases.

A) Observations on Larvae

Newly hatched veliger larvae of *T. cancellata* show a number of FMRFamide immunoreactive cells and fibres. Two prominently labelled somata are located apically in the region just below the ciliary crown that is found in newly hatched larvae (Fig. 13A). A fluorescent image overlain on a Nomarski DIC image shows these cells extending up into the epidermis that underlies the ciliary crown (Fig. 13B). These two cells send projections into the area of the cerebral commissure or the apical neuropil, it is difficult to tell which, but both are labelled prominently (Fig. 13 A,B).

The visceral loop connective, thrown into a characteristic figure-eight by the effects of torsion, is also strongly labelled. A single soma in the supraoesophageal ganglion shows FMRFamide-like immunoreactivity as well (Fig. 13C).

Tracts extend anteriorly and ventrally from the cerebral commissure into the base of each velar lobe (Fig. 13D,E). Three somata lying along the ventral portion of the metatroch in each velar lobe are immunoreactive to FMRFamide, and the tracts extending from the cerebral commissure or apical neuropil come into contact with them (Fig. 13E). A fibre running over the distal larval oesophagus joins the projections proceeding into each velar lobe (Fig. 13D).

Figure 13. Anti-FMRFamide immunoreactivity in newly hatched *Trichotropis cancellata* larvae.

A. Oblique frontal view showing two apical FMRFamide immunoreactive cells (arrowheads), as well as labelled neurites within the apical neuropil and cerebral commissure. Scale Bar = 100μm.

B. Same specimen as in A with the fluorescence image overlain on a DIC/Nomarski image. Note that the two apical cells immunoreactive to FMRFamide (arrowheads) extend up into the ciliary crown. Scale Bar = 100μm.

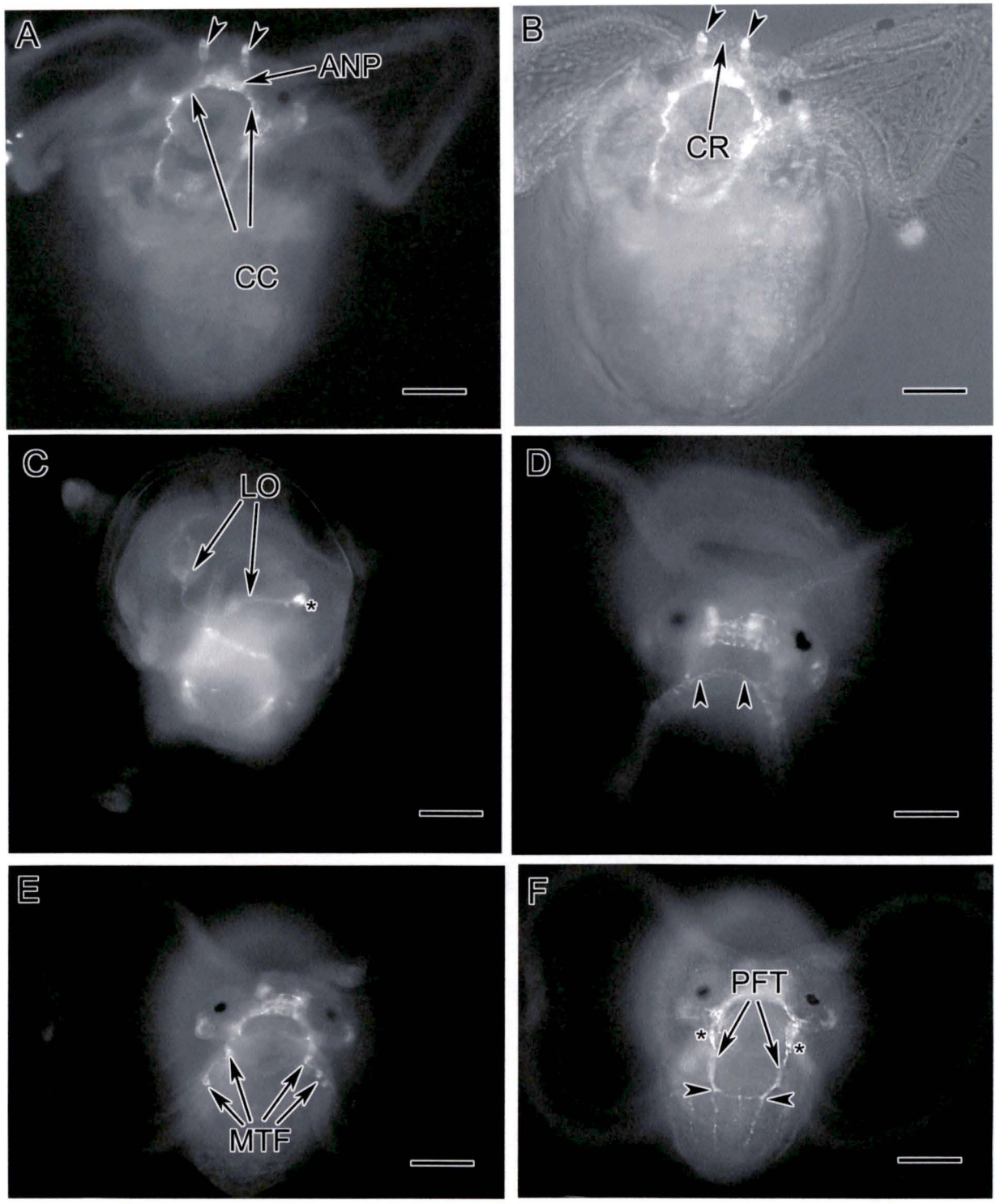
C. Anterior view showing labelled tracts within the visceral loop connective. Asterisk indicates a labelled cell within the supraoesophageal ganglion. Scale Bar = 100μm.

D. Anterior view showing a labelled tract extending over the distal larval oesophagus and along the metatroch into the ventral portion of each velar lobe. Scale Bar = 100μm.

E. Anterior view revealing labelled cells lying along the metatroch in the ventral portion of each velar lobe. Scale bar = 100μm.

F. Anterior view showing labelled tracts extending down the lateral sides of the foot. The lateral tracts bifurcate (arrowheads) approximately half the distance to the distal end of the metapodium and join near the midline. Asterisks indicate cells labelled within each of the pedal ganglia. Scale Bar = 100μm.

Lettering
ANP = apical neuropil
CC = cerebral commissure
CR = ciliary crown
LO = visceral loop connective
MTF = metatrochal FMRFamide immunoreactive cells
PFT = pedal FMRFamide tracts


A
ANP
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MTF
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PFT
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*

FMRFamide fibres run along the cerebropedal connectives and a single soma in each pedal ganglion was often labelled in hatching larvae. Tracts extending from each pedal ganglion extend along the length of the foot (Fig. 13F). At approximately half the distance to the distal end of the metapodium, each of these pedal tracts bifurcates and runs centrally, meeting up near the midline of the foot (Fig. 13F).

By the midpoint of the obligate larval stage, several changes have occurred. The two, large apical cells associated with the ciliary crown are gone, having disappeared shortly after hatching along with the crown itself (Fig. 14A). They are replaced by a cluster of four, smaller apical cells. It is difficult to assess whether these cells are in the apical ganglion or not, though they are in it's general location. These four apical cells are grouped into two bilaterally symmetrical pairs: one medial pair and one slightly more dorsolateral pair (Fig. 14A inset). A variable number of FMRFamide immunoreactive cells also appear in each of the cerebral ganglia.

The velar tracts of FMRFamide immunoreactive axons have also undergone some changes. The tracts proceeding along the metatroch have lengthened and the number of immunoreactive somata has increased to 6 or 7 on each side (Fig. 14B). The base of each of these cells comes directly into contact with, or sends a fine projection to, the FMRFamide tracts running along the metatroch. The apical end of each of these cells is elaborated into a fine projection that comes into contact with a second FMRFamide velar tract that runs along the prototroch (Fig. 14B-D). Both the prototrochal and metatrochal velar

Figure 14. Anti-FMRFamide immunoreactivity in mid- and late-stage *Trichotropis cancellata* veliger larvae.

A. Anterior view of a 15 day old (approximately halfway through the larval phase) veliger. Arrowheads indicate a cluster of four apical cells. The two large apical cells present in newly hatched veligers are no longer evident. Scale Bar = 150μm.

   Inset shows neurites from the apical cells extending into the region of the apical neuropil or cerebral commissure. Scale Bar = 30μm.

B. Oblique anterior view of a 15 day old veliger showing six labelled cells (arrowheads) along the ventral metatroch in the left velar lobe. The pattern is repeated on the right side as well. Scale Bar = 150μm.

C. Close up of the labelled cells and tracts within the ventral portion of the left velar lobe of a 15 day old veliger. Projections from two of the labelled cells in the metatroch are shown (arrowheads) extending to join with the prototrochal tract. Scale Bar = 15μm.

D. Same as in C but viewed in a different focal plane showing the labelled cells and tracts along the metatroch. Scale Bar = 15μm.

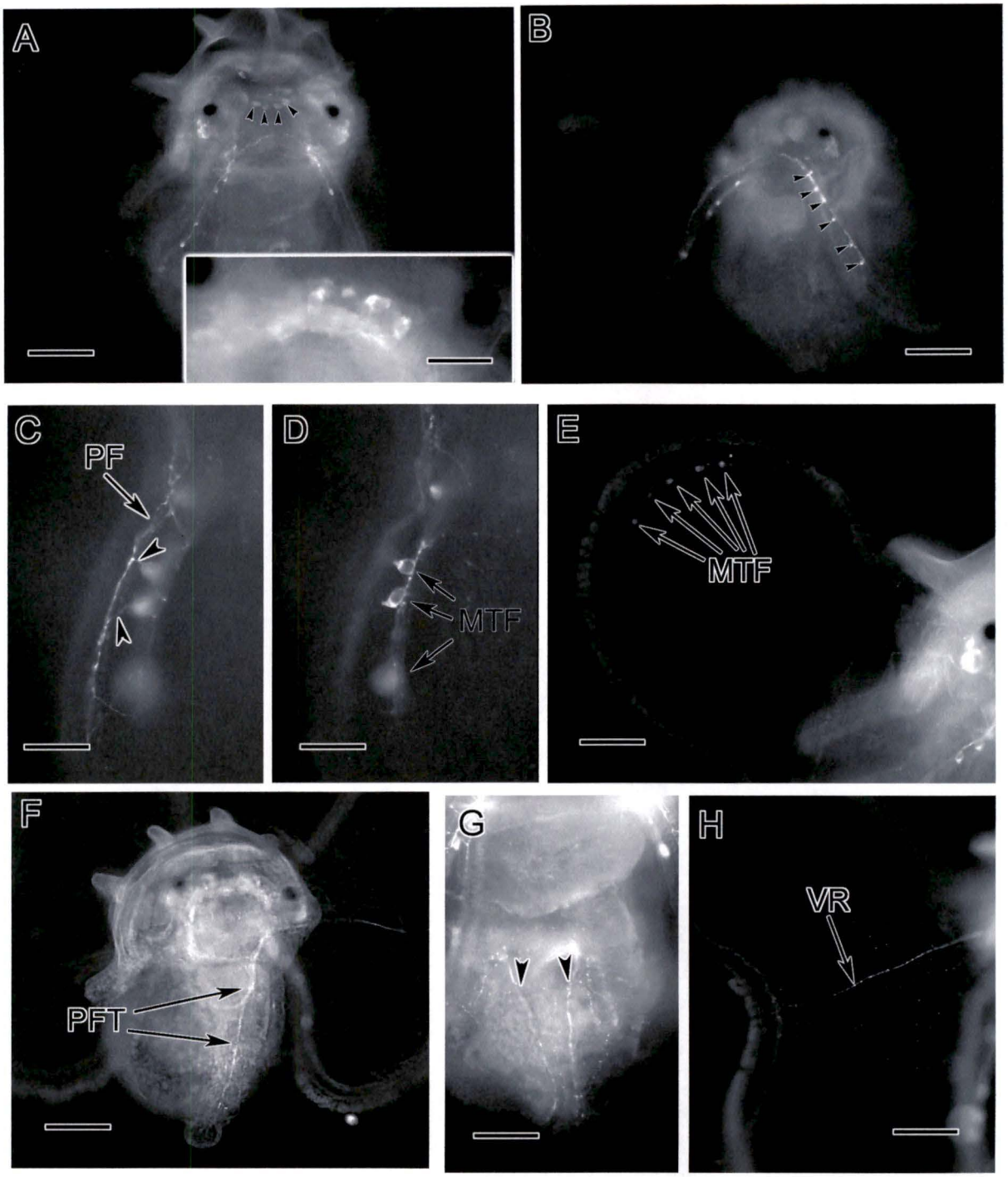
E. FMRFamide immunoreactive cells in the metatroch of the dorsal velar lobe of a 15 day old veliger. Scale Bar = 75μm.

F. Anterior view of a metamorphically competent veliger showing labelled tracts in the cerebral commissure, cerebropedal connectives and extending into the foot. Scale Bar = 200μm.

G. Close up of the foot in a metamorphically competent veliger showing two lateral labelled tracts (arrowheads). Scale Bar = 100μm.

H. The right velar lobe of a metamorphically competent veliger showing a labelled radial tract. Scale Bar = 75μm.

   Lettering
   MTF = metatrochal FMRFamide immunoreactive cells
   PF = prototrochal FMRFamide immunoreactive tract
   PFT = pedal FMRFamide tract
   VR = radial velar tract


A
B
C
PF
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MTF
E
MTF
F
PFT
G
H
VR

tracts originate in the cerebral commissure and terminate near the ventral edge of each velar lobe (Fig. 14B). In addition, in some specimens similar tracts and cells appear in the dorsal portions of each velar lobe (Fig. 14E).

Pedal innervation by FMRFamide projections remains much the same as in newly hatched specimens, with the addition of more cells in the pedal ganglia (Fig. 14F,G). A cell immunoreactive to FMRFamide also appears at the distal end of the tracts running into the metapodium.

