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Effects of ocean acidification combined with hypoxia, elevated temperature, or restricted food supply on early life stages of the forage fish *Menidia beryllina*, *Menidia menidia,* and *Cyprinodon variegatus*

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Abstract of the Thesis

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Estuarine organisms are experiencing many stressors related to climate change at an accelerated pace when compared to the open ocean. The co-occurrence of acidification and hypoxia has been observed during warmer months when many fish species spawn in temperate estuaries. Concurrently, estuarine systems can experience extreme temperatures during summer and dynamic levels of plankton. This study assessed the tolerance of early life stage estuarine fish to the co-occurrence of acidification with hypoxia, elevated temperatures, and varying levels of planktonic prey. Time to hatch, hatching rates, survival, and growth were quantified for larval *Menidia beryllina*, *Menidia menidia*, and *Cyprinodon variegatus* exposed from the egg through the larval stages to water with a low pH (7.4 versus 7.9, total scale) and dissolved oxygen concentration (2.5 mg L^{-1} versus 9.0 mg L^{-1}), while embryos of *M. beryllina* were also exposed to elevated temperatures and varying levels of prey. Hypoxia significantly delayed hatching of

embryos by one to three days and reduced hatching success of all three species by 24 - 80%. Acidification and hypoxia had an additive negative effect on survival of *M. beryllina*, a synergistic negative effect on survival of *M. menidia* spawned in May but not June, and no effect on survival of *C. variegatus*. Acidification and hypoxia had an additive negative effect on length of larval *M. beryllina* while hypoxia alone significantly reduced length of *M. menidia* and *C. variegatus,* with reductions ranging from 15 - 45%. As abundant forage fish in estuaries along the Atlantic coast of the US, the tolerance of these three species to acidification and hypoxia may strongly influence the success of the coastal ecosystems and fisheries that depend on them as prey. Acidification and restricted food each significantly reduced survival (by 26% and 33%, respectively) and length (by 15% and 20%) of *M. beryllina*, and when combined they had an additive negative effect on survival and an antagonistic effect on length. Acidification and elevated temperature each significantly reduced survival (by 18% and 85%, respectively) of *M. beryllina* while the combined stressors had an antagonistic effect. This study contributes to the growing body of research that aims to predict how multiple climate change stressors will affect marine organisms and, in turn, ocean ecosystems.

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INTRODUCTION

The burning of fossil fuels since the industrial revolution has led to an increase in atmospheric concentrations of CO_2, and seawater pH has declined as this gas dissolves into the ocean and forms carbonic acid (Caldeira and Wickett 2003). While ocean acidification over the past 200 years has resulted in an average pH decline of ~0.1 units, coastal pH is much more dynamic, in particular due to the influence of eutrophication, upwelling, river discharge, and cycles in biological productivity (Feely et al. 2010, Hofmann et al. 2011, Duarte et al. 2013, Waldbusser and Salisbury 2014). Coastal nutrient loading is known to cause increased algal growth, which is followed by an increase in microbial respiration and, in some cases, hypoxia (Cai et al. 2011). This microbial activity also produces CO_2 that can temporally depress pH levels. The co-occurrence of hypoxia and acidification is known to take place seasonally (Feely et al. 2010, Cai et al. 2011, Baumann et al. 2014), yet the combined effects of these stressors on coastal marine organisms have rarely been evaluated in the laboratory (Gobler et al. 2014).

Ocean acidification and its associated impacts on ocean chemistry, such as a decrease in the concentration of carbonate ions, can pose a significant physiological challenge for calcifying organisms. Numerous studies have evaluated the effects of acidified water on marine calcifiers and especially invertebrates (Ries et al. 2009, Talmage and Gobler 2009, Gazeau et al. 2013). *Mytilus edulis* (blue mussel) larvae exhibited reduced shell length and thickness when exposed to acidified water (Gazeau et al. 2010). *Mercenaria mercenaria* (hard clam), *Crassostrea virginica* (eastern oyster), and *Argopecten irradians* (bay scallop) all exhibited reduced growth and survival when reared from the larval stage under elevated pCO_2 conditions (Talmage and Gobler 2009, 2010). Not all calcifiers respond negatively to elevated pCO_2 exposure, however, as organisms from a broad range of taxa can experience increased, decreased, as well as unchanged

rates of calcification after exposure to pCO_2 equivalent to ~2, 3, and 10 times pre-industrial levels (~280 ppm) (Ries et al. 2009).

Fewer studies have evaluated the effects of ocean acidification on marine teleosts. Such studies have had differing outcomes, from no discernible effects to reduced survival at elevated CO_2 levels. Olfaction was impaired in the reef fish *Amphiprion percula* (orange clownfish) and *Pomacentrus wardi* (Ward's damsel) when exposed to elevated pCO_2, potentially altering the ability of larvae to find appropriate settlement sites or distinguish predators from non-predators (Munday et al. 2009, 2010). Additionally, clownfish and damselfish reared from the larval stage under elevated pCO_2 displayed riskier behavior leading to higher rates of predation (Munday et al. 2010). Dixson et al. (2010) found that larval clownfish olfaction was not impaired by exposure to elevated pCO_2 during the egg stage, and olfaction became impaired only after larvae were exposed to acidified water.

Atlantic cod (*Gadus morhua*) from the Norwegian coast experienced temporary tissue damage when exposed from the egg stage to acidified water, and the same species of cod from the Baltic showed no effects on hatch rate, growth rate, or protein synthesis (Frommel et al. 2012, 2013). These results illustrate the variable effects of acidification and the importance of assessing tolerance on a case-by-case basis. Baumann et al. (2012) found that *Menidia beryllina* (inland silverside) was most susceptible to acidification during the egg stage, with the greatest effects on larval growth (18% reduction) and survival (74% reduction) occurring when exposure to acidified water was initiated in the egg stage rather than the larval stage. Smaller larvae are more susceptible to predation and starvation, and thus the impact of acidification on fish could be amplified by poor recruitment of individuals from the larval to juvenile stage (Houde 1989, Sogard 1997, Kamler 2005).

The effects of hypoxia on marine organisms have been studied extensively. Much focus has been on spreading hypoxic "dead zones" that result from excessive nutrient loading and how they affect marine life, in particular the benthos that are unable to survive under such low oxygen conditions (Rabalais et al. 2002, Diaz and Rosenberg 2008, Vaquer-Sunyer and Duarte 2008). Ecosystem composition is greatly impacted by dead zones, with many organisms being replaced or migrating out of their preferred habitat (Rabalais et al. 2002). Reduced oxygen can be lethal to fish at concentrations above the generally accepted hypoxia threshold of 2 mg L^{-1} and can elicit a range of sublethal responses, including decreased activity, swimming speed, and growth rate (Breitburg 2002, Vaquer-Sunyer and Duarte 2008, Ekau et al. 2010).

Exposure to low dissolved oxygen during somitogenesis of *Pagrus major* (red sea bream) and *Seriola dumerili* (amberjack) resulted in centrum defects that in turn led to morphological defects post hatch (Hattori et al. 2004, Sawada et al. 2006). Larval *M. beryllina* experienced 90% mortality when exposed to dissolved oxygen of 1.3 mg L^{-1}, 50% mortality at 1.4 mg L^{-1}, and 10% mortality at 1.7 mg L^{-1} (Miller et al. 2002). Many adult fish appear to have developed tolerance for low dissolved oxygen, with 31 species of adult reef fish that could regularly encounter hypoxia in tide pools or near respiring corals relying solely on aerobic metabolism down to O_2 concentrations from 13 - 34% of air saturation (Nilsson and Ostlund-Nilsson 2004). The egg stage of several fishes, however, has proven to be highly susceptible to hypoxia with numerous deleterious effects after hatching, including reduced length and impaired swimming abilities (Ekau et al. 2010). These studies, coupled with the prevalence of hypoxia in estuaries, confirm the need to examine the effects of hypoxia on estuarine fish.

Estuaries are susceptible to hypoxia and acidification due to eutrophication from stormwater and sewage drainage in densely populated urban areas and fertilizer runoff from

heavily farmed areas (Feely et al. 2010, Cai et al. 2011). Considering the growing body of research on the individual effects of acidification and hypoxia on marine life, it is important to study the combined effects of these co-occurring stressors on the estuarine organisms most likely to encounter them. The interactions between environmental stressors are increasingly being studied, and often the results are not predictable from the effects of the individual stressors (Pörtner et al. 2005, Melzner et al. 2011, Pansch et al. 2013). For example, tolerance of hypoxia decreases for organisms outside their optimal temperature range; and acidification can result in a narrowing of thermal tolerance (Pörtner 2008, 2010).

As forage fish, *Menidia menidia*, *Menidia beryllina,* and *Cyprinodon variegatus,* or the Atlantic silverside, inland silverside, and sheepshead minnow, are important to estuarine ecosystems that extend from Florida to Prince Edward Island, Canada for Atlantic silversides and from Texas to Massachusetts for inland silversides and sheepshead minnows (Able and Fahay 1998). Like all forage fish, silversides and sheepshead minnows occupy an important position in the food web by serving as the energetic link between zooplankton and larger, carnivorous fish, as well as controlling the plankton population (Present and Conover 1992, Conover et al. 2005, Pikitch et al. 2014). Given their importance to the ecosystem, the demonstrated susceptibility of silversides to acidification, and the potential for temporary exposure to both acidification and hypoxia, larval silversides and sheepshead minnows are ideal experimental organisms for this study.

In addition to acidification and hypoxia, climate change can result in several other stressors including elevated temperatures and reduced food availability (Pörtner et al. 2005, Pörtner 2008, Doney et al. 2009). Rising levels of CO_2 in Earth's atmosphere have caused average global temperatures to rise by 0.74°C between 1906 and 2005, a process that in turn

warms the ocean and is expected to continue this century (IPCC 2007). Marine organisms exposed to acidified water may also experience a narrowing of thermal tolerance, which means ecosystems undergoing both warming and acidification could face major shifts in population structure (Pörtner 2008).

Food supply is impacted by climate change in a number of ways. Studies suggest that ocean warming during the past century has reduced planktonic food availability (Behrenfeld et al. 2006, Boyce et al. 2010). Warming can also impact food availability within specific ecosystems, as in the case of the North Sea where warmer waters are responsible for the double threat of increasing the metabolism of cod while causing reductions in mean size of their primary food source (Beaugrand et al. 2003). Acidification results in undersaturation of important shell minerals that can force prey populations out of low pH water, resulting in limited food availability for zooplanktivores (Doney et al. 2009). Additionally, changes in seawater chemistry or temperature can result in match-mismatch scenarios that temporally separate predators and prey during important feeding periods (IPCC 2007). Prior research has demonstrated that the susceptibility of marine organisms to acidification can be dependent on their food supply (Melzner et al. 2011, Pansch et al. 2013). Experiments testing the effects of acidification with these additional co-occurring stressors will add to a fuller understanding of the impact of climate change on estuarine fish.

