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Chapter 1

NEUROTRANSMITTERS, BENTHIC DIATOMS AND METAMORPHOSIS IN A MARINE SNAIL

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ABSTRACT

Many marine snails have a biphasic life cycle. They may live in diverse habitats on the ocean floor as adults, but are planktonic in the larval phase where they are subject to oceanic tides and currents. The link between these two disparate life history stages is metamorphosis, a dramatic and irreversible developmental event that transforms a larva physically, physiologically and behaviorally into a juvenile, a tiny, sexually immature adult. This conversion occurs only when animals are competent, physiologically able to respond to environmental cues that are indicative of a favorable juvenile habitat. Such cues can include biological odorants as well as physical features of the juvenile ecosystem. The idea that environmental stimuli, such as the presence of conspecific adults or useful food sources, could trigger metamorphosis in marine invertebrates was first promulgated about 90 years ago (Mortensen 1921). Since then, the sources of metamorphic stimuli have been identified for a variety of molluscs, including some of the caenogastropods, the more evolutionarily advanced snails. Our experiments on the induction of

metamorphosis in the intertidal mud snail, *Ilyanassa obsoleta,* by benthic diatoms have demonstrated that like other marine larvae, our model organism can display positive, negative or neutral responses to various diatom species.

Investigations into the regulation of metamorphosis have moved in the last 30 years from morphology and ecology to include the cells and signaling molecules that are active in the underlying neural pathways. Investigations with *I. obsoleta* have demonstrated that two important neurotransmitters, serotonin (5-HT) and nitric oxide (NO), play opposing roles in the neural network that controls metamorphosis. A third neurotransmitter, γ-aminobutyric acid (GABA), can induce metamorphosis in abalone, primitive molluscan relatives of *I. obsoleta,* by binding to GABAergic receptors located on epidermal projections of chemosensory neurons. Experimental evidence indicates that GABA mimicks the action of a natural algal ligand in this situation. In contrast, results of our recent experiments suggest that in *I. obsoleta,* GABA functions along with NO to inhibit metamorphosis. Experimental confirmation of this idea is proceeding in our laboratory.

Serotonergic neurons are retained in the anterior portion of the larval brain of most marine invertebrates, including the marine snails. Serotonin triggers or promotes metamorphosis in several other molluscs, but is not a universal metamorphic inducer. In contrast, NO preserves the larval state in *I. obsoleta* and although comparative work is limited, NO plays similar, although not necessarily identical roles in a few related species. Thus, metamorphic actions of NO and 5-HT generally appear to be conserved, especially within related molluscan taxa. However, we speculate that interactions between different gastropod larvae and diverse arrays of biological and physical features within their native ecosystems have led to the divergent evolutionary adaptations seen in the use of GABA and its receptors.

INTRODUCTION

The gastropod molluscs, the group that includes the snails and their close relatives, generally experience two distinct types of environments during their lives. The adults typically inhabit a particular region in the benthos, such as a soft sediment mudflat, a rocky intertidal zone or a subtidal coral reef. In contrast, the developing larval stages are planktonic, floating freely and swimming weakly in the water column. Metamorphosis is the dramatic developmental phenomenon that links these two disparate life history phases, transforming a tiny (<1mm) larva into a juvenile or miniature, sexually immature adult (Chia 1974; Crisp 1984).Metamorphosis is also a process that

unites the members of the Phylum Mollusca, even though many of the most advanced species, including a number of the caenogastropods (Bouchet and Rocroi 2005), have relegated vestigial forms of this process to their encapsulated embryos. Metamorphosis in true biphasic species is a complex eco-physiological process that begins with responses of larval sensory receptors to the external environment (Hadfield 1986).These responses change internal neural activity patterns (Leise and Hadfield 2000) and lead to alterations in bodily form and function. Metamorphosis ends with a newly transformed juvenile that may have distinctive food or microhabitat requirements, but is nonetheless capable of surviving in its final adult ecosystem (Hadfield 2000).

Marine organisms, adults as well as their early life history stages, face increasing stresses as anthropogenic effects proliferate, particularly in coastal waters (Deschaseaux et al. 2010; Kerr 2010). Marine organisms face subtle but potentially significant interference from anthropogenic pollutants because they often rely on chemical cues to initiate metamorphosis and recruitment and to find food and mates (Croll 1983; Atema 1996; Sidorov and Polyanina 2003; Hoegh-Guldberg et al. 2007; Guinotte and Fabry 2008; Kuffner et al. 2008; Tamburri et al. 2008; Crim et al. 2011). Thus, it is imperative that we understand how marine invertebrates develop and interact within their natural ecosystems.

Although we still have much to learn, research into the development of a number of molluscan species, some with significant commercial or ecological importance, has helped us to comprehend not only the mechanisms underlying the ontogeny of these individual species, but the patterns of molecular, cellular and organismal interactions that allow animals to succeed in complex nearshore environments (Beiras and Widdows 1995; Atema 1996; Boettcher and Targett 1996; Jackson et al. 2002; Croll 2009). We work with one of the more widespread and ecologically important gastropods, the eastern mud snail *Ilyanassa obsoleta* (Figure 1).

Because of its wide geographical range (Gosner 1971; Ruppert and Fox 1988) and the ease with which it can be reared in the laboratory (Gharbiah et al. 2008), this species has been used for over 130 years as a model system for explorations into molluscan development (Collier 2002).*I. obsoleta* is also a major agent of sediment disturbance, impacting the structure and complexity of temperate western Atlantic mudflats (Hunt et al. 1987; Kelaher et al. 2003). Our research with this snail has yielded major insights into the neurobiology and ecophysiology of invertebrate metamorphosis, and in this chapter we review our findings as well as describe our current research directions.

Figure 1. (A) Adult *l. obsoleta* exposed at low tide on a mudflat at the UNC Wilmington Center for Marine Science.(B) Adult female laying egg capsules on the side of a laboratory aquarium.

In the laboratory larval *I. obsoleta* hatch from egg capsules after 6 -10 days and then must grow in culture, feeding on single-celled algae and more than doubling in shell length before they acquire the ability to metamorphose (Scheltema 1962; Gharbiah et al. 2008). During the planktonic period, the free-swimming larval phase of *I. obsoleta* is the same as that of other advanced gastropod molluscs. This is the veliger larva, which possesses an anterior head

bearing sensory structures, much of the larval central nervous system (CNS) and a distinctive bilobed velum, the ciliated flaps of tissue that are the larval swimming and feeding structures (Figure 2). Behind the head is the visceral mass housed in a delicate shell and below is the ciliated and muscular foot upon which the animal can crawl (Fretter and Graham 1962). Larval *I. obsoleta* reach metamorphic competence some 2-3 weeks after hatching, depending upon the temperature and larval and algal food density in culture (Scheltema 1961; 1962; Couper and Leise 1996; Gharbiah et al. 2008). At competence a larva contains rudiments of all of the major adult organs and tends to spend more time crawling than swimming, apparently searching for an appropriate site for metamorphosis and its post-larval, juvenile existence (Scheltema 1961; 1962; Bishop et al. 2006a; Heyland and Moroz 2006).

