Interspecific variation in metamorphic competence in marine invertebrates: the significance for comparative investigations into the timing of metamorphosis

Cory D. Bishop,[1,*] Megan J. Huggett,[*] Andreas Heyland,[†,§] Jason Hodin,[¶] and Bruce P. Brandhorst[**]

*Kewalo Marine Laboratories, 41 Ahui St. Honolulu, HI 96813 USA; [†]Friday Harbor Laboratories University of Washington, 620 University Road, Friday Harbor, WA 98250 USA; [§]Whitney Laboratory for Marine Biosciences, University of Florida, 9505 Ocean Shore Blvd, FL 32080 USA; [¶]Hopkins Marine Station, Stanford University, Oceanview Boulevard, Pacific Grove, CA, 93950 USA; and **Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC V5A 1S6, Canada

Synopsis Metamorphosis in marine invertebrate larvae is a dynamic, environmentally dependent process that integrates ontogeny with habitat selection. The capacity of many marine invertebrate larvae to survive and maintain metamorphic competence in the absence of environmental cues has been hypothesized to be an adaptive convergence (Hadfield and others 2001). A survey of the literature reveals that a single generalized hypothesis about metamorphic competence as an adaptive convergence is not sufficient to account for interspecific variation in this character. In an attempt to capture this variation, we discuss the "desperate larva hypothesis" and propose two additional hypotheses called the "variable retention hypothesis" and the "death before dishonor hypothesis." To validate these additional hypotheses we collected data on taxa from the published literature and performed a contingency analysis to detect correlations between spontaneous metamorphosis, habitat specificity and/or larval life-history mode, three characters relevant to environmentally induced settlement and metamorphosis. In order to account for phylogenetic bias in these correlations, we also constructed a phylogeny of these taxa and again performed a character-correlation analysis. Both these tests suggest that juvenile habitat specificity is correlated to the capacity of individuals to retain the competent larval state in the absence of substrate cues and therefore validate the existence of more than one hypothesis about metamorphic competence. We provide new data from the sea urchin *Lytechinus pictus* that suggest that nitric oxide (NO) and thyroxine hormone signaling interact to determine the probability of settlement in response to a settlement cue. Similarly, we provide evidence that thyroxine signaling in the sand dollar *Dendraster excentricus* increases spontaneous metamorphosis in the absence of cues from adult conspecifics in a manner that is independent of larval age.

Introduction

In marine invertebrates, hatching results either in a small version of the adult or an intermediate stage, usually called a larva. In the latter case, both larval and presumptive juvenile tissues continue to grow and develop to varying degrees until a stage referred to as "competence" is reached. Competence is operationally defined as the capacity of a developing individual to initiate settlement and complete morphogenetic transformations associated with metamorphosis. In many cases this requires reception of environmental cues (for example, substrate-derived physical or chemical cues that indicate habitat quality). We use the term metamorphosis to refer to the point at which an environmentally mediated irreversible commitment to transform from the larval to the juvenile stage has occurred.

In order for habitat selection to coincide with metamorphosis, the competent larval state must be retained until suitable substrates are found. The competent stage, and the corresponding retention of the larval state in the absence of cues, therefore represents an example of developmental plasticity. Accordingly, the term "delay of metamorphosis" is used extensively (Pechenik 1990 for review) to reflect this plasticity in the developmental process. Here we use "retention of the larval state" synonymously with

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[1] E-mail: cbishop@hawaii.edu

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"delay of metamorphosis" or any other such terms describing this phenomenon. In our use of the word "retention" we are not referring to the physical retention of larvae close to the hatching site.

A principal point of interest in this work is the existence of broad patterns in the response of competent larvae to the absence of cues, as a function of time. Upon close examination of the many examples in the literature of developmental plasticity that fall under the rubric of delay of metamorphosis, one finds that the dynamics of this phenomenon show considerable diversity. In particular, there is variation among taxa in the degree to which larvae can retain the competent larval state in the absence of cues and food, and correspondingly, the degree to which the specificity of cues required for metamorphosis changes over time. The evolutionary and ecological ramifications of these differences in the retention of the competent larval state have been the subject of much theoretical consideration and speculation (for example, Pechenik 1990, 2006; Pechenik and others 1998). Based upon our own ideas, as well as those in the literature, we have organized these differences into three hypotheses. We explore the idea that, as a character, competence varies in the degree to which it is continuous, and that this variation is ultimately interpretable both in the context of ecological factors (such as juvenile habitats and larval feeding mode) and of underlying signaling systems that control the timing of the metamorphic "decision."

"Desperate larva" hypothesis

Tested recently by Marshall and Keough (2003) and Toonen and Pawlik (2001), but suggested originally by the data of Wilson (1953) and Knight-Jones (1953), the desperate larva hypothesis refers to the idea that, as energetic reserves decline during the competent period, metamorphosis occurs in response to a non-specific cue or even in the absence of any known substrate cue (for example, clean plastic or glass or the air/water interface). Marshall and Keough (2003) indirectly verified this idea by showing that larvae from two bryozoan and one ascidian species having lecithotrophic development settled spontaneously in a size-specific manner: larger larvae had longer swimming periods, indicating greater capacity (presumably because of greater maternal provisioning) for retaining the larval state. Inherent in this hypothesis is the idea that the mechanism that initiates metamorphosis must be epigenetically linked to metabolic processes such that a depletion of energy modulates mechanisms that control the timing of metamorphosis ("unmasking the metamorphic factor") (Chia 1978) instead of simply leading to death. When should energetic limitations lead to a decrease in selectivity or spontaneous metamorphosis?

If one assumes that desperate larval behavior in general is adaptive, it should be selected to arise during the competent period at the point when the benefits of retaining the larval state are offset by any combination of (1) larval mortality, (2) insufficient reserves to complete metamorphosis even if suitable habitats are encountered, (3) decreases in post-metamorphic performance, or (4) diminishing returns for continued dispersal. Additional support for the desperate larva hypothesis will come from (1) a correlation of larval size and energetic content; (2) an understanding of the mechanism by which a decrease in energetic content is transduced into a morphogenetic output; (3) integration with data such as those from Eri and others (1999), who demonstrated that in the ascidian *Herdmania curvata*, competence is correlated with the anterior expression of Hemps, an EGF-like protein; and (4) evidence that ecologically meaningful delays result in changes in larval selectivity.

The "variable retention" hypothesis

It is clear from several laboratory studies (see Table 2) that feeding larvae can also display a tendency toward spontaneous settlement and metamorphosis as a function of time in the competent state. This may involve the gradual increase in an internal stimulatory factor, or the gradual decrease of an inhibitory factor, or both. Again, this general idea is not new, but details of the identity and function of such stimulatory and inhibitory factors that can account for observed behaviors have thus far been slow to accumulate (but see Berking 1988; Welborn and Manahan 1995; and data below). In contrast to the "desperate larva" hypothesis, the "variable retention" tendency is less likely to be linked to food limitation. Nevertheless, it is possible for feeding larvae to become energy-limited either because of increases in metabolic demands from developing juvenile tissues or because of patchy food resources.

Fenaux and Pedrotti (1988) provided documentation of planktonic metamorphosis in four species of echinoids, indicating a low capability of these species to retain the larval state in the absence of cues from juvenile habitats. When data from all species and sampling points were pooled, post-larvae (that is, juveniles) represented an average of 9% of the total sample collected from the plankton over an 8-week period coincident with seasonal algal blooms. Thus, at least in some cases, metamorphosis in the absence of obvious cues appears to be independent of food limitation. Decreases in juvenile performance

correlating with delays in metamorphosis are not restricted to lecithotrophic larvae (Pechenik and others 1998), so finite energy reserves are not the only basis for desperate larval behavior. Although the division between the desperate larva hypothesis and the variable retention hypothesis is not sharp, we argue that the latter is a valid hypothesis if only for the distinction in larval nutrition, and we provide data below that support this contention.