The objective of my thesis was to evaluate the effects of acidification coupled with hypoxia, food limitation, or elevated temperature on the early life stages of forage fish. Wild *M. menidia*, commercially obtained *M. beryllina*, and commercially obtained *C. variegatus* were exposed from the egg to the early larval stage to acidification and hypoxia in order to assess the tolerance of, and potential interactions between, these co-occurring stressors. Additionally, *M.*

beryllina was exposed to acidification and reduced food availability, as well as acidification and elevated temperature, in order to test the tolerance of an acidification-sensitive fish to these climate change stressors.

METHODS

Seawater chemistry

Hypoxia and acidification were examined as two treatments in a 2x2 experimental design resulting in four sets of replicate (n = 4) 8 L, opaque polyethylene experimental vessels with lids: Control, hypoxic, acidified, and combined hypoxic and acidified. Target pCO_2 levels were ~450 µatm (control) and ~2000 µatm (acidified), and target dissolved oxygen levels were ~8.5 mg L^{-1} (control) and ~2 mg L^{-1} (hypoxic). These 16 experimental vessels were filled with 0.45µm filtered seawater from Old Fort Pond in Southampton, NY, which generally maintains a salinity of 30, a recommended salinity for all three species (Middaugh et al. 2009). Vessels were placed in troughs filled with water and maintained at the recommended temperature for these species ~22 - 24°C by use of commercially available chiller-heaters (Middaugh et al. 2009). Gas proportioners (Cole-Parmer) were used to control the rate of gas flow into each vessel, depending on the treatment. The control vessels were bubbled with compressed air, the hypoxic vessels were bubbled with air and tanked N_2 gas pre-mixed with 400 µatm CO_2, the acidified vessels were bubbled with air blended with tanked 5% CO_2, and the acidified/hypoxic vessels were bubbled with air, tanked N_2, and tanked 5% CO_2.

Daily measurements of pH were made with a Honeywell© Durafet Ion Sensitive Field Effect Transistor pH sensor calibrated with Tris buffer in synthetic seawater on the total pH scale (Dickson et al. 2007). pH measurements made with this electrode were not significantly

different from those made spectrophotometrically using m-cresol purple (Dickson et al. 2007). Daily dissolved oxygen measurements were made with a Hach optical dissolved oxygen probe calibrated in water-saturated air. Oxygen measurements made with these sensors have been found to be indistinguishable from those made via Winkler titrations (Grashoff et al. 1983, Gobler et al. 2014).

To determine carbonate chemistry in the experimental vessels, seawater was bubbled with gases as described above for at least 24 hours before being analyzed at the beginning and end of each experiment using a Liqui-Cel® Membrane (Membrana) to separate the gas phase from seawater and an EGM-4 Environmental Gas Analyzer® (PP Systems) to quantify total dissolved inorganic carbon (DIC). Measured levels of DIC, pH_T, temperature, salinity, and first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) were used in the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/) to calculate pCO_2, alkalinity, $\Omega_{calcite}$, $\Omega_{aragonite}$, and concentration of CO_3^{2-}. As a quality assurance measure, certified reference material for oceanic pCO_2 measurements from the Marine Physical Laboratory at Scripps Institution of Oceanography (Batches 117, 123, and 132) was analyzed during each analytical DIC run (mean percent recovery: 102 ± 4%).

Study organisms

A series of acidification-hypoxia experiments was performed on newly fertilized *Menidia* spp. and *Cyprinodon variegatus* eggs from several sources. Previous acidification research has been conducted with commercially available *M. beryllina* eggs from Aquatic Research Organisms (ARO) in Hampton, New Hampshire (Baumann et al. 2012). For this project, experiments were performed with *Menidia* eggs from two sources: *M. beryllina* from ARO and

strip-spawned eggs from adult *M. menidia* seined from Setauket Harbor in Poquot, New York. Since the egg stage of *M. beryllina* has been shown to be negatively affected by acidification (Baumann et al. 2012), <24-hour-old eggs were used for these experiments.

For *M. menidia*, after seining, adults were held overnight in filtered seawater supplied with air and strip spawned the next day. The strip-spawning technique involved gently squeezing sperm from adult males into plastic dishes containing clean seawater and cut-out sections of window screen (1 mm mesh size). The sperm was mixed with, and activated by, the seawater; then the adult females were gently squeezed to release their eggs into the mix of sperm and seawater. Within 15 minutes, fertilized eggs attached to the window screen via chorionic filaments, while unfertilized eggs sank to the bottom of the dish. Successfully fertilized eggs could be easily counted with the naked eye, and pieces of window screen containing 80 eggs were suspended in each replicate experimental vessel within four hours of fertilization. *M. beryllina* eggs from ARO were attached to clusters of yarn and counted so that strands containing 80 eggs were suspended in each replicate vessel. *C. variegatus* eggs were obtained from Aquatic BioSystems in Fort Collins, Colorado, and 80 loose eggs were distributed per replicate vessel.

Daily triplicate counts of live fish were made during experiments by placing a plastic divider into the experimental vessels to separate the fish into smaller groups. After hatching, *M. beryllina* larvae were fed *Brachionus plicatilis* (rotifer) *ad libitum* for the first five days and newly hatched *Artemia salina* (brine shrimp) nauplii for the remainder of the experiment, following Middaugh et al. (1987). *M. menidia* were fed newly hatched *Artemia* nauplii from the day of hatch as well as powdered food (Otohime Marine Weaning Diet, size A, Reed Mariculture®) during the first three days. *C. variegatus* were fed newly hatched *Artemia* nauplii

from the day of hatch (Middaugh et al. 2009). Half of the water in each experimental vessel was replaced with new filtered seawater twice weekly, and detritus was siphoned off the bottom of each vessel daily.

Experiments generally lasted for 10 days after the majority of fish had hatched in each treatment. At the end of the experiment, remaining fish were preserved in 10% buffered formalin (final concentration 3%) and photographed with a digital camera, and standard lengths were measured with image analysis software (ImageJ 1.45s, Media Cybernetics). Time to hatch was calculated in days from the start of the experiment until the first eggs hatched in each experimental vessel and averaged across treatments. Percent hatching was calculated by dividing the maximum number of larvae in each vessel by the initial total number of eggs (80) and averaging for each treatment. Percent survival was calculated by dividing the number of survivors at the end of an experiment by the maximum number of larvae to hatch in each vessel and averaged across the treatments.

Temperature and food limitation experiments

Experiments were conducted with *M. beryllina* as described above with alterations made to the feeding regime or temperature and type of pH electrode. Treatments in the acidification-food limitation experiment included control (ambient pH ~8.0_{NBS}, *ad libitum* feeding), acidified (reduced pH ~7.8_{NBS}, *ad libitum* feeding), reduced feeding (ambient pH, feeding at 20% of *ad libitum* treatment), and acidified with reduced feeding (reduced pH, feeding at 20% of *ad libitum* treatment). Treatments in the acidification-elevated temperature experiment included control (ambient pH ~8.0_{NBS}, 22°C), elevated temperature (ambient pH, 28°C), acidified (reduced pH ~7.7_{NBS}, 22°C), and acidified with elevated temperature (reduced pH, 28°C). For the

acidification-temperature experiment, 50 eggs were distributed to 1 L polypropylene beakers, and experimental vessels (n = 6) were placed in a temperature-controlled water bath set to either 22°C or 28°C. Daily pH measurements were made with a Thermo Scientific Orion Star Series™ Benchtop pH meter calibrated using NIST traceable standards on the NBS scale. All other aspects of the experiments proceeded as outlined above.

Statistical analyses

Statistical analyses were performed with SigmaPlot 11.0©. Percent survival and hatching values were arcsine square root transformed before statistical analyses. Two-way ANOVAs were performed to assess the effects of and interactions between each pH, dissolved oxygen, temperature, and food level treatment with respect to hatch timing, hatching success, survival, and size and were followed by Tukey's multiple comparison tests. Significant interactions included synergistic and antagonistic effects, wherein the results could not have been predicted by the effects of the individual stressors; and non-significant interactions were additive, wherein the effects of the co-occurring stressors could be predicted from their individual effects. Significance was determined with 95% confidence.

RESULTS

Hypoxia-acidification experiments with M. beryllina

Hypoxia significantly delayed hatching of *M. beryllina*, where eggs hatched after ~10 days when exposed to low dissolved oxygen of ~1.6 mg L^{-1} and after ~7 days when exposed to ambient levels of dissolved oxygen ($p<0.001$; Tables 1, 2; Fig. 2a). Hatching was not significantly delayed in an experiment with slightly higher levels of dissolved oxygen (~2.7 mg

L^{-1}; Tables 5, 6; Fig. 3a). Hypoxia also had a significant negative effect on hatching success. In two experiments, *M. beryllina* exposed from the egg stage to low dissolved oxygen of ~1.6 mg L^{-1} and ~2.7 mg L^{-1} had significantly lower hatching success compared to eggs exposed to ambient levels of dissolved oxygen ($p<0.001$; Tables 3, 7). With a dissolved oxygen level of ~1.6 mg L^{-1}, hatching success was 16 ± 14% compared to 82 ± 7% in the control treatment (Fig. 2b). With a slightly higher dissolved oxygen level of ~2.7 mg L^{-1}, hatching success was 71 ± 11% compared to 94 ± 4% in the control treatment (Fig. 3b). Exposure to ~2000 µatm CO_2 ($pH=7.4_T$) affected neither the timing nor the success of hatching in *M. beryllina* (timing: $p=0.147$, 0.781; success: $p=0.367$, 0.157), and there was no interaction between the stressors with regard to hatching (timing: $p=0.828$, 0.781; success: $p=0.676$, 0.367).

After hatching, survival of *M. beryllina* was significantly reduced in both experiments by elevated CO_2 concentrations of ~2000 µatm compared to ambient concentrations of ~550 µatm ($p=0.017$ and $p<0.001$; Tables 4, 8). Control treatment survival was 75 ± 6% and 81 ± 9% while acidified treatment survival was 48 ± 9% and 27 ± 10%, respectively (Figs. 2c, 3c). When dissolved oxygen levels were ~1.6 mg L^{-1}, survival was significantly reduced from 75 ± 6% in the control to 9 ± 12% in the hypoxic treatment ($p<0.001$). When dissolved oxygen levels were ~2.7 mg L^{-1}, survival was not significantly different from the control ($p=0.16$). There was no interaction between acidification and hypoxia with regard to larval survival ($p=0.415$, 0.812).

There were too few survivors for complete length analysis in the lower dissolved oxygen experiment with *M. beryllina*. Survivors in the higher oxygen experiment were measured at nine days post hatch, and both hypoxia and acidification had a significant negative effect on standard length (SL), with an additive effect from the combined stressors (low dissolved oxygen, $p<0.001$; low pH, $p=0.007$; interaction, $p=0.866$; Table 9). *M. beryllina* from the control

treatment measured 5.6 ± 0.03 mm while individuals in the acidified and hypoxic treatments grew to 5.1 ± 0.2 mm and 4.7 ± 0.4 mm, respectively; individuals from the hypoxic-acidified treatment measured 4.2 ± 0.5 mm (Fig. 3d).

