

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

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**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 2 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 1 of the 5 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.

**KEY WORDS:** Settlement cue · Helical swimming · Benthic · Desperate larva hypothesis

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## 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 have focused 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 im-

portant 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, Whitman & Reidenbach 2012, Wheeler et al. 2013, 2015).

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

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swimming or passive diving towards a substratum (Burke 1986, Pawlik 1992, Hadfield & Paul 2001). Moving toward the seafloor in response to these cues may benefit the larvae, as they are often indicators of preferred benthic habitat. Physical signals, including light and turbulence, can also influence larval behavior and are important to consider when understanding settlement (Koehl 2007, Fuchs et al. 2013, 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 to 3 wk (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).

*C. 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 settled 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 ob-

served 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). *C. 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 interested larval ecologists. The 'desperate larva' hypothesis was first proposed over 60 yr ago (Knight-Jones 1951, 1953, Wilson 1953) and states that lecithotrophic larvae respond to sub-standard settlement cues as they age and their energy supplies become depleted. Older larvae may be induced to settle by cues that have no effect on younger, more selective larvae (Gibson 1995); older larvae may also settle in the presence of settlement inhibitors (Gribben et al. 2006) or no cue at all (Bishop et al. 2006). Larval body size (Marshall & Keough 2003), planktonic mortality (Elkin & Marshall 2007), and prior exposure to settlement cues (Botello & Krug 2006, Swanson et al. 2007) can influence larval responses to cues with age (for an extreme example, see Strathmann & Strathmann 2007). Delayed metamorphosis and settlement in a variety of habitats may allow for gene flow among isolated populations, ultimately providing a safeguard against extinction (Gibson 1995).

Since its inception, the 'desperate larva' hypothesis has been expanded to include some planktotrophs (Botello & Krug 2006), with the caveat that planktotrophs, unlike lecithotrophs, become 'desperate' to settle because of a reduced capacity to maintain the competent larval swimming state (as opposed to a depletion of maternal resources) (Bishop et al. 2006). However, both cases are equivalent from an ecologi-

cal perspective, as they both involve delayed metamorphosis, more time spent swimming and searching for a substratum, and the eventual acceptance of sub-optimal settlement sites.

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

## MATERIALS AND 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 h of onset). The pilot experiment showed significant responses to a chemical settlement cue but no differences among larval ages in early competency (for results see Tables S1–S3 & Figs. S1–S5 in the Supplement at [www.int-res.com/articles/suppl/m599p093\\_supp.pdf](http://www.int-res.com/articles/suppl/m599p093_supp.pdf)) and motivated 2 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 d after onset). Because young competent larvae (time = 0 h, 50% with eyespots; see next section) displayed distinct settlement behaviors, we investigated the behaviors of pre-competent larvae in a second experiment, the 'Pre and post' (competent) experiment. For this experiment, we compared measurements among larvae at pre-competent (3 d before onset), competent, and post-competent (2 d after onset) stages. All experiments involved larval exposure to seawater with and without a chemical settlement cue.

### Study species handling practices

We obtained larvae from 2 spawns for use in our 2 experiments. Larvae were provided by the Aquaculture Research Corporation in August 2015 and July 2016 for the Time series and Pre and post experiments, respectively. The hatchery provided mature veligers retained on a 180  $\mu\text{m}$  sieve for the Time series experiment and younger veligers retained on a 120  $\mu\text{m}$  sieve for the Pre and post experiment involving pre-competent larvae. These younger veligers were approximately 3 d pre-competent based on subsequent eyespot development in the laboratory. Prior to experiments, all larvae were maintained in 1  $\mu\text{m}$ -filtered, aerated seawater at ambient field temperature (20 to 22°C) and salinity (33 psu), in covered 16 l plastic buckets. Larvae were kept at low densities (<3 larvae  $\text{ml}^{-1}$ ) to minimize interactions, and fed daily a suspension of the haptophyte *Isochrysis* sp. ( $\sim 9 \times 10^5$  cells  $\text{ml}^{-1}$  in filtered seawater). Larvae approaching competency were maintained in these conditions (2 to 5 d) until competency onset, as defined by ~50% of larvae exhibiting eyespots. Eyespots were identified by microscopic examination of a random sample of larvae, and observational experiments immediately commenced when this competency threshold had been reached. The pre-competent larvae were retained in identical conditions for a shorter time period (2 h) prior to starting experiments, in order to ensure larvae were not yet competent to settle during the experiment. Immediately prior to each experiment, a random sub-sample of larvae was preserved in a solution of 95% ethanol. These preserved larvae were microscopically examined for shell size measurements and eyespot identification. We recorded larval body sizes because larvae may continue to grow throughout the competency period (Kennedy 1996), and size could impact swimming ability (Hidu & Haskins 1978). Larval size was estimated by measuring shell height (straight-line dis-

tance perpendicular from the shell hinge to edge of the shell) and length (straight-line distance along the hinge axis).

