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RESEARCH ARTICLE

Nitric oxide signaling differentially affects habitat choice by two larval morphs of the sea slug *Alderia willowi*: mechanistic insight into evolutionary transitions in dispersal strategies

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SUMMARY

In many marine animals, adult habitat is selected by lecithotrophic (non-feeding) larvae with a limited lifespan. In generalist species, larvae may increasingly accept sub-optimal habitat over time as energy stores are depleted ('desperate larva' hypothesis). If the fitness cost of suboptimal habitat is too high, larvae of specialists may prolong the searching phase until they encounter a high-quality patch or die ('death before dishonor' hypothesis). In generalists, starvation is hypothesized to lead to a decline in inhibitory nitric oxide (NO) signaling, thereby triggering metamorphosis. Here, we document alternative functions for identified signaling pathways in larvae having 'desperate' *versus* 'death before dishonor' strategies in lecithotrophic clutches of a habitat specialist, the sea slug *Alderia willowi*. In an unusual dimorphism, each clutch of *A. willowi* hatches both non-selective larvae that settle soon after hatching and siblings that delay settlement in the absence of cues from the alga *Vaucheria*, the sole adult food. Pharmacological manipulation of NO signaling induced metamorphosis in non-selective but not selective stages. However, decreased NO signaling in selective larvae lowered the threshold for response to habitat cues, mimicking the effect of declining energy levels. Manipulation of cGMP or dopamine production induced metamorphosis in selective and non-selective larvae alike, highlighting a distinct role for the NO pathway in the two larval morphs. We propose a model in which NO production (1) links nitrogen metabolism with sensory receptor signaling, and (2) shifts from a regulatory role in 'desperate larva' strategies to a modulatory role in 'death before dishonor' strategies. This study provides new mechanistic insight into how the function of conserved signaling pathways may change in response to selection on larval habitat choice behaviors.

Key words: dimorphism, *Alderia*, desperate larva hypothesis, larval settlement, metamorphosis, mollusc.

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INTRODUCTION

Planktonic larvae of marine invertebrates are under strong selection to locate a suitable juvenile habitat prior to settlement, as the chosen site must support post-metamorphic growth and survival (Marshall and Morgan, 2011). Non-feeding larvae of generalist species may settle preferentially in response to physical and chemical cues of habitat suitability early in their planktonic period, but commonly accept suboptimal habitats if the competent (habitat searching) period is prolonged (Wilson, 1953; Elkin and Marshall, 2007; Toonen and Tyre, 2007; Burgess et al., 2012; reviewed in Pechenik, 1990). For species with more restrictive niches, competent larvae typically discriminate among potential habitat patches and delay settlement in the absence of optimal conditions such as obligate prey species (Pawlik, 1992; Bishop et al., 2006). However, larvae of some specialist taxa can also become less selective over time, highlighting the interplay of risk and reward that shapes the evolution of larval behavior.

Larvae that settle quickly after reaching competence may escape planktonic mortality (Morgan, 1995), locate settlement sites before space becomes preoccupied (Blythe and Pineda, 2009) and maintain energy reserves for post-metamorphic performance (Marshall et al., 2003; Bennett and Marshall, 2005). However, prolonging the larval searching phase can increase the odds of colonizing high-quality habitat with consequent fitness payoffs. Theory predicts that feeding larvae should remain selective for good habitat if juvenile fitness varies greatly among patches, whereas non-feeding larvae that become energetically limited over time should also become less discriminating (Elkin and Marshall, 2007; Toonen and Tyre, 2007). Empirical work in some systems confirms model predictions (e.g. Botello and Krug, 2006), but we have little mechanistic understanding of how life history, adult ecology or developmental constraints influence the evolution of larval settlement behaviors and the attendant regulatory systems.

Two alternative search behaviors predicted by theory are the 'desperate larva' and 'death before dishonor' hypotheses (Toonen and Pawlik, 2001; Bishop et al., 2006). The long-standing 'desperate larva' hypothesis posits that non-feeding larvae should become less choosy as a function of time in the competent state: 'desperate' behavior is widespread among habitat generalists (Knight-Jones, 1951; Knight-Jones, 1953; Wilson, 1953; Pechenik, 1990; Gibson, 1995; Hadfield et al., 2001; Marshall and Keough, 2003). In contrast, the 'death before dishonor' hypothesis posits that in species with highly specialized niches, larvae should swim until encountering suitable habitat or dying. For example, larvae of the coral-specialized nudibranch *Phestilla sibogae* swim until they die unless induced to settle by cues from the adult prey *Porites*

compressa (Miller and Hadfield, 1990; Miller, 1993). Similarly, less than 0.5% of larvae of the sacoglossan *Elysia tuca* metamorphosed in the absence of their host alga *Halimeda* (Krug, 2009). The two hypotheses are not mutually exclusive, and indeed some species exhibit a mixed strategy in which some larvae settle spontaneously and others settle in response to a specific cue (Toonen and Pawlik, 1994; Krug, 2001). Intra-clutch variation in settlement preferences can represent diversified bet hedging, and may be selectively favored when habitat quality varies unpredictably among patches or over time (Crean and Marshall, 2009). Moreover, 'death before dishonor' larvae may become less selective with age, as measured by increased sensitivity to required settlement cues (Botello and Krug, 2006).

If older larvae indeed become generally less selective, accepting poorer-quality habitat ('desperate' larvae) or weaker signals of high-quality habitat ('death before dishonor' larvae), the same endogenous systems may regulate larval response to environmental cues in generalists and specialists. Bishop and colleagues proposed that nitric oxide (NO) and cyclic guanine monophosphate (cGMP) signaling could explain the settlement dynamics observed in diverse taxa (Bishop et al., 2006; Bishop et al., 2008). Production of NO by enzymatic conversion of L-arginine to L-citrulline is intrinsically linked to nitrogen metabolism (Hobbs et al., 1994). NO inhibits life-history transformations in diverse multicellular eukaryotes (Bishop and Brandhorst, 2003). Among invertebrates, NO acts as an endogenous inhibitor of larval metamorphosis in habitat generalists including three ascidians (Bishop et al., 2001; Comes et al., 2007), two gastropods (Froggett and Leise, 1999; Pechenik et al., 2007), a sea urchin (Bishop and Brandhorst, 2001), an annelid (Biggers et al., 2012) and a crustacean (Zhang et al., 2012). Pharmacological suppression of NO signaling is thus sufficient to induce metamorphosis in larvae of diverse generalists, indicating a regulatory role for the NO/cGMP pathway in 'desperate' larvae.

A recent study tested the generality of the NO/cGMP signaling hypothesis in a trophic specialist, the nudibranch *Phestilla sibogae* (Bishop et al., 2008). Contrary to results with generalists, pharmacological inhibition of NO/cGMP signaling did not induce metamorphosis in *P. sibogae*, but rather potentiated larval response to the coral cue. Thus, NO/cGMP signaling modulated the sensitivity of *P. sibogae* larvae to extrinsic cues, but was not sufficient to induce metamorphosis. Here, we refer to this difference in function as modulatory *versus* regulatory. The differential function of NO/cGMP signaling in larvae of *P. sibogae* (modulatory) *versus* generalist taxa (regulatory) suggests this pathway may underlie differences in 'desperate larva' *versus* 'death before dishonor' behavior. However, whether experimentally or *via* comparative methods, no explicit tests have contrasted the function of identified signaling systems among taxa employing alternative larval strategies, or among larval morphs with different settlement requirements.

The sea slug *Alderia willowi* (Krug et al., 2007) (Heterobranchia: Sacoglossa) is a specialist herbivore with bet-hedging larval strategies that provide an opportunity to investigate how signaling pathways control settlement in larvae with differing habitat requirements (Krug, 2007; Krug, 2009). Slugs produce clutches of either planktotrophic larvae, which are long-lived and feeding, or short-lived lecithotrophic larvae, which can metamorphose without feeding (Krug, 1998). Moreover, lecithotrophic clutches hatch two larval types with differing settlement requirements. Non-selective larvae undergo spontaneous metamorphosis within 2 days of hatching, and thus behave like the ageing 'desperate' larvae of generalist taxa, settling in the absence of any extrinsic habitat cues (Krug, 2001). The remaining larvae from a clutch swim for up to 2 weeks in filtered seawater, delaying metamorphosis until either encountering dissolved or adsorbed cues from the adult host alga *Vaucheria longicaulis* Hoppaugh, or dying. These older, selective larvae conform to predictions of the 'death before dishonor' hypothesis, remaining dependent on induction by *Vaucheria* cues as they age. However, selective larvae eventually respond to lower doses of the algal cue if unable to sustain their energy levels by facultative feeding, revealing a connection between larval metabolism and cue sensitivity (Botello and Krug, 2006).