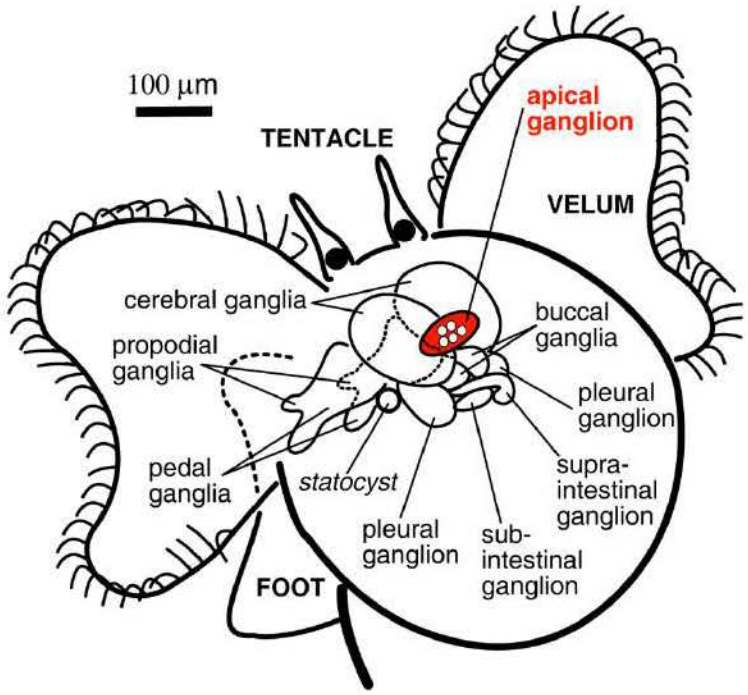

Figure 2.Diagram of the CNS (osphradial ganglion omitted for clarity) of a competent larva of *Ilyanassa obsoleta*. The apical ganglion (AG), also known as the apical or cephalic sensory organ, contains 5 serotonergic neurons and innervates the velum, 2 ciliated flaps of tissue used for swimming and feeding. The AG contains 26 - 28 neurons, all of which appear to produce NO (Thavaradhara and Leise, 2001; Dickinson and Croll, 2003).The velum and AG (Gifondorwa and Leise 2006) are lost during metamorphosis (modified from Lin and Leise, 1996a).

Settlement, the reversible behavior during which swimming ceases (Chia and Koss 1988), generally precedes metamorphosis in gastropod molluscs. Biological odorants are major triggers for metamorphosis in marine snails and typically they arise from the juvenile habitat, but physical features can also affect the metamorphic induction process (Rittschof et al. 1998; Pineda et al. 2010). Inductive odorants can have multiple origins, including conspecifics, prospective mates, preferred prey or microbial biofilms (reviewed in Rodríguez et al. 1993; Qian et al. 2007; Leise et al. 2009a).The specific sources of some environmental odorants have been identified, such as juvenile food for the conch *Strombusgigas*(Boettcher and Targett 1996), barnacles for *Concholepasconcholepas*(Manríquez et al. 2004), and diatoms and bacterial films for various abalone species (Kawamura 1996; Roberts 2001; Gallardo and Buen 2003; Daume 2006), but in many cases the inductive molecules are still being discovered (Steinberg et al. 2002; Harder 2008). Scheltema (1961) postulated that odorants arising from benthic diatoms might induce metamorphosis in larval *I. obsoleta*. Juveniles several months old can survive and grow on a diet of diatoms (Brenchley 1987), suggesting that diatoms may indeed be important to metamorphosing larvae in their natural habitat. Surprisingly, when we began our research (circa 1992), knowledge of any diatom inducers for *I. obsoleta* was still hypothetical. We discuss our confirmation of Scheltema's hypothesis below and plans for future investigations into this topic.

Larval responses to stimuli at the commencement of metamorphosis include actions of some of the classic mammalian neurotransmitters, such as serotonin (5-HT), dopamine (DA), norepinephrine (Nep) and epinephrine (Pires and Hadfield 1991; Couper and Leise 1996; Croll et al. 1997; Pires et al. 1997; 2000; Croll and Dickinson 2004) as well as more novel ones such as the gas nitric oxide (NO, Froggett and Leise 1999; Bishop et al. 2006b; Heyland and Moroz 2006). The neurotransmitter γ-aminobutyric acid (GABA) can elicit metamorphosis in a number of abalone species, but generally does so as an external ligand (Morse and Morse 1984; Morse 1985; 1990; Roberts 2001; Laimek et al. 2008; Yu et al. 2010b). Results of our recent experiments are in contrast with this action and suggest that GABA may have internal inhibitory functions in *I. obsoleta* (Leise et al. 2009b). Neuropeptides in the FMRFamide family and leu-enkephalin are also produced by gastropod CNSs (Croll and Voronezhskaya 1995; 1996; Dickinson et al. 1999; Croll 2000; Croll and Dickinson 2004; Wollesen et al. 2007) and along with 5-HT, DA and Nep are collectively involved in controlling the beat frequency of velar cilia, ciliary arrests and muscular contractions of the velar lobes (Braubach et al. 2006;

Croll 2009). These neurotransmitters then, govern behaviors that allow larvae to interact with and perhaps select potential metamorphic sites. Metamorphic actions of other peptides, such as small cardioactive peptide, are still undefined (Barlow and Truman 1992).

Metamorphosis can take 24-48 hours to complete in cultured *I. obsoleta*. While molluscs may metamorphose more rapidly under natural field conditions (Hadfield 2000; Hadfield and Koehl 2004), such situations are less conducive for investigations into the mechanisms underlying this important developmental event.

Larval-specific structures are lost or resorbed during metamorphosis, while adult organs are elaborated (Hadfield 2000; Hadfield et al. 2001). In *I. obsoleta*, loss of the velar lobes has been used to indicate that metamorphosis has occurred (Scheltema 1962). As in other molluscs, major organ systems such as the gut and nervous system (NS) undergo rearrangement (Bonar and Hadfield 1974; Bickell et al. 1981; Bickell and Kempf 1983; Lin and Leise 1996a), but in the caenogastropods, including *I. obsoleta*, no hard parts (shell, radula or operculum) are lost. Also, larval *I. obsoleta* will continue to grow after reaching competence if they do not encounter a suitable metamorphic substrate. However, they will eventually lose specificity, metamorphosing spontaneously in the absence of any known inducer, an adaptive response to eventual predation or advection from suitable habitats (Scheltema 1956; 1961; 1971; Doyle 1975; Pechenik 1980).Such larvae have been useful for investigations into the actions of stimuli that inhibit metamorphosis (Froggett and Leise 1999; Leise et al. 2009a; b).

Adult *I. obsoleta* rely on olfactory cues to find food items, especially the carrion they require for reproductive success (Carr 1967; Atema and Burd 1975; Hurd 1985; Zimmer et al. 1999). Adults of *Nassariusreticulatus,* a closely related species (Bouchet and Rocroi 2005), have chemosensory neurons in the osphradium, an adult olfactory organ, and in other locations, such as the siphon, tentacle and edge of the foot (Crisp 1971; 1973; 1976). While the NS in competent larvae may be considered rudimentary, larvae display sensory neurons in similar regions, including the edges of the foot and mantle cavity and in the apical ganglion (AG), the anterodorsal-most subdivision of the larval brain, also referred to historically as the apical or cephalic sensory organ (Bonar and Hadfield 1974; Kempf et al. 1997; Marois and Carew 1997a; Leise et al. 2001; 2004; Thavaradhara and Leise 2001; Dickinson and Croll 2003). Ganglia that would receive axons from these sensory neurons are also present in larvae; competent larvae contain rudiments of all adult ganglia (Lin and Leise 1996a).Whether the osphradium is

functional in larvae is unknown, but larvae do possess a siphon (to direct seawater into the mantle cavity where the osphradium lies) and a sizeable osphradial ganglion that could function in the detection of inductive odors (Lin and Leise 1996a; b). Larvae have two large cerebral ganglia at the bases of the tentacles, and the AG that lies atop the cerebral commissure has been experimentally and structurally implicated in the reception of metamorphic cues in other molluscs (Bonar 1978; Kempf et al. 1997; Marois and Carew 1997a; b; c; Hadfield et al. 2000; Leise and Hadfield 2000; Page 2002). Competent larvae thus have the neural equipment necessary for processing biological odorants that would reveal the relative merits of potential metamorphic, and thus juvenile, habitats.

