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Temperature-dependent stress response in oysters, *Crassostrea virginica*: Pollution reduces temperature tolerance in oysters

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Abstract

Combined effects of temperature and a toxic metal, cadmium (Cd), on energy metabolism were studied in a model marine bivalve, the eastern oyster *Crassostrea virginica*, acclimated at 20, 24 and 28 °C and exposed to 50 μg l^{-1} of Cd. Both increasing temperature and Cd exposure led to a rise in standard metabolic rates, and combined stressors appeared to override the capability for aerobic energy production resulting in impaired stress tolerance. Oysters exposed to elevated temperature but not Cd showed no significant change in condition, survival rate and lipid peroxidation, whereas those exposed to both Cd and temperature stress suffered high mortality accompanied by low condition index and elevated lipid peroxidation. Furthermore, RNA/DNA ratios indicative of protein synthesis rate, and levels of glutathione, which is involved in metal detoxification, increased in Cd-exposed oysters at 20 °C but not at 28 °C. Implications of the synergism between elevated temperatures and cadmium stress on energy metabolism of oysters are discussed in the light of the potential effects of climate change on oyster populations in polluted areas.

Keywords: Oyster; Temperature; Cadmium; Aerobic scope; Lipid peroxidation; RNA/DNA ratios

1. Introduction

Marine ectotherms inhabiting coastal shallow waters and estuaries such as oysters are exposed to a wide range of environmental stressors including anthropogenic (such as water and sediment pollution) and natural ones (such as daily and seasonal fluctuations in temperature, oxygen or CO_2 levels). Temperature plays a key role among these environmental factors due to its direct impact on all physiological processes. Most estuarine ectotherms are eurythermal organisms and can survive a broad range of body temperatures. However, at the temperature extremes on both sides of their thermal optimum (so-called pejus temperatures), estuarine ectotherms will exhibit a progressive decrease in whole-organism aerobic scope due to a mismatch between oxygen supply and demand (see Pörtner, 2001, 2002). Beyond the thermal pejus range, at so-called critical temperatures, the aerobic scope of an organism disappears and transition to anaerobic metabolism occurs due to insufficient oxygen supply via ventilation and/or circulation (Pörtner et al., 1998; Frederich and Pörtner, 2000; Sartoris et al., 2003; Lannig et al., 2004). Reduced aerobic scope may negatively affect an organism's fitness due to physiological tradeoffs, which can divert energy from essential processes such as growth, reproduction or locomotion towards maintenance (Sibly and Calow, 1986; Pörtner et al., 2001). Adjustment of the components of energy balance by acclimation may partially counteract these effects and allow an organism to maintain positive scope for growth, activity and reproduction; however, if thermal acclimation is incomplete or impossible, stress and reduced fitness can ensue.

In estuarine ectotherms, environmental stressors such as pollutants may negatively affect energy balance either due to an increase in energy expenditure and/or the reduction of metabolic energy capacity thereby reducing aerobic scope. In light of the concept of oxygen-limited thermal tolerance (Pörtner, 2001), such a reduction of aerobic scope would result in decreased thermal tolerance by the organism. By the same token, reduced aerobic scope at elevated temperatures may decrease tolerance for toxins due to limitations in energy supply for detoxification, elimination of the toxin and/or repair of the toxin-induced

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cellular damage. This creates a physiological basis for synergism between the effects of elevated temperature and pollution stresses, which may have important consequences for inhabitants of anthropogenically polluted estuarine and coastal waters.

Accumulation of pollutants such as trace metals is a global phenomenon, and high metal levels have been reported in many estuarine and coastal areas (http://www.oceansatlas.com/unatlas/-ATLAS-/chapter12; GESAMP, 1987). Cadmium (Cd) is among the most common metal pollutants in estuaries. It has no known biological function in animals and can interfere with various physiological processes in organisms from invertebrates to mammals (Shore and Douben, 1994; Ramachandran et al., 1997; Roméo et al., 2000). In particular, energy metabolism, membrane transport and protein synthesis are affected, and Cd may also act on DNA directly or indirectly by interference with genetic control and repair mechanisms (Hassoun and Stohs, 1996; Prusky and Dixon, 2002; Sokolova, 2004). In marine bivalves, Cd causes oxidative cellular damage by interfering with the antioxidant defense systems (Viarengo et al., 1990; Géret et al., 2002a), inducing lipid peroxidation (Géret et al., 2002b; Company et al., 2006) and ultimately causing apoptosis (Sokolova et al., 2004). It has also been shown that exposure to Cd may increase cellular oxygen demand in bivalves indicating elevated maintenance costs, mostly due to the elevated cost of the proteins synthesis (Cherkasov et al., 2006). In contrast, mitochondrial capacity for ATP synthesis does not increase in parallel to compensate for the elevated cellular energy demand and may in fact be inhibited at higher Cd levels (Sokolova, 2004; Sokolova et al., 2005; Cherkasov et al., 2006, in press). Taken together, these data clearly indicate that trace metal pollution may have detrimental effects on various aspects of metabolism in aquatic organisms and thus can conceivably result in an energy-deficient condition (Sokolova, 2004; Barata et al., 2005; Campbell et al., 2005; Cherkasov et al., 2006, in press).

