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The marine mud snail, *Ilyanassa obsoleta*, displays a biphasic life cycle. The initial phase consists of weakly swimming larval animals with ciliated feeding structures on the head, the velar lobes. The second phase consists of adult animals residing on intertidal mudflats as obligate omnivores. Metamorphosis, the event that links these life history stages, is initiated by external stimuli but coordinated by internal signaling molecules. Internally, molluscan metamorphosis includes neurotransmitters that modify the behavior of cells in neural circuits. For several species of molluscs, including abalone and scallops, larval metamorphosis can be induced by the external application of the neurotransmitter, γ-aminobutyric acid (GABA). In this situation, GABA is presumed to mimic the actions of food-related ligands that bind to external sensory receptors. GABA is the only neurotransmitter known to act in such a fashion. In contrast, results of our preliminary experiments with *I. obsoleta* strongly support the hypothesis that GABA works internally in this species as a neurotransmitter in the larval central nervous system (CNS) to inhibit metamorphosis. External application of GABA to metamorphically competent larvae of *I. obsoleta* does not elicit metamorphosis, but does repress metamorphic induction. I utilized a battery of GABAergic reagents to modify internal GABAergic activity to address the hypothesis that GABA inhibits metamorphosis due to its actions within the larval CNS. My results strongly suggest that GABA inhibits metamorphosis in this molluscan model organism. My results indicate that GABA acts

downstream from or directly on serotonergic neurons to decrease the metamorphosis-inducing effects of serotonin.

GABA IS AN INHIBITORY NEUROTRANSMITTER IN THE NEURAL CIRCUIT REGULATING METAMORPHOSIS IN *ILYANASSA OBSOLETA*

by

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Approved by

_____
Committee Chair

Dedicated to Rolito, Jazmin and Lorna Biscocho for all of their support

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

The marine snail *Ilyanassa obsoleta* is an agent of sediment disturbance that can greatly influence intertidal mudflat ecosystems. *Ilyanassa obsoleta* largely spends its adult life feeding and mating. Its ecological roles as both a decomposer and a scavenger influence microfaunal community biomass and species diversity in marine environments as the snails graze on the top layers of soft sediment in their habitat (Harper 1969; Connell 1975). This grazing behavior displaces sediment particles, thus promoting nitrogen cycling and algal growth (Connor 1982). The snails also influence larval settlement and juvenile recruitment of amphipods, copepods and polychaetes by ingesting them or by burying them in the marine sediment (Hunt et al. 1987). In early spring, females lay egg capsules on hard benthic substrates (Ruppert and Fox 1988). The embryos develop for a few days within egg capsules until they hatch as freely swimming veliger larvae in the warming waters above the mudflats (Scheltema 1961).

Metamorphosis in Molluscan Gastropods

When gastropod larvae are physiologically capable of, or competent to metamorphose, an encounter with the proper environmental cues or compounds activates chemosensory receptors that evoke neural changes that initiate this developmental

transformation (Leise and Hadfield 2000). Metamorphosis in gastropods is a complex and irreversible process requiring multiple changes in anatomy and cellular functions. During this process, the animal undergoes reorganization of the brain ganglia and loses its velar lobes that had been essential to its larval mobility and feeding (Scheltema 1961). Other morphological transformations occur in portions of the digestive tract, mantle, and structures no longer necessary in juveniles (Bickell and Kempf 1983). Metamorphosis in *Ilyanassa* is typical of the general gastropod pattern although these snails retain their larval shells during this process. *Ilyanassa* has been a model organism for the study of molluscan development for over a century (Collier 2002), but the neuronal circuitry responsible for initiating its metamorphosis from planktonic larva to benthic juvenile has not been fully mapped.

Signaling Molecules and Neurotransmitters

Metamorphosis in marine invertebrates is initiated by external stimuli and coordinated by internal signaling mechanisms. To begin metamorphosis, larvae of many marine molluscs typically respond to both physical and biological features of their environments (Scheltema 1961). For instance, sediment grain-size (a physical factor), can be an important factor for the initiation of metamorphosis in *Ilyanassa*. Also for *Ilyanassa,* species of diatoms in their natural environments release water soluble compounds, or kairomones (biological factors), that are linked to the induction of metamorphosis (Leise et al. 2009a). Internally, molluscan metamorphosis is undoubtedly controlled by hormonal activity and the action of dedicated neural circuits (Hadfield

1986; Couper and Leise 1996; Abbott et al. 1997; Fingerman 1997; Froggett and Leise 1997). It is critical to identify and understand the role of metamorphic signaling molecules and how they work in conjunction with one another, because anthropogenic pollutants in the intertidal waterways could interfere with natural cues, leading to undesirable developmental problems in populations of *I. obsoleta* (Deschaseaux et al. 2010). While knowledge of the neural circuits that control metamorphosis of gastropods is limited, there are several neurotransmitters, signaling molecules synthesized endogenously in neurons and responsible for their chemical communication with target cells, that play an integral role in the promotion and inhibition of metamorphosis (Pires and Hadfield 1993; Couper and Leise 1996; Froggett and Leise 1999). To date, we have characterized the metamorphic functions of two neurotransmitters, serotonin (5-HT) and nitric oxide (NO); this thesis addresses the function of yet another neurotransmitter, γ-aminobutyric acid (GABA).

Pharmacological Studies

Results of earlier pharmacological work have shown that a major excitatory neurotransmitter, serotonin (5-HT), can induce metamorphosis in *I. obsoleta* and related organisms of the same class (Levantine and Bonar 1986; Leise and Cahoon 2012). The apical ganglion (AG), a highly conserved mass of neuronal cell bodies in the larval CNS, contains serotonergic neurons that innervate the velar lobes (Lacalli 1994; Kempf et al. 1997; Marois and Carew 1997a; Leise et al. 2004). Larvae can internalize numerous small molecules from seawater (Preston 1993). Exogenous 5-HT is apparently

transported into the larva where it appears to mimic the release of endogenous 5-HT from serotonergic neurons to initiate natural metamorphosis (Couper and Leise 1996; Leise et al. 2004). Histological experimentation with *I. obsoleta* has demonstrated that the larval AG disappears during metamorphosis (Gifondorwa and Leise 2006). Serotonergic neurons in the AG innervate the velar lobes (Marois and Carew 1997a; Leise et al. 2004).

Experiments on the nudibranch, *Phestilla sibogae,* revealed deterioration of labeled serotonergic axons during metamorphosis. These serotonergic neurons are contained within the AG and innervate the velar lobes attached to the external head case of the animal (Ruiz-Jones and Hadfield 2011). This type of structural loss and neural reorganization can be attributed to programmed cell death (Marois and Carew 1997b; Gifondorwa and Leise 2006).

Evidence to date supports the idea that the gaseous neurotransmitter nitric oxide (NO) opposes the action of 5-HT in the metamorphic pathway in *I. obsoleta* (Froggett and Leise 1999). To locate the NO-releasing neurons, screens for its catalytic enzyme, nitric oxide synthase (NOS), were conducted by NADPH diaphorase histochemistry (NADPHd) and NOS immunocytochemistry on tissue sections of larval and juvenile *I. obsoleta* (Lin and Leise 1996; Thavaradhara and Leise 2001). These authors detected strong signals from NADPHd in neurites and NOS-like immunoreactivity (NOS-IR) in somata in the AG during the larval period, which declined during metamorphosis, as expected. These findings were supported by expression analysis of a partial gene transcript of neuronal nitric oxide synthase (nNOS). RNA extracts from cultured larvae

exposed to 5-HT for 0-24 hours were utilized for reverse transcriptase polymerase chain reaction. Levels of nNOS RNA dropped after 6 hours of exposure to 5-HT (Hens et al. 2006). In addition, pharmacological manipulation of NOS activity and NO release indicate that the activity of NO represses metamorphosis (Froggett and Leise 1999). NO-donors significantly inhibited serotonergically induced metamorphosis, while injection of NOS inhibitors elicited metamorphosis in the absence of other inducers. Together, these results indicate that NO in the larval CNS acts to retain the larval state.

A Novel Role for GABA as a Neurotransmitter in *I. obsoleta*

Investigations of these conserved neurotransmitters, 5-HT and NO, have demonstrated that each plays a critical role in the neural control of metamorphosis in our molluscan model organism. To more fully describe neural mechanisms in metamorphosis, I have investigated the role of GABA, another neurotransmitter in this pathway.

The neurotransmitter GABA is the principal inhibitory neurotransmitter in adult vertebrate CNSs where it generally evokes a hyperpolarizing response from target cells (Reichert 1993). GABA is made from the amino acid glutamate by the enzyme glutamic acid decarboxylase (Fig. 1). When GABA binds to $GABA_A$ or $GABA_c$ receptors, ion channels open, allowing chloride ions to flow into the target neuron, causing a negative change in membrane potential. $GABA_B$ receptors are metabotropic and activate intracellular second messenger systems. Among its cellular functions, GABA has been

linked to the regulation of cellular differentiation and migration of cellular processes in programmed cell death (Ganguly et al. 2001).

Previous molluscan studies have demonstrated that GABA can induce larval metamorphosis in the abalone, *Haliotis rufescens,* as it mimics a natural ligand present in the rocky intertidal and subtidal microalgal environment (Morse et al. 1979). Results of our experiments with *I. obsoleta* are in contrast to those obtained from this abalone. Bath application of GABA to larval *I. obsoleta* elicited no metamorphosis. Moreover, GABA inhibited serotonergically induced metamorphosis (Leise et al. 2009a; Leise et al. 2009b).

Hypothesis and Specific Aims

I hypothesized that in *Ilyanassa obsoleta*, GABA acts as an inhibitory neurotransmitter in the larval CNS to repress metamorphosis in serotonergically induced larvae.

