



## Interactive effects of elevated temperature and CO<sub>2</sub> levels on energy metabolism and biomineralization of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*



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### ABSTRACT

The continuing increase of carbon dioxide (CO<sub>2</sub>) levels in the atmosphere leads to increases in global temperatures and partial pressure of CO<sub>2</sub> (P<sub>CO2</sub>) in surface waters, causing ocean acidification. These changes are especially pronounced in shallow coastal and estuarine waters and are expected to significantly affect marine calcifiers including bivalves that are ecosystem engineers in estuarine and coastal communities. To elucidate potential effects of higher temperatures and P<sub>CO2</sub> on physiology and biomineralization of marine bivalves, we exposed two bivalve species, the eastern oysters *Crassostrea virginica* and the hard clams *Mercenaria mercenaria* to different combinations of P<sub>CO2</sub> (~400 and 800 μatm) and temperatures (22 and 27 °C) for 15 weeks. Survival, bioenergetic traits (tissue levels of lipids, glycogen, glucose and high energy phosphates) and biomineralization parameters (mechanical properties of the shells and activity of carbonic anhydrase, CA) were determined in clams and oysters under different temperature and P<sub>CO2</sub> regimes. Our analysis showed major inter-species differences in shell mechanical traits and bioenergetics parameters. Elevated temperature led to the depletion of tissue energy reserves indicating energy deficiency in both species and resulted in higher mortality in oysters. Interestingly, while elevated P<sub>CO2</sub> had a small effect on the physiology and metabolism of both species, it improved survival in oysters. At the same time, a combination of high temperature and elevated P<sub>CO2</sub> led to a significant decrease in shell hardness in both species, suggesting major changes in their biomineralization processes. Overall, these studies show that global climate change and ocean acidification might have complex interactive effects on physiology, metabolism and biomineralization in coastal and estuarine marine bivalves.

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### 1. Introduction

Global change, driven by the increase in atmospheric CO<sub>2</sub> concentrations, has a major impact on marine ecosystems due to a concomitant increase in seawater temperatures and ocean acidification (Cicerone et al., 2004; Cao and Caldeira, 2008; Doney et al., 2009). Current models predict an average increase in global surface temperatures of 1.8–4.0 °C (with some estimates reaching as high as 6.4 °C) and a decline in the ocean pH by 0.3 to 0.4 units by the year 2100, depending on the CO<sub>2</sub> emission scenario (Intergovernmental Panel on Global Climate Change; IPCC, 2007). This change in pH is accompanied by a decrease in the carbonate concentration and saturation levels of calcium carbonate (CaCO<sub>3</sub>) in seawater. Estuarine and

coastal habitats, which represent the ocean's reservoirs of biological diversity and productivity, are vulnerable to ocean acidification and global climatic change. Brackish estuarine waters have lower capacity to buffer pH and are subject to large fluctuations in pH and carbonate chemistry due to the land run-off, upwelling of CO<sub>2</sub>-enriched acidified waters and biological CO<sub>2</sub> production (Mook and Koene, 1975; Burnett, 1997; Cai and Wang, 1998; Rabalais et al., 2009). Lower capacity of thermal buffering of the shallow waters and the thermal exchanges with the land and fresh waters also predispose estuarine and coastal zones to rapid warming and temperature extremes (Helmuth et al., 2002; Gilman et al., 2006). The long-term temperature and pH recordings show that warming of the estuarine and coastal waters have outpaced those observed in the surface waters of the open ocean (Talmage and Gobler, 2011) and that rate of acidification of estuarine waters in the past 50 years closely follows the increase in atmospheric CO<sub>2</sub> (Cooley and Doney, 2009; Doney et al., 2009; Waldbusser et al., 2011).

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Marine calcifiers such as bivalve mollusks are keystone species in estuarine and coastal ecosystems as well as an important resource for marine fisheries and aquaculture. Elevated partial pressure of CO<sub>2</sub> (P<sub>CO2</sub>) can affect acid–base balance, physiology and biomineralization in mollusks leading to decreased rates of growth, higher mortality, as well as changes in shell structure, composition and mechanical properties (Michaelidis et al., 2005; Gazeau et al., 2007; Kurihara et al., 2007; Beesley et al., 2008; Kurihara, 2008; Ellis et al., 2009; Beniash et al., 2010; Dickinson et al., 2012). Physiological effects of elevated P<sub>CO2</sub> may be modulated by the environmental temperature because of the temperature-dependent changes in solubility of gases, pH and inorganic carbon speciation, and most importantly, due to the strong effects of temperature on the rates of all physiological and biochemical reactions (Lewis and Wallace, 1998; Pörtner, 2012). Moreover, ocean acidification may reduce thermal tolerance of marine organisms by shifting energy balance and reducing their aerobic scope (Pörtner, 2012; Sokolova et al., 2012) as was shown in crabs, bivalves and echinoid larvae (Metzger et al., 2007; O'Donnell et al., 2009; Lannig et al., 2010). However, the interactive effects of elevated P<sub>CO2</sub> and temperature stress under environmentally realistic scenarios of global climate change are not well understood in marine mollusks, hampering our ability to predict the consequences of the global climate change on marine ecosystems.

The aim of the present study was to determine the interactive effects of elevated temperature and CO<sub>2</sub> levels on energy metabolism and biomineralization of two common ecologically and economically important bivalves, the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791) and the hard clam *Mercenaria mercenaria* (Linnaeus, 1758). Clams and oysters are ecosystem engineers in the western Atlantic estuaries and common inhabitants of the intertidal and upper subtidal zones. Both species can experience large seasonal and diurnal change of temperature that can exceed 15–20 °C (Helmuth et al., 2002; Cherkasov et al., 2007). Hard clams are an infaunal species periodically exposed to corrosive, acidified conditions in the sediments due to accumulation of CO<sub>2</sub> and other metabolic acids, while oysters, as an epifaunal species, live in the habitats less prone to extreme acidification (Kennedy et al., 1996; Kraeuter and Castagna, 2001; Green et al., 2009). Clams and oysters differ in shell composition and mineralogy; clam shells are made of a more soluble isoform of CaCO<sub>3</sub>, aragonite, but their outer surface is protected by a thick proteinaceous periostracum (Kraeuter and Castagna, 2001). In contrast, shells of adult oysters consist of a much less soluble low-Mg calcite and have a poorly developed periostracum (Digby, 1968; Kennedy et al., 1996). We hypothesized that elevated P<sub>CO2</sub> will lead to elevated energy costs of basal maintenance and impaired biomineralization in clams and oysters, and that elevated temperature will exacerbate these negative effects of P<sub>CO2</sub> due to the increased energy demand and reduced aerobic scope (Pörtner, 2012; Sokolova et al., 2012). We also hypothesized that the effects of elevated P<sub>CO2</sub> will be stronger in oysters than in clams reflecting the adaptation of the latter species to extreme environmental acidosis common in sediments. To test these hypotheses, we determined survival, oxygen consumption rates, tissue and cellular energy status and mechanical properties of the shells in *M. mercenaria* and *C. virginica* after short-term (2 weeks) and long term (8–15 weeks) acclimation at different temperature and P<sub>CO2</sub> levels corresponding to the present-day conditions and the predictions of near-future scenarios of global climate change.

