Reference: *Biol. Bull.* **213:** 160–171. (October 2007)

Nitric Oxide Inhibits Metamorphosis in Larvae of *Crepidula fornicata,* the Slippershell Snail

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Abstract. This paper concerns the role of nitric oxide (NO) in controlling metamorphosis in the marine gastropod *Crepidula fornicata*. Metamorphosis was stimulated by the nitric oxide synthase (NOS) inhibitors AGH (aminoguanidine hemisulfate) and SMIS (*S*-methylisothiourea sulfate) at concentrations of about 100–1000 μmol l^{-1} and 50–200 μmol l^{-1}, respectively. Metamorphosis was not, however, induced by the NOS inhibitor L-NAME (L-N^G-nitroarginine methyl ester) at even the highest concentration tested, 500 μmol l^{-1}. Moreover, pre-incubation with L-NAME at 20 and 80 μmol l^{-1} did not increase the sensitivity of competent larvae to excess K^+, a potent inducer of metamorphosis in this species; we suggest that either L-NAME is ineffective in suppressing NO production in larvae of *C. fornicata*, or that it works only on the constitutive isoform of the enzyme. In contrast, metamorphosis was potentiated by the guanylate cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one) in response to a natural metamorphic inducer derived from conspecific adults. Because NO typically stimulates cGMP production through the activation of soluble guanylate cyclase, this result supports the hypothesis that NO acts as an endogenous inhibitor of metamorphosis in *C. fornicata*. The expression of NOS, shown by immunohistochemical techniques, was detected in the apical ganglion of young larvae but not in older larvae, further supporting the hypothesis that metamorphosis in *C. fornicata* is made possible by declines in the endogenous concentration of NO during development.

Received 17 October 2006; accepted 12 April 2007.

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Abbreviations: ACSW, adult-conditioned seawater; AGH, aminoguanidine hemisulfate; FSW, filtered seawater; IO, instant ocean artificial seawater; L-NAME, L-N^G-nitroarginine methyl ester; LTC, L-thiocitrulline; NO, nitric oxide; NOS, nitric oxide synthase; NOS-LIR, NOS-like immunoreactivity; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; SMIS, *S*-methylisothiourea sulfate.

Introduction

The larvae of marine invertebrates are competent to metamorphose either at or soon after their release into the plankton (*e.g.,* bryozoans and ascidians: Woollacott *et al.*, 1989; Wendt, 1996; Marshall *et al.*, 2003; Zega *et al.*, 2005) or they become competent to metamorphose after swimming (and, in many species, feeding) for days or weeks (Chia, 1978; Pechenik, 1990; Hadfield *et al.*, 2001). Competent larvae are then stimulated to metamorphose upon encountering certain external physical or chemical cues that apparently serve to indicate habitat suitable for juveniles or adults (Thorson, 1950; Hadfield, 1978; Pawlik, 1990; Pechenik, 1999). Although a stimulatory model of metamorphosis has been investigated for many years (*e.g.,* Hadfield, 1978; Morse *et al.*, 1980; Baloun and Morse, 1984; Yool *et al.*, 1986), growing evidence suggests that for the larvae of at least some invertebrate species from several phyla (Pechenik and Qian, 1998), metamorphosis is instead under inhibitory control (Bishop *et al.*, 2001; Bishop and Brandhorst, 2001, 2003; Leise *et al.*, 2001; Pechenik *et al.*, 2002), as first suggested by Chia (1978); thus, competent larvae may be held back from metamorphosing by the presence of an endogenous inhibitor, with metamorphosis taking place only when concentrations of that inhibitor are sufficiently reduced. There is growing evidence that in a number of invertebrate species the endogenous inhibitor of metamorphosis is nitric oxide (NO) (Froggett and Leise, 1999; Bishop *et al.*, 2001; Bishop and Brandhorst, 2001, 2003; Leise *et al.*, 2001; Pechenik *et al.*, 2002; Hens *et al.*, 2006), a gaseous neurotransmitter produced from L-arginine by the enzyme nitric oxide synthase (NOS) (Moroz, 2001; Bishop

et al., 2001; Bishop and Brandhorst, 2001; Serfözö and Elekes, 2002).

The evidence that NO inhibits metamorphosis in larvae of the marine gastropod *Crepidula fornicata* (Linnaeus, 1758) has so far been indirect: competent larvae metamorphosed during exposure to the nonspecific NOS inhibitor chlorpromazine (Pechenik *et al.*, 2002). However, chlorpromazine can do more than inhibit NOS. It can, for example, act as a histamine H_1 antagonist or inhibit calmodulin-stimulated cyclic nucleotide phosphodiesterase (Bennett, 1998; Hardman *et al.*, 2001; reviewed in Pechenik *et al.*, 2002), so that its recorded action on larvae of *C. fornicata* may not have reflected an effect on NOS. Indeed, the failure of the NOS inhibitor L-NAME (L-N^G-nitroarginine methyl ester) to stimulate metamorphosis in *C. fornicata* (Pechenik *et al.*, 2002) argued against the hypothesis that NO serves as an endogenous inhibitor of metamorphosis.

In the present research, we further investigated the hypothesis that metamorphosis in larvae of *C. fornicata* requires a decline in endogenous NO concentration, and that NO serves as a natural inhibitor of metamorphosis in this species. To do this, we tested the effects of the specific NOS inhibitors aminoguanidine hemisulfate (AGH) and *S*-methylisothiourea sulfate (SMIS), and examined whether responses to these NOS inhibitors changed as competent larvae aged. We also tested the effect of the NOS inhibitor L-NAME at higher concentrations than we had used previously (10–20 μmol l^{-1}: Pechenik *et al.*, 2002), in light of the possibility that those lower concentrations may simply not have reduced NO production enough to trigger metamorphosis. In addition, we determined whether larvae of *C. fornicata* become responsive to NOS inhibitors coincidently with their becoming responsive to excess K^+ (Pechenik and Heyman, 1987; Pechenik and Gee, 1993; Pechenik *et al.*, 2002), in an attempt to determine whether NOS inhibitors and excess K^+ stimulate metamorphosis through the same pathways. Competent larvae of *C. fornicata* metamorphose in response to excess K^+ within about 6 h (Pechenik and Heyman, 1987; Pechenik and Gee, 1993). We also tested whether exposure to low concentrations of NOS inhibitors alters the subsequent sensitivity of larvae to excess K^+. Because NO may function by activating guanylate cyclase (Lincoln and Cornwell, 1993; Salter *et al.*, 1996; Durham *et al.,* 2000; Bishop and Brandhorst, 2001; Serfözö and Elekes, 2002), we also asked whether metamorphosis could be potentiated by the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ). Finally, we localized NOS-like immunoreactivity (NOS-IR) in larvae of different ages, both to find putative endogenous sources of NOS and to see if the occurrence and distribution of such sources changed as larvae aged (Moroz *et al.*, 1994; Newcomb and Watson, 2001).

