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Cellular biomarker responses to hypoxia in eastern oysters and Atlantic ribbed marsh mussels

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ABSTRACT: Hypoxic zones in coastal ecosystems are rapidly expanding as global temperatures and anthropogenic inputs of nutrients continue to increase. These conditions can affect cellular homeostasis and pose serious threats to the fitness of a wide variety of organisms, including ecologically important bivalve species. The overall purpose of this study was to evaluate the effects of hypoxia on antioxidant status and tissue damage in two co-occurring bivalve species, eastern oysters *Crassostrea virginica* and Atlantic ribbed marsh mussels *Geukensia demissa*. We examined oysters and mussels collected from a reference site and exposed to different hypoxia regimes in the laboratory as well as bivalves collected from multiple field sites with varying hypoxia regimes. Glutathione (GSH) and lipid peroxidation levels were measured in hepatopancreas (HP) tissues as markers of overall antioxidant status and tissue damage, respectively. Increases in the concentrations of GSH, which is the most abundant cellular antioxidant and a reactive oxygen species scavenger, as well as elevated lipid peroxidation were observed in HP tissues of hypoxia-exposed bivalves. Species-specific differences in responses to hypoxia were also observed, and marsh mussels were found to be much more sensitive to hypoxia than oysters, as there were significant mortalities and higher lipid peroxidation levels in hypoxia-exposed mussels. The results and patterns from the field sites were found to be similar to those obtained from the laboratory studies. These studies suggest that hypoxia can cause increases in cellular damage, alter antioxidant status, and pose significant risks to estuarine bivalves. Assessments of species-specific differences using sublethal biomarkers are essential for developing habitat health criteria that are sufficiently protective for bivalves and other species.

KEY WORDS: Hypoxia · Biomarker · Bivalve mollusk · Oyster · Mussel · Estuary

INTRODUCTION

Dissolved oxygen (DO) has been regarded as one of the most drastically changing ecological variables in marine ecosystems over the past few decades (Diaz & Rosenberg 1995, Diaz 2001, Vaquer-Sunyer & Duarte 2008). Hypoxia is often defined as DO ≤ 2 mg l^{-1}, which is approximately 20% oxygen saturation at 25°C for 35 ppt salinity water (Rabalais et al. 2002). Hypoxia is a global environmental stressor in aquatic ecosystems and continues to be a threat to wildlife. In 2008, the number of hypoxic dead zones in the world was reported to be over 400, covering an area of 245 000 km^2 (Diaz & Rosenberg 2008), and by 2011, the number of hypoxic sites had increased to 500 worldwide (Conley et al. 2011). Increasing temperatures in coastal ecosystems and eutrophication due to anthropogenic inputs contribute to decreasing oxygen saturation potential (Rabalais et al. 2009, 2014). More recent reports on dead zone expansions suggest that climate change is going to further exacerbate hypoxic conditions worldwide (Altieri & Gedan 2015).

Coastal ecosystems support a wide variety of organisms and also serve as nurseries to larval stages of numerous species. They are exposed to diel and seasonal hypoxia (Brown-Peterson et al. 2008, Tyler et al. 2009), which is almost always accompanied by

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hypercapnia or high partial pressure of carbon dioxide, causing a decrease in pH (Burnett 1997, Ringwood & Keppler 2002). Therefore, hypercapnic hypoxia typically reflects an imbalance between photosynthetic and respiratory processes in nutrient-rich estuaries. One of the ways organisms can respond to hypoxia is to migrate away from the area, but even mobile animals like fish may face massive die-offs if the expanse and severity of the hypoxic area are large (Paerl et al. 1999, Breitburg 2002). Animals with carbon skeletons and shells are particularly susceptible to the effects of hypoxia and acidification (Middelburg & Levin 2009). Sessile organisms with limited or no movement, such as bivalves, cannot escape such environmental changes, so they reflect localized conditions and long-term effects of water quality degradation. Various mechanisms of coping with periodic hypoxia including metabolic depression and energy production via anaerobic pathways have been studied in vertebrates as well as invertebrates (Wang & Widdows 1993, Burnett 1997, Le Moullac et al. 2007, Ramirez et al. 2007, Aragonés et al. 2009) and contribute to some level of hypoxia tolerance. Even when conditions do not cause dramatic die-offs, periodic or chronic hypoxia can contribute to sublethal stress and reduced fitness. Ultimately, hypoxia can change the structure of benthos by affecting species and functional diversity as well as the behavior and ecology of invertebrate communities (Middelburg & Levin 2009).

There is evidence for changes in gene and protein expression, developmental abnormalities, and reduced fitness in estuarine animals exposed to hypoxia (Baker & Mann 1994, Ross et al. 2001, David et al. 2005, Brown-Peterson et al. 2008, Cheek 2011, Patterson et al. 2014). Induction of oxidative stress as a response to limited oxygen availability has been reported in many organisms (Hermes-Lima & Zenteno-Savín 2002, Zenteno-Savín et al. 2006, Lushchak 2011) and may be a major underlying mechanism that contributes to cellular damage and malfunction. Oxidative stress occurs when there is an imbalance in the generation and removal of reactive oxygen species (ROS) in cells, as excess ROS can damage biological macromolecules and disrupt cellular homeostasis (Kelly et al. 1998, Halliwell & Gutteridge 2007, Auten & Davis 2009). This balance can be perturbed under hyperoxic conditions, inflammation, or ischemia and reperfusion (damage related to reoxygenation after hypoxia) or when antioxidant defenses are limited or impaired. The mitochondrial electron transport chain (ETC) is typically one of the biggest sources of ROS production, but other sites such as endoplasmic reticulum-bound enzymes, cytoplasmic enzyme systems, NADPH oxidase of phagocytes, P450 systems, soluble oxidases, and autoxidation also contribute to the generation of ROS (Halliwell & Gutteridge 2007, Auten & Davis 2009, Tahara et al. 2009). It has been suggested that under hypoxic conditions, ETC carriers are more reduced, which facilitates the escape of electrons from the ETC that react with oxygen to produce excess ROS (Clanton 2005, Lushchak 2011). Additionally, increased ROS production has been observed during hypoxia possibly due to the effect on cytochrome oxidase (Chandel et al. 1998). Another process contributing to oxidative stress under hypoxia can be due to the xanthine reductase/xanthine oxidase system, which can be an effective ROS producer via limited oxidation steps (Lushchak 2011). Although the mechanisms are not fully understood in marine invertebrates, studies suggest that lack of oxygen can induce antioxidant defense responses, perhaps via regulation of gene expression by the hypoxia-inducible factor (a gene transcription regulator) and associated pathways (Haddad & Land 2000, Michiels et al. 2002). Identification of sublethal biomarkers of hypoxia in sentinel bivalve species is essential for predicting the effects on coastal wildlife (Patterson et al. 2014).

