Desperate planktotrophs: decreased settlement selectivity with age in competent eastern oyster (*Crassostrea virginica*) larvae

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Settlement cue, helical swimming, benthic, desperate larva hypothesis

ABSTRACT

For larvae of benthic marine invertebrate species, settlement from planktonic to benthic life is a critical transition. The "desperate larva" concept describes the tendency of larvae to accept suboptimal settlement habitats as they age. We quantified swimming behavior in planktotrophic larvae of the eastern oyster, *Crassostrea virginica*, to determine whether settlement behaviors, such as swimming downward and remaining on the bottom, increased with age and whether these ontogenetic changes were more apparent in larvae exposed to suboptimal

conditions than to preferred conditions (settlement cue absent or present, respectively). In two experiments, the proportion of competent larvae remaining near the bottom of experimental flasks (indicating settlement) increased with larval age, but only in larvae that were not exposed to the settlement cue. This result is consistent with the hypothesis that larvae encountering suboptimal habitat become "desperate" (i.e. more likely to settle) as they age. Exploratory behaviors, such as upward swimming, meandering, or helices, were expected to decrease with age, especially in the absence of the settlement cue, but this pattern was detected in only one of the five swimming metrics tested (helices in downward swimming larvae). Surprisingly, pre-competent larvae exhibited settlement behavior when exposed to the cue, raising the question of whether a response at this stage would have positive or negative consequences. Acceptance of suboptimal settlement habitats by aging larvae may increase the resilience of a species by allowing populations to persist in variable environmental conditions.

INTRODUCTION

Settlement of larvae on seafloor substrata is a critical stage in the life-cycle of benthic invertebrates. Most studies examining the transition from the planktonic larval stage to benthic life focus on larval behavior near and on the substratum (reviewed by Rodriguez et al. 1993, Abelson & Denny 1997, Hadfield & Paul 2001), where attachment and metamorphosis take place. However, competent larval behavior higher in the water column is also an important component of settlement and is much less understood (Johnson 2017). In particular, it remains unclear to what extent larvae can actively control their downward motion when competent to settle (Fuchs et al. 2004, 2007, 2013; Hadfield & Koehl 2004; Koehl & Reidenbach 2010; Wheeler et al. 2013, 2015; Whitman & Reidenbach 2012).

Many larvae respond to chemical or physical cues in the water column. Chemical signals from prey species, adult conspecifics, or associated biofilms can induce behavioral changes in larvae, including active swimming or passive diving towards a substratum (Burke 1986, Hadfield & Paul 2001, Pawlik 1992). Moving toward the seafloor in response to these cues may benefit the larvae, as they often are indicators of preferred benthic habitat. Physical signals, including light and turbulence, can also influence larval behavior and are important to consider when understanding settlement (Fuchs et al. 2013, Koehl 2007, Wheeler et al. 2017).

The eastern oyster, *Crassostrea virginica*, is a shallow water mollusc native to the North Atlantic. Adults commonly form reefs in the intertidal and sub-tidal zones and are broadcast spawners, producing planktotrophic, free-swimming larvae with a pelagic duration of 2-3 weeks (Kennedy 1986, 1996). The penultimate and final larval stages are the veliger and pediveliger stages, respectively. Larvae in both stages are characterized by a ciliated, retractable velum extending from a calcareous shell, which they use to swim and feed. Larvae become pediveligers upon the development of a foot and pronounced eyespot, at which point they are competent to settle out of the plankton and metamorphose (Thompson et al. 1996).

Crassostrea virginica larvae respond to chemical signals from conspecific adults and can be induced to settle gregariously in both still-water and flume experiments in the laboratory. However, the presence of adult oysters may not be necessary to induce settlement. Crisp (1967) demonstrated that larvae preferentially settled on adults shells with an intact biofilm, avoiding shells that had been bleached, suggesting a biofilm may be even more important than the presence of an adult oyster. Tamburri et al. (1992) went one step further by demonstrating that the presence of adult shells was unnecessary: larvae had a similar settlement response when exposed to “oyster bathwater”, a homogeneous solution of filtered seawater that had been

exposed to either adult oysters (without biofilms) or shell biofilms alone. In addition, various soluble chemical signals have been found to induce settlement, attachment, and metamorphosis in *C. virginica*. The peptide glycyl-glycyl-L-arginine (GGR) induces *C. virginica* larval attachment to substrata with an almost identical dose-response curve to adult-conditioned seawater in homogeneous solutions (Zimmer-Faust & Tamburri 1994). Larvae exposed to both homogeneous adult-conditioned seawater and GGR solutions in flume flow settle out of the flow significantly more than larvae that were not exposed to a chemical cue, over a range of mean flow velocities (Tamburri et al. 1996). Additionally, settlement in related oyster species has also been observed in response to bacterial supernatants (Fitt et al. 1990) and ammonia (Fitt & Coon 1992).

In the field, *C. virginica* larvae will settle on whelk shell restoration reefs and other non-oyster sites (Whitman & Reidenbach 2012), indicating that settlement can occur without chemical cues put forth by adult oysters. However, field observations suggest that chemical signaling from adult oysters does play a role in settlement patterns, as larvae will preferentially settle on living oyster reefs over broken oyster shells (Nestlerode et al. 2007, Schulte et al. 2009, Whitman & Reidenbach 2012). Furthermore, heavily-biofouled tiles are preferred to tiles with thinner biofilms (Campbell et al. 2011), indicating the role of bacterial biofilms in the field.

As larvae age and reach competency, they develop new behavioral responses which may facilitate settlement into suitable benthic habitats (e.g., Kingsford et al. 2002). *Crassostrea virginica* larvae experience and respond to a variety of cues, including light (Kennedy 1996, Wheeler et al. 2017), turbulence (Fuchs et al. 2013, Wheeler et al. 2013, 2015), and sound (Lillis et al. 2013), in addition to the previously-described chemical signals. Larval responses to settlement cues may also change over the competency period, a phenomenon that has long

92 interested larval ecologists. The "desperate larva" hypothesis was first proposed over sixty years
93 ago (Knight-Jones 1951, 1953; Wilson 1953) and states that lecithotrophic larvae respond to sub-
94 standard settlement cues as they age and their energy supplies become depleted. Older larvae
95 may be induced to settle by cues that have no effect on younger, more selective larvae (Gibson
96 1995); older larvae may also settle in the presence of settlement inhibitors (Gribben et al. 2006)
97 or no cue at all (Bishop et al. 2006). Larval body size (Marshall & Keough 2003), planktonic
98 mortality (Elkin & Marshall, 2007), and prior exposure to settlement cues (Botello & Krug 2006,
99 Swanson et al. 2007) can influence larval responses to cues with age (for an extreme example,
100 see Strathmann & Strathmann 2007). Delayed metamorphosis and settlement in a variety of
101 habitats may allow for gene flow among isolated populations, ultimately providing a safeguard
102 against extinction (Gibson 1995).

103 Since its inception, the "desperate larva" hypothesis has been expanded to include some
104 planktotrophs (Botello & Krug 2006), with the caveat that planktotrophs, unlike lecithotrophs,
105 become "desperate" to settle because of a reduced capacity to maintain the competent larval
106 swimming state (as opposed to a depletion of maternal resources) (Bishop et al., 2006). Both
107 cases are equivalent from an ecological perspective, however, as they involve delayed
108 metamorphosis, more time spent swimming and searching for a substratum, and the eventual
109 acceptance of suboptimal settlement sites.

