EFFECTS OF CADMIUM EXPOSURE ON CRITICAL TEMPERATURES OF AEROBIC METABOLISM IN EASTERN OYSTERS
CRASSOSTREA VIRGINICA (GMELIN, 1791)

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Abstract. Cadmium (Cd) and elevated temperatures are common stressors in estuarine and coastal environments. Elevated temperature can sensitize estuarine organisms to the toxicity of metals such as Cd and *vice versa*, but the physiological mechanisms of temperature-Cd interactions are not well understood. We tested a hypothesis that synergistic effects of elevated temperature and Cd stress involve Cd-induced reduction of the aerobic scope of an organism thereby narrowing the thermal tolerance window of oysters. We determined the effects of prolonged Cd exposure (50 µg Cd l⁻¹ for 30 days) on the upper critical temperature of aerobic metabolism (assessed by accumulation of anaerobic end products L-alanine, succinate and acetate), cellular energy status (assessed by the tissue levels of adenylates, phosphagen/aphosphagen and glycogen and lipid reserves) and oxidative damage during acute temperature rise (20 - 36°C) in the eastern oysters *Crassostrea virginica*. The upper critical temperature (T_{cII}) was shifted to lower values (from 28 to 24°C) in Cd-exposed oysters in spring and was lower in both control and Cd-exposed groups in winter (24 and <20°C, respectively). This indicates a reduction of thermal tolerance of Cd-exposed oysters associated with a decrease of the aerobic scope of the organism and early transition to partial anaerobiosis. Acute warming had no negative effects on tissue energy reserves or parameters of cellular energy status of oysters (except a decrease in adenylate content at the extreme temperature of 36°C) but led to an increase in oxidative lesions of proteins at extreme temperatures. These data show that transition to partial anaerobiosis (indicated by the accumulation of anaerobic end products) is the most sensitive biomarker of temperature-induced transition to energetically non-sustainable state in oysters, whereas disturbances in the cellular energy status (i.e. adenylate and phosphagen levels) and oxidative stress ensue at considerably higher temperatures, nearing the lethal range. This study indicates that long-term exposure to environmentally relevant levels of Cd may increase their sensitivity to elevated temperatures during seasonal warming and/or the global climate change in polluted estuaries.

INTRODUCTION

Temperature is an important environmental factor that affects survival and distribution of marine organisms by directly influencing rates of all physiological and biochemical reactions (Hochachka and Somero, 2002), and is a key driver of the global climate change-induced shifts in distribution and persistence of marine populations (Brierley and Kingsford, 2009; Burrows *et al.*, 2011). In many aquatic ectotherms (including invertebrates and fish), temperature tolerance is limited by the negative effects of sub- and supra-optimal temperatures on energy homeostasis as reflected in the reduction of the organism's aerobic scope, i.e. the amount of energy and aerobic capacity available to support fitness-related functions such as activity, growth and reproduction (Sokolova *et al.*, 2012; Bozinovic and Pörtner, 2015). As the environmental temperature deviates from the optimum, the aerobic scope declines due to the progressive mismatch between the energy demand and supply, and at certain threshold temperatures (called critical temperatures, or T_c), all metabolic capacity is devoted to the basal maintenance ensuring time-limited survival of an organism (Pörtner, 2002; Sokolova *et al.*, 2012b). This transition is typically associated with the activation of anaerobic component of metabolism to compensate for energy deficiency (Pörtner, 2002; Sokolova *et al.*, 2012b). The critical temperatures define the environmental thermal tolerance window of an organism and are correlated with the biogeographical distribution limits in aquatic ectotherms (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Bozinovic and Pörtner, 2015).

Earlier studies have shown that elevated temperatures can increase toxicity of environmental pollutants (including trace metals) in ectotherms (review in: Schiedek *et al.*, 2007; Sokolova and Lannig, 2008); however, the mechanisms of this effect are not fully understood. It has been proposed that the interactive effects of elevated temperature and pollutants involve disturbances of energy homeostasis (Pörtner, 2010; Sokolova *et al.*, 2012b). Trace metal pollutants such as cadmium (Cd) are strong candidates for causing the bioenergetic-dependent shifts of thermal tolerance of marine ectotherms. Cd is a common persistent pollutant in estuarine waters entering the environment from both anthropogenic and natural sources (Nriagu and Sprague, 1987). Cd is toxic at low concentrations and can strongly affect energy metabolism by suppressing the mitochondrial function and increasing basal energy demand of an organism to cover the energy costs of detoxification and damage repair

(Sokolova, 2004; Valko *et al.*, 2005; Cherkasov *et al.*, 2006; Ivanina *et al.*, 2008b; Cannino *et al.*, 2009; Ivanina *et al.*, 2009). This may result in the reduced aerobic scope of an organism and therefore narrow its thermal tolerance limits. However, the effects of metal exposure on thermal tolerance of marine ectotherms have not been extensively studied and the role of the Cd-induced energy imbalance in sensitizing an organism to the combined temperature and Cd stress is not well understood.

Eastern oysters, *Crassostrea virginica*, are a useful model to study the effects of metal pollutants on thermal tolerance of marine ectotherms. Oysters are common species serving as ecosystem engineers in estuarine and coastal zones of the western Atlantic. They are exposed to wide seasonal and diurnal fluctuations of temperature in their habitats and can bioaccumulate high levels of trace metals in their bodies making them susceptible to the metal toxicity (Kennedy *et al.*, 1996; O'Connor and Lauenstein, 2006). Earlier studies showed that Cd has a strong effect on aerobic capacity of oyster mitochondria inhibiting ATP synthesis, leading to elevated proton leak, increased production of reactive oxygen species and lower mitochondrial efficiency (Sokolova, 2004; Cherkasov *et al.*, 2006; Cherkasov *et al.*, 2007; Kurochkin *et al.*, 2011). Cd exposure also results in elevated basal energy demand of oysters due to the elevated costs of cellular protective mechanisms such as formation of metallothioneins, heat shock proteins and ATP-dependent efflux pumps (Cherkasov *et al.*, 2006; Ivanina *et al.*, 2008b; Ivanina *et al.*, 2009). This may lead to a mismatch between energy supply and demand in Cd-exposed oysters and a reduction of the upper limit of thermal tolerance of oysters. We tested this hypothesis by determining the effects of Cd exposure on thermal tolerance window of oysters (as defined by the upper critical temperatures (T_{cII}) of aerobic metabolism) using accumulation of anaerobic end products, shifts in cellular energy status and oxidative damage as markers of temperature-induced bioenergetics stress (Pörtner, 2010; Sokolova *et al.*, 2012b; Sokolova, 2013).

