Light stimulates swimming behavior of larval eastern oysters (*Crassostrea virginica*) in turbulent flow

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Running page head: Oyster larvae respond to light

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Abstract

Planktonic larvae of the eastern oyster (*Crassostrea virginica*) are able to regulate their vertical position in the water, but the environmental cues responsible for this regulation, particularly in turbulent settings, remain unclear. We quantified swimming responses of late-stage oyster larvae in a grid-stirred turbulence tank to determine how light affects the swimming behavior of larvae over a range of hydrodynamic conditions similar to their natural coastal environments. We used particle image velocimetry and larval tracking to isolate larval swimming from local flow and to quantify three behavioral metrics: vertical swimming direction, proportion of larvae diving, and proportion of larvae swimming helically. We compared these metrics across

turbulence levels ranging from still water ($\varepsilon = 0$ cm^2 s^{-3}) to estuarine-like conditions ($\varepsilon = 0.4$ cm^2 s^{-3}) in light and dark. In all turbulence levels, light had no effect on the proportion of upward swimming larvae, but elicited detectable increases in the proportion of helical swimming and diving behaviors. We further examined the effect of light and turbulence on specific characteristics of helical trajectories, and found that these environmental cues induce changes to both vertical and horizontal velocities of helically swimming larvae, changing the helix geometry. The increased prevalence of these behaviors in light likely plays an ecological role: increased diving in light (in conjunction with turbulence) is a potential mechanism to enhance settlement success, while changes to helical swimming in light may serve an anti-predatory function. Together, these behaviors provide insight into potentially complex larval responses to multiple simultaneous environmental cues.

1 Introduction

The eastern oyster (*Crassostrea virginica*), like many benthic marine invertebrates, is spawned into the water column and develops through a series of free-swimming planktonic larval stages prior to settlement to the benthos. Adult populations of oysters have high economic value through shellfisheries and aquaculture (Newell 1988, Breitburg et al. 2000), as well as less readily quantifiable benefits such as large-scale water filtration (Nelson et al. 2004) and shoreline stabilization (Currin et al. 2010). Oyster populations have declined to 1% of historical biomass due to a combination of overharvesting and long-term environmental changes (Rothschild et al. 1994, Kemp et al. 2005), and efforts at population restoration and conservation require us to

study oysters at the vulnerable larval stages. Understanding larval behavior during planktonic stages is important for both dispersal modelling (North et al. 2008, Metaxas and Saunders 2009, Kim et al. 2013) and effective population restoration via larval supply; the competent-to-settle larval stage is of particular interest, as successful larval recruitment is crucial to adult survival and reproduction (Butman 1987, Bartol et al. 1999, Nestlerode et al. 2007). Larval oyster recruitment in particular relies on larvae locating preferred settlement sites in shallow water on rough substrate (NOAA 2007).

Previous studies have shown strong correlations between physical habitat and oyster larval recruitment (Fredriksson et al. 2010, Whitman and Reidenbach 2012), suggesting that settlement habitats impart variable mortality or that environmental cues in the water column above suitable settlement habitats may mediate larval behavior. Both explanations likely factor into larval recruitment success, and the second explanation has been a continuing source of interest to larval ecologists. Indeed, larval oysters have long been known to use chemical cues released by adult oysters to initiate settlement (Tamburri et al. 1996), and more recent work suggests a possible role of acoustic signatures typical of oyster reefs (Lillis et al. 2013). Additionally, oyster larvae appear to respond to turbulence with a range of behaviors: larval eastern oysters have been reported to increase downward swimming (Fuchs et al. 2013), upward swimming (Wheeler et al. 2013) and diving (Wheeler et al. 2015) with changes in local flow conditions.

Whether light plays a role in the regulation of larval oyster swimming and settlement behavior remains unclear. Larval oysters are negatively buoyant and need to swim upwards to

maintain position in the water column, exhibiting negative gravitaxis and possibly positive phototaxis (Hidu and Haskin 1978, Kennedy 1996). Responses to light have been widely reported in larvae of other marine groups such as gastropods (Bingham and Young 1993), crustaceans (Forward and Cronin 1980, Wu et al. 1997), and ascidians (Svane and Young 1989, Vazquez and Young 1998). Further, responses to light vary with ontogeny (Young and Chia 1982, Vazquez and Young 1998). Oyster larvae may exhibit ontogenetic switching in phototactic responses: while early stage larvae remain high in the water column, late-stage pediveligers that are competent to settle into a benthic habitat could potentially display negative phototaxis to move downward in the water column. It is unclear at present if light influences settlement success and metamorphosis in larval oysters; confounding effects such as temperature and turbidity may account for contradictory results in the literature (see Kennedy (1996) for review).

As addressed above, most investigations of larval behavioral changes in light focus on vertical swimming direction as a positive or negative phototactic response. A less well studied question is whether other non-directional characteristic behaviors of larvae change significantly with light, as these responses can likewise affect larval positioning in the water column. Competent larval oysters are especially useful for investigating this question, due to distinct behaviors such as helical swimming (exploratory corkscrew swimming trajectories) and diving (transient rapid downward acceleration) that can be readily observed and compared between light and dark regimes. Our study aims to quantify the swimming responses of oyster pediveligers to light, and determine whether these responses vary over a range of turbulence conditions typical of their natural coastal environment. This dual-factor approach allows us to explore turbulence

thresholds of light-induced behaviors, and to evaluate whether particular larval responses might occur more commonly in day or night time conditions. We also investigate the potential utility of light as a cue to enhance settlement success in larvae. Larval behavior is quantified by observing the proportion of larvae: 1) swimming upward, 2) diving, and 3) swimming helically. Larval vertical swimming is of interest because it provides a broad indicator for active settlement. Diving is an active behavior that larvae may use for either settlement or predation escape (e.g., Finelli and Wethey 2003, Wheeler et al. 2015), whereas helical swimming may be used in exploration or feeding (e.g., Jonsson et al. 1991, Visser 2007).

2 Methods

2.1 Larval Culture

Larval eastern oysters used for this experiment were obtained from the Aquaculture Research Corporation in Dennis, Massachusetts at a size of 200-300 μm. A domestication effect from rearing larvae from multi-generation brood stock cannot be ruled out, but the benefits of commercial larvae instead of wild-caught larvae include their good-health, known history, and availability in large quantities (10^5-10^6 larvae). Larvae were maintained in 3 μm-filtered, aerated seawater at ambient field temperature (20-22 °C) and salinity (33 psu), in covered 16 L plastic buckets. Larvae were kept at low densities (< 3 larvae mL^{-1}) to minimize interactions and harmful metabolite build-up (Helm et al. 2004) and fed daily a suspension of haptophyte *Isochrysis* sp. (~9 x 10^5 cells mL^{-1} in filtered seawater). Experimental trials were conducted

within 2 days of larval acquisition, during which > 80% of the larvae were observed to have eyespots (a common indicator of competency, Thompson et al. 1996).

2.2 Experimental setup

The experiments were conducted in a grid-stirred turbulence tank (44.5 x 44.5 x 90 cm; described in Wheeler et al. 2013), filled with 3 μm-filtered seawater at ~20 °C, in a temperature-controlled chamber at 20 °C. The two horizontal grids, separated vertically by 45 cm, were constructed of 1 x 1 cm acrylic bars spaced 5 cm apart. The grids were attached to a drive rod that oscillated them vertically in phase with an amplitude of 5 cm at a specified frequency. While grid-stirred turbulence lacks the strong vertical shear of the bottom boundary layer, it is a good system for characterizing larval behavior >10 cm above the bottom and investigating responses in the absence of large scale velocity gradients. In the light treatment, our visible light source (2700K, PAR of 40.93 $\mu E\ m^{-2}\ s^{-1}$ at water surface) was placed on top of the tank and directed downwards to emulate the direction of light experienced by larvae in nature. This irradiance is characteristic of larval phototaxis studies (e.g., Forward and Cronin 1980, Bingham and Young 1993, Fuchs and Dibacco 2011) although likely lower than would be experienced by larvae in the field (Frouin et al. 2012).

