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_{O2}<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 Cd (0–20 μmol l^{−1}) 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 states 4 and 4ol (proton leak) respiration, thus reducing phosphorylation and coupling efficiencies. Low doses of Cd (≤5 μmol l^{−1}) 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, 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 a hormetic 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, and 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), the 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; Krumshnabel et al., 1996; Boutilier, 2001).

Hypoxia often co-occurs with other stressful conditions including metals pollution. A metal of particular importance because of its persistence, wide environmental distribution and high toxicity to aquatic organisms is cadmium (Cd) (Byczkowski and Sorensen, 1984; Hattink et al., 2005; Kamunde, 2009). Cd enters the environment from both natural and anthropogenic sources and is...
readily accumulated by aquatic organisms (Kraemer et al., 2005; Kraemer et al., 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 very few studies on hypoxia-tolerant aquatic species, 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 and 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).

As 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 [Oncorhynchus mykiss (Walbaum 1792)], 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. Because 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.

### RESULTS

#### Effect of duration of hypoxia on mitochondrial bioenergetics

An increase in the duration of exposure to hypoxia resulted in a marked decrease ($F_{4,20}=86, P<0.0001$) in state 3 respiration (Fig. 1A). 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. 1B) 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$_d$ (where ‘$d$’ indicates the addition of oligomycin; see Materials and methods), albeit with greater percent stimulation by hypoxia/reoxygenation (supplementary material Fig. S1A). Here, the state 4$_d$ 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 state 4 rates of respiration (Fig. 1C), leading to a precipitous decline in estimates of mitochondrial coupling and phosphorylation efficiency (Fig. 2). In this regard, the phosphorylation efficiency (P/O ratio) (Fig. 2A) 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, respectively. Similarly, the respiratory control ratio (RCR) was reduced by 47 and 76% after 5 and 60 min (Fig. 2B), respectively, 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

Cd exposure alone (Fig. 3A) inhibited mitochondrial state 3 respiration dose-dependently ($F_{4,20}=78, P<0.0001$), with 6 and 48% reduction for the lowest (1 μmol l$^{-1}$) and highest (20 μmol l$^{-1}$) Cd doses, respectively. In contrast, the state 4 respiration rate was stimulated dose-dependently ($F_{4,20}=8, P<0.0001$), resulting in a 26% higher respiratory rate (Fig. 5B) with the 20 μmol l$^{-1}$ Cd exposure relative to the control. A similar significant stimulatory trend was observed for the effect of Cd exposure (the lower control) on state 4$_d$ (supplementary material Fig. S2A). Consequent to these Cd-induced changes on states 3, 4 and 4$_d$, the RCR ($F_{4,20}=148, P<0.0001$) and RCR$_{ol}$ ($F_{4,20}=218, P<0.0001$) declined with maximal reductions of 59% (Fig. 3C) and 51% (supplementary material Fig. S2B) for the 20 μmol l$^{-1}$ Cd exposure, respectively.

#### Interactions of hypoxia and Cd on liver mitochondrial respiration

The effects of combined 5 min hypoxia and Cd (0–20 μmol l$^{-1}$) on state 3 respiration show that hypoxia exacerbates the inhibitory effect of Cd ($F_{2,60}=257, P<0.0001$; Fig. 3A). 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 μmol l$^{-1}$) and highest (20 μmol l$^{-1}$) 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 state 4 ($F_{2,60}=122, P<0.0001$) and state 4$_d$ ($F_{2,60}=131, P<0.0001$) respiration rate with respect to the controls (Fig. 3B).