Materials and Methods

Care and maintenance of larvae

One experiment, testing the involvement of guanylate cyclase in the signal transduction pathway, was performed at the Friday Harbor Laboratories, University of Washington. Adults of C*repidula fornicata* were collected in June 2005 from Totten Inlet, Thurston County, Washington, and kept at 20–23 °C in 1-gallon (~3.78-l) jars of unfiltered seawater that was changed daily until larvae were released. All other pharmacology experiments were conducted using females collected from Nahant, Massachusetts, or Wickford, Rhode Island. The larvae used in these experiments were released from at least seven different females over the course of 2 months between June and August 2005. Ages of all larvae in all experiments are expressed as days post-release. Once released from adults, larvae were collected on 150-μm-mesh Nitex and transferred to glass jars containing 1-μm or 0.45-μm filtered seawater with a practical salinity of about 30. Larvae were reared at room temperature (20–23 °C) on a diet of the naked flagellate *Isochrysis galbana* (clone T-ISO) using standard techniques (Pechenik, 1980; Pechenik *et al.*, 2002; Pires *et al.*, 2000a). Larvae were subsampled periodically and measured nondestructively (Pechenik *et al.*, 1996) at a magnification of 50×; larvae were used for experiments when they were about 900 to 1000 μm in mean shell length, as they tend to become competent to metamorphose in this size range at room temperature (Pechenik and Heyman, 1987).

Testing for metamorphic competence

Experiments were run only when at least 70% of subsampled larvae (one to three replicates of 8–10 larvae each) were competent to metamorphose on the previous day. Larvae were tested for competence by elevating the K^+ concentration of seawater by 15–20 mmol l^{-1} (Pechenik and Gee, 1993). Metamorphosis was signaled by loss of the ciliated larval swimming organ, the velum (Pechenik and Heyman, 1987; Pechenik and Gee, 1993; Pires *et al.*, 2000a).

General experimental protocol

All pharmacology experiments except the one involving a guanylate cyclase inhibitor (see below) were conducted using 12-well tissue-culture plates, with 4 ml of test solution per well. In each experiment, we included three to four replicates per treatment, with eight larvae per replicate. When subsampling larvae from batch cultures for use in experiments, we selected the largest individuals. We then measured (nondestructively, at 50× magnification) the shell lengths of 10–15 individuals subsampled from the population of larvae to be tested. Larvae used in experiments were always pipetted into a 30–50-ml bath of the proper test solution before being pipetted into the test wells, to mini-

mize the effect of larval transfer on the final concentrations of the chemicals being tested.

In all experiments, filtered seawater (FSW) served as a negative control, and seawater whose KCl concentration had been elevated by 20 mmol l^{-1} served as a positive control, to assess the percentage of larvae that were competent to metamorphose on the day of the experiment (Pechenik and Heyman, 1987; Pechenik and Gee, 1993).

The percentage of larvae that metamorphosed in each solution was assessed after about 6 h and 20 h, by visual inspection at 32–50×; metamorphosis was signaled by loss of the ciliated larval swimming organ, the velum (Pechenik and Heyman, 1987; Pechenik and Gee, 1993; Pires *et al.*, 2000a; Leise *et al.*, 2001).

Once an experiment was set up, the tissue culture plates were kept in the dark. Details for each experiment, including the age and mean shell length of tested larvae, are given in Table 1.

Testing effects of nitric oxide synthase inhibitors on larval metamorphosis

To test the hypothesis that metamorphosis occurs only when endogenous nitric oxide (NO) concentrations decline below some threshold level, we tested the effects of three NOS synthase (NOS) inhibitors; NOS inhibitors inhibit NO production and should therefore reduce endogenous concentrations of NO. If NO prevents metamorphosis, NOS inhibitors should allow metamorphosis to occur. The NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) was tested at eight concentrations: 10, 20, 40, 60, and 80, 150, 250, and 500 μmol l^{-1}. Aminoguanidine hemisulfate (AGH) was tested in one experiment at concentrations of 500 μmol l^{-1} and 1000 μmol l^{-1}. Lower concentrations (12.5–400 μmol l^{-1}) were tested in subsequent experiments (see Results), in an attempt to determine a threshold concentration. *S*-methylisothiourea sulfate (SMIS) was tested in three experiments at concentrations ranging from 20–200 μmol l^{-1}. Finally, in one experiment we also tested the NOS inhibitor LTC (L-thiocitrulline) at a concentration of 25 μmol l^{-1}. Tested concentrations were chosen on the basis of the published EC-50 values for the different inhibitors (Calbiochem) and on previously published findings for the response of larvae of this and other species (Bishop *et al.*, 2001; Bishop and Brandhorst, 2001; Pechenik *et al.*, 2002).

Larvae were examined after 6, 12, and 24 h to determine mean percent metamorphosis.

Effects of pre-incubation in nitric oxide synthase inhibitors on larval metamorphosis

Competent larvae of *C. fornicata* metamorphose in response to 15–20 mmol l^{-1} excess K^+ in seawater within 6–8 h (Pechenik and Heyman, 1987; Pechenik and Gee, 1993; Pechenik *et al.*, 2002). Four experiments were conducted to test whether pre-incubation in the NOS inhibitors L-NAME and AGH would potentiate the larval response to excess K^+; *i.e.,* whether pre-incubation would increase the sensitivity of competent larvae to that inducer. Larvae were pre-incubated with NOS inhibitors at various concentrations (see Results) overnight and then transferred into either a solution of 20 mmol l^{-1} excess K^+ in seawater or filtered seawater (negative control). A control group of larvae was pre-incubated in filtered seawater overnight and then transferred into either a 20 mmol l^{-1} excess KCl solution (positive control) or filtered seawater (negative control). Larvae were first pipetted into a bath of filtered seawater to remove

Table 1

Summary of experiments conducted on the metamorphosis of Crepidula fornicata*; each designated batch of larvae was released from a different female*

Experiment (Figure number)	Larval batch	Larval age (days)	Mean shell length (μm) ± SD (*n*)	Chemical(s) tested*
I (1a)	A	14	1144 ± 117.2 (10)	AGH
II (1b)	B	17	897.9 ± 138.4 (10)	AGH
III (2a)	C	27	1078.2 ± 75.71 (10)	SMIS
IV (2b)	D	28	944.1±94.1 (19)	AGH, SMIS
V (3)	A	13	1132.1 ± 91.5 (8)	AGH, L-NAME
VI (4a, 4b)	D	18	787.6 ± 112.2 (10)	AGH, SMIS
	D	20	819.1 ± 90.6 (10)	AGH
	D	21	883.4 ± 72.87 (15)	AGH
	D	22	860.5 ± 158.6 (10)	AGH
	D	24	938.6 ± 87.3 (15)	AGH
	D	26	984.5 ± 93.3 (10)	AGH
VII (5)	A	13	1132.4 ± 91.53 (10)	L-NAME
VIII (6)	B	24	1161.6 ± 183.5 (17)	AGH, L-NAME
IX (7)	E–K (7 batches)	12–16	822.0 ± 56.6 (10) to 1034.4 ± 56.5 (10)	ODQ

* AGH, aminoguanidine hemisulfate; L-NAME, L-N^G-nitroarginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; SMIS, *S*-methylisothiourea sulfate.

all of the test chemical before they were then transferred to the solutions of control seawater or excess K^+.

