Adaptive Variation of Mitochondrial Function in Response to Oxygen Variability in Intertidal Sculpins (Cottidae, Actinopterygii)

by

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Abstract

Variation in environmental oxygen (O₂) poses a significant physiological challenge to animals, not only because of the impact on aerobic metabolism, but also because it can lead to generation of potentially harmful reactive oxygen species (ROS). In this thesis, I aimed to investigate the interplay between two aspects of O₂ use at the mitochondria, aerobic respiration and ROS metabolism, using species of intertidal sculpins (Cottidae, Actinopterygii) which are distributed along the marine intertidal zone, exposed to varying O₂ conditions and vary in their tolerance to low O₂ (hypoxia).

I first hypothesized that there would be a relationship between whole animal hypoxia tolerance and mitochondrial and cytochrome c oxidase (COX) O₂-binding affinity, whereby hypoxia tolerant sculpins would have higher mitochondrial and COX O₂-binding affinity than less hypoxia tolerant sculpins. This hypothesis was supported with functional analysis. In silico modelling of the COX catalytic core revealed that the variation in O₂ binding was related to interspecific differences in the interaction between COX3 and membrane phospholipid, cardiolipin, which could impact O₂ diffusion to its binding site.

I then investigated whether intact mitochondria from hypoxia tolerant sculpins were able to use O₂ more efficiently such that phosphorylation efficiency was improved and ROS generation was reduced compared to mitochondria from less hypoxia tolerant sculpins. Although there were relationships between hypoxia tolerance and complex I and II dependencies, there were no interspecies differences in phosphorylation or mitochondrial coupling that would indicate differences in aerobic metabolism. Moreover, mitochondria from hypoxia tolerant sculpins generated more ROS under resting conditions and were more perturbed by in vitro redox and anoxia-recovery challenges.

Finally, I confirmed consistent responses of mitochondria to in vivo responses with a whole animal study comparing ROS metabolism (redox status, mitochondrial H₂O₂, oxidative damage and scavenging capacity) between two sculpin species with different hypoxia tolerance to hypoxia, hyperoxia, with normoxia-recovery exposures.

Taken together, my thesis demonstrates that hypoxia tolerance is associated with improved O₂ binding at the mitochondria and COX. Further, hypoxia tolerance in sculpins is associated with
higher ROS generation compared to less tolerant species, suggesting a potentially important role of ROS in mediating hypoxia tolerance.
Lay Summary

Animals are highly dependent on oxygen (O₂). The use of O₂, however, comes with both advantages and disadvantages. On one hand, O₂ is key to the process that produces chemical energy within mitochondria inside the cell, on the other hand the use of O₂ generates reactive oxygen species (ROS), which are harmful byproducts. In this thesis, I investigated these two aspects of O₂ use at mitochondria from a group of sculpin fishes that are distributed along the marine intertidal zone and are naturally exposed to daily fluctuations of O₂. I found that more hypoxia tolerant sculpins improved O₂ binding at the level of mitochondria. However, this increased O₂ binding was not associated with increased aerobic energy production, and counterintuitively, there was higher ROS generation in more hypoxia tolerant sculpins. It is possible that higher ROS generation in hypoxia tolerant sculpins is part of the strategy in surviving the O₂ variable intertidal.
Preface

A version of Chapter 2 has been published as “Lau, G.Y., Mandic, M. and Richards, J.G. (2017). Evolution of cytochrome c oxidase in hypoxia tolerant sculpins (Cottidae, Actinopterygii). Molecular Biology and Evolution. 34, 2153-2162”. I designed and carried out the protocols for the high-resolution respirometry experiments with input from Dr. Jeff Richards. I also carried out the in silico protein analyses. Dr. Milica Mandic isolated mitochondrial samples that were used for the COX \( K_{m,app} O_2 \) determination and also provided advice on statistical analyses. I wrote the manuscript with editorial input from Drs. Mandic and Richards.

I designed and carried out the protocols for the high-resolution respirometry and fluorometry in Chapter 3. I wrote the manuscript with editorial input from Dr. Richards.

I designed and carried out the whole animal exposure in Chapter 4 with input from Drs. Sabine Arndt, Michael Murphy and Richards. Dr. Arndt also performed mass spectrometry analysis on the MitoB samples. I performed the biochemical analyses on all tissue samples. I wrote the final manuscript with editorial input from Drs. Arndt, Murphy, and Richards.

All experiments performed for the various sculpin species were approved by the UBC Animal Care Committee (Protocol A13-0309).
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>[E]</td>
<td>enzyme concentration</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike's Information Criterion</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>cytochrome c oxidase</td>
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<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethyl ether)-N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ETS</td>
<td>electron transport system</td>
</tr>
<tr>
<td>FADH₂</td>
<td>flavin adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone</td>
</tr>
<tr>
<td>GPDH</td>
<td>glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide; oxidized glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>Hb-O₂ P₅₀</td>
<td>hemoglobin-O₂ binding affinity</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethesulfonic acid</td>
</tr>
<tr>
<td>HIF-₁α</td>
<td>hypoxia inducible factor 1, alpha</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>intermitochondrial space</td>
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<tr>
<td>K₂HPO₄</td>
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</tr>
<tr>
<td>k₉ₜ</td>
<td>catalytic rate</td>
</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>KH₂PO₄</td>
<td>potassium phosphate, monobasic</td>
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<tr>
<td>Kₘ</td>
<td>Michaelis Menten constant</td>
</tr>
<tr>
<td>Kₘ,₉ₜ</td>
<td>apparent Michaelis-Menten constant</td>
</tr>
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<td>kPa</td>
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</tr>
<tr>
<td>LOE</td>
<td>loss of equilibrium</td>
</tr>
<tr>
<td>LOE₅₀</td>
<td>time to loss of equilibrium</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
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</table>
min  minute
Mitochondrial $P_{50}$  mitochondrial oxygen binding affinity
mRNA  messenger ribonucleic acid
NAD$^+$  nicotinamide adenine dinucleotide
NADH  nicotinamide adenine dinucleotide, reduced
NADP$^+$  nicotinamide adenine dinucleotide phosphate
NADPH  nicotinamide adenine dinucleotide phosphate, reduced
O$_2$  oxygen
O$_2^-$  superoxide radical
°C  degrees celsius
OH$^-$  hydroxyl radicals
OLS  ordinary least squares
PaO$_2$  arterial partial pressure of oxygen
$P_{crit}$  critical oxygen tension of metabolic rate
PGLS  phylogenetically generalized least squares
PO$_2$  partial pressure of oxygen
RCR  respiratory control ratio
RNAi  RNA interference
ROS  reactive oxygen species
Rot  rotenone
s.e.m.  standard error of mean
sec  second
SUIT  substrate utilization inhibitor titration
TBA  thiobarbituric acid
TBARS  thiobarbituric acid reactive substances
TMPD  $N,N,N',N'$-tetramethyl-p-phenylenediamine
TOSC  total oxidative scavenging capacity
TPP$^+$  tetraphenylphosphonium ion
UCP  uncoupling proteins
$V_{max}$  maximum enzyme activity
$\Delta G$  change in Gibbs free energy
$\Delta p$  protonmotive force
$\Delta \Delta G$  change in $\Delta G$
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Having been at UBC for awhile, it is not an exaggeration to say I grew up a lot here. I have the deepest gratitude for everyone that has made graduate school such a great experience. I will miss being a part of UBC Zoology.

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Chapter One: General Introduction

1.1 Oxygen and the evolution of aerobic metabolism

The rise of O$_2$ in the atmosphere 2.4-2.1 billion years ago coincided with the rise of eukaryotes and complex life on earth. The success of eukaryotes in the newly oxic environment is thought to be due to the endosymbiotic event whereby an α-proteobacterium was engulfed by a methanogen (Lane, 2006; Martin and Müller, 1998). This merger not only provided the methanogen with an intracellular mechanism to metabolize toxic O$_2$, but it eventually provided the methanogen with the protein toolkit to harness energy from carbon substrates using O$_2$ in what is now referred to as the mitochondrion. Compared to O$_2$-independent energy metabolism, aerobic metabolism in eukaryotes allows for a more complete oxidation of carbon substrates, thus transferring a greater proportion of their chemical energy to ATP. This greater capacity to generate ATP supported the greater energy requirements of life on earth as organisms evolved to grow in complexity, in size, and to explore more diverse environmental niches (Lane, 2006).

Modern eukaryotes are highly dependent upon O$_2$ to sustain energy metabolism and in most eukaryotes even short periods of O$_2$ lack can threaten the maintenance of cellular energy balance (Boutilier and St-Pierre, 2000). Indeed, over 90% of O$_2$ delivered to tissues was estimated to be used by mitochondria for aerobic respiration (Rolfe and Brown, 1997). Under O$_2$ limiting conditions, cellular ATP levels can fall when ATP supply no longer matches ATP demand, resulting in the failure of ion-motive ATPases (among other ATPases), membrane potential depolarization, calcium ion influx, and the initiation of necrotic cell death (Boutilier and St-Pierre, 2000). As such, even short disruptions of O$_2$ supply to tissues can have dire consequences. In mammals, which are generally sensitive to low O$_2$ exposure (hypoxia), seconds of disrupted blood supply to critical tissues such as the brain can cause ATP levels to fall over the course of 5-10 sec resulting in irreparable cellular damage (Hansen, 1985; Lutz et al., 2003).

Though modern eukaryotes have evolved a heavy dependence on O$_2$ for energy metabolism, the threat of O$_2$ toxicity still looms. Oxygen can react with electrons to form reactive oxygen species (ROS) and this occurs primarily at sites within the mitochondrial electron transport system (ETS). Low levels of ROS are constantly produced by mitochondria through electron slip, which are believed to play an important role in cell signaling (D’Autréaux and Toledano, 2007), and are scavenged by mitochondrial and cellular antioxidant mechanisms (Turrens 2003). When ROS are
produced at high levels, which can occur under highly reducing redox conditions, they can overwhelm the cell’s antioxidant capacity and cause cellular damage by reacting with lipids, proteins, and DNA (Cadet, 2003; Gutteridge, 1995; Reznick and Packer, 1994). Therefore, O₂ use by the cell is a proverbial “double-edged sword”, where both the good and bad of O₂ use must be kept in careful balance. A steady substrate and O₂ supply to mitochondrial complexes must be maintained to sustain ATP production, meanwhile electron slip must be avoided in order to prevent increases in ROS generation. Indeed, mitochondrial aerobic and ROS metabolism have been the focus of numerous studies because of the pathological conditions that can arise from or cause variable O₂ supply to tissues (e.g. heart attack, stroke, sleep apnea; Chouchani et al., 2014; Piantadosi and Zhang, 1996; Troncoso Brindeiro et al., 2007)

The vast majority of work examining the interaction between aerobic metabolism and ROS generation has been conducted in mammalian models that have evolved to function under fairly constant O₂ conditions. Animals in nature, however, can be found in environments that vary immensely in O₂ from hypoxia or anoxia, to hyperoxia, over various time periods. For example, animals are able to survive months in cold anoxia during the winter months (Nilsson, 2001), and animals that inhabit intertidal environments can experience wide variability in O₂ due to tidal cycle (Richards, 2011). Compared with studies focused on hypoxia-sensitive mammalian systems, few studies have taken advantage of the organisms that inhabit different O₂ environments to examine putative adaptations of mitochondrial O₂ use for aerobic and ROS metabolism. There are a few exceptions with a study on intertidal elasmobranchs (Hickey et al. 2012) and laboratory bred deer mice of high altitude ancestry (Peromyscus maniculatus; Mahalingham et al. 2017). A comparative approach investigating mitochondria from multiple species that naturally experience O₂ variability would help to illuminate the underlying mechanisms essential to inhabiting these challenging environments. Further, the impacts of natural O₂ oscillations on aerobic and ROS metabolism are unclear, and hypoxia and hyperoxia both can pose very different challenges on mitochondrial function. Thus, the goal of this thesis is to elucidate the balance and trade-offs between the two aspects of mitochondrial O₂ use, aerobic respiration and ROS metabolism, in animals that inhabit different O₂ environments and vary in hypoxia tolerance. In this introduction, I will first briefly describe how mitochondria use O₂ and what affects aerobic and ROS metabolism, followed by what is currently known from well-studied mammalian models and the lesser-studied comparative models of adaptation to hypoxia.
1.2 The Mitochondrion
Mitochondria are often described as the “powerhouses of the cell” because of their critically important role in energy metabolism. Briefly, the mitochondrial electron transport proteins capture free energy from substrate oxidation in the form of a proton gradient that is used to facilitate the phosphorylation of ADP to ATP in a process termed oxidative phosphorylation (Mitchell 1961, 1966). Processes involved in substrate oxidation (namely glycolysis, the Kreb’s cycle and β-oxidation) generate reducing equivalents (NADH, succinate, FADH₂) which donate electrons to the protein components of the mitochondrial ETS (complexes I to IV; Fig.1.1). Complexes I (electrons from NADH), II (electrons from succinate), glycerol-3-phosphate dehydrogenase (electrons from glycerophosphate oxidation), electron-transferring flavin protein (electrons from fatty acid beta-oxidation) all donate electrons to the Q (ubiquinone) cycle at complex III. Another electron carrier, cytochrome c, receives electrons at complex III. Reduced cytochrome c then docks and donates electrons to complex IV (cytochrome c oxidase or COX). The electrons transferred to COX are eventually used to reduce O₂ to form water. As electrons move along the ETS, the resulting difference in free energy from the redox reactions is used by complexes I, III and IV to remove and/or transport protons from the mitochondrial matrix into the intermembrane space (IMS), to form a proton electroconcentration gradient or protonmotive force (Δp) across the inner mitochondrial membrane (IMM). In the final step of oxidative phosphorylation, complex V (F₁F₀-ATP synthase) couples the movement of protons back into the matrix with ADP phosphorylation to ATP (Fig.1.1). ATP is then transported out of the mitochondria into the cytoplasm via the adenine nucleotide transporter (ANT) to be used by the cell.

1.3 Factors affecting mitochondrial function
The proton gradient generated by the mitochondrial ETS complexes drives complex V activity to phosphorylate ADP into ATP. There are several factors that can influence ETS function and ultimately the rate and efficiency of phosphorylation of mitochondria, which include proton leak, electron leak, and O₂ availability.

1.3.1 Proton leak
Changes to proton movement from the matrix to the IMS could affect mitochondrial phosphorylation efficiency, which is empirically assessed by the ADP/O or P/O. Proton leak from the IMS into the matrix can occur via ANT (which exchanges ATP out of the matrix for ADP and a
proton into the matrix), uncoupling proteins (UCPs; Jastroch et al., 2011), and due to general mitochondrial membrane leakiness (Brand et al., 1994). This is commonly referred to as mitochondrial proton leak and results in ETS electron flux, proton pumping, and O₂ use without ATP generation. This futile cycling of protons has a role in heat generation in endotherms and can account for up to 15% of basal metabolic rate (Rolfe et al., 1999). It is unlikely, however, that proton leak functions solely for thermogenesis as it also occurs in ectotherms and in some cases can account for 20-30% of routine metabolic rate in lizards and frogs hepatocytes (Brand et al. 2000). As this proton leak incurs higher metabolic costs, it might be assumed that a lower proton leak would be beneficial and result in a higher phosphorylation efficiency and in a high protonmotive force. This would, however, also require maintaining the ETS complexes in a more reduced redox state which could stimulate ROS production at sites on the ETS complexes (discussed below). Thus, it has been suggested that the dissipation of the proton gradient would reduce mitochondrial ROS generation and relieve oxidative stress, which has been coined the ‘uncouple to survive’ hypothesis (Brand, 2000; Brand and Esteves, 2005).

1.3.2 Electron leak
Some of the electrons that enter the ETS leak out and react with O₂ to form ROS at a rate that accounts for 1-2% of total O₂ used by mitochondria (Boveris and Chance, 1973). Mitochondrial ROS have been observed to be generated from eleven sites along the ETS where one leaked electron reduces O₂ to form superoxide radicals, or two leaked electrons form the more stable H₂O₂ (Murphy, 2009; Quinlan et al., 2013; Brand 2016). Electrons that become substrates for ROS generation do not participate in the ETS redox reactions, which reduces the protons pumped to contribute to the protonmotive force. As a result, changes in ETS flux that increase ROS generation would impact phosphorylation efficiency. Additionally, changes in ETS flux that occur due to dysfunction or inhibition of ETS complexes that lead to increases in ROS generation can overwhelm cellular antioxidant mechanisms and lead to ROS accumulation (Ott et al., 2007; Sies, 1997). ROS accumulation can cause oxidative damage to the ETS and the phospholipid membrane, negatively impacting ETS flux (Paradies et al., 2004; Petrosillo et al., 2003).

ROS generation from the various ETS sites differ depending on the protonmotive force, redox environment, and energy status of the mitochondrion (Aon et al., 2010; Barja, 2002; Quinlan et al., 2013), which has made it challenging to assess ROS emission under physiologically relevant
conditions. ROS scavenging processes are also highly dependent upon the cellular and mitochondrial redox environment (which is the sum of various redox couples in the different cellular compartments). In order to better understand and predict the relationship between ROS generation/scavenging and redox environment, Aon et al. (2012) developed a hypothesis that ROS accumulates under both highly reduced and oxidized redox states. Under normal physiological conditions, ROS is generated at low rates, plays a role in cell signaling (D’Autréaux and Toledano, 2007) and is scavenged by cellular antioxidant defences (via a combination of small molecular weight antioxidants and antioxidant enzymes; Point 1 in Fig.1.2; Pamplona and Costantini, 2011). Under conditions of low ETS flux and high protonmotive force (point 2 in Fig 1.2), such as in hypoxia, electrons can build up at ETS complex redox centres which result in more reduced ETS complexes and lead to a high rate of ROS generation. This high rate of ROS generation can overwhelm the scavenging capacity of mitochondria and increase net ROS emission. Under conditions of high ETS flux and lower protonmotive force (point 3 in Fig.1.2), lower electron availability can cause the mitochondrial environment and ETS to become relatively more oxidized which can lower overall redox buffering capacity (e.g. by lowering NADPH/NADP⁺ redox couple and reducing recycling of other antioxidant redox couples) and compromise cellular scavenging capacity, also culminating in elevated ROS emission (Aon et al., 2010; Munro and Treberg, 2017).

While better coupled mitochondria with low proton leak results in efficient ATP production, this also leads to an increased rate of ROS generation by promoting a more reduced state in the ETS. As such, there is a clear trade-off with aerobic metabolism whereby improved phosphorylation efficiency can result in a mitochondrial environment conducive to ROS generation. Thus, ETS function must be carefully regulated to maintain ATP production to support cellular activities under

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1 The main redox couples within the cell are NADH/NAD⁺, NADPH/NADP⁺, and GSH/GSSG. NADH/NAD⁺ is an important redox couple functioning in electron transport, for example, donating to ETS complex I. The NADPH/NADP⁺ pair is involved in maintaining redox balance, for example, NADPH is important for the regulation of the GSH/GSSG redox couple as it donates electrons to the reaction of glutathione reductase to recycle GSSG into GSH. GSH/GSSG is a dominant redox couple as well and is associated with maintaining important redox-linked reactions in different cellular compartments, such as maintaining critical protein sulphhydrils in the nucleus for DNA repair and maintaining an oxidizing environment in the endoplasmic reticulum for protein disulfide bond formation (reviewed in Mari et al., 2013).
a range of physiological and environmental conditions while also preventing excessive ROS generation that would lead to oxidative stress.

