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Interactive effects of elevated temperature and CO_2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*)

Omera B. Matoo [a], Anna V. Ivanina [a], Claus Ullstad [a], Elia Beniash [b], Inna M. Sokolova [a,*]

[a] Department of Biology, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, USA
[b] Department of Oral Biology, University of Pittsburgh, 589 Salk Hall, 3501 Terrace Street, Pittsburgh, PA 15261, USA

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ABSTRACT

Marine bivalves such as the hard shell clams *Mercenaria mercenaria* and eastern oysters *Crassostrea virginica* are affected by multiple stressors, including fluctuations in temperature and CO_2 levels in estuaries, and these stresses are expected to be exacerbated by ongoing global climate change. Hypercapnia (elevated CO_2 levels) and temperature stress can affect survival, growth and development of marine bivalves, but the cellular mechanisms of these effects are not yet fully understood. In this study, we investigated whether oxidative stress is implicated in cellular responses to elevated temperature and CO_2 levels in marine bivalves. We measured the whole-organism standard metabolic rate (SMR), total antioxidant capacity (TAOC), and levels of oxidative stress biomarkers in the muscle tissues of clams and oysters exposed to different temperatures (22 and 27 °C) and CO_2 levels (the present day conditions of ~400 ppm CO_2 and 800 ppm CO_2 predicted by a consensus business-as-usual IPCC emission scenario for the year 2100). SMR was significantly higher and the antioxidant capacity was lower in oysters than in clams. Aerobic metabolism was largely temperature-independent in these two species in the studied temperature range (22–27 °C). However, the combined exposure to elevated temperature and hypercapnia led to elevated SMR in clams indicating elevated costs of basal maintenance. No persistent oxidative stress signal (measured by the levels of protein carbonyls, and protein conjugates with malondialdehyde and 4-hydroxynonenal) was observed during the long-term exposure to moderate warming (+5 °C) and hypercapnia (~800 ppm CO_2). This indicates that long-term exposure to moderately elevated CO_2 and temperature minimally affects the cellular redox status in these bivalve species and that the earlier observed negative physiological effects of elevated CO_2 and temperature must be explained by other cellular mechanisms.

1. Introduction

Estuarine and coastal zones are the most productive and biologically diverse areas of the ocean. These regions experience strong impacts of natural and anthropogenic stressors including fluctuations in temperature, salinity and dissolved levels of oxygen and carbon dioxide (CO_2). In the recent decades, global climate change and ocean acidification have been recognized as key factors affecting life in estuarine and coastal zones. Ocean acidification refers to the CO_2-driven decrease in seawater pH that is accompanied by a strong shift in the seawater chemistry including changes in dissolved inorganic carbon and calcium carbonate saturation state (Andersson et al., 2005; Doney et al., 2009). The continued release of CO_2 into the atmosphere has caused a reduction in ocean pH value by approximately 0.1 pH units with respect to the pre-industrial levels, and a further reduction by 0.3–0.5 pH units is predicted before the end of the 21st century (Caldeira and Wickett, 2005; Raven et al., 2005; IPCC, 2007). The increasing level of carbon dioxide (CO_2) in the atmosphere also leads to other global changes, including an increase of the average air and surface ocean temperatures (IPCC, 2007). The mean global temperatures have increased by approximately 0.7 °C during the last century and the recent climate models forecast a further increase of the mean global temperature by approximately 1.8 to 4 °C by the year 2100 (IPCC, 2007; Mann et al., 2008).

Marine calcifiers, including bivalve mollusks, are among the most sensitive groups to ocean acidification and global climate change (review in: Pörtner, 2002, 2008b; Guinotte and Fabry, 2008; Cooley et al., 2009; Doney et al., 2009). CO_2-driven changes in the temperature and chemistry of the ocean's waters have been shown to strongly affect development, growth and metabolism of marine bivalves leading to elevated mortality and decreased growth of larvae and juveniles, impaired biomineralization, elevated costs of basal maintenance and, at extreme pH, metabolic rate depression (Michaelidis et al., 2005a; Dove and Sammut, 2007; Gazeau et al., 2007; Beesley et al., 2008; Talmage and Gobler, 2009; Beniash et al., 2010; Gazeau et al., 2010; Lannig et al., 2010; Dickinson et al., 2012). The cellular mechanisms of CO_2-induced changes in the physiology of mollusks are not yet

* Corresponding author. Tel.: +1 704 687 8532; fax: +1 704 687 1488.
E-mail address: isokolov@uncc.edu (I.M. Sokolova).

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fully understood but are likely to involve multiple pathways of metabolism, biomineralization and acid–base balance (Pörtner, 1993; Pörtner and Bock, 2000; Pörtner, 2008a). Metabolic effects of elevated P_{CO_2} vary between different species (Dupont et al., 2010; Hendriks et al., 2010; Kroeker et al., 2010) and depend on the CO_2 concentration in seawater. In marine bivalves, moderate increases in P_{CO_2} (<1200 μatm) have little effect on metabolic rates, while higher P_{CO_2} levels have been shown to result in elevated rates of oxygen consumption (at ~2000–3500 μatm P_{CO_2}) and metabolic rate depression at extremely high levels P_{CO_2} levels (>5000 μatm) (Michaelidis et al., 2005b; Beniash et al., 2010; Hendriks et al., 2010; Thomsen and Melzner, 2010). Oxidative stress is a common consequence of the metabolic and acid–base disturbance in animals, and thus can be implicated in physiological responses to elevated temperature and CO_2 levels. An earlier study in the eastern oyster *Crassostrea virginica* showed that exposure to elevated CO_2 levels induces a strong oxidative stress response in gill tissues indicated by upregulation of major antioxidant proteins (Tomanek et al., 2011). Elevated temperature also often leads to oxidative stress in marine molluscs due to a mismatch between generation and detoxification of reactive oxygen species (ROS) (Abele et al., 1998, 2001, 2002; Heise et al., 2003; Pohlmann et al., 2011). This indicates that disturbances of the cellular redox balance may be a common mechanism contributing to the negative impacts of elevated temperature and CO_2 stress on marine mollusks. However, the interactive effects of elevated temperature and CO_2 levels that are relevant to the near-future global change scenarios (IPCC, 2007) are not well understood in marine mollusks and require further investigation.

The eastern oysters, *C. virginica*, and the hard shell clams, *Mercenaria mercenaria*, are common bivalve that serve as ecosystem engineers in estuaries of the southeastern U.S. (Kennedy et al., 1996; Kraeuter and Castagna, 2001). They are often exposed to variations of temperature, pH and CO_2 levels in estuarine habitats, where seawater pH can fluctuate from 8.1 to 6.9–6.5 (and P_{CO_2} can change from ~400 μatm to more than 10,000–40,000 μatm) due to biological CO_2 production and/or freshwater inflow, and temperature fluctuations can exceed 15–20 °C during diurnal, tidal and seasonal cycles (Cochran and Burnett, 1996; Burnett, 1997; Ringwood and Keppler, 2002; Cherkasov et al., 2007; Chapman et al., 2011). Ocean acidification and global climate change will shift the baseline pH and temperature levels in estuarine and coastal waters and thus can exacerbate the effects of natural variations in temperature and pH in these habitats (Cooley and Doney, 2009; Doney et al., 2009; Najjar et al., 2010; Waldbusser et al., 2011). Like other marine calcifiers, clams and oysters are expected to be sensitive to ocean acidification and global climate change but physiological mechanisms of their responses to the combined effects of elevated P_{CO_2} and temperature are not yet fully understood. Earlier studies have established an important role for changes in energy metabolism and cellular redox status in responses of marine bivalves to temperature stress (Viarengo et al., 1998; Pörtner, 2002; Dahlhoff, 2004; Abele et al., 2007), and recent studies indicate that metabolic and redox status changes may also be implicated in response to elevated P_{CO_2} (Beniash et al., 2010; Tomanek et al., 2011). However, our knowledge of the effects of elevated CO_2 levels on oxidative status of mollusks is currently limited to extreme P_{CO_2} exposures (Tomanek et al., 2011) and nothing is known about the potential involvement of oxidative stress in their responses to moderate hypercapnia such as expected in the case of ocean acidification. The aim of this study was to determine the effects of elevated temperature and CO_2 levels on oxidative stress and antioxidant defense of clams in oysters in order to assess whether shifts in the cellular redox balance are implicated in physiological response of these organisms to the combination of elevated temperature and CO_2 levels such as expected in the case of the global climate change. We hypothesized that exposure to elevated temperature would induce oxidative stress in the bivalve tissues, and that elevated CO_2 may potentiate the pro-oxidant effects of the elevated temperature. To test this hypothesis, we have measured whole-organism oxygen consumption rates, as well as the total antioxidant capacity (TAOC), and levels of biomarkers indicative of the oxidative damage to proteins and lipids in the muscle tissues of clams and oysters exposed to different temperatures (22 and 27 °C) and CO_2 levels (~400 and 800 ppm CO_2).

