

## Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses

Anton S. Cherkasov<sup>1</sup>, Pradip K. Biswas<sup>1</sup>, Daisy M. Ridings<sup>2</sup>, Amy H. Ringwood<sup>1</sup> and Inna M. Sokolova<sup>1,\*</sup>

<sup>1</sup>Biology Department, University of North Carolina at Charlotte, 9201 University City Blvd, Charlotte, NC 28223, USA and <sup>2</sup>Carolinas Medical Center, Cannon Research Center, 1542 Garden Terrace, Charlotte, NC 28203, USA

\*Author for correspondence (e-mail: insokolo@uncc.edu)

Accepted 12 January 2006

### Summary

In order to understand the role of metabolic regulation in environmental stress tolerance, a comprehensive analysis of demand-side effects (i.e. changes in energy demands for basal maintenance) and supply-side effects (i.e. metabolic capacity to provide ATP to cover the energy demand) of environmental stressors is required. We have studied the effects of temperature (12, 20 and 28°C) and exposure to a trace metal, cadmium (50 µg l<sup>-1</sup>), on the cellular energy budget of a model marine poikilotherm, *Crassostrea virginica* (eastern oysters), using oxygen demand for ATP turnover, protein synthesis, mitochondrial proton leak and non-mitochondrial respiration in isolated gill and hepatopancreas cells as demand-side endpoints and mitochondrial oxidation capacity, abundance and fractional volume as supply-side endpoints. Cadmium exposure and high acclimation temperatures resulted in a strong increase of oxygen demand in gill and hepatopancreas cells of oysters. Cd-induced increases in cellular energy demand were significant at 12 and 20°C but not at 28°C, possibly indicating a metabolic capacity limitation at the highest temperature. Elevated cellular demand in cells from Cd-exposed oysters was associated with a 2–6-fold increase in

protein synthesis and, at cold acclimation temperatures, with a 1.5-fold elevated mitochondrial proton leak. Cellular aerobic capacity, as indicated by mitochondrial oxidation capacity, abundance and volume, did not increase in parallel to compensate for the elevated energy demand. Mitochondrial oxidation capacity was reduced in 28°C-acclimated oysters, and mitochondrial abundance decreased in Cd-exposed oysters, with a stronger decrease (by 20–24%) in warm-acclimated oysters compared with cold-acclimated ones (by 8–13%). These data provide a mechanistic basis for synergism between temperature and cadmium stress on metabolism of marine poikilotherms. Exposure to combined temperature and cadmium stress may result in a strong energy deficiency due to the elevated energy demand on one hand and a reduced mitochondrial capacity to cover this demand on the other hand, which may have important implications for surviving seasonally and/or globally elevated temperatures in polluted estuaries.

Key words: mitochondrial respiration, cellular respiration, energy budget, mitochondrial membrane potential, proton leak, protein synthesis, cadmium, temperature, bivalve.

### Introduction

Metabolic regulation plays a key role in maintaining the optimal energy balance of the organism and matching energy demand with sufficient energy supply. Environmental stress often results in a reduction of net energy balance due to a reduction in assimilation of energy and/or its conservation in the form of ATP and other high-energy phosphates ('supply-side effects'), increases in basal metabolic demands ('demand-side effects') or a combination of both (Koehn and Bayne, 1989; Baird et al., 1990). The resulting energy deficit can have adverse effects on survival and performance of organisms and on the long-term persistence of their populations in the

stressful environment. To date, case studies analyzing stress-induced changes of energy budgets have mostly focused on single environmental stressors (Widdows, 1978; Dorigan and Harrison, 1987; Koehn and Bayne, 1989; Li et al., 2002), whereas effects of multiple stressors are not well understood. Because multiple stresses often have non-additive effects on physiology, analysis of environmentally relevant combinations of stressors is important to obtain a realistic picture of the impact of stress on animal bioenergetics in nature.

Trace metals (such as cadmium) and temperature are common stressors in estuaries, and their importance is increasing due to global climate change and accumulation of

persistent metal pollutants in coastal habitats (Helmuth et al., 2002; GESAMP, 1987). Cadmium (Cd) is a toxic metal that acts as a potent inhibitor of mitochondrial function in a variety of plant and animal models at concentrations as low as  $10^{-6}$  mol l<sup>-1</sup> (Kesseler and Brand, 1994; Kesseler and Brand, 1995; Korotkov et al., 1999; Dorta et al., 2003; Sokolova, 2004 and references therein). Typically, Cd exposure *in vitro* results in decreased mitochondrial efficiency, reduced rate of ATP synthesis and progressive uncoupling (for a review, see Brierley, 1977; Byczkowski and Sorenson, 1984; Miccadei and Floridi, 1993).

Our recent studies showed that elevated temperatures strongly enhance the adverse effects of Cd on mitochondrial ATP synthesis and coupling in a model marine poikilotherm, *Crassostrea virginica*, suggesting synergism between these two environmental stressors (Sokolova, 2004; A. S. Cherkasov, A. H. Ringwood and I. M. Sokolova, manuscript submitted for publication). Earlier studies have also shown that elevated temperatures and exposures to metals may result in elevated standard metabolic rates (SMR) in poikilotherms (Barber et al., 1990; Rowe, 1998; Rowe et al., 1998; Hopkins et al., 1999; Rowe et al., 2001; Willmer et al., 2000; Lannig et al., 2006). This suggests that poikilotherms exposed to elevated temperature and toxic metals may face a dilemma of elevated energy demand combined with reduced aerobic capacity to produce ATP. However, mechanisms underlying an increase in SMR in metal-exposed poikilotherms are not well understood and it is not known whether elevated energy demand can be partially compensated *in vivo* (e.g. by increases in mitochondrial abundance or efficiency). A comprehensive analysis of the combined effects of environmental temperature and Cd exposure on energy demand and aerobic capacity for energy supply will provide key information for mechanistic understanding of metabolic effects of these stressors and will further our knowledge of the role of bioenergetics in stress tolerance.

Eastern oysters, *C. virginica* Gmelin (Bivalvia: Ostreidae), are a useful model for studies of the interactive effects of temperature and Cd stress on the cellular energy budget of poikilotherms. Oysters are exposed to varying Cd concentrations in their habitats and have an ability to accumulate Cd in soft tissues to concentrations exceeding the environmental levels by orders of magnitude (Roesijadi, 1996). Like all intertidal organisms, oysters may experience extreme temperature fluctuations in their habitats, with a change in body temperature as large as 20°C within a few minutes during summer low tides and up to 35°C during more gradual seasonal variation in ambient temperatures (Helmuth et al., 2002; I.M.S., unpublished data). These temperature changes may strongly affect the SMR of oysters (Shumway, 1996; Hutchinson and Hawkins, 1992; Lannig et al., 2006) as well as the sensitivity of their mitochondria to Cd (Sokolova, 2004; A. S. Cherkasov, A. H. Ringwood and I. M. Sokolova, manuscript submitted for publication), thereby increasing the potential for interactive effects of those stressors on both the demand side and the supply side of the energy budget.

The aim of our study was to examine the effects of acclimation temperature and Cd exposure on cellular energy budget and mitochondrial capacity in *C. virginica* and to analyze which parts of the cellular energy demand are most strongly affected by temperature and Cd stress. We have studied the effects of temperature (12, 20 and 28°C) and exposure to Cd (50 µg l<sup>-1</sup>) on the energy budget of oysters using oxygen demand for ATP turnover, protein synthesis, mitochondrial proton leak and non-mitochondrial respiration in isolated cells as demand-side endpoints and mitochondrial oxidation capacity, abundance and fractional volume as supply-side endpoints. This study, for the first time, provides evidence that Cd stress adversely affects both demand and supply sides of the cellular energy balance in *C. virginica* and that Cd-induced metabolic costs are mostly due to the elevated rates of protein synthesis.

## Materials and methods

### *Animal collection and maintenance*

Adult oysters (70–120 mm shell length) were collected in Fall to Winter 2004 from two adjacent sites in the New River watershed (North Carolina, 34°28'27"N, 77°28'48"E). Water temperature at the times of collection varied between 12 and 17°C; average salinity was 30‰. The study sites have very low background concentrations of pollutants (Mallin et al., 1999; J. Swartzenberg, personal communication). Animals were transported to the University of North Carolina at Charlotte within 8 h and placed in tanks with artificial seawater (SW) (Instant Ocean®; Kent Marine, Acworth, GA, USA) at 15±1°C and 30±0.5‰. Oysters were allowed to recover for 3–5 days, and temperature in the tanks was then gradually changed to reach the required experimental temperatures (12, 20 and 28°C). The rate of the temperature change was <2°C day<sup>-1</sup>, and the duration of the pre-acclimation period was 8–10 days for all treatments.