MATERIALS AND METHODS

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.

Oyster maintenance and experimental exposures. Adult North Carolina oysters were purchased from J & B Aquafood (Jacksonville, NC, USA) and shipped to UNC Charlotte within 24 h of collection. Winter and spring oysters were collected in January (winter oysters) and March (spring oysters), respectively, in an intertidal location in Stump Sound, NC. At the time of collection, water salinity varied between 26 and 33 practical salinity units, and temperatures were 12-15°C in water, up to 22°C in air in January, and 15-17°C in water, up to 23°C in air in March. The study site in Stump Sound, NC is used for commercial oyster culture and has low background levels of metals and other pollutants.

All experimental animals were acclimated for 7 days at common temperature (20±1°C) which is close to the optimum for North Carolina oysters (Sokolova, unpublished data). This temperature was slightly above the water temperatures and close to the air temperatures experienced by the intertidal oysters at the study sites. After preliminary acclimation, the oysters were randomly divided into two groups and exposed to either clean artificial seawater (ASW; control group) or ASW with 50 µg L⁻¹ of Cd added as CdCl₂ (Cd-exposed group) for 30 days at 20±1°C. Salinity was maintained at 30±2 and the natural light regime (10:14 and 12:12 light to dark in January and March, respectively) was used during the preliminary acclimations and experimental exposures. Water volume in the tanks was 3-5 L per oyster. A static-renewal design was used with a weekly water change using clean ASW or Cd-supplemented ASW for control and Cd-exposed oysters, respectively, and a mid-week supplementation of Cd in Cd-exposed tanks to the target value of 50 µg L⁻¹ Cd. The amount of Cd added to the tanks between the water changes was calculated based on the known daily uptake rates of Cd in North Carolina oysters and oyster biomass in experimental tanks (Cherkasov *et al.*, 2010). Oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 mL per oyster) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* (DT's Live Marine Phytoplankton, Premium Reef Blend, Sycamore, IL, USA).

After the 30-day acclimation period, oysters were transferred into two large tanks (120 L each, 30-40 oysters per tank) containing aerated, re-circulated ASW (20°C, salinity 30) with the same Cd concentration as during the acclimation (0 and 50 µg L⁻¹ for control and Cd-exposed oysters, respectively). Oysters were allowed to remain in the tanks for 24 h at 20°C to reduce the effects of handling, and a subsample of oysters (N=8-16) was collected to represent the

acclimation temperature (Fig. 1). The water in the tanks was then gradually warmed in 4°C increments using a standard heating rate of 1°C h⁻¹ as described in earlier studies on T_c (Frederich and Pörtner, 2000; Sommer and Pörtner, 2002; Sokolova and Pörtner, 2003). After the first 4°C increment was completed and a target temperature of 24°C reached, the oysters were maintained at this temperature for 48 h (Fig. 1). This time is sufficient to reduce the acute effects of rapid temperature change but not sufficient for full thermal acclimation (Sokolova and Pörtner, 2003; Lannig *et al.*, 2010). After 48 h of exposure at a target temperature, another subsample of 8-16 oysters was collected, and the temperature was again increased by 4°C followed by a 48-h exposure period as described above. Water was changed in experimental tanks every 48 h using clean ASW or ASW supplemented 50 µg L⁻¹ (for control and Cd-exposed oysters, respectively) equilibrated to the same temperature as the current exposure temperature in experimental tanks. As a result of this stepwise warming procedure, subsamples of oysters were collected at 20°C, 24°C, 28°C, 32°C and 36°C (Fig. 1). Immediately after sampling, experimental oysters were dissected, their gills, hepatopancreas, muscle and mantle tissues shock-frozen and stored in liquid nitrogen until further analyses.

Tissue metabolite determination. Tissue metabolite concentrations were measured in deproteinized perchloric acid (PCA) extracts of oyster tissues. Biomarkers of cellular energy status including the levels of high energy phosphates and anaerobic end products (indicative of the transition to partial anaerobiosis) were measured in the gills which is the main organ involved in oxygen uptake in oysters. Glycogen content was measured in the gills, hepatopancreas and adductor muscle which are all involved in the glycogen storage in bivalves. Lipid content was only determined in the gills and hepatopancreas due to the limited amount of sample from the muscle.

For metabolite extraction, 200-300 mg of frozen tissue were ground under liquid nitrogen and extracted using ice-cold 0.6 M perchloric acid (PCA) containing 150 mM ethylenediaminetetraacetic acid (EDTA) (Sokolova *et al.*, 2000). Neutralized, deproteinized PCA extracts were stored at -80 °C and used to determine concentrations of tissue metabolites using standard spectrophotometric NADH- or NADPH-linked enzymatic assays at 340 nm absorbance wavelength (Grieshaber *et al.*, 1978; Bergmeyer, 1985; Kurochkin *et al.*, 2009; Ivanina *et al.*, 2010). Briefly, the assay conditions were as follows:

L-alanine: 80 mM Tris buffer, pH 7.6, 7 mM 2-oxoglutarate, 0.24 mM NADH, 260 U ml⁻¹ of lactate dehydrogenase (LDH), 10,000 U ml⁻¹ alanine aminotransferase;

Acetate: 100 mM triethanolamine (TRA) buffer, pH 7.6, 0.2 M magnesium chloride, 18 mM NADH, 91 mM ATP, 150 mM PEP phosphoenolpyruvate (PEP), 5 U ml⁻¹ of pyruvate kinase (PK), 5 U ml⁻¹ LDH, 50 U ml⁻¹ of acetate kinase (AK);

ATP: 38.5 mM TRA buffer, pH 7.6, 0.04 mM NADP, 7 mM MgCl₂, 0.462 U ml⁻¹ glucose-6-phosphate dehydrogenase, 50 mM glucose, 1.8 U ml⁻¹ hexokinase;

ADP and AMP: 58 mM TRA buffer, pH 7.6, 3 mM PEP, 0.09 mM NADH, 24 U ml⁻¹ LDH, 18 U ml⁻¹ PK, 16 U ml⁻¹ myokinase;

L-arginine: 170 mM glycylglycin, pH 7.6, 13 mM MgCl₂, 6.5 mM pyruvate, 0.26 mM ADP, 0.79 mM NADH, 1 U ml⁻¹ octopine dehydrogenase.

