

## ABSTRACT

Title of Document: THE EFFECTS OF THE DIEL-CYCLING OF DISSOLVED OXYGEN AND pH ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN), CLEARANCE RATES AND HEMOLYMPH pH

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Chronic hypoxia and hypercapnia affect *Crassostrea virginica*. Less is known about how the co-cycling of these stressors, as occurs in shallow waters worldwide, affects this filter feeder. I used laboratory experiments and age-specific models to test how diel-cycling hypoxia and hypercapnia affect algal clearance rates by *C. virginica* and *C. virginica* hemolymph pH. Clearance rates were reduced during periods of low dissolved oxygen, but older oysters compensated by clearing faster when DO returned to normoxia. Models indicated that this compensatory feeding may allow older oysters to avoid decreases in average summertime clearance rates. Low hemolymph pH has been linked to decreased immune function in marine invertebrates and low water pH decreases the hemolymph pH of oysters. My hemolymph experiment also showed that hemolymph pH decreased with decreasing water pH and indicated that oysters may begin to compensate for declining water pH at water pH values between 7.60 and 7.36.

THE EFFECTS OF THE DIEL-CYCLING OF DISSOLVED OXYGEN AND pH  
ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN),  
CLEARANCE RATES AND HEMOLYMPH pH

By

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## Dedication

To my wonderful parents, who sacrificed to provide ceaseless support and without whom I would not have come this far. Thank you for being there!

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# **Chapter I: The Effects of the Diel-Cycling of Dissolved Oxygen and pH on the Clearance Rates of Algae by the Eastern Oyster, *Crassostrea virginica* (Gmelin)**

## ***Introduction***

Organisms often experience single and multiple stressors in cycles, reflecting temporal variations in the environment. These cycles can be daily, such as the diel-cycling of dissolved oxygen (DO); seasonal, such as salinity, temperature, and nutrient changes experienced in systems with high springtime river flow; or can span even longer time scales, such as climactic variations caused by the El Niño Southern Oscillation. Organisms may respond differently to cycling conditions than to constant exposure to stressors. For example, larval mud crabs exposed to daily cycles of temperatures outside of their tolerance range have better survival than those constantly exposed to the same temperatures (Costlow & Bookhout 1971), while brown shrimp exposed to daily cycles of hypoxia have a poorer survival rate than those exposed to chronic hypoxia of the same severity (Brouwer et al. 2005).

The diel-cycling of DO and pH occurs in shallow waters, as a result of the day/night variation in photosynthesis and respiration. During daylight, when photosynthesis produces more oxygen than is consumed through respiration, high DO concentrations, including super-saturated (i.e., >100% air-saturation) conditions, can occur. During the night and early morning, conditions can become hypoxic or anoxic as respiration by both autotrophs and heterotrophs depletes water column oxygen

(D'Avanzo and Kremer 1994). The peaks and troughs of this cycle are intensified in eutrophic systems (D'Avanzo et al. 1996). Carbon dioxide, produced by respiration and consumed through photosynthesis, follows the opposite daily cycle of DO, reducing pH in the hours of dark and increasing pH in daylight (Ringwood & Keppler 2002).

The diel-cycling of DO occurs globally in a variety of habitats (Tyler et al. 2009). Daily DO cycles reaching hypoxic conditions have been found in habitats ranging from shallow commercial shrimp ponds in Hawaii (Szyper & Ebeling 1993), to a French Polynesian coral reef (Gattusso et al. 1993), and to a lagoon on the Iberian Peninsula (Cobelo-García 2012). In the Chesapeake Bay, DO and pH cycles can vary in amplitude with DO concentrations approaching anoxia near dawn and exceeding 11 mg L<sup>-1</sup> (roughly 150% air-saturation) during the day, and daily pH falling to 6.25 and rising to 9.00 in some locations (Fig. 1). I use an operational definition of hypoxia here as <30% air-saturation, or about 2.25 mg L<sup>-1</sup> given the water temperatures and salinities in the experiments I conducted.

In estuarine ecosystems along the Atlantic and Gulf Coasts, the eastern oyster, *Crassostrea virginica* (Gmelin), is an ecosystem engineer, a keystone species, and supports an economically important fishery. Oyster reefs create a diverse habitat that provides food and spawning grounds for a wide variety of organisms (Coen et al. 1999; Peterson et al. 2003; Breitburg & Fulford 2006; Grabowski & Peterson 2007). Filtration by oysters and similar benthic filter feeders also couples pelagic and benthic processes, removing nutrients from local waters and transporting them to the bottom sediments (Peterson et al. 2003; Porter et al. 2004; Newell et al. 2002). As filter feeders, oysters and other bivalves consume phytoplankton (Newell 1988) and alter the biomass and the

species composition of phytoplankton (Huang et al. 2008; Li et al. 2012). Models applied to the Chesapeake Bay have shown that filtration by restored oyster beds may help improve water quality by removing phytoplankton at the tributary scale (Fulford et al. 2007), increase water clarity and seagrass density and distribution (Newell and Koch 2004; Cerco and Noel 2007), increase summer bottom water DO (Cerco and Noel 2005, 2007), decrease Bay wide summer chlorophyll a concentration (Cerco and Noel 2005, 2007), and enhance denitrification (Kellogg et al. 2013). However, these effects will depend on the density of the local oyster population, the bathymetry and hydrodynamics of the habitat, and other factors like hypoxia (Fulford et al. 2007).

Both hypoxia and hypercapnia are known to affect this environmentally valuable species. Hypoxia alters oyster transcriptomes, changing the proteins oysters produce (Chapman et al. 2011) and affects a wide range of factors in both larval and adult oysters including feeding, growth, and susceptibility to other stressors. Hypoxia decreases larval growth (Baker & Mann 1992; Gobler et al. 2014) of bivalves, and the ingestion rates of small (436  $\mu\text{m}$ ) post-settlement oysters (Baker & Mann 1994). Hypoxia increases oyster susceptibility to other stressors such as cadmium (Ivanina et al. 2011). Low DO also increases oyster susceptibility to pathogens like bacteria (Macey et al. 2008) and *Perkinsus marinus* (Anderson et al. 1998; Boyd and Burnett 1999; Breitbart et al. in review), the protistan parasite that has contributed to high rates of oyster mortality in the Chesapeake Bay since the 1980's (Burreson & Ragone-Calvo 1996). Hypoxia increases vulnerabilities to these pathogens by decreasing the production of reactive oxygen intermediates (Boyd & Burnett 1999).

Hypercapnia also affects oysters in a variety of ways. On the molecular scale, pH, along with salinity, has the greatest effect on *C. virginica* transcriptomes out of all the environmental factors tested (Chapman et al. 2011). Hypercapnia decreases larval and juvenile biocalcification (Miller et al. 2009; Beniash et al. 2010; Waldbusser et al. 2011) successful larval development (Kurihara et al. 2008), and larval survivorship (Gobler et al. 2014) in bivalves. Effects of hypercapnia on bivalve feeding are poorly understood, with some studies indicating that lower pH increases clearance rates (Li et al. 2012) and others indicating no effect at all (Chen et al. 2007). Additionally, hypercapnia may affect oyster innate immune defenses, making them more susceptible to other stressors. External low pH (7.1) can cause a correlated reduction in hemolymph pH (Boyd & Burnett 1999); this reduction is noteworthy because some studies indicate that hemolymph pH affects *C. virginica* immune functions. As hemolymph pH decreases, so does production of reactive oxygen intermediates (Boyd & Burnett 1999), and clearance of *Vibrio campbellii* (Macey et al. 2008).

In general, far less is known about the effects of the diel-cycling of DO than about exposure to constant conditions. Additionally, co-occurring stressors often have interactive effects (Breitburg et al 1998; Folt et al. 1999; Egilisdottir et al. 2009) and the co-cycling of DO and pH, as occurs in the field, may alter the response of oysters to exposure to extreme DO and pH. Hypoxia and acidification are of immediate concern in the Chesapeake Bay and other coastal systems where symptoms of eutrophication, have been increasing in frequency and severity (Kemp et al. 2005; Diaz & Breitburg 2009). Additionally, the pH of ocean surface water is predicted to decrease 0.3–0.5 units by year

2100 due to a rise in atmospheric carbon dioxide (Caldeira and Wickett 2005) and estuarine waters are exposed to the same increase in atmospheric CO<sub>2</sub>.

In the present study, I examined the effects of the diel-cycling of DO and pH on the filtration of algae by *C. virginica*. I used the rate of removal or clearance of algae (hereafter referred to as clearance rate) from the water by *C. virginica* as a proxy for filtration. Specifically, I asked the following questions: 1) Do the diel-cycling of DO and the diel-cycling pH affect clearance rates separately and interactively during any part of the daily cycle? 2) Do oysters exhibit decreased effects of cycling conditions on their clearance rates after repeated exposures? 3) What do these experimental clearance rates predict under fluctuating DO conditions found in the field?

## **Methods**

From 2010 to 2012, I ran 5 experiments to test the effects of the diel-cycling of DO and pH on algal clearance rates by *C. virginica*. DO and pH were manipulated by adjusting air, CO<sub>2</sub>-free air, N<sub>2</sub>, CO<sub>2</sub>, and O<sub>2</sub> gasses delivered to the experimental tanks. Control treatment tanks were bubbled continuously with air to maintain air-saturated conditions. For the purpose of these experiments normoxia was defined as DO concentrations near 100% air-saturation and normcapnia was defined as 7.8, which was the pH consistently achieved through vigorous aeration of the water in our experimental systems. In general, each 24-h treatment cycle had 13 h of normoxia/normcapnia ('normoxia plateau'), a 4- h descent to the low targets of DO/pH ('descent to low targets'), 3 h of low DO/pH at these targets ('low plateau'), and a 4-h return to normoxia/normcapnia ('return to normoxia'). Day/night light cycles were altered in these experiments so that low DO and dark photo-periods co-occurred during the day when conditions could easily be monitored. I also performed a single experimental cycle consisting of 19 h of normoxia, a 2 h ascent to supersaturated DO (ie., >100% air-saturation) targets, maintenance of that DO for 3 h ('supersaturated DO plateau') followed by a quick return to normoxia. A summary of the methods by experiment can be found in Table 1.

## **Oyster Collection and Holding**

Oysters of 2 age classes, 1-year-olds and 3-year-olds in 2010 and 2012, and 1-year-olds and 4-year-olds in 2011, were purchased each year from Marinetics Inc. (Cambridge, MD). Starting standard shell height (hinge to bill) of all the experimental



oysters was measured with a flexible ruler following the protocol used by the Maryland Department of Natural Resources. Salinity at the Marinetics facility was within 5 units of Rhode River water. At the Smithsonian Environmental Research Center (SERC), oysters were placed in a raceway filled with water of the same salinity from which they had been taken, and Rhode River water was slowly dripped in.

### **The ‘Discrete DO Experiment’**

The Discrete DO Experiment tested the effect of the diel-cycling of DO on algal clearance rates by oysters using 3 treatments that each had a specific DO target for the ‘low plateau’ phase of the experiment. pH was not manipulated. DO treatments were used as class variables in statistical analyses.

DO was manipulated with gas proportioners that controlled the N<sub>2</sub>:Air ratio delivered to the 30 tanks. Oysters were placed in tanks that were arranged in a randomized block design with 5 replicates of each of 3 treatments: a control in which DO was held at normoxia, and treatments with low plateau targets of moderate and severe DO (1.5 mg L<sup>-1</sup> and 0.5 mg L<sup>-1</sup> DO, respectively; Fig. 2). Daily cycles were maintained for about 3 mo for 5 d per week, beginning mid-July. DO in all tanks was held at normoxia for the remaining 2 d per week. This was done so that the low DO periods occurred when conditions could be monitored and approximates the frequency of low DO cycles found at some field sites. DO concentration, salinity, and water temperature were measured 2 to 3 times per day in all tanks with a YSI Professional Plus (Yellow Springs Instrument Company). Peristaltic pumps continuously supplied raw water from the Rhode River supplemented with cultured live algae, *Chaetoceros muelleri*, from Horn Point Laboratory (Cambridge, MD. As part of a larger disease transmission experiment

(Breitburg et al., submitted), peristaltic pumps also transferred water from the 3-year-old oyster tanks to the corresponding 1-year-old oyster tanks. During the last week of the experiment, water flow rates to the tanks were halved to help maintain summer water temperatures. Oysters were removed from their tanks for cleaning once every 14 d during the DO uptrend. Five weeks into the experiment, 30 oysters from each tank were removed and dissected as part of the larger disease experiment.

I measured clearance rates at the end of the normoxia plateau and at the end of low plateau on 18 separate dates and when conditions had just returned to normoxia ('recovery point') on a single date. Clearance rates at the end of the normoxia plateau and at the end of the low plateau were based on measurements taken while oysters were in their experimental aquaria and were calculated using the flow-through method (see below). This method was inappropriate for measuring clearance rates at the recovery point when conditions had changed rapidly in the preceding 3 h. Therefore, on a single date, I removed half of the oysters in each tank (between 39 and 45 individuals) at the recovery point and placed them into 20 L containers of water that were bubbled with air to maintain normoxia. The number of oysters was noted and clearance rates were measured using the clearance rate method (see below). After 1 h, oysters were returned to their experimental tanks.

### **The 'Continuous DO Experiment'**

The Continuous DO Experiment tested the effects of a broader range of DO concentrations on clearance rates and used DO as a continuous variable in analyses. Eighteen low DO plateau time periods (Fig. 3) were tested. pH was not manipulated in this experiment.

One-year-old oysters were placed in 18 tanks, each with a different pattern of cycling DO, with DO concentration of the low DO plateau period ranging in from 0.28 mg L<sup>-1</sup> to 7.30 mg L<sup>-1</sup>. DO was manipulated using gas proportioners to control the N<sub>2</sub>:Air on each day of this 4 d experiment. The 1-year-old oysters were then removed, tanks were cleaned and 3-year-old oysters were placed in the 18 tanks for another 4 d of experimentation. Water supplied to tanks was heated to help maintain consistent summertime water temperatures.

I measured clearance rates using the clearance method at the at the end of normoxia plateau, at the beginning and end of low plateau, as DO returned to normoxia, and at the beginning of and 2 h after the recovery point (Fig. 3). I paused water flow during clearance rate measurements, and injected 5 mL of a concentrated commercial phytoplankton (DT's Live Marine Phytoplankton, Innovative Marine Aquaculture, Inc.) into each tank when water flow was stopped before taking IVF measurements. A YSI 85 (Yellow Springs Instrument Company) was used to measure salinity, water temperature, and DO immediately after clearance rate measurements were made. Total alkalinity of incoming Rhode River water was taken on 3 separate dates and determined through titration using a Corning pH Analyzer 350 (APHA 1992). pCO<sub>2</sub> was then calculated using CO2SYS (Pelletier et al. 2007) with the constants of Cai and Wang (1998).

### **The 'Discrete DO and pH Experiment'**

The Discrete DO and pH Experiment tested the separate and combined effects of cycling DO and pH on clearance rates using an incomplete factorial design combining 3 levels of cycling DO with 2 levels of cycling pH (Fig. 4). Each of the 5 treatments had

discrete DO and pH targets for the low plateau phase of the cycle. Treatments were used as class variables in statistical analyses.

Methods repeated those used in the Discrete DO Experiment with the exceptions listed here and in Table 1. The ratio of air, CO<sub>2</sub>-stripped air, N<sub>2</sub>, CO<sub>2</sub>, and O<sub>2</sub> gasses delivered to the tanks were manipulated to achieve target DO and pH. Two new treatments were added: 1) co-cycling moderate (1.5 mg L<sup>-1</sup>) DO and pH and 2) co-cycling severe (0.5 mg L<sup>-1</sup>) DO and pH. In both cases pH cycled from 7.8 during simulated daytime to 7.0 during simulated night (Fig. 4). To simulate the full daily cycle often seen in the field, the cycling treatments included a supersaturated DO phase (Fig. 2). Raw water was supplemented with a mixture of cultured (*Chaetoceros muelleri* from Horn Point) and concentrated (DT's Live Marine Phytoplankton) algae. Measurements of pH in all tanks coincided with the other water quality measurements and were made with an Acorn pH 5 Meter (Oakton Instruments) with a single-junction pH electrode and temperature probe. Total alkalinity of incoming Rhode River water was taken on 6 dates.

I estimated algal clearance rates by oysters at the end of the normoxia plateau and at the end of the low plateau on 5 dates using the flow-through method. I also estimated clearance rates using the clearance method at the recovery point on 2 dates by removing oysters from their tanks and placing them into 20 L containers for 60 min. The Discrete DO and pH experiment ran for 2 mo, starting in early August, and was terminated because of low salinity caused by Hurricane Irene and Tropical Storm Lee.

### **The 'Continuous DO, Discrete pH Experiment'**

The Continuous DO, Discrete pH Experiment tested effects of diel-cycling pH on clearance rates under a wide range of DO cycles to supplement data from the Discrete

DO and pH Experiment. Fifteen constant pH treatment replicates and 10 cycling pH treatment replicates were exposed to different patterns of cycling DO, with DO concentration of the low DO plateau period ranging in from 0.06 mg L<sup>-1</sup> to 8.62 mg L<sup>-1</sup> (Fig. 3) and run for 4 d in 25 tanks for each age class. I analyzed pH as a class variable and DO as a continuous variable.

pH was controlled and monitored continuously in 1 replicate tank of each treatment using Honeywell Durafet pH probes and gas flow rates were adjusted using a LabView (National Instruments Corps) program (Burrell et al. unpublished). As in the Discrete DO and pH Experiment, I used an Oakton Acorn pH 5 Meter to measure water pH, and a heater to help maintain summertime water temperatures. I measured algal clearance rates by oysters using the clearance method at the end of normoxia plateau, at the beginning and end of low plateau, and at the recovery point and 2 h after.

### **The ‘Supersaturated DO Experiment’**

The Supersaturated DO Experiment tested effects of supersaturated (i.e., >100% air saturation) DO on clearance rates to simulate conditions that are common in the field during mid-day, especially during algal blooms. One-year-old and 3-year old oysters were each tested on a separate date. Five oysters were placed in each of 25 tanks, each with a different supersaturated target. The LabView system was used on a single date for each oyster age class to increase DO from air-saturated to supersaturated concentrations over 2 h and hold DO at supersaturation for 3 h. I measured clearance rates using the clearance method at the end of the supersaturated plateau (Fig. 3).

## Clearance Rate Calculations

The flow through method (Hildreth and Crisp 1976) of clearance rate calculations was performed directly in experimental tanks supplied with flowing water during measurement periods. I quantified algal concentrations in the inflow water and in the oyster tanks using in vitro fluorometry (IVF) measured in the Discrete DO Experiment and the Continuous DO Experiments with a 10AU Field Fluorometer (Turner Designs) and in the Discrete DO and pH Experiment with an AquaFluor™ Hand Held Fluorometer (Turner Designs). These IVF data were converted to chlorophyll- $\alpha$  concentrations using spectrophotometric analysis (Jeffrey and Humphrey 1975). Clearance rates in each tank were calculated as:

$$CR = Fl \times \left( \frac{C_2 - C_1}{C_2} \right),$$

where CR is the oyster's clearance rate, Fl is the flow rate of water in  $L h^{-1}$ ,  $C_1$  is the inflow concentration of algae in  $\mu g L^{-1}$ , and  $C_2$  is the in-tank concentration of algae in  $\mu g L^{-1}$  (Hildreth & Crisp 1976). Data on water flowing from the older oyster tanks was used to correct flow rate and inflow algal concentration for the 1-year-old tanks.

I calculated clearance method clearance rates at time points where water was not flowing to the oysters:

$$CR = \frac{V}{nt} \times \ln \left( \frac{C_0}{C_t} \right),$$

where V is the volume of water, n is the number of oysters, t is time,  $C_0$  is the initial algal concentration, and  $C_t$  is the concentration of algal cells at time t (Riisgård 2001). Algal

concentrations for the clearance method were estimated in the same way as for the flow through method.