1.3.3 O₂ delivery to mitochondria
The supply of terminal electron acceptor, O₂, to the ETS is crucial in maintaining ETS flux. As the terminal electron acceptor, O₂ limitation to COX could lead to slowing of overall ETS flux, lower the maintenance of the proton gradient and ultimately affect complex V activity to phosphorylate ADP. The process of transporting O₂ from the environment to mitochondria involves both diffusive and convective movement along the O₂ transport cascade. Central to the efficient movement of O₂ across the steps of the O₂ transport cascade is the maintenance of partial pressure gradients so that O₂ readily diffuses from higher to lower PO₂. When O₂ comes into contact with the respiratory surface via ventilation, it diffuses across the respiratory surface into blood (typical mammalian arterial PO₂ is 10 to 13kPa; Malatesha et al., 2007), where convective movement of blood with O₂-bound hemoglobin is circulated to tissues. Oxygen then diffuses across the capillary walls into the interstitial fluid (mammalian PO₂ estimates of 1.3 to 2.3kPa; reviewed in Mik et al. 2009) and across the cell membrane into the cytoplasm (PO₂ of 0.4 to 0.9kPa; reviewed in Mik et al. 2009), and finally diffusing to mitochondria (PO₂ of 0.3 to 0.7kPa; Jones 1986, Whalen and Nair 1967) where binding to COX facilitates ATP generation. It is estimated that 90% of O₂ is used by mitochondria and COX, while the remainder is attributed to O₂ use at peroxisomes and endoplasmic reticulum that can contribute to ROS generation (Brown and Borutaite, 2012). Recent estimates of mitochondrial PO₂ in rat hearts reported a much higher mean mitochondrial PO₂ 4.7kPa (~46µM O₂)² than previously reported values, although there appears to be great variation in mitochondrial PO₂ with values as low as 0 to 1.3kPa (0 to 14µM O₂) reported in the same tissue (Mik et al. 2009). This variation is likely dependent upon the location of mitochondria within the cell. In comparison, mitochondrial P₅₀ (i.e. O₂ concentration at half-maximal O₂ consumption rate) is many fold lower than most of the estimated mitochondrial PO₂ values, and range between 0.05-0.1kPa (0.1-0.2µM) at rest and 0.15-0.24kPa (0.3-0.5µM) in phosphorylating mitochondria (Gnaiger et al. 1998) and the Michaelis-Menten constant (K_m; an indicator of substrate affinity) of mammalian COX for O₂ is estimated to be between 0.5-1µM in bovine COX (reviewed in Brunori et al. 1987 and Nicholls and

² At 37°C, barometric pressure of 100.7kPa, O₂ concentration at air saturation is 208.87µM.
Thus, normal intracellular physiological \( PO_2 \) are many fold above what is required for ETS to function maximally, suggesting that mitochondria and COX do not lack \( O_2 \) under normal physiological conditions. However, reductions in environmental \( PO_2 \) or disruption of blood supply to tissues can compress diffusion gradients such that \( O_2 \) does become limiting to mitochondria which causes ETS flux to slow as it is without a terminal electron acceptor. This slowing of electron flux subsequently slows the redox-linked proton pumping reactions at complexes I, III, and IV, which lowers the proton gradient and ultimately reduces the drive for complex V to phosphorylate ADP. Thus, complex feedback mechanisms involving \( O_2 \) levels, ATP turnover, and substrate oxidation ensure that cellular energy demands are matched with aerobic ATP supply (Balaban 1990; Brown 1992), such that increases in cellular activity that reduce intracellular \( O_2 \) levels will stimulate tissue blood flow to maintain \( PO_2 \) levels (Hogan 2001; LaManna et al. 2004). This feedback regulation results in tight coupling that attempts to maintain tissue \( PO_2 \) levels in order to sustain mitochondrial ETS function. However, if environmental hypoxia worsens, \( O_2 \) will eventually become limiting for mitochondria and threaten energy and redox balance.

1.4 Mammalian mitochondrial responses to \( O_2 \) variability

Much of what is currently known of how mitochondria respond to \( O_2 \) variability is from work conducted on hypoxia-sensitive mammals to investigate the implications of various \( O_2 \) related pathologies. The pathologies and tissue damage associated with ischemia-reperfusion injury (e.g. heart attack and stroke) are thought to be due to the ROS surge that occurs when \( O_2 \) supply to the tissue recovers after hypoxia (Murphy and Steenbergen, 2008). As a result, tremendous research efforts have focused on how to mitigate ROS generation and also to alleviate downstream effects to improve prognosis (e.g. Chouchani et al., 2014).

1.4.1 Mammalian mitochondrial responses to acute changes in \( O_2 \)

**Hypoxia**

The main response of mammalian mitochondria to acute hypoxia exposure is a reduction in respiration rate via a decrease in ETS capacity (Kuroda et al., 1996; Magalhães et al., 2005; Schumacker et al., 1993; Sims and Pulsinelli, 1987). The reduction in mitochondrial aerobic metabolism then necessitates an increase in \( O_2 \)-independent glycolytic flux in attempts to maintain cell function, which has a lower ATP yield. The hypoxia-induced reduction in state III ADP-stimulated respiration rate appears to be reversible and mediated through direct modifications of the
ETS complexes (Schumacker et al. 1993) causing them to adopt a more reduced redox status (Chandel et al., 1996; Vollmar et al., 1997). This lowered ETS capacity has been shown to be, in part, mediated through COX. Oxygen can allosterically modify COX in bovine mitochondria and lower its activity under hypoxic conditions (Chandel et al., 1996). Other mechanisms of modifying COX activity have been observed in hypoxia (6-12hrs at 0.13kPa), such as a reduction in COXI, IV, and Vb subunit mRNA content that is associated with an overall loss of COX enzyme in mice macrophages, and decreases in COX activity with a slight increase in catalytic turnover observed in rat PC12 cells (Vijayasarathy et al., 2003). The other ETS complexes also play a role in coordinating the mitochondrial response to hypoxia. Complex I-linked respiration was inhibited to a greater extent (32-46%) than complex II-linked respiration (25%) in rat parietal cortex mitochondria exposed to 7% O2 ischemic conditions (Gilland et al., 1998). These rapid modifications to ETS complexes can occur via post-translational modifications. For instance, S-nitrosation (R-SNO) of mitochondrial protein thiols at sites on complexes II, III, IV, and electron-transferring flavoproteins (ETF), have been observed to exert protective effects on mouse hearts during ischemia (Chouchani et al., 2017). Also, multiple phosphorylation sites have been identified on COX (Helling et al., 2012) and are believed to play a role in modifying COX function in response to hypoxia (Prabu et al. 2005). Thus, mammalian mitochondria inhibit ETS flux in response to low O2 supply.

Normoxic-recovery from hypoxia

As cellular PO2 ranges between 0.4 to 0.9kPa (Mik et al. 2009), studies that utilize normoxic-recovery following hypoxia exposure are in fact exposing mitochondria to non-physiological oscillation in O2 levels. Indeed, the vast majority of mitochondrial studies, including many performed in this thesis, undertake mitochondrial analysis under air-saturated conditions which does not replicate in vivo conditions. From studies of this nature, however, it is possible to understand the effects of large changes in O2 on overall mitochondrial function. For instance, ischemic-tissue that is reoxygenated experience a large increase in O2 levels. Upon normoxia-recovery, hypoxia-induced reduction in ETS flux needs to be reversed in order to restore aerobic ATP production and full cellular function. However, a quick return to normoxic and relatively high O2 levels can cause a surge in ROS generation. High electron flux along with high phosphorylation rate lowers the protonmotive force leading to a relatively oxidized redox environment, which with high O2 availability favors an increase in ROS generation from ETS sites (Brand 2000). It appears that an
inhibition of ETS flux upon hypoxia-reoxygenation may be part of a slow controlled recovery to reduce ROS generation. In fact, pharmacological inhibitions of both complex I \((via\ S\text{-nitrosation modification;}\ Chouchani\ et\ al.,\ 2013)\) and II \((via\ malonate\ inhibition;\ Chouchani\ et\ al.,\ 2014)\) have been shown to have cardioprotective effects and alleviated ischemic-reperfusion injury in murine models. Also, there may be temporal differences of \textit{in vivo} regulation of the different ETS complexes. In gerbils recovering from cerebral ischemia, complex II-linked state III respiration rate recovered more quickly (5min) compared to complex I-linked state III respiration rate (30min; Almeida\ et\ al.,\ 1995). It is also possible that the reduction in ETS complex activities is caused by a secondary effect of oxidative damage to the lipid bilayer in mammals. After ischemia exposure, an increase in \(\text{H}_2\text{O}_2\) generation was accompanied by a loss of cardiolipin (Chen and Lesnefsky, 2006; Paradies\ et\ al.,\ 2004), which is a mitochondrial phospholipid important for ETS complex assembly and for stabilizing respiratory supercomplexes (Mileykovskaya and Dowhan, 2014; Pfeiffer\ et\ al.,\ 2003).

1.4.2 Mammalian mitochondrial responses to chronic changes in \(O_2\)

\textbf{Hypoxia}

Many mammals have the ability to acclimate to longer term exposure to moderate hypoxia. Acclimation or acclimatization to prolonged exposure to new \(O_2\) environments are mediated by large-scale changes in gene and protein expression, epigenetic factors which have roles in stabilizing hypoxia inducible factor (HIF) and activating HIF directly (Watson\ et\ al.,\ 2010), and \textit{via} post-translational modifications of proteins (Kumar and Klein, 2004), primarily to reduce \(O_2\)-dependent pathways and to increase \(O_2\)-independent energy production. The transcription factor HIF-1\(\alpha\) plays a key role in coordinating cellular responses to hypoxia. HIF-1\(\alpha\) is targeted for degradation in normoxia, but is stabilized when \(O_2\) levels become limiting and acts on nuclear targets. HIF-1\(\alpha\) has also been shown to be stabilized by \(\text{H}_2\text{O}_2\) released by complex III, pointing to a role of \(\text{H}_2\text{O}_2\) in hypoxic signaling (Chandel\ et\ al.,\ 2000; Guzy\ et\ al.,\ 2005). Stabilized HIF-1\(\alpha\) then targets a number of genes that generally coordinate an increase in \(O_2\)-independent ATP producing pathways and reduce substrate supply to the mitochondria, including upregulation of glycolytic genes (\textit{e.g.} glucose transporter GLUT1), \textit{lactate dehydrogenase-}A to increase glycolytic flux (Kim\ et\ al.,\ 2006; Semenza, 2007), and \textit{pyruvate dehydrogenase kinase} that phosphorylates pyruvate dehydrogenase to inhibit pyruvate entry into the Kreb’s cycle and mitochondrial respiration (Papandreou\ et\ al.,\ 2006). HIF-1\(\alpha\)
also targets COX nuclear-encoded subunit 4 and initiates the switching of COX4-1 to COX4-2 during hypoxia. While COX protein with subunit 4-1 under normoxic conditions is inhibited by high ATP levels which would lower ETS flux, COX protein with 4-2 is not sensitive to high ATP levels and allows ETS flux to be maintained (Fukuda et al., 2007; Horvat et al., 2006). This COX4 subunit switch is thought to optimize efficiency of electron flux and minimizes ROS generation during hypoxia. Acclimation to moderate hypoxia has also been shown to result in morphological changes such as increased capillary density, increased mitochondrial volume density, and altered tissue mitochondrial distribution (in liver and heart mitochondria; Costa et al., 1997; Hoppeler et al., 2008; Howald et al., 2008), which would presumably reduce O₂ diffusion distances and improve O₂ delivery to mitochondria.

**Hyperoxia**

When PO₂ levels increase beyond normoxic levels, the increase in substrate (O₂) causes the rate of mitochondrial ROS generation to also increase (Jamieson et al., 1986). Although there are no natural exposures to hyperoxia in mammals, O₂ therapy is often used in clinical settings when the benefits of treatment outweigh the damaging effects of hyperoxic exposure (Thomson et al., 2002). The typical mammalian mitochondrial response to high O₂ tensions is an overall reduction in ETS flux that reduces ROS generation and accumulation. Chinese hamster ovarian cells exposed to hyperoxia (98% O₂, 2% CO₂) showed an 80% decrease in respiration rate within 3 days of exposure which was related to the selective inhibition of complexes I and II, although the cell line eventually died due to ATP depletion (Schoonen et al., 1990). ROS generated from hyperoxia exposure also caused cardiolipin loss (mice exposed to 100% O₂ for 72hrs; Tyurina et al., 2010), which may also negatively impact ETS complex activities similar to the response to hypoxia-recovery as discussed above. An increase in COX activity, however, may be important under high O₂ levels. HeLa cells maintained at 80% O₂ increased COX capacity by two-fold which was associated with two-fold lower ROS levels (Campian et al., 2007) indicating that COX may have a role in helping to mediate ETS flux to mitigate oxidative damage experienced in hyperoxia.

**1.5 O₂ variability in the natural environment**

Most mammals rarely experience hypoxic environments and are generally hypoxia sensitive as they have not evolved mechanisms to survive O₂ limiting conditions. In fact, many organisms, especially those in aquatic environments, are exposed to natural patterns of environmental O₂ variability that
can differ in spatial and temporal patterns and vary from hypoxia to hyperoxia (Diaz and Breitburg, 2009).

Timescale of O\(_2\) changes can vary from minutes to hours, to seasonal changes. Environments such as the marine intertidal zone, estuaries, swamps, and marshes (Diaz and Breitburg, 2009; Nikinmaa, 2002; Richards 2011), show fluctuations in the span of hours due to the tidal cycle. These quick changes are due to periods of high mixing at high tide that would bring about well-mixed oxygenated water, and periods of low tide that may create isolated areas with little to no mixing. In tropical freshwater systems, the rainy season can bring about increased water flow and oxygenation compared to during the dry season. There are also environments that are chronically hypoxic. Animals found living at high altitudes, in poorly ventilated burrows, and in the oceans’ O\(_2\) minimum zone are constantly under O\(_2\) limiting conditions (Storz et al., 2007; Larson et al., 2004; Seibel, 2011; Diaz and Breitburg, 2009).

Spatial variability of O\(_2\) can be driven by biotic and abiotic factors. Stagnant waters with high amount of organic matter and high biomass can become severely hypoxic and even anoxic due to high O\(_2\) consumption. Also, eutrophic environments that are abundant in green plants are prone to periods of hyperoxia as high photosynthetic rates generate high O\(_2\) levels (e.g. up to 300% air saturation was measured in high tidepool in the intertidal zone; Richards, 2011). Hypoxia or anoxia can occur easily in aquatic environments as O\(_2\) solubility is lower in water and diffusion rate is also slower in water than in air (Graham 1990). Both O\(_2\) solubility and diffusion rate vary with abiotic factors such as temperature and salinity (Diaz and Breitburg, 2009). Additional variation in O\(_2\) can be due to differences in mixing that could depend on density, current, wind, and also depth of the different layers (Diaz and Breitburg 2009).

As O\(_2\) variability occurs frequently in aquatic environments, many organisms have been shown to display diverse responses and mechanisms, behavioural, morphological, physiological, and biochemical, that enable them to inhabit and thrive in these O\(_2\) environments. Thus, aquatic organisms show wide variation in hypoxia tolerance.

1.6 Defining hypoxia tolerance

In defining hypoxia tolerance, we should first consider what happens when environmental PO\(_2\) level is reduced. As PO\(_2\) is reduced from air saturation, O\(_2\) content in the blood is maintained with high
blood hemoglobin concentration and increased O$_2$ binding in circulation. Many aquatic animals will exhibit behaviors such as avoidance and aquatic surface respiration to seek oxygenated water to respire, and also reduced spontaneous swimming to lower metabolic demand (although the thresholds for these behaviors are species and even individual dependent; reviewed in Chapman and Mckenzie, 2009). The hypoxic ventilatory response is observed in many fish species where there are changes in ventilation (volume, frequency, amplitude, and/or stroke volume depending on species under investigation), ultimately to increase oxygenated water flowing pass gill lamella to increase O$_2$ extraction (reviewed in Perry et al. 2009). Eventually when P$_{O2}$ has been impacted, aerobic scope is lowered and with it a number of physiological processes (e.g. growth, reproduction) are suppressed but standard metabolic rate is maintained. At the point where aerobic scope is reduced to zero, O$_2$ supply is no longer able to support standard metabolic rate and animals switch from being oxy-regulating to oxy-conforming at the critical O$_2$ tension of O$_2$ consumption rate (P$_{crit}$). Below P$_{crit}$, animals increase their reliance on anaerobic respiration in attempts to maintain cellular energy balance and extend survival time in hypoxia (Farrell and Richards, 2009).

Species with a lower P$_{crit}$ are presumed to be better able to extract O$_2$ from their environment so that standard metabolic rate is maintained to a lower environmental PO$_2$ and the animal is thus more hypoxia tolerant. Thus, P$_{crit}$ has been frequently used in literature as an indicator of hypoxia tolerance (Deustch et al. 2015; Regan and Richards, 2017; Speers-Roesch et al., 2013). Hypoxia tolerant organisms typically have larger respiratory surface area (Mandic et al., 2009; Nilsson, 2007), higher hemoglobin-O$_2$ binding affinity (Jensen and Weber, 1982), and lower tissue O$_2$ demands, all of which facilitate the maintenance of aerobic function even during hypoxia and would lower P$_{crit}$.

There are a couple other metrics commonly used to assess hypoxia tolerance. Animals that have a longer time to loss of equilibrium (LOE$_{50}$) at a hypoxic PO$_2$ are deemed more hypoxia tolerant, as they are able to survive long periods of O$_2$ limitation (Chapman et al., 1995; Mandic et al., 2013). The 50% effective/lethal concentration (EC$_{50}$/LC$_{50}$), which is the O$_2$ concentration that causes 50% reduction in hypoxic survival has been used to determine hypoxia tolerance as well (Andrade et al., 2017; Irving et al., 2004; Wu et al., 2002). These two metrics assess the species’ overall ability to maintain activity using both aerobic and anaerobic processes. A strong relationship between LOE$_{50}$ and P$_{crit}$ has been shown in species of intertidal sculpin fishes, where species with a longer time to LOE$_{50}$ also exhibit a lower P$_{crit}$ (Mandic et al., 2013), supporting that the maintenance of aerobic respiration is an important strategy to survive hypoxia exposure.
Further, the ability of organisms to recover from hypoxia should also be considered as a part of hypoxia tolerance. The reintroduction of O\(_2\) signifies that the processes which have redirected substrates away from mitochondria towards O\(_2\)-independent energy pathways can now be reversed. As I mentioned above, the reoxygenation of mammalian ischemic tissue causes a burst of ROS generation that can lead to extensive tissue damage. The extent of recovery of state III ADP-stimulated mitochondrial respiration after ischemia, the amount of ROS generated, the oxidative damage sustained upon normoxic recovery, and the ability to repair oxidative damage are indicators that have been used to assess the ability to recovery from O\(_2\) limitation (Almeida et al., 1995; Chouchani et al., 2014; Shiva et al., 2007). For example, mouse heart mitochondria exposed to 30min \textit{in vitro} anoxia typically recovers only 50% of state III respiration rate in normoxia, which is thought to be due to ROS damage to the ETS (Shiva et al., 2007). Hypoxia tolerant animals would presumably have strategies to deal with the challenges of exiting the hypoxia bout as well (Bickler and Buck, 2007), but it is unclear what adaptive traits at the mitochondrial level are associated with coordinating recovery from hypoxia.

