Vol. 571: 109–120, 2017
https://doi.org/10.3354/meps12106

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 17

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

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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 3 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. At 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.

KEY WORDS: Larval invertebrate ecology · Larval swimming · Environmental cues · Hydrodynamics

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

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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 modeling (North et al. 2008, Metaxas & 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 FEORT 2007).

Previous studies have shown strong correlations between physical habitat and oyster larval recruitment (Fredriksson et al. 2010, Whitman & 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 & Haskin 1978, Kennedy 1996). Responses to light have been widely reported in larvae of other marine groups such as gastropods (Bingham & Young 1993), crustaceans (Forward & Cronin 1980, Wu et al. 1997), and ascidians (Svane & Young 1989, Vazquez & Young 1998). Further, responses to light vary with ontogeny (Young & Chia 1982, Vazquez & 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 whether 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 aimed 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 nighttime conditions. We also investigated 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 & Wethey 2003, Wheeler et al. 2015), whereas helical swimming may be used in exploration or feeding (e.g. Jonsson et al. 1991, Visser 2007).

MATERIALS AND METHODS

Larval culture

Larval eastern oysters used for this experiment were obtained from the Aquaculture Research Corporation (Dennis, MA, USA) at a size of 200 to 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. (~9×10^5 cells ml^{-1} in filtered seawater). Experimental trials were conducted within 2 d of larval acquisition, during which >80% of the larvae were observed to have eyespots (a common indicator of competency, Thompson et al. 1996).

Experimental setup

The experiments were conducted in a grid-stirred turbulence tank (44.5 × 44.5 × 90 cm; described by Wheeler et al. 2013), filled with 3 µm-filtered seawater at ~20°C, in a temperature-controlled chamber at 20°C. The 2 horizontal grids, separated vertically by 45 cm, were constructed of 1 × 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 µE 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 & Cronin 1980, Bingham & Young 1993, Fuchs & 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 × 1024 pixel resolution) was focused on a ~3 × 3 cm field of view in the center of the tank, equidistant from the grids. Larval diameters were approximately 2 orders of magnitude smaller than the dimensions of the field of view, where individual larvae were ~10 pixels wide. A near-infrared laser (Oxford Lasers, Firefly 300 W, 1000 Hz, 808 nm), oriented perpendicularly to the camera, illuminated the field of view with a laser sheet unaffected by the presence or absence of visible light. The e-folding depth of the laser sheet was approximately 1 mm, and the detection depth of the sheet for clear imaging of the large, bright larvae was approximately 2.5 mm.

The larvae were subjected to either dark or light conditions under 5 turbulence levels, ranging from unforced flow ($\varepsilon = 0$ cm^2 s^{-3}) and low turbulence ($\varepsilon =$ 0.002 cm^2 s^{-3}) to conditions similar to coastal estuarine zones ($\varepsilon = 0.4$ cm^2 s^{-3}), with energy dissipation rates estimated as in Wheeler et al. (2013). After larvae and particles were introduced, the tank was permitted a 20 min relaxation period, with the still water (unforced) treatment conducted after this period. Video sequences, recorded at 60 frames s^{-1}, were collected for each turbulence level. These video sequences ranged from 135 s total duration in the highest turbulence level to 225 s duration in the lowest (where larval paths through the field of view were least frequent). In each turbulence level, the record was broken into 45 s intervals, separated by 5 min, to allow the camera to download the images.

Four replicate trials for the light and dark conditions, each with a separate batch of larvae, were conducted by cycling through all 5 turbulence levels. The turbulence levels were sequenced in a different order, in a Latin square configuration post-unforced flow, in each trial (Table 1, and see Table S1 in the Supplement at www.int-res.com/articles/suppl/m571p109_supp.pdf) to reduce possible confounding temporal effects.