For each experimental trial, larvae were gently introduced into the tank at densities of 0.36-0.6 larvae mL^{-1}. The tank was then seeded with neutrally buoyant polystyrene particles (3.0-3.4 μm diameter, Spherotech) to a density of ~ 4.2×10^4 particles mL^{-1} for flow characterization by particle image velocimetry (PIV). A monochrome high-speed camera (Photron Fastcam SA3, 1024 x 1024 pixel resolution), was focused on a ~ 3 x 3 cm field of view

149 in the center of the tank, equidistant from the grids. Larval diameters were approximately 2
150 orders of magnitude smaller than the dimensions of the field of view, where individual larvae
151 were ~10 pixels wide. A near-infrared laser (Oxford Lasers, Firefly 300W, 1000Hz, 808 nm),
152 oriented perpendicularly to the camera, illuminated the field of view with a laser sheet unaffected
153 by the presence or absence of visible light. The e-folding depth of the laser sheet was
154 approximately 1mm and the detection depth of the sheet for clear imaging of the large, bright
155 larvae was approximately 2.5mm.

156 The larvae were subjected to either dark or light conditions under 5 turbulence levels,
157 ranging from unforced flow ($\varepsilon = 0$ cm^2 s^{-3}) and low turbulence ($\varepsilon = 0.002$ cm^2 s^{-3}) to conditions
158 similar to coastal estuarine zones ($\varepsilon = 0.4$ cm^2 s^{-3}), with energy dissipation rates estimated as in
159 Wheeler et al. (2013). After larvae and particles were introduced, the tank was permitted a 20-
160 minute relaxation period, with the still water (unforced) treatment conducted after this period.
161 Video sequences, recorded at 60 frames per second, were collected for each turbulence level.
162 These video sequences ranged from 135 s total duration in the highest turbulence level to 225 s
163 duration in the lowest (where larval paths through the field of view were least frequent). In each
164 turbulence level, the record was broken into 45-s intervals, separated by 5 min, to allow the
165 camera to download the images.

166 Four replicate trials for the light and dark conditions, each with a separate batch of
167 larvae, were conducted by cycling through all 5 turbulence levels. The turbulence levels were
168 sequenced in a different order, in a Latin square configuration post-unforced flow, in each trial
169 (Table 1, Table S1) to reduce possible confounding temporal effects.
170

171 **2.3 Local flow subtraction to isolate larval swimming velocities**

172

173 Larval swimming velocity was calculated for each individual by subtracting local flow
174 velocity from the larval motion at each step in the recorded larval trajectory. The essentials of
175 this procedure are described here; full detail is presented in Wheeler et al. (2013). To track larval
176 motion, larval centroid positions were first identified in each frame using custom LabVIEW
177 (National Instruments) software with user-specified tolerances on larval size and pixel intensity.
178 Larvae were then tracked from frame to frame using a custom MATLAB script with a specified
179 maximum search radius in subsequent frames, and frame-to-frame instantaneous velocities were
180 thereby calculated.

181

182 To calculate flow velocities local to larvae, flow fields first were estimated using PIV
183 with DaVis v.7.2 (LaVision) software to a spatial resolution of ~0.04 cm and velocity vector
184 fields were imported into MATLAB. We identified annuli (inner radius ~0.04 and outer radius
185 ~0.2 cm) of flow vectors around each larva and averaged the flow velocity within each annulus.

186

187 To isolate larval swimming velocities, we subtracted the mean annulus flow velocity
188 from observed larval velocity at each time step for each larva. Individual instantaneous larval
189 swimming velocity time series were then used to compute the proportion of upward swimming
190 larvae. Individual mean larval velocities were computed by averaging instantaneous velocities
191 over the observed larval trajectory, and a larva was classified as upward swimming if its mean
192 vertical swimming velocity was positive.

193

194 **2.4 Identification of dives**

195

196 The dive response is a distinct behavior characterized by a rapid downward burst in
197 speed. Dives were identified using larval instantaneous vertical swimming velocity and
198 acceleration time-series, where acceleration was computed from the velocity time-series data
199 using a central difference scheme. A dive was characterized by a sudden (within 1/30 s) drop in
200 vertical velocity, typically lasting approximately one second, during which time the larva slowed
201 its descent and eventually reached near-zero vertical velocity (Fig. 1a). Larval trajectories were
202 classified as dives if they reached an instantaneous acceleration of 3.0 cm s^{-2} (~100 body lengths
203 s^{-2}) for more than one time step (1/60 s), and achieved an instantaneous negative vertical velocity
204 of at least -0.4 cm s^{-1}.

205

206 **2.5 Identification of helical swimming**

207

208 The corkscrew shaped path of helically swimming larvae results in a near-sinusoidal
209 curve in horizontal velocities with respect to time. We searched for occasions of helical
210 swimming by detecting sinusoidal-like motion in time-series of larval horizontal swimming
211 velocities. A larva was categorized as helically swimming if it contained at least one sinusoidal
212 peak in horizontal velocity or corrected horizontal position (in which corrected horizontal
213 position was numerically integrated from swimming velocity time series, in order to strip the
214 effects of flow on position). These peaks were determined by visual inspection, and were only
215 accepted as part of a helix if they exhibited a minimum horizontal velocity magnitude of 0.05 cm
216 s^{-1} (Fig. 1b).

217

2.6 Analysis of behavioral data

The effects of light and turbulence on the proportion of upward swimming larvae were analyzed in the turbulence regimes and the unforced regime using two separate general linear models. Data were separated into unforced and turbulence analyses because the unforced observations were taken prior to any turbulence observations in all trials, and the turbulence treatments between the trials were amenable to a Latin squares analysis. The purpose of the analysis was to detect effects of light and turbulence, as well as their interaction, on vertical swimming, but we also incorporated unavoidable potential influences on larval behavior, including larval age and time spent in the tank. Within each trial, we assume our estimates for vertical swimming in each turbulence treatment were independent, as the total number of larvae in the tank in each trial (~5 – 10 x 10^4) was several orders of magnitude larger than the number of trajectories observed (Table 1, Table S1). Further, the time delay between each video observation within a turbulence treatment increased the likelihood that new larvae were constantly being observed.

The model for *Y*, the proportion of upward swimming larvae, for the turbulence regime data was

$$Y = \mu + \text{light} + \text{turb.level} + \text{turb.level} \times \text{light} + \text{trial(light)} + \text{time} + \text{time} \times \text{light} + \text{error}.$$

Here μ and error denote the mean and normally distributed error, respectively. The model terms of primary interest consist of “light”, denoting light versus dark tank conditions, and “turbulence level”, denoting tank oscillating grid frequency. “Trial” denotes the replicate tank fill (4 in total

241 for each light regime) which also unavoidably encompasses larval aging, due to the time required
242 to conduct the full experiment (approximately 12 hours). "Time", denotes the variable
243 controlling the turbulence treatment order (that is, each turbulence level occurred at a different
244 time within each trial, as the turbulence levels were reordered for each new trial).

