Hydroides elegans (Annelida: Polychaeta): A Model for Biofouling Research

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Abstract The small serpulid polychaete *Hydroides elegans* is a problem fouling organism in warm water marine harbors around the world. Often the first significant animal biofouler on newly submerged surfaces, its calcareous tubes can accumulate rapidly and create serious problems for ships. *H. elegans* is easily adapted for laboratory biofouling research because of its rapid generation time (~3 wks) and ease of propagation. The dioecious adult worms spawn readily in the laboratory, and their metamorphically competent larvae develop in ~5 d at 25 °C. The larvae of *H. elegans* settle in response to natural biofilms or films formed by many, but not all, single marine bacterial species. Tubes of *H. elegans* adhere very tightly to surfaces and are more resistant to dislodgement than many barnacles. Thus, *H. elegans* is an excellent model organism for experimental studies, including tests of newly formulated marine coatings.

1 Introduction

The fouling communities that occur on ships and other man-made structures submerged in the sea are diverse assemblages of organisms (Carlton and Hodder 1995; Gollasch 2002; Godwin 2003). Due to that diversity, the variety of adhesives that fouling organisms utilize to cement themselves to settlement substrata are equally diverse (Naldrett and Kaplan 1997; Brady and Singer 2000; Wiegemann 2005; Smith and Callow 2006), posing a significant challenge for the development of new coatings to combat biofouling processes (Holm et al. 2006). Minimizing fouling on ship hulls is important because of the negative influence fouling has on hull performance (Woods Hole Oceanographic Institution 1952), expenses associated with dry-docking, scraping and re-painting hulls, and the substantial costs from propulsive fuel losses required to overcome the increased drag created by hull fouling (Townsin 2003). Research to find new coatings to combat biofouling has

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Springer Series on Biofilms, doi: 10.1007/7142_2008_15

two major thrusts, one in chemically formulating experimental coatings and another in testing these coatings in both field and laboratory settings. The serpulid polychaete *Hydroides elegans* (Haswell 1883) has proven to be an excellent organism for testing experimental coatings under both field and laboratory conditions.

Hydroides elegans is a common member of fouling communities throughout tropical and subtropical seas (ten Hove 1974; Hadfield et al. 1994; Unabia and Hadfield 1999; Bastida-Zavala and ten Hove 2002, 2003). *H. elegans* is a problematic fouling organism because: (1) it quickly colonizes newly submerged surfaces (Unabia and Hadfield 1999; Holm et al. 2000); (2) it grows as much as 1.5 mm day^{-1} (Paul 1937); (3) it reaches sexual maturity in as short a time as 9 days in a tropical harbor (Paul 1937); (4) it has a short larval period (Hadfield et al. 1994; Carpizo-Ituarte and Hadfield 1998); and (5) aggregations of its calcified tubes can accumulate to several centimeters thick on submerged surfaces in as short a time as 1–2 months in Pearl Harbor, Hawaii (Edmondson 1944) (Fig. 1).

Fig. 1 Dense accumulation of tubes of *Hydroides elegans* on Vexar after a 1-month submersion in Pearl Harbor, HI

Due to its importance as a fouler of ship hulls, there is a growing body of research concerned with the natural inductive cues for recruitment of *H. elegans* (Hadfield et al. 1994; Hadfield and Strathmann 1996; Walters et al. 1997; Unabia and Hadfield 1999; Hadfield and Paul 2001; Lau and Qian 2001; Harder et al. 2002; Lau et al. 2002; Huang and Hadfield 2003; Lau et al. 2005; Shikuma and Hadfield 2006), as well as the metamorphic cascades that are triggered by this process (Carpizo-Ituarte and Hadfield 1998; Holm et al. 1998; Carpizo-Ituarte and Hadfield 2003). Additionally, it has been employed in studies of neurogenesis (Nedved and Hadfield 1998, and unpublished), muscular development (Nedved and Hadfield 2001), segment development (Seaver and Kaneshige 2006; Seaver et al. 2005), and the heritability of egg size (Miles et al. 2007). *H. elegans* will undoubtedly find use as a research model in many other types of studies due to the ease with which is can be maintained and reared in the laboratory.

