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Settlement of the tube worm *Hydroides dianthus* (Polychaeta: Serpulidae): cues for gregarious settlement

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Abstract The larvae of many benthic marine invertebrates settle to form conspecific aggregations and are thought to rely on chemical cues associated with adults as indicators of habitat suitability, although the identification of inductive compounds has proven difficult. Still-water laboratory assays carried out during the summers of 1992 and 1993 with larvae of the serpulid polychaete, *Hydroides dianthus* (Verrill, 1873), demonstrate that unidentified water-borne compound(s) were responsible for gregarious settlement of competent larvae. Unlike inductive compounds associated with other tube-dwelling polychaetes, the settlement cue was soluble in water and was not associated with the tube, but rather with the body of live adults. In assay chambers divided by a 52-μm mesh barrier, a greater percentage of larvae settled on biofilmed substrata when adult worms were present on the other side of the barrier than when adults were absent. Settlement in response to conspecific adults, live worms removed from their tubes, and amputated tentacular crowns of live worms was significantly greater than settlement in response to dead worms, empty tubes, or biofilmed slides. The settlement inducer appears to emanate from the openings of occupied tubes; settlement was greatest along the anterior two-fifths of the tube of living conspecific adults. A single adult was equally capable of eliciting a gregarious response as were five or 25 conspecifics, and newly settled juveniles began to elicit gregarious settlement after approximately 96 h. Extraction of aggregations of adult worms with organic solvents removed the inductive capacity of the tissue, and activity was found in both nonpolar and polar fractions of an extraction series.

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Introduction

Larvae of benthic marine invertebrates were once thought to metamorphose in the water column and then sink passively to the bottom (e.g., Yonge 1937), however, considerable evidence has accumulated over the past few decades that larvae are capable of behavioral responses to substrata which may influence the local distribution of adults (reviewed by Meadows and Campbell 1972; Burke 1986; Pawlik 1992). The observation that many sessile invertebrate species are found primarily in monospecific aggregations has prompted studies to examine possible mechanisms leading to aggregated distributions. Settlement on or near conspecific adults (gregariousness) has obvious benefits; adults derive reproductive benefits from being within aggregations (e.g., Levitan 1991, 1993), and larvae that settle near adults benefit from choosing a habitat likely to support post-larval growth (reviewed by Crisp 1979; Pawlik 1992).

Studies of gregarious settlement of invertebrate larvae have repeatedly implicated chemical cues associated with adult conspecifics as being responsible for larval settlement responses, although the identification of specific cues has proven elusive (reviewed by Burke 1986; Pawlik 1992). Gregarious settlement has been reported in at least 35 marine invertebrate species representing 8 phyla, and in 18 of these studies, there was evidence for a chemical inducer of settlement (e.g., Crisp 1967; Keck et al. 1971; Larman et al. 1982; reviewed by Burke 1986; Pawlik 1992). Despite abundant evidence implicating the existence of chemical cues that mediate settlement, the chemical structures of substratum-derived, naturally occurring compounds that stimulate settlement are known for only five species of marine invertebrates: the hydroid *Coryne uchidai*

(Nishihira 1968; Kato et al. 1975), the echiuran *Bonellia virdis* (e.g., Jaccarini et al. 1983), the bivalve *Pecten maximus* (Yvin et al. 1985; Cochard et al. 1989), two subspecies of the sabellid polychaete *Phragmatopoma lapidosa* (Pawlik 1986, 1988a, b; Pawlik and Faulkner 1986), and the bivalve *Crassostrea virginica* (Zimmer-Faust and Tamburri 1994). Only the last of these examples is not the subject of some disagreement regarding the effectiveness of the isolated cue under natural conditions (Pawlik 1992).

Hydroides dianthus is a common fouling species, occurring from New England south through the West Indies (Hartman 1969), which is often found in large aggregations (dozens to hundreds of individuals) attached to the underside of floating docks, ships and other submerged hard substrata. Individuals are gonochoristic and reproduce sexually, with gametes broadcast every 2 to 4 wk at 23 °C (Zuraw and Leone 1968). Females release an average of 30 000 ova, and males an average of 6×10^7 sperm per spawn (Zuraw and Leone 1972). Larvae develop from fertilized eggs very rapidly; trochophore larvae begin feeding after 18 to 24 h, and become competent to settle after about 5 d at 23°C (Scheltema et al. 1981). Settlement occurs only on biofilmed surfaces (Toonen 1993; the present study), allowing larvae to be cultured in clean vessels over an extended planktonic lifespan. Larvae are ~300 µm in length at competency and undergo obvious morphological changes during metamorphosis that distinguish settled individuals from attached larvae. Gregarious settlement by larvae of the tube-dwelling polychaete worm *H. dianthus* was first described by Scheltema et al. (1981), and although chemical cues were suspected, none were characterized or isolated. We have further examined patterns of settlement in this species, and have hypothesized that *H. dianthus* produces larvae of at least two distinct behavioral classes that we term founders and aggregators (Toonen 1993; Toonen and Pawlik 1994). The majority of larvae are strongly gregarious (the type we term aggregators – these larvae will delay metamorphosis until they die in culture if not presented with adult conspecifics, see Toonen 1993; Toonen and Pawlik 1994), and chemical inducers of settlement are likely to be responsible for this settlement preference.