1.7 Adaptation and plasticity of mitochondria to O\(_2\) variability

Hypoxia tolerance in animals is often associated with modifications to the O\(_2\) transport cascade that improve O\(_2\) extraction from the environment and increase supply to the mitochondria. There are several detailed studies investigating mitochondrial characteristics from laboratory-selected lines of flies maintained at 4% O\(_2\) (herein referred to as hypoxic flies; Ali et al., 2012; Yin et al., 2013), laboratory-bred lines of deer mice with highland ancestry (herein referred to as highland deer mice; Mahalingam et al., 2017), and elasmobranchs that inhabit the marine intertidal environment (Hickey et al., 2012). However, the results from these studies do not suggest a unified evolutionary strategy of mitochondria to O\(_2\) variability. In this section of the Introduction, I will summarize what is currently known of three aspects of mitochondrial function as they relate to aerobic and ROS metabolism from organisms that are characterized as hypoxia tolerant or show variation in hypoxia tolerance.

1.7.1 O\(_2\) binding at mitochondria and COX

Although few studies have attempted to directly determine whether there are differences in mitochondrial and COX O\(_2\) binding in animals that vary in hypoxia tolerance, the few that do exist suggest that adaptation and acclimation to hypoxia exposure can result in modifications to
mitochondrial $P_{50}$ and COX $K_m$ in order to improve mitochondrial function at a lower $PO_2$. For example, highland deer mice have lower mitochondrial $P_{50}$ under resting respiration state conditions compared with a lowland population (Mahalingham et al., 2017). This difference of mitochondrial $P_{50}$ between populations were not modified by hypoxia acclimation. Similarly, killifish (*Fundulus heteroclitus*) showed no changes in mitochondrial $P_{50}$ when acclimated to hypoxia (Du et al., 2016). In overwintering frogs (*Rana temporaria*), however, hypoxia acclimation after 1-month reduced mitochondrial $P_{50}$ compared to normoxic and 4-month acclimated frogs. These studies suggest species-specific responses in plasticity of mitochondrial $O_2$ binding to $O_2$ limitation.

These shifts in mitochondrial $P_{50}$ are likely due to differences in COX activity. Higher COX enzyme content within mitochondria is thought to increase mitochondrial $O_2$ binding affinity (Gnaiger et al., 1998). Hypoxic flies and highland deer mice show higher COX activity or respiration rates compared to normoxic flies and lowland deer mice population (Ali et al., 2012; Mahalingam et al., 2017), which would be consistent with increased mitochondrial $O_2$ binding (although this was only empirically measured in deer mice and not in flies). In elasmobranchs, however, the opposite trend was observed where less hypoxia tolerant shovelnose rays (*Aptychotrema rostrata*) had higher COX respiration rate compared to more hypoxia tolerant epaulette sharks (*Hemiscyllum ocellatum*; Hickey et al. 2012), suggesting higher COX enzyme content in the less tolerant elasmobranch. Although there have not been direct measurements of COX $O_2$ binding across species or populations, there has been strong evidence for adaptive changes in COX substrate affinity in hypoxia tolerant organisms. In both high-altitude bar-headed geese (*Anser indicus*) and Tibetan locusts (*Locusta migratoria*), COX affinity for its electron donor, reduced cytochrome $c$, was observed to be higher than in their low-altitude counterparts (Scott et al., 2011; Zhang et al., 2013). The differences in cytochrome $c$ binding, at least in bar-headed geese, appeared to be due to a single amino acid residue difference on the COX3 subunit (Scott et al., 2011). While these studies allude to differences in COX function that may be associated with hypoxia tolerance, in fact, COX modeling studies did not show a clear mechanistic link between cytochrome $c$ and $O_2$ binding (Krab et al., 2011), so variation in cytochrome $c$ binding may not reflect differences in $O_2$ binding. Thus, whether there are functional differences in $O_2$ binding of COX in animals varying in hypoxia tolerance needs to be empirically determined.
1.7.2 Mitochondrial ETS function

Animals that inhabit $O_2$ variable environments appear to differ in mitochondrial ETS function. In hypoxic flies, there was lower state III ADP-stimulated respiration rate compared to normoxic flies, which was associated with 30% lower complex II activity, and 20% higher complex III and IV activities (Ali et al., 2012). This result suggests a shift to an increased dependence on complex I, which is a proton pump contributing to the proton gradient, rather than on complex II, which does not contribute to the proton gradient. An increased complex I dependency would presumably increase ADP/O leading to more efficient use of $O_2$. There was also an increase in leak respiration in hypoxic flies which indicates higher uncoupling and possibly associated with lower ROS generation (see discussion in 1.6.3). These observations, however, contrast what was observed in a comparison of elasmobranchs that inhabit different $O_2$ environments. There were no interspecies differences in state III respiration rates (both complex I and/or complex II fuelled) of permeabilized ventricular fibres, but the more hypoxia tolerant epaulette shark had lower leak respiration rates, resulting in more coupled mitochondria (higher respiratory control ratio; RCR) compared to the less hypoxia tolerant shovel nose ray (Hickey et al. 2012). Comparisons between highland and lowland populations of deer mice, however, showed no differences in leak respiration rate, and state III respiration rate was higher in the highland population compared to the lowland population indicating higher respiration capacity (Mahalingham et al. 2017). These results reveal divergent characteristics of mitochondrial ETS from different animal models that inhabit hypoxic environments.

Hypoxia tolerant species generally respond to hypoxia exposure by lowering of ETS activity and increasing their dependence on anaerobic respiration. Overwintering frogs submerged for 4 months in hypoxia lowered state III ADP-stimulated and state IV resting respiration rates by 60% in skeletal muscle which was mediated by a lowering of the proton motive force (St-Pierre et al. 2000a, b). A reduction in mitochondrial capacity was accompanied by an increase in glycolytic capacity as evidenced by an increase in transcription of *lactate dehydrogenase* and *phosphofructokinase* in liver and muscle of longjaw mudsucker (*Gillichthys mirabilis*; Gracey et al., 2001) and increased in activities of a number of glycolytic enzymes in tench (*Tinca tinca*; Johnston and Bernard, 1982). However, hypoxia-induced plasticity of mitochondria ETS appears to be species-specific as some species are also able to maintain ETS function even in the face of severe hypoxia. In killifish *Fundulus heteroclitus*, hypoxia, whether intermittent or chronic exposures for 28-33 days at 5kPa, did not affect liver mitochondrial
respiratory capacities or mitochondrial O$_2$ kinetics indicating that ETS flux was maintained (Du et al., 2016). Similarly, ventricular fibres from epaulette sharks showed no significant changes in mitochondrial function after whole animal hypoxia exposure, whereas fibres from the shovelnose ray exposed to hypoxia had 50% lower complex I and II fluxes rates and 33% lower COX flux rates compared with fibres from normoxia-acclimated rays. These results suggest that while there are animals that reduce ETS function in response to O$_2$ limitation, certain species of hypoxia tolerant fish were able to maintain mitochondrial function in response to acute hypoxia.

1.7.3. ROS metabolism
While mammals sustain extensive ROS damage after ischemia-reperfusion, it has been proposed that hypoxia tolerant animals would be able minimize the oxidative damage by either lowering ROS generation or increasing in ROS scavenging during normoxia recovery from hypoxia. Despite this intuitive expectation, neither whole animal studies looking at responses to O$_2$ challenges or mitochondrial studies in hypoxia tolerant or adapted animals have yielded consistent trends. The hypoxia tolerant epaulette shark generated 60-70% less ROS/O$_2$ at the mitochondria when examined under both working or resting conditions than the less hypoxia tolerant shovelnose ray (Hickey et al., 2012). Mitochondria from hypoxic flies showed reduced superoxide generation (with complex I substrates only), likely due to the increased uncoupling (measured higher leak respiration) that would decrease the protonmotive force and reduce the drive for ROS generation (Ali et al. 2012). In contrast, mitochondria from highland and lowland deer mice populations did not show differences in ROS emission under both state III and IV conditions (Mahalingham et al. 2017). Further, no study has shown consistent ROS responses at both the mitochondria and the tissue level during whole animal hypoxia exposure. The difference between diverse species is possibly due to variation in antioxidant defenses (Leveelahti et al., 2014). For instance, elasmobranchs are well known to have lower antioxidant activities than teleosts (Filho and Boveris, 1993; Gorbi et al., 2004) and thus could result in species differences in ROS accumulation kinetics.

Our current understanding of mitochondrial function in animals tolerant to natural variations in O$_2$ is scattered. First of all, previous studies have focused primarily on hypoxia exposure and characterizing hypoxia tolerance, and far less is known of hyperoxia tolerance. While it has been suggested that mitochondria and COX from hypoxia tolerant animals may be better able to function under low PO$_2$, this has not been explicitly measured. Further, while there is strong evidence that
mitochondria have a role in determining whole animal hypoxia tolerance, it has only been addressed in a few species that live in hypoxia environments (where hypoxia tolerance has not been determined), that are distantly related, exposed to different O₂ levels, under substrate and air-saturating, supraphysiological O₂ conditions, and also studied at various levels of biological organization (whole animal tissue responses vs permeabilized tissue vs isolated mitochondria). Therefore, characterizing mitochondrial traits of multiple closely-related species with known whole animal hypoxia tolerance would be insightful.

1.8 Thesis objectives and chapter hypotheses
The overall goal of this thesis is to determine the mitochondrial traits that have shaped whole animal tolerance to environmental O₂ variability. In particular, I will address the three aspects of mitochondrial function that I have discussed above: O₂ use at mitochondria and COX (Chapter 2), and mitochondrial ETS flux and ROS metabolism (Chapter 3 & 4) in a group of fish species commonly called sculpins that live along the highly variable marine near-shore environment.

1.8.1 Using intertidal sculpins (Cottidae, Actinopterygii) as model
The near-shore marine intertidal environment is heavily influenced by the daily tidal cycle, resulting in an environmental gradient of variability in a number of abiotic factors. In the rocky intertidal, ebb tide causes pools of water to become isolated from bulk seawater forming tidepools, which can occur at different levels within the intertidal zone. The longer the tidepool is emerged from the bulk ocean, the greater the fluctuations in environmental characteristics (0 to 400% O₂ air saturation, pH 7.0 to 9.5, 12 to 24°C; Richards, 2011). In contrast, the lower intertidal may still be submerged during low tide and as a result experience less dramatic fluctuation in environmental variables (0 to 200% O₂ air saturation, pH 7 to 8.5, and 12 to 18°C; Richards 2011). The subtidal and offshore environments stay relatively homogenous with the bulk ocean.

The variation in environmental gradient has a strong effect on species distribution along the marine near-shore environment, including multiple species of sculpins (Cottidae, Actinopterygii; Knope, 2013). Intertidal sculpins display high site fidelity and therefore they experience the environmental fluctuations particular to their habitat for prolonged periods of time, including drastic fluctuations in O₂ levels (Green, 1971; Knope et al., 2017). As such, species that inhabit the higher intertidal which are more prone to both hypoxic and hyperoxic exposures have been shown to be more hypoxia tolerant with longer time to LOE than species found in the lower intertidal and subtidal
environments (Mandic et al., 2013). This greater hypoxia tolerance in higher intertidal sculpins was significantly correlated with a lower $P_{\text{crit}}$ which was further correlated with a higher gill surface area and higher hemoglobin $O_2$-binding affinity (Hb-$P_{50}$); both traits increase $O_2$ movement from the environment into the animal’s circulation (Mandic et al., 2009). At the biochemical level, sculpins from the intertidal environment had higher brain LDH activity, which increases their anaerobic capacity and also their ability to buffer redox imbalances in the tissue (Mandic et al. 2013).

The sculpin system is ideal for the studies in this thesis for multiple reasons. First, evidence of adaptive variation in $O_2$ extraction and delivery has been observed at multiple steps of the sculpin $O_2$ transport cascade (Mandic et al., 2009; Mandic et al., 2013); however, whether there is similar interspecific variation in $O_2$ use at the mitochondrial level has not been studied. Second, while traits to enhance $O_2$ extraction and delivery would be beneficial during periods of $O_2$ lack, the daily tidal cycle also brings about periods of hyperoxia, and tolerance to hyperoxia has yet to be investigated in sculpins. Hypoxia and hyperoxia place very different demands on processes involved in aerobic respiration and ROS metabolism. As the natural variation in $O_2$ in the different intertidal zones have shaped sculpin hypoxia tolerance, these species provide an ideal system to study the interplay and potential evolutionary trade-offs between mitochondrial aerobic and ROS metabolism. Finally, a cross-lineage approach with closely-related species allows for the correlation of measured traits to the corresponding environmental factor while considering phylogenetic relationships. Where possible, I have chosen to characterize mitochondrial traits in multiple species (vs two species comparison) so as to draw conclusions and to form additional hypothesis about evolutionary processes that have shaped physiological traits (Garland and Adolph 1994). This has proven to be a valuable approach to account for the effects of phylogeny and identifying relationships of traits potentially adaptive in challenging environments. As such, with the sculpin model I will be able to assess the relationship between mitochondrial traits and whole animal hypoxia tolerance while incorporating phylogenetic relationships.

1.8.2 Chapter hypotheses

In the following three chapters, I assessed aspects of mitochondrial function using the intertidal sculpin model with the hypothesis that mitochondria from hypoxia tolerant species would show putatively adaptive traits that improve $O_2$ binding, maximize phosphorylation efficiency, and
minimize ROS emission compared to less tolerant species, resulting in an overall more O₂-efficient ETS (Fig.1.3).

**Hypothesis:** Hypoxia tolerant sculpins exhibit higher O₂ binding affinity at the mitochondria and COX compared with less hypoxia tolerant species.

In Chapter 2, I studied the O₂ kinetics of intact brain mitochondria and semi-purified COX. As mentioned earlier in the Introduction, few studies have attempted to examine whether there is variation in mitochondrial function as it relates to hypoxia tolerance and those that have, have generally focused on differences in how COX interacts with its other substrate, electron donor cytochrome c (Scott et al., 2011; Zhang et al., 2013), and have not directly studied COX O₂ kinetics. In fact, it appears that there is little evidence for a mechanistic link between the binding affinities of cytochrome c and O₂ (Krab et al., 2011). As the maintenance of O₂ binding to COX is likely paramount in sustaining aerobic metabolism in hypoxia, I developed a protocol to investigate O₂ kinetics of isolated brain mitochondria and semi-purified COX in 12 species of sculpins. Further, I used *in silico* protein modeling techniques to investigate possible underlying explanations for the interspecific variation in COX O₂ binding affinity, potentially via modifications to COX3 subunit interaction with membrane phospholipid, cardiolipin (Lau et al., 2017).

**Hypothesis:** Mitochondria from hypoxia tolerant sculpins would have more efficient O₂ use showing higher phosphorylation efficiency and lower ROS emission compared to less tolerant species.

In Chapter 3, I explored how the variation of mitochondrial and COX O₂ binding affinities in Chapter 2 is manifested in intact mitochondrial function in regards to phosphorylation efficiency and ROS emission. Aerobic and ROS metabolism in mitochondria are tightly linked. While O₂ usage powers ATP production and supports cellular work, at the same time it leads to generation of ROS which when accumulated can be damaging. With hypoxia tolerant animals having adapted mechanisms to improve O₂ delivery and enhance O₂ use, the question remains whether there are evolutionary trade-offs between aerobic and ROS metabolism. In this chapter, I investigated differences in mitochondrial respiration and ROS emission in isolated brain mitochondria from multiple sculpin species in order to identify adaptive traits that relate to whole animal ability to tolerate O₂ variability. I hypothesized that mitochondria from hypoxia tolerant sculpins would have
more efficient O$_2$ use which would be reflected in higher phosphorylation efficiency (P/O) as a result of increased complex I dependency (point (2) in Fig.1.3), better mitochondrial coupling (higher RCR due to reduced proton leak; point (3) in Fig.1.3), and lower overall ROS emission (lower ROS/O$_2$) compared to less tolerant species (point (4) in Fig.1.3).

**Hypothesis:** Hypoxia tolerant sculpins would reduce ROS accumulation and show less effects of ROS in response to hypoxia, hyperoxia, and normoxia recovery from both exposures compared to less tolerant species.

Previous studies have found species- and tissue-specific responses of ROS generation and scavenging capacities during exposure to O$_2$ variability (Leveelahti et al., 2014). Building upon the characterization of isolated mitochondria (Chapter 3), in Chapter 4 I investigated whether there are consistent responses of ROS metabolism observed at the whole animal level. I exposed two sculpin species to 6hrs of hypoxia or hyperoxia followed by a quick normoxic recovery, the timing of which mimics the duration of a typical tidal cycle. I then assessed different aspects of ROS metabolism, including redox status, mitochondrial ROS, oxidative damage, and scavenging capacity. In order to assess mitochondrial H$_2$O$_2$ levels, I used a mitochondria-targeted mass spectrometry probe, MitoB, to measure *in vivo* changes in ROS (Logan et al., 2014).

Finally, in the General Discussion (Chapter 5), I summarize the major findings from my thesis research, discuss how my findings extend our understanding of mitochondrial physiology, and also pose future research questions generated from my work.
**Figure 1.1 Vertebrate electron transport system (ETS).** The ETS protein complexes are imbedded in the inner mitochondrial membrane (IMM), where NADH donates electrons to complex I (NADH oxidoreductase; CI) and succinate donates electrons to complex II (succinate dehydrogenase; CII), and along with glycerol-3-phosphate dehydrogenase (GPDH) and electron-transferring flavin protein (ETF) donate electrons to ubiquinone/ubiquinol (UbQ). UbQ subsequently donates electrons to complex III (cytochrome bc1 complex/cytochrome c reductase; CIII) which donates its electrons to cytochrome c (Cyt c). Reduced cytochrome c binds to complex IV (cytochrome c oxidase; CIV) which is also where oxygen receives electrons and is reduced to water. Complexes I, III, and IV removes and/or pump protons (H⁺) from the mitochondrial matrix to the intermembrane space (IMS) to form the electrochemical gradient. The movement of protons back into the matrix via complex V (F₁Fo-ATP synthase; CV) is used to drive the phosphorylation of ADP into ATP. More details of this process of oxidative phosphorylation is in section 1.2.
Figure 1.2. Balance of ROS scavenging rate and generation rate that results in accumulation of ROS. Under a normal redox environment (1), a high ROS scavenging rate with a low ROS generation rate results in a low net ROS level. Under highly reduced redox environment (2), high ROS generation rate exceeds ROS scavenging rate, resulting in an increase in ROS levels. Under highly oxidized redox environment (3) which lowers the cell’s redox buffering capacity and thus lowers ROS scavenging rate, results in an accumulation of ROS. Detailed description of figure in section 1.3.2. This figure is modified from Aon et al. (2010).
Figure 1.3. A hypothesis for an $O_2$ efficient electron transport system. I hypothesized that the increased $O_2$ delivery and binding upstream in the $O_2$ transport cascade observed in the intertidal sculpin model (Mandic et al., 2009), would extend to the level of mitochondria such that there would be higher mitochondrial and COX $O_2$ binding affinity (1). A higher dependency on complex I, which is a proton pump, would result in higher phosphorylation efficiency (assessed as P/O; 2). Further, lower proton leak (3) would result in higher mitochondrial coupling (assessed with respiratory control ratio; RCR) and lower futile proton cycling. Finally, lower ROS generation (4) would lower the amount of $O_2$ used to generate a potentially harmful byproduct, ROS.
2.1 Introduction

Environmental hypoxia in marine ecosystems is increasing in both severity and duration due to climatic shifts and wide-spread eutrophication (Diaz and Breitburg, 2009). The increasing prevalence of hypoxia threatens to compress viable marine habitats, but whether a species will be impacted is dependent upon their hypoxia tolerance, which among fish is known to vary to a great degree (Chapman and Mckenzie, 2009). Indeed, naturally occurring hypoxia has been an important evolutionary driving force in fish, resulting in both convergent and divergent selection of physiological traits that enhance hypoxia tolerance (Richards, 2009; Richards, 2011). Understanding the determinants of variation in physiological function as it pertains to hypoxia tolerance is increasingly important in order to parametrize predictive models that seek to define how organisms will respond to the greater prevalence of hypoxia in the marine environment.

