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Evidence for stage-based larval vulnerability and resilience to acidification in *Crassostrea virginica*

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ABSTRACT

Using image analysis of scanning electron micrographs (SEMs), we compared differences in growth of D-stage veligers [i.e. prodissoconch I and II (PI and PII) larvae] of eastern oysters *Crassostrea virginica* grown in mesohaline water under high- and low-CO_2 conditions. We found SEMs to reveal no evidence of dissolution or shell structure deformity for larval shells in either of the CO_2 treatments but detected prominent growth lines in the PII regions of larval shells. The number of growth lines closely approximated the duration of the experiment, suggesting that growth lines are generated daily. Mean growth line interval widths were 20% greater for larval shells cultured in low- *vs* high-CO_2 conditions. *Crassostrea virginica* veliger larvae were shown to tolerate high CO_2 levels and aragonite saturation states (Ω_{arag}) < 1.0, but larval growth was slowed substantially under these conditions. Differences in growth line interval width translate into substantial changes in shell area and account for previously observed differences in total shell area between the treatments, as determined by light microscopy and image analysis. Other studies have documented high mortality and malformation of D-stage larvae in bivalves when pre-veliger life stages (i.e. eggs, gastrula and trochophores) were exposed to elevated CO_2. Our experiments revealed statistical differences in rates of larval survival, settlement and subsequent early-stage spat mortality for veligers reared in high- and low-CO_2 conditions. Although each of these rates was measurably affected by high CO_2, the magnitude of these differences was small (range across categories = 0.7–6.3%) suggesting that the impacts may not be catastrophic, as implied by several previous studies. We believe the apparent disparity among experimental results may be best explained by differential vulnerability of pre-veliger stage larvae and veligers, whereby PI and PII larvae have greater physiological capacity to withstand environmental conditions that may be thermodynamically unfavourable to calcification (i.e. Ω_{arag} < 1.0).

INTRODUCTION

As humans continue to enrich Earth's atmosphere with CO_2, oceans are absorbing roughly 30% of excess CO_2 from the atmosphere and undergoing changes in chemistry (Pachauri & Mayer, 2015). These changes, termed ocean acidification, include an overall reduction in oceanic pH, decreased availability of CO_3^{2-}, and lower $CaCO_3$ saturation states (Caldeira & Wickett, 2003; Orr *et al.*, 2005; Doney *et al.*, 2009; Pachauri & Mayer, 2015). Our understanding of acidification in fully marine systems is extensive and mature compared with our knowledge of carbonate chemistry dynamics in nearshore ecosystems (e.g. estuaries and bays). Carbonate chemistry in nearshore coastal systems is naturally much more variable and heterogeneous than in purely marine systems, and is driven by chemical, physical and biological processes (Duarte *et al.*, 2013; Baumann *et al.*, 2015; Lowe, Bos & Ruesink, 2019). This natural variability has important implications for the biota that live in these environments. Importantly, species with evolutionary histories in such locations are expected to be well adapted to chemically dynamic environmental conditions. Here, we describe experimental results that demonstrate normal growth and survival of fully formed D-stage (prodissoconch I = PI) larvae of the eastern oyster *Crassostrea virginica* (Gmelin, 1791) cultured under high CO_2 and water that is undersaturated for aragonite (Ω_{arag} < 1.0). From these results, we also seek to reconcile the seemingly disparate findings yielded by other bivalve larval acidification studies.

Understanding species' responses to changing carbonate chemistry conditions is an important focus of ocean acidification research (Doney *et al.*, 2009; Waldbusser & Salisbury, 2014; Stevens & Gobler, 2018). Estuaries commonly experience high CO_2 and

low pH, including conditions that are undersaturated with respect to aragonite (i.e. $\Omega_{arag} < 1.0$), the more soluble form of $CaCO_3$ used by many shell-building organisms in their larval shells. Among shelled molluscs, and oysters in particular, there are a variety of apparent emergent themes, including increased energetic costs, reduced rates of growth and calcification due to $\Omega_{arag} < 1.0$ conditions (Miller *et al.*, 2009; Parker, Ross & O'Connor, 2010; Gazeau *et al.*, 2013; Timmins-Schiffman *et al.*, 2013).

Several experimental studies have demonstrated that bivalve larvae reared under high CO_2 and low pH (in isolation or crossed with other environment stressors such as temperature and dissolved oxygen) can experience exceptionally high mortality (Kurihara, Kato & Ishimatsu, 2007; Talmage & Gobler, 2010; Barton *et al.*, 2012; Waldbusser *et al.*, 2013; Gobler *et al.*, 2014). Waldbusser *et al.* (2015) determined that both the Pacific oyster *C. gigas* and the Mediterranean mussel *Mytilus galloprovincialis* were sensitive to aragonite saturation state and that such conditions resulted in abnormal PI development and increased larval mortality. Talmage & Gobler (2010) identify malformation in the development of the PI hinge as the hallmark and likely cause of larval failure in *C. virginica* and the northern quahog *Mercenaria mercenaria* at high CO_2. Indeed, it appears that investigations that have imposed chemical challenges at the earliest embryological and developmental life stages (i.e. fertilized eggs, gastrula and trochophores or 'pre-veliger' stages) have revealed a critical developmental susceptibility of oyster and other bivalve larvae to high CO_2/low pH and $\Omega_{arag} < 1.0$.

The above results contrast with our own findings (Miller *et al.*, 2009), where exposing fully developed D-stage (PI) veliger larvae of two oyster species, *C. virginica* and the Suminoe oyster *C. ariakensis*, to differential levels of CO_2, including $\Omega_{arag} < 1.0$, did not induce strong differential mortality of larvae. Likewise, Ginger *et al.* (2013) observed no reduced survivorship when PI larvae from *C. gigas* were exposed to high CO_2 and $\Omega_{arag} < 1.0$. We hypothesize that differences in mortality across experiments are based on the timing of larval exposure to challenge conditions due to differences in susceptibility between early pre-veliger stages and later D-stage (PI) and prodissoconch II (PII) veliger larvae. Further, such differences are logically explained by differences in shell calcification mechanisms of gastrula and trochophores (shell field cells) that produce PI shell and mantle tissues that generate shell growth of PII larvae (Malchus & Sartori, 2013).

In the face of widespread natural variation in carbonate chemistry and other water quality characteristics, especially in estuaries where spatial and temporal heterogeneity can be especially pronounced (Frankignoulle, Borges & Biondo, 2001; Abril, Richard & Guérin, 2006; Joesoef *et al.*, 2015), planktotrophic larvae that spend weeks in the water column are likely to experience a variety of chemical environments throughout their development. Indeed, pre-veliger stages may be dispersed on tides and by currents far from their natal grounds in the first 12 h of life. Understanding the relative susceptibility of pre-veliger and veliger larvae to elevated CO_2, including conditions that are undersaturated for aragonite, will be important for clarifying how species with such life histories may react to a changing environment.