Upon submersion in seawater, surfaces undergo a well-characterized progression from initial coating of adsorbed organic molecules (Zobell and Allen 1935), to the formation of biofilms (Marshall 1981; Baier 1984) composed of a wide variety of microorganisms that form highly organized communities (Costerton et al. 1999). These complex biofilms provide settlement cues for larvae of many sessile marine invertebrate species (reviewed by Hadfield and Paul 2001). Biofilm bacteria produce the inductive cue for settlement of competent larvae of *H. elegans* (Hadfield et al. 1994; Unabia and Hadfield 1999; Lau and Qian 2001; Lau et al. 2002; Huang and Hadfield 2003; Lau et al. 2005; Shikuma and Hadfield 2006). Laboratory evidence that other biofilm organisms may produce inductive cues for larvae of *H. elegans* (e.g. diatoms: Harder et al. 2002) remain provisionary, given the difficulty of producing absolutely axenic cultures of such organisms for testing.

Larvae of *H. elegans* require a minimum bacterial density for the induction of metamorphosis, and increased larval settlement positively correlates with the density of bacteria in a biofilm (Hadfield et al. 1994; Huang and Hadfield 2003). Settlement by *H. elegans* is greatly reduced or eliminated when multi-species biofilms are treated with a variety of agents that act either as fixatives or antiseptics, demonstrating that the microorganisms within the biofilms must also be alive for induction to occur (Unabia and Hadfield 1999). Recent studies by Lau et al. (2005) and Shikuma and Hadfield (2006) using denaturing gradient gel electrophoresis (DGGE) have examined the effect that changes in the bacterial assemblages of biofilms have on the induction of metamorphosis of *H. elegans*, both demonstrating a stronger positive correlation between settlement of *H. elegans* and bacterial density than between settlement and differences in natural community composition. However, the effectiveness of a bacterial biofilm as an inducer of metamorphosis of *H.elegans* is not solely due to the sheer number of bacteria residing in it. Huang and Hadfield (2003) demonstrated that single-strain, low-density biofilms of *Pseudoalteromonas luteoviolacea* induced metamorphosis of *H. elegans* (Fig. 2), while mono-specific biofilms of *Flexibacter* sp. and *Cytophaga* sp. were non-inductive even though the cell densities of these biofilms were 7–12 times greater (Fig. 3). These data indicate that induction of metamorphosis is due to specific chemical characteristics of *P. luteoviolacea* (Huang and

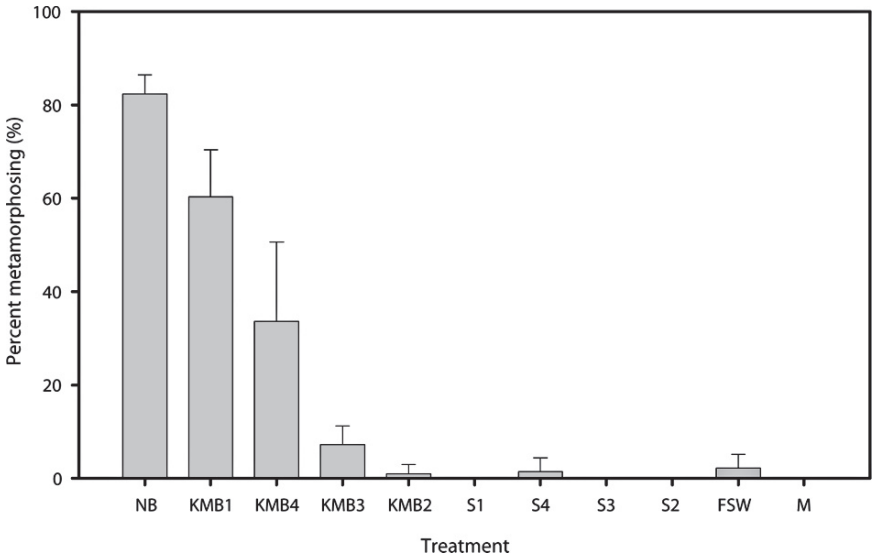

Fig. 2 Induction of metamorphosis of *Hydroides elegans* by mono-specific biofilms on plastic Petri dishes prepared from bacterial strains KMB1, KMB2, KMB3 and KMB4. Controls include: (1) dishes similarly treated with filter-sterilized culture medium from each bacterial strain (*S1*, *S2*, *S3*, *S4*); (2) natural biofilms (*NB*) allowed to accumulate on Vexar mesh in flowing seawater and placed in a Petri dish of filtered seawater (*FSW*); (3) untreated Petri dishes filled with FSW; and (4) dishes rinsed with fresh culture medium (*M*). *KMB1 Pseudoalteromonas luteoviolacea*, *KMB2 Flexibacter* sp, *KMB3 Cytophaga* sp, *KMB4 Cytophaga lytica*. Inoculation density of all strains was approximately 10^{-8} cells mL^{-1}. *Bars* represent mean percent of larvae that metamorphosed in 24 h + SD (n = 5 replicates per treatment) (reproduced from Huang and Hadfield 2003)

Hadfield 2003). Furthermore, production of this metamorphic cue is strain-specific; a different strain of *P. luteoviolacea* obtained from the American Type Culture Collection (Manassas, VA) does not induce settlement of larvae of *H. elegans* (unpublished personal observations).

