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Inhibitors of nitric oxide synthase induce larval settlement and metamorphosis of the polychaete annelid *Capitella teleta*

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The neurotransmitter nitric oxide (NO) has been implicated in the inhibitory control of metamorphosis of some marine gastropods, echinoderms, and ascidians. We have explored whether or not metamorphosis of metatrochophore larvae of the polychaete annelid *Capitella teleta* is also regulated by NO. Immunohistochemical analysis revealed a bilateral group of three large nitric oxide synthase (NOS) immunoreactive cells that lie dorsal to the pharynx and extend ventral processes toward the pharynx in the region of the dorsal pharyngeal pad. Smaller NOS-immunoreactive cells were distributed widely throughout the body but concentrated in the prostomium and pygidium. Histological analysis for NO, using the NO detector diaminofluorescein-FM, showed that NO concentration was high in the larval midgut, although diffuse amounts of NO were detected throughout the body. Inhibitors of NOS, including s-methylisothiourea sulfate, aminoguanidine hemisulfate, 7-nitroindazole, and N-methyl-L-arginine all induced settlement and metamorphosis of the *Capitella* larvae in a concentration-dependent manner. The NO donor nitroprusside prevented the induction of settlement and metamorphosis induced by the NOS inhibitor N-methyl-L-arginine, but did not prevent settlement and metamorphosis induced by a marine sediment extract, exogenous serotonin, or by the serotonin reuptake inhibitor fluoxetine. Pre-incubating larvae with the serotonin receptor antagonist ketanserin also inhibited settlement and metamorphosis in response to NOS inhibitors. These results suggest that endogenous production of NO maintains the larval state in *C. teleta*, and that endogenous serotonin stimulates metamorphosis in a way similar to that described previously for larvae from other major invertebrate phyla.

Keywords: nitric oxide; serotonin; diaminofluorescein-FM; development

Introduction

The settlement and subsequent metamorphosis of the larvae of benthic marine invertebrates represent a critical point in their life cycles at which dispersive larvae must recognize and then recruit to suitable habitats that favor juvenile growth and survival (reviewed in: Chia and Rice 1978; Pawlik 1992; Pechenik 1999; Hadfield and Paul 2001). Habitat discrimination by marine larvae resembles pheromone chemoreception in terrestrial animals in that specific environmental chemical cues are sensed by chemosensory receptor cells (reviewed in: Rittschof and Bonaventura 1986; Scheuer 1990; Pawlik 1992). The chemical cues usually vary among species, but are commonly derived from food required by post-metamorphic juveniles, from conspecific adults, or from another species that is specifically associated with appropriate adult habitat (reviewed in: Hadfield 1978; Pawlik 1992; Hadfield and Paul 2001; Bishop et al. 2006). Trochophore larvae of the polychaete annelid *Phragmatopoma californica*, for instance, detect the presence of natural chemical settlement cues, such as adult-derived polyunsaturated fatty acids, with sensory tufts of immotile cilia present on their tentacles that presumably bear chemosensory membrane receptors (Eckelbarger and Chia 1976; Amieva, et al. 1987). Similarly, cilia that bear receptors for facilitators of metamorphosis have been found on larvae of the abalone *Haliotis rufescens* (Baxter and Morse 1992) and larvae of the nudibranch *Phestilla sibogae* sense a cue released by the juvenile food source, using receptors located in an apical sensory organ (ASO; Hadfield et al. 2000). Nitrergic neurons, which may be involved with sensing or ''tasting'' sediment cues, have in addition been described in the adoral lobe in larvae of the sea urchin *Lytechinus pictus* (Bishop and Brandhorst 2007; Bishop and Hall 2009).

The binding of chemical cues that act as ligands to their chemosensory receptors initiates a cascade of events that lead to eventual settlement and

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metamorphosis (reviewed in: Hadfield 1978; Morse 1990; Leitz 1997). Of special interest for the regulation of metamorphosis in marine invertebrates is nitric oxide (NO). NO is an ancient gaseous signaling molecule (Freelisch and Martin 1995) that functions in such diverse roles as thermoregulation in protists (Malvin et al. 2003), modulation of feeding responses in *Hydra* (Colasanti et al. 1995, 1997) and planarians (Eriksson 1996), regulation of swimming behavior in jellyfish (Moroz et al. 2004), ink release in cuttlefish (Palumbo et al. 1999), repair of CNS injury in leeches (Shafer et al. 1998), and synaptic modulation associated with learning and memory in mammals (Snyder 1992; Murad 2006). NO also appears to function as a negative regulator of metamorphosis in marine invertebrate larvae from a variety of taxa, including ascidians, echinoderms, and gastropod mollusks: endogenous NO is thought to inhibit settlement and metamorphosis until the larvae come into contact with appropriate chemical settlement cues, whereupon synthesis of NO diminishes and metamorphosis are allowed to proceed (Bishop and Brandhorst 2003; Hens et al. 2006; Comes et al. 2007; Pechenik et al. 2007).

We have carried out investigations to determine if NO may also serve as a negative regulator of settlement and metamorphosis in polychaete annelid larvae as well, by investigating its presence and effects on metatrochophore larvae of the marine polychaete annelid *Capitella teleta* (previously designated *Capitella* sp. I), which is a member of the *Capitella capitata* Fabricius 1780 species complex (Grassle JF and Grassle JP 1976; Blake et al. 2009). The metatrochophores of *C. teleta* are lecithotrophic, segmented larvae possessing eye spots and prototrochal, telotrochal, and neurotrochal bands of cilia for swimming (Figure 1(A)). These larvae settle and metamorphose upon contacting chemical cues present in marine sediments (Grassle JF and Grassle JP 1976; Dubilier 1988; Biggers and Laufer 1992; Cohen and Pechenik 1999), which may be polyunsaturated fatty acids (Biggers and Laufer 1992; Biggers 1994), or proteins (Thiyagarajan et al. 2005). Shortly after settlement, metamorphosis is marked by loss of the swimming cilia (prototroch, telotroch, and neurotroch) and protrusion of the capillary setae necessary for movement through sediments. The prostomium also becomes more conical in shape (Figure 1(B)). The signal transduction process by which these larvae settle and metamorphose in response to chemical cues may be mediated by serotonin (Biggers and Laufer 1992) and through protein kinase C activation (Biggers and Laufer 1999). The data presented here indicate that NO and nitric oxide synthase (NOS) are both present in these larvae, and that NO may act to inhibit settlement and metamorphosis until the larvae come into contact with a chemical cue, in a way similar to that reported previously for larvae from some other invertebrate groups. Our data also indicate that this inhibition by NO can be suppressed by increased serotonergic function or by exposing the larvae to an exogenous natural chemical cue, and that serotonergic signaling is required for settlement and metamorphosis to proceed after NOS inhibitors lower NO levels.