For determination of phospho-L-arginine (PLA) levels, PLA in PCA extracts of gill tissues was subjected to acid hydrolysis yielding L-arginine. L-arginine was determined as described above and PLA levels calculated as a difference in L-arginine contents of the sample before and after the acid hydrolysis (Morris *et al.*, 2005; Kurochkin *et al.*, 2009; Ivanina *et al.*, 2010). The relative amount of phosphagen (R_{PLA}) in the total phosphagen/aphosphagen pool was calculated as follows:

$$R_{PLA} = \frac{[PLA]}{[PLA] + [L-arginine]} \quad [1],$$

where [PLA] and [L-Arginine] are the tissue concentrations of phospho-L-arginine and L-arginine, respectively in $\mu\text{moles g}^{-1}$ wet tissue mass.

Succinate was measured in PCA extracts of gill tissues using succinic acid kit (Boehringer Mannheim, R-Biopharm kit Darmstadt, Germany) according to the manufacturer's instructions. Glycogen concentration was measured in PCA extracts of gill, hepatopancreas and muscle tissues after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase (Keppler and Decker, 1984). D-glucose was measured using the following assay conditions: 38.5 mM

TRA buffer, pH 7.6, 0.04 mM NADP⁺, 7 mM MgCl₂, 0.462 U ml⁻¹ glucose-6-phosphate dehydrogenase, 1.8 U ml⁻¹ hexokinase.

Tissue lipid content was measured in gill and hepatopancreas tissues using a standard method of chloroform extraction (Folch *et al.*, 1957; Iverson *et al.*, 2001). Oyster tissues (~100 mg of wet mass) were homogenized in chloroform/methanol mixture (2:1 v:v) using tissue to chloroform/methanol ratio of 1:20 (w:v). Samples were sonicated for 1 min (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), vortexed for 2 min and centrifuged for 5 min at 13,000 x *g*. The supernatant was transferred into a new tube and the chloroform/methanol extraction was repeated on the tissue pellet. The supernatants of two extractions were pooled, mixed with water (25% of the total volume of supernatant), and centrifuged for 5 min at 13,000 x *g*. The lower phase (chloroform) was transferred to a pre-weighed tube and the chloroform was evaporated to determine the mass of the extracted lipids. Concentrations of glycogen and lipids were expressed in mg g⁻¹ wet tissue mass, and all other metabolites – as μmoles g⁻¹ wet tissue mass.

Oxidative stress markers. Levels of malondialdehyde (MDA) were assayed as the total concentration of the thiobarbituric acid-reactive substances (TBARS) in the mantle tissues (Li and Chow, 1994). Briefly, the mantle tissue was powdered under liquid nitrogen, homogenized in 50 mM potassium phosphate buffer (pH 7.0 at 20°C) (tissue to buffer (w:v) ratio 1:4), sonicated for 5 seconds (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA) and centrifuged for 5 min at 13 000 x *g* and 4°C. Sample supernatants as well as blanks or MDA standards were mixed with 0.375% thiobarbituric acid (TBA) and 2% butylated hydroxytoluene (BHT) (v:v:v 1:14:0.14), heated for 15 min at 100°C and centrifuged for 5 min at 13 000 x *g* at room temperature. The formation of a pink chromagen by reaction between MDA and TBA was measured spectrophotometrically at 532 nm, and tissue levels of MDA expressed in μmoles TBARS g⁻¹ wet mass.

Levels of the carbonyl groups in mantle tissue proteins were measured spectrophotometrically (Sukhotin *et al.*, 2008). Briefly, frozen mantle tissues were ground under liquid nitrogen and homogenized in the buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 125 mM KCl, 1.1 mM EDTA and 0.6 mM MgSO₄ (pH

7.4) and protease inhibitors [leupeptin ($0.5 \mu\text{g mL}^{-1}$), pepstatin ($0.7 \mu\text{g mL}^{-1}$), phenylmethylsulfonyl fluoride ($40 \mu\text{g mL}^{-1}$) and aprotinin ($0.5 \mu\text{g mL}^{-1}$)]. Samples were centrifuged at $100,000 \times g$ for 15 min, supernatant collected and incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl. The blanks were incubated with HCl without DNP. After incubation, proteins were precipitated by adding 100% trichloroacetic acid (TCA) and centrifuged at $11,000 \times g$ for 10 min. The pellet was collected, washed with ethanol ethylacetate (1:1) and dissolved in 6 M guanidine hydrochloride in 20 mM in KH_2PO_4 (pH 2.5). The absorbance was measured at 360 nm on a spectrophotometer (Cary 50, Varian, Cary, NC, USA) using guanidine HCl solution as reference. Protein content of the samples was determined using Biuret assay, and the amount of carbonyls expressed as nmoles carbonyls mg^{-1} protein.

Cd determination. Mantle tissues (~50-100 mg wet mass) were freeze-dried and digested in Teflon bottles with 52.5% nitric acid (trace metal grade; Fisher Scientific, Suwanee, GA, USA) using 3-5 cycles of microwave heating and cooling on ice. Water samples from experimental tanks were acidified with nitric acid (1% final concentration) for Cd determination. Cd concentrations in seawater and tissue digests were determined with an atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800, Shelton, CT, USA) equipped with graphite furnace and Zeeman background correction. The detection limit of this method was $0.5 \mu\text{g Cd L}^{-1}$ sample or 2.5 ng Cd g^{-1} dry tissue mass. National Institute of Standards and Technology (NIST) oyster tissue (1566b) was analyzed to verify the metal analyses; the percent recoveries over all batches were $94.6 \pm 6.6\%$ (mean $\pm$ standard deviation).