## 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

The experimental animals and exposure conditions were the same as in our companion study (Matoo et al., 2013). Adult eastern oysters *C. virginica* and hard clams *M. mercenaria* were purchased from a commercial supplier (Cuttyhunk Shellfish Farms, Cuttyhunk, MA, USA), shipped on ice by an overnight delivery service to the University of North Carolina at Charlotte and acclimated for 10 days in tanks with recirculating artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 22 ± 1 °C and 30 ± 1 salinity (practical salinity units), aerated with ambient air. Clams and oysters were then randomly assigned to four treatment groups, and each group was exposed to one of the four possible combinations of two levels of CO<sub>2</sub> and two temperatures. The two selected CO<sub>2</sub> levels were representative of the present-day conditions (~400 ppm CO<sub>2</sub>; normocapnia) and atmospheric CO<sub>2</sub> concentrations predicted by a moderate scenario of the Intergovernmental Panel for Climate Change (IPCC, 2007) for the year 2100 (~800 ppm CO<sub>2</sub>; 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). Both experimental temperatures are within the environmentally relevant range for these bivalves in the south-eastern U.S. estuaries. Two replicate tanks were set for each experimental treatment. Water in normocapnic treatments was bubbled with the ambient air whereas for hypercapnic treatments the CO<sub>2</sub>-enriched air (certified gas mixtures containing 21% O<sub>2</sub>, 0.08% CO<sub>2</sub> and balance N<sub>2</sub>; Roberts Oxygen, Charlotte, NC, USA) was used. The flow rates were regulated to maintain a steady pH throughout the exposures. To avoid potential variations in water chemistry, ASW for all exposures was prepared using the same batch of Instant Ocean® salt. Carbonate chemistry of seawater was determined periodically during experimental exposures as described elsewhere (Beniash et al., 2010). Seawater temperature and chemistry data are shown in Table 1.

For the groups exposed to elevated temperature, the water temperature in the tanks was raised by 1 °C per day until 27 °C was achieved. This time was considered time zero for all experimental exposures. Experimental exposures lasted for 2 and 15 weeks (short-term and long-term exposure, respectively). 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. Due to the high mortality of oysters exposed to the elevated temperature (27 °C) after 15 weeks of exposure, the experiment was repeated with a new batch of clams and oysters for 2 and 8 weeks (short-term and intermediate exposure, respectively). 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, the experimental data from the two exposures were pooled for analysis.

Water in experimental tanks was changed every other day using ASW pre-equilibrated to the respective temperature and pH. Throughout the experiment, mollusks were fed *ad libitum* every other day with a commercial algal blend 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 (5 mL per 30 L tank, ~15% of dry mass of animals; Espinosa and Allam, 2006) were added to the tanks following each water change. 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.

**Table 1**

Summary of water chemistry parameters during experimental exposures. The exposure conditions are the same as in our companion study (Matoo et al., 2013). Salinity, temperature, pH, and dissolved inorganic carbon (DIC) were determined in samples from experimental tanks as described in Materials and Methods. Other parameters were calculated using co2sys software. Data are presented as means  $\pm$  S.E.M. N = 22–27 for DIC and total alkalinity (TA), and 95–119 for other parameters.

	Exposure temperature			
	22 °C		27 °C	
	Normocapnia	Hypercapnia	Normocapnia	Hypercapnia
pH	8.14 $\pm$ 0.01	7.95 $\pm$ 0.01	8.08 $\pm$ 0.01	7.94 $\pm$ 0.02
Temperature (°C)	22.4 $\pm$ 0.1	22.2 $\pm$ 0.1	26.7 $\pm$ 0.1	26.7 $\pm$ 0.1
Salinity	30.4 $\pm$ 0.1	30.3 $\pm$ 0.1	30.4 $\pm$ 0.1	30.8 $\pm$ 0.1
P <sub>CO2</sub> ( $\mu$ atm)	429.75 $\pm$ 16.60	744.49 $\pm$ 30.63	535.52 $\pm$ 26.57	796.37 $\pm$ 47.28
DIC ( $\mu$ mol kg <sup>-1</sup> SW)	2439.28 $\pm$ 84.29	2554.09 $\pm$ 99.41	2571.64 $\pm$ 103.26	2549.93 $\pm$ 89.09
TA ( $\mu$ mol kg <sup>-1</sup> SW)	2683.17 $\pm$ 100.04	2735.44 $\pm$ 115.21	2824.94 $\pm$ 119.37	2725.84 $\pm$ 97.70
$\Omega$ Cal	6.33 $\pm$ 0.15	4.52 $\pm$ 0.15	6.50 $\pm$ 0.17	5.03 $\pm$ 0.16
$\Omega$ Arg	4.10 $\pm$ 0.10	2.92 $\pm$ 0.10	4.26 $\pm$ 0.11	3.29 $\pm$ 0.11

### 2.3. Micromechanical testing of the shells

After 15 weeks of experimental exposures, shells of experimental clams and oysters were collected and shipped to the University of Pittsburgh for micromechanical testing. Residual tissue from the shell valves was removed by incubation in sodium hypochlorite (NaOCl; three cycles of 10 min in NaOCl followed by 5 min sonication in a saturated CaCO<sub>3</sub> solution, pH 7.8), and shell valves carefully separated. We conducted the mechanical testing along the narrow (2 mm) area of the outermost edge of the shell where the growing region is situated. However, we were not able to clearly separate shell growth that occurred during experimental exposure from what was present at the start of the experiment. Therefore, results of these analyses should be interpreted as encompassing both the newly deposited shell material and changes in the existing shell due to differences in the seawater chemistry.

Micromechanical testing was conducted on 5–8 shells of oysters and 9–11 shells of clams per treatment. Testing on shells of oysters exposed to normocapnia at 27 °C could not be conducted due to high mortality in this treatment. Left valves of clam or oyster shells were manually trimmed with a bone-cutting tool. An approximately 3 x 3 cm region of the shell, which included its most distal (posterior) edge was mounted in epoxy resin (Epofix, ESM, Hatfield, PA, USA) and polymerized for 24 h at room temperature. Embedded shells were cut longitudinally, transecting the posterior edge, using a slow-speed water-cooled diamond saw (IsoMet, Buehler, Lake Bluff, IL, USA). A second cut was made parallel to the first to produce a 3-mm-thick section. Sections were ground and polished with Metadi diamond suspensions (6, 1 and 0.25  $\mu$ m diamond particles in saturated CaCO<sub>3</sub> solution, pH 7.8) on a grinder-polisher (MiniMet 1000, Buehler). No etching was observed during grinding or polishing.

Vickers microhardness tests were carried out using a micro-indentation hardness tester (IndentaMet 1104, Buehler) on polished shells at a load of 0.245 N and a dwelling time of 5 s. Five to ten indentations per shell were made at least 30  $\mu$ m (but no more than 2 mm) away from the shell edge. Vickers microhardness values were averaged for each shell sample. Digital photographs were taken before and immediately after each indentation. This enabled quantification of the longest crack produced by each indent, which was measured using Adobe Photoshop (ver. 4.0, San Jose, CA, USA) as the radius of a circle radiating from the center of the indent and enclosing all visible cracks. The crack radius for a shell sample was obtained by averaging the crack radii for all indents on that sample. There are a number of empirical equations used to calculate fracture toughness ( $K_c$ ) from the length of cracks generated by micro-indentation (Anstis et al., 1981; Baldassarri et al., 2008). However, because the empirical constants used in these equations were not determined for oyster shells, we chose to use the crack length as a

proxy for  $K_c$ . The term 'fracture resistance' is used in place of  $K_c$  to avoid confusion.

### 2.4. Carbonic anhydrase (CA) activity

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) activity was measured in the mantle (a 2–3 mm wide edge along the ventral shell margin) and gill tissues of clams and oysters exposed to different temperature and CO<sub>2</sub> conditions. Tissues were homogenized in Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA) in ice-cold buffer (1:10 w/v) containing 250 mM sucrose, 40 mM Tris-H<sub>2</sub>SO<sub>4</sub> pH 7.5 and 80  $\mu$ g L<sup>-1</sup> of a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) using an overhead motor (IKA Works RW 16 Basic, Fisher Scientific, Suwanee, GA, USA) at 800 rpm. The homogenate was centrifuged at 4 °C and 10,000 g for 10 min to remove cell debris, and the supernatant was used to determine activity of soluble CA. Enzyme extracts were stored at -80 °C for less than two weeks before activity assays; pilot studies showed that CA activity was not affected by this storage (data not shown).

