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Ammonia Induces Settlement Behavior in Oyster Larvae

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Abstract. Oyster larvae exposed to solutions of NH_4Cl exhibit stereotypical settlement behavior similar to that which normally precedes cementation and metamorphosis. Un-ionized ammonia is the active chemical species. At pH = 8.0, the threshold concentration of NH_4Cl (pH = 8.0) for newly competent larvae is 2.5 m*M*; maximum activity is at 7.9 m*M*, corresponding to calculated NH_3 concentrations of 100 μM and 310 μM, respectively. Induction of settlement behavior is rapid, with >90% of larvae exposed to 310 μM NH_3 responding within less than 5 min. After 15 to 30 min, larvae become habituated to NH_3 and resume swimming so that the percent exhibiting settlement behavior after 30 min is <10%. Other weak bases, such as methylamine and trimethylamine, induce similar behavior suggesting that NH_3 acts by increasing intracellular pH. Evidence that NH_3 and L-3,4-dihyrodxyphenylalanine (L-DOPA) induce settlement behavior through different mechanisms is presented. Ammonia may be a natural environmental cue that promotes oyster settlement behavior and, ultimately, recruitment.

Introduction

Many marine invertebrates, including oysters, have planktonic larvae that are recruited preferentially to habitats suitable for subsequent survival (Thorson, 1950). Recruitment of invertebrate larvae often involves a stereotyped series of search and crawl behaviors that is called settlement, followed by a morphogenetic phase called metamorphosis (Burke, 1983). The settlement behavior of oyster larvae has been well characterized and includes swimming with the foot extended forward followed by a series of increasingly localized crawling maneuvers (Prytherch, 1932; Cranfield, 1973; Coon *et al.*, 1985). If the habitat in which the larva has settled is suitable, the larva will cement permanently to the substratum and metamorphose. Settlement is reversible and does not necessarily culminate in metamorphosis once initiated; if the habitat is unsuitable, the larva may resume swimming and repeat the process elsewhere.

Invertebrate larvae are often induced to settle and metamorphose by environmental cues, typically chemical, associated with the adult habitat (Crisp, 1974; Chia and Rice, 1978). Microbial films play an important role in the development of many invertebrate assemblages (Zobell and Allen, 1935; Meadows and Campbell, 1972; Scheltema, 1974; Bonar *et al.*, 1986). Both soluble and surface-associated bacterial products are important in recruiting invertebrate larvae to surfaces containing bacterial films (Wilson, 1955; Scheltema, 1961; Gray, 1967; Muller, 1973; Neumann, 1979; Kirchman *et al.*, 1982), although some larvae prefer unfilmed surfaces (Crisp and Ryland, 1960). A bacterium, *Alteromonas colwelliana* (originally called LST), was found to enhance the recruitment of oyster larvae to colonized substrates (Weiner *et al.*, 1985; 1989). Supernatants from cultures of *A. colwelliana,* as well as other bacteria, contain one or more soluble factors that induce settlement behavior in oyster larvae of the genus *Crassostrea.* Preliminary studies showed that the soluble inducer has a low molecular weight (<300 daltons), and that supernatants have increased inductive potency commensurate with the age of the bacterial culture (Fitt *et al.*, 1990).

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Experiments reported in this paper demonstrate that solutions of NH_4Cl induce settlement behavior, and that NH_3, not NH_4^+, is the active chemical species. Additional experiments further explore the relationship between the mechanism of NH_3 induction and induction of settlement behavior by L-3,4-dihydroxyphenylalanine (L-DOPA), another known soluble inducer of oyster settlement behavior (Coon *et al.*, 1985, 1990). Preliminary results of this work have been presented (Coon *et al.*, 1988; Bonar *et al.*, 1990).

Materials and Methods

Obtaining and maintaining larvae

Larvae of the Pacific oyster, *Crassostrea gigas*, were obtained from the Coast Oyster Company of Quilcene, Washington, and maintained in the laboratory (Coon *et al.*, 1990). Larvae were used within one week of arrival.

