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ORIGINAL PAPER

Biofilm cue for larval settlement in *Hydroides elegans* (Polychaeta): is contact necessary?

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Abstract Larvae of many sessile marine invertebrates settle in response to surface microbial communities (biofilms), but the effects of soluble compounds from biofilms in affecting larval behavior prior to settlement, attachment, and metamorphosis have been little studied. This question was addressed by videotaping the behavior of competent larvae of the serpulid, *Hydroides elegans*, above settlement-inducing biofilms. Adult worms were collected in Pearl Harbor, Hawaii, USA in November 2012 and spawned almost immediately. Six-day old larvae were placed in five replicated treatments in small cups: (1) with a natural biofilm; (2) with a natural biofilm on an 8-μm screen, 1 mm above the bottom of a clean cup; (3) with a natural biofilm beneath a clean screen; (4) in a clean cup; and (5) in a clean cup with a clean screen. Using the videotapes, larval swimming speeds and trajectories were quantified within 5 min of the larvae being placed in a treatment. Only larvae that touched a biofilm, i.e., in treatments (1) and (2), slowed their swimming speed and increased the amount of time spent crawling rather than swimming. This shows that under these conditions, any soluble cues emanating from a biofilm do not affect settlement behavior. Furthermore, after 24 h close to 100 % of larva in the two accessible biofilm treatments had metamorphosed and <15 % in treatments that included a biofilm under a clean screen and no biofilm at all, strongly suggesting that soluble cues for settlement were not produced by the biofilms over the longer time period.

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Introduction

Because of their role in establishing benthic marine communities and biofouling of ships, settlement and metamorphosis of marine invertebrate larvae have drawn great interest almost since planktonic larvae were first recognized to be the progeny of invertebrates. Early studies of invertebrate reproduction assumed that the massive production of eggs by many marine invertebrates allowed larval settlement to be a random process, i.e., the few larvae fortunate enough to descend to a suitable site at the end of larval life were sufficient to establish and maintain communities. However, by the 1950s, the studies of Wilson (1952, 1953a, 1953b, 1954, 1955) and others had revealed that larvae of some polychaetes settle selectively on suitable substrata and avoid unsuitable substrata. Subsequent investigations have focused on selective settlement by larvae of nearly every major phylum of marine invertebrates, and it is now well established that the larvae of many species settle in response to specific environmental cues (reviewed by Pawlik 1992; Hadfield and Paul 2001). For many species, it is clear that the cues are associated with requisite plant or animal prey, conspecific adults, or surface biofilms composed of bacteria, diatoms, and other microorganisms.

Despite an abundance of studies on invertebrate larval settlement, the nature of the often very specific cues to which larvae respond remains poorly known except for a few examples. Although larvae of some invertebrate species

are induced to settle and metamorphose by dissolved cues arising from benthic organisms (e.g., the nudibranch *Phestilla sibogae*, Hadfield and Pennington 1990; the echinoid *Heterocidaris erythrogramma*, Swanson et al. 2006; the oyster *Crassostrea virginica*, Tamburri et al. 1996), larvae of many other species settle in response to surface-bound cues (e.g., barnacle cyprids, Crisp and Meadows 1963; spirorbid polychaetes, Kirchman et al. 1982a, b; the mud snail *Ilyanassa obsoleta*, Scheltema 1961).

A traditional approach to understanding the nature of settlement cues is to examine the behavior of larvae that are competent to metamorphose and in close proximity to a requisite cue. Early studies on the behavior of settling larvae of sessile species such as oysters, barnacles, bryozoans, and other groups demonstrated the efficacy of such an approach. Crisp (1974) and others described a two-stage process, defining the first step as "settlement," when a larva leaves the plankton to explore a surface, a reversible step, and the second as "fixation and metamorphosis," an irreversible event. We will adhere to these definitions. While the pioneering studies of the behavior of minute larvae employed either manual tracking with microscopes and camera lucidas, or, subsequently, by tracing tracks from ciné films [Crisp (1974) reviewed these results], modern approaches allow greater ease of tracking and detailed analyses from digital videotapes and image-analysis software (e.g., Zimmer-Faust et al. 1996; Marechal et al. 2004). We utilized the latter approach.

Most investigations of settlement stimuli have relied on simple metamorphosis assays, i.e., larvae are exposed to various suspected settlement stimuli (solid or dissolved) for a set period after which the percentage of the larvae that metamorphosed is determined and compared with a control surface without a stimulus. Some studies of behavior in response to suspected dissolved cues suggest that separate cues may stimulate settlement behavior (i.e., descent from the water column and surface exploration) from those that cause final attachment and metamorphosis (e.g., Krug and Zimmer 2000; Santagata 2004; Swanson et al. 2006).