The effects of pre-incubation in L-NAME were tested in two experiments. In one experiment, larvae were pre-incubated in 20 μmol l^{-1} L-NAME for 14 h. Larvae were then transferred into either a solution of 20 mmol l^{-1} excess KCl or filtered seawater and checked at 2, 4, and 8 h to determine mean percent metamorphosis. Control larvae subsampled from the same culture were transferred directly to a solution of excess K^+ for comparison (positive control) while other larvae remained in filtered seawater to serve as negative controls. In a second experiment (Experiment VIII, Table 1), larvae were pre-incubated in L-NAME at 80 μmol l^{-1} for 14 h. Larvae were then transferred into either a solution of excess KCl in seawater or to filtered seawater, and examined for metamorphosis after 3 and 6 h.

The effects of pre-incubation in AGH on responsiveness to excess K^+ were tested in two experiments. Larvae were pre-incubated in either 100 μmol l^{-1} or 300 μmol l^{-1} AGH for 12 h, and then transferred into either 20 mmol l^{-1} excess KCl or FSW. They were checked for metamorphosis several times over the next 8 h. Both positive and negative controls were included for comparison.

Potentiation by a soluble guanylate cyclase inhibitor

We tested whether metamorphosis could be potentiated by the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ). Experiments were performed on seven occasions with competent larvae from seven different larval cultures (12–16 days old) in 8 ml of test solution in 6-well polystyrene multiplates, 20 larvae per well. Treatments were as follows: 1 μmol l^{-1} and 10 μmol l^{-1}ODQ in FSW, 1 μmol l^{-1} and 10 μmol l^{-1} ODQ in adult-conditioned seawater (ACSW, a natural inducer of metamorphosis), FSW, and ACSW. The three treatments with ACSW were prepared by placing a small adult (~1.5 cm in shell length) in 8 ml of FSW in each of three multiplate wells. The adults were rotated between wells, so that each well was exposed to each adult for 1 h. Adults were removed from wells and ODQ was added immediately before the trials. Larvae in all trials were scored for metamorphosis after 24 h. Tested larvae were drawn from batches that ranged in mean size [mean ± SD (*n*)] from 822 ± 56.6 μm (10) to 1034.4 ± 56.5 μm (10).

Comparing the onset of competence in response to three chemical cues

Over the course of 2 weeks, seven experiments were conducted to compare the larval response to the NOS inhibitors AGH and SMIS with their response to 20 mmol l^{-1} excess K^+ as larvae aged. This was done to determine whether the onset of responsiveness to excess K^+ within the population corresponded to the rate at which larvae in the population also became responsive to NOS inhibitors. For all experiments, there was a negative control group of larvae transferred to FSW and a positive control group transferred to 20 mmol l^{-1} excess KCl. Larvae were generally checked at 6 and 24 h. The effects of SMIS were tested at concentrations up to 200 μmol l^{-1}, and the effects of AGH were tested at concentrations up to 1000 μmol l^{-1}. As before, there were four replicates per treatment in each experiment, with eight larvae per replicate.

Data analysis

Pharmacology data were analyzed using Student's *t*-tests or one-way analysis of variance (ANOVA) using Prism ver. 3.03 software from GraphPad, Inc. To improve normality, percentage data were arcsin-transformed before analysis (Sokal and Rohlf, 1995). When significant differences among means were found by ANOVA, treatment means were compared against both the negative and positive control means by using Bonferroni's method, corrected for multiple comparisons.

Immunohistochemistry

In this part of the study we sought to localize putative sites of larval NO synthesis, using an antibody directed against a highly conserved peptide sequence common to different NOS isoforms. After initial labeling experiments revealed NOS-like immunoreactivity (NOS-LIR) in a location consistent with that of the apical ganglion, a neural structure implicated in the control of metamorphosis (Hadfield *et al.*, 2000), additional immunolabeling experiments were performed with antibodies directed against tubulin and serotonin, both of which are markers for the apical ganglion in larvae of gastropods (Page and Parries, 2000; Kempf and Page, 2005). For these experiments adult *C. fornicata* were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, in January–March 2006 and maintained at Dickinson College in 1-gallon (~3.78-l) jars of Instant Ocean artificial seawater (IO), in an incubator on a 14:10 light/dark cycle. They were warmed to 21 °C over a period of 2 weeks, and fed varying amounts of *I. galbana*. The IO was changed every other day. Larvae were collected as above and cultured in IO at 21 °C, as described by Pires *et al.* (2000a).

Larvae were relaxed in a 1:1 mixture of FSW and 7.5% magnesium chloride, and fixed for 2–12 h in cold 4% paraformaldehyde in FSW with 50 mmol l^{-1} Tris buffer. They were then washed in phosphate-buffered saline (PBS), and decalcified with 5% EDTA in PBS. Larvae were incubated overnight at 4 °C in a blocking medium containing 5% goat serum, 4% Triton X-100, and 0.1% sodium azide. Larvae were then exposed to one or a pair of primary antibodies, for 24–48 h at 4 °C. The primaries were universal anti-NOS rabbit polycolonal antibody (Affinity Bioreagents PA1-039), 1:100; anti-β-tubulin monoclonal antibody (Developmental Studies Hybridoma Bank E7),

1:50–100; anti-serotonin rabbit polyclonal antibody (Immunostar 20080), 1:500—all diluted in blocking medium. Larvae were washed 4–5 times over 12–24 h with PBS containing 4% Triton X-100 and 0.1% sodium azide (PTA), and transferred to secondary antibodies for 24–48 h at 4 °C. Secondary antibodies were goat anti-rabbit Alexafluor 633 (Invitrogen/Molecular Probes A21070) and goat anti-mouse Alexafluor 488 (Invitrogen/Molecular Probes A11001), both at 1:200 dilution in blocking medium. Preparations were washed in PTA as above, dehydrated through ethanol to xylene, and mounted in DPX (Fluka). Immunohistochemistry preparations were analyzed using an Olympus Fluoview 500 inverted confocal microscope with argon (488-nm) and helium-neon (633-nm) laser excitation and 505–550-nm bandpass and 660-nm longpass barrier filters on the detection photomultipliers. Double-label preparations were scanned sequentially with the two lasers to avoid any artifact from overlapping fluorescent emission spectra. ImageJ ver. 1.34 (National Institutes of Health) was used to construct z-series projections of optical sections.