Elevated temperatures due to seasonal changes and/or long-term trends of global climate change may pose additional threats to survival of estuarine ectotherms (review in Walther et al., 2002) both through lethal effects of extreme temperatures and most importantly, due to sublethal chronic effects on energy metabolism and susceptibility to other stressors such as pollution. Therefore, combination of exposure to trace metals and elevated temperatures is an environmentally relevant scenario in many estuaries, which is expected to become more widespread in the future with the global warming. Currently, there are few studies, which address interactive effects of toxic metals and elevated temperatures in aquatic ectotherms (Denton and Burdon-Jones, 1981; Sokolova, 2004; Hallare et al., 2005; Dunca et al., 2005; Cherkasov et al., 2006). In this study, we investigate how an environmentally relevant stressor such as heavy metal pollution affects aerobic metabolism and interferes with temperature-dependent performance in a model marine mollusk, the eastern oyster, *Crassostrea virginica*. The objectives of this study were to find out: (1) which physiological parameters are responsible for the synergistic effect between elevated temperature and Cd stress on energy metabolism such as earlier reported in *C. virginica* (Sokolova, 2004) and (2) if temperature rise due to seasonal warming and/or global climate change may pose a more significant threat to ectothermic organisms inhabiting polluted rather than pristine areas. To this end, we determined standard metabolism as an indicator for the metabolic cost of maintaining physiological integrity, as well as general condition and survival rate of the experimental animals exposed to temperature, Cd or combination of both. Furthermore, temperature- and Cd-induced changes in cellular indices of metabolic activity and oxidative stress were determined by measuring glutathione content (GSH), which is an important modulator of cellular redox homeostasis, malondialdehyde levels (MDA) as an indicator for lipid peroxidation due to oxidative stress and RNA/DNA ratios, which reflect the transcriptional activity and rates of protein synthesis.

2. Materials and methods

2.1. Experimental set-up

Wild-cultured oysters (*C. virginica*, 2–3 years old, 103 ± 16 mm shell length) were collected from Stump Sound, NC (USA) in Fall 2004–Winter 2005. Water temperature at the times of collection varied between 10 and 20 °C; average salinity was 30 ppt. Study sites have very low background concentrations of pollutants (Mallin et al., 1999; Swartzenberg, personal communication). Oysters were transported to the University of North Carolina at Charlotte within 8 h of collection and placed in recirculating aerated tanks with artificial seawater (Instant Ocean®, Kent Marine, Acworth, USA) at 18 ± 2 °C and 30 ppt salinity. After 5 days, several replicate tanks were randomly designated as 20, 24 and 28 °C treatments, and the temperature in those tanks was changed at 2 °C day^{-1} to reach the target temperatures. Temperature in the tanks was maintained within 0.5 °C of the respective target temperature. Following pre-acclimation (at least 10 days), during which no mortalities occurred, 50 μg l^{-1} Cd (as $CdCl_2$) was added to half of the replicate tanks at each temperature (Cd-exposed oysters), while the other half was maintained in clean seawater (controls). Oysters were fed three times per week with commercial algal blend (PhytoPlex®, Kent Marine, Acworth, GA). Water was changed every other day. In order to avoid Cd depletion in Cd-exposed tanks, a static-renewal design was used, with Cd supplementation to the nominal concentration of 50 μg l^{-1} during each water change. Each water tank contained 25–30 oysters, and water volume was approximately 4–5 l per oyster. Cd levels were measured in water samples at least three times a week immediately before and 1 h after water changes. There was no difference in Cd levels before and after water change indicating that the maintenance conditions were adequate to prevent Cd depletion. The average Cd concentration in experimental tanks was 41.1 ± 5.6 μg l^{-1} (mean + S.D., $n = 54$), and Cd levels in the control tanks were below the detection limits of the method used (0.5 μg l^{-1}). Measurement of whole-organism oxygen consumption and tissue sampling for nucleic acid and metabolite analysis were conducted at intervals during the 40 days (20 days for 28 °C) of experimental exposures. Due to the high mortality rates in Cd-exposed oysters at 28 °C, the longest experimental exposure was 20 days for this group.

2.2. Whole-organism oxygen consumption and condition index

Standard metabolic rate (SMR) was measured as resting oxygen consumption (MO_2) in control and Cd-exposed oysters after 20 and 40 days of exposure at their respective acclimation temperature using microfiber optic oxygen probes (Tx-Type, PreSens GmbH, Germany, http://www.presens.de). Two-point calibration was performed at each temperature. Oyster shells were carefully scrubbed and cleaned of fouling organisms. Oysters were placed into flow-through respiration chambers and allowed to recover over night. To avoid interference with postprandial metabolism and feces excretion, animals were kept unfed for 24 h prior to the start of MO_2 recordings. Water flow (30–74 ml min^{-1}) was adjusted so that animals consumed less than 25% of O_2 at all times to avoid potential inhibitory effects of low oxygen levels on respiration rate. After measurements, oysters were dissected and tissue dry mass determined. SMR was calculated as follows:

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

where SMR is the normalized oxygen consumption (μmol O_2 g^{-1} dry mass h^{-1}), ΔP_{O_2} the difference in partial pressure between in- and out-flowing water (kPa), β_{O_2} the oxygen capacity of water (μmol O_2 l^{-1} kPa^{-1}), V_{fl} the flow rate ($l\,h^{-1}$), M the oyster dry tissue mass (g) and 0.8 is the allometric coefficient for *Crassostrea gigas* (Bougrier et al., 1995). Due to the limited size range of oysters used in the present study, the allometric coefficient for scaling of MO_2 on body mass could not be determined. However, this relationship is highly conserved in marine bivalves (von Bertalanffy, 1957; Bayne and Newell, 1983). Therefore, we have used the allometric coefficient from a closely related oyster species *C. gigas* to correct for potential size effects on metabolism in *C. virginica*.

In order to compare differences in activity levels between different exposure groups, respiratory time activity (RTA) was determined as the percentage of time spent active by the oyster (open valves for re-oxygenation of the fluids within the shell) relative to the total experimental time as described in detail by Bougrier et al. (1998).