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma-Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Animal collection and maintenance

Eastern oysters, *C. virginica*, and hard shell clams, *M. mercenaria*, were purchased from a commercial supplier (Cuttyhunk Shellfish Farms, Cuttyhunk, MA, USA). Oysters and clams were collected subtidally to avoid the potential effects of intertidal exposure on physiological characteristics. Oysters and clams were shipped overnight to the University of North Carolina at Charlotte within 48 h of their collection and acclimated for 10 days in aerated tanks with recirculating artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 22 ± 1 °C and 30 ± 1 practical salinity units (PSU) salinity. During the preliminary acclimation period, tanks were aerated with the ambient air.

After the preliminary acclimation, clams and oysters were exposed in a factorial design with two levels of P_{CO_2} [~400 ppm (normocapnia) and ~800 ppm (hypercapnia)] and two temperature levels [22 °C (control) and 27 °C (elevated)], making for a total of four treatment groups. The two selected CO_2 levels were representative of the present-day conditions (~400 ppm CO_2; normocapnia) and atmospheric CO_2 concentrations predicted by one of the moderate scenarios of the Intergovernmental Panel for Climate Change (IPCC, 2007) for the year 2100 (~800 ppm CO_2; hypercapnia). The two selected temperatures were chosen to represent the average water temperature in clam and oyster habitats at the time of collection (22 °C), and a +5 °C increase predicted for the year 2100 by an IPCC scenario (27 °C). It is worth noting that both experimental temperatures are within the environmentally relevant range for these bivalves that can experience temperature fluctuations of 10–20 °C during summer low tides in their habitats. Two replicate tanks were set for each experimental treatment. Water in normocapnic treatments was bubbled with the ambient air whereas in hypercapnic treatments the CO_2-enriched air (certified gas mixtures containing 21% O_2, 0.08% CO_2 and balance N_2; Roberts Oxygen, Charlotte, NC, USA) was used. The flow rates were regulated to maintain the target pH of the seawater at a steady level throughout the exposures. For the bivalves exposed to elevated temperature, the water temperature in the tanks was slowly raised from 22 °C by 1 °C per day until 27 °C was achieved and the experimental exposures began. The interactive effects of P_{CO_2} and temperature were tested after short-term (two weeks) and long-term (8–15 weeks) exposures. The two-week time point was selected because it is considered the minimum time required for full acclimation in mollusks (Prosser, 1958, 1991; Berger and Kharazova, 1997) and is a typical duration for many short-term physiological studies. Long-term exposure lasted for 15 weeks in the first experiment; however, due to the high mortality of oysters exposed to the elevated temperature (27 °C), the experiment was repeated with a new batch of clams and oysters for two and eight weeks. Clams and oysters were obtained from the same populations and matched in size to the first experimental batch. Preliminary acclimation and all exposure conditions were the same as during the first experiment. No

differences in the studied traits were observed between the two batches of oysters or clams after two weeks of exposure indicating similarity in physiological parameters between the two batches. Therefore, we have combined the data from the two replicate experiments for further analyses.

Throughout the experiments, animals were fed ad libitum every other day with a commercial algal blend (5 mL per 30 L tank) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Algae were added to the tanks following each water change. Water was changed every other day using ASW at the respective experimental temperature pre-equilibrated with the gas mixtures to achieve the required treatment pH. Experimental tanks were checked for mortality daily, and animals that gaped and did not respond to a mechanical stimulus were recorded as dead and immediately removed.

2.3. Seawater chemistry

To avoid potential variations in water chemistry, ASW for all exposures was prepared using the same batch of the Instant Ocean® salt throughout the experiment. Carbonate chemistry of seawater was determined as described in an earlier study (Beniash et al., 2010). Briefly, water samples were collected periodically from the experimental tanks during the time course of the experiment in air-tight containers without headspace, preserved by mercuric chloride poisoning and stored at +4 °C till further analysis. Water pH was measured at the time of collection using a pH electrode (pH meter, Pinpoint®, American Marine Inc., Ridgefield, CT, USA) calibrated with National Institute of Standards and Technology standard pH buffer solutions (NBS standards; American Marine Inc., Ridgefield, CT, USA). Water temperature and salinity were recorded at the same time. Total dissolved inorganic carbon (DIC) concentrations were measured within a week of collection by Nutrient Analytical Services (Chesapeake Biological Laboratory, Solomons, MD, USA) as described elsewhere (Beniash et al., 2010). Temperature, salinity and pH were measured at the time of collection and, along with the total DIC levels, were used to calculate P_{CO_2}, alkalinity and the saturation state (Ω) for calcite and aragonite in seawater using co2sys software (Lewis and Wallace, 1998). For co2sys settings, we used the NBS scale of seawater pH, dissociation and solubility constants from Millero et al. (2006), the KSO_4^- constant from Dickson et al. (2007) (cited in Lewis and Wallace, 1998), and concentrations of silicate and phosphate for Instant Ocean® seawater (silicate: 0.17 μmol kg^{-1} and phosphate: 0.04 μmol kg^{-1} at salinity of 30 PSU). Seawater chemistry data for the experimental exposures are given in Table 1.

2.4. Whole-organism oxygen consumption

Standard metabolic rate (SMR) was measured as resting oxygen consumption ($\dot{M}O_2$) in clams or oysters at their respective acclimation temperature and CO_2 levels using microfiber optic oxygen probes (Tx-Type, PreSens GmbH, Germany, www.presens.de). Two-point calibration was performed at each temperature and CO_2 concentration. Oyster and clam shells were carefully scrubbed and cleaned of fouling organisms. Mollusks were placed into flow-through respiration chambers and allowed to recover overnight. To avoid interference with post-prandial metabolism and feces excretion, animals were fasted for 24 h prior to the start of $\dot{M}O_2$ recordings. Water flow (20–25 mL min^{-1}) was adjusted so that animals consumed less than 25% of O_2 at all times to avoid potential inhibitory effects of low oxygen levels on respiration rate. After measurements, bivalves were dissected and wet tissue mass immediately determined. Dry tissue mass was calculated from the wet tissue mass assuming an average water content (80%) earlier measured in clam and oyster tissues (data not shown). Bivalve tissues were flash-frozen and stored in liquid nitrogen for analysis of oxidative stress markers and total tissue antioxidant capacity. SMR was calculated as follows:

$$\mathrm{SMR} = \left(\frac{\Delta P_{O_2} \times \beta_{O_2} \times V_{fl}}{M^{0.8}}\right),$$

where SMR — normalized oxygen consumption (μmol O_2 g^{-1} dry mass h^{-1}) normalized to 1 g dry mass, ΔP_{O_2} — difference in partial pressure between in- and out-flowing water (kPa), β_{O_2} — oxygen capacity of water (μmol O_2 L^{-1} kPa^{-1}), V_{fl} — flow rate (L h^{-1}), M — bivalve dry tissue mass (g) and 0.8 — allometric coefficient (Bougrier et al., 1995).