Local flow subtraction to isolate larval swimming velocities

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

To calculate flow velocities local to larvae, flow fields first were estimated using PIV with DaVis v.7.2

Table 1. Example of experimental design of Trial 4. Light treatments were randomized and turbulence orders were assigned by Latin square. See Table S1 in the Supplement at www.int-res.com/articles/suppl/m571p109_supp.pdf for complete experimental design of Trials 1 to 4

Treatment	Energy dissipation rate ($cm^2 s^{-3}$)	No. of 45 s datasets	No. of 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

(LaVision) software to a spatial resolution of ~0.04 cm, and velocity vector fields were imported into MATLAB. We identified annuli (inner radius ~0.04 and outer radius ~0.2 cm) of flow vectors around each larva and averaged the flow velocity within each annulus.

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

Identification of dives

The dive response is a distinct behavior characterized by a rapid downward burst in speed. Dives were identified using larval instantaneous vertical swimming velocity and acceleration time series, where acceleration was computed from the velocity time-series data using a central difference scheme. A dive was characterized by a sudden (within 1/30 s) drop in vertical velocity, typically lasting approximately 1 s, during which time the larva slowed its descent and eventually reached near-0 vertical velocity (Fig. 1a). Larval trajectories were classified as dives if they reached an instantaneous acceleration of 3.0 cm s^{-2} (~100 body lengths s^{-2}) for more than 1 time step (1/60 s), and achieved an instantaneous negative vertical velocity of at least −0.4 cm s^{-1}.

Identification of helical swimming

The corkscrew shaped path of helically swimming larvae results in a near-sinusoidal curve in horizontal velocities with respect to time. We searched for occasions of helical swimming by detecting sinusoidal-like motion in time-series of larval horizontal swimming velocities. A larva was categorized as helically swimming if it contained at least 1 sinusoidal peak in

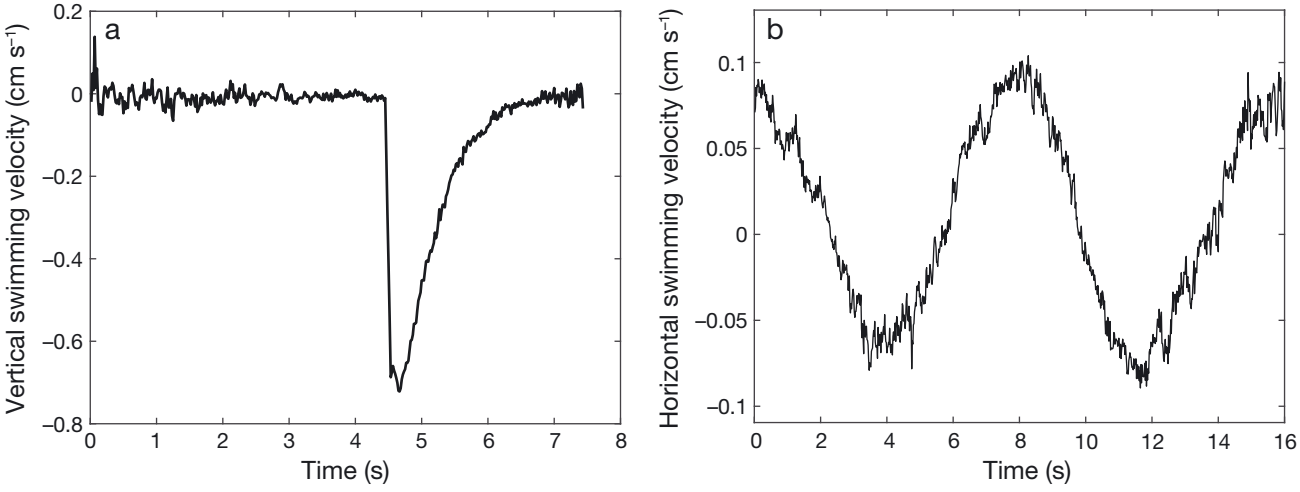

Fig. 1. Distinct eastern oyster *Crassostrea virginica* larval swimming behaviors observed in flow-subtracted larval swimming velocity time series at 60 frames s^{-1}, observed in a light, unforced flow regime. (a) A dive, as characterized by a sudden drop in vertical velocity. In this instance, the dive occurs at ~4.5 s, with the larva achieving a downward swimming velocity of −0.7 cm s^{-1}. (b) 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 8 s period in this example is relatively long

horizontal velocity or corrected horizontal position (in which corrected horizontal position was numerically integrated from swimming velocity time series, in order to strip the effects of flow on position). These peaks were determined by visual inspection, and were only accepted as part of a helix if they exhibited a minimum horizontal velocity magnitude of 0.05 cm s^{-1} (Fig. 1b).

