

Cadmium exposure affects mitochondrial bioenergetics and gene expression of key mitochondrial proteins in the eastern oyster *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae)

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Abstract

Cadmium is a ubiquitous and extremely toxic metal, which strongly affects mitochondrial function of aquatic organisms in vitro; however, nothing is known about the in vivo effects of sublethal concentrations of this metal on mitochondrial bioenergetics. We have studied the effects of exposure to 0 (control) or 25 $\mu\text{g L}^{-1}$ (Cd-exposed) Cd^{2+} on mitochondrial function and gene expression of key mitochondrial proteins in the eastern oyster *Crassostrea virginica*. Cadmium exposure in vivo resulted in considerable accumulation of cadmium in oyster mitochondria and in a significant decrease of ADP-stimulated respiration (state 3) by 30% indicating impaired capacity for ATP production. The decrease in state 3 respiration was similar to the level of inhibition expected from the direct effects of cadmium accumulated in oyster mitochondria. On the other hand, while no effect on proton leak was expected based on the mitochondrial accumulation of cadmium, Cd-exposed oysters in fact showed a significant decline of the proton leak rate (state 4 + respiration) by 40%. This suggested a downregulation of proton leak, which correlated with a decrease in mRNA expression of a mitochondrial uncoupling protein UCP6 and two other potential uncouplers, mitochondrial substrate carriers MSC-1 and MSC-2. Expression of other key mitochondrial proteins including cytochrome *c* oxidase, adenine nucleotide transporter and voltage dependent anion channel was not affected by cadmium exposure. Adenylate energy charge (AEC) was significantly lower in Cd-exposed oysters; however, this was due to higher steady state ADP levels and not to the decrease in tissue ATP levels. Our data show that adjustment of the proton leak in cadmium-exposed oysters may be a compensatory mechanism, which allows them to maintain normal mitochondrial coupling and ATP levels despite the cadmium-induced inhibition of capacity for ATP production.

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1. Introduction

Trace metals are ubiquitous components of marine environments and may greatly vary in availability in different habitats due to natural and anthropogenic

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sources (GESAMP, 1987). Cadmium is a highly toxic trace metal with no known biological function in animals, which is widespread in estuarine and coastal environments. Anthropogenic sources of cadmium in estuarine and coastal habitats due to pollution of the estuaries have received most attention (Roesijadi, 1996); however, natural ones are also very important and may be due to the river run-off from cadmium-rich soils, leaching of the rocks or diatom deposition in marine sediment (GESAMP, 1987; Frew et al., 1997). At high concentrations, cadmium is extremely toxic to aquatic organisms, but even low sublethal levels may significantly affect their physiology (Sokolova, 2004; Sokolova et al., 2004).

Marine bivalves including oysters are exposed to varying levels of cadmium in their natural habitats in estuaries and coastal areas. They have an ability to concentrate cadmium in soft tissues from water and sediments to the concentrations exceeding the environmental levels by orders of magnitude (Roesijadi, 1996; Frew et al., 1997). Body levels of cadmium in natural oyster population's range from 0.4 to 40 $\mu\text{g g}^{-1}$ dry weight (Roesijadi, 1996; Frew et al., 1997). During acute exposure to elevated cadmium concentrations in water or sediments, oysters can accumulate even higher loads of this metal up to 300–400 $\mu\text{g g}^{-1}$ dry weight (Roesijadi, 1996).

Resistance to the toxic effects of cadmium and other trace metals is provided primarily by binding to metallothioneins and deposition of insoluble metal-containing granules (Roesijadi, 1996). However, these detoxification mechanisms are imperfect, and from 10 to 90% of the total heavy metal pool in the cytosol is bound to low molecular weight compounds and other proteins depending on the species and the regime of exposure to heavy metals (Bracken et al., 1984; Giguere et al., 2003). Interestingly, recent studies have shown that although metallothionein-bound metals are less cytotoxic than free ions, they nevertheless can strongly affect mitochondrial function (Simpkins et al., 1994, 1998). Our current studies also showed strong accumulation of cadmium in oyster mitochondria to the levels 2–3 times exceeding cadmium levels in other organelles such as lysosomes and microsomes (Sokolova, Ringwood, Johnson, unpublished data). Thus, both metallothionein-bound and -unbound (“excess”) metal ions have a potential to exert physiological effects on

bivalves, in particular on their mitochondria and energy metabolism.

Trace metals including cadmium have long been known as powerful modulators of mitochondrial function (Skulachev et al., 1967). For instance, mammalian and plant mitochondria are very sensitive to cadmium, which strongly affects mitochondrial bioenergetics in vitro at concentrations as low as 10^{-6} M (Skulachev et al., 1967; Kessler and Brand, 1994, 1995; Ye et al., 2001). Our current studies have shown that oyster mitochondria are also highly sensitive to cadmium (Sokolova, 2004). Low levels of cadmium (1–5 μM) stimulated proton leak and decreased coupling of oyster mitochondria indicating high cost of mitochondrial maintenance and reduced mitochondrial efficiency. Higher cadmium levels (10–50 μM) further reduced mitochondrial coupling due to strong inhibition of the ADP-stimulated respiration (Sokolova, 2004). Interestingly, there was no reduction in the mitochondrial membrane potential by cadmium indicating that the activity of electron transfer chain was sufficient to counteract cadmium-stimulated proton leak (Sokolova et al., 2004).

Despite the strong evidence that cadmium impairs mitochondrial function in vitro, effects of this metal on mitochondrial bioenergetics of aquatic organisms in vivo at low sublethal concentrations are not well understood. Several lines of evidence suggest that mitochondria are a key intracellular target for this metal in vivo. Thus, long-term exposure to high cadmium levels resulted in mitochondrial swelling, reduction of the density of cristae and severe reduction of glycogen fields indicating activation of glycolysis in freshwater clams *Elliptio complanata* and *Anodonta cygnea* (Hemelraad et al., 1990a). Moreover, exposure to high levels of cadmium 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 (Hemelraad et al., 1990b). These studies using high lethal cadmium concentrations indicate that disturbance of mitochondrial bioenergetics is critically implicated in the acute cadmium toxicity in bivalves. However, it is not clear whether low sublethal cadmium concentrations can also affect their mitochondrial physiology and bioenergetics.

