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FEATURE ARTICLE

Diurnal fluctuations in CO_2 and dissolved oxygen concentrations do not provide a refuge from hypoxia and acidification for early-life-stage bivalves

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ABSTRACT: This study assessed the effects of constant and diurnally fluctuating acidification and hypoxia on the survival, growth, and development of larval stages of 3 bivalves indigenous to the east coast of North America: bay scallops *Argopecten irradians*, hard clams *Mercenaria mercenaria*, and eastern oysters *Crassostrea virginica*. Bivalves were exposed to ideal (pH = ~7.9, dissolved oxygen [DO] = ~7 mg l^{-1}), acidified (pH = ~7.2, DO = ~7 mg l^{-1}), hypoxic (pH = ~7.9, DO = ~2 mg l^{-1}), and acidified and hypoxic (pH = ~7.2, DO = ~2 mg l^{-1}) conditions, as well as treatments that fluctuated between ideal conditions by day and acidified, hypoxic, or acidified and hypoxic conditions by night. Continuously acidified conditions reduced survival of larvae of all 3 species, slowed growth of larval bay scallops and eastern oysters, and delayed the development of bay scallop larvae. Continuously hypoxic conditions reduced the survival, growth, and development of larval bay scallops and slowed the development of larval hard clams. Simultaneous exposure to continuously low pH and DO yielded more negative effects than each factor independently. Diurnal exposure to low pH and/or low DO rarely altered, and never fully mitigated, the negative effects of hypoxia and/or acidification despite significantly higher mean pH and DO levels. This suggests that pH and DO fluctuations were too intense, and/or the durations of normoxic and normcapnic conditions were not long enough for bivalve larvae to overcome the physiological stress of hypoxia and acidification. Therefore, the diurnal fluctuations of pH and DO in this study did not provide a temporal refuge from hypoxia and acidification for North Atlantic bivalve larvae, suggesting that such fluctuations in an ecosystem setting can be a significant threat to these larvae.

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Periods of higher DO and pH within diurnal fluctuations do not provide a temporal refuge from hypoxia and acidification for larvae of bivalves such as the adult bay scallop *Argopecten irradians* shown here. Such fluctuations can represent a significant environmental threat at the ecosystem level.

Photo: Stephanie Talmage-Forsberg

KEY WORDS: Ocean acidification · Hypoxia · Estuary · Bivalve · Larvae · Ecosystem metabolism · Diurnal patterns

INTRODUCTION

During the past 2 centuries, the ocean has assimilated nearly 30% of anthropogenic CO_2 emissions, leading to declining levels of pH, carbonate (CO_3^{2-}) ions, and saturation state of calcium carbonate (Sabine et al. 2004, Caldeira & Wickett 2003). Coastal ecosystems are also susceptible to excessive CO_2 from eutrophication (Wallace et al. 2014). Algal growth stimulated by excessive nutrients delivers organic matter to

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bottom waters, where microbial respiration driven by the degradation of this organic matter can deplete oxygen, produce CO_2, and acidify the water (Cai et al. 2011, Melzner et al. 2013, Wallace et al. 2014). While hypoxia in coastal zones has been studied for decades, the concurrent acidification of hypoxic zones has only recently been well-documented (Cai et al. 2011, Melzner et al. 2013, Baumann et al. 2015).

Although there is consensus that hypoxia in coastal systems is exacerbated by anthropogenic nutrient loading (Diaz 2001, Rabalais et al. 2002, Breitburg et al. 2009), there is some debate regarding how atmospherically driven ocean acidification will manifest in coastal regions. Unlike the open ocean, where pH remains relatively constant, coastal systems are typically less buffered (Cai & Wang 1998, Wang et al. 2013) and biological activity, combined with other variables, can drive pH fluctuations on diurnal and/or seasonal timescales (Ringwood & Keppler 2002, Wootton et al. 2008, Waldbusser & Salisbury 2014). During the day, photosynthetic activity produces oxygen, consumes CO_2, and increases pH, whereas at night, respiration becomes the dominant metabolic reaction consuming oxygen, producing CO_2, and decreasing pH (Yates et al. 2007, Wootton et al. 2008). Some have hypothesized that this metabolic control on pH is so great that the effects of ocean acidification will be small relative to the fluctuating diurnal acidification in estuaries and coastal waters (Duarte et al. 2013), or that metabolic activity driven by eutrophication may overwhelm the effects of ocean acidification in coastal surface waters (Borges & Gypens 2010). Alternatively, it has been suggested that ocean acidification will affect the magnitude of metabolically driven fluctuations in pH by decreasing the baseline pH of coastal systems (Miller et al. 2009, Feely et al. 2010). Coastal systems, being less buffered (Cai & Wang 1998, Wang et al. 2013), may then experience changes from ocean acidification before changes are observed in the open ocean (Waldbusser et al. 2011). In addition, Sunda & Cai (2012) predicted that future CO_2 concentrations in eutrophic coastal systems will increase nonlinearly from the combined delivery of CO_2 from the atmosphere and respiration. Cai et al. (2011) compared models of the pre-industrial and present-day conditions in the Gulf of Mexico and reported a decrease in pH of 0.45 units, noting that acidified ocean waters and eutrophication-driven respiration contributed to the decrease by 0.11 and 0.29 pH units, respectively. Records of pH in the waters surrounding Tatoosh Island, WA, USA, showed a significant decrease from 2000 to 2007 despite the presence of large diurnal fluctuations as a result of metabolism (Wootton et al. 2008).

Hypoxia increases stress and mortality in shellfish and other organisms (Diaz & Rosenberg 1995, Vaquer-Sunyer & Duarte 2008, Breitburg et al. 2009). Tolerance to hypoxia, however, can vary among shellfish species and age, and can interact with other stressors (Wu 2002, Zhang et al. 2010). Earlier life stages of bivalves are generally more vulnerable to low oxygen (Wang & Widdows 1991, 1993, Gobler et al. 2014). In addition, some bivalves undergo anaerobic respiration as dissolved oxygen (DO) concentrations decline, an adaptation that may permit greater resistance to the effects of low DO (Wu 2002). Importantly, however, the potential importance of anaerobiosis for bivalves increases with age and can only comprise 10% of total metabolism within post-larval individuals (Wang & Widdows 1991, 1993), a fact that may further account for the higher vulnerability of early life stages to hypoxia and acidification (Gobler et al. 2014).

Acidification can be inhibitory to calcifying bivalves, potentially inducing mortality, delayed metamorphosis, and slowed growth and calcification rates in early life stages (Talmage & Gobler 2009, 2010, Gazeau et al. 2013, Waldbusser et al. 2013, White et al. 2013). Less is known regarding the effects of the simultaneous exposure of low DO and low pH on shellfish. Some studies have found that low oxygen effects tend to dominate over pH effects (Kim et al. 2013), while others have found that the combined effects of acidification and hypoxia are more severe than the effects of each individual stressor, and that impacts in bivalves can be age-dependent (Gobler et al. 2014, Steckbauer et al. 2015). Keppel et al. (2015) examined how diurnal cycling of DO and pH affected disease acquisition in eastern oysters *Crassostrea virginica*, and found that diurnal cycling of DO increased the acquisition and progression of *Perkinsus marinus* infections during exposure and had a legacy effect the next year.