Here, we present the results of field and laboratory studies on the effects of hypoxia on two ecological engineer and bioindicator species, eastern oysters *Crassostrea virginica* and Atlantic ribbed marsh mussels *Geukensia demissa*. We hypothesize that exposure to hypoxia will induce oxidative stress and tissue damage in bivalves and that hypercapnic hypoxia will be more damaging than nitrogen (N_2)-induced hypoxia. Oxidative stress biomarkers were measured in hepatopancreas (HP) tissues, as they are composed of a high percentage of polyunsaturated fatty acids (PUFAs) that are a primary target of ROS during lipid peroxidation. Malondialdehyde (MDA) is one of the products of the oxidation of PUFAs found in membranes and other cellular lipids, and an increase in total cellular MDA is a valuable biomarker of oxidative stress (Kelly et al. 1998, Halliwell & Gutteridge 2007). To evaluate antioxidant capacity, glutathione (GSH) levels were measured. Glutathione is the most abundant antioxidant in living cells, and the role of GSH as an antioxidant in the regulation of oxidative stress has been widely studied (Meister & Anderson 1983, Kelly et al. 1998). Therefore, changes in GSH and MDA concentrations are valuable indicators of antioxidant status.

MATERIALS AND METHODS

Laboratory exposures

Eastern oysters *Crassostrea virginica* and Atlantic ribbed marsh mussels *Geukensia demissa* were collected from a clean reference site in Bogue Sound (34° 41′ 48.1″ N, 76° 56′ 08.9″ W) in Carteret County, NC, USA, which forms a portion of the Atlantic Intracoastal Waterway, and were acclimated in the lab for 7 to 10 d. After the acclimation period, 10 to 12 bivalves were transferred to pre-conditioned polypropylene exposure buckets, which contained 15 l of 25 psu water. A mixture of natural, low-organic oceanic seawater and artificial seawater prepared using the Marine Biological Laboratory formula (Cavanaugh 1975), filtered through a 0.45 µm filter, was used for all exposures. The bivalves were fed 2 phytoplankton species, *Isochrysis galbana* (dinoflagellate) and *Skeletonema costatum* (diatom), which are routinely used for the culture and feeding of bivalves. The original algal stocks were purchased from Bigelow Laboratory for Ocean Sciences at the National Center for Marine Algae and Microbiota, ME, USA, and laboratory cultures were maintained at 10^5 to 10^6 cells ml^{-1}. Live phytoplankton mixtures (50 ml of each species per 12 bivalves) were fed to the bivalves every day. Partial water changes were done on Day 4 for the 8 d exposures. Continuous N_2 and cyclical N_2 hypoxia treatments were maintained using ultrahigh-purity (UHP) N_2. Carbon dioxide (CO_2; 5% CO_2-air mix) was used to maintain continuous hypercapnic and cyclical hypercapnic hypoxia. All gas tanks were purchased from Roberts Oxygen. CO_2-induced hypoxia led to a concomitant decrease in pH, but N_2-induced hypoxia did not cause a decrease in pH. For both types of cyclical hypoxic exposures, treatment buckets were aerated with filtered house air for 8 h during the daytime, and then UHP N_2 or CO_2 gases were used to aerate during the night. Control normoxic exposures were conducted along with every experiment, and filtered house air was used to keep the DO levels above 60 to 70% and pH above 7.5 throughout the exposures. Hach® Hydrolab dataloggers (Minisonde 4a) were used to record water quality parameters (DO, pH, salinity, and temperature) every 30 min throughout the duration of exposures. Dataloggers were serviced and calibrated using certified thermometers, pH and salinity standards, and saturated air for DO per manufacturer's instructions. All calibrations were within 1% of standards. Each datalogger was also verified using standards at the end of the experiments. Representative datalogger profiles and averaged water quality parameters as described in 'Results: Field and laboratory water quality' are shown in Fig. 1 and Table 1, respectively. At the end of 4 and 8 d, 6 bivalves were shucked using a knife, and HP tissues were separated, rinsed with seawater and frozen at –80°C for further analyses. Data were collected from 6 different experiments over a period of 3 yr in the months of June, July, and August in 2010, 2011, and 2013.

Field sample collections

Bivalves were collected from 3 field sites in coastal North Carolina, USA, and representative datalogger

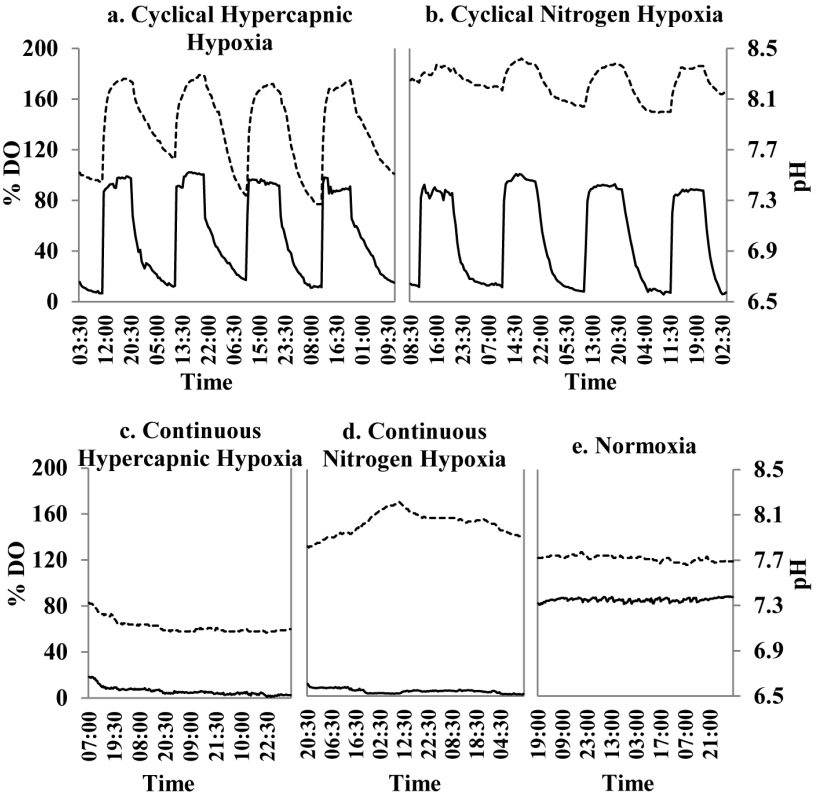

Fig. 1. Representative datalogger profiles measured from laboratory hypoxia exposures. (a) Cyclical hypercapnic hypoxia and (c) continuous hypercapnic hypoxia were induced by 5% carbon dioxide. (b) Cyclical N_2 hypoxia and (d) continuous N_2 hypoxia were induced by ultrahigh-purity nitrogen. (e) Normoxia was induced by air. Solid lines represent percent dissolved oxygen (DO), and dashed lines represent pH