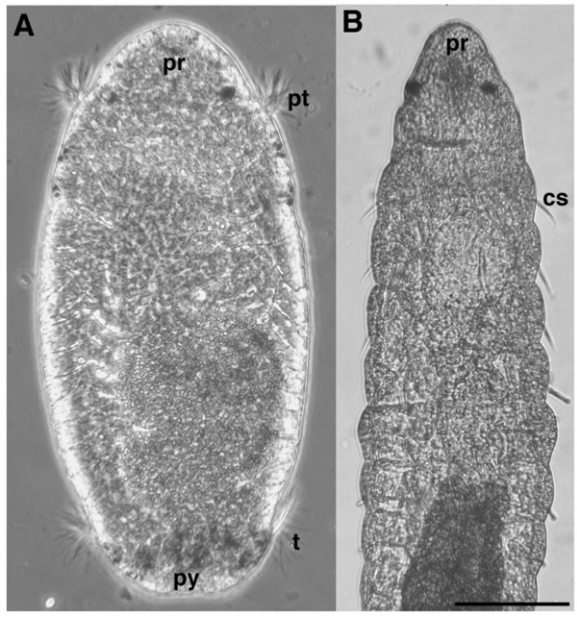


Figure 1. Light micrographs of (A) metatrochophore larva of *C. teleta*, showing the prototroch (pt), telotroch (t), prostomium (pr), and pygidium (py), and (B) a newly metamorphosed juvenile of *C. teleta* showing a more conical prostomium (pr) and developed capillary setae (cs). Anterior is at top and scale bar is 100 μm.

Materials and Methods

Animals

Stock cultures of *C. teleta* were kindly supplied by Dr Judith Grassle (Rutgers University) and maintained in the laboratory in a temperature- and photoperiod-controlled incubator at a photoperiod of 12 hL : 12 hD, and a temperature of 18°C. The cultures were maintained in 2 L plastic containers containing sea sand and artificial seawater (ASW, Instant Ocean) and fed ground Tetramin *ad libitum*. To obtain larvae, brood tubes containing females with developing larvae were collected from the laboratory cultures and placed in 60 mm glass Petri dishes with 10 mL ASW and kept at 18°C in a laboratory incubator (four to five parental brood tubes/Petri dish). Swimming metatrochophore larvae released within 24 h from at least three brood tubes were then collected and combined before use; all experiments were done on the day of collection. The larvae of this species are competent to settle and metamorphose almost immediately after their release from the mother's brood tube (Grassle JF and

Grassle JP 1976; Pechenik and Cerulli 1991; Hill and Boyer 2003).

Bioassays of settlement and metamorphosis

Swimming metatrochophore larvae were used in bioassays of settlement and metamorphosis within 24 h of their release from parental brood tubes. Bioassays were conducted in 60 mm glass Petri dishes each containing 10 larvae and 10 mL ASW. The effects of NOS inhibitors, S-methylisothiourea sulfate (SMIS), aminoguanidine hemisulfate (AGH), 7-nitroindazole (7-NI), N-methyl-L-arginine (L-NMMA), and N-nitro-L-arginine methyl ester (L-NAME) were tested by adding aliquots (10–100 μL) of inhibitor stock solutions to the Petri dishes; fluids were then mixed by swirling. SMIS was obtained from Calbiochem, prepared as a 100 mM stock solution in deionized water, and tested at final concentrations of 50, 100, 250, and 500 μM. AGH was obtained from Sigma–Aldrich, prepared as a 1 M stock solution in deionized water, and tested at final concentrations of 1, 2.5, 5, 7.5, and 10 mM. The competitive NOS inhibitor L-NMMA was obtained from Sigma–Aldrich, prepared as a 100 mM stock solution in deionized water, and tested at final concentrations of 200, 400, 600, and 800 mM. The competitive NOS inhibitor L-NAME was obtained from Sigma–Aldrich, prepared as a 1 M stock solution in deionized water, and tested at final concentrations of 0.5, 1, 5, and 10 mM. Also, 7-NI was obtained from Sigma–Aldrich, prepared at a stock solution of 250 mM using 95% ethanol, and tested at final concentrations of 0.5, 1, 1.5, 2, and 2.5 mM). Control dishes of larvae received up to 100 μL of solvent vehicle.

The NO donor nitroprusside was obtained from Sigma–Aldrich, prepared at a stock solution of 100 mM in deionized water, and tested at final concentrations of 100, 250, and 500 μM. In experiments with nitroprusside, larvae were pre-incubated in ASW containing nitroprusside for 3 h before adding NOS inhibitors, serotonin (2 mM final concentration), fluoxetine (300 nm final concentration), or 50 mg marine sediments (delivered to dishes as 50 μL aliquots) containing a natural chemical cue, to these same dishes. In these experiments, serotonin (Sigma–Aldrich) was prepared at a stock solution of 1 M in deionized water, and the serotonin reuptake inhibitor fluoxetine (Sigma–Aldrich) was prepared at a stock solution of 100 μM in deionized water. Marine sediments used in the experiments, which are effective and natural inducers of settlement and metamorphosis, were kindly provided by Dr Judith Grassle and collected from Sippewisset Marsh, Barnstable Co., MA.

In another series of experiments, we studied the effects of ketanserin, a serotonin 5-HT2 receptor antagonist, for its effect on settlement and metamorphosis stimulated by NOS inhibitors. Ketanserin was obtained from Sigma–Aldrich and prepared at a stock solution of 2 mM in 95% ethanol. In these experiments, larvae were pre-exposed to ketanserin at a final concentration of 2 μM for 3 h before addition of NOS inhibitors to the same assay dishes. In all the above bioassays, dishes were then kept at 18°C in a photoperiod-controlled incubator (12 hL:12 hD) and the number and percentage of larvae that had actively settled to the bottom of the Petri dish and metamorphosed into viable juveniles were then determined hourly with a dissecting microscope for 7 h, and then again after 24 h of exposure to the chemicals. Criteria for metamorphosis consisted of the new juveniles being elongated and crawling on the bottom of the Petri dish, having protruded prostomiums and capillary setae, and having lost prototrochs and telotrochs. Effects of NOS inhibitors and other treatments were assessed by one-way ANOVA with Bonferroni correction for multiple comparisons.