To verify that shell growth was normal and without detectable malformation when exposing otherwise healthy feeding D-stage veligers to differential CO_2 challenge conditions for 26 d, we used scanning electron microscopy (SEM) to examine the larval shells of *C. virginica* reared under high- and low-CO_2 conditions, when $\Omega_{arag} < 1.0$ and $\Omega_{arag} > 1.0$. Under SEM, we observed conspicuous concentric growth lines generated at the growing edge of the larval shell. Malchus & Sartori (2013) described subtle 'commarginal growth lines' in PI larvae but noted them as far more pronounced in the PII stage. These features suggest continuous deposition of calcium carbonate that is punctuated by a prominent ridge on an apparent daily frequency. Here, we combine shell height and area, along with these larval shell growth lines, to help substantiate differences in the tempo and extent of growth of *C. virginica* veliger larvae under differential CO_2. We also present data on larval survival, settlement rate and early-stage spat mortality under low and high CO_2.

MATERIAL AND METHODS

Larval life history

The PI stage is the first shelled stage of the oyster larva. Also known as the D-stage or straight hinge veliger, it is recognizable by its D-shape and smooth, transparent valves that are produced rapidly (6–24 h) by the embryonic shell field as the larvae transition from trochophore to the PI stage (Ockelman, 1965; Kniprath, 1981; Eyster & Morse, 1984; Wassnig & Southgate, 2012; Malchus & Sartori, 2013; Haley *et al.*, 2018; Zhao *et al.*, 2018). As an oyster enters the PII stage, it begins to deposit shell at the growing edge, extending outwards from the PI valves, and is distinguished visibly by striated, concentric growth lines. With the transition to the PII stage, the remnant of the PI stage is preserved at the umbo end of the larval shell. Importantly, calcification in trochophores that generates the D-stage (PI) larval shell is considered the product of calcification by the embryonic shell field; however, the site and mechanism of calcification shift to the mantle tissues at the shell's growing edge when the larva transforms from PI to PII stage (Weiss *et al.*, 2002; Moueza, Gros & Frenkiel, 2006; Kurihara *et al.*, 2007; Malchus & Sartori, 2013; Aranda-Burgos *et al.*, 2014). The pediveliger stage (late PII stage) is reached as the larval foot is developed and the larva becomes competent to settle and metamorphose into a juvenile oyster (dissoconch).

Larval incubations

The general aim of our experimental conditions was to simulate mesohaline conditions in the Chesapeake Bay, USA, during summer months when oysters reproduce and then expose larvae to differential CO_2/Ω_{arag} conditions. In so doing, we simulated a scenario in which spawning and early development of larvae (i.e. pre-veliger development) occur in favourable conditions and share a common embryological experience, but where half of the larvae continue their larval life cycle in favourable conditions (low CO_2 and $\Omega_{arag} > 1.0$, hereafter 'low-CO_2' conditions or larvae) and the other half experience harsher conditions, high CO_2 and $\Omega_{arag} < 1.0$ (hereafter 'high-CO_2' conditions or larvae). Given the natural spatial and temporal heterogeneity of carbonate chemistry and other water quality factors in estuaries and coastal ecosystems, combined with the diverse locations of oyster beds/reefs in these systems, the common immigration/emigration of larvae and extended planktotrophic life stage of oysters and numerous other bivalves, the experience of PI and PII larvae is expected to be decoupled in many instances.

We conducted two larval incubation experiments, one in 2008 (experiment 1) to assess larval shell condition and growth rates using SEM as well as to measure larval survival rates and a second in 2009 (experiment 2) to test for differential rates of larval survivorship, settlement and early-stage spat mortality. Experiments 1 and 2 began a few days following fertilization but after early embryological shell field calcification had yielded healthy PI larvae. We obtained D-stage (PI) larval *Crassostrea virginica* from the experimental shellfish hatchery at the Virginia Institute of Marine Sciences, Eastern Shore Laboratory, Wachapreague, VA, USA, in July 2008 and September 2009. We transferred D-stage veliger larvae (72 and 96 h post-fertilization in 2008 and 2009) to the Smithsonian Environmental Research Center, Edgewater, MD, USA, and acclimatized them to 18 ppt seawater at room temperature for 24 h (experiment 1) and 48 h (experiment 2) prior to moving larvae to one of two experimental CO_2/pH treatments (pre-industrial revolution atmospheric = 280 ppm; conservative

Table 1. Carbonate chemistry parameter values/sources and culture conditions of *Crassostrea virginica* for experiment 1 (April 2008; after Miller *et al.*, 2009) and experiment 2 (September 2009).

Parameter	Experiment 1		Experiment 2		Parameter source
Simulation	Pre-IR	Year 2100	Pre-IR	Year 2100	
Target pCO_2 (ppm)	280	800	280	800	
Mean pCO_2 (ppm)	284	840	311	846	CO2sys calc.
SEM	4.8	17.4	14.7	41.1	
Mean hourly pH	8.16	7.76	8.12	7.72	Direct meas.
SEM	0.002	0.005	0.001	0.001	
Mean TA (µmol/kg)	1,229	1,289	1,211	1,204	CO2sys calc.
SEM	15.7	15.7	34.4	35.8	
Mean TDIC (µmol/kg)	1,126	1,265	1,115	1,184	Direct meas.
SEM	15.1	15.7	33.3	36.4	
Mean CO_2 (µmol/kg)	8.8	25.9	9.6	26.1	CO2sys calc.
SEM	0.15	0.54	0.45	1.35	
Mean HCO_3^-	1,045	1,206	1,039	1,129	CO2sys calc.
SEM	14.1	14.9	31.6	34.7	
Mean CO_3^{2-}	72.4	32.7	66.9	29.0	CO2sys calc.
SEM	0.8	0.6	2.5	1.1	
Mean Ω_{arag}	1.2	0.6	1.1	0.5	CO2sys calc.
SEM	0.01	0.01	0.04	0.02	
Salinity (psu)	18.2	18.2	18.3	18.3	Direct meas.
SEM	0.04	0.04	0.08	0.08	
Duration (d)	26	26	24	24	

Larvae were grown under CO_2 conditions simulating equilibration with atmospheric CO_2 in the pre-industrial era (280 ppm) and 2100 ce (800 ppm). All experiments were conducted at 25 °C and under a 14 h:10 h light:dark cycle, simulating summer growing conditions in the Chesapeake Bay, USA. Abbreviations used: IR, industrial revolution; TA, total alkalinity; TDIC, total dissolved inorganic carbon. Parameters were measured under the SEM, by direct measurement or calculated using CO2sys_macro_PC.xls.

end of 21st century = 800 ppm CO_2). To ensure identical starting conditions (i.e. D-stage larval size and concentration), we seeded each of three replicate 4-l polycarbonate aquaria per treatment. We pipetted D-stage larvae from a common larvae suspension of known starting concentration: 15,000 larvae per replicate aquaria in experiment 1 and 19,950 larvae per replicate in experiment 2.