The aim of this study was to investigate the effects of in vivo exposure to cadmium on mitochondrial function and bioenergetics of a model marine bivalve, the eastern oyster *Crassostrea virginica*. We exposed oysters for 3 weeks to 0 or 25 $\mu\text{g L}^{-1}$ (0.22 μM) Cd^{2+} and determined oxidation rates and coupling of their mitochondria, as well as key parameters of tissue energy status. In particular, we measured ADP-stimulated (state 3) respiration rate of isolated mitochondria, which is indicative of the maximum rate of ATP synthesis, respiration of resting mitochondria (state 4 respiration) and proton leak (state 4+ respiration). Low levels of proton leak are normal for mitochondria and are considered a mechanism, which regulates production of reactive oxygen species by “mild uncoupling” (Goglia and Skulachev, 2003) and plays a role in determining the basal metabolic rate of an organism (Hulbert and Else, 1999). However, elevated proton leak would result in low mitochondrial efficiency and high cost of mitochondrial maintenance, which negatively impacts whole-organism bioenergetics (Pörtner et al., 1998, 2001). We have also studied the effects of cadmium exposure on gene expression of key mitochondrial proteins involved in substrate transport and oxidation. In particular, we analyzed gene expression of cytochrome *c* oxidase which is a key enzyme of the electron transfer chain, adenine nucleotide transporter (ANT) involved in mitochondrial ATP/ADP exchange, a voltage-dependent anion channel (VDAC), a novel uncoupling protein (UCP6) and two other mitochondrial substrate carriers, which may be involved in mitochondrial proton leak (MSC-1 and MSC-2). This study for the first time provides evidence that mitochondrial function and gene expression of mitochondrial proteins in marine mollusks is strongly affected by low sublethal concentrations of cadmium, and emphasizes potentially strong negative impact which trace metal pollution can have on bioenergetics and energy metabolism of estuarine mollusks.

2. Materials and methods

2.1. Animal collection and maintenance

Adult oysters (2 years, 95–120 mm shell length) were collected from Stump Sound, NC in April 2004. Water temperature at the time of collection was

18–20 °C 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; Swartzenberg, personal communication). Animals were transported to the University of North Carolina at Charlotte within 8 h after collection and placed in recirculated aquaria with artificial sea water (SW) (Instant Ocean[®], Kent Marine, Acworth, GA) at 20 ± 1 °C and 650 ± 20 mOsm. Animals were acclimated in the laboratory for 1 week prior to experimentation. During 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). No mortality was detected during the preliminary acclimation period. After the preliminary acclimation, oysters were randomly assigned to plastic Nalgene[®] trays (six oysters per tray), and incubated for 3 weeks in 5 L of SW (control) or of SW with 25 $\mu\text{g L}^{-1}$ Cd^{2+} (Cd-exposed oysters). Four trays were assigned to each experimental treatment (control and Cd-exposed). Oysters were fed with PhytoPlex[®] blend on alternate days, and water was changed 18 h after feeding. Less than 2% mortality was detected during the experimental incubations, and there were no significant differences in mortality between control and Cd-exposed oysters (data not shown).

2.2. Chemicals

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

2.3. Isolation of mitochondria

Mitochondria were isolated from oyster gills using a method modified from Ballantyne and Moyes (1987). In aquatic organisms, gills are a primary site of uptake of trace metals and a major target for trace metal toxicity (Xie and Klerks, 2004), which makes them a tissue of choice for the studies of cadmium effects on metabolic physiology. Isolation buffer consisted of 300 mM sucrose, 50 mM KCl, 50 mM NaCl, 8 mM EGTA, 1% bovine serum albumin (BSA, essentially fatty acid free), 2 $\mu\text{g mL}^{-1}$ of a protease inhibitor aprotinin and 30 mM HEPES (pH 7.5) (Ballantyne and Moyes, 1987). Gills of two or three animals were re-

moved, blotted dry and placed in 15 mL of ice-cold isolation medium. The tissue was homogenized with five passes (200 rpm) of a Potter–Elvehjem homogenizer using a loosely fitting Teflon pestle. The homogenate was centrifuged for 10 min at $2000 \times g$ and 4°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 $2000 \times g$, and supernatants from the two centrifugations were pooled. The supernatant was then centrifuged at $7500 \times g$ and 4°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 re-suspended in the ice-cold EGTA-free isolation buffer with 0.1% BSA to give a mitochondrial protein content of $5\text{--}10\text{ mg mL}^{-1}$. This method of isolation routinely yielded mitochondria with the respiratory control ratios of 2.5–4 (Sokolova, 2004).

It is worth noting that pure BSA solutions bind approximately $2\text{ }\mu\text{g Cd}^{2+}\text{ mg}^{-1}$ in high ionic strength media at pH 7.5 (Miller et al., 1973). However, the effects of this binding on the Cd^{2+} availability are controversial and not fully understood. We have conducted a series of preliminary experiments, in which we tested Cd^{2+} effects on oyster mitochondria in BSA-free media and found that Cd^{2+} effects were only about 10% greater in the absence of BSA at low Cd^{2+} concentrations ($\leq 5\text{ }\mu\text{M}$). No effect of BSA was detected at high Cd^{2+} levels in assay medium. Similar results were earlier reported in other mitochondrial studies (Miller et al., 1973). Because Cd^{2+} effect on oyster mitochondria was not changed appreciably by 0.1% BSA in assay medium, and because BSA improved mitochondrial coupling, we have used it in all further experiments.

2.4. Mitochondrial oxidation

Oxygen uptake by mitochondria was measured in 1 mL water-jacketed glass chambers using Clarke-type oxygen electrodes at 20°C (Qubit Systems, Kingston, Ont., Canada). Two-point calibration of electrodes was performed, and continuous data acquisition was made using a BIOPAC Data acquisition system (BIOPAC, USA). Temperature in mitochondrial respiration chambers was maintained constant using Fisher Isotemp refrigerated water circulator. All assays were completed within 2 h of isolation of the mitochondria. Preliminary experiments have shown that there was no change in

mitochondrial respiration or coupling during this period. The assay medium consisted of 150 mM KCl, 150 mM NaCl, 10 mM KH_2PO_4 , 20 mM sucrose, 0.1% bovine serum albumin (BSA, essentially fatty acid free), $2\text{ }\mu\text{g mL}^{-1}$ of aprotinin, $5\text{ }\mu\text{M}$ of a myokinase inhibitor AP_5A and 30 mM HEPES (pH 7.2). Succinate was used as a substrate at saturating amounts (10–15 mM) in the presence of $5\text{ }\mu\text{M}$ of rotenone. Maximal respiration rates (state 3) indicative of the maximum capacity for ATP synthesis in mitochondria 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 oxygen consumption rate after addition of $2.5\text{ }\mu\text{g mL}^{-1}$ 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; Kessler and Brand, 1995). In a preliminary study, KCN (100 μM) and salicylhydroxamic acid (SHAM, 200 μM) were added to the mitochondria at the end of assay to inhibit mitochondrial respiration and to measure non-mitochondrial rate of oxygen consumption. SHAM was used in order to avoid the 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 negligible (less than 0.5% of the state 3 respiration), and therefore was not taken into account in the subsequent measurements. Oxygen electrode drift was measured in assay medium containing no mitochondria, and respiration rates in states 3, 4 and 4+ were corrected for the electrode drift.