Hypoxia-acidification experiments with wild M. menidia

Eggs from wild-caught *M. menidia* were sensitive to hypoxia, experiencing a significant delay in hatching and reduction in hatching success when dissolved oxygen concentrations were ~2.5 mg L^{-1} versus ~8.5 mg L^{-1} in the control treatment (Tables 10, 15). In two experiments conducted in May and June, hatching was delayed from six days in the ambient dissolved oxygen treatment to ~7.5 days in the hypoxic treatment, while low pH ~7.46 versus control pH ~7.85 had no effect on hatch timing and there was no interaction between the stressors (low dissolved oxygen, $p<0.001$; low pH, $p=0.663$, 0.525; interaction, $p=0.663$, 0.525; Tables 11, 16; Figs. 4a, 5a). The percentage of eggs hatching in May was reduced from 79 ± 5% in the control to 47 ± 9% in the low oxygen treatment, and in June the reduction was from 93 ± 9% in the control to 55 ± 16% in the low oxygen treatment, whereas acidification had no effect on hatching success and there was no interaction between low dissolved oxygen and low pH (low dissolved oxygen, $p<0.001$; low pH, $p=0.898$, 0.916; interaction, $p=0.626$, 0.24; Tables 12, 17; Figs. 4b, 5b).

Survival at eight days post hatch of offspring from *M. menidia* born in May was significantly reduced by low dissolved oxygen and low pH, with a synergistic negative effect of these two stressors (low dissolved oxygen, $p<0.001$; low pH, $p=0.002$; interaction, $p=0.004$; Table 13). Control and low pH treatments had survival of 75 ± 5% and 72 ± 7%, while low dissolved oxygen treatment survival was 57 ± 18% and survival in the low dissolved oxygen/low pH treatment dropped to 17 ± 6% (Fig. 4c). For offspring of *M. menidia* born in June, survival

was significantly reduced by low dissolved oxygen but not low pH, and there was no interaction between the stressors (low dissolved oxygen, $p<0.001$; low pH, $p=0.784$; interaction, $p=0.331$; Table 18). Survival was high in the control and low pH treatments at 84 ± 12% and 89 ± 4%, while the hypoxic and hypoxic/low pH treatments both had substantially lower survival at 22 ± 12% and 15 ± 7%, respectively (Fig. 5c).

Low dissolved oxygen yielded significant reductions in the lengths of *M. menidia* larvae from both experiments while acidification did not, and there was no interaction between these factors (low dissolved oxygen, $p<0.001$; low pH, $p=0.822$, 0.964; interaction, $p=0.269$, 0.388; Tables 14, 19). Fish from hypoxic and hypoxic/acidified treatments in May measured 5.9 ± 0.5 mm and 5.6 ± 0.3 mm SL, respectively, while fish from control and acidified treatments measured 9.3 ± 0.4 mm and 9.5 ± 0.3 mm (Figs. 4d). In June, fish from hypoxic and hypoxic/acidified treatments measured 5.5 ± 0.6 mm and 5.6 ± 0.2 mm, and fish from control and acidified treatments measured 10.1 ± 0.3 mm and 9.9 ± 0.2 mm (Fig. 5d).

Hypoxia-acidification experiment with C. variegatus

In a manner consistent with both species of *Menidia*, hypoxia significantly delayed hatching of *C. variegatus* from ~5 days in the treatments with dissolved oxygen of ~8.5 mg L^{-1} to ~8.5 days in the low dissolved oxygen treatment at ~2.4 mg L^{-1} ($p<0.001$; Tables 20, 21; Fig. 6a). Acidification had no effect on the time to hatch with low pH ~7.46 and ambient pH ~7.90, and there was no interaction between hypoxia and acidification ($p=0.611$, 0.143). Hypoxia also reduced hatching success in *C. variegatus* from 62 ± 4% in the control to 27 ± 13% in the low dissolved oxygen treatment ($p<0.001$; Table 22; Fig. 6b). Hatching success in acidified water was similar to the control at 64 ± 8%, while that number was again reduced by half in the

hypoxic/acidified treatment to 31 ± 5% (Fig. 6b); there was no interaction between the factors with regard to hatching success (p=0.71).

Survival of *C. variegatus* at 10 days post hatch was not significantly affected by low dissolved oxygen, low pH, or the combination of the two stressors, as all treatments had survival exceeding 95% (Table 23; Fig. 6c). The standard length of *C. variegatus* at the end of the experiment, however, was significantly reduced in the hypoxic treatment (p<0.001; Table 24). Fish from the control and acidified treatments averaged ~9 mm while individuals from the hypoxic and hypoxic/acidified treatments averaged ~5 mm (Fig. 6d). There was no effect due to acidification and no interaction between hypoxia and acidification (p=0.474, 0.839).

Reduced feeding and acidification experiment with M. beryllina

Reduced food availability and low pH both had significant negative effects on larval survival, with an additive effect from the combined stressors (food, p=0.002; low pH, p=0.01; interaction, p=0.398; Table 26). When fed *ad libitum*, survival was higher in the control treatment than the acidified treatment at 81 ±11% versus 60 ± 11% (Fig. 7a). Under a restricted diet, survival was reduced to 54 ± 13% at ambient pH ~8.06 and 41 ± 9% at low pH ~7.78.

The standard length of *M. beryllina* at 24 days post hatch was significantly reduced by both the low food and acidified treatments, while the combined stressors had a significant antagonistic interaction (low food, p<0.001; low pH, p=0.001; interaction, p=0.004; Table 27). Fish in the control treatment averaged 9.5 ± 0.2 mm while the low food and acidified treatments had lower mean lengths of 7.5 ± 0.2 mm and 8.0 ± 0.4 mm, respectively (Fig. 7b). The low food, acidified treatment standard length average was 7.4 ± 0.6 mm.

Elevated temperature and acidification experiment with M. beryllina

Elevated temperature (28°C versus 22°C) and low pH (~7.70 versus ~8.06) had significant negative effects on the survival of *M. beryllina* at four days post hatch, while the co-occurring stressors had a significant antagonistic effect (temperature, $p<0.001$; low pH, $p=0.014$; interaction, $p=0.032$; Table 29). Survival decreased from 89 ± 8% in the 22°C treatment to 13 ± 7% in the 28°C treatment, while survival in the acidified treatment decreased to 73 ± 8% (Fig. 8). The combined elevated temperature, acidified treatment had 12 ± 6% survival, a higher percentage than would be predicted based on the effects of the individual stressors.

DISCUSSION

Marine organisms face a multitude of stressors due to climate change, some of which are exacerbated by anthropogenic processes in the coastal environment. Here, the impacts of acidification on three species of estuarine fish were studied in conjunction with co-occurring hypoxia, restricted food availability, and elevated temperature. Hypoxia significantly delayed hatching and reduced hatching success for *M. beryllina*, *M. menidia,* and *C. variegatus*. Hypoxia and acidification significantly reduced survival for both *Menidia* species, with responses including sensitivity to one or both of the stressors as well as sensitivity to the interaction of the two stressors. Length of *M. menidia* and *C. variegatus* was significantly reduced by hypoxia, while the length of *M. beryllina* was significantly reduced by both hypoxia and acidification. Limited food availability and acidification each significantly reduced survival and length of *M. beryllina*, while elevated temperature and acidification also reduced survival in this species.

Most experiments investigating the effects of hypoxia on marine organisms to date have bubbled N_2 gas to remove oxygen from seawater (e.g. Breitburg et al. 1997, Jordan and

Steffensen 2007, Clark et al. 2013), a process that also removes CO_2 and results in an elevated pH rather than low pH as is found in hypoxic ecosystems (Gobler et al. 2014). The hypoxia experiments presented here mixed N_2 gas with CO_2 and air to maintain pH at ambient or acidified levels, isolating the effects of low oxygen from the combined effects of low oxygen and low pH and thus mimicking seawater chemistry that exists in hypoxic systems (Gobler et al. 2014). The additively negative effect of hypoxia and acidification on survival of *M. beryllina* and the synergistically negative effect of the two stressors on survival of *M. menidia* demonstrate the importance of assessing hypoxia effects under the pH conditions most likely to occur in nature. Hypoxia, for example, could lead to a reduction in aerobic performance and thereby impair an organism's ability to cope with the additional stress of acidification (Pörtner et al. 2005, Pörtner and Knust 2007, Pörtner 2008). Prior studies examining the effects of hypoxia on fish using N_2 gas and thus higher pH may have, therefore, misidentified the true effects of hypoxic zones on fish populations.

Hypoxic marine ecosystems have become more prevalent in recent decades (Diaz and Rosenberg 2008), and the effects of hypoxia on marine fish populations can be profound (Breitburg 2002, Vaquer-Sunyer and Duarte 2008, Ekau et al. 2010). Hypoxia delayed hatching and reduced hatching success for all three study species, and the reduction in hatching was as high as 80% compared to the control treatment in one *M. beryllina* experiment, a result that could lead to catastrophic population losses. Short-term exposure to hypoxia near the end of the egg stage is known to induce hatching in fish (Oppen-Berntsen et al. 1990, Czerkies et al. 2001, Ciuhandu et al. 2005), however this is likely due to the presence of hatching enzymes in near-mature embryos. Exposure to hypoxia during the egg stage can inhibit growth (Ciuhandu et al. 2005, Ekau et al. 2010), and a prolonged egg stage and smaller larval size increase the risk of

predation on fish (Sogard 1997). While adult fish may migrate out of hypoxic zones, their eggs and larvae are likely more susceptible to the changes in seawater chemistry experienced in coastal zones during the spawning season (Feely et al. 2010, Gobler et al. 2014, Baumann et al. 2014).

Hypoxia also reduced lengths of all three study species. For most marine fish species, larval populations lose biomass during the early larval stage, as weight-specific growth rates are unable to keep pace with instantaneous mortality rates (Houde 1997). Furthermore, instantaneous mortality rates in early life stages of fish are tied to larval body size and growth rate, with the mortality rate dropping as size increases (Houde 1997). This suggests that the reductions in growth resulting from hypoxia for our three study species could have a negative impact on recruitment success, with increased losses of biomass and higher risk of mortality than would already be expected due to factors like predation.

The egg stage for all three species was prolonged in the hypoxic treatments, and the resulting shorter larval period likely contributed to the large difference in size between low and ambient oxygen treatments. However, while *M. beryllina* from the hypoxic treatment ~10 days post hatch were significantly larger than day-of-hatch larvae from the control (4.7 ± 0.4 mm compared to 3.4 ± 0.2 mm), and *C. variegatus* from the hypoxic treatment were significantly larger than day-of-hatch lengths from the literature (5.2 ± 0.8 mm compared to 4 mm, Kuntz 1916), *M. menidia* from the hypoxic treatment were not significantly larger than day-of-hatch lengths from the literature (5.9 ± 0.5 mm and 5.5 ± 0.6 mm compared to 5.4 ± 0.4 mm, Murray et al. 2014). It is possible, therefore, that *M. menidia* in the hypoxic treatment did not grow at all for the duration of these experiments.

