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Effects of co-varying diel-cycling hypoxia and pH on disease susceptibility in the eastern oyster *Crassostrea virginica*

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ABSTRACT: Diel-cycling hypoxia co-occurs with diel-cycling pH in shallow waters that are typically considered as refuge from deep-water hypoxia and are, therefore, targeted for restoration. These areas also tend to be heavily impaired by eutrophication from nutrient over-enrichment which increases the occurrence and severity of hypoxia and pH cycles. We used laboratory experiments to investigate the effects of diel-cycling dissolved oxygen (DO) and co-varying diel-cycling pH on infections of *Perkinsus* spp. and hemocyte activity in the eastern oyster *Crassostrea virginica*. *Perkinsus marinus* is the protistan parasite that causes Dermo disease in oysters. Severe diel-cycling DO increased the acquisition and progression of *Perkinsus* infections during exposure, and had a legacy effect the next year. Diel-cycling pH did not significantly affect infection dynamics either on its own or in combination with diel-cycling DO. Diel-cycling DO and pH both individually and in conjunction stimulated hemocyte activity, although this stimulated activity may not be effective at preventing *Perkinsus* infection. The magnitude of cycling conditions is an important consideration when choosing restoration sites, as severe cycling may hinder the re-establishment of oysters by creating areas that serve as reservoirs for parasites that can infect nearby populations.

KEY WORDS: Hypoxia · pH · Diel cycle · Multiple stressors · Disease · Oyster

INTRODUCTION

Shallow waters in estuaries and coastal zones traditionally are considered a refuge from deep-water benthic hypoxia (Bartol et al. 1999, Eby & Crowder 2002, Bell & Eggleston 2005) and often are targeted for species restoration (Lenihan et al. 2001, Byers et al. 2006); however, shallow waters are characterized by their own set of stressors. Diel cycles occur naturally in shallow waters, including those minimally affected by human activities, and are driven by daily or tidal cycles of respiration, photosynthesis, and other environmental factors (Nixon & Oviatt 1973, Kemp & Boynton 1980, Tyler et al. 2009). The magnitude of these cycles can vary from day to day, and may result in periods of hypoxia (dissolved oxygen [DO] concentrations below saturation) and environmental hypercapnia (elevated pCO_2 resulting in reduced pH) (Fig. 1) (Burnett & Stickle 2001, Tyler et al. 2009). The magnitude of DO and pH cycles are exacerbated by eutrophication (Boynton et al. 1996, Diaz & Rosenberg 2008) and are expected to worsen with increasing atmospheric CO_2 and consequent increases in global temperatures (Boynton et al. 1996, Diaz & Rosenberg 2008, Rabalais et al. 2010). Cycling DO/pH has the potential to create local variation in conditions available throughout a system, and may have sub-lethal effects upon individuals with

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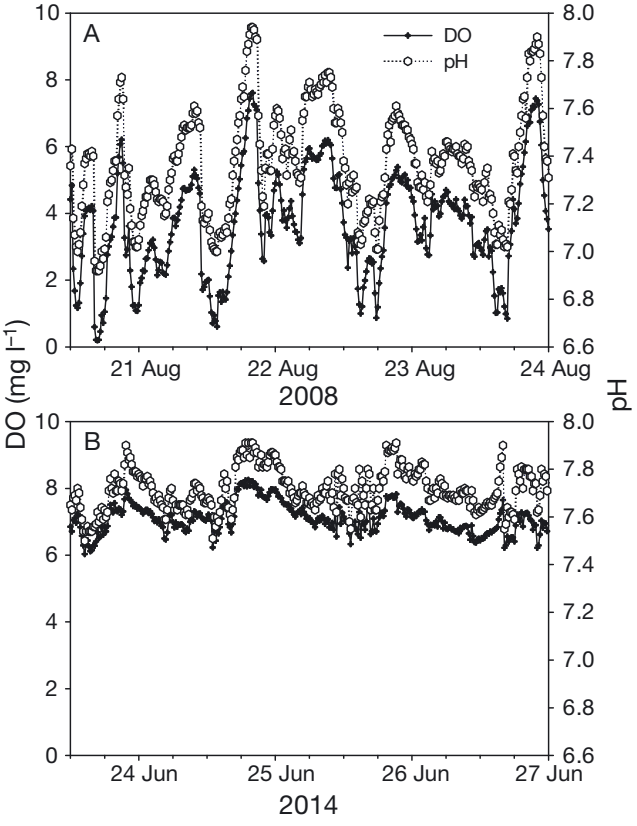

Fig. 1. DO and pH measured every 15 min in (A) the St. Mary's River showing diel-cycles during ca. 1 week in 2008 and (B) the Harris Creek Downstream continuous monitoring station during 1 week in 2014. Data from MD-DNR Shallow Water Monitoring Program available at www.eyesonthebay.net

negative consequences for populations (Sagasti et al. 2001, Eby et al. 2005, Tanner et al. 2006, Breitburg et al. 2015).

Acidification caused by elevated atmospheric CO_2, nutrient enrichment, and other sources is predicted to decrease the relatively stable pH of open ocean systems but the impacts on shallow water coastal systems are less predictable (Anthony et al. 2008, Yamamoto-Kawai 2009, Cai 2012). In shallow waters, the magnitude of daily fluctuations in pCO_2 ranges widely, from minimal fluctuations as in the Gironde estuary in France (Frankignoulle et al. 1998) to a factor of 10 or more in systems such as the Thames estuary in the United Kingdom (Frankignoulle et al. 1998) or the Anacostia River in the USA (Bala Krishna Prasad et al. 2013). These systems also experience large daily fluctuations in pH, although the relationship between pCO_2 and pH is controlled by the carbonate chemistry of the system (Doney et al. 2009). In the eutrophic Chesapeake Bay, a network of near-bottom shallow-water sensors has shown pH values can cycle one full unit or more per day (Breitburg et al. 2015). These meters also record large amplitude cycles of hypoxia, in some cases as large as 10 mg l^{-1} or more in a single day. Although diel-cycling DO and pH are common and mechanistically linked (Portner 2008, Levin et al. 2009), most laboratory research has focused on continuous hypoxia or cyclical DO without manipulating pH (e.g. Baker & Mann 1992, Dwyer & Burnett 1996, Lenihan & Peterson 1998, Burnett & Stickle 2001). Similarly, open-ocean constant pH has been a major focus of acidification research, with far less research replicating cyclical conditions (e.g. Bamber 1987, Burnett 1997, Waldbusser et al. 2011).