I used multiple GABAergic reagents to verify preliminary experiments which strongly suggested that GABA acts as an internal neurotransmitter that represses metamorphosis. To do this I used two major types of experimental protocols. I either added reagents alone to target GABAergic receptors or I weakly induced larval metamorphosis and applied reagents to modify this response. I applied the GABAergic agonists muscimol and isoguvacine to demonstrate that these reagents simulate GABAergic effects at $GABA_A$ receptors (Fig. 1). I also applied the chloride channel blocker picrotoxin to demonstrate that this reagent inhibits GABAergic effects at ionotropic receptors (Fig. 1).

Finally, I exposed larvae to artificial seawater with various concentrations of chloride ions. This demonstrated that reductions in the inward chloride current that passes through the ionotropic receptors promote metamorphosis.

A five hour exposure to 0.1mM 5-HT induces metamorphosis weakly. That is, fewer larvae metamorphose than after a full 48 hour exposure. After the five hour exposure, serotonin was removed and larvae were exposed to a $GABA_A$ receptor agonist (isoguvacine or muscimol) or to the GABA-T inhibitor aminooxyacetic acid (AOA, Fig.1) to modify the metamorphic induction. All of the successful experiments that I describe in this thesis resulted from bath application of reagents. I attempted injection experiments that included numerous procedural modifications. None of these injection experiments produced significant results, so they are not included here. However, because I used multiple injection procedures and parameters, I included them here as well as a brief discussion of their effects.

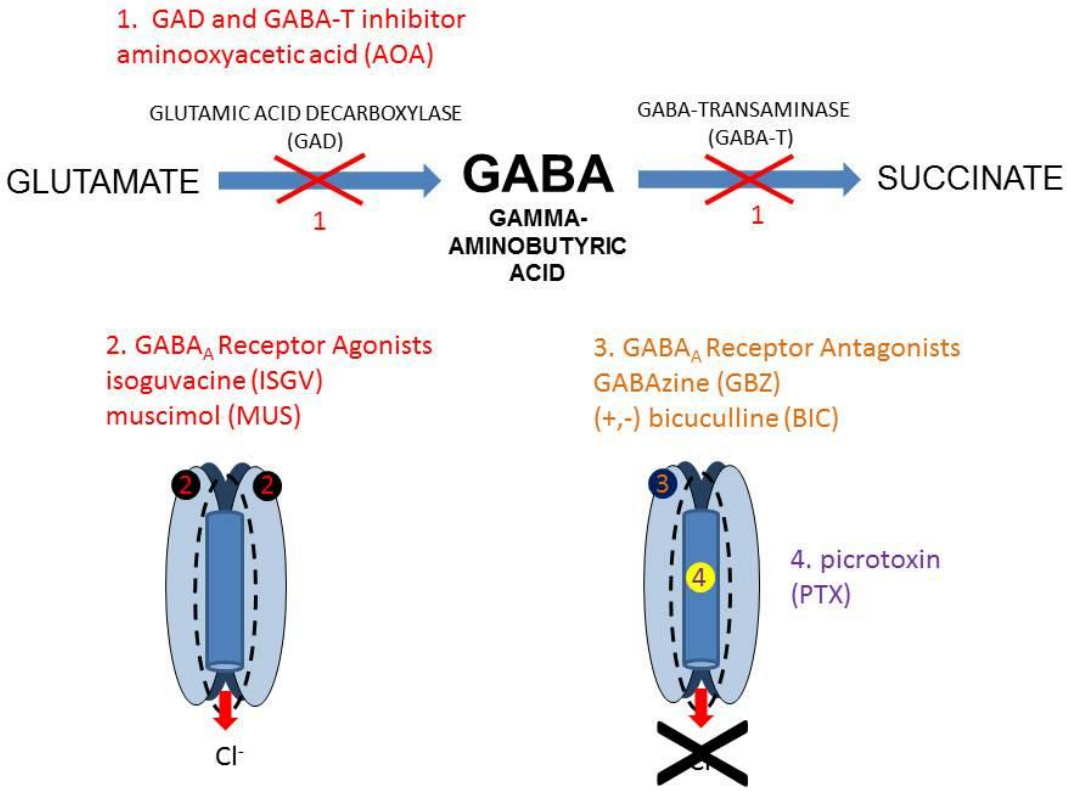

Figure 1. Pathway for GABA Synthesis and Metabolism. Aminooxyacetic acid (AOA) inhibits both glutamic acid decarboxylase (GAD) and GABA-transaminase (GABA-T) (1). Inhibition of GAD typically results in an increase in intracellular glutamate concentration while inhibition of GABA-T increases intracellular GABA concentration. B. Reagents Affecting Post-Synaptic $GABA_A$ Receptors. Both $GABA_A$ agonists and antagonists bind to GABA receptor sites (2, 3). PTX blocks the chloride channel (4). Pharmacological reagents listed above were utilized to target GABA receptors. The use of $GABA_A$ receptor agonists simulates the actions of GABA. In contrast, the $GABA_A$ receptor antagonists inhibit GABAergic action.

CHAPTER II

MATERIALS AND METHODS

Animal Care and Maintenance

Adult *Ilyanassa obsoleta* were collected from the mudflats of Wilmington, North Carolina in the winter at the CREST Research Park (Center for Marine Sciences) at UNCW. Animals were transported to UNCG laboratories and placed into aquaria with controlled salinity (~31 ppt), temperature (~26°C), and pH (~8.1). One hundred animals were placed into each aquarium and within two to three days they started laying egg capsules. Animals were also placed in reserve tanks in an environmental chamber at 6°C, thus simulating the conditions of winter, inhibiting egg laying behavior (Lambert et al. 1997). This winterizing process appears to have no affect on larval metamorphic responses (Leise, unpublished data), but in all bath application experiments, a control of 0.03mM 5-HT was conducted to ensure that larvae responded appropriately to experimental treatments. After 5-6 months at 6°C, gametes degenerate and while their larvae appear normal, they lose responsiveness to low concentrations of 5-HT. The additional control allows me to eliminate experiments with degenerate larvae. All egg capsules were collected by hand using a pipette and a single-edged razor blade and were transferred into a beaker containing artificial seawater (IO).

Egg capsules were rinsed daily in 70% ethanol followed by 0.2 µm filtered artificial seawater (FIO) to minimize bacterial growth. Newly hatched larvae were collected in a 100 µm mesh filter. Larvae were then backwashed into finger bowls for inspection and counting. Under a dissecting microscope, approximately 750 larvae were pipetted into a plastic beaker whose bottom had been replaced with 130 µm mesh plankton netting. The modified plastic beaker was placed inside a one liter pyrex beaker, allowing for easy transfer of larvae from one container to another (Gharbiah et al. 2008).

Approximately 650 ml of 0.2 µm filtered larval seawater (1:1 FIO and ocean water) was added to each of the 1 L pyrex beakers housing larvae. An air pump and plastic tubing was attached to each culture beaker to aerate the seawater (Miller and Hadfield 1986; Gharbiah et al. 2008). Newly seeded cultures were fed 40 ml of the unicellular alga, *Isochrysis galbana*. On subsequent days they were fed a mixture of 10 ml of *Isochrysis galbana* and 10 ml of *Duneliella tertiolecta*. Larvae do not metamorphose in response to these unicellular algal species. (Scheltema 1961; Pechenik and Fisher 1979)

Larval cultures were maintained for two and a half to three weeks (as described above), until larvae were competent to metamorphose. Cultures were given fresh larval sea water with streptomycin and penicillin antibiotic weekly to inhibit bacterial growth (Miller and Hadfield 1986). Metamorphic competence was estimated by measuring the longest shell lengths on 15-20 animals oriented operculum down. Animals were sized using a calibrated Olympus SZ40 dissecting microscope. The median size and range were

calculated and recorded. Only cultures with average sizes ≥550µm were used for bath experiments. Injection experiments were conducted on larger veligers, with shell lengths ≥600µm.

Preparation of Reagents

The following reagents were purchased from Sigma-Aldrich Inc. or Tocris Co.: GABA; isoguvacine and muscimol, ionotropic $GABA_A$ receptor agonists; picrotoxin, a chloride channel blocker; aminooxyacetatic acid (AOA), an inhibitor of GABA-transaminase and glutamic acid decarboxylase; gabazine and (-,-) and (+,-) bicuculline, $GABA_A$ receptor antagonists (Fig. 1) and 5-HT. Artificial seawaters (ASWs) were prepared with 100%, 75%, 50%, or 25% of normal chloride ion concentrations (Cavanaugh 1956). This ASW incorporated the substitution of sodium acetate for NaCl to maintain normal osmolarity of seawater.

All solutions with pharmacological reagents were prepared fresh, the day of the experiment, several minutes before application to animals. Calculations were also double checked. (-,-) and (+,-) bicuculline, GABAzine and picrotoxin were initially dissolved in dimethyl sulfoxide (DMSO), because of low solubility in water. Whenever DMSO was required, all solutions, including controls, contained 0.5% DMSO, which has no effect on metamorphosis in larval *Ilyanassa obsoleta* (Leise, unpublished data).

General Experimental Paradigm for Bath Experiments: Agonists and Antagonists

Two types of experiments were conducted. In standard bath application experiments, larvae were exposed to reagents for 48 hours, with no change of solution. Numbers of larvae and juveniles were counted at 24 and 48 hours to detect levels of metamorphosis elicited by experimental treatments.

A second experimental paradigm was used to determine if GABA or its agonists could inhibit serotonergically induced metamorphosis before larvae become irrevocably committed to this process. Larvae were exposed to 0.1mM 5-HT for 5 hours, a suboptimal metamorphic induction and then placed in FIO or various concentrations of GABAergic agonists. Responses were compared to those from a 0.1mM 5-HT to FIO control. I used multiple bath concentrations of GABA antagonists to develop dose-response curves. Results were analyzed as described below.

