

**COMBINED EFFECTS OF CADMIUM, HYPOXIA AND TEMPERATURE ON
MITOCHONDRIAL BIOENERGETICS IN RAINBOW TROUT (*Oncorhynchus mykiss*)**

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University of Prince Edward Island

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ABSTRACT

Aquatic organisms are exposed to diverse and dynamic combinations of stressors in their environment that may interact to alter mitochondrial function. It is important to understand mechanisms of joint effects of stressors to more accurately monitor and predict adverse biological outcomes. I studied the mechanisms of interactions of cadmium (Cd), hypoxia-reoxygenation (H-R) and temperature induced stress on mitochondrial bioenergetics. My overall hypothesis was that when present together Cd, hypoxia and temperature affect common target sites exacerbating single stressor-induced effects on mitochondrial function. In the first investigation I studied the effects of hypoxia-cadmium interactions on rainbow trout (*Oncorhynchus mykiss*) mitochondrial bioenergetics and showed that H-R enhances the sensitivity of mitochondria to Cd-induced stress. Interestingly, I observed that Cd at low dose attenuates H-R-induced proton leak. In the second study, I investigated how temperature modulates cadmium-induced mitochondrial dysfunction and volume changes. I showed that high temperature exacerbates Cd-induced mitochondrial dysfunction and volume changes in part by increasing metal uptake through the mitochondrial calcium uniporter. In the third study, I investigated the effect of H-R on the thermal sensitivity of complex I oxidative capacity. I showed that effects of H-R on mitochondrial function are exacerbated by thermal stress. My fourth study investigated the combined effects of cadmium, temperature and hypoxia-reoxygenation on mitochondrial function. I found that the ternary interactions of Cd, H-R and temperature exacerbate their binary effects on mitochondrial function. I linked the alterations in mitochondrial function to impaired volume homeostasis, complex I A-D transition, dissipation of mitochondrial membrane potential, increased ROS production and loss of structural integrity. Although the effects of Cd and/or H-R were greater at both high and low temperatures, this was not explained by increased Cd accumulation. Overall oxidative stress could explain to a large

extent the effects of Cd, H-R and temperature on mitochondrial structure and function. In the fifth study I investigated the role of mitoK_{ATP} and the effects of pharmacological modulators on H-R-induced mitochondrial dysfunction. I found that in the presence of Mg-ATP both the opening of mitoK_{ATP} channels and bioenergetic effects of diazoxide were protective against the deleterious effects of H-R while in the absence of Mg-ATP only the bioenergetic effects of diazoxide was protective. Overall, my research unearthed previously unknown mechanisms of interactions of Cd, hypoxia and temperature on mitochondrial bioenergetics and increased our understanding of the impact of multiple stressors on cellular energy metabolism in aquatic organisms.

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DEDICATION

To my late parents Samuel Onukwufor and Grace Onukwufor

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LIST OF ABBREVIATIONS

5-HD	5-hydroxydecanoate
AAS	Atomic absorption spectrophotometry
ADP	Adenosine diphosphate
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BKA	Bongkreikic acid
BSA	Bovine serum albumin
CAT	Carboxyattractyloside
Cd	Cadmium
CS	Citrate synthase
CSA	Cyclosporin A
Cyt bc ₁	Cytochrome bc ₁
Cyt c	Cytochrome c
DCIP	2,6-dichlorophenol indophenols
DMT-1	Divalent metal transport-1
DOC	Dissolved organic carbon
DTNB	5'5'-dithiobis-(2-nitrobenzoic acid)
EGTA	Ethylene glycol-bis (2-aminoethylether)-N, N, N',N'-tetraacetic acid
ETS	Electron transport system
FAD	Flavin adenine dinucleotide (oxidized)
FADH ₂	Flavin adenine dinucleotide (reduced)
Fe-S	Iron-sulphur cluster
FMN	Flavin mononucleotide
GSH	Glutathione (reduced)

GSSG	Glutathione (oxidized)
H-R	Hypoxia-reoxygenation
HRP	Horseradish peroxidase
IPC	Ischemic preconditioning
IR	Ischemia reperfusion
K^+/H^+	Potassium ion/proton exchanger
IMM	Inner mitochondrial membrane
IMS	Inter membrane space
MCU	Mitochondrial calcium uniporter
MIB	Mitochondrial isolation buffer
MitoK _{ATP}	Mitochondrial ATP-sensitive potassium channel
MPTP	Mitochondrial permeability transition pore
MRB	Mitochondrial respiration buffer
MT	Metallothionein
NAC	N-acetylcysteine
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
OMM	Outer mitochondria membrane
OXPHOS	Oxidative phosphorylation
PCO ₂	Partial pressure of carbon dioxide
P/O ratio	Phosphorylation efficiency
PO ₂	Partial pressure of oxygen
Q	Ubiquinone
QH ₂	Ubiquinol
RCR	Respiratory control ratio

Rh123	Rhodamine 123
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TEA ⁺	Tetraethylammonium ion
TEM	Transmission electron microscopy
UCP	Uncoupling protein
VDAC	Voltage dependent anion channel
$\Delta\Psi_m$	Mitochondrial membrane potential
Δp	Proton-motive force

CHAPTER 1

INTRODUCTION

1.1 THESIS SCOPE

Aquatic organisms are exposed to varied and dynamic combinations of environmental stressors as a regular part of their life. Empirical evidence shows that physiological outcomes can be altered by sequential and/or simultaneous exposure to multiple environmental stressors (Hertzberg and Teuschler, 2002; Sexton and Hattis, 2007). Current methods of risk assessment utilize data from individual stressors to predict effects of multiple stressors (U.S. Environmental protection Agency [EPA] 1998) and therefore may not be accurate. Although there is a growing knowledge base of joint and single effects of stressors on aquatic systems (Bryce et al., 1999; Dale et al., 1998; US EPA, 1998), sufficient and proper data are seldom obtainable to perform a thorough assessment of the impact of multiple stressors on aquatic organisms. Among the stressors of major concern to the aquatic environment are temperature fluctuation in part due to climate change, hypoxia (low O₂) and the presence of metals (e.g., cadmium: Cd). These three stressors commonly occur together in the aquatic environment and may interact to either reduce or exacerbate their individual effects in aquatic organisms. Therefore understanding the mechanisms of the interactions of metals (Cd), temperature and hypoxia would help in predicting their effects on aquatic organisms.

The central goal of my project was to investigate the combined effects of Cd, hypoxia and temperature on cellular energy balance with a focus on mitochondrial physiology. My thesis was that the effects of stressors will be different than when applied alone, and that results will depend on the metric used because the stressors would act differently when combined together. I first determined the individual effects of the stressors on mitochondrial bioenergetics, and thereafter determined their binary and ternary interactions (Fig. 1.1). Second, I studied how different reporters of the mitochondrial function such as volume regulation, membrane potential, reactive

oxygen species (ROS) production and structure are altered by the three stressors individually and in combination. Lastly, I used pharmacological agents to elucidate the pathways of stressor-stressor interactions and ways to mitigate stressor-induced mitochondrial dysfunction. Overall my research advanced our knowledge of how multiple stressors interact to alter mitochondrial function.

1.2 CADMIUM

Cd is a highly toxic ubiquitous trace metal with a relatively long biological half-life (Byczkowski and Sorenson, 1984; Kamunde, 2009). Cd enters aquatic systems through both natural and anthropogenic processes with the latter being the major source (McGeer et al., 2012). Cd occurs in nature mainly in mineral form in association with Zn, Pb and Cu ore deposits, and is released via weathering/leaching of rocks and volcanic activity (Mortvedt and Osborn, 1982; ATSDR, 2008; Pan et al., 2010). Anthropogenic sources include mining and smelting of ores, cement, paint (as a stabilizer), Ni-Cd batteries, sewage, municipal waste and phosphate fertilizers (Moore and Luoma, 1990; International Cadmium Association, 2000; Panagapko, 2007; Pan et al., 2010). In the aquatic environment, Cd in solution either adsorbs on components such as particulate matter and iron oxide leading to Cd removal from solution into the sediments (Thornton, 1995; Lawrence et al., 1996; Skeaff et al., 2002), or is taken up by aquatic biota such as fish.

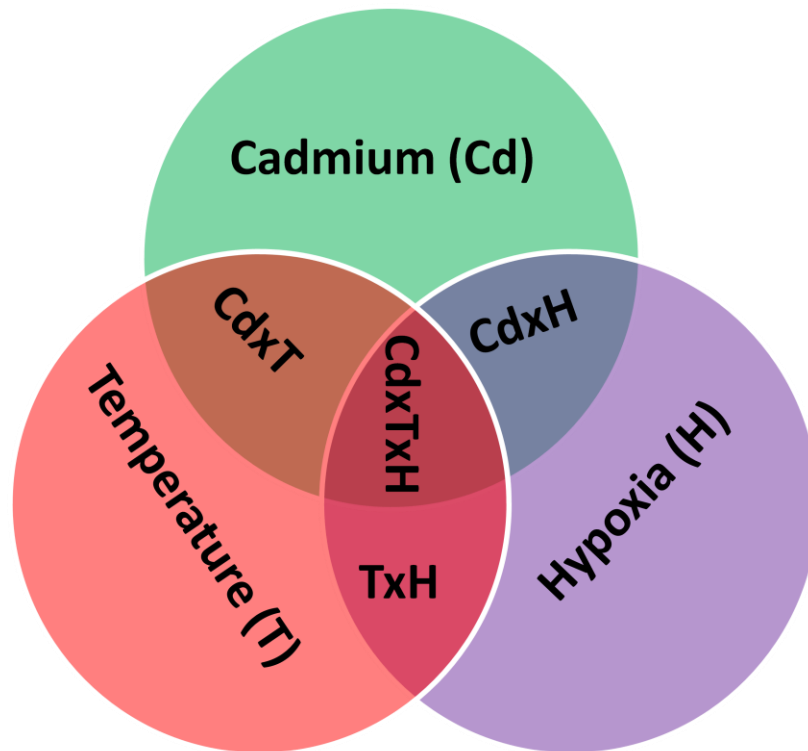


Figure 1.1: Diagram showing the stressors and interactions investigated in this thesis: T: temperature, Cd: cadmium, H: hypoxia, Cd×H: cadmium hypoxia interaction, Cd×T: cadmium temperature interaction, T×H: temperature hypoxia interaction and Cd×T×H: cadmium temperature and hypoxia interaction

1.2.1 Uptake of Cd by fish

The two main uptake routes of Cd in fish are the gastrointestinal tract for dietary and the gill for waterborne metal (Hollis et al., 1999; McGeer, et al., 2012). Waterborne Cd uptake occurs through the lanthanum-sensitive voltage-independent epithelial Ca channel (Galvez et al., 2006) and the divalent metal transporter-1 (DMT-1) (Cooper et al., 2007). Following epithelial Cd uptake, some of the metal is sequestered by low molecular weight proteins (Olsson and Hogstrand, 1987; Zalups and Ahmad, 2003). The metal may be transported across the basolateral membrane via the high-affinity Ca-ATPase and the Na/Ca exchanger into the blood stream as free Cd or as a conjugate (metallothionein (MT)-Cd, glutathione (GSH)-Cd) (Verbost et al., 1989; Flik, 1990; McGeer et al., 2012). Following uptake in fish, Cd is rapidly distributed in plasma to various internal organs with the highest percent accumulation occurring in the liver followed by the kidney (McGeer et al., 2012). The accumulation of Cd in fish tissues may result in toxicity. Generally the toxicity of Cd in aquatic organisms depends on the concentrations of its bioavailable form, which is determined by the total dissolved concentration of Cd and water chemistry. Specifically, it is the concentration of the free Cd ions that is associated with toxicity (Di Toro et al., 2001). In this regard, Cd complexation with other ions and natural organic matter reduces the amount of free Cd thus reducing toxicity. For example, dissolved organic carbon (DOC) complexes Cd and reduces the amount of the free metal available to bind to fish gill and cause toxicity (Playle et al., 1993; Niyogi et al., 2008).

1.2.2 Toxic effects of Cd in aquatic environment

A key mechanism of action of Cd is the disruption of ion homeostasis that has been demonstrated in several fish species including the European flounder (Larsson et al., 1981), trout (Giles, 1984), Atlantic salmon (Rombough and Garside, 1984), tilapia (Kalay, 2006) and carp

(Reynders et al., 2006). All of these fish show reduced plasma Ca, Na and Cl with Cd exposure. The disruption of ionic balance is transient in nature and has been suggested to be part of the classic damage-repair hypothesis (McDonald and Wood, 1993). Following chronic Cd exposure, two types of responses are initiated in response to Cd-induced damage. The first response is to assemble and recruit metal binding thiols/proteins such as GSH/MT that sequester and detoxify Cd (Mason and Jenkins, 1995; Chowdhury et al., 2005) while the second response includes the processes that mend or compensate for the physiological disorder caused by Cd damage.

Depending on the exposure concentration and duration, Cd may affect the growth and survival of aquatic organisms (Hollis et al., 1999; Hansen et al., 2002). Furthermore, Cd accumulation has been suggested to increase production of reactive oxygen species (ROS) resulting in oxidative damage of biomolecules such as proteins, membrane lipid and DNA (Livingstone, 2001).

1.2.3 Mitochondria as one target of Cd toxicity

While the potential sites of action of Cd within the cell are numerous, mitochondria appear to be one of the principal target sites of this metal (Kurochkin et al., 2011; Adiele et al., 2012a). The toxic effects of Cd on this organelle include promotion of ROS production, alteration of their membrane permeability and potential, and respiration in mammals (Dorta et al., 2003; Li et al., 2003; Lopez et al., 2006). Despite appreciable work on the mechanisms of Cd-induced mitochondrial dysfunction in aquatic organisms (Sokolova, 2004; Adiele et al., 2010) our knowledge of the combined effect of Cd with other environmental stressors such as temperature and/or hypoxia on mitochondrial bioenergetics is sparse.

1.3 HYPOXIA

The majority of eukaryotic aquatic organisms require O₂ to sustain life because of their dependence on aerobic respiration (Diaz and Breitburg, 2009). When O₂ supply declines or its

demand exceeds supply, dissolved O₂ levels in water bodies decline (Diaz and Breitburg, 2009) resulting in hypoxia. Hypoxia in water bodies 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; Richards, 2011). In order to protect water bodies, regulatory authorities have classified the levels of dissolved O₂ concentrations in freshwater and marine environments. Concentrations of O₂ <2-3 mg O₂/l in marine/estuarine environments and <5-6 mg O₂/l in freshwater are considered hypoxic (Diaz and Breitburg, 2009). The drawback of this classification of dissolved O₂ concentration is that fish vary in their sensitivity to hypoxia, making what is hypoxic for one fish not so for another (Vaquer-Sunyer and Duarte, 2008). Because of this variability, the terms functional and environmental hypoxia has been proposed to describe hypoxic conditions in aquatic systems. Environmental hypoxia is defined as the water PO₂ at which physiological function is compromised dependent upon the system under examination (Richards, 2009) while functional hypoxia denotes conditions at which tissue O₂ demands surpass circulatory O₂ supply, for example, during severe exercise (Richards et al., 2002; Steinhausen et al., 2008), temperature extremes (Clark et al., 2008; Portner and Lannig, 2009), anemia (Axelsson, 2005; Simonot and Farrell, 2007), acidosis (Nikinmaa, 2006; Wells, 2009) or disruption of gill structure (Gonzalez and McDonald, 1992; Matey et al., 2008).

1.3.1 Responses of fish to environmental hypoxia

To preserve biological function in the face of hypoxia fish use a suite of mechanisms ranging from behavioural to the molecular level, geared toward oxyregulating or oxyconforming (Richards, 2011). These mechanisms increase O₂ transfer from the environment to tissues and/or

avoid problems associated with hypoxia (Perry et al., 2009). Some of these responses can be initiated swiftly (e.g., min, hours), acclimation responses take longer (days) while others are fixed genetically and/or developmentally (e.g., generations) (Chapman and McKenzie, 2009). If circumstances permit, many fish species employ adaptive behavioural responses such as surface respiration (Lam et al., 2006; Mandic et al., 2009) and spontaneous swimming activity (Herbert and Steffensen, 2006) to facilitate O₂ uptake and enhance survival or move to areas that are normoxic (Chapman and McKenzie, 2009).

A key physiological response of fish to low environmental O₂ is hyperventilation to increase the supply of O₂ to the tissue (Perry et al., 2009). During hyperventilation improved water flow across the gill surface maintaining blood-to-water PO₂ slope and ultimately increasing the arterial PO₂ (Perry et al., 2009). Additionally, hyperventilation causes respiratory alkalosis as the arterial PCO₂ drops, because more CO₂ is removed from the blood at the gills (Gilmour, 2001). The ensuing rise of red blood cell pH boosts the affinity of haemoglobin for O₂ through the Bohr effect, leading to enhanced O₂ uptake at the gills (Jensen, 1991; Jensen et al., 1998). Although hyperventilation response increases branchial O₂ transfer contributing to the maintenance of oxygen supply under hypoxic conditions in oxyregulators, it also increases energy expenditure (Edwards, 1971; Steffensen, 1985). Thus the maintenance of metabolic rate under these conditions becomes a fight of diminishing benefits, in which the cost of increasing ventilation to maintain O₂ uptake from an environment of reduced O₂ availability eventually exceeds the returns of the O₂ so obtained (Perry et al., 2009). The ventilation response is different between hypoxia tolerant and sensitive species in that the latter increase the ventilation to sustain O₂ delivery (Boutilier, 2001; Richards, 2011) while the former down-regulate ventilation to conform

with low environmental O₂ levels (Boutilier and St-Pierre, 2000; Bickler and Buck, 2007; Richards, 2011).

1.3.2 Hypoxia-reoxygenation (H-R)

Aquatic organisms commonly encounter intermittent environmental hypoxia especially during the night and return to normal during the day time (Richards, 2011; McBryan et al., 2013). For example, at night the levels of O₂ drop because of absence of photosynthetic activity by plants and return to normal during the day time as plants resume photosynthesis that releases O₂ to the water body. Fluctuation in levels of environmental O₂ implies that resident organisms have to deal with consequences of both hypoxia and reoxygenation, collectively referred to as hypoxia-reoxygenation (H-R). H-R is comparable to ischemia-reperfusion (IR) wherein a brief period of pause in tissue blood supply resulting in a drop or stoppage of tissue O₂ delivery is followed by the return of blood flow and tissue O₂ (Kalogeris et al., 2012). Unlike IR that has been widely studied (Yellon and Hausenloy, 2007; Linfert et al., 2009; Baines, 2011), H-R (episodic hypoxia) in the aquatic environment has received limited attention (Borowiec et al., 2015) with most of the studies focussing on general physiological parameter such as growth, swimming rates and substrate utilization (Speers-Roesch et al., 2010; Burt et al., 2013; Yang et al., 2013). In aquatic organisms very few studies have investigated the effects of intermittent hypoxia on mitochondrial function (Hickey et al., 2012).

1.4 TEMPERATURE

Global climate change is associated with increased temperature variability and frequency of temperature extremes. Ectotherms such as fish are particularly vulnerable to changes in temperature because their body temperatures vary closely with that of the environment (Stevens and Fry, 1974). Importantly, physiological processes in fish including swimming, metabolic rate,

growth and reproduction are highly affected by temperature fluctuations. To cope with environmental temperature change, aquatic organisms have evolved a wide array of mechanisms. In fish many of these mechanisms entail modulation of energy metabolism (Guderley and St-Pierre, 2002; Kraffe et al., 2007; Lockwood and Somero, 2012).

1.4.1 Responses of fish to changing temperature

Temperature is a measure of the heat energy present in a system; thus the kinetic energy of molecules in a system changes in parallel with temperature. In general, biochemical processes are modulated by temperature through the alterations of protein structure and membrane properties (Hofmann and Todgham, 2010). At optimum environmental temperatures enzymes maintain functional structural configuration for proper substrate and cofactor binding but also permit the conformational changes required to catalyze reactions (Somero, 1995). These changes are geared towards enhancing the function of the enzyme depending on the temperature by first modifying the constituent amino acids (Fields and Somero, 1998) or changing to isoforms that are more suited for function at different temperatures (Baldwin and Hochachka, 1971). These two mechanisms affect both the catalytic rate and temperature dependency of substrate and cofactor affinity (Hofmann and Todgham, 2010).

Membrane lipids are not only critical for the function of proteins but are highly sensitive to temperature induced-stress (Hazel, 1995; Somero, 2011). Temperature stress alters the capacity of the lipid membrane environment to carry out its functions (Hazel, 1995; Portner et al., 2007) that include acting as a barrier for the transport of molecules, aiding in the formation of ion gradients across cellular compartments, and serving as a platform for the assembly of multi-constituent metabolic and signal transduction pathways (Hazel, 1995). To protect against temperature-induced alteration in membrane function, aquatic organisms modify their membrane

environment, particularly the acyl chain and the head groups of the lipids, for function at the prevailing temperature (Hazel, 1995; Logue et al., 2000; Somero, 2011). Changes in the lipid head groups involve alteration in the ratio of the phospholipids class between phosphatidylcholine and phosphatidylethanolamine (Hazel and Carpenter, 1985). In contrast, changes in the acyl chain usually entail the modification of chain length, double bond content, double bond positions, *cis* and *trans* configuration of the double bonds and the position of the acyl chain in the lipid molecule (Okuyama et al., 1979; Crockford and Johnston, 1990; Hazel et al., 1992). These acclimation changes occur over periods of days to weeks and may not apply to this thesis which studies acute responses.

1.5 CELLULAR ENERGY METABOLISM

All living things need energy for growth, reproduction, maintenance and other cellular functions (Berg et al., 2007). Cellular energy (ATP) is obtained from the catabolism of energy stored in food (Berg et al., 2007) through glycolysis, Krebs cycle, β -oxidation and oxidative phosphorylation (OXPHOS) (Berg et al., 2007). In eukaryote because the mitochondria is responsible for over 90% of the ATP production for cellular function, any alteration in their structure and function would affect cellular energy homeostasis.

1.5.1 Mitochondrion

Mitochondria comprise roughly 20% of the cytoplasmic volume of eukaryotic cells. These organelles have a diameter of 0.5-1 μm , and they are highly dynamic, moving about in the cell, regularly fusing and dividing, and varying in their shape and size (Berg et al., 2007; Rich and Marechal, 2010; Alberts et al., 2015). The mitochondrion (Fig. 1.2) contains two membranes, the inner and outer membrane, composed of phospholipid bilayers and proteins (Berg et al., 2007; Nelson and Cox, 2009; Rich and Marechal, 2010). The composition of the proteins and

phospholipids in these two membranes vary noticeably leading to distinct properties for the inner and outer membrane (Rich and Marechal, 2010). The outer mitochondrial membrane (OMM) that surrounds the whole organelle is about 60-75 Å thick and has a protein to phospholipid ratio of 1:1 by weight (Alberts et al., 2015). The OMM has porins that allow molecules of about 5 kDa to diffuse freely (Berg et al., 2007; Rich and Marechal, 2010). The intermembrane space (IMS) is the gap between the inner membrane and OMM. Because of the permeability of the OMM to smaller molecules, the concentrations of ions in the IMS and cytosol are similar (Berg et al., 2007). In contrast, the inner mitochondrial membrane (IMM) is highly specialized and impermeable to molecules except via specialized transport mechanisms. It contains high amounts of cardiolipin and has a high protein to phospholipid ratio (3:1 by weight; one protein for every 15 phospholipids) (Alberts et al., 2015). The IMM is folded into several cristae that significantly amplify its surface area thereby enhancing its ability to produce ATP (Berg et al., 2007; Rich and Marechal, 2010). The IMM contains three types of proteins specifically, those that carry out the redox reactions of the electron transport system (ETS), the ATP synthase that makes ATP, and transport proteins that allow the passage of substances and metabolites into and out of the matrix (Berg et al., 2007; Rich and Marechal, 2010). The matrix is a large space surrounded by the IMM and contains approximately 2/3 of the total mitochondrial proteins, the mitochondrial DNA, ribosomes, tRNAs, and enzymes that are involved in gene expression, and β-oxidation and Krebs cycle redox reactions (Berg et al., 2007; Rich and Marechal, 2010).

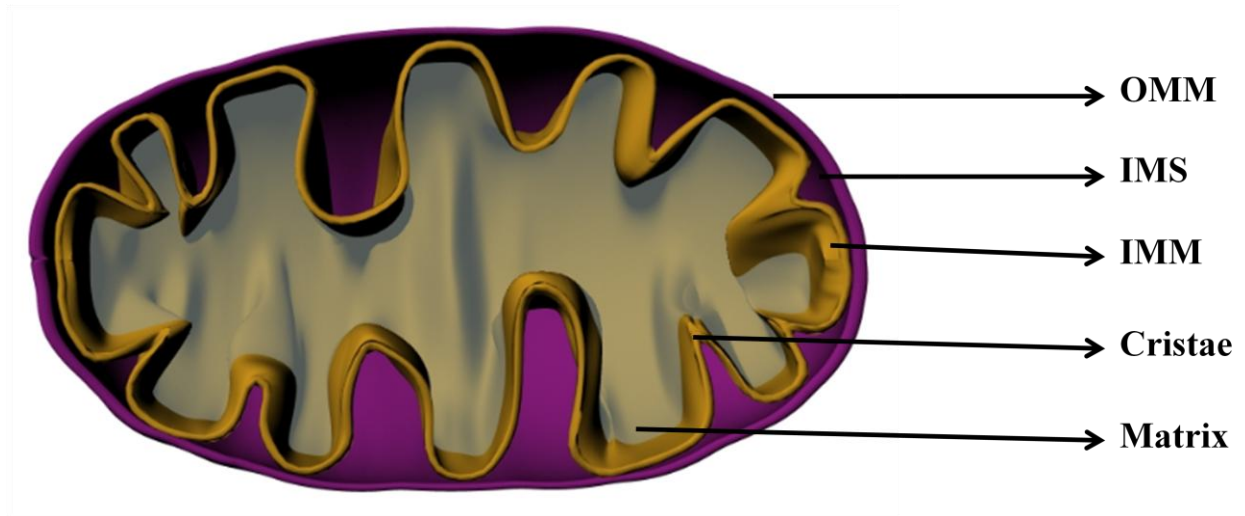


Figure 1.2: Structure of mitochondrion. Outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), intermembrane space (IMS). (Illustration generously provided by Dr. L. Bate).

1.5.2 Electron transfer system (ETS) and oxidative phosphorylation (OXPHOS)

The ETS consists of enzyme complexes in the IMM that allow the transfer of electrons from one complex to another in a tightly regulated manner. The ETS can be physically separated by mild treatment with detergents enabling the resolution of four distinct electron carrier protein complexes namely complex I (CI), complex II (CII), complex III (CIII) and complex IV (CIV) as shown in Fig. 1.3. Each of these complexes catalyzes electron transfer through a portion of the ETS (Baradaran et al., 2013; Nicholls and Ferguson, 2013).

CI, also known as NADH:ubiquinone oxidoreductase or NADH dehydrogenase, has 44 subunits and a molecular weight of 980 kDa (Efremov and Sazanov, 2011; Baradaran et al., 2013; Nicholls and Ferguson, 2013). Structurally, CI has an L shape that consists of hydrophilic matrix side and hydrophobic membrane domain (Hunte et al., 2010; Nicholls and Ferguson, 2013). CI receives reducing equivalents (NADH) from the Krebs cycle, and then in a redox reaction pumps out four protons into the IMS and transfers two electrons to flavin mononucleotide of complex I as a hydride anion (Nelson and Cox, 2009; Hunte et al., 2010; Nicholls and Ferguson, 2013). These two electrons are then transferred one by one along a chain of seven Fe-S clusters and then to ubiquinone (Q) reducing it to ubiquinol (QH₂) (Efremov and Sazanov, 2011; Baradaran et al., 2013; Nicholls and Ferguson, 2013). QH₂ diffuses in the IMM from CI to CIII where it is oxidized back to Q with the pumping of protons to the IMS (Rich and Marechal, 2010).

CII (Succinate dehydrogenase) the only membrane bound enzyme in the Krebs cycle catalyzes the oxidation of Q to QH₂ without pumping of protons into the IMS (Rich and Marechal, 2010; Maklashina and Cecchini, 2010). CII contains four distinct subunits, two are located in the hydrophobic domain (subunits C and D) and the other two in the hydrophilic domain (subunit A and B) that extends into the matrix (Rich and Marechal, 2010; Maklashina and Cecchini, 2010).

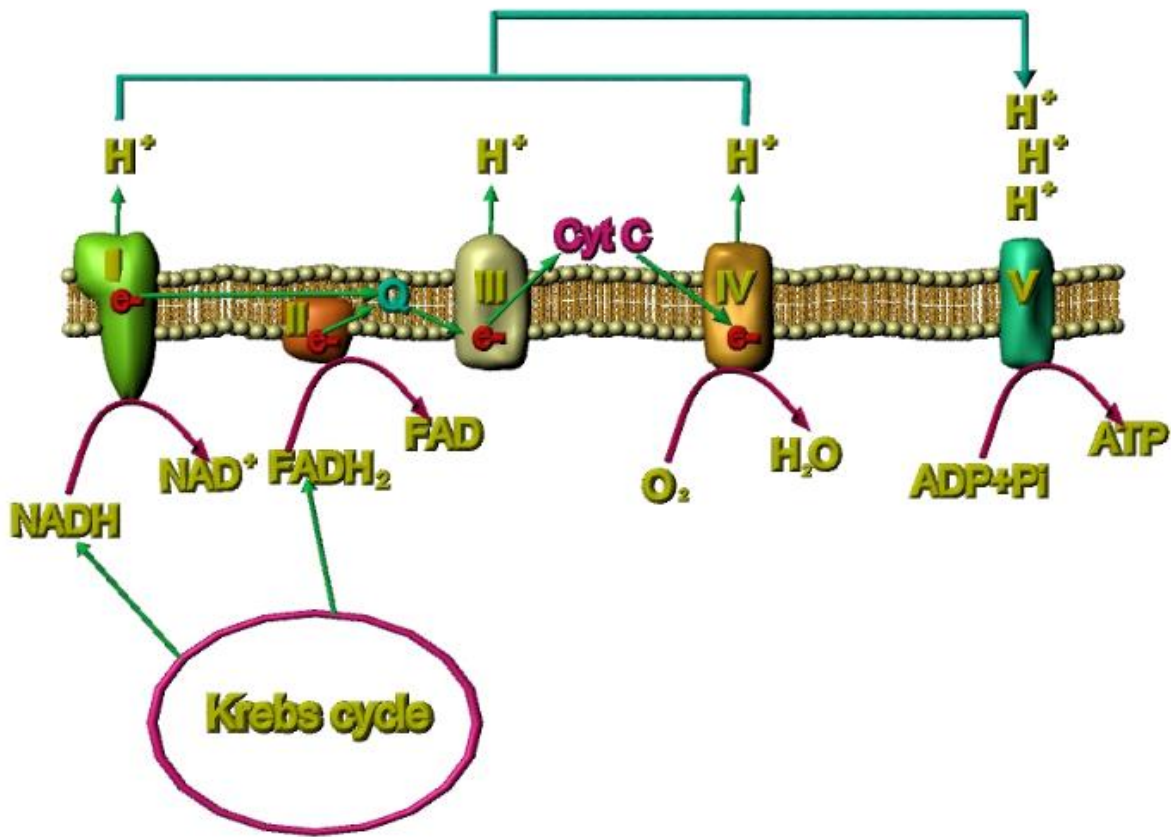


Figure 1.3: Diagram showing mitochondrial electron transport system: (I) complex I, (II) complex II, (III) complex III, (IV) complex IV, (V) complex V, (e^-) electron, (Q) ubiquinone, (cyt c) cytochrome c, (H^+) proton, NADH reducing equivalent for complex I and FADH₂ reducing equivalent for complex II. (Drawing by Dr. L. Bate).

Subunits A and B contain a binding site for the substrate succinate, a covalently bound FAD and three 2Fe-2S centers (Nelson and Cox, 2009). Subunits C and D are integral membrane proteins with six transmembrane domains comprising three helices for each subunit. Subunit C also contains heme b and a binding sites for ubiquinone, the last acceptor of electron transported by CII (Nelson and Cox, 2009; Rich and Marechal, 2010).

CIII, also called cytochrome bc_1 (cyt bc_1) or ubiquinone:cytochrome c oxidoreductase, couples the transfer of electrons from QH_2 to cytochrome c (cyt c) with the pumping of four protons from the matrix to the IMS (Crofts, 2004; Nicholls and Ferguson, 2013). The net effect of the transfer is that QH_2 is oxidized to Q and two molecules of cyt c are reduced (Nelson and Cox, 2009; Sweierczek et al., 2010; Nicholls and Ferguson, 2013). Cyt c is a soluble protein of the IMS (Nicholls and Ferguson, 2013) that upon receiving electrons from CIII moves to CIV to donate the electrons to copper A (Cu_A) center (Lange and Hunte, 2002; Kaila et al., 2010; Rich and Marechal, 2010). The final enzyme of the ETS is CIV, also referred to as cytochrome c oxidase that moves electrons from cyt c to molecular O_2 reducing the latter to H_2O . For every four electrons passing through this complex, the enzyme consumes four protons from the matrix in converting O_2 to H_2O and pumps an additional two protons to the IMS (Kaila et al., 2010; Nicholls and Ferguson, 2013). Overall, for each pair of electrons transferred to molecular O_2 , four protons are pumped out by CI, four by CIII and two by CIV.

The protons generated as a result of the redox reactions described above are used for ATP synthesis by CV, ATP synthase (Walker, 2013). CV is also capable of ATP hydrolysis linked to proton translocation from the matrix to the cytosolic side of the IMM (Walker, 2013; Nicholls and Ferguson, 2013). CV is composed of two principal domains that are connected together by a central stalk (Nicholls and Ferguson, 2013); these domains are called F_1 and F_o (Rich and

Marechal, 2010; Muench et al., 2011; Walker, 2013). F_o is the membrane domain that contains a pore through which protons leak to F_1 , the non-membrane bound section located in the matrix and catalyzes the reaction of ADP with P_i to form ATP (Berg et al., 2007; Rich and Marechal, 2010; Nicholls and Ferguson, 2013; Walker, 2013). The process of transfer of electrons along the ETS coupled with transport of protons from the matrix into the IMS to create a proton-motive force (membrane potential and pH gradient) across the IMM that drives the synthesis ATP from ADP and inorganic phosphate is known as OXPHOS (Mitchell, 1966).

1.5.3 Mitochondrial reactive oxygen species (ROS)

Mitochondria are a major site of ROS production (Kowaltowski et al., 2009; Dröse and Brandt, 2012) and this ROS (superoxide and H_2O_2) generation is closely associated with the primary function of the mitochondria (Murphy, 2009). The generation of superoxide radical ($O_2^{\cdot-}$) by the mitochondria is in part due to one-electron reduction of O_2 (Murphy, 2009; Dröse and Brandt, 2012). Under normal conditions O_2 has two unpaired electrons in its outer shell that are aligned side by side making O_2 to only accept one electron at a time, thus creating the chance for $O_2^{\cdot-}$ to be formed. Although thermodynamically small-molecules such as electron carriers (NADH, QH_2 , NADPH and GSH) have the potential to reduce O_2 to $O_2^{\cdot-}$ most of them do not react with O_2 to produce $O_2^{\cdot-}$. Instead, $O_2^{\cdot-}$ production occurs once electron carriers (QH_2) bind to proteins or at the redox-active prosthetic clusters inside the proteins (Murphy, 2009).

1.5.3.1 Sources of mitochondrial ROS

The mitochondria are thought to be the major site of ROS production in the cell with about 0.2-0.5% of the O_2 consumed by these organelles converted to ROS (Murphy, 2009; Dröse and Brandt, 2012). The amount of ROS produced by mitochondria depends on the redox state, substrate oxidized, metabolic state, O_2 saturation, and the concentration and nature of the

enzymes that generate the ROS (Murphy, 2009). Thermodynamically all four complexes of the ETS are capable of generating ROS; however, CI and CIII (Fig. 1.4) are regarded as the main sites of ROS generation in the mitochondria (Turrens and Boveris, 1980; Adam-Vizi and Chinopoulos, 2006). CIII releases ROS on both sides of the membrane (Murphy, 2009; Dröse and Brandt, 2012). The inhibition of Q_i site of CIII by antimycin leads to the production of large amounts of ROS as a result of O_2 reacting with ubisemiquinone bound to the Q_o site (Adam-Vizi and Chinopoulos, 2006; Murphy, 2009; Dröse and Brandt, 2012). In the absence of antimycin, production of ROS at CIII Q_o site is minimal because ubisemiquinone is not stable (Murphy, 2009; Zorov et al., 2014). The loss of cyt c or the inhibition of CIV by cyanide does not result in appreciable increase in CIII ROS production raising questions about the significance of ROS produced by CIII (Sipos et al., 2003; Adam-Vizi and Chinopoulos, 2006). On the other hand, ROS produced by CI is more physiologically relevant because the modulation of key bioenergetics factors such as NADH:NAD ratio and the proton motive force ($\Delta\Psi_m$ and ΔpH) alters ROS production at CI (Adam-Vizi and Chinopoulos, 2006, Murphy 2009; Zorov et al., 2014). This suggests that any factor that regulates the membrane potential and/or the ratio of the NADH:NAD could alter the rate of ROS production at CI. For example, ADP increases the rate of state 3 respiration thus depleting the pool of reduced NADH and membrane potential and reducing ROS production at CI (Adam-Vizi and Chinopoulos, 2006). Apart from CI and CIII there are other mitochondria ROS producing sites namely: 2-oxoglutarate dehydrogenase (Starkov., et al., 2004; Tretter and Adam-Vizi, 2004), dihydrolipoamide dehydrogenase (Bunik and Sievers, 2002; Tretter and Adam-Vizi, 2004), pyruvate dehydrogenase (Starkov et al., 2004), electron transfer flavoprotein (Eaton, 2002; Zorov et al., 2014), dihydroorotate dehydrogenase (Andreyev et al., 2005; Murphy, 2009), palmitoyl-CoA (Murphy, 2009), α -glycerophosphate

dehydrogenase (Zorov et al., 2014), succinate dehydrogenase (Andreyev et al., 2005; Zorov et al., 2014), monoamine oxidase (Andreyev et al., 2005; Zorov et al., 2014), aconitase (Zorov et al., 2014) and NADPH-oxidase (Zorov et al., 2014). ROS produced by the mitochondria can be beneficial or detrimental to mitochondrial and cellular function depending on the amount.

1.5.3.2 Physiological and pathological roles of ROS

ROS produced at physiological levels has been reported to play key roles in mitochondrial function. These roles include modulating signalling pathways important for mitochondrial survival, fission/fusion and mitophagy, a mechanism for removal of abnormal mitochondria (Droge, 2002; Dai et al., 2012). On the other hand excessive ROS levels have been linked with oxidative stress that damage biomolecules such as proteins, membrane lipids and DNA (Livingstone, 2001) as well as several human disorders including neurodegenerative diseases, diabetes and senescence (Adam-Vizi and Chinopoulos, 2006; Dröse and Brandt, 2012). ROS is usually produced in excess as a result of stressful conditions that overwhelm the capacity of the antioxidant system. Under normal conditions mitochondria produce low levels of ROS that is adequately detoxified by antioxidant enzymes located in the mitochondria and the cytosol (Turrens, 2003, Zorov et al., 2014). Specifically, antioxidant enzymes such as superoxide dismutase (SOD), with isoforms located in the matrix, extra cellular space and IMS, convert the $O_2^{\cdot -}$ to H_2O_2 (Inoue et al., 2003). The H_2O_2 is further reduced to water by the antioxidant enzymes including catalase and glutathione peroxidase (Fig. 1.4) (Turrens, 2003).

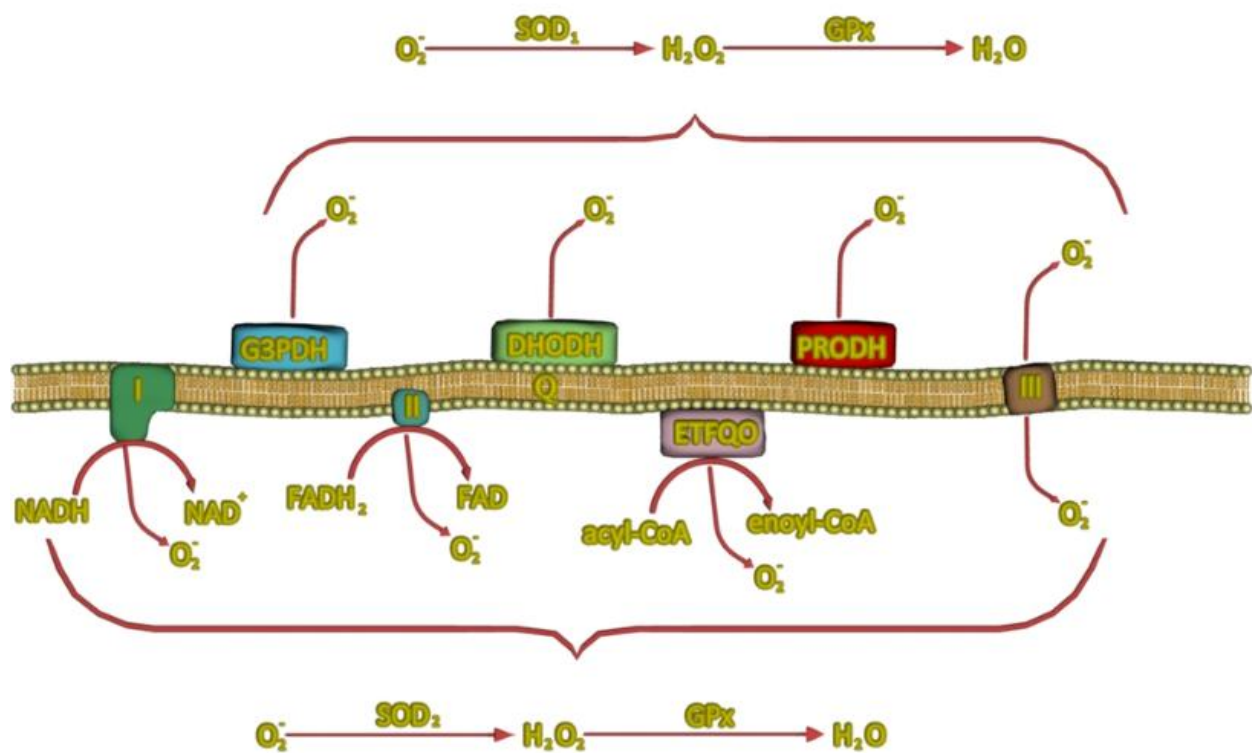


Figure 1.4: Diagram showing sources of mitochondrial ROS. Complex I, II and III, G3PDH (glycerol-3-phosphate dehydrogenase), DHODH (dihydroorotate dehydrogenase), PRODH (proline dehydrogenase) and ETFQO (electron transfer flavoprotein oxidoreductase) are sites of ROS production in the ETS. O_2^- : superoxide radical, H_2O_2 : hydrogen peroxide, SOD: superoxide dismutase, GPx: glutathione peroxidase. Other potential sites of ROS are mentioned in the text. (Drawing by Dr. L. Bate).

1.5.4 Mitochondrial volume

The regulation of mitochondrial volume is critical for ATP production and many physiological and pathophysiological conditions are known to impose volume changes on these organelles (Guerrieri et al., 2002; Fujii et al., 2004). Mitochondrial matrix volume is regulated by the IMM, which acts as the main barrier for molecules moving into and out of the organelle (Bernardi, 1999; O'Rourke, 2000; Lee and Thevenod, 2006). This tight control of IMM permeability enables the mitochondria to create a high proton gradient that drives the production of ATP that in turn supports functions important for cellular maintenance and survival. Alteration in IMM permeability leads to unguarded flux of solutes and water disrupting the function of the mitochondria (Belyaeva et al., 2001; Li et al., 2003; Orlov et al., 2013). Additionally, volume dysregulation can affect the activities/function of mitochondrial membrane channels that provide selective transport of ions and solutes across the IMM.

1.5.5 Mitochondrial membrane channels and transporters

Mitochondria possess different types of channels and transporters (Fig. 1.5) that facilitate the movement of materials between the mitochondria and the cytosol. The transporters and channels of particular interest to my research include the mitochondrial calcium uniporter (MCU), the mitochondrial potassium ATP sensitive channel (mitoK_{ATP}), the mitochondrial permeability transition pore (MPTP), the voltage dependent anion channel (VDAC), and the adenine nucleotide translocator (ANT).