Bioassay procedure

Experiments were conducted as previously described (Coon *et al.*, 1990). Aliquots of 20–50 larvae were assayed in 24-well tissue culture plates (Falcon #3047) in a final volume of 1.0 ml. Antibiotics were not used, but all experiments were conducted in 0.2 μm filtered seawater. Each treatment was duplicated or triplicated, and results are expressed as the mean ± standard error. All chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

Larval settlement behavior was defined as in Coon *et al.* (1990), the basic criterion being active foot extension beyond the ventral margin of the shell. Behavior in each well was monitored with a dissecting microscope for 30 s at the times noted. The length of each experiment was between 30 and 40 min, as noted.

Statistical tests were performed on arcsine-transformed data using a one-way analysis of variance (ANOVA) within time points. The ANOVA was followed by a Student-Newman-Keuls pair-wise comparisons test when significant differences were detected (Zar, 1974). Differences were considered significant if $P < 0.05$.

Effects of ammonia, ammonium, and pH

Larvae were exposed to a range of concentrations of NH_4Cl, in the first series of experiments. Stock solutions of NH_4Cl were made in seawater at twice the final concentration and adjusted to pH = 8.0 with NaOH. At the beginning of each bioassay, 0.5 ml of stock solution was added to an equal volume of seawater (pH = 8.0) containing swimming larvae. This experiment was repeated using $(NH_4)_2SO_4$ and other chloride salts (NaCl, KCl) at concentrations up to 10 m*M*.

Approximately 96% of the total ($NH_3 + NH_4^+$) in seawater at pH = 8.0 is present as the ammonium ion, NH_4^+ (Bower and Bidwell, 1978). To determine whether NH_3 or NH_4^+ was the active chemical species, larval settlement responses were observed while the concentrations of NH_3 and NH_4^+ were varied under two different regimes. In the first, pH was held constant at 8.0 and the total ($NH_3 + NH_4^+$), as NH_4Cl, was varied as described for the initial experiments above. In the second regime, total ($NH_3 + NH_4^+$) was held constant at 5.0 m*M* and the proportion of NH_3 to NH_4^+ was varied by altering the pH. The absolute concentrations of NH_3 and NH_4^+ were calculated by means of a hydrolysis constant for ammonium ion in seawater of $pK_a^s = 9.39$, at 30‰ salinity, 23°C and 1 atm pressure (Bower and Bidwell, 1978).

Because ammonia is a weak base ($pK_a = 9.25$), its effects might result from an increase in intracellular pH (pH_i). Therefore, two other weak bases, methylamine ($pK_a = 10.7$) and trimethylamine ($pK_a = 9.81$), were tested for their ability to induce settlement behavior. The inductive activities of these two compounds, along with those of NH_4Cl, were investigated according to the original protocol described above; concentration was varied while the pH remained constant at 8.0.

Relationship of NH_3-induction to L-DOPA-induction of settlement behavior

To determine whether NH_3 and L-DOPA induce settlement behavior through the same mechanisms, we tested sulpiride, a dopaminergic receptor antagonist (Stoof and Kebabian, 1984) and potent inhibitor of L-DOPA-induced settlement behavior (Coon and Bonar, 1987), for its ability to block NH_3-induced settlement behavior. Ammonium chloride stock solutions were made 10 times the final concentration in filtered seawater and adjusted to pH = 8.0. Solutions of L-DOPA and sulpiride were made 10 times their final concentrations in 0.002 *N* HCl. All larvae were pre-incubated in either 100 μM sulpiride or seawater for 12 min, then exposed to either 10 m*M* NH_4Cl or 100 μM L-DOPA. The effects of seawater and 0.002 *N* HCl were appropriately controlled. The pH of the final solutions was 7.7, yielding a calculated NH_3 concentration of 270 μM.

In other experiments, larvae that were "habituated" to NH_3 (see Results) were tested to see whether they would still respond to L-DOPA. Larvae were exposed to 5.0 m*M* NH_4Cl for 18 min until they began to habituate. They were then removed, rinsed, and exposed to either: (1) 100 μM L-DOPA; (2) fresh 5.0 m*M* NH_4Cl; (3) the NH_4Cl solution from which they had just been removed; or (4) filtered seawater. Control groups were pre-exposed to filtered seawater instead of NH_4Cl, then rinsed and put in