In general, bath application experiments were conducted as follows: 5 competent larvae were exposed to 2 ml of solution in each well of a 24-well Falcon plate, with 12-18 wells (replicates) per treatment. This was equivalent to 60-90 animals per treatment in each experiment. Animals for experimentation were given an anti-microbial rinse in a solution of 90% FIO and 10% povidine iodine, then four subsequent washes in FIO. The animals were moved into a fingerbowl filled with FIO and then distributed to experimental wells. Only healthy swimming veliger larvae were used. Larvae swam freely in FIO until it was replaced with an experimental reagent. Before the addition of any reagent, all residual FIO was removed from each well and the times of addition of

experimental reagents were recorded. Every bath experiment included an FIO control, a 0.03mM Low 5-HT control, and 0.1mM 5-HT control.

Larvae used for low chloride experiments were acclimated to artificial seawater (ASW) for a minimum of 24 hours. All low chloride experimental solutions were also prepared using some form of ASW.

General Experimental Paradigm: Microinjection Experiments

I injected approximately 20 competent animals per treatment in each experiment. Larvae were prepared as for bath application experiments, then decalcified in Low pH Seawater-Decalcifying Buffer (Cavanaugh 1956) overnight. This solution removed their shells, facilitating injections. The next day, animals were reaclimated to FIO for 1-2 hours. I distributed healthy, swimming larvae into small, 35 mm petri dishes for embedding in agarose. I heated a solution of 3% agarose in FIO in the microwave until the agarose dissolved. As the agarose cooled, I removed the residual FIO from the petri dishes and added approximately 2 ml of agarose with a micropipetter. I swirled the petri dish to keep the animals from sinking to the bottom of the dish until the agarose gelled. Animals were immobilized in this 3% low melting agarose at a temperature of 38-40°C. After the agarose polymerized, I added 1-2 ml of FIO to keep it hydrated. The animals were then ready for injections. I used the micropipette designs described below.

Glass micropipettes were made from 10cm standard borosilicate glass capillary tubing with filament, on a Flaming/Brown Micropipette Puller (Sutter Instruments Model P-87). There were five parameters to consider when designing micropipettes. The heat

and pull influenced the tip size and taper. An increase in heat and or pull made the tips smaller and the taper longer. An increase in velocity also made the tips smaller and an increase in time made the taper shorter. Finally an increase in the pressure or air flow produced a shorter taper (Oesterle 2011). The design of the micropipette was changed by manipulating one parameter at a time. I made gradual changes in increments of 1 to 5 units on each parameter and performed a RAMP test before each daily session. I inspected each micropipette under a dissecting microscope. Some micropipettes were beveled on a Narashige EG-40 beveler. I used the following 2 sets of parameters to pull micropipettes for injections:

Micropipette Design A: Heat=Ramp +1, Pull=28, Velocity=33, Time=105 Pressure=465

Micropipette Design B: Heat-Ramp +1, Pull-28, Velocity-33, Time-105 Pressure-465

These micropipette designs yielded thin but not wispy tips. The taper was long, but short enough that it remained fairly stiff. The tip bore was too small to be measured with a dissecting microscope. The injection parameters for Micropipette Design A were: 50 PSI, 500msec injection time, 2-3 Pulses. The injection parameters for Micropipette Design B varied due to different bore sizes resulting from beveling.

The tips of some micropipettes were sharpened by hand beveling after they were pulled. The micropipettes were attached to an adjustable Narishige coaxial manipulator at a 45 degree angle, several centimeters above a circular grinding stone. The beveling apparatus (Narishige model EG-40) was placed under an Olympus SZ-60 dissecting

microscope so that I could view the beveling process. The micropipette was then lowered until it touched the grinding stone which spun at approximately 60 rpm. The diameter of the micropipette tip or bore, was not very reproducible, but each micropipette was tested for volume delivery per injection as follows: a small droplet of deionized water was dispensed onto a parafilm-covered 3.5cm petri dish and a 1µl capillary tube was touched to the droplet. Amount of fluid in the droplet was calculated by measuring the length of the water-filled capillary tube. A small bore micropipette with the parameters of 50 PSI, 500msec injection time, 3 puffs, delivered about 10 nanoliters of reagent. A larger bore micropipette had parameters of 50 PSI, 300 msec injection time and 1 puff to deliver the same 10 nanoliters of reagent.

To successfully inject larvae, I targeted a single animal in the petri dish. I carefully removed as much agarose as possible from around its body and head exposing the animal and giving the micropipette access to the side of the head. Removal of agarose in layers also prevented clogging of micropipette tips. The micropipette was mounted on a Narishige micromanipulator and was advanced towards the animal from anterior to posterior, until contact was made at the side of the head located approximately 100 µm dorsal and 150 µm anterior to the statocyst. As the micropipette moved towards the site of injection, it made a visible indentation in the larval skin and I gently tapped the micromanipulator with my finger, easing the micropipette through the epidermis into the animal. I delivered 2 to 3 pulses and withdrew the micropipette. Injection into this site allowed the hemolymph to circulate the reagent to the CNS. I then freed the animal from

any residual agarose and transferred it by pipette into a well containing FIO. Procedures for injections into the area ~50μm dorsal to statocyst were similar, with the exception of moving the micropipette to the target site.

Statistical Analysis

Animals were counted at both 24 and 48 hours to determine numbers of larvae and juveniles. Percent metamorphosis was calculated and significant results of each experiment were determined as needed, by analyzing raw data in two way chi-square contingency table analysis at P=0.05 (Sokal and Rohlf 1995). I used Bonferroni's method (Sokal and Rohlf 1995) to correct for multiple comparisons. Percentage data were normalized with an arcsine transformation and standard deviations were calculated on the transformed data. Data were then transformed back to percentages for graphing. Graphs were generated on Graphpad Prism 5.0

CHAPTER III

RESULTS

Two types of unpublished, preliminary experiments with GABA were performed by undergraduate members of the Leise laboratory. I included them here (Figs. 2, 3) to provide continuity with my own experiments and because they support my hypothesis. They demonstrate that GABA alone had no effect on larval metamorphosis (Fig. 2) and that it could attenuate levels metamorphosis induced by serotonin. The initial experiment with GABA applied alone was repeated twice to yield the same results (Fig. 2).

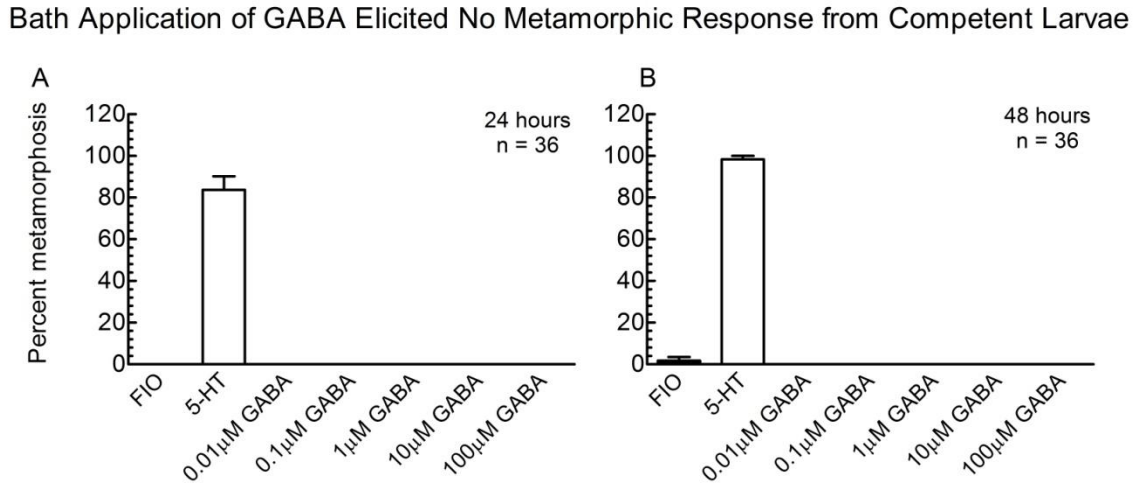

Figure 2. Bath Application of GABA Elicited No Metamorphic Response from Competent Larvae. Competent larvae that were exposed to multiple physiological concentrations of GABA, from 0.1µM-100µM, displayed no metamorphosis in response to this reagent at 24 (A) and 48 hours (B). The positive control (0.1mM 5-HT) induced nearly all larvae to metamorphose by 48 hours (Leise et al. 2009b).

The second paradigm of GABA application after a suboptimal exposure to 5-HT was replicated once, again yielding similar results.

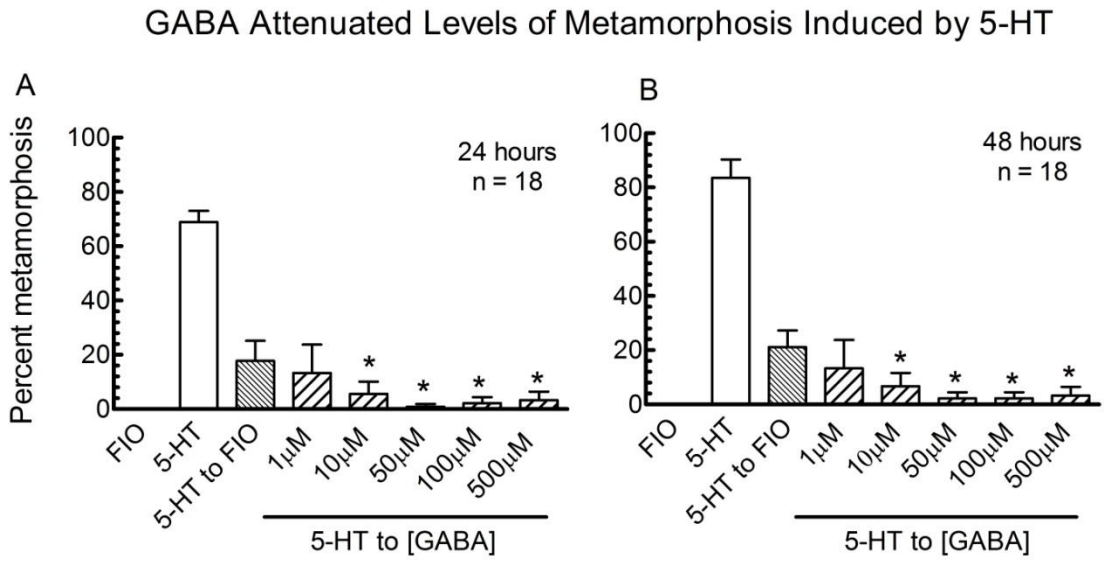

Figure 3. GABA Attenuated Levels of Metamorphosis Induced by 5-HT. An example of an experiment in which several concentrations of GABA inhibited serotonergically induced metamorphosis. Larvae that were first exposed to 0.1mM 5-HT for 5 hours then to four concentrations of GABA displayed statistically significant attenuation of levels of metamorphosis (*) when compared to the 5-HT to FIO control ($\chi^2_{0.001(1)}$ =7.149 or 5.569 for 5-HT to 10µM at 24 (A) and 48 (B) hours respectively, Leise et al. 2009b).