Exposure to hypoxia can negatively affect survival, growth, and reproduction of organisms (Boyd & Burnett 1999, Burnett & Stickle 2001, Breitburg et al. 2009, Vaquer-Sunyer & Duarte 2010), and has the potential to increase susceptibility to pathogens (Smolarz et al. 2006). Exposure to acidified water has also been associated with a wide range of biological effects, including increased mortality, altered production of reactive oxygen intermediates, decreased growth, reduced tissue energy stores, and decreased calcification rates (Boyd & Burnett 1999, Ringwood & Keppler 2002, Gazeau et al. 2007, Dickinson et al. 2012). For example, Dickinson et al. (2012) found increased mortality, reduced tissue energy stores, and negative soft tissue growth in the eastern oyster *Crassostrea virginica* (Gmelin, 1791) exposed to a pCO_2 of 800 ppm for 11 wk when compared to a pCO_2 of 400 ppm.

Invertebrate immune systems are affected by hypoxia and acidification, sometimes positively and sometimes negatively (Boyd & Burnett 1999, Burnett & Stickle 2001). Studies have also shown that higher bacterial loads can be found in organisms exposed to hypoxia and acidified water, including the blue crab *Callinectes sapidus* (Holman et al. 2004) and eastern oyster *C. virginica* (Macey et al. 2008). Predicted end-of-century ocean acidification levels increased *Vibrio tubiashii* infections in the blue mussel *M. edulis* (Asplund et al. 2014), and *Vibrio paraehaemolyticus* infections in the Norway lobster *Nephrops norvegicus*, and, when combined with hypoxia, reduced hemocyte counts in the Norway lobster (Hernroth et al. 2015).

The eastern oyster *C. virginica* is an important fishery species and ecosystem engineer throughout its range in the western Atlantic from Brazil to Canada's St. Lawrence River (Hargis & Haven 1999, Mann & Evans 2004). In Chesapeake Bay, stocks are estimated to be at or below 1% of historic levels (Newell

1988, Wilberg et al. 2011). Post-settlement oysters cannot move to avoid hypoxic events, and, in spite of tolerance to low DO, constant hypoxia reduces feeding, metabolism, and growth (Widdows et al. 1989, Baker & Mann 1992, Burnett & Stickle 2001) and delays and reduces larval settlement (Widdows et al. 1989). Early post-settlement *C. virginica* are susceptible to exposure to episodic hypoxia that does not cause mortality in older juveniles or adults (Osman 1994).

Reactive oxygen species (ROS) produced by hemocytes are an important part of the immune response in *C. virginica*. ROS production following pathogen or proxy challenge is commonly measured as a determinant of immune capacity. Unstimulated ROS production measures the innate levels of ROS produced by cell metabolism, whereas measurement of stimulated ROS production can indicate the ability of the cell to kill pathogens. High unstimulated production of ROS is an indicator of stress, and may be energetically costly and physically damaging to the organism. Anderson et al. (1998) found no effect of hypoxia on unstimulated ROS production in *C. virginica*, although an increase in unstimulated ROS production has been seen in other invertebrates (Moss & Allam 2006). Boyd & Burnett (1999) found that both hypoxia and hypercapnia reduced stimulated production of reactive oxygen intermediates by oyster hemocytes indicating that hypoxia exposure may reduce the ability of oysters to respond to a challenge.

Dermo and MSX are 2 diseases that are particularly damaging to oysters in the Chesapeake Bay region. *Perkinsus marinus,* a protistan parasite that causes Dermo disease in oysters, was initially identified in the Gulf of Mexico and first observed in Chesapeake Bay in the 1940's although it is thought to be endemic to the Chesapeake Bay region. *P. marinus* is one member of a genus of parasites that affect mollusks worldwide (Goggin & Lester 1987, Goggin & Barker 1993, Pecher 2007). Along with overharvesting, loss of hard bottom substrate, and water quality declines, *Perkinsus* infection is one of the major factors limiting eastern oyster populations and restoration efforts today (Ford & Tripp 1996, Harvell et al. 1999, Reece et al. 2001, Carnegie & Burreson 2009, Beck et al. 2011).

Previous laboratory and field studies indicate that diel-cycling DO increases the acquisition and progression of *P. marinus* infections in eastern oysters (Breitburg et al. 2015). Stronger effects of DO on *P. marinus* infection in the field than in the lab suggested the possibility that a co-occurring stressor increased the effect of DO. We postulated that the stressor unaccounted for in laboratory experiments was pH, which shows a tight correlation with DO in the field (Burnett 1997) and has been shown to reduce production of stimulated ROS in *C. virginica*, but which was not controlled in the study by Breitburg et al. (2015). In the present study, we examined whether repeated, short term, co-occurring stressors affected immune responses and acquisition and progression of protist infections by exposing oysters to diel-cycling DO and pH, as well as to each stressor individually, along with water containing *Perkinsus* for 3 mo. Our expectation was that exposure to repeated, brief periods of hypoxia and low pH would increase *Perkinsus* acquisition and progression and disturb the immune response more severely than either stressor independently.

MATERIALS AND METHODS

We tested the effects of diel-cycling DO and pH on *Perkinsus* infection acquisition and progression, as well as hemocyte status, in 1 yr-old eastern oysters (35–70 mm initial length) at the Smithsonian Environmental Research Center (SERC), in Edgewater, MD, USA, during July–September 2012, and the carryover effects of cycling conditions during a field deployment in the Rhode River, MD, USA, during September 2012 to July 2013. Older oysters (4–5 yr) were used as a source of *Perkinsus* in the laboratory portion of the experiment. All oysters were purchased from an aquaculture facility (Marinetics) on the Choptank River, MD, USA in April–May 2012, and held on flow-through Rhode River water at SERC until the experiment commenced. Salinity and temperature at the Marinetics facility were within 2 PSU and 1°C, respectively, of Rhode River ambient conditions at the time oysters were purchased.

Initial *Perkinsus* infection prevalence and intensities were determined in 100 individuals of each age class using Ray's Fluid Thioglycollate Medium (RFTM) assay (Ray 1952, Ray 1954) on rectal tissue. Intensity of infections was scored on the modified Mackin scale which ranges from 0 to 5 (Mackin 1962). A score of 0 indicates no detectable *Perkinsus* in the tissue sample, 0.5 or 1 represent light infections, with few cells in the tissue sample, 2 is a moderate infection, a 3 or 4 is a heavy infection, while a 5 represents a lethal level of infection with all sample tissue full of *Perkinsus* cells. Scores of 2 or higher are considered to reflect moderate-to-heavy infections and represent levels of infection which result in an energetic cost to the oyster.