In the present study we examined settlement preferences of *Hydroides dianthus* larvae to determine the nature of adult-associated cues to which settling larvae respond. Further, we attempted to isolate and characterize the compounds responsible for inducing gregarious settlement of *H. dianthus*.

Materials and methods

Spawning of *Hydroides dianthus* (Verrill, 1873)

Adult *Hydroides dianthus* were collected from large (≈125 000 liter) settling tanks at the former Wrightsville Beach desalination plant, or from Banks Channel under the drawbridge access to Wrightsville Beach, North Carolina, USA during the summers of 1992 and 1993. Water in the settling tanks was exchanged every 2 to 3 d, with replacement water pumped in from Banks Channel, a tidally flushed inlet of the Atlantic Intracoastal Waterway. Aggregations of *H. dianthus* were brought into the laboratory, where they were kept in running, unfiltered seawater until spawned.

For each experiment, spawning was induced by removing individual worms from their calcareous tubes (also see Strathmann 1987 for spawning and fertilization techniques). To collect gametes, each female was placed into a 10-cm diameter dish containing 50 ml of 1-µm-filtered seawater, and each male was placed on a watch glass without seawater. Eggs from 15 to 25 females were pooled prior to fertilization by sperm from a single male. Approximately 1 ml of sperm from each male was suspended in 20 ml of filtered seawater and checked for motility. The male with the highest density of motile sperm was selected to fertilize all females in a given replicate culture. Eggs were fertilized by placing dishes containing eggs on a shaker table rotating at 50 rpm and by initially adding 5 drops of sperm suspension, followed with two more additions, each of doubled volume, at 10-min intervals thereafter.

Larval culture technique

Trochophore larvae of *Hydroides dianthus* were cultured at 10 000 larvae l^{-1} using techniques adapted from Pawlik (1986). Each experiment was performed on a different batch of ≈90 000 larvae pooled from ~25 females. Larvae were cultured in three 4-liter wide-mouth glass jars containing 3 liter of 1-µm-filtered seawater. Culture jars were maintained in 21 ± 2 °C constant-temperature water baths beneath two 40 W fluorescent lamps set for a 15 h light : 9 h dark photoperiod (Pawlik 1986). The contents of each jar were gently agitated with filtered air bubbling at approximately 2 bubbles per second from the tip of a 23-cm Pasteur pipette resting on the bottom of the jar.

Larval cultures were cleaned every second day by filtering the entire culture through a 153-µm-mesh prefilter (to collect any particulate debris) followed by a 52-µm mesh to retain larvae. Filters were kept submerged during transfer to minimize damage to larvae. Larvae were resuspended in a clean jar filled with 3 liter of 1-µm-filtered seawater drawn from the settling tanks from which the adults were obtained; used jars were scrubbed in hot freshwater and allowed to air-dry prior to reuse. If larval cultures were to be maintained longer than 7 d, cultures were cleaned daily after 6 d. Larvae were fed monospecific cultures of the diatom *Phaeodactylum tricornutum* at 10^5 cells ml^{-1} each time larval cultures were cleaned. Algal culture methods were adapted from Guillard (1975).

Settlement substrata

Substrata presented to larvae to assess settlement preference were all biofilmed prior to being used in experimental assays. Biofilmed substrata were made by placing etched glass microscope slides (75 × 25 mm) in running, unfiltered seawater for at least 5 d, so that they became coated with an organic/microbial film. Experimental treatment slides were similarly submerged, but after 5 d, a potential cue source was attached to them using Thick-gel Superglue (cyanoacrylate adhesive). The slides were then returned to running seawater for at least 1 h before use in an assay. Biofilmed and experimental treatment slides used concurrently were submerged in close proximity for the same period of time and kept in running, unfiltered seawater for subsequent assays. Before adding slides to assay dishes, each slide was scraped with a straight-edge razor to remove any macroscopic organisms (such as algae or protozoans). Experimental slides were similarly scraped, but a toothbrush was also used to remove any organisms from the tubes of adults, if appropriate.

Experimental substrata included a variety of potential cue sources attached to biofilmed microscope slides: (1) conspecific worms in their tubes (*adults*); (2) intact live worms removed from their tubes (*live worms*); (3) intact worms removed from their tubes and killed by freezing (*dead worms*); (4) empty worm tubes (*tubes*); (5) tentacular crowns amputated from live worms (*crowns*); (6) worms removed from their tubes and with tentacular crowns amputated by dissection (*bodies*); or (7) plain biofilmed microscope slides (*biofilm*). In cases where extracts or isolated fractions were assayed, they were resuspended in methanol and ≈50 µl of extract (a volume equivalent to 1 mg of extract) was added to biofilmed slides; for controls in these assays 50 µl of methanol was also added to adult and biofilmed slides (see below). Conspecific worms, tubes or body portions were all attached to slides using Thick-gel Superglue, and any treatments in which body portions died during the experiment (did not respond to being touched with a probe after the assay period) were not used for analysis.