Hypoxia is known to cause prolonged states of elevated metabolism after feeding, costing fish more energy to assimilate their food and grow, which may also have contributed to the smaller size of fish in those treatments (Jordan and Steffensen 2007). Additionally, hypoxia can result in elevated ventilation rates and cardiovascular stress as well as reduced mobility, placing higher metabolic demands on fish while decreasing the time spent seeking food (Hughes and Saunders 1970, Randall 1982, Jordan and Steffensen 2007, Pörtner 2010). The physiological and behavioral impacts of hypoxia, especially when combined with acidification, reduce the competitiveness of affected organisms in the wild, where survival under climate change will depend on physiological performance and adaptability (Pörtner and Farrell 2008).

Differences between broodstock sources may explain the range in responses to hypoxia between species, as ~2.4 - 2.7 mg L^{-1} of dissolved oxygen did not significantly delay hatching or reduce survival of *M. beryllina*, while it delayed hatching and reduced survival of *M. menidia* and delayed hatching of *C. variegatus*. However, these results correlate with prior research with young-of-the-year fish showing that of the three species studied, *M. menidia* is the least tolerant of hypoxia (Able and Fahay 1998, Smith and Able 2003).

Acidification has become increasingly recognized as a potential threat to many marine fish (Munday et al. 2009, 2010; Baumann et al. 2012; Frommel et al. 2012; Chambers et al. 2013). While Baltic cod larvae appear to have adapted to their spawning sites that are enriched in CO_2, Atlantic cod larvae that are spawned near surface waters with ambient CO_2 levels experienced temporary tissue damage after long-term exposure to acidified water (Frommel et al. 2012, 2013). These studies reflect the variability in fish responses to acidification that was also found in this study, as *C. variegatus* was resistant to acidification but *M. beryllina* and *M. menidia* were not. In some cases, differences may be based in part on the environmental

conditions already experienced in the wild. *M. menidia* eggs were obtained from wild adults in May and June. While acidification and hypoxia had a negative effect on *M. menidia* survival in May, acidification had no effect on these fish in June. These results may reflect the differing environmental conditions encountered by the spawning adults in May when pH values are higher compared to June when pH values are lower (Baumann et al. 2014). Consistent with the results presented here, Murray et al. (2014) found that eggs spawned in mid-May are more resistant to acidification than those spawned earlier in the season when pH levels in the environment are higher. Given that *M. beryllina* and *C. variegatus* were obtained from hatchery broodstock, further experiments should be conducted with eggs from wild-caught adults to remove the effects due to domestication (Bobe and Labbé 2010).

Maternal effects may have affected the quality of eggs used in the *M. menidia* experiments and therefore the tolerance of acidification in May versus June, for example via spawner physiology and nutrition (Green 2008, Bobe and Labbé 2010). Measurements of the wild-caught *M. menidia* adults showed no correlation between length of spawners and survival of offspring between experiments, however female size is not a good predictor of egg composition e.g. lipid and protein concentration, which can contribute to egg survival (Kamler 2005). Other maternal effects that may be a factor in determining the hardiness of eggs include spawning behavior and parental care, which do not apply in the laboratory setting, and egg provisioning (Green 2008). There was no visible difference (i.e. size, color) between eggs as the season progressed, but many iteroparous fish produce eggs of varying quality through the spawning season (Kamler 2005, Green 2008). Further analysis is necessary to determine differences in egg quality as the spawning season progresses, as egg size and supply of lipid droplets may be correlated to egg survival or larval fitness (Kamler 2005, Bobe and Labbé 2010). Parental

encounter with acidified water may account for the resistance to acidification seen in June, however other environmental factors can affect egg quality, such as temperature; therefore, further research is necessary before attributing the change in tolerance of acidification to a single factor (Bobe and Labbé 2010).

C. variegatus was resistant to the levels of acidification used in this study. Like both *Menidia* species, *C. variegatus* spawns in estuaries. However, this fish is generally known to be tolerant of a wide range of seawater conditions (Able and Fahay 1998). *C. variegatus* is known to lay demersal eggs in shallow pools or marsh areas (Able and Fahay 1998), which generally have lower levels of pH and dissolved oxygen compared to the water column (Wallace et al. in review) where both *Menidia* species lay their eggs (Conover and Ross 1982, Able and Fahay 1998). These differences in life history traits may produce a high tolerance for acidification and hypoxia in early life *C. variegatus* (Ekau et al. 2010), which should be explored further through additional dual-stressor experiments with this and other species of fish that lay benthic and pelagic eggs.

Reduced growth rates of larval fish can impact fishery recruitment, and fluctuations in temperature or food availability can both significantly alter larval growth (Houde 1989). Food availability is known to affect recruitment of fish stocks, with both the quality of individual prey items and abundance contributing to the likelihood of success (Beaugrand et al. 2003), while reduced food availability can result in higher sensitivity to acidification in invertebrates (Melzner et al. 2011, Crook et al. 2013, Pansch et al. 2013, Thomsen et al. 2013). Survival and standard length of *M. beryllina* were significantly reduced by both acidification and food limitation, while the reduction was additive for survival and antagonistic for length. An acidified, low-food ecosystem would likely yield fewer and smaller fish larvae than a normal ecosystem, and these

results would have important ramifications for future fish stocks and ecosystem stability.

Acidification can alter the physiology of marine organisms, depressing protein synthesis, increasing ventilation, and re-directing resources toward acid-base regulation (Pörtner 2005, 2008, 2010). Such enhanced performance creates an energy demand that is less likely to be met under conditions of restricted food availability (Pörtner 2008, Melzner et al. 2011, Crook et al. 2013). In some cases, climate change can directly reduce food availability (Behrenfeld et al. 2006, Boyce et al. 2010) and in turn exacerbate the physiological impacts. Warming waters can increase the metabolism of fish while causing reductions in mean size of their primary food source (Beaugrand et al. 2003), and acidification results in undersaturation of shell minerals that may lower the abundance of some planktonic prey (Doney et al. 2009). Given the additive effect of acidification and reduced food on *M. beryllina* survival and the antagonistic effect on length, further research will be important to better understand how reduced food will affect fish populations' tolerance of climate change stressors.

Warmer ocean temperatures have resulted in major ecosystems shifts, specifically by forcing the ranges of some organisms toward the poles (Parmesan and Yohe 2003). Additionally, acidification can cause the narrowing of an organism's thermal tolerance, leading to further restricted ecosystem suitability, the loss or introduction of species to ecosystems based on new geographic ranges, and even the loss of an entire ecosystem (Pörtner and Knust 2007, Pörtner 2008). The effects of elevated temperature on survival of *M. beryllina* were drastic, with a smaller effect of acidification and no difference in thermal tolerance between pH treatments. Further research could test the effects of these stressors at a lower temperature than was used here, which may have been too high for any interaction to present itself with so few survivors. The importance of experiments assessing tolerance of elevated temperatures will continue to

increase, as average global sea surface temperatures taken from 1850 to 2005 show that the warmest five years on record all occurred after 1995 (IPCC 2007). The average global surface temperature increased by 0.6°C during the 20th century and will increase by 1.4 – 5.8°C over the next century (Houghton et al. 2001; IPCC 2007). While sea surface temperatures are rising, coastal waters are warming at a faster pace despite large regional variability (Nixon et al. 2004, Baumann and Doherty 2013).

The results presented here regarding the combined and interactive effects of acidification, hypoxia, warming, and food restriction on three fish species have significant implications for the future of coastal fisheries, which are dependent on the successful recruitment of early life stage fish to adulthood (Houde 1989). The future health of coastal ecosystems is also at risk, as forage fish hold an important place in the food web as both consumers of plankton and prey for larger fish (Present and Conover 1992, Conover et al. 2005, Pikitch et al. 2014). Reductions in the size and survival of forage fish due to limited food, hypoxia, and elevated temperature may result in major biomass losses, and the co-occurrence of acidification may further compromise an organism's capacity to cope with those stressors (Pörtner et al. 2005, Pörtner 2008). Given coastal ecosystems are often low in oxygen and more acidified during months when fish spawn, it will be important to refine our understanding of the effects of these and other climate change stressors on fish to best predict how fisheries will be impacted by climate change and implement management plans to sustain them. Some biological impacts of these stressors on fish may not be noticeable in the timeframe of these experiments (Pörtner 2008). Further studies should assess the long-term effects of climate change stressors on fish to determine if any mechanisms or behaviors are detrimentally affected in adulthood or first-generation offspring.

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APPENDIX

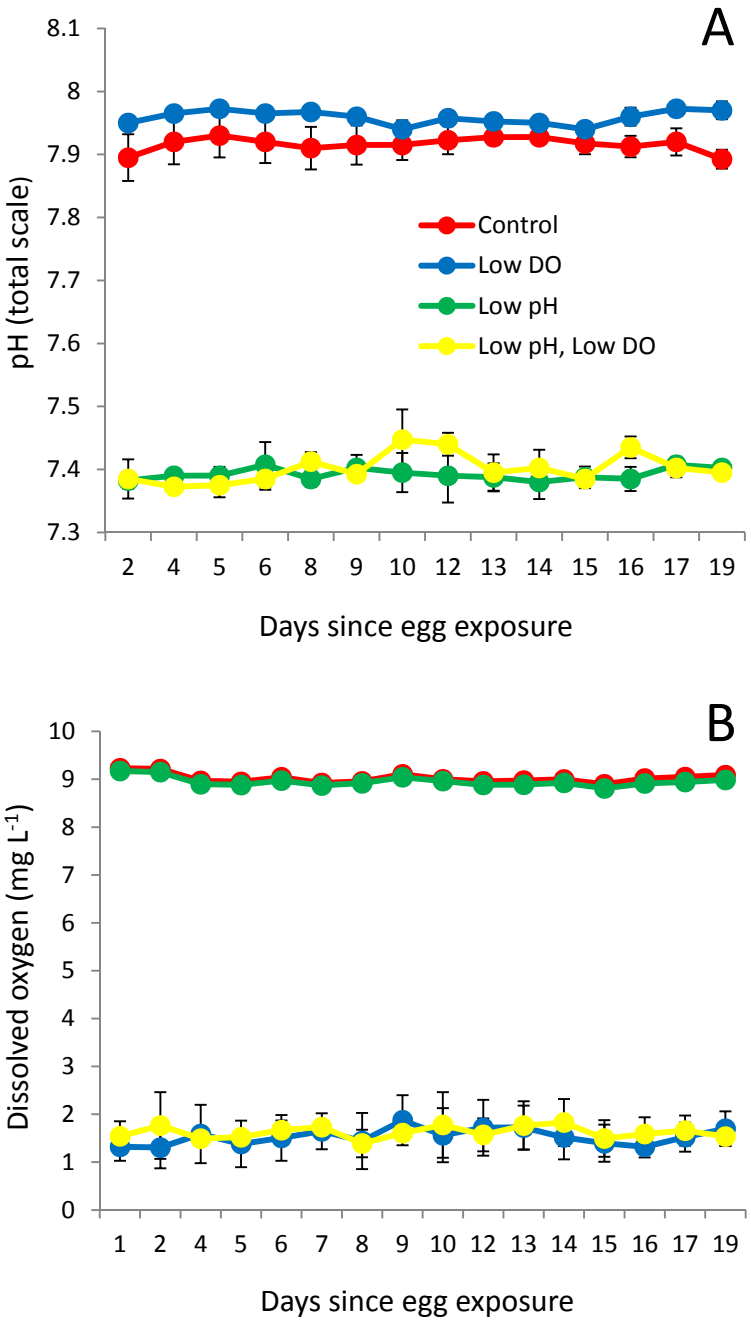

Figure 1. Time series of daily mean A) pH and B) dissolved oxygen from a *Menidia beryllina* experiment. Points represent means ± standard deviation (*n*=4).

