RESEARCH ARTICLE

Effects of temperature and cadmium exposure on the mitochondria of oysters (*Crassostrea virginica*) exposed to hypoxia and subsequent reoxygenation

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SUMMARY

Intertidal bivalves are commonly exposed to multiple stressors including periodic hypoxia, temperature fluctuations and pollution, which can strongly affect energy metabolism. We used top-down control and elasticity analyses to determine the interactive effects of intermittent hypoxia, cadmium (Cd) exposure and acute temperature stress on mitochondria of the eastern oyster *Crassostrea virginica*. Oysters were acclimated at 20°C for 30 days in the absence or presence of 50 μg l^{-1} Cd and then subjected to a long-term hypoxia (6 days at <0.5% O$_2$ in seawater) followed by normoxic recovery. Mitochondrial function was assessed at the acclimation temperature (20°C), or at elevated temperature (30°C) mimicking acute temperature stress in the intertidal zone. In the absence of Cd or temperature stress, mitochondria of oysters showed high resilience to transient hypoxia. In control oysters at 20°C, hypoxia/reoxygenation induced elevated flux capacity of all three studied mitochondrial subsystems (substrate oxidation, phosphorylation and proton leak) and resulted in a mild depolarization of resting mitochondria. Elevated proton conductance and enhanced capacity of phosphorylation and substrate oxidation subsystems may confer resistance to hypoxia/reoxygenation stress in oyster mitochondria by alleviating production of reactive oxygen species and maintaining high aerobic capacity and ATP synthesis rates during recovery. Exposure to environmental stressors such as Cd and elevated temperatures abolished the putative adaptive responses of the substrate oxidation and phosphorylation subsystems, and strongly enhanced proton leak in mitochondria of oysters subjected to hypoxia/reoxygenation stress. Our findings suggest that Cd exposure and acute temperature stress may lead to the loss of mitochondrial resistance to hypoxia and reoxygenation and thus potentially affect the ability of oysters to survive periodic oxygen deprivation in coastal and estuarine habitats.

Key words: hypoxia, metal pollution, mitochondria, mollusc, proton leak, temperature.

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INTRODUCTION

Oxygen deficiency is a common stressor in estuarine and coastal environments, and occurs when respiration by the resident biota (especially micro-organisms) outstrips the oxygen input into the water from photosynthesis, diffusion from the nearby oxygenated areas and atmospheric exchange (Tilman et al., 2001; Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008). In the past decade, the frequency of hypoxic events and the spatial extent of coastal hypoxic and anoxic 'dead zones' have been increasing at an alarming rate due to the eutrophication and warming of coastal waters (Tilman et al., 2001; Rabalais et al., 2009; Deutsch et al., 2011). Hypoxia is especially prominent in the near-bottom water layers and can strongly affect benthic invertebrates, including molluscs. Most benthic animals have low mobility that prevents their escape from unfavourable conditions, leading to adverse effects on their physiology, growth and reproduction, and often resulting in mass mortalities (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008).

Intertidal molluscs are among the animal champions of hypoxia tolerance owing to a suite of metabolic adaptations that allows them to survive prolonged periods without oxygen. Among these adaptations, metabolic arrest (a co-ordinated suppression of ATP production and consumption), alternative glycolytic pathways (increasing ATP yield per unit of metabolized substrate and mitigating the metabolic proton load) and high tissue-buffering capacities have been well studied (de Zwaan, 1983; Eberlee and Storey, 1984; Grieshaber et al., 1994; Sokolova et al., 2000a; Sokolova et al., 2000b). In contrast, the potential mechanisms that allow molluscs to preserve mitochondrial capacity during prolonged oxygen deficiency and subsequent reoxygenation are not fully understood. Mitochondria are the major ATP producers, providing >95% of aerobic ATP supply. During hypoxia, mitochondrial ATP synthesis ceases and mitochondrial integrity is maintained at the expense of ATP hydrolysis (St-Pierre et al., 2000). Post-hypoxic reoxygenation restores the mitochondrial ATP supply but represents an acute stress to mitochondria due to the increased generation of reactive oxygen species (ROS), as has been shown during ischaemia–reperfusion stress in mammalian models (Ambrosio et al., 1993; Blomgren et al., 2003; Levraut et al., 2003; Korge et al., 2008). Unlike mammals, hypoxia-tolerant intertidal molluscs can survive frequent hypoxia–reoxygenation events during the tidal cycles of air exposure and the following submersion, during diurnal cycles of oxygen concentrations, or during the seasonal development and disappearance of 'dead zones'. Functional changes in mitochondria are likely candidates for the involvement in the exceptional tolerance to hypoxia–reoxygenation stress in intertidal molluscs, and are the focus of the present study.

Top-down control analysis is a useful approach to study the control of mitochondrial respiration and function. This approach conceptually partitions mitochondrial reactions into three

interconnected blocks – phosphorylation, proton leak and substrate oxidation subsystems – linked by a common intermediate, the protonmotive force Δp (Brand, 1997; Brand, 1998; Suarez, 2004). The substrate oxidation subsystem creates Δp and involves the activities of the tricarboxylic acid cycle, electron transport chain, and substrate transporters. The phosphorylation and proton leak subsystems dissipate Δp. The phosphorylation subsystem (including F_o,F_1-ATPase, adenylate and inorganic phosphate transporters) uses Δp to synthesize ATP, and the proton leak subsystem involves all futile cation cycles that dissipate Δp without ATP production. Proton leak reactions reflect inherent inefficiency of mitochondria, but can also fulfil important physiological functions such as control of ROS production (Brand, 2000; Miwa and Brand, 2003; Rolfe and Brand, 1997). The top-down control analysis involves determining the kinetic response of these three subsystems (measured as changes of O_2 consumption) to an experimentally induced change in their common intermediate, Δp. This provides a way to quantify the control that each subsystem confers over mitochondrial respiration under different physiological conditions and to determine critical mitochondrial reactions modulated by external effectors. Top-down control analysis has been previously used to dissect the mechanisms of mitochondrial responses to temperature (Chamberlin, 2004b; Dufour et al., 1996), cadmium (Cd) exposure (Kesseler and Brand, 1994a; Kesseler and Brand, 1994b; Kesseler and Brand, 1994c; Kesseler and Brand, 1995; Kurochkin et al., 2011) and developmentally induced apoptosis (Chamberlin, 2004a). However, this approach has not been used to explore how rapid changes in oxygen levels affect the mitochondrial function of animals that often experience transient environmental hypoxia.