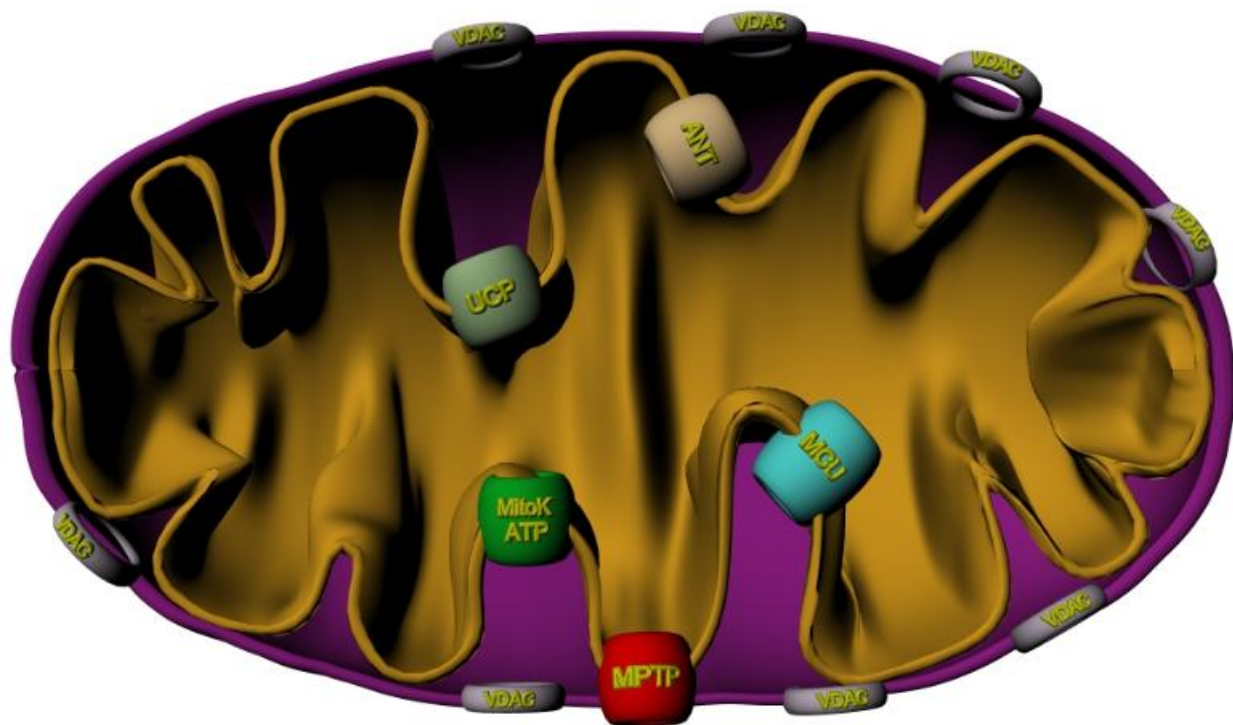


Figure 1.5: Diagram showing mitochondrial membrane channels: VDAC: voltage dependent anion channel, UCP: uncoupling protein, ANT: adenine nucleotide translocator, mitoK_{ATP}: mitochondrial potassium ATP channel, MPTP: mitochondrial permeability transition pore and MCU: mitochondrial calcium uniporter. (Illustration by Dr. L. Bate).

1.5.5.1 Mitochondrial calcium uniporter (MCU)

The major role of the MCU (Fig. 1.5) is to transport Ca from the cytosol down its electrochemical gradient into the mitochondrial matrix (Gunter et al., 2000; Pendin et al., 2014; Kevin Foskett and Philipson, 2015). This transport is not coupled with any other ion or molecule and is highly dependent on $\Delta\Psi_m$ (Gunter et al., 2000; Pendin et al., 2014) and is inhibited by ruthenium red (Gunter et al., 2000, Nicholls and Ferguson, 2013). Although the MCU has high affinity for Ca (Kirichok et al., 2004), it has been shown to transport other ions with the selectivity order of $\text{Ca} > \text{Sr} > \text{Mn} > \text{Ba} > \text{Fe} > \text{La}$ (Drahota et al., 1969; Vainio et al., 1970). This implies that the MCU can be an important uptake pathway for divalent cations into the mitochondria. Indeed, the MCU has been shown to be involved in Cd uptake by mitochondria since its inhibition with ruthenium red reduces Cd uptake by the mitochondria (Lee et al., 2005; Adiele et al., 2012b).

1.5.5.2 Mitochondrial potassium ATP sensitive channel (mitoK_{ATP})

The mitoK_{ATP} channel (Fig. 1.5) was first identified in 1991 by Inoue et al. (1991) who used mitoplasts from rat liver mitochondria to show that a highly selective small conductance K channel exists in the IMM. Since then the mitoK_{ATP} has been identified in mitochondria from the heart (Paucek et al., 1992; Wojtovich and Brookes, 2009), brain (Bajgar et al., 2001; Debska et al., 2001), skeletal muscle (Gurke et al., 2000; Debska et al., 2002), and kidney (Cancherini et al., 2003). Numerous studies have suggested a role for the mitoK_{ATP} channel in ischemic preconditioning (IPC) a process in which short ischemic periods protect against damage from subsequent longer episodes of ischemia (Kloner et al., 1998; Cohen et al., 2000). In addition to the IPC, pharmacological modulation of the mitoK_{ATP} has been instrumental in elucidating the pathophysiology of ischemia-reperfusion (IR) injury in mammals (Garlid et al., 1997; Grover et

al., 2001). In this regard the pharmacological mitoK_{ATP} opener (diazoxide) was found to protect against IR injury (Garlid et al., 1997) while 5-hydroxydecanoate (5-HD), a blocker of the channel (Jaburek et al., 1998) reduced the protection.

The exact mechanisms through which mitoK_{ATP} protects the mitochondria against IR injury are not clear but several hypotheses have been proposed (Ardehali and O'Rourke, 2005). The first hypothesis posits that opening mitoK_{ATP} causes mild ROS production that serve as a signal that activates kinases, which initiate cellular defence against IR (Zweier et al., 1987). The second is that opening of mitoK_{ATP} results in increased mitochondrial matrix swelling (Kowaltowski et al., 2001; Heinen et al., 2007; Szabo et al., 2012). It has been proposed that matrix swelling induced by mitoK_{ATP} opening brings the IMM and OMM closer to each other thereby promoting contact between proteins on these membranes which in turn facilitates the transport of ADP and enhances ATP synthesis (Kowaltowski et al., 2001). However, this hypothesis has been disputed by Lim et al. (2002) who argued that since diazoxide inhibits mitochondrial respiration, activation of OXPHOS by matrix swelling is unlikely to be the mechanism by which mitoK_{ATP} induces protection. The third hypothesis is that opening mitoK_{ATP} attenuates Ca uptake by mitochondria (Holmuhamedov et al., 1999; Liu et al., 1998; Wang et al., 2001; Korge et al., 2002) subsequently preventing the opening of the MPTP during reoxygenation thereby preserving mitochondrial integrity.

1.5.5.3 Mitochondrial permeability transition pore (MPTP)

Mitochondrial permeability transition pore (MPTP) as shown (Fig. 1.5) denotes a condition wherein mitochondria become leaky and swell, characteristically after exposure to high concentrations of Ca (Halestrap, 2010; Bernardi, 2013). Haworth and Hunter (1979) first suggested the name permeability transition pore (PTP) and showed that it involved the opening

of a non-specific channel permeable to molecules <1.5 kDa. Earlier this phenomenon was thought to be due to the activation of endogenous phospholipase A₂ leading to disintegration of phospholipids within the IMM (Gunter and Pfeiffer, 1990). Later Crompton et al. (1988) demonstrated that the pore could be blocked by sub-micromolar concentrations of cyclosporin A (CsA) an inhibitor of cyclophilin D that led to the identification of one of the components of MPTP. In addition to CsA, MPTP is inhibited by adenine nucleotides (ADP and ATP) and opened by IR injury, temperature stress and metals (e.g., Cd) that cause oxidative stress (Clarke et al., 2002; Leung et al., 2008; Di Lisa and Bernardi, 2009). Although the molecular identity of the MPTP is yet to be determined, several proteins have been implicated in its formation and regulation including, ANT (Halestrap, 2009; Bernardi, 2013), phosphate carrier (Halestrap, 2009; Bernardi, 2013), ATP synthase (Bonora et al., 2013; Alavian et al., 2014), cyclophilin D (Bernardi, 2013) and VDAC (Bernardi, 2013; Halestrap and Richardson, 2015).

1.5.5.4 Voltage dependent anion channel (VDAC)

VDAC (Fig. 1.5), also known as the mitochondrial porin, is a 31 kDa protein (Benz, 1985) that surrounds the OMM and regulates the movement of material across the OMM (Lemasters and Holmuhamedov, 2006; Shoshan-Barmatz et al., 2010). The channel possesses the capacity for both voltage dependence and ion selectivity with anions being favoured over cations (Gincel et al., 2000; Lemasters and Holmuhamedov, 2006; Shoshan-Barmatz et al., 2010). Apart from small lipophilic compounds such as short chain fatty acids, acetaldehyde and O₂ that are permeable to membranes, all other molecules pass across OMM through VDAC (Lemasters and Holmuhamedov, 2006). Thus, VDAC has been reported to participate in the transport of a wide array of compounds including cholesterol, heme precursors and ATP/ADP (Rebeiz et al., 1996; Rostovtseva et al., 2002). Moreover, the closure of VDAC stops the passage of organic anions

such as Pi, ATP, ADP, creatine phosphate and substrates for OXPHOS (Rostovtseva et al., 2002; Lemasters and Holmuhamedov, 2006).

1.5.5.5 Adenine nucleotide translocator (ANT)

The primary function of ANT (Fig. 1.5) is the trafficking of adenine nucleotides (ATP/ADP) across the IMM (Halestrap and Brenner, 2003). In addition to this basic role, ANT together with other proteins (VDAC, cyclophilin D and ATP synthase) has been implicated in the formation of MPTP (Halestrap and Brenner, 2003). Structurally, ANT exists in two conformations as revealed by the inhibitors of the protein, carboxyatractyloside (CAT) and bongkreikic acid (BKA). These inhibitors induce two different conformational states of the ANT. Notably, BKA binds ANT on the matrix side resulting in the 'M' conformation while CAT binds ANT from the cytosolic side leading to the 'C' conformation (Klingenberg, 1989). Although it is certain that CAT and BKA stabilise different conformations in the translocation cycle, it remains unclear whether or not these conformations are identical to an outward facing and inward facing single binding site. What is clear is that ANT shuttle the electrogenic exchange of ATP^{4-} with ADP^{3-} ; the charge disparity enables the $\Delta\Psi_m$ to drive ATP out of the mitochondria into the cytosol in exchange for ADP entering the matrix (Klingenberg, 1980). When the ETS is inhibited, the ANT can work in reverse and allow the entry of ATP into the mitochondria (Halestrap and Brenner, 2003). Under these conditions the mitochondria become consumers of the energy due to hydrolysis of the internalized ATP. The hydrolyzed ATP is used to pump protons from the mitochondrial matrix into the inter-membrane space in an attempt to maintain the $\Delta\Psi_m$ (Boutillier and St-Pierre, 2000; St-Pierre et al., 2000).

1.6 HYPOTHESIS AND OBJECTIVES

In the preceding sections I have discussed the stressors of interest for my thesis research: Cd, hypoxia and temperature. I demonstrated that these stressors compromise cellular energy balance and discussed how cellular energy (ATP) is produced by the mitochondria and the machinery that enables mitochondria to carry out this function. Importantly, it became clear to me that our knowledge of interactive effects of multiple stressors on energy metabolism is limited. Therefore the goal of my research was to unveil the interactions of Cd, hypoxia (with reoxygenation) and temperature –stressors that present immediate and increasing threat to aquatic systems– on mitochondrial physiology. The overall hypothesis was that when present together Cd, temperature and hypoxia affect common mitochondrial pathways exacerbating single stressor-induced structural-functional alterations. I tested this hypothesis with the following objectives:

- 1) Investigate the mechanisms of stressor-stressor interactions on mitochondrial bioenergetics following exposure to Cd, H-R and/or temperature.
- 2) Investigate how different reporters of mitochondrial function are altered following Cd, H-R and/or temperature exposure.
- 3) Elucidate ways to mitigate stressor-induced mitochondrial dysfunction using pharmacological agents.

CHAPTER 2

EFFECT OF HYPOXIA-CADMIUM INTERACTIONS ON RAINBOW TROUT (*Oncorhynchus mykiss*) MITOCHONDRIAL BIOENERGETICS: ATTENUATION OF HYPOXIA-INDUCED PROTON LEAK BY LOW DOSES OF CADMIUM

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Author contributions

C.K. conceived the project, C.K. and J.O.O 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.

2.1 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 ($0 > PO_2 < 2$ 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 μ M) 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 4_{ol} (proton leak) respirations, thus reducing phosphorylation and coupling efficiencies. Low doses of Cd (≤ 5 μ M) 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 system (ETS) 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), administered 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. These results indicate that hypoxia-reoxygenation impairs the ETS and sensitizes

mitochondria to Cd via mechanisms that involve, at least in part, ROS. Moreover I provide, for the first time in fish, evidence for 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.

2.2 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 (Krumschnabel 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 (Kessler and Brand, 1994a; Belyaeva and Korotkov, 2003; Cannino et al., 2009; Kurochkin et al., 2011; Adiele et al., 2012a; 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 I 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. I 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. My initial experiments focused on the effects of hypoxia alone and then I studied the interactive effects of hypoxia and Cd. By focusing on the mitochondria I 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, I measured mitochondrial respiration after hypoxia and subsequent reoxygenation.

2.3 MATERIALS AND METHODS

2.3.1 Fish

Rainbow trout (142 ± 10 g) were obtained from Ocean Farms Inc, Brookvale, PEI, and maintained in a 400-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 10 ± 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.

2.3.2 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 KH_2PO_4 , 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 $800 \times g$ for 15 min at 4 °C. The supernatant was collected and spun at $13,000 \times g$ for 10 min at 4 °C and the mitochondrial pellet was washed twice by re-

suspending in MIB and centrifuging at $11,000 \times g$ 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 KH_2PO_4 , 100 mM KCl, 1 mg/ml BSA (fatty acid free), 2 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.3] and used in the subsequent experiments.

2.3.3 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 (Pallotti and Lenaz, 2001; Barrientos, 2002; 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 ($\epsilon_{412} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Figure 2.1 shows that my 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 cyt c and NADH during state 4 did not stimulate respiration indicating that the OMM and IMM were intact, respectively (Fig. 2.2).

Figure 2.1

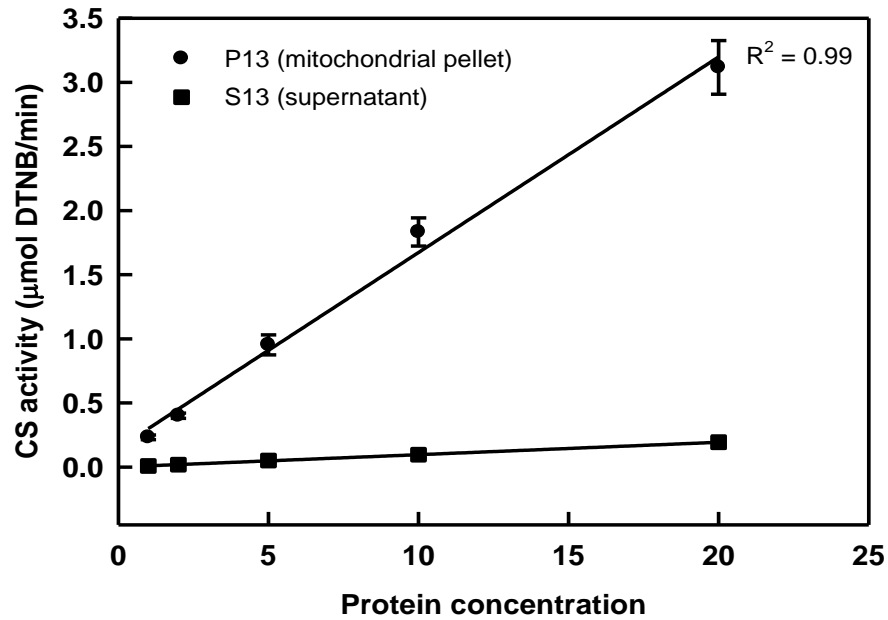


Figure 2.1: Mitochondrial content: citrate synthase (CS) activity in 13,000 $\times g$ pellet (P13, mitochondria) and 13000 $\times g$ supernatant (S13). P13 has high CS activity indicating high mitochondrial content whereas S13 supernatant has low CS activity indicating negligible amount of mitochondria and/or minimal disruption of mitochondria during isolation and purification. Data are means \pm SEM (n = 5).

Figure 2.2

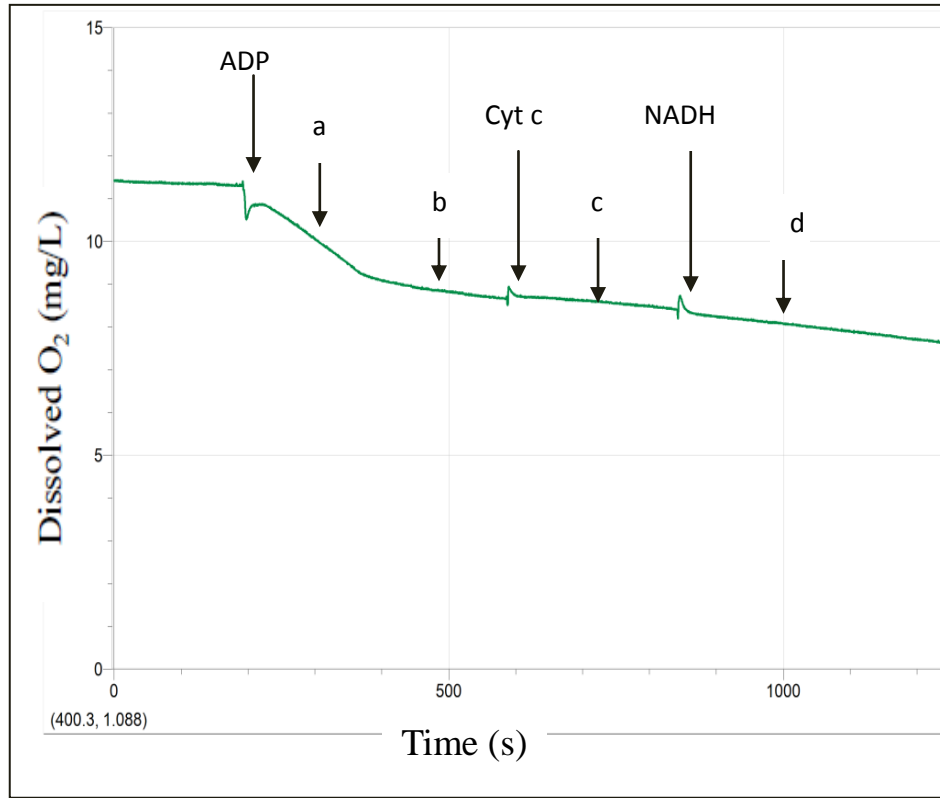


Figure 2.2: Mitochondrial integrity: representative polarographic tracing showing results of cytochrome c (Cyt c) and NADH tests of mitochondrial membrane integrity. The oxygen consumption slopes for the respective segments are: state 3 (a) = -0.013; state 4 (b) = -0.0016; + Cyt c (c) = -0.0017; + NADH (d) = -0.0017. The lack of stimulation of oxygen consumption indicates that the outer (Cyt c) and inner (NADH) mitochondrial membranes are intact

2.3.4 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 250 µmole 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; Kessler 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.

2.3.5 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. 2.3). 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 (PO_2) to <2 torr but > 0 torr (0.002-0.003 mg O_2/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). Mitochondria failed to regain functionality on reoxygenation if incubated at 0 torr. Once the PO_2 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 (250 μmol) 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.

Figure 2.3

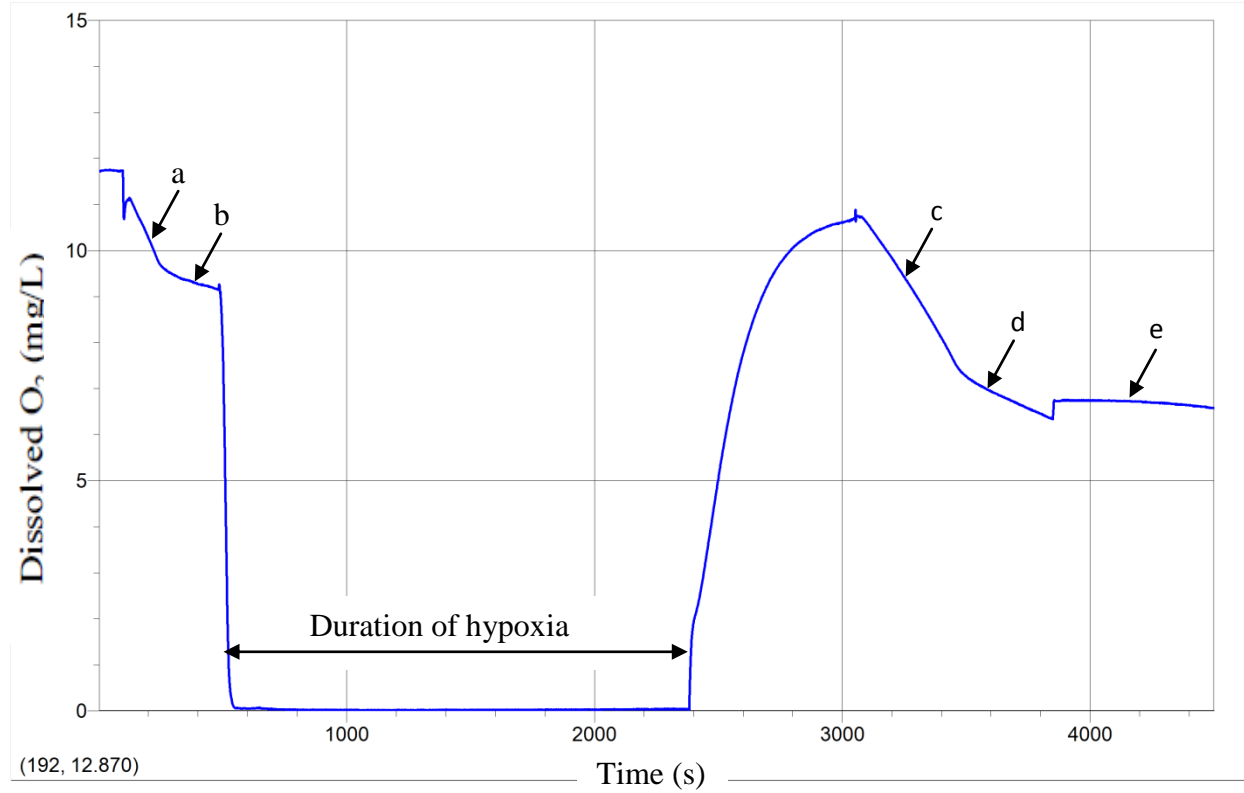


Figure 2.3: Protocol for the exposure of rainbow trout liver mitochondria to hypoxia-reoxygenation *in vitro*. Initially a two-point calibration at 0 and 100% O₂ saturation was done and normoxic (control) mitochondrial respiration parameters were measured after addition of mitochondria, substrates (malate 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.

2.3.6 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 $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ (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 $0 < \text{PO}_2 < 2$ 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 O_2) 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 sulfhydryl 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.

2.3.7 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 $10,000 \times g$ 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 $10,000 \times g$ 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 μ l 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 240 μ l of complex I enzyme assay buffer (25 mM potassium phosphate, 3.5 mg/ml BSA, 100 μ M DCIP, 70 μ M decylubiquinone, 0.6 mg/l antimycin A, and 200 μ M NADH, pH 7.3) was added to wells of a 96-well microplate. To initiate the reaction, 60 μ g of mitochondrial protein were added to all wells except the blanks and each sample was analyzed in triplicate with and without 2 μ M 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 $19.1 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.3.8 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). If the data did not pass the normality test, they were submitted to Box-Cox transformation; all data passed after transformation. An ANOVA is only slightly affected by inequality of variance using our models (equal sample sizes and all factors fixed). 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).

2.4. RESULTS

2.4.1 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. 2.4A). 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. 2.4B) 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 hypoxia-reoxygenation (Fig. 2.5A). 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. 2.3C) leading to a precipitous decline in estimates of mitochondrial coupling and phosphorylation efficiency (Fig. 2.6A, B). In this regard, the phosphorylation efficiency (P/O ratio) (Fig. 2.6A) 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. 2.6B) 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} (Fig. 2.5B).

Figure 2.4

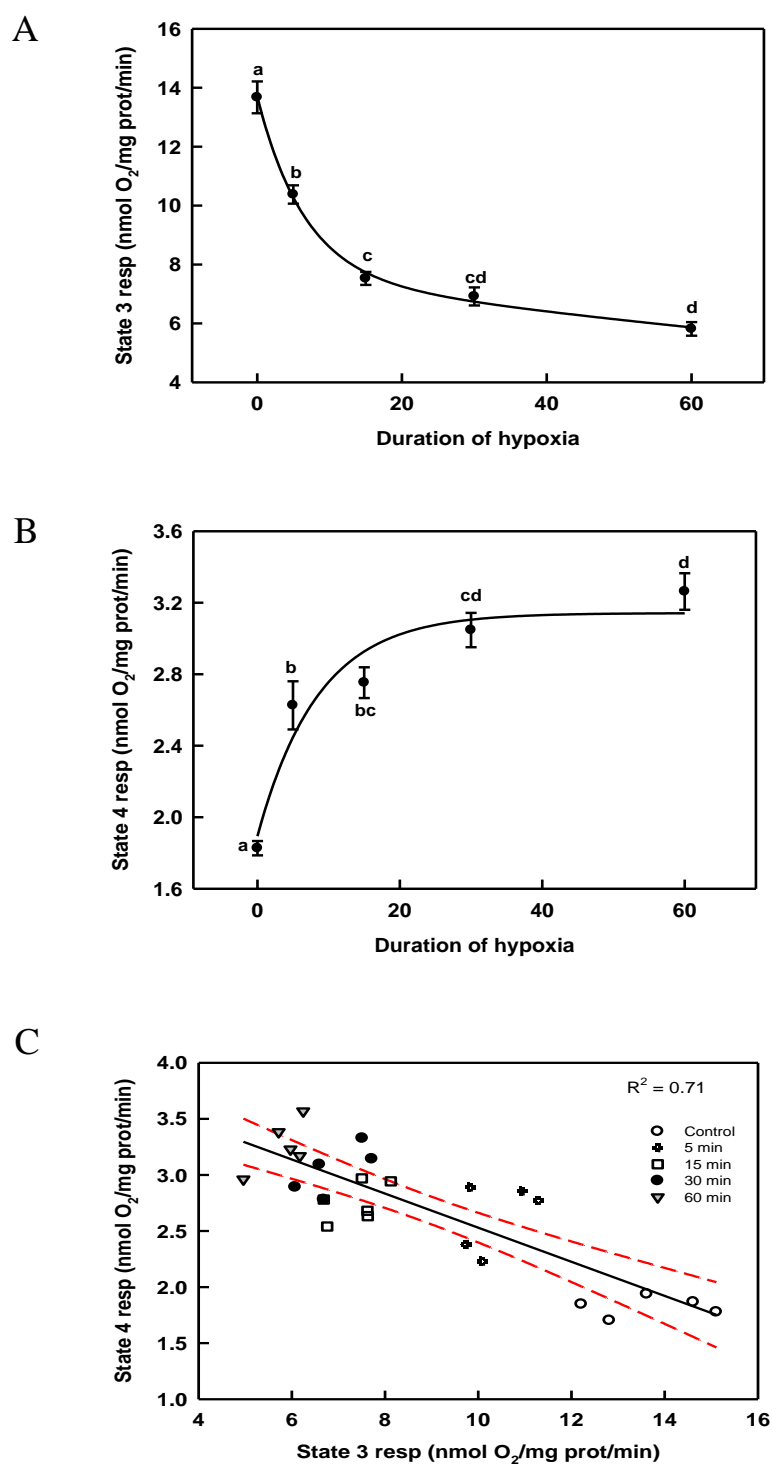
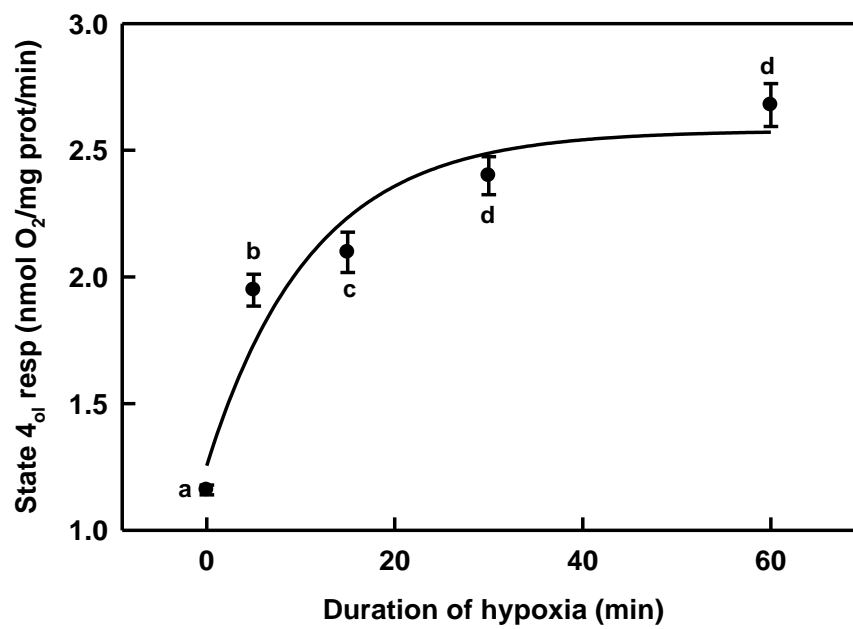


Figure 2.4: The effect of duration of hypoxia exposure on state 3 (**A**) and 4 (**B**) respirations rates in isolated rainbow trout liver mitochondria. (**C**): the relationship between state 3 and 4 respiration rates. Mitochondria were incubated under hypoxic conditions for 0, 5, 15, 30, and 60 min followed by reoxygenation (100% O₂ saturation) and measurement of oxygen consumption rates. Data are means \pm SEM (n = 5). Points with different letters are significantly different from each other (one-way ANOVA with Tukey's HSD, P<0.05).

Figure 2.5

A



B

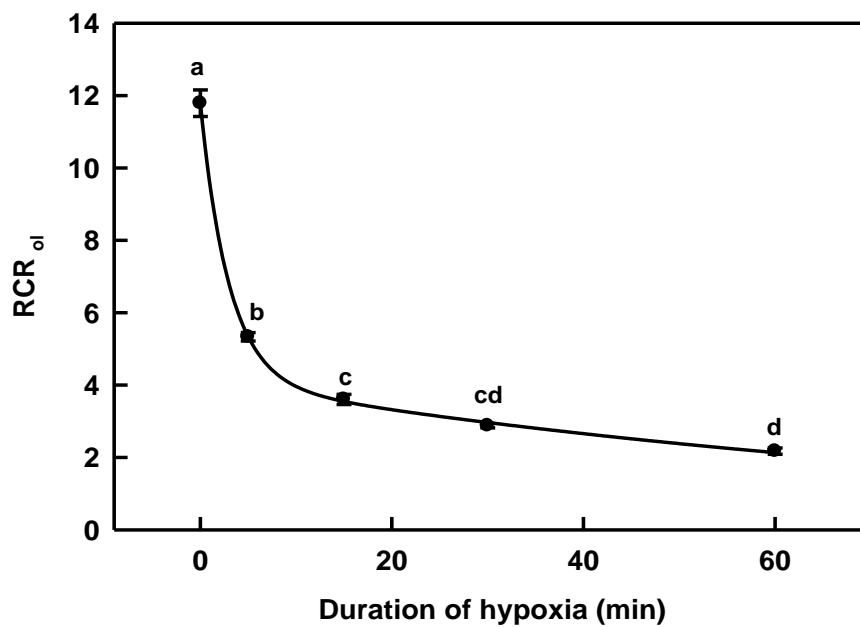
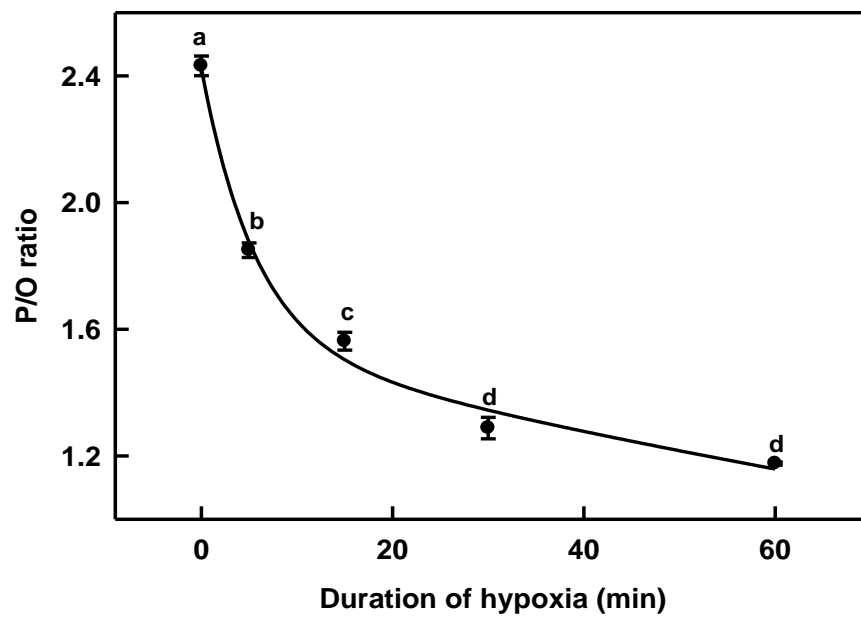


Figure 2.5: Effect of duration of hypoxia exposure on state 4_{ol} (**A**) and RCR_{ol} (**B**) in isolated rainbow trout liver mitochondria. Mitochondria were incubated under hypoxic conditions for 0, 5, 15, 30, and 60 min followed by reoxygenation (100% O_2 saturation) and measurement of oxygen consumption rates. Data are means \pm SEM ($n = 5$). Points with different letters are significantly different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 2.6

A



B

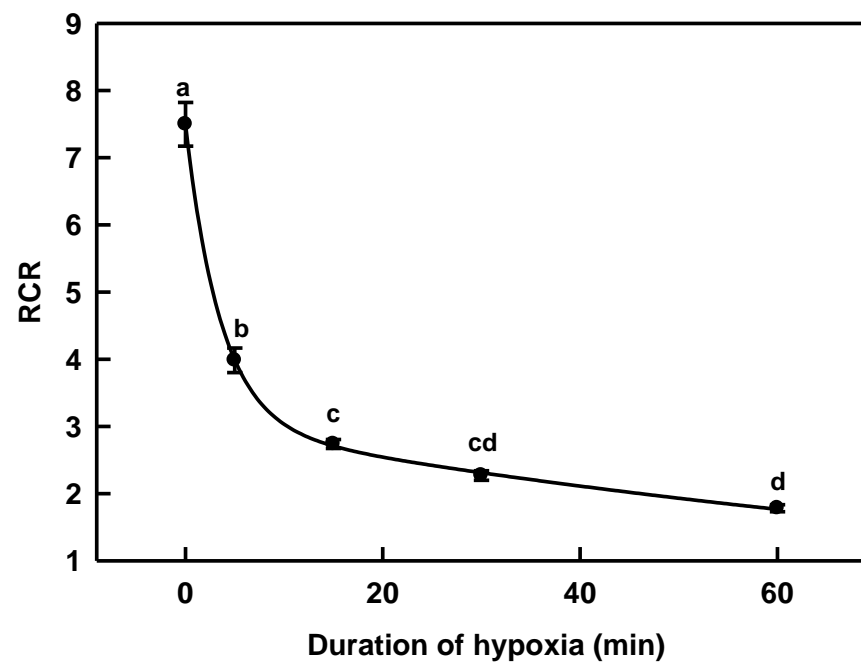


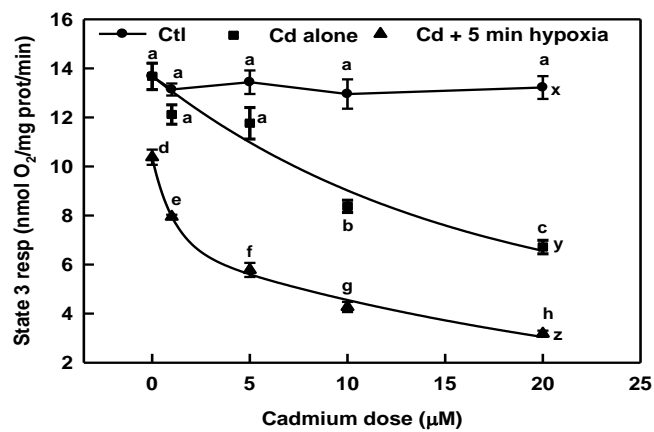
Figure 2.6: The effect of duration of hypoxia on P/O ratio (**A**) and RCR (**B**) in isolated rainbow trout liver mitochondria. Mitochondria were incubated under hypoxic conditions for 0, 5, 15, 30, and 60 min followed by reoxygenation (100% O₂ saturation) and measurement of oxygen consumption rates. Data are means \pm SEM (n = 5). Points with different letters are significantly different from each other (one-way ANOVA with Tukey's HSD, P<0.05).

2.4.2 Interactions of hypoxia and cadmium on liver mitochondrial respiration

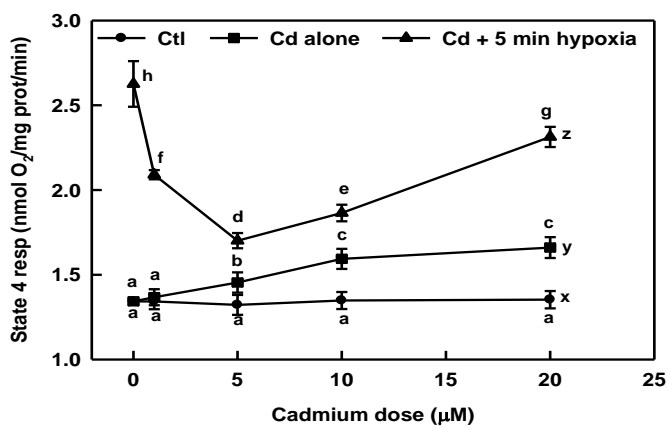
The effects of combined 5-min hypoxia and Cd (0–20 μ M) on state 3 respiration in Fig. 2.7A show that hypoxia exacerbates the inhibitory effect of Cd ($F_{2,60}=257$, $P<0.0001$). Moreover, the interaction between hypoxia level and Cd exposure was highly significant ($F_{8,60}=26$, $P<0.0001$) indicating 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 μ M) and highest (20 μ M) 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 μ M) inhibited but higher (>5 μ M) doses of Cd stimulated these respiration rates (Fig. 2.7B; Fig. 2.8A). Similar to state 3 respiration, the interaction terms of hypoxia and Cd on state 4 ($F_{8,60}=11$, $P<0.0001$) and state 4_{ol} ($F_{8,60}=33$, $P<0.0001$) 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 μ M Cd exposure reduced the RCR and RCR_{ol} by 82 and 85%, compared with the 59 and 51% reductions caused by Cd alone, respectively (Fig. 2.7C; Fig. 2.8B). There was a significant 2-way interaction on both the RCR ($F_{8,60}=47$, $P<0.0001$) and RCR_{ol} ($F_{8,60}=62$, $P<0.0001$) indicating co-dependence of the reduction in coupling on duration of hypoxia and Cd dose.

Figure 2.7

A



B



C

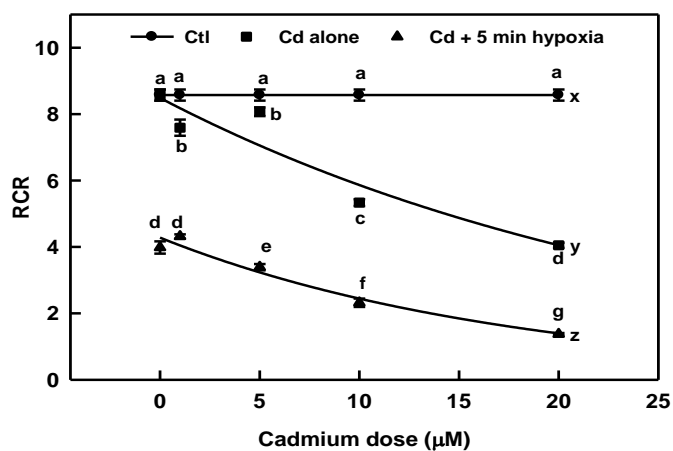
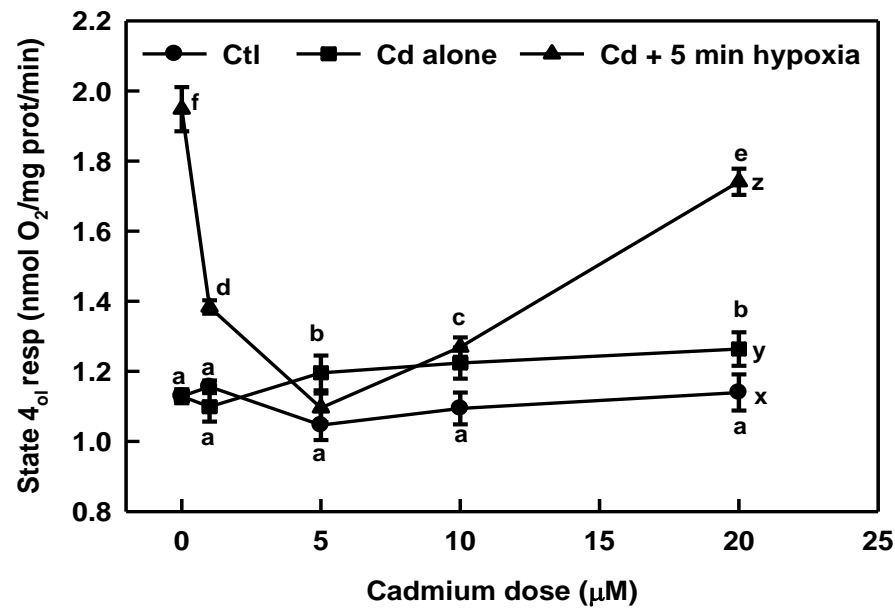


Figure 2.7: The interactions of hypoxia and Cd on rainbow trout liver mitochondria function: (A), state 3 respiration; (B), state 4 respiration; (C) RCR. Mitochondria were exposed to Cd (0, 1, 5, 10, and 20 μ M) with and without 5 min hypoxia. Data are means \pm SEM (n = 5). Points with different letters are significantly different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 2.8

A



B

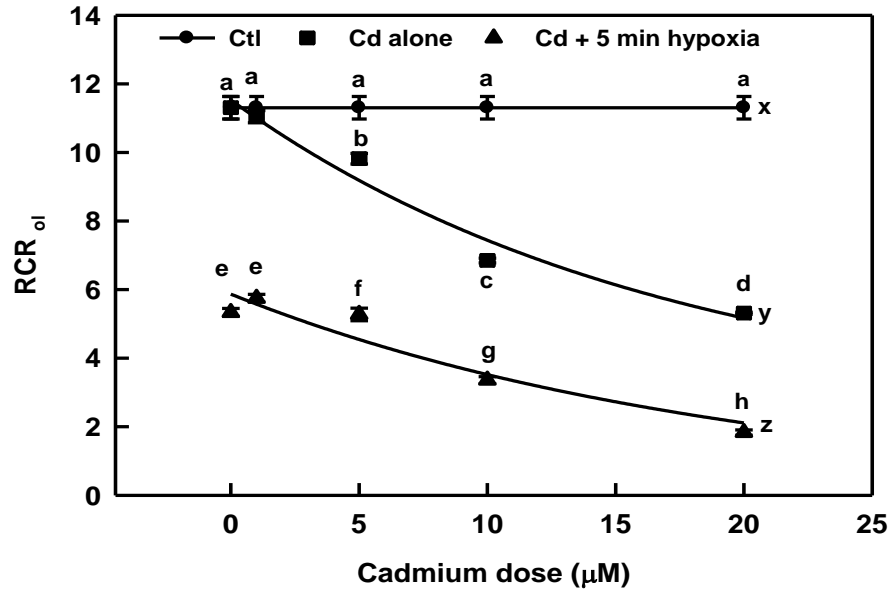


Figure 2.8: The interactions of hypoxia and Cd on rainbow trout liver mitochondria state 4_{ol} respiration (**A**) and RCR_{ol} (**B**). Mitochondria were exposed to Cd (0, 1, 5, 10, and 20 µM) with and without 5 min hypoxia. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (two-way ANOVA with Tukey's HSD, P<0.05).

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 ($F_{6,28}=42$, $P<0.0001$) 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. 2.9A). Similarly there was a significant effect of experimental group ($F_{6,28}=24$, $P<0.0001$) on state 4 (Fig. 2.8B) 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 NAC had no effect. For state 4_{ol}/proton leak (Fig. 2.10) 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 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. 2.9C) 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 μ M) 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.

2.4.3 Complex I activity

The effect of hypoxia and the interaction with Cd on complex I activity assessed using 5-min of hypoxia and 5 μ M Cd (Fig. 2.9D) 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 μ M Cd alone had no effect on the enzyme, it partially (22%) rescued hypoxia-induced complex I activity inhibition.