2.5. Oxidative markers

About 200–300 mg of the adductor muscle tissue of clams or oysters from different experimental treatments was homogenized in ice cold phosphate-buffered saline (PBS) with protease inhibitors [50 μg L^{-1} aprotinin and 40 μM of phenylmethylsulfonyl fluoride (PMSF)] in tissue-to-buffer proportion of 1:5 w:v using Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Samples were centrifuged at 15,000 *g* for 10 min at 4 °C. Protein concentration was measured in the supernatant using the Bio-Rad Protein Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Supernatants were diluted with PBS to a final concentration of 1 mg L^{-1} of protein. Protein conjugates of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured as biomarkers

Table 1
Summary of water chemistry parameters during experimental exposures. Salinity, temperature, pH, and DIC were determined in samples from experimental tanks as described in Materials and methods. Other parameters were calculated using co2sys software. P_{CO_2} levels were significantly different between normocapnia and hypercapnia within each temperature treatment ($p<0.01$ after sequential Bonferroni corrections) but not between 22 °C and 27 °C treatments within the respective CO_2 groups. Data are presented as means ± SEM. N = 22–27 for DIC and TA, and 95–119 for other parameters.

	Exposure temperature			
	22 °C		27 °C	
	Normocapnia	Hypercapnia	Normocapnia	Hypercapnia
pH	8.14 ± 0.01	7.95 ± 0.01	8.08 ± 0.01	7.94 ± 0.02
Temperature (°C)	22.4 ± 0.1	22.2 ± 0.1	26.7 ± 0.1	26.7 ± 0.1
Salinity, PSU	30.4 ± 0.1	30.3 ± 0.1	30.4 ± 0.1	30.8 ± 0.1
P_{CO_2} (μatm)	429.75 ± 16.60	744.49 ± 30.63	535.52 ± 26.57	796.37 ± 47.28
DIC (μmol kg^{-1} SW)	2439.28 ± 84.29	2554.09 ± 99.41	2571.64 ± 103.26	2549.93 ± 89.09
TA (μmol kg^{-1} SW)	2683.17 ± 100.04	2735.44 ± 115.21	2824.94 ± 119.37	2725.84 ± 97.70
HCO_3^- (μmol kg^{-1} SW)	2127.67 ± 20.53	2320.04 ± 27.09	2159.68 ± 28.37	2260.54 ± 25.89
CO_3^{2-} (μmol kg^{-1} SW)	255.71 ± 6.15	182.49 ± 6.13	259.71 ± 7.03	201.16 ± 6.45
CO_2 (μmol kg^{-1} SW)	13.35 ± 0.51	23.25 ± 0.96	14.88 ± 0.74	22.03 ± 1.29
Ω Ca	6.33 ± 0.15	4.52 ± 0.15	6.50 ± 0.17	5.03 ± 0.16
Ω Arg	4.10 ± 0.10	2.92 ± 0.10	4.26 ± 0.11	3.29 ± 0.11

of lipid peroxidation using enzyme immunoassays (MDA OxiSelect™ MDA adduct ELISA Kit and HNE OxiSelect™ HNE-His adduct ELISA Kit, respectively) according to the manufacturers' protocols (Cell Biolabs, Inc., CA, USA).

Protein carbonyl content was determined following the protocol of Levine et al. (2000). About 200–300 mg of adductor muscle tissue was homogenized using Kontes Duall tissue grinders (Fisher Scientific) in a homogenization buffer (50 mM HEPES, pH 7.4, 125 mM KCl, 0.5 μg mL^{-1} leupeptin, 0.7 μg mL^{-1}pepstatin A, 40 μg mL^{-1} PMSF and 0.5 μg mL^{-1} aprotinin). Ethylenediaminetetraacetic acid (1.1 mM EDTA) and $MgSO_4$ (0.6 mM) were freshly added to the buffer prior to homogenization. Homogenates were centrifuged for 10 min at 22,000 *g* and 4 °C. Streptomycin sulfate (1% v:v) was added to the supernatant, incubated for 15 min at room temperature and centrifuged at 6000 *g* for 10 min to remove possible nucleic acid contamination. Tissue extracts were stored at −80 °C.

Carbonyl content of extracted proteins was determined using the 2,4-dinitrophenyl hydrazine (DNPH) assay as described elsewhere (Levine et al., 2000). Two technical replicates were measured for each sample, and a sample blank was prepared using 2 M HCl instead of DNPH. Proteins in the DNPH-stained samples and HCl-treated sample blanks were precipitated with trichloroacetic acid (TCA), collected by centrifugation at 22,000 *g* for 10 min and the pellet washed three times in ethanol:ethyl acetate mixture (1:1 v:v). The pellets were dried, dissolved with 6 M guanidine HCl in 20 mM KH_2PO_4, pH 2.4, centrifuged at 11,000 *g* for 5 min, and the absorbance of the supernatants was determined at 360 nm (Cary 50 UV–Visible spectrophotometer, Cary, NC, USA). Carbonyl concentrations were calculated using an extinction coefficient of ε = 22,000 M^{-1} cm^{-1} for dinitrophenylhydrazone. Protein content of the samples was determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) standard prepared in 6 M guanidine HCl and 20 mM KH_2PO_4 (pH 2.4). Carbonyl content was normalized to the protein concentration of the tissue samples.

2.6. Total antioxidant capacity (TAOC)

Samples of adductor muscles were homogenized in ice cold PBS with protease inhibitors prepared as described above for the MDA and 4-HNE assays. Total antioxidant capacity was measured using colorimetric microplate assay for total antioxidant power (Oxford Biomedical Research, Oxford, MI, USA) following the manufacturer's protocol. TAOC was expressed in Cu^{2+}-reducing equivalents against a uric acid standard and normalized for the protein content of the sample.

2.7. Statistics

Effects of the factors temperature, P_{CO_2}, time of exposure and their interactions were assessed for all studied physiological and biochemical traits using generalized linear model (GLM) ANOVA after testing for the normality of data distribution and homogeneity of variances. All factors were treated as fixed and had two levels for temperature and P_{CO_2} (22 °C and 27 °C for temperature, and normocapnia and hypercapnia for P_{CO_2}), and three levels for exposure time (2, 8 and 15 weeks) (Table 2). Post-hoc tests (Fisher's Least Significant Difference) were used to test the differences between the group means; only planned contrasts were used for the comparison of all studied biological traits. For unplanned comparisons of correlated variables (e.g. parameters of seawater chemistry), a sequential Bonferroni correction was used to adjust the conventional probability levels to ensure the experiment-wise error did not exceed 5%. Sample sizes were 5–11 for all measured traits and experimental groups, except for oysters after 8–15 weeks of exposure at 27 °C where sample size was reduced due to mortality. Each biological replicate represents a sample from an individual clam or oyster. Unless otherwise indicated, data are represented as means ± standard errors of means (SEM). The

Table 2
ANOVA for the effects of temperature, CO_2 levels and exposure time on the studied physiological and biochemical traits of *C. virginica* and *M. mercenaria*. F-Values are given with degrees of freedom for the factor and the error in subscript. Significant effects are highlighted in bold.

