­­Title. Gregarious and non-gregarious larval settlement in the aggregation-forming annelid *Ficopomatus enigmaticus*

**Author list**. Alex Mendelson1, Keomonyroth Nuon2, and Bruno Pernet\*

**Affiliation**. Department of Biological Sciences, California State University Long Beach, Long Beach, CA 90840, USA

**\*Corresponding author**. [bruno.pernet@csulb.edu](mailto:bruno.pernet@csulb.edu)

**Running page head**. Settlement in a reef-forming annelid

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1Current address: Department of Biology, California State University Northridge, 18111 Nordhoff Street, Northridge, CA 91330-8303; alex.mendelson.698@my.csun.edu

2Current address: Department of Microbiology and Immunology, Thomas Jefferson University, 233 S. 10th Street, BLSB 731, Philadelphia, PA 19107; keomonyroth.nuon@jefferson.edu

**ABSTRACT**

Gregarious larval settlement – where cues associated with adult conspecifics induce larval settlement – plays a role in the growth of existing aggregations of many aggregation-forming sessile marine invertebrates. The formation of new aggregations, however, requires larvae to settle in response to other cues. The mechanism underlying this variation in larval settlement responses is unknown for most species with gregarious settlement. In this study we first present evidence that larvae of the serpulid annelid *Ficopomatus enigmaticus* settle gregariously. In no-choice experiments, a much higher percentage of larvae settled after 24 hrs of exposure to adult conspecific tube than after exposure to mussel shell collected from the same habitat. We then tested the hypothesis that larvae of *F. enigmaticus* display a genetically determined dimorphism in settlement behavior like the serpulid *Hydroides dianthus*, with most larvae settling only in response to adult conspecific cue, but a small percentage of larvae settling only in response to biofilm cue. A prediction of this hypothesis is that the sum of the percentages of larvae that settle in response to adult conspecific cues and those that settle in response to biofilm cues cannot exceed 100% (since each larva can only accept one of the two cue types throughout its competent period). Our data on *F. enigmaticus* are not consistent with this prediction, suggesting that individual larvae do not display fixed behaviors as either founders or aggregators, but instead can respond to multiple types of settlement cues during their competent period. This difference in how new aggregations form has significant implications for how frequently larvae can form new aggregations, a topic of special importance for *F. enigmaticus* and *H. dianthus*, both of which are well-known invasive species in marine habitats around the globe.

INTRODUCTION

Many sessile marine invertebrates occur primarily in aggregations (Burke 1986; Pawlik 1992). In some of these species, a specific larval behavior, gregarious settlement, plays a role in the growth of aggregations. In such species, the presence of dissolved or surface-bound cues associated with juveniles or adults induces settlement and metamorphosis of conspecific larvae, thus enlarging existing aggregations (Knight-Jones 1953; Scheltema et al. 1981; Burke 1986; Pawlik 1992).

In species with gregarious settlement, new aggregations must at least occasionally be formed at locations where no conspecific juveniles or adults initially occur. The establishment of a new aggregation in such a location likely depends on at least one larva settling and metamorphosing in the absence of any conspecific cues (Raimondi and Keough 1990; Pawlik 1992). The resulting juvenile can then serve as a cue for other larvae to settle, leading to the formation of a new aggregation.

For aggregation-forming species with gregarious larval settlement, then, there is likely variation in settlement behavior among conspecific larvae, with some settling gregariously on or immediately adjacent to existing juveniles or adults, and others settling at locations where no juveniles or adults are present (Raimondi and Keough 1990). At least three hypotheses might explain such intraspecific variation in larval settlement behavior:

**1)** **broadening of settlement preferences over time**. Here all larvae in a population initially settle only in response to juvenile or adult conspecific cues, but after some period of failing to encounter such cues, they begin to accept other cues (e.g., biofilm). This is also known as the “desperate larva” (Toonen & Pawlik 1994) or “variable retention” (Bishop et al. 2006) hypothesis.