Further elaboration of FMRFamide immunoreactivity in the ganglia associated with the visceral loop connective was not documented for the same reasons described in the serotonin section above.

Competent larvae exhibit few changes in overall FMRFamide immunoreactive patterning. The four apical cells remain. More cells are added to each cerebral and pedal ganglion, and the prototrochal and metatrochal velar tracts remain as they were at the halfway point of larval development. A radial FMRFamide tract, similar to the major radial serotonergic tracts described above, often extends into each velar lobe (Fig. 14H). The cephalic tentacles show FMRFamide immunoreactive tracts as well.

B) Observations on Post-Larvae

After metamorphosis, juveniles have many FMRFamide immunoreactive cells in their cerebral and tentacular ganglia (Fig. 15A-C). Due to the similarity in size and morphology of the four apical cells with cells of the cerebral ganglion, it is difficult to tell if the four apical cells degenerate at metamorphosis, or are incorporated into the cerebral ganglia. The apical neuropil, however, no longer

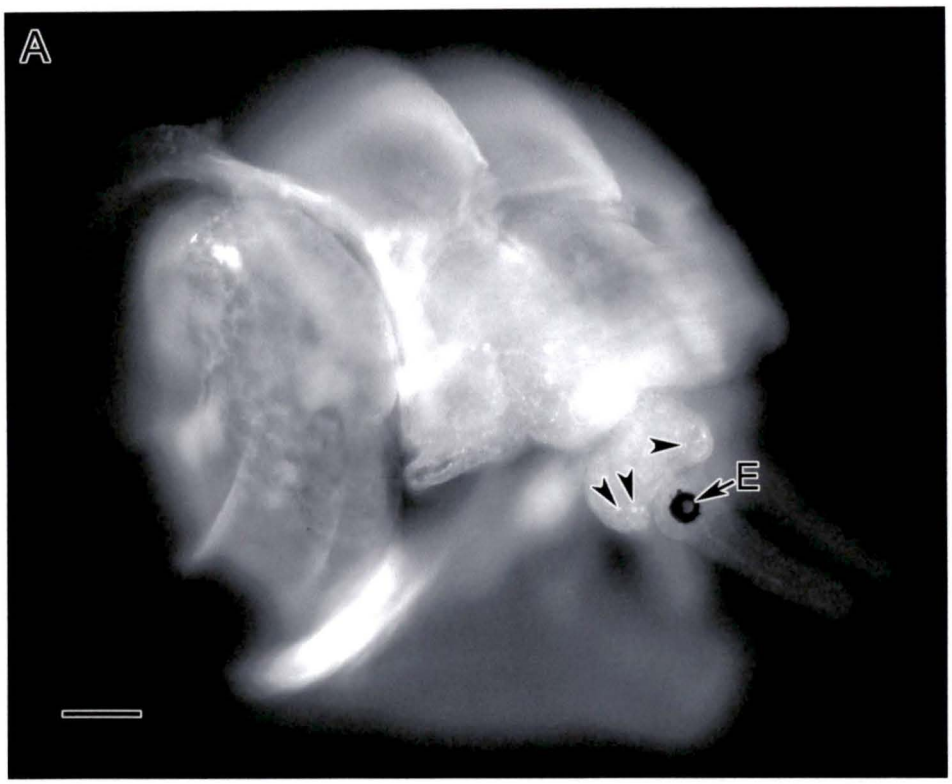
Figure 15. Anti-FMRFamide immunoreactivity in the early juvenile (24 hours post-metamorphosis) of *Trichotropis cancellata.*

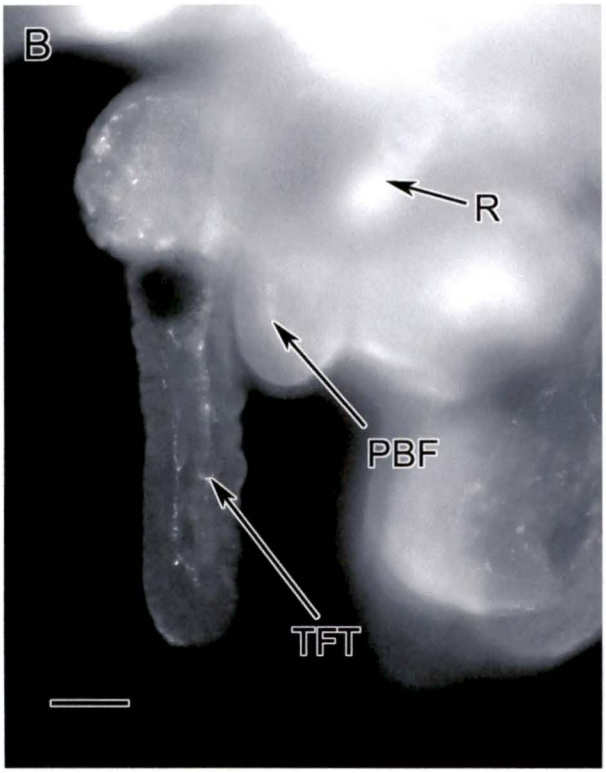
A. Oblique whole-mount right lateral view. Arrowheads indicate labelled cells within the right tentacular and cerebral ganglia. Faintly labelled tracts can be seen in the cephalic tentacles as well. Scale Bar = 75μm.

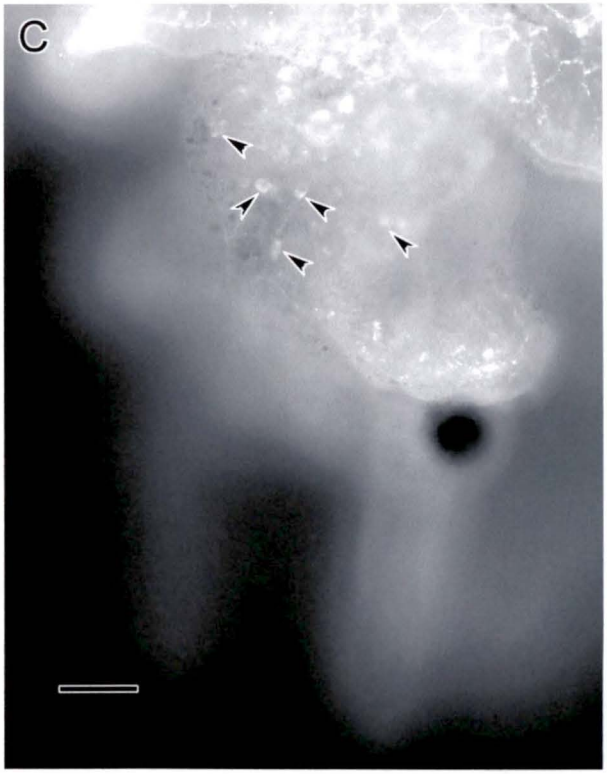
B. Dorsal view of the head. Labelled tracts can be seen in the right cephalic tentacle and pseudoproboscis. Also note the autofluorescent radula. Scale Bar = 50μm.

C. Same as in B viewed in a different focal plane showing several cells centrally in the cerebral ganglia. Scale bar = 50μm.

Lettering
E = eyespot
PBF = pseudoproboscis FMRFamide immunoreactive tracts
TFT = tentacular FMRFamide immunoreactive tracts
R = radula


A
E


B
R
PBF
TFT


C

appears in specimens labelled for FMRFamide. Fine projections can be seen proceeding into each cephalic tentacle and into the pseudoproboscis (Fig. 15B). Satisfactory observations were not made on the post-metamorphic foot or pedal ganglia.

III. Dopamine ß-Hydroxylase

All attempts to label *T. cancellata* with antibodies to dopamine ß-hydroxylase failed. Potential reasons will be discussed below.

IV. Nitric Oxide Synthase – NADPH diaphorase histochemistry

The application of NADPH diaphorase histochemistry to the various developmental stages in the larvae of three species of caenogastropod, *T. cancellata*, *A. columbiana*, and *M. stearnsii*, revealed similar results in all cases. A cluster of cells in the foot labelled very darkly with reaction product (Fig. 16A,B). At first glance, these cells appear to be potentially sensory in nature, having a distinctive projection extending to the surface of the sole of the foot (Fig. 16B). Controls however, labelled the same cells in all cases and subsequent histological sections revealed these cells to be of a glandular or secretory nature (Fig. 16C). No reaction product was observed in any ganglia, neuropils or nerve tracts in any of the species examined. Potential reasons will be discussed below.

Larval Musculature of *T. cancellata*

A) Phalloidin Labelling

The application of a fluorescent dye for filamentous actin reveals a well-developed larval retractor muscle in newly hatched veligers of *T. cancellata*

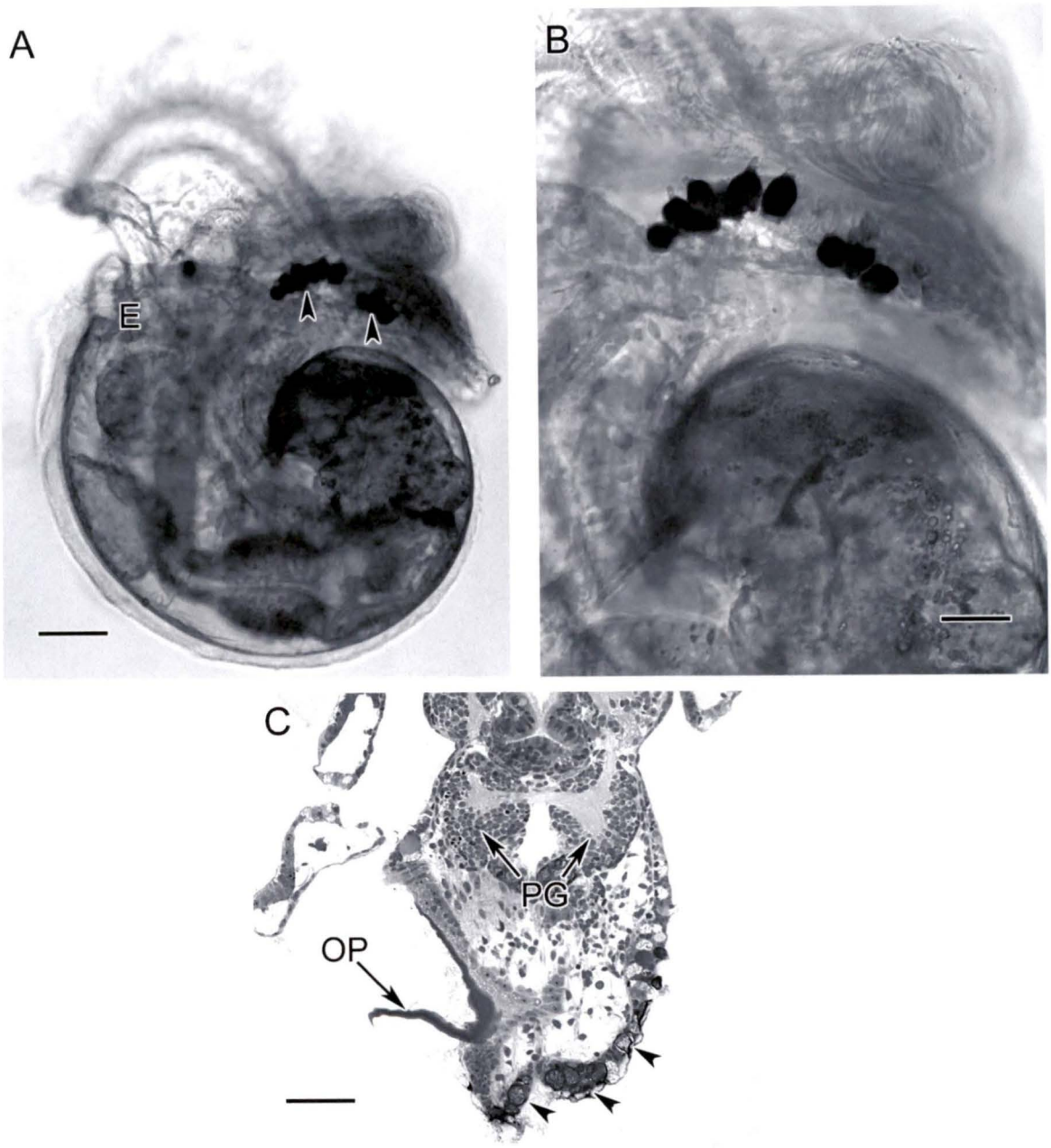
Figure 16. NADPH-diaphorase histochemical assay for nitric oxide synthase in newly hatched veligers of *Trichotropis cancellata*. The results are negative.

A. Right lateral view showing two clusters of labelled cells (arrowheads) in the foot. These cells also labelled in the control experiments indicating that this is a negative result. Scale Bar = 75μm.

B. Enlargement of the cells labelled in A. Scale Bar = 30μm.

C. Slightly oblique cross section through a newly hatched veliger revealing the cells labelled during the NADPH-diaphorase experiments (arrowheads) to be secretory in nature (pink staining with Richardson's stain). The pedal ganglia and operculum are also evident. Scale Bar = 75μm.

Lettering
PG = pedal ganglion
OP = operculum


A
E
B
C
PG
OP

(Fig. 17A,B). This muscle is attached to the inner wall of the shell on the left side (Fig. 17A). Large pedal branches are particularly evident (Fig. 17B).

Phalloidin staining also reveals a series of f-actin containing fibres in the velar lobes (Fig. 17A-D). There are particularly robust fibres running circumferentially about the velar perimeter just below the ciliated cells of the prototroch, as well as a brightly staining radial tract running across the midline of each velar lobe (Fig. 17D). Many finer fibres can also be seen crossing the velar radius to join with the thick circumferential ring. In older specimens, the brightly fluorescent fibres meet with the velar perimeter at the location where each velar lobe subdivides into two broad, blunt lobes (Fig. 17D). Staining remains consistent in all stages examined.