The goal of this study was to quantify the effects of static and diurnally fluctuating low DO and low pH on larval bay scallops *Argopecten irradians*, hard clams *Mercenaria mercenaria*, and eastern oysters *C. virginica* native to North Atlantic estuaries. While the effects of hypoxia or acidification on early-life-stage bivalves are well-known, their combined effects have been poorly studied, despite their frequent co-occurrence in estuaries (Cai et al. 2011, Melzner et al. 2013, Wallace et al. 2014). As coastal systems often experience metabolically driven, diurnal cycles of pH and DO, this study investigated how early-life-stage bivalves responded to diurnal patterns of acidification and hypoxia. Although high partial pressure of CO_2 (pCO_2) and low DO concentrations are known to

negatively affect larval bivalves, it was hypothesized that the negative effects would be lessened or mitigated when exposure to such conditions was in the form of repeated, short-term diurnal cycles. Physiological adaptations of shellfish, such as acid–base regulation and metabolic depression (Wu 2002, Michaelidis et al. 2005), may allow these bivalves to tolerate excursions into hypercapnic and hypoxic conditions, and once favorable conditions return, compensatory growth may occur and development may continue uninhibited.

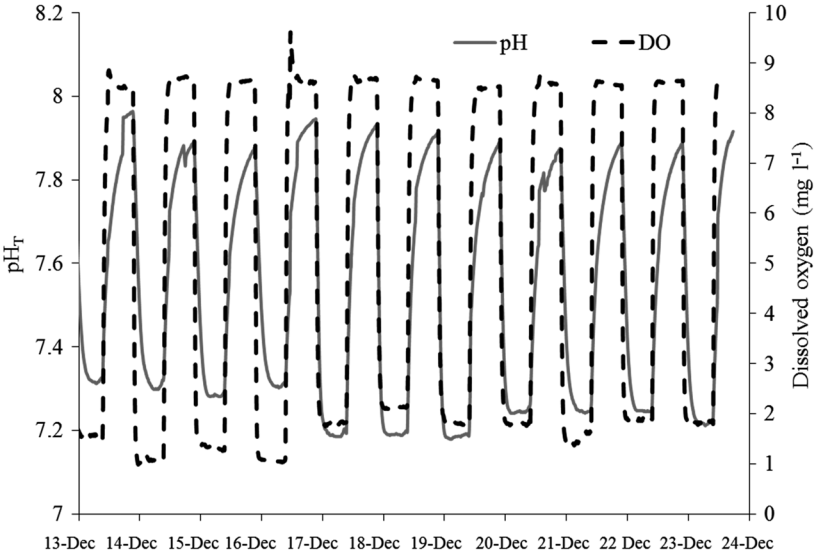

Fig. 1. Example of diurnal fluctuations in pH (on the total scale) and dissolved oxygen (DO) concentrations from the experiment with *Argopecten irradians* larvae

MATERIALS AND METHODS

Manipulation of pH and DO

Replicate (n = 4) 8 l polyethylene vessels were used for experiments and filled with UV-sterilized, 0.2 µm filtered seawater from Old Fort Pond in Shinnecock Bay, NY, USA (salinity = 30). A constant temperature of 23 to 24°C was maintained by partially submerging the experimental vessels in a water bath heated by a Delta Star heat pump. Experiments manipulating pH and DO were performed with each species of bivalve larvae, whereby control (pH = ~7.9, DO = ~7.0 mg l^{-1}), low pH and normal DO (~7.2, ~7.0 mg l^{-1}, respectively), normal pH and low DO (~7.9, ~2.0 mg l^{-1}, respectively), and combined low pH and DO (~7.2, ~2.0 mg l^{-1}, respectively) treatments were maintained by bubbling mixtures of air and tanked 5×10^4 ppm CO_2, pure N_2, and a 400 ppm CO_2+N_2 mix into experimental vessels (Gobler et al. 2014). Additional treatments of diurnally fluctuating pH, diurnally fluctuating DO, and combined diurnally fluctuating pH and DO were also carried out, where pH, DO, or both parameters oscillated between control and low conditions every 12 h (details below; Fig. 1), creating a mean pH and DO exposure level higher than the continuously low DO and/or pH exposures. Both the absolute levels of pH and DO, and the diurnal patterns of pH and DO applied in experiments, were consistent with prior observations in eutrophic estuaries (Ringwood & Keppler 2002, Melzner et al. 2013, Wallace et al. 2014, Baumann et al. 2015). Further, the level of DO and pH selected were previously found to be harmful to bivalve larvae individually (Wang & Widdows 1991, 1993, Vaquer-Sunyer & Duarte 2008, Talmage & Gobler 2010), but have yet to be explored in unison under static or fluctuating conditions.

The delivery rate of gases was controlled with a series of Cole-Parmer gas regulators, single-tube flowmeters, and/or multi-tube gas proportioners. Carbon dioxide gas was used to control pH and nitrogen gas to control DO concentrations (Gobler et al. 2014). To produce diurnal changes in pH and DO concentrations, ITT Alcon solenoid valves were attached to the compressed gas tanks and ambient air lines, and were controlled with a Rain Bird timer. During the day cycle (09:00 to 21:00 h), the valves on the ambient airlines were opened and the valves on the other mixes of gas were closed to create control pH and DO conditions. At night (21:00 to 09:00 h), the valves on the appropriate CO_2, N_2, or CO_2+N_2 gas tanks were opened to create low pH and/or DO conditions.

Measuring environmental conditions

Salinity was measured using a YSI 600QS multiparameter water quality sonde and temperature logged every 15 min on a HOBO U-002-64 data logger (Onset). The relative standard deviation of replicated temperature and salinity measurements was <1%, whereas the accuracy was ±0.15°C and 0.1 psu, respectively. Daily measurements of pH were made with a Honeywell Durafet Ion Sensitive Field Effect Transistor (ISFET)-based pH sensor calibrated with a seawater pH standard (Dickson 1993) and logged every 15 min in the diurnal treatments with a

Thermo-Scientific Orion STAR A321 pH meter. The relative standard deviation of replicated Durafet and Orion pH measurements was <1%, whereas the accuracy was ±0.005 and 0.002 units, respectively. A Clark-type electrode YSI 5100 oxygen meter was used to make daily DO measurements in all experimental vessels and DO was logged every 15 min on a HOBO U26 dissolved oxygen logger (Onset) in the diurnal treatments. The relative standard deviation of replicated dissolved oxygen measurements was <1%, whereas the accuracy was <0.1%. Previous studies have found that these instruments measure levels of DO that are indistinguishable from discrete measurements made with Winkler titrations (Gobler et al. 2014).

Dissolved inorganic carbon (DIC) measurements were made at the beginning and end of each experiment using a Liqui-Cel Membrane (Membrana) to separate the gaseous DIC from the seawater, which was then quantified with an EGM-4 Environmental Gas Analyzer (PP Systems) system. For all diurnal fluctuation treatments, samples were collected and analyzed from the end of both a day and night cycle. To determine the precision and accuracy of this technique, Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material for DIC was analyzed during each analytic run (percent recovery of DIC across all analytical runs: 103 ± 6%, mean ± SD). DIC levels, along with pH, temperature, salinity, pressure, phosphate, silicate, and carbonic acid dissociation constants recommended for estuarine waters (Millero 2010), were analyzed with the CO2SYS program (http://cdiac.ornl.gov/ftp/co2sys/) in order to quantify levels of pCO_2, saturation states of calcite and aragonite ($\Omega_{calcite}$, $\Omega_{aragonite}$, respectively), carbonate, and total alkalinity. Since pH values in some experimental treatments were made to fluctuate widely each day, and as pH is on a log scale, mean pH values were determined by first converting pH to $[H^+]$ concentrations. Mean $[H^+]$ concentrations were then converted back to pH.