We blocked CO_2 experimental treatments (280 and 800 ppm CO_2) using two environmental chambers (Percival I-36 Controlled Environment Chambers with Philips 700 series 32 W fluorescent bulbs), each containing three replicate aquaria. A pH-stat system monitored pH independently and continuously in each replicate aquarium, and each system was gently bubbled with CO_2-stripped air to continuously oxygenate and circulate water. Each aquarium contained a pH probe connected to an independent pH controller that automatically opened and closed a solenoid valve to a 1% CO_2/air mixture, based on upper and lower pH threshold settings that corresponded to predetermined CO_2/pH targets. According to hourly recorded pH measurements, the negative feedback control system maintained pH (and CO_2) at near-constant levels for up to 4 weeks. We collected *c.* 225 l of natural seawater from Sinepuxent Bay, MD, USA, for use in the experiments. After collection, we filtered the seawater through a 0.2-µm filter and diluted it to 18 ppt salinity using distilled/deionized water. We measured total alkalinity (TA) with open-cell two-point titration to establish starting conditions and to identify target pH and CO_2 values. In addition to hourly records of pH, we analysed dissolved inorganic carbon (DIC) every 2–3 d to verify CO_2 target levels. We calculated TA and CO_2 using CO2sys_macro_PC.xls (Pelletier, Lewis & Wallace, 2007). Table 1 summarizes the treatment conditions for experiments 1 and 2. More detailed methods for larval incubation and CO_2 control system can be found in Miller *et al.* (2009).

We reared all replicate larval suspensions at 25 °C under a 14 h:10 h light:dark cycle, and fed the larvae with a controlled daily diet of *Isochrysis galbana* until they reached the pediveliger stage, signifying that the larvae were competent to settle (experiment 1) or until extensive settlement began (experiment 2). We changed the water every 48 h, at which time we captured larvae on nylon mesh sieves before resuspending in clean, pre-conditioned water. Prior to each water change, we subsampled one replicate per treatment by pipette to determine larval stage, estimate survivorship and measure height (umbo to shell edge).

We terminated experiment 1 when most larvae were shown to be eyed pediveligers but prior to the onset of settlement. Experiment 1 yielded larval survival rates to the eyed pediveliger stage and we subsampled specimens for SEM analysis (details below). In experiment 2, we continued the incubation until larvae had begun to settle extensively, but not completely, since some swimming veligers were still present. We counted spat from all surfaces (i.e. PVC settlement plates, container walls and pH probes) to determine % settlement. We examined the spat that had settled on standardized settlement plates under a dissecting microscope and categorized them as live or dead to compare early post-settlement mortality between the CO_2 treatments. We also counted swimming larvae, as in experiment 1, for comparison with the total number of settled spat (live + dead), which enabled us to gauge whether the tempo and extent of settlement was different in high- *vs* low-CO_2 treatments.

Survival rate measurements

We measured larval survival rates by comparing the number of live larvae at the end of experiment 1 to the starting number of inoculated live larvae per treatment (two treatments × three replicates). Following gentle agitation to uniformly suspend larvae, we pipetted

three 1-ml water samples onto Sedgewick Rafter slides from each replicate, examined the larvae for condition and then immobilized them using a drop of Lugol's solution. At this point, we counted larvae with a binocular microscope under a 20× objective.

On day 26 (experiment 1) and day 24 (experiment 2), we poured all swimming larvae that were suspended in the water column for each replicate through a 132-µm sieve. We then immediately suspended the sieved larvae in 450–500 ml water, subsampled by pipette, and counted them under a binocular microscope to calculate the mean number of living larvae. When determining larval survivorship, we subsampled each treatment replicate three times and averaged the results. We examined materials at the bottom of each replicate container separately under the microscope to detect and count any unsettled living larvae. We averaged final live larvae counts for each treatment (i.e. low and high CO_2).

Settlement rate measurements

We assessed three aspects of larval settlement: (1) total number of settled spat relative to the starting larval number as an overall measure of % settlement by treatment; (2) total number of settled spat relative to the number of remaining swimming larvae by treatment to investigate the tempo at which larvae reached the spat stage; and (3) a standardized measure of early-stage spat mortality was achieved by comparing all live and dead spat on settlement plates by treatment. To quantify larval settlement, we suspended five 6.35 cm (length) × 6.35 cm (width) × 0.32 cm (thickness) roughened PVC plates in replicate containers from each treatment. We pre-seasoned plates in filtered seawater for several days to develop a natural biofilm to induce preferential settlement to plates *vs* other available surfaces (i.e. container wall, pH probe, aeration/gas delivery hose and air stone). On day 18, sampling and microscope analysis indicated that *c.* 25% of larvae had reached the eyed stage and seasoned settling plates were introduced to the six replicate containers across the two CO_2 treatments. On day 24 of experiment 2, after apparent extensive larval settlement, we terminated the experiment and determined survival and settlement rates. We also examined settlement plates, air stone/hose and pH probes and aquaria surfaces with a binocular dissecting microscope or magnifying glass to achieve complete spat counts. We examined spat on settlement plates individually and deemed them as live or dead.