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 2 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 assumed that 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 $\times$ 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

$$Y = \mu + \text{light} + \text{turb.level} + \text{turb.level} \times \text{light} + \text{trial(light)} + \text{time} + \text{time} \times \text{light} + \text{error} \quad (1)$$

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

The model for the unforced regime data was

$$Y = \mu + \text{light} + \text{trial(light)} + \text{video seq.} + \text{light} \times \text{video seq.} + \text{error} \quad (2)$$

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

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

The proportion of diving larvae in turbulence was also analyzed using the turbulence 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 2 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.

RESULTS

Vertical swimming

In both light and dark, larvae generally swam downward in the unforced flow regime, upward in moderate turbulence, and displayed decreased upward swimming in high turbulence (Fig. 2). This effect of turbulence was significant in the ANOVA (Table 2) and the post hoc tests (Table S2 in the Supplement). In the turbulence regimes, light had no significant effect on upward swimming, either by itself or in interaction with time or turbulence level (Table 2), which suggests that larvae did not respond phototactically.

Table 2. Results of ANOVA for proportion of upward swimming eastern oyster *Crassostrea virginica* larvae in forced flow, testing for effects of light, time (turbulence treatment order), turbulence level, and trial (aging). Significant results are in **bold**, with a significance level of α = 0.05

Source	df	MS	*F*	p
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 × Light	3	0.004	2.05	0.16
Turbulence level × Light	3	0.006	3.02	0.07
Trial (Light)	6	0.03	15.94	**<0.001**
Error	12	0.002		