RTFM assay is not capable of distinguishing *P. marinus* from other species of *Perkinsus*, and previous research has shown other species of *Perkinsus* infecting *C. virginica* (namely, *P. chesapeaki*) (Coss et al. 2001, Reece et al. 2008); therefore, it is possible that multiple species of *Perkinsus* were present in these experiments. However, Reece et al. 2008 found very low prevalence of other species of *Perkinsus* in *C. virginica* and no infections of other species without a co-infection of *P. marinus*. Coss et al. (2001) found 1 in 3 infected oysters harbored both *P. chesapeaki* and *P. marinus*, while a third of the population was infected with solely *P. marinus* and another third with solely *P. chesapeaki*.

Although RFTM assay may fail to detect very light infections (Robledo et al. 1998), it allows for a rapid and cost-effective analysis of infection in a large number of individuals. We define prevalence as the proportion of individuals with detectable infections out of the entire population analyzed; the change in prevalence over the course of the experiment was used as an index of infection acquisition. Mean infection intensity was the average Mackin score among only those oysters with detectable levels of infection (Mackin 1962). One year-old oysters were chosen for this study because they tend to have very low levels of infection due to their relatively short cumulative lifetime exposure and small volume of water filtered during the time of peak transmission in their first summer. Infections in older oysters can range widely depending on exposure but infections tend to increase with age (Paynter et al. 2010).

We ran 6 replicates each of 5 experimental treatments, for a total of 30 experimental units (75 l aquarium). Ninety 1 year-old oysters were assigned to each replicate aquarium, for a total of 2700 individual oysters. Aquaria were arranged in a randomized block design, with 1 replicate from each treatment clustered together, in case laboratory position affected results. Older oysters serving as the infection source were held in an air-bubbled 400 l tank. Experimental oysters were acclimated to aquaria, feeding regime, and light availability at normoxia/normocapnia for 5 d prior to commencing treatment conditions.

Treatments

A factorial design was used crossing 2 pH treatments: constant normocapnia pH (pH 7.8) and cycling pH (between pH 7.0 and 7.8), with 2 DO treatments: constant normoxia (7.0 mg l^{-1}) and severe cycling hypoxia ranging from 0.5 mg l^{-1} to a supersaturated value of 10.0 mg l^{-1} (Fig. 2). A fifth 'moderate hypoxia' treatment was also used, with DO cycling from a low of 1.7 mg l^{-1} to a supersaturated value of 10.0 mg l^{-1} along with cycling pH. For the purpose of these experiments, we defined normocapnia as a pH of approx. pH 7.8, which is a typical near-maximum pH at shallow-water field sites that do not experience severe cycling and have salinity similar to those in the Rhode River (e.g. Fig. 1b). The factorial structure of this design allowed for an estimate of the combined effects of cycling pH and severe cycling hypoxia, as well as the individual main effects. The additional moderate cycling treatment allowed for an estimate of the effects of a more moderate cycling hypoxia when compared to the 'normoxia-cycling pH' treatment. Our experimental facility precluded our ability to run additional treatments to test a full factorial design.

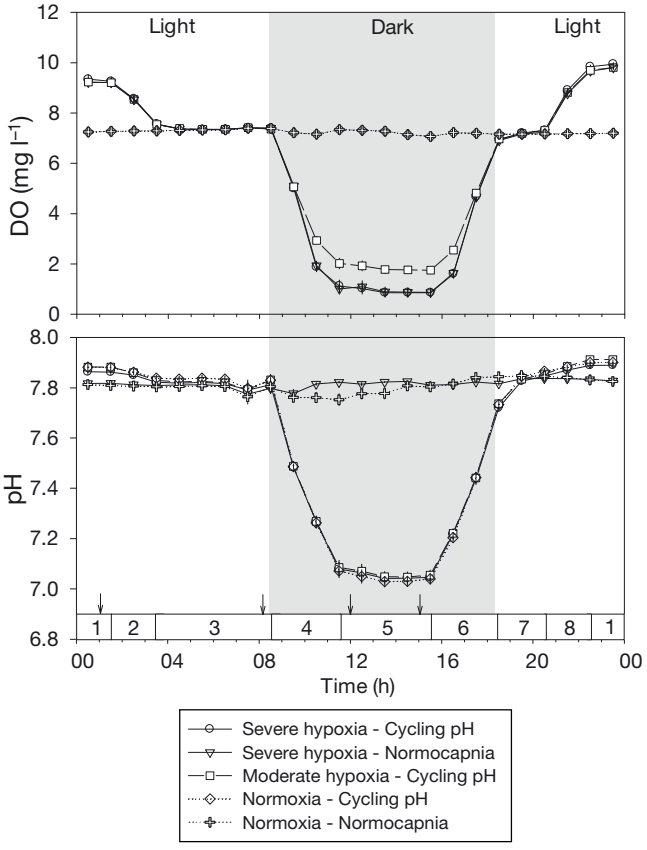

Fig. 2. Mean ± SE (non-visible error bars are obscured by symbols) dissolved oxygen (DO) and pH during 51 d when conditions were cycled. Data are from the 1 replicate of each treatment measured by the LabVIEW control program. Arrows represent times at which DO and pH were measured in all aquaria. Numbers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

One year-old oysters were exposed to cycling conditions 4–5 days per week from 5 July through 27 September 2012 (54 total cycles over 12 weeks). In the cycling treatments, DO and/or pH were decreased over 3h (ramp-down), held at continuous low values for 4h (low-plateau), brought back to normoxia/normocapnia over 3h (ramp-up), held for 2 h (normoxia), taken to supersaturated DO/normocapnia values over 2h (up-to-supersat), held at high values for 2h (supersat-plateau), brought back to normoxia over 2h (down-to-normoxia) and held at normoxia/normocapnia (normoxia) until the next day's cycle commenced (Fig. 2). Photoperiod regime was maintained in a 14 h light:10 h dark cycle, 7 days per week, using incandescent 5V rope-lighting. Light conditions in the tanks simulated those at 2 m depth in the Rhode River on a sunny day as measured with an LI-190 Quantum Sensor (Li-Cor). On the 2–3 days per week on which DO/pH cycling conditions were not applied, treatments were bubbled with air and CO_2 stripped air to maintain target values equivalent to those of the control (constant normoxia/normocapnia) treatment: this resulted in a DO of 7.44 ± 0.00 mg l^{-1} and pH of 7.83 ± 0.00. Water from infected 4–5 yr-old oysters was not transmitted to experimental oysters on these days.