**2)** **stable** **ranked settlement preferences**. Here all larvae in a population can settle in response to multiple potential settlement cues from the beginning of metamorphic competence. They always have high probabilities of settling in response to juvenile or adult conspecific cues, but lower, stably ranked probabilities of settling in response to other cues. This differs from the first hypothesis in that preferences do not change over time.

**3)** **genetically determined settlement dimorphism**. Here some larvae in a population will settle only in response to juvenile or adult conspecific cues, and other larvae will settle only in response to other cues. This differs from both previous hypotheses in that a particular larva will only respond to one type of settlement cue for its entire competent period.

For species with gregarious larval settlement, the only one of these hypotheses with any supporting evidence is that of genetically determined settlement dimorphism. Toonen and Pawlik (1994, 2001) showed that larvae of the serpulid *Hydroides dianthus* respond to potential settlement cues in one of two ways – either settling in response to a biofilm cue (but not in response to the presence of adults; these “founders” make up only a small proportion of the larvae in each family) or settling in response to the presence of conspecific adults (but not in response to a biofilm cue; most of the larvae in each family are such “aggregators”). Any given larva is either a founder or an aggregator for its whole larval life. Toonen and Pawlik (2001) showed that these dimorphic behaviors in larvae of *H. dianthus* were genetically determined.

We do not know how widespread this type of genetically determined dimorphism in settlement behavior is among aggregation-forming species. To our knowledge it has never been observed (or even looked for) in any other aggregation-forming species, not even another serpulid (among which there are numerous aggregation-forming species: Ten Hove 1979, Kupriyanova et al. 2001).

Here we report on the settlement behavior of the serpulid *Ficopomatus enigmaticus*, a widespread invasive species that often forms “reefs” of many thousands of adults, especially in coastal brackish-water habitats (e.g. Bianchi and Morri 1996; Schwindt and Iribarne 1998; Dittman et al. 2009; Pernet et al. 2016). We first present results of a simple laboratory experiment that strongly suggests that larvae of this species settle gregariously. We then report on an experiment designed to determine if larvae of *F. enigmaticus* have a genetically determined settlement dimorphism similar to that of *H. dianthus*. For species with such a dimorphism, in any given population of larvae, the sum of the percentages of larvae that settle in response to juvenile or adult conspecific cues and those that settle in response to a biofilm cue cannot exceed 100% (since each larva can only accept one of the two cue types throughout its competent period). In *H. dianthus*, this condition has been met in ~90 distinct experiments (Toonen and Pawlik 1994, 2001). This condition is not met in our experiment on *F. enigmaticus*, suggesting that individual larvae of that species do not display fixed behaviors as either founders or aggregators, but instead can accept multiple types of settlement substrate. Our data are most consistent with the hypothesis that larvae of *F. enigmaticus* show stable ranked settlement preferences. This difference in how aggregation-forming serpulids form new aggregations may have implications for the colonization of new habitats by serpulids like *H. dianthus* and *F. enigmaticus*, both of which are known as invasive species in many parts of the world.

**METHODS**

**Collection of adults**. Adults of *Ficopomatus enigmaticus* were collected from the mouth of the Los Angeles River adjacent to the Golden Shore Marina Biological Reserve in Long Beach, California (33.763°N, -118.202°W). A population of *F. enigmaticus* was first detected at this site in 2014 (Pernet et al. 2016). At low tide, clusters of intact tubes were detached from rocks and transported to the laboratory in seawater from the collection site (~20-25 psu), where they were stored at 16°C with bubbled air for no longer than 5 d before use in experiments.

**Spawning, fertilization, and algal and larval culture**. Spawning, fertilization, algal and larval culture, and all experiments were carried out in filtered (0.2 µm pore size) seawater adjusted to 20 psu by addition of deionized water (hereafter, FSW). Salinity was verified with a calibrated handheld refractometer. Spawning was induced by carefully extracting intact adult worms from their tubes. Extracted adults were rinsed with FSW to remove tube fragments and other debris, then transferred to 6-well culture plates (one worm per well) containing FSW. Fertilization was carried out by adding sperm from a single male to eggs from a single female, creating a full-sib family. This process was repeated as necessary to obtain the number of families needed for each experiment (see below). A small (but unmeasured) number of cells of *Isochrysis galbana* (cultured as described below) was added to each dish of zygotes so that when larvae began to feed there would be food available. Fertilized eggs were then left unstirred at room temperature (~21°C) to develop for 24 h.