NOS and acetylated tubulin immunohistochemistry

For localization of NOS in larvae, immunohistochemistry experiments were carried out using a universal polyclonal antibody that recognizes a conserved sequence of both inducible and constitutive forms of NOS. Because NOS in other marine invertebrate larvae is associated with neural structures, the larvae were also treated with an anti-acetylated tubulin monoclonal antibody that binds with neurofilaments, in order to clarify the neuroanatomical context of NOS-like immunoreactivity.

Before fixation larvae were relaxed in a 1:1 mixture of 0.45 μm filtered ASW and 7.5% $MgCl_2$. Larvae were then fixed for 24 h at 4°C in a 1:3 mixture of 16% aqueous paraformaldehyde and 50 mM Tris buffered seawater, and then washed three times in phosphate buffered saline (PBS). Larvae were then incubated for 24 h in a blocking medium consisting of 5% goat serum in PTA (PBS containing 4% Triton detergent and 0.1% sodium azide). Next, larvae were exposed to a primary antibody (AB) solution of 1:100 rabbit anti-NOS polyclonal AB (Affinity Bioreagents PA1-039) and 1:500 mouse anti-acetylated tubulin monoclonal AB (Sigma–Aldrich T7451), in blocking medium for 40–45 h at 4°C. Primary antibodies were omitted in preparations serving as controls for nonspecific labeling and autofluorescence. Larvae were then washed four to five times with PTA at 4°C over the course of 2 days, and then incubated with the appropriate secondary antibody for 24 h at 4°C (goat anti-mouse Alexafluor 488 and/or goat anti-rabbit Alexafluor 568, Invitrogen/Molecular probes), both diluted 1:200 in blocking medium. The larvae were washed in PTA

as above, dehydrated through ethanol to xylene, and mounted on slides in DPX medium (Sigma).

Detection of NO by DAF-FM DA

To detect NO, larvae were exposed within 24 h of their release to 100 nM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA; Invitrogen/Molecular Probes D-23844) in ASW for 2 h in total darkness. Control larvae were not exposed to DAF-FM DA. Non-fluorescent, cell-permeant DAF-FM DA is hydrolyzed by intracellular esterases to yield DAF-FM. The DAF-FM then reacts specifically with NO to produce a fluorescent triazolofluorescein derivative that is relatively impermeant to cell membranes (Kojima et al. 1998; Itoh et al. 2000). The larvae were then washed in ASW and pipetted into a 2:2:1 mixture of ASW: 7.5% MgCl: 4% paraformaldehyde for 20 min. Fixation for this period of time was necessary to both immobilize the larvae and to quench autofluorescence. The larvae were then cleared and mounted on slides in glycerol.

Confocal microscopy

All preparations were imaged with an Olympus Fluoview 500 inverted confocal microscope. Double-labeled immunohistochemical preparations were scanned sequentially with 488 nm argon and 568 nm krypton lasers to avoid artifacts from overlapping fluorescence emission spectra. Controls (not shown) exhibited only very weak and diffuse fluorescence at these wavelengths. The imaging of preparations labeled with DAF-FM DA was conducted with the 488 nm argon laser at 0.4% power. Fluorescent confocal microscopy of control (unlabeled) larvae (not shown) revealed no fluorescence at 1% laser power and only trace fluorescence even up to 8% laser power.

Results

Effects of inhibitors of NOS

All the known inhibitors of NOS that were tested, except L-NAME, were found to significantly stimulate settlement and subsequent metamorphosis of larvae of *C. teleta* in a concentration-dependent manner (ANOVA, $p < 0.001$). L-NMMA, which is a well known inhibitor of NOS and a competitive inhibitor that inhibits all three known isoforms of NOS (Southan and Szabo 1996; Alderton et al. 2001), was an effective inducer of settlement and metamorphosis of these larvae having a 24 h EC_{50} of 0.5 mM (Figure 2(A), Table 1). L-NAME, however, failed to induce settlement and metamorphosis even at a high concentration of 10 mM (data not shown). Of the different inhibitors tested, SMIS was found to be the most potent inducer of settlement and metamorphosis having a 24 h EC_{50} of 0.15 mM (Figure 2(B), Table 1). SMIS has been recognized to be a selective inhibitor of NOS II, an inducible type of NOS, and is more potent than L-NMMA (Southan and Szabo 1996; Alderton et al. 2001) in several cell types. Settlement and metamorphosis was also induced by 7-NI, an inhibitor considered to be selective for NOS 1, or neural NOS (nNOS; Southan and Szabo 1996; Alderton

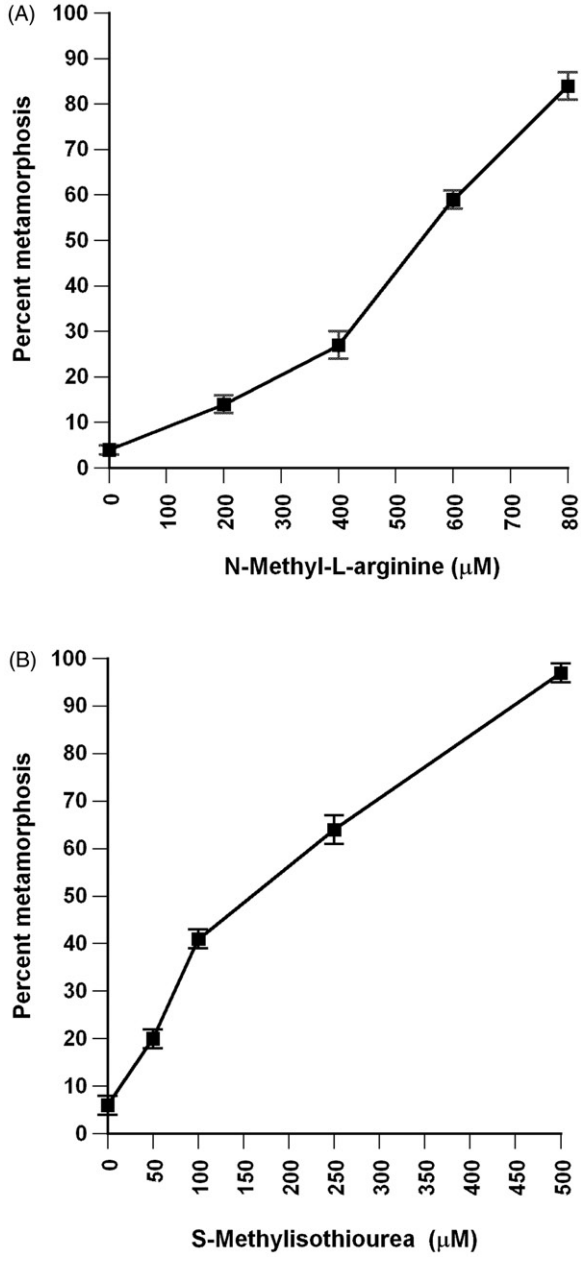
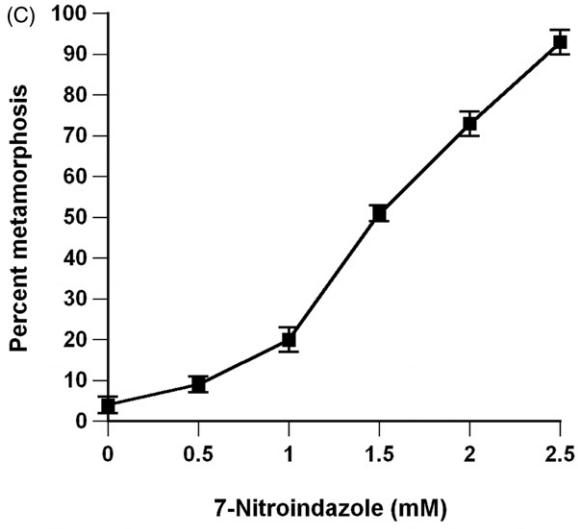