Adaptive modifications of the vertebrate oxygen (O$_2$) transport cascade are well described in various groups of organisms that encounter hypoxia including fish (Chapman and Mckenzie, 2009), high altitude geese (Scott et al., 2009) and deer mice (Lui et al., 2015; Natarajan et al., 2015; Storz et al., 2009). Hypoxia tolerant organisms typically have larger respiratory surface areas (gills, Nilsson, 2007; lungs in birds, Scott et al., 2011), higher haemoglobin-O$_2$ binding affinity (Jensen and Weber, 1982), and lower tissue O$_2$ demands (Hopkins and Powell, 2001) all of which facilitate the maintenance of aerobic function even in the presence of sometimes severe environmental hypoxia. Indeed, among sculpins (diverse species of fish from the family Cottidae; Actinopterygii) that live along the marine near-shore environment, an environment typified by strong spatial and temporal variation in hypoxia exposure, Mandic et al. (2009) demonstrated a phylogenetically-independent relationship between the critical PO$_2$ for O$_2$ consumption rate ($P_{cr}$; the environmental PO$_2$ below which animal O$_2$ consumption rate conforms to decreasing environmental PO$_2$), and several traits along the O$_2$ transport cascade. Hypoxia tolerant sculpins have larger mass specific gill surface area, higher

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hemoglobin-O₂ binding affinity (low Hb-O₂ P₅₀), and generally lower metabolic rates compared with less hypoxia tolerant species. Although it is well established that modifications to the O₂ transport cascade are important determinants of hypoxia survival in fishes and other organisms, much less is known about how variation in hypoxia tolerance is related to mitochondrial function and the actions of cytochrome c oxidase (COX), which is the protein responsible for the majority of whole-animal O₂ use during respiration.

Cytochrome c oxidase is an ancient, multi-subunit enzyme that is thought to be older than the surge in atmospheric O₂ that occurred 2.4-2.1 billion years ago and led to the explosion of eukaryotic biodiversity (Castresana et al., 1994; Lyons et al., 2014). Although the original function of COX remains unknown, COX in oxic mitochondria catalyze the final transfer of electrons from the electron transport system (ETS) to O₂, reducing it to water while simultaneously pumping protons to generate a proton electrochemical gradient for ATP synthesis via the F₁F₀ ATP-synthase. The subunits that compose the COX catalytic core, COX1, 2, and 3 are all coded for by the mitochondrial genome which is typified by a high mutation rate that can be ~10 times greater than that of the nuclear genome (Brown et al., 1979; Pierron et al., 2012). These COX subunits could thus be hotspots of genetic and hence functional variation upon which natural selection can act. Indeed, non-synonymous substitutions in COX 1 and 2 have been identified between low and high-altitude pika (Ochotona curzoniae) (Luo et al., 2008) and functional analysis of COX orthologues among geese (Scott et al., 2011) and locusts (Zhang et al., 2013) has revealed putatively-adaptive variation whereby COX from the more hypoxia tolerant, high-altitude species or populations have a higher binding affinity for cytochrome c than the lower altitude organisms. Although these studies suggest that aspects of COX function are under selection in organisms inhabiting hypoxic environments, no study has yet to examine whether there is adaptive variation in the kinetics of COX interactions with O₂, which is the critical element limiting survival in the hypoxic environments.

The goal of this study is to determine if there is functional variation in the kinetics of mitochondrial and COX interactions with O₂ among species of marine sculpins that vary in their ability to withstand O₂ deprivation. We chose to focus on mitochondria and COX from the brain because of its importance to the maintenance of whole-animal function in hypoxia. Further, to provide a mechanistic understanding of COX functional differences in the absence of being able to generate
recombinant protein of the complex multi-subunit COX enzyme, in silico analyses of deduced protein sequences were performed to highlight the putatively important amino acid sites on COX under selection by hypoxia.

2.2 Materials and methods

2.2.1 Chemicals
Cytochrome c (from equine heart; Sigma-Aldrich) was reduced via dialysis with ascorbate in 50mM Tris-HCl buffer, pH8.0. The concentration of reduced to oxidized cytochrome c was determined by spectrophotometry as A_{550}/A_{280} (between 1.1 to 1.3) and was stored in aliquots at -80°C until use. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Canada).

2.2.2 Species collection and holding
Sculpins were collected near Bamfield Marine Sciences Centre (British Columbia, Canada) at Ross Islets (48°52.4’N, 125°9.7’W) and Wizard’s Rock (48°51.5’N, 125°9.4’W) using either handheld nets or pole seines at the lowest tidal cycle. Animals were either sampled after one week of housing in flow-through seawater (12°C) or transported to The University of British Columbia (UBC) and housed in a recirculation system with artificial seawater (12°C) and maintained on a diet of shrimp, Atlantic krill, and bloodworms for at least 3 weeks. All experimental procedures were reviewed and approved by the UBC Animal Care Committee (A13-0309).

2.2.3 Mitochondrial isolation
Mitochondria were isolated from whole brain of 8 species of sculpins including (Oligocottus maculosus, Myxoceplistus polyacanthocephalus, Clinocottus globiceps, Hemilepidotus hemilepidotus, Leptocottus armatus, Artedius fenestralis, Artedius lateralis, Blepsias cirrhosus). Briefly, fish were stunned via concussion, euthanized by spinal severance and the brain was dissected and minced with a razor blade on ice. The minced tissues were transferred to a glass homogenizer containing isolation media (in mM: 25 KH₂PO₄, 50 KCl, 10 HEPES, 0.5 EGTA, 250 sucrose, 0.5% bovine serum albumin (BSA), pH 7.4) and homogenized with 3 full passes. The resulting homogenate was transferred to centrifuge tubes and centrifuged at 600g for 10min at 4°C. The supernatant was collected, filter though glass-wool, and centrifuged at 9000g for 10min at 4°C. The pellet was suspended in isolation media and centrifuged again at 9000g for 10min at 4°C. The final pellet was suspended in isolation media
without BSA and either used immediately (for whole mitochondrial measurements) or frozen in aliquots in liquid nitrogen (for COX $K_{m,app}$ O$_2$) and stored at -80°C for later analysis.

2.2.4 COX $K_{m,app}$ O$_2$

Frozen mitochondria were freeze-thawed three times and assayed after the third thaw in the Oroboros oxygraph (Innsbruck, Austria) that was calibrated daily to 100% air saturation and anoxia at 12°C in assay buffer (in mM at pH 7.2: 25 K$_2$HPO$_4$, 5 MgCl$_2$, 100 KCl, 2.5mg/mL BSA). An assay temperature of 12°C was chosen for this analysis because it is the typical marine water temperature off the coast of British Columbia and the temperature to which these animals were acclimated. Buffer pH was kept consistent across species to enable comparisons of COX $K_{m,app}$ O$_2$ under constant conditions for all species. Once the air calibration signal stabilized, chamber O$_2$ was reduced to ~100µM by passing a stream of nitrogen gas over the surface of an open oxygraph chamber. The chamber was then sealed and 4mM ascorbate, 0.5mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), and 100µM reduced cytochrome c were added sequentially, followed by the addition of the freeze-thawed mitochondria. The progression of O$_2$ consumption was then monitored until anoxia.

Background auto-oxidation was determined in separate trials (without freeze-thawed mitochondria), fit with a two-phase decay non-linear curve, and used to correct experimental O$_2$ consumption rate. The corrected O$_2$ consumption curves were then fit with a two-phase decay and the fast phase half-life was taken as the COX $K_{m,app}$ O$_2$.

2.2.5 Mitochondrial $P_{50}$

Mitochondrial respiration was measured with the Oroboros oxygraphy, which was calibrated daily to 100% air saturation and anoxia at 18°C in assay buffer (MiR05; in mM at pH 7.1: 0.5 EGTA, 3 MgCl$_2$·6H$_2$O, 60 lactobionic acid, 20 taurine, 10 KH$_2$PO$_4$, 20 HEPES, 110 D-sucrose, 1g/L BSA). We chose to perform the analysis of mitochondrial $P_{50}$ at 18°C instead of at their acclimation temperature of 12°C because of the higher signal-to-noise-ratio at the warmer temperature. Once the air calibration signal stabilized, mitochondria (~0.1mg protein) was introduced into the chamber and the chamber was then sealed. To fuel complex I, 5mM pyruvate, 2mM malate, and 10mM glutamate were added, following which 10mM succinate was added to fuel complex II. 1mM ADP was then added to stimulate maximum state III respiration rate. The progression of O$_2$ consumption
was then monitored until anoxia. The analyses of mitochondrial P50 was performed with DatLab2 (Oroboros; Innsbruck, Austria).

2.2.6 COX respiration rate (ascorbate-TMPD/FCCP respiration rate)
Samples of whole brain mitochondria were introduced into the calibrated Oroboros chamber set to 18°C. After complex I (5mM pyruvate, 2mM malate, and 10mM glutamate) and II (10mM succinate)-fuelled state III respiration was established (data not shown), the ETS was uncoupled with titration of two to three 0.05µM steps of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). After mitochondria were fully uncoupled (when respiration rate no longer increased with FCCP titration), 0.5µM rotenone and 2.5µM antimycin A were added to the chamber to inhibit complexes I and III respectively, followed by 2mM ascorbate and 0.5mM TMPD that were added to donate electrons to cytochrome c and maximally stimulate COX respiration rate. The COX respiration rate was corrected for the empirically determined chemical background of ascorbate-TMPD autoxidation at various PO2 values. COX respiration rate was normalized to the uncoupled respiration rate determined in the presence of FCCP and complex I and II substrates (an estimate of ETS capacity). The fact that the normalized values are close to or slightly below 1 (but not significantly; one sample t-test with Bonferroni correction for multiple comparisons) does not affect the relationship between COX respiration between species as the same trend is observed when COX respiration is expressed to complex I and II-fuelled state III ADP-stimulated respiration rate (data not shown).

2.2.7 COX voltage recovery rate
Membrane potential was monitored in freshly isolated mitochondria using tetraphenylphosphonium (TPP+) ion selective electrodes connected to the oxygraph unit. Four additions of 0.5mM TPP were used for calibration.

After TPP+ calibration, mitochondria were injected into the chamber. Complex I (5mM pyruvate, 2mM malate, and 10mM glutamate) and II (10mM succinate) substrates were used to induce state II respiration. 4mM ADP was added to stimulate state III respiration. The O2 inside the chamber was then allowed to deplete and during the resulting anoxic period (not exceeding 10min), the following were introduced into the 2mL oxygraph chamber: 560U catalase, 0.35µM ascorbate, 0.1µM TMPD, 1µM rotenone, 5µM antimycin A, 5µM oligomycin to ensure the other ETS complexes were inhibited, and that COX had saturating levels of electron donors to achieve maximal activity when
O₂ was reintroduced to the chamber. To reoxygenate, 0.08µM (in 3µL) H₂O₂ was injected into the chamber and in the presence of catalase it quickly reintroduced O₂ into the chamber.

To estimate membrane potential recovery rate, the initial velocity of TPP signal recovery was calculated per trial, and normalized to the maximum TPP signal to which the sample recovered to after reoxygenation. In order to account for varying concentrations of COX enzyme between samples, this recovery rate was then expressed relative to the ascorbate-TMPD stimulated respiration rate (expressed as sec⁻¹ max COX respiration rate⁻¹).

2.2.8 cox1 and cox3 sequencing
cox1 and cox3 genes which make up the catalytic core were sequenced from six sculpin species including Oligocottus maculosus, Artedius lateralis, Artedius fenestralis, Myoxocephalus polyacanthocephalus, and Blepsias cirrhous. These species were chosen because they are found on different clades of the sculpin phylogeny (Knope, 2013), and they show a range in whole animal hypoxia tolerance (Pcrit) and COX Kₘ,app O₂ (Figure 1). Mitochondrial DNA was extracted from muscle of 2-3 individuals per species using DNeasy Tissue Kit (Qiagen). Degenerate and specific primers (Supplementary Table 3) for PCR amplification were designed for cox1 and cox3 using GeneTool Lite 1.0 (BioTools). PCR products were purified with QIAquick PCR purification kit (Qiagen), and sequenced using an Applied Systems 3730 DNA Analyzer. A consensus sequence for each species was determined using Geneious (Drummond et al. 2012). Sequences were submitted to Genbank (accession numbers KY356329-KY356352).

2.2.9 Protein in silico analyses
The deduced consensus cox1 and cox3 sequences for each species were translated in Geneious (with BLOSUM matrix) and modeled separately using bovine heart COX structure (3ABM PDB) as template. Swiss-PdbViewer was used to view and manipulate protein data bank (PDB) files. The 3D structures were created using PyMOL. Comparisons of functional domains were determined after aligning to annotated bovine heart COX (3ABM)

Protein stability analyses
FoldX (Schymkowitz et al., 2005) (with YASARA view (Krieger and Vriend, 2014)) was used to calculate protein stability (ΔG). To prepare data for FoldX analyses, modeling results from Swissmodel were exported as PDB files, which were first repaired using FoldX (using the
REPAIRPDB function to undergo energy minimization of overall protein structure. The FoldX algorithm was then applied to calculate protein stability ($\Delta G$). Subunit and phospholipid interactions were investigated using PDBePISA (EMBL-EBI) after modeling all three subunits to the 3ABM catalytic core. ABS-Scan (Anand et al., 2014) online platform was used to carry out *in silico* alanine scanning mutagenesis on COX3 and cardiolipin (CDL270 in 3ABM PDB file) interaction. The larger the $\Delta \Delta G$ value indicates larger $\Delta G$ between wild-type and mutated protein, and the more the alanine mutation disrupted protein stability.

2.2.10 Statistical analyses
Correlative analysis was performed using both ordinary least squares (OLS) and phylogenetically generalized least squares (PGLS) using ape (Paradis et al., 2004), Geiger (Pennell et al., 2014) and nlme (Pinheiro et al., 2014) packages in R (Team, 2016). For the phylogenetic analyses we used the most updated phylogenetic tree of the marine species of Superfamily Cottoidei (Knope, 2013) and dropped the tips of the tree for species with no available data. Each set of regressions from our data set was tested under OLS and PGLS (Pagel, 1999). Pagel’s $\lambda$ of 0 indicated that the correlation was independent of phylogeny, whereas $\lambda$ value of 1 is consistent with the constant-variance model (or Brownian motion model). The model with the lower Akaike’s Information Criterion (AIC) value represented the better fitting model (Table 1).

2.3 Results and Discussion

2.3.1 Interspecific variation in COX function and mitochondrial $P_{50}$
In order to assess whether there is adaptive variation in how COX interacts with $O_2$, we assessed the kinetic properties of COX orthologues from the brain of eight species of sculpins, previously shown to vary in hypoxia tolerance (assessed as time to loss of equilibrium) and $P_{crit}$ (Mandic et al., 2009; Mandic et al., 2013). Among the eight species of sculpins, there was large variation in the apparent Michaelis-Menten constant for $O_2$ binding to COX ($K_{m,app} O_2$) and this variation was significantly correlated with previously determined $P_{crit}$ values (Fig.2.1A; Table 2.1). COX from hypoxia tolerant sculpins (those with lower $P_{crit}$ values) had a lower $K_{m,app} O_2$ than the less hypoxia tolerant species. These empirically determined values for COX $K_{m,app} O_2$ are generally lower than the values reported in mammals (ranging between 0.25 to 0.66 $\mu$M in this study compared to 0.5-1$\mu$M $K_m$ of $O_2$ in bovine COX (pH 7.4 at 25°C; reviewed in Brunori et al., 1987, and Nicholls and Chance 1974), which may not be surprising considering the general sensitivity of most mammals to $O_2$ deprivation.
Notwithstanding the generally lower COX $K_{m,app} O_2$ in fish relative to mammals, this is the first study to show that the evolution of hypoxia tolerance is directly associated with functional modifications to COX that increase $O_2$ binding affinity and facilitate the maintenance of aerobic metabolism in hypoxia.

In addition to the adaptive variation in COX $K_{m,app} O_2$ observed herein, previous comparative studies have shown a lower apparent binding affinity of COX for cytochrome $c$ in high altitude species or populations compared with lower altitude species and populations (Scott et al., 2011; Zhang et al., 2013). Although it is tempting to think that the apparent binding affinity of COX for both cytochrome $c$ and $O_2$ would co-vary, there appears to be no clear mechanistic link of steps in the COX redox cycle that involve cytochrome $c$ electron transfer to Cu$_A$ on COX2 exerting control over $K_m$ for $O_2$ (Krab et al., 2011). As such, the mechanistic origin of the adaptive variation in COX $K_{m,app} O_2$ does not appear to be linked to cytochrome $c$ binding.

COX $K_{m,app} O_2$ can be modified by the energy and redox states of the ETS (Krab et al., 2011). In order to investigate whether respiratory and redox states affect the interspecific relationship between $P_{crit}$ and COX $K_{m,app} O_2$ we assessed brain mitochondrial $P_{50}$ in five species of sculpins under state III phosphorylating conditions with complex I and II fuels. Under these strongly reducing conditions, we observe a significant relationship between $P_{crit}$ and mitochondrial $P_{50}$, with the most hypoxia tolerant sculpin having the lowest mitochondrial $P_{50}$ (Fig. 2.1A, Table 2.1).