After 24 h (1 d post-fertilization, dpf), swimming larvae from each family were decanted into a new container to separate them from any unfertilized eggs or dead embryos, and the concentration of larvae in each of these stock suspensions estimated by counting larvae in five 500 µl samples in a Bogorov tray. Larvae in these samples were killed with dilute formalin before counting. The appropriate volume of stock suspension from a given family was then added to a 500 mL beaker of FSW to reach a final concentration of 1 larva mL-1. A stock suspension of cells of *I. galbana* was added to each beaker to achieve a final concentration of 30,000 algal cells mL-1. Beakers were held at room temperature and continuously stirred with a paddle system (Strathmann 1987). At 3 dpf, larvae were filtered onto a submerged Nitex mesh (35 µm pore size) then returned to their beaker (which had been washed with hot tap water) containing fresh FSW; enough cells of *I. galbana* were then added to each beaker to achieve a final concentration of 30,000 algal cells mL-1. The beakers were then replaced on the paddle system. Experiments began when larvae were 4 dpf.

*Isochrysis galbana* was cultured at room temperature in natural light in sterile f/2 medium made up in FSW. Prior to use in feeding, cultured algae were centrifuged, the supernatant discarded, and the algal cells resuspended in FSW. The concentration of algae in the resulting stock suspension was then determined with an Accuri C6 flow cytometer (BD Biosciences), and that estimate used to calculate the volume of stock suspension to add to beakers to yield a final concentration of 30,000 cells mL-1.

**Experiment 1: Do larvae of *Ficopomatus enigmaticus* settle gregariously?** This no-choice settlement experiment was designed to determine if substrates associated with adults (chips of adult tube) induce settlement of larvae of *F. enigmaticus* more effectively than another hard substrate from the same environment (chips of mussel shell). We compared two substrates from the same brackish-water habitat to minimize salinity-driven differences in biofilm composition on the two substrates (e.g., Lau et al. 2005; Caruso 2020; Pinnell and Turner 2020).

To prepare for this experiment, we used sperm from a single male to fertilize eggs of a single female, creating a full-sib family. We repeated this process three more times with new males and females to create a total of four full-sib families, each with unique male and female parents. These four families of larvae were reared separately as described above until 4 dpf, when they were exposed to treatments. The experiment was carried out in four 24-well tissue culture plates whose wells had previously been coated with 0.2% gelatin to prevent larvae from sticking to their walls. Wells were rinsed with FSW before use. At 4 dpf, the day before most larvae become competent to metamorphose (Gabilondo et al. 2013), haphazardly chosen larvae from each of the four families were transferred to tissue culture plates (one family per plate; one larva per well) with pulled glass pipettes. Each well contained 1 ml of FSW, 30,000 cells of *I. galbana*, and one of the three substrate treatments: chips of adult tube, chips of mussel shell, or clean plastic (as a negative control). Each of the three treatments occurred in eight wells within a plate. We studied the responses of individual larvae instead of those of groups of larvae because in species with gregarious settlement, the decisions of one larva in a group may affect the decisions of other larvae (Gotelli 1990; Elbourne et al. 2008).

