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Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae)

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Summary

Marine intertidal mollusks, such as oysters, are exposed to multiple stressors in estuaries, including varying environmental temperature and levels of trace metals, which may interactively affect their physiology. In order to understand the combined effects of cadmium and elevated temperature on mitochondrial bioenergetics of marine mollusks, respiration rates and mitochondrial volume changes were studied in response to different cadmium levels (0–1000 μmol l^{-1}) and temperatures (15, 25 and 35°C) in isolated mitochondria from the eastern oyster *Crassostrea virginica* acclimated at 15°C. It was found that both cadmium and temperature significantly affect mitochondrial function in oysters. Elevated temperature had a rate-enhancing effect on state 3 (ADP-stimulated) and states 4 and 4+ (representative of proton leak) respiration, and the rate of temperature-dependent increase was higher for states 4 and 4+ than for state 3 respiration. Exposure of oyster mitochondria to 35°C resulted in a decreased respiratory control and phosphorylation efficiency (P/O ratio) compared to that of the acclimation temperature (15°C), while an intermediate temperature (25°C) had no effect. Cadmium exposure did not lead to a significant volume change in oyster mitochondria *in vitro*. Low levels of cadmium (1–5 μmol l^{-1}) stimulated the rate of proton leak in oyster mitochondria, while not affecting ADP-stimulated state 3 respiration. In contrast, higher cadmium levels (10–50 μmol l^{-1}) had little or no effect on proton leak, but significantly inhibited state 3 respiration by 40–80% of the control rates. Elevated temperature increased sensitivity of oyster mitochondria to cadmium leading to an early inhibition of ADP-stimulated respiration and an onset of complete mitochondrial uncoupling at progressively lower cadmium concentrations with increasing temperature. Enhancement of cadmium effects by elevated temperatures suggests that oyster populations subjected to elevated temperatures due to seasonal warming or global climate change may become more susceptible to trace metal pollution, and *vice versa*.

Key words: mitochondria, cadmium, temperature, proton leak, phosphorylation rate, P/O ratio, mitochondrial volume, bivalve mollusk, *Crassostrea virginica*.

Introduction

Regulation of mitochondrial function plays a crucial role in adaptation and environmental stress tolerance. Recent studies of mitochondrial function under a variety of physiological and stress conditions have led to a realization that the mitochondrion is highly tuned and prone to malfunction in response to even moderate stress, rather than operating reliably in the background as was previously believed (for a review, see Nicholls, 2002). Marine organisms, including molluscs, are exposed to a variety of environmental stressors in their habitats, which can strongly affect their metabolism and bioenergetics. It has been shown that regulation and constraints of mitochondrial function play a critical role in setting the tolerance limits of mollusks to temperature, salinity and oxygen deficiency (Pörtner et al., 1998, 2001; Sokolova et al., 2000a; Pörtner, 2001; Sokolova and Pörtner, 2001, 2003). However, most studies conducted so far have concentrated on the effect of a single environmental factor on the mitochondrial bioenergetics of marine mollusks. In contrast, the effects of multiple stressors and their interactions, which are common in marine environments, on mitochondrial bioenergetics are poorly understood.

Temperature and heavy metals are common stressors in estuarine and coastal habitats, and their importance is increasing due to the global climate changes and continuing pollution of the coastal waters (Helmuth et al., 2002; GESAMP, 1987). Cadmium is a common inorganic contaminant of coastal sediments and waters, and the levels of this metal can vary greatly, due to both anthropogenic pollution

and to natural sources such as river run-off from cadmium-rich soils, leaching of the rocks or diatom deposition in marine sediment (GESAMP, 1987; Roesijadi, 1996; Frew et al., 1997). As are most trace metals, cadmium is toxic at high concentrations, and increasing evidence points toward mitochondrial dysfunction as an important mechanism of cytotoxicity of cadmium (for reviews, see Brierley, 1977; Byczkowski and Sorenson, 1984; Miccadei and Floridi, 1993; Wallace and Starkov, 2000). However, cadmium also affects mitochondrial function at non-toxic concentrations as low as 10^{-6} mol l^{-1} (Skulachev et al., 1967; Kesseler and Brand, 1994a–c, 1995; Ye et al., 2001). In terrestrial plants and mammals, cadmium is known as a powerful modulator of mitochondrial function, inhibiting electron transport chain, increasing generation of reactive oxygen species (Miccadei and Floridi, 1993; Wallace and Starkov, 2000), and stimulating proton leak through the inner mitochondrial membrane (Kesseler and Brand, 1994a–c, 1995; Korotkov et al., 1996a,b, 1999; Al-Nasser, 2000; Belyaeva et al., 2001). These data strongly suggest that mitochondria are key intracellular targets for cadmium; however, nothing is known about effects of this metal on mitochondrial function in marine mollusks.

In poikilotherms, a change in environmental temperature leads to a direct change in the rate of all physiological and biochemical processes and in the steady-state levels of metabolic intermediates (for a review, see Hochachka and Somero, 2002). Increasing the environmental temperature results not only in elevated respiration rates of mitochondria, but also in a considerable rise in mitochondrial proton leak and in progressive mitochondrial uncoupling in marine fish and bivalves (Pörtner, 2001). Therefore, cadmium and temperature can interact in their effects on bioenergetics of marine poikilotherms, because the mitochondrion is likely to be a common intracellular target for these environmental factors. So far, however, there are no data about temperature-dependent effects of cadmium on mitochondrial function in marine poikilotherms, and the mechanisms and potential effects of these multiple stressors on molluscan mitochondrial function are not known. This research intends to close this gap by studying the combined effects of cadmium and temperature on mitochondrial function using eastern oysters *Crassostrea virginica* as a model.

