NEURAL AND MUSCULAR DEVELOPMENT IN A GASTROPOD LARVA

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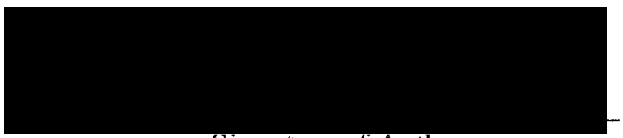

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This thesis is dedicated to my Parents and Grandparents:

Louis and Heather Dickinson

Dr. Charles and Bernadette Graham

and Jean Dickinson

and in memory of:

Magdalene Porter and Herbert Dickinson

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ABSTRACT

The goal of this thesis was to examine neural and muscular development in the well studied gastropod *Ilyanassa obsoleta*. A relatively sophisticated larval nervous system (LNS) was revealed using immunocytochemical techniques combined with confocal microscopy. The LNS first appeared in the trochophore stages and became more elaborate during veliger stages. Neurons containing monoamines and neuropeptides were observed in the apical organ, peripheral regions and developing ganglia destined to become the adult central nervous system. Furthermore, neural elements were also associated with ciliary bands and muscle fibers in the velum and near the mouth cavity suggesting roles in swimming and feeding. Exposure to exogenous monoamines, synthetic analogues and neuropeptides had significant effects on such behaviours. Distal-less was expressed in the ectoderm and then later in neural ectodermal regions from which the developing ganglia were formed. The expression pattern suggested a conserved role of the gene in ectoderm specification and development of migratory neurons. Distal-less was not expressed in other regions of the developing larval nervous system and therefore, separate mechanisms likely govern the differentiation of different parts of the larval nervous system. This hypothesis was further examined in experiments in which the D macromere was ablated. The resultant larvae lacked distal-less in the ectoderm and neural ectoderm and only neurons outside the developing central ganglia were observed. Finally, using F-actin labeling, the development and anatomy of the larval musculature was examined. The larval musculature was relatively extensive. Two major muscle systems were identified, the larval retractor and pedal retractor muscles. These muscles differed in their anatomy, timing of appearance and fates during metamorphosis. Cell lineage tracing indicated that these major muscle groups had different cellular origins. Additionally, cell ablations and chemical block of early inductive signals, indicated that induction signals were also necessary to form organized muscle systems.

LIST OF ABBREVIATIONS

1. General

analysis of variance	ANOVA	litre	L
central nervous system	CNS	lucifer yellow dextran	LYD
Celsius	°C	magnesium chloride	$MgCl_2$
dimethyl sulfoxide	DMSO	microlitre	μL
distal-less protein	Dll	micron	μm
distal-less gene	dll	millilitre	mL
dopamine	DA	millimetre	mm
Dopamine-beta-hydroxylase	DβH	millimolar	mM
ethanol	ETOH	millisecond	ms
ethylenediaminetetracetic acid	EDTA	mitogen-activated protein kinase	MAPK
excitatory post synaptic potentialsEPSPs	EPSP	moles per litre	M
extracellular signal-regulated protein kinases	ERK	nanometre	nm
fluorescein ithiocyanate	FITC	norepinephrine	NE
Phe-Met-Arg-Phe-NH2	FMRF--amide	percent	%
formaldehyde glutaraldehyde	FaGlu	phosphate buffer saline	PBS
grams	g	pounds per square inch	psi
hertz	Hz	probability	P
high performance liquid chromatography	HPLC	serotonin	5-HT
larval nervous system	LNS	seconds	s
laser scanning microscope	LSM	standard deviation	SD
like immunoreactive	LIR	tetramethlyrhodamine B isothiocyanate	TRITC
		tyrosine hydroxylase	TH

2. Identified Cells

Catecholamine Cerebral	CC	FMRFamide-LIR Velar	FV
Catecholamine Dorsal Mouth	CDM	FMRFamide-LIR	FSp
Catecholamine Distal Velar	CDV	Supra-intestinal	EA
Catecholamine Foot	CF	leu-Enkephalin-LIR Apical	EV
Catecholamine Osphradial	COs	leu-Enkephalin-LIR Velum	mSA
Catecholamine Proximal Velum	CPV	medial Serotonin-LIR Apical	mFA
Catecholamine Propodium	CPr	medial FMRFamide-LIR Apical	SAL
Catecholamine Ventral Mouth	CVM	Serotonin-LIR Apical Lateral	SA
Dopamine--hydroxylase-LIR	DF	Serotonin-LIR Apical	SC
Foot		Serotonin-LIR Cerebral	SOs
FMRFamide-LIR Apical	FA	Serotonin-LIR Osphradial	SPe
FMRFamide-LIR Cerebral	FC	Serotonin-LIR Pedal	SSp
FMRFamide-LIR Distal Foot	FDF	Serotonin-LIR Supra-intestinal	SSb
FMRFamide-LIR Osphradial	FOs	Serotonin-LIR Sub-intestinal	SV
FMRFamide-LIR Pedal	FPe	Serotonin-LIR Velar	
FMRFamide-LIR Posterior	FP		
FMRFamide-LIR Proximal Foot	FPF		
FMRFamide-LIR Sub-intestinal	FSb		

3. Identified Muscles

accessory muscle	ACM	pedal retractor muscle	PRM
dorsal head muscle	DHM	propodial muscles	PrM
dorsal mouth muscle	DMM	velar muscles	VM
foot muscle	FM	ventral mouth muscle	VMM
larval retractor muscle	LRM		

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LIST OF FIGURES AND ILLUSTRATIONS

* - schematic representation
\+ - graphical representation

CHAPTER 1

INTRODUCTION:

REVIEW OF GASTROPOD NEURAL

AND MUSCLE DEVELOPMENT

Part 1: Review of Gastropod Larval Nervous Systems

1. A) Why Study Neural Development in Molluscs?

Gastropods belong to the phylum Mollusca, one of the largest and most diverse groups of Protostomes. Thus a further understanding of the anatomy and development of the nervous system in this taxon may provide valuable insights into general conserved mechanisms of neural development and possibly how nervous systems have evolved in the Metazoa.

While little is known about neural development in gastropods, they have been very well studied in other aspects of development. For example, their cleavage patterns have been comprehensively investigated and detailed information is known about the cell lineages of many species. Gastropod species such as *Ilyanassa obsoleta* and *Patella vulgata* often appear as models in general textbooks of animal development (for example see Gilbert, 1998) to explain spiral cleavage patterns and the fates of cells during development.

Aspects of the adult nervous systems of gastropods have also been very well studied. In fact, the gastropod *Aplysia californica* has served as a primary model for investigations of the neural basis of behavior, especially in terms of learning and memory. For example, details about specific molecular activities within identified nerve cells known to be involved in learning and memory have been remarkably well investigated (for review see Kandel and Pittenger, 1999). Indeed, a Nobel prize was awarded to E.

Kandel for his work in this field.

While there is a wealth of knowledge concerning both the early development and the structure and function of the adult nervous system in gastropods very little information is available to bridge these fields. Such a bridge would involve studies of the mechanisms that govern neural development and how the developing nervous system functions in controlling larval behaviors. However, these studies have not been widely undertaken since even the anatomy of the larval nervous system is not well understood.

Several limitations have prevented extensive investigations of the development and anatomy of the gastropod larval nervous systems in the past. Firstly, a marker for all neurons does not exist for this taxon and therefore many investigations have focused on subsets of neurons expressing only one particular transmitter. Other studies have concentrated only on specific components of the larval nervous system such as the ganglia that persist into the adult or the larval apical organ. Finally due to the difficulty in obtaining and/or raising these small plankotrophic larvae, many studies have been limited to specific larval periods or to species with a direct intracapsular form of development. Together, all these limitations have prevented us from obtaining a clear picture of the complete larval nervous system in gastropods. Yet, reviews of the literature indicate that general patterns can be identified, and thus a hypothesis that a general neural plan for gastropod larval nervous systems exists. Therefore, the purpose of the first part of Chapter 1 is to provide a review of the similarities among gastropod larval nervous systems.

1. B) The Gastropod LNS: Review of the Literature

Recent investigations of gastropod larval nervous systems (LNS) in various species reveal distinct similarities in their morphology and development. Such similarities include the presence of an apical organ, and peripheral neurons and axons associated with the velum and foot. Furthermore, the ganglia which form the central nervous system of the adult begin to develop in early larval stages and likely function in the veliger as well as in the juvenile and adult. Therefore, these ganglia are also considered a component of the gastropod LNS. The purpose of this introduction is to review these three major components of gastropod larval systems as revealed by previous studies (see Fig.1A,B for summary diagram of the gastropod LNS). These studies included investigations of several different species representing various gastropod groups such as patellogastropods, vetigastropods, caenogastropods and heterobranchs (see Appendix 1 for summary of gastropod phylogeny). Furthermore, various techniques have been used to image elements of the LNS including those from classical light, electron and fluorescence microscopy, as well as immunohistochemical, pharmacological and physiological experiments that have been used to identify its components.

i. The Apical Organ

Historically, a sensory structure located at the anterior most region of gastropod larvae has been referred to as the apical or cephalic sensory organ (Bonar, 1978b), and recently the neural elements associated with the apical organ have been termed the apical

ganglion (Lin and Leise, 1996a; Lin and Leise, 1996b; Marois and Carew, 1997b; Marois and Carew, 1997a) or apical sensory organ (Kempf *et al.*, 1997). In this review, the apical organ is referred to as the collective group of sensory-like cells, associated neurons and a neuropil. The apical organ has been the best studied neural component of the gastropod LNS, having been identified in most gastropod species examined. The goal of this section is to illustrate the importance of the apical organ in gastropod larvae.

The Structure of the Apical Organ: Cell Types, Transmitters, Projection Patterns

A classical description of the development of the apical organ was provided by Raven (1966) and reviewed by Verdonk and Cather (1983). Spiral cleavage in molluscs characteristically gives rise to a specific pattern of cells, known as the molluscan cross. The cells at the center of this cross further divide to form the apical rosette cells which surround the animal pole and later form the apical plate (Raven, 1966; Verdonk and Cather, 1983). The apical plate was observed to undergo proliferation and later invagination to form the apical organ in the caenogastropod *Crepidula fornicata* (Conklin, 1897). Once formed, it has been generally reported to consist of a collection of 15-25 cells clustered dorsal to an apical neuropil that lies directly over the cerebral commissure. Furthermore the apical organ has consistently been observed in an anterior region, located along the mid-line dorsal to the mouth and often associated with a tuft or patch of cilia (for review see Raven, 1966). The morphological characteristics of the apical cells themselves also show distinct similarities in the gastropod species examined.

One striking similarity among gastropod apical organs is the consistent presence of vase-shaped cells making up the majority of the apical organ. These cells have a basal

portion that is sub-epithelial, and a slender apical portion that often extends through the epidermis, a morphology typical of sensory-like cells of adult molluscs (Croll, 1983; Boudko *et al.*, 1999). The vase-shaped cells of the apical organ were first described using electron microscopic investigations in the heterobranchs *Phestilla sibogae* (Bonar, 1978b) and *Rostanga pulchra* (Chia and Koss, 1984) (see Fig.1C for summary diagram of apical cell types). Three distinctly different types of vase-shaped cells were described; ciliated tuft cells, ampullary, and parampullary cells. Ciliated tuft cells each possess 20 or more cilia which protruded above the epidermal layer contributing to the apical tuft. Ampullary cells had deep ciliated lumens, however no cilia extended beyond the epidermal region. On the other hand, parampullary cells had a few cilia in the lumen and numerous very small cilia which extended above the epidermis (Chia and Koss, 1984). Additional electron microscopic studies have shown that the apical organ of other gastropod species also contain ampullary, parampullary and ciliated tuft cells (Bonar, 1978b; Uthe, 1995; Kempf *et al.*, 1997; Marois and Carew, 1997a; Page, 2002a; Page and Parries, 2000; Ruthensteiner and Schaefer, 2002).

In addition to vase-shaped cells, two to four round cells lacking dendritic projections to the epidermis have also been reported in the apical organ of gastropods (Dickinson *et al.*, 1999; Page and Parries, 2000) (Fig. 1C). Such cells were termed type II parampullary neurons (Kempf *et al.*, 1997) or interneurons (Marois and Carew, 1997a). Much less is known about the morphology of these round cells, however they usually have axons which project into the apical neuropil.

While various different transmitters have been observed in apical cells of

gastropods, serotonin-like immunoreactive (LIR) apical cells have been the best studied and appear in the majority of species examined. The number and arrangement of serotonin-like immunoreactive apical cells show a high degree of similarity in several species of nudibranchs. These serotonergic apical cells form a consistent pattern of five neurons, a medial central vase-shaped cell and two pairs of cells on each side. The cells of the first pair were suggested to be parampullary while the other were type II parampullary cells or interneurons (Kempf *et al.*, 1997; Marois and Carew, 1997a). A similar pattern of serotonergic cells has also been reported in the caenogastropod *Euspira lewisii* (Page and Parries, 2000). However, variations of this five-cell pattern were observed in other caenogastropod species. Such variations were suggested to reflect evolutionary changes from a common ancestral larval form (Page and Parries, 2000). Regardless of the differences in serotonin-LIR cell numbers, the predominance of this transmitter in apical cells probably reflects its importance in some aspect of neural control of larval behaviors. One such possibility is that these serotonin apical cells innervate the velum and control locomotion and feeding (see below).

Other neural markers such as catecholamines (Kempf et al. 1992; Voronezhskaya *et al.*, 1999; Pires *et al.*, 2000a), nitric oxide (Lin and Leise, 1996b; Thavaradhara and Leise, 2001), and neuropeptides including FMRFamide (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996a; Croll and Voronezhskaya, 1996b; Voronezhskaya and Elekes, 1996), small cardiac peptide-B (SCP-B) (Barlow and Truman, 1992b) have also been identified within apical cells in gastropod species. The numbers and types of apical cell show fewer similarities than do those cells containing serotonin. For example,

two vase-shaped FMRFamide-LIR cells were found in the pulmonate, *Lymnaea stagnalis* (Croll and Voronezhskaya, 1996a) while in the caenogastropod, *C. fornicata*, one vase-shaped and four round FMRFamide-LIR cells were observed (Dickinson *et al.*, 1999). Moreover, in the heterobranch *Aplysia californica,* there were no FMRFamide-LIR cells in the hatchling stage (Dickinson *et al.*, 2000). However, fewer comparative studies utilizing these markers have been undertaken, especially in closely related species, and therefore comparisons are difficult to assess.

Axons originating from the cells constituting the apical organ or the apical neuropil have been observed to project to peripheral regions of the veliger. One such peripheral region is the velum, the major organ of locomotion and feeding in the gastropod larva. Many gastropod veligers contain two bands of cilia on the velum edge that are responsible for creating swimming and feeding currents. Furthermore, the velum is supplied with many muscle fibers that can contract and pull the velar lobes into the mantle cavity during escape behaviors (for review of velum morphology see Raven, 1966; Bayne, 1983; Moor, 1983). Using light (Carter, 1926; Carter, 1928), electron microscopy and immunohistochemistry (for examples see Mackie *et al.*, 1976 and Marois and Carew, 1997c) velar nerves have been shown to radiate throughout the velum. Such velar nerves have also been found to contain classical transmitters such as serotonin and/or catecholamines as well as various neuropeptides such as FMRFamide (Goldberg and Kater, 1989; Marois and Carew, 1997c; Kempf *et al.*, 1997; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). These axons have often been found associated with ciliated bands and/or muscle fibers of the velum. (Kempf *et al.*, 1997; Page and Parries, 2000).

For example, Marois and Carew (1997c; 1997a; 1997b) showed ultra-structural evidence that serotonergic projections from the apical organ directly contacted ciliated cells in the velum and were closely associated with velar muscle fibers of the heterobranch *A. californica*. Such morphological evidence suggests that there is neural control over ciliary activity and velar retraction and relaxation. Thus, the apical organ may participate in the positioning of the velum, swimming patterns and escape behaviors in gastropods. Experimental studies further support these morphological studies. Arkett et al. (1987), and Mackie et al. (1976) presented electrophysiological evidence for the neural control of ciliary bands in the vetigastropod *Calliostoma ligatum* and the caenogastropod *Magelia* sp. respectively. For example, electrical recordings from ciliated cells in the velum of *C. ligatum* revealed that calcium mediated action potentials correlated with ciliary arrests and that small depolarizations resulted in fine changes in ciliary beat frequency (Arkett *et al.*, 1987).

In addition to the velum, axons originating in the neuropil and/or cells of the apical organ have also been reported to project into the foot of most gastropod species examined (Goldberg and Kater, 1989; Voronezhskaya and Elekes, 1993; Kempf *et al.*, 1997; Marois and Carew, 1997c; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000;). The gastropod foot develops during early veliger stages and has been suggested to be involved in locomotion, movement of food particles, respiration and mucus secretion (Moor, 1983, also see below). Innervation of the foot in most gastropods consists predominantly of serotonin-LIR and/or FMRFamide-LIR fibers. For example, in the pulmonate *Helisoma trivolvis* a pair of serotonergic apical cells were observed to have axons which projected

to the ciliated epithelial cells on the sole foot (Goldberg and Kater, 1989; Diefenbach *et al.*, 1995; Diefenbach *et al.*, 1998). These ciliated epithelial cells make up the pedal ciliary bands reported to be responsible for rotation of the larva within its egg capsule. Endogenous serotonin, likely from the axons originating from the serotonergic apical cells, were shown to affect the activity of the foot cilia and in turn the speed of rotation (Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994). In another experiment, laser perturbation of the apical cells, caused depolarization of the cells and release of the transmitter. Such perturbation, in turn, increased ciliary activity and rotational movements of larvae of *H. trivolvis* (Kuang and Goldberg, 2001). Therefore, the apical cells themselves were shown to influence directly the ciliary movement on the foot of *H. trivolvis*. Further experiments on this species uncovered the second messenger pathways by which serotonin influences ciliary movements of the pedal epithelial cells (Christopher *et al.*, 1996; Christopher *et al.*, 1999).

Sensory Role of the Apical Organ

The apical organ has been suggested to have a chemo- or mechanosensory function (Bonar, 1978a; Chia and Koss, 1984) based on the morphology of its cells. The vase-shaped apical cells contain cilia and numerous mitochondria, both of which are characteristics of sensory cells (Boudko *et al.*, 1999). The presence of many mitochondria in apical cells has been shown using electron microscopy in *A. californica* (Marois and Carew, 1997a). In addition, numerous mitochondria have also been revealed by the accumulation of the dye 4-(N,N-dimethylamino)-2,2,6,6,-tetramethyl-piperidine (DASPEI) in apical cells. DASPEI, a known marker for mitochondrial activity (Boudko

et al., 1999), was found to accumulate in putative ampullary apical cells of the caenogastropod *I. obsoleta* (Leise, 1996) and heterobranch *P. sibogae* (Hadfield *et al.*, 2000).

The apical organ has been suggested to be ideally located for sensing details about the external environment and then transmitting this information to velar ciliary bands, and muscle fibers. In this way, the apical organ probably regulates locomotion, feeding and escape responses. The best studied sensory role of the apical organ has been the transduction of the metamorphic signal (Hirata and Hadfield, 1986). Hadfield and coworkers (Hadfield *et al.*, 2000) provided experimental evidence to support the role of the apical organ in controlling metamorphosis. When cells in the apical organ of the heterobranch *P. sibogae* were irradiated, the larva was no longer able to respond to the usual metamorphic cue. In addition, in the same species, the chemical cue was shown to increase electrophysiological activity in a region corresponding to the apical organ (Leise and Hadfield, 2000). Further pharmacological and physiological studies are needed to clarify the sensory role of the apical organ in metamorphosis.

Fate of the Apical Organ

During and after metamorphosis, the apical organ undergoes significant changes. In most species examined, the apical organ has been reported to disappear during or soon after metamorphosis or before the embryo reached a juvenile stage. It is possible that the apical cells may either cease to express neural markers, migrate, or undergo cell death. Marois and Carew (1997b; 1997c) provided evidence to support the latter view. They examined the fate of the serotonin-LIR cells in the apical organ of *A. californica* and

observed cellular swelling, apparent vacuolization and disruption of membrane integrity. Similar observations indicative of apoptosis were also observed in apical cells in the caenogastropod *Ilyanassa obsoleta* (Leise and Thavaradhara, personal communication). On the other hand, evidence that the apical cells may not disappear after metamorphosis has also been reported. For example, in the heterobranch *P. sibogae,* the apical organ was still observed up to two days after the initiation of metamorphosis (Bonar, 1978b). Although its ultimate fate has not been determined in this species, Bonar (1978b) suggested that the apical cells could be incorporated in the developing cephalic tentacles. Furthermore, in the patellogastropod *Tectura scutum*, a medial vase-shaped apical cell ceased to express serotonin in metamorphically competent larvae, although the cell was still present after metamorphosis, postmetamorphic animals were not examined (Page, 2002a). Similar observations of transient expression of serotonin in apical cells have also been reported in the pulmonate *H. trivolvis* (Diefenbach *et al.*, 1998). However, the final fate of these apical cells in both *T. scutum* and *H. trivolvis* have not been investigated and therefore these apical cells may undergo apoptosis later in juvenile stages.

ii. Peripheral Nervous System

In addition to the apical organ and nerves originating from this structure, other central and peripheral neural elements have also been reported during larval stages in a variety of gastropods. Such elements included neurons and fibers that appeared to be associated with the mouth, esophagus and foot. In addition, a peripheral neuron has been observed near the telotroch and/or anus of some gastropod larvae. Some of the similarities in the morphology and the possible roles of the peripheral neural elements are

discussed in the following section.

Posterior FMRFamide-LIR Neuron

A notable similarity in the LNS of numerous gastropod species is the presence of a single cell positive for FMRFamide (Phe-Met-Arg-Phe-NH2) and/or other related peptides in the posterior region. This posteriorly located cell was first described in the trochophore stage of *L. stagnalis* (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996a) and similarly located cells have since been described in other heterobranchs such as *Biomphalaria glabarus* (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996b; Croll and Voronezhskaya, 1996a), *A. californica* (E.E. Voronezhskaya, personal communication) and the caenogastropod *C. fornicata* (Dickinson *et al.*, 1999). In all these species, anteriorly directed axons projected from the posterior FMRFamide-LIR cell and met the visceral connectives of the developing adult CNS. The role of the posterior cell is currently unknown, however the location may allow for some speculation. This cell was consistently observed near the telotroch, a patch of cilia near the anal cells or anus. The telotroch has been suggested to be involved in locomotion in many invertebrate larvae (Strathmann, 1993). Therefore, the posterior FMRFamide-LIR neuron may function in controlling ciliary movements of the telotroch and anterior projections from this neuron may allow coordination of the telotroch with other ciliary bands involved in locomotion. Pharmacological and electrophysiological experiments are necessary to determine the role of the posterior FMRFamide cell in molluscs.

In addition to its location, the developmental timing and fate of this posterior cell

appears to be similar in these gastropods. The posterior cell has been consistently reported in very early stages and was often the first or among the first neural cells observed. Furthermore, the cell also seemed to disappear during the mid or late veliger stages (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996b; Croll and Voronezhskaya, 1996a; Voronezhskaya and Elekes, 1996; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). However, the exact fates of the posterior FMRFamide-LIR cells and fibers were not examined in detail and it is therefore possible that the cells may either have ceased to express the neural marker, migrated, or have undergone cell death.

Neurons Associated with the Mouth and Esophagus

The mouth cavity and esophagus develop from the stomodaeum, an ectodermal structure formed after gastrulation (reviewed by Raven, 1966; Moor, 1983). Cilia exist around the mouth that are possibly involved in food particle selection and/or rejection (Raven, 1966; Bayne, 1983). In caenogastropods the region just ventral to the mouth, the mentum may also have a sensory function (Fretter, 1967; 1972).

In many species of gastropods peripheral vase-shaped catecholaminergic cells have been reported near the mouth. For example, in caenogastropods and heterobranchs (Dickinson *et al.*, 1999; Dickinson *et al.*, 2000; Pires *et al.*, 2000b) such catecholaminergic vase-shaped cells surrounded the ventral and lateral portion of the mouth and similar cells were also observed around the lips in the pulmonate *L. stagnalis* (Voronezhskaya *et al.*, 1999). The location of these catecholaminergic cells around the mouth suggests the possibility that they control different aspects of feeding.

Putative neurons associated with the esophagus have been identified in at least

one gastropod species. The esophagus is lined with smooth muscle and cilia (personal observations, also see chapter 5) and likely participates in controlling the movement of food particles to the stomach (Strathmann and Leise, 1979). In the caenogastropod *I. obsoleta*, cells and processes revealed by an antibody to histamine were observed to surround the esophagus during the free swimming veliger stage (Z. Zahrodney, unpublished observations). Up to five bilaterally symmetrical pairs of multipolar cells were observed to have fibers which extended from the cells and encircled the esophagus. Therefore, based on location, these histamine-LIR cells may function in the ciliary activity and/or muscle contractions of the esophagus and thus in moving food toward the stomach. Current studies in the lab are underway to test the role of histamine in feeding and locomotion in larval gastropods.

Neurons in the Foot

The foot begins to develop early in the larval period of gastropods and is often first observed during trochophore stages. The larval foot has been reported to have several functions such as feeding and locomotion and it can also participate in the escape behaviors (for reviews see Bayne, 1983; Moor, 1983). For example, many gastropod species have muscles that attach near the operculum on the posterior of the foot and can contract and pull the foot into the shell (Fretter, 1967; Fretter, 1969; Fretter, 1972). The foot contains a median row of cilia along the sole and such pedal cilia have been reported to be involved with food particle movements in planktotrophic forms (for reviews see Bayne, 1983; Moor, 1983) and in the rotational movements of intracapsular larvae (Goldberg and Kater, 1989; Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994). In addition,

the foot also contains pedal glands that secrete mucus. This mucus secretion has been reported to be necessary for crawling in the juvenile and also for anchoring the larva to the substratum during metamorphosis (Bonar and Hadfield, 1974). Furthermore, many pulmonates also have a specialized sinus at the tip of the foot called the podocyst. This structure has been suggested to participate in circulation, gas exchange and uptake of the albumen from capsular fluid (for reviews see Raven, 1966; Moor, 1983).

From the numerous functions of the gastropod foot it is not surprising that it also has peripheral vase-shaped cells in many species, in addition to innervation by the apical organ. Vase-shaped catecholaminergic cells were observed in the foot of caenogastropods *C. fornicata* (Dickinson *et al.*, 1999), and heterobranchs *L. stagnalis* (Voronezhskaya *et al.*, 1999), *A. californica* (Dickinson *et al.*, 2000) and *P. sibogae* (Pires *et al.*, 2000a). In all species examined, axons from these putative sensory cells appeared to converge and project dorsally toward the developing pedal ganglia and/or the apical region. Also in the heterobranch *Onchidoris bilamellata*, a unique ganglionic pair, named the anterolateral ganglia, was identified in the propodium, a region of the larval foot that becomes the anterior portion of the adult structure (Arkett *et al.*, 1989). Epithelium overlying the anterolateral ganglia had an ovoid patch of cilia and cells resembling sensory neurons were identified as part of the ganglion (Arkett *et al.*, 1989).

The morphology of the identified cells in the foot of gastropods suggests sensory roles possibly involving chemo- and/or mechano-reception. Such sensory roles, specifically concerning the process of metamorphosis, receive support from experimental evidence. For example, Arkett and coworkers (1989) revealed using electrophysiological

recordings that the sensory neurons near the foot in *O. bilamellata* were depolarized in the presence of metamorphic cues. Furthermore, exposure of larvae to catecholamines such as dopamine and norepinephrine analogs was found to modulate the metamorphic response in the heterobranch *P. sibogae* (Pires *et al.*, 2000b; Pires *et al.*, 2000a) and caenogastropod *C. fornicata* (Pires *et al.*, 2000c; Pechenik *et al.*, 2002). Also in another heterobranch, *L. stagnalis*, depletion or receptor blockage of dopamine were used to demonstrate that this transmitter was essential for normal development, past the stages equivalent to metamorphosis, in this intracapsular species (Voronezhskaya *et al.*, 1992; 1993). Further experiments are necessary to determine if these putative sensory cells in the foot are indeed involved with other functions of the foot in larval stages.

iii. Development of the Central Ganglia

The adult gastropod CNS consists of a collection of ganglia and connectives. While the number and arrangement of the ganglia can vary, most species have paired cerebral, pedal, pleural, buccal and intestinal ganglia. In some groups there are also paired parietal and single visceral and osphradial ganglia. The names of the ganglia generally correspond to the organ or region of the body which they innervate. Many investigations have focused on the development of the ganglia to understand possible homologies that may have become masked in adult stages, in which some of the ganglia are often fused and concentrated (for examples see Page, 1992; Page, 1993; Lin and Leise, 1996a). In addition, developmental studies on the heterobranch *A. californica* have been undertaken to determine the origins of well known neurons of the future adult CNS (Kandel *et al.*, 1981; Jacob, 1984). Since the ganglia appear early in larval stages and

likely function in larvae behaviors, the developing ganglia are considered a component of the LNS. What is known about the earliest development of the CNS and possible roles in gastropod veligers are discussed.

Descriptions of the basic mechanisms and timing of gangliogenesis were reported by Raven as early as 1949 (Raven, 1949). It has been well established that ganglia which form the adult CNS originate in proliferative regions of the ectoderm (for review see Moor, 1983). For example, the cerebral ganglia develop from the paired cephalic plates, each located laterally to the apical plate. Also the pedal ganglia develop from similar ectodermal proliferative zones on the lateral aspects of the ventral surface of the foot rudiment. These proliferative zones undergo delamination or invagination (Moor, 1983) and then finally individual cells migrate inward to form the ganglia (Jacob, 1984). The major ganglia in species representing caenogastropods and heterobranchs have been shown to develop in a specific temporal sequence from anterior to posterior, with the cerebral ganglia developing first, followed by the pedal ganglia and then the posterior ganglia (Kriegstein, 1977; Lin and Leise, 1996a). Commissures and connectives of the CNS were thought to form by secondary outgrowth from the ganglia (Raven, 1966; Demian and Yousif, 1975; Kandel *et al.*, 1981; but also see Schacher *et al.*, 1979). Furthermore, it has been demonstrated that continual addition of neurons to the ganglia occurred throughout embryonic and juvenile stages of the heterobranchs *L. stagnalis*, *H. trivolvis* and *A. californica* (Croll and Chiasson, 1989; Goldberg and Kater, 1989; Hickmott and Carew, 1991; Marois and Croll, 1992; Nolen and Carew, 1994).

More recent studies have focused on determining the location and morphology of

neural elements which express neuroactive substances early in development (Kruglyanskaya and Sakharov, 1973; Kempf *et al.*, 1987; Barlow and Truman, 1992a; Kempf *et al.*, 1992; Marois and Croll, 1992; Elekes *et al.*, 1996). These studies have allowed neurotransmitters (serotonin, FMRFamide, catecholamines, small cardioactive peptide and octopamine) to be used as markers permitting specific subsets of cells, developing within the ganglia, to be followed and their projections traced. For example, in *L. stagnalis* the first serotonergic cells appeared during the veliger stage in the cerebral ganglia and soon afterward additional cells arose in the more posterior ganglia. The timing and sequence of the developing serotonergic neurons was consistent with previous reports of the anterior to posterior sequence. In contrast several other studies, indicated that the development of the ganglia may not be so straightforward. For example, in the gastropods *C. fornicata*, *A. californica* and *L. stagnalis*, some of the earliest cells detected with anti-FMRFamide were located in regions which corresponded to the future intestinal, abdominal and parietal ganglia respectively (Croll and Voronezhskaya, 1996a; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). Therefore, the general theory that the ganglia develop in and anterior to posterior sequence may not be entirely correct (see above and Raven, 1966; Demian and Yousif, 1975; Kandel *et al.*, 1981). Furthermore, FMRFamide-LIR axon tracts that formed many of the connectives, including the posterior connectives appeared very early in the larval stages in these gastropods (Croll and Voronezhskaya, 1996a; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). Such results are consistent with previous reports in *A. californica*, where serial sections revealed connectives prior in embryonic veliger stages (Schacher *et al.*, 1979). Together these

studies support the notion that all connectives may not necessarily be formed as secondary outgrowths as predicted (Raven, 1966; Demian and Yousif, 1975; Kandel *et al.*, 1981). Together, this evidence indicates a need for some reinterpretation of the current views on the development of the CNS.

Possible roles of the developing ganglia in larval functioning have not been investigated, however the locations of the earliest cells might offer some clues. For example, the early appearance of immunoreactivity in neurons in the posterior ganglia (abdominal and parietal) implies that there is neural control over aspects of digestion, circulation (heart beat) and respiration. Observations of serotonin immunoreactive cells in the cerebral ganglia during early veliger stages of many species (Marois and Croll, 1992; Kempf *et al.*, 1997; Marois and Carew, 1997a; Dickinson *et al.*, 1999; Page, 2002a) suggests moreover that such neurons, along with apical neurons, could possibly innervate the velum and/or foot and modulate muscle contractions and ciliary activity. Investigations focused on the role of the first neurons in the developing ganglia are necessary to support these ideas.

iv. Summary and Future Studies

In summary, this section has revealed that the development, morphology and possible functions of the components of the LNS is similar across the gastropods. Such similarities may in fact reflect a general plan in the overall anatomy of the LNS. This general plan includes an apical organ which innervates peripheral structures such as the foot and velum and controlling aspects of locomotion and feeding as well as possibly being involved in metamorphosis. The gastropod LNS also consists of peripheral

neurons located near the foot and mouth that might be able to detect chemical or mechanical cues. Finally, the developing ganglia that form the future adult CNS are also a component of the gastropod LNS. These early developing neurons in the central ganglia also conceivably have roles in larval function. The investigations reviewed here have revealed that gastropods potentially have a more extensive LNS than previously thought. However, many more studies are necessary to show the extent of such a nervous system in a variety of gastropod species. Additional markers for other transmitters may uncover more neural elements. Such markers for other classical transmitters, such as acetylcholine (Twarog, 1954; Hanley and Cottrell, 1974; Chrachri and Williamson, 1998), GABA (Osborne *et al.*, 1972; Walker *et al.*, 1975; Morse *et al.*, 1980; Cooke and Gelperin, 1988; Vitellaro-Zuccarello and De Biasi, 1988) and glutamate (Bolshakov *et al.*, 1991; Dale and Kandel, 1993; Nesic *et al.*, 1996; Michel *et al.*, 2000) as well as various peptides (Croll and Van Minnen, 1992; Santama *et al.*, 1994; Willows *et al.*, 1997) are utilized in studies of adult molluscan nervous systems but have not yet been used for investigations of larval stages. Furthermore, studies which examine at least several markers throughout the entire larval period are necessary to make more adequate comparisons of the LNS in gastropods as well as other molluscan groups.

1. C) Comparisons of Gastropod Larval Nervous Systems

The previous section revealed that there are significant similarities in the LNS of various gastropods, so much so that a general plan has been postulated in this review (see Fig. 1A, B). It is tempting to speculate that such a general neural plan reflects a common

evolutionary origin for gastropod nervous systems. However, several obstacles indicate that such a speculation is premature. The first obstacle is the controversial status of the phylogenetic relationships of the gastropods (for summary of gastropod phylogeny see appendix 1). Without a well resolved phylogeny, comparisons across the class become problematic. Such problems become further compounded since natural selection can act separately on larval and adult stages (for review see Hickman, 1999) and therefore even closely related gastropod species may have very different larval forms. Thus a difficulty in comparing gastropod larvae is that variation in modes of larval development may have a significant effect on the complexity and developmental timing of neural elements constituting the LNS. The possibility that evolutionary convergence is quite prevalent in larval forms (Strathman, 1993) raises the question: to what extent are similarities in larval nervous systems the result of shared ancestry or of convergent modifications? Examples of these problems in making comparisons are addressed below with reference to specific neural elements observed in gastropod LNS.

The cellular arrangements and types of neurons constituting the gastropod LNS show many similarities possibly indicating a common origin/ancestor. A common ancestor for all gastropods seems likely (Ponder and Linderberg, 1997) and therefore some neural elements presumably reflect evolutionary relationships. This ancestral basis for the design of the gastropod LNS may be demonstrated in the apical organ in which the similar cell types, arrangement of cells, transmitter phenotypes and projection patterns all point to a common origin. Differences in the numbers of cells seem also to reflect modifications in life history. For example, species with long-lived, large planktotrophic

larvae have apical organs which consist of numerous cells with several different transmitters. To illustrate, Ruthensteiner and Schaefer (2002) examined the apical region in both planktotrophic and intracapsular species the pulmonate group Ellobiidae. In the planktotrophic species, *Laemodonta octanfracta*, an apical organ was observed to consist of up to eleven vase-shaped cells. On the other hand in a direct developing species, *Ovatella myosotis*, no characteristic vase-shaped apical cells were observed (Ruthensteiner and Schaefer, 2002). Such a correlation is further supported by the relatively few apically located vase-shaped cells observed in direct developing pulmonates such as *L. stagnalis* (Voronezhskaya *et al.*, 1999) and *H. trivolis* (Goldberg and Kater, 1989; Diefenbach *et al.*, 1998). Such a loss or reduction of the apical organ in these direct developing, intracapsular species correlates with a reduction in the typical larval molluscan structures such as the ciliated velum. Therefore, such drastic differences in the LNS due to lifestyle can make comparisons difficult and thus may obscure comparisons

On the other hand it is more difficult to assess relationships in other components of gastropod larval nervous systems. For example, similarities in the neural elements associated with feeding structures may be due to a common origin or convergent modifications. Support for convergence is derived from the fact that feeding larvae have evolved independently at least twice in gastropods (Ponder and Linderberg, 1997; Hickman, 1999). Therefore, neural elements associated with feeding, such as the sensory catecholaminergic cells surrounding the mouth, may have also arisen independently. Such evolutionary convergence of feeding structures may have been due to the functional

advantage of having control over the quantity of food ingested.

Comparisons of gastropod larval nervous systems may be useful in determining the interplay between evolution and larval life history on the development and anatomy of the neural components of the gastropod LNS. Furthermore, such studies provide insights into the extent to which convergent evolution has influenced neural development. However, further investigations of the development and anatomy, as well as developmental origins and physiology of neural elements, will be necessary before determining how the LNS evolved in gastropods and in other invertebrate larvae.

1. D) Comparisons of the Gastropod LNS with Other Molluscs and Invertebrate Larvae

Other molluscan groups, as well as several invertebrate taxa, with comparable free swimming larval stages, have larval nervous systems which share certain features with gastropods. For example, an apical organ has been reported in groups such as cnidarians, (Chia and Bickell, 1978), turbellarians, (Lacalli, 1982), echinoderms (Burke, 1983a; Burke, 1983b; Burke *et al.*, 1986; Bisgrove and Burke, 1987; Nakajima, 1988; Bisgrove and Raff, 1989; Moss *et al.*, 1994), bryozoans, (Reed, 1988), phoronids, (Hay-Schmidt, 1990c; Hay-Schmidt, 1990b) and nemertines, (Hay-Schmidt, 1990a) and other molluscan taxa such as bivalves (Croll *et al.*, 1997; Plummer, 2002) and polyplacophorans (Haszprunar *et al.*, 2002; Voronezhskaya *et al.*, 2002). In these groups the apical organ was shown to be associated with an apical tuft or patch of cilia in the anterior most region of the body. Additionally, the cellular arrangement and transmitters localized within the

apical cells in these diverse invertebrate species were also similar (for example see Hay Schmidt, 2000). Furthermore, axons were usually reported beneath the apical cells and were often associated with the larval brain (Lacalli, 1994). In addition to the apical organ, neurons and axons located in peripheral regions which may be involved with feeding and propulsion have been described in larval stages of bivalves (Croll *et al.*, 1997; Plummer, 2002) and polyplacophorans (Haszprunar *et al.*, 2002; Voronezhskaya *et al.*, 2002) as well as in other invertebrate phyla (for example see Lacalli, 1986). For example, catecholaminergic, serotonergic or FMRFaminergic elements have been consistently observed in association with ciliary bands in many invertebrate groups (Lacalli, 1982; Burke, 1983a; Burke, 1983b; Lacalli, 1986; Bisgrove and Burke, 1987; Bisgrove and Raff, 1989; Hay-Schmidt, 1990b; Hay-Schmidt, 1990c; Hay-Schmidt, 1990a; Hay-Schmidt, 1995; Chee and Byrne, 1999).

The presence of these similarities in the LNS from diverse taxa has been suggested to possibly reflect a common ancestral larval form for all Metazoa (Lacalli, 1981; Lacalli, 1994; Hay Schmidt, 2000). However, since larval forms have been postulated to have arisen independently in many taxa, such similarities are more likely to be attributed to convergent evolution due to similar functional requirements (Strathmann, 1978; Strathmann, 1993). For example, an anteriorly located sensory structure is well placed to provide information about the external environment ahead of the larva and therefore independent evolution of apical sensory organs would not be surprising (Strathmann, 1993). In addition, the ciliary bands located on ridges or posterior edges of the larvae meet a common functional requirement for propulsion and are expected to be

similar due to convergence, probably arising numerous times in evolution (Strathmann, 1993). Strathmann (1993), also notes that similar functional requirements would result in comparable nervous systems in larval forms and therefore the neural elements found associated with ciliary bands probably exist due to convergent evolution in at least some invertebrate groups. These examples demonstrate the likelihood that larval nervous systems appeared similar in many taxa due to convergent modifications and thus further investigations are necessary to test this hypothesis.

1. D) Conclusions and Thesis Goals

The previous section clearly demonstrated that additional investigations which integrate, morphology, physiology and developmental processes are necessary to make effective comparisons of the gastropod LNS. Therefore, the overall purpose of this thesis was to take a comprehensive approach to the study of gastropod neural development by addressing aspects such as the anatomy, roles of specific neural components, and cellular and molecular mechanisms governing development of the LNS.

While there seems to be a general organization of the neural components which constitute the gastropod LNS (Fig. 1A,B), these components have not been investigated in their entirety in a single species throughout the complete larval period. Thus, a goal of this thesis was to provide such an anatomical description of the LNS in the well studied caenogastropod *I. obsoleta*, using several different neural markers combined with confocal microscopy. Chapter 2 consisted of a detailed description of the development

and anatomy of larval neurons. Markers that have previously identified molluscan neurons were utilized, such as antibodies for serotonin, FMRFamide, leu-enkephalin, tyrosine hydroxylase and dopamine-beta-hydroxylase.

Once the morphology of many of the neural components was established, the roles of specific neurons were investigated in a larval stage of *I. obsoleta*. In chapter 3, study of the possible roles of neural elements regulating swimming and feeding behaviors was focussed on hatchling larvae. An integrative approach was used which combined investigations of: the anatomy and types of transmitters within neural elements associated with locomotory and feeding structures, and testing the effects of synthetic analogues of such transmitters on known behaviors of whole larvae and on individual ciliated cells.

The caenogastropod *I. obsoleta* has been a relatively well studied in terms of cell lineage and cleavage patterns (Clement, 1962; Verdonk and van den Biggelaar, 1983; Render, 1991; Damen and Dictus, 1994; Dictus and Damen, 1997; Render, 1997; Lambert and Nagy, 2001). Therefore, chapter 4 takes advantage of the established knowledge and techniques concerning early development in this species to explore possible cellular and molecular mechanisms governing the development of components of the LNS. To gain insight into molecular mechanisms in neural development, the expression of a protein of the well conserved gene, *distal-less*, was examined. Furthermore, the role of early inductive signaling in the development of neural elements was also investigated. Techniques to block such signaling have been well defined and therefore provided an interesting way of exploring how such signaling might specify

neural tissue.

The results of my studies on the anatomy and the roles of the LNS indicated that a relatively extensive system of neurons existed in this species. While characteristics of how this system might regulate ciliary bands have been studied to some extent virtually nothing has been reported concerning the other major effector system, the musculature. Furthermore, the nervous system has been reported to be closely associated with muscles in larval stages of many other invertebrates and thus investigations of muscle development could potentially offer insights into how nervous systems function and have evolved. Preliminary studies in *I. obsoleta* demonstrated that in many cases axons were associated with muscle fibers (see appendix 2). Understanding the development and the anatomy of the musculature in gastropods prompted the final study of this thesis. Investigations of the musculature also brought additional evidence to bear. For example, among the Metazoa neurons and muscle cells have been shown to have different embryological origins and different molecular mechanisms governing their development. Hence, understanding how developmental events give rise to different tissue types in gastropods will be useful for making comparisons with other taxa and thus understanding how developmental modifications may cause evolutionary changes.

Part 2: Review of Gastropod Larval Muscle Systems

2. A) Why study muscle development?

Garstang (1929) was among the first to suggest that developmental modifications result in evolutionary change. This hypothesis was based at least in part on studies of gastropod development, including the larval musculature. Subsequently, evolutionary and developmental biologists became particularly interested in the role of larval muscles in generating unique morphological characteristics in gastropods such as torsion and shell coiling. While many of the theories that were proposed concerning the role of larval muscle systems have since been overturned, such studies have established a good basis for the development of muscle in gastropods.

2. B) Review of the Literature

It has been suggested that a gastropod predecessor had two bilaterally symmetrical retractor muscles and that with the evolution of gastropods this pair of muscles lost its symmetry. This asymmetry of the larval muscles was thought to cause the ontogenetic twisting of the larval head and foot relative to the visceral mass, mantle and shell, also known as torsion. Subsequent studies on *Haliotis tuberculata* and several other species of patellogastropods and vetigastropods (formerly the "archaeogastropods") supported this hypothesis (Crofts, 1937; Crofts, 1955). In these species, two larval shell muscles were identified and the development of the right larval retractor prior to the left was believed to cause torsion.

In addition to providing an explanation for torsion, the development of gastropod muscles was also studied to provide insight into the evolution of muscle systems in both larvae and adults. The most influential thoughts came from Crofts (1937; 1955) whose studies on species of patellogastropods and vetigastropods revealed that the right and left larval retractors became the two adult muscles. Furthermore, the variations in the larval and adult muscle systems in other gastropods could be explained by evolutionary changes to the muscles groups that were observed in archaeogastropods. For example, it was hypothesized that the single larval retractor muscle present in caenogastropods was a homologue of the post-torsional left larval retractor muscle in archaeogastropods (Fretter. While Crofts' hypotheses have been a major influence in theories of gastropod development and evolution several less well known studies have suggested contrasting ideas of muscle development, especially concerning the fates of larval muscles. For example, the adult muscles of the patellogastropod *Patella vulgata* (Smith, 1935) and the shelled opisthobranch *Retusa obtusa* (Smith, 1967) were reported to develop independently from the larval retractor muscles. Thus the literature concerning gastropod muscle development has, until recently, been difficult to interpret.

With improved techniques to study the anatomy and development of muscles in gastropod larvae, many of the above mentioned controversies have been addressed. Firstly, new evidence indicates that the larval muscles may not solely be responsible for torsion in gastropod veligers. Hickman and Hadfield (2001) postulated that in order for muscles to be able to cause such twisting of the larval body, the muscle would have to be attached to a rigid shell. However, observations on the larvae of the "archaeogastropod"

Margarites pupillus undergoing torsion, revealed a dimpling of the shell at the site where the main larval muscle was attached. This dimpling indicated that the shell was still flexible at this point in development and therefore, could not generate the tension necessary for torsion. In another study, experimental conditions prevented the attachment of muscles to the shell in developing larvae of *C. ligatum* and *Diodora aspera* (Page, 2002b). In these "archaeogastropod" larvae, torsion was still observed. Furthermore, detailed histological examinations of the larvae in still another "archaeogastropod", *Haliotis kamtschatkana*, revealed that differential growth may primarily be responsible for torsion in this species (Page, 1997b).

Detailed developmental studies of muscle systems and their fates during metamorphosis clearly indicate that in at least some species the adult muscles are independent from the main larval retractor(s) (Page, 1995; Page, 1997a; Degnan *et al.*, 1997b; Page, 1998; Wanninger *et al.*, 1999). For example, the main larval retractor muscles in the caenogastropod *Polinices lewisii* (Page, 1998), the patellogastropod *P. caerulea* (Wanninger *et al.*, 1999) and the vetigastropod *Haliotis rufescens* (Degnan *et al.*, 1997b) were observed to disappear during or soon after metamorphosis and therefore could not have contributed to the adult musculature as Crofts suggested.

In addition to beginning to clarify some of the controversies discussed above, recent studies of the development of gastropod larval musculature have revealed marked similarities in the morphology of the main larval retractor. Such similarities include the branching patterns, insertion sites, attachment locations and the presence of striations (Page, 1995; Page, 1997a; Page, 1998; Wanninger *et al.*, 1999). Thus based on these

morphological studies it has been suggested that the main larval retractor muscle is homologous in gastropods (Page, 1998) (see Fig 2A-C).

In addition to the main larval retractor muscles, other muscle groups develop during the larval stages of gastropods. At least some of these other muscles were observed to be associated with the foot and were shown to persist into juvenile stages in gastropods including the caenogastropod *Polinices lewisii* (Page, 1998), the patellogastropod *Patella caerulea* (Wanninger *et al.*, 1999) and the vetigastropod *Haliotis rufescens* (Degnan *et al.*, 1997b). These pedal muscles, while differing in number, also shared several characteristics. For example, the pedal muscles were not obviously striated, and inserted on sub-opercular epithelium. Thus, it was suggested that these pedal muscles may be a separate homologous group and that variations in the pedal muscle number could be attributed to evolutionary modifications (see Fig 2A-C).

In shell-less adult heterobranchs (such as most opisthobranchs) there are no major adult shell muscles (for review see Moor, 1983). While all the larval muscles were observed to disappear at metamorphosis, these muscles resembled similarly located larval muscles observed in other gastropods. For example, in the nudibranchs *Tritonia diomedea, Rostanga pulchra, Doridella steinbergae, Dendronotus subramosus* and *Melibe leonina*, there is a main larval retractor muscle and two muscles that each project into the foot (Page, 1995). Page (1995) proposed that the main larval retractor of nudibranchs were the equivalent to the right retractor in *Phestilla sibogae*. Additionally, the right and left pedal muscles in nudibranchs were the same as the opercular and left retractor muscles respectively in *P. sibogae* (Bonar and Hadfield, 1974). Thus, based on

developmental and anatomical characteristics hypotheses have been formed about homologous muscle groups in gastropod larva. However further investigations that integrate morphology with both the function and developmental mechanisms of gastropod musculature will be useful to test these hypotheses further.

While much is presently known about the anatomy and development of larval muscle groups very little is known about the origins and specification of muscles in this taxon. Lineage tracing investigations have revealed that larval muscles may originate from more than one blastomere during early cleavage stages. There is a general agreement that a specific cell formed during cleavage, called the mesentoblast (4d), gives rise to the main larval retractor muscles in several species (Crofts, 1955). On the other hand, muscles located in the foot may originate from the 3a and 3b micromeres as in the patellogastropod, *P. vulgata* (Damen, 1994), or from the 2b micromere as has been reported in *I. obsoleta* (Render, 1997).

2. C) Conclusion and Thesis Proposal

One of the goals of this thesis was to test the hypothesis, developed by previous anatomical studies, that the main larval retractor muscle is independent from the muscles that persist into the juvenile and contribute to the major adult muscles in gastropods. Firstly, a detailed examination of muscle development was performed in *I. obsoleta.* Muscles were visualized with a technique to reveal F-actin combined with confocal microscopy. In addition, the fates of the muscles were also determined by examining the changes in the musculature during and after metamorphosis. Second, the cellular origins

and specification governing the formation of the major larval muscles were investigated. In this experiment, techniques such as injections of a lineage tracer and cell ablations and chemical block of inductive signals were utilized. These studies have the potential to provide important insights for elucidating the mechanisms governing muscle development and will aid in determining homologous muscle systems and evolutionary relationships within the gastropods.

Figure 1: General schematic diagrams of components of the LNS in a representative gastropod. **A)** Right lateral view and **B)** dorsal view of a whole larva. Both figures show the general location of the apical organ (AO), and ganglia that form the future adult CNS such as the cerebral (CG), pedal (PeG), and posterior (PG) ganglia. Peripheral neurons and axons (arrows) are also indicated in the posterior region, velum, foot and surrounding the mouth cavity. **C)** General schematic of the cell arrangement and cell types within the apical organ of gastropods.