CA activity was determined at the respective acclimation temperature (22 °C or 27 °C) as acetazolamide (AZM)-sensitive esterase activity following a standard method (Gambhir et al., 2007). The esterase activity was monitored at 348 nm (VARIAN Cary 50 Bio UV-Vis spectrophotometer, Cary, NC, USA) in an assay medium (50 mM Tris-H<sub>2</sub>SO<sub>4</sub>, pH 7.5) containing 1.5 mM of *p*-nitrophenyl acetate as a substrate. Total activity of CA was determined as the difference in the reaction slopes in the absence and presence of a specific inhibitor of carbonic anhydrase (acetazolamide, or AZM, 20  $\mu$ M) and background-corrected for spontaneous hydrolysis of *p*-nitrophenyl acetate. The extinction coefficient of for *p*-nitrophenol (5 mmol<sup>-1</sup> cm<sup>-1</sup> at 348 nm, pH 7.5) was used in all calculations. Protein concentrations of tissue homogenates were determined using Bradford assay (Bradford, 1976), and specific activity of CA was expressed as U g<sup>-1</sup> protein.

In a separate experiment, thermal sensitivity of CA activity was determined in different tissues of the two studied species. Gill, hepatopancreas, muscle and mantle tissues were collected from clams and oysters maintained under normocapnia at 20 °C, and CA activity was measured in tissue homogenates at different temperatures in an environmentally relevant range (5–35 °C) as described above. The apparent activation energy ( $E_a$ ) was determined from an Arrhenius plot of  $\ln(V_{max})$  against  $1/T$  (K<sup>-1</sup>) and used as a measure of the temperature dependence of CA activity. Arrhenius breakpoint temperature (ABT) was determined as a point when the activation energy (i.e. the slope of the Arrhenius plot) significantly changed using an algorithm for the multi-segment linear regression proposed by Oosterbaan (2008) (SegReg software downloaded at <http://www.waterlog.info/>).

## 2.5. Total lipid content

Total lipid content was determined in the mantle tissue using a chloroform extraction method (Folch et al., 1957; Iverson et al., 2001). Briefly, about 50 mg of mantle tissue was homogenized in chloroform/methanol mixture (2:1 v/v) using tissue: solvent proportion of 1:20 w/v. Samples were sonicated for 1 min (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), incubated overnight at 4 °C and centrifuged for 5 min at 13,000 g. The supernatant was transferred in a new tube, mixed with ultrapure water (0.25 volumes of the supernatant), vortexed for 2 min and centrifuged for 5 min at 13,000 g. The lower phase (chloroform) was transferred into a pre-weighed microcentrifuge tube and chloroform was allowed to evaporate to determine the dry mass of extracted lipids.

## 2.6. Total protein content

Mantle tissue (50) mg was homogenized in ice-cold homogenization buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5 µg mL<sup>-1</sup> leupeptin, 0.7 µg mL<sup>-1</sup> pepstatin, 40 µg mL<sup>-1</sup> PMSF and 0.5 µg mL<sup>-1</sup> aprotinin) using Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated three times for 10 s each (output 69 W, Sonicator 3000, Misonix), with cooling on ice between sonications, centrifuged for 10 min at 20,000 g and 4 °C, and supernatants were used for protein determination. Protein content was measured using the Bio-Rad Protein Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

## 2.7. Tissue levels of metabolites

Mantle tissue was powdered with mortar and pestle under liquid nitrogen and homogenized with five volumes of ice-cold 0.6 M perchloric acid (PCA) with 150 mM EDTA to maximize ATP recovery (Sokolova et al., 2000). An aliquot of the homogenate was reserved for glycogen determination, and the remaining homogenate was centrifuged to remove precipitated protein and neutralized with 5 M potassium hydroxide to pH 7.2–7.5. Precipitated potassium perchlorate was removed by a second centrifugation and extracts were stored at –80 °C. Concentrations of metabolites were measured in neutralized PCA extracts using standard NADH- or NADPH-linked spectrophotometric tests (Bergmeyer, 1985). Briefly, assay conditions were as follows:

ATP: 38.5 mM triethanolamine (TRA) buffer, pH 7.6, 0.04 mM NADP<sup>+</sup>, 7 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 50 mM glucose, 0.462 U mL<sup>-1</sup> glucose-6-phosphate dehydrogenase, 1.8 U mL<sup>-1</sup> hexokinase.

ADP and AMP: 58 mM triethanolamine buffer, pH 7.6, 3 mM phosphoenolpyruvate (PEP), 0.09 mM NADH, 24 U mL<sup>-1</sup> lactate dehydrogenase (LDH), 18 U mL<sup>-1</sup> pyruvate kinase (PK), 16 U mL<sup>-1</sup> myokinase (MK).

D-glucose: 38.5 mM TRA buffer, pH 7.6, 0.04 mM NADP<sup>+</sup>, 7 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.462 U mL<sup>-1</sup> glucose-6-phosphate dehydrogenase, 1.8 U mL<sup>-1</sup> hexokinase.

Glycogen concentration was measured in PCA extracts after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase (Keppler and Decker, 1984) and determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Concentrations of glycogen, lipids and proteins were expressed in mg g<sup>-1</sup> wet tissue mass, and concentrations of adenylates and D-glucose in µmol g<sup>-1</sup> wet tissue mass.

Adenylate energy charge (AEC) was calculated using the formula:

$$\text{AEC} = \frac{[\text{ATP}] + 0.5 \times [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]},$$

where [AMP], [ADP] and [ATP] are concentrations (µmol g<sup>-1</sup> wet weight) of the corresponding compounds.

## 2.8. Statistics

Experimental data sets were tested for the presence of potential outliers using Grubbs' test (extreme studentized deviate, or ESD, method), as implemented in GraphPad Prism ver. 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). A small number of statistically significant outliers was detected and removed from the analysis; the outlier occurrence was random among the treatment groups.

Effects of the factors "Temperature", "P<sub>CO2</sub>", "Time of exposure" and their interactions were assessed for all studied physiological and biochemical traits using a 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<sub>CO2</sub> (22 °C and 27 °C for temperature, and normocapnia and hypercapnia for P<sub>CO2</sub>), and three levels for exposure time (2, 8 and 15 weeks). Post-hoc tests (Fisher's Least Significant Difference) were used to compare the group means; only planned contrasts were used for the post-hoc comparisons. For micromechanical tests, only factors "Temperature" and "P<sub>CO2</sub>" were assessed since all tests were performed with animals exposed for 15 weeks. Cumulative mortalities were compared among treatments using Fisher's exact test. The best fit and significance of linear regressions for the Arrhenius plots of the temperature-dependent CA activity were determined using GraphPad Prism ver. 5.03. E<sub>a</sub> of CA was compared between different tissues and species by comparing the slopes of the respective Arrhenius plots using an algorithm implemented in GraphPad Prism 5.03. Sample sizes for all experimental groups were 5–11 except for oysters after 8–15 weeks of exposure at 27 °C where sample size was reduced due to mortality. Each biological replicate represented a sample from an individual bivalve. Unless otherwise indicated, data are represented as means ± standard errors of means.