Eastern oysters are a useful model for the study of interactive effects of cadmium and temperature on mitochondrial bioenergetics. Like all intertidal organisms, they may experience rapid and extreme temperature fluctuations in their habitats, with a change in body temperature as large as 10–20°C within a few minutes during summer low tides (Sokolova et al., 2000b; Helmuth et al., 2002; Sokolova and Boulding, 2004). Oysters are also exposed to varying cadmium concentrations in their habitats, and have an ability to concentrate cadmium in soft tissues to concentrations exceeding the environmental levels by orders of magnitude (Roesijadi, 1996; Frew et al., 1997). Body levels of cadmium in natural oyster populations range from 0.4 to 40 μg g^{-1} dry mass (Roesijadi, 1996; Frew et al., 1997), corresponding to the intracellular concentrations of ca. 1–90 μmol l^{-1}. During acute exposure to elevated cadmium concentrations in water or sediments, oysters can accumulate even higher loads of this metal, up to 300–400 μg g^{-1} dry mass, which corresponds to intracellular concentrations of ca. 670–900 μmol l^{-1} (Roesijadi, 1996). About 80% of the accumulated cadmium in bivalves is sequestered by metallothioneins (Roesijadi, 1996; Giguere et al., 2003), which were previously believed to remove cadmium from the physiologically active pool. However, recent studies have shown that both free and metallothionein-bound cadmium ions have a potential to strongly affect mitochondrial function (Simpkins et al., 1994, 1998). Therefore, both temperature and cadmium are potent modulators of mitochondrial function of oysters in nature.

The aims of the present study were (1) to investigate the combined effects of elevated temperature and cadmium on mitochondrial function in *Crassostrea virginica*, including respiration rate, phosphorylation efficiency and proton leak in isolated mitochondria, and (2) to study the effects of cadmium on mitochondrial volume, which is essential for the maintenance of controlled mitochondrial function. The results of this study support the hypothesis that cadmium effects on mitochondrial function can be considerably modified by environmental temperature in marine poikilotherms, and that these two environmental stressors can have synergistic effects on oyster mitochondria. This study reports for the first time in a marine poikilotherm the sensitivity of different aspects of mitochondrial metabolism to a trace metal and temperature, and is a first contribution to a series of studies examining the effects of trace metals and temperature on oyster bioenergetics *in vitro* and *in vivo*.

Materials and methods

Animal collection and maintenance

Adult oysters *Crassostrea virginica* Gmelin (80–120 mm shell length) were collected from Hewletts Creek near Wilmington, NC, USA in May (‘spring oysters’) and July (‘summer oysters’) of 2003. Water temperature at the times of collection was 14–15°C and 25–27°C, in spring and summer, respectively, and salinity varied between 22 and 30‰. The study site has very low background concentrations of heavy metals and organic pollutants (Mallin et al., 1999), with average cadmium concentrations of 0.02 mg kg^{-1} of dry sediment. Animals were transported on ice to the University of North Carolina at Charlotte within 8 h after collection and placed in recirculated aquaria with artificial seawater (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 16±1°C and 650+20 mOsm. Animals were acclimated in the laboratory for 3–4 weeks prior to experimentation. During the acclimation period, oysters were fed on alternate days with a commercial algal blend (0.5 ml l^{-1}) containing *Nannochloropsis*, *Tetraselmis* and *Isochrysis* spp. ranging in size from 2 to 15 μm (PhytoPlex©, Kent Marine, Acworth, GA, USA). No mortality was detected during the preliminary acclimation period.

Isolation of mitochondria

Mitochondria were isolated from oyster gills using a method modified from Ballantyne and Moyes (1987). Isolation buffer had osmolarity of 730 mOsm and consisted of 300 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA, 1% bovine serum albumin (BSA, essentially fatty acid free), 2 μg ml^{-1} of a protease inhibitor aprotinin and 30 mmol l^{-1} Hepes (pH 7.5). Previous studies have shown that isolation of oyster mitochondria in a slightly hyperosmotic medium maximizes coupling and yields superior quality mitochondria as compared to iso- or hypoosmotic media (Ballantyne and Moyes, 1987).

Gills of 6 or 7 animals were removed, blotted dry and placed in 15 ml of ice-cold isolation medium. The tissue was homogenized with three passes (200 r.p.m.) of a Potter–Elvenhjem homogenizer using a loosely fitting Teflon pestle. The homogenate was centrifuged for 10 min at 2000 ***g*** and 2°C. The supernatant was collected, and the tissue pellet re-homogenized in 15 ml of ice-cold isolation buffer. The second homogenate was centrifuged at 2000 ***g***, and supernatants from the two centrifugations were pooled. The supernatant was then centrifuged at 10 500 ***g*** and 2°C for 12 min. The resulting mitochondrial pellet was washed twice with ice-cold EGTA-free isolation buffer to minimize cadmium binding by the chelator and resuspended in the ice-cold EGTA-free isolation buffer to give a mitochondrial protein content of 5–10 mg ml^{-1}.