After the preliminary acclimation, half of the experimental tanks were randomly selected, and Cd was added to the nominal concentration of 50 µg l<sup>-1</sup>. The remaining tanks were used as controls. Oysters were incubated for 20 or 40 days at 50 µg l<sup>-1</sup> Cd (Cd-exposed oysters) or in clean artificial seawater (controls) at each temperature. To avoid pseudoreplication, at least two replicate tanks were set up for each treatment. Oysters were fed daily with a commercial algal blend (2 ml per oyster) containing *Nannochloropsis*, *Tetraselmis* and *Isochrysis* spp. ranging in size from 2 to 15 µm (PhytoPlex®; Kent Marine). Water was changed every other day. In order to avoid Cd depletion in Cd-exposed tanks, a static-renewal design was used, with cadmium supplementation to the nominal concentration of 50 µg l<sup>-1</sup> during each water change. Cd levels were measured in water samples at least twice a week immediately before and 1 h after water changes. There were no differences in Cd levels before and after water changes, indicating that the maintenance conditions were adequate to prevent Cd depletion. The average Cd concentration in experimental tanks was 42.9±7.10 µg l<sup>-1</sup>.

(mean  $\pm$  s.d.,  $N=37$ ), and Cd levels in the control tanks were below the detection limits of the method used ( $0.5 \mu\text{g l}^{-1}$ ). It is worth noting that Cd concentrations in our experimental exposures ( $50 \mu\text{g l}^{-1}$ ) were at the upper end of Cd concentrations found in polluted estuaries (Crompton, 1997; Hackney et al., 1998). However, due to relatively short exposure times, the tissue Cd burdens in our experiments were well within the range found in oysters from polluted sites in the nature (see Results below and compare with Roesijadi, 1996 and references therein). This indicates that our exposure regime resulted in environmentally realistic tissue Cd burdens and thus that physiological changes observed in response to Cd exposure in our experiments are environmentally relevant.

#### Chemicals

Type I collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA), and TMRM (tetramethylrhodamine methyl ester) was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). All other chemicals were purchased from Sigma Aldrich (St Louis, MO, USA) or Fisher Scientific (Suwanee, GA, USA) and were of analytical grade.

#### Mitochondrial oxidation

Mitochondria were isolated from gills of control and Cd-exposed oysters after 20 days of exposure at 12, 20 and 28°C. In oysters, gills are a primary site of uptake of trace metals, which are characterized by early accumulation of Cd in the mitochondrial fraction (Sokolova et al., 2005b), and therefore this tissue is an appropriate model to study effects of Cd on mitochondria. Isolation was performed as described previously (Sokolova, 2004).

Mitochondrial oxygen uptake was measured in 1 ml water-jacketed chambers using Clarke-type oxygen electrodes (Qubit Systems, Kingston, ON, Canada) at the respective acclimation temperatures (12, 20 and 28°C). Mitochondrial assay conditions were as described previously (Sokolova, 2004), and calibration of oxygen electrodes, data acquisition and rate of oxygen consumption ( $M_{O_2}$ ) calculations were performed as described previously (Sokolova et al., 2005a). Succinate was used as a substrate at saturating amounts ( $10\text{--}15 \text{ mmol l}^{-1}$ ) in the presence of  $5 \mu\text{mol l}^{-1}$  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 previously (Chance and Williams, 1955). State 4+ respiration was determined as oxygen consumption rate after addition of  $2.5 \mu\text{g ml}^{-1}$  of the 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). 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. Respiration rates were corrected for

electrode drift and non-mitochondrial respiration (see Sokolova, 2004) and expressed as  $\text{natom O min}^{-1} \text{ mg}^{-1}$  mitochondrial protein. Respiratory control ratio (RCR) was determined as a ratio of state 3 over state 4 respiration (Estabrook, 1967).

#### Mitochondrial membrane potential (MMP)

MMP was determined as described in Cherkasov et al. (in review). Briefly, mitochondrial suspensions were diluted to  $2 \text{ mg ml}^{-1}$  mitochondrial protein in the standard assay medium (AM) containing  $0.5 \mu\text{mol l}^{-1}$  of the potentiometric dye TMRM,  $20 \text{ mmol l}^{-1}$  succinate and  $5 \mu\text{mol l}^{-1}$  rotenone. Preliminary studies have shown that  $0.5 \mu\text{mol l}^{-1}$  TMRM does not affect respiration of oyster mitochondria (data not shown). TMRM fluorescence in mitochondrial suspension was measured under constant stirring at an excitation wavelength of 573 nm and emission wavelength of 590 nm (excitation and emission slits 10 nm) using a fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan). The mitochondrial uncoupler CCCP (carbonyl cyanide-chlorophenyl hydrazone;  $50 \mu\text{mol l}^{-1}$ ) was added to the mitochondrial suspension to collapse the membrane potential, and fluorescence was measured again. The degree of quenching of TMRM fluorescence by energized mitochondria at 573 nm excitation wavelength ( $1/F_{573}$ ) was used as an index of MMP and was normalized to the fluorescence level in CCCP-uncoupled mitochondria. Scaduto and Grotoyohann have previously demonstrated that the degree of quenching of TMRM fluorescence at 573 nm is directly and linearly proportional to MMP (Scaduto and Grotoyohann, 1999).

#### Mitochondrial abundance and fractional volume

Hepatopancreas and gill tissues ( $1\text{--}2 \text{ mm}^3$ ) were fixed in 2.5% glutaraldehyde solution in  $0.1 \text{ mol l}^{-1}$  cacodylate buffer (pH 7.5) containing  $150 \text{ mmol l}^{-1}$  NaCl and  $300 \text{ mmol l}^{-1}$  sucrose. Post-fixation in 1% osmium tetroxide buffered solution was followed by the standard dehydration procedure with increasing concentrations of ethyl alcohol and acetone (Hayat, 2000). Tissues were embedded in Embed-812–Araldite mixture, and an Ultracut ultramicrotome (Ultracut UCT, Deerfield, IL, USA) was used to cut ultra-thin sections ( $60\text{--}90 \text{ nm}$ ). Sections were mounted on 200-mesh copper grids and double-stained with uranyl acetate and lead citrate according to a standard protocol (Hayat, 2000). Sections were analyzed with a transmission electron microscope (Philips CM 10; Philips Export B.V., Eindhoven, Netherlands) at 60 kV.

Mitochondrial numbers per unit area were calculated using the unbiased stereology approach on randomly chosen sections of oyster gill and hepatopancreas cells as follows:

$$N_v = (\Sigma Q) / (alf N_f), \quad (1)$$

where  $N_v$  is an estimate of numerical density or abundance,  $alf$  is the area per frame,  $\Sigma Q$  is the sum of mitochondria counted within all frames and  $N_f$  is the number of frames counted (Howard and Reed, 1998).

The fractional volume of mitochondria in gill and hepatopancreas tissue was estimated using multi-purpose combined point grid (ratio of fine to coarse points = 25:1) as described previously (Howard and Reed, 1998). Fractional volume [ $V_v(Y,ref)$ ] was determined as:

$$V_v(Y,ref) = [P(Y)] / 25 P_c(ref) \times 100, \quad (2)$$

where  $P(Y)$  is the number of fine points hitting mitochondria, and  $P_c(ref)$  is the number of coarse points hitting the reference space. Mitochondrial abundance and fractional volume were estimated using 9–11 random tissue sections from each of 36 oysters (total  $N=363$ ).

#### Cell isolation and respiration rates

Cells were isolated from gills and hepatopancreas of control and Cd-exposed oysters after 20 or 40 days at 12, 20 and 28°C. Oyster shells were scraped and surface cleaned with 1% bleach. Gills or hepatopancreas tissues from 3–5 oysters were pooled on ice in 5 ml of the digestion buffer containing 24.72 g l<sup>-1</sup> NaCl, 0.68 g l<sup>-1</sup> KCl, 1.36 g l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.18 g l<sup>-1</sup> NaHCO<sub>3</sub> and 30 mmol l<sup>-1</sup> Hepes at pH 7.5. Tissues were minced and washed twice with 10 ml of the digestion buffer. Tissue fragments were digested for 10 min at room temperature in 0.125% trypsin in balanced Hank's solution (Fisher Scientific, Suwanee, GA, USA) adjusted to 720 mOsm with sucrose. Tissue fragments were carefully triturated to release cells and washed twice with the digestion buffer. The supernatant was filtered through 100 µm sterile nylon mesh and centrifuged for 10 min at 400 g to pellet the cells. Cells were washed three times in cell suspension medium (CSM), containing 24.72 g l<sup>-1</sup> NaCl, 0.68 g l<sup>-1</sup> KCl, 1.36 g l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.18 g l<sup>-1</sup> NaHCO<sub>3</sub>, 4.66 g l<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O, 6.29 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 15 mmol l<sup>-1</sup> glucose and 30 mmol l<sup>-1</sup> Hepes at pH 7.5, and re-suspended in 2 ml of the same medium. Tissue fragments remaining from the trypsin digestion were additionally digested with 0.125% of Type I collagenase (Worthington Biochemical Corporation) dissolved in Mg-free digestion buffer for 40 min at room temperature, triturated to release cells, washed twice, and filtered through 100 µm sterile nylon mesh to remove undigested tissue. Cells were collected by centrifugation as described above, washed three times and re-suspended in 2 ml of the CSM. Cell suspensions from both digestions were pooled, enumerated using a hemacytometer, and cell density adjusted to 10×10<sup>6</sup> cells ml<sup>-1</sup>. Cell viability was determined using a standard Trypan Blue exclusion assay and was found to be >90% (mean 95.9±1.10%).