Settlement assays

Bioassays to determine inductive activity of extracts were performed by removing 25 larvae from a culture and placing them in a covered 10-cm plastic petri dish containing 25 ml of 1-µm-filtered seawater and a single slide; dishes were then placed into a constantly illuminated 20 °C incubator for 24 h. Larvae were assayed 7 to 8 d after fertilization, because that was the period of peak larval settlement (Toonen 1993; Toonen and Pawlik 1994). Replicate samples were run simultaneously for each treatment on larvae from a single mixed-parentage (≈25 females) batch, but larval batches differed among experiments. Slides were rinsed gently with filtered seawater after 24 h, and settled juveniles were counted as they were removed.

Effect of adult density and juvenile age on settlement

To assess the effect of adult density on larval settlement, we ran assays with either 0, 1, 5 or 25 small (3 to 5 cm long) adult conspecifics per slide. We also wanted to know how soon after settlement juveniles could elicit gregarious settlement. We exposed competent (7-d-old) larvae to biofilmed slides for 1 h, selected 60 slides with five or more metamorphosed larvae, and then removed excess individuals to leave five juveniles per slide (*recruits*). Eight slides were immediately placed into petri dishes, and settlement assays were run as previously described, while the remaining recruit slides were returned to the water table. At intervals of 12, 24, 48, 72, 96 and 168 h we removed eight slides from the water, checked that they each had five live individuals still attached, and then assayed the response of larvae from the same batch to *recruits* of increasing age.

Solubility of settlement cue

In order to determine whether the cue was soluble, we ran assays in which larvae were free to contact only biofilmed slides, but other substrata were also present to which larvae were denied access by a 52-µm mesh. Larvae were contained in an 800-ml Tripour beaker with the bottom cut out and covered with 52-µm Nytex mesh. This 800-ml beaker was then suspended in a 1000-ml beaker filled with filtered seawater that contained an air-lift tube that circulated water from the larger beaker into the smaller (see diagram of "airlifted-droplet stirrer," p. 16, Strathmann 1987). A biofilmed slide was placed on the mesh within each 800-ml beaker, and either a biofilmed slide (*biofilm*), 5 live worms in their tubes (*adults*), 5 live worms removed from their tubes (*worms*), or empty tubes (*tubes*) were placed beneath the mesh on the bottom of the 1000-ml beaker. In this way, larvae contained within the internal (800 ml) beaker had access to a biofilmed substratum, but were prevented from contacting the source of a potential cue by the 52-µm mesh. Larvae (800) were added to the 800-ml beakers and left for 24 h before settlement was scored. Settlement assays, as described above, were run simultaneously with larvae from the same batch to confirm the batch was settling "normally" during these assays.

Crude extraction and stability of the settlement inducer

To determine whether an inducer of settlement could be extracted from adult conspecifics, we performed a series of crude aqueous and organic solvent extractions of an aggregation of live *Hydroides dianthus* (≈50 ml displacement volume) collected from the field. Worms and tubes were finely ground with a mortar and pestle with 10 ml of filtered seawater, and the resultant slurry of ground tissue and tubes was divided into three equal portions (20 ml) and frozen. The first portion was thawed, and the mixture was used to treat biofilmed and clean (new) slides (*homog*). The second portion was freeze-dried, then rehydrated with distilled water to the original volume and thoroughly mixed. The resultant mixture was used to treat biofilmed and clean slides (*FD homog*). The final portion was extracted in ≈50 ml of a 1:1 mixture of dichloromethane and methanol for 24 h at 4 to 5 °C with periodic agitation. The organic extract was filtered off, dried down on a rotary evaporator, and then resuspended in 4 ml of methanol. This extract in methanol was subsequently used to treat biofilmed slides (*crude org*). For each replicate, 50 µl of extract or homogenate was spread onto clean or biofilmed slides and allowed to dry for about 30 s before slides were placed into disposable plastic petri dishes with 25 ml of 1-µm-filtered seawater and 25 larvae for settlement assays. The volume of extract added (50 µl) was selected because this concentration translates to approximately 0.25 ml displacement volume from the initial aggregation, roughly equivalent to a single large adult. To control for the effect of methanol, adult and biofilmed slides were treated in the same way with 50 µl of pure methanol, and assayed at the same time.

Extraction of cue with organic solvents

Results from body portion settlement assays (above) indicated that it was the worm itself, and not the tube, that induced gregarious settlement. Therefore, after determining that we had an active cue in crude organic extracts, we used worms removed from their tubes prior to extraction for all subsequent assays. Extracts (50 ml) were prepared from isolated adult worms, which had been washed briefly in 1-µm-filtered seawater and frozen in a plastic scintillation vial. Because no adverse effect of freeze drying was detected in previous settlement assays, worms were all freeze-dried prior to extraction, and the dry weight of a 20 ml displacement volume of worms was recorded. The freeze-dried worms were then extracted with a series of solvents consisting of respectively: dichloromethane (*DCM*), 1:1 DCM:methanol (*1:1*), methanol (*MeOH*), and water (*water*). It is important to note that this extraction series was done sequentially, so that, unlike the first crude extraction, worms were extracted in DCM to remove lipophilic compounds, then the solvent was decanted and replaced with 1:1 DCM:methanol to extract more hydrophilic compounds, etc. Extracts were then filtered off, dried down on a rotary evaporator and resuspended in methanol as previously described. Extracts (≈50 µl) were spread onto slides so that 1 mg of extract was assayed in each case. These extracts were assayed along with *adult*, *biofilm*, and *homog* slides for controls.