The interspecific relationships between $P_{crit}$ and both mitochondrial $P_{50}$ and COX $K_{m,app} O_2$ were roughly parallel (Fig. 2.1A), but the mitochondrial $P_{50}$ was 1.6 to 2-fold higher than the associated COX $K_{m,app} O_2$, which may be due to greater diffusion distances for $O_2$ in the intact mitochondria compared with the semi-purified COX protein. Cellular $O_2$ levels are estimated to be 2-5µM $O_2$ (~0.14-0.35kPa), and below 2µM $O_2$ (~0.14kPa) closer to mitochondrial cluster (Jones, 1986). These $O_2$ concentrations are many fold higher than what the mitochondrial ETS normally requires to maintain function, suggesting that mitochondria and COX do not lack $O_2$ under normal physiological conditions but when environment $O_2$ is diminished, cellular PO$_2$ will decrease necessitating adaptations at the mitochondrial and COX level to improve $O_2$ kinetics in species inhabiting these environments. The similar interspecific relationships between $P_{crit}$ and both COX $K_{m,app} O_2$ and mitochondrial $P_{50}$ lead us to conclude that the variation in COX $K_{m,app} O_2$ among
sculpins is due to intrinsic properties of the COX enzyme and not due to modifying effects of mitochondrial respiration or redox state.

In combination with previous studies (Mandic et al., 2009; Mandic et al., 2013), we have now characterized five steps of the O₂ transport cascade for multiple species of sculpins that differ in whole animal hypoxia tolerance (represented as time to loss of equilibrium (LOE) in Supplementary Fig.2.1), from the extraction of environmental O₂ to the level of O₂ interactions with COX in mitochondria. Our interspecific analysis reveals a strong relationship between Pcrit, whole red blood cell P₅₀, and stripped hemoglobin-O₂ P₅₀, and also between mitochondrial P₅₀ and COX Kₘ,app O₂, whereby hypoxia tolerance sculpins show coordinated changes at multiple steps in the O₂ transport cascade which would serve to not only improve O₂ extraction from the hypoxic environment, but also improve or sustain O₂ delivery to mitochondria.

In addition to COX Kₘ,app O₂, COX Vₘₐₓ and respiration rate also shows interspecific differences related to hypoxia tolerance. Hypoxia tolerant sculpins with a lower mitochondrial P₅₀ have a lower COX Vₘₐₓ (determined on semi-purified COX; Fig.2.1B, Table2.1), but higher COX respiration rate relative to total ETS capacity (ascorbate-NN,NN’,NN’-tetramethyl-p-phenylenediamine (TMPD)/ carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) respiration rate of whole brain mitochondria; Fig.2.1C; Table2.1; note that no value in Fig.2.1C is lower than 1 according to one-sample t-test with Bonferroni correction for multiple comparisons). The lower COX Vₘₐₓ (Fig.2.1B) in hypoxia tolerant sculpins with lower mitochondrial P₅₀ suggests that these species have a less powerful COX or lower COX protein content (Vₘₐₓ = kcat x [E]), but the higher COX respiration rate determined in uncoupled mitochondria (Fig.2.1C) suggests that the COX protein operates at a higher level relative to total ETS flux in hypoxia tolerant sculpins compared with hypoxia intolerant sculpins. These divergent relationships between COX Vₘₐₓ and COX respiration rate suggest that other mitochondrial components, perhaps the phosphorylation system or allosteric regulation by ATP/ADP (Ludwig et al., 2001), may contribute to regulating COX activity in the intact mitochondria and that hypoxia tolerant sculpins appear to have COX proteins that can function to a greater extent of mitochondrial maximal capacity than do hypoxia intolerant sculpins. Excess COX capacity is widely acknowledged in the mammalian literature and several hypotheses have been put forward to explain the potential benefit of higher, or even excess COX capacity, toward aerobic metabolism under O₂ limiting conditions. First, as PO₂ is reduced, the flux control coefficient of
COX over ETS flux increases such that the total ETS flux is increasingly controlled by COX. As such, in organisms that frequently encounter hypoxic conditions, higher COX enzyme capacity could lower the flux control coefficient and reduce COX control over ETS flux, which would support relatively higher ETS flux even when PO\textsubscript{2} is decreasing (Gnaiger et al., 1998; Suarez et al., 1996). Second, it has been hypothesized that a greater mitochondrial COX capacity could serve to store excess electrons and reduce reactive O\textsubscript{2} species generation, which could potentially alleviate oxidative stress during recovery from hypoxia, although this hypothesis has not been empirically tested (Campian et al., 2007). A similar relationship in COX V\textsubscript{max} as the one in sculpins was observed when comparing the hypoxia tolerant epaulette shark (Hemiscyllium ocellatum) and to the less tolerant shovelnose ray (Aptychotera rostrata) where the less tolerant species exhibited higher COX activity (Hickey et al., 2012). However, the opposite trend was observed relating hypoxia tolerance and COX V\textsubscript{max} among triplefin fishes (families Bellapiscis and Forsterygion; Hilton et al., 2010), another group of fish that inhabit the marine near-shore environment, and also in highland deer mice that have higher COX V\textsubscript{max} than their lowland counterparts (Cheviron et al., 2014; Lui et al., 2015). These conflicting results suggest that a lower COX V\textsubscript{max} is not a universal evolutionary strategy for surviving environments prone to fluctuating PO\textsubscript{2}.

Our interspecific comparison of sculpins inhabiting the near-shore marine environment clearly suggests that a low mitochondrial P\textsubscript{50} and low COX K\textsubscript{m,app} O\textsubscript{2} are putative adaptations that enhance hypoxic survival in the more hypoxia tolerant sculpin species found in the O\textsubscript{2} variable intertidal zone, compared with species from the more O\textsubscript{2} stable subtidal environment. However, O\textsubscript{2} is not the only abiotic factor that varies in the intertidal environment. Tidepool temperatures can vary from ~12°C during high tide (bulk ocean temperature near collection sites) or low tide at night to upwards of 24°C or higher during daytime emergence (Richards, 2011). In ectotherms, increases in temperature necessitate increases in O\textsubscript{2} uptake in order to support a higher metabolic rate, but for many enzymes, increases in temperature are known to increase K\textsubscript{m} for substrate binding (Holland et al., 1997). Furthermore, temperature-induced changes in cellular pH (alpha-stat) may also impact protein function and K\textsubscript{m} (reviewed in Burton, 2002). Thus, it is also tempting to speculate that temperature variation may also serve as a selective pressure underlying the interspecific variation in COX K\textsubscript{m,app} O\textsubscript{2} whereby species that experience warm temperatures have evolved a COX protein with lower K\textsubscript{m,app} O\textsubscript{2} to offset temperature/pH dependent effects. However, the natural patterns of O\textsubscript{2} and temperature fluctuations experienced by our most hypoxia tolerance species do not support
temperature as an important variable underlying the low COX $K_{m,app} O_2$. For example, daytime emergence of tidepools is associated with both warm temperatures and hyperoxia (up to $\sim 400\%$ air saturation; Richards 2011), thus any warm temperature induced increases in COX $K_{m,app} O_2$ would be offset by higher $O_2$ availability. In contrast, nighttime emergence is associated with often severe hypoxia, but at temperatures that are near the bulk ocean temperatures of 12°C. Thus, based on the natural fluctuations in $O_2$ and temperature experienced by fish living in tidepools, it seems likely that the interspecific variation in COX $K_{m,app} O_2$ is due to variability in $O_2$, not temperature, but this assertion requires validation.

2.3.2 COX protein *in silico* analyses

In order to gain insight into the mechanistic underpinnings of the adaptive variation in COX $K_{m,app} O_2$ among sculpins, we adopted an *in silico* approach based on protein modelling of deduced amino acid sequences of the mitochondrial-encoded COX core. The catalytic core of the COX is comprised of three mitochondrial-encoded subunits: subunit 1 (COX1) contains the binuclear $O_2$ binding site (containing heme $a_3$ and Cu$_b$), subunit 2 (COX2) contains the docking site for reduced cytochrome $c$ and Cu$_A$, and subunit 3 (COX3) which is essential for maintaining protein function (Fig.2A&B; Bratton et al., 1999; Wikström et al., 2015), serves as the putative entry point for $O_2$, and protects the entrance of the D proton transfer pathway at the COX1/COX3 interface (Hosler, 2004; Sharma et al., 2015). Given the lack of a clear mechanistic link between cytochrome $c$ binding to COX2 and $O_2$ binding (Krab et al., 2011), we elected to focus our analysis on COX1 and COX3.

Variation in the apparent binding affinity of $O_2$ to COX could be due to differences in the fast trapping of $O_2$ by COX1 through the rapid electron transfer from the heme $a$ and $a_3$ (Verkhovsky et al., 1996) or due to differences in the rate of $O_2$ diffusion from outside of this multimeric protein to COX1 heme $a_3$ (Riistama et al., 1996). As all vertebrates contain the same $aa_3$-type COX1 (with heme $a$ and heme $a_3$) it is unlikely that the variation that we measure in COX $K_{m,app} O_2$ is due to variation in $O_2$ trapping, thus we focus on the role of variation in the $O_2$ diffusion pathways through the COX protein. A putative COX $O_2$ diffusion pathway was elucidated in the crystal structure of bovine heart COX and also via site-directed mutagenesis studies of $aa_3$-COX from *Paracoccus denitrificans*, which involves a hydrophobic channel that extends from the COX surface through a COX3 v-cleft structure to the COX1 binuclear $O_2$ binding site (Riistama et al., 1996; Tsukihara et al., 1996). We therefore hypothesized that amino acid variation in COX1 and/or COX3 subunits could
affect the pathway of O$_2$ diffusion and thus sculpin K$_{m,app}$O$_2$. To investigate this possibility, we evaluated sequence-based differences of COX1 and 3 subunits by aligning and modeling the two COX subunits from six sculpin species that varied in COX K$_{m,app}$O$_2$ using bovine heart COX protein crystal structure as template.

2.3.3 COX1 protein structure
In COX1, all residues important for heme $a$, $a_3$ binding were conserved as were all known residues participating in the proton pumping D-pathway, through which chemical and pumped protons are transferred (Wikström et al., 2015). Indeed, our sequence analysis demonstrated a high degree of conservation in COX1 sequence among the sculpins investigated and two species that differed in COX K$_{m,app}$O$_2$ (Myoxocephalus polyacanthocephalus and Blepsias cirrhosus; species 2 and 8 from Fig.2.1) had identical deduced amino acid sequences. At the four positions where we observe variation in amino acid sequence, the residues extend into both the intermembrane space and matrix and not towards the binuclear catalytic site (Supplementary Table1), suggesting that their role in defining COX K$_{m,app}$O$_2$ is minimal.

2.3.4 COX3 protein structure and stability
It was previously thought that the only role of COX3 was to impart structural integrity to the catalytic core, but it is now recognized that COX3 plays an essential role in maintaining COX activity at high pH by protecting the microenvironment of the proton acceptor of the D-pathway, as well as preventing suicide inactivation during the catalytic cycle (Bratton et al., 1999; Hosler, 2004). In addition, COX3 is the putative site for O$_2$ entry into the large COX complex en route to the COX1 catalytic site via a distinct v-cleft structure formed from two bundles of seven transmembrane helices of COX3. Three histidine residues near the N-terminus of COX3 have been shown to play a critical role in accepting and donating protons for possible use by D-pathway (Alnajjar et al., 2014). Combined, these findings suggest that COX3 not only has an important role in maintaining O$_2$ diffusion into the protein core but also proton uptake.

Cumulative point mutations on a protein surface can affect its interactions with the surroundings and alter protein stability/protein function (Eijsink et al., 2004; Strickler et al., 2006). As COX3 does not contain any catalytic residues, we first estimated in silico protein stability of the COX3 orthologues to determine whether interspecific amino acid substitutions could potentially affect COX3 protein surface and be related to COX K$_{m,app}$O$_2$. This analysis revealed a significant
relationship between COX3 protein stability with brain COX $K_{m,app}$ O$_2$ (estimated as free energy of unfolding; Fig.2.2B; Table2.1), suggesting that COX3 protein may play a role in determining $K_{m,app}$ O$_2$. A more stable COX3 protein in more hypoxia tolerant sculpin suggests that they either have a more compact and rigid protein, and/or they differ in how COX3 interacts with its surroundings due to surface mutations (e.g. with other protein subunits or phospholipids). The COX3 models reveal that its interface with COX1 is well conserved across sculpin species, whereas the opposing interface with the phospholipid bilayer showed interspecific amino acid residue differences (Fig.2.2D). In particular, there were three residues showing interspecies variation in amino acid functional groups on helix 2 which forms one arm of the previously thought highly conserved COX3 v-cleft structure (Fig.2.2B&D, Supplementary Table 2.2). Sequence analysis further suggests that interspecific differences in COX3 residues could also affect interactions with a high affinity bound cardiolipin adjacent to the v-cleft, and also with nuclear subunits 5b, 6a, 6b, and 7a (Supplementary Table 2.2).

2.3.5 Interspecific variation in mitochondrial kinetics does not affect proton pumping
Since differences in COX3 could potentially affect proton uptake to the D-pathway in COX1, we assessed whether the interspecific variation in O$_2$ kinetics was associated with differences in COX proton pumping, estimated as the speed with which anoxic brain mitochondria recovered the proton gradient upon re-oxygenation. However, this analysis did not reveal a significant relationship between COX $K_{m,app}$ O$_2$ and the rate of proton gradient recovery (Supplementary Fig.2.2). There may, however, be interspecific variation in the rate of recovery of post-anoxia membrane potential suggesting possible modifications to proton transfer unrelated to D-pathway residues in COX1 and these modifications deserve more in-depth study.

2.3.6 COX3 protein stability may affect cardiolipin interactions and mitochondrial function
Cardiolipin is a membrane phospholipid found exclusively in mitochondria and plays important roles in the function of COX (Alnajjar et al., 2015; Hofacker and Schulten, 1998). Four cardiolipin molecules are directly associated with COX, and one in particular, situated within the COX3 v-cleft with its head group in contact with COX7a (Arnez et al., 2013), is thought to be critical for O$_2$ uptake by the protein due to the higher partition coefficient of O$_2$ in phospholipids than aqueous media (Hofacker and Schulten, 1998). Indeed, Sedlak and Robinson (2015) demonstrated that the cardiolipin associated with COX7a (and COX3) is critical for the function of COX. Given the
interspecific variation in COX O₂ kinetics (Fig. 2.1A), COX3 protein stability (Fig. 2.2C) and COX3 residues that interact with COX7a (Fig. 2.2A; Supplementary Table 2.2), we hypothesized that residues important for cardiolipin interaction would vary between sculpin species. We carried out *in silico* alanine scanning mutagenesis to mutate residues between COX3 and cardiolipin on the bovine COX crystal structure to alanine to identify important sites involved in the protein-ligand (COX3-cardiolipin) interaction. These analyses yield a ΔΔG, representing the difference in ΔG between the native protein and a mutated protein where the native amino acid is replaced with an alanine residue (with a small methyl side chain) to investigate protein-ligand interactions. The results of this analyses predicted that alterations in both positions 55 and 224 would have the greatest impact on COX3-cardiolipin interactions, pointing to both positions having key roles in cardiolipin recognition by forming hydrogen bonds with the structure (Fig. 2.2E). Mapping of residues 55 and 224 onto a simplified sculpin phylogeny indicates a pattern that suggests that variation in this cardiolipin interaction may have been important in sculpins invading the upper intertidal environment (Fig. 2.3, Supplementary Fig. 2.3), which is typified by daily bouts of hypoxia at night. Two exceptions to this pattern can be observed in *M. polyacanthocephalus* and *O. maculosus*, both of which have similarly low COX Kₘ,opt O₂ but differ at residues 55 and 224. Additional comparison between *M. polyacanthocephalus* and *B. cirrhosus*, our most hypoxia intolerant species, reveals two of the four amino acid differences (positions 41 and 47) are also found on helix 2 (same as position 55) which forms an arm of the COX3 v-cleft (Fig. 2.3, Supplementary Table 2.2). This provides further evidence for a role of the COX3 v-cleft in determining COX Kₘ,opt O₂ in sculpins and that the low COX Kₘ,opt O₂ seen in hypoxia tolerant sculpins can be achieved via genetic mechanisms. As such, we provide the first mechanistic hypothesis for a previously unrecognized, but potentially critical adaptation that ensures hypoxia tolerant organisms maintain mitochondrial function and aerobic metabolism to a lower PO₂ than in hypoxia intolerant species.

### 2.4 Summary

The present study provides novel evidence of adaptive variation in the function of COX, arguably the most important protein in aerobic respiration, where organisms that have evolved a higher degree of hypoxia tolerance possess a high O₂ affinity COX that functions to a greater extent of its maximal activity. These adaptive modifications to COX and mitochondria in hypoxia tolerant sculpins translates into these species being better able to maintain mitochondrial function to a lower cellular PO₂ than in hypoxia intolerant sculpins. As such, we provide strong interspecific evidence
that the mitochondrion and O₂-binding COX protein are under selection for improved function in organisms that experience hypoxia more frequently in their natural environment. Furthermore, we have identified several amino acid residues on the sculpin COX3 structure that are strong candidates for explaining the adaptive variation in COX $K_{m,app}$ O₂ through modulation of the high-affinity interaction between COX3 and cardiolipin.
Figure 2.1 (A) Relationship between whole animal hypoxia tolerance ($P_{crit}$) and brain mitochondrial $P_{50}$ (Data are mean ± s.e.m.; phylogenetic generalized least squares (PGLS), $p=0.03$, $y=40.07x + 2.71$; hollow squares) and COX $K_{m, app} O_2$ (PGLS, $p<0.0001$, $y=37.12x + 3.75$; black circles) and relationship between brain mitochondrial $P_{50}$ and (B) COX $V_{max}$ enzyme activities among sculpins (PGLS, $p<0.0001$, $y=4607.04x + 173.74$), and (C) ascorbate-TMPD stimulated mitochondrial respiration (COX respiration rate; ordinary least squares (OLS), $p=0.015$, $y=-17.80x + 1.90$).