Figure 2. Effect of NOS inhibitors on metamorphosis of larvae of *C. teleta*. Frequency of metamorphosis of larvae after 24 h bath exposures to: (A) L-NMMA; (B) SMIS; (C) 7-NI; and (D) AGH. Treatments began less than 24 h after release of larvae from brood tubes. Each data point represents the mean, (+/− 1 SEM), for combined data from three trials with three replicates of 10 larvae per trial ($n = 90$ larvae).



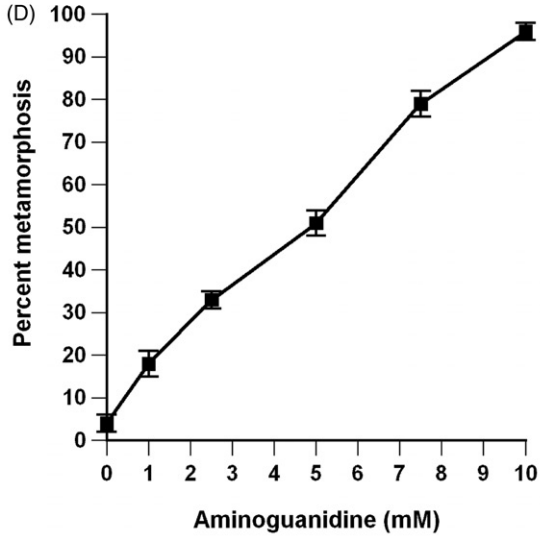


Figure 2. Continued.

Table 1. Relative efficacies of tested NOS inhibitors on *C. teleta* larvae.

NOS inhibitor	EC_{50} (mM)
S-methylisothiourea sulfate	0.15
N-methyl-L-arginine	0.50
7-Nitroindazole	1.50
Aminoguanidine hemisulfate	3.50

Note: Effective concentrations that stimulate settlement and metamorphosis of 50% of test animals after 24 h of exposure in bath culture are given.

et al. 2001), with an EC_{50} of 1.5 mM (Figure 2(C), Table 1). The NOS inhibitor AGH was the least potent of the inhibitors tested, having an EC_{50} of 3.5 mM (Figure 2(D), Table 1). All the above drugs are inhibitors

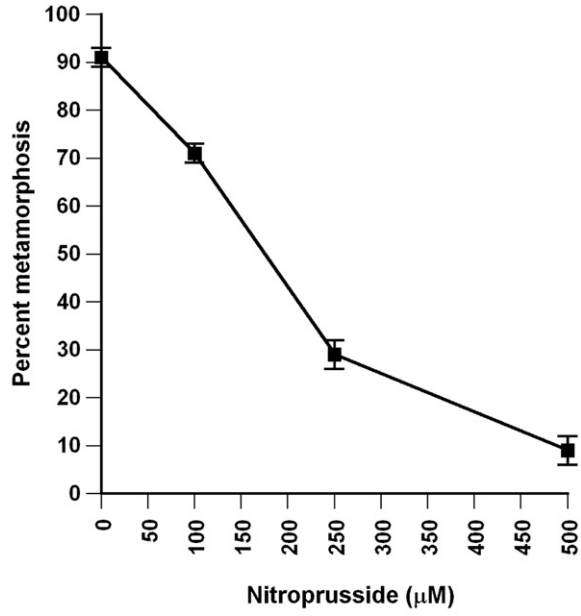


Figure 3. Effect of the NO donor nitroprusside on settlement and metamorphosis induced by L-NMMA. Metatrochophore larvae less than 1 day old post-release from brood tubes were incubated for 3 h in the presence of varying concentrations of the NO donor nitroprusside before addition of the NOS inhibitor L-NMMA (800 μM final concentration) to the dishes. Frequency of metamorphosis of larvae after 24 h in L-NMMA (together with nitroprusside) is shown. Each data point represents the mean, (+/− 1 SEM), for combined data from three trials with three replicates of 10 larvae per trial ($n = 90$ larvae).

of NOS (Southan and Szabo 1996; Wolff et al. 1997; Alderton et al. 2001). However, AGH is known to have inhibitory effects on other enzymes as well (reviewed in Southan and Szabo 1996).

The effects of the NO donor nitroprusside were also tested on settlement and metamorphosis of *C. teleta*. Exposing larvae within 24 h of their release to nitroprusside for 3 h significantly prevented the metamorphic inducing effect of L-NMA in a concentration-dependent manner during further incubation for 24 h (ANOVA, $p < 0.001$; Figure 3). These results provide further evidence that NO is able to inhibit settlement and metamorphosis of the larvae of *C. teleta* in the absence of a chemical cue and verifies the L-NMA experiments.