Larvae

This study examined the effects of low pH and low DO on larval stage bay scallops *Argopecten irradians*, hard clams *Mercenaria mercenaria*, and eastern oysters *Crassostrea virginica* obtained from the East Hampton Town Shellfish Hatchery located in Montauk, NY, USA. Broodstock were collected from mesotrophic regions of eastern Long Island estuaries (Shinnecock and Peconic Bays) in accordance with New York State Department of Environmental Conservation Collector's Permits. Adults were conditioned following the Food and Agriculture Organization of the United Nations' (FAO) protocol for shellfish aquaculture, being fed ~3% of their dry weight in algae for 6 to 8 wk and maintained at a temperature of 18°C (Helm et al. 2004). The algal diet included a mixture of the phytoplankton *Isochrysis galbana*, *Tetraselmis suecica, T. chuii, Chaetoceros muelleri, C. calcitrans*, and *Pavlova lutheri* in equal biovolumes (Helm et al. 2004). Adult shellfish were then temperature-spawned, and fertilized embryos were collected.

Each experimental vessel was stocked with 10 000 D-stage larvae less than 24 h old. All larvae were fed a diet of 4×10^4 cells ml^{-1} of *I. galbana* daily (Carriker 2001, Helm et al. 2004, Cragg 2006). Full water changes were performed twice per week, during which all contents of each experimental vessel were poured through a 64 µm sieve. Larvae collected on the sieve were condensed into a 50 ml container from which 2 ml were removed and preserved with a 3% solution of buffered formalin phosphate to assess mortality, size (distance from tip of the umbo to ventral side), and developmental stage (veliger, pediveliger, or metamorphosed) at each time point using a dissecting microscope with Nikon DigiSight Color Digital Camera System (DSVi1) and ImageJ software. Larvae that were alive at the time of preservation were counted to determine the rate of mortality in each treatment, and were distinguishable from dead larvae by pigmentation and morphology. Percent metamorphosis was calculated, based on the total number of surviving larvae at each time point, and larval experiments continued until all individuals in the control treatment had metamorphosed. Owing to the propensity of *C. virginica* larvae to set irreversibly on surfaces when metamorphosed, this experiment was ended after 14 d, prior to the larvae metamorphosing into juveniles, and thus metamorphosis was not documented.

Data analysis

Statistical analyses were performed in RStudio. Survival and development data were arcsine square root transformed before analysis. Two-way ANOVAs were performed on the survival, development, and growth rate data, where pH and DO exposure levels (normal, chronically low, or diurnal) were the main treatment effects. While the experimental design was

not fully balanced, all assumptions were met for these parametric tests. The Shapiro-Wilks test was used to assure all data were normally distributed and Bartlett's test was used to assure homogeneity of variance among each dataset. Two-way ANOVAs reporting significant effects from treatments were followed with Tukey's HSD test for all pairwise comparisons.

RESULTS

Argopecten irradians (bay scallop) larvae

There was a significant negative effect of pH (2-way ANOVA: $p < 0.001$) and DO ($p < 0.001$) on survival of *Argopecten irradians* larvae, and an interaction between the factors ($p < 0.05$), with all manipulated conditions significantly reducing survival relative to the control condition (Table 1, Fig. 2). Percent survival for the control, low pH, low DO, low pH–DO, diurnal pH, diurnal DO, and diurnal pH–DO conditions was 38 ± 2, 15 ± 4, 25 ± 6, 5 ± 6, 12 ± 3, 17 ± 5, and 7 ± 2%, respectively (Fig. 2A). Survival under continuously low DO was higher than the low pH ($p = 0.006$), diurnal pH ($p < 0.001$), and diurnal pH–DO ($p < 0.001$) conditions, though still significantly lower than the control treatment ($p = 0.009$). The interaction between DO and pH was most apparent in the diurnal pH–DO treatment, where the survival (7 ± 2%) was higher than would have been predicted by the reductions in survival in the individual diurnal pH and diurnal DO treatments (12 ± 3 and 17 ± 5%, respectively).

Growth rates of *A. irradians* larvae were affected by both pH (2-way ANOVA: $p < 0.001$) and DO ($p < 0.001$), but there was no interaction between the factors. Larvae experienced significantly slowed growth under all manipulated conditions, except for the diurnally fluctuating pH treatment (Fig. 2B). Control larvae grew at a rate of 13 ± 1 µm d^{-1}, while rates were slowed in the low pH–DO ($p < 0.001$), low pH ($p < 0.001$), diurnal pH–DO ($p < 0.001$), diurnal DO ($p = 0.018$), and low DO ($p = 0.024$) treatments. Both pH ($p < 0.001$) and DO ($p < 0.001$) slowed development of *A. irradians* larvae (2-way ANOVA). Fourteen days post-fertilization, 67 ± 5% of larvae had metamorphosed under control conditions (Fig. 2C). Continuously low pH ($p < 0.001$) and continuously low DO ($p = 0.026$) significantly reduced metamorphosis

Table 1. Mean (±SD) pH (pH_T = pH on the total scale), dissolved oxygen (DO), pCO_2, saturation states of calcite ($\Omega_{calcite}$) and aragonite ($\Omega_{aragonite}$), total dissolved inorganic carbon (DIC), carbonate, total alkalinity (TA), salinity, and temperature for larval *Argopecten irradians* diurnal acidification and hypoxia experiment. For diurnal treatments, conditions measured in the middle of the day and middle of the night cycles are depicted, along with a mean of the entire experiment

Parameter	Continuous				Diurnal pH			Diurnal DO			Diurnal pH–DO		
	Control	Low pH	Low DO	Low pH–DO	Day	Night	Mean	Day	Night	Mean	Day	Night	Mean
pH_T	7.91 ± 0.02	7.20 ± 0.09	7.91 ± 0.02	7.24 ± 0.11	7.83 ± 0.13	7.16 ± 0.11	7.47 ± 0.23	7.95 ± 0.06	7.95 ± 0.06	7.90 ± 0.02	7.90 ± 0.06	7.28 ± 0.08	7.58 ± 0.25
DO (mg l^{-1})	6.87 ± 0.33	6.96 ± 0.74	2.33 ± 0.64	2.19 ± 0.92	6.84 ± 0.19	6.77 ± 0.53	6.80 ± 0.57	6.82 ± 1.70	1.43 ± 0.34	4.24 ± 2.80	7.24 ± 0.12	1.30 ± 0.28	4.28 ± 2.90
pCO_2 (µatm)	516 ± 9.0	3480 ± 190	548 ± 56	3290 ± 234	586 ± 37	3180 ± 181	1880 ± 1390	573 ± 91	515 ± 21	544 ± 69	623 ± 41	2930 ± 241	1770 ± 1250
$\Omega_{calcite}$	3.00 ± 0.12	0.60 ± 0.07	2.93 ± 0.23	0.61 ± 0.08	2.61 ± 0.11	0.65 ± 0.11	1.63 ± 1.10	2.78 ± 0.09	2.80 ± 0.12	2.79 ± 0.10	2.48 ± 0.02	0.69 ± 0.13	1.59 ± 0.96
$\Omega_{aragonite}$	1.94 ± 0.08	0.39 ± 0.05	1.90 ± 0.15	0.39 ± 0.05	1.69 ± 0.07	0.42 ± 0.07	1.05 ± 0.68	1.79 ± 0.07	1.81 ± 0.08	1.80 ± 0.07	1.60 ± 0.02	0.45 ± 0.09	1.03 ± 0.62
DIC (µmol l^{-1})	1800 ± 31	2060 ± 147	1830 ± 91	2010 ± 40	1770 ± 16	2050 ± 129	1910 ± 169	1810 ± 117	1730 ± 31	1770 ± 55	1770 ± 45	2010 ± 140	1890 ± 169
CO_3^{2-} (µmol l^{-1})	120 ± 5.0	24.0 ± 3.0	117 ± 9.4	24.3 ± 3.2	104 ± 4.4	26.2 ± 4.5	65.2 ± 55	111 ± 4.3	112 ± 5.4	111 ± 0.57	99.1 ± 1.5	27.7 ± 5.4	63.4 ± 51
TA (µmol l^{-1})	1970 ± 36	1990 ± 147	1990 ± 93	1950 ± 52	1920 ± 15	1990 ± 137	1950 ± 53	1960 ± 104	1890 ± 34	1930 ± 51	1910 ± 40	1960 ± 150	1940 ± 42
Salinity	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6	29.5 ± 0.6
Temperature (°C)	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3	23.1 ± 0.3