Statistical Analyses. Statistical analysis was performed using generalized linear model (GLM) analysis of variance (ANOVA) after testing for normality of the data and homogeneity of variance. To determine the effects of temperature, Cd exposure and season on tissue metabolite concentrations, energy-related indices and oxidative stress biomarkers, three-way ANOVAs were used with “Temperature” and “Cd exposure” as fixed factors, and “Season” as a random factor. All models included the main factor effects and all factor interactions. Post-hoc tests (Fisher’s Least Square Difference) were used for planned comparisons among the group means. Unless otherwise indicated, data are represented as means $\pm$ standard errors of means (SEM). Statistical Analysis Software (SAS 9.2) (SAS Institute, Cary, NC, USA) was used for the

statistical analyses. Pearson correlations (R) of average values of studied biological traits in individual experimental groups were calculated using Origin 8.6 software package (OriginLab, Northampton, MA). The following parameters were included: tissue levels of L-alanine, acetate, PLA, L-arginine, R_{PLA} , ATP, ADP, AMP, MDA and carbonyls; season, temperature, and Cd exposure were used as potential explanatory variables. Principal component (PCA) analysis was conducted using Origin 8.6 software package (OriginLab, Northampton, MA) using the same list of parameters as for the correlation analysis.

RESULTS

Cd accumulation. In control oysters, low Cd levels were detected ($8.1 \pm 2.3 \mu\text{g g}^{-1}$ dry mass, N=18 and $3.4 \pm 0.4 \mu\text{g g}^{-1}$ dry mass, N=24 in winter and spring, respectively). Cd exposure resulted in a significant accumulation of Cd in the mantle tissue of oysters (Table 1; $P < 0.0001$ for the effects of Cd exposure). Cd burdens accumulated after 30 days of Cd exposure were ~50% higher in winter than in spring oysters (113.5 ± 8.0 vs. $75.0 \pm 5.7 \mu\text{g g}^{-1}$ dry mass, N= 45 and 32, respectively; $P = 0.003$ for the effects of season) but did not significantly change during the acute temperature rise ($P = 0.808$) (Table 1).

Anaerobic end product accumulation. Acute temperature rise led to accumulation of anaerobic end products in gill tissues of oysters; however, the temperature at which significant accumulation of anaerobic end products was detected, differed between control and Cd-exposed oysters as well as between the seasons. Tissue levels of L-alanine and acetate were significantly affected by the interactions of the factors “Cd exposure”, “Temperature” and “Season” (Table 2) indicating that temperature-induced changes in tissue levels of these metabolites were modulated by the season and Cd exposure. Succinate levels in gill tissues of oysters were significantly different among the two studied seasons but not affected by elevated temperature, Cd exposure or any factor interactions (Table 2). In winter oysters, the average tissue levels of succinate were $2.44 \pm 0.14 \mu\text{mole g}^{-1}$ wet tissue mass (N=86) compared to $3.82 \pm 0.22 \mu\text{mole g}^{-1}$ wet tissue mass in their spring counterparts ($P < 0.05$).

In winter, temperature rise led to a significant accumulation of L-alanine and acetate at temperatures $\geq 24^\circ\text{C}$ in control oysters indicating onset of anaerobiosis (Fig. 2A, C). In winter, L-alanine levels were significantly higher in Cd-exposed oysters than in their control

counterparts at the acclimation temperature (20°C) but did not change in response to the acute temperature rise (Fig. 2). Tissue levels of succinate did not change in response to Cd exposure and/or the acute temperature rise in winter oysters (data not shown).

In spring, acute temperature rise led to a significant accumulation of L-alanine at $\geq 28^\circ\text{C}$ in control oysters, and at $\geq 24^\circ\text{C}$ in their Cd-exposed counterparts consistent with the temperature-induced onset of cytosolic anaerobiosis (Fig. 2A, B). There was no temperature-induced accumulation of mitochondrial anaerobic end products (acetate or succinate) in spring oysters regardless of Cd exposure levels (Fig. 2, Table 1).

Phosphagen. At the acclimation temperature of 20°C, the levels of phosphagens (PLA) were considerably higher in gill tissues of winter oysters than in their spring counterparts ($P < 0.05$) (Fig. 3). In contrast, tissue concentrations of aphosphagen (L-arginine) were similar in winter and spring oysters at 20°C ($P > 0.05$). As a result, the relative proportion of phosphagen in the total phosphagen/aphosphagen pool (R_{PLA}) was significantly higher in the winter oysters compared to those collected in spring (0.8-0.9 vs. 0.3-0.5, respectively; $P < 0.05$) (Fig. 3).

Acute temperature rise had no effect on the tissue levels of phosphagens and aphosphagens in control or Cd-exposed oysters collected in winter (Fig. 3A & C; Table 2). In contrast, in spring-collected oysters, elevated temperatures (at or above 28°C) were associated with increased levels of L-arginine and reduced R_{PLA} values in control oysters but not in their Cd-exposed counterparts that had generally lower PLA content and R_{PLA} ratios (Fig. 3).

Adenylate content. At the acclimation temperature (20°C), the steady-state ATP levels were similar in the gills of winter and spring oysters ($P > 0.05$), whereas ADP and AMP concentrations were higher in spring-collected oysters compared to their winter counterparts (Table 3; Fig. 4). As a result, total adenylate concentrations were significantly lower in winter oysters compared to their spring counterparts when measured at the acclimation temperature (1.9-2.6 vs. 3.8-4.1 $\mu\text{mol g}^{-1}$ wet mass in winter and spring, respectively; $P < 0.05$).

In winter oysters, acute temperature rise had no effects on tissue adenylate levels (Fig. 4), whereas in spring oysters acute warming led to significant shifts in tissue adenylate contents (Fig. 4. B, D & F). In control oysters collected in spring, tissue ATP levels were not affected by

the acute warming while ADP and AMP levels decreased at elevated temperatures ($\geq 28^{\circ}\text{C}$ and 36°C for ADP and AMP, respectively) (Fig. 4B, D & F). As a result, the total adenylate pool was decreased in control oysters at or above 28°C ($P < 0.05$). In Cd-exposed spring oysters, elevated temperatures (28°C and above) led to a slight increase in ATP levels and a decline in tissue AMP content (Fig. 4B & F) with no significant change in the steady-state total adenylate content ($P > 0.05$; data not shown).

