Investigations of Neural Ontogeny in the Larval Oyster *Crassostrea virginica* and the nudibranch *Berghia verrucicornis*: A Histological and Immunohistochemical Approach

by

Ivey Rebecca Ellis

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 14, 2010

Keywords: bivalve, larva, nervous system, gastropod, histology, small cardioactive peptides

Approved by

Stephen Kempf, Chair, Associate Professor Biological Sciences
Christine Sundermann, Professor Biological Sciences
Kevin Fielman, Assistant Professor Biological Sciences

Abstract

Bivalves are a diverse group of molluscs that inhabit both marine and freshwater habitats. These animals play a vital role in the success of entire aquatic ecosystems by filtering the water and providing structural complexity to the benthos. Bivalves are also economically significant. Aquaculture practices rearing various bivalve species has expanded worldwide, which in turn has created a renewable food resource and millions of dollars in revenues. Due to anthropogenic and natural occurring events, bivalve populations have recently declined. In order to restore existing populations and facilitate aquaculture practices, research has shifted towards broadening our knowledge of bivalve biology including neurological morphology. Such information is vital to our understanding of how the nervous system effects and modulates behavior. This is particularly true during the larval period of development where behaviors such as setting and metamorphosis are critical for the production of viable adults.

Bivalve ontological research concerned with morphologic and, more recently, immunohistochemical analyses have occurred over the past century; however, none of these studies has provided a detailed histological analysis of nervous system development. The first chapter of my thesis provides a review of previous research concerned with bivalve ontogeny from fertilization through the mature larval stage with references to research on adult bivalves where applicable. Emphasis is placed on nervous system development. In addition, studies relating to potential cues for larval settlement and subsequent metamorphosis are discussed as they relate to the nervous system. This

review emphasizes the fact that a detailed investigation of bivalve nervous system development is still lacking in the extant literature.

Chapter 2 provides a histological analysis of the ontogeny of the central and peripheral nervous systems during the three major larval stages of *Crassostrea virginica* (the Eastern oyster). *C. virginica* was chosen as my model organism due to it's ecological and economic significance. This study characterizes the larval nervous system, reports the presence of novel ganglia and describes the peripheral nerves that innervate specific larval organs and tissues.

Based on the facts that small cardioactive peptides (SCPs) have been documented to modulate ciliary beating, feeding and / or gut motility in adult gastropods and in a few adult bivalves and that they have been reported as present in a few larval gastropods, chapter 3 of my thesis uses immunological techniques to examine the presence and location of SCPs in the larval stages of *C. virginica* and *Berghia verrucicornis*. The location of SCPs within the central nervous system and peripheral tissues is discussed along with proposed functions of SCPs in molluscan larvae.

Collectively, this thesis is a significant addition to our understanding of the bivalve larval nervous system, This new information will assist future studies in forming a connection between larval neural morphology and critical life history behaviors. As such, these findings will potentially aid in facilitating bivalve restoration efforts and advances in aquaculture techniques.

Acknowledgments

I want to first recognize my advisor Dr. Stephen Kempf for his guidance, encouragement and patience during my graduate years at Auburn University. Thank you for making my graduate career an adventurous and fun experience! I also appreciate the comments of my MS committee members, Drs. Christine Sundermann and Kevin Fielman. In addition, I would like to that Dr. Sundermann for her supervision and laboratory supplies throughout my mussel glochidium analysis. I want express my gratitude to The American Museum of Natural History, the American Microscopical Society, and the Auburn University Graduate School for providing some of the funds necessary to complete this project. Thank you to my friends and fellow graduate students, Shanna Hanes and Maria Mazzillo who have given advice and supported me throughout my masters. Thank you to my parents, Andy and Pam Ellis, and my siblings, Zach, Sumner and Worth Ellis, because without my family I would not be the individual I am today. Lastly, I want to thank my other half, Daniel Holt, for supporting me unconditionally.

Table of Contents

List of Figures

Chapter 1:

A Review of Bivalve Embryonic and Larval Life Stages with an Emphasis on Development of the Larval Nervous System

Ivey Ellis* and Stephen C. Kempf

Department of Biological Sciences, Auburn University, 331 Funchess Hall Auburn University, AL 36849-5407

*ellisir@auburn.edu (corresponding author)

key words: bivalve, larvae, development, and nervous system

List of Abbreviations

5-HT = serotonin

a = anus

aa = anterior adductor

ao = apical organ

aob = adoral ciliary band

bd = byssal gland duct

bg = byssal gland

cg = cerebral ganglion

cs = crystalline style

cvc = cerebro-pleural-visceral connective

DA = dopamine

DAB = Diaminobenzadine

dg = digestive gland

es = esophagus

eye = eye

g = gill primordium

gb = gill bridge

gc = gill cavity

gs = gastric shield

H = hinge

hf = heel of foot

hk = primordium of heart and kidney

i = intestine

m = mouth

mc = mantle cavity

ml = mouth lobe

pa = posterior adductor

pg = pedal ganglion

plg= pleural ganglion

pn = protonephridium

pob = postoral ciliary band

pr = pedal rectractor

prb = preoral ciliary band

pt = postanal ciliary tuft

s = stomach

SCP = small cardioactive-like peptides

Sh = shell

ss = style sac

st = statocyst

tf = toe of foot

u = umbo

U = umbo

v = velum

V = velum

vg = visceral ganglion

vr = velar retractor

Abstract

The class Bivalvia is the second largest class within the phylum Molllusca, and includes a multitude of both ecologically and economically valuable species. Due to recent declines of bivalve populations and an expansion of worldwide aquaculture practices, it is important to broaden our knowledge of bivalve morphology and our understating of how structure modulates behavior. While adult bivalves have gathered the most attention, investigations concerning bivalve larval nervous systems have recently gained interest. Therefore, this review describes the literature pertaining to bivalve ontogeny with an emphasis on nervous system development. First, a thorough synopsis of the major developmental stages from fertilization up to the mature larval stage is discussed, followed by a brief introduction to adult bivalve neural morphology. The majority of literature presented concentrates on larval nervous systems and the presence and potential functions of neurotransmitters and neuropeptides. Additionally, previous research proposing potential cues for larval settlement and eventually metamorphosis are discussed. Collectively, this review organizes our knowledge of bivalve neurological research and suggests areas for future investigations.

Significance of Bivalve Research

Molluscs are excellent model organisms for neurobiological studies for several reasons. Certain genera, including *Tritonia* and *Aplysia*, have become of special interest to neurobiologists due to the immense size of their neurons and nervous fibers (Halon, 1991). This allows for the nervous system to be examined and manipulated quiet easily. Also, there is a wide range of variation in neurological morphology within this phylum. Polyplacophorans, the chitons, are known for their simple nervous systems (Haszprunar et al., 2002), whereas the class Cephalopoda, which includes octopuses and squids, are known for their advanced and complex nervous systems (Abbott et al., 1995; Young, 1965). This variation allows for the possibility of comparative studies within the same phylum.

The class Bivalvia, which is the second largest class within the phylum Mollusca, includes an assortment of both marine and freshwater clams, oysters, scallops and mussels. Bivalves assist in maintaining balanced ecosystems by acting as nature's water filters, regulating phytoplankton levels (as this is their main food source), and diversifying the benthos with nooks and crevices, which provides refuge for many other aquatic organisms (National Research Council, 2004). Specifically, oysters are recognized as a keystone species or a species that functions as an indicator of the status and condition of the entire estuarine habitat (National Research Council, 2004). Moreover, shellfish significantly impact the seafood industry worldwide; therefore the majority of bivalve larvae studied today are commercially viable species. For example, *Crassostrea virginica*, the Eastern oyster, *C.gigas*, the Pacific oyster, and *Ostrea edulis*, the European oyster, are a few examples of oyster species that are commercially

significant. In fact, one oyster hatchery facility produces as many as 37.5 billion eyed larvae per year (National Research Council, 2004) for commercial purposes.

Scallops are another commercially pertinent group of bivalves. Species such as *Pecten maximus*, the great scallop, *Placopecten magellanicus*, the sea scallop, and *Chlamys opercularis*, the queen scallop, are of particular interest. In 1999, the scallop industry produced approximately 1.7 million metric tones of meat worldwide (O'Bannon, 1999). Although the United States is lagging behind in terms of scallop aquaculture, recent commercial fisheries have been established (Shumway and Parsons, 2006). In contrast, clam aquaculture has flourished throughout the United States, particularly in Virginia and Florida. In 2004, Virginia was ranked as the number one producer of the hard clam *Mercenaria mercenaria* in the United States (Murray and Kirkley, 2005). That same year hard clams were the 2nd most valuable aquatic reared crop and approximately 72.5 million seed clams were produced (Murray and Kirkley, 2005). Lastly, mussel species, including the blue mussel *Mytilus edulis*, the Mediterranean mussel *M. galloprovinicalis*, and the Baltic mussel *M. trossulus*, are also commercially significant bivalve species. In 2007, approximately 630,000 tons of mussel meat was cultivated worldwide creating nearly 610 million US dollars in revenues (FAO, 2007).

In summation, it is evident that bivalve culture fuels a sizeable multi-million dollar industry that has both economical and nutritional advantages. Although bivalve aquaculture practices are thriving, there is continuing research to improve these techniques. For example, Norwegian hatcheries have experienced high mortality rates in the production of *P. maximus* and have found that experimenting with different rearing techniques could assist in alleviating this trend (Torkildsen and Magnesen, 2004).

Unfortunately, many naturally occurring bivalve populations have recently suffered due to overharvesting for commercial purposes, increased sedimentation, and general habitat degradation (Williams et al., 1993; Nestlerode et al., 2007; Williams et al., 2008). Additionally, as in the case of the bay scallop *Argopecten irradians* in 1985, natural causes, such as mass algal blooms, can result in decimation of previously viable communitues (Blake and Shumway, 2006). A decline in adult populations may have serious life history implications. It has been reported that oyster larvae prefer to settle and metamorphose on existing oyster shells (Nestlerode et al., 2007). Also the presence of conspecifc chemical cues released from adults (discussed in more detail below) may signal or assist in signaling larval settlement and metamorphosis (Rodriguez et al, 1993). Therefore, a decline in the adult population would also decrease the proper substratum and/or chemical stimulation for larval settlement. Expanding our knowledge of the biology of larval bivalves can provide information that will facilitate restoration efforts and also assist in commercial rearing of bivalves.

While there is a wealth of neurobiological information concerned with gastropod larvae, little attention has been paid to the nervous system in larval bivalves. Research directed toward an understanding of bivalve larval nervous system morphology will assist in defining a connection between structure and function as it relates to larval behaviors such as settling and metamorphosis. Such investigations should also provide useful information concerning evolutionary relationships within the phylum Mollusca (Nielsen, 2004).

Before discussing the literature concerned with bivalve larval nervous systems, it is important to first review the development of bivalve larvae to provide a functional and

anatomic basis that may be related to larval nervous system structure. Thus, a detailed overview of the bivalve life history through the metamorphically competent larval stage will be discussed, followed by a review of previous investigations concerned with bivalve larval and adult nervous systems. While what might be called general bivalve development is discussed, there is variation within the class Bivalvia and some, but not all, of those variations are considered. Collectively, this review will enable readers to not only gain an appreciation of bivalve ontogeny, but also to detect potential avenues for future research that will bridge the link between structure and function of the larval nervous system as it relates to larval behaviors such as settling and metamorphosis.

Bivalve Embryonic and Larval Ontogeny

Reports concerned with the existence of oyster larvae date as far back as the late 1600's (Leeuwenhoek, 1665 as cited in Brach, 1960). Subsequently, Horst (1883-1884) and Huxley (1883) provided the first descriptions of bivalve development. Since then, general bivalve ontogeny has been examined in most of the major bivalve groups including studies of scallops (Culliney, 1974; Hodgson and Burke, 1988), several clam species (Ansell, 1962; Gros et al., 1997; Moueza et al., 1999; Silberfeld and Gros, 2006; Moueza et al., 2006; Da Costa et al., 2008), mussels (Harms, 1909; Flyachinskaya and Kulakovsky, 1991) and oyster species (Brooks, 1905; Erdmann, 1935; Galtsoff, 1964; Elston, 1980; Waller, 1981).

The typical bivalve life cycle begins with fertilization (Figure 1), followed by an embryonic period. The location of fertilization can vary depending on the species. Several bivalve species are hermaphroditic and therefore contain both sex organs.

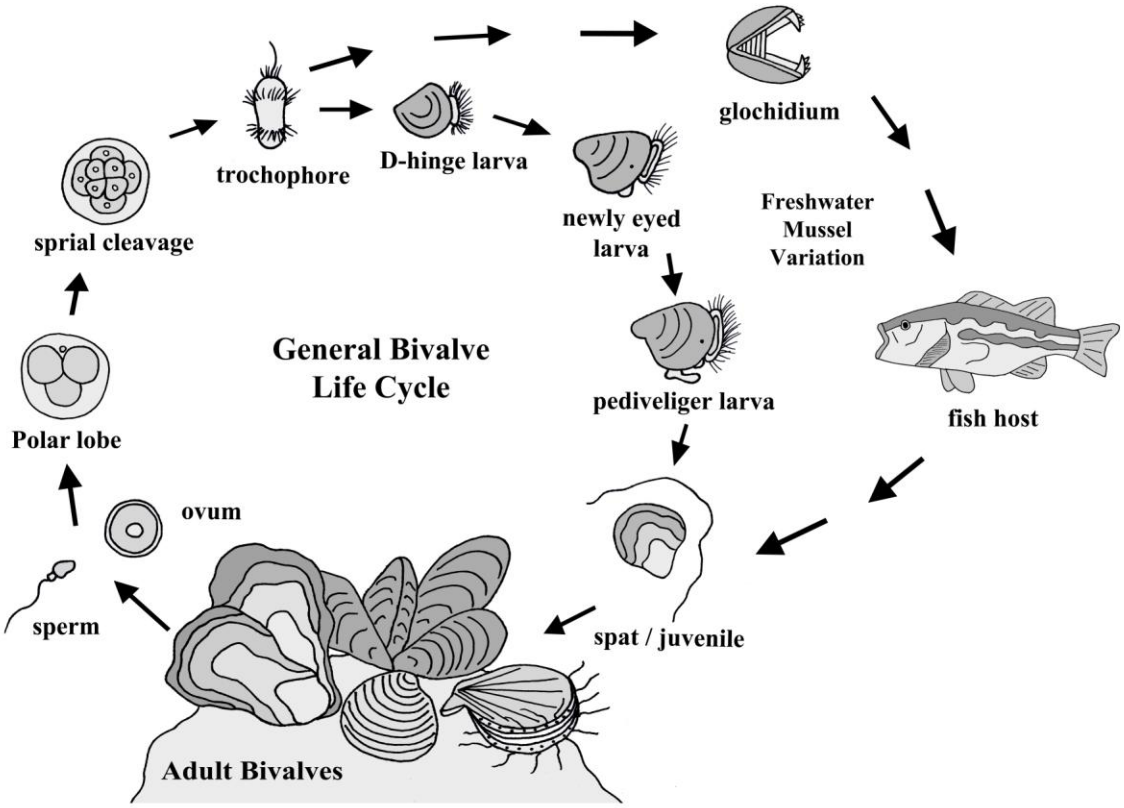


Figure 1: General bivalve and freshwater mussel life cycles

Cross-fertilization is the norm; however self-fertilization has been documented (Heller, 1993). Some species, such as *C. virginica* and *C. gigas,* are oviparous and expel their eggs and sperm into the water column resulting in external fertilization (Waller, 1981). Other species, e.g., the oyster *O. edulis*, and the mussels *Truncilla truncata, Lampsilis cardium and Amblema plicata plicata*, release radially organized spermatozeugmata composed of many spermatozoa that subsequently disassociate from this structure and move into the female oysters brood chamber where the eggs are fertilized internally (Waller, 1981; Foighil, 1989; Waller and Lasee, 1997; Willams et al., 2008).

Soon after fertilization subsequent cleavage occurs. During the first cleavage a polar lobe becomes evident. As the first cleavage progresses the polar lobe will fuse with one of the blastomeres creating two unequal cells: a smaller blastomere, the AB cell and a larger blastomere, the CD cell (Da Costa et al, 2008). The time it takes to reach the two-cell stage can vary. In the case of the razor clam, *Ensis aruatus*, completion of the first cleavage occurs one-hour post fertilization (Da Costa et al, 2008) versus approximately 3 hours post fertilization in the spiny scallop, *Chlamys hastata* (Hodgson and Burke, 1988). The second cleavage produces 4 cells (A, B, C and D). Subsequent cell divisions continue in the process of spiral cleavage, which produces alternately rotated sets of micromeres toward the animal pole that sit atop a single set of 4 large vegetal macromeres (Horst, 1883-1884). As development continues, the embryo reaches the bastula stage where soon thereafter, the blastopore becomes evident specifying the location of the future mouth. Epiboly and invagination of the blastopore continues as the gastrula develops. In a study by Flyachinkskaya and Kulakovskiy (1994) the gastrula has also been called a “conchostome”.

The next major developmental stage within the bivalve life cycle is that of the motile trochophore. The time it takes the embryo to reach the trochophore stage relative to fertilization varies greatly among taxa. In an investigation by Silberfeld and Gros (2006) the tropical clam *Tivela mactroides* reached the trochophore stage 6 hours post fertilization whereas the spiny scallop, *Chlamys hastata* (Hodgson and Burke, 1988), and the lucinid clam, *Codakia orbicularis* (Gros et al., 1997), did not reach the trochophore stage until 18-21 hours and 24 hours post fertilization respectively. This difference may be the result of different developmental programming. In addition, temperature differences may also lead to slight changes in the developmental timeline. For example, the time to reach the trochophore stage in *Ensis directus* while cultivated at 27-30 degrees Celsius was faster than that of *E. arcuatus* at 19 degrees Celsius (Costello and Henely, 1971).

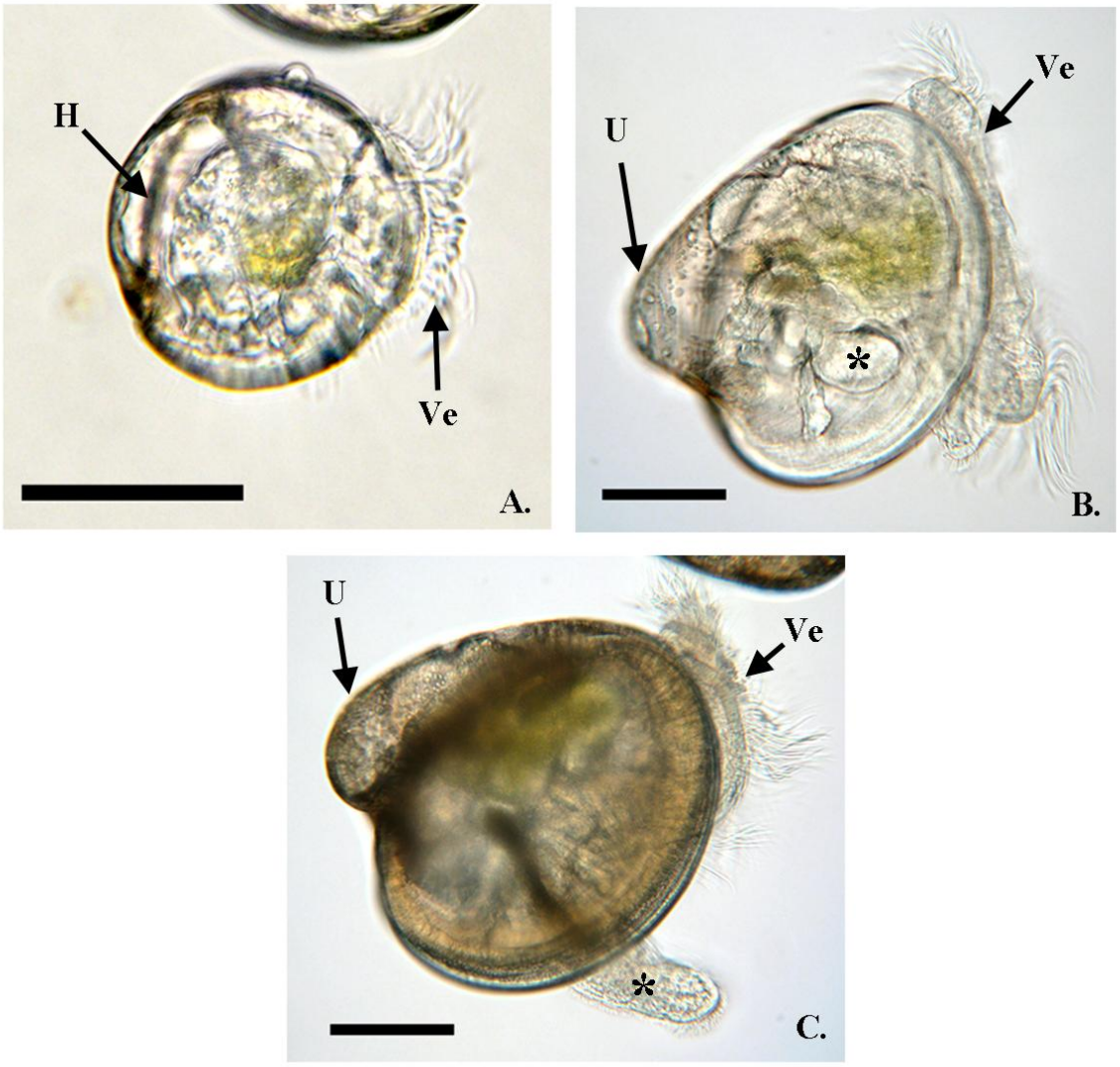
The trochophore stage possesses several novel structures. Two major ciliary bands, the prototroch and the teleotroch, develop that enable swimming. The prototroch is positioned anterior to the blastopore whereas the teleotroch is found near the anus (Nielsen, 2004). In addition, a structure called the apical or ciliary tuft composed of long non-motile cilia is present at the animal pole (Hodgson and Burke, 1998; Flyachinskaya and Kulakovskiy, 1991; Moueza et al., 1999; Moueza et al., 2006; Silberfeld and Gros, 2006; Da Costa et al., 2008). This ciliary tuft is an outgrowth of cells of the apical sensory organ (ASO) or apical ganglion, a structure whose histology and possible function have been best described in gastropod larvae (Kempf et al., 1997; Marois and Carew, 1997; Hadfield et al., 2000; Page and Parriers, 2000; Kempf and Page, 2005; Kempf, 2008). Recent investigations by Hadfield *et al.* (2000) have established that in the

larva of the opisthobranch gastropod *Phestilla sibogae*, the apical ganglion contains the sensory receptor for the settlement cue (Hadfield et al., 2000). The apical ganglion of bivalve larvae has also been suggested to have sensory functions (Da Costa et al., 2008); however, data to support this hypothesis is lacking.

The trochophore also has a large invagination on the surface of the presumptive dorsal side. This depression (the shell field) consists of the shell gland that will eventually produce the non-calcified precursor of an encompassing larval shell. The shell gland is composed of multiple cell types (T1, T2, and T3) that produce the periostricum, the shell material, hinge and ultimately the calcified prodissoconch (the larval shell) (Moueza at al., 2006). For a more extensive review of larval shell development and morphology, refer to the work Thomas Waller on *O. edulis* larvae (1981). The duration of the trochophore stage can vary in duration among species. In *C. virginica,* this stage has been documented within a laboratory setting to be a very brief, only lasting about 48 hours (Galtsoff, 1964).

The trochophore will undergo further differentiation and develop into a veliger larva. As this occurs, the prodissoconch I or the first larval shell will form. Additionally, the shell hinge becomes very distinct creating the larva's characteristic bivalve bilateral symmetry (Moueza et al., 2006). This denotes the first of three major veliger stages, the D-hinge or straight hinge larva (Rees, 1950) (Figure 2A). At this time, the larva also becomes laterally compressed. This movement causes the displacement of the prototroch to form the velum, which is the locomotive and feeding organ of the veliger (Moueza et al., 2006).

Figure 2: Light micrographs of the three larval stages of *C. virginica*. By using Adobe Photoshop these photographs were assembled as a compilation of photographs taken at different focus levels. A) D-hinge stage. B) Eyed stage. C) Pediveliger: H = hinge, U = umbo, V = velum, * or asterisk = foot. Scale bar = 100 μm.


H
Ve
A.
U
Ve
B.
U
Ve
C.

In *Ostrea edulis* the larva develops into the eyed or umbo larval stage approximately 15 days after fertilization (Waller, 1981) (Figure 2B). This stage is characterized by the formation of prodissconch II (the second larval and eventual juvenile shell) that results from shell secretion by the mantle. At this stage the umbones or bulges in the shell shield the hinge. There is the presence of a larval foot, but the foot is not yet specialized for crawling on the substratum. As the name of this stage implies, the larval pigmented eyespot also develops at the base of the velum (Galtsoff, 1964), although as seen in *C. virginica*, the eyespot may not always be clearly detectable at 15 days.

Upon further development the pediveliger stage is reached (Figure 2C). At this larval stage the major morphological characteristic is the larval foot. Eventually, the larval foot differentiates a distinctive heel or metapodium and toe or propodium for crawling on the benthos (Lane and Nott, 1975; Waller, 1981; Moueza et al., 2006). In the giant scallop, *P. magellanicus*, it took 28 days post spawning for more than half of the larvae to develop functional foot behaviors (Culliney, 1974). Foot maturation allows the larva to settle and metamorphose on the substratum. Metamorphosis will produce a juvenile or spat, which eventually matures into a reproductive adult.

Although this describes a general bivalve life cycle, variations have been documented. The most extreme case of deviation is the fascinating life cycle of freshwater mussels, which have evolved a parasitic larval stage to increase reproductive success. Williams and a multitude of colleagues (2008) have recently summarized the freshwater mussel life history which begins with the parent mussel brooding the embryos in a marsupium made of either gill or mantle tissue. In some cases, portions of the marsupium proliferate to produce a conglutinate or superconglutinate, that mimics larval

fish or aquatic insects that can lure potential fish hosts. These conglutinates house hundreds of veliger larvae called glochidia. Once a potential fish host approaches the mussel, the glochidia are expelled from the marsupium and latch on to either the fish's gills or fins. Due to this critical infection process, the mussel larvae have adapted a range of shell appendages that range from small micropoints to large hooks ("teeth") that aid in attachment to the fish host. In 1999, Hoggarth described the morphology of approximately 80 Unionidae glochidia via scanning electron microscopy, which included descriptions of various shell and appendage types. Upon attachment, the larvae are encapsulated in a cyst by fish epithelial tissue, and a maturation process or metamorphosis occurs. After some time, the juveniles will rupture out of the cysts and attach to the substratum for the remainder of the life cycle (Williams et al., 2008). The complex life history and the decline of many freshwater mussel populations has excited recent research interest in mussel larval ontogeny and morphology.

Bivalve Adult Nervous Systems

In order to make certain hypotheses about the larval nervous system of bivalves, it is helpful to analyze the nervous system in adults. Generally, the bivalve adult nervous system is simple with paired cerebro-pleural, pedal and visceral ganglia, which are connected by commissures, and cerebro-visceral and cerebro-pedal connectives (Brusca and Brusca, 2003). Although the nervous system is straightforward, variations have been documented. For example, the scallop *Patinopecten yessoensis* was reported to have an additional small pair of ganglia called the accessory ganglia (Matsutani and Nomura, 1986). These ganglia are positioned ventral and lateral to the visceral ganglia.

More recent bivalve nervous system investigations have focused on the presence and location of certain neurotransmitters / modulators within tissues and / or organs of both adult and larval bivalves. Understanding the location of these neuronal compounds and what nervous structures and tissues they are present in may suggest possible functionality relative to specific behaviors. Several studies of adult bivalves have explored the presence and location of serotonin or 5-hydroxytryptamine (5-HT) and have postulated that 5-HT modulates ciliary beating within the gills (Stephens and Prior 1992, Carroll and Catapane 2007) and neuromuscular regulation of both the heart and siphon (Kuwasawa and Hill, 1997; Ram et al., 1999, in Siniscalchi et al., 2004). In addition, a study by Siniscalchi et al. (2004) and Garnerot et al. (2006) indicated the presence of serotonin within the gonads of the bivalves *Venus verrucosa* and *Mya arenaria* respectively. Immunohistochemical labeling by Garnerot et al. (2006) failed to find evidence of the presence of 5-HT in organs within close proximity to the gonads, such as the digestive gland. These results have led investigators to hypothesize that 5-HT may also assist in gametogenesis, but that it does not modulate digestive function.

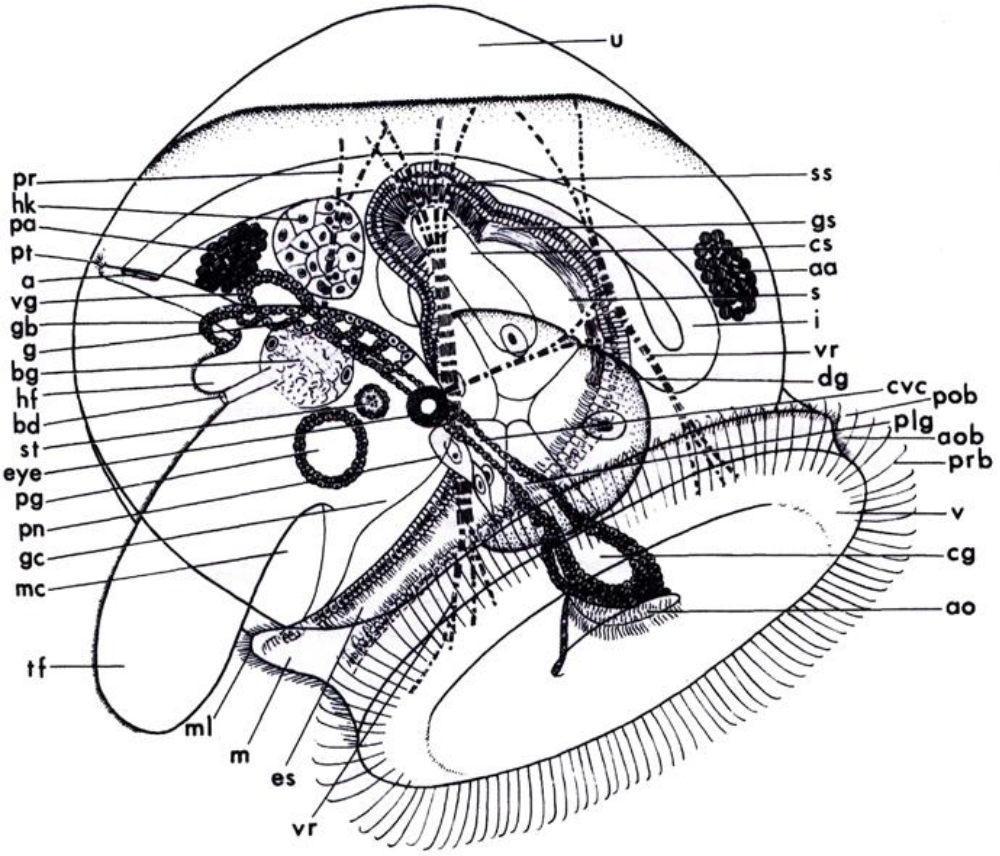
Bivalve Larval Nervous Systems

While the studies mentioned above describe the nervous system of adult bivalves, the literature lacks a proper analysis of nervous system structure and ontogeny within larval bivalves. An early study by Erdmann (1935) suggested the pediveliger nervous system of *O. edulis* was actually complex. He described pre-metamorphic *O. edulis* larvae as possessing an apical sensory organ (ASO) positioned between the two velar lobes with two cerebral ganglia directly posterior to the ASO (Figure 3). Short

connectives from each cerebral ganglion extended to respective pleural ganglia. The larvae also possessed paired pedal and visceral ganglia with pleural-visceral connectives on the left and right sides. Commissures joined each of the paired cerebral, pedal and visceral ganglia. In 1971, Hickman and Gruffydd described an alternate pediveliger central nervous system in *O. edulis* as containing one large cerebral ganglion, paired pedal ganglia and a single visceral ganglion. Furthermore, the pleural ganglia described by Erdmann (1935) were reported as the eye nerve trunks instead of actual ganglia (Hickman and Gruffydd, 1971). Bayne (1971) characterized the central nervous system in *M. edulis* as having paired cerebral, pedal and visceral ganglia, but the connectives of these ganglia were not identified. Lastly, the most detailed histological study of bivalve larvae (*C. virginica*) by Elston (1980) fails to recognize the larval nervous system components and suggests that in the early larva, nervous control may be accomplished by "neuroid transmission", a concept suggested by Carter (1926) where there is general cell to cell transmission of nervous impulses ion the absence of ganglia and nerves.

