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Response of early life stage bivalves to diurnal changes in carbon dioxide and dissolved oxygen concentrations

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Abstract of the Thesis

Response of early life stage bivalves to diurnal changes in carbon dioxide and dissolved oxygen concentrations

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Highly productive and shallow coastal systems often experience metabolically-driven, diurnal variations in pH and dissolved oxygen (DO) concentrations. It has been suggested that worsening acidification and eutrophication-driven hypoxia will intensify the magnitude of diurnal changes by decreasing baseline pH and DO levels. Few studies, however, have investigated the concurrent effects of low pH and low DO on ecologically and socioeconomically important marine organisms inhabiting coastal ecosystems. My thesis was designed to assess the effects of diurnal patterns in acidification and hypoxia on the survival, growth, and development of the early life stages of three 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 both continuous and diurnal fluctuations in low levels of pH and DO. Continuously acidified conditions reduced survival of juvenile bay scallops as well as larvae of all three species studied, slowed growth of larval bay

scallops and eastern oysters, and delayed the development of bay scallop larvae, while continuously hypoxic conditions reduced the survival, growth, and development of larval bay scallops and development of larval hard clams. Though simultaneous exposure to both factors had significantly more negative effects than each factor independently, the effects on survival of bay scallop and hard clam larvae, hard clam development, and eastern oyster growth were antagonistic. The effects of diurnal exposure to acidified and hypoxic conditions were more complex. In some cases, diurnally acidified conditions eliminated or mitigated the negative effects of survival for larval bivalves. These benefits were sometimes lost when both pH and DO varied diurnally suggesting the fluctuations in both factors at the same time were too energetically costly and/or occurred too rapidly for the bivalves to physiologically compensate without experiencing adverse effects. Collectively, this study provides a more accurate representation of the responses of early life stage bivalves to future acidification and hypoxia in shallow, coastal systems and demonstrates that diurnal fluctuations in pH and DO represent a significant threat to the North Atlantic bivalve populations.

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Introduction

Atmospheric carbon dioxide (CO_2) concentrations have been rising primarily as a result of fossil fuel combustion (Doney et al. 2009). The Intergovernmental Panel on Climate Change (IPCC), projects CO_2 emissions will continue to increase and atmospheric CO_2 concentrations could exceed 1,000 ppm by 2100 (IPCC 2014; Scenario RCP8.5). The ocean is a sink for CO_2 and has taken up nearly 30% of anthropogenic CO_2 emissions (Sabine et al. 2004; Canadell et al. 2007). When CO_2 enters the ocean, it reacts with water to form carbonic acid (H_2CO_3) before dissociating into bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, subsequently releasing hydrogen ions (H^+; Eq. 1). Therefore, as atmospheric CO_2 increases, the pCO_2 of seawater increases and pH decreases.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad (1)$$

Collectively, these changes in ocean chemistry have been termed, ocean acidification. There has already been a decrease in oceanic pH of 0.1 unit since the beginning of the Industrial Era and an additional reduction of 0.3-0.4 units is expected to occur by 2100 (Caldeira and Wickett 2003, 2005; Orr et al. 2005; Solomon et al. 2007). This rate of change in pH is 100 times faster than the ocean has experienced in the past 300 million years (Caldeira and Wickett 2003; Doney and Schimel 2007). Increases in CO_2 concentrations also result in changes in carbonate chemistry by reducing the saturation state of calcium carbonate, specifically calcite and aragonite, which poses a threat to calcifying marine organisms (e.g. Kleypas et al. 2006).

Coastal ecosystems are susceptible to anthropogenic nutrient loading (Cloern 2001; Rabalais et al. 2002; Valiela 2006; Breitburg et al. 2009). Algal growth stimulated by excessive nutrients delivers organic matter to 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; Wallace et al. 2014). Hypoxia is loosely defined as low oxygen concentrations causing physiological stress to organisms (Rabalais et al. 2002) and thresholds for hypoxic water are usually 2-3 mg L^{-1} (Diaz 2001; Rabalais et al. 2002). There are over 400 known hypoxic systems worldwide and future climate change predictions suggest an increase in the size, severity, and frequency of hypoxic events (Diaz and Rosenburg 2008; Rabalais et al. 2009; Keeling et al. 2010). For example, ocean warming and increased stratification of the upper ocean caused by global climate change will likely lead to declines in dissolved oxygen (DO) in the ocean interior (Keeling et al. 2010). In addition, nutrient delivery to water bodies like the Gulf of Mexico is projected to increase. Such an increase to the Gulf of Mexico will further decrease bottom water DO concentrations (Justić et al. 2002) in what is already the second largest hypoxic zone in the world (Rabalais et al. 2002). While hypoxia in coastal zones has been well-studied for decades, it is only recently that the concurrent acidification of hypoxic zones has been documented (Cai et al. 2011; Wallace et al. 2014; Baumann et al. 2015).

While there is consensus that hypoxia in coastal systems is exacerbated by anthropogenic nutrient loading (Diaz 2001; Rabalais et al. 2002, 2009; Breitburg et al. 2009), there is debate regarding how ocean acidification will affect coastal regions. Unlike the open ocean where pH remains relatively constant, coastal systems are typically less buffered (Cai and Wang 1998) and biological activity, combined with other variables, can drive pH fluctuations on daily and/or seasonal timescales (Wootton et al. 2008; Waldbusser and Salisbury 2014). Net ecosystem metabolism can alter pH, as well as DO levels in seawater on diurnal timescales (e.g. Ringwood and Keppler 2002, Yates et al. 2007; Baumann et al. 2015). During the day, photosynthetic activity produces oxygen, consumes CO_2, and increases pH. At night, respiration becomes the dominant metabolic reaction consuming oxygen, producing CO_2, and decreasing pH (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 and 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 and Wang 1998), may then experience changes from ocean acidification before changes are observed in the open ocean (Waldbusser et al. 2011). Furthermore, there is evidence that ocean acidification has already begun to affect coastal pH. 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 diurnal fluctuations as a result of metabolism (Wootton et al. 2008). In addition, Sunda and Cai (2012) predicted that future CO_2 concentrations in eutrophic coastal systems will increase nonlinearly from the combined delivery of atmospheric CO_2 and respiration.

In the United States, the shellfish industry generates more than $2 billion in revenue annually (Cooley and Doney 2009) and contributes to 100,000 jobs supported by the seafood industry in New York State alone (Gall 2001). Most shellfish production occurs in coastal systems and is therefore vulnerable to eutrophic-driven diurnal changes in pH and DO. Because mortality of larval stage bivalves is high (Grosberg and Levitan 1992), any additional mortality as a result of acidification or hypoxia could significantly impact the distribution and abundance

of adult populations (Pechenik 1999; Guinotte and Fabry 2008; Gaylord et al. 2011) having large-scale ecological consequences (e.g. Diaz and Rosenburg 1995, 2008; Vaquer-Sunyer and Duarte 2008; Levin et al. 2009; Ekau et al. 2010). Understanding how early life stage bivalves respond to acidification and hypoxia in coastal systems is therefore vital to preserving their ecological and socioeconomic benefits.

Hypoxia increases stress and mortality in shellfish and other organisms (Diaz and Rosenberg 1995; Breitburg 2002; Vaquer-Sunyer and Duarte 2008; Breitburg et al. 2009; Levin et al. 2009), especially those in early life stages as they are less tolerant to low oxygen conditions than adults (Zhang et al. 2010). Tolerance to hypoxia, however, varies among shellfish species. Gobler et al. (2014) found hypoxic water inhibited growth and development in bay scallop (*Argopecten irradians*) larvae and increased mortality in early juvenile hard clams (*Mercenaria mercenaria*), though later stage clams were tolerant to hypoxia. Slowed growth has also been observed in late stage blue mussel (*Mytilus edulis*) larvae in response to hypoxic conditions (Wang and Widdows 1991). Basso et al. (2015) found that the juvenile Mediterranean pen shell (*Pinna nobilis*) was tolerant of hypoxia even when combined with another stressor, high temperature.

Acidification can be harmful to calcifying bivalves. Calcification rates of larval hard clams, *M. mercenaria* (Gobler and Talmage 2013), bay scallops, *A. irradians* (Gobler and Talmage 2013), mussels, *M. edulis* (Gazeau et al. 2007), and oysters, *Crassostrea gigas* and *Crassostrea virginica* (Gazeau et al. 2007; Miller et al. 2009; Gobler and Talmage 2013), decrease with increasing pCO_2. High CO_2 concentrations can also induce mortality, delay metamorphosis, and slow growth in larval hard clams, eastern oysters and bay scallops (Miller et al. 2009; Talmage and Gobler 2009, 2010; White et al. 2013). Abnormal development in

oyster (*C. gigas*) and mussel (*Mytilus galloprovincialis*) larvae has been observed at high pCO_2 concentrations (Kurihara 2008). Mussel (*Mytilus californianus*) larvae reared under acidified conditions have been shown to grow smaller shells that were thinner and weaker than those of larvae reared under current ambient conditions (Gaylord et al. 2011). Fewer studies have been conducted on juvenile shellfish, although Green et al. (2004, 2009) reported mortality and shell dissolution of newly-settled juvenile clams (*M. mercenaria*) in sediment undersaturated with respect to calcium carbonate. Dickinson et al. (2012) found that juvenile oysters (*C. virginica*) experienced increased mortality and energy deficiency under elevated pCO_2 conditions. Little is known about the effects of the simultaneous exposure of low DO and low pH on shellfish, though Gobler et al. (2014) reported that the combined effects of acidification and hypoxia reduced survival, growth, and delayed metamorphosis in bay scallop (*A. irradians*) larvae and slowed growth in juvenile hard clams (*M. mercenaria*).

The goal of this thesis was to quantify the effects of static and diurnally fluctuating low DO and low pH on larval and juvenile bay scallops (*A. irradians*), hard clams (*M. mercenaria*), and eastern oysters (*C. virginica*) that inhabit North Atlantic estuaries. While the effects of hypoxia or acidification on early life stage bivalves is well known, their combined effects have been poorly studied, despite their frequent co-occurrence in estuaries (Cai et al. 2011; Wallace et al. 2014; Baumann et al. 2015). Because coastal systems often experience metabolically-driven, diurnal cycles of pH and DO, I investigated, for the first time, how early life stage bivalves respond to diurnal patterns of acidification and hypoxia. Although high concentrations of pCO_2 and low DO concentrations are known to negatively affect early life stage bivalves, I hypothesized that the negative effects could be lessened or mitigated when exposure to such conditions was in the form of repeated, short-term diurnal cycles. Physiological adaptions of

shellfish, such as acid-base regulation and metabolic depression (Michaelidis et al. 2005), may allow these bivalves to tolerate excursions into hypercapnic and hypoxic conditions and once favorable conditions return, growth and development may continue uninhibited.

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 23-24°C was maintained by partially submerging the experimental vessels in a water bath heated by a Delta-® Star heat pump. Three types of experiments were conducted. The first involved the manipulation of pH and the second and third involved the simultaneous manipulation of pH and DO. For the pH experiments, treatments of control (~7.9), intermediate (~7.5), and low pH (~7.2) were maintained by bubbling mixtures of tanked 5% CO_2 gas and air into the experimental vessels. An additional treatment of diurnal pH was used where pH oscillated between control and low pH conditions every 12 h (*details below;* Fig. 1). For the pH and DO experiments, two different types of experiments were performed. For one type, both pH and DO levels were altered in unison and four treatments were established: A control treatment (pH ~ 7.9, DO ~7.0 mg L^{-1}), an intermediate pH-DO treatment (~7.5, ~4.0 mg L^{-1}), a low pH-DO treatment (~7.2, ~2.0 mg L^{-1}), and a diurnally fluctuating pH-DO treatment that experienced the control pH and DO by day and the low pH and DO at night, resulting in mean levels similar to the intermediate pH-DO treatment (~7.5, ~4.0 mg L^{-1}). For the second type of pH-DO experiment, treatments of control (~7.9, ~7.0 mg L^{-1}), low pH (~7.2, ~7.0 mg L^{-1}), low DO (~7.9, ~2.0 mg L^{-1}), and combined low pH and DO (~7.2, ~2.0 mg L^{-1}) were maintained by bubbling mixtures of air and tanked 5% CO_2, N_2, and a 400 ppm CO_2/N_2 mix

into experimental vessels. Additional treatments of diurnal pH, diurnal DO, and combined diurnal pH and DO were also used where pH, DO, or both parameters oscillated between control and low conditions every 12 h (*details below;* Fig. 1). The range of pH and DO changes in all diurnal treatments was consistent with changes observed within temperate estuaries in recent studies (Wallace et al. 2014; Baumann et al. 2015).

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 (Table 1; 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 (0900 – 2100 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 (2100 – 0900 h), the valves on the appropriate CO_2, N_2, or CO_2N_2 gas tanks were opened to create low pH and/or DO conditions.

Measuring salinity, temperature, pH, and DO

Salinity was measured using a YSI 600QS multi-parameter water quality sonde and temperature logged every 15 minutes on a HOBO® U-002-64 Data Logger (Onset). 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 minutes in the diurnal treatments with a Thermo-Scientific Orion STAR A321 pH meter. A Clark-type electrode YSI 5100 oxygen meter was used to make daily DO measurements and DO was logged every 15 minutes on an HOBO® U26 dissolved oxygen logger (Onset) in the diurnal treatments. Prior studies have found these instruments measure

levels of dissolved oxygen 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 (mean percent recovery of DIC across all analytical runs: 103 ± 6%). DIC levels, along with pH, temperature, salinity, pressure, phosphate, silicate, and carbonic acid dissociation constants recommended for estuarine waters (Millero 2009) were analyzed with the CO2SYS program (http://cdiac.ornl.gov/ftp/co2sys/) in order to quantify levels of pCO_2, $\Omega_{calcite}$, $\Omega_{aragonite}$, carbonate, and total alkalinity. Since pH values in some experimental treatments were made to fluctuate widely each day and since 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.