Experimental conditions were monitored and manipulated using a custom-developed LabVIEW (National Instruments) program that used input from Standard DO probes (Oxyguard) and Durafet III pH sensors (Honeywell) and controlled ratios of 5 gases (air, CO_2-stripped air, oxygen, nitrogen, and carbon dioxide) through mass flow controllers (Dakota Instruments). Soda lime CO_2 scrubbers were used to create CO_2-stripped air. DO and pH sensors were checked at least once daily, calibrated weekly, and recalibrated if they were outside of published accuracy ranges. pH probes were 2-point calibrated (NBS scale, Thermo Fisher Scientific) and DO probes were calibrated in water-saturated air. One DO probe and one pH probe were placed in 1 replicate of each treatment and used to control all 6 replicates. One gas mix (30 l min^{-1}) was created per treatment and then split via gas manifolds to deliver 5 l min^{-1} of mixed gas to each replicate aquarium through 2 glass-bonded silica air diffusers (3.75 × 1.25 cm) resting on the bottom at the middle of the aquarium. For details on operation and performance of this system see Burrell et al. (in press).

In addition to continuous monitoring of DO and pH in one replicate, DO, temperature, salinity, and pH were measured 3 to 4 times per day in all aquaria using a YSI ProfessionalPlus (Yellow Springs Instruments), and an Acorn pH 5 meter (Oakton Instruments). This ensured that treatment variables were similar among replicates and that non-controlled variables (temperature and salinity) did not vary among treatments (authors unpubl. data). In-tank partial pressure of carbon dioxide (pCO_2) was measured 3–4 days per week via equilibration in 1 replicate of the control treatment and 1 day per week in 1 replicate of each of the other 4 treatments during the low-plateau part of the cycle using an 840A CO_2/H_2O gas analyzer (Li-Cor). pCO_2 could not be measured more frequently due to the changing pH targets inherent in cycling treatments, requiring individual equilibrators and gas analyzers for each treatment. Alkalinity was determined by titration 3 times per week in 1 replicate of the control treatment using a Tazo Schott-Gerate piston burette titrator and a Corning pH Analyzer 350 according to Standard Method 2320 (APHA 1992). Variation in alkalinity was tightly linked to variation in salinity (see 'Results' section), as is common in estuarine systems. We monitored salinity continuously and were prepared to take additional alkalinity samples if a salinity fluctuation was observed during the experiment, but salinity did not fluctuate widely enough during our experiment to require more frequent measurements of alkalinity. Measurements of pCO_2, alkalinity, and pH provide 3 of the 4 parameters of the carbonate system commonly considered in acidification studies, and were used in pairs to confirm the third measurement using CO2SYS (Pelletier et al. 2007).

Each aquarium received 1 l min^{-1} of flow-through, unfiltered, Rhode River water supplemented with 0.093 mL of stock algal diet (DT's Reef Blend) mixed into the inflow water every 8 minutes, 24 hours per day throughout the experiment, with the exception of a 10 day period in August when the timer controlling the algae system was under repair. While this would have reduced food availability, there was ambient phytoplankton in the SERC raw sea water system, and all treatments experienced the same reduction in phytoplankton availability during this period. Each aquarium also received 75 ml min^{-1} of water from the tank containing infected adult oysters. Both water inputs were located just above the air diffusers in the aquaria to promote mixing. The infected oyster tank was provided a constant 5 l min^{-1} of flow-through Rhode River water. All effluent water from the infected oyster tank and treatment aquaria was UV-sterilized before release to the Rhode River. Oysters were removed from aquaria and washed gently each week to remove mud, feces, pseudofeces, and polychaetes. Aquaria were drained and scrubbed bi-

weekly on a day when conditions were not cycled to remove waste products and bio-fouling.

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

Just before the end of the experiment, 2 oysters were removed from each replicate of each of the 5 treatments at the end of the low-plateau phase on 25 September 2012. Oysters were measured and hemolymph was removed from the adductor-muscle sinus of each oyster using a 1 ml syringe fitted with a 23-gauge needle inserted through a small notch cut into the ventral shell edge. Following hemolymph extraction, oysters were shucked, and a sample of rectal tissue was taken for infection analysis by RFTM assay. The hemolymph from each oyster was held on ice in an Eppendorf tube until being distributed into Falcon flow-cytometer tubes for analyses. In one Falcon tube, counts, mean sizes, and percentages of granular and agranular dead hemocytes were determined with an Accuri C6 flow cytometer (BD BioSciences) using the methods of Hégaret et al. (2003a). In another tube, percentages of total and granular phagocytic hemocytes were determined using 2-µm, plastic microbeads (Hégaret et al. 2003b). In a third tube, reactive-oxygen species (ROS) production by hemocytes was determined using the oxidation of non-fluorescent DCFH-DA to green-fluorescent DCFH (Hégaret et al. 2003b). For this analysis, cells were not stimulated with chemical or particulate inducers of oxidative burst, so values reported (in relative, dimensionless detector units) represented constitutive oxidative activity. Finally, in a fourth tube, percentages of live or dead apoptotic hemocytes were determined using the green-fluorescent probe Annexin V and propidium iodide following the manufacturer's instructions (Product V13241, Life Technologies).

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

To examine carryover effects of cycling conditions on infection acquisition and intensity, 17 oysters from each aquarium were placed in 3000 cm^3 cages constructed of 2 cm square mesh and suspended from SERC piers in the Rhode River approx. 0.5 m above the bottom. Cages were deployed 2 m apart at each site to minimize *Perkinsus* transmission, and in such a way that they were unlikely to be exposed to hypoxia as severe as that seen in the lab and that all treatments would experience similar field conditions. Approximately 9 months later, these cages were collected from the field sites on 18 and 19 July 2013. All oysters were measured and weighed, and infection was assayed.

Statistics

All data were tested for homogeneity of variance using an F-max test and normality using a Shapiro-Wilks test. Percentage data were logit transformed. Unless otherwise noted, data are presented as means ± SE.

The Proc Mixed procedure (SAS v. 9.2) was used to compare salinity, temperature, DO, and pH among the 5 treatments with nested ANOVAs during 2 time points: the end of low plateau and just prior to the ramp-down phase (see Fig 2). For these analyses we used values measured in all 30 experimental aquaria. Effects of DO and pH on *Perkinsus* prevalence and intensity from the laboratory experiment were analyzed using randomized complete block design ANOVAs. *Perkinsus* prevalence and intensity from the field deployment were analyzed using replicated block design ANOVAs with deployed field site as the blocking factor. Least square means comparisons were used to test *a priori* hypotheses that severe cycling DO and cycling pH would increase disease metrics, in combination and independently, and that moderate cycling DO would increase disease metrics in comparison to constant, normoxic treatments. The Proc Mixed procedure was also used to reveal main effects and interactions of the 2 independent variables (cycling DO and pH) upon each hemocyte variable.