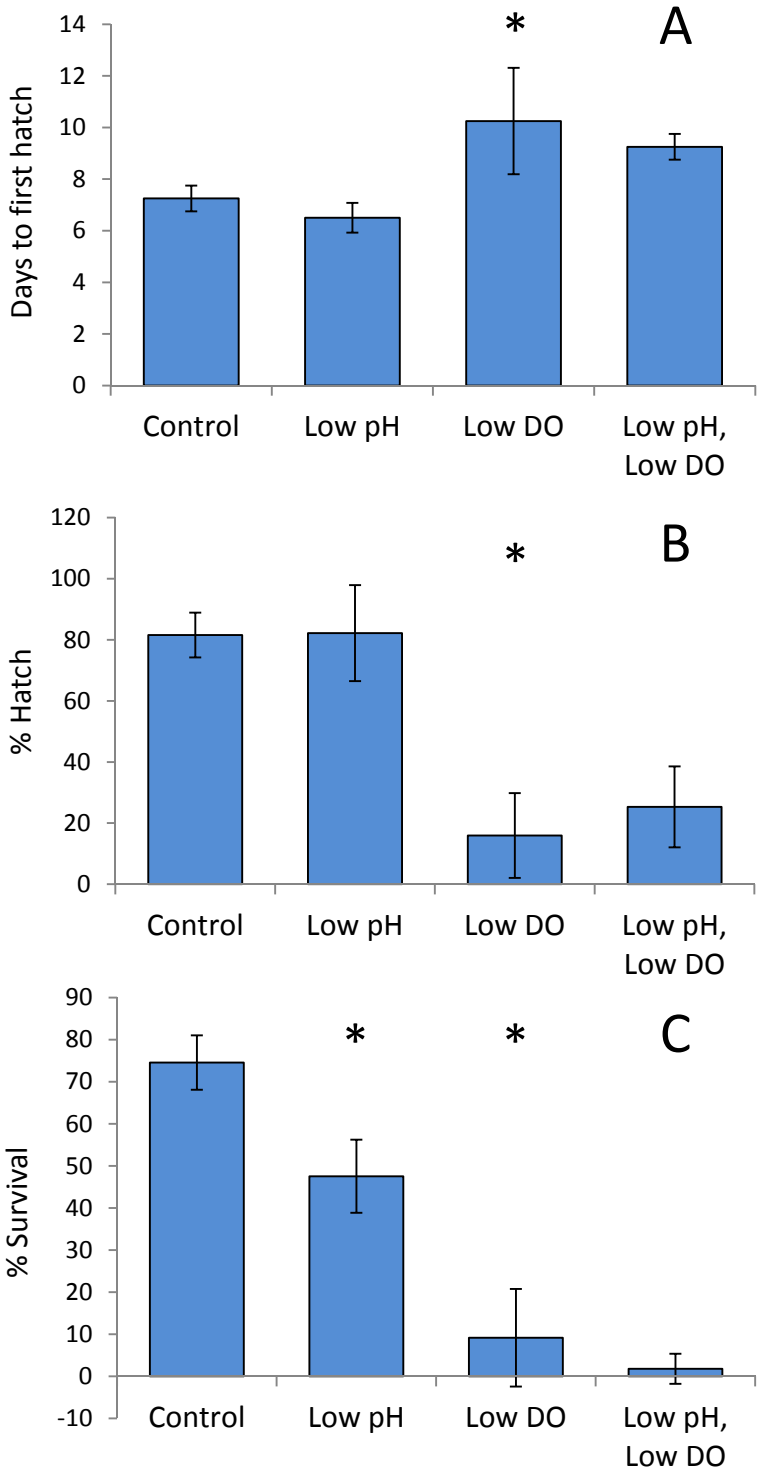

Figure 2. A) Number of days from start of January experiment until first *Menidia beryllina* eggs hatched. Low dissolved oxygen significantly delayed hatching ($p<0.001$). B) Percent of eggs to hatch from an initial 80 eggs. Low dissolved oxygen had a significant negative effect on hatching success ($p<0.001$). C) Percent survival of larvae at 10 days post hatch. Low pH and low dissolved oxygen both had a significant negative effect on survival ($p=0.017$, $p<0.001$, respectively), and their combined effects were additive. Bars represent means ± standard deviation ($n=4$).

Table 1. Seawater chemistry for January *Menidia beryllina* acidification and hypoxia experiment. Mean and standard deviation for temperature (°C), pH (total scale), dissolved oxygen (mg L^{-1}), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Control	**Low DO**	**Low pH**	**Low pH, Low DO**
Temperature	21.9 (0.2)	21.9 (0.2)	21.9 (0.2)	21.9 (0.2)
pH_T	7.92 (0.02)	7.96 (0.01)	7.39 (0.01)	7.40 (0.01)
DO	9.02 (0.03)	1.53 (0.29)	8.95 (0.02)	1.62 (0.11)
pCO_2	549 (9)	511 (28)	2187 (72)	2135 (99)
Ω calcite	3.84 (0.35)	3.96 (0.18)	1.26 (0.07)	1.22 (0.10)
Ω aragonite	2.49 (0.23)	2.57 (0.12)	0.82 (0.04)	0.79 (0.07)
TA	2290 (72)	2270 (62)	2310 (86)	2240 (79)
CO_3^{2-}	158 (13)	163 (7)	51.8 (2.6)	50.2 (3.6)
DIC	2080 (57)	2050 (56)	2300 (88)	2240 (80)
Salinity	32.5 (0.5)	32.5 (0.5)	32.5 (0.5)	32.5 (0.5)

Table 2. Two-way ANOVA for number of days until the first eggs hatched in January *Menidia beryllina* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	33.063	33.063	26.016	<0.001
pH	1	3.063	3.063	2.41	0.147
Dissolved oxygen x pH	1	0.0625	0.0625	0.0492	0.828
Residual	12	15.25	1.271		
Total	15	51.438	3.429		

Table 3. Two-way ANOVA for hatching success in January *Menidia beryllina* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	1.983	1.983	56.78	<0.001
pH	1	0.0307	0.0307	0.879	0.367
Dissolved oxygen x pH	1	0.00639	0.00639	0.183	0.676
Residual	12	0.419	0.0349		
Total	15	2.439	0.163		

Table 4. Two-way ANOVA for survival of *Menidia beryllina* larvae at 10 days post hatch in January acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	2.306	2.306	93.245	<0.001
pH	1	0.189	0.189	7.649	0.017
Dissolved oxygen x pH	1	0.0176	0.0176	0.713	0.415
Residual	12	0.297	0.0247		
Total	15	2.81	0.187		

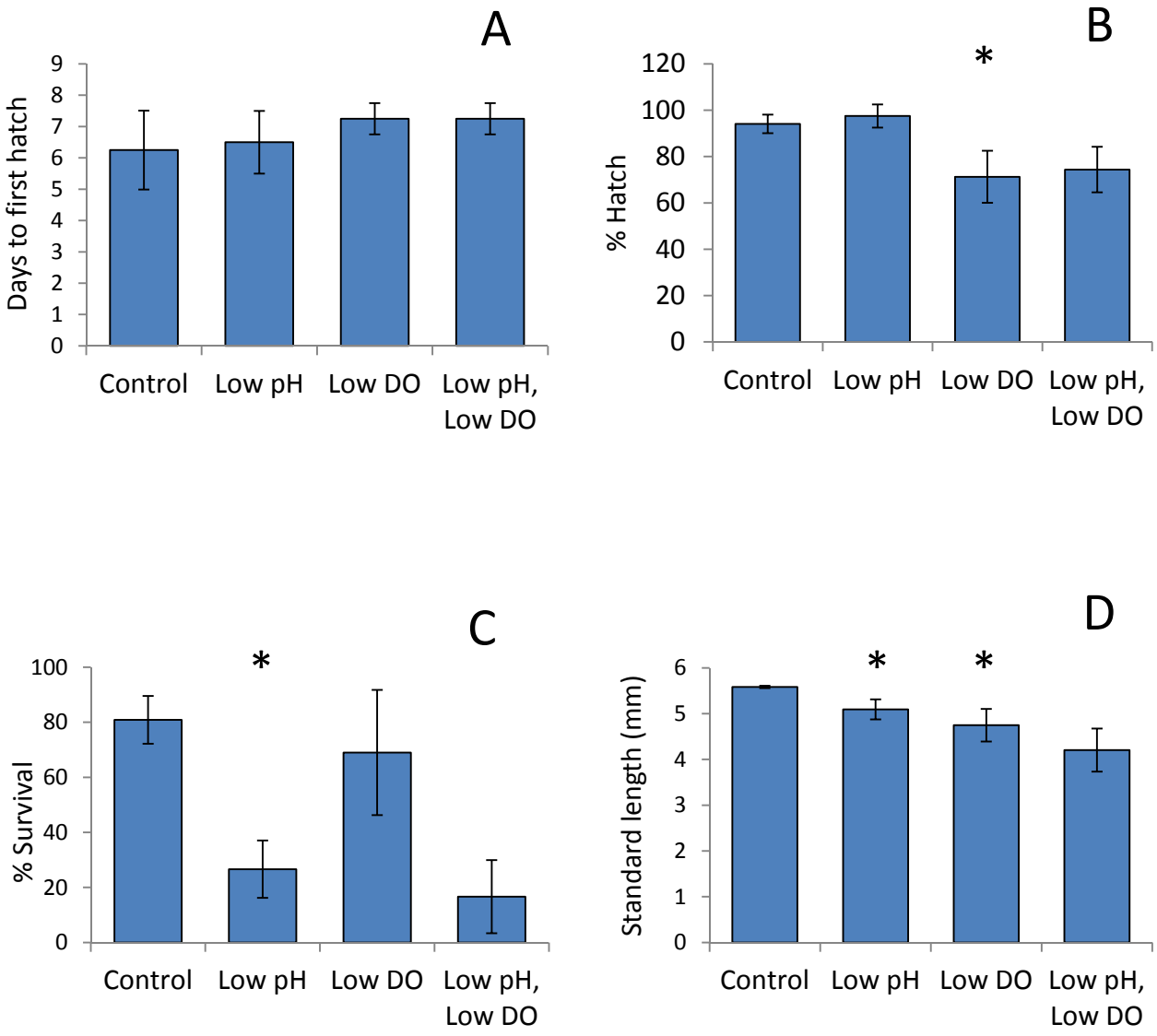

Figure 3. A) Number of days from start of March experiment until first *Menidia beryllina* eggs hatched. B) Percent of eggs to hatch from an initial 80 eggs. Low dissolved oxygen had a significant negative effect on hatching success ($p<0.001$). C) Percent survival of larvae at nine days post hatch. Low pH had a significant negative effect on survival ($p<0.001$). D) Standard length of nine-day-old larvae. Low pH and low dissolved oxygen both had a significant negative effect on length ($p=0.007$, $p<0.001$, respectively), and their combined effects were additive. Bars represent means ± standard deviation ($n=4$).