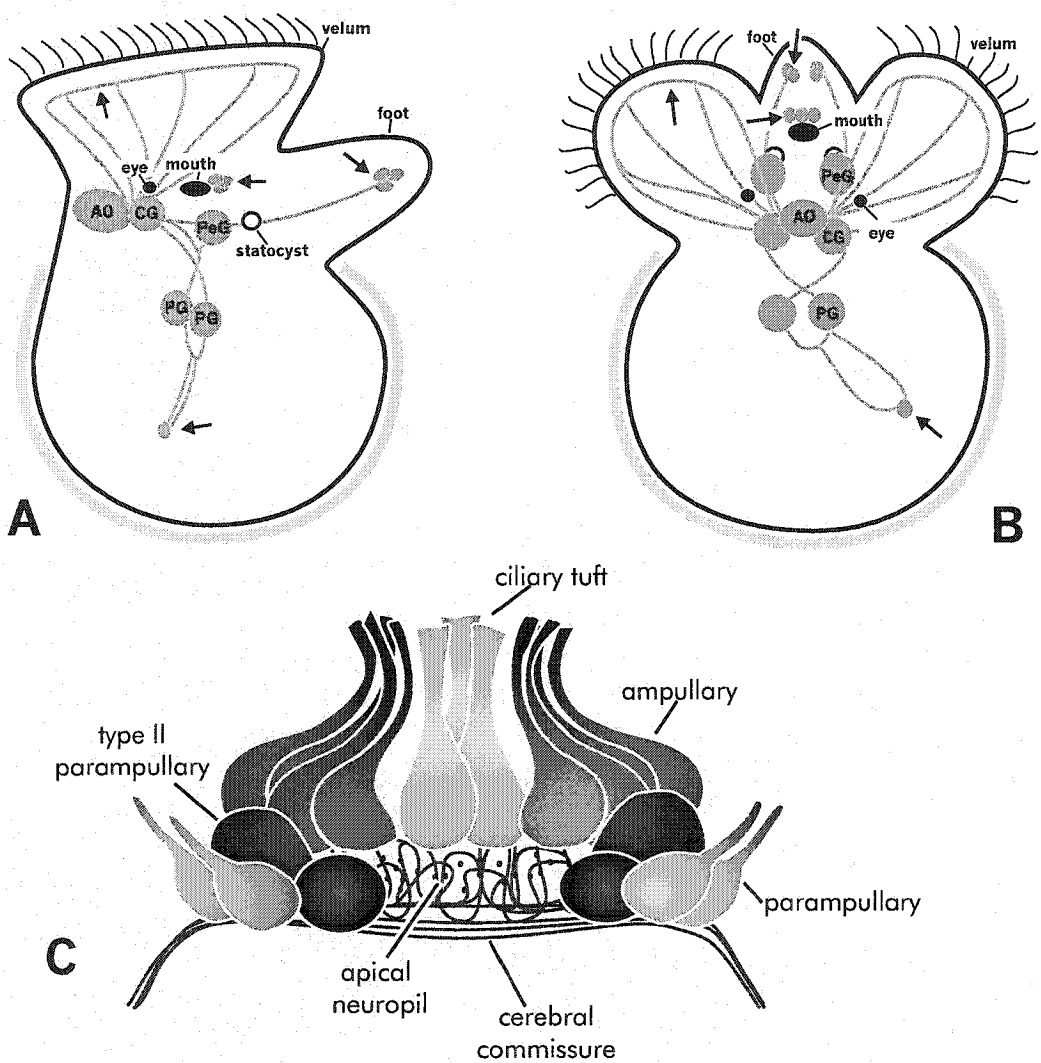
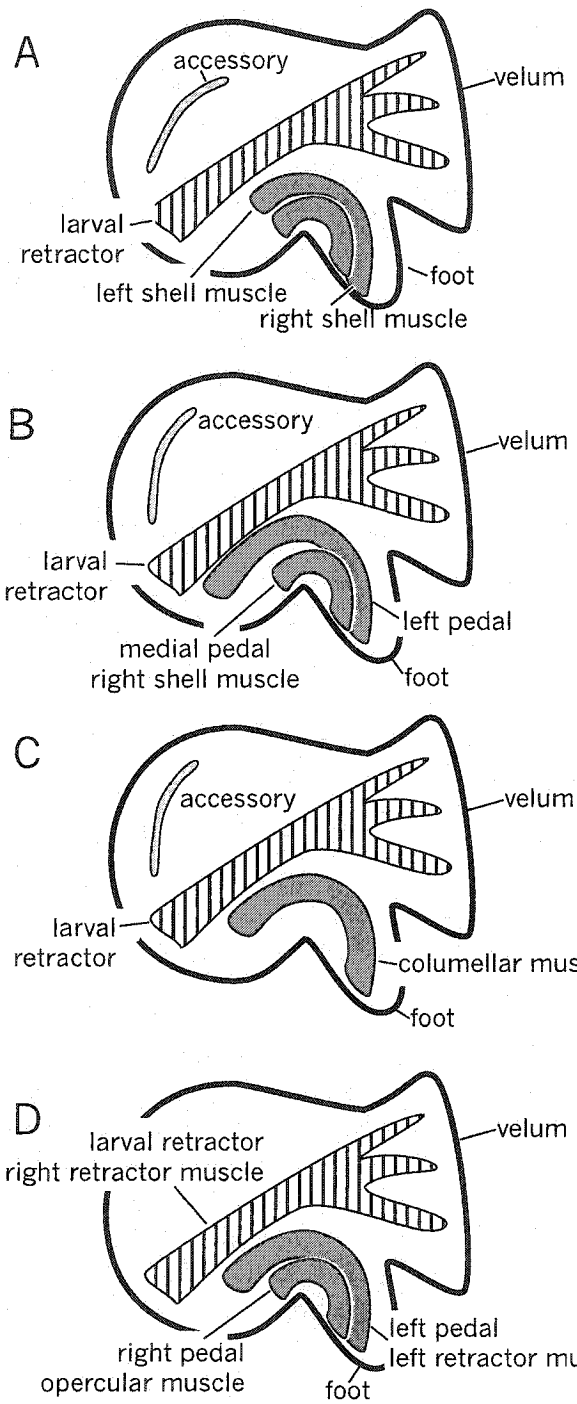

velum
foot
eye
mouth
AO
CG
PeG
statocyst
PG
PG
A
foot
velum
mouth
PeG
AO
CG
eye
PG
B
ciliary tuft
ampullary
type II
parampullary
parampullary
C
apical
neuropil
cerebral
commissure

Figure 1

Figure 2: Schematics of the larval muscles in representatives from several groups of gastropods. The striped areas represent the main larval retractor and the shaded areas are muscles in the foot. **A)** Patellogatropoda **B)** Vetigastropoda **C)** Caenogastropoda **D)** Heterobranchia.



Patellagastropoda

Patella caerulea
Wanninger et al , 1999

Vetigastropoda

Haliotis kamtschatkana, Page, 1997
Haliotis rufescens, Degan et al, 1997

Caenogastropoda

Polinices lewisii, Page, 1997

Heterobranchia

Tritonia diomedea, *Rostanga pulchra*,
Doridella steinbergae,
Dendronotus subramosus,
Melibe leonina,Page, 1997;
Phestilla sibogae, Bonar 1978

Figure 2

CHAPTER 2

DEVELOPMENT OF THE LARVAL NERVOUS SYSTEM OF THE GASTROPOD *ILYANASSA OBSOLETA*

Introduction

Gastropods have been well studied in terms of their cleavage patterns and cell lineages and have also become an important model for studying the neural bases of adult behaviors. However, very few investigations have been done to bridge these two fields and provide an understanding of the mechanisms governing neural development in this taxon. Therefore, in the present study we provide the groundwork for such experimental studies with a detailed examination of the development and anatomy of the larval nervous system in a well studied gastropod.

One of the major limitations of previous investigations of molluscan neural development has been that they have only concentrated on specific neural components. One example concerns the extent of the larval nervous system. For example, development of the central ganglia that become the adult central nervous system has been well studied in certain gastropod species (Kandel *et al.*, 1981; Jacob, 1984). However, the roles of the different ganglia in the larval stages and in the possible integration with other larval neural elements has not been investigated. In addition to the central ganglia, the apical organ has also been the focus of many isolated investigations in larval gastropods (for examples see Bonar, 1978b; Chia and Koss, 1984; Marois and Carew, 1997b; Page and Parries, 2000; Page, 2002a). Comparative studies of particular subsets of cells in the apical organ have been undertaken with the goal of learning more about evolutionary relationships between molluscan taxa. For example, the pattern of serotonin

containing cells in the apical organ has been shown to be similar in closely related groups of heterobranchs (Kempf *et al.*, 1997). Additionally, the role of this sensory organ in the process of metamorphosis has also been investigated (Hadfield *et al.*, 2000; Leise and Hadfield, 2000). However, the function of the apical organ during larval life and how it is integrated into a larval nervous system has not been well studied.

Recent studies have also shown that neural elements also exist outside the central ganglia and apical organ in peripheral regions such as in the velum, foot, mantle and mouth. Such peripheral neurons have been revealed with markers for different neuropeptides and transmitters in early embryonic stages. For example, cells and axons containing peptides related to Phe-Met-Arg-Phe-NH_2 (FMRFamide) and serotonin have been observed in peripheral regions in embryonic stages of several gastropods (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996a; Croll and Voronezhskaya, 1996b; Voronezhskaya and Elekes, 1996; Voronezhskaya and Elekes, 1997; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). Using a formaldehyde glutaraldehye (Faglu) technique (Furness *et al.*, 1977) and an antibody to tyrosine hydroxylase (TH) have been shown to uncover catecholamine-containing peripheral cells have also been revealed in molluscan larvae (Croll *et al.*, 1997; Dickinson *et al.*, 1999; Voronezhskaya *et al.*, 1999; Dickinson *et al.*, 2000; Pires *et al.*, 2000c).

Based on these previous studies we hypothesize that a comprehensive examination will reveal that the gastropod larval nervous system is relatively extensive and consists of

all of the above mentioned components; the apical organ, developing central ganglia as well as peripheral cells and axons. We test this hypothesis in the present study by examining the details of neural elements in larval stages of the caenogastropod *Ilyanassa obsoleta*, a relatively well studied species in terms of cell lineage and cleavage patterns (Clement, 1956; Clement, 1962; Render, 1991; Render, 1997; Lambert and Nagy, 2001). Several different markers have been utilized, combined with confocal microscopy to uncover a large proportion of the larval nervous system. While Leise and coworkers have previously described the development of the central ganglia destined to become the adult CNS, together with aspects of the apical organ in mid and late larval stages of *I. obsoleta*, we have focused on additional details of these neuronal components such as their first appearance and associations with other neuronal components. Also examined in this study was the development of neural elements throughout the embryonic, larval and metamorphic periods.

Together the comprehensive analysis of the entire larval period presented here allows for better comparisons between molluscan species, thus, helping to resolve phylogenetic relationships and further our understanding of the evolution of larval forms. Finally, knowing when neurons appear and which elements disappear during metamorphosis will also be necessary for studies concerning the general mechanisms by which gastropod nervous systems develop and are remodeled.

Materials and Methods

Adult and Larval Culture

Adult specimens of *I. obsoleta* were collected at Evangeline Beach, Grand Pre, Nova Scotia or were generously provided by L. Milstead from the Whitney Marine Lab (University of Florida, St. Augustine, FL) and were maintained in salt water aquaria. Egg masses were collected soon after oviposition and kept in separate containers of artificial salt water (Crystal Sea, Baltimore, MD) at 23-25 °C. Under these conditions embryos required approximately 7-10 days to develop from the first cleavage stage to hatching. Upon hatching the larvae were kept in culture systems described previously by Miller and Hadfield (1986) and fed daily *Isochrysis galabana* (Clone T.ISO) at a final concentration of approximately 15,000-20,000 cells/ml. The hatched veliger required another 2-2.5 weeks before becoming competent to metamorphose into juvenile snails (Lin and Leise, 1996a).

Developmental stages of *I. obsoleta* and closely related species have been previously described (Fretter, 1967; Fretter, 1969; Fretter, 1972; Tomlinson, 1987). In the present study, morphological and behavioral features were examined regularly from first cleavage to competency and the development was divided into several stages based on such features. The stages examined included trochophore (2.5-4 days), embryonic veliger (4.5-7 days), hatchling (7-10 days), and free swimming veliger (14-17 days), competent veliger (21-25 days). In addition, we examined the metamorphosing veliger.

Competent larvae were induced to undergo metamorphosis by exposing them to 10^{-4} M serotonin for 24 hours (Couper and Leise, 1996). Based on morphological changes, the process was divided into early metamorphosis (0-24 hours post induction) and late metamorphosis (24-48 hours post induction). Since the changes in the positions of the ganglia constituting the developing central nervous system during metamorphosis have been examined in detail by Lin and Leise (1996a), our observations focused on the changes or losses of the cells and axons in regions outside the ganglia.

Antibodies

Antibodies included polyclonal anti-FMRFamide and anti-serotonin (both obtained from Diasorin, Stillwater, MI), a monoclonal anti-tyrosine hydroxylase (TH) (Diasorin, Stillwater, MI) polyclonal anti-leu-enkephalin (Chemicon, Temecula, CA), polyclonal anti-alpha-tubulin (DM1A clone from Sigma Chemical Co., Mississauga, Ontario) and polyclonal anti-dopamine beta hydroxylase (DβH) (Diasorin, Stillwater, MI).

Immunocytochemistry

Larvae were initially relaxed using an isotonic solution of magnesium chloride. Larvae were then fixed with either of two different solutions, depending on the antibody being used. For most antibodies animals were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS;50mM $Na_2HPO_4.7H_20$ and 140mM NaCl in distilled

water adjusted to pH 7.2) for 10-30 minutes at room temperature (21-24 °C). For TH and DβH antibodies larvae were fixed in cold 100% methanol for 10-30 minutes at -20 °C. DβH labeling was observed after either 4% PFA or methanol fixation, however while axons were clearly labeled with PFA, cells were not, therefore we based our results on the labeling in methanol fixed larvae.

After fixation embryos were washed in PBS twice for 5 minutes and then one last time for at least 1 hour. The shells of older embryos (> day 5; early veliger stages) were then decalcified with 80% of 0.23M ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., Mississauga, Ontario) with 20% 0.1 M sodium acetate (Sigma Chemical Co., Mississauga, Ontario) for several hours. Embryos were next incubated overnight in 4% Triton X-100 in PBS at 4°C.

The embryos were then incubated in one of the antibodies listed above. All these antibodies were diluted 1:500 -1:1000 in PBS with the addition of 1.0% normal goat or sheep serum and 1.0% Triton X-100. Incubation periods in primary antibodies lasted 3-7 days at 4°C. After primary incubation the embryos were rinsed twice (5 minutes each) with PBS and given a final wash for 3-6 hours before incubating for 1-2 days in secondary antibody consisting of goat anti-rabbit or sheep anti-mouse antibodies conjugated to Alexa Fluor (Molecular Probes, Eugene. OR) or FITC (Bio/Can Scientific, Mississauga, Ontario) and/or rhodamine (Bio/Can Scientific, Mississauga, Ontario). These secondary antibodies were diluted 1:50 in PBS. The secondary antibody was removed by rinsing in PBS twice for 5 minutes and then a final wash of at least one hour.

Finally, some larvae were immersed in 10% hydrogen peroxide for 5-6 hours to remove pigmentation.

Double labeling of cells in certain larvae was performed sequentially. In some cases animals were labeled with one monoclonal and one polyclonal antibody and the appropriate secondary antibodies. In other cases two polyclonal antibodies, for example, anti-serotonin and anti-FMRFamide, were utilized and numbers of cells counted to determine likely co-localization.

Pre-absorption controls were performed for anti-FMRFamide and anti-leu-enkephalin. Synthetic FMRFamide (Sigma) was added to a 1:500 dilution of anti-FMRFamide to a final peptide concentration of 200ug/ml. Synthetic leu-enkephalin (Sigma) was added to a 1:500 dilution of anti-leu-enkephalin to a final concentration of 200ug/ml. The antibodies were pre-absorbed for 24 hours at 4°C and then centrifuged for 10 minutes at 5000rpm. Hatchling larvae, incubated in the pre-absorbed antibody solution and processed as described above, did not exhibit immunoreactivity.

Larvae processed for immunocytochemistry were mounted on glass slides in a 3:1 mixture of glycerol to PBS with 2% propyl gallate added to prevent fading (Longin *et al.*, 1993). Larvae were viewed on a Leitz Aristoplan microscope equipped for epifluorescence with filters appropriate for Alexa Fluor, FITC and rhodamine and on a Zeiss Axiovert LSM 510 confocal laser scanning microscope viewed at 488nm or 543nm. As negative controls, embryos were processed without incubation in primary antibody; such specimens exhibited no detectable fluorescence. Positive controls included parallel

processing of other molluscan larvae with known labeling patterns (Marois and Croll, 1992; Croll and Voronezhskaya, 1995; Marois and Carew, 1997b). At least 30 animals at each stage were examined for each immuno-label.

Catecholamine Histofluorescence

The Faglu technique (Furness *et al.*, 1977) was also used to localize catecholamines. Embryos were fixed in a solution consisting of 4% paraformaldehyde, 0.5% glutaraldehyde and 35% sucrose in PBS for at least 12 hours at 4°C. Similar results were also obtained when specimens were stored in this solution for several weeks. Embryos were then placed on glass slides, air dried for several hours at room temperature and then mounted in Fluoromount (BDH, Toronto, ON). Positive controls were performed by parallel processing of embryonic *L. stagnalis* which exhibited typical blue-green fluorescent staining as described elsewhere for catecholamines (Voronezhskaya *et al.*, 1999). Negative controls were performed by omitting the glutaraldehyde from the Faglu solution which eliminated the characteristic fluorescent staining. At least 30 animals at each stage were examined using this technique.

Photography and Cell Measurements

Photographs were created using the Zeiss confocal microscope by superimposing stacks of 10-80 images obtained through stepped sequences of focal planes at 0.10-0.80 μm intervals. Projections were created with Zeiss LSM 510 software using maximum

transparency. Some embryos were also photographed through the Leitz compound microscope equipped with a Orthomat E camera and Kodak 200 Select Color film. Negatives were digitally scanned with an HP Photosmart Scanner. All the images were then assembled into plates and labeled using Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). Contrast and brightness of the images were adjusted to provide consistency within plates. In some cases montages of confocal projection subsets were created. Measurements of the animals and of individual cells were achieved using the overlay function of the Zeiss LSM 510 software on randomly selected projections. Schematic diagrams were drawn using Corel Draw 8 (Corel Corporation Limited).

Results

Trochophore stage (Day 2.5-4)

Approximately 24 hours after gastrulation the embryo entered the trochophore stage (Fig. 3A). At this time the body became more elongated and rudiments of the velum and foot developed. Patches of cilia appeared on these rudiments forming prototrochal and pedal bands respectively. The shell field evaginated just prior to the initiation of shell secretion. By the end of this stage the shell was displaced to the right. Ventral and slightly to the left of the shell field was another patch of cilia, the telotroch. Trochophores rotated within the capsule and cilia beat constantly. When the capsule was cut open, rotational, forward and backward movement were observed, but embryos were not capable of swimming. During the trochophore stage the embryo was approximately 220 μm in length and 130-150 μm in width.

At the beginning of the trochophore stage (day 2.5) a single FMRFamide-like immunoreactive (LIR) Posterior (FP) cell was observed lateral to the developing shell field, near the telotroch (Fig. 4A and Fig. 5A). The FP cell was 15 μm in length with a large nucleus that was devoid of labeling (Fig. 5A). A single axon projected from the FP soma anteriorly on the left side of the embryo toward the left kidney (Fig. 5A, inset). Later another axon extended from the FP cell to the right side of the embryo (Fig. 4A). Also during the early trochophore stage a pair of medial FMRFamide-LIR Apical (mFA) cells appeared at the anterior most region (Fig. 4A, Fig 5B). These cells were 20 μm in

length and flask shaped. The distal tapered ends appeared more immunoreactive than the soma. Processes extended from each mFA cell body into a neuropil located just ventral and posterior (Fig. 5B). FMRFamide-LIR axons also extended posteriorly from the lateral edges of the neuropil and ran along the outer edge of the yolk mass. By the end of the trochophore stage, the posteriorly directed axons reached the midline near the larval kidneys and branched into the foot rudiment (Fig. 4A).

By day 3, two Serotonin-LIR Apical cells (SA1 cells) were located posterior and lateral to the mFA cells (Fig. 4A, Fig. 5B, C, D). These cells were approximately 10 μm in length and although irregular in shape, no apical dendrites were observed. An axon from each SA1 cell projected into a neuropil of serotonin-LIR varicosities and exited on the opposite side where it extended laterally and posteriorly along the outside of the yolk mass (Fig. 5C, D). By the end of the trochophore stage, these posterior projections terminated near the kidneys and branches from each projection entered the foot rudiment (Fig. 4A).

Also by Day 3, two pairs of leu-Enkephalin-LIR Apical (EA) cells were detected (Fig. 4A and Fig. 5E, F). Both pairs of EA cells appeared lateral to the mFA cells and dorsal to the SA1 cells. The EA cells were vase-shaped with dorsally directed dendrites and were smaller (10 μm in length) than the mFA cells but more intensely labeled. Faint enkephalin-LIR labeling of axons also appeared ventrally and medially to the pairs of EA cells (Fig. 5F).

Embryonic Veliger Stage (Day 4.5-7)

During the embryonic veliger stage (Fig. 3B) the animal was 220-250 μm in length and the velar lobes, rimmed by large (20 μm in length) ciliated cells, were evident. The foot grew in size and the operculum was secreted. At the beginning of this time torsion began with migration of the anus to a more dorsal-anterior position. As the embryo developed, the telotroch moved forward and became a ciliated region of the dorsal mantle lip after torsion. A continuous digestive tract, larval heart, statocysts and eyespots also appeared during this stage. The embryo rotated within the egg capsule, but when cut free from the capsule it swam vertically in the water column and the pre-oral cilia arrested intermittently allowing the larva to sink.

By Day 4, the posteriorly directed FMRFamide-LIR and serotonin-LIR projections from the apical organ met at the center of the embryo just posterior to the kidneys. As torsion proceeded these processes were twisted right over left to form a figure-eight, which later formed the connectives of the visceral and intestinal ganglia (and therefore will be referred to as visceral/intestinal connectives) (Fig. 4B, 6A, C). The apical neuropil also became more intensely labeled by both anti-serotonin and anti-FMRFamide (see Fig. 6B, D).

Projections from the FP cell met the FMRFamide-LIR axons forming the visceral/intestinal connectives (Fig. 6A). The FP cell migrated anteriorly along with the telotroch and by the end of the embryonic veliger stage, the FP cell was located at the dorsal lip of the mantle on the right side (see Fig. 8A). A new bilaterally symmetrical pair

of FMRFamide-LIR Proximal Foot (FPF) cells were located near where the foot meets the body (Fig. 4B, 6A). The FPF cells each had a dendritic process, which became more evident in later stages, and an axon that projected into the region of the developing pedal ganglia which was also apparent at later stages (see Fig. 12D, 15B). Also two additional FMRFamide-LIR cells appeared along the visceral/intestinal connectives and corresponded to locations of the future supra- and sub-intestinal ganglia. The FMRFamide-LIR Supra-intestinal (FSp) cell was located on the left and the FMRFamide-LIR Sub-intestinal (FSb) cell was located on the right (Fig. 4B, 6A). Also during this stage FMRFamide-LIR axons projected into each of the velar lobes (Fig. 4B) either directly from each side of the apical neuropil or indirectly passing first ventrally and posteriorly before entering the posterior portions of the velum. Each of these axons branched repeatedly within the velum by the end of this stage.

Two new Serotonin-LIR Apical (SA2s) cells were observed in the apical organ on either side of the SA1 cells (Fig. 4B, 6C, D). The SA2 cells were 10-15 μm in length and were less intensely labeled than the SA1 cells. In addition, the SA2 cells had apical dendrites which extended toward the epithelial surface which were more evident at hatching (see Fig. 9E). Axons from the SA2 cells also entered the apical neuropil, however they could not be traced further. Serotonin-LIR axons projected from the apical neuropil into the velum where they branched several times (Fig. 4B, 6C). While visceral-intestinal connectives were also labeled by anti-serotonin, there were no serotonin-LIR cells along these connectives (Fig. 4B, 6C).

During this stage the two pairs of EA cells became more elongated, reaching 20 μm in length. The nuclei of these cells were devoid of labeling at this stage (Fig. 4B, 6E, F).

The first catecholamine-containing cells appeared during the embryonic veliger stage. Catecholamines were detected using both the Faglu technique and antibodies to TH. Approximately 3-5 catecholamine-containing cells appeared in distal portions of each velar lobe. These Catecholamine Distal Velar (CDV) cells were round and 5-7 μm in diameter (Fig. 4B, 7A,B).

Hatchling Stage (Day 7.5-10)

After 7-10 days the embryos hatched from their capsules and began swimming in the water column. The size of the hatchling was 250-300 μm in length. The remainder of the yolk mass disappeared by the day after hatching. Pigmentation appeared along the rims of the velar lobes and along the mantle edge. The rudiment of the propodium appeared as a small protrusion on the anterior surface of the foot. The right tentacle also developed soon after hatching, at least one to two days prior to the left tentacle (Fig. 3C).

During the hatchling stage the general pattern of the nervous system was very similar to the embryonic veliger with some additions of cells and a more intricate system of axons projecting into peripheral regions. FMRFamide-LIR, serotonin-LIR and leu-enkephalin-LIR axons radiated into the velum, branching repeatedly. It appeared that axons circled the entire rim of the velar lobes at the base of the large ciliated epithelial

cells. Also the axons within the velar lobes had many swellings along their length (Fig. 8A-C, 9C, D, I).

In addition to the above mentioned labeling, new cells and distinct axon tracts appeared immunoreactive for FMRFamide. A new pair of round FMRFamide-LIR Apical (FA1) cells were located on each side of the mFA cells in the same location as the SA1 cells (Fig. 8A, 9B, C) and later, double labeling indicated that these are likely the same cells (see later stage, Fig. 12K). However, at this stage the SA2 cells were not detected with anti-FMRFamide (but see FA2 at later stages, Fig. 12C, 12K). Also, an unpaired FMRFamide-LIR Apical (FA3) cell was observed lateral to the left FA1 cell (Fig 8A, 9B, C). Double labeling at later stages revealed that the FA3 cell was also positive for anti-serotonin (see SA3 at later stages; Fig 12E, 12K). During the hatchling stage the mFA cells became faintly immunoreactive and were sometimes undetectable. At the tip of the foot a bilaterally symmetrical pair of FMRFamide-LIR Distal Foot (FDF) cells was observed (Fig 8A, 9A). These FDF cells were very small (5-7 μm in length) and faintly labeled. An axon from each of the FDF cells projected dorsally along parallel nerve tracts extending into each side of the apical organ (Fig 8A, 9C). Another FSb cell appeared along the visceral/intestinal connectives (Fig. 8A, 9A). Processes also radiated from the visceral/intestinal connectives along the edge of the mantle (not shown).

During the hatchling stage the first cells were observed in the region of the future cerebral and pedal ganglia. A bilateral pair of FMRFamide-LIR Cerebral (FC) cells were located ventral to the apical organ in the region of the cerebral ganglia (Fig 8A, 9A, C).

Also faintly labeled serotonin-LIR cerebral (SC) cells appeared in the cerebral ganglia ventral and lateral to the SA2 cells (Fig 8B, 9E). There were two SC cells on the left and one cell on the right at this stage. A pair of bilaterally symmetrical Serotonin-LIR Pedal (SPe) cells was observed just below the statocysts, in the location of the developing pedal ganglia (Fig 8B, 9F). Parallel axon tracts spanning the length of the foot, from the tip to each of the developing cerebral ganglia, were labeled with anti-FMRFamide and anti-serotonin. From these pedal tracts, many branches including axons forming the pedal commissure existed (Fig 8A, B, 9C, F).

Soon after hatching, a single unpaired medial Serotonergic Apical (mSA) cell with a dendritic process appeared (Fig 8B, 9E). This cell was very faintly labeled and like the mFA cells was not detected in all specimens. A group of three round Serotonin-LIR Osphradial (SOs) cells surrounded a neuropil on the right side of the animal below the mantle cavity corresponding to the region of the osphradial ganglion. At least one axon connected the osphradial ganglion to the visceral/intestinal connectives (Fig 8B, 9D).

An additional two pairs of EA cells were observed (Fig. 8C, 9G, H). By this stage there were two bilaterally symmetrical groups of leu-enkephalin-LIR cells in the apical region with each group containing four cells (Fig. 9G, H). The vase-shaped EA cells were 20 μm in length. In addition, faintly labeled processes formed a neuropil just ventral and medial to each group of cells. From this neuropil, processes extended into

the velum and foot and mantle (Fig. 8C, 9I). During this stage, the EA cells were also intensely immunoreactive for acetylated tubulin (see later stages, Fig.12L, M).

During the hatchling stage additional catecholamine-containing cells were present. Both TH labeling and Faglu technique revealed the same cells, however, TH-like immunoreactivity also allowed axons and deeper positioned cells to be viewed more easily. Additional CDV cells were observed at this stage so that a total of 9-10 cells were observed in each lobe (Fig 8D, 10A). The cells were vase-shaped and that axons extended from each cell (or the vicinity of each cell) to neighboring cells and to the base of the large ciliated epithelial cells lining the edge of the velar lobes (Fig. 8D, 10C). Also a catecholamine-containing cell was observed at the base of each of the velar lobes (Fig. 8D, 10C). These **C**atecholamine **P**roximal **V**elar (CPV) cells were round and approximately10 μm in diameter. Axons radiated from the regions of the CPV cells into the velar lobe terminating near the CDV cells (see later stages, Fig. 12D). TH-LIR axons were also located in the cerebral commissure and a pair of bilaterally symmetrical **C**atecholamine **C**erebral (CC) cells were observed on the lateral ends of the cerebral commissure (Fig. 8D, 10C). These cells were 7-10 μm in diameter. Another bilaterally symmetrical pair of catecholamine-containing cells was located just anterior and ventral to the cerebral commissure and dorsal to the mouth. These **C**atecholamine **D**orsal **M**outh (CDM) cells were 20 μm in length and vase-shaped (Fig. 6D, 8C). **C**atecholamine **V**entral **M**outh (CVM) cells were also observed ventral and lateral to the mouth (Fig.8D and 10B, C). The CVM cells were 15 μm in length and also vase-shaped with dendrites

orientated toward the opening of the mouth. The number of CVM cells increased from four to eight within one day after hatching (compare Fig. 10C and Fig 10B). A bilaterally symmetrical pair of Catecholamine Foot (CF) cells was observed near the tip of the foot (Fig. 8D, 10C, D). Each cell was vase-shaped and 10 μm in diameter. An axon projected dorsally from each CF cell toward the cerebral commissure (Fig. 8D and 10C).

Antibodies for DβH labeled neural elements for the first time in the hatchling stage. DβH-LIR axons radiated throughout the velum and foot (Fig. 8C and 10E). There were two bilateral symmetrical pairs of DβH-LIR Foot (DF) cells (Fig. 8C, 10F). One pair of DF cells was located near the tip of the foot; double labeling with anti-TH at later stages revealed they were different than the CF cells (see Fig 13B, C). Another pair of DF cells was located dorsally and medially to the first pair. The DF cells were irregular in shape and approximately 10 μm in diameter (Fig. 10F).

Free Swimming Veliger (Day 14-17)

The overall morphology during the free swimming veliger stage was similar to the hatchling stage (Fig. 3D). However, since the animal doubled in size within a week from hatching the most apparent change during the free swimming veliger stage was growth. The velum became as large or larger than the length of the larval body. The propodium became more prominant but was not motile until competence.

During this time additions to the larval nervous system, especially to the developing ganglia, were apparent. Neuropils of the cerebral, pedal ganglia, intestinal

and the osphradial ganglion were identified using antibodies to FMRFamide, serotonin and alpha-tubulin. Additional cells were also observed in the ganglia as well as in peripheral regions (Fig. 11A, B, C, D).

At 14 days, additional fibers and cells immunoreactive to FMRFamide were observed. Axons extended from the regions of each cerebral ganglion into the tentacles (Fig. 12A). In the middle of the apical neuropil at least one faintly labeled flask shaped cell, possibly one of the mFA cells, was observed (Fig. 12B). A pair of bilaterally symmetrical **F**MRFamide **A**pical (FA2) cells existed in the apical organ in the same location as the SA2 cells and double labeling indicated these were the same cells (Fig. 11A, 12C, 12K). An axon extended from the middle of the pedal commissure to the very distal tip of the foot, parallel to the pedal tracts (Fig. 11A, 12D). Along the axons forming the pedal tracts near the pedal commissure (Fig. 12D), were faintly labeled **F**MRFamide-LIR **Pe**dal (FPe) cells, one cell on the right and two cells on the left (Fig. 11A, 12D). An additional fiber tract existed ventral and parallel to the pedal commissure, referred to as the sub-pedal commissure (Fig. 11A, 12D). In the middle of, and just ventral to, this sub-pedal commissure were two cell-like structures (Fig. 11A,12D, large arrow). Additional FDF cells were observed at the tip of the foot (Fig. 11A, 12D). Axons were also seen to project from the pedal tracts and branched throughout the propodium (not shown). Axons connected the intestinal ganglia to a region ventral to the cerebral ganglia forming the first evidence of the pleuro-intestinal connectives (Fig. 11A, 12C, arrows). Several FMRFamide-LIR cells and many branching axons were also observed in the

posterior of the larva near the digestive structures, the osphradium, and the mantle edge (Fig. 11A). FMRFamide-LIR axons in the velar lobes also became more intricate (Fig. 11A, 12H)

During this stage, additional serotonin-LIR neurons and axons were also observed. A single unpaired Serotonin-LIR Apical (SA3) cell existed on the left in a similar location to FA3, labeling with both serotonin and FMRFamide indicated that these were the same cells (Fig. 11B, 12E). SC cells (Fig 9B, 10E) supra-intestinal ganglia Serotonin-LIR- Supra-intestinal (SSp) , Sub-intestinal (SSb) and Visceral (SVi) cells were all observed (Fig. 11B, 12F). Also at least three additional SOs cells appeared in the osphradial ganglion and axons were observed to connect the osphradial neuropil to the sub-intestinal ganglia (Fig. 11B, 12F). Several new SPe cells were located near the pedal commissure (Fig. 11B,12F, G). Serotonin-LIR cells and axons were also observed along the mantle edge (Fig, 11B).

Additional pairs of EA cells appeared in the apical organ during the free swimming veliger stage. There were a total of seven to eight vase-shaped cells in each of the two groups (Fig. 11C, 12 I, J) which were also revealed with anti-alpha-tubulin (Fig. 12 L,M). Axons labeled with leu-enkephalin antibodies in the foot and velar lobes were also more extensive at this stage.

During this period the pattern of catecholamine containing cells appeared very similar to the previous stage with the addition of several new cells. Approximately 19-20 CVM cells existed under the mouth arranged in two rows (Fig. 9D, 13A). Additional CF

cells were observed at the tip and along the outer edge of the foot, and axons projected dorsally from these cells (Fig. 11D, 13B). An additional five to seven CDV cells were located along the post-oral band of each velar lobe (Fig. 11D, 13C). Approximately 10-15 cells existed in the propodium, most of which were concentrated along the distal surface (see competent stage, Fig.16F).

Additional cells were also labeled with anti-DβH during the free-swimming veliger stage. Approximately six to eight new DF cells appeared in the middle of the foot and double labeling indicated that all the cells were different from the CF cells (Fig. 11C, 13B,C). Also axons in velum and head regions were detected with anti-DβH (Fig 11C, 13D).

Competent Veliger (12-16 days post hatching)

When larvae were competent to metamorphose (see Fig. 3E) the major indicator was the movement of the propodium and the larvae often alternated between swimming and crawling. The propodium was 200 μm and protruded from the foot proper or mesopodium. The velum had become bilobate and was capable of more complex movements. The average size during this stage was 600-700 μm in length. All the ganglia constituting the developing central nervous system (CNS) were evident using immunohistochemical (for examples see Fig. 15A, B, F, G, I) and Faglu techniques (for examples see Fig. 16A, B) and observations here were consistent with studies by Lin and Leise (1996a).

Additional FMRFamide-LIR cells and axons were observed in the central ganglia. At least five to six cells were present in each cerebral ganglion, two to three cells in each pedal ganglion, six to eight cells in each intestinal ganglion and seven to eight in the visceral ganglion (Fig 14A and also see 15A, B). At least one to two new cells were observed along the mantle edge (Fig. 14A and 15C) and near the osphradial ganglion (not shown). Also new FMRFamide-LIR cells existed in the middle of each lobe of the velum. Fibers from each of the FMRFamide-LIR Velar (FV) cells projected toward the base of the large ciliated cells along the edges of the velum (Fig. 14A, 15D). More FDF cells were observed near the tip of the foot so that at least seven to eight cells existed at this time (Fig. 14A).

The most conspicuous addition during this stage was a new pair of bilaterally symmetrical serotonin-LIR cells observed anterior and lateral to the cerebral-pedal connectives. These Serotonin-LIR Apical Lateral (SAL) cells were bipolar with one end that terminated near the outer edges of the cerebral ganglia and the other ending in the apical neuropil (Fig. 14B,15E). Additional serotonin-LIR cells were also observed in the developing ganglia of the future adult CNS. At least four to five cells were located in each of the cerebral ganglia, ten to twelve cells in each pedal ganglion, eight to ten cells in the supra-intestinal ganglion, four to six cells in the sub-intestinal ganglion and seven to eight cells in the visceral ganglia (Fig. 14B, 15F, G). The osphradial ganglion became very prominent, spanning 150-200 μm at this stage and consisted of seven to ten cells surrounding a neuropil (Fig. 14B and 15G, I). Also Serotonin-LIR Velar (SV) cells also

existed in the middle of each lobe of the velum (Fig. 14B,15H). Innervation to the peripheral areas became much more extensive, especially along the ciliated mantle cavity and newly developed siphon (Fig 15I).

By competency three new pairs of EA cells existed in the apical organ, so that a total of ten cells was observed in each bilateral group (Fig. 14C, 15J). New leu-Enkephalin-LIR Velar EV cells also existed in the velum, one in the middle of each lobe (Fig. 14C, 13K).

The catecholamine labeling was also much more extensive when larvae were competent to metamorphose. A new group of three to four cells appeared in the cerebral ganglia (Fig 14D, 16A). By this time many CVM cells were also observed ventral to the mouth (Fig 14D, 16B). Several catecholamine containing cells were also observed in the tentacles, (Fig 14D, 16C, arrows), lining the mantle cavity (Fig 14D, 16D, arrows), and in the osphradial ganglion (**C**atecholamine-containing **Os**phradial cells; COs) (Fig 14D, 14D). Additional catecholamine-containing cells existed in the velar lobes (Fig 14D, 14E), propodium (Fig 14D, 16F) and along the edge of the mesopodium (Fig 14D, 16G). Fibers existed within the pedal and propodial ganglia, which were likely efferent axons from the cells in the foot and propodium (Fig. 16B). Many catecholamine-containing cells were scattered over the surface of the body just beneath the epithelial layer (not shown).

Labeling by anti-DβH was similar to previous stages with the addition of DF cells in the mesopodium. Approximately 35-40 DF cells were present in the middle of the mesopodium at competency (Fig 14C, 16H).

Metamorphosis

During metamorphosis, some characteristic changes in morphology and behavior were observed. During early metamorphosis (first 24 hours) the larvae no longer swam vertically. While larvae ceased to swim, the velum remained outstretched and orientated upwards in a swimming posture. The velar lobes contracted more often and the large cilia began to beat rapidly. Also during this time sections of velar cilia were lost. During late metamorphosis (24-48 hours post induction), the velum became reduced to small mounds of un-ciliated tissue and then disappeared completely. Major changes also took place in the structure of the head due to the reorganization of the feeding structures, which also resulted in subsequent changes in the location of the CNS (reviewed by Lin and Leise, 1996b; Lin and Leise, 1996a). The eyes were more anteriorly located, moved closer together and were found at the base of the tentacles.

The apical cells labeled by FMRFamide and serotonin antibodies were difficult to follow throughout metamorphosis, although the largest of the cells (FA1/SA1, FA2/SA2, FA3/SA3) were still observed during early metamorphosis (Fig.17A, and also 17H). Also during early metamorphosis, the borders of the EA cells were not as sharp and the cells were less intensely labeled (Fig. 17C, F). The pattern of FMRFamide-LIR cells and

axons in the foot appeared very different during early metamorphosis. The FDF and FPF cells were no longer observed. Also by this time the distinct pedal tracts and branching pattern were no longer evident, instead the pattern appeared very homogeneous in the foot (Fig. 17B). This was also true of the serotonin- and leu-enkephalin-like immunoreactivity in the foot (not shown). The pedal gland cells, located along the outer edge of the mesopodium, were positive for FMRFamide (Fig 17B), serotonin (not shown) and leu-enkephalin antibodies (not shown). The reason for transient labeling of the pedal gland cells with the antibodies is unknown.

By late metamorphosis the velar lobes were reduced to small mounds and FMRFamide-, serotonin- and leu-enkephalin- like immunoreactivity was observed in these velar mounds, however it was unorganized and punctate (Fig. 17A, C, F). Also the CDV cells still existed at this time (Fig. 17G). By the end of this stage, the velum and all the associated cells and axons were no longer present. During late metamorphosis the EA cells disappeared but in a few animals remnants of the cells could be observed (Fig. 16E). In addition, while anti-TH labeling appeared in cells in the propodium the cells also seemed abnormal and disorganized (Fig. 17H). Catecholamine-containing cells surrounding the ventral portion of the former mouth were no longer observed during late metamorphosis (not shown). On the other hand, catecholamine-containing cells in the mesopodium were still present and many more catecholamine-containing cells covering the surface of the larvae were observed (not shown). No changes in DβH labeling in the foot was observed during metamorphosis (not shown).

Summary

The major cells and groups of cells were summarized in Figure 18. The time of their first appearance, disappearance, location and transmitter phenotype are described in this diagram.

Discussion

The larval nervous system of *I. obsoleta* includes an apical organ, developing central ganglia and peripheral neurons associated with the velum, foot and mantle. Neurons were first observed during the embryonic trochophore stage and the nervous system became increasingly extensive during the larval period. During metamorphosis some elements seemed to degenerate while others were incorporated into the adult nervous system. Neurons contained several different transmitters including monoamines and neuropeptides. While we likely uncovered a great proportion of the larval nervous system in *I. obsoleta*, still other neurons may exist. This seems especially likely since additional classical transmitters, such as acetylcholine (Twarog, 1954; Hanley and Cottrell, 1974; Chrachri and Williamson, 1998), GABA (Osborne *et al.*, 1972; Walker *et al.*, 1975; Morse *et al.*, 1980; Cooke and Gelperin, 1988; Vitellaro-Zuccarello and De Biasi, 1988) and glutamate (Bolshakov *et al.*, 1991; Dale and Kandel, 1993; Nesic *et al.*, 1996; Michel *et al.*, 2000) as well as various peptides (Croll and Van Minnen, 1992; Santama *et al.*, 1994; Willows *et al.*, 1997) are utilized in adult molluscan neurons. Furthermore, we have recently localized histamine in larval stages of *I. obsoleta* (unpublished observations). Even so, this study is the first to reveal what appears to be an elaborate gastropod larval nervous system.

The Apical Organ

The first four cells of the apical organ appeared during the trochophore stage, and the number of such cells increased to approximately 26-28 by the time the larva was competent to metamorphose. Using serial sections of late larval stages of *I. obsoleta* Lin and Leise (1996a) reported fewer cells suggesting that we have identified most of the cells in the apical organ. Many of the cells within the apical organ of *I. obsoleta* were vase-shaped with distal dendrites that extended to the epithelial surface. The morphology of these vase shaped cells varied slightly so that, for example, two FMRFamide medial vase-shaped cells had wider necks and large terminals at the epithelial surface. On the other hand many of the other vase-shaped cells had more slender dendrites. In addition to vase-shaped cells, there were also several round cells in the apical organ of *I. obsoleta.*

Similar types of cells have also been reported in the apical organ of other gastropod larvae. In several species, electron microscopic techniques have been used to characterize the morphological details of these apical cells. Such studies have shown that various sensory-like cell types exist including ciliary tuft cells which are responsible for the apical tuft, ampullary cells which have a deep ciliated lumen and para-ampullary (type I; Kempf *et al.*, 1997) cells that have numerous microcilia that extend above the epidermis (Bonar, 1978b; Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000; Page, 2002a). In the present study the apical cells of *I. obsoleta* were arranged in three bundles, a medial and two lateral bundles surrounding a

neuropil that lay dorsal to the cerebral commissure. Such a tripartite arrangement of apical cells has also been shown in many other gastropod species (Bonar, 1978b; Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000; Page, 2002a).

In addition to the arrangement and cell types, the transmitters localized within apical cells of *I. obsoleta* are also similar to those reported in other gastropods. The best studied cells in the apical organ of gastropods have been those immunoreactive to serotonin (Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000; Page, 2002a). All nudibranch species examined have an identical pattern of five serotonergic cells within the apical organ. Such a pattern consists of a medial vase-shaped cell, a pair of lateral vase-shaped cells and a pair of round cells. Other gastropod species had variations of this five cell pattern (Dickinson *et al.*, 1999; Dickinson *et al.*, 2000; Page and Parries, 2000; Page, 2002a). In the present study we have shown that *I. obsoleta* has five cells arranged as in nudibranchs with the addition of a single round cell on the right side. The same six cell arrangement was also reported in another caenogastropod, *Amphissa versicolor* (Page and Parries, 2000), and it was suggested that the presence of this unpaired right lateral cell might be related to a larger right velum and/or the earlier development of the right tentacle. Both of these characteristics also existed in *I. obsoleta*, however the role of the additional right cell must be investigated to understand the reason for such a correlation.

FMRFamide-like immunoreactivity was also observed in several apical cells of *I. obsoleta* and similar cells have also been reported in other gastropod species. For example, vase-shaped FMRFamide-LIR cells existed in the apical region of *Lymnaea stagnalis* (Croll and Voronezhskaya, 1996a) and *C. fornicata* (Dickinson *et al.*, 1999). In *I. obsoleta*, FMRFamide-like peptides were co-localized with serotonin in many of the apical cells and similar observations were reported in the polyplacophoran *Ischnochiton hakodadensis* (Voronezhskaya *et al.*, 2002). On the other hand, FMRFamide positive cells have not been detected in the apical organ of all molluscan species (Dickinson *et al.*, 2000 and Plummer, 2002). This may be in part due to the limited stages observed in such species or to evolutionary changes of the apical cells.

The most numerous and conspicuous group of cells within the apical organ of *I. obsoleta* were those positive for leu-enkephalin antibodies. These leu-enkephalin-LIR cells were arranged in two bilaterally symmetrical groups dorsal to the apical neuropil. Thavaradhara and Leise (2001) previously identified similar, likely the same, cells in late larval stages of *I. obsoleta* using antibodies to nitric oxide synthase. Leise and coworkers (Lin and Leise, 1996a; Lin and Leise, 1996b; Froggett and Leise, 1999) have also provided further evidence that nitric oxide is important in metamorphosis in this species. In the present study we also revealed tubulin immunoreactivity within these apical cells and therefore they likely contained many microtubules. Vase-shaped cells with numerous microtubules within the lumen are characteristic of sensory cells of the ampullary type (Bonar, 1978b; Chia and Koss, 1984; Kempf *et al.*, 1997). While the role of these cells

during larval life is presently unknown their morphology suggests that they are sensory cells, possibly chemoreceptors. Therefore, these cells may function to modulate locomotion in response to chemical substances in the environment. While the presence of leu-enkephalin-LIR apical cells has not been investigated in many gastropod species, at least one other caenogastropod species, *Crepidula fornicata*, also contains similar cells (unpublished observations). While the specificity of the antibody must be verified in *I. obsoleta*, enkephalin-like peptides have been localized in adult stages of other gastropods (Stefano and Martin, 1983; Stefano and Leung, 1984; Leung *et al.*, 1986; Dyakonova *et al.*, 1995). In addition, behavioral responses were observed when *I. obsoleta* larvae were exposed to the synthetic leu-enkephalin peptide (unpublished observations).

An apical sensory organ located at the anterior of the larva and associated with the apical tuft has been reported to be well conserved, in many invertebrate larval forms (for examples see Lacalli, 1981; Lacalli, 1994; Hay-Schmidt, 1995). Therefore, comparisons of the cell types, cell arrangements, and transmitter phenotypes in the apical organ may offer insight into the evolution of groups within the molluscan phylum as well as of other invertebrate taxa. It is likely that differences in the numbers and cell types have arisen during evolution due to the loss of cells, gain of new cells or by switching transmitters. Such changes may have also been influenced by larval lifestyles. For example, in pulmonates only one pair of sensory cells in the apical region has been observed (Goldberg and Kater, 1989; Croll and Voronezhskaya, 1996a; Voronezhskaya *et al.*, 1999). Since these larvae have an encapsulated and reduced larval mode of development

they may have lost most of the cells within the apical organ that were no longer necessary.

Peripheral Neurons

Peripheral cells were identified in posterior regions, and then later in the foot, the mantle edge, surrounding the mouth and throughout the velum of *I. obsoleta.* In the posterior region of the trochophore, an FMRFamide-LIR cell was identified. FMRFamide (or FMRFamide related peptides) have also been localized within a similar posterior cell in several gastropod and bivalve species (Croll and Voronezhskaya, 1995; Croll and Voronezhskaya, 1996a; Dickinson *et al.*, 1999; Plummer, 2002 E.E.Voronezhskaya, personal communication). In the trochophore stages of the gastropods, *I. obsoleta*, *A. californica* and the bivalve *Placopecten magellanicus* the posterior FMRFamide-LIR cell was observed near patches of cilia (personal observations, and Plummer, personal communication). Based on its location, this cell may therefore be involved in the control of the cilia. However, further investigation is necessary to confirm such a postulate.

In *I. obsoleta*, several different groups of peripheral cells were observed in the foot, many of which had morphologies consistent with a sensory function. Foot cells were positive for FMRFamide, TH and DβH. Catecholamine-containing and FMRFamide-LIR cells have also been reported in the foot of other gastropods (Croll *et al.*, 1997; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). Based on their locations and

innervation patterns, cells in the foot may have roles in ciliary movements of the pedal band, muscular contractions and pedal gland functioning. In addition, catecholaminergic foot cells have been suggested to be involved in sensing settlement cues during metamorphosis (Croll *et al.*, 1997). Support for the role of catecholamine cells in metamorphosis has been shown in both gastropods (Pires *et al.*, 2000b) and bivalves (Coon and Bonar, 1986). Furthermore, similarly located cells have also been found to be responsive to the chemical cues inducing metamorphosis in the heterobranch *Onchidoris bilamellata* (Arkett *et al.*, 1989).

In *I. obsoleta* many catecholamine containing cells were associated with ciliary bands along the velum. Similarly located cells were reported in the bivalves *M. edulis* and *Placopecten magellanicus* (Croll *et al.*, 1997). These cells may be important in regulating feeding currents or detecting food (unpublished data). Sensory-like catecholamine cells were also observed in the oral region in *I. obsoleta* and similar cells were also reported in this region in other gastropod and bivalve species (Croll *et al.*, 1997; Dickinson *et al.*, 1999; Voronezhskaya *et al.*, 1999). The location of these cells suggests that they also might influence feeding behavior (Baldwin and Newell, 1995). Fretter (1967; 1969; 1972) reported that the region ventral to the mouth, also called the mentum, has stiff cilia with a sensory function in related caenogastropod species. Therefore the catecholamine containing-cells ventral to the mouth in *I. obsoleta* may have a sensory role, perhaps sensing the presence or quality of food particles.

In the present study we also examined cells revealed by antibodies for the enzyme dopamine-beta-hydroxylase (DβH). This enzyme is utilized in the conversion of dopamine to norepinephrine in vertebrates. However, in the present study the immunoreactivity to DβH was found in cells different from those positive for catecholamines. In some invertebrates the antibody for DβH has been suggested to cross react with tyramine-beta-hydroxylase, an enzyme involved in the formation of octopamine (Osborne *et al.*, 1982; Klemm *et al.*, 1985). Furthermore, Crisp et al. (2002) reported that anti-DβH revealed octopamine containing neurons in the leech, *Hirudo medicinalis*. Therefore, it was likely that DβH-LIR cells also contained octopamine. While octopamine has been reported in other molluscs (Elekes *et al.*, 1996; Michel *et al.*, 2000), its presence in *I. obsoleta* must be verified. Regardless, the DβH antibody revealed a new group of peripheral neurons in the foot and axons in the velum in larval stages of *I. obsoleta*. The role of such cells and axons is unknown but based on their locations and roles of octopamine in insects and leeches they may modulate muscle contractions during swimming and later crawling (Hashemzadeh-Gargari and Friesen, 1989; Ramirez and Pearson, 1991; Orchard *et al.*, 1993; Johnston *et al.*, 1999).

The Developing Central Ganglia

In the caenogastropod *I. obsoleta*, the central ganglia include the paired cerebral, pedal, buccal, pleural ganglia and an unpaired visceral and osphradial ganglion. Lin and Leise (1996a; 1996b) described the development of these central ganglia in larval stages

of *I. obsoleta*. They reported that the cerebral, pedal and osphradial ganglia appeared by 6 days post hatching and the other more posterior ganglia, pleural, intestinal and visceral ganglia developed as the larva approached competency. In the present study we have shown additional details of the developing ganglia in *I. obsoleta*. The first cells identified in the developing CNS of *I. obsoleta* were FMRFamide-LIR neurons in the posteriorly located intestinal ganglia. These cells appeared during early veliger stages well before cells in the cerebral, pedal and osphradial ganglion. In other gastropod species similar observations have also been reported. For example, in the gastropods *C. fornicata*, *A. californica* and *L. stagnalis*, some of the earliest cells detected were FMRFamide-LIR neurons in regions that corresponded to the future intestinal, abdominal and parietal ganglia respectively (Croll and Voronezhskaya, 1996a; Dickinson *et al.*, 1999; Dickinson *et al.*, 2000). Therefore, previous suggestions that the ganglia develop in an anterior to posterior sequence may not be entirely correct (see Raven, 1966; Demian and Yousif, 1975; Kandel *et al.*, 1981).

