

Role of Chemical Inducers in Larval Metamorphosis of Queen Conch, *Strombus gigas* Linnaeus: Relationship to Other Marine Invertebrate Systems

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Abstract. Chemical cues are important in the exogenous and endogenous control of metamorphosis in many marine invertebrate larvae. In the queen conch, *Strombus gigas* Linnaeus, larval metamorphosis is induced by low molecular weight compounds associated with dominant species of red algae found in conch nursery grounds; these species include the foliose rhodophyte *Laurencia poitei* (Lamouroux). The responses of conch larvae to the algal-associated cues are dependent on concentration and length of exposure, with the initial events of metamorphosis occurring within 10 min of treatment with an aqueous extract of *L. poitei*. The free amino acids valine and isoleucine mimic the effects of the natural inducer, and they may bind to and be recognized by the same sites on the larvae as the algal cues. Hydrogen peroxide, vanadate, and γ -aminobutyric acid (GABA), as well as elevated K^+ concentrations (*i.e.*, above ambient seawater levels), also induce larval metamorphosis. Acetylsalicylic acid decreases the responses of conch larvae to the algal-associated cues and to the free amino acids, but it has no effect on the induction triggered by hydrogen peroxide. The chemical induction of metamorphosis in conch larvae shares many general features with chemoreception in aquatic invertebrates. The natural inducers of metamorphosis, like the cues involved in olfactory responses in other marine organisms, are of low molecular weight and water soluble. In addition, the results of the experiments with hydrogen peroxide, vanadate, and GABA suggest that second messenger pathways are involved in conch metamorphosis.

Introduction

Queen conch, *Strombus gigas*, are marine benthic gastropods found in seagrass beds and sand flats throughout the tropical Atlantic (Randall, 1964; Brownell and Stevely, 1981). As juveniles, conch occur primarily in seagrass beds of medium shoot density and in surrounding sandy areas where they feed on macrophytes and macrophyte epibionts (Ray and Davis, 1989; Stoner and Sandt, 1991; Stoner and Waite, 1991; Wickland *et al.*, 1991; Sandt and Stoner, 1993; Stoner and Ray, 1993; Ray and Stoner, 1994; Stoner *et al.*, 1994). The mechanisms by which conch larvae find their nursery grounds and metamorphose to juveniles are not well understood, but recent work has shown that a variety of chemical cues associated with nursery-ground substrates induce queen conch metamorphosis (Davis, 1994; Davis and Stoner, 1994; Boettcher and Targett, 1996; Stoner *et al.*, 1996). The most consistent and effective inducers are of low molecular weight (less than 1 kDa), stable, water soluble, and associated with the red algal species *Laurencia poitei* and *Fosliella* sp. (Boettcher and Targett, 1996). Crude aqueous extracts of the rhodophytes and a low molecular weight fraction (less than 1 kDa) of those extracts induce larval metamorphosis at levels comparable to those induced by the intact algae (Boettcher and Targett, 1996). These results suggest that chemical cues used by conch larvae during metamorphosis share many of the characteristics of those used by adult aquatic invertebrates. Marine invertebrates are sensitive to compounds of low molecular size, including the small peptides and amino acids that in adults serve as important cues in feeding, habitat selection, and mating, and in larvae as natural inducers of settlement and metamorphosis (Burke, 1984, 1986; Carr, 1988; Carr *et al.*, 1989; Tegtmeyer and Rittschof, 1989;

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Morse, 1990; Rittschof, 1990; Morse, 1992; Leitz *et al.*, 1994; Zimmer-Faust and Tamburri, 1994).

The effects of chemical cues on metamorphosis are ideally studied with queen conch larvae. Moreover, several research facilities and one commercial facility (Caicos Conch Farm, Turks and Caicos, British West Indies) are currently involved in the culture of queen conch. By providing a better understanding of the biology of this species, detailed studies of metamorphosis in *S. gigas* could lead to improvements in the culture of this valuable mollusc (Heyman *et al.*, 1989; Boettcher *et al.*, 1997). In turn, the commercial culture of queen conch makes large numbers of larvae available for research.

In this study, the responses of *S. gigas* larvae to chemical cues were further characterized: the concentration dependency and the optimal exposure time to aqueous extracts of *L. poitei* were determined; the order of behavioral and gross morphological changes that occur in conch larvae during metamorphosis were described; and the effects of low molecular weight compounds (*e.g.*, amino acids, ions, and neuroactive compounds) on conch larvae metamorphosis were established. The results of these experiments are discussed in terms of models that have been developed to explain the transduction of cues involved in invertebrate larval metamorphosis (Burke, 1983; Baloun and Morse, 1984; Coon *et al.*, 1985; Baxter and Morse, 1987; Bonar *et al.*, 1990; Freeman and Ridgeway, 1990; Beiras and Widdows, 1995; Clare *et al.*, 1995).

Materials and Methods

Collection of algae and preparation of extracts

Specimens of *Laurencia poitei* were collected off Pine Cay, Turks and Caicos, British West Indies, and the crude aqueous extract was prepared as described in Davis (1994) and Boettcher and Targett (1996). Briefly, the alga was chopped into small pieces and ground in seawater (0.6 g alga/ml seawater) with a mortar and pestle. The sample was centrifuged at low speed in a table-top centrifuge for 10 min so that the algal pieces would pellet out of solution. The supernatant was decanted and used in all metamorphosis assays.

For amino acid analysis, the algal extract was prepared in distilled water and fractionated with a 400-ml Amicon (Amicon Division, W.R. Grace, Danvers, MA) stirred cell with 76-mm membranes. Amicon Diaflo ultrafiltration membranes (YM1) were used to separate the extract into nominal molecular sizes of less than and greater than 1 kDa (Boettcher and Targett, 1996). The fraction containing molecules smaller than 1 kDa was hydrolyzed in 6 M hydrochloric acid, phenol, and trifluoroacetic acid and analyzed with a single-column Beckman 6300 auto-analyzer (Tsugita *et al.*, 1987). Hereinafter, we refer to this as the < 1 kDa component of the algal extract.

Metamorphosis assay procedures

All metamorphosis assays were conducted at the Caicos Conch Farm, Providenciales, Turks and Caicos, British West Indies, according to the methods described in Boettcher and Targett (1996). Competent *Strombus gigas* larvae (19–24 days post-hatch) were provided by the Caicos Conch Farm. Techniques for their culture were as described in Davis (1994). Metamorphosis assays were run as static, no-choice experiments in 500-ml polyethylene vessels containing either 300 ml of seawater or seawater to which the appropriate treatment had been added. Seawater used in all experiments was sterilized with ultraviolet light and filtered (10 μ m). Experiments were conducted at the ambient temperature (28°–29°C) and salinity (*ca.* 39 ppt), and under natural light conditions (*ca.* 12 h light: 12 h dark). The pH of the seawater was adjusted to between 8 and 8.5 with NaOH when necessary. For each assay, five replicates per treatment were used with either 15 or 25 larvae per replicate, depending on the experiment. Unless otherwise noted, the exposure time for all treatments was 5 h, after which the larvae were transferred to a fresh volume of seawater. Percent metamorphosis was determined after 24 h, and was calculated as $100 \times (\text{total number of larvae metamorphosed} / \text{total number recovered})$ (Pearce and Scheibling, 1990). A larva was considered to have undergone metamorphosis when it lost its velar lobes and began to use its foot to crawl (Davis, 1994). Each assay included a positive control (an aqueous extract of *L. poitei* [0.01 g wet weight/ml seawater = 20 μ l extract/ml seawater]) as a measure of larval competency and a negative control (seawater only) as a test for spontaneous metamorphosis.