Pearson correlation and principal component (PC) analyses were conducted using Origin 8.6 software package (OriginLab, Northampton, MA, USA) on values of all biochemical traits including concentrations of adenylates (ATP, ADP, AMP, total adenylate content) parameters of energy status (ADP/ATP ratio, AEC), tissue levels of glucose, glycogen, lipids and proteins, carbonic anhydrase activities in mantle and gill tissues and mortality. We also included standard metabolic rates (MO<sub>2</sub>) and parameters of tissue oxidative status (total antioxidant capacity, TAOC; and levels of oxidative markers–protein carbonyls, malondialdehyde (MDA)–protein adducts, and 4-hydroxynonenal (HNE)–protein adducts) that were determined in the same experimental animals in our companion study (Matoo et al., 2013). Since all studied parameters cannot be measured in the same individual, we used mean parameter values for the respective treatments and exposure time points. Seawater parameters (pH, P<sub>CO2</sub>, calcite (Ω<sub>CaI</sub>) and aragonite saturation (Ω<sub>Arg</sub>) and temperature), exposure duration and species were included into the correlation and PC analyses as potential explanatory variables.

## 3. Results

### 3.1. Survival

Temperature had a significant effect on survival of clams and oysters (Fig. 1A, B). In normocapnia, mortality was higher at 27 °C compared to 22 °C in both studied species (*p* < 0.05 after 5, 8 and 15 weeks of

exposure). Survival was more strongly compromised by elevated temperatures in oysters compared to clams (Fig. 1A, B). Hypercapnia did not affect survival of clams or oysters at 22 °C ( $P > 0.05$ ), while at 27 °C, hypercapnic exposure improved survival in oysters ( $p = 0.02$ ) but not in clams ( $p > 0.05$ ) (Fig. 1A, B).

### 3.2. Shell mechanical properties

Mechanical properties of the shells differed substantially between the two studied species. Clam shells (aragonite) had considerably higher hardness and fracture resistance than oyster shells (predominately calcite). On average, shell microhardness (measured as Vicker's hardness number) was  $219.8 \pm 2.2$  in oysters and  $331.2 \pm 1.8$  in clams (Fig. 1C, D). Crack radius formed at 0.245 N load and 5 s dwelling time was  $>50\%$  larger in oysters compared to clams ( $15.1 \pm 0.63 \mu\text{m}$  vs.  $9.27 \pm 0.30 \mu\text{m}$ , respectively). After 15 weeks exposure, fracture resistance (estimated by the crack radius) was not significantly affected by exposure to different  $\text{CO}_2$  and temperature levels in clams or oysters (Supplement Table 1). In contrast, there was a significant decrease in the shell microhardness in clams and oysters exposed to hypercapnia at 27 °C (Fig. 1C and D). Microhardness of clams exposed to normocapnia at 22 °C was  $334.2 \pm 3.6$  versus  $323.2 \pm 2.9$  in animals exposed to hypercapnia at 27 °C ( $p = 0.025$ ). For oysters, microhardness of animals exposed to normocapnia at 22 °C was  $225.4 \pm 2.9$  versus  $212.1 \pm 2.1$  in those exposed to hypercapnia at 27 °C ( $p = 0.018$ ).

### 3.3. Carbonic anhydrase (CA) activity

In clams, the apparent  $E_a$  of CA from all studied tissues was  $40\text{--}43 \text{ kJ mol}^{-1} \text{ K}^{-1}$  indicating the temperature dependence typical for metabolic enzymes of ectotherms, and no Arrhenius breakpoint temperature, indicative of a significant change in  $E_a$ , was detected in the range of 5–35 °C (Table 2). In oysters, the apparent  $E_a$  of CA was

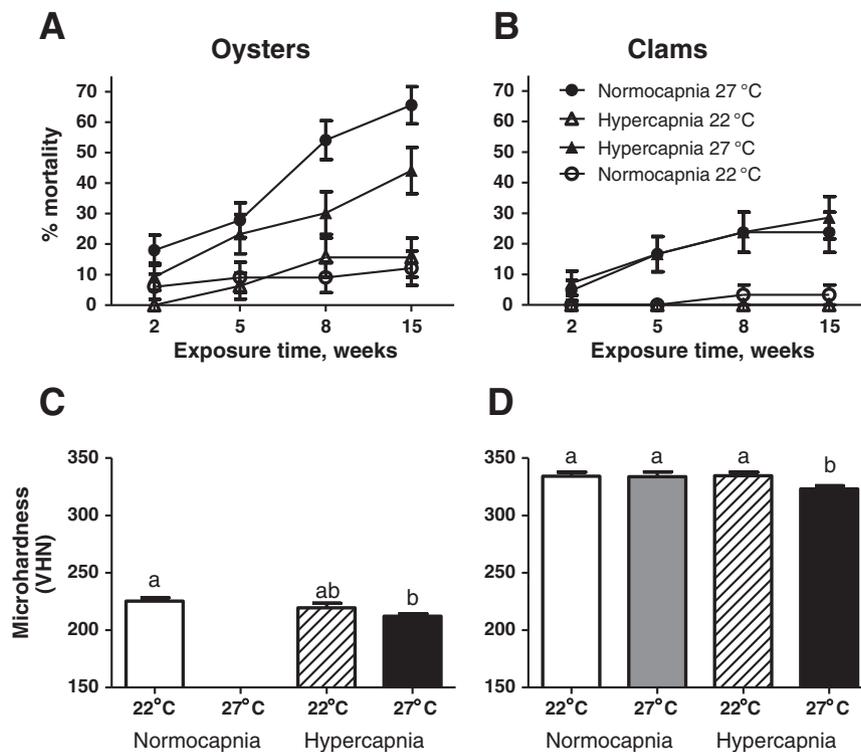
significantly lower than in clams in all studied tissues except hepatopancreas, and CA activity from the oyster gills and adductor muscles was essentially temperature-independent (Table 2).

Long-term acclimation to different temperatures exerted a strong effect on CA activity in mantle and gill tissues of the studied bivalves leading to a notable increase in the specific CA activity at 27 °C (Fig. 2). This effect was observed in gills and mantle tissues of both species but was more pronounced in clams than in oysters (Fig. 2). Notably, in oysters there was a slight decrease in CA activity after 8 and 15 weeks of exposure under the control conditions (22 °C, normocapnia) but in clams CA activity was stable throughout to the control exposures (Fig. 2). Elevated  $\text{CO}_2$  levels alone had little or no effect on the specific CA activity in gills and mantle of the studied bivalves (Fig. 2; Supplemental Table 1).

### 3.4. Tissue composition and energy reserves

In oysters, glycogen levels in the mantle tissues were maintained during acclimation under the control conditions (Fig. 3A). Hypercapnia at 22 °C resulted in a significant increase in glycogen levels after 2 weeks of exposure followed by a gradual decline after 8 and 15 weeks (Fig. 3A). Acclimation at 27 °C led to a significant reduction of the tissue glycogen content of oysters, and this reduction was especially pronounced when elevated temperature was combined with hypercapnia (Fig. 3A). In clams, glycogen content was maintained at the normal levels regardless of the exposure temperature and  $\text{P}_{\text{CO}_2}$ , except for a slight but significant transient increase after 8 weeks of exposure at 22 °C in both normo- and hypercapnia (Fig. 3B).

Tissue glucose levels varied over time and in response to elevated temperature in the two studied species (Fig. 3C, D). In clams, significantly elevated tissue glucose levels were found after 8 and 15 weeks of exposure to elevated temperature (27 °C) at both normo- and hypercapnia indicating enhanced glycolysis (Fig. 3D). A similar but less pronounced increase was found after 8 weeks of exposure to 22 °C at normo- and



**Fig. 1.** Mortality and mechanical characteristics of the growing edge of oyster (*C. virginica*) and clam (*M. mercenaria*) shells after 15 weeks of exposure to different temperature and  $\text{CO}_2$  levels. A, B – Mortality of oysters and clams, respectively. C, D – microhardness of the growing edge of shell expressed as Vickers microhardness number (VHM) in oysters and clams, respectively. Different letters in C and D denote significantly different values ( $p < 0.05$ ) while the columns that share the same letter are not significantly different ( $p > 0.05$ ).  $N = 10 - 11$  for mortality and 5–11 for microhardness. Testing of microhardness on shells of oysters exposed to normocapnia at 27 °C could not be conducted due to high mortality in this treatment.