Cellular respiration was determined in 1 ml water-jacketed chambers using Clarke-type oxygen electrodes (Qubit Systems, Kingston, ON, Canada) at the respective acclimation temperatures (12, 20 and 28°C). Total  $\dot{M}_{O_2}$ , and  $\dot{M}_{O_2}$  in the presence of 3 µg ml<sup>-1</sup> oligomycin (to inhibit mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase), 100 µmol l<sup>-1</sup> cycloheximide (to inhibit cytosolic protein synthesis) and 100 µmol l<sup>-1</sup> KCN and 200 µmol l<sup>-1</sup> salicylhydroxamic acid (SHAM) (to inhibit

Table 1. Calculations of  $\dot{M}_{O_2}$  related to oxygen- and/or energy-requiring processes in isolated cells of *C. virginica*

| $\dot{M}_{O_2}$ related to:   | Calculated as:                                   |
|-------------------------------|--|
| ATP turnover                  | $\dot{M}_{O_2, total} - \dot{M}_{O_2, oligo}$    |
| Proton leak                   | $\dot{M}_{O_2, oligo} - \dot{M}_{O_2, KCN+SHAM}$ |
| Protein synthesis             | $\dot{M}_{O_2, total} - \dot{M}_{O_2, cyclo}$    |
| Non-mitochondrial respiration | $\dot{M}_{O_2, KCN+SHAM}$                        |
| Mitochondrial respiration     | $\dot{M}_{O_2, total} - \dot{M}_{O_2, KCN+SHAM}$ |

$\dot{M}_{O_2, total}$ , oxygen consumption in the absence of inhibitors. Other respiration rates ( $\dot{M}_{O_2}$ ) were as follows: in the presence of oligomycin (oligo), cycloheximide (cyclo), KCN and salicylhydroxamic acid (KCN+SHAM).

mitochondrial respiration), were determined. Cycloheximide is widely used as an inhibitor of cytoplasmic protein synthesis in ectotherms including marine mollusks (Giuditta et al., 1968; Alkon et al., 1987; Fuery et al., 1998; Joyner and Peyer, 2003). In our preliminary studies, we tested different cycloheximide concentrations and found that concentrations between 75 and 750 µmol l<sup>-1</sup> showed a similar and consistent degree of inhibition of cellular  $\dot{M}_{O_2}$  (by 10–15%). This agrees with earlier studies showing that these levels of cycloheximide specifically and effectively block protein synthesis in isolated molluscan cells and tissues (Giuditta et al., 1968; Alkon et al., 1987). Effects of cycloheximide on cell respiration in oysters were maximal after 1 h of incubation, with no further decrease in  $\dot{M}_{O_2}$  up to 5 h of incubation (data not shown). Therefore, in all further experiments we incubated cells with 100 µmol l<sup>-1</sup> of cycloheximide for 1 h on ice. Control cells were incubated on ice for the same time without cycloheximide addition. Pilot experiments showed that there was no change in cell viability or  $\dot{M}_{O_2}$  during this incubation. Cellular responses to oligomycin, KCN and SHAM were immediate (within a few minutes). All respiration rates were corrected for electrode drift. Oxygen demand for different cellular processes was calculated as shown in Table 1.

#### Cadmium determination

Gill samples were freeze-dried and digested in Teflon bottles with 52.5% nitric acid (trace metal grade; Fisher Scientific) using 3–4 cycles of microwave heating and cooling until the tissues were fully digested. Cell suspensions were mixed 1:1 with 70% nitric acid and digested as described above. Water samples were acidified with 0.7% nitric acid, incubated overnight on a shaker at room temperature and diluted 1:5 with deionized water. Cd concentrations were determined with an atomic absorption spectrometer (Perkin Elmer AAnalyst 800; Norwalk, CT, USA), equipped with a graphite furnace and Zeeman background correction. NIST oyster tissue (1566b; National Institute of Standards and Technology, Gaithersburg, MD, USA) was analyzed with the samples to verify the metal analyses; the percent recoveries over all batches were 94.6±6.6% (mean ± s.d.).



### Protein concentrations

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

### Statistics

General linearized model (GLM) analyses of variance (ANOVAs) were used to test the effects of exposure duration (random effects), and acclimation temperature and cadmium exposure (fixed effects) after testing the assumptions of normality of data distribution and homogeneity of variances. Mitochondrial abundance and fractional volumes were analyzed using repeated-measures ANOVA with tissue sections nested within individual oysters (random effect) and tissue, acclimation temperature and cadmium exposure as fixed effects. Dunnett tests were used for *post-hoc* comparisons, and LSD (least squared difference) tests for planned comparisons of sample means as appropriate. Preliminary tests were run to analyze the effect of replicate tanks within each treatment on the studied variables (data not shown). No significant differences were observed between the tanks and the data were pooled to increase the power of analysis. Statistical analyses were performed using SAS 9.1.3 software (SAS Institute, Cary, NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05, and Bonferroni correction was used to adjust significance levels for multiple ANOVA tests.

## Results

### Cadmium accumulation

Exposure to cadmium resulted in a significant time-dependent accumulation of this metal in oyster tissues (Fig. 1A), while Cd levels were below the detection limit in control oysters. Temperature had no significant effect on Cd accumulation in oyster tissues ( $F_{2,69}=1.46$ ,  $P=0.24$  and  $F_{2,67}=0.15$ ,  $P=0.86$  for gills and hepatopancreas, respectively). Cd accumulation rates were higher in gills than in hepatopancreas. Notably, Cd burdens in whole gills were similar to those in isolated gill cells, indicating that accumulated Cd was mostly intracellular (Fig. 1A).

### Cellular respiration and energy budget

Protein content per  $10^6$  cells was higher in hepatopancreas than in gills ( $0.58\pm 0.01$  vs  $0.62\pm 0.01$  mg  $10^{-6}$  cells $^{-1}$  in gills and hepatopancreas, respectively;  $F_{1,142}=5.64$ ,  $P=0.02$ ,  $N=37-38$ ) and was not significantly affected by acclimation temperature ( $P>0.50$ ) or duration of experimental acclimation ( $P>0.25$ ). Cd exposures resulted in a slight but statistically significant decrease in cellular protein content in gills by 9.8% ( $F_{1,70}=6.22$ ,  $P=0.02$ ,  $N=37-38$ ). In hepatopancreas, Cd exposures had no effect on cellular protein content ( $F_{1,71}=1.15$ ,  $P=0.29$ ).

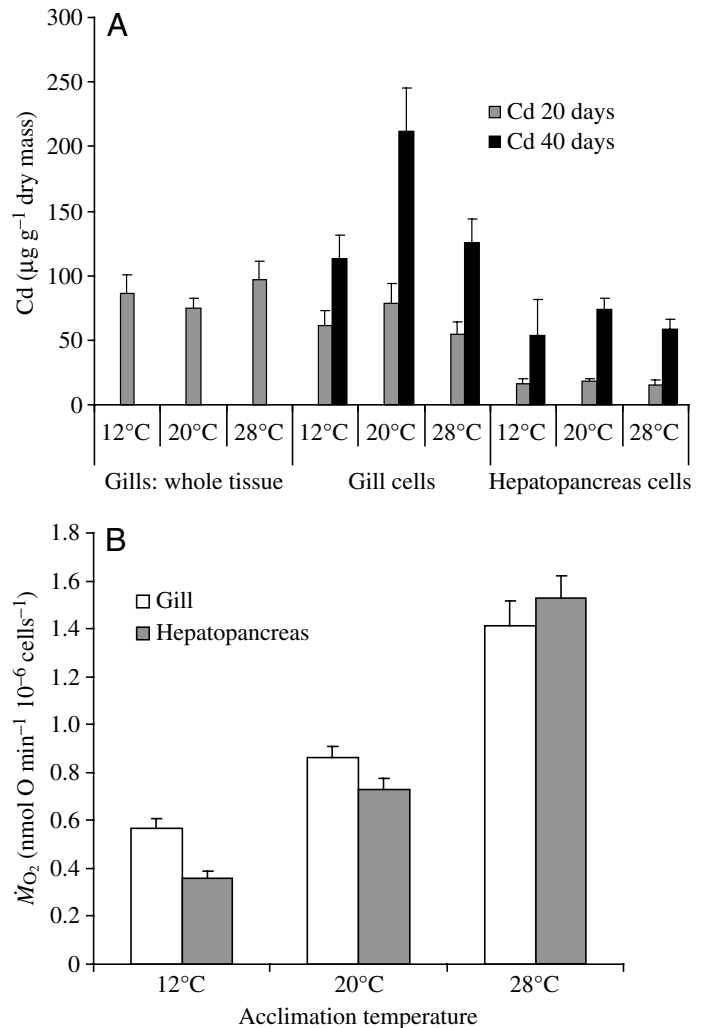


Fig. 1. Effects of acclimation temperature on Cd accumulation (A) and oxygen consumption (B) in isolated gill and hepatopancreas cells of *C. virginica*. Oysters were exposed for 20 or 40 days to clean artificial seawater (ASW) or  $50 \mu\text{g l}^{-1}$  Cd in ASW. Cd levels in tissues and isolated cells of control oysters were below the detection limits of the method used in this study ( $5 \text{ ng Cd g}^{-1}$  dry tissue mass).  $N=6-12$  in each group. Total oxygen consumption was measured in isolated cells of control oysters at their respective acclimation temperatures.  $N=6-12$  in each group.

