ABSTRACT

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Diel-cycling hypoxia and pH co-occur in shallow waters world-wide. Eutrophication tends to increase the occurrence and severity of diel cycles. We used laboratory experiments to investigate effects of diel-cycling DO and pH on acquisition and progression of infections by *Perkinsus marinus*, the protistan parasite which causes Dermo disease, as well as hemocyte activity, growth, and feeding in the eastern oyster, *Crassostrea virginica*, an important estuarine species. Diel-cycling DO increased *P. marinus* infection and cycling DO and pH stimulated hemocyte activity and reduced oyster growth. However, ambient environmental conditions and oyster age modulated some of these effects. Co-varying DO and pH cycles sometimes had less severe effects than either cycle independently. Oysters may acclimate to, or compensate for, effects of cycling conditions on growth. Variation in magnitude and spatial extent of cycling conditions is an important consideration when choosing restoration sites, as severe cycling conditions may hinder re-establishment of estuarine populations.

THE EFFECTS OF CO-VARYING DIEL-CYCLING HYPOXIA AND pH ON DISEASE SUSCEPTIBILITY, GROWTH, AND FEEDING IN *CRASSOSTREA VIRGINICA*

By

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Preface

Parts of the research described here represent a collaborative effort. In particular, Gary Wikfors, of the NOAA Fisheries Service, Northeast Fisheries Science Center, Milford, CT, USA provided the impetus for and made possible the hemocyte portion of the work described in Chapter 1. He provided the necessary tools and supplies for these analyses, performed the assays, and analyzed the hemocyte results presented in Chapter 1. I assisted with the hemocyte portion of the study and suggested the interpretation of the results.

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Chapter I. Effects of co-varying diel-cycling hypoxia and pH on disease susceptibility in *Crassostrea virginica*

Introduction Shallow waters in estuaries and coastal zones traditionally are considered a refuge from deep-water benthic hypoxia (Bartol et al. 1999; Eby and Crowder 2002; Bell and Eggleston 2005) and are often targeted for species restoration (Lenihan et al. 2001; Byers et al. 2006). These same shallow areas, however, often experience diel-cycling dissolved oxygen (DO) and co-occurring diel-cycling pH, resulting in periods of hypoxia (DO below saturation) and environmental hypercapnia (elevated pCO_2 resulting in low pH) (Burnett and Stickle 2001). Diel-cycling conditions occur naturally in shallow waters, including those minimally affected by human activities, and are driven by daily cycles of respiration and photosynthesis (Nixon and Oviatt 1973; Kemp and Boynton 1980; Tyler et al. 2009). The magnitude of diel cycling is affected by a variety of other environmental characteristics and therefore may vary in amplitude from day to day (Fig. 1) (Tyler et al. 2009). Although these cycles occur naturally, they are exacerbated by eutrophication and are expected to worsen with climate change (Boynton et al. 1996; Diaz and Rosenberg 2008; Rabalais et al. 2010). Cycling DO/pH has the potential to create landscape-level variation in the conditions seen by aquatic organisms, and may have sub-lethal effects upon individuals with negative consequences for populations , (Sagasti et al. 2001; Eby et al. 2005; Tanner et al. 2006; Breitburg et al., submitted).

Diel-cycling pH is of particular interest as awareness of acidification from elevated atmospheric CO_2, nutrient enrichment, and other sources, raises concern about how acidification affects marine and estuarine systems (Anthony et al. 2008; Yamamoto-

Kawai 2009; Cai 2012). In shallow waters, daily fluctuations in dissolved CO_2 concentrations range widely, from doubling, as in the Gironde estuary in France (Frankignoulle et al. 1998), to ranging by a factor of 10 or more, as in the Thames in the UK (Frankignoulle et al. 1998) or the Anacostia in the USA (Bala Krishna Prasad et al. 2013). Although these systems are also experiencing large daily fluctuations in pH, the relationship between pCO_2 and pH is indirect in that the effect of pCO_2 on pH is controlled by the carbonate chemistry of the system (Doney et al. 2009). In the Chesapeake Bay, a network of shallow-water sensors mounted 0.3-0.5m off bottom have shown pH values cycling one unit or more per day in some severely eutrophic systems (Breitburg et al., submitted). These severely eutrophic waters also tend to be the systems with cycling hypoxia of the largest amplitude (MDNR 2013).

Although diel-cycling pH and DO are intertwined closely (Portner 2008; Levin et al. 2009), most laboratory research has focused primarily upon continuous hypoxia or cyclical DO without manipulating pH (e.g. Baker and Mann 1992; Dwyer and Burnett 1996; Lenihan and Peterson 1998; Burnett and Stickle 2001). Acidification research primarily has focused upon current, or future predictions of, open-ocean pH, with less published research replicating cyclical conditions that shallow waters currently experience, (e.g. Bamber 1987; Burnett 1997; Waldbusser et al. 2011). Exposure to hypoxia can negatively affect survival, growth, and reproduction of organisms (Boyd and Burnett 1999; Burnett and Stickle 2001; Breitburg et al. 2009; Vaquer-Sunyer and Duarte 2010) as well as potentially increase susceptibility to pathogens (Smolarz et al. 2006). Exposure to acidified water also has been associated with a wide range of biological

effects, including increased mortality, altered production of reactive oxygen intermediates (ROIs), decreased growth, reduced tissue energy stores, and decreased calcification rates (Boyd and Burnett 1999; Ringwood and Keppler 2002; Gazeau et al. 2007; Dickinson et al. 2012). For example, Ringwood and Keppler (2002) found that mean pH below 7.5 or very short term exposures to pH below 7.2 decreased *Mercenaria mercenaria* growth more than 50% when compared with clams held above 7.5. Dickinson et al. (2012) found that a constant pCO_2 of 800 ppm increased mortality, reduced tissue energy stores, and caused negative soft tissue growth of the eastern oyster, *Crassostrea virginica,* after 11 weeks when compared to pCO_2 of 400 ppm. Effects of hypoxia and acidification upon the immune systems of invertebrates, some positive and some negative, also have been observed (e.g. Boyd and Burnett 1999; Burnett and Stickle 2001), and studies have shown that higher bacterial loads can be found in organisms exposed to hypoxia and acidified water can have (e.g. *Callinectes sapidus* (Holman et al. 2004) and *C. virginica* (Macey et al. 2008)).

The eastern oyster, *C. virginica* (Gmelin) naturally inhabits the western Atlantic from Brazil to Canada's St. Lawrence River. It is an important fishery species throughout much of its range, including the Chesapeake Bay (Hargis and Haven 1999; Mann and Evans 2004), where stocks are estimated to be at or below 1% of historic levels (Newell 1988; Wilberg et al. 2011). As sessile organisms, post-settlement oysters cannot move to avoid hypoxic events, and in spite of wide tolerance of low DO, constant hypoxia reduces feeding, metabolism, and growth (Widdows et al. 1989; Baker and Mann 1992; Burnett and Stickle 2001) and delays and reduces larval settlement (Widdows et al. 1989).

Reactive oxygen species (ROS) produced by hemocytes are an important part of the immune response in *C. virginica*. ROS production following pathogen or proxy challenge is commonly measured as a determinant of immune capacity. Unstimulated ROS production measures the innate levels of ROS produced by cell metabolism, whereas measurement of stimulated ROS production indicates the ability of the cell to kill pathogens. High unstimulated ROS is an indicator of stress, and may be energetically draining and physically damaging to the organism. Hemocytes from lobsters collected from hypoxic areas had higher unstimulated ROS production than those from higher-oxygen areas (Moss and Allam 2006). Anderson et al. (1998) found no effect of hypoxia upon unstimulated ROS production in *C. virginica*. Boyd and Burnett (1999) found that both hypoxia and hypercapnia reduced stimulated production of reactive oxygen intermediates by oyster hemocytes after stimulation with zymosan. This indicates that hypoxia may stimulate one aspect of the immune response, but may limit the ability of cells to respond to further stress and expose cells to possible oxidative damage.

Two diseases, Dermo and MSX, are particularly damaging to oysters in the Chesapeake Bay region. *Perkinsus marinus*, a protistan parasite that causes Dermo disease in oysters, was first observed in Chesapeake Bay in the 1940s after initially being discovered in the Gulf of Mexico. It is thought to be endemic to the Chesapeake Bay region. *P. marinus* is one member of a genus of parasites that affect mollusks worldwide (Goggin and Lester 1987; Goggin and Barker 1993; Pecher 2007). In the Chesapeake Bay, a period of drought in the 1980s (Dauer et al. 2000; Mann and Evans 2004; Murphy et al. 2011) increased Dermo prevalence (Burreson and Andrews 1988; Harvell et al. 1999; Kimmel

and Newell 2007). Along with overharvesting, loss of hard bottom substrate, and water quality declines, Dermo is one of the major factors limiting eastern oyster populations today (Ford and Tripp 1996; Harvell et al. 1999; Reece et al. 2001; Carnegie and Burreson 2009; Beck et al. 2011).

Previous laboratory and field studies indicate that diel-cycling DO increases the acquisition and progression of *P. marinus* infections in eastern oysters (Breitburg et al., submitted). Stronger effects of DO on *P. marinus* infection in the field than in the lab suggested the possibility that a co-occurring stressor increased DO effects. We postulated that the co-occurring stressor unaccounted for in previous laboratory experiments was pH, which shows a tight correlation with DO in the field (Burnett 1997) but which was not controlled in Breitburg et al. (submitted). The mechanism behind these infection differences may be an effect of cycling DO upon immune function, similar to the effects of constant hypoxia seen in Boyd and Burnett (1999).

The objective of this research was to examine the effects of repeated, short term, co-occurring stressors by exposing eastern oysters with no detected infection initially to both diel-cycling DO and diel-cycling pH, as well as to either of these stressors individually, along with water containing waterborne *P. marinus* for approximately 3 months and examining acquisition and progression of infection and immune status of the oysters. This builds upon previous work by examining the effects of cycling DO and the previously unstudied co-occurring cycling pH, as well as directly examining immune status under these conditions. Our expectation was that exposure to repeated, brief

periods of hypoxia and low pH would increase *P. marinus* acquisition and progression and disturb immune response more severely than either stressor independently.

Methods We tested the effects of diel-cycling DO and pH upon infection acquisition and progression as well as hemocyte status in 1 year old (yo) eastern oysters (35-70 mm initial length) at the Smithsonian Environmental Research Center (SERC), in Edgewater, Maryland, USA, during July-September 2012. Older oysters (4-5 yo) were used as a source of *P. marinus* in the experiments (initially 72% prevalence with an infection intensity of 1.35 $\pm$ 1.00). All oysters were purchased from Marinetics, Inc., an aquaculture facility on the Choptank River, MD, USA in April/May 2012, and held on flow-through Rhode River water at SERC until the experiment commenced. Salinity and temperature at the Marinetics facility were within 2 and 1 °C, respectively, of Rhode River ambient conditions at the time oysters were purchased.

Initial *P. marinus* infection prevalence and intensities were determined in 100 individuals of each age class using Ray's Fluid Thioglycollate Medium (RFTM) assay (Ray 1952; Ray 1954) on rectal tissue. Although RFTM assay may miss very light infections, it allows for a rapid and cost-effective analysis of infection in a large number of individuals. We define prevalence as the percentage of individuals with detectable infections out of the entire population analyzed; the change in prevalence over the course of the experiment was used as an index of infection acquisition. Mean infection intensity was the average modified Mackin score among only those oysters with detectable levels of infection (Mackin 1962; Craig et al. 1989; Lenihan et al. 1999).

Ninety 1yo oysters per 75 L aquaria were assigned to six replicates of five treatments arranged in a randomized block design, with one replicate from each treatment clustered

together in case room position affected results. Older oysters serving as the infection source were held in an air-bubbled 400 L tank. Experimental oysters were acclimated to aquaria, feeding regime, and light/dark cycles at normoxia/normcapnia for five days prior to commencing treatment conditions.

Treatments

A factorial design was used crossing two pH treatments: a constant "normcapnia" pH (7.8) and cycling pH between 7.0 and 7.8, with two DO treatments: constant "normoxia" (7.0 mg L^{-1}), and severe cycling hypoxia ranging from 0.5 mg L^{-1} to a supersaturated value of 10.0 mg L^{-1} (Fig. 2). Normcapnia is defined herein as a pH of approximately 7.8, which is reasonable for the field-site pH conditions that we replicated (Fig. 1). A fifth "moderate hypoxia" treatment also was run consisting of DO cycling from a low of 1.7 mg L^{-1} to a supersaturated value of 10.0 mg L^{-1} with cycling pH. The factorial structure of this design allowed for an estimate of the interaction of cycling pH and severe cycling hypoxia, as well as the individual main effects. The additional moderate cycling hypoxia treatment allowed for an estimate of the effects of a more moderate cycling hypoxia when compared to the constant high DO/cycling pH treatment. Our experimental facility precluded our ability to run additional treatments to test a full factorial design.

One-year-old oysters were exposed to cycling conditions 4-5 d wk^{-1} from July 5 through September 27, 2012 (54 days of cycles total). In the cycling DO and/or pH treatments, DO and pH were decreased over 3hrs (ramp-down), held at continuous low values for 4h

(low-plateau), brought back to normoxia/normcapnia over 3h(ramp-up), held for 2h (normoxia), taken to supersaturated DO/normcapnia values over 2h (up-to-supersat), held at high values for 2h (supersat-plateau), brought back to normoxia over 2h (down-to-normoxia) and held at normoxia/normcapnia (normoxia) until the next day's cycle commenced (Fig. 2). Photoperiod regime was maintained in a 14:10 light:dark cycle 7 d wk^{-1}, using incandescent 5V rope-lighting. Light conditions in the tanks simulated those at 2 m depth in the Rhode River, MD, USA on a sunny day as measured with a Li-Cor LI-190 Quantum Sensor (Li-Cor Inc., Lincoln, NE, USA). On the 2-3 d wk^{-1} on which DO/pH cycling conditions were not applied, treatments were bubbled with air and CO_2 stripped air to maintain target values of the control (constant normoxia/normcapnia) treatment: this resulted in a DO of 7.44 ± 0.004 mg L^{-1} and pH of 7.83 ± 0.002. Potentially *P. marinus*-contaminated water was not transmitted to experimental oysters on these days.

Experimental conditions were monitored and manipulated using a custom-developed LabVIEW (National Instruments Corp., Austin, TX, USA) program which used input from Oxyguard Standard DO probes (Oxyguard International A/S, Birkeroed, Denmark) and Honeywell Durafet III pH sensors (Honeywell International, Morristown, NJ, USA) and manipulating ratios of five gasses (air, CO_2-stripped air, oxygen, nitrogen, and carbon dioxide) through mass flow controllers (Dakota Instruments, Orangeburg, NY, USA). Soda lime CO_2 scrubbers were used to create CO_2-stripped air. Oxyguard DO and Honeywell pH sensors were checked for calibration weekly, and recalibrated if they were outside of published accuracy ranges. Honeywell pH probes were 2-point

calibrated (NBS scale, Thermo Fisher Scientific, Waltham, MA, USA) and Oxyguard DO probes were calibrated in water-saturated air. One DO probe and one pH probe were placed in 1 replicate of each treatment and used to control all six replicates. One 30 L min^{-1} gas mix was created per treatment and then split via gas manifolds to deliver 5 L min^{-1} of mixed gas to each replicate aquaria through 2 glass-bonded silica air diffusers (3.75cmx1.25cm) resting on the bottom at the middle of the aquarium. For details of this system, see Burrell et al. (submitted).

In addition to continuous monitoring of DO and pH in one replicate, DO, temperature, salinity, and pH were measured 3 to 4 times per day in all aquaria using a YSI ProfessionalPlus (Yellow Springs Instruments, Yellow Springs, OH, USA), and an Oakton Acorn pH 5 meter (Oakton Instruments, Vernon Hills, IL, USA). This ensured that treatment variables were similar among replicates and that non-controlled variables (temperature and salinity) did not vary among treatments. In-tank partial pressure of carbon dioxide (pCO_2) was measured 3-4 d wk^{-1} via equilibration in one replicate of the control treatment and 1 d wk^{-1} in one replicate of each of the other four treatments during the low-plateau part of the cycle using a Li-Cor 840A CO_2/H_2O gas analyzer (Li-Cor Inc., Lincoln, NE, USA). Alkalinity was determined by titration three times per week in one replicate of the control treatment using a Tazo Schott-Gerate piston burette titrator and a Corning pH Analyzer 350 according to Standard Method 2320 (APHA 1992).

Each aquarium received 1 L min^{-1} of flow-through, unfiltered, Rhode River water supplemented with 0.093 mL of stock algal diet (DT's Reef Blend,

http://www.dtplankton.com/) mixed into the inflow water every 8 minutes, 24 h d^{-1}, throughout the experiment, with the exception of a 10 day period in August during which the timer controlling the algae system was under repair. While this would have reduced food availability, there would have been some ambient phytoplankton in the SERC sea water system, and all treatments would have experienced the same reduction in phytoplankton availability during this period. Each aquarium also received 75 mL min^{-1} of water from the infected oyster tank. Both water inputs were located just above the air diffusers to promote mixing. The infected oyster tank was provided a constant 5 L min^{-1} of flow-through Rhode River water. All effluent water from the infected oyster tank and treatment aquaria was UV-sterilized before release to the Rhode River. Oysters were removed from aquaria and washed gently each week to remove mud, feces, pseudofeces and polychaetes. Aquaria were drained and scrubbed bi-weekly to remove waste products and bio-fouling on a day when conditions were not cycled.

Infection and growth metrics were measured half-way through the experiment and at the end of the experiment. At the midpoint, 30 oysters were removed haphazardly from each aquarium on August 8-9 2012, and infection prevalence and intensity (determined using the RFTM assay), shell length, and wet tissue weight were measured.

Just before the end of the experiment, 2 oysters were removed from each replicate of the 4 factorial treatments at the end of the low-plateau phase on September 25, 2012. Oysters were measured and hemolymph was removed from the adductor-muscle sinus of each oyster using a 1 mL syringe fitted with a 23-gauge needle inserted through a small

notch cut into the ventral shell edge. Following hemolymph extraction, oysters were shucked, and a sample of rectal tissue was taken for infection analysis by RFTM assay. Any oyster that did not provide enough hemolymph for analysis in a timely manner was discarded, resulting in a sample size of 10 for each treatment. The hemolymph from each oyster was held on ice in an Eppendorf tube until being distributed into Falcon flow-cytometer tubes for the several analyses conducted. In one Falcon tube, counts, mean sizes, and percentages of granular and agranular dead hemocytes were determined with an Accuri C6 flow cytometer (BD BioSciences, San Jose, CA) using the methods of Hégaret and colleagues (2003). In another tube, percentages of total and granular phagocytic hemocytes were determined using 2-µm, plastic microbeads (Hégaret et al. 2003). In a third tube, reactive-oxygen species production by hemocytes was determined using the oxidation of non-fluorescent DCFH-DA to green-fluorescent DCFH (Hégaret et al. 2003). For this analysis, cells were not stimulated with chemical or particulate inducers of oxidative burst, so values reported (in relative, dimensionless detector units) represented constitutive oxidative activity (ROS). Finally, in a fourth tube, percentages of live or dead apoptotic hemocytes were determined using the green-fluorescent probe Annexin V and propidium iodide following the manufacturer's instructions (Life Technologies, Carlsbad, CA, Product V13241).

At the end of the experiment on September 26-27, 2012, an additional 28 oysters were removed from each replicate. For each oyster, shell length was measured, tissue assayed for Dermo infection, and wet tissue weight was determined gravimetrically. All

remaining oysters were removed from the experiment, measured, and any mortality was recorded.

To examine latent effects of cycling conditions on infection acquisition and intensity, 17 oysters from each aquarium were placed in 3,000 cm^3 cages constructed of 2 cm square mesh and suspended from SERC piers in the Rhode River approximately 0.5 m above the bottom. Cages were deployed 2 m apart at each site to minimize *P. marinus* transmission, and in such a way that they were unlikely to be exposed to hypoxia as severe as that seen in the lab and that all treatments would experience similar field conditions. Approximately nine months later, these cages were collected from the field sites on July 18 and 19, 2013. All oysters were measured and weighed, and infection was assayed to examine any latent effects of cycling conditions experienced the previous year upon infection.

Statistics

All data were tested for homogeneity of variance using an F-max test and normality using a Shapiro-Wilkes test. Percentage data were logit transformed. Unless otherwise noted, data are presented as means $\pm$ standard error.

Using the proc mixed procedure (SAS Institute Inc., Cary, NC, USA), salinity, temperature, DO, and pH among treatments were compared with nested ANOVAs among the 5 treatments using data collected at the end of the low-plateau phase each day. Treatment effects also were tested prior to the ramp-down phase each day. Tukey post-hoc tests were used on any variables shown significant by ANOVA ($p<0.05$). Effects of

DO and pH on *P. marinus* prevalence and intensity from the laboratory experiment were analyzed using randomized complete block design ANOVAs. *P. marinus* prevalence and intensity from the field deployment were analyzed using replicated block design ANOVAs with deployed field site as the blocking factor. Least square means comparisons were used to test *a priori* hypotheses that severe cycling DO and cycling pH would increase disease metrics, in combination and independently, and that moderate cycling DO would increase disease metrics in comparison to constant, normoxic treatments.

MANOVA was used to reveal main effects and interactions of the two independent variables (DO and pH) upon each hemocyte variable (Statgraphics Plus, Statpoint Technologies, Inc., Warrenton, VA). As hemocyte variables are not necessarily independent of each other (e.g., an oyster with fewer granular hemocytes is likely to have a lower percentage of phagocytic hemocytes), we have found it useful to develop overall hemocyte profiles using correlation matrices calculated by Principal Components Analysis (PCOMP) and to test effects of experimental variables upon hemocyte profiles defined by the first and second Principal Component values (Hégaret et al. 2004). Thus, the following variables were entered into a PCOMP matrix: T (transformed) % live granular hemocytes, T% dead granular hemocytes, T% live agranular hemocytes, T% dead agranular hemocytes, T% apoptotic live hemocytes, T% apoptotic dead hemocytes, T% phagocytic hemocytes, T% phagocytic granular hemocytes, ROS granular hemocyte population 1, and ROS granular hemocyte population 2 (see Results). Principal

Components 1 and 2 were used in a MANOVA testing for main effects and interactions of experimental treatments as described above for individual hemocyte variables.

