Helical swimming as an exploratory behavior in competent larvae of the eastern oyster (*Crassostrea virginica*)

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ABSTRACT

Helical swimming is a common behavior in larvae of many marine invertebrate species that may facilitate either exploration or feeding. Swimming in helices may increase exposure of larvae to settlement cues localized to the seafloor by enhancing their horizontal scanning motion near potential settlement sites. Alternatively, helical swimming may increase feeding efficiency by allowing an organism to maximize time spent in vertically-constrained food patches. In this study, we investigated whether the

prevalence and geometry of helical swimming in competent larvae of the eastern oyster (*Crassostrea virginica*) vary in response to a settlement cue or to food. We performed two experiments, one examining helical swimming behavior in larvae exposed to different concentrations of a chemical settlement cue ("Cue Experiment") and the other examining helical swimming of fed and starved larvae in conditions with and without algal food ("Feeding Experiment"). In the Cue Experiment, the proportion of larvae swimming in helices increased with decreasing cue concentration, and helices became wider, which suggests that helices may be an exploratory behavior that is curtailed when preferred habitat is detected. In the Feeding Experiment, neither the proportion of larvae performing helices nor helix geometry varied with food availability or satiation. Our results indicate that variations in helical swimming likely enhance the ability of *C. virginica* larvae to detect lateral variation in waterborne cues and locate suitable habitat prior to settlement.

KEYWORDS

Mollusc larvae, veliger, settlement cue, swimming behavior, helices, feeding, benthic, video analysis

1. INTRODUCTION

Planktonic larvae of benthic marine invertebrates display dynamic swimming behaviors in response to environmental cues. These behaviors affect larval dispersal patterns, survival, and ultimately settlement success (reviewed by Cowen and Sponaugle, 2009). Larvae of many different marine species can control their vertical

position in a variety of ways, including helical swimming, active swimming along a straight trajectory, passive sinking (reviewed by Chia et al., 1984), and in some bivalve species, active diving (Finelli and Wethey, 2003; Wheeler et al., 2015). Helical swimming is a particularly interesting behavior because it has been documented across a range of taxa, including protozoans (reviewed by Jahn and Votta, 1972) and invertebrate larvae (reviewed by Knight-Jones, 1954) such as mollusc veligers (Cragg, 1980). Helical swimming is characterized by swimming along a corkscrew-like path and is thought to result from asymmetries in body shape or from the movement of locomotory structures such as flagellae and cilia (Jennings, 1901). Many bivalve larvae can modify helix shape by altering ciliary beat and velar angle (Cragg, 1980) in response to environmental cues (Buckham, 2015; Jackson, 1999; Mann and Wolf, 1983; Wheeler et al., 2017).

The mechanics of helical swimming have been well-described (Crenshaw, 1989); however, the function of this swimming pattern remains unknown (Chan, 2012). For many species, helical swimming is unlikely to be merely a by-product of body asymmetry, because of the active control individuals demonstrate over their helix geometry when responding to environmental conditions. Helical swimming has been proposed as a strategy for directional swimming to orient an organism towards environmental cues (Crenshaw, 1996; Jennings, 1901). Helix geometry may also be varied as a response to environmental conditions such as temperature (Chan and Grünbaum, 2010). Variations in helices may be used to avoid predators (Visser, 2007), while maximizing both prey capture (Gittleson et al., 1974) and exposure to settlement cues (Meyer et al., 2018), especially in the presence of light (Wheeler et al., 2017).

One potential benefit of helical swimming is to increase the time a larva spends in a particular stratum. This behavior would allow larvae to scan horizontally for settlement cues close to a substratum or feed on vertically-constrained patches of food. Prior to attachment and metamorphosis, larvae explore potential settlement sites (Doyle, 1975) and identify a proper site using both physical and chemical cues that are localized at the substratum (reviewed by Pawlik, 1992). “Exploration” is used in the literature to describe multiple distinct larval behaviors. It can be used to describe a larva surveying a settlement substratum by crawling along the surface (e.g., Mullineaux and Butman, 1991; Walters et al., 1999), and to describe a larva abandoning a poor settlement site and swimming up into the water column in search of an alternative settlement location (e.g., Butman, 1986). In this study, we refer to exploration as a behavior in which larvae increase their horizontal swimming motion and decrease their vertical motion to scan the water close to a substratum. Exploration behavior may be used by invertebrate larvae to select specific microhabitats for settlement based on cues such as light level (Maldonado and Uriz, 1998) and fluid shear (Mullineaux and Garland, 1993), and these small-scale choices could impact the survival of recruits. Increasing the scanned area near a potential settlement site by modifying helical swimming could allow a larva to detect subtle physical and chemical cues and better select suitable microenvironments for settlement.

Helical swimming may also serve to increase food capture by increasing the water volume an organism is exposed to in stratified food patches (Gittleson et al., 1974) and by increasing the time an organism spends within a food patch (Raby et al.,

1994). In copepods, slow swimming in helices has been shown to increase in response to prey availability (Caparroy et al., 1998).

We investigated the helical swimming response of larvae to a chemical settlement cue and food in the eastern oyster, *Crassostrea virginica* (Gmelin, 1791). This mollusk forms large reefs in the intertidal and subtidal west Atlantic (Bahr and Lanier, 1981). Adults reproduce by broadcast spawning from spring to fall (Hayes and Menzel, 1981). Larvae are free-swimming for 2 to 3 weeks post-fertilization (Kennedy, 1996) and are commonly reared for aquaculture, making *C. virginica* an accessible model organism that is maintainable in laboratory cultures. Because *C. virginica* larvae settle gregariously (Dame et al., 1984) in response to conspecific cues (Crisp, 1967), their behaviors can be examined in laboratory experiments. Additionally, these larvae are planktotrophic (Kennedy, 1996), which allowed us to investigate their swimming response to food in the water column.

Oyster larvae alter their helical swimming in response to physical conditions (Wheeler et al., 2017) and chemical cues (Meyer et al., 2018), so helical swimming is an inducible behavior in this species, rather than a passive result of asymmetries in body shape (as proposed by Jennings, 1901). In oyster larvae, feeding is morphologically linked to swimming behavior, as the ciliated velum is used for both. Beating bands of cilia serve both to propel larvae through the water and to create a feeding current to allow larvae to capture suspended algae (reviewed by Waller, 1981). Since algae are vertically stratified in the water column, a helical swimming pattern that maximizes horizontal area covered while minimizing vertical net movement could increase food capture.

We tested two separate hypotheses: 1) larvae increase their helical swimming in response to decreasing concentrations of chemical settlement cue and (2) larvae increase their helical swimming in response to food availability. In the first experiment, we exposed *C. virginica* larvae to serial dilutions of a conspecific settlement cue, similar to Zimmer-Faust & Tamburri (1994), and recorded their behavior. If helical swimming is an exploratory behavior used to improve the chance of finding a suitable settlement site, we would expect larvae to increase their time swimming in helices and to swim in wider helices when they are exposed to a weak cue, indicative of nearby habitat. When they are exposed to a strong cue, we would expect them to swim more directly downward. In the second experiment, we observed helical swimming behaviors of starved and fed larvae in conditions with and without algae available. We predicted that when food was available, larvae would spend more time swimming in helices and would swim in wider helices to maximize food capture. Starved larvae were expected to perform more helices than fed larvae in both the presence and absence of algae, as they would be more desperate for food and therefore engaging in more feeding behavior.