Detection and localization of NOS in larvae

Metatrochophore larvae of *C. teleta* were double-labeled with antibodies directed against NOS and against acetylated tubulin (Figure 4). NOS-like immunoreactivity (NOS-LIR) was observed in small cells distributed throughout the larval body, but more concentrated in the prostomium and pygidium. NOS-LIR was also present in a group of three bilateral pairs of larger cells with neuronal morphology, located dorsal

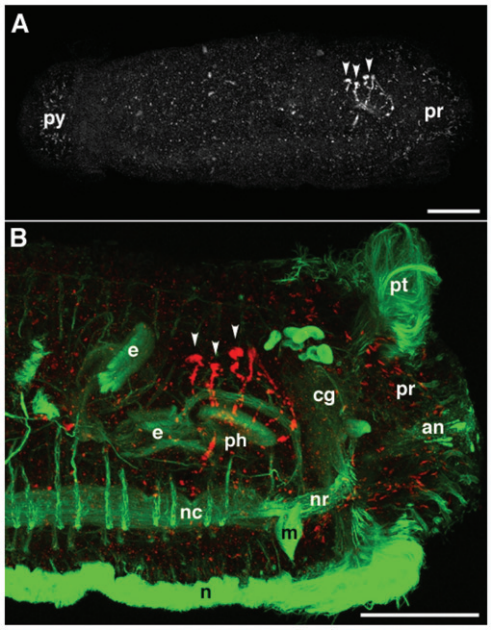


Figure 4. NOS-LIR in a metatrochophore larva of *C. teleta*. Anterior is to right, scale bars are 50 µm. (A) Confocal micrograph of entire larva, showing fluorescence indicating NOS-LIR. Arrowhead points to three pairs of bilateral cells with processes extending ventrally toward the pharynx. Pr, prostomium; py, pygidium. (B) Details of anterior portion of same larva shown in Figure 4(A). Two channels of fluorescence indicate NOS-LIR (red) and acetylated-tubulin-like immunoreactivity (green). An, anterior nerve fibers; cg, cerebral ganglia; e, esophagus; m, mouth; n, neurotroch; nc, ventral nerve cord; nr, circumesophageal nerve ring; ph, pharynx; pr, prostomium; pt, prototroch. Arrowheads indicate three pairs of NOS-LIR cells, as in A. Several optical sections were omitted from the right side of the larva, including portions of the prototroch, to avoid obscuring underlying label in the prostomium.

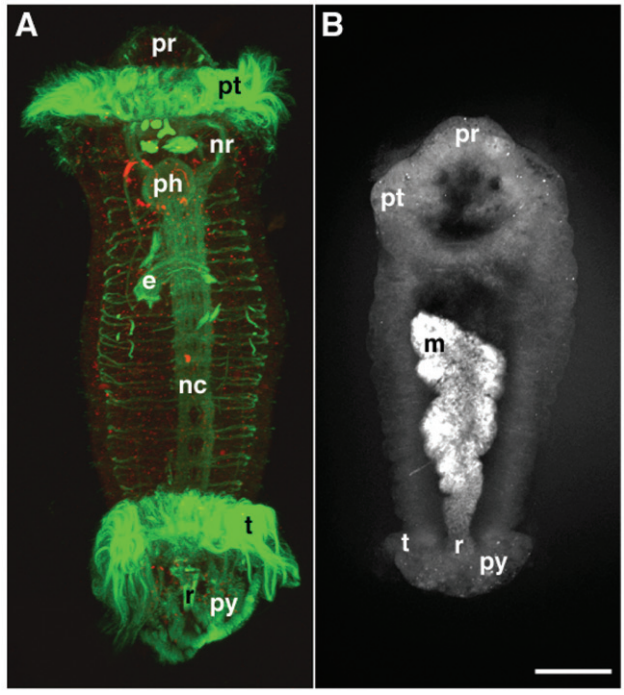


Figure 5. Comparison of NOS immunohistochemistry and 4-amino-5-methylamino-2′,7′-difluoroluorescein diacetate (DAF-FM DA) labeling of NO in whole larvae of *C. teleta*. Anterior is at top, scale bar is 50 µm. (A) Confocal image of larva labeled with antibodies against NOS (red) and acetylated tubulin (green). (B) Confocal image of larva labeled with DAF-FM DA. e, esophagus; m, midgut; nc, ventral nerve cord; nr, circumesophageal nerve ring; ph, pharynx; pr, prostomium; pt, prototroch; py, pygidium; r, rectum; t, telotroch.

to the pharynx. These cells extended processes ventrally toward the dorsal pharyngeal pad, which was itself marked by both punctate and diffuse NOS-LIR. Cilia of the dorsal pharyngeal pad, as well as other beds of cilia in the esophagus and a dense tuft of cilia at the mouth, were labeled by antibodies against acetylated tubulin. These antibodies strongly labeled cilia of the prototroch, neurotroch, and telotroch, and also revealed major features of the central nervous system, including large cell bodies and neuropil of the cerebral ganglia, the circumesophageal nerve ring, and the ventral nerve cord. Acetylated tubulin antibodies also marked bilateral groups of nerve fibers extending from the apical surface of the prostomium toward the cerebral ganglia.

Detection of NO in larvae

In preparations made with the NO-reactive fluorescent probe, DAF-FM DA (Figure 5(B)), diffuse NO labeling was seen throughout the larvae. The most intense signal, indicating the highest NO concentration, was observed in the midgut, suggesting that this is the area of greatest NO synthesis, although few NOS-immunopositive cells were detected there. While both NOS immunohistochemistry and direct detection of NO with DAF-FM DA revealed both diffuse and punctate labeling, cell-specific labeling was much more apparent in the immunohistochemical preparations (compare Figure 5(A) and (B)). As DAF-FM DA is a non-fixable probe, specificity of that label relies on its poor penetration of cell membranes after its diacetate moiety has been cleaved by intracellular enzymes. Therefore, some of the diffuse labels in those preparations may represent DAF-FM that was not retained in cells where it reacted with NO. Alternatively, NO can be produced non-enzymatically by release from natural NO donors such as nitrosoglutathione (Nozik-Grayck et al. 2002).

Ketanserin inhibits settlement and metamorphosis induced by NOS inhibitors

We have previously found that exogenous serotonin and also fluoxetine, which inhibits reuptake of serotonin, are able to induce settlement and metamorphosis

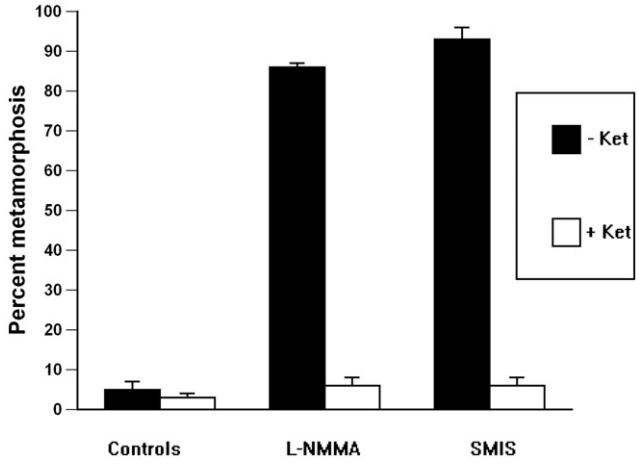


Figure 6. Effect of ketanserin on settlement and metamorphosis induced by L-NMMA and SMIS. Metatrochophore larvae less than 1 day old post-release were pre-incubated in seawater containing 2 μM ketanserin (+Ket) or 10 μL 95% ethanol (control) (−Ket) for 3 h before addition of L-NMMA (800 μM final concentration) or SMIS (500 μM final concentration) to the same dishes. Control larvae received 100 μL of solvent vehicle (deionized water) in seawater. Frequency of metamorphosis of larvae observed after 24 h after addition of L-NMMA or SMIS is shown. Each bar represents the mean, (+/− 1 SEM), for combined data from three trials with three replicates of 10 larvae per trial ($n = 90$ larvae).