A general condition index (CI) of the experimental oysters was calculated as follows (see Lucas and Beninger, 1985; Cruz-Rodríguez et al., 2000):

$$\mathrm{CI} = \left(\frac{\text{tissue dry mass (g)}}{\text{shell dry mass (g)}} \right) \times 100.$$

For oysters used in MO_2 determinations, both wet and dry tissue mass was determined to the nearest 0.1 mg, and the wet/dry mass ratios calculated for different experimental conditions. For determination of the dry tissue mass, samples were dried for 36–48 h at 70 °C until the tissue mass stabilized, cooled in a desiccator and weighed. Oysters whose tissue was used for determination of RNA/DNA ratio and tissue metabolite levels could not be dried; therefore, dry tissue mass of those samples was calculated from the wet tissue mass using wet/dry tissue ratios determined in MO_2 experiments.

2.3. Cadmium determination

Cd concentrations were determined in water samples and tissue digests with an atomic absorption spectrometer (AAnalyst 800; Perkin-Elmer) that was equipped with a graphite furnace and Zeeman background correction as described in Cherkasov et al. (2006, in press). The detection limit of this method was 0.5 $\mu g\,l^{-1}$ water sample and 5 ng g^{-1} dry mass tissue.

2.4. Tissue metabolite levels

Gill tissues were quickly removed, immediately shock-frozen in liquid nitrogen and stored in liquid nitrogen until further investigation. In oysters, gills are a primary site of uptake of trace metals characterized by early accumulation of Cd in mitochondrial fractions (Sokolova et al., 2005), making this tissue appropriate for a study of physiological and metabolic effects of Cd.

RNA/DNA ratio was determined as described in Caldarone et al. (2001). Briefly, tissue powdered under liquid nitrogen was homogenized in at 55 °C for 2–24 h in 7.5 volumes of STE (sodium-tris-EDTA) buffer containing 100 mM NaCl, 10 mM Tris–HCl and 1 mM EDTA, pH 7.7 at 20 °C with 1% sodium dodecyl sulfate and 5 mg/ml proteinase K. Following this, the homogenate was incubated for 20 min at 75 °C to halt proteinase K activity and centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was removed for analysis. Nucleic acids were estimated spectrophotometrically at 525/600 nm (excitation/emission) with ethidium bromide as the fluorophore. RNA and DNA values were determined by calculating the loss of fluorescence following treatment with RNAse and DNAse, respectively.

Tissue levels of malondialdehyde (MDA), a by-product of lipid peroxidation, were determined using a thiobarbituric acid assay (Uchiyama and Mihara, 1978; Ringwood et al., 1999). Briefly, gill tissue powdered under liquid nitrogen was homogenized in four volumes of 50 mM potassium phosphate buffer (pH 7.0 at 20 °C), sonicated for 3–5 s (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) and centrifuged for 5 min at 13,000 × g and 4 °C. Sample supernatants as well as blanks and MDA standards of known concentrations were mixed with 0.375% thiobarbituric acid (TBA) and 2% butylated hydroxytoluene (BHT) in the following ratio: 1:14:0.14, heated for 15 min at 100 °C and again centrifuged (13,000 × g, 5 min, room temperature). The formation of a pink chromagen by reaction between MDA and TBA was measured spectrophotometrically at 532 nm. Tissue levels of MDA were expressed in μmol g^{-1} wet mass.

Glutathione (GSH) concentration was determined enzymatically by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)–gluthatione disulfide (GSSG) reductase recycling procedure following the rate of formation of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm (Griffith, 1980; Anderson, 1985). Gill tissue ground under liquid nitrogen was homogenized in 10 volumes of 5% sulfosalicylic acid (SSA), sonicated for 5 s (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA), and centrifuged (13,000 × g, 5 min, 4 °C). Supernatant was filtered

through glass wool prior to measurements of total GSH. Subsamples were taken to measure the amount of oxidized GSH (GSSG) by blocking reduced GSH (redGSH) with 2-vinylpyridine and adjusting pH with triethanolamine (6 < final pH < 7). These steps were processed rapidly to maintain redGSH/GSSG ratio. After 60 min of incubation at room temperature, GSSG levels were measured. Results are expressed as redGSH/GSSG ratios and total GSH content in μmol g^{-1} wet mass.

2.5. Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific (Suwanee GA, USA) and were of analytical grade or higher. Enzymes were purchased from Sigma–Aldrich.

2.6. Data analysis

Effects of temperature and Cd exposure on the studied variables were tested using generalized linear models analysis of variance (ANOVA, SigmaStat or InStat, SAS) after testing the assumptions of normality of data distribution and homogeneity of variances. Post hoc procedures (Tukey's honest significant difference, Dunnett's tests and *t*-test or Dunn's multiple comparisons test if nonparametric method was required) were used to compare sample means as appropriate. Factor effects and differences between the means were considered significant if the probability of Type II error was less than 0.05. Values are given as means (±standard error). Since data of the measured parameters did not change significantly over time in control animals values were pooled and presented as one value in the figures unless indicated otherwise.

3. Results

Increasing acclimation temperature had a moderate impact on survival rates of oysters (Fig. 1A). After 30 days, control oysters showed no mortality at 20 °C and only 4% and 25% mortality at 24 and 28 °C, respectively. When oysters were additionally challenged by Cd, only 54% of oysters survived to 20 days of exposure at 28 °C (Fig. 1A); therefore, we terminated this treatment and killed animals to take tissue samples. In contrast, oysters exposed to Cd at 20 and 24 °C had a 96% and 92% survival rate, respectively, after 40 days of Cd exposure.