Table 5. Seawater chemistry for March *Menidia beryllina* acidification and hypoxia experiment. Mean and standard deviation for temperature (°C), pH (total scale), dissolved oxygen (mg L^{-1}), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Control	**Low DO**	**Low pH**	**Low pH, Low DO**
Temperature	21.1 (0.3)	21.1 (0.3)	21.1 (0.3)	21.1 (0.3)
pH_T	7.89 (0.00)	7.92 (0.01)	7.40 (0.02)	7.40 (0.01)
DO	9.01 (0.02)	2.70 (0.06)	8.95 (0.01)	2.66 (0.17)
pCO_2	577 (41)	543 (53)	1954 (180)	2027 (242)
Ω calcite	3.82 (0.28)	3.95 (0.30)	1.40 (0.06)	1.40 (0.20)
Ω aragonite	2.48 (0.18)	2.56 (0.20)	0.91 (0.04)	0.91 (0.13)
TA	2310 (80)	2290 (67)	2280 (122)	2320 (148)
CO_3^{2-}	156 (11)	161 (12)	57.2 (2.3)	57.2 (7.9)
DIC	2110 (76)	2080 (69)	2260 (128)	2300 (148)
Salinity	32	32	32	32

Table 6. Two-way ANOVA for number of days until first eggs hatched in March *Menidia beryllina* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	3.063	3.063	3.973	0.069
pH	1	0.0625	0.0625	0.0811	0.781
Dissolved oxygen x pH	1	0.0625	0.0625	0.0811	0.781
Residual	12	9.25	0.771		
Total	15	12.438	0.829		

Table 7. Two-way ANOVA for hatching success in March *Menidia beryllina* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.592	0.592	37.171	<0.001
pH	1	0.0363	0.0363	2.281	0.157
Dissolved oxygen x pH	1	0.014	0.014	0.881	0.367
Residual	12	0.191	0.0159		
Total	15	0.833	0.0555		

Table 8. Two-way ANOVA for survival of *Menidia beryllina* larvae at nine days post hatch in March acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.0886	0.0886	2.249	0.16
pH	1	1.497	1.497	37.989	<0.001
Dissolved oxygen x pH	1	0.00233	0.00233	0.0592	0.812
Residual	12	0.473	0.0394		
Total	15	2.06	0.137		

Table 9. Two-way ANOVA for standard length of nine-day-old *Menidia beryllina* in March acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	2.743	2.743	31.11	<0.001
pH	1	0.985	0.985	11.168	0.007
Dissolved oxygen x pH	1	0.00264	0.00264	0.0299	0.866
Residual	11	0.97	0.0882		
Total	14	4.485	0.32		

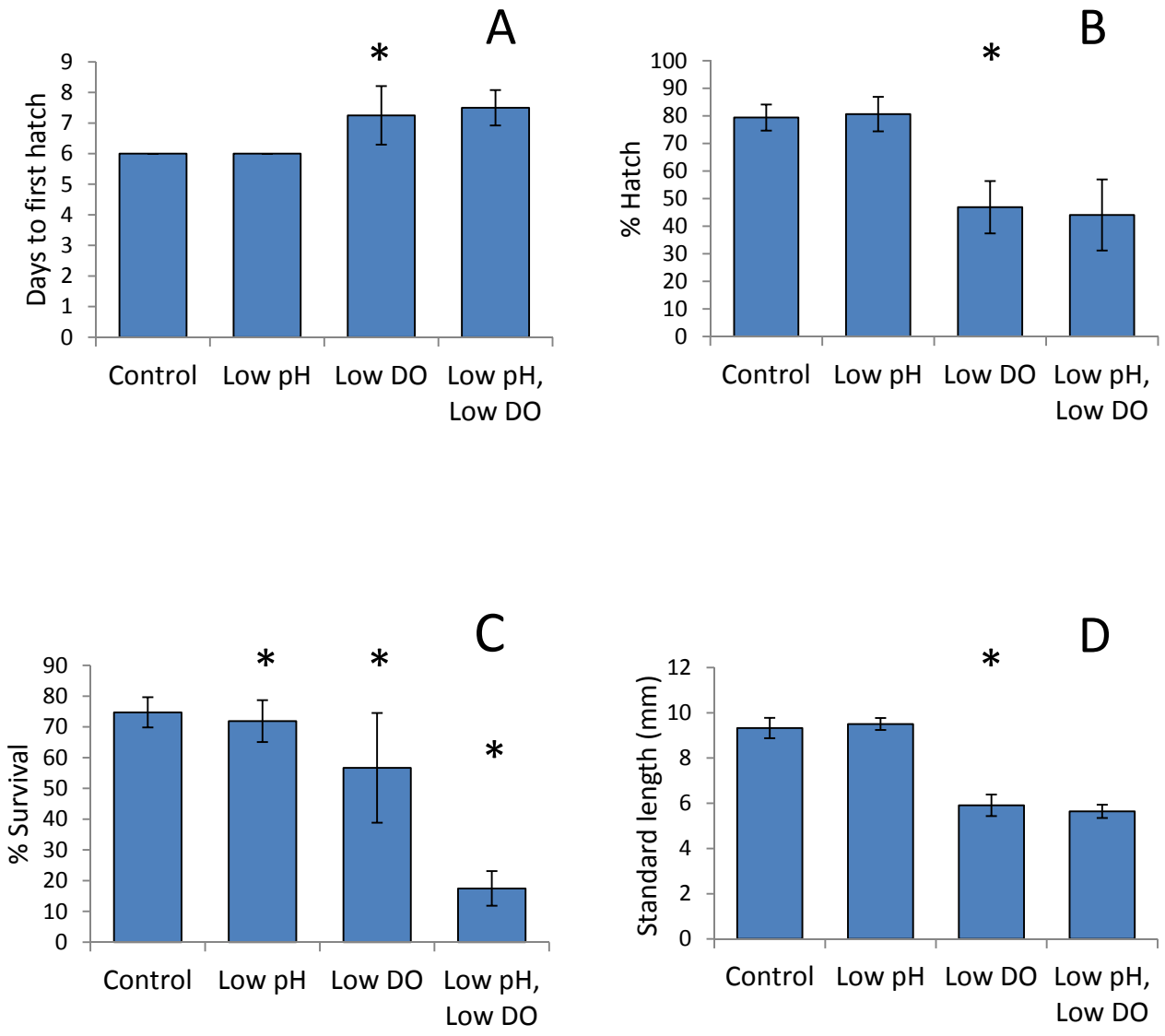

Figure 4. A) Number of days from start of May experiment until first *Menidia menidia* eggs hatched. Low dissolved oxygen significantly delayed hatching ($p<0.001$). B) Percent of eggs to hatch from an initial 80 eggs. Low dissolved oxygen had a significant negative effect on hatching success ($p<0.001$). C) Percent survival of larvae at eight days post hatch. Low pH and low dissolved oxygen each had a significant negative effect on survival ($p=0.002$, $p<0.001$, respectively), and combined low pH and low dissolved oxygen had a synergistic negative effect ($p=0.004$). D) Standard length of eight-day-old larvae. Low dissolved oxygen had a significant negative effect on length ($p<0.001$). Bars represent means ± standard deviation ($n=4$).

Table 10. Seawater chemistry for May *Menidia menidia* acidification and hypoxia experiment. Mean and standard deviation for temperature (°C), pH (total scale), dissolved oxygen (mg L^{-1}), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Control	**Low DO**	**Low pH**	**Low pH, Low DO**
Temperature	23.9 (0.2)	23.9 (0.2)	23.9 (0.2)	23.9 (0.2)
pH_T	7.85 (0.01)	7.87 (0.02)	7.48 (0.01)	7.47 (0.01)
DO	8.56 (0.04)	2.71 (0.34)	8.52 (0.03)	2.41 (0.01)
pCO_2	560 (4)	530 (2)	1599 (68)	1701 (38)
Ω calcite	3.36 (0.52)	3.27 (0.44)	1.31 (0.16)	1.19 (0.10)
Ω aragonite	2.16 (0.35)	2.11 (0.30)	0.84 (0.11)	0.76 (0.07)
TA	2030 (180)	1960 (145)	1930 (76)	1890 (56)
CO_3^{2-}	132 (23)	129 (20)	51.4 (7.1)	46.7 (4.7)
DIC	1860 (150)	1790 (118)	1910 (64)	1880 (47)
Salinity	29.5 (0.5)	29.5 (0.5)	29.5 (0.5)	29.5 (0.5)

Table 11. Two-way ANOVA for number of days until first eggs hatched in May *Menidia menidia* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	7.563	7.563	24.2	<0.001
pH	1	0.0625	0.0625	0.2	0.663
Dissolved oxygen x pH	1	0.0625	0.0625	0.2	0.663
Residual	12	3.75	0.313		
Total	15	11.438	0.762		

Table 12. Two-way ANOVA for hatching success in May *Menidia menidia* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.55	0.55	60.793	<0.001
pH	1	0.000157	0.000157	0.0173	0.898
Dissolved oxygen x pH	1	0.00227	0.00227	0.251	0.626
Residual	12	0.109	0.00905		
Total	15	0.661	0.0441		

Table 13. Two-way ANOVA for survival of *Menidia menidia* larvae at eight days post hatch in May acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.6	0.6	46.515	<0.001
pH	1	0.215	0.215	16.647	0.002
Dissolved oxygen x pH	1	0.16	0.16	12.405	0.004
Residual	12	0.155	0.0129		
Total	15	1.129	0.0753		

Table 14. Two-way ANOVA for standard length of eight-day-old *Menidia menidia* in May acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	52.97	52.97	361.129	<0.001
pH	1	0.00776	0.00776	0.0529	0.822
Dissolved oxygen x pH	1	0.197	0.197	1.343	0.269
Residual	12	1.76	0.147		
Total	15	54.935	3.662		