Mean percentages of metamorphosis among or between treatments in each experiment were compared with either a Model 1 ANOVA and Tukey's multiple comparison test or Student's *t* test ($\alpha = 0.05$). Plots of residuals were examined to assure that the underlying assumptions of these tests were met. Treatments in which percent metamorphosis was equal to zero for all replicates were not included in the statistical analyses.

Metamorphosis assays with algal extracts

The effects of concentration and duration of treatment on the metamorphogenic actions of crude aqueous extracts of *L. poitei* were tested according to procedures described above. In the first experiment, larvae were treated for 5 h with 11 dilutions of the *L. poitei* extract, ranging from 0.8 to 67 μ l extract/ml seawater. In the second experiment, the larvae were exposed to 20 μ l extract/ml seawater for 0.5, 1, 2, 3, and 5 h. As in all experiments, percentages of metamorphosis were determined after 24 h.

In an additional experiment, behavioral and morpho-

logical changes occurring in the larvae in response to the algal extract were monitored. Larvae treated with 20 μl extract/ml seawater were monitored with a compound microscope every 20 min, from time (t) = 0 to t = 3 h.

Metamorphosis assays with free amino acids

The effects of 11 free amino acids, all L-isomers (valine, leucine, isoleucine, cysteine, glycine, serine, threonine, lysine, arginine, histidine, and glutamic acid), were tested in assays of larval metamorphosis. Two criteria were used in choosing the amino acids to be tested: amino acids with basic, acidic, and neutral side chains were included, and all, except cysteine, were present in hydrolyzed samples of the less than 1 kDa component of the algal extract. Concentrations of amino acids tested ranged from 1 μM to 10 mM. The free amino acid isoleucine (10 μM) was also presented in combination with serine, histidine, and lysine (each at 10 μM), and in combination with valine (each at 50 μM).

Metamorphosis assays with acetylsalicylic acid

The effects of acetylsalicylic acid (aspirin, 0.1–5 mM), were examined alone and in combination with the algal extract (20 μl extract/ml seawater); treatments also included acetylsalicylic acid (1 mM) in combination with isoleucine and valine (each at 100 μM). The effect of salicylic acid (1 mM) on the induction of metamorphosis by the algal extract was also tested. The responses of the larvae to *N*-acetyl-L-valine and to valine (both at 100 μM) were compared. In an additional experiment, conch larvae were first treated with acetylsalicylic acid (1 mM) in combination with isoleucine (100 μM), valine (100 μM), or the extract, then rinsed in filtered seawater, and finally retreated for 5 h with isoleucine, valine, or extract. As a further examination of the effects of acetylsalicylic acid, the acetylsalicylic acid treatment was combined with exposure to 3-isobutyl-1-methyl xanthine (IBMX). The specific treatments in this experiment were IBMX (0.1 mM) or extract alone; IBMX plus acetylsalicylic acid (1 mM); acetylsalicylic acid plus extract; a combination of IBMX, acetylsalicylic acid, and the algal extract; and acetylsalicylic acid alone.

Metamorphosis assays with ion manipulations and neuroactive compounds

The effects on metamorphosis of elevated ion concentrations and of neuroactive compounds were examined in a series of experiments. The concentrations used were based on tests with other marine invertebrates (Baloun and Morse, 1984; Yool *et al.*, 1986; Davis *et al.*, 1990; Pires and Hadfield, 1991; Ilan *et al.*, 1993; Beiras and

Widdows, 1995). Elevated concentrations of K^+ , Ca^{2+} , Na^+ , and Mg^{2+} (respectively 20 mM, 60 mM, 60 mM, and 60 mM above ambient seawater levels) were tested individually for their ability to induce metamorphosis. Two additional experiments focused on the concentration dependence of the response to increased K^+ concentrations (5–30 mM).

The responses of the larvae to the neuroactive compounds 3,4-dihydroxyphenylalanine (DOPA, 1–100 μM), epinephrine (EP, 1 μM), γ -aminobutyric acid (GABA, 0.1–20 mM), and hydrogen peroxide (50 and 100 μM) were then examined. In addition, we tested the effects of compounds known to block the larval metamorphosis of other marine invertebrates (4 acetamino-4'isothioisilbene-2,2'-disulfonic acid [SITS, 10 and 50 μM] and tetraethylammonium chloride [TEA, 100 and 500 μM]) for their effect on metamorphosis induced by algal extract (20 μl extract/ml seawater), hydrogen peroxide (50 μM), and elevated K^+ concentrations (20 mM). SITS is an inhibitor of anion transport, and TEA is a K^+ channel blocker.

The larval responses to hydrogen peroxide (50 μM) and to sodium orthovanadate (1 and 2 mM) were also compared. The effects of bovine catalase (5 $\mu\text{g/ml}$ seawater) and acetylsalicylic acid (1 mM) on metamorphosis induced by hydrogen peroxide were compared with their effects on metamorphosis induced by the algal extract (20 μl extract/ml seawater).

Results

Metamorphosis assays with algal extracts

The concentration-function relationship of the response of conch larvae to the extract appeared to be hyperbolic (Fig. 1). Concentrations of algal extract lower than 5 $\mu\text{l/ml}$ seawater had no significant effect on conch metamorphosis (Fig. 1). At levels greater than 13 $\mu\text{l/ml}$ seawater, metamorphosis reached a plateau at about 85%. Concentrations of five μl extract/ml seawater and 6.7 $\mu\text{l/ml}$ seawater induced increasing levels of conch metamorphosis (10% \pm 6.0% and 37% \pm 12% respectively). These two responses were significantly different from one another and from all higher ones.

The response to algal extract (20 $\mu\text{l/ml}$ seawater) increased with duration of exposure (Fig. 2). The responses to the longest exposures, 3 h (76% \pm 14%) and 5 h (91% \pm 10%), were not significantly different. Thus, the exposure required to produce the maximal response is between 3 and 5 h.

Within 10 min of exposure to 20 μl extract/ml seawater, the velar cilia were arrested, and the conch larvae sank to the bottom of the experimental containers. Although the edges of the velar lobes started to curl, the lobes remained expanded and the cilia resumed beating

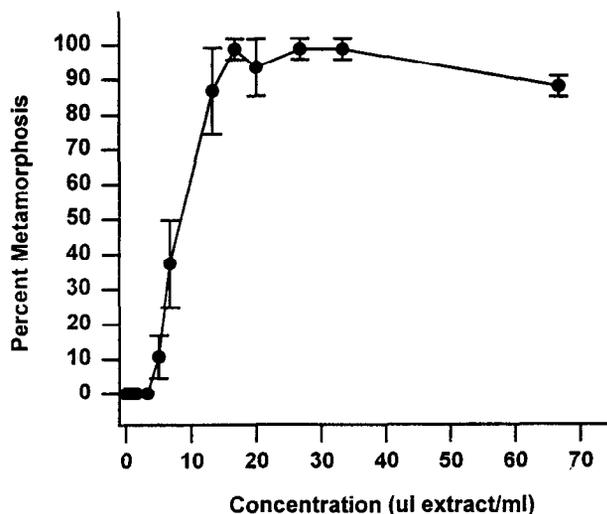


Figure 1. Percent metamorphosis of queen conch larvae in response to specific concentrations of *Laurencia poitei* extract. Points are means \pm SD; $n = 5$.

after the larvae contacted the bottom. After 30 min, all larvae were on the bottom and their lobes showed increased curling; after 90 min, individual cilia and portions of the velar lobes began to drop off. Cilia on the isolated lobes continued to beat for at least 10 min. At 110 min, some of the larvae had completed metamorphosis. At this time, many still retained remnants of their velar lobes, but they had begun using the foot to crawl on the bottom of the containers. By 2 h, more than 50% of the larvae had undergone complete metamorphosis.