Oyster bathwater was prepared in a similar method to that described by Tamburri et al. (1992). Live, unwashed, adult *Crassostrea virginica* were purchased from a local vendor (The Clam Man, Falmouth, MA, USA) from harvests of farmed oysters from Washburn Island in Waquoit Bay, MA. Individuals were measured to estimate shell surface area, and a set of oysters totaling 1600 cm<sup>2</sup> surface area were placed in a sterile plastic bucket with aerated seawater (4 l) filtered to 1 μm (ambient 20°C, 33 psu) for the Time series experiment. This protocol was modified slightly (3320 cm<sup>2</sup> surface area in 12 l seawater) for the Pre and post experiment. The buckets were covered and left undisturbed in a temperature-controlled environmental chamber for 4 h. Subsequently, adult oysters were removed, and the bathwater was filtered to <1 μm with a glass microfiber filter. The bathwater was then divided into 1 l aliquots and frozen at –20°C until immediately prior to use in experiments.

### 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 to 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; 12 V, 2 A, 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 2 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 h post-competency. For the Pre and post experiment, we included pre-competent larvae (without eyespots, time = –72 h), as well as larvae at 0 and 48 h 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 2 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 replicate consisted of a unique set of larvae in a new, unused flask. Larval swimming behaviors were recorded for 5 min at 30 frames s<sup>-1</sup> in each replicate.

### Larval tracking

The methods for larval identification and tracking were adapted from Wheeler et al. (2013, 2015) and are briefly summarized here. Video recordings of each replicate were saved as high-resolution TIFF

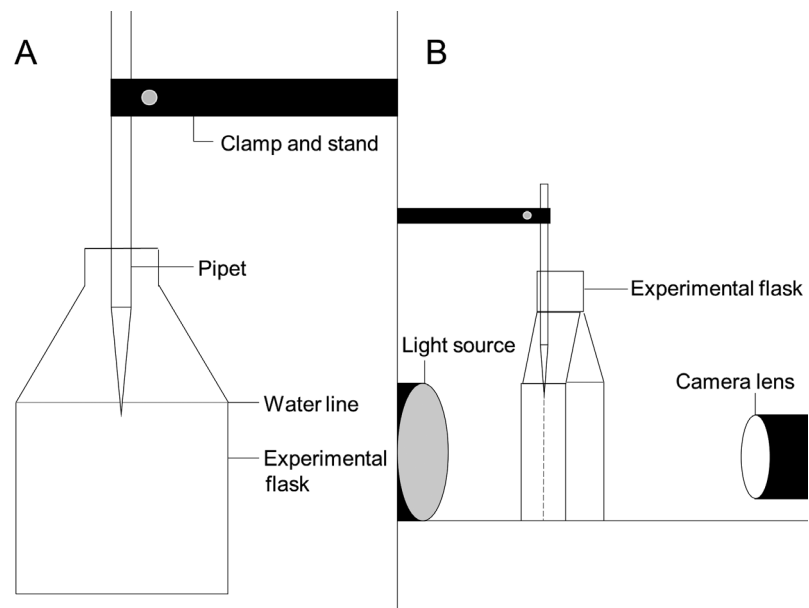


Fig. 1. Experimental setup. (A) Experimental flask from the direction of camera view; (B) flask and camera arrangement. Dashed vertical line: the camera's field of view plane

Table 1. Average size and percentage of *Crassostrea virginica* larvae with eyespots at each age in each experiment. n: number of individuals sub-sampled; intervals represent standard deviation. Pre-competent larvae are denoted as -72 h post-competency

Experiment	Hours post-competency	n	Length ( $\mu\text{m}$ )	Height ( $\mu\text{m}$ )	% with eyespots
Time series	0	28	260 $\pm$ 11	256 $\pm$ 11	64
	10	30	260 $\pm$ 9	254 $\pm$ 12	70
	22	30	273 $\pm$ 14	267 $\pm$ 10	90
	43	30	287 $\pm$ 13	284 $\pm$ 11	97
	64	30	285 $\pm$ 18	293 $\pm$ 21	100
Pre and post	-72	20	176 $\pm$ 16	181 $\pm$ 11	0
	0	24	285 $\pm$ 22	275 $\pm$ 25	38
	48	22	290 $\pm$ 23	281 $\pm$ 14	95

images (1040  $\times$  1390 pixels) for subsequent larval tracking. TIFF images were imported into LabVIEW 2013 (National Instruments), and average background pixel intensity was subtracted. Using a fixed-threshold particle size and intensity, larval centroid positions (x, z) were recorded in the frame in which they appeared. Centroid positional data were reconstructed into individual larval trajectories using a MATLAB script which tracked a larva from frame to frame according to a subsequent-frame tolerance distance radius set by the user. Larval trajectories were truncated by 5 frames at each end of the trajectories to avoid poor centroid estimates in cases where larvae passed laterally into and out of the focal plane. Instantaneous swimming velocities were computed using a central difference scheme of larval centroid positions in time, so that the velocity is defined as centered in time between 2 adjacent frames. Unlike the experiments of Wheeler et al. (2013, 2015), flow velocities in the small flasks in the present study were minimal, and therefore no effort was made to subtract local flow from observed larval velocities.

