Metamorphosis in the Marine Snail *Ilyanassa obsoleta*, Yes or NO?

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Abstract:

Metamorphosis is a crucial life-history event that can change an organism's form, function, behavior, and ecological interactions. In the Mollusca, several neurotransmitters and neuromodulators play inductive or inhibitory roles in the pathways that govern larval metamorphosis. Nitric oxide (NO) has been implicated in developmental processes in vertebrates and arthropods, but not previously in molluscs. We determined that NO donors block pharmacologically induced metamorphosis in the mud snail *Ilyanassa obsoleta*, whereas injections of inhibitors of nitric oxide synthase (NOS) allow competent larvae to become juveniles. We describe a new developmental role for NO, as an endogenous inhibitor of molluscan metamorphosis.

Article:

Introduction

Marine molluscs are an ecologically and economically significant group of organisms, yet the regulatory mechanisms underlying their metamorphosis, a key developmental process, are still poorly understood. Environmental factors that influence or directly induce metamorphosis in physiologically competent larvae are known for a handful of species, but often not in specific molecular detail (Hadfield and Pennington, 1990; Pawlik, 1992; Morse, 1994; Zimmer-Faust and Tamburri, 1994). The downstream neuroendocrine actions that control the events of larval metamorphosis have been studied in some of these same organisms, but in no instance are the molecular mechanisms that sub-serve an entire pathway understood. The actions of several neurotransmitters in this process have been investigated, but no previous report implicates NO as playing a role in molluscan development. Early reports of this work have been published only in abstract format (Froggett and Leise, 1996; 1997).

Several neuroactive compounds are known to be involved in the induction of metamorphosis in marine molluscs. For example, catecholamines, such as norepinephrine and dopamine, regulate metamorphosis in oysters of the genus *Crassostrea* (Coon and Bonar, 1986; Bonar *et al.*, 1990) and in the nudibranch *Phestilla sibogae* (Pires *et al.*, 1996, 1997). Elevated levels of endogenous serotonin (5-HT) also activate metamorphosis in the prosobranch gastropod *Ilyanassa obsoleta* (Couper and Leise, 1996). Immunocytochemical and high performance liquid chromatographic studies have demonstrated that these neurotransmitters and others, such as FMRFamide and the small cardioactive peptides, are present in the central nervous systems (CNSs) of several marine gastropods during larval development (Coon and Bonar, 1986; Bonar *et al.*, 1990; Barlow and Truman, 1992; Kempf *et al.*, 1992, 1997; Marois and Carew, 1997).

Lin and Leise (1996b) used NADPH diaphorase (NAD-PHd) histochemistry to describe a gradual increase in the occurrence of NOS (Dawson *et al.*, 1991) in the CNS of *Ilyanassa* during larval development. During metamorphosis, NADPHd staining in all ganglia drops dramatically, particularly in the apical ganglion. This ganglion, also de-scribed as the apical or cephalic sensory organ (Bonar, 1978; Lin and Leise, 1996a; Marois and Carew, 1997), innervates the velum, the larval swimming and feeding organ. During post-metamorphic development, juvenile NADPHd levels increase to produce a different staining pattern in all ganglia but the apical one. This ganglion is lost by the fourth day after metamorphic induction (Lin and Leise, 1996a). The

distinct changes in NADPHd staining patterns observed by Lin and Leise suggested that NO might play an important role in the regulation of metamorphosis in *Ilyanassa* and are consistent with three hypotheses: (a) that NO promotes metamorphosis, (b) that NO is necessary for the maintenance of the larval state, or (c) that NO is uninvolved in metamorphosis, having other larval and juvenile actions. To distinguish between these possibilities, we employed nitrergic reagents that allowed us to manipulate the function of the NOS enzyme and larval exposure to NO.