110 *Crassostrea virginica* is a good model species for studies on larval responses to
111 settlement cues with age because *C. virginica* larvae respond to a range of settlement cues and
112 have a relatively long competency period (> 24 hours) (Kennedy 1996). In this study, we
113 investigate how larval oyster swimming behaviors change, as the larvae become competent to
114 settle, in the presence and absence of a chemical settlement cue. We expect settlement behaviors

of competent larvae, such as downward directional swimming and remaining on the benthic substratum, to increase with age, and for this ontogenetic change to be more apparent in suboptimal conditions (settlement cue absent) than in preferred conditions (settlement cue present). In contrast, we expect exploratory behaviors (directional swimming away from the bottom, meandering, or helices) to decrease with age, particularly in suboptimal conditions. Competent larvae can be expected to exhibit settlement behavior in the presence of a cue regardless of age, so the change in behavior would be most strongly expressed in the absence of the cue, reflecting greater acceptance of sub-optimal habitat with age. We expect settlement behaviors in pre-competent larvae to be rare under both preferred and suboptimal conditions. The characteristics of the behavioral responses and the time-scales of their ontogenetic change should inform our understanding of the adaptive significance of becoming desperate and accepting suboptimal habitat.

METHODS

Experimental design

The present study was designed to quantify ontogenetic behavioral changes spanning several days before the onset of metamorphic competency to several days after. We conducted a pilot experiment with measurements at high-resolution time-points during early competency (within 12 hours of onset). The pilot experiment showed significant responses to a chemical settlement cue but no differences among larval ages in early competency (results in Supplementary Material, Tables S1-S3, Figs. S1-S5) and motivated two additional experiments, the results of which we report here. The first, the "Time Series Experiment," included measurements of larval behavior throughout competency (up to ~3 days after onset). Because

138 young competent larvae (time = 0 hours, 50% with eyespots, see below) displayed distinct
139 settlement behaviors, we investigated the behaviors of pre-competent larvae in a second
140 experiment, the "Pre and Post Experiment." For this experiment, we compared measurements
141 between larvae at pre-competent (3 days before onset), competent, and post-competent (2 days
142 after onset) stages. All experiments involved larval exposure to seawater with and without a
143 chemical settlement cue.
144
145 Study species handling practices
146 We obtained larvae from two spawns for use in our two experiments. Larvae were
147 provided by the Aquaculture Research Corporation (Dennis, MA, USA) in August 2015 and July
148 2016 for the Time Series and Pre and Post Experiments, respectively. The hatchery provided
149 mature veligers retained on a 180 μm sieve for the Time Series Experiment and younger veligers
150 retained on a 120 μm sieve for the Pre and Post Experiment involving pre-competent larvae.
151 These younger veligers were approximately 3 days pre-competent based on subsequent eyespot
152 development in the laboratory. Prior to experiments, all larvae were maintained in 1 μm-filtered,
153 aerated seawater at ambient field temperature (20-22° C) and salinity (33 psu), in covered 16 L
154 plastic buckets. Larvae were kept at low densities (< 3 larvae mL^{-1}) to minimize interactions and
155 fed daily a suspension of haptophyte *Isochrysis* sp. (~9×10^5 cells mL^{-1} in filtered seawater).
156 Larvae approaching competency were maintained in these conditions (2 – 5 days) until
157 competency onset, as defined by ~50% of larvae exhibiting eyespots. Eyespots were identified
158 by microscopic examination of a random sample of larvae, and observational experiments
159 immediately commenced when this competency threshold had been reached. The pre-competent
160 larvae were retained in identical conditions for a shorter time period (2 hours) prior to starting

161 experiments, in order to ensure larvae were not yet competent to settle during the experiment.
162 Immediately prior to each experiment, a random sub-sample of larvae was preserved in a
163 solution of 95% ethanol. These preserved larvae were microscopically examined for shell size
164 measurements and eyespot identification. We recorded larval body sizes because larvae may
165 continue to grow throughout the competency period (Kennedy 1996), and size could impact
166 swimming ability (Hidu & Haskins 1978). Larval size was estimated by measuring shell height
167 (straight line distance perpendicular from the shell hinge to edge of the shell) and length (straight
168 line distance along the hinge axis).
169 Oyster bathwater was prepared in a similar method to that described by Tamburri et al.
170 (1992). Live, unwashed, adult *C. virginica* were purchased from a local vendor (The Clam Man,
171 Falmouth, MA, USA) from harvests of farmed oysters from Washburn Island in Waquoit Bay,
172 Massachusetts. Individuals were measured to estimate shell surface area, and a set of oysters
173 totaling 800 cm^2 surface area were placed in a sterile plastic bucket with aerated seawater (4 L)
174 filtered to 1 µm (ambient 20° C, 33 psu) for the Time Series Experiment. This protocol was
175 modified slightly (1660 cm^2 surface area in 12L seawater) for the Pre and Post Experiment. The
176 buckets were covered and left undisturbed in a temperature-controlled environmental chamber
177 for 4 hours. Subsequently, adult oysters were removed, and the bathwater was filtered to < 1 µm
178 with a glass microfiber filter. The bathwater was then divided into 1L aliquots and frozen at -20°
179 C until immediately prior to use in experiments.
180
181
182 Experimental setup

All experimental observations were conducted in the environmental chamber at a constant temperature of 20° C in the dark, in order to minimize convective currents in the experimental flasks and light cues that may influence larval behavior. The observational tanks were 50 mL flat-sided plastic flasks with an open top, filled with either ambient seawater (20° C, 33 psu) filtered to 1 μm, or oyster bathwater at identical temperature and salinity. The bottom of the flask consisted of a smooth plastic surface with no biofilm, a poor settlement surface for oyster larvae (Su et al. 2007). Each flask was held stationary to minimize flow within the flask prior to the addition of larvae. Larvae swam into flasks via a gravity-assisted 1 mL pipette, with the pipette tip just breaching the water surface; this method of introduction imposed no external downward momentum on larvae (Fig. 1). Approximately 40-50 larvae were introduced to a new flask for each experimental replicate. Flasks were illuminated from behind with a near-infrared LED array light source (Olymstore, 12V, 2A, 850 nm). A monochrome camera (Hitachi KPF-120) facing the front of the flask recorded a 4×5 cm 2-dimensional field of view, which encompassed a vertical cross-section of the entire flask volume.

The two different experiments examined swimming behaviors in different, but overlapping, stages in larval development. In the Time Series Experiment, we observed changes in larval behavior over a long time-scale, exposing larvae at 0, 10, 22, 43, and 64 hours post-competency. For the Pre and Post Experiment, we included pre-competent larvae (without eyespots, time = -72 hours), as well as larvae at 0 and 48 hours post-competency. The average size of larvae and percentage with eyespots in each experiment are reported in Table 1.

For both experiments, larvae were exposed to two treatments: filtered seawater (control, no settlement cue) and oyster bathwater (a settlement cue). At each time-point, 5 replicate observations were conducted for each of the control and settlement cue treatments (n = 5). A

206 replicate consisted of a unique set of larvae in a new, unused flask. Larval swimming behaviors
207 were recorded for 5 minutes at 30 frames per second in each replicate.
208
209 Larval tracking
210 The methods for larval identification and tracking were adapted from Wheeler et al.
211 (2013, 2015) and are briefly summarized here. Video recordings of each replicate were saved as
212 high-resolution TIFF images (1040×1390 pixels) for subsequent larval tracking. TIFF images
213 were imported into LabVIEW 2013 (National Instruments), and average background pixel
214 intensity was subtracted. Using a fixed-threshold particle size and intensity, larval centroid
215 positions (x, z) were recorded in the frame in which they appeared. Centroid positional data were
216 reconstructed into individual larval trajectories using a MATLAB script which tracked a larva
217 from frame to frame according to a subsequent-frame tolerance distance radius set by the user.
218 Larval trajectories were truncated by five frames at each end of the trajectories to avoid poor
219 centroid estimates in cases where larvae passed laterally into and out of the focal plane.
220 Instantaneous swimming velocities were computed using a central difference scheme of larval
221 centroid positions in time, so that the velocity is defined as centered in time between two
222 adjacent frames. Unlike the experiments of Wheeler et al. (2013, 2015), flow velocities in the
223 small flasks in the present study were minimal, and therefore no effort was made to subtract local
224 flow from observed larval velocities.
225
226
227
228 Behavioral metrics