The results of the present study also suggest that the developing ganglia may be integrated with other components of the larval nervous system. Support for this integration is evident in the morphological associations of the developing CNS and transient peripheral neurons in *I. obsoleta*. For example, the apical neuropil lies directly above the cerebral commissure and many varicosities within this neuropil indicate the possible synaptic connections with axons of the cerebral commissure. In addition, transient neurons in the foot projected axons along the cerebro-pedal connectives.

Furthermore the transient posterior most FMRFamide-LIR cell has axons which project anteriorly and meet the visceral-intestinal connectives in early veliger stages.

Metamorphosis and Changes in the Larval Nervous System

Remodeling of nervous systems during metamorphosis has been the focus of numerous studies of other invertebrate and amphibian larva. However, less is known about how nervous systems change during this process in gastropods, especially since many components of larval nervous system had not been previously investigated. Lin and Leise (Lin and Leise, 1996b; Lin and Leise, 1996a) have reported changes in the central nervous system and apical organ during metamorphosis of *I. obsoleta.* We have supplemented this study by observing the fates of specific peripheral cells within the larval nervous system of *I. obsoleta.*

Our results indicate that many elements of the larval nervous system including cells in the foot and velum, as well as in the apical organ seem to disappear after metamorphosis. There has been some debate over the fate of the apical cells. For example, we observed that many apical cells in *I. obsoleta* disappeared as the velum degenerated, as has also been reported previously (Lin and Leise, 1996b; Lin and Leise, 1996a). Leise and coworkers have recently observed evidence of degeneration of the apical cells (personal communication). Similar observations of degeneration (cellular swelling, vacuolization and disruption of membrane integrity) of specific apical cells were also reported in another gastropod species (Marois and Carew, 1997c; Marois and

Carew, 1997a; Marois and Carew, 1997b). However, other more specific techniques to reveal apoptotic cells, such as TUNEL (TdT-mediated dUTP digoxigenin nick end labeling), have not been successful (see appendix). On the other hand, the disappearance of apical cells during metamorphosis may also be due to the loss of transmitter expression (Diefenbach *et al.*, 1998; Kuang and Goldberg, 2001). Therefore, it is possible that some of the apical cells undergo cell death while others are retained and function in the adult nervous system. We also observed that specific cells in the foot disappeared while others were retained during metamorphosis of *I. obsoleta.* For example, the FMRFamide labeled sensory cells were absent while catecholamine containing sensory-like neurons were present during late metamorphosis. These results provide the groundwork for further investigations of how the nervous system functions and is remodeled during metamorphosis.

Conclusion

In conclusion, we have shown that the gastropod *I. obsoleta* has a larval nervous system consisting of an apical organ, numerous peripheral neurons and the developing ganglia. The larval nervous system consists of many different cell types exhibiting immunoreactivity to transmitters such as serotonin, catecholamines, possibly octopamine, enkephalins and FMRFamide. Previous studies have also shown the possible presence of nitric oxide (Lin and Leise, 1996b; Froggett and Leise, 1999; Thavaradhara and Leise, 2001) within the larval nervous system of *I. obsoleta.* It is also likely that at least some

additional elements exist containing other transmitters. Future studies will be necessary to reveal the full extent of the larval nervous system of *I. obsoleta* and other gastropod species. Such morphological information will be useful for future studies of the role of neurons in larval behaviors. For example, axons from the apical neuropil of *I. obsoleta* innervate the velum, foot and posterior regions. Therefore, these cells likely control aspects of locomotion, feeding and escape responses. Finally, this study also lays the groundwork for investigations of the cellular and molecular mechanisms governing neural development in this taxon.

Figure 3. Schematic representations of the general morphology of *I. obsoleta* at the major larval stages. Figures are not to scale. **A)** Trochophore stage, 2.5- 4 days post first cleavage. **B)** Embryonic veliger stage, 4.5- 7 days post first cleavage. **C)** Hatchling stage, 7.5- 10 days post first cleavage. **D)** Free swimming veliger stage, 14-17 days post first cleavage. **E)** Competent stage, 21-25 days post first cleavage.

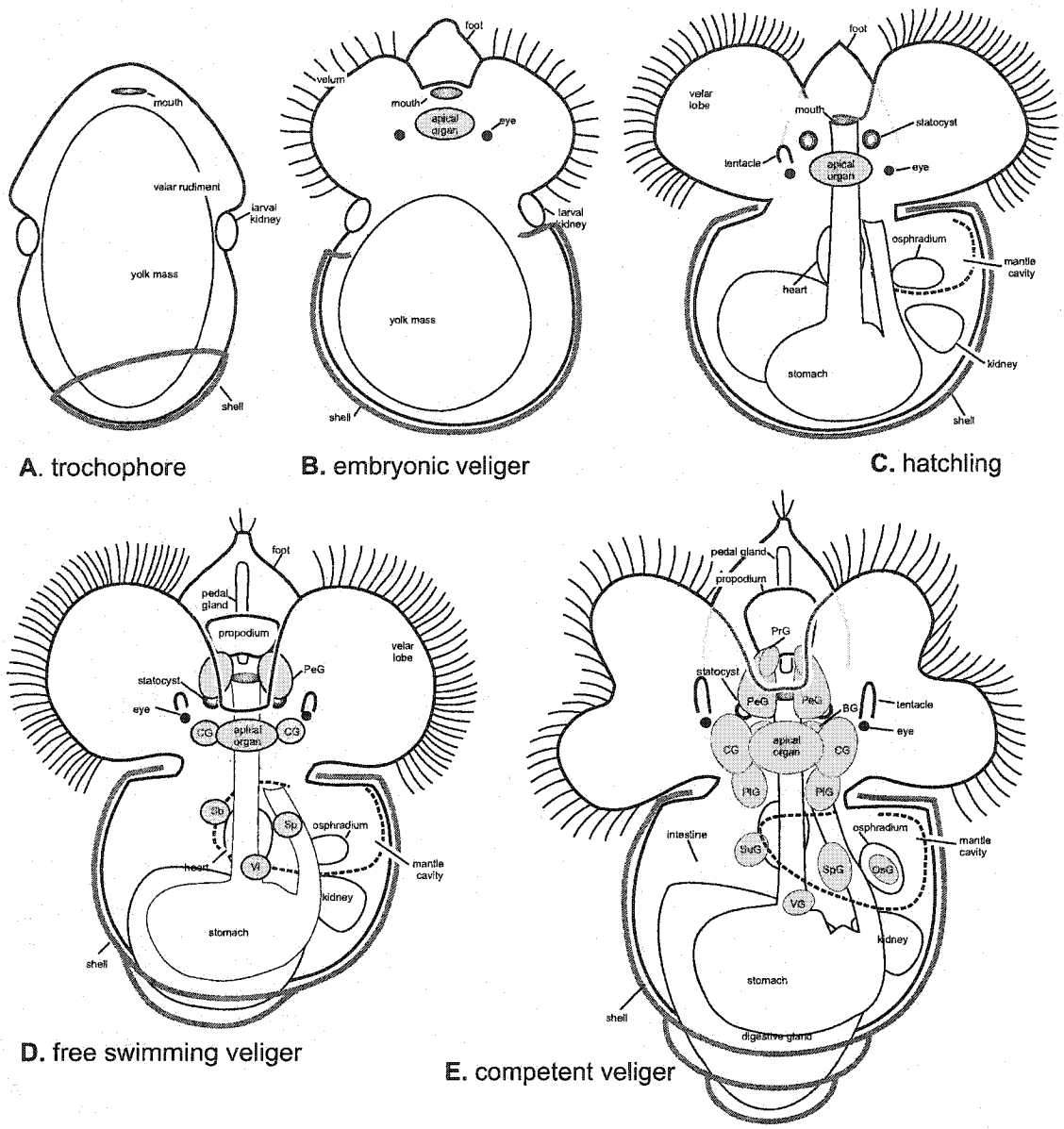

mouth
velar rudiment
larval kidney
yolk mass
shell
A. trochophore
velum
foot
mouth
apical organ
eye
larval kidney
yolk mass
shell
B. embryonic veliger
velar lobe
foot
mouth
statocyst
tentacle
apical organ
eye
osphradium
mantle cavity
heart
stomach
kidney
shell
C. hatchling
foot
pedal gland
propodium
velar lobe
statocyst
PeG
eye
CG
apical organ
CG
Sb
Sp
osphradium
heart
Vt
mantle cavity
kidney
stomach
shell
D. free swimming veliger
pedal gland
propodium
PrG
statocyst
PeG
PeG
BG
tentacle
eye
CG
apical organ
CG
PlG
PlG
intestine
SvG
osphradium
mantle cavity
SpG
OsG
VG
kidney
stomach
shell
digestive gland
E. competent veliger

Figure 3

Figure 4. Schematic representations of embryos of *I. obsoleta.* Anterior is at the top in each figure. **A)** Cells and fibers labeled with antibodies to FMRFamide, serotonin and leu-enkephalin in the trochophore stage. **B)** Cells and fibers labeled with antibodies to FMRFamide, serotonin and leu-enkephalin and positive for catecholamines in the embryonic veliger stage.

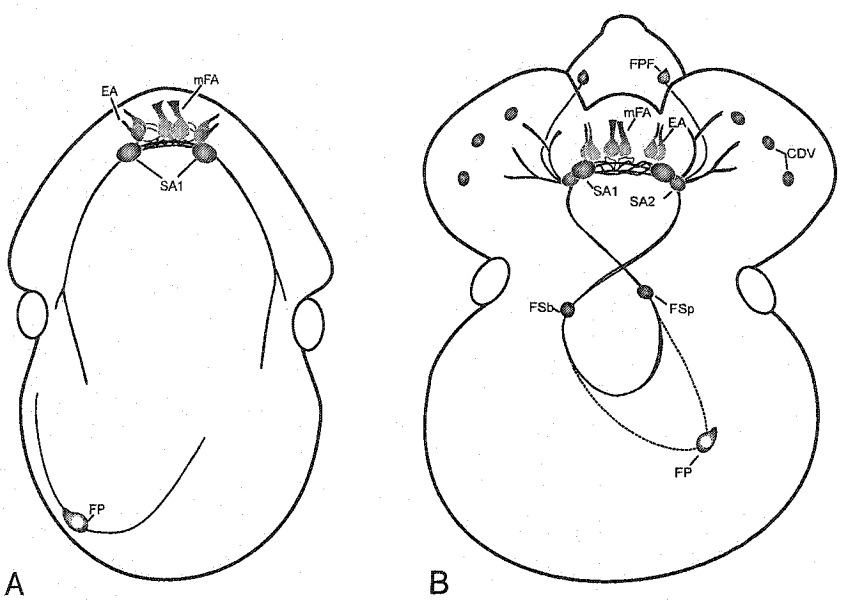


Figure 4

Figure 5. *I. obsoleta* during trochophore stages. Anterior is at the top in each figure. **A)** Dorsal view of a trochophore labeled with anti-FMRFamide, showing the FP cell and the mFA cells. The inset shows the FP cell and anteriorly directed axon. Scale bar is approximately 35 μ. **B)** Dorsal view of the anterior most region of a late trochophore labeled with both anti-FMRFamide and anti-serotonin. Note that the distinct mFA cells are located dorsal to the apical neuropil and the laterally located SA1 cells. Scale bar is approximately 10μm. **C)** Dorsal view of a late trochophore labeled with anti-serotonin, showing the SA1 cells and posteriorly directed axons. Scale bar is approximately 35μm. **D)** Dorsal view of the anterior most region of a late trochophore labeled with anti-serotonin showing the SA1 cells. Scale bar is approximately 10μm. **E)** Dorsal view of a late trochophore labeled with leu-enkephalin, showing the first EA cells in the apical region. Scale bar is approximately 35μm. **F)** Dorsal view of the anterior most region of a late trochophore labeled with anti-leu-enkephalin showing the EA cells and neuropilar fibers. Scale bar is approximately 10μm.

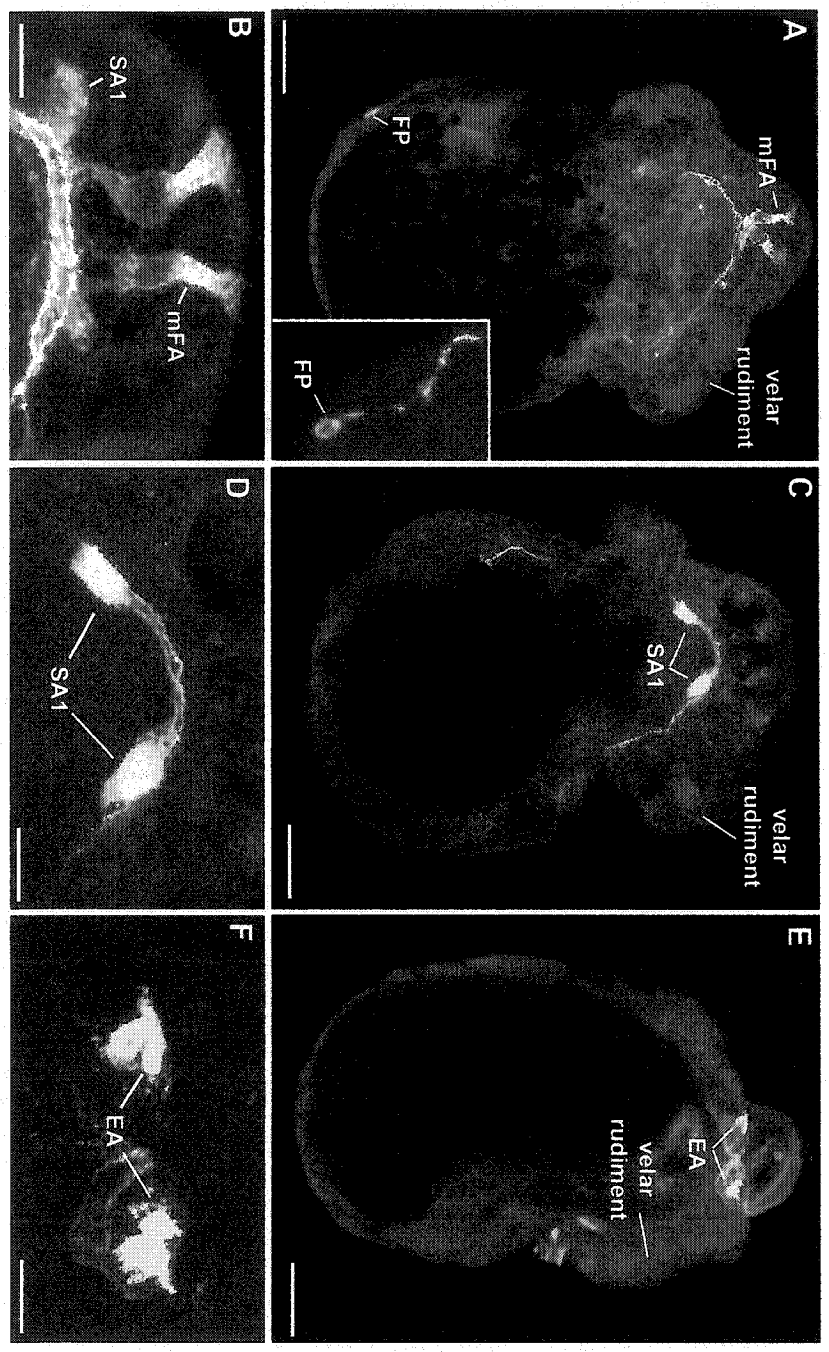

A
mFA
velar rudiment
FP
FP
B
SA1
mFA
C
SA1
velar rudiment
D
SA1
E
EA
velar rudiment
F
EA

Figure 5

Figure 6. *I. obsoleta* during embryonic veliger stages. Anterior is at the top in each figure. **A)** Dorsal right lateral view of an embryonic veliger labeled with anti-FMRFamide. Note the mFA cells in the apical organ, the right FPF cell, the intestinal/visceral connectives forming a figure eight (arrow), the FP cell and the FSb cell. Scale bar is approximately 40μm. **B)** Dorsal view of the anterior most region of an early embryonic veliger labeled with anti-FMRFamide showing the mFA cells and apical neuropil. Scale bar is approximately 10μm. **C)** Ventral view of an early embryonic veliger labeled with anti-serotonin. Note the SA1 and SA2 cells, axons forming the intestinal/visceral connectives (arrow) and axons extending into velar lobes (arrowhead). Scale bar is approximately 40μm. **D)** Dorsal view of the anterior most region of an early embryonic veliger labeled with anti-serotonin showing the SA1 and SA2 cells. Scale bar is approximately 10μm. **E)** Dorsal view of an early embryonic veliger labeled with anti-leu-enkephalin showing the first EA cells in the apical region. Scale bar is approximately 42μm. **F)** Dorsal view of the anterior most region of an embryonic veliger labeled with anti-leu-enkephalin showing the EA cells and neuropilar fibers (arrow). Scale bar is approximately 10μm.

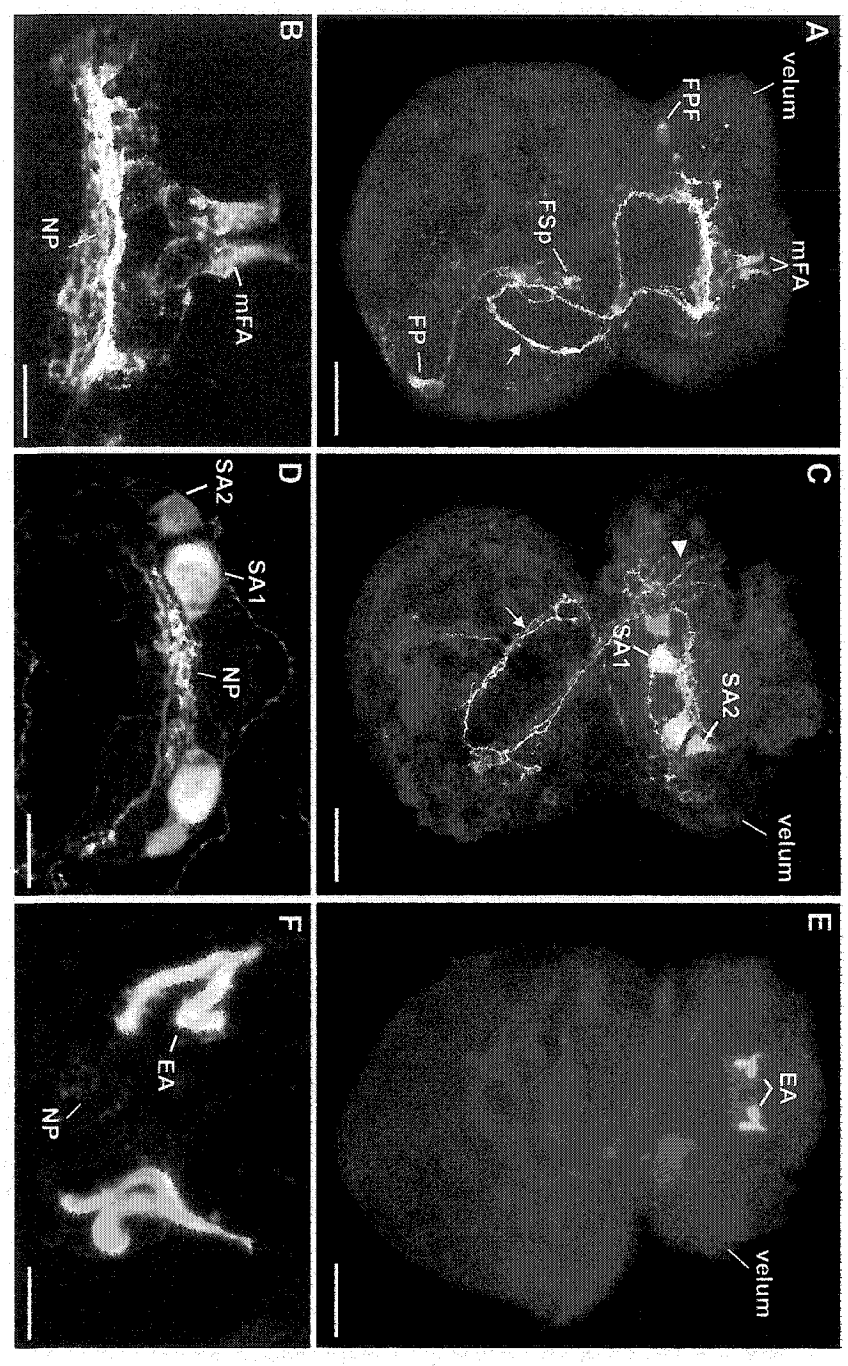

A
velum
FPF
mFA
FSp
FP
B
NP
mFA
C
SA2
SA1
velum
D
SA2
SA1
NP
E
EA
velum
F
EA
NP

Figure 6

Figure 7. *I. obsoleta* during embryonic veliger stages. Anterior is at the top in each figure. **A)** Ventral view of the anterior half of the embryonic veliger showing CDV cells using the Faglu technique. Scale bar is approximately 40μm. **B)** Ventral view of the anterior half of the embryonic veliger showing CV cells labeled with anti-TH. Scale bar is approximately 40μm.

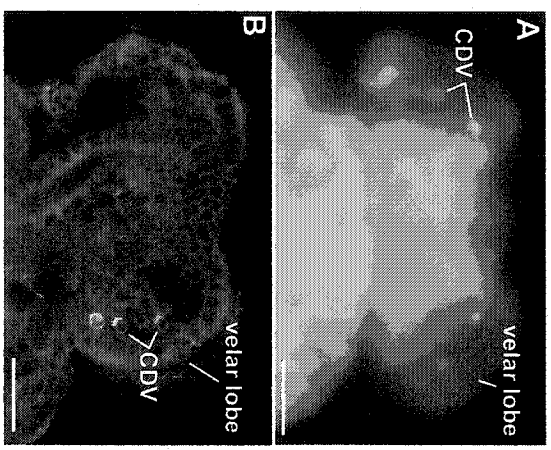


Figure 7

Figure 8. Schematic representations of cells and fibers in hatchling *I. obsoleta.* Anterior is at the top in each figure. **A)** Cells and fibers labeled with antibodies to FMRFamide. **B)** Cells and fibers labeled with antibodies to serotonin. **C)** Cells and fibers labeled with antibodies to leu-enkephalin and DβH. **D)** Cells and fibers containing catecholamines as revealed by labeling with anti-TH and the Faglu technique.

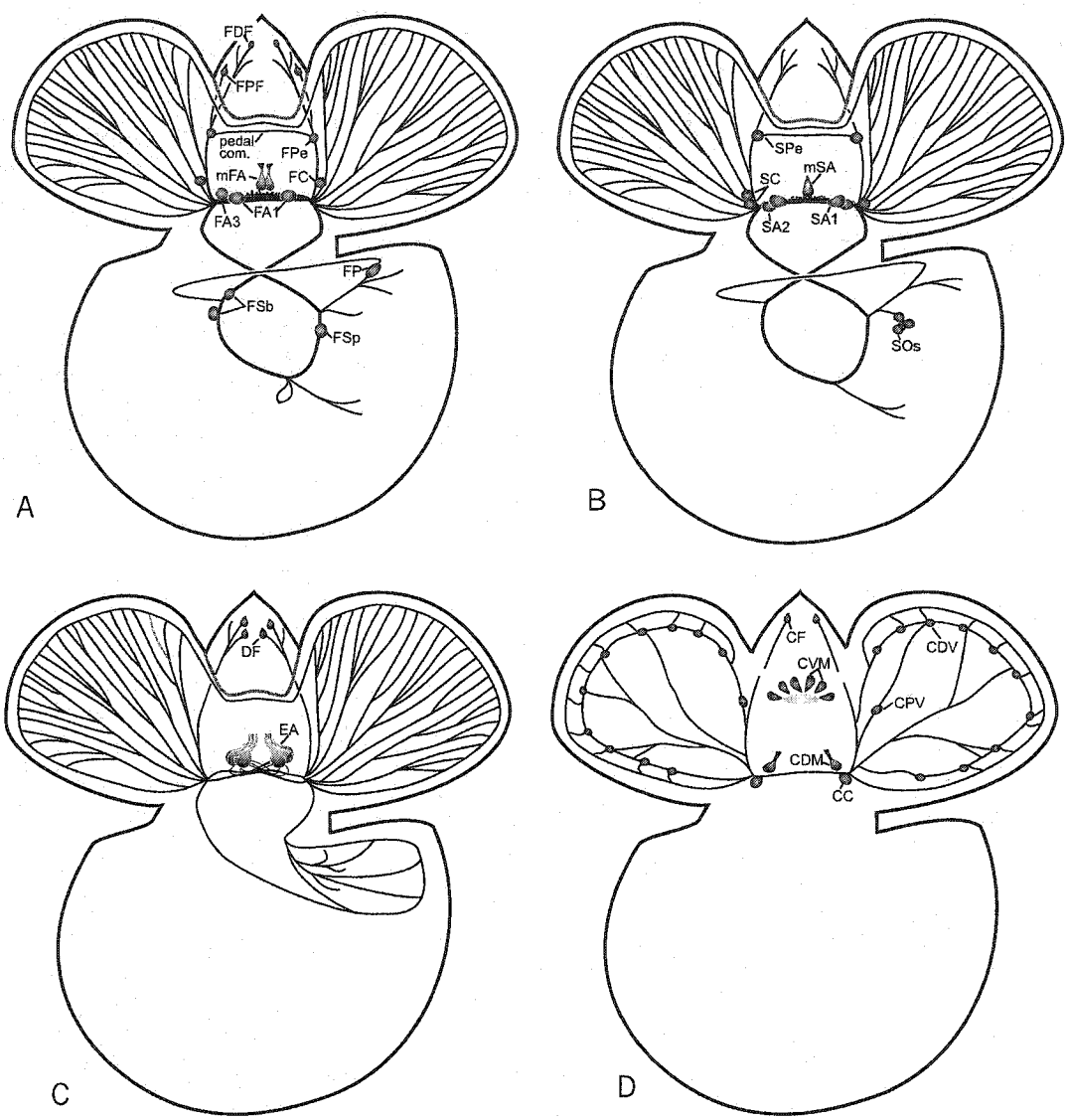

FDF
FPF
pedal com.
FPe
mFA
FC
FA3
FA1
FP
FSb
FSp
A
SPe
mSA
SC
SA2
SA1
SOs
B
DF
EA
C
CF
CVM
CDV
CPV
CDM
CC
D

Figure 8

Figure 9. *I. obsoleta* during hatchling stages. Anterior is at the top in each figure. **A)** Dorsal view, labeled with anti-FMRFamide, showing FC cells in cerebral ganglia, FSp and FSb cells of the intestinal ganglia, FDF and FPF cells in the foot, the intestinal/visceral connectives (arrow), and the FP cell. Scale bar is approximately 55μm. **B)** Dorsal view of the anterior most region labeled with anti-FMRFamide. Note that the mFA cells, FA1 cells and the unpaired FA3 cell in the apical organ. Scale bar is approximately 10μm. **C)** A frontal view, showing cells in the apical organ (FA1, FA3), in the cerebral ganglia (FC), innervation in the velum and foot, including the pedal tracts (arrows). Scale bar is approximately 40μm. **D)** Dorsal view of the whole animal labeled with anti-serotonin showing the cells in the apical organ (SA1 and SA2 cells), axons forming the intestinal/visceral connectives (arrow) and axons extending into velar lobes (arrowhead). Scale bar is approximately 55μm. **E)** Dorsal view of the anterior most region, labeled with anti-serotonin showing cells in the apical organ (mSA, paired SA1, SA2) and in the cerebral ganglia (SC). Scale bar is approximately 10μm. **F)** Frontal view of the foot showing SPe cells, pedal tracts (arrows) and pedal commissure (arrowhead). Scale bar is approximately 20μm. **G)** Dorsal view of the whole animal labeled with anti-leu-enkephalin showing the EA cells in the apical region and innervation of the velum (arrows). Scale bar is approximately 55μm. **H)** Dorsal view of the anterior most region, labeled with anti-leu-enkephalin showing the EA cells. Scale bar is approximately 10μm. **I)** Frontal view of the larva labeled with anti-leu-enkephalin

showing the EA cells in the apical organ, axons in the velum (arrows), foot (arrowhead) and the mantle cavity region. Scale bar is approximately 40μm.

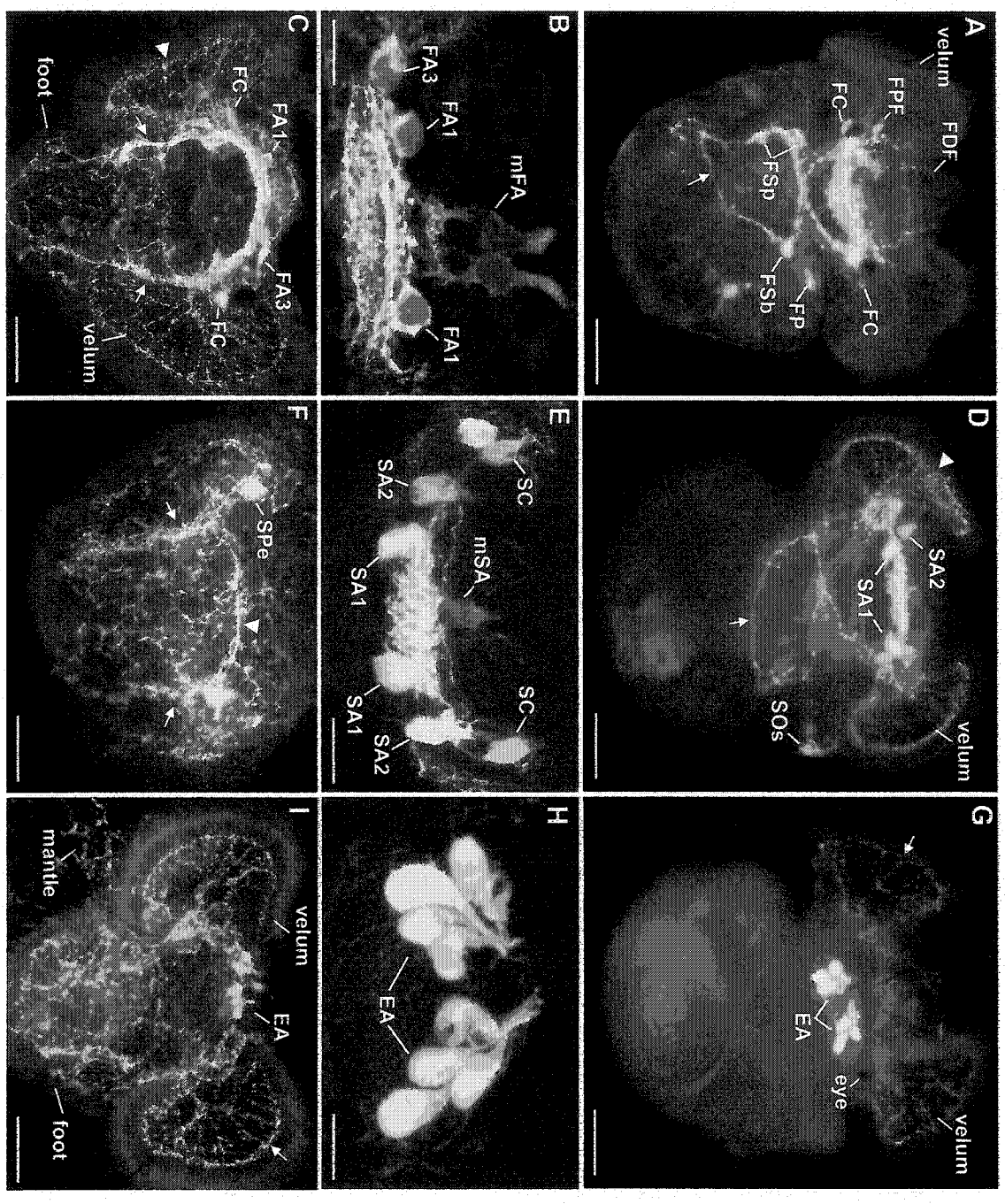

A
velum
FDF
FPF
FC
FSp
FSb
FP
FC
B
mFA
FA3
FA1
FA1
C
FA1
FC
FA3
FC
foot
velum
D
SA2
SA1
velum
SOs
E
SC
mSA
SA2
SA1
SC
SA2
SA1
F
SPe
G
velum
EA
eye
H
EA
I
velum
EA
mantle
foot

Figure 9

Figure 10. *I. obsoleta* during hatchling stages. Anterior is at the top in each figure. **A)** Lateral view of the whole animal showing catecholamine-containing cells using the Faglu technique showing the CDV cells in the velum. Scale bar is approximately 55μm. **B)** Frontal view of a larva showing CVM cells using the Faglu technique. Scale bar is approximately 20μm. **C)** Frontal view of a larva, labeled with anti-TH showing cells in the cerebral ganglia (CC) near the mouth (CDM, CVM), at the tip of the foot (CF) and in the velum (CDV, CPV). Scale bar is approximately 40μm. **D)** Frontal view of the foot labeled with the Faglu technique, showing the CF cells. Scale bar is approximately 25μm. **E)** Lateral view of the velum and foot labeled with anti-DβH, revealing axons (arrows). Scale bar is approximately 35μm. **F)** Frontal view of the foot labeled with anti-DβH showing the DF cells. Scale bar is approximately 25μm.

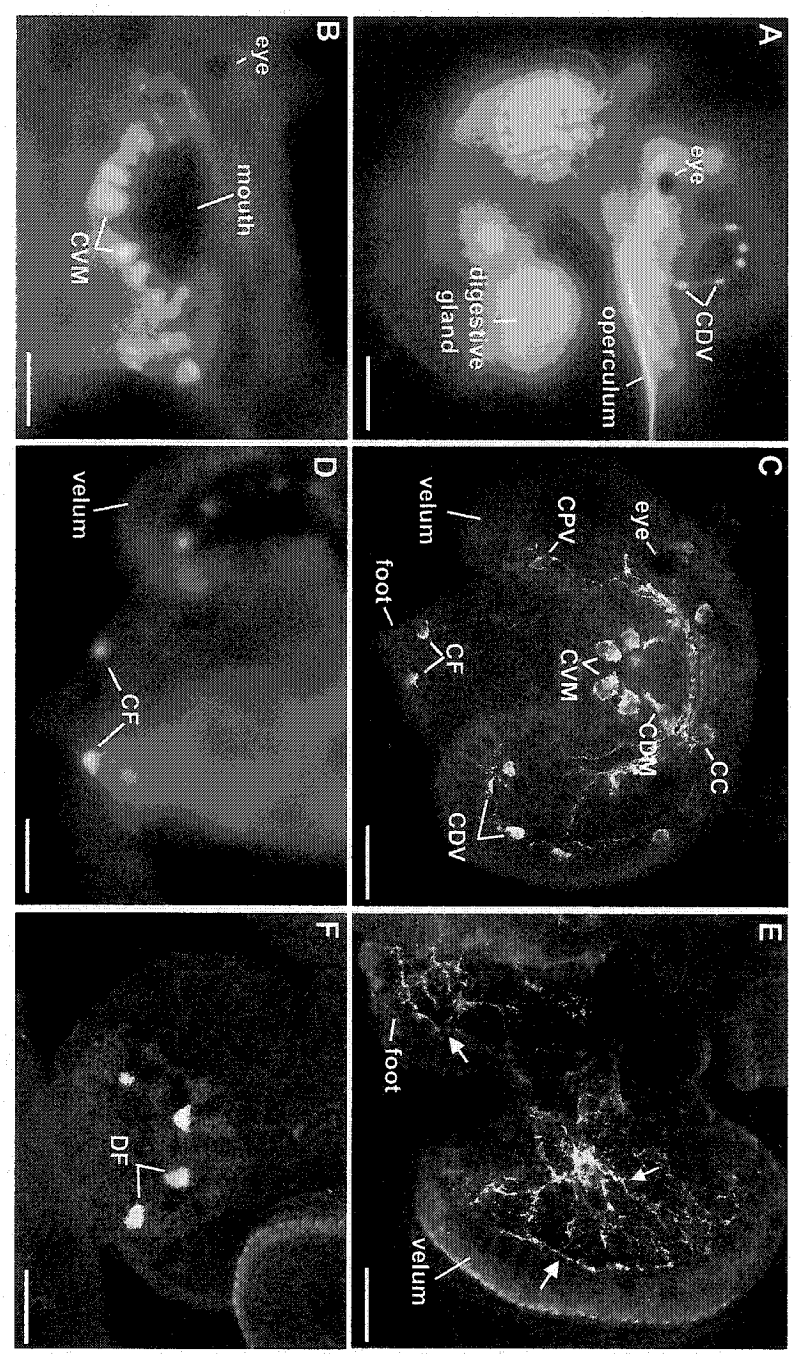
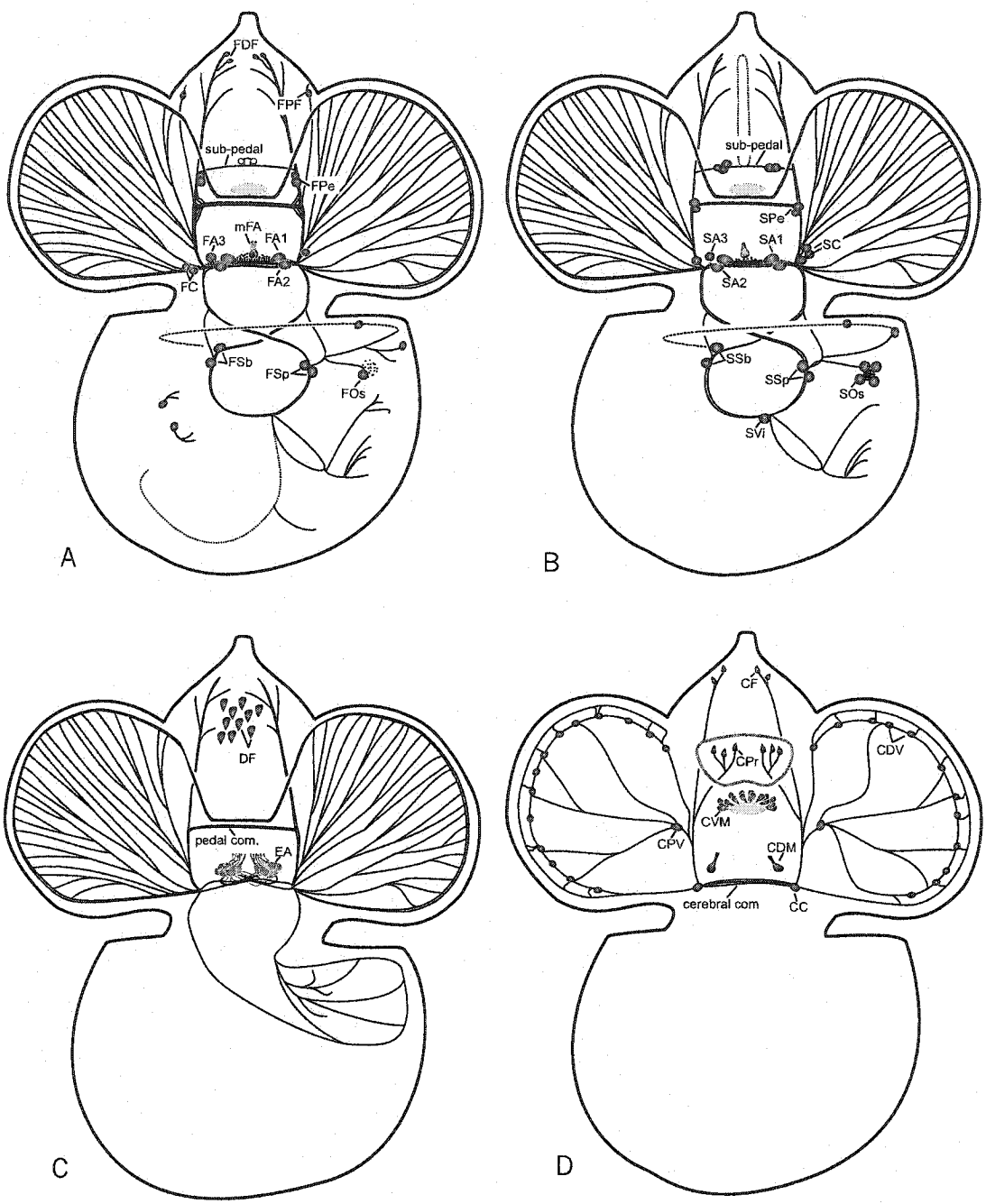

A
eye
CDV
operculum
digestive gland
B
eye
mouth
CVM
C
eye
CC
CDM
CPV
CVM
velum
foot
CF
CDV
D
velum
CF
E
foot
velum
F
DF

Figure 10

Figure 11. Schematic representations of cells and fibers in the free swimming veliger stage of *I. obsoleta.* Anterior is at the top in each figure. **A)** Cells and fibers labeled with antibodies to FMRFamide. **B)** Cells and fibers labeled with antibodies to serotonin. **C)** Cells and fibers labeled with antibodies to leu-enkephalin and DβH. **D)** Cells and fibers labeled with antibodies to TH and the Faglu technique.


FDF
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FPe
mFA
FA3
FA1
FC
FA2
FSb
FSp
FOs
A
sub-pedal
SPe
SA3
SA1
SC
SA2
SSb
SSp
SOs
SVi
B
DF
pedal com.
EA
C
CF
CPr
CDV
CVM
CPV
CDM
cerebral com
CC
D

Figure 11

Figure 12. *I. obsoleta* during free swimming veliger stage, 14-17 days post first cleavage. Anterior is at the top in each figure. **A)** FMRFamide-LIR axons in the tentacle. Scale bar is approximately 25μm. **B)** Flask shaped medial cell dorsal to the apical neuropil, labeled with anti-FMRFamide. Scale bar is approximately 20μm. **C)** Dorsal view of the apical organ, developing ganglia and the connectives of the future adult central nervous system labeled with anti-FMRFamide. Note the apical cells (paired FA1, FA2, and unpaired FA3), cells in the cerebral (FC) and intestinal (FSp, FSb) ganglia and pleural-intestinal connectives (arrows). Scale bar is approximately 40μm. **D)** Frontal view of the foot labeled with anti-FMRFamide showing the FPF (magnified 2X in the inset) and FDF cells, the pedal tracts, pedal commissure (arrowhead), cells in the pedal ganglia (FPe), the sub-pedal commissure and medial cell like structures (arrow) and medial axon (small arrow). **E)** Frontal view of the apical organ labeled with anti-serotonin, showing cells in the apical organ (SA1, SA2, SA3) and in the cerebral ganglia (SC). Scale bar is approximately 30μm. **F)** Dorsal view of the developing ganglia and connectives of the developing central nervous system labeled with anti-serotonin not including the apical organ. Note the cells in the pedal (SPe), cerebral (SC), intestinal (SSp, SSb), visceral (SVi) and osphradial ganglion (SOs) (shown in the inset are the cells and neuropil of the osphradial ganglion, magnified 2X). Scale bar is approximately 50μm. **G)** Frontal view of the foot labeled with anti-serotonin showing cells in the pedal ganglia (SPe), the pedal commissure (arrowhead) and the pedal tracts (arrows). Scale bar is approximately 45μm. **H)** Lateral view of FMRFamide-LIR axons (arrow) within the

right velar lobe. This pattern of innervation in the velum is also similar to larvae labeled with anti-serotonin and anti-leu-enkephalin. Scale bar is approximately 45μm. **I)** Dorsal view of the anterior half of the larvae showing the position of the leu-enkephalin labeled cells (EA). Scale bar is approximately 70μm. **J)** High magnification of the EA cells labeled with anti-enkephalin. Scale bar is approximately 20μm. **K)** High magnification of the apical cells labeled with both anti-FMRFamide and anti-serotonin. Scale bar is approximately 20μm. **L)** Apical cells (EA cells) labeled with anti-leu-enkephalin **M)** The same cells as shown in L labeled with anti-alpha-tubulin. Scale bars in L and M are approximately is approximately

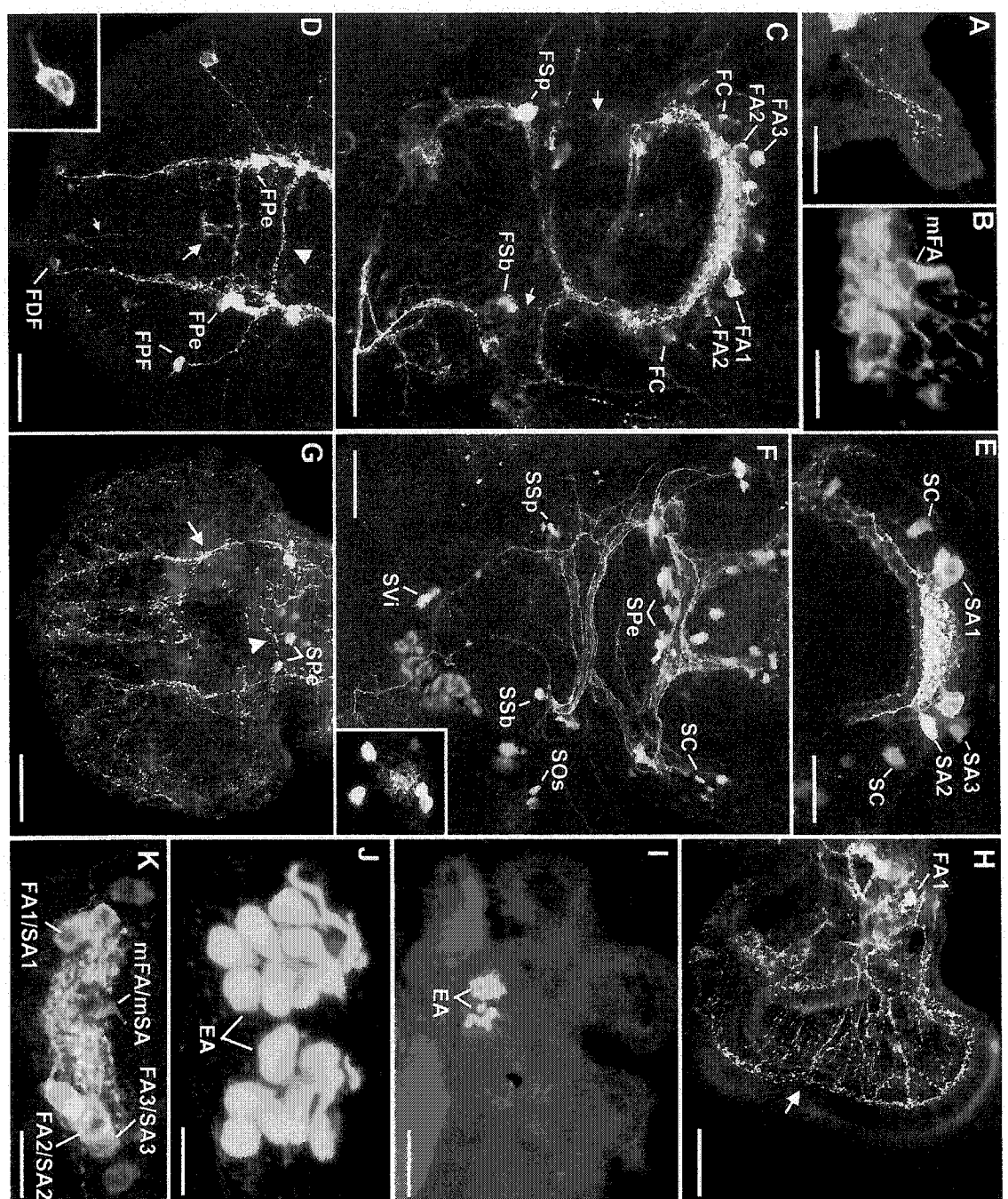

A
B
mFA
C
FA3
FA2
FC
FSp
FSb
FA1
FA2
FC
D
FPe
FPe
FPF
FDF
E
SC
SA1
SA3
SA2
SC
F
SSp
SVi
SPe
SSb
SC
SOs
G
SPe
H
FA1
I
EA
J
EA
K
mFA/mSA
FA3/SA3
FA1/SA1
FA2/SA2

Figure 12a

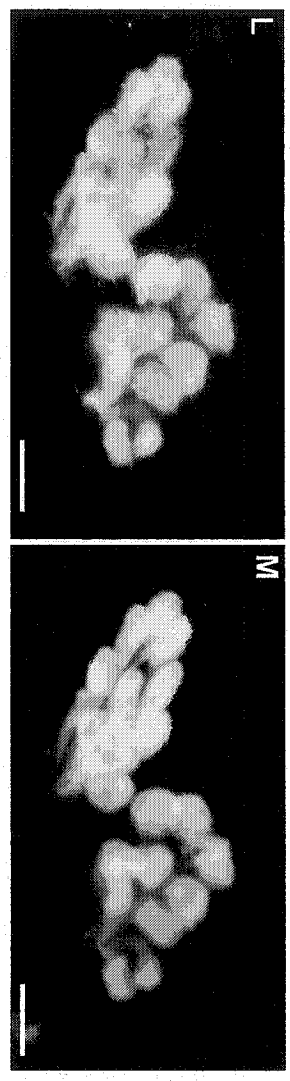


Figure 12b

Figure 13. *I. obsoleta* during free swimming veliger stage,14-17 days post first cleavage. Anterior is at the top in each figure. **A)** Frontal view labeled with Faglu technique showing the CVM cells in the ventral portion of the mouth. Scale bar is approximately 50μm. **B)** Frontal view of the foot labeled with anti-DβH. Note that the DF cells, asterisks: location of the CF cells in the same animal. Scale bar is approximately 25μm. **C)** Frontal view of the foot labeled with anti-TH showing the CF cells and asterisks represent the location of the DF cells in the same animal. Scale bar is approximately 25μm. **D)** Lateral view of the right velum labeled with anti-TH. Axons (arrow) radiate to the CDV cells. Additional axons are also located at the base of the large ciliated epithelial cells (arrowhead). Scale bar is approximately 40μm. **E)** Lateral view of the right velum and the foot labeled with anti-DβH. Axons (arrow) radiate throughout velum. Scale bar is approximately 40μm.

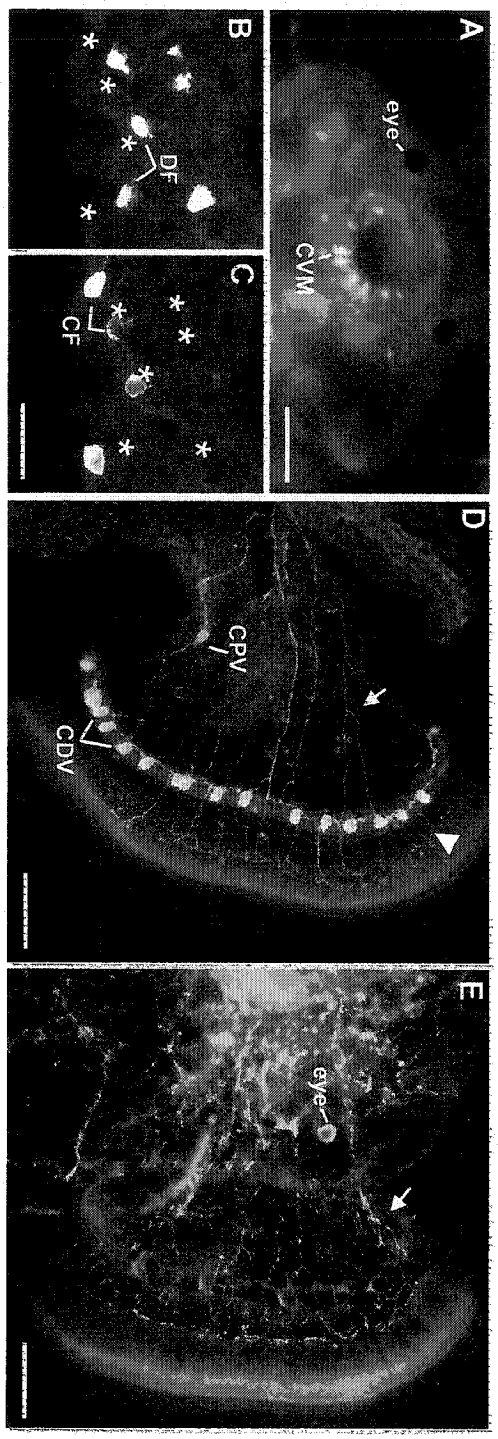


Figure 13

Figure 14. Schematic representations of cells and fibers in competent veligers of *I. obsoleta.* Anterior is at the top in each figure. **A)** Cells and fibers labeled with antibodies to FMRFamide. **B)** Cells and fibers labeled with antibodies to serotonin. **C)** Cells and fibers labeled with antibodies to leu-enkephalin and DβH. **D)** Cells and fibers labeled with antibodies to TH and the Faglu technique.