Materials and Methods

Detailed experimental protocols have previously been published (Couper and Leise, 1996). Briefly, in bath application experiments, competent larvae were placed in 2 ml of solution in wells of 24-well plastic Falcon tissue culture plates, at 10 larvae/well. In all experiments, Instant Ocean, filtered to 0.2 μm (FIO), and 10^{-4} *M* 5-HT served as negative and positive controls, respectively. In most cases, experimental and control conditions were simultaneously replicated three times, yielding a typical sample size of 30 larvae for each treatment. Graphs with more than 30 animals per treatment indicate additional experimental replications. Larvae in experimental treatments and control groups were usually obtained from one culture. If more than one culture was used, animals from both cultures were distributed among all conditions, but in separate wells so that any significant differences resulting from culture conditions could be detected. The number of larvae and metamorphosed individuals were counted at 24 and 48 h in all experiments. In all treatments in which metamorphosis was induced, some animals became juveniles by the end of the experiment (2 days after induction). However, even in a natural inducer (Leise *et al.*, 1996) or 10^{-4} *M* 5-HT, all larvae do not begin metamorphosis simultaneously. As a result, specific criteria that indicated the initiation of this irreversible process were used to evaluate larval response to each treatment. Metamorphosis was determined by an examination of the morphology of each larva. Animals that had partially or totally lost velar cilia or the velar lobes were scored as having metamorphosed.

Nitrergic reagents were obtained from Cayman Chemical Co., Tocris Cookson, Inc., or Sigma Chemical Co. and prepared just before use. During the bath experiments, mortality in control solutions was insignificant, but it was near 10% in the higher concentrations of SNAP.

Direct injection of compounds into the larval hemocoel allowed us to avoid confounding factors, such as metabolism of reagents in the seawater bath or variable uptake across the larval epidermis, that can occur in bath application experiments (Couper and Leise, 1996). To prepare competent larvae for injection experiments, they were rinsed with FIO four times, then decalcified in Ca^{2+}-free seawater for 12 h (Pires and Hadfield, 1993). Decalcified larvae were then embedded in 1% low melting point agarose (Type VII, Sigma Chemical Co.) in FIO to impede movement. Larvae were freed from the agarose and pressure injected with approximately 6 nl of experimental solution delivered subepidermally through a glass micropipette connected to a Picospritzer II (General Valve Corp). Larvae were then placed in FIO at a density of 10 larvae/2 ml. Controls always included bath applications and injections of FIO and 10^{-4} *M* 5-HT.

Animals that died during experiments were not scored. Numbers of animals (n = X) on the graphs indicate numbers at the beginning of an experiment for all concentrations unless otherwise indicated in the legend. Injection experiments had variable rates of mortality, from 2.5% to 30%, but occasionally higher (40% in L-NAME experiments). Mortality rates in Carboxy-PTIO solutions were relatively low (0%-7%). In all instances of higher mortality, rates were similar in experimental and control groups, suggesting that larval death occurred because the nervous system was inadvertently damaged during the injection procedure.

Results from each experiment were pooled and tested for statistical significance (P = 0.05) in 2-way chi-square contingency tables (Zar, 1974; Sokal and Rolf, 1981). We used the Bonferroni method to correct for multiple comparisons (Bland, 1995). If the corrected α level fell between published values, we rounded down to the nearest α value. Raw percentage data were normalized by an arcsine transform, and standard deviations were calculated on the transformed data. Data were transformed back to percentages for graphing. Graphs were produced with Deltagraph 4.0 software.

Results

Initial bath experiments utilized either S-nitroso-N-acetyl-D,L-penicillamine (SNAP) or 3-morpholino-sydnonimine (SIN-1), both NO donors, alone or in combination with the metamorphic inducer 5-HT, to determine whether metamorphosis was affected by exogenously generated NO. SIN-1 or SNAP alone in bath solutions did not significantly affect the percentage of larvae undergoing metamorphosis (Fig. 1).

Bath application of the NO-donors SIN-1 and SNAP at high concentrations reduced the ability of 5-HT to induce metamorphosis (Fig. 2). Because of these results, NOS inhibitors were injected into competent larvae to determine whether experimentally manipulated levels of endogenous NO would promote metamorphosis in the absence of any known inducer.

Injections of the NOS inhibitors N-nitro-L-arginine methyl ester (L-NAME) and N-methyl-L-arginine acetate (L-NMMA), or the NO scavenger 1*H*-imidazol-yloxy,2-(4- carboxyphenyl)- 4,5 - dihydro- 4,4,5,5 - tetramethyl -3-oxide (Carboxy-PTIO) directly into the larval hemocoel allowed us to determine how a decrease in endogenous levels of NO affects metamorphosis. Both L-NAME and L-NMMA induced significant levels of metamorphosis by 24 h when injected in the absence of 5-HT (Figs. 3, 4). Injections of the inactive isomer D-NAME did not significantly affect the percentage of larvae undergoing metamorphosis (Fig. 3B). In a final set of experiments, we attempted to induce metamorphosis by injecting Carboxy-PTIO into competent larvae (Fig. 5). These results were insignificant after application of Bonferroni's method.