**Table 2**

Activity, activation energy and Arrhenius breakpoint temperature (ABT) for carbonic anhydrase (CA) in different tissues of clams and oysters.  $E_a$  values highlighted in bold and marked with an asterisk are significant after the sequential Bonferroni corrections ( $p < 0.05$ ).  $Q_{10}$  temperature coefficients were calculated for the complete range of the studied temperatures (5–35 °C). Specific enzyme activities measured at 22 and 27 °C are shown for the enzymes isolated from clams and oysters acclimated at 20 °C and subjected to temperature rise *in vitro*; this was done to enable comparison of the acute response of CA to the temperature rise with the effects of the long-term acclimation to the respective temperatures (shown in Fig. 2). Different letters denote the specific activities and/or apparent  $E_a$  values that are significantly different between different tissues of the same species ( $p < 0.05$ ). Comparisons of  $E_a$  between species for the respective tissues are given in the lower portion of the table; “=” sign indicates that the respective  $E_a$  values are not significantly different ( $p > 0.05$ ), “>” or “<” signs indicate significant differences ( $p < 0.05$ ). Cv – *C. virginica*, Mm – *M. mercenaria*.

Tissue	Carbonic anhydrase					
	ABT, °C	$E_a$ , kJ mol <sup>-1</sup> K <sup>-1</sup>	$Q_{10}$	Specific activity, U g <sup>-1</sup> protein		
				22 °C	27 °C	
	Below ABT	Above ABT				
<i>C. virginica</i> (eastern oysters)						
Mantle	N/a	<b>18.46</b> * <sup>a</sup>	–	1.32	10.05 ± 2.30 <sup>a</sup>	11.64 ± 0.85 <sup>a</sup>
Gills	17.0	25.77 <sup>b</sup>	–8.73	1.01	15.15 ± 3.37 <sup>a</sup>	12.67 ± 1.80 <sup>a</sup>
Hepatopancreas	N/a	<b>30.84</b> * <sup>b</sup>	–	1.62	49.53 ± 6.45 <sup>b</sup>	54.67 ± 10.28 <sup>b</sup>
Muscle	23.5	–13.96 <sup>a</sup>	27.60	1.00	4.58 ± 1.34 <sup>a</sup>	4.90 ± 0.93 <sup>a</sup>
<i>M. mercenaria</i> (hard shell clam)						
Mantle	N/a	<b>42.31</b> * <sup>a</sup>	–	1.78	18.92 ± 1.54 <sup>a</sup>	24.43 ± 2.22 <sup>a</sup>
Gills	N/a	<b>43.06</b> * <sup>a</sup>	–	1.69	15.93 ± 0.65 <sup>a</sup>	19.13 ± 1.09 <sup>a</sup>
Hepatopancreas	N/a	<b>39.65</b> * <sup>a</sup>	–	1.69	42.54 ± 3.99 <sup>b</sup>	49.80 ± 5.54 <sup>b</sup>
Muscle	N/a	<b>43.48</b> * <sup>a</sup>	–	1.78	14.37 ± 1.06 <sup>a</sup>	17.92 ± 1.27 <sup>a</sup>
Between-species comparisons of $E_a$			Between-species comparisons of CA activity			
Mantle	Cv < Mm ( $p < 0.05$ )			$p = 0.088$	<b><math>p = 0.015</math></b>	
Gills	Cv = Mm			$p = 0.880$	$p = 0.213$	
Hepatopancreas	Cv = Mm			$p = 0.179$	$p = 0.348$	
Muscle	Cv < Mm ( $p < 0.05$ )			$p = 0.060$	<b><math>p = 0.013</math></b>	

hypercapnia in clams and oysters but it disappeared after 15 weeks (Fig. 3C, D).

The total lipid content of mantle tissue was not affected by acclimation temperature or  $P_{CO_2}$  in oysters (Fig. 3E). The lipid content in oyster mantle was reduced after 15 weeks of exposure in all treatments (no data available for normocapnia at 27 °C) (Fig. 3E). In clams, tissue lipid levels were elevated after 15 weeks at 27 °C and hypercapnia (Fig. 3F). At other exposure times, no significant effects of temperature or  $P_{CO_2}$  on the lipid content of clam mantle was observed (Fig. 3F).

The protein content of mantle tissues was not significantly affected by acclimation time, temperature or  $P_{CO_2}$  in oysters except for a significant increase after 15 weeks at 22 °C and hypercapnia (Fig. 3G). No significant effects of temperature or  $P_{CO_2}$  on the protein content were found in clam tissues (Fig. 3H) but protein content of mantle tissues slightly decreased over time in all experimental treatments except hypercapnia at 27 °C (Fig. 3H).

### 3.5. Cellular energy status

In oyster tissues, ATP content was stable over time and among different treatments, except for a significant decline after 8 and 15 weeks of exposure to hypercapnia at 22 °C (Fig. 4A). A transient decrease in ADP and increase in AMP levels were observed in oyster tissues after 8 weeks of exposure in all treatments except elevated temperature and  $P_{CO_2}$  (Fig. 4C & E). In the latter treatment, ADP and AMP concentrations declined over time during the 15 weeks exposure period (Fig. 4C). A similar decline in AMP levels over time was also observed in oysters exposed to elevated  $P_{CO_2}$  at 22 °C (Fig. 4E).

In clams, tissue ATP levels declined over time in the control exposure but increased in both high temperature treatments; however, the variations in ATP levels among treatments and experimental exposure times were modest (Fig. 4B). In contrast, tissue concentrations of ADP and AMP remained stable over time and among the experimental treatments except a moderate increase of ADP levels after 15 weeks of exposure at 27 °C and normocapnia and a transient increase in AMP concentrations after 8 weeks at 22 °C and elevated  $P_{CO_2}$  (Fig. 4D and F).

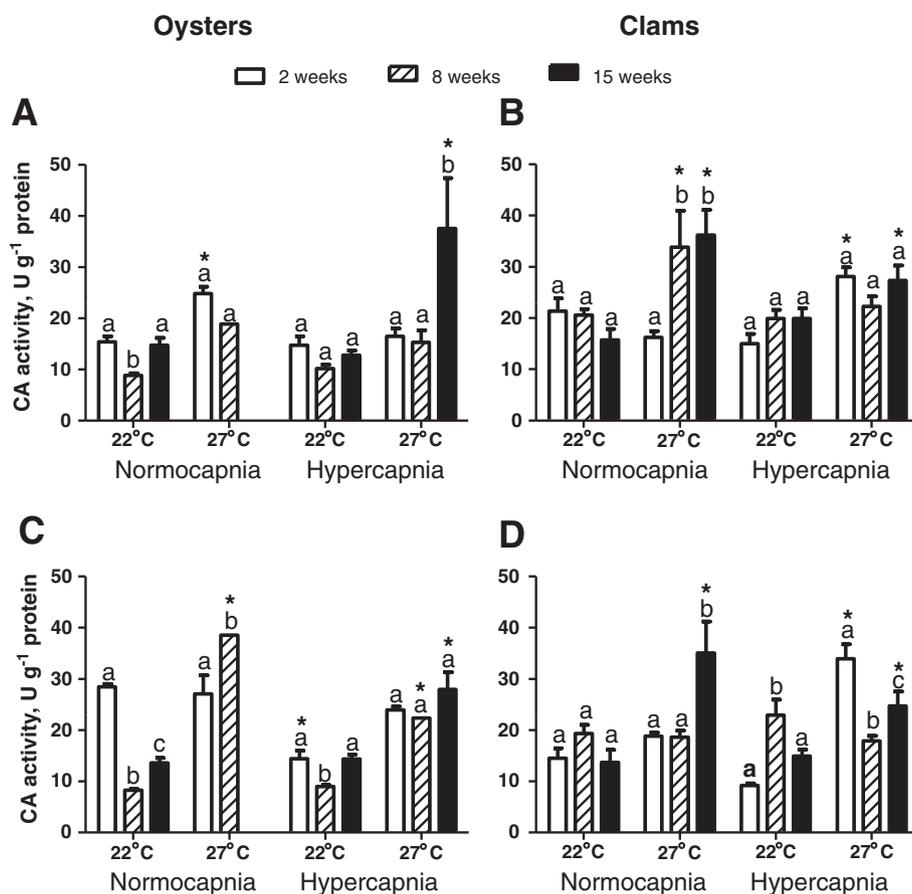
Adenylate energy charge (AEC) decreased after 2 weeks of exposure to elevated temperature and  $P_{CO_2}$  (both in clams and oysters) and after 2 weeks of exposure to normocapnia at 27 °C (in clams only) (Fig. 4G,H). At later time points AEC returned to the control levels in both species and all experimental treatments (Fig. 4G, H).