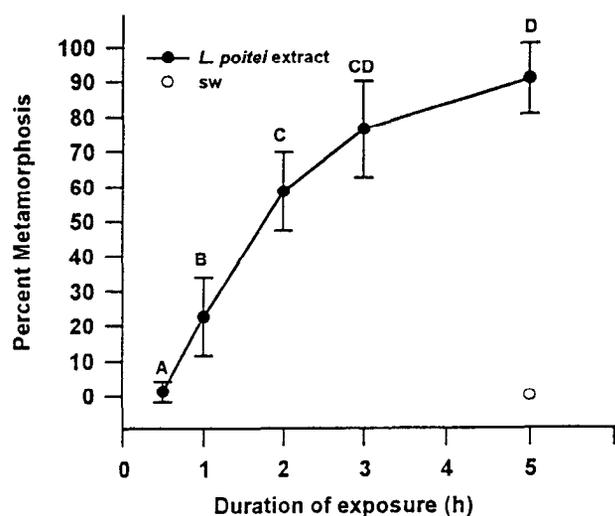


Figure 2. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control) for 5 h and an extract of *Laurencia poitei* (20 μ l extract/ml seawater) for 0.5, 1, 2, 3, and 5 h. Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

Amino acid composition of the algal extract

Of the 17 amino acids detected in the hydrolyzed <1 kDa component of the algal extract, the principal ones were glutamine/glutamate, glycine, alanine, and asparagine/aspartate (Table I).

Metamorphosis assays with amino acids

Of the 11 amino acids tested, only five (serine, histidine, leucine, isoleucine, and valine) induced significant levels of metamorphosis, and only two (isoleucine and valine) induced normal metamorphosis that was accompanied by normal behavior, as described above (Table II). Moreover, valine and isoleucine were present in the hydrolyzed extract at 6.6 and 4.2 μ M respectively (Table I).

At 50 μ M and 100 μ M, the levels of metamorphosis induced by isoleucine and valine were not significantly different from those induced by the algal extract, although at the lower concentration the response to isoleucine was significantly lower than that to valine. Isoleucine at 1 mM and 10 mM, the two highest concentrations tested, was again equipotent with the extract and showed no abnormal effects. At 10 μ M, isoleucine induced a high level of metamorphosis in one experiment (64% \pm 18%), but a low level in a repeat of this experiment (14% \pm 18%); and at the lowest concentration isoleucine had no effect (Table II), so the threshold is probably between 1 and 10 μ M.

Serine, histidine, and leucine also induced significant

Table I

Concentration of amino acids detected in the hydrolyzed less than 1 kDa fraction of an aqueous *Laurencia poitei* extract

Compound	Concentration (μ M)
Glutamine/Glutamate	46
Glycine	33
Alanine	19
Asparagine/Aspartate	18
Serine	14
Threonine	12
Homoserine	8.2
Valine	6.6
4-Hydroxyproline	6.2
Histidine	5.4
Leucine	4.4
Isoleucine	4.2
Hydroxylysine	3.6
Lysine	3.6
Arginine	3.0
Phenylalanine	2.8
Methionine	1.4

Only amino acids detected at concentrations greater than 1 μ M are listed.

Table II

Mean percent metamorphosis of *Strombus gigas* larvae treated with specific concentrations of amino acids or an aqueous *Laurencia poitei* extract (20 μ l/ml seawater)

Concentration (μ M)	Serine	Histidine	Leucine	Isoleucine	Valine	Extract
10,000	85 \pm 9.9A*	dead	na	93 \pm 6.5A	na	87 \pm 4.7A
1,000	69 \pm 14A	dead	na	86 \pm 9.4A	na	65 \pm 25A
100	0	88 \pm 9.9A*,†	na	77 \pm 5.8A	na	76 \pm 9.2A
100	na	na	dead	61 \pm 11A	68 \pm 16A	82 \pm 8.0A
100	na	na	na	64 \pm 8.1A	85 \pm 5.9B	84 \pm 14AB
100	na	na	na	73 \pm 13A	73 \pm 6.7A	87 \pm 9.4A
50	na	na	na	23 \pm 11A	53 \pm 10B	33 \pm 19AB
10	0	4.0 \pm 6.0A	na	14 \pm 18A	na	96 \pm 8.9B
10	na	na	68 \pm 15A*	na	na	84 \pm 8.9B
10	na	na	na	64 \pm 18A	na	92 \pm 5.6A
1	0	0	na	0	na	96 \pm 8.9

Data presented as mean \pm SD, $n = 5$; na indicates that the compound was not applied. Treatment results with the same letter adjacent to the mean are not significantly different at $P \leq 0.05$; each row represents a separate experiment. Unless otherwise indicated the metamorphosis responses were normal.

* Abnormal behavior (slow moving, not using foot to crawl).

† Many dead.

levels of metamorphosis, but the behavior of the larvae in response to these amino acids did not parallel that seen in response to the natural cue (Table II). The larvae tended to be slower moving and did not attach or begin crawling on the bottom as rapidly as with the algal inducer, isoleucine, or valine, if at all. Glutamic acid, arginine, threonine, and cysteine did not induce significant levels of metamorphosis at any of the concentrations tested. At 100 μ M, cysteine was toxic to the larvae. Glycine induced low levels of metamorphosis (20%–30%) at concentrations between 100 μ M and 1 mM. Lysine also induced low levels of metamorphosis at 1 mM (38% \pm 13%), but was toxic at concentrations greater than 1 mM.

The responses of the larvae to isoleucine (10 μ M) in combination with serine (10 μ M, 50% \pm 25%) or histidine (10 μ M, 58% \pm 20%) were not significantly different than the response of the larvae to isoleucine alone (64% \pm 18%). Isoleucine (10 μ M) in combination with lysine (10 μ M) was toxic; all larvae in this treatment appeared to undergo metamorphosis and then die. The response to a combination of isoleucine and valine (each at 50 μ M, 27% \pm 4.2%) was significantly lower than the response to valine alone (55% \pm 7.3%), but not significantly different than the response to isoleucine alone (23% \pm 11%).