Organisms

Larval and juvenile *A. irradians, C. virginica,* and *M. mercenaria* were obtained from the East Hampton Town Shellfish Hatchery located in Montauk, NY, USA, the Cornell Cooperative Extension of Suffolk County in Southold, NY, USA, or were spawned at the Stony Brook—Southampton Marine Sciences Center, Southampton, NY, USA. In all cases, 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 and were fed ~3% of their dry weight in algae for six to eight weeks and maintained at a temperature of 18°C (Helm et al. 2004). The algal diet included a biovolume equal mixture of the phytoplankton, *Isochrysis galbana, Tetraselmis suecica, Tetraselmis chuii, Chaetoceros muelleri, Chaetoceros calcitrans,* and *Pavlova lutheri* (Helm et al. 2004). Adult shellfish were then temperature-spawned as described in Deming et al. (1998) and fertilized embryos were collected.

Each experimental vessel was stocked with either 10,000 D-stage larvae less than 24 hours old or 15 juveniles (initial size of juvenile *A. irradians* = 4 mm; *M. mercenaria* = 1.5 mm). All experimental shellfish were fed a diet of 4×10^4 cells mL^{-1} of *Isochrysis galbana* daily (Carriker 2001; Helm et al. 2004; Cragg 2006). Full water changes were performed twice weekly for experiments involving larval shellfish. All contents of the 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 timepoint 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 the pigmentation present and whether or not the individual was intact. Percent metamorphosis was calculated based on the total number of surviving larvae at each timepoint and larval experiments continued until all individuals in the control treatment had metamorphosed. Due to the propensity of *C.*

virginica larvae to set-irreversibly on surfaces when metamorphosed, this experiment was ended after 14 days, but prior to the larvae metamorphosing into juveniles.

For juvenile shellfish, one and a half full water changes were performed weekly over the 3-4 week experiments. During the full water change, mortality was documented and size was measured with calipers to calculate growth rates. Dead individuals, deemed so by their gaping shells and/or lack of response to stimuli, were removed.

Data analysis

Statistical analyses were performed in RStudio-®. Survival and development data were arcsine square root transformed before analysis. A One-Way Analysis of Variance (ANOVA) test was performed on the survival, development, and growth rate data from diurnal acidification experiments and the four treatment diurnal acidification and hypoxia experiments (Table 1). Two-Way ANOVAs were performed on the survival, development, and growth rate data from the seven treatment diurnal acidification and hypoxia experiments where the type of pH and DO exposure (control, low, or diurnal) were the main treatment effects (Table 1). All assumptions were met for these parametric tests. Shapiro-Wilk's test was used to assure all data were normally distributed and Bartlett's test were used to assure homogeneity of variance among each dataset. ANOVAs reporting significant effects from treatments were proceeded with Tukey HSD test for multiple comparisons.

Results

Diurnal acidification experiments

Larval shellfish

Survival of larval *A. irradians* was significantly reduced by acidification (One-way ANOVA; $p < 0.001$; Tables 2, 3; Fig 2A). Larvae exposed to the control and intermediate levels

of pH (pH = 7.94 ± 0.06 and 7.60 ± 0.03, respectively) experienced 36 ± 2% and 21 ± 11% survival (± standard deviation), respectively, whereas the percentage of larvae that survived in the low pH treatment (pH = 7.33 ± 0.07; survival = 7 ± 5%) was significantly lower than the intermediate ($p = 0.026$) and control ($p = 0.003$), but not different than the diurnal treatment (mean pH = 7.47 ± 0.43; survival = 15 ± 3%). Low pH conditions slowed larval growth (One-way ANOVA; $p < 0.001$) and delayed metamorphosis (One-way ANOVA; $p < 0.001$) relative to the control treatment, but there was no effect of intermediate and diurnal pH treatments on growth or development (Tables 4, 5). Larvae grew at a rate of 11 ± 5 μm day^{-1} in the low pH treatment, 26 ± 4 μm day^{-1} in the control treatment, 20 ± 2 μm day^{-1} in the intermediate pH treatment, and 22 ± 3 μm day^{-1} in the diurnal pH treatment (Fig. 2B). Twelve days post-fertilization, 88 ± 5%, 58 ± 22%, and 61 ± 19% of *A. irradians* larvae had metamorphosed in the control, intermediate, and diurnal treatments while only 13 ± 19 % had metamorphosed in the low treatment (Fig. 2C).

Low pH (pH = 7.29 ± 0.06) reduced survival (One-way ANOVA; $p = 0.015$) of larval *M. mercenaria* though there was no effect of intermediate (pH = 7.58 ± 0.04) or diurnally fluctuating pH (mean pH = 7.54 ± 0.36; Tables 6, 7). Larvae reared under control (pH = 7.91 ± 0.02) , intermediate, and diurnal pH conditions had survival rates of 27 ± 3%, 23 ± 2%, and 25 ± 5% respectively whereas survival of larvae in the low pH treatment was 15 ± 5%, significantly lower than all other treatments (Fig. 3A). Growth and development of *M. mercenaria* larvae did not differ significantly among experimental treatments (Tables 8, 9; Fig. 3B, C).

Juvenile shellfish

Survival was significantly reduced in juvenile *A. irradians* exposed to low pH (pH = 7.13 ± 0.03; One-way ANOVA; $p = 0.016$), intermediate pH (pH = 7.49 ± 0.04; $p = 0.003$), and

diurnally fluctuating pH conditions (mean pH = 7.57 ± 0.43; $p = 0.001$; Tables 10, 11). In the control treatment (pH = 7.92 ± 0.05), 92 ± 6 % of individuals survived whereas low, intermediate, and diurnally fluctuating pH treatments experienced 57 ± 25%, 47 ± 9%, and 40 ± 12% survival, respectively, but were not significantly different from each other (Fig. 4A). There were no pH effects on the growth rates of juvenile *A. irradians* (Table 12; Fig. 4B). The differing levels of pH (Table 13) used in experiments did not significantly alter the survival and growth of juvenile *M. mercenaria* (Table 14, 15; Fig. 5A, B).

Diurnal acidification and hypoxia experiments, four treatments

Diurnal exposure to low pH and low DO (mean pH = 7.61 ± 0.26; mean DO = 4.11 ± 2.80 mg L^{-1}) significantly reduced survival of larval *A. irradians* (One-way ANOVA; $p = 0.023$), but chronically low and intermediate pH and DO conditions (pH = 7.22 ± 0.05; DO = 1.38 ± 0.45 mg L^{-1} and pH = 7.48 ± 0.05; DO = 4.08 ± 0.41 mg L^{-1}, respectively) had no effect (Tables 16, 17). The percent survival of larval *A. irradians* in the control (pH = 7.89 ± 0.00; DO = 6.87 ± 0.25 mg L^{-1}), intermediate, and low pH and DO treatments was 15 ± 10%, 17 ± 6%, and 6 ± 4%, respectively, while survival in the diurnally fluctuating treatment was 3 ± 1% (Fig. 6A). Both the low and diurnal treatment slowed growth (One-way ANOVA; $p < 0.001$; Table 17) from 13 ± 1 µm day^{-1} and 13 ± 1 µm day^{-1} in the control and intermediate pH-DO treatments to 7 ± 1 µm day^{-1} and 10 ± 0.4 µm day^{-1} in the low and diurnal pH-DO treatments (Fig. 6B). Continuously low pH and DO also significantly delayed development (One-way ANOVA; $p = 0.016$), while exposure to intermediate and diurnally fluctuating pH and DO did not (Table 18). After 15 days, 32 ± 8%, 36 ± 4%, and 32 ± 13% of larvae had metamorphosed in the control, intermediate, and diurnal pH-DO treatments, whereas only 13 ± 3% had metamorphosed in the low pH-DO treatment (Fig. 6C).

Exposure of larval *M. mercenaria* to low (pH = 7.24 ± 0.04; DO = 1.32 ± 0.30 mg L^{-1}) and diurnally fluctuating pH-DO (mean pH = 7.41 ± 0.34; mean DO = 4.02 ± 3.00 mg L^{-1}) significantly reduced their survival to 7 ± 2% and 5 ± 2% compared to the control (pH = 7.87 ± 0.03; DO = 6.92 ± 0.13 mg L^{-1}) and intermediate (pH = 7.43 ± 0.03; DO = 3.92 ± 0.34 mg L^{-1}) pH survival which was 13 ± 2% and 8 ± 2%, respectively (One-way ANOVA; $p = 0.032$; $p = 0.001$; Tables 20, 21; Fig. 7A). Continuously low pH and DO conditions slowed the growth of *M. mercenaria* larvae to 9 ± 2 µm day^{-1} compared to 13 ± 0.4 µm day^{-1}, 12 ± 1 µm day^{-1}, and 12 ± 2 µm day^{-1} in the control, intermediate (pH = 7.43 ± 0.03; DO = 3.92 ± 0.34 mg L^{-1}), and diurnally fluctuating pH-DO treatments (One-way ANOVA; $p < 0.05$; Table 22; Fig. 7B). Delays in development were observed when *M. mercenaria* larvae were exposed to lowered pH and DO (One-way ANOVA; $p < 0.05$; Table 23). Eleven days post-fertilization, 61 ± 4% of larvae had metamorphosed in the control treatment, whereas only 41 ± 7%, 28 ± 9%, and 24 ± 7% had metamorphosed in the intermediate ($p = 0.019$), diurnal ($p < 0.001$), and low pH-DO ($p < 0.001$) treatments respectively (Fig. 7C).

Diurnal acidification and hypoxia experiments, seven treatments

There was a significant negative effect of pH (Two-Way ANOVA; $p < 0.001$), and DO ($p < 0.001$) on survival of *A. irradians* larvae and an antagonistic interaction between both factors ($p < 0.05$) with all manipulated conditions significantly reducing survival relative to the control condition (Tables 24, 25). 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% (Fig. 8A). Survival under continuously low DO (pH = 7.91 ± 0.02; DO = 2.33 ± 0.64 mg L^{-1}) was higher than both the continuously low pH (pH = 7.20 ± 0.09; DO = 6.96 ± 0.74 mg L^{-1}; $p = 0.006$) and diurnal pH-DO (mean pH = 7.58 ± 0.25; mean

DO = 4.28 ± 2.90 mg L^{-1}; $p < 0.001$) conditions, though still significantly lower than the control treatment (pH = 7.91 ± 0.02; DO = 6.87 ± 0.33 mg L^{-1}; $p = 0.009$). The antagonistic 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 diurnal pH and diurnal DO treatments separately (12 ± 3% and 17 ± 5%, respectively).

Growth rates of *A. irradians* larvae were affected by both pH (Two-way ANOVA; $p < 0.001$) and DO ($p < 0.001$). There was no interaction between the factors (Table 26). Larvae experienced significantly slowed growth under all manipulated conditions, except for the diurnally fluctuating pH treatment (mean pH = 7.47 ± 0.23; DO = 6.80 ± 0.57 mg L^{-1}; growth rate = 10 ± 2 µm day^{-1}; Fig. 8B). Control larvae grew at a rate of 13 ± 1 µm day^{-1} while rates were slowed to 6 ± 1 µm day^{-1} in low pH-DO ($p < 0.001$), 7 ± 1 µm day^{-1} in low pH ($p < 0.001$), 8 ± 1 µm day^{-1} in diurnal pH-DO ($p < 0.001$), 9 ± 1 µm day^{-1} in diurnal DO ($p = 0.018$), and 9 ± 2 µm day^{-1} in low DO ($p = 0.024$). Both pH ($p < 0.001$) and DO ($p < 0.001$) affected development of *A. irradians* larvae (Two-way ANOVA; Table 27). Fourteen days post-fertilization, 67 ± 5% of larvae had metamorphosed under control conditions (Fig. 8C). Continuously low pH ($p < 0.001$) and continuously low DO ($p = 0.026$) reduced metamorphosis to 35 ± 9% and 48 ± 10% respectively, but diurnal exposure of low pH and of low DO did not alter the fraction of larvae that had metamorphosed. Metamorphosis was 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%; $p < 0.001$).

Survival of larval *M. mercenaria* was significantly reduced by pH (Two-way ANOVA; $p < 0.001$) but not DO, and there was an antagonistic interactive effect of these two factors ($p < 0.05$; Tables 28, 29). Under control (pH = 7.97 ± 0.07; DO = 7.13 ± 0.20 mg L^{-1}), chronically

low DO (pH = 7.92 ± 0.08; DO = 2.64 ± 0.40 mg L^{-1}), and fluctuating low DO conditions (pH = 7.92 ± 0.06; mean DO = 4.44 ± 2.70 mg L^{-1}), 28 ± 2%, 24 ± 3%, and 27 ± 5% of larvae 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 (pH = 7.21 ± 0.10; DO = 7.14 ± 0.22 mg L^{-1}; $p < 0.001$), diurnal pH (mean pH = 7.43 ± 0.65; DO = 7.47 ± 0.24 mg L^{-1}; $p < 0.001$), low pH-DO (pH = 7.22 ± 0.10; DO = 1.90 ± 0.38 mg L^{-1}; $p < 0.001$), and diurnal pH-DO (mean pH = 7.55 ± 0.56; mean DO = 4.49 ± 2.60 mg L^{-1}; $p < 0.001$) treatments (Fig. 9A). There was antagonism between pH and DO as the survival in the combined diurnal treatment was higher than would have been predicted by either individual treatment for both the static and diurnal treatments.

