Hypoxia-cadmium interactions on rainbow trout (*Oncorhynclus mykiss*) mitochondrial bioenergetics: attenuation of hypoxia-induced proton leak by low doses of cadmium

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Abstract

The goal of the present study was to elucidate the modulatory effects of cadmium (Cd) on hypoxia-reoxygenation-induced mitochondrial dysfunction in light of the limited understanding of the mechanisms of multiple stressor interactions in aquatic organisms. Rainbow trout (*Oncorhynchus mykiss*) liver mitochondria were isolated and energized with complex I substrates, malate-glutamate, and exposed to hypoxia (0>P*O*₂ <2 torr) for 0–60 min followed by reoxygenation and measurement of coupled and uncoupled respiration and complex I enzyme activity. Thereafter, 5 min hypoxia was used to probe interactions with cadmium (Cd) (0–20 µM) and to test the hypothesis that deleterious effects of hypoxia-reoxygenation on mitochondria were mediated by reactive oxygen species (ROS). Hypoxia-reoxygenation inhibited state 3 and uncoupler-stimulated (state 3u) respiration while concomitantly stimulating state 4 and 4ol (proton leak) respirations, thus reducing phosphorylation and coupling efficiencies. Low doses of Cd (≤ 5 µM) reduced, while higher doses enhanced, hypoxia-stimulated proton leak. This was in contrast to the monotonic enhancement by Cd of hypoxia-reoxygenation-induced reductions of state 3 respiration, phosphorylation efficiency and coupling. Mitochondrial complex I activity was inhibited by hypoxia-reoxygenation, hence confirming the impairment of at least one component of the electron transport chain (ETC) in rainbow trout mitochondria. Similar to the effect on state 4 and proton leak, low doses of Cd partially reversed the hypoxia-reoxygenation-induced complex I activity inhibition. The ROS scavenger and sulfhydryl group donor, N-acetylcysteine (NAC), administrated immediately prior to hypoxia exposure, reduced hypoxia-reoxygenation-stimulated proton leak without rescuing the inhibited state 3 respiration suggesting that hypoxia-reoxygenation influences distinct aspects of mitochondria via different mechanisms. Our results indicate that hypoxia-reoxygenation impairs the ETC and sensitizes
mitochondria to Cd via mechanisms that involve, at least in part, ROS. Moreover we provide, for
the first time in fish, evidence for hormetric effect of Cd on mitochondrial bioenergetics –the
attenuation of hypoxia-reoxygenation-stimulated proton leak and partial rescue of complex I
inhibition by low Cd doses.

Key words: Hypoxia, Reoxygenation, Cadmium, Interactions, Mitochondrial Bioenergetics,
Reactive oxygen species (ROS), Proton leak, Fish
Introduction

Aquatic organisms face multiple stressful conditions in their natural environments and their combined effects may not be predicted accurately using current single-stressor-data-based risk assessment procedures (Callahan and Sexton, 2007; Sexton, 2012). The difficulty in predicting effects of multiple stressors hinges on the fact that the mechanisms underlying interactive responses such as additivity, synergy or antagonism (Callahan and Sexton, 2007; Sexton, 2012) are not well known. Among the aquatic systems stressors, hypoxia and metals pollution are commonly encountered. Hypoxia denotes reduced dissolved oxygen levels in water bodies and occurs naturally as a result of poor circulation, high natural organic matter loads, diurnal and seasonal thermal stratification and anthropogenically through activities such as agriculture and discharge of domestic and industrial organic wastes that promote eutrophication (Wu, 2002; Hattink et al., 2005). Although low levels of oxygen in aquatic ecosystems have been associated with a range of deleterious effects including mass mortality, alteration in biodiversity, reduced growth, slowed development, and impaired reproduction of aquatic organisms (Wu, 2002; Hattink et al., 2005; Diaz and Rosenberg, 2008), fish do have mechanisms that, to variable extents, enable them to respond to and adapt to hypoxic conditions. These mechanisms include behavioral, physiological and biochemical adjustments and are geared initially at sustaining oxygen delivery to tissues and later to energy conservation with improved efficiency of ATP generation (Hochachka et al., 1996; Boutilier, 2001; Wu, 2002; Richards, 2011).

The metabolic response to hypoxia varies among aquatic organisms depending on their hypoxia sensitivity. Hypoxia-tolerant organism, e.g., oysters (Storey and Storey, 1990), African lungfish (Dunn et al., 1983), goldfish (Krumshnabel et al., 1996), eel (Busk and Boutilier, 2005) and carp (Bickler and Buck, 2007) possess the capacity for metabolic suppression (hypometabolism),
ability for anaerobic fermentative ATP production to sustain reduced ATP turnover, mechanisms for handling toxic by-products of anaerobic metabolism, and the capacity to avoid and/or repair cellular injury following reoxygenation after hypoxia (Boutilier and St-Pierre; 2000; Bickler and Buck 2007). In contrast, hypoxia sensitive organisms such as rainbow trout generally lack these adaptive mechanisms. When oxygen becomes limiting these organisms can reduce metabolic costs behaviorally but do not adapt by suppressing metabolism at the cellular level (Ferguson and Boutilier, 1989; Krumschnabel et al., 1996; Boutilier, 2001).

Hypoxia often co-occurs with other stressful conditions including metals pollution. A metal of particular importance due to its persistence, wide environmental distribution and high toxicity to aquatic organisms is Cd (Byczkowski and Sorenson, 1984; Hattink et al., 2005; Kamunde, 2009). Cadmium enters the environment from both natural and anthropogenic sources and is readily accumulated by aquatic organisms (Kraemer et al., 2005, 2006). Although the cellular targets and toxic effects of Cd are numerous, the mitochondrion is arguably the most important target site of its toxic action. In this regard, extant literature indicates that several aspects of the three mitochondrial subsystems –phosphorylation, substrate oxidation and proton leak– are impacted by Cd (Kesseler and Brand, 1994; Belyaeva and Korotkov, 2003; Cannino et al., 2009; Kurochkin et al., 2011; Adiele et al., 2012; Ivanina et al., 2012).