### 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 aver-

age vertical velocity of larvae, the proportion of larvae swimming in helices, and the net-to-gross distance ratio (NGDR; see next paragraph). 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 (gross-to-net ratio: GNDR = 1/NGDR) 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  $\gg$  1 indicates exploration. We report GNDR for oyster larvae in this paper.

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 portion of the water column (Crenshaw 1996). This spiral swimming pattern has multiple hypothesized functions, including directional swimming in asymmetrical organisms (Crenshaw 1996), regulating vertical position in the water column (Cragg 1980, Wang & Xu 1997), feeding, exploration (Chia et al. 1984), and maximizing prey encounter rate while minimizing predator encounter rate (Visser 2007). Helically swimming larvae represent a subset of high-GNDR larvae; however, we chose to focus on this subset because of the ubiquity of helical swimming in zooplankton and the tendency for oyster larvae to modify helical behavior in the presence of environmental cues (Wheeler et al. 2017).

Because the field of view was largely 2-dimensional, an unavoidable experimental constraint involved larvae laterally leaving the field of view. It is conceivable that a larva swam in one direction (downward or upward), exited the field of view, re-



turned to its original position (top or bottom of the flasks), and the re-entered the field of view. However, every effort was made to only count downward- and upward-swimming larvae once, so we consider this source of error to be very small.

The number of larvae performing dives was quantified according to the methods of Wheeler et al. (2015). Diving behavior is induced by local fluid acceleration in turbulence (Wheeler et al. 2015), and as such, diving was seldom found in our study, likely due to the lack of ambient flow in the experimental flasks.

### Statistical analyses

Significant differences in behavioral metrics between cue and no-cue treatments and among larval ages were examined using 2-way analysis of variance (ANOVA). We tested for 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 non-parametric tests do not assess interactions between larval age and cue treatments. Non-parametric results for heteroscedastic cases are reported in Table S4 in the Supplement. 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 *Crassostrea virginica* larvae

The proportion of no-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 no-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 ( $p = 0.002$  and  $0.023$ , respectively; 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 larvae, it was expected to be weaker in pre-competent larvae. The strength of the cue effect for pre-competent larvae (Fig. 2B), and the high proportion of pre-competent larvae showing settlement behavior, whether exposed to cue or not, was a surprise.

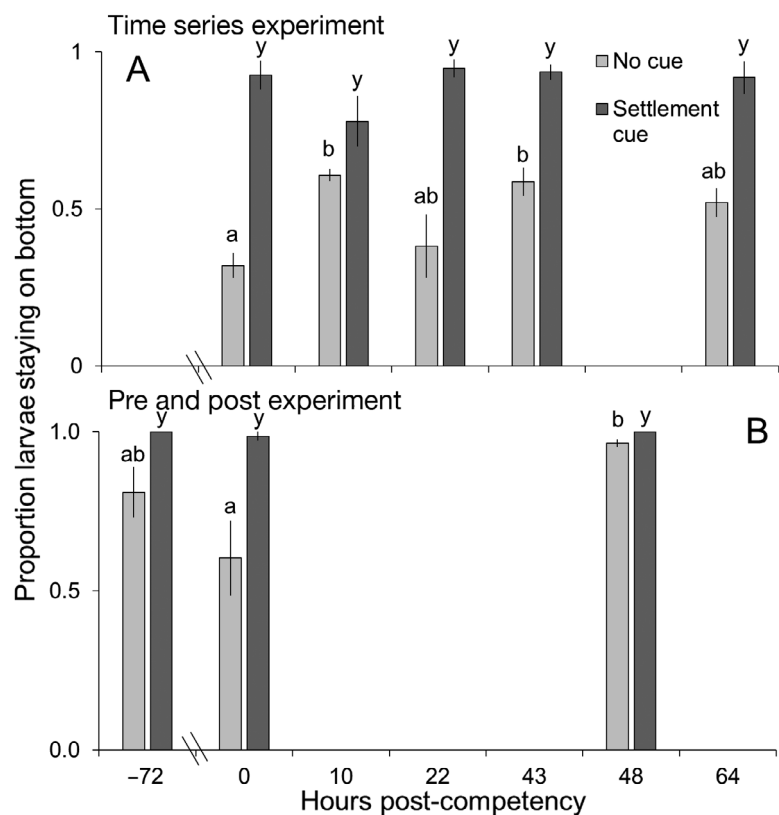


Fig. 2. Proportion of *Crassostrea virginica* larvae remaining on the bottom after swimming down, as a function of larval age. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical settlement cue (dark bars) in (A,B) 2 separate experiments. Larvae of age -72 h are pre-competent. Error bars: SE. Dissimilar letters indicate significant post hoc differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments

Table 2. Settlement behaviors of *Crassostrea virginica* 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	<b>&lt;0.001</b>
		Age	1.87	0.135
		Cue × Age	5.16	<b>0.002</b>
	Pre and post	Cue	17.9	<b>&lt;0.001</b>
		Age	5.17	<b>0.013*</b>
		Cue × Age	4.39	<b>0.023</b>
Velocity of downward-swimming larvae	Time series	Cue	1.01	0.321
		Age	0.84	0.509
		Cue × Age	1.01	0.413
	Pre and post	Cue	30.7	<b>&lt;0.001</b>
		Age	10.6	<b>&lt;0.001</b>
		Cue × Age	1.38	0.271

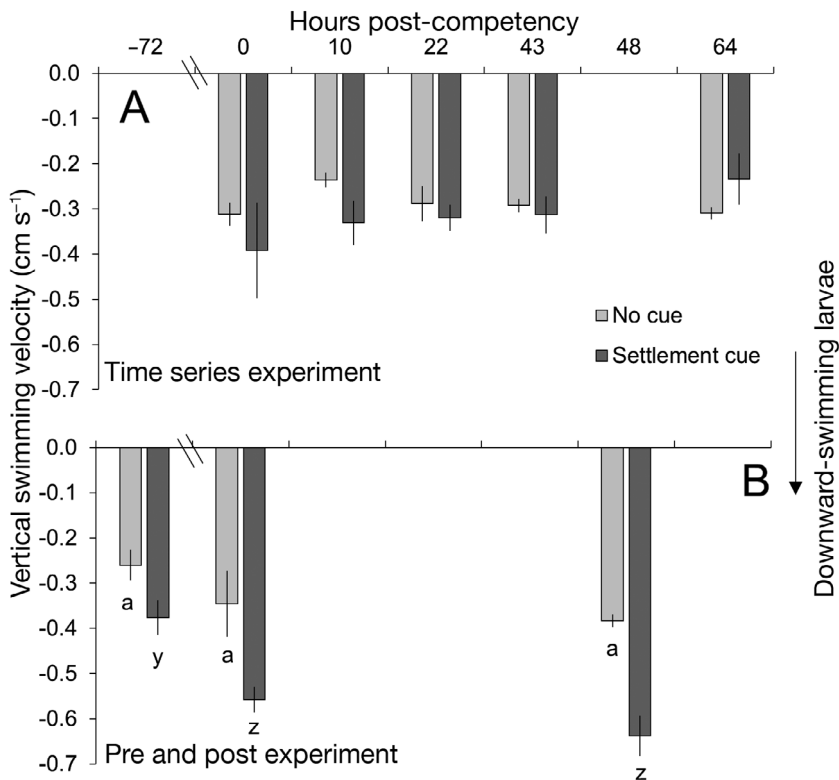


Fig. 3. Vertical swimming velocity of downward-swimming *Crassostrea virginica* larvae, as a function of age. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical settlement cue (dark bars) in (A,B) 2 separate experiments. Larvae of age -72 h were pre-competent. Error bars: SE. Dissimilar letters indicate significant post hoc differences between ages, tested separately for no-cue (a, b, c) and cue (y, z) treatments

The downward swimming velocity of competent larvae did not vary with larval age, whether they were exposed to settlement cue or not (Fig. 3, Table 2). Downward velocity was expected to be higher in cue-exposed larvae than no-cue-exposed larvae, but this difference was observed only in the Pre and post experiment ( $p < 0.001$ ; Table 2, Fig. 3B), while in the Time series experiment, larvae maintained velocities over the competency period (Fig. 3A). The lack of an ontogenetic change in downward velocity of no-cue-exposed larvae is not consistent with our expectations for aging larvae. In pre-competent larvae, the settlement response was weaker than in competent larvae, in both cue and no-cue conditions (Fig. 3B), as expected.

#### Exploratory behaviors in *C. virginica* larvae

The upward swimming velocity of competent no-cue-exposed larvae showed opposite trends with larval age in the 2 different experiments. The velocity of larvae swimming up off the bottom decreased with age in the Pre and post experiment but increased with age in the Time series experiment (Fig. 4, Table 3). All cue-exposed larvae remained on the bottom in the Pre and post experiment, while cue-exposed larvae showed a similar pattern to no-exposed larvae in the Time series experiment. The opposing ontogenetic patterns in the 2 experiments were significant ( $p = 0.002$  and  $0.035$ , respectively; Fig. 4), and there were no interaction effects between cue and age (Table 3). The observation of a decrease in exploratory behavior with larval age (Pre and post experiment) was consistent with our expectations, but the ontogenetic pattern in the Time series experiment was not. The observed effect of the cue in reducing exploratory behavior (Table 3) was expected for competent larvae, but the strength of the effect in pre-competent