Chips of adult tube were generated the day the experiment was started (that is, 4 dpf) from an aggregation of tubes collected from the field two days prior and maintained in the laboratory at 16°C with aeration.From these aggregations, we removed ~1 mm2 chips of the cleanest white tube material available (this was always the material at the growing tip of the tube). Tube chips were then inspected with a stereomicroscope, and any debris or algae removed with forceps. Chips of mussel shell were generated from living mussels (*Mytilus galloprovincialis*) collected two days prior to the start of the experiment at the same tidal elevation and within 30 cm of the aggregation of *F. enigmaticus* that was the source of adult tube chips. Small mussels whose shells had not obviously been colonized by macroalgae or sessile animals were targeted during collection. Mussels were maintained in the laboratory until use in a separate container from that containing adults of *F. enigmaticus*. Chips of mussel shell were generated the day the experiment was started. Mussels were killed by cutting their adductor muscles, and all body tissue was removed with forceps. Small irregular fragments of shell were removed from the posterior ends of the shells with cutting pliers. Shell fragments were inspected with a stereomicroscope, and any remaining mantle tissue, debris, or algae removed with forceps. Shell fragments were then further broken into ~1 mm2 chips. At all stages of this process care was taken to avoid touching the outer surfaces of the shell to avoid disrupting natural biofilm. For the clean plastic treatment, which served as a negative control, black acrylic sheets (1.5 mm thick) were cut into strips ~1 mm in width, then these were cut into 1 mm2 chips with cutting pliers. Clean plastic chips were soaked in deionized water for 1 d prior to the start of the experiment.

Once the experiment was set up, tissue culture plates were stored at room temperature in dim natural light. At 5 dpf, 24 h after starting the experiment, individuals in all wells were observed with a stereomicroscope. Individuals were scored as settled (if they were attached to a substrate and encased in a calcified tube) or not settled (in one of two categories: still swimming, or missing from the well). Percent settlement in the three treatments after 24 h was compared using a linear mixed model with family as a random effect followed by Tukey’s post-hoc comparisons. Assumptions of normality and equal variance were assessed visually. Because the assumption of equal variance was violated, we also analyzed these data with a non-parametric Kruskal-Wallis test followed by post-hoc comparisons using Dunn’s tests. All analyses were carried out using R version 4.4.3 (R Core Team 2025).

**Experiment 2: Do larvae of *Ficopomatus enigmaticus* have a genetically determined settlement dimorphism?** This no-choice settlement experiment was designed to determine if larvae of *F. enigmaticus* have fixed differences in settlement, responding to either cues associated with conspecific adults (chips of adult tube) or other substrates not associated with conspecific adults (chips of mussel shell, or biofilmed plastic chips), but not both. To accomplish this, we examined the settlement decisions of competent larvae of *F. enigmaticus* over six days of exposure of competent larvae to settlement cues. Each replicate of the experiment involved larvae of a single full-sib family. We replicated this experiment a total of six times over the course of eight months, each time using larvae from a different full-sib family.

For each family, adult collection and spawning was carried out as described above.At 4 dpf, individual larvae were exposed to one of four substrates (chips of adult tube, chips of mussel shell, lab-biofilmed plastic chips, or clean plastic chips) and their settlement tracked daily over the next six days. Experiments were carried out in 24-well tissue culture plates whose wells had previously been coated with 0.2% gelatin. At 4 dpf, haphazardly chosen larvae from a full-sib family were transferred to tissue culture plates (one larva per well) with pulled glass pipettes. Each well contained 1 ml of FSW, 30,000 cells of *I. galbana*, and one of the four randomly assigned substrate treatments. Treatments were evenly distributed among plates (six wells of each of each of four treatments in each plate), and treatments were randomly distributed among wells within each plate. Five tissue culture plates (120 wells; four treatments, 30 wells per treatment) were used for the experiment on Family 1; six tissue culture plates (144 wells; four treatments, 36 wells per treatment) were used for experiments on each of Families 2-6.

Adult tube chips were generated as described above the day each run was started (that is, 4 dpf) from the same aggregation of tubes from which adults had been obtained for spawning for that run. Chips of mussel shell were generated from living mussels (*M. galloprovincialis*) collected from a floating dock in Alamitos Bay in Long Beach, California (33.750°N, -118.122°W) the day each run was started. Mussels from this population were used because they were easier to access than those in the Los Angeles River population. Adults of *F. enigmaticus* have never been observed in Alamitos Bay (Yee et al. 2019; B. Pernet, personal observation). Mussel shell chips were generated as described above. Plastic chips (generated as described above) for the lab-biofilmed treatment were left in a plastic tea infuser (Toby Teaboy) floating in a recirculating seawater tank (35 psu, 16°C) to accrue a biofilm for 7 d prior to the start of an experiment. Clean plastic chips (generated as described above) for the negative control treatment were soaked in deionized water for 1 d prior to experiments.