Results

Effects of nitric oxide synthase inhibitors

The nitric oxide synthase (NOS) inhibitor aminoguanidine hemisulfate (AGH) stimulated metamorphosis at concentrations of 100–1000 μmol l^{-1} (Fig. 1a, b; Fig. 2b; Fig. 3); the extent of the effect varied among experiments with different batches of larvae (compare results for 500 μmol l^{-1} AGH in Figs. 1b and 3, relative to the effect of excess K^+). AGH failed to stimulate metamorphosis of *Crepidula fornicata* at concentrations of 50 μmol l^{-1} or less (mean percent response to excess K^+ = 91.3% ± 19.6 % (SD), data not shown). The NOS inhibitor *S*-methylisothiourea sulfate (SMIS) was very effective at inducing metamorphosis at concentrations as low as 50 μmol l^{-1} (Fig. 2). As with the response to AGH, sensitivity to SMIS varied among experiments (compare data for 100 μmol l^{-1} in Fig. 2a and b). In another experiment (data not shown), an SMIS concentration as low as 20 μmol l^{-1} was somewhat effective, inducing about 12% of larvae to metamorphose (SD = 15%, three replicates of eight larvae each) with no larvae metamorphosing in the control treatment, and an average of 96% of larvae metamorphosing (SD = 11%) in response to excess K^+.

In contrast, the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) failed to induce any larvae to metamorphose during 24-h exposures at even the highest concentrations tested: 80 μmol l^{-1} in one experiment (Fig. 3, note the high response to AGH in the same experiment), and 150, 250, and 500 μmol l^{-1} in two additional experiments (data not shown, mean percent metamorphosis–± SD–in response to excess K^+ was 52.2 ± 14.1% in one experiment and 81.2 ± 21.6 % in the second experiment, four replicates with eight larvae per replicate). Similarly, the NOS inhibitor

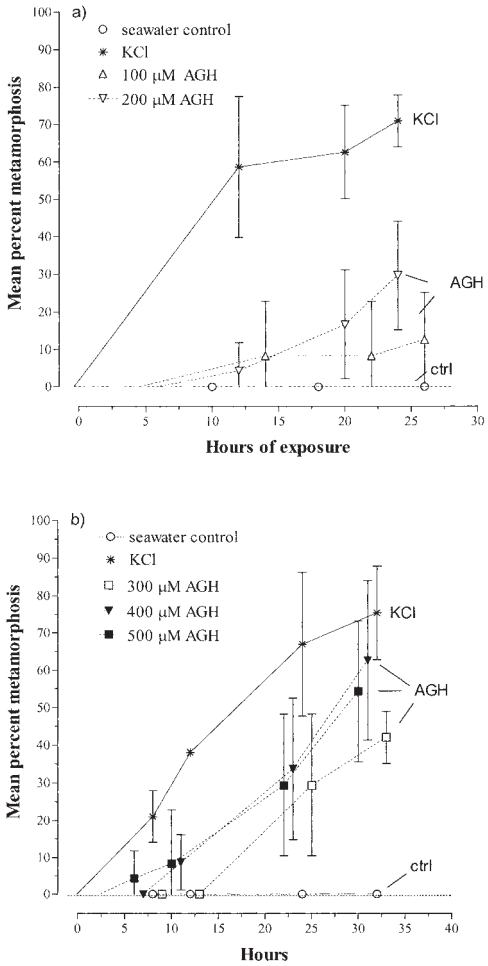

Figure 1. Induction of metamorphosis in larvae of *Crepidula fornicata* by the NOS inhibitor aminoguanidine hemisulfate (AGH). Each point represents the mean response (±1 SD) of four replicates of eight larvae each. Excess KCl (20 mmol l^{-1} in seawater) served as a positive control. See Table 1 for data on larval age and shell length for these experiments (Experiment I for "a," Experiment II for "b").

L-thiocitrulline (LTC) at a concentration of 25 μmol l^{-1} failed to stimulate any metamorphosis over 24 h (data not shown, mean ± SD response to excess K^+ = 96% ± 11%)

Although some larvae in the tested population first became responsive to the NOS inhibitors AGH and SMIS at about the same age that they became responsive to excess K^+, the responses to the NOS inhibitors were typically lower (*t*-tests, $P < 0.05$) than the response to excess K^+ for larvae of any particular age (Fig. 4a, b); these differences may simply reflect differences in the dose-response curves for the different substances tested.

Pre-incubating competent larvae in L-NAME at concentrations of 20 (Fig. 5) and 80 μmol l^{-1} (Fig. 6) did not accelerate the subsequent response of larvae to 20 mmol l^{-1} excess K^+ (*t*-tests, $P > 0.10$); in both experiments, the percentages of control and pre-incubated larvae that responded to excess K^+ were nearly identical at all time-points sampled (Figs. 5, 6). At least 90% of larvae were

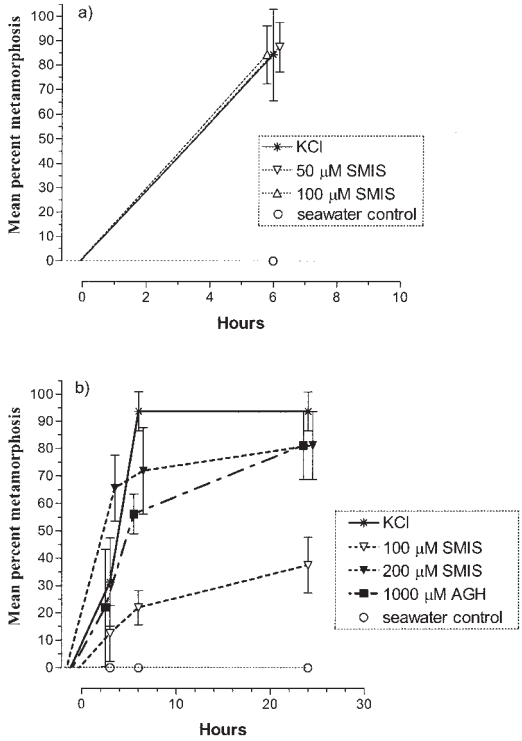

Figure 2. Induction of metamorphosis in larvae of *Crepidula fornicata* by the NOS inhibitor *S*-methylisothiourea sulfate (SMIS) tested at two concentrations. Each point represents the mean response (±1 SD) of four replicates of eight larvae each. Excess KCl (20 mmol l^{-1} in seawater) served as a positive control. See Table 1 for data on larval age and shell length for these experiments (Experiments III and IV)

competent to metamorphose in those experiments, as shown by their response to 20 mmol l^{-1} excess K^+, and no control larvae metamorphosed during the 6–9-h incubations,

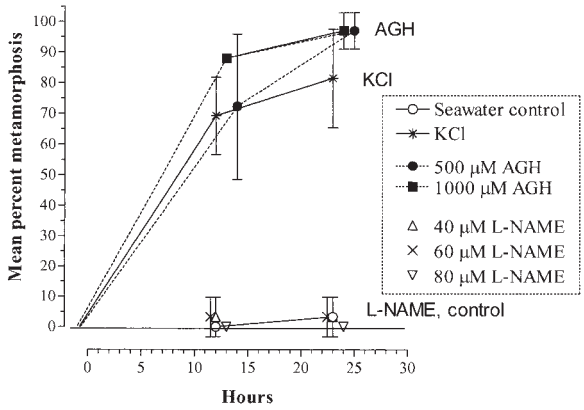

Figure 3. Effects of the nitric oxide synthase inhibitors aminoguanidine hemisulfate (AGH) and L-N^G-nitroarginine methyl ester (L-NAME) on metamorphosis of *Crepidula fornicata* larvae. Each point represents the mean response (±1SD) of four replicates of eight larvae each. Excess K^+ served as a positive control. No larvae metamorphosed when tested at an L-NAME concentration of 10 or 20 μmol l^{-1} (data not shown). See Table 1 for data on larval age and shell length for these experiments (Experiment V).