To verify these results and determine whether they were generated by GABA acting at ionotropic ($GABA_A$ or $GABA_C$) or metabotropic ($GABA_B$) receptors, larvae were exposed to two $GABA_A$ agonists, isoguvacine and muscimol, after suboptimal 5-HT induction (Figs. 4, 5). As expected, exposure of competent larvae to isoguvacine or muscimol after a 5 hour bath application of 5-HT demonstrated that these $GABA_A$ agonists could attenuate levels of serotonergically induced larval metamorphosis, although clearly isoguvacine was the more effective drug (Figs. 4, 5). Isoguvacine

effectively reduced metamorphosis for 24 hours at 0.2mM and 0.6mM concentrations. However, by 48 hours, considerable animal death among the experimental groups occurred, making the 0.4mM concentration appear to inhibit metamorphosis more effectively than at 24 hours (o in Fig. 4). The 0.6mM concentration remained insignificant because so few animals were alive at 48 hours. A repetition of this experiment yielded similar results. The $GABA_A$ agonist muscimol was effective at blocking serotonergically induced metamorphosis at concentrations of 1.0mM - 4.0mM only for 24 hours. This experiment was replicated three times, again yielding comparable results.

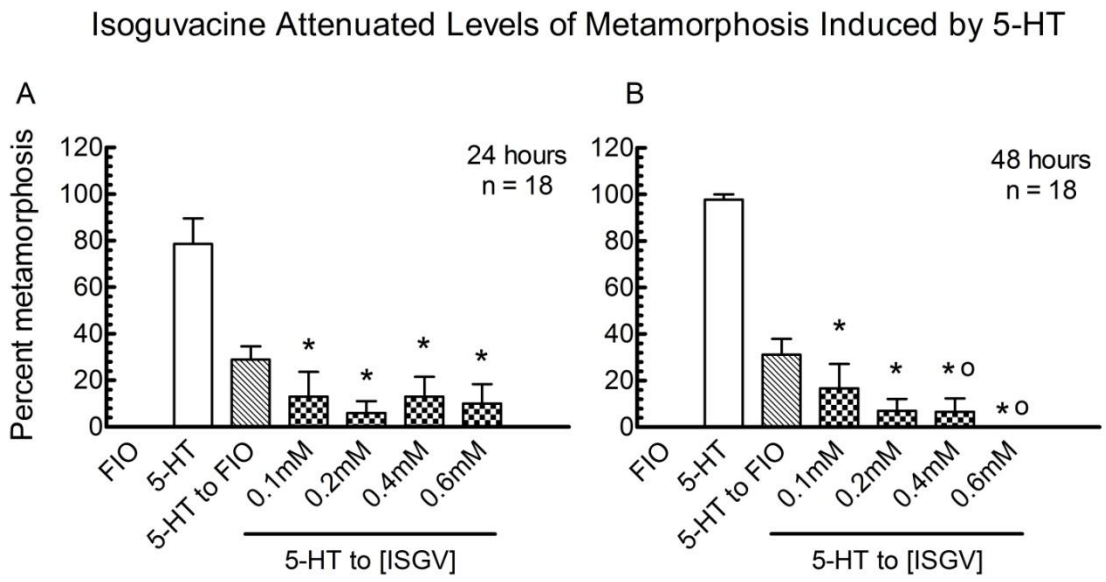

Figure 4. Isoguvacine Attenuated Levels of Metamorphosis Induced by 5-HT. $GABA_A$ receptor agonist isoguvacine attenuated serotonergically induced metamorphosis in competent larvae at 24 hours (A) and 48 hours (B). Larvae were bathed in 0.1µM 5-HT for five hours. The 5-HT was then replaced by various concentrations of isoguvacine for the remainder of the experiment. (*) Indicates statistically significant attenuation in levels of metamorphosis compared to the 5-HT to FIO control ($\chi^2_{0.001(1)}$ =10.03 for 0.6mM at 24 hours). (o) Indicates experimental groups with high numbers of animal death. This is an example of an experiment that was repeated 3 times with similar results.

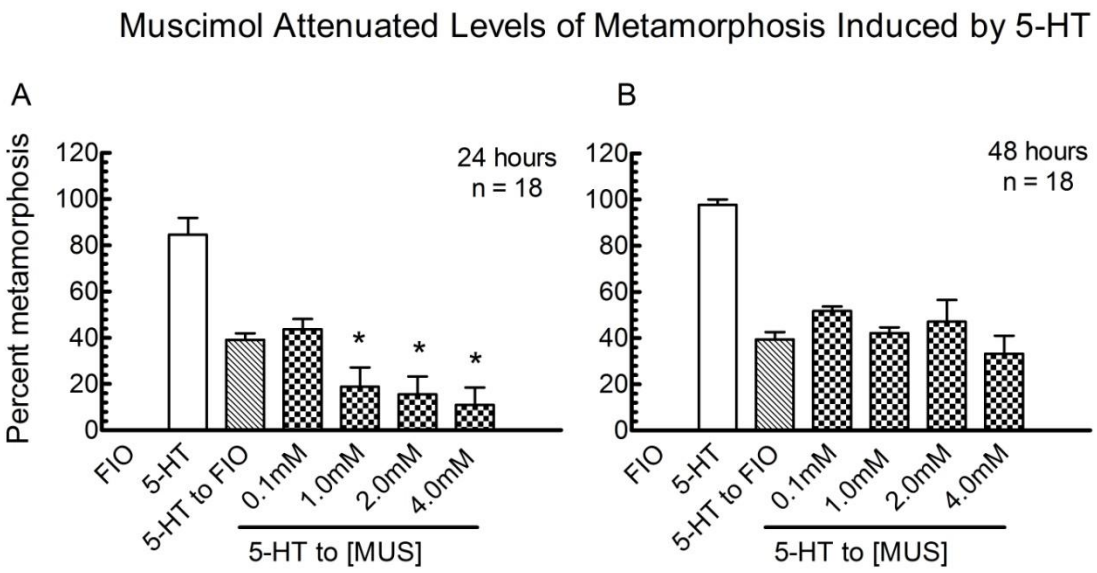

Figure 5. Muscimol Attenuated Levels of Metamorphosis Induced by 5-HT. The $GABA_A$ receptor agonist muscimol significantly attenuated serotonergically induced metamorphosis in competent larvae at 24 hours only (* in A), compared to the 5-HT to FIO control. ($\chi^2_{0.001(1)} = 9.067$ for 1.0mM). This is an example of an experiment that was repeated 3 times with similar results.

Another way to demonstrate that $GABA_A$ receptors are involved in the repression of metamorphosis is to manipulate the inward chloride ion current. Previous researchers have used the chloride channel blocker picrotoxin (PTX) to decrease this current (Leise et al. 2009b). PTX induced 11.1% of larvae to metamorphose at 24 hours (* in Fig. 6), significantly more metamorphosis than occurred in FIO. However, larvae did not reliably respond to PTX in additional experiments that I conducted. It elicited metamorphosis in only 1 out of 6 bath experiments. The successful experiment is shown here (Fig. 6).

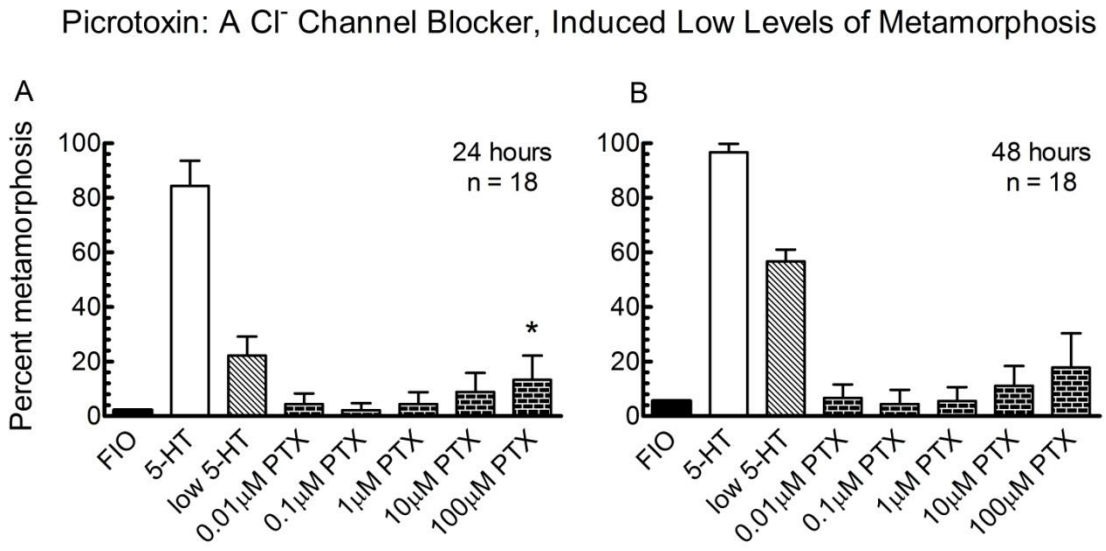

Figure 6. Picrotoxin: A Cl^- Channel Blocker, Induced Low Levels of Metamorphosis. The chloride channel blocker, picrotoxin (PTX), applied alone in the bath induced significant levels of metamorphosis in competent larvae at 24 (* in A) but not at 48 hours. A statistically significant increase in metamorphosis occurred in 100µM PTX compared to larvae in FIO. ($\chi^2_{0.001(1)}$ =7.745 at 24 hours).