The aim of this study was to elucidate the effects of prolonged hypoxia (such as can occur in the coastal 'dead zones') and post-hypoxic recovery on mitochondrial function in a hypoxia-tolerant intertidal mollusc, the eastern oyster (*Crassostrea virginica* Gmelin 1791), and to determine whether mitochondrial sensitivity to hypoxia and reoxygenation is modulated by other environmental stressors such as temperature and exposure to a toxic metal, cadmium. The distribution of the oyster populations in the western Atlantic overlaps with the coastal 'dead zones' where these organisms can be exposed to oxygen-deficient conditions for days to weeks (Diaz and Solow, 1999; Rabalais et al., 1999), making them an excellent model to investigate the mitochondrial effects of transient hypoxia. Temperature fluctuations and metal pollution are also common stressors in oyster habitats. Like all intertidal organisms, oysters experience frequent and rapid fluctuations in temperature with up to 10–20°C change within a few hours during the diurnal and tidal cycles (Helmuth et al., 2002; Cherkasov, A. S., et al., 2007). Oysters can also bioaccumulate high levels of trace metals such as Cd, rendering them susceptible to their toxic effects (Roesijadi, 1996; Sokolova et al., 2005a). Both temperature and Cd can strongly affect mitochondrial function in oysters, decreasing mitochondrial efficiency, negatively affecting ATP synthesis capacity and increasing oxidative stress (Sokolova, 2004; Cherkasov et al., 2006b; Cherkasov, A. A., et al., 2007; Kurochkin et al., 2011). Due to high sensitivity of mitochondria to temperature and toxic metals (Kurochkin et al., 2009; Ivanina et al., 2010a; Ivanina et al., 2010b; Ivanina et al., 2011; Kurochkin et al., 2011), these stressors can potentially impact the ability of oysters to endure transient hypoxia and successfully recover upon reoxygenation in polluted estuaries; however, such impacts are not yet fully understood. This study investigates the combined effects of transient hypoxia, temperature and Cd stress on mitochondrial capacity and control of

mitochondrial function in order to provide insights into the potential mechanisms of stress-induced injury and protection of molluscan mitochondria in the face of multiple environmental stressors.

MATERIALS AND METHODS
Animal collection and maintenance
Adult *C. virginica* (7–12 cm shell length) were obtained from a commercial supplier (Cuttyhunk Shellfish Farms, Cuttyhunk, MA, USA). Oysters were shipped within 48 h of collection to the University of North Carolina at Charlotte and placed in tanks with aerated artificial seawater (ASW) (Instant Ocean, Kent Marine, Acworth, GA, USA) at 20°C and 30‰ salinity. Temperature and salinity were maintained within 1°C and 1‰ of their respective target values. All oysters were allowed to recover for 10 days. After that period of time, half of the tanks were randomly selected, and Cd (as $CdCl_2$) was added at a nominal concentration of 50 µg l^{-1}. The remaining tanks were used as controls. Oysters in all tanks (with and without Cd addition) were acclimated for 30 days prior to hypoxic exposure. Mortality during this acclimation period was less than 5% and did not significantly differ between control and Cd-exposed groups. Oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 ml per oyster) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of 2–20 µm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). At least three tanks were set for control and Cd exposure, and oysters were randomly sampled from these tanks for each experiment.

After the preliminary acclimation, oysters were placed in plastic trays fitted with a lid (10–12 oysters in 5 litres ASW), and hypoxic conditions were created by bubbling ASW with nitrogen (Robert Oxygen, Charlotte, NC, USA). Oxygen concentrations were measured daily and ranged between 0.2 and 0.5% O_2 throughout the duration of hypoxic exposure. Water in each tray was changed every day using ASW pre-equilibrated with nitrogen. After 6 days of hypoxia, oysters were returned to well-aerated tanks with ASW and allowed to recover for 1 h. This time was chosen because our previous studies indicated that this is when the strongest change in mitochondrial characteristics is expected (Kurochkin et al., 2009). Cd levels during hypoxic exposure and recovery periods were maintained at the same levels as during the previous 30 day acclimation period (0 and 50 µg l^{-1} Cd for control and Cd-exposed oysters, respectively). Mitochondria were isolated from oysters exposed to 6 days of hypoxia without reoxygenation (later referred to as hypoxic oysters) and from oysters exposed to 6 days of hypoxia followed by 1 h normoxic recovery (referred to as hypoxia/reoxygenation treatments). Control and Cd-exposed oysters were maintained for the same duration of time under normoxic conditions (19–21% O_2) in the absence or presence of 50 µg l^{-1} Cd, respectively (referred to as normoxic oysters). Mitochondria isolated from normoxic oysters served as controls for the respective hypoxia and hypoxia/reoxygenation treatments. Oysters were fed *ad libitum* with DT's Live Marine Phytoplankton during normoxia and post-hypoxic reoxygenation but not during hypoxic exposure. Our pilot studies showed that oysters ceased feeding in hypoxia even if provided with food (I.M.S., unpublished data); therefore, no food was added during hypoxic exposures to prevent potential artifacts due to the algal decay and bacterial proliferation.

Mitochondrial isolations
Mitochondria were isolated from oyster gills using a method modified from Sokolova (Sokolova, 2004). Briefly, 2–4 g of gills were placed in an ice-cold buffer containing 100 mmol l^{-1} sucrose,

$200 \, \text{mmol} \, l^{-1}$ KCl, $100 \, \text{mmol} \, l^{-1}$ NaCl, $8 \, \text{mmol} \, l^{-1}$ EGTA and $30 \, \text{mmol} \, l^{-1}$ Hepes at pH 7.5 and homogenized with several passes (200 r.p.m.) of a Potter–Elvenhjem homogenizer with a loosely fitting Teflon pestle. EGTA was used during the isolations of mitochondria from both control and Cd-exposed oysters to ensure that there was no carry-over of cytosolic trace metals to the mitochondrial assays. The homogenate was centrifuged at $2000 \, \boldsymbol{g}$ for 8 min to remove cell debris, and the supernatant was centrifuged at $8500 \, \boldsymbol{g}$ for 8 min to obtain a mitochondrial pellet. The mitochondrial pellet was resuspended in homogenization buffer without EGTA, centrifuged again at $8500 \, \boldsymbol{g}$ for 8 min, and resuspended in 0.5 ml of ice-cold assay medium consisting of $150 \, \text{mmol} \, l^{-1}$ sucrose, $250 \, \text{mmol} \, l^{-1}$ KCl, $10 \, \text{mmol} \, l^{-1}$ glucose, $10 \, \text{mmol} \, l^{-1}$ KH$_2$PO$_4$, $10 \, \text{mg} \, \text{ml}^{-1}$ bovine serum albumin (BSA; fatty acid free), and $30 \, \text{mmol} \, l^{-1}$ Hepes at pH 7.2. Protein concentrations in mitochondrial isolates were measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) in the presence of 0.1% Triton X-100 to solubilize mitochondrial membranes.

Measurements of mitochondrial respiration and membrane potential

Respiration of mitochondrial suspensions ($2 \, \text{mg} \, \text{ml}^{-1}$ protein) was measured in a water-jacketed, temperature-stabilized four-port chamber (World Precision Instruments, Sarasota, FL, USA) at one of the two studied temperatures (20 or 30°C) using a fibre-optic oxygen sensor connected to the Microx TX3 oxygen monitor (Precision Sensing, Dusseldorf, Germany). A two-point calibration (0 and 100% of air saturation using a saturated Na$_2$SO$_3$ solution and an air-saturated assay medium, respectively) was performed prior to each measurement at the respective assay temperature (20 or 30°C). It is worth noting that all oysters were acclimated at 20°C and therefore mitochondrial exposure to 30°C represents an acute temperature stress. Such acute temperature stress is commonly experienced by intertidal bivalves including oysters, whose body temperature can fluctuate by 10–20°C within a short period of time (minutes to hours) during the tidal cycle (Helmuth et al., 2002; Cherkasov, A. S., et al., 2007).