Mitochondrial oxidation

Oxygen uptake by mitochondria was measured in 3 ml water-jacketed glass chambers using Clarke-type oxygen electrodes (YSI, Yellow Springs OH, USA). Two-point calibration of electrodes was performed at each experimental temperature, and continuous data acquisition was made using a BIOPAC Data acquisition system (BIOPAC, Santa Barbara, CA, USA). Temperature effects on mitochondrial respiration were determined during acute exposure of mitochondria of oysters acclimated at 15°C to different temperature increases (15, 25 and 35°C). This acute exposure to elevated temperatures is an environmentally realistic situation for intertidal animals including oysters, which may experience fast and acute rise in body temperature by 10–20°C in a few minutes during spring and summer low tides (Sokolova et al., 2000b; Helmuth et al., 2002; Sokolova and Boulding, 2004). Temperature in mitochondrial respiration chambers was maintained constant at 15, 25 or 35°C (+0.1°C) using a Fisher Isotemp (Suwanee, GA, USA) refrigerated water circulator. 3–5 volumes of assay medium were mixed with one volume of isolation medium containing the mitochondria. The assay medium had an osmolality of 650 mOsm and consisted of 150 mmol l^{-1} KCl, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} sucrose, 0.1% BSA, 2 μg ml^{-1} of aprotinin and 30 mmol l^{-1} Hepes (pH 7.2). All assays were completed within 2–3 h of isolation of the mitochondria. Preliminary experiments showed that there was no change in mitochondrial respiration or coupling during this period. A myokinase inhibitor AP_5A (5 μmol l^{-1}) was added to the assay buffer to prevent spontaneous regeneration of ADP from ATP by mitochondria. Sodium succinate was used as a substrate at saturating amounts (10–15 mmol l^{-1}) in the presence of 5 μmol l^{-1} of rotenone. Maximal respiration rates (state 3) were achieved by addition of 200–300 nmol ADP, and state 4 respiration was determined in ADP-conditioned mitochondria as described by Chance and Williams (1955). State 4+ respiration was determined as the oxygen consumption rate after addition of 2.5 μg ml^{-1} of an ATPase inhibitor oligomycin. State 4+ respiration in the presence of oligomycin is considered as a good upper limit estimate of mitochondrial proton leak measured at high mitochondrial membrane potential (Brand et al., 1994; Kesseler and Brand, 1995). At the end of the assay, KCN (100 μmol l^{-1}) and salicylhydroxamic acid (SHAM, 200 μmol l^{-1}) were added to the mitochondria to inhibit mitochondrial respiration and to measure the non-mitochondrial rate of oxygen consumption. SHAM was used in order to avoid overestimation of non-mitochondrial respiration rate due to the presence of an alternative oxidase in bivalve mitochondria (Tschischka et al., 2000). In all cases, non-mitochondrial oxygen consumption rate was less than 1–5% of the state 3 respiration. Respiration rates in states 3, 4 and 4+ were corrected for non-mitochondrial respiration and oxygen electrode drift.

Effects of cadmium on mitochondrial respiration *in vitro* were determined in oysters collected during June 2003. Each mitochondrial suspension was divided into 8 portions, and each portion was incubated for 10 min in the absence of cadmium (control) and at different concentrations of $CdCl_2$ in the range 1–50 μmol l^{-1}. After incubation, the respiration rates of state 3, 4 and 4+ were determined as described above. Addition of the highest concentration of cadmium used in this study (50 μmol l^{-1}) did not detectably change the pH of the assay buffer (i.e. the pH change was less than 0.01 units).

Oxygen solubility (β_{O_2}) for the assay medium at each experimental temperature was calculated as described in Johnston et al. (1994), and respiration rates were expressed as natom O min^{-1} mg^{-1} mitochondrial protein. Respiratory control ratio (RCR) was determined as a ratio of state 3 over state 4 respiration as described by Estabrook (1967), and RCR+ was determined as a ratio of state 3 respiration over state 4+ respiration (in the presence of oligomycin). P/O ratios were calculated by dividing the amount of added ADP by the amount of oxygen consumed in state 3 respiration (Hinkle, 1995). Inhibition constants for cadmium (K_i) were calculated assuming a noncompetitive inhibition model (Segel, 1976).

Mitochondrial swelling

Mitochondrial swelling was determined using a method modified from Li et al. (2003). Mitochondria were isolated as described above, and 100 μl of mitochondrial suspension added to 0.9 ml of the standard assay medium containing 20 mmol l^{-1} sodium succinate. Absorbance of the mitochondrial suspension was measured at 520 nm and 25°C using a UV/Vis Cary 50 spectrophotometer with a water-

jacketed cuvette holder (Varian, Victoria, Australia). After initial measurements, different concentrations of cadmium chloride were added to the cuvette. Mitochondria were incubated with cadmium for 20 min on ice, the cuvettes were then equilibrated for 3 min at 25°C, and changes in mitochondrial volume were monitored by measuring absorbance at 520 nm under constant stirring. Excess ADP (600 μmol) was then added to the cuvettes, incubated for 1 min at 25°C, and absorbance was measured at 520 nm under constant stirring. A reduction in absorbance of a mitochondrial suspension indicates mitochondrial swelling. For a positive control, mitochondria were incubated for 5 min in a hypo-osmotic assay buffer (375 mOsm).

Protein concentrations

Protein concentrations in mitochondrial suspensions were measured using a modified Biuret method with added 1% Triton-X to solubilize the mitochondria (Bergmeyer, 1988). BSA was used as the standard. Protein content was measured for each batch of the isolation medium and subtracted from the total protein content of the mitochondrial suspension to determine the mitochondrial protein concentration.

Statistics

Effects of the factors 'Temperature' and 'Cadmium concentration' and their interactions were analyzed using split-plot repeated-measures analysis of variance (ANOVA) after testing the assumptions of normality of data distribution and homogeneity of variances. Effects of factor interactions were significant for all studied variables except RCR+ (P<0.01, data not shown), thus preventing analysis of the effects of single factors. Therefore, we performed separate repeated-measures ANOVAs for each experimental temperature to test the effects of cadmium on the studied mitochondrial parameters. A sequential Bonferroni test was used to adjust probability levels for multiple ANOVAs. To analyze the effect of season (spring *vs* summer) and temperature on respiration rates and respiratory control ratios in oyster mitochondria, a mixed model ANOVA was used with 'Season' as a random factor and 'Temperature' as a fixed one. Dunnet tests were used for *post hoc* pairwise comparisons, and least-square difference test (LSD) for planned comparisons. Statistical analyses were performed using SAS 8.1 software (SAS Institute, Cary, NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05.

Results

Mitochondrial respiration

Temperature had significant effects on respiration rates, phosphorylation efficiency and respiratory control ratio (RCR) of oyster mitochondria (Table 1). Respiration rates increased with increasing temperature (Fig. 1A), with Q_{10} values generally higher for state 4 and 4+ respiration than for state 3 (Fig. 2). Notably, the temperature dependence of mitochondrial respiration was affected by cadmium, and at the highest cadmium concentration (50 μmol l^{-1}) state 3 and state 4+ respiration rates were independent of the temperature (Q_{10}=~1). Respiratory control ratios (RCR) and P/O ratios were similar at 15°C and 25°C but decreased significantly at 35°C (Fig. 1B). Notably, elevated temperatures led to a considerably higher variation of P/O ratios between mitochondria isolated from different oysters, so that the coefficient of variation for the P/O ratio increased from 8–15% at 15°C to 25–50% at 35°C. Variability of other mitochondrial parameters did not change with the temperature. RCR and P/O ratios were higher in spring oysters as compared to summer ones measured at the same temperature, whereas state 3, 4 or 4+ respiration rates did not significantly differ between oysters collected in spring and summer (Table 1, Fig. 1).