Once the experiment was set up, tissue culture plates were stored at room temperature in dim natural light. Starting at 5 dpf, individuals in all wells were observed with a stereomicroscope daily through (including) 10 dpf. Individuals were scored as settled (if they were attached to a substrate and encased in a calcified tube) or not settled (in one of five categories: incompletely settled, swimming, stuck to the well, missing, or dead). Individuals were scored as incompletely settled if they were attached to a substrate but encased in only a mucus tube. Individuals could be classified as incompletely settled for at least two reasons – first, they may have settled very recently and had not had time to build calcareous tube before our observations, or second, they had settled but failed to complete metamorphosis. Water was not changed during the experiment, and algal food was not supplemented. Percent settlement in the four treatments at completion of the experiment (10 dpf) was compared using a linear mixed model with family as a random effect followed by Tukey’s post-hoc comparisons. Assumptions of normality and equal variance were assessed visually.

**RESULTS**

**Experiment 1: Do larvae of *F. enigmaticus* settle gregariously?** After 24 hr of exposure to potential settlement cues, larvae of *Ficopomatus enigmaticus* from all four families had settled at much higher rates on conspecific adult tube than on mussel shell or clean plastic (Fig. 1). On average, 81.20% (±25.70 95% confidence intervals) of larvae exposed to adult tube settled within 24 h, compared to 9.38% (±19.0) of larvae exposed to mussel shell, and 3.12% (±9.95) of larvae exposed to the negative control (clean plastic chips). The linear mixed model analysis showed that substrate significantly affected settlement (F2,6=86.71, p<0.001). Settlement on adult tubes was more common than on mussel shell (p<0.001) and the negative control (p<0.001), and there was no difference in settlement between mussel shell and the negative control (p=0.404). The random effect of family accounted for 32.3% of total variance. Results of a Kruskal-Wallis analysis were consistent with those of the linear mixed model.

Settlement in the adult tube treatment always occurred directly on the adult tube chip. In the mussel shell treatment, one of three settled individuals settled on the bottom of the tissue culture plate, and the only settled individual in the negative control treatment settled on the bottom of the tissue culture plate. In almost all cases, larvae that had not settled were observed still swimming in the well of the tissue culture plate. Only one larva (from a mussel chip well) was scored as missing at the end of the experiment.

**Experiment 2: Do larvae of *F. enigmaticus* have a genetically determined settlement dimorphism?** Observed settlement patterns varied little among the six families (Fig. 2). The first settlement was usually observed at 5 dpf in conspecific adult tube and field-biofilmed mussel shell treatments, but usually delayed by one day in the lab-biofilmed plastic treatment. Settlement was extremely uncommon in the negative control (clean plastic). Throughout the observation period and at 10 dpf, the most settlement was induced by conspecific adult tube (89.0% on average at 10 dpf, ±9.90 95% confidence intervals); then field-biofilmed mussel shell (59.10%±17.8); then lab-biofilmed plastic (30.70%±13.9); then the negative control (3.70%±6.82). The only exception to this pattern was in Family 4, where larvae settled on lab-biofilmed plastic at a slightly higher frequency than on field-biofilmed mussel shell. Larvae in Family 4 also had unusually high settlement in the negative control. The linear mixed model analysis of cumulative settlement at 10 dpf was consistent with the general pattern described above: substrate had a significant effect on settlement (F3,15=63.52, p<0.001), and post-hoc comparisons using Tukey’s HSD test revealed that settlement differed significantly among all substrate pairs (all p<0.001). The random effect of family accounted for 14.5% of total variance.

The among-family average distribution of various types of “not settled” individuals at 10 dpf is shown in Fig. 3. As expected, frequencies of all types of “not settled” classes were low in the two treatments that elicited the highest cumulative settlement (conspecific adult tube and field-biofilmed mussel shell), and high in the two treatments that elicited the least cumulative settlement (lab-biofilmed plastic and the negative control). In those latter two treatments, the majority of “not settled” larvae were still swimming at 10 dpf.