There was an effect of pH (Two-way ANOVA; $p < 0.05$) and DO ($p < 0.05$) on the growth of *M. mercenaria* larvae, with the growth rates in the control, low pH, low DO, low pH-DO, diurnal pH, diurnal DO, and diurnal pH-DO treatments being 9 ± 0.4 µm day^{-1}, 9 ± 1 µm day^{-1}, 8 ± 1 µm day^{-1}, 8 ± 0.4 µm day^{-1}, 8 ± 1 µm day^{-1}, 9 ± 0.4 µm day^{-1}, and 8 ± 1 µm day^{-1} although post-hoc multiple comparisons did not identify differences among treatments (Table 30; Fig. 9B). Development of *M. mercenaria* larvae was affected by pH (Two-way ANOVA; $p < 0.001$), DO ($p < 0.001$), and there was an antagonistic interaction between these two factors ($p < 0.001$; Table 31). Fewer larvae metamorphosed 17 days post-fertilization in all pH and DO treatments compared to 80 ± 6% that had metamorphosed in control conditions ($p < 0.001$; Fig. 9C). There was an antagonistic 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 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%; $p < 0.001$) treatments than

the control, there were significantly more metamorphosed larvae in these two treatments than the diurnal pH (9 ± 4%; $p < 0.001$; $p = 0.002$), low pH-DO ($p < 0.001$; $p < 0.001$), and diurnal pH-DO (11 ± 3%; $p = 0.001$; $p = 0.034$) treatments.

DO and pH significantly reduced the survival of *C. virginica* larvae (Two-way ANOVA; ($p < 0.05$ and $p < 0.001$, respectively; Tables 32, 33) and there was no interaction between these factors. Survival was reduced from 15 ± 5% under control conditions (pH = 7.85 ± 0.04; DO = 7.04 ± 0.16 mg L^{-1}), to 3 ± 1%, 5 ± 4%, and 5 ± 2% in low pH (pH = 7.16 ± 0.07; DO = 6.98 ± 0.16 mg L^{-1}; $p < 0.001$), low pH-DO (pH = 7.18 ± 0.07; DO = 1.87 ± 0.41 mg L^{-1}; $p = 0.015$), and diurnal pH-DO (mean pH = 7.50 ± 0.23; mean DO = 4.36 ± 2.80 mg L^{-1}; $p = 0.017$) treatments (Fig. 10A). There was, however, no significant differences between control conditions and diurnal fluctuations in pH (mean pH = 7.54 ± 0.26; mean DO = 7.54 ± 0.18 mg $L^{-1;}$ survival = 6 ± 1%) as well as between diurnal and continuous low DO on survival percentages (mean pH = 7.99 ± 0.09; mean DO = 5.14 ± 2.10 mg L^{-1}; survival = 14 ± 5%; pH = 7.83 ± 0.05; DO = 2.50 ± 0.71 mg L^{-1}; survival = 21 ± 7%, respectively); these survival rates were all significantly higher than the low pH, low pH-DO, and diurnal pH-DO treatments. Finally, the percent survival of *C. virginica* larvae was significantly higher in the low DO treatment than the diurnal pH treatment ($p = 0.002$). There was an overall effect of pH (Two-way ANOVA; $p < 0.001$), DO ($p < 0.05$), and an antagonistic interactive effect of pH and DO ($p < 0.05$) on the growth rates of *C. virginica* larvae (Table 34). Growth rates were reduced from 1 ± 0.1 µm day^{-1} in control conditions to 0.7 ± 0.1 µm day^{-1}, 0.3 ± 0.2 µm day^{-1}, 0.2 ± 0.1 µm day^{-1}, and 0.4 ± 0.4 µm day^{-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 (Fig. 10B). Growth rates of *C. virginica* larvae exposed to chronically low or diurnal fluctuations of DO (1 ± 0.4 and 1 ± 0.2 µm day^{-1},

respectively) did not differ from the control treatment. The antagonistic 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.

Discussion

Ocean acidification and hypoxia are expected to worsen as a result of anthropogenic activity (Diaz and Rosenberg 2008; Breitburg et al. 2009; Doney et al. 2009) and both are known to negatively impact a multitude of marine species (e.g. Diaz and Rosenberg 2005; Kleypas et al. 2006). Furthermore, biotic controls of pH and DO in highly-productive coastal ecosystems complicate predictions on how ocean acidification and hypoxia will manifest in such environments (Miller et al. 2009; Borges and Gypens 2010; Feely et al. 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. To my knowledge, no prior study has investigated the diurnal vs. continuous as well as independent and combined effects of hypoxia and ocean acidification on bivalves. I found that continuously low pH and low DO negatively impact survival, growth, and development of early life stage bivalves and the concurrent continuous exposure had both additively negative and antagonistic effects. Additionally, diurnal exposure to these conditions alleviated some, but not all, negative effects.

Continuously acidified conditions reduced survival in juvenile *A. irradians* and larvae of all three bivalve species more so than under low oxygen conditions. Acidification also slowed the growth of larval *A. irradians* and *C. virginica* and the development of *A. irradians*. Of the three species in this study, *M. mercenaria* was the most resistant to high CO_2 as its growth was generally unaffected by pH. Development of *M. mercenaria* larvae was, however, negatively

affected by pH in one experiment, but unaffected in another with similar pH levels possibly reflecting the differences in time points assessed for metamorphosis between these two experiments or varied tolerances among cohorts (Byrne 2012; Murray et al. 2014). Shell formation and development of bivalve larvae is energetically costly and requires an even larger energy input when elevated CO_2 promotes 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 hours of development. Additionally, 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 (Gobler and Talmage 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 (Barnhart and McMahon 1988; Kwast and Hand 1996; Guppy and Withers 1999; Langenbuch and Pörtner 2002; Pörtner et al. 2005; Fabry et al. 2008; 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 and Rosenberg 2008; Rabalais et al. 2009, 2010). The results of this study show that continuously hypoxic conditions negatively affected survival, growth, and development of *A. irradians* larvae and development of larval *M. mercenaria*, demonstrating that while bivalves are some of the more hypoxia-tolerant

marine organisms (Diaz and 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 and Widdows 1991; 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 organisms (Grieshaber et al. 1994; Guppy and Withers 1999; Hochachka and Lutz 2001). Though 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* (Kraeuter and Castagna 2001; Kennedy 2006; Shumway and Parsons 2006).

Overall, acidification more negatively affected early life stage bivalves during this study, a finding consistent with a prior study of pH and DO effects on *A. irradians* larvae (Gobler et al. 2014). All three larval species and juvenile *A. irradians* were negatively affected by acidification in all parameters measured. Conversely, though hypoxia negatively affected *A. irradians* survival, growth and development, it had no effect on *C. virginica* larvae and only delayed development in larval *M mercenaria.* My research shows that concurrent exposure to

acidified and hypoxic water more negatively affected larvae than each factor alone. Low pH-DO reduced larval survival in all three bivalve species examined, inhibited growth in *A. irradians* and *C. virginica* larvae, and repressed development in *A. irradians* and *M. mercenaria* larvae. Again, *M. mercenaria* larvae were the most tolerant as growth was unaffected by diurnal pH-DO and by continuously low pH-DO in the seven treatment diurnal acidification and hypoxia experiment. Reduced growth that was observed under low pH-DO conditions in the four treatment diurnal acidification and hypoxia experiment may, again, reflect slightly lower levels of pH and DO in this experiment, differences in the duration of the experiment, or variations in tolerance between cohorts (Byrne 2012; Murray et al. 2014).

The antagonistic effects of simultaneously low pH and low DO on the survival of *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 milder 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 additive. Instead, the antagonistic effects observed suggest there is some level

of overlap in the physiological impacts of these stressors, a hypothesis that can be supported by prior studies. Acidification has been shown to reduce the lipid content and RNA:DNA ratios of bivalve larvae (Gobler and 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 and Rosenberg 1995; Vaquer-Sunyer and 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 antagonistic 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 and Farrell 2008).

Diurnal fluctuations in pH and DO driven by ecosystem metabolism have been observed in shallow estuaries (Ringwood and Keppler 2002, Yates et al. 2007; Baumann et al. 2015) and coastal acidification and hypoxia will likely make these fluctuations more extreme in the future (Diaz 2001; Rabalais et al. 2002; Miller et al. 2009; Feely et al. 2010). The extent to which the daily exposure to near normal levels of pH and DO may provide a temporal refuge for animals that protects them from the potential negative effects of these conditions is presently unknown. It is important to address this question separately for the four treatment and seven treatment experiments given that the four treatment experiments provided nearly identical mean pH-DO conditions in the diurnal exposure and the intermediate pH-DO exposure treatments while the seven treatment experiments were designed to contrast the individual effects of pH and DO, but in doing so, created conditions in the diurnal exposures that were, on average, less extreme than the chronic exposures. In some of the seven treatment experiments, the deleterious effects of

low pH and low DO were ameliorated by diurnal exposure. For example, the effects of low pH and combined low pH and low DO on the development of *A. irradians* larvae were mitigated or eliminated entirely when the exposure was ephemeral on diurnal timescales. Diurnal acidified conditions eliminated negative effects of low pH on survival in *M. mercenaria* and *C. virginica* larvae. Growth of *M. mercenaria* larvae in the combined continuously low pH-DO conditions was slowed, but was uninhibited when these conditions occurred diurnally. Again, however, these outcomes may be related to the higher mean pH and DO levels in these experiments compared to the continuous treatments, rather than the simple fluctuations.

Within the four treatment experiments, where the intermediate treatments matched the mean pH and DO levels in the diurnal treatment, outcomes depended on whether pH alone or both pH and DO fluctuated. When pH was the only variable examined, the intermediate and diurnal pH treatments yielded similar outcomes for both bivalves studied, indicating that the diurnal variation in pH alone had a neutral physiological impact and that performance was a function of mean pH exposure levels rather than the manner in which it was experienced (diurnal vs. continuous). However, when both pH and DO fluctuated, the survival of *M. mercenaria* and *A. irradians* larvae was significantly lower than the intermediate pH-DO treatment with identical mean pH and DO levels and was more similar to the low pH-DO treatment which, on average, had significantly lower pH and DO levels. Collectively, this suggests that while fluctuating pH alone may provide an outcome similar to static low pH exposure, when both variables change in unison, the outcomes are worse than static exposure and as bad as chronic exposure to even lower levels of pH and DO. This suggests that the rapidly changing pH and DO may not allow enough time for bivalves to acclimate to the levels of pH and DO present during experiments. This may be particularly important in the case of diurnal DO changes as it may force bivalves to

switch between aerobic and anaerobic metabolism (Grieshaber et al. 1994; Guppy and Withers 1999; Hochachka and Lutz 2001), an endeavor that may prove energetically costly and thus yield outcomes worse than when they are, on average, chronically exposed to the same conditions. Nearly all studies on ocean acidification and hypoxia to date have exposed organisms to continuously high CO_2 and/or low DO in laboratory settings (e.g. Talmage and Gobler 2009; Waldbusser et al. 2013; Gobler et al. 2014; Basso et al. 2015), conditions that misrepresent the manner in which bivalves are exposed to acidification and hypoxia in many of the shallow estuaries they inhabit. Our findings suggest previous studies focusing on continuous exposure to hypoxia and acidification may portray a more positive response of bivalves to low pH and DO conditions as constant physiological adaptations to diurnal hypoxia and acidification may be physiologically costly.

Differences in the responses of the three bivalves examined in this study may reflect species-specific tolerances to hypoxia and acidification or may stem from varying abilities to adapt based on life stage or cohort origin within a species. Because larvae tend to be the most sensitive life stage of bivalves (Widdicombe and Spicer 2008), it is unsurprising that, for example, that acidification and hypoxia negatively impacted hard clam larvae, but juveniles were unaffected or that bay scallop and hard clam larval survival rates were lower than those of juveniles. In a more complex response, larval hard clam survival was significantly reduced by diurnal (mean pH = 7.4) and continuously low pH (mean pH = 7.2) in one experiment, but only by continuous low pH (mean pH = 7.3) and not diurnal pH exposure (mean pH = 7.6) in another. These differences are likely attributable to the higher pH in the later experiment's diurnal pH treatment as larval hard clams are sensitive to minor changes in pH (Talmage and Gobler 2009). Furthermore, the two cohorts of larvae were spawned from different broodstock and their

dissimilar response may also reflect variation in genetics and phenotypic plasticity (Byrne 2012; Murray et al. 2014), a hypothesis also supported by minor differences in larval survival rates under ideal conditions among cohorts used in different experiments. Regardless, given that in the experiment where there were differences, survival of larvae during chronic exposure to low pH was lower than diurnal acidification, these findings collectively suggest that, as anticipated, chronic exposure to low pH has a more insidious effect on larval hard clams than diurnal exposure when the pH levels are, on average, higher. Lastly, juvenile *M. mercenaria* were markedly tolerant to acidification, while juvenile *A. irradians* experienced significant mortality in acidified conditions, a finding consistent with prior studies of these species (Talmage and Gobler 2011). Juvenile hard clam tolerance to low pH may be a result of their position in the seabed relative to bay scallops. Scallops, as epifauna, live on the sediment surface and are exposed to the overlying water column chemistry, whereas hard clams, as infauna, burrow in coastal sediments that are naturally acidic and often have porewaters that are undersaturated in calcium carbonate (Green et al. 2004).