Bicuculline, a $GABA_A$ receptor antagonist, induced no larval metamorphosis when applied alone in 3 experiments (Fig. 7). A second $GABA_A$ receptor antagonist, GABAzine, similarly induced no metamorphosis in competent larvae (Fig. 8).

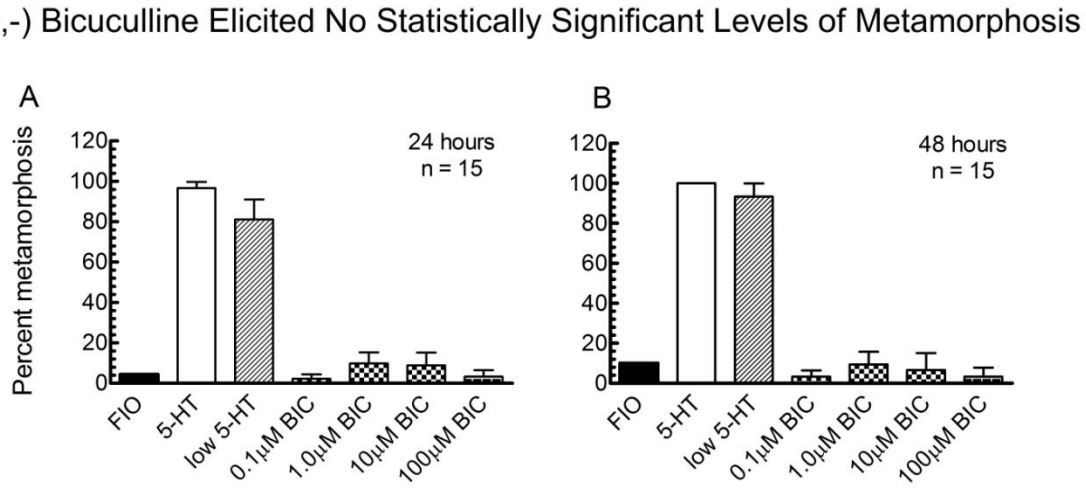

Figure 7. (+,-) Bicuculline Elicited No Statistically Significant Levels of Metamorphosis. Applied alone in the bath, $GABA_A$ receptor antagonist (+,-) bicuculline (BIC) induced no statistically significant levels of metamorphosis in competent larvae compared to the FIO control at 24 (A) or 48 (B) hours.

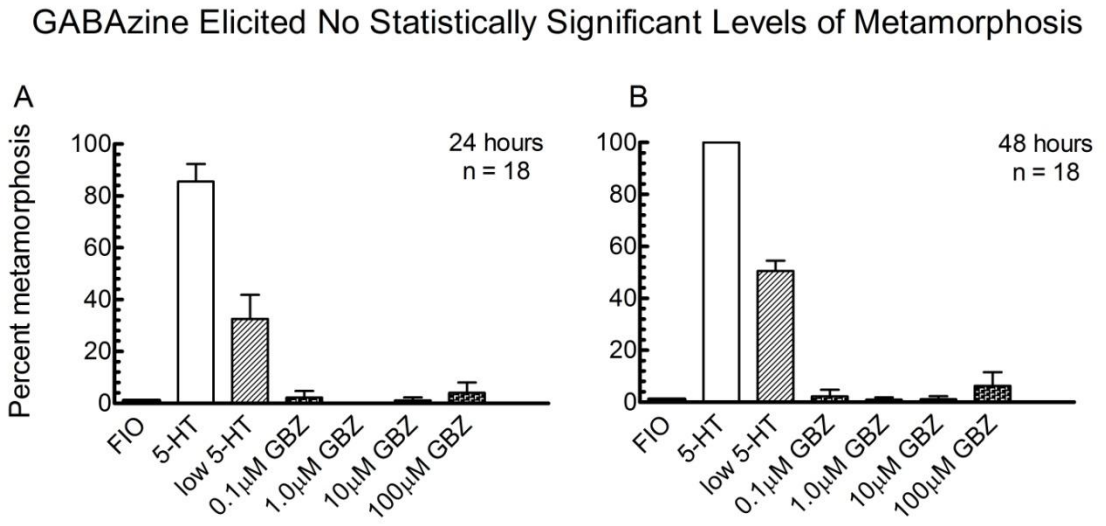

Figure 8. GABAzine Elicited No Statistically Significant Levels of Metamorphosis. Applied alone in the bath, $GABA_A$ receptor antagonist gabazine elicited no statistically significant levels of metamorphosis from competent larvae when compared to the FIO control at 24 (A) or 48 (B) hours.

Because activation of $GABA_A$ receptors opens their chloride channels, I investigated the effect of chloride concentration on metamorphosis (Fig. 9). ASW solutions with chloride ion concentrations at 25% of normal elicited metamorphosis in significant numbers of larvae at 24 hours. By 48 hours, solutions with either 25% or 50% of normal Cl^- ion concentration induced significant levels of metamorphosis, indicating that as chloride ion concentration decreased percent metamorphosis rose. The induction of 77% metamorphosis in 25% Cl^- at 48 hours was close to the level induced by 0.1mM 5-HT in this experiment.

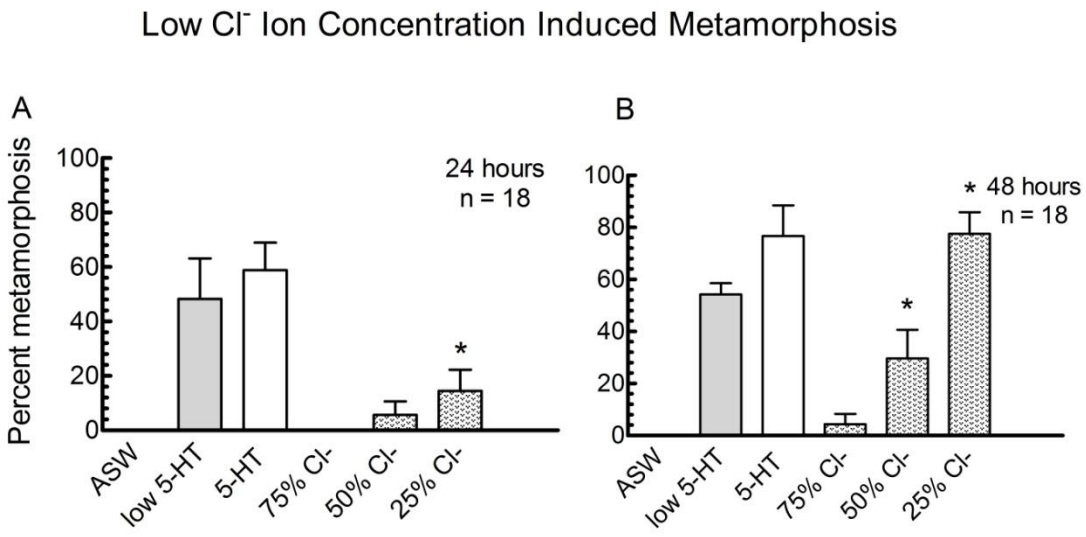

Figure 9. Low Cl^- Ion Concentration Induced Metamorphosis. Low chloride Artificial Seawater (ASW) significantly (*) increased percent metamorphosis in a dose dependent fashion at 24 (A) and 48 hours (B). $\chi^2_{0.001(1)}$ =14.011 for 25% Cl- at 24 hours and $\chi^2_{0.001(1)}$ = 31.139 for 50% Cl^- at 48 hours, both compared to the ASW control. Sodium acetate was substituted for NaCl to maintain ASW at appropriate osmolarity.

Aminooxyacetic Acid (AOA) is an inhibitor of GABA-Transaminase, the enzyme involved in breaking down GABA into succinate and L-glutamate (Fig. 1). It also functions, albeit more weakly, as an inhibitor of glutamic acid decarboxylase (GAD), the enzyme responsible for synthesizing GABA. AOA promoted serotonergically induced metamorphosis at low concentrations, but appeared to keep levels of metamorphosis low at higher concentrations (+ in Fig. 10A). By 48 hours, AOA appeared to induce only high levels of metamorphosis. This experiment was replicated three times with similar results.