SEM and image analysis

In experiment 1, after 26 d of incubation (larval age = 30 d), we captured larvae on filters and fixed them in 95% ethanol. We differentiated live larvae from dead shells and counted them for each of three replicate aquaria from both the 280 and 800 ppm CO_2 treatments. We mounted specimens on aluminium SEM stubs with sticky carbon tabs, and sputter coated the stubs with gold prior to observing and photographing them under 250× magnification at the SEM Laboratory of the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA. We positioned preserved larvae individually with a fine bristled paint brush under a binocular microscope and shells were segregated across SEM stubs by replicate (i.e. six stubs, each containing several dozen shells). While larval shells were positioned horizontally (in profile) on the tab and as normal to the vertical axis as possible, no measure of off-axis tilt was quantified. We used a JEOL JSM-6100 SEM to examine and select approximately equal numbers of 'live' larval specimens from the 280 ppm ($n = 58$) and 800 ppm ($n = 60$) treatments for imaging. Afterwards, we imported the images into ImageJ 1.52d software (Rasband, 1997–2018) to measure (1) height and area of PI portions of the shells, (2) total shell area (PI + PII), (3) number of growth lines of PII (the leading growth edge beyond the most recently generated growth line was a partial day of growth and was thus not counted) and (4) growth line interval widths across shell height axis (Fig. 1A–C). We measured

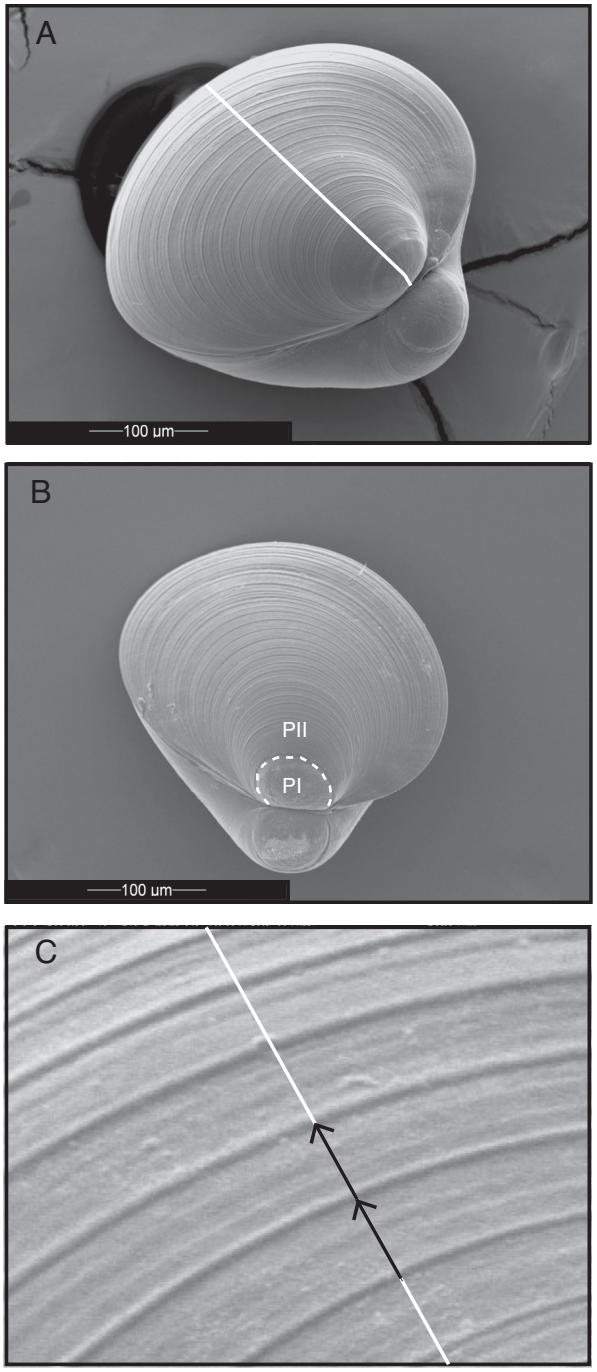

Figure 1. Thirty-day-old *Crassostrea virginica* veligers reared under differential CO_2/Ω_{arag} conditions for 26 d (larvae were 96-h old at start of experiment). **A.** Treatment with 280 ppm CO_2 and $\Omega_{arag} = 1.2$. **B.** Treatment with 800 ppm CO_2 and $\Omega_{arag} = 0.6$. PI and PII larval stages are highlighted by white dotted line in (**B**). Orientation of growth line interval measurements specified by the white line in (**A**), and growth line interval widths depicted with black arrows in (**C**). Specimens are representative of mean-sized larvae from the respective CO_2 treatments. Lengths and areas were measured relative to the 100-µm scale bar, where 5.1937 pixels = 1 µm.

total shell area by tracing the upper valve's perimeter with ImageJ's polygon tool and calculating the area. We measured PI areas in a similar way and measured heights using ImageJ's straight line tool. Because it was not possible to align shells in precisely the same orientation and normality to the vertical camera angle (due to idiosyncratic differences in shell shape and morphology), area and

linear measurements may not be exact and may be affected to some degree by foreshortening. Nevertheless, because shell valves are convex, plan view area measurements are conservative and should provide consistent, if slightly underestimated, values of the true curvilinear area; however, this should not affect comparisons between treatments because we were interested in the relative, not absolute differences. Furthermore, although shells were not randomly chosen, but instead selected according to most favourable orientation, these selections were made from among a much larger number of shells that were mounted one by one on the SEM aluminium stubs. As such, we had no a priori expectation of any inherent selection bias associated with either shell size or treatment for the PI or PII measurements. To confirm that starting shell sizes were similar between treatments, we measured D-stage portions of the larval shells that were generated prior to transition from PI to PII.

Data analysis

We compared larval survivorship rates (D-stage to pediveliger) in high- and low-CO_2 treatments in experiment 1 using a χ^2 test (2×2 contingency table). Likewise, we compared survivorship rates, spat settlement rates, settlement tempo and early spat stage in experiment 2 using χ^2 tests. We took SEMs of the 118 larvae deemed suitable for analysis based on whether the orientation of the shell was sufficiently in profile to yield the most reliable measurements ($n_{280} = 58$, $n_{800} = 60$). We used the Welch's *t*-test (i.e. two-sample *t*-test assuming unequal variances) to compare shell area, height, number of growth lines and growth line interval width measurements across the 280 and 800 ppm CO_2 treatments. We log-transformed measures for shell areas (both D-stage and total shell area) to better conform to normality requirements. Furthermore, to compare the rates at which discrete major growth lines were generated in the two treatments (i.e. the number of lines deposited across 26 d of the experiment), we assumed Poisson probabilities and applied a two-tailed large sample size Z-test (Mathews, 2010). Description and results from all statistical comparisons are summarized in Table 2. All contingency table comparisons can be found in the Supplementary Material.

RESULTS

Neither shell area nor height of D-stage (PI) larvae was statistically different between low-CO_2 (280 ppm) and high-CO_2 (800 ppm) treatments ($t_{Area} = 0.517$, $P = 0.6105$, df = 20; $t_{Height} = 1.048$, $P = 0.3046$, df = 25), confirming that the size and geometry of PI larvae were similar at the onset of the experiment (Fig. 2A, B). The pooled PI starting height was 51.5 ± 0.62 µm (mean ± 1 SE) and mean starting area was 2,654 ± 39.7 µm^2 ($n = 45$). At high optical resolution (250×), we did not discern any abnormalities in shell formation for either CO_2 treatment, despite an undersaturation state for aragonite in the high-CO_2 treatment.