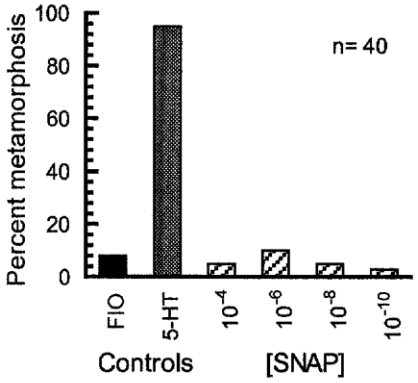

Figure 1. SNAP alone induces no metamorphosis in competent larvae by 48 h. Bath application of SNAP without 5-HT induced rates of metamorphosis similar to those induced by filtered Instant Ocean (FIO). The positive control (10^{-4} *M* 5-HT) induced 95% of the larvae to metamorphose by 48 h, a typical result. Concentrations of SNAP greater than 10^{-4} *M* were toxic to larvae, causing abnormal behavior and decalcification but no loss of velar tissue or any indication of metamorphosis (data not shown). Data from SIN-1 experiments (also not shown) were similar to those obtained with SNAP.

Discussion

All of our pharmacological data support the idea that NO acts to inhibit metamorphosis in *Ilyanassa.* Injections of NOS inhibitors into competent larvae allowed metamorphosis to proceed. Active concentrations of the NO reagents that we used were similar to or lower than those found to be active on other molluscan neural preparations (Gelperin, 1994a, b; Jacklet and Gruhn, 1994; Elphick *et al.*, 1995; Huang *et al.*, 1998). Unlike the NOS inhibitors, Carboxy-PTIO was not effective (Fig. 5). The levels of metamorphosis detected after injections of Carboxy-PTIO were significantly different from those obtained with 5-HT but not from those in the FIO controls, suggesting that the concentrations we used may have incompletely scavenged the available NO. Results with injections of the NOS inhibitors L-NAME and L-NMMA were more robust; a range of concentrations of both reagents was effective by 24 h (Figs. 3, 4). Interestingly, the FIO controls in the L-NMMA injection experiments (Fig. 4) showed unusually high levels of metamorphosis, especially by 48 h. Larvae in cultures older than 3 weeks will normally begin to metamorphose spontaneously (Leise *et al.*, 1996; unpubl. data), and the high levels of metamorphosis seen in the FIO controls at 48 h in the L-NMMA experiments (Fig. 4) suggested that spontaneous metamorphosis was occurring. In these experiments, bath application of 5-HT elicited nearly 100% metamorphosis by 24 h, suggesting that the 48-h results were

irrelevant; most metamorphosis that would occur had done so within 24 h. In the field, *Ilyanassa* larvae are probably induced to metamorphose by diatoms or associated organisms that occur naturally in their littoral habitats (Leise *et al.,* 1996), but we have no understanding of the time course for metamorphosis in that situation. Our results have led us to hypothesize that NO production is necessary for the maintenance of the larval state until an appropriate metamorphic cue is detected. Preliminary data from experiments on *Phestilla sibogae* suggest that NO may be active in the metamorphic pathway in this species as well (Meleshkevitch *et al.,* 1997). The ubiquity of NO in molluscan metamorphosis and its specific actions in this process remain to be determined.