ADP-stimulated (state 3) respiration of oyster mitochondria was inhibited by cadmium in a dose-dependent manner (Fig. 3). Effects of cadmium on state 3 respiration were significant at all temperatures ($F_{6,24}$=7.71, P=0.0001, $F_{7,35}$= 15.30, P<0.0001, and $F_{5,20}$=33.70, P<0.0001 for 15°, 25° and 35°C, respectively). Notably, at 15°C only the

Table 1. *Effects of temperature and season (spring* vs *summer) on respiration rates, respiratory control ratio and phosphorylation efficiency of oyster mitochondria*

	Effect					
	Season		Temperature		Season×Temperature	
	$F_{(1,47)}$	P	$F_{(2,47)}$	P	$F_{(2,47)}$	P
State 3	0.03	0.86	83.07	<0.0001	0.34	0.71
State 4	5.95	0.02*	118.42	<0.0001	1.24	0.30
State 4+	2.6	0.11	90.55	<0.0001	0.04	0.96
RCR	34.7	<0.0001	42.8	<0.0001	0.89	0.42
RCR+	28.05	<0.0001	30.40	<0.0001	5.05	0.01*
P/O	9.16	0.004	8.28	0.0009	2.13	0.13

RCR, respiratory control ratio; P/O, phosphorylation efficiency.

F-ratio with degrees of freedom for the factor and error, and probability levels for Type II error are given. *Non-significant after sequential Bonferroni correction.

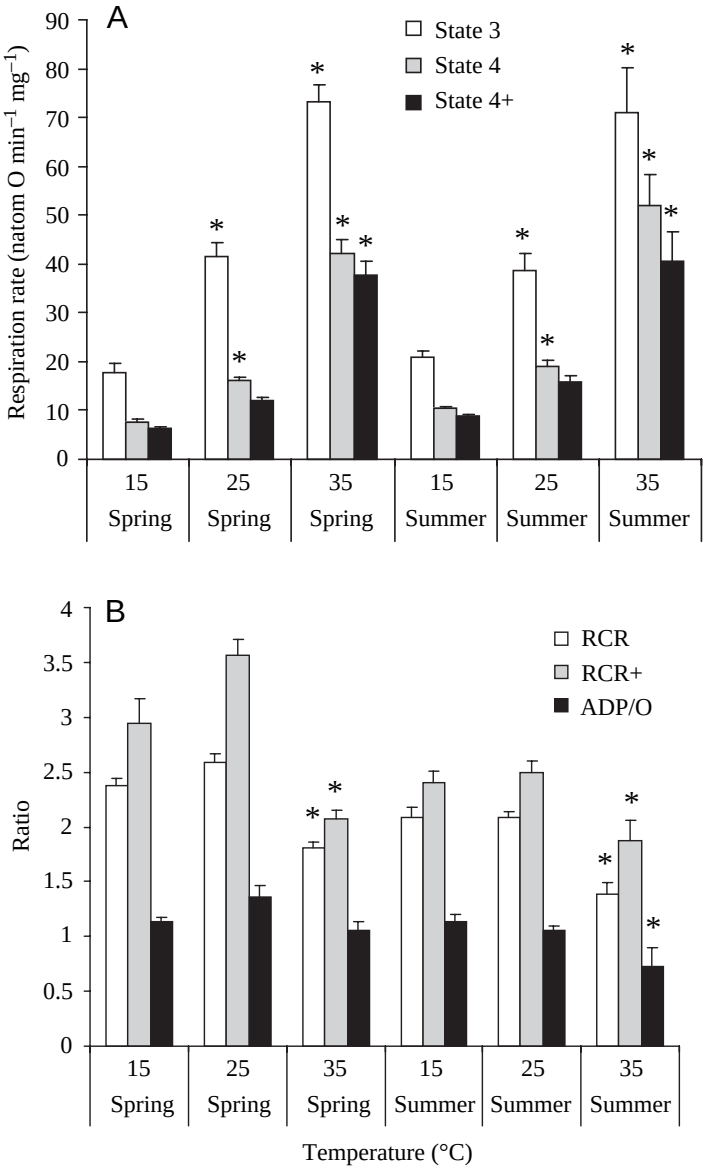

Fig. 1. Temperature effects on respiration rates (A), respiratory control and P/O ratio (B) of isolated oyster mitochondria. Respiration rates were measured after addition of ADP (state 3), after all ADP was used up (state 4) and in the presence of oligomycin (state 4+) in isolated oyster mitochondria at different temperatures. Respiratory control ratios were determined as the ratio of state 3 over state 4 respiration (RCR) or state 4+ respiration (RCR+) at different temperatures, and phosphorylation efficiency (ADP/O). Asterisks denote values that are significantly different from those of the respective controls measured at 15°C. Values are means ± S.E.M., N=6–8.