### List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>13P</td>
<td>13,000 g pellet</td>
<td>mitochondrial fraction</td>
</tr>
<tr>
<td>13S</td>
<td>13,000 g supernatant</td>
<td>cytosolic fraction</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
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<tr>
<td>Cd</td>
<td>cadmium</td>
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</tr>
<tr>
<td>CS</td>
<td>citrate synthase</td>
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</tr>
<tr>
<td>DCIP</td>
<td>2,6-dichlorophenol indophenol</td>
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<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
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<tr>
<td>DTNB</td>
<td>5,5′-dithiobis-(2-nitrobenzoic acid)</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<tr>
<td>MIB</td>
<td>mitochondrial isolation buffer</td>
<td></td>
</tr>
<tr>
<td>mitoK$_{ATP}$</td>
<td>mitochondrial ATP-sensitive potassium channel</td>
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</tr>
<tr>
<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
<td></td>
</tr>
<tr>
<td>MRB</td>
<td>mitochondrial respiration buffer</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
<td></td>
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<tr>
<td>P/O</td>
<td>phosphorylation efficiency</td>
<td></td>
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<tr>
<td>$P_{O_2}$</td>
<td>partial pressure of oxygen</td>
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<tr>
<td>RCR</td>
<td>respiratory control ratio</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>UCP</td>
<td>uncoupling protein</td>
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<td>$Δ$P</td>
<td>proton-motive force</td>
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**Note:** The symbols and abbreviations listed above are used throughout the text to denote various biochemical and physiological processes and conditions. For a comprehensive understanding, refer to the original research article for detailed definitions and methodologies.
respiration. Interestingly, Cd imposed a biphasic response on hypoxia/reoxygenation-stimulated states 4 and 4 ol, whereby low (≤5 μmol l⁻¹) doses of Cd inhibited but higher (>5 μmol l⁻¹) doses stimulated these respiration rates (Fig. 3B; 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 4 ol (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 μmol l⁻¹ Cd exposure reduced the RCR and RCR ol by 82 and 85%, compared with the 59 and 51% reductions cause by Cd alone, respectively (Fig. 3C; supplementary material Fig. S2B). There was a significant two-way interaction on both the RCR (F₈,₆₀=47, P<0.0001) and RCR ol (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 reactive oxygen species (ROS) in mediating the effects of hypoxia/reoxygenation and Cd was assessed by adding 5 mmol l⁻¹ N-acetylcysteine (NAC), an 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: (1) hypoxia/reoxygenation-induced inhibition persisted in the presence of NAC, (2) Cd alone had no significant effect, and (3) synergistic inhibition by combined hypoxia/reoxygenation and Cd was partially rescued by NAC (Fig. 4A). Similarly, there was a significant effect of experimental group (F₆,₂₈=24, P<0.0001) on state 4 (Fig. 4B), wherein: (1) NAC
The effects of hypoxia and its interaction with Cd on complex I activity were assessed using 5 min of hypoxia and 5 µmol l⁻¹ Cd (Fig. 4D), and results show an overall highly significant treatment group effect (F_{3,12}=77.8, P<0.0001). It is worth noting that just 5 min of hypoxia inhibited complex I enzyme activity by a massive 70%. Interestingly, while 5 µmol l⁻¹ 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 end point 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, which 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. (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-term (Ivanina et al., 2012) and short-term (Sussarellu et al., 2013) in vivo hypoxia exposure and reoxygenation in seawater, Kurochkin et al. (Kurochkin et al., 2009) observed a significant state 3 respiration overshoot relative to normoxic controls within the first 1–6 h 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 non-existent in mitochondria from rainbow trout and probably other hypoxia-sensitive species.

The clearly elevated state 4/4u respiration observed following hypoxia and reoxygenation of rainbow trout liver mitochondria is in stark contrast with the findings in hypoxia-resistant species such as oysters, in which hypoxia/reoxygenation in seawater, Kurochkin et al. (Kurochkin et al., 2009), and differences with the results obtained in the present study. Whereas reduced mitochondrial state 3 respiration occurs following both long-term (Ivanina et al., 2012) and short-term (Sussarellu et al., 2013) in vivo hypoxia exposure and reoxygenation in seawater, Kurochkin et al. (Kurochkin et al., 2009) observed a significant state 3 respiration overshoot relative to normoxic controls within the first 1–6 h 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 non-existent in mitochondria from rainbow trout and probably other hypoxia-sensitive species.

