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Gangliogenesis and Embryonic Development in Biomphalaria glabrata

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Lawrence University ‘23

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I hereby reaffirm the Lawrence University honor code.

ABSTRACT

Biomphalaria glabrata is an intermediate host for a parasitic species, *Schistosoma mansoni*, which can infect humans causing schistosomiasis. The majority of research conducted on these snails focuses on host-parasite relationships. Thus, very little is known regarding their nervous system, especially during embryonic and juvenile development. This project aims to investigate the transition from the embryonic nervous system consisting of a network of neurons to a developed ganglionic nervous system consisting of a separation between clusters of nerve cell bodies, or ganglia, and connectives. To investigate when this process, known as gangliogenesis, occurs, immunofluorescence is used to visualize the serotonergic nervous system at various ages, thus, providing a better understanding of the timeline for gangliogenesis. Additionally, this study aims to discern changes in gene expression throughout embryonic and early juvenile development by identifying differential gene expression patterns through RNA sequencing. Though we will be doing the transcriptome analysis with whole embryos and juveniles rather than just the isolated nervous system, we hypothesize that we will still be able to identify transcripts associated with the nervous system and its development. This process will also allow for the identification of candidate genes putatively involved in gangliogenesis in *B. glabrata*.

INTRODUCTION

Biomphalaria glabrata is a freshwater snail that is found in neotropical areas and is studied as a model for host-parasite interactions due to its role as an intermediate host of *Schistosoma mansoni*. *S. mansoni* is a trematode species of flatworm which are among the primary causes of human schistosomiasis, one of the most neglected tropical diseases affecting people in many parts of Africa, Asia, and South America (Portilho et al., 2019). The World Health Organization estimated that more than 218 million people in 78 countries required preventative treatment for schistosomiasis in 2015 (Mansour et al., 2017). Due to its epidemiological importance, many studies have been conducted regarding the complex relationship between the molluscs and the trematode; however, very little is known regarding the *B. glabrata* nervous system, especially during embryonic and juvenile development.

As it belongs to the phylum Mollusca and class Gastropoda, adult *B. glabrata* have a ganglionic nervous system comprised of neuronal cell bodies concentrated into collections, known as ganglia, which are localized within the head-foot region (Fig 1). Most ganglia come in pairs which are connected by lateral connections, called commissures, while differing ganglia are connected by longitudinal connections, called connectives. The adult *Biomphalaria* nervous system, in total, consists of five paired ganglia – cerebral, pedal, pleural, parietal, and buccal – and one unpaired visceral ganglion. The ganglia are arranged in an epiathroid organization around the feeding organ, or the radula, in the head-foot region of the snail (Mansour et al., 2017; Fig 1.C). Figure 1 gives a visual representation of the ganglionic central nervous system (CNS) in a *Biomphalaria* species, corresponding to the ganglionic CNS found in *B. glabrata*. This is useful for visualizing how this is localized within the snail's head-foot region, which is noted in the anatomical diagram of *Biomphalaria* (Fig 1.C).

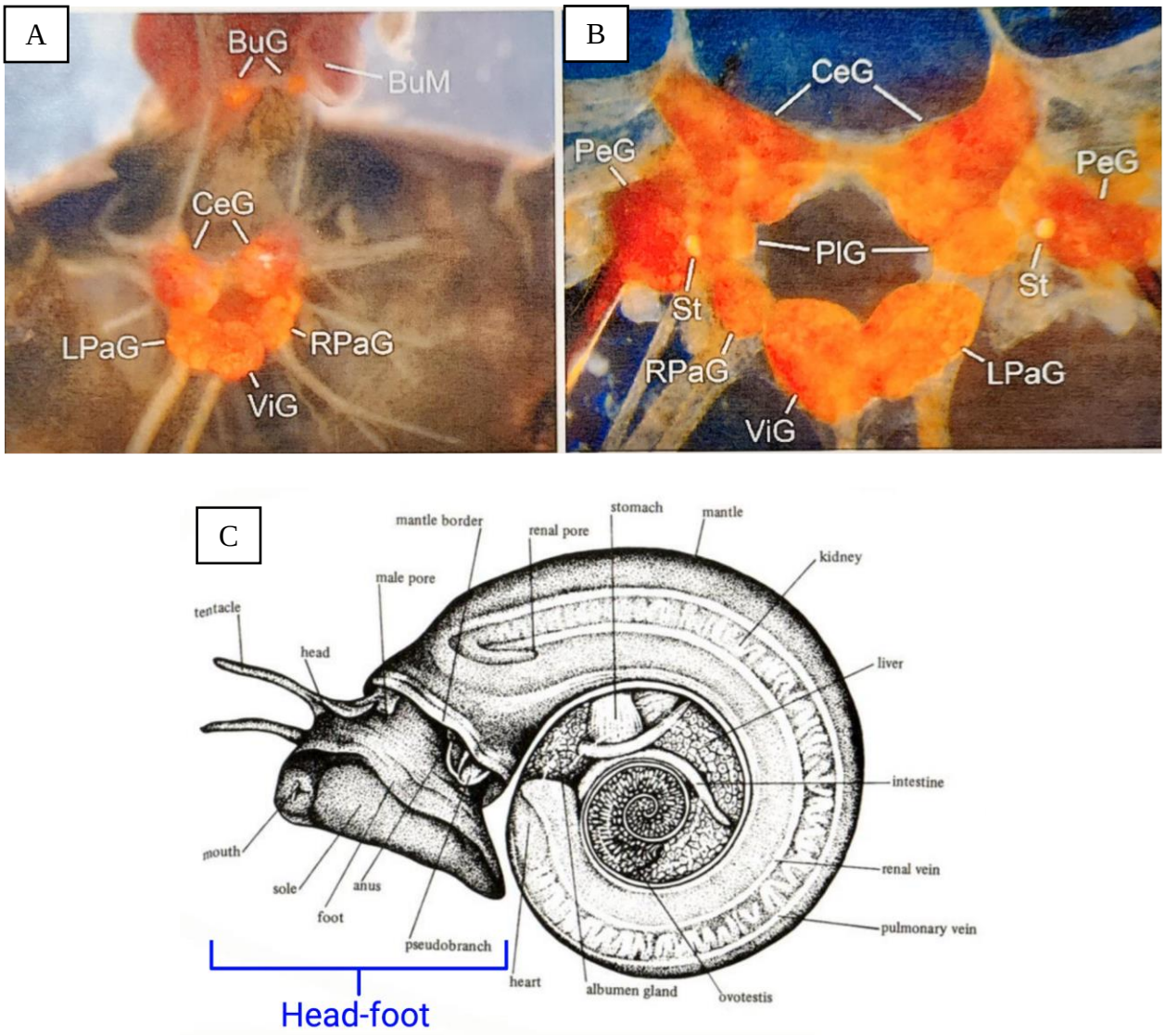

Figure 1. The central nervous system of *Biomphalaria alexandrina* in various stages of dissection alongside a reference diagram of *Biomphalaria* spp. (A) Dorsal perspective of a semi-isolated CNS following removal of the esophagus. The pedal ganglia (PeG) are obscured by the overlaying cerebral ganglia (CeG). (B) Ventral perspective of an isolated CNS with the pedal ganglia pinned laterally (BuG = buccal ganglia; BuM = buccal mass; LPeG = left parietal ganglion; PlG = pleural ganglia; RPaG = right parietal ganglion; St = statocyst; ViG = visceral ganglion). [From Acker et al., 2019]. (C) Anatomical diagram of *Biomphalaria* spp. modified to clearly describe the head-foot region. Image was modified in BioRender. [From Perez et al., 2004].

Gastropod & Molluscan Nervous System Development

Even though the nervous systems of several invertebrates have been used to extensively study fundamental neurodevelopmental processes, very little research has focused on CNS development in molluscs. However, large strides have been made to obtain a better understanding of molluscan neural development over the past several decades. Molluscan gastropod CNS development is referring to an extensive process which could be split into three stages of development: early neurogenesis, gangliogenesis, and the adult CNS. While these stages may not be distinct from one another with regards to when they occur, they each focus on a specific subset of molluscan CNS development.

Early neurogenesis

Early neurogenesis specifically examines the beginning of CNS development which is necessary to lay out the framework for the rest of the process. Overall, the first elements of the molluscan CNS seem to appear during the trochophore stage of embryonic development. Some of the very first putative neurons are detected in a structure known as the apical sensory organ (ASO). The ASO is a part of the nervous system and comprised of a patch of cilia within the anterior regions in the developing embryo (Richter et al. 2010; Page, 2002). These vase-like cells were initially discovered through electron microscopy in a gastropod larva, and subsequent studies demonstrated the existence of similar cells in the ASO of various gastropod groups (Bonar, 1978; Chia & Koss, 1984; Marois & Carew, 1997; Page, 2002; Page & Parries, 2000; Ruthensteiner & Schaefer, 2002). The ASO is shown to display serotonin-like immunoreactive (LIR) apical cells which seem to be generally consistent regarding their arrangement and number across various species (Page & Parries, 2000; Kempf, 2008). The ASO's morphological similarity to typical sensory cells within adult molluscs and clear association with serotonin had

suggested that the serotonergic component of the ASO could sense environmental stimuli (Bonar, 1978; Chia & Koss, 1984; Kempf et al., 1998). Alongside these anterior cells, posterior cells expressing FMRFamide-LIR have been shown to also develop during the trochophore stage.

Croll and Voronezhskaya (1996) found a novel view of the sequence of events occurring in early molluscan neurogenesis. Using immunohistochemical staining of FMRFamide in the embryos of the pond snail *Lymnaea stagnalis*, they looked at cells that were centralized, appeared early, and projected anteriorly throughout multiple stages of development and saw that the first elements of the nervous system appear during the trochophore stage in the embryo. Figure 2 represents the images they obtained of these cells. Croll & Voronezhskaya (1996) saw that the first three cells are located posteriorly within the embryo, not anteriorly, which was in contrast with the previous understanding of gastropod neurogenesis (Croll & Voronezhskaya, 1995; 1996; Dickinson et al., 2000; Dickinson & Croll, 2003).

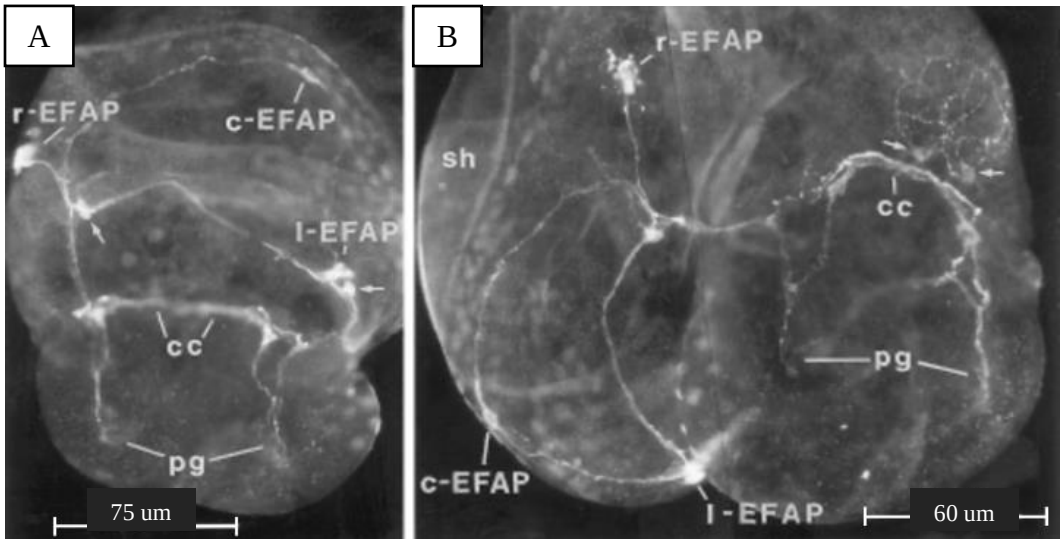

Figure 2. FMRFamide-like immunoreactive (Fa-LIR) cells in the early veliger of *L. stagnalis*. (A) Anterior view of an embryo showing that fibers from the three EFAP (Early, Fa-LIR, Anteriorly Projecting) cells project to the anterior of the embryo, cross the midline, and project ventrally. The c-

EFAP (central EFAP) develops earliest at around 25-28% of embryonic development. Smaller Fa-LIR cells (small arrows) are present near the r- and l-EFAP cells (r- & l- EFAP = right & left EFAP cell) which develop by 28-30% of embryonic development. Although no central ganglia have previously been detected in such early embryos, immunoreactive fibers clearly mark locations of the future cerebral ganglia, cerebral commissure (cc), and pedal ganglia (pg). (B) Numerous short projections partially obscure the soma of r-EFAP. An additional pair of Fa-LIR somata (small arrows) with fibers branching below the ciliary apical plate is also detectable. The early developmental stage of this embryo is clearly reflected in the small size of the developing shell (sh). [From Croll & Voronezhskaya 1996]

The extensive, anteriorly directed fibers from these FMRFamide-LIR cells project anteriorly along the side of the body wall, meeting near the ASO and the later developing cerebral and pedal ganglia (Croll & Voronezhskaya 1995; 1996; Dickinson et al., 1999; Dickinson & Croll, 2003). These early cells expressing FMRFamide-LIR could potentially play a role in forming the scaffold upon which the central ganglia and interconnecting pathways of the adult CNS may later develop (Croll & Voronezhskaya 1996; Croll 2009). This contrasts with the previous belief that the interconnecting commissures and connectives originated from fibers from differentiated neurons in the developing central ganglia. However, these cells appear to predate neural differentiation within these central ganglia.