High-CO_2 larvae grew more slowly over 26 d of incubation and attained just 77.9% of the total shell area as low-CO_2 larvae. A comparison of cumulative shell area frequencies revealed a consistent size shift across the two treatments (Fig. 3). High-CO_2 larvae measured 48,476 ± 1,451 µm^2 compared with 62,256 ± 1,311 µm^2 for low-CO_2 larvae ($t_{\ln(Area)} = 6.889$, $P < 0.0001$, df = 105; Fig. 2C). Likewise, total shell height was greater for low-CO_2 larvae (271.7 ± 7.05 µm) than high-CO_2 larvae (238.8 ± 7.03 µm; $t_{Ht} = 3.173$, $P < 0.0019$, df = 115; Fig. 2D).

Survivorship of low-CO_2 larvae (16.1 ± 0.66%) was somewhat greater than high-CO_2 larvae (13.4 ± 1.39%) over 26 d of experiment 1 ($\chi^2 = 8{,}791.8$, df = 1, $P < 0.001$). Likewise, larval survivorship of low-CO_2 larvae was also slightly greater than in high-CO_2 larvae (32.4% *vs* 31.7%; $\chi^2 = 7.3901$, df = 1, $P = 0.0066$) over 24 d in experiment 2.

Larval settlement rate was greater in the low-CO_2 treatment than the high-CO_2 treatment (23.9% *vs* 17.6%; $\chi^2 = 710.65$, df = 1, $P < 0.001$) in experiment 2. The tempo of settlement was compared using the following two measures:

$$\%\ \text{spat} = \text{number of spat} / (\text{number of spat} + \text{number of swimming larvae})$$

$$\%\ \text{veliger} = \text{number of live larvae} / (\text{number of spat} + \text{number of live larvae})$$

This comparison showed that the percentage of spat compared with veligers was significantly higher (73.6%) in the low-CO_2 treatment than in the high-CO_2 treatment (55.6%) ($\chi^2 = 1{,}363.1$, df = 1, $P < 0.001$). Finally, early-stage spat mortality was significantly lower in low-CO_2 conditions than in high-CO_2 conditions (2.69% *vs* 4.55%; $\chi^2 = 49.593$, df = 1, $P < 0.001$).

The mean number of major growth lines in both treatments approximated the 26 d of incubation in experiment 1: 22.9 ± 0.30 in the low-CO_2 treatment ($n = 58$) *vs* 23.9 ± 0.33 in the high-CO_2 treatment ($n = 60$). The Welch's *t*-test indicated a statistically significant, albeit small, difference between the CO_2 treatments, and high-CO_2 larvae had more growth lines than low-CO_2 larvae ($t_{lines} = -2.354$, $P = 0.02$, df = 115). The rates at which growth lines were generated were calculated as

$$\lambda = \textit{total number of lines} / (\text{number of shells} \times \text{number of days})$$

Thus, λ_{280} = 1,327 lines/(58 shells × 26 d) = 0.88 lines per shell-day and λ_{800} = 1,436 lines/(60 shells × 26 d) = 0.92 lines per shell-day. The values of λ were not significantly different between treatments ($Z = 1.183$, $P = 0.24$, $n = 3{,}068$ shell-days) and, despite the apparently large sample size, the test did not discern the slight fractional difference in deposition rates.

The mean distance between adjacent major growth lines (i.e. mean daily growth increments of 118 larval shells; $n = 2{,}767$ increments) was *c.* 1.2 times greater in the low-CO_2 treatment (12.1 ± 0.36 µm) compared to the high-CO_2 treatment (10.2 ± 0.27 µm; $t = 8.7995$, $P < 0.0001$, $n = 2{,}767$).

These results indicate that when incubations were initiated with fully formed D-stage larvae (PI, as opposed to trochophores or fertilized eggs), veligers reared in the low-CO_2 treatment grew significantly more per day than those reared in the high-CO_2 treatment, and that these rates of growth were recorded clearly in the shells of the growing larvae. These results agree with and reinforce those achieved via extensive light microscopy and image analysis in an earlier study (Miller *et al.*, 2009). Furthermore, high-CO_2/low-pH conditions did not induce shell malformation or any obvious shell dissolution. Larval survivorship and settlement rates, and the tempo of settlement were all lower in high-CO_2 conditions, while early-stage spat mortality was greater in high-CO_2 conditions. Importantly, despite factors that conceivably have effects on the ecology of oyster populations (i.e. modestly slowed growth and development, lower settlement and slightly higher spat mortality under high-CO_2 conditions), there were no indications that such rate differences would result in catastrophic failure of larvae grown under elevated CO_2 conditions.

DISCUSSION

Our SEM analyses of *Crassostrea virginica* veligers confirm significant differences in growth rates of the PII-stage larvae when identically sized D-stage (PI) larvae are cultured under differential CO_2 and Ω_{arag} conditions. Moderately high CO_2 levels that were undersaturated with respect to aragonite (800 ppm and $\Omega_{arag} = 0.6$) resulted in shells with just 78% the area of larvae grown under pre-industrial

Table 2. Summary of statistical contrasts between *Crassostrea virginica* D-stage and PII larvae incubated under the low-CO_2 (280 ppm) and high-CO_2 (800 ppm) treatments in experiments 1 and 2.