Cellular  $\dot{M}_{\text{O}_2}$  increased with increasing temperature (Fig. 1B). In controls,  $\dot{M}_{\text{O}_2}$  of gill cells was notably higher than in hepatopancreas at 12 and 20°C but not at 28°C (Fig. 1B). Non-mitochondrial respiration accounted for 12–15% and for 16–31% of total  $\dot{M}_{\text{O}_2}$  in gill and hepatopancreas cells, respectively. In gills, temperature acclimation had no effect on the proportion of non-mitochondrial oxygen consumption ( $F_{2,70}=0.85$ ,  $P=0.43$ ). In hepatopancreas cells, the proportion of non-mitochondrial respiration decreased with increasing temperature, from 31% in 12°C-acclimated oysters to 16% in the 28°C-acclimated group ( $F_{2,69}=15.64$ ,  $P<0.001$ ,  $N=6-12$ ). Cd exposure had no effect on non-mitochondrial oxygen consumption ( $P>0.10$ ).

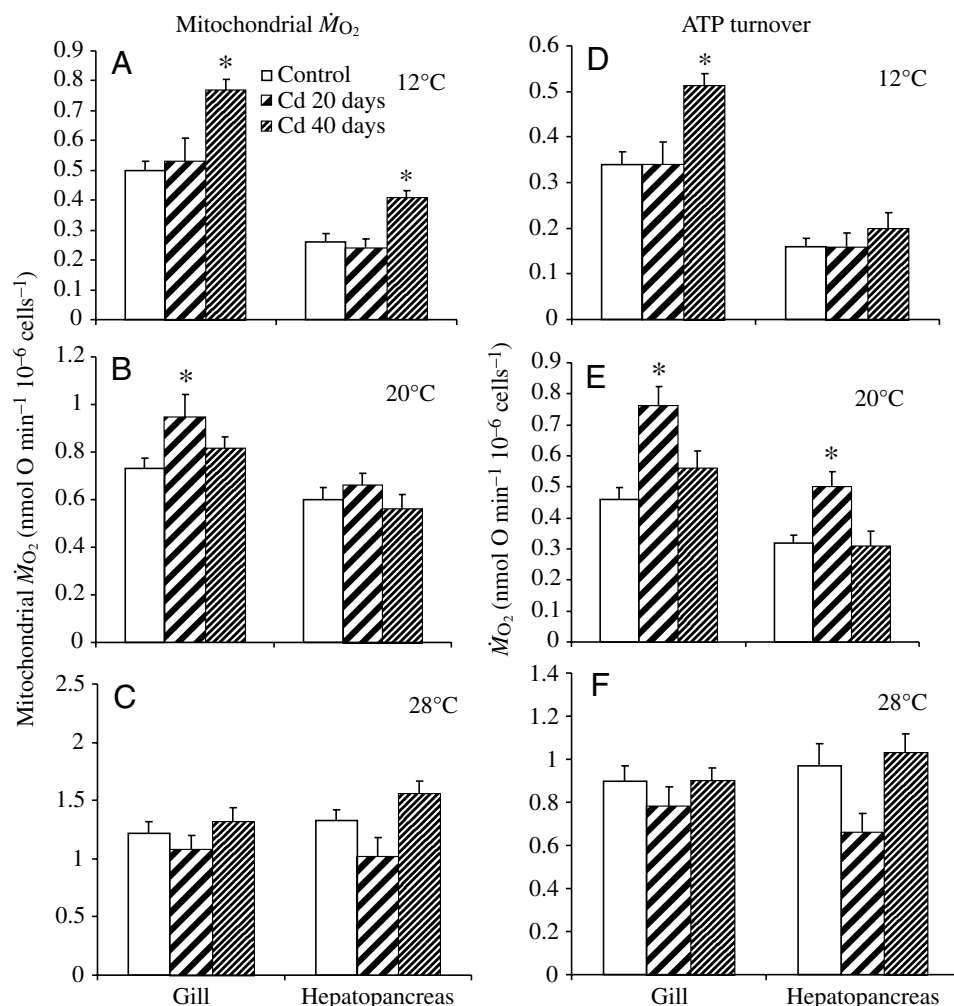


Fig. 2. Effects of acclimation temperature (12, 20 and 28°C) and cadmium exposure on mitochondrial oxygen consumption rate ( $\dot{M}_{O_2}$ ) (A–C) and  $\dot{M}_{O_2}$  related to ATP turnover (D–F) in isolated gill and hepatopancreas cells of *C. virginica*. Data for control oysters and for those exposed for 20 or 40 days to 50  $\mu\text{g l}^{-1}$  Cd are shown ( $N=6-12$ ). Asterisks mark values that are significantly different from the respective controls ( $P<0.05$ ).

control oysters, protein synthesis was responsible for 7–23% of total ATP turnover rate, whereas in oysters exposed to Cd for 40 days this proportion increased to 41–48%. Similarly, in hepatopancreas cells from control oysters, protein synthesis accounted for 23–49% of ATP turnover, whereas in Cd-exposed oysters this proportion rose to 54–71% (Fig. 4). The proportion of  $\dot{M}_{O_2}$  due to proton leak or ATP turnover did not change with temperature acclimation or Cd exposure (Fig. 4). Proton leak accounted for 21–33% of total  $\dot{M}_{O_2}$  in gills and for 23–37% in hepatopancreas. Oxygen demand for ATP turnover was 53–65% of total  $\dot{M}_{O_2}$  in gill cells at all studied

temperatures and in hepatopancreas cells at 28°C. At 12°C and 20°C, the proportion of ATP turnover in total  $\dot{M}_{O_2}$  of hepatopancreas cells was slightly lower (44–45%) due to a higher percentage of non-mitochondrial respiration.

Cd exposure resulted in elevated rates of mitochondrial  $\dot{M}_{O_2}$ , although this elevation was only statistically significant in gills and hepatopancreas cells at 12°C and gill cells at 20°C (Fig. 2A–C). Similarly, oxygen demand for ATP turnover significantly increased in response to Cd exposure at 12 (in gills) and 20°C (in both tissues) but not at 28°C (Fig. 2D–F). Energy demand for protein synthesis was strongly enhanced by Cd exposure, especially at 12 and 20°C (Fig. 3A–C). In 12°C- and 20°C-acclimated oysters, Cd-induced increase in protein synthesis was significant in gill and hepatopancreas cells, whereas in 28°C-acclimated ones it was only significant in hepatopancreas. On average, Cd exposure resulted in a 2–2.5-fold increase in protein synthesis rates after 40 days of exposure, with the exception of gill cells at 12°C, where protein synthesis rates in Cd-exposed oysters were six times higher than in the respective controls. Rates of mitochondrial proton leak were elevated in gill and hepatopancreas cells of oysters after prolonged (40 days) Cd exposure at 12°C (Fig. 3). At 20 and 28°C there were no significant changes in proton leak rates with Cd exposure (Fig. 3E,F).