A final set of neurons known to develop early in molluscan CNS development are the cone-shaped catecholaminergic cells. These appear to develop ventrally and laterally to the mouth, with the location suggesting a potential role in feeding (Dickinson & Croll, 2003; Bayne, 1983). Additionally, catecholamine-containing cells and axons have been associated with ciliary bands along the velum, a thick and lobular structure known as the swimming organ (Dickinson & Croll, 2003). This specific localization, again, suggests that catecholamines might play a role the muscle contractions of the velum and subsequent swimming behavior (Croll, 2009).

Overall, within the early stages of development, there seems to be an extensive nervous system in gastropods. However, this might yet be incomplete, as a majority of this research has been dependent on immunohistochemical methods to identify specific localization of neurotransmitters. There are still quite a few classical neurotransmitters which have yet to be tested in embryos, even though they have been explored in the adult molluscan nervous system. These include acetylcholine, GABA, glutamate, and various other peptides (Croll, 2009).

Gangliogenesis

As aforementioned, molluscan CNS development encompasses a wide range going from neurogenesis to a fully formed adult CNS, but gangliogenesis specifically refers to the process through which scattered neuronal cell bodies come together to form a tight cluster, known as a ganglion. Though the early CNS in *B. glabrata* embryos has been detected (Fig. 5), the process of gangliogenesis has yet to be characterized within these snails. In fact, there is very minimal research done into this specific avenue of CNS development in molluscs overall.

Nevertheless, it is generally understood that the development of ganglia within the CNS, or gangliogenesis, in gastropods progresses from an anterior to a posterior direction following this sequence: cerebral ganglia develop first, followed by the pedal ganglia, and then the more posterior ganglia of the abdominal loop (Croll & Voronezhskaya, 1996). For instance, this was demonstrated to be true in the gastropod species, *Aplysia californica*, which has been used in various studies on development of the nervous system. In *Aplysia*, the paired cerebral and pedal ganglia form first, having already appeared by late embryonic development (Kriegstein, 1977; Jacob, 1984; Croll, 2009). The ganglia seem to develop through zones of high cell proliferation in the absence of rapid migration, leading to areas of thick cellular layers in the ectoderm. The

ectoderm is amongst the primary germ layers seen in embryonic development and is the region from which neural tissue arises. As cell division continues within these proliferative zones, the newly divided cells migrate inward to join the developing cerebral ganglia (Jacob, 1984). Figure 3 represents this developmental model in *Aplysia*.

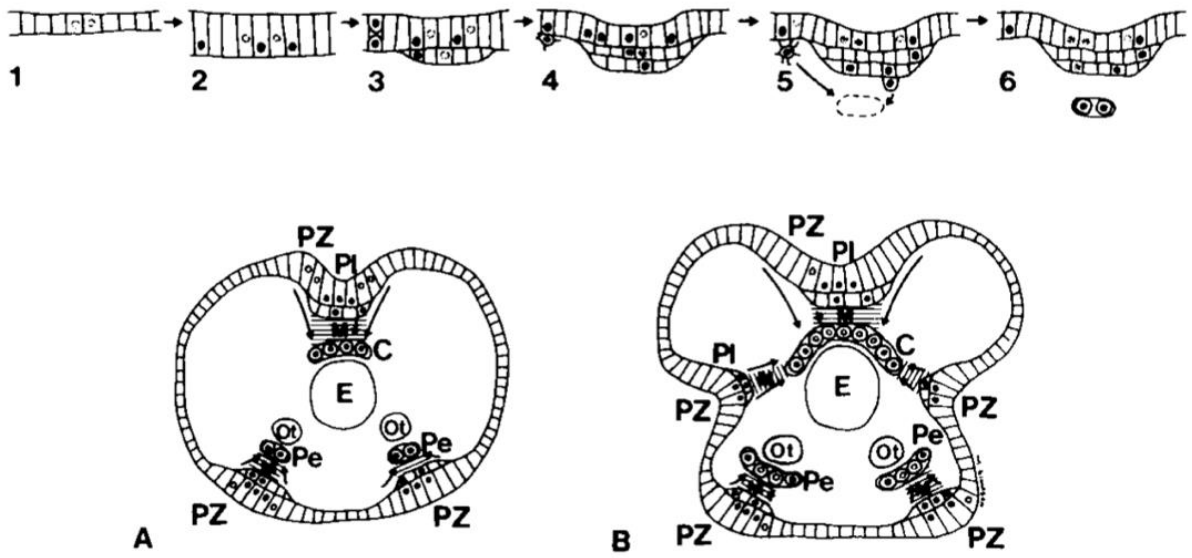

Figure 3. Schematic representation of neurogenesis in *Aplysia*. Parts 1-6 illustrates magnified views of the body wall of the ectoderm as it changes during neurogenesis, and (A) and (B) are cross sections of two stages of the whole animal (A = gastrula; B = trochophore stage embryos). Proliferative zones (PZ) appear in regions of the body wall adjacent. Multilayered placodes (Pl) form in a central region of each proliferative zone, these being composed of ectodermal cells that have elongated into columnar shapes. Each ganglion is formed from a unique adjacent proliferative zone, cells migrating from the surface inward along paths indicated by the arrows (C = cerebral ganglion; Pe = pedal ganglion; E = esophagus; Ot = otocysts). [From Jacob 1984].

This model for the development of the cerebral ganglia in gastropods seems to stay relatively consistent throughout various species, even if the timeline might differ per species. To reiterate, cerebral ganglia develop from these areas of high cell division, concurrent with an envelopment of the ectoderm. These are among the first pair to develop and are evident during hatching in *Aplysia*, *Ilyanassa obsoleta*, and related species (Raven, 1966; Hyman, 1967; Lin &

Leise, 1996). Functionally, the cerebral ganglia in adult gastropods are thought to innervate various muscles and sensory regions in the head including the ASO, eyes, tentacles, and skin. They also share connectives with the buccal, plural, and pedal ganglia (Bullock, 1965; Raven, 1966; Hyman, 1967; Voltzow, 1994). Furthermore, the development following an anterior to posterior sequence is shown to apply generally within gastropods as the development of the central ganglia seems to be followed by the pedal ganglia, which are also apparent at hatching. The pedal ganglia are positioned in the proximal part of the foot and are shown to innervate the foot and its derivatives (Bullock, 1965; Hyman, 1967). They are also connected to the pedal ganglia, along with the central ganglia.

The timeline for development of the other ganglia seems to be more varied between the various gastropod species which have been studied. For instance, the buccal ganglia have been observed to arise in embryos before hatching (Bedford, 1966; Demian & Yousif, 1975), in larvae after hatching (D'Asaro, 1969), and during the middle of larval development (Smith, 1935). However, the buccal ganglia do seem to develop from the wall of the foregut within most gastropods. Moreover, the structures these ganglia innervate in adulthood seems to be common among most gastropods. The buccal ganglia in adults innervate the walls of the pharynx, muscles of the buccal mass, radula, stomach, and occasionally other viscera (please refer to Fig. 4 for to better understand the morphology of the structures listed).

The pleural ganglia seem to innervate the mantle and the columellar muscles, which run from the inside of the mantle to the operculum. The parietal ganglia are thought to arise during the later stages of larval development and are often asymmetrical within adults. Their position can greatly vary due to torsion which occurs during the early veliger stage of embryonic development leading to the crossing of nerve cords (Raven, 1966; Hyman, 1967; Page 2003).

These ganglia, when fully developed, innervate the kidney, gills, and the chemosensory organ known as the osphradium (Hyman 1967).

Finally, each of the adult parietal ganglia share a connective with the visceral ganglion which is located at the beginning of the visceral mass (Raven, 1966; Hyman, 1967). This ganglion is generally depicted to arise from ectodermal thickening which occurs during the late veliger stage in embryos at the posterior end of the mantle cavity. In adult gastropods, the visceral ganglion innervates posterior regions of the gut, anus, reproductive organs, kidney, liver, and heart (Bullock, 1965; Hyman, 1967; D'Asaro, 1969).

A B C

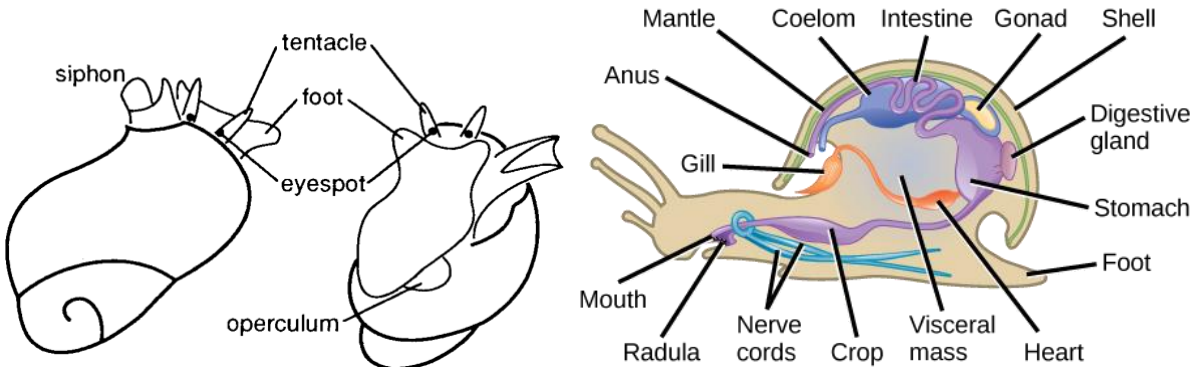

Figure 4. Diagrams of the external and internal morphology of a juvenile and adult gastropod, respectively. The dorsal (A) and ventral (B) views of the juvenile are shown [From Lin & Leise, 1996]. (C) shows the internal morphology within a gastropod species [From Biology for Majors II: phylum Mollusca].

Still, there is an overall lack of knowledge of gangliogenesis in *B. glabrata*. Lin & Leise's (1996) paper "Gangliogenesis in the Prosobranch Gastropod *Ilyanassa obsoleta"* was one of the only studies that could be found specifically exploring gangliogenesis within gastropods in the literature. They state that in *I. obsoleta*, a majority of the ganglia—cerebral, pedal, pleural, buccal, and apical—were formed by 8 days after hatching, with the exception of the visceral ganglia (Lin & Leise, 1996). However, this information was obtained from snails which have a very different process of early development compared to *B. glabrata*. *I. obsoleta* is

among a group of gastropods which undergo a more complex process of embryonic and larval development which differs greatly from direct-developing species like *B. glabrata*. Essentially, snails similar to *B. glabrata* undergo the entirety of embryonic development in an egg capsule and hatch as young juveniles (Croll, 2009), while snails like *I. obsoleta* have a more indirect process during which snails exit the egg capsule during the veliger stage of embryonic development and undergo various larval stages before they reach their final adult morphology (Raven, 1966; Marois & Carew, 1990). Therefore, while these results about the approximate stages of development during which ganglia in *I. obsoleta* arise did provide a hypothesis for the timeline during which gangliogenesis might occur within *B. glabrata* snails, the difference in their respective development should be acknowledged.

Hormones & neuropeptides in *B. glabrata* nervous system development

Certain key hormones, neuropeptides, and neurotransmitters have been discovered to be heavily concentrated within the adult nervous system of gastropods like *B. glabrata.* Some of these have been shown to be integral for molluscan nervous system development, amongst other major processes. As described earlier, various neurotransmitters and neuropeptides have been used as markers to visualize the nervous system and to describe neurogenesis in gastropods. These include serotonin, FMRFamide, dopamine, & neuropeptide Y (NPY). Immunofluorescence with these neurotransmitters provided one of the earliest pieces of evidence against the common model of gastropod neurogenesis from the 20th century, as previously described.