State 3 (ADP-stimulated) respiration was determined following addition of 200 nmol ADP, and state 4 (resting) respiration was measured when ADP was depleted. After state 4 respiration was measured, a specific inhibitor of mitochondrial F$_0$,F$_1$-ATPase, oligomycin ($2 \, \mu\text{g} \, \text{ml}^{-1}$), was added to ensure the complete cessation of ATP synthesis, and respiration was again determined. The difference in the rates of state 4 respiration in the presence or absence of oligomycin was less than 5%. To maintain consistency among the kinetic and bioenergetic assays, therefore, we report only values for state 4 in the presence of oligomycin. All mitochondrial respiration rates were corrected for non-mitochondrial respiration and oxygen sensor drift by subtracting the residual slope of the oxygen consumption in the presence of $8 \, \mu\text{mol} \, l^{-1}$ KCN. Non-mitochondrial respiration and sensor drift were negligible in these experiments (data not shown). Respiratory control ratios (RCR) were calculated by the ratio of state 3 over state 4 respiration rates, as described elsewhere (Kurochkin et al., 2011).

Mitochondrial membrane potentials ($\Delta\psi$) were determined simultaneously with respiration using a tetraphenyl phosphonium (TPP$^+$)-selective electrode (KWIKTPP-2) and a Super Dri-Ref reference electrode (World Precision Instruments) connected to a pH meter (model 1671, Jenco Instruments, San Diego, CA, USA) (Kurochkin et al., 2011). It is worth noting that mitochondrial protonmotive force (Δp) consists of the electrical membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) across the inner mitochondrial membrane. In this study, we added an H$^+$/K$^+$ exchanger, nigericin ($123 \, \text{nmol} \, l^{-1}$), to the assay media in order to collapse the pH gradient (ΔpH) and convert all of Δp into the electrical gradient ($\Delta\psi$). Therefore, throughout this study we refer to $\Delta\psi$ as a measure of the mitochondrial protonmotive force.

The tetraphenylphosphonium (TPP$^+$) electrode was calibrated before each measurement using stepwise additions of TPP$^+$ (2–$10 \, \mu\text{mol} \, l^{-1}$). Corrections for the non-specific binding of TPP$^+$ were conducted after fully collapsing the mitochondrial membrane potential with $0.4 \, \text{mmol} \, l^{-1}$ KCN and $0.5 \, \text{mmol} \, l^{-1}$ 2,4-dinitrophenol, as described by Lötscher (Lötscher et al., 1980) and Chamberlin (Chamberlin, 2004a). Non-specific binding of TPP$^+$ was $0.75 \pm 0.04 \, \mu\text{mol} \, \mu l^{-1} \, \text{mg}^{-1}$ protein and did not significantly differ between mitochondria of the control *versus* the Cd-exposed oysters at different temperatures. TPP$^+$ concentrations in the assay media were monitored using Logger Pro 3.2 with a Vernier LabPro interface (Vernier Software and Technology, Beaverton, OR, USA). The mitochondrial membrane potential was calculated using the Nernst equation, assuming a mitochondrial matrix volume of $1 \, \mu l \, \text{mg}^{-1}$ protein (Kurochkin et al., 2011).

Kinetic responses of the mitochondrial subsystems

The kinetic responses of the three mitochondrial subsystems (substrate oxidation, proton leak and oxidative phosphorylation) were measured as changes in the oxygen consumption in response to the experimentally induced changes in their common intermediate Δp (expressed as $\Delta\psi$) following a standard protocol of top-down control analysis (Brand, 1997; Brand, 1998; Suarez, 2004). For different subsystems, experimental manipulation of $\Delta\psi$ was achieved in different ways so that it did not directly affect the activity of the subsystem being measured. For proton leak and phosphorylation subsystems, a change in $\Delta\psi$ was achieved by titration with malonate, which affects $\Delta\psi$ *via* inhibition of the substrate oxidation subsystem but does not affect the proton leak or phosphorylation subsystems. For the substrate oxidation subsystem, manipulation of $\Delta\psi$ was achieved by the gradual addition of a mitochondrial uncoupler [(3-chlorophenyl)hydrazono]malononitrile (CCCP)], which affects $\Delta\psi$ *via* stimulation of the proton leak but does not directly affect the substrate oxidation subsystem. Titration with either malonate or CCCP led to a decrease in $\Delta\psi$.

The kinetic responses of proton leak, substrate oxidation and phosphorylation subsystems were measured in the presence of $0.5 \, \text{mmol} \, l^{-1}$ ADP, $20 \, \mu\text{mol} \, l^{-1}$ rotenone, $10 \, \text{mmol} \, l^{-1}$ succinate and 1% BSA (fatty acid free) (Kurochkin et al., 2011). Our pilot studies showed that $10 \, \text{mmol} \, l^{-1}$ succinate is a saturating concentration for all three subsystems under the assay conditions of this study (data not shown). The kinetic response of the oxidation subsystem was determined by a stepwise addition of small amounts of a mitochondrial uncoupler (0.2–$1.8 \, \mu\text{mol} \, l^{-1}$ CCCP) in the presence of $5 \, \mu\text{g} \, \text{ml}^{-1}$ oligomycin. The kinetic response of the proton leak subsystem was determined in the presence of oligomycin ($5 \, \mu\text{g} \, \text{ml}^{-1}$) by sequential addition of small amounts of malonate (2–$18 \, \text{mmol} \, l^{-1}$) to the respiring mitochondria. The phosphorylation subsystem was titrated by malonate (2–$18 \, \text{mmol} \, l^{-1}$) in the absence of oligomycin to achieve the maximum respiration rate. In these titrations, we added $0.15 \, \mu\text{mol}$ of ADP per 1 ml of the assay medium during each malonate addition to maintain the maximum mitochondrial respiration rates. In order to obtain the kinetics of the phosphorylation subsystem only, oxygen consumption measured during the titration of the phosphorylation subsystem was corrected by subtracting the oxygen consumption due to the proton leak calculated at the respective $\Delta\psi$ values.

Data analysis and statistics

We first examined the distributions of state 3 and 4 respiration, Δψ and RCR for normality and homogeneity of variances among the treatment groups to test the basic assumptions of the ANOVA. We used a three-way linear model ANOVA to test for the effects of three factors: temperature (two groups: 20 and 30°C), oxygen levels with three groups (normoxia, hypoxia and reoxygenation) and Cd exposure (two groups: control and Cd-exposed). The model included these three main effects as well as all three two-way interactions and the three-way interaction of these factors. We also used a *post hoc* procedure [Fisher's least significant difference (LSD) test for unequal *N*] to test differences between various pairs of means of interest.

The data from the kinetic analyses (i.e. plots of the oxygen consumption rates *versus* Δψ for each subsystem) were described using second- or third-order polynomials. The best fit was determined by the significance of the second- or third-order coefficients of the regressions using a standard procedure as described elsewhere (Kurochkin et al., 2011). Second-order polynomials were used for kinetics of the substrate oxidation subsystem, and third-order polynomials were used for kinetics of the phosphorylation and proton leak subsystems. Polynomial regressions were used to correct the kinetic response of the phosphorylation subsystem by subtracting the contribution of the proton leak to oxygen consumption at a given Δψ. All regressions were significant ($P<0.05$) and provided an excellent fit to the experimental data ($R^2=0.84$–0.99) except for the substrate oxidation subsystem in mitochondria from hypoxic oysters ($R^2=0.59$–0.61).

To test for significant differences between the kinetic responses of the mitochondria from different treatment groups, we used contrasts generated for the polynomial curves for each of the three subsystems using the generalized linear model (GLM) procedure of SAS (SAS Institute, Cary, NC, USA). These contrasts tested the collective differences between the curves (including both the intercepts and slopes, with 3 degrees of freedom for the quadratic polynomials and 4 degrees of freedom for the cubic polynomials). Separate statistical comparisons of intercepts and slopes of the respective curves were not conducted, because we were interested in the overall differences in the kinetic responses of different subsystems between the treatments, rather than in the individual estimated parameters of the empirical curves. The α levels for multiple comparisons were adjusted using sequential Bonferroni corrections (Sokal and Rohlf, 1995). The polynomial regressions were also used to calculate elasticities and the flux control coefficients as described previously (Brand et al., 1988; Hafner et al., 1990). Elasticities and the flux control coefficients, as well as the respiration rates at the common membrane potential, were calculated at the highest Δψ for states 3 and 4 of mitochondria common to all experimental treatments.