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. Though marine invertebrates naturally experience more than 99% mortality during early life stages (Bayne 1976; Gosselin and Qian 1997), additional stress on survival from acidification and hypoxia could significantly affect the success of adult populations (Schneider et al. 2003; Green et al. 2004; Guinotte and Fabry 2008). While survival in some experimental treatments during this study were low, I note that unlike prior experimental studies that reported larval survival when only a fraction of larvae had metamorphosed (Padilla et al. 2006; Talmage and Gobler 2009), the experiments presented here persisted until all larvae had metamorphosed

in all treatments with the larval *C. virginica* experiment being the sole exception. Gallager and Mann (1986) deem a range from 5-20% as excellent survival of hatchery-reared hard clam and eastern oyster larvae to the pediveliger stage. Similarly, Helm et al. (2004) note typical survival of scallop larvae in hatcheries from the D-stage through metamorphosis can be as low as 15%. Survival of bivalve larvae in the field can be <1% as a result of environmental stress and predation (Bayne 1976). Further, while growth rates of larvae within control treatments were normal (e.g. Gallager and Mann 1986, Cahalan, et al. 1989, Helm et al. 2004), slower growth rates and delayed development leading to smaller organisms under acidic and hypoxic conditions may ultimately yield higher rates of predation and mortality in an ecosystem setting (André and Rosenberg 1991; Tamburri and Zimmer-Faust 1996; Gosselin and Qian 1997; Kraeuter 2001). Such direct and indirect reductions in survival would not only be detrimental to the shellfish industry in the United States, but could also have significant ecological consequences. Bivalves provide many ecosystems services, including water filtration and subsequent control of coastal eutrophication (Officer et al. 1982; Petersen et al. 2015; Sebastiano et al. 2015). Beyond the measurements made during this study, longer-term effects of acidification and hypoxia on individuals surviving early life stage exposure may have "carry over" or "legacy" effects on subsequent life stages. High CO_2 elicited reduced growth in Olympia oyster (*Ostrea lurida*) larvae and this reduction in growth continued into the juvenile stage even when CO_2 conditions returned to normal (Hettinger et al. 2012). Similarly, bay scallop larvae grown under high CO_2 were smaller than those reared under higher CO_2 concentrations and the differences in size between these cohorts persisted for more than eight months into juvenile stages even in the absence of hypercapnia (Gobler and Talmage 2013).

Further research is needed to understand not only long term effects of acidification and hypoxia on shellfish, but also how anthropogenically-driven acidification and hypoxia will manifest in coastal ecosystems. In addition, studies examining larger and smaller ranges and more gradual diurnal transitions in pH and DO are warranted. 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 diel changes in pH of 0.22 units and DO concentration of ~2.0 mg L^{-1} in Tampa Bay, FL, USA, and Ringwood and Keppler (2002) reported average changes in pH on a diurnal cycle of 0.45 units in Charleston Harbor, SC, USA. Baumann et al. (2015) demonstrated that the range of diurnal changes in pH and DO are seasonally dependent, 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}, changes generally reflective of the conditions used during the experiments presented here (Fig 1). 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 upwelling may regularly expose organisms to continuously acidified but normoxic conditions (Feely et al. 2008, 2010). 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; Diaz and Rosenburg 2008; Rabalais et al. 2010; Wallace et al. 2014).

In conclusion, I found that diurnal exposure to low pH and low DO is more severe than chronic exposure when the mean levels of pH and DO are identical. These findings suggest previous studies focusing only on continuous exposure to similar factors may represent unrealistic responses of these organisms to coastal acidification and hypoxia. Regardless of the

manner in which these organisms are exposed to acidic and hypoxic conditions, it is obvious they will suffer deleterious effects. Because shellfish are ecologically and economically important marine species, it is necessary to implement environmental regulations that will protect them from worsening anthropogenic acidification and hypoxia.

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Figures and Tables

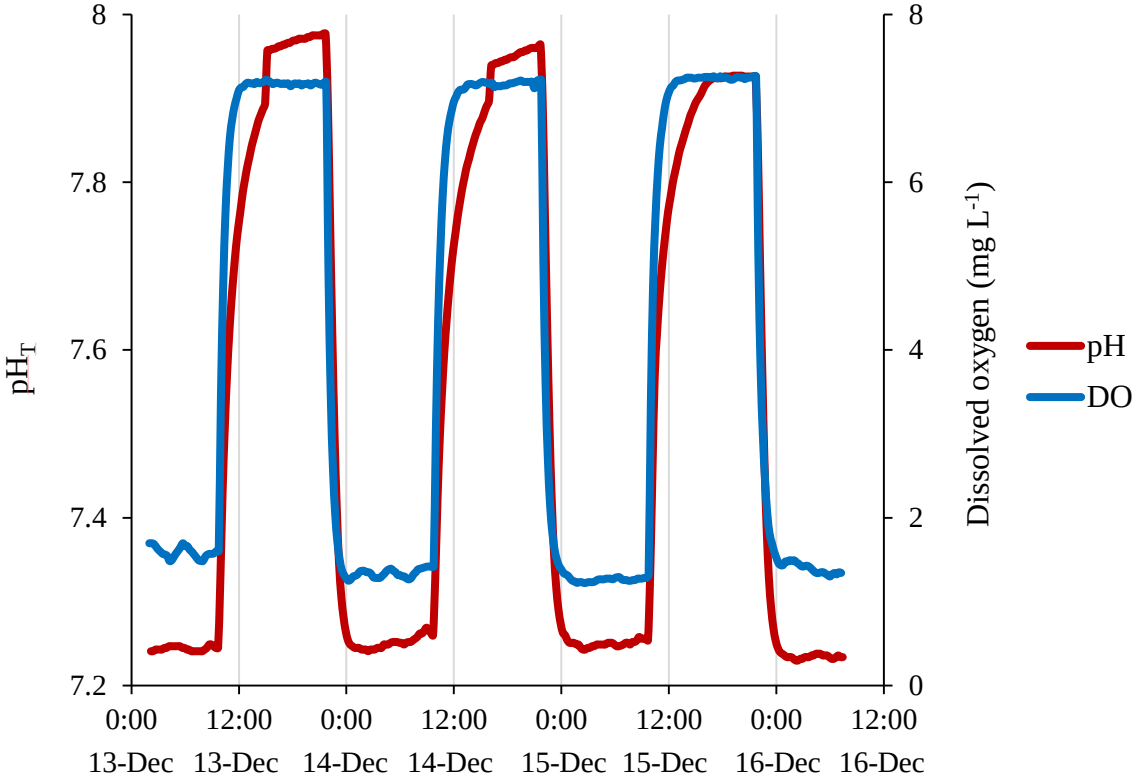

Figure 1. Example of diurnal fluctuations in pH and dissolved oxygen (DO) concentrations from the seven treatment acidification and hypoxia experiment with *Argopecten irradians* larvae. Each day from 0900-2100, ambient air was bubbled into the experimental vessels to maintain a pH of ~7.9 and DO concentrations of ~7 mg L^{-1}. From 2100-0900, CO_2 and N_2 gases were used to create acidic and hypoxic conditions with a pH of ~7.2 and DO concentrations of ~2 mg L^{-1}.

Table 1. Treatments and corresponding gas mixtures for three different acidification and hypoxia experiments.

Experiment	Treatment	Ambient air	5% CO_2	N_2	400 ppm CO_2/N_2
Acidification experiment	Control	✓			
	Low pH	✓	✓		
	Intermediate pH	✓	✓		
	Diurnal pH Day	✓			
	Diurnal pH Night	✓	✓		
Four treatment acidification and hypoxia experiment	Control	✓			
	Low pH-DO	✓	✓	✓	
	Intermediate pH-DO	✓	✓	✓	
	Diurnal pH-DO Day	✓			
	Diurnal pH-DO Night	✓	✓	✓	
Seven treatment acidification and hypoxia experiment	Control	✓			
	Low pH	✓	✓		
	Low DO	✓			✓
	Low pH-DO	✓	✓	✓	
	Diurnal pH Day	✓			
	Diurnal pH Night	✓	✓		
	Diurnal DO Day	✓			
	Diurnal DO Night	✓			✓
	Diurnal pH-DO Day	✓			
	Diurnal pH-DO Night	✓	✓	✓	

Table 2. Mean (± standard deviation) pH, pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the larval *Argopecten irradians* diurnal acidification experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.94 ± 0.06	7.60 ± 0.03	7.33 ± 0.07	7.87 ± 0.02	7.32 ± 0.08	7.47 ± 0.43
pCO_2 (µatm)	516 ± 18	1200 ± 134	2340 ± 384	662 ± 148	2700 ± 907	1680 ± 527
$\Omega_{calcite}$	3.44 ± 0.82	1.61 ± 0.10	0.90 ± 0.16	3.15 ± 0.69	0.99 ± 0.20	2.07 ± 0.45
$\Omega_{aragonite}$	2.21 ± 0.53	1.04 ± 0.06	0.58 ± 0.10	2.03 ± 0.44	0.64 ± 0.13	1.33 ± .03
TDIC (µmol L^{-1})	1930 ± 269	1970 ± 94	2030 ± 82	2090 ± 473	2290 ± 467	2190 ± 470
CO_3^{2-} (µmol L^{-1})	136 ± 33	63.7 ± 3.9	35.4 ± 6.1	125 ± 28	39.2 ± 7.9	81.9 ± 18
TA (µmol L^{-1})	2110 ± 307	2030 ± 89	2010 ± 80	2250 ± 495	2260 ± 444	2250 ± 469
Salinity	28.3 ± 0.58	28.3 ± 0.58	28.3 ± 0.58	28.3 ± 0.58	28.3 ± 0.58	28.3 ± 0.58
Temperature (ºC)	23.5 ± 0.52	23.5 ± 0.52	23.5 ± 0.52	23.5 ± 0.52	23.5 ± 0.52	23.5 ± 0.52

Table 3. One-way ANOVA results for survival of *Argopecten irradians* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.229	0.0762	10.43	0.00387
Residuals	8	0.0584	0.0073		

Table 4. One-way ANOVA results for growth rates of *Argopecten irradians* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	515	171.67	12.84	0.000648
Residuals	11	147.1	13.37		

Table 5. One-way ANOVA results for development of *Argopecten irradians* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	1.932	0.644	12.46	0.000735
Residuals	11	0.569	0.0517		

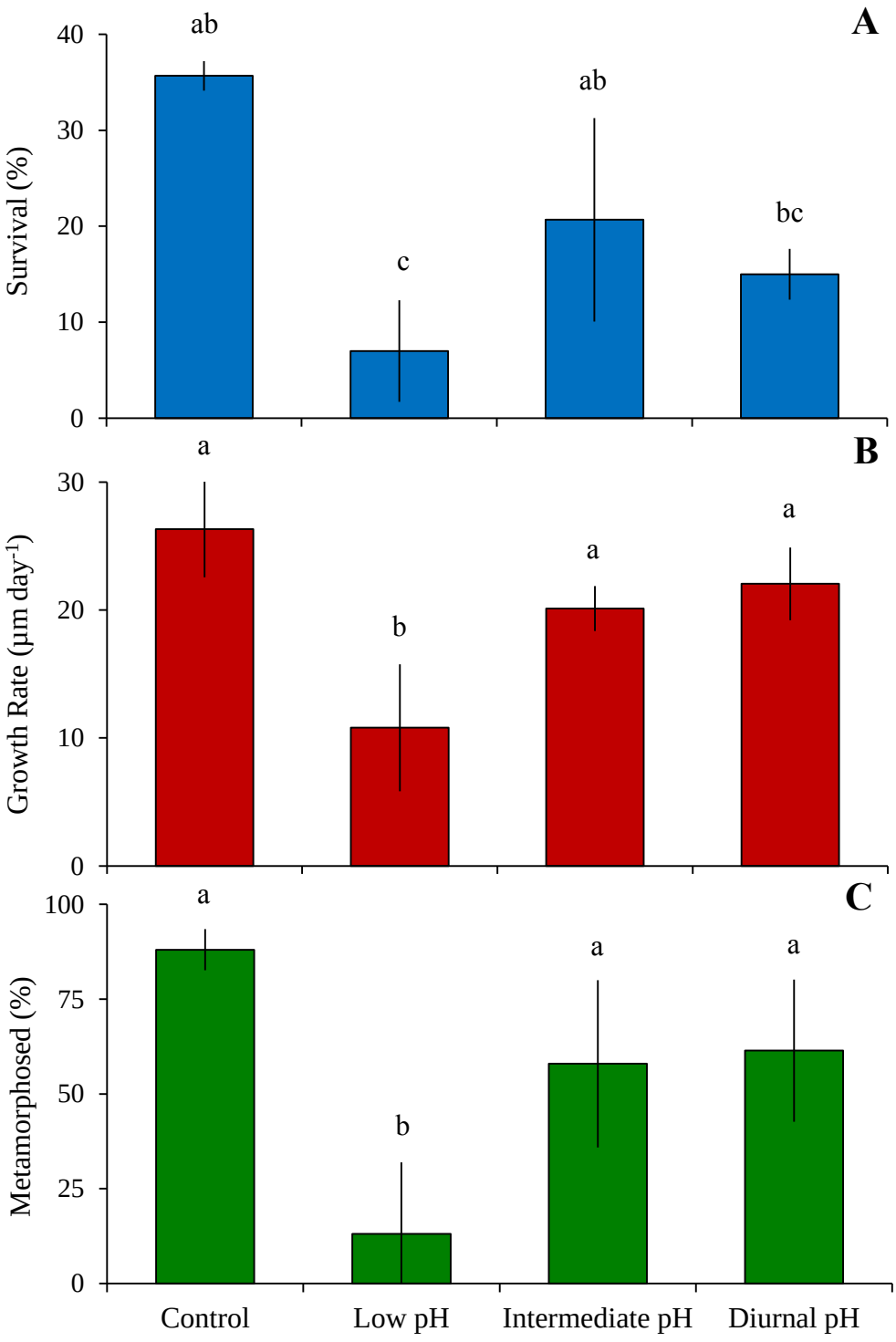

Figure 2. Survival (A), growth (B), and development (C) of *Argopecten irradians* larvae in the diurnal acidification experiment (Table 2). Percent metamorphosis was calculated 12 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 3, 4, 5).