The "death before dishonor" hypothesis

In this conception of developmental plasticity, larvae retain the competent state and do not initiate metamorphosis until a specific substrate cue is encountered. Juveniles from larvae of this type include habitat specialists. Larvae of this type should, under assumptions of optimality, only metamorphose in the presence of cues, even if starved. The aeolid nudibranch *Phestilla sibogae*, a specialist grazer of scleractinian coral in the genus *Porites* (Ritson-Williams and others 2003), very rarely metamorphoses in the protracted absence of its coral cue [reviewed by Hadfield (1998)]. As such, *P. sibogae* exemplifies the "death before dishonor" hypothesis. Miller and Hadfield (1990) found no difference in either life span or fecundity of juveniles of *P. sibogae* reared from larvae that were induced to metamorphose 1 week versus 4 weeks after hatching, although Miller (1993) reported negative effects of extended larval periods on post-larval weights, survival, metamorphic success, and reproductive output. Despite these negative effects, *P. sibogae* larvae do not decrease their specificity requirements or undergo increasing levels of spontaneous metamorphosis as a function of time in culture without a cue. Dramatic decreases in mitosis are associated with competence in *P. sibogae* (McCauley 1997), suggesting that when the larval state needs to be retained, adaptations distinct from those operating in the larvae with "desperate" behavior may be required. Nevertheless, comparative data on rates of metabolism, cell division, and transcription are needed before this finding of apparent adaptations to protracted, pre-metamorphic development can be considered significant in the context of this hypothesis. Other examples of larvae that do not metamorphose after protracted periods in the competent state, and are thus additional "death without dishonor" candidates, can be found in Table 2.

Settlement dimorphisms as special cases

There are two examples in which an actual settlement dimorphism has been observed, involving qualitatively distinct behavioral classes of larvae (Toonen and Pawlik 1994, 2001; Krug 2001; Krug and Zimmer 2004). In both cases, a bet-hedging strategy is inferred. Toonen and Pawlik (1994) described such a strategy for the reef-building polychaete worm *Hydroides dianthus*. As gregarious settlers, most *H. dianthus* larvae will settle upon sensing adult chemicals, but a small fraction of each clutch settles soon after competence is reached, and in response to biofilm, a general benthic cue. Toonen and Pawlik (1994) accounted for this pattern as follows: if new habitats are to be exploited, some fraction of the clutch must be capable of responding to a non-conspecific cue, but one that may nonetheless indicate quality of habitat. Consequently, there are distinct larval types within a clutch, the founder and gregarious types being distinguished by differences in as-yet-unidentified underlying mechanisms controlling behavioral and morphogenetic responses to substrate signals. Although Toonen and Pawlik (2004) rejected the desperate larva hypothesis as an explanation for larval settlement behaviors in *H. dianthus*, we argue that since these larvae can feed after acquiring competence, they may not be subject to the pattern ascribed to lecithotrophs under the desperate larva hypothesis.

The second example of a settlement dimorphism has been observed with the sacoglossan *Alderia modesta* (Krug 2001). In contrast to *H. dianthus*, *A. modesta* does not have gregarious settlement, but each clutch contains a fraction of larvae that will metamorphose in the absence of a cue, whereas the remainder of the clutch retains the larval state until they encounter a host-specific cue. Recently, Botello and Krug (2006) have shown that, if starved, larvae in the clutch that require the host-specific settlement cue display an *increased sensitivity* to that cue as a function age. If fed, their sensitivity to the cue remains unchanged through time. This result supports the central tenet of the desperate larva hypothesis: a connection between energy budgets and sensory physiology that is presumably adaptive. The unfed larvae, however, apparently do not become "desperate" enough to settle in response to alternate settlement cues. This case is especially interesting in light of the three hypotheses outlined above, because it does not fit cleanly into any of them. Nevertheless, in the complete absence of a cue, *A. modesta* larvae do die instead of metamorphosing, suggesting a behavior more akin to "death before dishonor." These fascinating examples of discrete levels of developmental plasticity among individuals of a single brood are bound to generate interesting new insights into how different control mechanisms correspond to differences in larval behaviors without the confounding effects of phylogeny.

A framework for comparing control mechanisms

The three hypotheses presented above make for a useful exercise in understanding the diversity of metamorphic processes across animal phyla. The full significance of these hypotheses will come from an understanding of the underlying mechanisms. For example, do distantly related animals such as bryozoans and corals with "desperate larvae" share similar underlying mechanisms that control metamorphosis? Or, are the larval mechanisms completely distinct, perhaps as a result of independent gains in metamorphic life histories? Currently, the diversity of mechanisms that can transduce both internal and external information into a "developmental decision" or "induction" to undergo settlement and metamorphosis are far from known. There is evidence that some known mechanisms are widely shared whereas others may be relatively taxonomically restricted; in many cases these data have arisen in the absence of a formal comparative study. For example, G-protein-mediated signal transduction of the inductive molecule GABA was inferred for the abalone *Haliotis rufescens* (Baxter and Morse 1987) and the scyphozoan *Cassiopea andromeda* (Wolk and others 1985), while no evidence for G-protein function was found in the polychaete *Hydroides elegans* (Holm and others 1998) or the bryozoan *Bugula stolonifera* (Betrand and Woolacott 2003).

Also interesting is evidence that the same neurotransmitter can function as an inhibitor of metamorphosis in one taxon, while having precisely the opposite function in others. Catecholaminergic signaling provides an instructive example. In the ascidian *Phallusia mammillata*, the barnacle *Balanus amphitrite*, and the bryozoan *Bugula neritina*, dopaminergic signaling was found to inhibit metamorphosis (Yammamoto and others 1999; Shimizu and others 2000; Zega and others 2005). In contrast, increasing endogenous dopamine in the nudibranch *P. sibogae* by incubation in L-DOPA resulted in a substantial sensitization of larvae to their natural inducer (Pires, Croll, and Hadfield 2000). Similarly, depleting endogenous dopamine in the prosobranch gastropod *Crepidula fornicata* attenuated responses to adult-conditioned seawater (Pires and Guilbault 2000). Such opposing functions of the same regulatory molecules may indicate an independent origin of metamorphosis in mollusks and bryozoans (as well as in arthropods and tunicates), and have interesting implications for notions about constraints on metamorphic life cycles (see Hodin 2006).

In addition to direct recordings of larval sensory responses (Satterlie and Cameron 1985; Arkett and others 1989; Leise and Hadfield 2000), there is good indirect evidence that neural control mechanisms are involved in processing sensory cues, with the diversity arising in mechanisms that control primary sensory responses to physical or biochemical cues, as well as their absence. The most widely reported and cited evidence for this comes in the form of cationic inductive mechanisms, such as elevated K^+, Rb^+, and Cs^+ in seawater. Despite the widespread capabilities of K^+ to induce metamorphosis, it is not a universal inducer (Pechenik and Rice 2001, and references therein) and can actually inhibit metamorphosis in an opisthobranch gastropod (Todd and others 1991) and a barnacle (Ritschoff and others 1986).

These considerations illustrate two important points about signaling systems that control the timing of metamorphosis in marine invertebrates: (1) systems functioning in a regulatory capacity will be shared by diverse taxa (having evolved in parallel, in some cases, depending on how many times metamorphosis evolved independently); (2) their particular function in a regulatory context can differ. In order to interpret similarities and differences at various taxonomic levels, explicit comparisons of the function of particular control mechanisms in the context of ecology and phylogeny are necessary.

Nitric-oxide signaling (in two cases via cGMP) has emerged as a shared mechanism that controls the timing of metamorphosis among phylogenetically disparate marine taxa (Froggett and Leise 1998; Bishop and others 2001; Bishop and Brandhorst 2001; Pechenik personal communication). Because a reduction in the output of nitric oxide (NO) signaling is sufficient to induce metamorphosis in the absence of cues, the function of NO in this context is inhibitory to metamorphic induction. Even more striking is the clear evidence that this role for NO is restricted neither to life history transitions of marine animals nor to a particular cellular mode of signal reception and transduction (see Hodin 2006). Thus, there appears to be a widespread inhibitory NO signaling as a component of systems whose function is to integrate external and internal signals into a life-history transformation. Bishop and Brandhorst (2003) attempted to explain this pattern with arguments about the utility of both NO as a second messenger in biological systems and of repressive systems that can respond to qualitatively distinct signals.