### **Additional Tests of Clearance Rate Methods**

To ensure that any difference in the algal concentrations of inflow and in tank water was due to the presence of oysters, I measured the 'clearance rate' of algae in a tank containing no oysters on 14 dates during the Discrete DO Experiment and used the flow through method clearance rate formula. To test for direct effects of the experimental DO and pH manipulations on phytoplankton, as well as any change in IVF not due to oysters, I also measured the initial and final IVF of 4 replicate tanks of a 4 treatments: a control held at normoxia/ normcapnia, a severe hypoxic treatment held at 0.5 mg L<sup>-1</sup> DO and 7.8 pH, a hypercapnic treatment held at 7.0 pH and normoxia, and a severe hypoxic, hypercapnic treatment held at 0.5 mg L<sup>-1</sup> DO and 7.0 pH. I also used the clearance method formula to measure any loss of algae over ten minutes in these empty tanks. Finally, I found an appropriate time to wait between measuring initial and final IVF measurements when using the clearance method to measure the algal clearance rates of five 1-year-old and five 3-year-old oysters. I set up a tank with five 1-year-old oysters held at normoxia and injected 5 mL DT's live Marine Phytoplankton (Innovative Marine Aquaculture, Inc.) and measured the initial IVF and the IVF of the water every 5 min for 25 min. I repeated this procedure with 3-year-old oysters.

### **Weights**

Wet weights of at least 30 oysters from each tank were measured to the nearest tenth of a gram at the end of each experiment. In the Discrete DO and pH Experiment,

100 oysters from the starting cohort of each age class had their meat dried for 48 hrs at 105°C (Mo & Neilson 1993) All oysters from both Continuous DO Experiments were dissected and the meat was dried. Dry weights were measured to the nearest ten thousandth of a gram. Dry Weights from the Discrete DO and pH and Continuous DO Experiments oysters were used to convert wet weights to dry weights for the Discrete DO Experiment oysters. Growth and weight loss during experiments was negligible (Keppel et al. Unpublished). All clearance rates were divided by the average dry weights of oysters in a tank; all reported clearance rates are weight specific.

### **Statistical Analyses**

All statistics were performed with Statistical Analysis Software 9.2 (SAS), unless otherwise noted, and  $p < 0.05$  was used as the significance level. Non-significant interaction terms were removed from models. When an overall model was significant, I used least squares means (LS means) for pre-planned pairwise comparisons of cycling treatments with controls. Additional treatment comparisons are described below.

To test my clearance rate methodology, I first used a t-test to compare the flow through method estimated 'clearance rate' of algae in an empty tank to the value zero; separate days were used as replicates. I used a paired t-test to assure that initial and final IVFs in empty tanks of non-flowing water were not significantly different and that the 'clearance rate' calculated by the clearance method in these tanks was not affected by DO and/or pH treatments.

I performed 1-way ANOVAs (Proc GLM) to determine whether oyster starting sizes, salinity, and temperature varied among treatments for any experiment. I also tested for an effect of salinity and temperature on clearance rate for the combined data from the



Discrete DO and Discrete DO and pH Experiments (Proc GLM). I did not test for effects of salinity or temperature on the Continuous DO or the Continuous DO, Discrete pH Experiments' clearance rate data, because individual oysters in these experiments did not experience a wide range of temperatures and salinities.

To analyze data from the Discrete DO Experiment I used repeated measures ANOVAs (Proc Mixed) on data taken at the end of the normoxia plateau and the end of the low plateau to test for the effect of treatment, date, and their interaction on algal clearance rates by oysters. Clearance rates by oysters at the recovery time point in this experiment were only measured on one date and I therefore used a 1-way ANOVA (Proc GLM) to test for differences among treatments. I used LS means pre-planned pairwise comparisons to compare the clearance rates by oysters in the moderate and severe cycling DO treatments to those in the controls for all three time points. Finally, to determine if the response of algal clearance rates by oysters to experimental treatments changed with the duration of exposure, I used a linear regression (Proc GLM) to test the effect of the number of days since the start of the experiment on the magnitude of the response (mean clearance rates of treatment / mean clearance rates of controls) for each treatment for the first 30 days of the experiment. Since clearance rates were measured regularly during the first 30 days but not again until day 62 and again on day 84, I used paired t-tests to compare the magnitude of the response for each treatment on day 62 and day 84 to the average of that in the first 30 days.

For the Continuous DO Experiment, I used a 1-way ANOVA (Proc Glm) for the 1-year-old clearance rates taken as DO was returning to normoxia because the low plateau DO these oysters had experienced only ranged from 0.79 to 1.10 mg L<sup>-1</sup> and a

linear regression would have been inappropriate. For all other time points in the Continuous DO Experiment, which tested a range of DO concentrations on clearance rates, I used DO as a continuous variable and used linear and non-linear regressions (SigmaPlot 11.0), selecting the best model based on corrected Akaike information criterion (AICc) scores.

For the Discrete DO and pH Experiment I used repeated measures ANOVAs (Proc Mixed) on data measured at all three time points, at the end of the normoxia plateau, at the end of the low plateau, and at the recovery time point, to test for the effect of treatment, date, and their interaction on clearance rates by oysters. To test the effects of cycling pH conditions on algal clearance rates by oysters under cycling DO conditions, I also compared the mean clearance rates by oysters in the severe DO cycling treatment to those in the severe DO treatment that co-cycled with pH, and compared the mean clearance rates by oysters in the moderate DO only cycling treatment to those in the moderate DO treatment that co-cycled with pH.

For the Continuous DO, Discrete pH Experiment, I used ANOVAs (Proc Glm), with DO concentration as a continuous variable and pH treatment as a class variable, to test for the effect of DO concentration, pH treatment, and their interaction on algal clearance rates by oysters for all time points. I also used linear and nonlinear regression (SigmaPlot 11.0) to test the effect of DO on clearance rates, selecting the best model based on AICc scores. Finally, I used quantile linear regression (Proc Quantreg) on the combined data from both Continuous DO Experiments to test for the effect of DO concentrations below  $2.5 \text{ mg L}^{-1}$  on clearance rates in the 90th, 50th, and 10th percentiles.

## Modeling Clearance Rates

I used clearance rate measurements from both Continuous DO Experiments to construct models to simulate the effects of cycling DO on 1-year-old and algal clearance rates by 3-year-old oysters. Parameters for each model were estimated concurrently by fitting the model to observed clearance rates and by minimizing the sum of squared errors. I chose to incorporate or exclude parameters in the model based on corrected Akaike information criterion (AICc) scores, only including those that lowered the models AICc score absolute value by at least 2.

The predicted weight specific clearance rate for a 1-year-old oyster in liters per hour per gram of dry meat was calculated as:

$$CR = CR_{temp} \times f(DO),$$

where  $CR_{temp}$  was the temperature dependent clearance rate and  $f(DO)$  was the proportional effect of DO on that temperature dependent clearance rate. The predicted algal clearance rate by 3-year-old oysters also included a factor to estimate the effect of previous exposure to low DO on current clearance rates. I included this because I found that 3-year-old oysters increased clearance rates above baseline levels after exposure to hypoxia and once they were returned to normoxia. I called this parameter ‘hypoxia exposure’ because it simulated persistent exposure to sub-optimal DO and its effect on the temperature dependent clearance rates; it was symbolized by  $f(E)$ . Therefore, the predicted weight specific clearance rate for a 3-year-old oyster in liters per hour per gram of dry meat was calculated as:

$$CR = CR_{temp} \times f(DO) \times f(E),$$

The model for 1-year-old oysters included a linear function of temperature dependent clearance rate

$$CR_{temp} = CR_{base} \times (Slope_{temp} \times T_t + Intercept_{temp}),$$

where  $CR_{temp}$  was the temperature dependent maximum clearance rate,  $T_t$  is the water temperature in degrees Celsius at time  $t$ ,  $CR_{base}$  was the average clearance rate of an oyster  $Slope_{temp}$  is the slope of the linear effect of temperature on clearance rate and  $Intercept_{temp}$  was the intercept of the linear function of temperature on oyster clearance rate. The model for 1-year-old oysters also included a logistic function describing the effect of DO (Fulford et al. 2007)

$$f(DO) = \frac{1}{1 + \exp(DO_{zero} \times \left( \frac{DO_{half} - DO_t}{DO_{half} - DO_{quarter}} \right))},$$

where  $f(DO)$  was the effect DO has on clearance rate,  $DO_{zero}$  was the DO concentration at which clearance would be zero,  $DO_{half}$  was the DO concentration at which clearance would be half the temperature dependent maximum clearance rate, and  $DO_{quarter}$  was the DO concentration at which clearance would be a quarter temperature dependent maximum clearance rate.

I estimated the correlation between hypoxia exposure and the increase in algal clearance rates by oysters previously exposed to low DO. This hypoxia exposure function was an exponential rise to a maximum

$$f(E) = E_{zero} + E_a \times (1 - \exp(-E_b \times E_{(t)})),$$

where  $E_{(t)}$  was the calculated amount of hypoxia exposure an oyster has experienced at time  $t$ ,  $E_{zero}$  was the level of hypoxia exposure below which there was no effect on clearance rates,  $E_a$  was the coefficient of the hypoxia exposure at time  $t$ , and  $E_b$  was the

exponential growth parameter. Hypoxia exposure accrued when DO fell below the fitted pivotal value of DO concentration ( $DO_{pivot}$ ), adding the difference between pivotal DO concentration and the current DO ( $DO_{(t)}$ ) to the previous hypoxia exposure ( $E_{(t-1)}$ ):

$$E = E_{(t-1)} + (DO_{pivot} - DO_{(t)})$$

Hypoxia exposure was paid off when DO rose above the critical oxygen concentration by subtracting the difference between the previous clearance rate and the previous temperature dependent clearance rate maximum from the previous hypoxia exposure. Paying off hypoxia exposure was controlled by the following formula, where  $CR_{(t-1)}$  was the previous clearance rate and  $CR_{temp(t-1)}$  was the previous temperature dependent clearance rate:

$$O_{2.debt} = O_{2.debt(t-1)} - (CR_{(t-1)} - CR_{temp(t-1)})$$

Hypoxia exposure was constrained to prevent it from becoming negative.

I applied my models to sites in the Maryland portion of the Chesapeake Bay, including those where Maryland DNR's constant monitoring program EyesontheBay.net had recorded summertime hypoxia at least as bad as those in our laboratory experiments. Several of these locations are near sites of historical or extant oyster bars or of oyster restoration efforts. I used the model to estimate average total daily algal clearance rates by oysters as a function of DO, temperature, basal clearance rate, and hypoxia exposure. I used the Maryland DNR EyesontheBay.net constant monitoring program's water quality data, which is taken every 15 minutes at sites around the Maryland portion of the Chesapeake Bay, to estimate the effect of cycling DO on oysters clearance rates at these sites. I used single measurements at time steps of 2 hrs in duration from these EyesontheBay.net data to make my predictions. This is similar to the time scale of water

quality sampling in my laboratory experiments. I only estimated clearance rates for the summer months of July and August because these were the months during which I ran my experiments and they are the months when the diel-cycling of DO and pH are most severe in the Chesapeake Bay. I applied the models for algal clearance rates by 1-year-old and 3-year-old oysters to these water quality data, summed the total liters filtered on each date and averaged these values for each site. I used a linear regression (Proc Reg) to compare these mean estimate clearance rates to the mean DO and the percent of time DO was less than  $1.5 \text{ mg L}^{-1}$  at each site.

## ***Results***

### **Tests of Clearance Rate Methods**

Within the Discrete DO Experiment, I used the flow through method to measure the algal ‘clearance rates’ in a tank containing no oysters and held at normoxia, and I found that these ‘clearance rates’ were not significantly different from zero ( $t(14)=1.02$ ,  $p=0.33$ ). Before the Continuous DO Experiment, I used the clearance method to measure the algal ‘clearance rates’, and found that there was no difference between starting and ending algal concentrations, as measured by IVF ( $t(15)=0.24$ ,  $p=0.81$ ) in tanks without oysters. In addition, there was no effect of the interaction between DO and pH ( $F(1,14)=0.03$ ,  $p=0.87$ ), DO alone ( $F(1,14)=0.03$ ,  $p=0.86$ ), or pH alone ( $F(1,14)=0.25$ ,  $p=0.63$ ) on the ‘clearance rates’ measured in tanks without oysters. I also measured algal clearance rates by oysters of both age classes using the clearance method every minute for 25 minutes. These clearance rate measurements indicated that the optimal time for measuring clearance rates by oysters in both age classes was around 10 minutes after algal injection. At 10 minutes, clearance rate measurements had reached their maxima and had not yet begun to decline. I therefore waited 10 minutes between initial and final algal concentration measurements for all clearance rates measured in the Continuous DO Experiment and the Continuous DO, Discrete pH experiment.

### **The Discrete DO Experiment**

Measured DO concentrations were near targets (Table 3). Mean starting standard shell heights for 1-year-old oysters and 3-year-old oyster in the Discrete DO Experiment were  $46 \pm 0.21$  SE and  $79 \pm 0.18$  SE mm, respectively. Mean starting standard shell

heights did not vary among treatments (1-year-olds:  $F(2,1348)=1.58$ ,  $p=0.21$ ; 3-year-olds:  $F(2,1348)=0.35$ ,  $p=0.35$ ). Salinity and temperature (Table 4) did not vary among replicate tanks (Salinity:  $F(2,847)=0.01$ ,  $p=0.99$ ; Temperature:  $F(2,769)=0.36$ ,  $p=0.69$ ). Salinity and temperature did, however, vary over the course of the experiment (Salinity:  $F(1, 848)=756.41$ ,  $p<0.0001$ ; Temperature:  $F(1,770)=2285.11$ ,  $p<0.0001$ ). I therefore allowed date to stand as a proxy for salinity and temperature in the repeated measures analyses reported. Both salinity and temperature were positively correlated with the clearance rates by oysters under near-saturation DO concentrations ( $n= 712$ ,  $p<0.0001$ ,  $\text{Adj. } R^2 = 0.18$ ). The mean measured algal clearance rate by control treatment oysters was  $2.4 \text{ L h}^{-1} \text{ g}^{-1} \pm 0.12 \text{ SE}$  ( $n=283$ ).

Algal clearance rates by oysters measured before DO descended each day did not differ among treatments. (Model 1-year-old:  $F(2,12)=3.00$ ,  $p=0.09$ ; 3-year-old:  $F(2,27)=0.43$ ,  $p=0.66$ ). Date did have a significant effect on the clearance rates by 1-year-old ( $F(1,179)=6.75$ ,  $p=0.01$ ) and 3-year-old oysters ( $F(1, 178)=48.72$ ,  $p<0.0001$ ), though clearance rates did not increase or decrease over the course of the experiment, but did differ among days.

Oysters exposed to 3 hours of low DO exhibited decreased clearance rates of algae (Fig. 5). For the 1-year old oysters, only DO reduced clearance rates ( $F(2,12)=27.61$ ,  $p<0.0001$ ) while the algal clearance rates by 3-year-old oysters were significantly affected by the interaction between date and DO treatment ( $F(2,188)=5.95$ ,  $p=0.003$ ). Mean clearance rates for 3-year-old oysters in the severe cycling DO treatment were lower than in moderate cycling DO treatment and both were lower than in the control each day; but the magnitude of the difference among the mean clearance rates for



each treatment varied among dates. The pre-planned pairwise comparisons of treatment clearance rate LS means to control clearance rate LS means showed that oysters of both age classes in the severe (1-year-olds:  $t(12)=-7.43$ ,  $p<0.0001$ ; 3-year-olds:  $t(12)=-13.25$ ,  $p<0.0001$ ) and moderate (1-year-olds:  $t(12)=-3.64$ ,  $p=0.003$ ; 3-year-olds:  $t(12)=-2.89$ ,  $p=0.01$ ) cycling DO treatments cleared algae slower than those oysters in the control.

Algal clearance rates by 1-year-old oysters did not differ among treatments at the recovery point ( $F(1,12)=1.63$ ,  $p=0.23$ ) (Fig. 6). However, 3-year-old oysters that had been exposed to both low DO treatments cleared algae faster than those in the control tanks (Model:  $F(2,12)=4.56$ ,  $p=0.03$ ; Moderate cycling treatment:  $t(12)=2.32$ ,  $p=0.0386$ ; Severe cycling treatment:  $t(12)=2.83$ ,  $p=0.01$ ; Fig. 6).

In the Discrete DO Experiment, oysters were exposed to cycling conditions of DO 5 days per week for about 3 months. The magnitude of the effect of low DO on clearance rates did not change within the first thirty days of the experiment (1-year-old severe cycling DO treatment:  $n=14$ ,  $p=0.82$ ,  $R^2=0.004$ ; moderate cycling DO treatment:  $n=14$ ,  $p=0.18$ ,  $R^2=0.18$ ; 3-year-old severe cycling DO treatment:  $n=14$ ,  $p=0.28$ ,  $R^2=0.10$ ; moderate cycling DO treatment:  $n=14$ ,  $p=0.27$ ,  $R^2=0.01$ ) or by the 62nd or 84th day (1-year-old: day 84: severe cycling DO treatment:  $t(5)=-0.08$ ,  $p=0.93$ ; 3-year-old: day 62: severe cycling DO treatment:  $t(5)=0.36$ ,  $p=0.72$ ; moderate cycling DO treatment:  $t(5)=1.39$ ,  $p=0.17$ ; day 84: severe cycling DO treatment:  $t(5)=1.26$ ,  $p=0.21$ ; moderate cycling DO treatment:  $t(5)=0.23$ ,  $p=0.81$ ), with one exception. Exposure of 1-year-old oysters to moderate cycling on day 62 caused slower clearance rates than similar exposure earlier in the experiment ( $t(5)=6.29$ ,  $p=0.01$ ).

## The Continuous DO Experiment

Mean starting standard shell heights for 1-year-old oysters and 3-year-old oyster in the Continuous DO Experiment were  $47 \pm 0.66$  SE and  $83 \pm 1.24$  SE mm, respectively and did not vary among treatments (1-year-olds:  $F(2,88)=0.39$ ,  $p=0.68$ ; 3-year-olds:  $F(2,88)=1.50$ ,  $p=0.23$ ). In the Continuous DO Experiment, temperature and salinity varied little (Table 3). The average clearance rate by control oysters in the Continuous DO Experiment was  $5.4 \text{ L h}^{-1} \text{ g}^{-1} \pm 0.25$  SE ( $n=139$ ). Total alkalinity in the Continuous DO Experiment averaged  $1564 \text{ } \mu\text{Eq L}^{-1}$ . This indicates that the  $\text{pCO}_2$  of control treatments held at a pH of 7.8 would have been approximately  $1081 \text{ } \mu\text{atm}$  while the  $\text{pCO}_2$  of treatments held a pH of 7.0 would have been approximately  $7030 \text{ } \mu\text{atm}$ .

Algal clearance rates by oysters did not differ among treatments at the end of the normoxia plateau (1-year-old:  $n=41$ ,  $p=0.86$ , Adjusted  $R^2 = -0.02$ ; 3-year-old:  $n=38$ ,  $p=0.5$ , Adjusted  $R^2 = -0.01$ ). However, by the beginning of the low plateau, clearance rates by oysters exposed to low DO had already decreased (1-year-old:  $n=35$ ,  $p=0.03$ , Adjusted  $R^2 = -0.10$ ; 3-year-old:  $n=18$ ,  $p=0.05$ , Adjusted  $R^2 = 0.18$ ; Fig. 7). This was also true for clearance rates measured at the end of the low plateau (1-year-old:  $n=72$ ,  $p=0.003$ , Adjusted  $R^2 = 0.11$ ; 3-year-old:  $n=71$ ,  $p=0.0001$ , Adjusted  $R^2 = 0.44$ ; Fig. 5). These results are similar to those found in the Discrete DO Experiment.

As DO was increasing toward near-saturated conditions in the cycling treatments, during the return to normoxia, no clear pattern for clearance rates could be discerned for either age class (1-year-olds:  $F(1,16)=1.49$ ,  $p=0.24$ ; 3-year-old:  $n=18$ ,  $p=0.49$ , Adjusted  $R^2 = -0.03$ ; Fig. 8). 1-year old oysters did not clear algae at different rates among the different treatment at the recovery point ( $n=18$ ,  $p=0.52$ , Adj  $R^2 = -0.03$ ; Fig. 6). Algal

clearance rates by 3-year-old oysters measured at the recovery point were also unaffected by the DO concentrations they had been held under during low plateau (n=42, p=0.28, Adjusted R<sup>2</sup>=0.004; Fig. 6); this result was inconsistent with those from all other experiments. However, the clearance rates of 3-year-olds that had experienced a low DO plateau were elevated 2 h after the recovery point (n=34, p=0.05, Adjusted R<sup>2</sup>= 0.09; Fig. 9).