Most larvae traveled to the bottom of the experimental flasks immediately after introduction, and then some larvae swam back up off the bottom of the flasks. Each larval track was viewed individually and identified as a larva swimming into the flask, a larva swimming into the flask helically, a larva swimming up off the bottom, or a larva swimming up off the bottom helically. We calculated behavioral metrics separately for downward- and upward-swimming larvae. For each swimming direction, we calculated the average vertical velocity of larvae, the proportion of larvae swimming in helices, and the net to gross distance ratio (NGDR, see below). We also calculated the proportion of larvae that remained on the bottom of the flask. For some replicates in the Pre and Post Experiment, all larvae exposed to the settlement cue remained on the bottom, so behavioral metrics based on upward-swimming larvae could not be calculated. We considered direct downward swimming and remaining on the bottom of the flask to be settlement behaviors, whereas direct upward swimming, meandering, and helical swimming were considered exploratory.

NGDR is a standard metric used in studies on swimming behavior (e.g. Buskey et al. 1983, Tiselius 1992). It is the ratio of net distance traveled (straight-line distance between the start and end points of a larval track) to gross distance traveled (total distance, including any curves in the larval track). However, because we investigated exploratory behavior of larvae, the inverse (1/NGDR = GNDR) is a more useful and intuitive metric for our study, increasing in magnitude with increasingly exploratory behavior. GNDR close to 1 indicates a relatively straight, direct path of travel, while GNDR >> 1 indicates exploration. We report GNDR for oyster larvae in this manuscript.

Helical swimming behavior produces a corkscrew-like trajectory and is characterized by sinusoidal patterns in both horizontal position and velocity of a larva, exposing it to a large

252 portion of the water column (Crenshaw 1996). This spiral swimming pattern has multiple
253 hypothesized functions, including directional swimming in asymmetrical organisms (Crenshaw
254 1996), regulating vertical position in the water column (Cragg 1980, Wang & Xu 1997), feeding,
255 exploration (Chia et al. 1984), and maximizing prey encounter rate while minimizing predator
256 encounter rate (Visser 2007). Helically-swimming larvae represent a subset of high-GNDR
257 larvae; however, we chose to focus on this subset because of the ubiquity of helical swimming in
258 zooplankton and the tendency for oyster larvae to modify helical behavior in the presence of
259 environmental cues (Wheeler et al. 2017).
260 Because the field of view is largely two-dimensional, an unavoidable experimental
261 constraint involves larvae laterally leaving the field of view. It is conceivable that a larva swam
262 in one direction (downward or upward), exited the field of view, returned to its original position
263 (top or bottom of the flasks), and the re-entered the field of view. However, every effort was
264 made to only count downward- and upward-swimming larvae once, so we consider this source of
265 error to be very small.
266 The number of larvae performing dives was quantified according to the methods of
267 Wheeler et al. (2015). Diving behavior is induced by local fluid acceleration in turbulence
268 (Wheeler et al. 2015) and as such diving was seldom found in our study, likely due to the lack of
269 ambient flow in the experimental flasks.
270
271 Statistical analyses
272 Significant differences in behavioral metrics between cue and non-cue treatments and
273 among larval ages were examined using 2-way analysis of variance (ANOVA). We tested for
274 homoscedasticity using Levene's tests, and in cases of heteroscedasticity, results were also

evaluated using non-parametric Kruskal-Wallis (K-W) and Mann-Whitney (M-W) tests. We report ANOVA results here, even for cases with heteroscedasticity, because the equal sample sizes make the ANOVAs robust and because the nonparametric tests do not assess interactions between larval age and cue treatments. Non-parametric results for heteroscedastic cases are reported in the Supplementary Material, Table S4. Pairwise post hoc Tukey tests were used when ANOVA revealed significant differences among larval ages or a significant age-cue interaction. Post hoc tests were conducted among ages within each treatment separately, to test our hypothesis that there would be stronger differences in larval behavior over time in no-cue treatments than in the presence of a settlement cue. All statistics were conducted in Matlab R2017b.

RESULTS

Settlement behaviors in *C. virginica* larvae

The proportion of non-cue exposed competent larvae remaining on the bottom of the flasks tended to increase with larval age (Fig. 2). In contrast, most or all of the cue exposed larvae remained on the bottom, and the proportion did not vary with age. This ontogenetic pattern in larvae in non-cue treatments was significant in the Pre and Post Experiment ($p = 0.013$, Fig. 2B), but not in the Time Series Experiment (Fig. 2A, Table 2). The lack of an ontogenetic pattern in the cue-exposed larvae was responsible for the significant interaction effect between cue exposure and larval age in both experiments ($p = 0.002, 0.023$; Table 2). These results are generally consistent with our expectation that settlement behavior in the absence of a cue would increase with larval age. Although the observed effect of the cue in increasing larval settlement behavior (significant, $p < 0.001$ in both experiments; Table 2) was expected for competent

298 larvae, it was expected to be weaker in pre-competent larvae. The strength of the cue effect for
299 pre-competent larvae (Fig. 2B), and the high proportion of pre-competent larvae showing
300 settlement behavior, whether exposed to cue or not, was a surprise.

301 The downward swimming velocity of competent larvae did not vary with larval age,
302 whether they were exposed to settlement cue or not (Fig. 3, Table 2). Downward velocity was
303 expected to be higher in cue exposed larvae than non-cue exposed larvae but this difference was
304 observed only in the Pre and Post Experiment ($p < 0.001$, Table 2, Fig. 3B), while in the Time
305 Series Experiment larvae maintained velocities over the competency period (Fig. 3A). The lack
306 of an ontogenetic change in downward velocity of non-cue exposed larvae is not consistent with
307 our expectations for aging larvae. In pre-competent larvae, the settlement response was weaker
308 than in competent larvae, in both cue and no-cue conditions (Fig. 3B), as expected.
309
310 Exploratory behaviors in *C. virginica* larvae

311 The upward swimming velocity of competent non-cue exposed larvae showed opposite
312 trends with larval age in the two different experiments. The velocity of larvae swimming up off
313 the bottom decreased with age in the Pre and Post Experiment but increased with age in the Time
314 Series Experiment (Fig. 4, Table 3). All cue-exposed larvae remained on the bottom in the Pre
315 and Post Experiment, while cue-exposed larvae showed a similar pattern to non-exposed larvae
316 in the Time Series Experiment. The opposing ontogenetic patterns in the two experiments were
317 significant ($p = 0.002, 0.035$; Fig. 4), and there were no interaction effects between cue and age
318 (Table 3). The observation of a decrease in exploratory behavior with larval age (Pre and Post
319 Experiment) is consistent with our expectations, but the ontogenetic pattern in the Time Series
320 Experiment is not. The observed effect of the cue in reducing exploratory behavior (Table 3) was