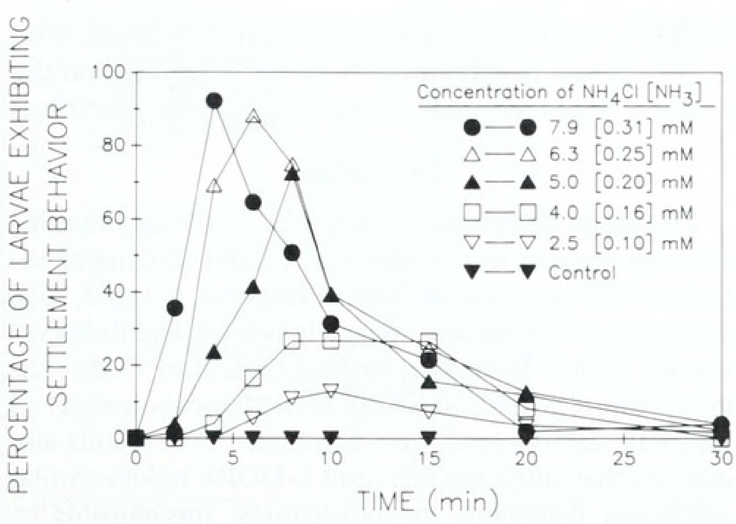


Figure 1. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of time exposed to specified concentrations of NH_4Cl at pH = 8.0. Calculated NH_3 concentrations are shown in brackets for reference. Data are means of duplicates followed through time. Controls contained only filtered seawater (pH = 8.0).

the same four treatments. In addition, some larvae were left in the NH_4Cl solution without rinsing, and were exposed to either: (1) the addition of another 5.0 m*M* NH_4Cl; (2) the addition of 100 μ*M* L-DOPA; or (3) no additional treatment. Larval settlement behavior was monitored for an additional 39 min.

Results

Ammonium chloride induces settlement behavior

Ammonium chloride induced high levels of settlement behavior in oyster larvae (Fig. 1). The percentage of larvae exhibiting settlement behavior reached >90% within 5 min of exposure to an NH_4Cl solution of 7.9 m*M* at pH = 8.0. Responses to higher concentrations of NH_4Cl are not shown because larvae in these solutions exhibited reduced activity levels after short exposures. Between 2.5 and 7.9 m*M* (pH = 8.0), the larval response to NH_4Cl was concentration dependent. As the NH_4Cl concentration increased, the percentage of larvae exhibiting settlement behavior increased, and the length of time required for the maximum percentage of larvae to respond decreased. Following the maximum larval response, the percentage of larvae continuing to exhibit settlement behavior rapidly declined so that 30 min after the initial exposure, almost all the larvae had "habituated" to the NH_4Cl solutions and had resumed normal swimming. No subsequent metamorphosis was observed after 24 to 48 h.

Larvae also exhibited high levels of settlement behavior in response to $(NH_4)_2SO_4$, indicating that either NH_3 or NH_4^+ was the active chemical species. This was corroborated by the observation that larval settlement behavior was not induced by Cl^-—as NaCl or KCl—at concentrations comparable to inductive NH_4Cl solutions (data not shown). Methylamine and trimethylamine, which are weak bases like NH_3, induced high levels of oyster settlement behavior (Table I).

The active species is NH_3 rather than NH_4^+

Ammonia has a pK_a^s of 9.39 in seawater and its calculated speciation as a function of pH is shown in Figure 2A. As the pH of the NH_4Cl solution decreases from 8.0 to 7.0, which is within the physiological tolerance range for oyster larvae, the NH_3 concentration changes much more dramatically (89.6% decrease) than the NH_4^+ concentration (3.6% increase) (Fig. 2B). Theoretically, the chemical species, NH_3 or NH_4^+, to which the larvae are responding, would have the same dose-response curve, whether the concentrations are adjusted by varying the NH_4Cl concentration under constant pH, or by keeping the NH_4Cl concentration constant and varying the pH.

This experiment shows that the maximal percentage of larvae exhibiting settlement behavior in response to NH_3 was independent of the regime used to vary the NH_3 concentration (Fig. 3A). The difference between these two curves represents less than 0.1 pH unit, which was within experimental error. In contrast, the larval response to NH_4^+ was highly dependent on the regime used to vary the NH_4^+ concentration; the larval response increased with increasing NH_4^+ concentration when the pH was held constant while the NH_4Cl concentration was varied, but the larval response decreased with increasing NH_4^+ concentration when the NH_4Cl was held constant while the pH was varied (Fig. 3B). These results indicate that, in these solutions, NH_3, not NH_4^+, was the active chemical species inducing settlement behavior in oyster larvae.

