**INTERACTIVE EFFECTS OF COPPER EXPOSURE AND ENVIRONMENTAL HYPERCAPNIA ON IMMUNE
FUNCTIONS OF MARINE BIVALVES *CRASSOSTREA VIRGINICA* AND *MERCENARIA MERCENARIA***

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Abstract. Estuarine organisms such as bivalves are commonly exposed to trace metals such as copper (Cu) and hypercapnia (elevated CO₂ levels) in their habitats, which may affect their physiology and immune function. This study investigated the combined effects of elevated CO₂ levels (~800-2000 µatm P_{CO2}, such as predicted by the near-future scenarios of global climate change) and Cu (50 µg l⁻¹) on immune functions of the sediment dwelling hard clams *Mercenaria mercenaria* and an epifaunal bivalve, the eastern oyster *Crassostrea virginica*. Clams and oysters were exposed for 4 weeks to different CO₂ and Cu levels, and tissue Cu burdens and immune parameters were assessed to test the hypothesis that hypercapnia will enhance Cu uptake due to the higher bioavailability of free Cu²⁺ and increase the immunomodulatory effects of Cu in marine bivalves. Exposure to Cu stimulated key immune parameters of clams and oysters leading to increased number of circulating hemocytes, higher phagocytosis and adhesion ability of hemocytes, as well as enhanced antiparasitic and antibacterial properties of the hemolymph reflected in higher activities of lysozyme and inhibitors of cysteine proteases. Lysozyme activation by Cu exposure was most prominent in normocapnia (~400 µatm P_{CO2}) and an increase in the levels of the protease inhibitors was strongest in hypercapnia (~800-2000 µatm P_{CO2}), but other immunostimulatory effects of Cu were evident in all P_{CO2} exposures. Metabolic activity of hemocytes of clams and oysters (measured as routine and mitochondrial oxygen consumption rates) was suppressed by Cu exposure likely reflecting lower rates of ATP synthesis and/or turnover. However, this metabolic suppression had no negative effects of the studied immune functions of hemocytes such as phagocytosis or adhesion capacity. Hypercapnia (~800-2000 µatm P_{CO2}) slightly but significantly enhanced accumulation of Cu in hemocytes, consistent with higher Cu²⁺ bioavailability in CO₂-acidified water, but had little effect on cellular and humoral immune traits of clams and oysters. These findings indicate that low levels of Cu contamination may enhance immunity of estuarine bivalves while moderate hypercapnia (such as predicted by the near future scenarios of the global climate change) does not strongly affect their immune parameters.

1. INTRODUCTION

Estuaries and coastal zones, the most productive areas of the ocean, experience high levels of abiotic stress from natural fluctuations in environmental conditions (such as temperature, salinity or pH), anthropogenic pollution, and biotic stress due to constant exposure to parasites and pathogens. Robust immunoprotection plays an important role in survival and health of estuarine organisms. Marine invertebrates including bivalves lack adaptive immunity and depend on the innate immune response to combat pathogens and parasites. Innate immunity in marine bivalves encompasses cell-mediated and humoral systems that operate in a coordinated way. Cellular responses are carried out by circulating hemocytes that can kill or isolate infectious agents through phagocytosis, encapsulation and various cytotoxic reactions (Allam *et al.*, 2001; Canesi *et al.*, 2002; Carla Pruzzo, 2005). Humoral factors released by hemocytes (including soluble lectins, lysozyme, protease inhibitors and antimicrobial peptides) play a key role in pathogen and non-self recognition, neutralization of the virulence factors (such as proteases) and aid in destruction of the pathogens and the parasites (Roch, 1999b; Yakovleva *et al.*, 2001; Armstrong, 2006). Coordinated cellular and humoral immune responses represent the critical first line of internal defense against pathogens and parasites in mollusks, and disturbance of this finely tuned system may affect survival and disease susceptibility of bivalves under stressful conditions (Roch, 1999b; a).

Environmental stressors, including trace metals, elevated temperature and reduced pH can modulate immune function in marine bivalves (Pipe and Coles, 1995; Wootton *et al.*, 2003; Giron-Perez, 2010; Dupont and Thorndyke, 2012; Matozzo *et al.*, 2012; Ivanina *et al.*, 2014). Many trace metals such as cadmium (Cd) and mercury (Hg) are known as potent immunosuppressors of bivalves (Troussett and White, 1990; Tsangaris and Tzortzatou-Stathopoulou, 1998; Auffret *et al.*, 2002; Hemdan *et al.*, 2006; Paul-Pont *et al.*, 2010; Chandurvelan *et al.*, 2013; Ivanina *et al.*, 2014) while the immune effects of Cu appear more variable as both inhibition and stimulation of the immune function by Cu have been reported (Stabel and Spears, 1989; Pipe *et al.*, 1999; Parry and Pipe, 2004; Jing *et al.*, 2007; Foster *et al.*, 2011; Silva-Aciaries *et al.*, 2013). Cu is a common environmental pollutant in estuaries, with dissolved Cu levels ranging from $\sim 0.25 \mu\text{g l}^{-1}$ in pristine areas to 250-1000 $\mu\text{g l}^{-1}$

l⁻¹ in polluted environments, and reaching up to 100 mg kg⁻¹ in the sediments (Hackney *et al.*, 1998; Blossom, 2015). Cu is an essential trace metal acting as a cofactor in many enzymes, most notably cytochrome c oxidase and Cu, Zn-superoxide dismutase which play a key role in aerobic metabolism and antioxidant protection. However, Cu is toxic at high concentrations, mostly due to strong pro-oxidant properties of this metal (Valko *et al.*, 2005). Moderately elevated levels of Cu can thus potentially aid in immune defense stimulating metabolism, increasing production of reactive oxygen species to kill pathogens and/or exerting direct toxic effects on the pathogens in molluscan blood. On the other hand, excessive levels of Cu can be cytotoxic to hemocytes negatively affecting their viability, secretion of the protective humoral factors and ability to execute phagocytosis. Thus, the immune defenses of bivalves exposed to elevated Cu levels in contaminated estuaries will depend on the balance between the stimulatory and inhibitory effects of Cu reflecting the accumulated Cu levels in the blood and hemocytes of the mollusks.

Biological accumulation of trace metals including Cu depends on bioavailability of the metal, metal uptake and excretion rates and extra- and intracellular sequestration of the metal (Hamelink, 1992; Rainbow, 2007). Bioavailability of Cu is sensitive to changes in the seawater chemistry caused by elevated levels of carbon dioxide (CO₂) and resulting in ocean acidification (Millero *et al.*, 2009). Elevated CO₂ levels (hypercapnia) commonly occur in estuaries due to the local factors (such as high respiratory CO₂ production by the resident biota coupled with the limited gas exchange between the seawater and the atmosphere) and globally due to the anthropogenic release of into the atmosphere leading to reduced seawater pH and lower concentrations of the carbonate (CO₃²⁻) ions (Millero *et al.*, 2009; Ivanina and Sokolova, 2015). Hypercapnia can increase bioavailability of Cu due to the higher solubility of Cu²⁺ in acidified seawater, mobilization of Cu²⁺ from rocks and sediments and an increase in the proportion of the most bioavailable free Cu²⁺ ion due to dissociation of Cu-carbonate complexes (Millero *et al.*, 2009). This, in turn, may affect uptake and accumulation of Cu by marine organisms and modulate the physiological and immune effects of this metal.