It has been shown that in several echinoid genera, application of exogenous thyroxine decreases the time between hatching and competence, whereas inhibition

of thyroxine synthesis increases this period (Chino and others 1994; Johnson 1998; Saito and others 1998; Heyland and Hodin 2004; Heyland and others 2004, 2006). Remarkably, this effect of treatment with L-thyroxine mimics adaptive developmental plasticity observed when food concentrations were manipulated (Strathmann and others 1992), leading to the idea that thyroxine bio-accumulates via ingestion of phytoplankton (Chino and others 1994; Saito and others 1998; Heyland and Hodin 2004; Heyland and Moroz 2005) and that it plays a key role in the allocation of energy between two life-history stages. From these studies we hypothesized that the accumulation of thyroxine in competent echinoderm larvae acts as a stimulatory modulator for the timing of settlement and metamorphosis. Given that Bishop and Brandhorst (2001) have previously shown that NO signaling inhibits this process in the sea urchin *Lytechinus pictus*, we also sought to determine whether, as a stimulatory signal, thyroxine was antagonistic to NO signaling and whether in this capacity thyroxine could account for the tendency of feeding larvae to become "more competent" or less selective as a function of time. We also asked whether manipulation of thyroxine levels in the sand dollar *Dendraster excentricus*, a species that settles gregariously, changed levels of metamorphosis in the absence of a conspecific cue.

L. pictus larvae respond to biofilm cues and are thus considered to have low habitat specificity, whereas *D. excentricus* larvae are gregarious settlers, responding to adult conspecific cues (Burke 1984), and can thus be considered to have higher habitat specificity.

Results

Is habitat specificity related to hypotheses of metamorphic competence?

It is clear that interspecific differences in the dynamics of metamorphic competence exist, even among closely related species. One possibility to account for these differences is that the tendency for larvae to display either desperate larval behavior or death before dishonor behavior is related to juvenile habitat specificity. We hypothesized that when juvenile habitats become specialized, the capacity of competent larvae to retain the larval state in the absence of cues increases. From the published literature we compiled data from 91 taxa with pelago-benthic life histories and scored: (1) whether competent larvae displayed substrate choice when initiating metamorphosis (as a coarse measure of habitat specificity); (2) larval nutritional mode (planktotrophy versus lecithotrophy); and (3) whether competent larvae initiated metamorphosis in the absence of cues (as a measure of the capacity of larvae to retain the larval state). Table 1 outlines our criteria for character-scoring, and Table 2 lists the taxa and their associated characters (see Methods for more details of this analysis). These data were used for a 3-way contingency-table analysis (Wilkinson 2002). This analysis, presented in Table 3, resulted in a single significant interaction: that between spontaneous metamorphosis and cue specificity independent of larval feeding mode ($p < 0.001$).

Table 1 Characters and corresponding criteria used for statistical analysis

	Character	Criterion
1	Specificity of cue required for metamorphosis (low = 0; high=1)	Scored as low if metamorphosis was induced by biofilms of all kinds or if multiple, qualitatively different cues were effective at inducing metamorphosis. Scored as high if induction was tested with multiple, qualitatively different cues and larvae demonstrated selectivity.*
2	Larval feeding mode (lecithotrophy = 0; planktotrophy = 1)	Lecithotrophy was scored for larvae that cannot feed. Planktotrophy was scored for larvae that can.
3	Spontaneous metamorphosis (0 = yes; no = 1)	Scored yes when metamorphosis in controls or culture vessels was above 5%.

See the discussion for rationale on scoring criteria for characters 1 and 3.
*For data from Fenaux and Pedrotti (1988), we are inferring low cue specificity and spontaneous metamorphosis from the fact that juveniles were found in the plankton.

To account for possible phylogenetic bias in this contingency analysis, we re-analyzed the data in Table 1 within a phylogenetic context. Using published literature, and unpublished input from researchers who are specialists for certain taxa (see legend of Fig. 1), we constructed a phylogeny of 69 informative taxa from Table 2 (that is, closely related species with the same character states were reduced to a single representative taxon). Then, using the pairwise comparison test (see Felsenstein 1985, 2003) as implemented in Mesquite 1.1 (Maddison and Maddison 2006), we specifically tested whether there was a significant correlation between spontaneous metamorphosis and cue specificity in each of 11 pairwise, independent cases in which these binary characters had differing states. This test revealed a significant interaction between these two characters ($P = 0.03$; Fig. 1), with no apparent correlation with larval nutritional mode.

Table 2 A list of species from 9 phyla and 4 associated life-history characters

Species	Cue(s)	Specificity	Feeding	Spont. met	Source
Cnidaria					
Acropora digitifera	Crustose coralline algae (CCA)	1	0	1	Morse and others (1996)
Acropora millepora	Biofilm, CCA	0	0	0	Hayward and Negri (1999); Negri and others (2001); Harrington and others (2004)
Acropora nasuta	CCA	1	0	1	Morse and others (1996)
Agaricia humilis	Coralline algae	1	0	1	Morse and others (1988); Morse and Morse (1991); Raimondi and Morse (2000)
Alcyonium siderium	Coralline algae	0	0	1	Kenneth Sebens (1983)
Alveopora japonica	Rock from adult habitat	0	0	1	Harii and others (2002)
Balanophyllia elegans	Biofilmed rock, moving water	0	0	0	Altieri (2003)
Dendronephthya hemprichi	Natural reef substrata	0	0	1	Ben-David-Zaslow and Benayahu (1998)
Heteroxenia fuscenscens	Natural reef substrata	0	0	0	Zaslow and Benayahu (1999)
Litophyton sp.	Natural reef substrata	0	0	0	Ben-David-Zaslow and Benayahu (1998)
Nephthea sp.	Natural reef substrata	0	0	0	Ben-David-Zaslow and Benayahu (1999)
Parerythropodium fulvum	Natural reef substrata	0	0	0	Ben-David-Zaslow and Benayahu (1998)
Porites asteroides	Coral rubble	0	0	1	Gleason and others (2006)
Siderastrea stellata	Coral rubble	0	0	1	Neves and da Silveira (2003)
Xenia umbelata	Natural reef substrata	0	0	0	Ben-David-Zaslow and Benayahu (2000)
Plexaura kuna	Coralline algae, biofilm	0	0	1	Butman and others (1988); Biggers and Laufer (1999)
Annelida					
Ditrupa arietina	Sediment of various sizes	0	1	0	Charles and others (2003)
Hydroides elegans	Biofilm, diatoms	0	1	0	Hadfield and others (1994)
Ophelia bicornis	Loose clean sand	1	0	1	Wilson (1948)
Phragmatopoma californica	Conspecific tube material	1	1	1	Jensen and Morse (1984); Pawlik (1988); Lasker and Kim (1996)
Pomatoceros lamarckii	Biofilm	0	1	1	Chan and Walker (1998); Hamer and others (2001)
Pomatoleios kraussii	Biofilm, adult conspecifics	0	1	1	Khandeparker and others (2005)
Sabellaria alveolata	Conspecific tube sand, non-specific substrata	0	1	0	Pawlik (1988)
Spirorbis borealis	Conspecifics and fucus serratus	0	0	0	Knight-Jones (1953)
Echinodermata					
Acanthaster planci	CCA, bacteria, thyroxine	0	1	1	Johnson and others (1991); Johnson and Sutton (1994); Johnson and Cartright (1996)
Anthocidaris crasspina	Biofilm, diatoms	0	1	0	Rahim and Kitamura (2004)
Arbacia lixula	Presumably biofilm	0	1	0	Fenaux and Pedrotti (1988)
Dendraster excentricus	Adults, sand conditioned with adults	1	1	0	Cameron and Rumril (1982); Burke (1984)
Echinocyamus pusillus	Presumably biofilm	0	1	0	Fenaux and Pedrotti (1988)
Echinometra spp.	Coralline algae, green and brown algae, rocks	0	1	1	Rahmani and Ueharai (2001)