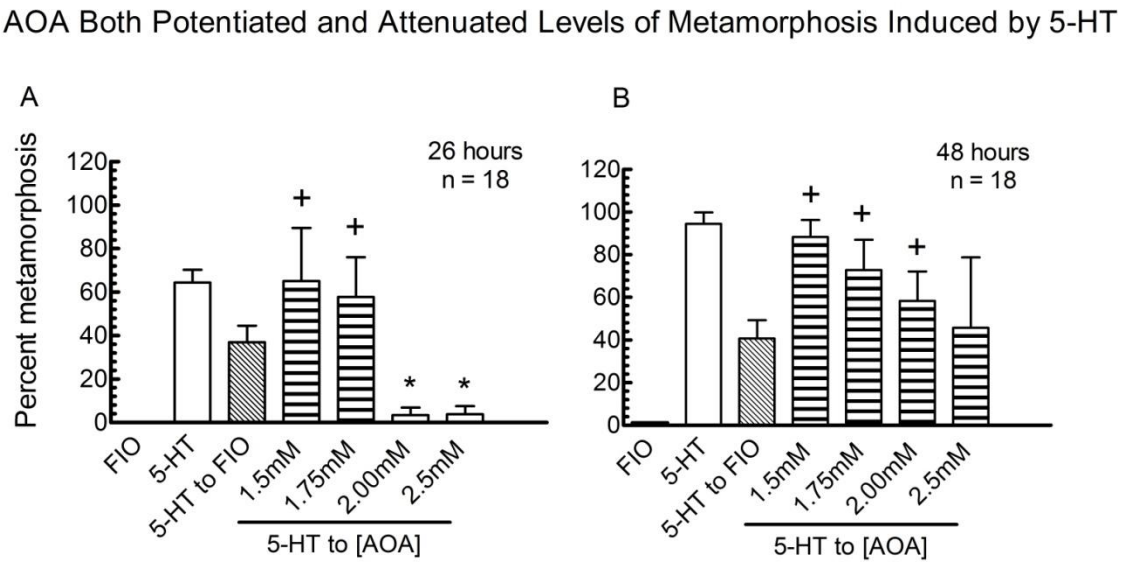

Figure 10. AOA Both Potentiated and Attenuated Levels of Metamorphosis Induced by 5-HT. AOA demonstrated dual activity at 24 hours as a promoter of metamorphosis at low concentrations (+) and an inhibitor of metamorphosis at high concentrations (* in A). Comparison of 1.5mM and 1.75 mM treatments to the 0.1mM 5-HT to FIO control revealed statistically significant promotion of metamorphosis($\chi^2_{0.001(1)}$ =13.578 and 8.128, respectively, + in A). In contrast, AOA inhibited metamorphosis at 2.0 mM and 2.5 mM ($\chi^2_{0.001(1)}$ =30.202 and 29.061 respectively) at 24 hours when compared to the same 5-HT to FIO control. At 48 hours, AOA promoted metamorphosis in all concentrations with the exception of 2.5mM when compared to the 5-HT to FIO control (+ in B, $\chi^2_{0.001(1)}$ = 6.837 for 2.0mM).

CHAPTER IV

DISCUSSION

One of the major goals in the study of metamorphosis in *Ilyanassa* is to understand the neural circuit that initiates this process. Previous work with this model organism has demonstrated that two neurotransmitters, 5-HT and NO, play opposing roles in this circuit. My research has allowed me to add GABAergic signaling to the properties of this circuit. Like NO, GABA appears to negatively regulate metamorphosis in *Ilyanassa obsoleta*. Importantly, results of my experiments strongly suggest that the role of GABA in this developmental event is unlike the role it plays in another well studied mollusc, specifically the red abalone *Haliotis rufescens*. GABA was identified over 30 years ago as a neurotransmitter that induced metamorphosis in *Haliotis rufescens* by mimicking algal ligands at epidermal GABA receptors. However, Figure 3 in Morse at al. (1980) depicts larval responses to GABA and suggests that high concentrations of this neurotransmitter inhibit, rather than induce metamorphosis. To my knowledge, these findings were not explored in subsequent publications. The preponderance of data from my pharmacological experiments supports the idea that GABAergic effects in *Ilyanassa* happen internally after sufficient reagents are transported into the CNS, as opposed to the external activity of GABA that evokes a rapid settlement response in *Haliotis*.

I tested my null hypothesis by using pharmacological reagents that alter neurotransmitter action and thus modify how larvae respond to reagents that induce or inhibit metamorphosis. Data acquired from the use of multiple reagents resulted in logically consistent results. GABA agonists appeared to simulate the effects of GABA and thereby attenuate levels of metamorphosis, while the Cl^- channel blocker PTX promoted metamorphosis, albeit unreliably. AOA is classified as a GABA-T inhibitor due to its tight binding affinity for the enzyme. However, AOA can also interact with glutamic acid decarboxylase, although with much lower affinity. Experiments involving GABA agonists have also demonstrated GABAergic inhibition of serotonergically induced metamorphosis. While we do not yet know the configuration of the putative GABAergic circuit, all of my evidence suggests that this neurotransmitter plays a key role in regulating the initiation of this important developmental process.

Modification of GABA Pathway by Bath Experiments

My research focused mostly on the modification of ionotropic $GABA_A$ receptors. Even in the Mollusca, these receptors are characterized as chloride permeable channels in which GABA binds to the active site of the receptor, changing the conformation of the channel and allowing the chloride ions to flow across the membrane. Results of the initial bath experiment (Fig. 2) demonstrated that GABA elicits no metamorphic response by itself. In contrast, exposure of animals to GABA after a brief application of 5-HT demonstrated that GABA can inhibit metamorphic induction for 48 hours (Fig. 3). Presumably, this short term exposure to 5-HT allowed the animals to internalize some

serotonin, but not enough for all larvae to commit themselves to metamorphosis. Furthermore, all of the experiments with GABAergic reagents allowed me to hypothesize that GABAergic neurons are downstream from the serotonergic ones.

Experiments performed using GABA alone (Fig. 2) provide only preliminary evidence that GABAergic neurons could inhibit metamorphosis in *Ilyanassa.* To further address my hypothesis, I exploited other GABAergic reagents; GABA agonists such as isoguvacine and muscimol mimic the effects of GABA and attenuate serotonergically induced metamorphosis. Isoguvacine simulated the effect of GABA (Fig. 4) by competitively binding to the $GABA_A$ receptor. Muscimol exhibited similar results and displayed a dose response curve at 24 hours (Fig. 5)

As expected, modification of the action of GABA with the chloride channel blocker picrotoxin (Fig. 6) elicited metamorphosis but only at a relatively high concentration. I was unable to replicate this effect in repeated experiments. Other $GABA_A$ receptor antagonists, bicuculline and GABAzine (Figs. 7, 8), yielded no statistically relevant results, most likely due to lipid insolubility of these compounds.

Production of GABA was modified with the use of aminooxyacetic acid (AOA), an inhibitor of GABA-Transaminase, the enzyme responsible for degrading GABA into succinate and L-glutamate (Fig. 1). Inhibition of GABA-T should have led to increased release of GABA from the presynaptic cell, and as expected AOA attenuated serotonergically induced metamorphosis at high concentrations, although for only 24 hours after exposure (Fig. 10). However, AOA displayed dual activity (Wu 1981),

promoting metamorphosis at low concentrations at 24 hours and 3 of 4 concentrations at 48 hours, indicating that the function of AOA is perhaps concentration dependent. Because AOA can have a dual function, it is possible that at low concentrations, AOA might function more in its capacity as an inhibitor of glutamic acid decarboxylase, which could explain its promotion of metamorphosis.

Finally, I manipulated larval chloride ion currents by exposing larvae to artificial seawaters made by substituting sodium acetate for sodium chloride at several concentrations. Exposure of competent animals to solutions with low [Cl^-] should decrease the chloride current that moves through the activated receptors (Reichert 1993). As expected, low [Cl^-] inhibited the functions of GABA and induced significant levels of metamorphosis in a dose dependent manner.

My experimental paradigms that used bath application of pharmacological reagents were probably effective because marine invertebrates can transport amino acids and related molecules across their epithelia by sodium dependent co-transporters that function against high intracellular to extracellular gradients of amino acids. Such transport across the epithelium and into different organ systems, including the CNS, may normally be used for osmoregulation, nutritional or chemoreceptive purposes (Preston 1993).

Modification of GABA Pathway by Microinjection Experiments

I attempted to supplement bath application results with microinjection experiments. In this technique, pharmacological reagents are injected into larvae, decreasing the chances of interactions between neuroactive compounds and epidermal sensory receptors. Pharmacological reagents were expected to act in the same manner, and indeed more efficiently, when compared to their action in bath application. This technique should have provided further support for the idea that GABAergic activity occurs internally, within the CNS and perhaps in the apical ganglion.

The results of our microinjection experiments varied, but were statistically insignificant. Numerous permutations of the injection protocols were performed. Both techniques and equipment were modified in attempts to yield significant results. Several factors may have affected the outcome of my injections, the first of which may have been the design of the micropipettes. I changed the shape of the tips in several ways by manipulating the heat, pull, velocity, time and pressure settings of the micropipette puller. These settings changed the taper, bore, length, and overall shape of the micropipettes. Although the micropipette puller consistently produced micropipettes of the same shape, the beveling procedure yielded tips with highly variable bore diameters.

Variability of tip size affected the injection parameters for each micropipette. The pressure, duration and number of injections were changed to normalize the volume and pressure of each pulse. Pressure was also adjusted in slight increments from low (10 psi)

to high (70 psi). Pulses ranged from 1-3, and duration from 100 milliseconds to 900 milliseconds. Yet all of these attempts yielded no increase in significant data.

Another factor that may have affected the results was the injection site. Animals were first injected into a site approximately 50μm dorsal to the statocyst, in the area adjacent to the cerebral ganglia (Couper and Leise 1996). Again, variations in injection sites yielded no change in results.

The lack of significant metamorphic data did not yield negligible physiological results. Injection of 5-HT caused the velar cilia to beat within a minute of delivery. This is consistent with the findings that serotonergic neurons innervate the velar lobes (Marois and Carew 1997a). This result suggests that the injection was initially successful in internalizing the reagent and that the reagent diffused into the apical ganglion to react with serotonergic receptors. The same type of behavior was observed with other pharmacological reagents, including the GABA antagonists, bicuculline and GABAzine, and the chloride channel blocker Picrotoxin.

The statistically insignificant results of injections may have occurred because the injection protocol did not expose larvae to enough reagent for a sufficiently long time. Also, damage at the injection site could have allowed reagents to leak out of the animal further minimizing exposure to the reagent.