The circum-globally distributed, warm-water serpulid *Hydroides elegans* is an excellent model for investigating factors that stimulate larval settlement and subsequent metamorphosis (e.g., Hadfield et al. 1994; Carpizo-Ituarte and Hadfield 1998; Nedved and Hadfield 2009). Planktotrophic larvae of *H.* elegans develop rapidly from small eggs (40–50 μm in diameter) to competent nectochaetes at a length of 225 μm in 5 days at 24–26 °C (Carpizo-Ituarte and Hadfield 1998). Larvae of *H. elegans* settle, attach, and metamorphose in response to complex marine biofilms or to biofilms formed by single species of bacteria isolated from biofilms (Unabia and Hadfield 1999; Huang and Hadfield 2003; Lau et al. 2005). Competent larvae typically attach to a surface, secrete a primary tube, and commence metamorphosis within ~15 min of contact with an inductive biofilm (Carpizo-Ituarte and Hadfield 1998). A bacterium shown to strongly induce settlement in larvae of *H. elegans* in single-species biofilms, *Pseudoalteromonas luteoviolacea*, has been investigated at the genome level to analyze the inductive factors (Huang and Hadfield 2003). Huang et al. (2012) identified a set of genes that are essential to the inductive capacity of *P. luteoviolacea*, and Shikuma et al. (2014) demonstrated that an expanded gene set produces complex arrays of organelles known as bacteriocins that must be present in their entirety for metamorphosis in larval *H.* elegans to occur. Shikuma et al. demonstrated that the bacteriocin clusters appear to be maintained by the complex layer of extracellular polymeric substances in which biofilm bacteria are embedded. Thus, although the bacterial source of a requisite, insoluble metamorphic cue for *H. elegans* is established, whether or not a separate and soluble bacterial metabolite stimulates settlement behavior leading to surface exploration and, ultimately, attachment and metamorphosis, remains in question. The present study sought to clarify whether a soluble bacterial cue that stimulates larval pre-attachment behavior in *H. elegans* is produced from biofilms or if the biofilms must be physically contacted by the larvae to elicit behavioral changes or induce metamorphosis.

Our goals were (1) to determine whether the behavior of larvae of *H. elegans* as they approach a surface is altered by detection of soluble metabolites arising from a biofilm, or only by contact with a biofilmed surface and (2) to ascertain whether dissolved chemicals from biofilms can induce metamorphosis, or whether contact with a biofilmed surface is required. We video-recorded and analyzed larval behavior in settings where larvae could make contact with a metamorphosis-inducing biofilm and where they were in water that bathes such a biofilm but were separated from it by a fine screen one millimeter above it. The experiments were performed in still water with the recognition that in ambient currents over fouling communities in harbors, water within several hundred microns from surfaces is swept away every few seconds by eddies in the boundary layer (Koehl at el. 2013), thereby dispersing soluble cues from a surface biofilm.

Materials and methods

Larval culture

Adult *H. elegans* were collected from our established field site on Ford Island in Pearl Harbor, Hawaii (21°21′25.5″N, 157°57′35.9″W) on November 19–20, 2012. Larvae of *H. elegans* were obtained by spawning the adults on November 21, and five batches of larvae, each parented

by a different group of 3–5 males and females per batch, were cultured using the methods of Nedved and Hadfield (2009). The embryos were raised in beakers (2 l) in 0.22-μm-filtered seawater (FSW) at a concentration of 10 larvae ml^{-1} at 25–26 °C. FSW in the larval cultures was changed daily when the larvae were fed the single-celled alga *Isochrysis galbana* (Tahitian strain) at a concentration of 6×10^4 cells ml^{-1}. The larvae used in our experiments were first assayed for metamorphic competence at day 5 post-fertilization by exposing them to natural biofilms on glass slides that had been conditioned for 1 week or longer in flowing, unfiltered seawater. Percent metamorphosis was determined after 24 h. Only larval batches that showed at least 75 % metamorphosis were included in the experiments described here, which were conducted when the larvae were 6 days old.

Plate preparation

Experiments were carried out in six-well Corning Transwell plates. Each well (34 mm diameter × 22 mm deep) held a removable cup the bottom of which was covered by a 10-μm thick screen with a pore size of 8 μm at a density of 10^5 pores cm^{-2}. When a cup was placed in a well, the screen was suspended ~1 mm above the bottom surface of the well. Some plates and their cup inserts were set aside and left clean and sterile, while others were submerged in Pearl Harbor so that natural biofilms could form on their surfaces. Plates and cups were mounted separately on Vexar screens and hung from a dock with their openings facing downwards. After a week in the field, the plates and cups were removed from Pearl Harbor and transported to the laboratory in a bucket of sea water collected at the field site. In the laboratory, the plates and cups were gently washed with FSW to remove loose debris. After rinsing, the biofilmed cups were submerged in a beaker of FSW, and 3 ml of FSW were added to each of the biofilmed wells. At the same time, 3 ml of FSW were also added to each of the wells in the clean sterile plates. Depth of water in cups without a screen at the bottom of the inner cup was 4.0 mm. Depth of water above the screens in those cups with a screen at the bottom of the inner cup was also 4.0 mm due to volume displacement by the cup.

Video recording of larvae over different substrata

The effects of different substrata on larval behavior and metamorphosis were measured. Larvae from each of the five batches were exposed to the following treatments: (1) a well in a biofilmed plate with no screen (so larvae could contact the biofilm); (2) a well in a clean plate covered by a biofilmed screen (to control for screen effects on behavior or metamorphosis when larvae could contact biofilm); (3) a well in a biofilmed plate covered by a clean screen (so larvae could not contact the biofilm, but were exposed to water potentially containing soluble metabolites from the biofilm); (4) a well in a clean plate; and (5) a well in a clean plate covered by a clean screen (to control for screen effects on behavior or metamorphosis when no biofilm was present). The appropriate cups with screens were inserted into the FSW-filled wells in which treatments 2, 3, and 5 were conducted, and clean cups from which the screens had been removed were inserted into wells in which treatments #1 and #4 were conducted.