Impact of Cd exposure on oyster general condition index and metabolism was strongly related to acclimation temperature. General condition index (CI) of oysters was in the range of values reported elsewhere (Chu and La Peyre, 1993), and was not affected by acclimation temperature alone ($P = 0.2584$ for controls at different temperatures) or by Cd exposure at 20 and 24 °C ($P > 0.05$ for control versus Cd-exposed oysters). However, condition index decreased significantly (by 31% after 20 days) in Cd-exposed oysters acclimated to high temperature 28 °C ($P = 0.0214$; Fig. 1B). Moreover, CI of Cd-exposed oysters at 28 °C was significantly lower than in their Cd-exposed counterparts at 20 or 24 °C (10 days of Cd exposure, $P = 0.0092$; 20 days of exposure, $P = 0.0019$).

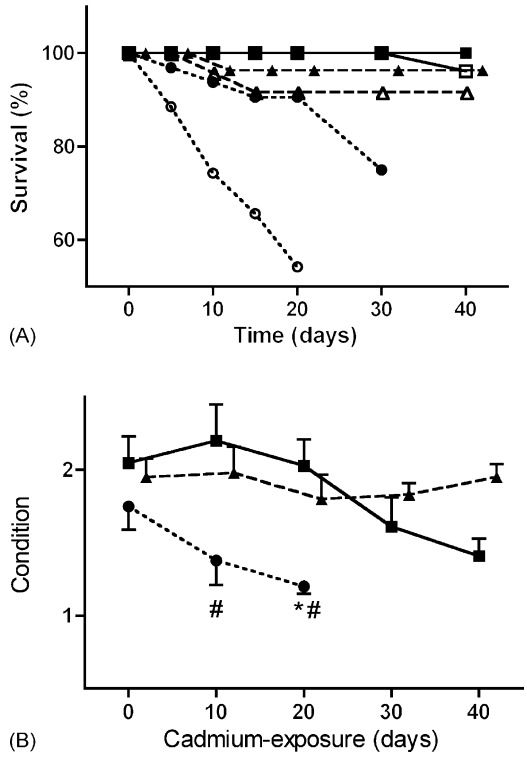

Fig. 1. Survival rate (A) and general condition (B) of oysters dependent on Cd exposure and acclimation temperature ((■) 20 °C, (▲) 24 °C and (●) 28 °C). In (A) controls are represented by closed symbols and Cd-exposed groups by open ones (controls: (■) 20 °C, (▲) 24 °C and (●) 28 °C; Cd-exposed: (□) 20 °C, (△) 24 °C and (◯) 28 °C; $n = 24$–50). In (B) values in control animals did not significantly change over time and thus were pooled and shown as 0 days of exposure ($n = 5$–15 (20 °C), $n = 6$–19 (24 °C) and $n = 5$–11 (28 °C)). (*) Values significantly different from respective controls; (#) values significantly different to values from the other acclimation groups when compared at the same Cd exposure time. For better viewing (▲) was shifted to the right.

Cadmium concentrations in control oysters were close to the AAS detection limit while in Cd-exposed oysters there was a time-dependent Cd accumulation in gill tissue (Fig. 2). Acclimation temperature had no effect on Cd accumulation resulting in similar values in 20- and 28 °C-acclimated oysters after 20 days of Cd exposure ($P = 0.4879$; Fig. 2).

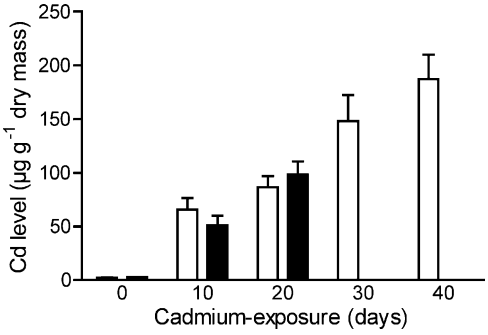

Fig. 2. Levels of Cd accumulation in gill tissue of oysters dependent on acclimation temperature ((□) 20 °C ($n = 3$–14) and (■) 28 °C ($n = 5$–12)). Values in control animals did not significantly change over time and thus were pooled and shown as 0 days of exposure. Cd accumulation in 24 °C-acclimated oysters was not determined.

Warming from 20 to 28 °C resulted in a doubling of standard metabolic rate (SMR). SMR at 28 °C was 68.3 ± 10.5 μmol O_2 h^{-1} g^{-1} dry mass compared to 40.6 ± 2.7 at 24 °C and 30.0 ± 6.3 at 20 °C ($P < 0.001$, Fig. 3A control animals). After 20 and 40 days of exposure to 50 μg l^{-1} Cd, SMR of Cd-exposed oysters strongly increased at 20 and 24 °C compared to the respective controls (Fig. 3A). Interestingly, the extent of Cd-stimulated increase in SMR was higher at 20 °C than at 24 °C (53–86% at 20 °C compared to 40% increase at 24 °C after 20–40 days of Cd exposure), so that the resulting SMR was similar in Cd-exposed oysters at 20 and 24 °C ($P > 0.05$). In contrast, Cd exposure resulted in no further increase in SMR at 28 °C after 20 days of exposure ($P = 0.8226$). No data are available for oysters exposed to Cd for 40 days at 28 °C due to high mortality.

Respiratory time activity (RTA, Fig. 3B) was similar in control groups irrespective of acclimation temperature ($P = 0.56$). In contrast, Cd-exposed oysters spent significantly more time open and actively ventilating at 28 °C than at 20 and 24 °C after 20 days Cd exposure ($P < 0.01$). When compared to the respective control groups Cd-exposed oysters showed elevated RTA at 28 °C ($P = 0.0054$) whereas at 20 and 24 °C no Cd-induced change in RTA was observed (20 °C, $P = 0.5662$; 24 °C, $P = 0.3312$).