The unusual life history of *A. willowi* provides a chance to explore how the ecology of settlement affects the function of signaling pathways in non-selective *versus* selective offspring of the same organism. Prior work suggests that inhibition of the NO/cGMP pathway should induce metamorphosis in non-selective larvae, but not in older, selective larvae. Here, we test the hypothesis that NO signaling acts as a regulator of metamorphosis in non-selective (early-settling) larvae of *A. willowi*, but as a modulator of habitat choice in older, selective larvae. We also tested the function of serotonin and dopamine to assess whether selective and non-selective larvae differ only in NO/cGMP signaling, or in other multiple pathways known to affect metamorphosis.

MATERIALS AND METHODS

Collection of specimens and larvae

Adult *A. willowi* and patches of the host alga *V. longicaulis* were collected from the high intertidal zone of mudflats in southern California from 2007 to 2008. Individual slugs were held overnight in dishes of 0.45 µm filtered seawater (FSW) for oviposition. Lecithotrophic clutches were identified by egg size (Krug, 1998) and transferred to individual dishes of FSW. Clutches were held at room temperature (22–25°C), and FSW was changed every other day, until hatching (~5 days). In *A. willowi*, lecithotrophic larvae are competent to metamorphose at hatching, but only a subset metamorphose spontaneously in the first 2 days post-hatching in FSW; typically a third of larvae are non-selective, but the percentage is highly variable among clutches (Krug, 2001). The remaining selective larvae are functionally non-feeding lecithotrophs in FSW, capable of completing metamorphosis but unable to supplement maternally endowed yolk reserves when held without food (Botello and Krug, 2006).

Patches of *V. longicaulis* from the Kendall-Frost Northern Wildlife Preserve (San Diego, CA, USA) were grown at 16°C under a 12 h photoperiod and used to prepare a stock solution of the algal carbohydrates that induce metamorphosis (Krug and Manzi, 1999; Krug and Zimmer, 2004). Algal tissue (1.34 g wet mass) was boiled in 50 ml distilled water for 10 min, and the resulting boiled *V. longicaulis* extract (BVE) was filtered through a 100 µm Nitex mesh and centrifuged to clarity. The BVE stock was diluted with FSW and used in dose–response experiments as described by Krug and Zimmer (Krug and Zimmer, 2000a), expressing 'dose' as a percentage of undiluted extract.

Pharmacological manipulation of spontaneous metamorphosis

Chemical modulators of the NO signaling pathway were tested for the ability to inhibit or stimulate spontaneous metamorphosis in non-selective larvae at the time of hatching. Reagents were obtained from Cayman Chemical (Ann Arbor, MI, USA) except as noted; stock solutions were prepared using ultra-pure water [or dimethyl sulfoxide (DMSO), where indicated], aliquoted and stored at −20°C, and thawed immediately before experiments. Stocks were diluted with FSW to achieve the desired final concentrations (see Results); an equivalent volume of water or DMSO was added to negative

control dishes to avoid any artifacts from carrier solvents. Reagents were bath-applied to egg masses to alter levels of NO in newly hatched larvae and test whether NO signaling maintains the larval state, and thus reduces spontaneous metamorphosis by non-selective larvae.

The enzyme nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline, producing NO gas as a byproduct. The NOS substrate L-arginine was added to increase levels of endogenous NO; the inactive isomer D-arginine was tested as a negative control for organic enrichment. Concentrations from 0.5 to 1.5 mmol l^{-1} were tested (n=9–19 clutches), and replicate runs of 1.0 mmol l^{-1} L-arginine were performed separately on two collections of clutches (Los Angeles Harbor, October 2007; Long Beach, November 2007). A competitive inhibitor of NOS, L-NAME (L-nitroarginine-methyl-ester), was also used to decrease NO concentrations. Of the available NOS inhibitors, L-NAME was chosen for two reasons: (1) treatments with the biologically inactive D-enantiomer control for exposure to a small molecule; and (2) L-NAME induced metamorphosis in other gastropods by bath application or injection [e.g. *Phestilla* (Bishop et al., 2008); *Crepidula* (Pechenik et al., 2007); *Ilyanassa* (Froggett and Leise, 1999)], thereby directly facilitating comparisons among experimental results. L- and D-arginine (Sigma-Aldrich, St Louis, MO, USA) and L-NAME were prepared as 100 mmol l^{-1} stocks.

Carboxy-PTIO [2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide] acts as a molecular sponge by reacting with NO, without affecting NOS (Akaike and Maeda, 1996). Carboxy-PTIO was prepared as a 20 mmol l^{-1} stock. Soluble guanylate cyclase catalyzes production of cGMP, a second messenger of the NOS signaling pathway (Ignarro, 1991). To test whether spontaneous metamorphosis was inhibited by cGMP, larvae were treated with the selective soluble guanylyl cyclase (sGC) inhibitor ODQ (IH-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) (Garthwaite et al., 1995; Bishop and Brandhorst, 2007; Zhang et al., 2012). ODQ was prepared as a 20 mmol l^{-1} stock in DMSO. Egg masses were either held in treatment solutions of ODQ continuously, or exposed to a transient pulse for 6 h and then transferred back to FSW, to avoid potential sub-lethal toxicity effects of prolonged exposure.

The neurotransmitters dopamine and serotonin typically have opposing effects on swimming and metamorphosis of molluscan larvae (Leise et al., 2004; Braubach et al., 2006; Yamamoto et al., 1999; Zega et al., 2005). Larvae were exposed to the dopamine precursor L-DOPA and to serotonin by diluting stock solutions to final concentrations of 10^{-5}–10^{-6} mol l^{-1}; assays were performed in the dark to prevent photochemical degradation of L-DOPA.

For a given experiment, encapsulated larvae were regularly inspected starting 5 days after oviposition for larval escape from individual egg capsules, indicating imminent hatching. At that time, each egg mass was cut in half using a sterile scalpel; one half was transferred to a Petri dish (35×10 mm) containing 4 ml FSW (control), while the other half was placed in 4 ml FSW plus one of the reagents (treatment). After 2 days, the percentage of larvae that had completed metamorphosis was scored in all dishes. Viability of juveniles was confirmed by allowing newly settled slugs to feed on *V. longicaulis* filaments; juveniles from all chemical treatments fed and grew normally. To confirm that physical disruption of the egg mass did not affect the tendency of larvae to undergo spontaneous metamorphosis, a pilot study was carried out wherein some egg masses were left intact, while others were split in half and both halves were held together in FSW until hatching. Two days post-hatching, there was no difference in percent metamorphosis between halved and intact egg masses (Student's t-test: d.f.=4, t=0.113, P=0.9155).

Each reagent assay tested the null hypothesis of no difference in percent spontaneous metamorphosis between treatment and control halves of the same clutch; this paired design is unaffected by the high among-clutch variance in the percentage of spontaneous metamorphosis in *A. willowi* (Krug, 2001). The number of replicate egg masses used to test a particular reagent ranged from eight to 20 clutches per treatment per collection of adults; where noted, treatments were tested using replicate collections of adult slugs. Percentages of metamorphosis were normalized with an arcsine square-root transformation prior to statistical analyses. For each reagent, there was an *a priori* expectation that metamorphosis would either increase (L-NAME, carboxy-PTIO, ODQ, L-DOPA) or decrease (L-arginine, serotonin), based on published data for other gastropods. Each treatment was therefore compared with the concurrent FSW control with a one-tailed, paired Student's t-test, given that the direction of change was predicted in advance. Notably, all differences remained significant using a two-tailed test with the sole exception of one of three trials testing 1.0 mmol l^{-1} L-NAME.