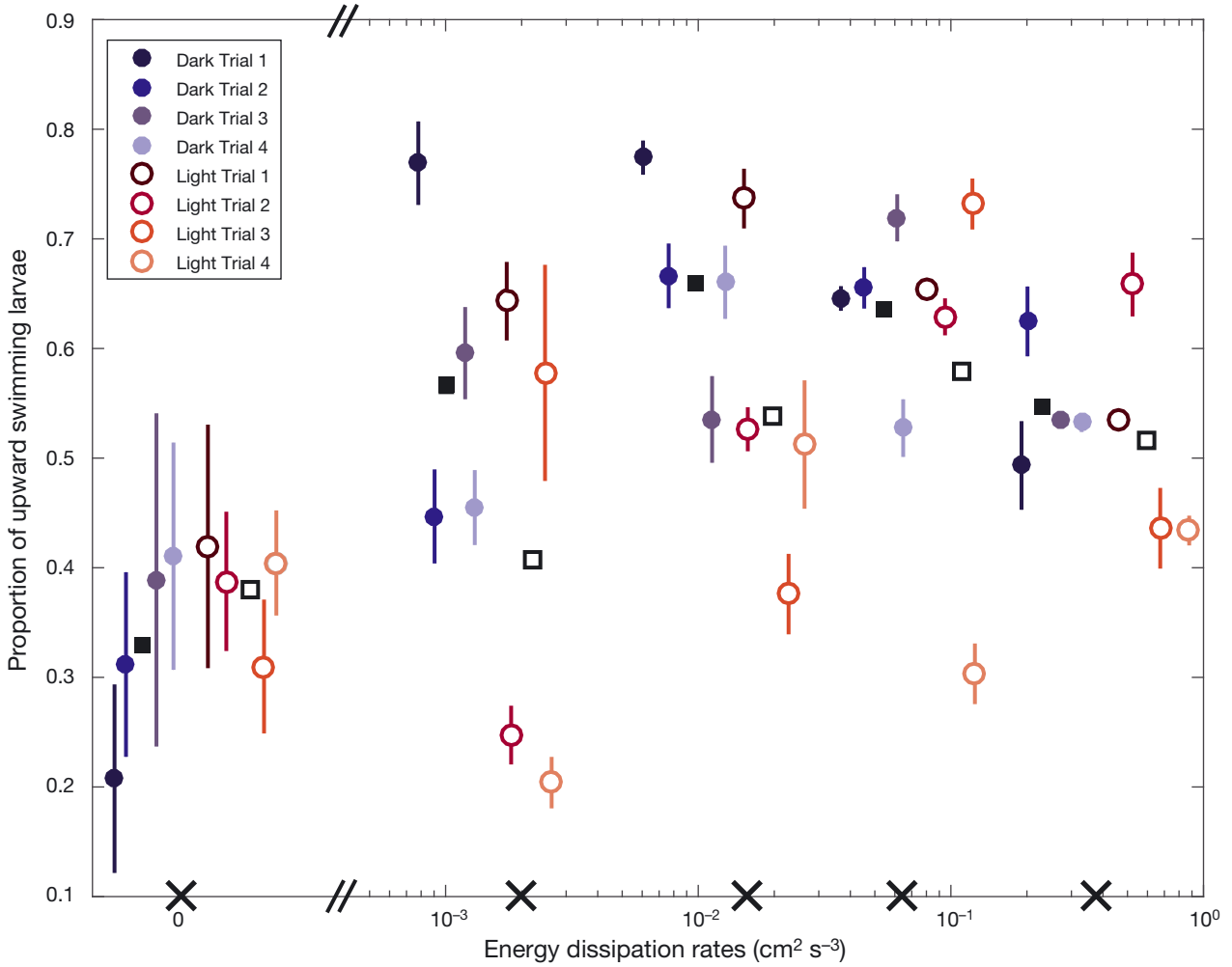

Fig. 2. Mean (±SE) proportion of upward swimming eastern oyster *Crassostrea virginica* larvae for each trial vs. turbulence level (energy dissipation rate), in dark (blue-toned closed circles) and light (red-toned open circles). Values at each turbulence level (denoted by × on the energy dissipation rate axis) are grouped by dark and light and are offset horizontally for clarity. Proportions are pooled across trial in both dark (closed squares) and light (open squares); as trial had a significant effect on upward swimming, these mean proportions are intended only to highlight the effects of light and turbulence. Larvae displayed primarily downward swimming in the unforced flow regime, and upward swimming in turbulence, although the prevalence of upward swimming decreased in high turbulence. Light had no significant effect on directional swimming

In the turbulent regimes, time (i.e. treatment order within a trial) and trial also had a significant effect on upward swimming (Table 2). Larvae exhibited decreased upward swimming in turbulence levels occurring later in the treatment order, regardless of what these turbulence levels happened to be (Table S3 in the Supplement). This could be a consequence of a larval response to an aggregative turbulence cue, acclimation to the tank, or fatigue. Upward swimming decreased over the full experimental time period (Fig. 2, dark to light points), with larvae generally exhibiting less upward swimming in later trials than in earlier trials.

In the unforced flow regime, light had no effect on the proportion of upward swimmers (Table 3). In contrast to the turbulence regimes, trial had no significant effect on upward swimming. Video sequence did have a significant effect but it was difficult to interpret. One might reasonably expect video sequence number to act as a proxy for time spent in the tank. However, in examining each trial, there was no robust temporal pattern in upward swimming over the full range of video sequences, and the post hoc comparison test showed no pairwise significant differences between sequences (Table S4 in the Supplement).