To determine the effects of cadmium on mitochondrial respiration of control and Cd-exposed oysters, each mitochondrial suspension was divided into 7–8 aliquots, and each aliquot was incubated for 5 min in the absence of cadmium (control) and at different concentrations of CdCl_2 in the range from 0.5 to 100 μM . After incubations, respiration rates of state 3, 4 and 4+ were determined as described above. Addition of the highest concentration of cadmium used in this study (100 μM) did not detectably change pH of the assay buffer (i.e. pH change was less than 0.01 U).

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 $\text{nmol O min}^{-1} \text{mg}^{-1}$ mitochondrial protein. Respiratory control ratio (RCR) was determined as a ratio of state 3 over state 4 respirations 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 non-competitive inhibition model (Segel, 1976).

2.5. Protein concentrations

Protein concentrations in mitochondrial suspensions were measured using a modified Biuret method with 1% Triton-X added to solubilize the mitochondria (Bergmeyer, 1985). 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.

2.6. Tissue energy status

Tissue levels of adenylates (ATP, ADP and AMP) were measured in protein-free perchloric acid extracts of the gill tissue of control and cadmium-exposed oysters as described in Sokolova et al. (2000). Briefly, gill tissue was powdered under liquid nitrogen, and ca. 300 mg of tissue powder were added to an excess ($5\times$) volume of pre-cooled 0.6 mol L^{-1} perchloric acid with 10 mmol L^{-1} EDTA, and homogenized. Precipitated protein was removed by centrifugation. The extract was neutralized with 5 mmol L^{-1} potassium hydroxide to pH 7.2–7.5. Precipitated potassium perchlorate was removed by a second centrifugation. Extracts were stored at -80°C .

ATP, ADP and AMP concentrations were measured enzymatically according to Bergmeyer (1985). Tissue levels of adenylates were expressed in $\mu\text{mol g}^{-1}$ wet weight.

Adenylate energy charge (AEC) was calculated from the tissue concentrations of adenylates as follows:

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

Adenylate kinase apparent equilibrium constant (K_{eq}) defined as

$$K_{\text{eq}} = \frac{[\text{Mg ATP}] \times [\text{AMP}]_{\text{free}}}{[\text{Mg ADP}] \times [\text{ADP}]_{\text{free}}}$$

was calculated from coordinated variation in AEC and ATP/ADP ratios. It has been previously shown that the relationship between the ATP/ADP ratio and AEC depends on the value of the apparent equilibrium constant of the adenylate kinase-catalyzed reaction thus allowing determination of K_{eq} based on the coordinated changes of the former two parameters (Thebault et al., 1996). K_{eq} was calculated as described in Thebault et al. (2000):

$$y = \frac{-x + 0.5 - \sqrt{x(x-1)(1-4K_{\text{eq}}) + 0.25}}{2x-2}$$

where $x = \text{AEC}$, $y = \text{ATP/ADP ratio}$ and $K_{\text{eq}} = \text{apparent equilibrium constant of adenylate kinase}$.

2.7. mRNA expression of key mitochondrial proteins

Total RNA was extracted from the gill tissue of control and cadmium-exposed oysters using TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. Tissue to TRI reagent ratio was kept below 1:10 (w/v). To improve RNA purity and to prevent co-precipitation of polysaccharides, RNA precipitation step was carried out using 1:1 mixture of isopropanol and RNA precipitation solution consisting of 1.2 mol L^{-1} NaCl and 0.8 mol L^{-1} disodium citrate. This method yielded high purity total RNA with 280/260 absorbance ratio ≥ 1.9 . mRNA was extracted from 150 to 200 μg of total RNA using Oligotex mRNA Mini Kit (QIAGEN, Valencia, CA, USA).

Semi-quantitative RT-PCR from *C. virginica* mRNA was performed using OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA) according to manufacturer protocol. Target fragments were amplified from 30 to 50 ng mRNA in multiplex reactions under the following conditions: reverse transcription step of 30 min at 50°C ; initial polymerase activation step of 15 min at 95°C ; 25–30 cycles of 45 s at 95°C , 45 s at 55°C , 45 s at 72°C ; final extension step of 10 min at 72°C . To check for possible DNA contamination of the mRNA samples, we performed RT-PCR with the same reaction mixture omitting the reverse transcription step.

Table 1
Primer sequences used in semi-quantitative RT-PCR to amplify gene fragments from *C. virginica*

Gene	Primer sequences	Source sequence #
β -actin	Act1F-263 5'-GAG ATT GGA TCT CGC TGG ACG TG-3' Act1R-470 5'-GCT CAT TTC CGA TGG TGA TTA CCT GA	AB071191
β -actin	Act-F63 5'-TGA TGA AGA AGT TGC AGC TTT AGT-3' Act-R393 5'-TTT CTC TGT TGG CCT TAG GG-3'	AB071191
Cytochrome <i>c</i> oxidase subunit IV (COIV)	COVI-F 5'-GGA GAC GAA ATC GGC AAG GAA ACA-3' COIV-R 5'-ATG GGG CAG TGG AAC AGG GAC TT-3'	BG624707
Adenine nucleotide transporter (ANT)	ANT-7F17 5'-AGC TTC GCA GAA AAC TTC GCA CTC-3' ANT-7R536 5'-ATG ATC CCC ACG CAA GAG ATG AC-3'	BG624882
Voltage dependent anion channel (VDAC)	VDAC-4F7 5'-CAG CGT GTG CTC CTG ATA AAG-3' VDAC-4R668 5'-CGG AGG ACC ATG AGA GTT TC-3'	CD649247
Uncoupling protein (UCP2)	UCP3-6F262 5'-CCA AAA CAA TGA AGG TGG GCG TCC-3' UCP3-6R574 5'-CAG TGG TCA CTC CCG CGA AGA CA-3'	AY736103
Mitochondrial substrate carrier 1 (oxoglutarate/malate carrier) (MSC-1)	OMC-1F76 5'-TTC ACC CCC AGC TGT ACT TTG-3' OMC-1R526 5'-CCG TGG GAA TAA CAC ATT AAA ACC-3'	CD650599
Mitochondrial substrate carrier 2 (MSC-2)	MSC-1F131 5'-GCC CCT CGT TGT AAG CAG CAC TAA-3' MSC-1R851 5'-TTG GTC AGG ACA GCA GCG ATT CT-3'	CD650087

GenBank accession numbers are given for the source sequences, which were used for primer design.