Hard clams *Mercenaria mercenaria* and eastern oysters *Crassostrea virginica* are useful models to study the interactive effects of Cu and hypercapnia on immunity of marine

bivalves. These bivalves are common in western Atlantic estuaries and serve as ecosystem engineers in their habitats. Clams and oysters are exposed to elevated levels of Cu in seawater and sediments, as well as to elevated CO₂ levels during seasonal and diurnal hypercapnia and anthropogenic ocean acidification (Chapman *et al.*, 2011; Hofmann *et al.*, 2011; Wallace *et al.*, 2014). Hard clams are an infaunal species often dwelling in metal-enriched sediments and chronically exposed to elevated levels of respiratory CO₂ due to the slow gas exchange between sediment and the overlaying water (Kraeuter and Castagna, 2001; Green *et al.*, 2009). Eastern oysters, as an epifaunal species, are also often exposed to hypercapnia and metal contamination in the near-bottom estuarine waters (Kennedy *et al.*, 1996; Chapman *et al.*, 2011) but typically experience lower metal and CO₂ levels than clams inhabiting the sediments. These ecological differences between clams and oysters may affect their susceptibility to the negative effects of Cu contamination and hypercapnia reflecting adaptations to different levels of metals and CO₂ in their environment.

The aim of this study was to investigate the combined effects of elevated CO₂ levels (~800-2000 µatm P_{CO2}) and Cu (50 µg l⁻¹) on immune functions and responses of *M. mercenaria* and *C. virginica*. We hypothesized that immune responses of clams will be more resistant to the negative effects of elevated Cu levels and hypercapnia than in oysters reflecting adaptation of clams to elevated metals and CO₂ levels in their habitats. We also anticipated that hypercapnia will enhance Cu uptake in hemolymph and hemocytes of clams and oysters due to the higher bioavailability of free Cu²⁺ in CO₂-enriched seawater. Enhanced Cu accumulation is expected to increase the immunomodulatory effects of Cu potentially shifting these effects from stimulation to inhibition if accumulated Cu levels cross the threshold from physiological to toxic. To test these hypotheses, we assessed the abundance and metabolic performance of hemocytes, and determined the key parameters of cellular and humoral immunity including phagocytosis and adhesion capacity of hemocytes, as well as the levels of protease inhibitors and lysozyme in the hemolymph in bivalves exposed to different Cu and CO₂ levels. Copper levels in hemolymph and hemocytes were measured as an index of bioaccumulation and biologically relevant Cu exposures under different CO₂ conditions.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

Animal maintenance. The eastern oysters, *C. virginica*, and the hard clams, *M. mercenaria*, were obtained from a local shellfish supplier (Inland Seafood, Charlotte, NC). Bivalves were acclimated for 10 days in tanks with recirculating artificial seawater (ASW; Instant Ocean®, Kent Marine, Acworth, GA, USA) at $20 \pm 1^\circ\text{C}$ and 30 ± 1 practical salinity units (PSU) and aerated with the ambient air. After the preliminary acclimation, clams and oysters were exposed for four weeks to different copper (Cu) and CO_2 levels in a full 3×2 experimental design. Three partial CO_2 pressures (P_{CO_2}) were used: $\sim 400 \mu\text{atm}$ (normocapnia) representative of the present-day conditions, $\sim 800 \mu\text{atm}$ P_{CO_2} (moderate hypercapnia) and $\sim 2000 \mu\text{atm}$ P_{CO_2} (extreme hypercapnia) predicted by a moderate scenario of the Intergovernmental Panel for Climate Change for the years 2100 and 2250, respectively (IPCC, 2014). Target P_{CO_2} levels were achieved by bubbling seawater with the ambient air or the ambient air mixed with CO_2 using precision mass flow controllers (Cole-Parmer, IL, USA). Flow rates of gases were set to maintain the target pH values during the exposures (Table 1). At each CO_2 level, bivalves were maintained either in clean ASW (control) or in ASW supplemented with $50 \mu\text{g l}^{-1}$ Cu added as CuCl_2 . Throughout the experiments, animals were fed every other day with a commercial algal blend (5 ml per tank) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of $2\text{--}20 \mu\text{m}$ (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Each tank contained 10-15 animals in 28 l of seawater. Water was changed twice a week using ASW pre-equilibrated with the respective gas mixtures to achieve target P_{CO_2} . A static-renewal design was used in Cu-exposed tanks, with Cu supplementation to the nominal concentration of $50 \mu\text{g l}^{-1}$ during each water change. The exposure conditions were identical to those described in our earlier published work (Ivanina *et al.*, 2015). Seawater Cu content could not be measured in our present experiment due to technical issues (sample contamination), and cellular Cu content is used as a marker of biological Cu

exposure. Experimental tanks were checked for mortality daily, and the cumulative mortalities over the exposure period were below 5% in all tanks.

Seawater temperature, salinity and pH were measured daily and adjusted as needed. Detailed description of the determination of seawater chemistry is given elsewhere (Beniash *et al.*, 2010; Matoo *et al.*, 2013). Seawater carbonate chemistry parameters (P_{CO_2} , total alkalinity, and the saturation state (Ω) for calcite and aragonite) were calculated using CO₂SYS software (Lewis and Wallace, 1998) using barometric pressure, DIC, pH, temperature and salinity values for the respective samples (Table 1). For calculations, we used NBS scale for seawater pH, published dissociation constants (Dickson, 1990; Millero *et al.*, 2006) and concentrations of silicate and phosphate for Instant Ocean® seawater (0.17 $\mu\text{mol kg}^{-1}$ and 0.04 $\mu\text{mol kg}^{-1}$, respectively).

After four weeks of experimental exposures, hemolymph was extracted from the adductor muscle of clams and oysters using sterile 10-ml syringe with a 21 gauge needle containing 1 ml ice-cold filtered artificial seawater (ASW) to prevent aggregation of the hemocytes. Each biological replicate represented hemolymph pooled from three oysters or from two clams; a total of 5-6 biological replicates was used for each experimental treatment. Concentration of the cells was determined using a Brightline hemacytometer.