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 in the Supplement). 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 in the supplement); that is, light became a significant effect

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

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

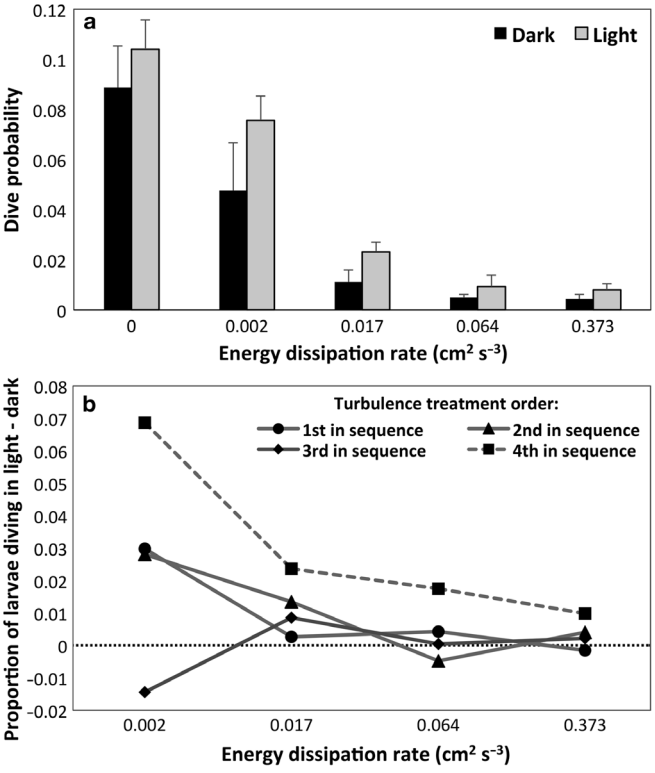

Fig. 3. (a) Proportion of eastern oyster *Crassostrea virginica* larvae diving (±SE) with respect to turbulence regime, as characterized by energy dissipation rate, in dark (black bars) and light (light grey bars), pooled across trials. Larvae exhibited non-significant increases in diving in light versus dark treatments and significant decreases in diving in increasing turbulence. Trial is also a significant factor for diving. Pooling across trials was done to highlight other effects and trials are not treated as replicates in the analysis; SE was calculated for this plot using trial means and indicates 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 panel a), with respect to turbulence regime, sub-divided into turbulence treatment order (when in the sequence of four turbulence regimes the given regime falls). Larvae in the fourth and final treatment (dashed line) dove more frequently in light than dark, irrespective of turbulence level, and previous turbulence history in the tank. Lines connecting turbulence levels are for visual clarity, not to imply quantitative interpolation

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.

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 significantly smaller proportion of larvae swimming helically was observed in the low forcing regime than in the unforced regime (Fig. 4a, Table 5). Trial had no impact on helical swimming, and the interactive effect of light and turbulence was also non-significant (Table 5). The decreased proportion of helical swimmers in turbulence appears to be a behavioral response, and not solely an effect of decreased detection as larvae are advected more rapidly through the thin laser sheet in the low forcing regime. Using PIV data from the unforced and low forcing regimes, we estimated average horizontal root mean square (rms) flow velocities of v_{rms} = 0.04 and 0.11 cm s^{-1}, respectively, and average flow autocorrelation timescales of τ = 7.2 and 3.6 s. Over the average time it took to visually identify a helix (~1.5 s), estimated ballistic displacements of larvae by turbulent fluctuations were 0.06 and 0.16 cm in the unforced and low forcing regimes, respectively. As these length scales are smaller than the depth of the laser sheet for larval imaging (0.25 cm), the helical trajectories are not likely to be systematically undetected in low intensity turbulence. Nevertheless, decreased detection may play a small role in the result and would certainly be exacerbated in more turbulent flow regimes.