Figure 2.9

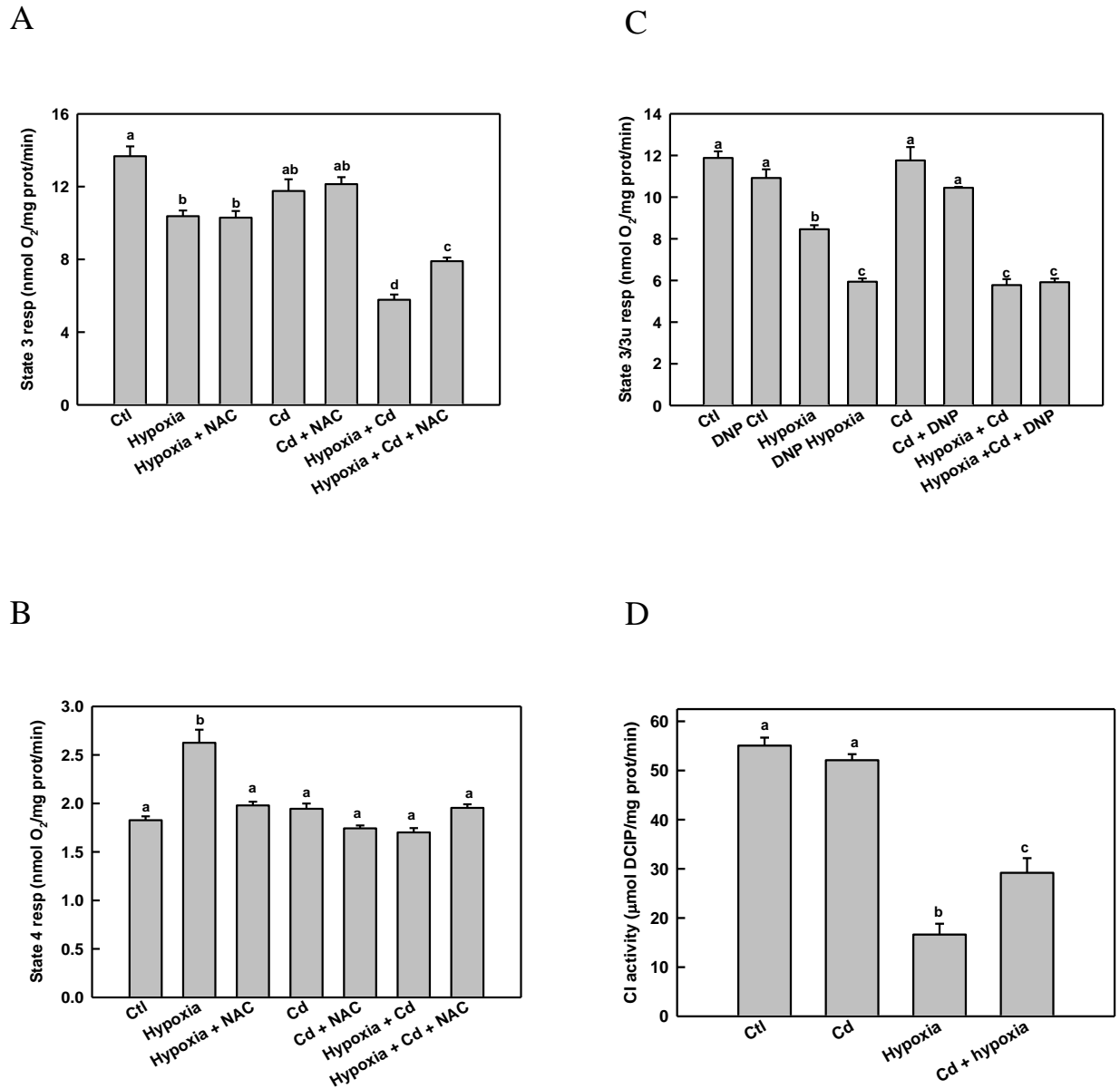


Figure 2.9: The effect of N-acetylcysteine (NAC) on hypoxia-, Cd-, and hypoxia + Cd-induced respiration disturbances in rainbow trout liver mitochondria. **(A)**: state 3 respiration; **(B)**: state 4 respiration. **(C)**: effect of 5 min hypoxia with and without 5 μm Cd on maximal coupled and 2,4-

dinitrophenol (DNP)-uncoupled respiration. (**D**): effect of 5 min hypoxia with and without 5 μ m Cd on mitochondrial electron transport chain complex 1 enzyme activity. Data are means \pm SEM (n = 5). Groups with different letters are significantly different from each other (one-way ANOVA with Tukey's HSD, P<0.05).

Figure 2.10

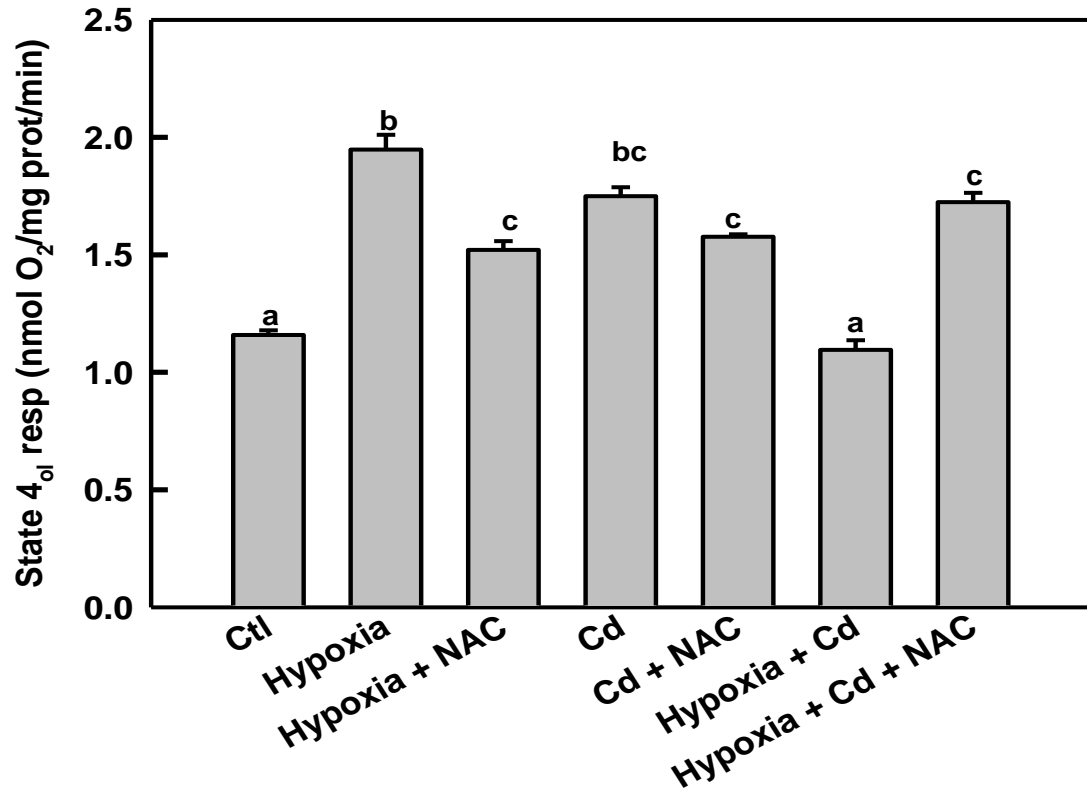


Figure 2.10: The effect of N-acetylcysteine (NAC) on Cd-, hypoxia-, and Cd + hypoxia-induced changes on proton leak (state 4_{ol} respiration). Data are means \pm SEM (n = 5). Groups with different letters are significantly different from each other (one-way ANOVA with Tukey's HSD, P<0.05).

2.5 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. I 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, my 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). My 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 4_{ol} respirations. It is also possible that the phosphorylation system (ATP synthase and phosphate and adenylyate 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 4/4_{ol}. 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. 2.3), 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, I tested the hypothesis that it entailed impairment of the electron transport system (ETS). First, I found that DNP-uncoupled respiration was inhibited (notably to a greater extent than state 3 respiration) following hypoxia-reoxygenation (Fig. 2.8C). Second, while 5 μ M 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 ETS. Thus inhibition of the uncoupler-stimulated respiration is indicative of impaired ETS (Belyaeva and Korotkov, 2003). Importantly, the impairment of ETS was directly confirmed by enzyme activity measurements that revealed a greatly reduced complex I activity (Fig. 2.6D) 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 ETS 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. I 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 4/4_{ol} 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-I mediated state 3 respiration by NAC does not preclude ROS-mediated damage involving the distal ETS 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 ETS complexes and complex-specific substrates would help identify if the distal enzymes were affected. In the apparent absence of ROS-mediated complex I damage, I 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, I demonstrated that rainbow trout liver mitochondria were impaired by this metal dose-dependently. Concentrations of Cd $\leq 5\mu\text{M}$ did not affect mitochondrial bioenergetics whereas concentrations $\geq 10\mu\text{M}$ 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; 2012b) 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, I 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 4_{ol} 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 my 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 MPTP, 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. 2.5B). 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) *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 *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 ETS at least at the complex I level. Lastly, I 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. I 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.

CHAPTER 3

MODULATION OF CADMIUM-INDUCED MITOCHONDRIAL DYSFUNCTION AND VOLUME CHANGES BY TEMPERATURE IN RAINBOW TROUT (*Oncorhynchus mykiss*).

A version of this chapter has been published with slight modification as:

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Author contributions

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

3.1 ABSTRACT

I investigated how temperature modulates cadmium (Cd)-induced mitochondrial bioenergetic disturbances and volume changes in rainbow trout (*Oncorhynchus mykiss*). In the first set of experiments, rainbow trout liver mitochondrial function and Cd content were measured in the presence of complex I substrates, malate and glutamate, following exposure to Cd (0-100 μ M) at three (5, 13 and 25 $^{\circ}$ C) temperatures. The second set of experiments assessed the effect of temperature on Cd-induced mitochondrial volume changes, including the underlying mechanisms, at 15 and 25 $^{\circ}$ C. Although temperature stimulated both state 3 and 4 rates of respiration, the coupling efficiency (respiratory control ratio [RCR]) was reduced at temperature extremes due to greater inhibition of state 3 at the lowest temperature and greater stimulation of state 4 at the highest temperature. On the other hand, co- exposure of Cd and temperature reduced the stimulatory effect of temperature on state 3 respiration, but increased that of state 4 respiration thus exacerbating mitochondrial uncoupling. I further found that the interaction of Cd and temperature yielded different responses on the Q_{10} of state 3 and 4 respiration wherein the Q_{10} values for state 3 respiration increased at low temperature (5-13 $^{\circ}$ C) while those for state 4 increased at high temperature (13-25 $^{\circ}$ C). The mitochondria accumulated more Cd at high temperature suggesting that the observed greater impairment of oxidative phosphorylation at 25 $^{\circ}$ C was due, at least in part, to a higher metal burden. Cadmium-induced mitochondrial volume changes were characterized by an early phase of contraction followed by swelling, with temperature intensifying these effects. Interestingly, at low doses of Cd (5 μ M), there was no initial swelling phase. Lastly, using specific modulators of mitochondrial ion channels, I demonstrated that the mitochondrial volume changes were associated with Cd uptake via the mitochondrial calcium uniporter (MCU) without significant contribution of the permeability transition pore and/or potassium channels. Overall, it appears that high temperature exacerbates

Cd-induced mitochondrial dysfunction and volume changes in part by increasing metal uptake through the MCU.

3.2 INTRODUCTION

In a natural environment aquatic ectotherms, such as fish, are particularly vulnerable to changes in temperature because their body temperatures are close to that of the environment (Stevens and Fry, 1974). Indeed, major physiological and biochemical processes in fish, including swimming, metabolic rate, growth and reproduction are highly affected by temperature fluctuations. To cope with environmental temperature change, aquatic organisms have evolved a wide array of mechanisms. In fish many of these mechanisms entail modulation of energy metabolism and include changes in mitochondrial membrane properties, density and enzyme activity (Guderley and St-Pierre, 2002; Kraffe et al., 2007; Lockwood and Somero, 2012; Oellermann et al., 2012). Within a zone of tolerance, these changes allow organisms to cope with the challenges associated with extreme temperatures. Because the mitochondria perform several other important functions such as cell signalling, redox regulation, Ca^{2+} homeostasis and control of apoptosis, temperature-induced mitochondrial dysfunction typically leads to loss of the cell function with cell death as the terminal sequel.

Environmental temperature stress is commonly encountered together with chemical pollutants including metals such as Cd. Cadmium is an important trace metal contaminant in aquatic systems due to its environmental persistence and high toxicity to aquatic organisms (Byczkowski and Sorenson, 1984; Hattink et al., 2005). When present at elevated levels in aquatic systems, Cd is readily taken up and accumulated in tissues of resident organisms resulting in toxicity (Kraemer et al., 2005; 2006). Although the toxic effects of Cd are numerous, the mitochondria are arguably the most important target site of its toxic action. In this regard, several authors have

demonstrated that many aspects of mitochondrial function are compromised by Cd in plants (Kesseler and Brand, 1994a), mammals (Belyaeva and Korotkov, 2003), invertebrates (Kurochkin et al., 2011) and fish (Adiele et al., 2012a; Chapter 2). The mechanisms through which Cd alters mitochondrial function include, formation of complexes with thiol proteins and the displacement of iron and/or Cu from their binding sites in key proteins of the respiratory chain (Rikans and Yamano, 2000; Dorta et al., 2003).

Although, it is evident from the foregoing that temperature and Cd affect cell/organismal function individually, our knowledge of their combined effects is limited to few studies that have to date failed to generate a consistent theme regarding temperature-metals interactions. For example, when *Daphnia magna* were exposed to Cd at increasing temperatures, the median and threshold lethal body burdens decreased suggesting that lower metals accumulation was needed to kill daphnids at higher temperature (Heugens et al., 2003). In contrast, Lannig et al. (2006) working with the eastern oyster (*Crassostrea virginica*) reported that exposure to Cd at 28 °C caused significantly higher mortality compared with exposure at 20 or 24 °C, even though both groups had the same tissue Cd burdens. With regard to interactions on energy homeostasis, Sokolova (2004) found that oyster mitochondria were more sensitive to Cd at high temperatures and concluded that temperature sensitizes mitochondria to Cd. Overall, the existing literature suggests that aquatic organisms may be vulnerable to metals levels that ordinarily would not be toxic in the absence of thermal stress.

The mechanisms through which thermal stress moderates metals-induced mitochondrial dysfunction have not been clearly elucidated but may include changes in mitochondrial volume. Mitochondrial volume (permeability) is critical for the activity of the electron transport system (i.e. ATP production) and many physiological and pathophysiological conditions are known to

impose volume changes on these organelles (Guerrieri et al., 2002; Fujii et al. 2004). Under normal physiological conditions the mitochondrial matrix volume is regulated by the inner mitochondrial membrane (IMM) which acts as the main barrier for molecules moving into and out of the organelle. However, the IMM is endowed with ion exchangers, uniporters and channels that impart selective permeability to specific molecules (Bernardi, 1999; O'Rourke, 2000; Lee and Thevenod, 2006). This tight control of IMM permeability enables the mitochondria to create a high proton gradient that drives the production of ATP that supports functions important for cellular maintenance and viability. Alteration in IMM permeability leads to unguarded passage of solutes and water disrupting the function of the mitochondria (Belyaeva et al., 2001; Li et al., 2003; Orlov et al., 2013). Cadmium and temperature stress may alter the permeability of IMM to solutes resulting in matrix volume changes. Indeed, evidence of Cd-induced mitochondrial volume changes has been provided, including swelling and contraction in mammals (Lee et al., 2005a), moderate swelling in rainbow trout (Adiele et al., 2012b) and contraction in oysters (Sokolova, 2004). These varied observations suggest that additional studies are required to clarify the mechanisms of Cd induced-mitochondrial volume changes. Furthermore, although there is evidence that temperature causes mitochondrial volume changes in both mammals and fish (Richardson and Tappel, 1962), little is known about the combined/interactive effects of thermal stress and Cd on mitochondrial volume.

In the present study the effect of temperature on Cd-induced mitochondrial dysfunction and volume changes were investigated. First, I sought to unveil the interactive effects of Cd and temperature on oxidative phosphorylation (OXPHOS) by testing the prediction that high temperature will increase mitochondrial Cd accumulation resulting in greater OXPHOS impairment. Second, given the importance of mitochondrial permeability in determining their

function, the mechanisms of Cd-induced mitochondrial volume changes and the effect of temperature on this phenomenon were investigated. I anticipate that findings from my study will highlight how temperature change in the context of the global climate change in combination with metals, in this case Cd, would impact energy homeostasis in fish.

3.3 MATERIALS AND METHODS

3.3.1 Ethics

All experimental procedures were approved by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

3.3.2 Experimental Animals

Rainbow trout were obtained from Ocean Farms Inc, Brookvale, PE, and maintained at the Atlantic Veterinary College Aquatic Facility in a 400-l tank containing aerated well-water with temperature of 10 ± 1 °C and a pH of 7.7. The fish were fed at 1% of their body weight daily with commercial trout chow pellets (Corey Feed Mills, Fredericton, NB) until sampled to isolate liver mitochondria used in the experiments. Fish weight ranged from 506 to 560 g during the experimental period.

3.3.3 Mitochondrial isolation

Fish were sacrificed by a sudden blow to the head and dissected to remove the liver and mitochondrial isolation was done according to the method described in chapter 2. Briefly, the livers were rinsed with mitochondrial isolation buffer (MIB: 250 mM sucrose, 10 mM Tris-HCl, 10 mM KH_2PO_4 , 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, ON)

running at 200 rpm were optimal for rainbow trout liver mitochondria isolation. The homogenate was then centrifuged at $800 \times g$ for 15 min at 4 °C. The supernatant was collected, centrifuged at $13,000 \times g$ for 10 min at 4 °C and the pellet (mitochondria) was washed twice by re-suspending in MIB and centrifuging at $11,000 \times g$ for 10 min at 4 °C. The pure mitochondrial pellet was finally re-suspended in a 1:3 (weight to volume) ratio of mitochondrial respiration buffer [MRB: 10 mM Tris-HCl, 25 mM KH_2PO_4 , 100 mM KCl, 1 mg/ml BSA (fatty acid free), 2 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.3]. The protein content of the isolated mitochondrial was measured spectrophotometry (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA) according to Bradford (1976) before respirometry.

3.3.4 Mitochondrial respiration

Mitochondrial respiration was measured using Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) in 1.5 ml cuvettes after a two-point calibration at 0 and 100% oxygen saturation. A traceable digital barometer (Fisher Scientific, Nepean, ON) was used to measure the atmospheric pressure and temperature was monitored and maintained at 5, 13 or 25 °C with the aid of a recirculating water-bath (Haake, Karlsruhe, Germany). Following the calibration, 1.45 ml of MRB and 100 μl of mitochondrial suspension containing 2.2-2.4 mg of protein (22-24 mg of mitochondrial mass, wet weight) were loaded into cuvettes and continuously stirred. Complex I substrates (5 mM malate and 5 mM glutamate) and 250 μM ADP were added to initiate state 3 respiration which transitioned to state 4 upon depletion of the ADP. Lastly, 2.5 $\mu\text{g}/\text{ml}$ oligomycin was added to inhibit ATP synthase activity in order to measure state 4_{ol} , a metric of mitochondrial proton leak (Brand et al., 1994; St-Pierre et al., 2000). To measure the combined effect of Cd and temperature, mitochondria isolated from each fish was tested at 5, 13 and 25 °C using 0-100 μM Cd for a total of $n = 5$ per temperature. Because the respiration rates varied

greatly across the 3 temperatures, Cd exposure times were synchronized by varying the durations of state 4 and state 4_{ol}. Thus, readings at all temperatures were taken after 2, 8 and 16 min of Cd addition for state 3, 4 and 4_{ol} respiration rates, respectively, thereby allowing valid comparison of the data. Note that the cuvettes remained well-oxygenated and never hypoxic after 16 min of Cd addition at the 3 temperatures. LabPro data acquisition software (Qubit Systems) was used to record and analyze all the oxygen consumption data. The phosphorylation efficiency (P/O ratio: ADP used/oxygen consumed) and the respiratory control ratio (RCR: ratio of state 3 to state 4 respiration) were calculated according to Estabrook (1967) and Chance and Williams (1955), respectively.

The temperature coefficients (Q₁₀ values) for state 3 and 4 respirations were calculated for the temperature ranges 5-13 and 13-25 °C using the equation: $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$, where R₂ and R₁ represent mitochondrial oxygen consumption rates at two temperatures, T₂ and T₁, and where T₂>T₁.

3.3.5 Mitochondrial Cd content analysis

Cadmium accumulation in the mitochondria was measured by atomic absorption spectrophotometry (AAS: PinAAcle 900T, Perkin Elmer, Woodbridge, ON). Briefly, after measuring the respiration rate, mitochondria samples were removed from the cuvettes and centrifuged at 10,000 ×g for 5 min at 4 °C. The supernatants were discarded and the pellets were washed with 500 µl of MIB to remove non-specifically bound Cd, with pelleting at 10,000 ×g for 5 min at 4 °C. The pellets were then stored at -80 °C until analyzed for Cd. For AAS, the pellets were oven-dried to constant weight at 70 °C (ISOTEMP, Fisher Scientific), weighed and digested with 500 µl of 30% H₂O₂ and 70% HNO₃ (trace metal grade, Fisher) in a 1:15 mixture for 24 h at room temperature. The digests were diluted appropriately using 0.2% HNO₃ and the

Cd concentrations were measured by AAS in furnace mode and expressed as $\mu\text{mol Cd/g}$ mitochondrial dry weight (mdw). All Cd analyses were done in the presence of modifiers ($\text{NH}_4\text{H}_2\text{PO}_4$ and $\text{Mg} [\text{NO}_3]_2$). Standard reference material (SRM: TMDA-70.2) and blanks were analyzed concurrently with the samples. Cadmium was not detected in blanks and the recovery rate of Cd from the SRM ranged between 95 and 106%.

3.3.6 Mitochondrial volume

Mitochondrial volume changes were measured as described by Lee et al. (2005a) and Sappal et al. (2014b). Briefly, mitochondria isolated as described above were re-suspended as 1 mg/ml protein in swelling buffer (100 mM KCl, 10 mM Tris-HCl, 25 mM KH_2PO_4 , 1 mg/ml BSA, 5 mM glutamate and 5 mM malate adjusted to pH 7.3). Volume changes were then measured at 15 and 25 °C using 0, 5, 50 and 100 μM Cd, with 200 μM Ca as a positive control. The cation (Cd or Ca) doses were added to microplate wells as 20 μl of appropriate stock solutions and brought to assay volume of 200 μl by adding 180 μl of the 1 mg/ml mitochondrial suspension equilibrated to test temperature. The changes in absorbance at 540 nm, wherein a decrease indicates swelling and an increase indicates contraction, were then monitored every 10 sec for 30 min. An additional study tested the effect of heat shock on Cd-induced mitochondrial volume changes by loading the 1 mg/ml mitochondrial suspension directly (i.e., without equilibration to assay temperature) from ice to microplate wells at 15 and 25 °C with the absorbance at 540 nm being monitored as above.

To unveil the mechanisms of Cd-induced volume changes, the effects of modulators of the mitochondrial permeability transition pore (MPTP: cyclosporin A (CsA), 1 μM), mitochondrial calcium uniporter (MCU: ruthenium red, 5 μM) and mitochondrial potassium channels (mitoK_{ATP}: diazoxide, 100 μM and 5-hydroxydecanoate (5-HD), 400 μM) on 100 (Cd)- and 200

μM (Ca)-induced volume changes were assessed. Here, the modulators were added to the wells as 10 μl of stock solutions and pre-incubated with 170 μl of 1 mg/ml of mitochondrial suspension for 5 min, after which 100 μM Cd and 200 μM Ca were added as 20 μl of appropriate stock solutions. Absorbance changes at 540 nm were then monitored every 10 sec for 30 min at 15 and 25 °C.

3.3.7 Data analysis

The data were tested for normality and homogeneity of variances (Cochran C) then submitted to one or two-way analysis of variance (ANOVA) (Statistica version 5.1, Statsoft, Inc., Tulsa, OK). If the data did not pass the normality test, they were submitted to Box-Cox transformation; all data passed after transformation. An ANOVA is only slightly affected by inequality of variance using our models (equal sample sizes and all factors fixed). In these analyses, “temperature” and “treatment” or “Cd dose” were the independent variables. Significantly different means were separated using Tukey’s post hoc test at $P < 0.05$. Linear regression analysis was performed using SigmaPlot 10 (Systat Software, San Jose, CA, USA).

3.4 RESULTS

3.4.1 Mitochondrial respiration

The effects of Cd on mitochondrial respiration were different at the 3 temperatures tested as clearly demonstrated by the significant 2-way interaction terms (Table 1). As well, temperature and Cd individually significantly altered all of the mitochondrial respiration indices except that thermal sensitivity (Q_{10} coefficient) of state 3 was not affected by Cd.

Temperature alone greatly stimulated ($F_{2,72}=671$, $P < 0.0001$) state 3 respiration resulting in an overall 2.8-fold increase in respiration between 5 and 25 °C (Fig. 3.1A) in the controls. In contrast, Cd dose-dependently inhibited ($F_{5,72}=222$, $P < 0.0001$) state 3 respiration at all the

temperatures, resulting in 3, 2.5 and 3-fold reductions in respiration rates relative to the corresponding controls in the 100 μ M Cd exposure for 5, 13 and 25 $^{\circ}$ C, respectively (Fig. 3.1A). Overall, a significant interaction ($F_{10,72}=18$, $P<0.0001$) between Cd and temperature on state 3 respiration was observed. The temperature sensitivity (Q_{10} coefficient) of state 3 respiration (Fig. 3.1B) was significantly higher at the low (5-13 $^{\circ}$ C) compared with the high (13-25 $^{\circ}$ C) temperature range ($F_{1,48}=558$, $P<0.0001$). In contrast there was no significant effect of Cd on state 3 Q_{10} ($F_{5,48}=1.08$, $P=0.3847$) but a significant temperature and Cd interaction ($F_{5,48}=5.6$, $P<0.0003$) was evident.

Table 3.1: Summary of 2-way ANOVA for mitochondrial respiration indices and Cd accumulation. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100 μ M) at 5, 13 and 25 °C. Data are means \pm SEM (n = 5). Two-way ANOVA with Tukey's HSD, P<0.05

Parameter	Main Effect								
	Cadmium (Cd)			Temperature (T)			Interaction (Cd \times T)		
	df	F	P	df	F	P	df	F	P
State 3	5,72	222	<0.0001	2,72	671	<0.0001	10,72	18	<0.0001
State 3 Q ₁₀	5,48	1.08	0.3847	1,48	558	<0.0001	5,48	5.6	0.0003
State 4	5,72	114	<0.0001	2,72	2455	<0.0001	10,72	53	<0.0001
State 4 Q ₁₀	5,48	47	<0.0001	1,48	2238	<0.0001	5,48	21	<0.0001
P/O ratio	3,48	22	<0.0001	2,48	76	<0.0001	6,48	9	<0.0001
RCR	5,72	919	<0.0001	2,72	657	<0.0001	10,72	24	<0.0001
[Cd]	5,81	357	<0.0001	2,81	7.85	<0.001	10,81	3.6	<0.001

Figure 3.1

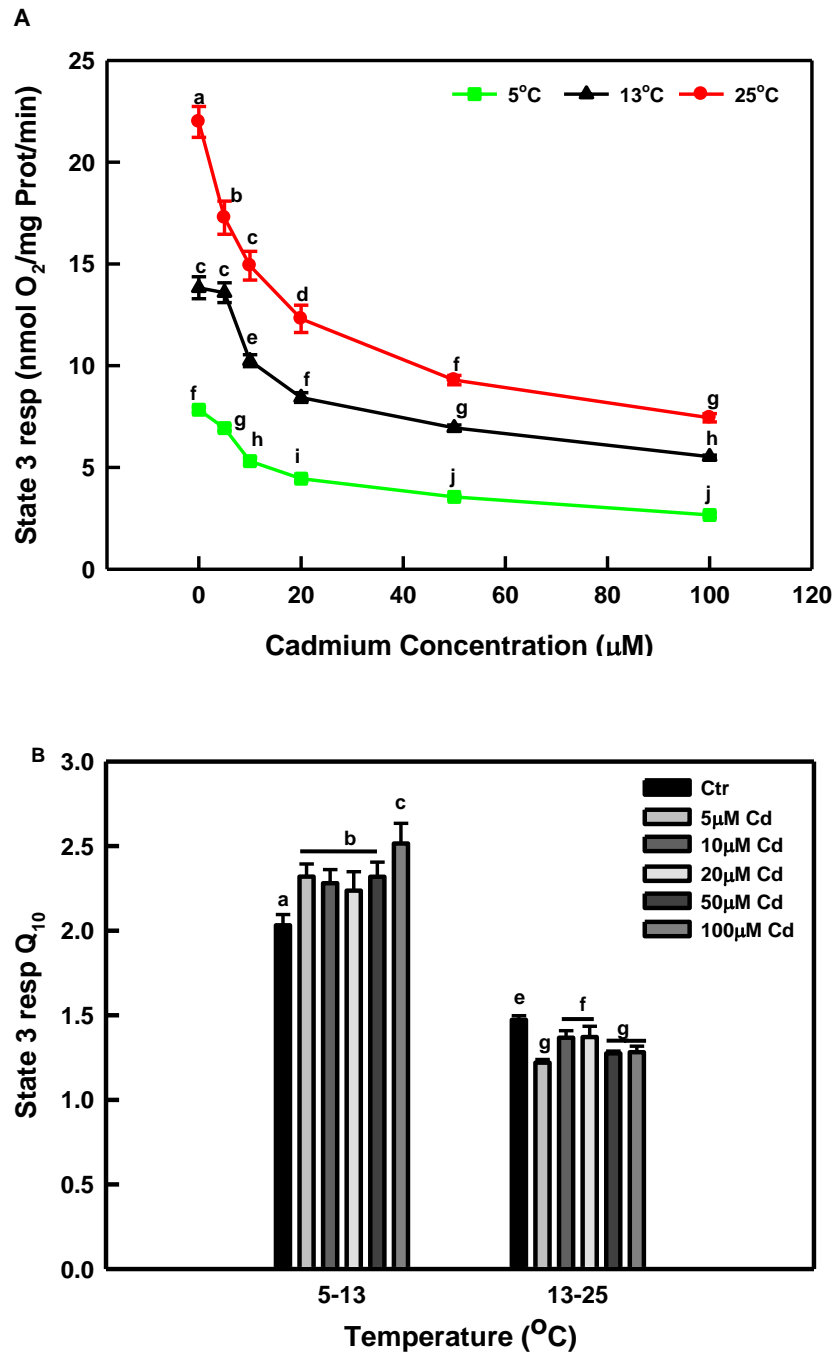


Figure 3.1: Interactive effects of temperature and Cd on (A) state 3 respiration and (B) Q_{10} of state 3. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100

μM) at 5, 13 and 25 °C. Data are means \pm SEM ($n = 5$). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

Akin to state 3, the resting (state 4) respiration increased significantly ($F_{2,72}=2455$, $P<0.0001$) with temperature, attaining a 2.3-fold increase between 5 and 25 °C in the controls (Fig. 3.2A). Cadmium ($F_{5,72}=114$, $P<0.0001$) acted in synergy with temperature to further stimulate state 4 respiration (Fig. 3.2A) culminating in a 3.6-fold increase in state 4 between 5 and 25 °C for the 100 µM Cd exposure. Moreover, the interaction between Cd exposure and temperature on state 4 respiration was significant ($F_{10,72}=53$, $P<0.0001$). In contrast with state 3 respiration, the control Q_{10} values for state 4 respiration (Fig. 3.2B) increased ($F_{1,48}=2238$, $P<0.0001$) with temperature from 1.1 to 1.8 for the temperature ranges 5-13 and 13-25 °C, respectively. While Cd exposure did not affect state 4 thermal sensitivity over the 5-13 °C range, the Q_{10} values were increased ($F_{5,48}=47$, $P<0.0001$) by Cd doses >20 µM over the 13-25 °C range. A significant ($F_{5,48}=21$, $P<0.0001$) interaction between temperature and Cd on state 4 thermal sensitivity was observed. Similar to state 4 respiration, state 4_{ol} (Fig. 3.3), a measure of proton leak, was highly stimulated by temperature ($F_{2,72}=1826$, $P<0.0001$) and Cd exposure ($F_{5,72}=104$, $P<0.0001$) intensified this leak as verified by the significant 2-way interaction ($F_{10,72}=46$, $P<0.0001$).

The P/O ratio, which indicates mitochondrial phosphorylation efficiency, was increased ($F_{2,48}=76$, $P<0.0001$) by temperature (Table 2) and reduced ($F_{3,48}=22$, $P<0.0001$) by Cd exposure. Overall, a significant ($F_{6,48}=9$, $P<0.0001$) 2-way interaction between Cd and temperature on P/O ratio was observed. Note that P/O ratios for Cd doses >20 µM were not calculated because the transition to state 4 could not be unambiguously estimated.

Figure 3.2

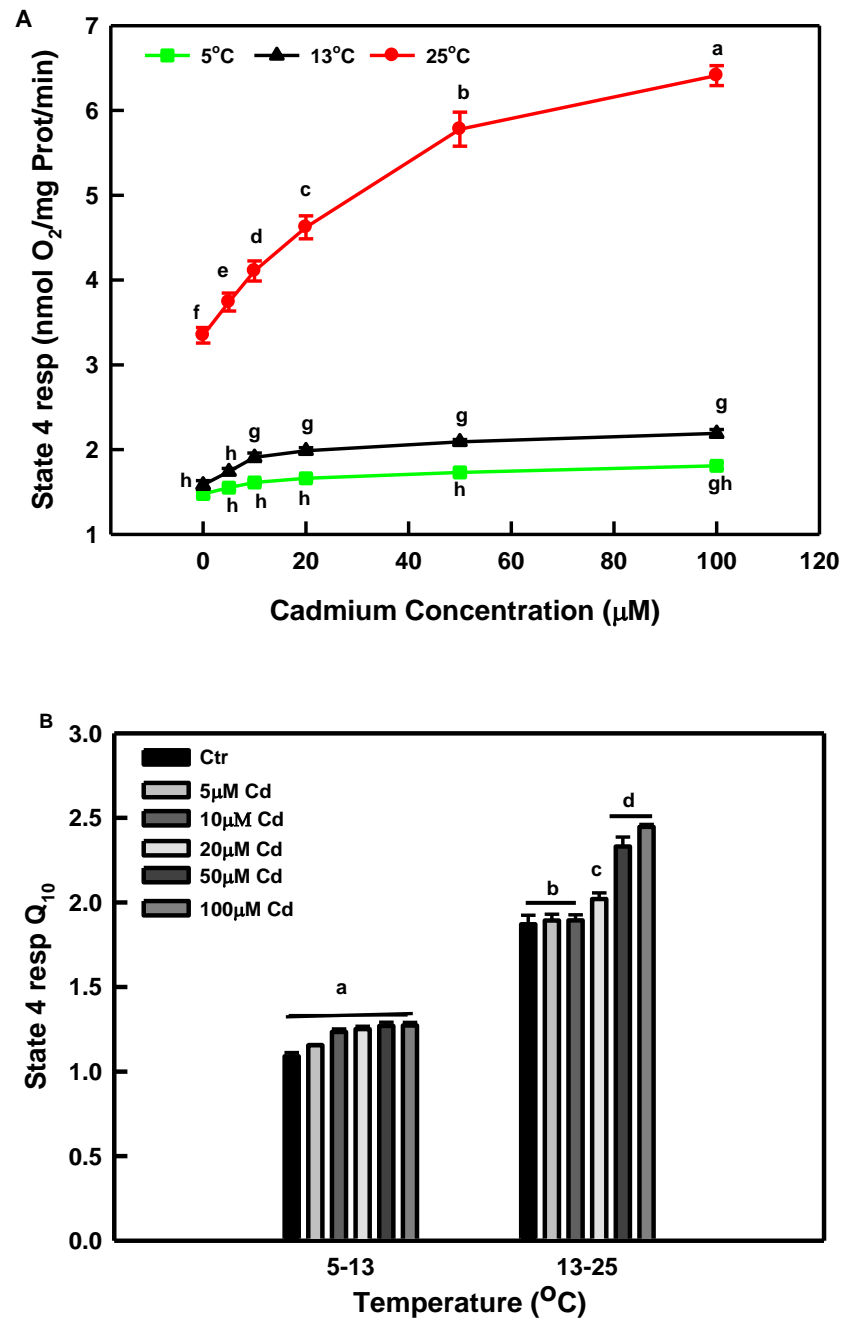


Figure 3.2: Interactive effects of temperature and Cd on (A) state 4 respiration and (B) Q_{10} of state 4. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100

μM) at 5, 13 and 25 °C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 3.3

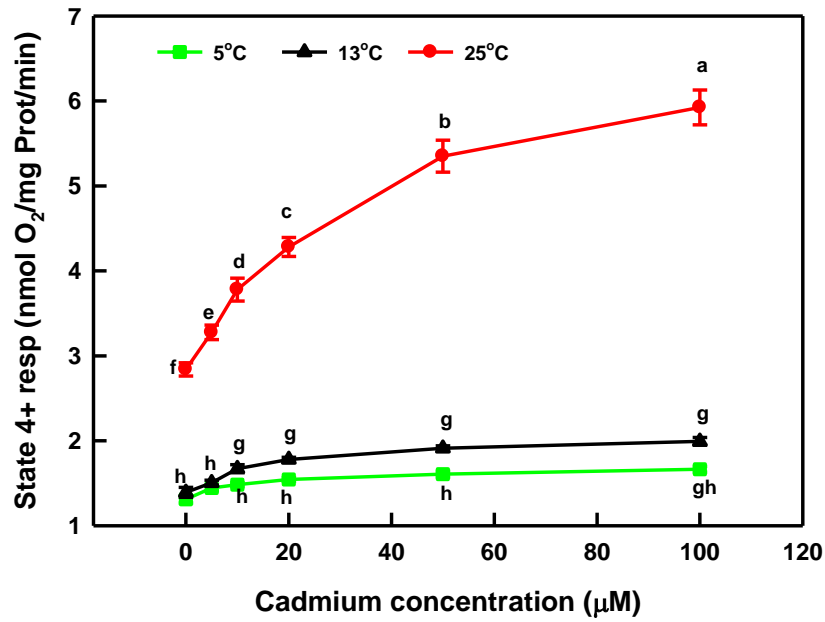


Fig 3.3: Interactive effects of temperature and Cd on state 4_{ol}. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100 μM) at 5, 13 and 25 °C. Data are means ± SEM (n = 5). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, P<0.005).

Table 3.2: Effects of Cd and temperature on phosphorylation efficiency (P/O ratio) in rainbow trout liver mitochondria. NM indicates not measured.

Cadmium (μM)	Temperature ($^{\circ}\text{C}$)		
	5	13	25
0	$2.51 \pm 0.07^{\text{cd}}$	$3.42 \pm 0.06^{\text{a}}$	$3.61 \pm 0.11^{\text{a}}$
5	$2.54 \pm 0.05^{\text{cd}}$	$3.11 \pm 0.07^{\text{b}}$	$3.50 \pm 0.14^{\text{a}}$
10	$2.43 \pm 0.12^{\text{d}}$	$3.00 \pm 0.08^{\text{b}}$	$3.29 \pm 0.07^{\text{ab}}$
20	$2.42 \pm 0.12^{\text{d}}$	$2.94 \pm 0.09^{\text{bc}}$	$2.42 \pm 0.02^{\text{d}}$
50	NM	NM	NM
100	NM	NM	NM

Table 3.2: Interaction of temperature and cadmium on P/O ratio of rainbow trout liver mitochondria. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100 μM) at 5, 13 and 25 $^{\circ}\text{C}$. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

The mitochondrial coupling efficiency (as estimated by the RCR) significantly influenced ($F_{2,72}=657$, $P<0.0001$) by temperature (Fig. 3.4) and was highest at 13 °C for all Cd concentrations. Exposure to Cd decreased ($F_{5,72}=919$, $P<0.0001$) the RCR at all temperatures, with mitochondria from fish held at high temperature (25 °C) showing the greatest reduction. The interaction between temperature and Cd on RCR was significant ($F_{10,72}=24$, $P<0.0001$).

3.4.2 Mitochondrial Cd accumulation

The amount of Cd accumulated by mitochondria increased dose-dependently ($F_{5,81}=357.75$, $P<0.0001$) with all exposures ≥ 20 μM Cd being significantly different from the control (Fig. 3.5A). Importantly, elevated temperature enhanced ($F_{2,81}=7.85$, $P<0.001$) Cd accumulation as affirmed by a significant 2-way interaction ($F_{10,81}=3.61$, $P<0.001$) between Cd and temperature. In addition, Cd accumulation was impacted by temperature as demonstrated by the clear inverse relation between state 3 (normalized to control values) and log mitochondrial Cd concentration with R^2 values of 0.93, 0.82, and 0.82 for 5, 13 and 25 °C respectively (Fig. 3.5B- D).

Figure 3.4

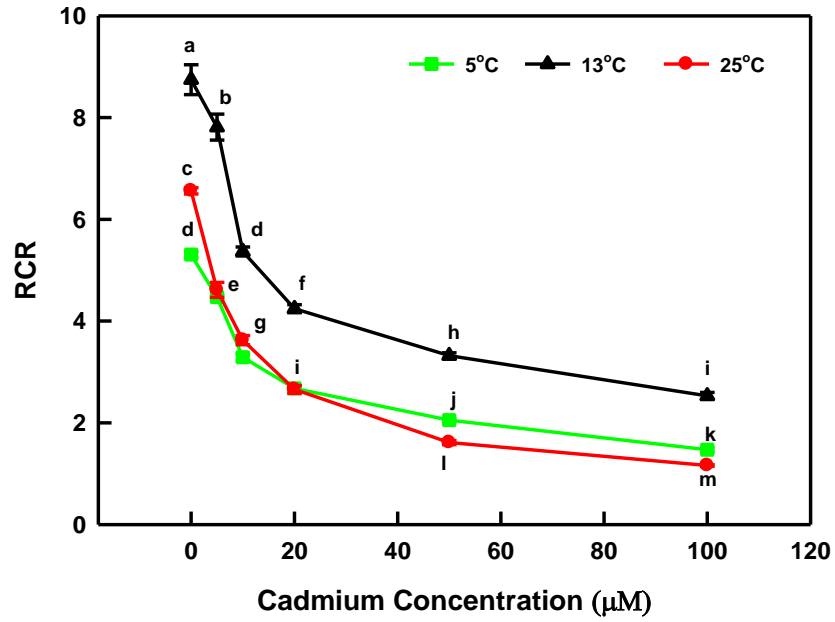


Figure 3.4: Interactive effects of temperature and Cd on RCR. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100 μM) at 5, 13 and 25 $^{\circ}\text{C}$. Data are means \pm SEM ($n = 5$). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 3.5

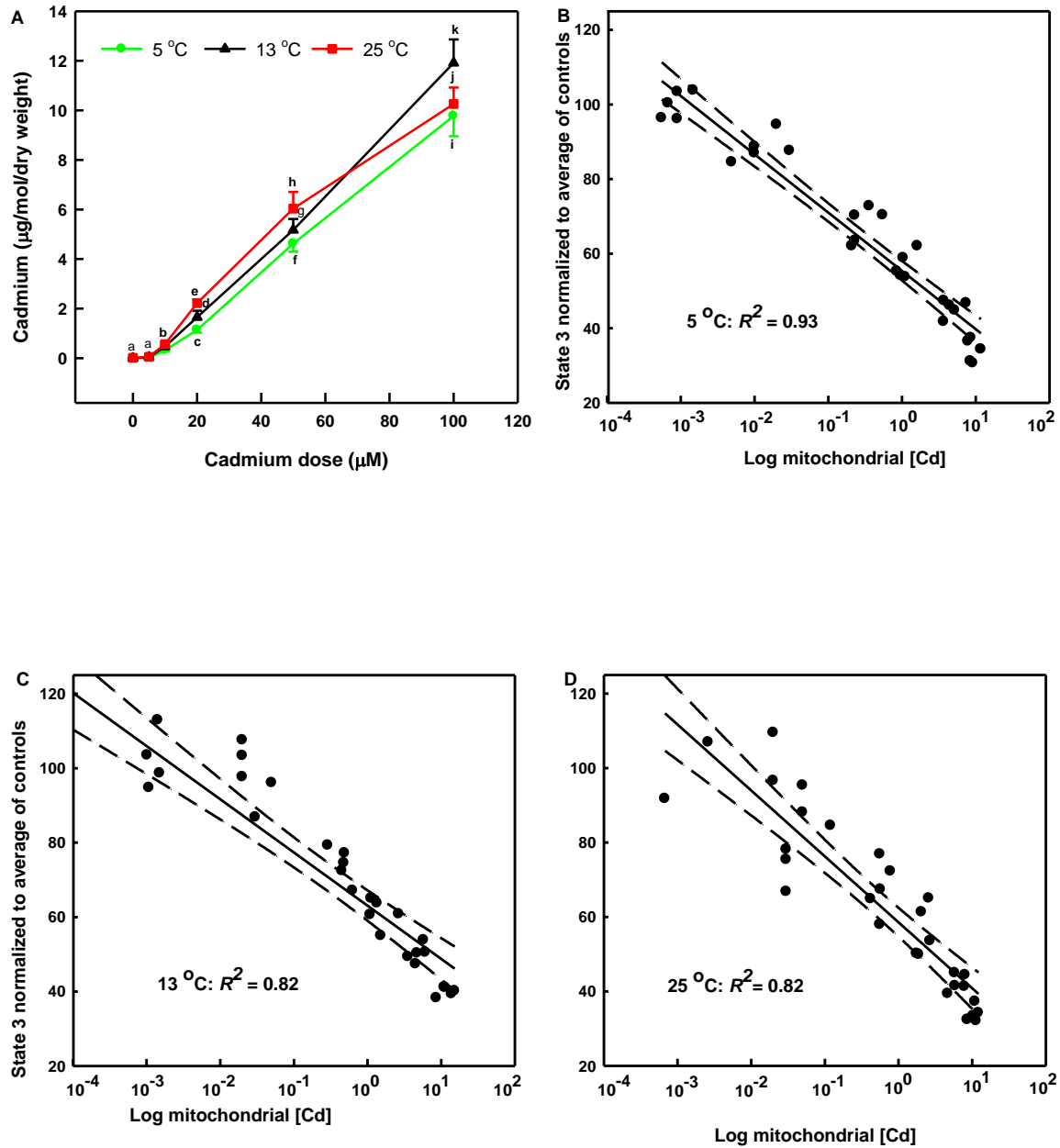


Figure 3.5: Interactive effects of temperature and Cd on (A) metal accumulation, and regression of state 3 and Cd accumulation at (B) 5 °C, (C) 13 °C and (D) 25 °C. Mitochondria isolated from each fish were exposed to Cd (0, 5, 10, 20, 50 and 100 µM) at 5, 13 and 25 °C.

Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

3.4.3 Mitochondrial volume

Cadmium at concentrations $\geq 50 \mu\text{M}$ initially caused the mitochondria to contract before inducing swelling (Fig. 3.6A and B) with an overall significant treatment effect at both 15°C (Fig. 3.6C: $F_{4,20}=11.51$, $P<0.001$) and 25°C (Fig. 3.6D: $F_{4,20}=27.59$, $P<0.0001$). Although the amplitude of swelling achieved after 30 min was not different from that in the controls for all the Cd doses. As expected, the positive control (Ca, $200 \mu\text{M}$) caused highly significant swelling, and unlike that caused by Cd, was not associated with an initial contraction phase. Moreover, temperature had no effect on the pattern (Fig. 3.6A vs 3.6B) or amplitude (Fig. 3.6C vs. 3.6D) of swelling. To test if the early contraction caused by high Cd doses was due to a chemical or physical interaction between Cd and buffer constituents, $100 \mu\text{M}$ Cd was added to the swelling buffer alone and absorbance monitored for 30 min; there were no changes in absorbance (Fig. 3.7). Finally, temperature shock resulted in greater volume changes at both 15°C ($F_{4,20}=58.04$, $P<0.00001$) and 25°C ($F_{4,20}=38.47$, $P<0.00001$) (Fig. 3.8) compared with mitochondria equilibrated to test temperature prior to the swelling assay (Fig. 3.6). Moreover, temperature-shocked mitochondria exhibited complex volume changes with two phases of contraction and swelling. Interestingly, the low dose ($5 \mu\text{M}$) of Cd reduced the amplitude of spontaneous swelling. Lastly, while Ca-induced swelling reached steady state in 4-5 min swelling evoked by Cd did not level out even after 30 min.

The prediction that Cd-induced mitochondrial volume changes are mediated by $\text{mitoK}_{\text{ATP}}$, MPTP and MCU were tested using specific modulators of these mitochondrial membrane pathways. We found that the $\text{MitoK}_{\text{ATP}}$ blocker, 5-HD, reduced Ca-induced swelling but had no effect on volume changes associated with Cd (Fig. 3.9A and B). However, a significant treatment effect on mitochondrial volume at 15°C (Fig. 3.9C: $F_{5,24}=228.29$, $P<0.00001$) and 25°C

(Fig 3.9D: $F_{5,24}=29.8$, $P<0.00001$) °C was observed. Diazoxide, a mitoK_{ATP} agonist, had no effect on both Ca- and Cd-induced volume changes (Fig. 3.10A-D), however, there were overall significant treatment effects on the volume changes both at 15 ($F_{5,24}=46.78$, $P<0.00001$) and 25 ($F_{5,24}=33.40$, $P<0.00001$) °C (Fig 3.10C and D).

With regard to the role of MPTP, CsA, an inhibitor of MPTP, reduced Ca-induced swelling but did not alter Cd-induced volume changes (Fig. 3.11A-D). A significant treatment effect was observed for tests done at both 15 ($F_{5,24}=31.98$, $P<0.00001$) and 25 ($F_{5,24}=18.05$, $P<0.00001$) °C (Fig. 3.11C and D). Lastly, ruthenium red, an MCU blocker, completely blocked Ca-induced swelling and abolished the Cd-induced contraction and spontaneous (control) swelling at both 15 and 25 °C (Fig 3.12A-D). Significant treatment effects were observed at both 15 °C ($F_{5,24}=74.58$, $P<0.00001$) and 25 °C ($F_{5,24}=46.94$, $P<0.00001$). Ruthenium red (Fig. 3.12A), and to some extent CsA (Fig. 3.11A), altered the kinetics of Cd-induced swelling at 15 °C.

Figure 3.6

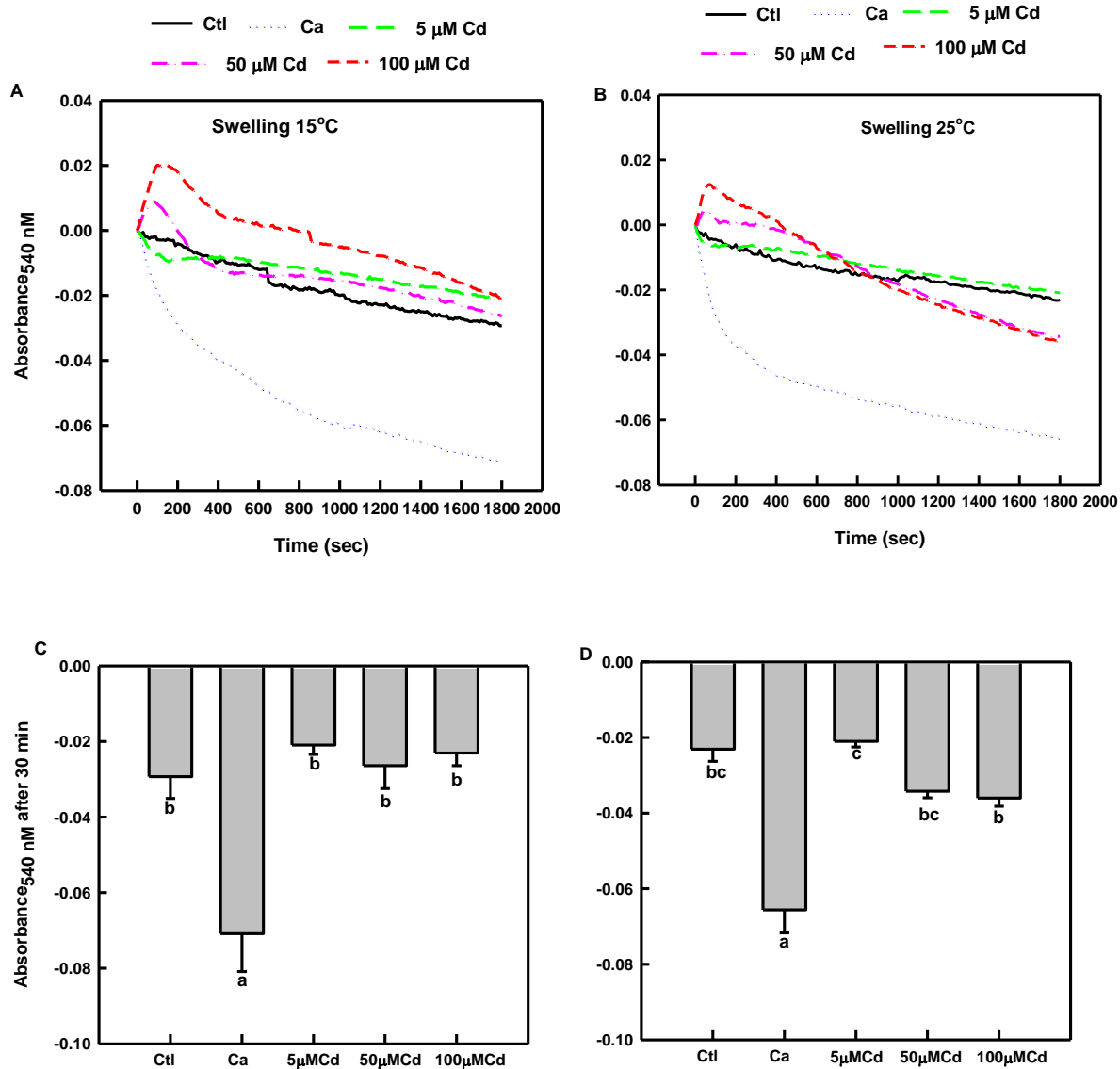


Figure 3.6: Dose response of Cd on mitochondrial volume changes. Mitochondrial suspensions were exposed to Cd (0, 5, 50 and 100 μ M) and 200 μ M Ca (positive control) and swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at (A) 15 $^{\circ}$ C and (B) 25 $^{\circ}$ C. The means \pm SEM (n = 5) amplitude of swelling after 30 min are shown in c (15 $^{\circ}$ C) and d (25 $^{\circ}$ C). Points with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 3.7

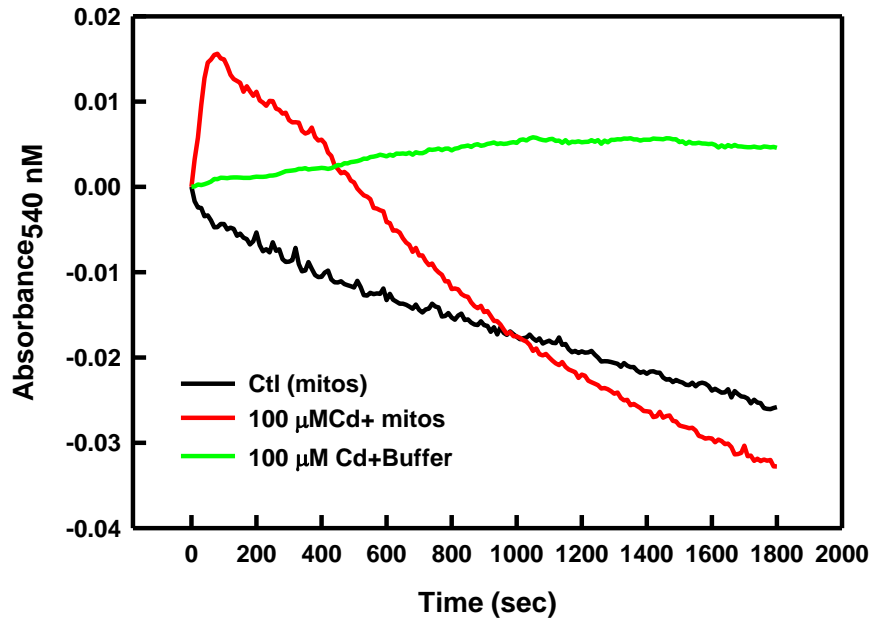


Fig 3.7: The effects of Cd induced mitochondrial volume changes on buffer and mitochondrial suspension. Mitochondrial suspension were exposed to (0 and 100 μ M) Cd and Buffer (without mitos) to 100 μ M Cd. Swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at 25 $^{\circ}$ C . Data are means \pm SEM (n = 5).

Figure 3.8

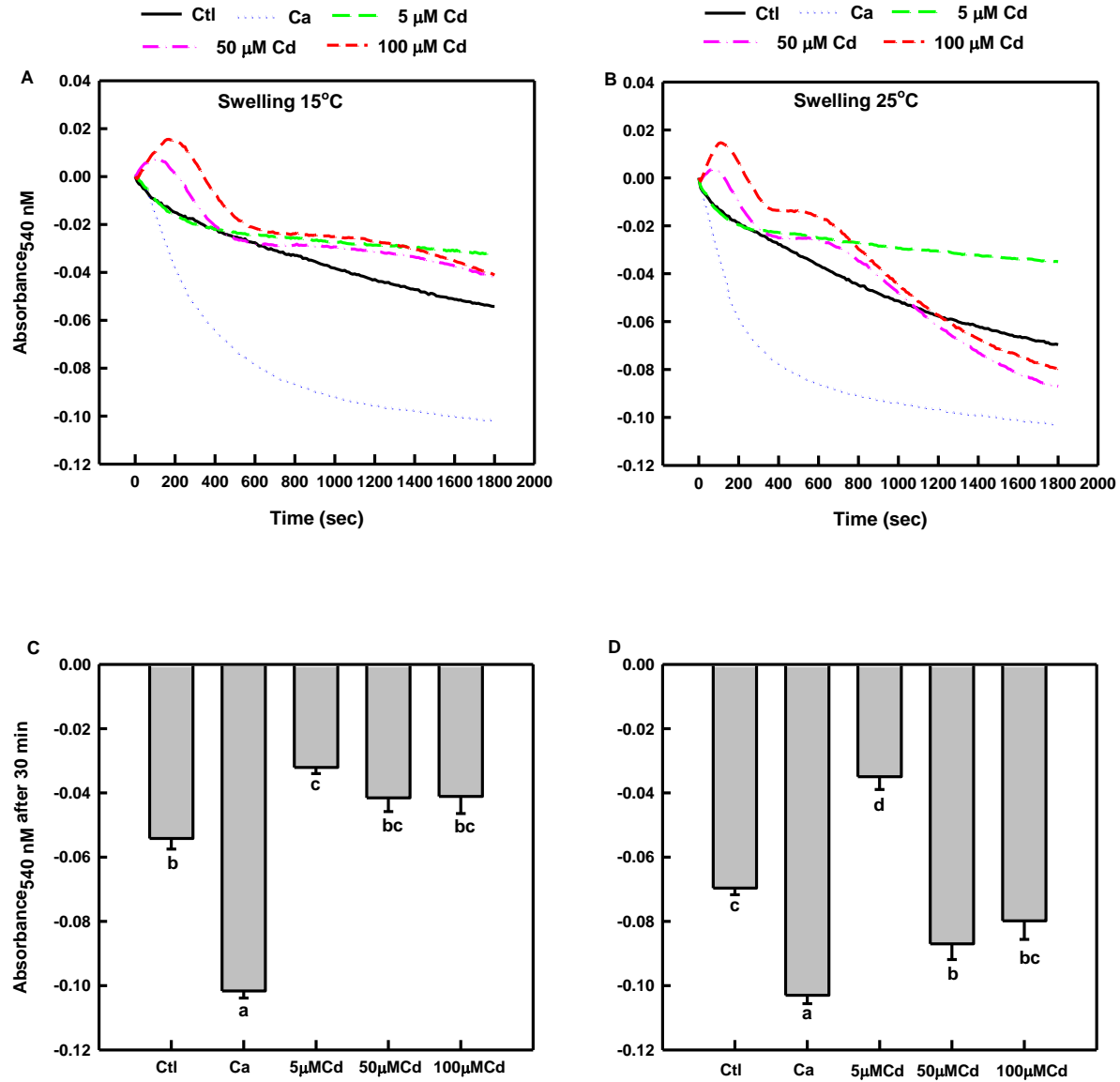


Figure 3.8: The effect of temperature shock (ice \rightarrow 15 or 25°C) on Cd-induced mitochondrial volume changes. Mitochondrial suspensions were exposed to Cd (0, 5, 50 and 100 μ M) and 200 μ M Ca (positive control) and swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at (A) 15 °C and (B) 25 °C. The means \pm SEM (n = 5) amplitude of swelling after 30 min are shown in c (15 °C) and d (25 °C). Points with different

letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 3.9

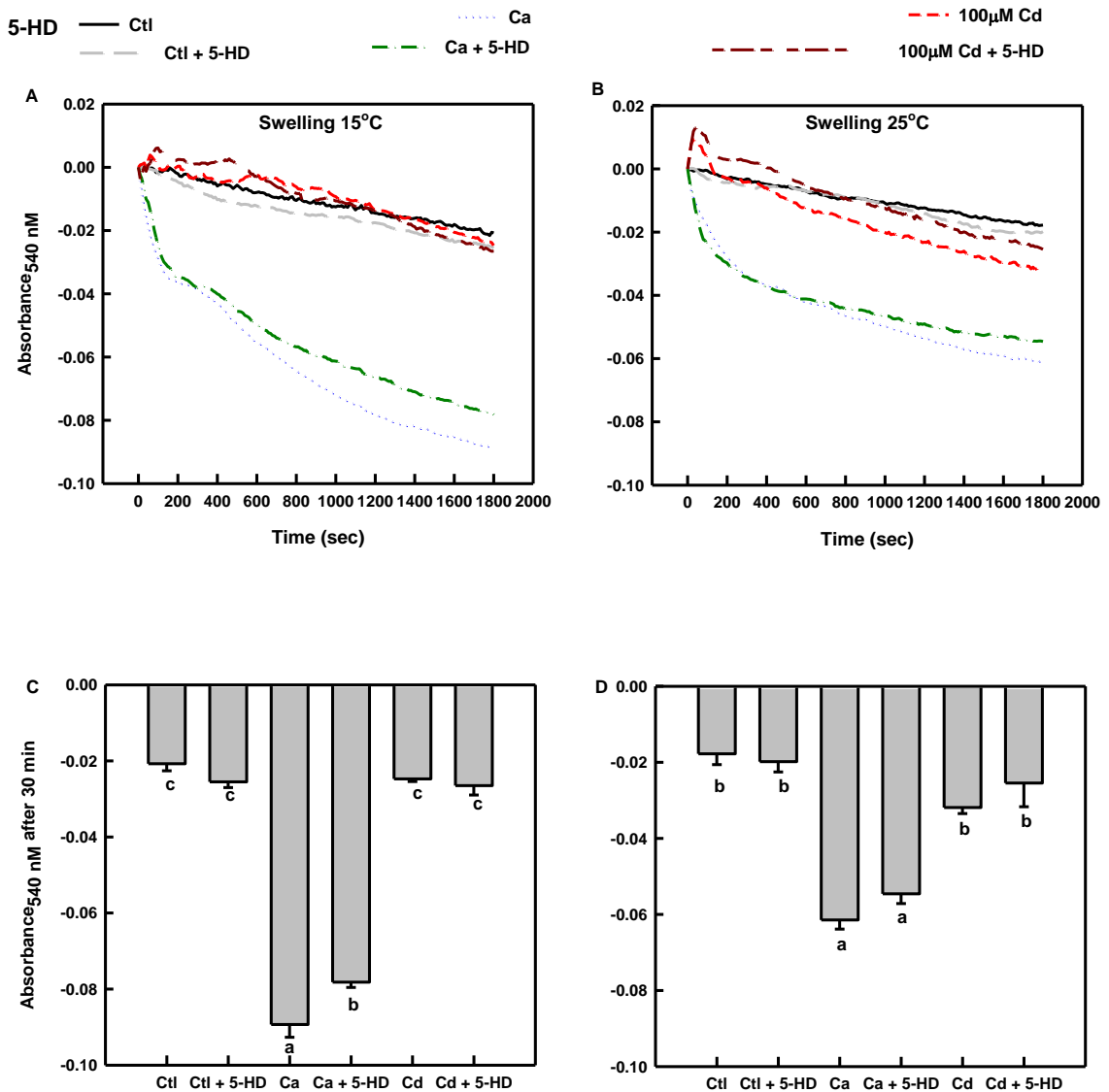


Figure 3.9: The effect of 5-hydroxydecanoate on Cd-induced mitochondrial volume changes. Mitochondrial suspensions were exposed to Cd (0 and 100 μ M) and 200 μ M Ca and swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at (A) 15 °C and (B) 25 °C. The means \pm SEM (n = 5) amplitude of swelling after 30 min are shown in c (15 °C) and d (25 °C). Points with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, P<0.05).

Figure 3.10

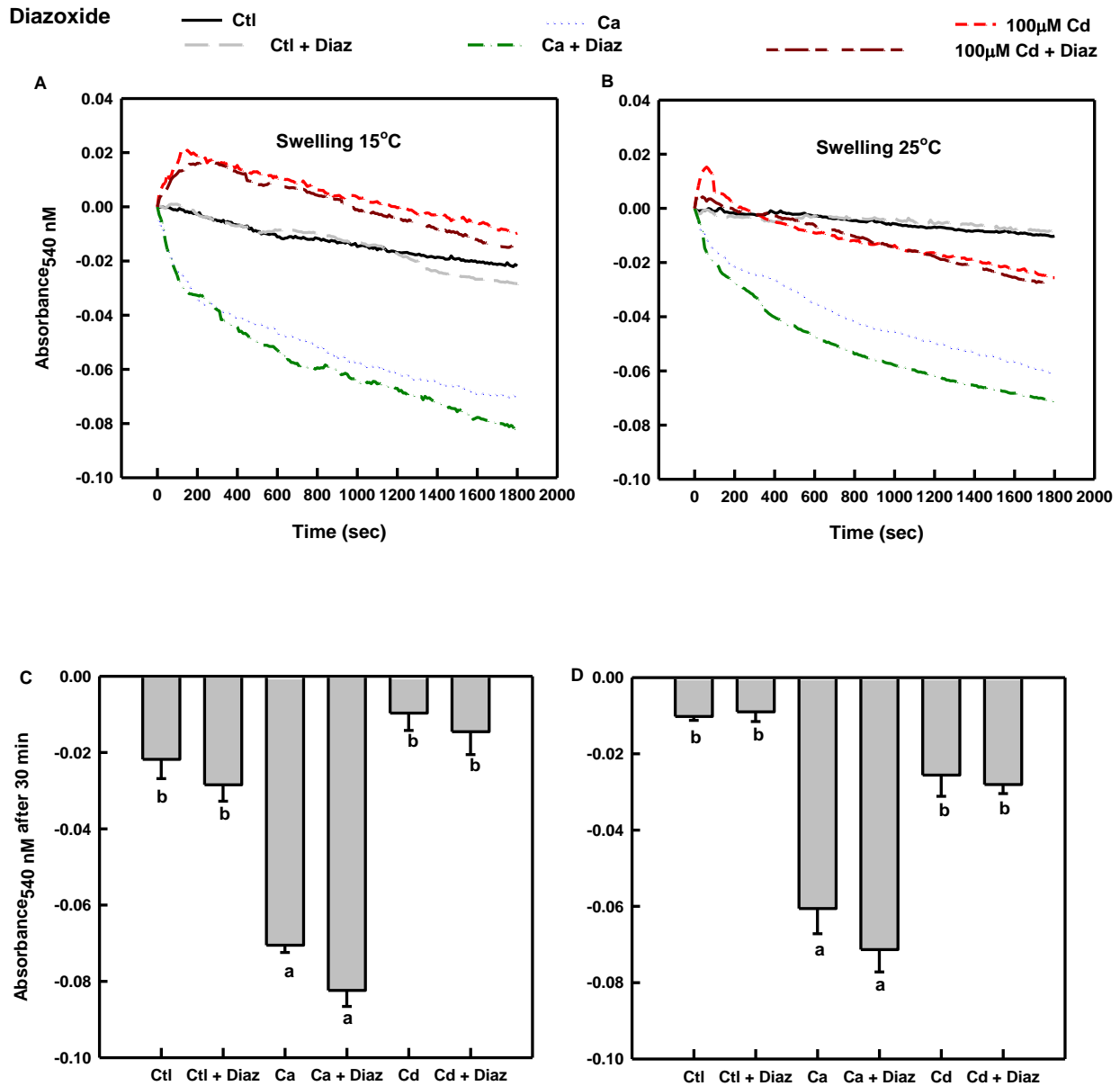


Figure 3.10: The effect of diazoxide on Cd-induced mitochondrial volume changes.

Mitochondrial suspensions were exposed to Cd (0 and 100 µM) and 200 µM Ca and swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at (A) 15 °C and (B) 25 °C. The means \pm SEM (n = 5) amplitude of swelling after 30 min are shown in C

(15 °C) and D (25 °C). Points with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 3.11

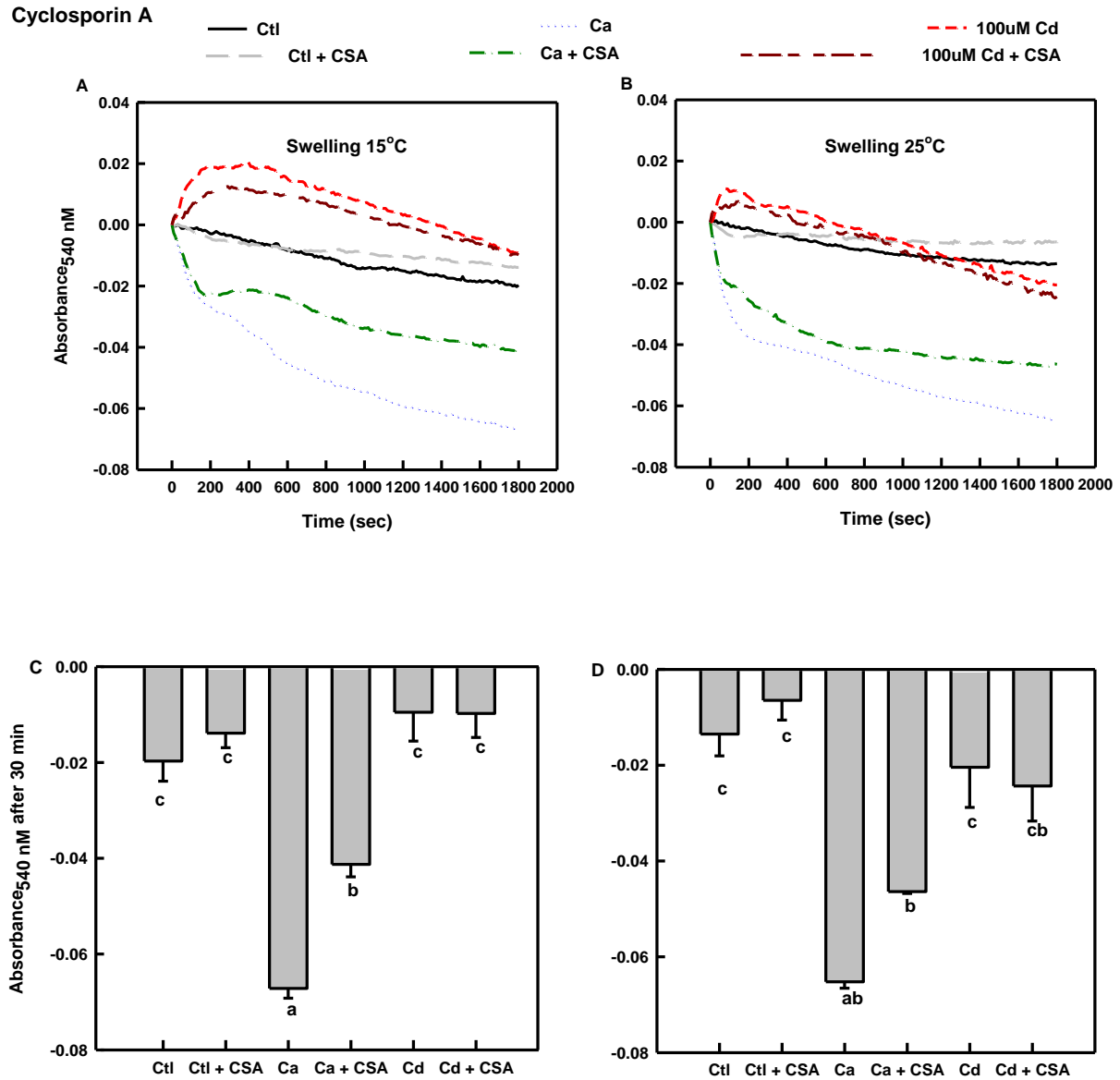


Figure 3.11: The effect of cyclosporin A on Cd-induced mitochondrial volume changes.

Mitochondrial suspensions were exposed to Cd (0 and 100 μM) and 200 μM Ca and swelling kinetics were monitored every 10 sec for 30 min as 540 nm absorbance changes at (A) 15 °C and (B) 25 °C. The means ± SEM (n = 5) amplitude of swelling after 30 min are shown in C

(15 °C) and D (25 °C). Points with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Ruthenium Red

— Ctl Ca - - - 100uM Cd - . - 100uM Cd + RR

A Swelling 15°C

B Swelling 25°C

C Absorbance₅₄₀ nM after 30 min

D

Time (sec)

Time (sec)

Ctl Ctl + RR Ca Ca + RR Cd Cd + RR

Ctl Ctl + RR Ca Ca + RR Cd Cd + RR

105

3.5 DISCUSSION

The co-occurrence of fluctuating temperatures and elevated metals concentrations in aquatic systems calls for increased understanding of their combined effects on the physiology of resident organisms in order to more accurately predict the environmental impacts. To identify the potential interactive effects of Cd and temperature on mitochondrial function and volume I assessed the effects of the metal at 3 temperatures. I demonstrate that all mitochondrial respiration indices except the thermal sensitivity of state 3 were significantly modulated by temperature and Cd exposure with significant 2-way interactions. Importantly, the inhibitory effect of Cd and its effects on volume were greater at high temperature suggesting that temperature increases as projected to occur naturally and due to global climate change may make fish energy generating systems more vulnerable to metals such as Cd.

Individually temperature had stimulatory effects on mitochondrial respiration, increasing the phosphorylating (state 3) respiration by approximately 3× between 5 and 25 °C. This finding is consistent with several other studies in a wide range endothermic and ectothermic animal species (Willis et al., 2000; Bouchard and Guderley, 2003; Birkedal and Gesser, 2003; Fangue et al., 2009; Lemieux et al., 2010; Zukiene et al., 2010; Schulte et al., 2011). At low temperatures the aerobic capacity of the mitochondria is believed to be limited by low substrate oxidation rates (Blier and Guderley, 1993) and reduced diffusion rate of substrates (Dunn 1988) that are in part caused by changes in membrane properties (Kraffe et al., 2007). In converse, raising the temperature increases the rates of substrate oxidation because of stimulation of activities of enzymes and decreased substrate binding affinities (Blier and Guderley 1993; Guderley and Johnston 1996; Somero, 2011), resulting in high respiration rate. Contrasting the stimulatory effect of temperature, Cd dose-dependently reduced state 3

respiration at all temperatures which is in agreement with several previous studies (Kessler and Brand, 1994b; Adiele et al., 2010; Kurochkin et al., 2011; Chapter 2). The mechanism through which Cd inhibits state 3 respiration include direct impairment of the activity of ETS enzymes and substrate transporters (Rikans and Yamano, 2000; Wang et al., 2004; Kurochkin et al., 2011), inhibition of substrate oxidation (Ivanina et al., 2008), increased mitochondrial membrane permeability (Belyaeva and Korotkov, 2003), and uncoupling of oxidative phosphorylation (Belyaeva et al., 2001; Adiele et al., 2010), leading to the overall inhibition of oxidative phosphorylation (Dorta et al., 2003; Wang et al., 2004). The combined exposure showed significant interaction between temperature and Cd wherein thermal stress exacerbated the inhibitory effect of Cd on state 3 mitochondrial respiration. This suggests, similar observation has been made in oysters (Sokolova, 2004), that elevation in environmental temperature would lead to greater disturbances of OXPHOS in fish.

The increase in state 4 respiration observed on raising the temperature from 5 to 25 °C is consistent with several other studies (Chamberlin, 2004; Sokolova, 2004; Fangue et al., 2009). This increase in state 4 was largely due to increase in state 4_{ol}, a measure of mitochondrial proton leak. It is likely that high temperature stimulates mechanisms that mediate proton leak such as the adenine nucleotide translocase, uncoupling proteins and other inner mitochondrial membrane proteins (Jastroch et al., 2010). Alternatively, temperature-induced increase in IMM permeability (Dahlhoff and Somero, 1993) would elevate inward flux of protons (Echtay et al., 2002; Goglia and Skulachev, 2003; Talbot et al., 2004). Interestingly, the combined exposure showed that temperature and Cd acted cooperatively in stimulating state 4/proton leak suggesting that aquatic organisms that are concurrently exposed to thermal stress and Cd would have increased mitochondrial

inefficiency. In addition to reducing ATP synthesis, high proton leak may lead to increased ROS production in inhibited mitochondria (Brookes, 2005) with damage to membrane lipids, proteins and DNA.

The Q_{10} values measured in the present study indicate that the thermal sensitivity of state 3 respiration was higher at low temperature which is in line with an earlier study by Chamberlin (2004). Interestingly, Cd exposure increased the Q_{10} values of state 3 respiration over the low temperature range. While the mechanisms underlying the high state 3 temperature coefficients at low temperature and their enhancement by Cd remain to be determined, this finding implies that mitochondria exhibit exaggerated changes in OXPHOS when faced with Cd stress at high temperature. Surprisingly unlike the maximal respiration, state 4 Q_{10} values were higher at high temperature and increased further on exposure to Cd indicating a synergistic interaction of temperature and Cd on basal mitochondrial respiration. While this is the first study to report that Cd increases state 4 thermal sensitivity, high Q_{10} values at the high temperature have been observed in earlier studies (Hulbert et al., 2002; Chamberlin, 2004). Because state 4 respiration is predominantly proton leak (Fig. 3.3) the enhancement of sensitivity of state 4 to temperature by Cd indicates decreased mitochondrial efficiency as confirmed by the lower RCR and P/O ratio in the high temperature-Cd exposure. Similar to this findings, temperature was shown to increase the sensitivity of oyster gill mitochondria to Cd (Sokolova, 2004), indicating that mitochondrial dysfunction would occur at lower concentration of Cd in the presence of thermal stress. Overall, the inhibition of state 3 coupled with preferential stimulation of 4 culminated in greatly reduced RCR in the combined exposure indicative of highly compromised OXPHOS.

The prediction that the greater mitochondrial dysfunction observed following Cd exposure at high temperature results from increased Cd accumulation was confirmed (Fig. 3.5). Notably, there were strong correlations between state 3 respiration and log mitochondrial Cd concentration at all temperatures indicating that low burdens of Cd impose big reductions in respiration while high burdens cause small reductions. Though the mechanisms of enhancement of mitochondrial Cd uptake at high temperature were not explored, it is possible that the inner mitochondrial membrane exhibited increased leakiness (Dahlhoff and Somero, 1993), thereby allowing greater influx of Cd. An alternative explanation for the increased Cd accumulation at high temperature is increased activity of mitochondrial transporters such as the MCU which my study and others (Lee et al., 2005a; Adiele et al., 2012b) have shown to be involved in Cd uptake by the mitochondria. Similar enhancement of Cd accumulation at high temperature was reported (Goncalves et al., 1988; Tessier et al., 1994; Bervoets et al., 1996; Heugens et al., 2003) suggesting that the augmenting effect of temperature on Cd accumulation cuts across different species of aquatic organisms.

The mitochondria functional integrity is guaranteed by the selective permeability of the IMM which regulates the flow of materials between the matrix and the cytosol/IMS and, importantly, allows the generation of a protonmotive force that drives ATP synthesis (Mitchell, 1966). Despite the importance of osmotic movement of water into and out of the mitochondria for mitochondrial function, the interactive effects of metals e.g., Cd and thermal stress on volume changes of these organelles have not been investigated. I therefore tested the idea that the deleterious effects of Cd and adverse temperature on mitochondria result from altered IMM permeability. By monitoring mitochondrial volume changes (i.e., changes in absorbance at 540 nm) over time as an indicator of changes in IMM permeability

I showed that Cd at doses $\geq 50 \mu\text{M}$ evoke early mitochondrial contraction before inducing swelling. Cadmium-induced swelling-contraction has been reported in mammalian mitochondrial by Lee et al. (2005a; b) who, similar to my study, monitored volume changes kinetically. A study that performed endpoint volume measurements detected only contraction in oyster (Sokolova, 2004) and suggested that oyster mitochondria do not undergo a mitochondrial permeability transition and associated swelling. The author speculated that oyster mitochondria retain sufficient proton pump activity to mitigate depolarization and swelling when exposed to Cd. More recently, Adiele et al. (2012b) observed mild swelling in an endpoint assay using rainbow trout liver mitochondria and argued that liver mitochondria in this species are recalcitrant to swelling. The present study, in particular the effect of Ca, indicates that these mitochondria are capable of substantial swelling, highlighting the importance of kinetic measurement of mitochondrial volume changes.

Mitochondria behave like osmometers capable of swelling and contraction due to water movement that accompanies the net transport of osmotically active solutes into and out of the matrix of these organelles (Beavis et al., 1985). Regarding the contraction I observed in the early phase of Cd exposure, the possibility that the change in absorbance was due to Cd or complexes formed by reaction of Cd with components of the swelling buffer were ruled out (Fig. 3.7). Furthermore, the possibility of an increase in refractive index due to Cd complexation with phosphate in the mitochondrial matrix that can be interpreted as contraction as observed following Ca exposure of brine shrimp, *Artemia franciscana* mitochondria (Menze et al., 2005; Holman and Hand, 2009), was ruled out because the contraction I observed was transient, whereas the formation of calcium phosphate in brine shrimp mitochondria was a permanent monotonic phenomenon. This suggests that the

mitochondrial contraction observed here was due to specific effects of Cd on the mechanisms or structures that regulate solute and water transport in these organelles. Therefore, a possible explanation of the volume changes is that Cd at high doses may initially activate mitochondrial K^+/H^+ exchanger leading not only to the dissipation of membrane potential but also contraction due to loss of water from the mitochondria associated with K^+ efflux. With time, however, matrix contraction would block the K^+/H^+ exchange creating an osmotic gradient that drives K^+ back into the matrix causing progressive osmotic mitochondrial swelling which may ultimately cause rupture of the outer membrane with leakage of the intermembrane space contents into the cytosol (Zoratti and Szabo, 1995; Bernardi, 1999; Halestrap et al., 2002). It is also possible that Cd activates mitochondrial aquaporins, channels that regulate the osmotic movement of water across IMM (Ferri et al., 2003), thus increasing influx of water leading to swelling. Indeed, blocking mitochondrial aquaporins with $AgNO_3$ abolished Cd-induced swelling (Lee et al., 2005a), a compelling indication that these channels are involved in the Cd-induced mitochondrial volume changes.

My mechanistic analysis revealed that contrary to my prediction, Cd-induced rainbow trout liver mitochondrial volume changes are not mediated by MPTP. Specifically, CsA, an MPTP inhibitor, did not stop or reduce Cd-induced swelling. Note that the induction of the MPTP occurs in these mitochondria (Adiele et al., 2012b) and was confirmed here by my finding that Ca-induced swelling was blocked by CsA. The inability of CsA to block mitochondrial swelling is not unique to my study having been observed in previous studies using Cd (Lee et al., 2005b), Hg (Eliseev et al., 2002) and long-chain fatty acid (Schönfeld et al., 2000) as inducers. However, Cd-induced volume changes were abolished by ruthenium red, an inhibitor of the MCU, indicating that Cd entry through this channel is an important

requirement for the observed volume changes. The involvement of MCU in Cd-induced swelling has previously been reported for rat mitochondria (Li et al., 2003; Lee et al., 2004; 2005b) although a role for the voltage-dependent anion channel (VDAC) cannot be ruled out because it is also sensitive to ruthenium red (Ryu et al., 2010). Lastly, neither diazoxide nor 5-HD had effect on Cd-induced volume changes, suggesting non-involvement of mitoK_{ATP} despite the importance of K⁺ fluxes in the regulation of mitochondrial volume (Garlid et al., 1996; Jaburek et al., 1998; Lee et al., 2005b).

The mitochondrial volume changes observed in my study, with a clear early contraction followed by two phases swelling were highly influenced by temperature. While mitochondria equilibrated to test temperature showed moderate contraction and swelling, those tested after temperature shock (abrupt transfer from ice→15 or 25 °C) had complex patterns of contraction and swelling with higher amplitudes. The complex swelling-contraction pattern is possibly associated with temperature-induced changes on mitochondrial membrane characteristics (Connell, 1961; Richardson and Tappel, 1962; Somero, 2011). Interestingly, 5 μM Cd prevented spontaneous swelling in both equilibrated and temperature-shocked mitochondria, suggesting that at low doses Cd blocks the mechanisms of solute and water movement across the inner mitochondrial membrane. Additional studies are required to unveil the mechanisms by which low Cd doses inhibit spontaneous swelling in isolated mitochondria.

In conclusion, I have shown that adverse temperature and Cd exposure interactively impair mitochondrial function and alter their volume. Whereas individually temperature stimulated both the maximal and basal/leak respirations, Cd inhibited the former while stimulating the later, leading to severe uncoupling and exacerbation of functional impairment in the

combined exposure. Interestingly, Cd increased the thermal sensitivity (Q_{10} values) of maximal respiration at low temperature and that of basal respiration at high temperature. Importantly, I show that the aggravation of Cd-induced mitotoxicity at high temperature was, at least in part, due to increased accumulation of the metal in the organelles. Additionally, my data suggest that Cd alters mitochondrial permeability leading to contraction and swelling that is aggravated by temperature change. It appears that mitochondrial volume alterations require Cd uptake via the MCU.

CHAPTER 4

HYPOXIA-REOXYGENATION DIFFERENTIALLY ALTERS THE THERMAL SENSITIVITY OF COMPLEX I BASAL AND MAXIMAL MITOCHONDRIAL OXIDATIVE CAPACITY

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Author contributions

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

4.1: ABSTRACT

Hypoxia-reoxygenation (H-R) transitions and temperature fluctuations occur frequently in biological systems and likely interact to alter cell function. To test how H-R modulates mitochondrial function at different temperatures I measured the effects of H-R on isolated fish liver mitochondrial oxidation rates over a wide temperature range (5-25 °C).

Subsequently, the mechanisms underlying H-R induced mitochondrial responses were examined. H-R inhibited the complex I (CI) maximal (state 3) and stimulated the basal (state 4) mitochondrial oxidation rates with temperature enhancing both effects. As a result, the thermal sensitivity (Q_{10}) for CI maximal respiration was reduced while that for basal respiration was increased by H-R. H-R reduced both the coupling and phosphorylation efficiencies more profoundly at high temperature suggesting that mitochondria were more resilient to H-R at low temperature. The H-R induced mitochondrial impairments were associated with increased reactive oxygen species (ROS) production and proton leak, dissipation of membrane potential, and loss of structural integrity of the organelles. Overall, my study provides insight into the mechanisms of H-R induced mitochondrial morphofunctional disruption and shows that the moderation of effects of H-R on oxidative phosphorylation by temperature depends on the functional state.

4.2 INTRODUCTION

Mitochondria produce the majority of the energy (ATP) needed for cellular function and maintenance through oxidative phosphorylation (OXPHOS) in which the transfer of electrons by the electron transport system (ETS) is coupled with transport of protons from the matrix into the intermembrane space creating a proton-motive force (membrane potential plus pH gradient) across the inner mitochondrial membrane (IMM) (Mitchell, 1966). These protons

are channeled back into the matrix via the ATP synthase to synthesize ATP from ADP and inorganic phosphate. Under stressful conditions, the ability of the mitochondria to synthesize ATP may be compromised in part by the disruption of the ETS and/or altered structural integrity with increased IMM permeability that typically manifests as changes in mitochondrial volume (Lee et al., 2005a, Orlov et al., 2013, Chapter 3). The regulation of mitochondrial volume is therefore important for ETS function and thus generation of the proton gradient for ATP synthesis.

Changes in temperature and oxygen fluctuations (hypoxia-reoxygenation, H-R) are common variables encountered by biological systems naturally or under experimental and clinical settings. Existing literature suggests that the disruption of biological systems by temperature and H-R results from altered mitochondrial function (Broderick 2006; Ivanina et al., 2012; Tissier et al., 2013; Sappal et al., 2015). For example, high temperature stress differentially increases the maximal and basal respiration rates that ultimately reduces the respiratory control ratio (RCR) in mitochondria from a variety of animal species (Zukiene et al., 2010; Galli and Richards 2012; Rodnick et al., 2014; Sappal et al., 2014b). Similarly, H-R can reduce RCR by inhibiting the ETS and/or increasing proton leak (da Silva et al., 2003; Navet et al., 2006; Hickey et al., 2012; Chapter 2). While the individual effects of temperature and hypoxia without and with reoxygenation on mitochondria are reasonably well known, it remains unclear how H-R modulates mitochondria function at different temperatures. Specifically, existing data are contradictory as to whether or not high/low temperature exacerbates/abrogates the effects of H-R (Yellon et al., 1992; Overgaard et al., 2004; Broderick, 2006; Tissier et al., 2010).

In my previous study (Chapter 2) I reported that a short period (5 min) of hypoxia followed by reoxygenation resulted in a decrease in state 3 and an increase in state 4 respiration. In the present study I reasoned that H-R damages one or more of the ETS complexes and I tested some predictions focused on this hypothesis. First, I predicted that the response would scale with the duration of the hypoxia. Second, I predicted that the response would be temperature sensitive but that state 3 alteration would be more sensitive to temperature change than state 4 alteration because the former reflects an active process (pumping protons) whereas the latter is a passive process (protons leaking back through the inner mitochondrial membrane). Third, I predicted that if H-R damaged all ETS proteins respiration rate in state 3 would ultimately drop to zero. Fourth, I predicted that if the decrease in state 3 respiration was due to damage to proton pumping proteins it would be associated with a decrease in membrane potential and if it was associated with damage to ATP synthase then it would result in a decrease in the P/O ratio. Finally, in as much as many mitochondrial insults result in disruption of mitochondrial membrane and matrix integrity, I predicted that H-R would be associated with changes in mitochondrial structure.

4.3 MATERIALS AND METHODS

4.3.1 Ethics

The procedures that experimental animals were subjected to were approved by the University of Prince Edward Island Animal Care Committee (Protocol # 11-034) in accordance with the Canadian Council on Animal Care.

4.3.2 Fish

Female rainbow trout (*Oncorhynchus mykiss*) were obtained from Ocean Farms Inc., Brookvale, PE, and kept in a 400-l tank supplied with flow-through aerated well water at the

Atlantic Veterinary College Aquatic Facility. Water temperature and pH were 10 ± 1 °C and 7.7, respectively. The fish were fed at 1% body weight daily with commercial trout chow (Corey Feed Mills, Fredericton, NB). At sampling the fish were about 6 months old and weighed 188 ± 17 g.

4.3.3 Mitochondrial isolation

To isolate mitochondria, fish were randomly sampled from the tank, stunned with a blow to the head, decapitated and immediately dissected to remove the liver. Mitochondria were isolated according to the method described in chapter 2. Briefly, the livers were rinsed with mitochondrial isolation buffer (MIB: 250 mM sucrose, 10 mM Tris-HCl, 10 mM KH_2PO_4 , 0.5 mM EGTA, 1 mg/ml BSA [free fatty acid], 2 µg/ml aprotinin, pH 7.3), blotted dry and weighed. Livers were diced and homogenized in 1:3 (weight to volume) ratio of liver to MIB in a 10-mls Potter-Elvehjem homogenizer (Cole Parmer, Anjou, QC). Three passes of the pestle mounted on a hand-held drill (MAS 2BB, Mastercraft Canada, Toronto, ON) running at 200 rpm were optimal for rainbow trout liver mitochondria isolation. The homogenate was then centrifuged at $800 \times g$ for 15 min at 4 °C. The supernatant was collected, centrifuged at $13,000 \times g$ for 10 min at 4 °C and the pellet (mitochondria) was washed twice by re-suspending in MIB and centrifuging at $11,000 \times g$ for 10 min at 4 °C. Finally, the mitochondrial pellet was re-suspended in a 1:3 (weight to volume) ratio of mitochondrial respiration buffer [MRB: 10 mM Tris-HCl, 25 mM KH_2PO_4 , 100 mM KCl, 1 mg/ml BSA (fatty acid free), 2 µg/ml aprotinin, pH 7.3]. Mitochondrial protein concentration was measured by spectrophotometry (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) according to Bradford (1976) with BSA as the standard.