Cellular oxygen consumption rates ($\dot{M}_{O_2}$). Hemocyte-containing samples of hemolymph (1.6–10.5 $\times 10^6$ cells per ml) were placed immediately after withdrawal into water-jacketed chambers at 20°C (OX1LP-1ml, Qubit Systems, Kingston, ON, Canada), and $\dot{M}_{O_2}$ was determined using Clarke-type oxygen electrodes. Two-point calibration with air-saturated seawater and saturated Na₂SO₃ solution was conducted prior to each measurement. We determined total respiration rate ($\dot{M}_{O_2\text{-total}}$) of hemocytes, and respiration rate in the presence of a specific inhibitor of mitochondrial F₀, F₁ ATPase, 3 $\mu\text{g ml}^{-1}$ oligomycin. Respiration rate in the presence of oligomycin ($\dot{M}_{O_2\text{-oligo}}$) is an estimate of the cellular energy demand to counteract the mitochondrial proton leak in the absence of ATP synthesis (Brand *et al.*, 1994; Brand, 1997). Non-mitochondrial respiration ($\dot{M}_{O_2\text{-KCN}}$) was determined in hemocyte suspensions in the presence of 100 $\mu\text{mol l}^{-1}$ KCN, a specific inhibitor of cytochrome c oxidase and (in clams) 200 $\mu\text{mol l}^{-1}$ salicylhydroxamic acid

(SHAM), an inhibitor of alternative oxidase (AOX). SHAM was not added to oyster hemocytes that do not exhibit AOX activity (data not shown). Total respiration ($\dot{M}_{O_2\text{-total}}$), mitochondrial respiration ($\dot{M}_{O_2\text{-mito}} = \dot{M}_{O_2\text{-total}} - \dot{M}_{O_2\text{-KCN}}$) and mitochondrial proton leak ($\dot{M}_{O_2\text{-leak}} = \dot{M}_{O_2\text{-oligo}} - \dot{M}_{O_2\text{-KCN}}$) were expressed as $\mu\text{mol O}_2 \text{ min}^{-1} 10^{-6} \text{ cells}$.

Phagocytosis. Phagocytosis assay was performed as described elsewhere (Ivanina *et al.*, 2014). Suspension of neutral Red-stained, heat-stabilized zymosan (Sigma Aldrich, St. Louis, MO, USA) was added to hemocytes at the final concentration of 200 zymosan particles per hemocyte. Hemocytes were incubated for 30 min at room temperature (RT), centrifuged at 1000 x g for 10 min and washed with ASW to remove extracellular zymosan. Cell-free suspensions of zymosan at known concentrations were used as calibration standards. Hemocytes or zymosan standards were incubated for 5 min with 1% acetic acid in 50% ethanol to extract the Neutral Red. The optical density (OD) of the extract was read at 550 nm on a microplate spectrophotometer (Multiscan GO, Thermo Scientific, Waltham, MA, USA). Results are expressed as the number of ingested zymosan particles per 100 cells.

Adhesion capacity. Isolated hemocytes (10^6 cells) were placed in 1 ml of ASW in the wells of a 12-well plate (Costar, Corning) and incubated for 2 h at room temperature. After the incubation, ASW was collected and the wells were surface-washed with 1 ml of ASW, which was added to the previously collected ASW, and centrifuged for 10 min 1000 x g to collect non-adhered cells. The non-adhered cells were enumerated using a Brightline hemacytometer (Sigma Aldrich, St. Louis, MO, USA), and the adhesion capacity expressed as the percentage of adhered hemocytes in each sample.

Lysozyme activity. Lysozyme activity was quantified in the cell-free hemolymph plasma as described elsewhere (Alunno-Bruscia *et al.*, 2011). Hemolymph samples were centrifuged at 1000 x g for 10 min. The supernatant (i.e. cell-free plasma) was collected and stored at -80°C until analysis. For measurement of the lysozyme activity, 50 μl of hemolymph were added in triplicate to the 96-well plates. 150 μl of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma Aldrich, St. Louis, MO, USA) in 66 mmol l⁻¹ phosphate buffer (pH 6.2) was added to the wells immediately before measurement. The decrease in absorbance was continuously recorded for 5 min at 450 nm using a microplate spectrophotometer

227 (Multiscan GO, Thermo Scientific, Waltham, MA, USA). Standard calibration solutions were
228 prepared from crystalline hen egg white lysozyme (Sigma Aldrich, St. Louis, MO, USA).
229 Protein concentrations were measured in hemolymph using Bio-Rad protein assay (Bio-
230 Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard. Lysozyme
231 activity was expressed as ng lysozyme mg⁻¹ protein.

232 *Protease inhibition assay.* Hemolymph samples from clams and oysters were analyzed for
233 inhibitory activities against the following proteases: papain (a cysteine protease) from
234 *Papaya latex*, thermolysin (a metalloprotease) from *Bacillus thermoproteolyticus rokko* and
235 trypsin (a serine protease) from bovine pancreases, all purchased from Sigma-Aldrich (St.
236 Louis, MO, USA). Stock solutions of proteases (1 mg ml⁻¹) were prepared in distilled
237 deionized water and kept on ice. Subsequent dilutions of protease stock solutions were
238 made in 0.2 M NaCl. Proteolytic activities were quantified with azure blue hide powder as a
239 substrate (Sigma Aldrich, St. Louis, MO, USA) as described elsewhere (Bernard *et al.*,
240 2011). The substrate was prepared as 2% (w/v) solution in assay buffer. The assay buffer
241 for thermolysin and trypsin contained 150 mM Tris, 30 mM CaCl₂, 0.05% Brij 35 and 20%
242 sucrose, pH 7.5. The assay buffer for papain consisted of 150 mM Tris, 300 mM NaCl, 0.05%
243 Brij 35, 2 mM EDTA, 5 mM cysteine and 20% sucrose, pH 6.2.

244 To determine the activity of the protease inhibitors, 20 µl of hemolymph were pre-
245 incubated for 20 min with 20 µl (5 µg) of the respective protease while shaking at room
246 temperature. A standard curve consisting of serial dilutions of the respective protease was
247 prepared for each assay. In order to identify background proteolytic level of hemolymph,
248 controls containing 20 µl hemolymph and no protease (substituted with 20 µl of 0.2 M
249 NaCl) were also prepared for each sample. All samples, standards and controls were
250 analyzed in triplicates. Following 20 min pre-incubation, 100 µl of hide powder azure
251 substrate was added to all samples, controls and standards, and resulting mixtures were
252 shaken for 3 h at 37°C. After incubation reaction was stopped by adding 100 µl of 10%
253 trichloroacetic acid. Samples were centrifuges at 12 000 x g for 5 min at +4°C to remove the
254 insoluble substrate. Absorbance of the supernatants (A₅₇₀) was measured at 570 nm in
255 microplate spectrophotometer (Multiscan GO, Thermo Scientific, Waltham, MA, USA). The

triplicate A_{570} values were averaged for each sample, and the level of protease activity determined by comparison to the standard curve and corrected for the intrinsic protease activity of the hemolymph sample.

The correction was carried out as follows:

$$\Delta A_{570} = A_{570\text{ prot}} - [A_{570\text{ prot+HL}} - A_{570\text{ HL}}],$$

where $A_{570\text{ prot}}$ – absorbance of the sample incubated with the respective protease, $A_{570\text{ prot+HL}}$ – absorbance of the samples co-incubated with the protease and the hemolymph, $A_{570\text{ HL}}$ - absorbance of the sample incubated with the hemolymph. The level of protease inhibition was determined by comparison of ΔA_{570} value to the standard curve. Protein concentrations were measured in hemolymph plasma using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard. Protease inhibitory activity was expressed as $\mu\text{g protease mg}^{-1}$ plasma protein.