FMRFamide (Phe-Met-Arg-Phe-NH_2) is one of a family of neuropeptides known to exist widely in invertebrates (Croll & Voronezhskaya 1996). Cells with FMRFamide-LIR have been

thought to innervate muscles developing in the trochophore and veliger stages of embryonic development in gastropods (Page, 1997). FMRFamide itself has been reported to increase velar contractions which is related to swimming activity in larval and juvenile snails (Braubach et al., 2006). Additionally, catecholamines, a class of aromatic amines such as dopamine and norepinephrine, have also been detected within the adult and embryonic gastropod CNS (Voronezhskaya et al., 1999; Marois, 1989). The presence of catecholamines have been reported to inhibit ciliary beating and generally seem to decrease swimming behavior and feeding rates within gastropods (Braubach et al., 2006).

Since these neurotransmitters are known to be present within the adult brain and some have been seen during embryonic development, they could perhaps also be present within juveniles. As such, any of these could be used as potential markers to visualize the juvenile *B. glabrata* CNS; however, this study specifically focused on serotonin.

Serotonin in B. glabrata

Serotonin (5-hydroxytryptamine or 5-HT) is one of the evolutionarily oldest and most widely distributed modulators of development, appearing in both vertebrates and invertebrates. It plays an important role in neuronal development, meiosis reinitiating, morphogenetic movements, cell divisions, and various other developmental processes within gastropods (Glebov et al., 2014). Serotonin has been observed to increase cilia-driven rotational behavior within various gastropods (Diefenbach et al., 1991), and prior research in the Humphries lab has observed this relationship in *B. glabrata* embryos. This rotational behavior seems to be an important behavior within embryos, and Goldberg et al., 2008 proposed an “embryo-stir bar hypothesis” attributing this behavior to help circulate oxygen and nutrients within the egg capsule as it seems to be triggered by hypoxia (Kuang et al., 2002). Overall, serotonin is a major

neurotransmitter and modulator within the gastropod CNS (Kempf et al., 1997; Dickinson et al., 2000; Kuang et al., 2002; Goldberg et al., 2008; Kempf, 2008). This prevalence of serotonin within the *B. glabrata* CNS in both adults and embryos makes it a potential marker to visualize the serotonergic nervous system in *B. glabrata* juveniles. This will allow us to visualize serotonin localization within the snail through fluorescence or confocal microscopy.

Embryonic development in *B. glabrata*

Embryonic development of molluscs has been studied since the end of the 19th century (Raven, 1966). There has been previous research conducted studying embryonic morphology and cell lineages during development in gastropods. This includes light microscopy studies to characterize cell lineages during early stages of development (Carney and Verdonk, 1970), analyses of the embryonic external morphology (Battenfeld et al., 1993), and ultrastructural details of the internal structure (Kikuchi et al., 1988) in *B. glabrata* embryos. However, there have been few studies on embryonic development, specifically embryonic CNS development, in *B. glabrata*, and the molecular mechanisms underlying this process remain largely unknown.

In *B. glabrata*, embryonic development begins with the initial laying of the egg mass which contains various embryos contained within their own egg chamber. This period of development spans until the snails emerge from their individual chambers with their young-adult, or juvenile, morphology. These stages go sequentially from the blastula stage to the gastrula, trochophore, veliger, and finally the juvenile stage. Figure 5 shows the progression of embryonic development in *B. glabrata*.

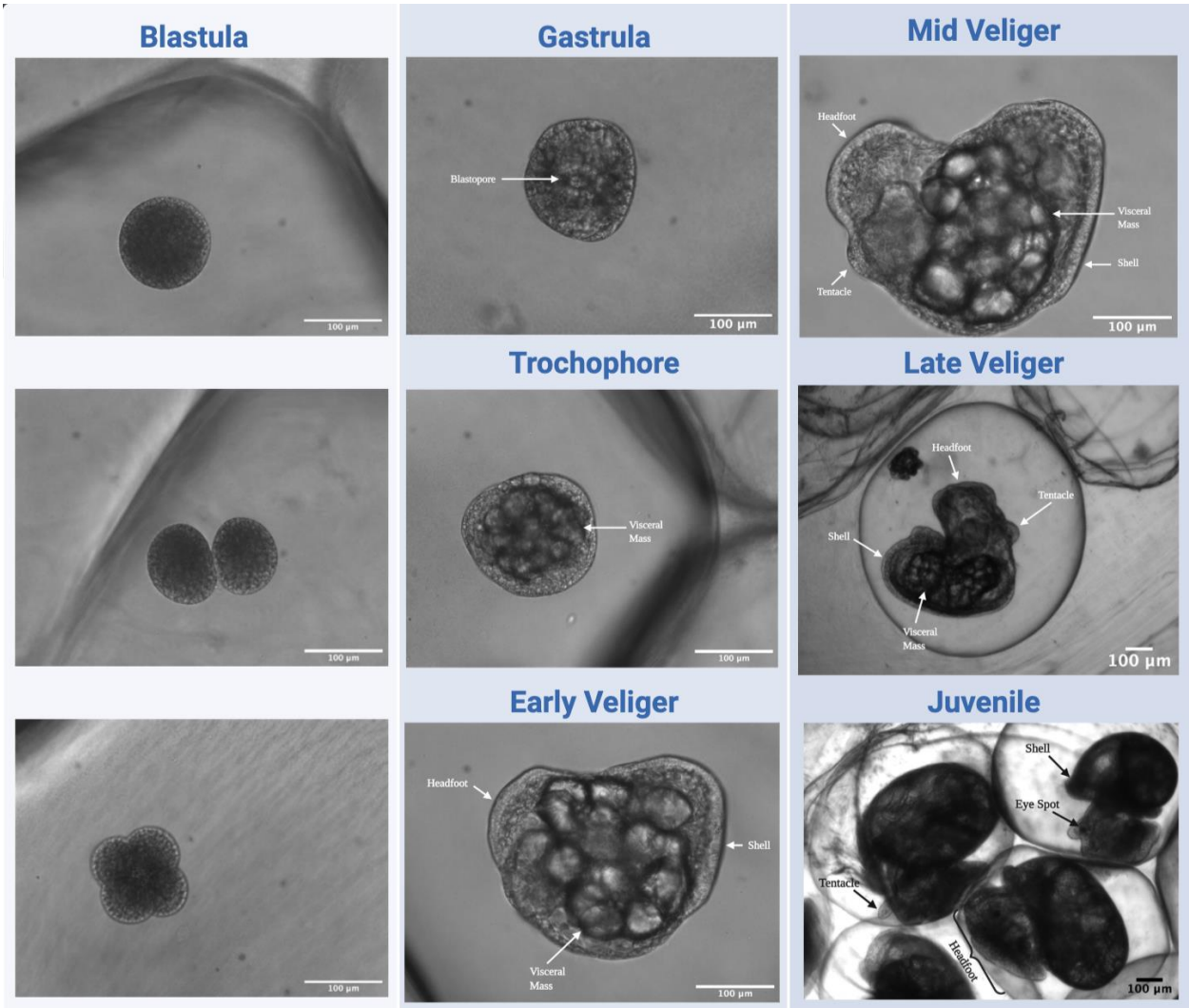

Figure 5. Embryonic development observed in *B. glabrata* snails. Various morphological transitions are depicted: initial cell cleavage in the blastula stage, the appearance of the blastopore in the gastrula embryo, developing visceral mass in the trochophore embryo, the development of the head-foot region and shell during the veliger stage, and the final stage of embryonic development—the pre-hatching juvenile [From Batista-Ruiz (2020)].

The blastula stage, which occurs during the first 0-2 days post-laying, is characterized by cell cleavage starting with the initial splitting of a single-celled egg. After multiple stages of cell division, the *B. glabrata* embryo transitions into the gastrula stage of development. This occurs at approximately the second day of development where the embryo develops distinct germ layers and the initial embryonic opening, known as the blastopore, which will form the anus or mouth

of the future snail. This stage is directly followed by the trochophore stage, occurring at approximately 2-3 days post-laying. There are two key events known to occur during this stage. The first is the development of the visceral mass which will become the snail's organs. Secondly, movement within the developing embryo is detected for the first time, consisting of the aforementioned rotational behavior within their individual egg capsules. From here, the embryo shifts onto the veliger stage which spans from 4-6 days post-laying. This stage is more prolonged than the others and encompasses the formation of the head-foot region, shell, and radula along with the growth of the visceral mass. The embryos now transition to their final stage of development, the juvenile stage, which occurs approximately 6-7 days post-laying. At this point, the snail is morphologically quite similar to a hatched, young adult snail as it has developed the head-foot region, their tentacles, a thickened shell, and their visceral mass which includes most of their major organ systems. They are ready to hatch from their chambers following this final stage.

Embryonic Nervous System Development in B. glabrata

Nervous system development also begins in the *B. glabrata* embryo, and past work from the Humphries lab has shown an extensive network of neurons (Fig 6). This shows a sequential representation of the preliminary serotonergic nervous system seen in embryos as they develop. We also see the beginning of the development of major axons within embryos. These images show CNS development beginning in embryos, and since the CNS in *B. glabrata* adults is drastically different from what can be seen in embryos, it is presumed that the gangliogenesis process of CNS development occurs post-hatching, during the juvenile stage of development. However, the specific details about when and how the actual gangliogenesis process occurs in *B. glabrata* are largely unknown.

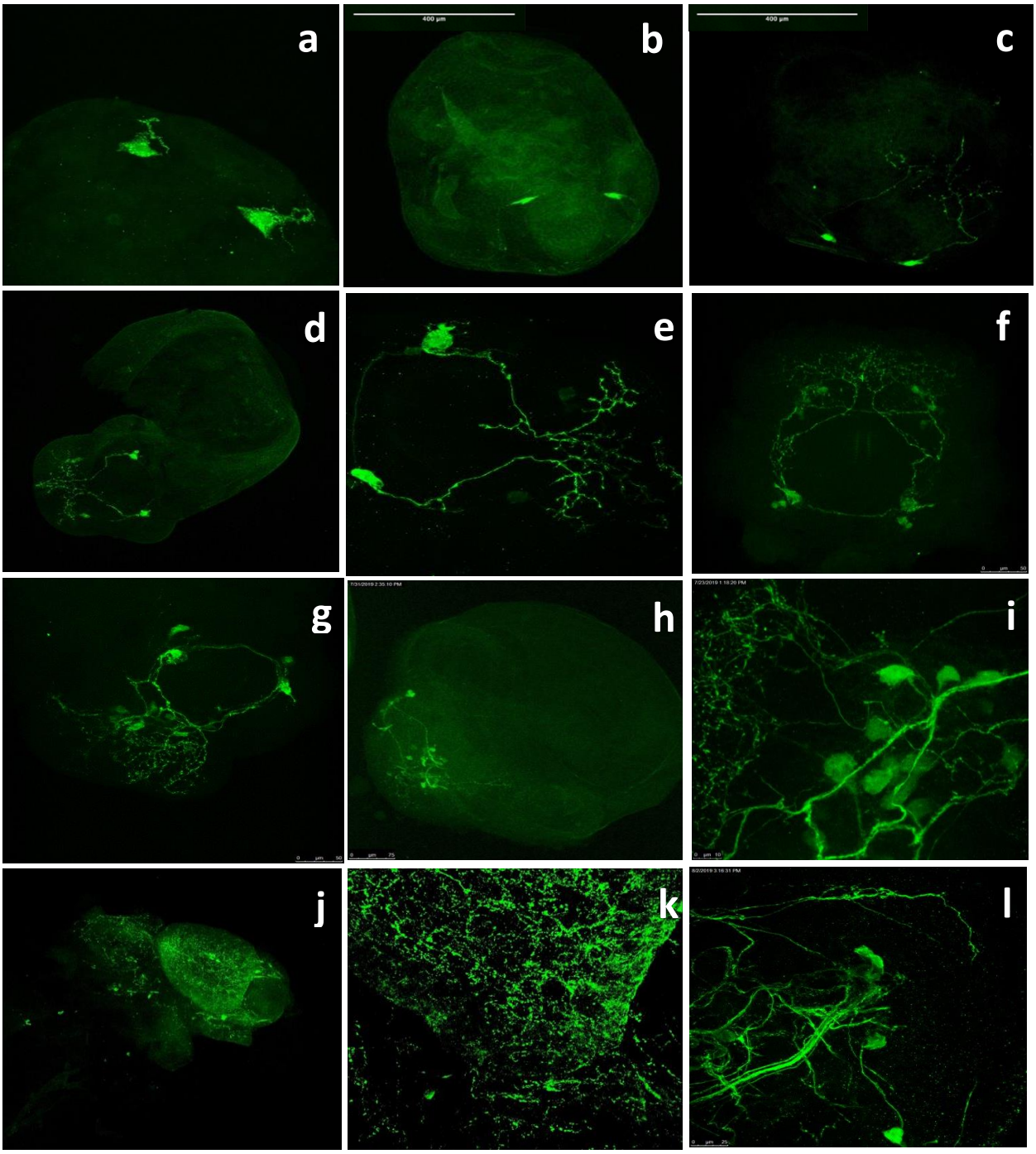

Figure 6. Serotonin immunoreactivity in *B. glabrata* embryos. (a) depicts serotonergic NS in 1–2-day old embryos. (b) & (c) depict serotonergic NS in 2–3-day old embryos. (d) & (e) depict serotonergic NS in 3–4-day old embryos. (f), (g), (h), & (i) depict serotonergic NS in 5–6-day old embryos. (j), (k), & (l) depict serotonergic NS in 5–6-day old embryos. Images were obtained on the confocal microscope. [Images from Helen Threkheld and Aparna Gajulapalli]. SCALE BARS

To reiterate, gangliogenesis refers to the specific process through which the nervous system transforms from this network of neurons seen in embryo to a developed ganglionic nervous system consisting of a separation between clusters of neuronal cell bodies, or ganglia, and connectives seen in adults. Additionally, since this development is controlled through gene expression and protein synthesis, gaining an understanding of how genes are being expressed within various stages of development will add to the current knowledge regarding the mechanisms underlying gangliogenesis. To do this, mRNA sequencing (mRNA-seq) with a next-generation sequencing technique (NGS) will be done with snails at various stages of development to better understand how gene expression is changing throughout this process.