321 expected for competent larvae, but the strength of the effect in pre-competent larvae, and the low
322 vertical velocities compared to 0-hour competent larvae (Fig. 4B), were unanticipated.
323 The gross-to-net distance ratio (GNDR) of downward-swimming larvae increased with
324 age in one of the experiments (Time Series) (Fig. 5A, Table 3) and remained constant in the
325 other (Pre and Post) (Fig. 5B, Table 3). Increased GNDR indicates increased exploration, and as
326 such, these results are not consistent with the expectation of decreasing exploratory behavior
327 with larval age. Exposure to the cue did not affect this behavior across larval ages (Table 3), but
328 an interaction between cue and age (borderline significant in the Time Series Experiment, $p =$
329 0.050) indicates that the increase in GNDR was apparent only in the cue-exposed larvae. In
330 downward-swimming pre-competent larvae, GNDR was no different than in competent larvae.
331 The gross-to-net distance ratio (GNDR) of upward-swimming larvae did not vary with
332 larval age in either experiment (Fig. 5C,D, Table 3). GNDR was higher in cue-exposed larvae
333 than in non-exposed larvae, a result that is not consistent with an expectation of reduced
334 exploration in response to a settlement cue. In upward-swimming pre-competent larvae, GNDR
335 was no different than in competent larvae.
336 The proportion of downward-swimming larvae performing helices decreased with larval
337 age, for both cue exposed and non-cue exposed larvae in the Time Series Experiment (Fig. 6A,
338 Table 3), but not in the Pre and Post Experiment (Fig. 6B, Table 3). Helices tended to be less
339 common in larvae exposed to the cue, but this effect was significant only in the Pre and Post
340 Experiment ($p < 0.001$). Both a decrease in helices with age and with exposure to a settlement
341 cue are consistent with our expectations, although we anticipated a more prominent ontogenetic
342 change in non-cue exposed larvae. In downward-swimming precompetent larvae, the proportion
343 exhibiting helices was no different than in competent larvae.

The proportion of upward-swimming larvae performing helices did not vary with larval age or exposure to the cue (Fig. 6C,D, Table 3). Precompetent larvae behaved no differently than competent larvae.

Differences between spawns

We tested for significant differences in larval behavioral metrics between the two spawns to explore whether inter-spawn variation might obscure differences in behavior between treatments or larval ages. GNDR was the only metric with a significant difference between spawns (downward-swimming larvae, t-test, $t = 3.46$, $p = 0.002$; upward-swimming larvae, M-W, $U = 77$, $p = 0.02$), being higher for larvae in the Time Series Experiment than the Pre and Post Experiment. Larvae in the first spawn thus had a greater overall propensity towards exploration.

DISCUSSION

We hypothesized that competent *Crassostrea virginica* larvae would exhibit settlement behaviors increasingly as they aged (descending directly to flask bottoms and remaining there) and exhibit fewer exploratory behaviors (upward swimming, meandering, or helices) in suboptimal conditions (non-cue treatments). Our results show higher proportions of older larvae remaining on the flask bottoms in non-cue treatments in both experiments. The trend of more larvae remaining near the bottom was only apparent in the absence of the settlement cue, fitting our expectations that *C. virginica* larvae become more willing to accept suboptimal habitats for settlement as they age.

366 Our second expectation, that exploratory behavior in competent larvae would decrease
367 with larval age in suboptimal conditions (non-cue) was supported in only a few cases. For
368 upward swimming velocity, there were opposite trends between the two experiments. It is
369 unclear why this was the case, though the faster velocities for older larvae in one
370 experiment/spawn may have induced a greater propensity towards exploration. For downward-
371 swimming larvae, there was a clear trend of fewer helices in older larvae, which was significant
372 in one experiment, supporting our expectation of less exploratory behavior in older larvae. The
373 GNDR of downward-swimming larvae increased with age in one experiment, indicating
374 increased exploration, but this trend was only apparent for larvae in the cue. Meandering (high-
375 GNDR) behavior may be subject to inter-spawn variation, as there were significant differences in
376 this metric for both downward- and upward-swimming larvae between the two
377 experiments/spawns. Exploratory behaviors of *C. virginica* larvae had mixed results and may be
378 influenced by factors other than habitat suitability as indicated by the presence of a settlement
379 cue. While the two spawns were held in comparable conditions throughout the experiments
380 (light conditions, temperature, salinity), it is possible that earlier developmental conditions for
381 the spawns introduced carry-over effects for our late stage observations. These exploratory
382 behaviors warrant further study to understand ontogenetic or environmental factors influencing
383 them.
384 This study is to our knowledge the first demonstration of increased settlement behavior
385 with age in a planktotrophic species. The acceptance of suboptimal settlement habitat by older
386 larvae lends support to the "desperate larva hypothesis" and contrasts with previous studies using
387 planktotrophic species. However, previous studies examined larval responses to settlement cues
388 with age, whereas we found increased settlement behavior in non-cue conditions. For example,

planktotrophic *Hydroides dianthus* larvae that were well-fed showed no change in response to settlement cues with age (Toonen & Pawlik 2001). Settlement behavior of a facultative planktotroph also depended on energy reserves, with no ontogenetic trend in settlement for well-fed individuals (Botello & Krug 2006). Larvae in the present study were fed daily prior to the experiments, so our observation of "desperation" and acceptance of suboptimal settlement substrata is novel for well-fed planktotrophic larvae. Nevertheless, older *C. virginica* in our study had mixed responses in exploratory behavior. Modelling efforts indicate that planktotrophic species have few deferred costs for prolonging the search for a suitable habitat, especially when food is plentiful, and larval behaviors are more strongly influenced by habitat availability and individual variation in size (Elkin & Marshall 2007). Our results fit with the previous finding that for planktotrophic species, the "desperate larva" effect is not as dramatic as for lecithotrophic species (i.e. Marshall & Keough, 2003) and may be influenced by individual variation. The exploratory behaviors we observed, especially for upward-swimming larvae, were exhibited by only a small proportion of the larval population, which may have had larger energy reserves or different intrinsic levels of settlement inhibitors (i.e. the "variable retention hypothesis;" Bishop et al., 2006).

Our observation of a decrease in helical swimming with age in *C. virginica* larvae is consistent with the results and interpretations of other studies on bivalve larval behavior. Under laboratory conditions, bivalves swim upward in helices and then sink passively back down, and this behavior is assumed to help maintain a preferable vertical position in the water column (Cragg, 1980; Troost et al., 2008; Wang & Xu, 1997). While helical swimming may be an adaptation for feeding or vertical swimming (i.e. Chan et al. 2011, Jonsson et al. 1991), in *C. virginica*, helical swimming behavior is modified by environmental cues involved in settlement

412 (Wheeler et al. 2017). The ontogenetic trend we observed supports the concept of helical
413 swimming as an exploratory behavior. Helical swimming is ubiquitous among microorganisms,
414 observed in bacteria, protists, fungi, sperm, and marine larvae (Crenshaw, 1996). Given this wide
415 phylogenetic spread, helical swimming may have multiple functions, and it warrants further
416 investigation in *C. virginica*.

417 Dive behavior was seldom observed in our experiments. Wheeler et al. (2015) showed
418 that dive behavior in *C. virginica* was triggered by turbulence, which was minimal to non-
419 existent in our experimental flasks. The relative absence of dive behavior in our still-water
420 experiments thus support the results of Wheeler et al. (2015) that dives are induced by
421 hydrodynamic signals in turbulent conditions.

422 One very surprising result from our study was the discovery that pre-competent larvae (t
423 = -72 hours, 0% with eyespots) also appeared to respond to the settlement cue, traveling to the
424 bottom of the flasks more quickly and directly than non-cue exposed larvae, and remaining near
425 the bottom. In many cases, the response of pre-competent larvae to the settlement cue was
426 similar to the response of competent (t = 0 hours) larvae. Pre-competent larvae had lower
427 swimming velocities than competent larvae in both downward and upward directions, most
428 likely because their smaller size. Swimming speed increases with age for many molluscan larvae
429 (Cragg, 1980), including *C. virginica* (Hidu & Haskins, 1978).