Table 6. Mean (± standard deviation) pH, pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the larval *Mercenaria mercenaria* diurnal acidification experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.91 ± 0.02	7.58 ± 0.04	7.29 ± 0.06	7.82 ± 0.06	7.27 ± 0.05	7.54 ± 0.34
pCO_2 (µatm)	643 ± 169	1390 ± 309	2800 ± 593	792 ± 320	2900 ± 710	1850 ± 515
$\Omega_{calcite}$	2.53 ± 0.09	1.24 ± 0.07	0.67 ± 0.08	1.99 ± 0.21	0.61 ± 0.07	1.30 ± 0.14
$\Omega_{aragonite}$	1.63 ± 0.06	0.80 ± 0.05	0.43 ± 0.06	1.28 ± 0.13	0.40 ± 0.05	0.84 ± 0.09
TDIC (µmol L^{-1})	1820 ± 238	1850 ± 205	1940 ± 253	1750 ± 294	1900 ± 269	1820 ± 282
CO_3^{2-} (µmol L^{-1})	100 ± 3.9	49.2 ± 3.2	26.5 ± 3.7	78.9 ± 7.7	24.4 ± 3.0	51.6 ± 5.4
TA (µmol L^{-1})	1950 ± 234	1880 ± 197	1890 ± 240	1850 ± 278	1850 ± 251	1850 ± 264
Salinity	28.5 ± 3.5	28.5 ± 3.5	28.5 ± 3.5	28.5 ± 3.5	28.5 ± 3.5	28.5 ± 3.5
Temperature (ºC)	23.0 ± 0.25	23.0 ± 0.25	23.0 ± 0.25	23.0 ± 0.25	23.0 ± 0.25	23.0 ± 0.25

Table 7. One-way ANOVA results for survival of *Mercenaria mercenaria* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.0434	0.0145	6.63	0.0146
Residuals	8	0.0174	0.00218		

Table 8. One-way ANOVA results for growth rates of *Mercenaria mercenaria* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	10.71	3.571	0.629	0.611
Residuals	11	62.43	5.675		

Table 9. One-way ANOVA results for development of *Mercenaria mercenaria* larvae in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.392	0.131	0.306	0.821
Residuals	5	2.135	0.427		

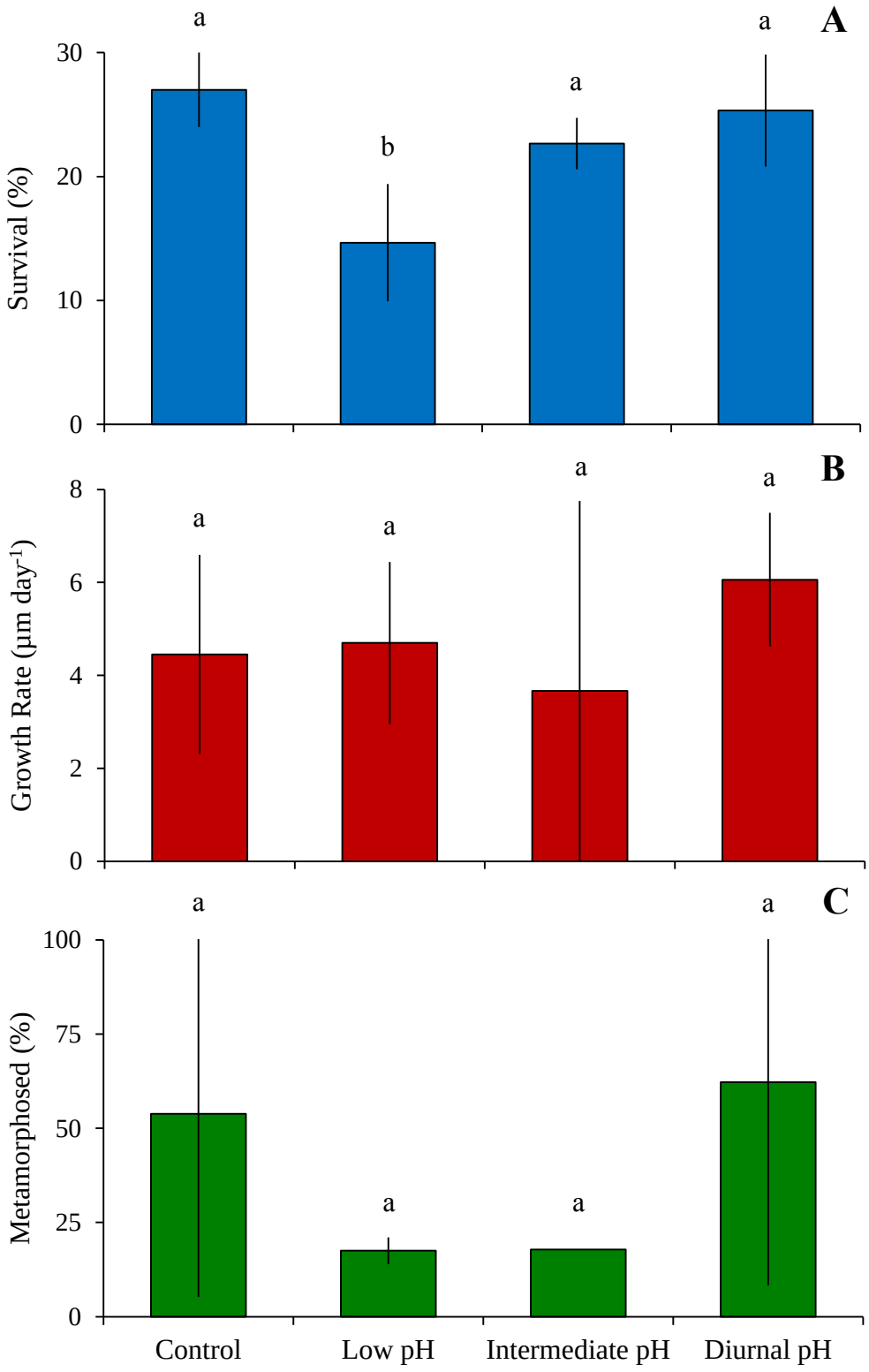

Figure 3. Survival (A), growth (B), and development (C) of *Mercenaria mercenaria* larvae in the diurnal acidification experiment (Table 6). Percent metamorphosis was calculated 18 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 7, 8, 9).

Table 10. Mean (± standard deviation) pH, pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the juvenile *Argopecten irradians* diurnal acidification experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.92 ± 0.05	7.49 ± 0.04	7.13 ± 0.03	7.87 ± 0.05	7.51 ± 0.10	7.57 ± 0.43
pCO_2 (µatm)	527 ± 132	1510 ± 146	3070 ± 375	990 ± 232	522 ± 50	756 ± 141
$\Omega_{calcite}$	3.32 ± 0.18	1.34 ± 0.15	0.52 ± 0.10	2.35 ± 0.41	3.15 ± 0.23	2.75 ± 0.32
$\Omega_{aragonite}$	2.16 ± 0.12	0.88 ± 0.09	0.34 ± 0.06	1.53 ± 0.27	2.05 ± 0.15	1.79 ± 0.21
TDIC (µmol L^{-1})	1860 ± 259	1950 ± 96	1760 ± 235	2090 ± 306	1810 ± 26	1950 ± 166
CO_3^{2-} (µmol L^{-1})	134 ± 7.4	54.2 ± 5.8	21.2 ± 3.9	94.6 ± 17	127 ± 9.3	111 ± 13
TA (µmol L^{-1})	2050 ± 254	1990 ± 98	1700 ± 230	2200 ± 305	1990 ± 21	2090 ± 163
Salinity	30.7 ± 0.58	30.7 ± 0.58	30.7 ± 0.58	30.7 ± 0.58	30.7 ± 0.58	30.7 ± 0.58
Temperature (ºC)	23.7 ± 0.48	23.7 ± 0.48	23.7 ± 0.48	23.7 ± 0.48	23.7 ± 0.48	23.7 ± 0.48

Table 11. One-way ANOVA results for survival of *Argopecten irradians* juveniles in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.984	0.328	10.32	0.00121
Residuals	12	0.381	0.0318		

Table 12. One-way ANOVA results for growth rates of *Argopecten irradians* juveniles in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	5367	1789	0.977	0.436
Residuals	12	21970	1831		

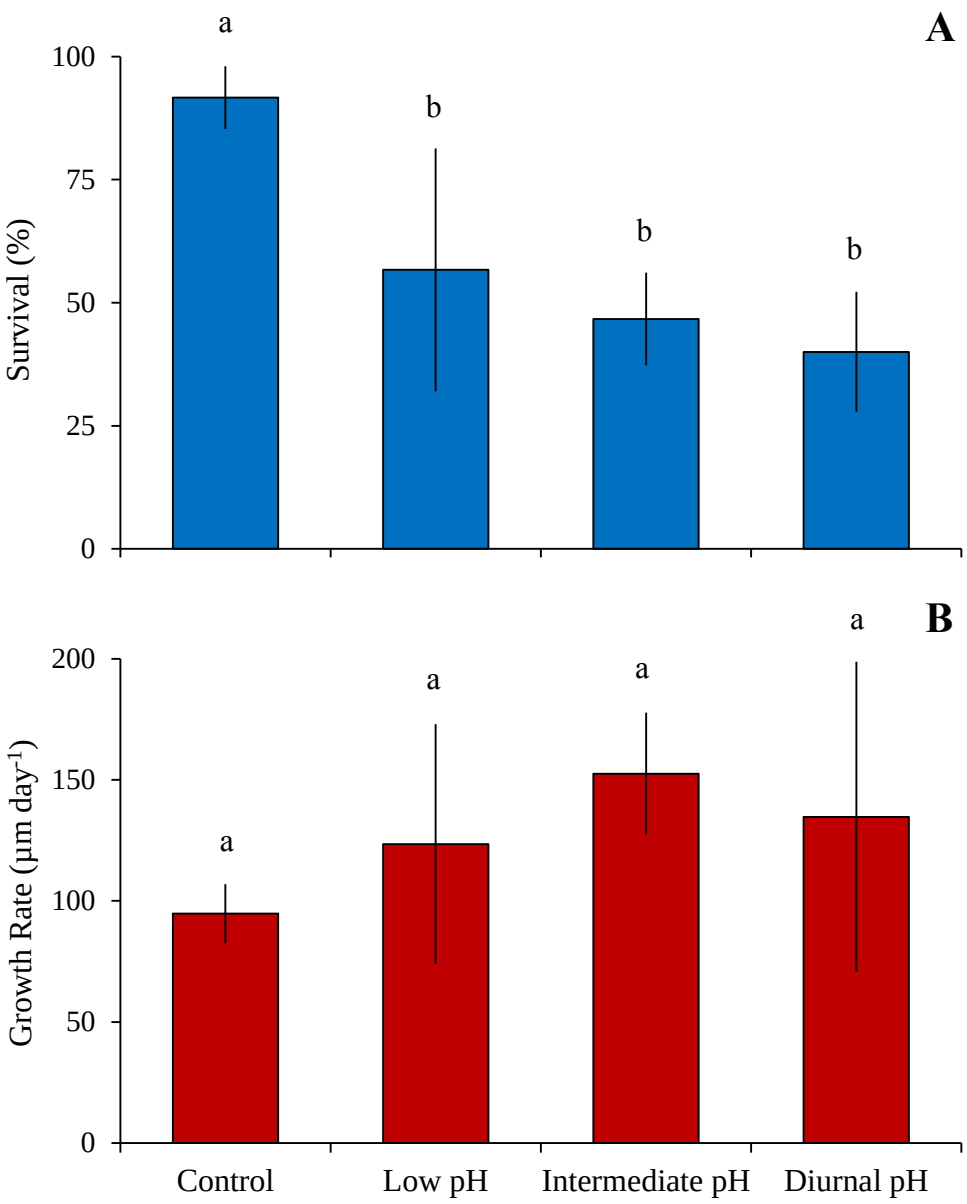

Figure 4. Survival (A) and growth (B) of juvenile *Argopecten irradians* in the diurnal acidification experiment (Table 10). Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 11, 12).