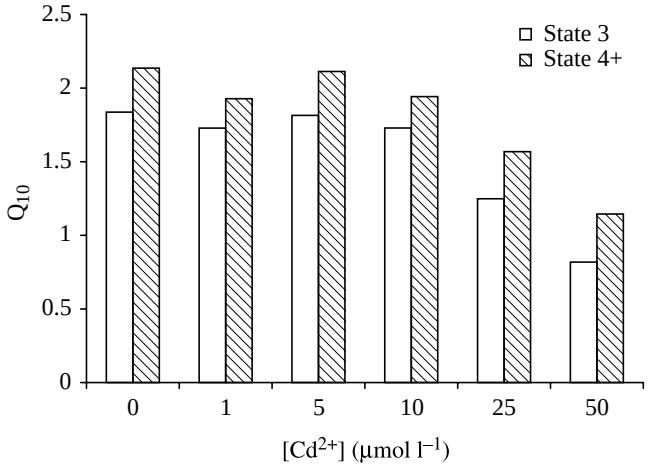

Fig. 2. Effects of cadmium concentration on temperature dependence (Q_{10}) of state 3 and state 4+ respiration in oyster mitochondria. Q_{10} of ~1 indicates a complete loss of temperature dependence of mitochondrial respiration at 25–50 μmol l^{-1} [Cd^{2+}].

highest cadmium concentrations (35–50 μmol l^{-1}) significantly inhibited state 3 respiration down to ca. 60% of control. In contrast, at 25° and 35°C a significant inhibition of state 3 respiration occurred at cadmium levels ≥25 μmol l^{-1} and ≥10 μmol l^{-1}, respectively, and at the highest cadmium concentration (50 μmol l^{-1}) state 3 respiration was only 10–30% of control rates. Higher cadmium sensitivity of state 3 respiration at elevated temperatures was reflected in the lower inhibition constants (K_i) for cadmium, which were 80, 24 and 3 μmol l^{-1} [Cd^{2+}] at 15°, 25° and 35°C, respectively.

State 4 respiration was significantly affected by cadmium at 25°C and 35°C but not at 15°C (15°C: $F_{6,23}$=1.90, P=0.125; 25°C: $F_{7,34}$=8.83, P<0.0001; 35°C: $F_{5,20}$=22.11, P<0.0001) (Fig. 4). Inhibition constants (K_i) for state 4 respiration were 172, 78 and 4 μmol l^{-1} [Cd^{2+}] at 15°, 25° and 35°C, respectively, indicating higher sensitivity of state 4 respiration to cadmium exposure at elevated temperatures.

State 4+ respiration in the presence of oligomycin, which is indicative of proton leak, was significantly and positively correlated with state 4 respiration while being ca. 10–20% lower than the latter. Effects of cadmium on state 4+ respiration were significant at all temperatures (15°C: $F_{6,24}$=4.71, P=0.003; 25°C: $F_{7,34}$=9.66, P<0.0001; 35°C: $F_{5,20}$=20.03, P<0.0001). Low concentrations of cadmium (″10 μmol l^{-1}) significantly stimulated state 4+ respiration of oyster mitochondria (Fig. 4). On average, exposure to 1–5 μmol l^{-1} Cd^{2+} led to 20–30% and 10–15% increase in state 4+ respiration at 15–25°C and 35°C, respectively, compared to control rates. Similar to states 3 and 4+, state 4 respiration of oyster mitochondria was increasingly more sensitive to cadmium as the temperature increased, as indicated by a decline of K_i values (154, 7 and 10 μmol l^{-1} [Cd^{2+}] at 15°, 25° and 35°C, respectively). ANOVA results and graphical analysis of the dose-dependent cadmium effects (Fig. 4) support the conclusion of higher sensitivity of state 4+ respiration to cadmium levels at elevated temperatures. Thus, at the highest cadmium level (50 μmol l^{-1}), state 4+ respiration was 83% of control rate at 15°C, but only 20% of control rate at 25–35°C.

Cadmium exposure resulted in partial uncoupling of oyster mitochondria, as indicated by a significant decrease in

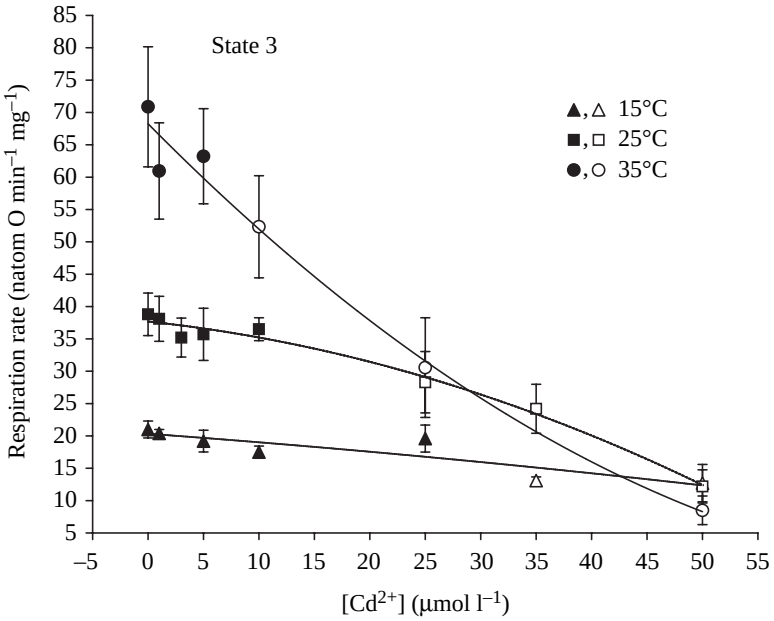

Fig. 3. Effects of cadmium concentration and temperature on state 3 respiration in isolated oyster mitochondria. Open symbols mark the values of state 3 respiration, which are significantly different from the respective controls (0 μmol l^{-1} of cadmium) measured at the same temperature. Values are means ± S.E.M., N=5–7.

respiratory control ratios ($F_{6,24}$=11.3, P<0.0001 and $F_{6,24}$=5.75, P=0.0007 for RCR and RCR+, respectively; Fig. 5). Uncoupling effects of cadmium on oyster mitochondria were observed at concentrations as low as 1 μmol l^{-1} and increased with increasing cadmium concentrations at all temperatures. Notably, elevated temperatures increased the sensitivity of mitochondria to cadmium-induced uncoupling, and the coupling was essentially abolished by exposure to 50 μmol l^{-1} Cd^{2+} at 25°C and 35°C, but not at 15°C.