NH_3 and L-DOPA induce settlement behavior through different mechanisms

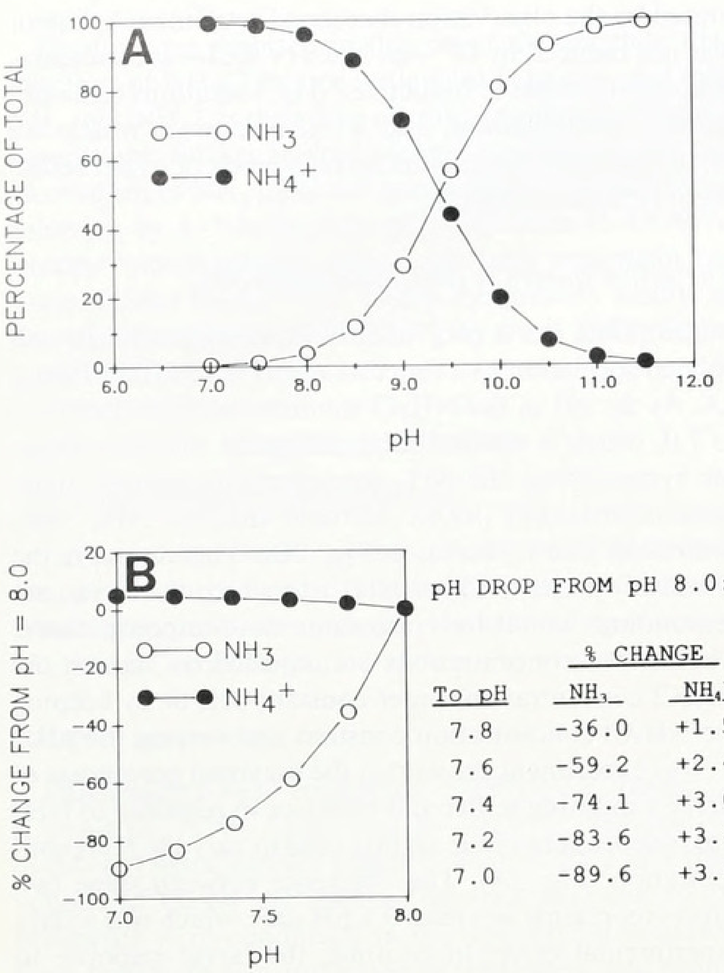
The dopaminergic antagonist, sulpiride, blocked the ability of L-DOPA to induce settlement behavior (Fig.

Table I

Maximal percentage of oyster larvae exhibiting settlement behavior in response to exposure to weak bases at pH = 8.0

	pK_a	1.0 m*M*	3.3 m*M*	10 m*M*
NH_4Cl	9.25	3.2 ± 0.4	51.2 ± 4.0	90.3 ± 2.3
Methylamine	10.7	4.4 ± 1.4	47.6 ± 14.2	94.6 ± 2.4
Trimethylamine	9.81	0	19.7 ± 7.6	91.6 ± 5.4

Data are means of duplicates ± standard error.



To pH	% CHANGE NH_3	% CHANGE NH_4^+
7.8	-36.0	+1.5
7.6	-59.2	+2.4
7.4	-74.1	+3.0
7.2	-83.6	+3.4
7.0	-89.6	+3.6

Figure 2. Calculated effect of pH on NH_3 and NH_4^+ speciation. (A) Percentage contribution to the total ($NH_3 + NH_4^+$) by each species as a function of pH. $pK_a^s = 9.39$. (B) Percentage change in NH_3 and NH_4^+ as the pH of the solution drops from pH = 8.0 to the specified value. Actual calculated changes in speciation are tabulated for clarity.

4A) but did not block the ability of NH_3 to induce settlement behavior (Fig. 4B). However, two small effects of sulpiride on NH_3-induced settlement behavior were noted: (1) settlement behavior was more rapidly expressed; and (2) the maximum percentage of larvae induced to exhibit settlement behavior was slightly lower (*t*-test; $P < 0.1$). The differential effects of sulpiride on the abilities of NH_3 and L-DOPA to induce settlement behavior indicate that NH_3 functions through a mechanism that does not require the dopaminergic receptor involved in the induction of settlement behavior by L-DOPA (Coon and Bonar, 1987).