Table 13. Mean (± standard deviation) pH, pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the juvenile *Mercenaria mercenaria* diurnal acidification experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.86 ± 0.05	7.57 ± 0.03	7.25 ± 0.03	7.83 ± 0.05	7.35 ± 0.12	7.57 ± 0.33
pCO_2 (µatm)	561 ± 93	1140 ± 65	2500 ± 267	605 ± 112	1940 ± 391	1270 ± 252
$\Omega_{calcite}$	2.52 ± 0.16	1.38 ± 0.16	0.69 ± 0.09	2.30 ± 0.30	0.88 ± 0.32	1.59 ± 0.31
$\Omega_{aragonite}$	1.62 ± 0.10	0.89 ± 0.12	0.44 ± 0.06	1.48 ± 0.20	0.57 ± 0.21	1.02 ± 0.21
TDIC (µmol L^{-1})	1690 ± 117	1760 ± 107	1850 ± 155	1670 ± 213	1770 ± 136	1720 ± 174
CO_3^{2-} (µmol L^{-1})	98.9 ± 6.3	54.3 ± 7.8	27.1 ± 3.7	90.6 ± 13	34.6 ± 13	62.6 ± 13
TA (µmol L^{-1})	1820 ± 116	1800 ± 117	1810 ± 154	1790 ± 222	1760 ± 166	1780 ± 194
Salinity	27.5 ± 2.1	27.5 ± 2.1	27.5 ± 2.1	27.5 ± 2.1	27.5 ± 2.1	27.5 ± 2.1
Temperature (ºC)	23.3 ± 0.61	23.3 ± 0.61	23.3 ± 0.61	23.3 ± 0.61	23.3 ± 0.61	23.3 ± 0.61

Table 14. One-way ANOVA results for survival of *Mercenaria mercenaria* juveniles in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.035	0.0117	0.45	0.722
Residuals	12	0.311	0.026		

Table 15. One-way ANOVA results for growth rates of *Mercenaria mercenaria* juveniles in the diurnal acidification experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	1081	360.2	1.847	0.192
Residuals	12	2341	195.1		

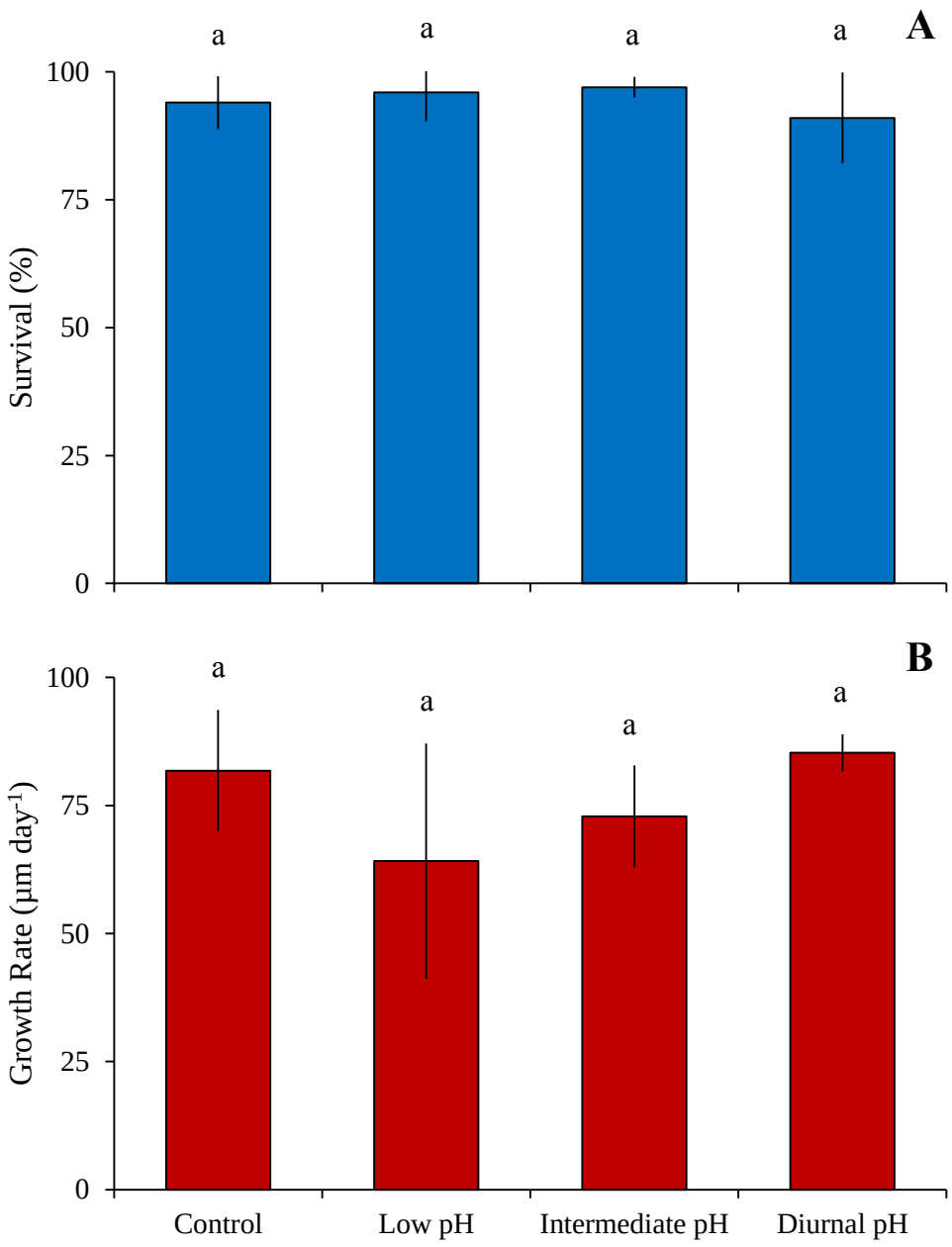

Figure 5. Survival (A) and growth (B) of juvenile *Mercenaria mercenaria* in the diurnal acidification experiment (Table 13). Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 14, 15).

Table 16. Mean (± standard deviation) pH, dissolved oxygen (DO), pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the four treatment larval *Argopecten irradians* diurnal acidification and hypoxia experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.89 ± 0.03	7.48 ± 0.05	7.22 ± 0.05	7.89 ± 0.06	7.28 ± 0.11	7.61 ± 0.26
DO (mg L^{-1})	6.87 ± 0.25	4.08 ± 0.41	1.38 ± 0.45	7.09 ± 0.12	1.32 ± 0.49	4.11 ± 2.80
pCO_2 (µatm)	542 ± 55	1590 ± 236	3030 ± 213	530 ± 44	2820 ± 336	1670 ± 1200
$\Omega_{calcite}$	2.83 ± 0.10	1.17 ± 0.08	0.67 ± 0.06	2.87 ± 0.18	0.68 ± 0.06	1.77 ± 1.10
$\Omega_{aragonite}$	1.83 ± 0.06	0.76 ± 0.06	0.43 ± 0.04	1.86 ± 0.12	0.44 ± 0.04	1.15 ± 0.74
TDIC (µmol L^{-1})	1790 ± 58	1920 ± 136	2020 ± 45	1780 ± 45	1960 ± 21	1870 ± 100
CO_3^{2-} (µmol L^{-1})	114 ± 3.6	47.0 ± 3.5	26.8 ± 2.6	115 ± 7.5	27.4 ± 2.3	71.3 ± 46
TA (µmol L^{-1})	1940 ± 56	1940 ± 131	1970 ± 46	1940 ± 49	1920 ± 11	1910 ± 35
Salinity	30.2 ± 1.2	30.2 ± 1.2	30.2 ± 1.2	30.2 ± 1.2	30.2 ± 1.2	30.2 ± 1.2
Temperature (ºC)	23.3 ± 0.84	23.3 ± 0.84	23.3 ± 0.84	23.3 ± 0.84	23.3 ± 0.84	23.3 ± 0.84

Table 17. One-way ANOVA results for survival of *Argopecten irradians* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.0978	0.0326	6.784	0.00631
Residuals	12	0.0577	0.0048		

Table 18. One-way ANOVA results for growth rates of *Argopecten irradians* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	110.01	36.67	87.83	1.96×10^{-8}
Residuals	12	5.01	0.42		

Table 19. One-way ANOVA results for development of *Argopecten irradians* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.156	0.052	4.824	0.0199
Residuals	12	0.129	0.0108		

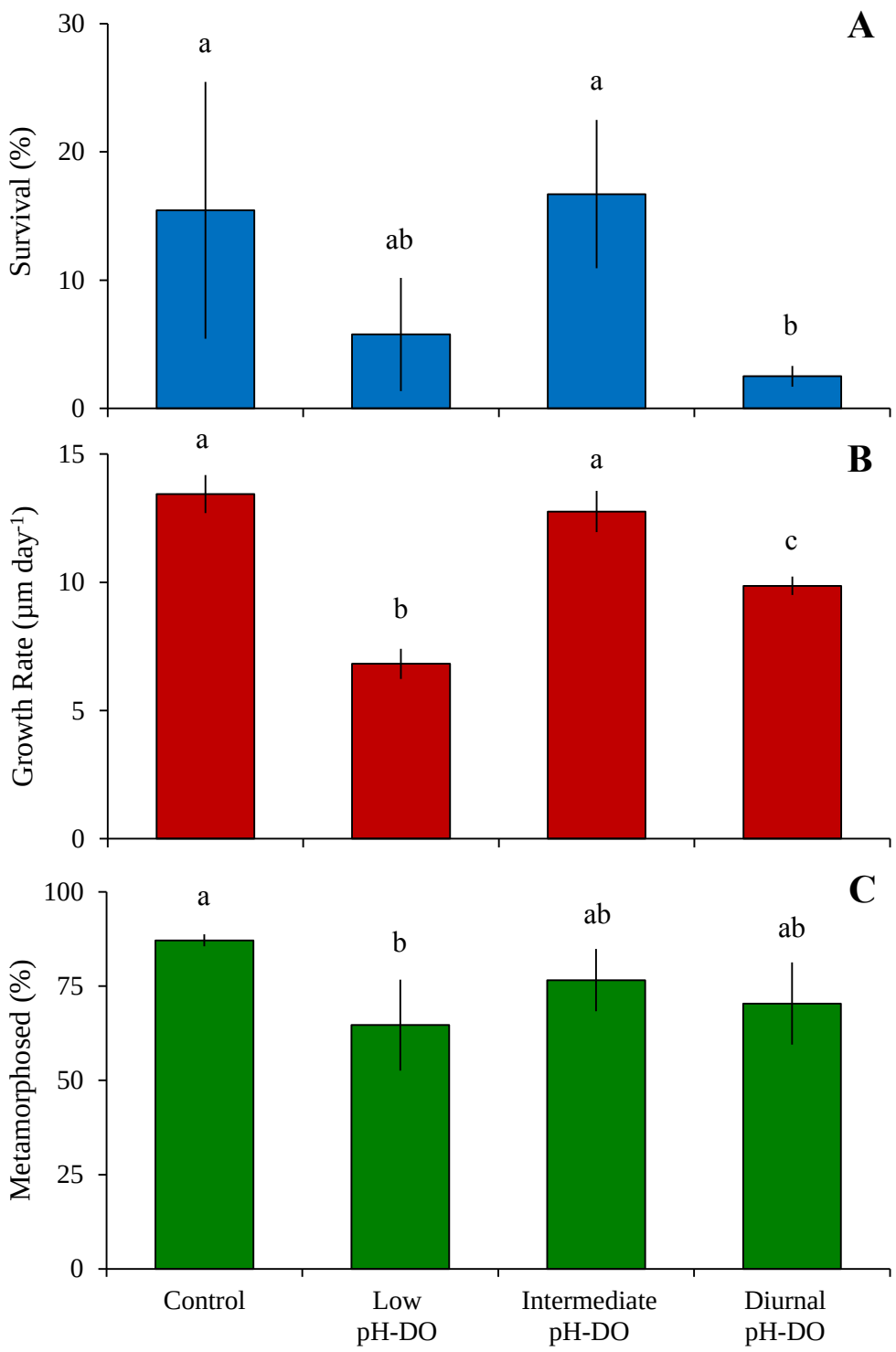

Figure 6. Survival (A), growth (B), and development (C) of *Argopecten irradians* larvae in the four treatment diurnal acidification and hypoxia experiment (Table 16). Percent metamorphosis was calculated 15 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 17, 18, 19).

Table 20. Mean (± standard deviation) pH, dissolved oxygen (DO), pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the four treatment larval *Mercenaria mercenaria* diurnal acidification and hypoxia experiment.