Copper determination. Hemocytes were collected by centrifugation (1000 x g for 10 min), washed twice in ASW to remove surface-associated Cu and re-suspended in 500 μl of distilled water. Cell suspensions and cell-free hemolymph were mixed 1:1 with 70% nitric acid and digested at 60°C for 24 h. Cu concentrations were determined with an atomic absorption spectrometer AAnalyst 800 (Perkin Elmer, Waltham, MA, USA), in flame mode and deuterium background correction. National Institute of Standards and Technology (NIST) oyster tissue (1566b) was analyzed with the samples to verify the metal analyses; the percent recovery for Cu was $84.75 \pm 10.10\%$ (mean $\pm$ standard deviation). Cu levels in hemocytes and hemolymph were expressed as $\text{ng Cu } 10^{-6}$ cells or $\mu\text{g Cu l}^{-1}$, respectively.

Statistics. Two replicate tanks were set for each experimental treatment, with clams and oysters co-exposed in each tank. Because no significant differences were found in the studied traits between bivalves from the replicate tanks, the data for each treatment condition were pooled across the tanks for statistical analysis. For all studied traits, the effects of the factors “ P_{CO_2} ”, “Cu exposure” and their interactions were assessed using generalized linear model ANOVA after testing for the normality of data distribution and

homogeneity of variances. All factors were treated as fixed and had three levels for P_{CO_2} (~400, ~800 and ~2000 $\mu\text{atm } P_{CO_2}$), and two levels for metal exposures (control and Cu-exposed). ANOVA results for all studied traits are given in Tables 3 and 4. Post-hoc tests (Fisher's Least Significant Difference) were used to test the differences between the group means. Sample sizes were 4–6 for all measured traits and experimental groups. Unless otherwise indicated, data are represented as means $\pm$ standard errors of means (SEM). The differences were considered significant if the probability of Type I error was less than 0.05.

3. RESULTS

3.1 Copper accumulation

Background levels of Cu were considerably (~500-7000 times) higher in the hemolymph plasma of clams compared to that of oysters (0.1-2.1 $\mu\text{g Cu l}^{-1}$ vs. 798-990 $\mu\text{g Cu l}^{-1}$ in oysters and clams, respectively; Fig. 1). Exposure to 50 $\mu\text{g l}^{-1}$ Cu led to a significant increase of Cu levels in hemolymph of oysters reaching ~480 $\mu\text{g Cu l}^{-1}$ (Fig. 1D, Table 3). In contrast, in clams exposure to Cu caused a significant decrease of Cu concentration in the hemolymph (~798-990 $\mu\text{g l}^{-1}$ in control vs. ~455-671 $\mu\text{g l}^{-1}$ in Cu-exposed animals) (Fig. 1C, Table 2). Cu level in hemolymph of oysters was not affected by elevated CO_2 regardless of Cu exposure (Fig. 1D, Table 3), while in clams, co-exposure to Cu and extreme hypercapnia (~2000 $\mu\text{atm } P_{CO_2}$) caused a decrease in Cu content of the hemolymph (Fig. 1C, Table 2).

Unlike hemolymph plasma, background Cu content of hemocytes was notably lower in clams compared to oysters (Fig. 1). In both species, Cu exposure led to Cu accumulation in hemocytes (Figure 1 A, B, Table 2, 3). However, clam hemocytes accumulated three orders of magnitude lower Cu burdens after 4 weeks of Cu exposure compared to hemocytes of oysters (Fig. 1). Due to large individual variation, Cu accumulation was only significant in hemocytes of clams co-exposed to Cu and moderate hypercapnia (~800 $\mu\text{atm } P_{CO_2}$) (Fig. 1A). In Cu-exposed oysters, the accumulated Cu burden in hemocytes tended to increase with increasing exposure P_{CO_2} (Fig. 1B).

3.2 Respiration of hemocytes

In control (non-Cu exposed) clams, total oxygen consumption as well as the mitochondrial respiration of hemocytes were stimulated by exposure to elevated P_{CO_2} , especially in extreme hypercapnia ($\sim 2000 \mu\text{atm } P_{CO_2}$) (Fig. 2A, C). In control (non-Cu exposed) oysters, total respiration or mitochondrial respiration of hemocytes was not affected by P_{CO_2} (Fig. 2B, D). In both studied species, there was no effect of elevated CO_2 levels on mitochondrial proton leak (Fig. 2E, Table 2).

Exposure to Cu suppressed respiration of bivalve hemocytes (Fig. 2, Table 2). In clams, this suppression was evident in hemocytes of Cu-exposed animals at all CO_2 exposure levels while in oysters, and a significant depression of hemocyte respiration was observed at $\sim 800 \mu\text{atm } P_{CO_2}$ (Fig. 2A, C, Table 2). These patterns were mirrored in the changes of the mitochondrial respiration rates of hemocytes of clams and oysters at different Cu and P_{CO_2} exposures (Fig. 2). Mitochondrial proton leak in bivalve hemocytes was not affected by Cu exposure or combination of Cu and elevated CO_2 levels (Fig. 2E, Table 2).

3.3 Cell-mediated immune response

The total concentration of circulating hemocytes in hemolymph of clams and oysters was not affected by exposure P_{CO_2} but significantly affected by Cu exposure (Table 2, 3). Overall, concentrations of circulating hemocytes were higher in Cu-exposed bivalves (Fig. 3). In oysters, this increase was significant in moderate hypercapnia ($\sim 800 \mu\text{atm } P_{CO_2}$) while in clams it was significant at ~ 800 and $\sim 2000 \mu\text{atm } P_{CO_2}$ (Fig. 3 A,B).

Adhesion capacity in clams in oysters hemocytes was not sensitive to exposure to elevated CO_2 levels (Table 2, 3, Fig. 3C,D). Exposure to Cu significantly increased adhesion capacity in clams hemocytes and this increase was similar at all CO_2 concentration (Table 2, Fig. 3C). In oysters, Cu exposure increased adhesion capacity of hemocytes under conditions of moderate and extreme hypercapnia (~ 800 and $\sim 2000 \mu\text{atm } P_{CO_2}$) (Table 3, Fig. 3D).

Phagocytic activity of hemocytes was not affected by elevated CO_2 in clams or oysters (Table 2, 3). In clams, Cu exposure combined with extreme hypercapnia ($\sim 2000 \mu\text{atm } P_{CO_2}$) significantly suppressed phagocytic activity of hemocytes (Fig. 3E). In oysters, exposure to

copper significantly stimulated phagocytic activity of hemocytes irrespective of exposure P_{CO_2} (Table 3, Fig. 3F).

3.4 Humoral response

Total protein content of the hemolymph was considerably higher in oysters ($\sim 3\text{--}4\text{ mg ml}^{-1}$) than in clams ($\sim 1\text{ mg ml}^{-1}$) and was not affected by P_{CO_2} or Cu exposure in either of the studied species (Table 2, Fig. 5).