Metamorphosis assays with acetylsalicylic acid

The response of the larvae to the algal extract was significantly decreased by 1 mM acetylsalicylic acid (Fig. 3). Acetylsalicylic acid concentrations of 0.1 mM and 0.5 mM had no effect either alone or in combination with the extract, and at 5 mM it was toxic alone or in combina-

tion with the extract. Acetylsalicylic acid at 1 mM also significantly decreased the responses to isoleucine and valine (Fig. 4). Salicylic acid, unlike acetylsalicylic acid, had no effect on the larval response to *L. poitei* extract (Fig. 5). As discussed above, valine induced significant levels of conch larval metamorphosis, but *N*-acetyl-L-valine had no effect on larval metamorphosis (Fig. 6). Treat-

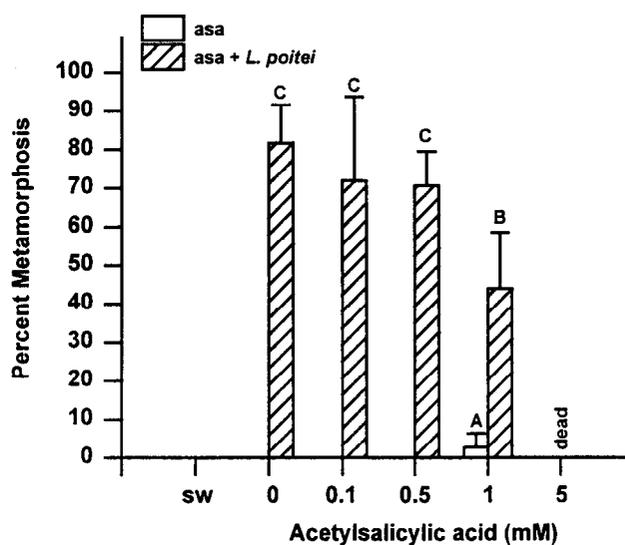


Figure 3. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), acetylsalicylic acid (asa, 0–5 mM) alone, and to asa in combination with an extract of *Laurencia poitei* (20 μ l extract/ml seawater). Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$. Dead indicates all larvae in these treatments were dead at $t = 24$ h.

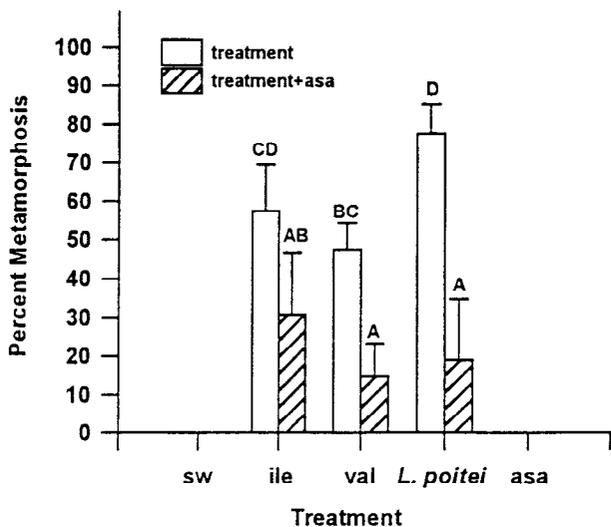


Figure 4. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isoleucine (ile, 100 μ M), valine (val, 100 μ M), and an extract of *Laurencia poitei* (20 μ l extract/ml seawater) alone, and in combination with acetylsalicylic acid (asa, 1 mM). Asa (1 mM) alone is also shown. Points are means \pm SD; n = 5. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

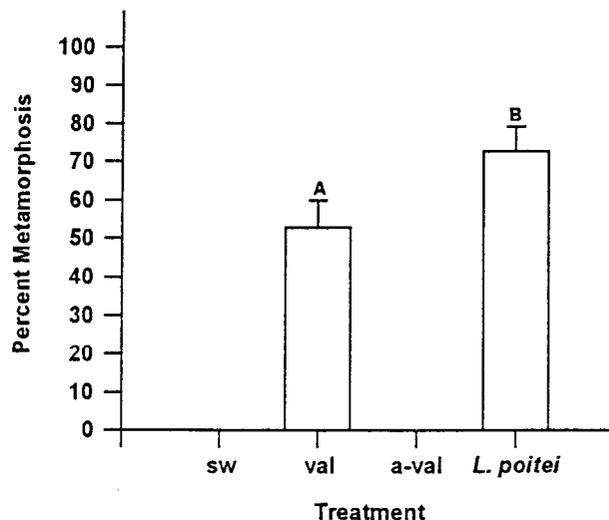


Figure 6. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), valine (val, 100 μ M), *N*-acetyl-L-valine (a-val, 100 μ M), and an extract of *Laurencia poitei* (20 μ l extract/ml seawater). Points are means \pm SD; n = 5. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

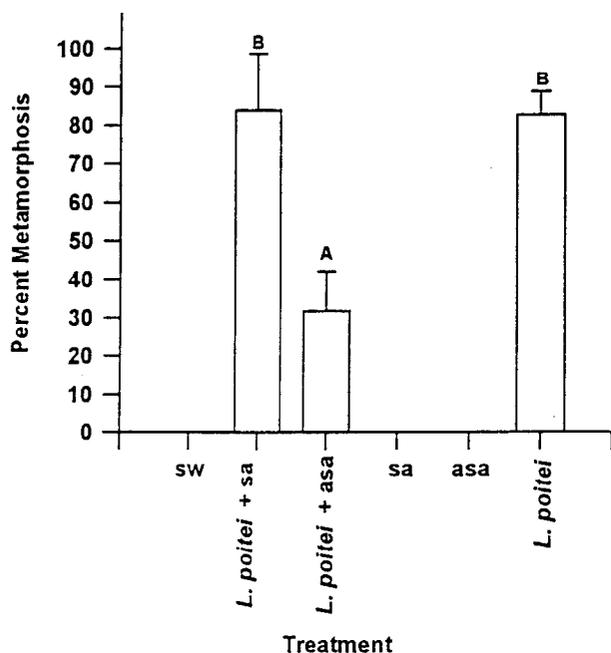


Figure 5. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), an extract of *Laurencia poitei* (20 μ l extract/ml seawater) in combination with salicylic acid (sa, 1 mM) or acetylsalicylic acid (asa, 1 mM). Sa, asa, and *L. poitei* extract presented alone are also shown. Points are means \pm SD; n = 5. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

ment of larvae with isoleucine, valine, or algal extract plus acetylsalicylic acid, followed by reexposure to the appropriate cue in the absence of acetylsalicylic acid, induced levels of metamorphosis equal to or greater than those induced by treatment with the cues only. Moreover, the levels of metamorphosis induced by reexposure were significantly higher than those induced by the same cues in combination with acetylsalicylic acid and without re-treatment (Fig. 7). In this experiment, the response to isoleucine alone was anomalously low.

IBMX had no effect when presented by itself or in combination with acetylsalicylic acid, but it reduced the effects of acetylsalicylic acid on the metamorphosis induced by the algal extract (Fig. 8). The response to the algal extract plus acetylsalicylic acid (26% \pm 12%) was significantly lower than the response to extract alone (88% \pm 9.1%). In the presence of IBMX, however, the response to extract plus acetylsalicylic acid (72% \pm 7.4%) was not significantly lower than that to the extract alone. Fewer larvae were attached and crawling in the IBMX combination treatment than in the treatment with only algal extract.