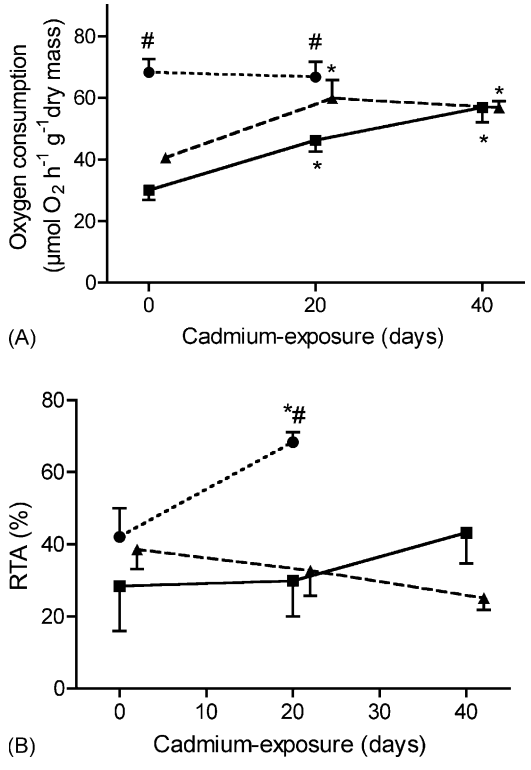

Fig. 3. Standard metabolism (A) and respiratory time activity (B) of oysters dependent on Cd exposure and acclimation temperature ((■) 20 °C ($n = 4$), (▲) 24 °C ($n = 4$–8) and (●) 28 °C ($n = 5$–7)). Values in control animals did not significantly change over time and thus were pooled and shown as 0 days of exposure. (*) Values significantly different from respective controls; (#) cases when values for 28 °C-acclimated oysters were significantly different from 20 to 24 °C-acclimated groups at the same exposure time except at 20 days cadmium exposure where 28 °C SMR was significantly different from 20 °C but not 24 °C value. For better viewing (▲) was shifted to the right.

At the cellular level, lipid peroxidation in the gills of oysters was unaffected by acclimation temperature, as indicated by similar malondialdehyde (MDA) levels in control groups at different temperatures ($P = 0.7839$, Fig. 4). Cd exposure did not alter lipid peroxidation at 20 °C (MDA levels: 0.49 ± 0.08 versus 0.46 ± 0.08 μmol g^{-1} wet mass after 30 days of Cd exposure; $P = 0.6548$), whereas the combination of elevated temperatures and Cd exposure was found to increase lipid peroxidation (Fig. 4). At 24 and 28 °C, MDA levels were notably higher in Cd-exposed oysters compared to respective controls already after 20 days Cd exposure (24 °C, 0.80 ± 0.11 versus 0.51 ± 0.04 μmol g^{-1} wet mass, $P = 0.0019$; 28 °C, 0.75 ± 0.08 versus 0.55 ± 0.05 μmol g^{-1} wet mass, $P = 0.0405$). Furthermore, MDA levels of Cd-exposed oysters tended to be higher at elevated temperatures (24 and 28 °C) when compared to their 20 °C-acclimated counterparts at the same exposure times.

The ratios of reduced GSH to GSSG (redGSH/GSSG) which indicate cellular redox status, were lower in warm-acclimated control oysters (28 °C) as compared to their 20- or 24 °C-acclimated counterparts (Fig. 5A), although these differences were only statistically significant between 24- and 28 °C-acclimated groups ($P = 0.0187$) but not between 20- and 28 °C-accimated ones ($P > 0.05$). Cd exposure did not significantly affect redGSH/GSSG ratios of oysters acclimated to 20 and 24 °C ($P = 0.1070$ and 0.2369, respectively). At 28 °C we found a transient increase in redGSH/GSSG ratio in Cd-exposed after 10 days of Cd exposure ($P = 0.003$) which after 20 days decreased back to the control levels ($P > 0.05$).

Total glutathione content (GSH_{total}) was significantly lower in control oysters at 24 °C compared to 20 or 28 °C (0.66 ± 0.03 ≠ 1.29 ± 0.08 and 1.11 ± 0.05 μmol g^{-1} wet mass, respectively, $P < 0.001$; Fig. 5B). This difference between 20- and 24 °C-acclimated animals was maintained throughout the Cd exposure period ($P < 0.01$). GSH_{total} increased during Cd exposure at 20 and 24 °C and was 34–63% above the respective controls after 30 days of Cd exposure ($P = 0.0263$ and $P < 0.0001$

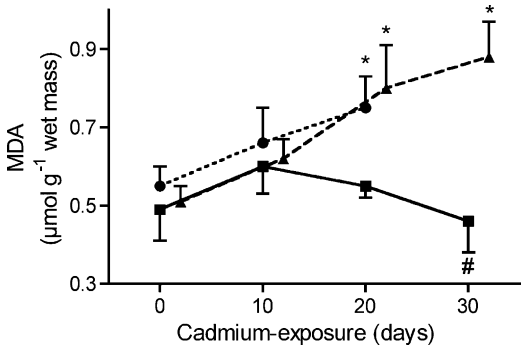

Fig. 4. Lipid peroxidation measured as malondialdehyde levels in gill tissue of oysters dependent on Cd exposure and acclimation temperature ((■) 20 °C ($n = 5$–10), (▲) 24 °C ($n = 6$–12) and (●) 28 °C ($n = 5$–9)). Values in control animals did not significantly change over time and thus were pooled and shown as 0 days of exposure. (*) Values significantly different from respective controls; (#) values significantly different to values from the other acclimation groups when compared at the same Cd exposure time. For better viewing (▲) was shifted to the right.