In order to test that the observed effects on mitochondria were due to cadmium, 2.5 mmol l^{-1} EGTA was added to the assay medium along with 5–50 μmol l^{-1} of cadmium. The cadmium effects on respiration of oyster mitochondria were completely abolished by addition of EGTA at all temperatures (data not shown).

The P/O ratio was not significantly affected by cadmium at the concentrations 1–25 μmol l^{-1} ($F_{2,27}$=0.94, P=0.484). Determination of P/O ratio at high cadmium concentrations (>25 μmol l^{-1}) was not possible due to low coupling of mitochondria and thus high error associated with determination of the transition to state 4.

Mitochondrial volume

Incubation with 5–1000 μmol l^{-1} of cadmium did not result in swelling of oyster mitochondria (Fig. 6). There was a trend to the increase in absorbance at 520 nm (A_{520}) of mitochondria in the presence of 5–100 μmol l^{-1} of Cd^{2+}, indicating mitochondrial contraction, although it was statistically non-significant due to high variation ($F_{5,20}$=2.49, P=0.065). Addition of ADP resulted in a decrease in absorbance at 520 nm, indicating swelling of phosphorylating mitochondria, which was similar in control and cadmium-incubated mitochondria ($F_{5,20}$=1.24, P=0.327). Incubation of mitochondria in hypo-osmotic buffer (375 mOsm) used as a positive control led to a significant decrease in A_{520} by 0.155±0.036 absorbance units.

Discussion

This research clearly demonstrates that mitochondrial function in oysters is highly sensitive to cadmium at physiologically and environmentally relevant low concentrations. Exposure of isolated oyster mitochondria to cadmium resulted in a progressive uncoupling, which was already evident at the lowest studied concentration (1 μmol l^{-1}) and increased with the increasing dose of cadmium. Notably, mechanisms of this uncoupling were different at different cadmium levels due to the dissimilar effects of cadmium on ADP-stimulated respiration and proton leak in oyster mitochondria. At low cadmium concentrations (<10 μmol l^{-1}), mitochondrial uncoupling was due to the elevated proton leak as indicated by the increase in state 4+ respiration, whereas the maximum respiration rate of state 3 remained unaffected. At cadmium levels of ≥10 μmol l^{-1} the partial uncoupling of mitochondria was mostly due to the inhibition of state 3 respiration, whereas state 4 and 4+ respiration rates were not affected except at the highest Cd^{2+} concentrations. Although physiological mechanisms of response to cadmium differed at high and low cadmium levels, all concentrations of this metal studied resulted in the impaired ability of mitochondria for phosphorylation due to diverting of a progressively increasing part of the flux from phosphorylation to proton leak. A similar response to increased cadmium levels was found in mammalian mitochondria (for a review, see Byczkowski and Sorenson, 1984; Belyaeva et al., 2001) and in isolated potato tuber and corn mitochondria (Miller et al., 1973; Kesseler and Brand, 1994a–c). Thus, in rat hepatic mitochondria, addition of 5–10 μmol l^{-1} [Cd^{2+}] elevated state 4 respiration by ca. 30–40% with succinate as a substrate, and 5 μmol l^{-1} [Cd^{2+}] resulted in an increase of ca. 20% in state 4 respiration with glutamate as substrate (Cameron et al., 1986; Belyaeva et al., 2001). Higher concentrations of cadmium (>10–15 μmol l^{-1}) resulted in significant inhibition of state 3 respiration, but had very little effect on state 4 respiration in rat mitochondria oxidizing succinate or glutamate (Cameron et al., 1986; Belyaeva et al., 2001). State 4 respiration is predominantly due to the proton leak and is typically only 10–20% higher than state 4+ respiration in the presence of oligomycin, used as a measure of proton leak in the present study. The small proportion of state 4 respiration in excess of state 4+ respiration can be due to ADP regeneration by ATP-consuming enzymes in mitochondrial suspension (e.g. ATPases and kinases) and/or proton slip at high membrane potential (Grabe et al., 2000). In general, the similarity of