430 Any explanations for why pre-competent larvae showed settlement behaviors can only be
431 speculative. Recent reviews on the adaptive significance of metamorphic competence in marine
432 invertebrate larvae have focused on habitat selection in the post-competent period, neglecting
433 pre-competent larvae (Hadfield et al., 2001; Bishop et al., 2006). Bishop et al. (2006) found a
434 relationship between the habitat specificity of juveniles of a species and the capacity of its larvae

435 to prolong the larval period until a suitable habitat is found. Here, we speculate that it may be
436 adaptive for hard-bottom invertebrates, especially those that inhabit island-like or specialized
437 habitats, to respond to settlement cues even when pre-competent. Delayed metamorphosis has
438 negative carryover effects on growth and fecundity for juveniles and adults (Marshall et al.,
439 2003; Pechenik, 2006). Therefore, if a larva responds to a settlement cue when pre-competent, it
440 may avoid these negative consequences of prolonged habitat search during the competency
441 period. On rocky shores, pre-competent sea urchin larvae respond to turbulence, a general
442 settlement cue, and even undergo accelerated development to become competent (Gaylord et al.,
443 2013). For *C. virginica*, we are unable to say whether pre-competent larvae would also undergo
444 accelerated development to competency, but the responses of pre-competent larvae and induction
445 of accelerated competence are avenues for future research. Some marine larvae possess
446 specialized organs for the transduction of settlement signals (Hadfield & Pennington 1990,
447 Hadfield et al. 2000), but much remains to be learned about the physiology of settlement
448 (Rodriguez et al. 1993).
449 Examining specific behavioral responses and the time-scales of their ontogenetic change
450 can help explain their adaptive significance. For example, differences in phototaxis between
451 newly-released and competent-to-settle larvae suggest adaptations for upward swimming for
452 dispersal and feeding early in the larval duration, followed by a return to the benthos when
453 competent (Miller & Hadfield, 1986; Montgomery et al. 2018). However, most studies
454 examining ontogenetic changes in larval behavior have focused on the period of larval
455 development prior to competency. It is important to study ontogenetic changes beyond the onset
456 of competency to understand behavioral changes influencing habitat selection and settlement.
457 Acceptance of suboptimal settlement habitats or acceptance of settlement habitats at suboptimal

458 development stages (i.e. when pre-competent) may decrease individual fitness, but it may also
459 increase the resilience of populations. If individual oysters are able to accept suboptimal
460 settlement habitats when no optimal habitat is available, for example following a major
461 disturbance (Livingston et al. 1999), this will allow the population to persist until optimal
462 settlement habitats are restored. Populations of *C. virginica* are adapted to variable
463 environmental conditions (Newkirk et al. 1977), which may allow them to recover quickly after
464 disturbances (Pollack et al. 2011).
465
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631 Table 1. Average size and percentage of larvae with eyespots at each age in each experiment. N,
632 number of individuals sub-sampled; intervals represent standard deviation. Pre-competent larvae
633 are denoted as -72 hours post-competency.

Experiment	Hours post-competency	N	Length (µm)	Height (µm)	% with eyespots
Time Series	0	28	260 ± 11	256 ± 11	64
	10	30	260 ± 9	254 ± 12	70
	22	30	273 ± 14	267 ± 10	90
	43	30	287 ± 13	284 ± 11	97
	64	30	285 ± 18	293 ± 21	100
Pre and Post	-72	20	176 ± 16	181 ± 11	0
	0	24	285 ± 22	275 ± 25	38
	48	22	290 ± 23	281 ± 14	95

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Table 2. Analysis of variance of settlement behaviors in response to settlement cue and larval age. Two-way ANOVA tests for differences in the proportion of larvae remaining on the bottom of experimental flasks and larval swimming velocities. Factors are Cue (presence or absence of a settlement cue) and Age (larval age in hours post-competency. Significant p-values (<0.05) shown in bold. * indicates heteroscedasticity and inconsistency between ANOVA and non-parametric analyses (non-parametric p = 0.253).

Behavioral metric	**Experiment**	**Factor**	**F**	**p**
Proportion of larvae staying on bottom	Time Series	Cue	145	**<0.001**
		Age	1.87	0.135
		Cue x Age	5.16	**0.002**
	Pre and Post	Cue	17.9	**<0.001**
		Age	5.17	**0.013***
		Cue x Age	4.39	**0.023**
Velocity of downward-swimming larvae	Time Series	Cue	1.01	0.321
		Age	0.84	0.509
		Cue x Age	1.01	0.413
	Pre and Post	Cue	30.7	**<0.001**
		Age	10.6	**<0.001**
		Cue x Age	1.38	0.271

Table 3. Analysis of variance of exploratory behaviors in response to settlement cue and larval age. Two-way ANOVA tests for differences in the upward swimming velocity of larvae, average gross:net distance ratio (GNDR), and proportion swimming in helices. Factors are Cue (presence or absence of a settlement cue) and Age (larval age in hours post-competency. Significant p-values (<0.05) shown in bold. * indicates heteroscedasticity and inconsistency between ANOVA and non-parametric analyses (non-parametric p = 0.164).

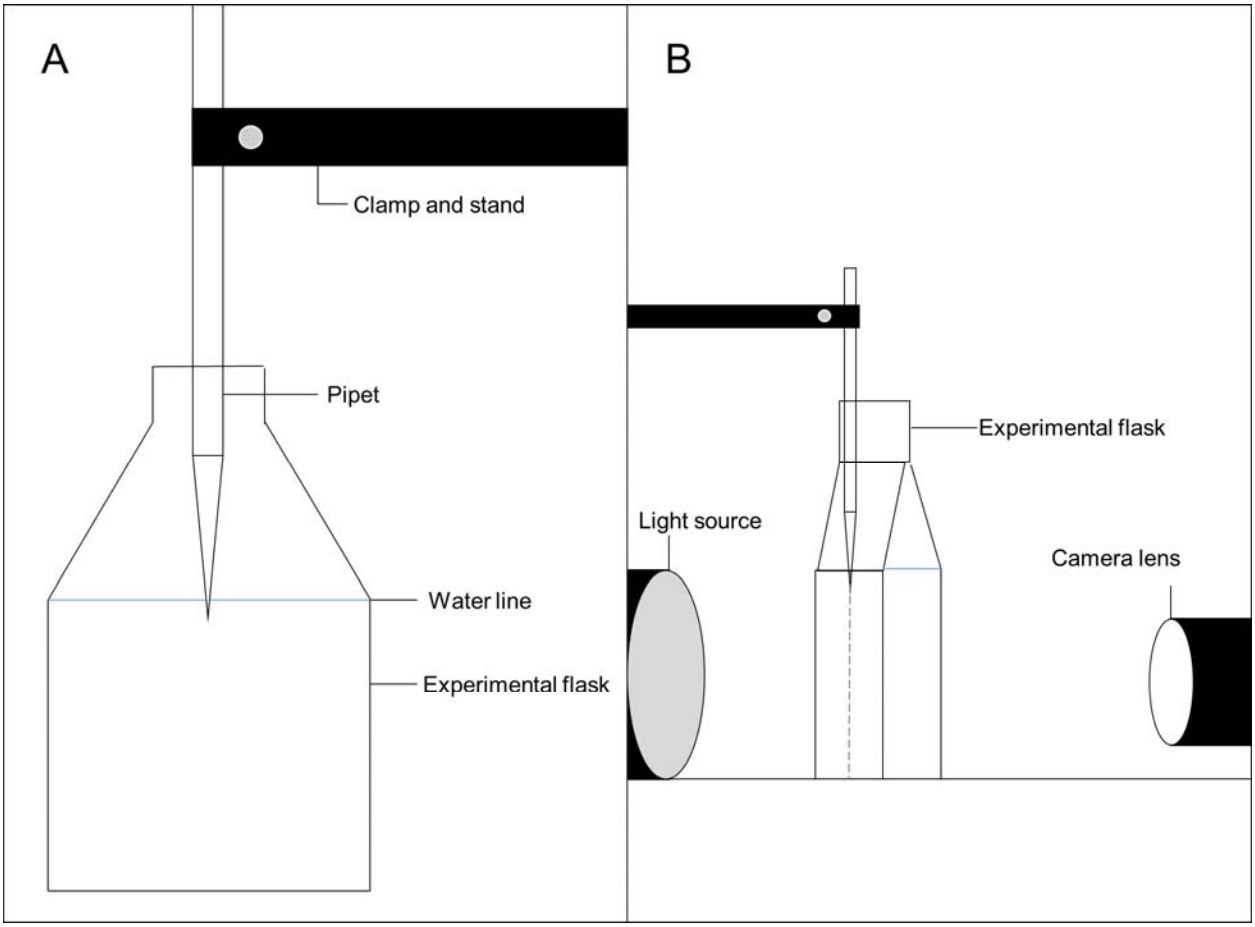
Behavioral metric	**Experiment**	**Factor**	**F**	**P**
Velocity of upward-swimming larvae	Time Series	Cue	28.2	<**0.001**
		Age	5.30	**0.002**
		Cue x Age	1.79	0.155
	Pre and Post	Age	4.38	**0.035**
GNDR of downward-swimming larvae	Time Series	Cue	0.01	0.943
		Age	2.76	**0.041**
		Cue x Age	2.61	0.050
	Pre and Post	Cue	2.79	0.107
		Age	1.21	0.315
		Cue x Age	0.65	0.531
GNDR of upward-swimming larvae	Time Series	Cue	10.4	**0.002***
		Age	1.19	0.334
		Cue x Age	0.67	0.617
	Pre and Post	Age	0.58	0.574
Proportion downward-swimming larvae performing helices	Time Series	Cue	3.78	0.059
		Age	4.27	**0.005**
		Cue x Age	0.21	0.931
	Pre and Post	Cue	39.3	**<0.001**
		Age	0.39	0.682
		Cue x Age	0.46	0.635
Proportion of upward-swimming larvae performing helices	Time Series	Cue	0.14	0.711
		Age	1.02	0.410
		Cue x Age	2.28	0.081
	Pre and Post	Age	1.03	0.386