### **The Discrete DO and pH Experiment**

Measured DO concentrations were near targets (Table 5). Measured pH was about 0.2 units lower than the pH targets for 4-year-old oyster treatments at the end of the normoxia/normcapnia plateau and for the 4-year-old control at the end of the low DO/pH plateau but was within 0.1 pH units of targets for all other treatments and time periods (Table 5). Mean starting standard shell heights for 1-year-old oysters and 4-year-old oysters in the Discrete DO and pH Experiment were  $45 \pm 0.12$  SE and  $74 \pm 0.17$  SE mm, respectively, and did not vary among treatments (1-year-olds: F(1,2249)=1.19, p=0.28; 4-year-olds: F(1,1998)=0.87, p=0.35). Salinity and temperature (Table 3) did not vary among replicate tanks (Salinity: F(4,365)=0.00, p=1.00; Temperature: F(4,366)=0.02, p=1.00). Salinity and temperature did vary over the course of the experiment (Salinity: F(1, 368)=546.62, p<0.0001; Temperature: F(1,369)=1592.46, p<0.0001). I therefore allowed date to stand as a proxy for salinity and temperature in the repeated measures analyses reported. Both salinity and temperature were positively correlated with the clearance rates by oysters under near-saturation DO concentrations (n= 712, p<0.0001, Adjusted R<sup>2</sup>= 0.18). The mean measured algal clearance rate by control treatment oysters in the Discrete DO and pH Experiment was  $1.3 \text{ L h}^{-1} \text{ g}^{-1} \pm 0.10$  SE (n=90).

Algal clearance rates by oysters did not differ among treatments before DO descended each day (Model: 1-year-old:  $F(4,20)=1.11$ ,  $p=0.38$ ; 4-year-old:  $F(4,20)=0.21$ ,  $p=0.93$ ), as in the Discrete DO Experiment. Date did affect clearance rates by 1-year-old ( $F(1,99)=154.22$ ,  $p<0.0001$ ) and 4-year-old oysters ( $F(1,99)=52.89$ ,  $p<0.0001$ ) at this point of the daily cycle.

Cycling treatments significantly reduced algal clearance rates by oysters of both age classes at the end of the low plateau (Model: 1-year-old:  $F(4,20)=4.59$ ,  $p=0.009$ ; 4-year-old:  $F(4,20)=4.59$ ,  $p=0.009$ ; Fig. 5). Date also affected algal clearance rates by 1-year-old oysters ( $F(1,49)=28.79$ ,  $p<0.0001$ ). Pre-planned comparisons of the LS means of treatments that only manipulated DO showed that cycling DO reduced clearance rates of oysters exposed to constant pH conditions. The severe DO only cycling treatments of both age classes had lower clearance rates than the controls (1-year-old:  $t(20)=-3.81$ ,  $p=0.001$ ; 4-year-old:  $t(20)=-5.25$ ,  $p<0.0001$ ). The 1-year-old oysters in the moderate DO only cycling treatment also had lower clearance rates than those in the controls ( $t(20)=-2.17$ ,  $p=0.04$ ) but the 4-year-old oysters in the moderate DO only cycling treatment did not ( $t(20)=-1.08$ ,  $p=0.29$ ). Pre-planned comparisons of the LS means of treatments that manipulated pH, showed that cycling pH, did not affect clearance rates by oysters exposed to cycling DO conditions. Oysters in the co-cycling severe DO and pH treatment did not have different clearance rates than those in the severe DO only cycling treatments (1-year-old:  $t(20)=-0.31$ ,  $p=0.76$ ; 4-year-old:  $t(20)=0.34$ ,  $p=0.74$ ). Neither did oysters in the co-cycling moderate DO and pH treatment have different clearance rates than those in the moderate DO only cycling treatments (1-year-old:  $t(20)=-0.27$ ,  $p=0.79$ ; 4-year-old:  $t(20)=-0.42$ ,  $p=0.68$ ).

As in the Discrete and Continuous DO Experiments, clearance rates by 1-year-old oyster did not differ among treatments at the recovery point ( $F(1,44)=0.56$ ,  $p=0.46$ ; Fig.6), but older oysters that had been exposed to low DO cleared algae faster than those in the control tanks (Fig. 6). Four-year-old oysters clearance rates of algae in the cycling DO, constant pH treatments were higher than those of control oysters at this time, as shown by pre-planned LS means comparisons (severe cycling DO treatment:  $t(44)=2.70$ ,  $p=0.01$ ; moderate cycling DO treatment:  $t(44)=3.41$ ,  $p=0.001$ ). Pre-planned comparisons of the LS means of treatments that manipulated pH once again showed that cycling pH did not affect clearance rates of 4-year-old oysters exposed to cycling DO conditions. Oysters in the co-cycling severe DO and cycling pH treatment did not have different clearance rates than those in the severe DO only cycling treatments ( $t(44)=-0.90$ ,  $p=0.37$ ). Neither did oysters in the co-cycling moderate DO and cycling pH treatment have different clearance rates than those in the moderate DO only cycling treatments ( $t(44)=-0.86$ ,  $p=0.39$ ).

### **The Continuous DO, Discrete pH Experiment**

Mean starting standard shell heights for 1-year-old oysters and 3-year-old oyster in the Continuous DO, Discrete pH experiment were  $49 \pm 0.66$  SE and  $85 \pm 0.96$  SE mm, respectively, and did not vary among treatments (1-year-olds:  $F(4,124)=0.14$ ,  $p=0.97$ ; 3-year-olds:  $F(4,124)=0.06$ ,  $p=0.67$ ). Temperature and salinity varied little (Table 3). The average clearance rate by control oysters in the Continuous DO, Discrete pH Experiment was  $3.0 \text{ L hr}^{-1} \text{ g}^{-1} \pm 0.17$  SE ( $n=157$ ). Total alkalinity in the Continuous DO, Discrete pH Experiment averaged  $1559 \mu\text{Eq L}^{-1}$ . This indicates that the  $\text{pCO}_2$  of control treatments

held at a pH of 7.8 would have been approximately 1081  $\mu\text{atm}$  while the  $\text{pCO}_2$  of treatments held a pH of 7.0 would have been approximately 7030  $\mu\text{atm}$ .

As in all other experiments, algal clearance rates by oysters did not differ among treatments at the end of the normoxia plateau in the Continuous DO, Discrete pH Experiment (Model: 1-year-old:  $F(2,44)=0.34$ ,  $p=0.72$ ; 3-year-old:  $F(2,49)=0.03$ ,  $p=0.98$ ). Similar to the Continuous DO Experiment, clearance rates by treatment oysters had already decreased with lower DO concentrations by the beginning of the low plateau (ANOVA Model: 1-year-old:  $F(2, 24)=15.77$ ,  $p<0.0001$ ; 3-year-old  $F(2,49)=14.47$ ,  $p<0.0001$ ; Fig. 7). The 1-year-old oysters in treatments that had just reached a pH of 7.0 exhibited faster algal clearance rates than those exposed to a continuous pH of 7.8 ( $F(1,24)=5.09$ ,  $p=0.03$ ) (Fig. 10), but 3-year-old oysters' clearance rates were unaffected by pH ( $F(1,49)=0.75$ ,  $p=0.39$ ). These results were echoed at the end of the low plateau, when cycling pH treatments increased clearance rates by 1-year-old oysters (ANOVA:  $F(1, 67)=5.35$ ,  $p=0.02$ ) (Fig. 11), but did not affect clearance rates by 3-year-old oysters ( $F(1, 38)=0.48$ ,  $p=0.49$ ).

Algal clearance rates by 1-year-old oysters never differed among treatments at the recovery point ( $F(1,22)=0.56$ ,  $p=0.46$ ). However, 3-year-old oysters previously exposed to low DO exhibited elevated clearance rates at this time. The ANOVA did not indicate a significant effect of DO ( $F(1,22)=0.23$ ,  $p=0.38$ ) or pH ( $F(1,22)=0.23$ ,  $p=0.63$ ) (Model:  $F(2,22)=0.46$ ,  $p=0.64$ ) on 3-year-old clearance rates at the recovery point. However, non-linear regression indicated recovery point clearance rates increased as the DO concentration oysters were exposed to during the low plateau decreased ( $n=25$ ,  $p=0.0009$ , Adjusted  $R^2=0.43$ ; Fig. 6). This was also true 2 h after the recovery point, when the

ANOVA did not indicate a significant effect of DO ( $F(1,22)=0.08$ ,  $p=0.76$ ) or pH ( $F(1,22)=0.87$ ,  $p=0.36$ ; Model:  $F(2,22)=0.48$ ,  $p=0.62$ ) on clearance rates, but the linear regression with DO showed algal clearance rates by 3-year-old oysters increased as the DO concentration oysters were exposed to during the low plateau decreased ( $n=25$ ,  $p=0.04$ ,  $\text{Adj } R^2=0.13$ ; Fig. 9).

I used quantile linear regression on the combined data from both the Continuous DO and the Continuous DO, Discrete pH Experiments to test for the effect of DO concentrations below  $2.5 \text{ mg L}^{-1}$  on maximal, median, and minimum clearance rates in (90th, 50th, and 10th percentiles). Low DO decreased the median and maximum algal clearance rates by oysters, but oysters were equally likely to exhibit a clearance rate of  $0 \text{ L h}^{-1} \text{ g}^{-1}$  across the range of DO tested (Fig 13). The highest percentile of clearance rates from both Continuous DO Experiments was significantly affected by DO concentrations under  $2.5 \text{ mg L}^{-1}$  (1-year-old:  $\chi^2(1,n=123)=5.10$ ,  $p=0.02$ ; 3-year-old:  $\chi^2(1,n=112)=9.72$ ,  $p=0.002$ ). The same was true of clearance rates in the median percentile for 1-year-olds ( $\chi^2(1,n=123)=8.00$ ,  $p=0.005$ ), but not for 3-year-olds ( $\chi^2(1,n=112)=0.31$ ,  $p=0.58$ ). The lowest percentile of clearance rates was not significantly affected in either age class (1-year-old:  $\chi^2(1,n=123)=0.08$ ,  $p=0.77$ ; 3-year-old:  $\chi^2(1,n=112)=0.00$ ,  $p=1.00$ ).

### **Modeling Clearance Rates**

I included only the effects of temperature and DO in the model for algal clearance rates by 1-year-old oysters; this model had an AICc score of -329.19. Including the effect of salinity only increased the absolute value of the AICc by 2, but I chose not to include salinity in the model for simplicity. Removing the temperature effect increased the

absolute value of the AICc by 17, while including an effect of hypoxia exposure increased the score by 12533.

I included the effects of temperature, DO, and hypoxia exposure in the model for algal clearance rates by 3-year-old oysters. This model had an AIC score of 607.13. As in the 1-year-old model, including the effect of salinity only increased the absolute value of the AICc by 2, but I chose not to include the model for simplicity. Removing the effect of temperature increased the AIC score by 23, while removing the effect of hypoxia exposure increased the AIC score by 63. The simpler models excluding salinity are below and Table 2 summarizes all the parameters.

The temperature dependent clearance rates for 1-year-olds was:

$$CR_{temp} = 4.1035 \times (0.0287 \times T_t + 0)$$

and for 3-year-olds was

$$CR_{temp} = 6.4426 \times (0.0303 \times T_t + 0.0041)$$

where  $T_t$  is the water temperature in degrees Celsius at time t.

For 1-year-old oysters the effect of DO on clearance rates was:

$$f(DO) = \frac{1}{1 + \exp(-0.7824 \times \left(\frac{1.4062 - DO_t}{1.4062 - 0.7267}\right))}$$

and for 3-year-old oysters was:

$$f(DO) = \frac{1}{1 + \exp(-1.3467 \times \left(\frac{0.9405 - DO_t}{0.9405 - 0.3871}\right))}$$

where  $DO_t$  is the DO at time t (Fig. 14). These functions were similar to the effect of DO used in Fulford et al.'s model (2007).



Algal clearance rates by 3-year-old oysters were also affected by previous exposure to low DO, an effect I call hypoxia exposure. Hypoxia exposure was allowed to accrue when DO was below the fitted concentration of  $7.81 \text{ mg L}^{-1}$  and was allowed to be paid off when DO rose above this same value by increasing clearance rates. Hypoxia exposure affected clearance rates through a non-linear function: a 3 parameter exponential rise to a maximum,

$$f(E) = 0.67 + 2.40 \times (1 - \exp(-0.004 \times E_{(t)})).$$

Hypoxia exposure accrued when DO fell below the fitted value of  $7.81 \text{ mg L}^{-1}$ , adding the difference between  $7.81 \text{ mg L}^{-1}$  and the current DO to the previous hypoxia exposure

$$E_{(t)} = E_{(t-1)} + (7.81 - DO_{(t)}).$$

I used the age specific oyster clearance rate models to estimate mean summertime clearance rates of 1 and 3-year-old oysters at sites around the Maryland portion of the Chesapeake Bay chosen for their varying degrees of the diel-cycling of DO (Fig. 15). The 3-year-old oyster model's estimated mean summertime clearance rates were unaffected by mean summertime DO ( $F(1,4)=0.58$ ,  $p=0.49$ ) or the percent of time DO was below  $1.5 \text{ mg L}^{-1}$  ( $F(1,4)=2.81$ ,  $p=0.17$ ). They were similar at all sites except one, the Mataponi, where DO remained below saturation for most of the summer (Fig. 16). In contrast, 1-year-old oyster mean predicted clearance rates decreased with increasing exposure to DO less than  $1.5 \text{ mg L}^{-1}$  ( $F(1,4)=38.79$ ,  $p=0.003$ ), though they were also unaffected by mean DO ( $F(1,4)=4.90$ ,  $p=0.09$ ). Mean 1-year-old clearance rates for Grey's Creek were predicted to be 75% that at Possum's Point, the "control" site where DO never fell below

1.5 mg L<sup>-1</sup>. The 1-year-old model also predicted that the lowest mean clearance rates would be at the Mataponi (Fig. 16).

## ***Discussion***

My results indicate that algal clearance rates by oysters decrease during the periods of low DO that occur nightly in systems experiencing diel-cycling DO (Figs. 5 & 7), that older oysters increase their clearance rates after brief exposure to low DO ('compensatory feeding'; Fig. 6), and that low pH can increase algal clearance rates by younger oysters (Figs. 10 & 11). Across all experiments, treatments had no effect on algal clearance rates by oysters at the end of the normoxia plateau, before DO/pH began to cycle for the day. By the beginning of the low plateau (Fig. 7), however, low DO concentrations decreased clearance rates by oysters in all age classes and this decrease continued to the end of the low plateau (Fig. 8). The Discrete DO Experiments showed that the DO concentration at which oyster's clearance rates are significantly reduced generally lies somewhere between 1.5 mg L<sup>-1</sup> DO and normoxia (Figs. 5 & 7). The Continuous DO Experiments helped better define the concentrations of DO at which clearance rates of each age class of oysters are affected and provided snapshots of how clearance rates are affected at more points in the daily cycle. As DO returned to normoxia, cycling treatments had no effect on clearance rates (Fig. 8), but when the DO reached normoxia, older oysters that had been exposed to low DO began clearing faster than control oysters (Fig. 6) and this continued for 2 h (Fig. 9). Supersaturated DO had no effect on algal clearance rates by oysters (Fig. 12).

Age specific models of the effects of DO on clearance rates indicate that diel-cycling DO concentrations measured near oyster reefs may halve the average summertime clearance rates of young oysters. However, the 'compensatory feeding' response of older oysters at the recovery point and 2 h later seems to prevent much of this

decrease in the older age class (Fig. 17). Although I did not test for the effects of the diel-cycling of DO on the long-term fitness of oysters, these results indicate that oysters, at least younger oysters, repeatedly exposed to low DO may experience nutritional deficits which may cause decreased fitness, reproduction, and survival.

### **Tests of Clearance Rate Methods**

Clearance rate (the volume of water cleared of particles over time) estimates provide an indirect measure of filtration (the volume of water pumped by a filter feeder over time). Its use assumes that the reduction in measured algae is due solely to filtration by oysters (Coughlan 1969). The clearance rates in tanks containing no oysters were not different from zero, confirming this assumption. This method assumes that the DO and pH treatments did not affect the algae concentration or IVF directly. Since tanks containing no oysters and exposed to the low DO/pH treatments did not differ in IVF, this assumption is also confirmed. Finally, use of clearance rate as a measure of filtration also assumes that 100% of the algae removed from suspension is retained by the oysters (Coughlan 1969). This is true for particles  $>5 \mu\text{m}$ , but retention gradually falls to 50% for  $2 \mu\text{m}$  size particles (Riisgard 1988).

The Discrete DO and the Discrete DO and pH Experiments were part of a larger experiment on the effects of diel-cycling DO and pH on the transmission and progression of *P. marinus* (Breitburg et al. Submitted; Keppel et al. Unpublished). This required that water flow to the oyster tanks during the low DO/pH portions of the cycle and this is why I chose to use the “flow through method” of clearance rate measurements at these time points in these experiments. However, as Riisgard (2001) points out, there are major pitfalls of this method. The foremost is the mixing of intake and exhalant waters that

cannot be accounted for in the formula. This problem, in conjunction with low salinities in the Discrete DO and pH Experiment, may be a reason that the clearance rates measured in these two experiments are lower than published rates. The average filtration rate of control oysters in the Discrete DO Experiment was  $2.4 \text{ L hr}^{-1} \text{ g}^{-1}$  and the average rate in the Discrete DO and pH Experiment was  $1.3 \text{ L hr}^{-1} \text{ g}^{-1}$  while published clearance rates are closer to  $5 \text{ L h}^{-1} \text{ g}^{-1}$  (Newell 1988).

In both the Continuous DO Experiments, I stopped water flow to experimental aquaria when taking clearance rate measurements and used the “clearance method” to avoid the water mixing problems inherent in the “flow through method” (Riisgard 2001). I did, however, allow water to flow to experimental tanks at  $300 \text{ mL min}^{-1}$  when I was not measuring clearance rates to eliminate the effects of starvation (Thompson et al. 1974) on bivalve feeding. The average filtration rate of control oysters in the Continuous DO Experiments was  $4.2 \text{ L hr}^{-1} \text{ g}^{-1}$ . I also maintained near optimum water temperatures with a heater/chiller and shortened the duration of the experiments to limit the effects of temperature and salinity on clearance rates. Tests of the methods used in the Continuous DO Experiments indicated that the treatments were not directly affecting algae and IVF measurements and that the time lapse for the “clearance method” of clearance rate measurements was appropriate.

### **Diel-cycling DO and Clearance Rates**

Data available for the effect of DO on *C. virginica* suggest that low DO decreases the ingestion rates of newly settled spat (Baker and Mann 1994). DO between 30 and 150% saturation is also positively correlated with clearance rates of adult oysters deployed in the field (Li et al. 2012). Furthermore, clearance rates of adult oysters in

nursery settings cycle with the co-occurring daily cycles of DO, pH, temperature and other diel-cycling factors (Li et al. 2012).

I examined the clearance rates of adult oysters of 1, 3, and 4 years of age under laboratory conditions as influenced by DO cycling alone and with cycling pH. DO in this study cycled as low as  $0.06 \text{ mg L}^{-1}$ . Both 1-year-old and older oysters in this study exhibited decreased clearance rates not only when they had been exposed to several hours of low DO, but also as soon as DO declined from normoxia to low concentrations. The combined 2012 clearance rate data that was used to create the age specific models indicated that  $1.4 \text{ mg L}^{-1}$  is the DO concentration at which algal clearance rates by 1-year-old oysters are halved (Fig. 14). This is a slightly lower concentration than the  $1.75 \text{ mg L}^{-1}$  DO previously chosen by modelers (Fulford et al. 2007), but is based on experiments specifically targeting this parameter. The DO concentration at which algal clearance rates by 3-year-old oysters are halved was even lower still,  $0.94 \text{ mg L}^{-1}$  (Fig. 14), indicating that these older oysters maintain their normoxic clearance rates at lower DO concentrations than younger oysters do.

Quantile regressions of 2012 clearance rate data indicate that median and maximum clearance rates decrease with decreasing DO but that DO concentrations do not affect the minimum clearance rates of oysters; a portion of oysters was not actively filtering at any given DO concentration (Fig. 13). The formulas created using combined 2012 data that relate DO to clearance rates also indicate that oysters of both age classes do not stop removing algae from the water entirely, even at anoxia (Fig. 15). These results may run counter to estimated effect of DO in previous models (Cercio & Noel 2005; Fulford et al. 2007). However, rhythmic periods of filtration and cessation in *C.*

*virginica* have been described before (Palmer, 1980) with these fluctuations occurring several times a day and not being influenced by tides, food availability, or light.