Results Severe cycling hypoxia increased *P. marinus* infection prevalence and intensity and also affected some metrics of the cellular immune status in *C. virginica* over the course of the 3-month exposure to cycling conditions. Moderate cycling hypoxia did not significantly affect infection prevalence or intensity; however there was a trend towards increased prevalence of more intense infections under these conditions. Cycling pH did not affect infection prevalence or intensity significantly. After a 9-month field deployment and respite from severe cycling conditions, the prevalence of infection in oysters previously exposed to severe cycling hypoxia was still elevated over the infection prevalence in oysters exposed to normoxia.

Water quality

Experimental conditions were within the environmental ranges for *P. marinus* transmission and proliferation (salinities above 8 and maximal summer temperatures) (McCollough et al. 2007) as well as the native range of *C. virginica* (Hargis and Haven 1999). Water quality measurements taken in experimental aquaria are shown in Table 1 (DO and pH), Figure 2 and Figure 3 (salinity, temperature, and alkalinity). Over the course of the experiment, salinity averaged 11.3 $\pm$0.0, with a range from 9.3-12.6; salinity did not differ among treatments (df=4, F=0.004, p=1.0). Temperature averaged 27.1 $\pm$0.1°C, ranging from 21.0°C to 30.5°C over the course of the experiment, and did not differ among treatments (df=4, F=0.038, p=0.997). Alkalinity averaged 1630$\pm$16 $\mu eqHCO_3^{-1} L^{-1}$, and ranged from 1454 $\mu eqHCO_3^{-1} L^{-1}$ on June 29, at the experiment's start, to 1758 $\mu eqHCO_3^{-1} L^{-1}$ on September 24, at the experiment's conclusion.

DO did not differ among treatments during the normoxic period prior to the ramp-down phase (df=4, F=0.31, p=0.8744). pH values varied among treatments (df=4, F=4.98, p=0.0005), but the variation was only a 0.02 unit range among treatments. The statistical significance of the difference in pH values reflected the very large sample size (6 replicates per treatment measured daily for 51 days), and is very small when compared to the 0.80 pH unit cycle of the applied treatment.

DO varied significantly among treatments at the end of the low-plateau phase (Table 1) (df=4, F=48708.5, p<0.0001). Severe DO cycles averaged within 0.07 mg L^{-1} of target values, and moderate DO cycles averaged within 0.03 mg L^{-1} of target values. Treatments also differed with regards to pH (df=4, F=12855.2, p<0.0001), with cycling pH treatment values averaged within 0.02 of the low target value, and normcapnic treatments averaged within 0.03 of target values.

During the low-plateau phase, there was a significant difference in pCO_2 between the 5 treatments (df=4, F=128.6, p<0.0001), with a Tukey HSD test indicating differences between all cycling pH treatments and all normcapnic treatments, and no differences within these treatments.

Disease

At the start of the experiment, 1yo oysters had no detectable *P. marinus* (i.e. 0% prevalence), and 4/5yos had 72% prevalence with an infection intensity of 1.35 $\pm$ 1.00. Disease parameters at the other time points of the experiment are summarized in Table 2.

Prevalence

Severe diel-cycling hypoxia increased overall prevalence of *P. marinus* infections compared to normoxia at both the mid- and endpoint of the experiment (Table 3A, Fig. 4A). After twelve weeks of exposure to cycling treatments prevalence of *P. marinus* in oyster populations exposed to periods of severe hypoxia (0.5 mg L^{-1}) 4-5 d wk^{-1} was nearly twice that of controls (51% vs. 26%). The main-effect of cycling pH was not significant at either time point, nor was the interaction of DO and pH. Moderate cycling DO did not increase *P. marinus* prevalence over that of the control.

When prevalence of just those infections scoring 2 or higher was examined (Table 3B, Fig. 4B), no difference was observed among treatments at the midpoint (very few oysters were this heavily infected), but by the end of the study nearly 20% of oysters exposed to severe hypoxia were scoring at 2 or higher, significantly more than the 5% of oysters held at constant normoxia. The lack of difference after six weeks, but the quadrupled prevalence of more heavily infected oysters at the endpoint may indicate stimulated infection progression under hypoxic conditions. There was also a trend towards higher prevalence of severe infections under moderate cycling hypoxia when compared to normoxic conditions.

After a period of field deployment during which all treatments experienced similar conditions, which were likely not as severe as those seen in the lab (Hondorp, unpublished data), *P. marinus* prevalence in oysters exposed to severe cycling hypoxia the previous summer was nearly double the prevalence in oysters exposed to continuous

normoxia the previous year (Table 4A, Fig. 5A). There was no difference between oysters exposed previously to moderate cycling DO and oysters exposed to normoxia. The prevalence of infections scoring 2 or higher on the modified Mackin scale, however, did not differ among treatments (Table 4B, Fig. 5B), perhaps indicating that these infections had yet to progress to severe infections at the time of assay after regressing during winter. After a complete season of exposure to conditions conducive to *P. marinus*, the latent effects of severe hypoxia might be even more serious.

Infection intensity

Neither cycling DO, cycling pH, nor the interaction of the two affected infection intensity after six weeks of exposure at the midpoint of the experiment. However, after twelve weeks of exposure to cycling conditions, severe-cycling DO significantly increased infection intensity as compared with normoxia with modified Mackin score infection intensities of 1.50 and 1.27 respectively (Table 3C, Fig. 4C). Like with prevalence, there was no significant effect of cycling pH on infection intensity, no interaction between cycling DO and pH, and no difference between constant normoxia and oysters exposed to 1.5 mg L^{-1} DO.

There was no effect of laboratory treatments upon infection intensity after a 9-month field deployment (Table 4C, Fig. 5C). Intensity among infected oysters was lower at the time of field collection than infected members of each cohort at the end of the experiment, possibly attributable to the water quality conditions and point in the summer at which infection was assayed.

Hemocytes

Hematology and immune function variables for oysters were all within ranges that can be considered "normal," as these variables tend to have wide ranges related to seasonal cycles and environmental conditions (Duchemin et al. 2007; Lambert et al. 2007). Severe cycling hypoxia increased phagocytosis and unstimulated ROS, but decreased apoptosis. pH cycling increased hemocyte phagocytosis, and significant interactive effects of DO and pH treatment were found for percent dead hemocytes, phagocytosis, and apoptosis (Appendix A).

Components 1 and 2 from the hemocyte-variable PCA are plotted in Fig. 6. Component 1 contrasted oysters having high granulocyte counts, but with low phagocytosis and accumulation of apoptotic and dead hemocytes in the hemolymph (positive scale), with oysters having the opposite profile (negative scale). In Component 2, oysters with high numbers of live agranular cells and dead granular hemocytes also are shown to have low phagocytosis and ROS production by the "active" sub-population of granular hemocytes found. A sub-population of live, granular hemocytes with very low ROS was found; these may be apoptotic hemocytes, but this could not be confirmed with the data collected.

When Component 1 (PCOMP1) was analyzed as the dependent variable in a MANOVA, significant main effects of DO, pH, and the interaction term were found (Fig. 7). These statistical results were driven mainly by the very different hemocyte profile of control treatment oysters kept at constant normoxic and normcapnic conditions (Fig. 7A). When

Component 2 was applied to the same MANOVA, only the interaction term was significant. Specifically, when normoxic oysters were in the cycled-pH treatment, PCOMP2 scores were lower than in normcapnic oysters; whereas, oysters in severe hypoxia had higher PCOMP2 scores when in the cycled pH treatment compared to normcapnia. The combination of constant normoxia and constant pH influenced immune status of oysters by repressing granulocyte phagocytic function, with a sub-population of these granular cells also showing low levels of ROS generation. Low phagocytic activity of hemocytes in these oysters also permitted the accumulation of apoptotic and dead hemocytes circulating in the hemolymph.

Discussion Results of this study indicated that exposure to brief periods (4 hr d^{-1}) of severe diel-cycling hypoxia, which are common in shallow-water systems globally, and eutrophic systems in particular, may increase acquisition and progression of infection and affect immune status. Contrary to our expectations, the combination of diel-cycling DO and diel-cycling pH did not affect infection acquisition or progression beyond those of diel-cycling DO alone. Although non-significant. diel-cycling pH may reduce infection prevalence and intensity slightly under normoxic conditions (Fig. 4). Severe diel-cycling hypoxia can increase the acquisition and subsequent progression of *P. marinus* infections in eastern oysters over the course of just one season, and prevalence may remain elevated through subsequent years. This effect was observed when DO cycled to 0.5 mg L^{-1} 4-5 d wk^{-1}, but not when DO minima approach 1.7 mg L^{-1}; however, moderate cycling hypoxia may have increased the number of more-intense infections slightly. These two findings in conjunction may indicate a threshold below which hypoxia increases susceptibility to infection.

Diel-cycling hypoxia and diel-cycling pH, as well as the two combined, were shown to up-regulate hemocyte phagocytosis and unstimulated ROS production and reduce apoptosis. All of these effects were seen at water quality levels at which oyster mortality was not affected over the 3 month duration of our laboratory experiment, and overall mortality was very low (<3.5%). Our experiment cannot distinguish effects of diel-cycling conditions on host versus pathogen, but the single infection source applied to all treatments, coupled with the effects on immune status lead us to believe that the altered disease dynamics are a product of effects upon oysters.

Neither moderate hypoxia (~1.7 mg L^{-1}) nor hypercapnia 4-5 d wk^{-1} significantly increased infection prevalence when compared to normoxia and hypercapnia during this experiment, although moderate hypoxia tended to result in higher prevalence of more intense infections. Previous work, however, found that diel-cycling DO significantly increased infection at a DO value of ~1.5 mg L^{-1} in some years but not in others (Breitburg et al., submitted). This may indicate that oysters, and perhaps some other estuarine organisms, have evolved to tolerate some degree of hypoxia or cycling DO. More severe cycles, which can be caused by eutrophication, may exceed tolerances of estuarine organisms and have negative effects. The effects of DO, and therefore the DO threshold which promotes infection, may interact with conditions which vary from year-to-year and were not controlled in our experimental apparatus (e.g. temperature, salinity, calcite saturation, etc.).

Previous work has primarily focused on the effects of constant hypoxia on disease dynamics. Anderson et al. (1998) found that previously-diseased oysters exposed to constant 2.86 mg L^{-1} DO experienced increased disease-related mortality, and Gray et al. (2002) found reduced growth below constant 1.5 mg L^{-1} DO, indicating physiological effects occurring near this concentration. Lack of disease effects until much more severe hypoxic values were reached in the present study may be an indication that oysters are more tolerant of hypoxia when it is interspersed with significant periods of normoxia, which may provide periods of recovery.

With the recent increase in concerns about acidification of aquatic systems, examining the effects of cycling pH seemed particularly relevant. In addition, although DO and pH are almost inextricably entwined in shallow-water systems, identifying individual effects may provide important information into the mechanisms by which these factors affect estuarine organisms. In contrast to our initial prediction, cycling pH did not increase infection susceptibility. Our pH cycles, although environmentally relevant, may not have led to increased acquisition and progression of infections in oysters because of the innate self-buffering ability of bivalves (Dwyer and Burnett 1996; Berge et al. 2006; Lannig et al. 2010) as well as the low natural pH of oyster hemolymph (Clark 2014). Periods of hypercapnia/low pH in the environment may require less energy because pH during periods of hypercapnia is closer to the internal pH of oysters (Croxton et al. 2011), which may require less regulation than during periods of normoxia/normcapnia. This could allow more energy to be allocated to immune response resulting in an overall slightly more infection-resistant condition; however, it is not known how these conditions may be affecting other aspects of oyster physiology and ecology (Ringwood and Keppler 2002; Miller et al. 2009; Lannig et al. 2010). Although cycling conditions employed in the present study are relevant to the environments that were replicated, more extreme conditions, in terms of both instantaneous values and magnitude of cycles, do occur (Boynton et al. 1996; Breitburg 2002) and might have effects not seen in this study.

Although DO and pH values are very tightly correlated (Burnett 1997; Tanner et al. 2006; Gobler et al. 2014), our results indicate that, within the range of pH values tested in this experiment and within the range of infection pressure, hypoxia is a better predictor of

infection in oyster populations than pH. Should pH values or other environmental parameters deviate from values tested here, or the magnitude of cycling exceed that of the cycles tested here, it is possible pH might have effects upon infection other than those observed in our results.

Field deployment of oysters that had been exposed previously to cycling conditions for a summer season allowed an estimate of how exposure to cycling conditions might continue to affect oysters after a period of respite from severe cycling conditions, and whether infections might return to these oysters with the same intensity. During the winter months, cycling conditions tend to break down as the magnitude of primary production decreases and water temperatures cool, thereby increasing DO saturation. During this period, *P. marinus* infections become more difficult to detect, and the infection may go in to remission (Oliver et al. 1998). Exposure to severe cycling hypoxia appeared to have a legacy effect on infection prevalence the following year, indicating that exposure to brief (4 hr), daily periods of hypoxia for one season may have lingering effects upon infection in oysters. At the time of collection, salinity was well below that at which *P. marinus* epizootics are likely, and infections are unlikely to have reached peak prevalence or intensity, yet there still was a difference in infection prevalence between treatments. If an oyster were to be exposed to cycling hypoxia during a second year, the infection increases might be additive, but this remains to be determined.

Cycling hypoxia and/or pH up-regulated cellular functions commonly considered to constitute the oyster immune response, but individuals exposed to cycling hypoxia also

had higher infection prevalence and intensity. These immune findings were counter to our expectation that the hemocyte activity would be degraded under hypoxic and/or hypercapnic conditions based upon previous research showing decreased ability to respond to immune stimulation under hypoxia and acidification (Boyd and Burnett 1999). While it is possible that the up-regulation of immune functions is an indication that environmental variation and "stressful" conditions stimulate immune activity, especially in granular hemocytes, as a "precaution" against opportunistic infection under challenging environmental conditions, the response may not be particularly or consistently effective against *P. marinus* (Chu and La Peyre 1993). *P. marinus* may instead benefit from this stimulated response by using the increased proportion of phagocytic granulocytes and reduced apoptosis as opportunities for infection (Sunila and LaBanca 2003; Goedken et al. 2005). The spread of *P. marinus* throughout the range of *C. virginica* is often attributed to warming temperatures (Andrews 1988; Ford 1996; Cook et al. 1998), but it is possible that one of the reasons *P. marinus* has been such a successful parasite is because of its ability to use the innate immune response of the oyster as a means for successful infection and proliferation. The up-regulated immune response still may be effective against other infectious agents that are not adapted to use the immune cells of the oyster as sites of infection. On the other hand, it also has been observed that *P. marinus* infection may stimulate phagocytic hemocytes (Anderson et al. 1992; Anderson et al. 1995; Samain et al. 2007). Although our infection scores are not correlated with immune variables, we cannot be certain that the immune responses are not influenced by differences in infection among treatments.

Other mechanisms also may result in higher infection levels under cycling hypoxia conditions. The restoration of oxygen after periods of hypoxia/anoxia results in the majority of tissue damage because ROS production spikes and the necessary anti-oxidants are not yet being produced (Anderson et al. 1992; Pannunzio and Storey 1998). This may occur daily under our cycling hypoxic conditions, as evidenced by the higher innate ROS levels in oysters from severe hypoxic treatments, resulting in more oxidative stress to the individual and possibly also in higher infection (Moss and Allam 2006). These negative effects may overwhelm any positive effects of the stimulated immune functions. This potentially also explains the slight trend for lower infection levels in oysters in cycling pH treatments which may benefit from the stimulated immune activity caused by exposure to fluctuating environmental conditions without experiencing the negative effects of sudden oxygen restoration. Finally, we only examined hemocyte variables on one day during the three-month experiment, and only during the most severe part of the cycle on that day, and *P. marinus* infections at the mid and end-points of the three month experiment; therefore, these effects may be occurring on different time scales and therefore not interacting as we had expected.

The treatments used in this experiment are representative of the range of conditions which are seen in the native range of the eastern oyster and might be found in shallow waters worldwide. Under current conditions, monitoring data suggest that few sites in the Chesapeake Bay experience daily periods of 0.5 mg L^{-1} DO and full pH unit cycles during the summer season (Breitburg 2002). These monitors are 0.3-0.5 meters off-bottom and may, therefore, underestimate the severity of bottom water conditions.

Furthermore, if eutrophication-driven phytoplankton blooms are not curbed such conditions may become more severe and more prevalent throughout shallow water areas during the summer months.

Periodic relief from stressors provided by cycling DO/pH may allow organisms to survive relatively brief periods of environmental conditions that are more severe than they could tolerate if exposure were continuous, and may in fact stimulate protective responses in organisms, although these may not always be effective. Eutrophication results in larger amplitude diel cycles and more extreme hypoxia, which may have harmful effects overwhelming any benefit of cycling conditions. These more severe conditions may also result in increased disease loads and epizootics, as shown by the greater proportion of animals scoring 2 or higher on the Mackin scale in this experiment. In this way, cycling conditions in one area may have widespread ramifications on a much larger geographic scale as increased higher-intensity infections result in higher disease loads to the environment. For these reasons, it is important to consider local environmental dynamics and their sub-lethal consequences, such as increased disease, when siting restoration, or setting water quality goals, to ensure that the maximum benefit in ecosystem services may be provided. It is also important to take these consequences into consideration when considering the importance of eutrophication as it is an important driver of cycle severity in near-shore shallow water systems. On the other hand, filter feeding organisms may ameliorate the negative impacts of eutrophication by reducing the magnitude and severity of algal blooms and simultaneously the amplitude of diel cycles; however restoration of filter feeders should not replace efforts at nutrient reduction

(Cerco and Noel 2007). For this reason, oyster restoration at sites in danger of severe algal blooms and large-amplitude cycles may be one step towards improving water quality.

Table 1. Mean±SE daily dissolved oxygen (DO), pH, and pCO_2 conditions in treatments at normoxia, at the end of the low-plateau phase, and at the end of the supersaturated plateau ('Supersat') phase. Measurements of DO and pH were made on 82 days in all six replicates of each treatment and at supersaturated-plateau ('Supersat') on six days in all six replicates of each treatment. Supersaturated measurements were only taken on six days due to logistical constraints. pCO_2 was measured by equilibration every minute for two hours during the low plateau one d wk^{-1} for six weeks due to constraints on number and functionality of equilibrators.

	DO (mg L^{-1})			pH			pCO_2 (ppm)
Treatment	Normoxia	Low-plateau	Supersat	Normoxia	Low-plateau	Supersat	Low-Plateau
Control	7.25± 0.02	7.36± 0.02	7.71± 0.11	7.81± 0.00	7.82± 0.01	7.93± 0.02	1043.2± 36.7
High DO-Cycling pH	7.24± 0.02	7.32± 0.02	7.68± 0.10	7.79± 0.01	6.98± 0.00	7.98± 0.02	7343.8± 606.1
Moderate DO-Cycling pH	7.25± 0.02	1.69± 0.01	10.20± 0.07	7.80± 0.00	7.02± 0.01	7.98± 0.02	6542.1± 380.6
Severe DO-Cycling pH	7.27± 0.02	0.57± 0.01	10.24± 0.07	7.81± 0.00	7.01± 0.00	7.97± 0.02	6583.8± 199.3
Severe DO-High pH	7.20± 0.02	0.56± 0.01	10.41± 0.02	7.79± 0.00	7.83± 0.00	7.92± 0.03	955.5± 49.3

Table 2. Mean±SE of (A) infection prevalence (proportion of total population assayed), (B) prevalence of infections scoring 2 or higher on the Mackin scale (proportion of population assayed), and (C) Mackin scale intensity of all individuals with detectable infections, after six weeks (Midpoint), twelve weeks (Endpoint), and nine month field deployment (Recovery).

A) Prevalence			
Treatment	Midpoint	Endpoint	Recovery
Control	0.089±0.025	0.262±0.017	0.210±0.036
High DO-Cycling pH	0.078±0.022	0.228±0.020	0.279±0.062
Moderate DO-Cycling pH	0.100±0.028	0.264±0.055	0.169±0.051
Severe DO-Cycling pH	0.194±0.043	0.507±0.049	0.041±0.080
Severe DO-High pH	0.235±0.023	0.567±0.023	0.277±0.058

B) Prevalence of 2+			
Treatment	Midpoint	Endpoint	Recovery
Control	0.033±0.000	0.065±0.015	0.043±0.031
High DO-Cycling pH	0.033±0.000	0.047±0.013	0.083±0.041
Moderate DO-Cycling pH	0	0.103±0.004	0.027±0.016
Severe DO-Cycling pH	0.033±0.000	0.209±0.027	0.067±0.035
Severe DO-High pH	0.034±0.001	0.185±0.022	0.066±0.030

C) Infection intensity			
Treatment	Midpoint	Endpoint	Recovery
Control	0.567±0.049	1.265±0.135	1.118±0.283
High DO-Cycling pH	0.604±0.166	1.009±0.130	1.158±0.258
Moderate DO-Cycling pH	0.554±0.041	1.182±0.097	0.979±0.086
Severe DO-Cycling pH	0.727±0.070	1.514±0.084	1.185±0.167
Severe DO-High pH	0.688±0.076	1.482±0.095	1.217±0.260

Table 3. Randomized complete block design ANOVA for (A) prevalence, (B) prevalence of infections scoring 2 or higher on the Mackin scale, and (C) infection intensity after 6 weeks of exposure to cycling conditions 4-5 d wk^{-1} (midpoint) and (D-E) the same three parameters after 12 weeks of exposure 4-5 d wk^{-1}. Tests are considered significant at a=0.05 and significant p values are bolded.

A) Prevalence at midpoint			
Source and Factor	df	F	p
Model	4	3.70	**0.022**
DO	20	3.41	**0.003**
pH	20	0.45	0.660
Interaction	20	0.58	0.572
Moderate hypoxia vs. normoxia	20	0.04	0.967

D) Prevalence at endpoint			
Source and Factor	df	F	p
Model	4	14.57	**<0.001**
DO	20	6.99	**<0.001**
pH	20	0.62	0.539
Interaction	20	0.77	0.449
Moderate hypoxia vs. normoxia	20	0.53	0.602

B) Prevalence of 2+ at midpoint			
Source and Factor	df	F	p
Model	4	0.65	0.633
DO	20	0.93	0.365
pH	20	0.13	0.896
Interaction	20	0.01	0.991
Moderate hypoxia vs. normoxia	20	0.68	0.504

E) Prevalence of 2+ at endpoint			
Source and Factor	df	F	p
Model	4	15.36	**<0.001**
DO	20	7.55	**<0.001**
pH	20	0.23	0.824
Interaction	20	1.25	0.225
Moderate hypoxia vs. normoxia	20	1.84	0.081

C) Infection intensity at midpoint			
Source and Factor	df	F	p
Model	4	0.69	0.608
DO	20	1.33	0.199
pH	20	0.42	0.679
Interaction	20	0.21	0.839
Moderate hypoxia vs. normoxia	20	0.39	0.701

F) Infection intensity at endpoint			
Source and Factor	df	F	p
Model	4	3.68	**0.021**
DO	20	3.28	**0.004**
pH	20	1.02	0.322
Interaction	20	1.65	0.116
Moderate hypoxia vs. normoxia	20	1.11	0.281

Table 4. Randomized complete block design ANOVA for (A) prevalence, (B) prevalence of infections scoring 2 or higher on the Mackin scale, and (C) infection intensity after retrieval from a nine month field deployment. Tests are considered significant at α=0.05 and significant p values are bolded.