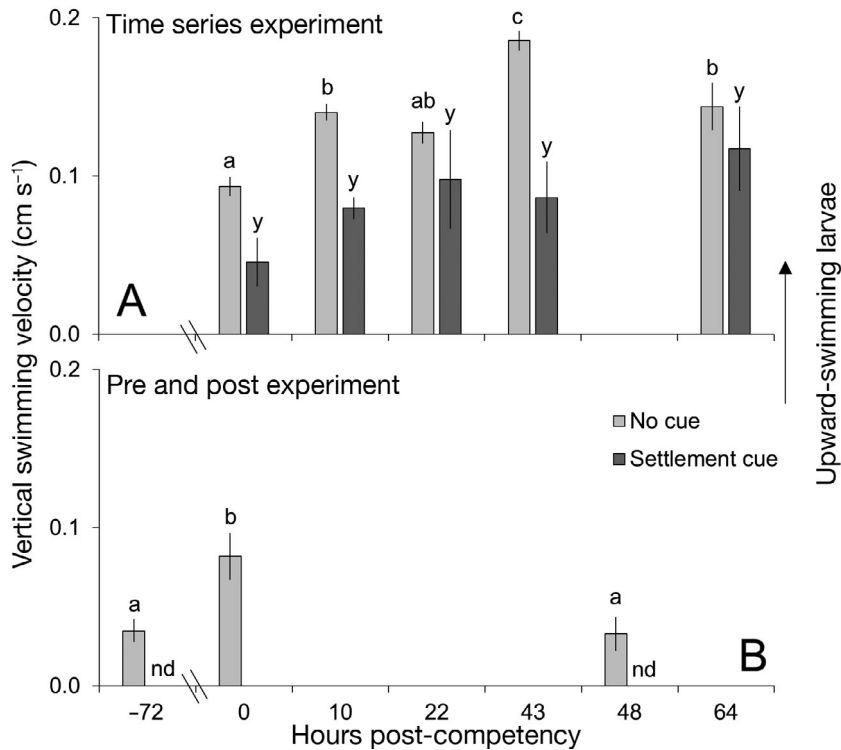


Fig. 4. Vertical swimming velocity of upward-swimming *Crassostrea virginica* larvae as a function of age. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical settlement cue (dark bars) in (A,B) 2 separate experiments. Larvae of age -72 h were pre-competent. Error bars: SE. Dissimilar 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 h swam upward off the bottom, so 'nd' indicates no data for upward swimmers

larvae, and the low vertical velocities compared to 0-hour competent larvae (Fig. 4B) were unanticipated.

The GNDR of downward-swimming larvae increased with age in one of the experiments (Time series) (Fig. 5A, Table 3) and remained constant in the other (Pre and post) (Fig. 5B, Table 3). Increased GNDR indicates increased exploration, and as such, these results are not consistent with the expectation of decreasing exploratory behavior with larval age. Exposure to the cue did not affect this behavior across larval ages (Table 3), but an interaction between cue and age (borderline significant in the Time series experiment,  $p = 0.050$ ) indicates that the increase in GNDR was apparent only in the cue-exposed larvae. In downward-swimming pre-competent larvae, GNDR was no different than in competent larvae.

The GNDR of upward-swimming larvae did not vary with larval age in either experiment (Fig. 5C,D, Table 3). GNDR was higher in cue-exposed larvae than in no-exposed larvae, a result that is not consistent with an expectation of reduced exploration in response to a settlement cue. In upward-swimming

pre-competent larvae, GNDR was no different than in competent larvae.

The proportion of downward-swimming larvae performing helices decreased with larval age, for both cue-exposed and no-cue-exposed larvae in the Time series experiment (Fig. 6A, Table 3), but not in the Pre and post experiment (Fig. 6B, Table 3). Helices tended to be less common in larvae exposed to the cue, but this effect was significant only in the Pre and post experiment ( $p < 0.001$ ). Both a decrease in helices with age and with exposure to a settlement cue are consistent with our expectations, although we anticipated a more prominent ontogenetic change in no-cue-exposed larvae. In downward-swimming pre-competent larvae, the proportion 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). Pre-competent larvae behaved no differently than competent larvae.