Porter et al. (unpublished) used strain gauges to detect the gaping of a subsample of 1-year-old oysters from a similar cycling DO and pH experiment. They found that 1-year-old oysters in the 0.5 mg L<sup>-1</sup> dissolved oxygen treatment were closed significantly more time than those in control tanks, but did open periodically. Additionally, they observed that all oysters in treatment tanks were open at the recovery point. Their data corroborate the decreased clearance rates I observed during the low DO plateau, my models' estimation that oysters would continue to clear at a low rate even at 0.5 mg L<sup>-1</sup> DO, and the increased clearance rates I observed at the recovery point and 2 h later. Their results also indicate that valve gape is at least part of the mechanism controlling clearance rates measured on a population of oysters.

Organisms, including invertebrates, are often capable of adjusting to stressors when they are repeatedly exposed. Intertidally exposed ribbed mussels adjust to decreased immersion time by increasing their gill area to body size ratio, possibly to compensate for shorter feeding time (Franz 1994). While I did not take allometric measures, my Discrete DO Experiment results indicate that no such adjustment occurred; the magnitude of the treatments effects on clearance rates taken at the end of the low DO plateau did not change as the 3 month long experiment progressed. Furthermore, even though *P. marinus* infection prevalence of oysters increased as the experiment progressed (Breitburg et al. Submitted), infection acquisition did not affect the magnitude of the treatments effects on clearance rates. It is possible that oysters would have adjusted to cycling conditions had they not acquired the disease but *P. marinus* infections are

widespread and so the conditions of our experiment mimic realistic conditions oysters would experience in the field.

My results for ‘compensatory feeding’ in older oysters suggest that oysters have developed behavioral responses to cope with the dynamic environment in which they evolved. Three and 4-year-old oysters in cycling treatments generally had increased clearance rates relative to control oysters at the recovery point (Fig. 6) and 2 h later (Fig. 9). Intertidally exposed ribbed mussels exhibit a similar increase in feeding when they re-submerge (Charles and Newell 1997) and can actually grow faster than their subtidal counterparts as a result (Gillmor 1982).

Given the inherent link between filtration of food particles and aeration of the gills (Stickle et al. 1989; Bayne et al. 1998), and that clearance rate is equal to filtration/ventilation rate when particle retention is 100% (Coughlan 1969), it is possible that the physiological factor driving this observed ‘compensatory feeding’ response stems from oxygen debt (de Zwaan 1977) rather than nutritional debt. Payment of oxygen debt via increased oxygen uptake from surrounding water has been observed in *C. virginica* (Widdows et al. 1979; Stickle et al. 1989). The term ‘hypoxia exposure’ I chose to describe the driving factor behind ‘compensatory feeding’ in my 3-year-old clearance rate model might be seen as analogous to oxygen debt.

Including this ‘compensatory feeding’ response in the 3-year-old clearance rate model allowed 3-year-old estimated mean summertime clearance rates for oysters to remain high across most sites (Fig. 17) in spite of variations in the frequency and severity of diel-cycling DO concentrations (Fig. 16). On the other hand, 1-year-old oyster estimated mean summertime clearance rates decreased steadily across stations with



increasing percent of time in which DO concentrations were below  $1.5 \text{ mg L}^{-1}$  (Fig. 17). Algal clearance rates by 1-year-old oyster at St. Mary's College, the station with the highest percent of time in which DO concentrations were below  $1.5 \text{ mg L}^{-1}$ , were half that of Possum Point, the station in which DO concentrations were never below  $1.5 \text{ mg L}^{-1}$  (MD DNR EyesOnTheBay.net). I modeled clearance rates at Mataponi, ignoring the station's low salinity and poor suitability as oyster habitat, to test a site where late summer DO concentrations, when higher temperatures would otherwise increase clearance rates, remain well below the critical DO concentration at which older oysters might begin their compensatory feeding. The Mataponi also had DO below  $1.5 \text{ mg L}^{-1}$  for a greater percent of time than all other sites except St. Mary's College; DO was less than  $1.5 \text{ mg L}^{-1}$  at the Mataponi for 32% of the time compared to 29% of the time at Harness Creek and 33% of the time at St. Mary's College (Fig. 16). DO often falling low enough to decrease 3-year-old clearance plus an inhibition of the ability of these oysters to clear faster when DO returns to normoxia causes their estimated mean summertime clearance rates at Mataponi to be relatively low compared to other sites. My model did not include an effect of salinity on clearance rates; this allowed me to use the DO data from Mataponi despite the low salinity of the site.

Results of my age specific models are corroborated by evidence from growth data of field-deployed oysters. One-year-old oysters deployed around the Chesapeake Bay exhibited decreased growth with increasing frequency and severity of diel-cycling DO. However, no such effect was observed for 3-year-old oysters placed concurrently at the same sites (Breitburg et al. Submitted).

The estimated pivotal DO concentration (below which oysters accrued hypoxia exposure and above which hypoxia exposure is paid off) was  $7.81 \text{ mg L}^{-1}$ . This is particularly high - near 100% saturation for the temperature and salinity of my experiments. I believe this is because the model needed two parameters here instead of one; one to estimate the DO concentration below which oysters accrued hypoxia exposure and one to estimate the DO concentration above which hypoxia exposure is paid off. From clearance rates taken as DO is returning to normoxia, I glean that oysters do not clear faster to 'pay off hypoxia exposure' at or below  $4.5 \text{ mg L}^{-1}$  (Fig. 9), therefore I do not think  $7.81 \text{ mg L}^{-1}$  (normoxia) is too high an estimate for the DO concentration above which hypoxia exposure is paid off. However, oysters did not exhibit decreased clearance rates until DO concentrations were below about  $3.0 \text{ mg L}^{-1}$  (Fig. 14), so the DO concentration below which oysters accrue hypoxia exposure is probably much lower than  $7.81 \text{ mg L}^{-1}$ .

Clearance rates of adult oysters in nursery settings exposed to daily cycles of DO increase linearly with increasing DO saturation up to 150% (Li et al. 2012). However, I did not observe an increase in clearance rates of 1-year-old or 3-year-old oysters exposed to 3 h of supersaturated DO conditions. Unlike oysters in the field, my supersaturated trial occurred outside of a normal daily cycle; oysters in this trial had been at near-saturation for at least 24 h beforehand. During a normal daily cycle, the increased feeding due to a return of relatively high DO concentrations and the period of supersaturated conditions might blend into one another. It is possible that field oysters exhibiting increased clearance rates during a period of supersaturated DO are actually compensating for the preceding period of low DO rather than responding to high DO concentrations.

My models did not include an effect of supersaturated DO on clearance rates and therefore the relatively high daytime DO concentrations at Grey's Creek and Bishopville Prong ( $>14 \text{ mg L}^{-1}$ ; MD DNR EyesOnTheBay.net; Fig. 16) did not increase estimated daytime clearance rates beyond normoxic levels to make up for low nighttime DO concentrations and clearance rates (Fig. 17).

### **Diel-cycling pH and Clearance Rates**

The low pH and  $\text{pCO}_2$  used in this study were approximately 7.0 and  $7001 \mu\text{atm}$ . This  $\text{pCO}_2$  is substantially higher than the oceanic  $\text{pCO}_2$  predicted for the future, but it is realistic and currently occurs in estuarine ecosystems where these oysters live and where pH can drop below 7.0. This study also does not distinguish between the effects of  $\text{pCO}_2$  and pH since the ratio of  $\text{CO}_2$  to other gasses was used to control the pH in these experiments.

The effect of pH on bivalve filtration rates is poorly understood. Within the range of pH for my experiments (7.0-7.8), some studies indicate that pH does not affect bivalve filtration (Chen et al. 2007) while others indicate that decreasing pH increases *C. virginica* filtration rates (Li et al. 2012). Results from my Discrete DO and pH and Continuous DO, Discrete pH clearance rate experiments fall into both of these categories as well; indicating either no effect of pH on algal clearance rates by oysters (Fig. 5 & 7) or that they were increased by decreasing pH (Fig. 10 & 11). Cycling pH treatments in the Discrete DO and pH Experiment did not affect the clearance rates of either age class of oysters at any time point measured. In the Continuous DO, Discrete pH Experiment, I observed that higher pH decreased clearance rates of 1-year-old oysters both at the beginning and end of the low DO plateau, but saw no effect on 3-year-old clearance rates.

While these results vary, a consistency among my experiments and the published data is that the low pH values tested do not appear to decrease clearance rates. I did not include an effect of pH in my clearance rate models.

### **Temperature and Salinity and Clearance Rates**

Both temperature and salinity significantly increase bivalve filtration rates (Loosanoff 1953; Newell and Koch 2004; Cerco and Noel 2005; Fulford et al. 2007). Results from the Discrete DO and the Discrete DO and pH Experiments also indicate that both increased temperature and increased salinity increase the clearance rates of oysters under near-saturated conditions. The Fulford et al. (Newell unpublished data in Fulford et al. 2007) model estimates that oyster filtration increases with increasing salinities between 5 and 12 PSU but that oysters cease to filter below 5 PSU. 2010 and 2011 clearance rates from this study were measured between 3.1 and 13.7 PSU and there was a linear increase in clearance rates of oysters exposed to  $DO > 5.0 \text{ mg L}^{-1}$  with salinity. However, oysters in this study continued to clear particles at salinities below 5 PSU. The optimal temperature for algal clearance rates by oysters is reported as 27°C (Newell & Langdon 1996), and the highest clearance rates of oysters in this study in 2010 and 2011 occurred around that temperature.

### **Conclusion**

Diel-cycling DO, which occurs globally in a variety of habitats (Tyler, Brady, & Targett 2009), can cause decreased clearance rates of algae by the eastern oyster during nightly periods of low DO. Daytime periods of high DO may not increase algal clearance rates by oysters sufficiently to completely make up for this in younger oysters. However,

older oysters increase their clearance rates for at least 2 h after DO concentrations return to near-saturation. Models indicate that this compensatory feeding may allow older oysters to avoid the decreases in average summertime clearance rates experienced by younger oyster. Clearance rates by oysters repeatedly exposed to cycling DO appear to be equally affected by the daily low DO concentrations throughout several months of repeated exposures. The nutritive stress that could result from by decreased clearance rates can have a number of negative impacts upon the health (Delaporte et al. 2003; Hegerat et al. 2004) and population dynamics (Davis and Hillman 1971; Barber and Blake 1991; Liu et al. 2010) of this environmentally and economically important species (Coen et al. 1999; Peterson et al. 2003; Breitburg & Fulford 2006; Grabowski et al. 2007). Understanding how diel-cycling DO affects keystone organisms like *C. virginica* is especially important as symptoms of eutrophication like exacerbated diel-cycling DO increase in frequency and severity in coastal systems worldwide (Kemp et al. 2005; Diaz & Breitburg 2009).

## Figures and Tables

Table 1. Summary of the methods used in each experiment.

Experiment	Year	Duration	Oyster ages (Years)**	Number of oysters per tank	Tank size (L)	Water Flow Rate (mL min <sup>-1</sup> )**	Algae supplementation	Clearance rate calculation method
Discrete DO	2010	3 mo	1	90	75	175 + 75*	<i>Chaetoceros muelleri</i>	Flow-through & Clearance
			3	90	35	500		
Continuous DO	2012	4 d	1	5	35	300	DT's Live Marine Phytoplankton	Clearance
			3	5	35	300		
Discrete DO and pH	2011	1 mo	1	90	75	200+50*	<i>Chaetoceros muelleri</i> & DT's Live Marine Phytoplankton	Flow-through & Clearance
			4	80	35	500		
Continuous DO, Discrete pH	2012	4 d	1	5	75	300	DT's Live Marine Phytoplankton	Clearance
			3	5	75	300		
Supersaturated DO	2012	1 d	1	5	75	300	DT's Live Marine Phytoplankton	Clearance
			3	5	57	300		

\*75 ml min<sup>-1</sup> of water was supplied to 1-year-olds from tanks containing older oysters. \*\*Note that differences among years in oyster age and water flow were primarily driven by requirements of the disease aspects of the experiments.

Table 2. Parameters used in the model.

Parameter	Definition	Value		Units
		1-year-olds	3-year-olds	
CR	Clearance rate of algae by oysters	.	.	mg L <sup>-1</sup>
CR <sub>temp</sub>	Temperature dependent clearance rate	.	.	L min <sup>-1</sup> g <sup>-1</sup>
CR <sub>base</sub>	Average clearance rate unaffected by temperature or DO	4.10	6.44	L min <sup>-1</sup> g <sup>-1</sup>
Slope <sub>temp</sub>	The slope of the linear effect of temperature on clearance rate	0.03	0.03	C <sup>o-1</sup>
T	Water temperature	.	.	C <sup>o</sup>
Intercept <sub>temp</sub>	The intercept of the linear effect of temperature on clearance	0.00	0.00	C <sup>o</sup>
DO <sub>zero</sub>	The DO at which clearance ceases	-0.78	-1.35	mg L <sup>-1</sup>
DO <sub>half</sub>	The DO at which clearance rates are halved	1.41	0.94	mg L <sup>-1</sup>
DO <sub>quarter</sub>	The DO at which clearance rates are quartered	0.73	0.39	mg L <sup>-1</sup>
f(DO)	The effect of DO on clearance rates	.	.	L mg <sup>-1</sup>
f(E)	The effect of oxygen debt on clearance rates	.	.	Unit-less
E <sub>zero</sub>	The level of debt below which there is no effect on clearance rates	n/a	0.67	Unit-less
E <sub>b</sub>	the exponential growth parameter of oxygen debt	n/a	0.00	Unit-less
E <sub>a</sub>	the coefficient of the oxygen debt	n/a	2.40	Unit-less
E	the effect of previous exposure to low DO on current clearance rates	.	.	Unit-less

Table 3: Measured DO within each treatment in the Discrete DO Experiment by cycle phase.

Cycle Phase	Oyster Age (years)	DO Treatment	n	DO (mean ± SE)
End of Normoxia Plateau	1	Control	65	7.25±0.05
		1.5 mg L <sup>-1</sup>	65	6.87±0.06
		0.5 mg L <sup>-1</sup>	65	6.74±0.07
	3	Control	69	7.02±0.06
		1.5 mg L <sup>-1</sup>	70	5.83±0.17
		0.5 mg L <sup>-1</sup>	70	6.17±0.10
End of Low Plateau	1	Control	70	6.97±0.04
		1.5 mg L <sup>-1</sup>	70	1.41±0.01
		0.5 mg L <sup>-1</sup>	70	0.47±0.01
	3	Control	69	6.76±0.06
		1.5 mg L <sup>-1</sup>	68	1.50±0.01
		0.5 mg L <sup>-1</sup>	69	0.67±0.02
Recovery Point	1	Control	5	7.28±0.03
		1.5 mg L <sup>-1</sup>	5	7.30±0.02
		0.5 mg L <sup>-1</sup>	5	7.29±0.04
	3	Control	5	7.29±0.03
		1.5 mg L <sup>-1</sup>	5	7.29±0.02
		0.5 mg L <sup>-1</sup>	5	7.30±0.02





Table 4. The salinity and temperature averaged for each experiment.

Experiment	Minimum Salinity	Maximum Salinity	Salinity (mean $\pm$ SE)	Minimum Temperature (C°)	Maximum Temperature (C°)	Temperature (mean $\pm$ SE) (C°)
Discrete DO	10.8	13.7	11.7 $\pm$ 0.02	21.3	29.5	27.1 $\pm$ 0.06
Continuous DO	11.2	11.6	11.6 $\pm$ 0.006	25.9	26.8	26.5 $\pm$ 0.02
Discrete DO and pH	3.1	10.7	7.7 $\pm$ 0.10	23.2	27.7	25.6 $\pm$ 0.07
Continuous DO, Discrete pH	12.2	14.4	13.0 $\pm$ 0.02	24.9	26.4	25.7 $\pm$ 0.05



Table 5: Measured DO and pH within each treatment in the Discrete DO and pH Experiment by cycle phase.

Cycle Phase	Oyster Age (years)	DO Treatment	pH Treatment	n	DO (mean $\pm$ SE)	pH (mean $\pm$ SE)
End of Normoxia Plateau	1	Control	Control	25	7.65 $\pm$ 0.06	7.74 $\pm$ 0.01
		1.5 mg L <sup>-1</sup>	Control	25	7.66 $\pm$ 0.06	7.75 $\pm$ 0.01
		1.5 mg L <sup>-1</sup>	Cycling	25	7.68 $\pm$ 0.06	7.74 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Control	25	7.66 $\pm$ 0.06	7.73 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Cycling	25	7.63 $\pm$ 0.06	7.72 $\pm$ 0.01
	4	Control	Control	25	7.61 $\pm$ 0.06	7.67 $\pm$ 0.02
		1.5 mg L <sup>-1</sup>	Control	25	7.60 $\pm$ 0.07	7.60 $\pm$ 0.02
		1.5 mg L <sup>-1</sup>	Cycling	25	7.59 $\pm$ 0.07	7.60 $\pm$ 0.02
		0.5 mg L <sup>-1</sup>	Control	25	7.55 $\pm$ 0.08	7.60 $\pm$ 0.02
		0.5 mg L <sup>-1</sup>	Cycling	25	7.55 $\pm$ 0.09	7.61 $\pm$ 0.02
End of Low Plateau	1	Control	Control	20	7.65 $\pm$ 0.09	7.87 $\pm$ 0.02
		1.5 mg L <sup>-1</sup>	Control	20	1.56 $\pm$ 0.04	7.75 $\pm$ 0.03
		1.5 mg L <sup>-1</sup>	Cycling	20	1.59 $\pm$ 0.05	7.03 $\pm$ 0.02
		0.5 mg L <sup>-1</sup>	Control	20	0.56 $\pm$ 0.02	7.75 $\pm$ 0.03
		0.5 mg L <sup>-1</sup>	Cycling	20	0.57 $\pm$ 0.02	7.10 $\pm$ 0.01
	4	Control	Control	20	7.51 $\pm$ 0.07	7.76 $\pm$ 0.03
		1.5 mg L <sup>-1</sup>	Control	20	1.60 $\pm$ 0.04	7.67 $\pm$ 0.03
		1.5 mg L <sup>-1</sup>	Cycling	20	1.77 $\pm$ 0.07	7.04 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Control	20	0.59 $\pm$ 0.02	7.82 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Cycling	20	0.58 $\pm$ 0.02	7.10 $\pm$ 0.01
Recovery Point	1	Control	Control	10	7.53 $\pm$ 0.01	7.87 $\pm$ 0.01
		1.5 mg L <sup>-1</sup>	Control	10	7.50 $\pm$ 0.01	7.90 $\pm$ 0.01
		1.5 mg L <sup>-1</sup>	Cycling	10	7.51 $\pm$ 0.01	7.92 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Control	10	7.49 $\pm$ 0.02	7.91 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Cycling	10	7.50 $\pm$ 0.01	7.91 $\pm$ 0.02
	4	Control	Control	10	8.13 $\pm$ 0.01	7.98 $\pm$ 0.004
		1.5 mg L <sup>-1</sup>	Control	10	8.13 $\pm$ 0.01	7.97 $\pm$ 0.01
		1.5 mg L <sup>-1</sup>	Cycling	10	8.14 $\pm$ 0.01	7.97 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Control	10	8.13 $\pm$ 0.01	7.95 $\pm$ 0.01
		0.5 mg L <sup>-1</sup>	Cycling	10	8.14 $\pm$ 0.01	7.95 $\pm$ 0.01

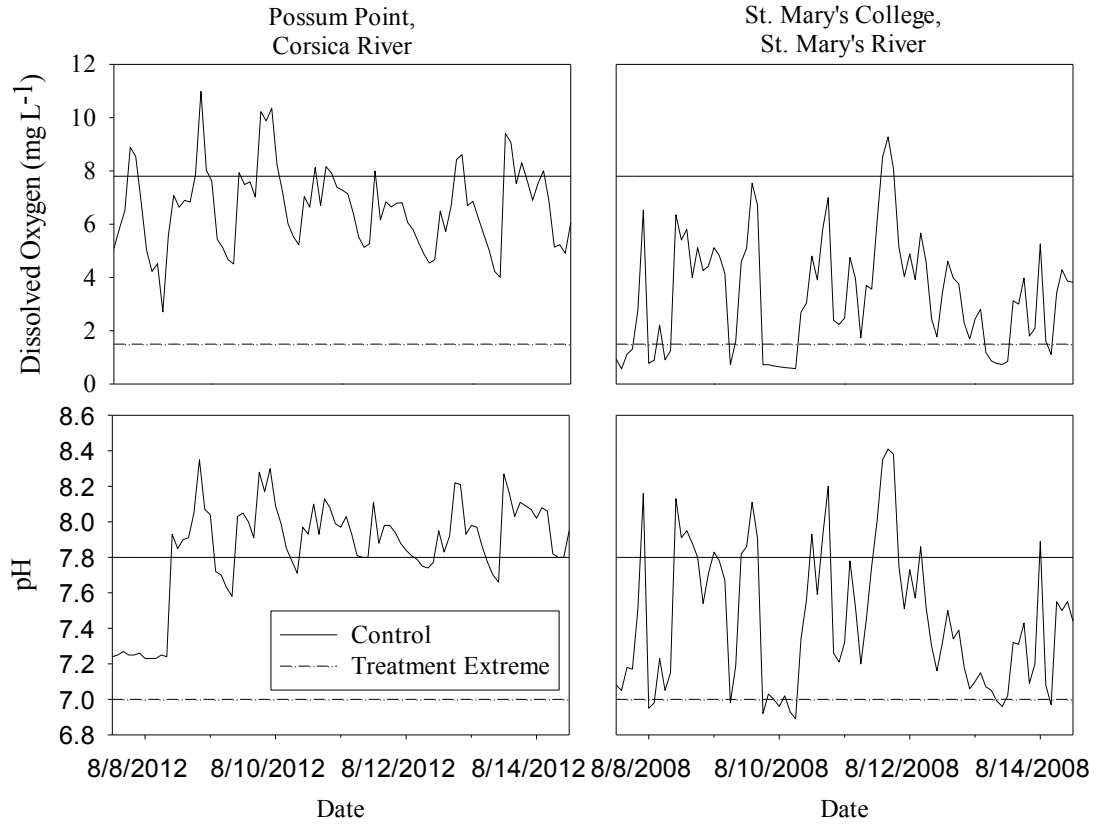


Figure 1. Examples of diel-cycling hypercapnic hypoxia at 2 sites near historic oyster beds (MD DNR EyesOnTheBay.net). Control treatments in my experiments were exposed to the DO and pH that occurs near air-saturation (solid lines) while the low end of the cycle in my discrete treatments were exposed to DO extremes of 0.5 mg L<sup>-1</sup> and pH extremes of 7.0 (dashed lines).