No product was obtained indicating that our samples were not contaminated with DNA (data not shown). Primer sequences and corresponding annealing temperatures are listed in Table 1. In order to normalize

mRNA level to an internal reference, a widely used housekeeping gene actin was selected, and expression of all studied genes was measured against the actin standard. RT-PCR reactions contained primers for both

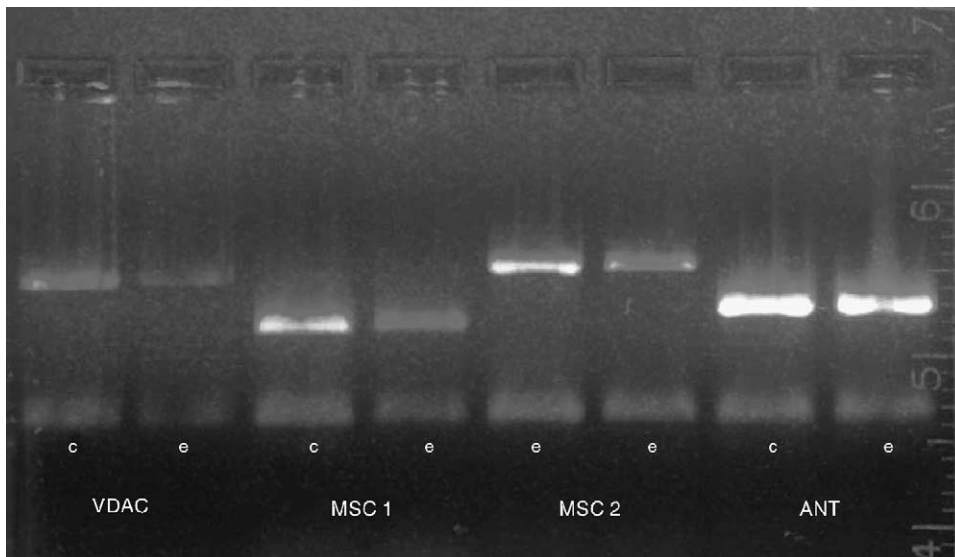


Fig. 1. A representative sample gel for semi-quantitative multiplex RT-PCR of selected gene fragments in *C. virginica*. Lanes (left to right): 1, 2—VDAC, 3, 4—MSC-1, 5, 6—MSC-2, 7,8—ANT, c—control oysters, e—cadmium-exposed oysters. The upper band in each lane corresponds to the target gene, and the lower band to the reference β -actin gene.

actin and a gene of interest. The concentrations of both primer pairs were selected experimentally for any given actin/target gene combination to keep signal intensity below saturation level (Fig. 1). Amplified fragments were resolved on 1.5% agarose gel, stained with ethidium bromide and analyzed using Kodak Documentation System EDAS 290 with Kodak 1D Image analysis software. In order to minimize effects of gel-to-gel variability in staining, all samples for a particular target mRNA were run on the same gel. Band intensities were automatically measured for both fragments in the amplificate, and relative expression level of target gene message was calculated as ratio against actin to give a semi-quantitative estimate of the relative change in gene expression in Cd-exposed oysters compared to the controls.

2.8. Statistics

In a preliminary analysis, the effect of replicate trays (four per each treatment) on experimental end points was tested by ANOVA and found to be not significant ($P > 0.10$). Therefore, we have omitted this effect from the final analysis in order to increase the statistical power of ANOVA. Effects of the factors “Cadmium exposure” and “Cadmium concentrations in vitro” and their interactions were analyzed using generalized linear model ANOVAs after testing the assumptions of normality of data distribution and homogeneity of variances. Dunnett’s tests were used for post-hoc pairwise comparisons, and Fisher’s least square difference test (LSD)—for planned comparisons. Statistical analyses were performed using SAS 8.2 software (SAS Institute, Cary, NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05.

3. Results

3.1. Mitochondrial function and energy status

Activity of citrate synthase, which is considered a marker of mitochondrial density, in total tissue homogenate or in the isolated mitochondrial fraction was similar in the control and Cd-exposed oysters indicating similar mitochondrial abundance in their tissues ($P > 0.84$) (Fig. 2). In contrast, respiration rate of

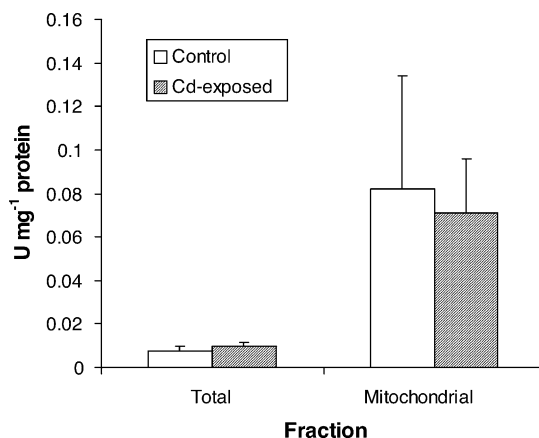


Fig. 2. Specific activities of citrate synthase in oyster gill tissue (Total) and the mitochondrial fraction (Mitochondrial) in control oysters and those exposed for 3 weeks to $25 \mu\text{g L}^{-1}$ $[\text{Cd}^{2+}]$. Vertical bars represent standard errors. $N = 5-7$.

isolated mitochondria was strongly affected by exposure of oysters to $25 \mu\text{g L}^{-1}$ $[\text{Cd}^{2+}]$ in vivo, as well as by cadmium addition to isolated mitochondria in vitro (Table 2). ADP-stimulated (state 3) respiration was significantly lower by ca. 30–35% in Cd-exposed oysters as compared to the control ones (Fig. 3). In

Table 2

ANOVA: effects of cadmium exposure in vivo (“Exposure”) and in vitro (“ Cd^{2+} level”) and their interaction on respiration rates and respiratory control ratios of isolated oyster mitochondria

	Cd^{2+} level	Exposure	Exposure \times Cd^{2+} level
State 3	$F_{7,94} = 4.56$ $P = \mathbf{0.0002}$	$F_{1,94} = 9.17$ $P = \mathbf{0.0032}$	$F_{7,94} = 0.14$ $P = 0.9952$
State 4	$F_{7,94} = 2.62$ $P = \mathbf{0.0163}$	$F_{1,94} = 16.13$ $P = \mathbf{0.0001}$	$F_{7,94} = 0.34$ $P = 0.9335$
State 4+	$F_{7,93} = 1.37$ $P = 0.2276$	$F_{1,93} = 5.50$ $P = \mathbf{0.0212}$	$F_{7,93} = 0.33$ $P = 0.9379$
RCR	$F_{7,95} = 6.54$ $P < \mathbf{0.0001}$	$F_{1,95} = 1.98$ $P = 0.1624$	$F_{7,95} = 0.85$ $P = 0.5466$
RCR+	$F_{7,94} = 2.49$ $P = \mathbf{0.0215}$	$F_{1,94} = 1.23$ $P = 0.2705$	$F_{7,94} = 1.03$ $P = 0.4141$
P/O ratio	$F_{6,77} = 1.44$ $P = 0.2145$	$F_{1,77} = 0.05$ $P = 0.8221$	$F_{6,77} = 0.27$ $P = 0.9780$

Oysters were exposed in vivo for 3 weeks to 0 or $25 \mu\text{g L}^{-1}$ $[\text{Cd}^{2+}]$ (nominal concentrations), and their isolated mitochondria were further exposed to 0–100 μM $[\text{Cd}^{2+}]$ in vitro. F -values with degrees of freedom and the respective significance levels (P) are given. Significant effects are highlighted in bold.