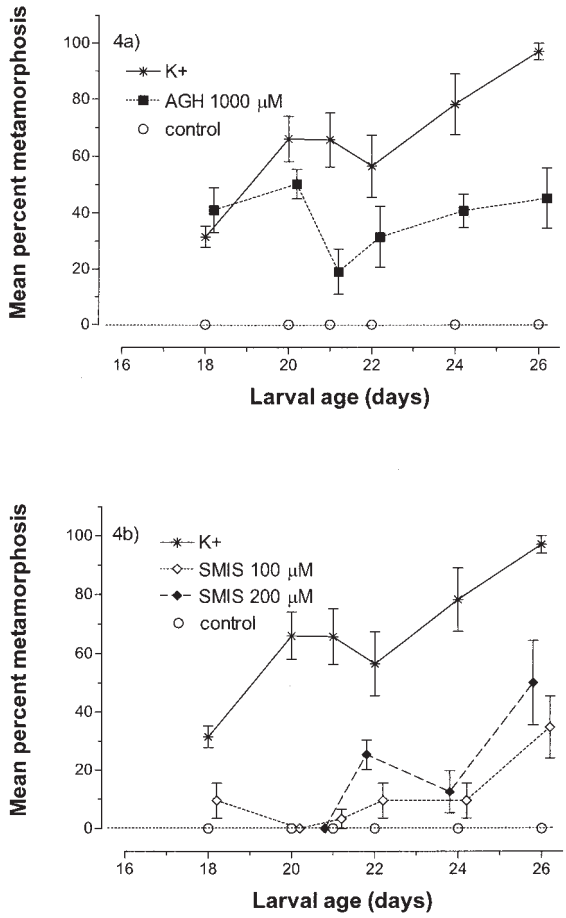

Figure 4. Development of responsiveness to excess K^+ and to the nitric oxide synthase inhibitors (a) AGH (aminoguanidine hemisulfate) at 1000 μmol l^{-1} and (b) *S*-methylisothiourea sulfate (SMIS) at 100 and 200 μmol l^{-1} in larval cultures of *Crepidula fornicata*. Data shown are the mean responses (±1SD) after 24 h of exposure. See Table 1 for additional details (Experiment VI).

whether or not they had been pre-incubated in L-NAME. Similarly, pre-incubation in AGH at either 100 μmol l^{-1} (data not shown) or 300 μmol l^{-1} (Fig. 6) did not sensitize larvae to subsequent stimulation by excess K^+.

Effects of soluble guanylate cyclase inhibitor

The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ), at a concentration of 10 μmol l^{-1}, potentiated metamorphosis in response to the natural inducer adult-conditioned seawater (ACSW); that is, exposure to the inhibitor greatly increased the proportion of larvae responding to the natural inducer (Fig. 7). Larvae given this drug along with ACSW metamorphosed in greater numbers than those in ACSW alone (one-way ANOVA followed by pairwise comparisons, $P < 0.05$). However, no significant ($P > 0.10$) potentiation of metamorphosis was observed when larvae were incubated at a lower concentration of ODQ (1 μmol l^{-1}) in ACSW, and

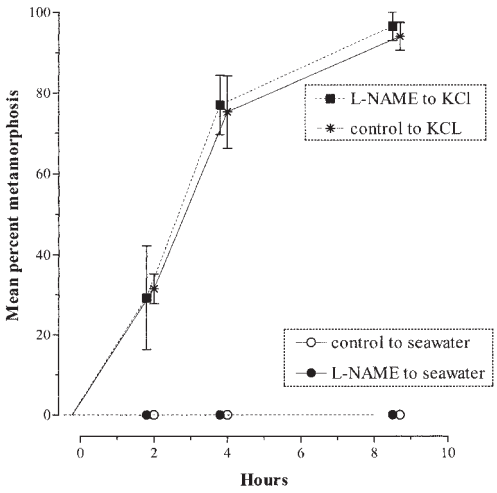

Figure 5. Effect of pre-incubation with the nitric oxide synthase inhibitor L-NAME on responsiveness to excess K^+ by larvae of *Crepidula fornicata*. Larvae were incubated for 14 h in darkness in 20 μmol l^{-1} L-NAME (L-N^G-nitroarginine methyl ester) and then transferred either to excess K^+ or control seawater. Other larvae were exposed only to excess K^+ (positive control) or control seawater. See Table 1 (Experiment VII) for additional details.

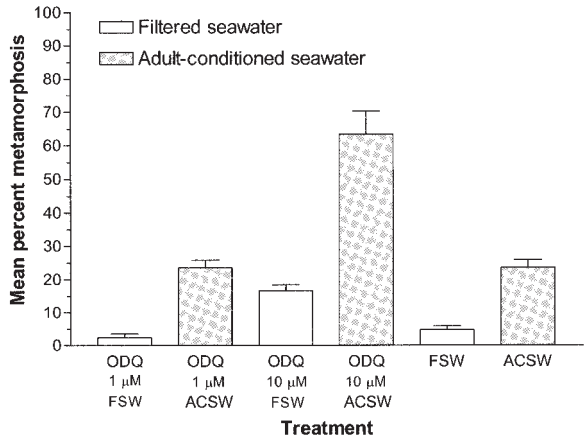

Figure 7. Potentiation of metamorphosis of larvae of *Crepidula fornicata* by the soluble guanylate cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one). Competent larvae were treated with 1 μmol l^{-1} or 10 μmol l^{-1} ODQ dissolved in filtered seawater (FSW) or adult-conditioned seawater (ACSW), or with FSW or ACSW alone, and assayed for metamorphosis after 24 h. Bars show mean percent metamorphosis (+SD, n = seven trials using larvae from seven different larval cultures), 20 larvae tested per treatment in each trial.

neither concentration of ODQ in filtered seawater (FSW) alone induced metamorphosis at levels significantly different from those recorded in FSW controls.

Immunohistochemistry

The universal anti-NOS antibody labeled cells in the region of the apical ganglion, the digestive gland, and the stomach (Fig. 8). The latter two structures also showed a variable degree of nonspecific labeling in control preparations from which the primary anti-NOS antibody had been omitted. NOS-like immunoreactivity (NOS-LIR) was observed in the region of the apical ganglion in 4-d and 5-d precompetent larvae, but not in precompetent 9-d nor competent 23-d larvae. To characterize these apical cells, some preparations were double-labeled with anti-NOS and anti-tubulin antibodies (Fig. 9). NOS-like and tubulin-like immunoreactivity were co-localized in a group of cells that usually could be resolved as two bilateral clusters of 10 monopolar neuronal somata with apical neurites extending toward the epithelial surface.