Conservation of GABA in other Phyla and in *Ilyanassa* Transcriptome

GABA occurs as a neurotransmitter in the CNSs of organisms throughout the animal kingdom, including the phylum Mollusca. Conserved transcripts of GABAergic receptors ($GABA_A$, $GABA_B$, $GABA_C$) and the enzyme producing it from glutamate (glutamic acid decarboxylase) have been reported for the gastropods *Haliotis rufescens*, *Aplysia californica*, and *Melibe leonine*, among others according to the National Center for Biotechnology Information. U.S. National Library of Medicine. 2012. (Nucletotide database collection [Online]. Available: http://www.ncbi.nlm.nih.gov/nuccore [2012, October 6]). Thus, it is quite likely that GABA is used as a neurotransmitter in *Ilyanassa obsoleta* as well. While it has not yet been located in our larvae, experiments with larval *Haliotis* show that these larvae express GABA receptors in their chemosensory cells (Morse 1990). Retention of neurotransmitters and their receptors is a common phenomenon; further research should demonstrate authentic GABA and its existence as a neurotransmitter in our model organism.

Recently, a partial transcriptome of early developing *Ilyanassa* has become available. Messenger RNA was isolated from embryos at different times past fertilization, between 0 and 48 hours, spanning the early to late gastrula stages. Expressed sequence tags (EST) from a transcription library of cDNA sequences were created for transcription analysis (Lambert et al. 2010). A search of this database for GABA transporters, receptors and its metabolic enzymes in this species yielded no results. To determine if the *Ilyanassa* EST library was of high quality, the sequence for a known housekeeping gene (actin) that had been described in the EST database was

BLASTed against the entire nucleotide collection at the National Center for Biotechnology Information, U.S. National Library of Medicine. 2012. (Nucletotide database collection [Online]. Available: http://www.ncbi.nlm.nih.gov/nuccore [2012, October 6]). The actin sequence yielded high output significance and high alignment scores across many other invertebrate species including gastropods in the phylum Mollusca. Thus the sequence output that was isolated from the *Ilyanassa* EST library appeared to be of high quality. A subsequent search for classical neurotransmitters receptors in the EST database yielded a sequence for a glutamate receptor. A search through the nucleotide database for GABAergic receptor sequences from *Haliotis* and *Aplysia* yielded $GABA_A$ receptor sequences from both organisms. However, when BLASTed against the EST library of *Ilyanassa*, the search yielded insignificant alignment scores. Thus the newly generated *Ilyanassa* EST library is either incomplete or our animal is not expressing any GABA related genes at such early stages. Given that neurogenesis has either not yet begun or is only just beginning, it is not surprising that the RNA isolated from the embryos showed no homology to any GABA related sequence.

Hypothetical Circuits

Research with *Ilyanassa obsoleta* supports the idea that environmental kairomones activate epidermal sensory neurons, some of which may be serotonergic (Couper and Leise 1996). Application of 5-HT is thought to mimic the action of serotonergic neurons in the apical ganglion (AG) to induce metamorphosis. In competent larvae, the production of NO inhibits metamorphosis and maintains the larval state

(Froggett and Leise 1999). Evidence presented here strongly suggests that GABA should be added to the neural circuit that regulates metamorphosis in *Ilyanassa obsoleta*. GABAergic neurons may provide tonic inhibition of metamorphosis by repressing the function of serotonergic neurons (Fig. 11 C). This would allow downstream nitrergic neurons in the AG to continue to produce NO, keeping *Ilyanassa* in its larval state. An alternate model places GABAergic neurons downstream of nitrergic ones (Fig. 11 B). In this hypothetical circuit, the production of NO helps to maintain GABAergic activity, likewise keeping the animal in its larval form. Activation of sensory neurons, followed by the activation of serotonergic neurons, would inhibit the production of NO causing an inactivation of GABAergic neurons, leading to metamorphosis. However, this scenario is unlikely because inhibition of NOS can directly activate programmed cell death, as occurs in the AG once metamorphosis in initiated. Thus, I suspect that GABA is more likely to be upstream of nitrergic neurons (Fig. 11 A). In the third hypothetical circuit (Fig. 11 C) co-repression of serotonergic neurons by both nitrergic and GABAergic ones may be required to maintain the larval state.

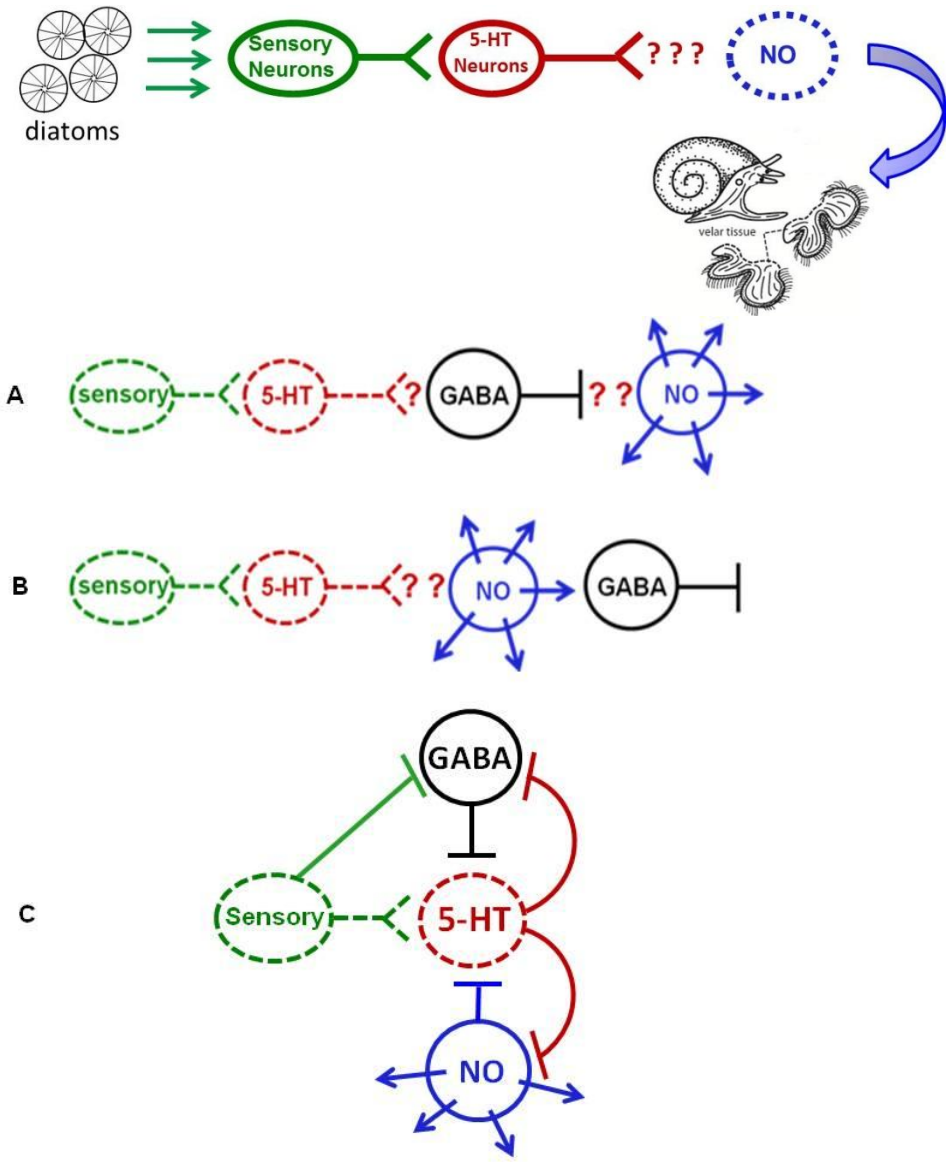

Figure 11. Hypothetical Metamorphic Circuits Regulating Metamorphosis in *I. obsoleta.* Results could support multiple circuits including (A) placement of GABAergic neurons downstream of serotonergic and upstream of nitrergic neurons, or (B) placement of GABAergic neurons downstream of both serotonergic and nitrergic neurons. (C) Co-repression of serotonergic neurons by both GABAergic and nitrergic neurons is also possible.

Evolutionary Modification of the GABAergic System in Molluscs

I have presented evidence that supports the hypothesis that the neurotransmitter GABA has an inhibitory function in the neural circuit regulating metamorphosis in the mud snail *Ilyanassa obsoleta*. What are some possible explanations behind the contrasting functions of GABA in *I. obsoleta* and the red abalone, *Haliotis rufescens*? The red abalone, as well as numerous related species of *Haliotidae*, reside in the rocky intertidal and subtidal zones of the Pacific rim, while the eastern mud snail inhabits soft sediment communities of the east Atlantic coast. These are two habitats with different plant biomass, microflora and microfauna. Like the adults, larvae of marine molluscs have evolved to respond to biological features of their distinctive environments.

Previous studies have shown that water soluble kairomones released by the diatom, *Coscinodiscus sp.*, can elicit metamorphosis in competent larvae of *Ilyanassa obsoleta* (Leise et al. 2009a). We postulate that external cues secreted by diatoms excite sensory neurons, which in turn excite serotonergic neurons, turning on the metamorphic pathway by turning off inhibitory downstream neurons.

In contrast, larval *Haliotis rufescens* are induced to metamorphosis by crustose red algae that release a kairomone that can bind to epidermal sensory neurons. Larvae that come into contact with this biochemical inducer near a food source will thus settle and metamorphose in favorable habitats. Bath applied GABA mimics this kairomone and can induce metamorphosis in statistically significant numbers of larvae (Morse et al. 1980). However, prolonged exposure to exogenous GABA decreased larval settlement.

Our recent findings suggest that after a prolonged period of exposure to GABA, the larvae of *Haliotis rufescens* may have internalized sufficient numbers of neurotransmitter molecules to inhibit settlement and metamorphosis.