Table 1. Water quality parameters from field sites and laboratory exposures. Overall means and average daily ranges are shown and represent averaged values from samplings and experiments done between 2010 and 2013. DO: dissolved oxygen

	DO		pH		Salinity	Temperature
	Overall mean (%)	Average range (%)	Overall mean	Average range	Overall mean (psu)	Overall mean (°C)
Field site						
Bogue Sound	78.1	60–111	8.0	7.8–8.1	34.8	29.2
Hoop Pole	53.7	24–93	7.8	7.6–8.0	35.5	29.7
Hoop Outfall	64.1	26–132	7.7	7.5–8.1	36.2	29.6
Laboratory exposure: Day 4						
Normoxia	76.9	63–85	7.9	7.7–8.0	26.7	21.3
Hypoxia, cyclical hypercapnic (CO_2)	49.7	6–92	7.4	6.6–8.0	25.9	21.9
Hypoxia, cyclical nitrogen (N_2)	43.4	14–95	7.9	7.8–8.0	28.8	22.2
Hypoxia, continuous hypercapnic (CO_2)	13.7	9–25	7.0	6.9–7.2	26.3	22.8
Hypoxia, continuous nitrogen (N_2)	11.5	5–33	7.6	7.4–7.8	26.8	22.3
Laboratory exposure: Day 8						
Normoxia	71.1	60–81	7.9	7.7–8.0	28.1	20.3
Hypoxia, cyclical hypercapnic (CO_2)	50.1	6–94	7.5	6.7–8.0	27.1	21.1
Hypoxia, cyclical nitrogen (N_2)	44.2	1–101	8.2	8.0–8.4	27.3	20.4
Hypoxia, continuous hypercapnic (CO_2)	4.8	1–18	7.3	7.2–7.5	28.6	21.6
Hypoxia, continuous nitrogen (N_2)	9.0	3–24	7.4	7.3–7.6	26.4	21.5

profiles are presented in Fig. 2. Bogue Sound is a reference normoxic site, where DO stays between approximately 60 and 100% and pH between 7.8 and 8.1. Two sites were sampled in Hoop Pole Creek (34° 42′ 06.3″ N, 76° 45′ 04.8″ W), which is located in the Hoop Pole Reserve. The Hoop Pole site receives limited nutrient inputs and gets moderately hypoxic. The Hoop Outfall site is located in the headwater region of the creek, near a storm drain that serves a small coastal town, and receives periodic elevated nutrient inputs. Hach® Hydrolab dataloggers were deployed at these sites, recording water quality every 30 min for a period of 7 to 10 d, and bivalves were collected at the end of the deployment period. All dataloggers were serviced, calibrated, and verified as described in 'Materials and methods: Laboratory exposures'. Bivalves were brought to the lab in site water and were kept cool and immediately sacrificed upon arrival, and HP tissues were frozen at −80°C for further analyses. The field studies were conducted in the month of August in 2010, 2011, and 2013.

Cellular biomarker analyses

Hepatopancreas tissues were chosen for the study because they serve major roles in digestion and nutrient processing and are a reservoir for toxins, contaminants, and bacteria in bivalves (Kennedy et al. 1996, Adami et al. 2002). As a deeper tissue, HP

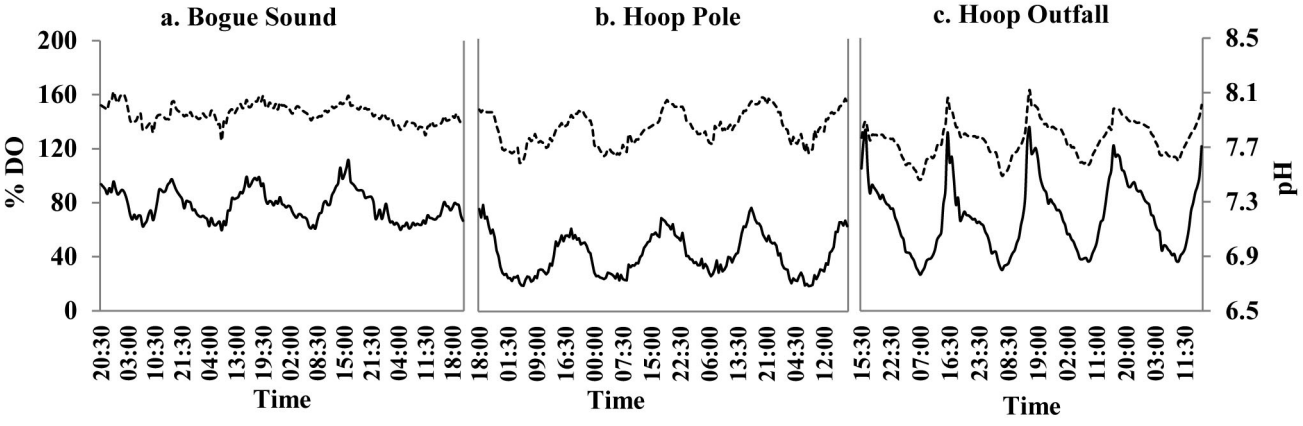

Fig. 2. Representative datalogger profiles for field sites at (a) Bogue Sound, (b) Hoop Pole, and (c) Hoop Outfall. Solid lines represent percent dissolved oxygen (DO), and dashed lines represent pH

could become more hypoxic than more superficial tissues under limited oxygen availability in water.

A lipid peroxidation assay was performed on the HP tissues to quantify total cellular MDA (Ringwood et al. 1999, 2003). This spectrophotometric assay uses the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product. Samples (50 to 100 mg frozen tissue) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 13 000 × *g* for 5 min at 4°C. A subsample of the supernatant was mixed with TBA, trichloroacetic acid, and butylated hydroxytoluene and was heated at 100°C for 15 min. The standards (ranging from 25 to 800 µM), blanks, and sample supernatants were read at 532 nm on a plate reader (µQuant, BioTek® Instruments, KCjunior software). MDA concentrations were estimated using a standard curve and were expressed as nmol g^{-1} tissue wet wt.