RESULTS

Severe cycling hypoxia increased *Perkinsus* infection prevalence and intensity and also affected some metrics of the cellular immune status in *C. virginica* over the course of the 3-month laboratory exposure to cycling conditions. Moderate cycling hypoxia did not significantly affect infection prevalence or intensity; however there was a trend towards increased prevalence of more intense infections under these

Table 1. Mean ± SE daily dissolved oxygen (DO), pH, and pCO_2 conditions in treatments at normoxia, at the end of the low-plateau phase, and at the end of the supersaturated plateau (Supersat) phase. DO and pH were measured towards the end of the normoxia and low plateau phases on 51 d in all replicates of each treatment, and at the supersaturated-plateau phase on 6 d only, due to logistical constraints, in all 6 replicates of each treatment. pCO_2 was measured during the low plateau by equilibration every minute for 2 h, 1 day per week for 6 wk

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

conditions. Cycling pH did not affect infection prevalence or intensity significantly. After a 9-month field deployment and respite from severe cycling conditions, the prevalence of infection in oysters previously exposed to severe cycling hypoxia was still elevated over the infection prevalence in oysters exposed to normoxia during the laboratory portion of the experiment.

Water quality

Experimental conditions were within the environmental ranges for *Perkinsus* transmission and proliferation (salinities above 8 and maximal summer temperatures) (McCollough et al. 2007) as well as the native range of *C. virginica* (Hargis & Haven 1999); see Table 1 for DO and pH and Figs. 2 & 3 for salinity, temperature, and alkalinity measurements in experimental aquaria. Over the course of the experiment, salinity averaged 11.3 ± 0.0 PSU, with a range from 9.3–12.6 PSU; salinity did not differ among treatments (df = 4, F = 0.004, p = 1.0). Temperature averaged 27.1 ± 0.1°C, ranging from 21.0°C to 30.5°C over the course of the experiment, and did not differ among treatments (df = 4, F = 0.038, p = 0.997). Alkalinity averaged 1622 ± 15 µmol kg^{-1} sw, and, following the trend of increasing salinity over the course of the experiment, ranged from 1449 µmol kg^{-1} sw on June 29, at the start of the experiment, to 1744 µmol kg^{-1} sw on 24 September at the experiment's conclusion.

DO did not differ among treatments during the normoxic period prior to the ramp-down phase (df = 4, F = 0.31, p = 0.874) (Table 1). pH varied among treatments during that experiment phase (df = 4, F = 4.98, p < 0.001), but the variation was only a 0.02 unit range among treatments (Table 1), which is within the error range of the sensor. The statistical significance of the difference in pH values reflected the very large sample size (6 replicates per treatment measured daily for 51 d), and is very small when compared to the 0.80 pH unit cycle of the applied treatment.

DO varied significantly among treatments at the end of the low-plateau phase (Table 1) (df = 4, F = 48708.5, p < 0.001). Severe DO cycles averaged within 0.07 mg l^{-1} of target values, and moderate DO cycles averaged within 0.03 mg l^{-1} of target values during the low plateau period. Treatments also differed with regards to pH (df = 4, F = 12855.2, p <

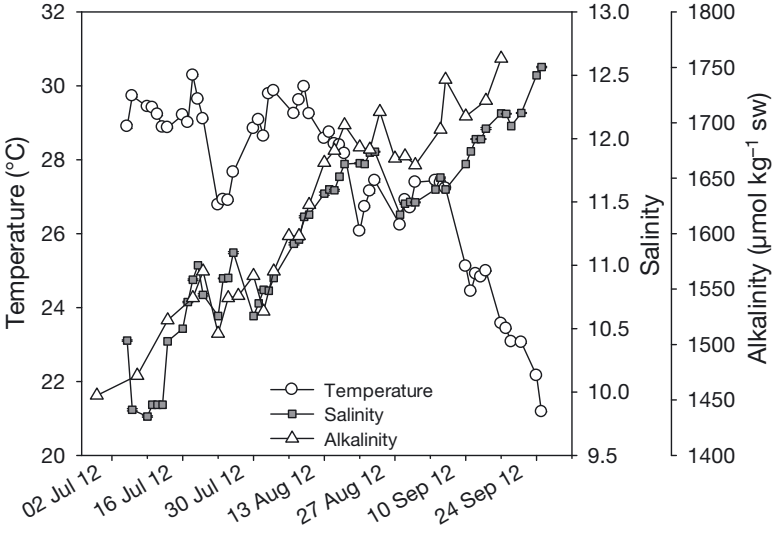

Fig. 3. Mean ± SE temperature and salinity (PSU) measured in all aquaria each day during the low-plateau phase (n = 30 aquaria over 51 d), and alkalinity of the SERC river-water system as measured approx. 3 times per week (n = 25 measurements). Non-visible error bars are obscured by symbols. sw: seawater

0.001), with cycling pH treatment values within 0.02 of the low target value on average, and normocapnic treatments within 0.03 of target values on average.

During the low-plateau phase, there was a significant difference in measured pCO_2 between the 5 treatments (df = 4, F = 128.6, p < 0.001), with a Tukey HSD test indicating differences between all cycling pH treatments and all normocapnic treatments, and no differences within these treatments.

Perkinsus infection patterns

At the start of the experiment, 1 year-old oysters had no detectable *Perkinsus* infections, and 4 and 5 year-olds had a prevalence of 0.72 with an infection intensity averaging 1.35 ± 1.00. Infection parameters at the other time points of the experiment are summarized in Table 2.

Prevalence

Severe diel-cycling hypoxia increased overall prevalence of *Perkinsus* infections compared to normoxia at both the midpoint (df = 20, F = 3.41, p = 0.003) and endpoint (df = 20, F = 6.99, p < 0.001) of the laboratory portion of the experiment. After 12 wk of exposure to cycling treatments, prevalence of *Perkinsus* infections in oyster populations exposed to periods of severe hypoxia (0.5 mg l^{-1}) over 4–5 days per week was nearly twice that of controls (0.51 vs. 0.26). The main-effect of cycling pH was not significant at either the midpoint (df = 20, F = 0.45, p = 0.660) or endpoint (df = 20, F = 0.62, p = 0.539), nor was the interaction of DO and pH at the midpoint (df = 20, F = 0.58, p = 0.572) and endpoint (df = 20, F = 0.77, p = 0.449). Moderate cycling DO did not increase *Perkinsus* prevalence over that of the control at either the midpoint (df = 20, F = 0.004, p = 0.967) or endpoint (df = 20, F = 0.53, p = 0.602).