While it is apparent that both hypoxia and Cd impact energy homeostasis as single stressors, our knowledge of their interactions is limited to a very few studies on hypoxia-tolerant aquatic species, the carp and oysters (Hattink et al., 2005; Kurochkin et al., 2009; Ivanina et al., 2012; Sussarellu et al., 2013). These interaction studies showed that while hypoxia-tolerant species are able to withstand the effect of hypoxia on mitochondrial function, concurrent Cd and hypoxia
exposure increased the Cd burden (relative to Cd alone exposure) in oysters (Kurochkin et al., 2009) but not in carp (Hattink et al., 2005) suggesting that different organisms respond to hypoxia-metals exposure differently. Moreover, Cd exposure impaired the mechanisms that oysters utilize to adjust their energy metabolism in response to hypoxia (Kurochkin et al., 2009; Ivanina et al., 2012).

In so far as we know, there are no studies on the interactive effects of hypoxia and Cd in hypoxia-sensitive aquatic species and the main goal of the present study was to fill that gap. We reasoned that mitochondria from a hypoxia-sensitive species, rainbow trout, would be more sensitive to hypoxia than those from hypoxia-tolerant species, and further that Cd would exacerbate the deleterious effects of hypoxia. Our initial experiments focused on the effects of hypoxia alone and then we studied the interactive effects of hypoxia and Cd. By focusing on the mitochondria we sought to unveil the mechanisms of interactions of multiple stressors (Cd and hypoxia) on energy homeostasis and improve our ability to extrapolate results to other species and different exposure scenarios. In as much as mitochondria in vivo are exposed to extremely low oxygen levels and that metabolic function of isolated mitochondria is technically impossible to measure at these low levels, we measured mitochondrial respiration after hypoxia and subsequent reoxygenation.

**Materials and methods**

Rainbow trout (142 ± 10 g) were obtained from Ocean Farms Inc, Brookvale, PEI, and maintained in a 250-l tank containing aerated well water at the Atlantic Veterinary College Aquatic Facility. The water contained (mg/l): Ca$^{2+}$ 72, Na$^+$ 119, K$^+$ 3.1, Mg$^{2+}$ 35.6, Cl$^-$ 289, SO$_4^{2-}$ 28.9, hardness (as CaCO$_3$) 326 and total alkalinity (as CaCO$_3$) 156. The temperature and
pH were 13 ± 1 °C and 7.7, respectively. The fish were fed 1% of their body weight daily with commercial trout chow pellets (Corey Feed Mills, Fredericton, NB) containing, according to the manufacturer: crude protein 48% (minimum), crude fat 22% (minimum), crude fiber 1.1% (maximum), calcium 1.2% (actual), phosphorous 1.1% (actual), sodium 0.80% (actual), vitamin A 3125 IU/kg (minimum), vitamin D3 3000 IU/Kg (minimum), and vitamin E 193 IU/Kg (minimum). The background Cd concentrations measured in the feed and water were 0.78 µg/g and below our limit of detection (0.03 µg/l), respectively. Trout were randomly sampled from the tank to isolate liver mitochondria for all experiments. All experimental procedures that fish were subjected to were approved by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

**Mitochondrial isolation**

Rainbow trout were sacrificed by a blow to the head and were dissected to remove the liver. Mitochondria isolation was done according to the method of Adiele et al. (2010). Briefly, the livers were rinsed with mitochondrial isolation buffer (MIB: 250 mM sucrose, 10 mM Tris-HCl, 10 mM KH2PO4, 0.5 mM EGTA, 1 mg/ml BSA [free fatty acid], 2 µg/ml aprotinin, pH 7.3), blotted dry and weighed. The livers were then diced and homogenized in 1:3 (weight to volume) ratio of liver to MIB in a 10-ml Potter-Elvehjem homogenizer (Cole Parmer, Anjou, QC). Three passes of the pestle mounted on a hand-held drill (MAS 2BB, Mastercraft Canada, Toronto) running at 200 rpm were found to be optimal for rainbow trout liver mitochondria isolation. The homogenate was then centrifuged at 800 g for 15 min at 4° C. The supernatant was collected and spun at 13,000 g for 10 min at 4 °C and the mitochondrial pellet was washed twice by re-suspending in MIB and centrifuging at 11,000 g for 10 min at 4 °C. The pure mitochondrial pellet was re-suspended in a 1:3 (weight to volume) ratio of mitochondrial respiration buffer...
[MRB: 10 mM Tris-HCl, 25 mM KH₂PO₄, 100 mM KCl, 1 mg/ml BSA (fatty acid free), 2 µg/ml aprotinin, pH 7.3] and used in the subsequent experiments.

**Determination of mitochondrial content and integrity**

Mitochondrial content in the samples used in the respiration experiments was estimated by measurement of the activity of citrate synthase (CS), a mitochondrial matrix enzyme of the tricarboxylic acid cycle that remains highly invariable in mitochondria and is considered a reliable marker of mitochondrial content (Barrientos, 2002; Pallotti and Lenaz, 2001; Wredenberg et al., 2002; Larsen et al., 2012). Here, the method of Spinazzi et al. (2012) was adapted to microplate and used for CS activity measurement. Briefly, an assay mixture (pH 8.1) containing 1M Tris-HCl buffer, 2 mM 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), 2 mM acetyl coenzyme A and 1% (v/v) Triton X-100 was made. To each well in a 96-well microplate were added 50 µl of the assay mixture, appropriate amount of mitochondria protein and the assay volume brought to 240 µl with Millipore water. Subsequently the reaction was started by the addition of 10 µl of 12.5 mM oxaloacetate (freshly made) and the reduction of DTNB was monitored at 412 nm every 15 sec for 10 min. Samples were run in triplicate with and without oxaloacetic acid and CS activity was calculated by subtracting the oxaloacetic acid controls from the samples with oxaloacetic acid added. Enzyme activity was measured in 1-20 µg of both the 13,000 g pellet (13P: mitochondrial fraction) and the 13,000 g supernatant (13S: cytosolic fraction) to check for potential damage to mitochondrial during isolation and purification. The final enzyme activities were expressed as µmol DTNB reduced/min (ε412 = 13.6 mM⁻¹ cm⁻¹).