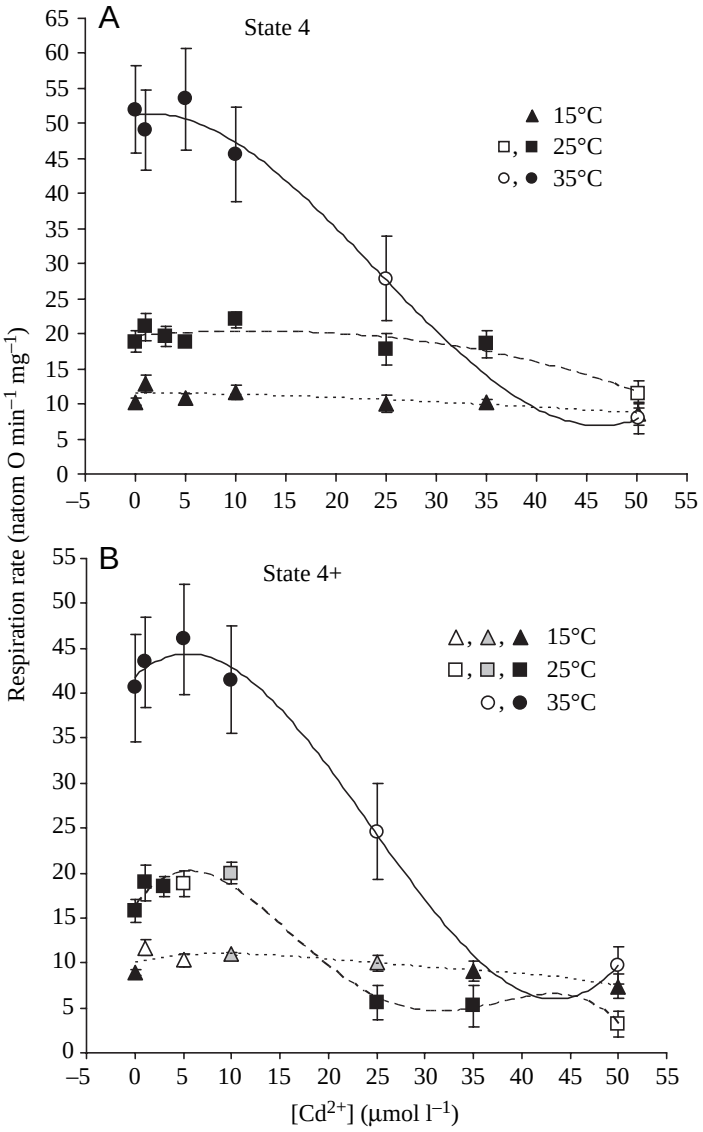

Fig. 4. Effects of cadmium concentration and temperature on (A) state 4 and (B) state 4+ respiration in isolated oyster mitochondria. Open symbols mark the values of state 4 or 4+ respiration, which are significantly different from the respective controls (0 μmol l^{-1} of cadmium) measured at the same temperature (P<0.05). Grey symbols mark marginally significant differences (P<0.06). Values are means ± S.E.M., N=5–7.

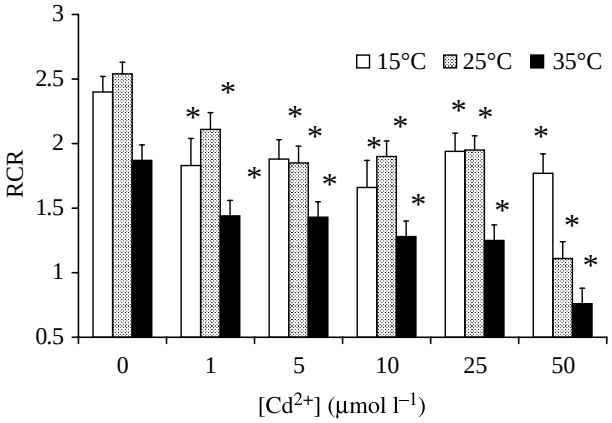

Fig. 5. Effects of cadmium concentration and temperature on respiratory control ratios (RCR) in isolated oyster mitochondria. Asterisks mark respiratory control ratios that are significantly different from the respective controls (0 μmol l^{-1} of cadmium) measured at the same temperature, are marked with asterisks. Values are means ± S.E.M., N=5-7.

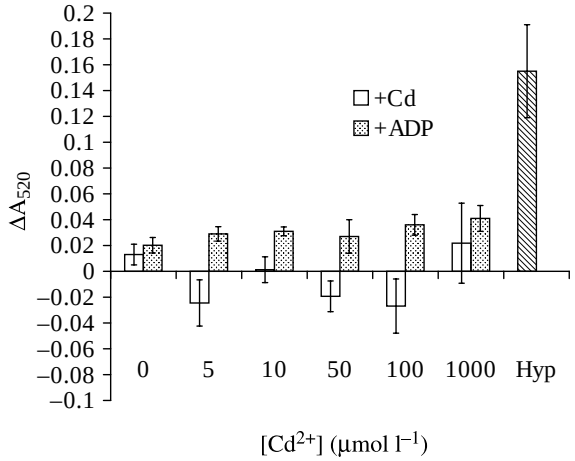

Fig. 6. Effects of cadmium concentration on *C. virginica* mitochondrial volume *in vitro*. Changes in mitochondrial volume were monitored as a change in absorbance of mitochondrial suspension at 520 nm at 25°C after 20 min of incubation with varying concentrations of cadmium (+Cd) and after subsequent addition of ADP (+ADP). Mitochondrial swelling in hypo-osmotic medium (375 mOsm, Hyp) was used as a positive control. This method only provides qualitative data about the relative change of mitochondrial volume (swelling *vs* contraction). Values are means ± S.E.M., N=5.

physiological response in cadmium-exposed mitochondria in mammals (Byczkowski and Sorenson, 1984; Belyaeva et al., 2001), plants (Kesseler and Brand, 1994a–c) and bivalves (this study) suggests that mitochondrial sites of cadmium action may be highly conserved across distant taxa. Interestingly, the *in vivo* effects of cadmium on mammalian mitochondria are very similar to the *in vitro* effects and involve impaired oxidative phosphorylation, decreased RCR and inhibited state 3 respiration, which occur in dose-dependent manner (Byczkowski and Sorenson, 1984). There are no published data concerning *in vivo* effects of cadmium on mitochondrial bioenergetics of marine bivalves. However, in freshwater bivalves *Elliptio complanata* and *Anodonta cygnea*, exposure to high levels of cadmium *in vivo* resulted in a decrease of aerobic respiration, significant depletion of glycogen reserves, decrease in intracellular ATP levels and transition to partial anaerobiosis, indicating severe disturbance of mitochondrial function and insufficient ATP production (Cheney and Criddle, 1996; Hemelraad et al., 1990b).