Species indicated in the figure are as follows: (1) *Oligocottus maculosus*, (2) *Myoxocephalus polyacanthocephalus*, (3) *Clinocottus globiceps*, (4) *Hemilepidotus hemilepidotus*, (5) *Leptocottus armatus*, (6) *Artedius fenestralis*, (7) *Artedius lateralis*, (8) *Blepsias cirrhosus*. $P_{crit}$ values used in panel A are taken from Mandic et al. 2009.
Figure 2.1
Figure 2.2 (A) Structure of whole COX enzyme (bovine heart 3ABM PDB structure) with COX1 (in orange), COX2 (in green), COX3 (in blue) and COX7a (in pink) highlighted; (B) COX1 (in orange) and COX3 (in blue) structures that were investigated for interspecific differences between sculpin species (heme $a_3$, a part of the binuclear site, is shown in pink in the COX1 structure); (C) Relationship between brain COX $K_{m,app} O_2$ and COX3 subunit protein stability (estimated as free energy of unfolding, in kcal/mol; data are means ± s.e.m.; OLS, $p= 0.029, y= 0.0052x +0.40$); (D) COX3 structure showing sculpin interspecific differences in amino acid residues. Cardiolipin (CDL270) is shown in green. Blue in panel D identifies residues where the least hypoxia tolerant species in our study Blepsias cirrhosus is different from the other species, and orange highlights positions where more hypoxia tolerant species Oligocottus maculosus, Artedius fenestralis, and Artedius lateralis are different from the others (refer to Supplementary Table 2.2); (E) $\Delta\Delta G$ of mutants from alanine mutagenesis analyses of COX3 interacting residues with cardiolipin (CDL270) in the bovine structure, showing particular importance of residues 55 and 224 for ligand recognition.
Figure 2.2
Figure 2.3 Phylogenetic transitions of two amino acid residues 55 and 224 on COX3 (shown here on bovine 3ABM chain C structure; UniProtKB accession number P00396) identified from the alanine mutagenesis analyses show that upper intertidal species have functionally different amino acid residues when compared to lower intertidal species. Each species is indicated with a number corresponding to those in Figure 2.1.
Figure 2.3
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Table 2.1 Regressions tested under ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) with Pagel’s model of evolution (Pagel 1999). Pagel’s λ is an indication of whether the phylogeny correctly predicts the patterns of covariance among species on a given trait. λ value of 0 indicates that the correlation is independent of phylogeny, whereas value of 1 is consistent with the constant-variance model (or Brownian motion model) being a good representation of the data. Model that best represented the data with the lowest Akaike’s Information Criterion (AIC) value is indicated with an asterisk.
Chapter Three: Hypoxia tolerance is associated with higher mitochondrial ROS emission in intertidal sculpins (Cottidae, Actinopterygii)

3.1 Introduction

Oxygen (O$_2$) use in eukaryotic cells can be a “double-edged sword”. On one side, O$_2$ binding to cytochrome $c$ oxidase (COX) serves as a critical step in the function of the mitochondrial electron transport system (ETS) to support respiration and aerobic ATP production. On the other side, stray electrons from ETS redox centres can bind O$_2$ to form reactive oxygen species (ROS; Hoffman and Brookes, 2009; Quinlan et al., 2013), which at low levels play a signaling role and are scavenged by cellular and mitochondrial antioxidant systems (Jones, 2006; Turrens, 2003). When ROS generation exceeds scavenging capacity however, ROS can accumulate and lead to oxidative damage (Ott et al., 2007; Sies, 1997). The relative rates of mitochondrial respiration and ROS generation by the ETS are dependent upon the redox environment (Aon et al., 2010), whereby mitochondrial ROS production is minimal in respiring mitochondria with a more oxidized redox environment. Mitochondrial ROS production increases as the ETS complexes become more reduced and the protonmotive force increases, as occurs in low O$_2$ (hypoxia) when respiration is inhibited. As such, mitochondrial redox environment serves as the mechanistic link explaining the inverse relationship between mitochondrial respiration and ROS generation and underlies the redox imbalance that occurs in mammalian systems during pathological states such as those experienced during ischemia-reperfusion (Aon et al., 2010). However, much less is known about the natural variation in mitochondrial respiration and ROS metabolism across organisms that have evolved to thrive in environments that experience fluctuations in O$_2$.

The evolution of hypoxia tolerance necessitates numerous modifications to the O$_2$ transport cascade that improves O$_2$ extraction from the environment and delivery to mitochondria (Mandic et al., 2009; Mandic et al., 2013; Scott et al., 2011). Hypoxia tolerance is often associated with a higher mitochondrial respiratory capacity as observed in intraspecific comparisons among high and low altitude native deer mice (Peromyscus maniculatus; Mahalingam et al., 2017) or higher ETS capacity as observed in interspecific comparisons in elasmobranchs inhabiting different intertidal environments (Hemiscyllum ocellatum vs. Aptychoteuthis rostrate; Hickey et al., 2012). Related to greater respiratory and ETS capacity in hypoxia tolerant organisms is a generally lower ROS/O$_2$ (Hickey et al., 2012; Mahalingam et al., 2017), which, if consistent across hypoxia tolerant species, may contribute to
explaining why mitochondria from hypoxia tolerant organisms tend to be more resistant to in vitro acute anoxia-reoxygenation stress and recover ADP-stimulated respiration to a greater extent after anoxia exposure than do hypoxia intolerant organisms (Hickey et al., 2012; Ivanina and Sokolova, 2016). The relative use of complexes I and II in supporting mitochondrial respiration can impact the efficiency of oxidative phosphorylation due to the higher ADP/O associated with complex I-fuelled respiration. Complexes I and II (along with other ETS sites) also contain important and well-studied sites of ROS generation but the magnitude of ROS generation from complex I can exceed that of complex II (Quinlan et al., 2013). Indeed, a laboratory-selected line of Drosophila melanogaster maintained at 4% O₂ had approximately 30% lower complex II activity relative to a normoxic strain of flies, indicating a higher dependency on complex I concomitant with a reduction in superoxide generation (with complex I substrates; Yin et al. 2013; Ali et al. 2012). As such, there still is a general lack of understanding of the relationship between respiratory capacity and ROS/O₂ across organisms that vary in hypoxia tolerance, also, the role of the redox environment in defining mitochondrial ROS generation as it relates to respiration has not been examined in a comparative context.

The goal of the present study was to assess the relationship between mitochondrial substrate preference, respiration, ROS/O₂ and redox environment across species that vary in hypoxia tolerance. To examine this relationship, we conducted four sets of analyses: first, we determined whether there was a relationship between respiratory capacity and hypoxia tolerance; second, we investigated whether differences in mitochondrial capacity were consistent with variation in ROS emission; third, in order to investigate ROS emission kinetics under redox-controlled conditions, we manipulated extramitochondrial redox environment to compare ROS/O₂ between species; and fourth, we compared the responses of mitochondria to in vitro anoxia-recovery. These experiments were conducted in mitochondria isolated from the brain of a well-characterized group of fish species, commonly called sculpins (Cottidae, Actinopterygii) that are distributed along the marine intertidal zone (Richards, 2011) and show interspecific variation in whole animal hypoxia tolerance (Mandic et al., 2013). Their ability to extract O₂ from the environment as assessed by P_{crit}, which is the PO₂ at which O₂ consumption rate transitions from oxy-regulating to oxy-conforming, which in turn is due to modifications to several steps in the O₂ transport cascade that enhances O₂ uptake and delivery to tissues (Mandic et al., 2009). Further, variation in hypoxia tolerance is also associated with modifications to the mitochondrion where cytochrome c oxidase (COX) of hypoxia tolerant
sculpins has an increased O$_2$ binding affinity than COX from hypoxia intolerant sculpins (Lau et al., 2017). The clear functional variation in mitochondrial O$_2$ binding among sculpins presents us with an opportunity to assess the relationship between mitochondrial respiration and ROS generation among multiple species that vary in hypoxia tolerance.

3.2 Methods

3.2.1 Species collection and holding
All species of sculpins used in this study were collected near Bamfield Marine Sciences Centre (British Columbia, Canada) at Ross Islets (48°52.4’N, 125°9.7’W) or Wizard’s Rock (48°51.5’N, 125°9.4’W) using either handheld dipnets or pole seines at lowest point in the daily tidal cycle. Animals were transported to The University of British Columbia (UBC) and housed in a recirculation system with artificial seawater (12°C) and maintained on a diet of shrimp, Atlantic krill, and bloodworms for at least 1 month before experimentation. All experimental procedures were reviewed and approved by the UBC Animal Care Committee (A13-0309).

3.2.2 Isolation of brain mitochondria
Individual fish from each of the following species, *Oligocottus maculosus* (6.6 ± 0.3g), *Artedius fenestralis* (38.2 ± 5.3g), *Artedius lateralis* (34.7 ± 7.7g), *Leptoocottus armatus* (91.3 ± 18.4g), *Scorpaenichthys marmoratus* (160.5 ± 40.0g), and *Blepsias cirrhosus* (30.0 ± 3.6g) were netted from their stock tank, weighed, stunned *via* concussion, euthanized by spinal severance, and the entire brain was quickly dissected for mitochondrial isolation. For fish <10g (*i.e.* *O. maculosus*), tissue from four animals were pooled to get sufficient yield for experiments, and for fish between 10 and 40g (*e.g.* *A. lateralis*), tissue from two animals were pooled. Each pooled sample is considered a single replicate for statistical analysis.

Briefly, brains were roughly chopped on an ice cooled surface with a razor blade in isolation buffer (250mM sucrose, 5mM Tris, 1mM EGTA, pH 7.4) and transferred into a Potter-Elvehjem tissue grinder and homogenized manually with 6-8 passes of polytetrafluoroethylene pestle. The tissue homogenate was then centrifuged at 1000g for 3min at 4°C and the supernatant was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000g for 10min at 4°C and the resulting supernatant was removed. The mitochondrial pellet was resuspended in cold isolation buffer and centrifuged again at 10,000g for 10min at 4°C to generate the final mitochondrial pellet.
which was resuspended in isolation buffer and kept on ice until analysis. Protein content of the mitochondrial suspension was determined with the Bradford’s assay (Sigma-Aldrich). An aliquot of the isolated mitochondria suspension was frozen in liquid N₂ and stored at -80°C for the determination of mitochondrial ETS complex maximal activity.

3.2.3 Part I: Mitochondrial respiration
Mitochondrial respiration was measured with an Oroboros oxygraph high-resolution respirometer (Innsbruch, Austria). The polarographic oxygen sensors were calibrated daily with air saturated and anoxic MiR05 buffer (in mM: 0.5 EGTA, 3 MgCl₂·6H₂O, 60 lactobionic acid, 20 taurine, 10 KH₂PO₄, 20 HEPES, 110 sucrose, and 1 g/L bovine serum albumin; pH 7.1; Gnaiger et al. 2000) at 18°C. We chose an assay temperature of 18°C to maximize signal to noise ratio in our respiration data, which is within the range of temperatures that sculpins are exposed to in the intertidal (Richards 2011).

To assess mitochondrial respiration, we employed a substrate utilization inhibitor titration (SUIT) protocol. Briefly, isolated mitochondria (~0.2 to 0.5mg mitochondrial protein) were first introduced into the respirometry chamber containing MiR05 buffer at 18°C. Following the introduction of mitochondria, complex I fuels were added (10mM pyruvate, 1mM malate, and 10mM glutamate; PMG) to yield state II (PMG) respiration rate. This was followed by addition of 0.75mM ADP to stimulate complex I-fueled state III respiration rate, then 10mM succinate was added to assess complex I and II fueled state III respiration rate (PMGS). State IV respiration was established with 2.5µM oligomycin. This was followed by titration of 2-3, 0.5µM steps of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) to fully uncouple the mitochondria. Once fully uncoupled, 0.5µM rotenone (Rot) was added to inhibit complex I, followed by addition of 2.5µM antimycin A to inhibit complex III.

Respiratory control ratio (RCR) was calculated as state III (both complex I and II fuelled) to state IV (oligomycin-induced) respiration rate. ADP/O was determined as the ratio of ADP phosphorylated divided by the O₂ consumed during the transition from state III to IV.

3.2.4 Part II: Mitochondrial respiration and simultaneous ROS measurements
To assess mitochondrial respiration simultaneously with ROS emission, mitochondrial respiration was assessed with steps described above and changes in ROS emission were assessed.
fluorometrically using Amplex Ultrared (ex 568nm/em 581nm; Invitrogen) with the Oroboros respirometer and fluorometer. Isolated mitochondria (~0.5-0.6mg mitochondrial protein) were introduced into the chamber calibrated with MiR05 maintained at 18°C, followed by the addition of 5µM Amplex Ultrared, 0.004U horseradish peroxidase (HRP), and 13U superoxide dismutase. To calibrate the ROS detection methods, four additions of H$_2$O$_2$ were sequentially added to the chamber to a total of 0.56µM H$_2$O$_2$ while monitoring changes in the product of the reaction between H$_2$O$_2$ and Amplex Ultrared. In order to confirm that ROS was the primary reactive species being detected (Amplex Ultrared is known to be sensitive to several reactive species, including reactive sulfur species (DeLeon et al., 2016)), we added catalase to catalyse the conversion of H$_2$O$_2$ to O$_2$ at the end of several experimental runs (after GSH titrations in Experimental Part III) which resulted in a 75 to 80% reduction in ROS/mg protein in all species examined, suggesting that the major reactive species detected in our experiments was H$_2$O$_2$. Thus, we have elected to refer to the reactive species measured in this study as ROS.

For this part of the study, isolated brain mitochondria were first incubated with 10mM pyruvate, 5mM malate (excess malate was used to ensure oxaloacetate removal to avoid inhibition of complex II; Hickey et al., 2012), and 10mM glutamate to establish complex I-fuelled state II respiration rate. This was followed by 10mM succinate to assess complex I and II fuelled state II, after which 16µM ADP was added to stimulate respiration to assess ADP/O. Once state IV respiration was obtained following the sub-maximal ADP addition, 0.75mM ADP was added to stimulate maximal state III respiration rate, followed by 2.5µM oligomycin to establish state IV respiration, and 0.5µM rotenone to inhibit complex I in state IV conditions.

3.2.5 Part III: In vitro redox challenge
In order to assess the effects of redox environment on mitochondrial ROS emission, we aimed to manipulate the redox environment of the mitochondrial matrix by adjusting the extra-mitochondrial GSH:GSSG while measuring mitochondrial GSH:GSSG and ROS emission under state II conditions. Briefly, the oxygraph was set up as described in Part II with Amplex Ultrared. Once the mitochondrial sample was introduced into the chamber and the H$_2$O$_2$ calibration was complete, complexes I and II substrates (PMGS) were added to establish stable state II respiration and ROS emission rates, after which the contents of the oxygraph chamber were removed and centrifuged at 10528g for 5min. The resulting mitochondrial pellet was washed twice with MiR05, and the
mitochondrial pellet was frozen in liquid nitrogen and stored at -80°C until analyses of GSH and GSSG (see below). To adjust the extra-mitochondrial GSH:GSSG, the oxygraph was set up as described above, but after state II (PMGS) respiration was established, four injections of 0.6mM GSH were added to the chamber to a final concentration of 2.4mM GSH. State II respiration rate and ROS emission were monitored simultaneously with the oxygraphy as GSH was titrated. At the end of the titrations, the entire mitochondrial suspension was sampled and processed as described previously for analysis of GSH and GSSG. GSH was below the level of detection in the supernatant from the last MiR05 wash.

3.2.6 Part IV: Recovery from in vitro anoxia

To assess potential interspecific differences in recovery from in vitro anoxia, the oxygraph was set up as described in Part II with Amplex Ultrared. Once the mitochondrial sample was introduced and H₂O₂ calibration was complete, complex I and II (PMGS) substrates were added followed by 0.75mM ADP to stimulate state III respiration. At this point, chamber O₂ concentration was adjusted to 100µM by passing a stream of nitrogen gas over the surface of the mitochondrial suspension. The chamber was then sealed and mitochondria allowed to deplete chamber O₂ to anoxia, at which point mitochondria were maintained in anoxia for 20min. The chamber was opened to reoxygenate with air, after which 0.5-1mM ADP was added to stimulate state III respiration rate. State IV was then induced with 2.5µM oligomycin, and finally 0.5µM rotenone was added to inhibit complex I.

3.2.7 Biochemical analyses

Mitochondrial GSH:GSSG

Mitochondrial GSH:GSSG was determined spectrophotometrically with the recycling method modified from Rahman et al. (2006) for isolated mitochondrial pellets. Briefly, frozen mitochondrial pellets were resuspended in 80µL buffer with 0.1M KH₂PO₄, 5mM EDTA disodium salt at pH7.5 (KPE buffer), with 0.1% triton X-100 and 0.6% sulfosalicylic acid and thawed in a room temperature water bath. The suspension was freeze-thawed twice and 50µL of the resulting suspension was used to determine glutathione disulfide (GSSG; oxidized glutathione) and the remainder of the sample for determining total glutathione (both reduced and oxidized forms). To determine GSSG, the GSSG samples and standards were incubated at room temperature with 1µL of vinylpyridine (diluted 1:10 v/v KPE) to derivatize endogenous GSH in the sample. After 1hr,
3µL of triethanolamine (diluted 1:6 v/v KPE) was added to the samples and standards and incubated for 10min at room temperature. This was followed by the addition of 3µL of 1M HCl to neutralize the sample. The GSSG and total glutathione samples were then assayed with the following protocol in which glutathione reductase (GR) converts GSSG into GSH. Briefly, equal volumes of 1.7mM [5,5'-dithio-bis(2-nitrobenzoic acid)] (DTNB) and glutathione reductase (3.33U/mL KPE) were mixed as the assay buffer, of which 125µL was added to 20µL of sample/standard. After 30sec to allow GR activity to convert GSSG into GSH, 60µL of 0.8mM β-NADPH was used to start the reaction and the rate of TNB formation was monitored at 412nm for 5min. GSH was calculated as the difference between total glutathione and GSSG. The redox status in (mV) was calculated using a simplified Nernst equation for GSH:GSSG: $E_{hc} = E_o + 30 \log([GSSG]/[GSH]^2)$ where $E_o$ is -264mV at pH 7.4 (Garcia et al. 2010; Jones 2002).

Mitochondrial complex maximal activities ($V_{max}$) on isolated mitochondria

To determine the maximal activity of complexes I, II, and V, frozen samples of isolated mitochondrial were thawed on ice and centrifuged at 15000g for 10min at 4°C. The pellet was resuspended in hypotonic medium (25mM K$_2$HPO$_4$, 5mM MgCl$_2$·6H$_2$O at pH7.2) and freeze-thawed thrice before proceeding with assay protocol described in Galli et al. (2013). To determine the maximal activity of complexes III, the frozen mitochondrial sample was thawed on ice and freeze-thawed twice before proceeding with assay described in Galli et al. (2013).

3.2.8 Calculations and statistical analyses

Due to the different body weights of sculpin species used in this study, we first confirmed using regression analysis that there were no relationships between body weight and measurements made in Part I.

To determine if there were interspecific differences in respiration rates and flux capacities (Part I and II), we performed one-way ANOVA followed by Tukey’s multiple comparisons tests. Correlative analysis in Part I was performed using both ordinary least squares (OLS) and phylogenetically generalized least squares (PGLS) using ape (Paradis et al., 2004), Geiger (Pennell et al., 2014) and nlme (Pinheiro et al., 2014) packages in R (Team, 2016). We used the most updated phylogenetic tree of the marine species of Superfamily Cottoidei (Knope, 2013) for the phylogenetic analyses and dropped the tree tips for species without available data. Each set of regressions from our data set
was tested under OLS and PGLS (Pagel, 1999). Pagel’s λ of 0 indicated that the correlation was independent of phylogeny, whereas λ value of 1 is consistent with the constant-variance model (or Brownian motion model). The model with the lower Akaike Information Criterion (AIC) value represented the better fitting model (Table 3.2).

The slopes and intercepts of the simple linear regressions in Part III were compared between species using the student’s t-test. The pre- and post-anoxia differences in respiration states for Part IV were tested using student t-test.

3.3 Results

3.3.1 Part I: Interspecific relationship between hypoxia tolerance and complex I and II respiratory flux capacity

The relative rate of complex I-fuelled ADP-stimulated respiration to ETS capacity differed between sculpins (one-way ANOVA; p=0.005) and was related to interspecific variation in Pcrit (OLS; p=0.05; Fig.3.1A, Table 3.2). Hypoxia tolerant sculpins with lower Pcrit had lower complex I-fuelled ADP-stimulated respiration rates than less tolerant species (Fig.3.1A). The relative rate of complex I and II-fuelled ADP-stimulated respiration to ETS capacity did not differ between species (p=0.12) and was not associated with Pcrit (data not shown; p=0.45). In uncoupled mitochondria, there were differences in complex I flux capacity between species (p=0.0035), which were related to Pcrit (OLS; p=0.03; Fig.3.1B, Table3.2). There were no differences in complex II flux capacity between species (p=0.074), but there was a strong inverse interspecific relationship with Pcrit (OLS; p=0.002; Fig.3.1C, Table 3.2). Overall, species with lowest Pcrit (O. maculosus) had lower complex I flux capacity (65% of ETS capacity) and higher complex II flux capacity (29%) than species with highest Pcrit (B. cirrhosus), which had higher complex I flux capacity (86%) and lower complex II flux capacity (8%; cf. Fig3.1B&C). These differences in complex flux capacities were not associated with differences in ADP/O (per mg mitochondrial protein), respiratory control ratio (RCR), or the maximal activity (Vmax) of complex I or II, all of which did not vary between species (Table 3.1).