On the final day of the experiment, 10 dpf, summed cumulative settlement percentages in two treatments – conspecific adult tube and field-biofilmed mussel shell – substantially exceeded 100% in all six families observed (Fig. 4).

**DISCUSSION**

*Ficopomatus enigmaticus* is well-known as an aggregating species, often forming “reefs” of many thousands of individuals (e.g. Bianchi and Morri 1996; Schwindt and Iribarne 1998; Dittman et al. 2009; Pernet et al. 2016). To our knowledge there are only two studies that address the question of whether these aggregations might form (at least in part) as a result of gregarious settlement. In no-choice experiments with groups of larvae, Yee (2019) showed that adult conspecific tube induced a higher percentage of larvae to settle than did any other substrate tested. However, in her experiments conspecific tube bore biofilm that had developed in brackish water (adult habitat), while the alternative substrates had biofilm that had developed in full-strength seawater from sites where adults of *F. enigmaticus* did not occur. Since salinity is known to affect biofilm composition (e.g., Lau et al. 2005; Caruso 2020; Pinnell and Turner 2020), it is possible that larvae in her experiments were not choosing conspecific tube because of some species-specific factor, but instead because it bore the biofilm indicative of their preferred habitat.

In the no-choice settlement experiments with individual larvae we report on here, we controlled for salinity-related differences in biofilm type by comparing conspecific tube to mussel shell collected from the same brackish-water habitat (indeed, from only a few cm away from aggregations of *F. enigmaticus*). We found a result very similar to that of Yee (2019) – in all four families we examined, adult conspecific tube chips induced larval settlement at much higher frequencies than did other substrates tested. Together, these two studies strongly suggest that gregarious settlement plays an important role in the growth of existing aggregations of *F. enigmaticus*.

New aggregations, however, are also regularly formed by settlement of larvae on uncolonized surfaces. Larvae of *F. enigmaticus* settle, for example, on settlement plates (Dittman et al. 2009), glass bottles, mollusk shells, and other surfaces from which adults are absent (Schwindt and Iribarne 1998, 2000). Further, *F. enigmaticus* is well-known as an invasive species, colonizing previously unoccupied locations many times since at least the 1920s (reviewed by Dittman et al. 2009). Initial colonization of a new location may occur via fragmentation of adult aggregations (e.g., on boat hulls) or by larval settlement, but any subsequent expansion of populations at new locations undoubtedly requires larval settlement on previously unoccupied surfaces. Thus there must be variation in the behavior of larvae of *F. enigmaticus*, with some settling on conspecific adults, but others settling on other substrates, founding new aggregations.

What might explain this variation in settlement behavior in larvae of *F. enigmaticus*? When discussing the spread of this species, prior workers have sometimes discussed work on the genetically determined settlement dimorphism in *Hydroides dianthus*, noting that it is unknown if a similar dimorphism exists in *F. enigmaticus* (e.g. Dittman et al. 2009). Our data suggest that such a dimorphism is unlikely in *F. enigmaticus*. In our experiments, the sum of cumulative settlement percentages on conspecifics and on field biofilm exceeded 100% in all six families tested (Fig. 3). This means that at least some larvae of *F. enigmaticus* are capable of settling in response to multiple types of cue. This result contrasts strongly with *H. dianthus*, where in ~90 experiments, cumulative settlement on conspecifics and biofilm (each estimated independently in no-choice experiments on groups of larvae) was always ≤100% (see Figure 1 in Toonen and Pawlik 1994, and Figures 2 and 4 in Toonen and Pawlik 2001). Thus, individual larvae of *H. dianthus* appear to be capable of responding either to conspecific cues or to biofilm, but not to both (Toonen and Pawlik 1994, 2001), but at least some individual larvae of *F. enigmaticus* appear to be capable of responding to both cues.