All differences were considered significant if the probability of type I error was less than 0.05. Data are expressed as means ± s.e.m. Sample size for all assays was 5–16, with each sample representing a separate mitochondrial isolate.

RESULTS
Effects of Cd, temperature and hypoxia on bioenergetic characteristics of mitochondria

The three-way ANOVA showed significant effects of two- and/or three-factor interactions of the studied factors on state 3 and state 4 respiration, and on state 4 Δψ (Table 1), thereby precluding the analysis of the single factor effects on these three traits. Therefore, significant treatment effects on these traits were determined using LSD *post hoc* tests (Fig. 1). For RCR and state 3 Δψ, the effects of the factor interactions were not significant. Temperature had a significant effect on state 3 Δψ of oyster mitochondria, while the effects of Cd exposure and hypoxia–reoxygenation were not significant (Table 1). Mitochondrial RCR was significantly affected by hypoxia–reoxygenation but not by Cd exposure (Table 1).

Long-term Cd exposure affected mitochondrial respiration and membrane potential (Δψ) of normoxic oysters at 20°C but not 30°C. At 20°C, mitochondria from normoxic Cd-exposed oysters had lower Δψ and decreased state 3 and 4 respiration rates (Fig. 1A,C), as well as reduced RCR (Fig. 1E). At 30°C, these differences in the bioenergetic characteristics between mitochondria from control and Cd-exposed oysters disappeared (Fig. 1B,D). Overall, elevated temperature (30°C) resulted in a reduced Δψ of state 3 and 4 mitochondria from control oysters, but not in their Cd-exposed counterparts (Fig. 1A,B). Due to the temperature-induced partial depolarization, state 3 and 4 respiration rate was similar at 20 and 30°C in mitochondria from control oysters while respiration rates of mitochondria from Cd-exposed oysters increased with increasing temperature (Fig. 1A,B).

Prolonged hypoxia had no effect on polarization of the state 4 mitochondria but led to a notable decrease of Δψ during state 3 in control oysters (Fig. 1A,B). In contrast, post-hypoxic recovery partially restored the state 3 Δψ in mitochondria of control oysters, but led to a significant decline of resting Δψ (Fig. 1A,B). In Cd-exposed oysters, hypoxia and post-hypoxic recovery led to elevated state 4 Δψ at 20°C, while at 30°C hypoxia slightly but significantly suppressed state 4 Δψ, which partially recovered during reoxygenation (Fig. 1B). A similar trend was found during state 3 in Cd-exposed oysters (Fig. 1A). Respiration rates of state 3 and

Table 1. ANOVA: effects of cadmium exposure, temperature and hypoxia–reoxygenation on bioenergetic characteristics of oyster mitochondria

	d.f.	State 3 respiration	State 4 respiration	State 3 Δψ	State 4 Δψ	RCR
		Probabilities from *F* tests				
Cd exposure (Cd)	2	0.427	0.399	0.480	0.374	0.117
Temperature (T)	2	0.698	**<0.0001**	**<0.0001**	**<0.0001**	n.a.
Oxygen levels (Oxy)	3	**0.002**	**0.016**	0.148	**0.021**	**<0.0001**
Cd × T	4	0.627	0.471	0.486	0.120	n.a.
Cd × Oxy	6	0.433	0.957	0.089	**<0.0001**	0.485
T × Oxy	6	0.188	**0.020**	0.469	**<0.0001**	n.a.
Cd × Oxy × T	12	**0.008**	0.163	0.237	**0.028**	n.a.

State 3: degrees of freedom (d.f.) and probability values from *F* tests are given for the effects of cadmium (Cd) exposure, temperature and oxygen levels, and their interactions on ADP-stimulated respiration; state 4: resting respiration in the presence of oligomycin; RCR, respiratory control ratio. Significant effects are highlighted in bold; n.a., the effect was not tested.
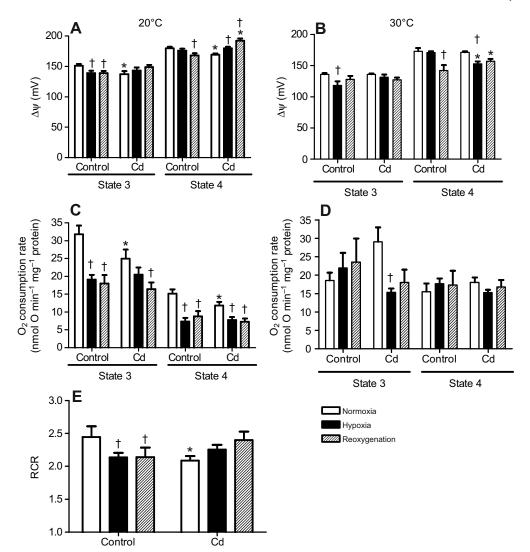
Fig. 1. Effects of cadmium (Cd), temperature and hypoxia–reoxygenation on basal functional parameters of oyster mitochondria. (A,B) Mitochondrial membrane potential (Δψ) at 20 and 30°C, respectively; (C,D) mitochondrial oxygen consumption rates at 20° and 30°C, respectively; (E) respiratory control ratios (RCR) at 20°C. All data in A–D are given for state 3 (ADP-stimulated) and state 4 (resting, in the presence of oligomycin) mitochondria. *Significant differences between the respective parameters in mitochondria from the control and Cd-exposed oysters (*P*<0.05). †Values are significantly different in the mitochondria from hypoxic oysters or those recovering after hypoxia compared with their normoxic counterparts (*P*<0.05). *N*=6–16.

state 4 mitochondria were significantly depressed at 20°C in control and Cd-exposed oysters exposed to hypoxia or post-hypoxic recovery; the effect disappeared at 30°C (Fig. 1C,D). Notably, state 4 respiration was suppressed to a lesser degree than state 3 respiration in mitochondria from the control oysters, resulting in lower RCR during hypoxia and subsequent recovery (Fig. 1E). In contrast, an opposite (albeit non-significant) trend was found in RCR of the mitochondria from Cd-exposed oysters (Fig. 1E).

The kinetic response and Δψ-dependent flux of the mitochondrial subsystems
Substrate oxidation subsystem

Long-term exposure to Cd had little effect on the kinetic response of the substrate oxidation subsystem to Δψ (Table 2, Figs 2, 3). Overall, there was only a 5–6% difference in the substrate oxidation system flux between normoxic mitochondria of control and Cd-exposed oysters when compared at a common membrane potential (Fig. 3). A temperature rise from 20 to 30°C led to a significant increase in the oxidative system flux by 50–70% (Table 2), and this increase was similar in mitochondria from control and Cd-exposed oysters.

The substrate oxidation flux in mitochondria of control oysters was ~20–30% higher after 6 days of hypoxia and subsequent reoxygenation compared with normoxia, when measured at 20°C at a common Δψ (Figs 2, 3, Table 2). In contrast, in Cd-exposed

oysters hypoxia and subsequent reoxygenation resulted in a significantly suppressed flux through the substrate oxidation subsystem at 20°C (Figs 2, 3). At 30°C, the substrate oxidation flux was significantly suppressed in mitochondria from hypoxic or recovering oysters in both control and Cd-exposed groups (Figs 2, 3).