Figure 1a shows that our isolation and purification protocol causes negligible damage to mitochondria because CS activity is high in 13P and minimal in 13S. Importantly, the CS
activity was highly correlated ($R^2=0.99$) with mitochondrial protein. Lastly, the integrity of mitochondrial membranes was confirmed polarographically (Lanza and Nair, 2009) wherein addition of cytochrome c and NADH during state 4 did not stimulate respiration indicating that the outer and inner mitochondrial membranes were intact, respectively (Fig. 1b).

**Normoxic mitochondrial respiration**

The protein content of the mitochondria was determined spectrophotometrically (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) by the method of Bradford (1976). Measurement of mitochondrial respiration under normoxic conditions was done using Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) in 1.5 ml cuvettes after a two-point calibration at 0 and 100% oxygen. A traceable digital barometer was used to measure the atmospheric pressure (Fisher Scientific, Nepean, ON) and temperature was monitored and maintained at 13 °C with the aid of a recirculating water-bath (Haake, Karlsruhe, Germany). After the calibration, 1.45 ml of MRB and 100 µl of mitochondrial suspension containing 2.3-2.7 mg of protein (23-27 mg of mitochondrial mass, wet weight) were loaded into cuvettes and continuously stirred. To initiate the Krebs cycle, 5 mM malate and 5 mM glutamate were added to the cuvettes. State 3 (ADP-stimulated) respiration rate was evoked by the addition of 375 nmol of ADP, the depletion of which imposed state 4 (ADP-limited) respiration. Addition of 2.5 µg/ml oligomycin to inhibit ATP synthase activity allowed the measurement of state 4 ol, an estimate of mitochondrial proton leak (Brand et al., 1994; Kesseler and Brand, 1995; St-Pierre et al., 2000). Finally, uncoupled respiration (state 3u) was measured after adding 0.5 mmoles of 2,4-dinitrophenol (DNP) into the cuvette during state 4 respiration. All of the oxygen consumption recordings were captured and analyzed using LabPro data acquisition software (Qubit Systems, ON). From the measured respiration rates, the phosphorylation efficiency (ratio of ADP used to oxygen consumed) as well
as the respiratory control ratio (RCR: the ratio of state 3 to state 4 respiration) were calculated according to Estabrook (1967) and Chance and Williams (1955), respectively.

**Mitochondrial respiration following hypoxia exposure**

The protocol used for the hypoxia experiment was based on the methods of Chandel et al. (1995) and Shiva et al. (2007) with modifications (Fig. 2). Initially, mitochondrial complex-1 driven oxygen consumption was measured under normoxic conditions as described above. Then, to make the MRB hypoxic, nitrogen gas was bubbled into the cuvettes depleting the partial pressure of oxygen (PO₂) to <2 torr but > 0 torr (0.002-0.003 mg O₂/l) at prevailing environmental conditions. This concentration is below the 2.25-3.75 torr intracellular level of oxygen typically encountered by mitochondria *in vivo* and therefore hypoxic but not anoxic (Gnaiger and Kuznetsov, 2002). We observed that mitochondria failed to regain functionality on reoxygenation if incubated at 0 torr. Once the PO₂ reached the desired level, the cuvettes were sealed to maintain the hypoxic conditions for the required hypoxia exposure durations. At the end of the hypoxic exposure period, the cuvettes were opened and fully re-oxygenated (100% air saturation) and ADP (375 nmol) was added to impose the second phosphorylation with measurements of a second (post-hypoxic) set of respiration parameters. The difference between the first and second set of respiration parameters represented the effect of hypoxia-reoxygenation on mitochondrial bioenergetics.

**Individual and combined effects of hypoxia and Cd on mitochondrial respiration**

In one set of experiments the effects of Cd alone were measured by adding pre-determined concentrations (0, 1, 5, 10 and 20 µM) as CdCl₂•2.5H₂O (Sigma-Aldrich, Oakville, ON) during state 3 respiration in actively phosphorylating mitochondria. Another experiment assessed the
effect of hypoxia duration alone on mitochondrial respiration following 5, 15, 30 and 60 min incubations at 0<PO2<2 torr oxygen. Based on the results of the duration of hypoxia study, 5 min hypoxia followed by reoxygenation (it took 10-15 min to re-saturate the MRB with O2) was selected to investigate the interactions with Cd. Here, required Cd doses (0, 1, 5, 10 and 20 µM) were added after 5 min hypoxia incubation and re-oxygenation and respiratory parameters measured as described above. For comparison with the state 3u respiration measured under normoxic conditions, the effect of hypoxia on uncoupler-stimulated mitochondrial respiration also was measured by adding DNP during state 4 respiration following 5 min of hypoxia incubation. Finally, to assess the involvement of oxidative stress in the observed hypoxia-reoxygenation effects, 5 mmoles of N-acetyl cysteine (NAC), a ROS scavenger and source of sulphhydryl groups (Zafarullah et al., 2003), were added to the cuvette at the beginning of hypoxia induction and incubated with the mitochondria for 5 min. The respiration parameters described above were again measured after reoxygenation.

Mitochondrial complex I (NADH:ubiquinone oxidoreductase) activity

At the end of the respiration experiments assessing the interaction of hypoxia and Cd, the mitochondria were removed from the cuvettes and centrifuged at 10,000 g for 5 min at 4 °C. The resultant supernatants were discarded and the pellets were washed twice with 500 µl of MIB with pelleting at 10,000 g for 5 min at 4 °C. The pellets were stored at -80°C and used for complex I assay within 2-3 weeks. Mitochondrial complex I assay was done according to the methods of Janssen et al. (2007) and Kirby et al. (2007) with significant modifications to accommodate microplate reader and fish liver mitochondria. Briefly, the mitochondrial samples were thawed and re-suspended in 100 µl of MRB and equal volumes of each sample and 2% Triton X-100 were mixed, sonicated on ice for 10 sec and the protein concentrations were measured.
Subsequently 240μl of complex I enzyme assay buffer (25 mM potassium phosphate, 3.5 mg/ml BSA, 100 μM DCIP, 70 μM decylubiquinone, 0.6 mg/l antimycin A, and 200 μM NADH, pH 7.3) was added to wells of a 96-well microplate. To initiate the reaction, 60 μg of mitochondrial protein were added to all wells except the blanks and each sample was analyzed in triplicate with and without 2 μM rotenone. The decrease in absorbance due to reduction of DCIP, the terminal electron acceptor in this assay, was monitored spectrophotometrically (Spectramax 384 Plus) at 600 nm for 5 min at 15 sec intervals. The complex I activity was then calculated by subtracting the rotenone-insensitive activity from the total activity and converted to micromoles of DCIP reduced using a molar extinction coefficient of 19.1 mM⁻¹ cm⁻¹.