A) Prevalence			
Source and Factor	df	F	p
Model	4	2.48	0.079
DO	19	2.5	**0.022**
pH	19	1.24	0.229
Interaction	19	0.19	0.854
Moderate hypoxia vs. normoxia	19	0.26	0.794

B) Prevalence of 2+			
Source and Factor	df	F	p
Model	4	0.54	0.708
DO	23	0.57	0.573
pH	23	0.71	0.484
Interaction	23	1.30	0.207
Moderate hypoxia vs. normoxia	23	0.33	0.743

C) Infection intensity			
Source and Factor	df	F	p
Model	4	0.17	0.949
DO	19	0.29	0.776
pH	19	0.11	0.916
Interaction	19	0.06	0.949
Moderate hypoxia vs. normoxia	19	0.58	0.571

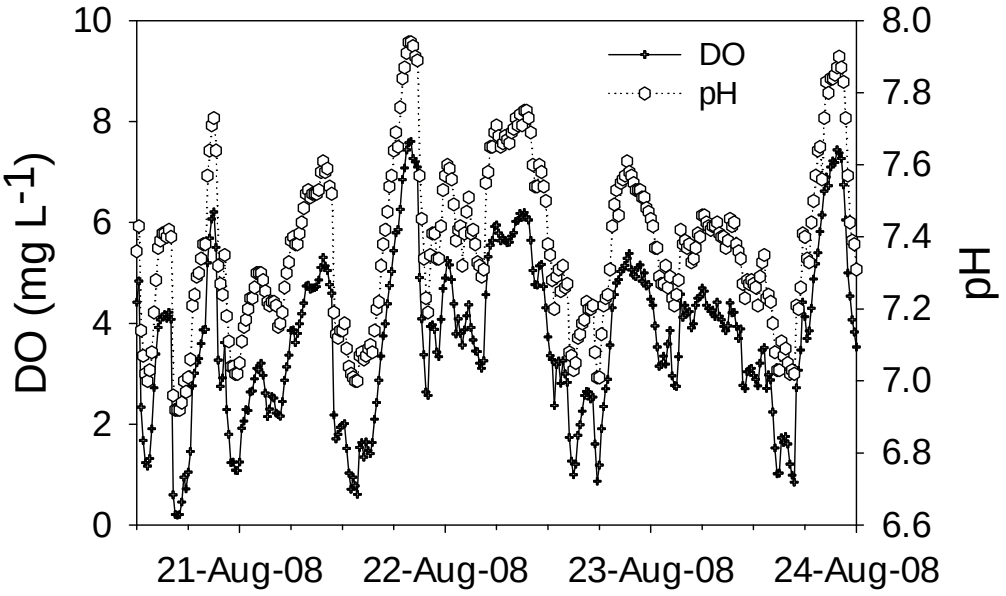

Figure 1. DO and pH in the St. Mary's River, MD, USA showing diel-cycles during circa one week in 2008. Data from MD-DNR Shallow Water Monitoring Program, eyesonthebay.net, station XCF1440, 38° 11.358' N, 76° 26.034' W.

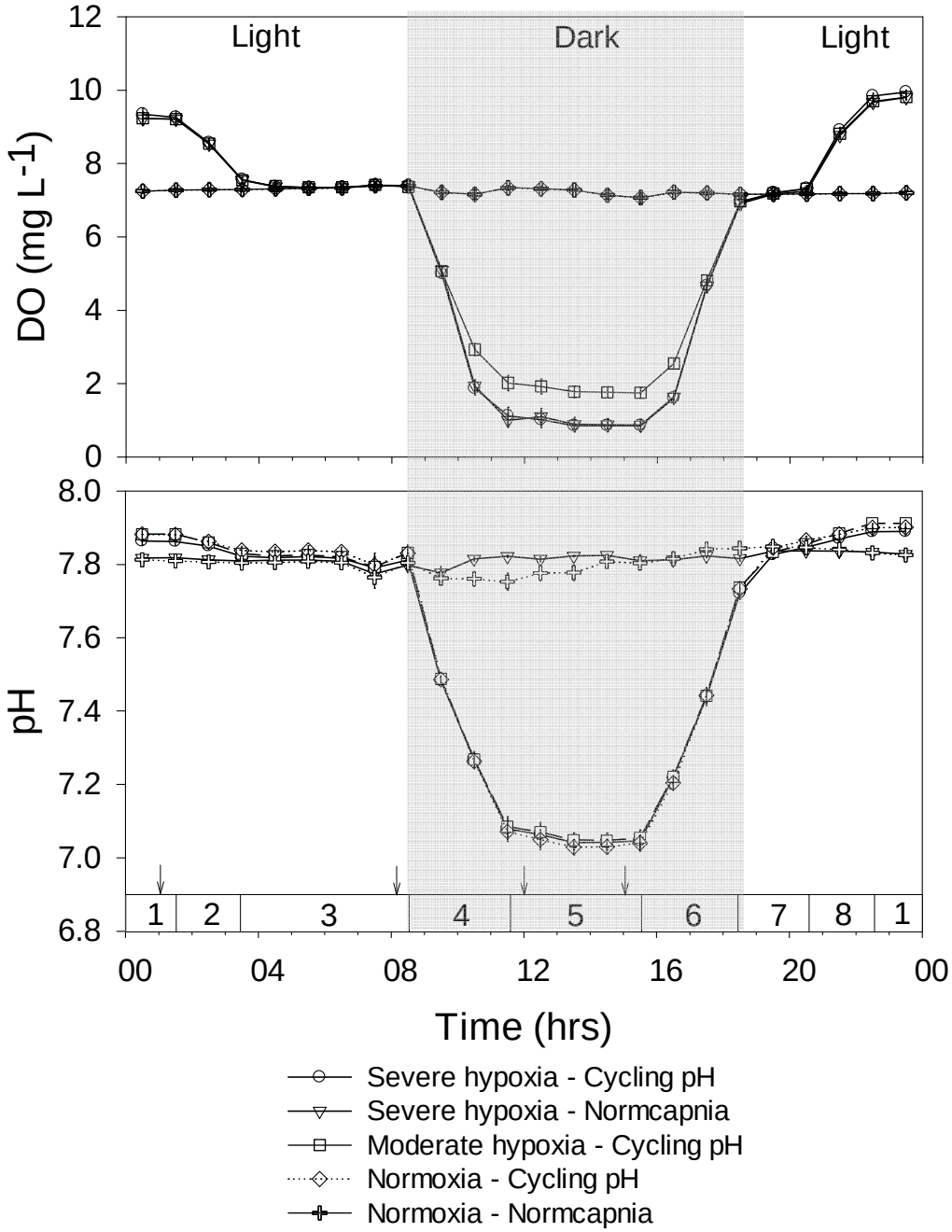

Figure 2. Mean ± SE (non-visible error bars are obscured by symbols) dissolved oxygen (DO) and pH during 51 days when conditions were cycled. Data are from the one replicate of each treatment measured by the LabVIEW control program. Arrows represents times at which DO and pH were measured in all aquaria. Numbers across the bottom represent cycle phases: 1-supersaterated-plateau, 2-down-to-normoxia, 3-normoxia, 4-ramp-down, 5-low-plateau, 6-ramp-up, 7-normoxia, 8-up-to-supersat.

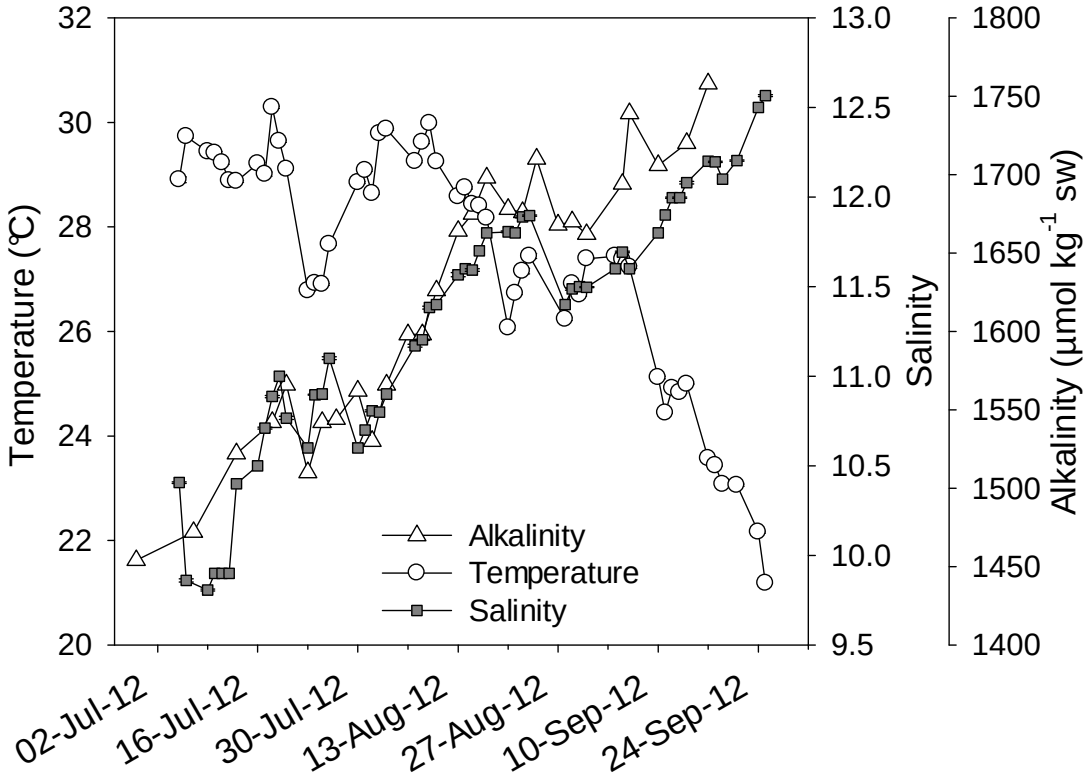

Figure 3. Mean ± SE temperature and salinity as measured in all aquaria each day during the low-plateau phase (51 days total), and alkalinity of the SERC river-water system as measured approximately 3 times per week at 0800H (25 measurements, non-visible error bars are obscured by symbols).

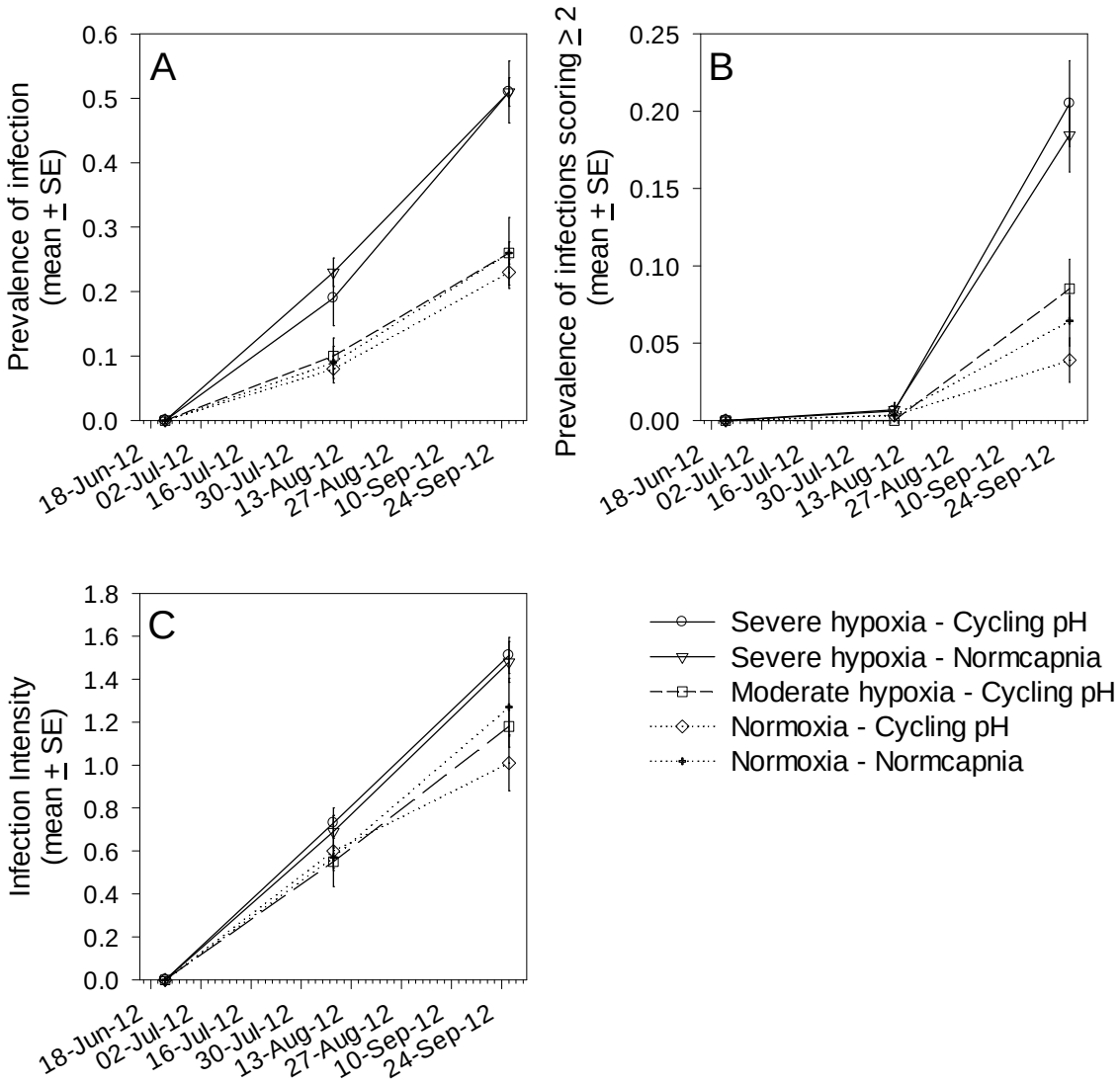

Figure 4. Mean ± SE of disease variables over the course of the laboratory experiment (mean of 6 replicates, 30 oysters from each replicate, non-visible error bars are obscured by symbols): (A) prevalence of infection (proportion of population assayed with detectable *P. marinus* infection), (B) prevalence of infections scoring ≥2 (proportion of population assayed with intensity scores of 2 or higher on the Mackin scale), and (C) infection intensity on the Mackin scale of detectable infections.

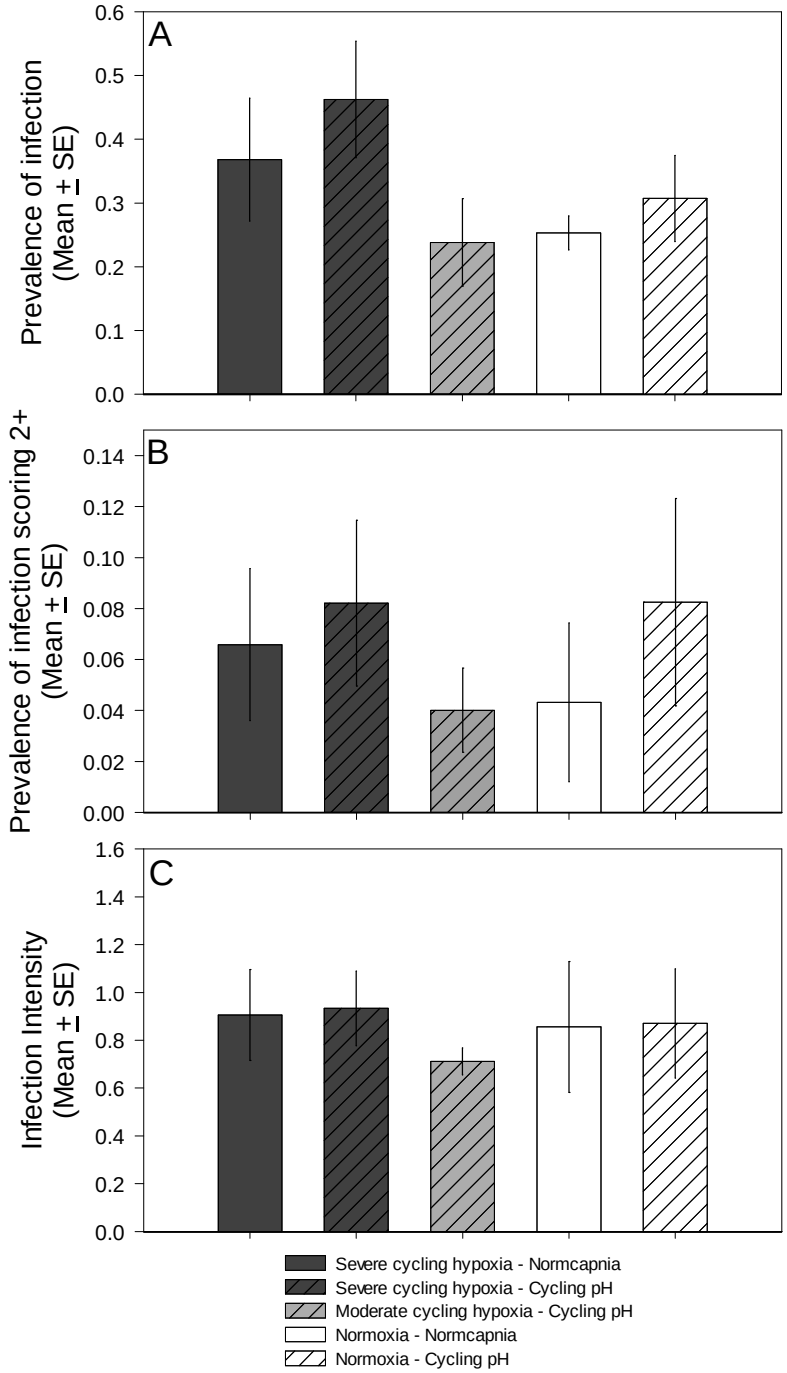

Figure 5. Mean ± SE of disease variables in oysters which were deployed in the field for nine months after the completion of the laboratory experiment and assayed in mid-July 2013 (mean of 6 replicates, approximately 17 oysters from each replicate, non-visible error bars are obscured by symbols): (A) prevalence of infection (proportion of population assayed with detectable *P. marinus* infection), (B) prevalence of infections scoring ≥2 (proportion of population assayed with intensity scores of 2 or higher on the Mackin scale), and (C) infection intensity on the Mackin scale of detectable infections.

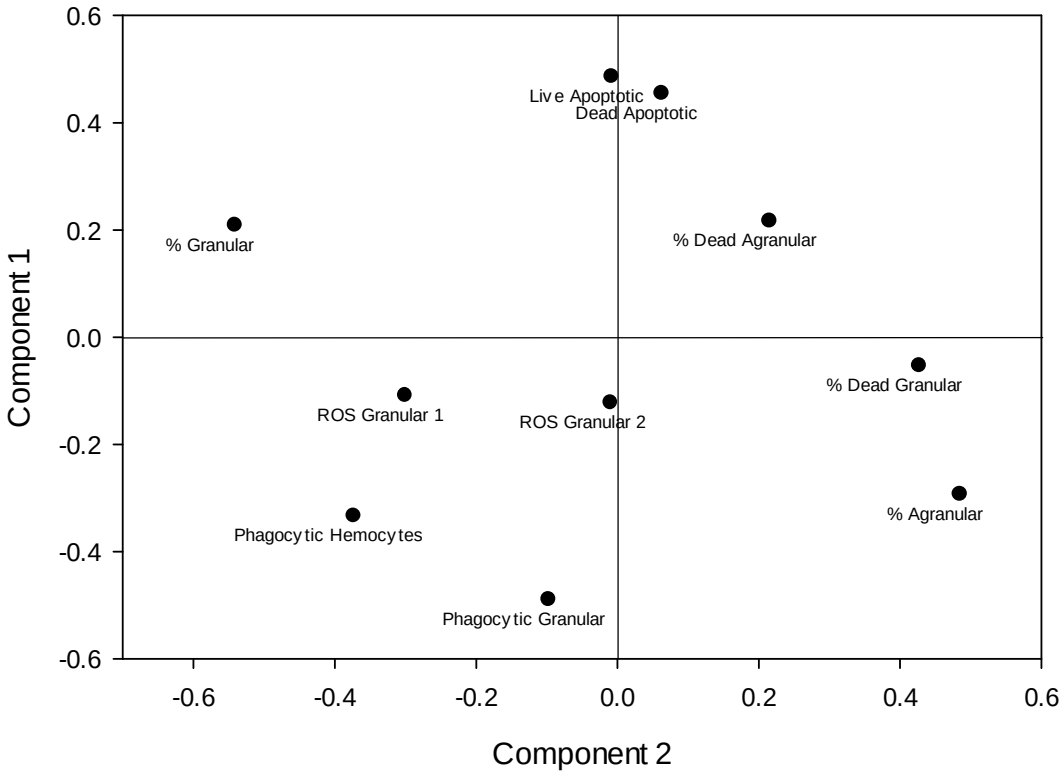

Figure 6. Principal Components Analysis of 10 hemocyte variables (see Materials and Methods) from 10 oysters in each of the following four experimental treatments: Normoxia, Normcapnia; Normoxia, cycling pH; Severe cycling hypoxia, Normcapnia; Severe cycling hypoxia, Cycling pH.