4.3.4 Mitochondrial respiration

Rainbow trout can experience temperatures ranging from 4 to 27 °C in their environment (Threader and Houston, 1983; Taylor et al., 1996; Aho and Vornanen, 2001). To capture this temperature range normoxic mitochondrial oxidation rates were assessed at 5, 13 and 25 °C using Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) in 1.5 ml cuvettes after a two-point calibration at 0 and 100% air saturation at the ambient atmospheric pressure. Temperature during the assays was maintained at the desired level using a recirculating water-bath (Haake, Karlsruhe, Germany). Although the system was equilibrated with atmospheric air initially and after reoxygenation, the concentration of O₂ was different at the different test temperatures because the solubility is different. The dissolved O₂ concentration was about 11.9, 9.9, and 7.8 mg/l at 5, 13 and 25 °C, respectively, when equilibrated with air. After the calibration, 1.45 ml of MRB and 100 µl of mitochondrial suspension containing 2.0-2.9 mg of protein were loaded into the cuvettes and continuously stirred. To spark the Krebs cycle, 5 mM malate was added and respiration was supported with saturating concentration (5 mM) of glutamate, a complex I substrate. The addition of 250 µM ADP initiated the maximal CI respiration (state 3) which changed to basal respiration (state 4) upon depletion of the ADP. To measure respiration after hypoxic-reoxygenation (H-R) the method in chapter 2 was used. Briefly, following normoxic respiration above, the MRB was made hypoxic by bubbling nitrogen into the cuvettes for about 2 min with the stirrer on until the O₂ concentration reached 0.1 mg/l, depleting the P_{O₂} to 1.3, 1.6, and 2.0 torr at 5, 13, and 25 °C, respectively. This P_{O₂} is below the 2.25-3.75 torr intracellular level of O₂ typically encountered by rat liver mitochondria *in vivo* (Gnaiger and Kuznetsov, 2002). Then the bubbling tube was removed, the cuvettes were sealed and mitochondria were maintained under hypoxic conditions for the required duration. At the end of the hypoxic period, the

cuvettes were opened and the contents were fully re-oxygenated to > 95% saturation with no bubbling but with the stirrer still on. Reoxygenation usually took 10 to 20 min. Thereafter ADP was added and a second set of respiration measurements post H-R was made in a similar manner as for normoxic conditions described above. The difference between the first and second set of respiration parameters represented the effects of H-R (exemplary trace shown in supplementary material, Fig. 4.1). To determine the interactions of temperature and H-R on mitochondrial oxidation rates, isolated mitochondria were tested at 5, 13 and 25 °C using (0, 5, 15 and 30 min) hypoxic conditions (n = 5 fish for each of the 12 treatments: 3 temperatures and 4 hypoxia durations). LabPro data acquisition software (Qubit Systems) was used to record and analyze all the O₂ consumption data. The phosphorylation efficiency (P/O ratio: ADP used/oxygen consumed) and the respiratory control ratio (RCR: ratio of state 3 to state 4) were calculated according to Estabrook (1967) and Chance and Williams (1955), respectively. The Q₁₀ values for state 3 and 4 respiration rates were calculated for the temperature ranges 5-13 and 13-25 °C using $Q_{10} = (R_2/R_1)^{[10/(T_2-T_1)]}$, where R₂ and R₁ represent mitochondrial oxygen consumption rates at two temperatures, T₂ and T₁, and where T₂>T₁.

I carried out a control experiment to test for the effect of bubbling in and of itself. In this case I bubbled with air rather than nitrogen but for the same length of time as for the H-R trails, 2 min. This test showed that there was no effect of bubbling with air on respiration rate during state 3 (Student's t-test, t = -0.56, p = 0.59) and state 4 (t = 0.00, p = 0.99), or RCR (t = -0.73, p = 0.50).

For all of the trials used to measure MMP, H₂O₂, and structural changes (transmission electron microscopy), the mitochondria were isolated in the same way, incubated and treated

the same way in the same cuvettes and the same solutions as they were for the respiration experiments. Substrates were not added in the cuvettes for MMP and H_2O_2 but were used in the experiments. ADP was not used in MMP, H_2O_2 and structural changes experiments. In all the experiments (MMP, H_2O_2 and structural changes) the mitochondria were subjected to the same normoxic or H-R protocols.

Figure 4.1

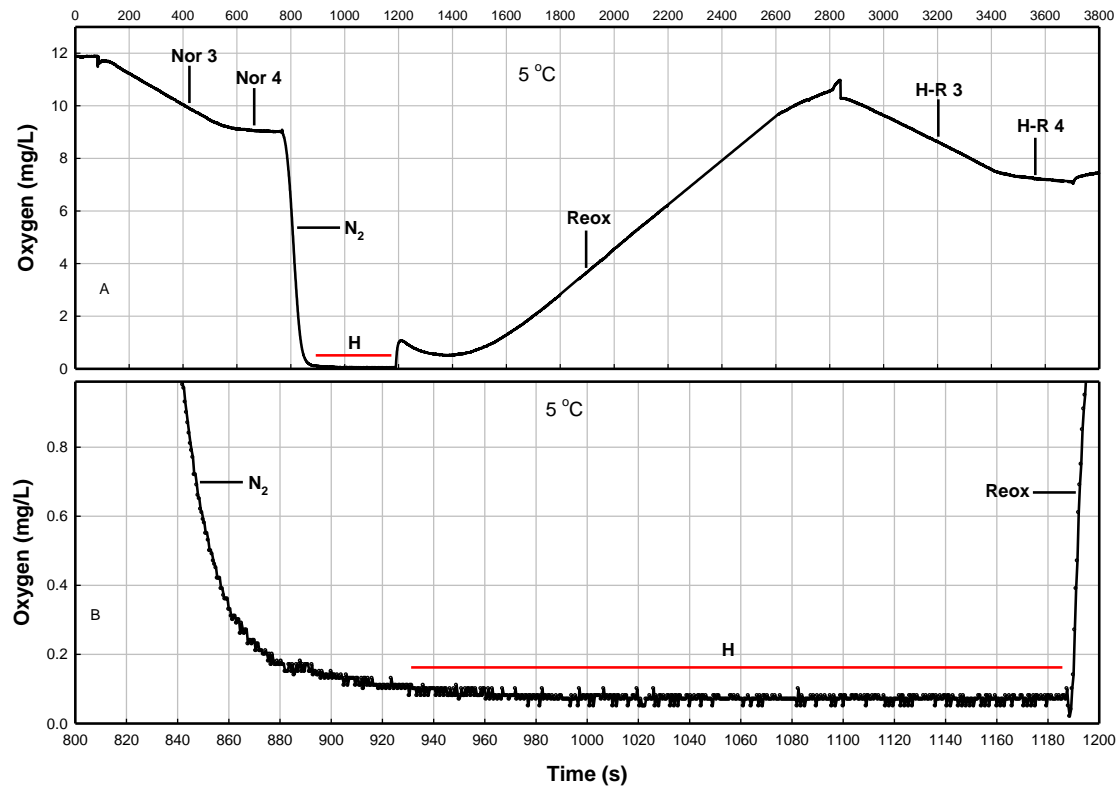


Figure 4.1: Exemplary trace showing hypoxia-reoxygenation (H-R) treatment and measurement of respiration rates in rainbow trout liver mitochondria. In this tracing obtained at 5 °C, the initial O₂ concentration was 11.9 mg/L when the respiration buffer was equilibrated with air. A: entire tracing. B: hypoxic zone of the trace revealing the O₂ concentration attained after bubbling the respiration buffer with N₂. Nor 3: normoxic state 3 respiration; Nor 4: normoxic state 4; N₂: nitrogen bubbling; H: hypoxia treatment; Reox: reoxygenation; H-R 3: post H-R state 3 respiration; H-R 4: post H-R state 4 respiration.

4.3.5 Mitochondrial membrane potential (MMP)

MMP changes under normoxia and after H-R were measured fluorimetrically (SynergyTM HT BioTek, US) using Rhodamine (Rh) 123 (excitation 485 nm; emission 586 nm) as described (Sappal et al., 2014a). Briefly, 1 mg/ml protein of normoxic and H-R treated mitochondria in MRB were added to microplate wells in duplicate. Thereafter, 5 μ M Rh123 was loaded into wells and the mitochondria were allowed to equilibrate to assay conditions for 15 min and an initial reading was taken. The mitochondria were then energized with 5 mM each of malate and glutamate then incubated for 5 min and a second reading was taken. For positive control, 0.5 μ M FCCP was added to another duplicate set of wells containing energized mitochondria to dissipate the membrane potential. An additional experiment tested the idea that vitamin E would protect against H-R induced dissipation of MMP by incubating the mitochondria for 5 min with vitamin E (20 μ M) under normoxia and after 15 min of H-R. Overall in this assay, a decrease in Rh123 fluorescence indicates a surge in MMP while an increase indicates its dissipation.

4.3.6 H₂O₂ production

Mitochondrial H₂O₂ production under normoxia and after H-R was determined fluorimetrically (SynergyTM HT) using Amplex red-horseradish peroxidase assay as recently described for rainbow trout liver mitochondria (Sharaf et al., 2015). Briefly, normoxic and H-R-treated mitochondria were resuspended as 1 mg/ml protein in MRB. Thereafter 10 μ l of 20 U/ml horseradish peroxidase and 5 μ l each of 200 mM of malate and glutamate were loaded to each microplate well followed by 170 μ l of 1 mg/ml normoxic or H-R-treated mitochondrial suspensions. In a different duplicate set of wells, 170 μ l of mitochondrial suspension and 10 μ l of 0.1 mg/ml antimycin A, complex III (CIII) Q_i site inhibitor to boost

H₂O₂ production, were added. To all the wells 10 µl of 1 mM Amplex red was added bringing the total assay volume to 200 µl, and the microplate was incubated in the dark at 20 °C for 30 min. Thereafter fluorescence was read at 15 min intervals for 30 min following excitation at 530 nm and emission at 590 nm. Known concentrations of H₂O₂ (0-5 µM) added to the assay buffer without mitochondria were used to generate a standard curve during each run for converting fluorescence data to actual H₂O₂ concentrations. Additionally, background fluorescence of assay components without mitochondria was determined during each run and subtracted from all fluorescence readings.

4.3.7 Mitochondrial structure

Mitochondrial ultrastructural changes were assessed in normoxic or H-R-treated mitochondria by transmission electron microscopy (TEM). Briefly, energized mitochondria were suspended in the cuvettes for 30 min under normoxic or H-R conditions. After pelleting by centrifugation at $10000 \times g$ for 5 min, the mitochondria were fixed with 1:1 volume of 6% glutaraldehyde in MRB at 4 °C overnight, washed with 0.1 M phosphate buffer and post-fixed in 1% OsO₄ for 1 h at room temperature. Subsequently the mitochondrial pellets were embedded in low melting point agar and dehydrated in a graded series (50-100%) of ethanol. The dehydrated pellets were cleared in propylene oxide, infiltrated with Epon and propylene oxide (sequentially in a ratio of 1:1 and 2:1) and embedded in 100% Epon resin. Thick (500 µm) and thin (80 nm) sections were obtained using an ultramicrotome (Reichert-Jung Ultracut E, Leica Microsystems, Richmond Hill, Canada). The thick sections were stained with 1% toluidine blue while thin sections were stained with uranyl acetate and Sato lead. The thin sections were then viewed at 80 kV using TEM (Hitachi BioTEM 7500; Nissei-Sangyo) and AMT XR40 digital camera (Advanced Microscopy Techniques, Danvers, MA,

USA) was used to photograph the mitochondria. Mitochondrial diameter was measured in five random fields per experimental group at 15000 \times . A total of 10 mitochondria in each of 5 fields ($n = 50$) for each treatment were measured to obtain the mean organelle diameter.

4.3.8 Data analysis

Data were tested for normality of distribution (chi-square test) and homogeneity of variances (Cochran C), before submission to one or two-way analysis of variance (ANOVA) (Statistica version 5.1, Statsoft, Inc., Tulsa, OK). If the data did not pass the normality test, they were submitted to Box-Cox transformation; all data passed after transformation. An ANOVA is only slightly affected by inequality of variance using our models (equal sample sizes and all factors fixed). Hypoxia and/or temperature were the independent variables and significantly different means were separated using Tukey's *post hoc* test at $P < 0.05$. Differences in diameters of control and H-R-treated mitochondria were tested using Student's t-test, $P < 0.05$. The data are reported as means \pm SEMs except the kinetics of membrane potential changes which are means without SEMs).

4.4 RESULTS

4.4.1 Effects of interactions of temperature stress and H-R on OXPHOS

Temperature and H-R individually and in combination had significant effects on all measures of mitochondrial oxidation assessed (Table 4.1). Increasing the temperature from 5 to 25 $^{\circ}\text{C}$ stimulated ($F_{2,48} = 367$, $P < 0.0001$), while H-R inhibited ($F_{3,48} = 99.5$, $P < 0.0001$) respiration rate during state 3 (hereinafter referred to as state 3) (Fig. 4.2A). Notably, the severity of H-R induced inhibition of state 3 increased with the duration of hypoxia. There was a significant ($F_{6,48} = 11.7$, $P < 0.0001$) interaction of the stress factors in which H-R reduced the stimulatory effect of temperature on state 3. The decreases in state 3 resulting from 30 min

H-R relative to normoxic controls were similar (51 to 62%) at all temperatures (Fig. 4.2A). The thermal sensitivity of state 3 (Fig. 4.2B) was altered by temperature ($F_{1,32}=4.44$, $P=0.04$) wherein the Q_{10} values for the low (5-13 °C) were higher than those for the high (13-25 °C) temperature range. H-R similarly had an overall significant inhibitory effect ($F_{3,32}=3.22$, $P=0.04$) on the thermal sensitivity because the Q_{10} values decreased with duration of hypoxia, but only in the low temperature range. A significant ($F_{3,32}=10.3$, $P<0.0001$) interaction of temperature and H-R on Q_{10} of state 3 was observed wherein the thermal sensitivity was reduced by H-R.

Temperature increased ($F_{2,48}=307$, $P<0.0001$) respiration rate during state 4 (hereinafter referred to as state 4) (Fig. 4.3A) whereas H-R stimulated ($F_{3,48}=29.9$, $P<0.0001$) state 4 and exacerbated the effects of temperature (Fig. 4.3A) as evidenced by the significant ($F_{6,48}=17.6$, $P<0.0001$) interaction. The increases in state 4 relative to normoxic controls were markedly different at different temperatures after 30 min hypoxia; the increase was only 22% at 5 °C (not statistically significant) but was 95% at 25 °C, and the differences were even greater after only 5 min H-R stress (Fig. 4.3A). Unlike state 3, the thermal sensitivity of state 4 increased with temperature ($F_{1,32}=20.2$, $P<0.0001$) and H-R ($F_{3,32}=11.6$, $P<0.0001$) (Fig. 4.3B). The effect of H-R at the high temperature range peaked after 5 min H-R and declined thereafter with increasing duration of hypoxia (Fig. 4.3B). There was a significant interaction between temperature and H-R on state 4 thermal sensitivity ($F_{3,32}=4.07$, $P=0.014$) (Fig. 4.3B) in that the state 4 Q_{10} was higher after H-R than for the normoxic control.

Table 4.1: Two-way ANOVA. Main effects and interactions of temperature and H-R on mitochondrial bioenergetics. Significant effects are highlighted in bold. F = F-statistic, df = degrees of freedom, and P = P-value.

Parameter	Main Effects and Interactions								
	Temperature (T)			Hypoxia (H)			Interaction (T×H)		
	df	F	P	df	F	P	df	F	P
State 3	2,48	367	< 0.0001	3,48	99.5	< 0.0001	6,48	11.7	< 0.0001
State 3 Q ₁₀	1,32	4.44	= 0.043	3,32	3.22	= 0.04	3,32	10.3	< 0.0001
State 4	2,48	307	< 0.0001	3,48	29.9	< 0.0001	6,48	17.6	< 0.0001
State 4 Q ₁₀	1,32	20.2	< 0.0001	3,32	11.6	< 0.0001	3,32	4.07	= 0.014
P/O ratio	2,48	113	< 0.0001	3,48	834	< 0.0001	6,48	24	< 0.0001
RCR	2,48	60.4	< 0.0001	3,48	463	< 0.0001	6,48	38.1	< 0.0001

Figure 4.2

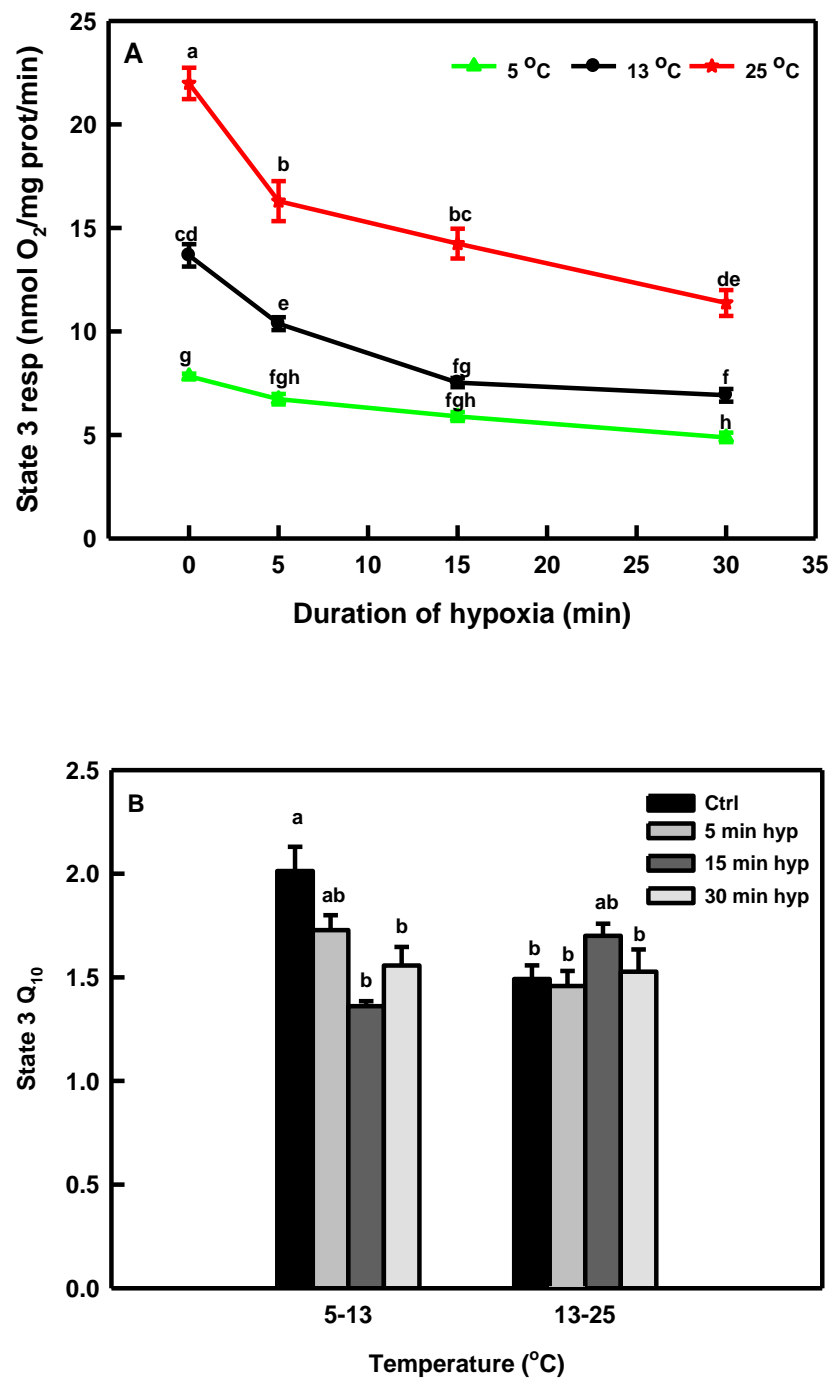


Figure 4.2: The effects of temperature and H-R on state 3 respiration (A) and Q₁₀ (B). Isolated mitochondria were exposed to H-R (0, 5, 15 and 30 min) at 5, 13 and 25 °C. Data are

means \pm SEM ($n = 5$). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 4.3

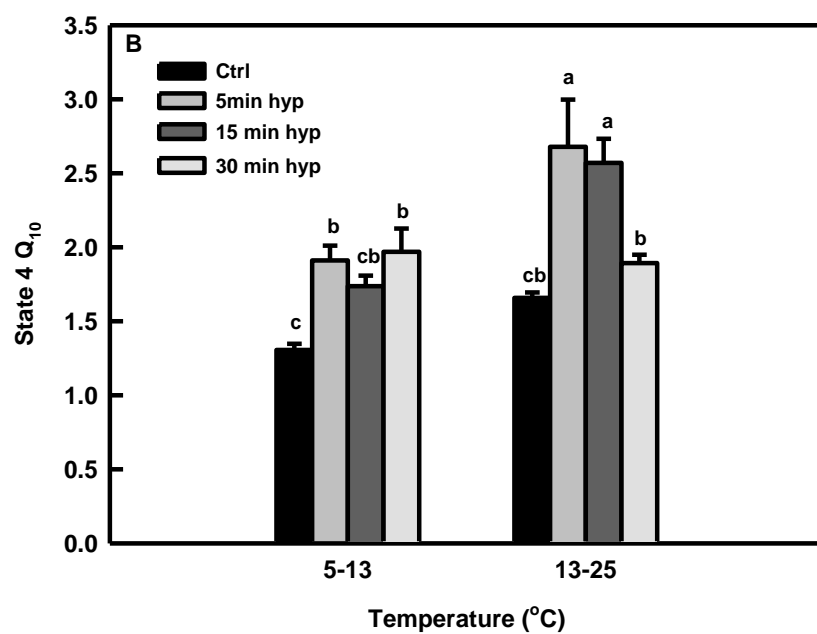
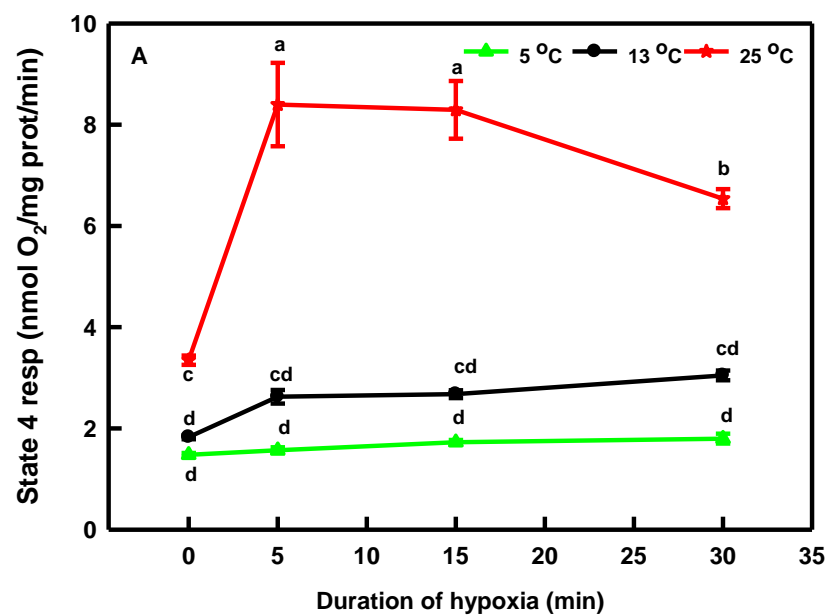


Figure 4.3: The effects of temperature and H-R on state 4 respiration (A) and Q_{10} (B).

Isolated mitochondria were exposed to H-R (0, 5, 15 and 30 min) at 5, 13 and 25 °C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

The phosphorylation efficiency (P/O ratio) was increased ($F_{2,48} = 43.6$, $P < 0.0001$) by temperature (Fig. 4.4A) and reduced by H-R ($F_{3,48} = 262$, $P < 0.0001$). The three temperature exposure regimes differently modulated the effects of H-R. Specifically, at 5 °C the initial drop in P/O ratio after 5 min H-R was not followed by additional reduction with increasing durations of hypoxia but for 13 and 25 °C the ratios decreased with increasing duration of H-R. Moreover, the reduction in the P/O ratio was greater at 25 compared with either 13 or 5 °C. A significant ($F_{6,48} = 11.6$, $P < 0.0001$) interaction was observed between temperature and H-R since H-R reduced the effect of temperature on P/O ratio.

While temperature increased ($F_{2,48} = 60.4$, $P < 0.0001$) the RCR (mitochondria coupling efficiency), the effect was not monotonic (Fig. 4.4B). In the controls, RCR values were lowest at 5 °C and the highest at 13 °C, with the 25 °C values falling in between. H-R greatly reduced the RCR ($F_{3,48} = 463$, $P < 0.0001$) but the effects were different at the 3 temperatures consistent with the significant ($F_{6,48} = 38.1$, $P < 0.0001$) two way interaction. It was evident that RCR was more resistant to H-R at 5 °C than at 13 or 25 °C. Additionally, while the RCR attained maximal reduction after only 5 min of exposure to H-R at 25 °C, it continued to decline with the duration of hypoxia at both 5 and 13 °C.

Figure 4.4

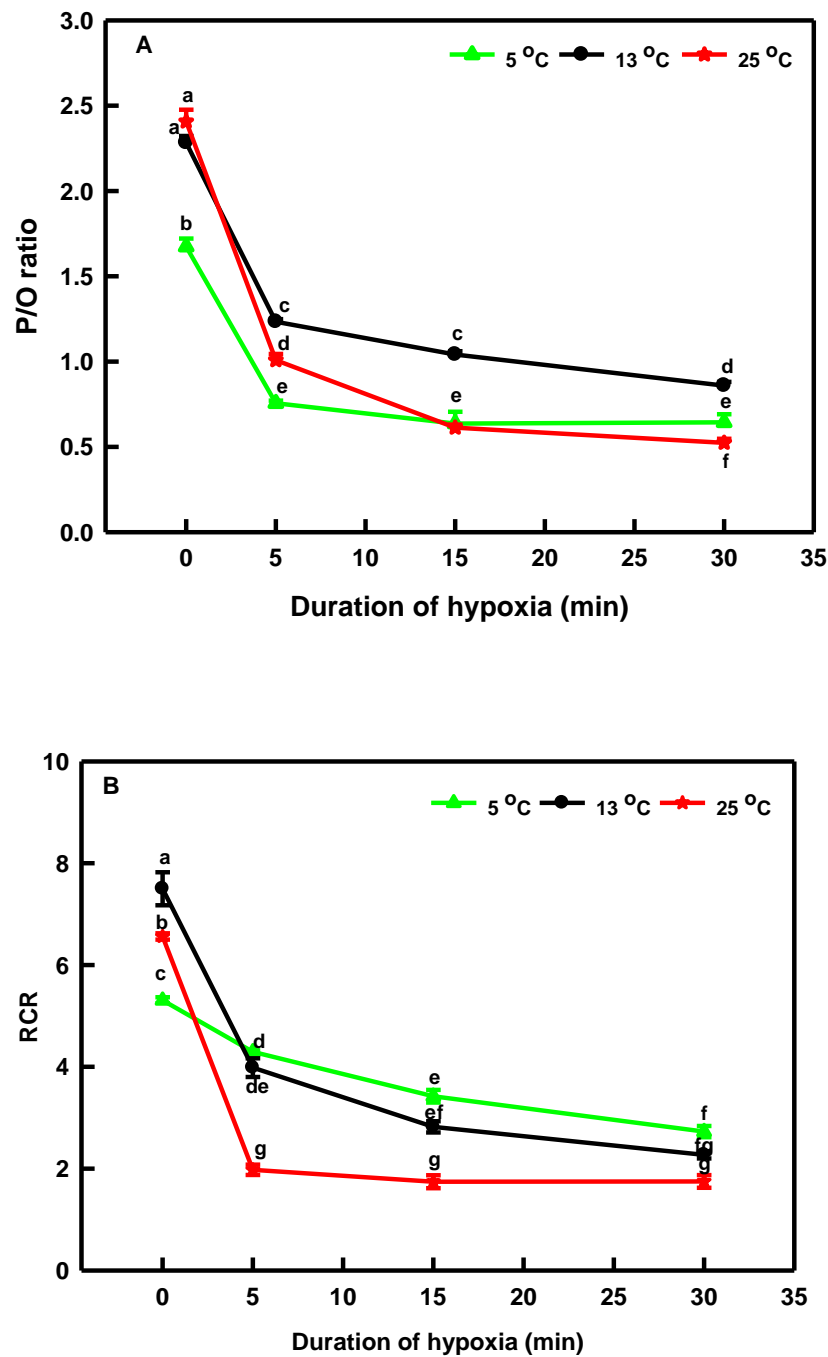


Figure 4.4: The effects of temperature and H-R on P/O ratio (A) and RCR (B). Isolated mitochondria were exposed to H-R (0, 5, 15 and 30 min) at 5, 13 and 25 °C. Data are means

\pm SEM ($n = 5$). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

4.4.2 Effects of H-R on mitochondrial membrane potential (MMP) and H₂O₂ production

Under normoxic conditions, un-energized mitochondria had minimal membrane potential; however, energization with malate and glutamate resulted in generation of MMP that was dissipated by the uncoupler, FCCP (Fig. 4.5). Energized mitochondria exposed to H-R had lower membrane potential relative to the normoxic energized mitochondria and vitamin E, an antioxidant, protected the mitochondria against H-R induced membrane potential dissipation. Direct measurement of mitochondrial H₂O₂ production showed it was stimulated by H-R when CIII was inhibited at its Q_i site with antimycin A (Fig. 4.6).

4.4.3 Effects of H-R on mitochondrial structure and size

Compared with the controls in which the outer and inner membranes were intact with visible cristae and electron dense matrix (Fig. 4.7A), mitochondria exposed to H-R were swollen and their membranes damaged (Fig. 4.7B). There was degeneration of the organelle characterized by cristolysis and loss of matrix density wherein the mitochondria appeared translucent (Fig. 4.7B). Notably, H-R-treated mitochondria had larger diameter than the controls (Fig. 4.7C).

Figure 4.5

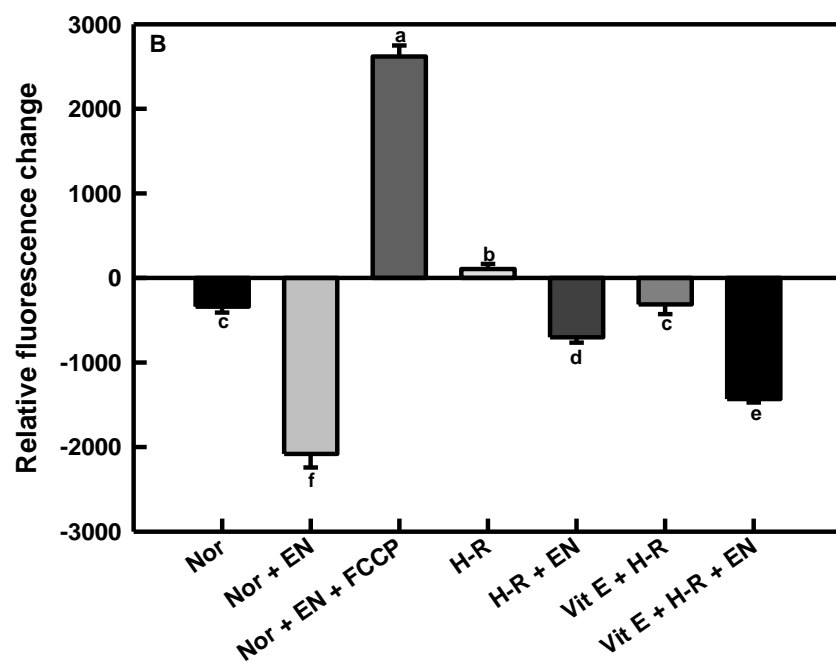
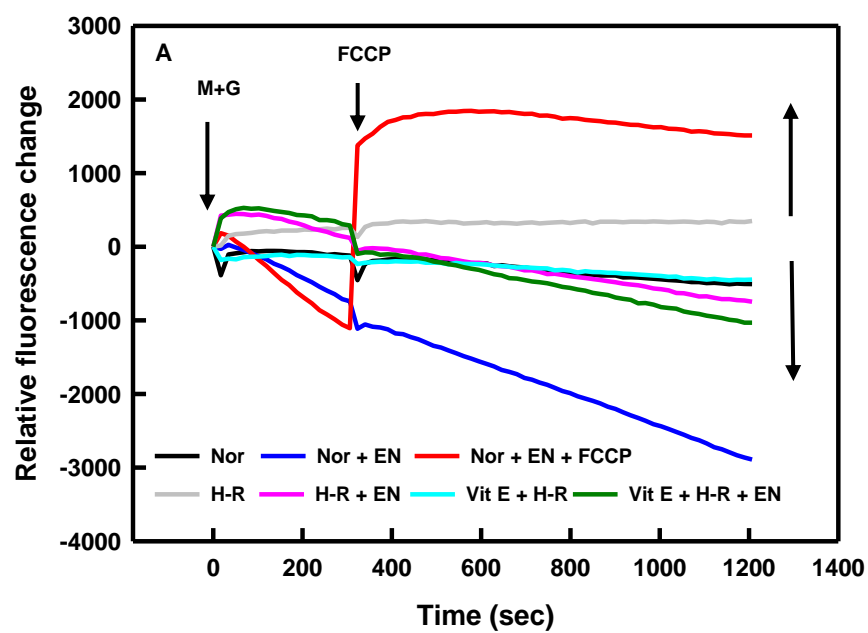


Figure 4.5: The effects of H-R on mitochondrial membrane potential. A: kinetics and B: amplitude of fluorescence change after 20 min under normoxia without energization (Nor), normoxia with energization (Nor+EN), normoxia with energization and FCCP (Nor+EN+FCCP), hypoxia-reoxygenation without energization (H-R), hypoxia-reoxygenation with energization (H-R+EN), vitamin E with hypoxia-reoxygenation without energization (Vit E+H-R), vitamin E with hypoxia-reoxygenation and energization (Vit E+H-R+EN). ↑ indicates dissipation of membrane potential and ↓ indicates increase in membrane potential. Trend lines (panel A) are mean values of 5 tracings obtained from mitochondria isolated from 5 different livers. Bar graphs (panel B) show means ± SEMs (n = 5) of amplitude of fluorescence change after 15 min. Bars with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $p < 0.05$).

Figure 4.6

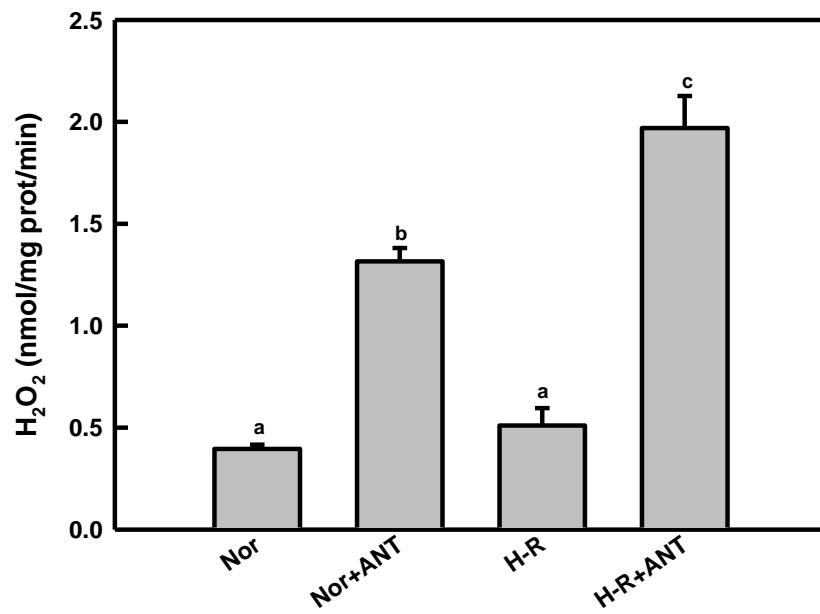


Figure 4.6: The effects of H-R on mitochondrial H₂O₂ production with and without antimycin A. Data are means \pm SEM (n = 5). Bars with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

Figure 4.7

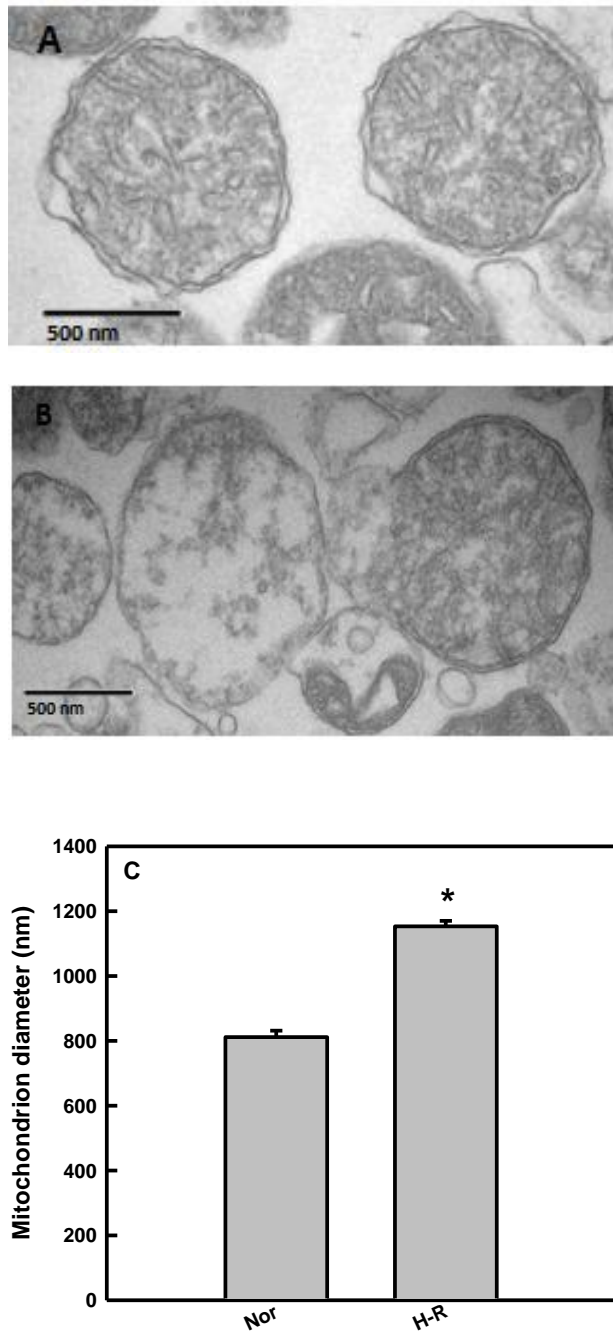


Figure 4.7: The effects of H-R on mitochondrial ultrastructure. A: normoxic (Nor) mitochondria with visible cristae and intact inner and outer membranes. B: H-R mitochondria showing swelling, damaged inner and outer membranes, reduced matrix density and loss of

cristae. C: Mitochondrial diameter (means \pm SEM, n = 50). Nor = normoxia and H-R = hypoxia-reoxygenation. The asterisk (*) indicates significant difference from the controls (*t*-test, $P < 0.05$)

4.5 DISCUSSION

In my previous studies (Chapter 2, and 3) I characterized the effects and interactions of Cd with temperature stress or H-R on mitochondrial bioenergetics. Here, I focused on the joint effects of temperature and H-R and demonstrated they modify each other's effects on mitochondrial function (Table 4.1). Different temperatures evoked different responses under H-R conditions with both additive/synergistic and antagonistic interactions that could impede attempts to generate a consensus regarding the combined effects of the two stressors on mitochondrial function.

4.5.1 H-R resulted in a smaller decrease in state 3 respiration at low temperature

While H-R was less detrimental to mitochondrial oxidation at low temperature, state 3 was significantly inhibited at the 3 temperatures tested contrasting a recent study (Tissier et al., 2013) that reported reversal of per-ischemic inhibition of state 3 by mild hypothermia in endothermic rabbit heart mitochondria. State 3 was more highly inhibited (48% inhibition at 25 °C vs. 38% inhibition at 5 °C) by H-R at high relative to low temperatures indicating that temperature enhanced the deleterious effects of H-R. However, my H-R treatment and per-ischemia (Tissier et al., 2013) are different procedures and my fish are ectotherms that normally experience temperature change whereas hypothermia in rabbits is abnormal; this could in part account for the discordant outcomes. It previously has been reported that H-R reduces state 3 by inhibiting the activities of the ETS enzyme complexes (Heerlein et al., 2005; Shiva et al., 2007; Di Lisa et al., 2011; Maruyama et al., 2013; Chapter 2). Although H-R has been shown to have specific effects on complex I (Galkin et al., 2009), the pervasive inhibition of the maximal mitochondrial respiration rate suggests that it may also act non-specifically. In this regard, my study showed that H-R increased the capacity for H₂O₂

production in isolated mitochondria. Interestingly, the thermal coefficient for state 3 was higher at low temperature and the severity of H-R-induced reduction of Q_{10} increased with the duration of hypoxia at low temperature. This reduction of state 3 Q_{10} at low temperature could have resulted from altered IMM structure and/or changes in the conformation of ETS enzymes. Another possible explanation is that control of mitochondrial function could switch from ETS and phosphorylation to dehydrogenases and redox processes upstream of the ETS at low temperature. That is, the lower Q_{10} at lower temperatures could reflect a change in an upstream rate limiting step.

At high temperature H-R induced a biphasic response on state 4 characterized by peak stimulation after a short duration (5 min) of hypoxia. The decrease of state 4 with longer durations of H-R suggests that the processes that mediate proton leak and/or generate the protonmotive force were impaired. In contrast, the monotonic and muted response at 5 °C suggests that low temperature preserves mechanisms that mediate proton leak and/or those that generate the protonmotive force during H-R. It is conceivable that reduced substrate oxidation (Blier and Guderley, 1993) and diffusion rates (Dunn, 1988) at low temperature altered the sensitivity of mitochondria to H-R. Thus, low temperature may stabilize mitochondria allowing them to better resist the deleterious effects of H-R. Moreover, the increase in thermal sensitivity for state 4 (proton leak) with temperature and its enhancement by H-R indicates that the combined stressors increase the permeability of IMM.

Although H-R reduced the stimulatory effect of temperature on the P/O ratio, the mitochondria apparently were more efficient in utilizing O_2 at low temperature. This implies that at low temperature the mitochondria would be able to maintain relatively stable ATP production under hypoxia. An alternative explanation is that the change in P/O ratio is due to

a change in proton leak. The reduction of RCR with temperature that I observed is consistent with previous studies (Rodnick et al., 2014, Sappal et al., 2014b; Chapter 3) and in my study it resulted from preferential stimulation of state 4. Moreover, the profound decrease in RCR after H-R treatment at high temperature and the resilience of mitochondria to this stressor at low temperature support the notion that low temperature insulates mitochondria from effects of H-R. While evidence that hypothermic conditions offer protection against ischemia-reperfusion has been presented (Khaliulin et al., 2007; Shao et al., 2010, Mochizuki et al., 2012, Tissier et al., 2012), the underlying mechanisms have not been fully elucidated. It has been proposed that reduced rate of biochemical activity at low temperature prevents mitochondrial Ca overload and reduces the production of ROS (Gambert et al., 2004) ultimately inhibiting the opening of mitochondrial permeability transition pore and preserving mitochondrial functional integrity.

4.5.2 H-R-induced mitochondrial membrane potential (MMP) dissipation and structural damage can be explained by increased ROS production

The dissipation of MMP by H-R observed in my study has been reported previously (Di Lisa et al., 1995; Honda et al., 2005; Pi et al., 2007) and is believed to result from inhibition of activities of ETS enzymes (Morin et al., 2004; Pi et al., 2007). However, this decrease in MMP could also be explained by a decrease in supply of reducing equivalents to the ETS. In my study, MMP dissipation was caused, at least in part, by ROS because I showed that H-R directly increased H₂O₂ production and that vitamin E partially conserved the MMP. Lastly, similar to previous studies (Ozcan et al., 2001; Solenski et al., 2002; Schild et al., 2003; Honda et al., 2005), H-R caused mitochondrial structural damage that also could be attributed to increased ROS production. These effects on MMP and structure likely contributed to the >40% loss of maximal complex I supported respiration following H-R in the present study.

4.5.3 CONCLUSIONS

H-R impaired OXPHOS by dissipating the MMP, stimulating proton leak (state 4), increasing ROS production and damaging mitochondrial structure. Temperature differentially modulated H-R-induced mitochondrial dysfunction wherein the sensitivity of OXPHOS to H-R was lessened and heightened by low and high temperatures, respectively. H-R reduced the stimulatory effects of temperature on state 3 and either increased (high temperature) or did not alter (low temperature) state 4. Furthermore, the phosphorylation efficiency was increased by temperature and reduced by H-R while both low and high temperatures exacerbated the RCR-reducing effect of H-R. Overall my study indicates that elevated temperature worsens the H-R-induced stress on rainbow trout mitochondria.

CHAPTER 5

COMBINED EFFECTS OF CADMIUM, TEMPERATURE AND HYPOXIA-REOXYGENATION ON MITOCHONDRIAL FUNCTION IN RAINBOW TROUT (*Oncorhynchus mykiss*)

A version of this chapter has been submitted for publication with slight modification as:

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Author contributions

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

5.1 ABSTRACT

Although aquatic organisms face multiple environmental stressors that may interact to alter adverse outcomes, our knowledge of stressor-stressor interaction on cellular function is limited. I investigated the combined effects of cadmium (Cd), hypoxia-reoxygenation (H-R) and temperature on mitochondrial function. Juvenile rainbow trout were exposed to Cd (0-20 μM) and H-R (0 and 5 min) at 5, 13 and 25 $^{\circ}\text{C}$ followed by measurements of mitochondrial Cd load, volume, complex I active (A) \leftrightarrow inactive (D) transition, membrane potential, ROS production and ultrastructural changes. At high temperature Cd exacerbated H-R-imposed reduction of maximal complex I (CI) respiration whereas at low temperature its lower doses (5 and 10 μM) stimulated maximal CI respiration post H-R. The basal respiration showed a biphasic response at high temperatures with low doses of Cd reducing the stimulatory effect of H-R and high doses enhancing this effect. At low temperature Cd monotonically enhanced H-R-induced stimulation of basal respiration. The thermal sensitivity for maximal CI respiration increased and that for basal respiration decreased at the high temperature range while the sensitivity of both maximal and basal respiration rates decreased at the low temperature range. Both the P/O ratio and the RCR were reduced at all of the 3 temperatures. Temperature rise alone increased mitochondrial Cd load and toxicity, while combined H-R and temperature exposure reduced mitochondrial Cd load but surprisingly exacerbated the mitochondrial dysfunction. Although mitochondrial dysfunction induced by Cd and H-R was associated with swelling of the organelle and conversion of CI A to D form, low doses of Cd protected against H-R induced swelling and enhanced CI A form. Both H-R and Cd dissipated mitochondrial membrane potential ($\Delta\psi_{\text{m}}$) and damaged mitochondria structure. I observed increased H_2O_2 production that together with the protection afforded by EGTA, vitamin E and N-acetylcysteine against the $\Delta\psi_{\text{m}}$ dissipation suggested involvement of the

metal and oxidative stress. Overall, my findings indicate that mitochondrial sensitivity to Cd toxicity was enhanced by the effects of H-R and temperature, and changes in mitochondrial Cd load did not always explain this effect.