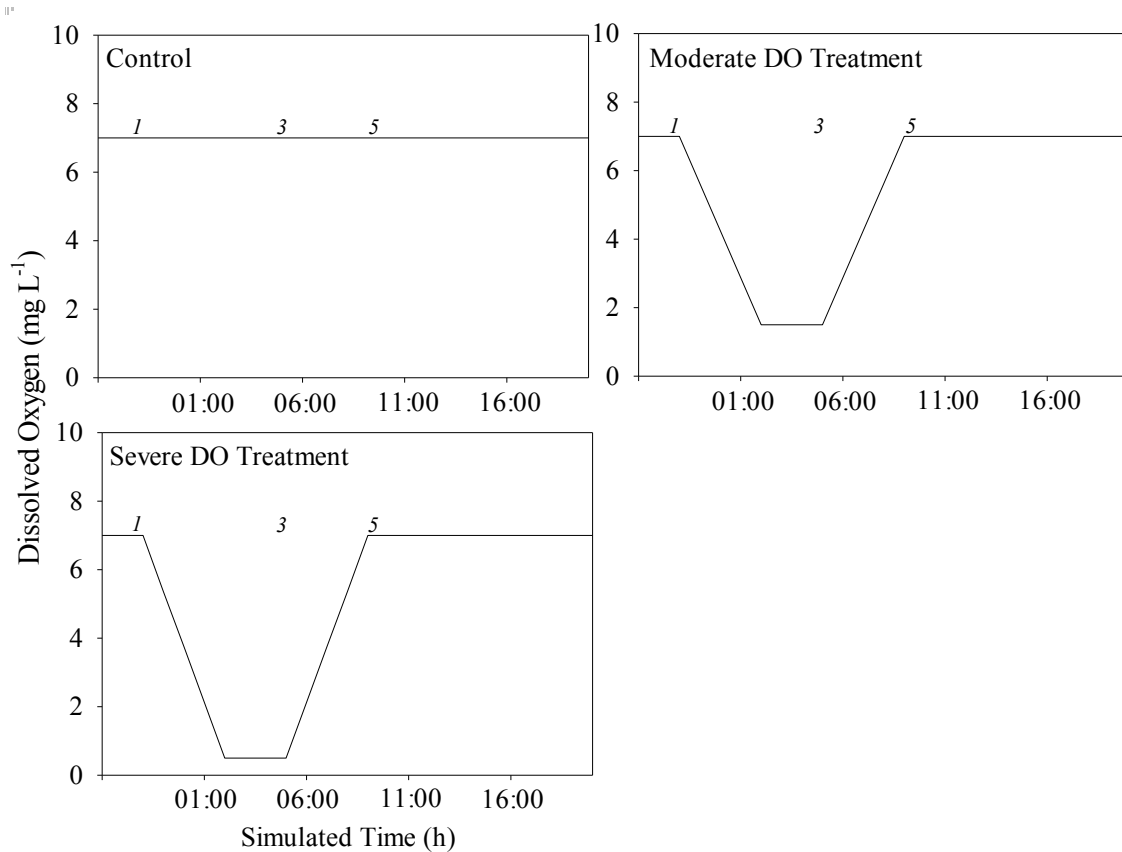


Figure 2. Treatments in the Discrete DO Experiment. Clearance rates were measured at the end of the normoxia plateau, before DO began to cycle for the day (Time Point 1; results not shown), at the end of the low plateau, when DO had been low for 3 h (Time Point 3; results shown in Fig. 5), and at the recovery point, when DO first returned to normoxia (Time Point 5; results shown in Fig. 6). Time points are indicated on the panels above with shaded and numbered columns.

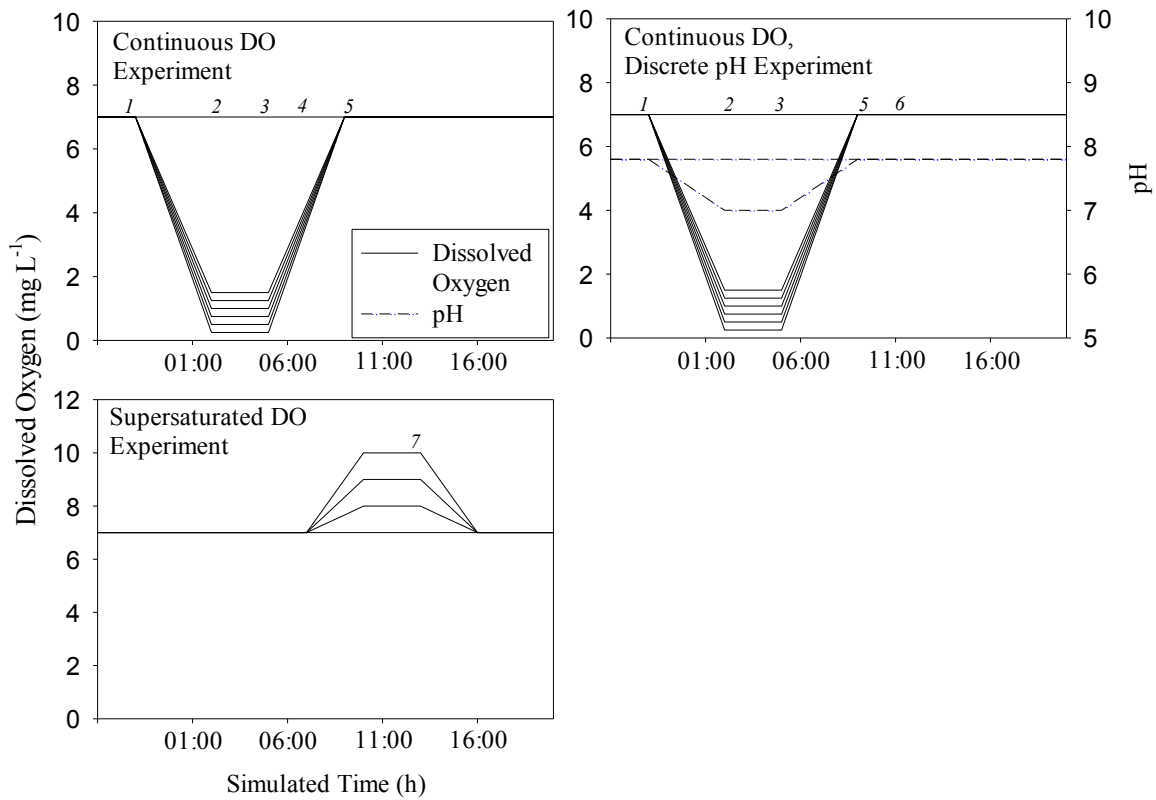


Figure 3. General patterns of DO and pH in the 3 Continuous DO Experiments (not exact DO and pH values). Clearance rates were measured at the end of the normoxia plateau, before DO began to cycle for the day (Time Point 1; results not shown), at the beginning of the low plateau, when DO and pH had first reached their low extremes (Time Point 2; results shown in Figs. 7 & 10), at the end of the low plateau, when dissolved oxygen had been low for 3 h (Time Point 3; results shown in Figs. 5 & 11), halfway into the rise of DO and pH (Time Point 4; results shown in Fig. 8), at the recovery point, when DO first returned to normoxia (Time Point 5; results shown in Fig. 6), 2 h after the recovery point (Time Point 6; results shown in Fig. 9), and at the end of the supersaturated plateau, when DO has been high for 3 h (Time Point 7; results shown in Fig. 12). Time points are indicated on the panels above with shaded and numbered columns.

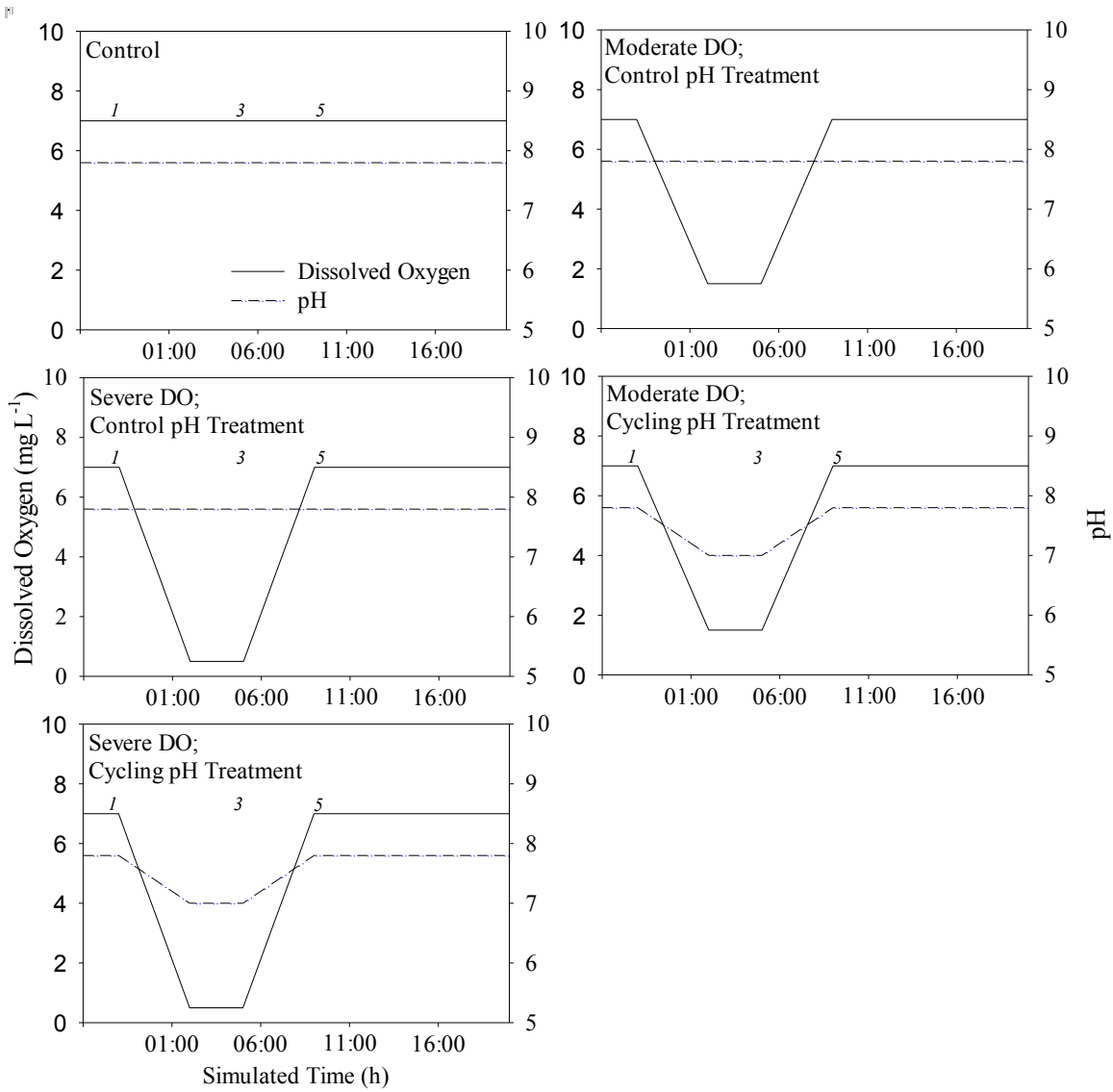


Figure 4. Treatments in the Discrete DO and pH Experiment. Clearance rates were measured at the end of the normoxic plateau, before DO and pH began to cycle for the day (Time Point 1; results not shown), at the end of the low plateau, when DO and pH had been low for 3 h (Time Point 3; results shown in Figs. 5 & 11), and at the recovery point, when DO and pH first returned to normoxia and normcapnia (Time Point 5; results shown in Fig. 9). Time points are indicated on the panels above with shaded and numbered columns.



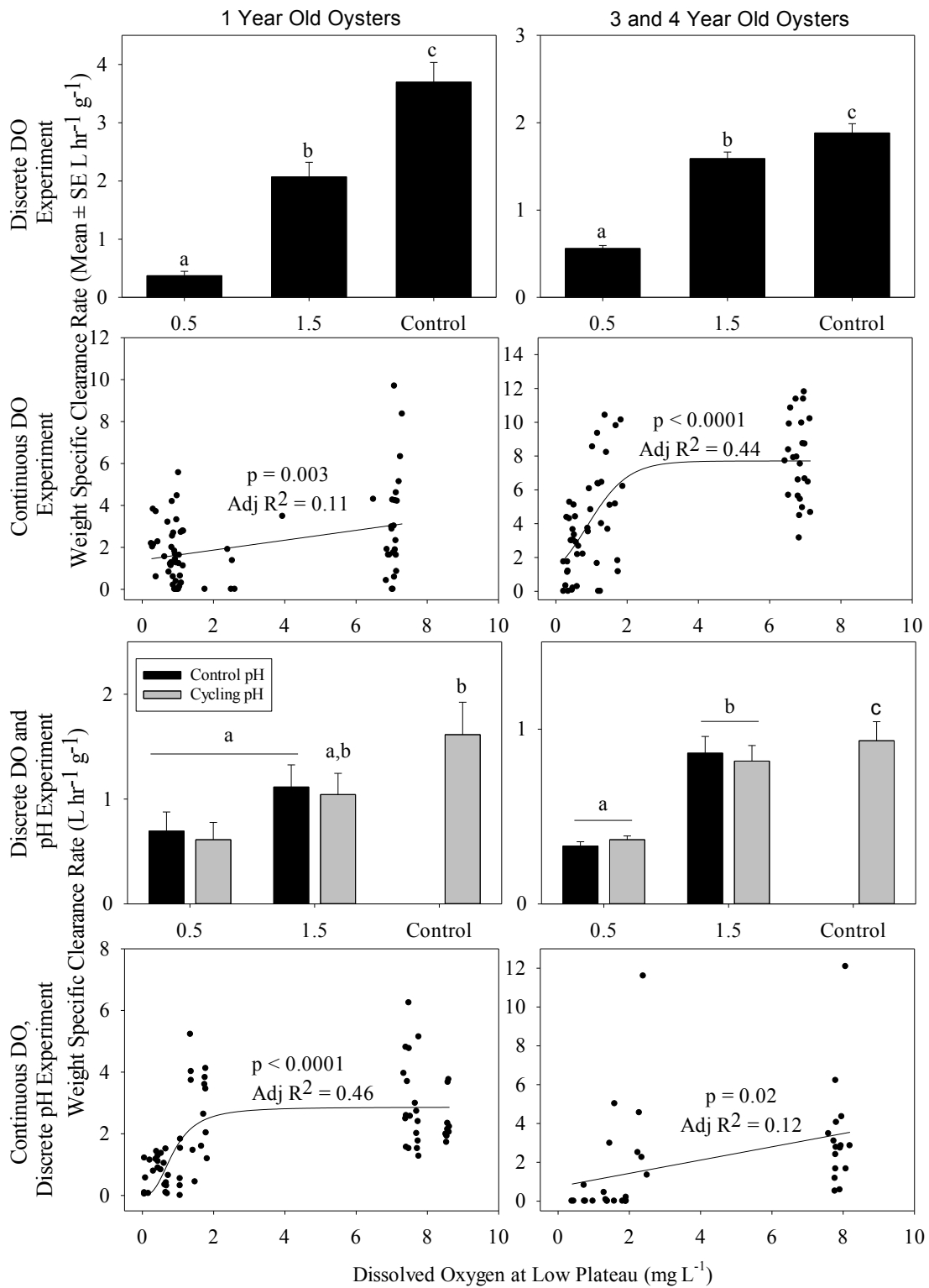


Figure 5. Low DO decreased clearance rates by oysters at the end of the low DO/pH plateau, when DO/pH had been at their low extremes for 3 h, data are shown by experiment and oyster age class.

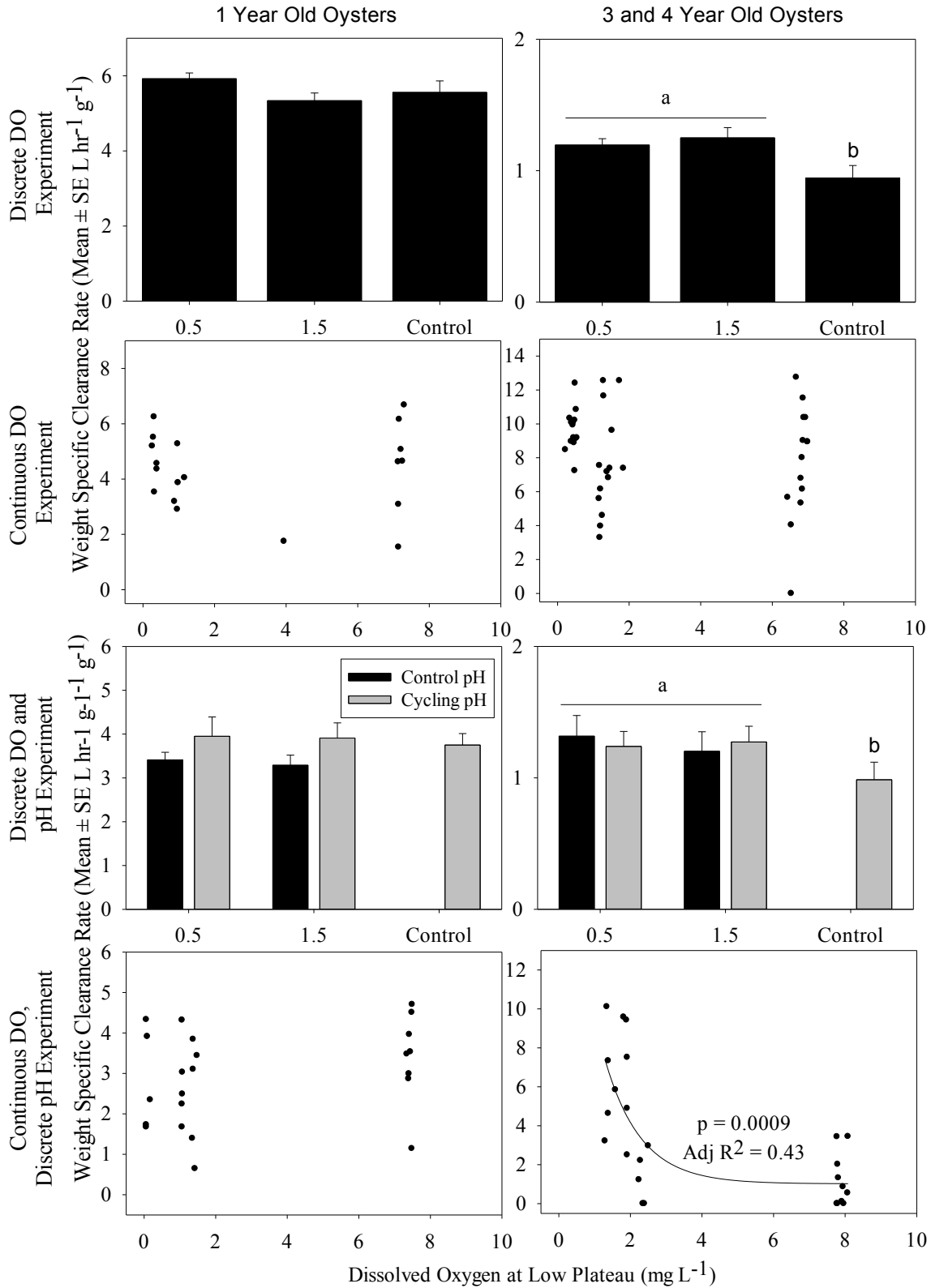


Figure 6. Prior exposure to low DO increased clearance rates by older oysters at the recovery point, when DO/pH first returned to normoxia/normcapnia, data are shown by experiment and oyster age class.