Energy reserves. The tissue distribution of glycogen was differently affected by season in different tissues of oysters ($P < 0.001$ for the Season x Tissue interactions; Table 4). At the acclimation temperature (20°C), glycogen levels were highest in the hepatopancreas of the winter oysters, while in the spring oysters the highest glycogen content was found in the gills (Fig. 5). In winter oysters, acute temperature rise and/or Cd exposure had no significant effect on the levels of energy reserves (glycogen or lipids) in either of the studied tissues (Fig. 5). In spring oysters, acute temperature rise led to an increase of the levels of glycogen ($P < 0.05$ in the hepatopancreas) and lipids ($P < 0.05$ in the gills and hepatopancreas) which peaked at 28°C and then dropped at the extreme temperature of 36°C (Fig. 5). Cd exposure partially suppressed the temperature-induced accumulation of glycogen in hepatopancreas of spring oysters, but had no effect on the temperature-induced changes in the lipid levels (Fig. 5).

Oxidative stress markers. The levels of oxidative stress biomarkers were higher in winter oysters compared to their spring counterparts (Fig. 6). Levels of MDA, an end product of lipid peroxidation, were significantly affected by Cd exposure and season and their interactions, but not by acute temperature rise (Fig. 6; Table 1). In contrast, the levels of protein carbonyls were affected by season and experimental temperature and their interactions, but not by Cd exposure (Table 1). The carbonyl content of the mantle tissues increased with increasing temperatures in winter oysters; this increase was significant at $\geq 28^{\circ}\text{C}$ in control oysters and at 36°C in the Cd-exposed group (Fig. 6). In spring, acute temperature rise did not affect the tissue carbonyl content of control or Cd-exposed oysters (Fig. 6).

Data integration. The principal component analysis (PCA) identified three principal components (PCs) with eigenvalues > 1 that explained 81% of the variation in the data set (Supplementary Table 1). The 1st PC (55% of variation) was mostly associated with the season

and had high loadings of PLA, R_{PLA}, ATP, AMP, carbonyl levels and a moderate loading of MDA levels. The 2nd PC (16% of variation) was linked to Cd exposure and had high loadings of acetate, arginine and MDA levels and a moderate loading of L-alanine levels. The 3rd PC (10% of variation) was highly correlated with the temperature and had high loadings of AMP levels.

Pearson correlation analysis revealed that L-alanine levels were negatively correlated with the levels of phosphagen (PLA) and R_{PLA} and positively correlated with aphosphagen (L-arginine), ATP and AMP (Supplementary Table 2; Fig. 7A). Tissue levels of acetate were positively correlated with oxidative lesions (MDA and carbonyls), and MDA and carbonyl levels were positively correlated with each other. PLA levels negatively correlated with the levels of adenylates (ATP, ADP and AMP) and positively linked to the oxidative lesions (MDA and carbonyls) levels. Tissue levels of adenylates (ATP, ADP and AMP) were positively correlated with each other and negatively linked to MDA and carbonyl levels.

DISCUSSION

Effects of Cd on T_c of aerobic metabolism

Rapid warming led to an induction of partial anaerobiosis in oysters (as indicated by accumulation of anaerobic end products) showing that oysters transitioned into the pessimum range at and beyond the critical temperatures (T_c) where only time-limited survival is possible (Pörtner, 2010; Sokolova, 2013). This temperature-induced activation of anaerobic pathways likely reflects tissue hypoxemia caused by mismatch between the elevated oxygen uptake of the tissues and insufficient capacity of ventilation and circulation systems to provide oxygen (Lannig *et al.*, 2008; Lannig *et al.*, 2010; Pörtner, 2010). The upper critical temperature (T_{cII}) was shifted by seasonal acclimatization in oysters as is typical for eurythermal ectotherms (Sommer *et al.*, 1997; Sokolova and Pörtner, 2003; Sommer and Pörtner, 2004). In winter-acclimatized oysters, transition to partial anaerobiosis occurred at lower temperatures than in spring (24°C vs 28°C, respectively). Moreover, in winter oysters the temperature-induced anaerobiosis encompassed cytosolic as well as mitochondrial pathways (shown by accumulation of L-alanine and acetate, respectively) indicating onset of deep mitochondrial anaerobiosis whereas in spring oysters only cytosolic anaerobic pathways were engaged (Grieshaber *et al.*, 1994; Tikunov *et al.*, 2014). Notably, a metabolomics study of the green

lipped mussels (*Perna canaliculus*) also showed induction of anaerobic pathways by heat stress leading to a depletion of aspartate and β -alanine, the metabolic precursors of L-alanine and acetate, respectively (Dunphy *et al.*, 2015). The season-induced differences in thermal tolerance persisted despite a prolonged (4 weeks) acclimation of oysters at a common garden temperature (20°C) indicating that the temperature is not the only driver of the seasonal physiological differences in thermal tolerance and other cues (such as light regime or reproductive status) may be involved. Notably, the T_{cII} of 28°C found in spring oysters is close to the long-term thermal tolerance limit of the species. Earlier studies showed that at 28°C and above, oysters enter the “no scope for growth” area where they do not deposit shell material and are not capable of withstanding additional stressors such as sublethal Cd stress (Lannig *et al.*, 2006b). Chronic exposure at 28°C also leads to a decline in the mitochondrial density suggesting a decrease in aerobic capacity of oysters (Cherkasov *et al.*, 2006a). Cessation of growth of oysters at the critical temperature of 28°C is consistent with the transition into the time-limited, bioenergetically unsustainable state where the aerobic scope for growth disappears. This indicates that *C. virginica* may be unable to survive in the environments where temperatures exceed 28°C for a prolonged period of time, making T_{cII} a good indicator of the ecologically relevant thermal limit of the species.

Exposure to the environmentally relevant, sublethal levels of Cd negatively affected the thermal tolerance of oysters. In spring oysters, Cd exposure led to a downward shift of T_{cII} from 28°C to 24°C. In winter Cd-exposed oysters we could not determine T_{cII} by the change in accumulation of anaerobic end products within the range of the studied temperatures (20-36°C) suggesting that the T_{cII} of Cd-exposed oysters was above 36°C or below 20°C. Of these explanations, the latter appears more plausible. It appears highly unlikely that T_{cII} in Cd-exposed oysters in winter was above 36°C, as this temperature was lethal to both control and Cd-exposed oysters after prolonged exposure. Furthermore, the finding of elevated levels of anaerobic end products (L-alanine and acetate) in winter Cd-exposed oysters is consistent with their higher dependence on anaerobiosis already at 20°C. The lack of further accumulation of L-alanine and acetate in Cd-exposed winter oysters during acute warming may reflect either suppression of anaerobic metabolism by the combined Cd and heat stress, or stimulation of aerobic pathways. The former explanation appears more likely, since Cd is

known to inhibit mitochondrial function as well as anaerobic glycolysis in mollusks (Sokolova, 2004; Kurochkin *et al.*, 2009; Ivanina *et al.*, 2011; Kurochkin *et al.*, 2011; Sokolova *et al.*, 2012a). Future studies using broader range and higher resolution of the experimental temperatures, as well as the concomitant measurements of aerobic and anaerobic metabolism, will be needed to unequivocally test these alternative hypotheses.