5.2 INTRODUCTION

Cadmium is a highly toxic ubiquitous trace metal (Byczkowski and Sorenson, 1984; Hattink et al., 2005; Kamunde, 2009). Due to its relatively long biological half-life Cd tends to accumulate in animal organs including kidney, liver, and gills in fish (McGeer et al., 2000; Hollis et al., 2001; Sokolova et al., 2005; Kamunde, 2009). Among numerous potential cellular sites of action, the mitochondria have emerged as key targets of Cd toxicity in a variety of animal species including eastern oysters (Sokolova, 2004; Kurochkin et al., 2011), rainbow trout (Adiele et al., 2012a; Chapter 2), and rat (Dorta et al., 2003; Belyaeva et al., 2006; Lopez et al., 2006). However, the mechanisms and effects of interactions of the metal and other environmental stress factors have not been comprehensively investigated. In this regard, hypoxia-reoxygenation (H-R) and temperature fluctuations are two environmental stressors that commonly co-occur in nature with Cd. There is some evidence that Cd, hypoxia (without and with reoxygenation) and temperature may act interactively to alter cellular and organismal function (Hattink et al., 2005; Kurochkin et al., 2009) but much remains unknown regarding their combined effects on mitochondrial function.

Mitochondrial structural integrity is critical for mitochondrial function (Kaasik et al., 2007). There is empirical evidence that the disruption of mitochondrial structure by Cd and/or H-R increases the permeability of the IMM to solute resulting in increased organelle volume (Lee et al., 2005a; Orlov et al., 2013; Chapter 3). Mitochondrial volume dysregulation has been shown to decrease mitochondrial membrane potential (Kaasik et al., 2007) the force that

drives ATP production. Furthermore, inhibition of the ETS by stressors could increase ROS production that then damage the mitochondrial structure and impair OXPHOS (Wang et al., 2004; Navet et al., 2006; Lopez et al., 2006).

Complex I (CI) is a key mitochondrial enzyme in OXPHOS responsible for the oxidation of NADH (produced in the Krebs cycle) with reduction of ubiquinone (Q) and transport of four protons across the IMM thus contributing to the proton-motive force ($\Delta\psi_m$ and ΔpH) used in ATP synthesis (Murphy, 2009; Hirst, 2013). Mitochondrial CI is known to adopt two catalytically and structurally distinct conformation –active (A) and de-active (D)– depending on the prevailing conditions (Babot et al., 2014). For example, under conditions of low oxygen (hypoxia), levels of the D-form increase rendering the enzyme incapable of ubiquinone reduction (Maklashina et al., 2004; Gorenkova et al., 2013). Other factors such as temperature, pH and presence of divalent metals also increase the levels of the D-form (Maklashina et al., 2002; Babot et al., 2014). However, the combined effects of H-R and divalent metals (Cd) on complex I A to D transition remain unknown.

In the present study I first tested the hypothesis that the ternary interactions of Cd, temperature and H-R would alter the individual and/or binary effects of the stressors on mitochondrial bioenergetics. I predicted that the alterations in mitochondrial function can be explained by differential Cd accumulation in the mitochondria. Second, I investigated the interactions of H-R and Cd on key indicators of mitochondrial functional integrity including volume homeostasis, complex I A \leftrightarrow D transition, $\Delta\psi_m$, ROS production and structure.

5.3 MATERIALS AND METHODS

5.3.1 Ethics

The University of Prince Edward Island Animal Care Committee approved all the experimental procedures that were used in this study in accordance with the Canadian Council of Animal Care.

5.3.2 Fish and mitochondrial isolation

Juvenile rainbow trout weighing (142 ± 24.5 g) were obtained from Ocean Farms Inc, Brookvale, PE, and maintained in the Aquatic Facility of Atlantic Veterinary College in a 400-l tank. The tank was supplied with flow-through water at a temperature of 11 ± 1 °C and pH of 7.7. The fish were fed at 1% of their body weight daily with commercial trout chow pellets (Corey Feed Mills, Fredericton, NB). The concentrations of Cd measured in the feed and water were $0.78\mu\text{g/g}$ and below our limit of detection ($0.003\mu\text{g/l}$), respectively. To isolate mitochondria, trout were randomly sampled from the tank and killed with a blow to the head. The liver was quickly removed, minced and homogenized and mitochondria were isolated as described (Chapter 2) and suspended in mitochondrial respiratory buffer (MRB) in a 3:1 ratio. Mitochondrial protein was determined spectrophotometrically (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA) according to the method (Bradford, 1976) with BSA as the standard.

5.3.3 Interactions of Cd, temperature and hypoxia on mitochondrial bioenergetics

Mitochondrial respiration was measured with Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) in 1.5 ml assay volume after a two-point calibration at 0 and 100% oxygen saturation. A traceable digital barometer (Fisher Scientific, Nepean, ON) was used to measure the atmospheric pressure, and the temperature was maintained at 5, 13 or 25 °C with

the aid of a recirculating water-bath (Haake, Karlsruhe, Germany). Following calibration, 1.45 ml of MRB and 100 μ l of the mitochondrial suspension containing 2.3-2.9 mg of protein (23-29 mg of mitochondrial mass, wet weight) were loaded into the cuvettes and mixed continuously. A saturating amount of complex I substrates (5 mM malate and 5 mM glutamate) were added to the cuvette and maximal (state 3) respiration rate was evoked by the addition of 250 μ M ADP. When the ADP was exhausted, the state 3 transitioned to basal (state 4) respiration rate. To measure respiration rate after H-R the method in chapter 2 was used. Briefly, after normoxic respiration, the MRB was made hypoxic by bubbling in N₂ to reduce PO₂ to < 2 torr (Note that in a control experiment we found that bubbling with air has no effect on mitochondrial state 3 and 4 respiration rates and the RCR). These PO₂ are below the 2.25-3.75 torr intracellular level of oxygen typically experienced by rat mitochondria *in vivo* (Gnaiger and Kuznetsov, 2002). After the hypoxia exposure period, the cuvettes were opened and the mitochondrial suspension was allowed to fully reoxygenate. The post H-R measurement of respiration with or without Cd addition was then carried out in the same fashion as the normoxic measurement. Ternary interactions of the stressor on OXPHOS were measured at 3 temperatures levels (5, 13 and 25 °C), 2 H-R durations (0 and 5 min) and 4 Cd exposure concentrations (0, 5, 10 and 20 μ M). The state 3 and 4 respiration were measured for all stressor combinations and the phosphorylation efficiency (P/O ratio, ADP/O) and respiratory control ratio (RCR, state 3/state 4 respiration) were calculated according to Estabrook (1967) and Chance and Williams (1955), respectively. In addition, temperature coefficients (Q₁₀ values) were calculated for state 3 and 4 for the temperature ranges 5-13 °C and 13-25 °C using the equation: $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$ where R₂ and R₁ represents mitochondrial oxygen consumption rates at two temperatures, T₂ and T₁ and where T₂>T₁.

5.3.4 Interactions of Cd, H-R and temperature on mitochondrial Cd accumulation

Cd accumulation in the mitochondria was measured by atomic absorption spectrophotometry (AAS) (PinAAcle 900T, Perkin Elmer, Woodbridge, ON). Briefly, after measuring the respiration rates, mitochondria suspensions were removed from the cuvettes and pelleted by centrifuging at $10000 \times g$ for 5 min at 4 °C. The supernatants were discarded, and the pellets were washed twice with 500 µl of mitochondrial isolation buffer (MIB) to remove non-specifically bound Cd, with re-pelleting at $10000 \times g$ for 5 min at 4 °C. The pellets were then stored at -80 °C until analyzed for Cd. For AAS, the pellets were oven-dried at 70 °C (ISOTEMP, Fisher Scientific) to a constant weight. After weighing, the pellets were digested with 500 µl of 30% H₂O₂ and 70% HNO₃ (trace metal grade, Fisher) in a 1:15 mixture for 24 h at room temperature. The resulting digests were appropriately diluted using 0.2% HNO₃, and the Cd concentrations were measured by AAS in furnace mode and expressed as µmol Cd/g mitochondrial dry weight (mdw). All of my Cd analyses were carried out in the presence of modifiers (NH₄H₂PO₄ and Mg [NO₃]₂). Standard reference material (SRM: TMDA-70.2:20, lot 0310 [Environment Canada]) and blanks were analyzed simultaneously with the samples. Cadmium was not detected in the blanks, and the recovery rate of Cd from the SRM ranged between 94 and 101%.

5.3.5 Interactions of Cd and H-R on mitochondrial volume homeostasis

The interactions of Cd and H-R on mitochondrial volume were assessed using a spectrophotometric method (Chapter 3). Briefly, isolated mitochondria were energized with 5 mM each of malate and glutamate in the respiratory cuvettes under normoxia and 5 min H-R. The mitochondrial suspensions were then diluted with air-saturated swelling buffer to a final assay concentration of 1 mg/ml of mitochondrial protein. Cd doses (0, 5 and 50 µM) or 200

μM Ca (positive control) were added to microplate wells as 20 μl of appropriate stock solutions and brought to assay volume of 200 μl by adding 180 μl of the 1 mg/ml mitochondrial suspension. Volume changes were monitored spectrophotometrically at 540 nm with reading every 10 s for 30 min at 25 °C. Here, a decrease in absorbance indicated mitochondrial swelling.

5.3.6 Effect of Cd and H-R on complex 1 conformation

Mitochondrial CI exists in two different states –the active (A) and de-active (D) conformation– that are catalytically and structurally distinct (Vinogradov, 1998; Maklashina et al., 2004; Babot et al., 2014). Only the A form of CI is capable of catalyzing ubiquinone reduction by NADH (Vinogradov, 1998; Maklashina et al., 2004). To quantify the proportions of A and D forms of CI we monitored NADH-induced oxygen consumption. Because NADH does not permeate intact mitochondria, isolated mitochondria were taken through 5 cycles of freeze (-80) and thaw (at room temperature) to disrupt their membranes allowing NADH access to CI and thus stimulate respiration. Thereafter, 100 μl of mitochondria with disrupted membranes were added to the cuvette containing 1.45 ml of MRB followed by 350 μmoles of NADH as substrate under normoxic conditions. In another set of experiments with the same mitochondrial prep on the same day, 1 mM N-ethylmaleimide (NEM) was added 5 min after the addition of 350 μM NADH. NEM permanently binds to the D-form preventing its conversion to A-form thus leaving only the A-form available to oxidize NADH during the trial (Gavrikova and Vinogradov, 1999). The addition of NEM allows estimation of the amount of CI in the A-form. To assess the effect of H-R on CI A \leftrightarrow D transformation, N₂ gas was bubbled into the cuvette after normoxic respiration to deplete the PO₂ exactly as in the respiration experiments. The disrupted

mitochondria were then incubated for 5 min under hypoxic conditions followed by reoxygenation and measurement of NADH-stimulated respiration. The effect of a low dose of Cd (5 μ M) was tested using normoxic or H-R conditions by adding the metal to the disrupted mitochondria 5 min after adding the NADH with O₂ consumption monitored for 5 min. These trials were carried out only at 13 °C.

5.3.7 Interactions of Cd and H-R on mitochondrial membrane potential

The effects of Cd and H-R individually and in combination on $\Delta\Psi_m$ were measured fluorometrically (SynergyTM HT BioTek, US) using rhodamine 123 (Rh123; excitation set at 485 nm; emission at 528 nm). Briefly, 170 μ l of 1 mg/ml normoxic or post H-R mitochondrial suspension in MRB and 5 μ M (final concentration as 10 μ l of appropriate stock solution) Rh123 were loaded into the microplate wells and fluorescence was read every 17 s for 15 min at room temperature to allow the mitochondria to equilibrate to assay conditions. Mitochondria were then energized with 5 mM each of malate and glutamate (total contribution of 10 μ l to assay volume) and fluorescence was read every 17 s for 5 min. The dose response of Cd on membrane potential was measured using 6 doses (0, 5, 10, 20, 50 and 100 μ M) of Cd added as 10 μ l of appropriate stock solutions with fluorescence reading every 17 s for 15 min. Thus the final total assay volume was 200 μ l. For positive control 0.5 μ M of FCCP (as 10 μ l) was added to another set of duplicate wells containing energized normoxic mitochondria and fluorescence was recorded as described above. To assess the interactions of Cd and H-R on $\Delta\Psi_m$, Cd (0, 5, 20 and 50 μ M) was added to the microplate wells containing post H-R mitochondria and fluorescence was monitored as described above. Additional tests assessed the effect of a metal chelator (EGTA) and antioxidants (N-acetylcysteine, NAC, and vitamin E) on $\Delta\Psi_m$ changes imposed by Cd (100 μ M), H-R, or H-R (5 min) + Cd (20 μ M). In

these experiments a decrease in Rh123 fluorescence indicates an increase of $\Delta\Psi_m$ while an increase indicates its dissipation.

5.3.8 Interactions of Cd and hypoxia-reoxygenation on mitochondrial H₂O₂ production

H₂O₂ production by the mitochondria following individual and combined exposure to Cd (0 and 20 μ M) and 5 min H-R was measured by fluorometry (SynergyTM HT BioTek) using Amplex Red-horseradish peroxidase (HRP) assay (Sharaf et al., 2015; Chapter 4).

Mitochondria were suspended as 1 mg/ml protein in MRB containing 1 mg/ml BSA and aprotinin. To measure H₂O₂ 10 μ l of 20 U/ml HRP and 5 μ l each of malate and glutamate (assay concentration 5 mM) were added to the microplate wells. Thereafter 170 μ l of 1 mg/ml mitochondrial suspension (normoxic or post H-R) and 10 μ l of each Cd dose (0-20 μ M) were added to the microplate wells. In other duplicate sets of wells with and without Cd, 170 μ l of the mitochondrial suspension and 10 μ l of 0.1 mg/ml antimycin A (complex III inhibitor to boost H₂O₂ production) were added. Lastly, 10 μ l of 1 mM Amplex Red were added bringing final volume to 200 μ l and the assay was incubated in the dark at room temperature for 30 min. In this assay HRP catalyzes the conversion of Amplex red by H₂O₂ to resorufin of which fluorescence (excitation 530 nm, 590 nm) was recorded at 15 min intervals for 30 min. Standard curves were obtained using known concentrations of H₂O₂ (0-5 μ M) in an assay with MRB alone and were used to convert fluorescence data to actual H₂O₂ concentrations. Background fluorescence of assay components (without mitochondria) was determined during each run and subtracted from all fluorescence readings.

5.3.9 Interactions of Cd and hypoxia-reoxygenation on mitochondrial ultrastructure

Ultrastructural changes of mitochondria were assessed by transmission electron microscopy (TEM) following exposure to Cd under normoxic and H-R conditions. Briefly, mitochondria

were suspended in the cuvettes for 30 min under normoxic and 5 min H-R with or without graded levels of Cd (0, 5 and 20 μM). After the exposure, mitochondrial suspensions were pelleted by centrifugation at $10000 \times g$ for 5 min and fixed with 1:1 volume of 6% glutaraldehyde in MRB at 4 °C overnight then washed with 0.1 M phosphate buffer and post-fixed in 1% OsO_4 for 1 h at room temperature. Mitochondrial samples were embedded in low melting point agar before dehydration in an ascending series (50-100%) of ethanol. The samples were cleared in propylene oxide, infiltrated with Epon and propylene oxide (1:1 and 2:1) and embedded in 100% Epon resin. Thin sections (80 nm) were cut using an ultramicrotome (Reichert-Jung Ultracut E, Leica Microsystems, Richmond Hill, Canada), stained with uranyl acetate and Sato lead and viewed using TEM (Hitachi BioTEM 7500; Nissei-Sangyo) under 80 kV. A digital (AMT XR 40) camera (Advanced Microscopy Techniques, Danvers, MA, USA) was used to take photographs of the mitochondria, and mitochondrial diameters were measured in five random fields per experimental group at $15000\times$. A total of 10 mitochondria in each of 5 fields ($n = 50$) for each treatment were measured to obtain the mean organelle diameter.

5.3.10 Statistical analysis

All of the data were first tested for normality of distributions and homogeneity of variances (Kolmogorov and Levene's tests respectively) before being subjected to one, two or three-way analysis of variance (ANOVA) (Statistica version 5.1, Statsoft, Inc., Tulsa, OK). If the data did not pass the normality test, they were submitted to Box-Cox transformation; all data passed after transformation. An ANOVA is only slightly affected by inequality of variance using our models (equal sample sizes and all factors fixed). Cd dose, hypoxia, and/or temperature were the independent variables. Significantly different means were separated

using Tukey's post hoc test at $P < 0.05$. Linear regression analysis was performed using Sigmaplot 10 (Systat Software, San Jose, CA, USA).

5.4 RESULTS

5.4.1 Effects of interactions of Cd, H-R and temperature on mitochondrial bioenergetics

For state 3 respiration, the 3-way interaction term, temperature \times H-R \times Cd, was significant ($F_{6,96}=5.8$, $P < 0.0001$) indicating that the 3 stressors modulated the effects of each other (Fig. 5.1A). Cd decreased state 3 respiration (main effect $F_{3,96}=169$, $P < 0.0001$) but the absolute state 3 rates decreased with a decrease in temperature and were greater after H-R. Although the absolute changes were different, the relative decreases in state 3 from control to 20 μ M Cd during normoxia were similar at the three temperatures: 43% at 5 $^{\circ}$ C, 38% at 13 $^{\circ}$ C, and 44% at 25 $^{\circ}$ C. The state 3 Q_{10} values presented in Table 5.1 show significant 3-way interactions of temperature \times Cd \times H-R ($F_{3,64}=16$, $P < 0.0001$), mainly because both Cd and H-R affected state 3 thermal sensitivity differently. For state 4 respiration, the 3-way interaction term, temperature \times H-R \times Cd, was significant ($F_{6,96}=15.4$, $P < 0.0001$) indicating that the 3 stressors altered the effects of each other (Fig. 5.1B). Cd increased state 4 respiration (main effect $F_{3,96}=9.8$, $P < 0.0001$) but the absolute and relative increases in state 4 increased with temperature and were greater after H-R. The state 4 Q_{10} values (Table 5.2) show that the effect of Cd \times temperature \times H-R interaction was not significant ($F_{3,64}=2.3$, $P=0.08$).

For the P/O ratio, the 3-way interaction term, temperature \times H-R \times Cd, was significant ($F_{6,96}=7$, $P < 0.0001$) showing that the 3 stressors altered the effects of each other (Fig. 5.2A).

While Cd decreased P/O ratio during normoxic conditions by 70% at 25 $^{\circ}$ C and 33% at 13 $^{\circ}$ C, there was no change at 5 $^{\circ}$ C. H-R markedly decreased but temperature stimulated the P/O

ratio. For RCR the 3-way interaction term, temperature \times H-R \times Cd, was significant ($F_{6,96}=20$, $P<0.0001$) showing that the 3 stressors altered the effects of each other (Fig. 5.2B).

RCR was highest with no Cd, during normoxia and at 13 °C. Cd and H-R decreased RCR, as did the high (25 °C) and low (5 °C) temperatures. The relative decrease in RCR with Cd during normoxic conditions was similar at the 3 temperatures (50% at 5 °C, 51% at 13 °C, and 60% at 25 °C) but greater after H-R (60% at 5 °C, 84% at 13 °C, and 78% at 25 °C).

5.4.2 Modulation of mitochondrial Cd accumulation by temperature and H-R

Mitochondrial Cd accumulation increased with Cd dose and increased with temperature but was lower after H-R than for normoxia at each dose (Fig. 5.3A). The 3-way interaction term, temperature \times H-R \times Cd, was significant ($F_{6,96}=7.9$, $P<0.0001$) showing that the 3 stressors altered the effects of one another. The decrease in RCR was associated with an increase in mitochondrial Cd concentration at all temperatures (Fig. 5.3B, C and D). The curves relating RCR to mitochondrial [Cd] were shifted down after H-R relative to those for normoxia at all temperatures; that is, normoxic mitochondria contained more Cd at the same RCR at all temperatures (Fig. 5.3B, C and D).

Figure 5.1

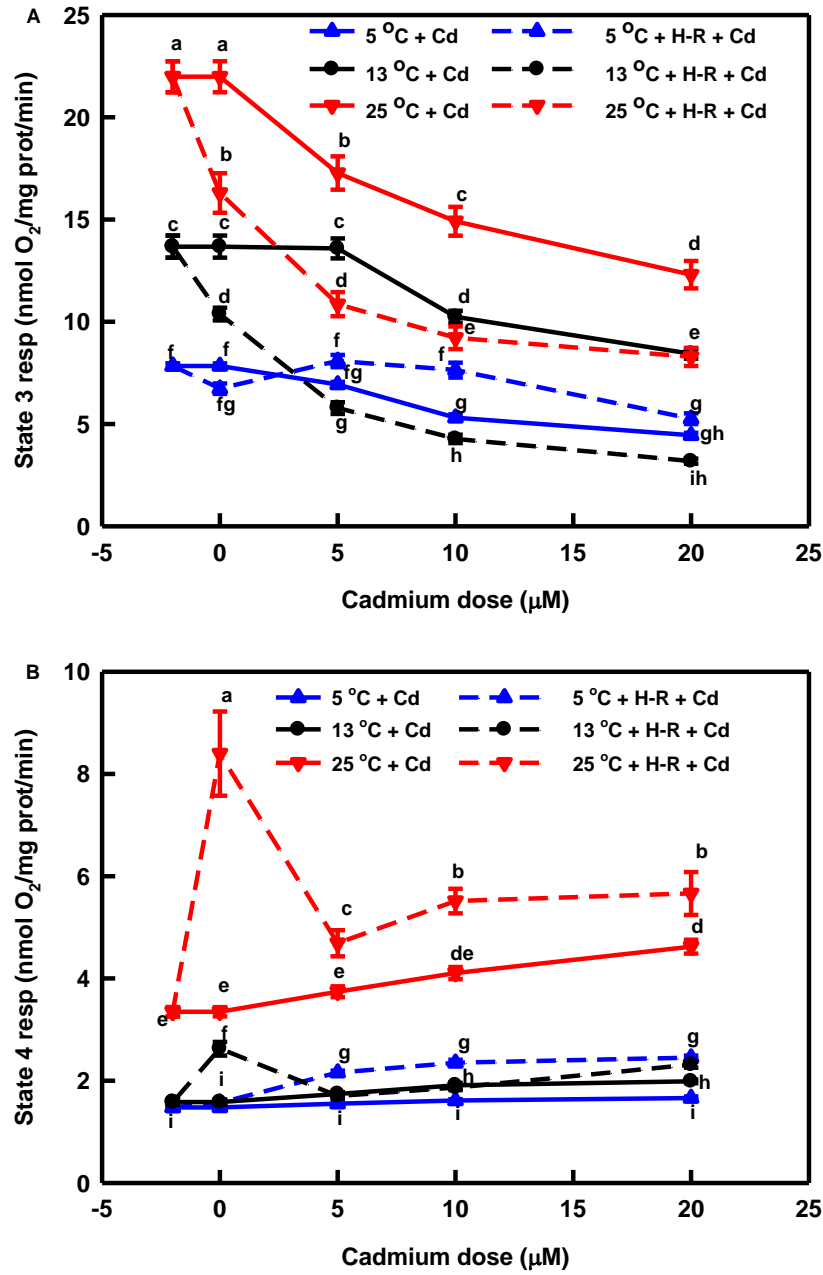


Figure 5.1: The interactions of temperature, H-R and Cd on state 3 (A) and state 4 (B) respiration rates. Isolated mitochondria were exposed to Cd (0, 5, 10 and 20 μM) and

Hypoxia (5 min) followed by reoxygenation (H-R) at 5, 13 and 25 °C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

Table 5.1: The interactions of temperature, H-R and Cd on state 3 Q_{10} . Isolated mitochondria were exposed to Cd (0, 5, 10 and 20 μ M) and Hypoxia (5 min) followed by reoxygenation (H-R) at 5, 13 and 25 $^{\circ}$ C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

	5-13 $^{\circ}$ C				13-25 $^{\circ}$ C			
	Control	Cd	H-R	Cd x H-R	Control	Cd	H-R	Cd x H-R
5 μ M Cd	2.03 \pm 0.06 ^a	2.32 \pm 0.07 ^a	1.73 \pm 0.07 ^b	0.66 \pm 0.03 ^e	1.47 \pm 0.02 ^{bc}	1.22 \pm 0.02 ^d	1.46 \pm 0.07 ^{bc}	1.71 \pm 0.11 ^b
10 μ M Cd	2.03 \pm 0.06 ^a	2.28 \pm 0.08 ^a	1.73 \pm 0.07 ^b	0.49 \pm 0.05 ^e	1.47 \pm 0.02 ^{bc}	1.37 \pm 0.04 ^c	1.46 \pm 0.07 ^{bc}	1.92 \pm 0.15 ^a
20 μ M Cd	2.03 \pm 0.06 ^a	2.24 \pm 0.11 ^a	1.73 \pm 0.07 ^b	0.54 \pm 0.03 ^e	1.47 \pm 0.02 ^{bc}	1.37 \pm 0.06 ^c	1.46 \pm 0.07 ^{bc}	2.24 \pm 0.16 ^a

Table 5.2: The interactions of temperature, H-R and Cd on state 4 Q₁₀. Isolated mitochondria were exposed to Cd (0, 5, 10 and 20 µM) and Hypoxia (5 min) followed by reoxygenation (H-R) at 5, 13 and 25 °C. Data are means ± SEM (n = 5). Points with different letters are statistically different from each other (three-way ANOVA with Tukey's HSD, P<0.05).

	5-13 °C				13-25 °C			
	Control	Cd	H-R	Cd x H-R	Control	Cd	H-R	Cd x H-R
5µM Cd	1.09±0.02 ^c	1.16±0.01 ^c	1.91±0.10 ^b	0.74±0.04 ^d	1.87±0.05 ^b	1.89±0.04 ^b	2.68±0.32 ^a	2.34±0.15 ^a
10µM Cd	1.09±0.02 ^c	1.23±0.02 ^c	1.91±0.10 ^b	0.75±0.04 ^d	1.87±0.05 ^b	1.89±0.03 ^b	2.68±0.32 ^a	2.47±0.11 ^a
20µM Cd	1.09±0.02 ^c	1.25±0.02 ^c	1.91±0.10 ^b	0.93±0.03 ^d	1.87±0.05 ^b	2.02±0.04 ^b	2.68±0.32 ^a	2.11±0.14 ^b

Figure 5.2

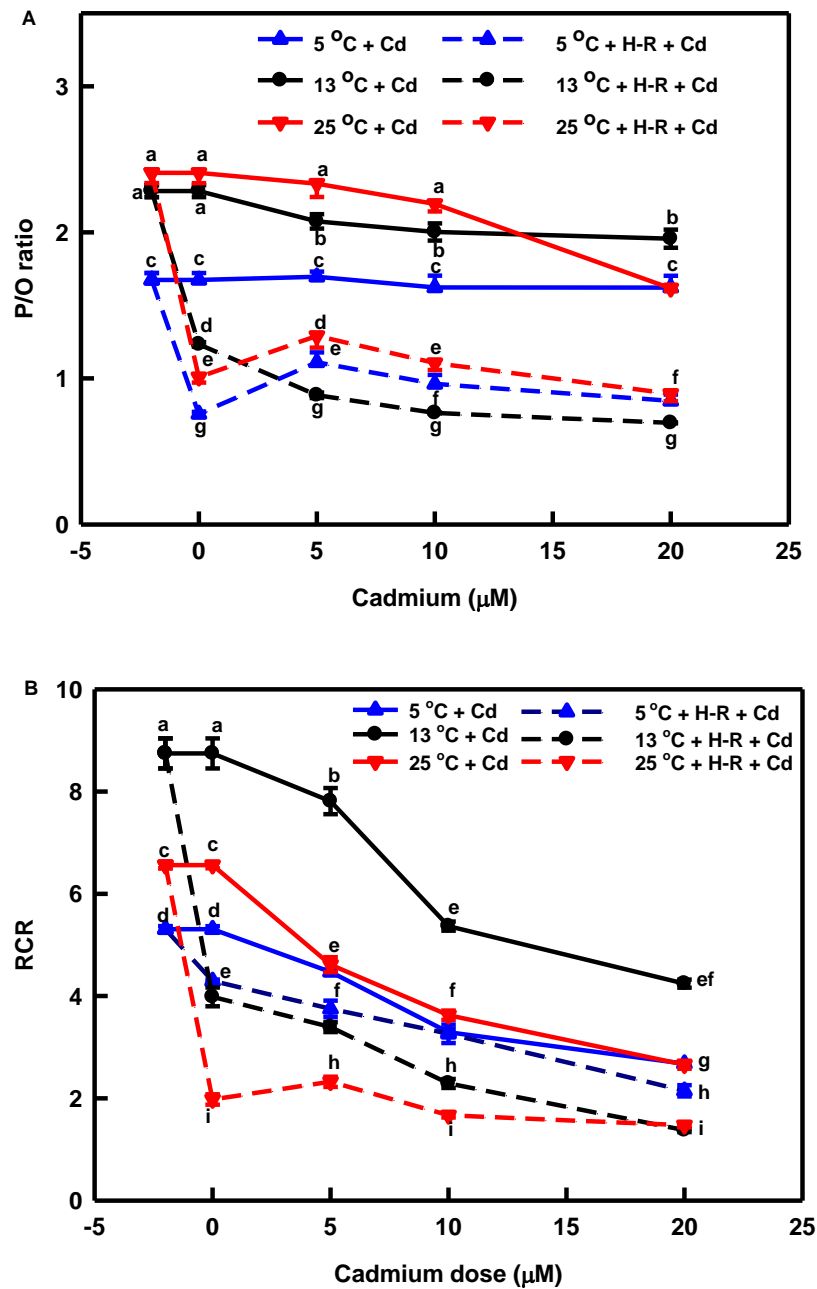


Figure 5.2: The interactions of temperature, H-R and Cd on P/O ratio (A) and RCR (B).

Isolated mitochondria were exposed to Cd (0, 5, 10 and 20 μM) and Hypoxia (5 min)

followed by reoxygenation (H-R) at 5, 13 and 25 °C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

Figure 5.3

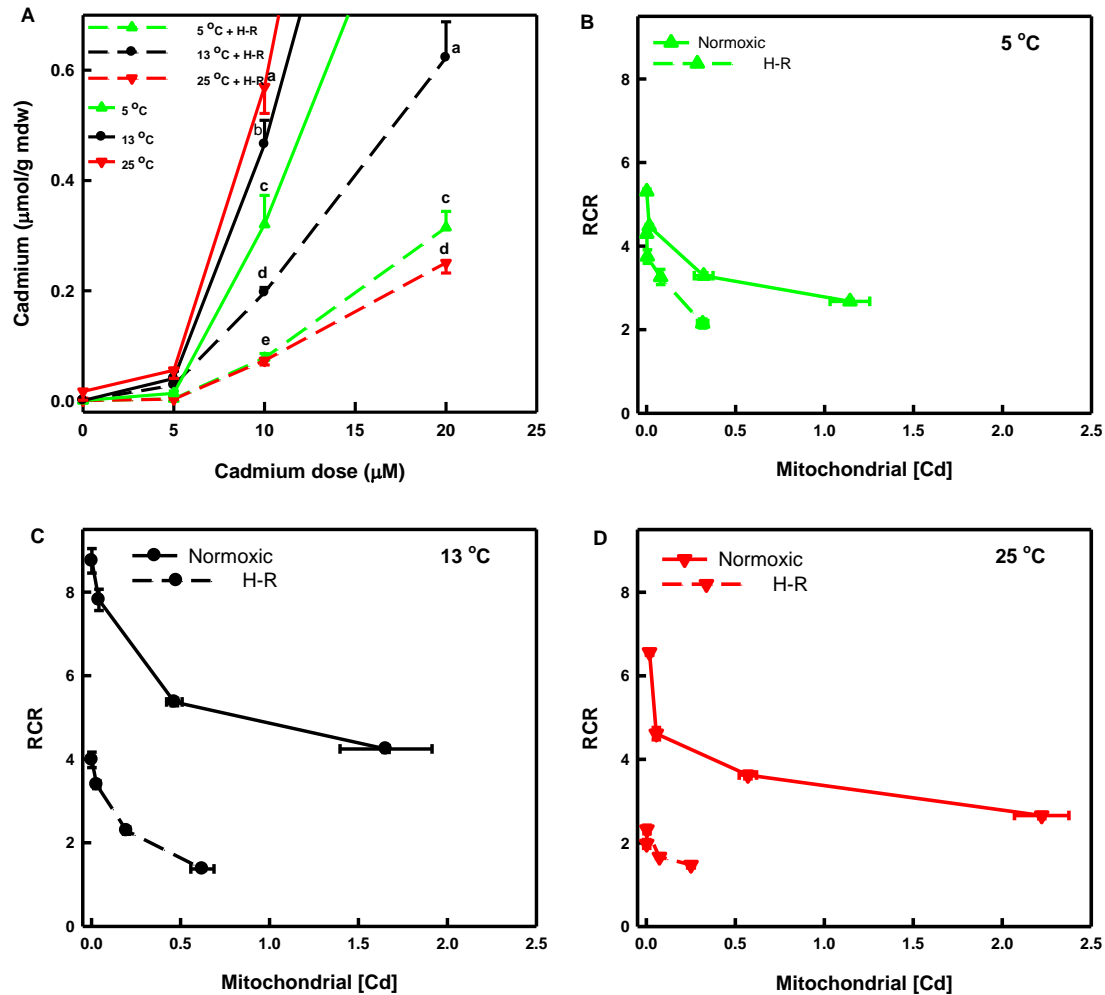


Figure 5.3: The interactions of temperature, H-R and Cd on metal accumulation (A) and relationship between RCR and Cd accumulation at 5 °C (B), 13 °C (C) and 25 °C (D).

Isolated mitochondria were exposed to Cd (0, 5, 10 and 20 μM) and hypoxia (5 min) followed by reoxygenation (H-R) at 5, 13 and 25 °C. Data are means \pm SEM ($n = 5$). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

5.4.3 Interactions of Cd and H-R on mitochondrial volume homeostasis

All treatments showed some swelling but those exposed to 50 μ M Cd (both normoxic and H-R) showed a short period of contraction before swelling (Fig 5.4A). Ca (positive control) caused rapid swelling that peaked earlier than all of the other treatments. H-R, Ca, H-R + Ca, and H-R + 50 μ M Cd caused the most swelling; these 4 treatments did not differ from one another at 30 minutes (Fig 5.4B). The interaction term 'H-R \times treatment' on the amplitude of mitochondrial swelling after 30 min was significant ($F_{3,32}=8.97$, $P=0.0002$). This significant interaction was due to the effect of 5 μ M Cd with H-R – 5 μ M would reduce the swelling caused by H-R to control levels.

5.4.4 Modulation of complex I A \leftrightarrow D transition by H-R and Cd

Fig. 5.5 shows the effect of H-R and Cd on CI A \leftrightarrow D transition. In the absence of NADH mitochondrial respiration was minimal. The addition of NADH stimulated respiration and the addition of NEM (blocking conversion of CI from the D \rightarrow A form) reduced respiration by 49% suggesting that about 49% of CI was in the A-form at the start of the trial and that 51% of the respiration during the trial was due to conversion of D \rightarrow A. H-R markedly reduced respiration and almost completely blocked the conversion of D \rightarrow A during the trial; only 15% of the respiration was due to D \rightarrow A. Cd (5 μ M) also reduced respiration but the fraction contributed by the conversion of D \rightarrow A during the trial (57%) was almost the same as for the control. Respiration after H-R when Cd was present was not as marked as with H-R alone. Moreover, presence of Cd increased the fraction contributed by the conversion of D \rightarrow A during the trial to 68% so that the absolute amount of activity was almost the same as the control (Fig. 5.5B). Thus H-R blocked the conversion of D \rightarrow A almost completely but 5 μ M Cd prevented this blockage. While respiration induced by H-R + NEM was similar to that

due to H-R alone, respiration resulting from Cd + NEM exposure was less than that due to Cd alone. Lastly, exposure to Cd + H-R + NEM resulted in lower respiration rate compared with Cd + H-R.

Figure 5.4

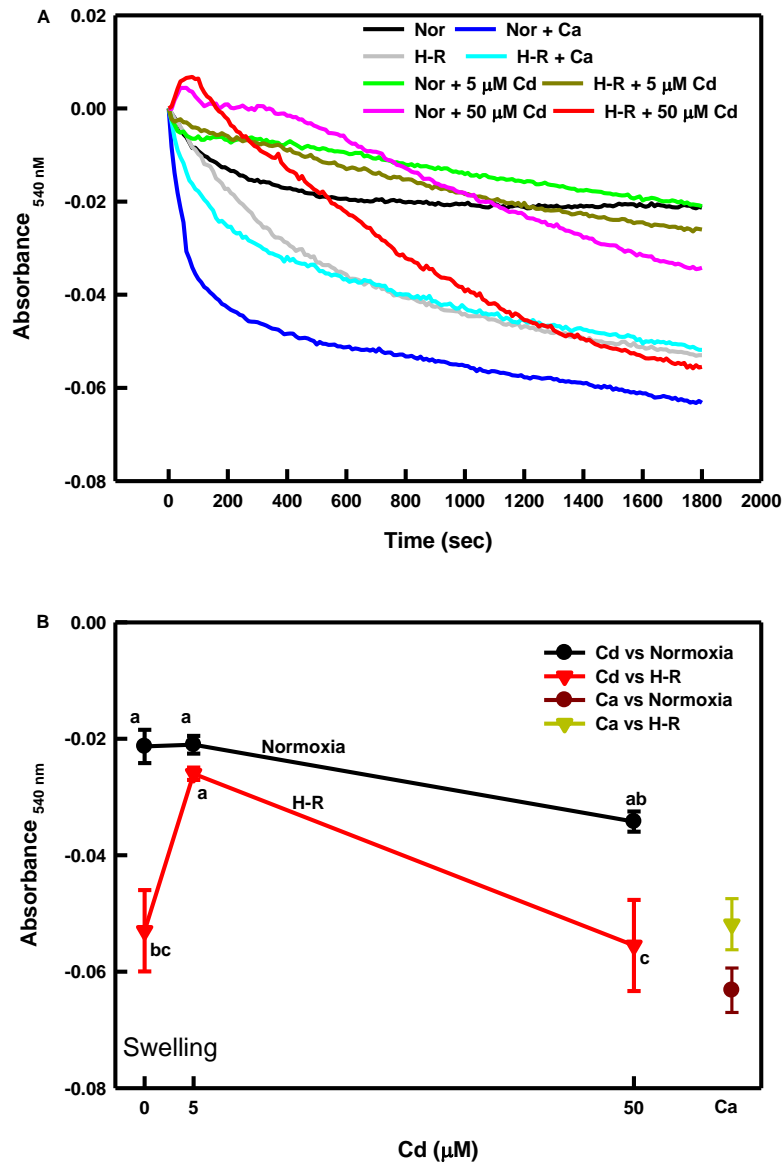


Figure 5.4: The interactions of Cd and H-R on mitochondria volume. Swelling kinetics (A) and swelling amplitude (B). Energized mitochondria in MRB were exposed to either normoxic or 5min of hypoxia followed by reoxygenation (H-R) at 13 °C in respirometry cuvettes. On the microplate to monitor swelling, they subsequently were exposed to Cd (0, 5 and 50 μM) or 200 μM Ca as positive control at 20 °C. Swelling was monitored every 10 s

for 30 min as absorbance changes at 540 nm and the kinetics and terminal amplitude of volume changes after 30 min shown. Data in panel A are means while in panel B they are means \pm SEM ($n = 5$). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

Figure 5.5

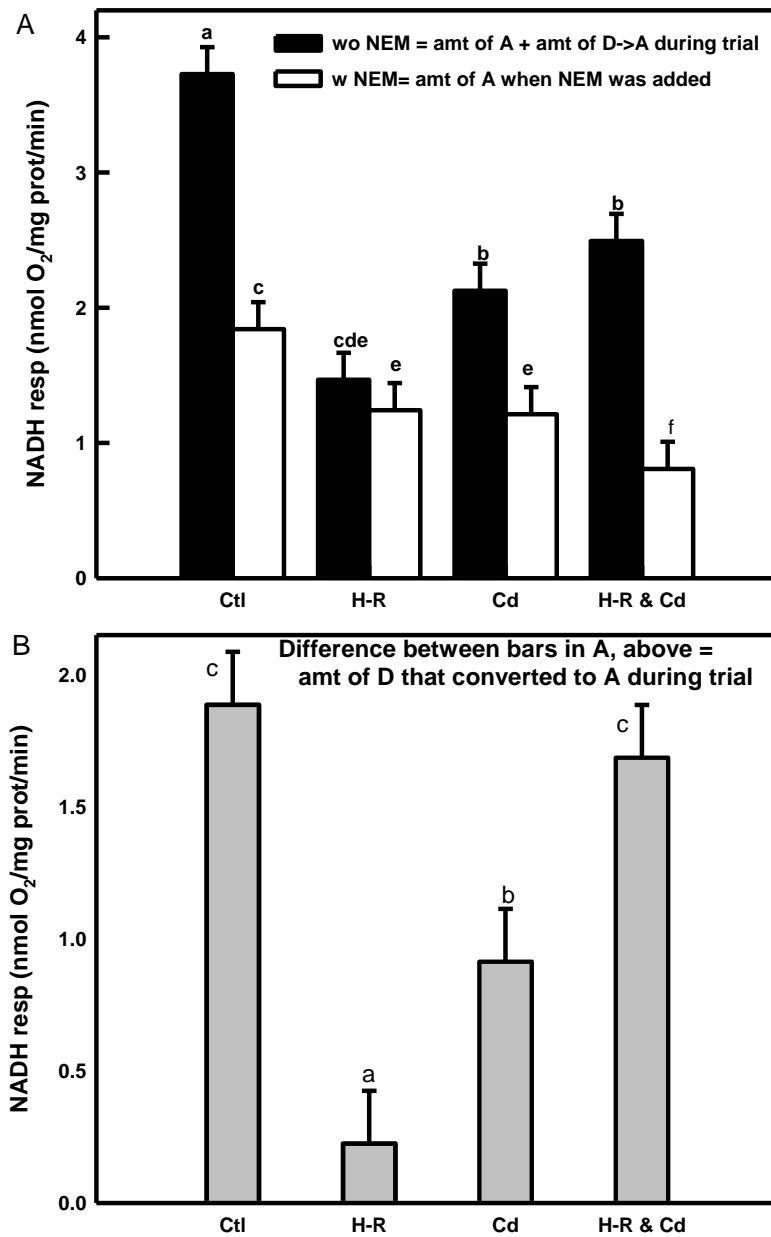


Figure 5.5: The effect of Cd and H-R on complex I A↔D form transition. NADH (350 μ M) stimulated respiration (with [w] and without [wo] NEM (A). Amount of D converted to A are shown in B. Mitochondria with disrupted membranes were exposed to normoxia (Ctl) wo NEM, Ctl w NEM, H-R wo NEM, H-R w NEM, Cd wo NEM, Cd w NEM, H-R + Cd wo

NEM and H-R + Cd w NEM, with the measurement of complex I activity. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

5.4.5 Interactions of Cd and H-R on mitochondrial membrane potential ($\Delta\Psi_m$)

Energizing mitochondria resulted in the development of the membrane potential that was dissipated by Cd dose-dependently (Fig. 5.6A and B) in a first-order manner. With 100 μ M Cd, the dissipation of the membrane potential was not significantly different from that caused by FCCP (Fig. 5.6B). The treatment of mitochondria with EGTA, NAC or vitamin E reduced ($F_{6,28}=76$, $P<0.0001$) 100 μ M Cd-induced dissipation of $\Delta\Psi_m$ with EGTA being the most protective (Fig. 5.6D).

Energized mitochondria post H-R exhibited slightly lower $\Delta\Psi_m$ relative to their normoxic counterparts (Fig. 5.7A and B). The interactions of Cd with H-R exacerbated ($F_{6,28}=105$, $P<0.0001$) the $\Delta\Psi_m$ dissipating effect of H-R (Fig. 5.7B). EGTA, NAC and vitamin E all protected against the $\Delta\Psi_m$ dissipating effect of combined H-R and Cd exposure with EGTA being the most protective in a manner very similar to Cd under normoxic conditions (Fig. 5.7C and D).

5.4.6 Interactions of Cd and H-R on mitochondrial H_2O_2 production

When antimycin A was absent (Fig. 5.8), neither the interaction term 'Cd \times H-R ($F_{1,19}=2.57$, $P=0.13$) nor the H-R effect were not significant ($F_{1,19}=0.82$, $P=0.38$). However there was a significant increase in H_2O_2 production with Cd ($F_{1,19}=52.35$, $P<0.001$). When antimycin A was present (Fig. 5.8), again the interaction term 'Cd \times H-R was not significant ($F_{1,19}=2.64$, $P=0.12$). However, in this case the Cd effect was significant ($F_{1,19}=42.87$, $P<0.001$) with an increase in Cd resulting in an increase in H_2O_2 production. H-R increased the effect of Cd so that H_2O_2 production was about 4 times higher after H-R ($F_{1,19}=41.21$, $P<0.001$) than for the normoxic condition (Fig. 5.8).