Data analysis

All of the data were first tested for normality (Kolmogorov-Smirnov) and homogeneity of variances (Cochran C) and submitted to one-or two-way analysis of variance (ANOVA) (Statistica version 5.1, Statsoft, Inc., Tulsa, OK). Specifically, the duration of hypoxia, uncoupler-stimulated respiration, ROS scavenger and complex I activity data were analyzed by one-way ANOVA with “duration of hypoxia” or “group” as independent variables as appropriate. The hypoxia-Cd interactions data were analyzed using a two-way ANOVA with “group” and “Cd concentration” as the independent variables. Significantly different means were separated using Tukey post hoc test at P<0.05. Linear regression analysis and curve fitting were done using SigmaPlot 10 (Systat Software, Inc., San Jose, CA).
Results

*Effect of duration of hypoxia on mitochondrial bioenergetics*

An increase in the duration of exposure to hypoxia resulted in a marked decreased ($F_{4,20}=86$, $P<0.0001$) in state 3 respiration (Fig. 3a). Surprisingly even the shortest hypoxia incubation (5 min) used caused a significant (22%) reduction in state 3 respiration relative to the controls whereas 60 min incubation caused 60% reduction in respiration. In contrast, hypoxia stimulated state 4 respiration rate (Fig. 3b) with a highly significant effect of hypoxia duration ($F_{4,20}=33$, $P<0.0001$). Specifically following 5 and 60 min of hypoxia, the respective state 4 respiration rates were 44 and 80% higher than the controls. A similar trend was observed for state 4$_{ol}$, albeit with greater % stimulation by hypoxiareoxygenation (Supplementary Material, Fig. S1a). Here, the state 4$_{ol}$ respiration rates were 68% and 131% higher than the controls after 5 and 60 min of exposure, respectively, with an overall highly significant effect of hypoxia duration ($F_{4,20}=70$, $P<0.0001$).

Hypoxia imposed a clear inverse relationship ($R^2 = 0.71$) between state 3 and 4 rates of respiration (Fig. 3c) leading to a precipitous decline in estimates of mitochondrial coupling and phosphorylation efficiency (Fig. 4a,b). In this regard, the phosphorylation efficiency (P/O ratio) (Fig. 4a) was reduced by hypoxia duration ($F_{4,20}=142$, $P<0.0001$) with 5 and 60 min incubation resulting in 24% and 51% reductions relative to the normoxic controls. Similarly, the respiratory control ratio (RCR) was reduced by 47 and 76% after 5 and 60 min (Fig. 4b) with a highly significant overall effect of duration of hypoxia ($F_{4,20}=165$, $P<0.0001$). Additionally, hypoxia duration had a highly significant inhibitory effect ($F_{4,20}=423$, $P<0.0001$) on RCR$_{ol}$ (Supplementary Material, Fig. S1b).
**Effect of Cd alone on mitochondria respiration**

Cadmium exposure alone (Fig. 5a) inhibited mitochondrial state 3 respiration dose-dependently \((F_{4,60}=78, P<0.0001)\) with 6% and 48% reduction for the lowest (1 µm) and highest (20 µM) Cd doses. In contrast, the state 4 respiration rate was stimulated dose-dependently \((F_{4,60}=8, P<0.0001)\) resulting in 26% higher respiration rate (Fig. 5b) with the 20 µM Cd exposure relative to the control. A similar significant stimulatory trend was observed for the effect of Cd exposure \((F_{4,60}=34, P<0.0001)\) on state 4_{ol} (Supplementary Material, Fig. S2a). Consequent to these Cd-induced changes on state 3, 4 and 4_{ol}, the RCR \((F_{4,60}=148, P<0.0001)\) and RCR_{ol} \((F_{4,60}=218, P<0.0001)\) declined with maximal reductions of 59 (Fig. 5c) and 51% (Supplementary Material, Fig. S2b) for the 20 µM Cd exposure, respectively.

**Interactions of hypoxia and cadmium on liver mitochondrial respiration**

The effects of combined 5-min hypoxia and Cd (0–20 µM) on state 3 respiration in Fig. 5a show that hypoxia exacerbates the inhibitory effect of Cd \((F_{2,60}=257, P<0.0001)\). Moreover, the interaction between hypoxia level and Cd exposure was highly significant \((F_{8,60}=26, P<0.0001)\) indicating that the effect of hypoxia-reoxygenation on state 3 respiration depended on the level of Cd the mitochondria were exposed to or vice versa. Thus while the lowest (1 µM) and highest (20 µM) Cd doses inhibited state 3 respiration by only 6 and 48%, respectively, superimposing a 5-min exposure of hypoxia-reoxygenation caused 42 and 77% inhibition, respectively. In contrast to the state 3 respiration inhibition, hypoxia-reoxygenation significantly stimulated the state 4 \((F_{2,60}=122, P<0.0001)\) and state 4_{ol} \((F_{2,60}=131, P<0.0001)\) respirations. Interestingly, Cd imposed a biphasic response on hypoxia-reoxygenation-stimulated state 4 and 4_{ol} whereby low \((≤5 \mu m)\) inhibited but higher \((>5 \mu M)\) doses of Cd stimulated these respiration rates (Fig. 5b;
Supplementary Material, Fig. S2a). Similar to state 3 respiration, the interaction terms of hypoxia and Cd on state 4 (F₈,₆₀=11, P<0.0001) and state 4ₒ₁ (F₈,₆₀=33, P<0.0001) were both significant indicating that the observed responses depended on the levels of the independent factors. The overall effect on mitochondrial functional integrity is that hypoxia exacerbated Cd-induced mitochondrial uncoupling (i.e., reduced RCR). Thus, while the control mitochondria were highly coupled with an RCR >8, combined 5-min hypoxia-reoxygenation and 20 µM Cd exposure reduced the RCR and RCRₒ₁ by 82 and 85%, compared with the 59 and 51% reductions cause by Cd alone, respectively (Fig. 5c; Supplementary Material, Fig. S2b). There was a significant 2-way interaction on both the RCR (F₈,₆₀=47, P<0.0001) and RCRₒ₁ (F₈,₆₀=62, P<0.0001) indicating co-dependence of the reduction in coupling on duration of hypoxia and Cd dose.