In addition, there have been a few histological investigations of the central nervous system in fresh-water mussel larvae. Examination of mature glochidia larvae of *Margaritifera auricularia* and *M. margaritifera* indicates that the establishment of ganglia, commissures and connectives has yet to occur and that the visceral organ primordia are not evident (Araujo and Ramos, 1998). On the other hand, in an analysis of an *Anodonta* species, Harms (1909) states that a single cerebral ganglion is well established at the mature larval stage and even a visceral ganglion can occasionally be found. Although the histological characterization of the glochidium is very interesting, the investigations do not allow one to make broad conclusions pertaining to bivalve larval

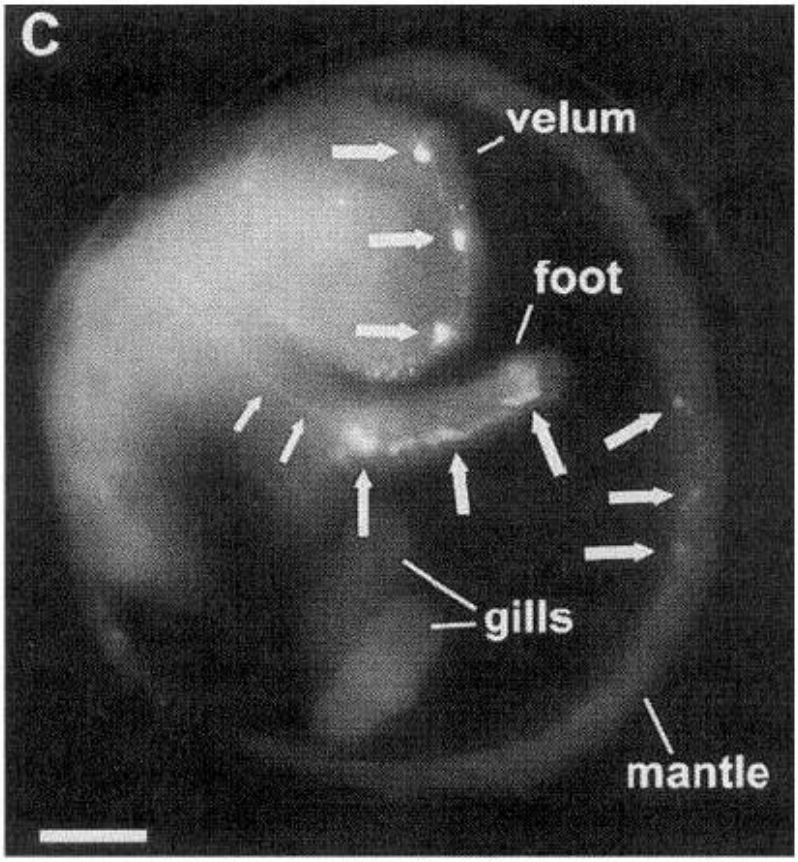
Figure 3: Erdmann (1935) diagram as revised by Waller (1981) of the pediveliger stage of *O. edulis*. a = anus; aa = anterior adductor; ao = apical organ; aob = adoral ciliary band; bd = byssal gland duct; bg = byssal gland; cg = cerebral ganglion; cs = crystalline style; cvc = cerebro-pleural-visceral connective; dg = digestive gland; es = esophagus; eye = eye; g = gill primordium; gb = gill bridge; gc = gill cavity; gs = gastric shield; hf = heel of foot; hk = primordium of heart and kidney; i = intestine, m = mouth; mc = mantle cavity; ml = mouth lobe; pa = posterior adductor; pg = pedal ganglion; plg= pleural ganglion; pn = protonephridium; pob = postoral ciliary band; pr = pedal rectractor; prb = preoral ciliary band; pt = postanal ciliary tuft; s = stomach; ss = style sac; st = statocyst; tf = toe of foot; u = umbo, v = velum; vg = visceral ganglion; vr = velar retractor.


u
pr
hk
pa
pt
a
vg
gb
g
bg
hf
bd
st
eye
pg
pn
gc
mc
tf
ml
m
es
vr
ss
gs
cs
aa
s
i
vr
dg
cvc
pob
plg
aob
prb
v
cg
ao

nervous systems because the mussel life cycle possesses a multitude of variations when compared to the general bivalve life cycle (Figure 1).

Additionally, immunohistochemical analyses have described the location and presence of neural compounds including serotonin (5-HT) (Croll et al., 1997; Voronezhskaya et al., 2008; Kreiling et al, 2001), FMRF-amide (Voronezhskaya et al., 2008), acetlycholinesterase (AChE) activity (Raineri, 1995; Raineri and Ospovat, 1994), and the catecholamines norepinephrine, epinephrine, and dopamine (Kreiling et al., 2001; Voronezhskaya et al., 2008). In 1997, Croll et al. conducted a study to determine the location of catecholaminergic neuronal cells. He and his team found twenty-two day old *Placopecten magellanicus* larvae possessed catecholaminergic cells within the velum and on the sides of the mouth (Croll et al., 1997). As the larva continued development, a number of catecholaminergic cells were also found along the edge of the mantle and within the abdominal ganglia (= visceral ganglia) (Croll et al., 1997) (Figure 4). This same study revealed similar results in *Mytilus edulis* larvae, as well as the presence of catecholaminergic cells within the numerous developing gill arches (Croll et al., 1997). There were a high number of fluorescent cells noted within the foot of both species and it appeared these cells extended through the epithelial layer and innervated long cilia that may act in a sensory function. Previous studies have determined that the larval foot must develop in order for metamorphosis to occur (Bonar, 1978; Bayne, 1971; Culliney, 1974). Although further investigation is needed, Croll et al (1997) loosely hypothesized that these catecholaminergic innervations within the foot may also function in the attainment of metamorphic competence.

Figure 4: Confocal micrograph labeling the presence of catacholamines in *P. magellanicus* pediveliger. From Croll et al. (1997). Large arrows = cells in mantle, velum and foot, small arrows = fluorescent processes innervating the foot. Scale bar = approximately 38 μm.


C
velum
foot
gills
mantle

Kreiling et al. (2001) have analyzed the serotnergic and dopaminergic innervations in various larval stages of the surf clam *Spisula solidissima*. The presence of 5-HT containing cells was found in 24 hour post fertilization (PF) early veligers in the apical ganglion and cerebral ganglia regions. At 48 hours PF, 5-HT continued to label as before with additional labeling in the visceral ganglion. By 96 hours PF labeling within a small cerebro-visceral connective was apparent. Dopamineric labeling was also seen early in development (24 hours PF), but the labeling was not localized to the nervous system. Rather the presence of dopamine labeled "structures" seemed to accumulate near the developing gut and eventually near the mouth and velum.

Similarly, Voronezhskaya et al. (2008) investigated changes in FMRFamide-like and 5-HT-like immunoreactivity and catecholamine histofluorescence in *Mytilus trossulus* embryos and larvae during development. The presence of FMRFamide and 5-HT immunolabeling initially occurred in the apical region of trochophore larvae. As the larvae reached the pediveliger stage, FMRFamide labeling (when compared to histological analyses of larval *M. trossulus* discussed above) was located in paired cerebral, pedal and visceral ganglia along with the various connectives and commissures of these ganglia as well as some peripheral processes. In contrast, 5-HT labeling continued to label only cells in the apical/cerebral ganglia region. The presence of catecholamines yielded a different pattern in that labeling did not occur until the D-hinge larval stage. At the pediveliger stage, catecholamines were found in clusters near the stomach and posterior adductor muscles. Also, labeling occurred along the periphery of the velum. Unfortunately, it could not be determined whether or not these clusters of

catecholaminergic cells were components of the central nervous system (Voronezhskaya et al., 2008).

Effect of the Nervous System on Settlement Behaviors:

Bivalve larval setting has been divided into two stages: 1) an explorative or searching stage in which the larva will search for the proper substratum and 2) an attachment stage where the larva secretes cement-like substances produced by the byssal gland (Beiras and Willows, 1995) in order to bind to the underlying substratum (Rodriguez et al., 1993). The actual physiological processes that take place during this period of the bivalve life cycle are not well understood (Rodriguez et al., 1993). Several studies have been conducted to test the initiating factors that direct the larva to begin the searching phase of settlement (e.g., Turner et al., 1994; Beiras and Widdows, 1995; Nestlerode et al., 2007). Rodriguez et al. (1993) discusses the literature on this particular topic. He separates cues that induce settlement into two major classes: 1) natural chemical cues and 2) artificial chemical cues. Conspecific chemical cues, microbial film cues, and prey signaling cues comprise those attributed to natural chemical inducers. As indicated earlier, it has been reported that oyster larvae respond to conspecific cues in that they prefer to settle on existing oyster shells (Nestlerode et al., 2007). Investigations of bacteria within the environment such as *Alteromonas colwelliana* provide evidence that bacteria may secrete the actual causative agent that initiates setting behaviors in oyster larvae (Weiner et al., 1985).

Artificial cues are those chemical compounds present on the substratum or in the water column that "mimic" the role of either a) the natural inducer or b) neurotransmitters

present within the larval nervous system that initiate or function as intermediaries in the metamorphic response (Rodriguez et al., 1993). One particular group of neurotransmitters thought to play a role in settlement and metamorphosis are catecholamines, which include norepinephrine, epinephrine, and dopamine. Turner et al. (1994) reported water borne concentrations of dopamine and varying concentrations of glycl-glycl-L-arginine (GGR peptide) can signal *C. virginica* larvae to approach the substratum. In addition, several tyrosine derivates such as dihydroxiphenylalanine (L-DOPA), a precursor for the neurotransmitter dopamine, and other catecholamines have been shown to possess inductive properties in *C. virginica* larvae (Pawlik, 1990). Cooper (1982) demonstrated in *Mytilus edulis* and *Crassostrea gigas* larvae that L-DOPA induced settlement on unusual substrates and on occasion were noted to skip the settlement stage and undergo metamorphosis within the water column. More recently, it has been documented in the nudibranch *Phestilla sibogae* that catecholamines may not be the actual inducer of metamorphosis, but instead the oxidized products of catecholamines, such as hydrogen peroxide, are actually effecting this behavior in larvae (Pires and Hadfield, 1991). Lastly, choline derivatives, precursors of the neurotransmitter acetylcholine, have been hypothesized to directly affect the nervous systems of *Phargmatipoma lapidosa californica* veliger larvae, instead of the larva possessing external receptors for these substances (Pawlik, 1990). These results support the hypotheses of Bonar et al. (1990) who suggested that oyster larvae control settlement behaviors through a dopaminergic pathway and induce metamorphosis through an adrenergic pathway. The organismal signaling pathways by which artificial inducers initiate settlement are still unresolved.

Candelario-Martinez et al. (1993) described the presence of SCP-like neuropeptides in several adult bivalve species including *Mercenaria mercenaria*, *Dinocardium robustrum*, and *Crassostrea virginica*. Using high-pressure liquid chromatography to identify the specific SCP neuropeptide sequences, they discovered a more diverse group of SCPs in bivalves than have been found in gastropods (Candelario-Marntinez et al., 1993). There were two SCP peptides shown to be present in *Crassostrea virginica*: APKYFYFPRMa and SAFYFPRMa. In addition, an immunohistochemical analysis identified SCP-like neuropeptide presence in the cerebral, pedal and visceral ganglia, where the concentration within the visceral ganglia was double the concentration found in the other ganglia. SCP innervation also extended to the gut, gills, posterior mantle and musculature. Bioassays conducted to indicate possible functionality of SCPs in bivalves provided additional evidence that one role of SCP-like neuropeptides in *M. mercenaria* may be to regulate digestion and feeding (Candelario-Marntinez et al., 1993). SCPs have also been reported to coexist with the regulatory peptide FMRF-amide within the motor neurons of the buccal ganglia of the gastropod *Aplysia* (Lloyd, 1989). Furthermore, SCP-like neuropeptides are not found in heart tissues of *Helix aspersa*, but do have an excitatory effect on the heart (Lloyd, 1978) Overall, these results suggest that SCP-like neuropeptides in bivalve and possibly other molluscan species may modulate aspects of gut motility and feeding and could possibly modulate heart rhythms.

Conclusions

In summation, the majority of the literature pertaining to larval nervous system has been conducted using immunohistochemical techniques to analyze presence and

possible location of neurotransmitters and neuropeptides such as serotonin, dopamine, FMRFamide and catecholamines. Although, knowledge of the presence and location of these neural compounds facilitate our efforts in hypothesizing functionality within the larval body, histological sectioning is needed to confirm the neural compound's location. It should be noted that all of the histological investigations that discuss nervous system structure provide only diagrammatic figures to visually present their descriptions. Therefore, the need for a detailed histological analysis of the bivalve larval nervous system such as those available for opisthobranch gastropod species (Kempf et al., 1987; Page, 1992a,b; Carroll and Kempf, 1994) still exists. This information is critical for understanding bivalve larval nervous systems because it would serve as a foundation for actually analyzing the function of specific nervous system components and neurotransmitters and neruomodulators in producing larval behaviors such as settlement and metamorphosis.

SCP-like neuropeptides have been described in adult bivalve species to possibly modulate gut motility and assist in feeding behaviors (Candelario-Martinez et al., 1993), but the presence of SCPs in bivalve larvae has yet to be described. Additional research will provide insight onto the presence of SCPs and how SCPergic neurons and neuronal processes modulate certain larval behaviors. It is essential to continue investigating the central and peripheral nervous system of bivalve larvae in order to gain a better understanding of how important larval behaviors such as settlement and metamorphosis are produced by nervous system structure and chemistry.

Acknowledgements

I would like to thank The American Museum of Natural History, the American Microscopical Society, and the Auburn University Graduate School for providing some of the funds necessary to complete this project. I also appreciate the comments of Drs. Christine Sundermann and Kevin Fielman that helped me formulate some of the ideas discussed in this paper. Thank you to the publishing companies who granted permission to use figures from various authors. In addition, I would like to recognize all of the researchers who are actively investigating bivalve larval development and analyses pertaining to bivalve larval nervous systems. This paper could not exist without your distinguished and greatly appreciated contributions to the field of molluscan ontogeny. Thank you and good luck in your future research endeavors!

Chapter 2:

Characterization of the Central Nervous System and Various Peripheral Innervations During Development of the Larval Oyster *Crassostrea virginica*

Ivey Ellis* and Stephen C. Kempf

Department of Biological Sciences, Auburn University, 331 Funchess Hall Auburn University, AL 36849-5407

*ellisir@auburn.edu (corresponding author)

key words: oyster, larvae, histology and nervous system

Abbreviations

A = anterior

ACG = accessory ganglion

AG = apical ganglion

BG = byssal gland

CP = cerebral portion of the cerebro-pleural ganglion

CC = cerebral commissure

CPG = cerebro-pleural ganglion/a

D = dorsal

DEN = dorsal esophageal nerve

DD = digestive diverticulm

diH2O = deionized water

Du = posterior duct

E = esophagus

EDTA = ethylenediaminetetraacetic acid

F = foot

GR = gill rudiment

HF = heel of foot

High Mg / Low Ca = High magnesium / Low calcium sea water

L = left

LP = lateral pouch

M = mouth

MC = mantle cavity

MN = mantle nerve

MT = mantle tissue

NaHCO3 = sodium bicarbonate

O = osphridium

P = posterior

PAM = posterior adductor muscle

PAN = posterior adductor nerve

PC = pedal commissure

PD = pedal depression

PG = pedal ganglion/a

PlPC = pleural-pedal connective

PlPN = pleural-pedal nerve

PlVC = pleural-visceral connective

PP = pleural portion of the cerebro-pleural ganglion

PPN = posterior pedal nerve

PVC = posterior visceral commissure

R = right

RM = retractor muscles

S = stomach

ST = statocyst

TF = toe of foot

V = ventral

VAC = visceral-accessory connective

VC = visceral commissure

Ve = velum

VG = visceral ganglion/a

VON = ventral osphridial nerve

VPN = ventral pedal nerve

VPN1 = ventral pedal nerve 1

VPN2 = ventral pedal nerve

Abstract

Although a few investigations have considered neuronal aspects of bivalve larval ontogeny, the central nervous system of bivalve veligers has yet to be described in detail. This study provides an in depth examination of the central and peripheral components of the larval nervous system in the Eastern oyster, *Crassostrea virginica*. Larvae at the D-hinge, newly eyed and pediveliger stages were analyzed at the light level by means of serial, 0.5 or 1.0 μm histological section sets. D-hinge larvae were found to have limited neurogenesis with only an anterior gangliar rudiment being identified. As development progressed to the newly eyed larval stage, a single apical ganglion and paired cerebro-pleural, pedal, and visceral ganglia were present forming a typical bivalve central nervous system loop. The nervous system of pediveligers was similar to that of newly eyed larvae, but added an additional accessory ganglion on the left and right sides, posterior to each visceral ganglion. A posterior visceral commissure was also present. Various peripheral innervations were also documented, including nerves extending from the pedal and visceral ganglia in both newly eyed and pediveliger larvae. Furthermore, several neuronal processes, such as the mantle, ventral osphridial and dorsal esophageal nerves were seen connecting to ganglia. In summation, this investigation provides an essential histological analysis characterizing the complex nervous system present in bivalve oyster larvae.

Introduction

Oysters play an essential role within marine estuarine ecosystems by regulating phytoplankton populations, providing reef habitats and acting as an indicator of the status and condition of estuarine ecosystems (Harding and Mann, 2001; National Research Council, 2004). Many species populations have recently suffered due to overharvesting for commercial purposes, increased sedimentation, and habitat degradation (Jackson et al., 2001; Nestlerode et al., 2007). Life history complications that result from decreased population sizes have multi-faceted consequences. Nestlerode et al. (2007) provided evidence that oyster larvae show a preference towards existing adult oyster shells for settlement and metamorphosis. Therefore, overharvesting oysters would not only decrease the number of viable adults, but also decrease the availability of proper substratum for larval settlement. Furthermore, oysters play a major role in aquaculture practices worldwide. For example, aquaculture operations in Australia comprised 1,004 oyster-farming facilities in the year 2000 (Nell, 2001). A single United States hatchery facility can produce up to 37.5 billion eyed larvae per year (National Research Council, 2004). Further investigations concerned with the morphology and behavior of larval oysters is essential for both restoration efforts and the continuation of innovative aquaculture practices. A better understanding of larval nervous system structure and the innervation of larval tissues and organs will facilitate future research concerned with how the nervous system modulates critical life history behaviors, such as settling and metamorphosis.

Although bivalves comprise the second largest class within the phylum Mollusca, literature dealing with the structure of the larval central nervous system is somewhat

meager. Erdmann (1935) described the larval nervous system of *Ostrea edulis* to be surprisingly complex with a single apical ganglion and paired cerebral, pleural, pedal and visceral ganglia. Subsequently, a few additional studies have examined the central nervous system of various bivalve species (Bayne, 1971; Hickman and Gruffydd, 1971; Waller, 1981), all of which briefly describe comparable nervous system organization but, as is the case for Erdmann (1935) lack histological micrographs to corroborate schematic diagrams. Recent immunohistochemical investigations comprise the majority of the information pertaining to bivalve larval nervous systems. These include studies indicating the presences and possible location of serotonin (5-HT) (Kreiling et al, 2001; Voronezhskaya et al., 2008), FMRF-amide (Voronezhskaya et al., 2008), acetlycholinesterase (AChE) activity (Raineri and Ospovat, 1994; Raineri, 1995), and catecholamines (Kreiling et al., 2001; Croll et al., 1997; Voronezhskaya et al., 2008). While confocal microscopy indicates extensive central and peripheral circuitry associated with these neuroactive substances, suggested locations for neurons and nervous processes are not verified with histological analyses. This, in turn, restrains investigators from developing hypotheses and possible conclusions about the functions of these various neurotransmitters and neuromodulators.

This study aims to strengthen the foundation of bivalve larval investigations by providing a detailed histological description of the central nervous system and portions of the peripheral nervous system in the larval Eastern oyster, *Crassostrea virginica*. Serial stained sections of the three major larval stages, the D-hinge, newly eyed and pediveliger, are examined at the light microscopic level. The spatial orientation of ganglia, commissures and connectives are discussed, along with an analysis of the tissues and

organs innervated. Overall, this investigation provides basic and essential histological data needed to corroborate the findings of recent immunohistochemical investigations and unveil possible future avenues for research.

Methods

The Auburn University Shellfish Laboratory (Mobile, Alabama) provided D-hinge, newly eyed, and pediveliger larvae of *C. virginica* during the summers of 2007, 2008 and 2009. Once in the laboratory, the larvae were prepped for histological sectioning through a sequence of relaxation (personal communication from Louise Page, University of Victoria, B.C.), fixation, decalcification, dehydration and embedment protocols. Moist larvae shipped on ice were transferred to 0.45 μm Millipore filtered aquarium seawater (MFSW) and allowed to acclimate for approximately 20 minutes at room temperature. Acclimated larvae were then transferred to vials containing a 3:1 solution of MFSW and High Mg / Low Ca MBL seawater (Audesirk and Audesirk. 1980). Every 15 minutes one part of the solution was removed and then replaced with an equal volume of High Mg / Low Ca solution. This process continued for a total of 8 High Mg / Low Ca solution additions. The volume of solution in each vial was then reduced to approximately 1.5 ml. Every 1.5 minutes, 3 drops of seawater saturated with chlorotone (chlorobutanol) were added to the solution for a total of 8 additions over 12 minutes. The larvae were then transferred to glass centrifuge tubes and briefly spun at low speed to sediment the relaxed larvae into a pellet. A Pastuer pipette was used to quickly transfer the larvae in a minimum volume of relaxation fluid to fixative.

Primary fixative consisted of 2.5% gluteraldehyde + 1.36 M sodium chloride + 0.2 M Millonigs phosphate buffer (Cloney and Florey, 1968). Fixation was carried out on ice for approximately 30 minutes, followed by 1 hr at room temperature. Afterwards, an equal volume of warm, freshly prepared, 20% ethylenediaminetetraacetic acid (EDTA) was added to the fixative vials to create an overall 10% EDTA solution. Decalcification was carried out at room temperature and monitored periodically starting at 7 hours post EDTA treatment by crushing a few larvae under a coverslip and scanning for shards of calcified shell. When no shards were detected, the larvae were deemed decalcified. Larvae were then rinsed three times for 10 minutes per rinse in a 1:1 mixture of 0.4 M Millonigs phosphate buffer and 0.34 M sodium chloride. Following the last rinse, the larvae were transferred to a secondary fixative of 2% osmium tertaoxide + 1.25% $NaHCO_3$ solution (Wood and Luft, 1965) at room temperature for 1 hour, followed by 3 washes in 2.5% sodium bicarbonate ($NaHCO_3$) in Nanopure water for 10 min each.

Following fixation, larvae were dehydrated through a series of 10 minute rinses beginning with Nanopure water and progressing to 30%, 50%, 70%, 80%, 90%, and 95% ethanol followed by 3 rinses in 100% ethanol. The ethanol was then removed from the larval tissues with three 10-minute rinses in propylene oxide. At the end of the third propylene oxide rinse an equal volume of Embed 812 plastic (Electron Microscopy Sciences) was added yielding a 1:1 solution. The plastic solution was allowed to infiltrate the tissue at room temperature for approximately 7-8 hrs and then, half of the solution was removed and an equal amount of plastic was added to yield a 3:1 solution which was allowed to infiltrate the tissues for another 8 hrs. This treatment was followed by two changes of 100% plastic for 7 hours each and then one 4 hour infiltration in catalyzed

100% plastic The larvae were then transferred into circular molds and the catalyzed plastic was polymerized overnight at 60^{o}C.

D-hinge (0.5 μm), newly eyed and pediveliger (1.0 μm) were sectioned using a Reichart ultramicrotome in either a sagittal, frontal or transverse plane of view and stained with 1% Thionin solution or methylene blue-Azure II (Richardson et al., 1960). Coverslips were mounted using catalysed Embed 812 plastic that was polymerized at 60 ^{o}C. Photographs were taken at the light level on a Nikon Optiphot microscope using a Canon PowerShot S5IS digital camera and a Martin Microscopes camera adaptor. In order to effectively follow the nervous system, serial section movies were created for sagittal, frontal and transverse planes of view for each larval stage using Adobe Photoshop CS4 and QuickTime Pro. Photoshop CS4 was also used to adjust level contrast of the photographs and occasionally to remove debris or air bubbles that were not contained within the tissue.

Results

Organization of the general structure of the larval body and the visceral organs of *Crassostrea virginica* are similar to the description of Eltson (1980) in what is the best previous histological study of the bivalve larva. Unfortunately, Eltson (1980) did not recognize the lava's central nervous system in his work. While the results discussed below support his findings, they also extend the histological description of larval *C. virginica* to include the development of central and peripheral nervous system structure.

The nomenclature chosen to identify commissures and connectives of the central nervous system in this study varies slightly from that in previous literature. These

processes are named based on the specific ganglion they exit and the ganglion they extend to. For example, a connective between the cerebro-pleural ganglion and the pedal ganglion is named the pleural-pedal connective because the connective exits the pleural portion of the cerebro-pleural ganglion. Previous literature called this the cerebro-pedal connective which is really misleading. Additionally, names used for peripheral nerves combine the orientation at which the nerve extends from the ganglion and the tissue it innervates, i.e., the posterior pedal nerve extends from the posterior region of the pedal ganglion and innervates the base of the larval foot. Peripheral nerves have not been identified in previous investigations.

Larval stages used in this study were examined in three different orientations: frontal, transverse and sagittal (Figure 1). The larva's axes of orientation are such that the velum occurs at the anterior pole while the hinge (or umbo) is posterior. The foot is on the ventral side of the larva and the stomach close to the dorsal surface. Movies of complete serial section sets of pediveliger larvae in all 3 plains of view are provided in Appendix A. For the purpose of description, I will often use the terms medial and lateral to describe structures towards the middle or the sides of the larva.

D-hinge Neuronal Structures

Histological sectioning revealed that D-hinge larvae have undergone only a limited amount of organogenesis. This is expected since the D-hinge is the youngest larval stage. The main structures seen were a mouth, esophagus, stomach, intestine, anus, a small digestive gland, adductor and retractor muscles, and various cells dispersed through out the visceral region. Additionally, the ciliated velum was present (Figure 2).

Figure 1: Axes and planes of view of *C. virginica* pediveliger histological sections. A) Sagittal view of whole larva indicating the anterior, posterior, dorsal and ventral sides. B) Frontal view looking down on the dorsal surface of larva. The rectangle denotes the plane of sectioning and then a section is shown with various orientations labeled. C). Similar schema as in B except the larva is being cut in the transverse plane of view. D). Sagittal plane of view. A = anterior; D = dorsal; L = left; P = posterior; R = right; V = ventral

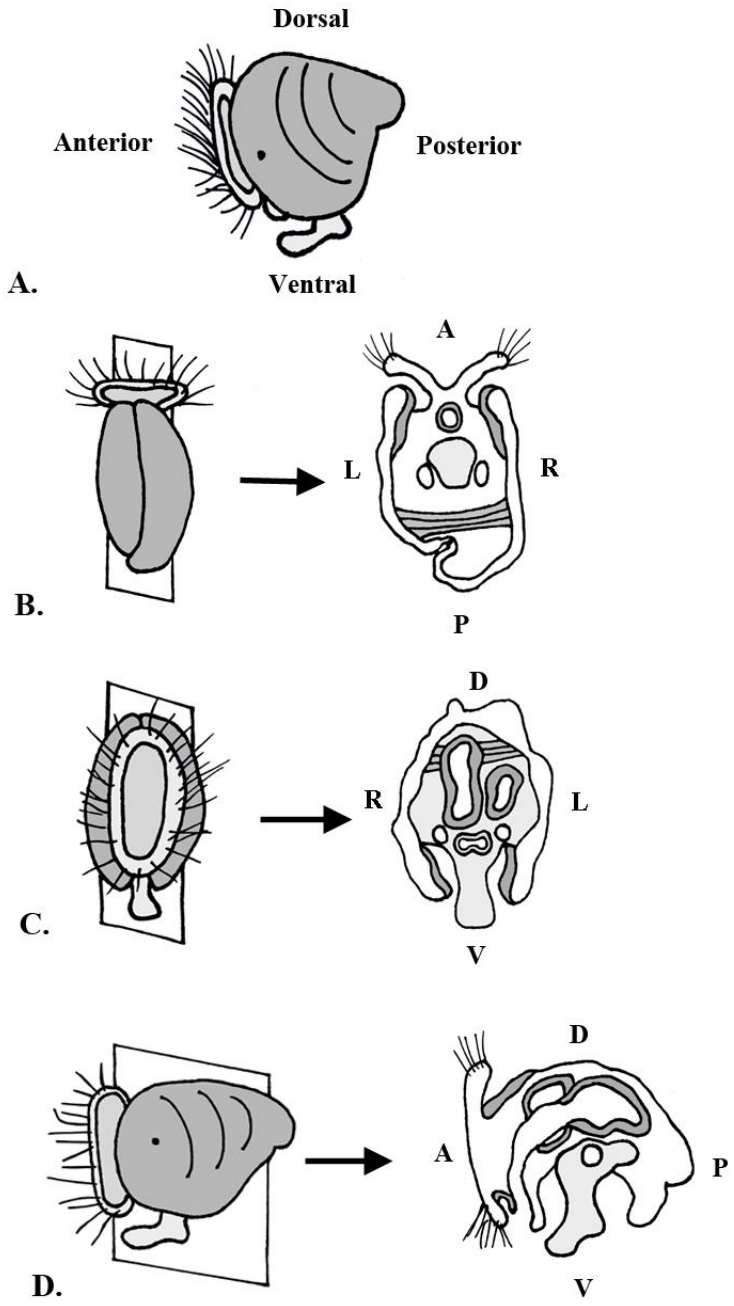
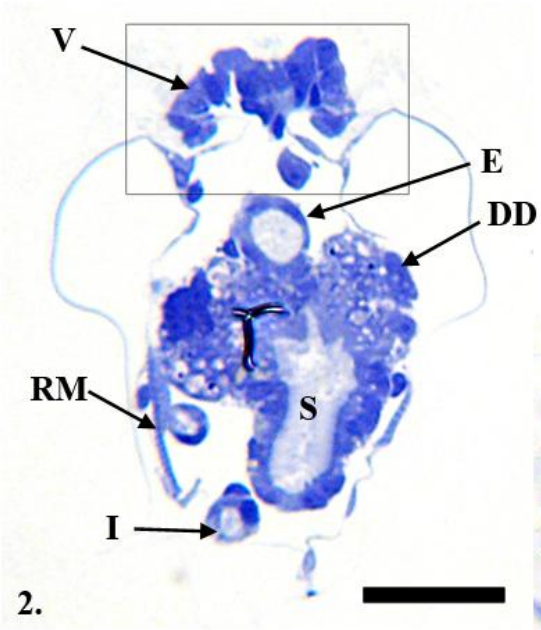

Dorsal
Anterior
Posterior
Ventral
A.
A
L
R
P
B.
D
R
L
V
C.
D
A
P
V
D.

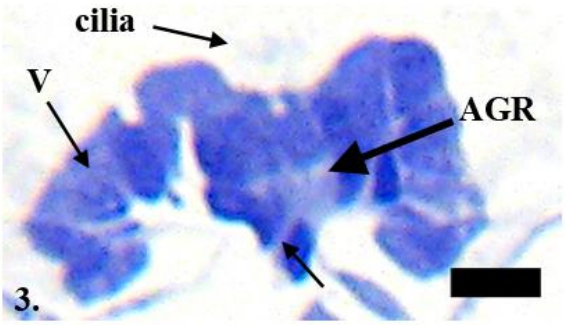
Figure 2 - 3: Frontal view of D-hinge *C. virginica* larva.

Figure 2: Anterior gangliar rudiment, velum, esophagus, stomach, digestive gland, intestine and retractor muscle are evident. Scale bar = 20 μm.