Factors/interactions	Traits				
	Oxygen consumption rate	TAOC	Protein carbonyls	MDA conjugates	HNE conjugates
C. virginica					
P_{CO_2}	$F_{1,84}=0.89$	$F_{1,75}=4.32$	$F_{1,47}=2.70$	$F_{1,76}=0.04$	$F_{1,76}=1.96$
	$p=0.349$	$p=0.415$	$p=0.109$	$p=0.836$	$p=0.166$
Temperature (T)	$F_{1,84}=3.39$	$F_{1,75}=0.02$	$F_{1,47}=0.01$	$F_{1,76}=0.35$	$F_{1,76}=0.28$
	$p=0.070$	$p=0.901$	$p=0.911$	$p=0.553$	$p=0.597$
Exposure time (Exp)	$F_{2,84}=2.31$	**$F_{2,75}=14.18$**	**$F_{2,47}=11.64$**	$F_{2,76}=0.07$	**$F_{2,76}=6.16$**
	$p=0.107$	**$p<0.0001$**	**$p=0.0001$**	$p=0.937$	**$p=0.004$**
$P_{CO_2}\times T$	$F_{1,84}=0.02$	$F_{1,75}=0.62$	$F_{1,47}=3.27$	$F_{1,76}=3.00$	**$F_{1,76}=5.22$**
	$p=0.897$	$p=0.434$	$p=0.078$	$p=0.088$	**$p=0.026$**
$P_{CO_2}\times Exp$	$F_{2,84}=0.28$	$F_{2,75}=0.02$	**$F_{2,47}=3.63$**	**$F_{2,76}=4.62$**	**$F_{2,76}=7.23$**
	$p=0.753$	$p=0.976$	**$p=0.036$**	**$p=0.013$**	**$p=0.001$**
$T\times Exp$	$F_{2,84}=1.18$	$F_{2,75}=1.62$	**$F_{2,47}=4.19$**	$F_{2,76}=0.67$	$F_{2,76}=0.70$
	$p=0.313$	$p=0.206$	**$p=0.023$**	$p=0.513$	$p=0.500$
$P_{CO_2}\times T\times Exp$	$F_{1,84}=0.73$	$F_{1,75}=0.88$	**$F_{1,47}=19.94$**	$F_{1,76}=1.88$	$F_{1,76}=0.00$
	$p=0.396$	$p=0.352$	**$p<0.0001$**	$p=0.175$	$p=0.949$
M. mercenaria					
P_{CO_2}	$F_{1,113}=0.13$	**$F_{1,111}=10.59$**	**$F_{1,81}=11.08$**	$F_{1,102}=2.88$	$F_{1,100}=0.24$
	$p=0.716$	**$p=0.002$**	**$p=0.001$**	$p=0.093$	$p=0.628$
Temperature (T)	**$F_{1,113}=30.35$**	$F_{1,111}=0.01$	**$F_{1,81}=4.01$**	$F_{1,102}=2.53$	$F_{1,100}=0.24$
	$p<0.001$	$p=0.937$	**$p=0.049$**	$p=0.115$	$p=0.628$
Exposure time (Exp)	$F_{2,113}=2.16$	**$F_{2,111}=5.18$**	$F_{2,81}=2.78$	$F_{2,102}=0.18$	$F_{2,100}=2.77$
	$p=0.121$	**$p=0.007$**	$p=0.069$	$p=0.839$	$p=0.068$
$P_{CO_2}\times T$	$F_{1,113}=0.14$	$F_{1,111}=2.45$	**$F_{1,81}=4.24$**	**$F_{1,102}=5.38$**	$F_{1,100}=3.31$
	$p=0.714$	$p=0.121$	**$p=0.043$**	**$p=0.023$**	$p=0.072$
$P_{CO_2}\times Exp$	$F_{2,113}=2.21$	$F_{2,111}=2.13$	**$F_{2,81}=5.04$**	**$F_{2,102}=3.37$**	**$F_{2,100}=5.11$**
	$p=0.114$	$p=0.125$	**$p=0.009$**	**$p=0.039$**	**$p=0.008$**
$T\times Exp$	**$F_{2,113}=3.67$**	$F_{2,111}=0.08$	**$F_{2,81}=5.03$**	**$F_{2,102}=5.26$**	**$F_{2,100}=4.56$**
	$p=0.029$	$p=0.927$	**$p=0.009$**	**$p=0.007$**	**$p=0.013$**
$P_{CO_2}\times T\times Exp$	$F_{2,113}=0.16$	$F_{2,111}=0.45$	**$F_{2,81}=6.84$**	$F_{2,102}=0.02$	**$F_{2,100}=3.38$**
	$p=0.851$	$p=0.641$	**$p=0.002$**	$p=0.983$	**$p=0.038$**

differences were considered significant if the probability of Type I error was less than 0.05.

3. Results

3.1. Mortality

At 22 °C, clams and oysters showed low mortality rates (~0–5% and ~30% after 15 weeks of exposure, respectively). Elevated temperature resulted in increased mortality in both species (Fisher's exact tests, $p<0.05$ for 22 °C vs. 27 °C contrasts at the respective P_{CO_2}). At 27 °C, mortality of oysters was significantly lower in hypercapnia than normocapnia (61% vs. 95%, $p<0.05$), while in clams P_{CO_2} did not affect mortality at 27 °C (~44% in normocapnia and hypercapnia, $p>0.05$). Exposure P_{CO_2} had no effect on mortality at 22 °C in either species (Fisher's exact tests, $p>0.05$).

3.2. Oxidative metabolism

Oysters had two- to three-fold higher rates of oxygen consumption ($\dot{M}O_2$) than clams regardless of the exposure conditions. In oysters, acclimation temperature and CO_2 levels had no effects on $\dot{M}O_2$ (Table 2; Fig. 1A). In clams, exposure to elevated temperature under the normocapnic conditions led to a transient increase in $\dot{M}O_2$ after 2 weeks with the subsequent return of $\dot{M}O_2$ to the control levels after 8–15 weeks of exposure (Fig. 1B). At high temperature (27 °C) and hypercapnia, oxygen consumption rates of clams were elevated throughout the experimental exposure (significantly at 2 and 15 weeks) (Fig. 1B).

3.3. Total antioxidant capacity (TAOC)

TAOC was higher in clam tissues compared with oysters under the control conditions (Fig. 1C, D). Exposure time significantly affected TAOC in both studied species, while CO_2 levels also significantly affected TAOC in clams (Table 2). In oysters, there was an increase of TAOC after 8 weeks of exposure at 22 °C both in normocapnia and hypercapnia that was not observed after 15 weeks (Fig. 1C). In clams, a similar transient increase was observed after 8 weeks of exposure at ~800 ppm CO_2 (Fig. 1D). Elevated temperature had no effect on TAOC in the two studied species when maintained in normocapnia (Fig. 1C, D). Prolonged exposure (15 weeks) at elevated CO_2 levels led to a significant decrease of TAOC in clam tissues (Fig. 1D). In contrast, a 15-week exposure to elevated CO_2 resulted in elevated TAOC of oyster muscle tissues; however, this increase was only significant when hypercapnia was combined with the elevated temperature (Fig. 1C).

3.4. Oxidative markers

Levels of oxidative stress biomarkers were significantly affected by interactions of the factors temperature, P_{CO_2} and/or exposure time in clams and oysters thereby precluding the analysis of the effects of single factors in ANOVA (Table 2). In control conditions, carbonyl levels in muscle proteins were similar in the two studied species (Fig. 2A, B). In oysters, carbonyl levels significantly but transiently increased after 2 weeks of exposure to elevated CO_2 at 22 °C, returning to the control levels after 8 and 15 weeks. In clams, carbonyl content remained at a stable low level regardless of the exposure conditions except for a