Metamorphosis assays with ions and neuroactive compounds

Elevations of Ca^{2+} , Na^+ , and Mg^{2+} concentrations (60 mM) over ambient seawater levels had no significant effect on metamorphosis. An increase in the concentration of K^+ to 20 mM over ambient, however, induced signifi-

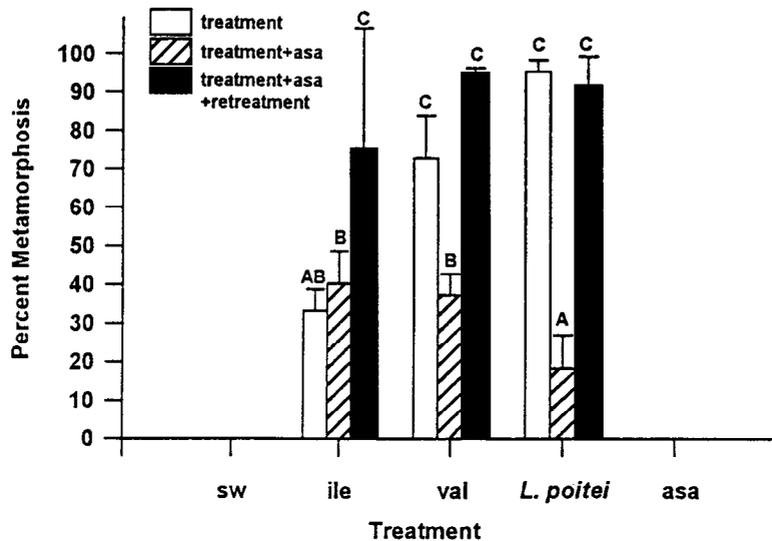


Figure 7. Mean percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isoleucine (ile, 100 μ M), valine (val, 100 μ M), and an extract of *Laurencia poitei* (20 μ l extract/ml seawater) alone, and in combination with acetylsalicylic acid (asa, 1 mM) with and without reexposure to the appropriate cue. Asa (1 mM) alone is also shown. In this experiment, the response to isoleucine is anomalously low. Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

cant levels of metamorphosis, although, as with other cues, the percentage varied among batches of larvae (Boettcher and Targett, 1996). Elevations in K^+ concentrations between 20 and 22 mM over ambient induced levels of metamorphosis equivalent to those induced by the algal extract. Concentrations ≤ 17 mM or ≥ 24 mM over ambient induced significantly lower levels of metamorphosis, and concentrations lower than 10 mM over ambient had no effect on metamorphosis.

DOPA and EP at 1 μ M had no effect on metamorphosis. However, at 10 μ M, DOPA induced significant levels of metamorphosis (87% \pm 14%), and at 100 μ M it had toxic effects. GABA induced significant levels of metamorphosis, but only at concentrations ≥ 5 mM (Fig. 9). The levels induced by GABA approached, but never were equal to, those induced by the algal extract. As reported previously, GABA at 1 and 100 μ M had no significant effect on larval metamorphosis (Boettcher and Targett, 1996). Hydrogen peroxide at 50 and 100 μ M induced levels of metamorphosis (54% \pm 16% and 96% \pm 4.0% respectively) equal to or greater than those induced by the algal extract (61% \pm 5.6% and 93% \pm 12%).

TEA at 100 and 500 μ M had no significant effect on induction of metamorphosis caused by elevated K^+ concentrations (20 mM), hydrogen peroxide (50 μ M), or the extract (20 μ l extract/ml seawater), and no significant effect when presented alone (Fig. 10). SITS at 10 μ M also had no effect on the above inducers; but at 50 μ M, it significantly decreased the response to hydrogen peroxide

and to elevated K^+ concentrations. SITS had no effect when presented alone (Fig. 10).

Vanadate (1 mM) induced levels of metamorphosis (53% \pm 8.3%) equal to those induced by hydrogen peroxide (32% \pm 33%) and *L. poitei* extract (56% \pm 20%); but at 2 mM it was toxic to the larvae. Unlike its effects on the induction of metamorphosis by the algal extract, isoleucine, and valine, acetylsalicylic acid had no significant effect on the response of the larvae to hydrogen peroxide. Bovine catalase, however, totally blocked the larval response to hydrogen peroxide, while having no effect on the response to the extract.

Discussion

The natural inducer of larval metamorphosis in queen conch is a water-soluble cue associated with species of red algae, including *L. poitei*, commonly found in conch nursery grounds (Davis and Stoner, 1994; Boettcher and Targett, 1996). Larval responses to this cue are dependent on both concentration and exposure time, with the initiation of metamorphosis occurring within 10 min of treatment. The free amino acids isoleucine and valine, elevations in external concentrations of K^+ , the neurotransmitters DOPA and GABA, as well as hydrogen peroxide and vanadate also induce larval metamorphosis.

Isoleucine and valine induce behavioral and morphogenic responses that mimic the effects of the natural inducer of conch metamorphosis. Valine and isoleucine are

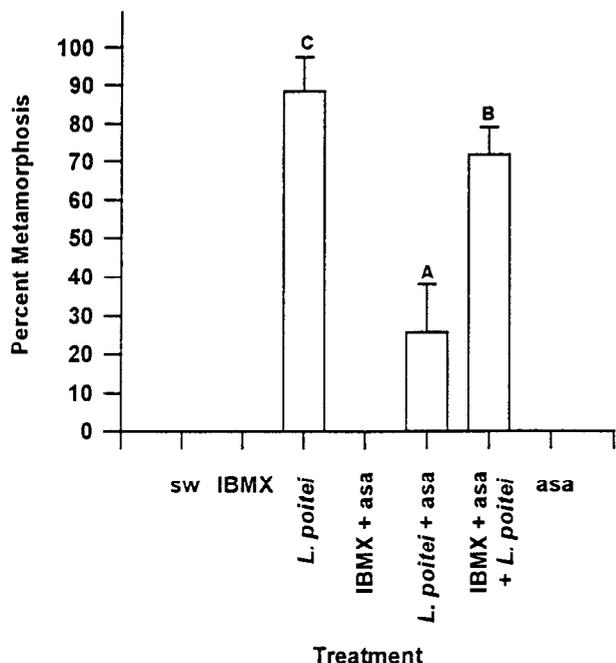


Figure 8. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isobutyl methyl xanthine (IBMX, 0.1 mM) and an extract of *Laurencia poitei* (20 μ l extract/ml seawater) alone, in combination with acetylsalicylic acid (asa, 1 mM), and in combination with one another and asa (1 mM). Asa (1 mM) alone is also shown. Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

similar in that they have neutral, hydrophobic, branched side chains that differ only in the length of one of the branches (Fig. 11). In leucine, the methyl group is displaced, and though this amino acid induces significant levels of metamorphosis, it does not induce normal larval behavior, and it is toxic at concentrations $\geq 100 \mu M$. Several other amino acids also induce partial metamorphosis, but not normal larval behavior (Table II). Glutamic acid, glycine, and threonine, although found at higher concentrations than valine, isoleucine, or leucine in the < 1 kDa component of hydrolyzed algal extract, induced only low levels or no metamorphosis.