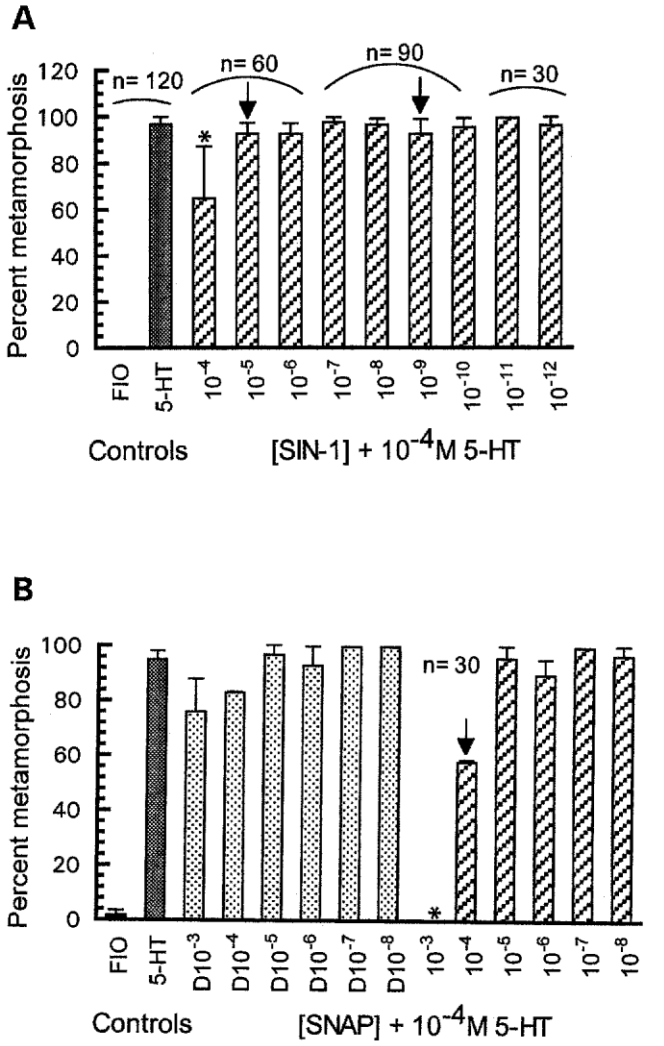

Figure 2. Inhibition of 5-HT-induced metamorphosis by SIN-1 (A) and SNAP (B) at 48 h. (A) Asterisk indicates concentration of unreplaced SIN-1 that significantly inhibited 5-HT-induced metamorphosis at 24 h (41% metamorphosis in 10^{-4} *M*, $\chi^2_{0.005(1)} = 15.5$) and 48 h (65% metamorphosis, $\chi^2_{0.005(1)} = 30.77$) compared to the 5-HT control. Arrows indicate concentrations that showed significant inhibition compared to 5-HT only at 24 h (*e.g.,* 59% metamorphosis in 10^{-9} *M* at 24 h, $\chi^2_{0.005(1)} = 13.9$), but not at 48 h. All SIN-1 solutions contained 10^{-4} *M* 5-HT. (B). Asterisk indicates concentration of SNAP that significantly inhibited 5-HT-induced metamorphosis at 24 and 48 h. Arrow indicates concentration that was inhibitory only at 24 h (40% metamorphosis in 10^{-4} *M*, $\chi^2_{0.005(1)} = 11.3$), but not at 48 h. Solutions of SNAP have a half-life of about 1 h and were changed every 6 h to maintain relatively steady concentrations of NO. $D10^{-x}$ = degassed solution of 10^{-x} *M* SNAP plus 10^{-4} *M* 5-HT; 10^{-x} = active solution of 10^{-x} *M* SNAP plus 10^{-4} *M* 5-HT.