Figure 5.6

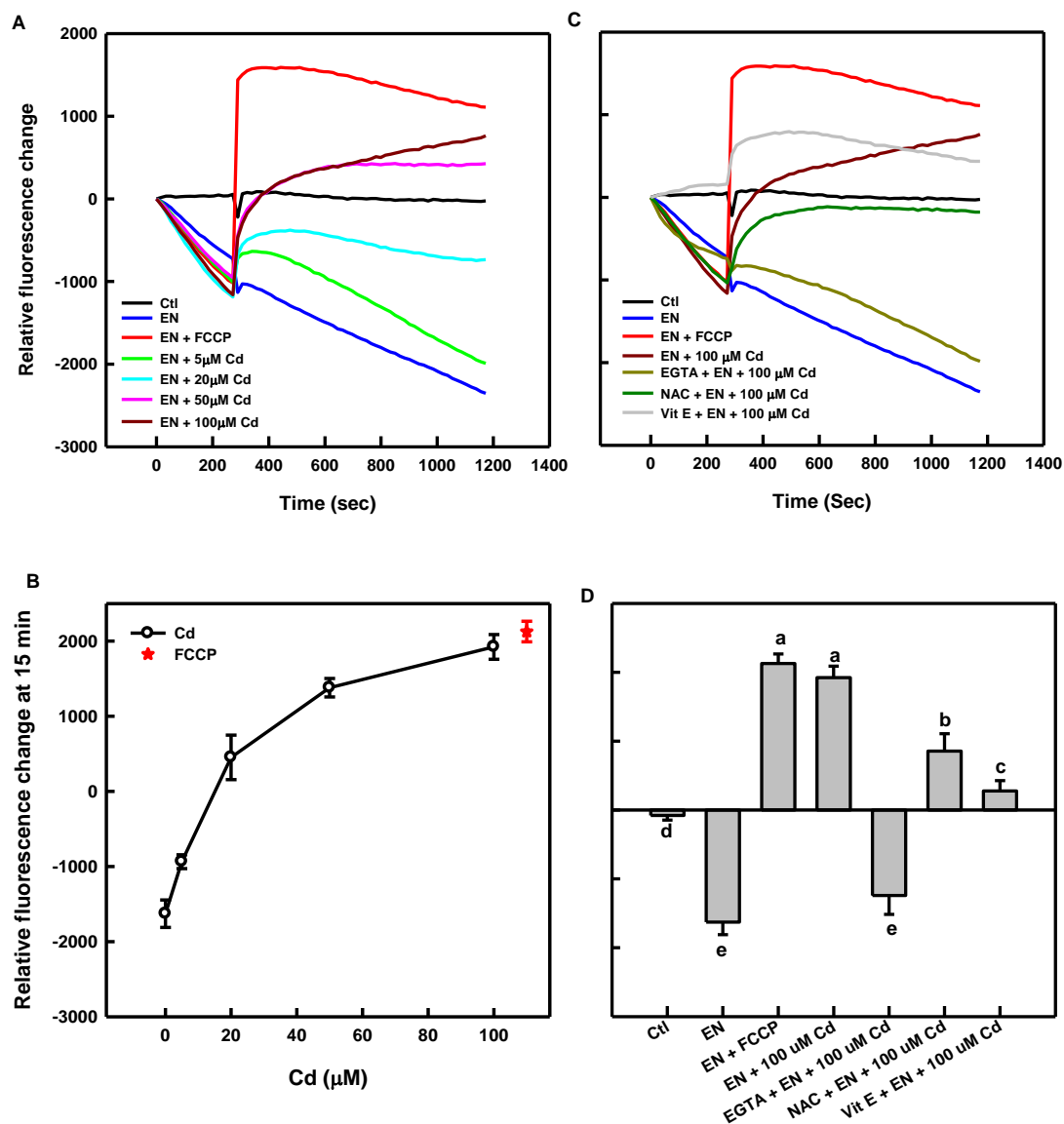


Figure 5.6: The effects of Cd on mitochondrial membrane potential. Kinetics of dose response of Cd (A), amplitude of fluorescence change for Cd dose response (B), kinetics of chelator and antioxidant effects on Cd dissipation of $\Delta\Psi_m$ (C) and amplitude of fluorescence change of chelator and antioxidant effects on Cd dissipation of $\Delta\Psi_m$ (D). Mitochondria were exposed to control without energization (Ctl), energization (EN), energization and FCCP (EN

+ FCCP), energization and 5 μM Cd (EN + 5 μM Cd), energization and 20 μM Cd (EN + 20 μM Cd), energization and 50 μM Cd (EN + 50 μM Cd), energization and 100 μM Cd (EN + 100 μM Cd), energization + EGTA + 100 μM Cd (EN + EGTA + 100 μM Cd), energization + NAC + 100 μM Cd (EN + NAC + 100 μM Cd) and energization + Vit E + 100 μM Cd (EN + Vit E + 100 μM Cd). Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, $P < 0.05$).

Figure 5.7

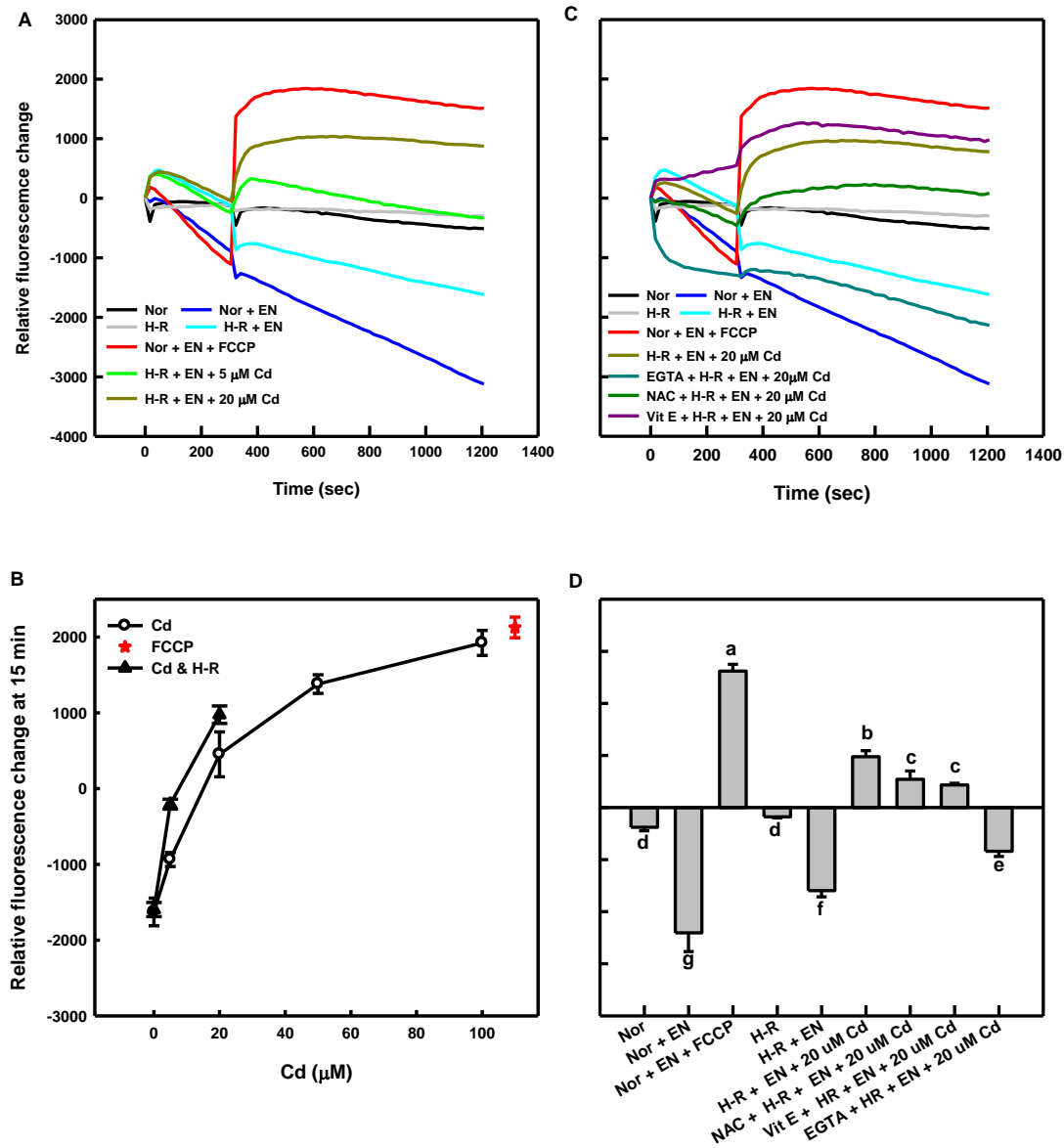


Figure 5.7: The interactions of Cd and H-R on mitochondrial membrane potential ($\Delta\Psi_m$).

Kinetics of $\Delta\Psi_m$ change (A), amplitude of $\Delta\Psi_m$ change (B), effects of EGTA and antioxidant on kinetics of $\Delta\Psi_m$ change (C) effects of EGTA and antioxidant on amplitude of $\Delta\Psi_m$ change (D). The treatment groups were normoxia without energization (Nor), normoxia with

energization (Nor + EN), normoxia with energization and FCCP (Nor + EN + FCCP), hypoxia reoxygenation without energization (H-R), hypoxia-reoxygenation with energization (H-R + EN), hypoxia-reoxygenation with energization and 5 μ M Cd (H-R + EN + 5 μ M Cd), hypoxia-reoxygenation with energization and 20 μ M Cd (H-R + EN + 20 μ M Cd), hypoxia-reoxygenation with energization + NAC + 20 μ M Cd (H-R + EN + NAC + 20 μ M Cd), hypoxia-reoxygenation with energization + Vit E + 20 μ M Cd (H-R + EN + Vit E + 20 μ M Cd) and hypoxia-reoxygenation with energization + EGTA + 20 μ M Cd (H-R + EN + EGTA + 20 μ M Cd). Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, P<0.05).

Figure 5.8

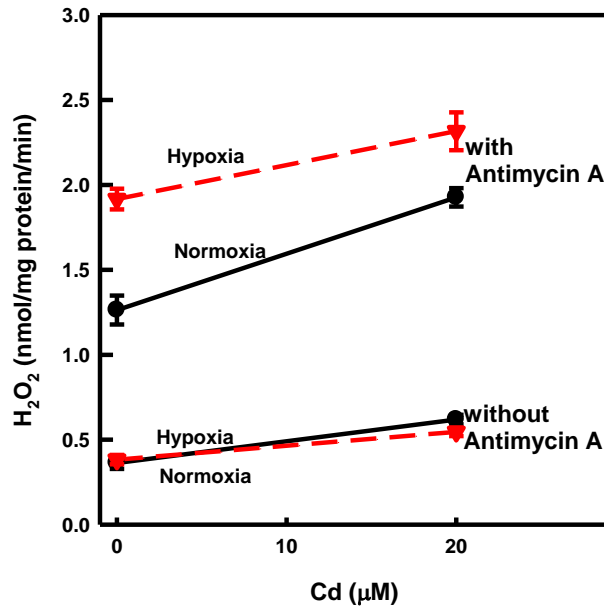


Figure 5.8: The interactions of Cd and H-R on mitochondrial H₂O₂ production with and without antimycin A (ANT). Treatment groups were control (Ctl), hypoxia-reoxygenation (H-R), 20 μM Cd, H-R + 20 μM Cd, ANT, H-R + ANT, 20 μM Cd + ANT and H-R + 20 μM Cd + ANT. Data are means ± SEM (n = 5). Points with different letters are statistically different from each other (ANOVA with Tukey's HSD, P<0.05).

5.4.7 Interactions of Cd and H-R on mitochondrial ultrastructure

Mitochondria exposed to Cd and/or H-R exhibited varied degrees of structural alterations depending on the stressor (Fig. 9). Specifically, mitochondria exposed to Cd at low concentration (5 μ M) showed signs of matrix contraction with the organelles assuming rod configurations. On the other hand, mitochondria exposed to 20 μ M Cd were donut shaped with electron dense matrix and visible signs of swelling. Exposure of mitochondria to 5 min H-R resulted in loss of matrix density and swelling. Co-exposure of mitochondria to H-R and 5 μ M Cd reduced the swelling and restored the matrix density. In contrast, co-exposure of H-R and 20 μ M Cd worsened the effects of H-R on mitochondrial structure producing swelling and translucent matrix. Overall, mitochondrial diameter increased with the exposure to Cd ($F_{2,114}=53$, $P<0.0001$) and H-R ($F_{1,114}=81$, $P<0.0001$). More importantly, the interaction term between Cd and H-R was significant ($F_{2,114}=14$, $P<0.0001$) largely because there was no difference between normoxic and H-R for 5 μ M Cd (Fig. 5.10).

Figure 5.9

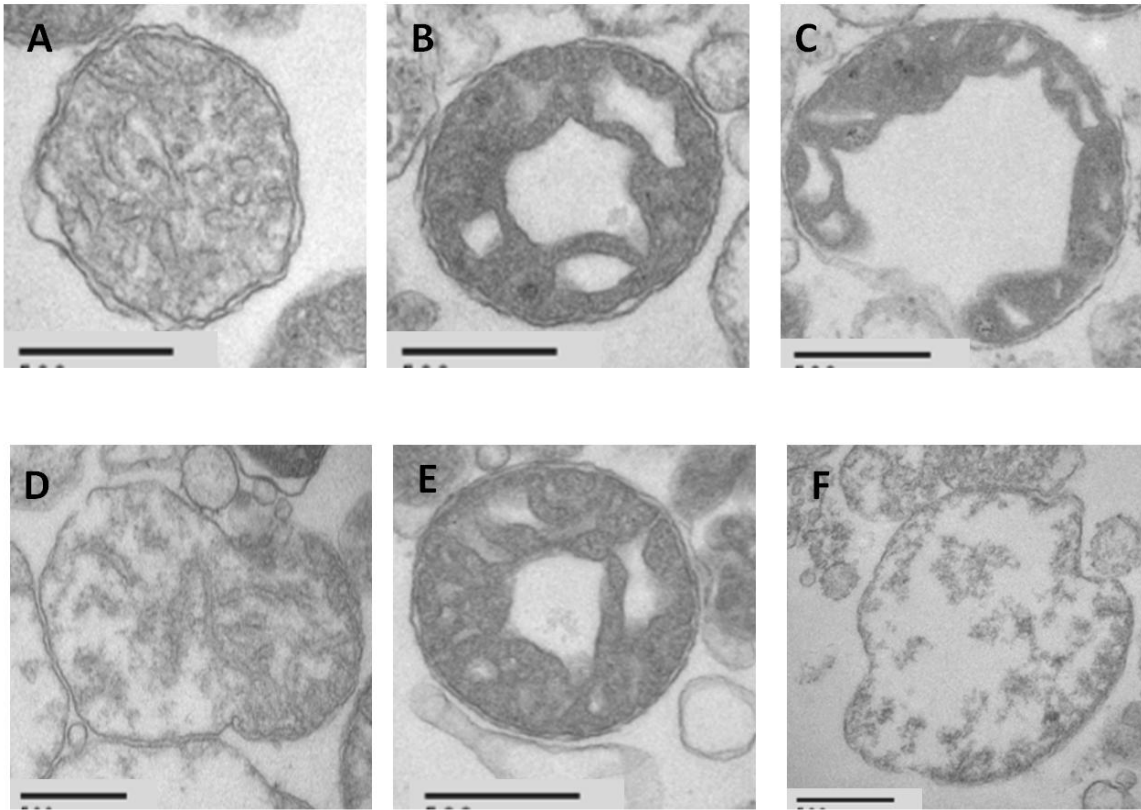


Figure 5.9: The interactions of Cd and H-R on mitochondrial structure. Control (Ctl) (A), 5 μM Cd (B), 20 μM Cd (C), H-R (D), H-R + 5 μM Cd (E) and H-R + 20 μM Cd (F). Data are means \pm SEM (n = 50).

Figure 5.10

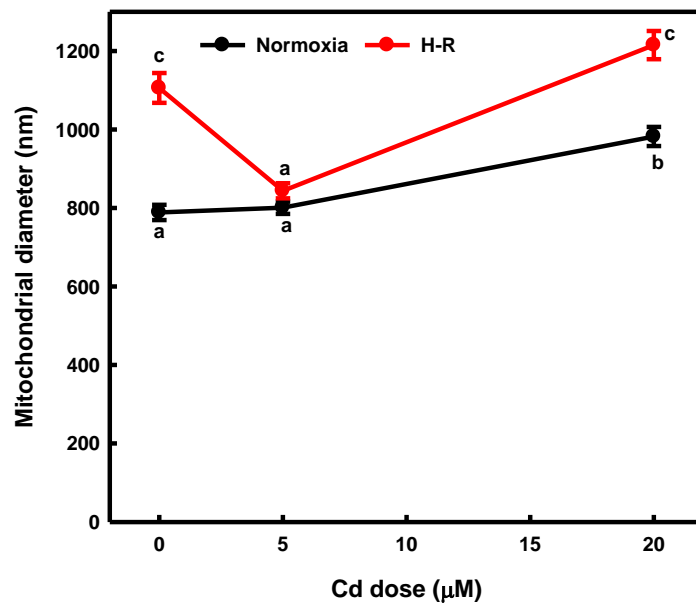


Figure 5.10: The interactions of Cd and H-R on mitochondrial diameter. Data are means \pm SEM (n = 50). Points with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, $P < 0.05$).

5.5 DISCUSSION

The combined effects of Cd, H-R and temperature were either greater or less than their individual or binary effects on mitochondrial function. To explain the ternary effects of Cd, H-R and temperature I included data on the binary interactions of temperature and Cd (Chapter 3). My analysis revealed that while exposure to these stressors differently altered a range of mitochondrial functional traits and structure, changes in metal accumulation explained only in part, the synergistic effect of Cd with temperature and/or H-R. Overall there were additive, antagonistic or synergistic interactive effects among the three stressors and the nature of these interactions depended on the endpoint measured.

5.5.1 Temperature modulates mitochondrial response to combined Cd and H-R-induced stress

Temperature significantly influenced mitochondrial response to individual and combined effects of Cd and H-R on maximal CI respiration. My data indicate that Cd and H-R synergistically reduced CI state 3 respiration and that the threshold doses for Cd toxicity progressively shifted to lower values at high temperature. A similar result has been observed with oysters (hypoxia-tolerant species) exposed to combined anoxia-reoxygenation and Cd stress *in-vivo* (Kurochkin et al., 2009). In my study, Cd alone, H-R alone and Cd + H-R markedly inhibited CI maximal respiration rate at 25 and at 13 °C. In contrast, at 5 °C, Cd + H-R did not alter state 3 respiration suggesting that at low temperature CI maximum respiration was resilient to the effects of Cd + H-R. It is possible that at low temperature mitochondrial membrane phospholipids and proteins were less sensitive to Cd + H-R induced stress (Jones, et al., 1986; Fadhaoui and Couture, 2016).

Individually, the stressors I tested stimulated CI basal respiration in agreement with my previous studies and those of others for Cd (Chapter 2), temperature (Abele et al., 2002; Chamberlin et al., 2004; Chapter 3) and H-R (Schild et al., 2003; Bosetti et al., 2004; Chapter 2). Stimulated basal respiration (proton leak) indicates Cd, H-R and thermal stress reduced the efficiency of ATP production (Bishop et al., 2002; Abele et al., 2007). I demonstrated that the combined effect of Cd and H-R on basal respiration depended on the temperature: at 13 and 25 °C the 5 µM Cd reduced the stimulation of basal respiration by H-R. In contrast, Cd at higher doses combined with H-R increased the basal respiration suggesting that Cd and H-R interact on basal respiration via a common mechanism, likely involving alteration of IMM permeability. It appears that low doses of Cd + H-R at high temperature reduce the permeability of the IMM to protons while high doses of the metal combined with H-R increase it. In contrast, at low temperature (5 °C) Cd acted in synergy with H-R to stimulate the basal respiration above their individual effects. This stimulation of basal respiration at low temperature may in part be due to the effect of Cd and/ or H-R on the mitochondrial membrane phospholipid and proteins (Jones et al., 1986; Fadhaoui and Couture, 2016). However, these changes were observed in acclimated conditions and may not apply to acute temperature change. Additional study on the membrane phospholipid and proteins is required to increase our understanding of the mechanisms of Cd and/or H-R induced stress following acute temperature change.

The observation that individually Cd, temperature, and H-R reduced P/O ratio is consistent with several earlier reports (Sokolova et al., 2005; Adiele et al., 2010; Sappal et al., 2015; Chapter 2, Chapter 3). Additionally, I showed that temperature exacerbated the inhibitory effect of combined Cd and H-R on phosphorylation efficiency (P/O ratio) possibly due to

greater inhibition of the ATP synthase and/or increased proton and electron leaks. Similar to the P/O ratio, RCR was reduced by all the 3 stressors individually in agreement with previous findings for Cd (Sokolova 2004; Adiele et al., 2010; Chapter 2), H-R (Navet et al., 2006; Hoffman et al., 2007; Chapter 2) and temperature (Lemieux et al., 2010; Iftikar and Hickey, 2013; Chapter 3). When combined, the stressors worsened the reduction of RCR suggesting additivity/synergy of their toxic effects. Previous studies with binary combination reported additive/synergistic interactions of Cd \times temperature (Sokolova, 2004; Cherkasov et al., 2010; Chapter 3), H-R \times Cd (Kurochkin et al., 2009; Chapter 2) and H-R \times temperature (Chapter 4). In this study the effects of Cd \times H-R \times temperature interaction reduced the RCR to a greater extent than the binary combinations. The greater reduction of RCR induced jointly by the 3 stressors suggests increased mitochondrial sensitivity to Cd in the presence of H-R and temperature or vice versa. Consistent with greater reduction of RCR, the dysregulation of mitochondrial volume, structural damage, dissipation of membrane potential and ROS production were all exacerbated by combined exposure to the stressors.

5.5.2 Mitochondrial Cd accumulation is modulated by Cd, temperature, and H-R

At the organismal (Heugens et al., 2003; Hallare et al., 2005; Khan et al., 2006; Sassi et al., 2010) and mitochondrial (Cherkasov et al., 2007; Chapter 3) levels, Cd toxicity was shown to increase with a rise in temperature. This effect has been associated with increased Cd uptake /accumulation (Köck et al., 1996; Heugens et al., 2003; Cherkasov et al., 2010; Dorts et al., 2012; Chapter 3) but not always (Hallare et al., 2005; Bao et al., 2008; Vergauwen et al., 2013). This discrepancy may be due to the differences in experimental conditions and the animal species/experimental models that were used in the investigations. In particular, in some studies the animals were acclimated to different temperatures whereas in others, like

my present study, the effects of acute temperature change were studied *in vitro*. Regardless, a possible mechanism for the increased Cd accumulation at high temperature is increased permeability of mitochondrial membranes to Cd (Chapter 3). Moreover, it is plausible that a rise in temperature would increase the activity of mitochondrial Ca^{2+} uniporter that has been shown to be involved in Cd uptake (Lee et al., 2005b; Adiele et al., 2012b).

My study also revealed that the combination of H-R and temperature stress increased the sensitivity of mitochondria to Cd-induced damage. Notably, my study clearly linked mitochondrial functional impairment with Cd accumulation in the organelles at the 3 temperatures I tested (Fig. 5.3B, C and D). While the mechanisms for the enhancement of Cd toxicity by combined temperature and H-R stress remain to be characterized, my study ruled out increased metal accumulation. It is possible that H-R and/or temperature alter the conformation of mitochondrial macromolecules e.g., membrane phospholipids and proteins (Kraffe et al., 2007), rendering them more metal-sensitive. Moreover, both H-R (Navet et al., 2006; Chen et al., 2008) and temperature (Abele et al., 2002; Iftikar and Hickey, 2013; Chung and Schulte, 2015) stimulate ROS production that would work in concert with Cd to exacerbate mitochondrial dysfunction.

5.5.3 Low doses of Cd protected against H-R-induced alterations of mitochondrial volume and preserved mitochondrial complex I A form activity

In the present study mitochondrial dysfunction induced by Cd and H-R was associated with volume changes indicating altered IMM permeability. These results are consistent with earlier studies with Cd (Lee et al., 2005a; Lee et al., 2005b; Chapter 3) and H-R (Ozcan et al., 2001; Chapter 4). Interestingly, low Cd doses protected against H-R-induced swelling while high doses exacerbated the mitochondrial swelling induced by H-R. The mechanisms through

which low doses of Cd protect against H-R-induced swelling are not known but it appears that low doses of this metal minimize changes in IMM permeability and/or reduce ROS-induced membrane phospholipids oxidation following exposure to H-R. This would ultimately reduce the influx of solute and water into the mitochondria thereby minimizing swelling. These findings, together with my previous report that Cd at low dose protected against H-R-induced mitochondrial dysfunction by enhancing the activity of CI (Chapter 2), cement the hypothesis that Cd at low doses may induce beneficial responses (hormesis) during stress.

Previously I speculated that the protective effect of low doses of Cd against H-R-induced mitochondrial impairment was due to the prevention of conversion of A-form of CI to the D-form (Chapter 2). First, I showed that rainbow trout liver mitochondrial CI undergoes $A \leftrightarrow D$ transformation (Fig. 5.5). While not universal, this transformation has been demonstrated in mitochondria of several animal species including the common carp (*Cyprinus carpio*) (Maklashina et al., 2003), pig (*Sus scrofa*) (Grivennikova et al., 2001), frog (*Rana spp*) (Maklashina et al., 2003), and rat (*Rattus rattus*) (Grivennikova et al., 2001; Maklashina et al., 2002). Second, H-R converted CI A- to D-form consistent with the observation that lack of oxygen promotes the accumulation of the D-form (Maklashina et al., 2004; Gorenkova et al., 2013). However, unlike other divalent metals (e.g., Ni, Co, Mn, Ca, Mg) that slow the conversion of the D- to the A-form (Kotlyar et al., 1992; Babot et al., 2014), Cd blunted the transition of the A- to D-form resulting in higher levels of the A-form, particularly after H-R. This is clearly demonstrated by comparing NADH-stimulated respiration evoked by NEM that permanently modifies the D-form preventing its transition to the A-form (Gavrikova and Vinogradov, 1999) with the respiration rates measured after Cd and H-R treatments (Fig.

5.5). However, the concentration of the other divalent cations used is usually in the mM range, 3 orders of magnitude greater than the low dose of Cd that I used. To the best of my knowledge this is the first report showing that low doses of Cd blunt conversion of CI A-form to D-form following H-R thereby improving CI activity (Chapter 2). While preservation of CI activity may generally be viewed as beneficial, it would be detrimental in mitochondria exposed to hypoxia because a high proportion of C1 D- relative to A-form reduces both the surge in respiration of downstream enzymes and ROS production on re-oxygenation (Babot et al., 2014). Lastly, my study showed that H-R inhibits the conversion of D→A but that 5 μ M Cd unblocks this inhibition so that the D form continues to convert to the A form during the trial.

5.5.4 Antioxidants and metal chelators protect against Cd and/or H-R induced dissipation of $\Delta\Psi_m$

The mitochondrial dysfunction induced by Cd and/or H-R resulted in part from $\Delta\Psi_m$ dissipation consistent with earlier studies (Li et al., 2003; Lopez et al., 2006; Belyaeva et al., 2006; Mao et al., 2011). Here I show that the combined effects of Cd and H-R exacerbated $\Delta\Psi_m$ dissipation, possibly due to synergistic impact on substrate oxidation and proton leak subsystems and/or IMM permeability. In this regard, Cd and H-R individually have been shown to inhibit enzymes involved in substrate oxidation (Lopez et al., 2006; Cherkasov et al., 2007; Adiele et al., 2012a; Chapter 2), increase proton leak (Bosetti et al., 2004; Navet et al., 2006; Chapter 2) and cause mitochondria to swell (Morin et al., 2004; Lee et al., 2005a; Chapter 3). The observation that EGTA inhibited Cd-induced $\Delta\Psi_m$ dissipation confirms a direct role of Cd. Moreover, the protection afforded by antioxidants (vitamin E and NAC) against Cd and/or H-R-induced dissipation of $\Delta\Psi_m$ suggests a role of oxidative stress probably due to inhibition of the ETS by ROS (Navet et al., 2006; Lopez et al., 2006;

Cherkasov et al., 2007; Adiele et al., 2012a; Chapter 2). I confirmed a role of oxidative stress by direct measurement of H_2O_2 production following exposure to Cd and/or H-R (Fig. 5.8). Furthermore, Cd and H-R acted in synergy to increase mitochondrial ROS production resulting in the worsening mitochondrial dysfunction I observed when the two stressors were combined in the presence of antimycin A. Cd and/or H-R are known to stimulate mitochondrial ROS production by inhibiting the activity and/or imposing conformational changes of the ETS enzymes (Wang et al., 2004; Chen et al., 2008; Gorenkova et al., 2013). Inhibition of the ETS would increase NADH/NAD^+ ratio resulting in a backup of electrons and formation of superoxide radicals (Murphy, 2009; Tomanek, 2015). The excess ROS thus produced would then oxidize membrane lipids and proteins including the TCA and ETS enzymes, resulting in structural damage, changes in IMM permeability and loss of mitochondrial oxidative function.

5.5.5 Exposure to Cd and H-R damages mitochondrial ultrastructure

Cd and H-R altered mitochondrial structure differently in that Cd at high concentration (20 μM) caused contraction of matrix, formation of donut-shaped mitochondria and overall swelling, while H-R caused loss of matrix and cristae resulting in translucent and swollen mitochondria. The formation of donut-shaped mitochondria has been described as a hallmark of mitochondrial oxidative stress (Ahmad et al., 2013), and is associated with reduced mitochondrial function (Hara et al., 2014). Similarly, loss of cristae contributes to reduced mitochondrial function. The combined Cd and H-R exposure revealed that at low dose Cd reduced the H-R induced mitochondrial swelling and restored organelle diameter to control levels while at high dose (20 μM) it exacerbated the structural damage. I speculate that ROS produced following Cd and H-R exposure (Fig. 5.8) were responsible for the mitochondrial

structural and functional changes I observed. For example, oxidation of membrane lipids could alter IMM permeability leading to swelling/contraction and cristolysis, while protein oxidation and loss of matrix content via leaky membranes could explain the reduced matrix density.

5.6 CONCLUSIONS

My hypothesis that the ternary interactions of Cd, temperature and H-R would alter their individual and/or binary effects on mitochondrial bioenergetics was validated in this study. I demonstrated that mitochondria at low temperature (5 °C) were more resilient than those at high temperature to the combined effects of Cd and H-R. The alterations in mitochondrial function were associated with impaired volume homeostasis, CI A to D transition, dissipation of $\Delta\Psi_m$, increased ROS production and loss of structural integrity. Although the effects of Cd and/or H-R were greater at both high and low temperatures, this was not explained by increased Cd accumulation. Overall oxidative stress could explain to a large extent the effects of Cd, H-R and temperature on mitochondrial structure and function.

CHAPTER 6

BIOENERGETIC AND VOLUME REGULATORY EFFECTS OF MITOK_{ATP} MODULATORS PROTECT AGAINST HYPOXIA-REOXYGENATION-INDUCED MITOCHONDRIAL DYSFUNCTION

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Author contributions

C.K., J.O.O. conceived the project, C.K., J.O.O., D.S., designed the study, J.O.O. carried out the experiments and data analysis and wrote the first draft of the article. All authors contributed to the interpretation of results and the editing of the article.

6.1 ABSTRACT

The mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel plays a significant role in mitochondrial physiology and protects against ischemic reperfusion injury in mammals. Although fish frequently face oxygen fluctuations in their environment, the role of mitoK_{ATP} channel in regulating the responses to oxygen stress is rarely investigated in this class of animals. To elucidate whether and how mitoK_{ATP} channel protects against hypoxia-reoxygenation (H-R)-induced mitochondrial dysfunction in fish, I first determined the mitochondrial bioenergetic effects of two key modulators of the channel, diazoxide and 5-hydroxydecanoate (5-HD), using a wide range of doses. Subsequently, the effects of low and high doses of the modulators on mitochondrial bioenergetics and volume under normoxia and after H-R using buffers with and without magnesium and ATP (Mg-ATP) were tested. In the absence of Mg-ATP (mitoK_{ATP} channel open) both low and high doses of diazoxide improved mitochondrial coupling but only the high dose of 5-HD reversed post H-R coupling enhancing effect of diazoxide. In the presence of Mg-ATP (mitoK_{ATP} channel closed) diazoxide at low dose improved coupling post H-R and this effect was abolished by 5-HD at low dose. Interestingly, both low and high doses of diazoxide reversed H-R-induced swelling under mitoK_{ATP} channel open conditions but this effect was not sensitive to 5-HD. Under mitoK_{ATP} channel closed conditions diazoxide at low dose protected the mitochondria from H-R-induced swelling and 5-HD at low dose reversed this effect. In contrast, diazoxide at high dose failed to reduce the swelling caused by H-R and the addition of high dose of 5-HD enhanced mitochondrial swelling. Overall my study showed that in the presence of Mg-ATP both opening of mitoK_{ATP} channels and bioenergetic effects of diazoxide were protective against H-R in fish mitochondria, while in the absence of Mg-ATP only the bioenergetic effect of diazoxide was protective.

6.2 INTRODUCTION

The mitochondrial ATP-sensitive K^+ (MitoK_{ATP}) channel was first identified in rat liver (Inoue et al., 1991) as a highly selective, small conductance K^+ channel located in the inner mitochondrial membrane. Since then it has also been identified in heart (Paucek et al., 1992; Wojtovich and Brookes, 2009), brain (Bajgar et al., 2001; Debska et al., 2001), skeletal muscle (Gurke et al., 2000; Debska et al., 2002), and kidney (Cancherini et al., 2003). Pharmacological modulation of the MitoK_{ATP} channel has been instrumental in elucidating the pathophysiology of ischemia- reperfusion (IR) injury in mammals (Garlid et al., 1997; Jaburek et al., 1998; Grover et al., 2001). In this regard the mitoK_{ATP} channel opener (diazoxide) was found to protect against IR injury (Garlid et al., 1997) and this protection was inhibited by 5-hydroxydecanoate (5-HD), a blocker of the channel (Jaburek et al., 1998). Although the exact mechanisms of protection are still debated, several potential explanations have been proposed (Garlid and Paucek, 2003; Ardehali and O'Rourke, 2005; Costa et al., 2006). First, it is hypothesized that opening of the MitoK_{ATP} channel increases mitochondrial matrix volume through uptake of K^+ from the cytosol into the matrix thus preventing intermembrane space (IMS) contraction (Garlid, 2000; Garlid et al., 2003). Second, opening mitoK_{ATP} channel is believed to cause mild mitochondrial uncoupling that triggers ROS with activation of kinases that protect against H-R/IR injury (Krenz et al., 2002; Andrukhiv et al., 2006). Support for this hypothesis is provided by the finding that ROS scavengers blocked the protection conferred by mitoK_{ATP} (Vanden Hoek et al., 1998; Pain et al., 2000; Baines et al., 2001; Cohen et al., 2001). Third, it has been argued that it is the reduction/inhibition of ROS production that causes protection (Ferranti et al., 2003; Facundo et al., 2007; Kulawiak et al., 2008) while others have suggested that the opening of mitoK_{ATP} channel would result in matrix alkalinisation triggering increased ROS production (Costa et al., 2006). Lastly, it

has been suggested that functions attributed to the mitoK_{ATP} channel may essentially be the effects of pharmacological modulators used to study the effect of the channel on mitochondrial function (Holmuhamedov et al., 1999; 2004; Dröse et al., 2006; Kopustinskiene et al., 2010).

Overall, empirical evidence from the use of pharmacological modulators of mitoK_{ATP} channel suggest two general bases of the mechanisms of protection against IR/H-R-induced deleterious effects: modulation of mitochondrial bioenergetics (Holmuhamedov et al., 1999; 2004; Dröse et al., 2006; Kopustinskiene et al., 2010) and/or volume (Garlid et al., 1997; Jaburek et al., 1998; Costa et al., 2006). However, Garlid (2000) argued that bioenergetic effects of mitoK_{ATP} channel modulators (diazoxide and 5-HD) were observed at high (toxic) concentrations with the channel already open because of the absence of Mg-ATP in the assay buffer. Given the controversies cast above, the present study sought to elucidate the relative contribution of bioenergetic and volume regulation modes of mitoK_{ATP} channel protection against H-R-induced stress with focus on mechanisms not mediated by ROS. First, I predicted that the effects of the mitoK_{ATP} opener (diazoxide) and blocker (5-HD) on mitochondrial respiration and volume would be diametrically opposite and dose-dependent. Second, I predicted that in the absence of Mg-ATP, diazoxide and 5-HD would modulate mitochondrial bioenergetics but not volume. Third, I predicted that low doses of diazoxide in the presence of Mg-ATP would protect against H-R-induced effects only by altering mitochondrial volume. Fourth, I predicted that high doses of both diazoxide and 5-HD in the presence and absence of Mg-ATP would alleviate H-R-induced damage by altering mitochondrial bioenergetics. The only previous studies on the mitoK_{ATP} channel in non-mammals have been concerned with the sarcolemmal mitoK_{ATP} channel; to the best of my

knowledge this is the first study to probe the role of mitoK_{ATP} channel in H-R-induced stress in isolated fish mitochondria.

6.3 MATERIALS AND METHODS

6.3.1 Ethics

The procedures that experimental animals were subjected to were approved (Protocol # 11-034) by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

6.3.2 Fish

Juvenile rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] weighing 150 ± 4.8 g (mean \pm SEM) at sampling were obtained from Ocean Farms Inc., Brookvale, PE, and kept in a 400-l tank supplied with flow-through aerated well water at the Atlantic Veterinary College Aquatic Facility. Water temperature and pH were 10 ± 1 °C and 7.7, respectively. The fish were fed at 1% body weight daily with commercial trout chow (Corey Feed Mills, Fredericton, NB, Canada).

6.3.3 Mitochondrial isolation

To isolate mitochondria, fish were randomly sampled from the tank, stunned with a blow to the head, decapitated and immediately dissected to remove the liver. Mitochondria were isolated according to the method described in chapter 2 and re-suspended in a 1:3 (weight to volume) ratio of mitochondrial respiration buffer [MRB: 10 mM Tris, 25 mM KH₂PO₄, 100 mM KCl, 1 mg/ml bovine serum albumin (BSA, fatty acid free), 2 µg/ml aprotinin, pH 7.3]. Mitochondrial protein concentration was measured by spectrophotometry (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) according to Bradford (1976) with BSA as the standard.

6.3.4 Measurement of mitochondrial respiration

Mitochondrial respiration rates were measured using Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) in 1.5 ml cuvettes after a two-point calibration at 0 and 100% air saturation at the ambient atmospheric pressure. Temperature during the assays was maintained at 13 °C using a recirculating water-bath (Haake, Karlsruhe, Germany). After the calibration, 1.45 ml of MRB and 100 µl of mitochondrial suspension containing 2.2-3.1 mg of protein were loaded into the cuvettes and continuously stirred. To spark the Krebs cycle, 5 mM malate was added and respiration was supported with saturating concentration (5 mM) of glutamate, a complex I substrate. The addition of 250 µM ADP initiated the state 3 respiration which transition to state 4 upon depletion of the ADP. All rates of oxygen consumption were monitored using Logger Pro 3 with Vernier Labpro interface (Vernier Software and Technology, Beaverton, OR, USA). The respiratory control ratio (RCR: ratio of state 3 to state 4) were calculated according to Chance and Williams (1955).

6.3.5 Exposure of mitochondria to hypoxia-reoxygenation (H-R)

The protocol used for the hypoxia-reoxygenation was based on the study in chapter 2. Briefly, mitochondrial complex-I driven oxygen consumption was measured under normoxic conditions as described above. To make the MRB hypoxic, nitrogen gas was bubbled into the cuvettes depleting the partial pressure of oxygen (PO_2) to <2 torr (actual concentrations were 0.002-0.003 mg O_2 /l) at prevailing environmental conditions. This concentration is below the 2.25-3.75 torr intracellular level of oxygen typically encountered by rat mitochondria *in vivo* (Gnaiger and Kuznetsov, 2002). Once the PO_2 reached the desired level, the cuvettes were sealed and the hypoxic conditions were maintained for 15 min. At the end of the hypoxia exposure period, the cuvettes were opened and fully re-oxygenated (100% air saturation) and

ADP (250 μ M) was added to impose the second phosphorylation with measurements of state 3 and 4 respiration rates. The difference between the first and second set of respiration parameters represented the effect of H-R on mitochondrial bioenergetics.

A control experiment was done to test the effect of bubbling with air on mitochondrial respiration. In this experiment I bubbled with air rather than nitrogen but for the same length of time (2 min) as the H-R trials. The results showed that there was no effect of bubbling with air on respiration rate during state 3 (Student's *t*-test $t = -0.56$, $p = 0.59$) and state 4 ($t = 0.00$, $p = 0.99$) or RCR ($t = -0.73$, $p = 0.50$).

6.3.6 Bioenergetics effects of mitoK_{ATP}

The prediction that diazoxide (mitoK_{ATP} channel opener) would protect against H-R damage by modulating mitochondrial bioenergetics and 5-HD (mitoK_{ATP} channel blocker) would reverse this effect was tested by measuring mitochondrial respiration before and after 15 min H-R at 13 °C. I first carried out dose response studies for diazoxide (10, 25, 100, 200 and 500 μ M) and 5-HD (50, 100, 200, 500 and 1000 μ M) and thereafter selected low (25 μ M diazoxide and 50 μ M 5-HD) and high (500 μ M diazoxide and 1 mM 5-HD) dose combinations and assessed their effects on post H-R respiration rates using respiration buffers with and without 1 mM MgCl₂ and 200 μ M ATP (Mg-ATP). The goal here was to delineate the direct effects of diazoxide and 5-HD on mitoK_{ATP} channels from their potential effects on oxidative phosphorylation (OXPHOS) by carrying out the assay with mitoK_{ATP} channel closed (with Mg-ATP) and open (without Mg-ATP). In these experiments, the mitochondria were pre-incubated with diazoxide alone and in combination with 5-HD for 5 min in the respiratory cuvettes after the measurement of normoxic respiration, and thereafter exposed to H-R followed by a second measurement of respiration rates.

6.3.7 Volume regulatory effects of mitoK_{ATP}

Mitochondrial volume was measured using spectrophotometric method (Chapter 3) under normoxic conditions and after H-R, without and with diazoxide alone and in combination with 5-HD using buffers with and without Mg-ATP. In this assay, 100 μ l of mitochondrial suspension was first energized with 5 mM glutamate and 5 mM malate in the cuvette and exposed to 15 min of hypoxia followed by reoxygenation. At the end of the reoxygenation the mitochondrial suspension was recovered and diluted with air-saturated buffer (10 mM Tris, 25 mM KH₂PO₄, 100 mM KCl) to give mitochondrial protein concentration of 1 mg/ml. Volume changes were then measured at 25 °C, with 200 μ M Ca as a positive control for swelling, by spectrophotometric monitoring of changes in absorbance at 540 nm every 10 s for 30 min. In this assay, a decrease in absorbance indicates swelling. To assess the role of mitoK_{ATP} channel on normoxic and H-R-induced mitochondrial volume changes, energized mitochondria were pre-incubated with 25 or 500 μ M diazoxide alone and in combination with 5-HD at either low (50 μ M) or high (1 mM 5-HD) dose, respectively, for 5 min, and changes in absorbance at 540 nm under normoxia were measured every 10 s for 30 min. For post H-R swelling, mitochondrial suspensions were incubated with diazoxide and 5-HD for 5 min and exposed to hypoxia for 15 min followed by full reoxygenation and measurement of changes in absorbance as described above. Lastly, to confirm that the swelling response was mediated by mitoK_{ATP} channel, K⁺ was replaced with tetraethylammonium (TEA⁺) and changes in absorbance at 540 nm in the presence of 25 μ M diazoxide alone and in combination with 50 μ M 5-HD were measured.

6.3.8 Data analysis

Data were tested for normality of distribution (chi-square test) and homogeneity of variances (Cochran C) before submission to one or two-way analysis of variance (ANOVA) or mixed model repeated measures general linear model (GLM) (Statistica version 13.0, Dell Statistica, Tulsa, OK). If the data did not pass the normality test, they were submitted to Box-Cox transformation; all data passed after transformation. An ANOVA is only slightly affected by inequality of variance using our models (equal sample sizes and all factors fixed). Significantly different means were separated using Tukey's *post hoc* test at $P < 0.05$. The data are reported as means \pm s.m.s. except the kinetics of volume changes which are means ($n = 5$) without s.m.s.

6.4 RESULTS

6.4.1 Effects of mitoK_{ATP} modulators on OXPHOS

I first measured the effects of diazoxide and 5-HD on OXPHOS with the mitoK_{ATP} channels open (i.e., in the absence of Mg-ATP) to characterize their bioenergetic effects. Diazoxide (0-500 μ M) did not alter state 3 respiration ($F_{5,24} = 0.92$, $P = 0.49$; Fig. 6.1A) but did stimulate state 4 respiration ($F_{5,24} = 4.69$, $P = 0.004$; Fig. 6.1B), leading to a significant dose-related reduction in RCR ($F_{5,24} = 6.64$, $P = 0.0005$; Fig. 6.1C). Similarly, 5-HD did not significantly alter state 3 respiration ($F_{5,24} = 2.47$, $P = 0.06$; Fig. 6.1D) but did reduce state 4 respiration ($F_{5,24} = 4.37$, $P = 0.006$; Fig. 6.1E). These effects of 5-HD on state 3 and 4 respiration resulted in dose related enhancement of RCR ($F_{5,24} = 4.61$, $P = 0.004$; Fig. 6.1F).