When prevalence of just those infections scoring 2 or higher was examined, no difference was observed among treatments at the midpoint (very few oysters were this heavily infected) (df = 4, F = 0.65, p = 0.633). By the end of the laboratory exposure, however, nearly 20% of oysters that had been exposed to severe hypoxia had a score of 2 or higher, significantly more than the 5% of oysters held at constant normoxia (df = 20, F = 7.55, p < 0.001). There was also a trend towards higher prevalence of severe infections under moderate cycling hypoxia when compared to normoxic conditions (df = 20, F = 0.84, p = 0.081).

Table 2. Mean ± SE of (A) infection prevalence (proportion of total population assayed), (B) prevalence of infections scoring 2 or higher on the Mackin scale (proportion of population assayed), and (C) Mackin scale intensity of all individuals with detectable infections, after 6 wk (Midpoint), 12 wk (Endpoint), and after 9 mo field deployment (Recovery). Prevalence and mean intensity of disease were calculated from the 30 individuals assayed for disease from each replicate aquarium at each time-point. Measures reported here are means and SE of the prevalence or mean intensity in the 6 replicates of each treatment

Treatment	Midpoint	Endpoint	Recovery
(A) Prevalence			
Control (Normoxia - Hypocapnia)	0.089 ± 0.025	0.262 ± 0.017	0.210 ± 0.036
Normoxia - Cycling pH	0.078 ± 0.022	0.228 ± 0.020	0.279 ± 0.062
Moderate cycling hypoxia - Cycling pH	0.100 ± 0.028	0.264 ± 0.055	0.169 ± 0.051
Severe cycling hypoxia - Cycling pH	0.194 ± 0.043	0.507 ± 0.049	0.041 ± 0.080
Severe cycling hypoxia - Normocapnia	0.235 ± 0.023	0.567 ± 0.023	0.277 ± 0.058
(B) Prevalence of 2+			
Control (Normoxia - Hypocapnia)	0.033 ± 0.000	0.065 ± 0.015	0.043 ± 0.031
Normoxia-Cycling pH	0.033 ± 0.000	0.047 ± 0.013	0.083 ± 0.041
Moderate cycling hypoxia - Cycling pH	0	0.103 ± 0.004	0.027 ± 0.016
Severe cycling hypoxia - Cycling pH	0.033 ± 0.000	0.209 ± 0.027	0.067 ± 0.035
Severe cycling hypoxia - Normocapnia	0.034 ± 0.001	0.185 ± 0.022	0.066 ± 0.030
(C) Infection intensity			
Control (Normoxia - Hypocapnia)	0.567 ± 0.049	1.265 ± 0.135	1.118 ± 0.283
Normoxia - Cycling pH	0.604 ± 0.166	1.009 ± 0.130	1.158 ± 0.258
Moderate cycling hypoxia - Cycling pH	0.554 ± 0.041	1.182 ± 0.097	0.979 ± 0.086
Severe cycling hypoxia - Cycling pH	0.727 ± 0.070	1.514 ± 0.084	1.185 ± 0.167
Severe cycling hypoxia - Normocapnia	0.688 ± 0.076	1.482 ± 0.095	1.217 ± 0.260

After a period of field deployment during which all treatments experienced similar conditions, and which would not have included repeated exposure to hypoxia as severe as that seen in the lab (Hondorp unpubl. data), *Perkinsus* prevalence in oysters exposed to severe cycling hypoxia the previous summer was nearly double the prevalence found in oysters exposed to continuous normoxia in the lab (df = 19, F = 2.5, p = 0.022). The prevalence of infections scoring 2 or higher on the modified Mackin scale, however, did not differ among treatments (df = 4, F = 0.54, p = 0.708). There was no difference in prevalence between oysters exposed previously to moderate cycling DO and oysters exposed to normoxia at the end of the field deployment (df = 19, F = 0.26, p = 0.794).

Infection intensity

Neither cycling DO, nor cycling pH, nor the interaction between DO and pH affected infection intensity after 6 wk of exposure to laboratory conditions. However, after 12 wk of exposure to cycling conditions, severe-cycling DO significantly increased infection intensity as compared with normoxia (df = 20, F = 3.28, p = 0.004) with modified Mackin score infection intensities of 1.50 ± 0.06 and 1.14 ± 0.10 (n = 12), respectively. As with prevalence, there was no significant effect of cycling pH on infection intensity (df = 20, F = 1.02, p = 0.322), no interaction between cycling DO and pH (df = 20, F = 1.65, p = 0.116), and no difference between constant normoxia and oysters exposed to 1.5 mg l^{-1} DO daily (df = 20, F = 1.11, p = 0.281).

There was no effect of laboratory treatments on infection intensity at the end of the 9-mo field deployment (df = 4, F = 0.17, p = 0.708). Intensity among infected oysters was lower at the time of field collection than at the end of the experiment.

Hemocytes

Hematology and immune function variables for oysters were all within ranges that can be considered 'normal', as these variables tend to have wide ranges related to seasonal cycles and environmental conditions (Table 3) (Duchemin et al. 2007, Lambert et al. 2007). Severe cycling hypoxia significantly increased the percentage of phagocytic hemocytes by nearly 100%, from 7.08% in the controls to 13.37% in the severe cycling hypoxia-normocapnia treatment (df = 54, F = 4.81, p < 0.001) and significantly decreased apoptosis by >50% compared to normoxic controls (live apoptotic cells: df = 54, F = 3.11, p = 0.001; dead apoptotic cells: df = 54, F = 5.05, p < 0.001). Severe cycling hypoxia with cycling pH increased unstimulated ROS (df = 54, F = 3.01, p = 0.004). Cycling pH increased hemocyte phagocytosis (df = 54, F = 4.73, p < 0.001) comparably to the increase under severe diel-cycling hypoxia (100% compared to normocapnic controls). Cycling pH also increased levels of ROS (df = 54, F = 2.31, p = 0.025) and decreased the rate of apoptosis (live: df = 54, F = 2.01, p = 0.050; dead: df = 54, F = 2.37, p = 0.022) and the percentage of dead agranular hemocytes by 40% (df = 54, F = 2.39, p = 0.021). Significant interactive effects of DO and pH treatment were found for percent dead hemocytes (df = 54, F = 2.33, p = 0.023), phagocytosis (df = 54, F =

Table 3. Mean ± SE immune response parameters as measured by flow-cytometry (n = 12 samples per treatment). ROS = reactive oxygen species