Energy allocation to protein synthesis in the cellular energy budget (as estimated by % of  $\dot{M}_{O_2}$  due to protein synthesis) strongly increased with Cd exposure. Thus, in gill cells from

control oysters, protein synthesis was responsible for 7–23% of total ATP turnover rate, whereas in oysters exposed to Cd for 40 days this proportion increased to 41–48%. Similarly, in hepatopancreas cells from control oysters, protein synthesis accounted for 23–49% of ATP turnover, whereas in Cd-exposed oysters this proportion rose to 54–71% (Fig. 4). The proportion of  $\dot{M}_{O_2}$  due to proton leak or ATP turnover did not change with temperature acclimation or Cd exposure (Fig. 4). Proton leak accounted for 21–33% of total  $\dot{M}_{O_2}$  in gills and for 23–37% in hepatopancreas. Oxygen demand for ATP turnover was 53–65% of total  $\dot{M}_{O_2}$  in gill cells at all studied

#### Mitochondrial oxidation capacity

Oxidation capacity of oyster mitochondria was significantly affected by acclimation temperature (Table 2). As expected, the lowest rate of mitochondrial respiration was found in cold-acclimated oysters at their acclimation temperature (12°C). However, contrary to our expectations, the highest rates of mitochondrial oxidation were found not in the 28°C-acclimated oysters but in the 20°C-acclimated ones when measured at their respective acclimation temperatures. This was true for both ADP-stimulated (state 3) respiration and proton leak (state 4+). Similarly, MMP was highest in the 20°C-acclimated oysters compared with the 12°C- and 28°C-acclimated groups (Table 2). Moreover, prolonged acclimation to cold (12°C) and warm (28°C) temperatures resulted in a reduced mitochondrial coupling compared with the 20°C-acclimated group, suggesting that, of the three studied temperatures, 20°C may be optimal for mitochondrial function. In 12°C- and 20°C-acclimated groups, Cd-exposed oysters

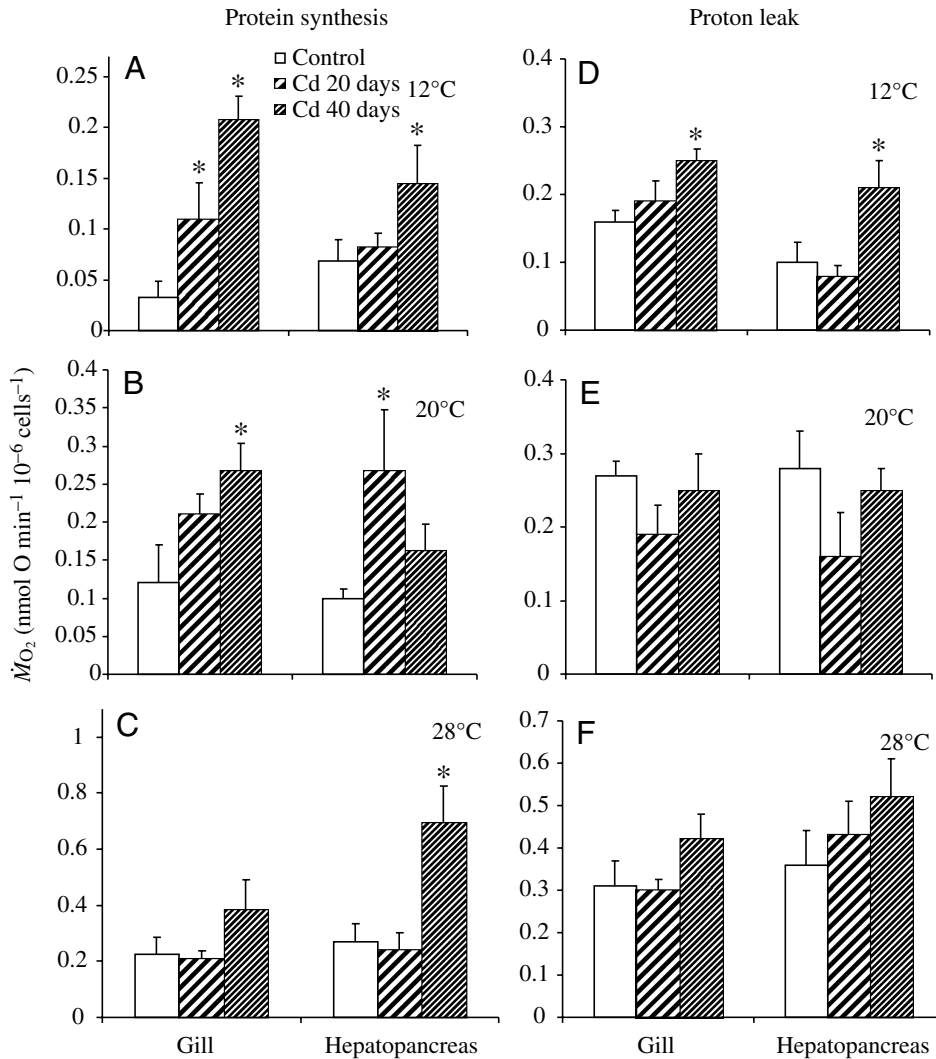


Fig. 3. Effects of acclimation temperature (12, 20 and 28°C) and cadmium exposure on mitochondrial  $M_{O_2}$  related to protein synthesis (A–C) and proton leak (D–F) in isolated gill and hepatopancreas cells of *C. virginica*. Data for control oysters and for those exposed for 20 or 40 days to 50  $\mu\text{g l}^{-1}$  Cd are shown ( $N=6-12$ ). Asterisks mark values that are significantly different from the respective controls ( $P<0.05$ ).

gills, interaction between temperature and Cd exposure had a significant effect on mitochondrial fractional volume ( $P=0.015$ ), thus preventing analysis of the effects of single factors. Therefore, effects of Cd exposure on the mitochondrial volume in gills were analyzed separately for each acclimation temperature; Cd effects were significant at 20°C ( $P=0.008$ ) but not at 12°C or 28°C ( $P=0.58$  and 0.24, respectively) (Table 4).

### Discussion

Cd exposure resulted in a significant increase in cellular  $M_{O_2}$  in oysters at 12 and 20°C, indicating elevated energy demand. By contrast, at 28°C there was no increase in cellular  $M_{O_2}$  in response to Cd. This agrees with our earlier studies showing elevated whole-organism SMR in Cd-exposed

tended to have higher mitochondrial oxidation rates and elevated MMP compared with their control counterparts (Table 2). In 28°C-acclimated oysters, Cd exposure had no significant effects on MMP or mitochondrial respiration rates (Table 2).

#### Mitochondrial abundance and fractional volume

Mitochondrial abundance was similar in gills and hepatopancreas ( $F_{1,354}=1.54$ ,  $P=0.2148$ ), whereas mitochondrial fractional volume was nearly twice as high in hepatopancreas than in gills ( $F_{1,356}=34.15$ ,  $P<0.0001$ ,  $N=29-31$ ), indicating the larger size of hepatopancreas mitochondria (Tables 3, 4). Cd exposure resulted in a decrease in mitochondrial numbers in both organs that was statistically significant in gills and marginally significant in hepatopancreas ( $F_{1,172}=4.50$ ,  $P=0.03$ ,  $N=29-31$  and  $F_{1,174}=2.68$ ,  $P=0.10$ ,  $N=29-31$ , respectively). This decrease was more pronounced at higher temperatures so that mitochondrial numbers dropped by 8–13% in Cd-exposed oysters at 12°C and by 20–24% at 28°C (Table 3). Mitochondrial volume was significantly reduced in Cd-exposed oysters in hepatopancreas ( $F_{1,177}=4.60$ ,  $P=0.03$ ,  $N=29-31$ ). In

oysters at 20°C but not at 28°C (Lannig et al., 2006) and suggests good correspondence between cellular and whole-organism metabolic response to Cd. Surprisingly, mitochondrial oxidation capacity (measured at the respective acclimation temperature) did not increase with increasing temperature of acclimation and was highest in 20°C-acclimated oysters compared with the 12°C- or 28°C-acclimated ones. In fact, mitochondrial capacity for ATP synthesis was >2 times lower in 28°C-acclimated oysters than in their 20°C-acclimated counterparts despite the fact that the whole-cell oxygen demand increased nearly twofold at 28°C. This indicates that oyster mitochondria may function close to their capacity limits at 28°C and could explain why SMR does not further increase in response to Cd at this temperature.

Analysis of cellular energy budgets in oysters showed that mitochondrial proton leak and protein synthesis represent major energy costs, accounting for 22–38% and 6–17% of the total oxygen consumption, respectively. These estimates are similar to the values reported in other poikilotherms (for reviews, see Brand et al., 1991; Hand and Hardewig, 1996; Hulbert and Else, 1999; Hulbert et al., 2002). An increase in acclimation

temperature did not significantly affect fractions of the total energy spent on proton leak, protein synthesis, overall ATP turnover or non-mitochondrial respiration despite an overall increase in cellular energy demand. This indicates a concerted change in the rates of different energy-requiring cellular processes during temperature acclimation in oysters. By contrast, Cd exposure had a disproportionate effect on some components of the energy budget (particularly protein synthesis and, to a lesser extent, proton leak) while not affecting the others (non-mitochondrial respiration and overall ATP turnover). Cd exposure resulted in a strong, 1.5-fold increase in mitochondrial proton leak in gill and hepatopancreas cells in 12°C-acclimated

oysters, whereas no change was observed at 20 and 28°C. This pattern closely corresponds to the data on isolated mitochondria, where Cd exposure resulted in a 1.5-fold increase in proton leak at 12°C but not 20 or 28°C. Thus, elevated proton leak may contribute to the observed increase in energy demand of Cd-exposed gill cells at 12°C.