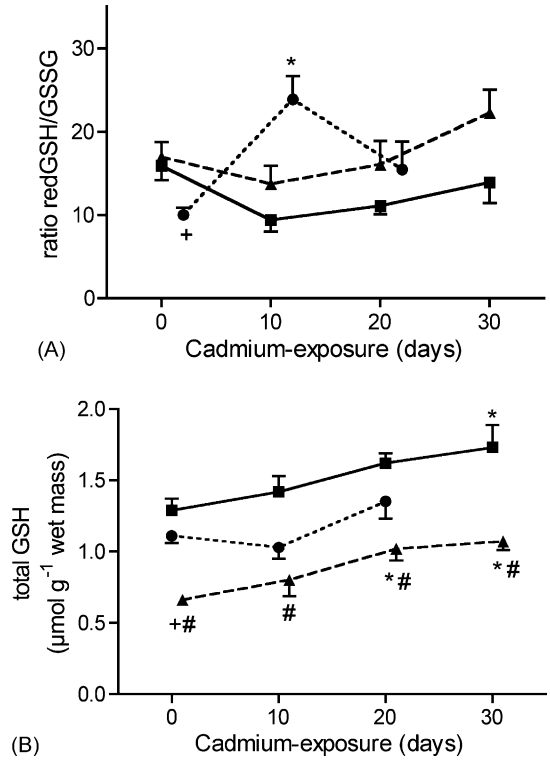

Fig. 5. Ratio of reduced GSH/GSSG (A) and total glutathione content (B) in gill tissue of oysters dependent on Cd exposure and acclimation temperature ((■) 20 °C (n = 4–14), (▲) 24 °C (n = 6–17) and (●) 28 °C (n = 5–11)). Values in control animals did not significantly change over time and thus were pooled and shown as 0 days of exposure. (*) Values significantly different from respective controls. Significant differences between values at 20 and 24 °C is marked by (#) and those between values at 24 and 28 °C by (+) for the respective Cd exposure times. For better viewing (▲) was shifted to the right.

for 20 and 24 °C, respectively). In contrast, no increase in GSH_{total} was found at 28 °C ($P > 0.05$).

Gill tissue RNA/DNA ratio, which serves as an index of instantaneous transcriptional activity, did not change over time during the 30 days of exposure in control or Cd-exposed oysters at either temperature ($P > 0.05$). Therefore, the respective values were pooled within control and Cd-exposed groups at each acclimation temperature to increase the power of analysis. In control oysters, RNA/DNA ratios were significantly lower at 20 °C than at 24 and 28 °C ($P < 0.02$ and $P < 0.0001$, respectively, Table 1). The RNA/DNA ratios at 28 °C were only marginally higher than at 24 °C ($P = 0.07$). Cd-induced changes in transcriptional activity were strongly related to the acclimation temperature. At 20 °C, a strong and significant increase in RNA/DNA ratio was found in Cd-exposed compared to control animals ($P < 0.0001$) whereas oysters acclimated to higher temperatures (24 and 28 °C) showed no Cd-induced rise in RNA/DNA ratios (24 °C, $P = 0.86$; 28 °C, $P = 0.43$). As a result, RNA/DNA ratios in Cd-exposed oysters were considerably higher at 20 °C than at 24 and 28 °C ($P = 0.02$). At 24 and 28 °C, the RNA/DNA ratios of the Cd-exposed oysters were not significantly different to each other ($P = 0.35$).

4. Discussion

Increasing acclimation temperature resulted in elevated SMR in control oysters indicating no metabolic compensation during warm-acclimation in these animals. The temperature quotient Q_{10} determined in the range of acclimation temperatures between 20 and 28 °C was 2.85 which is similar to the values ($Q_{10} \approx 3$) determined during acute temperature rise in oysters (Newell et al., 1977; Buxton et al., 1981). Q_{10} values were lower between 20- and 24 °C-acclimated groups of oysters (2.19) then between 24- and 28 °C-acclimated groups (3.59) indicating that warming to 28 °C may be metabolically more stressful than the moderate temperature increase to 24 °C.

Our study revealed a significant impact of Cd on oyster metabolic rate, which is in agreement with earlier studies showing elevated SMR in response to pollutant exposure (review in Calow, 1991; Roesijadi, 1996). It is worth noting that our exposure regime resulted in tissue Cd burdens similar to those found in oysters from polluted estuaries (Cherkasov et al., 2006; this study) indicating that the observed Cd-induced impact on metabolic rate occurs in response environmentally relevant Cd burdens. Notably, the extent of the impact of Cd on SMR was clearly temperature-dependent: the Cd-induced increase in SMR was highest at 20 °C, intermediate at 24 °C and absent at 28 °C. This temperature-dependent pattern closely follows Cd-induced changes in cellular oxygen demand at different temperatures (Cherkasov et al., 2006) and suggests that Cd-induced increase in whole-organism energy demand is to a large extent driven by the elevated cost of cellular maintenance. The observed differences in metabolic response to Cd cannot be attributed to differential tissue Cd accumulation but likely results from temperature–Cd interactions. Indeed, this study and earlier studies from our lab (Cherkasov et al., 2006, in press) revealed similar levels of Cd accumulation in gill and hepatopancreas tissues at different acclimation temperatures.