Statistical analysis

Differences between mean larval settlement in response to experimental substrata were tested with the parametric *t*-test (Sokal and

Rohlf 1981). Percentage settlement data were arcsine-transformed prior to testing, with the exception of a few cases where data required the more extreme $-\log[1/(x+1)]$ transformation to meet assumptions of normality (where x is the absolute number of larvae that settled in each assay). In cases where the assumption of homoscedasticity was violated after transformation (using a log-ANOVA test for homogeneity of variances), the data were analyzed with the t-test corrected for unequal variances (Sokal and Rohlf 1981). Differences between the mean larval settlement for each substratum within multiple-cue assays were tested with the nonparametric Kruskal–Wallis test, with p determined using a χ^2 approximation (Sokal and Rohlf 1981). Pairwise comparisons of means among assay treatments were tested using Bonferroni's correction for multiple comparisons (Snedecor and Cochran 1989).

Results

Settlement in response to adult density

Assays run to determine the effect of varying densities of conspecifics on the substratum indicated that the presence of a single adult was sufficient to elicit gregarious settlement of *Hydroides dianthus* (Fig. 1). Settlement responses to *adults* (1, 5 and 25) were not significantly different (Kruskal–Wallis $H = 2.4135$, $p > 0.05$, $df = 29$), but all were significantly greater than responses to biofilm ($t' = 2.88$ to 4.10, $p < 0.05$, $df \approx 12$ to 18). *Recruits* were capable of eliciting settlement after 96 h (Fig. 2). There were significantly more larvae responding to recruit slides at 96 h than to *biofilm* ($t = 5.42$, $p < 0.05$, $df = 4$), but no significant difference between settlement in response to *adults* and *recruits* ($t' = 3.77$, $p > 0.05$, $df \approx 2$). Settlement in response to *recruits* prior to 96 h was not significantly different from *biofilm* ($t = 0.19$ to 0.58, $p > 0.05$, $df = 9$).

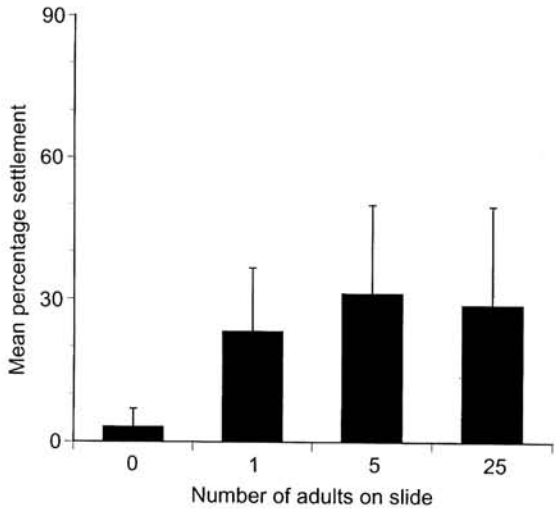

Fig. 1 *Hydroides dianthus.* Mean percentage settlement ($n = 10$, + 1 SE) of larvae in response to biofilmed slides with 0, 1, 5 and 25 adult conspecifics attached in 24-h assays

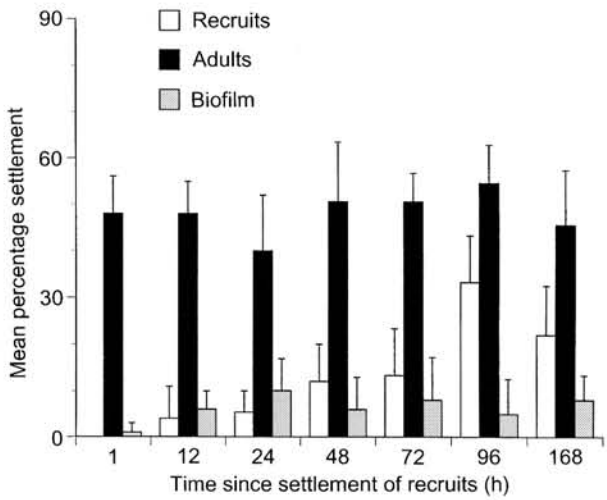

Fig. 2 *Hydroides dianthus.* Mean percentage settlement ($n = 8$, + 1 SE) of larvae in response to *recruit*, *adult* and *biofilm* slides in 24-h assays. Assays were started 1, 12, 24, 48, 72, 96 and 168 h after initial settlement of recruits on biofilmed slides