	Control (Normoxia - Normocapnia)	Normoxia - Cycling pH	Moderate cycling hypoxia - Cycling pH	Severe cycling hypoxia - Cycling pH	Severe cycling hypoxia - Normocapnia
Shell height (mm)	58.6 ± 1.2	58.8 ± 1.8	57.83 ± 1.3	62.8 ± 2.6	61.9 ± 2.5
Infection intensity	0.96 ± 0.39	0.25 ± 0.17	0.71 ± 0.35	0.64 ± 0.23	0.54 ± 0.11
Granular hemocytes (%)	14.8 ± 3.2	17.3 ± 3.4	15.1 ± 3.6	13.4 ± 2.3	21.7 ± 3.2
Dead granular hemocytes (%)	16.2 ± 3.4	10.12 ± 1.3	11.8 ± 2.3	14.5 ± 1.0	9.6 ± 1.7
Agranular hemocytes (%)	71.7 ± 3.3	70.7 ± 3.4	74.3 ± 4.3	74.8 ± 2.8	69.0 ± 4.0
Dead agranular hemocytes (%)	4.9 ± 0.7	3.0 ± 0.3	3.2 ± 0.5	3.8 ± 0.5	3.0 ± 0.6
Phagocytic granular hemocytes (%)	14.1 ± 1.8	24.4 ± 2.1	17.8 ± 2.3	25.3 ± 1.7	27.5 ± 2.6
Phagocytic hemocytes (%)	7.1 ± 0.7	13.3 ± 1.2	8.8 ± 0.8	11.7 ± 1.0	13.4 ± 1.2
ROS production - granular population 1	7901 ± 489	9357 ± 785	8929 ± 946	8506 ± 708	9269 ± 1315
ROS production - granular population 2	343 ± 21	278 ± 12	319 ± 26	365 ± 21	359 ± 18
Apoptotic dead cells	0.10 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.01
Apoptotic live cells	0.07 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00

4.85, $p < 0.001$), and apoptosis (live: df = 54, $F = 2.71$, $p = 0.009$; dead: df = 54, $F = 2.11$, $p = 0.040$). Infection intensity was not a significant covariate in preliminary ANCOVA analyses of hemocyte measures and was removed from all final models (all $p > 0.05$).

DISCUSSION

Results of this study indicated that exposure to diel-cycling hypoxia, which is common in shallow-water systems globally, and particularly in eutrophic systems, may increase acquisition and progression of parasite infections and affect immune status. These effects were seen at water quality levels that did not increase mortality during the 3 mo duration of our laboratory experiment; overall mortality in our experiments was <3.5% in all treatments. Severe diel-cycling hypoxia increased the acquisition and subsequent progression of *Perkinsus* infections in eastern oysters over the course of just one season, and prevalence remained elevated in the following year. Although diel-cycling pH (which accompanies diel-cycling hypoxia in the field) stimulated several measures of immune function, it did not have significant effects on acquisition or progression of *Perkinsus* infections. These results indicate that, within the range of pH values and infection pressure tested in this experiment, hypoxia is a better predictor of infection in oyster populations than pH. Results of our laboratory manipulations therefore agree with those based on statistical analyses of environmental factors at sites where oysters were deployed in the field in Chesapeake Bay (Breitburg et al. 2015).

Hypoxia increased infection prevalence and intensity when DO cycled to 0.5 mg l^{-1} 4–5 days per week, but not when DO minima averaged 1.7 mg l^{-1}; although there was a trend for moderate cycling hypoxia to slightly increase the number of moderate-to-lethal infections. These findings, in conjunction with those of 2 lab experiments reported in Breitburg et al. (2015), may indicate a hypoxia threshold around 1.5 mg l^{-1} below which susceptibility to infection increases. Between the 2 studies, mean exposure levels of 1.46–1.70 mg l^{-1} 4–5 days per week increased prevalence or intensity of infections significantly in 1 of 3 experiments. In contrast, repeated exposure to 0.5 mg l^{-1} consistently increased infection prevalence or intensity in all 3 experiments. Inter-annual variation in results of the diel-cycling moderate hypoxia treatment may have reflected slight differences in actual oxygen concentrations achieved or factors that were not controlled by our experimental apparatus (e.g. temperature, salinity, calcite saturation, *Perkinsus* dosage, etc.).

Monitoring data suggest that only a few sites in the Chesapeake Bay currently experience daily periods of 0.5 mg DO l^{-1} (Breitburg et al. 2015). However, monitors now in use are deployed 0.3–0.5 m off-bottom and may, therefore, underestimate the severity of bottom water conditions experienced by oysters. Other habitats not typically inhabited by oysters in Maryland waters, such as saltmarsh creeks, may also commonly experience DO concentrations ≤0.5 mg l^{-1} (Burrell et al. unpubl.). Furthermore, if eutrophication-driven phytoplankton blooms are not curbed, severe cycling conditions may become more prevalent in shallow water areas during the summer months.

In this study, all experimental units received similar doses of waterborne *Perkinsus* from a single tank of moderately diseased adult oysters. This design allowed us to clearly relate variation in infection acquisition and progression to experimental treatments. If the experiment had continued for a longer period of time, or disease inoculation had been higher, it is possible that disease prevalence in all treatments would have converged near 100%. However, cycling conditions could have meaningful impacts on acquisition and progression of infections in locations such as restoration sites where bar cleaning has removed older, diseased oysters, locations where oyster populations are low, and also those sites where ambient infection levels are not extreme.

Contrary to expectations based on work showing a decreased immune response in *C. virginica* under both hypoxia and acidification (Boyd & Burnett 1999), and higher rates of disease acquisition and progression in the field than in laboratory studies where only dissolved oxygen cycled (Breitburg et al. 2015), the combination of co-varying diel-cycling DO and pH did not increase infection acquisition or progression beyond that of diel-cycling DO alone. In fact, although differences were not statistically significant, the normoxia-cycling pH treatment tended to have the lowest total prevalence, lowest prevalence of moderate-to-lethal infections, and lowest mean infection intensity of all treatments (Table 2). Thus, cycling pH may have some slight protective effect against infection in oysters by stimulating the immune response.

Diel-cycling hypoxia and diel-cycling pH, as well as the 2 factors combined, stimulated phagocytosis and the production of ROS by unstimulated hemocytes, and reduced apoptosis. Our experiment cannot

distinguish whether effects of diel-cycling conditions on host versus pathogen were responsible for increased prevalence and intensity of infections we found. However, the effects of cycling DO and pH on measures of hemocyte function indicate that the altered infection dynamics are a product of the effects of these cycling conditions on oysters. *Perkinsus* infections can stimulate phagocytic hemocytes (Anderson et al. 1992, Anderson et al. 1995, Samain et al. 2007), potentially confounding effects of treatments on infections and hemocyte function. In our study, however, individual disease scores did not correlate with hemocyte variables suggesting that the effects seen here are a product of exposure to experimental treatments rather than to *Perkinsus* infection.