Species	Cue(s)	Specificity	Feeding	Spont. met	Source
Evechinus chloroticus	Coralline algae, biofilm, rubble	0	1	0	Lamare and Barker (2001)
Genocidaris maculata	Presumably biofilm	0	1	0	Fenaux and Pedrotti (1988)
Heliocidaris erythrogramma	Biofilm and various algae	0	0	0	Huggett and others (2006)
Holopneustes purpurascens	Histamine (from algae); several algal species	0	0	1	Williamson and others (2000); Swanson and others (2004); Williamson and others (2004)
Lytechinus pictus	Biofilm	0	1	0	C.B. personal observations
Ophiomastix venosa	No apparent cue	0	1	0	Fourgon and others (2005)
Ophiothrix fragilis	Adult conspecifics	0	1	0	Morgan and Jangoux (2005)
Paracentrotus lividus	Detrital particles	0	1	0	Fenaux and Pedrotti (1988)
Pseudocentrotus depressus	Biofilm, diatoms	0	1	0	Rahim and Kitamura (2004)
Strongylocentrotus droebachiensis	Various algae, biofilms	0	1	0	Pearce and Scheibling (1991)
Tetrapygus niger	Biofilm	0	1	1	Fuentes and Barros (2000)
Sipunculan					
Apionsoma misakianum	Conspecific conditioned S.W. and sediment	0	1	1	Pechenik and Rice (2001)
Mollusca					
Aplysia californica	Various algae	0	1	0	Pawlik (1989); Nadeau and others (1989); Pennings (1990, 1991)
Aplysia juliana	*Ulva* spp.	1	1	1	Switzer-Dunlap and Hadfield (1977)
Chorus giganteus	No cue	0	0	0	Gallardo and Sanchez (2001)
Concholepas concholepas	Biofilms, diatoms, adult shells with barnacles	0	1	0	Rodriguez and others (1993); Manriquez and others (2004)
Conus pennaceus	Biofilm	0	0	0	Perron (1981)
Crassostrea virginica	Peptides from adults	0	1	?	Zimmer-Faust and Tamburri (1994); Newell and others (2000)
Crepidula fornicata	Adult pheremones, biofilm	0	1	0	Pechenick and others (2002)
Dendronotus frondosus	Adult prey (hydroid)	1	1	1	Sisson (2005)
Doridella obscura	*Electra crustulenta*—bryozoan	1	1	1	Perron and Turner (1977)
Elysia viridis	Algae, conspecific adults, biofilm	0	1	1	Towbridge and Todd (2001)
Haliotis asinina	Diatoms, biofilm	0	1	0	Gapasin and Polohan (2005)
Haliotis discus hannai	GABA, algal films	0	0	0	Takami and others (2002)
Haliotis diversicolor	Diatoms, mucus, biofilm	0	0	0	Bryan and Qain (1998)
Haliotis iris	Coralline algae	1	0	0	Roberts and Lapworth (2001)
Haliotis laevigata	Diatoms, coralline algae	0	0	0	Daume and others (1999)
Haliotis rubra	Algae, diatoms	0	0	0	Daume and others (1999, 2000); Huggett and others (2005)
Haliotis rufescens	Various red algae, cyanobacteria	0	0	0	Morse and Morse (1984); Morse and others (1984); Boxshall (2000)
Haminoea callidegenita	Seagrass, sediment, egg mass jelly	0	0	0	Gibson (1995)
Hermissenda crassicornis	Hydroids and anemones	0	1	0	Avila (1998)
Littorina scutulata	Biofilm, adult conspecifics, algae, rocks	0	1	0	Hohenlohe (2002)
Lottia digitalis	Rock, alga, barnacle *Pollicipes polymerus*	0	0	1	Kay (2002)
Mercenaria mercenaria	Sediment, glass beads	0	1	0	Butman and others (1988); Buchelet and others (1992)

Table 2 Continued

Species	Cue(s)	Specificity	Feeding	Spont. met	Source
Mytilus edulis	Filamentous algae	0	1	0	Bayne (1965)
Phestilla sibogae	Porites sp	1	1	1	Hadfield (1977)
Phestilla minor	Corals from the family Poritidae	1	0	0	Ritson-Williams and others (2003)
Polinices pulchellus	Sediment from adult habitat	1	1	1	Kingsley-Smith and others (2005)
Okenia zoobotryon	Bryozoan prey *Zoobotryon verticillatum*	1	1	1	Robinson (2000)
Strombus gigas	Algae, sediment, seagrass and detritus	0	1	1	Davis and Stoner (1994); Boettcher and Target (1996, 1998)
Trochus niloticus	Biofilm; advanced stage of succession	0	0	0	Heslinga (1981)
Bryozoa					
Bugula flabellata	No cue	0	0	0	Maki and others (1989); Dahms and others (2004)
B. turrita	Filmed chicken eggshell membrane	0	0	0	Wendt and Woollacott (1995)
Watersipora subtorquata	Biofilmed rough surface	0	0	0	Marshall and Keough (2003)
Chordata					
Boltenia villosa	No cue added	0	0	0	Bishop and others (2000)
Ciona intestinalis	No cue added	0	0	0	Weizoreck and Todd (1997)
Ciona savigny	No cue added	0	0	0	Kimura and others (2003)
Cnemidocarpa finmarkiensis	No cue added	0	0	0	Bishop and others (2000)
Diplosoma listerianum	Biofilmed rough surface	0	0	0	Marshall and Keough (2003)
Herdmania curvata	No cue added	0	0	0	Degnan and Johnson (1999)
Herdmania curvata	No cue added	0	0	0	Degnan and others (1997)
Phallusia mammilata	No cue added	0	0	0	C. Bishop personal observation
Crustacea*					
Balanus amphitrite	Biofilms	0	0	0	Maki and others (1990, 1994); Faimali and others (2004); Patil and Anil (2005)
Balanus improvisus	No cue	0	0	0	Nylund and Pavia (2003)
Chasmagnathus granulata	Adult conditioned sediment, algae, mussel shell	0	1	0	Gebauer and others (1998, 1999, 2004)
Panopeus herbstii	Adult conspecifics, biofilm, rock, shell	0	1	0	Andrews and others (2001)
Sesarma curacaoense	Water conditioned by adults	0	1	0	Gebauer and others (2002, 2005)
Echiura					
Urechis caupo	Adult conditioned sediment	1	1	1	Suer and Phillips (1983)
Phoronida					
Phoronis 'architecta'	Metamorphose in plankton: general cue inferred	0	1	0	Santanaga personal communication
Phoronis pallida	Thallasinid shrimp	1	1	1	Santanaga (2004, personal communication)
Phoronopsis harmeri	Metamorphose in plankton: general cue inferred	0	1	0	Santanaga personal communication

See Table 1 for a description of the characters and the criteria by which they were scored.
*Barnacles are considered lecithotrophs since they stop feeding and transform to a cyprid prior to settlement and metamorphosis.

Experimental insight into the developmental progression toward decreasing selectivity

NO

Previous studies with *L. pictus* show that the NOS inhibitor L-nitro-arginine methyl ester (L-NAME) treatment is sufficient to induce metamorphosis in the absence of settlement cues, and that this effect can be rescued by application of exogenous NO (Bishop and Brandhorst 2001). In a variation of those experiments, a sub-threshold dose of the NOS inhibitor L-NAME potentiated the response of competent larvae to biofilm, a natural settlement cue. This treatment, while inducing no metamorphosis after 24 h in the absence of cues, caused a significant decrease in the time required for 50% of the larvae to metamorphose in response to biofilm (Fig. 2). See Bishop and Brandhorst (2001) for a more comprehensive set of experiments that test the role of NO in repressing metamorphosis in *L. pictus*.

Elevated K^+ is a widespread method of non-specifically inducing metamorphosis in marine invertebrates (Müller 1973; Müller and Buchal 1973 as cited by Baloun and Morse 1984; Yool and others 1986;

Table 3 Results from a 2 × 2 × 2 contingency analysis to detect associations between spontaneous metamorphosis, cue specificity, and feeding mode

Interaction	Chi	df	P
Spont × cue × feeding	0.6418	1	0.42307
Spont × cue	19.0036	1	<0.001
Feed × cue	0.5434	1	0.461
Spont × feed	1.0381	1	0.3083