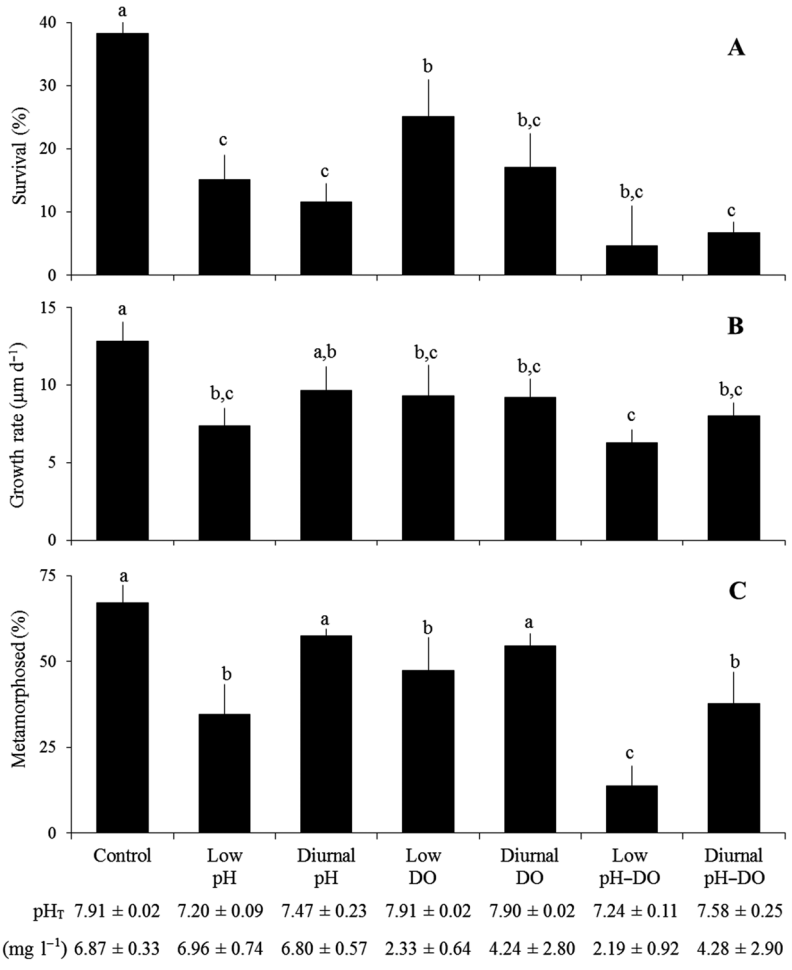

Fig. 2. (A) Survival, (B) growth, and (C) development of *Argopecten irradians* larvae. Percent metamorphosis was calculated 14 d post-fertilization. Error bars represent SD of the mean (n = 4). Mean pH and DO values during the experiment for each treatment are shown, whereas detailed experimental conditions appear in Table 1. Letters above bars denote significant groupings of treatments as indicated via post hoc Tukey's HSD tests

to 35 ± 9 and 48 ± 10%, respectively, but diurnal exposure to low pH and to low DO did not alter the fraction of larvae that had metamorphosed. Metamorphosis was significantly delayed in the low pH–DO treatment ($p < 0.001$) to 14 ± 6%, and to a significantly lesser extent in the diurnal pH–DO treatment (38 ± 9%, $p < 0.001$).

Mercenaria mercenaria (hard clam) larvae

Survival of larval *Mercenaria mercenaria* was significantly reduced by pH (2-way ANOVA: $p < 0.001$) but not DO, and there was an interactive effect of these 2 factors ($p < 0.05$; Table 2, Fig. 3). Under control, chronically low DO, and fluctuating low DO conditions, 28 ± 2, 24 ± 3, and 27 ± 5% of larvae, respectively, survived to the end of the experiment, while survival was significantly reduced to 1 ± 0.4, 2 ± 1, 1 ± 1, and 5 ± 3% in the low pH ($p < 0.001$), diurnal pH ($p < 0.001$), low pH–DO ($p < 0.001$), and diurnal pH–DO ($p < 0.001$) treatments, respectively (Fig. 3A). There was an interaction between pH and DO as the survival in the combined diurnal treatment was higher than would have been predicted by the individual treatments for both the static and diurnal treatments.

There was an effect of pH (2-way ANOVA: $p < 0.05$) and DO ($p < 0.05$) on the growth of *M. mercenaria* larvae, although post hoc multiple comparisons did not reveal differences among treatments (Fig. 3B). Development of *M. mercenaria* larvae was affected by pH (2-way ANOVA: $p < 0.001$) and DO ($p < 0.001$), and there was an interaction between these 2 factors ($p < 0.001$). Fewer larvae metamorphosed 17 d post-fertilization in all pH and DO treatments compared to 80 ± 6% that had metamorphosed in control conditions ($p < 0.001$; Fig. 3C). There was an interactive effect of pH and DO on development, with 21 ± 6 and 31 ± 7% of larvae reaching metamorphosis in the chronically low pH ($p < 0.001$) and chronically low DO ($p < 0.001$) treatments, respectively, and 7 ± 2% in the chronically low pH–DO ($p < 0.001$) treatment, a value higher than would have been predicted by the individual treatments. Although fewer larvae developed to metamorphosis in the low DO and diurnal DO (25 ± 5%, $p < 0.001$ for both) treatments than in the control, there were significantly more metamorphosed larvae in these 2 treatments than in the diurnal pH (9 ± 4%, $p < 0.001$, $p = 0.002$), low pH–DO (7 ± 2%, $p < 0.001$, $p < 0.001$), and diurnal pH–DO (11 ± 3%, $p = 0.001$, $p = 0.034$) treatments.