3.3.2 Part II: Interspecific differences in ROS/O2 in state IV

We propose that the interspecific differences in complex dependency could potentially drive differences in ROS accumulation from mitochondria. In light of this, we compared ROS metabolism in three species of sculpin: O. maculosus, which has lowest Pcrit among the species...
investigated and the more hypoxia tolerant, with two other species *A. lateralis* and *S. marmoratus* both with higher *P* crit values and which are less hypoxia tolerant (though not different from one another).

Consistent with our previous findings in Part I (Supplementary Fig.3.1), there were no differences in state II respiration rates (PMG or PMGS) between the three sculpin species tested in this part of the study (Fig.3.2A). *O. maculosus* had lower state III respiration rate (Fig.3.2A; one-way ANOVA, *p*=0.018) and lower oligomycin-induced state IV respiration rate (*p*=0.042) than *S. marmoratus*. There were no differences in rotenone-inhibited state IV respiration rates between the three species. Simultaneous measurement of ROS emission rate expressed relative to O₂ consumption rate (ROS/O₂) showed no differences between species in all states of respiration, except under state IV conditions where *O. maculosus* showed higher ROS/O₂ than the other two species (*p*=0.016; Fig.3.2B). There were no differences in ROS generated per mg mitochondrial protein in all states of respiration (Supplementary Table 3.1).

3.3.3 Part III: Effects of manipulating the redox environment on ROS/O₂

To investigate the effects of redox environment on ROS generation, the glutathione redox environment was manipulated by titrating GSH (to reduce the assay buffer environment) to relate ROS/O₂ to the measured mitochondrial GSH:GSSG. This part of the study was carried out with two species, *O. maculosus* and *S. marmoratus*, which differed in state IV ROS/O₂ in Part II (Fig.3.2B). As the redox environment shifted towards a more reduced state with GSH titrations (a more negative GSH:GSSG in mV), both species increased ROS/O₂ (Fig.3.3A) and ROS/mg protein (Supplementary Fig.3.1). The slopes of the relationship between mitochondrial GSH:GSSG and ROS/O₂ were -0.0030±0.00033 and -0.0016±0.00020 for *O. maculosus* and *S. marmoratus*, respectively, which were significantly different (Analysis of covariance; *p*=0.011), where the same concentration of extramitochondrial GSH reduced *S. marmoratus* mitochondrial GSH:GSSG more (more negative mV value) than in *O. maculosus* (by 11mV). Overall, these results indicate that *O. maculosus* produce more ROS/O₂ than *S. marmoratus* across various mitochondrial GSH:GSSG.

3.3.4 Part IV: *In vitro* anoxia-reoxygenation exposure

We compared the responses of brain mitochondria from *O. maculosus*, *A. lateralis*, and *S. marmoratus* to 20min of *in vitro* anoxia followed by reoxygenation. Anoxia-reoxygenation significantly reduced state III respiration rate in *O. maculosus* and *A. lateralis*, but not in *S. marmoratus* (Fig.3.4A). There was no effect of anoxia-reoxygenation on state IV or rotenone-inhibited state IV respiration rate in any
species examined (Fig.3.4A). The ROS/O₂ under state III conditions was not affected by anoxia-reoxygenation and did not differ between species, whereas ROS/O₂ in state IV was significantly reduced following anoxia-reoxygenation in S. marmoratus. Further, there were no differences in rotenone-inhibited ROS/O₂ following anoxia-reoxygenation (Fig.3.4B). ROS/mg protein under state III conditions was not affected by anoxia-reoxygenation, but it was reduced in O. maculosus and S. marmoratus under state IV conditions. Further, S. marmoratus reduced rotenone-inhibited ROS/mg protein (Fig.3.4C).

3.4 Discussion
The present study clearly demonstrates that interspecific variation in hypoxia tolerance among sculpins is related to how mitochondria from the brain utilized carbon substrates to support respiration. This variation, however, was not related to improving mitochondrial phosphorylation efficiency or reducing ROS emission. In fact, we show that the hypoxia tolerant O. maculosus generated more ROS/O₂ under reducing state IV conditions than the less tolerant A. lateralis and S. marmoratus. O. maculosus was more capable of buffering mitochondrial redox environment under conditions where extra-mitochondrial redox was reduced, but when ROS/O₂ was examined under similar mitochondrial GSH:GSSG conditions, brain mitochondria from O. maculosus had a higher ROS/O₂ than S. marmoratus. Finally, unlike that previously shown in elasmobranchs (Hickey et al., 2012) and clams (Ivanina and Sokolova, 2016), the hypoxia tolerant O. maculosus experienced a reduction in state III respiration rate after in vivo anoxia-recovery, which could be a sign of mitochondrial damage, following anoxia-reoxygenation compare to S. marmoratus which showed no changes in respiration rate. Both O. maculosus and S. marmoratus showed significant reduction in ROS/mg protein in state IV, which in S. marmoratus was due to a reduction in ROS/O₂. These outcomes suggest that hypoxia tolerance in sculpins is associated with a reduced reliance on complex I and higher mitochondrial ROS emission.

3.4.1 Hypoxia tolerant sculpins utilize less of complex I in overall ETS flux
Among six species of sculpin, there were strong but opposite relationships between complex I and II flux capacities and Pcrit, where hypoxia tolerant species had reduced complex I and increased complex II dependency and intolerant species increased complex I and reduced complex II dependency. This variation suggests that these differences are associated with whole animal hypoxia tolerance (Fig.3.1). Complex I is a proton pump fuelled by NAD⁺-linked substrates and is composed
of both nuclear and mitochondrial-encoded subunits, whereas complex II is nuclear-encoded, fuelled by succinate and does not directly contribute to proton pumping. It is therefore not surprising that the selective pressures of routine hypoxia exposure in sculpins has resulted in variation in how these two complexes are used. However, it was unexpected that the hypoxia tolerant species would show reduced complex I and increased complex II dependency, as opposed to relying more on complex I which would result in a higher ADP/O and be consistent with increased efficiency of O₂ use in generating the electrochemical gradient. In the present study, however, the interspecific shifts in complex flux capacity did not affect the measured phosphorylation efficiency (ADP/O) or mitochondrial coupling as assessed by RCR (Table 3.1). There are several possible explanations for why the reduced complex I flux capacity in the hypoxia tolerant sculpins does not result in lower phosphorylation efficiency. First, the phosphorylation efficiency was determined in coupled mitochondria whereas complex I and II flux capacities are determined in uncoupled mitochondria, thus there is a possibility that the complexes are differential regulated in the coupled and uncoupled states, which may minimize the effects of differences in flux capacity on ADP/O between the species. This is supported by the fact that state III respiration rates (PMG or PMGS; Supplemental Fig. 3.1) were not significantly different among the species. Second, it is also possible that other ETS proton pumps (complex III and IV) compensate for the reduced complex I flux capacity in the hypoxia tolerant sculpins. Complex III Vₘₐₓ (Table 3.1 and antimycin-A inhibited respiration rate; Supplementary Fig. 3.1) did not vary among sculpins, but in our previous work, we demonstrated that complex IV (cytochrome c oxidase; COX)-specific respiration rate was higher, with a lower Kₘ,app for O₂ in hypoxia tolerant sculpins than intolerant sculpins (Lau et al., 2017), which may point to an increased role of the O₂-binding ETS complex in the maintenance of ADP/O across the species of sculpins. The lack of an interspecific effect of variation in complex I flux capacity on ADP/O strongly suggests that the variation in complex I has little to do with its role as a proton pump, thus suggesting that there is another explanation for why hypoxia tolerant sculpins have a reduced complex I flux capacity compared with hypoxia intolerant sculpins.

While there are a number of sites along the ETS that generate ROS, complexes I sites are considered to be the major ROS generation sites (Quinlan et al., 2011; Quinlan et al., 2013; Treberg et al., 2011). It is possible that the lower dependency on complex I reduces ROS generation. Additionally, even though the present study is the first to show putatively adaptive variation in ETS substrate
dependency across species that vary in hypoxia tolerance, previous studies have shown that hypoxia exposure differentially affects complex I and II flux rates. For example, in murine brain mitochondria, complex I and II were inhibited to different degrees in response to hypoxia (complex II-fuelled state III was lowered by 25% compared to complex I-fuelled state III lowered more by 32-46%), and upon reoxygenation complex II-linked state III respiration recovered faster than complex I-linked state III respiration (Almeida et al., 1995; Gilland et al., 1998). Similarly, permeabilized heart fibres from cold anoxia acclimated turtles lowered complex I-IV flux, but not complex II-IV flux compared with normoxia-acclimated turtles (Galli et al., 2013). Thus, complexes I and II may be regulated differently in response to hypoxia exposure with complex I flux being inhibited to a greater extent and slower to recover than complex II. These acute responses to hypoxia are consistent with the innate interspecific differences observed in sculpins, where hypoxia tolerance is associated with reduced complex I and increased complex II dependency.

3.4.2 Hypoxia tolerant sculpins emit higher ROS/O2 with lower state IV respiration rate

The marine intertidal zone is heavily influenced by the daily ebb and flow of the tide, which results in a strong gradient of spatial and temporal oscillations in abiotic factors including O2. Isolated tidepools from the upper intertidal zone experience greater fluctuations in O2 (from hypoxic to hyperoxic levels), more so than the homogenous subtidal environments (Richards 2011). In hypoxia tolerant sculpins, enhanced steps of the sculpin O2 transport cascade (lower whole animal O2 consumption rate, higher gill surface area, lower Hb-P50, lower mitochondrial and COX Km,app O2; Lau et al., 2017; Mandic et al., 2009; Mandic et al., 2013) would presumably increase O2 extraction and delivery to tissues, and illustrates the importance of efficient O2 use in determining whole animal hypoxia tolerance. Mechanisms to increase the efficiency of O2 use would presumably also minimize ROS generation in order to restrict the use of already limited O2 towards the generation of a potentially harmful by-product. We thus investigated whether hypoxia tolerant sculpins would have lower ROS/O2, indicating that less O2 is reacting with electrons to form ROS.

We chose three species that varied in Pcrit and complex dependency in Part I to investigate the relationship between respiration and mitochondrial ROS emission. Among the three species, O. maculosus had a significantly lower state IV respiration rate, which is consistent with lower futile proton cycling and thus better mitochondrial coupling (although our six species comparison in part I showed no relationship between Pcrit and RCR). Increased mitochondrial coupling brings about a
higher proton gradient which is typically associated with higher ROS generation (Brand, 2000; Brand and Esteves, 2005), and indeed, *O. maculosus* had higher ROS/O$_2$ in state IV compared to *S. marmoratus*. This pattern of higher ROS/O$_2$ with lower leak respiration only emerged under state IV conditions (with high protonmotive force) rather than in state II, which indicates intrinsic differences in the capacity to generate ROS between sculpin species.

Although we hypothesized that variation in complex I flux capacity would be associated with reduced ROS emission, the interspecies differences in ROS/O$_2$ was not observed in rotenone-inhibited state IV (Fig. 3.2B). Under state IV conditions (high protonmotive force, high reduced redox environment) and in the presence of rotenone and complex I and II substrates, the ROS generated is likely from the quinone-binding I$_Q$ site on complex I (Quinlan et al., 2013). A more detailed study would be required to also assess the ROS generation capacity from the complex I NADH-binding flavin I$_F$ site.

Overall, ROS levels detected in sculpins are comparable to other studies. In state II (PMGS), *O. maculosus* and *S. marmoratus* emitted 2pmol H$_2$O$_2$/s/mg protein (Supplementary Fig. 3.2), and similar levels were measured in isolated killifish *Fundulus heteroclitus* liver mitochondria in leak state (Du et al., 2016). Similar ranges of values were also observed in mammalian mitochondria (rat skeletal muscle and guinea pig heart mitochondria; Aon et al., 2010; Munro et al., 2016). Relative to respiration rate (2pmol H$_2$O$_2$/s/mg protein and simultaneous 0.09nmol O$_2$/s/mg protein), sculpin H$_2$O$_2$ emission rates are consistent with the estimation that about 1-2% of O$_2$ used by mitochondria is emitted as ROS (Turrens, 2003).

3.4.3 Hypoxia tolerant sculpins buffer mitochondrial redox changes better but generate higher ROS/O$_2$

Mitochondrial ROS generation is highly affected by mitochondrial energy status and the redox environment (Aon et al., 2010; Munro and Treberg, 2017), both of which can be profoundly affected by the availability of O$_2$. Hypoxia results in a reduction in ETS flux and leads to a highly reduced redox environment, whereby excess electrons in the ETS complex redox centres favor ROS generation even though O$_2$ levels are low. Hyperoxia can lead to an oxidized mitochondrial redox environment due to high O$_2$ availability, also favoring ROS generation. At either extreme of the redox environment, the combined effects of ROS generation and scavenging capacity can result in higher net ROS emission (Aon et al., 2010). We hypothesized that the more hypoxia tolerant sculpin
would lower ROS emission under a stressful redox environment when compared to a less tolerant species.

To manipulate the mitochondrial redox environment, we altered the GSH pool as it is a major redox couple within the cell and is also easily assessed by monitoring concentrations of GSH and GSSG (Rahman et al., 2006; Schafer and Buettner, 2001). Mitochondria contains two GSH pools, the intermembrane space (IMS) and matrix pools and although the communication between these two pools is not understood (e.g. Kojer et al., 2012; Mari et al., 2013), it is generally recognized that mitochondria are not capable of synthesizing GSH, thus there must be a transport mechanism between the IMS and the matrix. Furthermore, we were effective at changing the combined mitochondrial GSH:GSSG by altering the extra-mitochondrial GSH:GSSG pool. Under conditions where we were able to adjust extra-mitochondrial GSH:GSSG, we predicted that the more hypoxia tolerant sculpin would (1) be able to maintain mitochondrial redox status with changes in extra-mitochondrial GSH:GSSG potentially due to better scavenging capacities, and (2) accumulate lower ROS/O\textsubscript{2} as GSH:GSSG became more reduced.

Indeed, mitochondrial redox environment was better buffered in the hypoxia tolerant *O. maculosus* (Fig.3.3) compared with *S. marmoratus* during titration of extra-mitochondrial GSH, but after each titration the same relative increase in ROS/O\textsubscript{2} were observed in both species. Further, when ROS/O\textsubscript{2} was examined at a single mitochondrial GSH:GSSG, it was consistently higher in *O. maculosus* than in *S. marmoratus*. *O. maculosus* experience more variable PO\textsubscript{2} in their environment (anoxia to 400% air saturation), which may translate to their mitochondria also experiencing large fluctuations in PO\textsubscript{2} and possibly variable redox conditions through the daily tidal cycle. Resistance to changes in the mitochondrial redox environment would ensure that the various redox-linked processes, such as thioredoxin/peroxiredoxin which are major antioxidant mechanisms (Drechsel and Patel, 2010) within the mitochondrial matrix are left unperturbed. One mechanism that would result in increased redox buffering capacity could be a higher NADPH/NADP\textsuperscript{+}, another redox couple that donates electrons to aid the recycling of GSSG back to GSH to maintain glutathione redox status (Rahman et al., 2006). This ability to maintain matrix GSH:GSSG, however, did not result in minimizing ROS emission, as indicated by the higher ROS/O\textsubscript{2} in *O. maculosus* compared to *S. marmoratus* at the same matrix GSH:GSSG. This difference suggests possible differences in ROS generation capacity from the ETS sites and/or differences in scavenging capacities in the matrix.
3.4.4 More hypoxia tolerant sculpins do not recover better from in vitro anoxia

Injury is sustained from ischemia-reperfusion due to surge of ROS generated in normoxic recovery following a period of O₂ deprivation. The oxidative damage caused by the transition from anoxia/hypoxia to normoxia results in incomplete recovery of respiration in mammalian mitochondria (Almeida et al., 1995; Du et al., 1998; Shiva et al., 2007). In fact, the interspecies difference in recovery from 20min of in vitro anoxia showed that hypoxia tolerant O. maculosus brain mitochondria aligned more with typical mammalian response in O₂ sensitive tissues, and a complete recovery of state III respiration was observed in less tolerant S. marmoratus (Fig.3.4). While both O. maculosus and S. marmoratus significantly reduced state IV ROS/mg protein, only S. marmoratus significantly reduced state IV ROS/O₂. This indicates that both tolerant and intolerant species reduced ROS generation, but the tolerant O. maculosus achieved this via an overall reduction in respiration capacity (as indicated by the reduction in state III respiration rate), and the less tolerant S. marmoratus achieved this by altering ROS generation capacity (ROS/O₂). Further, this lower ROS generation capacity in S. marmoratus appears to be in part mediated by changes in ROS generated from the I_Q site as evident by the reduced rotenone-induced ROS/mg protein.

3.4.5 A potential role for ROS in response to environmental O₂ variability

Our study in sculpin mitochondria paints a different picture from previous studies showing that hypoxia tolerant animals generally lower mitochondrial ROS emission. Our interspecies comparison shows that, in fact, hypoxia tolerance may be associated with higher ROS generation under state IV conditions (Fig.3.2B), higher sensitivity of ROS generation to changes in mitochondrial GSH:GSSG but better ability to maintain matrix redox status (Fig.3.3), and reduced ability to recover from in vitro anoxia (Fig.3.4). While more ROS accumulation is often associated with oxidative damage, perhaps an increase in ROS levels may potentially be an important response to O₂ variability. ROS has been shown to act as an agonists to covalently modify proteins (such as iron-sulfur clusters) which can be part of specific signaling processes mediating cellular responses to oxidative damage (D’Autréaux and Toledano, 2007). For instance, the Kreb’s cycle enzyme aconitase is targeted by reactive superoxide to reduce Kreb’s cycle flux (Powell and Jackson, 2003). ROS generated from complex III has also been shown to play a role in stabilizing the transcription factor hypoxia inducible factor 1 (HIF-1) during hypoxia, which subsequently acts on downstream targets to coordinate cellular responses to hypoxia (Chandel et al., 2000; Guzy et al., 2005; Klimova and Chandel, 2008). There is also an emerging view that mitochondria are not only generators of ROS, but also act as regulators
to maintain steady state ROS levels for the purpose of cell signaling (Munro and Treberg, 2017; Starkov, 2008). It is possible that an increase in ROS generation with relatively small changes in mitochondrial redox status is a part of the strategy of hypoxia tolerant sculpins to deal with variations in O$_2$ in the intertidal zone.