674 Fig. 1. Experimental set-up. A, experimental flask from the direction of camera view; B, flask
675 and camera arrangement. Dashed vertical line indicates the camera's field of view plane.
676 Fig. 2. Proportion of *Crassostrea virginica* larvae remaining on the bottom after swimming down
677 as a function of larval age. Larvae were exposed to filtered seawater (no cue; light bars) or a
678 chemical settlement cue (dark bars) in two separate experiments (A, B). Larvae of age -72 hours
679 are pre-competent. Error bars show standard error. Dissimilar letters indicate significant post hoc
680 differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments.
681 Fig. 3. Vertical swimming velocity of downward-swimming *Crassostrea virginica* larvae as a
682 function of age. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical
683 settlement cue (dark bars) in two separate experiments (A, B). Larvae of age -72 hours were pre-
684 competent. Error bars show standard error. Dissimilar letters indicate significant post hoc
685 differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments.
686 Fig. 4. Vertical swimming velocity of upward-swimming *Crassostrea virginica* larvae as a
687 function of age. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical
688 settlement cue (dark bars) in two separate experiments (A, B). Larvae of age -72 hours were pre-
689 competent. Error bars show standard error. Dissimilar letters indicate significant post hoc
690 differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments. In the
691 Pre and Post Experiment, no larvae exposed to the cue at t = -72 or 48 hours swam upward off
692 the bottom, so nd indicates no data for upward swimmers.
693 Fig. 5. Gross to net distance ratio (GNDR) of *Crassostrea virginica* larvae as a function of age
694 for larvae swimming downward to the bottom of the flask following introduction (A, B) and
695 larvae swimming back up off the bottom (C, D). Larvae were exposed to filtered seawater (no
696 cue; light bars) or a chemical settlement cue (dark bars) in two separate experiments. Larvae of

age -72 hours were pre-competent. Error bars show standard error. Dissimilar letters indicate significant post hoc differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments. In the Pre and Post Experiment, no larvae exposed to the cue at t = -72 or 48 hours swam upward off the bottom, so nd indicates no data for upward swimmers. The missing error bar at t = 0 hours in the Pre and Post Experiment indicates only one replicate in that treatment had larvae swimming back up off the bottom.

Fig. 6. Proportion of *Crassostrea virginica* larvae swimming in helices as a function of age for larvae swimming downward to the bottom of the flask following introduction (A, B) and larvae swimming back up off the bottom (C, D). Larvae were exposed to filtered seawater (no cue, light bars) or a chemical settlement cue (dark bars) in two separate experiments. Larvae of age -72 hours were pre-competent. Error bars show standard error. Dissimilar letters indicate significant post hoc differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments. In the Pre and Post Experiment, no larvae exposed to the cue at t = -72 or 48 hours swam upward off the bottom, so nd indicates no data for upward swimmers. The missing error bar at t = 0 hours in the Pre and Post Experiment indicates only one replicate in that treatment had larvae swimming back up off the bottom.

720 Fig. 1



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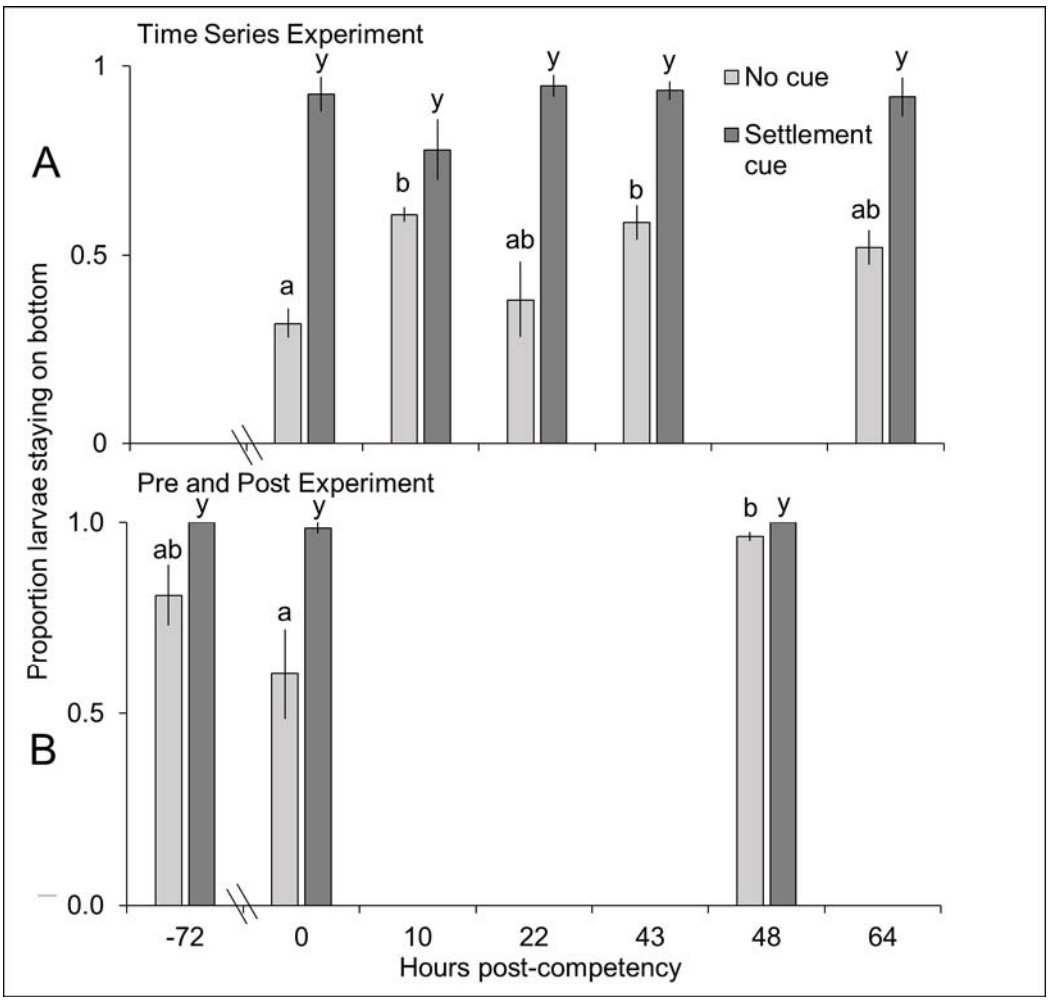
732 Fig. 2