2. MATERIALS AND METHODS

2.1. Experimental design

We conducted two experiments to determine what environmental cues influence helical swimming behavior in *C. virginica* larvae. In the first experiment, hereafter called the “Cue Experiment,” we exposed larvae to five different concentrations of a chemical settlement cue from adult conspecifics. The second experiment, the “Feeding Experiment,” followed a 2x2 factorial design. We varied algal availability, by either

adding an algal suspension or filtered seawater to the experimental flask, and larval satiety, by either starving or feeding the larvae for 24 hours prior to the trials.

2.2. Larval cultures

Crassostrea virginica larvae for this study were obtained from the Aquacultural Research Corporation (Dennis, MA, USA) in July 2017. Larvae were maintained in ~1 L of aerated, 10 µm-filtered seawater (salinity of 33 ppt, 19-20°C) in covered glass jars at low densities (< 5 larvae mL^{-1}) in a dark, temperature-controlled environmental chamber for 24 – 48 hours prior to the experiments. The use of the 10 µm filter was inadvertent (1 µm is standard) and may have allowed small (< 10 µm) algae into larval cultures.

All larvae for the Cue Experiment were fed 20 mL of an *Isochrysis* sp. suspension (10^6 cells mL^{-1}) per liter of culture once per day. In the Feeding Experiment, larvae were split into two groups: fed or starved. The starved group was introduced to increase the motivation for feeding behavior in oyster larvae, as it has been shown to do so in echinoderm larvae (Metaxas and Young, 1998). We chose to starve them 24 hours because larvae of a similar species, *Crassostrea gigas*, starved for more than 3 days begin to display negative effects on vitality (His and Seaman, 1992), and we wanted to increase the motivation to feed in our larvae without negatively affecting their health. Fed larvae were treated identically to the larvae in the Cue Experiment and received 20 mL of *Isochrysis* sp. suspension per liter of culture per day. *Isochrysis sp.* was used for consistency with rearing conditions in the hatchery, and because this species of algae is commonly used in feeding studies of larval oysters (e.g., Rhodes and Landers, 1973). Twenty-four hours prior to experiments, each group of larvae had

their filtered seawater replaced, and the fed larvae received their *Isochrysis* sp. suspension, while starved larvae received only seawater. For both experiments, water was replaced just prior to the trials to prevent the accidental addition of algae from the larval cultures into the experimental flasks.

Prior to beginning the experiments, a subsample of larvae was preserved in 95% ethanol for size measurements and eyespot identification (Table 1). Eyespots are a commonly-used visual indicator of competency to settle in *C. virginica* (Thompson et al., 1996). Size was quantified as shell width (length of shell parallel to hinge) and height (perpendicular to hinge). It was important to use larvae of the same approximate ontogenetic stage in both experiments because development affects feeding (Gerdes, 1983) and settlement behavior (Meyer et al., 2018) in *C. virginica* larvae. All trials were completed within 12 hours of sub-sampling, and settlement behavior typically does not change significantly over this time period in this species (Meyer et al., 2018).

2.3. Chemical cue preparation and serial dilution

The chemical settlement cue was prepared using live adult *C. virginica* oysters harvested from Duxbury, MA, USA in June 2017 purchased from a local vendor (The Clam Man, Falmouth, MA, USA). The surface area of adult oysters was calculated following the methods of Tamburri et al. (1992). A total of 22 oysters with 1592 cm^2 in surface area were soaked in 4L of aerated 10 µm-filtered seawater (salinity of 33 ppt) in a sterile bucket. Oysters were not rinsed prior to soaking, as the biofilms on their shells have been shown to induce settlement in addition to the chemicals released by the oyster tissues (Fitt et al., 1990). After 4 hours, the oysters were removed, and the

solution was filtered through a 0.2 µm glass microfiber filter using vacuum filtration. Filtered cue water was subsequently frozen in 250 mL aliquots at -20°C and thawed just prior to the experiments, similar to the freezing procedure of Tamburri et al. (1992). Aliquots of seawater filtered to 10 µm (FSW, salinity of 33 ppt) were collected the same day as the chemical cue preparation and were similarly frozen for use as the no-cue solution in the Cue Experiment and for preparing dilutions of the cue. This preparation method ensured that the water chemistry would be identical for all FSW used in dilutions of the cue and the no-cue solutions.

The chemical cue was diluted using a 1:4 dilution series to 0.25x, 0.0625x, 0.0156x, and 0.0039x the original concentration (1x) using prepared FSW. The no-cue solution (referred to as 0x) was FSW with no cue. This dilution series was based on concentrations found to affect the percent settlement of *C. virginica* by Zimmer-Faust and Tamburri (1994), although our original solution was more concentrated than theirs by roughly 6x, as calculated by shell area and seawater volume. Dilutions of the chemical cue were relative to the original concentration, but it was impossible to calculate the exact concentration of the settlement-inducing compound in these solutions, as the identity of the active chemical(s) remains unknown (reviewed by Hadfield and Paul, 2001). The chemical cue is likely a peptide with arginine and lysine residues at the C-terminus (Zimmer-Faust and Tamburri, 1994).

2.4. Experimental setup

All experiments were conducted in an environmental chamber at a constant temperature of 19 – 20□C. To record swimming behaviors, larvae were first taken from

low-density culture conditions (< 5 larvae mL^{-1}), retained on a 100 µm sieve and condensed to approximately 40 – 50 larvae mL^{-1}. Larvae were then introduced into a flat-sided flask (Corning, 25 cm^2 cell culture flask, canted neck) by suspending a sub-sample of larvae in a 1000 µL micropipette above the flask opening and allowing the larvae to swim passively into the flask. This introduction method prevented any flow in the flask that could impact larval movement. Additionally, convective currents in the flask were minimized by pre-filling the flasks and allowing them to equilibrate to the temperature of the environmental chamber before the introduction of larvae.

In the Cue Experiment, flasks were filled with 50 mL of either FSW or diluted chemical cue. Each cue concentration had five replicate flasks (n=5), with approximately 30 larvae per replicate, except 1x and 0.0156x, which each had 6 replicates. The order of trials was randomly assigned.

In the Feeding Experiment, flasks were filled with 50 mL of either FSW or an *Isochrysis sp.* suspension (salinity of 33 ppt, 19°C). The algae in the suspension were taken from the same culture used to feed larvae prior to the experiments and diluted to the desired concentration ($5x10^4$ cells mL^{-1}). The density of the algal culture was determined with a hemocytometer (Hausser Scientific, 0.100 mm deep) just prior to the experiment. The algal density utilized in the experimental flasks was based on the typical density of small (< 10 µm) algae in estuaries (10^4 to 10^5 cells mL^{-1}; E. Brownlee, personal communication) that are in the size-fraction typically consumed by competent-to-settle oyster larvae (Baldwin and Newell, 1995). Algal flasks were treated identically to flasks with the chemical cue or FSW, with all flasks being permitted to settle prior to the introduction of larvae in order to minimize flow. Nevertheless, algae likely remained

unstratified in the flasks because *Isochrysis sp.* are motile (Liu et al., 2011) and were not observed in batch culture to aggregate at any particular depth.