By extracting and processing just the messenger RNA (mRNA), we will be able to gain a better understanding of which parts of the *B. glabrata* genome are actively being transcribed, during which stage of development certain genes are being "turned-on," and how this data differs between snails of various ages. This differential data will give us insight into the specific molecular mechanisms which underlie embryonic and juvenile development in *B. glabrata*. A whole genome analysis of *B. glabrata* was performed (Adema et al., 2017), which was used as the reference genome for analysis of the mRNA-seq data.

Examination of the embryonic transcriptomic data will help us understand embryonic development and possibly also development of the embryonic nervous system. Meanwhile, an examination of juvenile transcriptomes will increase our understanding of gangliogenesis, assuming the snails which are sequenced cover the time period during which gangliogenesis is occurring.

Experimental design

The gap in knowledge regarding ganglionic development in *B. glabrata* compels the focus of this current study which (1) examines when gangliogenesis occurs. To investigate this, immunofluorescence with serotonin was used to visualize the serotonergic nervous system. As serotonin-immunoreactivity (IR) can be detected in *B. glabrata* embryos and adults, utilizing this method with *B. glabrata* juveniles at various ages should presumably provide a better understanding of the timeline in which this process occurs. With this established timeline, we can begin to address the question of how this process occurs.

This study, additionally, aims to (2) discern changes in gene expression throughout embryonic and early juvenile development by identifying differential gene expression patterns through RNA sequencing and (3) develop hypotheses regarding the mechanisms controlling gangliogenesis. This method allows for the analysis of RNA transcripts that are actively being transcribed at that point in development; this will subsequently give insight into when specific genes are being expressed and at what level. Though we will be doing the transcriptome analysis with whole embryos and juveniles, we hypothesize that we will still be able to identify transcripts associated with the nervous system and its development. This process will also allow for the identification of candidate genes putatively involved in gangliogenesis in *B. glabrata*, such as the genes encoding for neurotransmitters known to be associated with the nervous system.

Furthermore, this project has the potential to identify novel neural markers that could be used in further research to visualize the whole nervous system, rather than just the serotonergic nervous system. From this, we can generate novel hypotheses for future experimental testing.

Overall, this study will add immensely to our current understanding of overall embryonic development and nervous system development in *B. glabrata*.

METHODS

Collection and isolation of *B. glabrata* embryos and juveniles

B. glabrata snails were housed at a temperature of 26°C on a 12-hour light/dark cycle in large glass dishes filled with artificial pond water (1.25mM $CaCO_3$, 3.75mM $MgCO_3$, 5.25mM NaCl, and 0.75mM KCl in autoclaved, aged tap water). The snails were fed romaine lettuce *ad libitum*, and the tanks were cleaned once a week. Embryos from the BS90 strain of *B. glabrata* were collected by placing a small piece of polystyrene (~1 x 1 in.) into the dish to act as a substrate for egg laying. The egg masses were continuously checked 1-2 times within a ~24 hour period. Once laid, they were moved to a separate dish with artificial pond water and allowed to develop to select stages of development.

When the embryos reached the desired age, the egg masses were then retrieved by using a scalpel to gently break the adhesions between the outer membrane of the egg mass and the surface of the polystyrene. They were then placed into a petri dish with a small amount (~ 1 mL) of artificial pond water. The individual embryos were isolated while being observed using a Nikon SMZ745 dissection microscope (Nikon Instruments Inc., Melville, NY) by breaking apart the membranes of the egg mass and then individual egg chambers with beading needles. The isolated embryos were transferred to 1.5 mL microcentrifuge tubes. To collect juveniles, the egg masses (collected in the same manner as above) were maintained in their separate dishes until

they hatched with continuous, daily monitoring. The date of hatching was noted to keep track of the snails' ages, as this would become their day 0 of development. When the juveniles reached the desired age of development, they were retrieved with a pipette and were isolated into 1.5 mL microcentrifuge tubes. Isolated embryos and juveniles were processed according to the immunofluorescence or RNA extraction protocols.

Immunofluorescence

Both the isolated embryos and juveniles were fixed in 4% paraformaldehyde (PFA) in a snail phosphate buffered solution (sPBS, 0.14M NaCl, 2.7mM KCl, 10mM NaH_2 PO4 , 1.8mM KH_2PO_4, pH 7.4) for approximately 2 hours at 4°C. Following fixation, the snails were washed with 0.1% Triton X-100 (TX) in sPBS twice 10 minutes each to thoroughly remove the PFA. They were then stored in sPBS at 4°C for future use.

The younger embryos (< 4 days old) were washed with 4% TX for 15 minutes in sPBS prior to incubation with antibodies. The older embryos (> 4 days old) were incubated with 0.5% trypsin from porcine pancreas (Sigma-Aldrich, St. Louis, MO) in sPBS for 1 minute and then washed with 4% TX in sPBS for 15 minutes. The juveniles underwent an extra step to remove the shell for better visualization (please refer to appendix I for the detailed procedure used for this process). Afterwards, the juveniles were incubated in 1% trypsin in sPBS incubation for 1 minute followed by a wash with 4% TX in sPBS for 15 minutes.

All of the snails, regardless of stage of development, underwent incubation with 5% bovine serum albumin (BSA; Sigma-Aldrich) in sPBS overnight at 4°C. This stage of blocking with BSA was used to prevent nonspecific binding of the antibody. Following this, the snails were incubated with anti-serotonin (Sigma-Aldrich) diluted with 5% BSA at a factor of 1:10,000.

The snails were left to incubate in this primary antibody for ~48 hours at 4°C. They were then washed 3 times for 10 minutes each (3x10 mins.) with 4% TX in sPBS and then incubated with a fluorophore-tagged secondary antibody, anti-rabbit Alexa 488-conjugated IgG (Cell Signaling Tech., Boston, MA, 1:2000). They were wrapped in aluminum foil to prevent light bleaching of the fluorophore and incubated for ~24 hours at 4°C. Following this, they were washed 2x10 mins. with 4% TX in sPBS, and a Hoechst dye was used to visualize DNA within the tissue (1:4500 in sPBS). Hoechst functions differently to the antibodies as it is a stain which binds to the DNA itself and is excitable with ultraviolet (UV) light. The snails were left in this Hoechst in sPBS solution for ~1 hour and then washed 2x10 mins. with 4% TX in sPBS. A final wash was performed with sPBS, following which the snails were mounted onto glass microscope slides with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). All of the slides were viewed using a Leica DM1000 LED microscope, and specimens were imaged using a Leica TCS SP5 II confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Confocal microscopy & analysis

To obtain Leica TCS SP5 II confocal images of *B. glabrata* snails expressing serotonin-IR, argon laser 488 was used at 15% capacity. Images of *B. glabrata* expressing the Hoechst dye were excited by UV light with the 405 diode and HeNe 543 lasers at 15% and 33% capacity respectively. The images were analyzed using ImageJ software to obtain Z-projections.

RNA extraction

Snails were isolated using the aforementioned protocol, but diethyl pyrocarbonate-(DEPC-) treated water was used instead of artificial pond water when extracting the embryos. This was done to maintain a nuclease-free workspace and prevent RNA degradation. Additional

care was taken to ensure that the embryos were kept intact and alive while isolating them, to prevent the RNA degradation following the release of RNases, enzymes which breakdown RNA, at the point of tissue death. RNA was extracted from whole snails using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, making sure to use < 5ug of snail tissue per sample. To ensure the removal of genomic DNA, the RNase-Free DNase Set (Qiagen) was utilized during the protocol according to the instructions provided.

Assessing RNA concentration & purity

RNA concentrations were measured using a Nanodrop 2000 Spectrophotometer (ThermoFisher, ND-2000). This provided absorbance results at various wavelengths, corresponding to the absorbance measurements of nucleic acids, proteins, lipids, and other such molecules. RNA, along with other nucleic acids, absorbs at 260 nm, and the total absorbance at this point will correspond to the total RNA present within the sample. RNA purity will be measured through a ratio of absorbance at 260 & 280 nm, and 260 & 230 nm. A 260/280 ratio of 1.8 to 2.2 is generally considered "pure," and a lower ratio is indicative of the presence of protein, phenol, and other such contaminants which absorb at 280 nm. The 260/230 ratio is typically used as a secondary measure of purity, and acceptable values range between 2.0 to 2.2. A considerably lower ratio is indicative of the presence of carbohydrates and phenols such as TRIzol and guanidine isothiocyanate, two reagents which are commonly used in RNA or DNA extraction protocols. Only values with these respective purity values were sent for sequencing.

Next-gen mRNA sequencing

The construction of RNA-Seq libraries using polyA enrichment method was performed by Novogene Corporation Inc. (Sacramento, CA, USA). These libraries were sequenced using

the Illumina NovaSeq 6000 platform, and the subsequent bioinformatics analyses were done by Novogene. The sequencing reads of each RNA-Seq library were aligned to the reference genome of *B. glabrata* (Adema et al., 2017).

RESULTS

Serotonin immunoreactivity in B. glabrata juveniles

The aim of this study was to explore CNS development in *B. glabrata* juveniles and examine whether gangliogenesis occurs within these young juveniles. Immunofluorescence with serotonin was utilized to study juveniles spanning from 3 to 16 days post-hatching. The images obtained were compared to the serotonin-IR shown in the later stage of embryonic development in *B. glabrata* (Fig 6).

In 3-to-4-day old juveniles (Fig 7; 8), serotonin-IR was seen throughout the snail body but was concentrated within the head-foot region of the snail. There is clear localization of the serotonin-LIR around the radula in the head-foot region (Fig 7.E; 8.D). Additionally, compared to what was shown in older embryos, the serotonergic CNS in these 3-to-4-day old juveniles appears to have condensed into a more circular structure. Various long, branching axons projecting away from the CNS also displayed serotonin-IR. These axons appear to innervate the foot (Fig 7.E) and eyespots (Fig 8.D) of the developing juveniles. Within the CNS, there appear to be oval-shaped structures located at the base of the head-foot region, which seem to almost be mirroring each other (Fig 8). These structures are connected by a bundle of axons, closely resembling a connective (Fig 7.E; 8.D).