Hydroides elegans is particularly well-suited for use in testing of experimental coatings. The adhesive that secures the calcareous tubes of *H. elegans* appears to be stronger than that of the balanoid barnacles *Balanus eburneus* and *B. amphitrite*, two species often employed in testing of marine coatings. The mean removal force for *H. elegans* that had settled on six different silicone coatings in Pearl Harbor was nearly three times greater than the mean removal force required to remove *B. eburneus* from replicate panels immersed in the Indian River Lagoon, FL (Fig. 4A, Stein et al. 2003). Additionally, more spat of *B. amphitrite* than newly settled juveniles of *H. elegans* are removed from the silicone coating RTV11 (General Electric, New York) by a 4-min exposure to a wall-shear force equivalent to 100 Pa (unpublished personal observations presented in Fig. 4B) in a turbulent flow apparatus (described in Schultz et al. 2003).

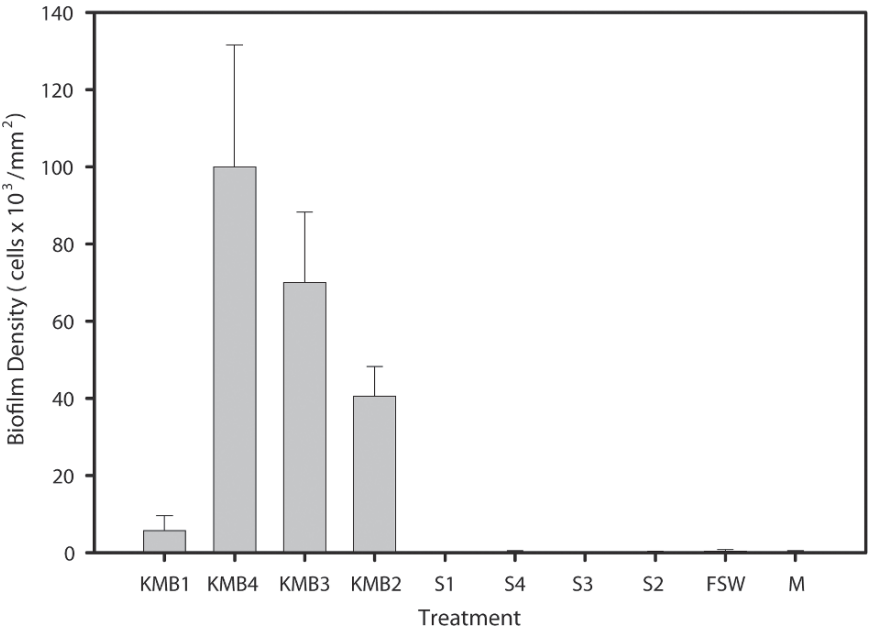

Fig. 3 Bacterial densities in single-species biofilms (see Fig. 2), counted under fluorescence microscopy after formalin fixation and DAPI staining. KMB1, KMB2, KMB3 and KMB4 are the bacterial strains studied; *S1–S4* are dishes treated with the supernatants from each strain, respectively; *FSW* and *M* are filtered seawater and culture-medium-only controls, respectively. *KMB1 Pseudoalteromonas luteoviolacea*, *KMB2 Flexibacter* sp, *KMB3 Cytophaga* sp, *KMB4 Cytophaga lytica*. Inoculation density of all strains was approximately 10^{-8} cells mL^{-1}. *Bars* represent bacterial cell numbers $\times 10^3$ mm^{-2} + SD (n = 25, consisting of five area counts per replicate and five replicate dishes per treatment; replicate effects were not significant (reproduced from Huang and Hadfield 2003)

Information on the occurrence and biology of *H. elegans* has been published under several taxonomic names (e.g. Edmondson 1944; Wisely 1958). This confusion has been resolved in taxonomic reviews by Zibrowius (1971), ten Hove (1974) and Bastida-Zavala and ten Hove (2003), who concluded that *H. norvegica* is a species of the northern Atlantic Ocean and the Mediterranean Sea and that the similar species in warm seas around the world should be referred to as *H. elegans*. There are, of course, other tropical species of *Hydroides*, and they may easily be confused with *H. elegans* without careful observation of the operculum and setae, which are well illustrated in ten Hove (1974), Bailey-Brock (1987) and Bastida-Zavala and ten Hove (2003). According to ten Hove (1974), *H. elegans* is the only *Hydroides* species that forms dense aggregations in warm water bays and estuaries worldwide.