of larvae of *C. teleta*, whereas ketanserin, a 5-HT2 serotonin receptor antagonist, inhibits this response (Biggers and Laufer 1992; Ricker et al. 2011). To determine if serotonin signaling is also needed for the induction of settlement and metamorphosis after NO is lowered by NOS inhibitors, larvae were pre-incubated with ketanserin for 3 h prior to adding NOS inhibitors to these dishes and the amount of settlement and metamorphosis was then observed after an additional 24 h. As shown in Figure 6, pre-exposing larvae to ketanserin significantly blocked the response to the NOS inhibitors L-NMMA and SMIS (ANOVA, $p < 0.001$). These results suggest that threshold levels of serotonin are needed to stimulate settlement and metamorphosis after endogenous levels of NO become lower due to NOS inhibition, and that the presence of NO inhibits this serotonergic signaling.

The NO donor nitroprusside does not inhibit settlement and metamorphosis in response to a chemical cue, exogenous serotonin, or fluoxetine

Pre-incubating the larvae for 3 h in nitroprusside at a concentration of 500 μM, which inhibits the response to L-NMMA, did not significantly inhibit the induction of settlement and metamorphosis by the concomitant addition of marine mud sediments containing a natural chemical cue, or by exogenous serotonin or fluoxetine (Figure 7; ANOVA, $p > 0.10$). These results suggest that inhibition of metamorphosis by NO can be overridden when a suitable chemical cue is detected and serotonergic activity may be increased.

Discussion

Relative effectiveness of different NOS inhibitors

As previously found for larvae from several other marine invertebrate phyla, NO appears to act as a negative regulator of settlement and metamorphosis in the polychaete annelid *C. teleta*. These results are the first to our knowledge that provide evidence for the regulation of settlement and metamorphosis by NO in an annelid, or in any lophotrochozoan outside the Gastropoda. We have demonstrated that four different NOS inhibitors were all able to induce settlement and metamorphosis in a concentration-dependent manner over 24 h of treatment, indicating that NO is acting to inhibit settlement and metamorphosis in these larvae.

The relative effectiveness of the different NOS inhibitors in inducing settlement and metamorphosis may be expected to be dependent on their ability to inhibit the different NOS isoforms, or on their specific mechanism of inhibition. In *C. teleta*, the NOS gene has been previously sequenced and found to be a NOS I isotype, 7.3 kb in size, with 23 exons, 22 introns, a Ca/CaM binding site, and an inhibitory loop (Joint Genome Initiative; Andreakis et al. 2011). The NOS I gene of protists and metazoans has been deduced from sequence analysis to be ancestral to the NOS II and III isoforms of vertebrates (Andreakis et al. 2011). Larvae of *C. teleta* were most sensitive to SMIS (Figure 2(B), Table 1), which has been considered a more or less selective inhibitor of inducible NOS (NOS II). SMIS, however, has also been demonstrated to inhibit constitutively active NOS 1 and III. In this regard, all three isoforms of NOS have been found to function either constitutively or inducibly depending on the tissue type

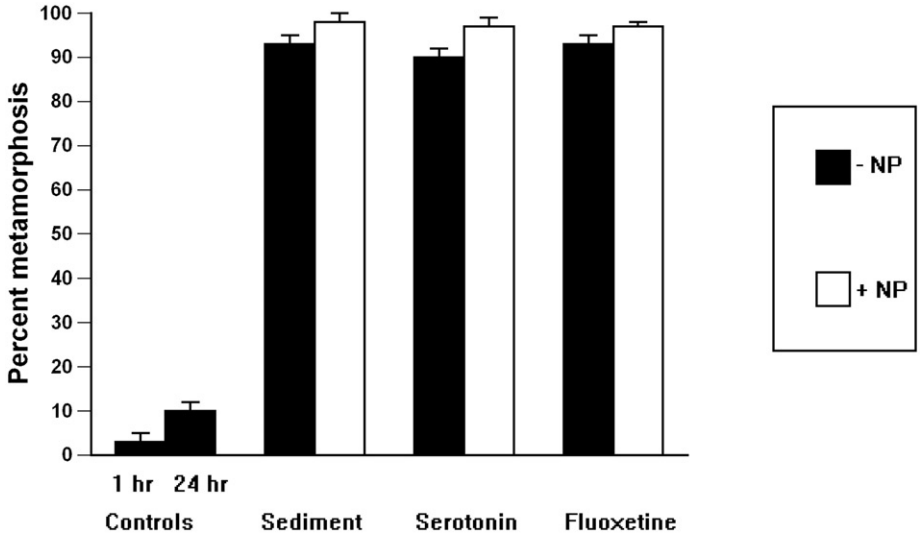


Figure 7. Effect of nitroprusside on settlement and metamorphosis induced by marine sediments, serotonin, and fluoxetine. Metatrochophore larvae were pre-incubated in seawater containing 500 μM nitroprusside (+NP) or 50 μL deionized water (controls) (−NP) for 3 h before addition of inducers of settlement and metamorphosis, including marine sediments (50 mg) (sediment), serotonin (2 mM), or fluoxetine (300 nM). Control larvae were treated with the appropriate amount of solvent vehicle for 1 h (Con-1) or 24 h (Con-24). The percent metamorphosis is shown after 1 h of exposure to marine sediments, or 24 h after addition of serotonin or fluoxetine. Each bar represents the mean ± SEM observed for 90 larvae.