Effects of neuromodulatory agents on spontaneous metamorphosis in 2-day-old (selective) larvae

Most lecithotrophic larvae of *A. willowi* that do not metamorphose spontaneously in the first 2 days after hatching will delay settlement until encountering the *V. longicaulis*-derived cue (Krug, 2001). We thus tested a suite of nitrergic reagents on 2-day-old larvae to determine whether NO signaling inhibits spontaneous metamorphosis in habitat-selective larvae. Lecithotrophic egg masses (N=20–30) were obtained as before, but upon hatching all larvae were held in FSW for 2 days to allow spontaneous metamorphosis to occur. To average over genotypic variants present in the maternal broodstock, the remaining veliger larvae were pooled and then sub-sampled for assays. Larvae (N=15 per dish) were transferred to replicate dishes (N=5–10 per treatment) containing solutions of L-arginine, L-NAME, c-PTIO or ODQ. A 2% BVE solution was used as a positive control for induction of metamorphosis by the natural cue, and FSW alone was the negative control. Transformed percentages of metamorphosis were compared for BVE and ODQ *versus* FSW by one-way ANOVA followed by *post hoc* Dunnett's tests (other treatments had no effect and were omitted from statistical analysis; see Results).

To control for the possibility that continuous exposure to ODQ triggered metamorphosis through sub-lethal toxicity, larvae were also transiently pulsed with ODQ prior to incubation in FSW or BVE solutions. A 2×2 design was used to test for an interaction between two main effects: pre-treatment with ODQ, and subsequent exposure to BVE. Larvae from one batch of ~40 egg masses were pooled after hatching and held for 2 days in FSW, and were then sub-sampled into 24 replicate dishes (N=20 larvae per dish). Half the dishes contained 0.05 mmol l^{-1} ODQ, while the remaining dishes contained FSW. After 6 h, larvae from each dish were transferred into a new dish containing FSW only (six out of 12 dishes pre-treated with ODQ, six out of 12 control dishes) or a 2% BVE solution (remaining six pre-treated and six control dishes). After 2 days, the percentage of larvae that had completed metamorphosis was scored for each dish, transformed, and treatments were compared using a two-way ANOVA.

We also tested whether L-DOPA could induce spontaneous metamorphosis in selective larvae. Two separate trials were run

comparing metamorphosis in FSW controls *versus* those in a 0.01 mmol l^{-1} solution of L-DOPA, a concentration effective on newly hatched larvae (see Results). Two days after hatching, pooled larvae were sub-sampled (N=15 per dish) and transferred to replicate dishes (N=10 per treatment); after 2 days, the percentage of metamorphosis was scored. A third trial tested 0.01 mmol l^{-1} serotonin, expected to inhibit metamorphosis, using the same experimental procedure but starting with a separate collection of adults. In all three trials, a one-tailed unpaired t-test compared the percentage of metamorphosis in the reagent treatment against the corresponding FSW control.

Modulation of sensitivity to habitat cues in 2-day-old, selective larvae

We next tested whether NO or dopamine pathways regulated sensitivity to habitat cues in 2-day-old larvae by testing whether reagents affected the slope of dose–response curves to algal extract. Lecithotrophic larvae from ~50 egg masses were held in FSW for 2 days after hatching, and then the batch culture was sub-sampled by transferring larvae to dishes with 4 ml FSW (N=15 larvae per dish). Aliquots of water, BVE and/or reagents were added to achieve the desired final concentration, with five replicate dishes per dose level. Dose–response curves were generated by testing four concentrations of BVE and a FSW-only control. To generate control (cue-only) curves, BVE stock was diluted with FSW to 0.25, 0.5, 1.0 and 2.0%, the range over which larval response to log(dose) is roughly linear (Krug and Zimmer, 2000a). In parallel, a treatment curve was constructed by adding one of four reagents to dishes containing the concentrations of BVE listed above: (1) 1.0 mmol l^{-1} L-arginine, (2) 0.5 mmol l^{-1} L-NAME, (3) 0.001 mmol l^{-1} L-DOPA or (4) 0.01 mmol l^{-1} serotonin. We predicted that the L-arginine and serotonin treatments would inhibit metamorphosis whereas the L-NAME and L-DOPA treatments would increase metamorphosis, based on results for other gastropods including two opisthobranchs (Avila et al., 1996; Couper and Leise, 1996; Bishop et al., 2008). Larvae were incubated at room temperature and metamorphosis was scored after 2 days. Results for serotonin treatments were not analyzed due to unusually high larval mortality.

A general linear model was used to test for effects of reagent treatment on the dose–response to algal cue. Treatment was a fixed categorical variable, and log(dose) a continuously distributed covariate; the response variable, percent metamorphosis, was arcsine square-root transformed prior to analysis. Non-significant terms were sequentially removed from the final model if P>0.25. A significant treatment × dose interaction indicates that the slopes of the best-fit lines differ between BVE-only controls and reagent + BVE treatments (Quinn and Keough, 2002); this is the biological response predicted if a reagent affects sensitivity to habitat cues. If a reagent instead triggered spontaneous metamorphosis across all doses of the BVE cue, then only the main effect of treatment would be significant (denoting a change in the y-intercept but not the slopes of best-fit lines).

Regulation of spontaneous metamorphosis in older larvae by dopamine and serotonin

We next tested whether the age of habitat-selective larvae affected the induction of spontaneous metamorphosis by L-DOPA, and whether spontaneous metamorphosis became more likely as larval age increased. Egg masses from one collection of adult slugs were pooled and hatched together in FSW; larvae were held for 6 days, and water was changed daily. Larvae were sub-sampled from the batch culture at 2, 4 and 6 days after hatching, and placed in 4 ml of 0.01 mmol l^{-1} L-DOPA in FSW (N=10 replicates, 15 larvae per dish). Controls for each age were FSW only, and percent metamorphosis was scored after 2 days. Assays were run in the dark to prevent photo-degradation of L-DOPA. Comparisons between L-DOPA and controls did not require statistical testing (see Results).

We also performed a fully factorial test of the effects of BVE and serotonin as larvae aged. Concentrated BVE was diluted to 1% with FSW, or with a solution of 0.01 mmol l^{-1} serotonin. At 2, 4 and 6 days after hatching, larvae (N=15) were sub-sampled and transferred to replicate dishes (N=10) containing one of four solutions: FSW (no cue, no serotonin), 1% BVE only, 0.01 mmol l^{-1} serotonin only or 1% BVE in 0.01 mmol l^{-1} serotonin. Metamorphic response was scored after 2 days, transformed and analyzed using a general linear model with serotonin treatment and cue as fixed factors and larval age as a covariate.

Constraints on offspring dispersal: spontaneous metamorphosis *versus* cue response

Starved slugs increase the proportion of lecithotrophic offspring that disperse rather than spontaneously metamorphosing, a maternal effect that may adaptively help offspring to escape locally deteriorating conditions (Krug, 2001). However, the same treatments that inhibited spontaneous metamorphosis in non-selective larvae also decreased sensitivity to algal cues in older larvae. Epistasis could affect habitat choice by selective larvae if the same pathway controls (1) initial odds of spontaneous metamorphosis, and (2) cue sensitivity later in larval life. Mothers may therefore experience a functional constraint: if they bias clutches towards higher NO levels, their offspring will be more dispersive yet less responsive to cues of suitable habitat.

To test for such a constraint, we compared the proportion of non-selective larvae with the cue responsiveness of the selective larvae from each of 39 replicate clutches. For each clutch, percent spontaneous metamorphosis was scored 2 days after hatching in FSW. The remaining veligers were then transferred to a dish containing 1% BVE, and after 2 days, percent metamorphosis was scored in response to the algal cue. The constraint hypothesis predicts a positive relationship if the same pathways influence both aspects of larval behavior. A non-parametric Spearman rank correlation procedure was used to test the relationship between paired values of percent spontaneous metamorphosis and percent metamorphosis induced by the cue. Spearman's rank correlation was chosen over Kendall's τ given the uncertainty in ranking close percentages for clutches of different size; the Spearman rank correlation coefficient is more sensitive to paired ranks that are far apart, minimizing errors in ranking percentages close in value (Sokal and Rohlf, 1995).