The remainder of this chapter provides a concise summary of methods developed in our laboratory for the culture of *H. elegans* for use in biofouling testing. Our techniques have been used to successfully culture *H. elegans* elsewhere (e.g. Bryan et al. 1997), including areas that do not have access to coastal waters.

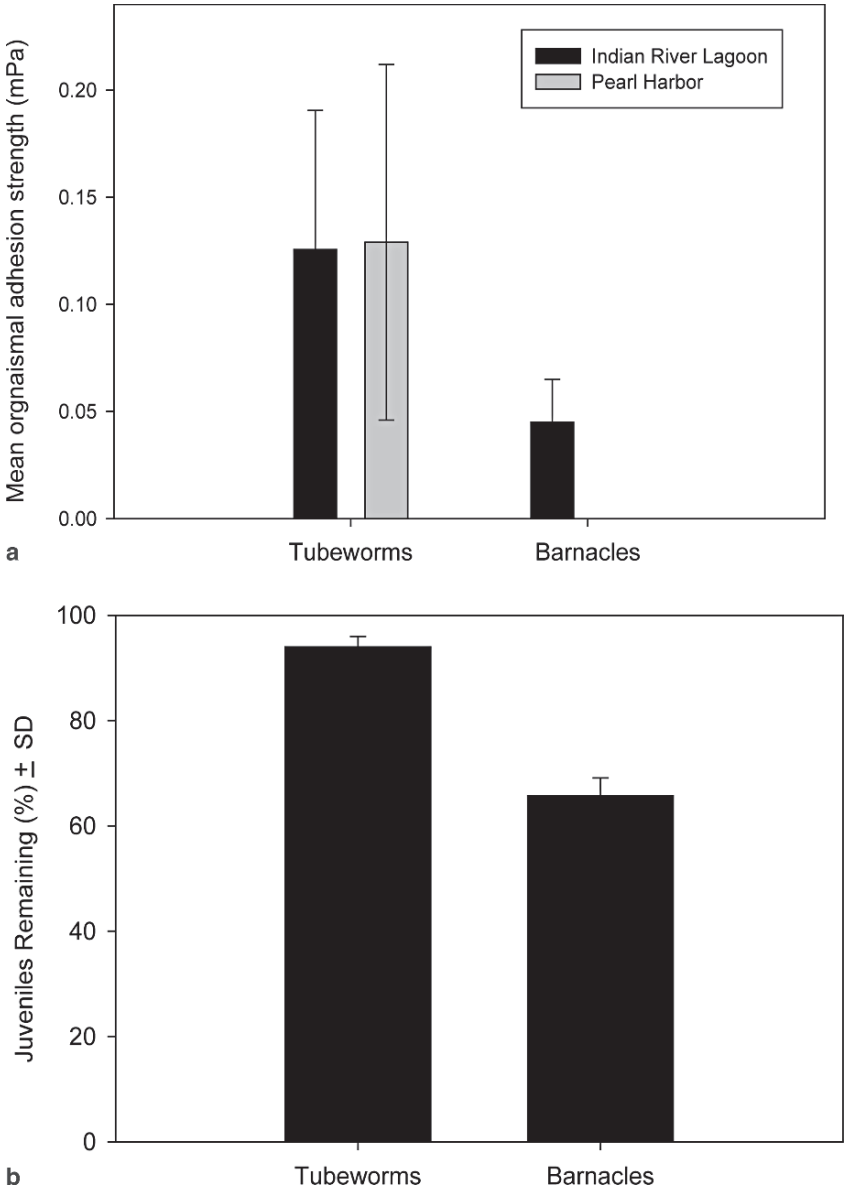

Fig. 4 Strength of adhesion of tubeworms and barnacles in the field (**a**) and a laboratory trial (**b**). **a** Mean attachment strength of barnacles and tubeworms on test coatings. Data are for *Hydroides dianthus* at Indian River and *H. elegans* at Pearl Harbor, and *Balanus eburneus* at Indian River (reproduced with permission from Stein et al. 2003). **b** Percent of juveniles remaining after exposure to a wall shear stress equivalent to 100 Pa for 4 min. Data are for *H. elegans* and *Balanus amphitrite* (previously unpublished data from our laboratory)