245 The model for the unforced regime data was

246

247 $$Y = \mu + \text{light} + \text{trial(light)} + \text{video seq.} + \text{light} \times \text{video seq.} + \text{error.}$$

248

249 In this model, the turbulence and time factors are no longer applicable, but an additional factor
250 "video sequence", was added, which specifies the 45s segment in a full set of video sequences
251 within a turbulence level. This factor was only considered in the unforced model because higher
252 flow regimes used different numbers of video sequences in each turbulence level (Table 1, Table
253 S1). The non-standardized number of video sequences was by design, in order to obtain a more
254 similar number of larval trajectories in each turbulence regime: fewer larval trajectories were
255 observed in lower turbulence treatments and hence more video sequences were taken.

256

257 Light (2 levels), turbulence level (4 levels), trial (4 levels), video sequence (4 levels), and
258 time (4 levels) were categorical variables, and light was tested between trials in the light and
259 dark treatments. Other effects were fixed and tested with the mean squares error of the ANOVA
260 within the light and dark treatments individually.

261

262 The proportion of diving larvae in turbulence was also analyzed using the turbulence
263 general linear model. The proportion of helically swimming larvae was tested using a modified

analysis, as helical swimming was only identified in the unforced and lowest forcing regime. This is due to the inherent challenge of identifying a multi-second behavioral pattern (a full helical period) when larvae are rapidly advected through the field of view in more highly turbulent flow. The unforced and low forcing regimes, in contrast, have individual larval trajectories sufficiently long to identify the helical swimming motion. For helix data, analysis on each variable was done with a split plots design with light as the main factor, trials nested within light and turbulence as the subplot factor. In addition to the proportion of helically swimming larvae, we also applied this model to two relevant characteristics of helix geometry: 1) vertical translational velocity (mean vertical swimming speed during an identified half helix) and 2) helix speed (instantaneous swimming speed averaged over a half helix period, or as long as the helix remained in the field of view).

In all analyses, the proportional behavioral metrics were not transformed as no transformations tested increased model fit. Residual analysis further determined that the general linear model was appropriate for our analysis. Factors deemed significant from the ANOVAs were compared post-hoc using Tukey HSD tests for least squares means of behavioral metrics.

3 Results

3.1 Vertical swimming

285 In both light and dark, larvae generally swam downward in the unforced flow regime,
286 upward in moderate turbulence, and displayed decreased upward swimming in high turbulence
287 (Fig. 2). This effect of turbulence was significant in the ANOVA (Table 2) and the post-hoc
288 tests (Table S2). In the turbulence regimes, light had no significant effect on upward swimming,
289 either by itself, or in interaction with time or turbulence level (Table 2), which suggests that
290 larvae did not respond phototactically.
291
292 In the turbulent regimes, time (i.e., treatment order within a trial), and trial also had a
293 significant effect on upward swimming (Table 2). Larvae exhibited decreased upward
294 swimming in turbulence levels occurring later in the treatment order, regardless of what these
295 turbulence levels happened to be (Table S3). This could be a consequence of a larval response to
296 an aggregative turbulence cue, acclimation to the tank, or fatigue. Upward swimming decreased
297 over the full experimental time period (Fig. 2, dark to light points), with larvae in later trials
298 generally exhibiting less upward swimming than earlier trials.
299
300 In the unforced flow regime, light had no effect on the proportion of upward swimmers
301 (Table 3). In contrast to the turbulence regimes, trial had no significant effect on upward
302 swimming. Video sequence did have a significant effect but it was difficult to interpret. One
303 might reasonably expect video sequence number to act as a proxy for time spent in the tank.
304 However, in examining each trial, there was no robust temporal pattern in upward swimming
305 over the full range of video sequences, and the post-hoc comparison test showed no pairwise
306 significant difference between sequences (Table S4).
307

3.2 Diving

We observed 367 dives in total, predominantly in the unforced and low turbulence regimes. The proportion of dives was distinctly and consistently higher in the light than dark regime, across all turbulence levels (Fig.3a), but the effect was not statistically significant (Table 4). The difficulty in ascertaining a light response may be due to the low power of the test and a significant variability among trials. The proportion of dives differed significantly between turbulence levels (Table 4), where the proportion of dives was highest in the lowest turbulence treatment and decreased with increasing turbulence (Fig. 3a, Table S5). Further, trial had a significant effect on diving (Table 4), with the proportion of dives increasing in the later trials.

While light alone was a (borderline) non-significant factor for diving, it interestingly was a significant effect in conjunction with time (Table 4). In the fourth (and last) turbulence treatment administered within a trial, the proportion of diving larvae was higher in light than in dark (Fig. 3b, Table S6); that is, light became a significant effect at the end of the series of turbulence treatments within a trial. Like with upward swimming, larvae appear to dive in response to an aggregative turbulence cue; in contrast with upward swimming, it also requires a light cue.

3.3 Helical swimming

Helical swimming was more common in light than dark treatments, but the difference was non-significant (Fig. 4a, Table 5). Turbulence negatively affected helical swimming, as a

331 significantly smaller proportion of larvae swimming helically was observed in the low forcing
332 regime than in the unforced regime (Fig 4a, Table 5). Trial had no impact on helical swimming,
333 and the interactive effect of light and turbulence was also non-significant (Table 5). The
334 decreased proportion of helical swimmers in turbulence appears to be a behavioral response, and
335 not solely an effect of decreased detection as larvae are advected more rapidly through the thin
336 laser sheet in the low forcing regime. Using PIV data from the unforced and low forcing
337 regimes, we estimated average horizontal root mean square (rms) flow velocities of $v_{\mathrm{rms}} = 0.04$
338 cm s^{-1} and 0.11 cm s^{-1}, respectively, and average flow autocorrelation timescales of $\tau = 7.2$ s and
339 3.6s. Over the average time it took to visually identify a helix (~1.5s), estimated ballistic
340 displacements of larvae by turbulent fluctuations were 0.06cm and 0.16cm in the unforced and
341 low forcing regimes, respectively. As these length scales are smaller than the depth of the laser
342 sheet for larval imaging (0.25cm), the helical trajectories are not likely to be systematically
343 undetected in low intensity turbulence. Nevertheless, decreased detection may play a small role
344 in the result and would certainly be exacerbated in more turbulent flow regimes.
345
346 While light did not impact the overall proportion of larvae swimming helically, it did
347 affect the mean helix speed, with borderline significance (Fig. 4b, Table 6). Turbulence did not
348 affect helix speed, nor did the interaction between turbulence and light (Table 6). Isolating and
349 testing the translational velocity (the vertical helical swimming velocity) yielded no effect of
350 light (Fig. 4c, Table 7), but a significant turbulence effect and interactive effect of light and
351 turbulence (Fig. 4c, Table 7). Overall, helically swimming larvae swam faster in light than
352 darkness, and vertical translational helical velocity increased with turbulence (Table S7).
353

4 Discussion

We found no evidence of direct phototaxis in competent-to-settle oyster larvae, as larvae exhibited no change in vertical directional swimming in either unforced flow or across a range of turbulence regimes. In contrast, both turbulence and larval age had strong impacts on larval swimming direction. We found that larvae exhibited distinct increases in dive frequency and increased speed in exploratory helical swimming behavior in the presence of light, suggesting that light encourages specialized exploratory, settlement, and predator-avoidance behavioral modes. Diving and helical swimming were less common in increased turbulence, suggesting a competing effect between light and turbulence in regulating these behaviors.