### 3.6. Correlation and principal component (PCA) analyses

As expected, different parameters of seawater chemistry (pH,  $P_{CO_2}$ ,  $\Omega_{Ca}$  and  $\Omega_{Arg}$ ) were significantly and strongly correlated with each other (Pearson correlation coefficient >0.9; Supplementary Table 2). Therefore, we used  $P_{CO_2}$  as a potential explanatory variable and a proxy for seawater chemistry in all further analyses. Based on the correlation analysis, species-specific differences were largely responsible for variations in mortality, tissue levels of proteins, adenylates (including ATP, ADP, AMP and total adenylate content) as well as the standard metabolic rate and tissue levels of MDA-protein conjugates (Supplementary Table 2). Notably, tissue levels of ATP were also strongly and positively associated with ADP and AMP content as well as the standard metabolic rate and MDA levels. Exposure time did not significantly correlate with any of the studied biological traits, while exposure temperature correlated with mortality, CA activity in gill and mantle tissues and tissue glycogen content (Supplementary Table 2). Exposure  $P_{CO_2}$  significantly and negatively correlated with AEC.

PCA including all studied physiological and biochemical traits as well as  $P_{CO_2}$ , temperature, exposure duration and species as potential explanatory variables, identified five principal components explaining 76% of the total variation of the studied data set (Supplementary Table 3). Tissue levels of adenylates (except AMP), protein content, overall metabolic rate ( $MO_2$ ) and MDA levels were associated with the 1st principal component (24% of variance) which reflects species-specific differences between clams and oysters (Supplementary Table 3). The 2nd and 3rd principal components (19 and 14% of variance, respectively) were associated with exposure temperature and had high loadings of the majority of the studied biological traits including CA activity in gills and mantle tissues, tissue levels of glucose, glycogen, ATP, AMP, total adenylates, AEC and ADP/ATP ratio, lipid content, as well as mortality, TAOC and tissue levels of HNE-protein conjugates. The 4th principal component (10% of variance) was correlated with  $P_{CO_2}$  and exposure time and linked to the parameters of cellular energy status (levels of AMP, ADP/ATP ratio and AEC). Lastly, the 5th principal component (9% of variation) was linked to the exposure duration and correlated with mortality, tissue content of lipids and protein carbonyls, and the parameters of cellular energy status (levels of AMP, ADP, as well as ADP/ATP ratio and AEC) (Supplementary Table 3).

Pearson correlation analysis conducted separately for each species revealed tight correlations between tissue levels of adenylates and energy-related indices (ADP/ATP ratio and AEC) in both studied species (Supplementary Table 4). Acclimation temperature significantly and positively correlated with CA activity in mantle and gill tissues of both clams and oysters, as well as with SMR of clams and mortality of oysters. In both species, oxidative stress biomarkers (tissue levels of carbonyls, MDA- and/or HNE-protein conjugates) were positively associated with the parameters of carbohydrate metabolism (glucose and glycogen content). Exposure  $P_{CO_2}$  was not strongly associated with any of the studied parameters except TAOC levels in clams



**Fig. 2.** Carbonic anhydrase (CA) activity in mantle edge and gill tissues of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO<sub>2</sub> levels. A, B – CA in mantle edge, C, D – CA in gills, A, C – *C. virginica*, B, D – *M. mercenaria*. X-axis – experimental conditions (temperature and CO<sub>2</sub> levels). CA activities were measured at the respective acclimation temperatures. Different letters indicate exposure times that are different within each experimental treatment group ( $p < 0.05$ ). Asterisk indicate 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  $N = 1–4$ .

where a significant positive correlation with  $P_{CO_2}$  levels was observed (Supplementary Table 4).