Following a 2x2 factorial design, the Feeding Experiment had 2 factors (algal availability and satiety) each with 2 levels (algae or no algae, and fed or starved, respectively). Larvae that had been fed the day prior to the experiments were split into two groups: one was introduced to flasks of FSW (fed, no algae), while the other was introduced to flasks containing the algal suspension (fed, algae). The starved larvae were similarly split into a group with FSW in the flasks (starved, no algae), and one with algae in the flasks (starved, algae). Each of the four treatments had five replicate flasks (n=5), with approximately 30 larvae per flask. The order of trials was again randomly assigned. Vitality of larvae was not expected to vary across treatments, or change over the course of the experiment, and was not measured at the end of the trials.

2.5. Video processing and statistical analysis

Larval swimming behaviors in each trial were recorded at 30 frames s^{-1} for 10 minutes. Videos were recorded in the dark because light influences oyster larval swimming (Wheeler et al., 2017) and *Isochrysis* sp. display phototactic responses (Kain and Fogg, 1958; Okauchi et al., 1997). Near-infrared lighting (Olymstore, 12V, 2A, 850 nm) and an IR-sensitive monochrome camera (Hitachi KPF-120) were used to record videos of larval swimming in a 2-dimensional full vertical cross-section of the flask (4 x 5 cm). Individual algal cells in the Feeding Experiment were too small to detect.

Video recordings of larvae were converted to a series of tiff files using LabVIEW 2013 (National Instruments), and larvae were identified by eliminating the average

254 background pixel intensity and then identifying larval centroids based on thresholds for
255 particle size and intensity. Larvae were subsequently tracked across successive frames
256 using a distance-traveled threshold using a custom MATLAB script. These video
257 processing methods were adapted from Wheeler et al. (2013; 2015), but because flow
258 in the flasks was minimal, larval swimming was quantified without subtracting local flow
259 conditions, following Meyer et al. (2018).

260 To analyze larval movements, we plotted each larval trajectory in (x,z) space and
261 categorized it as an “upward” or “downward" moving track. The majority of larvae
262 entering the flask exhibited one of two behaviors—swimming directly to the bottom and
263 remaining there, outside the camera's view (one downward track), or swimming to the
264 bottom, and then swimming back up into the water column (one downward and
265 one upward track). When a larva swam into the flask, stayed visible on the bottom and
266 then swam back up, the trajectory was separated into discrete downward and upward
267 tracks. In rare cases when a larva swam up off the bottom and then swam downward,
268 the downward section of the track was separated, but not counted as an additional larva
269 entering the flask (as in Meyer et al. 2018). When an upward-moving larva hovered in
270 the flask (making short, repeated, upward and downward motions), it was categorized
271 as a single upward track. Therefore, we were able to approximate the number of larvae
272 introduced into the flask as the number of downward tracks. The number of downward
273 tracks counted over each observation period was approximately 20-40 (mean=29.9,
274 s=16.1). The number of upward tracks counted was typically less than 10 (mean=8.4,
275 s=8.9). Both of these numbers could be biased by a few larvae making multiple

excursions up and down, while disappearing from view at the top or bottom of the trajectory, but we did not observe this behavior in visual examinations.

Given the accounting of downward and upward tracks above, a good estimate of the proportion of larvae remaining on bottom is 1 – (the number of upward tracks counted) / (the number of downward tracks counted). This proportion was interpreted as an indicator of settlement, and therefore lack of exploration in the Cue Experiment or a lack of swimming and food-searching behavior in the Feeding Experiment. For ease in reporting, we refer to the metric as “proportions of larvae” rather than “proportions of tracks,” following Meyer et al. (2018). Tracks showing helical motion anywhere along the path were categorized as upward-helical or downward-helical and were used to calculate the proportion of tracks in each direction exhibiting helices (displayed as “proportion of larvae performing helices”). Helix geometry (width W, height H, and elongation ratio H/W) was calculated from the helical segments of those tracks.

Since recordings were 2-dimensional, helical trajectories appeared sinusoidal in *(x,z)* space. The time series of *x* position of helical trajectories were likewise sinusoidal, and the peaks of these time series were identified using MATLAB local extrema-finding algorithms. The distances between the peak *x* positions were used to calculate the average width of each helix. The time points of identified peaks were then used to determine *z* positions at the beginning and end of each helix, and from these the average helix height was calculated (Fig. 1).

Behavioral metrics were compared between treatments using a 1-factor ANOVA (Cue Experiment) or a 2-factor ANOVA (Feeding Experiment) after confirming

299 homoscedascity with Levene's tests. Post-hoc Tukey HSD tests were used for pair-wise
300 comparisons. All analyses were conducted in JMP Pro 12 statistical software. The
301 Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was applied to p-
302 values from both 1-factor and 2-factor ANOVAS with a false discovery rate of 5%.
303 Pearson correlation coefficients between the measured metrics and treatments in each
304 experiment were calculated in JMP and are reported in the Supplementary Material.
305
306 3. RESULTS
307 *3.1. Cue Experiment*
308 The proportion of larvae remaining on the bottom of the flask decreased in a
309 dose-dependent manner as cue concentration decreased (Fig. 2, Table 2). Significantly
310 more larvae settled on the bottom in full-strength cue (1x) than in 0.0039x cue, and the
311 response to other concentrations of cue showed a strong trend, with the exception of
312 the no-cue (0x) solution.
313 The proportion of larvae performing helices when swimming downward increased
314 significantly with decreasing cue concentration (with the exception of 0x), with a
315 maximum at 0.0039x (Fig. 3A, Table 2). The proportion of larvae performing helices
316 when swimming upward also increased with decreasing cue concentration, resulting in
317 a strong, but not significant, trend for an effect of cue in the ANOVA (Fig. 3B, Table 2).
318 Helix width did not vary significantly with cue concentration for larvae swimming
319 in either direction (Fig. 3C and D, Table 2). Helix height in larvae swimming downward
320 also did not vary significantly across cue concentrations (Fig. 3E, Table 2). However,

helix height of larvae swimming upward did vary significantly, but not monotonically, with cue concentration (Fig. 3F, Table 2), and was greatest in larvae exposed to 0.25x.

The elongation ratio in downward-swimming larvae showed an increasing trend with decreasing cue concentration (except for 0x) (Fig. 3G, Table 2), but the pattern was not significant, possibly due to the high variation between replicates. The elongation ratio in larvae swimming upward varied significantly with cue concentration (except for 0x), with the lowest values in the most dilute cue concentration (0.0039x) and highest in the most concentrated cue (1x) (Fig. 3H, Table 2).

3.2. Feeding Experiment

Neither the algal availability nor satiety of larvae had a significant effect on the proportion of larvae remaining on the bottom of the flask (Fig. 4, Table 3). The proportion of larvae performing helices when swimming downward also did not vary with either algal availability or satiety (Fig. 5A, Table 3). The proportion of larvae performing helices when swimming upward was lowest in the No-Algae/Starved treatment (Fig. 5B), but neither algal availability nor satiety had a significant main effect (Table 3). The interaction effect resulted in a trend, showing a decrease in helical swimming when food was unavailable, but only for starved larvae.