The potential role of ROS in mediating the effects of hypoxia-reoxygenation and Cd was assessed by adding 5 mM NAC, a ROS scavenger, at the initiation of hypoxic conditions in the cuvettes. The results show a significant effect of group (F₆,₂₈=42, P<0.0001) on state 3 respiration in which (i) hypoxia-reoxygenation-induced inhibition persisted in the presence of NAC, (ii) Cd alone had no significant effect and (iii), synergistic inhibition by combined hypoxia-reoxygenation and Cd was partially rescued by NAC (Fig. 6a). Similarly there was a significant effect of experimental group (F₆,₂₈=24, P<0.0001) on state 4 (Fig. 6b) wherein (i), NAC reduced the hypoxia-reoxygenation-induced stimulation of state 4, (ii) Cd alone and Cd + NAC had no effect and (iii), hypoxia and Cd with without NAC had no effect. For state 4ₒ₁/proton leak (Supplementary Material, Fig. S3) a highly significant effect of experimental group (F₆,₂₈=65, P<0.0001) was observed. Here NAC reduced the hypoxia-reoxygenation-induced stimulation and, surprisingly, the reduction of proton leak caused by hypoxia + Cd was reversed by NAC.
To test the hypothesis that hypoxia impairs the electron transport, DNP, an uncoupler of mitochondrial respiration, was added with and without hypoxia-reoxygenation incubation. Additionally, the effects of Cd alone and in combination with hypoxia-reoxygenation on DNP-stimulated respiration were assessed. The results (Fig. 6c) indicate that the groups analyzed were significantly different ($F_{7,32}=68$, $P<0.0001$). It was evident that hypoxia inhibited uncoupler-stimulated respiration to a greater extent (45 vs. 29%) than it did the coupled state 3 respiration. Although Cd (5 µM) alone had no effect on state 3 and 3u respirations, marked inhibition (>50%) of both states was observed when Cd was combined with hypoxia-reoxygenation.

**Complex I activity**

The effect of hypoxia and the interaction with Cd on complex I activity assessed using 5-min of hypoxia and 5 µM Cd (Fig. 6d) show an overall highly significant treatment group effect ($F_{3,12}=77.8$, $P <0.0001$). It is worth noting that 5 min only of hypoxia inhibited complex I enzyme activity by a massive 70%. Interestingly, while 5 µM Cd alone had no effect on the enzyme, it partially (22%) rescued hypoxia-induced complex I activity inhibition.

**DISCUSSION**

The present study clearly demonstrates that rainbow trout liver mitochondria are highly sensitive to hypoxia-reoxygenation and that depending on the measured endpoint and dose, Cd either exacerbates or attenuates the deleterious effects of hypoxia-reoxygenation. We show that a brief (5 min) hypoxia exposure reduced state 3 respiration by 22% and within 60 min of incubation, only 40% of the pre-hypoxia respiration rate was preserved. These findings are similar to the observations made in hypoxia-sensitive mammalian mitochondria that typically exhibit reduced oxidative phosphorylation following hypoxia-reoxygenation (Schumacker et al., 1993; da Silva,
2003; Shiva et al., 2007). Indeed, our results are not only strikingly similar to the study by da Silva et al. (2003) who reported 25% inhibition of NADH-driven rat heart mitochondrial respiration after two 5-min ischemic exposures but also are consistent with the consensus that hypoxia-reoxygenation imposes severe mitochondrial stress in hypoxia-sensitive animals. In contrast, studies carried out in vivo with hypoxia-resistant species such as oysters show both similarities and differences with the results obtained in the present study. Whereas reduced mitochondrial state 3 respiration occurs following both long (Ivanina et al., 2012) and short (Sussarellu et al., 2013) term in vivo hypoxia exposure and reoxygenation in seawater, Kurochkin et al. (2009) observed a significant state 3 respiration overshoot relative to normoxic controls within the first 1-6 hours of reoxygenation following air-exposure-induced anoxia in the same species. This overshoot, thought to assist oysters in recovery from the oxygen debt and attendant energy (ATP) deficit incurred during the anoxic period, is apparently nonexistent in mitochondria from rainbow trout and probably other hypoxia-sensitive species.