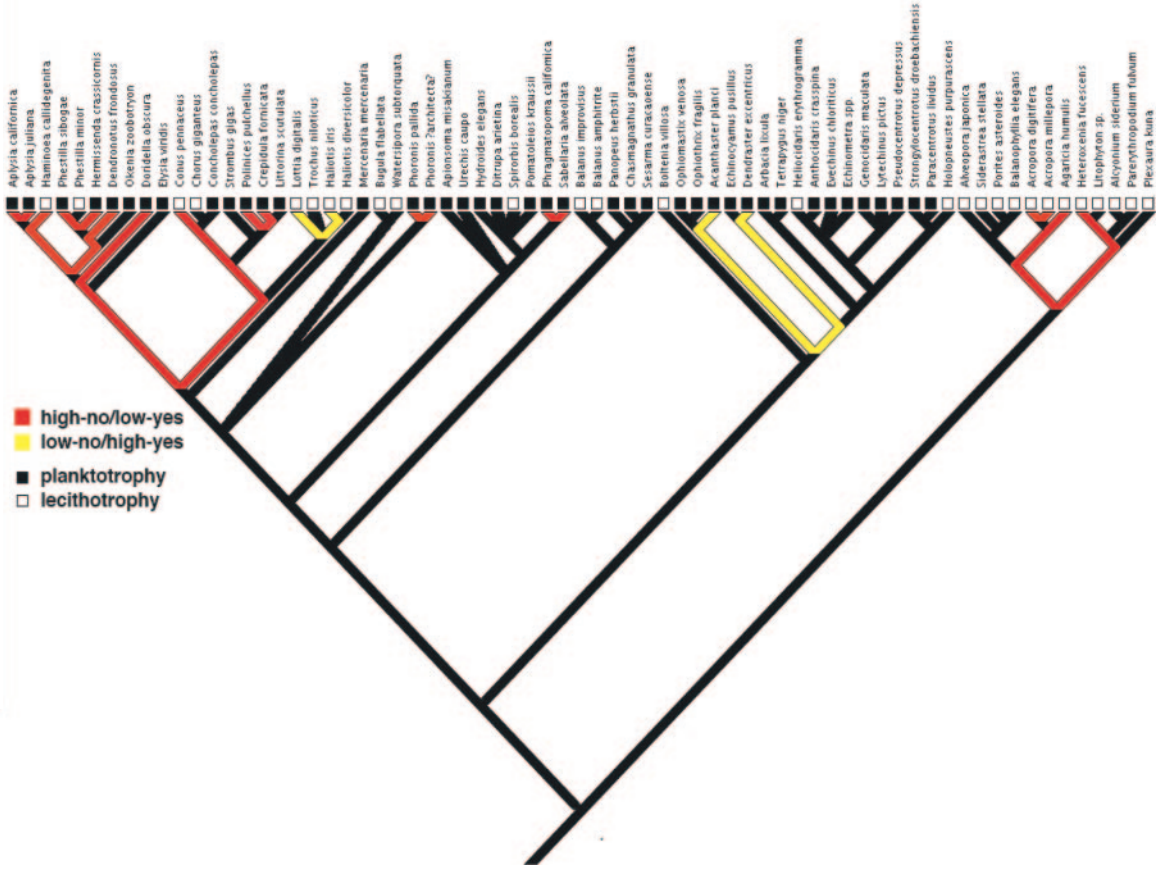


Fig. 1 A hodge-podge phylogenetic hypothesis for 69 informative taxa from Table 1. A pairwise analysis of 11 independent taxonomic pairs which differed in both of their states for characters **1** and **3** (see Table 1) revealed a significant interaction: in 9 of the 11 pairs (shown in red), high cue specificity was associated with no spontaneous metamorphosis, and low specificity was associated with spontaneous metamorphosis; in the other two pairs (shown in yellow), high specificity was associated with spontaneous metamorphosis, and low specificity was associated with no spontaneous metamorphosis. This imbalance of 9-to-2 in favor of the red taxonomic pairs was significant ($P = 0.03$). There are 5 possible sets of 11 contrasting pairs in this phylogeny (only one of the five is shown here), but each gives the same numerical and statistical result. Tree constructed from Littlewood and Smith (1995), Aguinaldo and others (1997), Smith (1997), Wollscheid and Gele (1999), Cameron and others (2000), Harasewych and McArthur (2000), McHugh (2000), Martin and Davis (2001), Giribet and Wheeler (2002), Grande and others (2004), Hayashi (2005), Regier and others (2005), McFadden and others (2006), Schubart and others (2006), and Williams (2006).

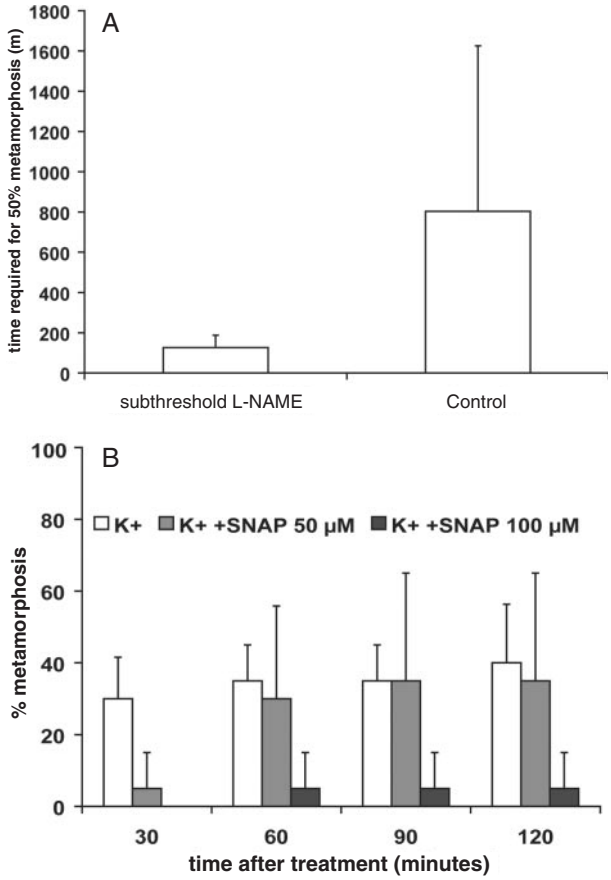


Fig. 2 A sub-threshold inhibition of NOS decreased the response time of competent *Lytechinus pictus* larvae exposed to biofilm. (A) 24 h dose of L-NAME (0.25 mM), insufficient to induce metamorphosis in the absence of cue, resulted in a 5.2 ± 3.4 fold decrease in the time required for 50% of the larvae to respond. (B) Induction of metamorphosis by treatment of *Lytechinus pictus* larvae with K^+ was suppressed by SNAP, an NO generator. An aliquot of 100 µM but not 50 µM SNAP suppressed the response of larvae to K^+, a non-specific inducer of metamorphosis over a period of 4 h. (for all time points $p < 2 \times 10^{-5}$; $n = 4$).

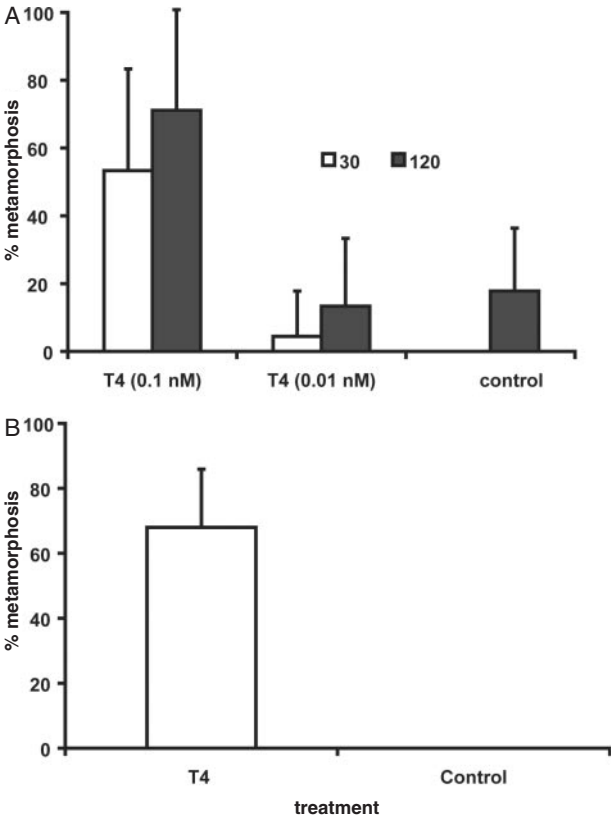


Fig. 3 Thyroxine potentiated the response of competent larvae to biofilm and L-NAME. A 2-day exposure to 10^{-9} M but not 10^{-10} M thyroxine (T4) increased the percentage of metamorphosis of competent *Lytechinus pictus* larvae after a 30 and 120 min exposure to (A) biofilm, a natural settlement cue ($p < 7 \times 10^{-5}$) and (B) an 18 hour sub-threshold dose of L-NAME, a NOS inhibitor ($p < 7 \times 10^{-4}$).

Muller and Leitz 2002). It is assumed that the mechanism of action is to disrupt the physiological homeostasis of nerves (that is, elicit changes in membrane potential, leading to a depolarization of nerves that process settlement cues) such that metamorphosis can be induced in the absence of cues. See Muller and Leitz (2002) for a more thorough explanation of this phenomenon. We tested the possibility that NO could suppress induction of metamorphosis via mechanisms that are subject to activation by elevated K^+ and found this to be the case (Fig. 2), further supporting a function for inhibitory NO signaling downstream from sensory perception.