2 Collection and Care of Adults

In Pearl Harbor, larvae of *Hydroides elegans* settle on biofilmed surfaces throughout the year and can reach high densities on both natural and man-made surfaces (Fig. 1). Several different materials have been used as artificial settlement substrata for the field collection of *H. elegans* (Walters et al. 1997; Lau and Qian 2001; McEdward and Qian 2001; Lau et al. 2002; Walters et al. 2003). We prefer to use small pieces of extruded plastic mesh (Vexar) as settlement substrata for collecting *H. elegans* in Pearl Harbor. These screens are hung from a pier approximately 1 m below the mean low tide line, and within 3–4 weeks thousands of recruits have settled on them and grown to reproductive maturity (Fig. 1). These dense populations of worms are then transported back to the laboratory and kept in continuously flowing, unfiltered seawater for several weeks without a noticeable decrease in fecundity.

Vexar is preferred over solid substrata, because: (1) it greatly increases the surface area of the material; (2) it provides crevices that may entrain larvae near the surface of screen facilitating settlement on its surface; and (3) it allows water flow through the mesh to bring food, oxygen, and remove wastes from settled worms (Walters et al. 1997).

Large populations of adult *H. elegans* can be kept in closed-system aquaria containing either natural or artificial seawater. Additionally, individual worms can be reared separately by allowing larvae to settle on small (1 × 1 cm) biofilmed chips of polystyrene, removing all but one juvenile worm from each chip, and maintaining the chips in individual wells of plastic ice cube trays (Eric Holm, personal communication; Miles et al.2007). In both settings, adult worms survive and continue producing gametes when fed *Isochrysis galbana* (6×10^4 cells mL^{-1}).

3 Spawning

Spawning of *H. elegans* can be achieved using either destructive or non-destructive methods. We typically use the destructive spawning method, because large numbers of gravid worms are available to us throughout the year in Hawaii. When using this method, we remove 30–40 worms from a piece of Vexar and place them in a small glass dish containing 100 mL of 0.22 μm Milipore-filtered seawater (FSW). To induce release of gametes, the calcareous tubes of the worms are broken in half using forceps, and the abdominal segments of the worms are exposed. This process causes release of thousands of small (~45 μm diameter) orange eggs or clouds of sperm from the abdominal segments of the worm. We then repeat this process until all the worms have been removed from their tubes. After 15–20 min, the fertilized eggs are separated from the adult worms and debris by passing them through a 200 μm sieve (Nitex) into a 500-mL beaker. Filtered seawater is added to achieve a volume of 200 mL. This addition of seawater dilutes the sperm concentration to prevent polyspermy. Fertilization occurs within minutes of exposure of eggs to sperm, and first cleavage occurs approximately 1 h after fertilization (23–26 C). Using this method it is easy to obtain tens of thousands of embryos at a time.

If it is not possible to sacrifice large numbers of adult worms to obtain gametes, a non-destructive method may be used. In this method, worms whose tubes are still attached to their substratum are placed in dishes containing FSW, and the aperture of the worm's tube is gently broken with fine forceps. This mechanical disturbance causes release of eggs and sperm into the tube, and the worm then expels the gametes from the tube by muscular peristaltic action. Generally, females induced to spawn using this method release fewer eggs than can be obtained with the destructive method. After the worms have spawned, they can be placed back into their individual containers where they will repair the apertures of their tubes, and can be induced to spawn again in 2–3 days (personal observations). We have also noted that when adult worms are kept individually in ice cube trays, they occasionally release gametes spontaneously after the FSW is changed in the wells of the trays.

4 Feeding and Care of Larvae

4.1 Seawater

In our laboratory, natural coastal seawater (salinity 35 ‰) filtered through a 0.22 µm Millipore filter (FSW) is used in larval culture to minimize bacterial contamination. We have raised larvae of *H. elegans* in "MBL artificial seawater" (Cavanaugh 1975; Bidwell and Spotte 1985; Strathmann 1987) with no deleterious effects. Antibiotics (60 µg mL^{-1} penicillin G and 50 µg mL^{-1} streptomycin sulfate) may be used if larval mortality is high, but this mixture is generally not required to maintain the larvae through metamorphic competence.