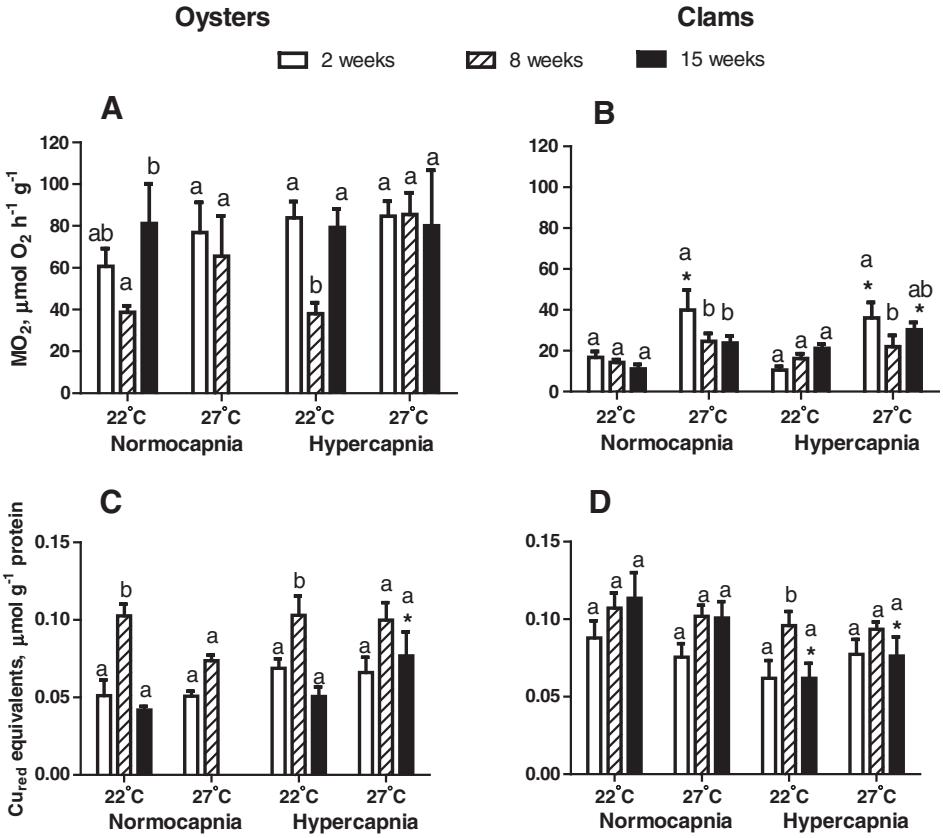

Fig. 1. Oxygen consumption rates and total antioxidant capacity (TAOC) of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperatures and CO_2 levels. A, B — oxygen consumption rates ($\dot{M}O_2$), and C, D — TAOC of oysters (A, C) and clams (B, D). X-axis — experimental conditions (temperature and CO_2 levels). Different letters indicate exposure times that are different within each experimental treatment group ($p<0.05$). Asterisk indicates values that are significantly different from the control (normocapnia at 22 °C) at the respective exposure period ($p<0.05$). For $\dot{M}O_2$, N = 8–10 except 8–15 weeks of exposure at 27 °C for oysters where N = 2–5. For TAOC, N = 7–11 except 8–15 weeks of exposure at 27 °C for oysters where N = 2–4.

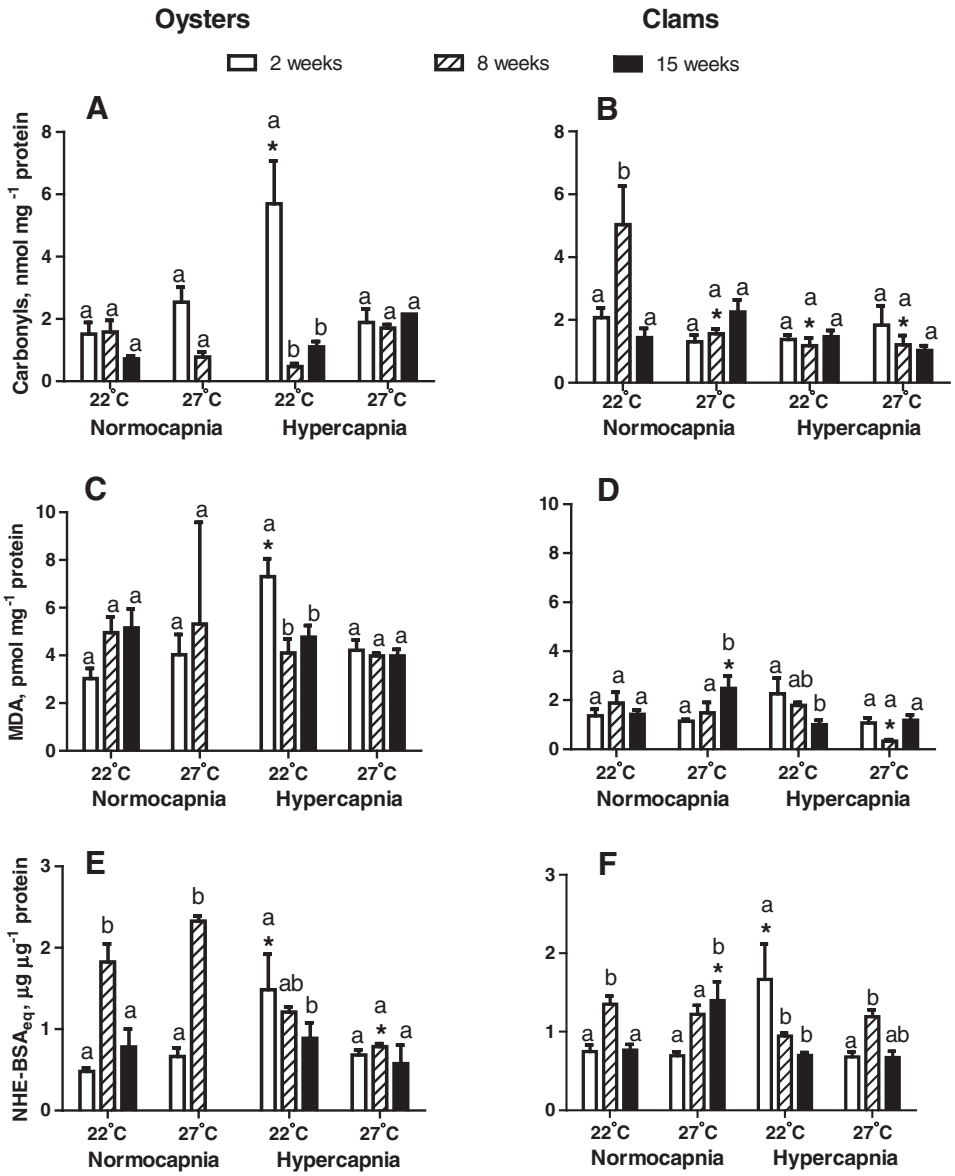

Fig. 2. Oxidative stress markers in gill tissues of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperatures and CO_2 levels. A, B — protein carbonyls, C, D — MDA–protein conjugates, E, F — HNE–protein conjugates. A, C, E — *C. virginica*, B, D, F — *M. mercenaria*. Carbonyl and MDA concentrations are expressed in nmol or pmol per mg protein. HNE concentrations are expressed in relative units compared to a NHE-conjugated BSA standard that was the same for all samples. X-axis — experimental conditions (temperature and CO_2 levels). Different letters indicate exposure times that are different within each experimental treatment group ($p<0.05$). Asterisk indicates values that are significantly different from the control (normocapnia at 22 °C) at the respective exposure period ($p<0.05$). N = 5–10 except 8–15 weeks of exposure at 27 °C for oysters where N = 1–4.

transient rise at 8 weeks of exposure under the control conditions (Fig. 2B).

Tissue levels of MDA–protein conjugates were considerably higher in oysters than in clams regardless of the experimental conditions (Fig. 2C, D). Similar to carbonyls, tissue levels of MDA conjugates in oysters showed a significant transient increase after 2 weeks of exposure to elevated CO_2 at 22 °C returning to the control levels after 8 and 15 weeks. In clams, elevated temperature (27 °C) resulted in a significant increase in the levels of MDA–protein conjugates after 15 weeks of exposure at normocapnia, while hypercapnia at 27 °C resulted in the suppressed levels of MDA–protein conjugates (Fig. 2D).

Levels of HNE–protein conjugates were similar in the muscle tissues of the two studied species under the control conditions (Fig. 2E, F). In oysters, exposure to elevated CO_2 led to a transient increase in the levels of HNE–protein conjugates after 2–8 weeks of exposure that returned to the control levels after 15 weeks (Fig. 2E). In clams, exposure to elevated temperature (27 °C) led to a significant increase of HNE–protein conjugates after 15 weeks of exposure in normocapnia but not in hypercapnia. A transient increase in HNE conjugate levels was also observed after a short-term (2 weeks) exposure of clams to elevated CO_2 at 22 °C that returned back to the control level after 8–15 weeks (Fig. 2E).

Notably, the levels of MDA and NHE conjugates in tissues of individual bivalves were significantly positively correlated (Pearson's $R = 0.34$, $p = 0.003$, $N = 74$ and $R = 0.71$, $p < 0.0001$, $N = 98$ for oysters and clams, respectively). In contrast, carbonyl concentration in muscle proteins did not significantly correlate with either NHE- or MDA-conjugate levels ($R = -0.25$–0.01, $p = 0.14$–0.94 for oysters and $R = -0.10$–0.02, $p = 0.37$–0.83 for clams).