B) Phalloidin Staining and Serotonergic Immunoreactivity in the Velum

Double labelling the veligers of *T cancellata* with phalloidin and antibodies to serotonin reveals a close association between the serotonergic tracts and the myofibres within the velum. Antibodies to serotonin and phalloidin both exhibit the strongest label in the circumferential and main radial velar branches. The circumferential serotonergic tract can be seen running just below the prototrochal ciliary band of the velum, with a number of varicosities occurring along its length (Fig. 18A-C). The circumferential myofilaments follow precisely the some route, as is particularly evident in the overlain photomicrographs shown in Figures 18A-C and 19A-C. The main radial tracts of serotonin immunolabelled axons and phalloidin-labelled myofibres are also very closely associated, as are a

Figure 17. Phalloidin staining of newly hatched *Trichotropis cancellata* veliger larvae.

A. Dorsal view showing the larval retractor muscle and myofibres within the velar lobes. Asterisk indicates the site of attachment for the larval retractor muscle on the left side of the shell. Scale Bar = 100μm.

B. Left lateral view showing the pedal tract of the larval retractor muscle. Scale Bar = 100μm.

C. Oblique anterior view showing the pedal tract of the larval retractor muscle and the myofibres running circumferentially around the velum. Also note the many finer radial myofibres. Scale Bar = 100μm.

D. Close up of the left velar lobe showing several radial myofibres and the robust circumferential myofibre. Scale Bar = 50μm.

Lettering
CM = circumferential myofibre
RM = larval retractor muscle

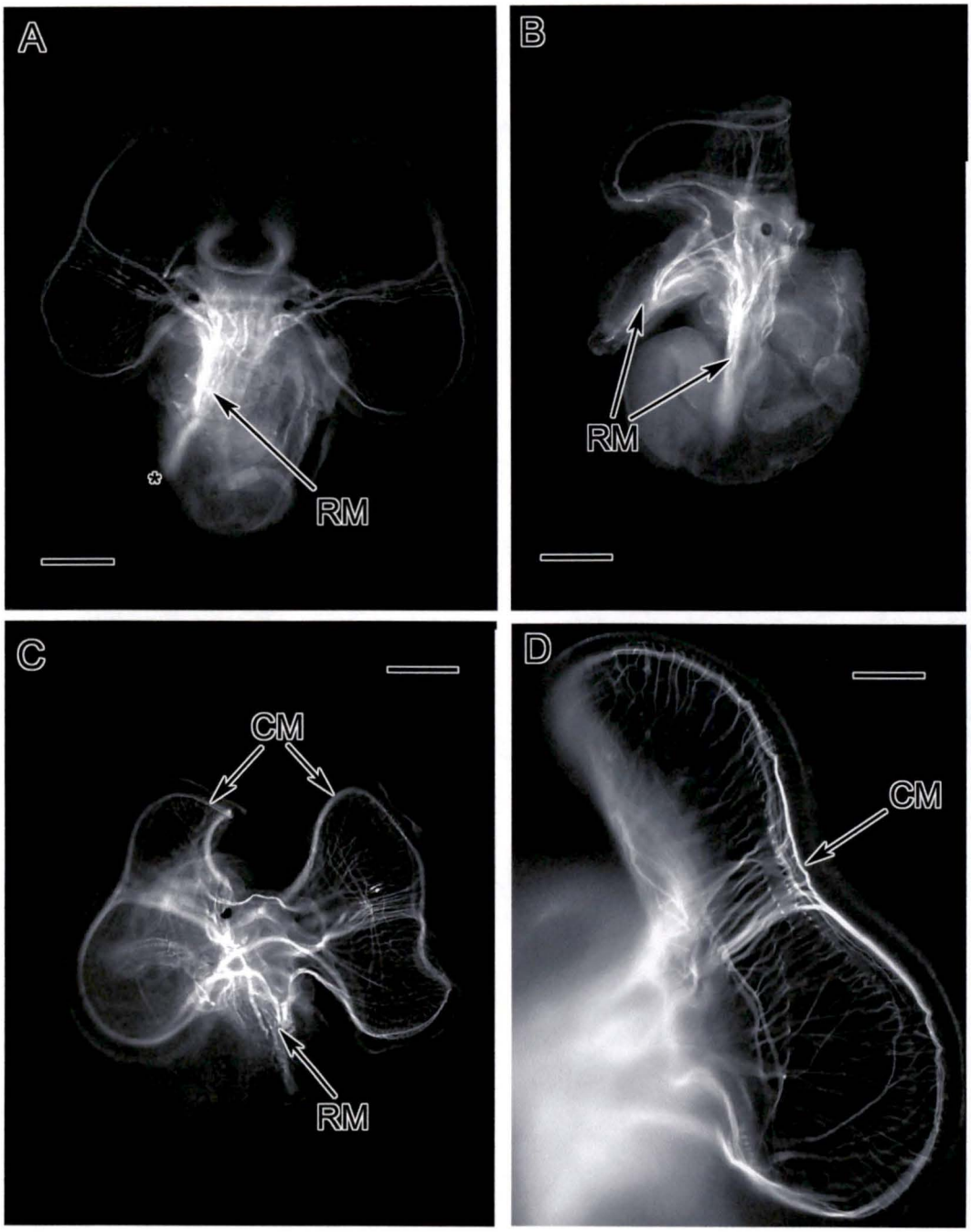

A
RM
B
RM
C
CM
RM
D
CM

Figure 18. Conventional epifluorescence images of anti-serotonin immunoreactivity and phalloidin staining in *Trichotropis cancellata* veligers. In all cases the left-most image shows the pattern of anti-serotonin immunoreactivity, the centre image shows the pattern of phalloidin staining, and the right-most image shows an overlay of the two. In the overlay, anti-serotonin immunoreactivity is represented in red and phalloidin staining is represented in green.

A. Whole-mount newly hatched veliger in dorsal view. Note the overlap of the velar serotonergic tracts with the main radial and circumferential myofibre tracts. Scale Bar = 100μm.

B. Right velar lobe of a 21 day old (three-quarters through the larval phase) veliger. Note the overlap of the velar serotonergic tracts with the main radial and circumferential myofibre tracts in the velum. Scale Bar = 120μm.

C. Close up of the ventral portion of the left velar lobe of a metamorphically competent veliger. Note the overlap of the circumferential serotonergic and myofibre tracts. Also note the prominent varicosities along the circumferential serotonergic tract. Scale Bar = 25μm.

Lettering
CM = circumferential myofibre
RM = larval retractor muscle
VC = circumferential velar tract
VR = radial velar tract

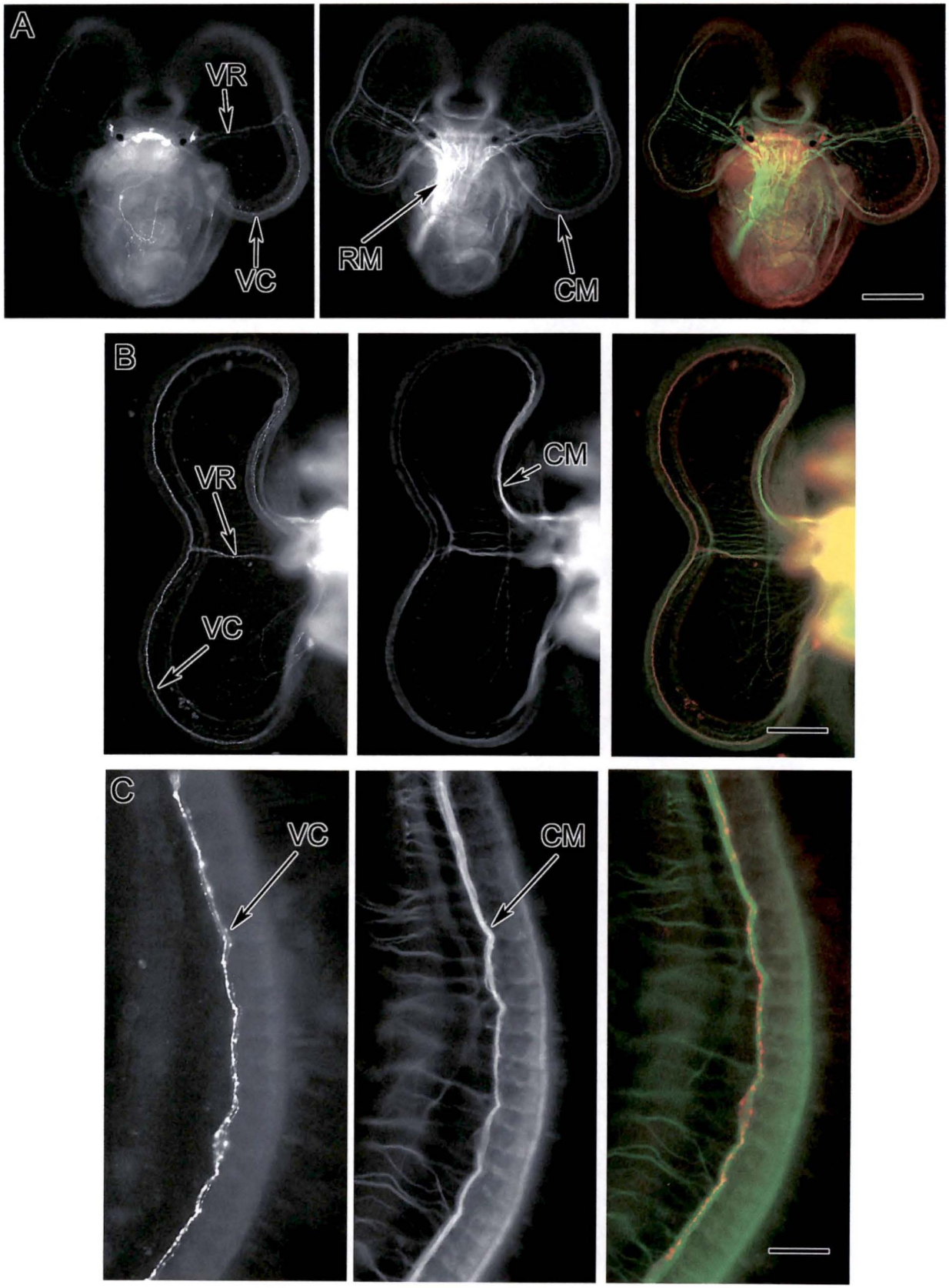
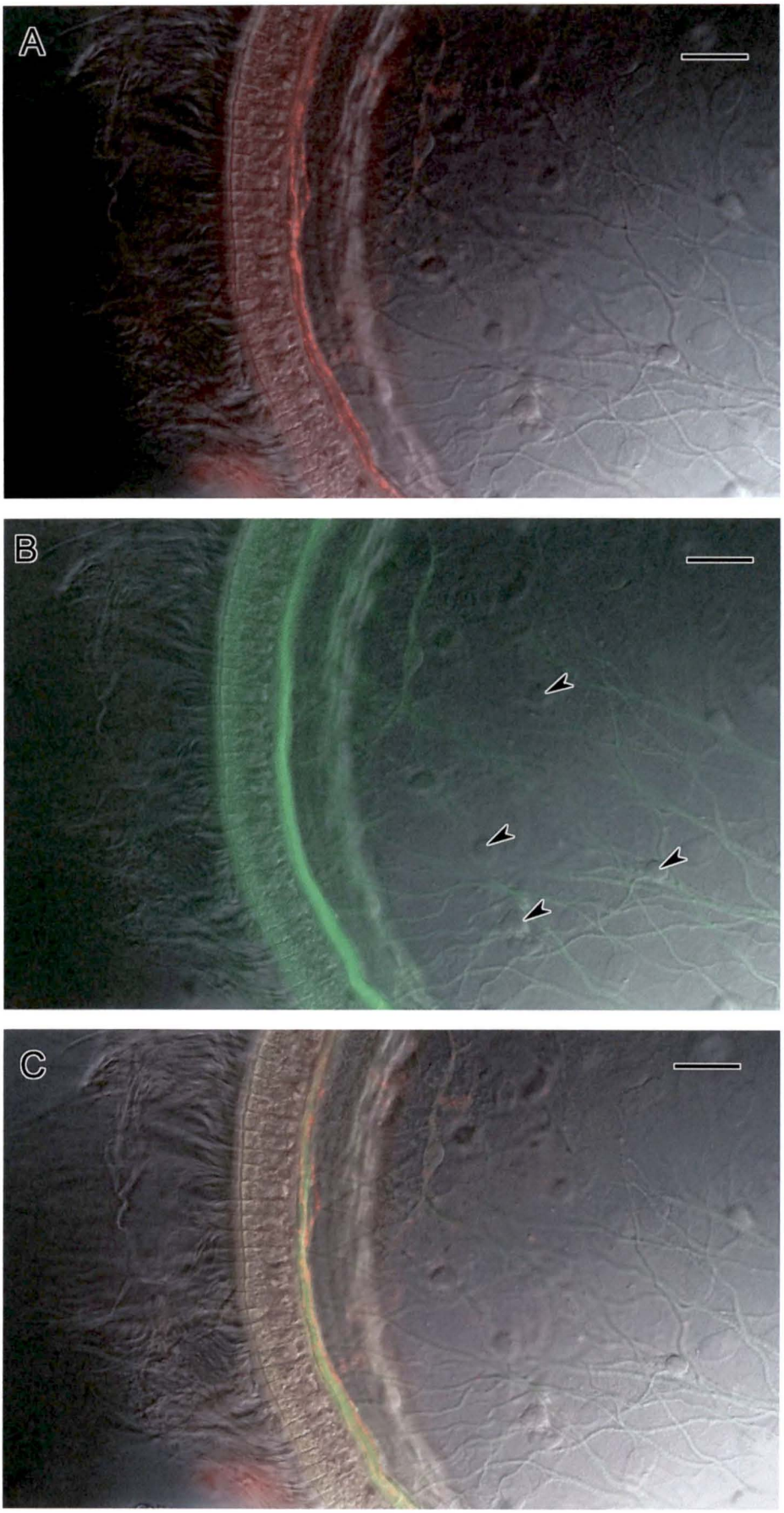

A
VR
VC
RM
CM
B
VR
VC
CM
C
VC
CM

Figure 19. Anti-serotonin immunoreactivity and phalloidin staining overlain on DIC/Nomarski images of the ventral portion of the right velar lobe of a 16 day old (approximately half way through the larval phase) *Trichotropis cancellata* veliger.