The effects of light on swimming behavior have several implications for larval ecology, specifically relating to settlement and predator-avoidance. Diving is an active downward acceleration that may enhance settlement (Fuchs et al. 2013, Wheeler et al. 2013, Wheeler et al. 2015); larval diving responses occur in the range of turbulence regimes consistent with flow several centimeters above rough bottom topographies (Wheeler et al. 2015, Pepper et al. 2015). Oyster larval settlement in the field has historically been observed to be higher during daylight hours (Medcof 1955); our observations suggest that diving was enhanced by the combined effects of light and an aggregative turbulence cue, wherein larvae in the light regime dove more frequently in the fourth and last turbulence regime experienced, regardless of turbulence intensity. Increased diving in response to a combined light and turbulence cue may help larvae in navigating flow fields over their preferred rough bottom settlement sites and in encountering

377 said sites during their preferred daylight settlement times. From an anti-predatory perspective,
378 many predators of larval invertebrates use visual cues to detect their prey (Iwasa 1982), and so
379 increasing predator-avoidance behaviors in light versus dark would be a useful survival strategy.
380 Indeed, oyster larvae dive more frequently when exposed to anomalously high local fluid
381 acceleration (Wheeler et al. 2015), which larvae may interpret as the presence of a suction
382 feeding predator (Kiørboe et al. 1999, Jakobsen 2001). Similarly, helical swimming may also act
383 as a predator-avoidance response while simultaneously allowing larvae to feed and explore the
384 water column: helical swimming clears large foraging volumes while presenting a minimal
385 hydromechanical presence to predators (Visser 2007). The increased occurrence of diving and
386 helical swimming in light may reflect the larval response to an increased predation risk during
387 daylight hours.
388
389 Alternatively, helical swimming may increase the precision of navigation during
390 directional swimming such as phototaxis, as demonstrated in simulations of annelid swimming
391 (Jékely et al. 2008, Jékely 2009). While no phototactic response is obvious in the proportion of
392 upward swimming larvae in our study, the change in helical swimming characteristics in light
393 demonstrates a photokinetic behavior of potential benefit to a directionally swimming larva.
394 Such results indicate the importance of considering multiple swimming metrics when
395 quantifying a behavioral response.
396
397 Moreover, the change in helix speed and vertical translational velocity in response to
398 light indicates that larval oysters have active control over helical swimming behavior. The
399 observation that helical swimming persists in turbulence indicates a robust larval control of

swimming, even in more energetic flows. Such control does not appear to be dictated by morphology alone, as commonly observed in some echinoid species (Chan and Grünbaum 2010); larval oysters display flexibility in their helix translational and angular velocity in response to environmental cues.

Light had no effect on the proportion of upward swimming larvae, which was surprising because we had expected to see some phototactic response in directional swimming. Despite the long-established prevalence of positive phototaxis in larvae (Thorson 1964) and observations of positive phototaxis in younger oyster larvae (Kennedy 1996), light had no observable effect on vertical swimming direction of our late-stage larvae. Our results demonstrate that oyster larvae may undergo a shift from positive to neutral phototaxis with age. Such ontogenetic changes in phototaxis have been widely documented in larvae of eels (Yamada et al. 2009), polychaetes (Young and Chia 1982, McCarthy et al. 2002), crabs (Forward and Costlow 1974), mussels (Fuchs and DiBacco 2011), nudibranchs (Miller and Hadfield 1986), conch (Barile et al. 1994), and both larval and juvenile sole (Champalbert et al. 1991). Competent larvae may cease to display positive phototaxic behavior because they no longer need to stay high in the water column. Further studies comparing phototactic behaviors of oyster larvae at various stages of development would be required to better characterize such an ontogenetic shift. A caveat to consider from our analyses, however, is the strong effect of time on larval swimming. Larvae exhibited considerable behavioral shifts over the full experimental time scale, and inter-trial variability may have masked an effect of light on upward swimming behavior.

An intriguing, though unexpected, result of our study was the strong effect of larval age on vertical swimming direction and dive frequency (through the trial variable). The full experimental time scale encompassed approximately 12 hours, during which the competent larvae persisted in culture and demonstrated all signs of good health. Our results suggest that over the competency window, larval behavior can change significantly, potentially impacting settlement success. In both light and dark, older larvae were less likely to swim upward in turbulence than younger larvae, which might help older larvae to passively encounter settlement sites. We speculate that the "young" competent-to-settle larvae in our experiment persisted in upward swimming because the environmental signals they experienced (light, turbulence) did not impart a strong settlement cue. The "desperate larva hypothesis", first proposed for lecithotrophic larvae, suggests that young competent larvae demonstrate strong selectivity in responding to potential settlement cues, but their finite energy supplies will ultimately force them to accept sub-standard settlement cues (Knight-Jones 1951). An extension of this hypothesis for planktotrophic larvae (like oysters) suggests that a reduced capacity to maintain the competent larval swimming state over time induces larvae to settle in the absence of preferred settlement cues (Botello and Krug 2006, Bishop et al. 2006). In the framework of this hypothesis, turbulence might act as a sub-standard settlement cue for oyster larvae, prompting newly competent larvae to persist in swimming while older larvae cease swimming in flow. Alternatively, the ontogenetic shift in vertical swimming could be due to energetic constraints of swimming in turbulence. Oyster larvae continue to grow throughout the competency period (Wheeler, *unpub. data*) and older, heavier larvae may reach the point where swimming in turbulence is energetically unfeasible. Because larvae are negatively buoyant, they will passively sink in the water column once they cease swimming, and as such the observed decrease

445 in upward swimming over the experimental period may be explained by the passive sinking of
446 older, heavier larvae. However, our observations demonstrate that upward swimming over time
447 only changes in turbulence, suggesting that it is a combination of turbulence and age, and not
448 merely age, which induces changes in larval swimming; in the unforced flow regime, older
449 competent larvae exhibited similar responses to newly competent larvae. In fact, both
450 interpretations (turbulence acting as a settlement cue or as an energetic constraint to swimming)
451 are supported by the observation that the effects of aging only impacted larvae swimming in
452 turbulence. The effect of age during the competency window on larval behavior may
453 furthermore explain previous conflicting results on larval oyster responses to turbulence
454 (Wheeler et al. 2013, Fuchs et al. 2013). Our results give a strong indication that ontogeny
455 should be more carefully considered in larval behavioral studies; while ontogenetic changes
456 across multiple larval stages are commonly studied, a focus on within-stage ontogenetic change
457 is rare.
458
459 Our study further strengthens the body of evidence documenting upward swimming of
460 larval oysters in moderate turbulence (Wheeler et al. 2013), which suggests that turbulence alone
461 does not act as a cue for larval settlement (with a possible exception for older, heavier, and/or
462 less selective larvae). Similarly, we observed no change in the proportion of larvae which swam
463 upward in response to light; the absence of an obvious negative phototactic response suggests
464 that neither light alone, nor light in conjunction with turbulence, are effective inducers of larval
465 settlement at a population level. Nevertheless, the observed changes in larval diving and helical
466 swimming in the presence of light suggest that they modify potentially exploratory and anti-
467 predatory behaviors in light versus darkness. Although light does not modify larval vertical

swimming direction on a population level (indicative of an active settlement response), it does induce behavioral changes in individuals. This shift towards exploratory swimming and rapid downward responses in light is consistent with, and offers a potential behavioral mechanism for, the enhanced settlement observed in the field during daylight hours. Ultimately, the importance of environmental cues to larval survivorship and settlement may only become clear when observing the effects of multiple drivers (like light and turbulence) on a range of larval behaviors throughout an ontogenetic window.