Maternal size *versus* proportion of spontaneous metamorphosis in clutches

To connect mechanisms controlling larval metamorphosis with ecological factors influencing the expression of maternal effects, we examined the link between maternal condition and spontaneous metamorphosis among clutches. Prior work showed that the percentage of larvae in a clutch that spontaneously metamorphosed decreased for starved slugs, but the effects of initial maternal size on offspring dispersal were not investigated (Krug, 2001). To test for a relationship between maternal size and the proportion of spontaneous metamorphosis, four successive field collections were made from Los Angeles Harbor spanning a 2-month period in summer 2009. Slugs were isolated overnight to allow egg laying, and then were weighed to the nearest 0.1 mg. Clutches were individually maintained in culture dishes until hatching. Two days

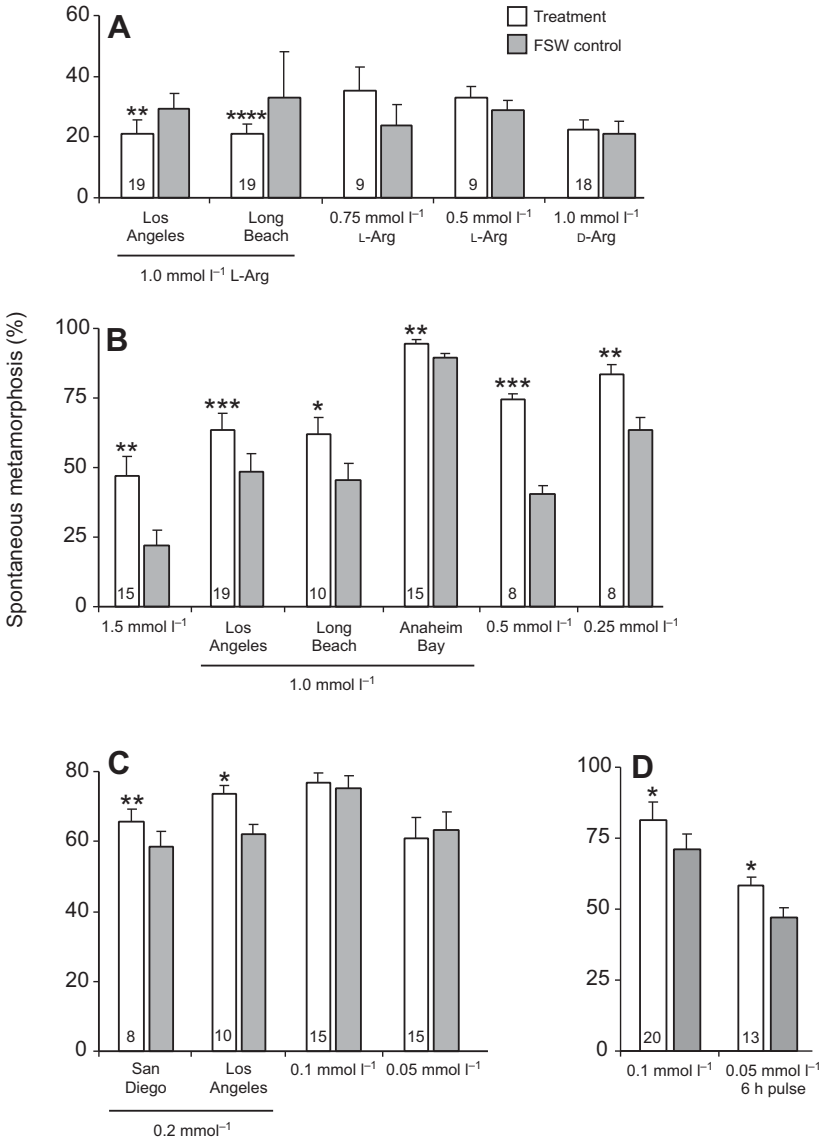

Fig. 1. The effect of pharmacological manipulation of the nitric oxide (NO) signaling pathway on the percentage of metamorphosis among newly hatched larvae of *Alderia willowi*. Data are mean (+1 s.e.m.) percentages of metamorphosis scored 2 days after clutches were split between treatment solutions and filtered seawater (FSW), with the number of egg masses given within the treatment bar. Where indicated, a given concentration of reagent was tested on independent batches of egg masses from separate adult collections (sites joined by a horizontal line). Mean responses of treated *versus* control halves were compared with one-tailed paired *t*-tests. (A) Metamorphic response to nitric oxide synthase (NOS) substrate L-arginine, or the inactive isomer D-arginine, at concentrations ranging from 0.5 to 1.0 mmol l^{-1}. (B) Response to 0.25–1.5 mmol l^{-1} L-NAME, a competitive inhibitor of NOS. (C) Response to 0.05–0.2 mmol l^{-1} carboxy-PTIO, which sequesters free NO. (D) Effect of ODQ, a selective inhibitor of the NO-binding enzyme soluble guanylyl cyclase. One set of egg masses was continuously exposed to 0.1 mmol l^{-1} ODQ; a second set was transiently incubated in 0.05 mmol l^{-1} ODQ for 6 h after hatching, then treatment halves were transferred into FSW. **P*<0.05; ***P*<0.005; ****P*<0.0005; *****P*<0.0001.

later, the percent spontaneous metamorphosis per clutch was scored. Simple linear regression was used to test whether log(maternal wet mass) predicted the percentage of spontaneous metamorphosis (arcsine square-root transformed) per clutch for each collection.

RESULTS

Regulation of spontaneous metamorphosis in hatching larvae

NO/cGMP pathway

The NOS substrate L-arginine was used to increase endogenous levels of NO for larvae near the point of hatching from egg masses, when most spontaneous metamorphosis occurs. Clutches hatching in 1 mmol l^{-1} L-arginine had significantly lower levels of metamorphosis than controls, in trials with clutches from both Los Angeles parents (one-tailed paired Student's *t*-test: d.f.=18, *t*=–3.21, *P*<0.0024) and Long Beach parents (d.f.=18, *t*=–5.18, *P*<0.0001; Fig. 1A). Lower doses of L-arginine did not affect metamorphic response, and the enantiomer D-arginine was inactive (one-tailed paired Student's *t*-test: d.f.=17, *t*=0.56, *P*=0.29). Conversely, addition of L-NAME, a competitive inhibitor of the NOS enzyme, significantly increased spontaneous metamorphosis at concentrations as low as 0.25 mmol l^{-1} (d.f.=7, *t*=4.96, *P*=0.0008; Fig. 1B). Levels of metamorphosis were significantly elevated in 1.0 mmol l^{-1} L-NAME in three trials with different source populations and collections of egg masses: Los Angeles (d.f.=18, *t*=4.08, *P*<0.0005), Long Beach (d.f.=9, *t*=1.88, *P*=0.046) and Anaheim Bay (d.f.=14, *t*=2.94, *P*=0.005; Fig. 1B). The molecular sponge carboxy-PTIO was also used to decrease levels of endogenous NO. Spontaneous metamorphosis increased significantly when larvae were exposed to 0.2 mmol l^{-1} carboxy-PTIO in separate trials with clutches from San Diego (one-tailed paired Student's *t*-test: d.f.=7, *t*=3.44, *P*=0.005) and Los Angeles (d.f.=9, *t*=2.39, *P*=0.02; Fig. 1C). Lower doses were ineffective.

In other systems, NO stimulates the enzyme sGC to produce the second messenger cGMP. Larvae were therefore hatched in the presence of ODQ, a selective inhibitor of the sGC enzyme predicted to increase spontaneous metamorphosis. At 0.1 mmol l^{-1}, ODQ significantly increased spontaneous metamorphosis in newly hatched larvae, relative to 0.1% DMSO in FSW controls (one-tailed Student's *t*-test: d.f.=19, *t*=2.72, *P*=0.007; Fig. 1D). A pulsed exposure to 0.05 mmol l^{-1} ODQ for 6 h also induced significantly more metamorphosis than controls (d.f.=12, *t*=2.71, *P*=0.009).