Figure 3: High magnification of boxed region in Figure 2 depicting the anterior gangliar rudiment (AGR). Note the cilia extending anteriorly from the AGR and the small process (arrow) projecting from the AGR. Scale bar = 5 μm.

AGR = anterior gangliar rudiment; DD = digestive diverticulum; E = esophagus; I = intestine; RM = retractor muscle; S = stomach; small arrow = process extending from AG; V = velum; cilia = cilia of AGR.


V
E
DD
RM
S
I
2.

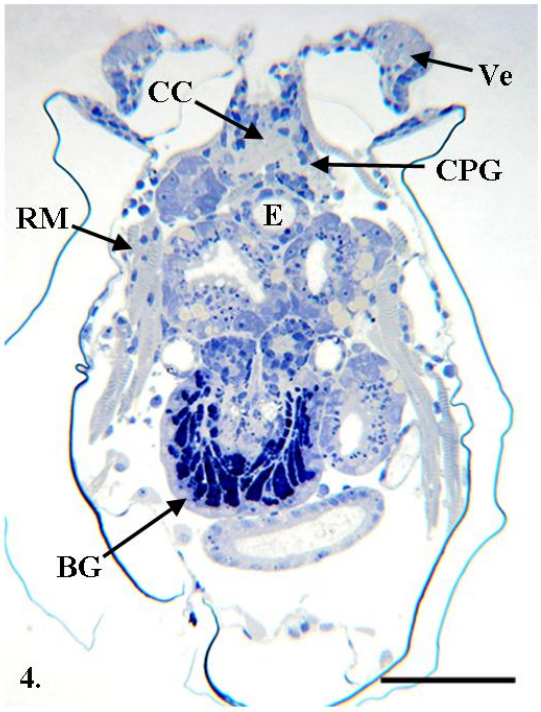

cilia
V
AGR
3.

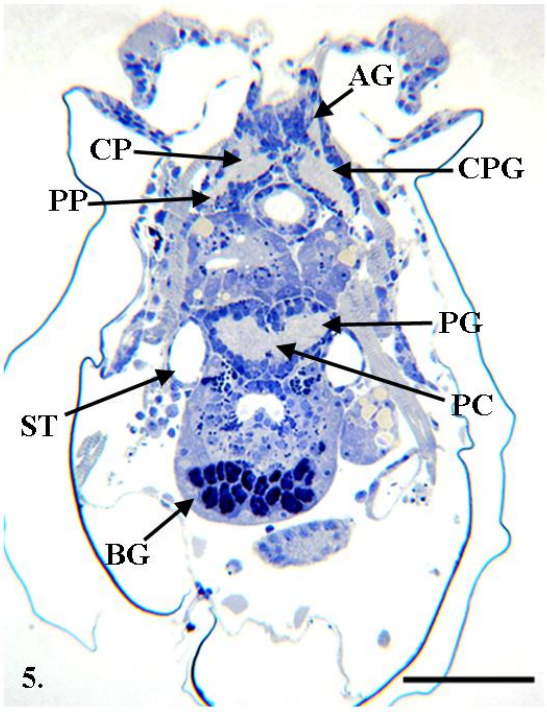
Although, there was no histological evidence of a complete central nervous system loop (however, see immunlabeling results in Chapter 3), some neuronal structures were present. Centered directly posterior to the velum was an area of differential staining (similar to that seen in stained tissues of later larval stages) where the apical ganglion will eventually develop (Figure 2 and 3). This appears to be what was described by Rainier (1995) as the anterior gangliar rudiment. Although short cilia and a cup-like structure are present, it was not clearly evident whether this structure is differentiating into the apical ganglion, the cerebro-pleural ganglia or both. The position of the structure suggests that at least the apical ganglion will arise from these tissues. In addition, a thin process extending from the anterior gangliar rudiment can be seen (Figure 3), but unfortunately the process could not be followed in serial sections due to the staining of surrounding tissues.

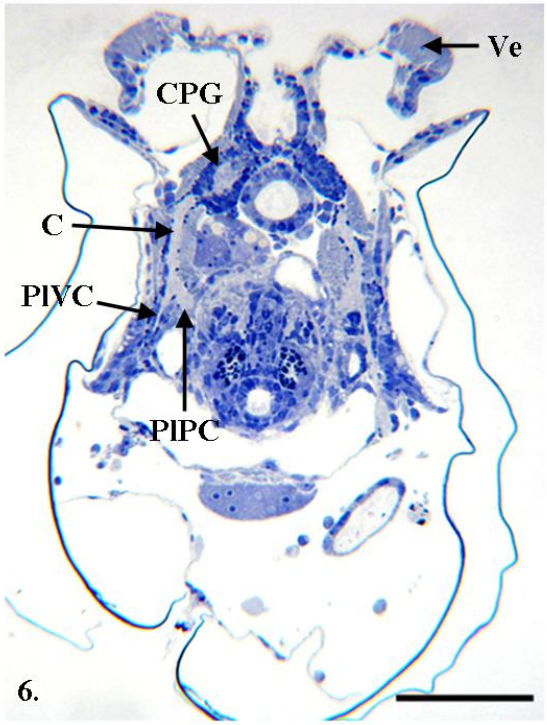
Newly Eyed Larval Central Nervous System

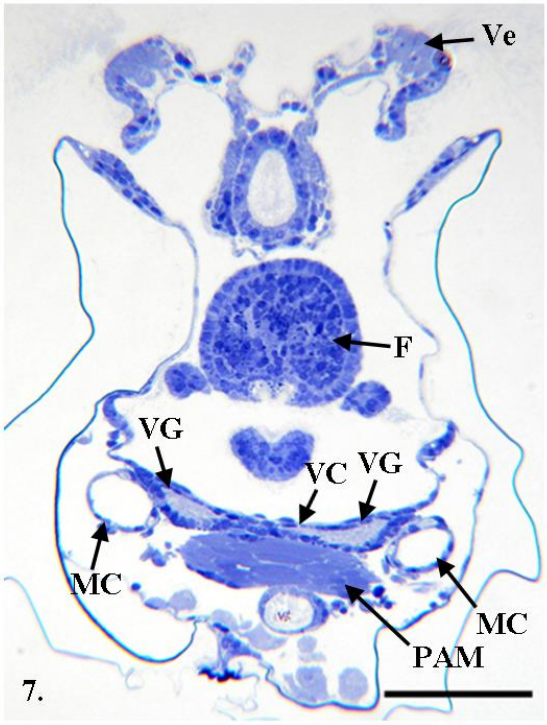
The organ systems of newly eyed larvae have undergone considerable differentiation and the central nervous system is easily observed in histological sections at the light level. In order to fully categorize the central nervous system of newly eyed larvae, it is necessary to analyze the neural tissues from multiple angles including the frontal (Figure 4-7), sagittal (Figures 8-10) and transverse views (Figures 11-14) because each view reveals significant morphological characteristics that would not be discerned with examination from only one angle. The frontal orientation generates the best general view of the organization of the central nervous system loop which includes a single apical ganglion and paired cerebro-pleural, pedal and visceral ganglia, and their various

Figure 4 - 7: Four sequential sections from the frontal serial set of a newly eyed larva. Scale bar in all = 50 μm. AG = apical ganglion; BG = byssal gland; C = connective; CC = cerebro-pleural commissure; CP = cerebral portion of the cerebro-pleural ganglion; CPG = cerebro-pleural ganglion; E = esophagus; F = foot; MC = mantle cavity; PAM = posterior adductor muscle; PC = pedal commissure; PG = pedal ganglion; PlPC = pleural-pedal connective; PlVC = pleural-visceral connective; PP = pleural portion of the cerebro-pleural ganglion; RM = rectractor muscles; ST = statocyst; Ve = velum; VC = visceral commissure; VG = visceral ganglion.


Ve
CC
CPG
RM
E
BG
4.


AG
CP
CPG
PP
PG
PC
ST
BG
5.


Ve
CPG
C
PlVC
PlPC
6.


Ve
F
VG
VC
VG
MC
MC
PAM
7.

commissures and the connectives. The sagittal view shows the position of the central nervous system loop with respect to the midline or center of the larva while transverse sectioning creates the most sections and therefore can give insight into the detail of neuronal innervation of tissues.

The apical ganglion (AG) is the most anterior portion of the nervous system (Figure 5). This structure resides directly posterior to the velum (Ve) in an epithelial cup that surrounds all but the exposed anterior surface. Short cilia extend from the anterior cells of the apical ganglion. The AG is also positioned anterior to and in close association with the cerebral commissure (Figure 4). Cell nuclei are apparent in the AG, but light microscopy does not provide sufficient magnification to allow investigation of the fine detail of this structure.

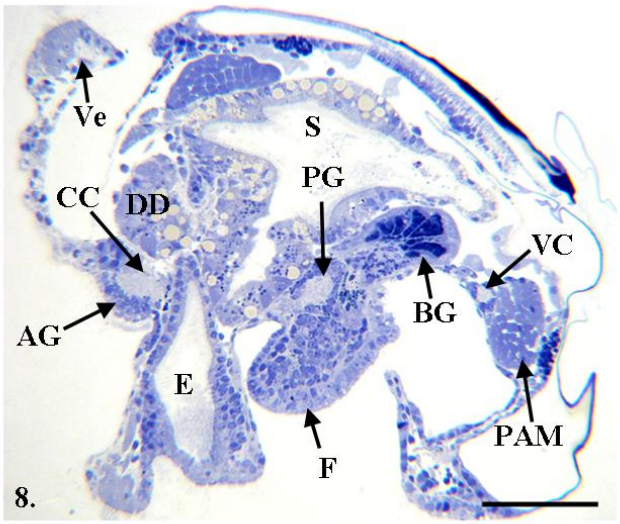
The cerebral and pleural ganglia have fused in larval *C. virginica* to create the left and right cerebro-pleural ganglia (CPG) (Figures 6 and 7). The cerebral portion of the ganglion (CP) is more anterior and circular while the pleural portion (PP) is posterior, narrow and elongated. The ganglia are found on either side of the esophagus and ventral to the lobes of digestive diverticulum (DD) (Figs. 8 and 9). The cerebral commissure (CC) connects the cerebral ganglia anterior to the esophagus (E) (Figure 4). The pleuro-visceral and pleural-pedal connectives exit the posterior region of each cerebro-pleural ganglion as one entity (C), which then splits near the region of the larval foot (F) (Figure 6). On each side, the pleural-pedal connective (PlPC) dives medial and ventral toward its respective pedal ganglion while each pleural-visceral connective (PlVC) continues to extend posterior to its respective visceral ganglion. The connective also runs in close association with the velar retractor muscle (RM) bundle (Figure 4).

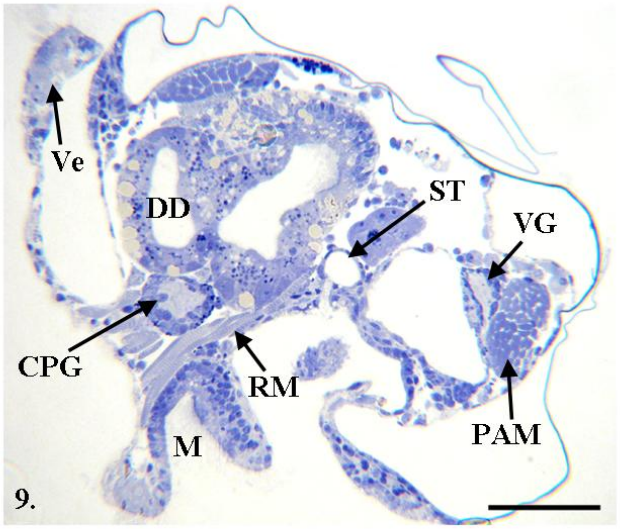
If not carefully examined the two pedal ganglia (PG) can be incorrectly identified as one large ganglion; however, serial sections reveal that this structure consists of two closely apposed ganglia that are connected by a thick pedal commissure (PC) (Figure 5) The two statocysts (ST) or gravitational sensing organs are latero-ventral to each PG (Figure 5). Various visceral organs are in close proximity to the PG. Dorsal to the PG is the digestive diverticulum (DD) and the anterior region of the stomach (S) (Figure 8). Below or ventral to the PG is the larval foot while the byssal gland (BG) is posterio-dorsal to the PG (Figure 8). At the newly eyed stage, the PG are similar in size to the cerebro-pleural ganglia.

The most posterior ganglia present within newly eyed larvae are the visceral ganglia (VG) (Figure 7). These ganglia are situated anterio-ventral to the posterior adductor muscle (PAM). A narrow visceral commissure (VC) connects the VG, and runs parallel to the posterior adductor muscle. This commissure is quite lengthy when compared to the other neural commissures previously described. Posterior projections of the mantle cavity (MC) extend lateral to each visceral ganglion. In frontal sections, these extensions are circular in appearance (Figure 7).

Figures 8-10 are sagittal sections that yield an alternate view of the structures described above. Briefly, Figure 8 shows the apical ganglion and one of the pedal ganglion, both of which reside towards the midline of the larva. Here, it can also be seen that the majority of large visceral organs are found lie dorsal to the central nervous system loop and that the nervous system itself actually lies ventrally within the larva. Moving from left to right in serial sagittal sections, the VG are encountered first indicating that these are the most laterally positioned ganglia (Figure 9). Figure 9

Figures 8 - 10: Three sections of a newly eyed larva in the sagittal plane of view. A small piece of debris was removed from the mantle area in Figure 10 using Photoshop. Scale bar in all = 50 μm. AG = apical ganglion; BG = byssal gland; CC = cerebro-pleural commissure; CPG = cerebro-pleural ganglion; DD = digestive diverticulum; E = esophagus; F = foot; M = mouth; PAM = posterior adductor muscle; PG = pedal ganglion; PlVC = pleural-visceral connective; RM = retractor muscles; S = stomach; ST = statocyst; Ve = velum; VC = visceral commissure; VG = visceral ganglion.


Ve
S
PG
CC
DD
VC
BG
AG
E
PAM
F
8.


Ve
DD
ST
VG
CPG
RM
PAM
M
9.

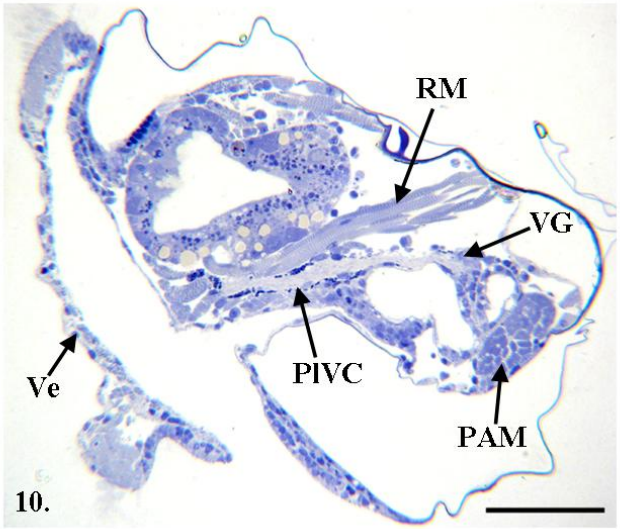
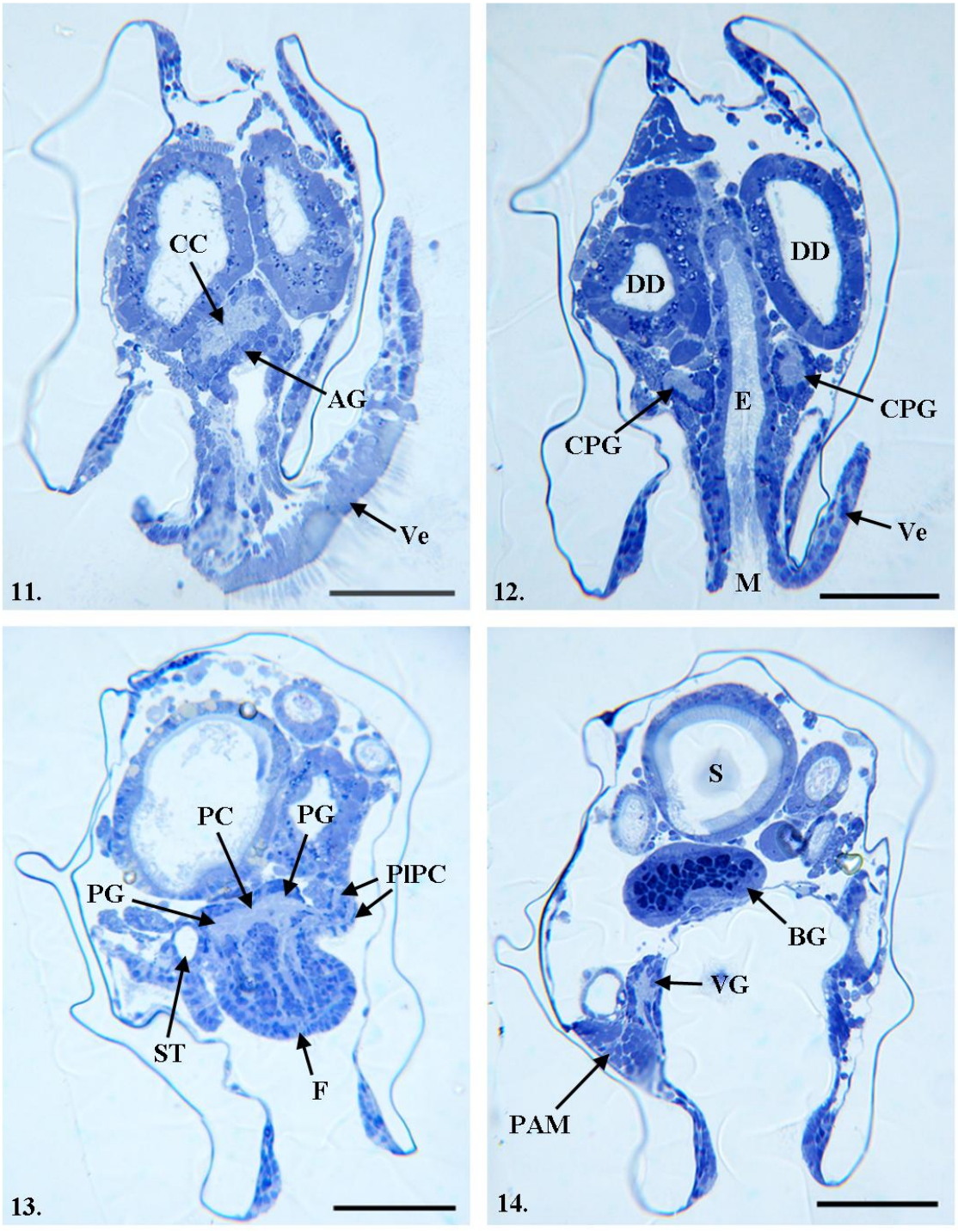

RM
VG
PIVC
Ve
PAM
10.

Figure 11 - 14: Four transverse sections of a newly eyed larva. In Figure 12, an air bubble was removed from the lumen of the digestive diverticulum for better clarity. Scale bar in all = 50 μm. AG = apical ganglion; BG = byssal gland; CC = cerebro-pleural commissure; CPG = cerebro-pleural ganglion; DD = digestive diverticulum; E = esophagus; F = foot; M = mouth; PAM = posterior adductor muscle; PC = pedal commissure; PG = pedal ganglion; PlPC = pleural-pedal connective; S = stomach; ST = statocyst; Ve = velum; VG = visceral ganglion.


CC
AG
Ve
11.
DD
DD
E
CPG
CPG
Ve
M
12.
PC
PG
PG
PIPC
ST
F
13.
S
BG
VG
PAM
14.

illustrates their position anterior and directly adjacent to the posterior adductor muscle. The close association of the velar retractor muscles to the CPG and the pleuro-visceral connectives is apparent (Figures 9 and 10).

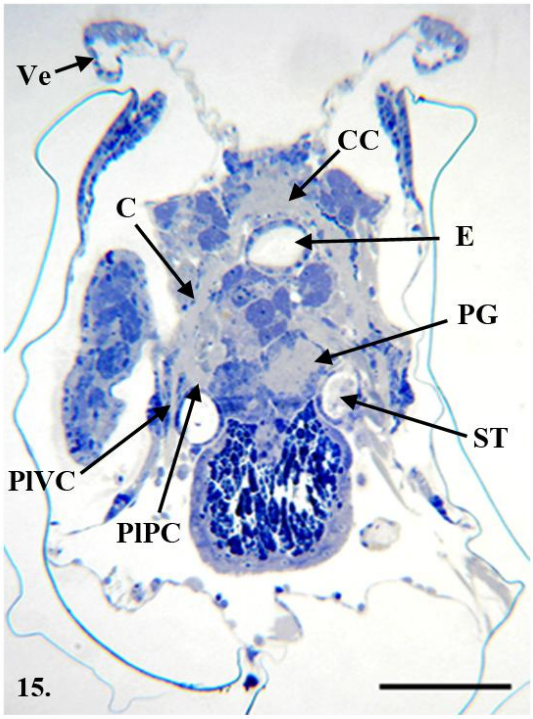
Transverse sections of the newly eyed larvae corroborated the structure of the central nervous system as discussed above for frontal views (Figures 11-14). Note that nerves (arrowheads) extending into peripheral tissues from the PG and VG are apparent in Figures 13 and 14. These will be discussed in more detail below.

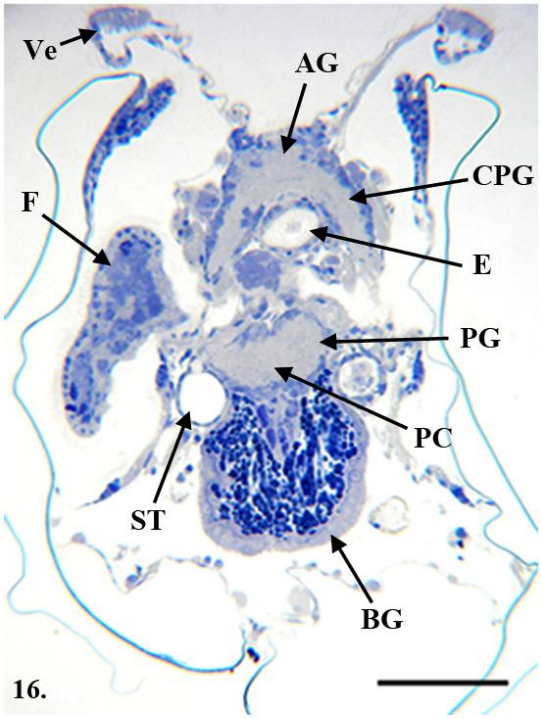
Pediveliger Larval Central Nervous System

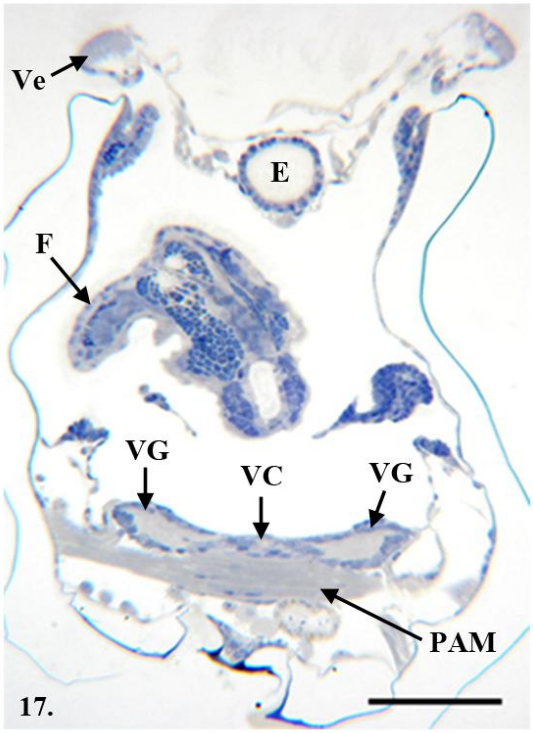
The pediveliger stage signifies that the larva is either at or approaching competence to metamorphose. Therefore the nervous system at this stage would reflect the most mature morphological characteristics found within the larval stage. Figures 15-25 are organized as frontal (Figures 15 – 18), sagittal (Figures 19 – 21) and transverse (Figures 22-250 views, as was done for the newly-eyed larval stage above. The morphology of the visceral organs in the pediveliger is similar to that of the newly eyed larva. The major exceptions are the larval foot (F), which has significantly elongated and is usually curled into one side of the mantle cavity (Figure 16 and 23) and the gill or ctenidial rudiment (GR) (Figure 18 and 19). Here, the gill ridges (or ctenidial crypts according to Moueza et al., 1999) have differentiated and are clearly evident.

The central nervous system of the pediveliger is very similar to that of the newly eyed larva. The AG, CPG, PG and the VG continue to reside in the same positions and orientations described above. However, two new ganglia, the paired visceral accessory ganglia, are present ventral to the VG and anterior to the PAM. These ganglia are also in

Figure 15 – 18: Four frontal sections from a pediveliger larva. Scale bar in all = 50 μm. AG = apical ganglion; BG = byssal gland; C = connective; CC = cerebro-pleural commissure; CPG = cerebro-pleural ganglion; E = esophagus; F = foot; GR = gill rudiment; PAM = posterior adductor muscle; PC = pedal commissure; PG = pedal ganglion; PlPC = pleural-pedal connective; PlVC = pleural-visceral connective; ST = statocyst; Ve = velum; VC = visceral commissure; VG = visceral ganglion.


Ve
CC
C
E
PG
ST
PlVC
PlPC
15.


Ve
AG
CPG
F
E
PG
PC
ST
BG
16.


Ve
E
F
VG
VC
VG
PAM
17.

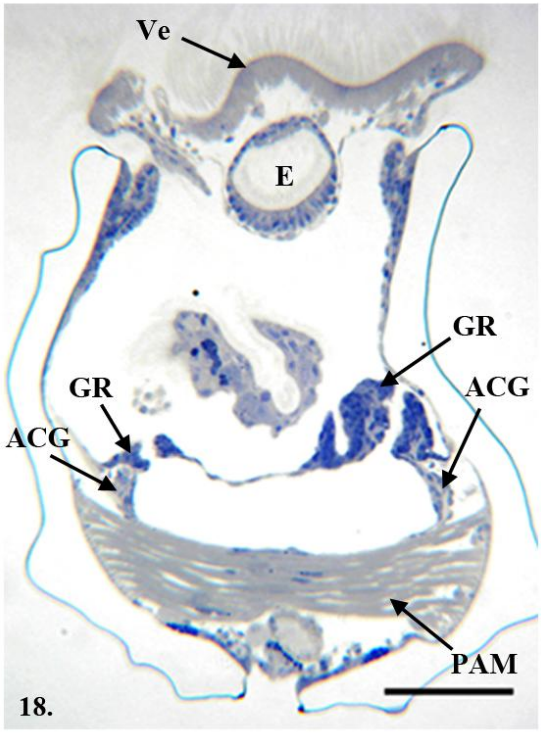

Ve
E
GR
GR
ACG
ACG
PAM
18.

Figure 19 – 21: Three sagittal sections from a pediveliger larva. Scale bar in all = 50 μm. ACG = accessory ganglia; AG = apical ganglion; BG = byssal gland; CPG = cerebro-pleural ganglion; E = esophagus; F = foot; GR = gill rudiment; PAM = posterior adductor muscle; PG = pedal ganglion; S = stomach; Ve = velum; VC = visceral commissure; VG = visceral ganglion.

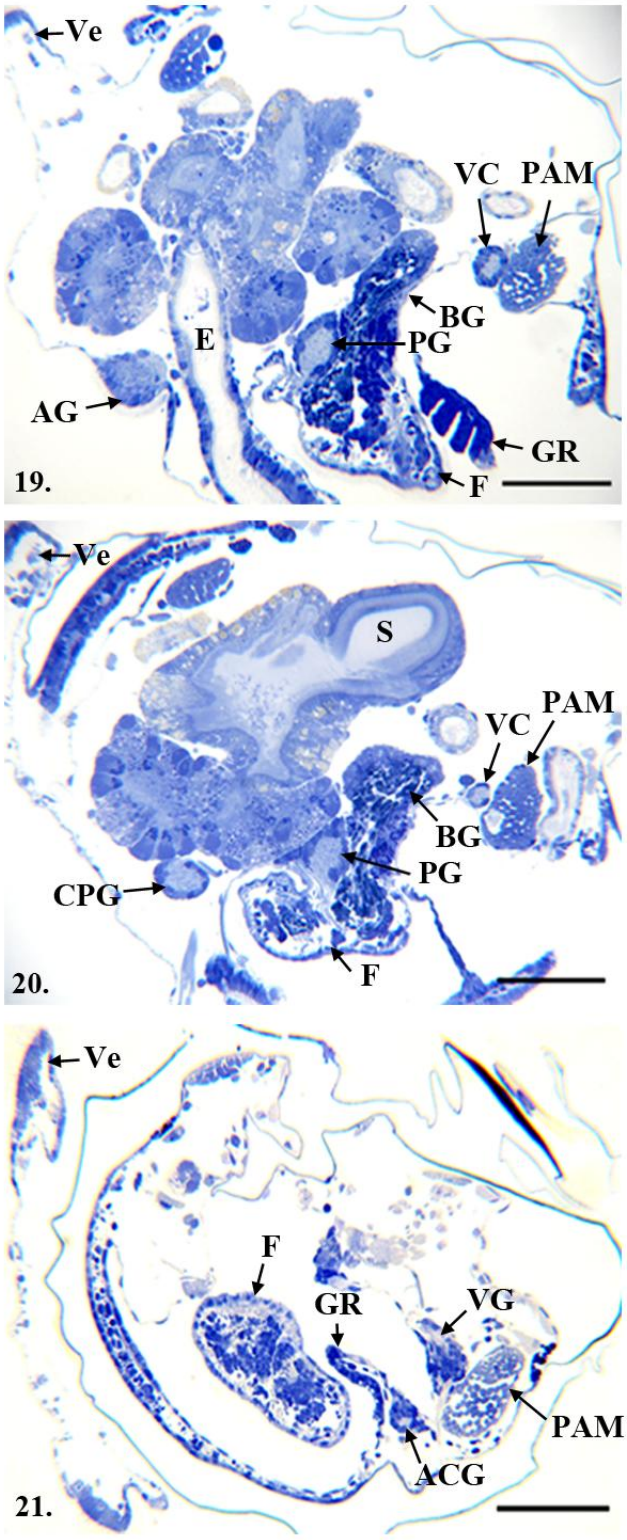
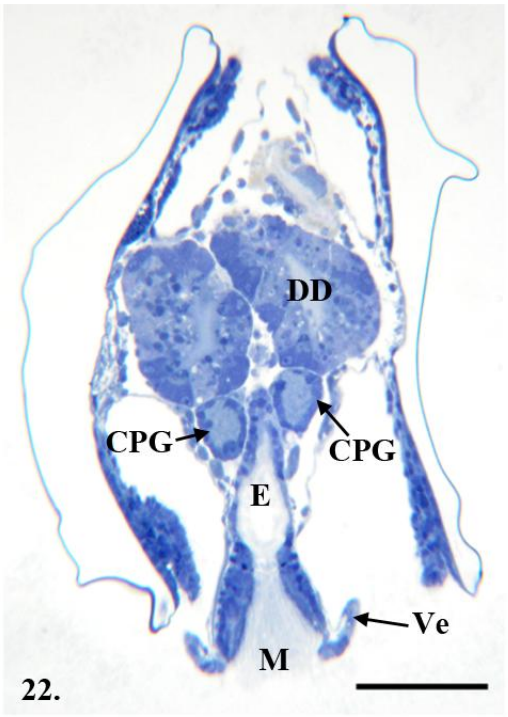
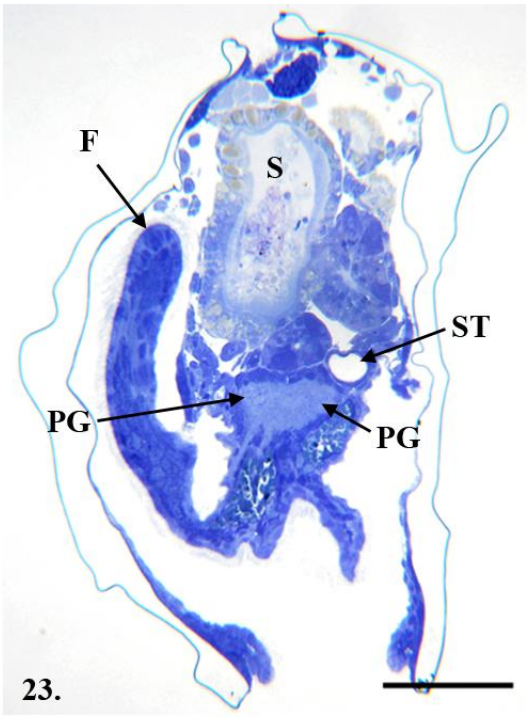

Ve
VC
PAM
BG
E
PG
AG
GR
F
19.
Ve
S
PAM
VC
BG
PG
CPG
F
20.
Ve
F
GR
VG
PAM
ACG
21.