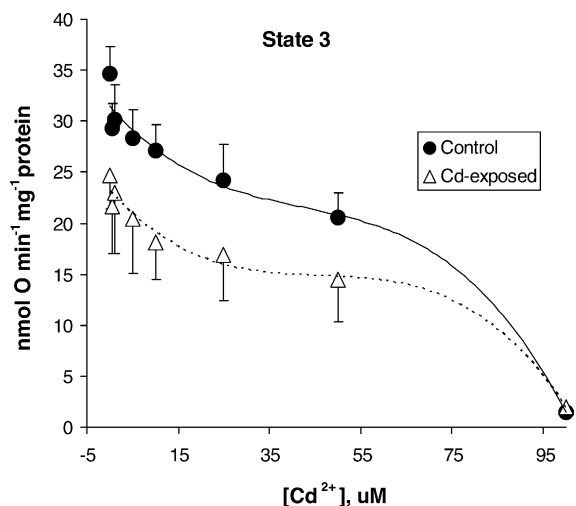


Fig. 3. Effect of cadmium exposure in vitro and in vivo on ADP-stimulated (state 3) respiration in mitochondria of *C. virginica*. Vertical bars represent standard errors. $N=7-8$. Horizontal axis— $[Cd^{2+}]$ in assay medium, vertical axis—state 3 oxygen consumption.

both groups of oysters, cadmium addition in vitro led to a gradual decrease of state 3 respiration rate. Inhibition constants for state 3 respiration (K_i) based on the non-competitive inhibition model were similar for control and Cd-exposed oysters (109 and 90 μM , respectively). Phosphorylation efficiency (P/O ratio) of oyster mitochondria varied between 1.3 and 1.7 and was not affected by long-term cadmium exposure in vivo and/or addition of 0.5–50 μM Cd^{2+} in vitro (Table 2).

Cadmium exposure in vivo led to a significant reduction in state 4 and 4+ respiration in isolated oyster mitochondria as compared to mitochondria from control oysters (Table 2, Fig. 4). The rates of state 4 and 4+ respiration in mitochondria from Cd-exposed oysters were ca. 40% lower than in their control counterparts when measured in the absence of cadmium in assay medium. State 4 respiration gradually declined with increasing cadmium levels in isolated mitochondria from control and cadmium exposed oysters, while state 4+ respiration was not significantly affected by cadmium addition in vitro except at the highest Cd^{2+} levels (100 μM). Interestingly, low levels of cadmium (0.5–10 μM) added to mitochondria in vitro stimulated state 4+ respiration by 12–33% in Cd-exposed oysters but not in their control counterparts. Inhibition constants for state 4 respiration (K_i) based on the non-competitive inhibition

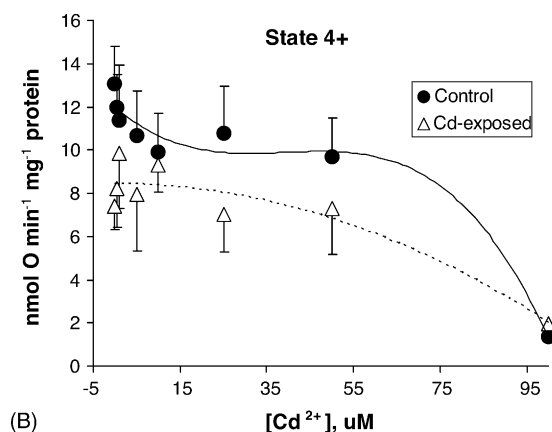
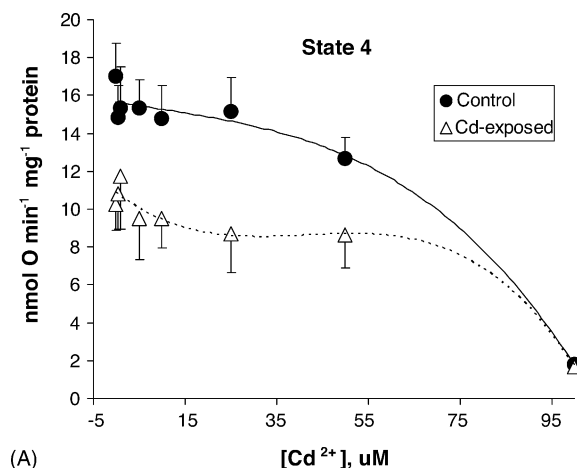


Fig. 4. Effect of cadmium exposure in vitro and in vivo on resting (state 4) respiration and proton leak (state 4+ respiration) in mitochondria of *C. virginica*. Vertical bars represent standard errors. $N=7-8$. Horizontal axis— $[Cd^{2+}]$ in assay medium, vertical axis—oxygen consumption.

model were similar for control and Cd-exposed oysters (211 and 192 μM , respectively), while K_i for the proton leak (state 4+ respiration) was considerably higher in Cd-exposed oysters (297 μM) as compared to their control counterparts (216 μM).