Proton leak subsystem

Temperature increase from 20 to 30°C led to a significant rise in the proton conductance in oyster mitochondria (Table 2, Figs 3, 4). Thus, in mitochondria from control normoxic oysters, the proton conductance increased from 0.17–0.18 $H^+ min^{-1} mg^{-1}$ protein mV^{-1} at 20°C to 0.24–0.25 $H^+ min^{-1} mg^{-1}$ protein mV^{-1} at 30°C, when measured at 130–140 mV (Figs 3, 4). In control oysters, prolonged hypoxia and post-hypoxic recovery resulted in elevated proton conductance of isolated mitochondria. At a low membrane potential (140 mV) this increase was only pronounced at 30°C where the proton conductance increased by ~65% during post-anoxic recovery (Fig. 3). At a higher membrane potential typical of non-stressed resting mitochondria (165 mV), the respective increase in the proton conductance was considerably stronger, with a 60–70% and 100–250% increase (at 20 and 30°C, respectively) in mitochondria from recovering oysters compared with their normoxic counterparts (data not shown). Long-term Cd exposure led to elevated mitochondrial proton conductance at 30°C but not 20°C (Table 2,

Table 2. Statistical comparisons of Δψ-dependent kinetic curves for the three studied mitochondrial subsystems

| Planned contrasts | Treatment conditions | | | Subsystem | | |
	Cadmium exposure	Oxygen levels	Temperature (°C)	Substrate oxidation	Proton leak	Phosphorylation
Control *vs* Cd	n.a.	Normoxia	20	*	*	n.s.
	n.a.	Normoxia	30	*	n.s.	*
20 *vs* 30°C	Control	Normoxia	n.a.	*	*	*
	Cd	Normoxia	n.a.	*	*	*
Hypoxia *vs* normoxia	Control	n.a.	20	*	*	*
	Control	n.a.	30	*	*	*
	Cd	n.a.	20	n.s.	*	n.d.
	Cd	n.a.	30	*	n.s.	*
Reoxygenation *vs* normoxia	Control	n.a.	20	*	*	n.s.
	Control	n.a.	30	*	*	n.s.
	Cd	n.a.	20	*	n.s.	n.s.
	Cd	n.a.	30	*	*	n.s.

Only planned contrasts are included. *Indicates contrasts where the differences between the kinetic curves are significant after the sequential Bonferroni correction for multiple comparisons; n.s., contrasts not significant after the Bonferroni corrections; n.d., contrasts cannot be computed due to the insufficient data; n.a., the effect was not tested.

Figs 3, 4). At 30°C, the Cd-induced increase in the proton conductance was ~15% at 130–140 mV (Fig. 3) and ~20% at 165 mV (data not shown). Notably, long-term Cd exposure abolished the increase of the proton conductance induced by exposure to prolonged hypoxia or reoxygenation in oyster mitochondria (Fig. 3).

Phosphorylation subsystem
Long-term Cd exposure had a significant effect on the phosphorylation subsystem kinetics of oyster mitochondria at 30°C, but not at 20°C (Table 2, Figs 3, 5). At 20°C, the differences in the flux rates of the phosphorylation subsystem were negligible between control and Cd-exposed oysters in normoxia. In contrast, at 30°C

the phosphorylation rate of mitochondria from Cd-exposed normoxic oysters was >40% lower than in their control counterparts when measured at a common Δψ of 130 mV (Fig. 3). The temperature increase from 20 to 30°C led to a significant rise in the Δψ-dependent phosphorylation flux (by 2- to 3-fold at 130 mV) in both control and Cd-exposed oysters (Fig. 3).

At 20°C, prolonged hypoxia resulted in a strong and significant increase of the flux through the phosphorylation subsystem of mitochondria in the control and Cd-exposed oysters (Figs 3, 5, Table 2). During 1 h of post-hypoxic recovery, the Δψ-dependent phosphorylation flux rate returned to normoxic levels (Figs 3, 5). In contrast, at 30°C long-term hypoxia led to a significant
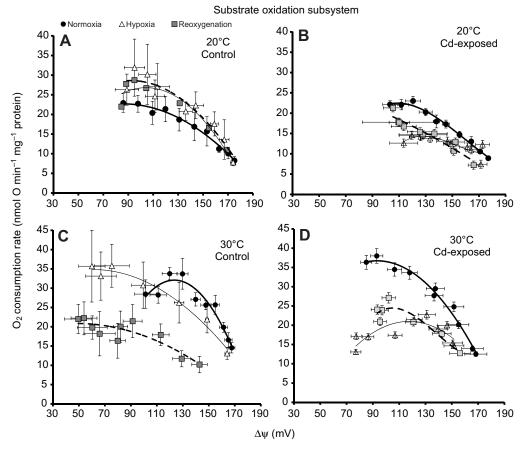

Substrate oxidation subsystem

Fig. 2. Kinetic response of the substrate oxidation subsystem to changes in mitochondrial membrane potential (Δψ) in control (A,C) and Cd-exposed (B,D) oysters. (A,C) Mitochondria from control oysters at 20 and 30°C, respectively; (B,D) mitochondria from Cd-exposed oysters at 20 and 30°C, respectively. Lines represent the respective best-fit polynomial regressions; statistical comparisons of these regressions are given in Table 2. Filled circles and continuous thick line represent data for mitochondria from normoxic oysters; open traingles and continuous thin line represent data from oysters after 6 days of hypoxia; grey squares and broken line represent data for mitochondria from oysters after 12 h of post-hypoxic recovery. Horizontal and vertical bars represent standard errors of the mean for Δψ and respiration rates, respectively. Note different scales of the vertical axes for 20 and 30°C data. N=5–7.
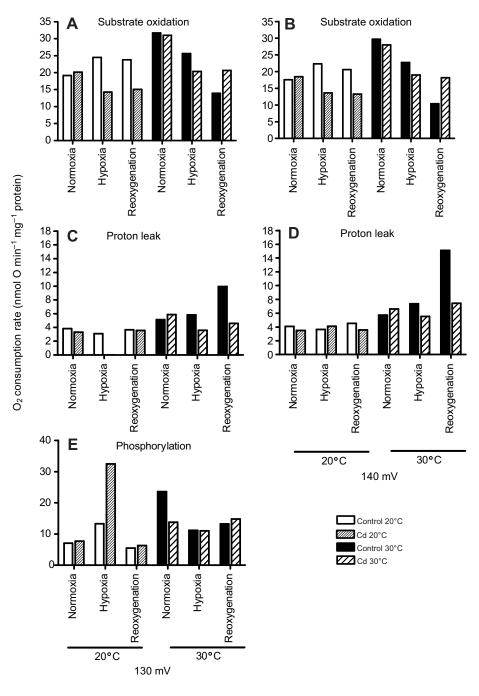
Fig. 3. Flux rates for the three studied mitochondrial subsystems calculated at the common mitochondrial membrane potentials. (A,C,E) Fluxes by the substrate oxidation (A), proton leak (C) and phosphorylation (E) subsystems at a common state 3 membrane potential (130 mV); (B,D) fluxes through the substrate oxidation (B) and proton leak (D) subsystems at a common state 3 membrane potential (140 mV). Flux control coefficients over state 3 and state 4 respiration were calculated at the respective state Δψ common to all experimental treatments (130 and 140 mV for states 3 and 4, respectively; cf. Fig. 1). The flux rates of the proton leak subsystem could not be calculated for mitochondria from Cd-exposed hypoxic oysters at 20°C and 130 mV, because 130 mV was outside the range of the Δψ values for the respective kinetic curve (cf. Fig. 3B). Membrane potentials for the flux calculations were chosen to represent the highest common value of Δψ of state 3 or state 4 respiration in all experimental treatment groups (cf. Fig. 1), to permit comparison across the treatments.

suppression of the phosphorylation flux rate in oyster mitochondria that was partially recovered upon reoxygenation (Figs 3, 5).