### Differences between spawns

We tested for significant differences in larval behavioral metrics between the 2 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, meander-



Table 3. Exploratory behaviors of *Crassostrea virginica* in response to settlement cue and larval age. Two-way ANOVA tests for differences in the upward swimming velocity of larvae, average gross-to-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	< <b>0.001</b>
		Age	5.30	<b>0.002</b>
		Cue × Age	1.79	0.155
	Pre and post	Age	4.38	<b>0.035</b>
GNDR of downward-swimming larvae	Time series	Cue	0.01	0.943
		Age	2.76	<b>0.041</b>
		Cue × Age	2.61	0.050
	Pre and post	Cue	2.79	0.107
		Age	1.21	0.315
		Cue × Age	0.65	0.531
GNDR of upward-swimming larvae	Time series	Cue	10.4	<b>0.002*</b>
		Age	1.19	0.334
		Cue × 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	<b>0.005</b>
		Cue × Age	0.21	0.931
	Pre and post	Cue	39.3	< <b>0.001</b>
		Age	0.39	0.682
		Cue × 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 × Age	2.28	0.081
	Pre and post	Age	1.03	0.386

ing, or helices) in suboptimal conditions (no-cue treatments). Our results showed higher proportions of older larvae remaining on the flask bottoms in no-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.

Our second expectation, that exploratory behavior in competent larvae would decrease with larval age in suboptimal conditions (no-cue) was supported in only a few cases. For upward swimming velocity, there were opposite trends between the 2 experiments. It is unclear why this was the case, though the faster velocities for older larvae in one experiment/spawn may have induced a greater propensity towards exploration. For downward-swimming larvae, there was a clear trend of fewer helices in older larvae, which was

significant in one experiment, supporting our expectation of less exploratory behavior in older larvae. The GNDR of downward-swimming larvae increased with age in one experiment, indicating increased exploration, but this trend was only apparent for larvae in the cue. Meandering (high-GNDR) behavior may be subject to inter-spawn variation, as there were significant differences in this metric for both downward- and upward-swimming larvae between the 2 experiments/spawns. Exploratory behaviors of *C. virginica* larvae had mixed results and may be influenced by factors other than habitat suitability as indicated by the presence of a settlement cue. While the 2 spawns were held in comparable conditions throughout the experiments (light conditions, temperature, salinity), it is possible that earlier developmental conditions for the spawns introduced carry-over effects for our late-stage observations. These exploratory behaviors warrant further study to understand ontogenetic or environmental factors influencing them.

This study is, to our knowledge, the first demonstration of increased settlement behavior with age in a planktotrophic species. The acceptance of suboptimal settlement habitat by

older larvae lends support to the 'desperate larva hypothesis' and contrasts with previous studies using planktotrophic species. However, previous studies examined larval responses to settlement cues with age, whereas we found increased settlement behavior in no-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. Modeling efforts indicate that planktotrophic species have few deferred