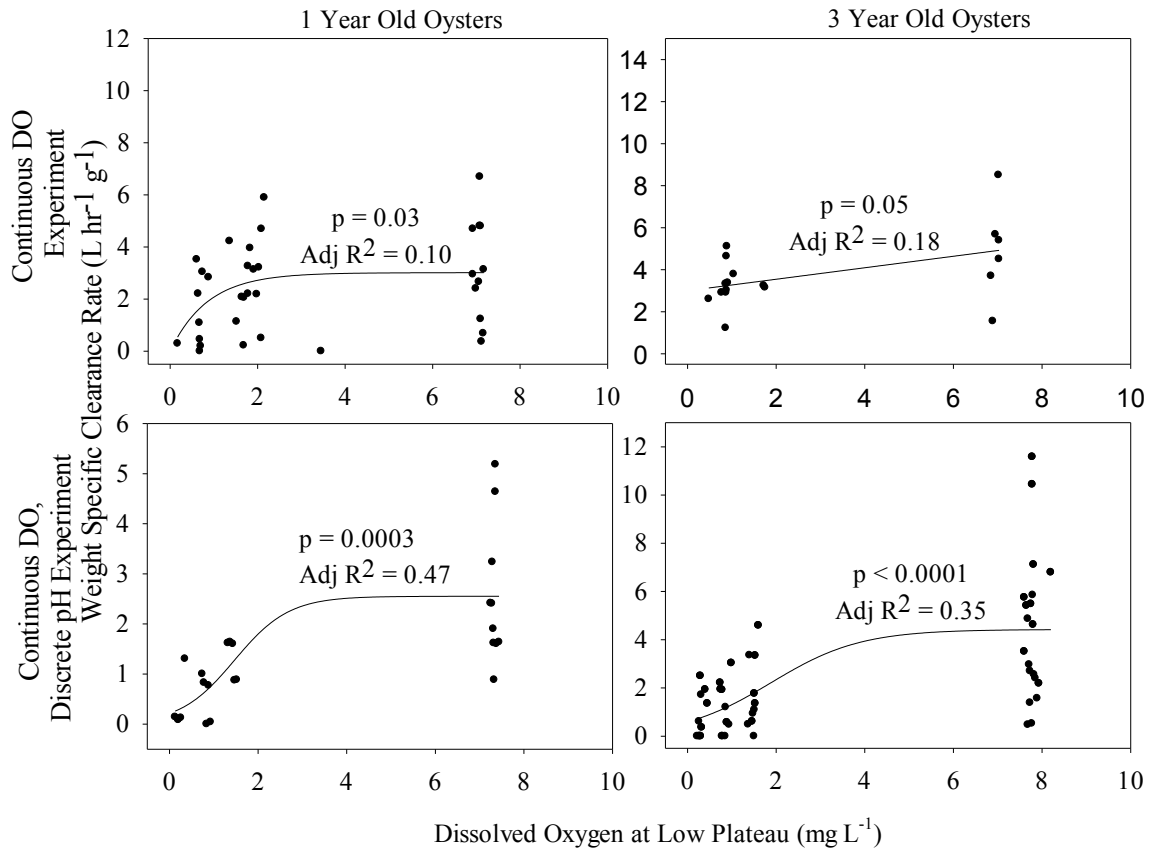


Figure 7. Low DO decreased clearance rates by oysters at the beginning of the low DO/pH plateau, when DO/pH first reached their low extremes, data are shown by experiment and oyster age class.

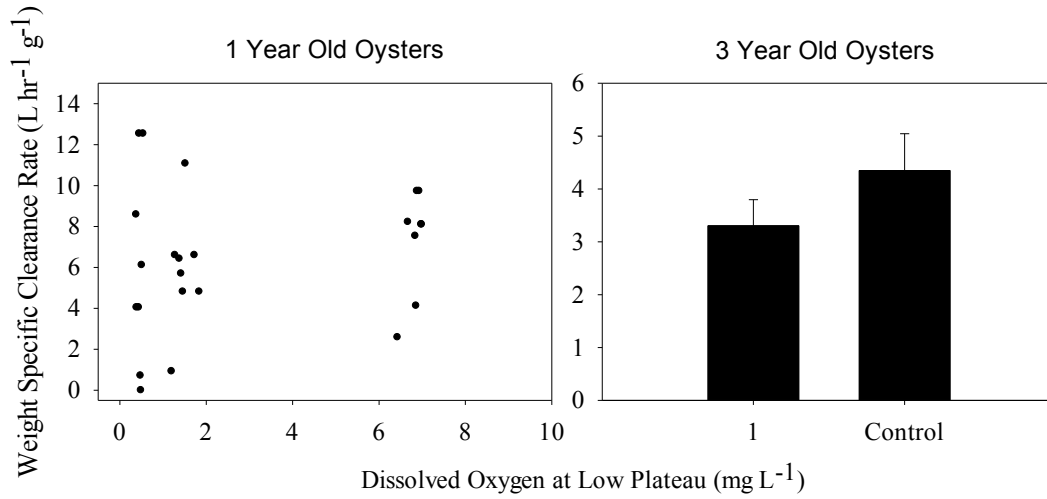


Figure 8. Prior exposure to low DO did not affect clearance rates by oysters as DO returns to normoxia in the Continuous DO Experiment.

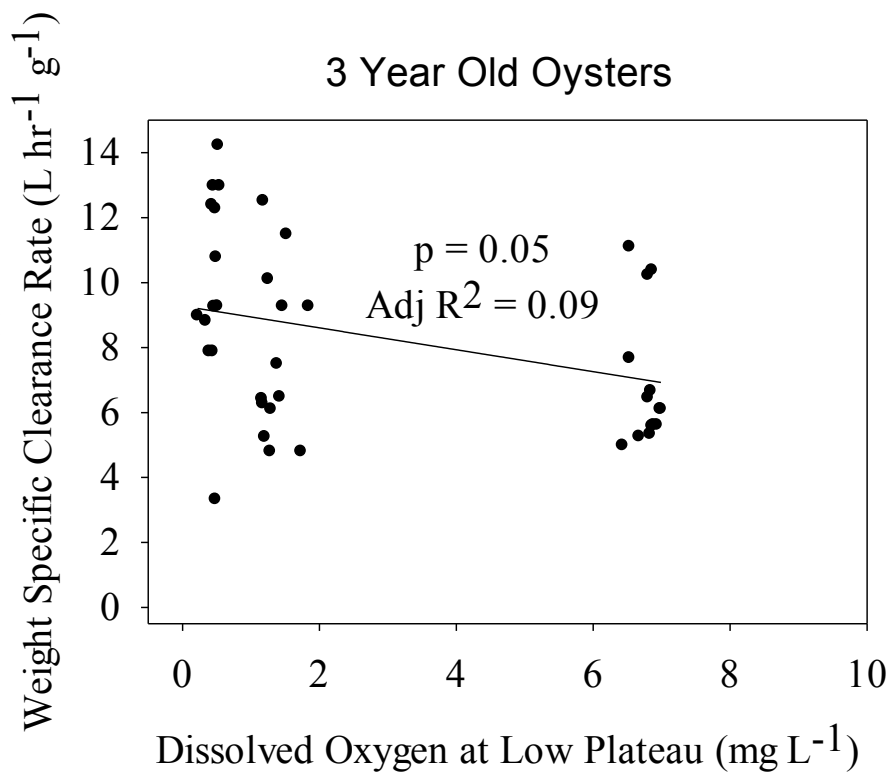


Figure 9. Prior exposure to low DO increased clearance rates by 3 year old oysters 2 h after the recovery point, 2 hours after DO/pH has returned to normoxia/normcapnia in the Continuous DO Experiment.

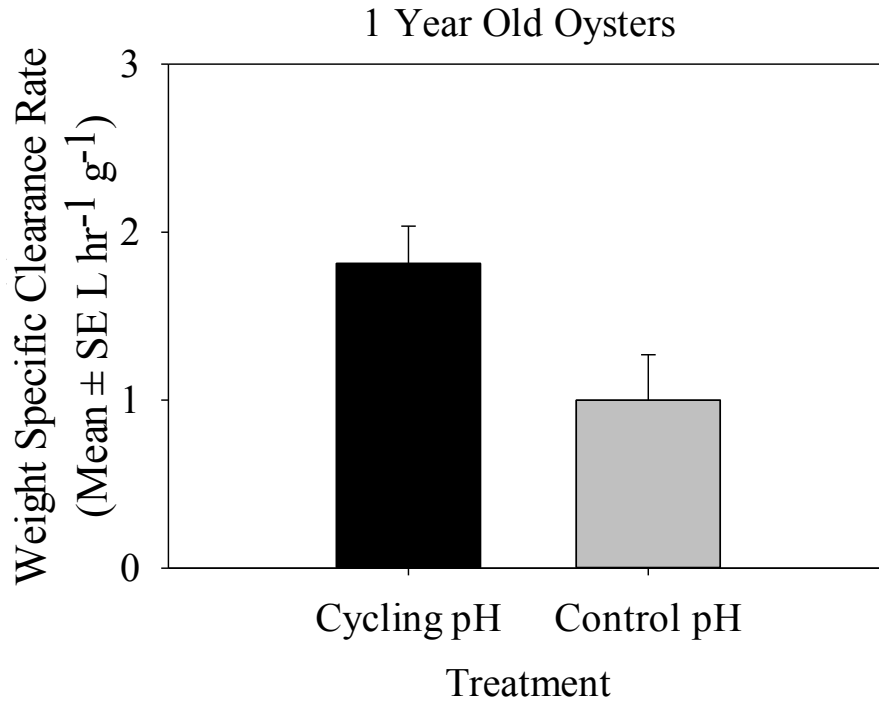


Figure 10. Low pH increased clearance rates by 1 year old oysters at the beginning of the low DO/pH plateau, when DO/pH first reached their low extremes in the Continuous DO, Discrete pH experiment.

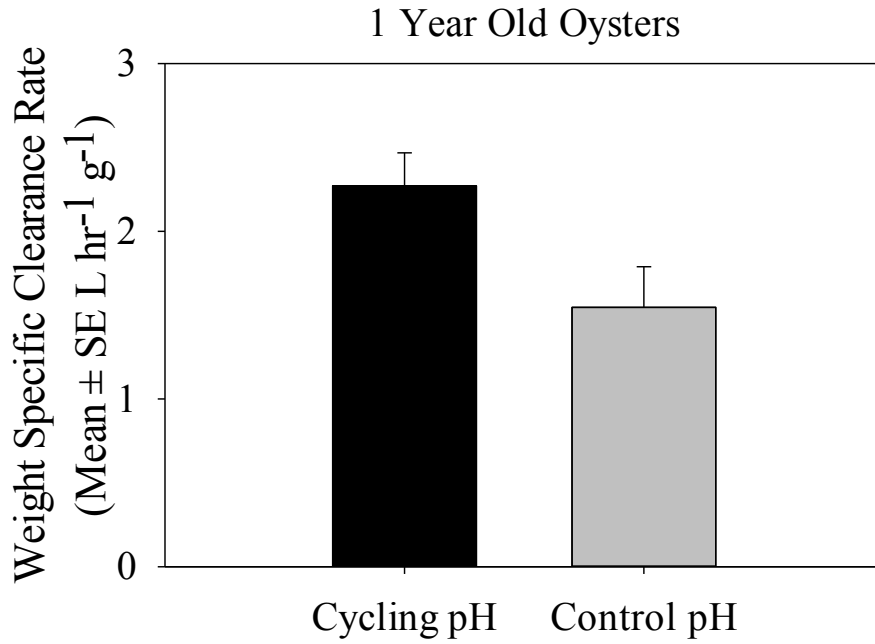


Figure 11. Low pH increased clearance rates by 1 year old oysters at the end of the low DO/pH plateau, when DO/pH had been at their low extremes for 3 h in the Continuous DO, Discrete pH experiment.

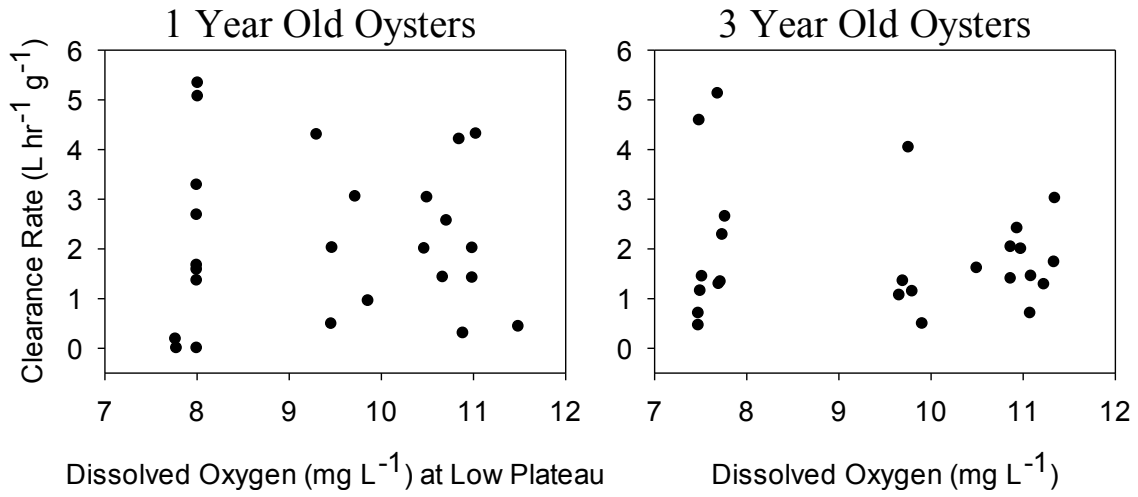


Figure 12. Clearance rates of oysters in the Supersaturated DO Experiment were unaffected by supersaturated dissolved oxygen conditions.



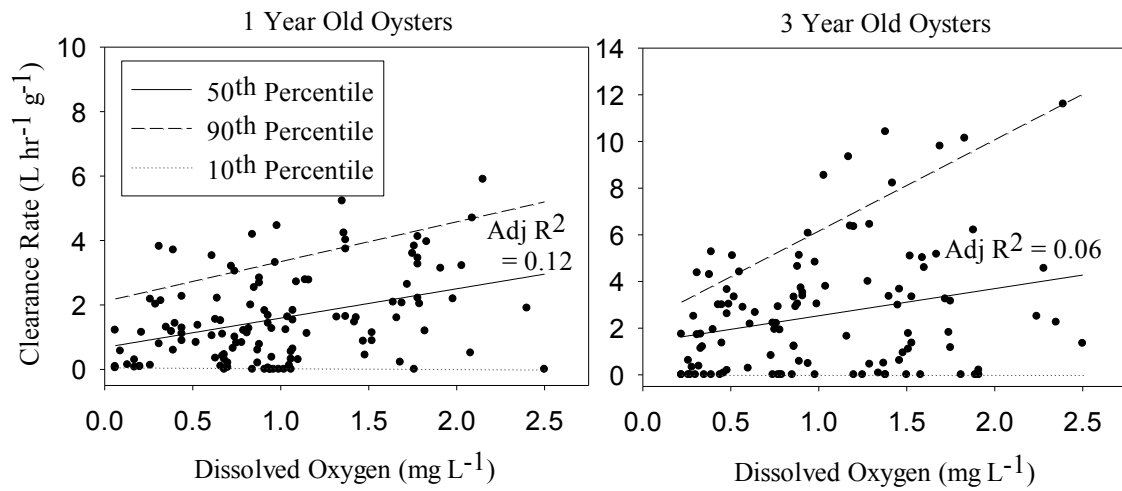


Figure 13. Low DO had no effect on clearance rates in the 10<sup>th</sup> percentile but reduced those in the 90<sup>th</sup> and 50<sup>th</sup> percentiles. Data are from the Continuous DO Experiments, quantile regression was used to examine the effect of DO on clearance rates of oysters exposed to dissolved oxygen concentrations less than 2.5 mg L<sup>-1</sup>.

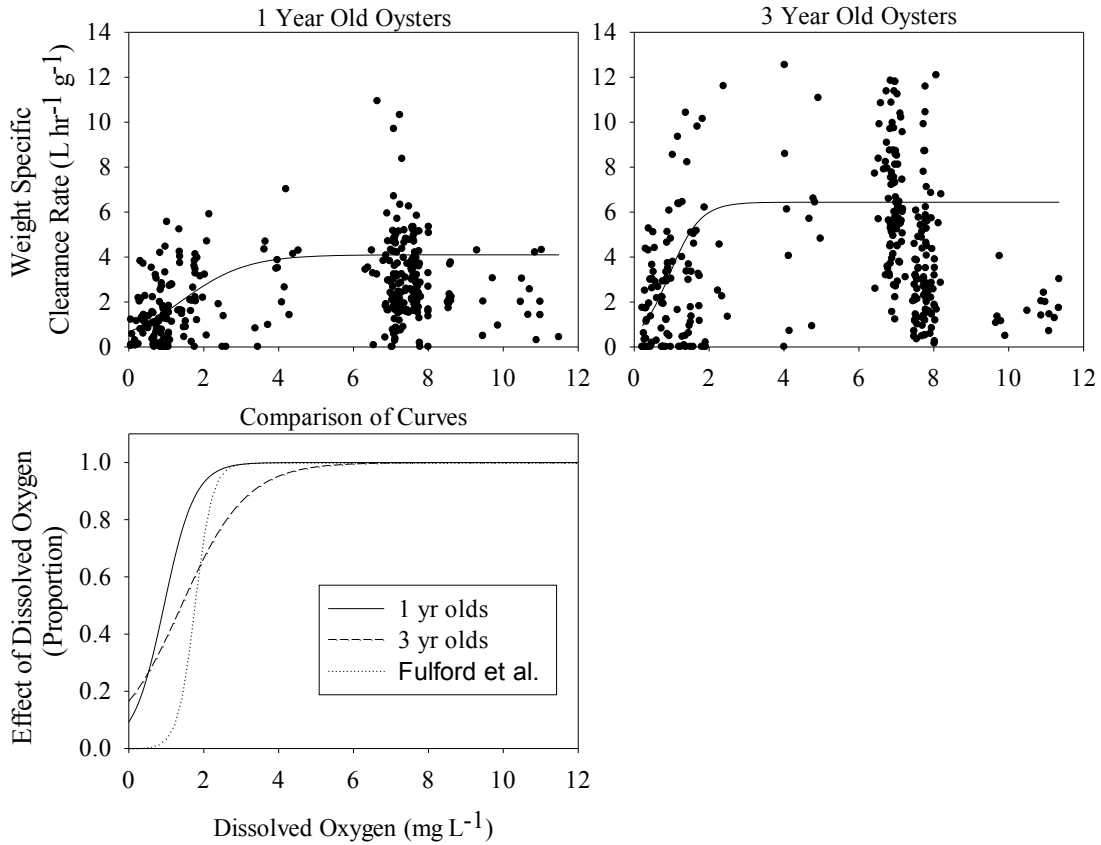


Figure 14. Plots of model formulas using dissolved oxygen to predict weight specific algal clearance rates by oysters. These curves were fitted to the data from the Continuous DO Experiments and then overlaid over that data for comparison. The curve used in the Fulford et al. (2007) model is also displayed for comparison.