Our data show that an increase in the cost of protein synthesis was the most consistent metabolic response to Cd, indicating that this mechanism may significantly contribute to the Cd-induced increase in energy demand. In gills, prolonged Cd exposure resulted in a >6-fold and >2-fold increase in the protein synthesis costs at 12 and 20°C, respectively. In Cd-exposed hepatopancreas cells, energy demand for protein synthesis increased by a factor of 2–2.5 across all studied temperatures. This increase is likely to reflect elevated rates of protein synthesis, which may include protein deposition (growth) as well as proteins synthesized for Cd detoxification, general stress protection and replacement of the damaged housekeeping proteins. There is no evidence that Cd exposure can result in elevated rates of protein deposition and growth; in fact, Cd exposure is known to stunt growth and lead to protein loss in oysters, especially at high Cd levels (reviewed in Roesijadi, 1996). Notably, this study also showed that cellular protein content decreased by approximately 10% in gills and did not change in hepatopancreas in Cd-exposed oysters, indicating that there was no increase in protein deposition in response to Cd in oyster cells.

Overexpression of inducible stress proteins is a particularly attractive hypothesis to explain the elevated rates of protein synthesis in response to Cd exposure. It has been shown that Cd exposure results in elevated expression of metallothioneins (MTs) and heat-shock proteins (HSPs) in oysters (Roesijadi et al., 1997; Piano et al., 2004; Moraga et al., 2005). Due to the short half-life of MTs and HSPs (Andersen et al., 1978; Mehra and Bremner, 1985; Pelham, 1990), high synthesis rates would be required to maintain elevated steady-state levels of these proteins in Cd-exposed oysters. On the other hand, Cd exposure can induce oxidative damage of proteins (Sandalio et al., 2001; Collen et al., 2003; Shi et al., 2005) and/or disrupt their native structure due to binding to the critical thiol and imidazol groups (reviewed in Valko et al., 2005). Thus, synthesis costs to replace the damaged housekeeping proteins may also add to the elevated energy demand in Cd-exposed oysters. Quantification of the relative contributions of stress protein expression and the replacement of the damaged housekeeping to the global costs of protein synthesis is an important question that requires further investigation in order to further our understanding of the mechanisms of metabolic response to stress.

It is worth noting that the above interpretation (i.e. that elevated protein synthesis costs in Cd-exposed oysters are reflective of the elevated rates of protein synthesis) assumes that the efficiency of protein synthesis (i.e. ATP demand per peptide bond) is the same in control and Cd-exposed oysters. If this does not hold true and the efficiency of the protein synthesis is adversely affected by Cd, an increase in energy demand for protein synthesis may reflect higher ATP demand per unit

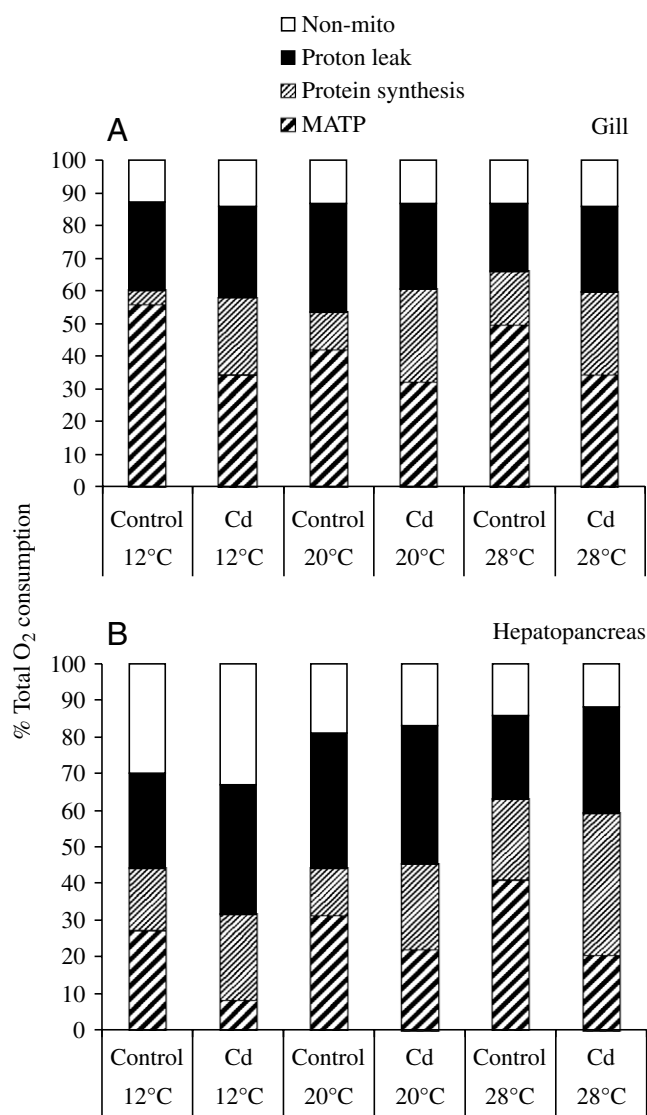


Fig. 4. Effects of acclimation temperature and cadmium exposure on cellular energy budgets of isolated gill (A) and hepatopancreas (B) cells of *C. virginica*. Data for control oysters and for those exposed for 40 days to  $50 \mu\text{g l}^{-1}$  Cd are shown ( $N=6-12$ ). Proportions of the total oxygen consumption in isolated cells due to non-mitochondrial respiration (non-mito), mitochondrial proton leak (proton leak), cytosolic protein synthesis (protein synthesis) and the remaining ATP turnover (MATP) are given.



Table 2. Mitochondrial oxidation capacity and MMP in isolated mitochondria from gills of control and Cd-exposed *C. virginica* acclimated at different temperatures

| $T_{acc}$ (°C) | Cd exposure ( $\mu\text{g l}^{-1}$ ) | Respiration rate (nmol O $\text{min}^{-1}$ $\text{mg}^{-1}$ protein) |            | RCR       | MMP index (RFU) |
|----------------|--------------------------------------|--|------------|-----------|-----------------|
|                |                                      | State 3  | State 4+   |           |                 |
| 12             | 0                                    | 7.39±1.80  | 3.73±0.90  | 1.62±0.16 | 2.05±0.17       |
|                | 50                                   | 10.12±0.95   | 5.67±0.47* | 1.59±0.15 | 2.70±0.20*      |
| 20             | 0                                    | 26.17±4.31   | 11.22±2.43 | 2.09±0.20 | 2.64±0.09       |
|                | 50                                   | 32.18±5.22   | 12.93±2.44 | 2.11±0.12 | 3.15±0.14*      |
| 28             | 0                                    | 10.41±2.40   | 6.48±1.42  | 1.65±0.21 | 1.69±0.06       |
|                | 50                                   | 10.57±3.15   | 4.65±1.54  | 1.57±0.18 | 1.51±0.05       |

Oysters were acclimated for 20 days at 12, 20 and 28°C without cadmium (control) or with the addition of 50  $\mu\text{g l}^{-1}$  cadmium (Cd-exposed). Oxidation rates, respiratory control ratios (RCR) and mitochondrial membrane potential (MMP) were determined in gill mitochondria at the respective acclimation temperatures ( $T_{acc}$ ). Asterisks denote values that are significantly different in Cd-exposed oysters (50  $\mu\text{g l}^{-1}$  Cd) compared with their respective controls (0  $\mu\text{g l}^{-1}$  Cd) ( $P < 0.05$ ).  $N = 5-13$  for each group.

Table 3. Mitochondrial abundance in gills and hepatopancreas cells of control and Cd-exposed *C. virginica* acclimated at different temperatures

| $T_{acc}$ (°C) | Cd exposure ( $\mu\text{g l}^{-1}$ ) | Mitochondrial density ( $10^5 \text{ mm}^{-2}$ ) |                | % decrease from control |                |
|----------------|--------------------------------------|--|----------------|-------------------------|----------------|
|                |                                      | Gills  | Hepatopancreas | Gills                   | Hepatopancreas |
| 12             | 0                                    | 5.54±0.85 (29)                                   | 6.24±0.96 (29) |                         |                |
|                | 50                                   | 5.08±0.62 (31)                                   | 5.45±0.71 (31) | 8.4                     | 12.7           |
| 20             | 0                                    | 7.36±0.71 (31)                                   | 5.71±1.05 (31) |                         |                |
|                | 50                                   | 5.94±0.57 (30)                                   | 4.74±0.75 (30) | 19.3                    | 17.0           |
| 28             | 0                                    | 5.99±0.56 (30)                                   | 5.23±0.52 (30) |                         |                |
|                | 50                                   | 4.55±0.45 (30)                                   | 4.16±0.42 (30) | 24.0                    | 20.5           |

Number of TEM sections analyzed for each tissue and temperature–cadmium combination is given in parentheses.

synthesized protein rather than elevated rates of synthesis. Few studies that have directly measured protein synthesis efficiency in marine invertebrates indicate that ATP demand per peptide bond is fairly constant over a broad range of environmental temperatures and at different stages of life cycles (Storch and Pörtner, 2003; Pace and Manahan, 2006). Unfortunately, the effects of Cd exposure on the ATP demand for protein synthesis have not yet been studied and clearly warrant further investigation. Irrespective of the molecular mechanisms, elevated energy expenditure for protein synthesis could divert energy from other energy-demanding cellular processes; indeed, the fraction of protein synthesis costs in the total ATP turnover increased by approximately 20% in Cd-exposed oysters.