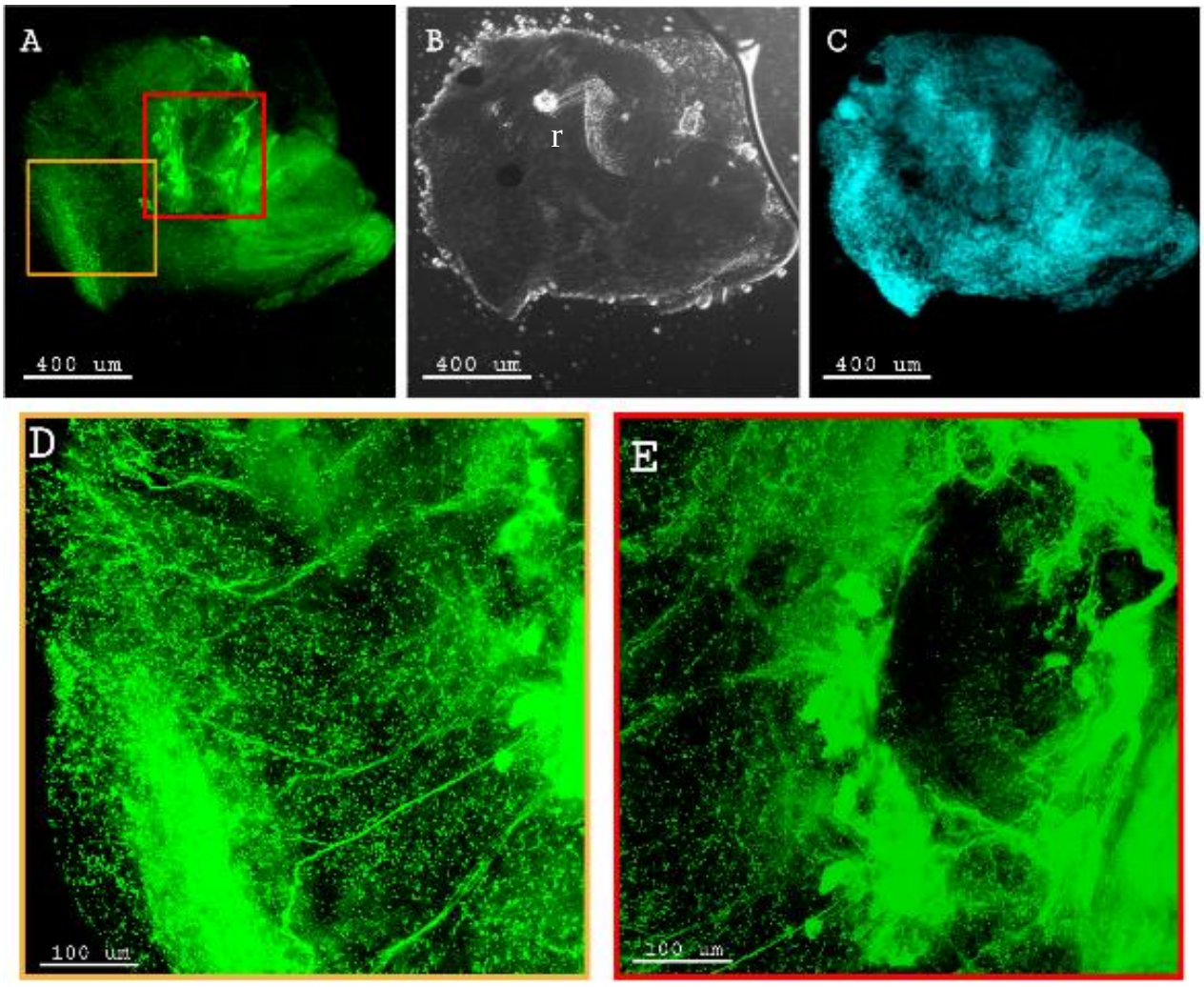

Figure 7. Confocal images of a 3–4 day old *B. glabrata* juvenile treated with anti-serotonin. r = radula. Serotonin-IR is shown in green, and Hoechst is shown with the blue. The top row consists of the various images of the snail taken using a 10x objective. A, B, & C respectively correspond to the serotonin-IR, white light, and Hoechst binding within the snail tissue. The boxes on (A) correspond to regions which have been magnified in the images in the bottom row, using a 40x oil lens. The orange box corresponds to (D), and the red box corresponds to (E).

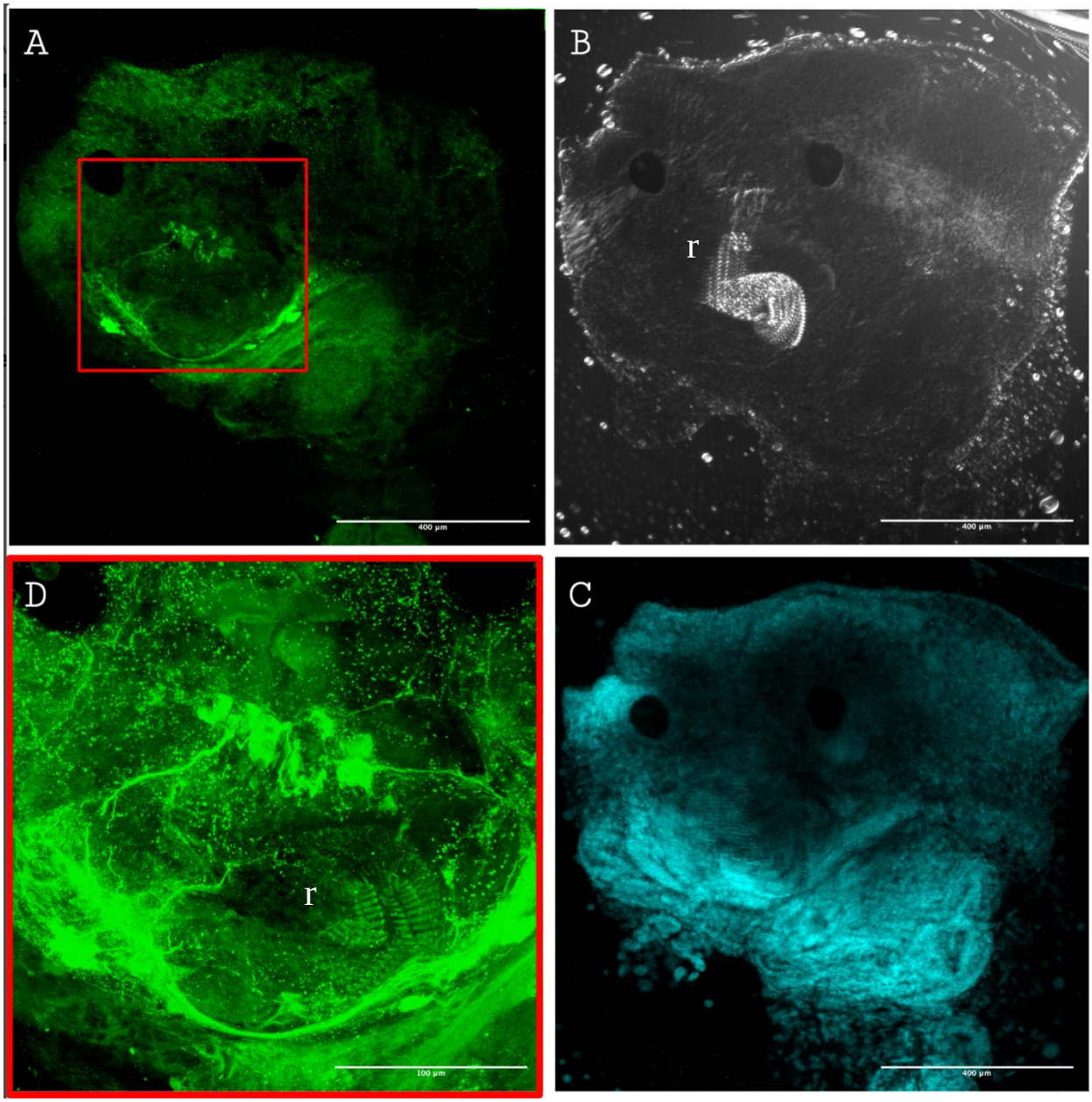

Figure 8. Confocal images of a 3–4 day old *B. glabrata* juvenile treated with anti-serotonin. r = radula. Serotonin-IR is shown in green, and Hoechst is shown with the blue. A, B, & C respectively correspond to the serotonin-IR, white light, and Hoechst binding within the snail tissue taken using a 10x objective. The red box on (A) corresponds to (D), magnifying the region with a 40x oil lens.

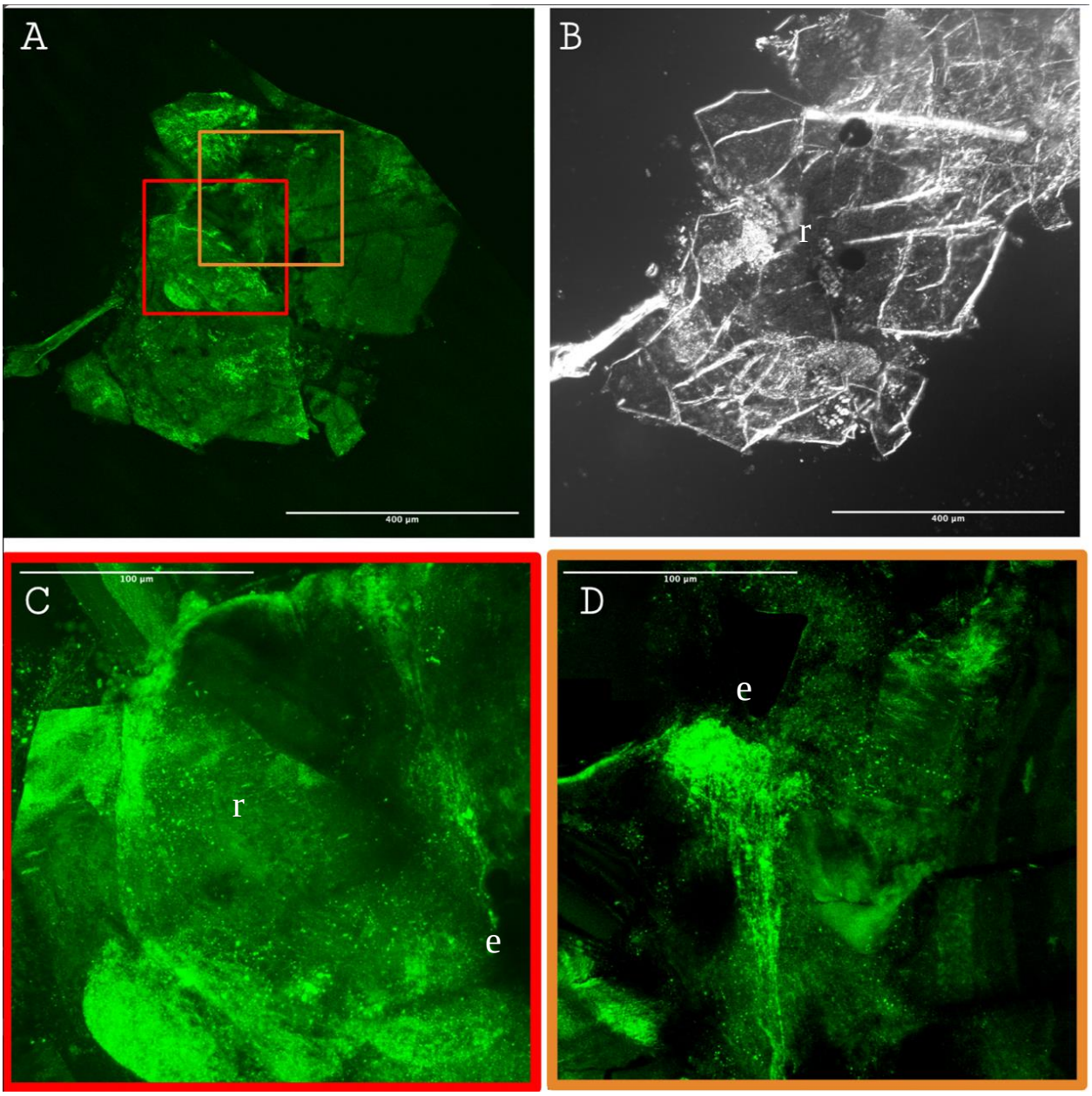

Figure 9. Confocal images of a 5-to-6-day old juvenile treated with anti-serotonin. r = radula. e = eyespot. Serotonin-IR is shown in green. The top row consists of the various images of the snail taken using a 10x objective. A & B respectively correspond to the serotonin-IR and white light images. The boxes on (A) correspond to regions which have been magnified in the images in the bottom row, using a 40x oil lens. The red box corresponds to (C), and the orange box corresponds to (D).

Though the serotonergic CNS in the 5-to-6-day old *B. glabrata* juvenile is not as extensive as the one in the 3-to-4-day old juveniles, the major circular structure seems to be maintained (Fig 9). The serotonin-IR seen at this age does not appear to be as complex as the CNS seen in the 3-to-4-day old juveniles (Fig 7; 8). However, the localization of the CNS organized in this circular manner around the radula appears to be conserved (Fig 9.C). Unfortunately, the head-foot region of this snail is partly covered by shell fragments, but the CNS is still able to be visualized. The serotonin-IR in this 5-to-6-day old juvenile depicts the connective-like structures spanning throughout the CNS. Additionally, the oval-shaped structures displaying serotonin-IR at the base of the head-foot region can also be seen at this age, though it appears to be more concentrated at this age. Though it is not as clear as with the younger snails, there does seem to be innervation of the eyespot in 5-to-6-day old juveniles as well.