Dopamine and serotonin

In two trials, 0.01 mmol l^{-1} L-DOPA induced significantly elevated levels of metamorphosis in hatching larvae (one-tailed paired

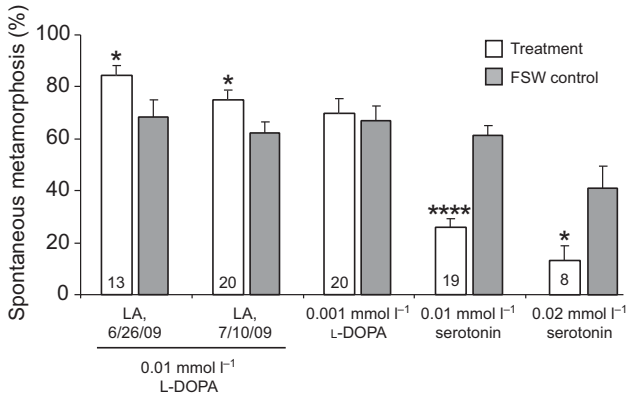

Fig. 2. Opposing effects of neurotransmitters L-DOPA and serotonin on spontaneous metamorphosis in newly hatched larvae of *A. willowi*. Data are mean (+1 s.e.m.) percentages of metamorphosis scored 2 days after clutches were split between treatment solutions and FSW, with the number of egg masses indicated on the treatment bar. Tests of 10^{-5} mol l^{-1} L-DOPA were run on clutches from two separate collections of adults from the Los Angeles harbor site. Mean response of treated *versus* control halves was compared with one-tailed paired *t*-tests; **P*<0.05; *****P*<0.0001.

Student's *t*-test: trial 1, d.f.=12, *t*=–2.75, *P*=0.009; trial 2, d.f.=19, *t*=–2.86, *P*=0.005; Fig. 2). At 0.001 mmol l^{-1}, L-DOPA had no effect on newly hatched larvae. Serotonin strongly inhibited spontaneous metamorphosis in two trials, at 0.01 mmol l^{-1} (d.f.=18, *t*=15.36, *P*<0.0001) and 0.02 mmol l^{-1} (d.f.=7, *t*=2.83, *P*=0.013; Fig. 2).

Induction of spontaneous metamorphosis in older, selective larvae

NO pathway

No metamorphosis occurred among 2-day-old larvae exposed to L-NAME and carboxy-PTIO at concentrations that affected newly hatched larvae (Fig. 3). Similarly, L-arginine did not lower spontaneous metamorphosis below background levels of FSW controls. In contrast to the inactivity of NO-pathway modulators, inhibition of the cGMP pathway by ODQ induced as much metamorphosis as the algal cue (one-way ANOVA: $F_{3,26}$=26.65, *P*<0.00001; *post hoc* Dunnett's test: 0.05 mmol l^{-1} ODQ *versus* FSW, *P*<0.00001; 0.1 mmol l^{-1} ODQ *versus* FSW, *P*=0.00001; 1% BVE *versus* FSW, *P*<0.00001; Fig. 3A).

When larvae were transiently pulsed with ODQ and then incubated in either algal cue or FSW, significant metamorphosis occurred in ODQ-only treatments (Fig. 3B). Levels of induced metamorphosis were equivalent in BVE treatments regardless of whether larvae had been exposed to ODQ. The greater effect of ODQ pre-treatment in the absence of any inductive cue produced a significant interaction term for ODQ and cue treatments in the two-way ANOVA (Table 1).

Dopamine and serotonin

Significant metamorphosis was induced by 0.01 mmol l^{-1} L-DOPA in 2-day-old larvae produced by adults from Los Angeles (one-tailed unpaired *t*-test: d.f.=18, *t*=7.51, *P*<0.0001) and Long Beach (d.f.=18, *t*=8.92, *P*<0.0001; Fig. 3C). Serotonin tended to decrease metamorphosis relative to controls (one-tailed unpaired *t*-test: d.f.=18, *t*=–1.51, *P*=0.075; Fig. 3C), but the low levels of spontaneous metamorphosis in FSW made it difficult to detect a further decrease due to serotonin.

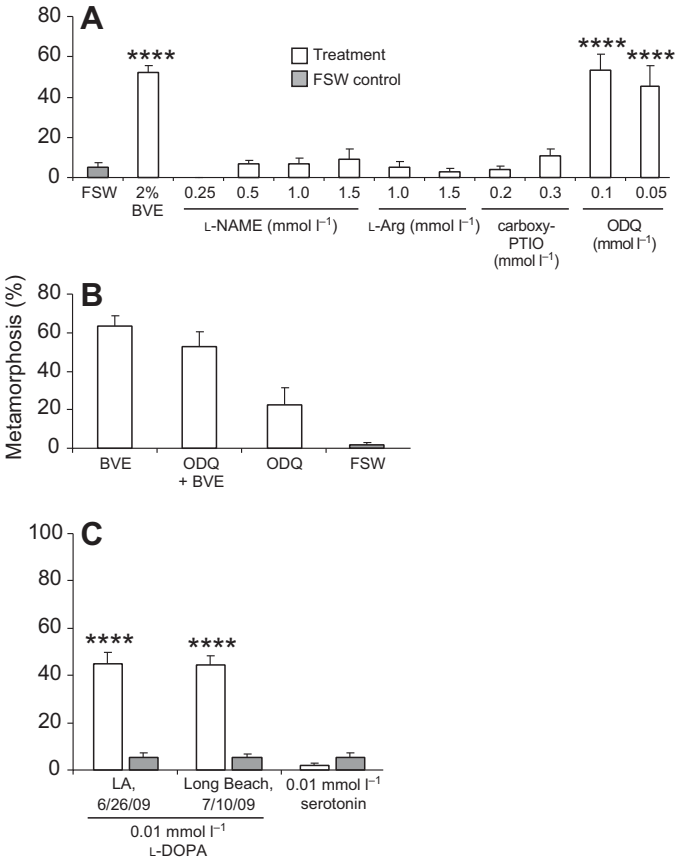

Fig. 3. Effects of pharmacological manipulation on spontaneous metamorphosis in 2-day-old, selective larvae of *A. willowi*. Data are mean (+1 s.e.m.) percentages of metamorphosis for replicate dishes. (A) Effects of manipulating NO signaling in selective larvae; *N*=5 replicates for all reagent treatments, *N*=10 for 2% BVE (solution of the natural settlement cue) and FSW. A range of concentrations of L-arginine, L-NAME and carboxy-PTIO were tested, with separate linear regressions run for each reagent to test for an increasing proportion of metamorphosis with dose. A one-way ANOVA was used to compare results for ODQ and 2% BVE against the FSW control with a one-way Dunnett's *post hoc* test. Larvae from a single collection of egg masses were pooled and then subsampled for use in all treatments and controls. (B) Effects of a 6-h pulsed exposure to 0.05 mmol l^{-1} ODQ, with or without subsequent incubation in 2% BVE, on selective larvae. Pairwise comparisons were not performed given the significant interaction between BVE and ODQ exposure (see Table 1). (C) Effects of neurotransmitters L-DOPA and serotonin on selective larvae. Larvae used in each comparison were from a separate collection of egg masses, and were pooled then subsampled and divided among treatment and control dishes; percent metamorphosis in each treatment was compared against the corresponding FSW control using a one-way unpaired *t*-test. *****P*<0.0001.

Modulation of sensitivity to habitat cues in 2-day-old, selective larvae

NO pathway

Increasing levels of endogenous NO with 1 mmol l^{-1} L-arginine resulted in a significant decrease in metamorphosis for 2-day-old larvae, at all doses of algal cue (Fig. 4A). There was a significant effect of treatment and cue dose, but no interaction, indicating an across-the-board suppression of metamorphosis (Table 2). Conversely, larvae showed an increased response to high doses of the cue in the presence of 0.5 mmol l^{-1} L-NAME (Fig. 4B). The synergistic effect of NO pathway inhibition combined with the two

Table 1. Effects of cGMP-pathway inhibition on spontaneous *versus* induced metamorphosis in older larvae of *Alderia willowi*

Source of variance	d.f.	MS	*F*	*P*
Natural inducer	1	7.575	182.654	<0.00001
ODQ	1	0.092	2.228	0.151
Natural inducer × ODQ	1	0.334	8.053	0.010
Residual	20	0.041		

Two-day-old larvae were incubated in filtered seawater (FSW) or 0.05 mmol l^{-1} ODQ, an inhibitor of soluble guanylyl cyclase; in a 2×2 design, larvae were then transferred from pre-treatments to either FSW or a solution of the algal metamorphic inducer (BVE). Percentages of metamorphosis were arcsine(square-root) transformed and compared by two-way ANOVA. Significant terms are in bold.

highest doses of algal cue drove the highly significant interaction between dose and reagent treatment in the general linear model (Table 2).