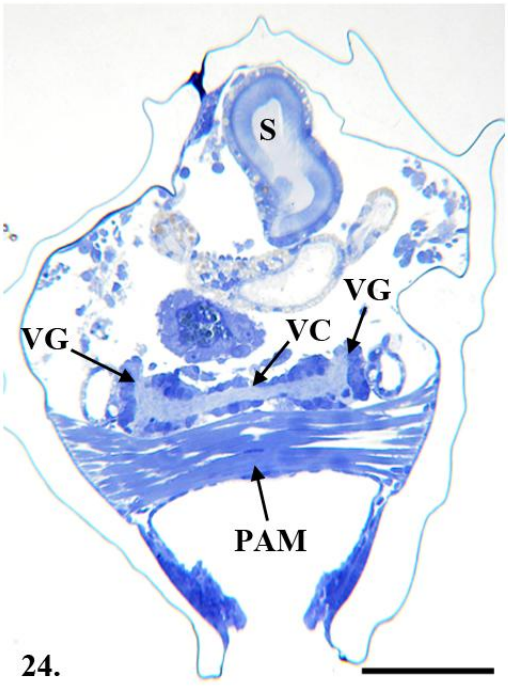
Figure 22 – 25: Four transverse sections at the pediveliger stage. Scale bar in all = 50 μm. ACG = accessory ganglia; CPG = cerebro-pleural ganglion; DD = digestive diverticulum; E = esophagus; F = foot; PAM = posterior adductor muscle; PG = pedal ganglion; S = stomach; Ve = velum; VC = visceral commissure; VG = visceral ganglion.



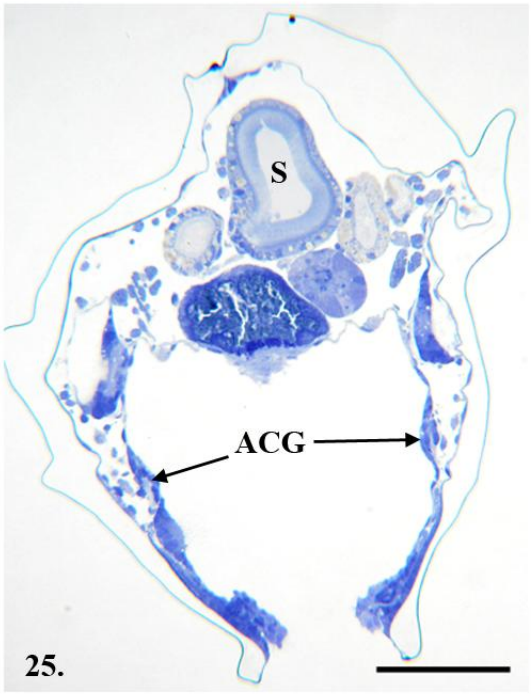
22.



23.



24.



25.

close association to the gill rudiment (Figure 18 and 21). I have identified these ganglia as the accessory ganglia (ACG) since they are in a similar location to the accessory ganglia described in various adult bivalves (Parker and Haswell, 1940). There appears to be a connective extending (depicted later in Figure 47) from the VG to the ACG, but there was no evidence of a commissure between them.

Peripheral Nerves and the Tissues Innervated

The observable presence of peripheral nerves in histological sections of *C. virginica* larvae is very dependent on the quality of the fixation. Fortunately, the fixation protocol used in this study was sufficient to reveal several aspects of not only the central nervous system, but also portions of the peripheral nervous system. In the following figures, the micrographs of the ganglia and associated peripheral innervations have been magnified and cropped, and therefore surrounding tissues are restricted to only those pertaining to the nervous processes themselves.

The apical ganglion did not appear to possess neuronal processes innervating the surrounding velar tissue; however, considering the very close association between the AG and the cerebral commissure, it is possible that nervous processes from this ganglion extend into the CC and exit into surrounding tissues via the cerbro-pleural ganglia. Also, nervous processes extending from the AG may be beyond the resolution limits of light microscopy and the particular staining method used.

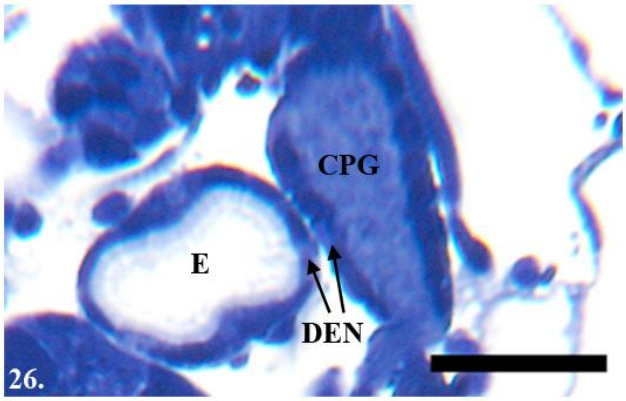
A neuronal process, the dorsal esophageal nerve (DEN) is present extending from the dorsal side of each CPG to the adjacent ciliated cuboidal epithelium lining the

Figure 26 and 27: Micrographs depicting the presence of the dorsal esophageal nerve.

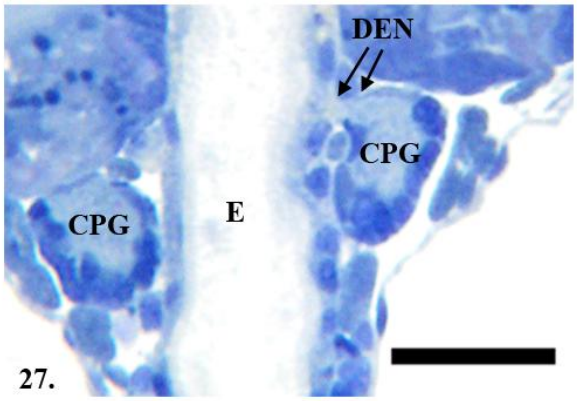
Figure 26: Frontal section from a newly eyed larva. CPG = cerebro-pleural ganglion; DEN = dorsal esophageal nerve; E = esophagus.

Figure 27: Transverse section in a pediveliger larva.

Scale bars = 20 μm. CPG = cerebro-pleural ganglion; DEN = dorsal esophageal nerve; E = esophagus.



26.



27.

esophagus (Figures 26 and 27). Careful examination of several serial section sets, suggests that there are actually several small DENs (see Figure 27) extending from each CPG, but only a handful of sections actually captured the exact angle and cut that allows observation of the DENs.

A large nerve, the posterior pedal nerve, can be seen extending into the foot from each pedal ganglion (Figures 28 and 29). This nerve projects along the lateral edge of the lateral pouches (LP) (described in Lane and Lott, 1975) toward the byssal gland at the base of the foot. Additional peripheral nerves, the ventral pedal nerves (VPN), extend from the ventral side of the PG (Figure 30-37). Both the PPNs and one of the VPNs are evident in the sagittal sections shown in Figures 30 and 31. Note the projection of the PPN toward the byssal gland. The trajectories of these nerves may be followed in the pediveliger serial section set movie provided in Appendix A.

Investigations in the transverse plane of view offer further enlightenment concerning the peripheral nerves associated with the PG. Figures 32-36 are serial sections of the PG in a newly eyed larva beginning at the anterior portion of the PG and progressing towards the posterior end. Here, the cross sections reveal that the pleural-pedal connective (PlPC) enters each ganglion from the lateral side. In addition, as the connective reaches the PG, it splits into two smaller processes. The more ventral process, the pleural pedal nerve (PlPN), bypasses the PG completely and innervates the foot (Figure 32-34). The second process is the actual pleural-pedal connective that extends laterally into the PG. The PlPN can appear to merge with either the PG or a nerve extending from the PG (Figure 34), but progressive sectioning reveals two ventral pedal

Figures 28 – 31: Histological sections from the newly eyed and pediveliger stages that allow visualization of the peripheral nervous system associated with the pedal ganglia.

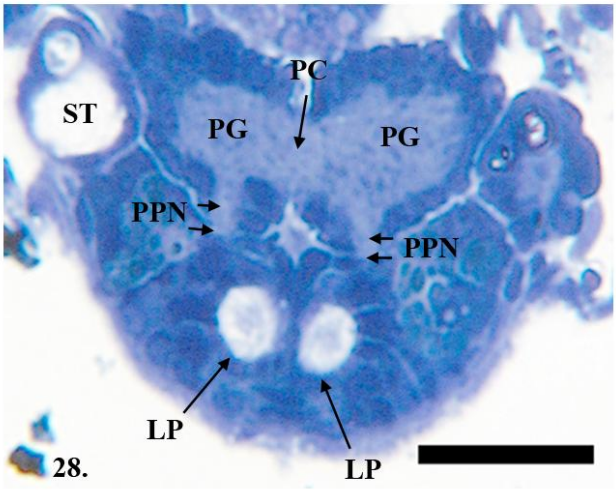
Figure 28: Frontal view in a newly eyed larva.

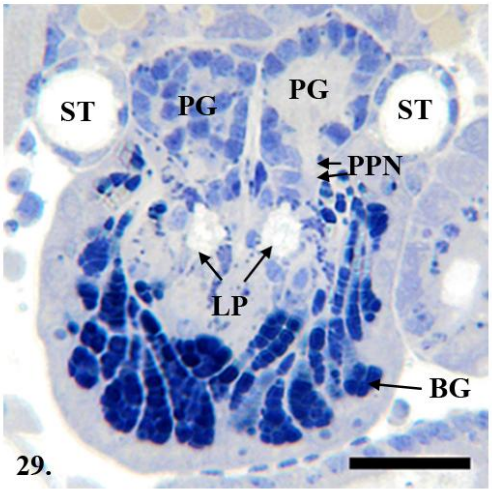
Figure 29: Frontal view of the pedal ganglia at the pediveliger stage.

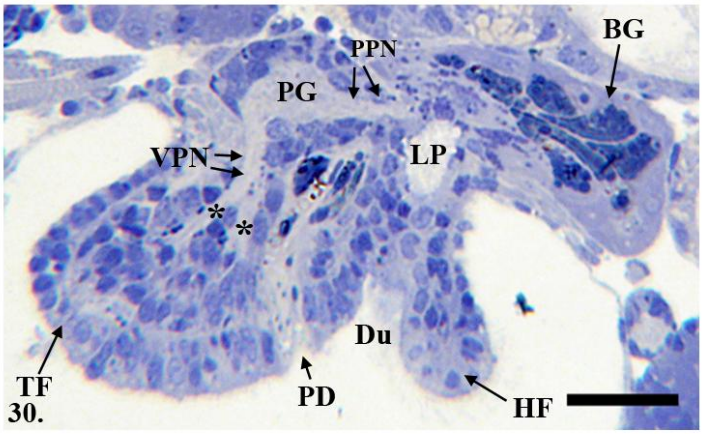
Figure 30: Sagittal section of a newly eyed larva.

Figure 31: Sagittal plane of view in a pediveliger larva.

Scale bars = 20 μm. * = indicate fork in VPN; BG = byssal gland; Du = posterior duct; F = foot; GR = gill rudiment; HF = heel of foot; LP = lateral pouch; PC = pedal commissure; PD = pedal depression; PG = pedal ganglion; PPN = posterior pedal nerve; ST = statocyst; TF = toe of foot; VPN = ventral pedal nerve


ST
PC
PG
PG
PPN
PPN
LP
LP
28.


ST
PG
PG
ST
PPN
LP
BG
29.


PPN
BG
PG
VPN
LP
*
*
Du
TF
PD
HF
30.

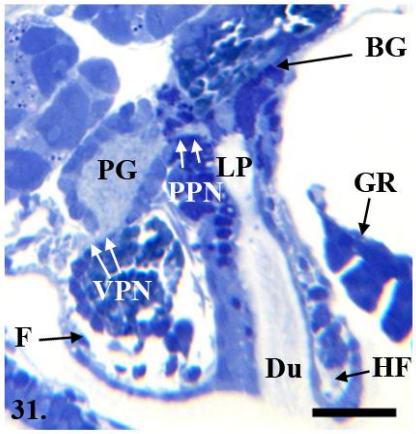
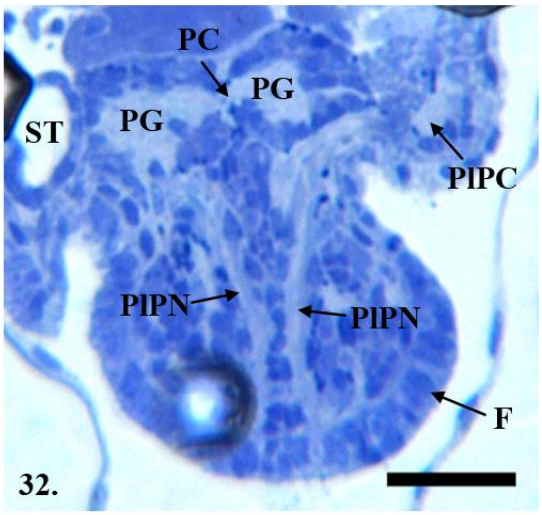
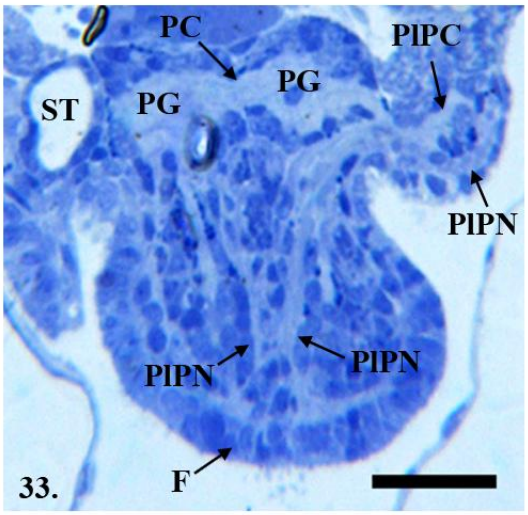

BG
PG
LP
PPN
GR
VPN
F
Du
HF
31.

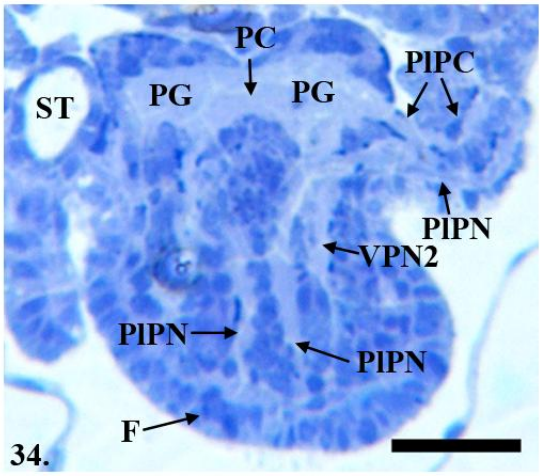
Figure 32 – 36: Five transverse sections of the pedal ganglia in a newly eyed larva showing various peripheral pedal nerves.

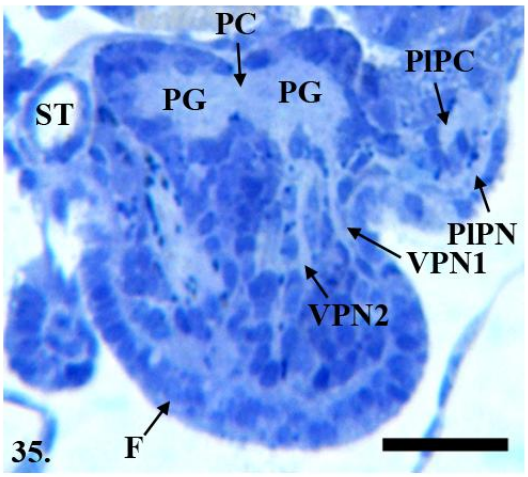
Figure 37: Similar transverse section in a pediveliger larva depicting the ventral pedal nerves 1 and 2.

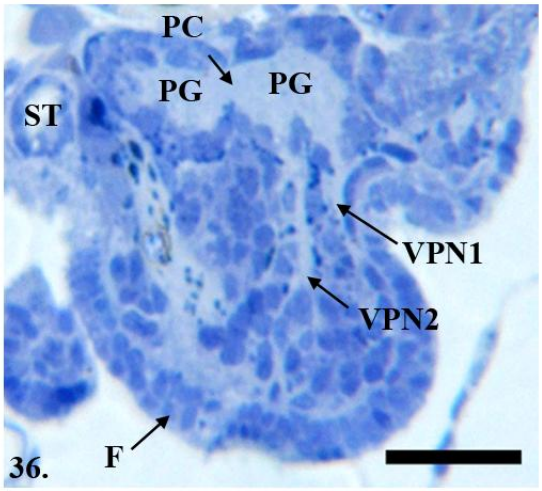
Scale bars = 20 μm. F = foot; PC = pedal commissure; PG = pedal ganglion; PlPC = pleural-pedal connective; PlPN = pleural-pedal nerve; ST = statocyst; VPN = ventral pedal nerve; VPN1 = ventral pedal nerve 1; VPN2 = ventral pedal nerve 2.

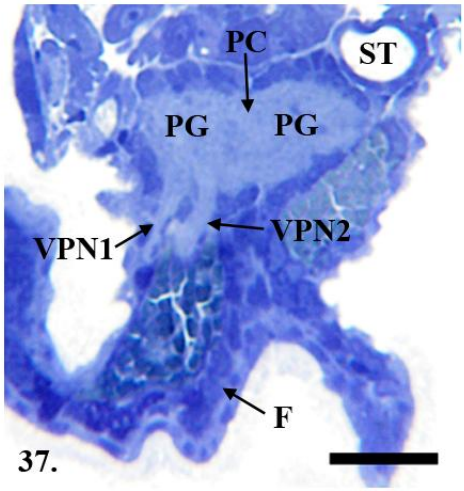

PC
PG
PG
ST
PlPC
PlPN
PlPN
F
32.


PC
PlPC
PG
PG
ST
PlPN
PlPN
PlPN
F
33.


PC
PlPC
ST
PG
PG
PlPN
VPN2
PlPN
PlPN
F
34.


PC
PlPC
ST
PG
PG
PlPN
VPN1
VPN2
F
35.


PC
PG
PG
ST
VPN1
VPN2
F
36.


PC
ST
PG
PG
VPN1
VPN2
F
37.

nerves (VPN1 and VPN2), which lay directly behind the PlPN. The presence of two ventral pedal nerves clarifies the fork-like appearance seen in sagittal sections (Figure 30). Figure 37 also depicts the two VPNs in a cross section of a pediveliger larva.

Each visceral ganglion has a thin proccess extending posterior underneath the posterior adductor muscle (Figure 38 and 39). Successive sectioning in the sagittal plane of view (Figure 39 and 40) depicts the process transitioning from a longitudinal to a circular morphology, indicating a change in direction. Frontal sections (Figure 41 and 42) reveal that the left and right processes extending from the visceral ganglia loop behind the rectum / anus and connect to each other forming an additional posterior visceral commissure (PVC). In transverse sections, (Figure 43) the PVC squeezes between the posterior adductor muscle and the wall of the mantle cavity. In addition, the VG directly innervate the posterior adductor muscle via the posterior adductor nerve (PAN) (Figure 44 and 45).

The viscero-accessory connectives (C) and the accessory ganglia undergo marked maturation between the newly eyed to the pediveliger larval stages (Figures 46 and 47-49). In pediveligers, the viscero-accessory connectives thicken, the accessory ganglia develop a distinct neuropil, and various nerves become evident (Figure 47-49). The viscero-accessory connectives on both the left and right sides of the larva are lateral to the posterior visceral commissure; therefore the PVC does not connect to the accessory ganglia. As it approaches the accessory ganglia, the viscero-accessory connective splits to yield a likely mantle nerve (MN, Figure 48). The exact target tissue was not confirmed; however, due to proximity, it is probable this process innervates the mantle tissue of the

Figures 38 – 42: Micrographs detailing the posterior visceral commissure associated with the visceral ganglia.

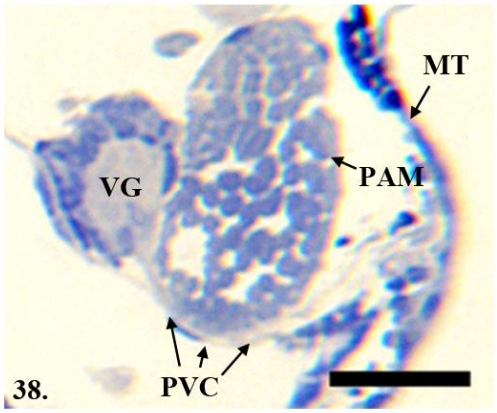
Figure 38: Sagittal section in a newly eyed larva.

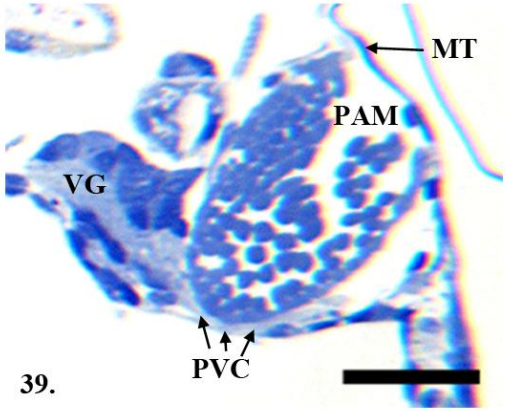
Figure 39 – 40: Serial sections in the sagittal plane of view in a pediveliger larva. Note the change in appearance of the posterior visceral commissure.

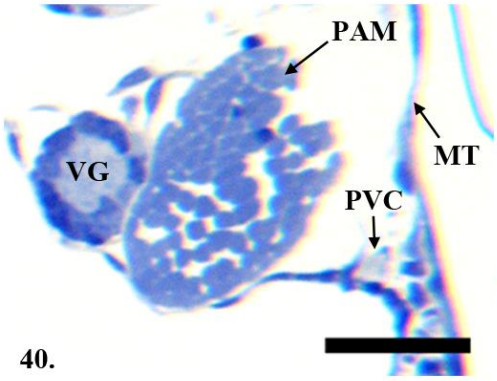
Figure 41: Frontal view in a newly eyed larva.

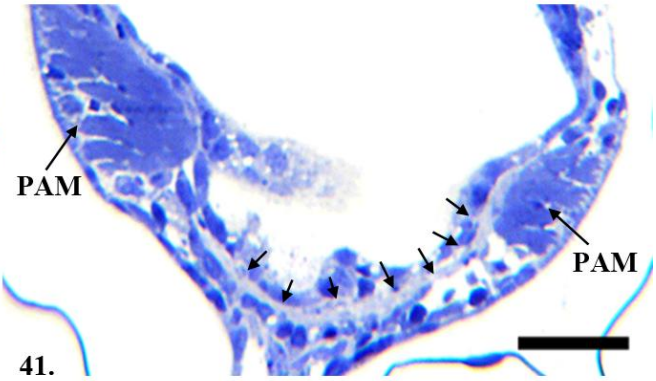
Figure 42: Frontal view in a pediveliger larva.

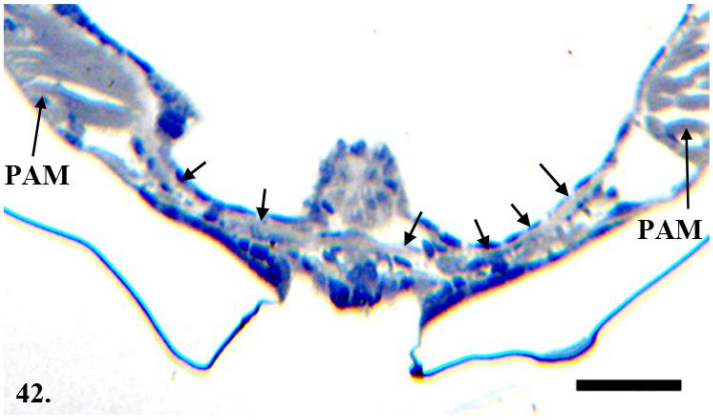
Scale bars = 20 μm. arrows = posterior visceral commissure; MT = mantle tissue; PAM = posterior adductor muscle; PVC = posterior visceral commissure; VG = visceral ganglion.


MT
VG
PAM
PVC
38.


MT
PAM
VG
PVC
39.


PAM
MT
VG
PVC
40.


PAM
PAM
41.

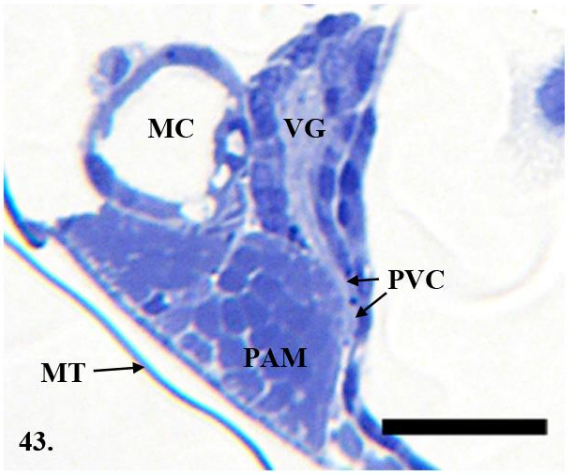

PAM
PAM
42.

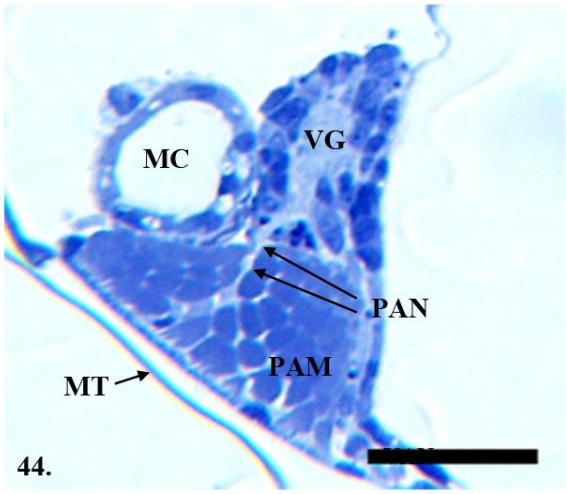
Figures 43 – 45: Micrographs of the visceral ganglion of a pediveliger stage larva in the transverse plane of view illustrating the posterior visceral commissure and the posterior adductor nerve.

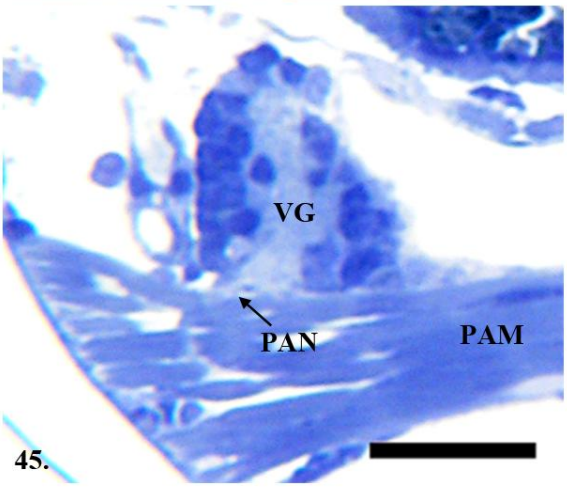
Figure 43 – 44: Transverse serial sections in a newly eyed larva.

Figure 45: Similar transverse section in a pediveliger larva as Figure 44.

Scale bars = 20 μm. MC = mantle cavity; MT = mantle tissue; PAM = posterior adductor muscle; PAN = posterior adductor nerve; PVC = posterior visceral commissure; VG = visceral ganglion.


MC
VG
PVC
PAM
MT
43.


MC
VG
PAN
PAM
MT
44.

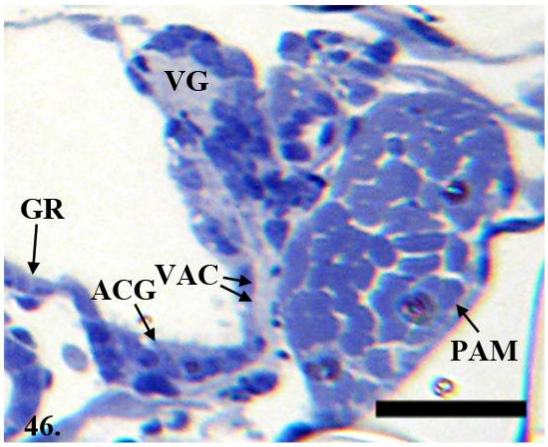

VG
PAN
PAM
45.

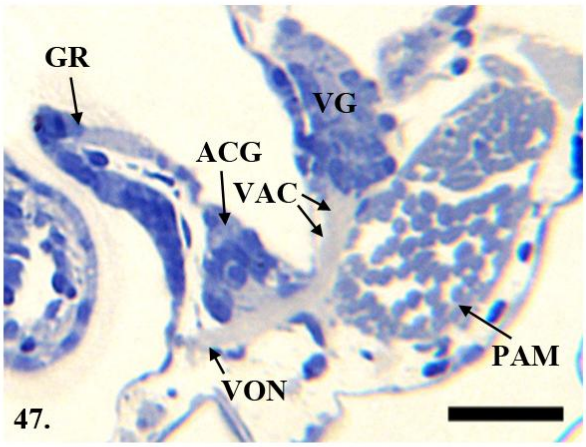
Figures 46 – 49: Histological Sections of the visceral and accessory ganglia in newly eyed and pediveliger *C. virginica* larvae.

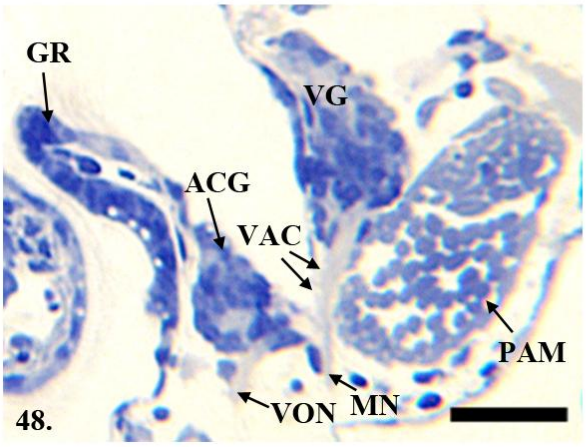
Figure 46: Sagittal micrograph of the ACG in a newly eyed larva.

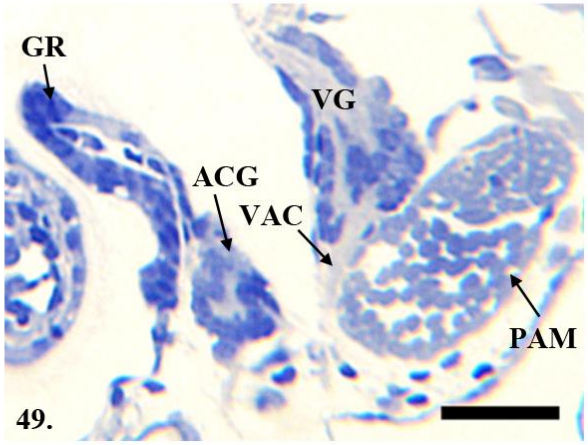
Figure 47 – 49: Three sections in the sagittal plane of view in a pediveliger larva. Note the distinct differences in morphology of the ACG between the newly eyed and the pediveliger larval stages. In the pediveliger a distinct neuropil is clearly present surrounded by a rind of neurons.

Scale bars = 20 μm. ACG = accessory ganglion; GR = gill rudiment; MN = mantle nerve; PAM = posterior adductor muscle; VAC = visceral-accessory connective; VG = visceral ganglion; VON = ventral osphridial nerve.


VG
GR
ACG
VAC
PAM
46.