Before videotaping each well with larvae, a cup was inserted with or without an attached screen and water within the well was allowed to equilibrate for 4 min. Then, ~50 larvae were pipetted into each well in a small volume of FSW (<0.5 ml). After filling all wells, the plate was gently swirled to distribute the larvae and dissolved substances throughout the cup. To standardize across treatments within each replicate, we used a randomized block design to select the order of video recording of the treatments within each replicate. Each replicate contained a different set of wells and screens.

A video record of larvae in each well was made using a Sony Handycam HDR-HC3 mounted on a Zeiss Stemi SV11 dissecting microscope. The camera was mounted on a camera tube on the top of the microscope, so that the view plane was straight down, perpendicular to the bottom of the dish. The wells were illuminated from below. Competent larvae of *H. elegans* are indifferent to the direction of illumination. The field plane in the video frames was 27.6 mm by 26.7 mm, but we analyzed only the arena within a well, a circle with a diameter of 24 mm, which was centered in the frame. Each video record was started 1 min after the larvae were added to a well and swirling motion of the water had ceased. Behavior was recorded for a period of 1.5 min. Because we could measure only the horizontal components of larval swimming velocity, we may have underestimated the velocities of larvae that also had a vertical component to their swimming direction

Assays of metamorphosis on different substrata

After the video recordings were completed, the experimental plates were set aside for 24 h under ambient day–night illumination. Then, the plates were observed under a dissecting microscope and the numbers of larvae that had settled and metamorphosed or were still swimming in each well were counted and recorded.

Video analysis of larval behavior

The video records for each replicate of each treatment were converted into digital.avi format using Windows Movie Maker 2012 software. Each frame was 1,920 × 1,080 pixels.

VirtualDub 1.9 software was used to select every third frame from the video to make a new video (900 frames long) with an inter-frame interval of 0.1 s. After this conversion, a larva occupied 20–60 pixels, depending on orientation. ImageJ software (version 1.47) was used to enhance contrast and to convert the video into a stack of .tif images. ImageJ (version 1.33i) with a PTV (Particle Tracking Velocimetry) manager plugin was used to digitize larval trajectories. The range of sizes of dots in the video frames to be tracked was determined by running a horizontal transect along the midline of the first frame of each video and measuring the length of each larva encountered. The positions in each video frame of all larvae that were visible in the video for more than 20 frames were digitized. These data were used to calculate locomotory speeds and quantify the straightness of larval trajectories. Based on the these data, larval movements were assigned to one of four categories—straight swimming, turning, circling or crawling—as described in detail in Results.

Data analysis

The proportions of larvae evincing different behaviors were transformed by the arcsine of the square root of the variate (Sokal and Rohlf 1981). One-way ANOVA's and Tukey–Kramer HSD pair-wise comparisons ($\alpha = 0.05$) were performed using JMP Pro 10 software (SAS Institute Inc., Cary, North Carolina, 1989–2007), and Mann–Whitney *U* tests were performed using StatView 5.0 software. Means and standard deviations were transformed back to proportions and converted to percentages for preparing graphs.

Results

Pre-attachment larval behavior

Examples of the trajectories of competent larvae of *H. elegans* over a biofilmed surface and over a clean surface are shown in Fig. 1. We categorized the movements of larvae at each instant along its trajectory using the following operational definitions based on speed and path curvature. As Fig. 1 illustrates, a single larva could engage in more than one type of movement.

"Straight Swimming": A larva's instantaneous speed was $\geq$60 μm s^{-1} and it moved along a straight line. (We measured the speeds of crawling larvae in higher magnification videos and found that crawling speeds varied with time but were always <60 μm s^{-1}. Therefore, we operationally defined swimming as moving at 60 μm s^{-1} or faster.)

"Turning": A larva's instantaneous speed was $\geq$60 μm s^{-1}, and its path had a curvature >9 cm^{-1} for a period $\leq$20 frames ($\leq$2 s). The curvature at each point in the trajectory was calculated from a three point discrete approximation where curvature $= (x'y'' - y'x'')/(x'^2 + y'^2)^{3/2}$. The result is the inverse of the radius of curvature and has the units cm^{-1}.

"Circling": A larva's instantaneous speed was $\geq$60 μm s^{-1}, and its path had a curvature >9 cm^{-1} for a period >20 frames (>2 s), which was long enough for the larva to swim in one complete circle.