4.2 Temperature and Light

Larval cultures of *H. elegans* are maintained on the bench-top at room temperature (23–26 C) and kept under the ambient lighting regime of the laboratory. However, maintaining larval cultures at 25 C in an incubator provides a greater degree of synchrony of early larval stages. At 24–25°C, larvae attain metamorphic competence after 5 days in culture. Lower temperatures increase time to competence, at 20 C, larvae become competent to metamorphose after 8–10 days in culture (Wisely 1958, as *H. norvegica*).

4.3 Culture Vessels

All glassware used for larval culture in our laboratory is scrubbed under running tap water, rinsed several times with deionized water, and allowed to air dry prior to use.

Additionally, all of our glassware is periodically soaked in a strong acid solution (25% HCl and 25% H_2SO_4) to destroy any organic films that may have developed on the interior surfaces of the containers. Adsorbed organic films hasten the development of a biofilm in the culture vessels, which can provide a metamorphic cue for competent larvae and may cause a substantial number of larvae to metamorphose on the walls of the glassware and the surface film. In our laboratory, larvae are cultured in 1- or 2-L beakers at an initial density of 5–10 larvae mL^{-1}. Cultures of larval *H. elegans* are maintained without stirring or aeration with high levels of larval survival through attainment of competence.

In order to prevent evaporation, beakers are covered with plastic wrap.

4.4 Changing Water in Cultures

After the second day, the larvae are transferred to clean beakers with fresh FSW. To do this, each larval culture is poured into a small plastic beaker whose bottom has been replaced by a piece of 41 μm Nitex sieve. The beaker is placed in a small bowl of seawater in a sink and, as the larval culture is poured through the sieve, the old culture water is allowed to run over the top of the bowl, and the larvae are confined above the screen (see Strathmann 1987). The concentrated larvae are then gently washed from the sieve into clean, acid-washed beakers containing fresh FSW and phytoplankton. Care is taken to retain a small volume of water in the sieve to prevent larvae from being crushed against it. This procedure is subsequently repeated daily until larvae attain competence. Once attaining competence, larvae can be maintained in this manner for several weeks (Unabia and Hadfield 1999).

4.5 Larval Food and its Culture

The unicellular alga *Isochrysis galbana* (Tahitian strain) is the most commonly used food source for larvae of *H. elegans* (Hadfield et al. 1994; Carpizo-Ituarte and Hadfield 1998; Holm et al. 1998; McEdward and Qian 2001; Carpizo-Ituarte and Hadfield 2003; Huang and Hadfield 2003; Lau et al. 2005; Shikuma and Hadfield 2006). However, other algal species have been utilized to raise larvae of this species through metamorphosis (Wisely 1958 as *H. norvegica*; Hadfield et al. 1994). We use *I. galbana* at a density of 6×10^4 cells mL^{-1}. Larval cultures of *H. elegans* are fed *I. galbana* daily by adding aliquots from our working alga cultures; we do not attempt to separate the alga from its culture media.

In our laboratory, *I. galbana* is grown in a commercially produced, modified Guillard's f/2 media (Micro Algae Grow, Florida Aqua Farms, Dade City, FL). We syringe filter (0.22 μm) Micro Algae Grow and use it at a working concentration of 1:1,000 in autoclaved seawater (salinity 25‰). A stock culture of *I. galbana* is maintained in 50-mL screw-top Erlenmyer flasks and recultured bi-weekly. Algal

cultures for larval cultures are started every 3 days and maintained in autoclaved culture containers described in Switzer-Dunlap and Hadfield (1981). These cultures are then used as a larval food source when the algal populations are in the later portion of their growth phase. All cultures are bubbled and kept in continuous light supplied by 20-W cool white fluorescent bulbs at room temperature.

4.6 Larval Development

Although the embryonic and larval development of *H. elegans* has been previously described (Wisely 1958 as *H. norvegica*), the timing of larval development is highly dependent on the culture conditions. Larvae cultured in our laboratory develop more rapidly due to the higher ambient temperatures of Hawaii. Cell division proceeds rapidly after first cleavage, and larvae hatch after about 4 h. Larvae of *H. elegans* begin feeding as early trochophores approximately 9 h after fertilization (25°C), and by 12 h they are well developed trochophores with an apical sensory organ (ASO), a single eyespot on the right side of the larva (ES), a prototroch (Pro), and a metatroch (Met) (Fig. 5a).