The downward shift of T_{cII} and an earlier onset of temperature-induced anaerobiosis in Cd-exposed oysters may be explained by the progressive tissue hypoxemia driven by a mismatch between the tissue oxygen demand and the oxygen supply capacity of respiratory and circulatory systems (Lannig *et al.*, 2006a; Lannig *et al.*, 2006b; Lannig *et al.*, 2008). This may be due to the Cd-induced increase in the basal cost of maintenance, impaired ventilation or circulation limiting oxygen supply to the tissues, interference of Cd with the mitochondrial function or combination of these factors (Butler and Roesijadi, 2000; Lannig *et al.*, 2008; Sokolova and Lannig, 2008; Muyssen *et al.*, 2010). Earlier studies showed that oysters exposed to Cd have elevated standard metabolic rate (SMR) due to increased energy costs of the synthesis of protective proteins such as heat shock proteins, metallothioneins and antioxidants (Livingstone, 2001; Cherkasov *et al.*, 2006; Ivanina *et al.*, 2008a). Cd exposure and elevated temperatures also increased ventilating activity and heart rate in oysters (Lannig *et al.*, 2006a; Lannig *et al.*, 2006b), but this increase was insufficient to compensate for the tissue oxygen uptake leading to reduced hemolymph oxygen content (Lannig *et al.*, 2008) that could eventually lead to the onset of partial anaerobiosis as seen in our present study. Similar to our present study, the negative effects of trace metals on thermal tolerance were documented in other aquatic organisms. Thus, exposure to Cd or chromium (Cr) reduced the CT_{max} (critical thermal maximum determined by onset of the neural damage) and decreased blood O_2 concentrations in marine prawn *Macrobrachium rosenbergii* and in stonefly nymph *Cliopepla clio* (Poulton *et al.*, 1989; Rosas and Ramirez, 1993). The metal-induced decrease in CT_{max} was also seen in fish (e.g. in coho salmon *Oncorhynchus kisutch* and steelhead trout *Salmo gairdneri* on exposure to nickel and in muskellunge fry *Esox masquinongy* on exposure to arsenic) (Paladino and Spotila, 1978; Becker and Wolford, 1980). A decrease in thermal tolerance due to exposure to toxic metals therefore appears a common phenomenon in aquatic

extotherms (including mollusks, fish and crustaceans) and can jeopardize survival of their populations during seasonal warming and/or global climate change.

Cellular energy status and oxidative stress

Phosphagen (PLA) serves as a cellular energy buffer rapidly restoring ATP in a one-step reaction during periods of intense energy demand. R_{PLA} ratio decreased in response to Cd exposure and acute warming in spring oysters consistent with the elevated energy demand and consumption of PLA to buffer ATP levels under the conditions of high ATP turnover. However, there was no clear correlation between PLA levels or R_{PLA} ratios and onset of anaerobic metabolism across all experimental groups showing that these markers have insufficient resolution to detect T_{cII} . No depletion of energy (glycogen or lipids) stores was observed at and beyond T_{cII} , and tissue ATP levels. Furthermore, adenylate energy charge and ADP/ATP ratios were maintained across all experimental temperatures in control and Cd exposed oysters. This indicates strong homeostatic mechanisms regulating cellular energy status not only in the optimum, but also in the pessimum temperature range. In fact, in spring oysters the glycogen and lipid content transiently increased at T_{cII} possibly reflecting metabolic rate depression and slow-down of the substrate oxidation during transition to the suboptimal temperatures. During extreme warming (36°C) a decrease in the tissue levels of ADP and/or AMP was observed indicating that the cellular energy status may be partially maintained by transamination of the adenylate pool. Overall, our data show that the negative shifts in the cellular energy status only occur when oysters are nearing the lethal temperature range. This agrees with the earlier findings showing that ATP levels and adenylate energy charge are maintained during severe energy stress, such as caused by prolonged oxygen deprivation in oysters and other facultative anaerobes (Grieshaber *et al.*, 1994; Kurochkin *et al.*, 2009; Ivanina *et al.*, 2010; Ivanina *et al.*, 2011). This indicates that unlike accumulation of anaerobic end products (which are an early sign of transition into the pessimum temperature range), parameters of the cellular energy status cannot serve as sensitive markers of the critical temperatures in oysters.

Acute temperature rise elicited oxidative stress in the cold-acclimatized, winter oysters (albeit at the temperatures exceeding T_{cII}), while in spring oysters no signs of the excess oxidative

lesions were detected at any temperature. Notably, the tissue levels of MDA and carbonyls were negatively correlated with the concentrations of adenylates suggesting that energy deficiency and negative shifts in the cellular energy status may contribute to higher susceptibility to oxidative stress either directly (e.g. via increasing metabolic rates to compensate for ATP deficiency) or indirectly, by limiting the amount of energy available for antioxidant production. A close relationship between the potential for ATP generation and the capacity to counteract oxidative stress has been earlier documented in other species (Dowd *et al.*, 2013) and may be a common feature of marine bivalves. The impressive resilience to oxidative damage in spring oysters may reflect upregulation of the antioxidant defenses in preparation for the warmer season and/or lower susceptibility of the warm-acclimated membranes to oxidation (Lau *et al.*, 2004; Regoli *et al.*, 2004b; Malanga *et al.*, 2007; Crockett, 2008). This is consistent with the overall lower levels of the oxidative lesions in spring compared to winter oysters acclimated at the same temperatures.