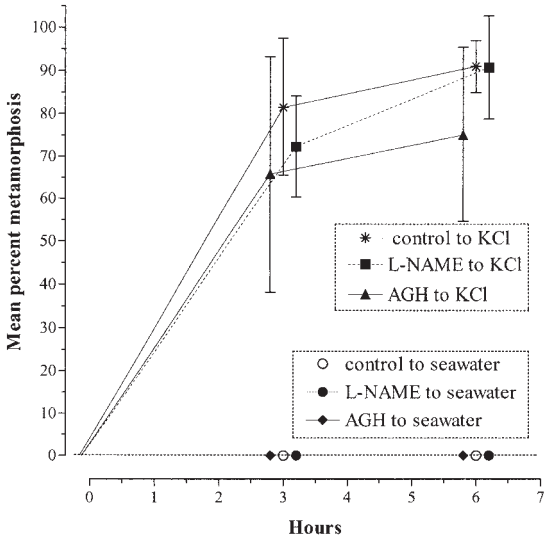

Figure 6. Effect of pre-incubation in nitric oxide synthase inhibitors on responsiveness to excess K^+ by larvae of *Crepidula fornicata*. Larvae were incubated for 12 h in darkness in either 80 μmol l^{-1} L-NAME (L-N^G-nitroarginine methyl ester) or 300 μmol l^{-1} AGH (aminoguanidine hemisulfate) and then transferred either to excess K^+ or control seawater. Other larvae were exposed only to excess K^+ (positive control) or control seawater. See caption to Fig. 1 and Table 1 (Experiment VIII) for additional details.

To further place these cells within the context of the larval nervous system, especially with respect to previously described cells of the apical ganglion, some preparations were double-labeled with anti-tubulin and anti-serotonin antibodies (Fig. 10); a NOS/serotonin double label was not attempted, because the available antibodies that work well in molluscs are derived from the same host species. Serotonin-like immunoreactivity was found in a group of five neuronal somata contributing to an apical neuropil. The two largest of these cells were a bilateral pair, each of which gave rise to an axon projecting across the midline into the contralateral velar lobe. All five serotonin-labeled somata and the associated apical neuropil were superficial to tubulin-labeled axons of the cerebral commissure and posterior to the tubulin-labeled somata described above.

Discussion

In general, our data support the hypothesis that competent larvae of *Crepidula fornicata* are prevented from metamor-

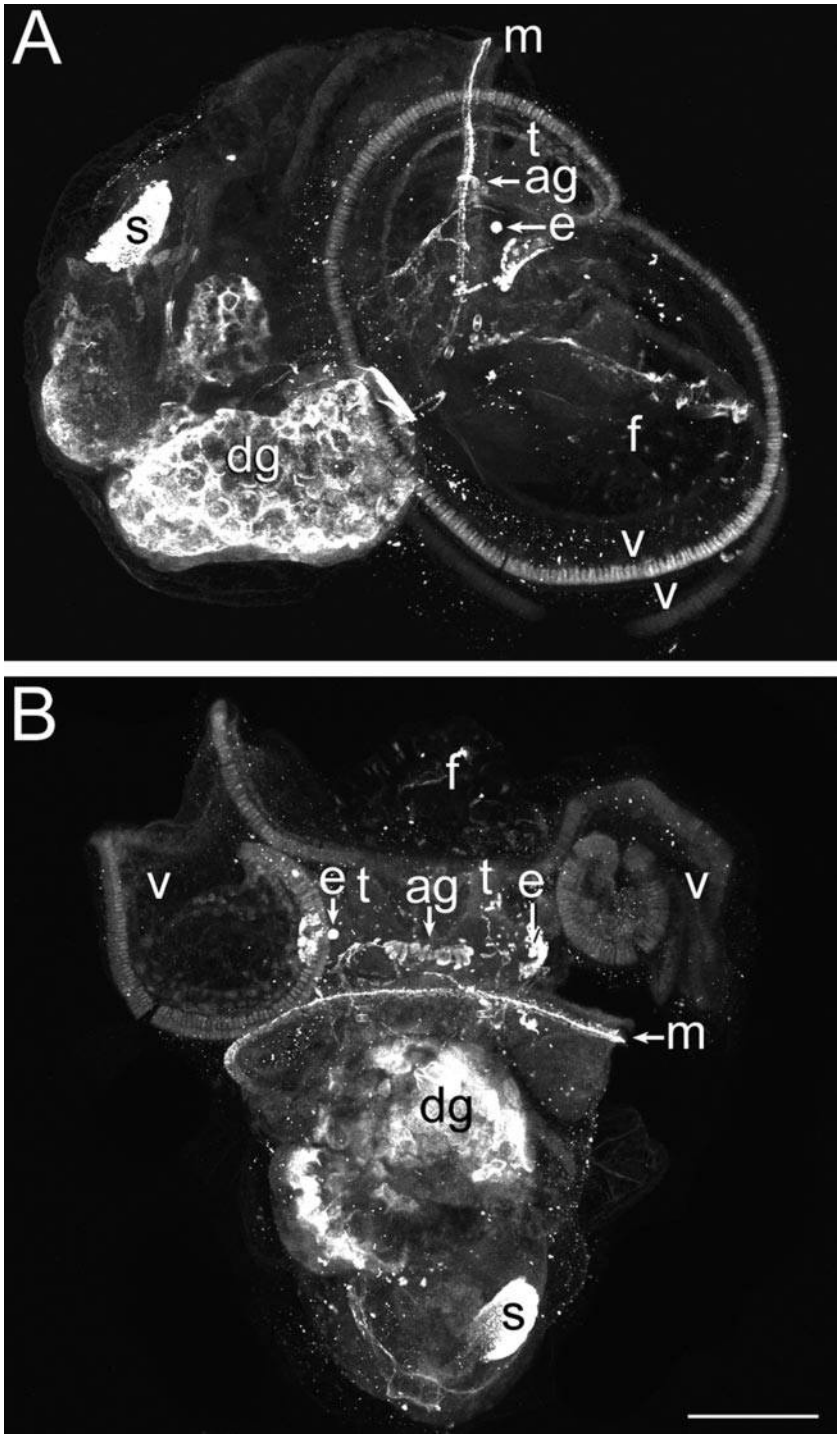

Figure 8. Nitric oxide synthase-like immunoreactivity in 4-d precompetent larva of *Crepidula fornicata*. (A) Lateral aspect; (B) dorsal aspect. Specific immunofluorescent labeling was found in the apical ganglion (ag), digestive gland (dg), and stomach (s). Other fluorescent cells, particularly in the foot (f), the surface of the head, the edge of the mantle (m), and ventrolateral to the eyes (e), as well as the eyes themselves, are the consequence of nonspecific labeling or autofluorescence. Velar lobes (v) and cephalic tentacles (t) are indicated for orientation. Scale bar, 100 μm.