Thyroxine

Results similar to those of the L-NAME potentiation experiments described above were obtained by incubating competent larvae in L-thyroxine. After a 48 h treatment, no larvae metamorphosed in the absence of biofilm, but a significant dose-dependent increase in metamorphosis upon exposure to biofilm was observed in the thyroxine-treated group (Fig. 3A). Thus, either an *increase* in levels of thyroxine or a *decrease* in NO generated the same metamorphic response to biofilm, supporting the possibility that these two signaling systems are acting antagonistically.

NO-Thyroxine interaction

In a direct test for an interaction between thyroxine and NO signaling, larvae were again incubated in thyroxine for 2 days and then treated with L-NAME at a concentration that was insufficient for inducing metamorphosis in the absence of cues. We sought

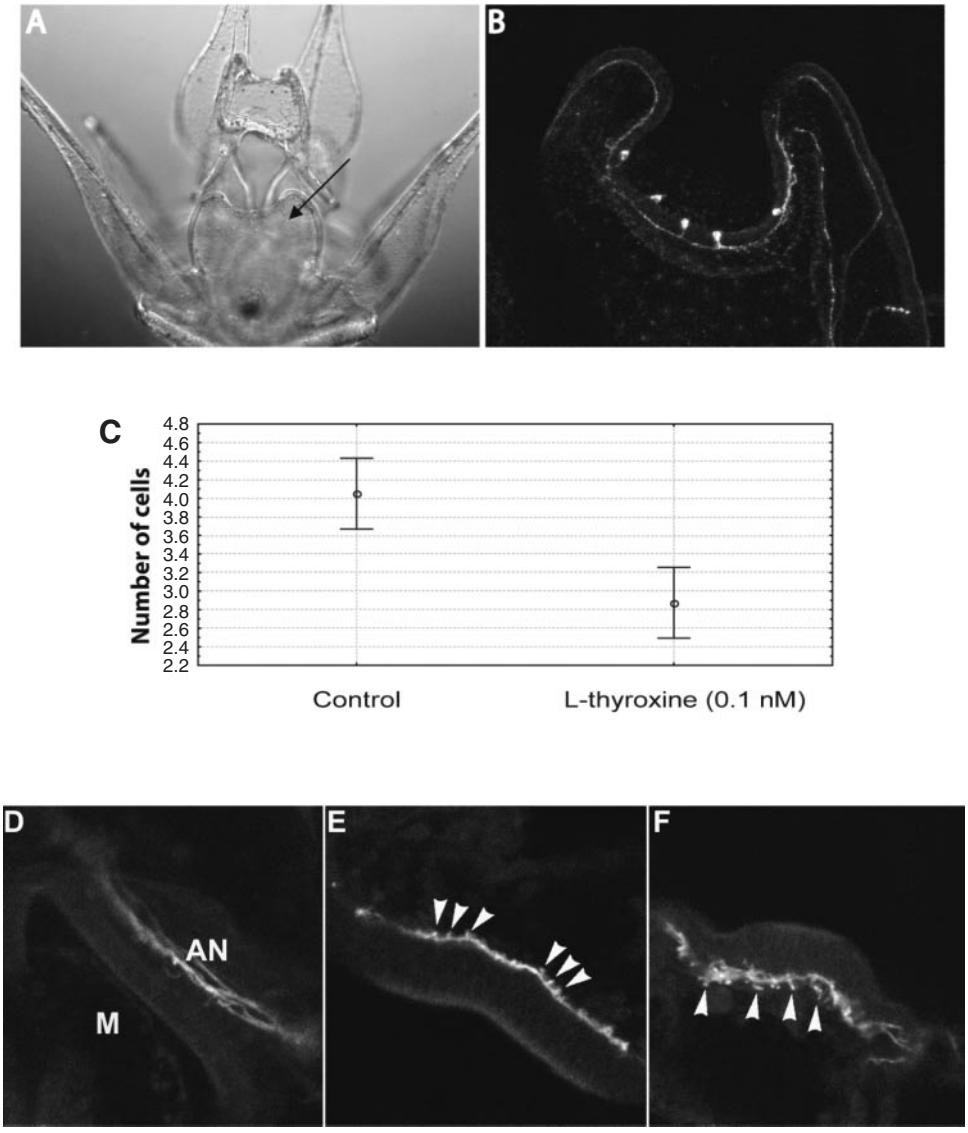


Fig. 4 A 6-day thyroxine treatment retarded development of NOS-defined putative neurons in *Lytechinus pictus* larvae. (A) A crypt forms in the transverse ciliary band of the late feeding larva (arrow). (B) Cell bodies immunostained for NOS develop sequentially in this crypt and thus their number is a proximate measure of the rate of development. (C) A 6-day treatment with 1 nM thyroxine delayed or arrested the formation of these NOS-defined cell bodies ($p = 0.00004$, $F = 1,78$). (D) A whole mount confocal image of NOS-defined putative axons in the apical neuropile. (E and F) These putative axons show abnormal ramifications and other irregularities in the apical neuropile after a 6-day treatment with thyroxine.

a synergistic effect of these two treatments. Indeed, only larvae that had been pre-treated with thyroxine metamorphosed in response to this concentration of L-NAME (Fig. 3B), suggesting that thyroxine and NO act antagonistically.

One explanation of these results is that thyroxine treatment is (either directly or indirectly) modulating the function of cells that produce NO. As part of an ongoing characterization of the spatial distribution of NO synthase (NOS), the enzyme responsible for NO production during larval development, CDB has identified a region of the ventral transverse ciliary band (Fig. 4A) that contains NOS- defined cell bodies of putative neurons (Fig. 4B) that may be chemosensory. Recent experiments indicate that this region of the ciliary band mediates behavioral and morphogenetic responses to substrate-derived biofilm cues (to be published elsewhere).

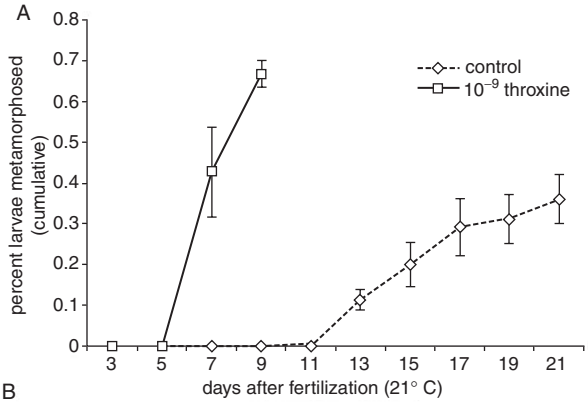
The NOS-defined cell bodies appear in the transverse ciliary band (between the post-oral arms) when the juvenile rudiment begins to develop and increase in number as the juvenile rudiment grows. We compared the accumulation of NOS-defined cells between thyroxine-treated and control larvae.

Significantly fewer NOS-defined cells were observed in the transverse ciliary band as a result of thyroxine treatment, indicating an effect of thyroxine on the development of this larval character known to be involved in settlement behavior (Fig. 4C). These cell bodies extend NOS-defined projections to the apical neuropile of the larva (Fig. 4D). Qualitative effects on the structure of these NOS-defined putative axons in the apical neuropile were also observed as a result of thyroxine treatment (Fig. 4E and F), further suggesting effects of thyroxine on the development, and possibly the function, of cells that produce NOS.

We performed a similar set of thyroxine analyses on the sand dollar *D. excentricus*. Compared to untreated larvae, chronic treatment of larvae with L-thyroxine resulted in both a decrease in elapsed time from hatching to metamorphosis and, over a much shorter time window, induction of significantly higher levels of metamorphosis in the absence of adult conspecific cues (Fig. 5A). Importantly, these differences are independent of the stage of development of the juvenile, as measured by the growth of five adult structures (Fig. 5B). These results show that the developmental stage of larvae in 10^{-4} thiourea (inhibitor), the control, and the two thyroxine treatments on day 28 are not significantly different from the stage of high thyroxine-treated larvae on day 23.

At a given developmental stage then, high thyroxine-treated larvae are more likely to spontaneously metamorphose than are larvae subjected to other treatments. Together, these data suggest that thyroxine promotes progression toward spontaneous metamorphosis independently of developmental stage. It is currently unknown whether inhibitory NO signaling is operating in *D. excentricus*, but preliminary data (CDB) suggests that a reduction in NO is not sufficient to induce metamorphosis in the absence of adult-derived cue.