The responses of conch larvae to the algal extract, valine, and isoleucine are blocked by acetylsalicylic acid (Fig. 4). In other systems (Hara, 1977), alterations of the α -amino or α -carboxyl group on amino acids through acetylation, methylation, or esterification block the activity of amino acids. Hara's results (1977) coupled with the results of our experiments on the effects of *N*-acetyl-L-valine suggest that acetylsalicylic acid may be modifying sites on either the inducer (*L. poitei* extract, valine, or isoleucine) or the larval receptors, possibly through acetylation. Acetylsalicylic acid, however, may also affect transduction of the metamorphic signal, since it indirectly affects levels of cAMP in cells (Hecker *et al.*, 1995; Payan and Katzung, 1995). Although the cAMP phosphodiesterase inhibitor IBMX does not, by itself, have an effect on conch larval metamorphosis, it does alleviate the negative effects of acetylsalicylic acid on metamorphosis (Fig. 8). Therefore, the ability of acetylsalicylic acid to modulate

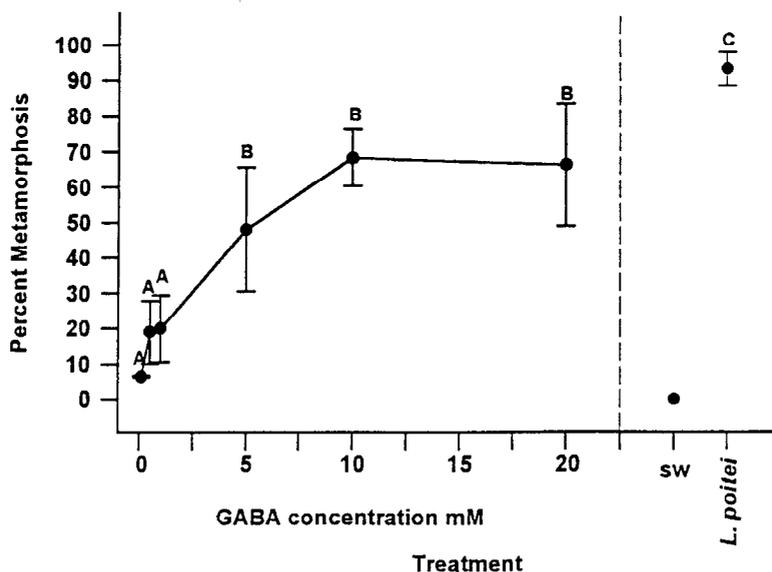


Figure 9. Percent metamorphosis of queen conch larvae in response to specific concentrations of γ -aminobutyric acid (GABA). Seawater only (sw, a negative control) and an extract of *Laurencia poitei* (20 μ l extract/ml seawater) are also shown. Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

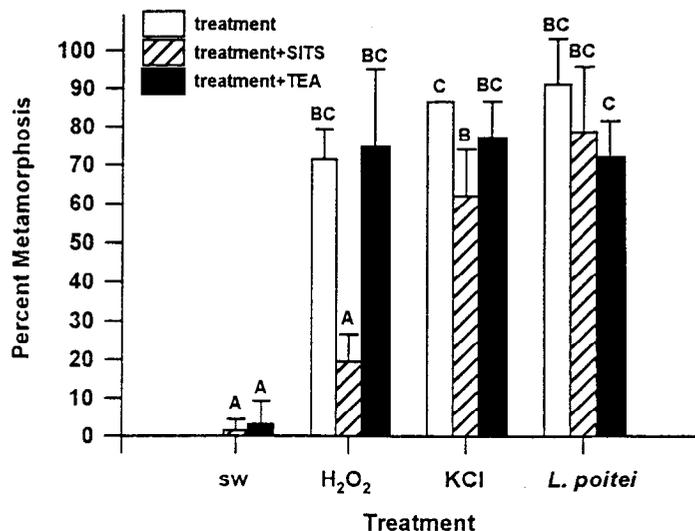


Figure 10. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), hydrogen peroxide (H_2O_2 , $50 \mu\text{M}$), increased KCl (20 mM) and an extract of *Laurencia poitei* ($20 \mu\text{l}$ extract/ml seawater) alone and in combination with 4-acetamino-4'isothiostilbene-2,2'-disulfonic acid ($50 \mu\text{M}$, SITS) or tetraethylammonium chloride ($500 \mu\text{M}$, TEA). Points are means \pm SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

cAMP levels during conch metamorphosis cannot be disregarded. However, since salicylic acid (a compound that, like acetylsalicylic acid, can affect cAMP levels) has no effect on conch larval metamorphosis, and since metamorphosis induced by hydrogen peroxide is unaffected by acetylsalicylic acid, it is unlikely that the effects of acetylsalicylic acid are due to its influence on the cAMP second messenger system.

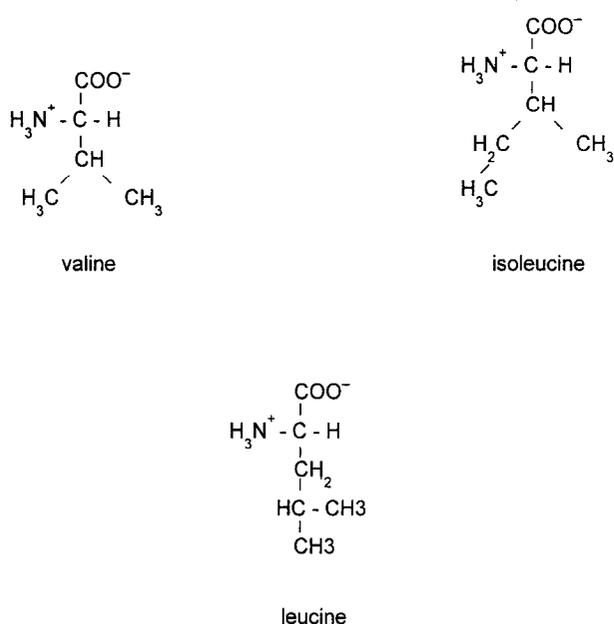


Figure 11. Structure of the amino acids valine, isoleucine, and leucine.

The responses of conch larvae to hydrogen peroxide, DOPA, vanadate, and GABA are consistent with the known roles of second messenger pathways in settlement and metamorphosis, as well as with the known activities of these compounds in other systems. Models for processes controlling the transduction of metamorphogenic signals in other marine larval systems have drawn on those developed for olfactory responses, involving primarily the adenylate cyclase (AC)/cAMP pathway, the phospholipase C (PLC)/inositol triphosphate (IP_3) pathway, or both (Leitz and Müller, 1987; Freeman and Ridgeway, 1990; Morse, 1990; Rittschof *et al.*, 1991; Anholt, 1992; Fadool and Ache, 1992; Michel and Ache, 1992; Clare *et al.*, 1995; Brunet *et al.*, 1996).

Hydrogen peroxide, vanadate, and DOPA all induce complete larval metamorphosis in the queen conch. The response of conch larvae to DOPA appears to be related to the production of hydrogen peroxide in the breakdown of this catecholamine rather than to any direct effect of DOPA itself; no response is initiated in the DOPA treatments until peroxide concentrations reach levels that alone induce metamorphosis (AAB, pers. obs.). This is similar to the response of *Phestilla sibogae* larvae to DOPA and hydrogen peroxide (Pires and Hadfield, 1991). Although hydrogen peroxide induces complete metamorphosis in the conch, only partial metamorphosis is included in *P. sibogae* (Pires and Hadfield, 1991). In other systems, hydrogen peroxide and vanadate have been shown to directly or indirectly activate Ca^{2+} release channels via oxidation of thiol groups, stimulate phospholipase C, and increase protein tyrosine phosphorylation (Paris

and Pouysségur, 1987; Ranjan and Goetz, 1990; Leitz and Wirth, 1991; Pires and Hadfield, 1991; Favero *et al.*, 1995). In addition, it has recently been hypothesized that hydrogen peroxide, like nitric oxide, may itself be an intracellular second messenger (Dong, 1995; Sundaresan *et al.*, 1995). One or several of these mechanisms may be responsible for the activities of hydrogen peroxide, DOPA, and vanadate in conch larval metamorphosis, and all suggest that these compounds influence metamorphosis by modulating second messenger pathways.