A. Anti-serotonin immunoreactivity is represented in red. The circumferential axonal tract runs directly below the columnar ciliated cells of the prototroch. Several varicosities occur along the tract and often directly contact the ciliated cells. Scale Bar = 40μm.

B. Phalloidin staining is represented in green. The circumferential myofibre tract runs immediately beneath the prototroch. Several of the radial myofibres are also faintly labelled. Arrowheads indicate muscle cell bodies visible in the velar lobe. Scale Bar = 40μm.

C. The overlay of the images in A and B show the close association of the circumferential serotonergic and myofibre tracts. Scale Bar = 40μm.


A
B
C

number of the less distinct branches. This association remains consistent throughout the veliger stage (Fig. 18A-C).

Comparative Aspects of the Caenogastropod Apical Ganglion

In order to better assess the nature of the apical ganglion in larvae of gastropod molluscs, comparative data are contributed on this structure as it appears in early post-hatching planktotrophic veligers of five species of caenogastropod, each from a different superfamily (*Trichotropis cancellata*, *Euspira lewisii*, *Lacuna vincta*, *Amphissa columbiana*, and *Marsenina stearnsii*). A basic ultrastructural description of the AG is given, but the majority of the observations will focus on comparative aspects of the serotonin-like immunoreactivity exhibited by the AG in each species.

A) Comparative Ultrastructure

In all species examined, the apical ganglion lies immediately below the apical epidermis, and just dorsal to the cerebral commissure (Fig. 20A,B). The apical ganglion is fully formed at hatching, and is larger than the still developing cerebral ganglia. Neuronal somata of the apical ganglion are gathered around, and send axonal projections into, a distinct neuropil of tangled, varicose neurites (Fig. 20C). There are sensory neurons, characterized by dendritic projections extending apically through the overlying epidermis, and non-sensory neurons in which dendritic extensions do not occur. The sensory neurons can be further categorized into two types: ampullary and parampullary neurons. Ampullary sensory neurons are distinguished by a deep apical invagination that forms a

Figure 20. Basic ultrastructural details of the apical ganglion in planktotrophic caenogastropod larvae. Sections for the transmission electron microscope were cut by Dr. Louise Page.

A. Cross section through the head of a late-stage (35 day old, 1999 culture) veliger of *Trichotropis cancellata* showing the location of the apical ganglion in relation to the cerebral ganglia and commissure. Scale Bar = 20μm.

B. Sagittal section just to the left of the median through a late-stage veliger of *T. cancellata* showing the apical ganglion between the apical epidermis and foregut. The left statocyst is also visible in this section. Scale Bar = 50μm.

C. Transmission electron micrograph (TEM) through a portion of the apical ganglion of a 6 day old *Euspira (Polinices) lewisii* veliger showing axons (asterisks) from two neuronal somata extending into the apical neuropil. Scale Bar = 1μm.

D. TEM (frontal section) through a portion of the apical ganglion of a 6 day old *E. lewisii* veliger showing two lateral dendritic bundles (arrows) extending into the overlying epidermis. Scale Bar = 4μm.

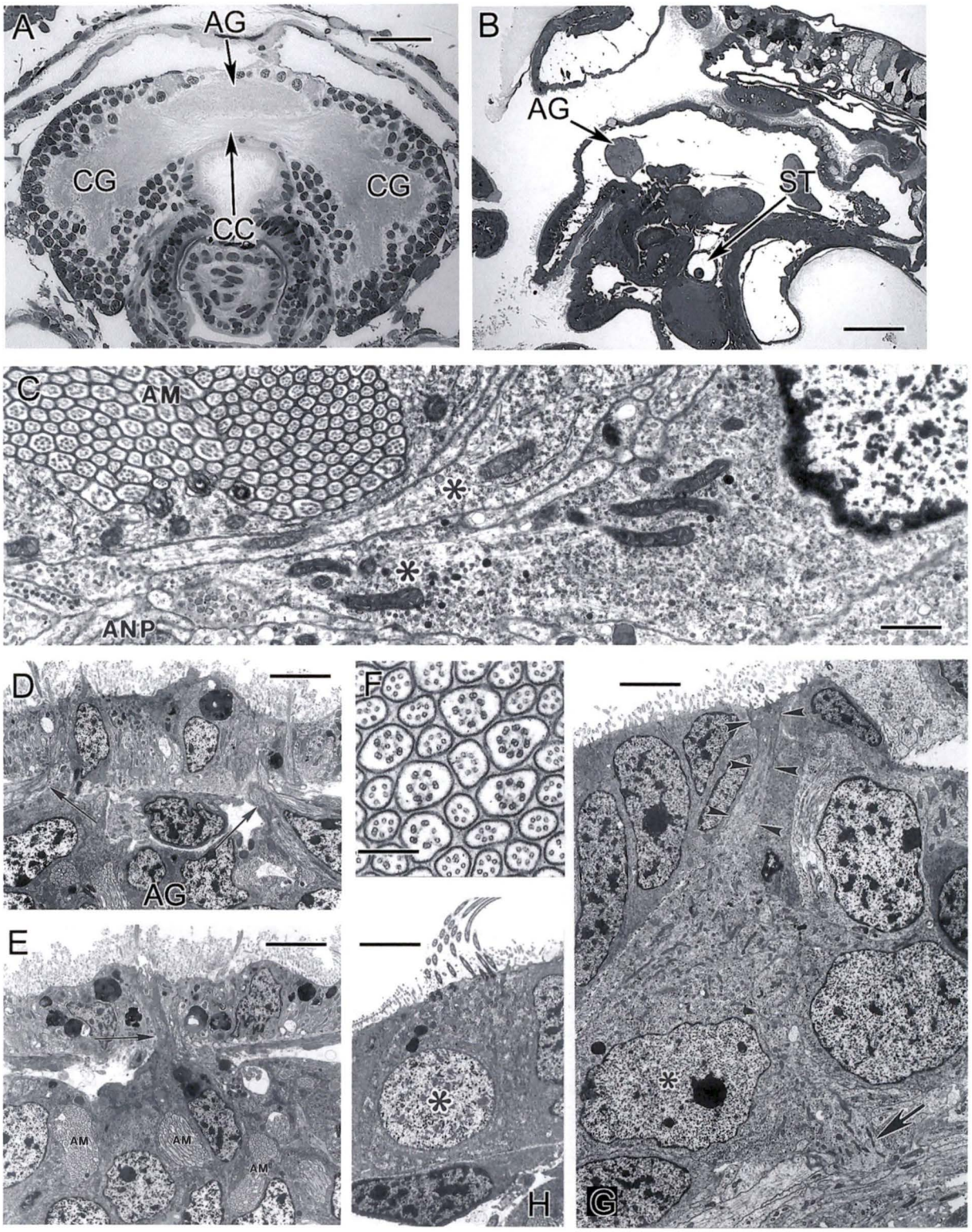
E. Section cut from a more dorsal location of the specimen in D showing the medial dendritic bundle (arrow). Also note the cilia-filled lumens of the ampullary sensory neurons. Scale Bar = 4μm.

F. Cross-sectional profiles of cilia from an ampullary neuron in the apical ganglion of an emergent *Amphissa columbiana* veliger exhibiting modified ciliary axonemes. Scale Bar = 0.25μm.

G. TEM (frontal section) through a portion of the apical ganglion of an emergent *T. cancellata* veliger. The apical dendrite (arrowheads), nucleus (asterisk), and basal axon (arrow) of a parampullary sensory neuron are visible. Scale Bar = 4μm.

H. TEM (frontal section) showing a ciliary tuft cell in a 17 day old *Lacuna vincta* veliger. Scale Bar = 5μm.

Lettering
AG = apical ganglion
AM = ampullary neuron
ANP = apical neuropil
CC = cerebral commissure
CG = cerebral ganglion
ST = statocyst


A
AG
CG
CG
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lumen packed tightly with cilia (Fig. 20D,E). The axoneme of these cilia is variously modified from the regular 9 + 2 arrangement of microtubules (Fig. 20F). Parampullary neurons have a similar morphology to the ampullary type, but lack the ciliated lumen (Fig. 20G). The dendrites of both sensory cell types are gathered into three bundles that penetrate the epidermis in three distinct locations; two lateral (Fig. 20D) and one medial (Fig. 20E). Caenogastropods may or may not have a ciliary tuft associated with the apical ganglion. If present, they can generally be seen externally in scanning electron micrographs. When present, the ciliary tuft arises from a distinct cell type: the aptly titled, ciliary tuft cells. In the present study, only *E. lewisii* and *L. vincta* had ciliary tufts. The ciliary tuft cells, unlike other cells associated with the apical ganglion, exist wholly within the apical epidermis and do not send axonal projections into the apical neuropil (Fig. 20H). These cilia do exhibit a normal 9 + 2 arrangement of microtubules in their axoneme, and they also have long ciliary rootlets (data not shown).

B) Serotonergic Immunoreactivity in the Caenogastropod Apical Ganglion

In all species examined, the serotonergic complex of the apical ganglion was complete at hatching and did not subsequently change during the course of larval development. All species also exhibited consistent labelling of serotonergic projections within the velar lobes. Changes did occur in other aspects of the larval serotonergic nervous system, but these are documented elsewhere (Page and Parries, 2000) or were not studied during the course of this investigation.

Trichotropis cancellata

The serotonergic complex of *T. cancellata* consists of three large neuronal somata surrounding a distinct, varicose neuropil (Fig. 21A-D). The two lateral neurons are non-sensory and send projections into the neuropil only (Fig. 21B,D). The medial neuron, as confirmed by histological sections of specimens labelled with DAB reaction product, is sensory, sending an apical dendrite up through the pretrochal epidermis (Fig. 21C). It also sends an axon into the neuropil.

Euspira lewisii

Five serotonergic cells are found in the apical ganglion of larval specimens of *E. lewisii*: a lateral pair of sensory cells (one on either side), a lateral pair of non-sensory neurons, and a medial sensory neuron (Fig. 22A-D). All of these cells send axonal projections into the prominent apical neuropil. Other slightly smaller cells can be seen quite near the apical ganglion in whole-mount fluorescence images (Fig. 22B). Histological sections of specimens labelled with DAB reaction product confirm that these cells are not a part of the apical ganglion (Fig. 22C). Also note the radiating velar tracts that merge with the circumferential velar tract running below the ciliated cells of the prototroch (Fig. 22A).

Marsenina stearnsii

The serotonergic complex of *M. stearnsii* is essentially identical to that exhibited by *E. lewisii*. It consists of a lateral pair of sensory neurons, a lateral pair of non-sensory neurons, and a medial sensory neuron, all extending axonal

Figure 21. Anti-serotonin immunoreactivity in newly hatched veliger larvae of *Trichotropis cancellata.*

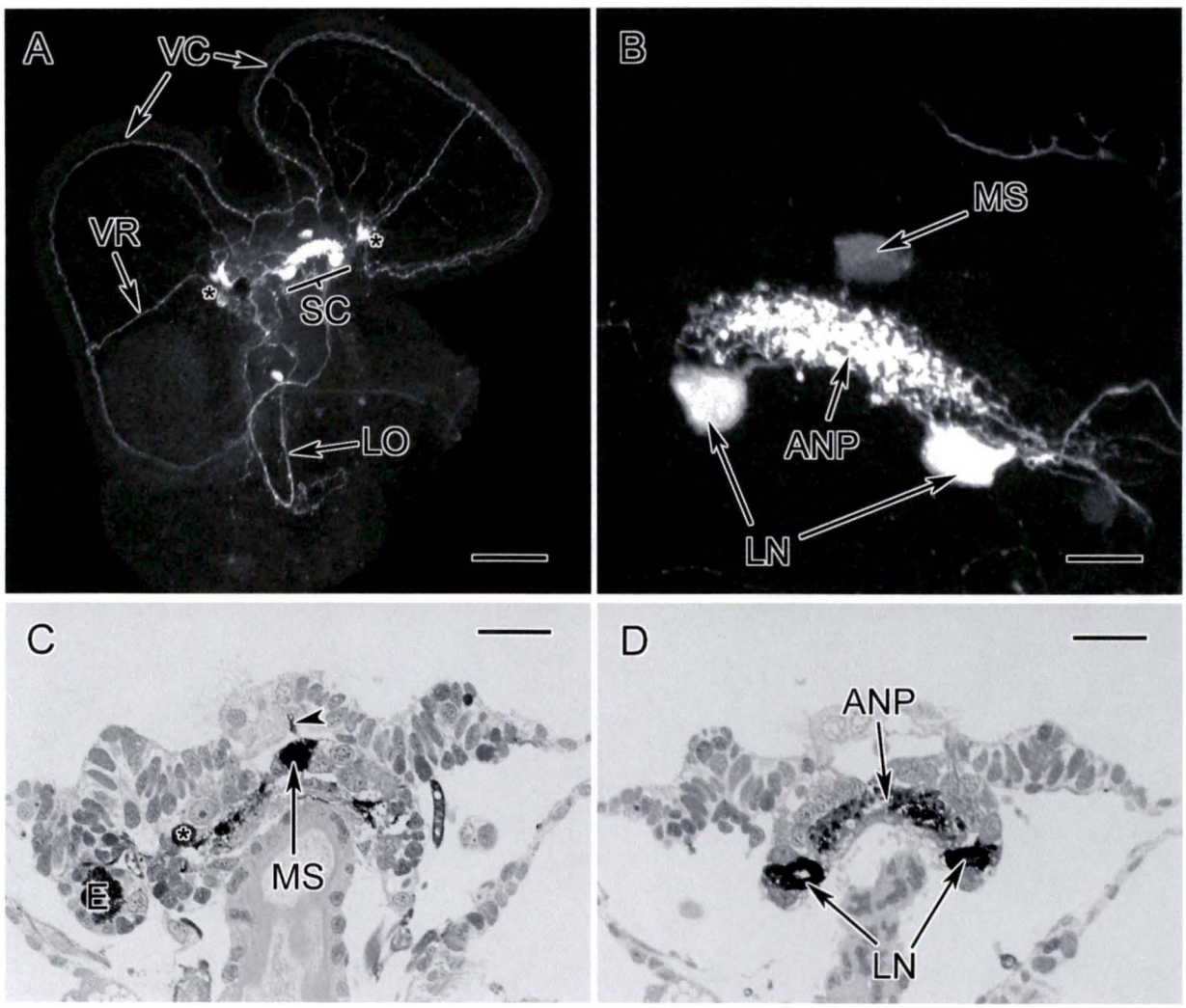
A. Wholemount dorsal view. The serotonergic complex of the apical ganglion is visible, as are circumferential and radial axon tracts in the velum, and axons within the visceral loop connective. Asterisks indicate lateral clusters of serotonin immunoreactive cells located outside of the apical ganglion. Arrowhead indicates an immunoreactive cell in the supraoesophageal ganglion. Scale Bar = 100μm.