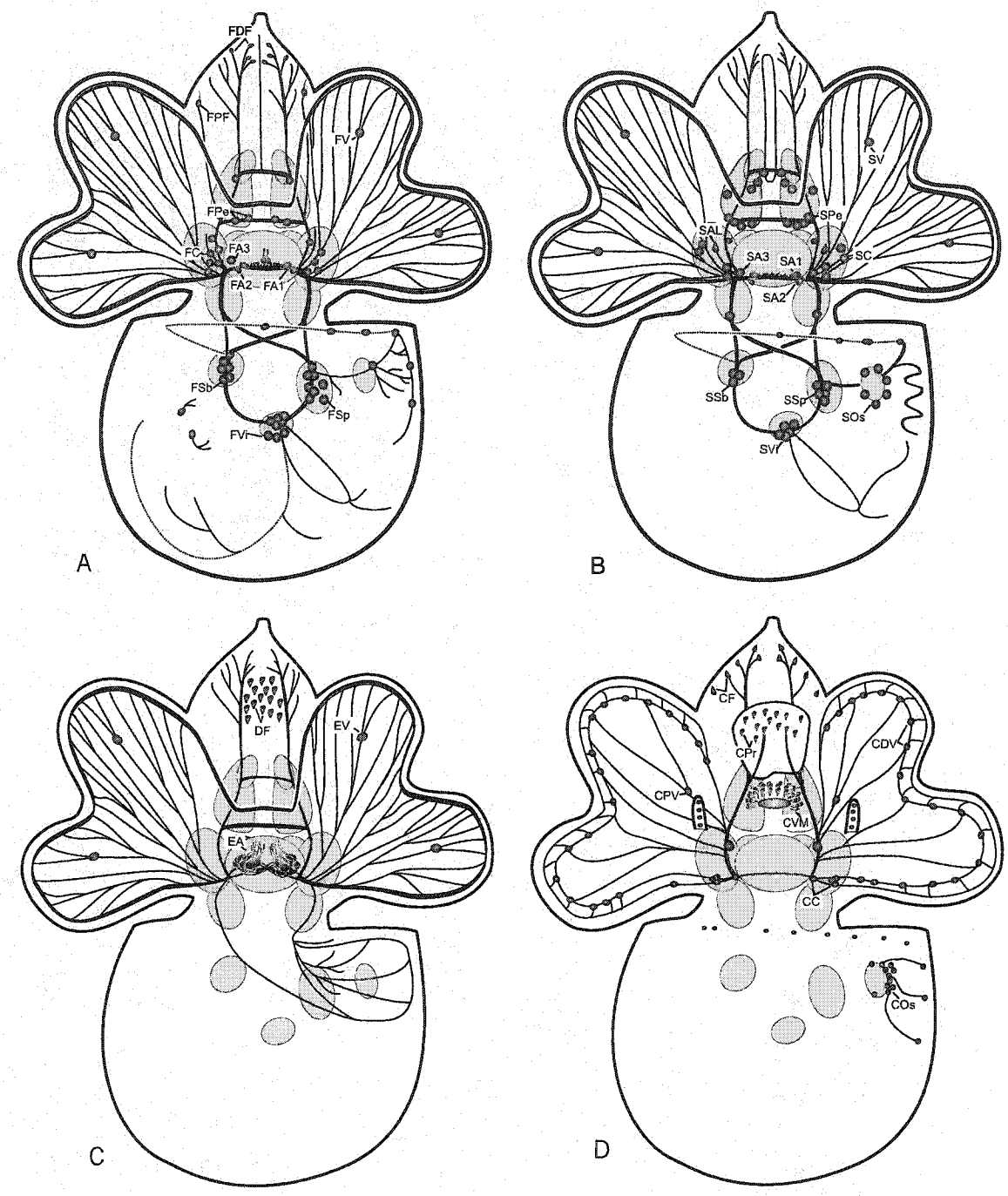

FDF
FPF
FV
FPe
FC
FA3
FA2
FA1
FSb
FSp
FVr
A
SV
SPe
SAL
SA3
SA1
SC
SA2
SSb
SSp
SOs
SVr
B
DF
EV
EA
C
CF
CPr
CDV
CPV
CVM
CC
COs
D

Figure 14

Figure 15. *I. obsoleta* during competent stages. Anterior is at the top in each figure, unless otherwise indicated. **A)** Frontal view labeled with anti-FMRFamide showing cells in the apical organ (FA1, FA2) and pedal ganglia (FPe), and also the neuropils of the cerebral ganglia (CG) and pedal ganglia (PG). Scale bar is approximately 40μm. **B)** Frontal view of the neuropil of the right pedal ganglia (PG), cells in the pedal ganglia (FPe) and the FPF cell. The inset shows a FPF cell magnified 2X. Scale bar is approximately 35μm. **C)** FMRFamide-LIR cells (arrow) along the outer wall of the mantle cavity. Scale bar is approximately 25μm. **D)** Magnified edge of a velar lobe labeled with FMRFamide showing axons (arrow) and velar cell (FV). Scale bar is approximately 15μm. **E)** Frontal view of the apical organ and associated cells labeled with anti-serotonin. Cells within the apical organ (SA1s, SA2s, SA3) and the left dorsal laterally located SAL cell with axon projecting into the apical organ. The inset shows the left SAL and dendritic projection terminating near cells (SC) at the base of the velum and outer edge of cerebral ganglion. Scale bar is approximately 40μm. **F)** Frontal view of the foot labeled with anti-serotonin showing numerous cells within pedal ganglia (SPe) and pattern of innervation in mesopodium and propodium. Scale bar is approximately 60μm. **G)** Dorsal view of the osphradial ganglion and supraintestinal ganglion labeled with anti-serotonin showing the SOs and SSp cells respectively. Anterior is to the right in the figure. Scale bar is approximately 60μm. **H)** Edge of a velar lobe labeled with anti-serotonin, shows a SV cell and pattern of innervation. Scale bar is approximately 35μm. **I)** Lateral view labeled with anti-serotoninshowing some cells in the apical organ (SA1),

right cerebral (SC), subintestinal (SSb), visceral (SVi), supraintestinal (SSp) and osphradial (SOs) ganglion. Also shows innervation along edge of mantle cavity (arrows), and siphon (arrowhead). Scale bar is approximately 70μm. **J)** Magnified view of the EA cells labeled with anti-leu-enkephalin. Scale bar is approximately 15μm. **K)** Edge of a velar lobe labeled with anti-leu-enkephalin, shows an EV cell body and pattern of innervation. Scale bar is approximately 25μm.

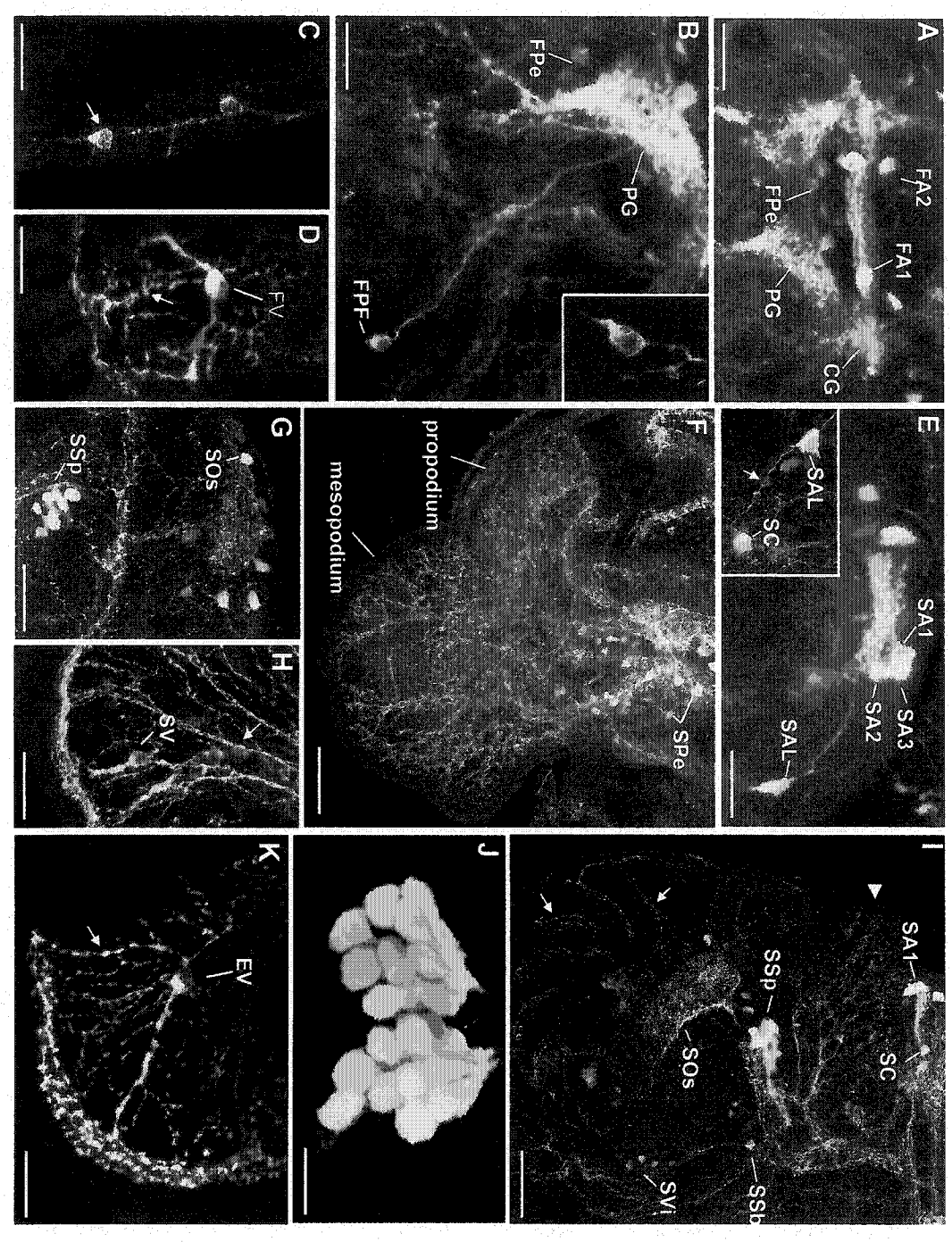

A
FA2
FA1
FPe
PG
CG
B
FPe
PG
FPF
C
D
FV
E
SA1
SA3
SA2
SAL
SAL
SC
F
propodium
mesopodium
SPe
G
SOs
SSp
H
sv
I
SA1
SC
SSp
SOs
SVi
SSb
J
K
EV

Figure 15

Figure 16. *I. obsoleta* during competent stages. Anterior is at the top in each figure. **A)** Frontal view, labeled with the Faglu technique showing the neuropil of the cerebral ganglia (CG), CC cells on either side of the cerebral commissure (arrow) and on the lateral edges of the ganglia. Scale bar is approximately 40μm. **B)** Frontal view, labeled with the Faglu technique showing many CVM cells in the mentum and the neuropils of the cerebral (CG) and pedal ganglia (PG). Scale bar is approximately 50μm. **C)** Tentacle labeled with the Faglu technique, shows several small cells (arrows). Scale bar is approximately 25μm. **D)** TH labeled cells (COs) and axons within the osphradial ganglion and along the outer edge of the mantle cavity (arrows). Scale bar is approximately 55μm. **E)** A portion of the velar lobe labeled with the Faglu technique showing the CDV cells and an axon (arrow) along the base of the large ciliated epithelial cells. Scale bar is approximately 30μm. **F)** Lateral view of the propodium labeled with anti-THshowing cells in the in the propodium (CPr). Scale bar is approximately 40μm. **G)** Frontal view of the mesopodium labeled with anti-TH showing CF cells at the tip and along the edge. Scale bar is approximately 45μm. **H)** Frontal view of the mesopodium labeled with anti-DβH, showing DF cells. Scale bar is approximately 40μm.

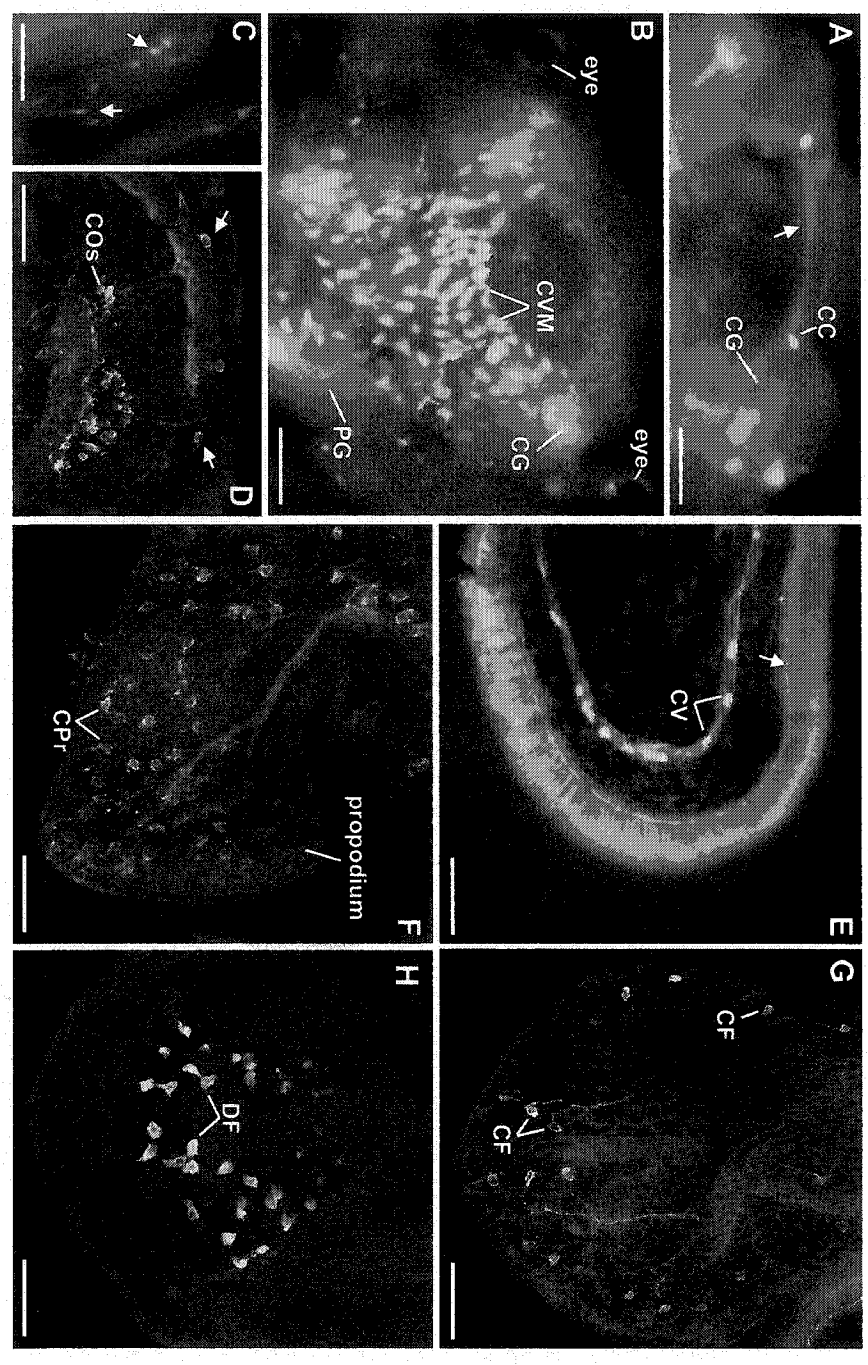


Figure 16

Figure 17. *I. obsoleta* during metamorphosis. **A)** Lateral view of anterior region labeled with anti-FMRFamide showing punctate labeling in the degenerating velum and the right FA1 cell (inset shows a FA1 cell magnified by 2X). Scale bar is approximately 40μm. **B)** Frontal view of the mesopodium labeled with anti-FMRFamide showing the pedal gland cells (pgl) along the edge (inset shows a pgl cell magnified 2X). Scale bar is approximately 30μm. **C)** Lateral view of the anterior region labeled with anti-leu-enkephalin showing labeling in the degenerating velum and the EA cells. Scale bar is approximately 45μm. **D)** Magnified dorsal view of the EA cells labeled with leu-enkephalin showing the uneven borders of the cells. Scale bar is approximately 25μm. **E)** Magnified dorsal view of the EA cells labeled with leu-enkephalin showing few cells still present. Scale bar is approximately 25μm. **F)** Frontal view of the anterior region labeled with anti-serotonin showing apical cells (SA1, SA2, SA3, SAL) and labeling in the degenerating velum. Scale bar is approximately 40μm. **G)** Lateral view of the anterior region labeled with anti-TH showing cells (arrow) in the degenerating velar lobe 15-24 hours post induction. Scale bar is approximately 40μm. **H)** Lateral view of the propodium also 15-24 hours post induction, revealing labeling (arrow). Scale bar is approximately 35μm.

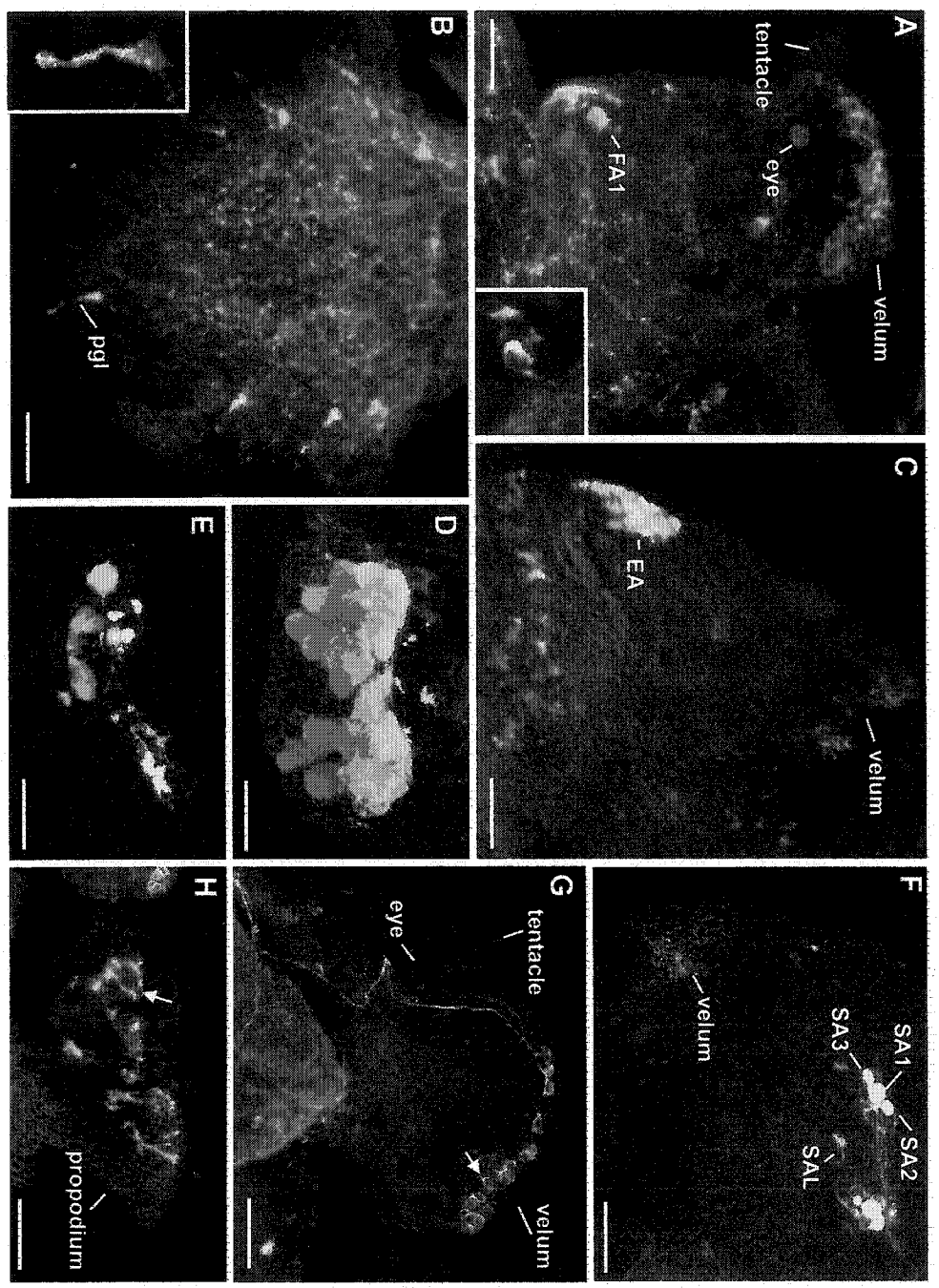

A
velum
tentacle
eye
FA1
B
pgl
C
velum
EA
D
E
F
SA1
SA2
SA3
SAL
velum
G
tentacle
eye
velum
H
propodium

Figure 17

Figure 18. Time line of the major cells and/or groups of cells revealed by antibodies to FMRFamide, serotonin, leu-enkephalin, DβH and TH as well as the Faglu technique. The cell phenotypes are shown with different fills of the boxes. The time of appearance is along the top and the locations are along the left side. Numbers of cells are not represented. Cell abbreviations are: Serotonin-LIR Apical (SA), FMRFamide-LIR Apical (FA), medial Serotonin-LIR Apical (mSA), medial FMRFamide-LIR Apical (mFA), leu-Enkephalin-LIR Apical (EA), Serotonin-LIR Apical Lateral (SAL), FMRFamide-LIR Posterior (FP), Catecholamine Distal Velar (CDV), Catecholamine Proximal Velum (CPV), Catecholamine Cerebral (CC), Serotonin-LIR Velar (SV), FMRFamide-LIR Velar (FV), leu-Enkephalin-LIR Velum (EV), FMRFamide-LIR Proximal Foot (FPF), FMRFamide-LIR Distal Foot (FDF), Catecholamine Foot (CF), Dopamine-beta-hydroxylase-LIR Foot (DF), Catecholamine Propodium (CPr), Catecholamine Ventral Mouth (CVM), Catecholamine Dorsal Mouth (CDM), FMRFamide-LIR Supra-intestinal (FSp), Serotonin-LIR Supra-intestinal (SSp), FMRFamide-LIR Sub-intestinal (FSb), Serotonin-LIR Sub-intestinal (SSb), Serotonin-LIR Cerebral (SC), FMRFamide-LIR Cerebral (FC), Serotonin-LIR Pedal (SPe), FMRFamide-LIR Pedal (FPe), Serotonin-LIR Osphradial (SOs), FMRFamide-LIR Osphradial (FOs), Catecholamine Osphradial (COs), Serotonin-LIR Pleural (SPl), FMRFamide-LIR Pleural (FPl).

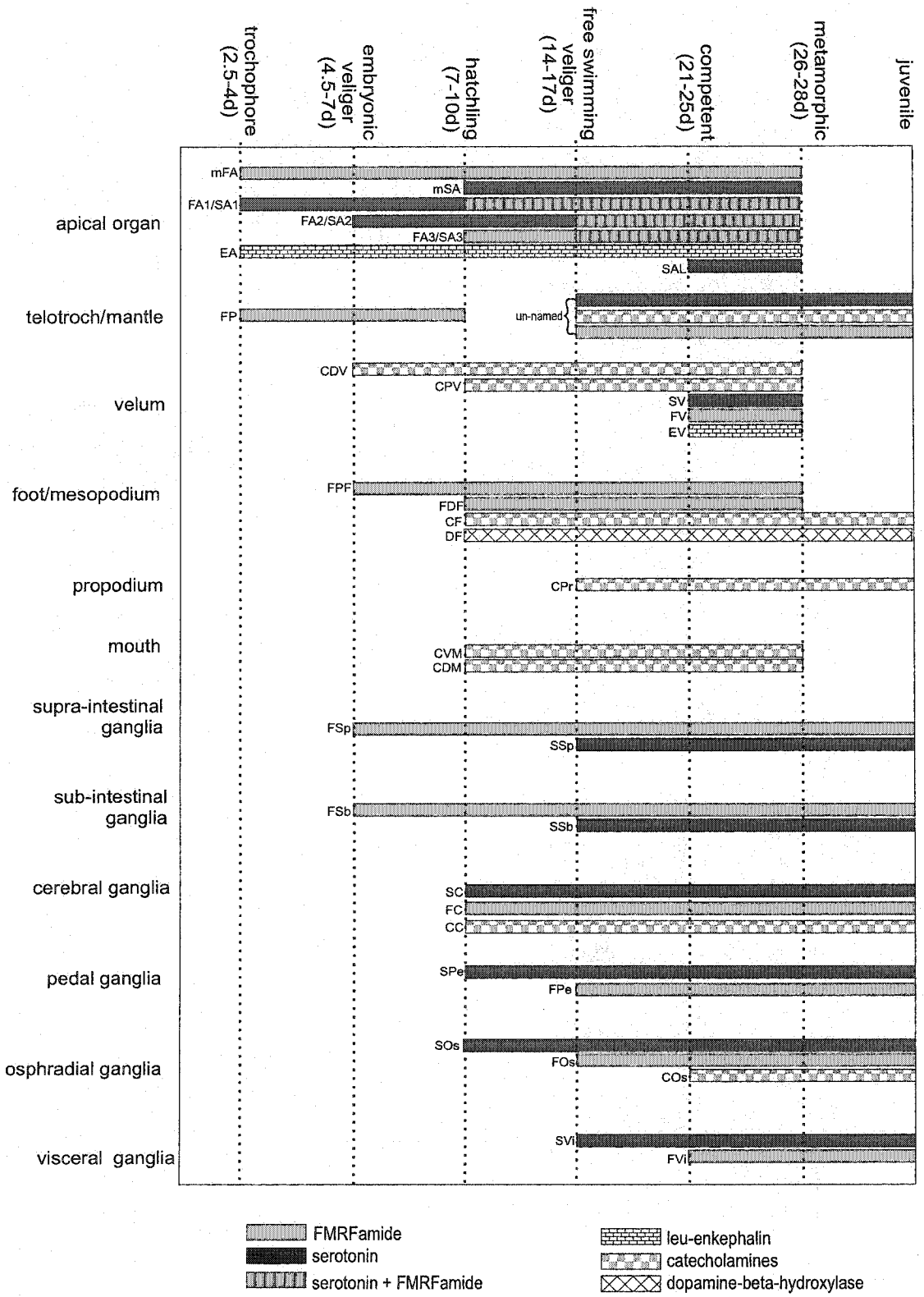

trochophore (2.5-4d)
embryonic veliger (4.5-7d)
hatchling (7-10d)
free swimming veliger (14-17d)
competent (21-25d)
metamorphic (26-28d)
juvenile
apical organ
telotroch/mantle
velum
foot/mesopodium
propodium
mouth
supra-intestinal ganglia
sub-intestinal ganglia
cerebral ganglia
pedal ganglia
osphradial ganglia
visceral ganglia
mFA
mSA
FA1/SA1
FA2/SA2
FA3/SA3
EA
SAL
FP
un-named
CDV
CPV
SV
FV
EV
FPF
FDF
CF
DF
CPr
CVM
CDM
FSp
SSp
FSb
SSb
SC
FC
CC
SPe
FPe
SOs
FOs
COs
SVi
FVi
FMRFamide
serotonin
serotonin + FMRFamide
leu-enkephalin
catecholamines
dopamine-beta-hydroxylase

Figure 18

CHAPTER 3

ROLES OF THE LARVAL NERVOUS SYSTEM IN REGULATING SWIMMING AND FEEDING BEHAVIORS OF THE GASTROPOD *ILYANASSA OBSOLETA*

Introduction

Planktotrophic molluscan larvae depend on their ability to swim through the water column to feed and find appropriate locations for settlement and metamorphosis. The swimming patterns, responses to food and light and escape behaviors have been relatively well studied in several molluscan species (for review see Chia and Buckland-Nicks, 1984). While anatomical studies have revealed that free swimming gastropod larvae have relatively extensive nervous systems (for examples see Lin and Leise, 1996a, Pires *et al.* 2000b) the neural control of behaviors in these larvae remains unknown. The present study, therefore, investigated the neural control of swimming and feeding behaviors in a gastropod by examining the morphology and transmitter contents within neural elements associated with locomotory and feeding structures. Furthermore, the effects of synthetic analogs of such transmitters on known behaviors of whole larvae and on ciliated cells were investigated.

Many behaviors of molluscan larvae depend on the structure and functioning of the swimming organ, the velum (for reviews see Strathmann and Leise, 1979; Chia and Buckland-Nicks, 1984). In gastropods, cilia are arranged in two bands on the edge of each velar lobe, enclosing the food groove (see Fig. 3A). The pre-oral band of cilia originates from large epithelial cells located on the outer edge of the velum. The post-oral band is located on a ridge on the ventral side of the velum and consists of shorter cilia that beat towards the pre-oral band. The combined effort of these two ciliary bands

collects suspended particles in the food groove and then transports them to the mouth. The long compound cilia of the pre-oral band are also largely responsible for locomotion.

Previous evidence that the nervous system controls aspects of swimming and feeding in gastropod larvae is based in part on morphological studies. For example, peripheral axons and neurons have been reported to be associated with the pre-oral ciliated cells, post-oral ciliary band and velar musculature. Neuronal processes have long been observed to ramify across the velum toward the pre-oral cells (Carter, 1926; Carter, 1928; Mackie *et al.* 1976). Axons radiating throughout the velum have also been reported in various molluscan groups such as the caenogastropods (Dickinson *et al.*, 1999; Dickinson *et al.*, 2000; Page and Parries, 2000), heterobranchs (Kempf *et al.*, 1997; Marois and Carew, 1997c; Marois and Carew, 1997a; Marois and Carew, 1997b; Page and Parries, 2000) and bivalves (Croll *et al.*, 1997; J. T. Plummer, unpublished data). In most species examined, such axons are immunoreactive to serotonin and/or FMRFamide. Furthermore, Marois and Carew (1997c; 1997a; 1997b) presented ultrastructural evidence that serotonin-LIR projections from the apical organ directly contacted the pre-oral, ciliated cells in the heterobranch *Aplysia californica.* It has also been reported that axons within the velum were closely associated with velar muscle fibers in various gastropods suggesting possible control of velar retraction (Kempf *et al.*, 1997; Page and Parries, 2000). In some species of gastropods several catecholaminergic cells, interconnected with axons, have been observed along the post-oral ciliary band of the velum (Dickinson *et al.*, 1999). Similar catecholaminergic cells have been observed in

the velum of various bivalves species, however, their association with specific ciliary bands was not clear (Croll *et al.*, 1997; Plummer, 2002). Together these studies provide significant morphological evidence that neural elements exist in the velum of molluscan larvae and may be associated with ciliary bands and muscles controlling swimming and feeding behaviors. In addition, in many species of molluscan larvae, putative neurons have been observed in regions near the mouth and pharynx. In several gastropods, (Dickinson *et al.*, 1999; Voronezhskaya *et al.*, 1999; Dickinson *et al.*, 2000; Pires *et al.*, 2000b) and bivalves (Croll *et al.*, 1997; Plummer, 2002), vase-shaped cells surround the mouth during larval stages. Such cells are often observed to lie directly ventral and lateral to the mouth and to contain catecholamines.

While anatomical studies have demonstrated that neural elements in the velum and near the mouth contain transmitters which include serotonin and catecholamines, other research indicates that such substances have a physiological role in locomotion and feeding in larval stages of various molluscs. For example, Beiras and Widdows (1995) have shown that administration of exogenous serotonin, dopamine and norepinephrine affected the larval swimming and feeding behaviors of the bivalve *Mytilus edulis*. Increased swimming activity and feeding rates were reported when these bivalve larvae were exposed to serotonin. In contrast, exposure to catecholamines had the opposite effects. In addition, Goldberg and coworkers have performed several experiments to demonstrate the cilio-excitatory role of serotonin in embryonic stages of the gastropod

Helisoma trivolvis (Goldberg and Kater, 1989; Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994).

Evidence that the nervous system controls aspects of swimming in gastropod larvae is also based on several physiological investigations of the large, pre-oral, ciliated cells, which appear capable of modulating ciliary beat frequency and exhibit complete arrests of ciliary beating (for review see Chia and Buckland-Nicks, 1984). These ciliary arrests have been correlated with action potentials recorded from the pre-oral cells in gastropods (Mackie *et al.*, 1976; Arkett *et al.*, 1987; Arkett, 1988). Intracellular recordings from the pre-oral ciliated cells showed that such action potentials were mediated by calcium and were elicited by summing excitatory post synaptic potentials (EPSPs), thus indicating the presence of neuronal input (Arkett *et al.*, 1987; Arkett, 1988). While there is considerable information about the neural control of the pre-oral ciliated cells, very little is known about the control of the post-oral ciliary band or effectors of the velum.

In the present study, we use a comprehensive approach to determine possible roles of the nervous system in controlling swimming and feeding behavior in the caenogastropod *Ilyanassa obsoleta*. Initially, using immunohistological methods, the location and types of transmitters within neural elements in the velum were determined. Then, whole animal behavioral tests were designed to assess aspects of swimming and feeding. Such tests measured vertical distributions, feeding rates, rates of locomotory arrests and touch responses of the larvae. The effects of transmitters, such as serotonin,

norepinephrine, dopamine and synthetic peptides, on these behaviors were measured. Furthermore, the effects of several agents that increase or decrease endogenous activity of these transmitters were tested. Finally, the effects of such chemicals were also examined using extracellular electrophysiological recordings from the cells located on the edges of the velar lobes. Such experiments have allowed us to formulate a working hypothesis of how specific neural elements control the velar cilia and musculature.

Materials and Methods

Adult and Larval Culture

Refer to Chapter 2, page 44

Immunohistochemistry, behavioral experiments and electrophysiological recordings were performed on newly hatched larvae, prior to commencement of planktotrophic feeding.

Morphological Preparations

Immunocytochemistry

Refer to Chapter 2, page 45

Antibodies

The larvae fixed with 4% PFA were then incubated in one of the polyclonal antibodies; anti-FMRFamide, anti-serotonin, anti-leu-enkephalin (all from Diasorin, Stillwater, MI). Larvae fixed with methanol were incubated in monoclonal anti-tyrosine hydroxylase (TH) (Diasorin, Stillwater, MI).

Labeling Muscle: F-actin labeling

Refer to Chapter 5, page 218

Larvae labeled with anti-serotonin, anti-FMRFamide or anti-leu-enkephalin, F-actin labeling was then used to reveal muscle fibers following methods described by Degnan et al. (1997b). Briefly the animals were incubated in 1:100 phalloidin labeled

with tetramethlyrhodamine B isothiocyanate (TRITC; Sigma Chemical Co., Mississauga, ON) for one to two hours. Larvae were washed in PBS twice for five minutes and then a final wash of at least 60 minutes.

Viewing and Photography

Refer to Chapter 2, page 47 and also Chapter 5, page 219

Behavioral Tests

Chemicals and Concentrations Tested

We tested the effects of 5-hydroxy-tryptamine hydrochloride (serotonin; Sigma Chemical Co., Mississauga, ON), dopamine hydrochloride (Sigma Chemical Co.), and arterenol bitartrate salt (norepinephrine; Sigma Chemical Co.). Mianserin hydrochloride (Sigma Chemical Co.) a vertebrate 5-HT_2 receptor antagonist (Willins *et al.*, 1999) was utilized since it has been shown to be an effective serotonin antagonist in adult (Baker and Croll, 1996) and larval (Diefenbach *et al.*, 1991; Uhler *et al.*, 2000) gastropods. Fluoxetine hydrochloride (Eli Lilly and Company, Toronto, ON), an established vertebrate serotonin re-uptake inhibitor (Fuller, 1996), was also used since previous studies have provided evidence that it enhanced serotonergic activity in adult (Fong *et al.*, 1998) and larval (Uhler *et al.*, 2000) molluscs. Spiperone hydrochloride (Sigma Chemical Co.) has been shown to block D_2 dopamine (Amenta *et al.*, 1999), alpha-adrenergic (Testa *et al.*, 1993) and 5-HT2/5-HT1 (Geerts *et al.*, 1999) receptors in

vertebrates. This substance has also been shown to be a possible antagonist for dopamine in larval and adult molluscs (Green *et al.*, 1996; Pechenik *et al.*, 2002), however its effect on other receptors has not been resolved in this phylum (for example see Goldberg *et al.*, 1994). Alprenolol (Sigma Chemical Co.), a vertebrate beta-adrenergic receptor antagonist (Surman and Doggrell, 1993) was utilized since it has also been suggested to affect this receptor type in a polychaete larva (Marsden and Hassessian, 1986). Haloperidol (Sigma Chemical Co.), a $D_{2\text{-}4}$ dopamine receptor and also possibly a 5-HT receptor antagonist (Seeman, 2002) in vertebrates was used since it has been demonstrated to be an effective dopamine antagonist in adult molluscs (Heiss *et al.*, 1976; Voronezhskaya *et al.*, 1993).

Stock solutions of most chemicals were made at concentrations of 10^{-2} M or 10^{-3} M in distilled water. However, spiperone hydrochloride was first dissolved in ethanol (EtOH) and haloperidol in dimethyl sulfoxide (DMSO) and each of these solutions was further diluted in natural sea water such that the initial solvents were at a final maximal concentration of 0.25%. Preliminary tests were performed to determine if 0.25% solutions of ethanol (EtOH) and dimethyl sulfoxide (DMSO) in sea water affected larval behaviors. In most experiments there were no significant differences (not shown). However, EtOH did have a slight effect on the vertical distribution of larvae. Therefore, a saturated solution of spiperone dissolved in water was used for this experimental test. This saturated spiperone solution was determined to have similar effects as 10^{-6} M when tested on other behaviors in preliminary experiments. Final concentrations ranging from

10^{-3} M to 10^{-7} M were made in filtered natural sea water and initially tested to determine the appropriate concentrations. Such concentrations were determined based on the observation of significant behavioral changes after a 10-15 minute drug exposure and the ability of larvae to recover from the drug effects within1 hour after removal of the drug. The only chemical in which larvae did not recover was haloperidol at 10^{-4} M and therefore lower concentrations were utilized for this drug. For acute tests, within 10 minutes (locomotory arrests, touch recovery, and electrophysiological recordings), higher concentrations were determined to be more effective and therefore concentrations of 10^{-4} M and 10^{-5} M were utilized. Some of the chemicals tested have been reported to break down chemically and the products may affect larval behaviors and cell physiology (Pires and Hadfield, 1991). Therefore, to avoid this possibility, stock solutions were made immediately before use, and experimental times were minimized where possible.

In addition to the chemicals listed above, synthetic neuropeptides, FMRFa and leu-enkephalin (both from Sigma Chemical Co.) were used in some experiments. The effects of the synthetic peptides were short lasting and therefore could only be used for acute tests. It was determined that concentrations of 10^{-4} M, 10^{-5} M and 10^{-6} M were also effective for these peptides.

Behavioral Tests and General Observations of Swimming and Feeding Behaviors

1) General Behavioral Observations

General observations of larval swimming behaviors such as location in the water column, velar ciliary movements and retractions, and swimming activity (in this chapter I use the term activity to indicate the appearance of speed, however since no quantitative measurements of swimming speeds were made I have used a more general descriptive term). Ten larvae were observed using a binocular stereo-dissecting microscope at 15X to 60X magnification in a 20mm diameter compartment of a Falcon multi-well culture dish. The appropriate amount of each chemical from stock solutions was added to 1 ml of salt water. Behavioral changes were recorded five minutes after the chemical was applied and all observations were made within 10-15 minutes.

2) Vertical Distribution Test

Larvae were pipetted into the top of a column, created with plastic pipettes (30 cm X 1 cm), containing 10 ml of seawater or seawater with the addition of one or more chemicals. The bottom of the column was sealed with Parafilm (American National Can., Menasha, WI). After 30 minutes, the animals were removed from the column by carefully removing the Parafilm seal and slowly emptying 5 ml of fluid containing larvae into a 40 mm compartment of a Falcon multi-well culture dish (bottom sample). The final 5 ml of fluid and larvae (top sample) was then emptied into another compartment of the culture dish. Sometimes larvae were located in a drop of water that was removed with the Parafilm. Therefore, the piece of Parafilm was also placed into another compartment of the culture dish and any larvae observed were counted and added to the

number in the bottom 5ml of the column. Enough EtOH was added to each compartment and over the Parafilm to immobilize the larvae and enable counting under a stereo-dissecting microscope at 60X magnification.

Preliminary experiments indicated that the distribution was not significantly altered in darkness compared with normal laboratory lighting and therefore we used laboratory overhead lighting for all the experiments. In addition, the relative distribution of 25 versus 50 and 100 larvae did not differ significantly and therefore, approximately 50 larvae was used for each trial. All trials were performed between 12 PM and 5 PM to avoid variations resulting from possible circadian migrations. The length of each experiment was 30 minutes and concentrations of drugs ranging from 10^{-4} M to 10^{-7} M were used for some chemicals to achieve a dose-dependent response. In addition, combinations of serotonin and mianserin as well as norepinephrine and spiperone were also used to test the specific actions of their antagonists. A total of 8 trials was performed for each drug concentration tested.

3) Feeding Rates

Pechenik (1979) reported feeding rates of 1000-4000 algal cells/animal/hr by larvae of *I. obsoleta* at concentrations ranging from 50,000-250,000 cells/ml at room temperature. Similar feeding rates were also recorded in the population of larvae used in the present study (Fig. 19). A solution of live *Isochrysis galbana* (Clone T.ISO, Provasolin Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor,

Maine) was diluted with seawater to a concentration ranging between 75,000 and 250,000 cells/ml. The algal solution (9 ml) was poured into each of the 50 ml plastic tissue culture flasks (VWR, Canlab, Mississauga, ON). The appropriate volume of the tested chemical was added to the culture bottles and then gently mixed. Preliminary experiments revealed a high degree of variability in feeding rates. Also chemical exposure caused changes in algal suspension and thus a matched pair experimental design was used. In one flask of each pair, 1.0 ml of sea water containing 25 larvae were added to one of the culture bottles. To the other flask, only 1.0 ml of sea water was added and this flask served as the control. At least two additional controls, with and without larvae but no chemicals added, were also set up on each day of the experiments. These controls served to normalize the data (see equation below). After 4 hours, each culture bottle was gently stirred and 1.0 ml samples were taken from it and 20 μl of 4% PFA was added. The algal concentrations from these samples were determined by standard counting methods using a Brightline hemacytometer (VWR, Canlab). The clearance rates in each matched pair were determined by subtracting the algal concentration in the flask containing algae and larvae from the algal concentration in the flask containing algae only. The final number of algae ingested per larvae per hour was calculated using the formula below; subtracting the algal concentration from each matched pair and then normalized by dividing by the average amount ingested by the controls. For each drug tested, 8 trials were performed.

$$\left(\frac{\text{\#cells/ml (algae + chemical) - \#cells/ml (larvae + algae + chemical)}}{\text{average \#cells/ml (algae) - average \#cells/ml (larvae + algae)}} \right) \times \frac{10 \text{ ml}/4 \text{hrs}}{25 \text{ animals}} = \text{\#cells/ml/hr/animal}$$

Equation 1.

4) Frequency of Locomotory Arrests: Ciliary Arrest and Velar Contractions

Approximately10 larvae were observed using a binocular dissecting microscope at 15X to 60X magnification in a 20mm diameter compartment of a Falcon multi-well culture dish. The appropriate amount of each chemical from stock solutions was added to 1 ml of salt water. The number of locomotory arrests, which consisted of both ciliary arrests and velar contractions, was counted over 1 minute period in an individual larva. In this test all the chemicals listed above including synthetic neuropeptides were utilized. A total of 10 larvae were tested for each drug concentration.

5) Touch Response and Recovery Time

Approximately10 larvae were tested using a binocular dissecting microscope at 15X to 60X magnification in a 20mm diameter compartment of a Falcon multi-well culture dish. The appropriate amount of each chemical from stock solutions was added to 1 ml of salt water. A light touch stimulus was applied to larvae by gently touching the velum with the edge of a glass capillary tube (outer diameter 1.0mm, 0.78 inner diameter; Warner Instrument Corp, Hamden, CT) that was pulled to a point with a Brown-Flaming micropipette puller (Model P-77, Sutter Instruments Co., CA). A response was recorded

if the cilia arrested and the velum and foot at least partially contracted. Recovery time was determined by recording the time from ciliary arrest and velar contraction to the time when normal swimming resumed. Each trial was ended at 15 seconds if prolonged arrest and contraction occurred. A minimum time of 0.50 seconds was used for extremely quick recoveries where the time was below the limits of my reaction time. A time of zero was recorded for larvae that did not respond to the stimulus. A total of 10 larvae was tested for each drug concentration.

Touch response was also tested on isolated velar lobes to provide additional information about the neural control. Using small dissecting scissors the velum was cut either anterior or posterior to the eyespots. The eyespots were used as a general guide for the location of the apical organ and cerebral ganglia since previous morphological studies have demonstrated that these structures were located between the eyes. The movements of the cilia were observed under a binocular dissecting microscope at 60X and the touch stimulus was applied as described above.

4. Extracellular Electrical Recordings

Larvae were pipetted into a depression in a glass slide with 1ml of seawater. A suction electrode was used to record compound action potentials generated along the edge of the velum. The electrode consisted of thin walled borosilicate glass capillary tubing (Warner Instrument Corp.) that was pulled to a sharp point with the Brown-Flaming micropipette puller (Sutter Instruments Co.). The tip of the tube was then partially

removed with fine grain sand paper (600 grit) to create an opening of 50-75 μm in diameter. The glass capillary was then fitted into one end of 1mm (inner diameter) plastic polyethylene tubing containing a silver wire electrode lead. Gentle suction permitted a portion of a velar lobe of an intact larva to fill the opening of the capillary tube. Recordings were amplified 1000X and filtered (bandpass of 10 Hz - 10 kHz) by an extracellular differential amplifier. Signals were acquired by a Digidata 1320A acquisition system (Axon Instruments, Inc., Union City, CA) in conjunction with Axoscope 8 software (Axon Instruments, Inc., Union City, CA) on continuous gap-free mode. A 10 μl volume of the stock solution of each chemical was added directly to 1.0 ml of sea water containing larvae to achieve a concentration of 10^{-4} M which was utilized for this test. An additional 1-2 trials using lower concentrations were also performed. For each trial on a single larva, a control recording was performed for 2 minutes, after which the chemical was administered and then 3-5 minutes later the recording was resumed for another 2-5 minutes. Finally, the chemical was washed out with several water changes over a 10 minute period and then a final recording was performed on the same larva for 2 minutes. The average amplitude and frequency of the recordings were calculated, from the last 2 minutes of each recording, using Clampfit 8 software (Axon Instruments, Inc., Union City, CA). A total of 5 larvae were sampled for each of the chemicals: serotonin, dopamine, norepinephrine and synthetic FMRFamide.

5. Statistics

Statistical analysis was performed using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA). Data were subjected to tests for normality. For normally distributed data a parametric one way analysis of variance (ANOVA) was conducted to detect significant effects of the chemicals. Alternatively, for those trials which failed to show normal distribution a Kruskal-Wallis one way ANOVA on ranks was conducted to test for significant differences between the control and drug trials. Dunnett's test for multiple pairwise comparisons ($P<0.05$) was used to identify the drug trials which differed significantly from the controls.

Results

Morphology of Neural Elements

The velum of *I. obsoleta* contained an elaborate network of axons and muscles at the hatchling stage. Axons labeled by anti-FMRFamide, -leu-enkephalin and -serotonin had similar appearances in the velum. These axons had numerous varicosities and radiated from the head region into each velar lobe along muscle fibers and along the base of the pre-oral ciliated cells (Fig. 20A-C, 21B). The discontinuous appearance of the axons in some confocal images (e.g. Fig. 20C) was due to the intense labeling of the varicosities. However, higher magnifications (Fig. 20E, F) revealed that these axons were indeed continuous throughout the velum. The axons appeared to originate from the neuropil of the apical organ or cerebral ganglia where cells containing such transmitters were located (not shown, but see chapter 2). TH-like immunoreactivity revealed 8-10 cells interconnected with axons along the post-oral ciliated band in each velar lobe (Fig. 20D, 21C). Furthermore, some axons labeled with anti-TH extended to the base of the pre-oral ciliated cells and others projected in the opposite direction toward the head (Fig. 20D, 21C). FMRFamide-LIR axons and F-actin labeled muscle fibers were evident at the base of the large pre-oral epithelial cells at higher magnifications (Fig. 20E). In addition, at higher magnification the locations of the TH-LIR neurons and axons were found along the post-oral band rather than the pre-oral band (Fig. 20F). Approximately 4-8 TH-like immunoreactive cells were also located around the ventral and lateral edges of the mouth.

Such cells were vase-shaped with the distal dendrites oriented toward the mouth opening (Fig. 20G).

General Behavioral Observations and Behavioral Tests

1) Description of Normal Swimming Behaviors and the Effect of Chemicals

Once the animal hatched, swimming activity began immediately. During active swimming the larvae were estimated to spend approximately 70-80 % of the time moving vertically with their vela directed upward until they reached the surface. The rest of the time was spent swimming horizontally with their vela directed laterally or toward the bottom. Arrests in locomotion were observed when the larvae contacted either the water surface or an object. These arrests were characterized by the cessation of beating by the pre-oral cilia, and contractions of the velar lobes and foot. When larvae contacted the surface, the animals retracted into their shells, fell in the water column and remained immobile for 2- 4 seconds before the pre-oral cilia began to beat again. Upon resumption of pre-oral ciliary beating the larvae emerged from their shells and started to swim. A less forceful stimulus (e.g. contact with small particulates within the water) resulted in briefer ciliary arrests and less robust contraction of the vela, causing only momentary interuptions in swimming and/or changes in swimming direction. Other cilia, including those constituting the post-oral band and surrounding the mouth, were never observed to stop moving.

In the presence of many of the chemicals tested here, the swimming behavior of hatchlings was altered. Serotonin and fluoxetine noticeably increased the swimming activity, the animals appearing to swim faster. These chemicals also caused more larvae to come to the water surface compared with the controls and fewer locomotory arrests were observed. The serotonin antagonist mianserin resulted in the opposite effect, decreasing swimming activity by what appeared to be a decreased swimming speed. At 10^{-5} M and 10^{-6} M concentrations of mianserin the larvae appeared to remain near the bottom of the compartment. At a higher concentration (10^{-4} M) of mianserin, the larvae lay on their sides with velar lobes partially contracted, while cilia remained beating. At high concentrations (10^{-4} M and 10^{-5} M) of both catecholamines, dopamine and norepinephrine, the swimming speeds also appeared to decrease and many larvae were located near the bottom of the compartment. Furthermore, hatchlings were sometimes swimming vertically very slowly with a slight wobble rather than the smooth upward motion observed in the controls. The catecholamine antagonist, spiperone, had the opposite effect of dopamine and norepinephrine by increasing the swimming activity and the larvae were gathered at the surface of the compartment. Additionally, ciliary arrests were never observed when larvae were exposed to spiperone. In contrast to behavioral effects of spiperone, the catecholamine antagonist decreased the swimming activity of hatchlings. At a high concentration (10^{-4} M) of this chemical , the larvae ceased swimming and velum and foot were contracted into the shell but cilia were still active. Many larvae did not recover from this concentration. At lower concentrations (10^{-5} M and

10^{-6} M) of haloperidol, 20-50% of the larvae stopped swimming for prolonged periods while the other larvae that continued swimming seemed to have decreased activity. Finally the last catecholamine antagonist, alprenolol caused cessation of swimming at a high concentration (10^{-4} M), while at lower concentrations (10^{-5} M and 10^{-6} M), very little change in swimming activity was observed.

When larvae were exposed to a high concentration (10^{-4} M) of synthetic FMRFamide the larvae ceased swimming, the cilia were arrested and the vela were partially contracted for prolonged periods lasting from 30 minutes to 2 hours. In smaller concentrations of this peptide the larvae also stopped swimming and the cilia were observed to arrest intermittently for a period of 5-20 minutes before they resumed normal swimming. Concentrations of 10^{-4} M and 10^{-5} M of synthetic leu-enkephalin had similar effects as 10^{-6} M synthetic FMRFamide, but to a lesser degree. Recovery times, from the effects of both synthetic peptides, were approximately proportional to the peptide concentration as well as to the number of larvae in the compartment.

Together, these qualitative observations strongly suggested that various chemicals tested here had profound affects on swimming behaviors and therefore the subsequent quantitative tests were utilized.

2. Vertical Test

Results of the Kruskal-Wallis test revealed an overall significant difference between the average vertical distribution, expressed as the percentage of animals in the

top half of the column (H=162.81, P=0.001). The control revealed an average distribution of 46.7% (± 6.63 SD) within the top of the water column. Serotonin increased the number of larvae in the top part of the water column in which concentrations of 10^{-5} M, 10^{-6} M resulted in percentages of 87.6 (SD ± 6.14) and 82.0 (SD ± 5.77) (Fig 22A). At 10^{-4} M of serotonin there was an increase in the number in the top of the column, however, this effect was not as high as 10^{-5} M. Fluoxetine also increased the average number of larvae in the top half of the column (68.4 ± 8.76) at a concentration of 10^{-7} M. Mianserin resulted in decreased numbers of larvae in the top of the vertical column also in a dose-dependent manner, with 5.42% (SD ± 4.52), 11.5% (SD ± 7.72) and 20.6% (± 6.93 SD) at concentrations of 10^{-5} M, 10^{-6} M, 10^{-7}M respectively (for example see Fig. 22A for results at 10^{-5} M). In addition, when different concentrations of mianserin were added to 10^{-6} M serotonin the distribution of larvae was attenuated. For example, 10^{-6} M of serotonin combined with 10^{-7} M mianserin resulted in 33.5% (± 12.07 SD) of the larvae in the top half of the column, a change of 48.5% compared with the percentage of larvae exposed to 10^{-6} M serotonin alone (Fig 22A).