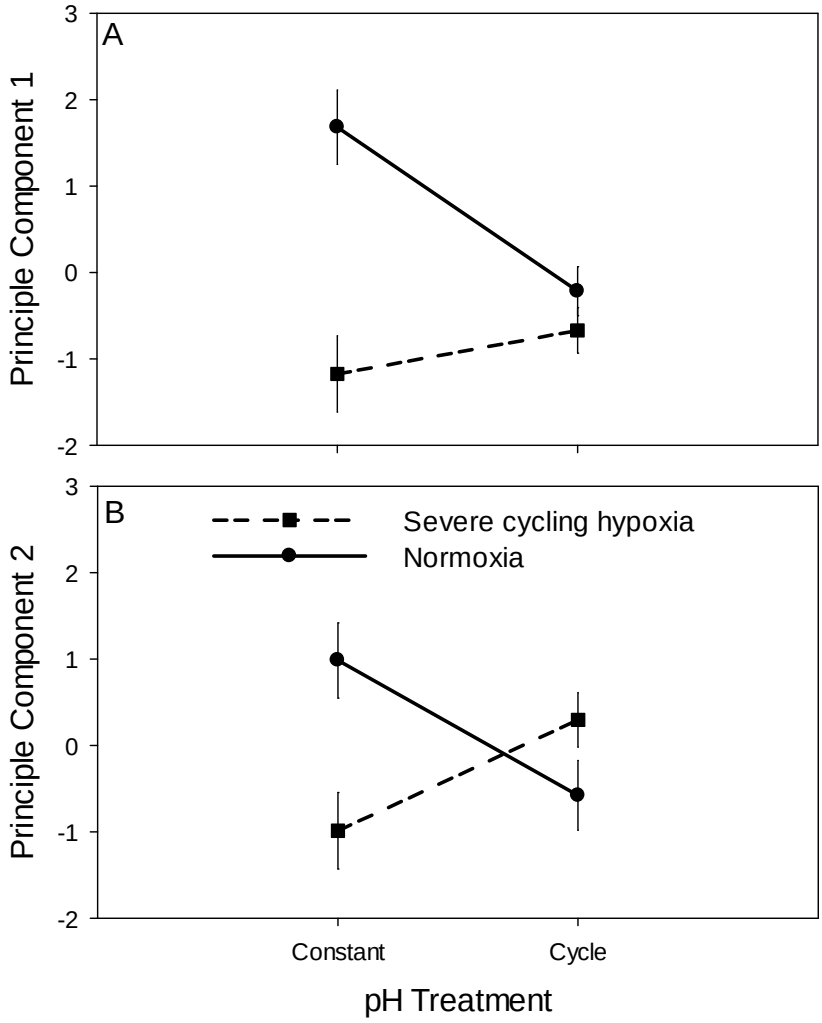

Figure 7. Mean and 95% least significant difference (LSD) intervals for A) Principle Components 1 and B) Principle Component 2 plotted for four dissolved oxygen (DO) and pH treatments.

Chapter II. Effects of co-varying diel-cycling hypoxia and pH on growth and feeding in the eastern oyster, *Crassostrea virginica*

Introduction Day-night patterns of photosynthesis and respiration occur naturally and, in combination with other environmental parameters, result in periods of hypoxia (low dissolved oxygen, or DO) and environmental hypercapnia (levels of CO_2 above those in equilibrium with the atmosphere resulting in low pH), as well as periods of supersaturation (oxygen levels above those at equilibrium with the atmosphere) and elevated pH in shallow water systems world-wide. The amplitude of these cycles can be increased by eutrophication, which results in higher biomass of photosynthetic organisms, microbes, and heterotrophs than typically occur under non-eutrophic conditions (Nixon and Oviatt 1973; Kemp and Boynton 1980; Tyler et al. 2009). In the eutrophic Chesapeake Bay, for example, pH values at some shallow water sites cycle one unit or more on a daily basis and DO levels can fluctuate from near anoxia to well above 100% saturated levels during a day (MDNR, 2013; Breitburg et al., submitted). In addition to the effects of increased metabolic CO_2 loads on pH in estuarine systems, increasing atmospheric CO_2 concentrations cause acidification (Melzner et al. 2013) and are predicted to increase the severity and duration of hypoxic events(Diaz and Rosenberg 1995; Rabalais et al. 2010) by increasing global temperatures (Diaz and Rosenberg 1995; Boynton et al. 1996; Rabalais et al. 2010).

Repeated exposure to brief periods of hypoxia and hypercapnia may be harmful to estuarine organisms in spite of adaptations to a wide range of environmental conditions (Eby et al. 2005; Tanner et al. 2006). The majority of previous research has focused on the effects of continuous hypoxia such as might be found in deeper bottom waters (e.g.

Rabalais et al. 2002; McNatt and Rice 2004; Brouwer et al. 2007) or continuous low pH (e.g. Miller et al. 2009; Waldbusser et al. 2011) and some studies have examined continuous exposure to the two stressors in combination (e.g. Melzner et al 2013; Boleza et al. 2001). Gobler et al. (2014) found additive and synergistic effects of continuous hypoxia and low pH on growth of larval scallops, *Argopecten irradians*. They found that acidification, but not hypoxia, reduced survivorship, while hypoxia but not acidification reduced growth. In addition, DO and pH had interactive effects on metamorphosis. Understanding the effects of co-varying hypoxia and pH on important members of shallow-water communities is vital to understanding the effects of eutrophication on estuarine systems as well as planning and protecting for future climate change.

Fewer studies have investigated the effects of hypoxia or hypercapnia under cycling conditions (Tyler et al. 2009; Bockmon et al. 2013), and replicating the two cycles under co-varying conditions is uncommon (but see Bogue 2013). Cycling conditions may have effects similar to those of continuous low conditions, or may affect organisms differently due to the rapid changes in DO and pH and the frequent periods of respite interspersed among the periods of potentially harmful conditions. Although mobile organisms will often relocate to avoid hypoxia exposure (Breitburg et al. 2003), Bell and Eggleston (2005) found reduced avoidance behavior in blue crabs, *Callinectes sapidus*, exposed to hypoxia associated with sudden upwelling events than in those exposed to long term hypoxic events. The authors attributed reduced avoidance to the rapid changes in DO associated with periodic upwelling. Taylor and Miller (2001) found that southern flounder, *Paralichtys lethostigma*, under diel-cycling hypoxia experienced similar

changes in hematrocrit levels to those under constant hypoxia; however, when growth rates were examined, flounder were found to acclimate to continuous hypoxia exposure, but not to cycling hypoxia.

Calcifying organisms are heavily dependent on the availability of calcium carbonate in the environment. Hydrogen ions produced by the dissolution of carbon dioxide in water bond with free carbonate ions and reduce their availability in the water column. Low alkalinity waters are more susceptible to pH changes than better buffered, higher alkalinity water. Extremely low pH results in calcium carbonate levels below saturation ($\Omega_{calcite} < 1.0$) and the dissolution of carbonate compounds (Feely et al. 2004). Even at values above but near the saturation point, calcification requires the expenditure of excess energy and calcification rates may be reduced. For example, calcification is reduced in the Pacific oyster, *Crassostrea gigas,* at calcite saturations of 3.1 and below (Gazeau et al. 2007). When calcite is under-saturated, not only must additional energy be expended to create shell, but calcium carbonate structures can dissolve, requiring compensation for dissolution as well as additional calcification to form shell. Low calcite saturation resulting from elevated CO_2 has been shown to reduce growth in larvae and adult eastern oysters in the lab and in the field (Miller et al. 2009; Barton et al. 2012). Olympia oysters, *Ostrea lurida*, raised under acidified conditions resulting in a pH ~7.8 did not have thinner shells, but were 29-40% smaller than control oysters reared at a pH of 8 (Sanford et al. 2014). Continuous hypercapnia resulting in a pH of ~7.5 increased the standard metabolic rates of eastern oysters, inhibited both shell and tissue growth, and increased mortality (Beniash et al. 2010). Under suboptimal conditions, Pacific oysters

preferentially put energy in to shell growth (Brown and Hartwick 1988; Jokela et al. 1997) to avoid predation and create a self-contained structure to avoid suboptimal external conditions (Shick et al. 1986; Riisgård et al. 2003; Sanford et al. 2014) so conditions that reduce shell growth or increase energetic cost of producing shell are likely to have severe effects on other processes affecting oyster survival and fitness.

In nature, diel-cycling DO and pH interact with other environmental conditions that may affect not only the severity of the cycles themselves, but may also moderate the way in which estuarine organisms respond. Increased temperature and salinity decrease oxygen solubility (Benson and Krause Jr 1984). Salinity and alkalinity are closely correlated due to the impacts on both by the presence and proportion of fresh water mixing (Lee et al. 2006). Low salinity and low alkalinity reduce the availability of calcite in aquatic systems (Millero 1979). It is difficult to tease apart main effects of hypercapnia versus low pH versus carbonate saturation due to the interactive nature of these three measurements in water (Gibson et al. 2011). Low salinity also reduces the assimilation rate of food in the Pacific oyster (Brown and Hartwick 1988). Conversely, food availability can influence the effects of DO or pH on organisms. For example, increased food availability can allow organisms to withstand the increased energy demands associated with acidification (Thomsen et al. 2013). When conditions approach the extremes of the natural range of an organism, metabolic processes are likely to be slowed or disrupted; for instance, lower salinity and temperature reduce growth rate in juvenile oysters (Osman 1994) and salinity and temperature can affect metabolism (Claireaux et al. 2000) and survival (McLeese 1956) in other marine organisms.

The sessile nature and economic and ecologic importance of *C. virginica* make them an excellent representative organism for studies of changes in estuarine water quality as they are unable to move to avoid periods of potentially harmful conditions, and anything negatively affects oysters is likely to have widespread impact on estuarine communities and the economies that depend on them. The eastern oyster is the foundation of a major fishery throughout the western Atlantic from Brazil to Canada in waters with salinity above 5 and temperatures below 32°C; although they may survive brief periods of conditions exceeding these bounds (Hargis and Haven 1999; Mann and Evans 2004). Oysters couple the benthic and pelagic environments, filter the water column, and engineer habitat (Grabowski and Peterson 2007). Overfishing, environmental degradation, and disease have resulted in severe population declines. For example, stocks in Chesapeake Bay are estimated to be below 1% of historic levels (Newell 1988; Wilberg et al. 2011). As with many other sessile organisms, oysters tend to be tolerant of hypoxia (Pörtner et al. 2005; Vaquer-Sunyer and Duarte 2010), but constant exposure to hypoxia has been shown to reduce feeding, metabolism, and growth (Widdows et al. 1989; Baker and Mann 1992; Burnett 1997). Hypoxia can also result in mortality of adult oysters, and change oyster reef community dynamics (Lenihan and Peterson 1998). As oyster larvae develop, they become more tolerant of hypoxic exposure (Widdows et al. 1989). Hypoxia does, however, reduce settlement and immediate post-settlement growth, and anoxia reduces settlement to near zero and stops post-settlement growth completely (Baker and Mann 1992). Exposure to diel-cycling hypoxia increases infection acquisition

and progression (Breitburg, et al., submitted; Chapter 1). Exposure to cycling hypoxia or cycling pH, as well as both cycles in conjunction, stimulates immune activity (Chapter 1).

The objective of this research was to examine the effects of diel-cycling DO and co-varying pH, as well as each stressor individually and under constant conditions, on growth and feeding of adult and juvenile eastern oysters, *Crassostrea virginica*. Although there is a plethora of pH and DO data available for shallow water environments in Chesapeake Bay (MDNR 2013); other carbonate chemistry parameters for these sites are not well measured. For this reason, we designed our experiment around pH targets; although we suspect that the availability of calcium carbonate is the primary driving force behind pH effects on oyster growth seen here.

Methods We tested the effects of diel-cycling DO and pH on growth and feeding of adult (1 year-old (yo)) and juvenile (≤ one month post-settlement, colloquially referred to as 'spat') eastern oysters at the Smithsonian Environmental Research Center (SERC), in Edgewater, Maryland, USA. Adult growth experiments were conducted during July-September 2012 and May-August 2013; experiments on the growth of several post-settlement ages of spat were conducted during the summers of 2012-2014. Adult growth for both years was measured as part of an experiment on the effects of diel-cycling hypoxia and pH on disease acquisition and progression and immune response (Chapter 1).

Five experiments were performed on either adult or juvenile oysters. Treatment names and mean DO and pH values at various parts of the cycle are described in Table 5. Briefly, the 2012 adult growth and juvenile growth experiments consisted of five cycling and control treatments with no constant hypoxia or hypercapnia treatments. In the 2013 adult growth experiment, a constant moderate pH treatment was added in order to help tease apart effects of cycling versus constant pH, and the DO target for the moderate cycling hypoxia treatment was adjusted from 1.5 mg L^{-1} to 1.3 mg L^{-1} to look for any threshold of DO at which effects might occur. An additional constant moderate hypoxia treatment at the same target DO level as the moderate cycling hypoxia treatment was added for the 2013 juvenile growth experiment. For the 2014 experiment, the target DO level of the constant hypoxia treatment was increased from 1.3 mg L^{-1} to 2.0 mg L^{-1}. In addition, all treatments were run at both ambient and supplemented chlorophyll levels to examine any interactive effect between food availability and effects of DO or pH.

Oysters were raised in replicate aquaria under DO and pH conditions controlled by a custom-developed LabVIEW (National Instruments Corp., Austin, TX, USA) based diel-cycling laboratory system described in Burrell et al. (submitted). Briefly, oysters were placed in 75 L aquaria and bubbled with a constant volume of gas comprised of N_2, CO_2, O_2, and either atmospheric or CO_2-stripped air, the ratios of which were varied using mass flow controllers (Dakota Instruments, Orangeburg, NY, USA) controlled by the LabVIEW program. One gas mix was created per treatment and then split equally among replicates. DO and pH were monitored in one replicate using Oxyguard Standard DO probes (Oxyguard International A/S, Birkeroed, Denmark) and Honeywell Durafet III pH sensors (Honeywell International, Morristown, NJ, USA). Because the LabVIEW program only has the ability to monitor and control 5 treatments, some non-cycling treatments were created separate from the program using flow meters and Saga pH-2002C Digital pH-ORP Controllers (Saga Electronic Enterprise Co., Ltd., New Taipei City, Taiwan).

During experiments, DO and pH cycled daily 4-6 d wk^{-1}. On days when conditions did not cycle, all treatments were bubbled with air and CO_2-stripped air to maintain DO and pH values similar to the control (constant normoxia/normcapnia) treatment. In the field, environmental conditions (winds, temperature, solar irradiance, etc.) can result in days on which hypoxia and environmental hypercapnia do not occur (Breitburg et al., submitted) which would be similar to the non-cycling days in these experiments. Normoxia is defined as oxygen saturation levels in equilibrium with oxygen in the atmosphere and normcapnia, for the sake of these experiments, is defined as pCO_2 levels resulting in a pH

between 7.8 and 8.1. Incandescent 5V rope-lighting was used to replicate light levels at a depth of two meters in the Rhode River on a sunny day: Photoperiod regime was maintained in a 14:10 light:dark cycle 7 d wk^{-1}.

To determine whether all replicates were similar to those being controlled by the LabVIEW system, DO, temperature, and salinity were measured 3 to 4 times per day in all replicate aquaria using a YSI ProfessionalPlus (Yellow Springs Instruments, Yellow Springs, OH, USA) and pH was measured at the same times using an Oakton Acorn pH 5 meter (Oakton Instruments, Vernon Hills, IL, USA).

Adult growth

All 1yo oysters were purchased from Marinetics, Inc., an aquaculture facility on the Choptank River, MD, USA. Oysters were purchased early in the season to avoid the onset of summertime hypoxia or hypercapnia in the Choptank and held under well-oxygenated flow-through conditions at SERC until the start of experiments. Adult oysters were individually labeled, measured to the nearest millimeter from hinge to bill on the right valve using a flexible ruler following shell contours, and weighed, after which 90 oysters were placed in 75 L aquaria (6 replicates of 5 treatments in 2012, 30 total aquaria; 6 replicates of 6 treatments in 2013, 36 total aquaria). Mean $\pm$ SE and minimum and maximum shell heights at the start of each experiment are presented in Table 6. Treatments were arranged in a randomized block design with one replicate from each treatment clustered together to account for room position in analysis of results.

Oysters were allowed to acclimate to laboratory conditions for four to six days before experiments began.

In order to measure mid-point growth rates, a haphazard subset of 30 oysters was removed from each aquarium after six weeks of the 2012 experiment and measured. At the end of the experiment, after twelve weeks, 30 additional oysters were removed from each aquarium and shell heights were measured. Any mortality among remaining oysters was noted and empty shells were discarded. Seventeen oysters from each tank were deployment from three piers owned by SERC on the Rhode River for later analysis of recovery. Oysters were deployed hanging from piers 0.5 m above the bottom in approximately 2 m of water to avoid periods of air exposure or bottom water hypoxia. Oysters from individual tanks were placed approximately 2 m apart to reduce disease transmission between oysters from individual tanks as these oysters were also part of a disease acquisition and progression experiment (Chapter 1). Three sites were necessary to find enough dock area to space out oysters. Although we describe field deployment as a respite from diel-cycles, field conditions are certainly not as stable as treatments under laboratory control; however we believe that field conditions were less severe than any of our laboratory cycling conditions (Hondorp et al., unpublished data), and all treatments would have been exposed equally to field conditions. Laboratory blocks were continued in the field, with two replicates going to each of two sites, and the fifth replicate deployed at the third site. In July of 2013, field-deployed oysters from the 2012 experiment were collected, measured, and weighed again.

In 2013, 30 oysters were removed from each tank after four weeks and shell height was measured. In addition, a tissue sample was taken from 15 of these oysters for analysis of fecundity (Steppe et al., unpublished data). After ten weeks, 30 additional oysters from each tank were measured, weighed, and then frozen for further processing. Any mortality was noted in the remaining oysters. Ten oysters from each aquarium were later defrosted and tissue was carefully separated from shells, dried at 65°C for 48 hours, and then weighed to the nearest 0.0001 g. The 2013 adult growth experiment oysters were also exposed to *P. marinus* spores throughout the experiment although salinity was below that at which Dermo epizootics typically occur.

In 2012, each aquarium received 1 L min^{-1} of flow-through, unfiltered, Rhode River water supplemented with 0.093 mL of stock algal diet (DT's Reef Blend, http://www.dtplankton.com/) mixed into the inflow water every 8 minutes, 24 h d^{-1}, throughout the experiment except for a 10 d period during the second half of the experiment while the system was under repair. In 2013, each aquarium received 0.5 L min^{-1} of flow-through, unfiltered, Rhode River water supplemented continuously with 0.088 mL min^{-1} of stock algal diet.

Juvenile growth

Eyed larvae were obtained from Horn Point Oyster Hatchery (Cambridge, MD, USA) for all juvenile growth experiments. Larvae were returned to SERC and placed in 0.25 m^3 raceways with roughened 12.7x12.7x0.5 cm PVC (2012) or ABS (2013-2014) tiles in 0.54 µm filtered Rhode River water modified using Coralife Scientific Grade Marine Salt

(Coralife, Central Aquatics, Franklin, WI, USA) to match the salinity at which larvae had been hatched. After three days, raceways were put on 0.54 µm filtered flow-through Rhode River water, and fed intermittently with stock algal diet. Larvae were set four weeks prior to the experiment's start in 2012, four weeks, two weeks, and one week prior to the experiment in 2013, and three weeks prior to the commencement of experiments in 2014. Mean $\pm$ SE, minimum and maximum shell area of spat at the start of experiments are presented in Table 6.

At the start of the juvenile growth experiments, all tiles were removed from settlement raceways, photographed, and 1-3 tiles per age class were placed into each of 30 (2012), 35 (2013), or 48 (2014) 75 L experimental aquaria on July 26, 2012, August 29, 2013, and May 29, 2014. Multiple tiles were used in cases where settlement was not dense enough to achieve target numbers of individuals per tank with single tiles. Tiles were oriented vertically with the bottom edge several centimeters above the bottom of the tanks in order to avoid sedimentation on top of juvenile oysters. In 2013, the three age classes of spat were all placed in the same experimental units. A randomized block design was used clustering one replicate from each treatment together to account for room position in analysis of results. Photographs were used to measure spat area using image analysis software (ImageJ, v. 1.37, National Institutes of Health, USA). In some cases (youngest age class in 2013 and all individuals in 2014), oysters were too small to be efficiently measured by photographing tiles. In these cases, a subset of the cohort was measured and found to be $\leq$ 1 mm^2. Spat were allowed to acclimate to water flow, light levels, and feeding regimes in experimental aquaria for four days at

normoxia/normcapnia, after which treatment cycles commenced. Treatments were cycled 4-6 d wk^{-1} for 4 weeks (2012), 5 weeks (2013), or 2 weeks (2014). Constant treatments were maintained continuously for the length of the experiment.

The 2012 spat were placed in the experiment with the adults described above. Because adults reduced algal concentrations in aquaria, and experimental treatments affected adult filtration rates, food availability likely varied among treatments: during hypoxic exposure, phytoplankton availability would have been higher, but lower during other parts of the cycle. The 2013 experiment did not include adults, and each aquarium received 0.3 L min^{-1} of Rhode River water supplemented with 0.088 mL min^{-1} of stock algal diet. In 2014, each aquarium received 0. 3 L min^{-1} of Rhode River water, aquaria in the supplemented food treatment received 0.109 mL min^{-1} of stock algal diet continuously.

All tiles from the 2012 and 2013 spat experiments were photographed again at the mid- (August 7, 2012, September 17, 2013) and end-points (August 27, 2012, October 8, 2013) of each experiment. Oysters from the 2014 spat experiment were not analyzed at the mid-point due to the brief duration of the experiment but were analyzed after two weeks, at the endpoint of the experiment (June 10, 2014). At the midpoint of the 2013 experiment, oysters of the youngest age class were thinned haphazardly to 6 individuals per replicate aquarium to avoid overcrowding. All tiles were then processed using image analysis software with the same methods as those at the start of experiments. Any mortality was noted at the end-point of each experiment.

After endpoint sampling in 2012, spat tiles from each aquarium were deployed in the Rhode River along with the corresponding adult oysters. Spat were collected after nine months of field deployment and again analyzed for size.

Other Measurements

During all 2013 and 2014 experiments, we estimated the relative amount of food available in each aquarium by *in-vivo* fluorescence measurement of chlorophyll levels in aquaria using a Turner Designs 10-AU Fluorometer (Turner Designs, Sunnyvale, CA, USA). Water samples (50 mL) were removed from aquaria at mid-depth by transfer pipette, placed in blackened containers, and processed immediately. Samples were taken at the end of hypoxia/hypercapnia (simulated dawn), return to normoxia/normcapnia (simulated mid-morning), end of the supersaturated oxygen period (simulated mid-afternoon), and end of normoxia/normcapnia (simulated dusk). Samples were taken on eleven days at the end of hypoxia/hypercapnia, six days at return to normoxia, five days at supersaturation, and three days at the end of normoxia/normcapnia. We were able to use the relative chlorophyll abundances as a proxy for differences in feeding among treatments in the 2013 experiment. Since this was done in flow-through aquaria, it is necessary to point out that chlorophyll (Chl*a*) measurements are in fact integrating a period of time as in-flow rates cause aquarium volumes to turn over approximately every two and a half hours, and inflow Chl*a* levels vary over time with ambient Rhode River conditions. Any phytoplankton removed by the oysters were being actively replaced by

the inflow water, but some of the water in the tanks was available for filtration up to 2.5 hours prior to time of sampling.