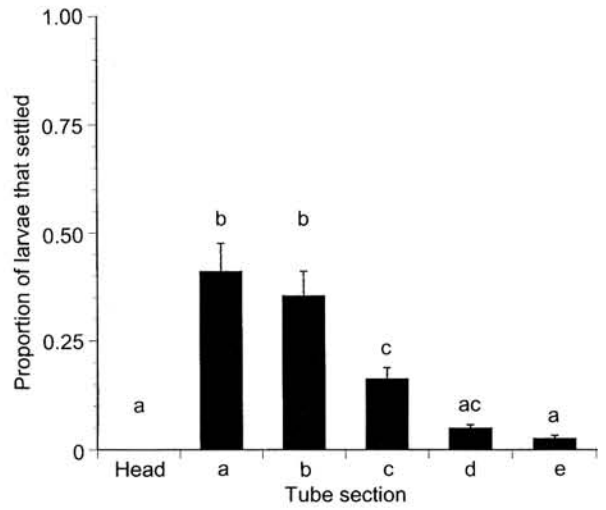

Fig. 3 *Hydroides dianthus.* Total proportion ($n = 25$, + 1 SE) of larvae that settled on each fifth (*a* to *e*) of the tube of conspecific adult worms and a sixth section extending anteriorly for the same length (*head*). The *head* section would normally be occupied by the appendages of a feeding worm. Larvae were assayed 7 d after fertilization for responses to adult slides in 24-h assays. *Letters above bars* designate groups among which the means did not differ significantly (multiple comparisons t-test with Bonferroni correction, $T_{CRIT} = 2.998$, $\alpha = 0.05$)

Settlement in response to location on adult tubes

To determine whether larval settlement responses to adult conspecifics were nonrandom with respect to position along the worm tube, we recorded the position of settlers along the tube in a separate set of assays. Most settlers on adult slides were concentrated along the anterior section of the tubes of adults (Fig. 3). The length of the tube was divided into five equal sections

(a to e, anterior to posterior), with an additional (sixth) section of the same length extending anteriorly from the tube opening (*head*). The head section would normally be occupied by the appendages of a feeding worm. The number of larvae found within each section was recorded at the end of the 24-h assay period. Significant differences in settlement were found among the tube regions (Kruskal–Wallis $H = 64.69$, $p < 0.01$, $df = 5$), but there was no settlement on or around the *head*. There were no differences in the responses of larvae between Sections a and b ($t = 0.72$, $p > 0.05$, $df = 38$), nor was there a difference between Sections d and e of the tube ($t = 0.78$, $p > 0.05$, $df = 38$). The middle of the tube (Section c) had intermediate settlement, being significantly lower than Section b ($t = 2.70$, $p < 0.05$, $df = 38$) and significantly higher than Section d ($t' = 2.24$, $p < 0.05$, $df \approx 33$). A Bonferroni pairwise comparison of means indicated three groups among which the means did not differ significantly (Fig. 3).

Settlement in response to whole worms, tubes and isolated portions of worms

The portion of the body in which the cue is elaborated was determined by assaying each portion separately (Fig. 4). Significant differences were found among the responses of larvae to these substrata (Kruskal–Wallis $H = 32.408$, $p < 0.01$, $df = 6$), but *adult*, *live worm* and *crown* were the only treatments with levels of settlement significantly higher than responses to *biofilm*. There were no significant differences in the responses of larvae to live conspecific *adults* in their tubes or to *live worms* removed from their tubes ($t = 0.96$, $p > 0.05$, $df = 10$), but settlement responses to both were significantly higher than to *biofilm* ($t = 11.08$ and 11.96, respectively, $p < 0.01$, $df = 10$). Settlement in response to *crown* was significantly lower than responses to *adult* ($t = 5.66$, $p < 0.01$, $df = 10$) and *live worm* treatments ($t = 5.33$, $p < 0.01$, $df = 10$), but was significantly higher than responses to *biofilm* ($t = 4.10$, $p < 0.01$, $df = 10$), *dead worm* ($t = 4.24$, $p < 0.01$, $df = 10$), *body* ($t = 2.54$, $p < 0.05$, $df = 10$), and *tube* treatments ($t = 3.96$, $p < 0.01$, $df = 10$). There were no differences in the responses of larvae to *dead worm*, *body*, *tube* and *biofilm* treatments (Kruskal–Wallis $H = 4.674$, $p > 0.05$, $df = 3$). A Bonferroni pairwise comparison of means found three groups among which the means did not differ significantly (Fig. 4).