A



B

age (days)	treatment	mean stage (std error)	percent spontaneous metamorphosis	
23	10^{-2}M thiourea	2.64 (0.39)	0.0 %	
23	10^{-4}M thiourea	5.00 (0.40)	0.0 %	
23	control	3.60 (0.23)	0.0 %	
23	10^{-11}M thyroxine	3.67 (0.44)	0.0 %	
23	10^{-9} M thyroxine	5.67 (0.33)	16.7 %	
				p-value (stage)
28	10^{-2}M thiourea	2.73 (0.29)	0.0 %	<0.001
28	10^{-4}M thiourea	5.67 (0.18)	1.8 %	1.000
28	control	5.40 (0.50)	0.0 %	0.525
28	10^{-11}M thyroxine	5.87 (0.13)	0.0 %	0.632
28	10^{-9} M thyroxine	6.00 (0.00)	69.8 %	0.429

Fig. 5 Thyroxine treatment leads to increases in the likelihood of spontaneous metamorphosis in larvae of the sand dollar *D. excentricus*. (A) More than 2/3 of thyroxine-treated larvae spontaneously metamorphosed over a 4-day window (days 5 through 9). The cohort of control larvae, in contrast, is much less synchronous: fewer than half of these larvae metamorphosed spontaneously over a 10-day period (days 11 through 21). (B) In a second experiment (14°C, larvae from a different mother and father), we first counted numbers of spontaneously metamorphosed larvae, and then staged 5 randomly chosen larvae per replicate (3 replicates/treatment) by the growth of adult skeletal structures. The *P*-values shown in the right-hand column are the result of a simple pairwise contrast of each of the staging values from day 28 *versus* the stage of 10^{-9}M ("high") thyroxine larvae on day 23. From Bishop and others (2006).

Discussion

Hadfield and others (2001) advanced the hypothesis that metamorphic life histories in the marine environment have evolved multiple times from direct-developing ancestors. There is an important corollary to this hypothesis: all characters associated with independently derived metamorphic life-history strategies are adaptations to (1) surviving in the plankton until and beyond the acquisition of metamorphic competence, (2) finding a suitable juvenile habitat, and (3) transforming into a feeding juvenile. A key character that Hadfield and others (2001) focus upon is the retention of the competent larval state in the absence of suitable cues. Interspecific differences in this character invoke three distinct hypotheses of metamorphic competence as a convergent adaptation (see Fig. 6 for illustration).

In order to ask whether these different hypotheses are in fact valid assessments of variation in metamorphic competence, we tested for an interaction between habitat specificity and levels of spontaneous metamorphosis of larvae in laboratory culture and in plankton samples. This analysis accounted for phylogenetic effects, and indicated a possible evolutionary relationship between juvenile habitat specificity and the capacity of competent larvae to retain the larval state in the absence of substrate cues. We suggest from this analysis that there are categorical differences

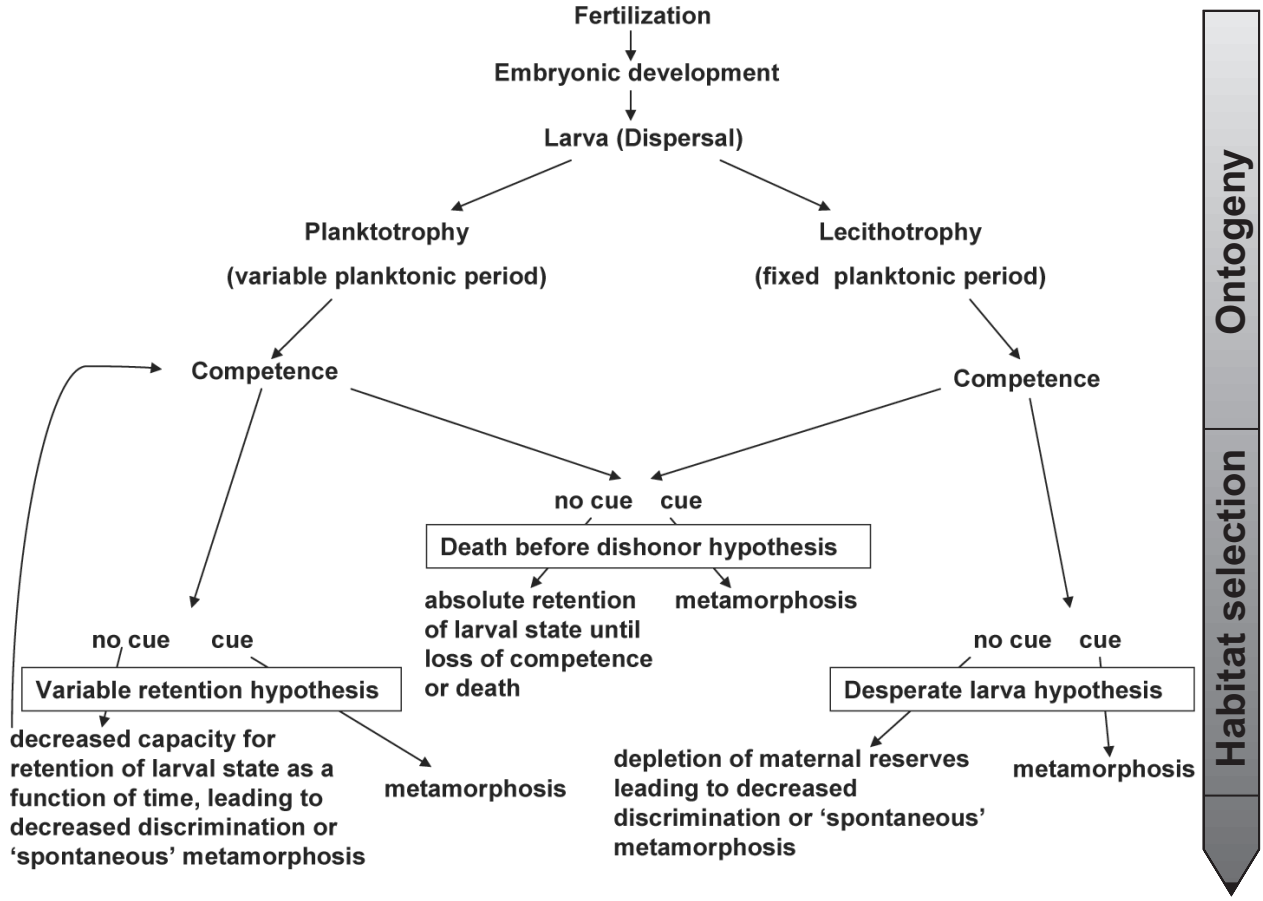


Fig. 6 A schematic representation of the 3 hypotheses described and tested in the text. The two known settlement dimorphisms presented in the Introduction are not included in this scheme.

in how natural selection acts upon metamorphic competence.

One method of verifying the validity of these individual categories of larval behavior is to understand and compare the function of control mechanisms, and to ask whether these mechanisms vary in ways that correspond to the behavioral differences described herein. This functional approach carries with it its share of difficulties and caveats. For example, how confident can one be of conclusions from bath-applied chemicals with varying degrees of specificity (Pawlik 1992)? When such experiments are carefully carried out, conservatively interpreted, and complemented with other approaches (expression studies, biochemical measurements, microsurgical manipulations), this comparative functional approach has ongoing potential to be fruitful.

We recognize that the results from our phylogenetic analyses rest squarely upon our scoring criteria and so, discuss them briefly here. Spontaneous metamorphosis was scored if more than 5% of the culture or experimental batch was observed to undergo metamorphosis in the absence of obvious cues. Although 0% is the most precise value for scoring "no spontaneous metamorphosis," we increased this to 5% to account for low background levels and batch-specific differences in percentages of non-induced metamorphosis. Because not all studies in the literature maintain competent larvae for protracted periods, the codings that we present here are conservative with respect to how often "spontaneous metamorphosis" is observed as a function of time in the competent state. A similar argument can be made for our assessments of habitat specificity: one can never expose larvae to all possible substrates that they would encounter in nature. Moreover, in the cases of characters 1 and 3, we are converting presumably continuous characters into discrete characters. For all these reasons, our analysis must be considered provisional. We hope that it will provide a comparative framework for ongoing investigations.