DIATOM ODORANTS CAN TRIGGER METAMORPHOSIS

Diatoms can trigger metamorphosis in larvae from at least 5 major invertebrate phyla: the Arthropoda (Le Tourneux and Bourget 1988; Patil and Anil 2005), Annelida (Harder et al. 2002), Ectoprocta (Dahms et al. 2004), Mollusca (Daume 2006) and Echinodermata (Ito and Kitamura 1997). Within the Mollusca, bivalves that respond to diatoms have been investigated because of their commercial importance in the food and pearl industries, as resources for shell-based materials, or because of their participation in biofouling communities (Qian et al. 2007; Tamburri et al. 2008; Yu et al. 2010a). Diatoms and their soluble extracts can induce larval metamorphosis in a variety of experimental regimes, including larval exposure to single or multiple diatoms in culture or to natural or artificial biofilms (Slattery 1992; Kawamura 1996; Kavouras and Maki 2003; Najmudeen and Victor 2004; Chiu et al. 2007; Roberts et al. 2007). A number of diatom species can produce metamorphically inductive compounds known, in some instances, to be extracellular polymers (Lam et al. 2005a; b; Patil and Anil 2005). Diatoms tend to co-occur with a typical bacterial assemblage that can affect both their growth and their exuded exopolymers(Grossart et al. 2005; Grossart and Simon 2007).Thus, most studies, including our own, utilize diatoms along with their associated microorganisms because cultures created in the presence of antibiotics can display reduced or limited inductive abilities (Chiu et al. 2007; Roberts et al. 2007).Larval-diatom interactions among the gastropods are well-studied in the herbivorous limpets and abalone, relatively primitive members of this class that are likewise commercially significant throughout the Pacific Rim (Kawamura 1996; Roberts 2001; Najmudeen and Victor 2004). The

caenogastropods include predatory species (Anderson 1998), some of which metamorphose in response to particular species of prey (e.g., *C. concholepas*, Manríquez et al. 2004). *I. obsoleta*, although a member of a major caenogastropod family (Nassariidae), is omnivorous as an adult (Curtis and Hurd 1979; 1981; Connor and Edgar 1982; Hurd 1985; Zimmer et al. 1999). Its ability to derive nutrition from diatom cells and other microorganisms differentiates it from its more strictly carnivorous relatives. Juvenile *I. obsoleta* can also thrive on diatoms (Brenchley 1987), adding to the relevance of the literature on diatom induction of metamorphosis, even on taxonomically remote species.

As mentioned, induction of metamorphosis among the Haliotidae, the family of abalone species, continues to elicit investigation. Diatoms in multiple genera, such as *Amphora, Cocconeis, Navicula, Nitzschia,* and *Pleurosigma,* are metamorphically inductive to larval abalone and often, though not always, are of high nutritional value for newly metamorphosed juveniles (Kawamura 1996). Evidently, newly metamorphosed juveniles can crack such diatoms and easily extract their cellular contents. Prostrate benthic diatoms appear to fulfill these requirements more readily than erect or upright species that create complex 3-dimensional communities (Kawamura et al. 1995; 1998b; Kawamura 1996; Roberts 2001). Preferred diatom prey also tend to promote rapid growth (Kawamura et al. 1995; Kawamura 1996; Yang 1998).

We note that larval (as opposed to juvenile) *I. obsoleta* do not eat benthic diatoms, feeding instead on small unicellular flagellated algae. Larval attraction to diatoms is thus part of the metamorphic process, not a predator-prey relationship. Scheltema(1956; 1961) postulated that benthic diatoms were a likely source for the water-soluble biological compounds that could induce larval metamorphosis in *I. obsoleta.* This was a well-reasoned hypothesis. Benthic diatoms are cells of high nutritional quality that occur abundantly on intertidal mudflats (Sakshaug and Holm-Hansen 1977; Cammen et al. 1982; McIntyre et al. 1996; Cahoon et al. 1999). They are also a known food source for benthic herbivores and omnivores (Haines and Montague 1979; Hughes and Sherr 1983; Sawatpeera et al. 1998), including adult and juvenile *I.obsoleta* several months old (Scheltema 1964; Connor and Edgar 1982; Brenchley 1987).

We tested Scheltema's idea in a series of laboratory experiments in which we exposed competent larvae to extracts of sediment, a mixed diatom culture, and clonal cultures, all isolated from mudflats inhabited by juvenile and adult snails. In short, we confirmed Scheltema's hypothesis (Leise et al. 2009a).

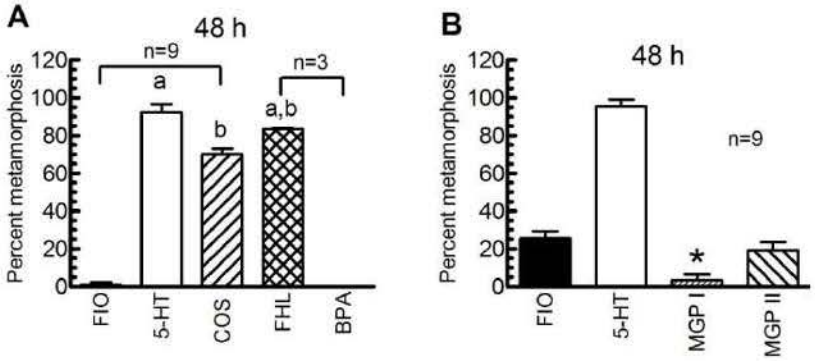

Figure 3.Metamorphic responses to diatom extracts after 48 hours of exposure.0.2μm filtered artificial seawater (FIO) and 0.1mM serotonin (5-HT) are negative and positive controls, respectively, in all experiments.(A) A tychopelagic diatom, *Coscinodiscus sp.* (COS), and a benthic pennate diatom isolated at the Friday Harbor Laboratories, Friday Harbor, WA (FHL) induced metamorphosis in competent larvae. Larvae were unresponsive to an attached benthic pennate species (BPA).Bars with common letters display statistically indistinguishable levels of metamorphosis ($P>0.05$, contingency table analysis, n is # of replicates with 10 animals per replicate; from Leise et al., 2009a).(B) Extracts of cultures of MGPI, a pennate diatom, significantly reduce metamorphosis below levels in FIO (*).Another extract, MGPII, has no activity on competent larvae (from Leiseet al. 2009a).