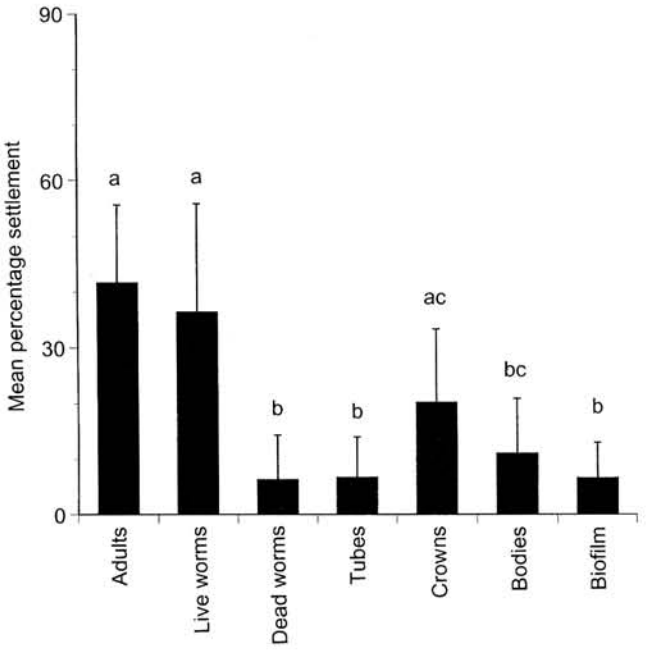

Fig. 4 *Hydroides dianthus*. Mean percentage settlement ($n = 6$, + 1 SE) of larvae in response to isolated portions of conspecific adults. Assays were run 7 d after fertilization to assess larval responses to worms or body portions attached to biofilmed slides: (1) conspecific worms in their tubes (*adults*); (2) intact live worms removed from their tubes (*live worms*); (3) intact worms removed from their tubes and killed by freezing (*dead worms*); (4) empty worm tubes (*tubes*); (5) tentacular crowns removed from live worms (*crowns*); (6) worm bodies removed from their tubes and with tentacular crowns amputated (*bodies*); or (7) plain biofilmed microscope slides (*biofilm*). *Letters above bars* designate groups among which the means did not differ significantly (multiple comparisons *t*-test with Bonferroni correction, $T_{CRIT} = 3.276$, $\alpha = 0.05$)

Cue solubility

The inductive compound(s) appear to be water soluble; larvae responded to biofilm in the presence of *adults*, even when prevented from contacting them by a 52-μm-mesh screen (Fig. 5). In assays in which larvae could contact the substratum, significantly more worms settled in response to *adult* and *worm* treatments than to *biofilm* and *tube* ($t = 3.45$ to 4.53, $p < 0.05$, $df = 6$). Patterns of settlement, however, were similar when larvae were permitted contact or denied contact with the experimental substrata (Fig. 5). There were no significant differences in the response of larvae to *biofilm* or *tube* treatments in either assay method ($t = 0.36$ and 0.97, $p > 0.05$, $df = 6$), but the total proportion of larvae settling was significantly greater when they could contact the experimental substrata (Kruskal–Wallis $H = 4.317$, $p < 0.05$, $df = 1$). A Bonferroni pairwise comparison of means confirmed three groups among which the means did not differ significantly (Fig. 5).

Cue stability and isolation in organic solvents

Crude organic extractions of a homogenized aggregation of adult *Hydroides dianthus* elicited gregarious settlement (Fig. 6). Freeze-drying the worms prior to extraction had no apparent effect on the activity of the extract; there were no differences among settlement

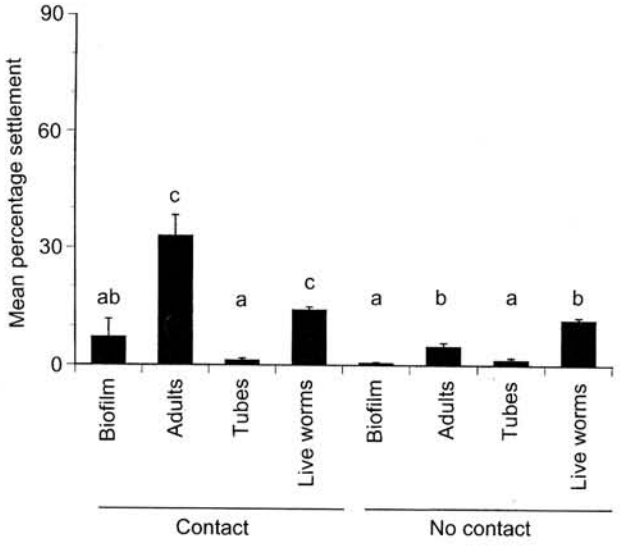

Fig. 5 *Hydroides dianthus.* Mean percentage settlement ($n = 5$, + 1 SE) of larvae that were able to contact experimental substrata (*contact*) and prevented from contacting experimental substrata by a 52-μm-mesh screen (*no contact*). Larvae were assayed 7 d after fertilization for responses to: *biofilm*, *adults*, *tubes* or *live worms*; in 24-h assays. *Letters above bars* designate groups among which the means did not differ significantly (multiple comparisons *t*-test with Bonferroni correction, $T_{CRIT} = 3.513$, $\alpha = 0.05$)