	Continuous			**Diurnal**		
Parameter	Control	Intermediate	Low	Day	Night	Mean
pH_T	7.87 ± 0.03	7.43 ± 0.03	7.24 ± 0.04	7.78 ± 0.06	7.24 ± 0.07	7.41 ± 0.34
DO (mg L^{-1})	6.92 ± 0.13	3.92 ± 0.34	1.32 ± 0.30	7.08 ± 0.15	1.34 ± 0.59	4.02 ± 3.00
pCO_2 (µatm)	612 ± 36	1750 ± 142	2710 ± 203	694 ± 96	2850 ± 544	1770 ± 1170
$\Omega_{calcite}$	2.81 ± 0.17	1.23 ± 0.14	0.77 ± 0.14	2.41 ± 0.36	0.76 ± 0.17	1.58 ± 0.90
$\Omega_{aragonite}$	1.82 ± 0.11	0.79 ± 0.09	0.50 ± 0.04	1.56 ± 0.25	0.49 ± 0.11	1.02 ± 0.58
TDIC (µmol L^{-1})	1880 ± 32	2060 ± 97	2050 ± 36	1840 ± 105	2050 ± 32	1940 ± 133
CO_3^{2-} (µmol L^{-1})	113 ± 6.9	49.2 ± 5.5	31.0 ± 2.2	96.6 ± 15	30.4 ± 6.9	63.5 ± 36
TA (µmol L^{-1})	2030 ± 38	2080 ± 101	2010 ± 34	1960 ± 117	2010 ± 34	1980 ± 87
Salinity	30.3 ± 0.35	30.3 ± 0.35	30.3 ± 0.35	30.3 ± 0.35	30.3 ± 0.35	30.3 ± 0.35
Temperature (ºC)	22.9 ± 0.16	22.9 ± 0.16	22.9 ± 0.16	22.9 ± 0.16	22.9 ± 0.16	22.9 ± 0.16

Table 21. One-way ANOVA results for survival of *Mercenaria mercenaria* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.0456	0.0152	9.156	0.00199
Residuals	12	0.0199	0.00166		

Table 22. One-way ANOVA results for growth rates of *Mercenaria mercenaria* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	33.08	11.027	4.994	0.0178
Residuals	12	26.5	2.208		

Table 23. One-way ANOVA results for development of *Mercenaria mercenaria* larvae in the four treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Between groups	3	0.363	0.121	21.52	4.05×10^{-5}
Residuals	12	0.0675	0.00562		

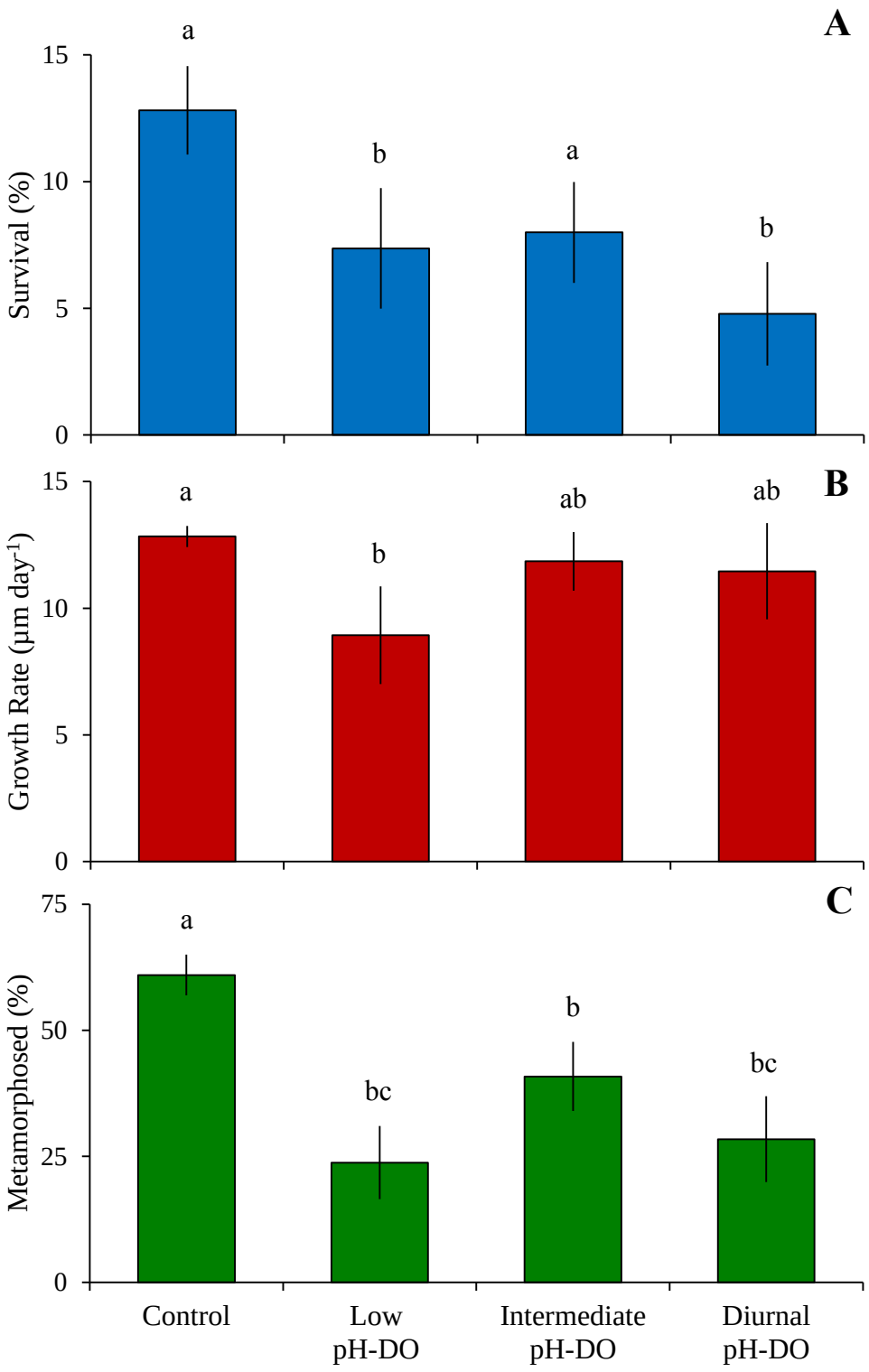

Figure 7. Survival (A), growth (B), and development (C) of *Mercenaria mercenaria* larvae in the four treatment diurnal acidification and hypoxia experiment (Table 20). Percent metamorphosis was calculated 11 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). Lowercase letters indicate significant differences (Tables 21, 22, 23).

Table 24. Mean (± standard deviation) pH, dissolved oxygen (DO), pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the seven treatment larval *Argopecten irradians* diurnal acidification and hypoxia experiment.

	Continuous				**Diurnal pH**		
Parameter	Control	Low pH	Low DO	Low pH-DO	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
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
pCO_2 (µatm)	516 ± 9.0	3480 ± 190	548 ± 56	3290 ± 234	586 ± 37	3180 ± 181	1880 ± 1390
$\Omega_{calcite}$	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
$\Omega_{aragonite}$	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
TDIC (µmol L^{-1})	1800 ± 31	2060 ± 147	1830 ± 91	2010 ± 40	1770 ± 16	2050 ± 129	1910 ± 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
TA (µmol L^{-1})	1970 ± 36	1990 ± 147	1990 ± 93	1950 ± 52	1920 ± 15	1990 ± 137	1950 ± 53
Salinity	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56
Temperature (ºC)	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31

	Diurnal DO			**Diurnal pH-DO**		
Parameter	Day	Night	Mean	Day	Night	Mean
pH_T	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})	6.82 ± 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)	573 ± 91	515 ± 21	544 ± 69	623 ± 41	2930 ± 241	1770 ± 1250
$\Omega_{calcite}$	2.78 ± 0.09	2.80 ± 0.12	2.79 ± 0.10	2.48 ± 0.02	0.69 ± 0.13	1.59 ± 0.96
$\Omega_{aragonite}$	1.79 ± 0.07	1.81 ± 0.08	1.80 ± 0.07	1.60 ± 0.02	0.45 ± 0.09	1.03 ± 0.62
TDIC (µmol L^{-1})	1810 ± 117	1730 ± 31	1770 ± 55	1770 ± 45	2010 ± 140	1890 ± 169
CO_3^{2-} (µmol L^{-1})	111 ± 4.3	112 ± 5.4	111 ± 0.57	99.1 ± 1.5	27.7 ± 5.4	63.4 ± 51
TA (µmol L^{-1})	1960 ± 104	1890 ± 34	1930 ± 51	1910 ± 40	1960 ± 150	1940 ± 42
Salinity	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56	29.5 ± 0.56
Temperature (ºC)	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31	23.1 ± 0.31

Table 25. Two-way ANOVA results for survival of *Argopecten irradians* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
pH	2	0.205	0.103	53.356	5.86×10^{-9}
Dissolved oxygen	2	0.0906	0.0453	23.577	4.28×10^{-6}
pH:Dissolved oxygen	2	0.0267	0.0133	6.932	0.00488
Residuals	21	0.0404	0.00192		

Table 26. Two-way ANOVA results for growth rates of *Argopecten irradians* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
pH	2	63.02	31.509	18.264	2.54×10^{-5}
Dissolved oxygen	2	34.42	17.208	9.975	0.000901
pH:Dissolved oxygen	2	7.88	3.939	2.283	0.127
Residuals	21	36.23	1.725		

Table 27. Two-way ANOVA results for development of *Argopecten irradians* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
pH	2	0.61	0.305	53.284	5.93×10^{-9}
Dissolved oxygen	2	0.284	0.142	24.85	2.91×10^{-6}
pH:Dissolved oxygen	2	0.0065	0.00326	0.569	0.574
Residuals	21	0.12	0.00572		

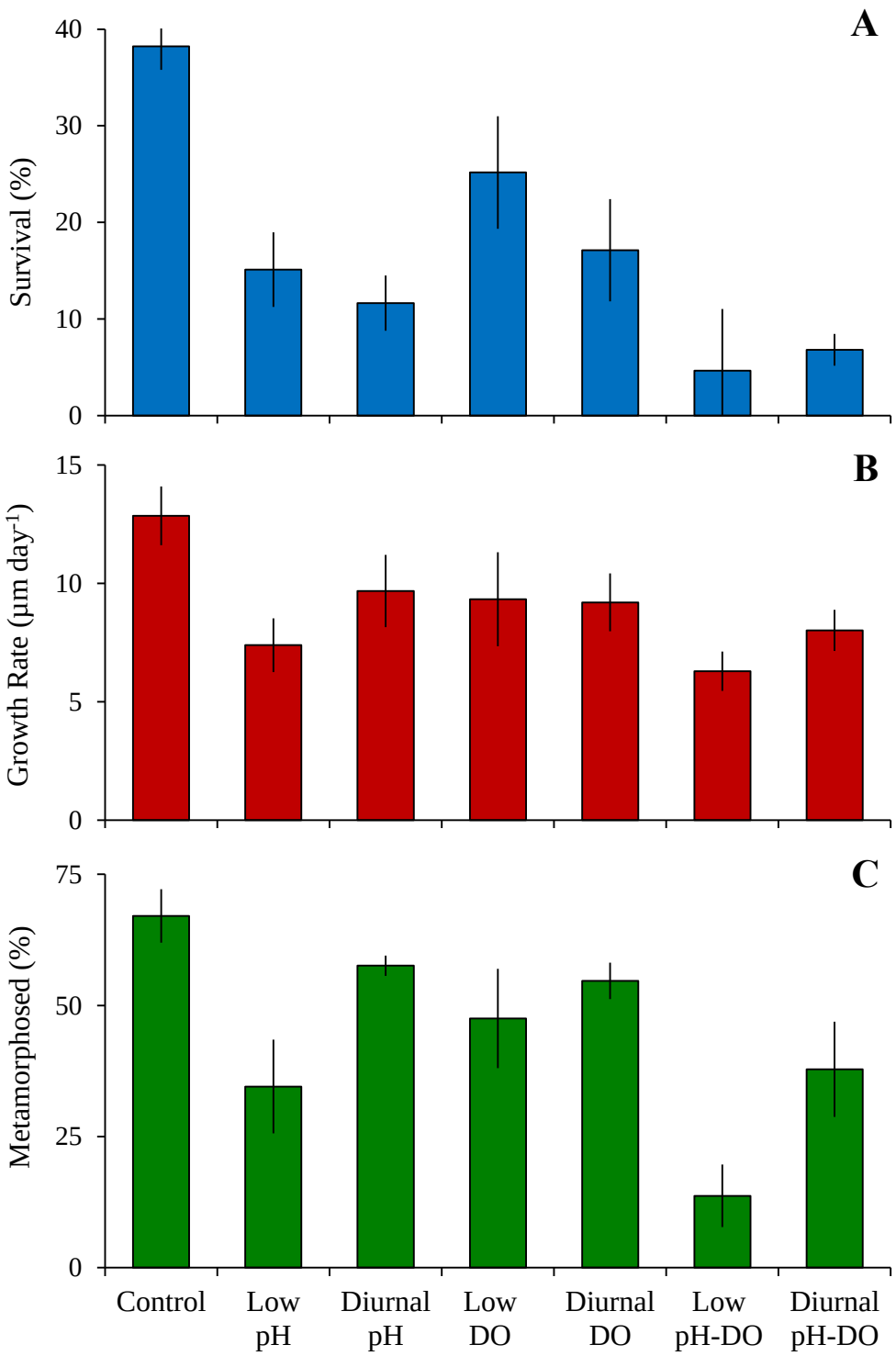

Figure 8. Survival (A), growth (B), and development (C) of *Argopecten irradians* larvae in the seven treatment diurnal acidification and hypoxia experiment (Table 24). Percent metamorphosis was calculated 14 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). Both pH and DO negatively affected survival (pH-$p < 0.001$; DO-$p < 0.001$; Table 25), growth (pH-$p < 0.001$; DO-$p < 0.001$; Table 26), and development (pH-$p < 0.001$; DO-$p < 0.001$; Table 27). There was an antagonistic negative effect of both factors on survival ($p < 0.05$).

Table 28. Mean (± standard deviation) pH, dissolved oxygen (DO), pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the seven treatment larval *Mercenaria mercenaria* diurnal acidification and hypoxia experiment.