Of the 7 species tested, one local species, the large centric diatom *Coscinodiscussp.* (COS), triggered metamorphosis (Figure3A)(Leise et al. 1996; 2009a). This tychopelagic species can be suspended in the plankton, but settles to the benthos under calm mudflat conditions. For a variety of reasons that we now discuss, we suspect that multiple diatom species contribute to natural induction, and we anticipate that in combination, multiple inductive species will elicit a more robust response from competent larvae than any single species. First, although COS can induce metamorphosis robustly, it often elicited significantly less metamorphosis than 0.1mM 5-HT (Figure 3A), the positive control we use in our experiments (Leise et al., 2009a). Second, multiple cryptic *Coscinodiscus* species occur along the North Carolina coast (Hustedt 1955).Congeneric species can differentially affect the process of metamorphosis (Kawamura 1996; Dobretsov et al. 2006; Leise et al. 2009a), and while we do not yet know if all *Coscinodiscus* species are equally inductive, they may be. *I. obsoleta* is widespread along the Atlantic coast and has even invaded Pacific coastal mudflats (Kozloff 1983; Ruppert and Fox 1988), hence its larvae have had the opportunity to interact successfully with multiple diatom species. Given the number of different diatoms that can

induce metamorphosis in species of *Haliotis*(Kawamura 1996; Najmudeen and Victor 2004; Roberts et al. 2007), we expected to find several species that were inductive to *I. obsoleta*. Extracts from a culture of an attached benthic pennate diatom that had been isolated at the Friday Harbor Laboratories in Washington state displayed significant inductive abilities (Figure 3A, FHL), supporting our idea of the attractiveness of multiple diatoms for competent larvae of this snail (Leise et al. 2009a).

Investigations of olfactory abilities in two major Arthropod taxa, the insects and the crustaceans, tell us that multi-component odorant mixtures are often more effective than individual compounds alone (Hildebrand 1995; Gentilcore and Derby 1998).Thus, we had anticipated that extracts of mixtures of diatom species would elicit high levels of metamorphosis. Intriguingly, this was not the case. Extracts of both raw sediment and a culture of mixed diatoms elicited low levels of metamorphosis, 26% and 27% respectively, compared to the 64-77% routinely elicited by COS after 48 hours of exposure (Leise et al. 2009a).The low levels of metamorphosis mentioned above could be explained as problems with our experimental protocol, but more informative rationales exist. The sediment chosen and the month during which the diatom mixture was in culture and thus changing in species composition, may have resulted in these extracts containing compounds from species that elicited neutral or even inhibitory responses from larval *I. obsoleta*, compounds that could decrease the effectiveness of any metamorphic inducers included in these extracts.

We were surprised to discover that genera that induce metamorphosis in abalone were ineffective in *I. obsoleta.* An unidentified species of *Navicula* and *Nitzschiaclosterium*, species that are congeneric to diatoms that are inductive for *Haliotisrufescens, H. discushannai, H. iris,* and *H. varia*(Kawamura 1996; Siqueiros-Beltrones and Voltolina 2000; Najmudeen and Victor 2004; Roberts et al. 2007) elicited no metamorphic response from competent *I. obsoleta*(Leise et al. 2009a).*N. closterium*and various species of *Navicula*occur broadly in east coast habitats as both planktonic forms and members of the benthic microflora(Hustedt 1955; Cahoon and Laws 1993).We suspect that either they are nutritionally poor food items for juveniles or that their ubiquity carries little useful information about the worth of prospective microhabitats. Our results also indicate that one cannot make assumptions about the inductive capabilities of various diatom species, even among closely related larval types. Various gastropods, even species within a single genus (e.g., *Haliotis*) can display widely different responses to the same diatom species (Kawamura 1996; Daume et al. 1999; Roberts et al. 2007), again

reflecting differential larval preferences for distinct metamorphic or post-larval microhabitats or juvenile nutritional requirements.

The low levels of metamorphosis elicited by the extracts from mixed sediment and diatom cultures mentioned earlier suggested that larval *I. obsoleta* could reject unfavorable microhabitats, as can other larval types (Woodin 1991; Walters et al. 1996; Woodin et al. 1998).One of our experiments serendipitously supported this hypothesis. A benthic pennate diatom species isolated from the Myrtle Grove area of the Wilmington, North Carolina coast (MGPI) elicited a level of metamorphosis significantly below that seen in artificial seawater (FIO, Figure 3B).The most parsimonious explanation for this low level of metamorphosis is that soluble compounds from MGPI inhibited spontaneous metamorphosis.

Newly metamorphosed gastropods often use adult, bacterial, and diatom mucus as a food source for a few days to a few weeks, before they are able to ingest and utilize diatom cellular contents (Slattery 1992; Kawamura and Takami 1995; Takami et al. 1997; Kawamura et al. 1998b; Gallardo and Buen 2003). We do not yet know if young *I. obsoleta* do likewise. Preliminary behavioral experiments with newly metamorphosed juveniles indicated that ~40% were attracted to fish routinely used to feed adults (Leise, unpublished data).However, we do not know if such juveniles can actually ingest and utilize this carrion for nutrition. We also do not know when the majority of snails might switch to their adult omnivorous diet. Other gastropods, such as *Haliotis discus hannai*, change to their adult food when longer than about 1.8 cm (Takami et al. 2003). Even among herbivores, juvenile feeding preferences change as animals grow and become capable of dislodging, ingesting, and fragmenting strongly adherent diatom cells or larger algae (Kawamura et al. 1998a; Roberts et al. 1999; Siqueiros-Beltrones and Voltolina 2000).As mentioned, sexually mature *I. obsoleta* require a high protein diet for reproductive success, so juveniles might be expected to make a dietary change before reproduction begins.

More experiments are clearly required before we will have definitive information about the importance of diatoms to metamorphosing larvae and the resultant juveniles. Unanswered questions include: do inductive diatoms represent the best juvenile food source? Do multiple diatoms inhibit metamorphosis? Can juveniles ingest and grow on inhibitory diatoms or are they poor food items? And, if some diatoms can inhibit spontaneous metamorphosis, can they also suppress pharmacologically induced metamorphosis? These are just a few of the questions we hope to address in future research projects. And, while we understand something about the

actions of both serotonergic and nitrergic neurons in this system, as we describe below, we do not yet know how the neural circuit that controls the initiation of metamorphosis responds to diatoms either as inducers or inhibitors.

SEROTONERGIC NEURONS ARE RETAINED IN LARVAL MOLLUSCS

Serotonin (5-HT) is a major excitatory neurotransmitter that is broadly expressed in adult brains throughout the animal kingdom. A small suite of serotonergic neurons also differentiates early in development in a number of invertebrates (Croll 2000). Most marine invertebrate larvae display a set of serotonergic neurons in an anterior region of the brain (Lacalli 1994) and within molluscan veliger larvae, these neurons generally occur as part of the apical ganglion (Kempf et al. 1997; Marois and Carew 1997a; Page and Parries 2000; Croll and Dickinson 2004). Five serotonergic neurons occur in this ganglion in larval *I. obsoleta* (Leise et al. 2004). Some form of an apical ganglion or organ is likewise retained by most marine invertebrate larvae (Lacalli 1994). This structure is generally an outgrowth of the apical tuft, an anterior group of cells with elongated cilia that appears to act as a sensory organ in a variety of larval types (reviewed in Croll and Dickinson 2004). In molluscan larvae, the apical tuft is well developed in the trochophore stage that precedes the veliger. The gastropod apical ganglion is often described as a sensory organ, but is more correctly a sensorimotor structure as it contains all 3 basic types of neurons (sensory, motor and interneurons) and is likely to be involved in the control of velar lobe motility (Mackie et al. 1976; Kempf et al. 1997; Marois and Carew 1997c; Hadfield et al. 2000; Croll and Dickinson 2004; Braubach et al. 2006).