The effects of NH_3 and L-DOPA are not completely independent. Although larvae that had habituated to NH_3 were almost completely refractory to fresh NH_3, they could still respond to L-DOPA (Fig. 5). However, fewer of these larvae exhibited settlement behavior, and they responded more slowly to L-DOPA than larvae that had not been habituated to NH_3. The larvae also showed an attenuated response to L-DOPA in additional treatments during which they were left in the presence of NH_3 when L-DOPA was added (data not shown). The small, transient, increase in settlement behavior following transfer to a new NH_4Cl solution was an artifact of the procedure.

Discussion

This study demonstrates that NH_3 in the surrounding medium induces oyster larvae to exhibit settlement behavior. The onset of settlement behavior is rapid, high percentages of larvae are induced to behave, and the larvae quickly resume swimming without cementing to the plastic culture plates (a suboptimal settlement surface) in which the experiments were conducted. The results also indicate that, although NH_3 and L-DOPA induce similar settlement behaviors, the biochemical mechanisms by which they do so are different.

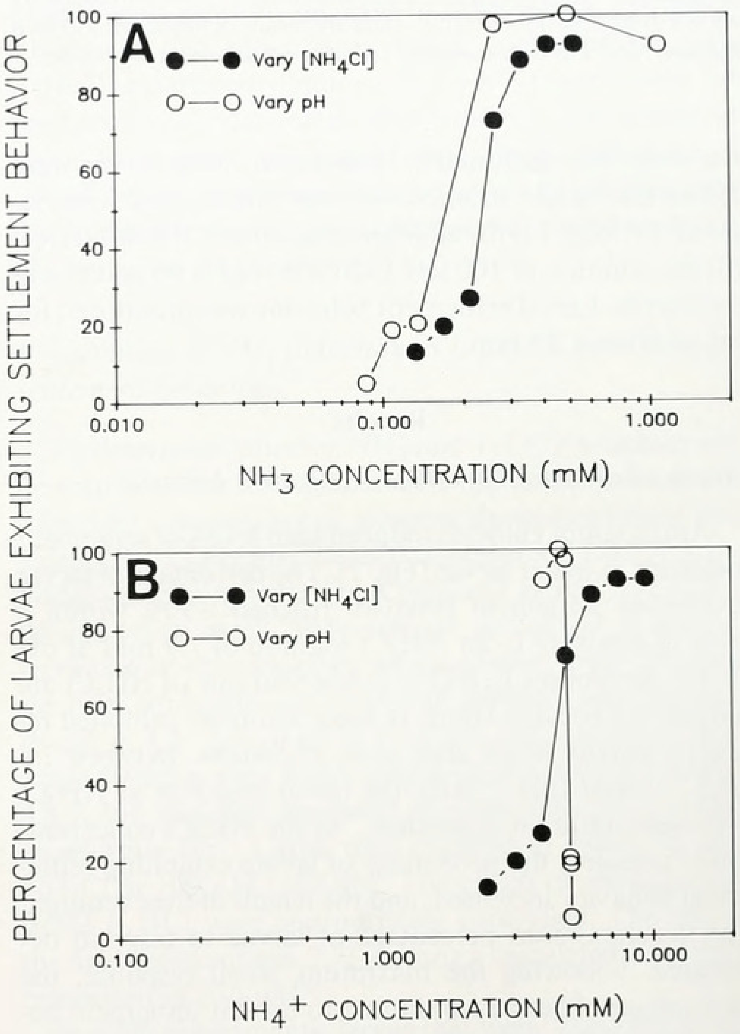


Figure 3. Maximal percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of the concentration of either NH_3 or NH_4^+. In one regime, pH was held constant while the NH_4Cl concentration was varied (data calculated from Fig. 1); in the other regime, NH_4Cl concentration was held constant while the pH was varied. The concentrations of NH_3 and NH_4^+ were calculated from pH values and NH_4Cl concentration. (A) Larval response as a function of the calculated concentrated of NH_3 under the two regimes. (B) Larval response as a function of NH_4^+ under the two regimes. Data are means of duplicates.