Due to the concomitant changes in ADP-stimulated respiration and proton leak in mitochondria from cadmium-exposed oysters, respiratory control ratios (RCR and RCR+) indicative of mitochondrial coupling were similar to those from the control oysters (Table 2, Fig. 5). Cadmium exposure of oysters in vivo resulted in a significant decrease in AEC and elevated

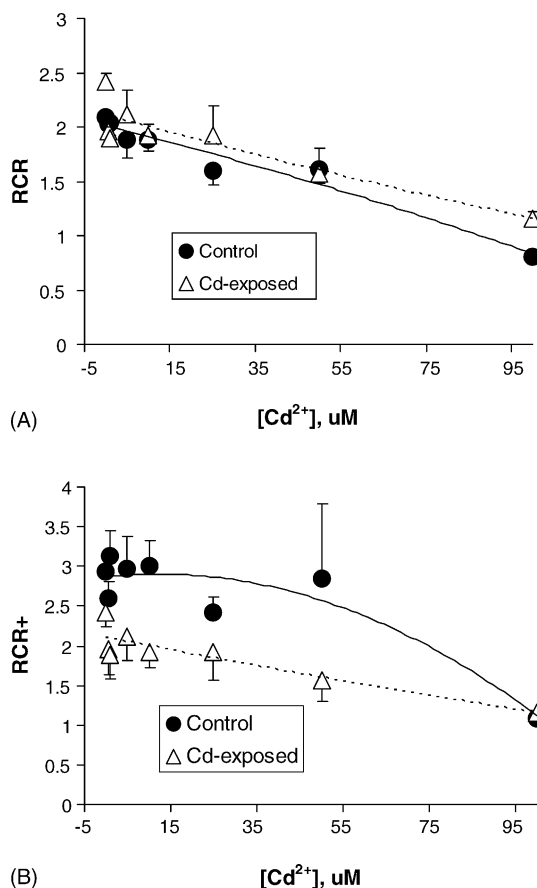


Fig. 5. Effect of cadmium exposure in vitro and in vivo on mitochondrial coupling in *C. virginica*. Vertical bars represent standard errors. $N=7-8$. Horizontal axis— $[Cd^{2+}]$ in assay medium, vertical axis—respiratory control ratios (RCR or RCR+).

ADP/ATP ratio indicating a shift in cellular energy balance (Tables 3 and 4; Fig. 6). Interestingly, ATP and AMP levels, as well as the total concentration of adenylates were similar in control and Cd-exposed oysters, while ADP levels were significantly higher in oysters exposed to cadmium as compared to the controls (Table 4, Fig. 7). Apparent equilibrium constant for adenylate kinase reaction was significantly lower in Cd-exposed oysters reflecting higher steady-state ADP levels (Table 4).

3.2. Gene expression of key mitochondrial proteins

Cadmium exposure resulted in a 15% decrease in expression of cytochrome *c* oxidase (subunit IV). Al-

Table 3

ANOVA: effects of cadmium exposure in vivo ("Exposure") on tissue levels of adenylates and parameters of tissue energy status (adenylate energy charge, or AEC, and ADP/ATP ratio, and adenylate kinase apparent equilibrium constant K_{eq}) in the gill tissue of *C. virginica*

Variable	d.f.	F-value	P
[ATP]	1, 11	0.41	0.537
[ADP]	1, 11	10.10	0.009
[AMP]	1, 11	0.04	0.847
Total adenylates	1, 11	0.01	0.907
AEC	1, 11	8.94	0.012
ADP/ATP	1, 11	8.79	0.013
K_{eq}	1, 9	7.06	0.026

Oysters were exposed in vivo for 3 weeks to 0 or 25 $\mu g L^{-1}$ $[Cd^{2+}]$ (nominal concentrations). F-values with degrees of freedom (d.f.) and the respective significance levels (P) are given. Significant effects are highlighted in bold.

though statistically significant (ANOVA: $F_{1,11} = 13.64$, $P=0.004$), this difference is considered to be biologically non-significant because such a small change is below the detection limits of the semi-quantitative method used in this study. Cadmium exposure had no effect on mRNA expression of adenyly nucleotide transporter or mitochondrial voltage-dependent anion channel (Fig. 8; ANOVA: $F_{1,10} = 0.11$, $P=0.750$ and $F_{1,10} = 0.05$, $P=0.826$ for ANT and VDAC, respectively). In contrast, gene expression levels of an uncoupling protein UCP6 and two mitochondrial carrier proteins (MSC-1 and MSC-2) were strongly decreased in Cd-exposed oysters as compared to the control ones (Fig. 8; ANOVA: $F_{1,11} = 11.92$, $P=0.006$, $F_{1,9} = 10.66$, $P=0.01$ and $F_{1,11} = 9.62$, $P=0.01$ for UCP2, MSC-1 and MSC-2, respectively).

Table 4

Adenylate energy charge (AEC), ADP to ATP ratio (ADP/ATP) and apparent equilibrium constant of adenylate kinase reaction (K_{eq}) in the gill tissue of *C. virginica* exposed to 0 or 25 $\mu g L^{-1}$ of cadmium for 3 weeks (control and Cd-exposed oysters, respectively)

Variable	Control oysters	Cd-exposed oysters
AEC	0.90 \pm 0.013 (7)	0.82 \pm 0.025* (6)
ADP/ATP	0.18 \pm 0.034 (7)	0.47 \pm 0.098* (6)
K_{eq}	0.562 \pm 0.129 (5)	0.211 \pm 0.056* (6)

Values that are significantly different from the respective controls are marked with asterisks (*). N is given in brackets.

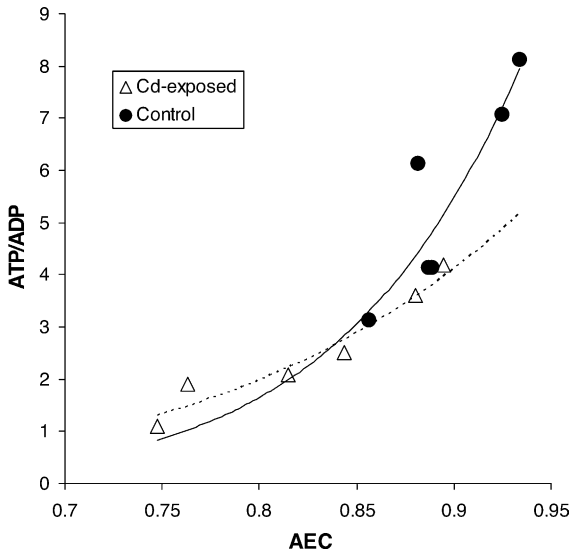


Fig. 6. Effect of cadmium exposure on adenylate energy charge and ATP/ADP ratios in gill tissue of *C. virginica*. Each point represents an individual sample. Lines represent power regressions ($R^2 = 0.78$ and 0.91 for control and cadmium-exposed oysters, respectively).