Measurement (units)	Treatment contrast	Statistical test	Sample size	Result, df	Experiment, year
Initial D-stage shell area [ln(μm^2)]	280 ppm *vs* 800 ppm	Welch's *t*-test, unequal variances	29, 16	NS, $t = 0.517$, df = 20, $P = 0.6105$	1, 2008
Initial D-stage height (μm)	280 ppm *vs* 800 ppm	Welch's *t*-test, unequal variances	29, 16	NS, $t = 1.048$, df = 25, $P = 0.3046$	1, 2008
Ending total shell area [ln(μm^2)]	280 ppm *vs* 800 ppm	Welch's *t*-test, unequal variances	58, 60	$t = 6.889$, df = 105, $P < 0.0001$	1, 2008
Ending total shell height (μm)	280 ppm *vs* 800 ppm	Welch's *t*-test, unequal variances	58, 60	$t = 3.173$, df = 115, $P < 0.0019$	1, 2008
No. of growth lines	280 ppm *vs* 800 ppm	Welch's *t*-test, unequal variances	58, 60	$t = -2.354$, df = 115, $P = 0.02$	1, 2008
Rate of growth line deposition	280 ppm *vs* 800 ppm	Large sample *Z*-test	3,068 shell-days	$Z = 1.183$, $P = 0.24$	1, 2008
Inter-line width (μm)	280 ppm *vs* 800 ppm	Two-sample *t*-test, unequal variances	58, 60	$t = 3.633$, df = 106, $P < 0.001$	1, 2008
Survival rate (D-stage to pediveliger) (no. of live larvae/no. of starting larvae)	280 ppm *vs* 800 ppm	Two-tailed χ^2 test	See Supplementary Material Table S1	$\chi^2 = 8{,}791.8$, df = 1, $P < 0.001$	1, 2008
Survival rate (D-stage to pediveliger + Spat) [(no. of larvae + no. of spat)/no. of starting larvae]	280 ppm *vs* 800 ppm	Two-tailed χ^2 test	See Supplementary Material Table S2	$\chi^2 = 7.3901$, df = 1, $P = 0.006558$	2, 2009
Percent settlement (no. of spat/no. of starting larvae)	280 ppm *vs* 800 ppm	Two-tailed χ^2 test	See Supplementary Material Table S3	$\chi^2 = 710.65$, df = 1, $P < 0.001$	2, 2009
Settlement tempo (no. of larvae *vs* no. of spat)	280 ppm *vs* 800 ppm	Two-tailed χ^2 test	See Supplementary Material Table S4	$\chi^2 = 1{,}363.1$, df = 1, $P < 0.001$	2, 2009
Early-stage spat mortality (no. of spat: live *vs* dead)	280 ppm *vs* 800 ppm	Two-tailed χ^2 test	See Supplementary Material Table S5	$\chi^2 = 49.593$, df = 1, $P < 0.001$	2, 2009

Nonsignificant results are indicated as 'NS'.

conditions (280 ppm and $\Omega_{arag} < 1.0$), despite identical dietary, temperature and light regimes. Larval shells showed no obvious signs of corrosion or dissolution in either treatment when examined under high magnification. Statistical results were equivocal regarding the rate of deposit and average number of prominent growth lines per shell under differential CO_2. The average number of growth increments for both treatments (23–24) approximates the length of the 26d experiment, suggesting that prominent striated growth lines are deposited daily, perhaps associated with or exacerbated by daily pulses of food. Differences in shell area cannot be accounted for by the number of growth lines, instead shell size differences are better explained by the amount of shell deposited per growth increment, revealed here by narrower daily growth increments of the larvae grown under high CO_2. Our results confirm the physiological capacity of PII larvae for net growth, albeit at slower rates, in environmental conditions that are thermodynamically unfavourable for calcification (i.e. $\Omega_{arag} < 1.0$). Our results also demonstrated differences in larvae survival, settlement and early spat mortality. Higher proportions of larvae grown under low CO_2/high pH survived to competency and settled successfully than under high CO_2. Furthermore, spat mortality was lower in low-CO_2 treatments. Importantly, despite such differences, our results do not suggest any sort of catastrophic failure of D-stage and PII larvae when exposed to conditions that are undersaturated with respect to aragonite, conditions that are common and widespread in estuaries.

These results stand in stark relief to previous experiments where shell malformation and extensive larval mortality were well documented when oyster larvae were exposed to high CO_2 and aragonite undersaturation (e.g. Kurihara *et al.*, 2007; Talmage & Gobler, 2010; Barton *et al.*, 2012; Waldbusser *et al.*, 2013; Gobler *et al.*, 2014). However, we contend that such disparities can be explained when the oyster larvae's life history is taken into consideration; fertilized eggs, gastrula and trochophores lack the physiological resiliency possessed by the D-stage (PI) and PII larvae, and these two important developmental stages possess fundamentally different mechanisms by which calcium carbonate shells are produced.

Shelled molluscs are among the most well studied of all marine invertebrates, yet we are still resolving aspects of larval development