Under control conditions at normocapnia, inhibitory activity against cysteine proteases was similar in the hemolymph of clams and oysters (Fig. 4). Exposure to elevated CO_2 had no significant effect on cysteine protease inhibition of the hemolymph of studied bivalves (Table 2, 3, Fig. 4 A, B). The only exception was elevated levels of cysteine protease inhibitors in hemolymph clams exposed to extreme hypercapnia ($2000\text{ }\mu\text{atm } CO_2$); however, it was not statistically significant due to large individual variation (Fig. 4A). In contrast, exposure to $50\text{ }\mu\text{g l}^{-1}$ Cu combined with moderate hypercapnia ($800\text{ }\mu\text{atm } CO_2$) significantly (by ~ 7 fold) increased the activity of cysteine protease inhibitors in clam hemolymph (Fig. 4A). In oysters, Cu exposure had no effect on cysteine protease inhibition by hemolymph (Fig. 4B, Table 3). Inhibitory activity of hemolymph plasma against thermolysin and trypsin proteases was considerably higher in clams than in oysters and was not affected by exposures to elevated CO_2 , Cu or their combinations (Fig. 4, Table 2,3). The only exception was a significantly higher level of trypsin protease inhibitors in hemolymph of Cu-exposed clams compared to their control counterparts at extreme hypoxia ($2000\text{ }\mu\text{atm } CO_2$). However, this difference reflected a modest decrease of cysteine protease inhibition by hemolymph of the control clams at $2000\text{ }\mu\text{atm } CO_2$ rather than Cu-induced stimulation of the cysteine protease inhibitors (Fig. 4E).

In control (non-Cu exposed) oysters, lysozyme activity in hemolymph plasma was significantly stimulated by exposure to extreme hypercapnia ($\sim 2000\text{ }\mu\text{atm } P_{CO_2}$) (Fig. 5C). Exposure to Cu also increased lysozyme activity of oyster plasma (by ~ 10 times) under normocapnia ($\sim 400\text{ }\mu\text{atm } P_{CO_2}$). However, combination of Cu exposure and elevated P_{CO_2} suppressed lysozyme activity of oyster plasma (by ~ 6 times at $\sim 2000\text{ }\mu\text{atm } P_{CO_2}$) (Fig. 5C,

Table 3). Lysozyme activity in hemolymph plasma of clams was under the detection limit in this study (data not shown).

4. DISCUSSION

4.1. Cu accumulation in hemolymph and hemocytes

The background levels and accumulation of Cu in hemolymph and hemocytes of clams and oysters showed strong species-specific patterns. The total Cu content was ~3 orders of magnitude higher in the hemolymph of clams than oysters and considerably higher than the ambient Cu concentration. This difference in the background Cu content of the hemolymph between clams and oysters is especially intriguing because neither species contains oxygen-carrying pigments in the hemolymph (such as hemocyanin that could explain large Cu loads) and the overall protein content of clam hemolymph is ~ 3 times lower than in oysters. This indicates that the clam hemolymph contains metal-binding proteins with high affinity and carrying capacity for Cu. Earlier studies identified metal binding proteins in hemolymph of bivalves including clams and oysters that can be involved in Cu sequestration including histidine-rich glycoproteins (HGRs), serum protein band 1 and other high molecular weight metal binding proteins (Abebe *et al.*, 2007). HRGs are the most abundant group of these proteins representing 44 and 58% of the total plasma proteins in *C. virginica* and *M. mercenaria*, respectively (Abebe *et al.*, 2007). Given a much higher Cu content despite the lower protein levels in clam plasma, it is likely that the metal binding plasma proteins of clams have higher binding capacity for Cu than those of oysters. This difference may reflect the ecological differences between the two studied species; the hard clams are an infaunal, sediment-dwelling species that can be exposed to much higher metal levels than the epifaunal species such as oysters due to the considerably higher metal levels in the sediments compared to the surrounding water (Hackney *et al.*, 1998; Riba *et al.*, 2003; Amiard *et al.*, 2007). This ecological difference is also reflected in the presence of highly specialized cellular mechanisms of the metal sequestration and excretion (such as brown cells) in clams that are absent in oysters (Zaroogian *et al.*, 1992; Zaroogian and Anderson, 1995; Zaroogian and Norwood, 2002). Interestingly, hemolymph Cu levels decreased in Cu-exposed clams, and this decrease was especially pronounced

(~50%) in extreme hypercapnia combined with Cu exposure. A similar decline in Cu content of the hemolymph caused by Cu exposure was found in a freshwater mussel *Anodonta cygnea* (Lopes-Lima *et al.*, 2012). The decline of Cu content was not due to a decrease in the overall protein content of the plasma of Cu-exposed *M. mercenaria* (present study) or *A. cygnea* (Moura *et al.*, 2000). The mechanisms of Cu loss from hemolymph of Cu-exposed clams are not known and may reflect the saturation of Cu-binding capacity of the hemolymph plasma and/or increase in the regulatory mechanisms such as Cu-sequestering brown cells (Zaroogian and Norwood, 2002) that excrete excess Cu from the hemolymph. In contrast, in oysters Cu exposure led to a considerable increase of Cu in the hemolymph plasma from very low concentrations to the levels far in excess of the ambient Cu concentration (~500 $\mu\text{g l}^{-1}$ Cu in the hemolymph vs. ~50 $\mu\text{g l}^{-1}$ Cu in the water). This indicates that the high binding capacity proteins sequestering Cu are inducible by Cu exposure in oysters, even though they appear to be absent under the control conditions. Unlike Cu, cadmium (Cd) does not accumulate in the hemolymph of clams or oysters beyond the ambient levels indicating that most of the plasma Cd is free or loosely bound to low affinity ligands (Ivanina *et al.*, 2014) and reflecting differences in handling of essential (e.g. Cu) and non-essential (e.g. Cd) metals in bivalve hemolymph.

In contrast to plasma levels of Cu, cellular content of Cu was considerably higher in hemocytes of oysters compared to clams. Higher background levels of Cu in oyster hemocytes may reflect the higher metabolic rates and thus higher levels of Cu-containing enzymes such as cytochrome c oxidase in oysters. This agrees with the observed difference in total and mitochondrial respiration rates of oyster hemocytes (which are approximately twice as high in oysters as in clams) and the earlier findings of the higher whole-organism metabolic rates of oysters compared with clams (Matoo *et al.*, 2013). Higher metabolic rates (and thus potentially higher activities of ion transport mechanisms) may also contribute to the higher rates of Cu accumulation in hemocytes of Cu-exposed oysters compared to clams (0.1 ng vs. 100 ng per 10^{12} cells of clams and oysters, respectively, after 4 weeks of Cu exposure). Elevated P_{CO_2} tended to enhance Cu accumulation in hemocytes of clams and oysters hemocytes, which peaked at 800 $\mu\text{atm } P_{\text{CO}_2}$ in clams and was highest at 2000 $\mu\text{atm } P_{\text{CO}_2}$ in oysters. This pattern agrees with an earlier *in vitro* study in isolated clam

hemocytes that showed an increase of intracellular Cu accumulation at elevated P_{CO_2} (Ivanina *et al.*, 2013). Low pH also increased ^{63}Cu accumulation in hemolymph of the blue mussels *Mytilus edulis* (Han *et al.*, 2014). This may reflect greater bioavailability of Cu^{2+} in CO_2 -acidified water (Millero *et al.*, 2009), increased expression of intracellular metal ligands such as metallothioneins (MTs) or ferritin at elevated P_{CO_2} (Ivanina *et al.*, 2013; Götze *et al.*, 2014) or combination of these factors.