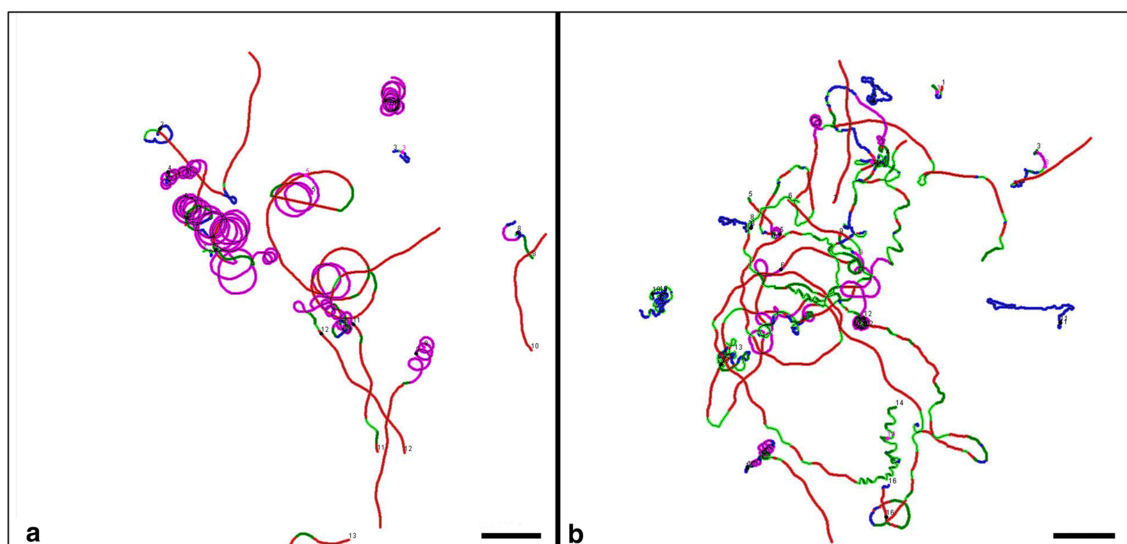

Fig. 1 Digitized trajectories of larval movements at the bottom of a well over **a**, a clean surface, and **b**, a biofilmed surface, video-recorded vertically through a dissecting microscope. *Red* straight swimming; *green* turning; *pink–purple* circling; *blue* crawling. *Scale bar* = 2 mm

"Crawling": A larva's instantaneous speed was <60 µm s^{-1}.

A. Effects on larval movement of exposure to water from a biofilm versus direct contact with a biofilm.

1. Is the speed of larval movement different when they can make physical contact with a biofilm versus when they are separated from a biofilm by a clean screen?

A single larva could engage in more than one type of movement (Fig. 1). For each larva, we calculated the mean of the speeds at which it moved during the portions of its trajectory when it was engaged in Straight Swimming, when it was Circling, and when it was Crawling. Then, for each replicate of a treatment, we calculated the mean of those mean speeds for each type of movement, and, finally, we calculated a grand mean for the speed at which larvae performed each of these movement types across all five replicates for that treatment. These data are graphically presented in Figs. 2, 3 and 4. For each type of movement, we tested whether the larval speeds were significantly different between the five treatments using a one-way ANOVA followed by a Tukey–Kramer HSD pair-wise comparisons (Fig. 5).

Larvae exposed only to dissolved substances from biofilms (i.e., larvae in the treatment in which they were separated from a biofilmed surface by a clean screen) moved at the same speeds as larvae in treatments with no biofilm present (i.e., clean well, and clean screen over a clean well) when they were engaged in Straight Swimming (Fig. 2), Circling (Fig. 3), and Crawling (Fig. 4); note that larvae whose predominant movements were behaviorally categorized as Swimming-and-Touching behavior, below and in Fig. 6, they had brief crawling episodes whose speeds were determined. Thus, under the described experimental conditions, there was no evidence that larval behavior indicative of exploration or settlement occurred in response to soluble substances arising from a biofilm.

When larvae could make physical contact with a biofilm, they swam more slowly, but crawled at the same speed as they did over a clean surface, which they did only briefly. Larvae in treatments in which they could touch a biofilm (biofilmed well, and biofilmed screen over a clean well) swam significantly more slowly, while Circling than did larvae over clean surfaces (clean screen over biofilmed well, clean well, and clean screen over clean well) (Fig. 3). Similarly, larvae engaged in Straight Swimming in biofilmed wells and over biofilmed screens swam more slowly than larvae over clean surfaces, although the difference was not significant (Fig. 2). There was no significant difference between the Crawling speeds of the larvae in any of the treatments (Fig. 4). Therefore, we cannot reject the possibility that contact with a biofilm induces larvae to swim more slowly, although it is clear that contact with a biofilm induces larvae to crawl.

2. Do larvae alter the straightness of their trajectories when over touchable biofilms?

For each larva, we calculated the straightness index of its entire trajectory, which could have included several types of behavior (Straight Swimming, Turning, Circling, and Crawling). The straightness index (Hadfield and Koehl 2004) is the ratio of the distance between the position of the larva at the start of the trajectory and the end of the trajectory, to the length of the path that the larva followed during its trajectory. For each replicate of a treatment, we calculated the mean of the straightness indices for all the larvae in that replicate. For each type

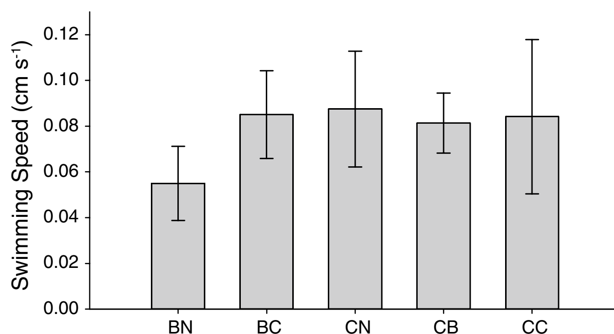

Fig. 2 Mean speed of larvae engaged in Straight Swimming in each of five treatments: *BN* biofilmed well, no screen; *BC* biofilmed well, clean screen; *CN* clean well, no screen; *CB* clean well, biofilmed screen; *CC* clean well, clean screen. *Error bars* one SD ($n = 5$ replicates). Swimming speeds not significantly different [ANOVA, $F(4,20) = 2.68$, $p = 0.061$]