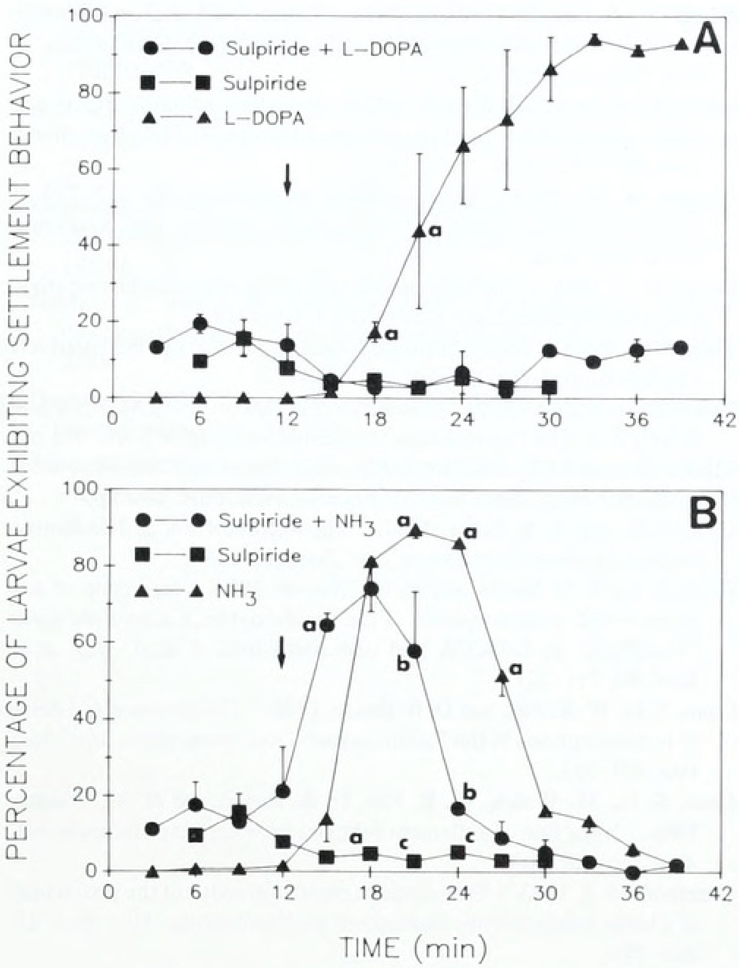


Figure 4. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of the duration of their exposure to NH_3 or L-DOPA in the presence of sulpiride. Sulpiride is a dopaminergic receptor antagonist. (A) Effect of sulpiride on the ability of L-DOPA to induce settlement behavior. (B) Effect of sulpiride on the ability of NH_3 to induce settlement behavior. Larvae were pre-incubated in sulpiride (100 μM) or seawater for 12 min, then L-DOPA (100 μM), NH_4Cl (10 mM), sulpiride (100 μM) or seawater were added as indicated by the arrows. Data are means ± standard error of triplicates followed through time. For each time point, treatments with the same letter, or no letter are not significantly different from each other. Only statistics for relevant time points are shown for clarity.

Ammonia, as a by-product of protein catabolism, is excreted by most marine bacteria and animals (Campbell, 1970; Billen, 1984). Therefore, in areas of high biological activity and reduced mixing (such as in boundary layers near surfaces), NH_3 might reach levels high enough to induce settlement behavior in oyster larvae. Total (NH_3 + NH_4^+) concentrations of 10 mM have been reported in interstitial waters from marine sediments (Bruland, 1983). Stevens (1983) found that total (NH_3 + NH_4^+) concentrations in association with oyster reefs may reach greater than 200 μM in sediment waters and 3 μM in overlying waters 10 cm above the sediment interface. The presence of high levels of NH_3 in the environment, the rapid induction of settlement behavior by NH_3, and the quick reversibility of inductive effects of NH_3, strongly suggest that NH_3 is a natural environmental cue for recruitment of oyster larvae. Its actual involvement in larval recruitment, however, has not yet been demonstrated.