Although the general physiological response of oyster

mitochondria to increased cadmium concentrations is similar to that of mammals, unlike homeotherms the mitochondrial function of poikilotherms is also strongly affected by the environmental temperature. Indeed, the most important novel finding of the present study is that environmental temperature strongly modulates cadmium effects on oyster mitochondria. The results of the present study clearly demonstrate that sensitivity of mitochondrial function to cadmium increases with increasing temperatures in the environmentally relevant range. This is reflected in a dramatic drop of K_i values for cadmium with increasing temperature, indicating that 50% inhibition of mitochondrial respiration rate requires 80–170 μmol l^{-1} [Cd^{2+}] at 15°C but only 3–4 μmol l^{-1} [Cd^{2+}] at 35°C. The temperature-dependent increase in cadmium sensitivity was especially prominent for ADP-stimulated (state 3) respiration and respiratory control ratio (RCR and RCR+), reflecting the degree of mitochondrial coupling. Thus, the threshold cadmium concentrations, below which no effect on state 3 respiration was observed, were progressively shifted to lower values with increasing temperature from ≥35 μmol l^{-1} to ≥25 μmol l^{-1} and ≥5 μmol l^{-1} at 15, 25 and 35°C, respectively. Similarly, cadmium-induced uncoupling was faster and more pronounced with increasing temperatures. At 15°C, cadmium exposure in the range 1–50 μmol l^{-1} led to a similar small decrease in RCR, so that mitochondria retained coupling with a RCR of ca. 2 at all cadmium levels. In contrast, at 35°C, cadmium concentrations as low as 10 μmol l^{-1} resulted in a completely abolished mitochondrial coupling. In the absence of cadmium, only a small reduction of RCR and RCR+ was observed at the highest temperature (35°C), and mitochondria still retained coupling. This implies that elevated environmental temperatures may result in the onset of mitochondrial dysfunction at lower cadmium concentrations, which are essentially non-toxic at lower temperatures. State 4+ respiration rate in the presence of an ATPase inhibitor oligomycin is considered to be a good upper limit estimate of proton leak at high mitochondrial membrane potentials typical of resting mitochondria (Brand et al., 1994; Kesseler and Brand, 1995). Proton leak in resting mitochondria is an important determinant of basal metabolic rate (BMR) accounting for 20–30% of BMR both in poikilo- and homeotherms (Brand et al., 1991; Hulbert and Else, 1999). Our study demonstrated that low concentrations of cadmium (1–5 μmol l^{-1}) elevated proton leak by 20–30%. High levels of proton leak were also maintained at higher cadmium levels, in contrast to a considerable inhibition of ADP-stimulated respiration.

Cadmium-induced stimulation of the proton leak may have important implications for the whole organism BMR, leading to an increased cost of mitochondrial maintenance and thus to a higher cost of basal metabolism in cadmium-exposed oysters. Decreased coupling and elevated proton leak are also known to negatively affect thermal tolerance of poikilotherms, in particular, the critical temperatures characterized by the transition to partial anaerobiosis (Pörtner, 2001, 2002; Sokolova and Pörtner, 2003). Cadmium-induced mitochondrial dysfunction may result in an earlier failure of aerobic scope and the onset of anaerobiosis at progressively lower temperatures as cadmium levels in the environment increase. In order to test these hypotheses, further research is required to determine the intracellular cadmium levels and the effects of trace metals on BMR and heat tolerance *in vivo*. To date, studies of the heavy metal effects on the BMR of poikilotherms are scarce and have yielded controversial results. Thus, Hopkins and co-authors (Hopkins et al., 1999) have demonstrated that BMR is elevated by 32% in banded water snakes *Nerodia fasciata* exposed to heavy metals in their environments as compared to their conspecifics from the reference site. On the other hand, routine metabolic rates of various fish and invertebrates, which in addition to BMR included variable non-quantified contributions from physical activity, were either unaffected or inhibited by exposure to cadmium (Coenen-Stass, 1998; Leung et al., 2000; McGeer et al., 2000; Knops et al., 2001; Rajotte and Couture, 2002). In order to understand the role of trace metals in metabolic costs and regulation of BMR, carefully designed studies measuring BMR over a wide range of trace metal concentrations are required. The finding that cadmium may significantly increase proton leak in mitochondria and thus the cost of mitochondrial maintenance, also cautions against unverified interpretations of the increased metabolic rate in response to a toxicant as a cost for detoxification or transmembrane transport of the toxicant, and emphasizes the importance of taking into account the direct non-adaptive effects of a toxicant on the components of BMR, which may lead to an increase in basal metabolism.

Cadmium exposure did not result in swelling of oyster mitochondria in isoosmotic, K^+- and sucrose-containing medium. This contrasts with the results in mammalian mitochondria, where addition of cadmium resulted in considerable swelling due to energy-dependent accumulation of K^+ in the matrix and/or opening of the mitochondrial permeability pore (Brierley, 1977; Rasheed et al., 1984; Li et al., 2003). *In vivo* studies demonstrated an inconsistent response of mitochondrial volume and morphology to cadmium exposure and both mitochondrial swelling and contraction were reported depending on the cell type and cadmium concentration (Hemelraad et al., 1990a; Early et al., 1992; Al-Nasser, 2000; Romero et al., 2003). This suggests that mitochondrial volume changes in response to cadmium vary considerably between species and cell types. In the present study, there was a notable trend towards cadmium-induced increase in optical density of suspended oyster mitochondria, suggesting mitochondrial contraction rather than swelling. This indicates that, unlike mammalian mitochondria, oyster mitochondria do not undergo a permeability transition, which is typically associated with considerable mitochondrial swelling, even at high cadmium concentrations that completely inhibit respiration. This suggestion is supported by our recent finding that cadmium exposure does not result in depolarization of the mitochondrial membrane (I. M. Sokolova, S. Evans, and F. M. Hughes, manuscript in review). This suggests that, despite an impaired ability for ATP

synthesis and increased proton leak, the activity of proton pumps is sufficient to maintain the mitochondrial membrane potential and prevent depolarization and swelling in cadmium-exposed oyster mitochondria.

As a corollary, mitochondrial function in oysters is highly sensitive to cadmium and temperature, which have synergistic effects on mitochondrial respiration (particularly on ADP-stimulated respiration) and coupling. Cadmium exposure results in elevated proton leak, and reduces phosphorylation rate and coupling in oyster mitochondria. Elevated temperatures increased sensitivity of oyster mitochondria to cadmium many-fold, suggesting that onset of mitochondrial dysfunction will occur at lower cadmium levels as the environmental temperature increases. These findings emphasize the importance of taking temperature into account when studying the effects of environmental toxicants on poikilotherms, or developing biomarkers of environmental stress using poikilothermic organisms as models. This also suggests that oyster populations subjected to one of the stressors in their natural environments (e.g. to elevated temperatures due to seasonal warming or global climate change) may become more susceptible to other stressors (such as trace metal pollution) and *vice versa*, and emphasizes the importance of analysing multiple stressors in estuaries. Currently, further studies are being conducted in order to elucidate the role of mitochondrial mechanisms and temperature–trace metal interactions on oyster bioenergetics *in vivo*.

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