Table 1
RNA/DNA ratios in gills of control and Cd-exposed oysters, *C. virginica* acclimated to different temperatures

Group	Acclimation			Temperature effect
	20 °C	24 °C	28 °C	
Control	8 ± 1 (n = 4)	22 ± 3 (n = 6)	41 ± 5 (n = 14)	20 < [24 = 28]
Cd treated	62 ± 8 (n = 15)	23 ± 2 (n = 4)	41 ± 7 (n = 20)	20 > [24 = 28]
Cd effect	Cd > control	Cd = control	Cd = control	

All (>/<) designations are significant at $P < 0.05$; all (=) designations are not significant. Ratios in control as well as in Cd-exposed animals did not significantly change over time and thus were pooled within the respective treatments. Values are means ± standard error.

Interestingly, exposure to Cd led to a decreased temperature sensitivity of SMR, at least after 20 days of exposure (later dates could not be tested due to the mortality of Cd-exposed oysters at 28 °C). The temperature quotient Q_{10} (20–28 °C) was 1.55 in Cd-exposed oysters, much lower than in control oysters (2.85). This was mainly due to the strong reduction in Q_{10} between 24 and 28 °C down to 1.32 in Cd-exposed oysters, compared to 3.59 in controls. The different Q_{10} values between control and Cd-exposed oysters may be partly explained by a Cd-induced loss of temperature dependence of mitochondrial capacities. Indeed, respiration rates of mitochondria were strongly temperature-dependent with a Q_{10} of ≈2 under control conditions, whereas addition of 25–50 μmol l^{-1} of Cd resulted a decrease of Q_{10} values down to ≈1 indicating complete loss of thermal sensitivity of mitochondrial function (temperature range 15–25 °C, Sokolova, 2004).

The above-discussed notion that Cd exposure results in elevated energy (and thus oxygen) demand especially at elevated temperatures, is further supported by the fact that oysters spent significantly more time open when exposed to combination of high temperature and Cd indicating increased proportion of time spent actively ventilating. Interestingly, it is known that oysters can isolate themselves from unfavorable environmental conditions (especially poor water quality) by closing their valves thereby surviving on anaerobic metabolism for prolonged periods of time (Dunnington, 1986). Our data show that at elevated temperatures the necessity to cover high metabolic demand by enhanced oxygen uptake may take precedence over this protective mechanism.

The inability of Cd-exposed oysters to further increase SMR at 28 °C despite enhanced ventilation suggests that the capacity of oxygen supply mechanisms may be overridden by combination of elevated temperatures and Cd exposure. This assumption is supported by our observation that hemolymph oxygen tensions significantly decreased in Cd-exposed compared to control animals during an acute temperature increase (4 °C per 36 h, G.L., unpublished data), which points to a mismatch between oxygen demand and supply in these oysters. In aquatic invertebrates including bivalves (Pörtner et al., 2006) and crustaceans (Frederich and Pörtner, 2000), impaired oxygen supply at elevated temperatures has been shown to result from the temperature-dependent failure in ventilatory and circulatory capacities. In contrast, during acute warming in teleost fish, oxygen supply is mostly limited by the circulatory system capacity (Lannig et al., 2004). It is not known which mechanism(s) may limit oxygen delivery in oysters at high temperatures and/or under conditions of Cd exposure. One of the possibilities may be the direct impairment of mitochondrial function in oyster gills by elevated temperatures and temperature/Cd combination (Sokolova, 2004; Sokolova et al., 2005; Cherkasov et al., in press and references therein) resulting in a reduced capacity for oxygen uptake. Other factors (such as insufficient circulation and/or cardiac failure) may also contribute to oxygen limitation and require further investigation. However, irrespective of the mechanisms, the mismatch between the energy demand and energy supply in Cd-exposed oysters is likely to be highest at the elevated temperatures resulting in synergism between these two stressors.

As a result of the mismatch between elevated energy demand and limited energy supply during exposures to elevated temperatures and Cd, energy-dependent cellular protective mechanisms involved in detoxification and damage repair are more likely to fail leading to elevated mortality and whole-organism physiological stress. Indeed, our data clearly show the degree of Cd-induced physiological stress is strongly increased by elevated temperature. At 20 and 24 °C no significant changes in general condition index and survival rate were observed in Cd-exposed animals implying that oysters were able to compensate for the increased energy demand to combat toxic stress. This is also indicated by significantly increased SMR and apparently positive net energy balance. Put in the perspective of the concept of oxygen-limited thermal tolerance (Pörtner, 2001), these data suggest that in the temperature range between 20 and 24 °C oysters are still in the optimal range of aerobic scope, which ensures efficient stress response and adequate physiological condition and survival performance. In contrast, 28 °C may be close the suboptimal (pejus) temperatures for this species. This is supported by the fact that *C. virginica* does not deposit shell material at water temperatures above 28 °C (Surge et al., 2001) suggesting no "scope for growth" (Warren and Davis, 1967), and by our data showing that oysters are not capable to sufficiently withstand sublethal Cd stress at this temperature. The absence of an increase in SMR in response to Cd (this study) and decreased mitochondrial aerobic capacity (Cherkasov et al., 2006, in press) in 28 °C-acclimated oysters also points towards the reduced aerobic scope. Overall, this study clearly suggests that a stressor that affects metabolic energy balance (such as Cd) becomes critical once an organism is in its thermal pejus range where the aerobic scope is limited. Given the importance of metabolic balance in determining thermal tolerance limits in aquatic ectotherms, we suggest that this finding may have broader implications and will hold true not only for Cd, but also for other pollutants and environmental stressors which require elevated energy demand for stress protection. This hypothesis, however, is outside the scope of the present work and requires further investigation.