If NH_3 is a natural inducer of settlement behavior, then it must be a relatively non-specific indicator of biologically rich environments. Ammonia alone could not account for the specificity observed in natural oyster settlement and metamorphosis. We hypothesize that NH_3 acts as a chemokinetic agent that induces settlement behavior once a threshold concentration is encountered, bringing oyster larvae into contact with substrates and other potential contact-dependent and soluble cues (*c.f.* Crisp, 1974). Once settlement behavior has been initiated, oyster larvae rely on other cues from the environment to indicate that the habitat is suitable for cementation and metamorphosis. If these secondary cues are not present, the larvae habituate to NH_3 and swim away. This scenario is consistent with models and observations of oyster settlement (Prytherch, 1934; Cranfield, 1973; Coon *et al.*, 1985; Weiner *et al.*, 1989; Coon *et al.*, 1990).

Although the mechanism by which NH_3 induces settlement behavior in oysters is unknown, NH_3 acts by increasing pH_i in other invertebrate systems (Boron and DeWeer, 1976; Roos and Boron, 1981; Dube and Guerrier, 1982; Ward *et al.*, 1983; Bibring *et al.*, 1984; Williams *et al.*, 1984; Busa, 1986). Weak bases, such as NH_3, raise pH_i by penetrating the cell membrane as the uncharged

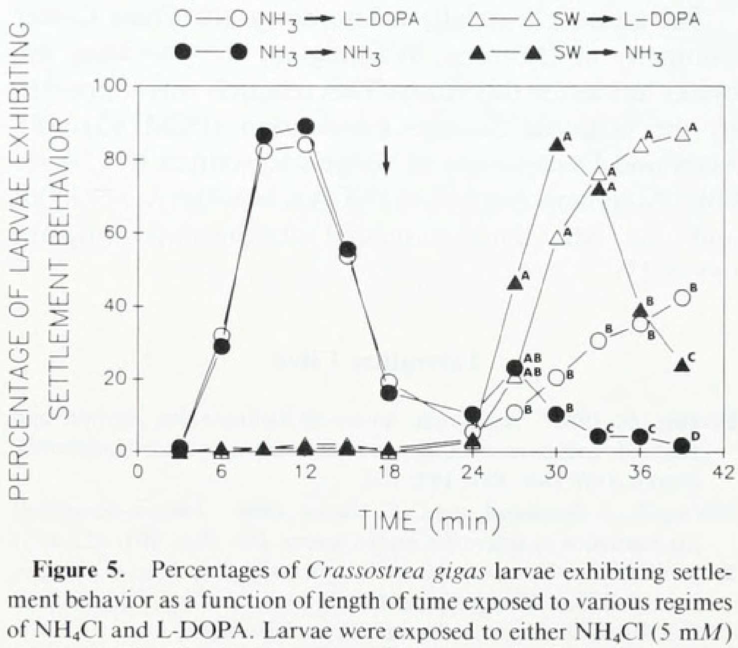


Figure 5. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of time exposed to various regimes of NH_4Cl and L-DOPA. Larvae were exposed to either NH_4Cl (5 mM) or seawater for 18 min, then removed (downward pointing arrow) and put into either NH_4Cl (5 mM) or L-DOPA (100 μM). Data are means ± standard error of triplicates followed through time. For each time point, treatments with the same letter, or no letter, are not significantly different from each other. Only statistics for relevant time points are shown for clarity.

species, then reprotonating in the cytoplasm (Roos and Boron, 1981). The induction of settlement behavior by other weak bases, such as methylamine and trimethylamine, is consistent with NH_3 acting by increased intracellular alkalization. An increase in pH_i would not be expected to be cell-type specific and so may affect a diverse range of cell types in the larvae. Larvae of the hydroid, *Hydractinia*, are induced to metamorphose by NH_4^+, not NH_3, through a mechanism that may involve regulation of intracellular transmethylation rather than pH_i (Berking, 1988).

Whatever its mode of action, induction of settlement behavior by NH_3 clearly involves a mechanism different from that of L-DOPA induction, though they are probably related. Ammonia and L-DOPA have different time courses. Larvae respond quickly to NH_3, then soon habituate to it; in contrast, larvae respond more slowly to L-DOPA, and the effects are longer lasting. Induction of settlement behavior by NH_3 is not mediated through the same dopaminergic receptors required for induction by L-DOPA, but is effected slightly by blocking these receptors with sulpiride. Conversely, larvae habituated to NH_3 can still respond to L-DOPA but to a lesser degree. There may be some interaction between pH_i and signal transduction through the dopaminergic receptors. Further experiments are underway to resolve the mechanism of NH_3-induction.

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