4. Discussion

Clams were considerably more tolerant of chronically elevated temperatures than oysters as shown by the lower mortality at 27 °C. Earlier studies showed that optimal temperature range is similar in clams and oysters (20–24 °C) but responses to chronically elevated temperatures are different (Kraeuter and Castagna, 2001; Lannig et al., 2006). Hard shell clams were capable of maintaining positive growth during prolonged exposures to 27–30 °C despite a decrease in the feeding rates (Kraeuter and Castagna, 2001). In contrast, the growth ceased and survival dramatically declined in oysters exposed to temperatures of 28 °C or above (Bushek and Allen, 1996; Surge et al., 2001; Cherkasov et al., 2006; Lannig et al., 2006; Li et al., 2007). Physiological mechanisms underlying differences in thermal tolerance of clams and oysters are presently unknown and may be related to the differences in ecology of these species. *M. mercenaria* is a typical infaunal species that lives buried below the sediment surface (Kraeuter and Castagna, 2001). In these habitats, sediment and interstitial water may buffer rapid thermal fluctuations but once the sediment warms up, evaporative cooling is limited potentially exposing clams to long-term temperature stress. In contrast, oysters are epibenthic species exposed to extreme, but typically short-term temperature fluctuations during the low tide (Kennedy et al., 1996; Cherkasov et al., 2007). Like other epibenthic intertidal organisms, they can use evaporative cooling and metabolic rate depression to survive short-term heat exposure but may be less capable of surviving chronic temperature stress (Cleland and McMahon, 1986; Sokolova and Pörtner, 2001; Fitzhenry et al., 2004). Determination of the exact physiological mechanisms of the species-specific differences in thermal tolerance was beyond the scope of this study and requires further investigation. Regardless of the underlying mechanisms, lower tolerance of oysters to chronically elevated temperatures may have important implications for survival of their populations in the face of the global climate change.

An increase in environmental temperature typically results in elevated SMR in ectotherms reflecting rate-enhancing effects of temperature on physiological and biochemical reactions such as activities of mitochondria, metabolic enzymes, ion channels and other important oxygen- and energy-demanding processes (Hochachka, 1973, 1988; Cossins et al., 1995; Johnston, 1996). However, the absolute rates as well as temperature-dependence of metabolism are subject to acclimatory or evolutionary changes and thus can differ within and between species depending on the environment, physiological state, activity levels and history of thermal adaptation or acclimation (Somero, 1995; Johnston and Bennett, 1996; Pörtner, 2010; Seebacher et al., 2010). In the present study, a 5 °C increase in temperature (from 22 to 27 °C) under the current ambient CO_2 conditions had no effect on the metabolic rates of clams and oysters after prolonged (8–15 weeks) acclimation. These temperatures are well within the range of the normal temperatures experienced by clams and oysters in their intertidal and shallow water habitats (Kennedy et al., 1996; Kraeuter and Castagna, 2001; Cherkasov et al., 2007). Notably, in clams the metabolic rate increased during the short-term (2 weeks) exposure to the elevated temperature whereas in oysters there was no change in SMR throughout the experimental exposure. A similar situation was found during the acute temperature rise in oysters where a 4 °C rise above the acclimation temperature (from 20 to 24 °C) had no effect on the oxygen consumption rate (Lannig et al., 2008). Such relative temperature independence of aerobic metabolism in the environmentally relevant temperature range is commonly found in intertidal and subtidal mollusks (Branch et al., 1988; McMahon, 1992; Sokolova and Pörtner, 2003). This may reflect metabolic adaptation to fluctuating temperature regime in intertidal and shallow water habitats. Earlier studies indicate that high thermal sensitivity of aerobic metabolism in the environmentally relevant range of temperatures can be associated with long-term metabolic costs and thus may be selected against in these thermally unstable environments (Hawkins, 1995). Notably, when elevated temperature was combined with moderate hypercapnia (~800 ppm CO_2), SMR remained elevated in clams even after the long-term acclimation indicating that a combination of these two stressors led to elevated metabolic costs in these organisms. In contrast, no significant elevation of SMR was found after prolonged exposure to hypercapnia and elevated temperatures in oysters. This may reflect a stronger metabolic regulation in oysters (a typical epibenthic species exposed to rapid and extreme thermal fluctuations) compared with hard shell clams from the more thermally buffered infaunal habitats, or may be due to the selective mortality of oysters at 27 °C.

Oysters had lower total antioxidant capacity and considerably higher rates of oxygen consumption associated with higher levels of MDA–protein conjugates in the adductor muscle tissues compared with clams. This suggests that oyster tissues may be more susceptible to lipid peroxidation than those of clams. In contrast, levels of carbonyls and HNE–protein conjugates were similar in the tissues of the two studied species. Within each species, levels of MDA- and HNE-conjugates were tightly correlated with each other but not with the carbonyl levels likely indicating different kinetics and/or susceptibilities of lipids and proteins to ROS-mediated oxidation (Avery, 2011). Lipid peroxidation biomarkers were also not linked to protein carbonyl content in tissues of aging clams and oysters (Ivanina et al., 2008). Similarly, in mussels *Mytilus gallprovincialis* an increase in carbonyl levels was induced by much lower exposures to metals (Cd and Zn) or organic pollutants (PAHs and lindane) compared with those that induced an elevation in MDA concentrations (Kaloyianni et al., 2009). In our earlier study we also found that an acute temperature rise from 20 °C to 28–32 °C induces a strong accumulation of protein carbonyls but not MDA in oysters (I. Sokolova, unpubl. data). This indicates that oxidative stress assessment should optimally rely on multiple biomarkers encompassing proteins, lipids and/or DNA as the oxidative damage levels can differ between different macromolecular targets (Avery, 2011). In marine bivalves, protein oxidation appears to be an earlier biomarker of oxidative stress than the lipid oxidation markers.

In this study, there was no persistent oxidative stress signal generated by the long-term exposure to moderate warming (+5 °C) and hypercapnia (~800 ppm CO_2) in the two studied bivalves. In oysters, there was a strong but transient increase in the levels of all three oxidative markers (carbonyls, MDA- and HNE-conjugates) after two weeks of exposure to hypercapnia at the normal temperature (22 °C). This agrees with the findings of an earlier study that identified an oxidative stress signature in proteome of oysters exposed for two weeks to elevated CO_2 (~3500 ppm) (Tomanek et al., 2011). An increase in the levels of oxidative stress biomarkers after two weeks of exposure to hypercapnia (~800 ppm CO_2) was followed by an increase in the tissue TAOC in oysters possibly reflecting a compensatory response. However, this response did not persist during the long-term (15 weeks) exposure of oysters to hypercapnia. This may indicate that ROS production and antioxidant defense reached a new steady-state after the long-term acclimation to hypercapnia in oysters. In oysters exposed to hypercapnia at the elevated temperature (27 °C) the changes in oxidative stress markers and antioxidant levels were much less pronounced than at 22 °C suggesting that elevated temperature may partially counteract the effects of elevated CO_2 on the redox status of these organisms. It is worth noting that the relative stability of the levels of antioxidants and oxidative stress biomarkers during exposures of oyster to the elevated temperature may also be explained by high mortality (and thus differential selection) in this experimental group; this hypothesis could not be tested in the present experimental regimes because the oxidative stress markers and TAOC could only be measured in survivors.

In clams, exposure to moderate hypercapnia (~800 ppm CO_2) had no effect on the oxidative status of the muscle tissue suggesting that this species is more capable of withstanding elevated CO_2 levels than oysters. Interestingly, exposure to 27 °C under the normocapnic

conditions led to a transient increase in metabolic rates as well as the tissue levels of MDA and HNE conjugates in clams; this increase was absent under the hypercapnic conditions indicating that elevated CO_2 may alleviate the negative effects of warming on tissue oxidative status in this species.