Alkalinity was measured thrice weekly during all experiments in order to calculate calcite saturation states using CO_2SYS.XLS (Pelletier et al. 2007). Samples were filtered to 0.45 µm and kept at 4°C until processing. In 2012 alkalinity samples were processed according to Standard Methods 2320 (American Public Health Association 1992), and in 2013 and 2014 according to the Guide to Best Practices for Ocean CO_2 Measurements (Dickson et al. 2007).

Statistics

Shell heights and total oyster weights of adult oysters and shell areas of juvenile oysters were used to calculate instantaneous growth rates. In cases where initial measurements could not be made (youngest age class of spat in 2013 and all spat in 2014), starting size was assumed to be 1 mm^2 for the sake of calculations. Statistics for experimental parameters only measured at one time were performed on means within aquaria. Unless otherwise noted, data are presented as means $\pm$ standard error. Any differences referred to as significant are significant at $p=0.05$.

All data were tested for homogeneity of variance using an F-max test and normality using a Shapiro-Wilkes test. All statistical analyses were performed in the proc mixed procedure in SAS (SAS Institute Inc., Cary, NC, USA). Effects of cycling treatments on mortality were examined for every experiment performed using a randomized complete block design (RCBD) ANOVA with laboratory position as the blocking factor. When

starting sizes were available, effects of DO and pH on growth rates during the laboratory experiments were analyzed as RCBD ANCOVAs with laboratory position as the blocking factor and starting size as the covariate. Growth rates during the first and second halves of each experiment as well as the overall growth rate were analyzed to allow for examination of differences in growth rates among treatments during different time periods, which might indicate acclimation to- or compensation for- exposure to experimental conditions. In the case of adult oysters sampled destructively, and juvenile oysters too small to individually identify at the start of experiments, individuals could not be tracked through all experimental periods. Growth rates during the recovery portion of experiments (2012 adult and spat growth) were also analyzed as ANCOVAs using shell height or spat area at time of deployment as the covariate. For the 2014 spat growth experiment, results were first analyzed using a two-way ANOVA testing for an interactive effect of food treatment with cycling treatments. Since the interaction was not significant, results were analyzed as an RCBD ANOVA with feeding treatment as the blocking factor in order to focus the analyses on detection of DO and pH treatment effects. Since chlorophyll was measured in all replicates on multiple days, chlorophyll content was analyzed using a repeated measures ANOVA.

Least square means contrasts were used to test for interactive effects of severe cycling DO and cycling pH as well as *a priori* hypotheses that cycling DO and cycling pH would reduce growth and feeding. *A priori* hypotheses that constant conditions would reduce growth and feeding as compared to the controls and that constant conditions would not differ from similar cycling conditions were also tested using least square means

comparisons. Pre-planned comparisons were performed regardless of overall test significance (Keppel 1991).

Results Effects of diel-cycling hypoxia and pH, and the interaction of the two on growth of eastern oysters varied from year-to-year but, particularly in combination with other environmental stressors, cycling conditions had the potential to reduce growth. Five experiments, conducted over the course of three summers, resulted in a range of ambient environmental conditions in addition to the experimental stressors. Dates of experiments and water quality parameters not manipulated in these experiments are presented in Table 7. Salinity and alkalinity were allowed to vary with ambient conditions in the Rhode River, as was temperature in 2012 and 2013. Due to the earlier experimental dates in 2014, incoming water was warmed to keep temperature close to that of previous experiments. Temperature during the 2012 experiment was warmer than during the other three experiments, but all were within the natural range of the eastern oyster (Mann and Evans 2004).

Salinity during the 2013 adult growth and 2014 spat growth experiments was lower than during the other experiments (Table 7), and was at the low end of the natural range for *C. virginica* (Mann and Evans 2004). The lowest recorded salinity during the 2013 adult growth experiment (5.68) was still in the nominal range of eastern oysters (Mann and Evans 2004) but well below the optimal range for eastern oysters in the state of Maryland (Shumway 1996). The mean salinity during the course of the experiment was lower than the lowest salinity recorded during the 2012 adult growth experiment. This lower salinity and alkalinity resulted in lower mean calcite saturation states in these experiments than in earlier experiments (Table 8). Mean calcite saturation during the 2013 adult experiment was just below saturation whereas mean calcite saturation in the 2012 experiment was

just above saturation. These conditions resulted in lower growth rates in the 2013 adult experiment than the 2012 adult experiment. Juvenile oysters grew at similar rates during the first two weeks of each experiment, perhaps indicating the prioritization of shell growth in spite of suboptimal conditions. Mean, standard error, minimum, and maximum shell height for adult oysters and shell area for juveniles are presented in Table 6.

Mortality

There were no differences in mortality among treatments during any of the laboratory experiments (Table 9). Mortality per treatment in the adult experiments ranged from 1.3% to 3.5%. Spat mortality per treatment ranged from 0.0% to 18.0%.

Adult growth

Adult oysters grew very little during the laboratory experiments but exhibited significant differences in growth in one of the two experiments. In 2012, adult oysters added an average of 5.64 mm in shell height during the laboratory experiment. Instantaneous shell growth rates were not significantly affected by either severe or moderate cycling hypoxia nor were they significantly affected by cycling pH conditions (Table 10, Fig. 8A, B). During a nine month field deployment, the oysters added an average of 18.83 mm of shell height. Prior exposure to cycling conditions had no latent effects on growth rates during the field deployment (Table 10, Fig. 8C).

Under slightly lower salinity/alkalinity conditions during 2013, adult oysters grew an average of 3 mm in shell height. Control oysters ended the tallest at 50$\pm$0 mm and the

normoxia/cycling pH treatment oysters ended the shortest at 49±0 mm. In spite of these small differences in ending size, there were statistically significant differences in growth rates among treatments over the course of the entire experiment. During the first 40 days of exposure, there were no differences in growth rate among treatments (Table 11, Fig. 9A). Over the course of the entire experiment, however, there was a significant interaction between the effects of severe cycling hypoxia and cycling pH on instantaneous growth rate. Shell growth was significantly reduced by cycling pH only under normoxic conditions, while cycling hypoxia reduced growth only under normcapnia (Table 11, Fig. 9B). Adult oysters exposed to co-varying severe cycling hypoxia and cycling pH grew at the same rate as control oysters. Constant moderate pH significantly reduced shell growth rates by 20% when compared to constant normoxia (Table 11, Fig. 9B). Growth rates under moderate cycling hypoxia were not significantly different from either constant normoxia or severe cycling hypoxia. There were no significant differences in dry tissue mass among treatments (Table 11, Fig. 9C).

Juvenile growth

Juvenile oysters grew substantially under laboratory conditions, and exhibited a variety of responses to cycling conditions. In 2012, spat grew an average of 428.3 mm^2 during the laboratory exposure. Juvenile oysters exposed to repeated, brief periods of severe hypoxia had significantly lower rates of growth in shell area (by nearly 10%) within the first two weeks of exposure (Table 12, Fig. 10A). Juvenile oysters exposed to moderate

cycling hypoxia also grew significantly slower than oysters exposed to normoxia during the first two weeks (Fig. 10A).

During the second two weeks of exposure in 2012, growth rates were lower than during the first two weeks, and there was an interactive effect of cycling hypoxia and pH. Oysters grown under co-varying diel-cycling hypoxia and pH exhibited compensatory growth, with significantly higher growth rates than oysters grown under normoxia and normcapnia (Table 12, Fig. 10B). Oysters exposed to moderate cycling hypoxia also exhibited compensatory growth and had significantly higher growth rates during the second two weeks than those of oysters exposed to normoxia during this time period (Table 12, Fig. 10B). During the second half of the experiment, severe cycling hypoxia under normcapnic conditions continued to reduce growth compared to the control treatment. Cycling pH had no effects on growth rate under normoxia during this time period. Over the course of the entire experiment, all oysters grew at similar rates except those exposed to severe cycling hypoxia, especially in combination with normcapnia (Table 12, Fig. 10C).

During a nine month period of deployment in the Rhode River, oysters added an additional 918.1 mm^2. Oysters that were previously exposed to severe hypoxia grew significantly faster than those that had experienced constant normoxia or moderate cycling hypoxia (Table 12, Fig. 10D). Cycling pH did not have any latent effects on growth rate during this period (Table 12). Mean spat area at the end of the recovery

period was 1348.3 mm^2 and shell areas were similar regardless of pH or DO treatments during the laboratory experiment (Table 8, 12).

In 2013, three separate age classes of spat, oysters settled 4-weeks, 2-weeks, and 1-week prior to the commencement of experiments, were grown under cycling conditions. The oldest spat grew an average of 517.5 mm^2, and instantaneous growth rates were not significantly affected by any cycling treatment (Table 13, Fig. 11 A,D,G). The juveniles that settled 2-weeks prior to the experiment added an average of 431.0 mm^2 of shell area over the course of the experiment. These oysters grew significantly more slowly under severe cycling hypoxia than under normoxia during the first two weeks of the experiment but not during the second two weeks (Table 13, Fig. 11B,E). However, neither moderate cycling hypoxia nor cycling pH significantly affected growth rate of these juvenile oysters during any portion of the experiment (Table 13, Fig. 11 B,E,H). The juveniles that settled 1-week prior to the experiment grew an average of 330.2 mm^2 in shell area over the course of the entire experiment. These juveniles displayed a trend towards reduced shell area under severe cycling hypoxia at the midpoint of the experiment, but there were no significant effects of moderate cycling hypoxia or cycling pH on growth (Fig. 11C). There were also no differences in growth among DO or pH treatments during the second two week period (Table 13, Fig. 11F). Over the course of the entire experiment, there was a trend towards reduced growth under severe cycling hypoxia, but no significant effects of any other cycling treatment (Table 13, Fig. 11I). All three age classes of juveniles experienced a 30-50% reduction in growth rate at a constant DO of 1.27 mg L^{-1} during the month-long course of the experiment (Table 13, Fig. 11G,H,I).

Salinity during the 2014 spat experiment was at the extreme low end of the eastern oyster's native range as a result of spring and early summer precipitation patterns. This relatively fresh water was also very low in alkalinity. These oysters may therefore have experienced carbonate stress even in normcapnia treatments (Fig. 12, Table 6, 7). Spat grew an average of 11.1 mm^2 over the two week course of this experiment. Although the oysters were three weeks post-settlement at the start of the experiment and had been kept under well aerated conditions, they were still $\leq$ 1 mm in shell area when placed into experimental aquaria (Table 8). Supplementing aquaria with a stock algal diet significantly increased growth rates of spat; however the difference between oysters receiving supplemented and ambient food was only on the order of one square millimeter and there were no interactive effects of food availability with DO/pH treatment (Table 14). Food level was therefore used as a blocking factor for further analysis in order to focus on DO and pH treatment effects. After two weeks of exposure to cycling conditions, there was a significant interaction between severe cycling hypoxia and cycling pH. Cycling pH reduced spat growth rates under normoxic conditions and hypoxia reduced growth under both normcapnia and cycling pH similarly (Table 14, Fig. 12). Constant mild hypoxia significantly reduced growth rate by 15% compared to constant normoxia. Juvenile oysters exposed to constant mild hypoxia and cycling moderate hypoxia grew at similar rates (Table 14).

Filtration

There was not a significant interaction between severe cycling hypoxia and cycling pH on chlorophyll levels in treatment aquaria at any part of the diel cycle during the 2013 adult growth experiment (Table 15, Fig. 13). Chlorophyll levels were 33% higher in aquaria containing adult oysters under severe hypoxia than in aquaria maintained continuously at normoxia: chlorophyll levels were 20% higher in aquaria under moderate hypoxia than normoxia (Table 15, Fig. 13A). During the normoxia and supersaturated oxygen portions of the cycle, chlorophyll was significantly higher in aquariums that had been exposed to hypoxia in the previous 24 hours than in those exposed to constant normoxia (Table 15, Fig. 13B,C,D). These differences were slight during the normoxic portions of the cycle, but, during the supersaturated portions of the cycle, chlorophyll levels were 15% lower in tanks previously exposed to hypoxia than in tanks continuously exposed to normoxia (Table 15, Fig. 13C). Chlorophyll levels in aquaria exposed to brief daily periods of moderate hypoxia were not significantly different during normoxic or supersaturated oxygen portions of the cycle from those in aquaria maintained at normoxia (Table 15, Fig. 13B,C,D).

In-tank chlorophyll was significantly reduced during exposure to low pH when compared to aquaria not exposed to low pH (Table 15, Fig. 13A). This difference appears to be driven primarily by the difference in filtration between cycling pH and normcapnia at hypoxia. There were no pH effects on chlorophyll levels during the normoxia or supersaturated parts of the cycle when pH would have been at normcapnia (Table 15, Fig.

13B,C,D). Constant moderate pH did not affect in-tank chlorophyll content compared to chlorophyll content in normcapnic aquaria (Table 15, Fig. 13).

No significant differences in chlorophyll were observed among treatments in the two spat growth experiments during which in-tank chlorophyll were measured (data not shown). It is unlikely that spat filtration rates were sufficient to measurably affect chlorophyll levels.

Discussion Results of this study indicate that exposure to diel-cycling conditions consisting of brief repeated periods of hypoxia and low pH can reduce adult and juvenile eastern oyster growth rates and affect adult filtration rates (Table 12). Oysters exposed to cycling conditions reduced feeding during hypoxic periods but increased feeding under high oxygen or low pH portions of the cycle, potentially compensating for some of the reduced feeding during hypoxia (see also Clark (2014)). Results also indicate that juvenile oysters can acclimate to, or compensate for, early reductions in growth either while still exposed to cycles or once removed from laboratory conditions. When the DO and pH cycles co-varied, effects on oysters were sometimes less than those of either cycle independently. For example, in 2013, adult oysters had very slightly but significantly reduced growth under diel-cycling hypoxia or pH, but the two in combination had no effects on growth. Inter-annual variation in salinity, as well as the timing of experiments, and oyster age may have modulated the effects of cycling hypoxia and pH on oysters, resulting in variation in the presence or magnitude of effects in different experiments. Although oysters were remarkably tolerant of cycling conditions, reduced growth and modified feeding patterns that were detected may alter the ecosystem services provided by oysters and have the potential to affect ecosystem functioning in shallow-water systems.

Filtration

Although these experiments were designed primarily to evaluate the effects of diel-cycling hypoxia and pH on oyster growth, the measurements of in-tank Chl*a* under flow-

through conditions can be used to infer information on feeding rates of oysters in cycling conditions. To do so, it is assumed that differences among time periods and treatments in Chl*a* reflect differences in filtration and ingestion by oysters.

In-tank Chl*a* was 30% higher on average under severe hypoxia than under normoxia, indicating decreased filtration by 1yo oysters. Clark (2014) also found reduced filtration in eastern oysters during periods of hypoxia. Shell closure in response to hypoxia (Shick et al. 1986; Riisgård et al. 2003) may result in reduced filtration under low DO concentrations. Reduced filtration during exposure to hypoxia was at least partially compensated for during periods of normoxia and supersaturated oxygen when Chl*a* levels in cycling tanks were lower than those exposed to constant normoxia. Previous work has also shown that oysters exposed to cycling conditions may increase filtration after DO concentrations return to normoxia to compensate for a preceding period of hypoxia (Clark 2014). Supersaturated oxygen alone does not increase oyster filtration rates (Clark 2014), but did increase filtration when periods of supersaturation occurred as part of a diel cycle. Clark (2014) suggested that oysters exposed to hypoxia accumulate an oxygen debt that is repaid at high oxygen. Therefore, the increased filtration observed under saturated and supersaturated DO concentrations may be a byproduct of the increased pumping necessary to repay oxygen debt (De Vooys and De Zwaan 1978; Stickle et al. 1989). This ability to compensate for reduced feeding under hypoxia at periods of high oxygen is a fundamental difference between cycling DO and constant hypoxia, and may impact the energy available to exposed organisms.

Adult oysters exposed to cycling low pH conditions filtered slightly more than oysters maintained at high pH, similar to the effect of pH seen under constant low oxygen and cycling pH in Clark (2014). The mechanism behind the stimulation of oyster filtration by cycling low pH is unclear. Previous research has shown higher metabolism in oysters exposed to a constant pH of 7.5 (Beniash et al. 2010), similar to that of our constant moderate pH treatment (~7.45) perhaps requiring increased energy uptake. However, our study showed no effects of constant moderate pH on filtration. This may indicate that the increased filtration we observed was stimulated by some aspect of the lower pH value used in our cycling treatments. In many species, elevated CO_2 concentrations are the stimulus for increased respiration (Bainton et al. 1978; Pörtner et al. 2004). The increased pumping associated with increased respiration may result in the increased filtration observed at lower pH. Whatever the cause, stimulated feeding during the low pH portion of pH cycles may help explain the higher growth rates in adult oysters exposed to cycling hypoxia and cycling pH as compared to those experiencing cycling hypoxia alone.

Ambient Conditions

The effects of low salinity and corresponding low alkalinity, as well as resulting low calcite saturation, almost certainly increased the susceptibility of oysters to the harmful effects of diel-cycling hypoxia and pH in these experiments. Due to the complexity of the natural environment, no organism will be exposed to a single stressor in isolation (Breitburg et al. 1998; Folt et al. 1999; Heugens et al. 2001). Results of this study

indicate that diel-cycling hypoxia and pH can affect growth in both juvenile and adult eastern oysters, but that the presence or magnitude of any effects may be influenced by other environmental variables.

Low calcite saturation states result in the dissolution of carbonate compounds (Dove and Sammut 2007; Fabry et al. 2008), increase energy requirements for shell production (Miller et al. 2009; Gazeau et al. 2010), and may have contributed to growth differences between experiments. In this study, adult oyster shell growth was slightly reduced when challenged continuously by low calcite saturation state conditions in the continuous moderate pH treatment in 2013, and in cycling pH treatments when pH and thus calcite saturation were lowest.

Under 2012 conditions, adult oysters showed no differences in growth whether exposed to normoxia and normcapnia. The 2013 adult growth experiment was performed under slightly lower salinity and alkalinity, slightly lower temperature, and increased phytoplankton supplementation (Table 6, 7) and produced significant negative effects of both cycling hypoxia and cycling pH on growth. Lower salinity likely reduced the rate of nutrient assimilation in oysters (Brown and Hartwick 1988), potentially eliminating any benefit of increased phytoplankton supplementation during this experiment. This difference in salinity also corresponded to a slight difference in alkalinity, the combination of which reduced calcite saturation. Calcification would have been energetically costly for the 2012 oysters, but, except for those oysters exposed to brief periods of calcite availability below saturation by cycling pH, shell dissolution should not

have occurred because mean calcite saturation states during the high pH portions of cycles was just above saturation. In the 2013 adult experiment on the other hand, all oysters, even those nominally not exposed to pH stress, may have experienced shell dissolution throughout most of the experiment (Feely et al. 2004; Orr et al. 2005; Fabry et al. 2008). Higher energetic costs in conjunction with shell dissolution may have contributed to negative effects of pH on growth; results that were also observed when salinity and alkalinity were even lower during the 2014 juvenile growth experiment

Our adult growth experiments are difficult to interpret because of extremely low growth rates; however, juveniles in 2012 also appeared to be less affected by cycling DO and pH than in other years: juvenile growth in 2012 was only reduced by exposure to severe cycling hypoxia (0.5 mg L^{-1}) 4-5 d wk^{-1}, and there were no negative effects of the pH cycle alone. Furthermore, during the second half of this experiment, juvenile oysters exposed to both cycling hypoxia and pH grew more quickly than control oysters. The 2013 spat growth experiment occurred under the lowest temperatures and highest salinities and alkalinities of the five experiments described here (Table 7), although temperature and salinity were still within the natural range of eastern oysters. Much like the 2012 juvenile growth experiment, another experiment under relatively high salinity, spat growth in 2013 was negatively affected by hypoxia, but not by cycling pH. In contrast, the 2014 juvenile growth experiment occurred under the lowest salinity and alkalinity conditions of any of these experiments, but under temperatures comparable to those of the 2013 juvenile growth experiment. Calculations of calcite saturation states (Table 8) indicate that juvenile oysters, even those not intentionally exposed to pH stress,

were constantly exposed to severe carbonate stress in 2014. Perhaps as a consequence, oyster growth was negatively impacted by *both* cycling hypoxia and cycling pH.

Food by DO/pH Treatment Interaction

Supplemented food availability increased oyster growth slightly, but, although Thomsen et al. (2013) found that ample food availability can mitigate harmful effects of acidification on juvenile blue mussels, *Mytilus edulis*, we did not find evidence that food availability modified effects of DO or pH on growth of juvenile oysters. There was a very slight (<1 square millimeter) increase in size of oysters supplied with supplemental algae over those grown at ambient conditions of the SERC sea-water system in spite of the average 50% increase in Chl*a* availability. It is possible that the two Chl*a* levels, both of which were below those of the Rhode River on most days, were not sufficiently different from each other to result in a biologically relevant interaction with other conditions. In addition, the 2014 juvenile growth experiment was performed under salinity conditions at the extreme low end of the eastern oyster's native range. Conditions for growth may have been so poor that the food provided could not compensate for the harmful effects of ambient conditions. Compounding any effects of food availability, low salinity may also have reduced the ability of oysters to assimilate any nutrients from the available food as was found by Brown and Hartwick (1988).

Hypoxia Effects

This study indicates that the severity and duration of hypoxia can influence the magnitude of effects on growth of exposed oysters. In 2014, juvenile growth was reduced by similar amounts when exposed to constant mild hypoxia (2.0 mg L^{-1}) or to brief periods of 0.5 mg L^{-1} DO 5-6 d wk^{-1}. However, exposure to constant moderate hypoxia (1.3 mg L^{-1}) in 2013 reduced juvenile growth far more than did exposure to severe cycling hypoxia (0.5 mg L^{-1}). Cycling conditions provide periods of respite at high oxygen which can allow for compensatory feeding and the repayment of oxygen debt (De Vooys and De Zwaan 1978; Taylor and Miller 2001), potentially allowing oysters to grow more quickly under cycling conditions than constant conditions even when minimum DO concentrations to which oysters are exposed are lower.