GR
VG
ACG
VAC
PAM
VON
47.


GR
VG
ACG
VAC
PAM
VON
MN
48.

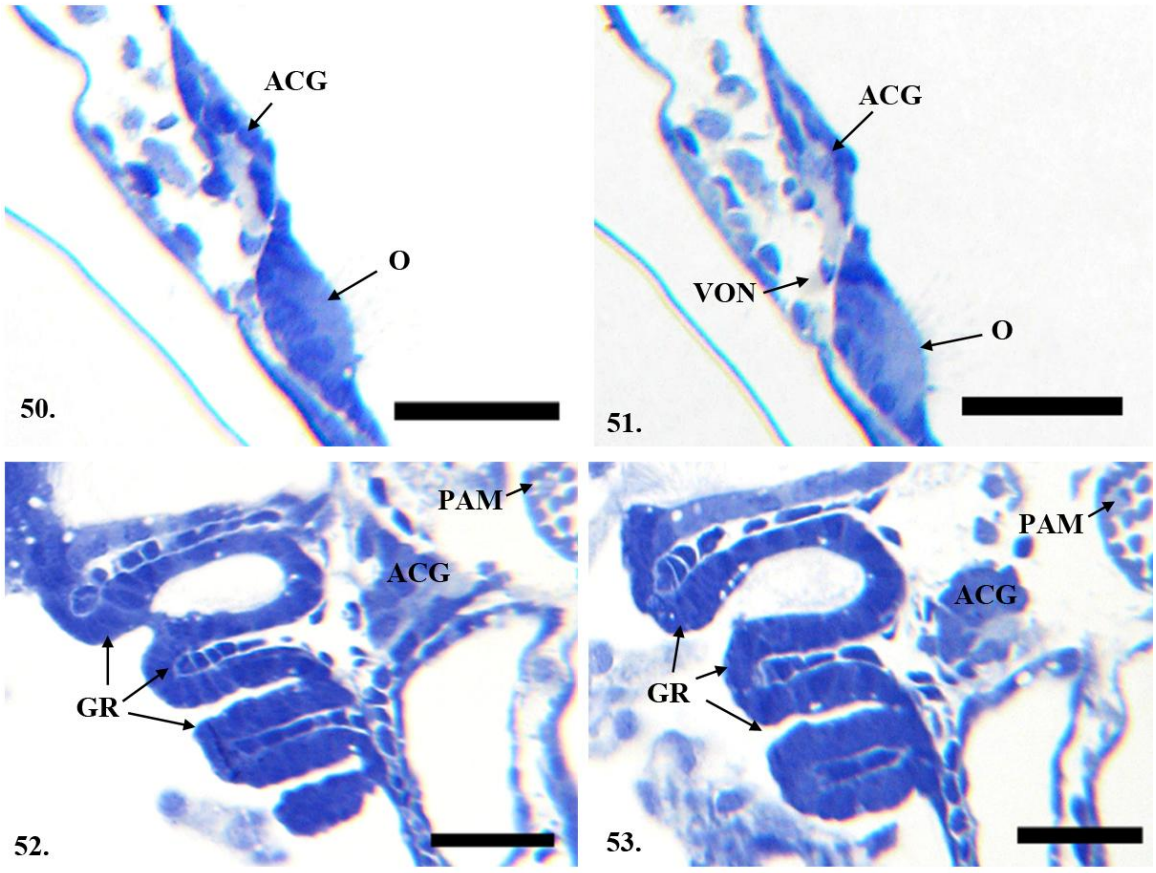

GR
VG
ACG
VAC
PAM
49.

Figures 50 – 53: Additional micrographs of the accessory ganglia.

Figures 50 and 51: Transverse sections in a pediveliger larva that show the ventral osphridial nerve extending downward towards the osphridium.

Figures 52 and 53: Additional sagittal sections of the ACG in a pediveliger larva. Note the close association of the ganglia to the developing gill rudiments.

Scale bars = 20 μm. ACG = accessory ganglion; C = connective; GR = gill rudiment; MN = mantle nerve; O = osphridium; PAM = posterior adductor muscle; VG = visceral ganglion; VON = ventral osphridial nerve.


ACG
O
50.
ACG
VON
O
51.
PAM
ACG
GR
52.
PAM
ACG
GR
53.

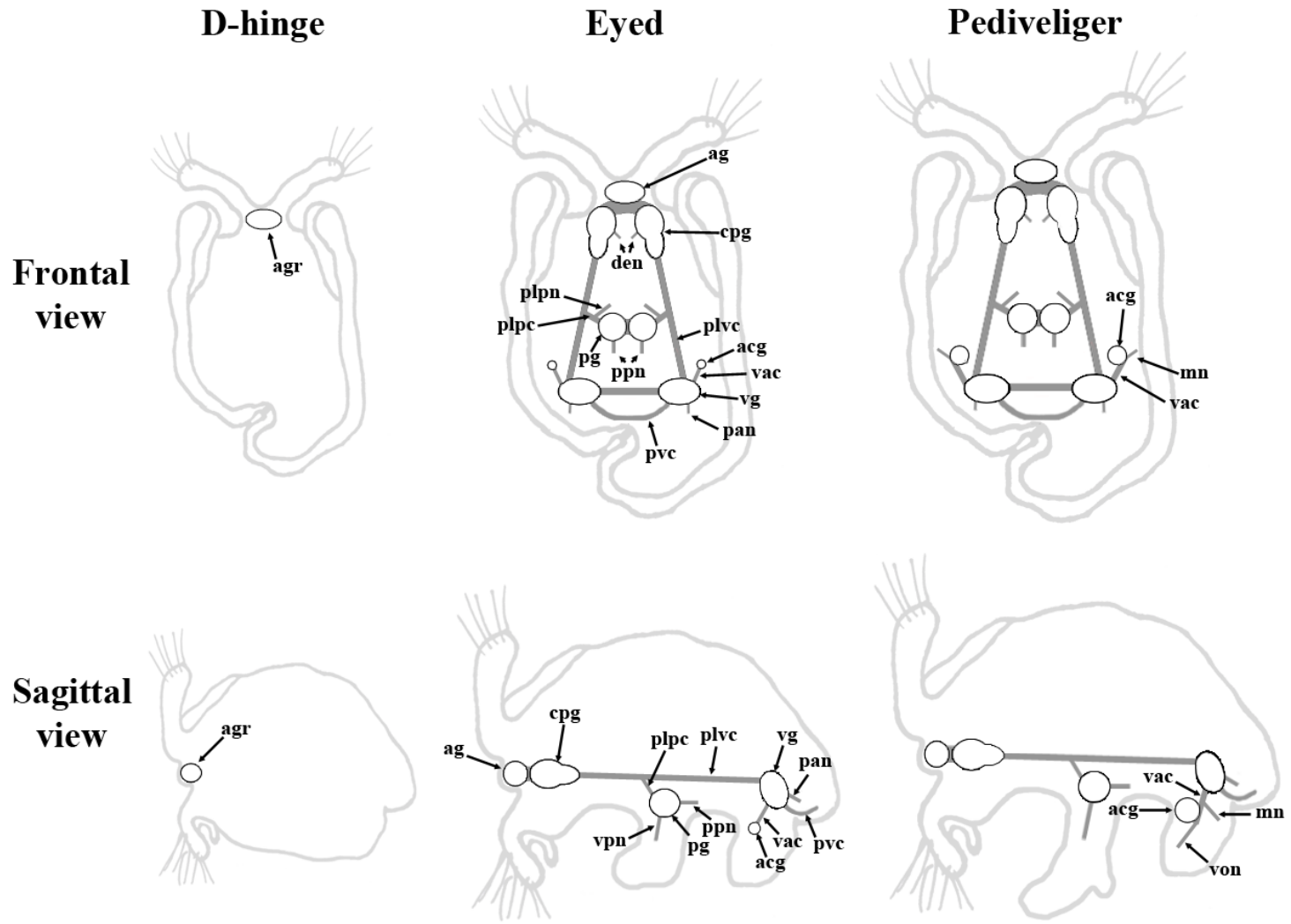
larva. Similarly, the target tissue of the probable ventral osphradial nerve (VON), which extends from either the accessory ganglion or directly from the connective, could not be verified at the light level. Transverse serial sectioning (Figure 50 – 51) demonstrates the VON extending ventrally in close association with the osphradium. Figures 52 and 53 offer additional views of an accessory ganglion and it's close association with the gill rudiment.

Discussion

The presence of a central nervous system in bivalve veligers has frequently been recognized (Erdman, 1935; Galtsoff, 1964; Bayne, 1971; Hickman and Gruffydd, 1971; Raineri and Ospovat, 1994; Raineri, 1995; Croll et al., 1997; Kreiling et al., 2001; Voronezhskaya et al., 2008). Erdmann (1935) documented the apical organ or apical pit as the only nervous system component in *Ostrea edulis* D-hinge larvae. This structure is homologous to the anterior gangliar rudiment described by Rainier in late trochophores of *M. galloprovincialis* (1995) and identified in this study in D-hinge *Crassostrea virginica*. The presence of ganglia, commissures and connectives was not detected with light microscopic histology in D-hinge larvae indicating that there is considerable neurogenesis yet to be accomplished at this point in development (Figure 54).

Although the larval central nervous system is not yet fully established, recent immunohistochemical studies indicate serotonergic, dopaminergic (Kreiling et al., 2001), cholinesterase (Raineri and Ospovat, 1994; Raineri, 1995) and FMRF-amide containing cells (Voronezhskaya et al., 2008) are present in areas outside the apical ganglion region early in development. Histological section sets recognize dispersed cells or miscellaneous

Figure 54: Diagrammatic sketch of the histology of the nervous system at various developmental stages in *C. virginica* larvae. Frontal and sagittal planes of view of the D-hinge, eyed and pediveliger larval stages are shown. White circles represent ganglia and grey lines represent commissures, connectives and nerves. At the D-hinge stage, only the presence of the anterior gangliar rudiment (agr) can be visualized histologically. Newly eyed larvae possess a single apical ganglion, and paired cerebro-pleural (cpg), pedal (pg), visceral (vg) and very small accessory ganglia (acg). In addition, connectives shown are the pleuro-visceral (plvc), pleuro-pedal (plpc) and the visero-accessory (vac) connectives. Only the posterior visceral commissure (pvc) is labeled although all commissures that are present between pairs of ganglia are drawn. The paired accessory ganglia lack a commissure. Nerves at this stage of development include the dorsal esophageal nerves (den), pleuro-pedal nerves (plpn), posterior pedal nerves (ppn), ventral pedal nerves (vpn), and posterior adductor nerves (pan). Only new structures are labeled in the pediveliger larvae, which include more prominent accessory ganglia and viscero-accessory connectives and the mantle (mn) and ventral osphridial nerves (von).


D-hinge
Eyed
Pediveliger
Frontal view
Sagittal view
agr
ag
cpg
den
plpn
plpc
plvc
pg
ppn
acg
vac
vg
pan
pvc
mn
vac
vpn
von

cells (Erdmann, 1935) within the visceral cavity. While the origin of the dispersed cells could not be verified, it is probable that some of these cells are neuronal. In opisthobranch embryos and larvae, progenitor cells separate from ectodermal placodes, migrate into the body and associate with each other to form the ganglia of the larval central nervous system (Page, 1992a,b). It is likely that development proceeds similarly in bivalve larvae and that some scattered cells within larval tissues and cavities are migrating neurons that will contribute to the various ganglia.

Central Nervous System in C. virginica Larvae

Erdmann (1935) and Galtsoff (1964) analyzed the general anatomy of *O. edulis* and *C. virginica* pediveliger larvae respectively and briefly described the central nervous system to be comprised of an apical sensory organ and paired cerebral, pleural, pedal and visceral ganglia. Similarly, this investigation reports the presence of an apical ganglion and paired cerebro-pleural, pedal and visceral ganglia in larvae *C. virginica* and adds the paired accessory ganglia as CNS components, as well as providing a description of peripheral innervation within the larval tissues (Figure 54). Additionally, unique to this study are detailed histological micrographs in various orientations that fully characterize the nervous system in *C. virginica* larvae.

The locations of the pleural ganglia can vary from species to species. The pleural ganglia are documented as separate unfused ganglia in *O.edulis* (Erdmann, 1935) and *M. trossulus* (Voronezhskaya et al, 2008) and appear to be entirely absent in *M. edulis* larvae (Bayne, 1971). In *C. virginica*, the pleural ganglia are fused with the cerebral ganglia.

Histological sections reveal the cerebral portion is anterior and more circular where as the pleural portion is positioned posterior and is elongated.

Also, *C. virginica* pediveligers have an additional pair of ganglia, which are designated as the accessory ganglia (ACG) based on the presence of similar ganglia in adult scallops (see below). These ganglia are positioned ventral to the VG and in close association with the gill rudiment. There has been no previous documentation of larval ganglia in this location; therefore these ganglia have either been overlooked in previous studies or they are a novel structure in the central nervous system of larval *C. virginica*. Croll et al. (1997) uses the unique term abdominal ganglia when describing immunolabeling in paired ganglia in the posterior region of *P. magellanicus* and *M. edulis*; however, the location and size of these ganglia suggests they are homologous to the visceral ganglia seen in *C. virginica* and not the accessory ganglia.

The location and morphology of the ACG in the adult scallop, *Nacula* (Parker and Haswell, 1940), suggest these ganglia are homologous to the ACG seen in larval stages of *C. virginica*. The ACG of *Naucla* adults attach to the central nervous system loop via a branch of the pleuro-visceral connective and are not reported to be connected by a commissure. Similarly, an accessory commissure is absent in *C. virginica* larvae; however the ACG connect directly to the VG instead of the pleuro-visceral connective (see pediveliger sagittal serial sections in Appendix A).

Interestingly, the ACG and viscero-accessory connectives are barely detectable in newly eyed *C. virginica* larvae. At the pediveliger stage, a distinct neuropil is observed, the viscero-accessory connectives thicken, and various nerves are apparent suggesting that this portion of the central nervous system is not fully functional until larval

competency or at some point thereafter. The ACG are closely associated with the gill rudiments, which differentiate during metamorphosis (Gros et al., 1997; Mouez et al., 1999). Moueza et al. (1999) suggests environmental cues may trigger gill maturation. Additionally, in adult scallops, the ACG connect to the brachial nerve, which innervates the gills (Gutsell, 1931; Bullock and Horridge, 1965). Therefore, it is tempting to hypothesize that maturation of the gill rudiments is mediated by innervation arising from the accessory ganglia in *C. virginica* larvae.

Peripheral Innervations

Erdmann (1935) described the larval nervous system of *O.edulis* as being more complex than the adult. The results described above indicate that not only the central nervous system, but also peripheral innervations add to the nervous system complexity in bivalve larvae. In *C. virginica* larvae, serial sectioning revealed nerves extending into the foot and posterior adductor muscle from the pedal and visceral ganglia, respectively. Investigations examining the presence and location of various neurotransmitters and neuropeptides in bivalve larvae have indentified several neuronal processes innervating tissues that likely extend through these nerves. For example, a catecholaminergic peripheral process originating at the pedal ganglia and innervating the foot has been documented in *P. magellanicus* pediveligers (Croll et al., 1997) Histological sectioning indicates this process to could be in either ventral pedal nerve 1 or 2 described in this study. Likewise, in *M. trossulus* pediveligers, a FMRF-amidergic process extended ventrally from a visceral ganglion into the caudal region of the larva (Voronezhskaya et al., 2008). This process did not label until the pediveliger stage of development. The

location and the onset of fluorescence correlates with the location and development of the viscero-accessory connective or the nerve branches associated with ACG.

This study also revealed information concerning the relationships between peripheral nerves and the connectives of the central nervous system loop. The pleuro-pedal connective splits as it approaches each pedal ganglion with one track entering the ganglion and the other innervating the foot. A similar phenomenon is seen in adult *Nacula* bivalves. Here, the neuronal innervations of the statocysts come from the pleuro-pedal connectives instead of the pedal ganglia (Parker and Haswell, 1940). The orientation of the approach of the pleural-pedal connective to the pedal ganglion and foot suggests that the nerve originating from this connective contains axons projecting from the cerebro-pleural ganglia. Thus, control of foot activities may result from direct involvement of neurons in both the pedal and cerebro-pleural ganglia.

A novel peripheral nervous component documented in this study was the posterior visceral commissure which extends from the VG, dives underneath the posterior adductor muscle, loops behind the rectum / anus and connects to the contralateral VG. Although, the VG have a larger and more anterior visceral commissure, this process is also considered a commissure because it also joins the right and left VG. Morphological differences were noted between the commissures. The posterior visceral commissure appears thin and extends deep into the surrounding tissues whereas the cerebral, pedal and visceral commissures are prominent and extend directly from left to right between the ganglia. Further investigations will be necessary in order to understand the functional purpose of multiple commissures connecting a single pair of ganglia.

The results reported herein provide the first detailed histological study of the development of a bivalve larval nervous system. The histology of both the central and peripheral nervous systems can be used to corroborate immunohistochemical analysis of the location of fluorescent neurons and axons relative to the central and peripheral nervous systems. This will facilitate future investigations concerned with nervous system control of critical life history behaviors, such as settling and metamorphosis. A better understanding of nervous system structure and how it modulates larval behaviors has the potential to significantly aid in both restoration efforts and aquaculture practices involving *C. virginica* and various other bivalve species.

Acknowledgements

I would like to thank The American Museum of Natural History, the American Microscopical Society, and the Auburn University Graduate School for providing some of the funds necessary to complete this project. I also appreciate the comments of my MS committee members and my fellow graduate students, Shanna Hanes and Maria Mazzillo that helped me formulate some of the ideas discussed in this paper.

Chapter 3:

The Presence and Location of Small Cardioactive-like Peptides (SCPs) in Larval *Crassotrea virginica* and Newly Hatched *Berghia verrucicornis*

Ivey R. Ellis* and Stephen C. Kempf

Department of Biological Sciences, Auburn University, 331 Funchess Hall Auburn University, AL 36849-5407

*ellisir@auburn.edu (corresponding author)

key words: oyster, gastropod, larvae, SCP and nervous system

Abbreviations

AAM = anterior adductor nerve

ACG = accessory ganglion/a

AG = apical ganglion

AGR = anterior gangliar rudiment

BG = byssal gland

BuG = buccal ganglion/a

CaN = caudal neuron

CC = cerebral commissure

CPG = cerebro-pleural ganglion/a

Du = posterior duct

E = esophagus

EDTA = ethylenediaminetetraacetic acid

EN = esophageal neuron

EtOH = ethanol

Ey = eye

F = foot

GR = gill rudiment

H = hinge

Ha = heart

HF = heel of foot

High Mg / Low Ca = high magnesium / low calcium solution

HN = heart neuron

M = mouth

Ma = mantle

M / E = oral / esophageal junction

MN = mantle nerve

O = operculum

PAN = posterior adductor nerve

PAM = posterior adductor muscle

PBS+ = phosphate buffered saline with 0.1% Triton X100 and 0.1% sodium azide

PC = pedal commissure

PG = pedal ganglion/a

PL? = possibly the pleural ganglion/a

PlPC = pleuro-pedal connective

PlVC = pleuro-visceral connective

PPN = posterior pedal nerve

R = rectum

SCP = small cardioactive-like peptide

TF = toe of foot

Va = varicosities

VAC = viscero-accessory connective

VC = visceral commissure

Ve = velum

VG = visceral ganglion/a

VON = ventral osphridial nerve

VP = velar process

VPN = ventral pedal nerve

Abstract

Small cardioactive peptides have been proposed to have several functions in adult molluscs. Although the literature for adult species is vast, little attention has been paid to the occurrence and function of small cardioactive peptides in larval molluscs. In this study, the presence and location of SCP-like neuropeptides were detailed in both the central and peripheral nervous system of D-hinge, newly eyed and pediveliger *Crassostrea virginica* larvae. Results indicate that SCPs are present early in development (D-hinge larvae) and increase towards competency (pediveliger larvae). Additionally, in newly eyed and pediveligers, SCPs were found in a varying number of neurons within all central ganglia, except the accessory ganglia. Varicose labeling was also documented within the commissures and connectives. Furthermore, several peripheral tissues were innervated by SCPergic axons including the velum, foot, esophagus, mantle and various musculatures. Due to the location of SCPs in *C. virginica* larvae, it is likely that these neuropeptides modulate muscle contraction and / or ciliary beating in larval molluscs. In addition, immunohistochemical labeling for SCPs was also examined in newly hatched *Berghia verrucicornis* larvae. Labeling was mainly centered within the central nervous system. Further examination is needed to make conclusive hypotheses concerning the functions of SCPs in *B. verrucicornis*.

Introduction

Cardioactive agents, such as FMRF-amide and small cardioactive peptides (SCPs), were first detected in molluscan nervous tissue homogenates (Price and Greenberg, 1977; Lloyd, 1978). Initially, SCP and FMRF-amide were thought to be the same neuropeptide (Lloyd, 1978). Further investigations revealed that when compared to FMRF-amide (Greenberg and Price, 1980), SCPs were higher in molecular weight and effective in increasing heart rate and beat amplitude (Lloyd, 1980).

Soon thereafter, SCPs were sequenced in the adult gastropod *Aplysia californica* and divided into distinctive compounds: small cardioactive peptide A (SCP_A) and B (SCP_B) (Morris et al., 1982; Lloyd et al., 1984). These neuropeptides vary by 4 amino acid residues. A multitude of research has identified the presence and location of SCP-like neuropeptides in numerous molluscan adults including the pulmonates *Helix aspersa* (Lloyd, 1978; Lloyd, 1980) and *Lymnaea stagnalis* (Perry et al., 1999; Masinovsky et al., 1988), the opisthobranchs *Aplysia californica* (Lloyd et al., 1985a, b; Lloyd et al., 1984; Fox and Lloyd; 1997), *Tritonia diomedia* (Lloyd, 1979; Lloyd, 1987; Masinovsky et al., 1988; Willows et al., 2000; Beck et al., 2000) and *T. festiva* (Masinovsky et al., 1988), the bivalves *Crassostrea virginica*, *Dinocardium robustum*, and *Mercenaria mercenaria* (Candelario-Martinez, 1993), and the cephalopod *Octopus vulagris* (Oct-SCP) (Kanda and Minakata, 2006).

Furthermore, SCP-like substances have been documented in more derived metazoans such as annelids (Reuter and Palmberg, 1989; Evans and Calabrese, 1989; Gustaffson and Wikgren, 1989) and arthropods (Callaway et al., 1987; Masinovsky et al.,

1988). Generally, findings in these phyla are comparable with molluscan studies in that SCP-like immunoreactivity was commonly found throughout the central nervous system.

The majority of investigations have focused on the neuronal networks of *A. californica* and *T. diomedea* due to the presence large, SCP positive neurons, which permit easily repeatable identification and efficient neurophysiological manipulation of the nervous system. In *A. californica*, SCPs were identified in all central ganglia but concentrations were markedly elevated in the buccal ganglia, suggesting SCPs may modulate feeding behaviors (Lloyd et al., 1985a). For example, in *Aplysia* the buccal B3 neuron innervates the accessory radular muscles (Lloyd et al., 1984) and the buccal muscles (Fox and Lloyd, 1997). In addition, B11 and B12 in *Tritonia* innervate the foregut (Perry et al., 1999; Masinovsky et al., 1988) while SCP containing cells in *Lymnaea* extend to the foregut and salivary glands (Masinovsky et al., 1988). Although the number of SCPergic neurons present in the buccal ganglia vary from species to species, all tissues innervated contribute to feeding and / or digestion.

Although there is an apparent association between SCPs and feeding structures, SCPs in molluscs appear to be multifunctional. Studies have also shown that SCPs modulate ciliary beating (Gainey et al., 1999; Willows et al., 2000). In order to fully assess functionality, investigators have directed their attention toward examining the presence of SCPs during ontogeny. The presence and location of SCP-like neuropeptides have been characterized in *T. diomedea* larvae (Kempf et al., 1987) and results are comparable in other larval opisthobranchs such as *Melibe leonia* (Kempf and Page, 2005). To date, ontogenetic analyses are limited to gastropod larvae.

To broaden our understanding in the larvae of other molluscan classes, this study examines the presence and location of SCP-like neuropeptides in larvae of the bivalve *Crassostrea virginica* and the gastropod opisthobranch *Berghia verrucicornis*. Confocal microscopy was used to analyze D-hinge, newly eyed and pediveliger larval stages of *C. virginica* and newly hatched veligers of *B. verrucicornis*. The findings in this study, along with the location of SCPs in adult *C. virginica* (Candelario-Martinez et al., 1993), suggest potential hypotheses concerning the functionality of this neuropeptide throughout the bivalve life history. Additionally, these results are compared to the distribution of SCPs in newly hatched larvae of the aeold nudibranch *Berghia verrucicornis*, providing comparative information relative to larval SCP innervation in a better studied group of mollouscs (e.g. Kriegstein, 1977; Bickell and Chia, 1979; Bickell and Kempf, 1983; Kempf et al., 1987; Page, 1992a,b; Page, 1993; Carroll and Kempf, 1994; Kempf et al., 1997).

Methods

The Auburn University Shellfish Laboratory (Mobile, Alabama) provided *Crassostrea virginica* larvae during the spawning seasons of 2007, 2008 and 2009. Upon arrival, larvae were immediately prepped for relaxation and fixation. In contrast, *Berghia verrucicornis* adults and numerous *Aiptasia pallida*, this seaslug's only food source, were collected from the Gulf of Mexico side of the Florida Keys in the summer of 2007, 2008 and 2009. The animals were then relocated to Auburn University and cultivated in artificial seawater aquaria. *B. verrucicronis* larvae were reared according to the protocol of Carroll and Kempf (1990).

Freshly shipped *C. virginica* larvae were placed in 0.45 μm Millipore filtered aquarium seawater (MFSW) to acclimate for approximately 20 minutes. Then, both species of larvae were anesthetized (personal communication from Louise Page, University of Victoria, B.C.) in scintillation vials using a 3:1 High Mg / Low Ca MBL seawater solution (Audesirk and Audesirk, 1980). Every 15 minutes, one part of the solution was removed and replaced with an equal volume of High Mg / Low Ca seawater for a total of 8 solution additions. The volume in each vial was then brought down to approximately 1.5 ml. after which 3 drops of chlorotone (chlorobutanol) saturated seawater were added to each vial every 1.5 minutes for a total of 8 additions over 12 minutes. The larvae were then gently spun into a pellet using a clinical centrifuge and transferred directly into fixative in a minimum volume of seawater.

Larvae were fixed in 4% paraformaldehyde + 0.2 M Millonigs Phosphate Buffer + 0.14M sodium chloride for approximately 1 hour at 5 degrees Celsius and then for 1 hour in a fume hood at room temperature. Afterwards, an equal amount of freshly made, warm, 20% ethylenediaminetetraacetic acid solution was added to each vial. Decalcification proceeded at room temperature overnight. Throughout the following day larval decalcification was monitored by scanning crushed larvae for calcified shards of larval shell. Periodic sampling was continued until the scanned larvae were fully decalcified.

Following decalcification, the larvae were rinsed 3 times in 20 mM phosphate buffered saline containing 0.1% Triton X100 and 0.1% sodium azide (PBS+), followed by successive 10-minute rinses in nanopure water and 30%, 50%, 70% ethanol (EtOH). Larvae were then rehydrated through 10-minute rinses in a descending series of the same

EtOH solutions and a nanopure water rinse. Following dehydration and rehydration, larvae were incubated in 5% heat inactivated goat serum in PBS+ for 1 hour on an orbital shaker table at 5 degrees Celsius. The blocking solution was then removed and replaced with a 1:1 solution of blocking medium and a primary SCP antibody solution (Masinovsky et al., 1988) overnight at 5 degrees Celsius on the orbital shaker. Larvae were then rinsed 4 times in PBS+ over an 8-hour period, followed by incubation in a 1:300 dilution of goat anti-mouse-IgG-Alexafluor 488 (Invitrogen) in blocking medium overnight at 5 degrees Celsius on the orbital shaker. Following secondary antibody incubation, the larvae were again rinsed 4 times with PBS+ over an 8 hour period.

Following antibody labeling, larvae were dehydrated in 10-minute steps in nanopure water and 30%, 50%, 70%, 80%, 90%, and 3X 100% EtOH rinses, The larvae were and transferred through three 10-minute toluene rinses. Larvae were cleared by transferring them from the last toluene rinse into a 1:1 mixture of DPX mounant (Electron Microscopy Sciences) and toluene in which they were allowed to infiltrate at room temperature overnight. After about 7 hours the larvae were transferred to 3:1 (DPX: toluene) solution for approximately 7 hours and then two changes of 100% DPX for 4 hours each. Cleared larvae were then mounted on slides in DPX under supported coverslips and the edges of the coverslips sealed with additional DPX. All slides were allowed to dry overnight.

The larvae were examined for SPC labeling with a Bio-Rad, MRC 1024, laser-scanning, confocal microscope. Serial optical sections at a z-step of either 0.5 or 1.0 μm were prepared and used for movies, 3 dimensional rotating reconstructions, and maximum intensity projections of larvae in frontal and sagittal planes of views. Negative

control samples were also generated for each larval immunohistochemical experiment (labeled with only secondary antibodies). In all cases, control samples exhibited only background fluorescence. Adobe CS4 Photoshop was utilized to adjust levels contrast of individual images and to prepare figures. QuickTime Pro was used to prepare serial section and rotating 3 dimensional movies for analysis of the data.

Results

SCP-like labeling in the CNS of C. virignica Larvae

Small cardioactive-like peptides were present in the youngest *C. virginica* larval stage (D-hinge). At this stage SCP labeling is limited to the apical gangliar rudiment (AGR) and the processes of the developing central nervous system loop (Figure 1). At higher magnification, the fluorescent structures located in the AGR and positioned directly posterior to the velum (Ve) are identified as varicosities (Va) instead of neuron perikarya, due to the lack of detectable nuclei (inset Figure 1). Varicosities also occur along the neural extensions of the central nervous system loop (Figure 1 arrows). On occasion a thin process could be seen connecting the right and left sides of the loop, which are probably the beginning of the development of the future pedal commissure are SCP positive. Additionally, a thin axonal extension labeled for SCPs in the dorsal portion of the velum (VP, Figure 2). This process was circular in appearance and did not innervate the ventral half of the velum.

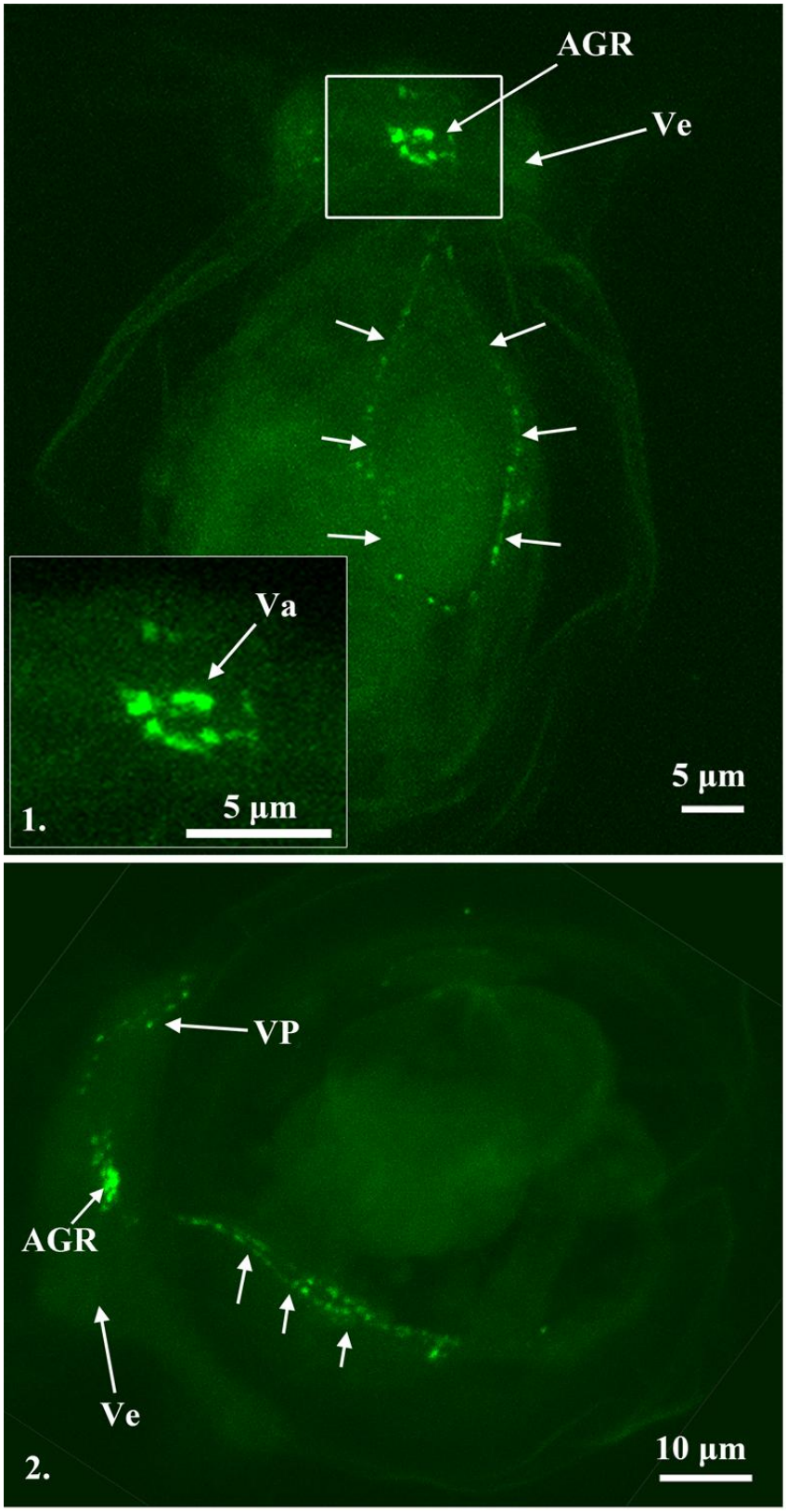
In contrast, newly eyed oyster larvae exhibit a considerable amount of SCP labeling. At this point in larval ontogeny, most components of the central nervous system are strongly labeled (Figure 3). Tiny varicosities along axons projecting through the

Figure 1 and 2: SCP labeling in D-hinge *C. virginica* larvae.