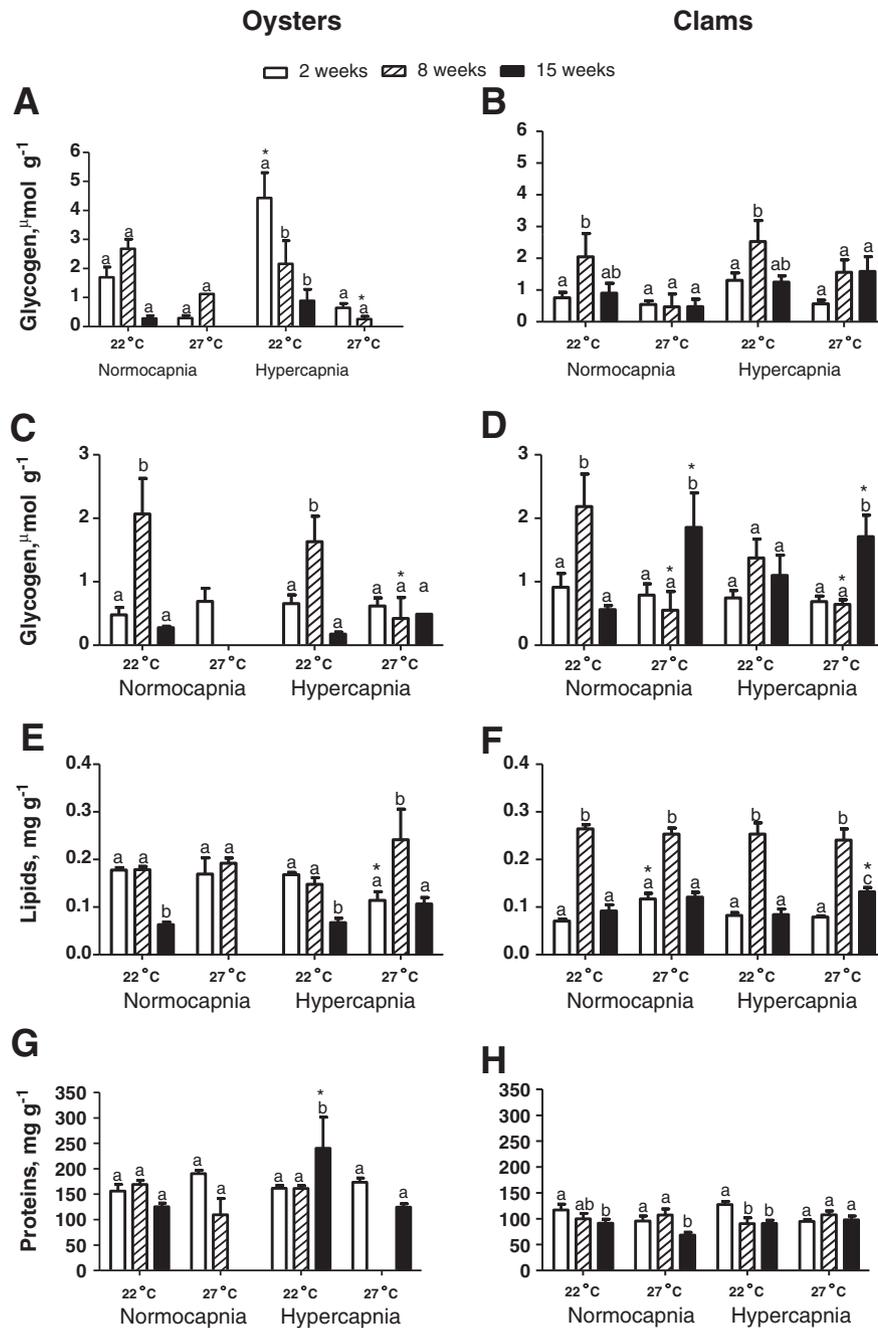
#### 4. Discussion

##### 4.1. Bioenergetic responses to temperature and $P_{CO_2}$

The effects of elevated temperature and  $P_{CO_2}$  on survival and energy metabolism of the studied bivalves were species-specific indicating different sensitivity of clams and oysters to these factors. Oysters were especially susceptible to elevated temperatures showing high mortality after prolonged exposure at 27 °C. This is consistent with earlier findings that oysters have no “scope for growth” and do not deposit shell material at and above 28 °C, and cannot withstand exposures to sublethal levels of pollutants such as cadmium at this temperature (Surge et al., 2001; Lannig et al., 2006). These data suggest that chronic warming such as expected in the case of global climate change may be deleterious for oyster populations, especially in the low-latitude parts of their distribution range. Of the two studied factors, temperature exerted much stronger effects on metabolism and survival of clams and oysters than  $P_{CO_2}$ . This is consistent with the results of earlier studies that showed the predominant effects of temperature on metabolic physiology and survival of marine invertebrates in different temperature/ $P_{CO_2}$  combinations within the environmentally relevant range (Lannig et al., 2010; Chapman et al., 2011; McElroy et al., 2012). While the effects of moderate hypercapnia on energy metabolism are usually less pronounced,  $P_{CO_2}$  can modulate metabolic responses to elevated temperature in bivalves and other aquatic ectotherms (Lannig et al., 2010;

Wood et al., 2011; Catarino et al., 2012; McElroy et al., 2012; present study).

Earlier studies showed that oysters (*C. virginica*) and clams (*M. mercenaria*) strongly differ in the rates as well as the temperature sensitivity of basal metabolism (Shumway and Koehn, 1982; Kraeuter and Castagna, 2001; Matoo et al., 2013 and references therein). Standard metabolic rates (SMR) of adult oysters are, on average, 2–4 times higher than in adult clams and do not change with acclimation to elevated temperature (27 °C) while in clams a significant temperature-induced increase in SMR is observed during long-term acclimation to 27 °C (Matoo et al., 2013). The higher SMR of oysters (as compared to clams) was associated with elevated steady-state levels of ATP and ADP (but not AMP) and higher total adenylate content in the former species. This indicates differences in the equilibrium constants of arginine kinase and/or adenylate kinase between these two species (Hardie and Hawley, 2001; Igamberdiev and Kleczkowski, 2006) and suggests that the overall metabolic activity may play a role in determining the set-points for cellular adenylate homeostasis of bivalves. As expected, tissue levels of different adenylates are tightly linked within each of the two studied species (Supplementary Fig. 1) reflecting physiological and biochemical coordination of adenylate metabolism. Despite the higher ATP and ADP levels as well as total adenylate content in oysters, the adenylate energy charge (AEC) was similar in the two studied bivalve species (~0.6) and within the range of the values reported for bivalves (Wijsman, 1976; Rainer et al., 1979; Barthel, 1984; Isani et al., 1997; Ivanina et al., 2011). AEC was not significantly affected by elevated temperature and  $P_{CO_2}$  in clams or in oysters (except for a modest and transient decrease after two weeks of exposure),

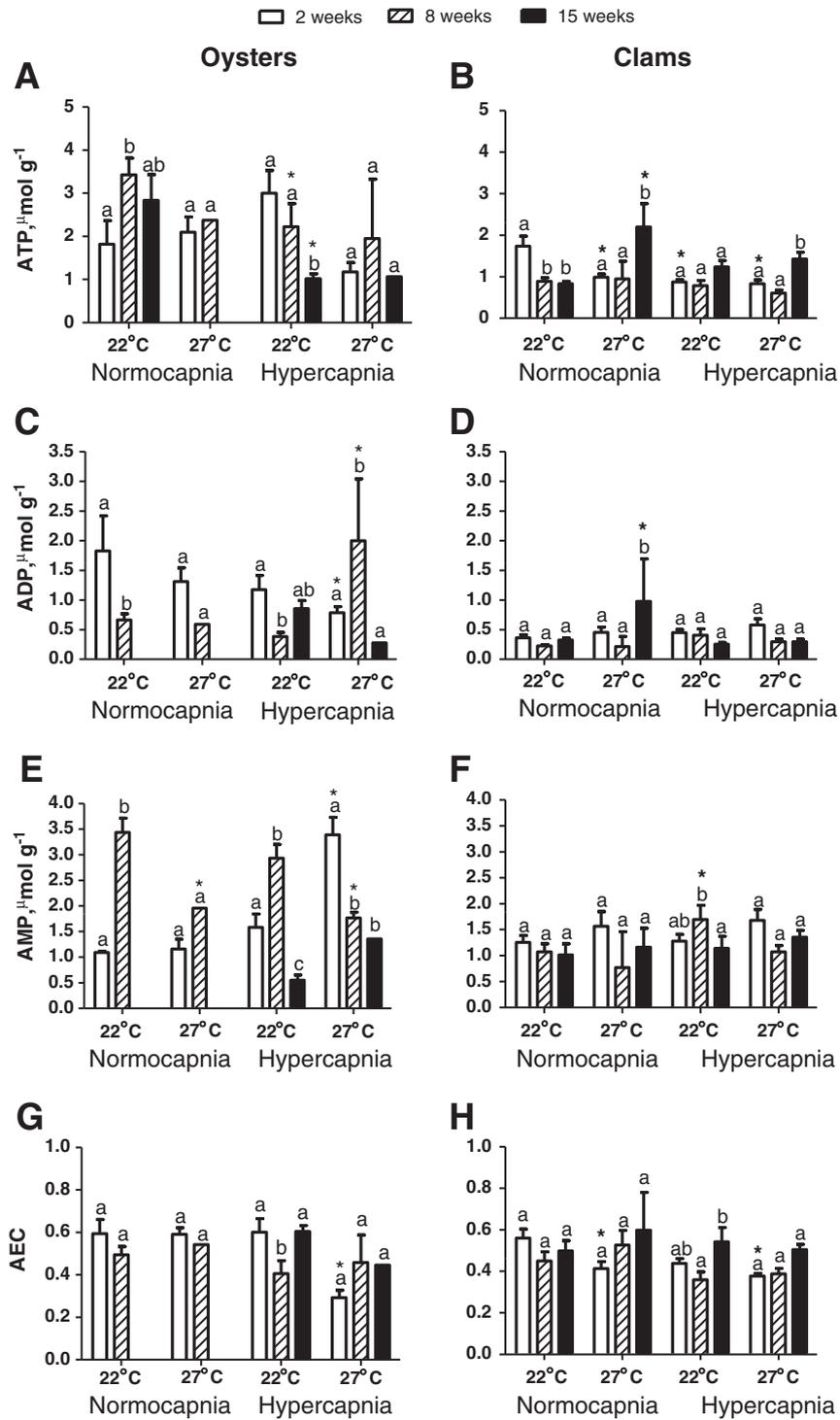


**Fig. 3.** Tissues concentration of major energy reserves in oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO<sub>2</sub> levels. A,B – glycogen, C,D – glucose, E,F – lipids, G,H – proteins. A,C,E,G – *C. virginica*, B,D,F,H – *M. mercenaria*. X-axis – experimental conditions (temperature and CO<sub>2</sub> levels). Different letters indicate exposure times that are different within each experimental treatment group ( $p < 0.05$ ). Asterisk indicate 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  $N = 1–4$ .

suggesting that it may not be a sensitive index to detect bioenergetic shifts during chronic stress exposures in these species.