Elevated SMR (and thus maintenance cost) in animals exposed to heavy metals such as Cd, may reflect increased costs of detoxification and cellular protection and repair. In particular, protein synthesis is known to increase during temperature or pollution stress due to induction of molecular chaperones such as heat-shock proteins and metal chelators including metallothioneins (Anderson et al., 1999; Viarengo et al., 1999; Butler and Roesijadi, 2000; Cruz-Rodríguez et al., 2000; Ettajani et al., 2001; Ahearn et al., 2004; Boutet et al., 2004). Additionally, protection against cell damage caused by metal-induced oxidative stress can involve a compensatory elevation of the antioxidative enzymes (Regoli et al., 1998; Livingstone, 2001; Basha and Rani, 2003). This increase in protein synthesis is expected to reflect in elevated transcriptional activity and thus, higher RNA/DNA ratios. As shown by Cherkasov et al. (2006) significant Cd-induced increases in gill protein synthesis rates were found in 12 and 20 °C but not in 28 °C-acclimated oysters. An increase in the protein synthesis rate was a major

contributor to the observed increase in cellular oxygen consumption due to Cd exposure. This finding agrees with a strong increase in RNA/DNA ratios indicating elevated transcriptional activity in Cd-exposed oysters at 20 °C found in this study. In contrast, Cd exposure did not result in a significant increase in RNA/DNA ratios at 24 and 28 °C suggesting that warm-acclimated oysters may be less capable of mounting an adequate transcriptional response to support detoxification needs. Correspondingly, Hallare et al. (2005) found an increase in stress protein (hsp70) expression in fish larvae acclimated to intermediate (26 °C) but not high temperature (33 °C) in response to Cd exposure concluding that the production of heat shock proteins (HSPs) could have reached its maximum and that both stressors (temperature and Cd) have overridden the capability of cells to generate more HSPs. Further studies are needed to determine whether Cd-induced transcriptional activation of other stress proteins (such as metallothioneins or antioxidant enzymes) is also impaired at elevated temperatures. This may have important implications for the ectotherms' capability to efficiently withstand toxic metals in their environment during seasonal warming and/or global climate change.

The suggestion that elevated temperatures impair defense mechanisms against Cd exposure was supported by the increased lipid peroxidation in Cd-exposed oysters at 24 °C and especially 28 °C. Unexpectedly, this increase in lipid peroxidation was not paralleled by a change in the glutathione redox status as indicated by the redGSH/GSSG ratios. Glutathione serves as a primary nucleophile in numerous protective detoxification reactions and in the maintenance of cellular redox status (Meister and Anderson, 1983). The response of GSH to pollution exposure appears to vary considerably depending on the pollutant, model species and exposure regime. Typically, exposures to high Cd levels result in gluthathione depletion (Regoli and Principato, 1995; Ringwood et al., 1998; Mitchelmore et al., 2003; Toplan et al., 2003), whereas at lower Cd concentrations no change or even increase in the GSH levels can be observed (Viarengo et al., 1990; Ringwood et al., 1999; Mitchelmore et al., 2003; Regoli et al., 2004; this study). Possibly, the Cd-induced increase in total glutathione levels at 20 and 24 °C reflects a compensatory mechanism, which allows oysters to maintain constant redGSH/GSSG ratios despite Cd-induced oxidative stress, especially if GSH-recycling enzymatic mechanisms are limiting. Alternatively, increased levels of GSH may serve as metal chelators and aid in detoxification of Cd. It has been previously shown that GSH depletion strongly enhances Cd toxicity in oysters (Ringwood and Conners, 2000), supporting the suggestion that an increase in the total GSH in Cd-exposed oysters may represent a cellular protective mechanism. Notably, this increase was stunned in warm-acclimated oysters at 28 °C, which went hand-in-hand with elevated oxidative stress, poor physiological condition and high mortality in this experimental group. Overall, this research in conjunction with previous studies shows that the direction and extent of the Cd-induced change in glutathione concentration and redox status, as well as the resulting oxidative stress (indicated by MDA accumulation) strongly depend not only on Cd concentrations but also on the environmental temperature. This may explain why some earlier studies failed to detect a consistent relation between lipid peroxidation and GSH levels in oysters at different polluted sites *in situ* (Ringwood et al., 1999).

In conclusion, elevated temperatures such as expected in the case of seasonal warming and/or global climate change may threaten oyster populations in polluted areas due to the interaction of Cd and high temperature on energy budget and metabolism. Cd exposure leads to elevated energy costs as well as impaired aerobic energy production due to a progressive mismatch between oxygen demand and oxygen supply and increasing mitochondrial dysfunction with rising temperature. In the context of oxygen-limited thermal tolerance in aquatic ectotherms (Pörtner, 2001), pollution-exposed marine ectotherms that experience high standard metabolic rates due to detoxification and repair responses will have reduced aerobic capacities for additional stress responses. Therefore, environmental warming will pose a higher threat for animals in polluted versus non-polluted areas, and even low levels of pollution remain a cause for concern for marine ecosystems especially in the light of the predicted increase in temperature extremes. With respect to this, an interesting but presently unexplored link is a potential impact of combined temperature and pollution stress on oyster reproduction. Since gametogenesis will depend on both temperature and the availability of stored nutrient reserves, the "decision" to invest in reproduction is related to surplus energy (Roff, 1983; Shpigel et al., 1992). Given that adult oysters appear incapable of metabolic compensation in response to temperature increase (Beiras et al., 1995; present study), even a moderate temperature rise can lead to spawning failure in polluted areas due to metabolic energy deficiency. Indeed, such failure has been earlier shown in freshwater mollusks where production and cumulative fecundity decreased gradually with increasing Cd concentrations (Perceval et al., 2002). Investigation of the effects of temperature and pollution stress on reproduction represent a fruitful future direction of studies to complement the emerging picture of potential ecological impacts of these combined stressors on populations of marine ectotherms.

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