Helix width (Fig. 5C and D) and height (Fig. 5E and F) did not vary significantly in response to either food availability or larval satiety in larvae swimming in either direction (Table 3). There was also no significant effect of food availability or satiety on the elongation ratio in either direction (Fig. 5G and H, Table 3).

4. DISCUSSION

In the Cue Experiment, we tested whether larvae increased the prevalence of helical swimming in decreasing concentrations of a chemical settlement cue. Swimming in helices may allow larvae to increase their horizontal motion, increasing the time larvae can remain close to a potential settlement substratum and survey for subtle chemical cues from conspecifics or biofilms. Our results provide evidence that helical swimming becomes more prevalent in conditions (a dilute cue) where horizontal exploratory behavior has the potential to increase settlement success.

Prior to settlement, larvae select a settlement site based on fine- and broad-scale environmental cues. Some broad-scale cues may include sound (Lillis et al., 2013) and turbulence (Fuchs et al., 2004) mediated by larval age (Wheeler et al., 2017). Navigating these cues ultimately permits larvae to move closer to the substratum, where they are exposed to fine-scale cues. We predicted that in low cue concentrations, larvae would perform more helices to better detect these fine-scale chemical cues. This prediction was supported by our data, as the proportion of larvae performing helices in both directions increased with decreasing chemical cue concentration (although only significantly in downward-swimming larvae), indicating that helices may be used to search for subtle chemical cues. Invertebrate larvae are known to display behavioral responses to chemical cues in the water column, resulting in movement towards the benthos (Hadfield and Koehl, 2004; Koehl et al., 2007), and they further explore chemical settlement cues once they have made contact with a surface (e.g. reviewed by Bourget, 1988).

Our study provides evidence that the detection of fine-scale cues, such as chemicals from adult oysters and their biofilms, affects helical swimming in a concentration-dependent manner that may increase the potential for locating a suitable settlement site. It also hints that larvae increase their exploratory behavior (e.g. swimming up off the bottom, performing helices) in response to a dilute cue relative to no cue, although these differences are non-significant trends. For instance, the proportion of larvae performing downward helices was higher in the most dilute (0.0039x) concentration than in the no-cue (0x) treatment. We also observed enhanced exploratory behavior in the dilute cue (0.0039x) relative to no cue, with fewer larvae remaining on the bottom when the settlement cue was most dilute. There was a similar decrease in the elongation ratio of upward-swimming larvae in dilute cue concentrations compared to no cue (indicative of a greater width to height ratio in the helices), but the differences were not significant in pair-wise tests. However, this same peak in exploratory behavior in 0.0039x cue was not seen in the proportion of helices performed by upward-swimming larvae or in the elongation ratio of downward-swimming larvae. We speculate that when a dilute cue is detected by downward-swimming larvae, they engage in helical swimming to explore the lateral variation in these chemicals. In contrast, when larvae swim upward after rejecting a settlement site, a dilute cue and no cue both elicit exploratory behavior (i.e. helices) because larvae are searching for higher cue concentrations or other environmental cues indicative of a better settlement site.

A concentration-dependent response to the chemical cue was also observed in the settlement behavior of larvae, with higher proportions remaining on the bottom in

higher cue concentrations, although the significant difference was observed only between the most dilute and most concentrated solutions. This result is in agreement with previous work by Tamburri et al. (1992), who found a linear increase in the settlement response of *C. virginica* larvae when exposed to increasing serial concentrations of a conspecific cue. In the field, turbulence mixes chemical cues emanating from the benthos, resulting in patchy, filamentous cue structures that are most concentrated close to the source (Hadfield and Koehl, 2004); in this case, the source is adult oyster populations. Oyster reefs are also patchily distributed, so distinguishing between different concentrations of settlement cue and responding accordingly may help larvae to home in on aggregates of adult oysters and aid in gregarious settlement. Although the majority of larvae in our experiment remained on bottom when the concentration of cue was high, a smaller proportion of larvae in dilute cue concentrations likewise remained on the bottom. This pattern of behavior would result in most larvae settling in the densest patches of oysters; however, some might respond to dilute cue and settle on sparser patches.

Bivalve larvae are known to modify their helix geometry in response to environmental conditions (Buckham, 2015; Jackson, 1999; Mann and Wolf, 1983; Wheeler et al., 2017), and therefore we expected that if larvae were using helices to stay near the bottom while exploring for cues of a preferred benthic habitat, those in the lower cue concentrations would have greater helix width and decreased helix height (i.e., a smaller elongation ratio) to improve detection. Such modifications to helix geometry might increase the probability of detecting spatially-variable cues released at the substratum by increasing the horizontal scanning motion of larvae near the benthos.

In our experiments, helix height and width in both upward- and downward-swimming larvae showed a high degree of variability. Although helix height and width did not vary monotonically with cue concentration when analyzed independently, when they were combined into the elongation ratio, the predicted pattern of a decrease in elongation ratio with decreasing cue concentration emerged. This result was detected only for upward-swimming larvae; the lack of pattern in downward-swimming larvae may have been due to high variation between replicates, especially in the lowest cue concentration. The difference in behavior between upward- and downward-swimming larvae could also be due to the fact that upward-swimming larvae were responding to a combination of their encounter with the bottom of the flask and the cues in the water column, whereas the downward-swimming larvae had not yet encountered the bottom. The responses of larvae in both the prevalence and geometry of their helical swimming follow, although not consistently, our expectations for increasing exploration with decreasing cue strength.

In the Feeding Experiment, we tested whether helical swimming was a response to food availability. We hypothesized that helical swimming was used by larvae to remain in vertically-stratified food patches and therefore increase feeding efficiency. We predicted that if helices were used to feed on algae, they would occur more frequently when larvae were starved and more desperate for food. Our results for the Feeding Experiment do not support our hypothesis that *C. virginica* larvae swim helically to increase food capture.

We expected that larvae would perform more helices when algae were available, and that this effect would be amplified when larvae were starved. Our results for the

proportion of larvae performing helices did not agree with our predictions. The lack of effect of algal availability on helical behavior was surprising, given the evidence in the literature suggesting that increased turning behavior increases food capture (Buskey and Stoeker, 1988; Caparroy et al., 1998; Menden-Deuer and Grünbaum, 2006; Gittleson et al., 1974). However, these studies describe turning behavior in copepods and protists, so helical swimming may serve alternative purposes in bivalve larvae. Additionally, a sufficiently high food concentration (Buskey and Stoeker, 1988) and stratification of food (Menden-Deuer and Grünbaum, 2006) were necessary to induce turning behavior in previous studies, and therefore may be necessary to induce a similar change in helical behavior in bivalve larvae. Because the algae in this study (*Isochrysis* sp.) were free-swimming (Liu et al., 2011) and are not known to have a preferred swimming direction, they were likely homogenously distributed, not stratified. Thus, it is possible that our experimental design did not support a robust test of our feeding hypothesis.

Satiety produced an effect opposite to our predictions, as starved larvae performed fewer helices than fed larvae in the presence of algae. In echinoid larvae, nutrient deprivation enhances the response to algal availability (Metaxas and Young, 1998), so the lack of increased helical swimming in starved larvae with algae available suggests that this swimming pattern may not be a feeding response. However, this result may also be due to insufficient nutrient deprivation, as larvae in our study were starved for only 24 hours. Additionally, the use of a 10 μm filter for filtering seawater may have further reduced the effects of starvation by inadvertently introducing algae

457 into the culture jars. Starved larvae were therefore not without food, but just had less
458 compared to the satiated larvae.