NO, thyroxine, and the variable retention hypothesis

Our results indicate that both NO and thyroxine can act as modulators in the response of the sea urchin

Lytechinus pictus larvae to substrate cues. An antagonistic interaction between these two signals is evident, and we provide data that suggest a possible cellular mechanism by which this is achieved. From the work of Heyland and others (2006), it may be assumed that many phytoplanktivorous larvae bioaccumulate thyroxine or its metabolic precursors. Because competence does not preclude feeding in the species examined here, thyroxine levels may continue to accumulate after competence is reached. In this way, increasing thyroxine levels may antagonize inhibitory NO signaling (or any other mechanisms that confer the capacity to retain the larval state) such that the latter is insufficient to retain the larval state indefinitely. Such a scenario could account for a "variable-rention"-like scenario in planktotrophic larvae. The observation by Fenaux and Pedrotti (1988) that four species of echinoid post-larvae were found in the upper layer of the water column in the bay of Villefranche sur Mer (France) is also consistent with the variable retention hypothesis. So, despite the fact that the variable retention hypothesis does essentially describe "desperate" larval behavior, it may not be for the same reasons. The above explanation indicates the potential utility of the combined approach of larval ecology and physiological mechanisms that we adopt herein and broadly advocate.

Because phytoplankton is a widespread diet of many marine zooplankton, the antagonistic relationship between thyroxine and NO that we describe here may be a general property of life histories under the variable retention hypothesis. In contrast, however, several examples of lengthy periods of delay in echinoids [reviewed by Strathmann (1978) and Pechenik (1990)] suggest intraspecific genetic variation in the capacity to retain the larval state or that our model may be limited in its relevance to all planktotrophs. Dimorphic settlement patterns (Krug 2001; Toonen and Pawlik 2001), and other polytypic life-history parameters (Gibson 1995; Hadfield and Strathmann 1996) may also factor heavily in ongoing interpretations of variability in larval settlement behaviors.

Methods

Data compilation, tree construction, and character correlation analyses

For a $2 \times 2 \times 2$ contingency table, there are 8 cells, and therefore at least 40 data points are required ($5 \times$ the number of cells) (Keough and Quinn 2002), and our data met this requirement. Another requirement for contingency-table analyses is that the expected frequency of each cell be at least five; our data met this requirement as well. The categories crossed in the three-way analysis were C × F × S, where C = cue specificity, F = feeding mode, and S = spontaneous metamorphosis (see Table 1 for the criteria used to code these characters). Log-linear models with different combinations of terms were fitted to the data using SYSTAT 10.2 (Wilkinson 2002) and the fit of each model was compared. The fit of each model was based on comparing observed and fitted cell frequencies and comparing the fit of each model to that of the saturated model with zero degrees of freedom.

For each species we coded these three characters as binary states 0 or 1 (specificity of habitat required to induce metamorphosis, feeding mode, and whether larvae metamorphosed in the absence of any obvious cue) (Tables 1 and 2). Using Mesqite 1.1 (Maddison and Maddison 2006) we constructed a phylogeny from data in the published literature, as well as from the input of several researchers who specialize in phylogenetics of various invertebrate taxa (see legend to Fig. 1 for references), and then tested for pairwise correlations between spontaneous metamorphosis and cue specificity in each of 11 pairwise, independent cases in which these binary characters had differing states. Congeneric taxa with the same character states were not used in the analysis. We did not have a reliable method for calculating branch lengths and therefore assumed them to be equal, a key caveat for the results presented herein (see Felsenstein 1985, 2003; Pagel 1994).

Larval culture

Lytechinus pictus larvae were cultured as described by Bishop and Brandhorst (2001) with the modification that larvae were raised exclusively on a mono-algal diet of *Dunaliella tertiolecta* (NEPCC strain 001, 5000 cells/ml/day) and were incubated in the dark at 16–24°C without stirring.

Solutions and treatments for *L. pictus* experiments

L-nitro-arginine-methyl ester (L-NAME) is a competitive inhibitor of NOS activity and *S*-Nitroso-*N*-acetylpenicillamine (SNAP) is an NO generator in aqueous solutions. L-NAME and SNAP solutions and biofilmed surfaces were prepared and administered as described by Bishop and Brandhorst (2001). L-thyroxine stocks were prepared at 1×10^{-4} M in 0.01 N NaOH and then stored frozen in aliquots until use. Elevated K^+ seawater was prepared by mixing equal volumes of FSW and 40 mM KCl-FSW. Larvae

from cultures containing competent individuals were incubated in 1×10^{-9} and 1×10^{-10} M L-thyroxine for 48–60 h in each of four (for each concentration) 500 ml plastic beakers with food. Therefore, each experimental replicate consisted of larvae of the same clutch (full sibs) exposed to L-thyroxine in four different beakers and then exposed to experimental solutions or surfaces in different Syracuse dishes or different wells of the same tissue-culture plate. Larvae treated with L-thyroxine were exposed to either biofilm, L-NAME or FSW by first reverse-filtering cultures to small volumes and then pipetting larvae into either glass Syracuse dishes coated with biofilm, or into wells of a 12-well plastic tissue-culture plate.

For assessing the effects of L-thyroxine on the NOS-defined nervous system, 50 larvae with small juvenile rudiments were incubated in each of four beakers (200 larvae total) containing 1×10^{-9} M L-thyroxine for 6 days under a normal feeding regime at a stage when the number of NOS-defined cells in larvae was increasing. At the end of this period, larvae were immunostained for NOS according to the methods described in Bishop and Brandhorst (2001) and examined with a Zeiss LSM410 confocal microscope. The anti-uNOS antibody was from Affinity Bioreagents Inc. (Boulder, CO). The number of immunostained cell bodies in the ventral transverse ciliary band of 10 randomly chosen larvae was counted. Results were subjected to a one-way ANOVA with a Tukey HSD *post-hoc* test to detect differences in mean numbers of cells due to effect of treatment with L-thyroxine.

Solutions and treatments for *D. excentricus* experiments

Adults were collected intertidally in the early summer of 2000 in East Sound, Orcas Island, Washington. We carried out larval cultures and experiments at Friday Harbor Laboratories, Friday Harbor. To obtain gametes, we injected one male and one female (different ones in each of the two experiments) with 0.55 M KCl and set up larval cultures after hatching [as described by Strathmann (1987)]. The cultures were gently stirred using a motor-driven stirring apparatus (Strathmann 1987) or a shaker table, and we changed the water every 2–3 days, at which time we fed the larvae 6000 cells/ml *Dunaliella tertiolecta*. We set up stock larval cultures at a maximal initial larval density of 1 larva/5 ml MFSW, and set up experimental treatments at the stage when the invaginating echinus rudiment contacted the left hydrocoel. The treatments were as follows: Experiment 1- control (MFSW), 10^{-9} M thyroxine (in MFSW); Experiment 2- high inhibitor (10^{-2}M thiourea in MFSW), low inhibitor (10^{-4} M thiourea in MFSW), control (MFSW), low thyroxine (10^{-11}M in MFSW); high thyroxine (10^{-9} M in MFSW). We set up these treatments in wide-mouthed glass flasks, three replicates per treatment. By later stages (due to subsampling of larvae throughout the course of the experiment), larval density was never greater than 1 larva/10 ml MFSW.

We checked larvae for spontaneous settlement at each change of water. We judged a larva as having settled either (1) if it had lost its larval arms and was an obvious juvenile or (2) if it was still a larva in form, but remained firmly adherent to the substrate (clean glass finger bowl) in the face of a strong suction challenge with a Pasteur pipette. Our experience is that all such *D. excentricus* larvae complete settlement in the following hour or two.

In order to compare developmental stages we staged larvae (modified from Heyland and Hodin 2004) as follows: 1 = pentameral symmetry; 2 = primary podia present; 3 = spicules present in rudiment; 4 = skeletal lattice (body plate primordia) present; 5 = tube feet present; 6 = spine primordia and/or spines present.

Temperatures and durations for the two experiments were as follows (first date is spawning; second date is end of experiment): Experiment in panel A, 21–23°C (8 July–12 August 2000; 34 days); Experiment in panel B, 11.5–14.0°C (30 June–12 August 2000; 42 days).

We prepared thyroxine (T-1775 Sigma-Aldrich, St Louis, MO) as described by Chino and others (1994), and thiourea (a thyroxine synthesis inhibitor which acts by blocking iodine peroxidase activity; Sigma-Aldrich) in MFSW (millipore-filtered seawater; 0.45 m) at appropriate concentrations.

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