Flux control coefficients of mitochondrial respiration in response to stress

State 3 (ADP-stimulated) mitochondria

Distribution of control over state 3 respiration among three mitochondrial subsystems (substrate oxidation, proton leak and phosphorylation) was qualitatively similar in mitochondria from control and Cd-exposed oysters at 20°C (Fig. 6). Under these conditions, the substrate oxidation subsystem exerted the highest degree of control (>75%) over the respiration of ADP-stimulated mitochondria (Fig. 6). Contributions from the proton leak and phosphorylation subsystems to the control of state 3 respiration were low at 2–5 and 8–14%, respectively (Fig. 6). At 30°C, the degree

of control exerted by the substrate oxidation subsystem on state 3 respiration increased to 80–95% in oysters (Fig. 6). Regardless of the experimental temperature, the substrate oxidation subsystem retained the predominant control over state 3 respiration in mitochondria from hypoxic oysters, while post-hypoxic recovery led to a considerable decrease in the degree of control over the state 3 respiration exerted by the substrate oxidation subsystem, and a respective increase in the control by the proton leak and phosphorylation subsystems (Fig. 6).

State 4 (resting) mitochondria

At 20°C, the strongest degree of control over the resting mitochondrial respiration was exerted by the proton leak subsystem (56–68%), with the remaining 32–44% controlled by the substrate oxidation subsystem (Fig. 6). Elevated temperature (30°C) led to a
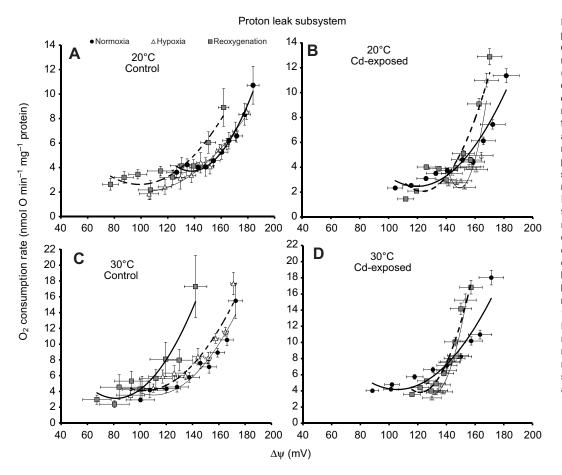
Fig. 4. Kinetic response of the proton leak subsystem to changes in mitochondrial membrane potential in control (A,C) and Cd-exposed (B,D) oysters. (A,C) Mitochondria from control oysters at 20 and 30°C, respectively; (B,D) mitochondria from Cd-exposed oysters at 20 and 30°C, respectively. Lines represent the respective best-fit polynomial regressions; statistical comparisons of these regressions are given in Table 2. Filled circles and continuous thick line represent data for mitochondria from normoxic oysters; open triangles and continuous thin line represent data from oysters after 6 days of hypoxia; grey squares and broken line represent data for mitochondria from oysters after 12 h of post-hypoxic recovery. Horizontal and vertical bars represent standard errors of the mean for $\Delta\psi$ and respiration rates, respectively. Note different scales of the vertical axes for 20 and 30°C data. N=5–8.

reversal of the flux control between the substrate oxidation and proton leak subsystems, with the substrate oxidation subsystem exerting most (57–60%) of the control over state 4 respiration (Fig. 6). This pattern was similar in mitochondria isolated from control and Cd-exposed oysters maintained in normoxia.

Prolonged hypoxia led to a notable increase in the degree of control over state 4 respiration exerted by the substrate oxidation system (to >60% of the state 4 flux) and a respective decrease in the flux control coefficients of the proton leak subsystem (Fig. 6). This trend was seen in the mitochondria from the control and Cd-exposed oysters at 20 and 30°C. Post-hypoxic reoxygenation resulted in a partial recovery of the distribution of flux control over state 4 respiration that was closer to the typical normoxic levels, but in most treatment groups this recovery was incomplete after 1 h of reoxygenation (Fig. 6).
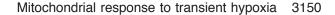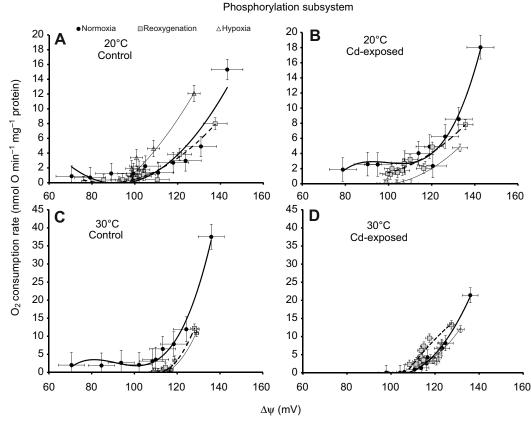
DISCUSSION
Oyster mitochondria are resilient to hypoxia–reoxygenation stress
In the absence of temperature stress and the toxic metal, oyster mitochondria exhibited significant resilience to the prolonged hypoxia and subsequent reoxygenation, and could endure these extreme fluctuations in oxygen levels without loss of the membrane potential or deterioration in their ATP-synthesizing capacity. Prolonged (6 days) hypoxia such as can be expected in the coastal 'dead zones' resulted in a mild depolarization of oyster mitochondria accompanied by a slight decrease in state 3 and state 4 respiration rates and reduced respiratory control ratios. The depolarization trend continued during the post-hypoxic recovery, and respiratory control ratios of mitochondria remained suppressed after 1 h of recovery. This situation is in stark contrast with mammalian mitochondria

where hypoxic events (such as ischaemia) result in a transient increase of $\Delta\psi$ followed by a strong hyperpolarization during reperfusion (Kadenbach et al., 2011). Mitochondrial hyperpolarization during the initial stages of recovery enhances production of ROS in mammalian mitochondria and leads to a cascade of ROS-mediated reactions including damage to the mitochondrial proteins [such as electron transport chain (ETC) complexes and aconitase], a decrease in the rate of oxidative phosphorylation, loss of mitochondrial integrity and membrane potential, and eventually cell death (Sadek et al., 2002; Blomgren et al., 2003; Lee et al., 2003; O'Rourke et al., 2005; Talbot and Brand, 2005; Navet et al., 2006). Thus mild mitochondrial depolarization observed during hypoxia and reoxygenation in oyster mitochondria may be a protective response to prevent excessive ROS formation during post-hypoxic reoxygenation, because mitochondrial ROS generation is very sensitive to $\Delta\psi$ and increases exponentially with increasing membrane potential (Miwa and Brand, 2003; Talbot and Brand, 2005). Interestingly, modest mitochondrial depolarization with pharmacological agents or by preischaemic conditioning attenuates ROS generation and confers protection from reperfusion-induced injury in mammals (Honda et al., 2005; Sack, 2006; Chen et al., 2007), resembling the situation normally observed in oyster mitochondria during hypoxia/reoxygenation.