Dopamine and serotonin

For 2-day-old larvae, 0.001 mmol l^{-1} L-DOPA induced a significant increase in metamorphic response to higher doses of the algal cue, but not to low doses or FSW only (Fig. 4C). There was a significant interaction between dose and treatment in the general linear model, consistent with the different slopes of the regression lines (Table 2). Effects of serotonin on dose–response could not be determined because of high mortality among larvae rafting on the surface tension, a likely by-product of accelerated swimming by serotonin-stimulated veligers.

Induction of spontaneous metamorphosis in older larvae by dopamine and serotonin

At 0.01 mmol l^{-1}, L-DOPA induced more than half of 2-day-old larvae to metamorphose in the absence of the algal cue, and 87.3% (±3.4% s.e.m.) of 6-day-old larvae (Fig. 5A). This contrasted markedly with the low mean level of spontaneous metamorphosis in older larvae, which gradually increased from 5% in 2-day-old larvae to 15% in 6-day-old larvae.

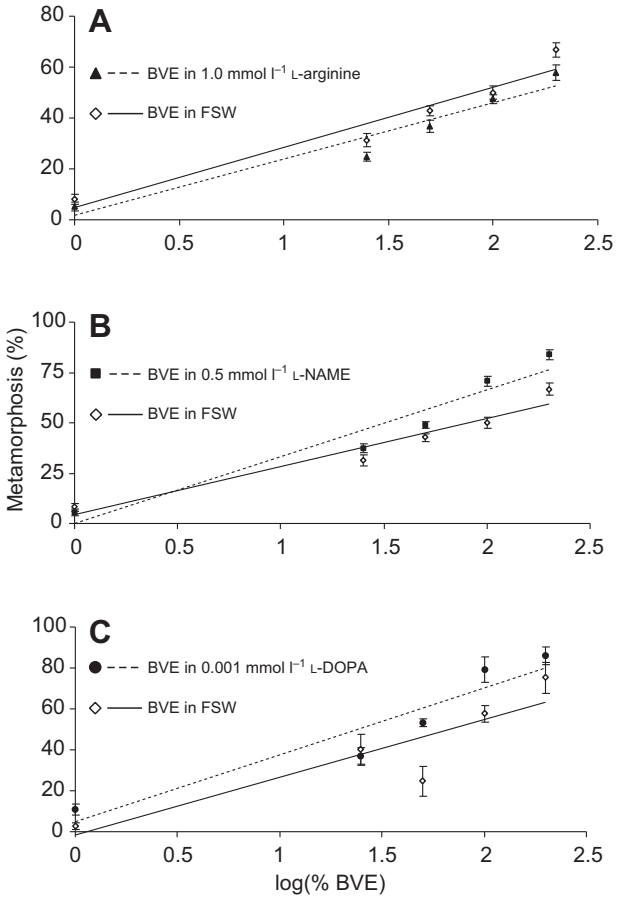

Fig. 4. Pharmacological agents affect dose–response to the natural settlement cue in selective larvae of *A. willowi*. Plotted data are mean (±1 s.e.m.) percentages of metamorphosis after 48 h when 2-day-old larvae were exposed to varying concentrations of BVE, in the presence or absence of (A) 1.0 mmol l^{-1} L-arginine, (B) 0.5 mmol l^{-1} L-NAME and (C) 10^{-6} mol l^{-1} L-DOPA. Best-fit lines were calculated for arcsine(square-root)-transformed percentages and log(% BVE); intercepts and slopes were compared in the presence or absence of each reagent by ANCOVA.

Table 2. Nitric oxide signaling and L-DOPA affect sensitivity to an environmental cue in selective larvae of *A. willowi*

Source of variance	d.f.	MS	*F*	*P*
L-Arginine (1.0 mmol l^{-1})				
Treatment	1	0.098	7.693	0.007
Dose	1	5.451	428.289	<0.00001
Residual	97	0.013		
L-NAME (0.5 mmol l^{-1})				
Treatment	1	0.021	1.396	0.240
Dose	1	8.172	542.204	<0.00001
Treatment × dose	1	0.263	17.437	<0.00001
Residual	96	0.015		
L-DOPA (10^{-6} mol l^{-1})				
Treatment	1	0.054	2.137	0.151
Dose	1	3.623	142.998	<0.00001
Treatment × dose	1	0.154	6.082	0.017
Residual	46	0.025		

Dose–response to the natural cue was compared in FSW *versus* the indicated neuromodulator by two-way ANCOVA, with treatment as a fixed factor and log(dose) as a covariate. Percentages of metamorphosis were arcsine(square-root) transformed for analysis. Significant terms are in bold.

In a separate trial, 0.01 mmol l^{-1} serotonin suppressed spontaneous metamorphosis in older larvae (Fig. 5B). The inhibition was particularly notable among 6-day-old larvae, which had an atypically high percentage of metamorphosis in FSW-only controls, and drove a highly significant serotonin × age interaction in the general linear model (Table 3). The algal cue triggered a 30% increase in metamorphosis over FSW controls at all three ages (Fig. 5B). There was no interaction between serotonin and the algal cue, as evidenced by the parallel slopes of BVE + serotonin and serotonin-only lines, and results of the general linear model (Table 3).

Constraints on offspring dispersal: spontaneous metamorphosis *versus* cue response

There was a significant but negative correlation between the percentage of spontaneous metamorphosis in newly hatched larvae from a given clutch and the metamorphic response to the algal cue of the remaining dispersive larvae (Spearman rank correlation: r_S=–0.363, P=0.025; Fig. 6). Thus, contrary to the constraint hypothesis, clutches with less spontaneous metamorphosis (suggesting upregulation of NO/cGMP signaling) had dispersive larvae that were more responsive to a given dose of habitat cue.

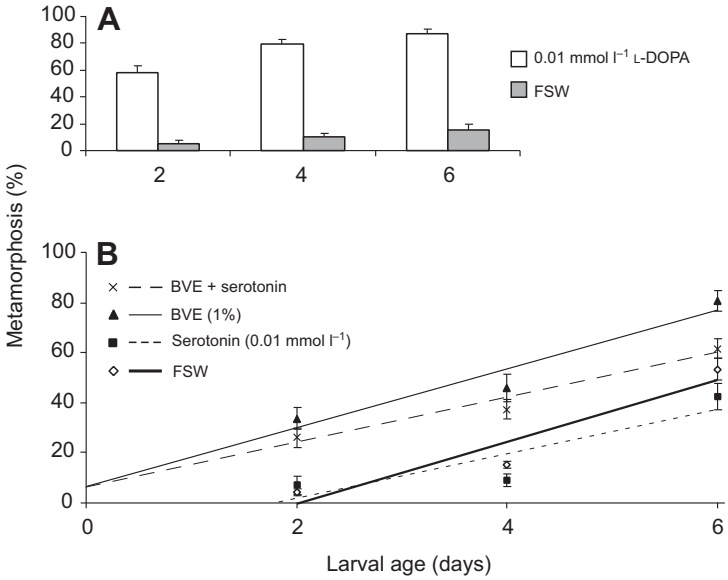

Fig. 5. Effects of L-DOPA and serotonin on metamorphosis as larval age increases. Larvae were 2, 4 or 6 days post-hatching at the beginning of the experiment. (A) Mean percentage of metamorphosis (+1 s.e.m.) after 48 h exposure to L-DOPA or FSW. (B) Mean percentage of metamorphosis (+1 s.e.m.) after 48 h in one of four treatments, in a 2×2 design that tested the effects of the algal cue (1% BVE) and serotonin, alone or in concert.

Maternal size *versus* proportion of spontaneous metamorphosis in clutches

In the first field collection (27 May), there was a borderline significant tendency for larger slugs to lay egg masses with a lower percentage of spontaneous metamorphosis upon hatching (Fig. 7). However, 1 month later (26 June) there was a highly significant positive relationship between maternal size and percent spontaneous metamorphosis. Two weeks thereafter (10 July), there was only a weak and non-significant positive relationship; by 23 July, there was no relationship between slug size and the proportion of non-dispersive offspring produced (Fig. 7).

DISCUSSION

Modeling and empirical tests of settlement theory have generated specific predictions about how larvae should behave to maximize recruitment as a function of larval lifespan, energetic resources and post-metamorphic performance in patches of varying quality (Bishop et al., 2006; Elkin and Marshall, 2007; Toonen and Tyre, 2007; Burgess et al., 2009). Just as theory aims to build a conceptual framework that can be generalized across taxa, so too should mechanistic studies identify endogenous pathways that regulate settlement behaviors in predictable ways among species and across life histories, both to enhance the predictive power of models and to identify targets of selection. Our goal was to bridge the gap between mechanism (signal transduction systems) and theory (models of life-history evolution) for marine larvae. Here, we tested the relationship between theoretical predictions of behavior and the function of identified signaling systems that operate downstream of sensory receptors.