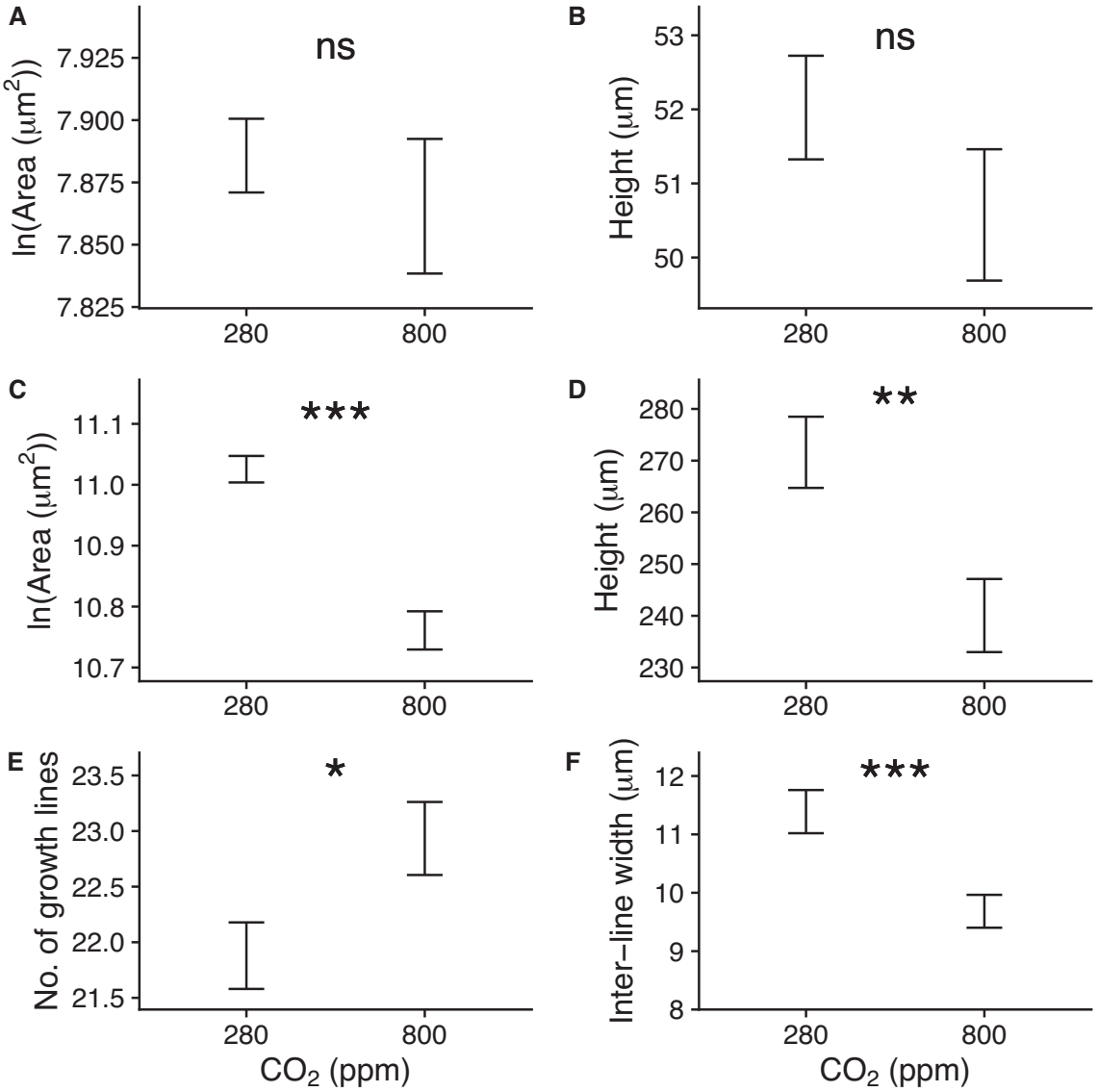

Figure 2. Comparison of larval shell metrics used in the study. **A.** D-stage larval shell ln(Area). **B.** D-stage larval height. **C.** PII total ln(Area). **D.** PII height. **E.** Number of growth lines/shell. **F.** Inter-line width. Plots include mean value ± 1 SEM for low-CO_2 (280 ppm) and high-CO_2 (800 ppm) treatments. Significance of results from Welch's two-sample *t*-tests is indicated by one ($P < 0.05$), two ($P < 0.01$) and three ($P < 0.001$) asterisks. Nonsignificant results are indicated as 'ns'.

(Aranda-Burgos *et al.*, 2014). In particular, the presence of growth lines in the PII stage in some taxa has received little attention. These features have been posited as diagnostic among larval types (i.e. planktotrophic, lecithotrophic and direct developers) by Ockelman (1965). The number of lines has been hypothesized to correspond to the age of larvae in *Ostrea chilensis* and *O. edulis*, although the influence of environmental conditions on growth line production in bivalves was reported as unknown by Millar (1968). Daily growth lines were observed in larvae of the sea scallop *Placopecten magellanicus* by Hurley, Tremblay & Couturier (1987), and larval growth lines were used to postulate planktotrophy in fossil inoceramid bivalves that flourished during the Cretaceous, with an estimated 50-d planktotrophic phase (Knight & Morris, 1996). More recently, major and minor growth lines were detected on larvae of the fan mussel *Atrina fragilis*, major lines being attributed to daily growth (Stirling *et al.*, 2018). Our results suggest that while the rate at which growth lines are generated may be an intrinsic larval shell characteristic, the distance between major growth lines (growth rate) is variable and can be clearly influenced by the environment. In this larger context, our findings suggest that growth lines may be an alternative and more precise indicator of larval age than shell height or area, the measurements commonly used to estimate age.

A variety of previous studies have observed distinctive embryonic larval shell abnormalities (e.g. deformed hinge development) and much higher than expected mortality in a range of bivalves exposed to high CO_2 (e.g. Talmage & Gobler, 2010; Barton *et al.*, 2012; Waldbusser *et al.*, 2013; Gobler *et al.*, 2014), but no such elevated mortality was found for either *C. virginica* or *C. ariakensis*, when healthy D-stage larvae were cultured under high CO_2 and $\Omega_{arag} < 1.0$ (Miller *et al.*, 2009). It must be noted that apart from Miller *et al.* (2009) these studies subjected larvae to high CO_2 and $\Omega_{arag} < 1.0$ from just minutes to hours after fertilization (i.e. prior to the transition from trochophore to veliger), highlighting the apparent pH susceptibility of early embryonic bivalve larvae, as was suggested by Kurihara *et al.* (2007). When Ginger *et al.* (2013) strip-spawned adult *C. gigas* taken from the Yellow Sea in

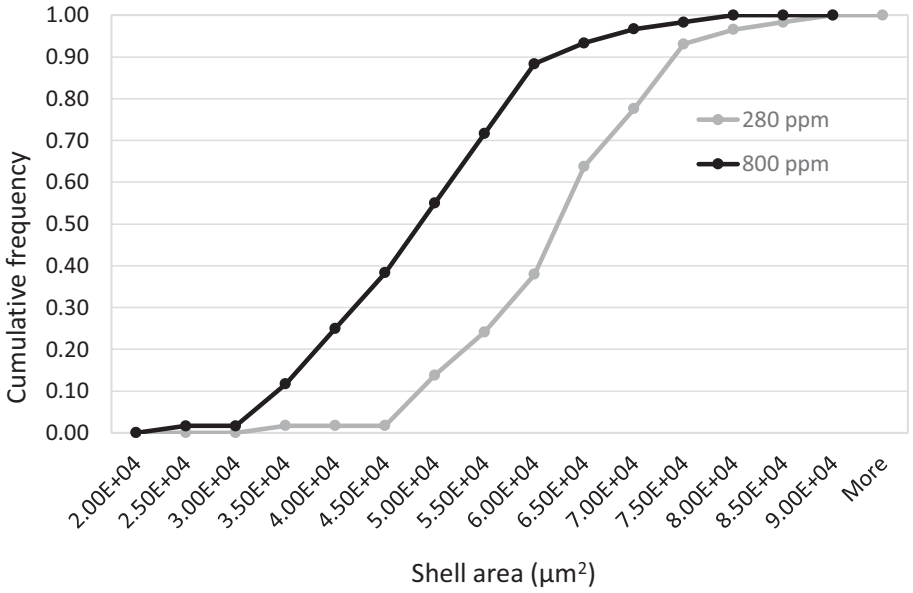

Figure 3. Cumulative frequency distributions of total shell areas (μm^2, one valve) of larvae grown under 280 ppm $pCO_2/\Omega_{arag} = 1.2$ ($n = 58$ shells) and 800 ppm $pCO_2/\Omega_{arag} = 0.6$ ($n = 60$ shells).

the Western Pacific and experimentally incubated D-stage larvae to settlement, they found no significant reduction in survivorship in low-pH treatments (pH = 7.66, $\Omega_{arag} < 0.82$). They concluded that *C. gigas* from the Yellow Sea, unlike populations from Australia, Europe, Japan and the USA, may be pre-adapted to a broader pH range and therefore tolerant of acidification conditions projected in the coming centuries.