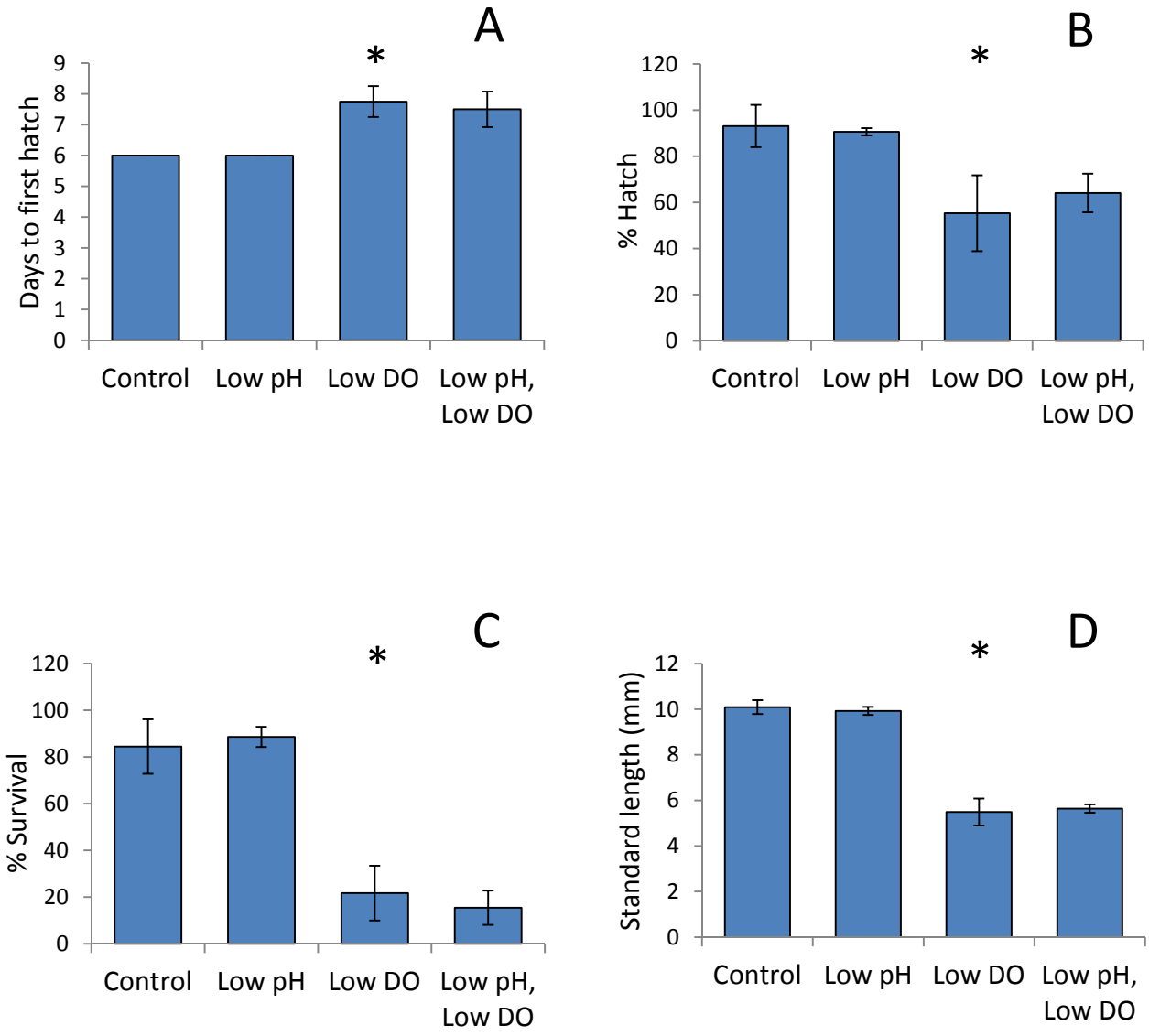

Figure 5. A) Number of days from start of June experiment until first *Menidia menidia* eggs hatched. Low dissolved oxygen significantly delayed hatching ($p<0.001$). B) Percent of eggs to hatch from an initial 80 eggs. Low dissolved oxygen had a significant negative effect on hatching success ($p<0.001$). C) Percent survival of larvae at eight days post hatch. Low dissolved oxygen had a significant negative effect on survival ($p<0.001$). D) Standard length of eight-day-old larvae. Low dissolved oxygen had a significant negative effect on length ($p<0.001$). Bars represent means ± standard deviation ($n=4$).

Table 15. Seawater chemistry for June *Menidia menidia* acidification and hypoxia experiment. Mean and standard deviation for temperature (°C), pH (total scale), dissolved oxygen (mg L^{-1}), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Control	**Low DO**	**Low pH**	**Low pH, Low DO**
Temperature	24.4 (0.5)	24.4 (0.5)	24.4 (0.5)	24.4 (0.5)
pH_T	7.85 (0.01)	7.88 (0.01)	7.44 (0.01)	7.45 (0.01)
DO	8.50 (0.03)	2.55 (0.26)	8.40 (0.03)	2.36 (0.05)
pCO_2	555 (2)	530 (2)	1726 (112)	1787 (84)
Ω calcite	3.12 (0.18)	3.03 (0.10)	1.19 (0.01)	1.17 (0.08)
Ω aragonite	2.01 (0.13)	1.95 (0.08)	0.76 (0.00)	0.75 (0.06)
TA	1940 (50)	1870 (22)	1900 (30)	1920 (94)
CO_3^{2-}	122 (8)	118 (5)	46.3 (0.0)	45.9 (3.6)
DIC	1780 (37)	1720 (13)	1880 (33)	1910 (92)
Salinity	26	26	26	26

Table 16. Two-way ANOVA for number of days until first eggs hatched in June *Menidia menidia* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	10.563	10.563	72.429	<0.001
pH	1	0.0625	0.0625	0.429	0.525
Dissolved oxygen x pH	1	0.0625	0.0625	0.429	0.525
Residual	12	1.75	0.146		
Total	15	12.438	0.829		

Table 17. Two-way ANOVA for hatching success in June *Menidia menidia* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.686	0.686	37.32	<0.001
pH	1	0.000213	0.000213	0.0116	0.916
Dissolved oxygen x pH	1	0.0282	0.0282	1.531	0.24
Residual	12	0.221	0.0184		
Total	15	0.936	0.0624		

Table 18. Two-way ANOVA for survival of *Menidia menidia* larvae at eight days post hatch in June acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	2.388	2.388	155.792	<0.001
pH	1	0.0012	0.0012	0.0782	0.784
Dissolved oxygen x pH	1	0.0157	0.0157	1.025	0.331
Residual	12	0.184	0.0153		
Total	15	2.589	0.173		

Table 19. Two-way ANOVA for standard length of eight-day-old *Menidia menidia* in June acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	78.982	78.982	620.75	<0.001
pH	1	0.00027	0.00027	0.00212	0.964
Dissolved oxygen x pH	1	0.102	0.102	0.804	0.388
Residual	12	1.527	0.127		
Total	15	80.612	5.374		

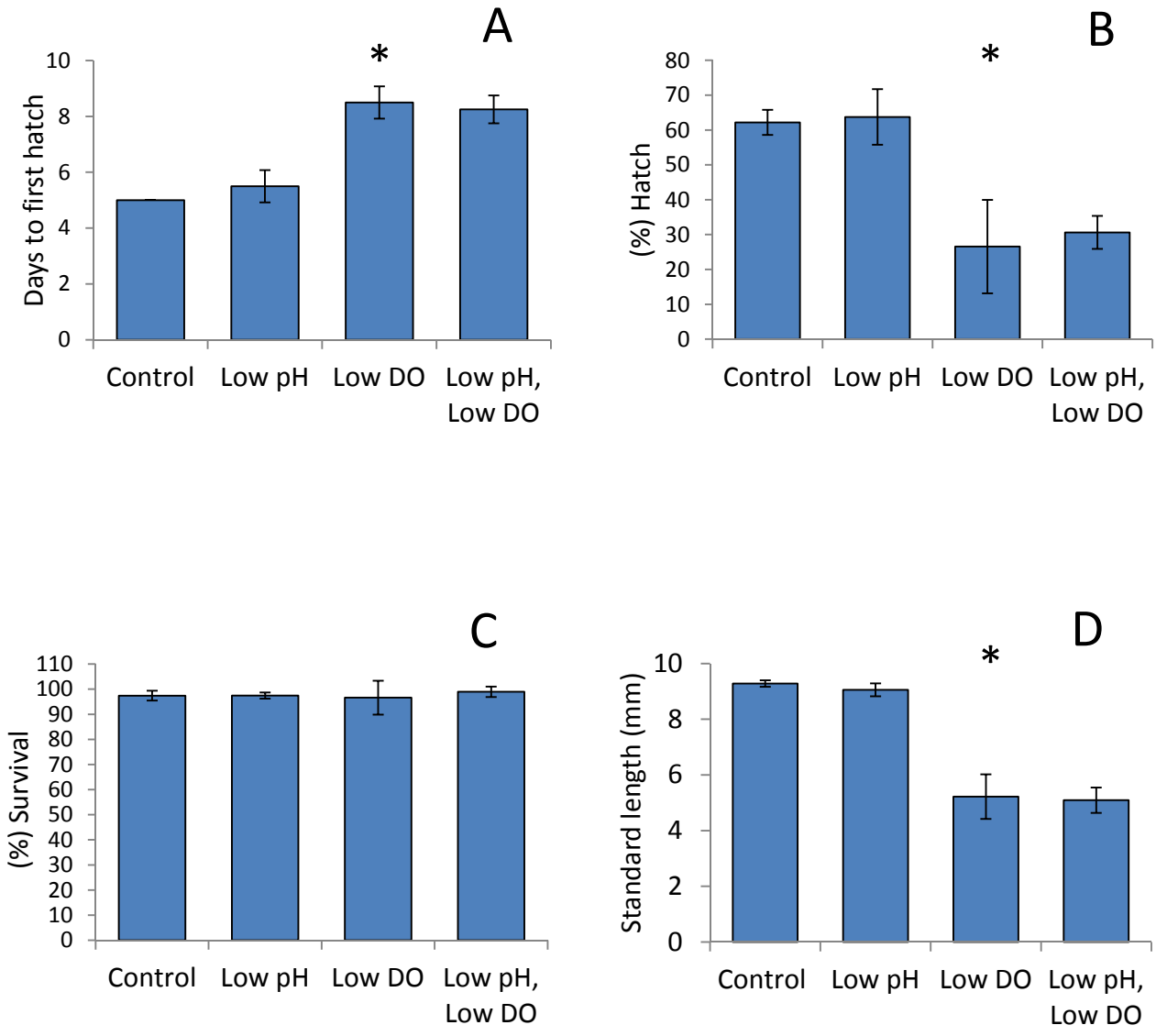

Figure 6. A) Number of days from start of experiment until first *Cyprinodon variegatus* eggs hatched. Low dissolved oxygen significantly delayed hatching ($p<0.001$). B) Percent of eggs to hatch from an initial 80 eggs. Low dissolved oxygen had a significant negative effect on hatching success ($p<0.001$). C) Percent survival of larvae at 13 days post hatch. D) Standard length of 13-day-old larvae. Low dissolved oxygen had a significant negative effect on length ($p<0.001$). Bars represent means ± standard deviation ($n=4$).