Table 3. Serotonin lowers the response to the metamorphic cue in older larvae of *A. willowi*

Source of variance	d.f.	MS	*F*	*P*
Cue	1	1.225	41.400	<0.00001
Age	1	6.550	221.410	<0.00001
Cue × age	1	0.115	3.899	0.050
Serotonin × age	1	0.568	19.198	0.00002
Residual	127	0.030		

Change in metamorphic response to the natural cue over time, in FSW *versus* 10^{-5} mol l^{-1} serotonin. Percentages of metamorphosis were arcsine(square-root) transformed and compared by a three-way ANCOVA, with cue (+/–) and serotonin (+/–) as fixed factors and age as a covariate. Significant terms are in bold; non-significant terms were removed from the final model if $P>0.25$.

Enzymatic production of NO is directly linked to levels of the NOS substrate L-arginine, the most nitrogen-rich amino acid and a potentially sensitive and reliable indicator of metabolic reserves. Thus, NO may inhibit life-history transitions in disparate taxa by linking nitrogen metabolism with larval sensory systems and their attendant signal transduction pathways (Bishop and Brandhorst, 2003). We hypothesize that this pre-existing link was co-opted during the evolution of 'desperate larva' strategies, to integrate information on endogenous metabolism with cues of habitat suitability received by external sensory receptors. Metabolic depletion reduces NO levels, and thereby triggers metamorphosis even in low-quality patches, an adaptive strategy for taxa that perform well even in sub-optimal habitat. This model predicts that the role of NO/cGMP signaling must change in 'death before dishonor' life histories, allowing the larval stage to persist despite declining energy, and extending the searching phase in the absence of suitable habitat.

Because embryonic development in *A. willowi* produces both non-selective and selective larvae in most lecithotrophic clutches, pathway functions could be compared across larval morphs without confounding phylogenetic effects. Our results showed that only NO signaling functioned differently in selective *versus* non-selective larvae of *A. willowi* (Table 4). Altered levels of cGMP, serotonin and dopamine all influenced metamorphosis by non-selective as well as selective larvae. In contrast, treatments that decreased NO levels triggered metamorphosis in non-selective larvae, but not in older, selective larvae, which remained dependent on the host-derived cue. Manipulation of NO concentrations did affect proportional response to habitat cues by selective larvae, however, indicating that the NO pathway shifted from a regulatory to a modulatory function. These findings support the hypothesis that evolutionary transitions from a 'desperate larva' to a 'death before dishonor' strategy require a partial decoupling of metamorphic induction from NO signaling and its link to nitrogen metabolism.

Prior work suggested lecithotrophic clutches of *A. willowi* contained two larval morphs: non-selective larvae that metamorphose within 2 days, and selective larvae programmed to

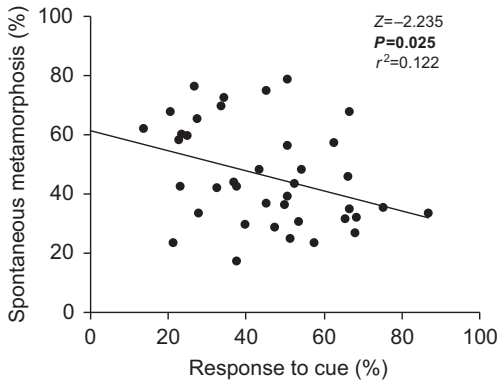

Fig. 6. Correlation between spontaneous metamorphosis over the first 2 days post-hatching, and response of the remaining larvae to a low concentration of the algal settlement cue.

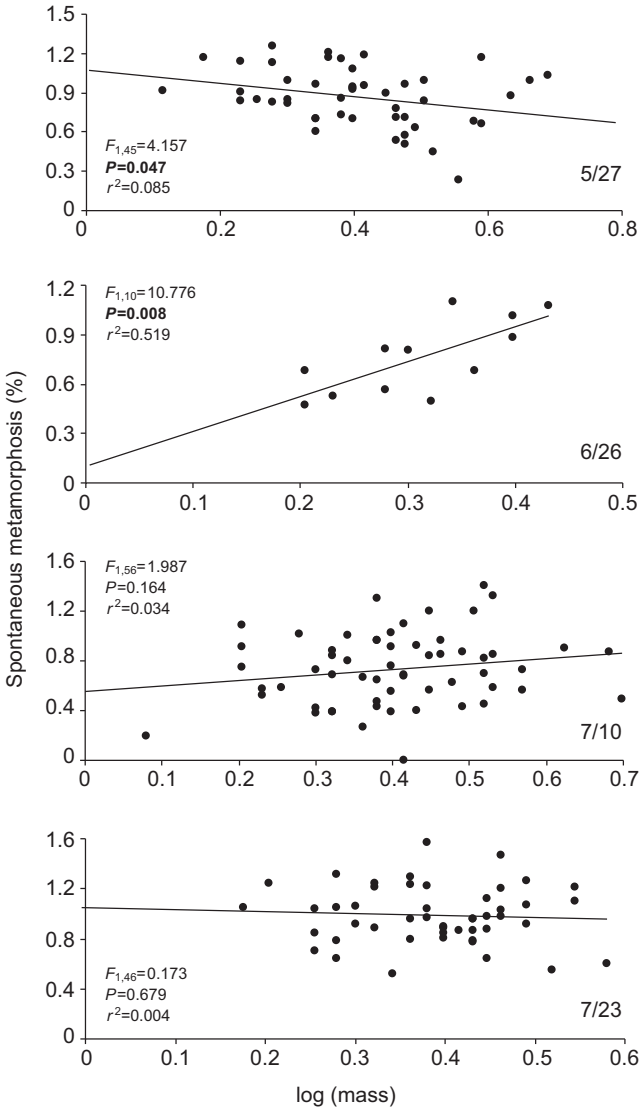

Fig. 7. Relationship between maternal mass (log-transformed) and percent spontaneous metamorphosis in the first deposited egg mass for slugs collected at four time points from Los Angeles, CA, USA. Best-fit lines and results of simple linear regressions are shown.

delay metamorphosis until cued by the adult host. However, up to a third of hatching larvae metamorphosed when NO signaling was suppressed, but developed as selective larvae in FSW controls. Thus, in a substantial fraction of larvae, metamorphosis is initially controlled by the NO pathway ('non-selective' response), yet metamorphosis does not normally occur at endogenous NO levels ('selective' behavior). After 2 days, these larvae cannot be induced to metamorphose by altering NO levels in the absence of the host cue. Manipulation of NO can thus skew the proportion of offspring that settle as non-selective *versus* selective larvae, which would not be predicted if there were functionally distinct morphs at hatching.

The settlement dimorphism in *A. willowi* may therefore be a threshold developmental trait that depends on a continuously varying level of NO among larvae. Individual variation in NO production (and the threshold required to inhibit metamorphosis) likely depends on a combination of larval genotype, developmental noise and epigenetics [including anticipatory maternal effects (Krug, 2001)]. We hypothesize that larvae with higher NO levels (or a lower threshold) pass through the non-selective stage and become 'selective', at which point the NO pathway switches to a modulatory role (Fig. 8). If this model is correct, there is actually only one 'morph' in lecithotrophic clutches of *A. willowi*; individual production of NO determines whether metamorphosis occurs before an ontogenetic shift in NO function, after which metamorphosis depends on chemoreception of an extrinsic signal.

Our data demonstrate that NO functions differently in non-selective *versus* selective larvae, although further work is needed to establish whether NO levels are responsible for maintaining the larval state in the selective stage, when metamorphosis must be induced by exogenous cues. We propose that when larvae cannot feed, metabolic depletion reduces the concentration of the NOS substrate L-arginine; as NO levels in turn decrease, larval response to habitat cues is potentiated. Thus, while older larvae still require some signal of suitable habitat, more dilute cues will suffice, allowing larvae to respond to more distant or lower-quality algal patches (Krug and Zimmer, 2000b; Botello and Krug, 2006). Future studies should explore the mechanistic basis behind the change in NO function, to determine why lower NO levels result in settlement for non-selective larvae but not in older, selective larvae.