Studies on the developmental functions of 5-HT in gastropods have illuminated a few key roles for this neurotransmitter. Serotonergic neurons may play pathfinding roles early in the development of the NS (Dickinson et al. 1999; Dickinson and Croll 2003) and identified serotonergic neurons regulate the early rotational behavior displayed by encapsulated embryos of the pulmonate snail *Helisoma trivolvus*(Goldberg and Kater 1989; Diefenbach et al. 1991; Goldberg 1995; Kuang et al. 2002). Levantine and Bonar (1986) reported that this neurotransmitter could induce metamorphosis in larval *I. obsoleta*, but the mode of action of 5-HT was unclear. That is, was 5-HT

inducing metamorphosis by binding to epidermal chemosensory receptors or by being taken up by larvae to act internally as a neurotransmitter, and thus mimicking the action of endogenous 5-HT at postsynaptic targets? A series of experiments in which 5-HT and related neuroactive compounds were injected into larvae demonstrated that pharmacological activation of serotonergic receptors and blockade of serotonergic membrane transporters could trigger metamorphosis in the absence of any other inductive agent (Couper and Leise 1996). Injection of neuroactive agents into larvae often produced somewhat less robust results than did bath application experiments, but these procedures allowed us to introduce reagents directly into larvae, observe their responses, and obtain statistically significant results from compounds that were otherwise ineffective when applied externally. For example, injected 0.1mM 5-HT generally induced 50-52% of larvae to metamorphose (Figure 4), compared to 70-100% in bath application experiments (Couper and Leise 1996). Injection of artificial seawater elicited metamorphosis from 1% of larvae or less. More importantly, 0.1mM α-methyl 5-HT, a 5-HT agonist, was inactive in bath application, but when injected, elicited metamorphosis from over 40% of larvae within 48 hours (Figure 4). Likewise, 1μM fluoxetine, a selective serotonin reuptake inhibitor later marketed as Prozac©, triggered 61% of larvae to metamorphose when injected. It too was inactive in bath application situations (Couper and Leise 1996). The preponderance of data from these experiments provided strong support for the idea that 5-HT applied in the bath was being taken up by larvae to act at specific internal membrane receptors.

Given the conservation of serotonergic neurons early in development and within the NSs of many marine invertebrate larvae, one might speculate that 5-HT would be a universal inducer of metamorphosis. Interestingly, it is not. Serotonergic actions in the inititation of metamorphosis have not been broadly studied, but where reported, the results are mixed (Table 1).A number of invertebrates do respond positively to 5-HT as a metamorphic inducer, but others respond only weakly or not at all. Additionally, where 5-HT elicits no response (e.g., in competent larvae of the nudibranch*Phestillasibogae*) results often remain unpublished (M.G. Hadfield, personal communication), so any compendium of serotonergic actions may be skewed towards positive responses. Clearly, while serotonergic neurons are broadly expressed in larval NSs, their functions have diverged over the course of evolutionary time as larvae came to interact with a diverse array of marine ecosystems.

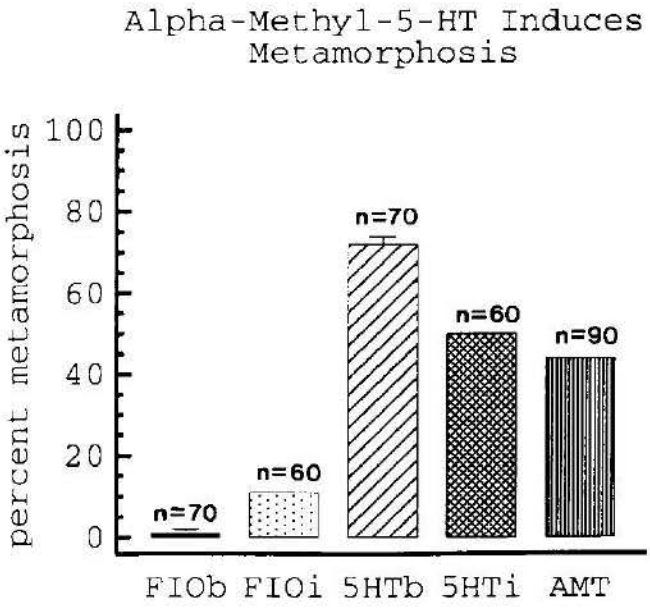

Figure 4. Injection of the 5-HT agonist α-methyl-5-HT (AMT) elicits 44% of larvae to metamorphose and is as effective an inducer as injected 5-HT (5HTi).AMT injection also elicited significantly more metamorphosis than did injection of artificial seawater (FIOi, 11%) in this experiment. Levels of metamorphosis in response to bath application of 5-HT (5HTb) indicate that most larvae have achieved competence by the start of this experiment (from Couper and Leise 1996).

NITRIC OXIDE INHIBITS METAMORPHOSIS

The gaseous neurotransmitter nitric oxide (NO) was originally identified as endothelial-derived relaxing factor (EDRF), a descriptor of its function in mammalian cardiovascular systems (Garthwaite et al. 1988; Furchgott and Vanhoutte 1989; Moncada et al. 1989; Ignarro 1990). Despite the novel nature of this signaling molecule, NO was rapidly found to be broadly retained within NSs of animals from all of the major phyla (Dawson and Snyder 1994; Garthwaite and Boulton 1995; Snyder 1995; Gelperin et al. 1996; Jacklet 1997; Palumbo 2005). In adult molluscs, NO has multiple functions, including activation of feeding circuits, modulation of chemosensory processing and odor discrimination (Gelperin and Tank 1990; Moroz et al. 1993; 2000; Elphick et al. 1995; Gelperin et al. 1996; 2001; Jacklet 1997; Jacklet and Tieman 2004; Watanabe et al. 2008). To the best of our knowledge, actions of

NO during molluscan development were unknown until we discovered the presence of its generative enzyme, nitric oxide synthase (NOS), within the CNSs of larval *I. obsoleta*(Lin and Leise 1994; 1996b). Neuronal NOS (nNOS) uses nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate, so this enzyme can be localized in tissue sections by NADPH diaphorasehistochemistry(Sheehan and Hrapchak 1980; Bredt et al. 1991; Dawson et al. 1991; Hope et al. 1991; Bredt 1995). NADPH diaphorase, and thus nNOS activity, occurs in all major ganglionic neuropils in larval *I. obsoleta* and staining intensity appears to increase as larvae develop metamorphic competence. At that time, the neuropil of the apical ganglion (AG) displays the most NADPH diaphorase activity (Lin and Leise 1996b).

Table 1. Responses of marine invertebrate larvae to serotonin. Serotonin induces a number of invertebrate larvae to metamorphose (+) but some larvae only respond weakly to it (+/-) or are inhibited by it (-)

Organism	Response	Citation
Cnidaria *Phialidiumgregarium*	+	(McCauley 1997)
Mollusca -*Mytilusgalloprovincialis* -*Ruditapesphilippinarum* -*Crassostreagigas* -*Pinctada maxima* -*Ilyanassa obsoleta* -*Hermissendacrassicornis* -*Haliotisasinina*	 + + +/- + + + +/-	 (Satuito et al. 1999) (Urrutia et al. 2004) (Beiras and Widdows 1995) (Zhao et al. 2003) (Levantine and Bonar 1986; Couper and Leise 1996) (Avila et al. 1996) (Wang et al. 2010a)
Arthropoda -*Balanusamphitrite*	+	(Yamamoto et al. 1996)
Ectoprocta -*Bugulaneritina*	--	(Shimizu et al. 2000)
Urochordata -*Phallusiamammillata*	+	(Zega et al. 2005)

Curiously, our specimens displayed no staining in any neuronal cell bodies, a situation that we suspect could be remedied by modifying our paraformaldehyde fixation protocol. The length and robustness of the aldehyde fixation can dramatically affect cellular staining quality (Vincent 1995). Later immunocytochemistry improved upon these histochemical results, displaying NOS-like immunoreactivity (NOS-IR) in nearly all of the neuronal somata of the AG as well as in sensory neurons on the edge of the mantle and foot. The number of cells in the AG that display NOS-IR also increase throughout the larval period (Thavaradhara and Leise 2001). Lin and Leise(1996b) also established that NADPH diaphorase staining intensity dropped during metamorphosis, to reappear in a somewhat different pattern in young juveniles. This discovery suggested that NO might play a role in the metamorphic pathway, but not what that role might be.