The 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-glutathione disulfide reductase recycling assay was used to measure total cellular glutathione levels, i.e. both oxidized and reduced forms (Anderson 1985, Ringwood et al. 1999, 2003). The samples (50 to 100 mg) were acidified in 5% sulfosalicylic acid (SSA) in a ratio of 10:1 (SSA volume to tissue weight), homogenized, and centrifuged at 13 000 × *g* for 5 min at 4°C. The supernatants were then diluted in a 1:2 ratio with 5% SSA and mixed with nicotinamide adenine dinucleotide phosphate buffer and 10 mM DTNB. An enzymatic reaction catalyzed by glutathione reductase (50 units) from baker's yeast was used to determine total glutathione concentrations with this kinetic assay. The conversion of glutathione back to the reduced form by the reductase is coupled with the recycling of DTNB, and the rate of formation of the chromophore 5′-thio-2-nitrobenzoic acid is proportional to the concentration of glutathione in the sample, which is quantified spectrophotometrically at 412 nm on a plate reader (µQuant, BioTek Instruments, KCjunior software). Blanks and standards (ranging from 6.25 to 200 µM) were used to generate a standard curve, and GSH concentrations were estimated as nmol g^{-1} tissue wet wt.

Data processing and statistics

Lipid peroxidation and GSH data were organized and summarized using Microsoft Excel. All statistical analyses were conducted using the SigmaStat® software program. One-way ANOVAs were used to compare different treatments, and the Student-Newman-Keuls method was used to perform pairwise comparisons. Normality and equal variance assumptions are automatically conducted as part of the ANOVA test by SigmaStat® (Kolmogorov-Smirnov test and *F*-test), and these were verified with more than 90% of the data; if these assumptions were violated, a Kruskal-Wallis non-parametric 1-way ANOVA was used (oyster field and mussel lipid peroxidation data were the only sets of data which required a non-parametric test). Multiple comparisons versus control normoxic group analyses were also conducted using the Holm-Sidak method. Bivalve mortality data were averaged over all experiments and are expressed as mean percentage mortality.

The data records from the dataloggers used for field and laboratory studies were downloaded to Microsoft Excel and summarized. Various metrics from the data—overall means and average daily ranges (identified for each 24 h period and averaged)—were calculated as presented in Table 1.

RESULTS

Field and laboratory water quality

The datalogger profiles from field sites show the characteristic coupling of pH and DO cycles (Fig. 2) and were consistent from year to year. Most shallow water estuaries experience moderate hypoxia with diel cycles of low amplitude like Hoop Pole (Fig. 2b), especially during summer months. More impacted sites such as Hoop Outfall (Fig. 2c) have dramatic DO cycles that range from severe hypoxia/anoxia to supersaturation each day.

Our normoxia controls for laboratory exposures had a mean DO of >70% and mean pH of 7.9 (representative example in Fig. 1e). The patterns of cyclical DO and pH regimes found in the field were successfully simulated in the laboratory (Fig. 1), the latter ranging from a low of 1% to a high of 100% DO (Table 1). The cyclical hypoxia N_2 exposure never experienced pHs below 7.8, unlike the cyclical hypercapnic hypoxia exposures, where pH cycles ranged from below 7 to 8. Both continuous hypoxia exposures had low DO (<15%, Table 1) throughout the exposures; however, higher pHs occurred with the N_2 treatments, but the hypercapnia treatments had lower pHs.

Cellular damage and mortality

In oysters, lipid peroxidation levels (based on MDA concentrations) were not significantly elevated for any of the laboratory hypoxia regimes after 4 d, but

lipid peroxidation levels were significantly higher in HP tissues after 8 d for the continuous hypercapnic hypoxia and cyclical N_2 hypoxia treatments (ANOVA $p < 0.01$); there were no significant increases in the continuous N_2 hypoxia treatment or the cyclical hypercapnic hypoxia treatment (Fig. 3a). No oyster mortalities were observed in any experiments for any of the hypoxia or control exposures. Mussels were found to be much more sensitive, as significant increases in oxidative damage were observed in 3 of the 4 hypoxia regimes after only 4 d (Fig. 3b), and while the baseline levels of mussels and oysters were similar, the lipid peroxidation levels in mussels tended to be higher when exposed to hypoxia. Moreover, unlike oysters, the mussels experienced significant mortalities, 100% in some treatments by 8 d, as shown in Table 2. The highest percent mortalities were observed in continuous hypercapnic hypoxia and cyclical N_2 hypoxia exposures.

In the field studies, Bogue Sound oysters and mussels had the lowest lipid peroxidation levels. Significantly higher lipid peroxidation levels were observed in bivalves from the Hoop Outfall site, the site with the most extreme hypoxic cycles (Fig. 4). Based on visual observations, Hoop Outfall had very sparse populations of oysters and mussels compared to any other site included in this study. Additionally, the damage levels of mussels from Hoop Outfall tended to be higher than the levels measured in oysters, and the mussels also had higher variation in these levels at all of the sites.

Table 2. Mussel mortalities after 4 and 8 d of exposure to normoxic and hypoxic regimes. Data represent average mean percentage values from all experiments conducted between 2010 and 2013 (n = 6 to 18)

Laboratory exposure	Cumulative mortality (%) 4 d	8 d
Normoxia	0	0
Hypoxia, cyclical hypercapnic (CO_2)	6	11
Hypoxia, cyclical nitrogen (N_2)	0	83
Hypoxia, continuous hypercapnic (CO_2)	33	100
Hypoxia, continuous nitrogen (N_2)	11	33

Antioxidant status

Laboratory studies indicated that total HP GSH levels were higher in hypoxia-exposed oysters than in control oysters (Fig. 5a, ANOVA $p < 0.01$). GSH levels were elevated in all hypoxia treatments after 4 d, and the levels remained high but did not increase further by 8 d, so no differences in HP GSH were observed between 4 and 8 d for any hypoxic treatment. The control data include GSH levels from bivalves exposed to 4 and 8 d of normoxia, as there were no changes in GSH levels for normoxic treatments. Mussels showed slightly different patterns of changes in GSH levels. No increases were observed by Day 4, but GSH levels for all hypoxia treatments were significantly higher (except for continuous hypoxia hypercapnia, where there was 100% mortality) by Day 8 (Fig. 5b, ANOVA $p < 0.01$).