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5 Figures

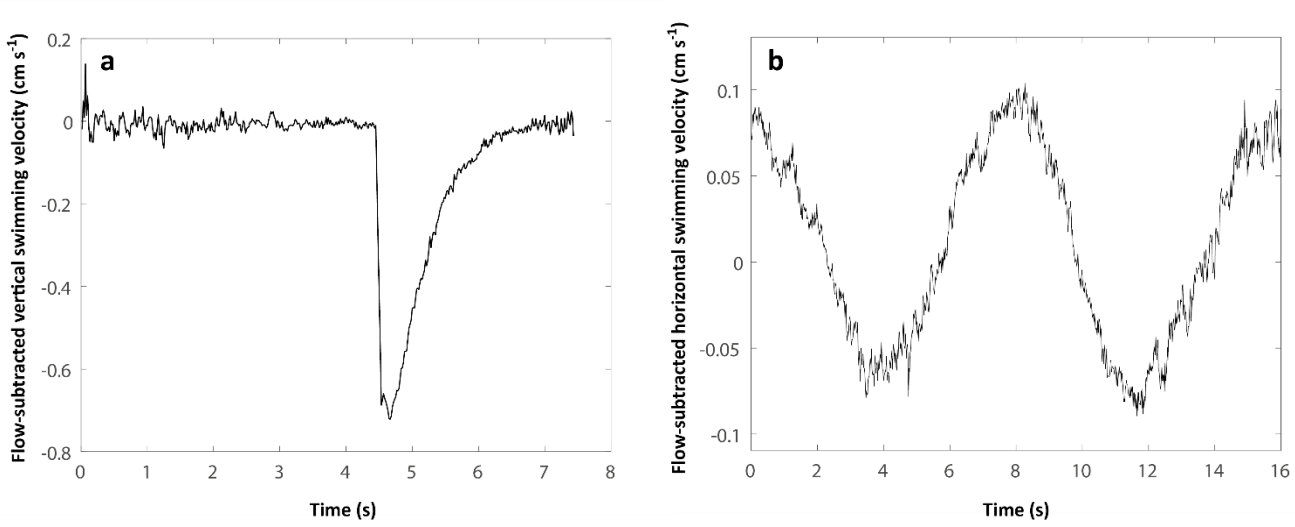
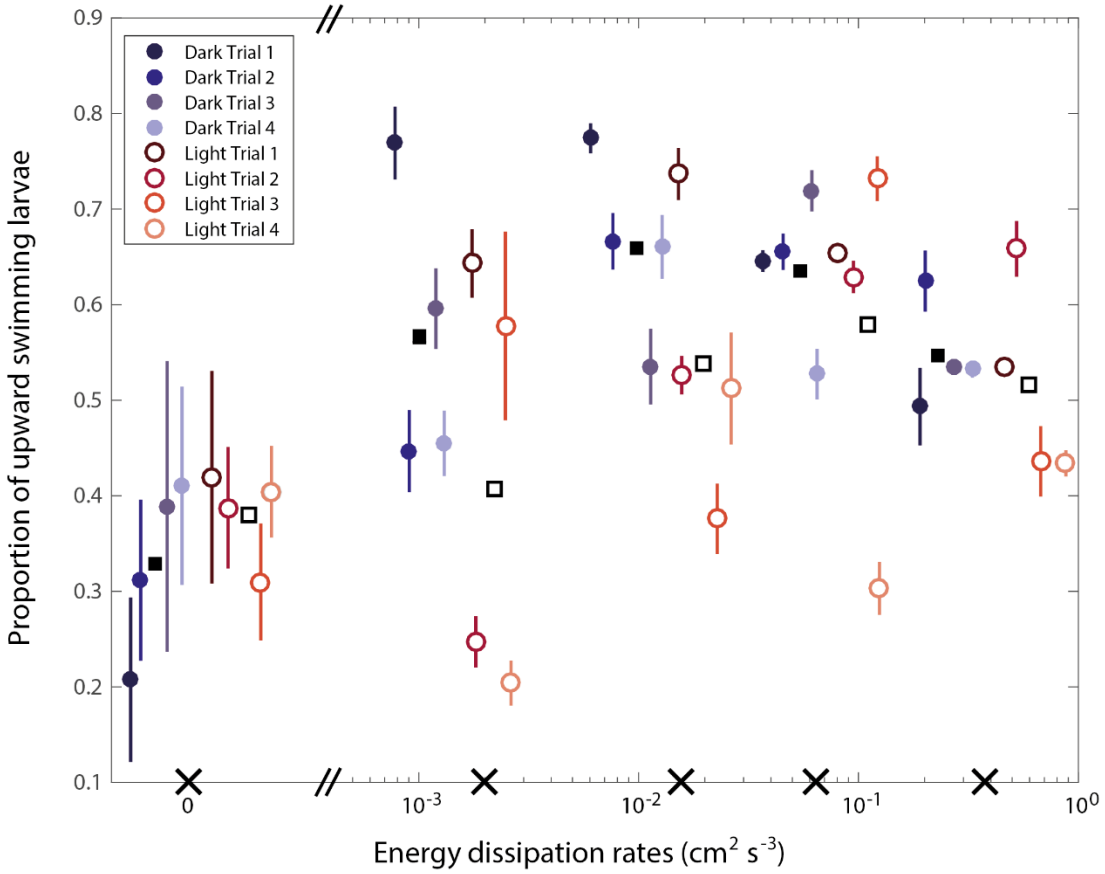


Figure 1: Distinct larval swimming behaviors observed in flow-subtracted larval swimming velocity time series at 60 frames per second, observed in a light, unforced flow regime. **(a)** Example of a dive, as characterized by a sudden drop in vertical velocity. In this instance, the dive occurs at ~4.5s, with the larva achieving a downward swimming velocity of -0.7 cm s^{-1}. **(b)** Example of a helically swimming larva, characterized by a sinusoid-like shape in horizontal velocity. The period of the oscillation has a wide larva-dependent range; the 8s period in this example is relatively long.



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512 **Figure 2:** Mean (±SE) proportion of upward swimming larvae for each trial vs. turbulence level
513 (energy dissipation rate), in dark (blue-toned closed circles) and light (red-toned open circles),
514 with trials 1-4 denoted for each by decreasing color intensity. Values at each turbulence level
515 (denoted by x on the energy dissipation rate axis) are grouped by dark and light and are offset
516 horizontally for clarity. Additionally, proportions are pooled across trial in both dark (black
517 closed squares) and light (black open squares): as trial was a significant effect on upward
518 swimming, these mean proportions are intended only to highlight the effects of light and
519 turbulence. Larvae displayed primarily downward swimming in the unforced flow regime, and
520 upward swimming in turbulence, although the prevalence of upward swimming decreased in
521 high turbulence. Light had no significant effect on directional swimming.