4.2. Interactive effects of Cu and P_{CO_2} on metabolism and immune response

Metabolic effects of hypercapnia on hemocytes differed in clams and oysters with respiration of clam hemocytes stimulated, and that of oysters – suppressed by elevated P_{CO_2} . These species-specific differences in the metabolic responses reflect mitochondrial sensitivity to acclimation P_{CO_2} and are likely driven by active processes (such as changing ATP demand and/or turnover rates in response to exposure P_{CO_2}) rather than the mitochondrial resting metabolism because the rates of the mitochondrial proton leak were the same in all exposure conditions. Moderate hypercapnia is known to stimulate metabolism in aquatic organisms including mollusks while extreme hypercapnia suppresses metabolic rates (Michaelidis *et al.*, 2007; Lannig *et al.*, 2010; Stapp *et al.*, 2015). The thresholds of what can be considered extreme hypercapnia are likely different among species reflecting evolutionary adaptations to the predominant CO_2 levels in their habitats, and infaunal species such as clams that are normally exposed to elevated CO_2 levels in the sediments are more tolerant to elevated CO_2 than epifaunal species such as oysters. Recent studies showed that physiology and biomineralization of oysters are more sensitive to hypercapnia than those of clams (Beniash *et al.*, 2010; Dickinson *et al.*, 2012; Dickinson *et al.*, 2013; Matoo *et al.*, 2013), and this may also be reflected in lower P_{CO_2} threshold for the metabolic suppression of hemocytes in oysters than in clams found in our present study.

Cu exposure suppressed the metabolic activity of hemocytes of clams and oysters, and similar to the effects of P_{CO_2} , these effects were driven by changes in the routine rather than the resting mitochondrial metabolism. Copper is known to adversely affect mitochondrial function of a variety of animals at low (micromolar) concentrations (Valko *et al.*, 2005; Valko *et al.*, 2006), and active respiration of phosphorylating mitochondria is more

sensitive to Cu than proton leak (Ivanina and Sokolova, 2013; Sappal *et al.*, 2014; Sappal *et al.*, 2015), which may explain why routine but not resting metabolism of the hemocytes was affected by Cu exposure. Notably, the degree of Cu-induced metabolic suppression was more pronounced in hemocytes of clams, despite the fact that clam hemocytes accumulated significantly lower Cu burdens than those of oysters. This emphasizes that cellular burdens of trace metals (such as Cu) are poor predictors of their physiological effects even in ecologically similar and taxonomically related groups such as different species of bivalves.

Notably, the Cu-induced suppression of hemocyte metabolism did not translate into the impairment of immune function in Cu-exposed clams or oysters. In both species, several important parameters of cellular immunity (including the number of circulating hemocytes, as well as phagocytosis and adhesion ability) as well as antiparasitic and antibacterial properties of the hemolymph (such as activities of lysozyme and levels of inhibitors of cysteine proteases) were stimulated by Cu exposure. Some of these immunostimulatory effects of Cu were evident in all P_{CO2} exposures, while others were most prominent in normocapnia (e.g. lysozyme activation) or only in hypercapnia (e.g. increase in the levels of the protease inhibitors). Enhanced phagocytosis in Cu-exposed bivalves may indicate alterations of the cell membrane properties such as decreased surface charge due to Cu²⁺ binding to the negatively charged group of the membrane proteins and phospholipids leading to an increased binding and subsequent phagocytosis on nonpolar particles (Brousseau *et al.*, 2000; Muller *et al.*, 2011). Immune stimulation by Cu is supported by the results of a recent transcriptomic study showing a significant upregulation of immune-related transcripts including inhibitors of metalloproteases, the tumor necrosis factor α pathway, as well as NOD-like and Toll-like receptor pathways in Japanese scallops *Mizuhopecten yessoensis* exposed to 100 $\mu\text{g l}^{-1}$ Cu (Meng *et al.*, 2015). This indicates that immunostimulation by sublethal Cu exposure may be a general feature for marine bivalves.

Increase in the hemocyte adhesion capacity, phagocytosis and hemolymph levels of the inhibitors of serine and cysteine proteases may explain the earlier findings that Cu exposure suppressed the development of infections by a protozoan parasite *Perkinsus marinus* in Cu-exposed oysters *C. virginica* (Foster *et al.*, 2011). *P. marinus* is a common

intracellular protozoan parasite in bivalves, that uses extracellular proteases (including serine and cysteine proteases) to enter the host cells (Koizumi *et al.*, 1994; La Peyre *et al.*, 1996; Faisal *et al.*, 1998; MacIntyre *et al.*, 2003; Casas *et al.*, 2004). Bacterial pathogens such as *Vibrio* spp. also express metalloproteases and serine proteases as virulence factors (Koizumi *et al.*, 1995). Molluscan protease inhibitors can directly inactivate extracellular pathogen-secreted proteases reducing the pathogen's virulence (Xue *et al.*, 2006). Thus, stimulation of the hosts' immune defense by Cu (reflected in the elevated activity of protease inhibitors and enhanced cellular defenses) is likely a contributing factor to the reduced infection levels by *P. marinus* in Cu-exposed oysters (Foster *et al.*, 2011). It is intriguing to speculate that elevated background levels of hemolymph Cu along with higher constitutive levels of protease inhibitors in *M. mercenaria* may similarly serve as immunoprotection explaining lower levels of infection by *P. marinus* in clams compared to oysters (Kennedy *et al.*, 1996; Kraeuter and Castagna, 2001). Notably, earlier studies showed that Cd exposure suppresses bivalve immunity and increases abundance of certain bacteria (such as a human pathogen *Vibrio vulnificus* for which oysters serve as a vector) (Ivanina *et al.*, 2011; Ivanina *et al.*, 2014). Immunosuppression by trace metals such as cadmium, lead and mercury have been also demonstrated in other species of marine bivalves (Oliver and Fisher, 1999; Gagnaire *et al.*, 2004; Jing *et al.*, 2007; Han *et al.*, 2014; Ivanina *et al.*, 2014) indicating that immunostimulatory effects of Cu may be exceptional among the metal contaminants. Furthermore, the protective effects of elevated Cu may differ depending on the pathogen; thus, Cu exposure sensitized rather than protected mussels against the infections caused by a bacterium *Vibrio tubiashii* (Pipe and Coles, 1995; Parry and Pipe, 2004). Future studies comparing the effects of elevated Cu levels on pathogen infectivity and disease progression in oysters and clams infected with different protozoan and bacterial pathogens would be needed to disentangle the potential effects of metals on the hosts' immunity and pathogen performance and provide insights into the ecological consequences of metal exposures on host-parasite interactions.