Crassostrea virginica (eastern oyster) larvae

DO and pH significantly reduced the survival of *Crassostrea virginica* larvae (2-way ANOVA: $p < 0.05$ and $p < 0.001$, respectively) and there was no interaction between these factors (Table 3, Fig. 4). Survival was reduced from 15 ± 5% under control conditions to 3 ± 1, 5 ± 4, and 5 ± 2% in low pH ($p < 0.001$), low

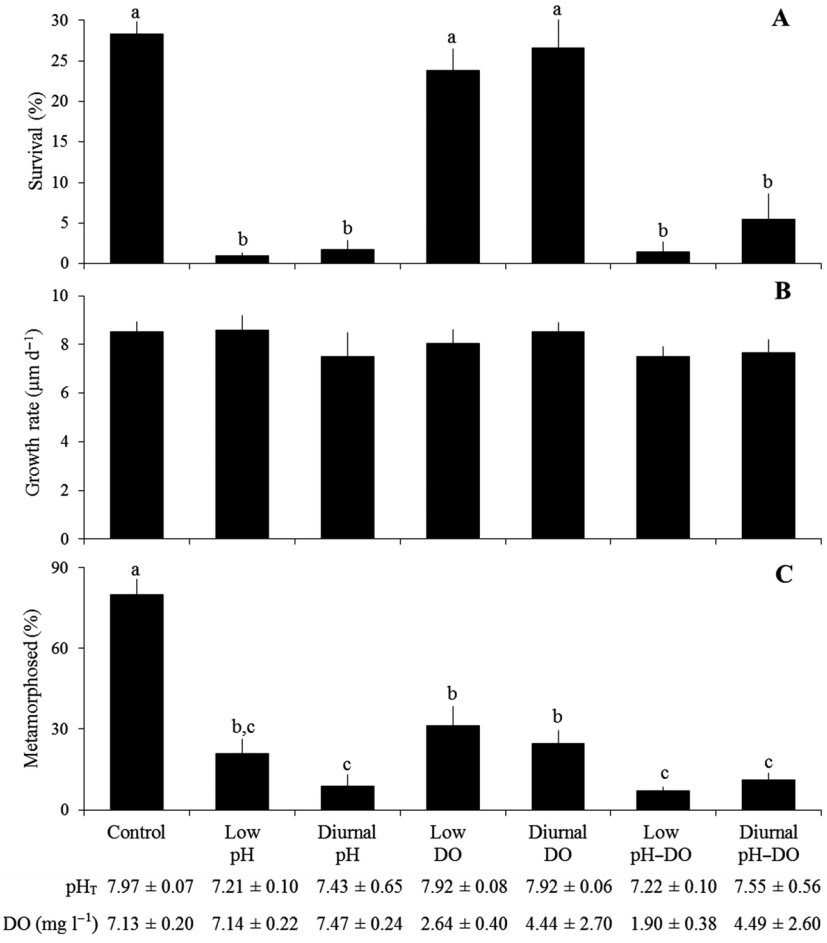

Fig. 3. (A) Survival, (B) growth, and (C) development of *Mercenaria mercenaria* larvae. Percent metamorphosis was calculated 17 d post-fertilization. Error bars represent SD of the mean (n = 4). Mean pH and DO values during the experiment for each treatment are shown, whereas detailed experimental conditions appear in Table 2. Letters above bars denote significant groupings of treatments as indicated via post hoc Tukey's HSD tests

pH–DO ($p = 0.015$), and diurnal pH–DO ($p = 0.017$) treatments, respectively (Fig. 4A). There were no significant survival differences between control conditions and the treatment with diurnal fluctuations in pH or between diurnal and continuous low DO treatment: these survival rates were all significantly higher than in the low pH, low pH–DO, and diurnal pH–DO treatments ($p < 0.05$ for all). Finally, the percent survival of *C. virginica* larvae was significantly higher in the low DO treatment than in the diurnal pH treatment ($p = 0.002$).

There was an effect of pH (2-way ANOVA: $p < 0.001$), DO ($p < 0.05$), and an interactive effect of pH and DO ($p < 0.05$) on the growth rates of *C. virginica* larvae. Growth rates were reduced from 1 ± 0.1 µm d^{-1} in control conditions to 0.7 ± 0.1, 0.3 ± 0.2, 0.2 ± 0.1,

Table 2. Mean (±SD) pH, DO, pCO_2, $\Omega_{calcite}$, $\Omega_{aragonite}$, DIC, carbonate, TA, salinity, and temperature for larval *Mercenaria mercenaria* diurnal acidification and hypoxia experiment. For diurnal treatments, conditions measured in the middle of the day and middle of the night cycles are depicted, along with a mean of the entire experiment

Parameter	Continuous				Diurnal pH			Diurnal DO			Diurnal pH-DO		
	Control	Low pH	Low DO	Low pH–DO	Day	Night	Mean	Day	Night	Mean	Day	Night	Mean
pH_T	7.97 ± 0.07	7.21 ± 0.10	7.92 ± 0.08	7.22 ± 0.10	7.94 ± 0.03	7.16 ± 0.07	7.43 ± 0.65	7.93 ± 0.04	7.92 ± 0.10	7.92 ± 0.06	7.95 ± 0.10	7.29 ± 0.06	7.55 ± 0.56
DO (mg l^{-1})	7.13 ± 0.20	7.14 ± 0.22	2.64 ± 0.40	1.90 ± 0.38	7.49 ± 0.18	7.51 ± 0.33	7.47 ± 0.24	7.35 ± 0.24	1.59 ± 0.38	4.44 ± 2.70	7.32 ± 0.29	1.58 ± 0.37	4.49 ± 2.60
pCO_2 (µatm)	509 ± 70	3580 ± 70	521 ± 62	3100 ± 61	601 ± 54	3130 ± 268	1870 ± 1470	500 ± 9.2	507 ± 9.2	504 ± 6.5	601 ± 54	2860 ± 118	1730 ± 1310
$\Omega_{calcite}$	3.04 ± 0.05	0.66 ± 0.01	3.03 ± 0.16	0.67 ± 0.01	2.60 ± 0.19	0.74 ± 0.06	1.67 ± 1.08	2.85 ± 0.04	2.89 ± 0.08	2.87 ± 0.06	2.50 ± 0.00	0.80 ± 0.02	1.65 ± 0.98
$\Omega_{aragonite}$	1.97 ± 0.03	0.43 ± 0.01	1.97 ± 0.01	0.44 ± 0.01	1.68 ± 0.12	0.48 ± 0.04	1.08 ± 0.70	1.85 ± 0.03	1.87 ± 0.05	1.86 ± 0.04	1.62 ± 0.00	0.52 ± 0.01	1.07 ± 0.64
DIC (µmol l^{-1})	1790 ± 0.56	2180 ± 43	1810 ± 152	2040 ± 8.3	1780 ± 7.5	2160 ± 14	1970 ± 216	1720 ± 2.0	1740 ± 24	1730 ± 24	1740 ± 2.1	2130 ± 21	1930 ± 281
CO_3^{2-} (µmol l^{-1})	122 ± 1.9	26.5 ± 0.52	122 ± 6.5	27.0 ± 0.34	104 ± 7.6	29.9 ± 2.3	67.1 ± 53	115 ± 1.6	116 ± 3.4	115 ± 1.0	100 ± 0.12	32.1 ± 0.76	66.2 ± 48
TA (µmol l^{-1})	1960 ± 3.6	2110 ± 41	1980 ± 155	1990 ± 5.9	1920 ± 5.6	2110 ± 2.8	2020 ± 128	1880 ± 0.77	1910 ± 28	1890 ± 16	1870 ± 2.2	2100 ± 16	1980 ± 157
Salinity	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8	29.2 ± 0.8
Temperature (°C)	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.21	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.21	22.8 ± 0.2

Table 3. Mean (±SD) pH, DO, pCO_2, $\Omega_{calcite}$, $\Omega_{aragonite}$, DIC, carbonate, TA, salinity, and temperature for larval *Crassostrea virginica* diurnal acidification and hypoxia experiment. For diurnal treatments, conditions measured in the middle of the day and middle of the night cycles are depicted, along with a mean of the entire experiment