The types of chemical cues involved in conch metamorphosis and the mechanisms controlling it share general features with chemoreception in adult aquatic invertebrates (Burke, 1983; Baxter and Morse, 1987; Arkett *et al.*, 1989; Carr *et al.*, 1989; Bonar *et al.*, 1990; Freeman and Ridgway, 1990; Morse, 1990; Pawlik, 1990; Leitz, 1993; Leitz *et al.*, 1994). The red algal cues, like those that induce olfactory responses in other marine organisms, are of low molecular weight and soluble in water. It appears that the molecules of the algal cue bind to sites that also recognize and bind particular amino acids (*i.e.*, valine and isoleucine). The results of the experiments with vanadate and hydrogen peroxide suggest that metamorphosis induced by the natural cue may be triggered through a second messenger pathway. As with other marine larvae, elevated K^+ concentrations (above ambient seawater) can directly activate the metamorphosis process, presumably by depolarizing sensory cells. Studies focusing on further characterization of the natural inducer, and on the potential involvement of the PLC/DAG/IP₃ and AC/cAMP second messenger systems in conch metamorphosis, will deepen our understanding of the metamorphic process in conch and in other marine invertebrates. Electrophysiological studies directly testing the effects of morphogens on potential larval chemoreceptors would contribute further to the understanding of how metamorphosis is induced and how the signals are transduced, and would also aid in the identification of the active components of the cues.

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Literature Cited

- Anholt, R. R. H. 1992. Molecular aspects of olfaction. Pp. 51–79 in *Science of Olfaction*, M. J. Serby and K. L. Chobor, eds. Springer-Verlag, New York.
- Arkett, S. A., F.-S. Chia, F. I. Goldberg, and R. Koss. 1989. Identified settlement receptor cells in a nudibranch veliger respond to specific cue. *Biol. Bull.* **176**: 155–160.
- Baloun, A. J., and D. E. Morse. 1984. Ionic control of settlement and metamorphosis in larval *Haliotis rufescens* (Gastropoda). *Biol. Bull.* **167**: 124–138.
- Baxter, G., and D. E. Morse. 1987. G protein and diacylglycerol regulate metamorphosis of planktonic molluscan larva. *Proc. Natl. Acad. Sci. USA* **84**: 1867–1870.
- Beiras, R., and J. Widdows. 1995. Induction of metamorphosis in larvae of the oyster *Crassostrea gigas* using neuroactive compounds. *Mar. Biol.* **123**: 327–334.
- Boettcher, A. A., and N. M. Targett. 1996. Induction of metamorphosis in queen conch, *Strombus gigas* Linnaeus, larvae by cues associated with red algae from their nursery grounds. *J. Exp. Mar. Biol. Ecol.* **196**: 29–52.
- Boettcher, A. A., C. Dyer, J. Casey, and N. M. Targett. 1997. Hydrogen peroxide induced metamorphosis of queen conch, *Strombus gigas*: tests at the commercial scale. *Aquaculture* **148**: 247–258.
- Bonar, D. B., S. L. Coon, M. Walch, R. M. Weiner, and W. Fitt. 1990. Control of oyster settlement and metamorphosis by endogenous and exogenous chemical cues. *Bull. Mar. Sci.* **46**: 484–498.
- Brownell, W. N., and J. M. Stevely. 1981. The biology, fisheries, and management of the queen conch, *Strombus gigas*. *Mar. Fish. Rev.* **43**: 1–12.
- Brunet, L. J., G. H. Gold, and J. Ngai. 1996. General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. *Neuron* **17**: 681–693.
- Burke, R. D. 1983. The induction of metamorphosis of marine invertebrate larvae: stimulus and response. *Can. J. Zool.* **61**: 1701–1719.
- Burke, R. D. 1984. Pheromonal control of metamorphosis in the Pacific sand dollar, *Dendraster excentricus*. *Science* **225**: 442–443.
- Burke, R. D. 1986. Pheromones and the gregarious settlement of marine invertebrate larvae. *Bull. Mar. Sci.* **39**: 323–331.
- Carr, W. E. S. 1988. The molecular nature of chemical stimuli in the aquatic environment. Pp. 3–27 in *Sensory Biology of Aquatic Animals*, J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga, eds. Springer-Verlag, New York.
- Carr, W. E. S., R. A. Gleeson, and H. G. Trapido-Rosenthal. 1989. Chemosensory systems in lower organisms: correlations with internal receptor systems for neurotransmitters and hormones. *Adv. Comp. Environ. Physiol.* **5**: 25–52.
- Clare, A. S., R. F. Thomas, and D. Rittschof. 1995. Evidence for the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. *J. Exp. Biol.* **198**: 655–664.
- Coon, S. L., D. B. Bonar, and R. M. Weiner. 1985. Induction of settlement and metamorphosis of the Pacific oyster, *Crassostrea gigas* (Thunberg), by L-DOPA and catecholamines. *J. Exp. Mar. Biol. Ecol.* **94**: 211–221.
- Davis, M. 1994. Mariculture techniques for queen conch (*Strombus gigas* Linne) eggmass to juvenile stage. Pp. 231–252 in *The Biology, Fisheries, Mariculture and Management of the Queen Conch*, R. S. Appeldoorn and B. Rodriguez, eds. Fundación Científica Los Roques, Caracas.
- Davis, M., and A. W. Stoner. 1994. Trophic cues induce metamorphosis of queen conch larvae (*Strombus gigas* Linnaeus). *J. Exp. Mar. Biol. Ecol.* **180**: 83–102.
- Davis, M., W. D. Heyman, W. Harvey, and C. A. Withstandley. 1990. A comparison of two inducers, KCl and *Laurencia* extracts, and techniques for the commercial scale induction of metamorphosis in queen conch, *Strombus gigas* Linnaeus, 1758, larvae. *J. Shellfish Res.* **9**: 67–73.
- Dong, X. 1995. Finding the missing pieces in the puzzle of plant disease resistance. *Proc. Natl. Acad. Sci. USA* **92**: 7137–7139.