Conclusions and perspectives

Our data indicate that long-term exposure to environmentally relevant levels of Cd ($50 \mu\text{g L}^{-1}$) negatively affects the thermal tolerance of oysters by shifting the upper critical temperature (T_{cII}) to lower values. This shift may be explained by the Cd-induced reduction of the aerobic scope caused by Cd-induced suppression of the mitochondrial function and an ensuing mismatch between elevated energy demand and reduced aerobic capacity for energy supply. This finding is in agreement with the earlier proposed concept of oxygen- and capacity-limited thermal tolerance (OCLTT) (Bozinovic and Pörtner, 2015) and indicates that environmental toxins (such as trace metals) that negatively affect aerobic metabolism may increase sensitivity of aquatic ectotherms to elevated temperatures during seasonal warming and/or global climate change. Our data also support the notion that transition to partial anaerobiosis (indicated by the accumulation of the anaerobic end products) is the most sensitive biomarker of transition to the energetically non-sustainable state at critical temperatures and is well correlated with the ecologically relevant thermal limits of the species (Pörtner and Knust, 2007; Sokolova, 2013). As the organism nears the thermal limits where only short-term survival is possible, the progressing energetic stress leads to a breakdown of the cellular energy and redox homeostasis indicated by the depletion of adenylate stores and elevated

oxidative stress. This study demonstrates the usefulness of the energy-based biomarkers for analyzing the effects of multiple stressors in aquatic ectotherms and has implications for understanding the potential impacts of seasonal heat stress or global warming on ectotherm populations from polluted estuaries.

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Table 1. ANOVA: Effects of season, Cd exposure and temperature, and their interactions on the levels of Cd and oxidative lesions in mantle tissues of *C. virginica*.

DF – degrees of freedom for the main factor or interaction effects. The degrees of freedom for error are given in brackets on the top of the F-value column for each metabolite. Significant effects are highlighted in bold.

Factors / Interactions	DF	Cd burdens		Carbonyls		MDA	
		F (99)	P	F (140)	P	F (132)	P
Cd exposure (Cd)	1	119.94	<.0001	0.01	0.757	7.1	0.009
Season	1	9.29	0.003	101.1	<.0001	40.49	<.0001
Temperature (T°)	4	0.4	0.808	2.63	0.037	0.67	0.616
Cd x Season	1	3.15	0.079	0.69	0.408	6.91	0.010
T° x Cd	4	0.3	0.880	1.54	0.215	0.18	0.947
T° x Season	4	0.44	0.778	2.66	0.035	0.38	0.823
T° x Cd x Season	4	0.66	0.620	0.9	0.468	1.23	0.301

Table 2. ANOVA: Effects of season, Cd exposure and temperature, and their interactions on metabolite levels in gill tissues of *C. virginica*.

DF – degrees of freedom for the main factor or interaction effects. The degrees of freedom for error are given in brackets on the top of the F-value column for each metabolite. Significant effects are highlighted in bold.

Factors / Interactions	DF	Acetate		Succinate		L-Alanine		L-Arginine	
		F (174)	P	F (161)	P	F (158)	P	F (123)	P
Cd exposure (Cd)	1	18.12	<.0001	0.24	0.625	4.5	0.035	10.04	0.002
Season	1	88.18	<.0001	22.96	<.0001	37.56	<.0001	10.35	0.002
Temperature (T°)	4	8.45	<.0001	0.76	0.551	2.17	0.074	1.93	0.110
Cd x Season	1	38.45	<.0001	1.23	0.268	0.5	0.482	10.62	0.001
T° x Cd	4	8.08	<.0001	0.31	0.872	1.3	0.274	2.71	0.033
T° x Season	4	6.88	<.0001	1.43	0.225	10.14	<.0001	0.8	0.529
T° x Cd x Season	4	13.41	<.0001	0.43	0.789	4.72	0.001	0.54	0.655

Factors / Interactions	DF	PLA		PLA + L-Arginine		R-PLA	
		F (110)	P	F (115)	P	F (113)	P
Cd exposure (Cd)	1	2.96	0.088	0.28	0.598	1.43	0.234
Season	1	134.06	<.0001	118.73	<.0001	228.04	<.0001
Temperature (T°)	4	0.27	0.894	0.47	0.759	2.62	0.039
Cd x Season	1	8.3	0.005	2.65	0.106	24.1	<.0001
T° x Cd	4	0.29	0.885	0.54	0.706	3.13	0.017
T° x Season	4	1	0.396	0.42	0.742	2.59	0.056
T° x Cd x Season	4	0.56	0.640	1	0.395	0.4	0.754

Table 3. ANOVA: Effects of season, Cd exposure and temperature, and their interactions on levels of adenylates and cellular energy indices in gill tissues of *C. virginica*.

DF – degrees of freedom for the main factor or interaction effects. The degrees of freedom for error are given in brackets on the top of the F-value column for each metabolite. Significant effects are highlighted in bold.

Factors / Interactions	DF	ATP		ADP		AMP		Total adenylates	
		F (169)	P	F (147)	P	F (147)	P	F (133)	P
Cd exposure (Cd)	1	0.18	0.669	0.81	0.370	0.29	0.590	0.9	0.344
Season	1	39.06	<.0001	27.3	<.0001	125.37	<.0001	10.13	<.0001
Temperature (T°)	4	0.8	0.528	1.61	0.176	8.13	<.0001	1.97	0.103
Cd x Season	1	5.4	0.021	1.89	0.171	2.22	0.138	0.29	0.590
T° x Cd	4	0.34	0.850	1.36	0.249	2.07	0.087	0.3	0.876
T° x Season	4	0.67	0.613	2.5	0.045	1.76	0.141	0.83	0.477
T° x Cd x Season	4	2.1	0.083	1.05	0.384	0.74	0.565	2.83	0.027

Table 4. ANOVA: Effects of season, Cd exposure, temperature, tissue type, and their interactions on levels of glycogen, glucose and lipids in tissues of *C. virginica*.

DF – degrees of freedom for the main factor or interaction effects. The degrees of freedom for error are given in brackets on the top of the F-value column for each metabolite. Significant effects are highlighted in bold. Glycogen and glucose were measured in gills, hepatopancreas and muscle tissues, while lipids were only measured in gills and hepatopancreas due to the limited amount of tissue samples from the muscle.