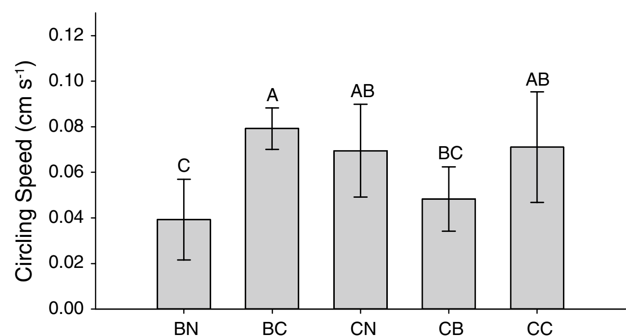

Fig. 3 Mean speed of larvae Swimming and Circling in five treatments: *BN* biofilmed well, no screen; *BC* biofilmed well, clean screen; *CN* clean well, no screen; *CB* clean well, biofilmed screen; *CC* clean well, clean screen. *Error bars* one SD ($n = 5$ replicates). *Letters above bars* treatments where means do not differ significantly from each other [ANOVA, $F(4,20) = 5.01$, $p = 0.006$, followed by Tukey–Kramer HSD pair-wise comparisons, $\alpha = 0.05$]

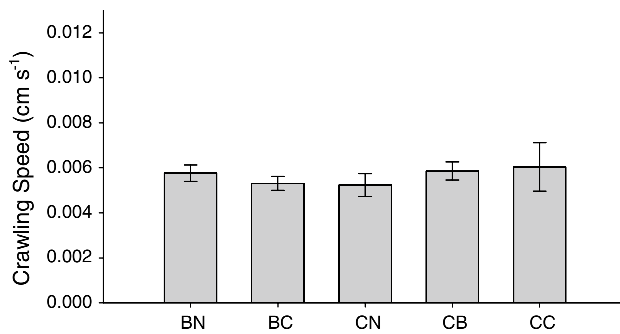

Fig. 4 Mean speed of larvae Crawling in five treatments: *BN* biofilmed well, no screen; *BC* biofilmed well, clean screen; *CN* clean well, no screen; *CB* clean well, biofilmed screen; *CC* clean well, clean screen. *Error bars* one SD ($n = 5$ replicates). Crawling speeds were not significantly different [ANOVA, $F(4,20) = 2.44$, $p = 0.082$]

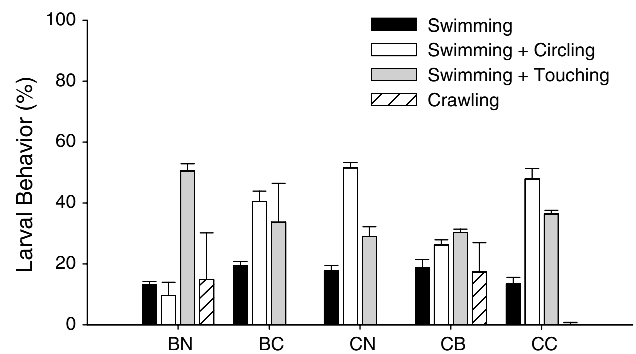

Fig. 6 Mean percent of larvae engaged in one of four behavioral categories during 1.5-min recording period in five treatments: *BN* biofilmed well, no screen; *BC* biofilmed well, clean screen; *CN* clean well, no screen; *CB* clean well, biofilmed screen; *CC* clean well, clean screen. *Error bars* one SD ($n = 5$ replicates)

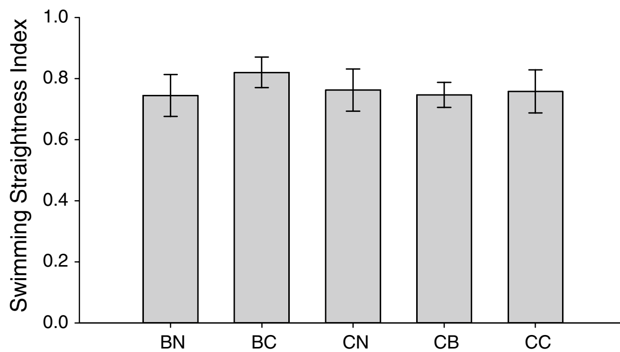

Fig. 5 Mean straightness indices of larval swimming trajectories in each of five treatments: *BN* biofilmed cup, no screen; *BN* biofilmed well, no screen; *BC* biofilmed well, clean screen; *CN* clean well, no screen; *CB* clean well, biofilmed screen; *CC* clean well, clean screen. *Error bars* one SD. ($n = 5$ replicates). No significant differences in swimming straightness indices [ANOVA, $F(4,20) = 1.99$, $p = 0.134$]

of behavior, we tested whether the trajectory straightness indices were significantly different among the five treatments using a one-way ANOVA followed by Tukey–Kramer HSD pair-wise comparisons. There was no significant difference between the straightness indices of larval trajectories in any of the treatments (Fig. 5). Therefore, although larvae engaged in different types of behaviors when they could touch biofilms versus when they could not (e.g., more crawling and less circling over touchable biofilms), the net straightness of their entire trajectories did not differ among treatments.