We used several types of nitrergic reagents in pharmacological experiments to determine how NO might act in the metamorphic pathway (Froggett and Leise 1999). Bath application of NO-donors, compounds that degenerate into NO and an inactive side-product in solution, had no effect on competent larvae. Larval exposure to several NOS inhibitors likewise resulted in no metamorphic responses. However, two NO-donors, S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and 3-morpholino-sydnonimine (SIN-1) significantly suppressed serotonergically-induced metamorphosis. Injection of the NOS inhibitors N-nitro-L-arginine methyl ester (L-NAME) and N-methyl-L-arginine acetate (L-NMMA) proved to be crucial, allowing both of these reagents to elicit metamorphosis in the absence of any other inducer (Froggett and Leise 1999). These experiments strongly supported the idea that NO was necessary for the maintenance of larval life and in its absence, competent larvae would undergo metamorphosis (Figure 5). Since our initial work with *I. obsoleta*, other researchers have studied NO actions in additional larvae. Pharmacological manipulation of NO levels can regulate larval metamorphosis in 4 phyla: the Mollusca, Annelida, Echinodermata and Urochordata (Bishop et al. 2001; 2008; Bishop and Brandhorst 2001; 2003; 2007; Gaudette et al. 2001; Pechenik et al. 2007). At least two NOS inhibitors, 7-nitroindazole (7-NI, Leise et al. 2004) and S-methylisothiourea sulfate (Pechenik et al. 2007) work well in bath application experiments, eliciting high levels of metamorphosis in competent larvae. In an opisthobranchmollusc, *Phestillasibogae*, NO appears to play a modulatory role, as inhibition of NO production promoted metamorphosis but was insufficient to induce it (Bishop et al. 2008). In its capacity as a regulator of metamorphosis, unlike other

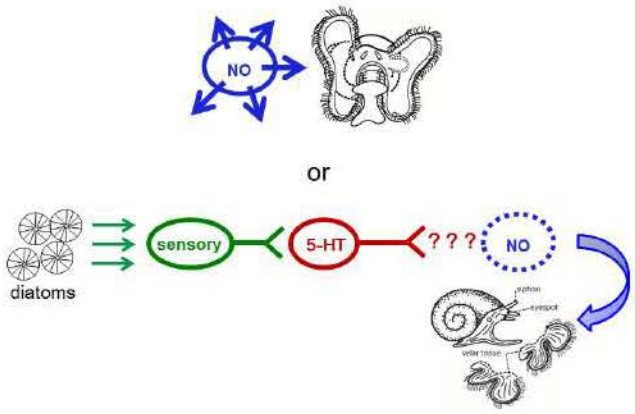

Figure 5.Diagrams illustrating current hypotheses about actions of diatoms, 5-HT and NO in the pathway that controls metamorphosis in larval *I. obsoleta*. (Upper) Production of NO maintains the larval state. (Lower) Interaction with diatoms, application of 5-HT or NOS inhibitors can all induce metamorphosis, as identified by loss of the velar lobes (Scheltema 1961; Couper and Leise 1996). Metamorphosis also includes a decrease in nNOS expression (c.f. Figures 6,7).Parts of this diagram were extracted from Lin and Leise (1996a) and Couper and Leise (1996).

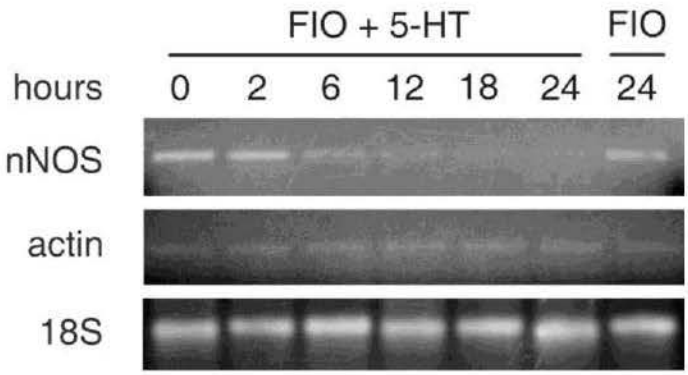

Figure 6. Levels of nNOS cDNAs drop following induction of metamorphosis by 0.1mM 5-HT as demonstrated by RT-PCR analysis. cDNAs were prepared from 1.0 µg of total RNA obtained from larvae cultured for the indicated number of hours in 0.1mM 5-HT.FIO/24 samples were cultured without 5-HT in 0.2µm filtered artificial seawater. Neuronal NOS gene expression decreases in specimens incubated for 6 - 24 hours in 5-HT.Larvae bathed in artificial seawater display no change in nNOScDNAs. Expression of actin was unchanged throughout the experimental period. 18S ribosomal RNA derived from electrophoresis of 1.0µg of total larval RNA on a formaldehyde-agarose gel demonstrates that equal amounts of RNA were used in the preparation of the template cDNAs (from Hens et al. 2006).

neurotransmitters such as 5-HT, NO's inhibitory function appears to have been conserved across phyletic boundaries.

More recent molecular studies have confirmed some of our ideas about the actions of NO in larval and metamorphosing *I. obsoleta*. The isolation and cloning of part of the gene encoding larval nNOS allowed us to conduct a semi-quantitative analysis of gene expression levels during metamorphosis with reverse transcription-based polymerase chain reaction (RT-PCR, Hens et al. 2006). NOS gene expression based on levels of nNOS complementary DNAs (cDNAs) prepared from larvae by RT-PCR drops noticeably within 6 hours of serotonergic induction, confirming our underlying hypothesis (Figures 5, 6).Unfortunately, similar semi-quantitative results did not confirm earlier observations of an increase in NADPH diaphorase activity or NOS-IR labeling during larval development (Hens et al. 2006). Creation of an antibody to the larval NOS protein did allow us to conduct a western blot analysis of changes in nNOS levels during metamorphosis (Weaver 2009).Again, NOS protein levels declined within 6 hours of metamorphic induction, giving evidence that NO production must undoubtedly drop as well (Figure 7). Weaver's (2009) results suggest that metamorphosis in *I. obsoleta* may be controlled by regulation of both the activity and amount of NOS present in the larval nervous system.

Studies on the actions of NO during neural development have yielded a variety of roles for NO, including regulation of DNA synthesis and rates of mitosis (Peunova and Enikolopov 1995; Enikolopov et al. 1999; Moreno-Lopez et al. 2004), coordination of axonal growth and synaptogenesis (Van Wagenen and Rehder 2001; Gibbs 2003; Bicker 2005) and the regulation of programmed cell death (PCD, Wang et al. 2002; 2010b; Brüne 2003). The presence of NOS-IR in the majority of the cells of the apical ganglion suggested that NO might be involved in the regulation of PCD in this part of the larval brain. Loss of the AG had been proposed previously to occur by some form of PCD (Barlow and Truman 1992; Marois and Carew 1997b). We examined larvae histologically at 12 hour intervals for 4 days after metamorphic induction, by both 5-HT and 7-NI, and used the terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assay and Hoechst 33342 staining in several attempts to demonstrate loss of the AG by PCD (Gifondorwa and Leise 2006).Our results supported this idea, but did not demonstrate early signs of the loss of this part of the brain. In *I. obsoleta,* PCD in the AG begins within 12 hours of metamorphic induction, before loss of the velar lobes occurs, and ends 3 days after induction. A recent investigation into the loss of sensory cells in the AG in the opisthobranch *P. sibogae*provided

evidence that cell death in this species begins within 3 hours of metamorphosis, overlapping in time with disintegration of the velar lobes. (Ruiz-Jones and Hadfield 2011).