We considered two alternative hypotheses to explain the variation in settlement behavior we observed in larvae of *F. enigmaticus*. One possibility is that this variation was generated by a broadening of larval settlement preferences over time – newly competent larvae might settle only in response to conspecific cues, but as time passes without encountering juvenile or adult conspecifics, they begin to respond to additional cues. This is also known as the “desperate larva” or “variable retention” hypothesis (Bishop et al. 2006). The mechanism underlying such a broadening of settlement preferences might be energy limitation (e.g., if algal food becomes scarce) or the accumulation or loss of some factor that stimulates or inhibits, respectively, competence to settle on alternative substrates (Bishop et al. 2006). However, in our experiments, larvae of *F. enigmaticus* settled in response to field-biofilmed mussel shell (i.e., a non-conspecific cue) immediately after reaching competence (5 dpf in four of the six families we examined; by 6 dpf, larvae from all six families had started settling in response to field-biofilmed mussel shell). Toonen and Pawlik (1994) observed a similar pattern in *H. dianthus*: newly competent larvae began settling on both conspecific and biofilmed surfaces roughly simultaneously. Like Toonen and Pawlik (1994), we interpret this as inconsistent with the hypothesis that larval settlement preferences are broadening over time. However, we note that we provided algal food to our larvae only at the start of experiments, at 4 dpf. Larvae of the co-occurring serpulid *Hydroides gracilis* are very similar in morphology to those of *F. enigmaticus*, and at 6 dpf these have maximum clearance rates on the order of 50 µl•h-1 (B. Pernet, unpublished data). Assuming that larvae of *F. enigmaticus* have similar maximum clearance rates, each larva in our experiment might have captured all its available food after 1 d of being in their experimental treatment. Thus larvae may have been energy-limited relatively early in the experiment. A better design would have been to offer each larva supplementary food daily to reduce the possibility of energy limitation.

Another possibility is that all larvae of *F. enigmaticus* are capable of settling in response to multiple potential settlement cues from the moment they are competent, but with different (but stably ranked) likelihoods of settling in response to different cues. A given larva might, for example, have a high probability of settling when it encounters juvenile or adult conspecific cues, but a lower probability of settling when it encounters other cues (e.g., biofilmed surfaces of various sorts). Our data are consistent with this hypothesis. Larvae of *F. enigmaticus* from five of six families had identically ranked preferences for four different settlement substrates, with conspecific tube inducing the greatest settlement across the whole observation period, followed by field-biofilmed mussel shell, lab-biofilmed plastic, and clean plastic (the negative control), respectively.

Based on the above evidence, we tentatively conclude that larvae of *F. enigmaticus* displayed stably ranked settlement preferences for the substrates we offered them (but we acknowledge that additional experiments must be carried out to rule out the hypothesis of an energy limitation-induced broadening of larval settlement preferences over time). How might such a system work? Perhaps the simplest hypothesis is that larvae respond to only one type of settlement-inducing chemical cue, and that different substrates present that cue at different intensities. For example, larvae of some serpulids are known to settle in response to contact with biofilm (e.g., Toonen and Pawlik 1994; Carpizo-Ituarte and Hadfield 1998; Hadfield et al. 2014; Shikuma et al. 2014). The settlement substrates we offered larvae – conspecific tube, field-biofilmed mussel shells, and lab-biofilmed plastic – all bore biofilm, but the inductiveness of the biofilm on each substrate might have differed predictably in relation to the density of one or more specific settlement-inducing bacterial taxa within it. Such differences in the composition and inductiveness of the biofilm in our experiment are plausible, because biofilms on the three substrates were formed in very different environments: conspecific tubes were from the mouth of the Los Angeles River, where salinities are typically ~20-25; field-biofilmed mussels were from Alamitos Bay, where salinities are typically ~35; and lab-biofilmed plastic was incubated in a recirculating system containing seawater (salinity 35) collected several kilometers offshore and then stored in the lab for months prior to use. As previously mentioned, salinity and other environmental parameters are known to affect biofilm community composition (e.g., Lau et al. 2005; Caruso 2020; Pinnell and Turner 2020), as well as the inductiveness of biofilm as a settlement cue for marine invertebrate larvae (Lau et al. 2005).