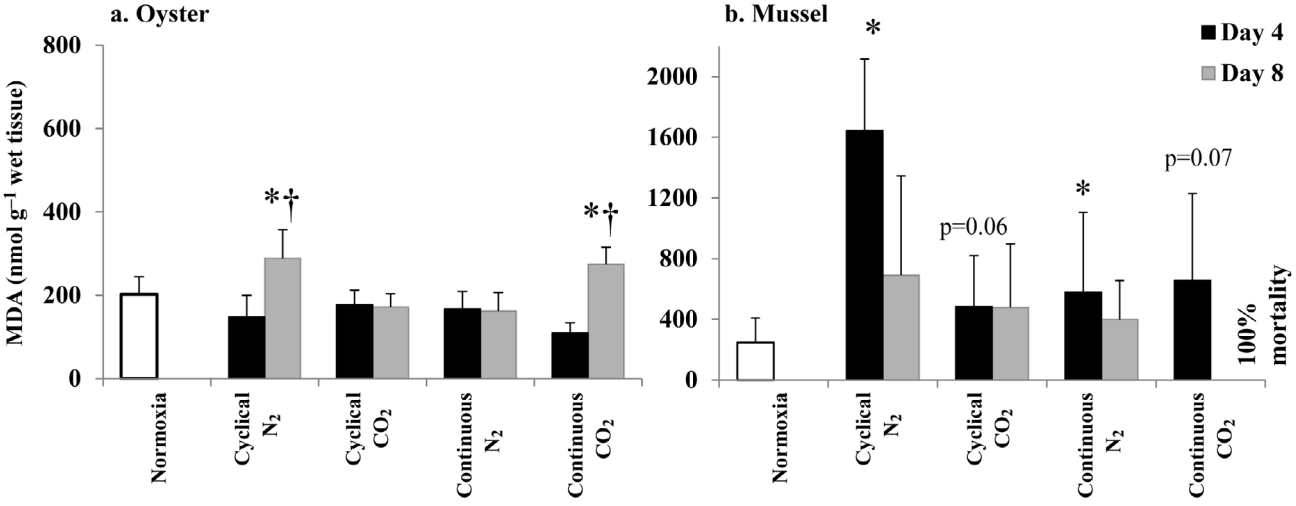

Fig. 3. Malondialdehyde (MDA) concentrations in hepatopancreas tissues of (a) oysters and (b) mussels. Data shown for 4 and 8 d of hypoxic exposure. Normoxic control organisms were exposed to normoxia throughout the exposures and are represented by white bars. Asterisks (*) indicate significant increases from normoxia ($p < 0.05$), and daggers (†) indicate significant differences between 4 and 8 d for a given hypoxic exposure ($p < 0.01$). Values are means +SDs, n = 6 to 28. Data were combined from 6 experiments conducted between 2010 and 2013. Detailed data for oysters and mussels from different years are shown in Figs. S1 & S2, respectively, in the Supplement at www.int-res.com/articles/suppl/m546p123_supp.pdf

In the field studies, site-specific differences in GSH levels in oysters and mussels were found that were consistent with our laboratory studies. Oysters and mussels used for the laboratory experiments were collected from a well-oxygenated field site in the open areas of Bogue Sound, characterized by DO patterns of normoxic conditions with very minimal cycles. The GSH levels of the oysters from this site have consistently been around 1000 to 1200 nmol g^{-1}. The GSH levels at the other 2 field sites targeted for these studies had elevated GSH, probably because of chronic cyclical DO stress (Fig. 6). Oysters from Hoop Outfall, which represents our most DO-stressed site, had the highest levels of GSH among all field studies. Glutathione levels tended to be lower in mussels than in oysters from the Bogue Sound and Hoop Outfall sites. However, the overall pattern of elevated GSH in mussels from hypoxia-stressed sites was similar to oysters. Mussels from Hoop Pole and Hoop Outfall had higher GSH than mussels from our reference normoxic site, Bogue Sound.

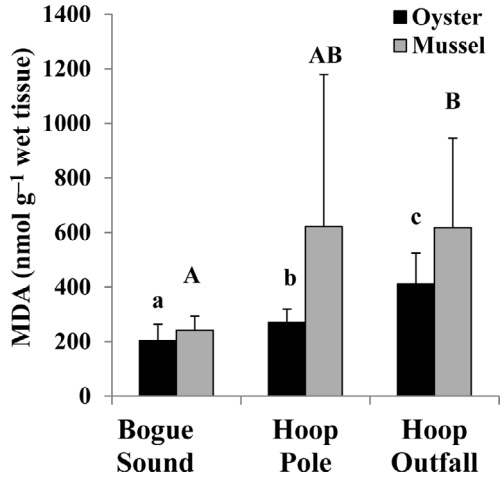

Fig. 4. Malondialdehyde (MDA) concentrations in hepatopancreas tissues of oysters (black) and mussels (gray) collected from 3 field sites. Different letters represent statistical differences between sites ($p < 0.05$); lowercase letters represent statistical differences between oysters, and uppercase letters represent statistical differences between mussels. Data were collected and averaged over multiple years between 2010 and 2013. Values are means +SDs, n = 8 to 24. Detailed data for oysters and mussels from different years are shown in Figs. S3 & S4, respectively, in the Supplement at www.int-res.com/articles/suppl/m546p123_supp.pdf

DISCUSSION

With an increase in the number of dead zones, higher global water temperatures, and nutrient loading in marine ecosystems, hypoxia has become a significant stressor for marine organisms over broad coastal regions (Diaz & Rosenberg 2008, Rabalais et al. 2009, 2014). Environmental hypoxia can induce cellular oxidative stress and alter antioxidant status (Lushchak et al. 2005, Lushchak & Bagnyukova 2007), leading to cellular dysfunction. The concept of elevated cellular ROS production during hyperoxic con-