The potential impact of the stress-induced energy demand would depend on the ability of the metabolic machinery to adequately increase energy output to compensate for it. Obviously, if the energy supply is adequate, elevated energy demand *per se* may not cause any adverse effects at the whole-organism level. Our data suggest that this is not the case in oysters exposed to combined Cd and temperature stress. No

Table 4. Mitochondrial fractional volume in gills and hepatopancreas cells of control and Cd-exposed *C. virginica* acclimated at different temperatures

| $T_{acc}$ (°C) | Cd exposure ( $\mu\text{g l}^{-1}$ ) | Mitochondrial fractional volume (%) |                |
|----------------|--------------------------------------|-------------------------------------|----------------|
|                |                                      | Gills                               | Hepatopancreas |
| 12             | 0                                    | 16.6±2.64 (29)                      | 33.3±4.37 (30) |
|                | 50                                   | 18.1±2.16 (33)                      | 23.8±2.14 (29) |
| 0              | 0                                    | 24.9±3.57 (29)                      | 27.2±3.17 (33) |
|                | 50                                   | 15.5±2.26 (29)                      | 27.1±2.41 (32) |
| 28             | 0                                    | 14.3±1.69 (29)                      | 32.8±5.01 (31) |
|                | 50                                   | 18.2±1.77 (30)                      | 23.7±2.05 (29) |

Mitochondrial fractional volume was calculated based on the relative area occupied by mitochondria to the total area occupied by cells on a tissue section (see Howard and Reed, 1998). Number of TEM sections analyzed for each tissue and temperature–cadmium combination is given in parentheses.

compensatory increase in mitochondrial oxidation rate, abundance or fractional volume was observed in Cd- and/or temperature-stressed oysters, indicating that the supply side of the cellular energy budget may become limiting under combined exposure to those stressors. In fact, there was a trend for decreased mitochondrial abundance in Cd-exposed oysters compared with their control counterparts, and this decrease was most pronounced in warm-acclimated oysters that experience the highest energy demand. Our earlier studies also showed that oyster mitochondria become increasingly more sensitive to the toxic effects of cadmium as the environmental temperature rises (Sokolova, 2004). Taken together, these data indicate that combined exposure to temperature and Cd stress may result in a decreased mitochondrial capacity of oyster tissues, which, in conjunction with elevated SMR, may narrow the aerobic scope in oysters.

As a corollary, our data show that Cd exposure and elevated temperatures strongly affect both the demand side and the supply side of cellular energy budgets in the model marine poikilotherm *Crassostrea virginica*. Elevated cellular energy demands in Cd-exposed oysters are due to the increased costs of protein turnover and, at cold acclimation temperatures, are also partially due to the elevated mitochondrial proton leak. Cellular aerobic capacity does not increase in parallel to compensate for elevated energy demand. This may result in a discrepancy between energy demand and energy supply and lead to the reduced aerobic scope for activity, growth and/or reproduction and thus to potential fitness costs in temperature- and Cd-stressed oysters. The disconnection between indices of energy supply and demand also has important methodological implications, suggesting that demand-side and supply-side endpoints should be considered separately in studies of energy budgets. It strongly cautions against the use of capacity indices such as the maximum activity of electron transfer system as a measure of energy demand, as was proposed recently (Fanslow et al., 2001; De Coen and Janssen, 2003; Smolders et al., 2004). Our study shows that both elevated temperatures and Cd exposure lead to high energy demand and adversely affect mitochondrial aerobic capacity despite different molecular mechanisms of action. This could explain strong synergism between these environmental stressors and suggests that elevated temperatures (as expected during seasonal acclimatization and/or global climate change) may strongly enhance pollution-related stress in marine poikilotherms.

This work was supported by funds provided by National Science Foundation (IBN-0347238) to I.M.S.

## References

- Alkon, D. L., Bank, B., Naito, S., Chen, C. and Ram, J. (1987). Inhibition of protein synthesis prolongs Ca<sup>2+</sup>-mediated reduction of K<sup>+</sup> currents in molluscan neurons. *Proc. Natl. Acad. Sci. USA* **84**, 6948–6952.
- Andersen, R. D., Winter, W. P., Maher, J. J. and Bernstein, I. A. (1978). Turnover of metallothioneins in rat liver. *Biochem. J.* **174**, 327–338.
- Baird, D. J., Barber, I. and Calow, P. (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. I. Chronic life-history effects. *Funct. Ecol.* **4**, 399–407.
- Barber, I., Baird, D. J. and Calow, P. (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. II. Physiological effects. *Funct. Ecol.* **4**, 409–414.
- Bergmeyer, H. U. (1985). *Methods of Enzymatic Analysis*. Weinheim: VCH Verlagsgesellschaft.
- Brand, M. D., Couture, P., Else, P. L., Withers, K. W. and Hulbert, A. J. (1991). Evolution of energy metabolism: proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile. *Biochem. J.* **275**, 81–86.
- Brand, M. D., Chien, L. F., Aiscow, E. K., Rolfe, D. F. S. and Porter, R. K. (1994). The causes and functions of the mitochondrial proton leak. *Biochem. Biophys. Acta* **1187**, 132–139.
- Brierley, G. P. (1977). Effects of heavy metals on isolated mitochondria. In *Biochemical Effects of Environmental Pollutants* (ed. S. D. Lee), pp. 397–411. Ann Arbor, MI: Anna Arbor Science.
- Byczkowski, J. Z. and Sorenson, J. R. J. (1984). Effects of metal compounds on mitochondrial function: a review. *Sci. Total Environ.* **37**, 133–162.
- Chance, B. and Williams, G. R. (1955). Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. Biol. Chem.* **217**, 383–393.
- Collen, J., Pinto, E., Pedersen, M. and Colepicolo, P. (2003). Induction of oxidative stress in the red macroalga *Gracilaria tenuistipitata* by pollutant metals. *Arch. Environ. Contam. Toxicol.* **45**, 337–342.
- Crompton, T. R. (1997). *Toxicants in Aqueous Ecosystem*. Chichester, UK: John Wiley and Sons.
- De Coen, W. M. and Janssen, C. R. (2003). A multivariate biomarker-based model predicting population-level responses of *Daphnia magna*. *Environ. Toxicol. Chem.* **22**, 2195–2201.
- Dorigan, J. V. and Harrison, F. L. (ed.) (1987). *Physiological Responses of Marine Organisms to Environmental Stresses*. Washington, DC: US Department of Energy.
- Dorta, D. J., Leite, S., DeMarco, K. C., Prado, I. M. R., Rodrigues, T., Mingatto, F. E., Uyemura, S. A., Santos, A. C. and Curti, C. (2003). A proposed sequence of events for cadmium-induced mitochondrial impairment. *J. Inorg. Biochem.* **57**, 251–257.
- Estabrook, R. W. (1967). Mitochondrial respiratory control and the polarographic measurements in mitochondria. *Meth. Enzymol.* **10**, 41–47.
- Evans, W. R. (1971). The effect of cycloheximide on membrane transport in *Euglena*: a comparative study with nigericin. *J. Biol. Chem.* **246**, 6144–6151.
- Fanslow, D. L., Nalepa, T. F. and Johengen, T. H. (2001). Seasonal changes in the respiratory electron transport system (ETS) and respiration of the zebra mussel, *Dreissena polymorpha* in Saginaw Bay, Lake Huron. *Hydrobiologia* **448**, 61–70.
- Fuery, C. J., Withers, P. C. and Guppy, M. (1998). Protein synthesis in the liver of *Bufo marinus*: cost and contribution to oxygen consumption. *Comp. Biochem. Physiol.* **119A**, 459–467.
- Giuditta, A., Dettbarn, W. D. and Brzin, M. (1968). Protein synthesis in the isolated giant axon of the squid. *Proc. Natl. Acad. Sci. USA* **59**, 1284–1287.
- GESAMP (1987). Land–sea boundary flux of contaminants: contributions from rivers. GESAMP Reports and Studies **32**, 1–172.
- Hackney, C. T., Grimley, J., Posey, M., Alphin, T. and Hyland, J. (1998). Sediment contamination in North Carolina's estuaries. Publication 198 of the Centre for Marine Science Research, University of North Carolina at Wilmington, Wilmington NC, USA. pp. 2–59.
- Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* **58**, 539–563.
- Hayat, M. A. (2000). *Principles and Techniques of Electron Microscopy: Biological Applications*. Cambridge: Cambridge University Press.
- Helmuth, B., Harley, C. D. G., Halpin, P. M., O'Donnell, M., Hofmann, G. E. and Blanchette, C. A. (2002). Climate change and latitudinal patterns of intertidal thermal stress. *Science* **298**, 1015.
- Hopkins, W. A., Rowe, C. L. and Congdon, J. D. (1999). Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. *Environ. Toxicol. Chem.* **18**, 1258–1263.
- Howard, C. V. and Reed, M. G. (1998). *Unbiased Stereology: Three-dimensional Measurement in Microscopy*. New York: Springer-Verlag/BIOS Scientific Publishers.
- Hulbert, A. J. and Else, P. L. (1999). Membranes as possible pacemakers of metabolism. *J. Theor. Biol.* **199**, 257–274.
- Hulbert, A. J., Else, P. L., Manolis, S. and Brand, M. (2002). Proton leak in hepatocytes and liver mitochondria from archosaurs (crocodiles) and allometric relationships for ectotherms. *J. Comp. Physiol. B* **172**, 387–397.