The decrease in $\Delta\psi$ found in mitochondria from hypoxic and recovering oysters was probably due to the elevated mitochondrial proton conductance rather than suppression of the activity or efficiency of the proton pumps in the ETC. Indeed, when corrected to a common $\Delta\psi$, the flux capacity of the substrate oxidation subsystem was significantly elevated in mitochondria

Phosphorylation subsystem

Fig. 5. Kinetic response of the phosphorylation subsystem to changes in mitochondrial membrane potential in control (A,C) and Cd-exposed (B,D) oysters. (A,C) Mitochondria from control oysters at 20 and 30°C, respectively; (B,D) mitochondria from Cd-exposed oysters at 20 and 30°C, respectively. Lines represent the respective best-fit polynomial regressions; statistical comparisons of these regressions are given in Table 2. Filled circles and continuous thick line represent data for mitochondria from normoxic oysters; open triangles and continuous thin line represent data from oysters after 6 days of hypoxia; grey squares and broken line represent data for mitochondria from oysters after 12 h of post-hypoxic recovery. Horizontal and vertical bars represent standard errors of the mean for $\Delta\psi$ and respiration rates, respectively. Note different scales of the vertical axes for 20° and 30°C data. N=6–8.

from hypoxic and recovering oysters compared with their normoxic counterparts. Notably, phosphorylation flux capacity measured at the common $\Delta\psi$ was also considerably higher in mitochondria from post-hypoxic, recovering oysters. This is in stark contrast with mitochondria of hypoxia-sensitive organisms such as mammals, which experience a strong reduction of oxidative flux during ischaemia and reperfusion (Zimmer et al., 1989; Blomgren et al., 2003; Boengler et al., 2007; Boengler et al., 2011; Di Lisa et al., 2011). The elevated flux capacity of the substrate oxidation and phosphorylation subsystems induced by hypoxia and reoxygenation may be an adaptive mechanism that allows oyster mitochondria to sustain ATP production at reduced $\Delta\psi$ during post-hypoxic recovery. Elevated proton conductance in mitochondria of recovering oysters may also be adaptive by reducing the generation of ROS (Miwa and Brand, 2003; Talbot and Brand, 2005). In mammalian and plant mitochondria, mitochondrial proton conductance has been found to increase in response to elevated superoxide levels; this is achieved by stimulation of uncoupling proteins and serves as a feedback mechanism to prevent excessive ROS production (Brand, 2000; Echtay et al., 2002; Considine et al., 2003; Miwa and Brand, 2003). In oyster, several isoforms of uncoupling proteins are expressed but do not appear to contribute to physiological proton leak (Cherkasov, A. A., et al., 2007; Sokolova and Sokolov, 2005), indicating that other molecular mechanisms (such as activities of substrate transporters or cation pumps) may be involved in regulating the mitochondrial proton conductance. Regardless of their exact molecular mechanisms, elevated proton conductance and enhanced capacity of phosphorylation and substrate oxidation subsystems can preserve aerobic capacity and protect mitochondrial integrity of oysters during periodic hypoxia/reoxygenation in the coastal and estuarine habitats,

especially if coupled with the hypoxia-induced up-regulation of antioxidants such as shown in other intertidal molluscs (Hermes-Lima et al., 1998; Pannunzio and Storey, 1998; English and Storey, 2003; Larade and Storey, 2009).

The characteristic distribution of control over state 3 and state 4 respiration among the three studied mitochondrial subsystems was preserved in mitochondria from oysters exposed to hypoxia–reoxygenation stress. In the absence of Cd or temperature stress, the substrate oxidation subsystem conferred the greatest degree of control over the state 3 respiration. The same trend is also found in insects (Chamberlin, 2004a; Chamberlin, 2004b), mammalian liver (Brown et al., 2007; Ciapaite et al., 2009) and plant mitochondria (Kesseler et al., 1992; Kesseler and Brand, 1994b). In marked contrast, control over state 3 respiration of mammalian muscle mitochondria is shared between the substrate oxidation and phosphorylation subsystems (34–44 and 51–54%, respectively) with a small contribution (7–8%) by the proton leak subsystem (Lombardi et al., 2000). This split control over state 3 respiration between the substrate oxidation and phosphorylation subsystems may be an adaptation to high and extremely variable rates of ATP turnover in mammalian muscles (Hochachka and McClelland, 1997) compared with invertebrate and plant tissues, or to the mammalian liver where fluctuations in the ATP turnover rates are less pronounced. The relatively small contribution of the proton leak subsystem to the control of state 3 respiration in all organisms studied thus far is consistent with reduced proton conductance at low membrane potentials characteristic of ADP-stimulated (state 3) mitochondria.

In resting (state 4) unstressed oyster mitochondria, respiration was predominantly controlled by the proton leak subsystem (~70%) and to a lesser degree by the substrate oxidation subsystem (~30%). A similar distribution of control over state 4 respiration was found in
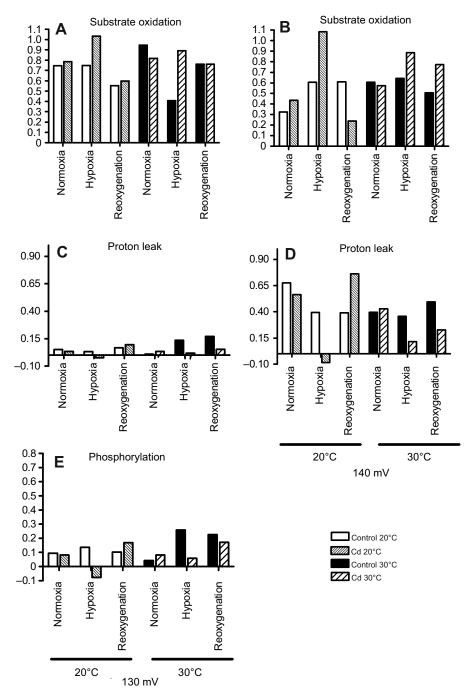
Fig. 6. Control of mitochondrial respiration in resting and ADP-stimulated oyster mitochondria exerted by the three mitochondrial subsystems. (A,C,E) Control over mitochondrial respiration in state 3 exerted by the substrate oxidation (A), proton leak (C) and phosphorylation (E) subsystems; (B,D) control over mitochondrial respiration in state 4 (in the presence of oligomycin) exerted by the substrate oxidation (B) and proton leak (C) subsystems. Membrane potentials for the calculations of the flux control coefficients were chosen to represent the highest common value of $\Delta\psi$ of state 3 or state 4 respiration in all experimental treatment groups (130 and 140 mV for states 3 and 4, respectively; cf. Fig. 1), to permit comparison across the treatments.

mitochondria from pig muscles (Lombardi et al., 2000), potato tubers (Kesseler et al., 1992; Kesseler and Brand, 1994b), and livers of torpid Siberian hamsters (Brown et al., 2007). In contrast, in the mitochondria from the mid-gut of tobacco hornworm and the liver of normothermic Siberian hamsters, the control of state 4 respiration was almost equally distributed between the proton leak and substrate oxidation subsystems (Chamberlin, 2004a; Chamberlin, 2004b; Brown et al., 2007). This resembled the situation found in mitochondria from hypoxic and recovering oysters where the substrate oxidation subsystem assumed a greater degree of control over state 4 respiration (~60%). Despite these modest variations, both the proton leak and substrate oxidation subsystems are quantitatively important in the control of state 4 respiration in all studied mitochondrial systems, including oyster mitochondria in the absence of temperature and Cd stress.