The effects of Cd alone and in combination with hypoxia/reoxygenation on DNP-stimulated respiration were assessed. The results (Fig. 4C) indicate that the groups analyzed were significantly different (F_{3,32}=68, P<0.0001). It was evident that hypoxia inhibited uncoupler-stimulated respiration (state 3u) to a greater extent (45 versus 29%) than it did the coupled state 3 respiration. Although Cd (5 µmol l⁻¹) alone had no effect on state 3 or 3u respiration, marked inhibition (>50%) of both states was observed when Cd was combined with hypoxia/reoxygenation.

Complex I activity

Reduced the hypoxia/reoxygenation-induced stimulation of state 4, 2 (2) Cd alone and Cd + NAC had no effect, and (3) hypoxia and Cd with or without NAC had no effect. For state 4u/proton leak (supplementary material Fig. S3), a highly significant effect of experimental group (F_{6,28}=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 electron transport, 2,4-dinitrophenol (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. 4C) indicate that the groups analyzed were significantly different (F_{3,32}=68, P<0.0001). It was evident that hypoxia inhibited uncoupler-stimulated respiration (state 3u) to a greater extent (45 versus 29%) than it did the coupled state 3 respiration. Although Cd (5 µmol l⁻¹) alone had no effect on state 3 or 3u respiration, marked inhibition (>50%) of both states was observed when Cd was combined with hypoxia/reoxygenation.
increased proton leak and a 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 (UCPs) 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, 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 the partial pressure of oxygen \( P_{O_2} \) decreases in isolated rat mitochondria (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). 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; 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).
al., 2003; Heerlein et al., 2005; Galkin et al., 2009) that implicated studies in mitochondria from hypoxia-sensitive species (da Silva et al., 2003; 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 respiration, 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 3/4a, 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 although there is wide acceptance of the notion that ROS production by the mitochondria increases in hypoxia (Bell et al., 2005; Wayapa 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-I-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 to complex III and by cytochrome c to complex IV. Thus complexes 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 whether 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) and a deactivated (D) form – have been identified (Vinogradov, 1998; Galkin and Korotkov, 2003) 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 ≤5 μmol l⁻¹ did not affect mitochondrial bioenergetics, whereas concentrations ≥10 μmol l⁻¹ 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; Adiele et al., 2011; Adiele et al., 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

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**Fig. 5. Mitochondrial content and integrity assessment.** (A) Citrate synthase (CS) activity in the 13,000 g pellet (P13, mitochondria) and 13,000 g supernatant (S13). P13 has high CS activity, indicating high mitochondrial content, whereas S13 supernatant has low CS activity, indicating a negligible amount of mitochondria and/or minimal disruption of mitochondrial content during isolation and purification. Data are means ± s.e.m. (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: (a) state 3=–0.013; (b) state 4=–0.0016; (c) +Cyt c=–0.0017; (d) +NADH=–0.0017. The lack of stimulation of oxygen consumption indicates that the outer (Cyt c) and inner (NADH) mitochondrial membranes are intact.
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 μmol l⁻¹ 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 Δψ, respiration 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 μmol l⁻¹ Cd while the greatest stimulation occurred at 20 μmol l⁻¹ Cd, the highest dose used in the present study. Whether 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 μmol l⁻¹ 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 a low dose of nitric oxide, 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: (1) Cd-induced opening of the mitochondrial permeability transition pore (MPTP) with an influx of protons, (2) inhibition of mechanisms that drive proton leak including, but not limited to, ANT and UCPs by low Cd doses, and (3) activation of the mitochondrial ATP-sensitive potassium channels (mitoKATP) or K⁺ cycling by low levels of Cd in the presence of ROS, leading to K⁺ influx, IMM depolarization and reduction in Δψ. In this regard, Cd is known to induce MPTP, inhibit ANT and activate mitochondrial K⁺ cycling (Li et al., 2003; Lee et al., 2005; Adiele et al., 2012), while opening of mitoKATP 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), which is consistent with our findings that NAC reversed hypoxia/reoxygenation-stimulated state 4 respiration and proton leak. Surprisingly, NAC attenuated the proton leak, decreasing the effect of 5 μmol l⁻¹ Cd, a result that can be attributed to the 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 μmol l⁻¹ Cd is lower than that of 5 μmol l⁻¹ Cd (Fig. 5B). It is, however, notable that the outcome of combined Cd and hypoxia exposure appears 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) 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 such as 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 in vitro. The ROS scavenger NAC partly reversed hypoxia-stimulated proton leak but not the state 3 inhibition, suggesting that 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 end point measured and the dose of Cd administered at which state 3 respiration, RCR and P/O ratio all were synergistically reduced, whereas Cd imposed a bi-phasic 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 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.