Parameter	Continuous				Diurnal pH			Diurnal DO			Diurnal pH-DO		
	Control	Low pH	Low DO	Low pH–DO	Day	Night	Mean	Day	Night	Mean	Day	Night	Mean
pH_T	7.85 ± 0.04	7.16 ± 0.07	7.83 ± 0.05	7.18 ± 0.07	7.95 ± 0.03	7.28 ± 0.06	7.54 ± 0.26	8.02 ± 0.04	7.96 ± 0.12	7.99 ± 0.09	7.93 ± 0.04	7.27 ± 0.03	7.50 ± 0.23
DO (mg l^{-1})	7.04 ± 0.16	6.98 ± 0.16	2.50 ± 0.71	1.87 ± 0.41	7.52 ± 0.13	7.59 ± 0.24	7.54 ± 0.18	7.24 ± 0.12	2.91 ± 1.50	5.14 ± 2.09	7.33 ± 0.05	1.66 ± 0.56	4.36 ± 2.79
pCO_2 (µatm)	522 ± 2.7	3380 ± 250	575 ± 52	3480 ± 134	570 ± 16	3230 ± 131	1900 ± 1540	645 ± 64	524 ± 31	585 ± 81	644 ± 16	2990 ± 380	1820 ± 1370
$\Omega_{calcite}$	2.96 ± 0.18	0.54 ± 0.01	2.83 ± 0.29	0.54 ± 0.01	2.62 ± 0.01	0.56 ± 0.00	1.59 ± 1.2	2.70 ± 0.00	2.70 ± 0.01	2.70 ± 0.01	2.46 ± 0.02	0.59 ± 0.08	1.52 ± 1.08
$\Omega_{aragonite}$	1.91 ± 0.11	0.35 ± 0.01	1.83 ± 0.19	0.35 ± 0.01	1.69 ± 0.01	0.36 ± 0.00	1.03 ± 0.76	1.74 ± 0.00	1.74 ± 0.01	1.74 ± 0.01	1.59 ± 0.01	0.38 ± 0.05	0.98 ± 0.70
DIC (µmol l^{-1})	1810 ± 53	1940 ± 53	1840 ± 22	1975 ± 15	1770 ± 15	1940 ± 48	1850 ± 104	1900 ± 91	1720 ± 44	1810 ± 125	1812 ± 14	1890 ± 3.0	1850 ± 56
CO_3^{2-} (µmol l^{-1})	118 ± 7.0	21.4 ± 0.50	113 ± 12	21.5 ± 0.57	104 ± 0.50	22.4 ± 0.18	63.3 ± 58	107 ± 0.14	107 ± 0.58	107 ± 0.10	97.9 ± 0.79	23.3 ± 3.1	60.6 ± 53
TA (µmol l^{-1})	1970 ± 62	1870 ± 44	1990 ± 39	1900 ± 9.6	1910 ± 17	1870 ± 44	1890 ± 23	2040 ± 87	1870 ± 41	1960 ± 119	1940 ± 12	1840 ± 13	1890 ± 74
Salinity	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3	29.3 ± 1.3
Temperature (ºC)	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2	22.8 ± 0.2

and 0.4 ± 0.4 µm d^{-1} in low pH ($p = 0.014$), diurnal pH ($p < 0.001$), low pH–DO ($p < 0.001$), and diurnal pH–DO ($p < 0.001$) conditions, respectively (Fig. 4B). Growth rates of *C. virginica* larvae exposed to chronically low or diurnal fluctuations of DO (1 ± 0.4 and 1 ± 0.2 µm d^{-1}, respectively) did not differ from the control treatment. The interactive effect of pH and DO on *C. virginica* growth rates was most obvious in the diurnal treatments, where exposure to diurnally low pH and DO yielded growth rates higher than would have been predicted by the individual treatments. Metamorphic state was not quantified for *C. virginica* larvae.

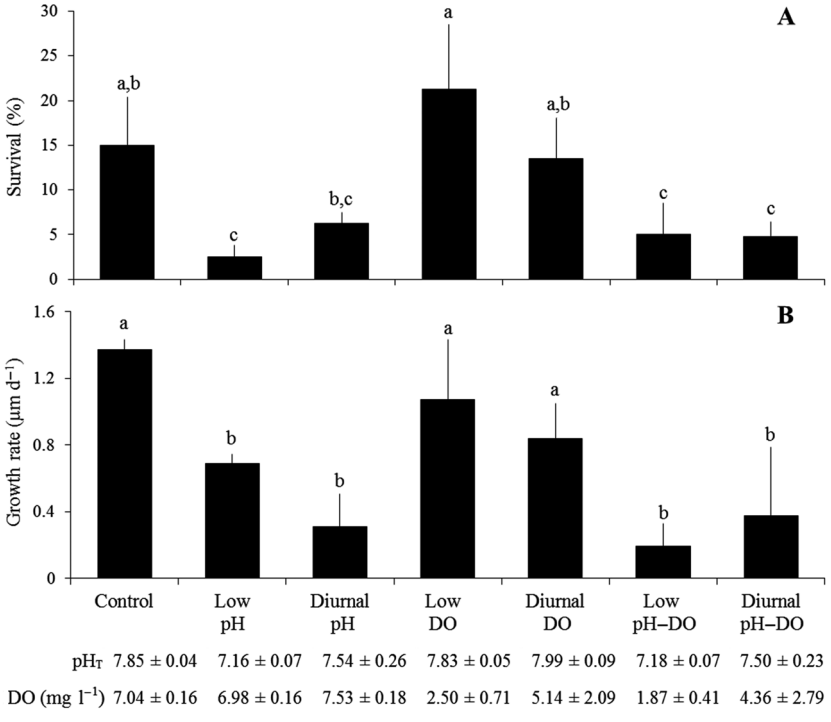

Fig. 4. (A) Survival and (B) growth of *Crassostrea virginica* larvae. Error bars represent SD of the mean (n = 4). Mean pH and DO values during the experiment for each treatment are shown, whereas detailed experimental conditions appear in Table 3. Letters above bars denote significant groupings of treatments as indicated via post hoc Tukey's HSD tests

DISCUSSION

Ocean acidification and hypoxia are expected to worsen as a result of anthropogenic activity (Diaz &

Rosenberg 2008, Breitburg et al. 2009, Doney et al. 2009). Both processes are known to negatively impact a multitude of marine species (e.g. Diaz & Rosenberg 2008, Doney et al. 2009). Biotic controls of pH and DO in highly productive, coastal ecosystems complicate predictions of how ocean acidification and hypoxia will manifest in such environments in the future (Miller et al. 2009, Borges & Gypens 2010, Duarte et al. 2013). To date, nearly all studies of bivalves have reported the independent effects of high CO_2 or hypoxia, with only a few investigating effects of simultaneous exposure. No prior study has investigated the diurnal compared to continuous, as well as independent and combined effects of hypoxia and ocean acidification on bivalves. This study found that continuously low pH and low DO negatively impacted survival, growth, and development of larval bivalves, and the concurrent continuous exposure had both additively negative and interactive effects. Additionally, diurnal exposure to these conditions rarely improved the performance of larvae, despite significantly higher mean pH and DO levels. Collectively, these findings have important implications for understanding the effects of estuarine acidification and hypoxia on marine bivalves.