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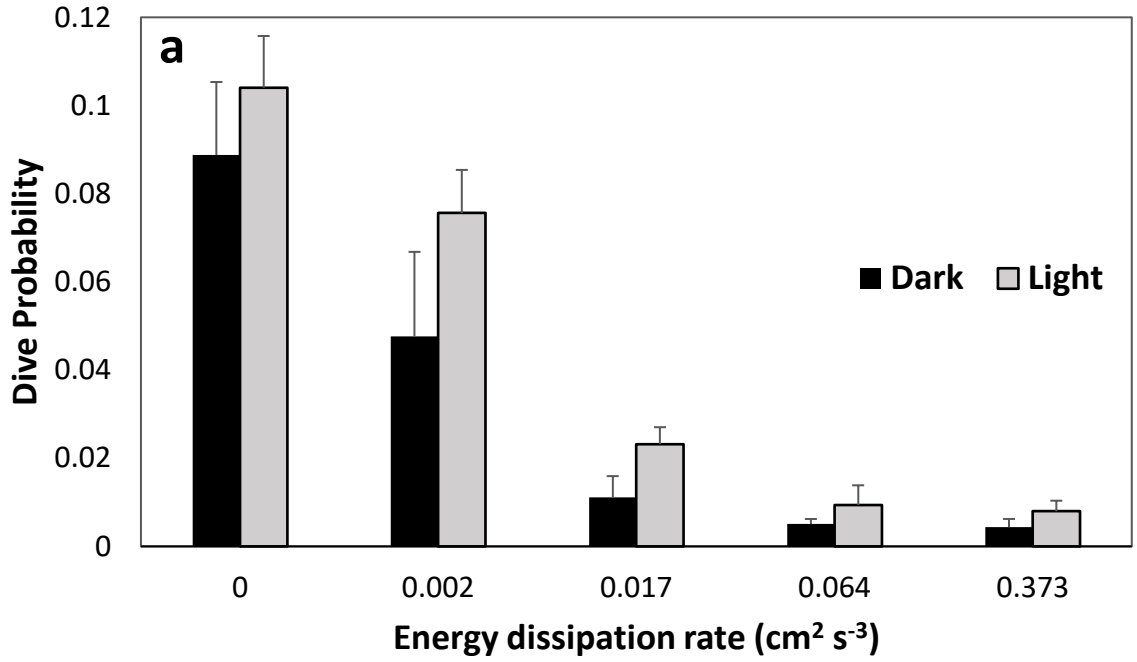

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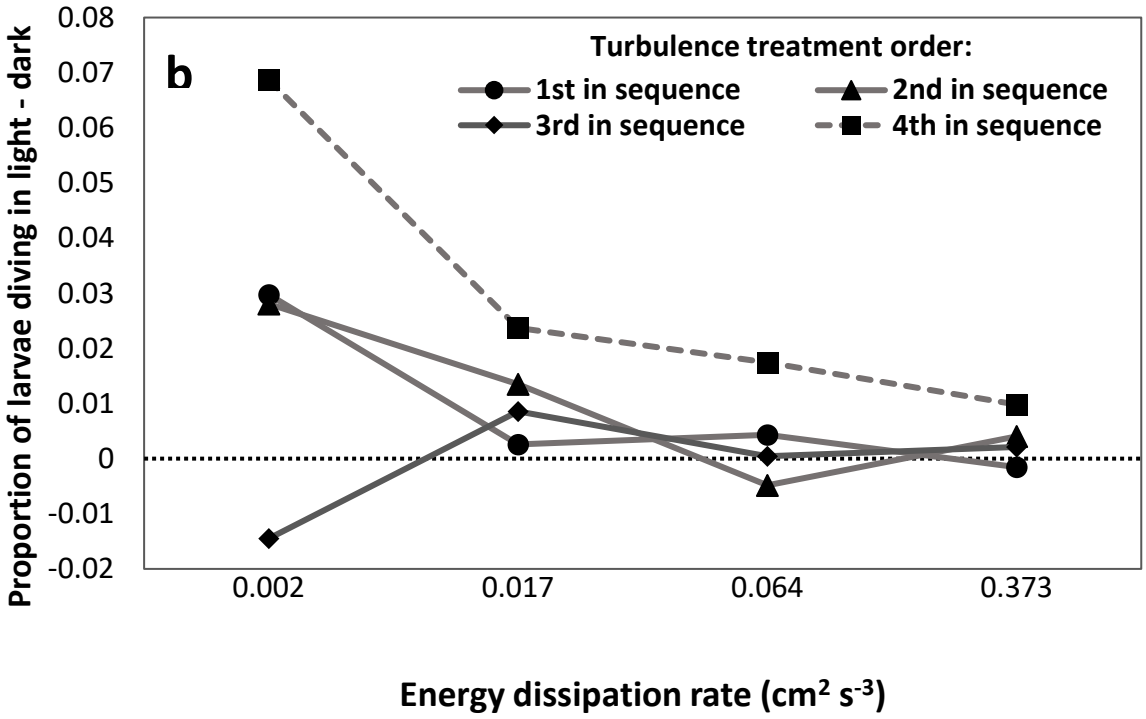

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529 **Figure 3: (a)** Proportion of larvae diving (± SE) with respect to turbulence regime, as
530 characterized by energy dissipation rate, in dark (black bars) and light (light grey bars), pooled

across trials. Larvae exhibited increases in diving in light versus dark treatments (albeit non-significant) and significant decreases in diving in increasing turbulence. Note that trial is also a significant factor for diving. Pooling across trial is done to highlight other effects and trials are not treated as replicates in the analysis; SE bars are calculated for this plot using trial means and indicate the inter-trial variability. **(b)** Difference in the proportion of larvae diving between light and dark regimes, where a positive proportion denotes higher dive proportion in light (i.e., points denote difference between grey and black bars in **(a)**), with respect to turbulence regime, sub-divided into turbulence treatment order. Treatment order denotes when in the sequence of four turbulence regimes a given turbulence regime fell. The dashed line denotes a significant interactive effect of treatment order and light: larvae in fourth and final turbulence treatment dive more frequently in light than dark, irrespective of what the turbulence level is, and what previous turbulence history they have experienced in the tank. Note that the lines connecting turbulence levels for each turbulence treatment order are for visual clarity in grouping treatment orders, not to imply quantitative interpolation.