	Continuous				**Diurnal pH**		
Parameter	Control	Low pH	Low DO	Low pH-DO	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
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
pCO_2 (µatm)	509 ± 70	3580 ± 70	521 ± 62	3100 ± 61	601 ± 54	3130 ± 268	1870 ± 1470
$\Omega_{calcite}$	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
$\Omega_{aragonite}$	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
TDIC (µmol L^{-1})	1790 ± 0.56	2180 ± 43	1810 ± 152	2040 ± 8.3	1780 ± 7.5	2160 ± 14	1970 ± 216
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
TA (µmol L^{-1})	1960 ± 3.6	2110 ± 41	1980 ± 155	1990 ± 5.9	1920 ± 5.6	2110 ± 2.8	2020 ± 128
Salinity	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75
Temperature (ºC)	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21

	Diurnal DO			**Diurnal pH-DO**		
Parameter	Day	Night	Mean	Day	Night	Mean
pH_T	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})	7.35 ± 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)	500 ± 9.2	507 ± 1.8	504 ± 6.5	601 ± 54	2860 ± 118	1730 ± 1310
$\Omega_{calcite}$	2.85 ± 0.04	2.89 ± 0.08	2.87 ± 0.06	2.50 ± 0.00	0.80 ± 0.02	1.65 ± 0.98
$\Omega_{aragonite}$	1.85 ± 0.03	1.87 ± 0.05	1.86 ± 0.04	1.62 ± 0.00	0.52 ± 0.01	1.07 ± 0.64
TDIC (µmol L^{-1})	1720 ± 2.0	1740 ± 24	1730 ± 15	1740 ± 2.1	2130 ± 21	1930 ± 281
CO_3^{2-} (µmol L^{-1})	115 ± 1.6	116 ± 3.4	115 ± 1.0	100 ± 0.12	32.1 ± 0.76	66.2 ± 48
TA (µmol L^{-1})	1880 ± 0.77	1910 ± 28	1890 ± 16	1870 ± 2.2	2100 ± 16	1980 ± 157
Salinity	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75	29.2 ± 0.75
Temperature (ºC)	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21	22.8 ± 0.21

Table 29. Two-way ANOVA results for survival of *Mercenaria mercenaria* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH	2	0.514	0.257	242.428	2.99×10^{-14}
Dissolved oxygen	2	0.0047	0.00234	2.21	0.137
pH:Dissolved oxygen	2	0.008	0.00398	3.761	0.042
Residuals	19	0.0201	0.00106		

Table 30. Two-way ANOVA results for growth rates of *Mercenaria mercenaria* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH	2	2.982	1.491	4.306	0.0271
Dissolved oxygen	2	2.541	1.271	3.67	0.043
pH:Dissolved oxygen	2	0.442	0.221	0.639	0.538
Residuals	21	7.27	0.346		

Table 31. Two-way ANOVA results for development of *Mercenaria mercenaria* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH	2	1.0667	0.533	128.5	3.86×10^{-12}
Dissolved oxygen	2	0.52	0.26	62.6	2.46×10^{-9}
pH:Dissolved oxygen	2	0.405	0.203	48.8	2.02×10^{-8}
Residuals	21	0.083	0.0042		

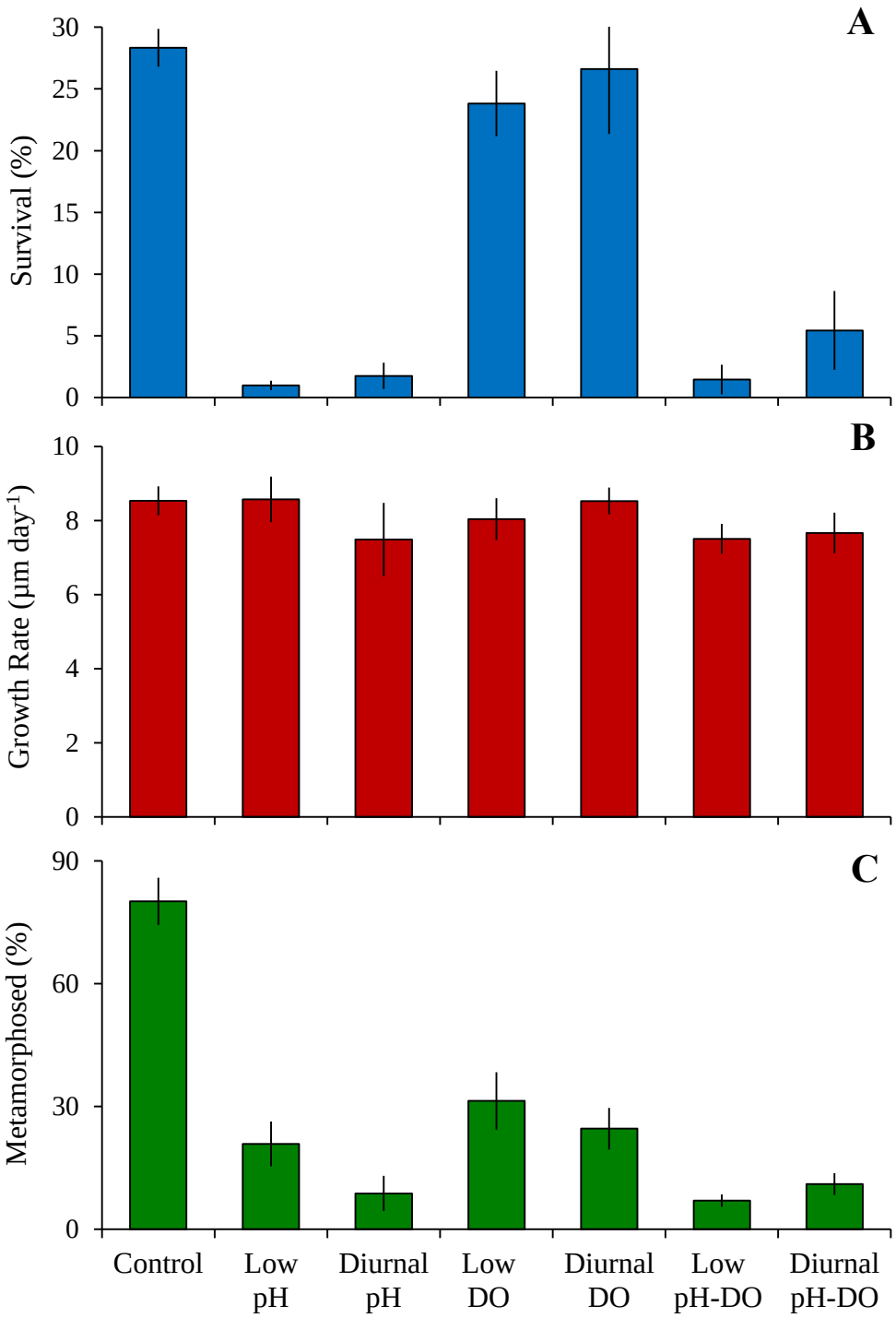

Figure 9. Survival (A), growth (B), and development (C) of *Mercenaria mercenaria* larvae in the seven treatment diurnal acidification and hypoxia experiment (Table 28). Percent metamorphosis was calculated 17 days post-fertilization. Error bars represent standard deviation of the mean (n = 4). There were no effects of DO on survival, but pH significantly reduced survival ($p < 0.001$; Table 29) and there was an antagonistic negative effect of both pH and DO ($p < 0.05$). Growth and development of larvae were affected by pH (growth-$p < 0.05$; development-$p < 0.001$; Table 30) and DO (growth-$p < 0.05$; development-$p < 0.001$; Table 31), and there was an antagonistic negative effect of pH and DO on development ($p < 0.001$).

Table 32. Mean (± standard deviation) pH, dissolved oxygen (DO), pCO_2, saturation states of calcite and aragonite, total dissolved inorganic carbon (TDIC), carbonate, total alkalinity (TA), salinity, and temperature for the seven treatment larval *Crassostrea virginica* diurnal acidification and hypoxia experiment.

	Continuous				**Diurnal pH**		
Parameter	Control	Low pH	Low DO	Low pH-DO	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 ± 0.0.06	7.54 ± 0.26
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
pCO_2 (µatm)	522 ± 2.7	3380 ± 250	575 ± 52	3480 ± 134	570 ± 16	3230 ± 131	1900 ± 1540
$\Omega_{calcite}$	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
$\Omega_{aragonite}$	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
TDIC (µmol L^{-1})	1810 ± 53	1940 ± 53	1840 ± 22	1975 ± 15	1770 ± 19	1940 ± 48	1850 ± 104
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
TA (µmol L^{-1})	1970 ± 62	1870 ± 44	1990 ± 39	1900 ± 9.6	1910 ± 17	1870 ± 44	1890 ± 23
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
Temperature (ºC)	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19

	Diurnal DO			**Diurnal pH-DO**		
Parameter	Day	Night	Mean	Day	Night	Mean
pH_T	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})	7.24 ± 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)	645 ± 64	524 ± 31	585 ± 81	644 ± 16	2990 ± 380	1820 ± 1370
$\Omega_{calcite}$	2.70 ± 0.00	2.70 ± 0.01	2.70 ± 0.01	2.46 ± 0.02	0.59 ± 0.08	1.52 ± 1.08
$\Omega_{aragonite}$	1.74 ± 0.00	1.74 ± 0.01	1.74 ± 0.01	1.59 ± 0.01	0.38 ± 0.05	0.98 ± 0.70
TDIC (µmol L^{-1})	1900 ± 91	1720 ± 44	1810 ± 125	1812 ± 14	1890 ± 3.0	1850 ± 56
CO_3^{2-} (µmol L^{-1})	107 ± 0.14	107 ± 0.58	107 ± 0.10	97.9 ± 0.79	23.3 ± 3.1	60.6 ± 53
TA (µmol L^{-1})	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
Temperature (ºC)	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19	22.8 ± 0.19

Table 33. Two-way ANOVA results for survival of *Crassostrea virginica* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
pH	2	0.153	0.0763	37.017	1.3×10^{-7}
Dissolved oxygen	2	0.0165	0.00826	4.011	0.0335
pH:Dissolved oxygen	2	0.00022	0.00011	0.054	0.947
Residuals	21	0.0433	0.00206		

Table 34. Two-way ANOVA results for growth rates of *Crassostrea virginica* larvae in the seven treatment diurnal acidification and hypoxia experiment.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
pH	2	3.434	1.717	29.676	7.6×10^{-7}
Dissolved oxygen	2	0.588	0.294	5.084	0.0158
pH:Dissolved oxygen	2	0.484	0.242	4.179	0.0297

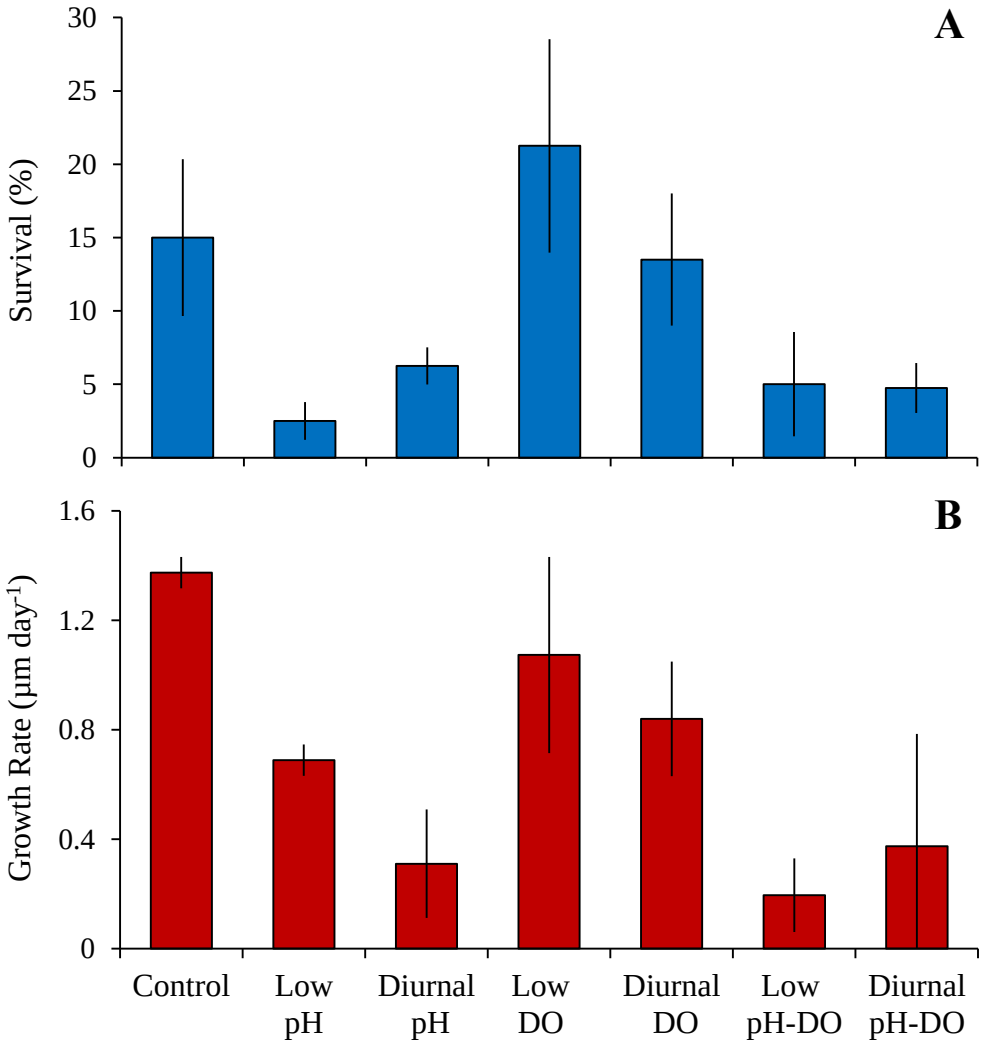

Figure 10. Survival (A) and growth (B) of *Crassostrea virginica* larvae in the seven treatment diurnal acidification and hypoxia experiment (Table 32). Error bars represent standard deviation of the mean (n = 4). Survival and growth were affected by pH (survival-$p < 0.001$; growth-$p < 0.001$; Tables 33, 34) and DO (survival-$p < 0.05$; growth-$p < 0.05$) and there was an antagonistic negative effect of pH and DO on growth ($p < 0.05$).