Under ambient conditions of the 2013 adult growth experiment, adult oysters grew shell more slowly under severe cycling hypoxia than under normoxia particularly in combination with normcapnia. In addition, moderate cycling hypoxia did not impact shell growth during this experiment. Spat growth in 2012 was reduced under severe diel-cycling hypoxia over the entire experiment, while moderate cycling hypoxia (1.71 mg L^{-1}) reduced growth in the first half of the experiment and increased growth in the second half of the experiment, resulting in no overall effect of moderate cycling hypoxia. In 2013, the middle age class of spat reduced growth rate slightly in the first two weeks of exposure to severe cycling hypoxia (0.5 mg L^{-1}) but not in the second two weeks or over the duration of the experiment. The youngest age class, on the other hand, experienced a

trend towards reduced growth throughout the experiment. In 2014, severe hypoxia exposure reduced growth of juvenile oysters. The absence of moderate cycling hypoxia (1.3 mg L^{-1}) effects in some experiments (2013 adult growth, 2013 spat), and changes in effects of moderate cycling hypoxia (1.71 mg L^{-1}) over time (2012 spat) even when there were negative effects of severe cycling hypoxia indicate that there may be a threshold of hypoxia somewhere between 0.5 and 1.5 mg L^{-1} at which oyster growth is affected, similar to the potential threshold of hypoxia for disease effects (Chapter 1) or thresholds for behavior in other estuarine species (Eby and Crowder 2002; Vaquer-Sunyer and Duarte 2010). The lack of effects of severe cycling hypoxia on adult growth in 2012 and variation in sensitivity among 2013 spat age classes indicates that the threshold for hypoxia effects likely varies with environmental conditions and oyster age.

Cycling pH and DO by pH Interactions

Cycling pH affected growth of oysters when salinity was at the lower end of the native range of the eastern oyster, resulting in low alkalinity and calcite saturation and carbonate stress as well as potential osmotic stress. The effect of pH also varied among DO treatments in several experiments. Negative effects of hypoxia but not pH on growth under otherwise optimal ambient conditions in oysters agree with the findings of Gobler et al. (2014) for scallops, although oysters experienced negative effects of pH when also experiencing other environmental stressors. There were also significant DO*pH interactions, the form of which varied between experiments. During the 2013 adult growth experiments, for example, severe diel-cycling hypoxia and hypercapnia in

combination had no effect on adult oyster shell growth even though growth was reduced by both cycling DO and cycling pH alone. Juvenile tubeworms, *Hydroides elegans* showed a similar effect with reduced expression of calcification related proteins under either hypoxia or hypercapnia, but protein expression restored to control levels when exposed to both stressors simultaneously (Mukherjee et al. 2013). In the 2014 spat growth experiment, the combination of cycling pH and severe cycling hypoxia resulted in growth reductions equivalent to those of severe cycling hypoxia or cycling pH independently. Given a longer exposure and the compensatory growth of oysters (see below), it is possible that the interaction of DO and pH effects observed in 2014 would have resulted in reduced or eliminated negative effects on growth given more time.

The combination of compensatory feeding during high oxygen portions of the cycle with increased feeding under low pH may have allowed oysters exposed to cycles of both DO and pH to grow similarly to oysters exposed to non-cycling conditions. Bayne (2002) demonstrated that other oyster species can modify feeding behaviors to maintain necessary energy uptake rates under fluctuating environmental conditions. This does not account for the negative effects on growth of diel-cycling pH under normoxic conditions in the 2013 adult growth and 2014 juvenile growth experiments. Further experimentation will be required to determine whether or not this mechanism is effective in juvenile oysters.

Acclimation/Compensation

In addition to compensatory feeding, oysters acclimated to, or compensated for, early reductions in growth under hypoxic exposure or combined exposure to cycling hypoxia and pH. Acclimation in this case is defined as declining severity of effects over the course of an experiment, while compensation is defined as stimulatory effects later in exposure that ultimately eliminate differences among treatments. Juvenile oysters compensated for moderate cycling hypoxia exposure as well as exposure to co-varying cycles of severe hypoxia and pH in the 2012 juvenile growth experiment. However, they did not acclimate to cycling severe hypoxia in the absence of cycling pH. In this experiment, early exposure to moderate cycling hypoxia resulted in reduced growth, but growth rates during the second two week period were similar to those of the other treatments and, over the full month, growth rates were no different from those of oysters under any other condition indicating that oysters had acclimated to and compensated for early reductions in growth. The oldest age class of spat in the 2013 juvenile growth experiment was not affected by any cycling treatment; however, the middle and youngest age classes of spat in 2013 acclimated to but did not compensate for the early negative effects of severe cycling hypoxia. The salinity and alkalinity in 2013 were much higher than in 2012, which perhaps allowed for acclimation to more severe cycling conditions than in the previous year due to a lower energetic cost of calcification. All three age classes of spat in the 2013 experiment acclimated to constant moderate hypoxia. These results suggest that oysters are well adapted to cycling conditions and exhibit enough plasticity to overcome exposure to negative conditions (Bayne 2002; Ivanina et al. 2011).

During the nine month respite from laboratory cycling conditions following the 2012 experiment, juvenile oysters exhibited compensatory growth, which resulted in similar sized oysters among treatments. Oysters with some chance to establish themselves before exposure to fluctuating conditions or oysters removed from severe cycling conditions may sometimes acclimate to, and compensate for, the effects of cycling hypoxia.

Marine organisms may have a wide variety of mechanisms for coping with exposure to hypoxia (Wu 2002). Oysters may develop increased gill tissue under potentially stressful conditions resulting in more efficient feeding (Bayne 1993). During metamorphosis, larval oysters require energy and are unable to feed, resulting in limited energy reserves after the completion of metamorphosis (Baker and Mann 1992; Baker and Mann 1994; Osman 1994). As oysters develop, they increase energy reserves (Baker and Mann 1992) and are better able to avoid potentially harmful conditions through shell closure (Shick et al. 1986; Riisgård et al. 2003). Eastern oysters modify metabolic profiles, including increasing activity of important mitochondrial enzymes when exposed to hypoxic conditions (Ivanina et al. 2011). Other bivalves are able to compensate for the effects of exposure to suboptimal environmental conditions on reproduction early in the season, but are not able to compensate for changes later in the season (Jokela 1996). Stressful conditions may have also impacted energy budgeting and left latent effects on energy allocation in the second year of growth.

Energy allocation

In addition to increasing the energetic costs of growth at low calcite saturations or limiting energy uptake at low DO, cycling hypoxia and pH may affect allocation of energy to maintenance and other biological functions (Stearns 1992). While some research indicates that shell growth is a prioritized activity in oysters (Brown and Hartwick 1988), other research has shown that freshwater clams, *Anodonta piscinalis*, preferentially reduce energy allocation to shell growth before sacrificing maintenance or reproduction (Jokela and Mutikainen 1995). For adults, effects of severe cycling hypoxia on growth in shell height were either not significant (2012) or extremely small (2013). Tissue mass was not affected by cycling conditions in the experiment in which it was measured, perhaps indicating the use of energy for maintenance rather than shell growth. One year old oysters in the 2013 experiment reduced reproductive effort under severe cycling hypoxia (Steppe et al., unpublished data), perhaps to preserve energy for other important functions (Jokela and Mutikainen 1995; Jokela et al. 1997). Oysters also up-regulate immune responses as a precaution against potential harm when challenged by diel-cycling conditions (Chapter 1). Energy allocation may also change with life stage; for instance, younger animals may prioritize growth, while an older animal may preferentially put energy towards reproduction (Jokela et al. 1997). This may help explain some differences in treatment effects between adult and juvenile oysters. The adult growth experiment in 2013 was performed earlier in the season to allow for an analysis of reproductive effort (Steppe et al., unpublished data), while the 2012 experiment was performed later in the season when energetic priorities might have been

shifting away from reproductive output to growth and maintenance. In their second summer, oysters previously exposed to cycling hypoxia may be putting energy in to compensatory growth, leaving less energy to allocate to reproduction in spite of being similar in size to oysters not previously exposed to hypoxia (Jokela et al. 1997), potentially reducing reproduction even after cycling conditions abate.

Implications

Reduced oyster size caused by exposure to diel-cycling hypoxia and pH may diminish important ecosystem services including provision of oyster bar habitat and water filtration (Hargis and Haven 1999), may reduce fecundity as smaller oysters produce fewer eggs or sperm each season (Davis and Chanley 1956), and may increase susceptibility to predation (Osman 1994; Sanford et al. 2014). However, results herein indicate that oysters have an ability to sometimes acclimate to, and compensate for, the negative effects of exposure to cycling hypoxia on growth as well as an ability under some circumstances to withstand exposure to co-varying cycling hypoxia as low as 0.5 mg L^{-1} and pH as low as 7.0 without reductions in growth. Nevertheless, long term effects on energy allocation including reduced fecundity under cycling conditions (Steppe et al., unpublished data) may have important effects on population viability.

Under global climate change, the Chesapeake Bay region is predicted to become warmer and drier (Diaz and Rosenberg 1995; Najjar et al. 2010). While the small range of temperatures tested did not appear to interact with cycling conditions in this experiment, higher temperature might both increase the severity of hypoxic events (Diaz and

Rosenberg 1995; Rabalais et al. 2010) and work as an additional interactive stressor on estuarine organisms (Gabbott and Bayne 1973; Lannig et al. 2006; Ivanina et al. 2009). Drier conditions will increase salinity in some areas, resulting in higher alkalinity and increasing calcite availability, which might, given the results here, reduce effects of cycling conditions on growth in oysters, but also increase the risk of disease (Vølstad et al. 2008). While this might help oysters mitigate some deleterious effects of climate change, the interactive effects of severe DO or pH cycles with increased temperatures are likely to have negative consequences for oyster populations (Davis and Calabrese 1964; Parker et al. 2009).

It would be interesting to look at extended periods of recovery after laboratory exposure to diel-cycling conditions to see how long growth rates in oysters previously exposed to severe cycling hypoxia might remain stimulated. If short-term exposure to brief periods of hypoxia stimulate growth beyond that necessary to compensate for previously reduced growth, such exposure might be a strategy of interest to aquaculture facilities interested in stimulating an oyster to reach market size sooner as well as to managers interested in restoring larger oysters more quickly. The latent effects of previous exposure to diel-cycling conditions on fecundity are also worthy of investigation, as the oysters which we have shown to grow faster in their second summer may be doing this at the expense of other metabolic processes such as reproduction. We also do not know how these cycling conditions might affect larval oysters and settlement. Younger individuals may be more susceptible to conditions which affect energy availability, so any impacts on this stage

may affect the size of oyster population as well as the compensatory behavior of individual oysters in locations experiencing these conditions.

Conclusion

The brief periods of hypoxia and environmental hypercapnia to which estuarine organisms are exposed under diel-cycling conditions particularly in eutrophic, shallow waters have the potential to reduce growth rates and change feeding patterns in eastern oysters; changing ecosystem structure and functioning. The effects of these cycles may be compounded by other environmental conditions such as low salinity/alkalinity, as well as seasonal period, and age of individuals exposed. Under optimal conditions, well established oysters may not be affected by cycling conditions. Juvenile oysters showed an impressive ability to acclimate to, and compensate for, exposure to hypoxia. The timing of severe cycles in DO and pH may influence effects on growth and reproduction of cycles as energetic priorities change throughout the season. Smaller oysters may be more susceptible to predation, reproduce less, and provide less water filtration and ecosystem engineering services, contributing to long term ecosystem degradation and potential loss of an economically viable fishery.

Table 5. Mean±SE (n) DO and pH in oyster growth experiments on days on which treatment conditions cycled. High dissolved oxygen, high pH (HDO, HpH)= DO and pH measured in aquaria at simulated late afternoon portion of the daily cycle when pH and DO were at or near their daily maxima in cycling treatments (i.e. high). Low dissolved oxygen, low pH (LDO,LpH) = DO and pH measured in aquaria at simulated dawn when pH and DO were at their daily minima in cycling treatments (i.e. low). Empty boxes are treatments which were not performed during the experiment in that column.

Treatment	DO & pH	2012 Adult	2013 Adult	2012 Spat	2013 Spat	2014 Spat
Normoxia, Normcapnia	HDO:	7.25±0.02(349)	7.53±0.02(224)	7.31±0.02(259)	7.80±0.02(10)	7.82±0.05(12)
	LDO:	7.36±0.02(320)	7.65±0.02(282)	7.42±0.03(236)	7.97±0.03(85)	7.88±0.02(121)
	HpH:	7.81±0.00(359)	7.92±0.00(264)	7.83±0.00(257)	8.09±0.01(120)	7.96±0.01(152)
	LpH:	7.82±0.00(326)	7.93±0.01(273)	7.84±0.01(242)	8.12±0.00(85)	7.98±0.01(128)
Normoxia, cycling pH	HDO:	7.24±0.02(348)	7.51±0.02(225)	7.31±0.02(258)	7.78±0.02(10)	7.84±0.06(19)
	LDO:	7.32±0.02(321)	7.59±0.02(282)	7.37±0.03(238)	7.92±0.03(85)	7.88±0.02(119)
	HpH:	7.79±0.00(359)	7.90±0.00(264)	7.82±0.00(257)	8.03±0.00(120)	7.92±0.01(151)
	LpH:	6.98±0.00(328)	7.01±0.02(265)	6.99±0.00(244)	7.00±0.01(85)	7.11±0.00(128)
Moderate cycling hypoxia, Normcapnia	HDO:		7.51±0.02(224)		7.78±0.02(10)	
	LDO:		1.35±0.00(281)		1.31±0.01(84)	
	HpH:		7.91±0.01(264)		8.04±0.01(120)	
	LpH:		7.96±0.00(273)		8.10±0.01(85)	
Moderate cycling hypoxia, cycling pH	HDO:	7.25±0.02(349)		7.30±0.02(259)		
	LDO:	1.69±0.01(321)		1.71±0.01(237)		
	HpH:	7.80±0.00(359)		7.81±0.00(257)		
	LpH:	7.02±0.00(327)		7.04±0.01(243)		
Severe cycling hypoxia, Normcapnia	HDO:	7.20±0.02(349)	7.51±0.02(224)	7.26±0.02(259)	7.77±0.02(10)	7.84±0.04(21)
	LDO:	0.56±0.01(320)	0.51±0.01(281)	0.59±0.01(236)	0.55±0.00(84)	0.51±0.01(128)
	HpH:	7.79±0.00(359)	7.90±0.00(264)	7.81±0.00(257)	8.03±0.01(120)	7.93±0.01(152)
	LpH:	7.83±0.00(326)	7.97±0.00(274)	7.84±0.00(242)	8.08±0.01(85)	8.05±0.01(128)
Severe cycling hypoxia, Moderate cycling pH	HDO:					7.84±0.05(21)
	LDO:					0.53±0.01(128)
	HpH:					7.91±0.01(152)
	LpH:					7.46±0.00(128)
Severe cycling hypoxia, cycling pH	HDO:	7.37±0.02(349)	7.48±0.02(224)	7.32±0.02(259)	7.76±0.02(10)	7.83±0.05(22)
	LDO:	0.57±0.01(322)	0.57±0.00(282)	0.57±0.01(238)	0.56±0.01(85)	0.52±0.01(128)
	HpH:	7.81±0.00(359)	7.90±0.00(264)	7.84±0.00(257)	8.03±0.00(120)	7.91±0.01(151)
	LpH:	7.01±0.00(328)	7.02±0.00(271)	7.02±0.00(244)	6.99±0.00(85)	7.09±0.00(126)
Normoxia, Constant low pH	HDO:		7.50±0.02(224)		7.79±0.01(10)	
	LDO:		7.59±0.02(282)		7.97±0.03(85)	
	HpH:		7.48±0.00(264)		7.41±0.01(119)	
	LpH:		7.45±0.00(271)		7.35±0.01(85)	
Constant moderate/mild hypoxia, Normcapnia	HDO:				1.28±0.01(85)	2.07±0.01(22)
	LDO:				1.28±0.01(130)	2.09±0.01(128)
	HpH:				8.05±0.01(85)	8.02±0.01(152)
	LpH:				8.05±0.00(199)	8.03±0.01(128)

Table 6. Starting, ending, and recovery shell heights (mm) or shell area of oysters for each experiment, mean±SE, sample size, and range (in parenthesis). Endpoint means are means of all replicates of all treatments. Empty boxes are time points not measured in that experiment.

	Starting (mm)	Ending (mm)	Recovery (mm)
2012 Adult Growth	43.6±0.14 2700 (30-70)	49.2±0.27 900 (31-78)	68.0±0.54 452 (31-99)
2013 Adult Growth	46.1±0.12 3240 (34-71)	49.2±0.15 1985 (34-75)	
2012 Spat Growth	52.6±1.29 359 (6.6-154.2)	430.2±7.79 344 (68.9-799.6)	1348.3±31.51 144 (168.8-2689.7)
2013 Spat Growth – 4 weeks post settlement	18.3±1.32 131 (1.72-70.14)	535.9±21.55 89 (104.49-981.12)	
2013 Spat Growth – 2 weeks post settlement	3.7±0.12 236 (0.59-13.14)	434.6±12.13 192 (85.36-858.27)	
2013 Spat Growth – 1 week post settlement	<1	331.2±9.74 251 (30.48-793.16)	
2014 Spat Growth	<1	12.1±0.22 515 (3.13-36.01)	

Table 7. Experimental dates, mean ± SE and range of water quality parameters in treatment aquaria during growth experiments 2012-2014. Chl*a* is the concentration of chlorophyll-a in the water column as measured by fluorescence. Empty boxes are variables not measured during that experiment.

	Dates	Salinity	Temperature (°C)	Total Alkalinity ($\mu mol\ kg^{-1}$ sw)	Chl*a* ($\mu g\ L^{-1}$)
2012 Adult Growth	7/5/12-9/26/12	10.95±0.01 (8.27-14.85)	28.56±0.01 (21.29-31.66)	1622.4±15.4 (1448.7-1745.2)	
2013 Adult Growth	6/7/13-7/30/13	8.20±0.01 (5.68-9.45)	26.37±0.03 (23.0-32.8)	1400.9±28.8 (1252.7-1586.1)	4.230±0.036 (0.810-10.846)
2012 Spat Growth	7/25/12-8/27/12	10.66±0.00 (9.25-12.28)	29.49±0.01 (24.83-31.36)	1614.7±15.8 (1524.0-1700.7)	
2013 Spat Growth	8/29/13-10/8/13	11.90±0.01 (9.2-12.93)	24.55±0.02 (21.97-27.03)	1678.9±4.97 (1664.6-1692.9)	4.075±0.137 (1.343-9.869)
2014 Spat Growth	5/29/14-6/27/14	6.10±0.01 (5.36-6.81)	25.08±0.02 (22.88-27.17)	1174.3±16.03 (1089.45-1273.31)	Algae added: 4.272±0.185 (0.722-13.422) Ambient: 2.829±0.116 (0.899-11.290)

Table 8. Mean ± SE (n), and range of calcite saturation states by treatment for each experiment during the simulated day and night periods, high DO/pH and the low DO/pH periods. Calcite saturation state calculated using CO2SYS.XLS (Pelletier et al. 2007) from ten minute average LabVIEW data. Calcite saturations for the treatments which were not monitored by the LabVIEW based system were calculated from mean alkalinity and pH for the treatments in question and therefore do not have errors or ranges. Empty boxes are treatments which were not used during the experiment in that column.

		Growth experiment (year, life stage)				
Treatment		2012 Adult	2013 Adult	2012 Spat	2013 Spat	2014 Spat
Normoxia, Normcapnia	HDO/pH:	1.08±0.001 23763 (0.32-2.05)	0.95±0.002 6849 (0.52-1.48)	1.05±0.001 17045 (0.61-2.03)	1.87	0.69
Normoxia, cycling pH	HDO/pH:	1.14±0.002 11508 (0.52-1.89)	0.93±0.003 5050 (0.45-1.51)	1.11±0.001 8071 (0.73-1.65)	1.94±0.003 2856) (0.93-2.15)	0.66±0.003 1099 (0.43-1.06)
	LDO/pH:	0.19±0.000 3012 (0.15-0.23)	0.12±0.000 1137 (0.10-0.16)	0.19±0.000 2207 (0.15-0.23)	0.18±0.000 552 (0.17-0.23)	0.10±0.000 528 (0.08-0.11)
Moderate cycling hypoxia, Normcapnia	HDO/pH:		0.90±0.003 4689 (0.46-1.25)		1.91±0.002 2694) (1.58-2.24)	
	LDO/pH:		1.06±0.005 1144 (0.61-1.48)		2.18±0.004 536 (2.02-2.46)	
Moderate cycling hypoxia, cycling pH	HDO/pH:	1.08±0.002 11140 (0.57-1.74)		1.04±0.001 7800 (0.57-1.71)		
	LDO/pH:	0.19±0.000 3006 (0.16-0.26)		0.194±0.000 2202 (0.16-0.26)		
Severe cycling hypoxia, Normcapnia	HDO/pH:	1.06±0.002 11154 (0.64-1.93)	0.91±0.003 4684 (0.47-1.41)	1.00±0.001 7800 (0.70-1.59)	1.98±0.001 2705 (1.69-2.24)	0.71±0.004 957 (0.43-1.11)
	LDO/pH:	1.12±0.001 2991 (0.78-1.41)	1.07±0.004 1156 (0.60-1.55)	1.13±0.001 2207 (0.99-1.24)	2.14±0.003 551) (1.95-2.31)	0.89±0.007 517 (0.58-1.18)
Severe cycling hypoxia, Moderate cycling pH	HDO/pH:					0.67±0.003 961 (0.45-0.91)
	LDO/pH:					0.24±0.001 528 (0.20-0.27)
Severe cycling hypoxia, cycling pH	HDO/pH:	1.08±0.001 (10914) (0.73-1.83)	0.88±0.003 4679 (0.42-1.35)	1.04±0.001 7578 (0.73-1.49)	1.87±0.003 2654 (0.89-2.23)	0.68±0.003 961 (0.46-1.08)
	LDO/pH:	0.19±0.000	0.12±0.000	0.19±0.000	0.18±0.000	0.10±0.000

		(2998) (0.12-0.22)	1151 (0.09-0.18)	2196 (0.12-0.22)	551 (0.15-0.23)	528 (0.08-0.11)
Normoxia, Constant low pH	HDO/ LpH:		0.35±0.001 6107 (0.11-2.45)		0.43±0.001 4402 (0.31-1.45)	
Constant moderate hypoxia, Normcapnia	LDO/ HpH:				1.84±0.003 4227 (1.29-2.76)	
Constant mild hypoxia, Normcapnia	LDO/ HpH:					0.75±0.002 2743 (0.49-1.06)

Table 9. Randomized complete block design ANOVA of mean tank mortality during each of the five growth experiments. Tests are considered significant at a=0.05 and significant p values are bolded.