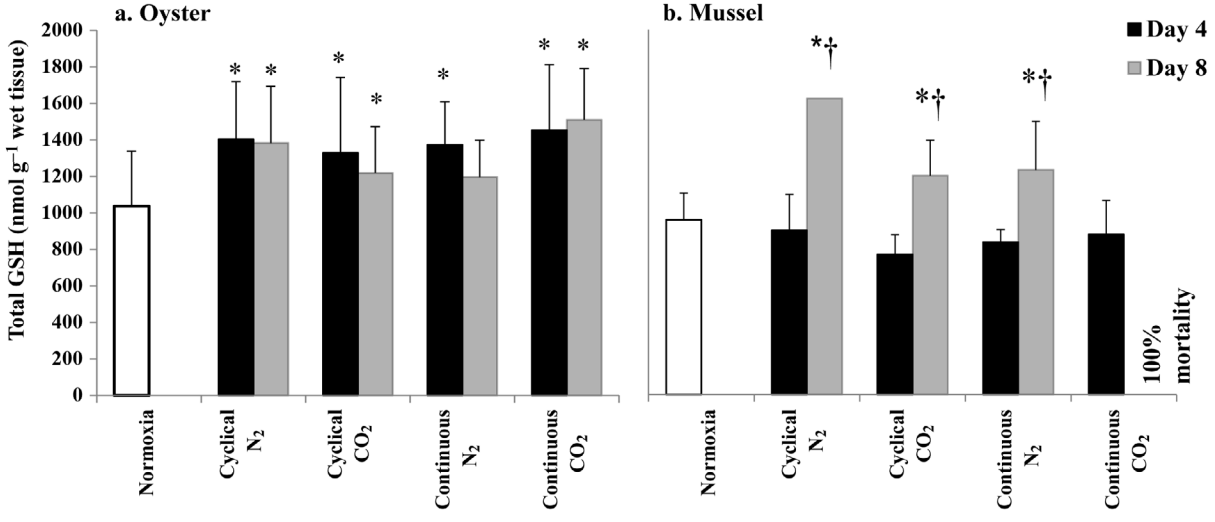

Fig. 5. Total glutathione (GSH) concentrations in hepatopancreas tissues of (a) oysters and (b) mussels. Data shown for 4 and 8 d of hypoxic exposure. Normoxic control organisms were exposed to normoxia throughout the exposures and are represented by white bars. Asterisks (*) indicate significant differences from normoxia ($p < 0.01$), and daggers (†) indicate significant differences between 4 and 8 d for a given hypoxic exposure ($p < 0.01$). Data were combined from 6 experiments conducted from 2010 to 2013. Values are means +SDs, n = 6 to 28. Detailed data for oysters and mussels from different years are shown in Figs. S5 & S6, respectively, in the Supplement at www.int-res.com/articles/suppl/m546p123_supp.pdf

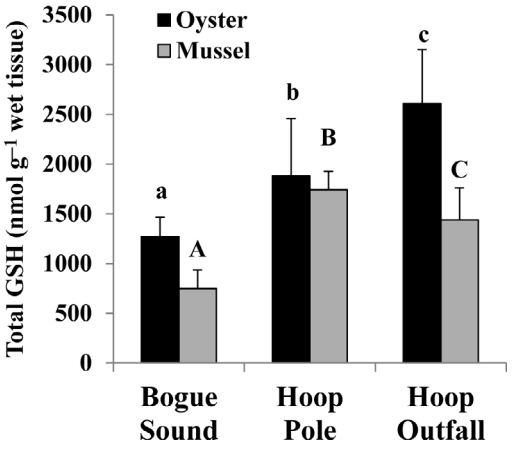

Fig. 6. Total glutathione (GSH) concentrations in hepatopancreas tissues of oysters (black) and mussels (gray) collected from 3 field sites. Different letters represent significant differences between sites (oysters: $p < 0.05$, mussels: $p < 0.01$); lowercase letters represent significant differences between oysters, and uppercase letters represent significant differences between mussels. Data were collected and averaged over multiple years between 2010 and 2013. Values are means +SDs, n = 6 to 26. Detailed data for oysters and mussels from different years are shown in Figs. S7 & S8, respectively, in the Supplement at www.int-res.com/articles/suppl/m546p123_supp.pdf

ditions and hyperoxia related to reperfusion following ischemia is widely accepted (Halliwell & Gutteridge 2007) and supports results from some clinical studies on aging and the role of oxidative stress in cardiovascular diseases (Finkel & Holbrook 2000, Buttemer et al. 2010, Jacob et al. 2013, Hristova & Penev 2014). Additionally, existing literature also supports the damaging effects of environmental cycles of DO, which include reoxygenation following hypoxia (Almeida et al. 2005, de Oliveira et al. 2005, Bickler & Buck 2007). A relatively new emerging complexity in free radical biology is the production of ROS during hypoxia, and several studies provide evidence for hypoxia-induced ROS formation (Chandel et al. 1998, Clanton 2005, 2007, Magalhaes et al. 2005, Lushchak & Bagnyukova 2007, Pialoux et al. 2009). Our studies fit this model and show that continuous hypercapnic hypoxia is the most damaging to bivalves among all other hypoxic regimes tested. Increases in cellular damage markers such as lipid peroxidation in the presence of elevated antioxidant levels such as GSH, as seen in our laboratory studies, can be indicative of the induction of compensatory mechanisms. Glutathione plays a dynamic role in ROS scavenging, metal detoxification mechanisms, drug metabolism, cell signaling pathways, and cellular homeostasis (Meister & Anderson 1983, Halliwell & Gutteridge 2007). Changes in GSH concentrations represent an altered antioxidant status, which can exacerbate oxidative stress and jeopardize normal cellular physiology. Although depletion of GSH has been primarily suggested as a marker of contaminant stress and increased susceptibility to toxicity in aquatic organisms (Ringwood et al. 1999, Conners & Ringwood 2000, Peña-Llopis et al. 2002), our studies presented here show that there is evidence that GSH levels can be affected by hypoxia. Elevated GSH levels have also been reported in fish exposed to cycles of DO (Lushchak et al. 2005, Lushchak & Bagnyukova 2006). Our results suggest that increases in ROS production and lipid peroxidation could stimulate compensatory antioxidant responses. The increased GSH concentrations in oysters and mussels represent short-term effects on the induction of low molecular weight thiols as an antioxidant defense. Glutathione synthesis is catalyzed by cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase, and these enzymes are transcriptionally and post-transcriptionally regulated. ROS and the ratio of reduced to oxidized glutathione can play important roles in the regulation of glutathione synthetases (Meister & Anderson 1983, Rahman & MacNee 2000, Dickinson & Forman 2002). Under longer-term hypoxia, the production of GSH and other antioxidants may be overwhelmed by ROS production or energetically constrained, leading to decreased antioxidant potential and increased cellular damage. Our laboratory studies showed elevated GSH levels in oysters as early as 4 d of exposure, and this elevation prevented increases in lipid peroxidation levels. However, after 8 d of exposure, the damage continued to increase, indicating that the elevated GSH levels were not sufficient to prevent oxidative damage. Field data, which represent more chronic responses, suggested a similar outcome, where we found tissue damage to be highest at Hoop Outfall, the site with the most dramatic and severe hypoxia. Hoop Outfall undergoes dramatic DO cycles diurnally, and although other factors such as nutrient inputs may also contribute to cellular damage and changes in adaptive strategies, the elevated tissue damage levels still reflect some effects of hypercapnic hypoxia.

Interestingly, under laboratory cyclical hypoxia, lipid peroxidation damage was not higher under hypercapnic conditions compared to higher pH conditions. The cyclical hypercapnic hypoxia exposure is an environmentally relevant simulation, and the organisms that live in habitats with moderate DO cycles are probably somewhat adapted to moderate diel shifts in DO and pH. Moderate decreases in pH may favor depression in metabolic rate (Larade & Storey

2002) and limit ROS production. In the absence of the moderate shifts in pH but presence of DO cycles such as our cyclical hypoxia N_2 exposure, there may not be a suppression of metabolic rate and, hence, more ROS-induced damage. Although the reduced production of ROS may minimize oxidative stress potential, hypercapnic hypoxia exposures may negatively affect immune responses of hemocytes in oysters (Boyd & Burnett 1999, Macey et al. 2008). Hemocytes play a critical role in the bivalve immune system, and ROS kill invading pathogens in bivalves. Under compromised immune health conditions, oysters may not be able to clear bacterial and parasitic infections, which may have severe impacts on fitness.