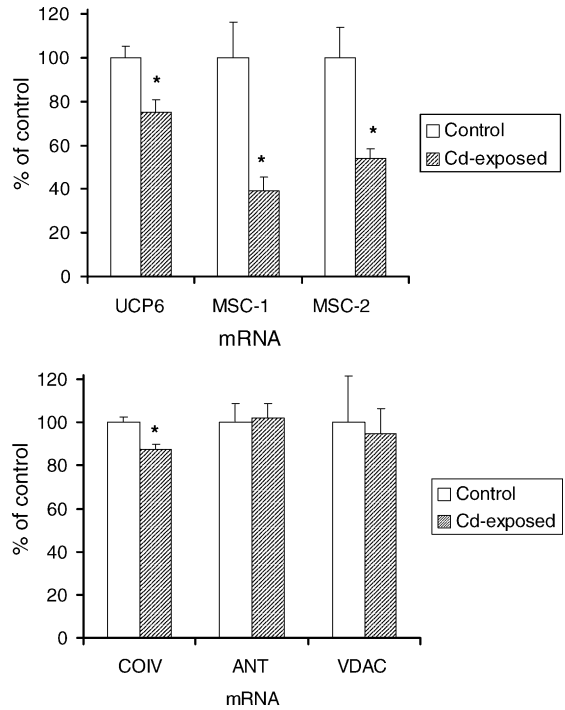


Fig. 8. Effect of cadmium exposure on mRNA expression levels of nuclear coded mitochondrial proteins in gill tissue of *C. virginica*. Ratios of band intensities of the target mRNA relative to the actin standard amplified in the same multiplex RT-PCR reaction were measured, and normalized to the mean control value. Target mRNAs: COIV—cytochrome *c* oxidase subunit IV; ANT—adenine nucleotide transporter; VDAC—voltage dependent anion channel; UCP6—uncoupling protein 6, MSC-1 and MSC-2—mitochondrial substrate carriers 1 and 2, respectively. Vertical bars represent standard errors. $N = 5-7$. Asterisks (*) denote statistically significant differences in mRNA expression between the control and Cd-exposed oysters.

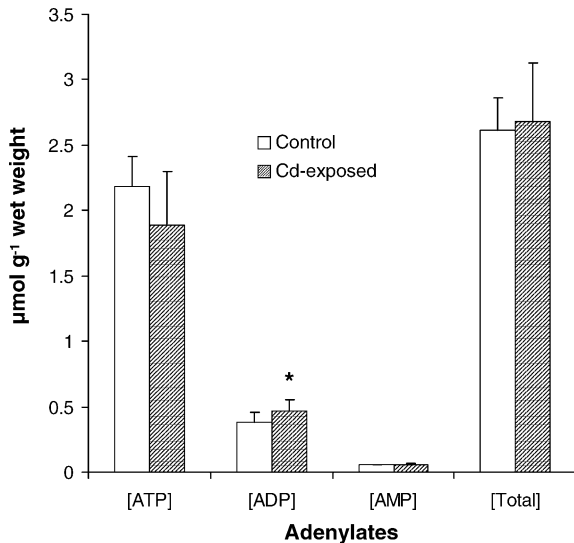


Fig. 7. Effect of cadmium exposure on adenylate levels in gill tissue of *C. virginica*. Vertical bars represent standard errors. [Total] represents the sum of all adenylate concentrations ([ATP] + [ADP] + [AMP]). Asterisk (*) represents significant differences between control and cadmium-exposed oysters. $N = 5-6$.

4. Discussion

Exposure of oysters for 3 weeks to low sublethal Cd^{2+} levels ($25 \mu g L^{-1}$) strongly affected their mitochondrial function and tissue energy status. Cadmium concentrations in our experimental exposures are above the typical levels found in the water column in polluted estuaries, which range between 1 and $6 \mu g L^{-1}$ (Crompton, 1997), although cadmium levels in estuarine sediments can be much higher and reach $1.5-2 mg kg^{-1}$ (Crompton, 1997; Hackney et al., 1998), thus considerably increasing cadmium exposure of bottom dwellers such as oysters. More

importantly, tissue burdens of cadmium detected in our experiments (31.9 ± 4.12 and $33.7 \pm 4.87 \mu\text{g g}^{-1}$ dry weight in hepatopancreas and gills, respectively) are close to the levels found in oysters from polluted sites in the nature ($20\text{--}40 \mu\text{g g}^{-1}$ dry weight) (Roesijadi, 1996; Frew et al., 1997) suggesting that our exposure regime resulted in environmentally relevant tissue cadmium burdens. Interestingly, cadmium concentration in mitochondrial fraction of oysters reached up to $11\text{--}14 \mu\text{g g}^{-1}$ dry weight after 3 weeks of exposure to $25 \mu\text{g L}^{-1} \text{Cd}^{2+}$ (Sokolova, Ringwood, Johnson, unpublished data). This burden is comparable to the exposure of isolated mitochondria to $25\text{--}30 \mu\text{M}$ of cadmium assuming that most cadmium in mitochondria is bound to protein under in vitro as well as in vivo conditions. Our present research shows that ADP-stimulated (state 3) respiration rate was ca. 30–35% lower in Cd-exposed oysters as compared to their control counterparts. Interestingly, addition of $25 \mu\text{M} [\text{Cd}^{2+}]$ to isolated mitochondria of control oysters resulted in a similar decrease of state 3 respiration by ca. 30% indicating similarity of the mitochondrial response in vitro and in vivo. This suggests that cadmium accumulation by oyster mitochondria in vivo results in a strongly impaired capacity for ATP synthesis even at low sublethal cadmium concentrations, and that this impairment is likely to be due to the direct effects of cadmium.

In contrast, a 40% decrease in the proton leak in Cd-exposed oysters cannot be explained by the direct effects of cadmium on oyster mitochondria. Thus, our data show that state 4+ respiration, which reflects proton leak had low sensitivity to cadmium, and addition of up to $50 \mu\text{M}$ of Cd^{2+} to isolated mitochondria had no significant effect on state 4+ respiration. Therefore, an observed decrease of state 4+ respiration in Cd-exposed oysters is not due to the cadmium accumulation in mitochondria. It is worth noting that a decrease in proton leak in state 4+ may be explained by two alternative mechanisms: (1) a decline in the capacity for proton leak (e.g. changes in the inner membrane permeability to protons or activity of the proton transporters); (2) a decrease in the mitochondrial membrane potential (Δp) and therefore protonmotive force. Our earlier studies have shown that mitochondrial membrane potential in oysters is not reduced by cadmium exposure in vivo or in vitro at similar or higher cadmium concentrations (Sokolova et al., 2004). Moreover, exposure of isolated oyster cells to cadmium resulted in a slight

but significant hyperpolarization of the mitochondrial membrane (Sokolova et al., 2004). If change in mitochondrial proton leak in cadmium-exposed oysters was mostly driven by protonmotive force, we would expect increase rather than decrease in state 4+ respiration. Therefore, an observed decline in state 4+ proton leak in Cd-exposed oysters cannot be explained by a decrease in Δp and most likely reflects a change in the capacity of the proton transport in vivo.