The rapid changes in conditions associated with the fluctuating nature of cycling conditions may also result in additional stress. The restoration of oxygen after periods of hypoxia/anoxia results in the majority of tissue damage because ROS production spikes and the anti-oxidants necessary to control harmful effects of ROS on the host do not return as quickly (Anderson et al. 1992, Pannunzio & Storey 1998). Under cycling oxygen conditions this may occur daily, as evidenced by the higher innate ROS levels in oysters from severe hypoxic treatments, resulting in more oxidative stress to the individual and possibly also in higher infection (Moss & Allam 2006). These negative effects may overwhelm positive effects of the stimulated immune functions. This mechanism potentially also explains the slight trend toward lower infection levels in oysters in the normoxic/cycling pH treatment, which may benefit from the stimulated immune activity (e.g. increased phagocytosis) caused by exposure to fluctuating environmental conditions without experiencing the harmful effects of sudden oxygen restoration. A caveat is that hemocyte variables were only examined on one day during the 3-mo experiment, and only during the most severe part of the cycle on that day, while infection prevalence and intensity may integrate responses to conditions over the entire experiment.

Cycling hypoxia and/or pH stimulated cellular functions are commonly considered to constitute the oyster immune response, but individuals exposed to cycling hypoxia also had higher infection prevalence and intensity. The up-regulation of immune functions may be an indication that environmental variation and stressful conditions stimulate immune activity, especially in granular hemocytes, as a precaution against opportunistic infection under challenging environmental conditions. The response may not be particularly or consistently effective against *P. marinus* (Chu et al. 1993), and *P. marinus* may instead benefit from this stimulated response by using the increased proportion of phagocytic granulocytes and reduced apoptosis as opportunities for infection (Sunila & LaBanca 2003, Goedken et al. 2005). It is possible that *P. marinus* has been such a successful parasite because of its ability to use the oyster's innate immune response as a means of infection and proliferation (Chu et al. 1993). The stimulated immune response still may be effective against other infectious agents that are not adapted to use the immune cells of the oyster as sites of infection.

Field deployment of oysters that had been exposed previously to cycling conditions for a summer season allowed an estimate of how exposure to cycling conditions might continue to affect oysters after a period of respite from severe cycling conditions, and whether infections might return to these oysters with the same intensity. During the winter months, cycling conditions subside as primary production decreases and water temperatures cool. During this period, *Perkinsus* infections become more difficult to detect, and infections may go into remission (Oliver et al. 1998). Prior exposure to severe cycling hypoxia had a legacy effect with higher prevalence of infection in oysters previously exposed to severe hypoxia. Given the low salinity (~6.5 PSU), the transmission of *Perkinsus* before collection was unlikely. Nevertheless, there was still a difference in infection prevalence between DO treatments. After a complete season of exposure to conditions conducive to *Perkinsus*, the carryover effects of severe hypoxia might be even more serious. In prior laboratory experiments, salinities as low as 3 did not eliminate infections, but did prevent intensification of pre-existing infections and reduced transmission (Chu 1996). Salinities below 10–14 were shown to delay *Perkinsus* development (Chu 1996) such that *Perkinsus* epizootics do not occur below salinities of 10–12. If an oyster were to be exposed to cycling hypoxia during a second year, the infection increases might be cumulative, but this remains to be determined.

Most previous work on hypoxia and disease in oysters has focused on the effects of constant exposure. For example, Anderson et al. (1998) found that previously-diseased oysters continuously exposed to 2.86 mg l^{-1} DO experienced increased Dermo-related mortality. Lack of disease effects in the present study until more severe hypoxic values were reached may be an indication that oysters are more tolerant of hypoxia when it is interspersed with significant periods of normoxia, which may provide periods of recovery from the harmful effects of hypoxia.

Cycling conditions may also modify oyster filtration, potentially altering rates of encounter with infective particles. At low oxygen, oyster filtration is reduced; but this reduction in filtration under hypoxia may be at least partially compensated for by increased filtration at high oxygen (Clark 2014, authors unpubl. data). Low pH on the other hand, may increase filtration (Clark 2014, authors unpubl. data). While reduced filtration may reduce rates of encounter with new infective particles, compensatory feeding at high oxygen may result in similar temporally averaged exposure to infective particles. Slowed filtration might also result in higher residence times in the gut, giving any previously filtered infective particle more chance to establish an infection.

Our pH cycles, although environmentally relevant, may not have increased acquisition and progression of infections in oysters because of the innate self-buffering ability of bivalves (Dwyer & Burnett 1996, Berge et al. 2006, Lannig et al. 2010) as well as the low natural pH of oyster hemolymph (Boyd & Burnett 1999). Periods of hypercapnia/low pH in the environment may require less energy for internal pH regulation because external pH is closer to the internal pH of oysters (Boyd & Burnett 1999). This could allow more energy to be allocated to immune responses resulting in an overall slightly more infection-resistant condition. However, environmental pH lower than normal internal values could require energy for pH regulation and hypercapnic or low pH conditions also negatively affect other aspects of oyster physiology and ecology (Ringwood & Keppler 2002, Miller et al. 2009, Lannig et al. 2010). Conditions more extreme than those tested here, in terms of both instantaneous values and magnitude of cycles, do occur (Boynton et al. 1996, Breitburg 2002, MDNR 2013), and might cause negative effects not observed in this study.

Periodic relief from stressors provided by the high DO/high pH phase of cycles may allow organisms to utilize habitat with relatively brief periods of severe environmental conditions that would negatively affect them if those conditions were continuous. Cycling conditions may also stimulate protective responses; however, our results suggest that these defenses may not always be effective. In spite of increased hemocyte activity, severe cycling hypoxia increased acquisition and progression of *Perkinsus* infections in oysters. Constant mild hypoxia has also been shown to increase mortality from *P. marinus* infections (Anderson et al. 1998). These results both suggest that even small areas of hypoxia may have ramifications on disease dynamics at larger spatial scales; heavily infected oysters in one area may act as a disease source for surrounding areas, potentially contributing to larger scale epizootics. It is important, therefore, to consider both the temporal and spatial scales at which hypoxia occurs when setting water quality standards in order to protect the health of aquatic organisms. Current water quality standards often average conditions over time, or permit a limited proportion of space to violate criteria without assessing the entire water body as being in violation. Requirements based on temporal averages will not protect for the negative effects of severe cycling conditions. Furthermore, standards that permit failure in some areas may result in small pockets of individuals with high disease levels that could increase disease loads for the entire system, including those areas that meet water quality standards. Additionally, restoration siting should consider environmental conditions and their sub-lethal consequences beyond the actual restoration sites as these conditions could indirectly influence disease dynamics and restoration success.

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