Both dopamine and norepinephrine decreased the percentage of larvae in the top portion of the water column in a dose-dependent manner. At concentrations of 10^{-4} M and 10^{-5} M, dopamine caused a significant decrease (8.79 % ± 3.43 SD; 28.9% ± 12.4 respectively)(Fig. 22B). Similarly, at concentrations of 10^{-4} M and 10^{-5} M of norepinephrine a significant decreases (6.81% ± 3.84 SD and 12.1% ± 4.90 SD respectively)(Fig. 22C). Both haloperidol and alprenolol at 10^{-5} M also resulted in a

significant decrease in the percentage of larvae (29.0% ± 10.01 SD and 14.1% ± 4.67 SD respectively) in the top part of the water column (Fig. 22B,C). On the other hand, the same concentration of spiperone resulted in a significant increase in the percentage of larvae (79.08% ± 3.17SD) in the top half of the column (Fig. 22C). When larvae were exposed to a combination of norepinephrine and spiperone, their vertical distribution was only slightly lower than the control animals (Fig. 22C).

3. Feeding Rate

Results of the ANOVA statistical test revealed an overall significant difference in feeding rates between treatment groups ($F= 23.15$, $P<0.001$). Results above 100% indicated an increased feeding rate and a number below this value indicated a decreased feeding rate. When animals were exposed to a serotonin concentration of 10^{-5}M the feeding rate increased to 186% (± 17.6 SD), while exposure to 10^{-5} M of the serotonin antagonist, mianserin, significantly decreased the feeding rate to 55.5% (± 41.1 SD) (Fig. 23). When larvae were immersed in a concentration of 10^{-5} M norepinephrine feeding rates decreased to the lowest recorded value of 29.1% (± 28.6 SD) (Fig. 23). Dopamine had no significant effect on feeding rates at 10^{-5} M concentration (Fig. 23). Spiperone significantly decreased feeding rates to 57.5 % (± 18.2 SD), while alprenolol had no significant effect on feeding rates (Fig. 23).

3) Locomotory Arrests: Ciliary Arrests and Velar Contractions

Results of the Kruskal-Wallis statistical test revealed an overall significant difference in the average number of locomotory arrests between treatment groups ($H=122.65$, $P<0.001$). In the control larvae the average was 1.0 arrests/minute (± 0.82 SD) (Fig. 24 A, B). While monoamines and their analogues did not significantly alter this behavior (see Fig. 24 A-C) several trends were observed that suggested effects on this behavior. For example, both serotonin and fluoxetine decreased the average frequency of locomotory arrests. The average number of arrests per minute for serotonin and fluoxetine (10^{-4} M) was 0.0 arrests/minute (± 0.0 SD). Mianserin (10^{-4} M) had the opposite effect with 1.9 arrests/minute (± 1.1 SD). Dopamine or norepinephrine (10^{-4} M) had no noticeable effect on the frequency of locomotory arrests, however, the catecholamine antagonists spiperone and alprenolol (10^{-4} M) decreased the frequency to undetectable levels. In contrast, haloperidol had the opposite effect of the other catecholamine antagonists by increasing the frequency of the arrests (1.8 ± 1.14) at a concentration of 10^{-5} M.

At 10^{-6} M, synthetic FMRFamide prominently increased the number of ciliary arrests to an average of 18.1 arrests/minute (± 1.91 SD) (Fig. 24C). Synthetic leu-enkephalin slightly increased the number of ciliary arrests per minute at 10^{-4} M (not shown), however, at a lower concentration (10^{-6} M) the number of arrests (0.5 arrests/minute ± 0.97 SD) was not significantly different than the controls (Fig. 24C).

4) Touch Response and Recovery Time

Results of the Kruskal-Wallis statistical test revealed a significant difference between treatment groups ($H=220.10$, $P< 0.001$). Larvae normally responded to the touch stimulus with a fast ciliary arrest and a velar contraction. General observations of the larvae indicated that length of time to recover from mechanical stimuli correlated with the degree to which the larva responded to such a stimulus. For example, a forceful contraction of the velum and the arrest of all of the pre-oral cilia resulted in a longer recovery time. On the other hand, a short recovery was always observed when fewer pre-oral cilia arrested and the velum only partially retracted. In control animals the time from the touch stimulus to re-commencement of swimming averaged 3.27 seconds (s) (± 0.710s SD) (Fig 25A-D). This recovery time was affected by several chemicals. Concentrations of 10^{-4} M and 10^{-5} M of serotonin caused significant decreases in recovery times (1.286s ± 0.711s SD and 1.964s ± 0.492s). Furthermore, fluoxetine (10^{-4} M) resulted in a significant decrease in recovery time of 0.50 (± 0.0s SD) (Fig. 25A). On the other hand, a concentration of 10^{-4} M of mianserin caused a substantial increase in the recovery time lasting at least 15s for each trial (Fig. 25A).

Larvae exposed to norepinephrine and dopamine showed no significant effect on the touch recovery time at any of the concentrations tested (Fig. 25B). The touch response was eliminated when larvae were exposed to the antagonist spiperone (Fig. 25C). Alprenolol (10^{-4} M) resulted in significantly decreased recovery times (0.30s ± 0.0s SD). On the other hand, haloperidol at 10^{-5} M caused a significant increase in the recovery time of 7.91 seconds (± 3.42s SD)

A 10^{-6} M solution of synthetic FMRFamide caused an increased recovery time of 7.16 seconds (± 2.468s SD) (Fig. 25D). On the other hand synthetic leu-enkephalin did not significantly affect the recovery time (Fig. 25D).

Isolated velar lobes without eyespots did not respond to touch stimuli. The cilia on these lobes continued to beat and the pre-oral cilia appeared to be more active. Isolated velar lobes with eyespots responded in a similar manner as whole intact larvae when a mechanical stimulus was applied.

Electrical Recordings

Visual observations of the animal while performing extracellular recordings from the edges of the velum revealed that compound action potentials correlated with ciliary arrests and muscle contraction. Muscle contractions that were independent of the ciliary arrests did not correlate with action potentials. Also, ciliary arrests and action potentials were sometimes observed to be independent of muscle twitches. The electrical recordings showed action potentials that had a positive peak that ranged from 0.5 mV to 4.0 mV and a peak-to-peak time of approximately 20-25 ms (see Fig. 26 A, for sample trace). The frequency of action potentials was variable, ranging from 15-50 spikes per minute (see Fig. 26 C, D controls). It was also noticed that a larger tip diameter in the suction electrode resulted in a larger amplitude in the action potential, presumably due to recording from a larger number of ciliated cells. When serotonin was added (10^{-4} M), the amplitude of the spikes decreased and then later the spikes were abolished (Fig. 26 B, C

and 27A, B). The frequency was also reduced to zero (Fig. 26A,C and 27A, B). Results from the Kruskal-Wallis test on the average amplitude and frequency of action potentials revealed an overall significant differences between treatment groups (H=17.88, P=0.001; H=14.87, P= 0.005, respectively). Single trials using serotonin and fluoxetine concentrations of 10^{-6} M resulted in decreased amplitudes and frequencies (not shown). The other chemicals tested had no significant effects on the amplitude and frequency of the compound action potentials (Fig. 26C and 27A, B).

Discussion

In the present study, we provided evidence that elements of the larval nervous system are involved in the control of specific larval behaviors, such as swimming, feeding and the escape response in the gastropod *I. obsoleta.* Morphological evidence consisted of neurons and axons containing monoamines and neuropeptides associated with ciliary bands and muscle fibers in the velum and with the mouth cavity. Furthermore, exposure to synthetic analogues of the monoamines and neuropeptides found within the neurons, in many cases had significant qualitative and quantitative effects on behaviors and cellular physiology. Together these experiments provided details about the types of endogenous transmitters and the receptors present in the gastropod larval nervous system. Moreover, the results have permitted the formulation of hypotheses concerning how these transmitters may work in concert to control the ciliary activity and muscle contractions that ultimately control larval behaviors.

1. Endogenous Actions of Neurotransmitters

The histological examinations indicated that serotonin immunoreactivity was present within axons associated with muscle fibers and the pre-oral ciliated cells of the velar lobe of *I. obsoleta.* In addition to the morphology, the pharmacological experiments in whole larvae are consistent with the endogenous role of serotonin. Application of exogenous serotonin caused increased swimming and feeding activity and

decreased recovery times following mechanical stimuli. These behavioral changes were mimicked by fluoxetine, a vertebrate serotonin re-uptake inhibitor that has also been shown to enhance serotonin mediated processes in other molluscs (Fong *et al.*, 1998; Uhler *et al.*, 2000). The actions of fluoxetine were possibly mediated by decreasing the re-uptake of serotonin and thus resulting in an increase in endogenous activity of this transmitter. Furthermore, mianserin, the vertebrate type 5-HT_2 receptor antagonist that is also known to act as an antagonist in molluscs (Diefenbach *et al.*, 1991; Baker and Croll, 1996; Uhler *et al.*, 2000), decreased swimming activity and feeding behaviors. The actions of mianserin were possibly mediated by blocking endogenously released serotonin. Together the effects of serotonin, fluoxetine and mianserin were evidence consistent with a role for endogenous serotonin in regulating swimming and feeding behaviors of *I. obsoleta.* Finally, the results also provide some insights into characteristics of the receptors and re-uptake mechanisms in the gastropod larva. Changes induced by mianserin indicated that serotonin possibly acted on receptors similar to those characterized in vertebrates and that the serotonin transporters were also comparable to those in vertebrates. Similar pharmacological results have also been reported in other gastropod larvae (Goldberg *et al.*, 1994; Uhler *et al.*, 2000).

The localization of the catecholamine generative enzyme, tyrosine hydroxylase (TH) (Cooper *et al.*, 1996) and the Faglu technique suggested the presence of catecholamines within axons and neurons associated with the ciliary bands of the velum in *I. obsoleta.* The ability of these techniques to identify catecholamines has been verified

in other molluscs by combining histological studies with high performance liquid chromatography (HPLC). Such studies have indicated the prevalence of both dopamine and norepinephrine in both the CNS and peripheral regions of adult (Pani and Croll, 1995; Pani and Croll, 1998; Pani and Croll, 2000) and larval molluscs (Pires *et al.*, 1998; Pires *et al.*, 2000b; Pires *et al.*, 2000c). Pharmacological experiments in the present study indicated that either or both dopamine and norepinephrine were active in the larval nervous system of *I. obsoleta*. However, the pharmacological results were difficult to interpret since the identity of the catecholamine, whether dopamine and/or norepinephrine, was not clear since neither histological method identified specific types of catecholamines. While it is difficult to determine which catecholamine was located in neurons and axons, in the velum of *I. obsoleta* some of the results of the study combined with evidence from other studies may provide some clues. For example, some of the experiments revealed that norepinephrine had more profound effects on swimming and feeding behaviors of *I. obsoleta* at lower concentrations than dopamine. These results suggest the possibility that norepinephrine was the primary transmitter located in cells regulating the behaviors tested. Similarly, larval behaviors governed primarily by norepineprine and not dopamine have been demonstrated in other molluscan larvae (Coon and Bonar, 1985). In contrast, HPLC studies have indicated that dopamine was present in considerably greater quantities in larval gastropods (Pires *et al.*, 1998; Pires *et al.*, 2000b; Pires *et al.*, 2000c). However, these studies did not focus on the location of dopamine and norepinephrine containing neurons and therefore do not preclude the possibility that

norepinephrine has a major role in controlling swimming and feeding in gastropod larvae. Also, it is possible that some of the dopamine measured in the HPLC studies served as a precursor to norepinephrine. It is clear from this study that further experiments are necessary to identify the types of catecholamines present in larval neurons associated with the ciliated bands of the velum in this species and other molluscan larvae.

Exposure of larvae to exogenous dopamine or norepinephrine generally decreased swimming activity and feeding rates suggesting a possible endogenous role of catecholamines in *I. obsoleta.* However, the behavioral changes observed when larvae were exposed to the putative antagonists spiperone, alprenolol and haloperidol were inconsistent. Such inconsistent specificity of these antagonists has also been reported in other larval and adult gastropods (for examples see Goldberg *et al.*, 1994; Pechenik *et al.*, 2002 and Heiss *et al.*, 1976; Magoski *et al.*, 1995; Green, 1997). Therefore, it is possible that these antagonists have multiple actions on catecholamine receptors as well as other receptors for serotonin or acetylcholine. For example, in the present study the antagonist alprenolol affected the touch recovery time in a similar manner as spiperone, possibly by affecting the same dopamine or norepinephrine receptor. On the other hand, alprenolol mimicked the effects of the serotonin antagonist, mianserin, in the vertical distribution test. Similar, mixed effects of alprenolol have also been shown in vertebrates (for example see Seeman, 2002). The effects of the catecholamine antagonist, spiperone, on behaviors of *I. obsoleta* were more consistent. For example, exogenous exposure of spiperone caused an increased swimming activity possibly by blocking dopamine or

norepinephrine receptors and preventing endogenous action of one or both of these transmitters. Furthermore, the results of the vertical distribution test also suggest that spiperone acts specifically on norepinephrine receptors since the distribution changes induced by norepinephrine were attenuated when larvae were also exposed to spiperone. Additional experiments are clearly necessary to determine if the actual release of norepinephrine and/or dopamine regulate the velar ciliary bands and to characterize the receptors in gastropods.

Labeling by FMRFamide and leu-enkephalin antibodies indicated the possible presence of these and/or related peptides within neurons associated with the pre-oral ciliary band and muscle fibers of *I. obsoleta.* However, previous immunohistochemical studies have shown that antibodies for at least FMRFamide also cross react with other similar peptides in gastropod larvae (Voronezhskaya and Elekes, 1997). Therefore, additional studies are necessary to determine the precise identity of these neuropeptides in larval stages of *I. obsoleta.*

While antagonists for these neuropeptides were not utilized in the present study, the effects of the synthetic peptides on ciliary arrests and muscle contractions suggested the possibility that these or related neuropeptides were normally acting endogenously in the velum of *I. obsoleta.* Exogenous application may mimic endogenous release of the peptides if the small synthetic FMRFamide and leu-enkephalin molecules were able to diffuse into cells and interact with receptors. Additional studies are necessary to determine the mechanisms by which these synthetic peptides enter the animal. Support

for the endogenous action of FMRFamide and leu-enkephalin related peptides in the larvae also comes from other studies in various molluscs which show that similar neuropeptides were active in the adult nervous systems of other molluscs (for examples see Stefano and Catpane, 1979; Leung *et al.*, 1986; Billy and Walters, 1989; Cawthorpe and Lukowiak, 1990; Muneoka *et al.*, 1991). However, the results presented here cannot exclude a non-specific response of larvae to these synthetic peptides and therefore additional characterization of the presence and endogenous role to neuropeptides in larval gastropods is necessary.

2. Neuronal Control of Pre-Oral Ciliary Activity

The large compound pre-oral cilia are responsible for propelling the larva through the water column. Morphological evidence in this study indicated that axons were closely associated with the base of the epithelial cells containing these pre-oral cilia. Also, many varicosities along these axons suggested possible synapses. Similar reports of neural association with these epithelial cells have been demonstrated with light and electron microscopy in other gastropod larvae (Mackie *et al.*, 1976; Arkett *et al.*, 1987; Marois and Carew, 1997d). In *I. obsoleta*, the axons associated with the cells containing the pre-oral cilia were labeled with serotonin and catecholamines.

<u>B) Role of Serotonin: Increased Ciliary Activity</u>

Morphological and pharmacological evidence in the present study suggests that serotonin normally functions to increase the velar ciliary activity, thus swimming activity in *I. obsoleta*. Serotonin has also been shown to increase swimming activity in other larvae. For example, exposure to serotonin caused increased swimming activity in the bivalve *M. edulis* (Beiras and Widdows, 1995). In that molluscan species, larval swimming activity was also controlled by a ciliated velum and it was suggested that serotonergic nerves associated with the velum had an excitatory effect. In another example, serotonin was shown to increase activity of a band of cilia located on the sole of the foot in the larval stages of the pulmonate gastropod, *Helisoma triviolus*. This species spends its entire larval life within a capsule and, while there is no functional velum, the larva can rotate by the movement of the pedal cilia. Morphological examinations revealed that axons from a pair of serotonin-LIR apical cells projected to the ciliated epithelial cells forming the pedal ciliary band (Goldberg and Kater, 1989; Diefenbach *et al.*, 1995). A series of experiments indicated that endogenous serotonin from these apical cells increased the rotational speed of the larva presumably by increasing the activity of the pedal cilia (Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994; Kuang and Goldberg, 2001). Similar results were also reported in another intra-capsular pulmonate species (Uhler *et al.*, 2000). Serotonin has also been shown to increase swimming activity in other larval invertebrates (Wada *et al.*, 1997a; Wada *et al.*, 1997b; Hay-Schmidt, 2000). In all of these experiments, the increased locomotory activities were attributed to accelerated ciliary beat frequencies, however no direct measurements were conducted of

the actual ciliary movements. On the other hand serotonin has been shown to affect ciliary beat frequency directly in adult preparations from molluscs and various other taxa (for examples see Audesirk *et al.*, 1979; Smith, 1982; Aiello, 1990; Nguyen *et al.*, 2001). While the behavioral observations of swimming speeds and tests of changes in locomotory activity were suggestive of changes in ciliary beat frequency of the pre-oral cilia, precise measurements of ciliary movements are necessary to confirm a cilio-excitatory role for serotonin in larvae of *I. obsoleta*.

B) Role of Catecholamines: Decreased Ciliary Activity

Morphological and pharmacological evidence in the present study also suggests that catecholamines normally function to decrease the ciliary activity in *I. obsoleta*. When larvae of *I. obsoleta* were exposed to dopamine or norepinephrine the swimming activity decreased and the animals rarely reached the surface. These general behavioral observations were also supported by the vertical distribution tests; both dopamine and norepinephrine also caused an increase in the number of larvae in the bottom half of the water column. Such alterations in the vertical distribution may have been due to decreased ciliary activity which resulted in the inability of larvae to generate enough force to swim upward (see review of larval swimming see Young, 1995). In contrast, the catecholamine antagonist, spiperone, resulted in increased swimming activity and a larger number of larvae in the top half of the column further supporting an endogenous role of catecholamines in regulating swimming activity.

Catecholamines have also been reported to decrease swimming activity in other larvae with swimming larval forms. For example, exposure to both dopamine and norepinephrine was reported to decrease velar ciliary activity in the larval stages of another mollusc, the bivalve *M. edulis* (Beiras and Widdows, 1995; Plummer, 2002). Dopamine also induced the reduction of swimming speed of sea urchin larvae (Wada *et al.*, 1997a). In sea urchin larvae the direct effect of catecholamines on ciliary movements to cause decreased swimming activity was attributed not to the actual ciliary beat frequency but to changes in the plane of ciliary movement. In adult preparations, the effect of catecholamines on ciliary movement has not been as consistent. For example, dopamine has been shown also to have a cilio-inhibity effect on the lateral gill cilia (Paparo and Aiello, 1970) and cilio-excitatory effect on the frontal gill cilia (Malanga, 1975) of the adult bivalve *Mytilus edulis* (also see Aiello, 1990). Precise measurements of ciliary movements are necessary to determine how dopamine and norepinephrine effect ciliary movements and in turn the swimming activity in *I. obsoleta*

3. Neural Control of Feeding

Feeding by planktotrophic gastropods occurs through the capture of algal cells in the food groove (reviewed by Bayne, 1983). Strathmann and Leise (1979) also suggested that the pre-oral cilia are used specifically for particle capture and the post-oral cilia retain particles in the groove possibly by slowing the current created by the pre-oral cilia. The presence of axons along both these ciliary bands suggested the possibility that there is

neural control over the coordination of pre and post-oral cilia. In addition, cilia located ventral to the mouth have also been suggested to be involved in food particle rejection and/or selection (Bayne, 1983). Therefore, the vase-shaped cells associated with the mouth cavity implicated further regulation of feeding by the larval nervous system in *I. obsoleta.*

Serotonin immunoreactive axons were observed chiefly to be associated with the pre-oral ciliary band and therefore this transmitter would be expected to alter feeding rates by regulating the activity of the cilia comprising this band. The results demonstrated that when larvae were exposed to elevated levels of serotonin the feeding rates in *I. obsoleta* larvae increased substantially. The increased swimming activities may have allowed larvae to come into contact with more algal cells and therefore indirectly increased feeding rates. Also if the cilia themselves were more active due to serotonin exposure then additional algal cells may have been captured. Both these possibilities were supported by the effects of the serotonin antagonist, mianserin, which reduced feeding rates, probably as a result of decreased ciliary activity and/or decelerated locomotory speeds.

Catecholamine-containing cells were observed in *I. obsoleta* along the post-oral ciliary band. Nerve fibers, possibly originating from these cells, spanned the food groove and ended at the base of the epithelial cells containing the pre-oral cilia. The locations of such cells and axons in the velum suggested their involvement in controlling both pre- and post-oral ciliary bands in *I. obsoleta.* Feeding was negatively affected by

norepinephrine in larvae of *I. obsoleta*. Such an effect may be due to a cilio-inhibitory effect of catecholamines (as suggested above). The decreased ciliary activity of either the pre- or post-oral cilia or of both bands may have caused decreased feeding rates for many reasons. For example, reduced pre-oral ciliary activity itself may have reduced food capture. Also decreased activity of the post-oral band may have prevented algal cells from remaining in the food groove. Another possibility is that decelerated swimming speeds diminished the contact and thus capture of algal cells.

Alternatively, catecholamines may normally function to coordinate the movements of the pre- and post-oral ciliary bands in *I. obsoleta*. Therefore, the similar effects of both exposure to exogenous norepinephrine and the possible antagonist, spiperone, may have been due to both chemicals disrupting the coordination of these two bands of velar cilia. Disrupted coordination may have then in turn prevented the capture and movement of food particles to the mouth. Loss of coordination in the pre- and post ciliary bands may also account for the decreased swimming activity and abnormal vertical distribution of larvae of *I. obsoleta* in the present study. Improper coordination of the velar ciliary bands may have impeded the creation of efficient swimming currents and thus prevented larvae from swimming upward (see reviews Chia and Buckland-Nicks, 1984; Young, 1995). Similar results have also been reported in sea urchins in which catecholamines caused decreased swimming activity by changing the coordination of the cilia used for locomotion. Such changes in coordination were achieved by altering the plane of ciliary beating (Wada *et al.*, 1997a). Changes in the mechanics of ciliary motion

have also been shown to be responsible for various patterns of locomotion in other free swimming larvae (for example see Sleigh and Barlow, 1982). The results presented here only suggest possible effects of catecholamine on ciliary movement. Additional studies are necessary to determine precisely how catecholamines affects ciliary movements of the pre- and post-oral bands in gastropod larvae.

The catecholamine, norepinephrine, may have also decreased feeding rates by affecting the ability to detect food particles. Such detection may be performed by the cells located along the food groove and/or by the cells surrounding the mouth. At least some of these catecholaminergic cells in the velum and surrounding the mouth were vase-shaped in larvae of *I. obsoleta*. Vase-shaped cells with dendritic processes which penetrate the epithelium have been postulated to be sensory cells in adult molluscs (Zylstra, 1972; Emery and Audesirk, 1978; Croll, 1983; Boudko *et al.*, 1999). Such sensory cells may therefore detect the presence and nutritional aspects of food particles. For example, the cells may have detected the size of the particles or even the species of algae both of which has been suggested possible in some molluscan larvae (Bayne, 1983). Therefore, in the presence of excess norepinephrine or the putative antagonist, spiperone, the vase-shaped catecholamine cells in larvae of *I. obsoleta* were not able to properly transmit sensory information and thus caused feeding rates to decline.

4. Locomotory Arrests: Ciliary Arrests and Muscle Contractions

Intermittent cessations of locomotion, allowing the larvae to sink rapidly in the water column, have been shown to be caused by arrests of the pre-oral cilia and contraction of the velar musculature (Mackie *et al.*, 1976; Arkett *et al.*, 1987). Such a behavior has been suggested to provide a mechanism of escape (Fretter, 1967) and/or to allow continuous vertical cycling in the water column for feeding (Chia and Buckland-Nicks, 1984). Moreover, the ability to regulate this behavioral response suggested the possibility that there is neural control governing ciliary arrests and muscle contractions and that these two actions are coupled together. Such a hypothesis is consistent with the histological evidence of serotonin, catecholamines, FMRFamide and leu-enkephalin within axons along the base of the cells containing the pre-oral cilia and along muscle fibers. Furthermore several of the behavioral tests performed in the present study provided quantitative details about the ciliary arrest and muscle contraction behavior. Such tests included feeding rates, vertical distributions, locomotory arrests and touch response and recovery times.

The frequency of ciliary arrests and muscle contraction would likely affect the distribution of larvae in the water column. Animals that have many locomotory arrest responses would be expected to be located at the bottom of the water column much of the time. Conversely, larvae that had fewer of these responses would be expected to sink less and thus more animals would be located at the top of the water column. For example, serotonin and fluoxetine both resulted in more larvae at the surface of the water column and therefore may have caused larvae to undergo fewer locomotory arrests. The serotonin

antagonist, mianserin caused the opposite effect and increased the number of larvae at the bottom of the water column, possibly by increasing the frequency of locomotory arrests. On the other hand, the catecholamine antagonist, spiperone increased the number of larvae located in the top of the water column which was attenuated by addition of norepinephrine. Therefore catecholamines may also regulate the frequency of locomotory arrests.

The locomotory arrest rate was also measured directly in larvae. However, due to the low incidence of arrests and high degree of variability in the average arrest rate in the control larvae many chemicals did not cause a significant effect on this behavior. In contrast, synthetic FMRFamide had a profound effect on the frequency of the locomotory arrests over a short time suggesting the possibility that neurons containing this peptide have a more direct role in regulating this behavior.

The touch response test indicated that mechanoreceptors located on the velar lobes and/or head were able to cause ciliary arrests and muscle contractions. Isolated velar lobes lacking the apical organ and cerebral ganglia were unable to respond to touch stimuli. This indicated that mechanoreceptors directly or indirectly regulated the ciliary arrests and muscle contractions via a pathway that went through the apical organ or cerebral ganglia. General observations of the larvae indicated that length of time for larvae to recover from mechanical stimuli correlated with the degree to which the larva responded to such a stimulus. Therefore, changes in the recovery times recorded in this study may have reflected effects on either the mechanoreceptors themselves or on the

ciliated cells and muscle fibers in which the response is seen. In either case, the numbers of activated cells or the degree to which cells responded may have been affected. Additional studies are necessary to determine how the specific neural elements regulate the pathway involved in touch responses. Regardless, the results of this test did indicate that catecholamines may be involved in the response to mechanical stimuli. The putative catecholamine antagonist, spiperone, eliminated the response to mechanical stimuli indicating that the transformation of sensory information may be mediated by catecholamine-containing neurons. Such neurons may be the TH-like immunoreactive cells identified in the velar lobes of *I. obsoleta.* Together the results of this study suggest multiple roles for catecholamines in *I. obsoleta.* Some catecholaminergic cells may have coordinated the cilia of the pre and post-oral bands and/or have decreased velar ciliary activity, while other cells may have functioned to mediate mechanical stimuli.

The results of the electrophysiolgical recordings from the edges of the velar lobe of larvae of *I. obsoleta* revealed action potentials that correlated with pre-oral ciliary arrests. These action potentials likely originated from the ciliated epithelial cells rather than muscle, since muscle twitches independent of ciliary arrests did not cause action potentials. Moreover, the origin of such action potentials from the epithelial cells containing the pre-oral cilia and not muscle or neurons has been well established using intracellular recordings in other gastropod larvae (Arkett *et al.*, 1987; Arkett, 1988). These studies have demonstrated that the ciliary arrests correlate with action potentials from the ciliated cells and such action potentials were mediated by calcium (Arkett *et al.*,

1987; Arkett, 1988). If the action potentials correlated with ciliary arrests then it would be expected that the frequency of action potentials reflected the number of ciliary arrests. Also, it would be likely that the amplitude of the compound action potentials were correlated with the number of ciliated cells recorded in *I. obsoleta*. This was supported by the general observation that the large tip opening in the suction electrode, and thus a larger portion of the velum filling the opening, resulted in larger action potentials. Furthermore, if these action potentials were regulated by the nervous system, then it would be expected that at least some of the chemicals tested would alter the properties, specifically the amplitude and frequency of electrical recordings in *I. obsoleta*.

Significant changes in the electrical recordings were achieved when larvae were exposed to serotonin and fluoxetine. These chemcials decreased amplitude and frequency of the compound action potentials and most significantly, at high concentrations, the action potentials were abolished. Such results may be due to an inhibitory action of serotonin on the ciliary arrests and muscle contraction. These results also support the suggestive (but non-significant) results of the rate of locomotory arrests. Therefore serotonergic neurons, appeared to have two roles: to increase ciliary activity and to inhibit pre-oral ciliary arrest and muscle contractions.

Unfortunately, the recordings failed to show any changes when larvae were exposed to synthetic FMRFamide. Since this neuropeptide was shown to increase the number of ciliary arrests it was expected that it would increase the frequency of the action potentials. These negative results, though, may be explained by technical constraints.

For example, the suction electrode used to capture the electrical properties of the velar cells probably caused a constant stimulation of mechanoreceptors and thus produced a ceiling effect on rates of ciliary arrests and numbers of cells activated. Such an idea is supported by the fact that in control recording the frequency ranged from 15 to 50 spikes per minute, where as the normal rate as shown in the locomotory rate test was on average 1.0 arrest per minute. Future studies that are designed so that the locomotory arrest response is not activated by the recording electrode. Furthermore, intracellular recordings would also be useful to determine how both FMRFamide and serotonin as well as other possible transmitters regulate the activity of pre-oral cilia.

5. Conclusions and Future Experiments

In the present study, it was shown that serotonin, catecholamines, FMRFamide and leu-enkephalin related peptides may have endogenous roles of in controlling locomotion and feeding. The results of the present study, including the anatomical locations of axons and neurons within the velum and the effects of transmitters and analogs on larval behaviors and physiological properties of ciliated cells have allowed the creation of a hypothetical model shown in figure 28 that explains neural control in the velum. While some specific roles of these transmitters were postulated, this study clearly shows additional studies concerning the physiology of muscle fibers and cells containing the pre-and post-oral cilia are necessary to elucidate how the nervous system regulates larval behaviors. Furthermore, measurements of the swimming velocity and ciliary

movements will also help to determine more precisely how the nervous system may function in controlling swimming and feeding behaviors. Finally, similar studies in other species would be useful to understand how generalized the functioning of nervous systems are in gastropod and other molluscan larvae.

Figure 19: Feeding rates versus algal concentration by larvae of *I. obsoleta*. The graph indicates that at higher concentrations of algae the feeding rates of the larvae increase. It also demonstrates the high degree of variability in feeding rates by larvae at the hatchling stage.

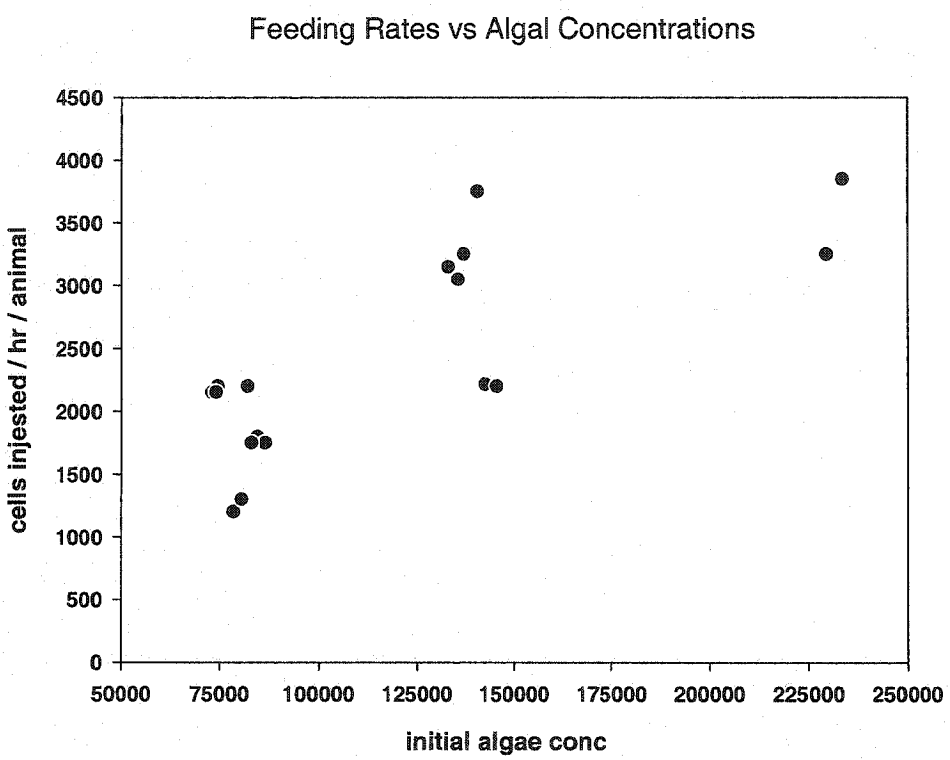

Feeding Rates vs Algal Concentrations
cells injested / hr / animal
4500
4000
3500
3000
2500
2000
1500
1000
500
0
50000
75000
100000
125000
150000
175000
200000
225000
250000
initial algae conc

Figure 19

Figure 20: Immunocytochemical localization of neurons and axons (green) and F-actin labeling of muscle (red) in *I. obsoleta* during veliger stages. **A)** Lateral view of a velar lobe showing FMRFamide-like immunoreactive axons (arrow) and muscle fibers (arrowhead). Scale bar is approximately 30 μm. **B)** Lateral view of a velar lobe showing leu-enkephalin-like immunoreactive axons (arrow) and muscle fibers (arrowhead). Scale bar is approximately 30 μm. **C)** Lateral view of a velar lobe showing serotonin-like immunoreactive axons (arrow) and muscle fibers (arrowhead). Scale bar is approximately 25 μm. **D)** Lateral view of a velar lobe showing TH-like immunoreactive cells and axons (arrows). Also indicates cells containing pre- and post-oral cilia. Scale bar is approximately 30 μm. **E)** High magnification of the edge of a velar lobe showing FMRFamide-like immunoreactive axons (arrow) and muscle fibers (arrowhead). Note the cells containing pre-oral cilia. Scale bar is approximately 10 μm. **F)** High magnification of the edge of a velar lobe showing TH-like immunoreactive cells and axons (arrows) and cells containing pre- and post-oral cilia. Scale bar is approximately 10 μm. **G)** High magnification of the vase-shaped TH-like immunoreactive cells and axons (arrows) surrounding the mouth. Scale bar is approximately 10 μm.

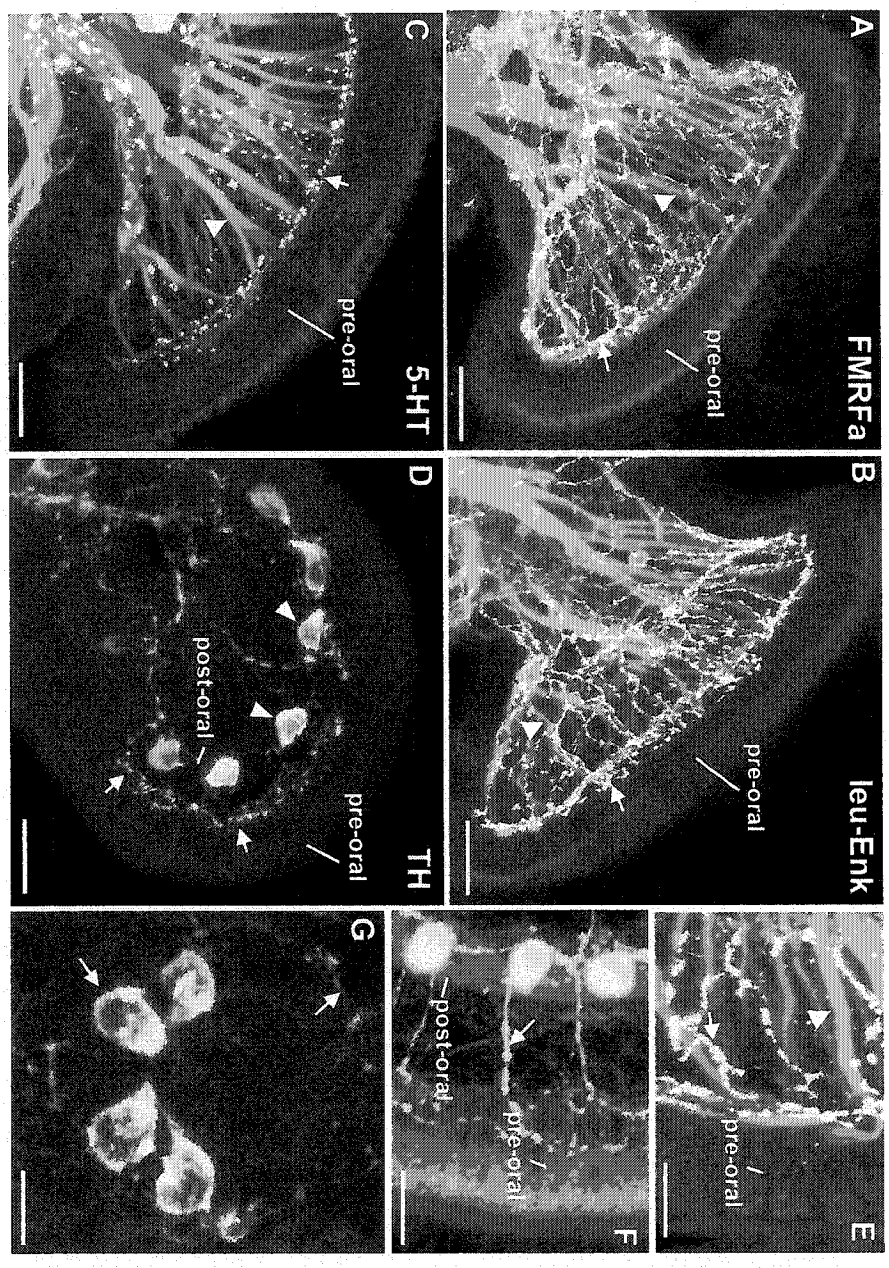

A
FMRFa
pre-oral
B
leu-Enk
pre-oral
C
5-HT
pre-oral
D
TH
pre-oral
post-oral
E
pre-oral
F
post-oral
pre-oral
G

Figure 20

Figure 21: Schematic diagram of a lateral view of one lobe of the velum of *I. obsoleta*. **A)** General arrangement of the ciliary bands in the velar lobe. **B)** Arrangement of muscle fibers (grey shaded regions) and axons (black dashed lines) labeled with FMRFamide, serotonin and leu-enkephalin in a velar lobe. **C)** Arrangement of catecholamine containing cells and axons (black) in a velar lobe.

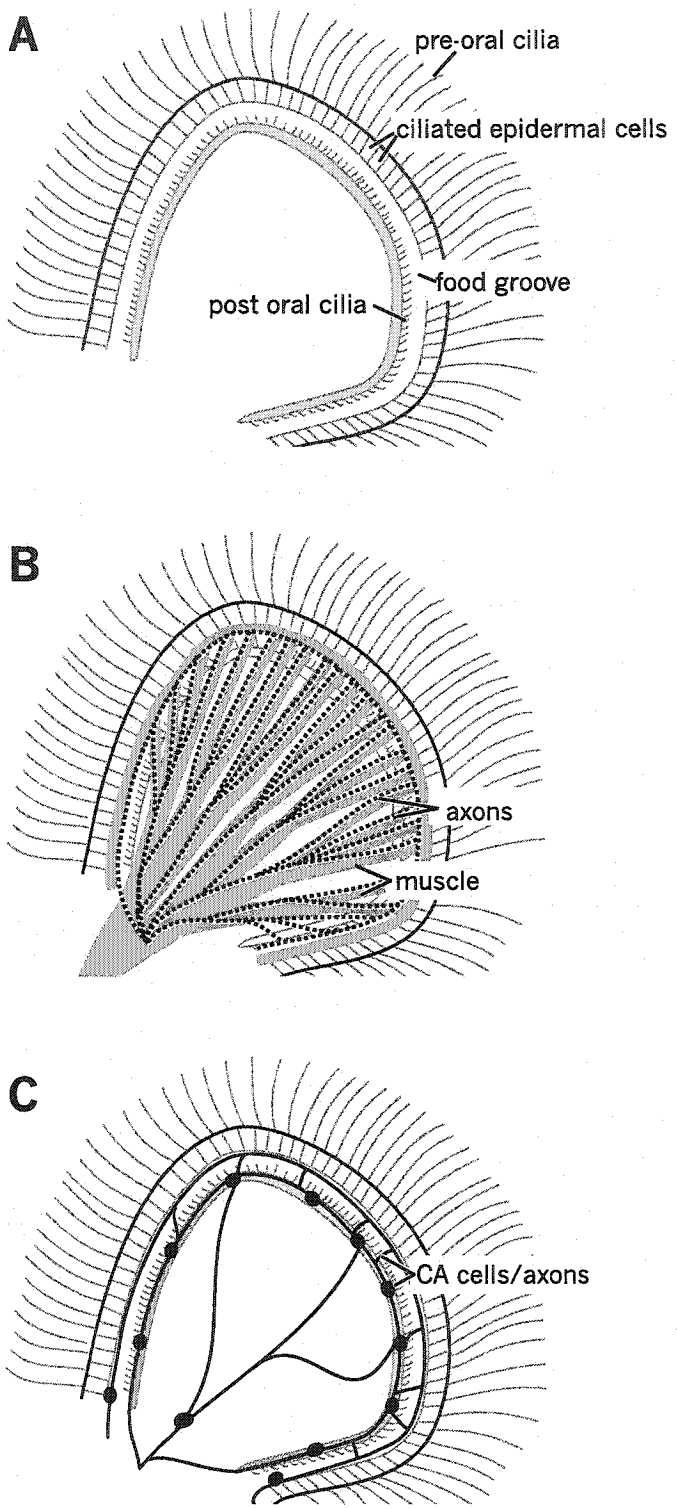
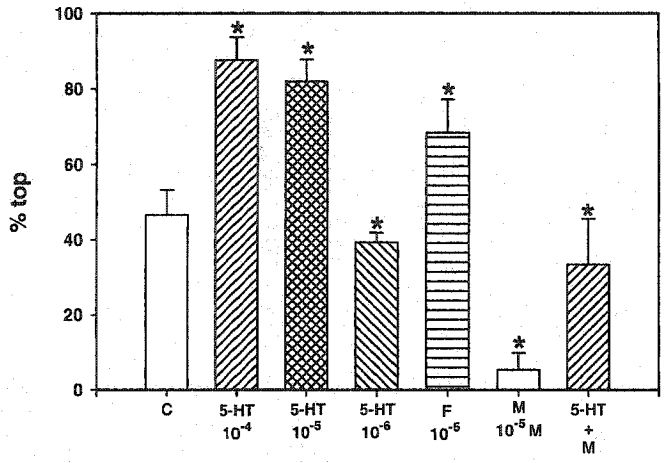

A
pre-oral cilia
ciliated epidermal cells
food groove
post oral cilia
B
axons
muscle
C
CA cells/axons

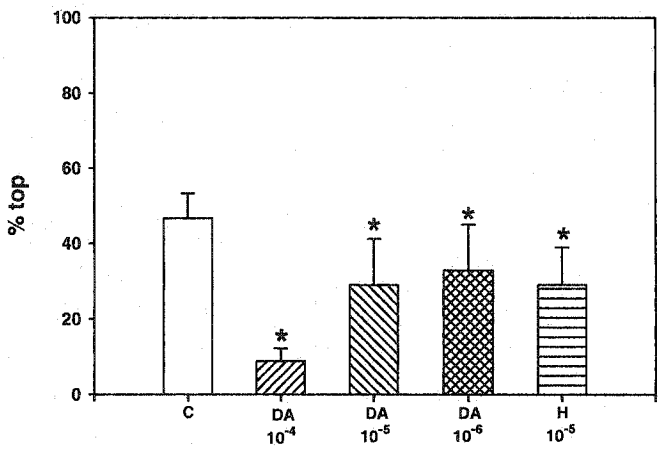
Figure 21

Figure 22: Vertical distribution test, the average percentage of larvae of *I. obsoleta* in the top of the water column. **A)** The percentage in the top when larvae were exposed to serotonin analogs. **B)** The percentage in the top when larvae were exposed to dopamine and haloperidol. **C)** The percentage in the top when larvae were exposed to norepinephrine, spiperone and alprenolol. Abbreviations: C, control; 5-HT, serotonin; F, fluoxetine; M, mianserin; DA, dopamine; H, haloperidol; NE, norepinephrine; A, alprenolol; S, spiperone; NE + S, norepinephrine (10^{-5} M) and spiperone (10^{-5} M); 5-HT+M, serotonin (10^{-6} M) and mianserin (10^{-7} M). Asterisks indicate significant difference ($P<0.05$) when compared with the control group.

A. Serotonin



B. Dopamine



C. Norepinephrine

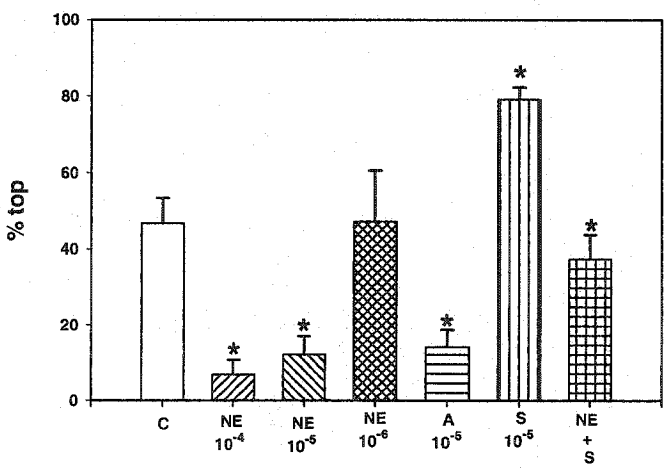


Figure 22

Figure 23: Feeding rates expressed a percentage of the control in larvae of *I. obsoleta*. The dotted line represents the control value of 100%. Abbreviations: 5-HT, serotonin; M, mianserin; DA, dopamine; NE, norepinephrine; S, spiperone; A, alprenolol. Asterisks indicate significant difference ($P<0.05$) when compared with the control group.

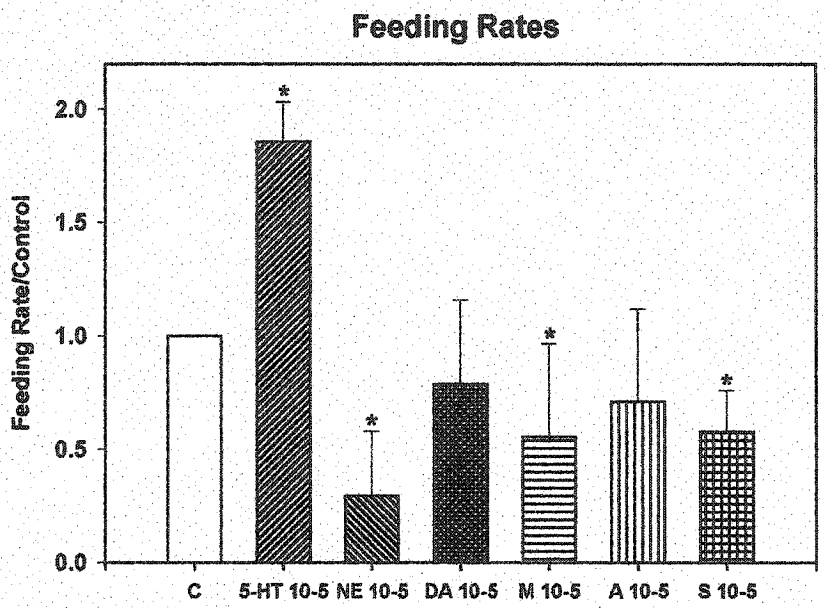
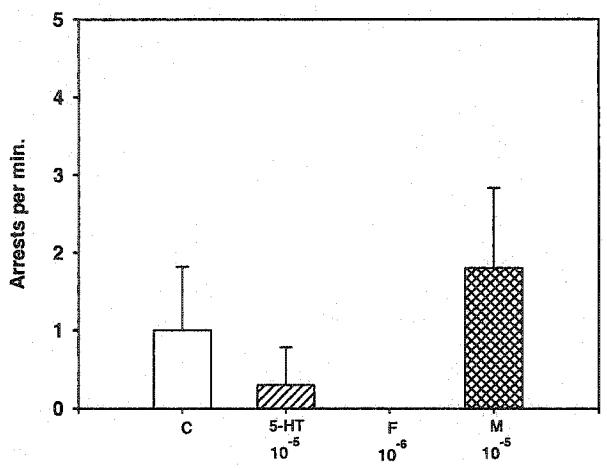

Feeding Rates
Feeding Rate/Control
2.0
1.5
1.0
0.5
0.0
*
*
*
*
C
5-HT 10-5
NE 10-5
DA 10-5
M 10-5
A 10-5
S 10-5

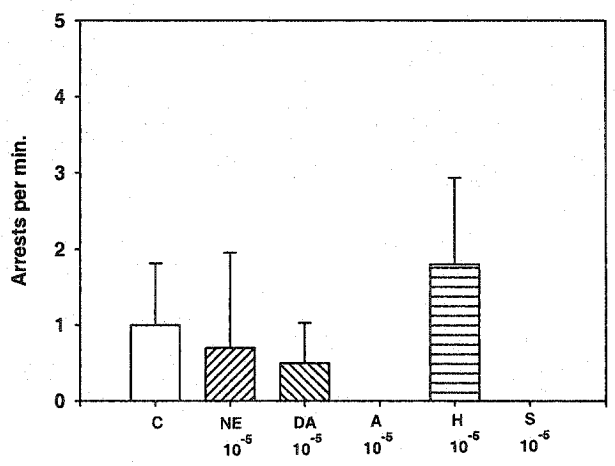
Figure 23

Figure 24: The average number of locomotory arrests per minute when larvae of *I. obsoleta* exposed to various chemicals. **A)** The average number of arrests when larvae were exposed to serotonin analogs. **B)** The average number of arrests when larvae were exposed to catecholamine analogs showed no significant changes compared to the control. **C)** The average number of arrests when larvae were exposed to 10^{-6} M synthetic FMRFamide was significantly greater than the control, whereas 10^{-6} M synthetic leu-enkephalin had no effect. Abbreviations: C, control; 5-HT, serotonin; F, fluoxetine; M, mianserin; DA, dopamine; H, haloperidol; NE, norepinephrine; A, alprenolol; S, spiperone; FM, synthetic FMRFamide; E, synthetic leu-enkephalin. Asterisks indicate significant difference ($P<0.05$) when compared with the control group.

A. Serotonin



B. Catecholamines



C. Peptides

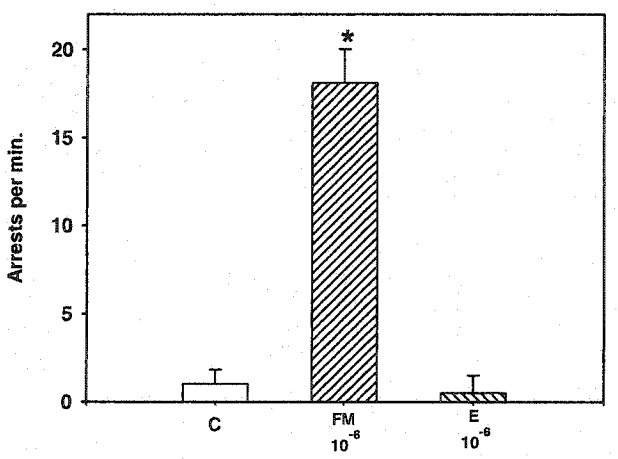


Figure 24

Figure 25: The average time for larvae of *I. obsoleta* to recommence normal swimming after a mechanical stimulus when exposed to various chemicals. **A)** The average recovery times when larvae were exposed to serotonin analogs. **B)** The recovery times when larvae were exposed to dopamine and haloperidol. **C)** The recovery times when larvae were exposed to norepinephrine, spiperone and alprenolol. Spiperone (10^{-4} M) was the only chemical that abolished touch responses. **D)** The recovery times when larvae were exposed to synthetic neuropeptides. Abbreviations: C, control; 5-HT, serotonin; F, fluoxetine; M, mianserin; DA, dopamine; H, haloperidol; NE, norepinephrine; A, alprenolol; S, spiperone; FM, synthetic FMRFamide; E, synthetic leu-enkephalin. Asterisks indicate significant difference ($P<0.05$) when compared with the control group.