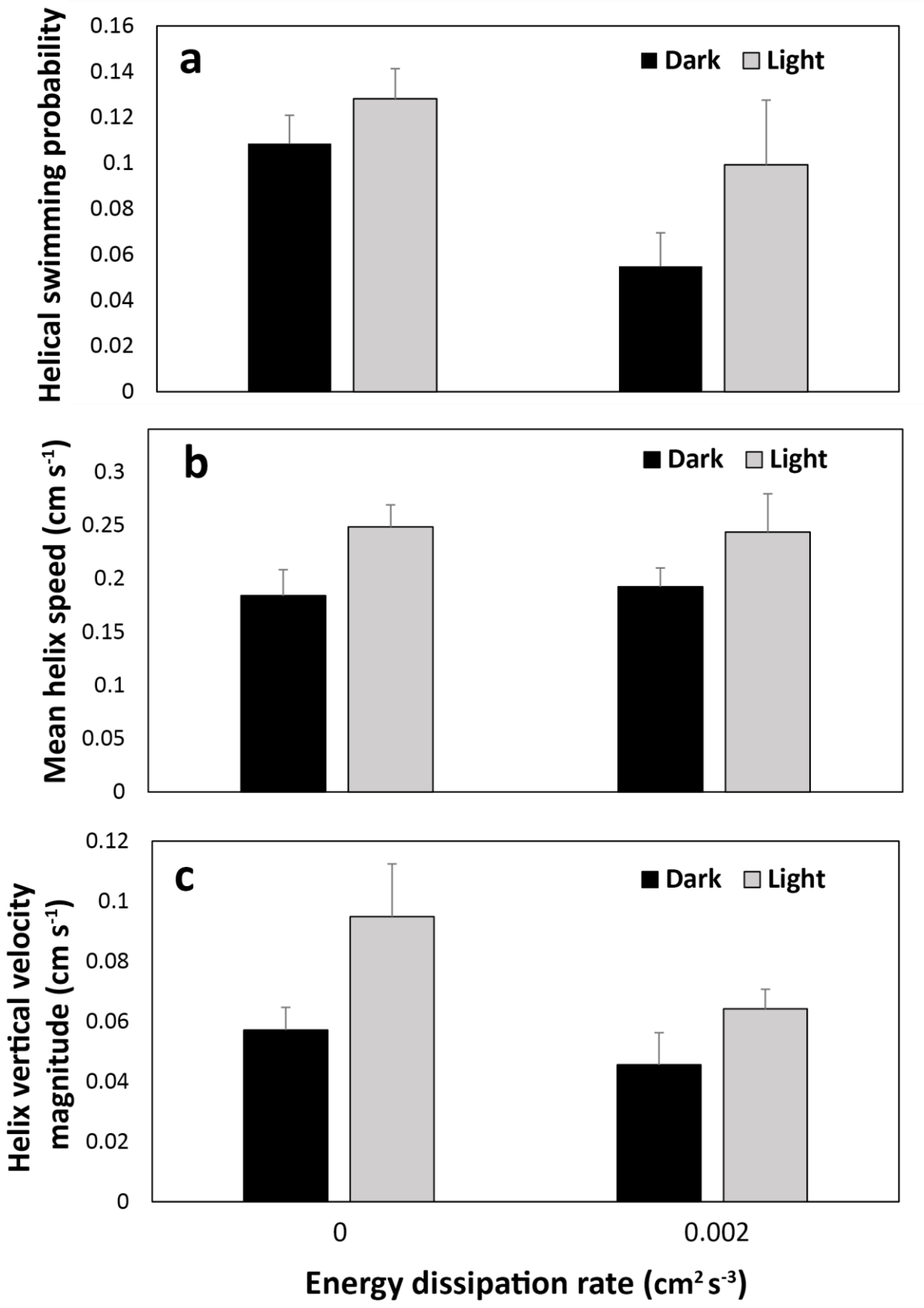
a
Dark
Light
Helical swimming probability
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
b
Dark
Light
Mean helix speed (cm s-1)
0.3
0.25
0.2
0.15
0.1
0.05
0
c
Dark
Light
Helix vertical velocity magnitude (cm s-1)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.002
Energy dissipation rate (cm2 s-3)

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Figure 4: (a) Proportion of larvae swimming helically, **(b)** helix speed, and **(c)** vertical translational velocity magnitude between unforced and low forcing turbulence regime ,as characterized by energy dissipation rate, in dark (black bars) and light (light grey bars), pooled across trials. Trials are not treated as replicates in the analysis, but pooling by trial is done here to highlight effects of light and flow on helical behavior. SE bars are calculated using trial means and indicate the inter-trial variability.

6 Tables

Table 1: Example of experimental design of Trial 4. Light treatments were randomized and turbulence orders were assigned by Latin square. Consult Supplemental Materials for complete experimental design of Trials 1-4 (Table S1).

Treatment	Energy dissipation rate (cm^2s^{-3})	# 45 s datasets	# Larvae tracked
Dark	0	5	168
Dark	0.027	4	512
Dark	0.373	3	368
Dark	0.002	4	207
Dark	0.064	3	390
Light	0	5	307
Light	0.027	4	465
Light	0.373	3	737
Light	0.002	4	230
Light	0.064	3	187

Table 2: Results of ANOVA for proportion of upward swimming larvae in forced flow, testing for effects of light, time (turbulence treatment order), turbulence level, and trial (aging). Significant results are bolded, with a significance level $\alpha = 0.05$.

Source	*df*	MS	*F*-ratio	*p*-value
Light	1	0.06	2	0.21
Time	3	0.08	43.59	**<0.001**
Turbulence level	3	0.02	12.35	**<0.001**
Time x Light	3	0.004	2.05	0.16
Turbulence level x Light	3	0.006	3.02	0.07
Trial (Light)	6	0.03	15.94	**<0.001**
Error	12	0.002		

Table 3: Results of ANOVA for proportion of upward swimming larvae in unforced flow, testing for effects of light, video sequence number, and trial (aging). Significant results are bolded, with a significance level $\alpha = 0.05$.

Source	*df*	MS	*F*-ratio	*p*-value
Light	1	0.02	0.95	0.36
Video sequence	3	0.1	3.2	**0.05**
Light x Video sequence	3	0.009	0.31	0.81
Trial (Light)	6	0.02	0.72	0.63
Error	18	0.03		

628 **Table 4:** Results of ANOVA for proportion of diving larvae in forced flow, testing for effects of
629 light, time (turbulence treatment order), turbulence level, and trial (aging). Significant results are
630 bolded, with a significance level $\alpha = 0.05$.
631

Source	***df***	**MS**	***F*-ratio**	***p*-value**
Light	1	0.01	4.32	0.08
Time	3	0.001	1.27	0.32
Turbulence level	3	0.04	50.34	**<0.001**
Time x Light	3	0.003	3.99	**0.03**
Turbulence level x Light	3	0.0009	0.94	0.44
Trial (Light)	6	0.003	3.53	**0.03**
Error	12	0.0009		

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634 **Table 5:** Results of ANOVA on split plots design for proportion of helically swimming larvae in
635 unforced and low forcing flow, testing for effects of light, turbulence level, and trial (aging).
636 Significant results are bolded, with a significance level $\alpha = 0.05$.
637

Source	***df***	**MS**	***F*-ratio**	***p*-value**
Light	1	0.004	2.01	0.20
Turbulence level	1	0.006	10.68	**0.02**
Turbulence level x Light	1	0.0006	0.96	0.36
Trial (Light)	6	0.002	3.22	0.09
Error	6	0.0006		

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640 **Table 6:** Results of ANOVA on split plots design for mean helix swimming speed in unforced
641 and low forcing flow, testing for effects of light, turbulence level, and trial (aging). Significant
642 results are bolded, with a significance level $\alpha = 0.05$.
643

Source	***df***	**MS**	***F*-ratio**	***p*-value**
Light	1	0.01	5.02	0.06
Turbulence level	1	0.00001	0.005	0.94
Turbulence level x Light	1	0.0002	0.068	0.80
Trial (Light)	6	0.003	1.03	0.48
Error	6	0.003		

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Table 7: Results of ANOVA on split plots design for mean helix vertical translational velocity in unforced and low forcing flow, testing for effects of light, turbulence level, and trial (aging). Significant results are bolded, with a significance level $\alpha = 0.05$.

Source	***df***	**MS**	***F*-ratio**	***p*-value**
Light	1	0.002	0.83	0.39
Turbulence level	1	0.01	16.4	**0.006**
Turbulence level x Light	1	0.005	6.72	**0.04**
Trial (Light)	6	0.002	3.98	0.06
Error	6	0.0007		

693 **Supplementary Materials**
694
695 **Table S1:** Experimental design: Light treatments were randomized and turbulence orders were
696 assigned by Latin square in an attempt to minimize the effects of time.
697

Trial	Treatment	Turbulence level (cm^2s^{-3})	Number of 45-second datasets	Number of larvae tracked
1	dark	0	5	69
1	dark	0.002	4	133
1	dark	0.027	4	464
1	dark	0.064	3	409
1	dark	0.373	3	379
1	light	0	5	91
1	light	0.002	4	191
1	light	0.027	4	450
1	light	0.064	3	566
1	light	0.373	3	510
2	light	0	5	298
2	light	0.373	3	896
2	light	0.064	3	967
2	light	0.027	4	767
2	light	0.002	4	323
2	dark	0	5	91
2	dark	0.373	3	376
2	dark	0.064	3	330
2	dark	0.027	4	424
2	dark	0.002	4	237
3	light	0	5	220
3	light	0.064	3	604
3	light	0.002	4	215
3	light	0.373	3	343
3	light	0.027	4	382
3	dark	0	5	68
3	dark	0.064	3	430
3	dark	0.002	4	210
3	dark	0.373	3	301
3	dark	0.027	4	398
4	dark	0	5	168
4	dark	0.027	4	512
4	dark	0.373	3	368
4	dark	0.002	4	207
4	dark	0.064	3	390
4	light	0	5	307
4	light	0.027	4	465

4	light	0.373	3	737
4	light	0.002	4	230
4	light	0.064	3	487

Table S2: Post-hoc Tukey's HSD test of differences in mean proportion of upward swimming larvae in flow, with respect to turbulence level, using least squares means and $df = 12$. Significant results are bolded, with a significance level $\alpha = 0.05$.