Unlike Cu, exposure to the elevated CO₂ (~800-2000 µatm) had no effect on the immune function of clams or oysters, except for a significant increase in the lysozyme activity in oysters exposed to ~2000 µatm P_{CO2}, which was completely abolished by co-exposure to

elevated CO₂ and Cu. This agrees with an earlier study showing stimulatory effects of elevated CO₂ on lysozyme levels in oysters as well as in clams (Ivanina *et al.*, 2014). In our present study the lysozyme levels in clam hemolymph were below the detection limits using the identical method of measurement. The reasons for such variation in the background lysozyme levels in the clams used in these two experiments are not known and may reflect seasonal or year-to-year differences in animal physiology, reproductive status or exposure to bacteria (Tokutake *et al.*, 1992; Miura *et al.*, 1994; Ciacci *et al.*, 2009). Notably, the stimulatory effects of hypercapnia on lysozyme activity do not appear to be universal in marine bivalves or other aquatic animals. Thus, in the mussels *Mytilus galloprovincialis* or clams *Chamelea gallina* the number of circulating hemocytes increased or decreased in response to elevated P_{CO2} depending on the temperature or salinity (Matozzo *et al.*, 2012). Lysozyme levels in hemolymph plasma of *M. galloprovincialis* and *C. gallina* remained stable at elevated P_{CO2} in some salinity-temperature combinations and were suppressed by elevated P_{CO2} in others (Matozzo *et al.*, 2012). In the mussels *Mytilus edulis*, exposure to hypercapnia (~1160-3320 µatm P_{CO2}) suppressed an increase in hemocyte phagocytosis observed during the long-term laboratory exposure to normocapnia, but did not alter other hemocyte parameters such as intracellular production of superoxide or fraction of eosinophils (Bibby *et al.*, 2008). Elevated CO₂ levels (14 mg l⁻¹) suppressed innate immunity in a freshwater fish *Oncorhynchus mykiss* including nitro blue tetrazolium (NBT), lysozyme, and myeloperoxidase activities and melano-macrophage frequency (Kaya *et al.*, 2013). Overall, these data indicate that the immune effects of moderate hypercapnia such as predicted by the near-future ocean acidification scenarios are likely to be modest and context-dependent in marine bivalves (varying depending on the animal's condition, reproductive status and environmental parameters such as temperature and salinity).

4.3 Conclusions and perspectives

Environmental copper is an important immune stimulant in marine bivalves. The direction and the effect size of Cu-induced immunostimulation were similar in clams and oysters despite their different abilities to accumulate and regulate intracellular Cu concentrations. Even though the effect size of Cu on immune parameters was moderate (10-30%, with the

exception of hemocyte counts and levels of cysteine protease inhibitors in clams that were enhanced by 50-500% by Cu exposure), the stimulatory effects of Cu were consistent across multiple immune-related traits and linked to the reduced disease severity in Cu-exposed bivalves (Foster *et al.*, 2011). This raises a possibility that Cu exposure can be used for immune enhancement and/or disease treatment in marine bivalves, especially in hatchery or aquaculture settings where Cu exposures can be carried out under controlled conditions and the pollutant can be contained. If needed, such treatments can be followed by a depuration period to flush out the excess of Cu from bivalve tissues. Hypercapnia (at least in the tested range of CO₂ levels predicted by the near future ocean acidification scenarios) has small and variable effects on molluscan immunity and is unlikely to significantly influence disease resistance in marine bivalves.

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Table 1. Summary of water chemistry parameters during experimental exposure. Salinity, temperature, pH_{NBS} and dissolved inorganic carbon (DIC) was measured in water samples collected during the exposure. Other parameters are calculated using co2SYS. Data is represented as means $\pm$ SEM. The same batch of sea water was used throughout the course of the experiment with an average total alkalinity (TA) of 3098.40 $\pm$ 83.13 mmol kg⁻¹ SW. N= 5 for DIC, and 22-28 for other parameters.

	Control exposure			Copper exposure		
Nominal P _{CO2}	400 μ atm	800 μ atm	2000 μ atm	400 μ atm	800 μ atm	2000 μ atm
Salinity	29.81 $\pm$ 0.14	30.36 $\pm$ 0.11	29.14 $\pm$ 0.18	30.27 $\pm$ 0.14	30.46 $\pm$ 0.13	30.13 $\pm$ 0.16
Temperature, °C	20.41 $\pm$ 0.11	20.41 $\pm$ 0.06	20.46 $\pm$ 0.07	20.31 $\pm$ 0.12	20.16 $\pm$ 0.10	20.29 $\pm$ 0.11
pH _{NBS}	8.22 $\pm$ 0.01	7.71 $\pm$ 0.04	7.66 $\pm$ 0.03	8.13 $\pm$ 0.02	7.83 $\pm$ 0.03	7.59 $\pm$ 0.02
P _{CO2} , μ atm	343.24 $\pm$ 7.87	1473.7 $\pm$ 138.10	1638.74 $\pm$ 107.54	450.42 $\pm$ 20.04	1062.26 $\pm$ 113.89	1846.76 $\pm$ 98.56
Ω_{calcite}	8.02 $\pm$ 0.10	3.22 $\pm$ 0.26	2.77 $\pm$ 0.21	6.83 $\pm$ 0.20	3.94 $\pm$ 0.23	2.36 $\pm$ 0.13
$\Omega_{\text{aragonite}}$	5.16 $\pm$ 0.07	2.07 $\pm$ 0.17	1.78 $\pm$ 0.13	4.40 $\pm$ 0.13	2.54 $\pm$ 0.15	1.52 $\pm$ 0.08

Table 2. ANOVA: Effects of P_{CO2} and Cu exposure on the hemocyte and hemolymph traits in *M. mercenaria*. Significant effects are highlighted in bold.