B. Effects of treatment on the percentage of larvae engaged in different behaviors

Although many of the larvae engaged in different movement categories during one trajectory, we put each larva into a single behavioral category based on its predominant activity so that we could compare the numbers of larvae behaving in different ways in the different treatments. We used the following operational definitions to categorize the type of behaviors used by each larva:

> "Swimming" behavior: A larva engaged in straight swimming or straight swimming and turning during its entire trajectory.
> "Swimming-and-Circling" behavior: A swimming larva circled one or more times during its trajectory.
> "Swimming-and-Touching" behavior: A larva that was swimming or circling, touched the substratum briefly and crawled for >3 frames (>0.3 s).
> "Crawling" behavior: A larva crawled for its entire trajectory.

The percentages of larvae engaged in each of these behaviors in the five treatments are plotted in Fig. 6.

1. Do larvae stop swimming and crawl in response to soluble settlement cues from biofilms?

Larvae of *H. elegans* always settle onto a surface and crawl before making a permanent attachment, secreting a primary tube, and completing metamorphosis within the primary tube (Carpizo-Ituarte and Hadfield 1998). We found that a significantly larger percentage of the larvae were Crawling on biofilmed surfaces (biofilm, and biofilmed screen) than on clean surfaces and clean permeable screens suspended directly above a biofilm (Fig. 6). In the treatment where larvae could not touch the biofilm but were exposed to dissolved substances from the biofilm, no larvae spent the entire recording period Crawling in any of the replicate experiments. The percentage of larvae exhibiting the two categories of swimming were significantly lower only when larvae could touch a biofilm. In the absence of evidence for alterations of larval behavior

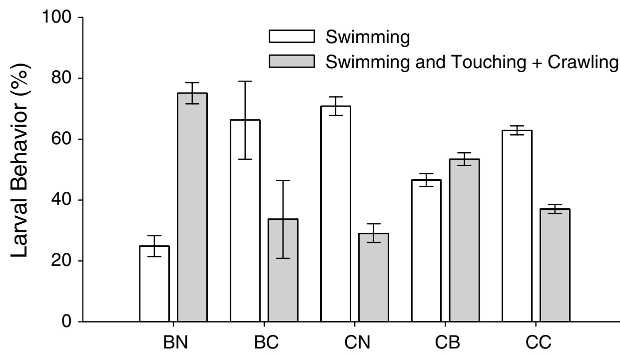

Fig. 7 Mean percent of larvae that only swam (sum of percent Swimming and percent Circling in each replicate) (*white bars*) versus mean percent of larvae touching the substratum (sum of percent Swimming-and-Touching and percent Crawling in each replicate) (*gray bars*). *BN* biofilmed cup, no screen; *BC* biofilmed cup, clean screen; *CN* clean cup, no screen; *CB* clean cup, biofilmed screen; *CC* clean screen, clean cup. *Error bars* one SD ($n = 5$ replicates)

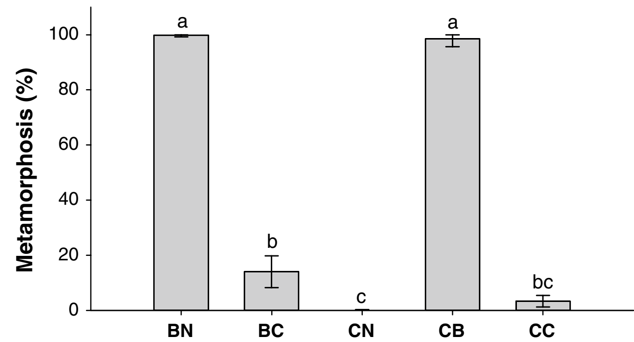

Fig. 8 Mean percentage of larvae completing all stages attachment and metamorphosis after 24 h in five treatments: *BN* biofilmed cup, no screen; *BC* biofilmed cup, clean screen; *CN* clean cup, no screen; *CB* clean cup, biofilmed screen; *CC* clean screen, clean cup. *Letters above bars* treatments for which means are not significantly different [ANOVA, $F(4,20) = 110.43$, $p < 0.0001$, followed by Tukey–Kramer HSD pair-wise comparisons]

based on biofilm-bathed water alone, we reject the hypothesis that larvae settle and crawl in response to dissolved substances arising from a biofilm. An alternative explanation of our results is that the 3.0 ml of water in each of our wells was not in contact with the biofilm long enough to accumulate a concentration of chemical cue high enough to induce behavioral changes. However, results of the 24-h metamorphosis assays given below are not consistent with this alternative explanation.