As a corollary, our study shows that exposure to moderately elevated temperature and CO_2 levels such as expected in the case of the global climate change does not lead to the persistent shifts in the oxidative status of tissues of clams and oysters and has a minimum impact on their energy metabolism. This may reflect adaptation of these intertidal and shallow water species to their environments that naturally experience high fluctuations in temperature and CO_2 levels. Temperature appears to have a stronger effect on metabolism and oxidative status of these species than moderate hypercapnia, especially in oysters where elevated temperature led to considerable mortality regardless of the exposure CO_2 levels. Our data indicate that cellular mechanisms underlying the negative physiological effects of elevated CO_2 and temperature in these bivalve species (including reduced growth, elevated mortality and disturbances in development and biomineralization; Michaelidis et al., 2005a; Dove and Sammut, 2007; Gazeau et al., 2007; Beesley et al., 2008; Talmage and Gobler, 2009; Beniash et al., 2010; Gazeau et al., 2010; Lannig et al., 2010; Dickinson et al., 2012) likely do not involve disturbances of the cellular redox status and must be explained by other mechanisms (such as impacts on metabolic and biomineralization enzymes, stress protection proteins and/or energy trade-offs) that are a subject of further investigations.

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References

Abele, D., Burlando, B., Viarengo, A., Pörtner, H.O., 1998. Exposure to elevated temperature and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. Comp. Biochem. Physiol. B 120, 425–435.

Abele, D., Tesch, C., Wencke, P., Pörtner, H.-O., 2001. How does oxidative stress relate to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*? Antarct. Sci. 13, 111–118.

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841.

Abele, D., Philipp, E., Gonzalez, P.M., Puntarulo, S., 2007. Marine invertebrate mitochondria and oxidative stress. Front. Biosci. 12, 933–946.

Andersson, A.J., Mackenzie, F.T., Lerman, A., 2005. Coastal ocean and carbonate systems in the high CO_2 world of the Anthropocene. Am. J. Sci. 305, 875–918.

Avery, S.V., 2011. Molecular targets of oxidative stress. Biochem. J. 434, 201–210.

Beesley, A., Lowe, D.M., Pascoe, C.K., Widdicombe, S., 2008. Effects of CO_2-induced seawater acidification on the health of *Mytilus edulis*. Clim. Res. 37, 215–225.

Beniash, E., Ivanina, A., Lieb, N.S., Kurochkin, I., Sokolova, I.M., 2010. Elevated levels of carbon dioxide affect metabolism and shell formation in oysters *Crassostrea virginica*. Mar. Ecol. Prog. Ser. 419, 95–108.

Berger, V.J., Kharazova, A.D., 1997. Mechanisms of salinity adaptations in marine molluscs. Hydrobiologia 355, 115–126.

Bougrier, S., Geairon, P., Deslous-Paoli, J.M., Bacher, C., Jonquieres, G., 1995. Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). Aquaculture 134, 143–154.

Branch, G.M., Borchers, P., Brown, C.R., Donelly, D., 1988. Temperature and food as factors influencing oxygen consumption of intertidal organisms, particularly limpets. Am. Zool. 28, 137–146.

Burnett, L.E., 1997. The challenges of living in hypoxic and hypercapnic aquatic environments. Am. Zool. 37, 633–640.

Bushek, D., Allen Jr., S.K., 1996. Host–parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. Mar. Ecol. Prog. Ser. 139, 127–141.

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110, C09S04.

Chapman, R.W., Mancia, A., Beal, M., Veloso, A., Rathburn, C., Blair, A., Holland, A.F., Warr, G.W., Didinato, G.U.Y., Sokolova, I.M., Wirth, E.F., Duffy, E., Sanger, D., 2011. The transcriptomic responses of the eastern oyster, *Crassostrea virginica*, to environmental conditions. Mol. Ecol. 20, 1431–1449.

Cherkasov, A.S., Biswas, P.K., Ridings, D.M., Ringwood, A.H., Sokolova, I.M., 2006. Effects of acclimation temperature and cadmium exposure on cellular energy budgets in a marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. J. Exp. Biol. 209, 1274–1284.

Cherkasov, A.S., Grewal, S., Sokolova, I.M., 2007. Combined effects of temperature and cadmium exposure on haemocyte apoptosis and cadmium accumulation in the eastern oyster *Crassostrea virginica* (Gmelin). J. Therm. Biol. 32, 162–170.

Cleland, J.D., McMahon, R.F., 1986. Upper thermal timit of nine intertidal gastropod species from a Hong Kong rocky shore in relation to vertical distribution and desiccation associated with evaporative cooling. In: Morton, B. (Ed.), Proceedings of the 2nd International Workshop on the Malacofauna of Hong Kong and Southern China. Hong Kong University Press, Hong Kong, pp. 1141–1152.

Cochran, R.E., Burnett, L.E., 1996. Respiratory responses of the salt marsh animals, *Fundulus heteroclitus*, *Leiostomus xanthurus*, and *Palaemonetes pugio* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, azinphosmethyl. J. Exp. Mar. Biol. Ecol. 195, 125–144.

Cooley, S., Doney, S., 2009. Anticipating ocean acidification's economic consequences for commercial fisheries. Environ. Res. Lett. 4, 024007.

Cooley, S.R., Kite-Powell, H.L., Doney, S.C., 2009. Ocean acidification's potential to alter global marine ecosystem services. Oceanography 22, 172–181.

Cossins, A.R., Schwarzbaum, P.J., Wieser, W., 1995. Effects of temperature on cellular ion regulation and membrane transport systems. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, 5, pp. 101–126.

Dahlhoff, E.P., 2004. Biochemical indicators of stress and metabolism: applications for marine ecological studies. Annu. Rev. Physiol. 66, 183–207.

Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M., 2012. Interactive effects of salinity and elevated CO_2 levels on juvenile eastern oysters, *Crassostrea virginica*. J. Exp. Biol. 215, 29–43.

Dickson, A.G., Sabine, C.L., Christian, J.R., 2007. Guide to best practices for ocean CO_2 measurements, 3. PICES Special Publication (191 pp.).

Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A., 2009. Ocean acidification: the other CO_2 problem. Ann. Rev. Mar. Sci. 1, 169–192.

Dove, M.C., Sammut, J., 2007. Impacts of estuarine acidification on survival and growth of Sydney rock oysters *Saccostrea glomerata* (Gould 1850). J. Shellfish Res. 26, 519–527.

Dupont, S., Dorey, N., Thorndyke, M., 2010. What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? Estuar. Coast. Shelf Sci. 89, 182–185.

Fitzhenry, T., Halpin, P.M., Helmuth, B., 2004. Testing the effects of wave exposure, site, and behavior on intertidal mussel body temperatures: applications and limits of temperature logger design. Mar. Biol. 145, 339–349.

Gazeau, F., Quiblier, C., Jansen, J.M., Gattuso, J.P., Middelburg, J.J., Heip, C.H.R., 2007. Impact of elevated CO_2 on shellfish calcification. Geophys. Res. Lett. 34, L07603.

Gazeau, F., Gattuso, J.P., Dawber, C., Pronker, A.E., Peene, F., Peene, J., Heip, C.H.R., Middelburg, J.J., 2010. Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*. Biogeosciences 7, 2051–2060.

Guinotte, J.M., Fabry, V.J., 2008. Ocean acidification and its potential effects on marine ecosystems. Year in Ecology and Conservation Biology, vol. 1134. Blackwell Publishing, Oxford, pp. 320–342.

Hawkins, A.J., 1995. Effects of temperature change on ectotherm metabolism and evolution: metabolic and physiological interrelations underlying the superiority of multi-locus heterozygotes in heterogeneous environments. J. Therm. Biol. 20, 23–33.

Heise, K., Puntarulo, S., Poertner, H.O., Abele, D., 2003. Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. Comp. Biochem. Physiol. C 134, 79–90.

Hendriks, I.E., Duarte, C.M., Alvarez, M., 2010. Vulnerability of marine biodiversity to ocean acidification: a meta-analysis. Estuar. Coast. Shelf Sci. 86, 157–164.