Figure 6.1

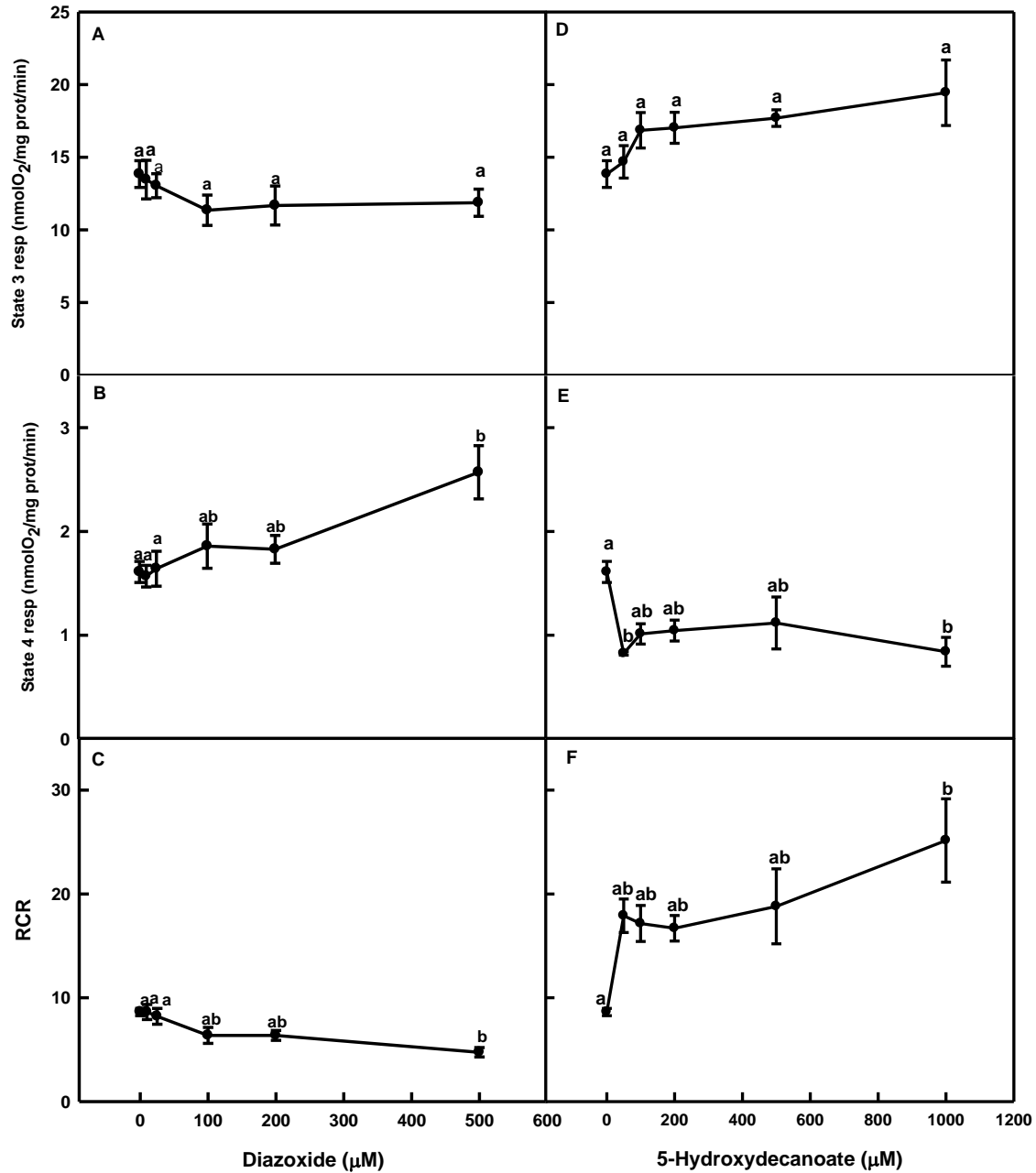


Figure 6.1: The dose response (D-R) of diazoxide and 5-hydroxydecanoate (5-HD) on normoxic mitochondrial respiration. A: D-R of diazoxide on state 3, B: D-R of diazoxide on state 4, C: D-R of diazoxide on RCR, D: D-R of 5-HD on state 3, E: D-R of 5-HD on state 4

and F: D-R of 5-HD on RCR. Isolated mitochondria were exposed to diazoxide (10, 25, 100, 200 and 500 μ M) or 5-HD (50, 100, 200, 500 and 1000 μ M). Data are means \pm SEM (n = 5). Bars with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$).

I then used combinations of low doses without bioenergetic effects (25 μ M diazoxide and 50 μ M 5-HD) and high doses with bioenergetic effects (500 μ M diazoxide and 1 mM 5-HD) and tested their effects on post H-R respiration using buffers without and with Mg-ATP. Mg-ATP blocks mitoK_{ATP} channels on the cytosolic (*cis*) side (Bednarczyk et al., 2005) and in its absence the channels are open (Garlid, 2000) and therefore not amenable to modulation by diazoxide or 5-HD. I found that state 3 and state 4 respiration were slightly higher while RCR was slightly lower during normoxia when tested in the absence of Mg-ATP; however, these results were from different experiments and were not compared statistically.

In the absence of Mg-ATP (Fig. 6.2A), H-R ($F_{1,20}=53.9$, $P<0.0001$), treatment with mitoK_{ATP} modulators ($F_{4,20}=8.21$, $P=0.0004$) and interaction ($F_{4,20}=6.18$, $P=0.002$) significantly altered state 3 respiration. This response was primarily driven by the inhibitory effect of H-R in controls and that of H-R combined with the low and high doses of diazoxide. Interestingly, diazoxide at both low and high doses in the presence of 5-HD resulted in the restoration of state 3 respiration to the normoxic level. Similarly, state 4 respiration (Fig. 6.2B) was altered by H-R ($F_{1,20}=121$, $P<0.0001$) and mitoK_{ATP} modulators ($F_{4,20}=37.8$, $P=0.0001$) as well as their interaction ($F_{4,20}=23.0$, $P<0.0001$). Specifically, H-R stimulated state 4 respiration, with the high but not the low dose of diazoxide inhibiting this stimulation. Moreover, diazoxide at the high dose in the presence of 5-HD (high dose) exacerbated the stimulatory effect on state 4 respiration. Consistent with their effects on state 3 and 4 respiration, H-R ($F_{1,20}=728$, $P<0.0001$) and mitoK_{ATP} modulators ($F_{4,20}=25.1$, $P<0.0001$) reduced the RCR and showed a significant interaction effect ($F_{4,20}=11.45$, $P<0.0001$; Fig. 6.2C). Here, except for the combined high doses of diazoxide and 5-HD, all of the treatments minimized the RCR-reducing effect of H-R.

With Mg-ATP in the buffer (Fig. 6.2D), the interaction between treatment with mitoK_{ATP} modulators and H-R was significant for state 3 ($F_{4,20}=3.50$, $P=0.03$) but their individual effects were not. Interestingly, low dose of diazoxide alone and with low dose of 5-HD did not affect state 3 respiration, but their combination at high doses reduced state 3 respiration relative to the controls. In contrast, H-R ($F_{1,20}=179$, $P<0.0001$) and treatment mitoK_{ATP} modulators ($F_{4,20}=6.55$, $P=0.0015$) and their interaction ($F_{4,20}=8.27$, $P=0.0004$) significantly altered state 4 respiration (Fig.6.2E). Here, as expected, H-R stimulated state 4 respiration, and the low but not the high dose of diazoxide reversed the H-R effect. Importantly, the low dose of 5-HD reversed the inhibitory effect of diazoxide on state 4 respiration, essentially restoring it to the H-R level. However, the high dose of 5-HD reduced the stimulatory effects of high dose of diazoxide on state 4 after H-R. Lastly, the RCR was significantly reduced by H-R ($F_{1,20}=897$, $P<0.0001$), treatment with mitoK_{ATP} modulators ($F_{4,20}=8.29$, $P=0.0004$) and their interaction ($F_{4,20}=13.89$, $P=0.0001$; Fig. 6.2F). The low dose of diazoxide partially protected against the RCR-reducing effect of H-R, this protection was blocked by low dose of 5-HD. In contrast, the high dose of diazoxide alone and in combination with 5-HD (high dose) did not alter the RCR-reducing effect of H-R.

Figure 6.2

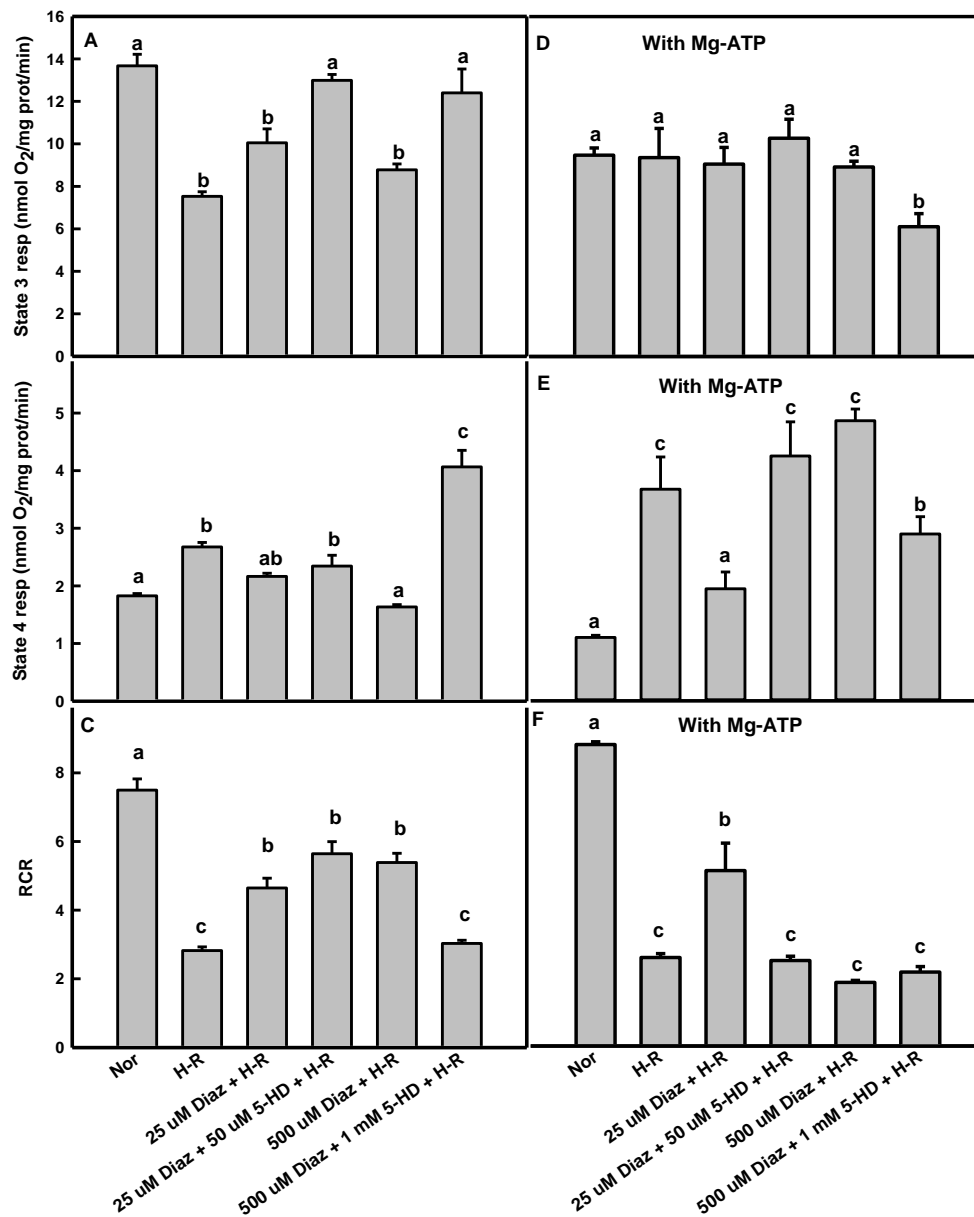


Figure 6.2: The effects of diazoxide and 5-HD on mitochondrial OXPHOS capacity during H-R in Mg-ATP free respiration buffer. A: state 3, B: state 4 and C: RCR and in buffer containing Mg-ATP, D: state 3, E: state 4, F: RCR. Low doses (25 μ M diazoxide and 50 μ M 5-HD) and high doses (500 μ M and 1 mM 5-HD) were tested under normoxia and after 15

min H-R at 13 °C. Data are means \pm SEM (n = 5). Points with different letters are statistically different from each other (mixed model repeated measures ANOVA with Tukey's HSD, $P < 0.05$).

6.4.2 Role of mitoK_{ATP} in mitochondrial function and volume homeostasis

Under normoxia without Mg-ATP (Fig 6.3A, B), high dose of diazoxide and Ca (positive control) caused mitochondrial swelling of similar form and amplitude. The low dose (25 μ M) of diazoxide without and with the low dose of 5-HD did not significantly alter mitochondrial volume, whereas its high dose induced significant swelling that was reversed by the high dose of 5-HD. Mitochondrial swelling following exposure to H-R alone was similar to that resulting from exposure to combined H-R and Ca or Ca alone (Fig. 6.3C, D). Incubating mitochondria with low and high doses of diazoxide before exposure to H-R resulted in less swelling relative to H-R alone, and 5-HD at both low and high doses did not reverse these effects of diazoxide (Fig. 6.3C, D).

In the presence of Mg-ATP, mitochondria displayed high-amplitude, swelling as shown by the Ca-positive control (Fig. 6.4A, B). Here, the low dose of diazoxide caused significant swelling that was reversed by the low dose of 5-HD. Moreover, the high dose of diazoxide resulted in greater swelling than that which resulted from its low dose, but this swelling was exacerbated by 5-HD at the high dose. Exposure of mitochondria to H-R resulted in swelling relative to normoxia; however, Ca-induced swelling after H-R was of similar magnitude as that observed under normoxia (Fig. 6.4). Importantly, the H-R-induced swelling was reversed by low dose of diazoxide, and 5-HD at low dose blocked this effect. In contrast, the high dose of diazoxide did not alter H-R-induced swelling, and addition of high dose of 5-HD exacerbated the swelling. The role of mitoK_{ATP} channel was corroborated by the observation that 5-HD reversed swelling in the presence of K⁺ but not TEA⁺ when Mg-ATP was present (Fig. 6.5).

Figure 6.3

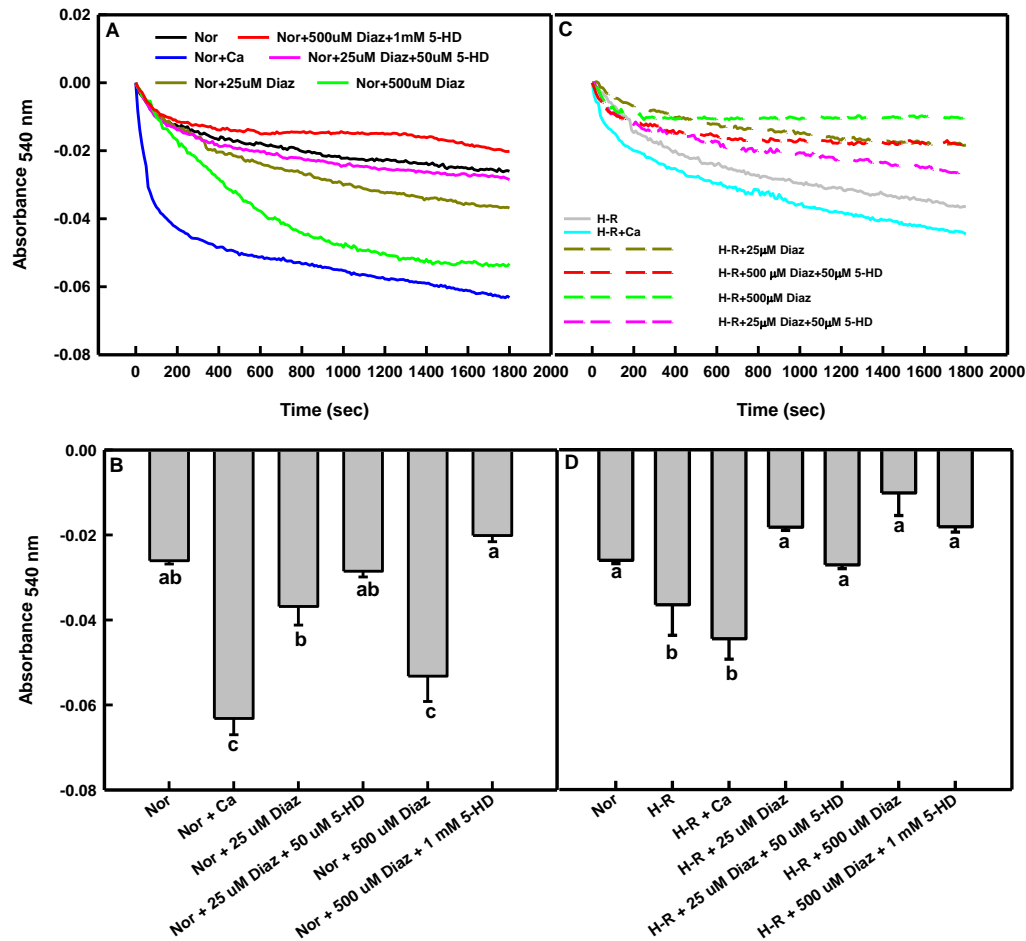


Figure 6.3: The effects of diazoxide and 5-HD on mitochondrial volume under normoxic (Nor) and after 15 min H-R in Mg-ATP free swelling buffer at 24 °C. A: Normoxic swelling kinetics, B: Normoxic swelling amplitude at 30 min, C: H-R swelling kinetics, D: H-R swelling amplitude at 30 min. Low doses (25 μ M diazoxide and 50 μ M 5-HD) and high doses (500 μ M and 1 mM 5-HD) were tested with 200 μ M Ca as positive control. Swelling was monitored every 10 s for 30 min as absorbance changes at 540 nm and the kinetics and terminal amplitude of volume changes after 30 min are shown. The trend lines represent

means of swelling data from 5 independent mitochondrial preparations (i.e., $n = 5$). Bars with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$, $n = 5$).

Figure 6.4

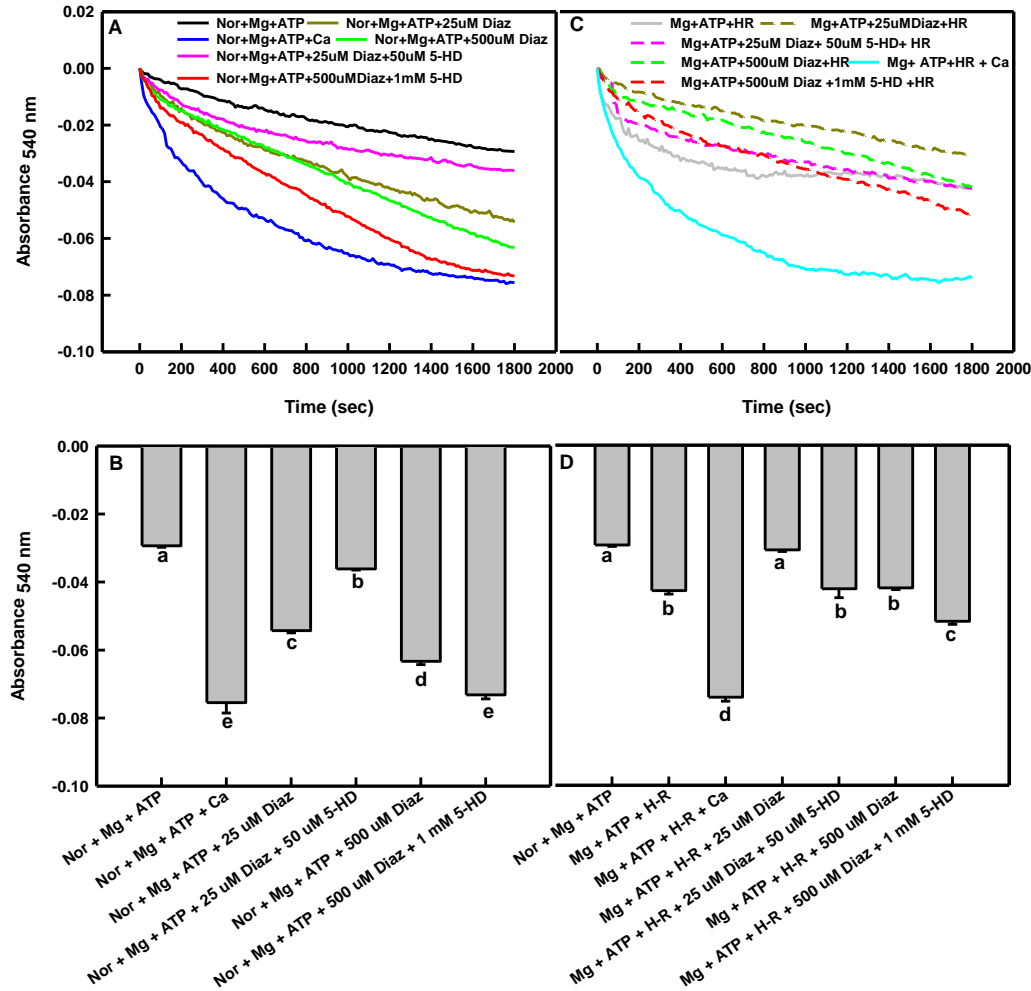


Figure 6.4: The effects of diazoxide and 5-HD on mitochondrial volume under normoxic (Nor) and after 15 min H-R in the presence of Mg-ATP in swelling buffer at 24 °C. A: Normoxic swelling kinetics, B: Normoxic swelling amplitude at 30 min, C: H-R swelling kinetics, D: H-R swelling amplitude at 30 min. Low doses (25 μ M diazoxide and 50 μ M 5-HD) and high doses (500 μ M and 1 mM 5-HD) were tested with 200 μ M Ca as positive control. Swelling was monitored every 10 s for 30 min as absorbance changes at 540 nm and

the kinetics and terminal amplitude of volume changes after 30 min are shown. The trend lines represent means of swelling data from 5 independent liver mitochondrial preparations (i.e., $n = 5$). Bars with different letters are statistically different from each other (one-way ANOVA with Tukey's HSD, $P < 0.05$, $n = 5$).

Figure 6.5

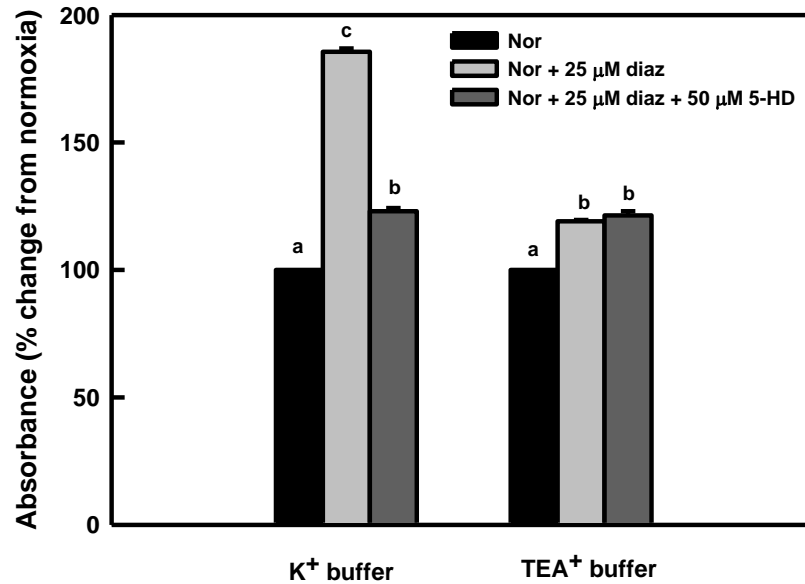


Figure 6.5: The percent change in mitochondrial volume from control in K⁺ and TEA⁺ buffers containing Mg-ATP under normoxia. Volume changes were measured following exposure of mitochondrial suspension to 25 μ M diazoxide and 25 μ M diazoxide + 50 μ M 5-HD. Swelling was monitored every 10 s for 30 min absorbance changes at 540 nm. Data are means \pm SEMs (n = 3) after 30 min. Bars with different letters are statistically different from each other (two-way ANOVA with Tukey's HSD, P<0.05)

6.5 DISCUSSION

Opening mitoK_{ATP} channels is believed to protect against the deleterious effects of IR; however, the effects of the two widely used mitoK_{ATP} channel modulators, diazoxide (channel opener) and 5-HD (channel blocker) are controversial. Specifically, Garlid (2000) argued that opening mitoK_{ATP} channel has minimal direct effects on mitochondrial bioenergetics and that bioenergetic effects observed by others (Holmuhamedov et al., 1999; 2004; Dröse et al., 2006; Kopustinskiene et al., 2010) reflect toxic effects secondary to the use of excessively high doses of the modulators. Thus I sought to shed light on the controversies surrounding the effects of diazoxide and 5-HD on OXPHOS and mitochondrial volume. I found that under mitoK_{ATP} channel open conditions (no Mg-ATP), both low and high doses of diazoxide improved mitochondrial coupling but only the high dose of 5-HD reversed the post H-R coupling-enhancing effect of diazoxide (Fig. 6.2C). Although 5-HD can be converted to 5-HD-CoA, which is metabolized via β -oxidation to provide NADH, which in turn supplies electrons to the ETS thus stimulating mitochondrial respiration (Lim et al., 2002; Hanley et al., 2005), its high doses inhibit both β -oxidation and succinate dehydrogenase/complex II (Hanley et al., 2002, 2005; Lim et al., 2002). The 5-HD dose range tested in my study did not significantly alter state 3 respiration of mitochondria respiring on a complex I substrate but inhibited state 4 respiration, thereby improving coupling. In tests carried out in the presence of Mg-ATP, the low dose of diazoxide alone had a bioenergetic effect evidenced by improved coupling post H-R, and this effect was abolished by 5-HD at low dose (Fig. 6.2F). Note that this low dose of diazoxide had no effect on complex-I-supported OXPHOS under normoxia (Fig. 6.1). That is, my findings are consistent with earlier reports on the beneficial effects of low dose of diazoxide on mammalian mitochondrial coupling (Iwai et al., 2000; Dos Santos et al., 2002).

The primary role of mitoK_{ATP} channel is believed to be mitochondrial volume regulation (Jaburek et al., 1998; Garlid, 2000; Costa et al., 2006). In my study, diazoxide at low dose had no effect in the absence of Mg-ATP under normoxic conditions but its high dose caused mitochondria to swell and 5-HD at high dose reversed this effect. Because mitoK_{ATP} channel were open throughout during this assay, these volume changes were likely mediated by mechanisms other than mitoK_{ATP} channel, e.g., opening of mitochondrial permeability transition pores (Hausenloy et al., 2004) and/or inhibition of K⁺/H⁺ exchanger. Under these conditions, the reversal of diazoxide effect by 5-HD can be explained by their diametrically opposite effects on OXPHOS (Fig. 6.1). Additional evidence of diazoxide altering mitochondrial volume via mechanisms independent of mitoK_{ATP} channel was that its reversal of H-R-induced swelling was not sensitive to 5-HD. In contrast, in the presence of Mg-ATP under normoxia, swelling induced by diazoxide (low dose) was partially reversed by 5-HD and could therefore in part be attributed to opening of the mitoK_{ATP} channel. The fact that diazoxide at high dose caused greater swelling relative to the low dose and the addition of 5-HD worsened this effect implies that this was a toxic response. Notably, low dose of diazoxide protected the mitochondria from H-R-induced swelling, an effect that was reversed by low dose of 5-HD thus suggesting a role of mitoK_{ATP} channel in this phenomenon and in preserving state 3 respiration. In contrast, diazoxide at high dose failed to reduce the swelling caused by H-R and the addition of 5-HD (high dose) enhanced mitochondrial swelling (Fig. 6.4). Because mitoK_{ATP} channel is closed by Mg-ATP, it is logical that H-R-induced swelling would be less pronounced in the presence of Mg-ATP than in its absence. Overall, my study showed that while both the opening of mitoK_{ATP} channels and bioenergetic effects of diazoxide were protective against H-R-induced swelling in the presence of Mg-ATP, only the

bioenergetic effect was protective in the absence of Mg-ATP. Furthermore, mitochondrial swelling was responsive to low doses of diazoxide and 5-HD in K^+ but not TEA^+ buffer confirming the involvement of mitoK_{ATP} channel in mitochondrial volume regulation (Beavis et al., 1993, Garlid et al., 1996, Jaburek et al., 1998).

Several previous studies have reported that mitochondria shrink/contract at high phosphorylation state/state 3 respiration (Packer, 1960; Garlid, 2000; Hackenbrock, 1968; Kowaltowski et al., 2001) and swell in state 4 respiration (Packer, 1960; Hackenbrock, 1968; Bosetti et al., 2004). In my study, the relationship between H-R-induced swelling and state 3 respiration was positive in the presence of Mg-ATP and negative in its absence. In contrast, state 4 respiration (proton leak) was positively correlated with mitochondrial volume both in the presence and absence of Mg-ATP but the leak was five times higher in the presence of Mg-ATP. A possible explanation of these findings is that during phosphorylation (state 3) the proton gradient that drives electrogenic K^+ entry into mitochondria matrix is consumed for ATP synthesis, thus reducing influx of K^+ , and subsequently that of osmotically obliged water (Garlid, 2000). Conversely, the swelling in state 4 respiration was possibly due to the high membrane potential (proton gradient) that supports high rates of K^+ and water influx. Overall, it appears that assay conditions and the procedure of inducing swelling define the mitochondrial volume-functional state relationship.

6.6 CONCLUSIONS

The prediction that modulators (diazoxide and 5-HD) of mitoK_{ATP} channel would exhibit a dose-dependent opposite effect on mitochondrial bioenergetics was confirmed in my study, and explains their antagonistic effects on mitochondrial responses not mediated by mitoK_{ATP} channel opening. The prediction that in the absence of Mg-ATP diazoxide and 5-HD would

modulate only mitochondrial bioenergetics was corroborated by my findings and highlighted the physiological role of Mg-ATP in blocking mitoK_{ATP} channel. Notably, low dose of diazoxide in the presence of Mg-ATP had both volume regulatory and bioenergetic effects. Finally, high doses of diazoxide and 5-HD alleviated H-R-induced damage by altering mitochondrial bioenergetics in the absence but not in the presence of Mg-ATP. Thus, both the opening of mitoK_{ATP} channel and bioenergetic effects of diazoxide protected against H-R-induced mitochondrial swelling and preserved their functional states.

CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

7.1 Discussion

My project probed the impact of multiple stressor factors on mitochondrial function. Because aquatic organisms are exposed to diverse and dynamic combinations of stressors in their environment it is important to understand mechanisms of joint effects of stressors to more accurately monitor and predict adverse biological outcomes. I utilized robust and sensitive techniques that enabled me to study the mechanisms of interactions of Cd, hypoxia (with reoxygenation) and temperature induced stress on mitochondrial bioenergetics. The overreaching hypothesis was that when present together Cd, hypoxia and temperature affect common target sites exacerbating single stressor-induced effects on mitochondrial function. My PhD research unearthed previously unknown mechanisms of interactions of Cd, hypoxia and temperature on mitochondrial bioenergetics and increased our understanding of the impact of multiple stressors on cellular energy metabolism in aquatic organisms.

7.1.1 Co-exposure of multiple stressors exacerbates their individual effects on mitochondrial function

My findings that mitochondrial respiration was altered following exposure to Cd and H-R (Chapter 2), Cd and temperature (Chapter 3), temperature and H-R (Chapter 4) and Cd, temperature and H-R (Chapter 5) on state 3 and 4 respiration rate, P/O ratio and the RCR supported the hypothesis that the combined effects of multiple stressors would exacerbate their individual effect on mitochondrial function. I showed that the responses induced by single stressors on mitochondrial function were different from those caused by binary and/or ternary combination of the same stressors. Importantly, the nature (additive, antagonistic or synergistic) of the interactive effects was unpredictable and depended on the assessment endpoint. For example in Chapter 5 I showed that Cd and H-R synergistically reduced maximal mitochondrial respiration and that the threshold doses for Cd toxicity progressively

shifted to lower values at high temperatures. A similar result has been observed with oysters (hypoxia-tolerant species) exposed to combined anoxia-reoxygenation and Cd stress *in-vivo* (Kurochkin et al., 2009). However, at low temperature Cd in synergy with H-R stimulated state 3 respiration rates, a response that could be attributed to alteration of membrane phospholipids and proteins with enhancement of the activity of ETS and substrate transporters (Hazel, 1995; Kraffe et al., 2007; Portner et al., 2007).

Furthermore, I demonstrated that at high temperature Cd at low dose antagonized the stimulation of basal respiration by H-R to a greater degree at 25 compared with 13 °C. Contrastingly, Cd at high doses in synergy with H-R increased the basal respiration indicating that Cd and H-R interact on basal respiration via a common mechanism, likely involving alteration of IMM permeability. It appears that low doses of Cd + H-R at high temperature reduce the permeability of the IMM to protons while high doses of the metal combined with H-R increase it. On the other hand, at low temperature (5 °C) Cd dose-dependently acted in synergy with H-R to stimulate the basal respiration above their individual rates.

I observed that individually Cd, temperature, and H-R reduced P/O ratio that is consistent with several earlier reports (Sokolova et al., 2005; Adiele et al., 2010; Sappal et al., 2015). Additionally, I showed that temperature exacerbated the inhibitory effect of combined Cd and H-R on phosphorylation efficiency (P/O ratio) possibly due to greater inhibition of the ATP synthase and/or increased proton and electron leaks. Similarly I showed that the RCR was worsened with subsequent addition of stressors that suggests additivity/synergy of their toxic effects. Previous studies with binary combination reported additive/synergistic interactions of Cd × temperature (Sokolova, 2004; Cherkasov et al., 2010; Chapter 3), H-R ×

Cd (Kurochkin et al., 2009; Chapter 2) and H-R \times temperature (Chapter 4). In Chapter 5 the effects of Cd \times H-R \times temperature interaction reduced the RCR to a greater extent than the binary combinations. The greater reduction of RCR induced jointly by the 3 stressors could result from increased mitochondrial sensitivity to Cd in the presence of H-R and temperature or vice versa. Consistent with greater reduction of RCR (Chapter 2, 3, 4 and 5), the dysregulation of mitochondrial volume (Chapter 3 and 5), structural damage (Chapter 4 and 5), dissipation of $\Delta\Psi_m$ (Chapter 4 and 5) and ROS production (Chapter 4 and 5) were all exacerbated by combined exposure to the stressors.

The interactive responses of temperature, Cd and/or H-R on mitochondrial bioenergetics are summarized in Fig. 7.1. I propose that the inhibition of ETS by the stressors results in excessive ROS production that damage biomolecules (membrane lipids and proteins) resulting in mitochondrial swelling and loss of structural integrity. The increased swelling and/or loss of structure together with the impaired ETS would result in decreased $\Delta\Psi_m$ leading to reduced RCR an indication of compromised mitochondria.

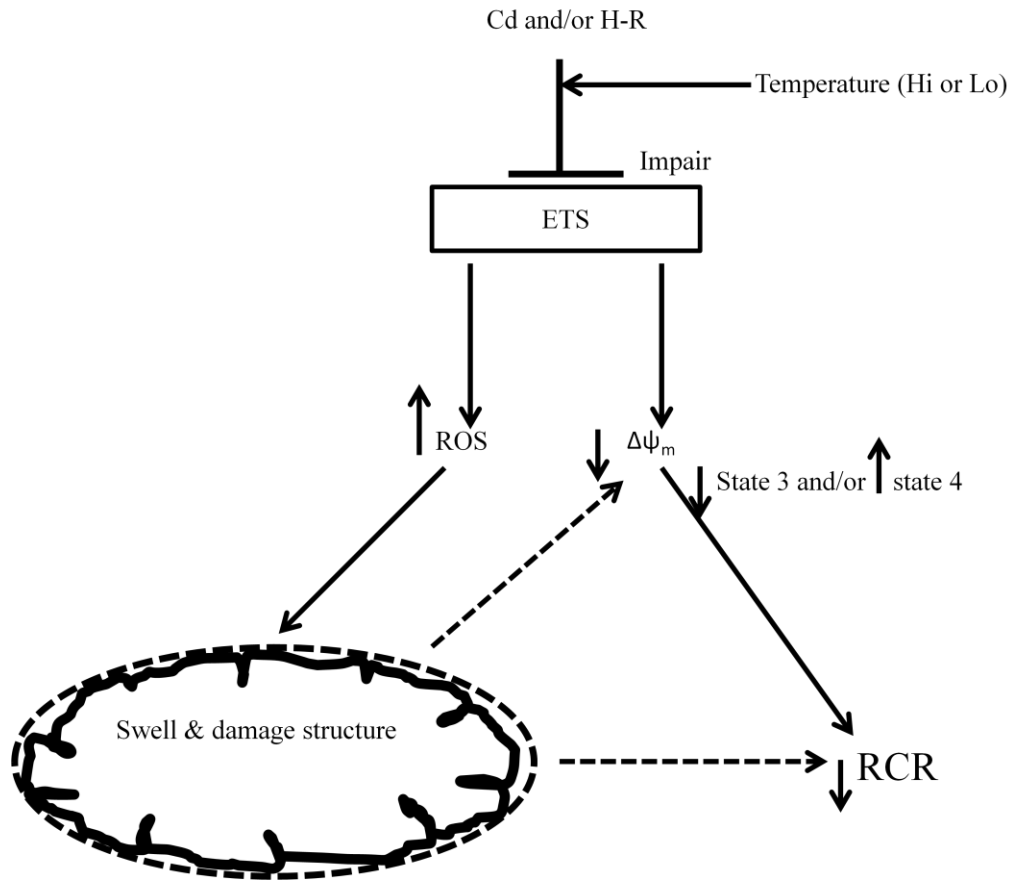


Figure 7.1: Temperature (high [Hi] or low [Lo]) exacerbates Cd and/or H-R induced ETS impairment. The impaired ETS increases ROS production and decreases mitochondrial membrane potential (MMP). The increase in ROS causes mitochondrial swelling and structural damage. The decrease MMP due to impaired ETS and/or swelling/structural damage results in a decrease in RCR

7.1.2 Low dose of Cd alleviate H-R induced mitochondrial dysfunction

The discovery that low doses of Cd were protective against H-R-induced mitochondrial dysfunction is another key highlight of my doctoral research. I showed that when in combination with H-R, Cd at low dose (5 μ M) protected mitochondria against H-R induced structural damage, swelling, complex I activity inhibition, complex I A \rightarrow D transition and proton leak (Chapter 2 and 5). The effects of Cd on H-R-induced stimulation of proton leak and complex I activity were 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. To the best of my knowledge, these are the first reports of possible beneficial effects of low Cd doses in attenuating mitochondrial proton leak and rescuing complex I from H-R-induced inhibition. In Chapter 2, I speculated that the protective effect of low doses of Cd against H-R-induced mitochondrial impairment of CI supported respiration emanated from the prevention of conversion of A-form of complex I to the D-form. I tested this hypothesis in Chapter 5 and showed that during combined Cd and H-R exposure, Cd at low doses converted complex I D- to A-form thus shielding the later from the effects of H-R. To the best of my knowledge this is the first study to show that low doses of Cd convert complex I D-form to A-form following H-R thereby improving enzyme activity.

With respect to swelling I observed that Cd at low doses protected against H-R-induced swelling while at high doses the metal exacerbated the mitochondrial swelling induced by H-R. While the mechanisms of this protection are not known, it is possible that at low doses Cd minimizes the changes in IMM permeability and/or reduces ROS-induced oxidative damage

following exposure to H-R. These findings were supported by the TEM work wherein 5 μ M Cd prevented mitochondrial swelling following H-R treatment.

7.1.3 Bioenergetic and volume regulatory effect of mitoK_{ATP} protect against H-R induced mitochondrial dysfunction

The long controversy as to whether mitoK_{ATP} bioenergetics and volume regulatory effects protect against H-R induced mitochondrial dysfunction was addressed in Chapter 6. It had been earlier (Garlid, 2000) argued that opening mitoK_{ATP} has minimal direct effects on mitochondrial bioenergetics and that bioenergetic effects observed by others (Holmuhamedov et al., 1999; 2004; Dröse et al., 2006; Kopustinskiene et al., 2010) reflect toxic effects secondary to the use of excessively high doses of mitoK_{ATP} modulators. Thus I sought to shed light on the controversies surrounding the effects of diazoxide and 5-HD on OXPHOS and mitochondrial volume. I found that low doses of diazoxide in the presence of Mg-ATP had both volume regulatory and bioenergetics effects that alleviated H-R-induced mitochondrial dysfunction. Because in the presence of Mg-ATP the channel is closed and low doses of diazoxide open the channel, my data suggested that the mechanisms of protection involve the opening of mitoK_{ATP} channel. On the other hand, my observation that in the absence of Mg-ATP high doses of diazoxide protected against H-R induced mitochondrial respiration impairment shows that it has bioenergetic effect but not volume regulation. Since under these conditions the channel was open the protection involved mechanisms not mediated by the opening of the mitoK_{ATP} channel.

7.1.4 Mechanisms of Cd-induced mitochondrial volume changes

Prior to my research, Sokolova (2004) observed only contraction in oyster's gills while Adiele et al. (2012b) reported mild swelling in rainbow trout liver following exposure of

isolated mitochondria to Cd. I found that Cd and/or temperature stress are capable of inducing both swelling and contraction depending on their levels. Specifically, Cd induced transient contraction before swelling and temperature modulated the contraction and swelling (Chapter 3). Regarding the contraction I observed in the early phase of Cd exposure, the possibility that the change in absorbance was due to Cd or complexes formed by reaction of Cd with components of the swelling buffer were ruled out. Furthermore, the possibility of an increase in refractive index due to Cd complexation with phosphate in the mitochondrial matrix that can be interpreted as contraction as observed with following Ca exposure of brine shrimp, *Artemia franciscana* mitochondria (Menze et al., 2005; Holman and Hand, 2009), was ruled out because the contraction I observed was transient, whereas the formation of calcium phosphate in brine shrimp mitochondria was a permanent monotonic phenomenon. Therefore this suggests that the mitochondrial contraction I observed was due to specific effects of Cd on the mechanisms or structures that regulate solute and/or water transport in these organelles.

Additionally, I showed that the mitochondrial volume changes I observed in my study, with a clear early contraction followed by two phases of swelling, were highly influenced by temperature (Chapter 3). While mitochondria equilibrated to test temperature showed moderate contraction and swelling, those tested after temperature shock (abrupt transfer from ice→15 or 25 °C) had complex patterns of contraction and swelling with higher amplitudes. The complex swelling-contraction pattern is possibly associated with temperature-induced changes on mitochondrial membrane characteristics (Connell, 1961; Richardson and Tappel, 1962; Somero, 2011). Interestingly, 5 µM Cd prevented spontaneous swelling in both equilibrated and temperature-shocked mitochondria, suggesting that at low doses Cd blocks

the mechanisms of solute and/or water movement across the inner mitochondrial membrane. Additional studies are required to unveil the mechanisms by which low Cd doses inhibit spontaneous swelling in isolated mitochondria.

7.1.5 Modulation of Cd accumulation by temperature and H-R

In view of the limited knowledge on mitochondrial Cd accumulation and the role of multiple stressors in enhancing or abating mitochondrial Cd load, I addressed three questions: (i) Does mitochondrial impairment increase with Cd accumulation? (ii) Is mitochondrial Cd accumulation dose-dependent? (iii) Do temperature and H-R affect Cd accumulation in the mitochondria? Additionally, I tested the prediction that the greater mitochondrial dysfunction I observed following Cd exposure to temperature and/or H-R results from increased Cd accumulation. I found that mitochondrial Cd accumulation is dose-dependent and is influenced by temperature. Notably, there were strong correlations between state 3 respiration rates and log mitochondrial Cd concentration at all temperatures indicating that low burdens of Cd impose relatively greater reductions in respiration compared with the high burdens. Though the mechanisms of enhancement of mitochondrial Cd uptake at high temperature were not explored, it is possible that the IMM exhibited increased leakiness (Dahlhoff and Somero, 1993), thereby allowing greater influx of Cd. An alternative explanation for the increased Cd accumulation at high temperature is increased activity of mitochondrial transporters such as the MCU that I (Chapter 3), that others (Lee et al., 2005a; Adiele et al., 2012b) have shown to be involved in Cd uptake by the mitochondria.

Surprisingly, my research also revealed that the combination of H-R and temperature stress increased Cd-induced mitochondrial impairment without a corresponding change in Cd accumulation (Chapter 5). It is possible that H-R and/or temperature alter the conformation

of mitochondrial macromolecules e.g., membrane phospholipids and proteins (Kraffe et al., 2007), rendering them more metal-sensitive. Overall my research revealed that temperature increases mitochondrial Cd accumulation and toxicity while combined temperature and H-R increased mitochondrial sensitivity to Cd but not accumulation.

7.2 Future directions

Despite the substantial new findings reported in this thesis, several issues do require additional research to strengthen our understanding of the mechanisms of multiple stressor interaction on mitochondrial physiology and energy metabolism in general. First, the mechanisms underlying the beneficial effects of low doses of Cd against H-R induced mitochondrial dysfunction need to be investigated. Specifically, there is a need to identify if and what parts of mitochondrial proteins and membrane lipids are altered by Cd to reduce the deleterious effects of H-R. Here proteomics and lipidomics would be useful. Additionally, the use of NMR, optical spectroscopy, mass spectrometry and X-ray crystallography could elucidate potential structural changes in proteins and phospholipids that explain the functional changes associated with low dose Cd and H-R exposures.

The finding that mitochondrial dysfunction observed in my project was due to increased ROS production is another area that could benefit from additional research to identify how ROS damage macromolecules. In this regard, using optogenetic techniques to target and localize ROS produced by the complexes would help to differentiate pathological and beneficial ROS.

Furthermore, the finding that the combined effects of the stressors exacerbate or antagonize their individual effects needs further investigation by focussing on measuring multiple

parameters (ROS, $\Delta\Psi_m$, ATP production, and respiration rates) of mitochondrial function at the same time. Ultimately there is a need to investigate the effects of the three stressors at the cellular and organism levels to obtain a more holistic picture of effects of multiple stressors on aquatic organisms.

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