Continuously acidified conditions reduced survival of larvae of all 3 bivalve species more than low oxygen conditions. Acidification also slowed the growth of larval *Argopecten irradians* and *Crassostrea virginica*, and the development of *A. irradians*. Of the 3 species in this study, *Mercenaria mercenaria* was the most resistant to high CO_2 as its growth was unaffected by pH, a finding consistent with past studies (Talmage & Gobler 2010). Shell formation and development of bivalve larvae is energetically costly and requires an even larger energy input when elevated CO_2 creates unfavorable conditions for the precipitation of calcium carbonate (Palmer 1992, Pörtner 2008, Waldbusser et al. 2013). Waldbusser et al. (2013) reported that *C. gigas* larvae precipitated 90% of their body weight in calcium carbonate within the first 48 h of development, and some bivalve larvae precipitate a form of calcium carbonate that is less stable and more soluble than aragonite and calcite, known as amorphous calcium carbonate (Weiss et al. 2002). The high energetic cost of shell formation under elevated CO_2 conditions may result in energy reallocation away from growth, resulting in smaller larvae with reduced lipid contents (Gobler & Talmage 2013, Waldbusser et al. 2013). Beyond promoting unfavorable conditions for the biomineralization of calcium carbonate, hypercapnia can create other physiological problems for marine invertebrates. Disturbances in acid–base regulation, protein synthesis, and metabolism occur as a result of high CO_2 exposure (Pörtner et al. 2005, Sokolova 2013, Waldbusser et al. 2015), and could further disrupt development and growth or induce mortality.

Coastal eutrophication is expected to worsen as a result of anthropogenic nutrient loading leading to increased size, duration, and severity of hypoxic regions (Diaz & Rosenberg 2008, Rabalais et al. 2009). The results of this study show that continuously hypoxic conditions negatively affected survival, growth, and development of *A. irradians* larvae and slowed the development of larval *M. mercenaria*, demonstrating that while bivalves are some of the more hypoxia-tolerant marine organisms (Diaz & Rosenberg 1995), early life stages are still susceptible to the deleterious effects of low oxygen conditions. Slowed growth has been observed in multiple species of early-life-stage bivalves exposed to hypoxia (Wang & Widdows 1991), including *A. irradians* (Gobler et al. 2014). These negative effects may be a result of tradeoffs encumbered by physiological adaptations employed to survive hypoxia. Reducing oxygen demand via metabolic depression and switching from aerobic to anaerobic metabolism are typical responses to low oxygen availability for many marine invertebrates (Grieshaber et al. 1994, Guppy & Withers 1999, Hochachka & Lutz 2001). Although reduced metabolism and more energetically costly anaerobic metabolic pathways enable an organism to survive hypoxia or anoxia, growth may be inhibited as a result (Wu 2002). Low DO concentrations used in these experiments (~2 mg l^{-1}), while considered hypoxic (Diaz 2001, Rabalais et al. 2002), may still be tolerable to some species. For example, although *A. irradians* larvae suffered reductions in survival, growth, and development under low DO conditions, there was no effect of low DO on survival or growth of *M. mercenaria* and *C. virginica* larvae, thus indicating species-specific tolerance of these conditions. These differences may be a function of the more rapid rates of growth and respiration in *A. irradians* compared to *M. mercenaria* and *C. virginica* (Kennedy et al. 1996, Kraeuter & Castagna 2001, Shumway & Parsons 2006).

Concurrent exposure to acidified and hypoxic water usually yielded a more negative outcome for larvae than each factor alone. Low pH–DO yielded reduced survival in all 3 bivalve species examined, inhibited growth in *A. irradians* and *C. virginica* larvae, and repressed development in *A. irradians* and *M. mercenaria* larvae. The interactive effects of simultaneously low pH and low DO on the survival of

Table 4. Overview of the effects of pH and DO on the survival, growth, and metamorphosis of *Argopecten irradians*, *Mercenaria mercenaria*, and *Crassostrea virginica* larvae. X: treatment that yielded a significantly lower performance compared to the level achieved in the control; –: treatment that yielded a performance that did not differ from the control; ↑: diurnal treatment that yielded a performance significantly better than the continuous treatment, but significantly lower than the control treatment. Diurnal exposure significantly improved performance in 25% of instances, but never fully ameliorated the significant negative effects of low pH and/or low DO on larvae, despite higher mean pH and DO levels

	Low pH	Diurnal pH	Low DO	Diurnal DO	Low pH, DO	Diurnal pH, DO
A. irradians						
Survival	X	X	X	X	X	X
Growth rate	X	↑	X	X	X	X
Metamorphosis	X	↑	X	↑	X	↑
M. mercenaria						
Survival	X	X	–	–	X	X
Growth rate	–	–	–	–	–	–
Metamorphosis	X	X	X	X	X	X
C. virginica						
Survival	X	↑	–	–	X	X
Growth rate	X	X	–	–	X	X

A. irradians and *M. mercenaria* larvae, development of *M. mercenaria* larvae, and growth of *C. virginica* larvae evidenced the complex physiological effects of these stressors on these bivalves. Interactions among multiple stressors may arise when the physiological pathways that the stressors act upon are not entirely independent. For example, exposure to low oxygen could reduce an organism's acid–base regulatory mechanisms, making them more susceptible to acidified conditions (Pörtner 2008). While the combined pH–DO treatments usually yielded an outcome more severe than the individual treatments, the antagonistic effects observed during some experiments indicated that the combined effects were less severe than would have been predicted by the individual variables. This outcome suggests that some of the negative effects of pH and DO emanated from action on similar, rather than independent, physiological pathways. For example, if low pH only affected calcification, and low DO only affected aerobic metabolism, and these pathways were wholly independent of each other, then the combination of low pH–DO would have been strictly additive. Instead, the interactive effects observed suggest there is some level of overlap in the physiological impacts of these stressors, a hypothesis supported by prior studies. Acidification has been shown to reduce the lipid content and RNA:DNA ratios of bivalve larvae (Gobler & Talmage 2013), suggesting a more universal, cascading physiological impact of low pH beyond simply inhibiting calcification. Given that low DO is also known to have large, overarching effects on bivalve physiology and metabolism (Diaz & Rosenberg 1995, Vaquer-Sunyer & Duarte 2008, Levin et al. 2009), it seems likely that some of the physiological impacts of low DO and low pH overlap, accounting for the interactive effects on some traits of the bivalves studied here. Regardless, the compounded effects of hypoxia and acidification in a changing climate will ultimately favor bivalve species whose early life stages have the ability to adapt and maintain performance under shifting conditions (Pörtner & Farrell 2008).

Diurnal fluctuations in pH and DO driven by ecosystem metabolism have commonly been observed in shallow estuaries (Ringwood & Keppler 2002, Yates et al. 2007, Baumann et al. 2015), and climate change may make these fluctuations more extreme in the future (Rabalais et al. 2002, Miller et al. 2009, Feely et al. 2010). The extent to which the daily excursions to near normal levels of pH and DO may provide a temporal refuge for animals suffering from potential negative effects of these conditions is unknown at present. Given that diurnal exposure treatments during this study provided mean pH and DO levels that were significantly less severe than the chronic exposures, it would be expected that performance within diurnal exposure treatments would be improved relative to chronic exposures. Indeed, in 25% of possible outcomes (Table 4), the significant reductions in performance experienced by exposure to low pH and/or low DO were ameliorated by diurnal exposure. For example, the negative effects of low pH and low DO on the development of *A. irradians* larvae were significantly lessened when the exposure was ephemeral on diurnal timescales. Diurnal acidified conditions lessened the negative effects of low pH on survival of *C. virginica* larvae and on the growth of

A. irradians. In all of these cases, however, the larval performance in these diurnal treatments was still significantly poorer than in the control, indicating that the diurnal exposure lessened, but did not eliminate, negative effects.