2. Do larvae respond to contact with biofilmed surfaces?

We compared the percentage of larvae engaged in each type of behaviors when they could touch a biofilm (biofilmed well and clean well with biofilmed screen) with the percentage of larvae engaged in each behavior when they could not make physical contact with a biofilm (clean screen over a biofilmed well, clean well, and clean screen over a clean well). A significantly greater percentage of the larvae were observed Crawling over biofilmed surfaces than over clean surfaces (Mann–Whitney *U* test, $p = 0.005$). In contrast, Swimming-and-Touching behavior was common in all of the treatments (Fig. 6) and was more prevalent in the dishes with a biofilmed surface or a biofilmed screen, i.e., where larvae could make physical contact with the biofilm (Fig. 7). There also was no significant difference between the percentages of larvae showing Swimming behavior when directly over biofilmed versus over clean surfaces (Mann–Whitney *U* test, $p = 0.488$) (Fig. 7). Although larvae swimming over biofilmed surfaces that they could touch often executed turns (e.g., see green segments of trajectories over a biofilm in Fig. 1), the percentage of larvae that swam in circles (Swimming-and-Circling behavior) was significantly lower over touchable biofilms than over clean surfaces (Mann–Whitney *U* test, $p = 0.0003$) (Fig. 6) (e.g., see pink segments of trajectories in Fig. 1). In addition, there were more larvae in the two behavioral categories that included surface contact (Swimming-and-Touching, Crawling) than in swimming categories only when the larvae had direct access to the biofilm (Fig. 7).

Metamorphosis

The percent of larvae that had completed metamorphosis and tube formation was determined 24 h after introduction to the treatment wells. Significantly greater numbers of larvae (>90 %) completed settlement, attachment, primary tube formation, and metamorphosis when in contact with a living biofilm, either on the dish bottom or an 8-μm screen, than when no biofilm was present (one-way ANOVA, Tukey–Kramer LSD) (Fig. 8). The small mean percent of larvae that metamorphosed on a clean screen suspended above a biofilm occurred in only one of the five replicates. This may be explained by a sufficient biofilm growing on the screen to induce some larval settlement during the period before metamorphosis was assayed.

Discussion

Analyses of the experiments described here appear to eliminate the possibility that soluble substances released by natural marine biofilms elicit changes in behavior of larvae of *H. elegans* that result in close approach and necessary contact with a biofilm, followed by definitive attachment and metamorphosis. Although dissolved organics were not

analyzed in these experiments, if such substances were present they did not affect larval behavior in the short term nor did their accumulation result in significant settlement and metamorphosis after 24 h (Figs. 7, 8).

There are numerous reports of larvae of a wide spectrum of marine invertebrates settling in response to dissolved cues, for example, oysters (e.g., Hidu 1969; Tamburri et al. 1992, 1996; Turner et al. 1994; Zimmer-Faust et al. 1996), phoronids (Herrmann 1979, 1995; Santagata 2004), the mud snail *I. obsoleta* Scheltema 1961; Leise et al. 2009), another gastropod, *Crepidula onyx* (Zhao and Qian 2002), and some barnacles (Elbourne et al. 2008; Elbourne and Clare 2010). However, physical contact with specific surfaces—in most cases another organism or a bacterial film—is reported to be necessary for metamorphosis to occur for many other invertebrate species (e.g., Matson et al. 2010; Penniman et al. 2013, earlier papers reviewed by Hadfield and Paul 2001). The importance of marine biofilms, and especially their bacterial members, for the settlement of larvae from many invertebrate phyla and many very different habitats has become increasingly apparent (Hadfield 2011). Because bacteria are so small (<1 µm) and frequently motile, they pass through all but submicron filters and establish biofilms quickly on previously clean surfaces. Thus, in conducting experiments, if the water to which larvae are experimentally exposed was passed through something other than a filter with a pore diameter < 1.0 µm (e.g., a "mesh bag," Hidu 1969; or fine screens or bolting cloth, Thompson 1958, Scheltema 1961), it undoubtedly contained millions of bacteria. Furthermore, if a metamorphosis assay ran for >24 h, there was more than sufficient time for a bacterial film to become established on the surfaces on which the larvae were settling. To exclude the possibility of biofilm buildup on the screens in our experiments, we separated larvae from a biofilm with a fine (8-µm pore diameter) screen and followed larval behavior within 5 min of the insertion of the screen into the well above the biofilm. Thus, our "clean screen over a biofilm" was essentially free of bacteria at the time larval behavior was videotaped, although not by the time, we counted total larvae metamorphosed at 24 h.

While it is established that competent larvae of *H. elegans* will settle and metamorphose with no cue other than a marine biofilm, even a mono-specific bacterial film (Hadfield et al. 1994; Unabia and Hadfield 1999; Lau et al. 2003; Huang and Hadfield 2003), there has remained uncertainty about whether soluble bacterial metabolites first stimulate larvae to make contact with a surface. Reports that larvae of *H. elegans* settle in response to soluble cues from adult conspecific worms to bring about gregarious recruitment (Harder and Qian 1999) are contradicted by experimental laboratory and field data (Walters et al. 1997) clearly showing that the larvae recruit equally beside live worms, empty worm tubes, and plastic worm-tube mimics. Thus, in the present study, we quantified and compared the behavioral responses of larvae of *H. elegans* when they were able to make physical contact with a biofilm versus when they were only 1 mm away from a settlement-inducing biofilm but separated from it by a screen. The screen should have allowed ready diffusion of a solutes from the biofilm into the water above the screen (4 mm deep), and the water in the well was well mixed and could pass through the screen when the larvae were added at the start of each experiment. Harder et al. (2002), in an effort to isolate bacterial metabolites from inductive bacteria, separated competent larvae from inductive biofilms by a 90-µm mesh in what they described as a "double-compartment test vessel" (not illustrated by the authors) and noted the absence of metamorphosis in the compartment lacking a bacterial film. These authors did not quantify larval behavior, and swirling their experimental dishes at 50 rpm to disperse bacterial metabolites to the "clean" side of the vessel seemingly prevented close observations on larval behavior.