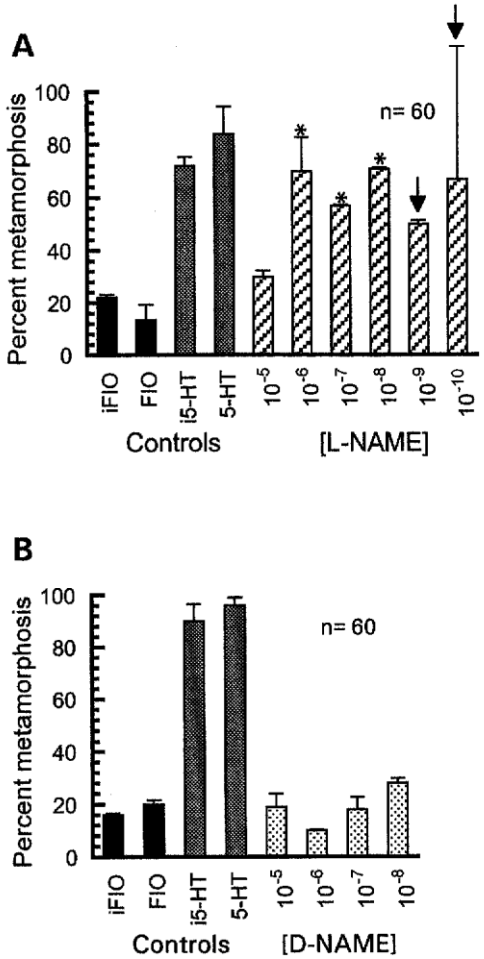

Figure 3. (A) Injections of L-NAME induced metamorphosis in competent larvae by 48 h in the absence of any inducer. Asterisks indicate levels of metamorphosis significantly different from those induced by injected FIO (iFIO) (*e.g.,* 10^{-7} *M*, $\chi^2_{0.005(1)} = 14.5$). Arrows indicate concentrations that were significantly effective by 24 h but not at 48 h. n = 30 for 10^{-9} and 10^{-10} *M* L-NAME. (B) Injections of the inactive isomer D-NAME induced no significant rates of metamorphosis by 48 h. (i5-HT = injected 5-HT).

Although the tranformations that invertebrate larvae un-dergo in reaction to metamorphic cues are among their most well known activities (Pawlik, 1992), unsuitable habitats can also elicit distinctly negative responses from

some larvae, such as those of the polychaetes *Nereis vexillosa* and *Capitella sp.* (Woodin, 1991). We recently found a similar effect on *Ilyanassa* larvae from one species of benthic diatom. Extracts of cultures of a sheathed pennate diatom species that were isolated from sediments obtained at Myrtle Grove, North Carolina, inhibit spontaneous metamorphosis in older (>3 weeks in culture) *Ilyanassa* larvae (Leise *et al.,* 1996; unpubl. data). Such negative metamorphic actions and the uncertainty of larval encounters with appropriate juvenile habitats suggest that the maintenance of the larval life-history phase is an integral component of the metamorphically competent state. For *Ilyanassa,* the production of NO by competent larvae appears to be necessary for this purpose. However, maintenance of the larval state is likely to depend upon more than one inhibitory compound. For example, Pires *et al.* (1996) suggested that norepinephrine might inhibit the circuits controlling metamorphosis in the slipper limpet *Crepidula fornicata.* We do not yet know how *Ilyanassa* larvae utilize dopamine or other catecholamines.

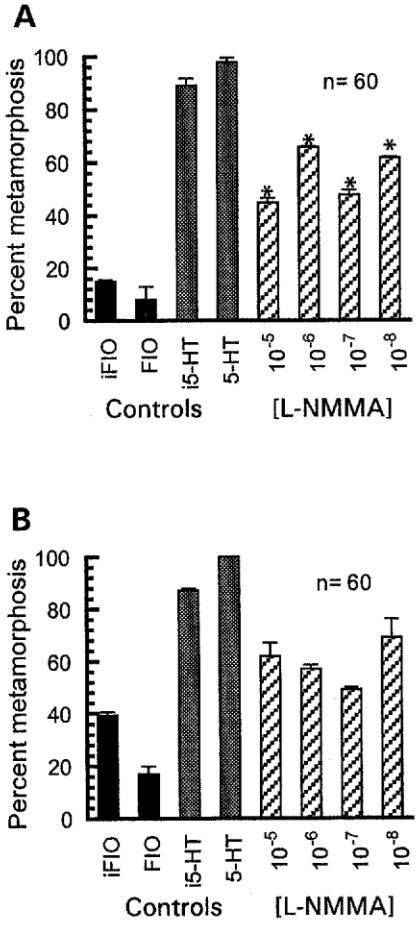

Figure 4. (A) Injections of all concentrations of L-NMMA induced significant rates of metamorphosis by 24 h in the absence of any inducer. Asterisks indicate concentrations that triggered rates of metamorphosis significantly different from those induced by iFIO (*e.g.*, at 10^{-5} *M*, $\chi^2_{0.01(1)} = 11.5$). Note unusually high levels of metamorphosis in both 5-HT controls. (B) Levels of metamorphosis detected at 48 h. No concentrations remained significantly different from iFIO ($\chi^2_{0.05(1)} = 6.2$). Note the unusually high levels of metamorphosis in both iFIO and FIO.