MATERIALS AND METHODS
Rainbow trout (142±10 g) were obtained from Ocean Farms (Brookvale, PE, Canada) and maintained in a 250 l tank containing aerated well water at the Atlantic Veterinary College Aquatic Facility. The water contained (in mg l⁻¹): Ca²⁺ 72, Na⁺ 119, K⁺ 3.1, Mg²⁺ 35.6, Cl⁻ 289, SO₄²⁻ 28.9, hardness (as CaCO₃) 326 and total alkalinity (as CaCO₃) 156. The temperature and pH were 13±0.1°C and 7.7, respectively. The fish were fed 1% of their body mass daily with commercial trout chow pellets (Corey Feed Mills, Fredericton, NB, Canada) 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⁻¹ (minimum), vitamin D₃ 3000 IU kg⁻¹ (minimum) and vitamin E 193 IU kg⁻¹ (minimum). The background Cd concentrations measured in the feed and water were 0.78 μg g⁻¹ and below our limit of detection (0.03 μg l⁻¹), 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 killed by a blow to the head and were dissected to remove the liver. Mitochondria isolation was carried out according to the method of Adiele et al. (Adiele et al., 2010). Briefly, the livers were rinsed with mitochondrial isolation buffer (MIB: 250 mmol l⁻¹ sucrose, 10 mmol l⁻¹ Tris-HCl, 10 mmol l⁻¹ KH₂PO₄, 0.5 mmol l⁻¹ EGTA, 1 mg ml⁻¹ BSA (fatty acid free), 2 μg ml⁻¹ aprotinin, pH 7.5), blotted dry and weighed. The livers were then diced and homogenized in a 1:3 (w/v) ratio of liver to MIB in a 10 ml Potter-Elvehjem homogenizer (Cole Parmer, Anjou, QC, Canada).
Three passes of the pestle mounted on a hand-held drill (MAS 2BB, Mastercraft Canada, Toronto, ON, Canada) running at 200 r.p.m. 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 (v/v) ratio of mitochondrial respiration buffer [MRB: 10 mmol l⁻¹ Tris-HCl, 25 mmol l⁻¹ KH₂PO₄, 100 mmol l⁻¹ KCl, 1 mg ml⁻¹ BSA (fatty acid free), 2 μg ml⁻¹ 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 et al., 2002; Pallotti and Lenaz, 2001; Wredenberg et al., 2002; Larsen et al., 2012). Here, the method of Spinazzi et al. (Spinazzi et al., 2012) was adapted for use on a microplate and used for CS activity measurement. Briefly, an assay mixture (pH 8.1) containing 1 mol l⁻¹ Tris-HCl buffer, 2 mmol l⁻¹ 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2 mmol l⁻¹ acetyl coenzyme A and 1% (v/v) Triton X-100 was made. To each well in a 96-well microplate we added 50 μl of the assay mixture and an appropriate amount of mitochondria protein, and the assay volume was brought to 240 μl with Millipore water. Subsequently, the reaction was started by the addition of 10 μl of 12.5 mmol l⁻¹ oxaloacetate (freshly made) and the reduction of DTNB was monitored at 412 nm every 15 s 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 mitochondria during isolation and purification. The final enzyme activities were expressed as μmol DTNB reduced min⁻¹ (ε412=13.6 (mmol l⁻¹)⁻¹ cm⁻¹). Fig. 5A shows that our isolation and purification protocol causes negligible damage to mitochondrial matrix enzyme of the tricarboxylic acid cycle that remains estimated by measurement of the activity of citrate synthase (CS), a mitochondrial content in the samples used in the respiration experiments 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 mitochondria during isolation and purification. The final enzyme activities were expressed as μmol DTNB reduced min⁻¹ (ε412=13.6 (mmol l⁻¹)⁻¹ cm⁻¹). Fig. 5A 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 (R²=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. 5B).