		Factors/interaction		
		CO ₂	Cu	CO ₂ x Cu
Metal accumulation	Hemocytes	F _{2,22} =0.78, P=0.473	F_{1,22}=5.46, P=0.029	F _{2,22} =0.76, P=0.478
	Hemolymph	F _{2,27} =0.003, P=0.997	F_{1,27}=4.20, P=0.050	F _{2,27} =0.72, P=0.497
Total protein content	Hemolymph	F _{2,24} =0.12, P=0.890	F _{1,24} =0.13, P=0.719	F _{2,24} =0.02, P=0.976
Cellular respiration	Total respiration	F _{2,27} =2.11, P=0.141	F_{1,27}=19.10, P=0.002	F _{2,27} =1.37, P=0.271
	Mitochondrial respiration	F _{2,27} =1.86, P=0.175	F_{1,27}=19.98, P=0.0001	F _{2,27} =1.18, P=0.322
	Proton leak	F _{2,26} =0.66, P=0.528	F _{1,26} =0.00, P=0.983	F _{2,26} =0.53, P=0.595
	Non-mitochondrial respiration	F _{2,27} =0.25, P=0.779	F _{1,27} =0.39, P=0.539	F _{2,27} =0.49, P=0.620
Immune-related functions	Hemocyte count	F _{2,27} =0.19, P=0.832	F_{1,27}=34.38, P<0.0001	F_{2,27}=3.75, P=0.037
	Zymosan uptake	F _{2,27} =1.33, P=0.282	F _{1,27} =2.85, P=0.103	F _{2,27} =1.87, P=0.173
	Adhesion	F _{2,26} =1.55, P=0.231	F_{1,26}=21.05, P<0.0001	F _{2,26} =0.12, P=0.890
	Cystein protease inhibition	F _{2,21} =2.43, P=0.113	F _{1,21} =1.17, P=0.291	F _{2,21} =1.83, P=0.185
	Metalloprotease inhibition	F _{2,24} =1.06, P=0.362	F _{1,24} =1.63, P=0.214	F _{2,24} =0.11, P=0.899
	Trypsin protease inhibition	F _{2,23} =0.69, P=0.512	F _{1,23} =2.22, P=0.150	F _{2,23} =0.31, P=0.737

Table 3. ANOVA: Effects of P_{CO2} and Cu exposure on the hemocyte and hemolymph traits in *C. virginica*. Significant effects are highlighted in bold.

		Factors/interaction		
		CO ₂	Cu	CO ₂ x Cu
Metal accumulation	Hemocytes	F _{2,24} =0.80, P=0.460	F_{1,24}=13.78, P=0.001	F _{2,24} =0.30, P=0.745
	Hemolymph	F _{2,22} =0.32, P=0.732	F_{1,22}=68.38, P<0.0001	F _{2,22} =0.32, P=0.729
Total protein content	Hemolymph	F _{2,24} =0.55, P=0.583	F _{1,24} =2.04, P=0.166	F _{2,24} =1.55, P=0.234
Cellular respiration	Total respiration	F _{2,23} =1.85, P=0.179	F_{1,23}=7.41, P=0.012	F _{2,23} =2.22, P=0.131
	Mitochondrial respiration	F _{2,24} =3.15, P=0.06	F_{1,24}=7.80, P=0.010	F _{2,24} =0.88, P=0.428
	Proton leak	F _{2,23} =0.22, P=0.806	F _{1,23} =0.30, P=0.591	F _{2,23} =0.53, P=0.597
	Non-mitochondrial respiration	F _{2,23} =0.42, P=0.659	F _{1,23} =0.60, P=0.446	F _{2,23} =3.12, P=0.063
Immune-related functions	Total hemocytes numbers	F _{2,24} =1.47, P=0.250	F_{1,24}=5.73, P=0.025	F _{2,24} =0.46, P=0.638
	Zymosan uptake	F _{2,24} =0.41, P=0.669	F_{1,24}=6.96, P=0.014	F _{2,24} =0.10, P=0.909
	Adhesion	F _{2,24} =0.10, P=0.909	F_{1,24}=14.79, P=0.0008	F _{2,24} =0.03, P=0.969
	Lysozyme activity in hemolymph	F _{2,24} =0.97, P=0.395	F _{1,24} =0.005, P=0.943	F_{2,24}=9.37, P=0.001
	Cysteine protease inhibition	F _{2,24} =0.18, P=0.837	F _{1,24} =1.79, P=0.194	F _{2,24} =1.13, P=0.340
	Metalloprotease inhibition	F _{2,24} =0.29, P=0.753	F _{1,24} =0.001, P=0.975	F _{2,24} =0.11, P=0.899
	Trypsin protease inhibition	F _{2,24} =0.80, P=0.459	F _{1,24} =0.575, P=0.456	F _{2,24} =0.50, P=0.614

FIGURE LEGENDS

Fig.1. Effect of P_{CO_2} and metal exposure on accumulation of Cu in hemolymph and hemocytes of clams (*M. mercenaria*) and oysters (*C. virginica*). A,B - Copper accumulation on hemolymph, C,D -copper concentration in hemocytes of clams (A,C) and oysters (B,D). Note different scales of the Y axis on C and D due to lower intracellular Cu content in clam hemocytes. Columns that share letters represent values that do not significantly differ among different CO_2 levels within the same Cu treatment group ($P<0.05$). Asterisks indicate values that are significantly different from the control (non-copper exposed) group at the respective P_{CO_2} ($P<0.05$). Vertical lines represent the SEM. N=4-6.

Fig.2. Effect of P_{CO_2} and metal exposure on cellular respiration of clams (*M.mercenaria*) and oysters (*C. virginica*). A, B-total MO_2 , C,D- Mitochondrial MO_2 , E,F-Mitochondrial proton leak for clams (A,C,E) and oysters (B,D,F). Columns that share letters represent values that do not significantly differ among different CO_2 levels within the same Cu treatment group ($P<0.05$). Asterisks indicate values that are significantly different from the control (non-copper exposed) group at the respective P_{CO_2} ($P<0.05$). Vertical lines represent the SEM. N=4-6.

Fig.3. Effect of P_{CO_2} and metal exposure on immune-related traits of hemocytes of clams (*M.mercenaria*) and oysters (*C. virginica*). A, B-Total blood cell count, C,D-cell adhesion rates, E,F-average rate of hemocyte phagocytosis for clams (A,C,E) and oysters (B,D,F). Columns that share letters represent values that do not significantly differ among different CO_2 levels within the same Cu treatment group ($P<0.05$). Asterisks indicate values that are significantly different from the control (non-copper exposed) group at the respective P_{CO_2} ($P<0.05$). Vertical lines represent the SEM. N=4-6.

Fig.4. Effect of P_{CO_2} and metal exposure on protease inhibitory activity of clams (*M.mercenaria*) and oysters (*C. virginica*). A, B - inhibition of a cysteine protease (papain), C,D - inhibition of a metalloprotease (thermolysin), E,F - inhibition of a serine protease (trypsin) for clams (A,C,E) and oysters (B,D,F). Columns that share letters represent values that do not significantly differ among different CO_2 levels within the same Cu treatment group ($P<0.05$). Asterisks indicate values that are significantly different from the control

(non-copper exposed) group at the respective P_{CO_2} ($P<0.05$). Vertical lines represent the SEM. N=4-5.

Fig.5. Effect of P_{CO_2} and metal exposure on protein concentration and lysozyme activity of clams (*M.mercenaria*) and oysters (*C. virginica*). A, B - total protein concentration in hemolymph, C-lysozyme activity in hemolymph for clams (A) and oysters (B, C). Columns that share letters represent values that do not significantly differ among different CO_2 levels within the same Cu treatment group ($P<0.05$). Asterisks indicate values that are significantly different from the control (non-copper exposed) group at the respective P_{CO_2} ($P<0.05$). Vertical lines represent the SEM. N=4-5.

Fig.1

Clams

Oysters

Fig.2

Clams

Oysters

Fig.3

626

627 FIG.4

Clams

Oysters

Fig.5

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