The clearly elevated state 4/4_{ol} respiration observed following hypoxia and reoxygenation of rainbow trout liver mitochondria is in stark contrast with the findings in hypoxia-resistant species wherein hypoxia-reoxygenation of oysters in seawater reduced state 4 respiration (St-Pierre et al., 2000; Ivanina et al., 2012; Sussarellu et al., 2013). Our results are nonetheless similar to those obtained following 6 days of anoxia by air exposure (Kurochkin et al., 2009) wherein state 4 respiration was elevated within the first 6 hours of post anoxia reoxygenation. Sussarellu et al. (2013) speculated that oysters employ different mechanisms to adjust energy metabolism depending on how hypoxia is experienced, i.e., via low dissolved oxygen in seawater or by air exposure. High state 4 respiration, and more specifically state 4_{ol}, indicates increased proton leak and high cost of mitochondrial maintenance (Bishop et al., 2002; Abele et al., 2007). Although
the actual mechanisms remain to be fully characterized, proton leak across the inner mitochondrial membrane (IMM) is believed to be mediated by adenine nucleotide translocase (ANT), uncoupling proteins (UPCs) and other IMM proteins (Parker et al., 2008; Jastroch et al., 2010). Unsurprisingly therefore, even the mechanisms via which hypoxia-reoxygenation activates proton leak pathways are not well known. Nonetheless, reactive oxygen species (ROS), together with resultant products of oxidation, stimulate mitochondrial proton leak (Jastroch et al., 2010), and the proportion of electrons redirected to ROS production increases as PO2 decreases in isolated rat mitochondria (Hoffman et al., 2007). The role of ROS in stimulating proton leak was, at least in part, substantiated in the present study by the finding that NAC, a ROS scavenger, attenuated hypoxia-reoxygenation-stimulated state 4 and 4ol respiations. It is also possible that the phosphorylation system (ATP synthase and phosphate and adenylate transport) was inhibited under the hypoxia-reoxygenation conditions in the present study, decreasing the utilization/dissipation of proton-motive force (Δp) and thus contributing to increased state 4 respiration and proton leak. Additionally, inhibition of oxidative phosphorylation likely caused the ATP synthase to function in reverse, hydrolyzing ATP and pumping protons from the mitochondrial matrix into the inter-membrane space in an attempt to maintain the mitochondrial Δp (Boutilier and St-Pierre, 2000; St-Pierre et al., 2000). This would conceivably be visualized polarographically as elevated oxygen consumption in state 4/4ol. Note that while hypoxia-tolerant species are able to reduce ATP hydrolysis by inhibiting ATP synthase and thus can withstand hypoxic conditions longer, hypoxia-sensitive ectothermic species lack this ability (Rouslin et al., 1995) and rapidly experience catastrophic cellular energy imbalance that can lead to cell death.
Because state 3 respiration decreased as state 4 increased (Fig. 3), the rainbow trout mitochondria became markedly uncoupled (reduced RCR) and inefficient (reduced P/O ratio) in line with previous findings in hypoxia-sensitive mammalian mitochondria (Gnaiger et al., 2000; Blomgren et al., 2003; Kim et al., 2003; Navet et al., 2006; Hoffman et al., 2007). In contrast, mitochondria from hypoxia-tolerant species maintain or increase the phosphorylation efficiency and coupling following hypoxia-reoxygenation (Storey and Storey, 1990; Kurochkin et al., 2009; Ivanina et al., 2012; Sussarellu et al., 2013). Thus these disparate responses are defensible, in part, based on hypoxia tolerance/sensitivity of the experimental animal species employed in various studies. It is noteworthy that reduced RCR has been linked with increased ROS production and with damage to mitochondria and impaired oxidative phosphorylation (Blomgren et al., 2003; Navet et al., 2006; Kurochkin et al., 2009).

To determine the potential mechanisms of the observed hypoxia-induced mitochondrial dysfunction, we tested the hypothesis that it entailed impairment of the electron transport chain (ETC). First, we found that DNP-uncoupled respiration was inhibited (notably to a greater extent than state 3 respiration) following hypoxia-reoxygenation (Fig. 6c). Second, while 5 μM Cd alone did not significantly affect DNP-stimulated respiration, it induced marked inhibition when combined with hypoxia-reoxygenation. Mitochondrial uncouplers such as DNP shuttle protons from the inter-membrane space into the matrix increasing oxygen consumption and dissipating the Δp without causing damage to the mitochondrial membrane or ETC. Thus inhibition of the uncoupler-stimulated respiration is indicative of impaired ETC (Belyaeva and Korotkov, 2003). Importantly, the impairment of ETC was directly confirmed by enzyme activity measurements that revealed a greatly reduced complex I activity (Fig. 6d) consistent with previous studies in
mitochondria from hypoxia-sensitive species (da Silva et al., 2003; Heerlein et al., 2005; Galkin et al., 2009) that implicated a role of complex I in hypoxia-reoxygenation-induced dysfunction. Irrespective of the cause, the quintessential effect of complex I inhibition is leakage of electrons from the ETC leading to increased production of ROS (Raha et al., 2000; Turrens, 2003; Galkin and Brandt., 2005, Shiva et al., 2007; Fato et al., 2009; Murphy, 2009), with oxidative damage of not only the enzyme itself but also other mitochondrial components. We therefore tested the hypothesis that inhibition of complex I-driven state 3 respiration was mediated by oxidative damage following over-production of ROS after hypoxia-reoxygenation. Surprisingly, NAC did not rescue the hypoxia-inhibited state 3 and 3u respirations, although ROS generation has previously been linked to complex I-driven respiration inhibition during ischemia-reperfusion (da Silva et al., 2003; Murphy, 2009). However, NAC did reduce hypoxia-reoxygenation-stimulated state 4/4ol suggesting that ROS-dependent mechanisms are involved in hypoxia-reoxygenation-imposed uncoupling and inefficiency. Although ROS scavengers are commonly used to implicate ROS in pathophysiological processes, unambiguous confirmation of ROS involvement in the stimulation of proton leak observed in the present study requires actual measurements of ROS generation. It is also worth noting that while there is wide acceptance of the notion that ROS production by the mitochondria increases in hypoxia (Bell et al., 2005; Waypa and Schumacker, 2002; Murphy, 2009), reduced ROS generation has also been demonstrated and convincingly justified (Weir et al., 2005; Hoffman et al., 2007).

The observed lack of protection of complex-1 mediated state 3 respiration by NAC does not preclude ROS-mediated damage involving the distal ETC complexes or other mitochondrial components. Typically, electrons from complex I are delivered to and ferried by co-enzyme Q (CoQ) to complex III and by cytochrome c to complex IV. Thus complex III and IV are active
and contribute to oxygen consumption when mitochondria are energized with malate-glutamate
and damage to these distal complexes also would manifest as reduced complex I driven
respiration. Employing a regimen of sequential inhibition of ETC complexes and complex-
specific substrates would help identify if the distal enzymes were affected. In the apparent
absence of ROS-mediated complex I damage, we speculate that hypoxia caused conformational
changes to the enzyme that interfered with NADH oxidation and thus impaired electron transport
and proton pumping. In this regard, two structurally and catalytically different forms of
mitochondrial complex I—an active (A-form) and a deactivated (D-form)—have been identified
(Vinogradov, 1998; Galkin et al., 2009) and, more importantly, hypoxia caused accumulation of
the D-form in human kidney epithelial cells (Galkin et al., 2009) and isolated mitochondria
(Murphy, 2009).