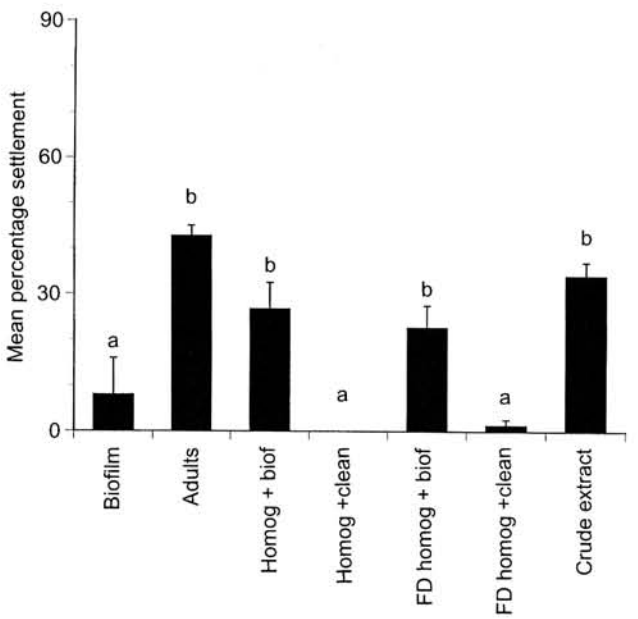

Fig. 6 *Hydroides dianthus.* Mean percentage settlement ($n = 12$, + 1 SE) of larvae in response to *biofilm*, *adult*, *homog*, *FD homog*, and *crude* extracts of whole aggregations on biofilmed slides. Responses to *homog* and *FD homog* were further assayed on new, unbiofilmed (*clean*) glass slides in 24-h settlement assays 7 d after fertilization. *Letters above bars* designate groups among which the means did not differ significantly (multiple comparisons *t*-test with Bonferroni correction, $T_{CRIT} = 3.151$, $\alpha = 0.05$)

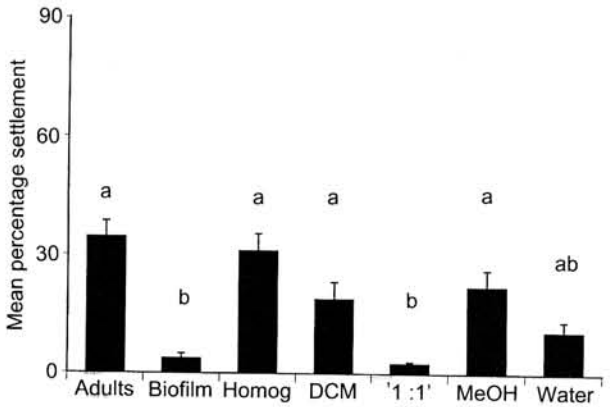

Fig. 7 *Hydroides dianthus.* Mean percentage settlement ($n = 17$, + 1 SE) of larvae in response to extracts of worms removed from tubes. Worms were extracted with a series of organic solvents consisting of respectively: dichloromethane (*DCM*), 1:1 dichloromethane:methanol (*1 : 1*), methanol (*MeOH*), and *water*. Larvae were assayed 7 d after fertilization for responses to *biofilm* (with and without worm extracts and homogenate) and *adult* slides in 24-h assays. *Letters above bars* designate groups among which the means did not differ significantly (multiple comparisons *t*-test with Bonferroni correction, $T_{CRIT} = 3.202$, $\alpha = 0.05$)

responses to *adult*, *FD homog*, *homog* or *crude* extract in the presence of biofilmed slides (Kruskal–Wallis $H = 6.11$, $p > 0.05$, $df = 47$). However, all four of these substrata elicited significantly greater settlement than did biofilmed slides ($t' = 3.78$ to 11.38, $p < 0.01$, $df \approx 6$ to 13). Extracts were not effective when applied to unfilmed (clean) slides (Fig. 6). Despite significant activity in the presence of *biofilm*, there were no differences between settlement responses to *biofilm* and to *FD homog* or *homog* applied to clean slides (Kruskal–Wallis $H = 3.86$, $p > 0.05$, $df = 35$).

Settlement responses were more variable in solvent-fractionated extracts than those seen in whole aggregation crude extractions. Assays (12 replicates) with solvent-partitioned extracts were repeated 17 times with different batches of larvae to overcome this increased variability. The means of these trials are presented (Fig. 7). There was no significant difference in the response to *adult*, *homog*, *DCM* and *MeOH* slides (Kruskal–Wallis $H = 4.8743$, $p > 0.05$, $df = 67$). Similarly, there was no significant difference in the response of larvae to *biofilm*, *1:1* or *water* slides (Kruskal–Wallis $H = 5.5504$, $p > 0.05$, $df = 50$). A Bonferroni pairwise comparison of means established that there were two groups among which the means did not differ significantly (Fig. 7).

Discussion

We have demonstrated previously that most larvae of the tube worm *Hydroides dianthus* settle gregariously, and that these larvae delay settlement in the absence of cues associated with conspecifics (Toonen 1993; Toonen and Pawlik 1994). The present study examines the nature of cues associated with adults that are

responsible for the gregarious settlement of larvae. We have discovered that, unlike other tube-dwelling polychaetes that have been studied to date (reviewed by Pawlik 1992), the inductive compounds responsible for gregarious settlement of *H. dianthus* are soluble in water and are associated with the body of living adults rather than the calcareous tubes they secrete. Moreover, production of the settlement inducer by juvenile worms does not begin until 4 d after metamorphosis. This delay in inducer production may be the result of maturation, or alternatively, it may be a consequence of small juveniles being incapable of producing sufficient concentrations of the inducer to affect larval settlement.

Our results suggest that there are at least two different cues to which gregariously settling larvae of *Hydroides dianthus* respond: biofilm on the substratum surface, and soluble chemical compound(s) released from adult or juvenile worms. Biofilm appears to be a prerequisite for larval settlement; in assays using adult homogenate (*FD homog* and *homog*) and crude organic extracts (*crude*), both of which induced significant levels of settlement on biofilmed slides, settlement was not significantly different from zero when an extracted cue was applied to clean (unfilmed) slides. A similar settlement requirement for biofilm has been demonstrated for the congeneric Pacific species, *H. elegans* (Hadfield et al. 1994). The crown-of-thorns starfish, *Acanthaster planci*, has been shown to metamorphose in response to a biofilm on the surface of crustose coralline algae (Johnson and Sutton 1994).