phosing by endogenous nitric oxide (NO), and that metamorphosis occurs only when perception of an external cue lowers NO concentrations. In particular, two NOS inhibitors— aminoguanidine hemisulfate (AGH) and *S*-methylisothiourea sulfate (SMIS)—stimulated substantial metamorphosis of competent larvae, and did so, in at least some

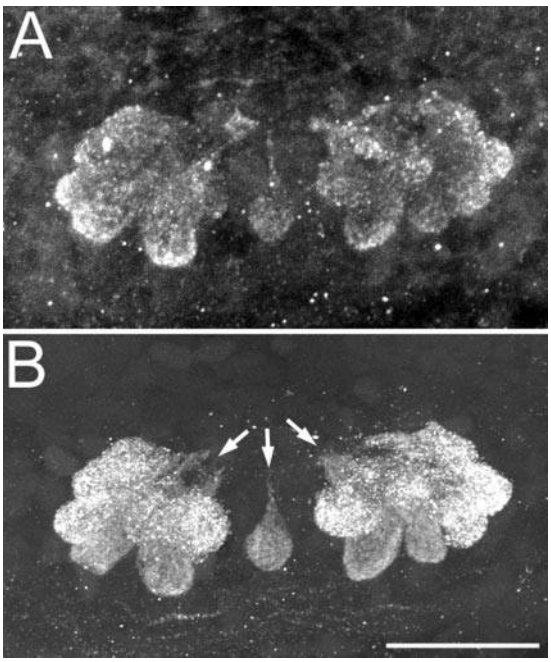

Figure 9. Co-localization of (A) nitric oxide synthase and (B) tubulin-like immunoreactivity in clusters of neurons within the apical ganglion of 5-d precompetent larva of *Crepidula fornicata*. Arrowheads indicate apical neurites projecting toward the epithelial surface. Scale bar, 20 μm.

experiments (*e.g.,* see Figs. 1b, 2), in about the same amount of time required for excess K^+ to stimulate the same amount of metamorphosis.

The potentiation of larval responsiveness to adult-conditioned seawater by the soluble guanylate cyclase inhibitor ODQ (Fig. 7) is also consistent with a role for NO as a natural inhibitor of metamorphosis. Activation of guanylate cyclase is the best-known mechanism of action of NO (Lucas *et al.*, 2000); we therefore expected that ODQ would antagonize endogenous NO and potentiate metamorphosis. However, we cannot exclude the possibility that guanylate cyclase in larvae of *C. fornicata* may be regulated by other signaling molecules, such as carbon monoxide (Lucas *et al.*, 2000; Ryter *et al.*, 2004).

We localized NOS-like immunoreactivity (NOS-LIR) in the apical ganglion of *C. fornicata* (Figs. 8, 9), as has been done previously in the caenogastropod *Ilyanassa obsoleta* (Thavaradhara and Leise, 2001). To confirm the localization of NOS-LIR in the apical ganglion, we determined the relationship between NOS-LIR cells and the serotonin-LIR parampullary neurons that characterize gastropod apical ganglia (Fig. 10; Page and Parries, 2000). We took advantage of the co-localization of β-tubulin-LIR and NOS-LIR in cells of the apical ganglion (Fig. 9) by performing a double-label for tubulin and serotonin (Fig. 10). It remains to be determined whether the cells that co-localize NOS-LIR and tubulin-LIR are homologous to the ampullary neurons designated by Chia and Koss (1984) and labeled with an anti-tubulin antibody by Kempf and Page (2005). The latter authors used an antibody against acetylated α-tubulin to label ciliary bundles of ampullary neurons in the apical ganglia of two opisthobranchs, but reported no such labeling when the same antibody was applied to larvae of four caenogastropod species. In addition, their observed pattern of labeling differed significantly from ours: neuronal somata were weakly labeled in their preparations, but strongly labeled in ours. The tubulin/serotonin double-label (Fig. 10) provides anatomical context for the NOS/tubulin-LIR cells: they are located immediately anterior to the serotonergic parampullary neurons of the apical ganglion and to the tubulin-labeled axons of the cerebral commissure.

The sensory function of the apical ganglion has been imputed on morphological grounds in a variety of gastropod veligers (Bonar, 1978; Chia and Koss, 1984; Marois and Carew, 1997; Kempf *et al.,* 1997; Page and Parries, 2000; Page, 2002; Kempf and Page, 2005) and members of other taxa (reviewed by Lacalli, 1994). Hadfield *et al.* (2000) demonstrated that photoablation of the apical ganglion in the nudibranch *Phestilla sibogae* abolished responses to the natural chemical inducer of metamorphosis. Although our hypothesis proposes that inhibitable NOS activity is involved in regulating metamorphosis in competent larvae of *C. fornicata*, we detected NOS-LIR in apical ganglia of young precompetent (4- and 5-d) larvae only, and not in apical ganglia of older precompetent (9-d) or competent (23-d) larvae. However, it is possible that such activity exists in older larvae but is not detectable by the methods that we used.

A decline in NOS expression with age is consistent with our hypothesis that NO serves as an endogenous inhibitor of metamorphosis in larvae of *C. fornicata*, and may underlie the phenomenon of "spontaneous metamorphosis" documented previously as competent larvae of this species age (Pechenik, 1980, 1984; Pechenik and Lima, 1984), as suggested by Pechenik *et al.* (2002). In previous studies (Pechenik *et al.*, 2002), the NOS inhibitor chlorpromazine stimulated metamorphosis in a greater proportion of older competent larvae than younger competent larvae, a result consistent with gradually declining NO concentrations in competent larvae as they postpone their metamorphosis. We are currently trying to document changes in NO production in aging larvae through direct measurement.

Our results suggest that larvae can be competent to respond to excess K^+ without yet being competent to respond to the effect of NOS inhibitors. Thus, in some experiments (*e.g.,* Figs. 1a, 4), more larvae at each particular age responded to excess K^+ than to either AGH or SMIS. However, as larvae of *C. fornicata* aged, they eventually became responsive to both of the NOS inhibitors that we tested, and eventually responded to those inhibitors about as quickly as they responded to excess K^+ (*e.g.,* Figs 2a, 3). Excess K^+ probably has a greater range of targets within larvae, whereas NOS inhibitors presumably act only to suppress NO production. In particular, although excess K^+ may shut