Hochachka, W.P., 1973. Basic strategies and mechanisms of enzyme adaptation to temperature. In: Wieser, W. (Ed.), Effects of Temperature on Ectothermic Organisms. Springer-Verlag, Berlin, pp. 69–81.

Hochachka, P.W., 1988. Channels and pumps — determinants of metabolic cold adaptation strategies. Comp. Biochem. Physiol. B 90, 515–519.

IPCC, 2007. IPCC — Intergovernmental Panel on Climate Change 4th Assessment Report. Cambridge University Press, New York.

Ivanina, A.I., Sokolova, I.M., Sukhotin, A.A., 2008. Oxidative stress and expression of chaperones in aging mollusks. Comp. Biochem. Physiol. A 150, 53–61.

Johnston, I.A., Bennett, A.F., 1996. Animals and Temperature: Phenotypic and Evolutionary Adaptation. Cambridge University Press, Cambridge.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, D.H., Koliakos, G., Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. Comp. Biochem. Physiol. C 149, 631–639.

Kennedy, V.S., Newell, R.I.E., Eble, A.F. (Eds.), 1996. The Eastern Oyster *Crassostrea virginica*. A Maryland Sea Grant Book, College Park, Maryland.

Kraeuter, J.N., Castagna, M.E., 2001. Biology of the Hard Clam. Elsevier Science.

Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol. Lett. 13, 1419–1434.

Lannig, G., Flores, J.F., Sokolova, I.M., 2006. Temperature-dependent stress response in oysters, *Crassostrea virginica*: pollution reduces temperature tolerance in oysters. Aquat. Toxicol. 79, 278–287.

Lannig, G., Bock, C., Cherkasov, A., Pörtner, H.O., Sokolova, I.M., 2008. Cadmium-dependent oxygen limitation affects temperature tolerance in eastern oysters (*Crassostrea virginica* Gmelin). Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R1338–R1346.

Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M., Bock, C., 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. Mar. Drugs 8, 2318–2339.

Levine, R.L., Wehr, N., Williams, J.A., Shacter, E., 2000. Determination of carbonyl groups in oxidized proteins. In: Keyse, S.M. (Ed.), Stress Response. Methods in Molecular Biology, 99. Springer, pp. 15–24.

Lewis, E., Wallace, D.W.R., 1998. Program Developed for CO_2 System Calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center.Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

Li, Y., Qin, J.G., Abbott, C.A., Li, X.X., Benkendorff, K., 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R2353–R2362.

Mann, M.E., Zhang, Z., Hughes, M.K., Bradley, R.S., Miller, S.K., et al., 2008. Proxy-based reconstructions of hemispheric and global surface temperature variations over the past two millennia. Proc. Natl. Acad. Sci. U. S. A. 105, 13252–13257.

McMahon, R.F., 1992. Respiratory response to temperature to temperature and hypoxia in intertidal gastropods from the Texas coast of the Gulf of Mexico. In: Grahame, J., Mill, P.J., Reid, D.G. (Eds.), Proceedings of the Third International Symposium on Littorinid Biology. Malacological Society of London, London, pp. 45–59.

Michaelidis, B., Ouzounis, C., Paleras, A., Portner, H.O., 2005a. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 293, 109–118.

Michaelidis, B., Ouzounis, C., Paleras, A., Pörtner, H.O., 2005b. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 293, 109–118.

Millero, F.J., Graham, T.B., Huang, F., Bustos-Serrano, H., Pierrot, D., 2006. Dissociation constants of carbonic acid in seawater as a function of salinity and temperature. Mar. Chem. 100, 80–94.

Najjar, R.G., Pyke, C.R., Adams, M.B., Breitburg, D., Hershner, C., Kemp, M., Howarth, R., Mulholland, M.R., Paolisso, M., Secor, D., Sellner, K., Wardrop, D., Wood, R., 2010. Potential climate-change impacts on the Chesapeake Bay. Estuar. Coast. Shelf Sci. 86, 1–20.

Pohlmann, K., Koenigstein, S., Alter, K., Abele, D., Held, C., 2011. Heat-shock response and antioxidant defense during air exposure in Patagonian shallow-water limpets from different climatic habitats. Cell Stress Chaperones 16, 621–632.

Pörtner, H.O., 2002. Climate change and temperature dependent biogeography: systemic to molecular hierarchies of thermal tolerance in animals. Comp. Biochem. Physiol. A 132, 739–761.

Pörtner, H., 2008a. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar. Ecol. Prog. Ser. 373, 203–217.

Pörtner, H.-O., 2008b. Meeresorganismen unter CO_2-stress — Grenzen der Zumutbarkeit? In: Gleich, A., Gößling-Reisemann (Eds.), Industrial Ecology. S. Vieweg & Teubner Verlag, pp. 26–40.

Pörtner, H.O., 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J. Exp. Biol. 213, 881–893.

Pörtner, H.O., Bock, C., 2000. A contribution of acid–base regulation to metabolic depression in marine ectotherms. In: Heldmaier, G., Klingenspor, M. (Eds.), Life in the Cold. Springer Verlag, Berlin, pp. 443–458.

Pörtner, H.O., 1993. Multicompartmental analyses of acid–base and metabolic homeostasis during anaerobiosis: invertebrate and lower vertebrate examples. In: Hochachka, P.W., Lutz, P.L., Sick, T., Rosental, M., van den Thillart, G. (Eds.), Surviving Hypoxia: Mechanisms of Control and Adaptation. CRC Press Inc., Boca Raton, FL, USA, pp. 139–156.

Prosser, C.L., 1958. Physiological Adaptation. Ronald Press, New-York.

Prosser, C.L., 1991. Environmental and Metabolic Animal Physiology. Wiley-Liss, New York (etc.).

Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C., Watson, A., 2005. Ocean Acidification Due to Increasing Atmospheric Carbon Dioxide. The Royal Society, Clyvedon Press Ltd., 68 pp.

Ringwood, A.H., Keppler, C.J., 2002. Water quality variation and clam growth: is pH really a non-issue in estuaries. Estuaries 25, 901–907.

Seebacher, F., Brand, M.D., Else, P.L., Guderley, H., Hulbert, A.J., Moyes, C.D., 2010. Plasticity of oxidative metabolism in variable climates: molecular mechanisms. Physiol. Biochem. Zool. 83, 721–732.

Sokolova, I.M., Pörtner, H.O., 2001. Physiological adaptations to high intertidal life involve improved water conservation abilities and metabolic rate depression in *Littorina saxatilis*. Mar. Ecol. Prog. Ser. 224, 171–186.

Sokolova, I.M., Pörtner, H.O., 2003. Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*, Gastropoda: Littorinidae) from different latitudes. J. Exp. Biol. 206, 195–207.

Somero, G.N., 1995. Proteins and temperature. Annu. Rev. Physiol. 57, 43–68.

Surge, D., Lohmann, K.C., Dettman, D.L., 2001. Controls on isotopic chemistry of the American oyster, *Crassostrea virginica*: implications for growth patterns. Palaeogeogr. Palaeoclimatol. Palaeoecol. 172, 283–296.

Talmage, S.C., Gobler, C.J., 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). Limnol. Oceanogr. 54, 2072–2080.

Thomsen, J., Melzner, F., 2010. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar. Biol. 157, 2667–2676.

Tomanek, L., Zuzow, M.J., Ivanina, A.V., Beniash, E., Sokolova, I.M., 2011. Proteomic response to elevated pCO_2 level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. J. Exp. Biol. 214, 1836–1844.

Viarengo, A., Abele-Oeschger, D., Burlando, B., 1998. Effects of Low Temperature on Pro-oxidant Processes and Antioxidant Defense Systems in Marine Organisms. Cambridge University Press, Cambridge.

Waldbusser, G., Voigt, E., Bergschneider, H., Green, M., Newell, R., 2011. Biocalcification in the eastern oyster (*Crassostrea virginica*) in relation to long-term trends in Chesapeake Bay pH. Estuar. Coasts 34, 221–231.