Turbulence levels ($cm^2\ s^{-3}$)	**Difference**	**p-value**	**95% CIS**
0.002 vs 0.027	-0.11	**0.002**	[-0.17, -0.04]
0.002 vs 0.064	-0.12	**0.001**	[-0.18, -0.05]
0.002 vs 0.373	-0.04	0.35	[-0.10, 0.02]
0.027 vs 0.064	-0.01	0.97	[-0.08, 0.06]
0.027 vs 0.373	0.07	**0.04**	[0.001, 0.133]
0.064 vs 0.373	0.08	**0.02**	[0.01, 0.14]

Table S3: Post-hoc Tukey's HSD test of differences in mean proportion of upward swimming larvae in flow, with respect to time within a trial, using least squares means and $df = 12$. Significant results are bolded, with a significance level $\alpha = 0.05$. The time comparisons here, *i* vs *j*, refer to the i^{th} and j^{th} times of four possible times in the treatment order.

Times	**Difference**	**p-value**	**95% CIS**
1 vs 2	0.05	0.19	[-0.02, 0.11]
1 vs 3	0.15	**<0.001**	[0.08, 0.22]
1 vs 4	0.23	**<0.001**	[0.17, 0.30]
2 vs 3	0.10	**0.003**	[0.04, 0.16]
2 vs 4	0.18	**<0.001**	[0.12, 0.25]
3 vs 4	0.08	**0.01**	[0.02, 0.15]

Table S4: Post-hoc Tukey's HSD test of differences in mean proportion of upward swimming larvae in unforced flow, with respect to video sequence number, using least squares means and $df = 18$. Significant results are bolded, with a significance level $\alpha = 0.05$.

Video Sequences	**Difference**	**p-value**	**95% CIS**
1 vs 2	0.08	0.81	[-0.16, 0.32]
1 vs 3	-0.12	0.47	[-0.37, 0.11]
1 vs 4	-0.16	0.30	[-0.40, 0.09]
2 vs 3	-0.20	0.12	[-0.45, 0.04]
2 vs 4	-0.23	0.07	[-0.48, 0.01]
3 vs 4	-0.02	0.99	[-0.27, 0.22]

719 **Table S5:** Post-hoc Tukey's HSD test of differences in mean dive proportion in flow, with
720 respect to turbulence level, using least squares means and $df = 12$. Significant results are
721 bolded, with a significance level $\alpha = 0.05$.
722

Turbulence levels (cm^2 s^{-3})	Difference	p-value	95% CIS
0.002 vs 0.027	0.12	**<0.001**	[0.07, 0.16]
0.002 vs 0.064	0.16	**<0.001**	[0.11, 0.21]
0.002 vs 0.373	0.17	**<0.001**	[0.12, 0.22]
0.027 vs 0.064	0.04	0.06	[-0.002, 0.09]
0.027 vs 0.373	0.05	**0.02**	[0.008, 0.10]
0.064 vs 0.373	0.01	0.92	[-0.03, 0.05]

723
724
725 **Table S6:** Post-hoc Tukey's HSD test of differences in mean dive proportion in flow, with
726 respect to interactive light and time within a trial, using least squares means and $df = 12$.
727 Significant results are bolded, with a significance level $\alpha = 0.05$. The time comparisons here, *i*
728 vs *j*, refer to the i^{th} and j^{th} times of four possible times in the treatment order, while 'L' and 'D'
729 refer to the light and dark treatments, respectively.
730

Light x Time	Difference	p-value	95% CIS
Dark Only			
D1 vs D2	0.02	0.95	[-0.06, 0.10]
D1 vs D3	-0.02	0.98	[-0.10, 0.06]
D1 vs D4	0.04	0.54	[-0.04, 0.12]
D2 vs D3	-0.04	0.55	[-0.12, 0.04]
D2 vs D4	0.02	0.98	[-0.06, 0.10]
D3 vs D4	0.06	0.17	[-0.02, 0.14]
Light Only			
L1 vs L2	0.01	0.99	[-0.07, 0.09]
L1 vs L3	-0.007	0.99	[-0.09, 0.07]
L1 vs L4	-0.04	0.56	[-0.12, 0.04]
L2 vs L3	-0.02	0.98	[-0.10, 0.06]
L2 vs L4	-0.05	0.31	[-0.13, 0.03]
L3 vs L4	-0.04	0.73	[-0.11, 0.04]
Dark vs Light			
D1 vs L1	-0.02	0.97	[-0.10, 0.06]
D1 vs L2	-0.01	0.99	[-0.09, 0.07]
D1 vs L3	-0.03	0.89	[-0.11, 0.05]
D1 vs L4	-0.06	0.15	[-0.14, 0.02]
D2 vs L1	-0.05	0.49	[-0.12, 0.03]
D2 vs L2	-0.03	0.77	[-0.11, 0.05]
D2 vs L3	-0.05	0.34	[-0.13, 0.03]
D2 vs L4	-0.09	**0.03**	[-0.17, -0.008]
D3 vs L1	-0.003	1.00	[-0.08, 0.07]
D3 vs L2	0.009	0.99	[-0.07, 0.08]

D3 vs L3	-0.01	0.99	[-0.09, 0.07]
D3 vs L4	-0.04	0.50	[-0.12, 0.03]
D4 vs L1	-0.06	0.14	[-0.14, 0.01]
D4 vs L2	-0.05	0.30	[-0.13, 0.03]
D4 vs L3	-0.07	0.09	[-0.15, 0.01]
D4 vs L4	-0.11	**0.006**	[-0.17, -0.03]

Table S7: Post-hoc Tukey's HSD test of differences in mean helix translational velocity in unforced and low forcing flow, with respect to interactive light and turbulence level, using least squares means and $df = 6$. Significant results are bolded, with a significance level $\alpha = 0.05$. The numbers associated with the turbulence levels are the reported energy dissipation rates for each flow regime (in $cm^2\ s^{-3}$), while 'L' and 'D' refer to the light and dark treatments, respectively.

Turbulence level x Light	**Difference**	**p-value**	**95% CIS**
D0 vs D0.002	-0.09	**0.01**	[-0.16, -0.02]
L0 vs L0.002	-0.02	0.74	[-0.09, 0.05]
D0 vs L0	-0.06	0.07	[-0.13, 0.007]
D0 vs L0.002	-0.08	**0.02**	[-0.14, -0.01]
D0.002 vs L0	0.03	0.45	[-0.10, 0.04]
D0.002 vs L0.002	0.01	0.94	[-0.06, 0.08]

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