and species (Southan and Szabo 1996; Alderton et al. 2001). Since the *Capitella* NOS is a NOS I isotype (nNOS), it would therefore be expected that 7-NI would be an effective inhibitor of the *Capitella* NOS and induce settlement and metamorphosis, which we indeed observed (Figure 2(C), Table 1). The NOS inhibitor AGH also induced settlement and metamorphosis in our study, but was effective only at much higher concentrations than SMIS (Figure 2(D), Table 1). Our results also demonstrate that the competitive NOS inhibitor L-NMA was an effective inducer of settlement and metamorphosis, which would also be expected since it inhibits both constitutive and inducible isoforms (Figure 2(A), Table 1). Our results further show that the NO donor, nitroprusside, is able to prevent the inducing effect of L-NMA, verifying the results from inhibition of NO production (Figure 3). The competitive NOS inhibitor L-NAME, however, did not induce settlement and metamorphosis at even a high concentration of 10 mM in bath application (data not shown). The inefficacy of L-NAME on these larvae is not likely to be due to too low a concentration being applied, or that L-NAME cannot enter the larvae in bath applications (Froggett and Leise 1999; Pechenik et al. 2002), since L-NAME at lower concentrations (1 mM) in bath applications was able to penetrate and induce metamorphosis in larvae of other species (Bishop and Brandhorst 2001). The reason for this inefficacy therefore remains to be determined. In relative efficacies, our results closely parallel previously published work on larvae of the caenogastropod *Crepidula fornicata*, in which SMIS was a more effective inducer than AGH, and L-NAME treatment was also ineffective (Pechenik et al. 2007). L-NAME did however induce metamorphosis in larvae of the caenogastropod *Ilyanassa obsoleta* (Froggett and Leise 1999; Leise et al. 2001), the sea urchin *L. pictus* (Bishop and Brandhorst 2001), and the ascidians *Cnemidocarpa finmarkiensis* and *Boltenia villosa* (Bishop et al. 2001), and L-NAME also potentiated responses to the natural inducer in *P. sibogae* (Bishop et al. 2008).

For all the NOS inhibitors tested in our study, except for L-NAME, the initiation of settlement behavior was only observed after 6 h of exposure, suggesting that it takes this long for levels of NO in the regulatory tissues or organs to be reduced to non-inhibitory levels. Since the larvae of *C. teleta* are metamorphically competent shortly after release from the brood tubes, and they settle and metamorphose within minutes in response to cues in natural mud (Grassle JF and Grassle JP 1976; Dubilier 1988; Pechenik and Cerulli 1991), this delayed response of larvae to NOS inhibitors does not indicate that they are artificially activating a chemosensory process; they are most likely to act by inhibiting the NOS enzyme. Similarly, AGH and SMIS did not induce settlement and metamorphosize immediately in the gastropod *C. fornicata*, but instead required several hours to act (Pechenik et al. 2007).

Localization of NOS-immunopositive positive cells

The cells that regulate settlement and metamorphosis in *C. teleta* may include the apical sensory cells and

associated neurons that are known to be present in these larvae and that are thought to play a role in sensing environmental cues (Bhup and Marsden 1982; Eckelbarger and Grassle 1987). In this regard, anti-acetylated tubulin immunolabeling revealed fibers in the prostomium that are most likely nerve tracts leading from the epithelia of the prostomium to the cerebral ganglia (Figure 4(B)). These nerve tracts may lead from chemosensory ciliated cells known to be present on the surface of the prostomium (Eckelberger and Grassle 1987), and may relay chemosensory information to the cerebral ganglia. In addition, many small NOS-immunoreactive cells were found in the prostomium (Figure 4). It is possible that some of these cells inhibit settlement and metamorphosis mediated by the apical sensory cells and/or cerebral ganglion until a chemical cue is detected. The nuchal organs in these larvae are also thought to be chemosensory and therefore may also be involved in chemoreception for settlement and metamorphosis (Bhup and Marsden 1982; Eckelbarger and Grassle 1987). The apical ganglia and ASO have been implicated in regulating settlement and metamorphosis in other taxa (reviewed by Lacalli 1994). In the larvae of *C. fornicata*, NOS-LIR in the apical ganglia declines prior to the onset of metamorphic competence (Pechenik et al. 2007). Leise and co-workers have shown that NOS (assayed as nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase) activity decreases in the apical ganglia of *I. obsoleta* during metamorphosis (Lin and Leise 1996), and that the induction of metamorphosis triggers programmed cell death in this organ before morphological indicators of metamorphosis are apparent (Gifondorwa and Leise 2006). Hadfield et al. (2000) demonstrated a chemosensory function of the apical ganglia in metamorphosis of the nudibranch *P. sibogae* by showing that its photoablation abolished responses to a natural inducer of metamorphosis. The existence of an apical ganglion in *C. teleta* has to our knowledge not been documented, however, and was not discernable in our confocal images.

Tissues and organs other than lophotrochozoan apical sensory structures have been implicated in nitrergic regulation of settlement and metamorphosis. In the sea urchin *L. pictus,* nitrergic neurons that project from the adoral lobe to the pre-oral neuropil appear to mediate complex settlement behaviors in response to biofilm-derived cues (Bishop and Brandhorst 2007). In these studies, NOS-immunopositive cells were also detected in the lower lip of the larval mouth, and pharmacological evidence strongly implicated NO as an inhibitor of metamorphosis in this species (Bishop and Brandhorst 2001, 2007).

In this study, NOS-LIR was localized in the pharynx and in three bilateral pairs of large cells that extended processes toward the dorsal pharyngeal pad (Figure 4). The dorsal pharyngeal pad is a densely ciliated structure that is located near the mouth and situated within the pharynx which is eversible in juveniles and adults, and is thought to be chemosensitive (Boyle and Seaver 2009). Nitrergic cells may regulate chemoreception of metamorphically inductive cues in the substrate by modulating extension of the pharynx or chemosensory transduction in the pharynx. In this regard, it is worth noting that NOS immunopositive neurons and NADPH diaphorase activity have been demonstrated to be present in the pharynx of the planarian *Dugesia tigrina* (Eriksson 1996) and in the nematode *Ascaris suum* (Bascal et al. 1995). A role for NO as a regulator of feeding motor patterns has been proposed for these animals, although direct physiological evidence for this was not obtained. In the freshwater heterobranch gastropod *Lymnaea stagnalis*, however, NO does appear to mediate chemosensory activation of feeding (Elphick et al. 1995). Among cnidarians, NO inhibits the feeding response of *Hydra* (Colasanti et al. 1995, 1997). It is therefore, possible, that in *C. teleta*, NO produced by NOS-immunopositive cells in the pharynx and mouth may inhibit feeding until the animals come into contact with chemical cues that stimulate their metamorphosis from lecithotrophic larvae to feeding juveniles. The dorsal pharynx in *C. teleta* is eversible in juveniles and adults (Boyle and Seaver 2009) and possibly also in metatrochophore larvae. It is therefore possible that cilia in the larval dorsal pharyngeal pad may sense chemical cues when in close proximity to bottom sediments, similar to the putative chemosensory cells in the adoral lobe of *Lytechinus* (Bishop and Brandhorst 2007; Bishop and Hall 2009). In this context, it is possible that contact with a chemical settlement cue by the apical sensory cilia may promote settlement behavior involving rapid spiraling and searching among bottom sediments. If the larvae then sense the appropriate sediment chemicals with their dorsal pharyngeal pads, they may be induced to complete settlement and metamorphose. Previous studies with *C. teleta* suggest that these larvae respond to organic molecules bound to sediment particles (Cohen and Pechenik 1999; Thiyagarajan et al. 2005), and it may be these particles that the larvae detect with their dorsal pharyngeal pads.