B. The serotonergic complex of the apical ganglion in *T. cancellata.* Two lateral non-sensory neurons, a medial sensory neuron, and the varicose apical neuropil are all visible. The apical dendrite of the medial neuron is not visible in this orientation. Scale Bar = 15μm.

C. Frontal section through the ventral portion of the apical ganglion in a DAB labelled *T. cancellata* veliger. The medial sensory neuron and its apical dendrite (arrowhead) are visible. Also note the smaller labelled cell (asterisk) located within the right cerebral ganglion. Scale Bar = 25μm.

D. Frontal section cut slightly more dorsal to that in C showing DAB label in two lateral non-sensory neurons of the apical ganglion. Scale Bar = 25μm.

Lettering
ANP = apical neuropil
E = eyespot
MS = medial sensory neuron
LN = lateral non-sensory neuron
LO = visceral loop connective
SC = serotonergic complex of the apical ganglion
VC = circumferential velar tract
VR = radial velar tract


A
VC
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MS
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ANP
LN

Figure 22. Anti-serotonin immunoreactivity in *Euspira (Polinices) lewisii* veligers. Histological sections in C and D cut by Dr. Louise Page.

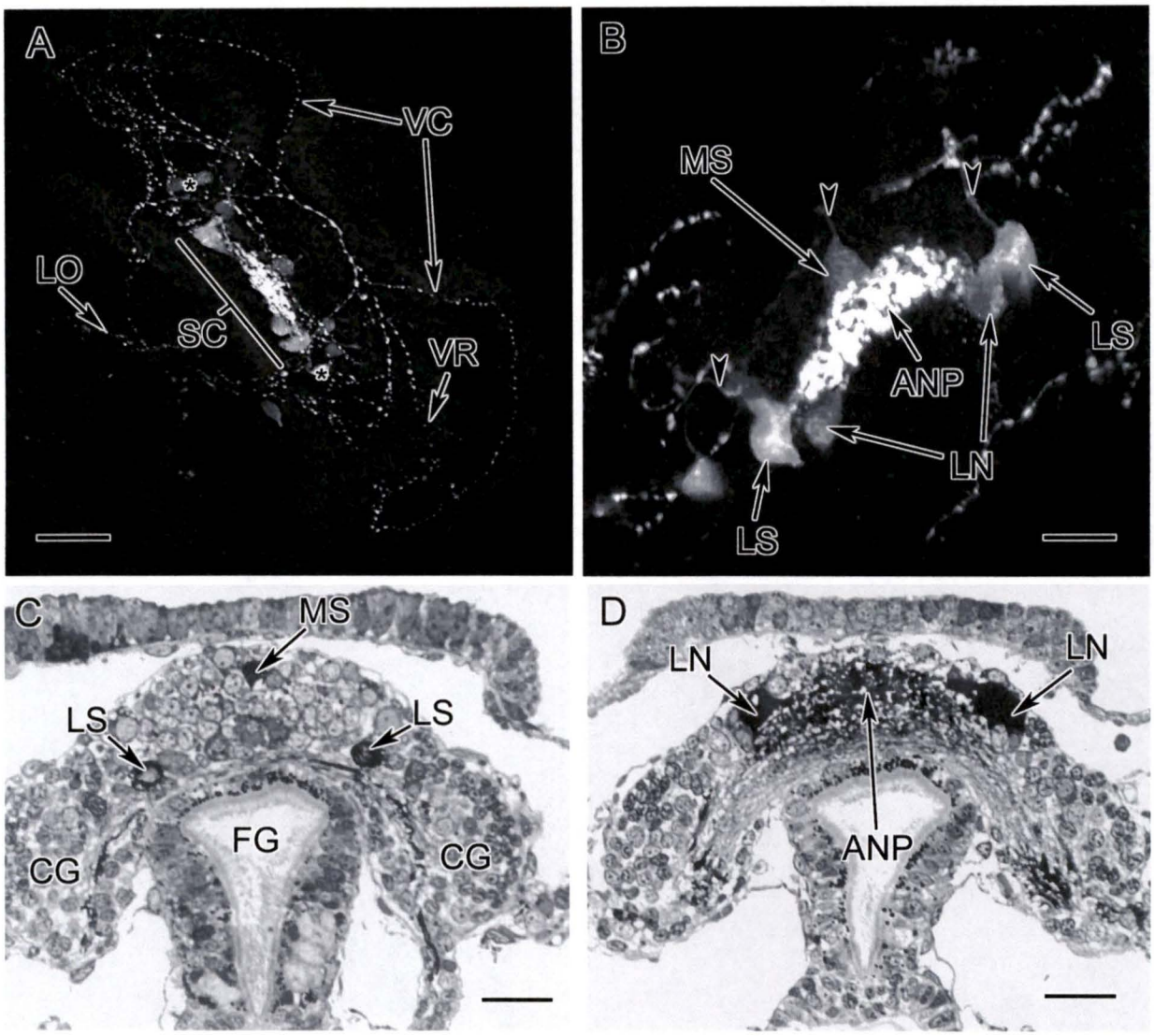
A. Wholemount dorsal view of a newly hatched veliger showing the serotonergic complex of the apical ganglion, radial and velar axonal tracts, and axons within the visceral loop connective. Asterisks indicate labelled clusters of neurons located outside of the apical ganglion. Scale Bar = 40μm.

B. The serotonergic complex of the apical ganglion in *E. lewisii.* A pair of lateral non-sensory neurons, a pair of lateral sensory neurons, and a medial sensory neuron are visible (arrowheads indicate the apical dendritic projections of the sensory neurons). Note that the apical neuropil contains many varicose neurites. Scale Bar = 15μm.

C. Transverse section through a 15 day old *E. lewisii* veliger showing three neurons labelled with DAB reaction product within the apical ganglion. Scale Bar = 15μm.

D. Transverse section cut slightly more posterior to that shown in C. DAB reaction product is localized in within two additional neurons of the apical ganglion. Also note the DAB reaction product in the apical neuropil. Scale Bar = 15μm.

Lettering
ANP = apical neuropil
CG = cerebral ganglion
E = eyespot
FG = foregut
MS = medial sensory neuron
LN = lateral non-sensory neuron
LO = visceral loop connective
LS = lateral sensory neuron
SC = serotonergic complex of the apical ganglion
VC = circumferential velar tract
VR = radial velar tract


A
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ANP

projections into a prominent apical neuropil (Fig. 23A,B). Well-defined velar and pedal tracts are also evident, as are a few cells in the pedal ganglia (Fig. 23A).

Lacuna vincta

The apical ganglion of *L. vincta* contains four serotonergic cells: a lateral pair of sensory neurons, and a lateral pair of non-sensory neurons (Fig. 23C,D). Each cell extends an axon into the apical neuropil. The overall pattern is very similar to that of *E. lewisii*, except that the medial sensory neuron is missing.

Amphissa columbiana

The hatching stage of *A. columbiana* is intriguing, and this is reflected in the pattern of serotonergicity exhibited in its apical ganglion. Externally these larvae show a distinct asymmetry with the right velar lobe being larger than the left, and with the right cephalic tentacle appearing quite prominently while the left does not. Internally, this asymmetry is reflected in the serotonergic complex of the apical ganglion. It consists of six cells: two on the left, one medially, and three on the right (Fig. 23E-G). Again the disproportions of the right side are apparent; the side with the larger velar lobe and tentacle also has an extra cell on the right side of its apical ganglion. The medial neuron is sensory, as is one of the left lateral cells and at least one of the right lateral cells (Fig. 23F). A second cell on the right is suspected to be sensory as well, but a dendritic extension was never satisfactorily resolved in fluorescence images and DAB labelled specimens were not prepared. The remaining neurons, one on each side, are non-sensory

Figure 23. Anti-serotonin immunoreactivity in newly hatched veligers of *Marsenina stearnsii*, *Lacuna vincta*, and *Amphissa columbiana.*

A. Wholemount anterior view of *M. stearnsii* showing the serotonergic complex of the apical ganglion, radial and velar axonal tracts, and highly branched pedal tracts. Scale Bar = 50μm.

B. The serotonergic complex of the apical ganglion in *M. stearnsii*. A pair of lateral non-sensory neurons, a pair of lateral sensory neurons, and a medial sensory neuron are visible (arrowheads indicate the apical dendritic projections of the sensory neurons). Note that the apical neuropil contains many varicose neurites. Scale Bar = 15μm.

C. Wholemount dorsal view of *L. vincta*. The serotonergic complex of the apical ganglion is visible, as are peripheral axonal tracts in the velum and visceral loop connective. Scale Bar = 40μm.

D. The serotonergic complex of the apical ganglion in *L. vincta.* A pair of lateral sensory neurons and a pair of lateral non-sensory neurons are labelled. Arrowheads indicate apical dendritic projections of the sensory neurons. Scale bar = 15μm.

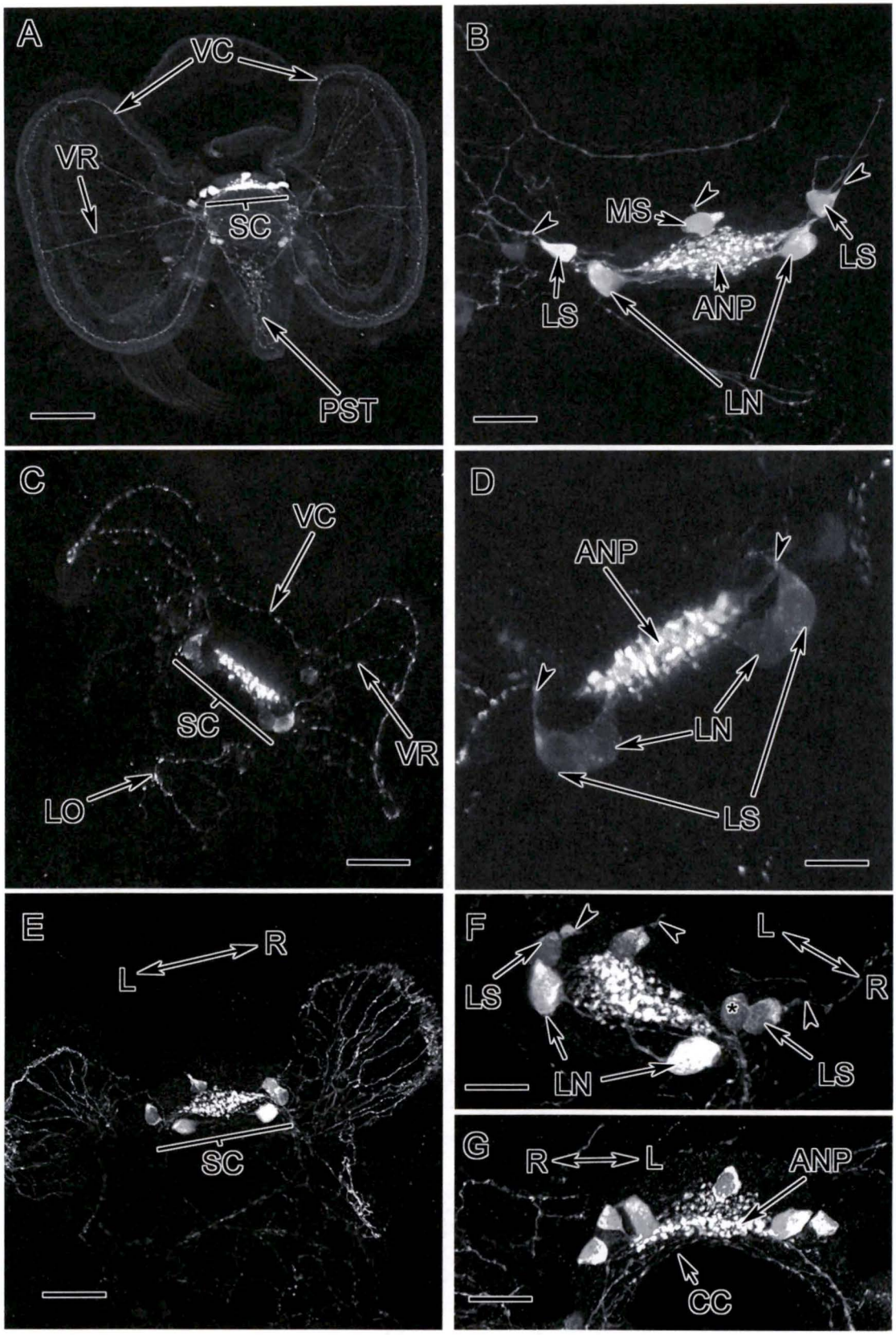
E. Wholemount dorsal view of *A. columbiana.* The asymmetrical serotonergic complex of the apical ganglion and many radial velar tracts are shown. Scale Bar = 50μm.

F. Oblique dorsal view of the serotonergic complex in *A. columbiana* showing the left-right asymmetry of labelled neurons within the apical ganglion. Asterisk indicates a presumed lateral sensory neuron and arrowheads indicate the apical dendritic projections of the confirmed sensory neurons. Scale Bar = 20μm.