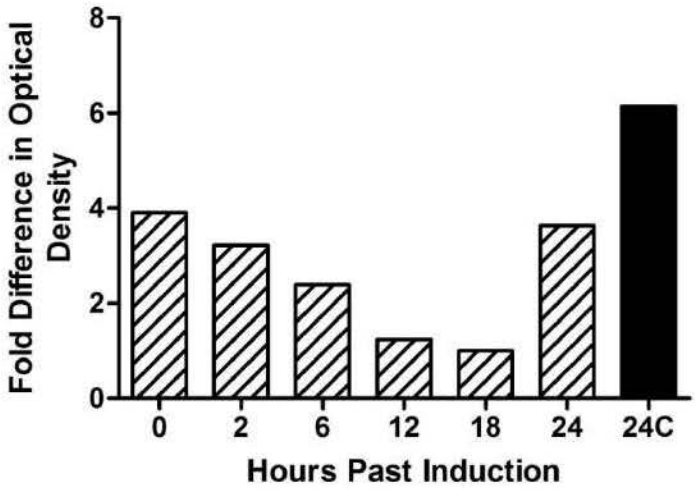

Figure 7.Optical density measurements of a 66 kDanNOS protein band. This band was identified by immune staining a western blot of larval proteins obtained at times indicated during metamorphosis, induced with 0.1mM 5-HT.Control protein (24C) was obtained from larvae not exposed to 5-HT. Within 6 hours of serotonergically induced metamorphosis, levels of NOS protein drop by nearly 1/3 of their original value. Twelve hours after induction, protein levels have decreased to less than ½ of that obtained without induction. Levels of NOS rise to pre-metamorphic levels 24 hours after induction (modified from Weaver, 2009).

As mentioned earlier, cultured larvae will tend to metamorphose spontaneously as they age. Investigations into the morphology of the AG in such larvae also demonstrated its degeneration before the velar lobes detach (Gifondorwa and Leise 2006). Whether spontaneous metamorphosis is the result of loss of NOS gene expression or depletion of enzymatic activity remains to be elucidated.

Prosobranch gastropods are not alone in their retention of nitrergic signaling pathways during development, and multiple aspects of embryonic and larval maturation may require NO. For example, pharmacological manipulation of endogenous NO production in embryos of the pond snail *H. trivolvus* have demonstrated nitrergic regulation of rotational surges (Cole et al. 2002). In another pond snail, *Lymnaeastagnalis,* NO governs several aspects of embryonic behavior, such as locomotion and feeding actions

(radular protrusions) as well as quantitative changes in the ultrastructure of their central ganglia (Serfözö and Elekes 2002).After *I. obsoleta* hatch, their central ganglia grow in size and cell number until metamorphic competence is achieved (Lin and Leise 1996a; Burrows 2005). Because NO can regulate neurogenesis in a variety of other animals (Peunova and Enikolopov 1995; Peunova et al. 2001; Moreno-Lopez et al. 2004), we wondered if that might be a function for NO in larval *I. obsoleta*. To determine how rates of neurogenesis change during larval development, Burrows (2005) incubated developing larvae in 5-bromo-2'-deoxyuridine (BrdU) for 3 hours to label cells that were undergoing replication and presumably, mitosis. BrdU was localized subsequently by immunocytochemistry. Larval specimens were then stained with 4',6-diamidino-2-phenylindole (DAPI) to facilitate the counting of neuronal nuclei in 9 major ganglia of the CNS. Numbers of nuclei labeled with DAPI and displaying BrdU immunoreactivity were compared (Figure 8)Levels of neurogenesis do indeed decline as development progresses, but such changes have yet to be correlated with a concomitant rise in NO production

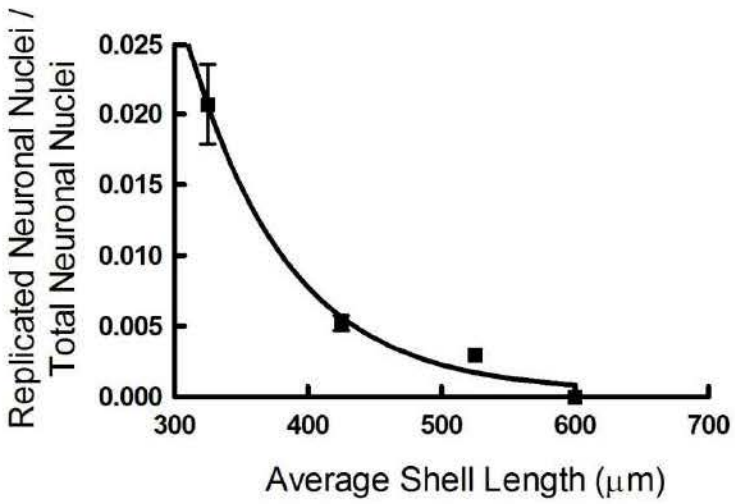

Figure 8. Neurogenesis in 9 major ganglia of the CNS decreases as larvae mature to competence. Index of neurogenesis was calculated from the average number of BrdU immunoreactive nuclei(n= 18, 19, 20 for the first 3 data points) divided by the average number of total ganglionic nuclei stained by DAPI (n=5).Ganglia used were the paired cerebral, pedal, pleural, buccal and single osphradial ganglia. Line is a non-linear regression fit with one phase exponential decay (r^2=0.9899). Ganglia were counted in larvae sectioned at 20μm (from Burrows, 2005).

(Burrows 2005; Hens et al. 2005).Experiments in which larvae were incubated in 7-NI to inhibit NOS and perhaps artificially increase neuronal numbers require further analysis.

GABA CAN INDUCE METAMORPHOSIS, BUT NOT IN *ILYANASSA*

Molluscan metamorphosis, like many developmental processes, is controlled by both permissive and inhibitory factors. This dual regulation is reflected in the behavior of competent larvae. They seek an environment with appropriate inductive cues, but can reject unfavorable habitats (Woodin et al. 1993; Walters et al. 1996), delaying metamorphosis until conditions are appropriate (Scheltema 1974; Pechenik 1990).Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter throughout the animal kingdom (Walker 1986; Kandel et al. 2000), but where molluscan metamorphosis is concerned, it is generally a permissive agent (Morse 1990; 1992; Rodríguez et al. 1993; Avila et al. 1996; Bryan and Qian 1998; Roberts et al. 1999; Roberts 2001; Garcia-Lavandeira et al. 2005; Yu et al. 2010b).In abalone, where it has