More complex possibilities are also possible, of course. Conspecific tube material may bear a qualitatively different (more inductive) type of cue than other substrates, and all other substrates may be ranked on the quality of a more general cue like biofilm. It is also possible that larvae may integrate multiple types of cues (e.g., both chemical and textural) when making settlement decisions.

A potentially complicating factor is that the adults of *F. enigmaticus* that we used as sources of gametes in these experiments likely included individuals of two very distinct (~19% uncorrected sequence difference) cytochrome B mitochondrial haplotype groups. Though members of these two haplotype groups are to the best of our knowledge morphologically indistinguishable, the two groups are so distinct molecularly that it has been suggested that they might represent cryptic species (Styan et al. 2017). In a 2018 survey of the Los Angeles River population of *F. enigmaticus* studied here, 39% of the adults collected had Clade 1 haplotypes, and 61% had Clade 2 haplotypes (Yee et al. 2019). Assuming similar proportions were present in 2022-2025, when the experiments described here were carried out, it seems possible that some of our larval families were of pure Clade 1 parentage, some of pure Clade 2 parentage, and some of mixed parentage. Results of both of our experiments were strikingly consistent among families, however, so it seems unlikely that this possible variation in parentage should affect our conclusions.

Together, the results of our experiments and those of Toonen and Pawlik (1994, 2001) suggest that there is variation in how new aggregations are established among species of aggregation-forming serpulids. This variation may have significant implications for the spread of *H. dianthus* and *F. enigmaticus*, both of which have been introduced and become established in many parts of the world (*H. dianthus*: Sun et al. 2017, Bastida-Zavala et al. 2017; *F. enigmaticus*: Dittman et al. 2009, Bastida-Zavala et al. 2017). Specifically, the genetically determined settlement dimorphism described in *H. dianthus* (Toonen and Pawlik 1994, 2001) may limit the rate at which this species can form new aggregations by larval settlement. Toonen and Pawlik (2001) estimated that the average frequency of “founder” larvae (those that settled and metamorphosed in response to a biofilm cue, but not in response to the presence of conspecifics) in families derived from 308 separate females was 4.3% (range 0-51%); further, the frequency of founder larvae was zero in ~120 of the 308 families. Thus in *H. dianthus*, only 4.3% of the larvae produced in a population (on average) are available to form new aggregations, and many families produce no founders at all. In *F. enigmaticus*, in contrast, many larvae from all families studied were capable of settling in response to biofilm cues. In our second experiment, we found that by 10 dpf, ~55% of larvae exposed only to field-biofilmed mussel shell had settled. This difference suggests that once introduced to a new location, *F. enigmaticus* might form new aggregations at a much higher rate than *H. dianthus*. Further, the rate of forming new aggregations in *H. dianthus* should be fixed genetically and independent of the density of aggregations in the surrounding habitat. In contrast, if aggregations of *F. enigmaticus* are rare or nonexistent (the expectation in a novel habitat), a greater percentage of larvae produced by that species will never encounter conspecific cues and so are more likely to settle in response to general biofilm cues, thus forming new aggregations. The hypothesis that such differences in how new aggregations are formed may affect the rate of colonization of new habitats in these species cannot yet be tested with comparative data on the rate of formation of new aggregations in nature because of the complete absence of such data, but its validity could be assessed using mathematical models.

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A diagram of a diagram of a family

AI-generated content may be incorrect.

**Fig. 1.** Mean settlement of larvae of *Ficopomatus enigmaticus* after 24 h exposure to each of three treatments. Error bars are 95% confidence intervals.

**A graph of different stages of growth

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**Fig. 2.** Cumulative settlement of larvae of *Ficopomatus enigmaticus* in each of four treatments from 5-10 days post-fertilization.

**A graph of different colored bars

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**Fig. 3.** The average (among the six families studied) distribution of states of larvae of *Ficopomatus enigmaticus* at 10 days post-fertilization.

**A graph with a line and numbers

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**Fig. 4.** Cumulative settlement of larvae of *Ficopomatus enigmaticus* at 10 days post-fertilization summed over the two most preferred substrates, conspecific tube and field biofilmed mussel shell. Summed settlement percentages were >>100% in all six families examined.