Table 20. Seawater chemistry for *Cyprinodon variegatus* acidification and hypoxia experiment. Mean and standard deviation for temperature (°C), pH (total scale), dissolved oxygen (mg L^{-1}), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Control	Low DO	Low pH	Low pH, Low DO
Temperature	24.0 (0.4)	24.0 (0.4)	24.0 (0.4)	24.0 (0.4)
pH_T	7.90 (0.02)	7.94 (0.01)	7.46 (0.01)	7.46 (0.00)
DO	8.53 (0.02)	2.41 (0.10)	8.39 (0.08)	2.36 (0.10)
pCO_2	483 (2)	452 (8)	1633 (69)	1705 (102)
Ω calcite	3.99 (0.11)	3.95 (0.38)	1.43 (0.12)	1.42 (0.06)
Ω aragonite	2.61 (0.08)	2.58 (0.26)	0.93 (0.08)	0.93 (0.04)
TA	2100 (42)	2040 (133)	2020 (127)	2050 (103)
CO_3^{2-}	162 (6)	161 (17)	58.3 (5.4)	57.7 (3.1)
DIC	1880 (32)	1810 (110)	1990 (122)	2020 (102)
Salinity	31.6 (0.5)	31.6 (0.5)	31.6 (0.5)	31.6 (0.5)

Table 21. Two-way ANOVA for number of days until first eggs hatched in *Cyprinodon variegatus* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	39.063	39.063	170.455	<0.001
pH	1	0.0625	0.0625	0.273	0.611
Dissolved oxygen x pH	1	0.563	0.563	2.455	0.143
Residual	12	2.75	0.229		
Total	15	42.438	2.829		

Table 22. Two-way ANOVA for hatching success in *Cyprinodon variegatus* acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.512	0.512	63.16	<0.001
pH	1	0.00477	0.00477	0.589	0.458
Dissolved oxygen x pH	1	0.00117	0.00117	0.145	0.71
Residual	12	0.0972	0.0081		
Total	15	0.615	0.041		

Table 23. Two-way ANOVA for survival of *Cyprinodon variegatus* larvae at 13 days post hatch in acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	0.0224	0.0224	1.581	0.233
pH	1	0.000602	0.000602	0.0425	0.84
Dissolved oxygen x pH	1	0.00371	0.00371	0.262	0.618
Residual	12	0.17	0.0142		
Total	15	0.197	0.0131		

Table 24. Two-way ANOVA for standard length of 13-day-old *Cyprinodon variegatus* in acidification and hypoxia experiment.

Source of Variation	DF	SS	MS	F	P
Dissolved oxygen	1	64.38	64.38	280.755	<0.001
pH	1	0.125	0.125	0.545	0.474
Dissolved oxygen x pH	1	0.00992	0.00992	0.0433	0.839
Residual	12	2.752	0.229		
Total	15	67.267	4.484		

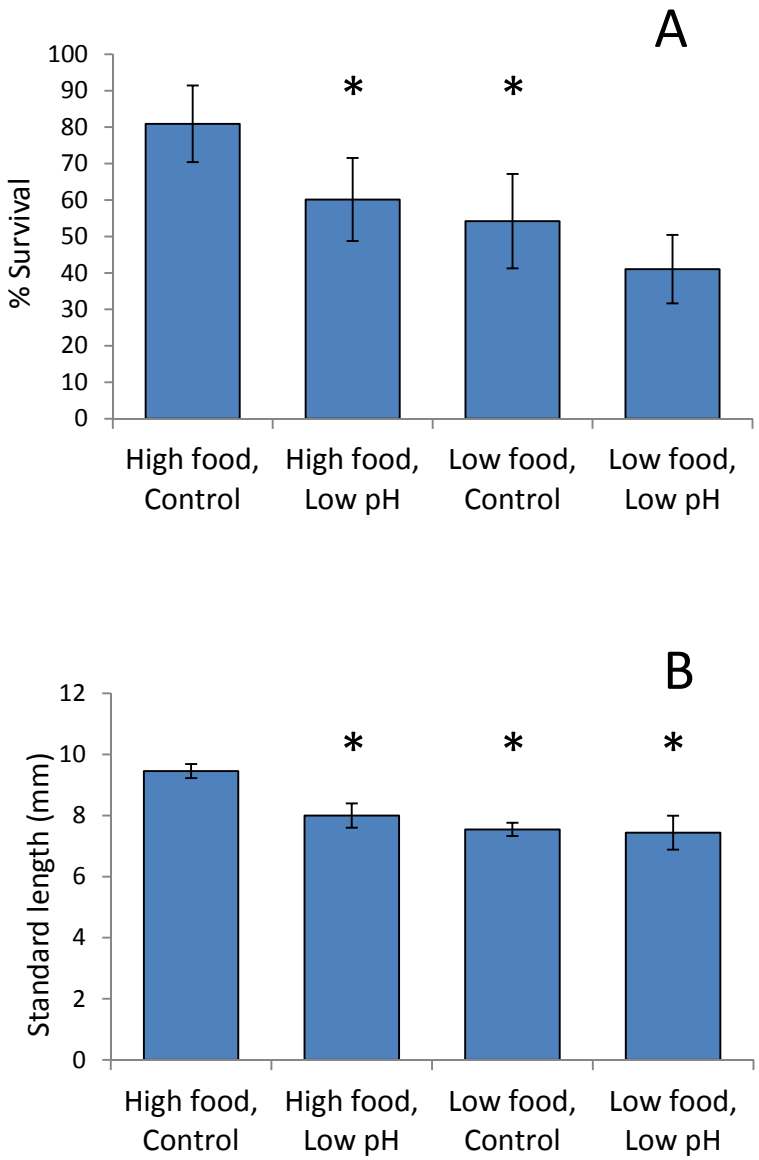

Figure 7. A) Percent survival of *Menidia beryllina* larvae at 24 days post hatch. Low pH and low food both had a significant negative effect on survival (p=0.01, p=0.002, respectively), and their combined effects were additive. B) Standard length of 24-day-old larvae. Low pH and low food both had a significant negative effect on length (p=0.001, p<0.001, respectively), and their combined effects were antagonistic (p=0.004). Bars represent means ± standard deviation (*n*=4).

Table 25. Seawater chemistry for *Menidia beryllina* acidification and restricted food experiment. Mean and standard deviation for pH (NBS scale), pCO_2 (µatm), total alkalinity (µmol kg^{-1}), CO_3^{2-} (µmol kg^{-1}), and total dissolved inorganic carbon (µmol kg^{-1}).

	Low food, Low pH	**High food, Low pH**	**Low food, Control**	**High food, Control**
pH_{NBS}	7.775 (0.013)	7.762 (0.010)	8.063 (0.010)	8.032 (0.019)
pCO_2	1165 (11)	1124 (156)	533 (4)	551 (58)
Ω calcite	1.94 (0.23)	2.00 (0.19)	3.44 (0.13)	3.48 (0.27)
Ω aragonite	1.25 (0.15)	1.29 (0.12)	2.22 (0.08)	2.24 (0.17)
TA	2220 (150)	2210 (168)	2170 (45)	2210 (111)
CO_3^{2-}	78.8 (9.4)	81.1 (7.7)	140 (5)	141 (11)
DIC	2140 (139)	2130 (169)	1990 (40)	2020 (107)
Salinity	30.7 (0.6)	30.7 (0.6)	30.7 (0.6)	30.7 (0.6)

Table 26. Two-way ANOVA for survival of *Menidia beryllina* larvae at 24 days post hatch in acidification and restricted food experiment.

Source of Variation	DF	SS	MS	F	P
pH	1	0.142	0.142	9.352	0.01
Food Level	1	0.248	0.248	16.297	0.002
pH x Food Level	1	0.0117	0.0117	0.767	0.398
Residual	12	0.183	0.0152		
Total	15	0.585	0.039		

Table 27. Two-way ANOVA for standard length of 24-day-old *Menidia beryllina* in acidification and restricted food experiment.

Source of Variation	DF	SS	MS	F	P
pH	1	2.448	2.448	17.241	0.001
Food level	1	6.088	6.088	42.871	<0.001
pH x Food level	1	1.826	1.826	12.861	0.004
Residual	12	1.704	0.142		
Total	15	12.068	0.805		

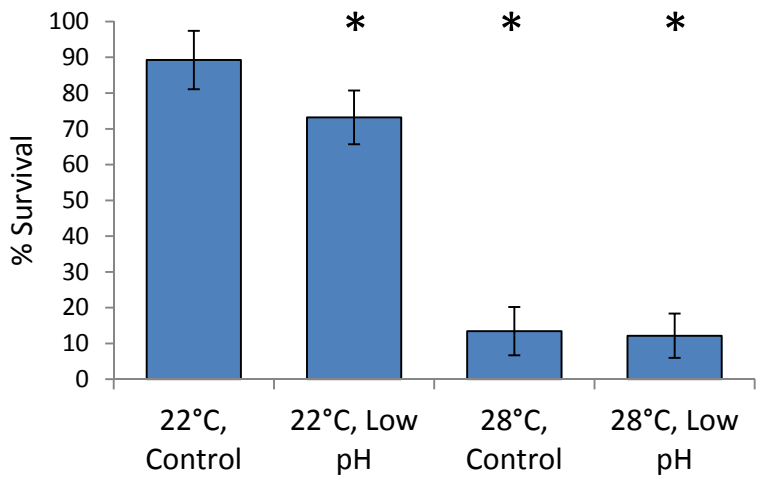

Figure 8. Percent survival of *Menidia beryllina* larvae at four days post hatch. Low pH and high temperature both had a significant negative effect on survival ($p=0.014$, $p<0.001$, respectively), and their combined effects were antagonistic ($p=0.032$). Bars represent means ± standard deviation ($n=6$).

Table 28. Seawater chemistry for *Menidia beryllina* acidification and elevated temperature experiment. Mean and standard deviation for pH (NBS scale).

	22°C, Control	**22°C, Low pH**	**28°C, Control**	**28°C, Low pH**
pH_{NBS}	8.041 (0.004)	7.682 (0.022)	8.085 (0.001)	7.740 (0.034)
Salinity	28	28	28	28

Table 29. Two-way ANOVA for survival of four-day-old *Menidia beryllina* in acidification and elevated temperature experiment.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Temperature	1	3.799	3.799	261.35	<0.001
pH	1	0.105	0.105	7.206	0.014
Temperature x pH	1	0.0773	0.0773	5.315	0.032
Residual	20	0.291	0.0145		
Total	23	4.272	0.186		