In contrast to the field studies, increased lipid peroxidation levels were not observed in oysters in our short-term cyclical hypercapnic hypoxia laboratory exposures. This may be due to the short-term nature of the laboratory studies compared to the field sites that have persistent hypoxia cycles for weeks to months during the summer. In both field and laboratory studies, GSH levels of oysters from hypoxic conditions were elevated, indicating oxidative perturbation. Our combination of laboratory and field studies provides insights for both short-term and long-term responses. In the short term, increased GSH levels can serve to prevent oxidative damage, but under longer-term and more extreme hypoxia cycles, GSH levels cannot compensate and the damage increases. As noted in 'Results: Cellular damage and mortality', our most hypoxia-stressed site, Hoop Outfall, has very small populations of oysters and mussels, while other regions in Bogue Sound have extensive oyster and mussel populations. While these studies indicated that hypoxic conditions tended to cause an increase in GSH levels and antioxidant capacity, studies with oysters and other bivalves indicate that exposure to contaminants such as metals or PAHs are often associated with reduced GSH levels (Regoli & Principato 1995, Ringwood et al. 1999). Elevated GSH levels in the absence of contaminants could be a valuable biomarker of hypoxia stress.

Another important finding of these studies was the differences in sensitivity between two co-existing bivalve species. Overall, there were some similarities in the responses of oysters and mussels to hypoxia; continuous hypercapnic hypoxia and cyclical N_2 hypoxia were the most damaging exposures to both species. However, we found that mussels were more sensitive to cellular homeostasis disruption by hypoxia. The overall MDA levels were higher in mussels, and mortalities were only reported in mussels after hypoxic exposures. Unlike mussels, oysters exposed to continuous N_2 hypoxia in the laboratory did not have elevated lipid peroxidation levels. It also suggests that pH plays an integral role in hypoxia-induced cellular damage. Current understanding of cellular mechanisms involved in responses to the interactions of simultaneous shifts in pH and DO in bivalves is limited. More studies on the effects of pH on bivalve physiological pathways are needed to completely evaluate the effects on environmentally relevant hypoxia.

Our short-term studies also suggest that oysters demonstrated a more rapid antioxidant response than mussels, which is represented by significant differences in GSH levels after only 4 d in oysters but not in mussels. The reduced ability to compensate for the oxidative damage hence makes mussels more vulnerable to hypoxia. Mussels also have a higher background variation between individuals exposed to similar water quality conditions, indicating greater differences in individual sensitivity. We conclude that although under well-regulated optimal laboratory conditions the baseline levels of GSH and MDA in both these estuarine bivalves are comparable, the responses to hypoxia are different. It must be noted that the interactions between co-varying environmental variables in the field such as pH, salinity, temperature, and hypoxia present complex environmental challenges. These parameters influence each other and can exacerbate overall effects on the organisms. Parameters such as temperature and salinity affect DO saturation in coastal ecosystems, and based on our field studies, mussels seem to be more sensitive to such interactive effects of environmental variables. Understanding the differences in sensitivity of two ecologically and evolutionarily related species is of critical importance for estimating the risks associated with an emerging and worsening environmental stressor like hypoxia.

In conclusion, we show that hypoxia stress, based on the concordance between field and laboratory studies, can affect antioxidant status and increase cellular oxidative damage. Our studies also show that even short-term exposure to hypoxia can affect GSH levels and induce cellular damage. The differences in sensitivity of different species suggest that a suite of biomarkers in multiple indicator species should be assessed to evaluate the effects of environmental stressors on coastal and marine ecosystems. Biomarkers such as GSH serve as valuable tools for evaluating the effects of abiotic stressors, as suggested by our current study. It is further suggested that biomarkers or short-term bioassays may be used in a diagnostic way to distinguish between hypoxia and

contaminant stress. These studies provide valuable new insights regarding the cellular responses of two ecological engineers to the growing threat of hypoxia worldwide.

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LITERATURE CITED

Adami G, Barbieri P, Fabiani M, Piselli S, Predonzani S, Reisenhofer E (2002) Levels of cadmium and zinc in hepatopancreas of reared *Mytilus galloprovincialis* from the Gulf of Trieste (Italy). Chemosphere 48:671–677

Almeida EA, Bainy ACD, Dafre AL, Gomes OF, Medeiros MHG, Di Mascio P (2005) Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. J Exp Mar Biol Ecol 318:21–30

Altieri AH, Gedan KB (2015) Climate change and dead zones. Glob Chang Biol 21:1395–1406

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548–555

Aragonés J, Fraisl P, Baes M, Carmeliet P (2009) Oxygen sensors at the crossroad of metabolism. Cell Metab 9: 11–22

Auten RL, Davis JM (2009) Oxygen toxicity and reactive oxygen species: the devil is in the details. Pediatr Res 66: 121–127

Baker SM, Mann R (1994) Description of metamorphic phases in the oyster *Crassostrea virginica* and effects of hypoxia on metamorphosis. Mar Ecol Prog Ser 104:91–99

Bickler PE, Buck LT (2007) Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu Rev Physiol 69:145–170

Boyd JN, Burnett LE (1999) Reactive oxygen intermediate production by oyster hemocytes exposed to hypoxia. J Exp Biol 202:3135–3143

Breitburg D (2002) Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25:767–781

Brown-Peterson NJ, Manning CS, Patel V, Denslow ND, Brouwer M (2008) Effects of cyclic hypoxia on gene expression and reproduction in a grass shrimp, *Palaemonetes pugio*. Biol Bull 214:6–16

Burnett LE (1997) The challenges of living in hypoxic and hypercapnic aquatic environments. Am Zool 37:633–640

Buttemer WA, Abele D, Costantini D (2010) From bivalves to birds: oxidative stress and longevity. Funct Ecol 24: 971–983

Cavanaugh GM (ed) (1975) Formulae and methods VI of the Marine Biological Laboratory chemical room. Marine Biological Laboratory, Woods Hole, MA

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 95:11715–11720

Cheek AO (2011) Diel hypoxia alters fitness in growth-limited estuarine fish (*Fundulus grandis*). J Exp Mar Biol Ecol 409:13–20

Clanton T (2005) Yet another oxygen paradox. J Appl Physiol 99:1245–1246

Clanton TL (2007) Hypoxia-induced reactive oxygen species formation in skeletal muscle. J Appl Physiol 102:2379–2388

Conley DJ, Carstensen J, Aigars J, Axe P and others (2011) Hypoxia is increasing in the coastal zone of the Baltic Sea. Environ Sci Technol 45:6777–6783

Conners DE, Ringwood AH (2000) Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassostrea virginica*). Aquat Toxicol 50:341–349