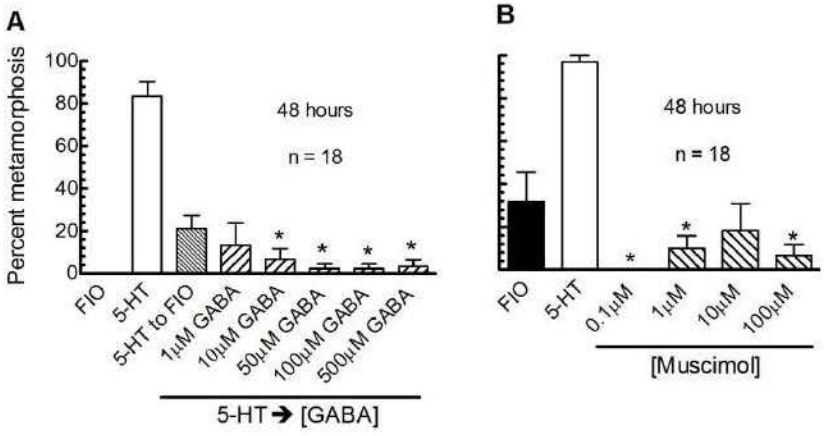

Figure 9.GABA and the $GABA_A$ agonist muscimol inhibit metamorphosis at 48 hours. (A) Larvae exposed to 0.1mM 5-HT for 5 hours, then incubated in GABA at various concentrations (5-HT→GABA) show significant reduction in levels of metamorphosis (*) compared to the 0.1mM 5-HT to FIO control. (B) Muscimol significantly inhibited (*) high levels of spontaneous metamorphosis (compare FIO in A and B) seen in this experiment. (X^2= 6.635, α=0.01, corrected for multiple comparisons, n is # of replicates, with 5 animals per replicate, from Leise et al., 2009b).

received much attention, GABA mimics a natural algal ligand that triggers metamorphosis by interacting with sensory receptors on the larval surface (Morse et al. 1979; Baxter and Morse 1992).

A few researchers have postulated that GABA might act internally as an inhibitory neurotransmitter in the control of metamorphosis (Morse et al. 1980; Feng et al. 2006), but to our knowledge, this line of evidence has not been systematically pursued (Morse 1990; Morse and Morse 1996; Croll 2009).Our recent experiments on the larval metamorphic response to GAB have led us to hypothesize that this neurotransmitter can inhibit molluscan metamorphosis(Leise et al. 2009b).

To date, manipulation of GABAergic pharmacology has yielded some intriguing results. Application of GABA to competent larvae elicits no response, except perhaps to slow swimming speed, an effect that we have not quantified. In contrast, application of GABA to larvae that have been exposed to 0.1mM 5-HT has demonstrated GABA's ability to inhibit suboptimal metamorphic induction (Figure 9A).Bath application of $GABA_A$ agonists such as muscimol can inhibit spontaneous metamorphosis (Figure 9B)and experiments are underway with further GABAergic reagents to determine whether such compounds can also affect pharmacological induction of metamorphosis. The preponderance of our current data suggests that GABA acts downstream from serotonergic neurons, but further experiments are needed to determine where GABAergic neurons might exert their effects in relation to nitrergic ones (Figure 10).Preliminary immunocytochemical procedures have labeled axons radiating across the velar lobes, but have not yet allowed us to characterize the full suite of larval GABAergic neurons.

GABA does mediate excitatory actions, particularly in developing nervous systems (Ganguly et al. 2001; Ben-Ari 2002). As neurons mature, changes in the developmental expression of membrane chloride ion transporters will alter the chloride equilibrium potential, transforming GABAergic opening of chloride channels from an excitatory action to an inhibitory one (Ben-Ari 2002). Excitatory GABAergic actions in *I. obsoleta* may occur early in development and are probably not germane to the metamorphic pathway.

I. obsoleta inhabits intertidal mudflats, whereas a number of the molluscs whose metamorphoses can be induced by GABA inhabit rocky intertidal or subtidal zones and require coralline red algae as a cue (Rodríguez et al. 1993; Roberts 2001; Mesías-Gansbiller et al. 2008; Yu et al. 2008; 2010b). *I. obsoleta* does not encounter such algae. We speculate that diverse habitats have influenced the evolution of divergent GABAergic pathways in marine molluscs.

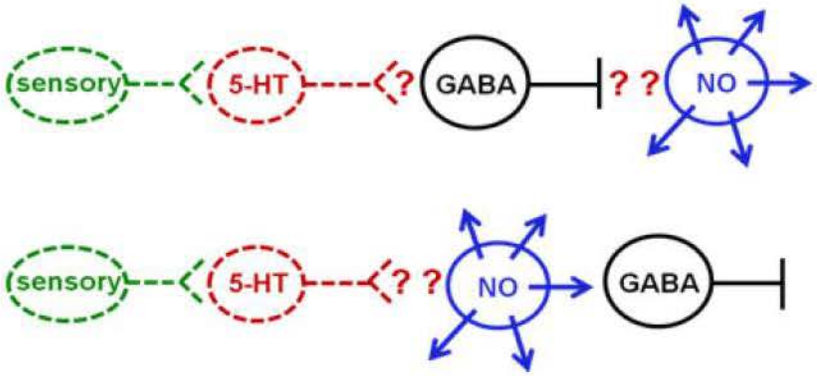

Figure 10.Two hypothetical circuits that might control metamorphosis in *I. obsoleta,* drawn in the larval state, with no 5-HT activity. (Upper) GABAergic neurons might provide tonic inhibition to the metamorphic pathway, upstream from nitrergic neurons, allowing cells of the apical ganglion to produce NO. In the presence of diatoms, activated sensory neurons excite serotonergic ones, leading to inhibition of GABAergic neurons and loss of NO production. (Lower) Alternatively, larval production of NO maintains inhibitory GABAergic activity. Activation of sensory, then 5-HT neurons might inhibit NO production, inactivating GABAergic ones, and thereby initiating metamorphosis.

CONCLUSIONS

Metamorphosis remains a complex developmental event that occurs in organisms at extremely small sizes, is often seasonal in the natural habitat and may require multiple environmental cues that can be difficult to fully replicate under laboratory conditions. Nonetheless, investigations into this process in model organisms like *I. obsoleta* have helped us to understand some of the external forces and internal circuits that drive this striking set of morphological and physiological events.

Several species of benthic diatoms can trigger larval metamorphosis in *I. obsoleta* and while we suspect that these diatoms would provide juveniles with high quality food sources, that hypothesis remains to be tested. Like other marine invertebrate larvae, *I. obsoleta* may display no response to some diatoms and appears to be inhibited by others. Again, the actions of inhibitory diatoms require further investigations into both their larval and juvenile effects.

Larval *I. obsoleta* display 5 serotonergic neurons in their apical ganglia and our pharmacological data support the idea that exogenous serotonin is

acting at internal neuronal receptors to trigger metamorphosis. The response of *I. obsoleta* to this neurotransmitter is similar to that of many marine larvae, but even though 5-HT is conserved broadly in the brains of larval marine invertebrates, it does not induce metamorphosis in all species, even within the Phylum Mollusca.

The actions of NO stand in direct contrast to those obtained for 5-HT.Multiple lines of evidence all indicate that NO inhibits metamorphosis and that in competent larvae of *I. obsoleta,* lack of NO production is sufficient to initiate this process. This may not be the case in all gastropods, or even all snails. However, NOS does appear to be expressed in most, if not all neurons of the apical ganglion, and levels of NOS mRNA and protein decrease dramatically once metamorphosis is initiated. These decreases appear to drive programmed cell death in the AG, a phenomenon that may be widespread among invertebrate larvae. Inhibitory actions for NO have already been detected in species from 3 other major invertebrate phyla.

Experimental explorations of the functions of GABA as a neurotransmitter in the metamorphic pathway are still in their infancy. Our pharmacological results thus far strongly suggest that GABA plays an inhibitory role in the metamorphic process, which may reflect its status as the major inhibitory neurotransmitter throughout the animal kingdom. Our results support the views of a few prior studies, but any systematic investigation of the actions of GABA in this role appears to be lacking. We anticipate that experiments with other methods will shed further light not just on the role of GABA, but on the phylogenetic diversity that exists with invertebrate metamorphic pathways.

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