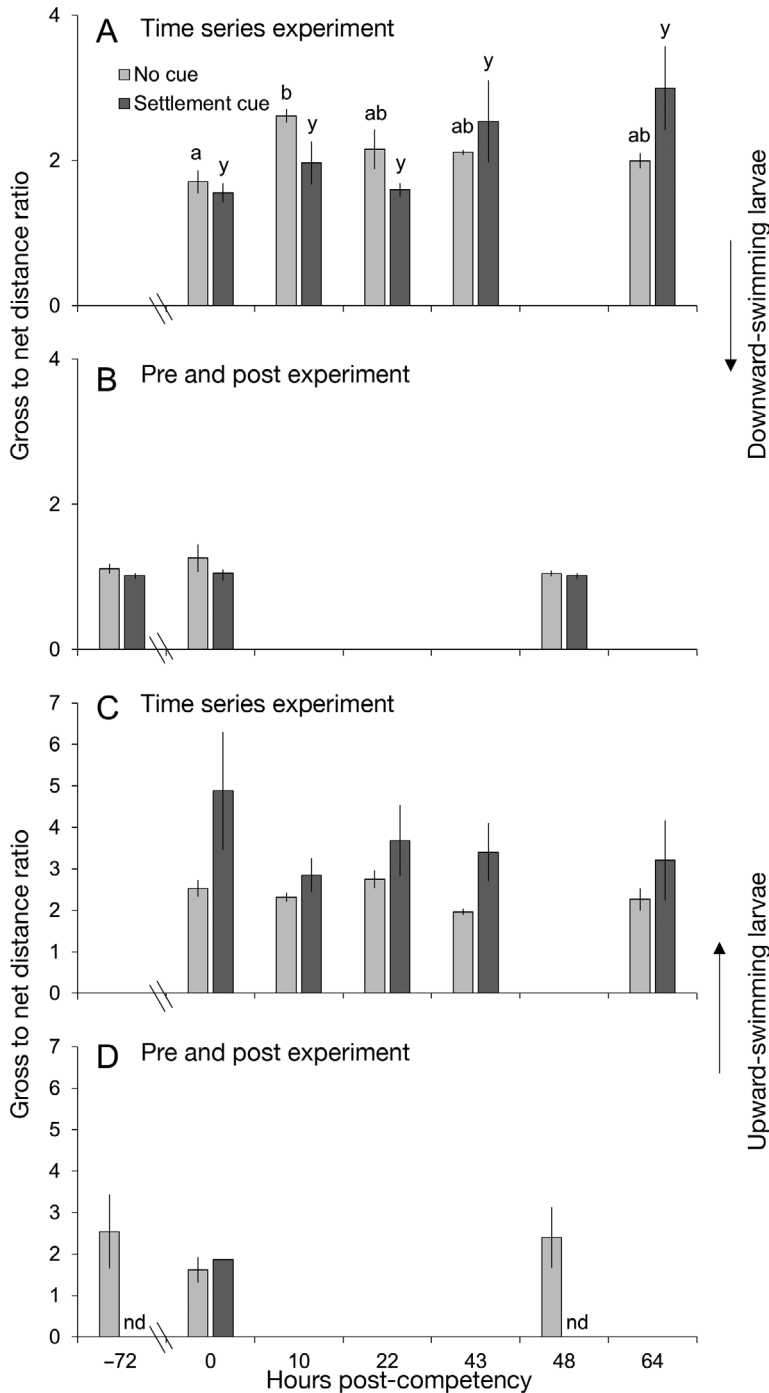


Fig. 5. Gross-to-net distance ratio (GNDR) of *Crassostrea virginica* larvae as a function of age for (A,B) larvae swimming downward to the bottom of the flask following introduction and (C,D) larvae swimming back up off the bottom. Larvae were exposed to filtered seawater (no cue; light bars) or a chemical settlement cue (dark bars) in 2 separate experiments. Larvae of age -72 h were pre-competent. Error bars: SE. 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 h swam upward off the bottom, so 'nd' indicates no data for upward swimmers. The missing error bar at  $t = 0$  h in the Pre and post experiment indicates only 1 replicate in that treatment had larvae swimming back up off the bottom

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, Wang & Xu 1997, Troost et al. 2008). While helical swimming may be an adaptation for feeding or vertical swimming (i.e. Jonsson et al. 1991, Chan et al. 2011), in *C. virginica*, helical swimming behavior is modified by environmental cues involved in settlement (Wheeler et al. 2017). The ontogenetic trend we observed supports the concept of helical swimming as an exploratory behavior. Helical swimming is ubiquitous among microorganisms, observed in bacteria, protists, fungi, sperm, and marine larvae (Crenshaw 1996). Given this wide phylogenetic spread, helical swimming may have multiple functions, and it warrants further investigation in *C. virginica*.