Temperature- and P<sub>CO<sub>2</sub></sub>-induced changes in bioenergetics were considerably more pronounced in oysters than in clams. In oysters, long-term exposure to elevated temperature (27 °C) resulted in the depletion of glycogen reserves indicating energy deficiency that goes hand-in-hand with elevated mortality. Hypercapnia partially alleviated the negative effects of elevated temperature on mortality but not on the glycogen depletion in oysters. Notably, exposure of oysters to hypercapnia at 22 °C resulted in an initial increase of the tissue glycogen content after two weeks of exposure followed by a

gradual decline, so that after 15 weeks the tissue glycogen content in this group reached the control levels. This early hypercapnia-induced rise in glycogen content could have gone undetected at elevated temperatures due to the opposing effects of high temperature and P<sub>CO<sub>2</sub></sub> on glycogen stores but may have contributed to the better survival of hypercapnia-exposed oysters at 27 °C. In fish (the rainbow trout *Salmo gairdneri*) and mammals (the brown rat *Rattus norvegicus*), external hypercapnia induced a rapid depletion of glycogen in the liver and the cerebral cortex, respectively; however, P<sub>CO<sub>2</sub></sub> levels used in these studies were selected to mimic the respiratory rather than environmental hypercapnia and were much higher than in the present



**Fig. 4.** Tissues concentration of adenylates in oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO<sub>2</sub> levels. A, B – ATP, C, D – ADP, E, F – AMP, G, H – adenylate energy charge (AEC). A, C, E, G – *C. virginica*, B, D, F, H – *M. mercenaria*. X-axis – experimental conditions (temperature and CO<sub>2</sub> levels). Different letters indicate exposure times that are different within each experimental treatment group ( $p < 0.05$ ). Asterisk indicate 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  $N = 1-4$ .

study (~10,000 and 100,000  $\mu\text{atm P}_{\text{CO}_2}$  for fish and rats, respectively) (Folbergrová et al., 1975; Mommsen et al., 1988; Perry et al., 1988). The effects of environmental hypercapnia on glycogen turnover in invertebrates have not been studied, and so the mechanisms of the hypercapnia-induced accumulation of glycogen in oysters remain presently unknown. Rapid changes in the glycogen content over time and in response to elevated temperature and  $P_{\text{CO}_2}$  in oysters (as opposed to

much more stable levels of lipid and protein content) are in agreement with the role of glycogen as a rapidly mobilizable fuel that can be used to buffer excessive energy demand and is quickly replenished when energy assimilation exceeds consumption (Kooijman, 2010; Sokolova et al., 2011).

In the less temperature-sensitive of the two studied species, *M. mercenaria*, the levels of tissue energy reserves (including glycogen,

lipids and proteins) were not affected by temperature and  $P_{CO_2}$  exposure, and a transient decline in ATP level after 2 weeks of exposure to elevated temperature and/or hypercapnia was restored or overcompensated after 8–15 weeks. In contrast to the present study that used adult clams and oysters, an earlier study in juvenile clams showed that moderate hypercapnia (~800  $\mu\text{atm}$ ) results in elevated mortality and a significant depletion of glycogen and lipid reserves after 11 weeks of exposure at the control temperature (22 °C) (Dickinson et al., 2012). Similarly, juvenile oysters were more sensitive to extreme hypercapnia (~3500  $\mu\text{atm}$ ) than adults as shown by a strong increase in standard metabolic rates and elevated mortality in hypercapnia-exposed juveniles (Beniash et al., 2010). This is in agreement with the generally higher sensitivity of early life stages of marine invertebrates to acidification and temperature stress (Kurihara, 2008; Dupont and Thorndyke, 2009; Ellis et al., 2009; Gazeau et al., 2010; Suwa et al., 2010; Zippay and Hofmann, 2010) which may reflect a priority of energy demands for growth and development over the basal maintenance during the early life stages (Sokolova et al., 2012).

#### 4.2. Temperature modulates the effects of $P_{CO_2}$ on biomineralization

Mechanical testing of the shells showed that aragonitic shells of *M. mercenaria* are considerably stronger (in terms of both hardness and fracture resistance) than calcitic oyster shells. Such mechanical differences in bivalves that produce different polymorphs of  $\text{CaCO}_3$  have been well established (Currey and Taylor, 1974; Currey, 1976) and reflect differences in the mechanical properties of calcite and aragonite and the nano- and microstructural organization of the shells. Moderate hypercapnia alone (~800  $\mu\text{atm}$ ) did not significantly affect shell mechanical properties (microhardness and fracture toughness) or carbonic anhydrase (CA) activity in the mantle tissues of the two studied bivalve species acclimated under the control temperature conditions (22 °C). In contrast, hypercapnia combined with the elevated temperature (27 °C) led to an increase in CA activity in the mantle tissues of clams and oysters. While in clams this increase may reflect the direct rate-enhancing ( $Q_{10}$ ) effects of the temperature, this is unlikely to be the case in oysters because of the low thermal sensitivity of CA enzyme from the mantle tissue (indicated by low  $E_a$ , Table 2) and the absence of a comparable increase in CA activity in the mantle tissues of oysters acclimated to 27 °C in normocapnia. The increase in CA activity in the mantle may indicate a compensatory response to increase the driving force towards calcium carbonate deposition and/or facilitate the gas exchange and  $\text{CO}_2$  release at the mantle surface in the bivalves exposed to the combination of elevated temperature and  $P_{CO_2}$ .

The temperature-augmented increase in CA activity, however, appears insufficient to fully prevent disturbance of biomineralization processes in the studied bivalves as indicated by the reduced hardness of the shells of clams and oysters exposed to hypercapnia at the elevated temperature. It is worth noting that the impact of elevated temperature and  $P_{CO_2}$  on the mechanical properties of the bivalve shells is not likely due to simple chemically induced shell dissolution because  $\Omega_{\text{Arg}}$  and  $\Omega_{\text{Cal}}$  remained well above saturation in all experimental treatments and were in fact higher in the hypercapnic treatments at 27 °C than at 22 °C (Table 1). It is therefore likely that hypercapnia, under our experimental conditions, influences the biological processes underlying shell deposition rather than physical chemistry of mineral precipitation, although at this point the exact mechanism remain elusive. Notably, synergistic negative effects of elevated temperature and hypercapnia on biomineralization were also described in a brittlestar *Ophiopecten sericeum*, where combined exposure to hypercapnia and elevated  $\text{CO}_2$  levels reduced the rates of arm regeneration (Wood et al., 2011).

In summary, elevated temperature (such as can be expected in the case of the global climate change) negatively affects bioenergetics of intertidal bivalves such as clams and oysters leading to energy

deficiency that can have an impact on their survival, growth, reproduction, immunity and other fitness-related functions. Compared to the dominant effects of temperature, metabolic consequences of moderately elevated  $P_{CO_2}$  appear mild and in some cases can slightly improve survival of temperature-stressed animals as was found in oysters exposed to elevated temperature and hypercapnia. On the other hand, the combination of elevated temperature and  $P_{CO_2}$  had an overall negative effect on biomineralization leading to a decrease in shell mechanical properties, suggesting a synergistic relationship between these environmental factors. These changes may have important implications for survival and performance of the populations of clams and oysters in the face of the global climate change especially under the conditions when the ambient temperatures are at the species-specific thermal tolerance limits (e.g. during summer heat waves and/or in the southernmost populations of the studied bivalves).

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpa.2013.05.016>.

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