- Fadool, D. A., and B. W. Ache. 1992. Plasma membrane inositol 1,4,5-trisphosphate-activated channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron* **9**: 907–918.
- Favero, T. G., A. C. Zable, and J. J. Abramson. 1995. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **270**: 25557–25563.
- Freeman, G., and E. B. Ridgway. 1990. Cellular and intracellular pathways mediating the metamorphic stimulus in hydrozoan planulae. *Roux's Arch. Dev. Biol.* **199**: 63–79.
- Hara, T. J. 1977. Further studies on the structure-activity relationships of amino acids in fish olfaction. *Comp. Biochem. Physiol. A* **56**: 559–565.
- Hecker, M., M. L. Foegh, and P. W. Ramwell. 1995. The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and related compounds. Pp. 290–304 in *Basic and Clinical Pharmacology*, B. G. Katzung, ed. Appleton and Lange, Norwalk, CT.
- Heyman, W. D., R. A. Dopperteen, L. A. Urray, and A. M. Heyman. 1989. Pilot hatchery for the queen conch, *Strombus gigas*, shows potential for inexpensive and appropriate technology for larval aquaculture in the Bahamas. *Aquaculture* **77**: 277–285.
- Ilan, M., R. A. Jensen, and D. E. Morse. 1993. Calcium control of metamorphosis in polychaete larvae. *J. Exp. Zool.* **267**: 423–430.
- Leitz, T. 1993. Biochemical and cytological bases of metamorphosis in *Hydractinia echinata*. *Mar. Biol.* **116**: 559–564.
- Leitz, T., and W. A. Müller. 1987. Evidence for the involvement of PI-signaling and diacylglycerol second messengers in the initiation of metamorphosis in the hydroid *Hydractinia echinata* Fleming. *Dev. Biol.* **121**: 82–89.
- Leitz, T., and A. Wirth. 1991. Vanadate, known to interfere with signal transduction, induces metamorphosis in *Hydractinia* (Coelenterata; Hydrozoa) and causes profound alterations of the larval and postmetamorphic body pattern. *Differentiation* **47**: 119–127.
- Leitz, T., K. Morand, and M. Mann. 1994. Metamorphosin A: a novel peptide controlling development of the lower metazoan *Hydractinia echinata* (Coelenterata, Hydrozoa). *Dev. Biol.* **163**: 440–446.
- Michel, W. C., and B. W. Ache. 1992. Cyclic nucleotides mediate an odor-evoked potassium conductance in lobster olfactory receptor cells. *J. Neurosci.* **12**: 3979–3984.
- Morse, A. N. C. 1992. Role of algae in the recruitment of marine invertebrate larvae. Pp. 385–403 in *Plant and Animal Interactions in the Marine Benthos*, D. M. John, S. J. Hawkins, and J. H. Price, eds. Systematics Association Special Vol. 46. Clarendon Press, Oxford.
- Morse, D. E. 1990. Recent progress in larval settlement and metamorphosis: closing the gap between molecular biology and ecology. *Bull. Mar. Sci.* **46**: 465–483.
- Paris, S., and J. Pouysségur. 1987. Further evidence for a phospholipase C-coupled G protein in hamster fibroblasts. *J. Biol. Chem.* **262**: 1970–1976.
- Pawlik, J. R. 1990. Natural and artificial induction of metamorphosis of *Phragmatopoma lapidosa californica* (Polychaeta: Sabellariidae), with a critical look at the effects of bioactive compounds on marine invertebrate larvae. *Bull. Mar. Sci.* **46**: 512–536.
- Payan, D. G., and B. G. Katzung. 1995. Nonsteroidal anti-inflammatory drugs, nonopioid analgesics; drugs used in gout. Pp. 536–559 in *Basic and Clinical Pharmacology*, B. G. Katzung, ed. Appleton and Lange, Norwalk, CT.
- Pearce, C. M., and R. E. Scheibling. 1990. Induction of metamorphosis of larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, by coralline red algae. *Biol. Bull.* **179**: 304–311.
- Pires, A., and M. G. Hadfield. 1991. Oxidative breakdown products of catecholamines and hydrogen peroxide induce partial metamorphosis in the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia). *Biol. Bull.* **180**: 310–317.
- Randall, J. E. 1964. Contributions to the biology of the queen conch, *Strombus gigas*. *Bull. Mar. Sci. Gulf Caribb.* **14**: 246–295.
- Ranjan, M., and F. W. Goetz. 1990. Orthovanadate and fluorialuminate stimulate inositol phosphate production and *in vitro* ovulation in goldfish (*Carassius auratus*) follicles. *Biol. Reprod.* **43**: 323–324.
- Ray, M., and M. Davis. 1989. Algae production for commercially grown queen conch (*Strombus gigas*). *Proc. Gulf Caribb. Mar. Fish. Inst.* **39**: 453–457.
- Ray, M., and A. W. Stoner. 1994. Experimental analysis of growth and survivorship at a juvenile queen conch aggregation: balancing growth with safety in numbers. *Mar. Ecol. Prog. Ser.* **105**: 47–59.
- Rittschof, D. 1990. Peptide-mediated behaviors in marine organisms, evidence for a common theme. *J. Chem. Ecol.* **16**: 261–272.
- Rittschof, D., A. R. Schmidt, I. R. Hooper, D. J. Gerhart, D. Gunster, and J. Bonaventura. 1991. Molecular mediation of settlement of selected invertebrate larvae. Pp. 317–330 in *Bioactive Compounds from Marine Organisms with an Emphasis on the Indian Ocean*, M-F. Thompson, R. Sarojini, and R. Nagabhushanam, eds. A. A. Balkema, Rotterdam.
- Sandt, V. J., and A. W. Stoner. 1993. Ontogenetic shift in habitat by early juvenile queen conch, *Strombus gigas*: patterns and potential mechanisms. *Fish. Bull. U.S.* **91**: 516–525.
- Stoner, A. W., and M. Ray. 1993. Aggregation dynamics in juvenile queen conch: population structure, growth, mortality, and migration. *Mar. Biol.* **116**: 571–582.
- Stoner, A. W., and V. J. Sandt. 1991. Experimental analysis of habitat quality for juvenile queen conch in seagrass meadows. *Fish. Bull. U.S.* **89**: 693–700.
- Stoner, A. W., and J. M. Waite. 1991. Trophic biology of *Strombus gigas* in nursery habitats: Diets and food sources in seagrass meadows. *J. Molluscan Stud.* **57**: 451–460.
- Stoner, A. W., M. D. Hanisak, N. P. Smith, and R. A. Armstrong. 1994. Large scale distribution of queen conch nursery habitats: implications for stock enhancement. Pp. 169–189 in *The Biology, Fisheries, Mariculture and Management of the Queen Conch*, R. S. Appeldoorn and B. Rodriguez, eds. Fundación Científica Los Roques, Caracas.
- Stoner, A. W., M. Ray, R. A. Glazer, and K. J. McCarthy. 1996. Metamorphic responses to natural substrata in a gastropod larva: decisions related to postlarval growth and habitat preference. *J. Exp. Mar. Biol. Ecol.* **205**: 229–243.
- Sundaresan, M., Z.-X. Yu, V. J. Ferrans, K. Irani, and T. Finkel. 1995. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* **270**: 296–299.
- Tegtmeyer, K., and D. Rittschof. 1989. Synthetic peptide analogs to barnacle settlement pheromone. *Peptides* **9**: 1403–1406.
- Tsugita, A., T. Uchida, H. W. Mewes, and T. Akata. 1987. Rapid vapor-phase hydrolysis of peptides and proteins. *J. Biochem.* **102**: 1592–1597.
- Wickland, R. I., L. J. Hepp, and G. A. Wenz. 1991. Preliminary studies on the early life history of the queen conch, *Strombus gigas*, in the Exuma Cays, Bahamas. *Proc. Gulf Caribb. Fish. Inst.* **40**: 283–298.
- Yool, A. J., S. M. Grau, M. G. Hadfield, R. A. Jensen, D. A. Markell, and D. E. Morse. 1986. Excess potassium induces larval metamorphosis in four marine invertebrate species. *Biol. Bull.* **170**: 255–266.
- Zimmer-Faust, R. K., and M. N. Tamburri. 1994. Chemical identity and ecological implications of a waterborne, larval settlement cue. *Limnol. Oceanogr.* **39**: 1075–1087.