Figure 1: A frontal of view of a D-hinge larva with the boxed region indicating the area of the inset. Note apical gangliar rudiment and the precursor of the nervous system loop.

Figure 2: Sagittal view. Note axons in velum (P).

AGR = apical gangliar rudiment; arrows = varicosities of the central nervous system loop; VP = velar process; Va = varicosities; Ve = velum; P = velar process; arrows denote precursor of nervous system loop.

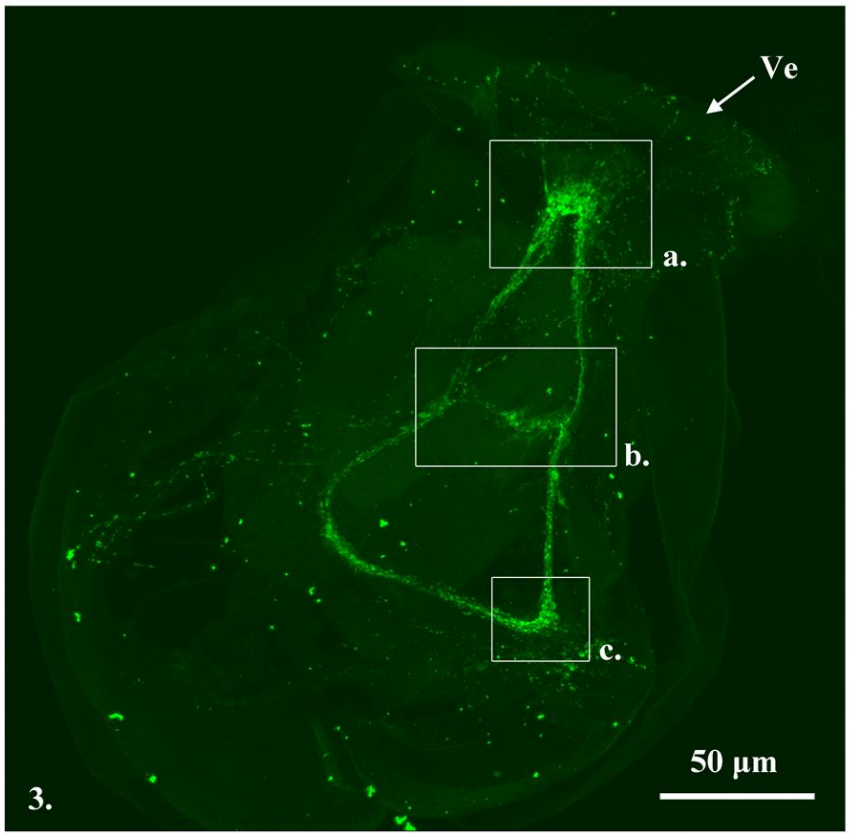

AGR
Ve
Va
5 µm
1.
5 µm
VP
AGR
Ve
2.
10 µm

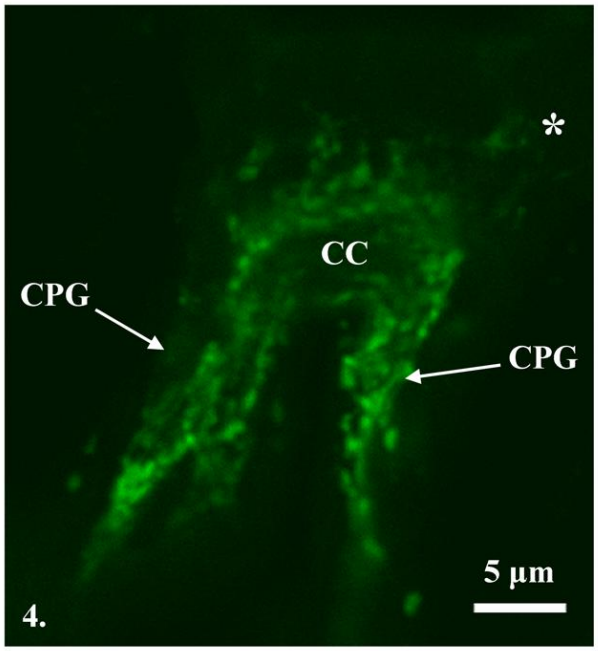
Figures 3 - 5: SCP labeling of a newly eyed *C. virginica* larva.

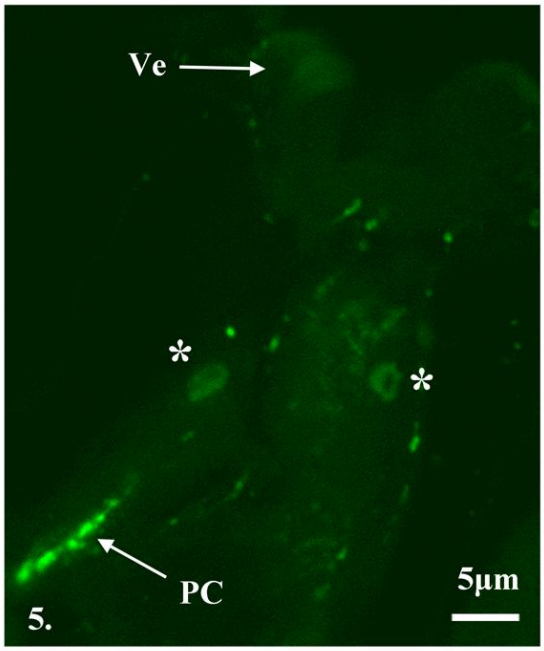
Figure 3: Confocal micrograph of a whole newly eyed larva. Nervous system loop is complete with labeling in all ganglia, commissures and connectives present at this stage. The boxes indicate regions of subsequent figures.

Figure 4 and 5: Oblique frontal sections of the apical and cerebro-pleural ganglia taken from box "a".

* = SCPergic neuron cell bodies; CC = cerebral commissure; CPG = cerebro-pleural ganglion/a; PC = pleural connective; Ve = velum


Ve
a.
b.
c.
50 µm
3.


*
CC
CPG
CPG
5 µm
4.


Ve
*
*
PC
5µm
5.

commissures, connectives and within ganglion neuropils label strongly for SCP. Figure 4 depicts this type of labeling seen in the neuropil of the apical and cerebro-pleural ganglia (Figure 4). In addition, the ganglia have neurons that now retain SCPs with in their perikarya. Two to three neurons of the apical ganglion are barely positive for SCPs, whereas each cerebro-plerual ganglion has one (and sometimes 2) prominent ventral SCPergic neurons (Figure 5). At the level of the larval foot, 0-1 neurons can be located ventrally in each pedal ganglion, although the pedal commissure and pleuro-pedal connectives are the chief source of SCP labeling (Figure 6). Lastly, 3-4 SCP positive neuron perikarya are found in the lateral portion of each visceral ganglion (Figures 7-8). These perikarya are closely arranged such that examination through serial confocal micrographs must be used to obtain precise counts (Figures 7-8).

The presence and location of SCP-like neuropeptides in pediveligers is similar to that of newly eyed larvae. The central nervous system loop continues to stain brightly, but there is also a discernable increase in the fluorescence of axons in the peripheral nervous system (Figure 9). At competency, there are approximately 6 SCPergic neurons in the apical ganglion (Figure 10). Three neurons are located on each side of the apical pit with the central neurons of each group of three exhibiting distinguishably stronger SCP labeling. The neuron perikarya are dispersed between epithelia cells, but there was no evidence that these neurons bear cilia. Additionally, the cerebro-pleural (Figure 11), pedal (Figure 12) and visceral ganglia (Figure 13) each possess 4-5, 2-3 and 7-8 SCPergic neurons respectively.

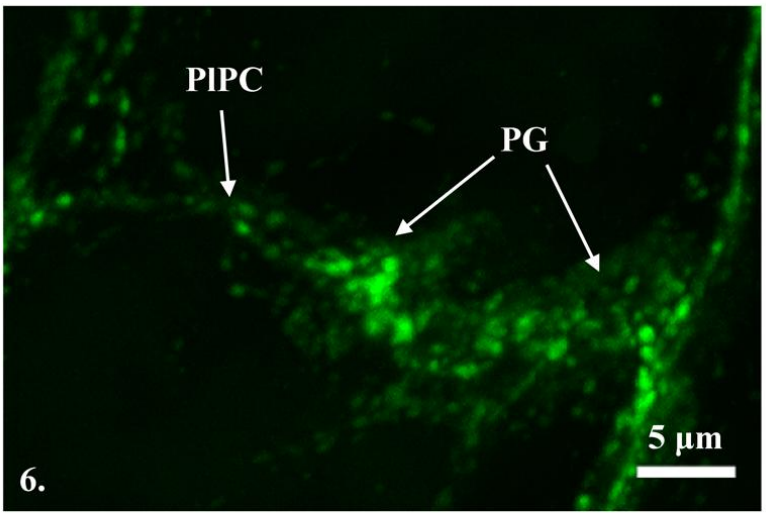
Peripheral SCPergic Innervations

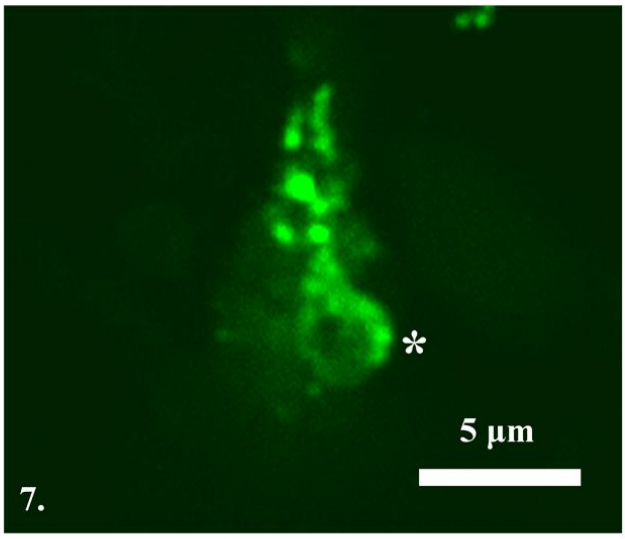
Figures 6 – 8: Pedal and visceral SCP antigenicity in a newly eyed *C. virginica* larva

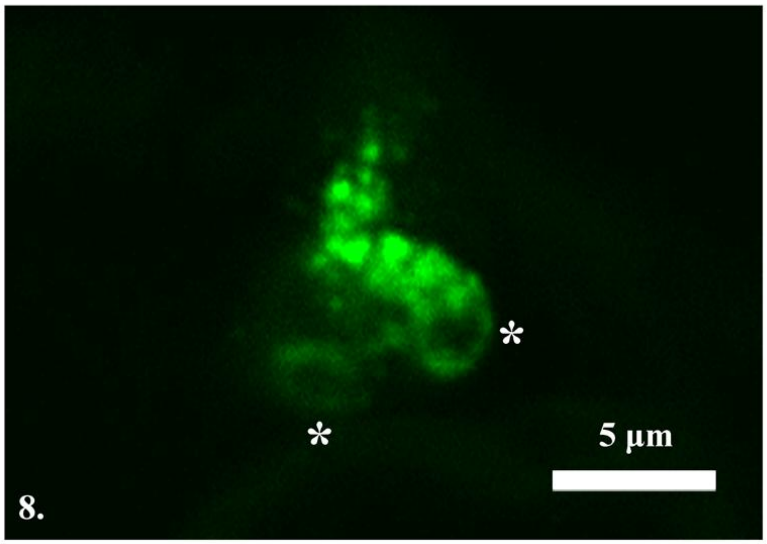
Figure 6: Boxed region "b" from Figure 3 which shows strong SCP labeling in the pedal commissure, neuropil and connectives. 0 – 1 SCP neurons were documented in each pedal ganglion at this stage of development.

Figure 7 and 8: Micrographs at different levels from the "c" box in Figure 3. Eyed larvae had a range of 3 – 4 SCP neurons in each visceral ganglion.

* = SCP positive neuron cell bodies; PG = pedal ganglion/a; PlPC = pleuro-pedal connective.


PIPC
PG
5 µm
6.


*
5 µm
7.


*
*
5 µm
8.

Figures 9 – 13: Central nervous system SCP labeling in a pediveliger *C. virginica* larva.

Figure 9: An oblique frontal view of the SCPergic innervations in a pediveliger. Boxes indicate areas of subsequent micrographs.

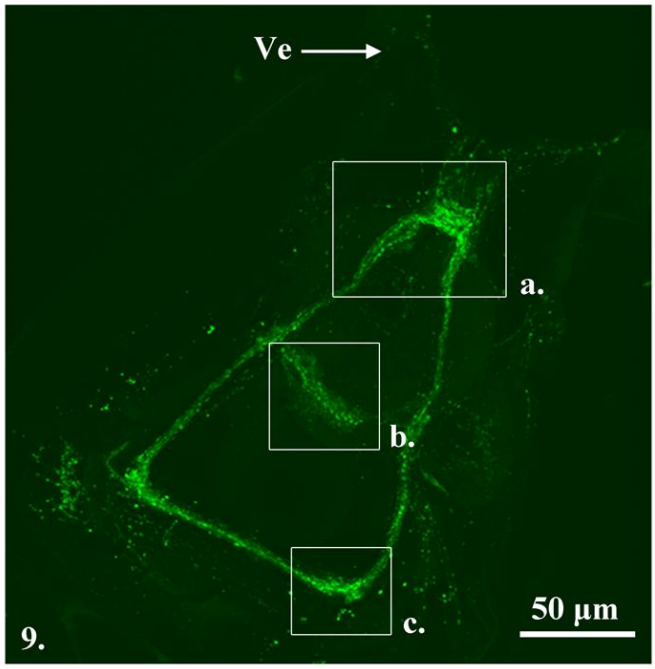
Figure 10: High magnification micrograph showing labeling in the apical ganglion (box "a" in figure 9). Although the positions of only 4 SCPergic neurons are visible in this micrograph, pediveligers normally had a total of 6 SCP neurons in the apical ganglion, three on the left and three on the right sides. The asterisks mark the positions of all 6 neurons.

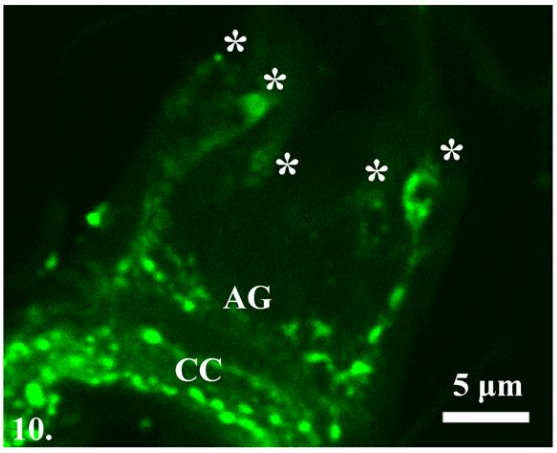
Figure 11: SCP labeling of the cerebro-pleural ganglia taken from box "a" in Figure 9. The left ganglion depicts two ventral strongly labeled neurons, whereas two lightly staining neurons (more dorsal) can be seen in the right ganglion. Usually, each of the cerebro-pleurals possessed 4 – 5 SCPergic neurons.

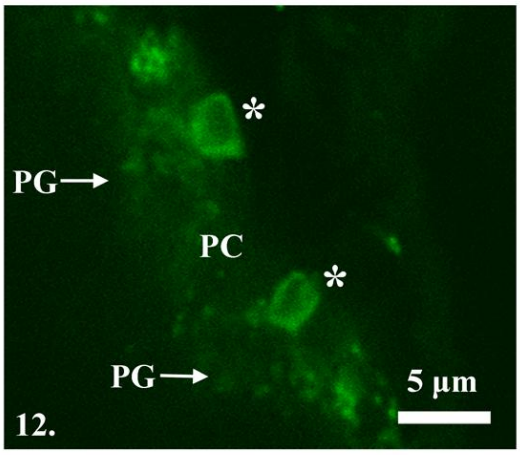
Figure 12: 2 SCP neurons towards the ventral portion of the pedal ganglia. SCP positive neurons can range from 2 – 3 in each pedal ganglion at the mature larval stage.

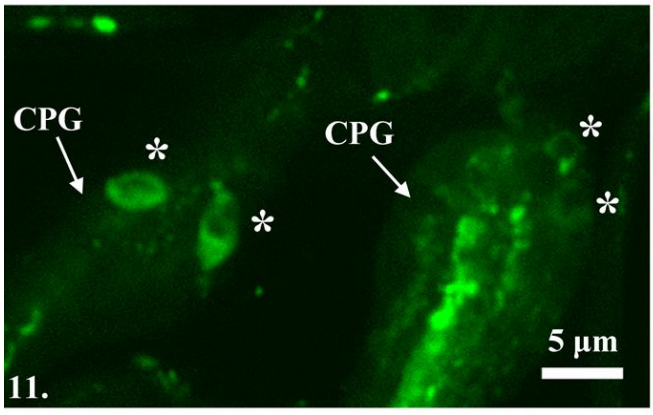
Figure 13: Maximum intensity projection of a visceral ganglion which indicates 4 or the SCPergic neurons present in each visceral ganglion at the pediveliger stage.

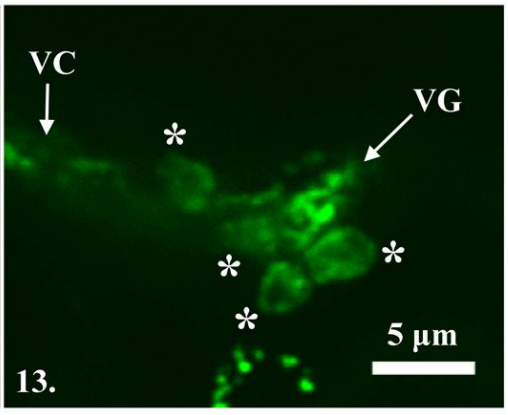
AG = apical ganglion; * = SCP positive neuron cell bodies; CC = cerebral commissure; CPG = cerebro-pleural ganglion/a; PC = pedal commissure; PG = pedal ganglion/a; VC = visceral commissure; Ve = velum; VG = visceral ganglion/a


Ve
a.
b.
c.
50 µm
9.


AG
CC
5 µm
10.


PG
PC
PG
5 µm
12.


CPG
CPG
5 µm
11.


VC
VG
5 µm
13.

The presence of small cardioactive peptides in the peripheral nervous system becomes detectable early in ontogeny. As detailed above, D-hinge *C. virginica* larvae possess an SCPergic process in the dorsal half of the velum (see Figure 2). Interestingly, a positive correlation is seen between larval development and peripheral SCP antigenicity. Extensive, but weakly labeled, peripheral innervations begin to appear in newly eyed larvae and increase in number and fluorescent strength in the pediveliger stage. Common peripheral SCP innervations include tissues such as the velum, foot, mantle, esophagus and various musculatures.

Velar SCP innervation is limited to the outer rim of the velum. In sagittal view, a SCPergic process extends from the antero-ventral portion of each cerebro-pleural ganglion into the ventral velum (Figure 14 arrows), while an additional extension from the antero-dorsal region of the cerebro-pleural ganglia innervates the dorsal velum (Figure 15 arrows). Similarly, SCPergic axons extend from the pedal ganglia into varying regions of the larval foot. For instance, the propodium of the foot (TF) receives SCP innervation from the pedal ganglia via the ventral pedal nerves (VPNs, Chapter 2) (Figure 16), whereas the posterior pedal nerves (PPNs, Chapter 2) dive ventrally to supply the heel of the foot (HF) (Figure 17).

SCP labeled axons that appear to project from the visceral ganglia provide extensive innervation to the mantle tissues. Sagittal micrographs indicate SCP labeled axons are located in the viscero-accessory connectives (VAC, Chapter 2), the mantle nerves (MN, Chapter 2) and the ventral osphridial nerves (VON, Chapter 2) (Figure 18 and 20). In nearly all cases, confocal images did not reveal the presence of SCPergic perikarya within the accessory ganglia; however, one exception was detected in a very

Figures 14 – 17: Peripheral SCPergic axons associated with the apical and pedal ganglia in mature *C. virginica* larvae.

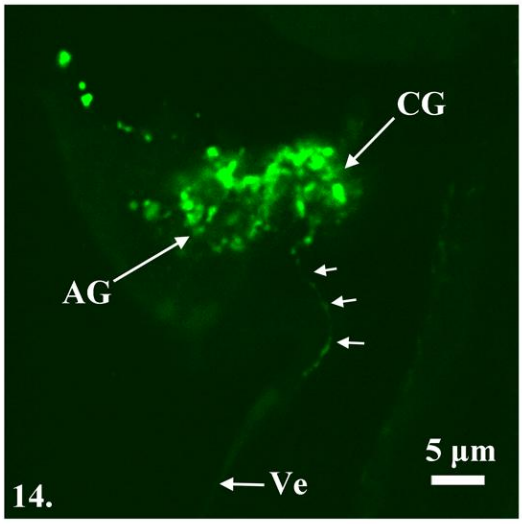
Figure 14: Sagittal view of the apical and cerebro-pleural ganglia indicating the ventral SCPergic axon which extends into the ventral velum.

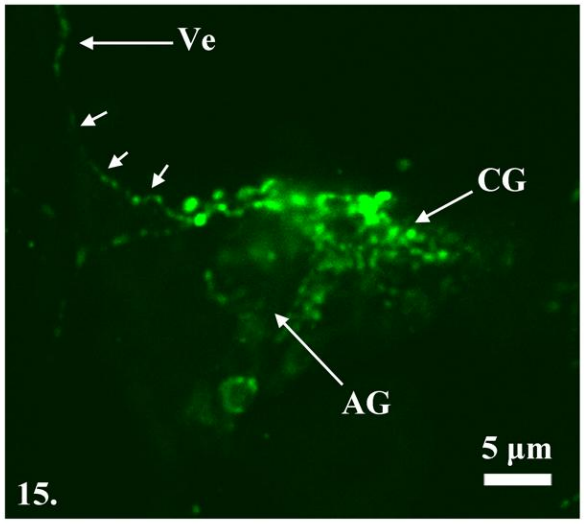
Figure 15: Micrograph depicting SCPergic innervation of the dorsal portion of the velum.

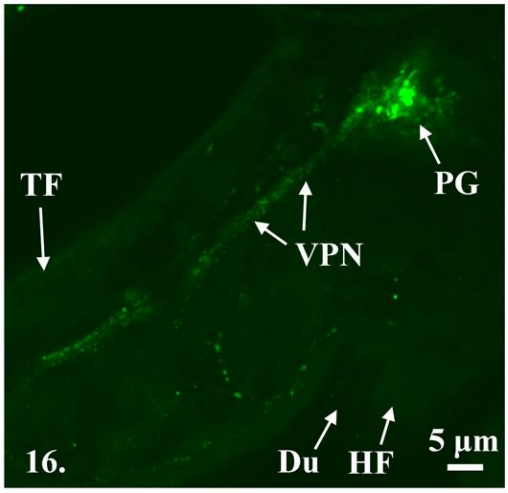
Figure 16: Sagittal view of SCP positive labeling in the ventral pedal nerve that innervates the larval foot of a pediveliger larva.

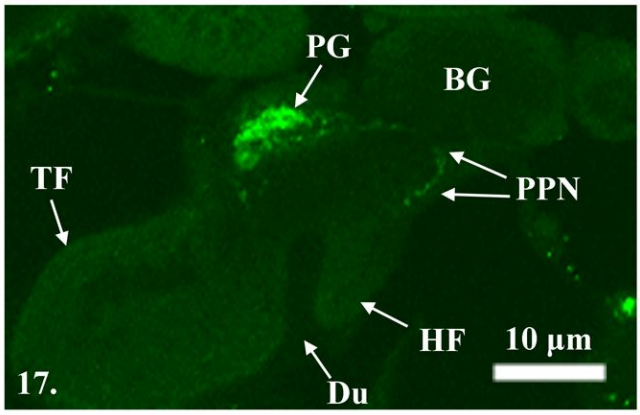
Figure 17: SCPergic labeling of the posterior pedal nerve (in a sagittal view) that extends posterior and then dives downward towards the heel of the foot.

AG = apical ganglion; * = the ventral and dorsal SCPnergic velar innervations; BG = byssal gland; CG = cerebro-pleural ganglion/a; Du = posterior duct; HF = heel of foot; PG = pedal ganglion/a; PPN = posterior pedal nerve; TF = toe of foot; VG = visceral ganglion/a; VPN = ventral pedal nerve


CG
AG
5 µm
Ve
14.


Ve
CG
AG
5 µm
15.


TF
PG
VPN
Du
HF
5 µm
16.


PG
BG
TF
PPN
HF
10 µm
17.
Du

Figures 18 – 21: SCPnergic processes associated with visceral and accessory ganglia in eyed and pediveliger larvae of *C. virginica*.

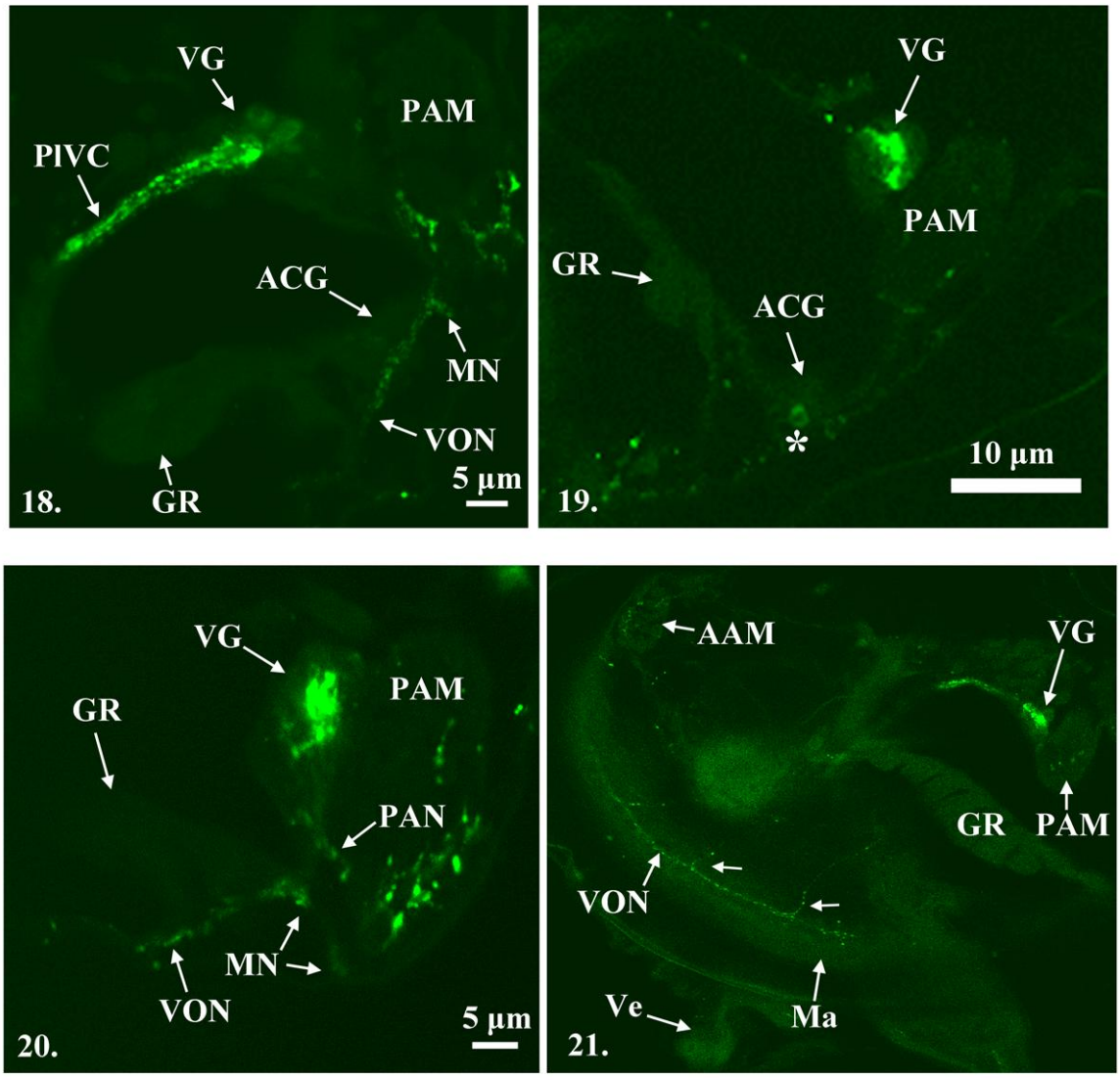
Figure 18: Sagittal view of the visceral and accessory ganglia in an eyed larva indicating the SCP positive labeling of the mantle nerve and the ventral osphradial nerve.

Figure 19: SCP positive labeling of a single neuron in the accessory ganglion of a very mature pediveliger larva.

Figure 20: Sagittal view of the SCPergic posterior adductor nerve in an eyed larva.

Figure 21: Micrograph depicting the SCP positive innervation of the mantle tissue that originates from the ventral osphradial nerve in a pediveliger larva. This nerve eventually supplies SCPergic axons to the anterior adductor nerve.

AAM = anterior adductor nerve; ACG = accessory ganglion/a; arrows = SCPergic branches of the ventral osphradial nerve; * or asterisk = SCP neuron cell bodies; GR = gill rudiment; Ma = mantle; MN = mantle nerve; PAM = posterior adductor nerve; PAN = posterior adductor nerve; PlVC = pleuro-visceral connective; Ve = velum; VG = visceral ganglion/a; VON = ventral osphradial nerve.


VG
PAM
PIVC
ACG
MN
VON
5 µm
18.
GR
VG
PAM
GR
ACG
*
10 µm
19.
VG
PAM
GR
PAN
MN
VON
5 µm
20.
AAM
VG
GR
PAM
VON
Ve
Ma
21.

mature pediveliger where 1-2 neurons in each accessory ganglion exhibited SCP labeling (Figure 19).

Serial optical sections revealed that mantle SCPergic innervation arises from two nerves on both the right and left sides of the larva: a single axon in the mantle nerve and the ventral osphridial nerve (Figure 20). Each ventral osphridial nerve extends anterior along the mantle tissue and eventually projects into the anterior adductor muscle (AAM). Along the way, small SCPergic processes branch from the VONs (Figure 21, arrows). The SCPergic axons in the mantle nerves dive ventrally to innervate the associated mantle tissue surrounding the mantle cavity (see Movie 4 in Appendix A).