Because larvae of *H. dianthus* settled in response to adults even when prevented from contacting them, they must respond to a cue that is soluble. In the present study, significantly more larvae settled in assays in which contact was allowed, however, suggesting that a soluble cue is likely diluted at distance from the source, and the response is concentration dependent. Alternatively, multiple ranked cues could be involved in settlement, and contact with the adult-occupied substratum may provide further inductive cues to which larvae respond. Wethey (1984, 1986) demonstrated that multiple ranked cues were responsible for the settlement of two barnacle species, *Chthamalus fragilis* and *Semibalanus balanoides*.

Soluble cues have been implicated in the settlement behavior of several other invertebrate species (reviewed by Crisp 1984; Pawlik 1992), with a range of cue sources including postmetamorphic prey organisms (Hadfield and Scheuer 1985; Chia and Koss 1988) and sediments suitable for adult survival (Scheltema 1961, 1974). There is good evidence to suggest that soluble cues are also responsible for gregarious settlement of sand dollars (Highsmith 1982) and oysters (Zimmer-Faust and Tamburri 1994). Recently, Lambert and Todd (1994) provided evidence that a water-borne cue is involved in inducing metamorphosis in the dorid nudibranch *Adalaria proxima*, and Stoner (1994) found that larvae of the colonial ascidian *Diplosoma similis* use a noncontact mode of substratum selection on coral reefs.

The present study represents the first demonstration of gregarious settlement of a polychaete worm in response to a soluble cue. Sabellariid worms of the genus *Phragmatopoma* settle with a high degree of specificity on the sand tubes of adult conspecifics (Pawlik 1986), but for these worms, perception of the cue is dependent on contact with the tube, and a biofilm is not required for metamorphosis (Jensen and Morse 1984; Pawlik 1988a, b). Contact with the substratum is also important for the spirorbid polychaete *Janua brasilensis*, which settles preferentially on microbial films cultured from the green alga *Ulva lobata* (Kirchman et al. 1982a, b). But soluble cues may be important for some polychaetes that settle associatively: Marsden (1987) discovered that even precompetent larvae of the serpulid worm *Spirobranchus giganteus*, an obligate coral associate, would swim toward some coral species in preference to other substrata in assays performed in small volumes of still water.

Crisp (1965, 1984) argued that larval cues for settlement and metamorphosis should be insoluble (reviewed by Pawlik 1992). First, turbulent water flow would dilute a soluble cue to negligible concentrations within a very short distance (Butman 1986). Second, the small sizes of most invertebrate larvae would make orientation and navigation in a concentration gradient largely ineffective. There appear to be two ways that larvae could perceive a chemical gradient: (1) by detecting a difference in concentration between receptors located on opposite ends of the body, or (2) by integrating concentration changes through time as the larva moves through the water column. The former appears implausible because a concentration gradient across the length of a larva seems likely to be imperceptible, and the latter seems equally unlikely because larvae are far more apt to travel along with a water mass than move through it (Denny and Shibata 1989).

In light of the theoretical difficulties with both the perception and response of larvae to soluble cues, it may be that gregariously settling larvae of *Hydroides dianthus* perceive soluble cues only at very short range. In both of our experiments in which half the larvae were allowed to contact the substrata and the others were not, significantly more larvae settled in response to living conspecific adults (within or removed from their tubes) than to either biofilm or uninhabited conspecific tubes. As previously stated, under natural conditions, a soluble cue would be diluted rapidly in turbulent flow. Therefore, it seems likely that the inducer would be present in appreciable concentrations only in the viscous boundary layer adjacent to the substratum. The thickness of the boundary layer under natural flow conditions would be similar to the size of an individual larva (Butman 1986), and therefore, larval responses to a diffusing cue may be virtually equivalent to responses to substratum-derived insoluble cues in

the field. Adult worms might also mediate larval settlement by entraining or catching larvae in their feeding tentacles and depositing them near the anterior ends of their tubes, where soluble cues should be at their highest concentrations.

Homogenates of whole adult worm aggregations elicited settlement from competent larvae, and freeze drying the samples did not significantly decrease the inductive activity, suggesting that the cue is relatively stable. The inductive activity of the homogenates and extracts indicates that a chemical cue is responsible for settlement, because no physical cues associated with adult conspecifics were available to the larvae in these assays. Extraction of washed worms, which had been previously removed from their tubes, confirmed that the cue was associated with the worm bodies, rather than the tubes. Activity was concentrated in both the nonpolar (DCM) and polar (MeOH) fractions of the extraction series, suggesting that the cue may consist of a mixture of compounds of differing polarities. Further characterization of the compounds that induce gregarious settlement of *Hydroides dianthus* is a subject for future research, but it is clear that the chemical induction of invertebrate larval settlement is not currently subject to broad generalizations, either in terms of cue solubility, or the structural class of inductive compound.

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