G. Ventral view of the serotonergic complex of the apical ganglion in *A. columbiana*. Also note the labelled neurites within the cerebral commissure. Scale Bar = 15μm.

Lettering

ANP = apical neuropil
CG = cerebral ganglion
E = eyespot
FG = foregut
MS = medial sensory neuron
L = left
LN = lateral non-sensory neuron
LO = visceral loop connective
LS = lateral sensory neuron
PST = pedal serotonergic tract
R = right
SC = serotonergic complex
VC = circumferential velar tract
VR = radial velar tract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G
R
L
ANP
CC

(Fig. 23F,G). There are many more prominent radial velar tracts apparent in this species as well (Fig. 23E).

Post-metamorphic Fate of the Apical Ganglion in *T. cancellata*

Previous research indicates that the apical ganglion rapidly degenerates post-metamorphically in gastropod larvae. This was examined in *T. cancellata* larvae with fluorescent preparations of serotonin-like immunoreactivity in pre- and post-metamorphic specimens. These were compared to histological sections of similar stages that were either conventionally fixed or labelled with DAB reaction product.

Fluorescence images show that the apical serotonergic complex of *T. cancellata* persists for the duration of the larval phase (Fig. 24A-D). Post-metamorphically, the characteristic features of the serotonergic complex have disappeared (Fig. 24E,F). The large serotonergic neuronal somata of the apical ganglion, normally so easily recognizable, are gone and only the smaller cells in the cerebral ganglia remain. The prominent neuropil has also disappeared in immunofluorescent preparations for serotonin. Histological sections confirm these observations, although structural aspects of the apical ganglion remain (Fig. 24G). Histological sections of juveniles four days after metamorphosis show complete degeneration of the apical ganglion (Fig. 24H).

Figure 24. Ontogeny and fate of the apical ganglion in *Trichotropis cancellata* veligers.

A. Wholemount oblique anterior view of a late-stage veliger (35 days post-hatching, 1999 cultures) showing immunoreactivity to serotonin in the peripheral velar tracts, pedal ganglia, cerebral ganglia and apical ganglion. Scale Bar = 200μm.

B. The serotonergic complex of the apical ganglion remains unchanged in late-stage veligers, except that the apical neuropil has flattened over the top of the now much larger cerebral commissure. Asterisks indicate two smaller labelled cells located in each of the cerebral ganglia. Scale Bar = 40μm.

C. Cross-section through a DAB labelled metamorphically competent veliger showing two lateral neurons within the apical ganglion. Scale Bar = 20μm.

D. Cross- section through a DAB labelled metamorphically competent veliger showing the medial neuron within the apical ganglion. Asterisks indicate labelled cells within the cerebral ganglia. Scale Bar = 20μm.

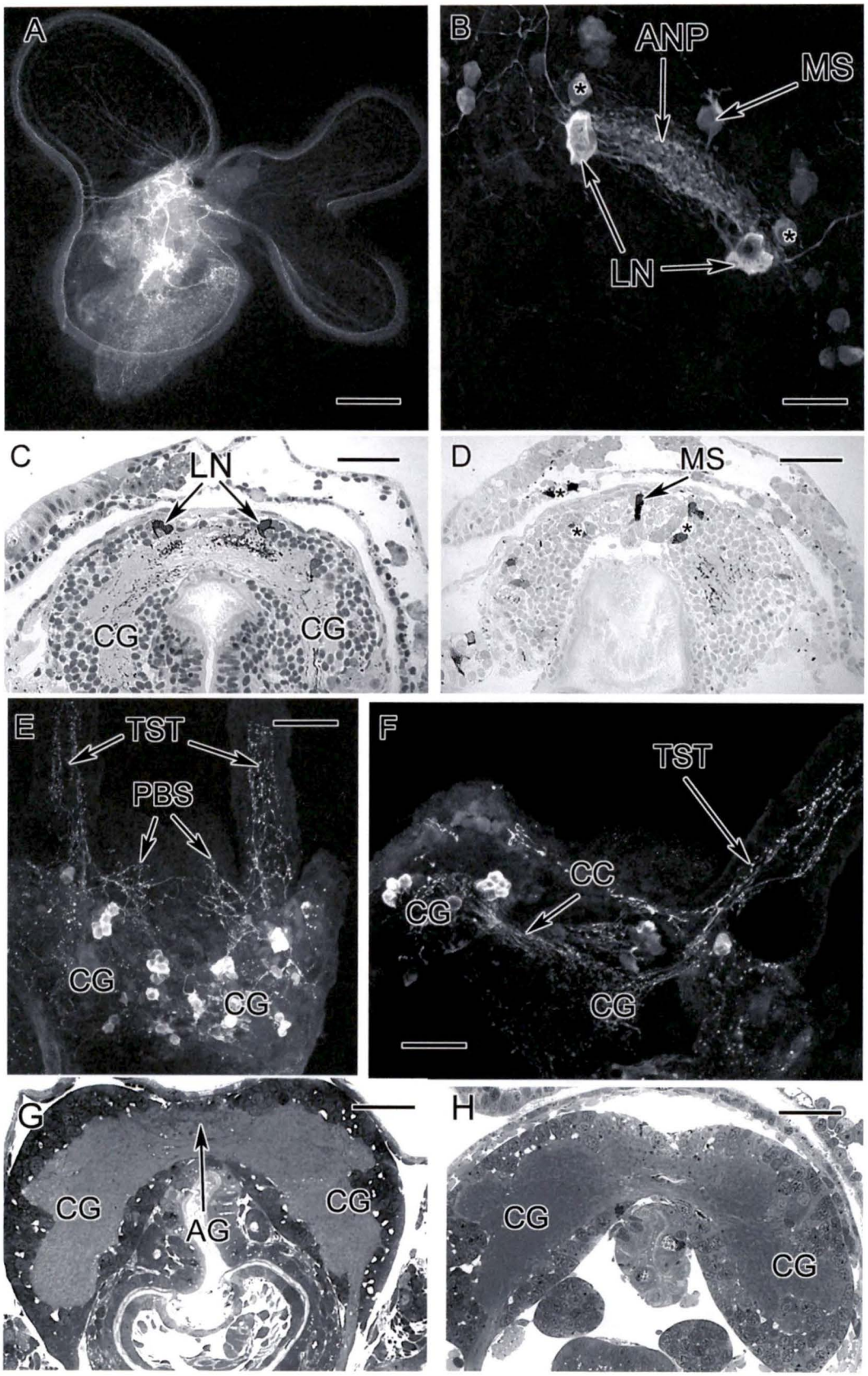
E. Wholemount dorsal view of the head of juvenile snail 24 hours post-metamorphosis. Several cells in the cerebral ganglia show immunoreactivity to serotonin, and serotonergic tracts extend into the pseudoproboscis and cephalic tentacles. The serotonergic complex of the apical ganglion is no longer evident. Scale Bar = 25μm.

F. Dorsal view of the head of juvenile snail 24 hours post-metamorphosis showing labelled neurites in the cerebral commissure. Scale Bar = 25μm.

G. Cross-section through the head of a juvenile 24 hours post-metamorphosis. Structural aspects of the apical ganglion remain, although serotonin is no longer being expressed. Scale Bar = 25μm.

H. Cross-section through the head of a juvenile 4 days post-metamorphosis. All aspects of the apical ganglion have disappeared. Scale Bar = 25μm.

Lettering
ANP = apical neuropil
CC = cerebral commissure
CG = cerebral ganglion
MS = medial sensory neuron
LN = lateral non-sensory neuron
PBS = pseudoproboscis serotonergic tracts
TST = tentacular serotonergic tracts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Discussion

Early Life History and the Veliger of *Trichotropis cancellata*

The life history of *T. cancellata* includes an initial period of encapsulated embryonic development within a benthic egg mass, followed by a free-living larval stage. The veliger larva is an obligate planktotroph, which exists pelagically for a variable amount of time. Life histories of marine invertebrates are often regarded as rigidly defined and species-specific, but this is not entirely accurate (Hadfield and Strathmann, 1996). The larval phase can last for a variable amount of time and, in some species, can show phenotypic plasticity (Boidron-Metairon, 1988; Strathmann et al., 1993). Flexibility in the timing of developmental events can depend on a number of factors, including but not limited to: egg quality, trophic mode, season, temperature, food quality, and availability of suitable settlement substrata (Hadfield and Strathmann, 1996). Flexibility during the larval stage in the life cycle may maximize both pre- and post-metamorphic survival. In this study, I identified two factors that influenced length of larval life for *T. cancellata*: food quality and availability of settlement substratum.

The difference in the duration of the larval stage in my 1999 (42 to 45 days) and my 2000 (28 to 32 days) cultures can most probably be accounted for by improved quality of the larval diet in the second year. The 42 to 45 days to metamorphic competence exhibited by my 1999 cultures was not inordinately long. Cultures of *T. cancellata* larvae reared at the Friday Harbor Laboratories in 1995 did not achieve metamorphic competence until 13 weeks after hatching (Dr. Bruno Pernet, Friday Harbor Laboratories, personal communication). The rate of

development shown by my cultures in both 1999 and 2000 is probably higher than what larvae in the field exhibit, because laboratory-cultured larvae were given a high concentration of algal species known to support rapid growth of marine larvae and the culture temperature (12°C) was at least 2°C higher than local oceanic temperatures during late winter and early spring. The egg-laying season of *T. cancellata* extends over several months (January to April) during which phytoplankton concentrations gradually increase. Sensitivity of larval growth rate to quantity and quality of phytoplankton may result in similar time of recruitment for larvae, regardless of their time of hatching during January through April.

Metamorphically competent larvae of *T. cancellata* will delay metamorphosis in the absence of a suitable settlement substratum. Rates of spontaneous metamorphosis in laboratory cultures were very low and larvae were maintained for up to six weeks after metamorphic competency had been reached. Larvae kept in culture for extended periods retained the ability to metamorphose when exposed to the appropriate stimulus. Metamorphosis was induced by placing the larvae on rocks encrusted with spirorbid or serpulid polychaetes, the preferred hosts for the post-metamorphic kleptoparasitic habit. The metamorphic event is rapid in *T. cancellata* and velar loss could be observed within an hour of exposure to the polychaete-encrusted substrate.

Larvae produced by *T. cancellata* resemble very closely those of *Trichosirus inornatus* (Pilkington, 1974, 1976), a species in the same family (Capulidae) as *T. cancellata. T. inornatus* also has an echinospira larva in which the scaphoconch is retained post-metamorphically and incorporated into the adult

shell. Most echinospira larvae, such as those of *Lamellaria*, *Marsenina*, and *Trivia* species, cast off the scaphoconch at metamorphosis (Fretter and Graham, 1994; personal observation for *M. stearnsii*). It is hypothesized that the echinospira shell is a buoyancy device, assisting the velum in keeping the animals afloat (McCloskey, 1973; Fretter and Graham, 1994). Histological sections through the scaphoconch of *Lamellaria rhombica*, as described by McCloskey (1973), and of *T. cancellata* reveal a highly vacuolated substructure. McCloskey (1973) suspected that the vacuoles were filled with a fluid making them very effective as incompressible, flotation devices.

Late embryos of *T. cancellata* have a ciliary crown that is lost either before, or shortly after, hatching. A similar ciliary crown has been reported in pre-hatching larvae of the caenogastropods, *Ilyanassa obsoleta* by Tomlinson (1987) and *Crepidula fornicata* by Conklin (1897). This feature probably helps circulate intracapsular fluid, thereby promoting diffusive gas exchange by preventing an anoxic boundary layer around late stage embryos. Studies by R. Strathmann and co-workers (Chaffee and Strathmann, 1984; Strathmann and Chaffee, 1984; R. Strathmann and M. Strathmann, 1995; Cohen and Strathmann, 1996; Lee and Strathmann, 1998) have shown that oxygen supply to encapsulated embryos is a major limiting factor for rate of development. Furthermore, the limiting effect of oxygen supply to encapsulated gastropod embryos is most acute in later stages of development, presumably due to a progressive increase in metabolic rate of embryos over the period of encapsulation (R. Strathmann and M. Strathmann, 1995).

A patch of microvilli at the apex of the larval head, covering the epidermis through which dendrites of the apical ganglion penetrate, has been reported in the veligers of *E. lewisii*, *L. vincta*, and *T. cancellata* (Page and Parries, 2000). The apical pit extending towards the apical ganglion has also been reported in *E. lewisii* (Page and Parries, 2000), and in the larvae of an opisthobranch, *Melibe leonina* (Kempf et al., 1997). The patches of microvilli lining the apical epidermis and the walls of these pits are good external indications that this area could function as a transport epithelium, possibly playing a role in the sensory nature of the apical ganglion. Further examination of the ultrastructure of these microvillar patches will certainly be worthwhile in continued investigations of the apical ganglion.

The elaboration of the ventral lip during the larval phase and its rapid transition into the pseudoproboscis post-metamorphically is very intriguing. Many gastropod larvae, particularly carnivorous forms, require a number of days after metamorphosis before feeding can occur (e.g. Bickell et al., 1981; Pedersen and Page, 2000; Page, 2000). This delay is due to morphological changes that occur during the metamorphosis, particularly in the foregut and stomach tissues. *T. cancellata* does not exhibit this delay; as soon as the velum was lost during metamorphosis (a process taking from one to several hours), snails were observed to migrate to the nearest polychaete host and began using their pseudoproboscis to ingest stolen food. Post-metamorphic feeding began within four hours after the loss of the velum. Pernet and Kohn (1998) noted that small snails without access to a host suffered high mortality, suggesting that the kleptoparasitic habit is

obligate for smaller individuals of *T. cancellata.* Indeed, ctenidial surface area in young juveniles may be too small to allow sufficient food capture. Nevertheless, the extremely rapid onset of post-metamorphic feeding in *T. cancellata* is surprising. A thorough investigation of foregut development, paying particular attention to features that allow rapid onset of post-metamorphic feeding, would be interesting, especially when compared to the results reported by Page (2000) in three other species of caenogastropod.