	ANOVA Source and Factor	df	F	p
2012 Adult Growth	Treatment	4, 20	1.11	0.381
2013 Adult Growth	Treatment	5, 25	1.88	0.134
2012 Spat Growth	Treatment	4, 20	0.82	0.529
2013 Spat Growth – Settlement 1	Treatment	6, 24	0.24	0.959
2013 Spat Growth – Settlement 2	Treatment	6, 24	0.61	0.719
2013 Spat Growth – Settlement 3	Treatment	6, 24	1.30	0.295
2014 Spat Growth	Treatment	11, 33	1.03	0.441

Table 10. 2012 adult growth experiment. Randomized complete block design ANCOVA of instantaneous growth rates (A) during first 45 days and (B) full 95 days of experiment using starting shell height as a covariate and laboratory position as a blocking factor. Randomized complete block design ANCOVA of (C) instantaneous growth rates during a nine month field deployment using deployment height as the covariate and laboratory position as the blocking factor. Tests are considered significant at a=0.05 and significant p values are bolded.

A) Instantaneous 6 week growth			
ANCOVA Source and Factor	df	F	p
Starting shell height	1, 15.58	19.98	**<0.001**
Treatment	4, 20.35	0.54	0.709
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	19	0.04	0.972
Severe cycling hypoxia vs. Normoxia	19	0.25	0.806
Severe cycling hypoxia vs. Moderate cycling hypoxia	19	0.69	0.497
Moderate cycling hypoxia vs. Normoxia	19	0.90	0.382
Cycling pH vs. Normcapnia	19	1.05	0.309
B) Instantaneous 3 month growth			
ANCOVA Source and Factor	df	F	p
Starting shell height	1, 14.58	21.89	**<0.001**
Treatment	4, 20.49	1.30	0.303
Contrasts	Df	t	p
Severe cycling hypoxia*Cycling pH Interaction	19	1.52	0.145
Severe cycling hypoxia vs. Normoxia	19	0.87	0.397
Severe cycling hypoxia vs. Moderate cycling hypoxia	19	0.42	0.681
Moderate cycling hypoxia vs. Normoxia	19	1.12	0.275
Cycling pH vs. Normcapnia	19	1.10	0.286
C) Instantaneous Recovery Growth			
ANCOVA Source and Factor	df	F	p
Deployment shell height	1, 17.61	9.83	**0.006**
Treatment	4, 17.31	0.30	0.871
Contrasts	Df	t	p
Severe cycling hypoxia*Cycling pH Interaction	16	0.23	0.822
Severe cycling hypoxia vs. Normoxia	16	0.64	0.528
Severe cycling hypoxia vs. Moderate cycling hypoxia	16	0.47	0.644
Moderate cycling hypoxia vs. Normoxia	16	1.01	0.328
Cycling pH vs. Normcapnia	16	0.23	0.824

Table 11. 2013 adult growth experiment. Randomized complete block design ANCOVA of (A) instantaneous rate of growth in shell height with starting shell height as the covariate, and laboratory position as blocking factor, during first six weeks of growth experiment and (B) during entire twelve weeks of experiment. (C) Randomized complete block design ANOVA of tissue dry weight from experiment using laboratory position as blocking factor. Tests are considered significant at a=0.05 and significant p values are bolded.

A) Instantaneous 6 week Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell height	1, 27.29	5.72	**0.024**
Treatment	5, 24.40	0.69	0.637
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	24	1.03	0.315
Severe cycling hypoxia vs. Normoxia	24	0.93	0.361
Moderate cycling hypoxia vs. Normoxia under Normcapnia	24	0.61	0.549
Cycling pH vs. Normcapnia	24	0.92	0.368
Constant pH vs. Normoxia/ Normcapnia	24	0.94	0.356
Normoxia/Cycling pH vs. Constant pH	24	0.52	0.605
B) Instantaneous 12 week Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell height	1, 24.85	4.99	**0.035**
Treatment	5, 26.10	6.52	**<0.001**
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	24	4.49	**<0.001**
Severe cycling hypoxia vs. Normoxia under Normcapnia	24	2.17	**0.040**
Severe cycling hypoxia vs. Normoxia under Cycling pH	24	2.83	**0.009**
Severe cycling hypoxia/Cycling pH vs. Normoxia/Normcapnia	24	1.28	0.212
Moderate cycling hypoxia vs. Normoxia under Normcapnia	24	1.08	0.293
Cycling pH vs. Normcapnia under Normoxia	24	4.41	**<0.001**
Cycling pH vs. Normcapnia under Severe cycling hypoxia	24	0.75	0.461
Constant pH vs. Normoxia/ Normcapnia	24	4.31	**<0.001**
Normoxia/Cycling pH vs. Constant pH	24	0.13	0.894
C) Tissue Dry Weight			
ANOVA Source and Factor	df	F	p
Treatment	5, 25	0.47	0.798
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	25	0.54	0.592
Severe cycling hypoxia vs. Normoxia	25	0.27	0.787
Severe cycling hypoxia vs. Moderate cycling hypoxia	25	1.38	0.179
Moderate cycling hypoxia vs. Normoxia	25	1.25	0.222
Cycling pH vs. Normcapnia	25	0.18	0.858
Constant pH vs. Normcapnia	25	0.5	0.620
Cycling pH vs. Constant pH	25	0.33	0.745

Table 12. 2012 juvenile growth experiment. Randomized complete block design ANCOVA of instantaneous rate of growth in shell area (A) during first two weeks and (B) full month of experiment using starting area as a covariate. Randomized complete block design ANCOVA of instantaneous rate of growth in shell area (C) during recovery using deployment area as a covariate and (D) randomized complete block design ANOVA of mean tank spat area at the end of the recovery period (post-nine month field deployment) with lab placement as the blocking factor. Tests are considered significant at a=0.05 and significant p values are bolded.

A) 2 week Instantaneous Growth			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 9.73	1.84	0.206
Treatment	4, 22.06	2.67	0.059
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	19	0.87	0.398
Severe cycling hypoxia vs. Normoxia	19	3.77	**0.001**
Severe cycling hypoxia vs. Moderate cycling hypoxia	19	0.90	0.379
Moderate cycling hypoxia vs. Normoxia	19	2.50	**0.022**
Cycling pH vs. Normcapnia	19	0.51	0.616
B) 2-4 week Instantaneous Growth			
ANOVA Source and Factor	df	F	p
Starting shell area	1, 11.34	3.88	0.074
Treatment	4, 21.42	6.05	**0.002**
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	19	3.00	**0.007**
Severe cycling hypoxia vs. Normoxia under Normcapnia	19	2.83	**0.011**
Severe cycling hypoxia vs. Normoxia under Cycling pH	19	2.20	**0.041**
Severe cycling hypoxia vs. Moderate cycling hypoxia	19	0.32	0.751
Moderate cycling hypoxia vs. Normoxia	19	2.75	**0.013**
Cycling pH vs. Normcapnia under Normoxia	19	1.37	0.186
Cycling pH vs. Normcapnia under Severe cycling hypoxia	19	3.84	**0.001**
C) 4 week Instantaneous Growth			
ANOVA Source and Factor	df	F	p
Starting shell area	1, 8.88	3.14	0.111
Treatment	4, 22.51	2.39	0.081
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	19	0.41	0.685
Severe cycling hypoxia vs. Normoxia	19	3.55	**0.002**
Severe cycling hypoxia vs. Moderate cycling hypoxia	19	2.40	**0.027**
Moderate cycling hypoxia vs. Normoxia	19	0.71	0.485
Cycling pH vs. Normcapnia	19	0.41	0.688
D) Recovery Instantaneous growth			
ANOVA Source and Factor	df	F	P
Deployment shell area	1, 14.79	9.04	**0.009**

Treatment	4, 19.87	4.43	**0.011**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	16	0.01	0.989
Severe cycling hypoxia vs. Normoxia	16	3.38	**0.004**
Severe cycling hypoxia vs. Moderate cycling hypoxia	16	2.33	**0.033**
Moderate cycling hypoxia vs. Normoxia	16	0.53	0.604
Cycling pH vs. Normcapnia	16	1.67	0.115
E) Recovery Area			
ANOVA Source and Factor	df	F	P
Treatment	4, 17	0.66	0.629
Contrast	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	17	0.32	0.751
Severe cycling hypoxia vs. Normoxia	17	0.62	0.542
Severe cycling hypoxia vs. Moderate cycling hypoxia	17	0.39	0.699
Moderate cycling hypoxia vs. Normoxia	17	0.10	0.919
Cycling pH vs. Normcapnia	17	1.41	0.178

Table 13. 2013 spat growth experiment. Randomized complete block design ANCOVA of instantaneous rate of growth in shell heights showing results from 2 age classes of spat; (A) 4 weeks post-settlement, (B) 2 weeks post-settlement during first 18 days of experiment. (C) ANOVA for 18 day shell area of spat from 1 week post-settlement oysters. (D,E) ANCOVA of instantaneous growth rate during entire 39 day experiment, and ANOVA of (F) instantaneous rate of growth in shell heights from settlement 3:1 week post-settlement during 39 day experiment. Tests are considered significant at a=0.05 and significant p values are bolded.

A) 4 weeks post-settlement – 2.5 week Instantaneous Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 25.97	31.63	**<0.001**
Treatment	6, 22.24	1.86	0.133
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	22	0.87	0.393
Severe cycling hypoxia vs. Normoxia	22	0.40	0.695
Severe cycling hypoxia vs. Moderate cycling hypoxia	22	0.28	0.780
Moderate cycling hypoxia vs. Normoxia	22	0.45	0.660
Cycling pH vs. Normcapnia	22	0.79	0.440
Constant moderate hypoxia vs. Cycling moderate hypoxia	22	2.15	**0.043**
Constant moderate hypoxia vs. Normoxia	22	2.14	**0.044**
Constant moderate pH vs. Normcapnia	22	0.79	0.436
Constant moderate pH vs. Cycling pH	22	0.03	0.974
B) 2 weeks post-settlement – 2.5 week Instantaneous Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 24.14	25.99	**<0.001**
Treatment	6, 23.03	6.80	**<0.001**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	23	0.87	0.395
Severe cycling hypoxia vs. Normoxia	23	2.07	**0.050**
Severe cycling hypoxia vs. Moderate cycling hypoxia	23	0.41	0.684
Moderate cycling hypoxia vs. Normoxia	23	1.08	0.289
Cycling pH vs. Normcapnia	23	1.12	0.273
Constant moderate hypoxia vs. Cycling moderate hypoxia	23	4.19	**<0.001**
Constant moderate hypoxia vs. Normoxia	23	4.94	**<0.001**
Constant moderate pH vs. Normcapnia	23	0.30	0.771
Constant moderate pH vs. Cycling pH	23	0.00	0.999
C) 1 week post-settlement – 2.5 week Shell Area			
ANOVA Source and Factor	df	F	p
Treatment	6, 23	6.18	**<0.001**
Contrast	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	23	0.16	0.874
Severe cycling hypoxia vs. Normoxia	23	2.02	0.056
Severe cycling hypoxia vs. Moderate cycling hypoxia	23	0.47	0.644

Moderate cycling hypoxia vs. Normoxia	23	1.59	0.126
Cycling pH vs. Normcapnia	23	0.33	0.745
Constant moderate hypoxia vs. Cycling moderate hypoxia	23	3.83	**<0.001**
Constant moderate hypoxia vs. Normoxia	23	5.13	**<0.001**
Constant moderate pH vs. Normcapnia	23	1.25	0.224
Constant moderate pH vs. Cycling pH	23	1.3	0.206
D) 4 weeks post settlement – 2.5-5.5 week Instantaneous Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 24.66	3.50	0.073
Treatment	6, 21.19	0.49	0.806
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	21	0.46	0.650
Severe cycling hypoxia vs. Normoxia	21	0.14	0.889
Severe cycling hypoxia vs. Moderate cycling hypoxia	21	0.37	0.718
Moderate cycling hypoxia vs. Normoxia	21	0.71	0.485
Cycling pH vs. Normcapnia	21	0.25	0.804
Constant moderate hypoxia vs. Cycling moderate hypoxia	21	1.31	0.206
Constant moderate hypoxia vs. Normoxia	21	1.06	0.303
Constant moderate pH vs. Normcapnia	21	0.22	0.825
Constant moderate pH vs. Cycling pH	21	0.90	0.377
E) 2 weeks post-settlement – 2.5-5.5 week Instantaneous Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 22.57	1.69	0.207
Treatment	6, 22.04	0.66	0.679
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	22	0.08	0.939
Severe cycling hypoxia vs. Normoxia	22	0.92	0.369
Severe cycling hypoxia vs. Moderate cycling hypoxia	22	0.79	0.437
Moderate cycling hypoxia vs. Normoxia	22	1.13	0.270
Cycling pH vs. Normcapnia	22	0.07	0.944
Constant moderate hypoxia vs. Cycling moderate hypoxia	22	0.66	0.519
Constant moderate hypoxia vs. Normoxia	22	0.28	0.786
Constant moderate pH vs. Normcapnia	22	0.95	0.351
Constant moderate pH vs. Cycling pH	22	0.88	0.389
F) 1 week post-settlement – 2.5-5.5 week Instantaneous Growth Rate			
ANOVA Source and Factor	df	F	p
Treatment	6, 23	0.31	0.926
Contrast	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	23	1.05	0.304
Severe cycling hypoxia vs. Normoxia	23	0.35	0.728
Severe cycling hypoxia vs. Moderate cycling hypoxia	23	0.38	0.709

Moderate cycling hypoxia vs. Normoxia	23	0.26	0.795
Cycling pH vs. Normcapnia	23	1.03	0.316
Constant moderate hypoxia vs. Cycling moderate hypoxia	23	0.58	0.565
Constant moderate hypoxia vs. Normoxia	23	0.80	0.434
Constant moderate pH vs. Normcapnia	23	0.05	0.941
Constant moderate pH vs. Cycling pH	23	0.83	0.415
G) 4 weeks post settlement – 5.5 week instantaneous growth rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 22.73	25.15	**<0.001**
Treatment	6, 20.66	2.43	**0.062**
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	20	1.10	0.284
Severe cycling hypoxia vs. Normoxia	20	0.23	0.820
Severe cycling hypoxia vs. Moderate cycling hypoxia	20	0.22	0.827
Moderate cycling hypoxia vs. Normoxia	20	0.20	0.842
Cycling pH vs. Normcapnia	20	1.00	0.330
Constant moderate hypoxia vs. Cycling moderate hypoxia	20	2.58	**0.018**
Constant moderate hypoxia vs. Normoxia	20	2.10	**0.049**
Constant moderate pH vs. Normcapnia	20	0.96	0.348
Constant moderate pH vs. Cycling pH	20	0.03	0.974
H) 2 weeks post-settlement – 5.5 week Instantaneous Growth Rate			
ANCOVA Source and Factor	df	F	p
Starting shell area	1, 23.55	27.35	**<0.001**
Treatment	6, 22.12	5.02	**0.002**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	22	1.71	0.102
Severe cycling hypoxia vs. Normoxia	22	0.41	0.684
Severe cycling hypoxia vs. Moderate cycling hypoxia	22	0.96	0.346
Moderate cycling hypoxia vs. Normoxia	22	0.20	0.844
Cycling pH vs. Normcapnia	22	1.58	0.129
Constant moderate hypoxia vs. Cycling moderate hypoxia	22	4.71	**<0.001**
Constant moderate hypoxia vs. Normoxia	22	3.76	**0.001**
Constant moderate pH vs. Normcapnia	22	0.44	0.668
Constant moderate pH vs. Cycling pH	22	2.03	0.055
I) 1 week post-settlement – 5.5 week Instantaneous Growth Rate			
ANOVA Source and Factor	df	F	p
Treatment	6, 23	8.28	**<0.001**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	23	0.43	0.673
Severe cycling hypoxia vs. Normoxia	23	1.95	0.063
Severe cycling hypoxia vs. Moderate cycling hypoxia	23	0.93	0.360

Moderate cycling hypoxia vs. Normoxia	23	1.02	0.318
Cycling pH vs. Normcapnia	23	0.70	0.492
Constant moderate hypoxia vs. Cycling moderate hypoxia	23	5.01	**<0.001**
Constant moderate hypoxia vs. Normoxia	23	5.84	**<0.001**
Constant moderate pH vs. Normcapnia	23	1.16	0.259
Constant moderate pH vs. Cycling pH	23	1.26	0.220

Table 14. 2014 spat growth experiment. Randomized complete block design 2-way ANOVA of DO/pH treatment by food treatment interaction (ANOVA 1) and ANOVA of shell area (ANOVA 2) from the end of the two week laboratory exposure. Tests are considered significant at a=0.05 and significant p values are bolded.

2 week shell area			
ANOVA 1 Source and Factor	df	F	p
Food Treatment*DO/pH Treatment	5, 33	0.54	0.747
DO/pH Treatment	5, 33	3.68	**0.009**
Food Treatment	1, 33	4.13	**0.050**
ANOVA 2 Source and Factor	df	F	P
DO/pH Treatment	5,41	3.90	**0.006**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	41	4.88	**0.033**
Severe cycling hypoxia vs. Normoxia under Normcapnia	41	13.58	**<0.001**
Severe cycling hypoxia vs. Normoxia under Cycling pH	41	1.33	0.256
Constant mild hypoxia vs. Normoxia under Normcapnia	41	11.79	**0.001**
Constant mild hypoxia vs. Cycling moderate hypoxia	41	1.57	0.217
Cycling pH vs. Normcapnia under Normoxia	41	5.78	**0.021**
Cycling pH vs. Normcapnia under Severe cycling hypoxia	41	0.02	0.898

Table 15. 2013 Adult growth experiment feeding results statistics. Chl*a* measured in all tanks on 3 days at the end of normoxia/normcapnia (simulated dusk), 8 days at the end of the low plateau (simulated dawn), 5 days at the return to normoxia/normcapnia (simulated mid-morning), and 5 days at supersaturated oxygen (simulated mid-afternoon). Tests are considered significant at a=0.05 and significant p values are bolded.

A) Low Plateau			
ANOVA Source and Factor	df	F	P
Julian	7, 235	543.51	**<0.001**
Treatment	5, 235	92.44	**<0.001**
Treatment*Julian	35, 235	9.94	**<0.001**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	235	0.02	0.988
Severe cycling hypoxia vs. Normoxia	235	19.20	**<0.001**
Moderate cycling hypoxia vs. Normoxia	235	3.95	**<0.001**
Cycling pH vs. Normcapnia	235	3.34	**0.001**
Constant pH vs. Normcapnia	235	1.16	0.246
B) Normoxia PM			
ANOVA Source and Factor	df	F	p
Julian	4, 165	561.86	**<0.001**
Treatment	5, 165	3.44	**0.006**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	165	0.84	0.401
Severe cycling hypoxia vs. Normoxia	165	2.11	**0.036**
Moderate cycling hypoxia vs. Normoxia	165	1.67	0.097
Cycling pH vs. Normcapnia	165	0.06	0.955
Constant pH vs. Normcapnia	165	0.30	0.766
C) Supersaturation			
ANOVA Source and Factor	df	F	P
Julian	4, 143	317.60	**<0.001**
Treatment	5, 143	6.66	**<0.001**
Treatment*Julian	20, 143	2.41	**0.002**
Contrasts	df	t	P
Severe cycling hypoxia*Cycling pH Interaction	143	1.51	0.133
Severe cycling hypoxia vs. Normoxia	143	4.67	**<0.001**
Moderate cycling hypoxia vs. Normoxia	143	0.00	0.996
Cycling pH vs. Normcapnia	143	0.45	0.650
Constant pH vs. Normcapnia	143	0.98	0.331
D) Normoxia AM			
ANOVA Source and Factor	df	F	p

Julian	2, 61	292.22	**<0.001**
Treatment	3, 61	1.94	0.133
Contrasts	df	t	p
Severe cycling hypoxia*Cycling pH Interaction	61	0.61	0.547
Severe cycling hypoxia vs. Normoxia	61	2.16	**0.035**
Cycling pH vs. Normcapnia	61	1.03	0.308

Table 16. Summary of water quality conditions and results from five oyster growth experiments. Calcite saturation states are for the pH control treatments. *'s indicate significant results at p=0.05 while #'s represent trends with 0.05<p<0.1. Empty boxes indicate effect was not examined in that experiment.