Factors / Interactions	DF	Glycogen		Glucose		DF	Lipids	
		F (243)	P	F (241)	P		F (136)	P
Cadmium (Cd)	1	3.21	0.074	4.81	0.029	1	0.32	0.574
Season	1	20.44	<0.0001	1.26	0.263	1	65.62	<.0001
Temperature (T°)	2	2.83	0.061	0.98	0.376	2	20.71	<.0001
Tissue	2	9.21	0.0001	12.13	<0.0001	1	0.26	0.611
Season x Cd	1	0.85	0.358	3.31	0.070	1	2.56	0.112
T° x Cd	2	0.97	0.380	1.01	0.365	2	1.15	0.320
Tissue*Cd	2	0.1	0.907	2.18	0.116	1	0.13	0.721
Season x T°	2	1.05	0.351	1.06	0.348	2	24.53	<.0001
Season x Tissue	2	17.07	<0.0001	1.97	0.142	1	5.66	0.019
Tissue x T°	4	2.82	0.026	0.57	0.682	2	1.31	0.272
T° x Season x Cd	2	1.87	0.1561	1.22	0.296	2	1.69	0.188
Tissue x Cd x Season	2	0.48	0.617	3.78	0.024	1	1.05	0.306
Tissue x Cd x T°	4	1.99	0.096	0.45	0.773	2	0.07	0.936
Season x Tissue x T°	4	3.05	0.018	1.28	0.280	2	1.72	0.182
Season x Cd x Tissue x T°	4	0.54	0.705	1.27	0.283	2	0.58	0.580

FIGURE CAPTIONS

Figure 1. Temperature profile during the acute warming of experimental oysters. The downward open arrow indicates the time when oysters were placed in the experimental tanks. The solid line corresponds to the temperature profile, and the upward facing arrows indicate the times when sub-samples of experimental oysters were collected for each of the five target temperatures.

Figure 2. Accumulation of anaerobic end products during acute temperature rise in gill tissues of control and Cd-exposed *C. virginica*.

Tissue levels of L-alanine (A, B) and acetate (C, D) are given. Winter oysters – A, C; spring oysters – B, D. Open columns – control oysters; black columns – Cd-exposed oysters. Asterisks indicate values that are significantly different from the respective value at the acclimation temperature (20°C) measured for the same season and Cd exposure condition ($P < 0.05$). Daggers indicate values that are significantly different between control and Cd-exposed oysters at the same experimental temperature and in the same season ($P < 0.05$). All concentrations are given in $\mu\text{moles per g}$ of wet mass. For winter oysters, $N=6-8$ except control oysters at 32°C where $N=2$. For spring oysters, $N=9-13$.

Figure 3. Effects of acute temperature rise and Cd exposure on concentrations of the phosphagen and aphosphagen in gill tissues of *C. virginica*.

Tissue levels of phosphagen (phospho-L-arginine)(A, B), aphosphagen (L-arginine) (C, D) and the proportion of the phosphagen in the total phosphagen/aphosphagen pool (R_{PLA}) (E, F) are given. Winter oysters – A, C, E; spring oysters – B, D, F. Open columns – control oysters; black columns – Cd-exposed oysters. Asterisks indicate values that are significantly different from the respective value at the acclimation temperature (20°C) measured for the same season and Cd exposure condition ($P < 0.05$). Daggers indicate values that are significantly different between control and Cd-exposed oysters at the same experimental temperature and in the same season ($P < 0.05$). N.d. – values not determined. All concentrations are given in $\mu\text{moles per g}$ of wet mass. $N=4-8$ and $7-8$ for winter and spring oysters, respectively.

Figure 4. Effects of acute temperature rise and Cd exposure on concentrations of adenylates in gill tissues of *C. virginica*.

Tissue levels of ATP (A, B), ADP (C, D) and AMP (E, F) are given. All concentrations are in $\mu\text{moles per g}$ of wet mass. Winter oysters – A, C, E; spring oysters – B, D, F. Open columns – control oysters; black columns – Cd-exposed oysters. Asterisks indicate values that are significantly different from the respective value at the acclimation temperature (20°C) measured for the same season and Cd exposure condition ($P < 0.05$). Daggers indicate values that are significantly different between control and Cd-exposed oysters at the same experimental temperature and in the same season ($P < 0.05$). For winter oysters, $N = 6-8$ except control oysters at 32°C where $N = 2$. For spring oysters, $N = 9-13$.

Figure 5. Effects of acute temperature rise and Cd exposure on tissue levels of glycogen and lipids in gills, hepatopancreas and adductor muscle of *C. virginica*.

A, B, G, H – gills; C, D, I, J – hepatopancreas, E, F – adductor muscle. Winter oysters – A, C, E, G, I; spring oysters – B, D, F, H, J. Open columns – control oysters; black columns – Cd-exposed oysters. Glycogen levels were measured in gills, hepatopancreas and adductor muscle tissues, while lipids were only measured in gills and hepatopancreas due to the limited amount of tissue samples from the muscle. Asterisks indicate values that are significantly different from the respective value at the acclimation temperature (20°C) measured for the same season and Cd exposure condition ($P < 0.05$). Daggers indicate values that are significantly different between control and Cd-exposed oysters at the same experimental temperature and in the same season ($P < 0.05$). All concentrations are given in mg per g of wet mass. For winter oysters, $N = 6-8$ except control oysters at 28°C and 32°C where $N = 3$ and 1 , respectively. For spring oysters, $N = 8-14$.

Figure 6. Effects of acute temperature rise and Cd exposure on the levels of oxidative stress markers in the mantle tissues of *C. virginica*.

Winter oysters – A, C; spring oysters – B, D. Open columns – control oysters; black columns – Cd-exposed oysters. Asterisks indicate values that are significantly different from the respective value at the acclimation temperature (20°C) measured for the same season and Cd

exposure condition ($P < 0.05$). Daggers indicate values that are significantly different between control and Cd-exposed oysters at the same experimental temperature and in the same season ($P < 0.05$). $N = 5-10$ except the winter control oysters at 32°C where $N = 3-4$.

Figure 7. A schematic representation of the relationships between the studied metabolic parameters and the critical temperatures (T_c) of metabolism in different treatment groups.

A – the relationships among the studied traits based on Pearson correlations. Black solid and red dotted connector lines represent relationships with positive and negative correlations, respectively. Only significant correlations (based on Pearson correlation analysis) are shown. Correlations that explain over 36% of covariation among the studied traits (the correlation coefficient > 0.6 or < -0.6) are shown by thick lines.

B – putative upper critical temperatures (T_{cII}) of aerobic metabolism in control and Cd-exposed oysters. Note that T_c could not be determined in winter Cd-exposed oysters.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7