The Larval Nervous System of *T. cancellata*

Serotonin

Serotonin is a biogenic monoamine derived from the amino acid tryptophan. It is found in virtually all animal phyla, making it an ancient neuroactive substance (see Hay-Scmhidt, 2000 for review). Its neurophysiological role has been particularly well studied in adult molluscan tissues where it acts as a mediator of extracardiac nerves (e.g. Erspamer and Boretti, 1951; Welsh and Moorhead, 1960; Twarog, 1967; Moroz et al., 1997). Serotonin is also a regulator of neuronal development in pulmonate gastropods; specifically, it can evoke an inhibition of neurite outgrowth and synaptogenesis in specific neurons within *Helisoma trivolvis* embryos (Goldberg and Kater, 1989; Goldberg et al., 1991).

Serotonin has been convincingly shown to have cilioexcitatory effects in a number of different molluscs. When bath applied to excised ctenidial lamellae of bivalves, for instance, serotonin has a pronounced stimulatory effect (Gosselin, 1961; Aiello, 1970; Saimi et al., 1983; Murakami, 1983). Koshtoyants et al.

(1961) showed that exogenous serotonin application increased the beat frequency of prototrochal cilia in gastropod larvae. Mackie et al. (1976) demonstrated the existence of neurociliary synapses onto prototrochal cells of gastropod veligers. According to Mackie et al. (1976), some of the presynaptic profiles showed many dense-core vesicles, which may have contained serotonin. The velum of gastropod larvae examined in the present and other studies (e.g. Barlow and Truman. 1991; Marois and Carew, 1997c; Kempf et al., 1997; Page and Parries, 2000) and the velum of bivalve larvae (Leask, 1991; Croll et al., 1997) shows a pronounced circumferential tract of one or more serotonergic axons that appear to contact ciliated cells of the prototroch. Synapses by serotonergic axons onto prototrochal cells of *Aplysia californica* veligers were found by Marois and Carew (1997) using an immuno-electron microscopy technique. The serotonergic velar neurites generally originate in the neuropil of the apical ganglion, suggesting that this ganglion may be the nervous centre responsible for velar coordination. All available evidence suggests that serotonin plays an important role in controlling the ciliary activity of the velum. It would be interesting to perform a series of pharmacological experiments with a variety of serotonin agonists and antagonists in order to further assess the role of serotonin in the control of ciliary locomotion in gastropod veligers.

The importance of serotonin in developing gastropods that lack a veliger stage has been shown by pharmacological studies on developing embryos of the pulmonate *H. trivolvis.* These embryos have a pair of identified neurons that transiently release serotonin onto ciliary cells of the prototroch, causing periodic

increases in embryonic movement (Diefenbach et al., 1991; Goldberg et al., 1994). Serotonin immunoreactive neurons have also been identified in encapsulated embryos of the pulmonate, *Lymnaea stagnalis* (Marois and Croll, 1992).

In adult *Tritonea diomedea* and *Lymnaea stagnalis*, serotonin release from neurons having somata within the pedal ganglia increases the rate of ciliary beating along the sole of the foot (Audesirk et al., 1979; Syed and Winlow, 1989). Both opisthobranch (Kempf et al., 1997) and caenogastropod (Page and Parries, 2000; present study) veligers have been shown to have a gradually increasing number of serotonergic neurons in their pedal ganglia, as well as an increasingly elaborate network of serotonergic fibres ramifying along the sole of the foot. The pseudoproboscis of *T. cancellata* is yet another heavily ciliated structure that is well supplied with serotonergic neurites, although the precise target of these tracts was not examined in this study.

In addition, serotonin has been shown to modulate muscle contractility in adult opisthobranchs (e.g. Satterlie, 1995; Satterlie and Norekian, 1995; Satterlie et al., 1995). Serotonergic neurites contact muscle fibres within the larval velar lobes of the opisthobranchs *Aplysia californica* (Marois and Carew, 1997) and *Melibe leonina* (Kempf et al., 1997), and fibres of the larval retractor muscle in *A. californica*. The present study showed a distinct relationship between velar serotonergic projections and trajectories of velar muscles. The velum in *T. cancellata*, and in virtually all other veliger larvae, can be contracted and contorted into a variety of shapes by the action of muscle fibres. The velar

perimeter can pull together as though it were on a drawstring, presumably through the action of the circumferential muscle fibres underlying the prototroch. However, data obtained using fluorescent labels for both muscles and serotonin immunoreactive axons do not conclusively demonstrate direct innervation of muscle by serotonergic axons. Velar axons may merely use muscle cells as guideposts as they progress to other targets, such as the ciliated cells of the prototroch. Serotonin is known to modulate a wide range of muscular activities in adult molluscs (see Walker, 1986 for review), and therefore it is not unreasonable to suggest that it could regulate larval muscular activities as well. It would be useful to explore this relationship further using immunocytochemical techniques suitable for transmission electron microscopy.

FMRFamide

FMRFamide is a neuropeptide first extracted and characterized from ganglia of the clam, *Macrocallista nimbosa* (Price and Greenberg, 1977). This original report characterized it as a cardioexcitatory peptide, but it has subsequently been shown to have both excitatory and inhibitory effects on the heart, depending on the species of bivalve to which it is applied (Painter and Greenberg, 1982). Since its original discovery, an entire family of FMRFamide-like peptides has been identified and these occur in many invertebrate organisms. The physiological role of FMRFamide is often as a neuromodulator involved with muscle contraction/relaxation (e.g. Muneoka and Matsura, 1985; Muneoka and Saitoh, 1986; Smith and Hill, 1987). Recently it has been implicated in the regulation of osmoregulation in the pulmonate, *Helisoma duryi* (Khan et al.,

1998). FMRFamide may also modulate the activities of the gut and salivary glands (for review see Greenberg et al., 1983).

A number of FMRFamide-like immunoreactive cells and processes were found in the nervous system of *T. cancellata* larvae. The two large apical cells associated with the ciliary crown in hatching larvae are intriguing. These cells extend projections into the area of the apical neuropil or cerebral commissure, although it is difficult to tell which. The shape of the somata does not conform to the morphology of other cells that have been identified in the apical ganglion, and unlike the serotonin immunoreactive cells of the apical ganglion, the apical FMRFamide neurons disappear shortly after hatching. All other data to date indicate that the apical ganglion is fully formed at hatching and undergoes little subsequent modification during the larval phase (Marois and Carew, 1997a; Kempf et al., 1997; Page and Parries, 2000). Transient expression of FMRFamide has been reported in the developing nervous system of *Lymnaea stagnalis* (Voronezhskaya and Elekes, 1996). It is possible that the two apical cells expressing FMRFamide-like immunoreactivity persist after the ciliary crown is gone, but stop expressing the peptide. Further investigation of the nature of these cells is certainly warranted. Histological sections of specimens labelled with DAB reaction product to determine the precise relationship of these cells to others in the region of the apical ganglion would be particularly valuable.

The presence of FMRFamide-like immunoreactive cells in the region of the apical ganglion has been reported in embryonic *Crepidula fornicata* (Dickinson et al., 1999). Five FMRFamide-like cells with a flask-shaped

appearance are described, and seem to resemble the serotonergic neurons present in the apical ganglion of opisthobranch larvae. Four cells expressing FMRFamide-like immunoreactivity appear in the region corresponding to the apical ganglion in *T. cancellata*, but only after the ciliary crown is gone. These cells do not match the morphology of other apical ganglion cells and it could not be determined if they were truly located within the apical ganglion, or if they were actually associated with the cerebral ganglia. Again, DAB labelled material could resolve this uncertainty. Serotonin and FMRFamide have been shown to have antagonistic actions in some tissues (Painter et al., 1982) and it is plausible that the apical ganglion has a dual control system underlying some of its functions.

The pattern of FMRFamide-like immunoreactive cells and fibres located along the metatrochal ciliary band in *T. cancellata* is markedly different from that reported in pre-hatching larvae of *C. fornicata* (Dickinson et al., 1999). *C. fornicata* extends FMRFamide fibres into the velar lobes in a pattern very similar to the patterns of serotonergic fibres described for other species (Marois and Carew, 1997a,b,c; Kempf et al., 1997). Dickinson et al. (1999) suggest that the FMRFamide-like processes extend from the apical ganglion to control aspects of velar locomotion and food capture in coordination with the serotonergic tracts. As mentioned earlier, bath applied serotonin increases the rate of ciliary beating in a number of opisthobranch species (Koshtoyants et al., 1961). Given that FMRFamide and serotonin have antagonistic effects in some tissues, it would be interesting to see what effect this peptide would have on ciliary beating. The

differences observed between *T. cancellata* and *C. fornicata* are not easily explained. It would be useful to label comparable stages in the two species, as the existing observations involve embryonic stages in the case of *C. fornicata*, and the free-swimming larva in the case of *T. cancellata.*

Feeding in planktotrophic veligers requires the coordinated activity of both the prototrochal and metatrochal ciliary bands (see Strathmann and Leise, 1979). The activity of the metatrochal (post-oral) ciliary band is regulated separately from that of the prototrochal (pre-oral) ciliary band. Mackie et al. (1976) noted, "the post-oral bands and food groove [cilia] continue normal metachronal beating when the preoral bands are arrested". The presence of FMRFamide-like immunoreactive fibres and cells in the metatroch implicates its involvement in the activity of this ciliated band.

Dopamine ß-hydroxylase

Catecholamines are present in the nervous systems of many invertebrate larvae. In molluscs there is evidence to suggest that catecholamines act as morphogens, directly influencing early development (Buznikov et al., 1996). Catecholamines may also play a role in controlling ciliary activity (Beiras and Widdows, 1995). Recent experimental work by Pires et al. (2000), suggests that catecholamines serve as endogenous modulators of metamorphosis in competent larvae of an opisthobranch gastropod, *Phestilla sibogae.* Methods for the direct immunocytochemical localization of catecholamines are not yet reliable, although antibodies to the enzymes responsible for catecholamine synthesis are available. Catecholamine presence is therefore inferred through the immunolocalization of

those enzymes. Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine synthesis. Commercially available antibodies against tyrosine hydroxylase are commonly used as a marker of catecholaminergic neurons (e.g. Dickinson et al., 1999; Pires et al., 2000). Dopamine ß-hydroxylase is another enzyme in the catecholamine synthesis pathway, converting dopamine to norepinephrine (Cooper et al., 1986). Antibodies recognizing dopamine ß-hydroxylase have been used to infer the location of catecholaminergic neurons in the veliger larva of the scallop, *Patinopecten yessoensis* (Leask, 1991). This author immunolocalized dopamine ß-hydroxylase cells in the lower lip of scallop larvae and suggested that they may play a chemosensory role, particularly in the detection and subsequent integration of the cue for metamorphosis.

All attempts to label the nervous system of *T. cancellata* with antibodies to dopamine ß-hydroxylase failed. This could be construed to indicate that this enzyme was not present, and by inference catecholamines were not present, in the larval nervous system of *T. cancellata.* However, considering the wide spread occurrence of catecholamines in the larval nervous systems of many other molluscs, this seems unlikely (e.g. Pires et al., 1997, 2000; Croll et al., 1997; Dickinson et al., 1999; Voronezhskaya et al., 1999). The simplest explanation of the failure of these antibodies to successfully label any tissues in *T. cancellata* lies simply in the fact that they were old and had potentially been frozen and thawed several times. It is also possible that these antibodies, having been produced and isolated from a mammalian system, do not recognize the appropriate antigen in

the gastropods examined during the course of this study. I believe that further experimentation with fresh antibodies would reveal more interesting results.

Nitric Oxide Synthase – NADPH diaphorase histochemistry

In the last decade, it has become firmly established that nitric oxide (NO) is a gaseous signalling molecule operating in both the central and peripheral nervous systems of vertebrates (e.g. Garthwaite et al., 1988; Bredt et al., 1990, 1991). Nitric oxide synthases (NOS) have NADPH-diaphorase activities and, consequently, histochemical techniques for NADPH-diaphorase can be used to locate the enzymes that produce NO. NO signalling, including neurotransmission and neuromodulation, is now know to be widespread throughout the invertebrate phyla as well (see Martinez, 1995; Jacklet, 1997 for review). Moroz and Gillette (1995) provided a comparative analysis of nitric oxide signalling in three classes within the Mollusca: the Polyplacophora, the Gastropoda and the Cephalopoda. Recent experimental work with the caenogastropod *Ilyanassa obsoleta*, indicates that NO may be an endogenous inhibitor of gastropod metamorphosis (Froggett and Leise, 1999). In *I. obsoleta* larvae, most nitric oxide synthase activity occurs within the apical ganglion (Lin and Leise, 1996b). It seemed appropriate to search for evidence of this extraordinary signalling system in other gastropod larvae.

Attempts in thus study to localize NADPH-diaphorase activity in the larvae of three species of caenogastropods were not successful. Results showed reaction product in a series of cells in the foot of all three species, but control experiments showed reaction product in precisely the same cells. Histological

sections revealed that these cells were glandular cells of the foot. Lin and Leise (1996a,b) indicate that a positive stain would manifest itself as a dark blue reaction product within the ganglia and neuropils of the nervous system. No such staining was evident in any of the specimens prepared in this study. The protocol that I used was the same as that used by Lin and Leise (1996a,b), except that I did not use as high a concentration of Triton X-100 (0.1% as opposed to 0.25%) in my incubation solutions. It is possible that the labelling reagents failed to penetrate in my specimens. The histological sections cut by Lin and Leise were also much thicker than those cut in this study (8 μm as opposed to 1 μm) and thicker sections might be necessary to adequately resolve the reaction product in stained tissues (Dr. E. Leise, University of North Carolina, personal communication). However, it seems likely that at least some staining should appear in 1μm sections. Regardless, further studies of this signalling system in gastropod larvae should be pursued.

Comparative Aspects of the Apical Ganglion

Ultrastructurally, the apical ganglion has a very consistent ground plan in all gastropod veligers examined to date. That plan consists of a cluster of sensory and non-sensory neurons, each sending an axonal projection into a central neuropil, lying just dorsal to the cerebral commissure. Bundles of the dendritic projections of the sensory cells are gathered together to penetrate the overlying epidermis in three distinct locations. There are, however, a few notable differences between the apical ganglion as it appears in opisthobranch larvae and in caenogastropod larvae.