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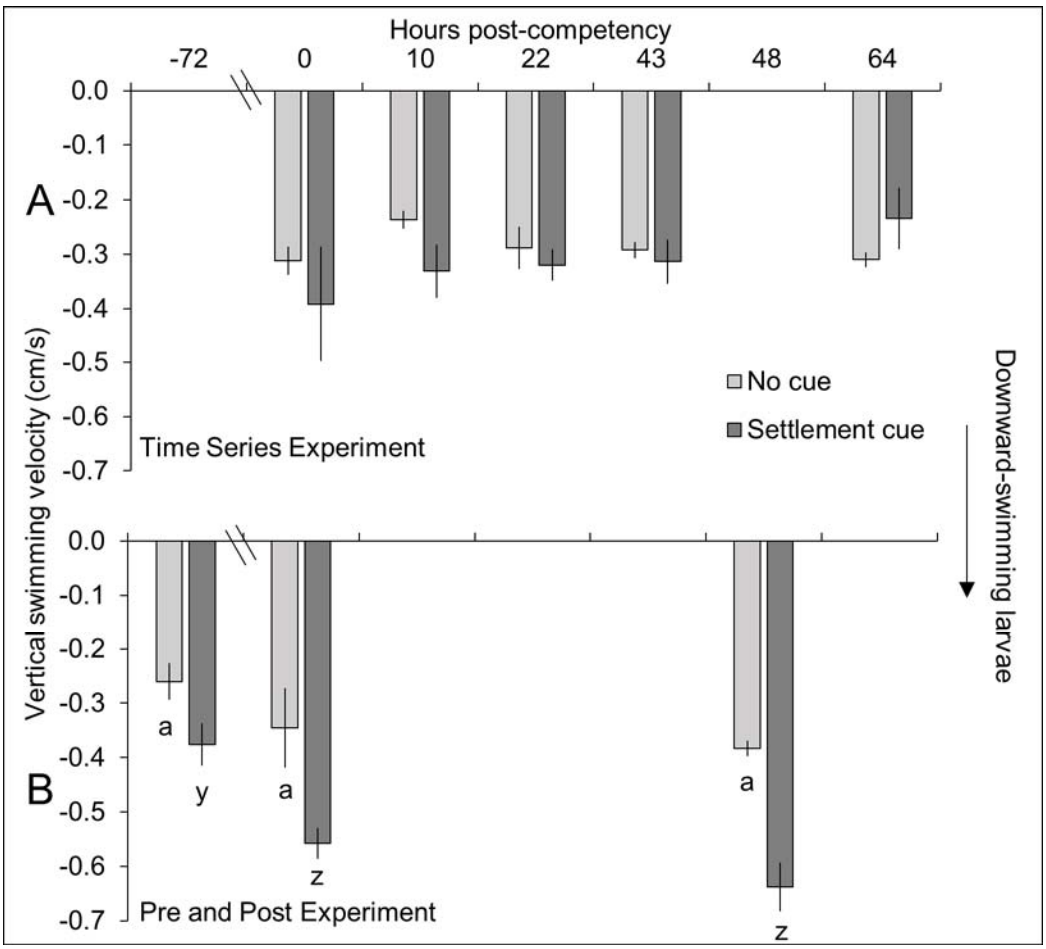
743 Fig. 3



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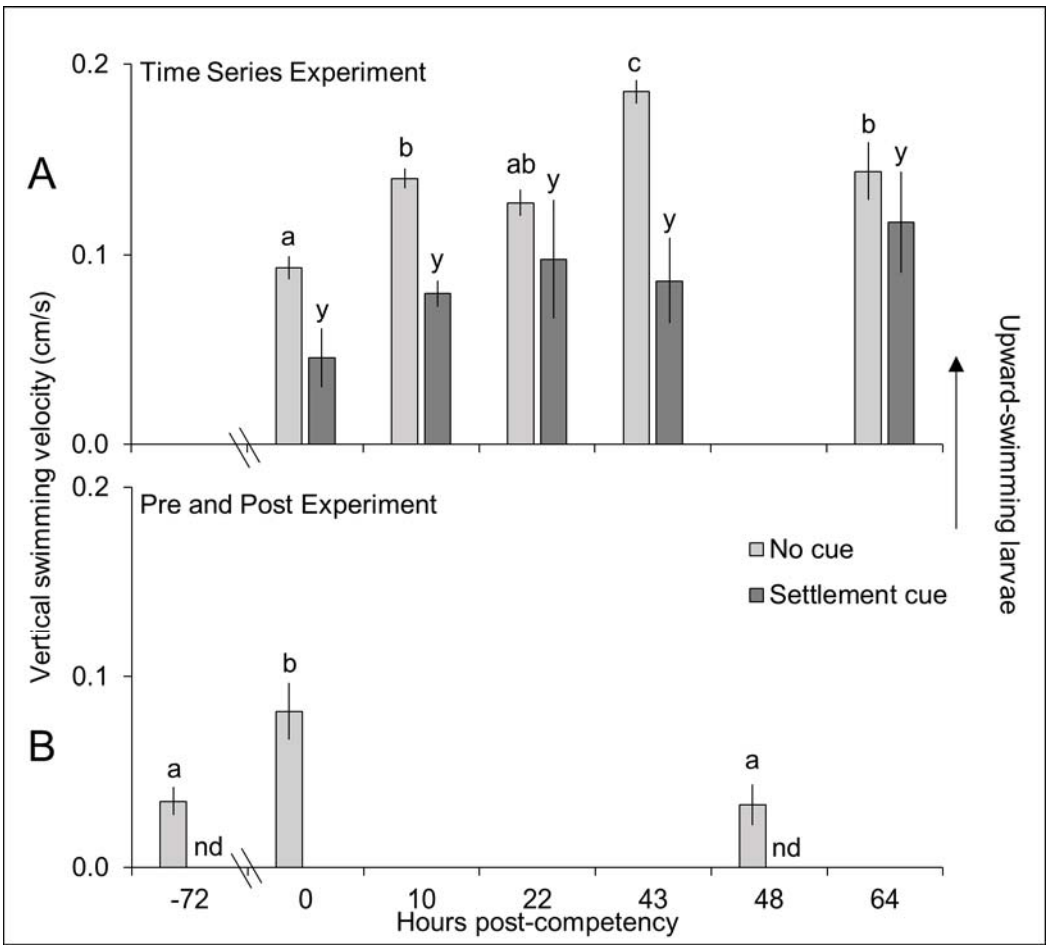
754 Fig. 4