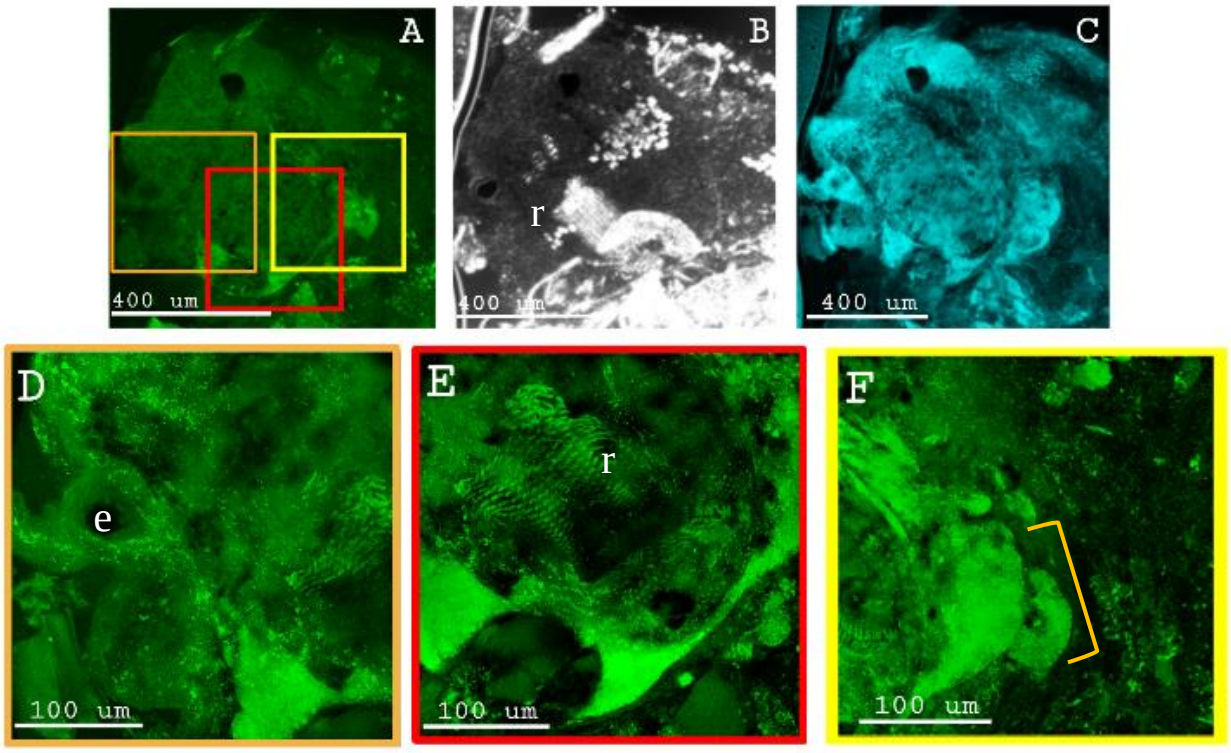

Figure 10. Confocal images of an 8–9 day old *B. glabrata* juvenile treated with anti-serotonin. r = radula. e = eyespot. Serotonin-IR is shown in green, and Hoechst is shown with the blue. The top row consists of

the various images of the snail taken using a 10x objective. A, B, & C respectively correspond to the serotonin-IR, white light, and Hoechst binding within the snail tissue. The boxes on (A) correspond to regions which have been magnified in the images in the bottom row, using a 40x oil lens. The orange box corresponds to (D), the red box corresponds to (E), and the yellow box corresponds to (F).

The condensing of the serotonergic CNS seen in the younger juveniles (Fig 7.E; 8.D; 9.C) does not seem to continue as juvenile development progresses. The serotonin-IR in the 8-to-9 day old juvenile snail (Fig 10) takes on a more rectangular shape while still being localized around the radula. However, these ganglion-like structures seen in the younger juveniles (Fig 8.D; 9.C) appear much more condensed at this stage. In the younger juveniles, there were multiple long axons passing throughout these whereas it seems that the axons have gotten shorter to correspond with the growth of these ganglion-like structures. They are still located along the base of the head-foot region but are now further apart from each other (Fig 10.D; F). These crescent-shaped areas of high serotonin-IR remain connected by the connective-like structures (Fig 10.E).

Finally, in figure 10.F, there seems to be a layering of ganglion-like structures atop one another. The yellow bracket is encompassing this smaller and rounder ganglion-like structure on figure 10.F and should help to visualize these two distinct structures. This rounder structure is located dorsally to the bigger, crescent-shaped structure. This indicates the growing complexity within the CNS as the juvenile progresses through development. In addition, there are axons which appear to arise from the ganglion-like structure (Fig 10.D). These appear to travel to the anterior end of the head-foot region to innervate the eyespot, the tentacle, and the foot.

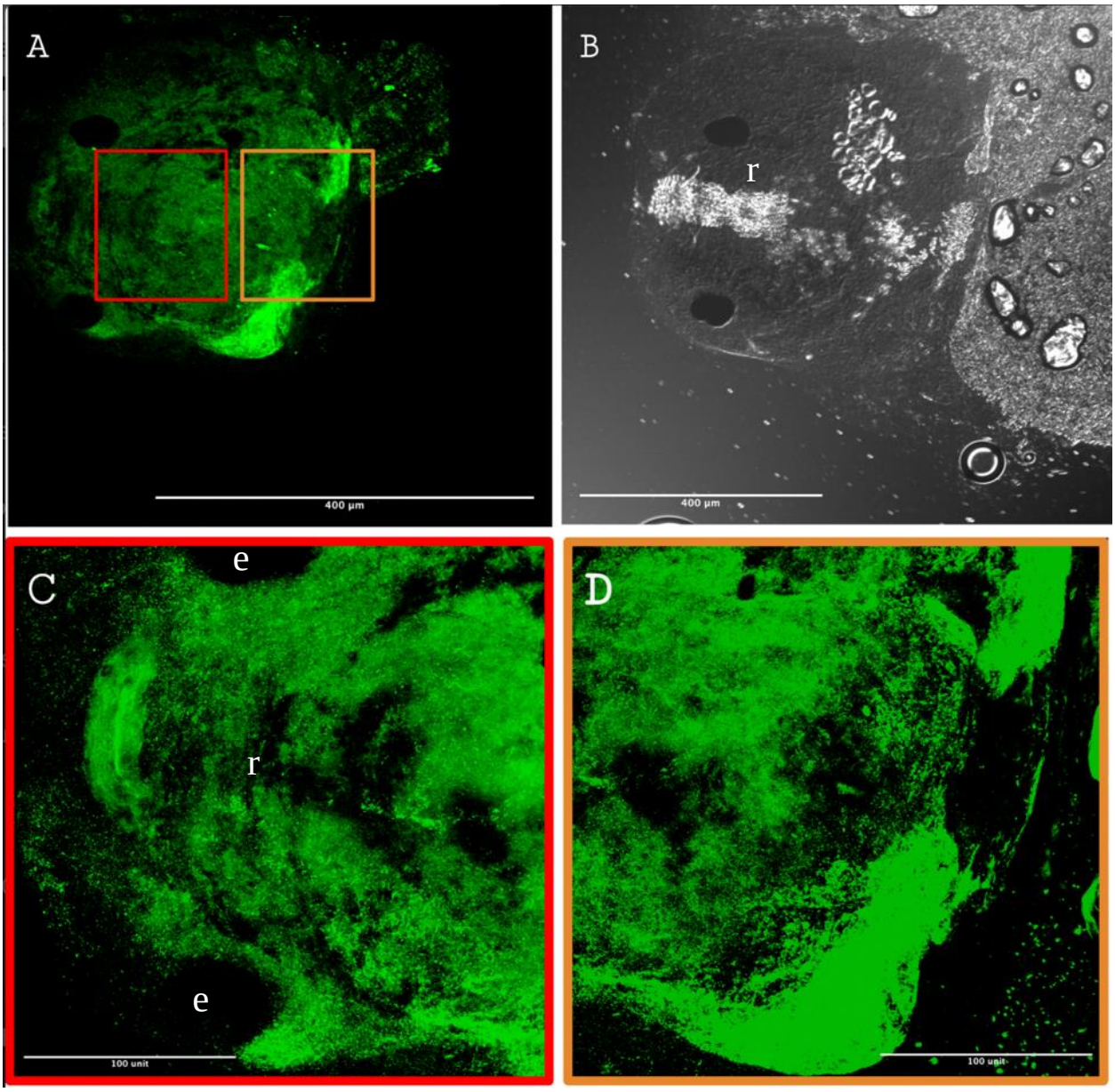

Figure 11. Confocal images of an 11-to-12-day old juvenile treated with anti-serotonin. r = radula. e = eyespot. Serotonin-IR is shown in green. The top row consists of the various images of the snail taken using a 10x objective. A & B respectively correspond to the serotonin-IR and white light images. The boxes on (A) correspond to regions which have been magnified in the images in the bottom row, using a 40x oil lens. The red box corresponds to (C), and the orange box corresponds to (D).

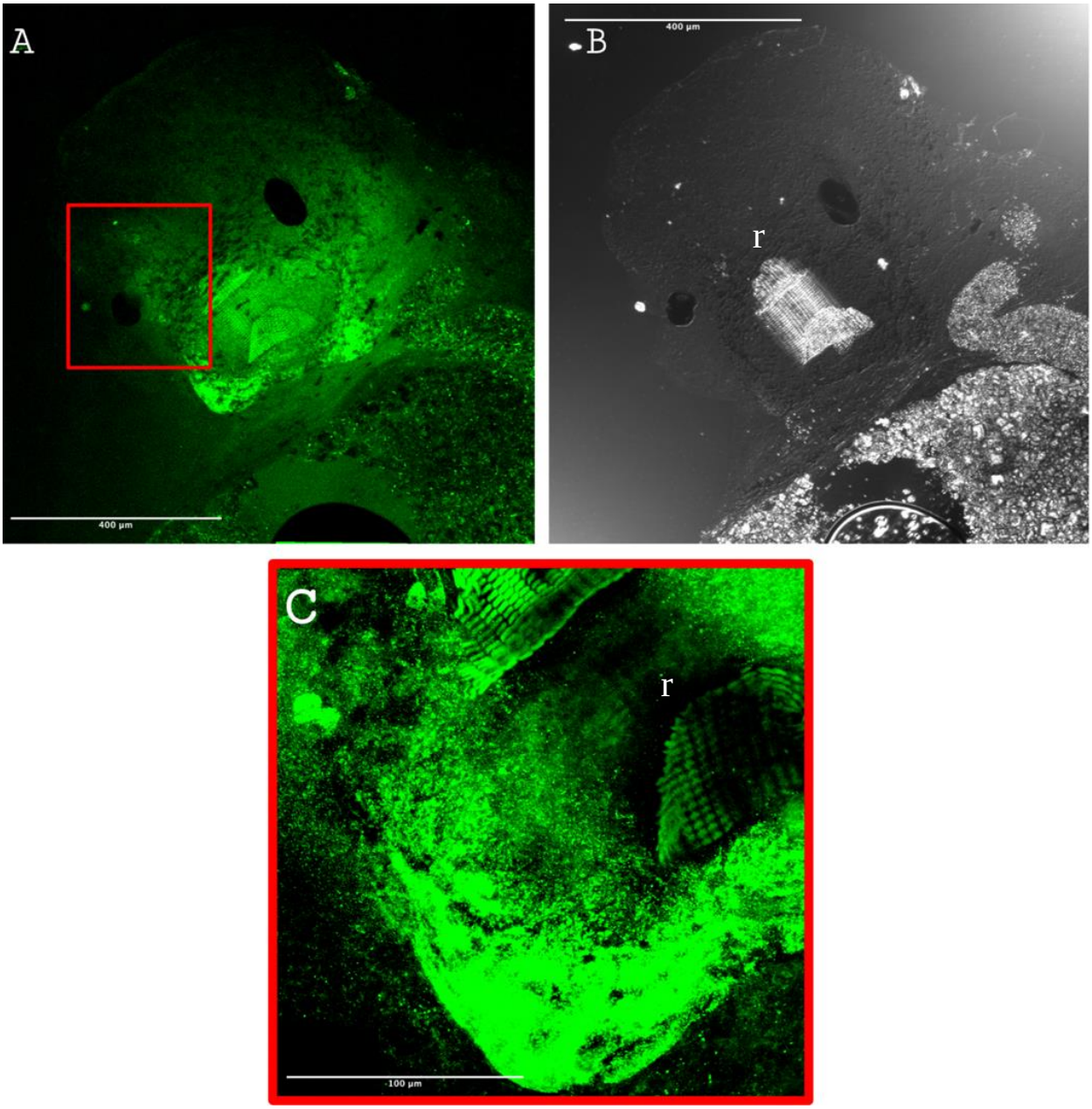

Figure 12. Confocal images of a 13-to-14-day old juvenile treated with anti-serotonin. r = radula. Serotonin-IR is shown in green. The top row consists of the various images of the snail taken using a 10x objective. A & B respectively correspond to the serotonin-IR and white light images. The box on (A) corresponds to a region which has been magnified in the image on the bottom row, using a 40x oil lens. The red box corresponds to (C).