459 We also predicted that larvae would alter their helix geometry to increase the
460 horizontal component (width) and decrease the vertical component (height), thereby
461 decreasing the elongation ratio of their helices when food was available, especially
462 when starved and more desperate for food. Helix geometry is certainly not fixed in *C.*
463 *virginica* larvae, based on the variations in helix geometry in response to different
464 chemical cue concentrations observed in our data and the previously documented
465 variability in geometry with variations in light conditions (Wheeler et al., 2017). However,
466 helix geometry did not vary in a predictable way when food availability and satiety were
467 varied, suggesting that *C. virginica* larvae do not respond to food variation (at least over
468 the range we tested) with alterations to helix geometry. In echinoderms, changes to
469 helix geometry may be a compensatory mechanism to conserve energy when larvae
470 are subjected to sub-optimal environmental conditions (Chan and Grünbaum, 2010).
471 Larvae in our experiment may not have altered their helix geometry because they were
472 not sufficiently starved and therefore did not need to conserve energy through
473 adjustments to behavior. Ontogeny also plays a role in feeding behavior (Gerdes,
474 1983), and therefore larvae at an earlier ontogenetic stage might have shown a more
475 distinct helical swimming response to food.

476 In our Cue Experiment, we found that larval helical swimming behavior occurred
477 more prominently in the presence of a dilute settlement cue than in a strong cue or no
478 cue. In dilute cue treatments, a lower proportion of larvae remained on the bottom, a
479 higher proportion of downward-swimming larvae performed helices, and the elongation

ratio in upward swimming larvae was reduced. These changes to behavior indicate that *C. virginica* larvae are most likely to reject a settlement site and engage in wider, shallower helices when a chemical settlement cue is detectable but weak. Chemical cues diffuse from benthic settlement habitats and are stirred by turbulent flow into filaments (Hadfield and Koehl 2004), with filament gradients of chemical signals likely persisting on micron scales (Taylor and Stocker 2012). Invertebrate larvae can rapidly cease swimming upon exposure to strong chemical settlement cues (eg. Hadfield and Koehl 2004), and as seen in this work, strong cues induce almost all *C. virginica* larvae to rapidly settle to the bottom and cease exploratory swimming activity. In weak cues, however, larvae reject the bottom and instead enhance horizontal helical swimming. Larvae may through this behavioral change seek to navigate chemical gradients in the near-bottom cue filaments, in order to reach more concentrated chemical signals, at which point they stop swimming and settle. In such a way, helical swimming in weak cues may lead larvae to increased benthic settlement success in the turbulent bottom boundary layer of the ocean.

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Table 1: Average size and percentage of larvae with eyespots for each experiment. N, number of larvae sub-sampled. Intervals represent standard deviation.

Experiment	N	Width (μm)	Height (μm)	% with eyespots
Cue	19	328 ± 18.0	265 ± 31.2	74
Feeding	12	343 ± 18.7	326 ± 17.8	92

680 **Table 2:** Results of 1-factor ANOVA testing the effect of settlement cue concentration
681 on behavioral metrics of upward- and downward-swimming *Crassostrea virginica* larvae.
682 The sum of squares (SS), mean squares (MS), and F-ratio are included for each
683 behavior. Degrees of freedom (df= groups, error) differed based on the number of
684 replicates with larvae displaying helical swimming behavior: proportion remaining on
685 bottom and performing helices in both directions (5, 26), helix width, height and
686 elongation ratio in downward-swimming larvae (5, 12), helix width, height and
687 elongation ratio in upward-swimming larvae (5, 18). P-values reported are from the
688 ANOVA. Significant p-values before correction ($p < 0.05$) are marked by an asterisk,
689 and significant values after application of the Benjamini-Hochberg correction are shown
690 in bold.

Behavioral metric	Swimming direction	SS	MS	F	p
Proportion of larvae remaining on bottom	None	0.37	0.07	4.21	**<0.01***
Proportion of larvae performing helices	Down	0.11	0.02	3.87	**0.01***
Helix width	Down	<0.01	<0.01	0.15	0.98
Helix height	Down	0.71	0.14	0.97	0.47
Elongation ratio	Down	2711.27	542.30	0.42	0.82
Proportion of larvae performing helices	Up	1.45	0.29	2.48	0.06
Helix width	Up	<0.01	<0.01	1.45	0.26
Helix height	Up	0.28	0.06	3.64	**0.02***
Elongation ratio	Up	86.11	17.22	4.71	**<0.01***

691

692

693 **Table 3**: Results of 2-factor ANOVA testing the effects of satiety and availability of algae
694 on behavioral metrics of upward- and downward-swimming *Crassostrea virginica* larvae.
695 The sum of squares (SS) and F-ratio (degrees of freedom for groups and error = 1 and
696 16, respectively) are included for each behavior. P-values reported are from the
697 ANOVA. Significant values before correction ($p < 0.05$) are marked by an asterisk; no
698 values remained significant after application of the Benjamini-Hochberg correction.

Behavioral metric	Swimming direction	Factor	SS	F(1,16)	p
Proportion of larvae remaining on bottom	None	Availability	<0.01	0.03	0.88
		Satiety	0.04	2.69	0.12
		Availability*Satiety	<0.01	0.26	0.62
Proportion of larvae performing helices	Down	Availability	<0.01	0.01	0.92
		Satiety	0.01	1.01	0.33
		Availability*Satiety	<0.01	<0.01	0.95
Helix width	Down	Availability	<0.01	1.07	0.32
		Satiety	<0.01	0.82	0.38
		Availability*Satiety	<0.01	0.05	0.82
Helix height	Down	Availability	0.13	3.53	0.08
		Satiety	0.04	1.13	0.30
		Availability*Satiety	0.02	0.60	0.45
Elongation ratio	Down	Availability	120.20	3.27	0.09
		Satiety	50.71	1.39	0.26
		Availability*Satiety	29.79	0.81	0.38
Proportion of larvae performing helices	Up	Availability	0.02	0.77	0.39
		Satiety	0.03	1.52	0.24
		Availability*Satiety	<0.01	4.80	0.04*
Helix width	Up	Availability	<0.01	0.14	0.72
		Satiety	<0.01	0.89	0.36
		Availability*Satiety	<0.01	0.12	0.74
Helix height	Up	Availability	<0.01	0.18	0.68
		Satiety	<0.01	<0.01	0.94
		Availability*Satiety	<0.01	<0.01	0.96
Elongation ratio	Up	Availability	0.63	0.05	0.82
		Satiety	3.23	0.26	0.61
		Availability*Satiety	5.49	0.45	0.51