Larvae remain in the unsegmented prototroch stage for approximately 60 h longer. Three days after fertilization, larvae have developed into metatrochophores with a second eyespot on the left side of the larval episphere, rudiments of the

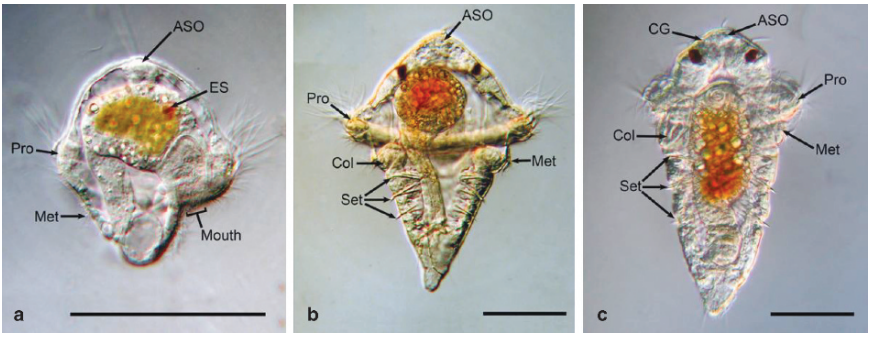

Fig. 5 Differential interference contrast microscopy images of larval development in *Hydroides elegans*. **a** Lateral view of trochophore-stage larva (12 h post-fert at 25°C). **b** Ventral view of metatroch-stage larva (72 h post-fert). Notice the appearance of a second eyespot in the episphere of the larva, and the precocious development of the collar and the first three abdominal segments. The segments are identifiable by the positioning of the paired setigers within each segment. **c** Ventral view of a competent larva (~120 h post-fert). The larva has grown considerably since the trochophore stage, and the hyposphere has become considerably longer. The horse-shoe shaped cerebral ganglion have become quite developed, and the mid-gut of the larva has almost been entirely displaced from the episphere of the larva. Larval mid-gut is easily visualized due to the pigmented algal cells in its lumen. *ASO* apical sensory organ; *CG* cerebral ganglia; *Col* collar; *ES* eyespot; *Met* metatroch; *Pro* prototrochal band; *Set* setae. *Scale bars:* 50 μm in all panels

collar (Col), and elements of the first three abdominal segments including setae (Set) (Fig. 5b).

Larvae of *H. elegans* become competent to metamorphose 5 days after fertilization (25°C). The hyposphere of the larvae has lengthened considerably and the gut has shifted posteriorly. The larval midgut (discernable by the algal cells within it, visible in Figs. 5a–c) has been almost entirely displaced from the episphere by the differentiating cerebral ganglia (CG, Fig. 5c). In competent larvae, the growth and differentiation of the cerebral ganglia is accompanied with a change in a shape of the larval episphere. The lateral margins of the episphere appear to constrict in the region immediately anterior of the prototroch (compare Figs. 5b and 5c), so that the previously hemispherical episphere becomes conical and provides a morphological landmark for the development of competence.

4.7 Metamorphosis

In addition to exposure to biofilm bacteria, larvae of *Hydroides elegans* can be artificially induced to metamorphose by the bath application of the cations K^+ and Cs^+ and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). IBMX (0.1 mM) induces 80% of the larvae exposed to it to undergo metamorphosis (Holm et al. 1998). Potassium ions (50 mM excess in FSW) typically induce metamorphosis in over 70% of larvae, but the response is much slower than the rate of metamorphosis induced by biofilms (Carpizo-Ituarte and Hadfield 1998). Maximal induction by caesium ions occurs when 10 mM excess Cs^+ is applied in a 3.0–4.5 h pulse.

Carpizo-Ituarte and Hadfield (1998) described the morphogenic events associated with metamorphosis in larvae of *H. elegans* (Fig. 6). Competent larvae of *H. elegans* may initiate metamorphosis almost immediately after contacting inductive surfaces and begin the process by excreting a sticky thread from their posterior end that serves to tether the larvae to the substratum. Almost immediately after, larvae lie flat on the surface and begin secreting a primary tube from most or all of the segments. They shape the newly secreted tube by rotating within it as they erect their setae to push the primary tube away from their bodies (Carpizo-Ituarte and Hadfield 1998). The secretion of the primary tube, which can be completed in as little as 10 min after contact with a surface, is an irreversible process that permanently attaches a larva to the substratum. As the primary tube is secreted, the collar is everted, the area immediately surrounding the collar constricts, the larval body elongates, and the metatroch is lost. Simultaneously, the pair of lobes that are the precursors to the branchial radioles become apparent on the anterolateral margins of the episphere of the juvenile. The primary tube is never calcified.