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

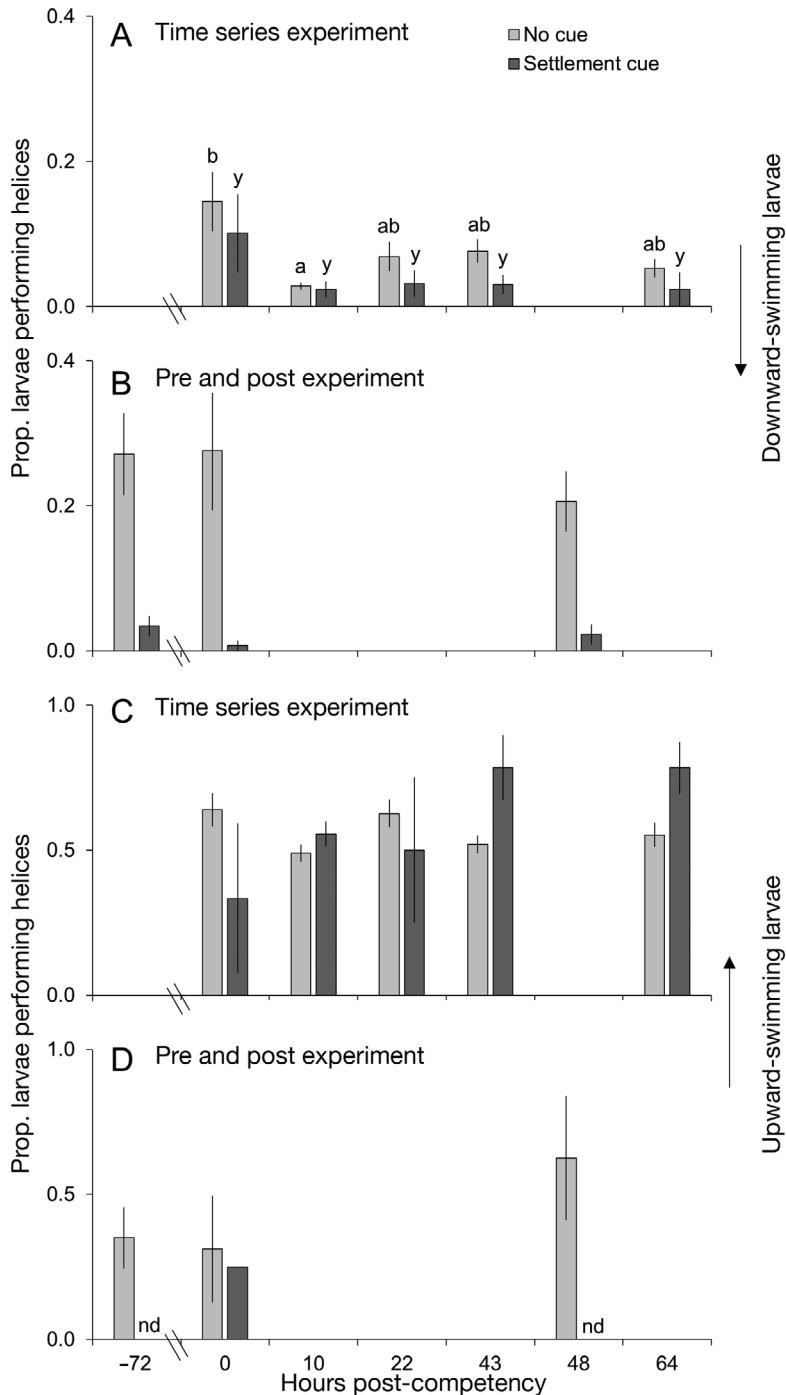


Fig. 6. Proportion of *Crassostrea virginica* larvae swimming in helices as a function of age for (A,B) larvae swimming downward to the bottom of the flask following introduction and (C,D) larvae swimming back up off the bottom. Larvae were exposed to filtered seawater (no cue, light bars) or a chemical settlement cue (dark bars) in 2 separate experiments. Larvae of age  $-72$  h were pre-competent. Error bars: SE. 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  $48$  h swam upward off the bottom, so 'nd' indicates no data for upward swimmers. The missing error bar at  $t = 0$  h in the Pre and post experiment indicates only 1 replicate in that treatment had larvae swimming back up off the bottom

One very surprising result from our study was the discovery that pre-competent larvae ( $t = -72$  h, 0% with eyespots) also appeared to respond to the settlement cue, traveling to the bottom of the flasks more quickly and directly than no-cue-exposed larvae and remaining near the bottom. In many cases, the response of pre-competent larvae to the settlement cue was similar to the response of competent ( $t = 0$  h) larvae. Pre-competent larvae had lower swimming velocities than competent larvae in both downward and upward directions, most likely because of their smaller size. Swimming speed increases with age for many molluscan larvae (Cragg 1980), including *C. virginica* (Hidu & Haskins 1978).

Any explanations for why pre-competent larvae showed settlement behaviors can only be speculative. Recent reviews on the adaptive significance of metamorphic competence in marine invertebrate larvae have focused on habitat selection in the post-competent period, neglecting pre-competent larvae (Hadfield et al. 2001, Bishop et al. 2006). Bishop et al. (2006) found a relationship between the habitat specificity of juveniles of a species and the capacity of its larvae to prolong the larval period until a suitable habitat is found. Here, we speculate that it may be adaptive for hard-bottom invertebrates, especially those that inhabit island-like or specialized habitats, to respond to settlement cues even when pre-competent. Delayed metamorphosis has negative carryover effects on growth and fecundity for juveniles and adults (Marshall et al. 2003, Pechenik 2006); therefore, if a larva responds to a settlement cue when pre-competent, it may avoid these negative consequences of prolonged habitat search during the competency period. On rocky shores, pre-competent sea urchin larvae respond to turbulence, a general settlement cue, and even undergo accelerated development to become competent (Gaylord et al. 2013). For *C. virginica*, we are unable to say whether pre-competent larvae would also undergo accelerated development to competency, but the responses of pre-competent larvae and induction of accelerated competence

are avenues for future research. Some marine larvae possess specialized organs for the transduction of settlement signals (Hadfield & Pennington 1990, Hadfield et al. 2000), but much remains to be learned about the physiology of settlement (Rodriguez et al. 1993).

Examining specific behavioral responses and the time-scales of their ontogenetic change can help explain their adaptive significance. For example, differences in phototaxis between newly released and competent-to-settle larvae suggest adaptations for upward swimming for dispersal and feeding early in the larval duration, followed by a return to the benthos when competent (Miller & Hadfield 1986, Montgomery et al. 2018). However, most studies examining ontogenetic changes in larval behavior have focused on the period of larval development prior to competency. It is important to study ontogenetic changes beyond the onset of competency to understand behavioral changes influencing habitat selection and settlement. Acceptance of suboptimal settlement habitats or acceptance of settlement habitats at suboptimal development stages (i.e. when pre-competent) may decrease individual fitness, but it may also increase the resilience of populations. If individual oysters are able to accept suboptimal settlement habitats when no optimal habitat is available, for example following a major disturbance (Livingston et al. 1999), this will allow the population to persist until optimal settlement habitats are restored. Populations of *C. virginica* are adapted to variable environmental conditions (Newkirk et al. 1977), which may allow them to recover quickly after disturbances (Pollack et al. 2011).

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