Proton transport across the membrane of animal mitochondria is a complex process and includes passive and active components. However, current studies indicate that passive diffusion of protons through the inner mitochondrial membrane is relatively unimportant for mitochondrial proton leak, the major role being played by mitochondrial enzymes and different anion carriers including so called uncoupling proteins (Sazanov and Jackson, 1994; Adams, 2000; Adams et al., 2001). Among the mitochondrial enzymes, which are involved in proton leak, NAD- and NADP-dependent isocitrate dehydrogenases (IDH) play a key role (Sazanov and Jackson, 1994). These enzymes are involved in a futile proton cycle coupled to a membrane enzyme H^+ -transhydrogenase and resulting in the net transport of protons into the mitochondrial matrix (Jackson, 1991; Sazanov and Jackson, 1994). Our studies have shown that activities of NAD- and NADP-dependent IDH are insensitive to cadmium in vitro and in vivo except at the extremely high Cd^{2+} concentrations ($>50 \mu\text{M}$) (Habinck and Sokolova, unpublished data). Therefore, inhibition of these enzymes is unlikely to explain the observed reduction of the mitochondrial proton leak in Cd-exposed oysters.

Interestingly, our study showed that cadmium exposure results in a significant downregulation of gene expression for an uncoupling protein UCP6 and two mitochondrial substrate carriers MSC-1 and MSC-2, which correlates with a 40% reduction of the proton leak. On the other hand, expression levels of mRNA for other key mitochondrial proteins including cytochrome *c* oxidase, voltage-dependent anion channel and adenylyl nucleotide transporter were not considerably affected by cadmium exposure. This indicates that the observed changes in mRNA levels of UCP6, MSC-1 and MSC-2 were specific to these proteins and did not result from an overall cadmium-induced inhibition of transcription. Although the function of the UCP6 and mitochondrial substrate carriers (MSC-1 and -2) has not been directly

studied in mollusks, high similarity of these proteins to the vertebrate uncoupling proteins and MSCs, respectively (>50% on the amino acid level), suggests that they may perform similar functions in invertebrates (Sokolova and Sokolov, 2005; Sokolova, unpublished data). Recent studies in uncoupling proteins and several mitochondrial substrate carriers (including oxoglutarate/malate carrier homologous to MSC-1) from vertebrates have shown that these proteins act as physiological uncouplers *in vitro* and *in vivo* facilitating mitochondrial proton leak, although they failed to attribute a major portion of proton leak to a single one of those proteins (Adams, 2000; Adams et al., 2001; Klingenberg et al., 2001; Goglia and Skulachev, 2003). Current consensus in the field is that proton leak may reflect the aggregate activities of multiple mitochondrial carriers each contributing some fraction of uncoupling activity towards the whole rather than predominant function of a single protein (Adams, 2000; Adams et al., 2001; Goglia and Skulachev, 2003). In view of this data, our finding about concomitant decrease of the proton leak and UCP6 and MSCs expression in oysters is very intriguing and provides the first evidence of correlation between putative uncouplers (including UCP6 first discovered in invertebrate genomes by our laboratory—Sokolova and Sokolov, 2005) and the rates of proton leak in molluscan mitochondria. This finding warrants further investigation in order to elucidate to which extent these proteins may be involved in physiological proton leak in oyster mitochondria and its decrease in cadmium-exposed oysters. In any case, our data unequivocally show that cadmium exposure at low environmentally relevant concentrations strongly and specifically affects gene expression levels of several key mitochondrial proteins supporting our view that mitochondria are among key cellular targets for this metal.

Notably, acclimation to elevated cadmium levels *in vivo* had no effect on sensitivity of ADP-stimulated (state 3) and resting (state 4) respiration of oyster mitochondria to cadmium. On the other hand, mitochondrial proton leak (state 4+ respiration) was less inhibited by Cd²⁺ addition to mitochondria from cadmium-exposed oysters as indicated by higher K_i values compared to their control counterparts. This reduced Cd²⁺ sensitivity may reflect an overall downregulation of mitochondrial proton leak in Cd-exposed oysters. Interestingly, one of us has earlier shown that acute temperature rise

results in a significant reduction of K_i in oyster mitochondria indicating higher sensitivity to cadmium at elevated temperatures (Sokolova, 2004). Thus, inhibition constant (K_i) by cadmium for state 3 respiration of mitochondria from 15 °C-acclimated spring oysters was 80, 24 and 3 μ M if measured at 15, 25 and 35 °C (Sokolova, 2004). Similar decrease in K_i with increasing temperature was shown for state 4 and 4+ respiration (Sokolova, 2004). In this study, K_i for state 3 respiration of mitochondria from summer oysters acclimated to 20 °C and measured at their acclimation temperature (90–109 μ M) was similar to that of the cold-acclimated spring oysters measured at 15 °C (80 μ M). The same holds true for cadmium inhibition constants of state 4 and state 4+ respiration, which were similar in 15 and 20 °C-acclimated oysters if measured at their respective acclimation temperatures (Sokolova, 2004; this study). This suggests that temperature-induced increase in sensitivity of mitochondrial function to cadmium may be a specific response to acute warming but it can be offset during the long-term acclimation and acclimatization to higher temperatures.

Cadmium exposure had significant effects on tissue energy status resulting in a decrease in AEC, ATP/ADP ratio and the apparent equilibrium constant for adenylate kinase reaction all reflecting higher steady-state ADP levels in cadmium-exposed oysters. On the other hand, tissue ATP levels were similar in control and cadmium-exposed oysters although the ADP-stimulated oxygen consumption and thus the maximum phosphorylation rate of mitochondria were significantly lower in the latter. This suggests that mitochondrial adjustments in cadmium-exposed oysters are effective in providing the levels of coupling and phosphorylation potential, which are needed to maintain normal levels of ATP production at least under the resting conditions. On the other hand, a decrease in the maximum attainable ATP production rate in Cd-exposed oysters suggests that this situation can change under conditions of high energy demand (such as during summer spawning and/or temperature rise) leading to energy deficiency in Cd-exposed oysters. This hypothesis is currently explored in our laboratory.

In summary, exposure of oysters to low sublethal cadmium levels had a significant effect on their mitochondrial function resulting in lower rates of mitochondrial oxidation including phosphorylation rate and

proton leak and in higher steady-state ADP levels. The observed decrease in phosphorylation rate may be due to the direct effects of cadmium on mitochondria, while a decrease in the proton leak more likely reflects a whole-organism acclimation response, which correlates with a downregulation of an uncoupling protein 6 and mitochondrial carrier proteins. Irrespective of its exact molecular nature, a downregulation of the proton leak in Cd-exposed oysters may be a compensatory mechanism, which allows them to maintain normal mitochondrial coupling and ATP levels despite the cadmium-induced decrease in phosphorylation rate. However, survival in this metabolically depressed state is only possible when the energy demand is low. Under conditions of high energy demand such as during reproduction or summer heat stress, oysters may experience energy shortage due to the insufficient ability of their mitochondria to produce ATP, which could threaten survival of oyster populations chronically exposed to cadmium in polluted estuaries.

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