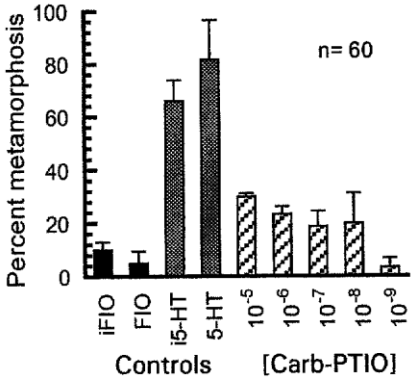

Figure 5. No concentrations of injected Carboxy-PTIO (Carb-PTIO), an NO scavenger, induced significant rates of metamorphosis by 48 h when compared to iFIO (10^{-5} *M* $\chi^2_{0.01(1)} = 5.76$, 10^{-6} *M* $\chi^2_{0.01(1)} = 3.84$). $n = 30$ for 10^{-5} and 10^{-9} *M* Carboxy-PTIO. Significance levels for 24 and 48 h data were the same.

Developing nervous systems in vertebrates and arthropods express NO transiently in a variety of areas (Bredt and Snyder, 1994; Truman *et al.,* 1996; Gibbs and Truman, 1998; Scholz *et al.,* 1998). NO has been reported to cause growth cone collapse (Renterfa and Constantine-Paton, 1996) and may act in the regulation of neuronal proliferation (Peunova and Enikolopov, 1995; Kuzin *et al.,* 1996), affecting the ability of axons to reach appropriate targets and initiate synaptogenesis (Bredt and Snyder, 1994; Wu *et al.,* 1994; Truman *et al.,* 1996; Gibbs and Truman, 1998; Scholz *et al.,* 1998). Comparable roles for this molecule in molluscs are just beginning to come under investigation.

How NO exerts its effects in larval *Ilyanassa is* still unknown. Typically, NO binds to guanylyl cyclase, stimulating the formation of cyclic guanosine 3',5' monophosphate (cGMP) (Murad *et al.,* 1978); the biochemistry of the nitrergic signaling pathway appears to remain applicable to both vertebrate and invertebrate systems (Dawson *et al.,* 1991; Elphick *et al.,* 1993; Elofsson *et al.,* 1993; Huang *et al.,* 1998). Recent work on the growth and survival of cultured neurons suggests that NO may also affect cGMP-independent intracellular signaling pathways (Gonzalez-Zulueta *et al.,* 1997). We cannot yet distinguish between these two mechanisms in our experimental animal. At present, we suggest that NO is produced within the developing molluscan nervous system and diffuses to its target cells to activate guanylyl cyclase, thereby increasing intracellular levels of cGMP. We hypothesize that high levels of cGMP are necessary for the maintenance of larval tissues. We anticipate that neuronal somata in the apical ganglion—the brain region that governs key larval functions—will express high levels of NOS. In the presence of a natural metamorphic inducer, nitrergic neurons are probably inhibited, either directly by serotonergic neurons or by feedback from activated NO targets. Activation of serotonergic neurons and the resultant inhibition of NOS activity would decrease levels of cGMP, allowing metamorphosis to proceed. Investigations into the downstream actions of NO are just beginning.

Given its widespread occurrence in behaviorally significant neural circuits throughout the animal kingdom, NO would appear to be a relatively ancient neurotransmitter. In adult molluscs, NO functions as an intercellular messenger in behaviorally important circuits. NO appears to be necessary for learning in cephalopods (Chichery and Chichery, 1994; Robertson *et al.,* 1994, 1995, 1996), olfaction in pulmonates (Gelperin, 1994a, b; Gelperin *et al.,* 1996), and feeding in several gastropods (Moroz *et al.,* 1993; Elphick *et al.,* 1995; Teyke, 1996). Our understanding of the importance of this molecule in developing organisms is still relatively immature, but the growing literature indicates that this molecule can be differentially activated to coordinate specific developmental events occurring throughout a field of maturing neural tissue (Edelman and Gally, 1992; Bredt and Snyder, 1994; Wu *et al.,* 1994; Peunova and Enikolopov, 1995; Kuzin *et al.,* 1996; Renteria and Constantine-Paton, 1996; Truman *et al.,* 1996; Gibbs and Truman, 1998; Scholz *et al.,* 1998). In larvae of marine molluscs, nitrergic pathways may have been exploited to regulate diverse target tissues, much as the ecdysteroids coordinate activity during insect metamorphosis (Riddiford and Truman, 1993). Ecdysteroid synthesis is inhibited in crustaceans by molt-inhibiting hormone (reviewed in Fingerman, 1997), which may have a molluscan analog in NO. Our comprehension of the mechanisms that drive molluscan metamorphosis will be aided by further explorations of this pathway.

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