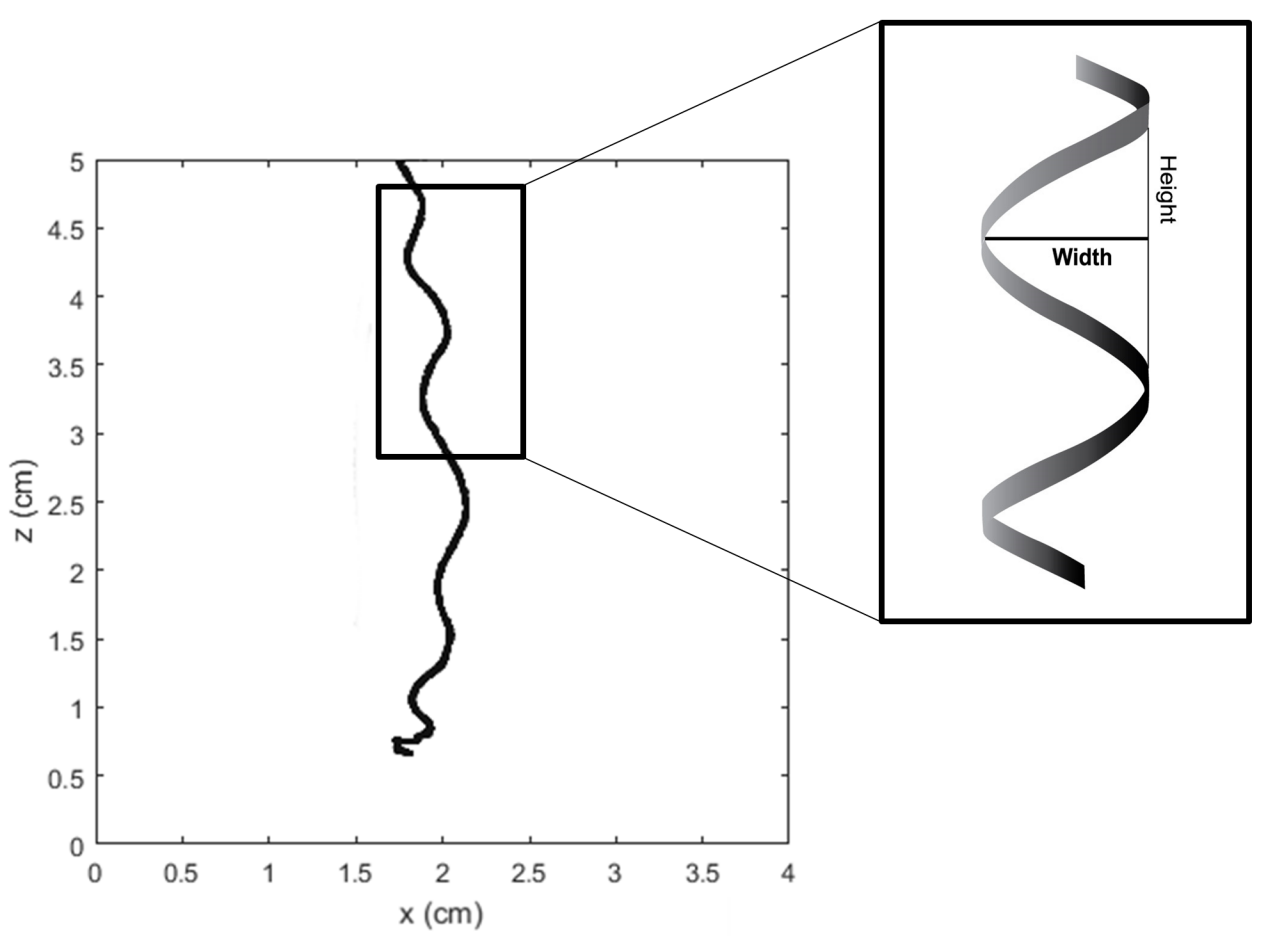
Figure 1: Example trajectory of a larva swimming upward in a helix. A zoomed-in schematic helical path shows the width and height components.

Figure 2: Proportion of larvae remaining on the bottom of the flask in the Cue Experiment. Error bars represent standard error of the mean. Statistical differences in the post-hoc Tukey test ($p < 0.05$) are denoted by different letters.

Figure 3: Helical swimming behavior in response to cue concentration in the Cue Experiment. Error bars represent standard error of the mean. Statistical differences in the post-hoc Tukey test ($p < 0.05$) are denoted by different letters. (A, B) The proportion of larvae performing helices when swimming downward (A) and upward (B); (C, D) Helix width of larvae swimming downward (C) or upward (D) in helices; (E, F) Helix height of larvae swimming downward (E) or upward (F) in helices; (G, H) Helix elongation ratio of larvae swimming downward (G) or upward (H) in helices. For downward-swimming larvae in the highest concentration of cue (1x), only one replicate had observations so there are no error bars shown for concentration “1” in panels A, C, E, and G.

Figure 4: Proportion of larvae remaining on the bottom of the flask in the Feeding Experiment. Error bars represent standard error of the mean.

Figure 5: Helical swimming behavior in response to algal availability and satiety in the Feeding Experiment. Error bars represent standard error of the mean. Statistical differences in the post-hoc Tukey test ($p < 0.05$) are denoted by different letters. (A, B) The proportion of larvae performing helices when swimming downward (A) and upward (B); (C, D) Helix width of larvae swimming downward (C) or upward (D) in helices; (E, F) Helix height of larvae swimming downward (E) or upward (F) in helices; (G, H) Helix elongation ratio of larvae swimming downward (G) or upward (H) in helices.