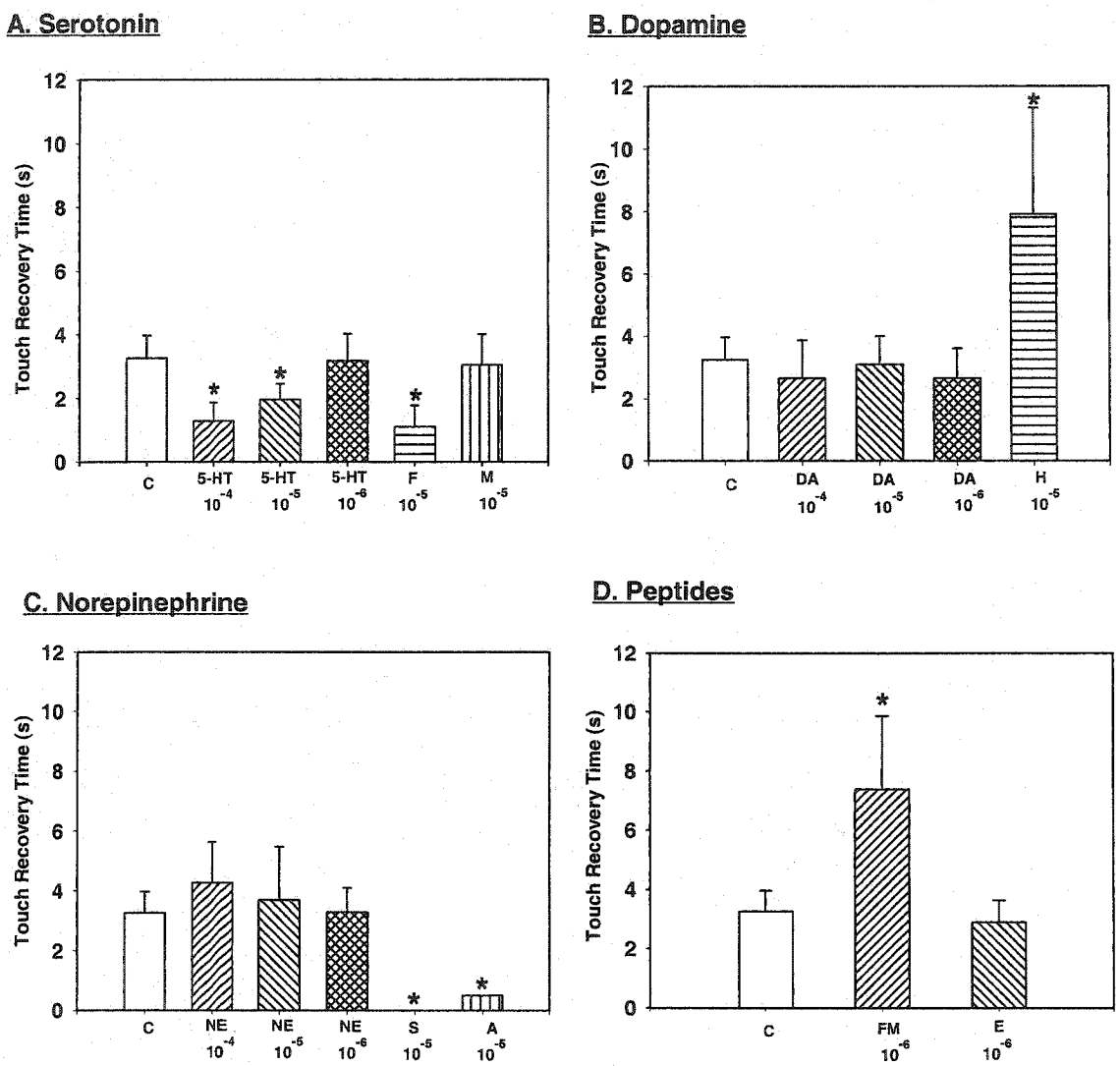

A. Serotonin
B. Dopamine
C. Norepinephrine
D. Peptides
Touch Recovery Time (s)
0
2
4
6
8
10
12
C
5-HT 10-4
5-HT 10-5
5-HT 10-6
F 10-5
M 10-5
DA 10-4
DA 10-5
DA 10-6
H 10-5
NE 10-4
NE 10-5
NE 10-6
S 10-5
A 10-5
FM 10-6
E 10-6

Figure 25

Figure 26: Sample traces from electrical recordings from the edge of velar lobes from larvae of *I. obsoleta.* **A)** Several action potentials superimposed from a control recording. **B)** Recording soon after serotonin (10^{-4} M) was added, showing the reduction in amplitude of the action potentials. **C)** The last 2 minutes of a single trial, showing control, after serotonin was added and then after the wash out. **D)** The last 2 minutes of a single trial, showing control, after dopamine was added and then after the wash out.

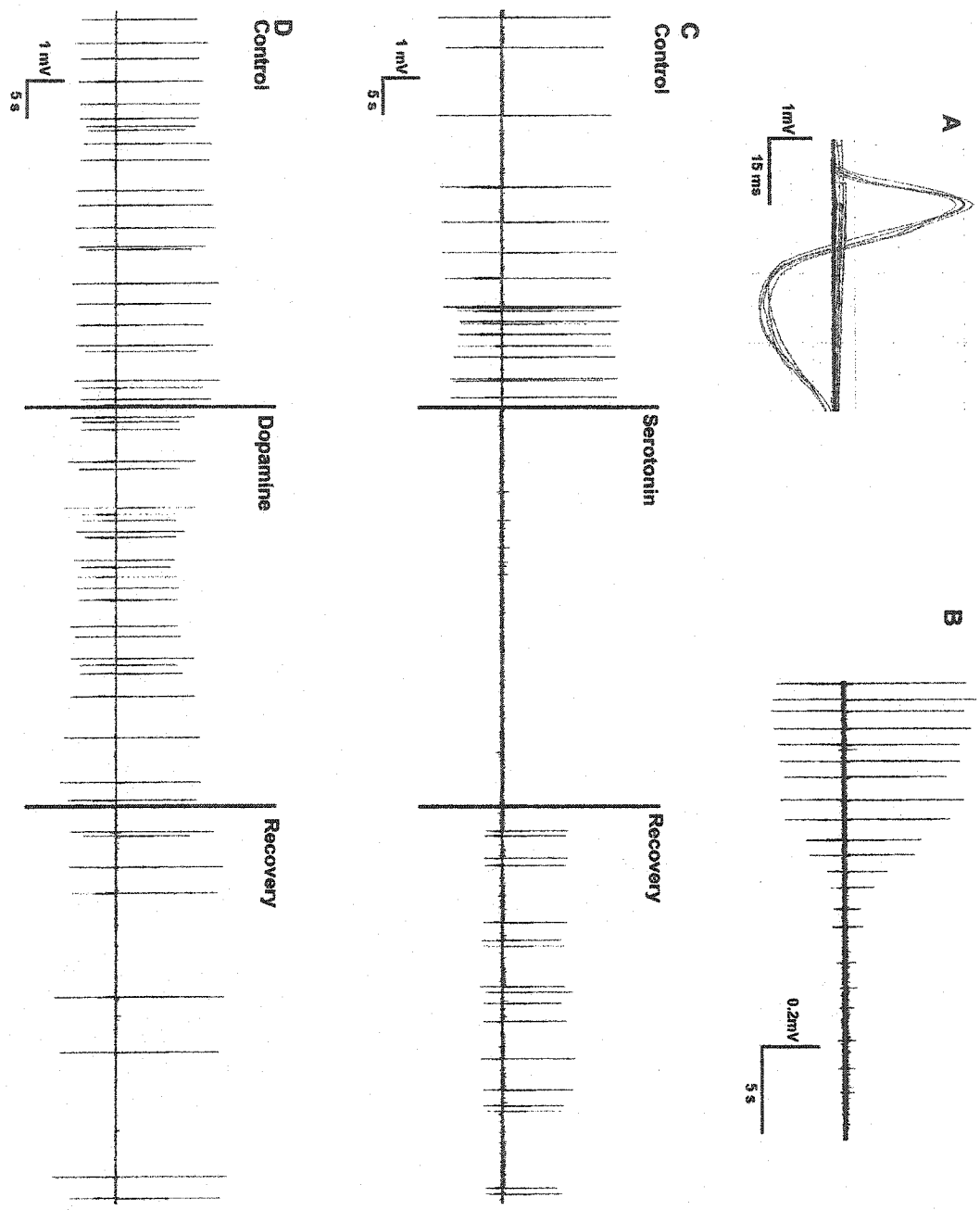

A
1mV
15 ms
B
0.2mV
5 s
C
Control
1 mV
5 s
Serotonin
Recovery
D
Control
1 mV
5 s
Dopamine
Recovery

Figure 26

Figure 27: Average amplitude (**A**) and frequency (**B**) expressed as a percentage of the control. All chemicals were used at 10^{-4} M. Abbreviations: C, control; 5-HT, serotonin; DA, dopamine; NE, norepinephrine; FM, synthetic FMRFamide. Asterisks indicate significant difference ($P<0.05$) when compared to the control group.

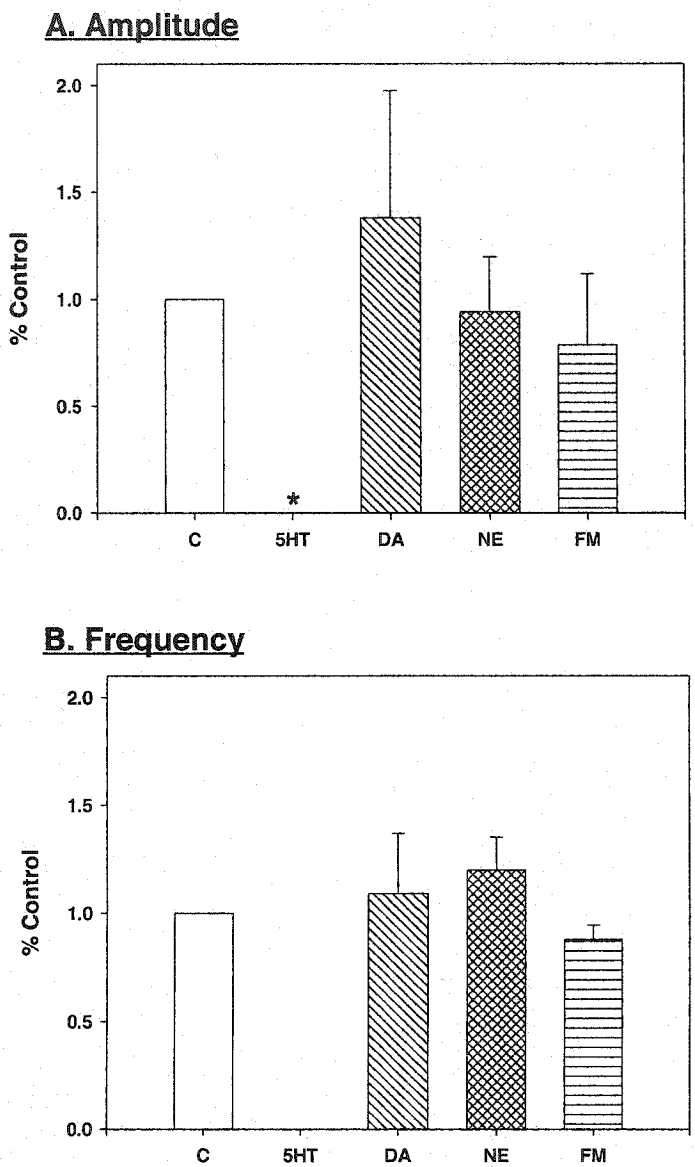

A. Amplitude
% Control
2.0
1.5
1.0
0.5
0.0
*
C
5HT
DA
NE
FM
B. Frequency
% Control
2.0
1.5
1.0
0.5
0.0
C
5HT
DA
NE
FM

Figure 27

Figure 28: Hypothetical model of the neuronal control of cilia and muscle in the velum of *I. obsoleta.* **A)** FMRFamide directly (or indirectly) controls ciliary arrest of the pre-oral cilia and muscle contraction. **B)** Leu-enkephalin modulates the action of FMRFamide. **C)** Serotonin increases ciliary activity, and inhibits ciliary arrests and muscle contractions. **D)** Catecholamines act to decrease and/or coordinate the ciliary activity of the pre and post-oral bands. In addition, some catecholamine containing cells also mediate responses from mechanical stimuli by directly (or indirectly) affecting FMRFamide neurons in the apical organ (or cerebral ganglia).

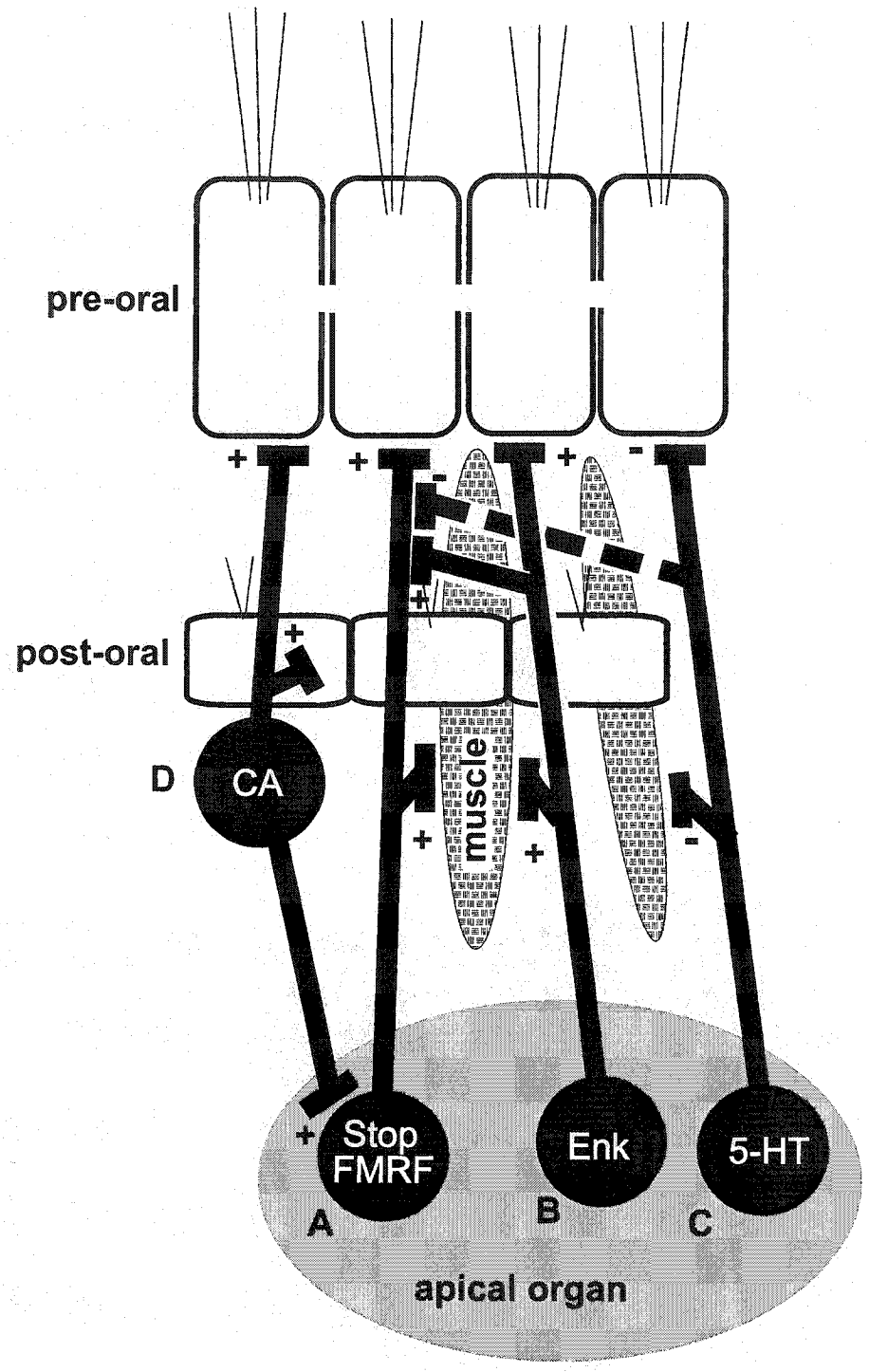

pre-oral
post-oral
D
CA
muscle
Stop
FMRF
Enk
5-HT
A
B
C
apical organ

Figure 28

CHAPTER 4

SPECIFICATION OF THE GASTROPOD LARVAL NERVOUS SYSTEM: DISTAL-LESS EXPRESSION AND THE ROLE OF THE D LINEAGE IN *ILYANASSA OBSOLETA*

Introduction

Recent investigations have uncovered sophisticated larval nervous systems in several species of molluscs. For example, the larval nervous system of the gastropod *Ilyanassa obsoleta* consists of an apical organ, peripheral neurons and the developing central ganglia that will eventually become the adult central nervous system (CNS) (see chapter 2). Cells within the larval nervous system are found to contain several different transmitters such as serotonin, catecholamines and peptides such as FMRFamide and enkephalin. However, while the anatomy and functions of such nervous systems are becoming increasingly clear, little is known about the mechanisms governing neurogenesis in this taxon.

What little is known about neural development in molluscs concerns the cellular origins of the ganglia that form the adult CNS and the apical organ. The central ganglia originate from specialized epithelial regions through the processes of proliferation followed by invagination or delamination (Raven, 1966). In addition, further growth of the ganglia occur through the migration of cells from the ectodermal proliferation zones (Jacob, 1984). The apical organ has been reported to develop from a specialized ectodermal region, the apical plate (Raven, 1966). Thus obvious questions arise involving the cellular and genetic mechanisms that govern the development of the different components of the larval nervous system.

In other phyla the molecular networks governing the patterning and differentiation of the nervous system have been the focus of numerous studies (for examples see Sasai, 2001b; Sasai, 2001a). It has become clear that many genes involved in neural development are conserved in diverse taxa. One such well conserved gene is *Distal-less*, a homeobox gene that codes for a transcription factor that is activated several times during development and participates in the differentiation of various organ systems, including the nervous system (for review see Merlo *et al.*, 2000). The first studies of *distal-less* focussed on the possible conserved role in the proximal-distal formation of limbs in several different taxa (for review see Panganiban *et al.*, 1994; Panganiban *et al.*, 1997). While previous investigations of the expression of the distal-less protein (Dll) during molluscan development did not demonstrate involvement in limb outgrowth, this protein was suggested to be involved in the establishment of embryonic polarity and differentiation of ectoderm (Lee and Jacobs, 1999). However, later larval stages of molluscs were not examined and therefore expression of Dll in the larval nervous system was not established. Thus whether *distal-less* or any other conserved genes operate in neural development in molluscs is unknown. It might be argued, however, that since molluscs represent a major bilaterian group, understanding the cellular and genetic interactions underlying the development of nervous systems may provide valuable insights into how nervous systems evolved.

Although relatively little is known about molecular aspects governing development in gastropod molluscs, other features of their development have been well

studied. For, example considerable cell lineage data exists for several gastropod species in particular, *I. obsoleta*. Molluscs undergo spiral cleavage and initially produce four macromeres A, B, C, and D, after the second cleavage. During the next three cleavage cycles, each macromere divides to form smaller micromeres at the animal pole (for review see Verdonk and van den Biggelaar, 1983). In general, these micromeres form the ectoderm which will develop into the apical tuft cells, eyes, tentacles cerebral ganglia, shell secreting cells, foot, velum, operculum secreting cells and mantle epithelium of *I. obsoleta* (Clement, 1967; Clement, 1986a; Clement, 1986b; Render, 1991; Render, 1997). The micromeres created after the fourth cleavage and all the macromeres generally form the mesoderm which will form the heart, kidney and muscle and the endoderm that will become digestive organs (Clement, 1967; Clement, 1986a; Clement, 1986b; Render, 1991; Render, 1997). Caenogastropods, including *I. obsoleta*, utilize a polar lobe to shunt maternal determinants into the CD blastomere after the first cleavage and then the D blastomere after the second cleavage (Verdonk and van den Biggelaar, 1983). Experiments have clearly demonstrated the importance of these maternal determinants contained within the D lineage in establishing axial properties and normal organization of the molluscan larva (Clement, 1962; Atkinson, 1971; Cather, 1973; Verdonk and Cather, 1983). Furthermore, the D lineage likely influences other cell fates via inductive mechanisms that involve the MAP kinase signaling pathway and conserved patterning genes (Lambert and Nagy, 2001; Lartillot *et al.*, 2002). Therefore, the role of

such early cell signaling in the specification of the larval nervous system has been investigated in the present study in the gastropod *I. obsoleta.*

Presently, the genetic mechanisms involved in neural development were explored by examining Dll protein expression at different stages of development in the well studied gastropod, *I. obsoleta.* Secondly, the role of early cell signaling in the specification of the nervous system was investigated by ablating the source of inductive signals, the D macromere. After D macromere ablations, changes in the formation of neurons and in the Dll expression pattern in the resultant larvae were examined. The results of this current study present among the first evidence of mechanisms that govern neural development in a mollusc. Furthermore, the possibility that molluscs share a conserved genetic program that is involved in the differentiation of components of the gastropod nervous system is explored.

Materials and Methods

Adult and Larval Culture

Refer to Chapter 2, page 44

The stages examined for normal Dll-like immunoreactivity (see below) included cleavage (1-6 hours after fertilization), trochophore (2.5- 4 days), embryonic veliger (4.5-7 days), hatchling (7-10 days), and free swimming veliger (14 days was used a representative).

D macromere ablations

The D macromere was ablated between the 3rd and 4th cleavages by puncturing the cell (Clement, 1956) with a borosilicate glass capillary tube (Warner Inst. Corp., outer diameter 1.0 mm, 0.78 mm inner diameter, Hamden, CT) pulled to a sharp point with Brown-flaming micropipette puller (Model P-77, Sutter Instruments Co., California). Preliminary experiments revealed similar results when the D macromere was ablated prior to 5th cleavage, which is consistent with findings by Clement (1962) and Lambert and Nagy (2001). The operations were performed in 10 ml glass Petri dishes under a binocular stereo-dissecting microscope. Experiments were performed on groups of animals from several egg masses and several larvae within the same egg mass were not operated upon to serve as controls. At least 30 animals were examined for each immuno-label. All operations for a single group were performed within 10-15 minutes, well

before the next set of cell divisions. After approximately 15-30 minutes, the entire D macromere separated from the other cells and later disintegrated (see appendix 3). The resulting larvae were then transferred into a new Petri dish containing 0.2 μm filtered natural seawater with 0.10 μg/ml penicillin G. The sea water was changed each day and after 7 days larvae were fixed and then processed for immunocytochemistry as described below. At least 20-30 operated larvae from several different egg masses were examined.

Immunocytochemistry

Refer to Chapter 2, page 45

Antibodies

The larvae fixed with 4% PFA were then incubated in one of the polyclonal antibodies; anti-FMRFamide, anti-serotonin, anti-leu-enkephalin (all from Diasorin, Stillwater, MI) or an antibody against the butterfly Distal-less homeodomain (Panganiban *et al.*, 1997) generously provided by Dr. Jhumku Kohtz (Northwestern University Medical School). Larvae fixed with methanol were incubated in monoclonal anti-tyrosine hydroxylase (TH) (Diasorin, Stillwater, MI). Anti-Dll was diluted 1:50 in PBS and all other antibodies were diluted 1:500 in PBS with the addition of 1.0% normal goat or sheep serum.

Catecholamine Histofluorescence

Refer to Chapter 2, page 48

Photography

Refer to Chapter 2, page 48

Results

1. A) Normal Dll immunoreactivity:

During the single cell and early cleavage stages, up to the 5th cleavage, all the nuclei were labeled with anti-Dll (Fig. 29A, B). In stages after the 5th cleavage Dll immunoreactivity was only observed in the micromeres (Fig. 29C) and while the intensity in each nucleus was not the same, no discernible pattern was observed. Later as the larva underwent gastrulation, the micromeres grew over the large endomeres by epiboly and only nuclei of the presumptive ectoderm were labeled. Confocal sections indicated that mesodermal and endodermal cells located in the middle of the gastrula were devoid of Dll-like immunoreactive (LIR) nuclei (see Fig. 30A). During the trochophore stage (2.5-4 days after first cleavage) cell nuclei of only specific ectodermal regions were labeled with anti-Dll. Such regions included bilaterally symmetrical regions in the dorsal ectoderm of the head that resembled the cephalic plates (see Fig. 30B, arrows), an area of dorsal post-trochal ectoderm (see Fig. 30B, arrowhead) and a region of ectoderm on the developing foot (not shown).

By 5-6 days after first cleavage, the larvae have entered the veliger stage and Dll-LIR nuclei existed in cells in the regions of the future cerebral, pedal and intestinal ganglia (Fig. 29D). Also anti-Dll labeled many axons which radiated into the velum and foot and formed ganglionic connectives at this stage (Fig. 29D). During free swimming larval stages of *I. obsoleta* (7-14 days after first cleavage) Dll immunoreactivity

continued to be observed in many axons and within nuclei of cells within the regions of the central ganglia (Fig. 29E). During these veliger stages, Dll labeling was not observed in nuclei in the region of the apical organ or in regions of previously identified peripheral neurons in the velum, foot or posterior (see chapter 2).

1. B) Dll immunoreactivity Following D Macromere Ablation

One day after ablation the controls had reached the gastrula stage and the nuclei of ectodermal cells were exclusively labeled with anti-Dll (Fig. 30A). In the D macromere-ablated larva, Dll immunoreactivity appeared in nuclei of all cells (Fig. 30D). By three days after ablation the control larvae had reached the trochophore stage and anti-Dll labeling was restricted to nuclei of cells within specific regions of the ectoderm (Fig. 30B). However, no Dll-LIR labeling appeared in the D macromere ablated larvae at the corresponding time (Fig. 30E). After seven days, control larvae had reached the hatchling stage. Nuclei of cells within the cerebral and intestinal ganglia and many axons were labeled with anti-Dll (Fig. 30C). However, the D macromere ablated larvae were devoid of Dll-LIR nuclei at this time (Fig. 30F)

2. The Presence of Neural Elements Following D Macromere Ablation

The normal pattern of neural elements containing FMRFamide, serotonin, leu-enkephalin and catecholamines have been described previously (see chapter 2). The first neurons appeared in the trochophore stage, approximately 3 days after first cleavage. By

the hatchling stage, seven days after first cleavage, the larval nervous system consisted of an apical organ, peripheral cells and processes in the foot, velum, mantle regions and the developing central ganglia. Neurons were first observed in D macromere-ablated larvae 4-5 days after ablation. However, the most consistent results were observed in larvae 7 days after ablation. Therefore, the results reported here are based on observations of larvae one week following ablations.

A) FMRFamide-like immunoreactivity

Anti-FMRFamide labeling appeared in the control larvae, 7 days after first cleavage, as described previously (see chapter 2). Briefly, a total of 14 FMRFamide-LIR cells existed in the hatchling (see Fig. 31A for a representative figure showing some of the cells described below). Two pairs of bilaterally symmetrical cells and one unpaired cell were observed in the apical organ. The central most pair of apical cells were distinctly vase-shaped while the remaining apical cells were round. Axons with many varicosities formed an apical neuropil just ventral to the apical cells. FMRFamide-LIR fibers extended posteriorly from this neuropil to form a figure eight. This pattern was characteristic of the connectives which form the abdominal ganglia of the adult CNS. Two FMRFamide-LIR cells lay along these abdominal connectives in the regions of the future sub- and supra-intestinal ganglia. A FMRFamide-LIR cell was also present in the regions of each of the future cerebral ganglia. Also peripheral neurons were observed, two bilaterally symmetrical pairs of cells in the foot and a single cell in the mantle.

FMRFamide-LIR processes originating from the apical neuropil and/or cerebral ganglia radiated into the foot, the velar lobes and mantle region.

In the D macromere ablated larvae, two to four FMRFamide-LIR cells were observed and at least two of these cells were always vase-shaped (Fig. 31D, arrows). Sometimes roundish FMRFamide-LIR cells were observed, however, their shape was often irregular when compared to the cells in control larvae (Fig. 31D, arrowhead). In addition, FMRFamide-LIR axons also existed throughout many larvae (Fig. 31D). These FMRFamide-LIR cells and axons were unorganized and the pattern of immunoreactivity varied in each of the animals examined.

B) Serotonin-like immunoreactivity

Serotonin-like immunoreactivity in the control larvae appeared similar to the normal larvae described previously (see chapter 2). There were a total of 13 serotonin-LIR cells in the hatchling stage (see Fig. 31B as a representative showing some of the cells described below). In the apical organ there were three serotonin-LIR vase-shaped cells, a bilaterally symmetrical pair and a single medial cell. Also a bilaterally symmetrical pair of round serotonin-LIR cells existed in the apical organ. Just ventral to these apical cells was a neuropil of processes with many varicosities. Serotonin-LIR fibers extended posteriorly from this neuropil to form a figure eight, characteristic of the pattern of connectives which form the abdominal ganglia of the adult CNS as described with anti-FMRFamide labeling. Three serotonin-LIR cells existed in the developing

osphradial ganglia. Also a bilaterally symmetrical pair of serotonin-LIR cells was also observed in the developing pedal ganglia. Serotonin-LIR process originating from the apical neuropil and/or cerebral ganglia radiated into the foot, the velar lobes and mantle region.

In the D macromere-ablated larvae there was consistently one bright serotonin-LIR cell (Fig. 31E, arrow). This cell often vase-shaped, with a short narrow apical dendrite. Also surrounding this cell was serotonin-LIR processes with many varicosities that resembled a neuropil (Fig. 31E). In approximately 80% of the larvae examined there were also 1-2 very faintly labeled serotonin-LIR cells located near the one brightly labeled cell (Fig. 31E, arrowheads). Also in some D macromere-ablated larvae, serotonin-LIR patches of tissue were also observed which were not obvious cells (Fig. 31E, asterisks).

C) Leu-enkephalin-like immunoreactivity

There was a total of eight leu-enkephalin-LIR cells in the control larvae as described previously (chapter 2) (see Fig 31C). Four pairs of bilaterally symmetrical vase-shaped leu-enkephalin-LIR cells were located in the apical organ. In addition, leu-enkephalin-LIR processes were observed radiating into the velar lobes and foot.

In D macromere-ablated larvae there was only one enkephalin-LIR cell observed. This cell appeared vase-shaped however the apical neck was narrower and longer than the

cells appeared in the controls (Fig. 31F, arrow). No leu-enkephalin-LIR axons were observed in the D macromere-ablated larvae.

D) Faglu reaction and TH-like immunoreactivity

In the hatchling stage, the Faglu technique and TH-like immunoreactivity revealed the same pattern of 28-30 catecholamine-containing cells (see Fig. 32A, B). Approximately 10-12 catecholamine-containing cells were located in each velar lobe. Also four vase-shaped cells surrounded the ventral and lateral edges of the mouth and pair cells were located near the tip of the foot. Catecholamine-containing cells existed in each of the developing cerebral ganglia and axons in the cerebral commisure were also labeled by both techniques.

The Faglu technique and labeling with anti-TH revealed up to 10-20 catecholamine-containing cells in the D macromere-ablated larvae (Fig 32C, D, arrows). Although there was a high degree of variability between each animal examined, some consistent patterns were observed. The cells were usually located in groups and were associated with large ciliated cells resembling cells that were normally located in the velum (not shown).

Discussion

In the present study, it was shown that the distal-less protein (Dll) was normally expressed throughout embryonic and larval stages, and consequently this gene/protein likely has multiple functions in molluscan development. One such role, in *I. obsoleta*, seems to be the involvement of Dll in differentiation and/or patterning the ectoderm, and the development of the nervous system. Dll expression in the nervous system was limited to the developing ganglia that persist into adult stages. The protein was not expressed in other regions of the larval nervous system such as the apical organ and velar peripheral cells that normally disappear at metamorphosis. Therefore, based on expression patterns of the protein, it is possible that separate genetic programs and cellular mechanisms govern the differentiation of the future adult CNS versus the other components of the larval nervous system. This hypothesis was further demonstrated in experiments in which the D macromere was ablated. The resultant larvae lacked the normal Dll expression pattern in the ectoderm and in the developing CNS and only neurons outside the developing central ganglia were observed.

Specificity of the Dll Antibody

Here, as in many other studies of invertebrate distal-less protein expression, we use an antibody developed against the homeodomain region of the butterfly distal-less protein (Panganiban *et al.*, 1994). Furthermore, A *distal-less* gene fragment has recently

been isolated and sequenced in two species of molluscs, the bivalves *Nutricola* and *Crassostrea gigas* and it was shown that these molluscan gene sequences and amino acid translations shared a high degree of similarity in the homeodomain regions of orthologues from other animals, especially with arthropods (Lee *et al.*, 2001) Therefore, it is likely that the antibody used in this study also recognizes the molluscan Dll protein.

Normal Distal-less Protein Expression

A) Early Expression and Ectoderm Differentiation

In this present report it was revealed that Dll expression patterns in early developmental stages of the mollusc, *I. obsoleta* were similar to distribution patterns previously reported in the gastropod *Lelletia kelletii* (Lee and Jacobs, 1999). In *I. obsoleta* and in other molluscs, Dll was found in the fertilized egg, and in the nuclei of all cells during early cleavage stages. Such an expression pattern has been suggested to reflect a conserved maternal transcript which has also been reported in vertebrates (Asano *et al.*, 1992; Merlo *et al.*, 2000). Later, Dll was observed in the presumptive ectoderm during late cleavage and gastrulation in *I. obsoleta,* similar to previous reports in the other molluscan species as well as a variety of other taxa. For example, ectodermal expression has been reported in the lower chordate, *Amphioxus* (Holland *et al.*, 1996) and in vertebrates, *Xenopus* (Luo *et al.*, 2001) and the mouse (Morasso *et al.*, 1996). These studies have suggested that the expression in the ectoderm reflects a role of Dll in regulating the initial differentiation of this germ layer into epidermal, neural plate and

neural crest tissue (Luo *et al.*, 2001). However, further experiments are necessary to determine the precise role of Dll in ectodermal differentiation in molluscs.

B) Dll and Nervous System Development

In the trochophore stage of *I. obsoleta* Dll was expressed in specific ectodermal areas which appeared to be the regions that generate the central ganglia of the future adult CNS. Jacob (Jacob, 1984) reported that in another gastropod *Aplysia californica*, the ganglia develop from proliferative zones of ectodermal cells, which were established in regions of the body wall adjacent to underlying mesodermal cells. These proliferative zones generated a population of cells which left the surface and migrate inward to join the nearby forming ganglia. Jacob (1984) suggested that this pattern of neurogenesis in the molluscan central nervous system resembled the proliferation of cells in the neural tube and the migration of neural crest and ectodermal placode cells in the vertebrate nervous system. *Distal-less* orthologues were also expressed in migratory regions in the developing vertebrate CNS (for review see Mayor and Aybar, 2001). Furthermore, in the lower chordate, Amphioxus, a *distal-less* orthologue was observed in migratory epidermal cells that bordered the neural plate, a region suggested to be homologous to the vertebrate neural crest. More recently, *distal-less* related genes have also been shown to be expressed in vertebrate sensory placodes. These placodes are epidermal thickenings that give rise to aspects of the sensory nervous system (eg. the inner ear or nose) (Solomon and Fritz, 2002). Therefore, *distal-less* may have a similar role in both

chordates and molluscs in the differentiation of neural structures formed from migratory cells from specialized ectodermal regions. Such a role in the differentiation of migratory neurons is also consistent with observations that *distal-less* expressing stem cells from the basal forebrain migrate into the cerebral cortex in the mouse (Anderson *et al.*, 1997). Moreover, Anderson *et al.* (1997) showed that this gene was necessary for migration to occur. Further experiments in molluscs are necessary to determine the role of *distal-less* genes and proteins in development of the central ganglia.

In *I. obsoleta*, the lack of expression of the Dll protein in the apical organ suggested that this component of the larval nervous system developed via a different mechanism than the central ganglia. The apical organ is believed to be a conserved structure in invertebrate larvae and the genetic mechanisms that govern its formation have not been examined. Finally Dll was absent in regions of the developing peripheral sensory neurons (eg. cells in velum) suggesting that Dll was also not involved in their differentiation. On the other hand Dll was reported to be expressed in developing peripheral mechano-sensory neurons in the horseshoe crab (Mittmann and Scholtz, 2001) as well as in the peripheral sensory regions in vertebrates (Solomon and Fritz, 2002). Therefore, the involvement of *distal-less* in the development of peripheral sensory systems may represent independently co-opted functions in other taxa or a loss of this function in molluscs. Comparisons of Dll expression and function in other molluscs and in other closely related taxa such as annelids are necessary to further understand of how the role of this gene and protein has changed during evolution.

D) Dll Expression in Axons

Dll-like immunoreactivity was primarily observed in the nuclei of cells in *I. obsoleta*, emphasizing its role as a transcription factor. However, this protein was also expressed within the cytoplasm of neurons (e.g. in axons). A similar cytoplasmic localization of Dlx proteins was recently reported in the mouse (Eisenstat *et al.*, 1999). Eisenstat et al, (1999) reported that *Dlx-5* produces at least three RNA transcripts and that at least two of these transcripts encode for shorter proteins either lacking the homeodomain or nuclear localization signals. Such modified distal-less proteins were suggested to possibly interact with other proteins to coordinate signal transduction from the extracellular environment to the nucleus in neurons of the mouse forebrain (Eisenstat *et al.*, 1999). Further experiments are necessary to determine how Dll proteins function in the cytoplasm of neurons in molluscs.

2) The Role of the D Lineage and Early Induction Signaling in Dll Expression and Neural Development

The expression pattern of Dll described above in *I. obsoleta* suggested that this protein has a role in the development of the ectoderm and a component of the nervous system. The experimental manipulations performed in this study further support these morphological observations. Such manipulations focussed on the removal of the D

macromere during early cleavage stages and thus altering the Dll-like immunoreactivity and development of neural elements.

The effects of D macromere or polar lobe removal on the development of *I. obsoleta* have been investigated previously to determine the roles of maternal components and signaling (Clement, 1956; Clement, 1962; Atkinson, 1971; Cather, 1973; Sweet, 1998; Lambert and Nagy, 2001). Such experiments have shown that ablation of the polar lobe or the D macromere prior to the 5th cleavage both result in larvae that lack axial properties (e.g. bilateral symmetry, dorsal-ventral axis) and many morphological structures characteristic of the larvae. These results are likely due to the removal of maternal determinant(s) within the polar lobe and later in the D macromere that is/are responsible for inducing cell fates of adjacent micromeres and thus the organization of the tissues. The induction was recently shown to act via the MAP kinase signaling pathway (Lambert and Nagy, 2001). Blocking this pathway also resulted in similar morphological changes as ablations to the D macromere or the polar lobe. Thus, it was suggested that removal of the polar lobe or D macromere somehow prevented proper activation of MAP kinase cascade and the necessary inductive signal for normal cell fates (Lambert and Nagy, 2001). Also these experiments by Lambert and Nagy (2001) provided evidence that the morphological abnormalities were caused by the lack of induction signaling and not the positional changes due to cell removal. Finally, the D lineage has also been shown to be important since it gives rise to specific embryonic tissues, contributing to part of the endoderm and all of the endomesoderm (Clement, 1962; Atkinson, 1971).

Therefore, without this lineage, or blocking its specification, presumably disrupted the formation and/or patterning of the germ layers.

A) Distal-less Protein Expression Following D Macromere Ablation

i) Gastrulation

During the time of gastrulation of *I. obsoleta,* Dll normally became restricted to the ectoderm, however, in D macromere-ablated larvae, the protein was observed in all cells. In the absence of the D macromere, many of the micromeres may not have received the cues necessary for proper specification. One such cue may be inhibitory in nature as suggested by Sweet (1998) and therefore in its absence all the cells in the D macromere-ablated larvae may have developed via a default program. This default program may involve expression of certain genes and proteins such as Dll. A similar finding was also reported by Mochel-Lynch (2002) where there was an increase in the expression of an orthologue of the gene *twist* in larvae of *I. obsoleta*, lacking the polar lobe. Another example of the inhibitory role of D macromere signaling can be observed in investigations of the development of specific ciliated cells in *I. obsoleta.* Ciliated ectodermal cells that were normally restricted to the dorso-anterior most regions of *I. obsoleta* were observed to cover the entire larva after the removal of the polar lobes (Cather, 1973). One might have assumed that since Dll was normally expressed in presumptive ectodermal cells then all cells in the D macromere-ablated larvae should have become ectodermal derivatives. However, this was not the case, since endodermal

tissues such as digestive gland and stomach cells have been reported in D macromere-ablated larvae (Atkinson, 1971). Therefore, Dll expression alone was not sufficient for the specification of ectodermal cell types and other intrinsic factors or inductive signals independent of the D lineage are likely involved in the differentiation of some cell types. Further studies of the *distal-less* gene expression and determination of the downstream targets of the Dll protein will be necessary for a complete understanding of the function of this gene and protein in ectodermal differentiation of molluscs.

ii) Trochophore Stage

During the trochophore stage Dll was normally expressed in specialized ectodermal regions, possibly in the regions that form the developing central ganglia of the future adult CNS. However, in the corresponding stage of larvae in which the D macromere was ablated there was no Dll labeling. These results indicated that the D lineage was necessary for the normal differentiation of specific ectodermal regions that were labeled with anti-Dll. Furthermore, such results suggested the possibility that Dll expression may also be necessary for the development of the future adult CNS. This hypothesis was corroborated in further results of the present study described below in which the development of the larval nervous system in D macromere-ablated larvae was examined.

B) Development of LNS in D Macromere-Ablated Larvae

The normal expression of Dll suggested that this protein may be involved in the differentiation of neurons forming the ganglia of the future adult CNS from specialized ectodermal regions. Therefore, since Dll was absent in these ectodermal regions following D macromere ablation, it was expected that the neurons that originated from these regions would not develop in later stages of these larvae. The results of the present study supported this expectation. Only a subset of neurons from the larval nervous system were observed in D macromere-ablated larvae. Based on the morphology of these neurons they appeared to be cells located outside the developing central ganglia. The FMRFamide-LIR, leu-enkephalin-LIR and serotonin-LIR cells in larvae following D macomere ablations possessed morphologies characteristic of cells that were normally located in the apical organ. Also the catecholamine-containing cells localized in D macromere-ablated larvae were likely peripheral velar cells. Neurons, neuropils and axon connectives immunoreactive for serotonin, and FMRFamide that were characteristic of the developing cerebral, pedal and intestinal ganglia, were not observed in these larvae following the removal of the D macromere.

The normal expression of Dll in neurons and axons of the ganglia in late veliger stages also indicated a possible role of this protein in continuous growth of the ganglia and possibly other roles such as signal transduction. In the corresponding stage, following D macromere ablation, the absence of Dll-like immunoreactivity further supported the other results that these larvae lacked neurons that form the developing CNS. Together these experiments indicated the importance of the D macromere in

normal expression of Dll and the development of the central ganglia that form the adult nervous system. Furthermore, the results of the present study also suggested that the apical organ and peripheral neurons develop via different mechanisms.

While some of the cell types that compose the apical organ and some peripheral neurons appeared in the larvae following D macromere ablation, there was an obvious lack of organization and symmetry of these neural elements. For example, only single leu-enkephalin- and serotonin-LIR cells were observed, when normally each cell was a part of a bilaterally symmetrical pair. Also such lack of symmetry and organization was observed in the morphology of muscle fibers in larvae following D macromere ablation (unpublished observations, see chapter 5). Togther these results strengthen previous suggestions that the signaling from the D macromere is necessary for organization of tissues and the formation of axial properties of the larva (Clement, 1962; Atkinson, 1971; Lambert and Nagy, 2001).

Conclusion

The results of this study indicated that Dll likely has several roles in the development of molluscs. One such role included the differentiation of specific ectodermal cells into neurons that contribute to the ganglia that eventually form the adult central nervous system. Furthermore, early inductive signaling and subsequent formation of the germ layers were shown to be important for the normal expression of Dll and formation of the central ganglia. On the other hand, the transient components of the

larval nervous system may have developed independently from Dll and early patterning of the larva. Thus these results suggested that differential cellular and molecular programs govern the formation of the various components of the larval nervous system. A better understanding of these programs in *I. obsoleta* and other gastropod species may provide insight into how nervous systems have evolved within this taxa.

Figure 29: Distal-less-like immunoreactivity in *I. obsoleta*. **A)** Single cell stage showing labeling in the nucleus (arrow). Scale bar is approximately 30 μm. **B)** Eight cell stage revealing nuclear labeling in all cells. Micromeres (1a-1d) and macromeres (1A-1D) are labeled for orientation. Scale bar is approximately 30 μm. **C)** Later cleavage stage (32 cell stage) showing labeling in most of the micromeres. The 4d and 3d micromeres and 4D macromere are labeled for orientation. Scale bar is approximately 30 μm. **D)** Dorsal view of an early veliger stage (4-7 days after first cleavage). Anterior is to the top. Labeling appears in axons and in cells near the developing cerebral ganglia (CG) and supra- (SpG) and sub- (SbG) intestinal ganglia. No labeling was observed in cells of the apical organ (arrow). Scale bar is approximately 32.9 μm. **E)** Dorsal view of the anterior of a free-swimming veliger stage (15 days after first cleavage), showing labeling in the cerebral ganglia (CG), pedal ganglia (PG) and in axons. Scale bar is approximately 35.7 μm.

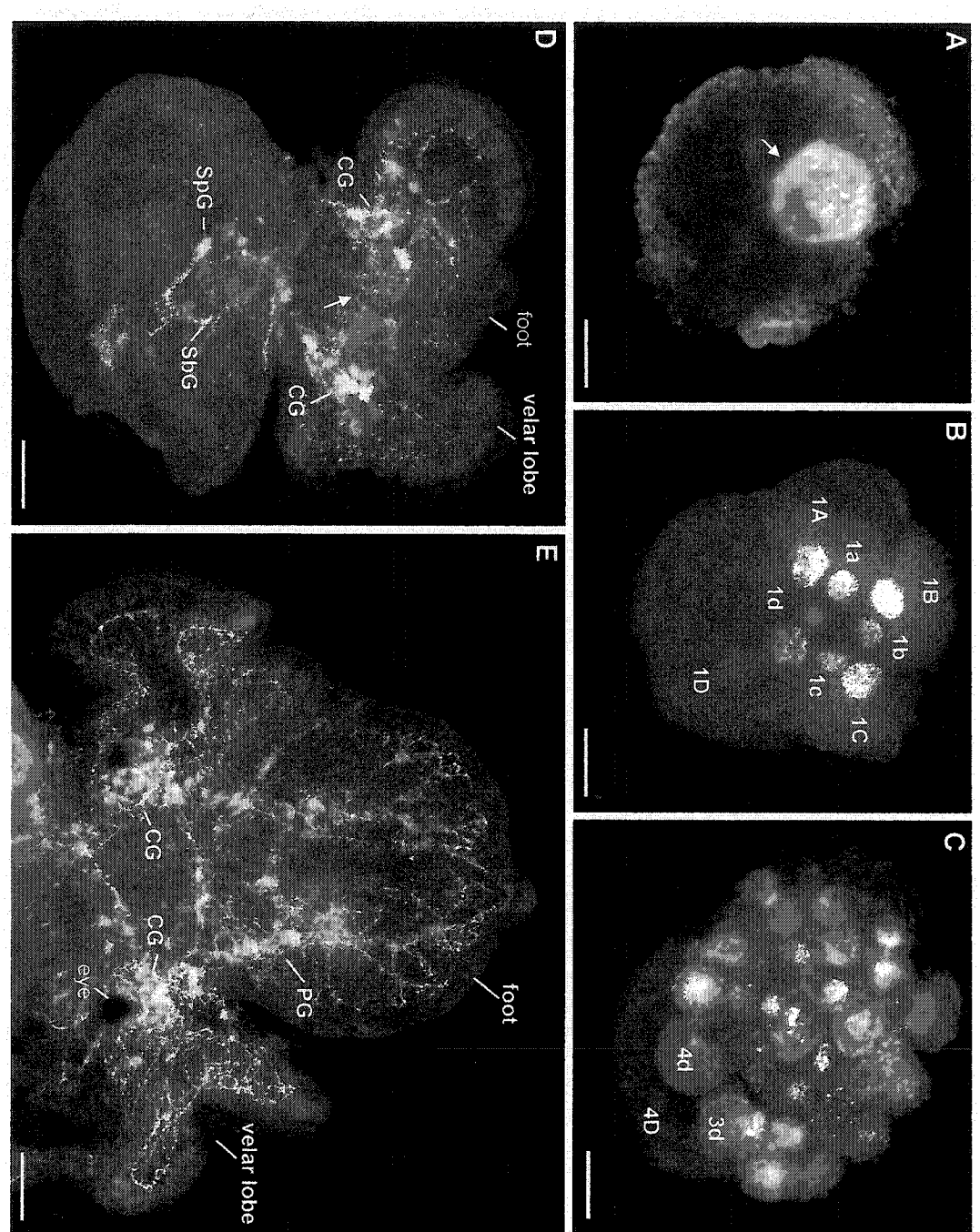

A
B
1B
1b
1A
1a
1d
1c
1C
1D
C
4d
3d
4D
D
foot
velar lobe
CG
SpG
SbG
CG
E
foot
CG
CG
PG
eye
velar lobe

Figure 29

Figure 30: Distal-less-like immunoreactivity in the larval stages of *I. obsoleta* 1 day, 3 days and 7 days after first cleavage. The top row represents the controls while the bottom row represents the larvae after D macromere ablation. **A)** Early gastrula stage, 1 day after first cleavage showing nuclei labeled in presumptive ectoderm and lack of labeling in the blastopore (arrow). Scale bar is approximately 35 μm. **B)** Dorsal view of the trochophore stage (approximately 3 days after first cleavage). Labeling appears in ectodermal regions of the cephalic plates (arrow) and post-trochal ectoderm (arrowhead). Scale bar is approximately 35 μm. **C)** Dorsal view of a hatchling, 7 days after first cleavage, showing labeling of nuclei in the cerebral ganglia (CG) and axons (arrow). Scale bar is approximately 45 μm. **D)** Experimental larva 1 day after D macromere ablation revealing labeling in all nuclei. Scale bar is approximately 30 μm. **E)** Experimental larva 3 days after D macromere ablation revealing the absence of labeling. Scale bar is approximately 30 μm. **F)** Experimental larva 7 days after D macromere ablation also revealing little or no labeling. Scale bar is approximately 30 μm.

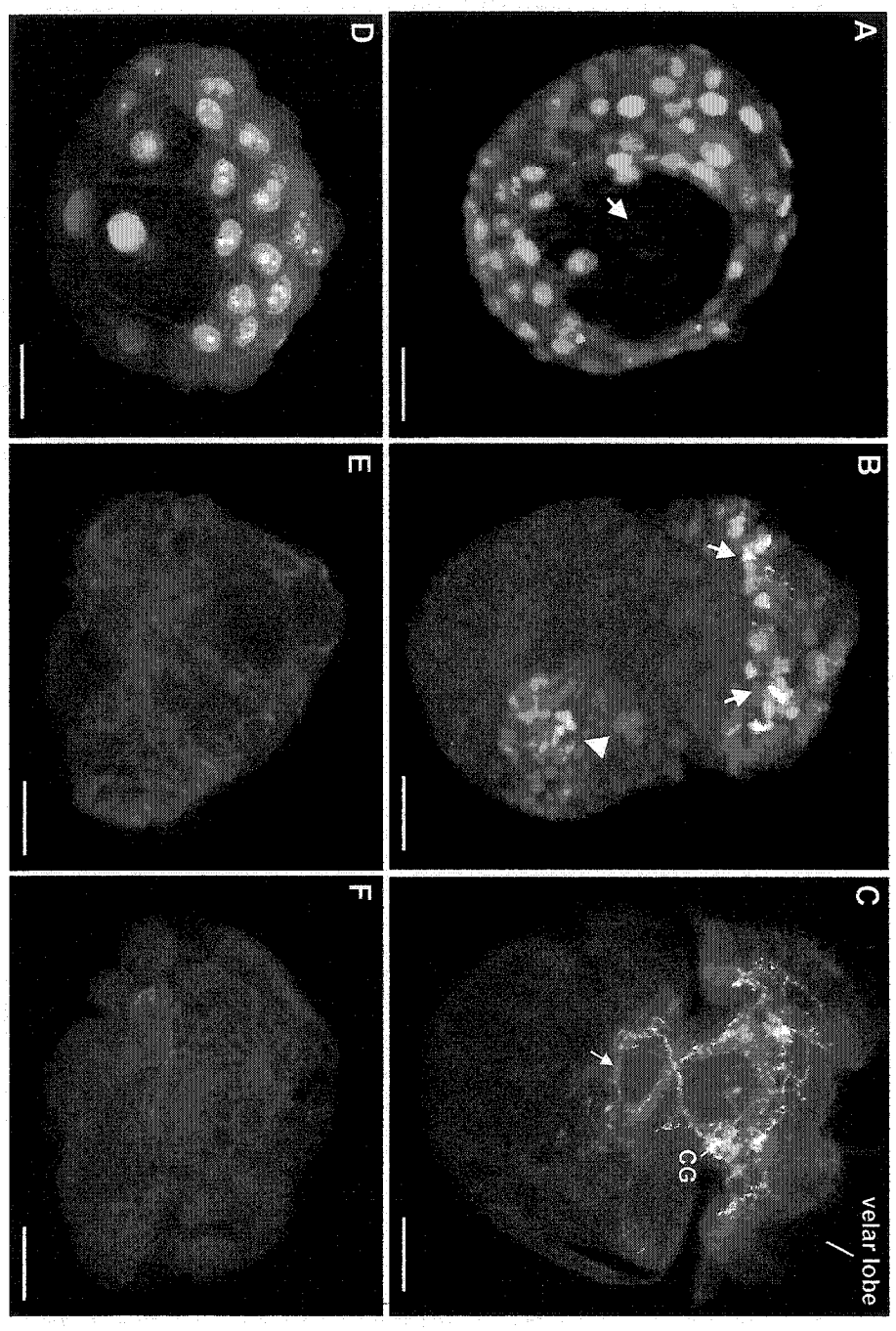


Figure 30

Figure 31: Neural elements labeled in the larval stages of *I. obsoleta* 7 days after first cleavage. The top row represents the controls while the bottom row represents the larvae after D macromere ablation. **A)** FMRFamide-like immunoreactivity in a right lateral view of a control larvashowing vase-shaped apical cell (arrow) neuropil of the apical organ (AO), region of the right cerebral ganglia (CG) and sub-intestinal ganglia (SbG). Scale bar is approximately 50 μm. **B)** Serotonin-like immunoreactivity in a dorsal view control larva revealing cells in the apical organ (AO) and cerebral ganglia (CG) Scale bar is approximately 50 μm. **C)** Leu-enkephalin-like immunoreactivity in a dorsal view of control larva showing cells in the apical organ (AO). Scale bar is approximately 50 μm. **D)** FMRFamide-like immunoreactivity in an experimental larvae revealing vase shaped cells (arrows), irregular shaped cell (arrowhead) . Scale bar is approximately 30 μm. **E)** Serotonin-like immunoreactivity in an experimental larva showing a single cell (arrow) very faint labeling (arrowheads) and labeled patches (asterisks). Scale bar is approximately 30 μm. **F)** Leu-enkephalin-like immunoreactivity in an experimental larva showing a single cell (arrow). Scale bar is approximately 30 μm.