	2012 Adult Growth	2013 Adult Growth	2012 Spat Growth	2013 Spat Growth – Set 1	2013 Spat Growth – Set 2	2013 Spat Growth – Set 3	2014 Spat Growth
Calcite Saturation State	1.08	0.95	1.05	1.87	1.87	1.87	0.69
Decreased growth – Severe cycling hypoxia	no effect	*	*	no effect	* (first half)	#	*
Decreased growth – Constant hypoxia				*	*	*	*
Decreased growth – Cycling pH	no effect	*	no effect	no effect	no effect	no effect	*
DO*pH interaction	no effect	* (same as control)	* (same as control in second half)	no effect	no effect	no effect	* (same as cycling pH or cycling hypoxia)
Decreased growth – constant hypercapnia		*		no effect	no effect	no effect	
Acclimation	no effect	no effect	*	no effect	*	#	
Compensation	no effect	no effect	*	no effect	no effect	no effect	

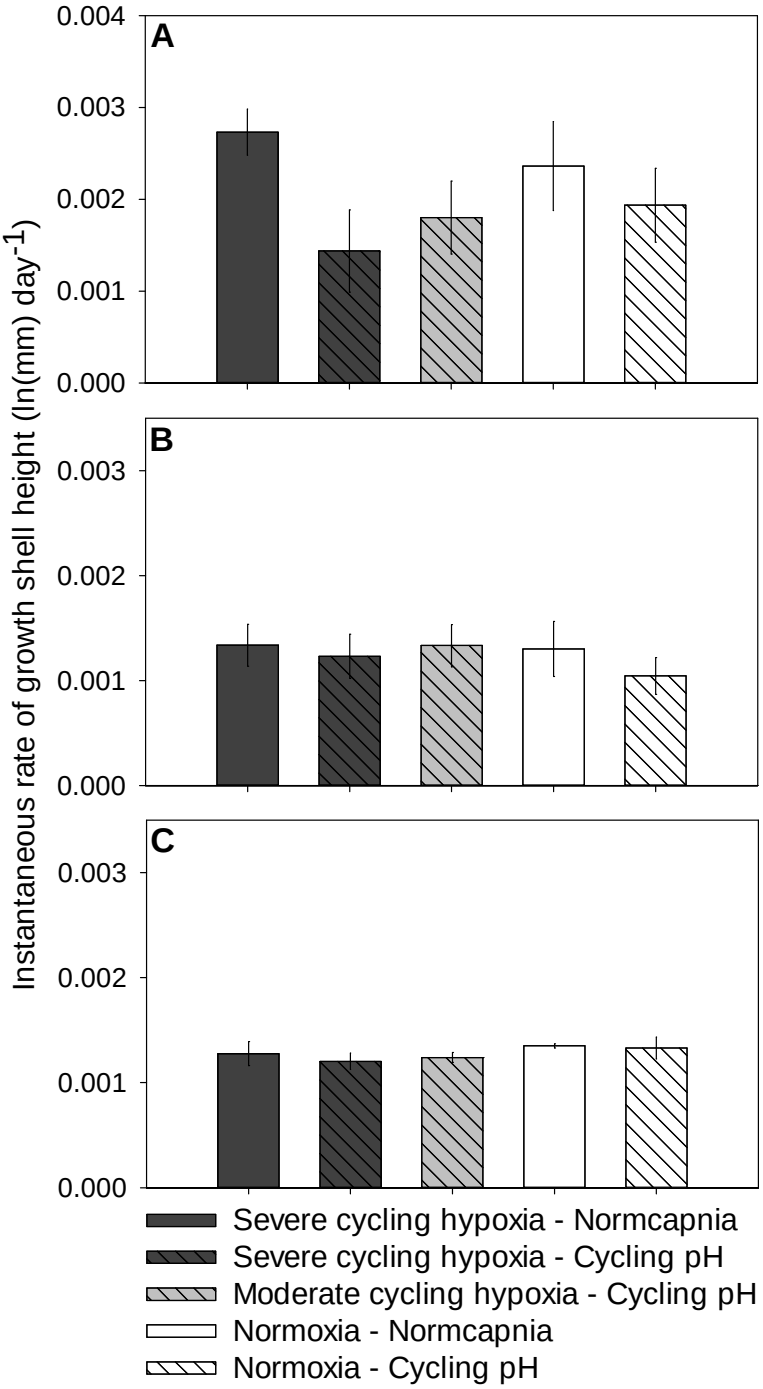

Figure 8. 2012 adult growth experiment. Mean ± SE instantaneous rate of growth in shell height by treatment of adult oysters exposed to diel cycles 4-5 d wk^{-1} during (A) the first six weeks, (B) the full twelve weeks, and (C) a nine month field deployment.

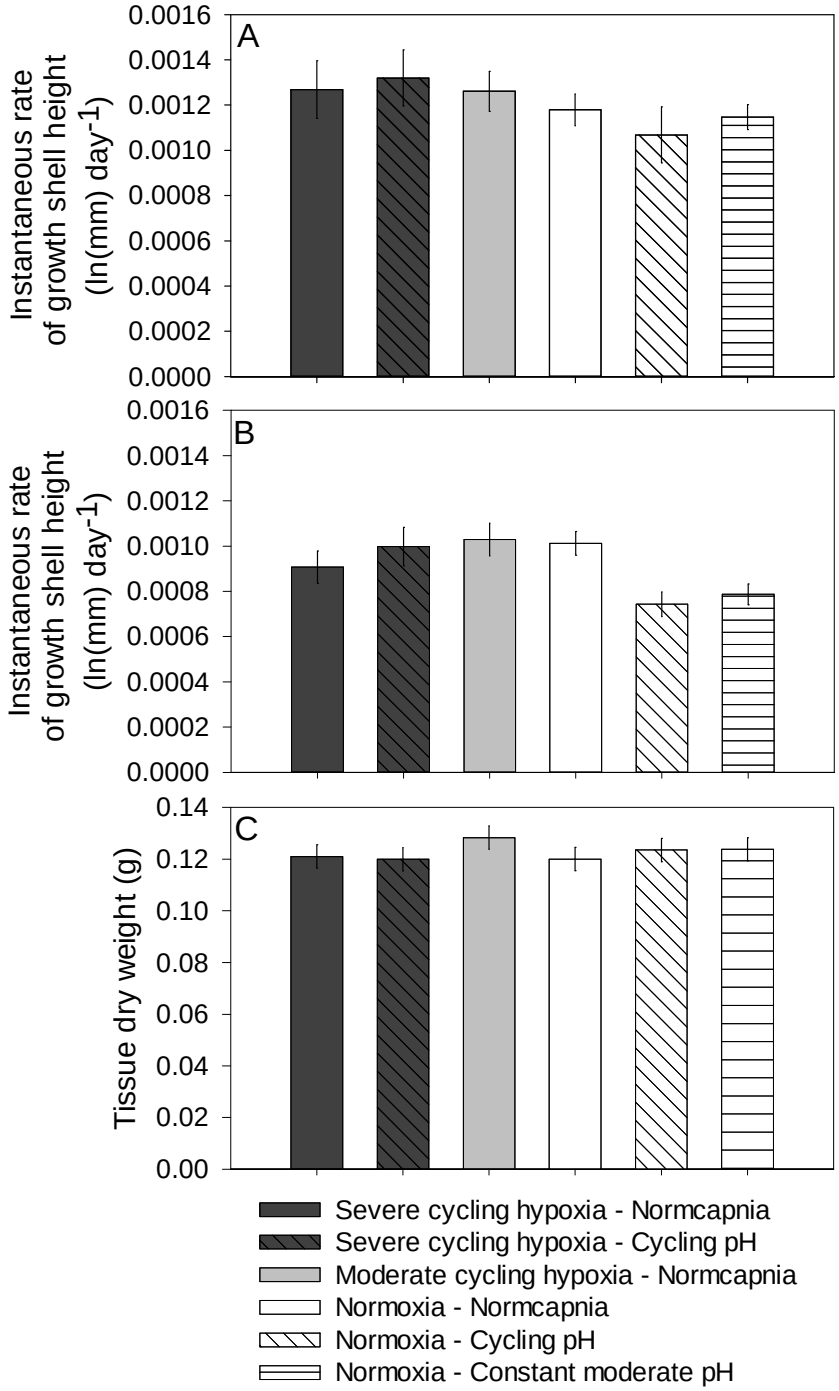

Figure 9. 2013 adult growth experiment. Mean± SE instantaneous rate of growth in shell height by treatment of adult oysters exposed to diel cycles 5 d wk^{-1} during (A) the first six weeks, (B) the entire experimental period, and (C) dry tissue weight at the conclusion of the experiment.

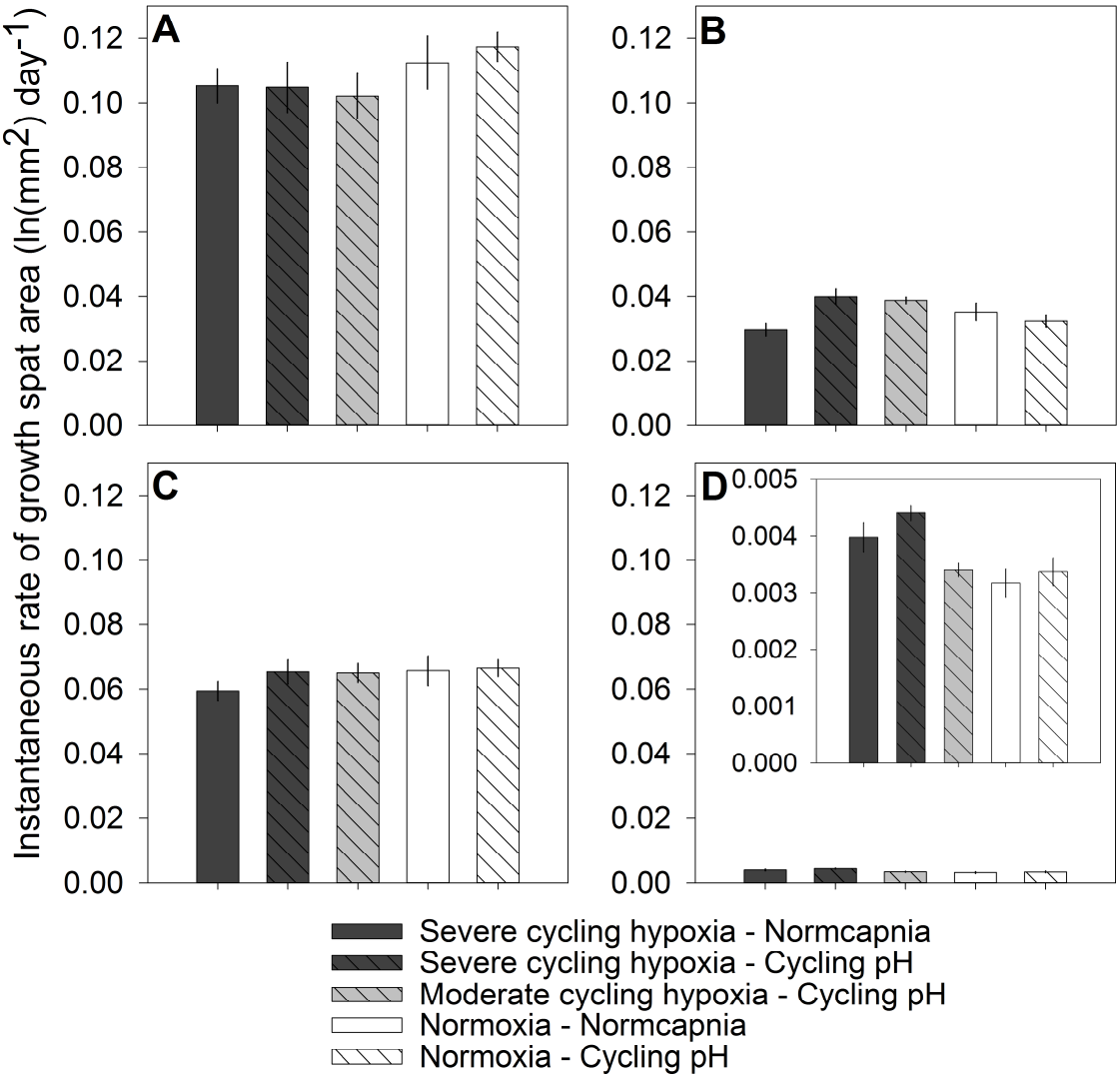

Figure 10. 2012 juvenile growth experiment. Mean ± SE instantaneous rate of growth in area by treatment of spat exposed to diel cycles 4-5 d wk^{-1} during (A) the first two weeks, (B) second two weeks, (C) the course of the entire experiment, and (D) during a 9-month field deployment (inset) with y-axis adjusted to increase visibility of differences among treatments.

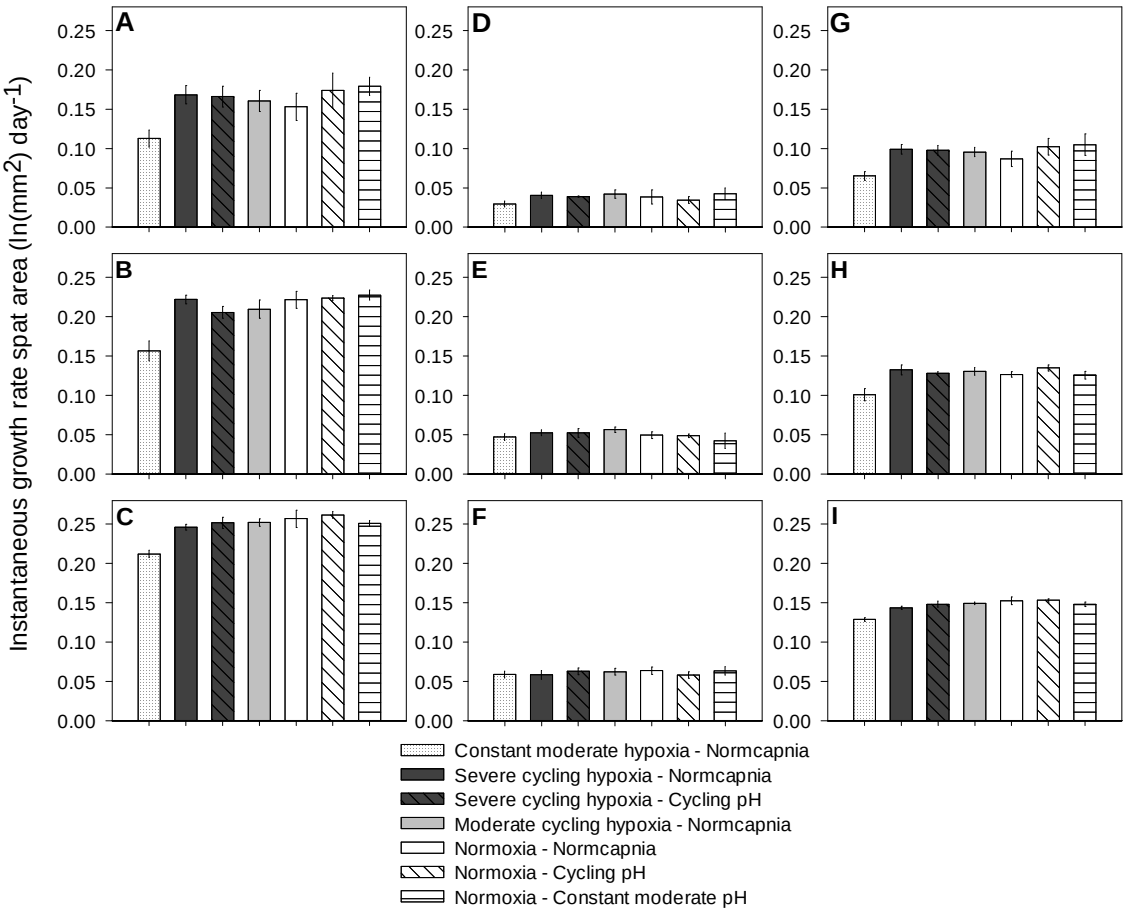

Figure 11. 2013 juvenile growth experiment. Mean ± SE instantaneous rate of growth in shell area during first two weeks of the experiment for three age classes of spat; (A) 4 weeks post settlement, (B) 2 weeks post-settlement, and (C) 1 week post-settlement, (D-F) during second two week experimental period, and (G-I) during full 4 weeks of experiment for the same three age classes.

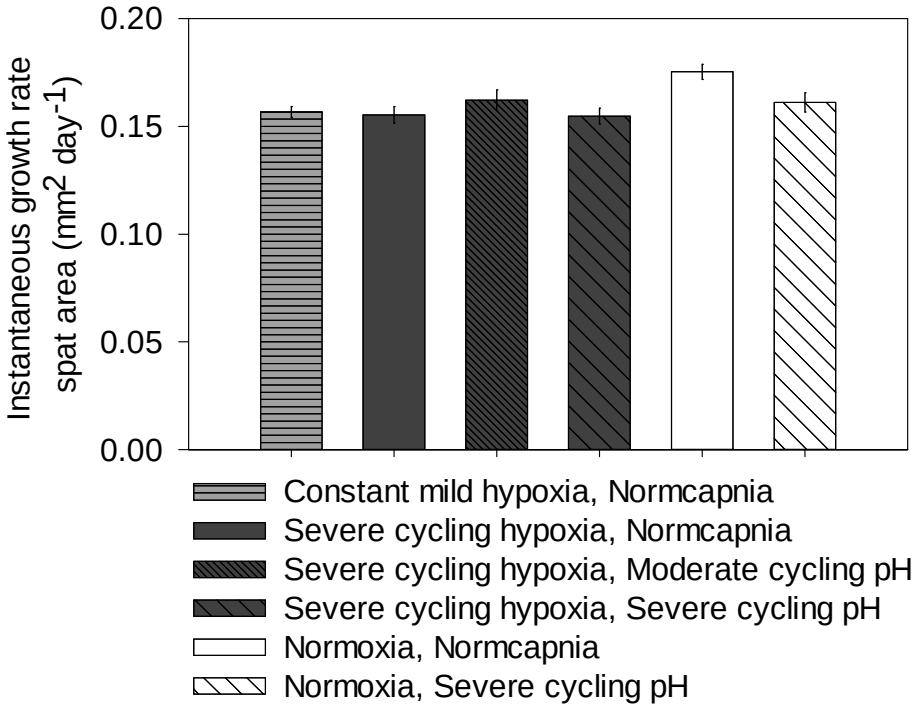

Figure 12. 2014 juvenile growth experiment. Mean ± SE instantaneous rate of growth by treatment of spat exposed to diel cycles 5-6 d wk^{-1} during two week laboratory experiment.

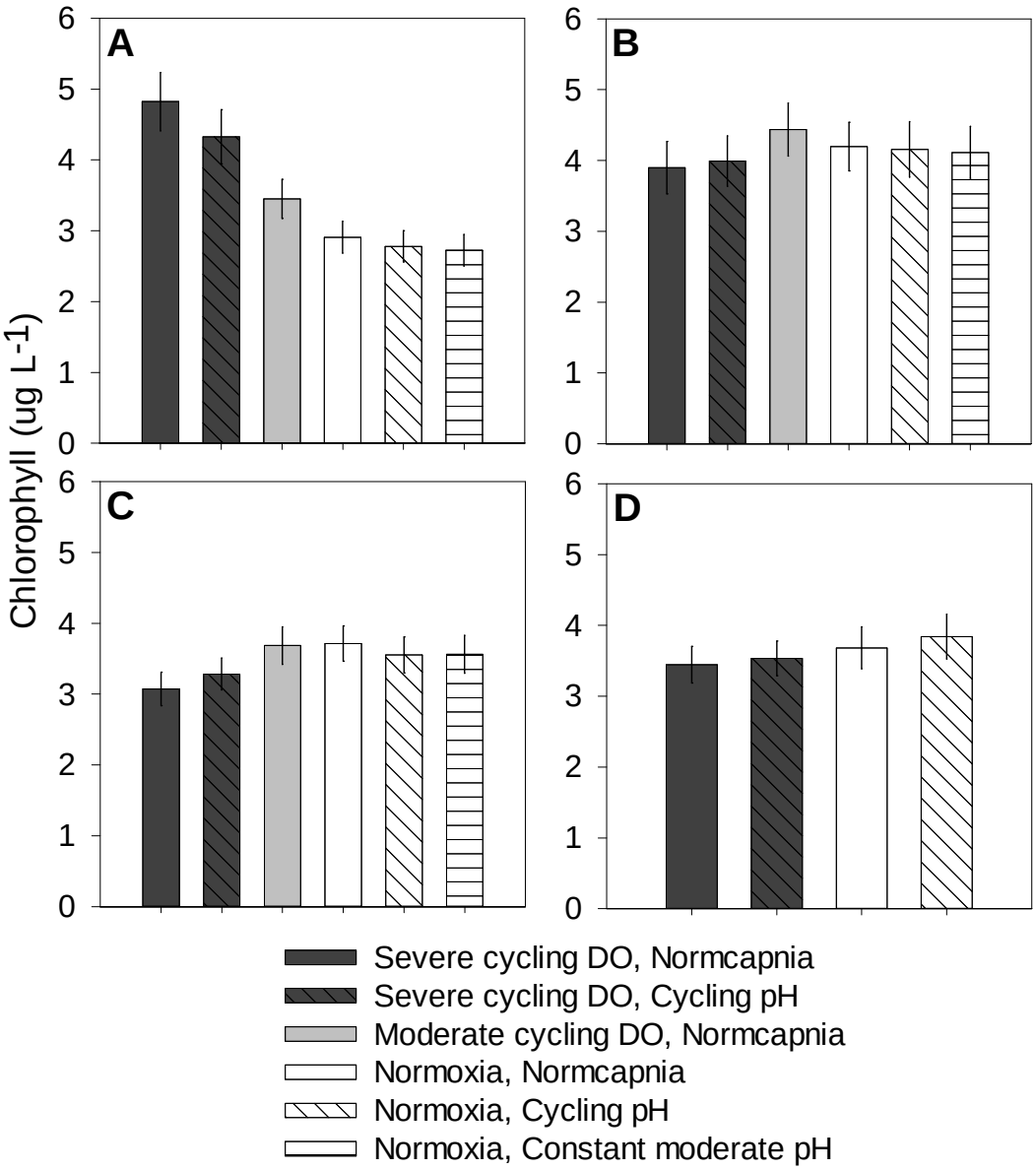

Figure 13. 2013 adult growth experiment. Mean ± SE in-tank chlorophyll *a* levels as measured by in-vivo fluorescence at different points in the cycle: (A) at low-plateau (simulated early morning), (B) immediately after returning to normoxia (simulated late morning), (C) during the supersaturated plateau (simulated mid-afternoon) and (D) at normoxia prior to reducing DO and/or pH (simulated late evening). Fluorescence was measured on 11 days at low oxygen/low pH, 6 days at return to normoxia, 5 days at supersaturation of days in all aquaria, and on 3 days at normoxia before low oxygen/low pH.

Appendix A. Mean±SE immune response parameters as measured by flow-cytometry. N=12 samples.

	Severe hypoxia Normcapnia	Severe hypoxia Cycling pH	Moderate hypoxia Cycling pH	Normoxia Normcapnia	Normoxia Cycling pH
Shell Height (mm)	61.92± 2.45	62.82± 2.61)	57.83± 1.33)	58.58± (1.23)	58.75± (1.80)
Infection Intensity	0.54±0.12	0.64±0.23	0.71±0.35	0.96±0.39	0.25±0.17
Percent granular hemocytes	0.20±0.00	0.12±0.00	0.13±0.00	0.13±0.00	0.16±0.00
Percent dead granular hemocytes	0.09±0.00	0.14±0.00	0.11±0.00	0.15±0.00	0.10±0.00
Percent agranular hemocytes	0.70±0.00	0.75±0.00	0.75±0.00	0.72±0.00	0.71±0.00
Percent dead agranular hemocytes	0.03±0.00	0.04±0.00	0.03±0.00	0.05±0.00	0.03±0.00
Percent phagocytic granular hemocytes	0.27±0.00	0.25±0.00	0.17±0.00	0.13±0.00	0.24±0.00
Percent phagocytic hemocytes	0.13±0.00	0.12±0.00	0.09±0.00	0.07±0.00	0.13±0.00
Reactive oxygen species (ROS) production - granular population 1	9268.77± 1315.08	8505.69± 708.06	8928.92± 945.56	7900.61± 489.09	9357.10± 785.06
Reactive oxygen species (ROS) production - granular population 2	359.28± 17.95	364.95± 21.08	318.51± 26.16	343.40± 21.08	278.53± 12.29
Apoptotic dead cells	0.04±0.00	0.05±0.00	0.06±0.00	0.10±0.00	0.06±0.00
Apoptotic live cells	0.02±0.00	0.03±0.00	0.04±0.00	0.07±0.00	0.04±0.00

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