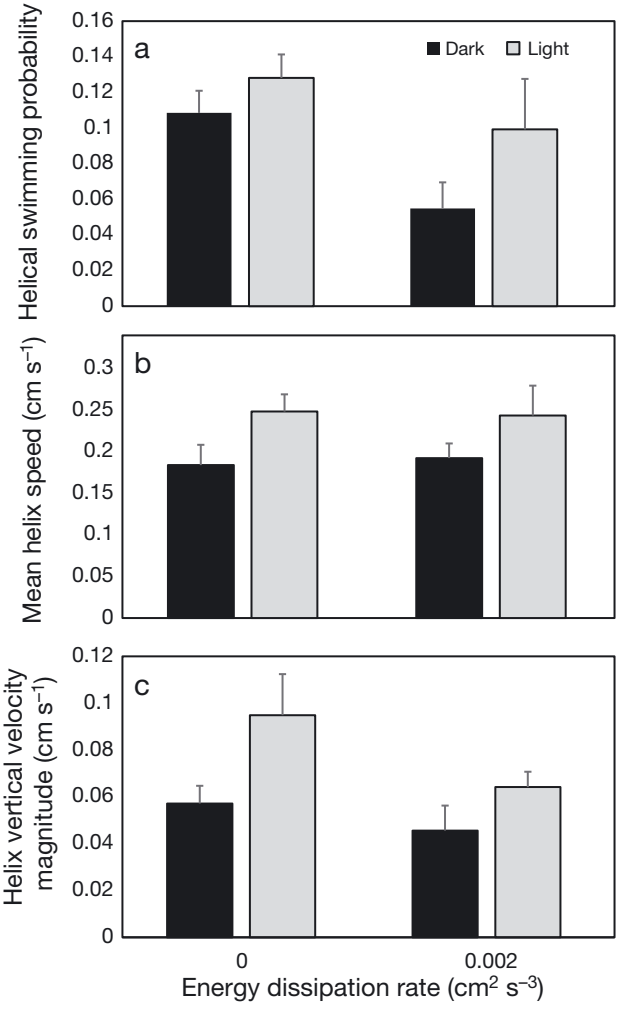

Fig. 4. (a) Proportion of eastern oyster *Crassostrea virginica* larvae swimming helically, (b) helix speed, and (c) vertical translational velocity magnitude between unforced and low forcing turbulence regime, as characterized by energy dissipation rate, in dark (black bars) and light (light grey bars), 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 was calculated using trial means and indicates the inter-trial variability

Table 4. Results of ANOVA for proportion of diving eastern oyster *Crassostrea virginica* larvae in forced flow, testing for effects of light, time (turbulence treatment order), turbulence level, and trial (aging). Significant results are in **bold**, with a significance level of α = 0.05

Source	df	MS	*F*	p
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 × Light	3	0.003	3.99	**0.03**
Turbulence level × Light	3	0.0009	0.94	0.44
Trial (Light)	6	0.003	3.53	**0.03**
Error	12	0.0009		

Table 5. Results of ANOVA on split plots design for proportion of helically swimming eastern oyster *Crassostrea virginica* larvae in unforced and low forcing flow, testing for effects of light, turbulence level, and trial (aging). Significant results are in **bold**, with a significance level of α = 0.05

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

While light did not impact the overall proportion of larvae swimming helically, it did affect the mean helix speed, with borderline significance (Fig. 4b, Table 6). Turbulence did not affect helix speed, nor did the interaction between turbulence and light (Table 6). Isolating and testing the translational velocity (the vertical helical swimming velocity) yielded no effect of light (Fig. 4c, Table 7), but a significant turbulence effect and interactive effect of light and turbulence (Fig. 4c, Table 7). Overall, helically swimming larvae swam faster in light than darkness, and vertical translational helical velocity increased with turbulence (Table S7 in the Supplement).

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.

Table 6. Results of ANOVA on split plots design for mean helix swimming speed of eastern oyster *Crassostrea virginica* larvae in unforced and low forcing flow, testing for effects of light, turbulence level, and trial (aging). Significance level: $\alpha = 0.05$