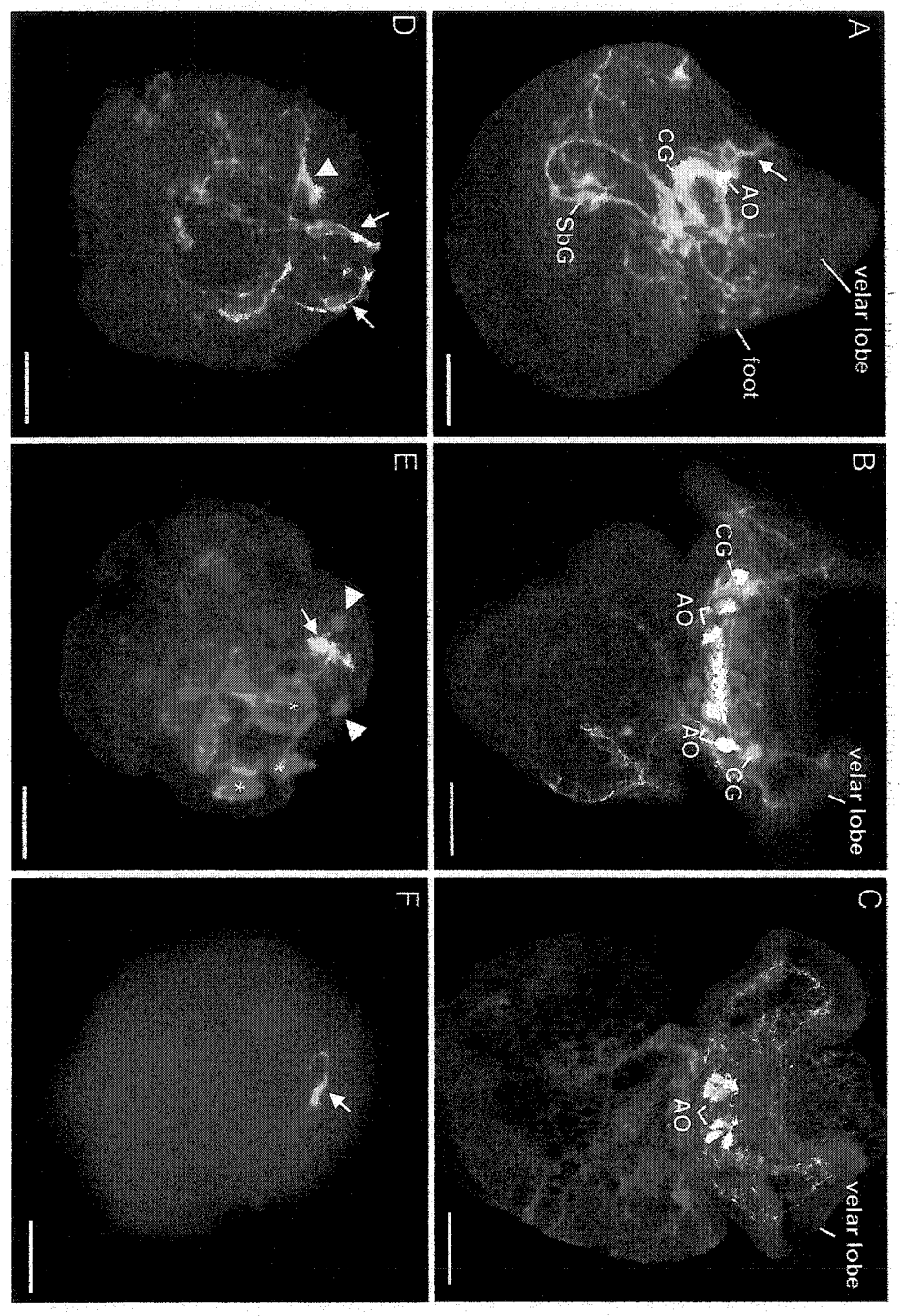

A
velar lobe
AO
CG
SbG
foot
B
CG
AO
AO
CG
velar lobe
C
AO
velar lobe
D
E
F

Figure 31

Figure 32. Neural Elements labeled in the larval stages of *I. obsoleta* 7 days after first cleavage. The top row represents the controls while the bottom row represents the larvae after D macromere ablation. **A)** TH-like immunoreactivity in a dorsal view of the anterior half of a control larva showing the peripheral velar cells (arrows) as well as other catecholamine cells near the mouth. Scale bar is approximately 30 μm. **B)** Catecholamine-containing cells (arrows) revealed by the FaGlu technique in the a velar lobe of a control larva. Scale bar is approximately 20 μm. **C)** TH-like immunoreactivity revealing cells (arrows) in an experimental larva. Scale bar is approximately 30 μm. **D)** Catecholamine-containing cells (arrows) revealed by the FaGlu technique in an expreimental larvae. Scale bar is approximately 30 μm.

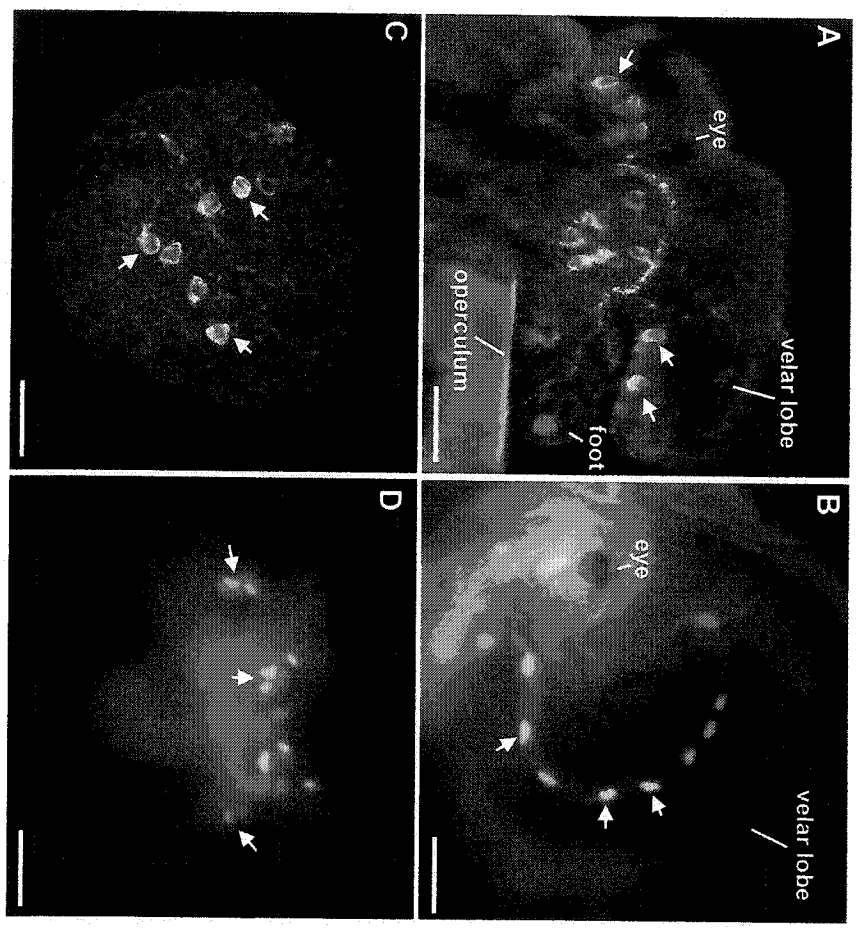


Figure 32

CHAPTER 5

DIFFERENTIAL DEVELOPMENT OF THE MAJOR MUSCLE SYSTEMS IN THE LARVAL GASTROPOD, *ILYANASSA OBSOLETA*

Introduction

Studies of molluscan larval musculature have historically played prominent roles in attempts to explain how evolutionary changes occur though developmental modifications (Garstang, 1929). However, a major issue in such studies has revolved around controversy over the homology of muscle groups in species of molluscs. Recently, with improved techniques, such as electron and confocal microscopy, such controversies have been resolved. It has been established that there are two major muscle systems in larval stages of gastropods; 1) the larval retractor muscle and 2) muscles in the foot that persist into the post-larval stages. Each of these two major larval muscle systems share many developmental and morphological similarities in species representing patellogastropods (Wanninger *et al.*, 1999), vetigastropods (Degnan *et al.*, 1997b; Page, 1997a) and caenogastropods (Page, 1998) and heterobranchs (Page, 1995). For example, in several gastropods the larval retractor muscle was striated, developed in early larval stages and degenerated during metamorphosis (Page, 1998; Wanninger *et al.*, 1999). While the pedal muscles differed in number in different taxa, these muscles appeared smooth, developed later in larval stages and persisted through metamorphosis (Page, 1998; Wanninger *et al.*, 1999). While aspects of the anatomy of larval musculature are becoming increasingly clear, the mechanisms of myogenesis have still largely been unstudied in molluscs. A better understanding of the molecular and cellular

mechanisms specifying the development of different muscle groups will aid in making phylogenetic comparisons.

Detailed cell lineage data exist for several species of gastropod (for review see (Clement, 1962; Verdonk and van den Biggelaar, 1983; Render, 1991; Damen and Dictus, 1994; Dictus and Damen, 1997; Render, 1997). From such studies, we know that muscle in gastropods is derived from the mesoderm which can be divided into ectomesoderm and endomesoderm based on the cellular origins. Endomesoderm is derived from the mesentoblast cell (4d) while ectomesoderm is derived from some of the cells from the second and third quartet of micromeres (for review see Verdonk and van den Biggelaar, 1983) . However, misidentifications of larval muscles in the past have led to confusion when determining the origins of muscle groups. Furthermore, the role of inductive mechanisms and axial patterning in the formation of muscle have not been examined. In the current study we have attempted to examine these questions by investigating the role of a specific cell lineage in the well studied caenogastropod *Ilyanassa obsoleta*. The cells arising from the D macromere (D lineage) have been shown in classical (Clement, 1962) and more recent studies (Lambert and Nagy, 2001) to play a role in inducing other cell fates and patterning the embryonic axis. In addition, the 3D macromere divides to form the mesentoblast cell which gives rise to the endomesoderm. Therefore, we can investigate the role of this macromere as well as the influence of inductive signals in the formation of the different muscle groups.

In the present study we test the hypothesis that the larval retractor muscle and the pedal muscle systems in the larva develop via different mechanisms in *I. obsoleta*. We examined: 1) the morphology of these major muscle systems, 2) the development and fates of the muscles and finally 3) the cellular origins and specification governing the formation of the larval retractor muscle and pedal muscles. The results provide important insights for determining mechanisms governing muscle development and will aid in determining homologous muscle systems and evolutionary relationships within the gastropods.

Materials and Methods

Adult and Larval Culture

Refer to Chapter 2, page 44

The stages examined for normal development of the muscle systems included trochophore (2.5- 4 days), embryonic veliger (4.5- 7 days), hatchling (7-10 days), free swimming veliger (14-17 days used a representative), and competent veliger (21-25 days). In addition we examined the metamorphosing veliger (24hrs post induction with 10^{-4}M serotonin (Lin and Leise, 1996a) and juvenile (48hrs post induction).

Role of the D Lineage: D macromere ablations and inhibition of MAPK signaling

Two methods to block the signaling induction by the D macromere in *I. obsoleta* have been established. The first method is to ablate the D macromere prior to the 5th cleavage as shown by Clement (1962). Lambert and Nagy (2001) showed that D macromere ablation was mimicked by blocking the mitogen-activated protein kinase (MAPK) signaling pathway also prior to the 5th cleavage. Therefore, we have utilized both these techniques to explore the role of early inductive signaling in specification of muscle systems. Furthermore, ablation of the D macromere also prevents the formation of the mesentoblast cell (4d) and therefore offers an experimental approach to examining the role of the endomesoderm in muscle development.

The D macromere was ablated by puncturing the D macromere at the 4 cell stage (see Clement, 1956) with a glass capillary tube (outer diameter 1.0mm) pulled to a sharp point with a Brown-flaming micropipette puller (Model P-77, Sutter Instruments Co., California). The operations were performed in 10ml glass Petri dishes under a stereo-dissecting microscope. Experiments were performed on groups of animals from two to three egg masses, totaling at least 30 animals and several embryos were unoperated, to serve as controls. All operations for a single group were performed within 10-15 minutes, well before the next set of cell divisions. After approximately 30 minutes, the entire D macromere separated completely from the other cells (personal observations). The embryo was then transferred into a new Petri dish containing 0.2 μm filtered natural seawater and 0.10 μg/ml penicillin G (Sigma Chemical Co., Mississauga, ON). The sea water was changed each day and after 7 days embryos were fixed in 4% paraformaldehyde (PFA) for 10 minutes and then processed for F-actin labeling as described below.

Inhibition of the MAPK signaling pathway was achieved by exposing 50-100 embryos at the 4 cell stage to 10^{-5}M of the drug U0126 (Promega, Madison,WI). This drug specifically inhibits MAPK kinase which phosphorylates extracellular signal-regulated protein kinases (ERK), or MAPK (Lambert and Nagy, 2001; Lartillot *et al.*, 2002). After three to four hours of U0126 exposure the embryos were washed three times in 0.2 μm filtered natural sea water and were then pipetted into10ml glass Petri dishes also containing filtered sea water and 0.10 μg/ml penicillin G. Embryos were

cultured for seven days as described above and then fixed and processed for F-actin labeling.

F-actin labeling

Labeling of muscle was achieved by staining F-actin following methods described by Degnan et al. (1997b). Briefly, animals were first relaxed and anesthetized with isotonic $MgCl_2$ for 30-60 minutes. Larvae were then fixed in 4% PFA in phosphate buffered saline (PBS; 50mM $Na_2HPO_4.7H_20$ and 140mM NaCl in distilled water adjusted to pH 7.2) for 10-30 minutes at room temperature. The animals were washed in PBS twice for 5 minutes and then given a final wash of 60 minutes. The shells of older embryos (>Day 5) were then decalcified with 80% of 0.23M ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., Mississauga, ON) and 20% of 0.1 M sodium acetate for four to 24 hours. Larvae were next treated with 4% Triton X-100 in PBS for 24 hours. The animals were then incubated in 1:100 phalloidin labeled with tetramethlyrhodamine B isothiocyanate (TRITC)(Sigma Chemical Co., Mississauga, ON) for one to four hours. Larvae were washed in PBS twice for five minutes and then a final wash of at least 60 minutes. Larvae were stored in PBS for one to seven days at 4°C before viewing. At least 30 of these animals were viewed at each stage.

Lucifer Yellow Dextran Injections

Lucifer Yellow Dextran (LYD) 10,000 MW (Molecular Probes, Eugene OR) was dissolved in distilled water at a concentration of 0.25mg/ml and was then centrifuged at 5000 rpm for 15 min. The LYD was backfilled into a thin walled borosilicate glass capillary tubing (Warner Instrument Corp. (outer diameter 1.0mm, 0.78 inner diameter), Hamden, CT) that was pulled to a tip diameter of 1.0 - 0.5 μm with the Brown-Flaming micropipette puller. Eggs that had completed the second division were placed onto a drop of 3% methyl cellulose on a glass slide in a slit created with the edges of two cover slips. The LYD was pressure injected into D macromeres using a Picospritzer II (General Valve Corp, Fairfield, NJ). The amount of LYD injected was adjusted by varying the pressure (30-50 psi) and injection time (10-100 ms) depending on the tip diameter. Embryos which survived injections were pipetted into a glass Petri dish containing 10ml of 0.2 μm filtered natural sea water and placed in the dark for 5-7 days. Embryos from the same egg mass that were not injected were also included in the same dish to serve as controls. A total of 10 animals were examined 7 days after LYD injection.

Viewing

Embryos were mounted on glass slides in a 3:1 mixture of glycerol to PBS with 2% propyl gallate to reduce fading (Longin *et al.*, 1993). Embryos were initially viewed on a Leitz Aristoplan microscope equipped for epifluorescence and some embryos were photographed with a Orthomat E camara and Kodak 200 Select Color film. In these cases the negatives were digitally scanned with an HP Photosmart Scanner. Most viewing and

scanning of embryos labeled with TRITC-phalloidin and LYD were performed on a Zeiss Axiovert confocal laser scanning microscope (model LSM 510) with a helium neon laser (543nm and 633nm). Optical sections of 0.20-0.80 μm were performed and projections of stacks were created with Zeiss LSM 510 software. As negative controls, embryos were processed without incubation in TRITC-phalloidin; such specimens exhibited no detectable fluorescence. Measurements of selected muscle systems were performed on selected preparations using the overlay function of the LSM 510 software. All the images were then assembled into plates and labeled using Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). Contrast and brightness of the images were adjusted to provide consistency within plates.

Results

Part 1: The Morphology of Larval Muscle Systems

The muscle groups described here are shown in simplified schematic diagrams in Figure 1 and the nomenclature was adapted from Page (1998) and Wanninger *et al.* (1999). All of the larval muscle systems were evident in the hatchling, 7-10 days after first cleavage. The larval retractor muscle (LRM) attached to the shell ventrally on the left side in the posterior end of the animal (Fig. 33A,B, 34A, D, I and also see 35A, C, 36A). The fibers of the LRM divided into dorsal and ventral components that extended anteriorly and inserted in regions in the head, velum and foot (Fig. 33A,B, 34A, D). Fibers from the dorsal component of the LRM extended to the head or into the dorsal portions of the left and right velar lobes contributing to the velar musculature (Fig. 34D, 35C). The ventral component of the LRM branched to the right and the left where the head and body met (Fig. 34A). Each left and right branch further divided into tracts which inserted on transverse muscle systems (see below) or extended into the velum or the foot. The LRM was distinguished from other muscle systems since it appeared striated (Fig. 34I and see inset). The LRM remained relatively unchanged throughout the free swimming larval stage. At the hatchling stage the diameter of the LRM was 25-30 μm at the base and was not significantly different in diameter two weeks later (compare 34D and 35A, C, 36A).

The other large muscle system in the larva was the pedal retractor muscle (PRM) system. At the hatchling stage this muscle system was very small consisting of one or two fibers (Fig. 33A,B, 34D) but became more substantial at later stages (Fig. 35A, 36A, B). By one week post hatching the PRM consisted of many fibers that together were approximately 50-70 μm in diameter at the base (Fig 36A). By competency the PRM was even more extensive and was 120-150 μm in diameter at the base (not shown). The PRM consisted of un-striated muscle and attached dorsal to the LRM attachment site (Fig. 33B, 35A). The majority of the fibers composing the PRM system extended anteriorly and then ventrally into the foot where cells inserted on the operculum in the middle of the mesopodium (Fig. 3A). Some fibers from the PRM system also extended dorsally to the mantle region and possibly into the head.

In addition to the LRM and PRM, several other smaller muscle systems were found. The accessory muscles (ACM) were located at the posterior of the larva and were orientated dorso-ventrally (Fig. 33A, B, 34A, D, H). The number of ACM fibers increased as the larva grew in size and encircled the stomach and digestive gland (Fig. 35A and 36D). There were also three systems of transverse muscles in the head of the larva. The dorsal most transverse system (dorsal head muscle; DHM) was located just posterior to the eyes (Fig 33A, B, 34B). The DHM system consisted of fibers that extended into the dorsal portion of each velar lobe and branched several times contributing to the velar musculature. Another transverse muscle system was located just dorsal to the mouth (DMM) and extended on each side along the edges of the velar lobes

(Fig. 33A, B, 34C, G). The third transverse muscle system was located ventrally to mouth (ventral mouth muscle; VMM). The VMM fibers extended into ventral portions of each velar lobe and also branched several times contributing to the velar musculature (Fig. 33A, B, 34A). The DMM and VMM were also the site for the insertion of fibers from the LRM and muscles intrinsic to the foot. Muscle, in the tentacles, had a ladder-like pattern of fibers (Fig. 33B, 35B). The velar and foot muscle systems were composed of fibers contributed from several sources. The velar muscle (VM) system consisted of radiating fibers originating from the LRM, DHM, DMM and VMM and intrinsic fibers that inserted below the outer layer of ciliated epithelial cells (Fig. 33A, B, 34G, 35A,C, 36C). During the late larval stages, additional muscle fibers appeared in the velum and the PRM may have also been the source of at least some of these fibers. Muscles in the foot were composed of fibers branching from the LRM, PRM as well as intrinsic muscles (foot muscles; FM) (Fig 33A, B, 34E, F, 35A, 36B). One intrinsic foot muscle extended from the operculum just right of the midline and inserted on or near the VMM (Fig. 33A,B, 34D, F). During later veliger stages a protrusion from the foot developed, called the propodium and the foot proper became the mesopodium. Muscles extended from the middle of the mesopodium into the propodium (propodial muscles; PrM)(Fig. 33B, 36B). Increased numbers of fibers extended into the propodium and branched near their terminus, by competency approximately 10-12 fibers were observed (Fig. 36F). Also near the end of the larval period, intrinsic small fibers formed a crisscrossing network in both parts of the foot (mesopodium and propodium) and a fine fiber lined their edges (Fig. 36

E, F). Fine muscle fibers were directed both longitudinally and transversely around the mantle cavity (Fig. 33A, B, 34D, 35A, C). Fibers were also observed to extend from the mantle cavity to the region of the base of the PRM and the ACM (Fig. 33A, B, 34D, 35A ,C). In addition, muscles lined the digestive tract (Fig 36D, arrow).

Part 2: Development and Fates of the Larval Muscle Systems

A) Development:

The first week of the larval period is spent within a capsule relying on yolk stores as nutritive sources. During this time the embryo undergoes cleavage, gastrulation, trochophore stage and the first few days of the veliger stage before hatching. F-actin labeling first became apparent during the trochophore stage. Fine muscle fibers ran dorsally along the right side of the embryo and fanned out in the anterior region (Fig. 37A). These muscle fibers corresponded to the developing LRM. Transverse muscle fibers also appeared in the anterior region, dorsal to the mouth and just posterior to a row of large columnar epithelial cells. These muscles appeared to be the developing DMM system (Fig. 37A). Several faintly labeled muscle fibers were also observed in the posterior regions corresponding to the developing ACM (Fig. 37A).

During the early veliger stage the embryo undergoes torsion, and develops eyes, velum and foot. Many of the larval muscle systems became recognizable soon after torsion and by the hatchling stage were consolidated and more brightly labeled. During the early veliger stage the right and left components of the LRM appeared to join together

posteriorly and ventrally, however they were still spread apart considerably in anterior regions relative to later stages (Fig. 37B). The branching pattern of the LRM was similar to that described above and the transverse muscle systems, DHM, DMM and VMM were evident in the early veliger stage (Fig. 37C, D). While muscle fibers appeared in the foot (Fig. 37D) fibers of the PRM were not apparent in the early veliger. Fine muscles were also observed near the mantle cavity and along the digestive tract during the early veliger stage (Fig. 37A, B).

C) Fates:

Metamorphosis often begins 3-4 weeks after first cleavage and takes up to 2 days to complete. During the first 24 hours of metamorphosis, the animal ceased to swim, and the velum was reduced. Later, major reorganization of the head took place as new feeding structures formed. At the beginning of metamorphosis the velar muscle became wavy in appearance indicating the loss of attachment (Fig. 38A, B). When the velum was reduced, velar muscle fibers appeared broken and unorganized (Figure 38C, D). At this time, extensions from the LRM to the velum, when present, appeared thin and broken (Fig, 38C, D). Blebs of actin appeared at the base of the PRM and in the foot during metamorphosis, likely remnants of the degenerating LRM (Fig 38C). By 48 hours post induction most of the LRM and all muscles associated with the velum, including the DMM and VMM disappeared (Fig. 38E, F). A few LRM fibers remained and inserted ventral to the radular sac (Fig 38E). From this insertion point several fibers radiated

dorsally toward the radular sac and head. The ACM, mantle musculature and the muscles associated with digestive organs remained in the newly metamorphosed juvenile. The intrinsic network of muscle in the foot (mesopodium and propodium) were also present in the juvenile. A similar network of muscle fibers were also seen in the head region (Fig. 38E). New muscle fibers appeared to surround the radular sac and extended to the base of the PRM.

Part 3: Specification of Muscle Groups: Role of the D lineage

A) Cell Lineage Tracing:

Injection into the D macromere (Fig. 39A) labeled many structures in the veliger derived from the endoderm and mesoderm, including digestive structures, heart and larval kidneys (Fig. 39B) In addition, some muscles were also labeled in *I. obsoleta.* The most conspicuous were anterior branches of the LRM that extended into the velum (Fig 39B). The posterior end and attachment site of the LRM was not labeled. In addition, the DMM was also clearly labeled (Fig. 39C). While several large glandular cells at the tip of the foot also appeared to contain lucifer yellow dextran (LYD), however, no PRM or other FM were labeled (Fig. 39D).

B) Cell Ablations and MAPK kinase inhibition

Ablations of the D macromere and exposure to the MAPK signaling pathway antagonist resulted in very similar morphological abnormalities. The embryos lacked

bilateral symmetry and many larval structures were absent. The living embryos exhibited the ability to contract their body indicating the possible presence of functional muscle (personal observation). F-actin labeling revealed extensive amounts of muscle in these embryos (Fig 39E, F). The muscle fibers appeared unorganized and radiated in all directions. Some bundles of fibers were observed but no muscle groups were identifiable (Fig 39E, F). In some cases muscle fibers circled structures or cavities, and appeared similar to the muscle surrounding the digestive tract (Fig 39E). All the muscle fibers in these ablated embryos appeared smooth, lacking striations (for examples see Fig 39G, H).

Discussion

The results of this study suggest the possibility that there are at least two different groups of larval muscles based on morphology, development, fates and cellular origins. The first group are muscles that only function in larval stages, degenerating at metamorphosis (most of the LRM, transverse muscles, and intrinsic muscles of the velum). The second group consists of muscle systems which contribute to adult musculature (PRM, muscles associated with the mantle and digestive organs, ACM and some fibers from the LRM). Together these results suggest that two different programs govern the development of the major larval muscles.

Muscle Anatomy: Comparisons with Other Gastropods

The results of this investigation revealed a much more complex system of larval muscles in this species compared with reports in other gastropods. Nevertheless, all the major muscle groups reported in other species were also identified in *I. obsoleta*. The LRM had anatomical characteristics similar to those described in other species such as striations and branches into the velum, foot and head (Page, 1995; Page, 1997a; Page, 1998; Wanninger *et al.*, 1999). Therefore, the anatomical information from the present study supported the hypothesis that the LRM is homologous in gastropods (Page, 1998; Wanninger *et al.*, 1999).

A muscle system similar to the pedal retractor muscle (PRM) of *I. obsoleta* has also been described in the caenogastropod species *Polinices lewisii* (Page, 1998). In this

species the single pedal muscle had long myosin filaments and was not obviously striated, and projected mainly into the foot inserting on sub-opercular epithelium. Similarly located muscle systems were also described in the patellogastropods *P. vulgata* and *P. caerulea* (Wanninger *et al.*, 1999) and the vetigastropods *Haliotis kamtschatkana* (Page, 1997a) and *Haliotis rufescens* (Degnan *et al.*, 1997b). However, in these species there were two pedal retractor muscles possibly reflecting the existence of the two adult muscles. In the heterobranchs *Phestilla sibogae* (Bonar, 1977; Bonar, 1978b; Bonar, 1978a), *Tritonia diomedea, Rostanga pulchra, Doridella steinbergae, Dendronotus subramosus* and *Melibe leonina* (Page, 1995) similarly located pedal muscles were also described. Page (1995) reported that the right and left pedal muscles of various heterobranchs were also likely homologues of the left retractor muscle and opercular muscles of *P. sibogae*. Page (1998) has also suggested that the pedal retractor muscle systems are a separate homologous group in gastropods. Nevertheless, it does appear that the pedal retractor muscles are anatomically distinct from the LRM in many gastropod larvae, indicating that there are at least two major, independent larval muscle systems in this taxon.

Other larval muscle systems also show similarities in gastropod larvae. For example, muscle fibers associated with the mantle have been reported in *Patella vulgata*, *Patella caerulea* (Wanninger *et al.*, 1999), and *H. kamtschatkana* (Page, 1997a). These muscles likely allow for changes in the size of the mantle cavity to accommodate the head and velum during escape behaviors. Transverse muscles systems described here in *I.*

obsoleta were not reported in other species. However, the velar rim muscle in *P. vulgata*, *P. caerulea* (Wanninger *et al.*, 1999) shows similarities with the dorsal mouth muscle (DMM) that also extends laterally along the edges of the velar lobes in *I. obsoleta.* In addition, Fretter (1972), describes a ventral velar muscle in caenogastropods that might correspond to the ventral mouth muscle (VMM). These transverse muscle systems may be necessary for more sophisticated movements in large, long-lived planktotrophic larvae. However, additional anatomical studies of the musculature are necessary in various other species with larvae of different sizes and swimming capabilities to determine if a correlation exists. In the present study, ACM were described in the posterior regions of *I. obsoleta* and similar muscle were also reported in *P. vulgata*, *P. caerulea* (Wanninger *et al.*, 1999) and *H. kamtschatkana* (Page, 1997a). In these other species the accessory muscles were striated and degenerated at metamorphosis. However, in *I. obsoleta*, the locations and morphology of the ACM were different than accessory muscles in the other species. Furthermore, the ACM of *I. obsoleta* were still intact after metamorphosis. Therefore, it is not likely that the ACM of *I. obsoleta* and accessory muscles of other species are homologous muscle groups.

Development and Fate of Larval Muscle Systems

The developmental timing and fates of the LRM and PRM were similar in several gastropods. In the present study of the caenogastropod *I. obsoleta* and in a previous study of patellagastropods, *P. vulgata* and *P. caerulea* (Wanninger *et al.*, 1999) the LRM was

the first muscle system observed in trochophore stages. While other investigators did not examine the trochophore stage, they also reported that the LRM developed prior to all other major larval muscle groups (Bonar and Hadfield, 1974; Bonar, 1977; Page, 1997a; Page, 1998). Most of the fibers composing the LRM and the transverse muscle systems associated with the velum and head degenerated during metamorphosis in *I. obsoleta*. The muscles were observed to become fragmented and then disappeared. Similar observations of degeneration of the LRM were also reported in other gastropod species (Degnan *et al.*, 1997b; Page, 1998) and therefore it would be interesting to determine if the LRM underwent programmed cell death.

In several caenogastropod species some LRM fibers did not degenerate. A few ventral fibers from the LRM inserted on the ventral portion of the buccal mass in *I. obsoleta* and in other caenogastropods, *P. lewisii* (Page, 1998) and *Lacuna vincta* and *Crucibulum spinosum* (Fretter, 1972). According to Fretter (1972) these persisting muscles became the protractors of the subradular membrane. Thus, at least in the caenogastropods, similarities also exist in which some fibers of the LRM are retained through metamorphosis and some degenerate. Further investigation of the mechanisms governing differential cell death in muscle groups may provide additional insights into the evolution of gastropod larval musculature.

The PRM developed after the LRM and contributed to the adult musculature in *I. obsoleta* and several other gastropod species. For example, in the caenogastropod, *Polinices lewisii* the pedal retractor muscle system appeared during the veliger stage and

became the adult columellar muscle (Page, 1998). In the patellagastropods *P. vulgata* and *P. caerulea* (Wanninger *et al.*, 1999) and the vetigastropod *H. kamtschatkana* (Page, 1997a) the two pedal muscle systems were still present in juveniles and migrated into positions that corresponded to the two major adult muscles (Page, 1997a). While the pedal muscles were not retained in the heterobranch adults they degenerated later than the LRM in the species examined (Page, 1995). Thus, the pedal muscle systems also may be homologous based on the developmental timing relative to the LRM.

Origins and Patterning of the Larval Muscle Systems

Cell lineage tracing in the present study indicated that the D macromere contributed to specific larval muscle groups in *I. obsoleta*. At least part of the LRM and the DMM originated from the D lineage. Muscles originating from the D lineage are likely derived from the mesentoblast cell (4d) which in turn forms the endomesoderm (for review see Verdonk and van den Biggelaar, 1983). Previous cell lineage studies of *I. obsoleta* (Render, 1997) and other gastropods (Verdonk and van den Biggelaar, 1983; Dictus and Damen, 1997) have also shown that the LRM or a similar muscle group originates from the mesentoblast cell. On the other hand, in the present study, lineage tracing revealed that the PRM system, that contributes to the major adult muscle, did not originate from the D lineage. In addition, we showed that ablations of the D lineage resulted in the presence of muscle fibers, suggesting that cells other than the mesentoblast are responsible for at least some muscle groups. Furthermore, the muscle fibers present in

D ablated embryos were not obviously striated and therefore not likely to be the LRM. Therefore, the PRM system is probably derived from one or more micromeres from the second and/or third quartet which contributes to the ectomesoderm. This hypothesis is also consistent with previous cell lineage investigations in *I. obsoleta* (Render, 1997) and other gastropods (Boyer *et al.*, 1996; Dictus and Damen, 1997). Together, the present results and previous studies indicate that the pedal retractor muscle groups that contribute to major adult muscle have different cellular origins than at least some of the muscle groups which degenerated during metamorphosis.

In the past it has been suggested that the ectomesoderm and the endomesoderm are precursors to larval and adult mesoderm respectively in many invertebrate groups (for review see Verdonk and van den Biggelaar, 1983). However, the present study revealed that the opposite is true in *I. obsoleta.* Generally we found that ectomesoderm likely contributed to the PRM that contributes to the adult muscle, whereas, the endomesoderm contributed to the transitory larval muscles. However, this correlation was not absolute since only a subset of the fibers composing the LRM were labeled in the D lineage tracing experiment. Therefore, the possibility exists that some fibers of the LRM may have been derived from the ectomesoderm. Several other studies have indicated that both the ectomesoderm and endomesoderm can contribute to both larval and adult mesoderm (for review see Verdonk and van den Biggelaar, 1983). For example, the mesentoblast cell contributes to both larval and adult mesoderm in polychaetes (Schroeder and Hermans, 1975). The differences in the contributions of the ectomesoderm and endomesoderm in

different taxa may reflect evolutionary changes that allowed for the great diversification of metazoan body plans (Martindale and Henry, 1995; Boyer *et al.*, 1996; Henry *et al.*, 2000). Further experiments examining the germ layer origins and contributions to specific larval and adult tissues will be valuable in understanding how evolutionary change occurred though developmental modifications.

Previous experiments have demonstrated the importance of the maternal components within the D macromere for inducing the other cell fates and in turn patterning the embryonic axis (Clement, 1962). The molecular elements involved in this induction event are currently being uncovered. Lambert and Nagy (2001) provided evidence that the MAPK signaling pathway is involved in the induction by the D macromere in *I. obsoleta.* When the MAPK signaling pathway was blocked the resultant embryo was identical to an embryo in which the D macromere was ablated. In the present study, the unorganized pattern of muscle fibers in the D ablated embryos and in embryos exposed to the MAPK signaling antagonist also appeared comparable. The un-striated appearance of the muscle in these embryos also indicates the probability that the early inductive signaling from the D macromere is not necessary for the formation of the PRM and possibly some other muscle groups. While this early signaling does not seem to affect mechanisms governing myogenesis it does appear to be necessary for the formation of discrete muscle groups in *I. obsoleta.* Since induction signals from the D macromere were necessary for the formation of the dorso-ventral axis and bilateral symmetry it was not surprising that these signals are in turn necessary to form the proper arrangement of

the muscles. Genes responsible for patterning the embryonic axis in other taxa are also necessary for the arrangement of muscle fibers (for example see Wigmore and Evans, 2002)

In the present study we hypothesize that separate programs govern the development of the major muscle systems in larval gastropods. This theory is also supported by recent molecular evidence. Degnan and coworkers (Degnan *et al.*, 1997a; Hinman and Degnan, 2002) identified a homeobox gene related to the vertebrate Mox genes which are expressed in the mesoderm and developing muscles. During the development of the vetigastropod *Haliotis asinina* the *Has-Mox* gene was restricted to the foot muscle (Hinman and Degnan, 2002). This foot muscle was reported to become part of the adult columellar pedal complex after metamorphosis (Degnan *et al.*, 1997b; Hinman and Degnan, 2002) and therefore may be similar to the pedal retractor muscle systems (PRM) reported here in *I. obsoleta*. Thus, differential expression of genes involved in myogenesis and arrangement of muscle fibers provides additional evidence of independent larval muscle systems. Future experiments, investigating genetic networks governing muscle development will also be useful for understanding how molecular and cellular changes influence evolution of different types of muscle.

Figure 33: Schematic of the muscles in a mid larval stage of *I. obsoleta.* Anterior is to the top in each figure **A)** Dorsal view. **B)** Right lateral view. Shown in black: LRM; larval retractor muscle, ACM; accessory muscles, MM; mantle muscles, DHM; dorsal head muscle, DMM; dorsal mouth muscle, VMM; ventral mouth muscles, FM; foot muscles, VM; velar muscles. Shown in grey: PRM; pedal retractor muscle.

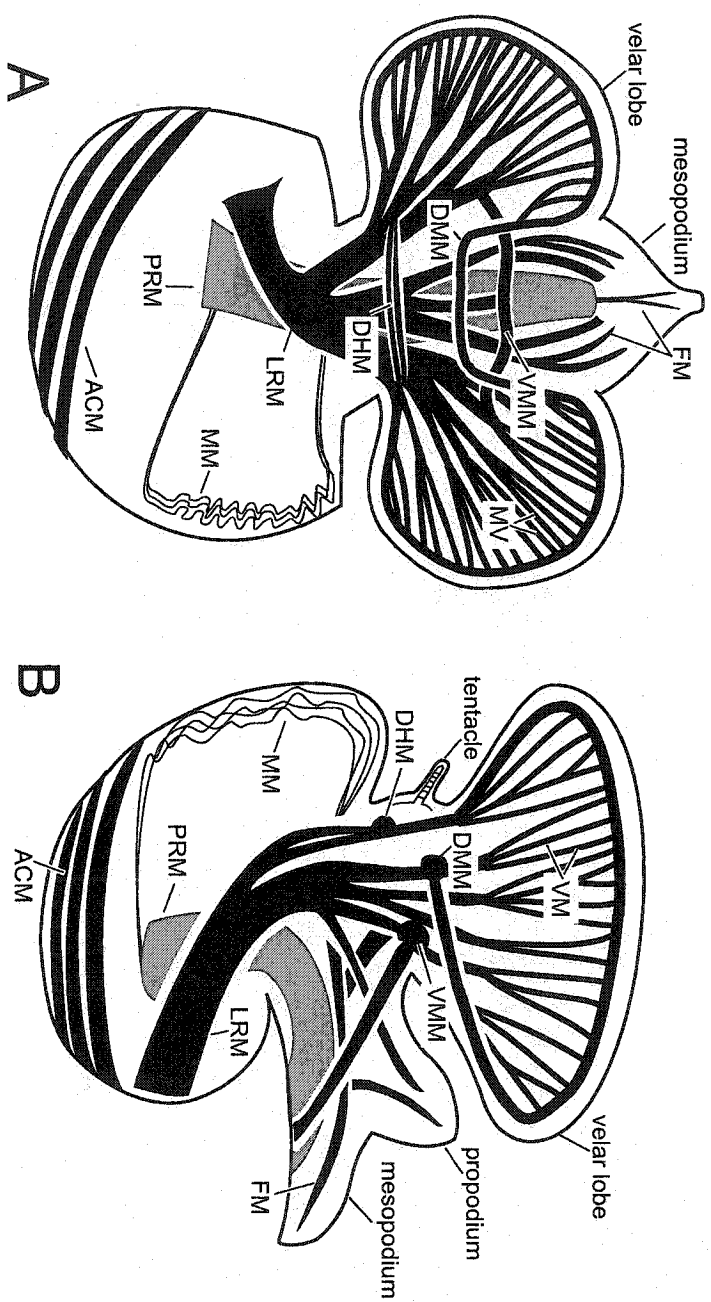

A
velar lobe
mesopodium
DMM
PRM
DHM
LRM
ACM
MM
VMM
FM
MV
B
tentacle
DHM
MM
PRM
ACM
DMM
VM
VMM
LRM
FM
velar lobe
propodium
mesopodium

Figure 33

Figure 34: TRITC-phalloidin labeling in the hatchling stage of *I. obsoleta.* Anterior is to the top in each figure. **A)** Dorsal view of the whole larva showing the LRM; larval retractor, ACM; accessory muscles, VMM; ventral mouth muscles and VM; velar muscles. Scale baris approximately 45 μm. **B)** Dorsal view of the anterior end showing the DHM. Scale bar is approximately 40 μm. **C)** Dorsal view of the anterior end showing the DMM; dorsal mouth muscle. Scale bar is approximately 40 μm. **D)** Lateral view of the whole larval showing the LRM; larval retractor muscle, ACM; accessory muscles, MM; mantle muscles, FM; foot muscles, VM; velar muscles and PRM; pedal retractor muscle. Scale bar is approximately 45 μm. **E)** Frontal view of the foot showing the FM; foot muscles Scale bar is approximately 35 μm. **F)** Lateral view of a muscle in the foot. Scale bar is approximately 10 μm. **G)** Lateral view of a velar lobe showing the VM; velar muscles. Scale bar is approximately 20 μm. **H)** Lateral view of the posterior end of the larva showing the ACM; accessory muscles. Scale bar is approximately 30 μm. **I)** Dorsal view of the posterior end of the larva showing individual fibers and striations of the LRM; larval retractor muscle. Scale bar is approximately 25 μm. Inset shows a 2X magnification of fibers of the LRM.

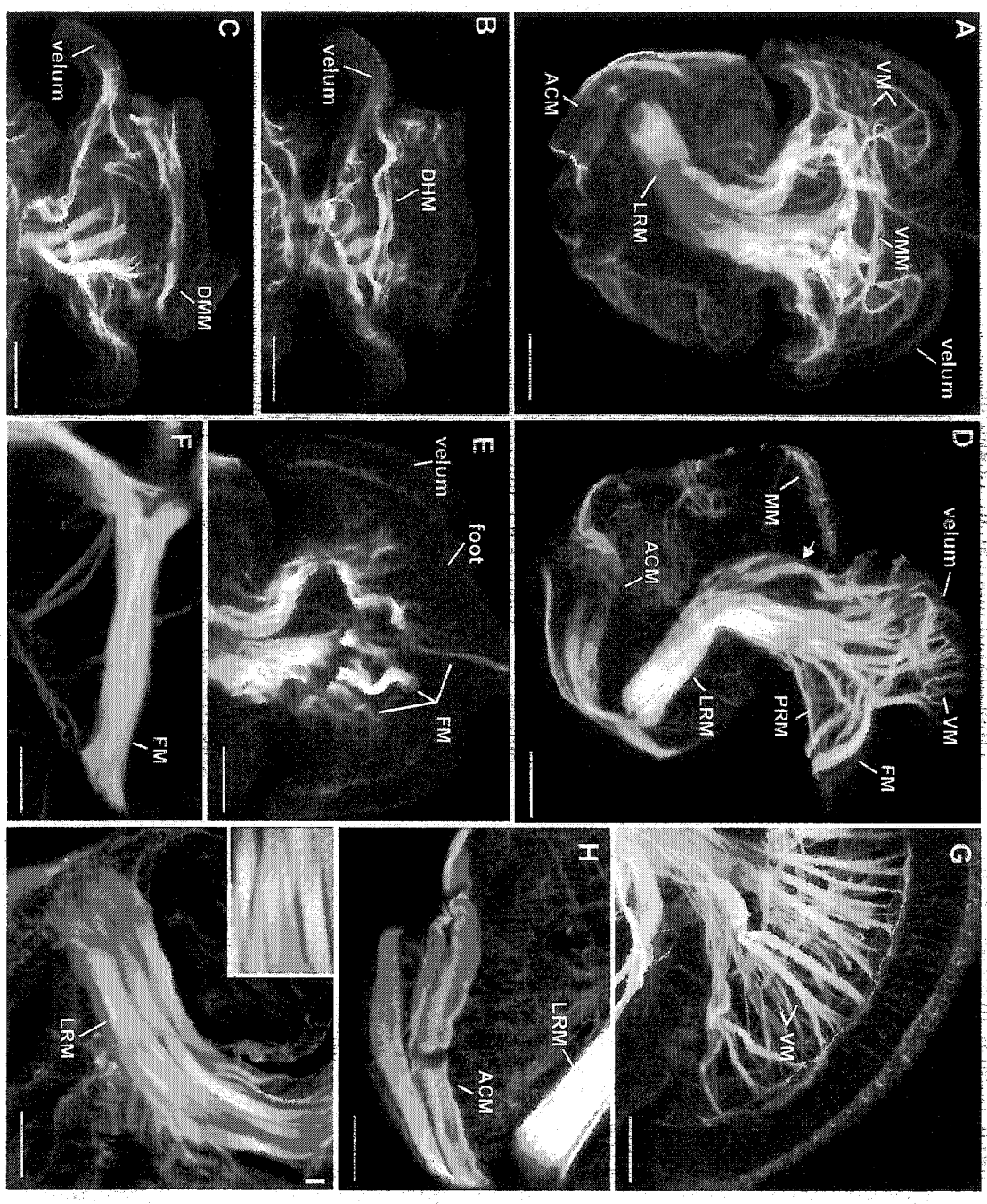

A
velum
VM
VMM
LRM
ACM
B
DHM
velum
C
DMM
velum
D
velum
VM
FM
MM
PRM
LRM
ACM
E
foot
velum
FM
F
FM
G
VM
H
LRM
ACM
LRM

Figure 34

Figure 35: TRITC-phalloidin labeling in mid to late larval stages of *I. obsoleta.* Anterior is to the top in each figure. **A)** Lateral view of a free swimming veliger 14 days post first cleavage showing the foot and left velar lobe. The LRM; larval retractor muscle, PRM; pedal retractor muscle, MM; mantle muscle, FM; foot muscles and VM; velar muscles are shown. Scale bar is approximately 60 μm. **B)** Magnified image of the right tentacle from a free swimming veliger 14 days post first cleavage showing the TM; tentacle muscles (2X magnification of C). **C)** Lateral view of the same animal shown in A showing the right velar lobe. The LRM; larval retractor muscle, MM; mantle muscle, VM; velar muscles and ACM; accessory muscles are shown. Scale bar is approximately 60 μm.

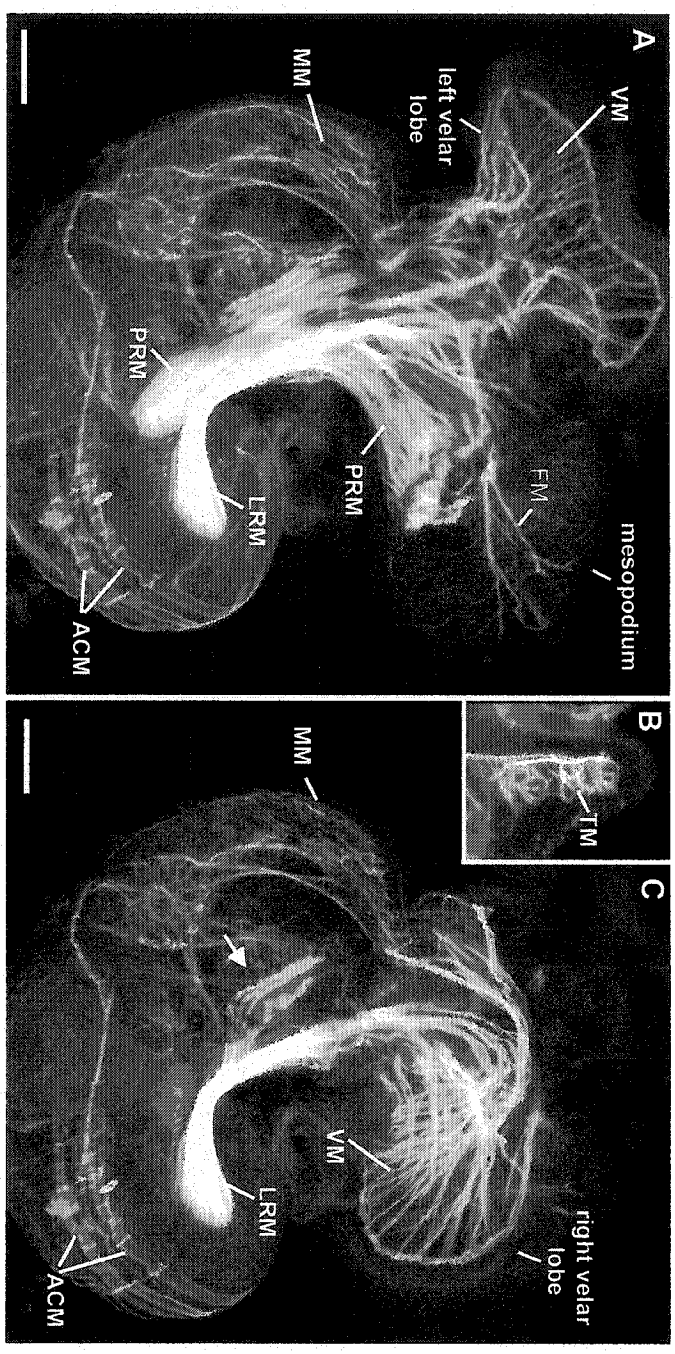

A
VM
left velar lobe
MM
PRM
PRM
FM
mesopodium
LRM
ACM
B
TM
C
MM
VM
LRM
ACM
right velar lobe

Figure 35

Figure 36: **A)** Lateral view of the posterior regions of the LRM; larval retractor muscles and PRM; pedal retractor muscles, in a veliger 14 days post first cleavage. Compare the diameters of the two muscle groups at the attachment sites. Scale bar is approximately 30 μm. **B)** Frontal view of the foot in a veliger 14 days post first cleavage showing the PRM; pedal retractor muscles, and the FM; foot muscles. Scale bar is approximately 30 μm. **C)** Lateral view of the right velar lobe in a veliger 14 days post first cleavage showing the VM; velar muscles. Scale bar is approximately 35 μm. **D)** Lateral view of the posterior end of a veliger 21-25 days post first cleavage showing numerous ACM; accessory muscles and muscle lining the esophagus (arrow). Scale bar is approximately 80 μm. **E)** Ventral view of the mesopodium in a veliger 21-25 days post first cleavage, shows the network of the FM; foot muscles and muscle lining the edge (arrow). Scale bar is approximately 60 μm. **F)** Ventral view of the foot in a veliger 21-25 days post first cleavage, shows the network muscles in the propodium (PrM; propodial muscles). Scale bar is approximately 65 μm.