David E, Tanguy A, Pichavant K, Moraga D (2005) Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. FEBS J 272:5635–5652

de Oliveira UO, da Rosa Araújo AS, Belló-Klein A, da Silva RS, Kucharski LC (2005) Effects of environmental anoxia and different periods of reoxygenation on oxidative balance in gills of the estuarine crab *Chasmagnathus granulata*. Comp Biochem Physiol B 140:51–57

Diaz RJ (2001) Overview of hypoxia around the world. J Environ Qual 30:275–281

Diaz RJ, Rosenberg R (1995) Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanogr Mar Biol Annu Rev 33:245–303

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321:926–929

Dickinson DA, Forman HJ (2002) Cellular glutathione and thiols metabolism. Biochem Pharmacol 64:1019–1026

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

Haddad JJ, Land SC (2000) O_2-evoked regulation of HIF-1α and NF-κB in perinatal lung epithelium requires glutathione biosynthesis. Am J Physiol Lung Cell Mol Physiol 278:L492–L503

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine. Oxford University Press, New York, NY

Hermes-Lima M, Zenteno-Savín T (2002) Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp Biochem Physiol C 133:537–556

Hristova M, Penev M (2014) Oxidative stress and cardiovascular diseases. Trakia J Sci 12:300–307

Jacob KD, Hooten NN, Trzeciak AR, Evans MK (2013) Markers of oxidant stress that are clinically relevant in aging and age-related disease. Mech Ageing Dev 134: 139–157

Kelly KA, Havrilla CM, Brady TC, Abramo KH, Levin ED (1998) Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Perspect 106:375–384

Kennedy VS, Newell RIE, Eble AF (eds) (1996) The eastern oyster: *Crassostrea virginica*. University of Maryland Sea Grant College, College Park, MD

Larade K, Storey KB (2002) A profile of the metabolic responses to anoxia in marine invertebrates. In: Storey KB, Storey JM (eds) Sensing, signaling and cell adaptation. Elsevier Press, Amsterdam, p 27–46

Le Moullac G, Quéau I, Le Souchu P, Pouvreau S, Moal J, Le Coz JR, Samain JF (2007) Metabolic adjustments in the oyster *Crassostrea gigas* according to oxygen level and temperature. Mar Biol Res 3:357–366

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30

Lushchak VI, Bagnyukova TV (2006) Effects of different

environmental oxygen levels on free radical processes in fish. Comp Biochem Physiol B 144:283–289

Lushchak VI, Bagnyukova TV (2007) Hypoxia induces oxidative stress in tissues of a goby, the rotan *Perccottus glenii*. Comp Biochem Physiol B 148:390–397

Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB (2005) Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. Int J Biochem Cell Biol 37: 1319–1330

Macey BM, Achilihu IO, Burnett KG, Burnett LE (2008) Effects of hypercapnic hypoxia on inactivation and elimination of *Vibrio campbellii* in the eastern oyster, *Crassostrea virginica*. Appl Environ Microbiol 74:6077–6084

Magalhaes J, Ascensao A, Soares JM, Ferreira R, Neuparth MJ, Marques F, Duarte JA (2005) Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. J Appl Physiol 99:1247–1253

Meister A, Anderson ME (1983) Glutathione. Annu Rev Biochem 52:711–760

Michiels C, Minet E, Mottet D, Raes M (2002) Regulation of gene expression by oxygen: NF-κB and HIF-1, two extremes. Free Radic Biol Med 33:1231–1242

Middelburg JJ, Levin LA (2009) Coastal hypoxia and sediment biogeochemistry. Biogeosciences Discuss 6:3655–3706

Paerl HW, Pinckney JL, Fear JM, Peierls BL (1999) Fish kills and bottom-water hypoxia in the Neuse River and Estuary: reply to Burkholder et al. Mar Ecol Prog Ser 186: 307–309

Patterson HK, Boettcher A, Carmichael RH (2014) Biomarkers of dissolved oxygen stress in oysters: a tool for restoration and management efforts. PLoS ONE 9:e104440

Peña-Llopis S, Ferrando MD, Peña JB (2002) Impaired glutathione redox status is associated with decreased survival in two organophosphate-poisoned marine bivalves. Chemosphere 47:485–497

Pialoux V, Mounier R, Brown AD, Steinback CD, Rawling JM, Poulin MJ (2009) Relationship between oxidative stress and HIF-1α mRNA during sustained hypoxia in humans. Free Radic Biol Med 46:321–326

Rabalais NN, Turner RE, Wiseman WJ Jr (2002) Gulf of Mexico hypoxia, aka "the dead zone". Annu Rev Ecol Syst 33:235–263

Rabalais NN, Turner RE, Díaz RJ, Justi D (2009) Global change and eutrophication of coastal waters. ICES J Mar Sci 66:1528–1537

Rabalais NN, Cai WJ, Carstensen J, Conley DJ and others (2014) Eutrophication-driven deoxygenation in the coastal ocean. Oceanography (Wash DC) 27:172–183

Rahman I, MacNee W (2000) Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 16: 534–554

Ramirez JM, Folkow LP, Blix AS (2007) Hypoxia tolerance in mammals and birds: from the wilderness to the clinic. Annu Rev Physiol 69:113–143

Regoli F, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat Toxicol 31:143–164

Ringwood A, Keppler C (2002) Water quality variation and clam growth: Is pH really a non-issue in estuaries? Estuar Coast 25:901–907

Ringwood AH, Conners DE, Keppler CJ, Dinovo AA (1999) Biomarker studies with juvenile oysters (*Crassostrea virginica*) deployed *in-situ*. Biomarkers 4:400–414

Ringwood A, Hoguet J, Keppler C, Gielazyn M, Ward B, Rourk A (2003) Cellular biomarkers (lysosomal destabilization, glutathione & lipid peroxidation) in three common estuarine species: a methods handbook. Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC

Ross SW, Dalton DA, Kramer S, Christensen BL (2001) Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp Biochem Physiol C 130:289–303

Tahara EB, Navarete FDT, Kowaltowski AJ (2009) Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. Free Radic Biol Med 46:1283–1297

Tyler R, Brady D, Targett T (2009) Temporal and spatial dynamics of diel-cycling hypoxia in estuarine tributaries. Estuar Coast 32:123–145

Vaquer-Sunyer R, Duarte CM (2008) Thresholds of hypoxia for marine biodiversity. Proc Natl Acad Sci USA 105: 15452–15457

Wang WX, Widdows J (1993) Metabolic responses of the common mussel *Mytilus edulis* to hypoxia and anoxia. Mar Ecol Prog Ser 95:205–214

Zenteno-Savín T, Saldierna R, Ahuejote-Sandoval M (2006) Superoxide radical production in response to environmental hypoxia in cultured shrimp. Comp Biochem Physiol C 142:301–308

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