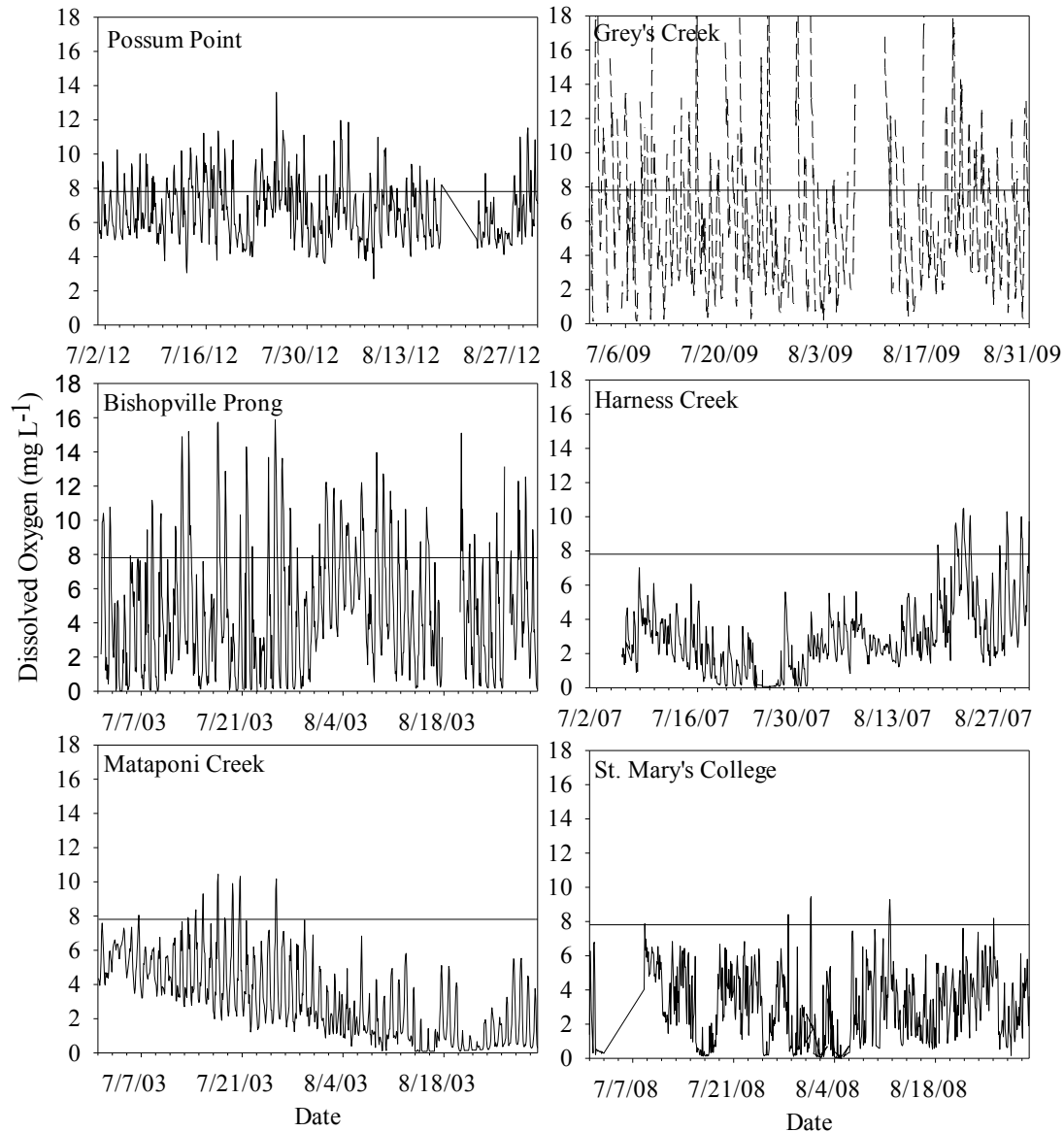


Figure 15. Summertime (July 1<sup>st</sup>-August 31<sup>st</sup>) dissolved oxygen concentrations at six sites continuously monitored by Maryland Department of Natural Resources' program, 'Eyes on the Bay' that were then used to predict clearance rates of oysters with our model. A) Possum's Point 2012, a point on near the mouth of the Corsica River which historically contained an oyster bar and is a restoration site and sanctuary. B) Grey's Creek 2009, a tidal creek off the coastal bays in Maryland. C) Bishopville Prong 2003, a site in the coastal bays of Maryland. D) Harness Creek 2007, A creek near the mouth of the South River used as an oyster restoration site. E) Mataponi 2003, a freshwater creek near the head of the Patuxent River, unsuitable for oyster habitation but included in this model to examine how oysters may react to prolonged periods of hypoxia. F) St. Mary's College 2008, a site on the Potomac River with historical and extant oyster bars.

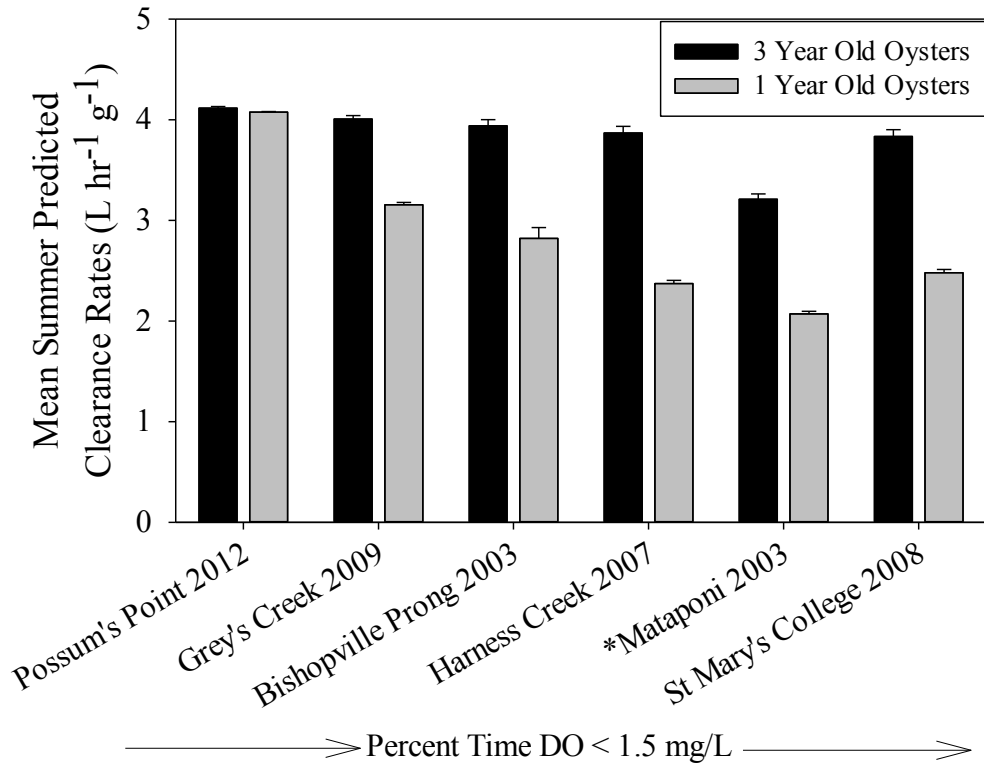


Figure 16. Model predicted clearance rates at six sites continuously monitored by Maryland Department of Natural Resources' program, 'Eyes on the Bay.' \*Mataponi is a freshwater creek included in this figure to examine the effects of dissolved oxygen concentration at a site with a high percent of time below 1.5 mg L<sup>-1</sup> that while also rarely rises above 7.8 mg L<sup>-1</sup>.

## **Chapter II: The Effects of the Diel-cycling of DO and pH on the Hemolymph pH of the Eastern Oyster, *Crassostrea virginica* (Gmelin)**

### ***Introduction***

Low pH (hypercapnia) and low dissolved oxygen (hypoxia) occur in shallow waters around the world on a diel cycle (Tyler, Brady, and Targett 2009) as a result of the day/night variation in total photosynthesis and total respiration. During daylight, the oxygen produced and carbon dioxide fixed by photosynthesis can be greater than the oxygen consumed and carbon dioxide produced by respiration. During the night and early morning, DO and pH can decline as respiration by both autotrophs and heterotrophs depletes water-column oxygen and produces carbon dioxide (D'Avanzo and Kremer 1994). The peaks and troughs of this cycle are intensified in eutrophic systems (D'Avanzo et al. 1996).

*Crassostrea virginica* (Gmelin), eastern oysters, are ecosystem engineers that provide diverse habitats, food, and spawning grounds to other organisms (Coen et al. 1999; Peterson et al. 2003; Grabowski et al. 2005; Breitburg & Fulford 2006). Additionally, filtration of the water by oysters can improve water quality (Newell 1988; Cerco and Noel 2005, 2007), especially at the creek (Dame & Libes 1993) and the tributary scales (Fulford et al. 2007). *C. virginica* populations in the Chesapeake Bay have declined to <1% of their historic populations (Newell 1988; Rothschild et al. 1994; Wilberg et al.

2011) and *C. virginica* fisheries have collapsed along the eastern coast of North America (Kirby 2004).

Hypercapnia and hypoxia affect oysters in a variety of ways. Both hypercapnia (Miller et al. 2009; Beniash et al. 2010; Waldbusser et al. 2011) and hypoxia (Widdows 1989; Baker & Mann 1992) can decrease oyster growth. Reduced pH resulting from hypercapnia as well as hypoxia can decrease oyster immune defenses by decreasing the production of reactive oxygen intermediates (Boyd & Burnett 1999). Hypoxia on its own can decrease feeding of newly settled oyster spat (Baker & Mann 1994) and adult oysters (Chapter 1), inhibit the growth and metamorphosis of other larval bivalves (Gobler et al. 2014), and can increase oyster susceptibility to other stressors (Anderson et al. 1998; Boyd & Burnett 1999; Macey et al. 2008; Ivanina et al. 2011; Breitburg et al. Submitted). Diel-cycling hypoxia can also slow the filtration rates of *C. virginica* (Chapter 1) and decrease how often *C. virginica* open their valves (gape) (Porter et al. Unpublished).

Emersion in water with continuous low pH driven by hypercapnia has been shown to cause an initial large decrease in the extracellular pH in bivalves (Boyd and Burnett 1999) and other marine invertebrates (Pörtner et al. 1998), followed by a partial compensation (Burnett 1997). In bivalves, this compensation has been shown to correlate with an increase in  $\text{Ca}^{2+}$  ions in the hemolymph derived from the dissolution of the shell (Crenshaw and Neff 1969; Lindinger et al. 1984). Because  $\text{Ca}^{2+}$  can take a several hours to accumulate in the hemolymph (Michaelidis et al. 2005), this process of partial compensation takes several hours (Burnett 1997).

Oysters respond to low dissolved oxygen (DO) of  $0.5 \text{ mg L}^{-1}$  by slowing their clearance rates of algal particles (Chapter 1) and closing their valves (Lombardi et al.

2013; Porter et al. unpublished). Forcing an oyster to remain closed decreases the pH of the oyster's hemolymph (Lombardi et al. 2013). Hypoxia may therefore decrease oyster hemolymph pH because of the decreased gaping. Furthermore, when oysters are exposed to hypoxia, they maintain most (75%) (Stickle et al. 1989) of their normoxic metabolic rate but switch to mostly anaerobic pathways (Willson & Burnett 2000) resulting in the production of acidic metabolites. Finally, Boyd and Burnett (1999) showed that exposure to a combination of hypercapnic and hypoxic water decreases oyster hemolymph pH.

Bivalve hemolymph pH affects the function of immune defenses. As hemolymph pH decreases, so does the production of reactive oxygen intermediates (Boyd and Burnett 1999), lysosome activity (Beesley 2008), phagocytic stress response (Bibby et al. 2008) clearance of *Vibrio campbellii* (Macey et al. 2008). Reduced cell signaling, phagocytosis, and lysosomal health, which may be at the root of these susceptibilities, may be decreased by the increased concentrations of calcium ions in the hemolymph of bivalves exposed to low environmental pH (Beesley et al. 2008; Bibby et al. 2008) that result from shell dissolution (Lindinger et al. 1984).

I measured the hemolymph pH in oysters exposed to constantly well-aerated conditions and those exposed to cycling DO and pH conditions and asked the following questions. 1) How does the cycling of water pH, as occurs in diel-cycling hypercapnic hypoxia, affect oyster hemolymph pH on an hourly time scale? 2) Does cycling hypoxia alone affect oyster hemolymph pH? 3) Can hypoxia and hypercapnia act interactively to affect oyster hemolymph pH ?

## ***Methods***

The hemolymph pH study was part of a larger experiment on the effects of diel cycling hypoxia and hypercapnia on the transmission and progression of *Perkinsus marinus* (Breitburg et al. Submitted; Keppel et al. Unpublished). I report disease measurements and consider the implications of disease levels in the Discussion.

### **Oyster Collection**

One-year-old oysters were purchased from a local oyster grower Marinetics Inc. (Cambridge, MD). Salinity at the Marinetics facility was within 2 PSU of Rhode River water at SERC. At the Smithsonian Environmental Research Center (SERC), oysters were placed in a raceway filled with water of the same salinity from which they had been taken, and Rhode River water was slowly dripped in.

### **Treatments**

In 2012, ninety 1-year-old oysters were placed in each of thirty 20 gallon tanks. Water flowed to the tanks at a rate of  $1 \text{ L min}^{-1}$ . Because these oysters were part of a larger disease transmission experiment (Keppel et al. unpublished), additional water was supplied to experimental tanks from a single raceway of diseased older oysters at  $66 \text{ mL min}^{-1}$ . Raw water was supplemented with about  $12 \text{ mL h}^{-1}$  DT's Live Marine Phytoplankton (Innovative Marine Aquaculture, Inc).

Tanks were arranged in a randomized block design with 6 replicates of each of 4 treatments: 1) a control, where DO was held near normoxia (defined here as near 100% air-saturation) and pH was 7.8 (the pH consistently achieved through vigorous aeration of the water in our experimental systems and used here as 'normcapnia'), and treatments in



which 2) DO was held at normoxia while pH cycled from 7.8 to 7.0, 3) DO cycled from normoxia down to  $0.5 \text{ mg L}^{-1}$  while pH was held near normcapnia, and 4) DO cycled down to  $0.5 \text{ mg L}^{-1}$  and pH cycled down to 7.0 (Fig. 1). Control treatment tanks were bubbled continuously with air to maintain normoxic conditions and this air switched between ambient air or  $\text{CO}_2$ -stripped air to maintain a pH of 7.8. In general, each 24 h treatment cycle had 6 h of near air-saturated conditions (the ‘normoxia/normcapnia plateau’), a 4 h descent to low oxygen and low pH (the ‘descent’), 3 h where DO/pH stayed low (the ‘low plateau’), a 4 h return to normoxia and 7.8 pH (the ‘ascent’), a point where near-air saturated conditions return (the ‘recovery point’), and 2 h of these near air-saturated conditions (Fig. 1). After this 2 h period of normoxia and normcapnia oysters in the cycling treatments were then also exposed 2 h of supersaturated DO (defined here as  $> 100\%$  air saturation) of  $10 \text{ mg L}^{-1}$  DO and 8.0 pH to mimic the mid-day portion of the daily cycle, though I did not measure hemolymph variables during this part of the cycle. Daily cycles described above were maintained for nearly 3 months for 5 days a week, beginning early-July. DO in all tanks was held at near saturation for the remaining 2 d a week so that hazardous conditions only occurred when they could be closely monitored and to mimic the day to day variability of DO/pH cycling. Day/night light cycles were altered so that low DO and dark photo-periods occurred during the day when conditions could easily be monitored. Oysters were removed from their tanks for cleaning once every 14 d, during the period of time when DO/pH in the treatment tanks was ascending.

DO and pH were continuously monitored in 1 replicate of each treatment using Oxyguard standard DO probe IIIs (Oxyguard International A/S, Birkerød, Denmark) and

Honeywell Ion Sensitive Field Effect Transistor (ISFET) Durafet III pH probes. These data were fed to a LabVIEW™ (National Instruments Corp.) program, which in turn controlled the flow rates of N<sub>2</sub>, Air, CO<sub>2</sub>-stripped Air, CO<sub>2</sub>, and O<sub>2</sub> gasses to tanks (Burrel et al. in draft). The ratio of these gasses changed the DO and pH in the tanks. A YSI ProPlus (Yellow Springs Instruments) and an Oakton Acorn pH 5 Meter with a single-junction pH electrode and temperature probe (Oakton Instruments) were also used to measure DO concentration, pH, salinity, and water temperature 2 to 3 times a day in all replicate tanks to assure that the 5 remaining replicate tanks of each treatment not measured by the Oxyguard and Durafet probes attached to the automated system were nonetheless experiencing the same DO and pH. Alkalinity was measured three days and determined through titration using a Corning pH Analyzer 350 (APHA 1992); pCO<sub>2</sub> was then calculated using CO<sub>2</sub>SYS (Pelletier et al. 2007) with the constants of Cai and Wang (1998).

### **Hemolymph Collection and Measurement**

In mid-September, when oysters had been exposed to this daily cycle for 6 weeks, I began to collect hemolymph samples at 6 specific times in the daily cycle (illustrated in Fig. 1): 1) at the end of the normoxia/normcapnia plateau, 2) 2 h (halfway) into the descent, 3) at the beginning and 4) end of the low plateau, 5) 2 h (halfway) into the ascent, and 6) the recovery point (Fig. 1). On each of 6 days, I collected water quality data and 1 oyster from a single replicate of each treatment at each of these 6 critical time points. Water temperature measurements were recorded with the same temperature probe used for pH measurements. Water salinity measurements were recorded with a YSI Pro+. Total alkalinity of the water (Dickson 2007) was measured on 3 of the 6 days on which

oyster hemolymph was sampled through titration using a Corning pH Analyzer 350 (APHA 1992). pCO<sub>2</sub> was then calculated using CO2SYS (Pelletier et al. 2007) with the constants of Cai and Wang (1998).

Glass syringes were prefilled with N<sub>2</sub> saturated deionized water and kept on ice until used. On the first day of hemolymph sampling, 1 oyster in each treatment was removed from one replicate tank of each treatment at each time point, its shell was notched, and approximately 0.5 mL of hemolymph was drawn anaerobically from the adductor muscle. Hemolymph was immediately and gently transferred to 1 mL microcentrifuge tubes that were covered in parafilm to limit gas exchange and held in a bath of water at the same temperature as the experimental tanks from which the oysters were held. The hemolymph pH was measured immediately using a semi-microelectrode (Thermo Scientific Orion 8115BNUWP ROSS Ultra Electrode) and an Oakton Acorn 5 handheld meter and temperature probe; this process took less than a minute. Temperature and pH of the tanks from which the oysters were taken were also recorded using the same devices; pH was calibrated with NIST standards at the same temperature as the tank water. This process was repeated on each of the 6 days, randomizing the order in which treatments were sampled for a randomized complete block design.

After collection and measurement of hemolymph, each oyster was measured for standard shell height (from the umbo to the farthest point on the bill) with a flexible ruler and dissected. I removed the anus and I used Ray's fluid thioglycollate culture method (RFTM) (Ray 1996) to detect *Perkinsus marinus* infections and to score them on the Mackin scale (Mackin 1962). Oysters with a score of zero were classified as uninfected

although this method can miss very light infections (Bushek et al. 1994). The remaining wet meat of the oyster was then weighed to the nearest tenth of a gram.