- Hutchinson, S. and Hawkins, L. E.** (1992). Quantification of the physiological response of the European flat oyster *Ostrea edulis* L. to temperature and salinity. *J. Molluscan Stud.* **58**, 215-216.
- Joyner, J. L., Peyer, S. M. and Lee, R. W.** (2003). Possible roles of sulfur-containing amino acids in chemoautotrophic bacterium–mollusc symbiosis. *Biol. Bull.* **205**, 331-338.
- Kessler, A. and Brand, M. D.** (1994). Effects of cadmium on the control and internal regulation of oxidative phosphorylation in potato tuber mitochondria. *Eur. J. Biochem.* **225**, 907-922.
- Kessler, A. and Brand, M. D.** (1995). The mechanism of the stimulation of state-4 respiration by cadmium in potato-tuber (*Solanum tuberosum*) mitochondria. *Plant Physiol. Biochem.* **33**, 519-528.
- Koehn, R. K. and Bayne, B. L.** (1989). Towards a physiological and genetical understanding of the energetics of the stress response. *Biol. J. Linn. Soc.* **37**, 169-171.
- Korotkov, S. M., Glazunov, V. V., Rozengart, E. V. and Suvorov, A. A.** (1996). Effect of hydrophobic organic complex of cadmium on ion permeability of mitochondrial membrane and on respiration of rat liver mitochondria. *Biol. Membr.* **13**, 178-184.
- Korotkov, S. M., Glazunov, V. V., Rozengart, E. V., Suvorov, A. A. and Nikitina, E. R.** (1999). Effects of Cd<sup>2+</sup> and two cadmium organic complexes on isolated rat liver mitochondria. *J. Biochem. Mol. Toxicol.* **13**, 149-157.
- Lannig, G., Cherkasov, A. S. and Sokolova, I. M.** (2006). Temperature-dependent effects of cadmium on mitochondrial and whole-organism bioenergetics in oysters. *Mar. Environ. Res.*, in press.
- Li, S.-C., Wang, W.-X. and Hsieh, D. P. H.** (2002). Effects of toxic dinoflagellate *Alexandrium tamarense* on the energy budgets and growth of two marine bivalves. *Mar. Environ. Res.* **53**, 145-160.
- Mallin, M. A., Ensign, S. H., Parsons, D. C. and Merritt, J. F.** (1999). Environmental quality of Wilmington and New Hanover county watersheds 1998-1999. ECMSR Report 99-02, Center for Marine Science Research, University of North Carolina at Wilmington, Wilmington NC. pp. 1-58.
- Mehra, R. K. and Bremner, I.** (1985). Studies on the metabolism of rat liver copper-metallothionein. *Biochem. J.* **227**, 903-908.
- Miccadei, S. and Floridi, A.** (1993). Sites of inhibition of mitochondrial electron-transport by cadmium. *Chem. Biol. Interact.* **89**, 159-167.
- Moraga, D., Meistertzheim, A.-L., Tanguy-Royer, S., Boutet, I., Tanguy, A. and Donval, A.** (2005). Stress response in Cu<sup>2+</sup> and Cd<sup>2+</sup> exposed oysters (*Crassostrea gigas*): an immunohistochemical approach. *Comp. Biochem. Physiol.* **141C**, 151-156.
- Pace, D. A. and Manahan, D. T.** (2006). Fixed metabolic costs for highly variable rates of protein synthesis in sea urchin embryos and larvae. *J. Exp. Biol.* **209**, 158-170.
- Pelham, H. R. B.** (1990). Functions of the hsp70 protein family: an overview. In *Stress Proteins in Biology and Medicine* (ed. R. I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 287-299. Cold Spring Harbor: Cold Spring Harbor laboratory Press.
- Piano, A., Valbonesi, P. and Fabbri, E.** (2004). Expression of cytoprotective proteins, heat shock protein 70 and metallothioneins, in tissues of *Ostrea edulis* exposed to heat and heavy metals. *Cell Stress Chaperones* **9**, 134-142.
- Roesijadi, G.** (1996). Environmental factors: response to metals. In *The Eastern Oyster Crassostrea Virginica* (ed. V. S. Kennedy, R. I. E. Newell and A. F. Eble), pp. 515-537. College Park, MD: Maryland Sea Grant Book.
- Roesijadi, G., Brubacher, L. L., Unger, M. E. and Anderson, R. S.** (1997). Metallothionein mRNA induction and generation of reactive oxygen species in molluscan hemocytes exposed to cadmium in vitro. *Comp. Biochem. Physiol.* **118C**, 171-176.
- Rowe, C. L.** (1998). Elevated standard metabolic rate in a freshwater shrimp (*Palaeomonetes paludosus*) exposed to trace element-rich coal combustion waste. *Comp. Biochem. Physiol.* **121A**, 299-304.
- Rowe, C. L., Kinney, O. M., Nagle, R. D. and Congdon, J. D.** (1998). Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiol. Zool.* **71**, 27-35.
- Rowe, C. L., Hopkins, W. A., Zehnder, C. and Congdon, J. D.** (2001). Metabolic costs incurred by crayfish (*Procambarus acutus*) in a trace element-polluted habitat: further evidence of similar responses among diverse taxonomic groups. *Comp. Biochem. Physiol.* **129C**, 275-283.
- Sandalio, L. M., Dalurzo, H. C., Gomez, M., Romero-Puertas, M. C. and del Rio, L. A.** (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp. Bot.* **52**, 2115-2126.
- Scaduto, R. C., Jr and Grotzoyhann, L. W.** (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* **76**, 469-477.
- Shi, H., Sui, Y., Wang, X., Luo, Y. and Ji, L.** (2005). Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. *Comp. Biochem. Physiol. C* **140C**, 115-121.
- Shumway, S. E.** (1996). Natural environmental factors. In *The Eastern Oyster Crassostrea Virginica* (ed. V. S. Kennedy, R. I. E. Newell and A. F. Eble), pp. 467-514. College Park, MD: Maryland Sea Grant Book.
- Smolders, R., Bervoets, L., De Coen, W. and Blust, R.** (2004). Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environ. Pollut.* **129**, 99-112.
- Sokolova, I. M.** (2004). Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *J. Exp. Biol.* **207**, 2639-2648.
- Sokolova, I. M., Sokolov, E. P. and Ponnappa, K. M.** (2005a). Cadmium exposure affects mitochondrial bioenergetics and gene expression of key mitochondrial proteins in the eastern oyster *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Aquat. Toxicol.* **73**, 242-255.
- Sokolova, I. M., Ringwood, A. H. and Johnson, C.** (2005b). Tissue-specific accumulation of cadmium in subcellular compartments of eastern oysters *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Aquat. Toxicol.* **74**, 218-228.
- Storch, D. and Pörtner, H. O.** (2003). The protein synthesis machinery operates at the same expense in eurythermal and cold stenothermal pectinids. *Physiol. Biochem. Zool.* **76**, 28-40.
- Valko, M., Morris, H. and Cronin, M. T.** (2005). Metals, toxicity and oxidative stress. *Curr. Med. Chem.* **12**, 1161-1208.
- Widdows, J.** (1978). Physiological indices of stress in *Mytilus edulis*. *J. Mar. Biol. Assoc. UK* **58**, 125-142.
- Willmer, P., Stone, G. and Johnston, I.** (2000). *Environmental Physiology of Animals*. Oxford, London: Blackwell Science.