Although there were few NOS-immunopositive cells in the midgut region of the larvae of *C. teleta* in our observations, this region showed the highest concentrations of NO when assessed by DAF-FM labeling (Figure 5(B)). This suggests that NO produced in other tissues may accumulate in the midgut region and possibly serve a physiological role there such as in digestion. In this regard, our results are similar to those reported for the ascidian *Ciona intestinalis*, in which high amounts of NO (also detected using DAF-FM) were found in the intestinal disc of late larvae and juveniles, pointing to a role in development or

functioning of the digestive system (Comes et al. 2007). Bishop and Brandhorst (2001) also demonstrated NOS activity in the larval midgut of *L. pictus* by NADPH-diaphorase staining. NO is also known to diffuse easily through membranes and into neighboring cells (Snyder 1992; Liu et al. 2008), and to play other physiological roles in annelids besides regulating metamorphosis. Licata et al. (2002) showed that NO regulates tissue homeostasis and salt-water balance in the epidermis of the earthworm *Lumbricus terrestris*, and endothelial NOS plays a role in repair of the nervous system in leeches (Shafer et al. 1998) as well as in neurotransmission in the ventral nerve cord in the earthworm *Eisenia fetida* (Kitamura et al. 2001).

Interaction of serotonin and NO signaling in regulating settlement and metamorphosis

Previous investigations by Couper and Leise (1996) and Leise et al. (2001) have demonstrated a regulatory relationship between the inductive effects of serotonin and the inhibitory effect of NO in regulating settlement and metamorphosis of larvae of the gastropod *I. obsoleta*. A similar phenomenon appears to occur for larvae of *C. teleta* that are stimulated to settle and metamorphose by exogenous serotonin (Biggers and Laufer 1992). Other results in our laboratory have demonstrated that the serotonin reuptake inhibitor fluoxetine induces settlement and metamorphosis, and that ketanserin, a 5-HT2 serotonin receptor antagonist prevents metamorphosis in response to mud sediment cues, serotonin, or fluoxetine (Ricker et al. 2011). These results suggest that binding of the chemical cue present in the sediments to chemosensory cilia in these larvae activates serotonergic neurons that then release stimulatory levels of serotonin for settlement and metamorphosis, and antagonizes the inhibition caused by NO.

In this study, we have demonstrated that settlement and metamorphosis induced by the NOS inhibitors L-NMMA and SMIS is completely inhibited by prior exposure to ketanserin (Figure 6). These results indicate that serotonin signaling is also required for settlement and metamorphosis to proceed after NO levels are lowered due to NOS inhibition. It is also possible that NO suppresses the inductive effect of tonic levels of serotonin that are released spontaneously in the absence of a chemical cue. In this regard, NO is known to inhibit serotonin signaling by causing nitrosylation of the serotonin 5-HT2 receptor, which is a metabotropic G-protein-coupled receptor (Nozik-Grayck et al. 2002). NO may also inhibit metamorphosis in *C. teleta* by activating soluble guanylate cyclase and raising intracellular cGMP concentrations, as suggested for other larvae (Bishop and Brandhorst 2001; Pechenik et al. 2007; Bishop et al. 2008) and possibly through the covalent nitrosylation of cysteine residues in other regulatory proteins such as caspases, IκB, and ion channels, which has been shown to occur in some other physiological systems (Mannick et al. 2001; Williams et al. 2003; Marshall et al. 2004; Raines et al. 2006).

Although the NO donor nitroprusside reversed the inhibition of metamorphosis caused by L-NMMA (Figure 3), pre-treatment of larvae with nitroprusside did not inhibit the response of the larvae to exogenous serotonin, fluoxetine, or a natural chemical cue present in marine mud sediments (Figure 7). An explanation for this may be that upon exposure to a chemical cue, endogenous serotonergic activity is increased, which increases excitatory signaling and overcomes inhibitory signaling caused by NO. The present results with *C. teleta* differ from those observed with larvae of the gastropod *I. obsoleta* and also with the sea urchin *L. pictus*.

In *I. Obsoleta,* NO donors were able to inhibit the response of these larvae to a chemical cue or serotonin (Leise et al. 2001), and similarly NO donors inhibited the response of *L. pictus* larvae to a chemical cue (Bishop and Brandhorst 2001), suggesting that NOS activity must be down-regulated in order for metamorphosis to proceed in those larvae. In the case of *I. obsoleta,* transcription of a nNOS gene is down-regulated by serotonin during metamorphosis; a current working model proposes that the excitation of serotonergic sensory neurons inhibits NOS activity in post-synaptic nitrergic neurons allowing metamorphosis to proceed in response to a natural cue (Leise et al. 2001; Hens et al. 2006). Our studies with *C. teleta* indicate that NO levels need not be lowered before larvae are able to metamorphose in response to an external chemical cue or to increased endogenous serotonergic activity. This conclusion is also supported by the fact that settlement and metamorphosis of *C. teleta* in response to a natural mud sediment chemical cue occurs within 5 min. It is unlikely that NOS activity would decrease significantly within this short period of time. An important unexplored issue in the life history of these short-term, lecithotrophic larvae is the potential role of NO in the extension of the competent period in the absence of appropriate cues for settlement and metamorphosis (''desperate larva hypothesis,'' reviewed in Bishop et al. (2006)). Larvae of *C. teleta* will metamorphose ''spontaneously'' in the apparent absence of a cue (Pechenik and Cerulli 1991; Biggers 1994), which may be a result of their energy stores running down (Marshall and Keough 2003), but the larvae also showed more rapid responses, the longer they were kept swimming before mud was added (Pechenik and Cerulli 1991), suggesting a gradual decrease in endogenous NO production as metamorphosis was delayed. A gradual decline in the nitrergic inhibition of metamorphosis

may be part of a mechanism that limits the larval period, as proposed for the larvae of planktotrophic gastropods (Pechenik et al. 2002, 2007; Gifondorwa and Leise 2006).

As demonstrated in this article, NO appears to act as a negative regulator of settlement and metamorphosis in *C. teleta* until the larvae come into contact with specific chemical cues, in a manner similar as that found for the larvae of some other animal groups (Bishop and Brandhorst 2003). Fully understanding the neural and biochemical regulatory systems that govern this process in *C. teleta*, however, still requires much further work, which will be the subject of future reports.

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