z (cm)
x (cm)
0
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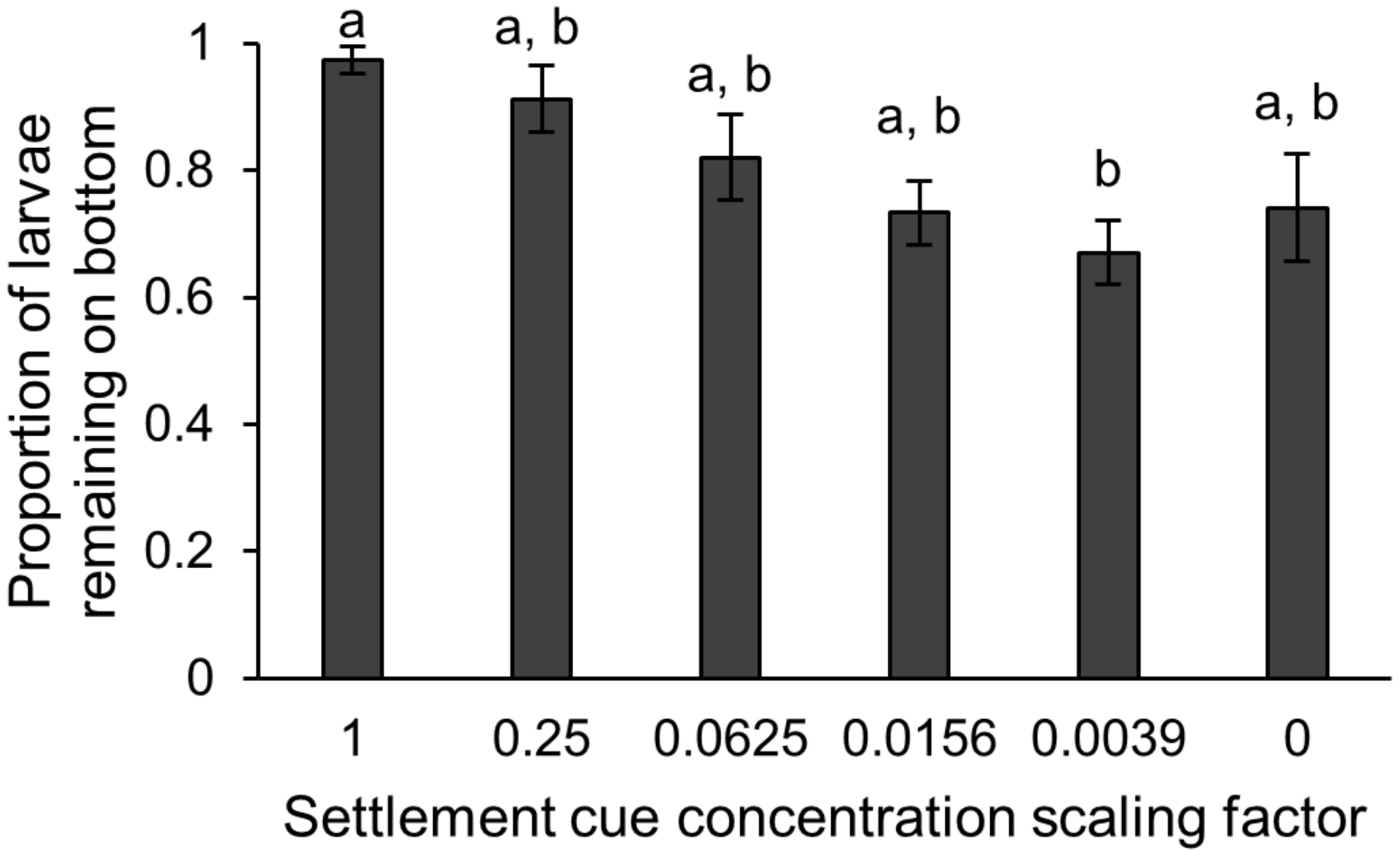
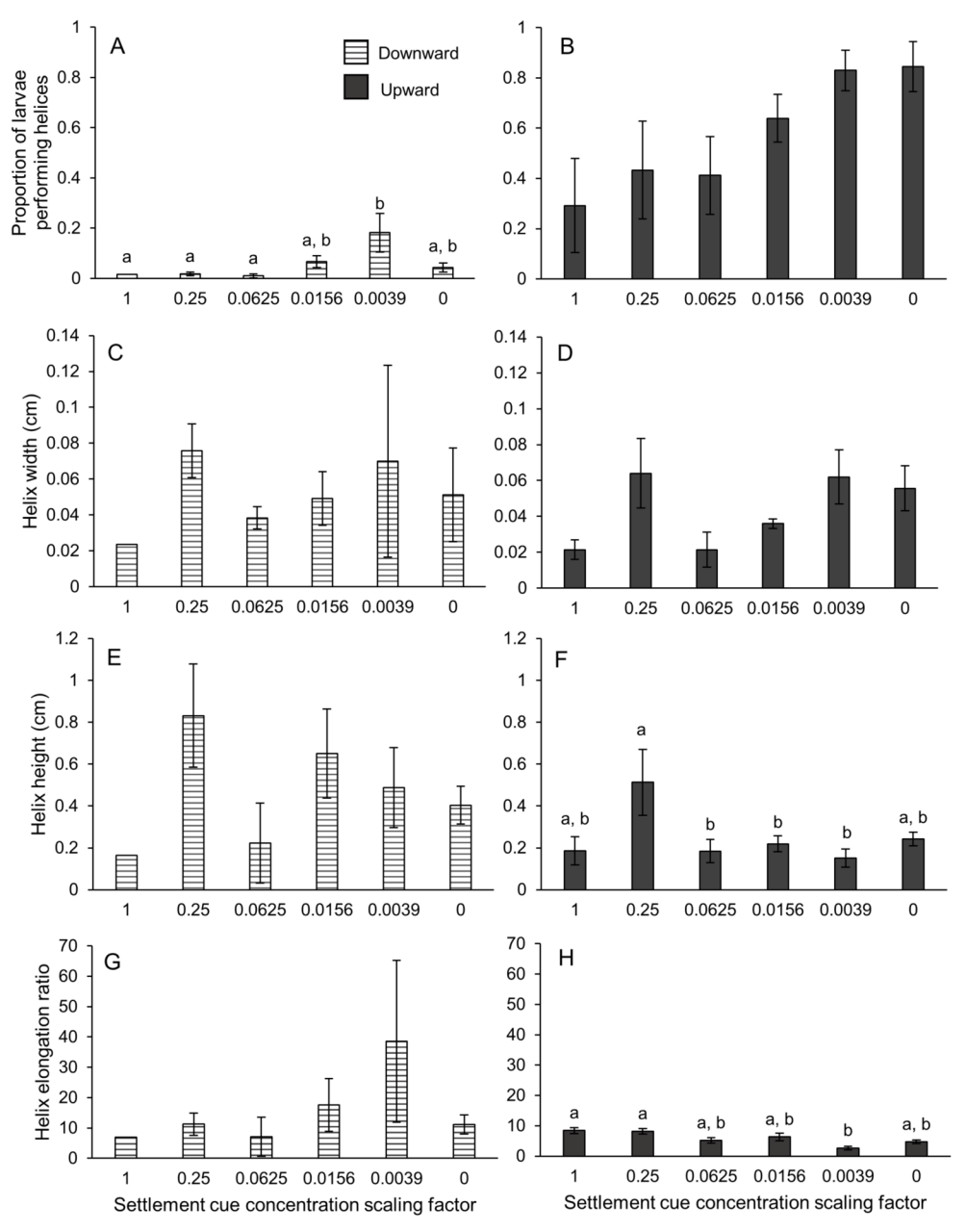
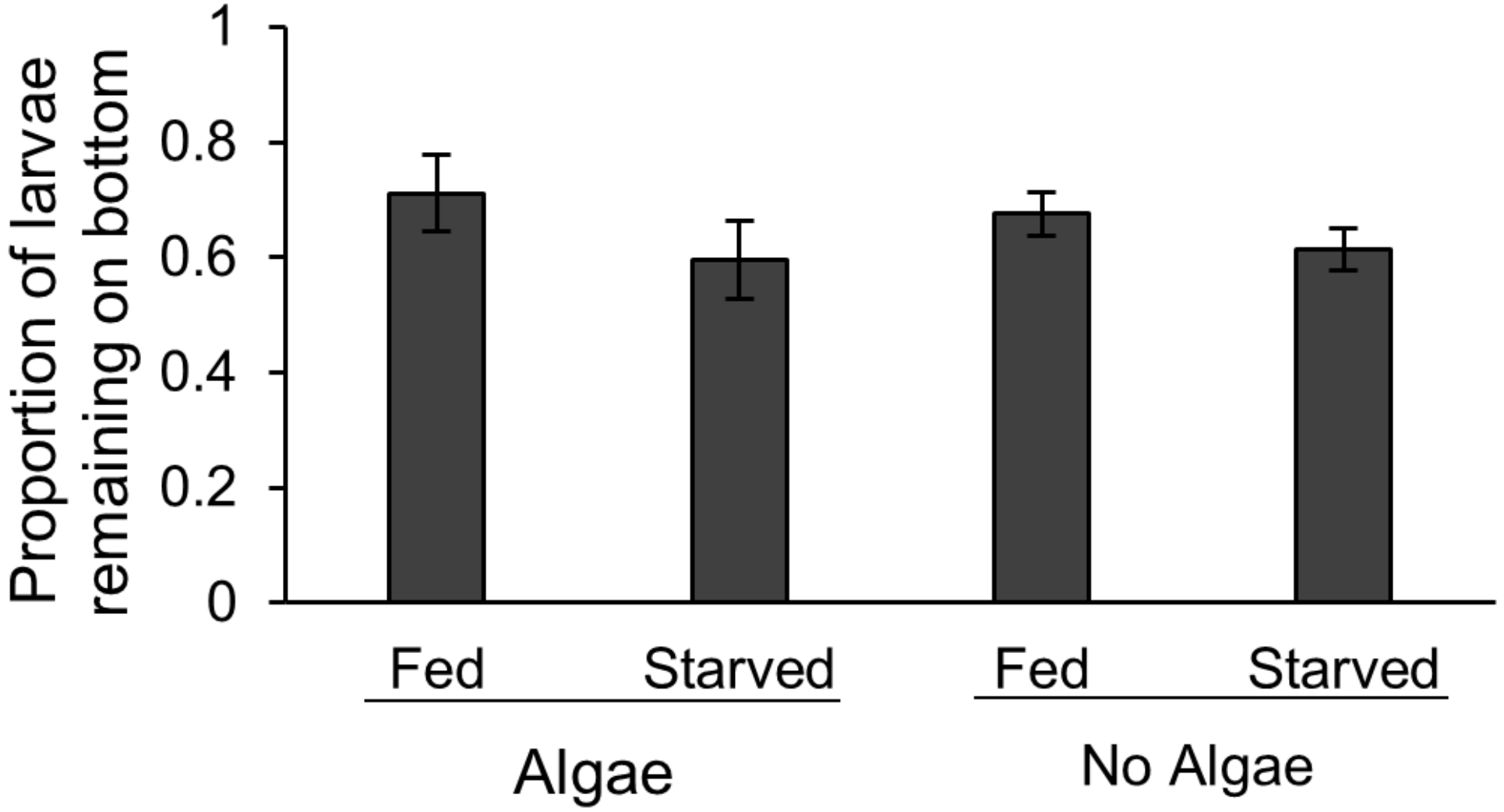
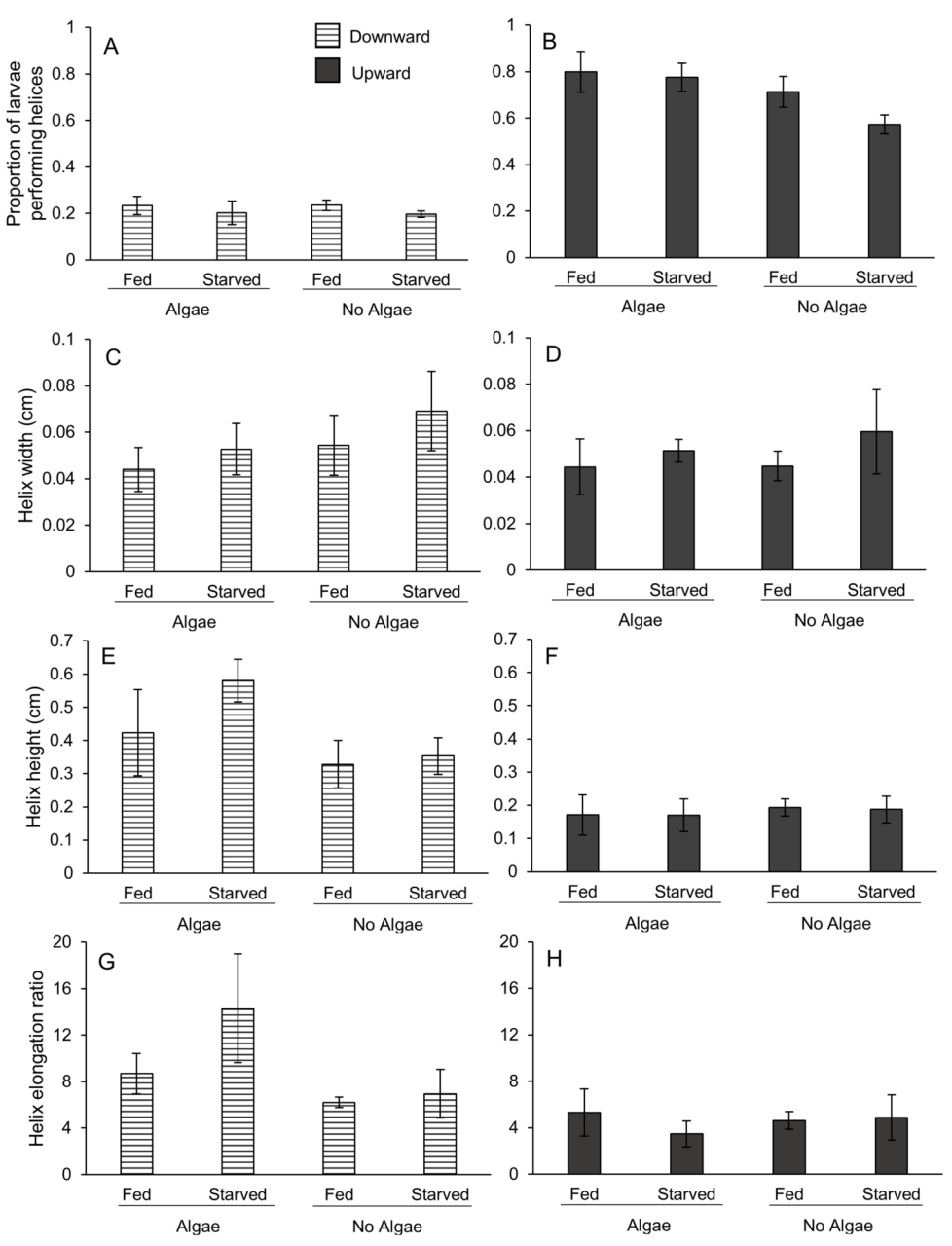

a
a, b
a, b
a, b
b
a, b
1
0.8
0.6
0.4
0.2
0
Proportion of larvae remaining on bottom
1
0.25
0.0625
0.0156
0.0039
0
Settlement cue concentration scaling factor


A
B
C
D
E
F
G
H
Downward
Upward
Proportion of larvae performing helices
Helix width (cm)
Helix height (cm)
Helix elongation ratio
a
a
a
a, b
b
a, b
a, b
a
b
b
b
a, b
a
a
a, b
a, b
b
a, b
1
0.25
0.0625
0.0156
0.0039
0
Settlement cue concentration scaling factor
Settlement cue concentration scaling factor


Proportion of larvae remaining on bottom
1
0.8
0.6
0.4
0.2
0
Fed
Starved
Fed
Starved
Algae
No Algae


A
B
C
D
E
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G
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Downward
Upward
Proportion of larvae performing helices
Helix width (cm)
Helix height (cm)
Helix elongation ratio
Fed
Starved
Algae
No Algae