On the effects of Cd, we demonstrated that rainbow trout liver mitochondria were impaired by
this metal dose-dependently. Concentrations of Cd \( \leq 5 \mu M \) did not affect mitochondrial
bioenergetics whereas concentrations \( \geq 10 \mu M \) reduced the maximal respiration and both
coupling and phosphorylation efficiencies, and increased state 4/proton leak respiration. These
results are consistent with our previous findings (Adiele et al., 2010, 2011, 2012) except the
stimulation of proton leak which is a novel finding in the present study for rainbow trout liver
mitochondria. Other effects of Cd on the mitochondria, which are beyond the scope of the
present study, are comprehensively discussed in a recent review (Cannino et al., 2009).

Therefore having confirmed that both hypoxia and Cd affect mitochondrial function, we sought
to understand their combined effects with the overarching hypothesis that they would act
additively or synergistically. The results indicate that the joint effects of hypoxia-reoxygenation
and Cd on mitochondria depend on the measured response and dose of Cd. Specifically, Cd at all
of the doses tested including those that had no effect alone, acted cooperatively with hypoxia-reoxygenation to impair mitochondria and reduce the coupling and phosphorylation efficiency. For example, 1 µM Cd alone did not impair mitochondrial function but when in combination with 5 min hypoxia it evoked a substantial (42%) inhibition of state 3 respiration, an effect significantly greater than the 22% inhibition caused by 5 min hypoxia alone. This can be taken to mean that hypoxia-reoxygenation sensitizes rainbow trout liver mitochondria to Cd damage or that Cd potentiates the effects of hypoxia.

Interestingly, Cd imposed a biphasic response on state 4 and proton leak wherein low doses of the metal attenuated hypoxia-reoxygenation-stimulated state 4 and 4ol while higher doses increased these rates to levels comparable to those caused by hypoxia alone. The greatest reduction in proton leak was seen at 5 µM Cd while the greatest stimulation occurred at 20 µM Cd, the highest dose used in the present study. Whether or not higher Cd doses combined with hypoxia would have resulted in stimulation of proton leak beyond that caused by hypoxia alone remains unknown. Nonetheless, the biphasic response observed in the present study is akin to hormesis (Calabrese and Baldwin, 2002; Calabrese and Baldwin, 2003; Nascarella et al., 2003) wherein low doses of stereotypically noxious (inhibitory) substances elicit beneficial (stimulatory) effects. A similar beneficial response was observed with regards to the combined action on complex I activity in that while hypoxia acting alone inhibited complex I activity, administration of 5 µM Cd partially reversed this inhibition. To the best of our knowledge, this is the first report of possible beneficial effects of low Cd doses in attenuating mitochondrial proton leak and rescuing complex I from hypoxia-reoxygenation-induced inhibition. However, among other potentially toxic compounds, the beneficial effect of low dose of nitric oxide (NO), a
reactive nitrogen species, in mitigating hypoxia-induced inhibition of complex I enzyme activity has been reported in mice mitochondria (Shiva et al., 2007; Murphy, 2009).

The fundamental mechanisms by which low doses of Cd attenuate proton leak and partially protect against hypoxia-reoxygenation-induced complex I inhibition remain unknown but likely entail modulation of both IMM permeability and complex I conformation. Thus, potential mechanisms may involve (i), Cd-induced opening of the mitochondrial permeability transition pore (MPTP) with influx of protons (ii), inhibition of mechanisms that drive proton leak including but not limited to ANT and UCPs by low Cd doses and (iii), activation of the mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) or K^+ cycling by low levels of Cd in the presence of ROS leading to K^+ influx, IMM depolarization and reduction in Δp. In this regard, Cd is known to induce MTPT, inhibit ANT and activate mitochondrial K^+ cycling (Li et al., 2003; Lee et al., 2005; Adiele et al., 2012) while opening of mitoK_{ATP} and ROS have been implicated in ischemia-reperfusion cytoprotection (da Silva, 2003; Shiva et al., 2007). It is also possible that low doses of Cd promoted the conversion of hypoxia-deactivated (D-form) complex I to the A-form thus alleviating the impediment of electron flow and promoting oxidative phosphorylation which subsequently consumed part of the proton gradient. Regardless of the actual causal mechanisms, reduction of proton leak/state 4 respiration decreases ROS production (Ramsey et al., 2000) and is consistent with our findings that NAC reversed hypoxia-reoxygenation-stimulated state 4 respiration and proton leak. Surprisingly, NAC attenuated the proton leak lowering effect of 5 μM Cd, a result that can be attributed metal-chelating property of NAC (Banner et al., 1986; Kadima and Rabenstein, 1990) lowering the effective (bioavailable) concentration of Cd. Indeed, the protective effective of 1 μM Cd is lower than that
of 5 μM Cd (Fig. 5b). It is, however, notable that the outcome of combined Cd-hypoxia exposure appear to depend on the level of hypoxia sensitivity of investigated species because when Cd exposure was overlain on hypoxia stress in oysters (hypoxia-tolerant organism) \textit{in vivo}, the hypoxia defense mechanisms were impaired and no beneficial effects were observed (Kurochkin et al., 2009; Ivanina et al., 2012). Additional research is clearly necessary to understand the mechanisms of reduction of proton leak by low doses of Cd following hypoxia-reoxygenation in oxygen-sensitive species like trout.