The musculature of newly eyed and pediveliger larvae labeled strongly for SCPs, particularly the adductor muscles. The posterior adductor muscle (PAM) receives SCP innervation from the posterior adductor nerves (PAN, Chapter 2), which extend from each visceral ganglion to the base of the PAM at the region of muscular attachment to the larval shell (Figure 22). Proceeding more ventral, SCP labeling at the base of the PAM increases and SCPergic processes run parallel to the muscle cells (Figure 23). A similar network of SCP-like labeling is seen in the anterior adductor muscle, except that innervation occurs through the mantle nerves. Furthermore, faint SCP labeling occurs in the bundles of velar and pedal retractor muscles (RM) (Figure 24). The origin of retractor muscle innervation could not be determined.

Isolated SCP Neurons in Oyster Larvae

Although the majority of SCP positive structures in the peripheral nervous system could be tracked to specific CNS structures, a few neuron cell bodies were noted to reside

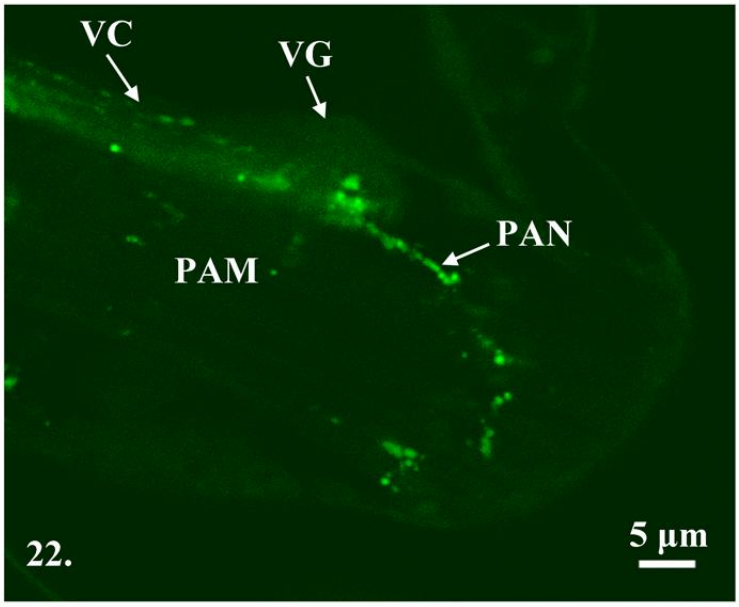
Figure 22 -24: Confocal micrographs depicting SCP labeling associated with various musculatures in both eyed and pediveliger *C. virginica* larvae.

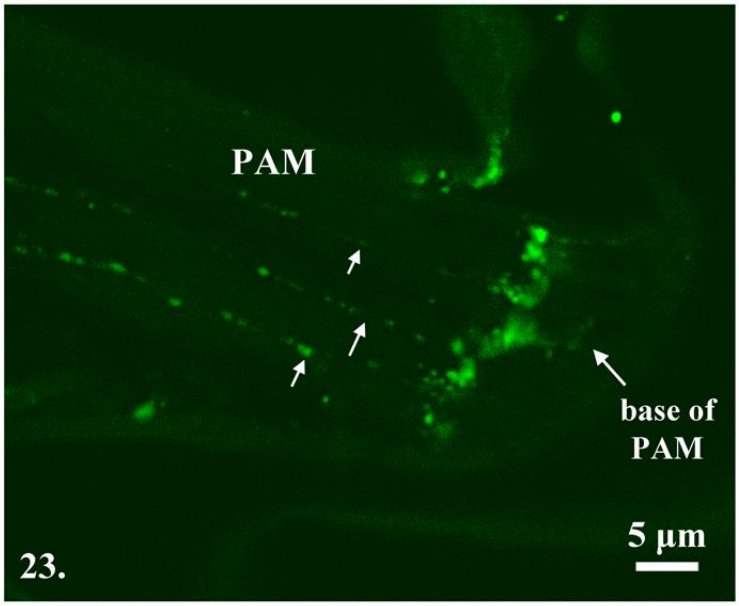
Figure 22: Frontal view of the posterior adductor nerve extending from a visceral ganglion. Note that the SCPergic posterior adductor nerve innervates the base of the posterior adductor muscle.

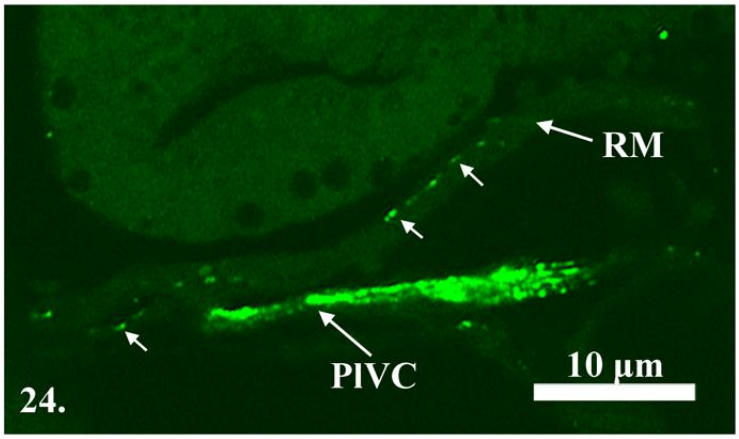
Figure 23: Continued confocal sectioning from the larva in Figure 22, which indicates several small SCPergic neurites parallel to individual muscle fibers.

Figure 24: Sagittal view of the SCP innervation of a retractor muscle.

arrows = SCPergic neurites; PAN = posterior adductor nerve; PAM = posterior adductor muscle; PlVC = pleuro-visceral commissure; RM = retractor muscle; VC = visceral commissure; VG = visceral ganglion


VC
VG
PAN
PAM
22.
5 µm


PAM
base of
PAM
5 µm
23.


RM
PlVC
10 µm
24.

apart from the central ganglia. For instance, in frontal micrographs, two caudal, SCP staining neurons (CaNs) are observed on either side of the distal intestine or rectum (R) (Figure 25). In some newly eyed larvae only one neuron can be detected. In contrast, all pediveliger larvae have four distinct SCPergic cell bodies that each extend an axon into the posterior visceral commissure (PVC, Chapter 2), which is not labeled for SCPs along the entire length (Figure 26). These neurites ultimately connect with the visceral ganglia. There was no evidence that these neurons directly associate with the accessory ganglia. The caudal neurons are also evident from the sagittal point of view, where they can be seen positioned posterior and ventral to the visceral commissure and posterior adductor muscle (Figure 27).

Two additional individual SCPergic neurons are directly associated with the left and right sides of the larval heart (Ha). The presence and strength of apparent fluorescence of these neuron perikarya increases from the newly eyed to the pediveliger stage (Figure 28 = newly eyed; Figure 29 = pediveliger). Thin SCPergic neurites extend between the heart neurons (HN) and the pleural-visceral connective near the area where the pleural-pedal connective dives toward the pedal ganglia (see Movie 4 in Appendix A). Even though the HNs are in close contact with the developing heart, there was no evidence of SCP labeling within heart tissues.

Lastly, extensive SCP neuronal innervation occurs along the length of the larval esophagus. Labeling of the esophagus is consistent in both newly eyed and pediveliger life stages. Here, SCP positive, varicose fibers are limited to the outer epithelium and extend from the mouth to the point of attachment to the stomach (Figures 30-32, arrows). SCPergic processes are not present in the stomach or digestive diverticula. Four SCP

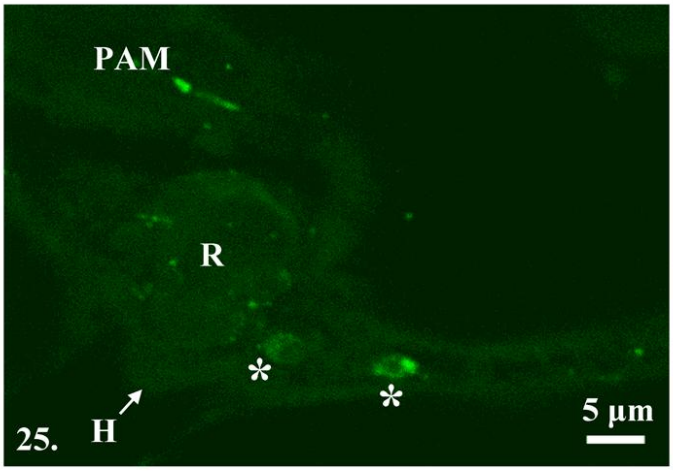
Figures 25 – 27: SCPergic neurons associated with the posterior visceral commissure in *C. virginica* larvae.

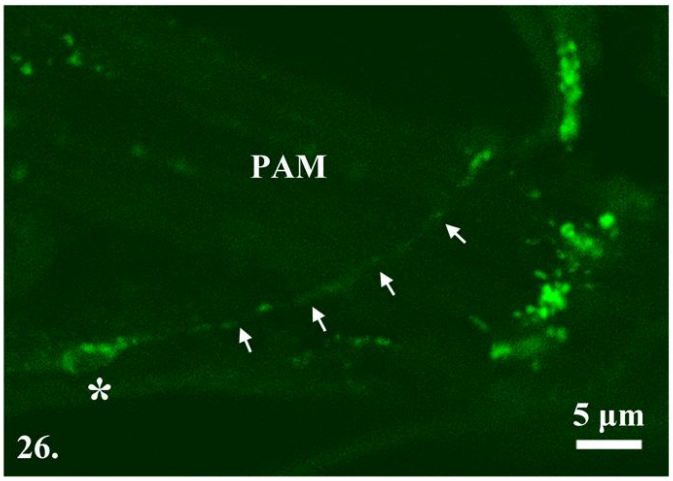
Figure 25: Frontal view of 2 SCP positive caudal neurons located in close proximity to the posterior visceral commissure.

Figure 26: Frontal micrograph depicting an axon connection via the caudal neuron to the visceral ganglion (not depicted, but would be seen in subsequent serial optical sections).

Figure 27: Sagittal view of the SCPergic caudal neurons.

arrows = caudal neuron axon; * = SCP neuron cell bodies; H = hinge; PAM = posterior adductor muscle; R = rectum; VC = visceral commissure


PAM
R
H
5 µm
25.


PAM
5 µm
26.

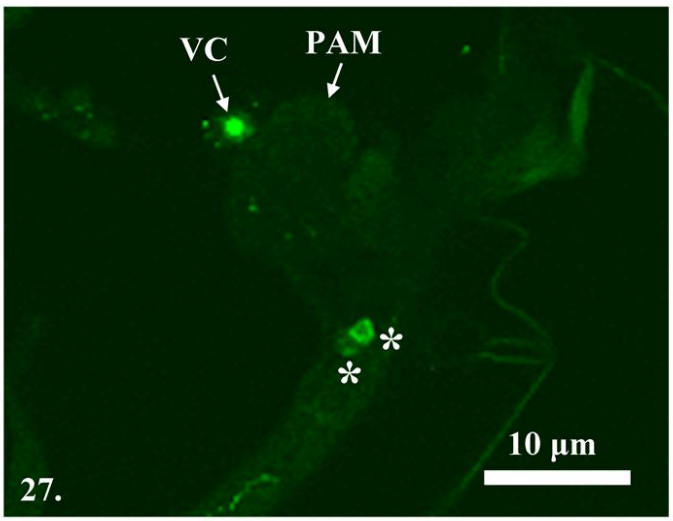
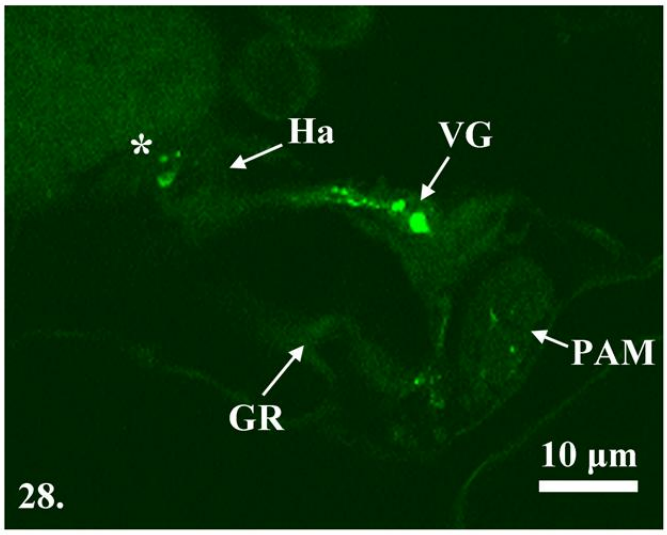

VC
PAM
10 µm
27.

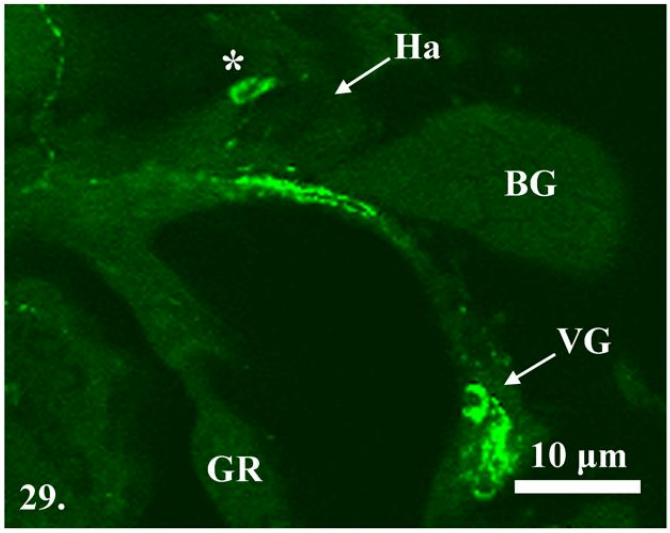
Figure 28 and 29: SCP labeling of two neurons in close proximity to the larval heart in *C. virginica* eyed and pediveliger larvae.

Figure 28: Sagittal view of a newly eyed larva indicating the presence of one of the two weakly labeling heart neurons.

Figure 29: Sagittal view of a heart neuron in a pediveliger larva. Note that the neuron now labels strongly for SCP.

* = SCP neuron cell bodies; BG = byssal gland; GR = gill rudiment; Ha = larval heart; PAM = posterior adductor muscle; VG = visceral ganglion/ia


*
Ha
VG
PAM
GR
10 µm
28.


*
Ha
BG
VG
GR
10 µm
29.

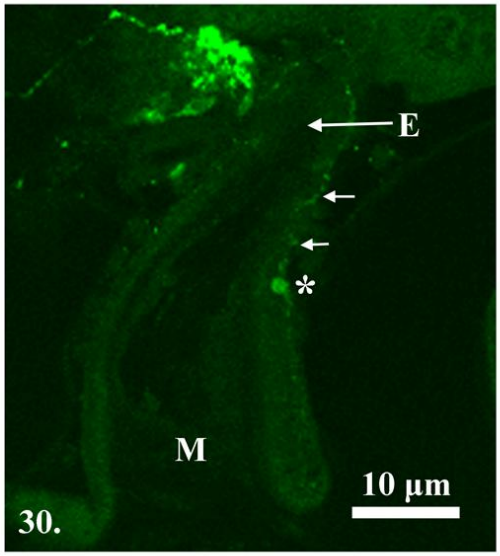
Figures 30 – 33: SCPnergic neurons and processes associated with the larval esophagus in *C. virginica* larvae.

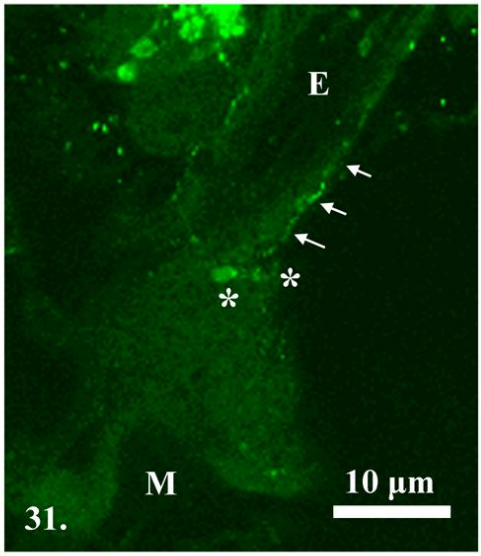
Figure 30 and 31: Sagittal confocal micrographs indicating the presence of SCPergic neurons along the posterior region of the oral / esophageal junction. Only three neurons are shown, but a total of 4 cells are present.

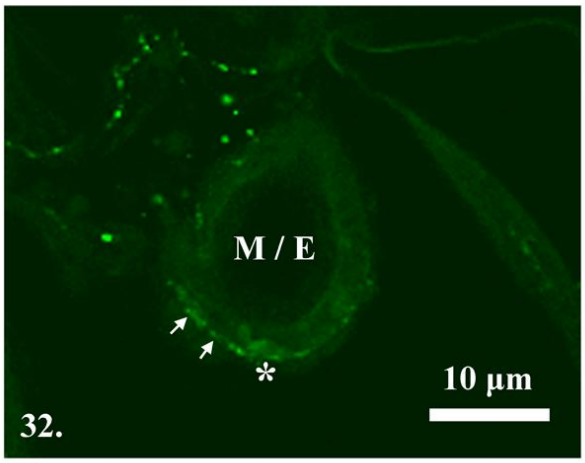
Figure 32: Frontal view of one esophageal neuron, which also shows SCPergic processes that innervate the periphery of esophageal epithelium.

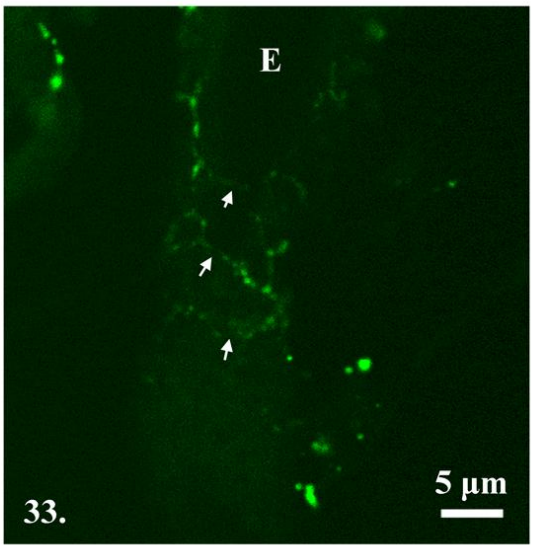
Figure 33: Sagittal view of the SCPergic neural network that encompasses the larval esophagus.

arrows = SCPnergic process innervating the larval esophagus; * or asterisk = SCP neuron cell bodies; E = esophagus; M = mouth; M / E = oral / esophageal junction


E
M
10 µm
30.


E
M
10 µm
31.


M / E
10 µm
32.


E
5 µm
33.

labeled neurons, the esophageal neurons (ENs), are found at the posterior region where the mouth meets the esophagus (oral / esophageal junction) (Figure 30 and 31). No SCP labeled cell bodies were identified in the anterior region of the oral / esophageal junction (Figure 32). Furthermore, the esophageal nerves, along with other SCPergic processes, project processes that coalesce to form a complex neurite network (arrows) around the entire circumference and length of the esophagus (Figure 33).

SCP-like Neuropeptides in Newly Hatched Berghia verrucicornis

The presence and locations of small cardioactive neuropeptide-like labeling in newly hatched *B. verrucicornis* show some similarity to that seen in *C. virginica* pediveligers. Both species have a velum and muscular foot (see encapsulated *B. verrucicornis* larva in Figure 34) and therefore SCP innervation could potentially be conserved relative to these structures. Although SCP neurites are seen innervating areas of a number of peripheral tissues, such as the larval foot, *B. verrucicornis* newly hatched, competent larvae do not appear to have as extensive an SCPergic peripheral innervation as that seen in *C. virginica*. Sagittal confocal micrographs indicate that SCP innervation is concentrated in the larval CNS (Figure 35). Numerous cells of the cerebral and pedal ganglia stain strongly for SCP-like neuropeptides (Figures 35 - 36). Additionally, there are a few weakly labeled neurons anterior to the cerebral ganglia on both the right and left sides of the larva that may be positioned within the optic ganglia or a portion of the apical ganglion. A plethora of brightly labeled varicose fibers extend through the neuropils, commissures and connectives associated with both cerebral and pedal ganglia. Furthermore, two to three neurons on the left and right sides that appear to be associated

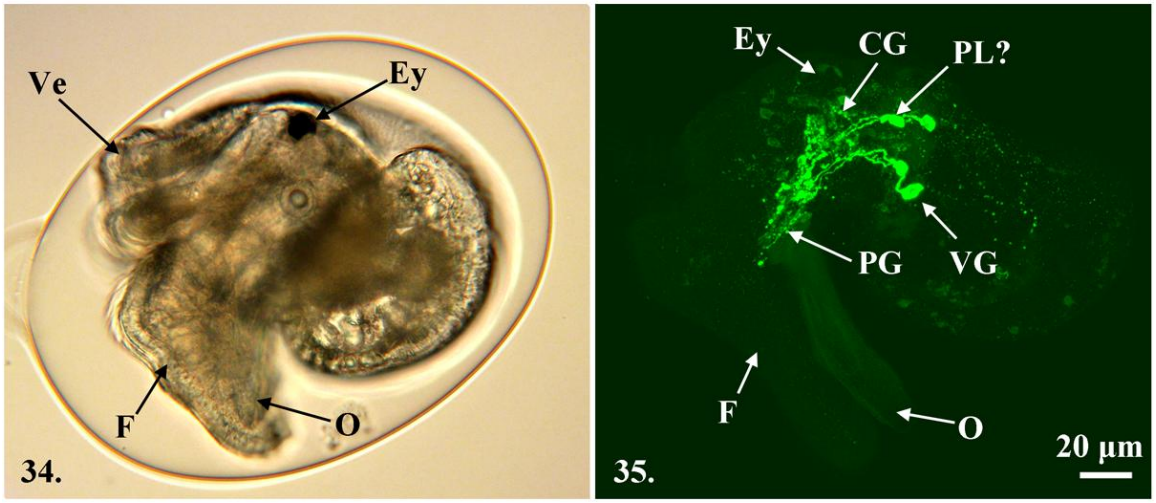
Figures 34 – 36: Light and Confocal micrographs of newly hatched *B. verrucicornis* larvae.

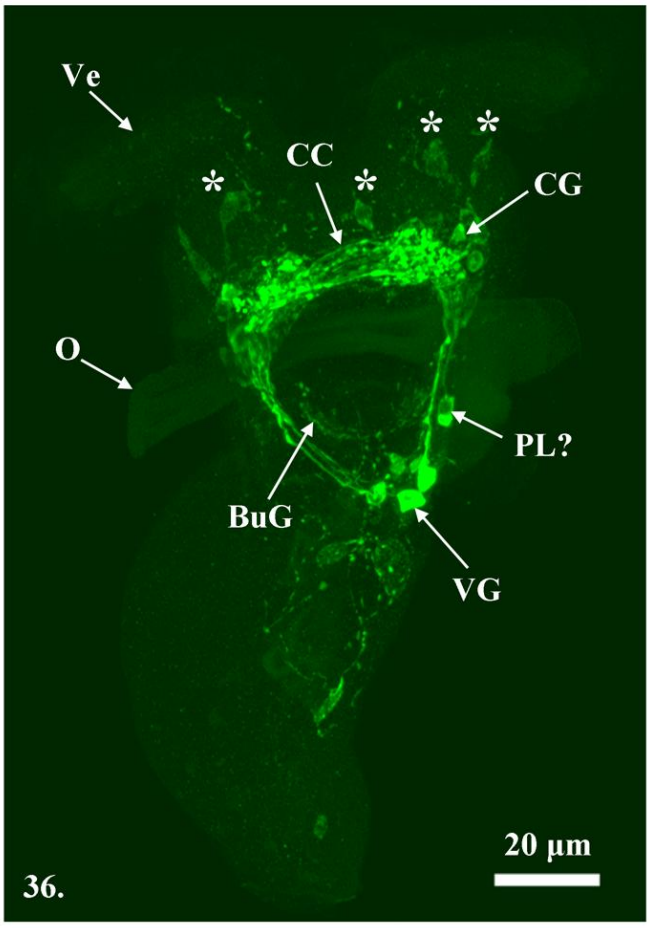
Figure 34: Light micrograph of a sagittally oriented newly hatched larva.

Figure 35: A sagittal maximum intensity projection of SCP labeling in the newly hatched larva.

Figure 36: Frontal maximum intensity projection of SCPergic neurons and processes. Note the weakly staining cells in the anterior region of the larvae that are thought to be present in either the optic or apical ganglia.

BuG = buccal ganglion/a; CC = cerebral commissure; CG = cerebral ganglion/a; Ey = eye; F = foot; O = operculum; PG = pedal ganglion/a; PL? = pleural ganglion/a; Ve = velum; VG = visceral ganglion/a





with the pleuroviseral loop exhibit significant SCP labeling (Figure 36). The exact location / ganglion where each of these neurons resides could not be determined solely

from immunofluorescent labeling; however these cells are likely present in the pleural, visceral and possibly the osphradial ganglia. Histological section sets with horseradish peroxidase staining (DAB kit) are needed to follow and confirm the location of these neurons.

Discussion

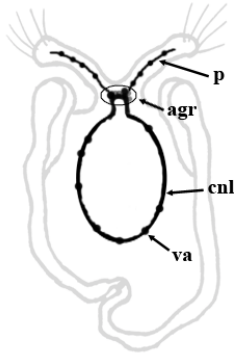
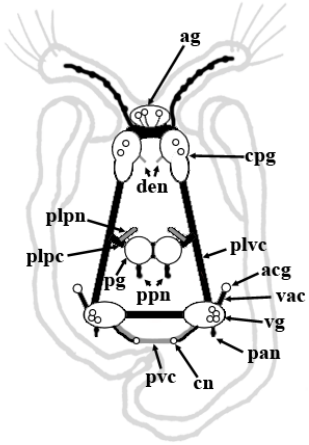
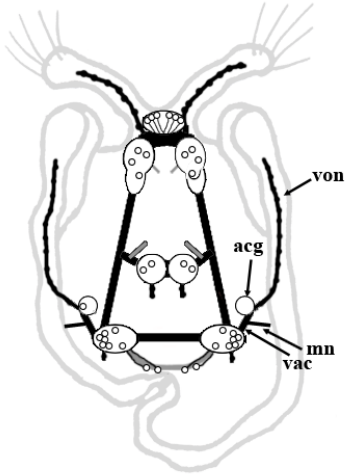
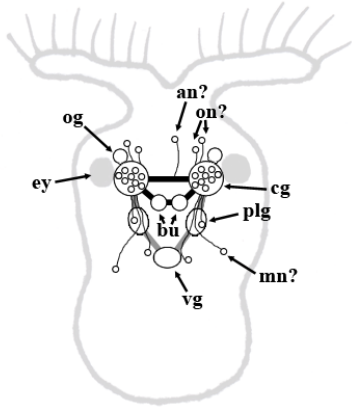
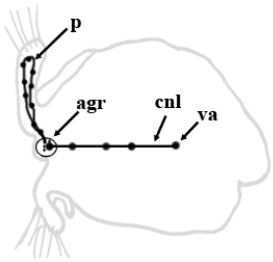
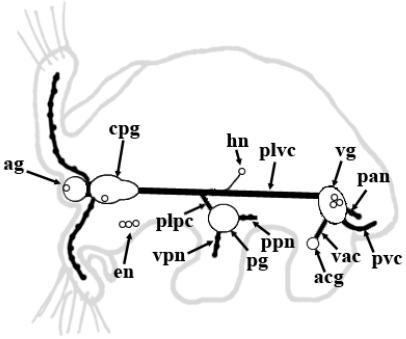
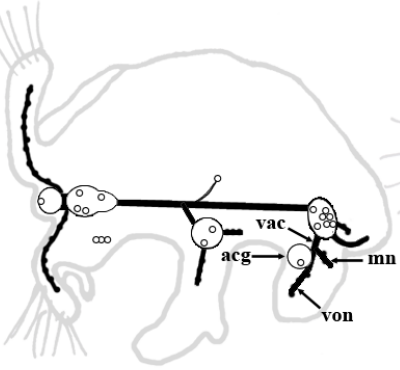
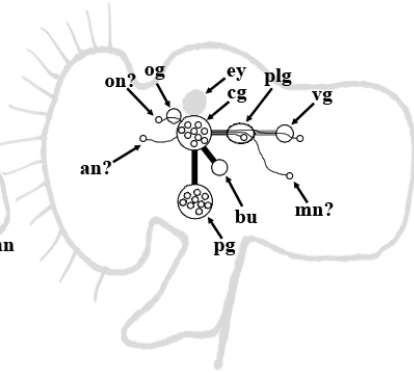
The presence of small cardioactive peptides in the adult bivalves *Crassostrea virginica, Dinocardium robustum*, and *Mercenaria mercenaria*, have been previously documented in the cerebral, pedal and visceral ganglia of the central nervous system (Candelario-Martinez et al., 1993). Additionally, SCPergic processes innervated the rectum and gills, and exhibited very weak fluorescence in the heart (Candelario-Martinez et al., 1993). The present study not only corroborates similar occurrence of SCPs in bivalve larvae, but also extends our understanding of SCP-like neuropeptides throughout the life history of *Crassostrea virginica*.

Immunohistochemical analyses revealed that SCP-like neuropeptides appear in all larval stages (Figure 37). D-hinge larvae have limited varicose SCP labeling in the anterior gangliar rudiment and the central nervous system loop. The presence of a single SCPergic process was also detected innervating the dorsal portion of the velum. No labeled neuron perikarya could be detected at the D-hinge stage. This suggests that SCP synthesis either initially occurs in the neuronal processes or that SCPs synthesized in the

Figure 37: Overview diagram of SCP labeling in *C. virginica* and *B. verrucicornis* larvae. Frontal and sagittal views depicting the progression of SCP innervation through

larval ontogeny are drawn for the D-hinge, eyed, and pediveliger larvae of *C. virginica* and the newly hatched (competent) larva of *B. verrucicornis*. Large outlined white structures indicate ganglia, small white circles indicate SCPergic neuron cell bodies, grey lines indicate non-labeled neural processes and black lines denote SCPergic neural processes. Note that SCPergic processes (easily seen in the D-hinge stage) have a stippled appearance to mimic the presence of varicosities within processes. Structures pointed out in the eyed stage were only labeled at the pediveliger stage if there was a noticeable change in the structure. In *B. verrucicornis* larvae, it was difficult to determine which ganglion or tissue structure several fluorescent cells were located in; therefore these cells are labeled with a proposed location indicated by a "?". acg = accessory ganglion/a; ag = apical ganglion; agr = anterior gangliar rudiment; an? = possible apical nerve within the apical ganglion; bu = buccal ganglion/a; cg = cerebral ganglion/a; cn = caudal nerve; cnl = central nervous system loop; cpg = cerebro-pleural ganglion/a; den = dorsal esophageal nerve; en = esophageal neuron; ey = eye; ha = heart neuron; mn = mantle nerve; mn? = possible mantle neuron; og = optic ganglion/a; on? = possible optic neuron; p = velar process; pan = posterior adductor nerve; plg = pleural ganglion/a; plpc = pleuro-pedal connective; plpn = pluero-pedal nerve; plvc = pleuro-visceral connective; ppn = posterior pedal nerve; pvc = posterior visceral commissure; va = varicosity; vac = viscero-accessory connective; vg = visceral ganglion/a; von = ventral osphridial nerve

C.virginica | *B. verrucicornis*

	D-hinge	Eyed	Pediveliger	Newly hatched
Frontal view				
Sagittal view				

neuron perikarya are rapidly transported into the neuronal processes and do not accumulate in the perikarya until later larval stages.

In contrast, newly eyed and pediveliger larvae have significant SCP labeling throughout the ganglia, commissures and connectives of the central nervous system. A plethora of SCPergic neurites also innervates peripheral tissues including the velum, mantle, foot, esophagus and various musculatures. Furthermore, the number of SCP labeled neurons and axons in the central ganglia, commissures and connectives increases as the larva develops from the newly eyed to the pediveliger stage of development. These findings indicate an observable correlation between the extent of SCP labeling and larval development. A similar trend was documented in *T. diomedea* larvae (Kempf et al., 1987). Interestingly, SCP innervations in *C. virginica* larvae are found in several strictly larval structures, such as the velum, foot, and anterior adductor muscle that degenerate during metamorphosis, indicating that at least some SCP positive innervation serves specific functions during larval life.