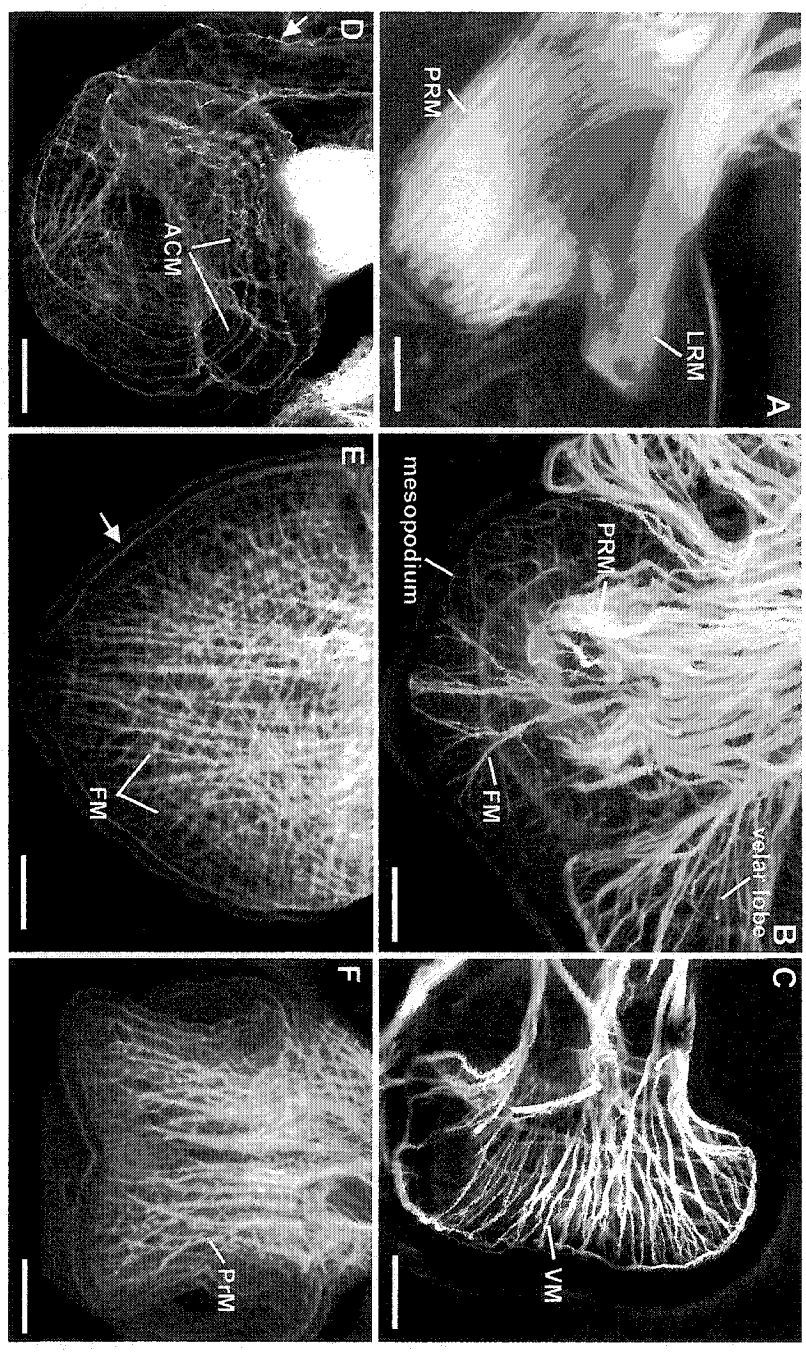


Figure 36

Figure 37: TRITC-phalloidin labeling in embryonic stages of *I. obsoleta*. Anterior is to the top in each figure. **A)** Dorsal lateral view of a trochophore stage. Diffuse labeling of the LRM; larval retractor muscle, ACM; accessory muscles and DMM; dorsal head muscles were evident. Scale bar is approximately 35 μm. **B)** Dorsal view of the whole animal at an early veliger stage. The VMM; ventral mouth muscles, DMM; dorsal mouth muscle, VM; velar muscles are shown. Scale bar is approximately 40 μm. **C)** Dorsal view of the anterior end of an early veliger stage showing a more dorsal view than in B revealing the DHM; dorsal head muscles. Scale bar is approximately 40 μm. **D)** Ventral view of the anterior end of an early veliger stage showing the VMM; ventral mouth muscle and the FM; foot muscles. Scale bar is approximately 40 μm.

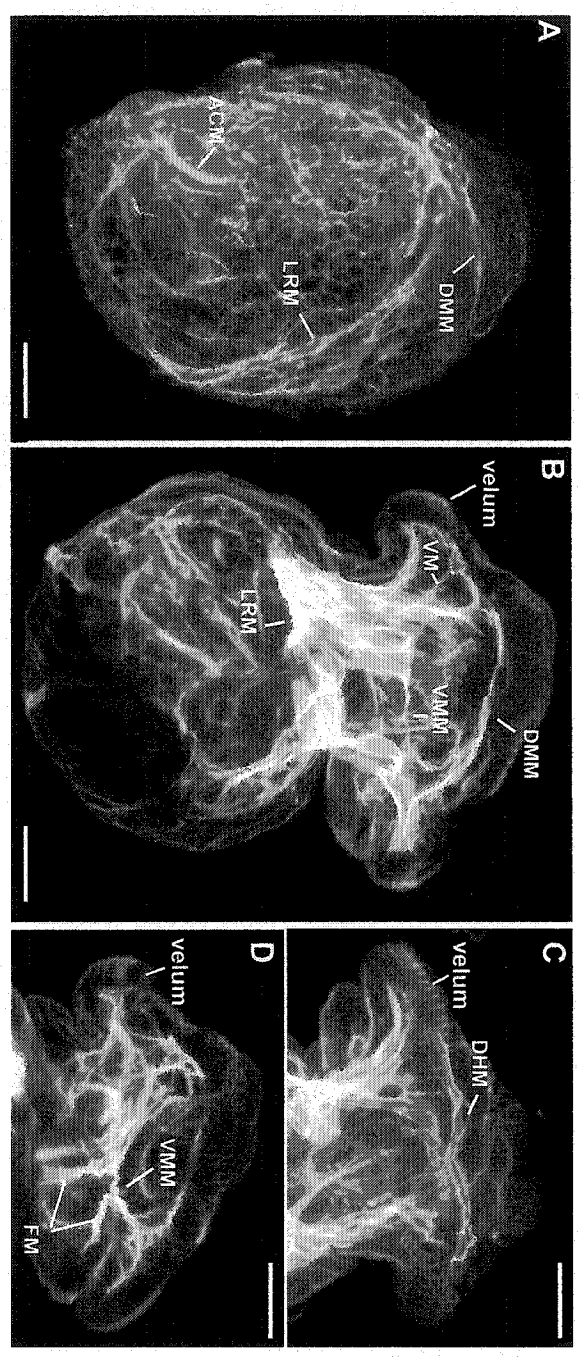


Figure 37

Figure 38: TRITC-phalloidin labeling in metamorphic and juvenile stages of *I. obsoleta*. Anterior is to the top in each figure. **A)** Lateral view of the anterior end of the animal showing the reduced velar lobe, condensed VM; velar muscles, PRM; pedal retractor muscle and the still evident LRM; larval retractor muscle. Blebs of labeled actin (arrowheads) are also evident. Scale bar is approximately 80 μm. **B)** Lateral view of the velar lobe showing loss of insertion/attachments of the VM; velar muscle fibers resulting in their wavy appearance. Scale bar is approximately 50 μm. **C)** Lateral view of the anterior end of the animal showing further reduction of the velar lobe and broken and fewer fibers extending from the LRM to the velum. Scale bar is approximately 90 μm. **D)** Higher magnification of the velar lobe shown in C revealing broken muscle fibers (arrows). Scale bar is approximately 45 μm. **E)** Lateral view of the anterior end of a juvenile snail showing the large PRM; pedal retractor muscle, the remnants of the LRM; larval retractor and its insertion (arrow) ventral to the radular sac. Scale bar is approximately 50 μm.

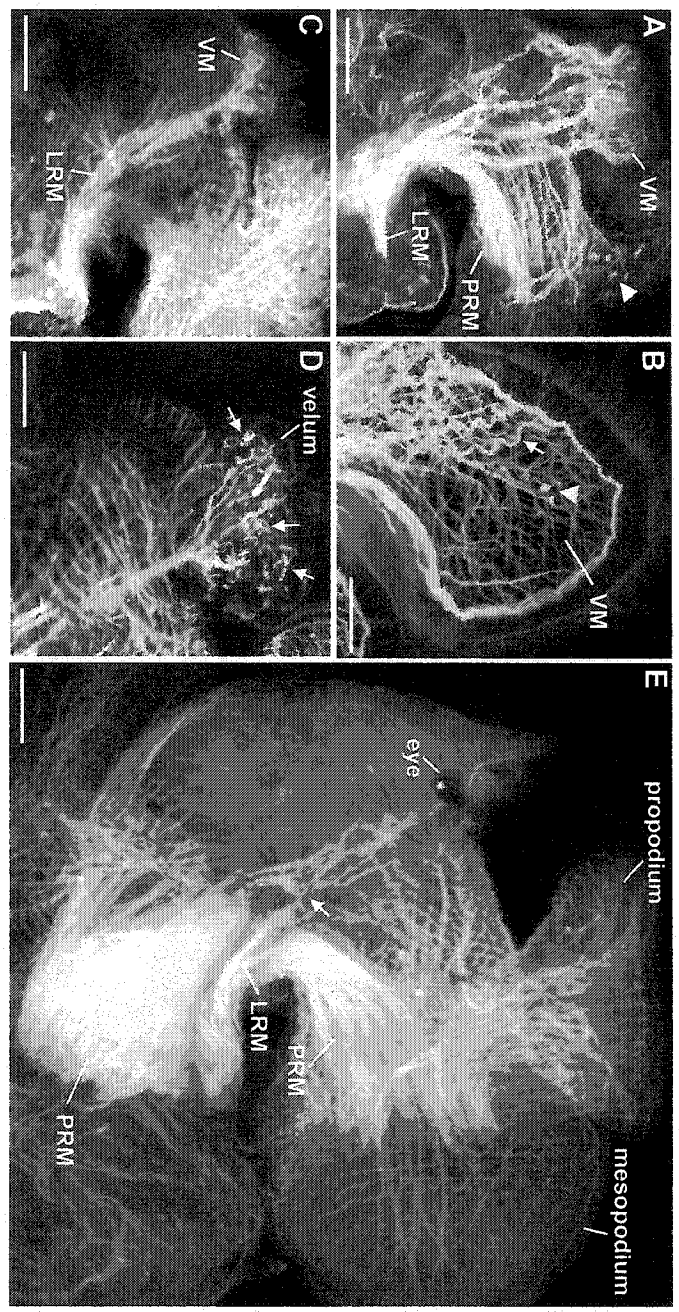

A
VM
LRM
PRM
B
VM
C
VM
LRM
D
velum
E
propodium
eye
mesopodium
PRM
LRM
PRM

Figure 38

Figure 39: D lineage tracing, ablation and block of MAPK signaling experiments in *I. obsoleta*. Anterior is to the top in each figure **A)** Lucifer Yellow dextran(LYD) labeling soon after injection into the D macromere Scale bar is approximately 35 μm. **B)** LYD labeling in the whole animal seven days after injection into the D macromere showing labeling of many larval structures, notably the anterior portion of the LRM; larval retractor muscle. Scale bar is approximately 50 μm. **C)** LYD labeling of the anterior end of the animal seven days after injection into the D macromere showing labeling of the DMM, dorsal mouth muscle. Scale bar is approximately 45 μm. **D)** LYD labeling of the foot 7 days after injection into the D macromere showing no muscle fibers were labeled in the foot region Scale bar is approximately 35 μm. **E)** TRITC-phalloidin labeling in embryos 7 days after D ablation. Note that unorganized muscle arrangement. Smooth circular muscles (arrow) was evident. Scale bar is approximately 35. **F)** TRITC-phalloidin labeling in embryos 7 days after U1026 treatment. Note that unorganized muscle arrangement. Scale bar is approximately 35 μm. **G** and **H)** TRITC-phalloidin labeling in embryos 7 days after U1026 treatment showing higher magnified image to reveal muscle fibers lack striations. Scale bar is approximately 15 μm.

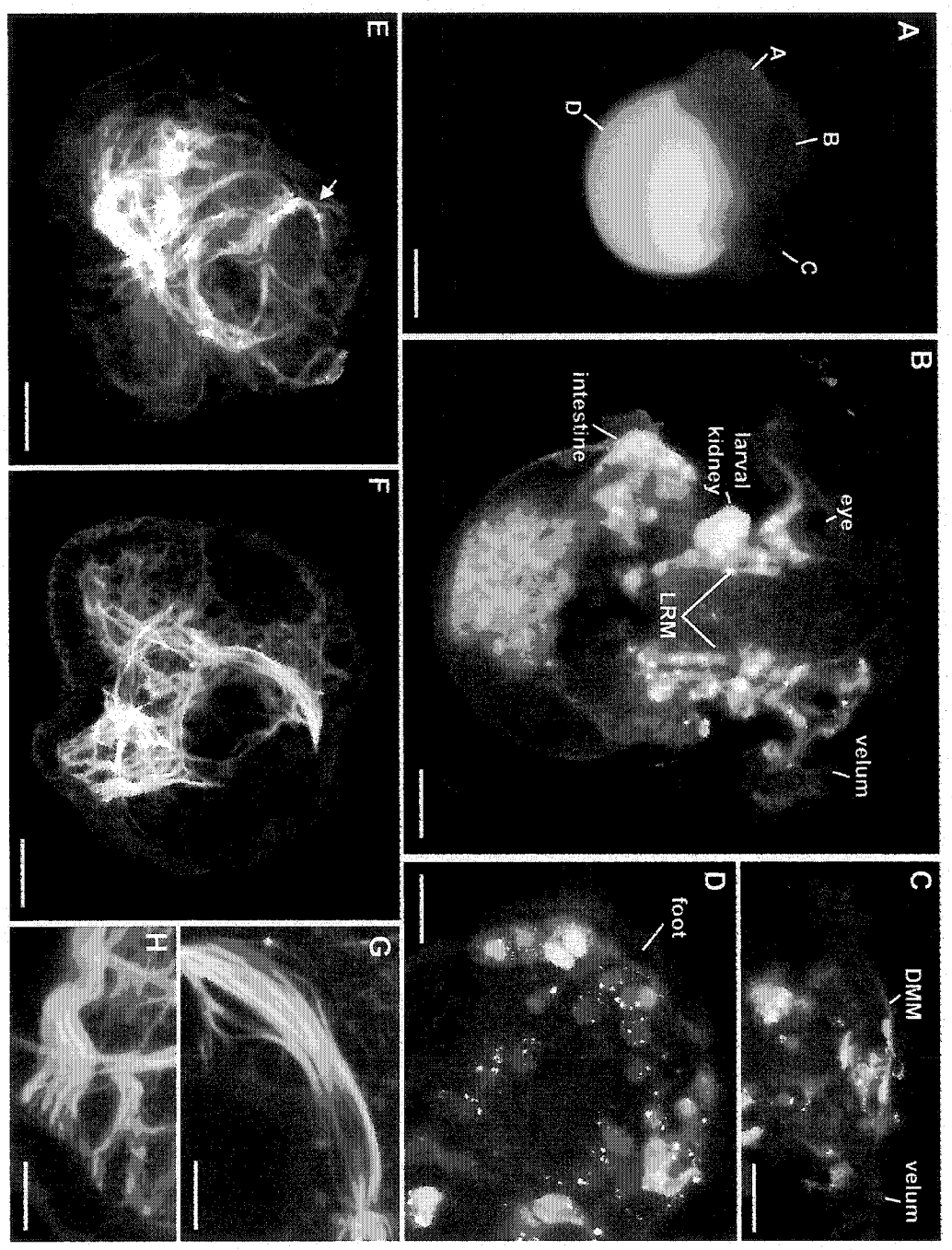

A
A
B
C
D
B
eye
larval kidney
intestine
LRM
velum
C
DMM
velum
D
foot
E
F
G
H

Figure 39

Concluding Remarks

In chapter 2, the components forming the LNS were examined and found to be consistent with the hypothesis, formed in chapter 1, that a general neural plan exists for gastropods. The larval nervous system of *I. obsoleta* include an apical organ, developing central ganglia and peripheral neurons associated with the velum, foot and mantle. Neurons were first observed during the embryonic trochophore stage and the nervous system became increasingly extensive during the larval period. During metamorphosis some elements disappeared, while others were incorporated into the adult nervous system. Neurons contained several different transmitters including monoamines and neuropeptides. Although a large portion of the larval nervous system in *I. obsoleta* was likely uncovered, still other neurons may exist. Possibly additional classical transmitters as well as various peptides are utilized in adult molluscan neurons. Preliminary studies using techniques to label acetylcholinesterase, glutamate, transforming growth factors,(see appendix 4A) revealed that other neural elements indeed exist in larval stages of *I. obsoleta*. Furthermore, histamine has also recently been localized in larval stages of *I. obsoleta* (unpublished observations). Future studies examining the fate of larval neurons will also be important. Few studies have even shown conclusive evidence of cell death in the molluscan nervous system. In collaboration with Dr. E. Leise and coworkers, I performed some initial studies label apoptotic cells using the TUNEL technique in larvae of *I. obsoleta*. Preliminary investigations have shown TUNEL positive cells in the

larvae of *I. obsoleta*. Preliminary investigations have shown TUNEL positive cells in the degenerating velum during metamorphosis (see appendix 4B). However, additional work is necessary to verify apoptosis in the TUNEL positive cells and to identify if cell death occurs in specific neurons. Nevertheless, this study has been the first study to reveal what appears to be an elaborate gastropod larval nervous system and has laid the groundwork for the investigations of the roles of the LNS and of the cellular and molecular mechanisms governing neural development in this taxon.

In chapter 3, evidence that elements of the larval nervous system control specific larval behaviors, such as swimming, feeding and the escape response was provided in the gastropod *I. obsoleta*. Such evidence consisted of anatomical data that showed neurons and axons containing monoamines and neuropeptides are associated with ciliary bands and muscle fibers in the velum and the mouth cavity. Furthermore, exposure to synthetic analogues of these monoamines and peptides had, in many cases, significant effects on qualitative and quantitative behaviors and cellular physiology. Together these experiments provided details about the types of endogenous transmitters and the receptors present in the gastropod larval nervous system. Moreover, the results have permitted the formulation of hypotheses concerning how these transmitters possibly work to control ciliary activity, muscle contractions, and cell physiology that ultimately control larval behaviors

This study has also shown that gastropod larvae have a more complex set of behaviors than previously anticipated. We have only begun to understand the neural

control of these behaviors. Since there are likely additional neurons and transmitter types in the LNS of *I. obsoleta*, additional studies are necessary to uncover these remaining neural elements and explore their roles. Further studies of the physiology of the pre-and post oral ciliated cells and muscle fibers are necessary to elucidate fully neural control these cells to control larval behaviors. For example, intracellular electrical recordings and imaging techniques using voltage sensitive ionic dyes will be useful to test how specific transmitters directly affect the physiology of these cells. Finally similar studies in other species would be useful to understand how generalized is the functioning of the larval nervous systems in gastropod and other molluscs. Details about the roles and physiology of particular components of the LNS in different species may also provide insights into how nervous systems evolved.

In chapter 4, it was shown that the distal-less protein (Dll) was normally expressed during embryonic and larval stages and thus likely has multiple roles in molluscan development. One such role in *I. obsoleta*, seems to be the involvement of Dll in differentiation and/or patterning the ectoderm, and the development of the nervous system. Dll expression in the larval nervous system was limited to the developing ganglia which persist into adult stages was not expressed in the apical organ or in peripheral neurons Therefore, based on expression patterns of this protein, it is possible that separate genetic programs and cellular mechanisms govern the differentiation of the future adult CNS versus the other components of the larval nervous system. This hypothesis was upheld in experiments in which the D macromere was ablated. The

resultant larvae lacked the normal Dll expression pattern in the ectoderm and in the developing CNS and only neurons outside the developing central ganglia were observed. Furthermore, early inductive signaling and subsequent formation of the germ layers were shown to be important for the normal expression of Dll and formation of the central ganglia. Finally the expression of Dll in the cells of the developing central ganglia suggest a conserved function for this protein in the development of migrating immature neurons. Such a function has been reported in vertebrate neural crest and basal forebrain. A better understanding of the genetic programs involved in the differentiation and patterning components of the LNS in *I. obsoleta* and other gastropod species may provide insight into how nervous systems have evolved within this taxon. Recent studies have shown other conserved genes expressed in components of the LNS in other gastropods, supporting the idea that genetic programs governing neural development are well conserved. For example, a *snail* orthologue was sequenced in *Patella vulgata* and was observed to be expressed in the apical organ as well as in other region of the trochophore (Lespinet *et al.*, 2002). In another species *Haliotus refescens*, a *Hox5* orthologue was sequenced and shown to be expressed in specific regions of the developing CNS (Giusti *et al.*, 2000). Experimental studies involving cell ablations and microinjections will also provide insights into the cellular origins of the different components of the LNS of *I. obsoleta*. Comparisons of cellular origins combined with details about genetic programs governing neural development, will be useful for understanding how developmental modifications may have influenced the evolution of nervous systems.

In chapter 5, the development of the musculature was examined in *I. obsoleta* and the results revealed a much more complex system of larval muscles in this species compared with reports in other gastropods. The striated larval retractor muscle (LRM) first appeared during the trochophore stage and most of its fibers degenerated during metamorphosis. The un-striated pedal retractor muscle (PRM) developed during the veliger stage and persisted though metamorphosis. In addition, three transverse muscle systems, intrinsic muscles in the velum, tentacles and foot, accessory muscles, mantle muscles and muscles lining the digestive tract existed in the veliger. These anatomical studies were consistent with studies in other gastropod species and thus indicated the possibility that at least two major groups of muscles existed in *I. obsoleta*

Furthermore in this chapter, investigations of the cellular origins and specification of the muscles were performed. Lineage tracing using Lucifer yellow dextran, cell ablations and a chemical block of early inductive signals, also provided important evidence to support the hypothesis that there are at least two different groups of larval muscles. The first group were muscles that only function in larval stages, degenerating at metamorphosis. The second group consisted of muscle systems which contributed to the adult musculature. Together, the morphology, development, fates and cellular origins, suggested the possibility that different programs govern the development of the major larval muscles. This theory was also supported by recent molecular evidence which have shown that there is differential expression of a *Mox* orthologue in larval muscles. *Mox* is homeobox gene that is expressed in the mesoderm and developing muscles in vertebrates

(Degnan *et al.*, 1997a; Hinman and Degnan, 2002). Future experiments, investigating genetic networks governing myogenesis will also be useful for understanding how molecular and cellular changes influenced the evolution of different types of muscle. Thus, the expression patterns of other conserved genes involved in mesoderm patterning and muscle differentiation will be useful to examine during the development of *I. obsoleta* and also in other gastropods. In an attempt to examine other such conserved molecules, I screened several antibodies from the Developmental Studies Hybridoma Bank. On such antibody was against Nkx 2.5 a gene that has been shown to be involved with mesoderm and muscle development in other taxa. The expression of anti-Nkx 2.5 in *I. obsoleta* did not reveal labeling of mesoderm or muscle specific tissue, but rather the developing pharynx. However, such experiments could provide insights in how genetic networks may have changed in various taxa and how genes have become co-opted for different functions.

In conclusion, the major goals of this thesis was to provide pioneering studies in neural and musculature development in gastropods providing information about anatomy, roles and developmental mechanisms in a single organism. From this research intriguing questions about how developmental modifications to the cellular origins, physiology, molecular networks and signaling pathways, reflect evolutionary changes in nervous and muscle systems. Gastropod larvae offer interesting models to test these questions, since their cleavage patterns are well known, lineage analysis is relatively easy to perform, and the larval nervous systems and musculature is comparatively simple. Furthermore, with

the recent advances in molecular sequencing in molluscs, there is the potential to better understand mechanisms governing development in this taxon.

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Appendix 1

Gastropod Phylogeny

The consensus, based on morphological characters, is that gastropods are monophyletic, that is they share a common ancestor. The most comprehensive review of gastropod phylogeny was published by Ponder and Lindberg (1997), who separated the gastropods into two major groups, the Eogastropoda and the Orthogastropoda. The Eogastropoda includes the Patellogastropoda (e.g. limpets) and their extinct ancestors while the Orthogastropoda comprises all other groups of gastropods. The major clades of orthogastropods include the Vetigastropoda, Neritopsina and Apogastropoda and the Hot vent taxa. The most basal clade, the vetigastropods, include many of the old "Archaeogastropoda" (e.g. abalone). The Neritopsina (bleeding tooth shells) were also called archaeogastropods in the past. The Apogastropoda are divided into the Caenogastropoda and Heterobranchia. The caenogastropods include the "Neogastropoda" (e.g. periwinkles, cowries, conchs, moon shells muricids, whelks, and cones) and most of the former "Mesogastropoda". The heterobranchs comprise of the Opisthobranchia and Pulmonata and a few groups previously classified as "mesogastropods" (e.g. sea slugs, land and fresh water snails and slugs).

Figure 40: A simplified summary diagram of gastropod phylogeny after Ponder and Lindberg (1997).

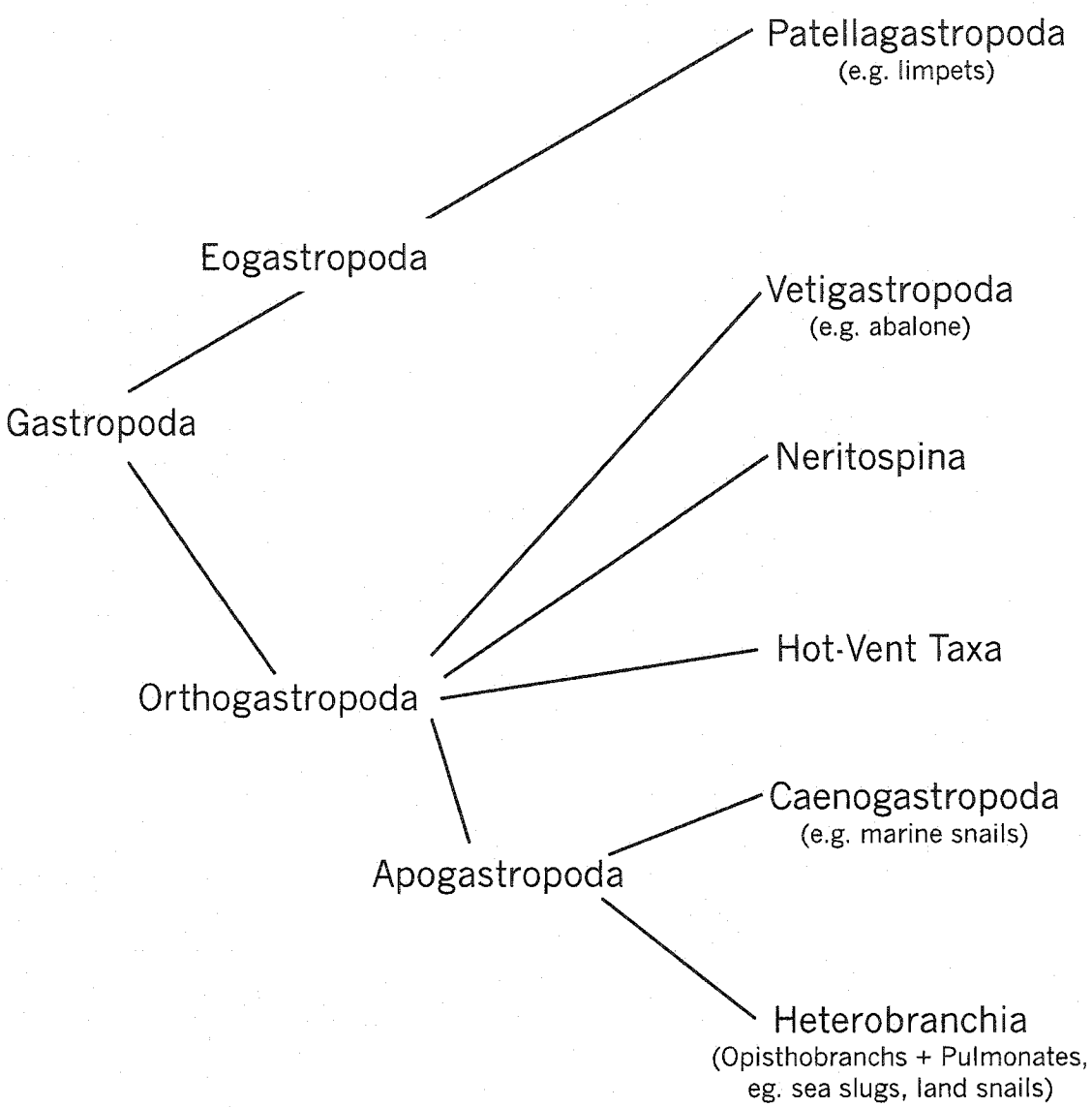
Patellagastropoda
(e.g. limpets)
Eogastropoda
Vetigastropoda
(e.g. abalone)
Gastropoda
Neritospina
Hot-Vent Taxa
Orthogastropoda
Caenogastropoda
(e.g. marine snails)
Apogastropoda
Heterobranchia
(Opisthobranchs + Pulmonates,
eg. sea slugs, land snails)

Figure 40

Appendix 2

Double Labeling of Serotonin- or FMRFamide-LIR Cells and Axons and Musculature in Hatchling Stages of *Ilyanassa Obsoleta*.

The close association of muscle fibers and axons is evident in the following images.

Figure 41

A) Frontal view of a larva showing serotonin-LIR axons (green) and F- actin labeled muscle fibers (red) in the velum, foot and above and below the mouth. Dorsal is to the top of the image. Scale bar is approximately15 μm. **B)** Right lateral view of a larva showing FMRFamide-LIR cells and axons in the head and foot. Anterior is to the right of the image. Scale bar is approximately 15 μm. **C)** Right lateral view of the mantle region showing the muscle fibers lining the mantle cavity and serotonin-LIR cells in the osphradium and axons also lining the mantle cavity. Scale bar is approximately 7.7 μm. Abbreviations: DMM, dorsal mouth muscle; VM, ventral mouth muscle; FM, foot muscle; LRM, larvale retractor muscle; FSb, FMRFamide-LIR sub-intestinal cell; FA FMRFamide-LIR apical cell; FC, FMRFamide-LIR cerebral cell ; MM, mantle muscles; SOs, Serotonin-LIR osphradial cell

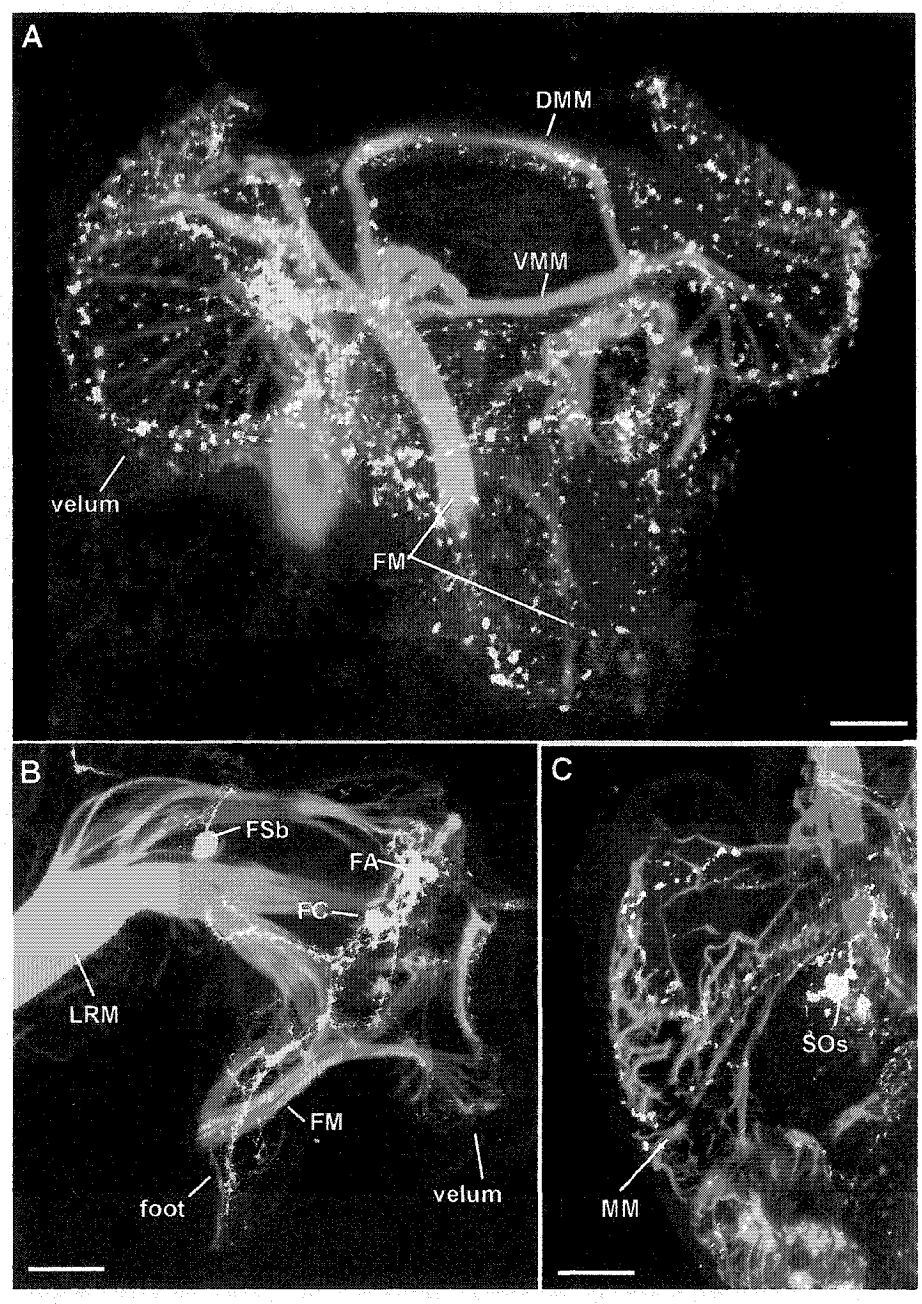

Figure 41

Appendix 3

Separation of the D macromere after Ablation

D macromere ablations were performed with a glass capillary pulled to a fine point (see Materials and Methods, Chapter 4 and 5).

Figure 42

A) Five minutes after D macromere ablation. The cell attachment site has become stretched and clear vesicles were extruded from around the edges. Scale bar is approximately 23 μm. **B)** Thirty minutes after D macromere ablation, shows that the cell becomes completely detached from the other cells by this time. Scale bar is approximately 23 μm.

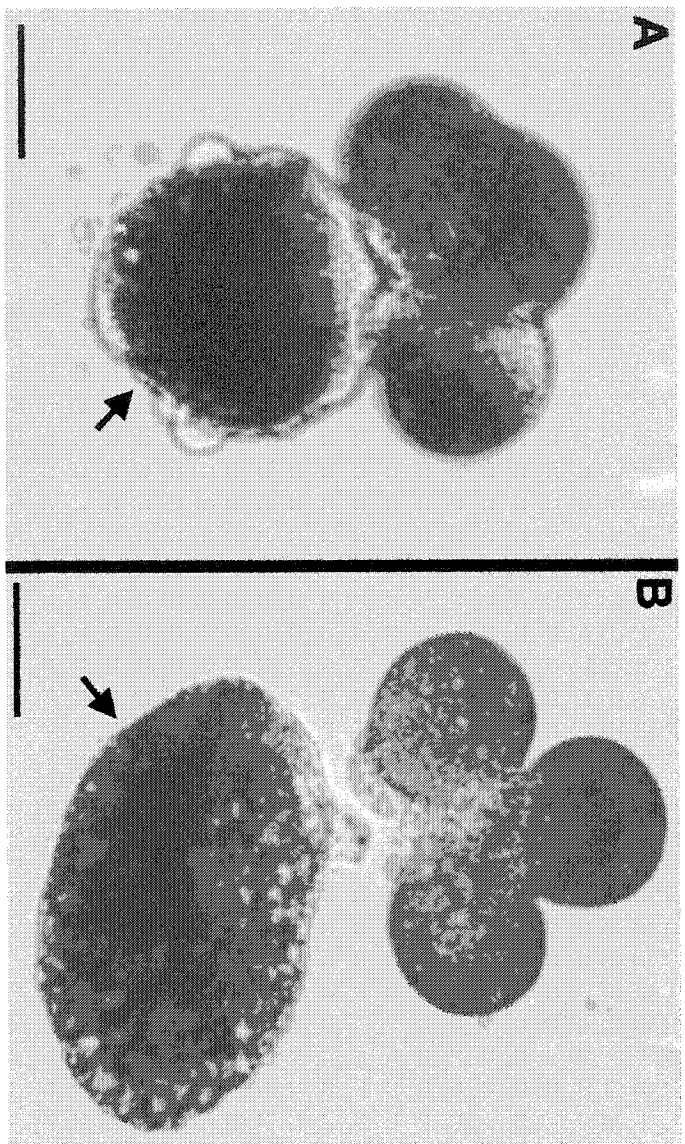

Figure 42

Appendix 4

Preliminary Results of Additional Experiments in *Ilyanassa obsoleta*

A: Acetylcholinesterase Labeling in *I. obsoleta*

Acetylcholinesterase (AChE), an enzyme which breaks down acetylcholine in the synapse was localized to reveal the possible existence of acetylcholine in *I. obsoleta*. A similar technique to localize this enzyme has also been utilized in other molluscs (Raineri, 1995; Raineri, 2000). Antibodies for choline acetyltransferase (Chat) did not reveal immunoreactivity during any stages and therefore specificity of this labeling technique could not be confirmed.

Figure 43.

A-B) AChE labeling in the trochophore stage appeared at the anterior most region in a pair of cell-like structures (arrows). Labeling also appeared along the margin of the rudiments of the velar lobes, between and under cells that become the pre-oral ciliated cells. Diffuse labeling of patches of cilia were also observed in region of the telotroch. **A)** Ventral view of a whole trochophore. Scale bar is approximately 25 μm. **B)** Right lateral view of the anterior half of a trochophore. Scale bar is approximately 25 μm. **C-E)** AChE labeling in the hatchling stage. **C)** Frontal view showing velum and head regions showing labeling of ciliated patches in the apical plate region (arrow) and mantle edge also along the base and between the pre-oral ciliated cells (arrow). Scale bar is approximately 20 μm. **D)** Ventral view of the foot showing AChE labeling of the epithelial surface of cells at the tip of the foot. Scale bar is approximately 15 μm. **E)**

epithelial surface of cells at the tip of the foot. Scale bar is approximately 15 μm. **E)** Lateral view of a velar lobe showing labeling along the base and between the pre-oral ciliated cells. Scale bar is approximately 15 μm.

These results revealed that labeling was often associated with patches of cilia on the foot, head and mantle edge and telotroch. In addition the labeling was also observed at the base of the pre-oral ciliated cells. Therefore, it is possible that acetylcholine containing axons may synapse on the ciliated epithelial cells throughout the larva. Additional studies are necessary to confirm this hypothesis.

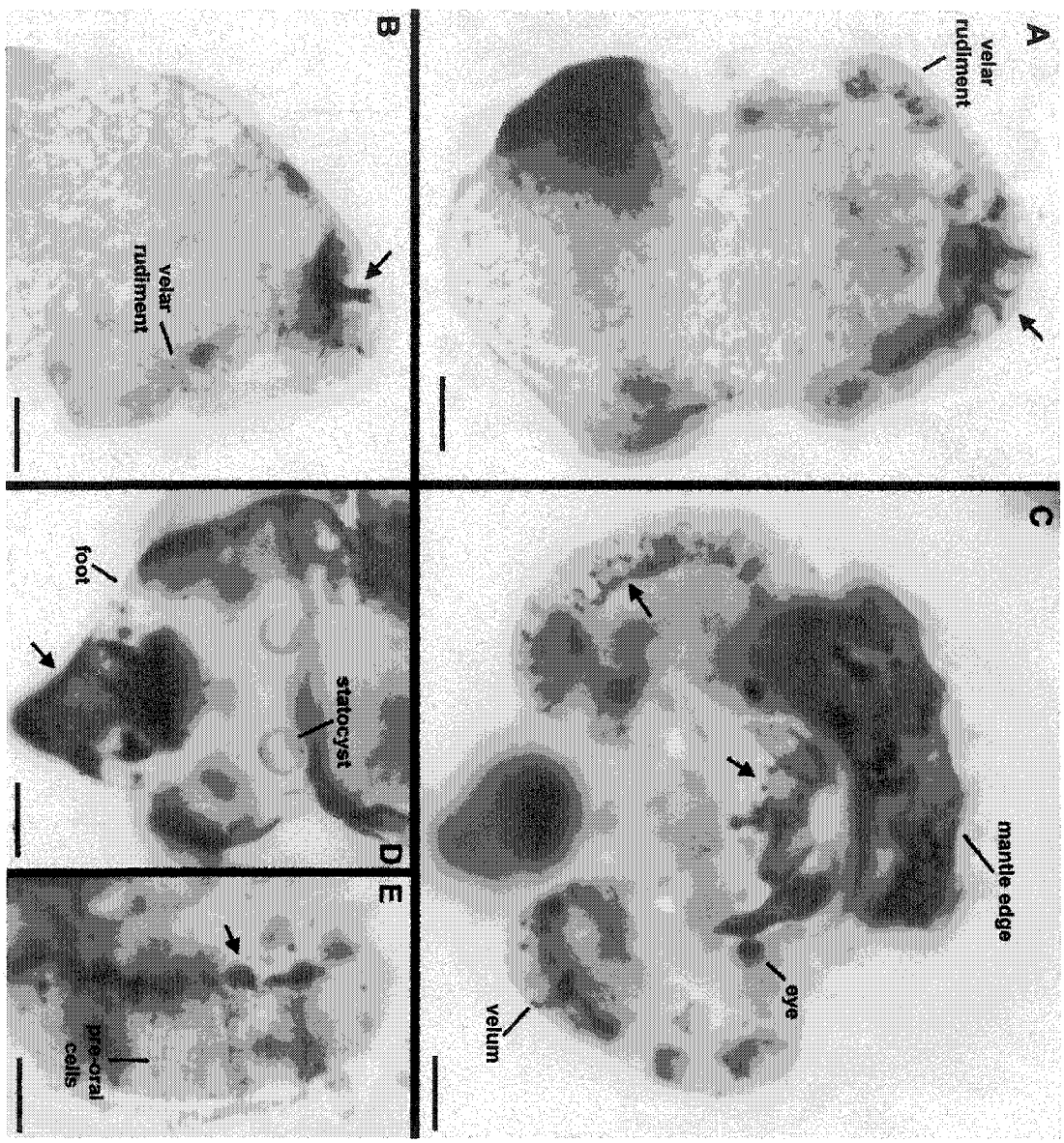
A
velar rudiment
B
velar rudiment
C
mantle edge
eye
velum
D
foot
statocyst
E
pre-oral cells

Figure 43

B) Labeling Axons and Cells by Various Antibodies in Hatching stages of *I. obsoleta.*

Various different antibodies were screened in hatchling stages of *I .obsoleta* and several of these antibodies revealed neural-like elements. However, the intensity of labeling was very faint, the identity of the structures labeled were questionable or unreliable and therefore were not included in the initial morphological study (Chapter 2). Some examples of antibodies that labeled neural-like structures are shown here.

Figure 44

A) The SV2 antibody (synaptic vesicles protein; K.M. Buckley) obtained from Developmental Studies Hybridoma Bank (University of Iowa) labeled many axons in the hatchling stage. Scale bar is approximately 45 μm. **B)** Anti-APGWamide (Chemicon) also labeled many axons in the hatchling stage. Scale bar is approximately 45 μm. **C)** Anti-glutamate (Signature Immunologics) labeling showing two cells in the velum of the hatchling stage. Scale bar is approximately 45 μm. **D)** The NF (Neurofilaments, RT97; J. Wood) antibody obtained from Developmental Studies Hybridoma Bank (University of Iowa). Revealed vase-shaped cells in the head region (arrow). Scale bar is approximately 45 μm.

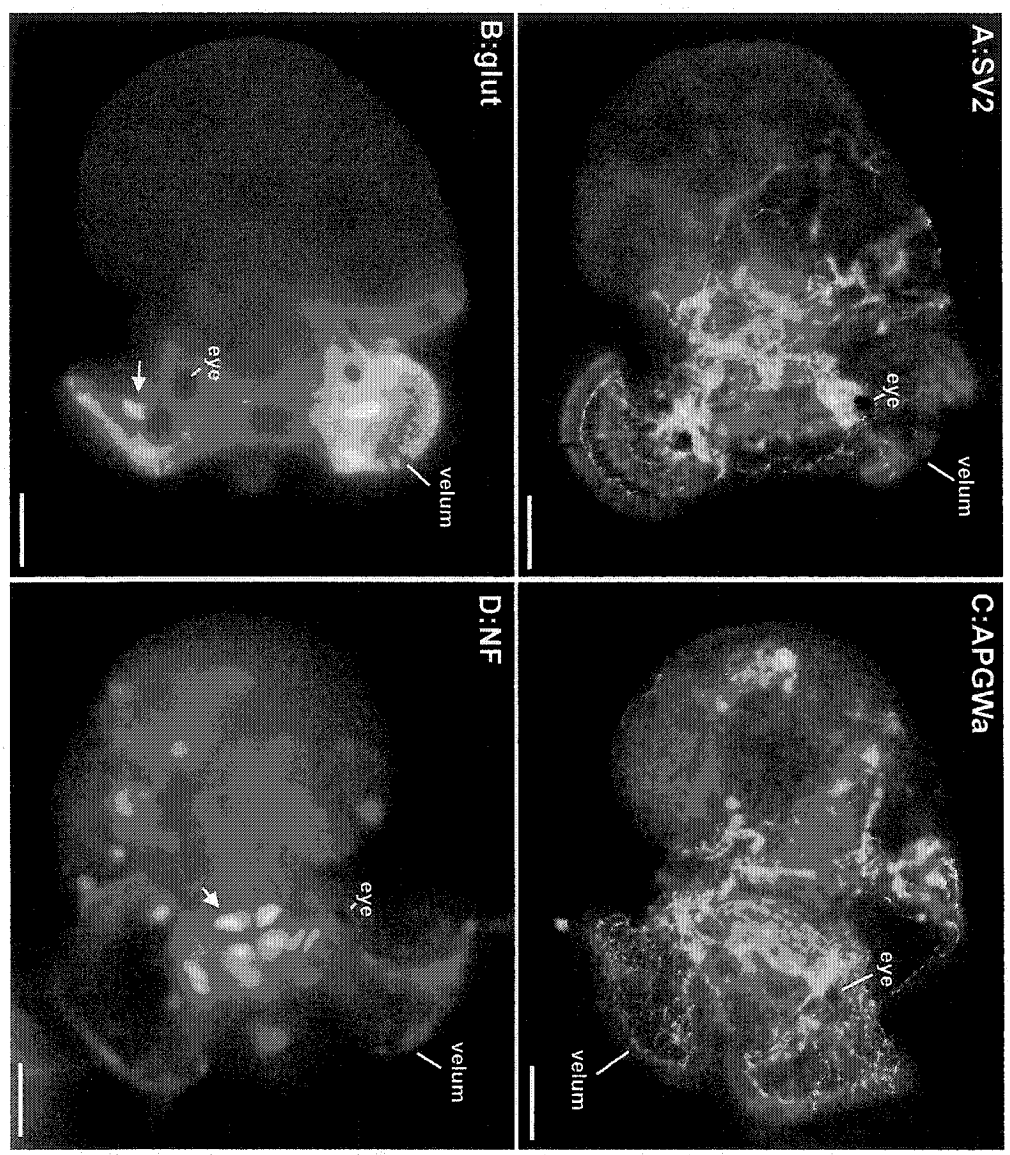
A:SV2
eye
velum
C:APGWa
eye
velum
B:glut
eye
velum
D:NF
eye
velum

Figure 44

C) TUNEL Labeling in *I. obsoleta*; Evidence of Programmed Cell Death During Metamorphosis

The TUNEL (TdT-mediated dUTP digoxigenin nick end labeling) technique uses the enzyme, terminal deoxynucleotidyl transferase (TdT) to label cells that have oligonucleosomal nicks/strand breaks in their DNA. An In Situ Cell Death Detection Kit from Boehringer (Mannheim), with directly labeled dUTP-FITC to reveal cells undergoing apoptosis during metamorphosis.

Figure 45

A) TUNEL labeling during metamorphosis shows several nuclei labeled in the mantle and degenerating velum (arrows). Scale bar is approximately 100 μm. **B)** TUNEL labeling during metamorphosis showing several nuclei labeled in the propodium and mantle regions. Scale bar is approximately 100 μm.

Surprisingly, a high degree of variability in the labeling pattern was observed in larvae at approximately the same stages. Furthermore, fewer cells than expected were labeled in each animal. Therefore, the TUNEL technique may only label dying cells during a discreet period of time. Other methods are needed to verify these results.

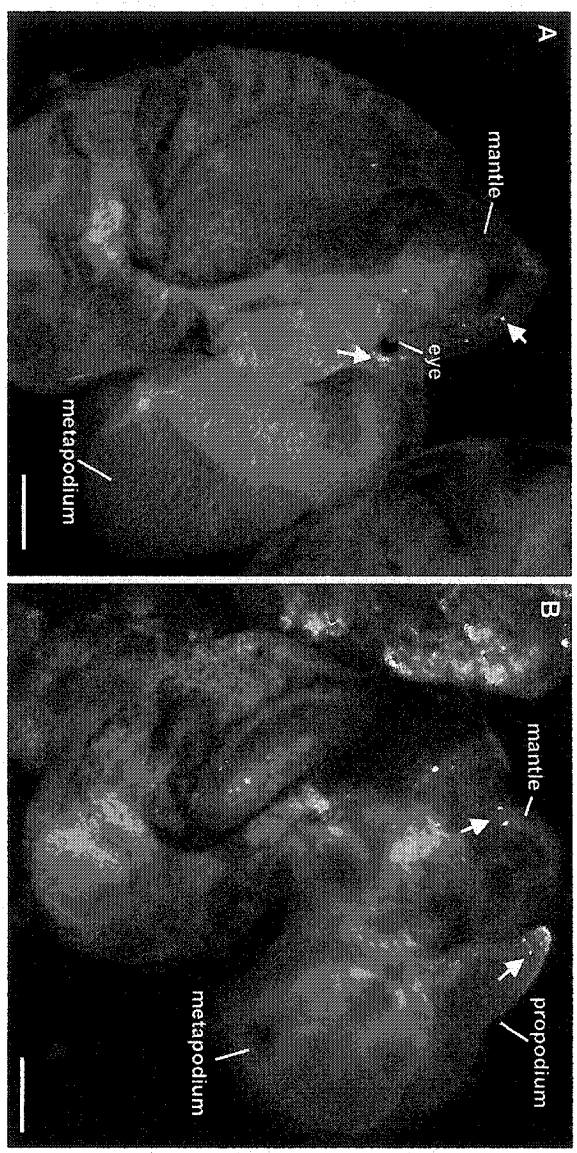

Figure 45

D) Nkx-like immunoreactivity in the veliger stage of *I. obsoleta*

Nkx genes code for a transcription factor and have been shown to have many roles in development including neural and muscle differentiation (for examples (Buckingham, 1994; Cornell and Ohlen, 2000). These genes have also been shown to exist in a variety of taxa and often have similar roles. An antibody against a chick Nkx protein (74.5A5, T.A. Jessell) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

Figure 46:

Nkx-like immunoreactivity was observed in cells of the pharynx or esophagus and surrounding the mouth. Scale bar is approximately 40 μm.

While, the labeling was not found in developing muscle or neurons, as hoped for, it does suggest an interesting avenue. *Nkx* orthologues have also been reported to be expressed in the developing pharynx in *Drosophila* (Zaffran *et al.*, 2000; Tanaka *et al.*, 2001).

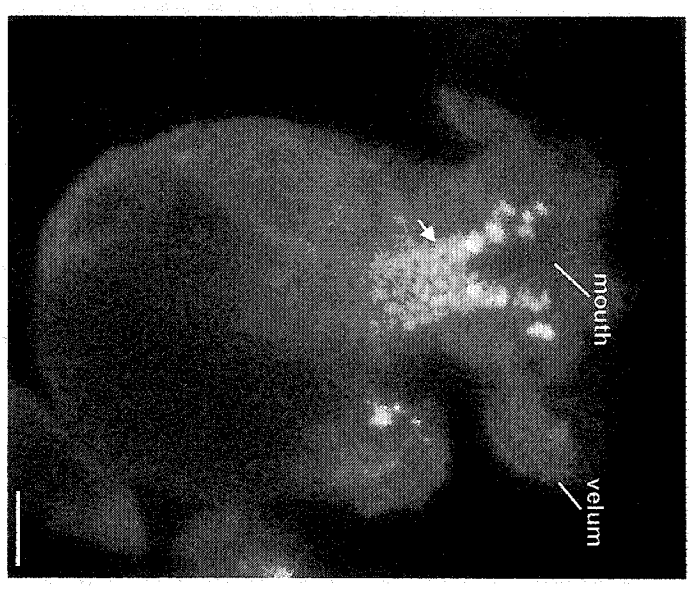

Figure 46