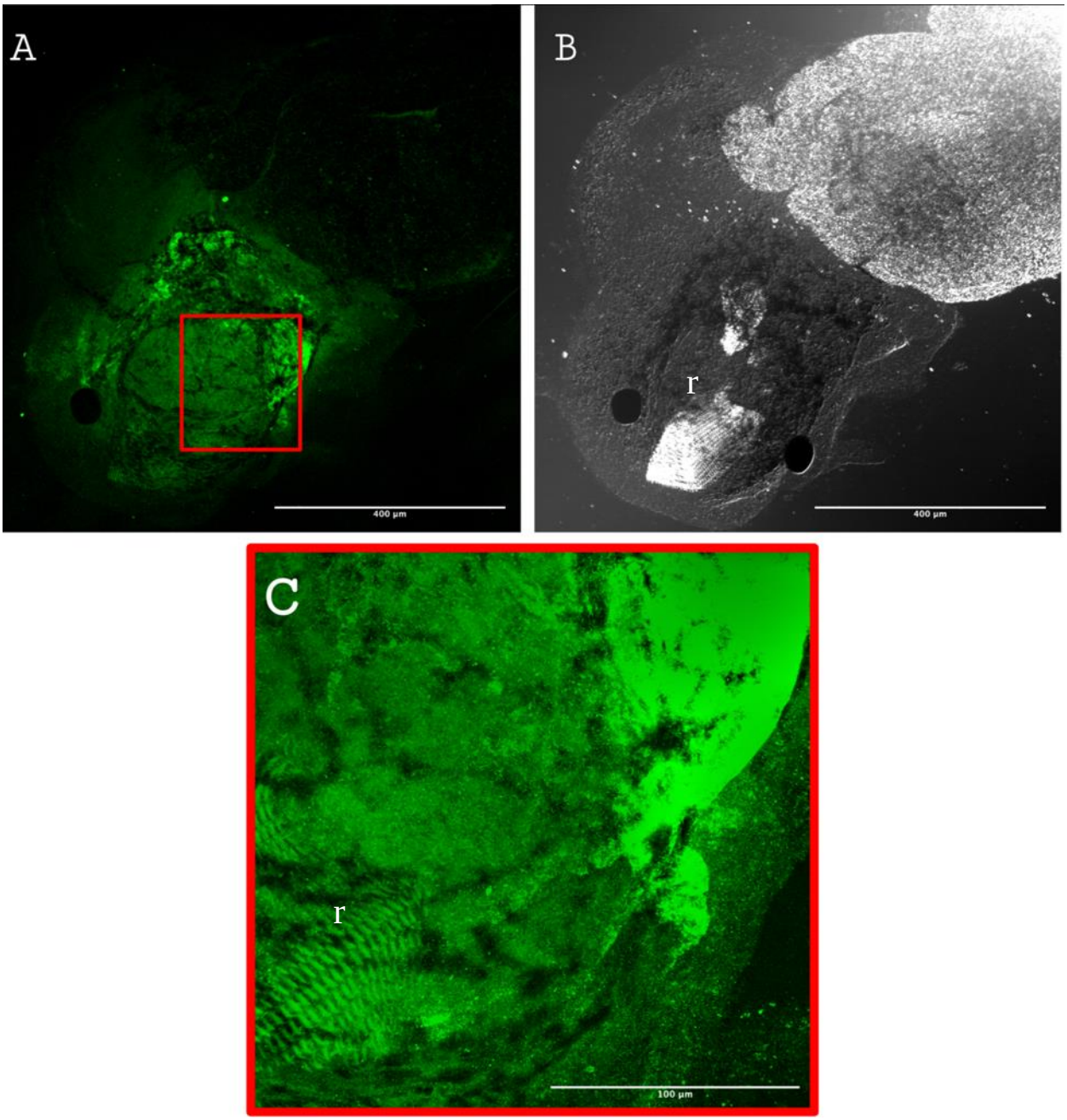

Figure 13. Confocal images of a 15-to-16-day old juvenile treated with anti-serotonin. r = radula. Serotonin-IR is shown in green. The top row consists of the various images of the snail taken using a 10x objective. A & B respectively correspond to the serotonin-IR and white light images. The box on (A) corresponds to a region which has been magnified in the image on the bottom row, using a 40x oil lens. The red box corresponds to (C).

Overall, there are multiple key features of the serotonin-IR that remain consistent within the older *B. glabrata* juveniles (11 to 16 days old). This includes the crescent-shaped ganglion-like structures at the base of the head-foot region (Fig 11.D; 12; 13), and the axons connect those to the eyespots. Finally, the localization of the CNS around the radula stays consistent throughout this period of juvenile development. However, a key difference that can be observed is that these ganglion-like areas get larger as the juvenile grows. The serotonin-IR increases in intensity as they grow, indicating that these structures seem to become denser. These structures are at their largest size and most-defined within the 15-to-16 day old juvenile comparative to the younger juveniles (Fig 13.C). Furthermore, as the older snails develop more pigment, visualization of the serotonin-IR underneath the tegument becomes difficult. This can be seen in the 15-to-16-day old snails (Fig 13.A) which has dark pigment throughout its head-foot region and cause shadows on the serotonin-IR.

Hoechst

In addition to the anti-serotonin, a Hoechst stain was also used to dye DNA in an effort to identify individual neuronal cell bodies within the serotonergic CNS. However, the Hoechst dye tended to stain all of the cells within the snail tissue, leading to poor specificity of the cells in the CNS. Nevertheless, there are a few confocal images included which show Hoechst binding within the tissue. The dye in these images appear to correspond to the regions showing high concentrations of serotonin-IR (Fig 7.C; 8.C; 10.C).

DISCUSSION

CNS development and gangliogenesis in B. glabrata *juveniles*

From the immunofluorescence data collected from juveniles at various stages of development, there were some key patterns uncovered regarding CNS development within these snails. This study found that the serotonergic CNS of early juveniles (Fig 7) resembles the serotonergic nervous system described in the late veliger stage embryo (Fig 6). Both depict an extensive network of neuronal cell bodies and long axons branching throughout the head-foot region. This provides preliminary evidence suggesting that *B. glabrata* may not undergo much neurobiological change within the CNS during the metamorphosis phase of development, during which embryos develop into juveniles. Within various other gastropod species, this time period is related to the loss of certain neural structures or even neuronal cell death (Croll, 2009).

Moreover, the appearance and development of certain structures within the developing *B. glabrata* juvenile should be noted. Namely, the crescent-shaped structures located at the base of the head-foot region have been described as ganglion-like within juveniles aged 8 to 9 days old and above (Fig 10). However, these structures can be seen within juveniles are young as 3-to-4-days old, although they look far less developed within these young juveniles (Fig 7. E; 8. D). The existence of these ganglion-like structures so early suggests that the development of certain ganglia might arise soon after hatching. The fact that the structures displaying distinct serotonin-IR develop alongside juvenile development leads me to believe that these might be the first ganglia to arise in *B. glabrata*.

This, however, conflicts with the current, accepted model for gastropod CNS development. To reiterate, the cerebral ganglia are thought to develop first followed shortly by

the pedal ganglia (Jacob, 1984). However, the position of the ganglion-like structures suggests that these might be the developing parietal ganglia. This contrast in data could be explained in two ways. One, the research that was used to summarize the order of ganglionic development was done in species like *Aplysia* and *I. obsoleta* whose embryonic development varies vastly from that of *B. glabrata* (Marois & Carew, 1990). This would suggest that direct-developing species might undergo a different sequence of gangliogenesis. On the other hand, the second theory is that the methods used to visualize the developing CNS in *B. glabrata* are biased as it only shows the serotonergic nervous system, which is only a specific portion of the *B. glabrata* CNS. A more thorough analysis of the developing *B. glabrata* nervous system should be conducted using various markers for the nervous system.

Even though no definitive conclusions can be made regarding the exact timeline for gangliogenesis, the results of this study uncovered a transition from a condensed, circular CNS structure seen in the younger juveniles (Fig 7; 8; 9) to the less extensive, rectangular-shaped CNS appearing in the juveniles from 8 to 16 days old (Fig 10; 11; 12; 13). The results of this study suggest that this transition period occurs sometime between 6 to 8 days of juvenile development. Following this transition period, older juveniles seem to consistently share this morphology of developing CNS which branches from one eyespot to another using axons arising from these ganglion-like structures. This process may perhaps be highlighting the start of gangliogenesis, but much future testing is required to get a more thorough understanding of this process within *B. glabrata* juveniles.

Serotonin-IR in B. glabrata *juveniles*

Prior to this study, serotonin-IR had been detected within the embryonic and adult *B. glabrata* CNS, but there was minimal research regarding whether serotonin was present within juveniles. This study showed that serotonin-IR can indeed be detected within the juvenile *B. glabrata* CNS. While immunofluorescence data was meant to give a better understanding of gangliogenesis, the results also provided specific information regarding which regions contained serotonin. Knowing the localization of serotonin along with the various areas innervated by serotonin-IR cells may be used to identify potential physiological functions that serotonin might be playing in the juvenile *B. glabrata* CNS.

Essentially all the juveniles that were used for immunofluorescence showed serotonin-IR surrounding the eyespot. This consistent expression of serotonin within this region suggests it may be an important neurotransmitter regulating vision within the *B. glabrata* juveniles. Additionally, serotonin-IR was displayed in axons innervating the foot suggesting that serotonin might play a role within motor control. This is supported by prior research done showing that serotonin increases ciliary-rotational behavior in *B. glabrata* embryos along with triggering an increase in velar contractions and subsequent swimming behavior (Diefenbach et al., 1991; Croll, 2009). Serotonin-IR is displayed within the tentacles of the older juveniles, and as the tentacles are important for olfactory sensing and detection of food, serotonin might play a role within this sensory mechanism.

Limitations

Due to the study's novel nature, there were quite a few limitations throughout this project. The greatest obstacle would be lack of research within the literature investigating gangliogenesis in gastropods. Since gangliogenesis has not been documented yet, analysis of the

immunofluorescence results was quite exploratory as it was unknown what the process should look like. There was an overall lack of information regarding the details of the pattern of immunofluorescence seen during gangliogenesis, and the process of analyzing data became much more prolonged and meticulous to ensure that no potentially important details were missed. Additionally, the lack of immunofluorescence done with juvenile snails led to many periods of trial and error to perfect the protocol (please refer to appendix I to learn more about the troubleshooting process). Optimizing the protocol took up much of the time spent working on this project. Overall, the lack of research conducted with *B. glabrata* juveniles was the cause of the limitations encountered within this project.

Future research

Future research should focus on developing a more comprehensive timeline for CNS development and gangliogenesis within *B. glabrata* juveniles. Immunofluorescence must be done with the various ages which were not included within this study. Examining the CNS of older juveniles should absolutely be an avenue for future testing. Moreover, molecular mechanisms implicated throughout various stages of embryonic and juvenile development must be explored. This should go in hand with any novel hypotheses that may arise from the RNA-seq data analysis. In general, there is much more to be studied regarding these snails and their complex nervous systems.

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Works Cited

Acker, M. J., Habib, M. R., Beach, G. A., Doyle, J. M., Miller, M. W., & Croll, R. P. (2019). An immunohistochemical analysis of peptidergic neurons apparently associated with reproduction and growth in *Biomphalaria alexandrina*. *General and Comparative Endocrinology*, 280: 1–8. https://doi.org/10.1016/j.ygcen.2019.03.017

Alavi, S. M. H., Nagasawa, K., Takahashi, K. G., & Osada, M. (2017). Pharmacology and molecular identity of serotonin receptor in bivalve mollusks. *Serotonin - A Chemical Messenger Between All Types of Living Cells*, Ch. 2: 7–31. London: Intech. https://doi.org/10.5772/intechopen.69680

Battenfeld, C., Bielefeld, U., Becker, W., & Bretting, H. (1993). Light- and electron-microscopical studies on the embryonic development of the snail *Biomphalaria glabrata*. *Invertebrate Biology,* 122: 313-325. https://doi.org/10.1111/j.1744-7410.2003.tb00096.x

Bautista-Ruiz, L. (2020). Guide for the Embryonic Development of *Biomphalaria glabrata* [PDF]. Appleton: Lawrence University.

Bedford, L. (1966). The electron microscopy and cytochemistry of oogenesis and the cytochemistry of embryonic development of the prosobranch gastropod *Bembicium nanum.* L. *Development,* 15 (1): 15-37. https://doi.org/10.1242/dev.15.1.15

Bonar, D. B. (1978). Ultrastructure of a cephalic sensory organ in larvae of the gastropod *Phestilla sibogae* (Aeolidacea, Nudibrachia). *Tissue & cell*, 10 (1): 153–165. https://doi.org/10.1016/0040-8166(78)90014-9

Braubach, O. R., Dickinson, A. J., Evans, C. C., & Croll, R. P. (2006). Neural control of the velum in larvae of the gastropod, *Ilyanassa obsoleta*. *Journal of Experimental Biology,* 209 (23): 4676–4689. https://doi.org/10.1242/jeb.02556

Bulloch, A. G. M. (1985). Development and plasticity of the molluscan nervous system. *The Mollusca,* 8: 335–408. https://doi.org/10.1016/B978-0-12-751408-6.50011-4

Bullock, T.H. (1965). *Structure and Function of the Nervous Systems of Invertebrates.* San Francisco: Freeman Press.