**Normoxic mitochondrial respiration**

The protein content of the mitochondria was determined spectrophotometrically (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA, USA) by the method of Bradford (Bradford, 1976). Measurement of mitochondrial respiration under normoxic conditions was performed using Clark-type oxygen electrodes (Quibit Systems, Kingston, ON, Canada) in 1.5 ml cuvettes after a two-point calibration at 0 and 100% oxygen. A traceable digital barometer (Fisher Scientific, Nepean, ON, Canada) was used to measure the atmospheric pressure 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 (wet mass)] were loaded into cuvettes and continuously stirred. To initiate the Krebs cycle, 5 mmol l⁻¹ malate and 5 mmol l⁻¹ glutamate were added to the cuvettes. State 3 (ADP-stimulated) respiration rate was evoked by the addition of 375 mmol of ADP, the depletion of which imposed state 4 (ADP-limited) respiration. Addition of 2.5 μg ml⁻¹ oligomycin to inhibit ATP synthase activity allowed the measurement of state 4o, an estimate of mitochondrial proton leak (Brand et al., 1994; Kessler and Brand, 1995; St-Pierre et al., 2000). Finally, uncoupled respiration (state 3u) was measured after adding 0.5 mmol of DNP into the cuvette during state 4 respiration. All of the oxygen consumption recordings were captured and analyzed using LabPro data acquisition software (Quibit Systems). From the measured respiration rates, the phosphorylation efficiency (ratio of ADP used to oxygen consumed) as well as the RCR (the ratio of state 3 to state 4 respiration) were calculated according to Estabrook (Estabrook, 1967) and Chance and Williams (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. (Chandel et al., 1995) and Shiva et al. (Shiva et al., 2007) with modifications (Fig. 6). 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 Pₒ₂ to <2 Torr (1 Torr=133.32 Pa) but >0 Torr 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 is 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 hypoxia exposure period, the cuvettes were opened and fully re-oxygenated (100% air saturation) and ADP (375 mmol) 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 μmol 1-1) of CdCl2: 2.5H2O (Sigma-Aldrich, Oakville, ON, Canada) 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–P5,2 Torr oxygen. Based on the results of the duration of hypoxia experiment, 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 μmol 1-1) were added after 5 min hypoxia incubation and reoxygenation and respiratory parameters measured as described above. For comparison with the state 3 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 mmol of NAC, an ROS scavenger and source of sulfhydryl groups (Zafarullah et al., 2003) was added. Mitochondria in 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 the complex I assay within 2–3 weeks. The mitochondrial complex I assay was performed according to the methods of Janssen et al. (Janssen et al., 2007) and Kirby et al. (Kirby et al., 2007), with significant modifications to accommodate the microplate reader and fish liver mitochondria. Briefly, the mitochondrial samples were thawed and resuspended in 100 μl of MRB and equal volumes of each sample and 2% Triton X-100 were mixed, sonicated on ice for 10 min at 4°C. The pellets were stored at −4°C. The complexes from the state 3 + rotenone (Complex I activity) was monitored spectrophotometrically (Spectramax 126A) at 550 nm at 15 s intervals. The complex I activity was then calculated by subtracting the rotenone-insensitive activity from the total activity and was converted to micromoles of DCIP reduced using a molar extinction coefficient of 19.1 (μmol 1-1 cm-1) 

Data analysis

All data were first tested for normality (Kolmogorov–Smirnov) and homogeneity of variance (Cochran’s C) and submitted to one- or two-way ANOVA (Statistica version 5.1, Statsoft, Tulsa, OK, USA). 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 interaction data were analyzed using a two-way ANOVA with group and Cd concentration as the independent variables. Significantly different means were separated using Tukey’s post hoc test at P<0.05. Linear regression analysis and curve fitting were performed using SigmaPlot 10 (Systat Software, San Jose, CA, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.K. conceived and designed the study. J.O.O. carried out the experiments and data analysis and wrote the first draft of the article. N.M assisted with enzyme analysis. D.S. and F.K. participated in the study design. All authors contributed to the interpretation of results and the editing of the article.

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