## **Statistics**

All statistics were performed with Statistical Analysis Software 9.2 (SAS) unless otherwise stated and  $p < 0.05$  was used as the general significance level. I used 1-way ANOVAs (Proc GLM) to test for differences among treatments in length, wet meat weight, and *P. marinus* infection status of oysters (See Keppel et al. unpublished for disease analysis of full experiment). To show the changes in oyster hemolymph pH relative to water pH across the treatments, I used a generalized linear model (Proc GLM) with disease status as a random categorical variable to test the effect of water pH (a continuous variable) and DO treatment (a class variable) on the difference between water pH and hemolymph pH. Highest order non-significant terms of the model were consecutively removed. I also used SigmaPlot12.3 to create a linear model to describe the relationship between water pH and hemolymph pH. Finally, I used a 1-way ANOVA (Proc GLM) to test for the effect of time (a class variable) on control oysters to test whether time of day, rather than cycling DO/pH conditions, affected oyster response; I used the same 1-way ANOVA on all three treatments.

## **RESULTS**

Mean length of oysters was 53 mm  $\pm$  0.6 SE, mean wet meat weight was 1.39 g  $\pm$  0.04 SE. *P. marinus* infection prevalence was 27.78% and RFTM scores ranged from 0 to 2, indicating low levels of infection. There were no significant differences among treatments in the length ( $F(3,140)=0.15$ ;  $p=0.88$ ), wet meat weight ( $F(3,140)=0.20$ ;  $p=0.68$ ), or RFTM score ( $F(3,140)=0.72$ ;  $p=0.49$ ) of the oysters used for this study. Mean water pH was within 0.10 of target pH (Table 1). Average pCO<sub>2</sub> in control tanks was 986  $\mu$ atm while the average treatment tank that cycled in pH cycled from 1010  $\mu$ atm to 7001  $\mu$ atm.

Both water pH ( $F(3,140)=219.95$ ;  $p<0.0001$ ; Fig. 18) and disease status (infected versus uninfected; ( $F(3,140)=21.74$ ;  $p<0.0001$ ; Fig. 19) significantly affected the difference between water pH and hemolymph pH. Hemolymph pH increased with increasing water pH ( $n=144$ ; Adjusted  $R^2=0.51$ ;  $p<0.0001$ ; Fig. 18) while infection with *P. marinus* decreased hemolymph pH ( $F(3,140)=21.74$ ;  $p<0.0001$ ; Fig. 19). DO treatment, water pH, and RFTM score did not interact to affect the difference between water pH and hemolymph pH (all  $p<0.05$ ) in the generalized linear model. The effect of DO treatment as a class variable was also not significant ( $F(3,140)=2.09$ ;  $p=0.15$ ).

In the control treatment, mean hemolymph pH averaged 7.62 and remained about 0.2 pH units ( $0.212 \pm 0.039$  SE) below mean environmental water pH throughout all 6 time points. Time of day did not affect the hemolymph pH of the control oysters ( $F(5,30)=0.74$ ;  $p=0.60$ ). Oysters in the DO-only cycling treatment also exhibited this consistency of hemolymph pH across time points ( $F(5,30)=0.19$ ;  $p=0.96$ ), averaging 7.60 and 0.13 pH units  $\pm$  0.02 SE below water pH. In contrast, hemolymph pH in the pH only

cycling ( $F(5,30)=9.68$ ;  $p<0.0001$ ) and combined pH and DO cycling treatments ( $F(5,30)=6.80$ ;  $p=0.0002$ ), varied with phase of the cycle. In these treatments, mean hemolymph pH averaged 7.57 and was 0.23 pH units  $\pm$  0.02 SE below mean water pH at the normcapnic parts of the cycle, but averaged 7.17 and was 0.14 pH units  $\pm$  0.04 SE higher than the mean water pH during the low plateau.

## ***DISCUSSION***

This study indicates that periods of low pH, as occur during the diel-cycling of DO and pH, decrease the hemolymph pH of *C. virginica*. It has already been established that constant low water pH decreases the hemolymph pH of oysters (Boyd and Burnett 1999) and other invertebrates (Pörtner et al. 1998) and this study shows that brief periods of low pH do the same, but only for the period when water pH is low (Figs. 18 & 20); recovery is rapid as water pH increases. The difference between hemolymph pH under normcapnic and hypercapnic conditions can be large, averaging 0.40 pH units in this study. Additionally, the present study indicates that oyster hemolymph pH can be higher than water pH even at water pH values between 7.36 and 7.60 (probe accuracy is 0.01 pH units; Fig. 20).

Boyd and Burnett (1999) observed hemolymph pH below water pH after only 1 h of constant exposure to hypercapnic water (1999) and these hemolymph pH measurements were lower than those taken after the oysters had been exposed to hypercapnic conditions for 2 days. In the present study, I did not observe an initial sharp decrease in hemolymph pH. Instead, hemolymph pH seemed to fall and rise more slowly than water pH, never getting as low as the water's low pH extreme (Fig. 20). The difference between results of the present study and of Boyd and Burnett's (1999) may be due to the gradual decline in water pH employed in the present study. The release of  $\text{Ca}^{2+}$  ions derived from the dissolution of the shell (Lindinger et al. 1984) into the hemolymph may have already begun during this decline of water pH, before my second measurement, preventing that initial drop. The elevation of hemolymph pH over water pH when water pH was declining supports this theory (Fig. 20).

As expected based on Dwyer and Burnett's (1996) results, oysters with *P. marinus* infections also had lower hemolymph pH at all points in the cycle (Fig. 4). *P. marinus* infections are prevalent among *C. virginica* in Atlantic and Gulf Coasts of North America (Cook et al. 1998, Wilberg et al. 2011), especially in adult oysters (Burreson 1991). Therefore, including infected oysters in this study realistically mimics how oysters in the field might react to the diel-cycling pH.

Oysters in my control tanks had an average hemolymph pH of 7.62, those in the cycling DO only treatment averaged 7.60, and those in the pH cycling treatment averaged 7.57 during the normcapnic portion of their cycle. These are similar to the 7.55 hemolymph pH of oysters under well aerated conditions in Boyd and Burnett's (1999) investigation. They are also similar to the 7.7 hemolymph pH of oysters that had been held under well aerated conditions and had yet to be aurally exposed in Dwyer & Burnett's (1996) study. The oysters in the present study subjected to cycling pH conditions had an average hemolymph pH of 7.17 during exposure hypercapnia. This is similar to the 7.12 hemolymph pH of oysters exposed to constant hypercapnia for 48 h but is higher than that of 6.9 in oysters exposed to constant hypercapnia for only 1 h (Boyd & Burnett 2000).

The low extreme pH and pCO<sub>2</sub> used in this study were 7.0 and 7001 μatm. This is substantially higher than the oceanic pCO<sub>2</sub> predicted for the future, but it is realistic and currently relevant in the estuarine ecosystems where these oysters live, where pH can drop below 7.0 (Chapter 1). This study also does not distinguish between the effects of pCO<sub>2</sub> and pH since ratios of CO<sub>2</sub> to other gasses were used to control the pH in this experiment.



I detected no effect of DO on hemolymph pH (Fig. 20), despite the fact that *C. virginica* close their valves for a greater percent of time under hypoxic conditions than under air-saturated conditions (Porter et al. Unpublished) which decreases hemolymph pH (Lombardi et al. 2013) and despite the reliance of oysters on anaerobic pathways when exposed to hypoxia (Stickle et al. 1989; Willson & Burnett 2000). This may be because these oysters are gaping sufficiently to prevent acidosis. Gaping under hypoxic conditions, as oysters in the present study may be doing, is different from gaping while aerially exposed. Under 25° and 35°C, aerially exposed *C. virginica* do not observably gape (Willson & Burnett 2000) and their hemolymph pH decreases (Dwyer & Burnett 1996). However, data on algal clearance rate by emersed oysters indicate that, while *C. virginica* filter less water at 0.5 mg L<sup>-1</sup> DO than under air-saturated conditions, they do continue to filter, indicating that they do, at least periodically, open their valves (Chapter 1).

## **Conclusion**

Diel-cycling hypercapnic hypoxia occurs in areas near historical oyster reefs and reef restoration sites and daily minimum pH can fall to 6.25 in some locations (MD DNR's EyesOnTheBay.net; Chapter 1). Because decreased hemolymph pH can decrease bivalve immune functions (Boyd and Burnett 1999; Beesley et al 2008; Bibby et al. 2008; Macey et al. 2008), it is important to understand how these daily cycles affect hemolymph pH. This study shows that even a short period of low pH can decrease oyster hemolymph pH by 0.40 units.

### ***Figures and Tables***

Table 6. Mean water pH measurements within the different treatments taken at the six time points (illustrated in Fig. 1) when hemolymph was measured (n=6).

Time Points	Water pH (mean $\pm$ SE)			
	Control	Cycling DO Treatment	Cycling pH Treatment	Co-cycling Treatment
End of the normoxia/normcapnia plateau	7.89 $\pm$ 0.02	7.87 $\pm$ 0.02	7.88 $\pm$ 0.02	7.88 $\pm$ 0.03
Halfway into the descent	7.85 $\pm$ 0.02	7.85 $\pm$ 0.01	7.38 $\pm$ 0.03	7.38 $\pm$ 0.05
Beginning of the low plateau	7.82 $\pm$ 0.03	7.81 $\pm$ 0.02	7.07 $\pm$ 0.03	7.09 $\pm$ 0.03
End of low plateau	7.83 $\pm$ 0.01	7.84 $\pm$ 0.03	7.08 $\pm$ 0.02	7.08 $\pm$ 0.02
Halfway into the ascent	7.81 $\pm$ 0.01	7.81 $\pm$ 0.01	7.36 $\pm$ 0.02	7.33 $\pm$ 0.03
Recovery point	7.81 $\pm$ 0.01	7.81 $\pm$ 0.01	7.79 $\pm$ 0.01	7.79 $\pm$ 0.01

Table 7. Water quality measurements in tanks on dates hemolymph samples were measured.

Date	Salinity	Temperature (°C)	Total Alkalinity ( $\mu\text{Eq L}^{-1}$ )
9/12/2014	12.6 $\pm$ 0.1	24.9 $\pm$ 0.3	1736
9/13/2014	12.7 $\pm$ 0.1	24.9 $\pm$ 0.3	1720
9/14/2014	12.7 $\pm$ 0.1	25.0 $\pm$ 0.2	.
9/18/2014	13.3 $\pm$ 0.0	23.6 $\pm$ 0.0	1708
9/19/2014	13.3 $\pm$ 0.1	23.2 $\pm$ 0.1	.
9/20/2014	13.4 $\pm$ 0.1	23.1 $\pm$ 0.3	.

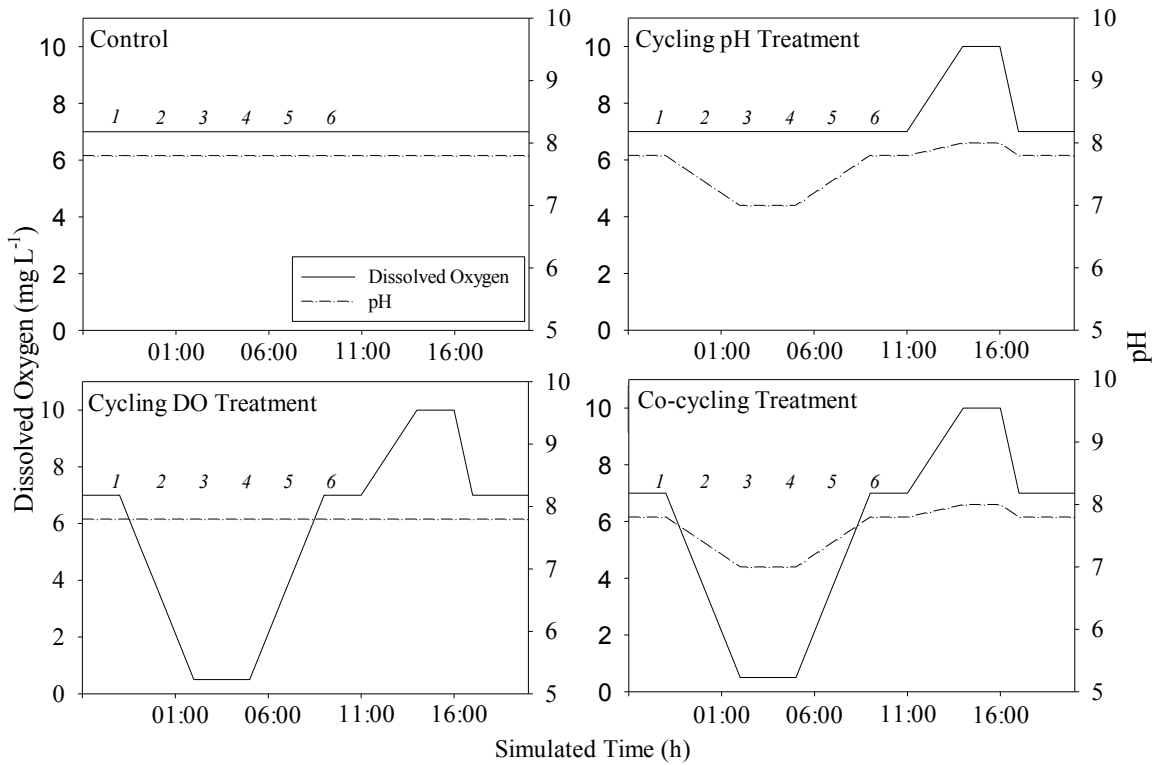


Figure 17. The dissolved oxygen and pH cycles of the control and 3 treatments. Hemolymph pH of oysters were measured at 6 time points: at the end of the normoxia plateau, before DO/pH began to cycle for the day (Time Point 1), halfway into the descent of DO/pH to their low extremes (Time Point 2), at the beginning of the low plateau, when DO/pH had first reached their low extremes (Time Point 3), at the end of the low plateau, when DO/pH had been low for 3 h (Time Point 4), halfway into the rise of DO/pH (Time Point 5), and at the recovery point, when DO/pH first returned to normoxia/normcapnia (Time Point 6). Time points are indicated on the panels above with shaded and numbered columns.

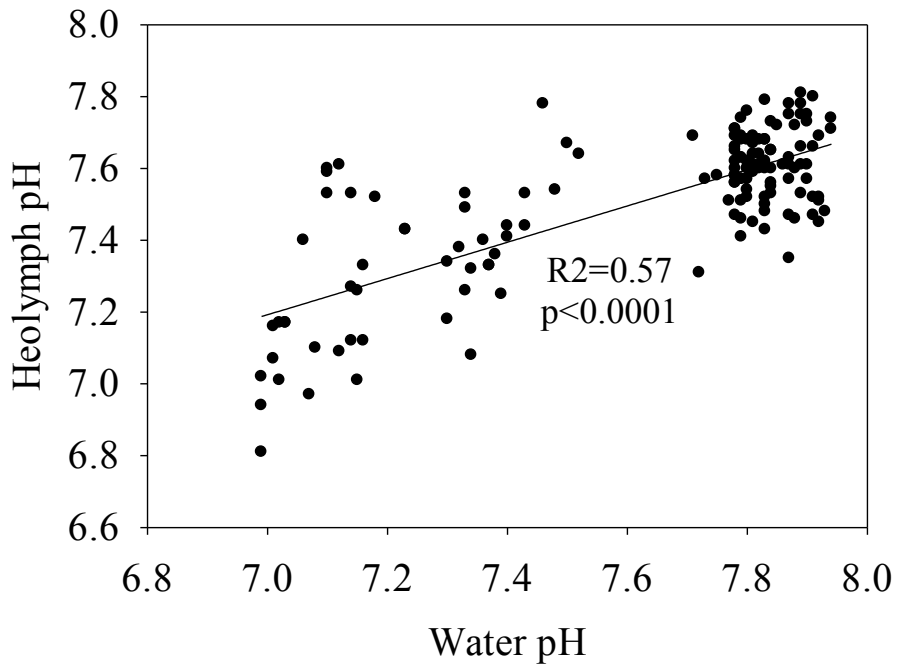


Figure 18. A scatter plot of individual measurements of water pH and hemolymph pH for all oysters sampled. The equation is  $f = 3.66 + 0.51x$  where  $f$  is hemolymph pH,  $x$  is the water pH.

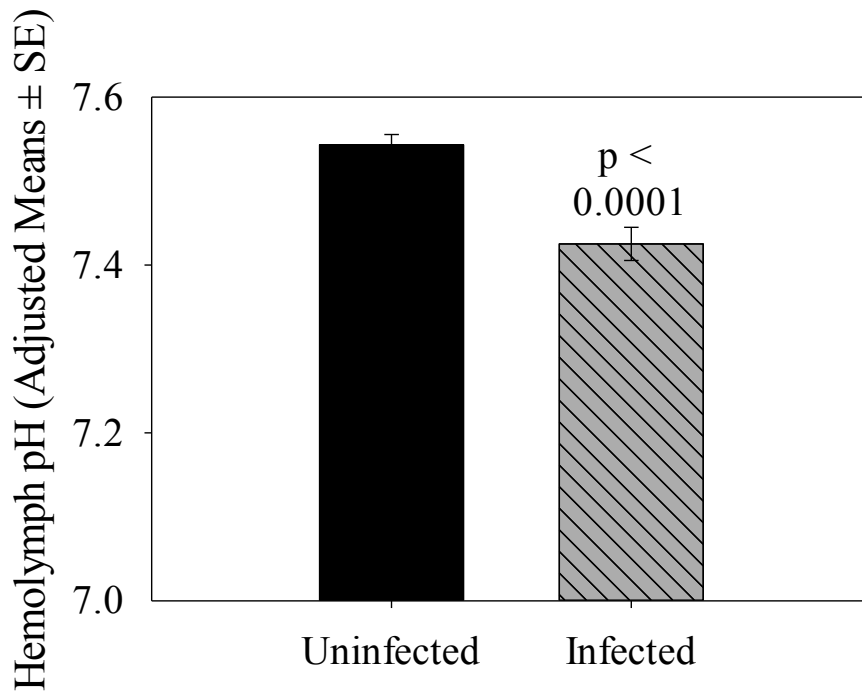


Figure 19. The mean hemolymph pH in oysters either infected or not infected with *Perkinsus marinus*. These means were adjusted for the influence of water pH (least squared means).

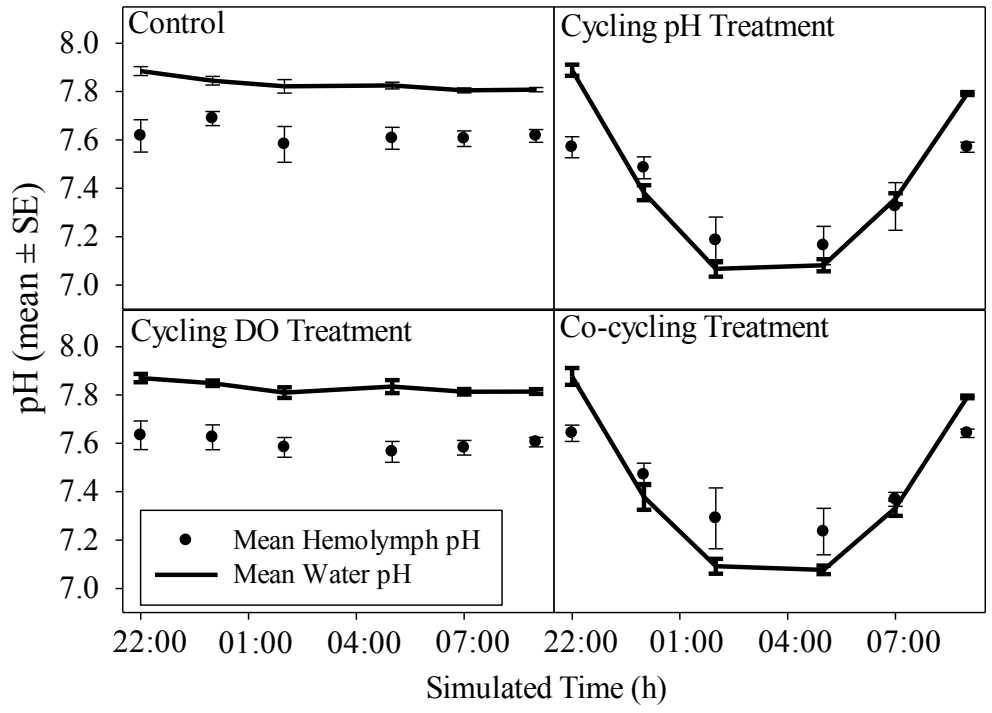


Figure 20. Mean hemolymph pH of oysters treatments measured at six time points in the control and cycling treatments. Mean water pH within the treatments is also shown for comparison, but not DO, as DO did not affect oyster hemolymph pH ( $p=0.15$ ).

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