Collectively, our results are consistent with a model of interactions among NO/cGMP, serotonin and dopamine signals that makes testable predictions about interactions among pathways regulating metamorphosis (Fig. 8). We propose that 'desperate' (non-selective) behavior results when the NO pathway exerts epistatic control over metamorphosis through its effects on cGMP production (Fig. 8A). The effects of NO signaling can be modulated by other pathways, but metamorphosis is regulated by production of NO. The transition to a 'death before dishonor' strategy in selective larvae (e.g. older larvae of *A. willowi*, all larvae of *P. sibogae*) involves an epistatic shift such that cGMP production determines when metamorphosis is initiated, conditional upon signal transduction of an environmental cue (Fig. 8B). Other signaling pathways including NO may converge to modulate sensitivity to extrinsic cues (e.g. the non-additive interaction between dopamine and the algal cue for *A. willowi*), or act directly on downstream targets of cGMP (e.g. dopamine triggering metamorphosis in selective larvae).

Dopaminergic and serotonergic signaling were manipulated to test whether altered response to NO in selective *versus* non-selective larvae reflected across-the-board differences in the signaling networks that control the onset of metamorphosis. In other gastropods, L-DOPA leads to an increase in dopamine production,

Table 4. Summary of results with non-selective *versus* selective larvae of *A. willowi*

Experimental treatment	Pharmacological effect	Predicted change in % metamorphosis		Observed change in % metamorphosis		Figure(s)
		Hatching larvae	Selective larvae	Hatching larvae	Selective larvae	
Hatching *versus* 2-day-old larvae						
L-Arginine	↑ NO production	Decrease	No difference	Decrease	No difference	1A, 3A
L-NAME	↓ NO production	Increase	No difference	Increase	No difference	1B, 3A
Carboxy-PTIO	↓ NO concentration	Increase	No difference	Increase	No difference	1C, 3A
ODQ	↓ cGMP production	Increase	No increase	Increase	**Increase**	1D, 3B
L -DOPA	↑ Dopamine production	Increase	No increase	Increase	**Increase**	2, 3C
Serotonin	↑ Serotonin	Decrease	No decrease	Decrease	Inconclusive	2, 3C
2-day-old larvae: reagent + cue						
L-Arginine + cue	↑ NO production	N/A	Decrease	N/A	Decrease	4A
L-NAME + cue	↓ NO production	N/A	Increase	N/A	Increase	4B
L-DOPA + cue	↑ Dopamine production	N/A	Increase	N/A	Increase	4C
Serotonin + cue	↑ Serotonin	N/A	Decrease	N/A	Inconclusive	5B
4- to 6-day-old larvae (selective)						
L-DOPA	↑ Dopamine production	N/A	No increase	N/A	**Increase**	5A
Serotonin	↑ Serotonin	N/A	No decrease	N/A	**Decrease**	5B

Bolded results contradict a model proposing that NO, cGMP, DOPA and serotonin would similarly modulate, but not regulate, metamorphosis in older (selective) larvae.

either changing larval response to natural cues (Pires et al., 2000) or the timing of metamorphosis. Serotonin generally has an opposite effect to that of dopamine (Braubach et al., 2006; Yamamoto et al., 1999; Zega et al., 2005). Dopamine and serotonin had the expected opposing effects on metamorphosis in *A. willowi*, but functioned the same way in selective and non-selective larvae. These results did not support the hypothesis that selective larvae would resist pharmacological induction of metamorphosis in the absence of habitat cues, regardless of the pathway being artificially manipulated (Bishop et al., 2008). The NO pathway may thus be uniquely attuned to the habitat requirements of a species, and affect larval behavior accordingly. To verify the generality of our findings, future experiments should test the proposed connection between nitrogen limitation and larval selectivity, explore the epistatic interactions proposed here for established signaling pathways, and perform analogous experiments with other species that express mixed strategies of larval habitat choice.

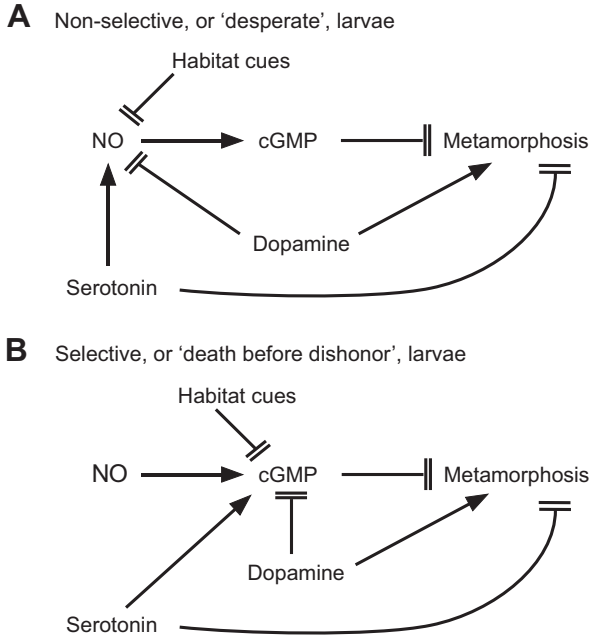

Fig. 8. Model of how identified signaling pathways could interact to regulate or modulate metamorphic response in larvae with different habitat choice behaviors. Lines ending in double hash marks indicate inhibitory or negative effects, while arrows indicate stimulatory effects. (A) Proposed epistatic interactions among signaling pathways in non-selective larvae that exhibit 'desperate larva' behavior, a positive relationship between larval age and likelihood of metamorphosis. (B) Hypothesized epistatic shift underlying the transition to a 'death before dishonor' dispersal strategy, in which larvae evaluate the strength of habitat cues but do not naturally settle without some level of external stimulation. Multiple pathways converge to affect cGMP levels, modulating responsiveness to cues of habitat suitability. The larval state is retained in the absence of habitat cues, but metamorphosis can be triggered by artificial stimulation of the dopamine pathway.

Constraints on maternal control over offspring phenotype

Over successive days of starvation, specimens of *A. willowi* increase the proportion of selective larvae in each clutch (Krug, 2001). This maternal effect is likely adaptive, reducing local recruitment of offspring into a deteriorating maternal environment. Given our results, maternal control over offspring phenotype could be directly mediated by the level of arginine (or indirectly, by the pool of other nitrogenous metabolites) invested in eggs. Nitrogen-rich embryos may develop into larvae with higher levels of NO production, and hence a reduced propensity for spontaneous metamorphosis. However, NO not only inhibited metamorphosis in newly hatched larvae, but also depressed the response to habitat cues of selective larvae. Carryover effects of high endogenous NO production could therefore inhibit dispersing larvae from responding when a marginal food patch is encountered. Mothers may therefore face an intrinsic constraint if clutches of dispersive larvae are thereby rendered highly selective, a maladaptive combination if conditions are deteriorating and good patches are rare.

Under this model, we hypothesized that maternal condition would affect both the proportion of spontaneous metamorphosis in a clutch and the sensitivity of selective larvae to settlement cues. Surprisingly, we instead found a negative correlation: selective larvae from clutches with high levels of spontaneous metamorphosis were less responsive to the algal cue. Although the relationship accounted for little of the variance in response (12%), no constraint was evident. This result suggests an adaptive decoupling of maternal and offspring phenotypes, allowing larvae to respond to diffuse cues of habitat suitability and accept the low-quality patches that are

available during times of environmental stress. There was also no consistent relationship between maternal size and the proportion of non-selective larvae; within 2 months, a field population showed a slight negative relationship, then a strongly positive relationship, and then no relationship. Factors other than maternal size, including recent feeding history or conspecific density, may influence the proportion of dispersive *versus* non-dispersive offspring that a slug produces. Future experiments could test for interactions between maternal condition and pharmacological manipulation, to begin to unravel the mechanistic underpinning of anticipatory maternal effects (Marshall and Uller, 2007; Marshall et al., 2008).

LIST OF ABBREVIATIONS

BVE	boiled *Vaucheria* extract
carboxy-PTIO	2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide
cGMP	cyclic guanosine monophosphate
DMSO	dimethyl sulfoxide
FSW	filtered seawater
L-NAME	L-nitroarginine-methyl-ester
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	IH-[1,2,4]oxadiazole[4,3a]quinoxalin-1-one

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