Although differences in physiological tolerance almost certainly exist among populations, we hypothesize that differences in pH susceptibility (or aragonite saturation state) of early- and late-stage larvae may better explain the discrepancies among acidification studies of bivalve larvae (Miller *et al.*, 2009; Talmage & Gobler, 2010; Barton et al., 2012; Ginger *et al.*, 2013; Waldbusser *et al.*, 2013; Gobler *et al.*, 2014). As such, the timing of a physiological challenge is critical to the larval response. In this light, Ginger *et al.*'s (2013) results correspond well with those from our present study and our past work (Miller *et al.*, 2009), indicating that larvae of at least three congeneric oyster species (*C. virginica*, *C. ariakensis* and *C. gigas*), once having reached the PI stage and ceased calcification of the embryonic shell field, may attain a stage refuge from low-pH conditions that can be lethal for pre-veliger larvae.

In the wild, planktotrophic larvae encounter a mosaic of environmental conditions, some favourable and others not. Such variability can be especially pronounced in estuaries, where carbonate chemistry is known to be far more variable and extreme than fully marine settings (Frankignoulle *et al.*, 2001; Abril *et al.*, 2006; Joesoef *et al.*, 2015), and frequently driven by biological forcing (Lowe *et al.*, 2019). Planktotrophic larvae sample the environment as they drift or swim through a gauntlet of variable water qualities prior to settlement and metamorphosis. Although fertilization and early larval development in high CO_2 and $\Omega_{arag} < 1.0$ may result in significant larval mortality, if larvae have stage-specific vulnerability/resilience, encountering these conditions during post-trochophore stages (Pl and PII) may pose lower risk to individuals than if they had been exposed to such conditions as trochophores. Daily growth lines recorded in wild-caught larval shells should capture the history of larval experience, reflecting both challenging and favourable environmental conditions.

Our results and conclusions highlight the importance of timing and larval stage when designing experiments and interpreting results. Although laboratory-based studies have the distinct advantage of controlling for multiple factors, they are always simplifications of what occurs in natural environments. Lab experiments often reflect the physiological capacity of an organism to respond to isolated chemical and/or physical drivers rather than providing definitive evidence for how an organism will respond in natural settings. There have been significant advances made in understanding how hatchery success can be optimized through closer attention and management of the carbonate chemistry of the water in which larvae are reared; however, caution should be exercised when extrapolating these (or any laboratory) results too extensively to the natural environment. The larval experience, and ultimately the success or failure of larvae to recruit, is expected to be shaped in part by the specific carbonate chemistry conditions and timing of encounter. Elucidation of the larval experience is a complex and thorny challenge. Connecting larval sources and experiences with adult benthic communities/larval sources (i.e. multiphasic lifestyles) has been a difficult and recurring theme in larval ecology for well over a century (see Grosberg & Levitan, 1992), yet these relationships may be critical to understanding and predicting how shellfish larvae will respond to the shifting carbonate chemistry regimes of the future.

Interrogation of trace and minor chemical element ratios in larval shells via inductively coupled plasma mass spectrometry has enabled researchers to robustly discriminate the natal origins of D-stage larvae of *Mercenaria mercenaria* that were supplied from three separate shellfish hatcheries in the Mid-Atlantic (Cathey, Miller & Kimmel, 2014). Haley *et al.* (2018) developed methods to isolate and measure trace metal (Sr/Ca, Mg/Ca) compositions in the PI portions of larval shells of *C. gigas* in relation to the ambient water they were reared in. Their findings indicate that trace metal ratios deviate from the ambient water at the early rapid PI calcification stage but converged as PII calcification proceeded over 20 d. Chemical finger printing and fine-scale analytical techniques show promise for distinguishing the natal origins of larvae collected in the wild and to better resolve larval sources and sinks. Such efforts may be further enhanced if combined with larval growth line structures to allow investigators to recreate the specific chemical histories of individual planktotrophic bivalve larvae.

Even in the absence of robust information on larval sources and sinks, the apparent stage-specific vulnerability of bivalve larvae may be an important consideration when contemplating oyster or other

shellfish habitat restorations. Just as juvenile and adult bivalves likely tolerate harsh environmental conditions in ways that larvae cannot, the choice of restoration location may be critical if early- and late-stage bivalve larvae respond differently to elevated CO_2. For example, in an estuary the location of a restored oyster reef will represent the natal habitat where local fertilization takes place. If the local conditions of a reef have elevated CO_2/poor Ω_{arag}, then developing pre-veliger larvae may be especially vulnerable, leading to an inhibition of self-recruitment. However, the same local conditions may pose far less risk to late-stage larvae whose natal grounds are at a distance that enjoy more favourable carbonate chemistry. We therefore encourage the inclusion of CO_2 or pH in the decision matrices used for siting oyster reef or other shellfish habitat restorations.

Upwelling of acidic water has been posited as the negative forcing function responsible for *C. gigas* larval failure in shellfish hatcheries in the Pacific Northwest (Feely *et al.*, 2008; Barton *et al.*, 2012). In this setting, *C. gigas* has been held up as a sort of canary in the coal mine for ocean acidification. Although *C. gigas* has existed in the Pacific coastal waters of the USA for a century, this is a non-native species and has a relatively limited evolutionary history there (Ruesink *et al.*, 2005). In contrast, the native Olympia oyster *Ostrea lurida* has evolved under natural upwelling conditions for millennia. Larval recruitment of *O. lurida* was quantified by Wasson *et al.* (2016) in estuaries across a 2,500-km stretch of the USA's Pacific coast. It was observed that recruitment was extremely variable across estuaries in any given year but also extremely variable within single estuaries across years. Nevertheless, recruitment synchrony tended to occur more frequently within estuaries that were more environmentally homogeneous and recruitment was correlated positively with upwelling of nutrient-rich (and acid-rich) waters. Unlike the planktotrophic *C. gigas*, *O. lurida* broods its early-stage larvae internally and then releases fully formed D-stage veligers. Pritchard *et al.* (2015) have hypothesized that internal fertilization and extensive incubation of early embryological development could improve the capacity of *O. lurida* to escape the negative effects of acidification, a notion that coincides with our stage-based larval vulnerability model. Whether or not larval brooding is an evolutionary strategy to avoid stage-based larval vulnerability in highly variable environments, such as upwelling coast lines and spatially heterogeneous estuaries, remains an open question.

As we contemplate the many ways in which coastal oceans and their biota may be affected by ocean acidification, it is clear that predictive coastal carbonate chemistry models for such ecosystems lag far behind those for open ocean settings. Recognition of stage-based vulnerability of larvae to acidification in *C. virginica* (and possibly a wider range of ecologically and economically important coastal shellfish species with planktotrophic larvae) reveals yet another layer of biological complexity that comes into play in dynamic coastal oceans. Life-stage considerations are crucial when attempting to scale up from individual to population responses and therefore should be taken into consideration when making biological and ecological predictions under future acidification scenarios.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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