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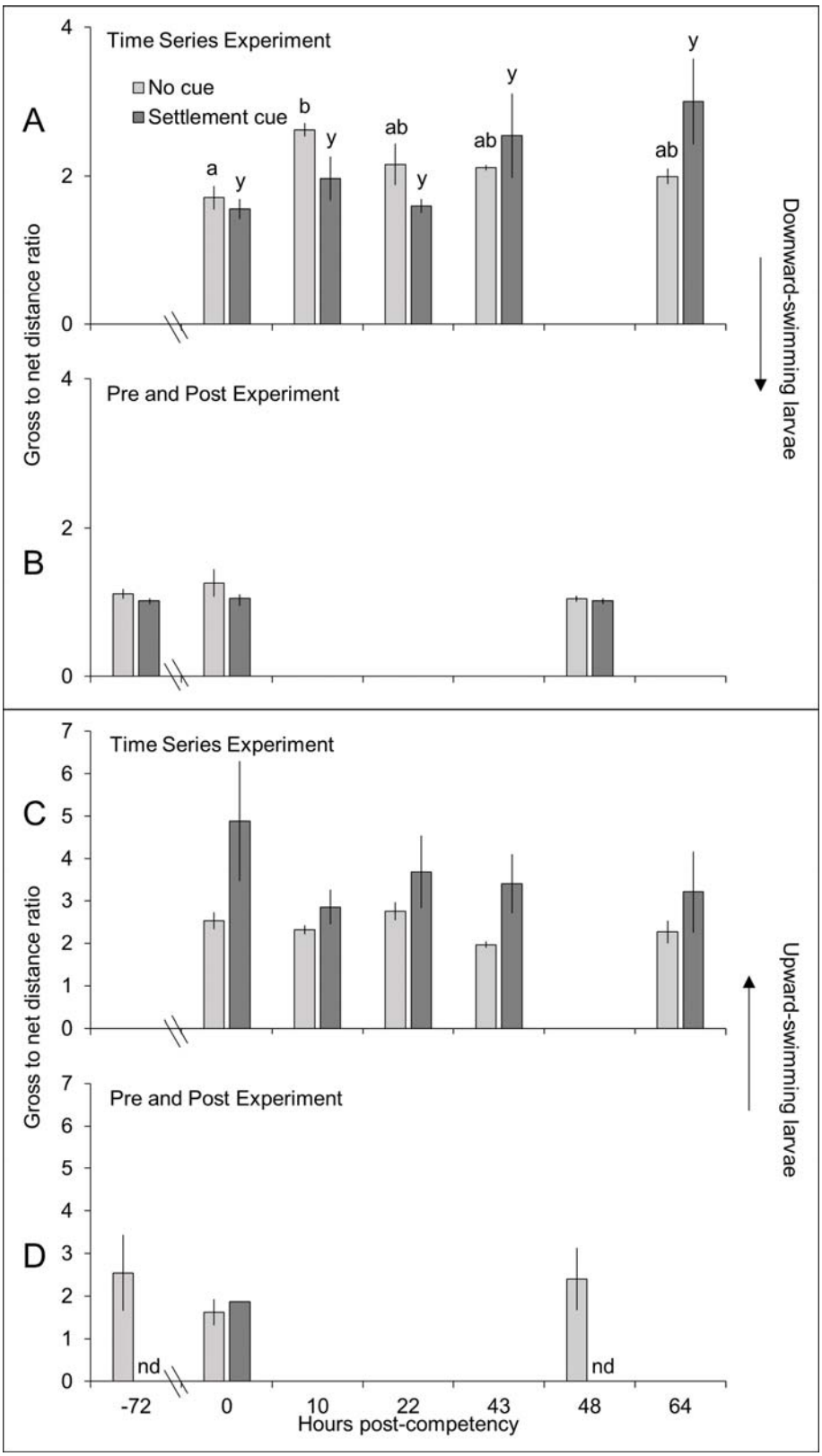
765 Fig. 5


Time Series Experiment
No cue
Settlement cue
A
B
C
D
Gross to net distance ratio
Pre and Post Experiment
Downward-swimming larvae
Upward-swimming larvae
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b
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Hours post-competency

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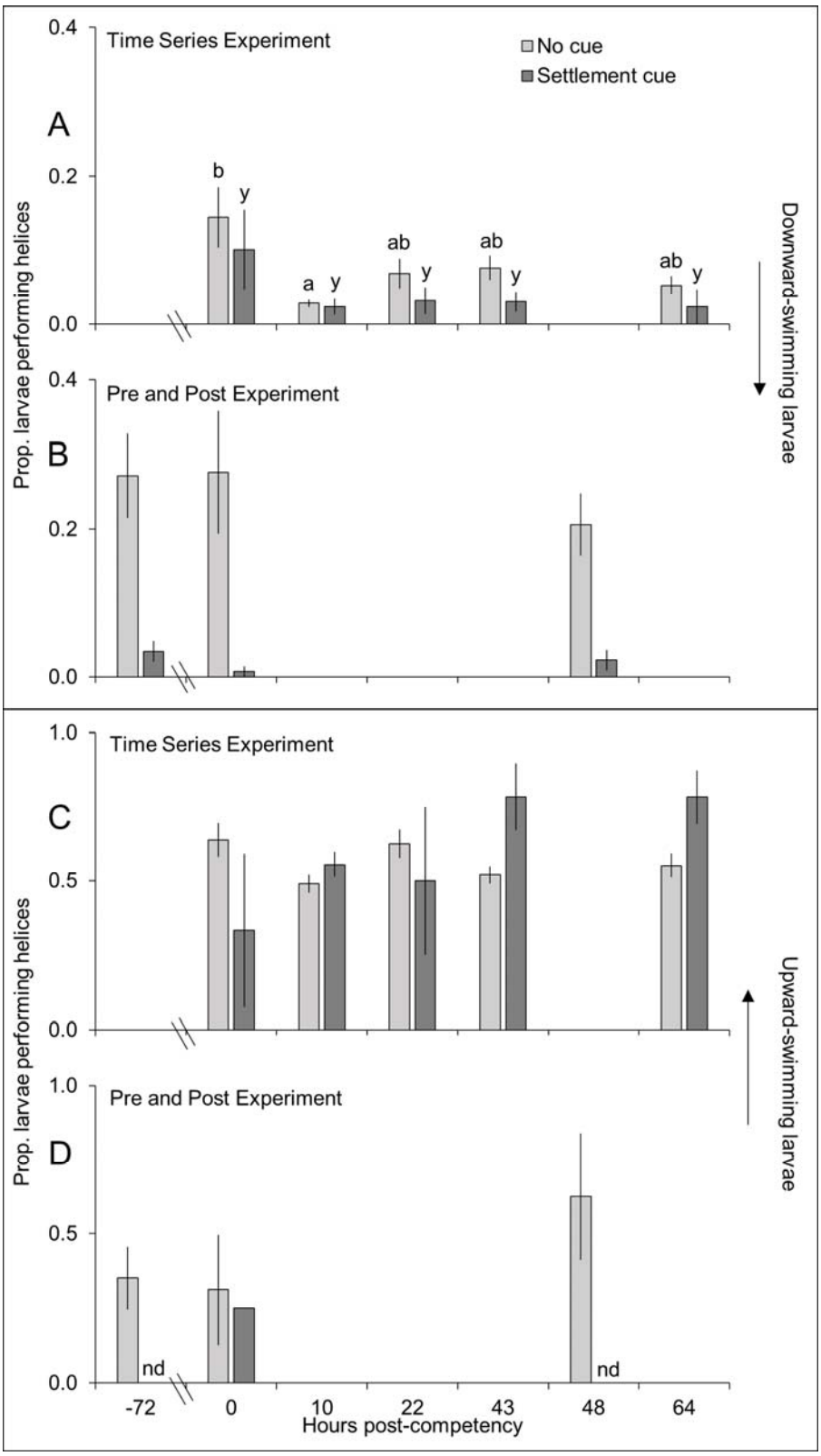
767 Fig. 6


Time Series Experiment
No cue
Settlement cue
A
Pre and Post Experiment
B
Time Series Experiment
C
Pre and Post Experiment
D
Prop. larvae performing helices
Downward-swimming larvae
Upward-swimming larvae
0.4
0.2
0.0
1.0
0.5
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a
y
ab
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ab
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ab
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nd
nd
-72
0
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43
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Hours post-competency

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