This study also reveals the first SCPergic innervation of the apical ganglion. The apical ganglion is hypothesized to have sensory capabilities that may detect metamorphic environmental chemical cues (Bonar, 1978; Chia and Koss, 1984). Recent work by Hadfield et al. (2000) has shown this to be the case in competent larvae of the opisthobranch, *Phestilla sibogae*. Additionally, investigators have turned their attention to comparing the structure of the apical ganglion of various species in order to make hypotheses concerning molluscan evolutionary trends (Page and Kempf, 2009; Kristof and Klussmann-Kolb, 2010).

As larvae of *C. virginica* developed toward competence, an increase in the number of SCPergic neurons, as well as axonal processes associated with these neurons was observed in the apical ganglion of *C. virginica* larvae suggesting that SCPs may have a functional role in modulating the metamorphic event. A sensory role for SCPergic neurons in the apical ganglion is also possible. A study by Ohsuga et al. (2000) supports this hypothesis by documenting the presence of 2 SCPergic, sensory, hair cells lining the statocysts in adult *Pleurobranchea japonica*. Unfortunately, literature concerned with the presence of SCPs in the molluscan apical ganglia is limited. Although two previous analyses have been conducted, SCPergic neurons or axons are not documented in the apical ganglion of gastropod larvae *T. diomedea* (Kempf et al., 1987), and *M. leonina* (Kempf and Page, 2005). In this study, a single SCPergic neuron might lie in the apical ganglion of newly hatched *B. verrucicronis* (see Figure 36). This potentially provides two species (*C. virginica and B. verrucicornis*) that may serve as organisms for comparative investigations of SCPergic innervations in the apical ganglion. For now, the possible role of SCPs in bivalve metamorphosis must remain a hypothesis that awaits further support by experimental analyses.

Previous literature in adult molluscs proposes that SCPs aid in gut motility and feeding (Candelario-Martinez et al., 1993; Perry et al., 1993). In *C. virginica* larvae, SCPergic peripheral innervation includes a complex network along the length of the esophagus. SCPergic neuron perikarya are also located on either side of the rectum. These results suggest that SCPs may also function in modulating muscles associated with the larval gut. The possible role of SCPs in modulating muscle contraction is also supported by the presence of SCP positive axonal processes in tissues of the adductor

muscles, foot and retractor muscle. In addition, SCP labeling along the margin of the velar lobe is consistent with the labeling of striated musculature (personal observation using a *C. virginica* muscle specific (3b5) antibody developed in our laboratory). Therefore, during the larval stage of *C. virginica*, it would appear that SCPs are not exclusively limited to modulating gut motility, but that they are more generally associated with larval musculature and serve muscle related function throughout the entire organism.

Perhaps, in the case of SCPergic innervations of the esophagus and velum in *C. virginica* larvae, an alternative function could be proposed. Previous research provides evidence that SCPs modulate (directly or indirectly) an increase in ciliary beating (Gainey et al., 1999; Willows et al., 2000). Therefore, due to presence of cilia associated with the tissues of both these structures, SCPs may act to regulate ciliary activity. Dual functions modulating both ciliary beat and muscle contractility are also possible.

Two SCPergic neurons were identified in close association with the larval heart. Although adult *Helix apsersa* heart tissues are documented to be void of SCPs (Lloyd, 1978), these neuropeptides have been documented to elicit cardio-excitatory responses in adult *Helix aspersa* and *Aplysia californica* (Lloyd 1978; 1980, 1985b). Direct SCPergic innervation of the larval heart was not detected in this study; however there is an SCPergic process that connects each heart neuron to the central nervous system loop. Potentially, two scenarios may explain the association between SCPs and the larval heart: (1) these neurons actually receive stimulation from surrounding visceral organs and relay information to the larval central nervous system or (2) the processes are actually axons that extend from other SCPergic neurons in the CNS and synapse on the heart neurons. In

second scenario, the heart neurons would actually have a neurosecretory function that could possibly release SCP into the heart musculature where it could have an excitatory effect.

From this study, small cardioactive peptides or similar molecules were found abundantly in larval tissues of *C. virginica* and are likely to modulate the activities of muscle and/or cilia. Although the location of SCP innervations assists in making hypothetical conclusions, we actually know very little about the exact functions of SCPs in molluscan larvae. Research detailing the larval effects of SCP-like neuropeptides, similar to those of gastropod and bivalve adults described above, is needed to elaborate our understanding of SCPs function and how it relates to critical larval behaviors, such as feeding, settlement and metamorphosis. Furthermore, continued examination of SCPs function(s) in larval stages may give insight onto the role of SCPs throughout the entire life history of mollusks. This could potentially reveal a common trend in functions or variations in functionality at different points along a developmental timeline.

Conclusions

The results of this thesis extend the work of earlier investigators and demonstrate that the larval nervous system of bivalves is even more complex than previously thought. *C. virginica* D-hinge larvae exhibit limited neurogenesis; however as competency is reached (pediveliger larval stage) the central nervous system is comprised of a single apical and paired cerebro-pleural, pedal, visceral and accessory ganglia. Each pair of ganglia, except for the accessory ganglia, has a commissure between them. Interestingly, the visceral ganglia possess an additional commissure, the posterior visceral commissure, that runs underneath the posterior adductor muscle and behind the anus / rectum of the larva. In addition, pleuro-visceral, pleuro-pedal and viscero-accessory connectives can be identified histologically.

Peripheral nerves extending from the pedal ganglia, including the posterior pedal and two ventral pedal nerves, innervate the larval foot. The posterior adductor nerves that originate from the visceral ganglia innervate the posterior adductor muscle. Also, several other neural processes can be seen extending from the central nervous system, but the actual target tissues could not be confirmed by means of light microscope, histological sectioning. These nerves are tentatively identified as the dorsal esophageal, mantle and ventral osphradial nerves based on their trajectories within the larva.

Immunohistochemical labeling for small cardioactive-like peptides revealed that SCPs are present early in nervous system development (D-hinge stage) and that the neural circuitry utilizing these peptides increases in complexity as the larva develops to

competency. This coincides with the increase in complexity of the larval nervous system as described above. SCPergic neurons and / or processes were present throughout the central nervous system, except for the accessory ganglia. In addition, peripheral nerves including the posterior pedal, ventral pedal, posterior adductor, mantle and ventral osphridial nerves contained axons that labeled for SCPs. These nerves along with other SCPergic processes innervated the velum, foot, mantle, esophagus and various musculatures. Lastly, two putative SCPergic neurons were identified in close proximity to the larval heart and four putative SCPergic neurons were seen to be associated with the esophagus at the oral / esophageal junction. SCPergic neurons and / or processes in newly hatched and competent *B. verrucicornis* larvae are present throughout the entire central nervous system, although further investigations are needed in order to identify the specific location of several SCPergic neurons.

Overall, the location of SCPs in *C. virginica* larvae indicate that SCPs are likely to modulate muscle function and possibly ciliary activities. These findings are consistent with the proposed and demonstrated functions of SCPs in adult molluscs, which suggest SCPs are functioning similarly in both larval and adult stages of development. However, the presence of SCPergic neurons and processes in the apical ganglion of larval C. virginica and B. verrucicornis indicate that SCPs also play an as yet unknown role that is purely larval in nature.

Literature Cited

Abbott, N. J., R. Williamson, and L. Maddock. 1995. *Cephalopod Neurobiology*. Oxford University Press, Oxford

Audesirk, G., and T. Audesirk. 1980. Complex mechanoreceptors in *Tritonia diomedea*. I. Responses to mechanical and chemical stimuli. *J. Comp. Physiol.* **141**: 101-109.

Ansell, A. D. 1962. The functional morphology of the larva and the post-larval development of *Venus striatula* (Da Costa). *J. Mar. biol. Ass. U.K.* **42:** 419-443.

Araujo, R., and M. A. Ramos. 1998. Description of the glochidium of *Margaritifera auricularia* (Spengler 1793) (Bivalvia, Unionoidea). *Philosophical Transaction of the Royal Society B: Biological Sciences*. **353:** 1553-1559.

Bayne, B. L. 1971. Some morphological changes that occur at the metamorphosis of the larvae *Mytilus edulis*. Pp 259 – 280 in *The Fourth European Marine Biology Symposium*. Cambridge University Press, London.

Beck, J. C., M. S. Cooper, and A. O. D. Willows. 2000. Immunocytochemical localization of pedal peptide in the central nervous system of the gastropod mollusc *Tritonia diomedea*. *The Journal of comparative Neurology.* **425:** 1-9.

Beiras, R., and J. Widdows. 1995. Induction of metamorphosis in larvae of the oyster *Crassostrea gigas* using neuroactive compounds. *Marine Biology.* **123**: 327-334.

Bickell, L. R., and F. S. Chia. 1979. Organogenesis and histogenesis in the

planktotrophic veliger of *Doridella steinbergae* (Opisthobranchia, Nudibranchia). *Marine Biology*. **52:** 291-313.

Bickell, L. R., and S. C. Kempf. 1983. Larval and metamorphic morphogenesis in the nudibranch Melibe leonine (Mollusca, Opisthobranchia). *Bio. Bull.* **165:** 119-138.

Blake, N. J. and S. E. Shumway. 2006. Bay Scallop and Calico Scallop Fisheries, Culture and Enhancement in Eastern North America. Pp 945-961 in *Scallops: Biology, Ecology and Aquaculture*. Elsevier, San Diego.

Bonar, D.B. 1978. Morphogenesis at metamorphosis in opisthobrach molluscs. Pp 165-175 in *Settlement and Metamorphosis of Marine Invertebrate Larvae*. Elsevier, New York.

Bonar, D. B, S. L. Coon, M. Walch, R. M. Weiner, and W. Fitt. 1990. Control of oyster settlement and metamorphosis by enogenous and exogenous chemical cues. *Bull. Mar. Sci.* **46:** 484-498.

Brach, J. 1960. Observ. CCIII, Deovis Ostrcorum. Ephemeidan Academia Leopoldina Naturae Curiosorum, VIII: 506-508.

Brooks, W. K. 1905. *The Oyster*. The Johns Hopkins Univ. Press, Baltimore.

Brusca, R. C. and G. J. Brusca. 2003. *Invertebrates 2nd edition*. Sinauer Associates, Inc, Publishers, Sunderland.

Bullock, T. H. and G. A. Horridge. 1965. Mollusca: Pelecypoda. Pp 1390 – 1431 in *Structure and function in the nervous systems of invertebrates. Vol II*. W.H. Freeman and Company, San Francisco.

Candelario-Martinez, A., D. M. Reed, S. J. Prichard, K. E. Doble, T. D. Lee, W. Lesser, D. A. Price, and M. J. Greenburg. 1993. SCP- related peptides from

bivalve mollusks: identification, tissue distribution and actions. *Biol. Bull.* **185:** 428 - 439.

Callaway J. C., B. Masinovsky, and K. Graubard. 1987. Co-localization of SCP_B-like and FMRFamide-like immunoreactivities in crustacean nervous systems. *Brain Research.* **405:** 295–304

Carroll, D., and S. C. Kempf. 1990. Laboratory culture of the aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): some aspects of its development and life history. *Biol. Bull.* **179:** 243-253.

Carroll, D., and S. C. Kempf. 1994. Changes occur in the central nervous system of the nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia) during metamorphosis. *Biol. Bull.* **186:** 202-212.

Carroll, M. A., and E. J. Catapane. 2007. The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology, Part A*. **148:** 445-450.

Carter, G. S. 1926. On the nervous control of the velar cilia of the nudibranch veliger larva. *J. Exper. Biol.* **4:** 1-26.

Chia, F. S. and R. Koss. 1984. Fine structure of the cephalic sensory organ in the larva of the nudibranch *Rostana pulchra* (Mollusca, Opisthobranchia, Nudibranchia). *Zoomorphology.* **104:** 131-139.

Cloney, R. A. and E. Florey. 1968. Ultrastructure of cephalopod chromatophore organs. *Z. Zellforesch. Mikrosk. Anat*. **89:** 250-280.

Cooper, K. 1982. Potential for application of the chemical DOPA to commercial bivalve setting systems. *J. Shellfish Res.* **3:** 110-111.

Costello, D. P., and C. Henley. 1971. *Methods for obtaining and handling marine eggs and embryos.* Woods Hole, MA.

Croll, R. P., D. L. Jackson, and E. E. Voronezhskaya. 1997. Catecholamine-containing cells in larval and post larval bivalve mollscs. *Biol Bull*. **193**: 116-124.

Culliney, J. L. 1974. Larval Development of the Giant Scallop *Placopecten Magellanicus* (Gmelin). *Biol. Bull*. **174:** 321-332.

Da Costa, F., S. Darriba, and D. Marinez-Patino. 2008. Embryonic and larval development of *Ensis arcuatus* (Jefferys, 1865) (Bivalvia: Pharidae). *Journal of Molluscan Studies*. **72:** 103-109.

Elston, R. 1980. Functional anatomy, histology, and ultrastructure of the soft tissues of the larval America oyster, *Crassostrea virginica*. *Proceedings of the National Shellfisheries Association*. **70:** 65-93.

Erdmann, W. 1935. Untersuchungen uber die Lebensgeschichte der Auster. Nr. 5 Uber die Entwicklung und die Anatomie der "ansatzreifen" Larve von *Ostrea edulis* mit Bermerkungen uber die Lebensgeschichte der Auster. Wissenschaftliche Meeresuntersuchungen herausgegeben von der Kommission zur wissenschaftlichen Untersuchung der deutschen Meere in Kiel und der Biologischen Anstalt auf Helgoland, Neue Folge, Band 19, Abteilung Helogland, Heft 3, Abhandlung Nr. 6, pp 1-25.

Evans, B. D. and R. L. Calabrese. 1989. Small cardioactive peptide-like immunoreactivity and it's colocalization with FMRFamide-like immunoreactivity in the central nervous system of the leech, *Hirudo medicinalis*. *Cell and Tissue Research.* **257:** 187-199.

FAO. 2007. Summary Tables of Fishery Statistics. Statistics. http://www.fao.org/fishery/statistics/en.

Flyachinskaya, L. P., and E. Y. Kulakovskiy. 1991. Larval development of the bivalve *Mytilus edulis* (Mytilida, Mytilidae). Originically published in *Zoologichskiy zhurnal*. **70:** 23-29.

Foighil, D. O. 1989. Role of spermatozeugmata in the spawning ecology of the brooding oyster *Ostrea edulis*. *Gamete Res.* **24:** 219-228.

Fox, L. E. and P. E. Lloyd. 1997. Serotonin and the dmall cardioactive peptides differentially modulate two motor neurons that innervate the same muscle fibers in *Aplysia*. *The Journal of Neuroscience.* **17:** 6064-6074.

Galtsoff, P. S. 1964. The American oyster. *U.S. Fisheries Bulletin Fish and Wildlife Serv.* 64: 1-480.

Gainey Jr., L. F., K. J. Vininv, K. E. Doble, J. M. Waldo, A. Canderlario-Martinez, and M. J. Greenberg. 1999. An endogenous scp-related peptide that modulates ciliary beating in the gills of a venerid clam, *Mercenaria mercenaria*. *Biol Bull.* **197:** 159-173.

Gros, O., L. Frenkiel, and M. Moueza. 1997. Embryonic, larval, and post-larval development in the symbiotic clam *Codakia orbicularis*. *Invertebrate Biology*. **116:** 86-101.

Gustaffsson, M. K. S., and M. C. Wikgren. 1989. Development of immunoreactivity to the invertebrate neruopeptide small cardiac peptide B in the tapeworm *Diphyllobothrium dendriticum*. *Parasitology Research*. **75:** 396-400.

Gutsell, J. S. 1931. Natural history of the bay scallop. *Bulletin of the Bureau of Fisheries (United States bureau of Fisheries)* **46:** 569-632.

Hadfield, M. G., E. A. Meleshkevitch, and D. Y. Boudko. 2000. The apical sensory organ of a gastropod veliger is a receptor for settlement cues. *Biol. Bull.* **198:** 67-76.

Hanlon, R. 1991. Integrative neurobiology and behavior of mollusks symposium: introduction, perspectives, and round-table discussion. *Biol. Bull.* **180:** 197-199.

Harding, J. M., and R. Mann. 2001. Oyster reefs as fish habitat: opportunistic use of restored reefs by transient fishes. *Journal of Shellfish Rearch.* **20:** 951-959.

Harms, W. 1909. Postembryonale Entwicklungsgeschichte der Unioniden. *Zool Jahrb Abt Anat Ont Tiere*. **28:** 325-386.

Haszprunar, G., S. Friedrich, A. Wanninger, and B. Ruthensteiner. 2002. Fine structure and immunochemistry of a new chemosensory system in the chiton larva (Molluscs: Polyplacophora). *J Morphol.* **251:** 210-218.

Heller, J. 1993. Hermaphroditism in molluscs. *Biological Journal of the Linnean Society*. **48:** 19 - 42.

Hickman, R. W., and L. L. D. Gruffydd. 1971. The histology of the larva of *Ostrea edulis* during metamorphosis. Pp 281-294 in the *Fourth European Marine Biology Symposium*. Cambridge University Press, London.

Hodgson, C. A., and R. D. Burke. 1988. Development and Larval Morphology of the Spiny Scallop, *Chlamys hastate*. *Biol. Bull*. **174:** 303-318.

Hoggarth, M. A. 1999. Descriptions of some of the glochidia of the Unionidae (Mollusca: Bivalvia). *Malacologia.* **41:**1-118.

Horst, R. 1883-1884. The Development of the Oyster (Ostrea edulis). [Translation from Dutch by Herman Jacobson.] United States commission of Fish and Fisheries, Report of the commissioner for 1884, part 12. [1886]: 891-910.

Huxley, T. H. 1883. Oyster and the oyster question. *The English Illustrated Magazine*. **1:** 47-55; 112-121.

Jackson, J. B. C., M. X. Kirby, W. H. Berger, K. A. Bjorndal, L. W. Botsford, B. J. Bourque, R. H. Bradbury, R. Cook, J. Erlandson, J. A. Estes, T. P. Hughes, S. Kidwell, C. B. Lange, H. S. Lenihan, J. M. Pandolfi, C. H. Peterson, R. S. Steneck, M. J. Tegner, and R. R. Warner. 2001. Historical overfishing and the recent collapse of coastal ecosystems. *Science.* **293:** 629-638.

Kanda, A., H. Minakata. 2006. Isolation and characterization of a novel small cardioactive peptide-related peptide from the brain of *Octopus vulgaris*. *Peptides.* **27:** 1755-1761.

Kempf, S. C. 2008. Serotonergic sensory modifications in the apical ganglion during development to metamorphic competence in larvae of the dendronotid nudibranch *Melibe leonia* and *Tritonia diomedea*. *Microscopy Research and Techniques.* **71:** 863-869.

Kempf, S. C., and L. R. Page. 2005. Anti-tubulin labeling reveals ampullary neuron ciliary bundles in opisthobranch larvae and a new putative neural structure associated with the apical ganglion. *Biol. Bull*. **208:** 169-182.

Kempf, S. C., L.R. Page, and A. Pires. 1997. Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *The Journal of Comparartive Neurology*. **386:** 507-528.

Kempf, S. C., B. Masinovsky, and A. O. W Willows. 1987. A simple neuronal system characterized by a monoclonal antibody to SCP neruopeptides in embryos and larvae of *Tritonia Diomedea* (Gastropoda, Nudibranchia). *J Neurobio.* **18:** 217-236.

Kreiling, J. A., K. Jessen-Eller, J. Miller, R. F. Seegal, and C. L. Reinisch. 2001. Early development of the serotonergic and dopaminergic nervous system in *Spisula solidissima* (surf clam) larvae. *Comparative Biochemistry and Physiology Part A.* **130:** 341-351.

Kristof, A., and A. Klussmann-Kolb. 2010. Neuromuscular development of *Aeolidiella stephanieae* Valdez, 2005 (Mollusca, Gastropoda, Nudibrnachia). *Frontiers in Zoology*. **7:** 1-24.

Kuwasawa, L., and R. Hill. 1997. Evidence for cholinergic inhibitory and serotonergic excitatory neuromuscular transmission in the heart of the bivalve *Mercenaria mercenaria*. *Journal of Experimental Biology.* **200:** 2123-2135.

Lane, D. J. W., and J. A. Nott. 1975. A study of the morphology, fine structure and histochemistry of the foot of the pediveliger of *Mytilus edulis* L. Journal of Marine Biological Associations of the UK. **55:** 477-495.

Leewenhoek, A. V. 1965. Arana Naturae detecta. Vol. II, letters 92 and 94.

Lloyd, P. E. 1978. Distribution and molecular characteristics of cardioactive peptides in

the snail, *Helix aspersa*. *J. comp. Physiol.* **128:** 269-276.

Lloyd, P. E. 1979. Central peptide containing neurons modulate gut activity in *Tritonia*. *Neruosci. Abstr.* **5:** 252.

Lloyd, P. E. 1980. Biochemical and pharmacological analyses of endogenous cardioactive peptides in the snail, *Helix aspersa*. *J. Comp. Physiol.* **138:** 265-270.

Lloyd, P. E., A. C. Mahon, I. Kupfermann, J. L. Cohen, R. H. Scheller, and K. R. Weiss. 1985a. Biochemical and immunocytological localization of molluscan small cardioactive peptides in the nervous system of *Aplysica californica*. *The Journal of Neuroscience.* **5:** 1851-1861.

Lloyd, P. E., I. Kumpfermann, and K. R. Weiss. 1985b. Two endogenous neuropeptides (SCPa and SCPb) produce a camp-mediated stimulation of cardiac activity in *Aplysia*. *J. Comp. Physiol A.* **156:** 659-667.

Lloyd, P. E., I. Kumfermann, and K. R. Weiss. 1984. Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in *Aplysia*. *Proc. Natl. Acad. Sci.* **81:** 2934-2937.

Lloyd, P. E., M. Frankfurt, P. Stevens, I. Kumpfermann, and K. R. Weiss. 1987. Biochemical and immunocytological localization of the neuropeptides FMRFamide, SCPa and SCPb, to neurons involved in the regulation of feeding in *Aplysia*. *The Journal of Neuroscience.* **7:** 1123-1132.

Lloyd, P. E. 1989. Peripheral actions of the SCPs in *Aplysia* and other gastropod molluscs. *Am. Zool.* **29:** 1265-1274.

Marios, R., and T. J. Carew. 1997. Fine structure of the apical ganglion and its serotonergic cells in the larva of *Aplysia californica*. *Biol Bull*. **192:** 388-398.

Matsutani, T., and T. Nomura. 1984. Localization of monoamines in the central nervous system and gonad of *Patinopecten yessoensis*. *Bull Jpn Soc Sci.* **50:** 425-430.

Matsutani, T., and T. Nomura. 1986. Serotonin-like immunoreactivity in the central nervous system and gonad of the scallop, *Patinopecten yessoensis*. *Cell and Tissue Research* **244:** 515-517.

Masinovsky, B. C., S.C. Kempf, J.S. Calloway, and A.O.D. Willows. 1988. Monolonal antibodies to the molluscan small cardioactive peptide SCP_B: Immunolabeling of neurons in diverse invertebrates. *J. Comp. Neurol.* **273:** 500 – 515.

Morris, H. R., M. Panico, A. Karplus, P. E. Lloyd, and B. Riniker. 1982. Eludication by FAB-MS of the structure of a new cardioactive peptide from *Aplysia*. *Nature.* **300:** 643-645.

Moueza, M., O. Gros, and L. Frenkeil. 1999. Embryonic, larval, and postlarval development of the tropical clam, *Anomalocardia brasiliana* (Bivavlia, Veneridae). *J. Moll. Stud*. **65:** 73-88.

Moueza, M., O. Gros, and L. Frenkiel. 2006. Embryonic development and shell differentation in *Chione cancellata* (Bivalvia, Veneridae): an ultrastructural analysis. *Invertebrate Biology*. **125**: 21-33.

Murrary, T. J., and J. E. Kirkley. 2005. Economic Activity Associated with Clam Aquaculture in Virginia – 2004. Virginia Institute of Marine Science.

National Research Council. 2004. Nonnative oysters in the Chesapeake Bay. The National Academies Press, Washington D.C.

Nell, J.A. 2001. The history of oyster farming in Australia. *Marine Fisheries Review*. **63:** 14-25.

Nell, J. A. 2002. Farming triploid oysters. *Aquaculture*. **210:** 69-88.

Nestlerode, J. A., M. W. Luckenbach, and F. X. O'Beirn. 2007. Settlement and survival of the oyster *Crassostrea virgininca* on created oyster reef habitats in Chesapeake Bay. *Restoration Ecology*. **15:** 273-283.

Nielsen, C. 2004. Trochophore Larvae: cell-linages, ciliary bands, and body regions. 1. Annelida and Mollusca. *Journal of Experimental Zoology (Mol. Dev. Evol.)* **302B:** 35-68.

O'Bannon, B.K. (Ed.) 1999. Fisheries of the United States, 1998. Current Fishery Statistics No. 9800. NOAA/NMFS. 130p.

Ohsuga, K., and M. Kurokawa. 2000. Mosaic arrangement of SCPb, FMRFamide, and histamine-like immunoreactive sensory hair cells in the statocyst of the gastropod mollusc *Pleurobranchaea japonica*. *Cell Tissue Res.* **300:** 165-172.

Page, L. 1992a. A new interpretation of the nudibranch central nervous system based on ultrastructural analysis of neurodevelopment in *Melibe leonina*. I. Cerebral and visceral loop ganglia. *Biol. Bull*. **182:** 348-365.

Page. L. 1992b. A new interpretation of the nudibranch central nervous system based on ultrastructural analysis of neurodevelopment in *Melibe leonina*. II. Pedal, pleural, and labial ganglia. *Biol. Bull*. **182:** 366-381.

Page, L. R. 1993. Developmental analysis reveals labial and subradular ganglia and the primary framework of the nervous system in nudibranch gastropods. *Journal of Neurobiology*. **24:** 1443-1459.

Page, L. R., and S. C. Kempf. 2009. Larval apical sensory organ in a neritimorph gastropod, and ancient gastropod lineage with feeding larvae. *Zoomorphology*. **128:** 327-338.

Page, L. R., and S. Parries. 2000. Comparative study of the apical ganglion in planktotrophic caenogastropod larvae: ultrastructure and immunoreactivity to serotonin. *The Journal of Comparative Neurology*. **418:** 383-401.

Price, D. A., and M. J. Greenberg. 1977. Structure of a molluscan cardioexcitatory neuropeptide. Science. **197:** 670-671.

Parker, T. J. and W. A. Haswell. 1940. A Text-book of Zoology 6th edition. Macmillan and Co. Limited, London.

Pawlik, J. R. 1990. Natural and artificial induction of metamorphosis of *Phargmatopoma lapidosa californica* (Polychaeta: Sabellariidae), with critical look at the effects of bioactive compounds on marine invertebrate larvae. *Bull. Mar. Sci*. **46:** 512-536.

Perry, S. J., A. C. Dobbins, M. G. Schofield, M. R. Piper, and P.R. Benjamin. 1999. Small cardioactive peptide gene: structure, expression and mass spectrometric analysis reveals a complex patter on co-transmitters in a snail feeding neuron. *Euro. J Neuroscience*. **2:** 655-662.

Piers, A., and M. G. Hadfield. 1991. Oxidative Breakdown products of cateholamines and hydrogen peroxide induce partial metamorphosis in the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opishobranchia). *Bio Bull.* **180:** 310-317.

Raineri, M. 1995. Is a mollusk an evolved bent meta tronchophore? A histochemical investigation of neruogenesis in Mytilus (Mollusca: Bivalvia). *J. Mar. Biol. Ass.*

U.K. **75:** 571-592.

Raineri, M, and M. Ospovat. 1994. The initial development of gangliar rudiments in a posterior position in *Mytilus galloprovincialis* (Mollusca: Bivalvia). *J. Mar. Biol. Ass. U.K.* **74:** 73-77.

Ram, J. L., D. Moore, S. Putchakayala, A. A. Paredes, D. Ma, and R. P Croll. 1999. Serotonergic responses of the siphons and adjacent mantle tissue of the zebra mussel, *Dresissena polymorpha*. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology, and Endocrinology*. **124:** 211-220.

Rees, C. B. 1950. The identification and classification of lamellibranch larvae. *Hull Bulletins of Marine Ecology*. **3:** 73-104.

Reuter, M. and I. Palmberg. 1989. Development and differentiation of neuronal subsets in asexually reporoducing *Microstomum lineare*. *Histochemical and Cell Biology.* **91:** 123-131.

Richardson, K. C., L. Jarett, and F.H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technology.* **35:** 313-323.

Rodriguez, S. R., F. P. Ojeda, and N. C. Inestrosa. 1993. Settlement of benthic marine invertebrates. *Marine ecology progress series*. **97:** 193-207.

Shumway, S. E., and G. J. Parsons. (Eds). 2006. Scallops: Biology, ecology and aquaculture 2nd Edition. Elsevier, San Diego.

Silberfeld, T., and O. Gros. 2006. Embryonic development of the tropical clam *Tivela mactroides* (Born, 1778) (Veneridae: subfamily Meretricinae): a SEM study. *Cah. Biol. Mar*. **47:** 243-251.

Siniscalchi, A., S. Cavallini, D. Sonetti, G. Sbrenna, S. Capuano, L. Barbin, E. Turolla, and R. Rossi. 2004. Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology*. **144:** 1205-1212.

Stephens, R. E. and G. Prior. 1992. Dynein from serotonin-activated cilia and flagella: extraction characteristics and distinct sites for camp-dependent protein phosphorylation. *J Cell. Sci*. **103:** 999-1012.

Torkildsen, L., Magnesen, T. 2004. Hatchery production of scalloplLarvae (Pecten maximus) – survival in different rearing systems. *Aquaculture International* **12:** 489-507.

Turner, E. J., R. K Zimmer-Faust, M. A. Palmer, M. Luckenbach, and N. D. Pentcheff. 1994. Settlement of oyster (*Crassostrea virginica*) larvae: Effects of water flow and a water-soluble chemical cue. *Limnol. Oceanogr*. **39:** 1579-1593.

Waller, T. R. 1981. Functional morphology and development of veliger larvae of the European oyster, *Ostrea edulis* Linne. *Smithson Contrib. Zool*. **328:** 70.

Waller, T. R. and B. A. Lasee. 1997. External Morphology of Spermatozoa and Spermatozeugmata of the Freshwater Mussel Truncilla truncata (Mollusca: Bivalvia: Unionidae). *Am. Midl. Nat.* **138:** 220-223.

Weiner, R. M., A. M. Segall, and R.R. Colwell. 1985. Characterization of a marine bacterium associated with *C. virginica* (the Eastern oyster). *Appl. Environ. Microbiol*. **49:** 83-90.

Williams, J .D., A. E. Bogan, and J. T. Game (Eds). 2008. Freshwater Mussels of Alabama and the Mobile Basin in Georgia, Mississippi and Tennessee. University of Alabama press, Tuscaloosa.

Williams, J. D., M. L. Warren Jr., K. S. Cummings, J. L. Harris, and R. J. Neves. 1993. Conservation Status of Freshwater Mussels of the United States and Canada. *Fisheries.* **18:** 6-22.

Willows, A. O. D., L. A. Nikitina, V. V. Bezuglov, N. M. Gretskaya, and G. A. Buznikov. 2000. About possible functional interaction between serotonin and neuropeptides in control processes of embryogenesis (experiments on embryos of Tritonia diomedea). *Russian Journal of Developmental Biology.* **31:** 106-112.

Wood, R. L., and J. H. Luft. 1965. The influence of buffer systems of fixation with osmium tetroxide. *J. Eltrastruct. Res.* **12:** 22-45.

Young, J. Z. 1965. The central nervous system of *Nautilus*. *Philosophical Transaction of the Royal Society of London.* **249:** 1-25.

Voronezhskaya, E. E., L. P. Nezlin, N. A. Odintsove, J.T. Plummer, and R. P. Croll. 2008. Neruonal development in larval mussel *Mytilus trossulus* (Mollusca; Bivalvia). *Zoomorphology*. **127:** 97-110.