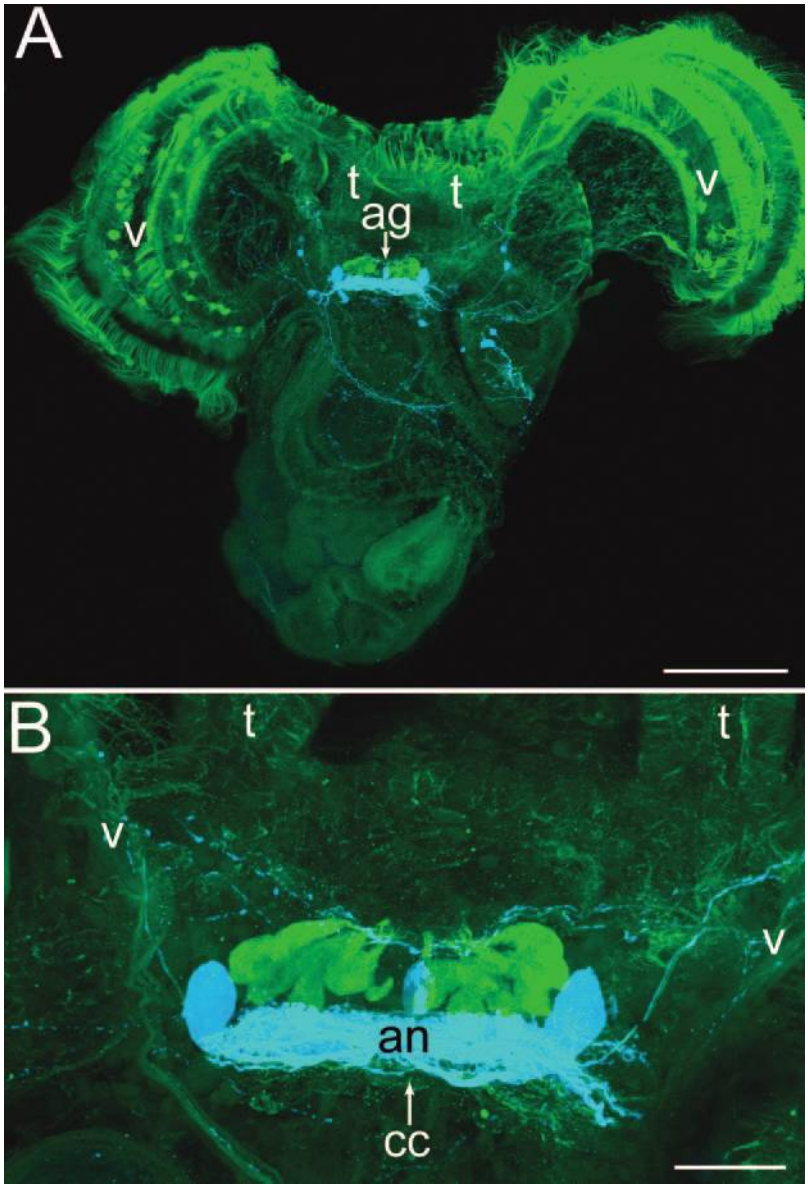

Figure 10. Co-localization of tubulin-like (green) and serotonin-like (blue) immunoreactivity in 5-d precompetent larva of *Crepidula fornicata*. (A) Whole larva. Tubulin antibody strongly labeled cilia in the velum (v), and neurons of the apical ganglion (ag). Cephalic tentacles (t) indicated for orientation. Serotonin antibody labeled other cells of the apical ganglion. Scale bar, 100 μm. (B) Detail of (A). Tubulin-like immunoreactivity was found in two clusters of 10 neurons of the apical ganglion, which also co-localized nitric oxide synthase-like immunoreactivity (Fig. 9). These cells were located anterior to a group of five neurons showing serotonin-like immunoreactivity, which formed an apical neuropil (an) dorsal to tubulin-labeled axons of the cerebral commissure (cc). In this view, two of the serotonin-labeled cells are obscured by the large cells at the lateral margins of the apical neuropil. Scale bar, 20 μm.

down NO production in competent larvae, it may also have other direct effects further along in the metamorphic pathway. Under this hypothesis, the shutdown of NO production is not the final step in the pathway, but instead triggers further events—possibly hormone or neurotransmitter release—to which younger larvae are not yet competent to respond. Excess K^+ may act directly on the target of such a signal, whereas AGH and SMIS can only reduce NO production. Indeed, excess K^+ might not be acting to suppress NO production at all, but rather acting only further downstream in the signal transduction pathway. Additional studies will be required to document the effects of AGH, SMIS, and excess K^+ on NO concentrations within larvae.

The NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) did not stimulate metamorphosis even at concentrations as high as 500 μmol l^{-1}, extending prior results

for this inhibitor (Pechenik *et al.*, 2002). L-NAME is reported (on the basis of assays using mammalian preparations) to work on both the constitutive and inducible isoforms of NOS (Schwartz *et al.*, 1997; Avontuur *et al.*, 1998; Sahrbacher *et al.*, 1998), with greater effectiveness against the constitutive isoform (Cayman Chemical Company, Ann Arbor, MI). The other two inhibitors tested in this study are reported to act selectively on the inducible isoform of NOS (Szabó *et al.*, 1994; Wildhirt *et al.*, 1996; Khatsenko and Kikkawa, 1997; MacFarlane *et al.,* 1999). Possibly L-NAME acts only on the constitutive isoform in *C. fornicata*, in which case our data would suggest that only the inducible isoform of NOS is active in preventing metamorphosis in larvae of this species. Alternatively, L-NAME becomes effective in inhibiting NOS only after hydrolysis by cellular esterases (Khatsenko and Kikkawa, 1997); such hydrolysis may occur less readily in tissues of *C. fornicata*. Note, however, that L-NAME has successfully induced metamorphosis in the sea urchin *Lytechinus pictus* (Bishop and Brandhorst, 2001), the ascidians *Boltenia villosa* and *Cnemidocarpa finmarkiensis* (Bishop *et al.*, 2001), and the gastropod *Ilyanassa obsoleta* (Leise *et al.*, 2001). In studies using the gastropod *Ilyanassa obsoleta*, metamorphosis could be induced by injection of L-NAME but not by bath application (Leise *et al.*, 2001); additional studies will be required to determine whether the larvae of *C. fornicata* respond to direct injection of L-NAME, or whether there is a fundamental difference in the control of metamorphosis for the two species.

Pre-incubation of larvae in the NOS inhibitors L-NAME and AGH did not increase their sensitivity to excess K^+. Such pre-incubation might, however, more successfully potentiate the response of competent larvae to adult-conditioned seawater (Pechenik and Gee, 1993; Pires *et al.*, 2000b). We are currently attempting to purify the active components in adult-conditioned seawater, which will facilitate such studies in the future.

Acknowledgments

Research supported by an NSF-REU grant to Tufts University, organized by Colin Orians, and by NSF-RUI grant IBN-0110832 to A.P. A.P. thanks A.O.D. Willows, R.R. Strathmann, and the staff of the University of Washington Friday Harbor Laboratories for access to their facilities. Diane Cooper of the Taylor Shellfish Co. kindly allowed collection of animals from Taylor property. The confocal microscope used in this research was funded by NSF MRI/RUI grant DBI-0320606 to J. Henson with T. Barber, A. Pires, and K. Guss.

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