Carney, T., & Verdonk, N. (1970). The early development of the snail *Biomphalaria glabrata* and the origin of the head organs. *Netherlands Journal of Zoology*, 20 (1): 93-121.

Chia, F. S., & Koss, R. (1984). Fine structure of the cephalic sensory organ in the larva of the nudibranch *Rostanga pulchra* (Mollusca, Opisthobranchia, Nudibranchia). *Zoomorphology,* 104: 131–139. https://doi.org/10.1007/BF00312131

Croll, R. P. (2000). Insights into early molluscan neuronal development through studies of transmitter phenotypes in embryonic pond snails. *Microscopy Research and Technique,* 49 (6): 570–578. https://doi.org/10.1002/1097-0029(20000615)49:6%3C570::aid-jemt7%3E3.0.co;2-q

Croll, R. P., & Voronezhskaya, E. E. (1995). Early FMRFamide-like immunoreactive cells in gastropod neurogenesis. *Acta Biologica Hungarica,* 46 (2-4): 295–303.

Croll, R. P. & Voronezhskaya, E. E. (1996). Early elements in gastropod neurogenesis. *Developmental Biology,* 173 (1): 344–347. https://doi.org/10.1006/dbio.1996.0028

D'Asaro, C. N. (1969). The comparative embryogenesis and early organogenesis of *Bursa corrugata* Perry and *Distorsio clathrata* Lamarck (Gastropoda: Prosobranchia). *Malacologia*, 9: 349-389.

Delgado, N., Vallejo, D., & Miller, M. W. (2012). Localization of serotonin in the nervous system of *Biomphalaria glabrata*, an intermediate host for schistosomiasis. *Journal of Comparative Neurology*, 520 (14): 3236–3255. https://doi.org/10.1002/cne.23095

Demian, E.S., & F. Yousif. (1975). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae) V. Development of the nervous system. *Malacologia*, 15 (1): 29-42.

Dickinson, A. J. G., & Croll, R. P. (2003). Development of the larval nervous system of the gastropod *Ilyanassa obsoleta*. *Journal of Comparative Neurology*, 466 (2): 197– 218. https://doi.org/10.1002/cne.10863

Dickinson, A. J. G., Croll, R. P., & Voronezhskaya, E. E. (2000). Development of embryonic cells containing serotonin, catecholamines, and FMRFamide-related peptides in *Aplysia californica*. *The Biological bulletin*, 199 (3): 305–315. https://doi.org/10.2307/1543187

Dickinson, A., Nason, J., & Croll, R. (1999). Histochemical localization of FMRFamide, serotonin and catecholamines in embryonic *Crepidula fornicata* (Gastropoda, Prosobranchia). *Zoomorphology*, 119: 49–62. https://doi.org/10.1007/s004350050080

Diefenbach, T. J., Koehncke, N. K., & Goldberg, J. I. (1991). Characterization and development of rotational behavior in *Helisoma* embryos: Role of endogenous serotonin. *Journal of Neurobiology*, 22: 922–934. https://doi.org/10.1002/neu.480220905

Herbert, D., & Kilburn, D. (2004). *Field guide to the land snails and slugs of eastern South Africa*. Pietermaritzburg, South Africa: Natal Museum.

Hyman, L.H. (1967). *The Invertebrates Volume IV: Mollusca I.* New York: McGraw-Hill Book Co.

Glebov K., Voronezhskaya E. E., Khabarova M. Y., Ivashkin E., Nezlin L. P., & Ponimaskin, E. G. (2014). Mechanisms underlying dual effects of serotonin during development of *Helisoma trivolvis* (Mollusca). *BMC Developmental Biology*, 14: 14. https://doi.org/10.1186/1471-213X-14-14

Glover, J. C., & Kramer, A. P. (1982). Serotonin analog selectively ablates indentified neurons in the leech embryo. *Science*, 216: 317–319. https://doi.org/10.1126/science.7063890

Goldberg, J. I., & Kater, S. B. (1989). Expression and function of the neurotransmitter serotonin during development of the *Helisoma* nervous system. *Developmental Biology*, 131: 483-495. https://doi.org/10.1016/S0012-1606(89)80019-3

Kempf, S.C. (2008). Serotonergic, sensory modifications in the apical ganglion during development to metamorphic competence in larvae of the dendronotid nudibranchs *Melibe leonina* and *Tritonia diomedea*. *Microscopy and Research Technique*, 71: 863-869. https://doi.org/10.1002/jemt.20630

Kempf, S. C., Page, L. R., & Pires, A. (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. *Journal of Comparative Neurology*, 386:

507-528. https://doi.org/10.1002/(SICI)1096-9861(19970929)386:3<507::AID-CNE12>3.0.CO;2-7

Kikuchi, K.K., Okazaki, K., Kawano, T., & Ribeiro, A. A. G. F. C. (1988). Estudos ultraestruturais de embrigo de *Biomphalaria glabrata*. *Publicaqilo IPEN*, 203:1-10.

Kuang, S., Doran, S. A., Wilson, R. J., Goss, G. G., & Goldberg, J. I. (2002). Serotonergic sensory-motor neurons mediate a behavioral response to hypoxia in pond snail embryos. *Journal of Neurobiology*, 52 (1): 73-83.

Marois, R. (1989). Embryonic development of *Lymnaea stagnalis*: General, neuronal and behavioral aspects. *M. Sc. Thesis, Halifax, N. S: Dalhousie University*.

Marois R., & Carew T. J. (1990). The gastropod nervous system in metamorphosis. *Journal of Neurobiology,* 21: 1053-1071.

Marois R., & Carew T. J. (1997). Fine structure of the apical ganglion and its serotonergic cells in the larva of *Aplysia californica*. *Biological bulletin,* 192: 388–398.

Mansour, T. A., Habib, M. R., Rodríguez, L. C. V., Vázquez, A. H., Alers, J. M., Ghezzi, A., Croll, R. P., Brown, C. T., & Miller, M. W. (2017). Central nervous system transcriptome of *Biomphalaria alexandrina*, an intermediate host for schistosomiasis. *BMC Research Notes*, 10: 729. https://doi.org/10.1186/s13104-017-3018-6

Page, L. R. (1997). Larval shell muscles in the abalone *Haliotis kamtschatkana. Biological Bulletin*, 193: 30–46.

Page, L. R. (2002). Apical sensory organ in larvae of the patellogastropod *Tectura scutum*. *Biological Bulletin*, 202: 6–22.

Page, L. R. (2003). Gastropod ontogenetic torsion: developmental remnants of an ancient evolutionary change in body plan. *Journal of Experimental Zoology. Part B, Molecular and developmental evolution*, 297 (1): 11–26. https://doi.org/10.1002/jez.b.12

Portilho, L. G., Duarte, B. C. D., Queiroz, F. R., Ribeiro, T. H. C., Jeremias, W. de J., Babá, E. H., Coelho, P. M. Z., Morais, E. R., Cabral, F. J., Caldeira, R. L., & Gomes, M. de S.. (2019). Genome-wide identification, characterisation and expression profiling of the ubiquitin-proteasome genes in *Biomphalaria glabrata*. *Memórias Do Instituto Oswaldo Cruz*, 114(Mem. Inst. Oswaldo Cruz, 2019 114), e190052. https://doi.org/10.1590/0074-02760190052

Raven, C.P. (1966). *Morphogenesis: The analysis of molluscan development.* Oxford: Pergamon Press.

Richter, S., Loesel, R., Purschke, G., Schmidt-Rhaesa, A., Scholtz, G., Stach, T., Vogt, L., Wanninger, A., Brenneis, G., Döring, C., Faller, S., Fritsch, M., Grobe, P., Heuer, C. M., Kaul, S., Møller, O. S., Müller, C. H., Rieger, V., Rothe, B. H., Stegner, M. E., & Harzsch, S. (2010). Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary. *Frontiers in Zoology*, *7*: 29. https://doi.org/10.1186/1742-9994-7-29

Santhanagopalan, V., & Yoshino, T. P. (2000). Monoamines and their metabolites in the freshwater snail *Biomphalaria glabrata. Comparative Biochemistry and Physiology. Part A: Molecular & Integrative Physiology*. 125: 469-478.

Smith, F. G. M. (1935). *The development of* Patella vulgata. London: Philosophical Transactions of the Royal Society B.

Voltzow, J. (1994). *Microscopic Anatomy of Invertebrates. Vol. V: Mollusca I: Gastropoda: Prosobranchia.* New York: Wiley-Liss, Inc.

Voronezhskaya, E. E., Hiripi, L., Elekes, K., & Croll, R. P. (1999). Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis* I: embryonic development of dopaminergic neurons and dopamine dependent behaviors. *Journal of Comparative Neurology*, 404: 297–309.

Appendix I: Methods

Protocol to remove the shell of juvenile snails:

By the late veliger to juvenile stage of development, the shell was hardened and was opaque and prevented proper visualization of the serotonin-IR cells within the snail tissue. This prompted the removal of the shell after fixation. This process was done under the Nikon SMZ745 dissection microscope using a variety of tools including a scalpel, flexible forceps, beading needles, and a small 10 mL beaker. The snails were pipetted onto a petri dish with a small volume of sPBS, and the protruding shell was first cut with the scalpel and separated from the rest of the snail's body. The bottom of the beaker was next used to place pressure and crack the hard shell very gently. With the shell now in broken pieces around the snail, the flexible forceps and the beading needles helped to carefully move the shell fragments away from the snail tissue. This was a delicate, meticulous process as the tissue itself was extremely soft and prone to tearing.

Troubleshooting the immunofluorescence protocol:

The immunofluorescence protocol detailed in the methods section was modified from an existing protocol to obtain serotonin-IR, and I received it from Helen Threkheld, a previous member of the Humphries lab. However, this protocol was meant for older embryos, and as there was a minimal amount of immunofluorescence work performed on juvenile snails, optimizing this protocol became an integral part of this project. Three parts of the original protocol were optimized with the general goal to obtain better permeabilization of tissue to aid antibody binding: (1) incubation time for the primary antibody, (2) concentration of the trypsin in sPBS, and (3) concentration of TX in sPBS for the various wash cycles throughout the protocol.

1. Primary incubation time

The initial protocol urged a 24-hour incubation with the primary antibody—the anti-serotonin in rabbit. While this incubation time worked well for embryos along with the rest of the protocol, there was poor serotonin-IR seen with juveniles. Thus, this time was modified from a 24-hour to a 48-hour incubation to encourage better permeabilization of the tissue. Doubling the amount of the time the snails were in the primary antibody solution worked in increasing the amount of serotonin-IR seen within the juveniles, so this step was incorporated into the protocol.

2. Trypsin concentration

The original protocol consisted of a 0.5% trypsin in sPBS incubation directly following fixation, and as this step is intended specifically for permeabilization of the tissue to aid antibody binding, I troubleshooted this to test whether a modification was necessary for better serotonin-IR. I tested the effects of doubling the concentration of trypsin in sPBS along with incremental increases in the incubation time. The trials consisted of these following conditions: (I - control) 0.5% trypsin in sPBS for 1 minute, (II) 0.5% trypsin in sPBS for 3 minutes, (III) 0.5% trypsin in sPBS for 5 minutes, (IV) 1% trypsin in sPBS for 1 minute, and (V) 1% trypsin in sPBS for 3 minutes.

The results of these trials showed better visualization as a result of increased permeability for conditions (III), (IV), and (V). However, the longer incubation time and higher trypsin concentration in sPBS for condition (V) made the tissue much more delicate, and the head-foot region of the snail was prone to become misshapen. With that, another trial was performed with just conditions (III) and (IV). These results showed that condition (IV) with double the

concentration of trypsin had the best permeabilization of the tissue and subsequent serotonin-IR and was able to maintain an intact head-foot structure.

3. Concentration of Triton X-100 in sPBS

The protocol asked for a 0.1% triton wash to be used during this process. However, it was discovered that washing with 4% triton was able to provide much better immunofluorescence results as it increased the permeability of the tissue for antibody binding.

	Treatment	**Implemented?**	**The original step**
Primary antibody incubation *(α-serotonin & α-synapsin diluted in bovine serum albumin)*	48-hour incubation	Yes	24-hour incubation
Triton-x 100 washes *(Triton-x 100 diluted in snail phosphate buffer saline)*	4% for washes	Yes	0.1% for washes throughout and using 4% only for initial permeabilization
Trypsin incubation *(Porcine trypsin diluted in snail phosphate buffer saline)*	0.5% for 3 mins	No	0.5% for 1 minute
	0.5% for 5 mins	No	
	1% for 1 min	Yes	
	1% for 3 mins	No	

Table 1. Various permeabilization treatments and whether they were implemented or not based off of ability to visualize serotonin with immunofluorescence.