Our results indicate that only when larvae of *H. elegans* made physical contact with a biofilm was their swimming speed affected, i.e., slowed. This was reflected in both straight-path swimming and circling behaviors. Because the depth of water over both the solid biofilmed surface and a biofilmed screen was only 4 mm, it is likely that all swimming trajectories would lead larvae to occasionally brush the surface and thus be "cued" to its presence, if it was coated by a bacterial film. The result of biofilm contact may result in a switch to swimming and touching behavior, which was more prevalent in the two settings where larvae could make contact with a biofilm (Fig. 7). Swimming and touching behavior would allow surface testing that, when a biofilm is present, stimulates larvae to actually settle onto the surface and crawl. The instances of larvae crawling throughout our filming interval, 90 s, were almost exclusively in settings where larvae could contact the biofilm on both solid surfaces and screens. That crawling behavior is the prelude to permanent attachment and metamorphosis is substantiated by the findings that these two processes were frequently observed in the wells with accessible biofilm.

The behavior of larvae of *H. elegans* swimming in an arena close to a metamorphosis-stimulating biofilm contrasts sharply with similar analyses of larval behaviors of many other invertebrates in similar settings. Crisp (1974), who was convinced that larvae could not use dissolved settlement cues because typical seawater flows exceed the swimming speed of larvae, summarized observations on larvae of a barnacle, a polychaete, and an oyster and concluded that all settlement behavior consisted of various stages of searching and attachment *after* initial contact with a stimulating surface. Several subsequent studies refute Crisp's opinion about soluble settlement-inducer

factors, for example, oyster larvae (Tamburri et al. 1992, 1996), a small opisthobranch (Krug and Zimmer 2000), the coral-eating nudibranch *P. sibogae* (Hadfield and Koehl 2004; Koehl and Hadfield, 2004; Koehl et al. 2007), and the barnacle *Amphibalanus* (*as Balanus) amphitrite* (Elbourne and Clare 2010). In contrast to these studies of larvae filmed or videotaped when swimming and then settling in the presence of soluble settlement cues, we found that larvae of *H. elegans* do not change their behavior in response to dissolved cues from biofilms. We suggest that swimming by the larvae of *H. elegans*, coupled with their transport in turbulent ambient flow, causes them to make contact with surfaces, and that contact with a biofilmed surface induces them to crawl upon it, attach, and metamorphose.

Studies of other serpulids provide evidence that their larvae respond to soluble cues for settlement and metamorphosis. In particular, prominent are studies that find soluble factors arising from conspecific worms bring about gregarious recruitment, reported for *Hydroides ezoensis* (Okamoto et al. 1998; Watanabe et al. 1998) and *H. dianthus* (Scheltema et al. 1981; Toonen and Pawlik 1996). However, the methods in the study of *H. ezoensis* did not exclude the possibility that biofilming bacteria were present in the settlement assays, and the 48-h duration of the assays makes a bacterial role likely. This possibility seems even more likely in the assays of worms and extracts of *H. dianthus*; Toonen and Pawlik (1996) reported an absolute requirement for bacterial films on surfaces before most of the larvae would settle in response to a soluble substance from living adult worms and organic extracts of adult worms. A small percentage of any cohort of larvae of *H. dianthus* settled on biofilms in the absence of living juvenile or adult worms; the authors concluded that these larvae provide the foundations for new populations (Toonen and Pawlik 1994, 2001).

Reports on larvae of some other serpulid species provide evidence that they are like those of *H. elegans* in requiring biofilm contact to stimulate settlement and metamorphosis. Larvae of two spirorbid species typically associated with particular algal species were found to settle preferentially on pieces of those same algae in still water, but also to settle heavily on the glass walls of the dishes and on the surface film, suggesting that biofilms played a major role in stimulating settlement (Wisely 1958). Kirchman et al. (1982a, b) found that cultured films of a bacterium isolated from the green alga *Ulva lobata* provided a strong stimulus for settlement of larvae of *Janua brasilensis*, and the bacterial factor did not appear to be dissolved in the overlying seawater. Thus, larvae of spirorbid polychaetes, like those of *H. elegans*, must make contact with a biofilm to be stimulated to attach and metamorphose.

Our experiments were done in still water, but larvae never live in a still-water environment in the field. Ocean turbulence disperses soluble metabolites emanating from benthic sources, although they can accumulate in hidden recesses of slowly moving water in complex substrata such as coral reefs (Koehl and Hadfield 2004; Hadfield and Koehl 2004; Reidenbach et al. 2006). However, in fouling communities, eddies that sweep through the boundary layer can sweep away water near the surface every few seconds (Koehl et al. 2013). Therefore, our still-water experiments provided the larvae of *H. elegans* with "extra time," both for the accumulation of soluble cues, if they exist, and for larvae to detect and respond to them. The absence in our experiments of any apparent response to solutes from metamorphosis-stimulating biofilms when larvae were prevented from touching the biofilmed surfaces strongly supports the conclusion that the larvae of *H. elegans* locate inducing bacterial films only by touching them.

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