In conclusion the present study revealed that rainbow trout liver mitochondria are highly sensitive to hypoxia and exhibit marked inhibitory and stimulatory effects on state 3 and state 4/proton leak respiration, respectively, following short term hypoxia exposures and reoxygenation \textit{in vitro}. The ROS scavenger, NAC, partly reversed hypoxia-stimulated proton leak but not the state 3 inhibition, suggesting different mechanisms underlie the two responses. Hypoxia-reoxygenation-induced mitochondrial dysfunction was associated with impairment of the ETC at least at the complex I level. Lastly, we show that the combined effects of hypoxia and Cd depended on the mitochondrial endpoint measured and the dose of Cd administered wherein state 3 respiration, RCR and P/O all were synergistically reduced whereas Cd imposed a biphasic response on hypoxia-stimulated proton leak and state 4 respiration. We believe that the attenuation of hypoxia-reoxygenation-induced proton leak and partial rescue of complex I activity inhibition by low Cd doses observed in the present study is the first report of potential beneficial effects of Cd on vertebrate aerobic energy metabolism.
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Figure legends

Figure 1: Mitochondrial content and integrity assessment. (a): citrate synthase (CS) activity in 13,000g pellet (P13, mitochondria) and 13000g supernatant (S13). P13 has high CS activity indicating high mitochondrial content whereas S13 supernatant has low CS activity indicating negligible amount of mitochondria and/or minimal disruption of mitochondria during isolation and purification. Data are means ± SEM (n = 5). (b): representative polarographic tracing showing results of cytochrome c (Cyt c) and NADH tests of mitochondrial membrane integrity. The oxygen consumption slopes for the respective segments are: state 3 (a) = -0.013; state 4 (b) = -0.0016; + Cyt c (c) = -0.0017; + NADH (d) = -0.0017. The lack of stimulation of oxygen consumption indicates that the outer (Cyt c) and inner (NADH) mitochondrial membranes are intact.

Figure 2: Protocol for the exposure of rainbow trout liver mitochondria to hypoxia-reoxygenation in vitro. Initially a two-point calibration at 0 and 100% O₂ saturation was done and normoxic (control) mitochondrial respiration parameters were measured after addition of mitochondria, substrates (malate and glutamate) and ADP. After normoxic respiration, nitrogen gas was bubbled into the respiratory cuvette to deplete the O₂ levels to <2 torr (hypoxic conditions). The mitochondria were then incubated under the hypoxic conditions for the desired duration (0, 5, 15, 30, and 60 min) followed by reoxygenation of the cuvette to 100% O₂ saturation. A second dose of ADP was added to initiate the second (hypoxic-reoxygenated) oxidative phosphorylation with taking of a second set of respiration parameters. A typical polarographic tracing with oxygen consumption slopes after a 30 min hypoxic episode is displayed: normoxic (control) state 3 (a) = -0.013; normoxic (control) state 4 (b) = -0.0015;
hypoxic-reoxygenated state 3 (c) = -0.0063; hypoxic-reoxygenated state 4 (d) = -0.0027; hypoxic-reoxygenated state 4_{ol} (e) = -0.0022.

**Figure 3:** The effect of duration of hypoxia exposure on state 3 (a) and 4 (b) respirations rates in isolated rainbow trout liver mitochondria. (c): the relationship between state 3 and 4 respiration rates. Mitochondria were incubated under hypoxic conditions for 0, 5, 15, 30, and 60 min followed by reoxygenation (100% O₂ saturation) and measurement of oxygen consumption rates. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (one-way ANOVA with Tukey’s HSD, P<0.05).

**Figure 4:** The effect of duration of hypoxia on P/O ratio (a) and RCR (b) in isolated rainbow trout liver mitochondria. Mitochondria were incubated under hypoxic conditions for 0, 5, 15, 30, and 60 min followed by reoxygenation (100% O₂ saturation) and measurement of oxygen consumption rates. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (one-way ANOVA with Tukey’s HSD, P<0.05).

**Figure 5:** The interactions of hypoxia and Cd on rainbow trout liver mitochondria function: (a), state 3 respiration; (b), state 4 respiration; (c) RCR. Mitochondria were exposed to Cd (0, 1, 5, 10, and 20 µM) with and without 5 min hypoxia. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (two-way ANOVA with Tukey’s HSD, P<0.05).

**Figure 6:** The effect of N-acetylcysteine (NAC) on hypoxia-, Cd-, and hypoxia + Cd-induced respiration disturbances in rainbow trout liver mitochondria. (a): state 3 respiration; (b): state 4 respiration. (c): effect of 5 min hypoxia with and without 5 µm Cd on maximal coupled and 2,4-dinitrophenol (DNP)-uncoupled respiration. (d): effect of 5 min hypoxia with and without 5 µm
Cd on mitochondrial electron transport chain complex 1 enzyme activity. Data are means ± SEM (n = 5). Groups with different letters are significantly different from each other (one-way ANOVA with Tukey’s HSD, P<0.05).
Figure 1

(a) Protein concentration

Figure 1 (a) shows a graph plotting CS activity (μmol DTNB/min) against protein concentration. The graph includes data points labeled as P13 (mitochondrial pellet) and S13 (supernatant), with the line of best fit having a slope of R^2 = 0.99.

(b) Dissolved O_2 (mg/L)

Figure 1 (b) illustrates the change in dissolved O_2 concentration over time. The graph includes labeled points such as ADP, Cyt c, and NADH, indicating the progression of respiratory processes. The dissolving O_2 is indicated with arrows labeled a, b, c, and d, corresponding to specific time points or processes.
Figure 2
Figure 3

(a) Duration of hypoxia versus State 3 resp (nmol O₂/mg prot/min) for different durations.

(b) Duration of hypoxia versus State 4 resp (nmol O₂/mg prot/min) for different durations.

(c) State 4 resp (nmol O₂/mg prot/min) versus State 3 resp (nmol O₂/mg prot/min) with R² = 0.71.

Legend:
- Control
- 5 min
- 15 min
- 30 min
- 60 min
Figure 4

(a) P/O ratio vs. Duration of hypoxia (min)

(b) RCR vs. Duration of hypoxia (min)
Figure 5

(a) 

(b) 

(c) 

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Figure 6

(a) State 3 resp (nmol O₂/mg prot/min)

(b) State 4 resp (nmol O₂/mg prot/min)

(c) State 3′3u resp (nmol O₂/mg prot/min)

(d) CI activity (μmol DCIP/mg prot/min)