All opisthobranchs examined to date share almost identical ultrastructural and serotonergic characteristics (Chia and Koss, 1984; Marois and Carew, 1997a; Kempf et al., 1997). Four cell types can be distinguished in the opisthobranch apical ganglion: ampullary sensory neurons, parampullary sensory neurons, non-sensory neurons and ciliary tuft cells. There are five serotonergic neurons associated with the apical ganglion: a bilateral non-sensory pair, a bilateral sensory pair, and a medial sensory neuron. The ampullary neurons, as described by several authors (Bonar, 1978, Chia and Koss, 1984; Kempf et al., 1997) are particularly distinctive, having a deep internal pocket that is packed tightly with internalized cilia, and is formed by an invagination of the distal dendritic terminal. The axonemes of the cilia in the ampullary cells of opisthobranchs show a typical 9 + 2 arrangement of microtubules.

All cells associated with the apical ganglion, with the possible exception of ciliary tuft cells, extend axons into the apical neuropil (Marois and Carew, 1997a; Kempf et al., 1997). This does not rule out a neuronal or sensory function for the ciliary tuft cells, however, as other ciliated neuronal types neither exhibit nor require axonal projections in order to perform their sensory function. Hair cells of the mammalian inner ear are an example (Kandel et al., 1991).

Kempf et al. (1997), noting the highly conserved structure of the apical ganglion in all opisthobranchs thus far examined, proposed that it might prove a useful character in the construction of phylogenetic hypotheses, particularly amongst the gastropod molluscs. Early observations did indeed suggest that there were clade specific differences exhibited by the apical ganglion in two sister

groups within the Gastropoda: the heterobranchs (pulmonates and opisthobranchs) and the caenogastropods. Uthe (1995) provided the first ultrastructural analysis of the apical ganglion in one species of caenogastropod, *Littorina littorea*, and she found both similarities and differences to the opisthobranch type. Ampullary neurons were identified, with essentially the same morphology as those of the opisthobranch type, although the ciliary axonemes were not of the typical 9 + 2 arrangement. Uthe (1995) also identified "accessory cells" which very closely resemble parampullary neurons, but she was unable to identify an axonal projection. The neuropil was also not identified in *L. littorea*, but the distinctive attributes of this tangled mass of neurites are visible in Uthe's Figure 11 (1995). Finally, only two bundles of dendritic projections were identified, and ciliary tuft cells did not appear.

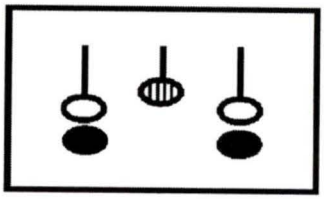
A study by Page and Parries (2000) examined the ultrastructure and serotonin-like immunoreactivity of the apical ganglion in four species of caenogastropod veligers. Results showed that the caenogastropod apical ganglion has much more in common with the opisthobranch type than was revealed by Uthe's (1995) investigation. The only clade-specific difference between the apical ganglion of the opisthobranch and caenogastropod type is the variously modified ciliary axoneme exhibited by the cilia of the ampullary cells in all caenogastropods thus far examined.

Caenogastropods do, however, show a greater degree of interspecific variability within the basic plan of the apical ganglion than do opisthobranchs. For instance, ciliary tuft cells may or may not be present. As well, the appearance

Figure 25. Summary diagram of the neuronal composition of the serotonergic complex within the apical ganglion of planktotrophic caenogastropod larvae. "Opisthobranchia " includes *Aplysia californica* (Marois and Carew, 1997a-c) and four species of nudibranchs (Kempf et al. 1997). The sensory or non-sensory nature of the third neuron on the right lateral side of *A. versicolor* is unknown. Classification into superfamilies after Ponder and Warén (1988). Diagram modified from Page and Parries (2000).

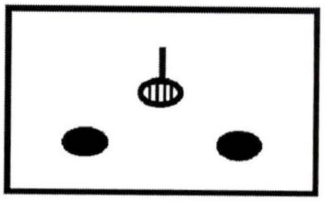
HETEROBRANCHIA

OPISTHOBRANCHIA

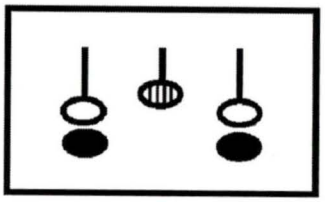


CAENOGASTROPODA

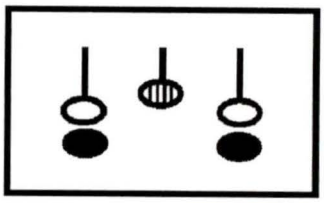
TRICHOTROPIS CANCELLATA
(CALYPTRAEOIDEA)



EUSPIRA LEWISII
(NATICOIDEA)



MARSENINA STEARNSII
(LAMELLAROIDEA)



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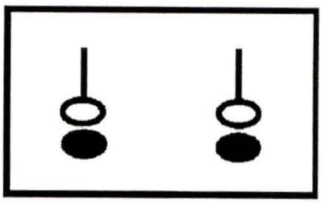
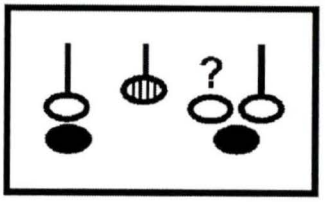
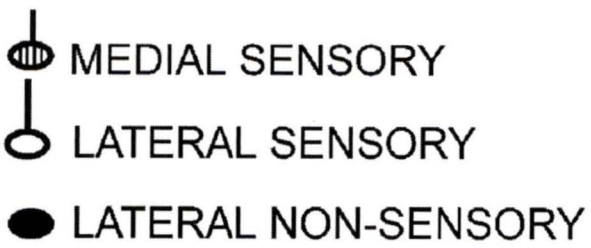
(LITTORINOIDEA)



AMPHISSA VERSICOLOR
(MURICOIDEA)





personal observation). At present, there is no readily apparent explanation for interspecific differences in the number of serotonergic cells in the apical ganglion of caenogastropod larvae, and until the precise function of the apical ganglion is elucidated, it will probably remain so.

Potential Functions of the Apical Ganglion

Previous studies regarding the cellular morphology of the apical ganglion in gastropod larvae have firmly established that it is sensory in nature (Bonar, 1978; Chia and Koss, 1984; Uthe, 1995; Marois and Carew, 1997a; Kempf et al., 1997; Page and Parries, 2000). The apical ganglion is positioned so that the epidermis overlying it is constantly bathed in water currents generated by the velar cilia. Bonar (1978) suggested that the apical ganglion's position placed it in an ideal location to sense the chemical stimulus that initiates metamorphosis. A study by Couper and Leise (1996) showed that direct injection of serotonin induced metamorphosis in the caenogastropod *Ilyanassa obsoleta,* and similar results were reported by Avila et al. (1996) for the nudibranch, *Phidiana [Hermissenda] crassicornis.* Other work has suggested that serotonergic neurons of the apical ganglion, modulated by the action of nitric oxide, might play an important role in the regulation of metamorphosis in *I. obsoleta* (Frogget and Leise, 1999). Recent work performed by Hadfield et al. (2000) provided the first experimental evidence that the apical ganglion is indeed a receptor for settlement cues in the larvae of the nudibranch *Phestilla sibogae*. When components of the apical ganglion were photoablated, larvae of *P. sibogae* lost the ability to metamorphose when exposed to the usual inducer, a soluble metabolite from the

coral prey of the adult animal. These results, combined with the fact that the apical ganglion rapidly degenerates post-metamorphically, certainly seem to imply that this structure plays an important role in the regulation of metamorphosis in gastropod veligers.

A question arises however; why would a structure involved in the last stage of larval life develop so early on? In all species examined to date, the apical ganglion is clearly distinguishable at hatching in both planktotrophic and lecithotrophic larvae. In *T. cancellata* (this study) and *C. fornicata* (Dickinson et al., 1999), serotonergic components of the apical ganglion begin forming in early embryonic stages. Detection of the signal for metamorphosis is certainly plausible, but it seems likely that the apical ganglion serves some other function(s) as well.

Chia and Koss (1984) suggest that the apical ganglion of *Rostanga pulchra* plays multiple roles, both chemo- and mechanosensory, in mediating larval responses to environmental stimuli. This proposal was based on the fact that the apical ganglion contained three distinct sensory cell types. In addition to detecting metamorphic inducing factors, the functions proposed by Chia and Koss (1984) include: monitoring the position and orientation of the larva within the water column, maintenance of mantle cavity water quality, co-ordination of retraction/protraction of the velum and cephalopedal mass into and out of the shell, and the sorting of food particles. They also suggested that the ampullary cells of the apical ganglion might function as vibration or pressure receptors based

on comparison with similar cells in *Octopus* (Emery, 1976; Altner and Prillinger, 1980).

Kempf et al. (1997) suggested a possible endocrine function for the apical ganglion. In young opisthobranch larvae, the apical ganglion bulges slightly into the larval hemocoel. The axonal varicosities within the apical neuropil are filled with vesicles and separated from the hemocoel only by a thin layer of connective tissue. The authors suggest that this may be the site for release of neurohormones affecting larval behaviour or development.

The velar lobes, in all species examined to date, are innervated both ipsilaterally and contralaterally by neurites originating within the apical neuropil suggesting that the apical ganglion plays a compensatory role in velar co-ordination. Ultrastructural analysis and phalloidin labelling of f-actin also reveal a close association of serotonergic axons with muscle fibres, indicating a possible function of the apical ganglion in modulating velar muscle activity.

Overall, the apical ganglion seems best regarded as a complex, multi-modal nervous structure playing no single or simple role in mediating larval behaviour. This is an area of potentially profitable future research. Expanding upon the work of Hadfield et al. (2000) would be especially interesting. Attempting to target and ablate cells in the apical ganglion earlier than at metamorphic competence might reveal insights into some of the other functions of the apical ganglion (i.e. what sort of behavioural changes occur when the apical ganglion is disabled?). It would also be interesting to obtain neurophysiological recordings from the cells of the apical ganglion and relate them to observed

ciliary or muscular activity within the velum. Increasing the scope of the experiments already carried out to incorporate a wider array of species would be especially useful in elucidating the functions of the apical ganglion and in increasing our understanding of the evolutionary origin of the apical ganglion in the Gastropoda.

Apical Organs Across the Invertebrate Phyla

To date, the apical organs of marine larvae have most often been associated with the very characteristic ciliary tuft. This is not an invariant feature however. In bivalve larvae, for instance, an apical ciliary tuft may or may not be present (see review in Cragg, 1996). A similar situation occurs in caenogastropod larvae (Page and Parries, 2000; present study). A number of recent studies have revealed that the presence of serotonergic neurons is also very typical of these apical organs. In addition to those found in gastropod species (Marois and Carew, 1997a,b,c; Kempf et al., 1997; Page and Parries, 2000; present study), the work of Hay-Schmidt has revealed serotonin immunoreactivity in the larval apical organs of two congeneric phoronids (*Phoronis mulleri* & *P. vancouverensis,* 1990a,b), a brachiopod (*Glottidia* sp., 1992), and a polychaete (*Polygordius lacteus,* 1995). Bisgrove and Burke (1986) have also found serotonin immunoreactive neurons in the apical ganglion of a deuterostome, the echinoderm *Strongylocentrotus purpuratus*. In all of these examples the neurons involved extend axons into a distinct apical neuropil and then on into other tissues of the larval body.

The evolutionary transition from trochophore to veliger larva has been a source of interest to developmental biologists for decades (Wilson, 1892; Conklin,

1897). During the embryonic development of the veliger larva, a stage distinctly resembling a trochophore larva can be observed and some basal gastropod groups have a free-swimming trochophore larval form. These observations have led to the generally accepted notion that the veliger of molluscs evolved from a trochophore-like ancestor.

The apical ganglion of the veliger larvae of molluscs is most similar to that of the trochophore larvae of polychaete annelids. Lacalli (1981, 1984) reported the presence of a ciliary tuft and ampullary-like cells in larvae of the polychaetes *Phyllodoce maculata* and *P. mucosa*. His ultrastructural studies (Lacalli, 1981, 1983, 1984) have shown that the apical organs of both polychaete and turbellarian larvae contain a characteristic axonal neuropil adjacent to the cerebral commissure. In addition, Hay-Schmidt (1995) has examined serotonin-like immunoreactivity in the trochophore of the polychaete *Polygordius lacteus.* The apical ganglion in these larvae is located between a pair of apical tentacles and includes three monopolar serotonergic cells, two lateral and one dorsal, which extend processes into a central neuropil. The apical ganglion innervates the tentacles, the two eyespots and also sends processes to the proto- and metatroch. Hay-Schmidt's study also revealed serotonergic nerves in close association with ciliary bands and muscles, which is another similarity between gastropod veligers and polychaete trochophores. Since serotonin is widespread, Hay-Schmidt suggests that it is a "phylogenetically ancient transmitter that may have retained some of its ancient physiological functions during the diversification of the main animal phyla".

Kempf et al. (1997) interpreted similarities in the structure and position of the apical ganglion among various spiralian phyla as evidence of homology. Criteria commonly applied by neurobiologists to infer neuronal homologies are: soma position and size, axon trajectory, neuritic branching patterns, synaptic connectivity, electrophysiological parameters, biochemical parameters, and developmental lineage. The larval apical ganglion among species of gastropod molluscs certainly conforms to many of these criteria and the potential for its use as a character in the dissection and understanding of phylogenetic relationships cannot be dismissed.

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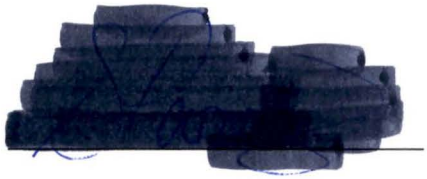
Author 

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