An explanation for the disparity between the between the two molluscan model organisms and their responses to GABA in bath experiments may lie in the evolution of their ancestor, an early lophotrochozoan. While the two marine molluscs retain similar genes, their use appears to have evolved in two different directions. They have evolved different uses for GABA, internal in *I. obsoleta* and external and perhaps internal in *Haliotis rufescens*. Like other organisms, molluscs probably retain useful genes that can be adapted for novel expression, giving them a selective advantage for survival in their diverse habitats. Thus, the use of GABA as an internal neurotransmitter has been retained as a common physiological function between both species, although they are separated by thousands of miles and millions of years of evolution. However, in one species, *Haliotis rufescens*, the GABAergic system has become adapted for other purposes. With the work that has been done to characterize the function of GABA, we can begin to confidently place this neurotransmitter in the neural circuit regulating metamorphosis in *Ilyanassa obsoleta*.

LITERATURE CITED

Abbott, L. F., J. A. Varela, K. Sen and S. B. Nelson (1997). "Synaptic Depression and Cortical Gain Control." *Science* **275**: 220-223.

Bickell, L. and S. Kempf (1983). "Larval and Metamorphic Morphogenesis in the Nudibranch *Melibe leonina* (Mollusca: Opisthobrancia)." *Biological Bulletin* **165**: 119-138.

Cavanaugh, G. (1956). *Formulae and Methods VI of the Marine Biological Laboratory Chemical Room*. Woods Hole, MA, Marine Biological Laboratory.

Collier, J. (2002). "A bibliography of the marine mud snail *Ilyanassa obsoleta*." *Invertebrate Reproduction and Development* **42**(2-3): 95-110.

Connell, J. H. (1975). "Some mechanisms producing structure in natural communities: a model and some evidence from field experiments." *Ecology and evolution of communities*: pp. 460–490.

Connor, M. S. (1982). "The effect of feeding by mud snails, *Ilyanassa obsoleta* (Say), on the structure and metabolism of a laboratory benthic algal community." *Journal of Experimental Marine Biology and Ecology* **65**(1): 29-45.

Couper, J. M. and E. M. Leise (1996). "Serotonin injections induce metamorphosis in larvae of the gastropod mollusc *Ilyanassa obsoleta*." *Biological Bulletin* **191**: 178-186.

Deschaseaux, E. S. M., A. M. Taylor, W. A. Maher and A. R. Davis (2010). "Cellular responses of encapsulated gastropod embryos to multiple stressors associated with climate change." *Journal of Experimental Marine Biology and Ecology* **383**(2): 130-136.

Fingerman, M. (1997). "Crustacean Endocrinology: A Retrospective, Prospective, and Introspective Analysis." *Physiological Zoology* **70**(3): 257-269

Froggett, S. and E. Leise (1997). "Endogenous Nitric Oxide Inhibits Metamorphosis in a Larval Mollusc." *Society for Neuroscience Abstracts* **23**: 1234.

Froggett, S. J. and E. M. Leise (1999). "Metamorphosis in the marine snail *Ilyanassa obsoleta*, yes or NO?" *Biological Bulletin* **196**: 57-62.

Ganguly, K., A. F. Schinder, S. T. Wong and M.-M. Poo (2001). "GABA Itself Promotes the Developmental Switch of Neuronal GABAergic Responses from Excitation to Inhibition." *Cell* **105**: 521-532.

Gharbiah, M., J. Cooley, E. M. Leise, A. Nakamoto, J. S. Rabinowitz, J. D. Lambert and L. M. Nagy (2008). The Snail *Ilyanassa*: A Reemerging Model for Studies in Development. *Emerging Model Organisms*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Gifondorwa, D. J. and E. M. Leise (2006). "Programmed cell death in the apical ganglion during larval metamorphosis of the marine mollusc *Ilyanassa obsoleta*." *Biological Bulletin* **210**(1): 109-120.

Hadfield, M. G. (1986). "Settlement and recruitment oif marine invertebrates: a perspective and some proposals." *Bulletin of Marine Science* **39**(2): 418-425.

Harper, J. L. (1969). "The role of predation in vegetational diversity." *Brookhaven Symp. Biol.* **22**: 48-61.

Hens, M. D., K. A. Fowler and E. M. Leise (2006). "Induction of metamorphosis decreases nitric oxide synthase gene expression in larvae of the marine mollusc *Ilyanassa obsoleta* (Say)." *Biological Bulletin* **211**(3): 208-211.

Hunt, J. H., J. W. G. Ambrose and C. H. Peterson (1987). "Effects of the gastropod, *Ilyanassa obsoleta* (Say) and the bivalve, *Mercenaria mercenaria* (L.), on larval settlement and juvenile recruitment of infauna." *Journal of Experimental Marine Biology and Ecology* **108**(3): 229-240.

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." *Journal of Comparative Neurology* **386**: 507-528.

Lacalli, T. C. (1994). "Apical organs, epithelial domains, and the origin of the chordate central nervous system." *American Zoologist* **34**(4): 533-541.

Lambert, J. D., X. Y. Chan, B. Spiecker and H. C. Sweet (2010). "Characterizing the Embryonic Transcriptome of the Snail Ilyanassa." *Integrative and Comparative Biology* **50**(5): 768-777.

Lambert, W. J., C. D. Todd and J. D. Hardege (1997). "Partial characterization and biological activity of a metamorphic inducer of the dorid nudibranch *Adalaria proxima* (Gastropoda: Nudibranchia)." *Invertebrate Biology* **116**(2): 71-81.

Leise, E. M. and L. B. Cahoon (2012). Neurotransmitters, Benthic Diatoms and Metamorphosis in a Marine Snail. *Snails: Biology, Ecology and Conservation*, Nova Science Publishers, Inc.**:** 1-43.

Leise, E. M., S. J. Froggett, J. Nearhoof and L. B. Cahoon (2009a). "Diatom cultures induce and inhibit larval metamorphosis in the marine gastropod *Ilyanassa obsoleta* (Say)." *Journal of Experimental Marine Biology and Ecology* **379**: 51-59.

Leise, E. M. and M. G. Hadfield (2000). "An Inducer of Molluscan Metamorphosis Transforms Activity Patterns in a Larval Nervous System." *Biological Bulletin* **199**: 241-250.

Leise, E. M., S. C. Kempf, N. R. Durham and D. J. Gifondorwa (2004). "Induction of metamorphosis in the marine gastropod *Ilyanassa obsoleta*: 5HT, NO and programmed cell death." *Acta Biologica Hungarica* **55**(1-4): 293-300.

Leise, E. M., J. G. Long, J. Washburn, N. M. Shah and J. Cook (2009b) GABA may repress metamorphosis in the marine snail *Ilyanassa obsoleta*. *Soc. Neurosci. Abstr. Viewer & Itinerary Planner* **889**,

Levantine, P. L. and D. B. Bonar (1986). "Metamorphosis of *Ilyanassa obsoleta*: Natural and artificial inducers." *American Zoologist* **26**: 14A.

Lin, M.-F. and E. M. Leise (1996). "NADPH-diaphorase activity changes during gangliogenesis and metamorphosis in the gastropod mollusc *Ilyanassa obsoleta*." *Journal of Comparative Neurology* **374**: 194-203.

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

Marois, R. and T. J. Carew (1997b). "Projection patterns and target tissues of the serotonergic cells in larval *Aplysia californica*." *Journal of Comparative Neurology* **386**: 491-506.

Miller, S. E. and M. G. Hadfield (1986). "Ontogeny of phototaxis and metamorphic competence in larvae of the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia)." *Journal of Experimental Marine Biology and Ecology* **97**: 95-112.

Morse, D. E. (1990). "Recent progress in larval settlement and metamorphosis: closing the gaps between molecular biology and ecology." *Bulletin of Marine Science* **46**(2): 465-483.

Morse, D. E., H. Duncan, N. Hooker, A. Baloun and G. Young (1980). "GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae." *Federation Proceedings of the Federation of the American Societies for Experimental Biology* **39**: 3237-3241.

Morse, D. E., N. Hooker, H. Duncan and L. Jensen (1979). "Gamma-aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis." *Science* **204**: 407-410.

Oesterle, A. (2011). "Pipette Cookbook Version G."

Pechenik, J. A. and N. S. Fisher (1979). "Feeding, assimilation, and growth of mud snail larvae, *Nassarius obsoletus* (Say), on three different algal diets." *Journal of Experimental Marine Biology and Ecology* **38**: 57-80.

Pires, A. and M. G. Hadfield (1993). "Responses of isolated vela of nudibranch larvae to inducers of metamorphosis." *Journal of Experimental Zoology* **266**: 234-239.

Preston, R. L. (1993). "Transport of amino acids by marine invertebrates." *Journal of Experimental Zoology* **265**(4): 410-421.

Reichert, H. (1993). Introduction. *Introduction to Neurobiology*, Thieme Publishing Group**:** 272.

Ruiz-Jones, G. J. and M. G. Hadfield (2011). "The loss of sensory elements in the apical sensory organ during metamorphosis in the nudibranch *Phestilla sibogae*." *Biological Bulletin*(220): 39-46.

Ruppert, E. and R. Fox (1988). *Seashore Animals of the Southeast: A Guide to Common Shallow-Water Invertebrates of the Southeastern Atlantic Coast*. Columbia, S.C., University of South Carolina Press.

Scheltema, R. S. (1961). "Metamorphosis of the veliger larvae of *Nassarius obsoletus* (Gastropoda) in response to bottom sediment." *Biological Bulletin* **120**(1): 92-109.

Sokal, R. R. and F. J. Rohlf (1995). *Biometry: The Principles and Practices of Statistics in Biological Research* New York, W.H. Freeman and Co.

Thavaradhara, K. and E. M. Leise (2001). "Localization of nitric oxide synthase-like immunoreactivity in the developing nervous system of the snail *Ilyanassa obsoleta*." *Journal of Neurocytology* **30**: 449-456.

Wu, J. Y. (1981). Assay methods, purification and characterization of L-glutamate decarboxylase and GABA transaminase. *Research Methods in Neurochemistry*. **5:** 129-177.