A substantially more common occurrence (75% of outcomes; Table 4) were cases when chronically low DO and/or pH significantly inhibited the larval performance and outcomes were not improved by diurnal exposure, despite substantially and significantly higher mean pH and DO levels. This was the case for *A. irradians* survival when exposed to diurnal pH, diurnal DO, and diurnal pH and DO; for *A. irradians* growth when exposed to diurnal DO and diurnal pH and DO; for *M. mercenaria* survival when exposed to diurnal pH and diurnal pH and DO; for *M. mercenaria* growth when exposed to diurnal pH, diurnal DO, and diurnal pH and DO; for *M. mercenaria* metamorphosis when exposed to diurnal pH, diurnal DO, and diurnal pH and DO; for *C. virginica* survival when exposed to diurnal pH and DO; and for *C. virginica* growth when exposed to diurnal pH and diurnal pH and DO. This outcome suggests that diurnally changing pH and DO may not allow enough time for bivalves to acclimate to the fluctuating conditions present during experiments. This may be particularly important in the case of diurnal DO changes, as it may cause bivalves to switch between aerobic and anaerobic metabolism (Grieshaber et al. 1994, Guppy & Withers 1999, Hochachka & Lutz 2001), an endeavor that may prove energetically costly. It is also possible that 12 h of exposure to suboptimal pH and/or DO conditions is enough to have detrimental physiological impacts. Nearly all studies of ocean acidification and hypoxia to date have exposed organisms to continuously high CO_2 and/or low DO in laboratory settings (e.g. Waldbusser et al. 2013, Gobler et al. 2014), conditions that do not represent the manner in which some bivalves are exposed to acidification and hypoxia in many of the shallow estuaries they inhabit. However, our findings suggest that in most cases, diurnal exposure to low pH and low DO does not provide a refuge from the negative physiological impacts of these conditions.

The results of this study provide evidence of the differential negative impacts of chronic and diurnal fluctuations in low pH and DO on bivalves, as well as the interactions between these stressors. While growth rates of larvae within control treatments were within the ranges previously published (e.g. Gallager & Mann 1986, Cahalan, et al. 1989, Helm et al. 2004), slower growth rates and delayed development leading to smaller organisms under acidified and/or hypoxic conditions may ultimately yield even higher rates of mortality in an ecosystem setting due to enhanced predation pressure (André & Rosenberg 1991, Tamburri & Zimmer-Faust 1996, Gosselin & Qian 1997). In addition, longer-term effects of acidification and hypoxia on individuals surviving early life stage exposure may have negative 'carry over' or 'legacy' effects on subsequent life stages (Hettinger et al. 2012, Gobler & Talmage 2013). All of these negative impacts could ultimately impact shellfish industries, as well as the many ecosystems services that bivalves provide (Officer et al. 1982, Petersen et al. 2015, Sebastiano et al. 2015).

Further research is needed to understand not only the long-term effects of acidification and hypoxia on shellfish, but also how anthropogenically driven acidification and hypoxia will manifest in coastal ecosystems. In the coming decades, progressive loading of anthropogenic CO_2 from the atmosphere will lower ocean pH (Doney et al. 2009), while the excessive loading of nutrients in coastal ecosystems may decrease DO (Diaz 2001) and synergistically lower pH levels (Sunda & Cai 2012). Alternatively, mitigation of nutrient loads could improve DO levels, while pH levels may be unchanged due to continued atmospheric loading. It has been proposed that anthropogenically driven acidification will depress the baseline, normal pH conditions in coastal ecosystems that go through diurnal changes (Miller et al. 2009, Feely et al. 2010), suggesting that even without changing nutrient loading, there may be some decoupling of DO and pH fluctuations in the future.

The diurnal ranges of pH and DO in estuaries depend upon many factors and differ among each coastal system. For example, Yates et al. (2007) reported diurnal changes in pH of 0.3 units and DO concentration of ~2.0 mg l^{-1} in Tampa Bay, FL, USA, while Ringwood & Keppler (2002) reported changes in pH on a diurnal cycle of 0.3 to 0.7 units and diurnal DO changes of 3 to 4 mg l^{-1} in Charleston Harbor, SC, USA. Baumann et al. (2015) demonstrated that the range of diurnal changes in pH and DO are seasonally dependent in temperate estuaries, being minimal in winter and maximal in late summer, with daily pH and DO changes of 0.7 units and 6.5 mg l^{-1}, respectively, changes reflective of the conditions applied during the experiments presented in this study. There are many important considerations when comparing the experimental diurnal changes in this study to those that occur in estuaries. While the rate of pH changes during our experiments was somewhat gradual and thus consistent with ecosystem observations (e.g. Ringwood & Keppler 2002, Bau-

mann et al. 2015), DO changes were more rapid, occurring in <1 h (Fig. 1), possibly decreasing the ability of larvae to adapt. Furthermore, while the experiments presented here returned larvae to ideal DO conditions for nearly 12 h each day, in some estuaries, the period of ideal conditions during summer can be significantly shorter (Baumann et al. 2015) or, in some cases, ideal conditions may never be achieved. This partly parallels our pH conditions, which were maximal for a shorter period of time than DO conditions (Fig. 1). This was also shown by Ringwood & Keppler (2002), who demonstrated that pH varied by 0.3 to 0.7 units daily within South Carolina estuaries, and that the daily pH maximum never exceeded 7.6 on the NBS scale. Regardless, given that providing larvae with ideal DO conditions for nearly 12 h each day, and ideal pH for several hours each day, did not provide a refuge, it is possible the actual negative effects of DO and/or pH on early-life-stage bivalves may be due to a duration of time below a critical threshold value. Ultimately, there are an infinite number of potential ecosystem and experimental combinations regarding exposure duration and severity of acidification and hypoxia, leaving much to be learned regarding the effects of diurnal pH and DO exposure on marine life. The levels and duration of low pH and low DO used in experiments presented here were, in many ways, consistent with prior ecosystem observations (Ringwood & Keppler 2002, Baumann et al. 2015), and were detrimental to the growth and survival of North Atlantic bivalve larvae.

Although this study focused on diurnal acidification and hypoxia, it is important to note that not all shallow coastal systems experience biotic-driven diurnal variations in pH and DO. Other factors, such as acidic and low salinity riverine discharge, can intermittently influence pH only (Salisbury et al. 2008), and tidal processes in estuaries can alter pH and DO on cycles that may act synergistically or antagonistically with diurnal cycles (Baumann et al. 2015). Additionally, in deeper stratified water, hypoxia and acidification are not alleviated via photosynthetic activity, and can persist for long periods of time (weeks to months; Rabalais et al. 2002, Diaz & Rosenberg 2008, Wallace et al. 2014).

In conclusion, diurnal exposure of larval bivalves indigenous to the Northwest Atlantic Ocean to low pH and low DO infrequently alleviated the effects of low DO and low pH, despite the higher mean pH and DO levels in those treatments. Regardless of the manner in which larval bivalves are exposed to acidic and hypoxic conditions, they experienced deleterious effects. Given that shellfish are ecologically and economically important marine organisms, implementing managerial criteria that will mitigate anthropogenic acidification and hypoxia seems warranted.

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