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

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

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

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, 2015); larval diving responses occur in the range of turbulence regimes consistent with flow several centimeters above rough bottom topographies (Pepper et al. 2015, Wheeler 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 said sites during their preferred daylight settlement times. From an anti-predatory perspective, many predators of larval invertebrates use visual cues to detect their prey (Iwasa 1982), and so increasing predator-avoidance behaviors in light versus dark would be a useful survival strategy. Indeed, oyster larvae dive more frequently when exposed to anomalously high local fluid acceleration (Wheeler et al. 2015), which larvae may interpret as the presence of a suction feeding predator (Kiørboe et al. 1999, Jakobsen 2001). Similarly, helical swimming may also act as a predator-avoidance response while simultaneously allowing larvae to feed and explore the water column: helical swimming clears large foraging volumes while presenting a minimal hydromechanical presence to predators (Visser 2007). The increased occurrence of diving and helical swimming in light may reflect the larval response to an increased predation risk during daylight hours.

Alternatively, helical swimming may increase the precision of navigation during directional swimming such as phototaxis, as demonstrated in simulations of annelid swimming (Jékely et al. 2008, Jékely 2009). While no phototactic response was obvious in the proportion of upward swimming larvae in our study, the change in helical swimming characteristics in light demonstrates a photokinetic behavior of poten-

tial benefit to a directionally swimming larva. Such results indicate the importance of considering multiple swimming metrics when quantifying a behavioral response.

Moreover, the change in helix speed and vertical translational velocity in response to light indicates that larval oysters have active control over helical swimming behavior. The 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 & Grunbaum 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 & Chia 1982, McCarthy et al. 2002), crabs (Forward & Costlow 1974), mussels (Fuchs & DiBacco 2011), nudibranchs (Miller & Hadfield 1986), conch (Barile et al. 1994), and both larval and juvenile sole (Champalbert et al. 1991). Competent larvae may cease to display positive phototactic 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 h, 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 substandard 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 (Bishop et al. 2006, Botello & Krug 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 (J. Wheeler unpubl. 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 in upward swimming over the experimental period may be explained by the passive sinking of older, heavier larvae. However, our observations demonstrate that upward swimming over time only changes in turbulence, suggesting that it is a combination of turbulence and age, and not merely age, which induces changes in larval swimming; in the unforced flow regime, older competent larvae exhibited similar responses to newly competent larvae. In fact, both interpretations (turbulence acting as a settlement cue or as an energetic constraint to swimming) are supported by the observation that the effects of aging only impacted larvae swimming in turbulence. The effect of age during the competency window on larval behavior may furthermore explain previous conflicting results on larval oyster responses to turbulence (Fuchs et al. 2013, Wheeler et al. 2013). Our results give a strong indication that ontogeny should be more carefully considered in larval behavioral studies; while ontogenetic changes across

multiple larval stages are commonly studied, a focus on within-stage ontogenetic change is rare.

Our study further strengthens the body of evidence documenting upward swimming of larval oysters in moderate turbulence (Wheeler et al. 2013), which suggests that turbulence alone does not act as a cue for larval settlement (with a possible exception for older, heavier, and/or less selective larvae). Similarly, we observed no change in the proportion of larvae which swam upward in response to light; the absence of an obvious negative phototactic response suggests that neither light alone, nor light in conjunction with turbulence, are effective inducers of larval settlement at a population level. Nevertheless, the observed changes in larval diving and helical swimming in the presence of light suggest that they modify potentially exploratory and anti-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.

Acknowledgements. We thank Susan Mills and Anthony Ritchie for experimental assistance, and Samuel Laney for light measurements. We gratefully acknowledge the Aquaculture Research Corporation (Dennis, MA, USA), who provided the larvae used in this study. Thanks to Jen Nguyen for facilitating data transfer. Funding was provided by NSF grant OCE-0850419, NOAA Sea Grant NA14OAR4170074, grants from WHOI Coastal Ocean Institute, discretionary WHOI funds, a WHOI Ocean Life Fellowship to L.S.M., a Grove City College Swezey Fund Grant to E.J.A., and a WHOI Summer Student Fellowship to E.L.

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Editorial responsibility: Paul Snelgrove, St. John's, Newfoundland and Labrador, Canada

Submitted: September 21, 2016; Accepted: March 2, 2017
Proofs received from author(s): April 14, 2017