Elevated temperature and Cd modulate mitochondrial bioenergetics and response to transient hypoxia

Elevated temperature led to an increase in mitochondrial respiration and the flux through the three studied mitochondrial subsystems in oysters, as is typical for ectotherm mitochondria (Blier and Guderley, 1993; Abele et al., 2002; Chamberlin, 2004b; Sokolova, 2004; Cherkasov et al., 2006a). Notably, the thermal sensitivity of the flux through the three studied mitochondrial subsystems was dependent on $\Delta\psi$. At a typical state 3 mitochondrial membrane potential of 130 mV, a 10°C temperature increase (from 20 to 30°C) had a considerably stronger rate-enhancing effect on the flux through the phosphorylation subsystem (resulting in a 235% increase) of mitochondria from control oysters compared with the substrate oxidation and proton leak subsystems (65 and 33% increase in flux, respectively). In contrast, in mitochondria from Cd-exposed oysters,

the temperature-induced increase in the flux at 130 mV was comparable for all three studied subsystems (55, 77 and 78% for the substrate oxidation, proton leak and phosphorylation subsystems, respectively). This finding is in agreement with earlier studies showing reduced thermal sensitivity of ETC enzymes in the presence of Cd (Ivanina et al., 2008) and lower susceptibility of the resting mitochondrial respiration (driven predominantly by the proton leak) to Cd inhibition at elevated temperatures compared with respiration of phosphorylating mitochondria (Sokolova, 2004). Our present study also found that at high $\Delta\psi$ of 165 mV typical of resting mitochondria, a 10°C temperature increase doubled the flux through the proton leak subsystem, whereas the substrate oxidation flux experienced a modest increase of 20–45%. This was true for mitochondria from both control and Cd-exposed oysters. This indicates that different mitochondrial subsystems will differentially respond to acute temperature rise depending on the physiological state of an organism and the status of its mitochondria. In the absence of external stressors such as Cd, acute temperature rise will preferentially enhance phosphorylation in state 3 mitochondria and proton leak in state 4 mitochondria of oysters. This can increase ATP synthesis capacity in the active state and prevent hyperpolarization and excessive ROS production under resting conditions.

Lower thermal sensitivity of the flux through the substrate oxidation subsystem (compared with other subsystems) at both low and high physiological $\Delta\psi$ resulted in redistribution of the control over mitochondrial respiration at elevated temperature, with an increased control exerted by the substrate oxidation subsystem at the expense of the phosphorylation and proton leak subsystems. Interestingly, in mammalian liver mitochondria, the degree of control exerted by the substrate oxidation subsystem over state 3 respiration was also higher at high temperatures (25–37°C) compared with the low temperature of 4°C, whereas the opposite was true for state 4 respiration (Dufour et al., 1996). In insect mid-gut mitochondria, the flux control coefficients for state 3 and 4 respiration were unaffected by temperature (Chamberlin, 2004b). Albeit limited, these data show that redistribution of the flux control between the three mitochondrial subsystems in response to thermal stress is probably species specific.

Long-term exposure to Cd in vivo also affected mitochondrial function of oysters. It is worth noting that in this study, mitochondria were isolated and assayed in the presence of a strong trace metal chelator (EGTA). Because this chelator ensured the virtual absence of free Cd^{2+}, the observed effects of Cd exposure probably represent acclimatory changes in mitochondrial structure and/or function, rather than direct toxic effects of Cd. Long-term Cd exposure resulted in a slight but significant depolarization of mitochondria, reduced state 3 and 4 respiration, and decreased degree of coupling, as indicated by lower RCR values. A decline in RCR in mitochondria of Cd-exposed oysters indicates that state 4 respiration is less sensitive to Cd effects than state 3 respiration. These changes are consistent with those found during in vitro Cd exposure of oyster mitochondria (Sokolova, 2004; Cherkasov et al., 2006b; Cherkasov et al., 2010). However, unlike the in vitro effects of Cd, long-term exposure to Cd in vivo did not considerably affect the flux through the three studied mitochondrial subsystems when measured at the same membrane potential. This indicates that the observed decrease in respiration of mitochondria from Cd-exposed oysters probably reflects their lower $\Delta\psi$ rather than changes in the intrinsic ETC capacity and proton conductance of the mitochondrial membrane.

Long-term Cd exposure in vivo resulted in an increase in the degree of control conferred by the substrate oxidation subsystem over the resting and ADP-stimulated respiration, similar to the situation found during in vitro exposure of plant and animal mitochondria to Cd^{2+} (Kesseler and Brand, 1994b; Ciapaite et al., 2009; Kurochkin et al., 2011). This similarity is remarkable given that the mitochondrial function we assayed in the oysters was done in the absence of free Cd^{2+}. This suggests that in addition to the direct effects of Cd present in the cytosol and mitochondria, there are also slight but persistent shifts in the properties of oyster mitochondria induced by long-term Cd exposure that can contribute to the disturbance of energy metabolism in Cd-exposed oysters (Sokolova et al., 2004; Sokolova et al., 2005b; Cherkasov et al., 2006a). The molecular mechanisms of these shifts are currently unknown but may involve ROS-induced damage to mitochondrial enzymes due to the pro-oxidant effects of Cd (Wang et al., 2004; Valko et al., 2005; Cherkasov, A. A., et al., 2007). Direct inhibition of ETC by Cd can also contribute to this change; however, isolated ETC and matrix enzymes of oysters are more resilient to Cd than intact mitochondria, indicating that Cd-induced inhibition of mitochondrial substrate oxidation is probably a complex systemic phenomenon rather than a consequence of a single-enzyme inhibition (Sokolova, 2004; Cherkasov, A. A., et al., 2007; Ivanina et al., 2008). Perhaps not surprisingly, the distribution of control over respiration was much less affected by chronic Cd exposure in vivo (present study) compared with the direct effects of Cd on isolated mitochondria in vitro (Kesseler and Brand, 1994b; Ciapaite et al., 2009; Kurochkin et al., 2011). The observed increase of the control exerted by the substrate oxidation subsystem in response to both long-term Cd exposure and temperature stress indicates that mitochondrial respiration in oysters facing these stressors is mostly limited by the substrate oxidation capacity.

Exposure to Cd or elevated temperature significantly altered the responses of phosphorylation and substrate oxidation subsystems to transient hypoxia in oysters. Elevated temperature and Cd exposure prevented an increase in the oxidation subsystem capacity in response to hypoxia and reoxygenation found in control oysters. Similarly, a strong increase in the $\Delta\psi$-dependent flux capacity through the phosphorylation subsystem induced by hypoxia at 20°C was not observed at 30°C. Mild mitochondrial depolarization induced by hypoxia and reoxygenation was ameliorated or completely abolished in oyster mitochondria exposed to Cd or elevated temperatures, despite the fact that the mitochondrial proton conductance was increased. This indicates that both Cd and elevated temperature stress disrupt the normal mitochondrial response to hypoxia–reoxygenation and may result in oxidative stress or bioenergetic disturbances when co-occurring with hypoxia in intertidal or coastal habitats. Earlier studies (Kurochkin et al., 2009) showing elevated oxidative damage during post-hypoxic recovery in Cd-exposed oysters but not in their control counterparts, support this hypothesis. This can make mitochondria of oysters exposed to Cd and/or elevated temperatures more susceptible to hypoxia–reoxygenation-induced damage and affect the ability of oysters to survive periodic oxygen deficiency in polluted estuaries.

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