Secretion of the calcified secondary tube begins at the anterior margin of the primary tube approximately 2 h after the commencement of metamorphosis, after which new material is added to the secondary tube continuously. As the secondary tube is secreted, the prototroch is resorbed, and the branchial radioles begin to

differentiate from the anterior lobes. Metamorphosis is complete and juvenile development has commenced by 11–12 h post-settlement (Carpizo-Ituarte and Hadfield 1998). Because both the primary tube and the early portions of the secondary tube are transparent, the events of metamorphosis and early juvenile development are easily observed with relatively low power microscopy (Fig. 6).

In summary, the major tropical marine fouler *H. elegans* has proven to be a near perfect laboratory-animal model for studies of biofouling processes.

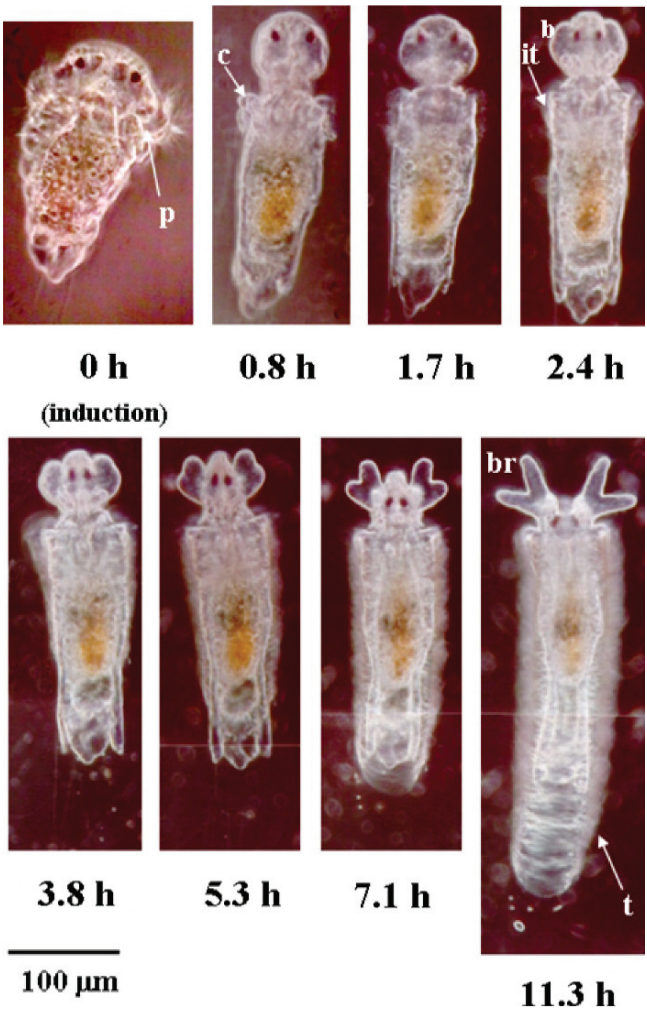

Fig. 6 Time-course of metamorphosis in *Hydroides elegans*. Frames represent a competent larva at the moment of induction to metamorphosis (0 h) and selected stages for the first 11.3 h after induction: *p* prototroch; *c* collar; *b* branchial lobes; *it* initiation point of calcareous tube; *br* branchial radioles; *t* calcareous tube covering the worm (Carpizo-Ituarte and Hadfield 1998)

The information provided above should make it possible to employ this organism for studies of biofouling or questions involving the development of polychaete worms in most laboratories. If scientists attempting to use *H. elegans* in their research find problems in its culture, we would be happy to communicate with them to find solutions.

Acknowledgments We are grateful to many former graduate students and postdoctoral fellows in the Hadfield laboratory who have contributed significantly to the development of the methods described here, especially E. J. Carpizo-Ituarte, E. R. Holm, S. Huang, Y. Huang, N. Shikuma and C. Unabia. The authors are grateful to M. J. Huggett and T. DuBuc for their assistance in creating the figures in this manuscript. Research reported here has been supported by the US Office of Naval research, currently grant no. N00014-05-1-0579 to MGH.

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