3.5 Summary
In animals that frequently encounter hypoxia in their natural environment, it has been observed that there are putative adaptations in the O$_2$ transport cascade presumably to maintain O$_2$ supply to mitochondria to maintain energy output from oxidative phosphorylation even at low environmental PO$_2$. Changes in ETS flux can cause a rise in ROS emission from mitochondria, which when accumulated can result in oxidative damage. Although it was previously proposed that hypoxia tolerant animals or animals that are frequently exposed to O$_2$ limitations would be able to reduce ROS generation, in fact, in the sculpin model this was not what we observed. In this study, we first provided evidence that there was a difference in reliance of carbon substrates between species of varying hypoxia tolerance, with more hypoxia tolerant species showing lower complex I and higher complex II dependency compared to less tolerant species. We then demonstrated that brain mitochondria from hypoxia tolerant species of sculpin in fact generated more ROS under state IV conditions and with changes in extramitochondrial redox environment. We also observed that species which vary in hypoxia tolerance have different strategies of reducing ROS/mg protein after in vitro anoxia exposure. It is possible that higher mitochondrial ROS emission has a role in determining hypoxia tolerance in intertidal sculpins, but this will require further investigation.
Figure 3.1. Relationship between whole animal hypoxia tolerance as assessed by critical oxygen tensions (P_{crit}; Mandic et al. 2009) and mitochondrial respiration rates (A) under ADP-stimulated respiration state with complex I substrates (10mM pyruvate, 1mM malate, and 10mM glutamate; expressed to FCCP-fully uncoupled respiration rate), (B) complex I flux capacity (with pyruvate, malate, and glutamate) determined as the rotenone-sensitive respiration rate in uncoupled mitochondria (expressed to FCCP-uncoupled respiration rate), and (C) complex II flux capacity (PMG with 10mM succinate; determined as the antimycin A-sensitive respiration rate in the presence of rotenone in uncoupled mitochondria; expressed to FCCP-uncoupled respiration rate). Data are means ± standard error. Species indicated in the figure are as follows: (1) Oligocottus maculosus, (2) Artedius fenestralis, (3) Artedius lateralis, (4) Leptocottus armatus, (5) Scorpaenichthys marmoratus, and (6) Blepsias cirrhosus.
Figure 3.1

**1A**

Complex I-fueled ADP-stimulated respiration (%; expressed to FCCP-uncoupled respiration)

**1B**

Complex I flux capacity (%; expressed to FCCP-uncoupled respiration)

**1C**

Complex II flux capacity (%; expressed to FCCP-uncoupled respiration)
Figure 3.2. Substrate-inhibitor titration protocol with simultaneous measurement of (A) oxygen consumption rate and (B) reactive oxygen species generated (expressed as ROS/O₂) in isolated brain mitochondria in three sculpin species (from left to right: *O. maculosus* (species 1 in Fig. 3.1) in squares, *A. lateralis* (species 3) in triangles, and *S. marmoratus* (species 5) in circles). State II PMG (with complex I fuels pyruvate, malate, and glutamate), state II PMGS (addition of complex II fuel succinate), state III (ADP maximally stimulated), state IV (oligomycin-induced), Rotenone (inhibition of complex I). Within each respiration state, symbols with different letters are significantly different (P<0.05 one-way ANOVA with Bonferroni multiple comparison corrections).
Figure 3.3. (A) Relationship between mitochondrial pellet GSH:GSSG and ROS/O₂ in *O. maculosus* (species 1; squares and solid lines; $y=-0.0030x-0.95$) and *S. marmoratus* (species 5; circles and dotted lines; $y=-0.0016x-0.48$). Mitochondrial GSH:GSSG was manipulated by varying extramitochondrial GSH:GSSG with four steps to a total of 2.4mM GSH (to make more reduced, GSH:GSSG more negative). All four GSH doses are shown but only first and last doses (solid symbols) had mitochondrial pellet GSH:GSSG measurements, whereas the middle doses (hollow symbols) are estimates.
Figure 3.4. The effect of 20min *in vitro* anoxia-recovery on O\(_2\) consumption and ROS emission in isolated brain mitochondria from three sculpin species (from left to right: *O. maculosus* in squares, *A. lateralis* in triangles, and *S. marmoratus* in circles) with measurements of (A) O\(_2\) consumption, (B) ROS/O\(_2\) measurement, and (C) ROS/mg protein of state III, IV, and rotenone addition to state IV (expressed relative to values in normoxic respiration states). Asterisks indicate significance from multiple student’s *t*-tests with Holm-Sidak method for multiple comparison corrections.
Figure 3.4
<table>
<thead>
<tr>
<th>Species</th>
<th>ADP/O (State III/IV)</th>
<th>RCR (State III/IV)</th>
<th>Mitochondrial complex maximal activities</th>
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<tr>
<td><em>Oligocottus maculosus</em></td>
<td>5.0 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.9 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>34.2 ± 3.1&lt;sup&gt;ef&lt;/sup&gt;</td>
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<tr>
<td><em>Artedius fenetralis</em></td>
<td>4.3 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.6 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.2 ± 3.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Artedius lateralis</em></td>
<td>5.2 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.7 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.6 ± 2.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Leptocottus armatus</em></td>
<td>5.4 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.9 ± 3.2&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Scorpaaenichthys marmoratus</em></td>
<td>2.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
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<tr>
<td><em>Blepsias cirrhosus</em></td>
<td>4.6 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.2 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>39.9 ± 2.3&lt;sup&gt;ef&lt;/sup&gt;</td>
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**Table 3.1.** ADP/O, respiratory control ratios (RCR; state III/state IV respiration rates), and mitochondrial complex maximal activities ($V_{max}$ of complexes I, II, III, and V; complex IV $V_{max}$ is published in Lau et al. 2017; nmole/min/mg mitochondrial protein) from five species of sculpins. Data are means ± standard error. Letters indicate the results from Tukey’s multiple comparisons test following one-way ANOVA. Mitochondrial complex $V_{max}$ for *S. marmoratus* is not available.
Regression
(x,y) | Test (OLS/PGLS) | Slope estimate | Slope p value | AIC | Pagel’s λ
---|---|---|----|----|------|
Complex I fueled ADP-stimulated respiration rate, $P_{crit}$ | OLS | 0.058 | 0.046 | 12.91* | 
FCCP-Rot/FCCP (Complex I flux capacity), $P_{crit}$ | OLS | 0.074 | 0.032 | 11.85* | 
PGLS | 0.057 | 0.055 | 22.50 | 0.31 |
Rot-Ama/FCCP (Complex II flux capacity), $P_{crit}$ | OLS | -0.11 | 0.0016 | 3.12* | 
PGLS | -0.11 | 0.0007 | 14.97 | -0.78 |

Table 3.2. Regressions were tested with ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) with Pagel’s model of evolution (Pagel 1999). Pagel’s $\lambda$ is an indication of whether the phylogeny correctly predicts patterns of covariance among species for a given trait. $\lambda$ value of 0 indicates that correlation is independent of phylogeny, whereas value of 1 indicates that correlation is consistent with constant-variance model (or Brownian motion model) being a good representation of the data. Model with the lowest AIC value represents the best fitting model (indicated with an asterisk).
Chapter Four: Whole animal responses of ROS metabolism to hypoxia- and hyperoxia-recovery

4.1 Introduction

Changes in environmental \( O_2 \) elicit not only changes in aerobic metabolism, but also in reactive oxygen species (ROS) metabolism. ROS can be generated from multiple sites along the mitochondrial electron transport system (ETS; Quinlan et al., 2013) under conditions of high protonmotive force where ETS complexes are in a reduced redox state and capable of donating electrons directly to \( O_2 \). Mitochondria continuously produces ROS at low rates, which have cellular signaling functions (D’Autréaux and Toledano, 2007) and are scavenged by antioxidant mechanisms. At high rates of production, ROS can overwhelm cell or mitochondria scavenging capacities, and then accumulate and cause oxidative damage to DNA, proteins, and lipids (Cadet, 2003; Gutteridge, 1995; Reznick and Packer, 1994). When \( O_2 \) becomes limiting, the disruption of electron flux through the ETS can result in an increase in ROS generation. Animals that are sensitive to changes in environmental \( O_2 \) (i.e. the majority of mammalian species), are sensitive because of devastating effects of low \( O_2 \) (hypoxia) on cellular energy status, but also because rapid changes in \( O_2 \) such as those that occur during reoxygenation after hypoxia can induce significant tissue injury due to the effects of ROS (i.e. ischemia-reperfusion injury; Chouchani et al., 2014).

There are animals that inhabit and thrive in environments that are prone to changes in \( O_2 \), which have potentially evolved strategies to minimize ROS production or mitigate the damaging effects of ROS. From the literature in this area, however, there does not appear to be a consistent response within ROS metabolism to changes in \( O_2 \), particularly hypoxia, and there are considerable tissue- and species-specific differences (Leveelahti et al., 2014). For example, several studies looking at mitochondrial ROS metabolism suggest that the evolution of hypoxia tolerance was associated with lower mitochondrial ROS generation (elasmobranchs, molluscs, and hypoxia-acclimated killifish \( Fundulus heteroclitus \); Du et al., 2016; Hickey et al., 2012; Ivanina and Sokolova, 2016) as typically expected; however, our recent study using isolated mitochondria from multiple species of intertidal sculpins (Cottidae, Actinopterygii) demonstrated the opposite where the more hypoxia tolerant species had higher ROS/O\(_2\) under state IV conditions and also under highly reduced glutathione redox status when compared to less tolerant species (Chapter 3). These studies suggest that even at the mitochondrial level the relationship between hypoxia tolerance and ROS metabolism is nuanced.
and not straightforward, possibly due to variation in the role of ROS in cell signaling and the role of mitochondria as regulators of ROS metabolism \textit{in vivo} (Munro and Treberg, 2017). In addition, it is unclear whether studies of ROS metabolism conducted \textit{in vitro} at the level of mitochondria apply to \textit{in vivo} responses during whole animal exposure to O\textsubscript{2} stress.

The goal of this present study was to investigate whether there are interspecific differences in aspects of \textit{in vivo} ROS metabolism in response to O\textsubscript{2} variation as it relates to whole animal hypoxia tolerance. In order to assess \textit{in vivo} relationships between redox environment and ROS emission, and to extend our interspecific investigation to \textit{in vivo} cellular damage and ROS scavenging, we exposed two species of sculpin, \textit{Oligocottus maculosus} and \textit{Scorpaenichthys marmoratus}, that differ in hypoxia tolerance and mitochondrial function (Chapter 3) to hypoxia (2.3kPa and 3.5kPa), hypoxia followed by reoxygenation, and hyperoxia (64kPa). Sculpin are distributed along the marine intertidal zones where depending on location, the species experience different fluctuation in O\textsubscript{2} due to the influence of the tidal cycle, with sculpins (e.g. \textit{O. maculosus}) located higher in the intertidal experiencing greater O\textsubscript{2} fluctuations than those in the more homogenous subtidal (e.g. \textit{S. marmoratus}). We chose a 6hr hypoxia and hyperoxia exposure duration followed by a quick normoxia recovery to mimic exposures patterns that occur in the higher intertidal environment (Richards, 2011). Following exposure to hypoxia, hyperoxia, and normoxic recovery, aspects of \textit{in vivo} ROS metabolism were assessed in liver, gill, and brain. Tissue redox status was assessed as the relative ratio of reduced and oxidized glutathione, which is a dominant redox couple within the cell (Rahman et al., 2006). For this study, we used the ratiometric mitochondrial-targeted mass spectrometry probe MitoB to measure changes in H\textsubscript{2}O\textsubscript{2}, which is boronic acid conjugated to a tetraphenylphosphonium ion (TPP\textsuperscript{+}; Logan et al., 2014). Once collected within the mitochondrial matrix, it reacts with H\textsubscript{2}O\textsubscript{2} to form a stable product, MitoP. We quantified oxidative damage as lipid peroxidation, which was measured as thiobarbaruturic acid reactive substances (TBARS) levels (Gutteridge, 1995). We also assessed tissue total oxidative scavenging capacity (TOSC).

4.2 Methods

4.2.1 Equipment
MitoB compounds used for this study were synthesized by Dr. Richard Hartley (University of Glasgow). Chemicals were purchased from Sigma-Aldrich unless otherwise specified.
4.2.2 Animals

*O. muculosus* (average 4g) and *S. marmoratus* (average 16g) were collected near Bamfield Marine Sciences Centre (British Columbia, Canada) at Ross Islets (48°52.4’N, 125°9.7’W) and Wizard’s Rock (48°51.5’N, 125°9.4’W) using either handheld nets or pole seines at the lowest tidal cycle. Animals were transported to the University of British Columbia (UBC) and housed in a recirculation system with artificial seawater at 12°C and maintained on a diet of shrimp, Atlantic krill, and bloodworms for at least 3 weeks before experimentation. All experimental procedures were reviewed and approved by the UBC Animal Care Committee under animal use protocol number A13-0309.

4.2.3 Validation of MitoB use in sculpins

Individual *O. muculosus* were retrieved from the stock tank and injected intraperitoneally with 50μL of MitoB (2nmol/g fish in phosphate-buffered saline) using a BD Ultra-Fine™ syringe (6mm needle). Following injection, fish were transferred to separate plastic mesh baskets (with three fish each) and held in a wet table with recirculating seawater at 12°C. Three fish at each timepoint (0.5, 1, 4, 8, 24, and 72hrs post-injection) were sampled. Animals were euthanized with 0.5g/L benzocaine. Gills, brain, muscle, and liver tissues were sampled at each of the timepoints, frozen in liquid nitrogen and stored at -80°C until processing (see below).

4.2.4 Experimental protocol and sampling

Both species of sculpin were exposed to normoxia, hypoxia and hypoxia-recovery, and hyperoxia and hyperoxia-recovery to assess the effects of different PO2’s on ROS metabolism. For hypoxia treatment, the less hypoxia tolerant species *S. marmoratus* was exposed to 3.5kPa, which is equivalent to 65% of the species’ Pcrit value (Mandic et al., 2009), whereas the more hypoxia tolerant species was exposed to two different hypoxia PO2 treatments of 2.3 and 3.5kPa, corresponding to 65% Pcrit and at Pcrit respectively. These hypoxic PO2 levels were chosen to facilitate the interspecies comparison at a common PO2 exposure (3.5kPa) and also at a hypoxic exposure that is relative to each species’ Pcrit (65% of Pcrit), which has been shown in other species to control for the level of hypoxemia (Speers-Roesch et al., 2013). For hyperoxia treatment, both species were exposed to 64kPa, which corresponds to a level of hyperoxia that is frequently encountered by *O. muculosus* in the higher intertidal (Richards 2011). Both species were also held in normoxia (21kPa) and normoxic control treatments which were run in parallel with each of the hypoxia and hyperoxia treatments. The treatments were set up by placing two covered aquaria (one for normoxic control animals and
the other for O\textsubscript{2} treatments) on a wet table with recirculating water chilled to 12°C. Small circulating water pumps were placed inside each aquarium to ensure adequate mixing throughout the experiment.

To initiate the experiment, sculpins of each species were taken from their stock tank and injected intraperitoneally with MitoB (2nmol/g fish) and placed into 1.1L plastic mesh baskets with gravel on the bottom (4-6 of \textit{O. maculosus} and 2-3 of \textit{S. marmoratus} in each basket) held within each of the treatment aquaria. The PO\textsubscript{2} was then adjusted to the desired level with either nitrogen gas (for hypoxia), 100% O\textsubscript{2} (for hyperoxia), or aerated with compressed air (normoxia) and maintained at these levels for 6hrs. Oxygen levels were monitored with a hand-held O\textsubscript{2} probe (Oakton; for hypoxia) or a FOXY Ocean Optics fluorescent O\textsubscript{2} probe (for hyperoxia). At 6hr, a total of 12 individuals of \textit{O. maculosus} and 8 individuals of \textit{S. marmoratus} were sampled as described below. Following sampling at 6hrs, the hypoxic aquaria at 2.3kPa or 3.5kPa were then quickly returned to normoxia (within 30min) by aeration and a total of 12 individuals of \textit{O. maculosus} and 8 individuals of \textit{S. marmoratus} were sampled at 1hr recovery. In order to account for excretion rates of MitoB and MitoP, paired normoxic controls were also sampled at the same time to compare to O\textsubscript{2} treatment samples. To sample fish, the mesh baskets were removed from the treatment aquaria and placed in a 3.7L container with anaesthetic (0.5g/L benzocaine). Once the fish were unresponsive to touch, they were removed from the anaesthetic bath and brain, gill, and liver were dissected and frozen in liquid N\textsubscript{2} and stored at -80°C until further analyses.

4.2.5 Purification of tissue samples for MitoP/MitoB
Briefly, tissues were homogenized in 200µL ice-cold 60% acetonitrile/0.1% formic acid in the bullet blender bead homogenizer (Next Advance). Homogenates were then centrifuged at 16000g for 10min at 4°C, after which the supernatant was transferred to a new vial. The tissue was resuspended in 200µL 60% acetonitrile/0.1% formic acid and homogenized and centrifuged as described above. The resulting supernatant was combined with the previous supernatant and 10µL of internal standard (10µM d\textsubscript{15}-MitoB/5µM d\textsubscript{15}-MitoP) was added to each sample, vortexed for 10sec, and incubated for 30min at 4°C. Samples were then centrifuged at 16000g for 10min, and the supernatant was filtered with the Millipore centrifugal filter plate (0.45µm hydrophilic, low protein binding Durapore membrane; centrifuged at 3000g for 10min). The filtrate was collected and the samples were dried in a vacuum speed centrifuge (Labconco Centrivap Concentrator). The dried
sample was then resuspended in 250µL 20% acetonitrile/0.1% formic acid, vortexed for 5min to resuspend and centrifuged at 16000g for 10min. 200µL was used for LC-MS/MS analysis. Individual sample variation was normalized to deuterated internal standards.

Standard curves for MitoB (0 to 1000pmol) and MitoP (0 to 1000pmol) were prepared with salmon tissue. Salmon tissue were homogenized in 60% acetonitrile/0.1% formic acid as described above. After two centrifugation steps, 10µL of internal standard and 10µL of standard was added to the supernatant. The standards were then purified with the same procedure as described above (standard curves in Supplementary Fig.4.1).

4.2.6 Tissue glutathione redox status (GSH:GSSG)
Reduced (GSH) and oxidized (GSSG) glutathione were assayed with the enzymatic recycling method described in Rahman et al. (2006). Briefly, tissues were homogenized in 80µL buffer with 0.1M KH₂PO₄ and 5mM EDTA at pH7.5 (KPE buffer) with 0.1% triton X-100 and 0.6% sulfosalicylic acid. The homogenized sample was then centrifuged at 8000g for 10min at 4°C. The supernatant was then divided for assessment of total GSH and GSSG. To determine GSSG, 50µL of the samples or GSSG standards were incubated for 1hr at room temperature with 1µL vinylpyridine (1:10 v/v in KPE buffer) to derivatize GSH. After 1 hr, 3µL triethan olamine (1:6 in KPE buffer) was added to the samples/standards and incubated for 10min at room temperature, which was followed by the addition of 3µL 1M HCl to neutralize the sample. The GSSG and total GSH samples were then assayed using the same protocol in which glutathione reductase (GR) converts GSSG into GSH. For the assay, the buffer was prepared with equal volumes of 1.7mM [5,5'-dithio-bis(2-nitrobenzoic acid)] (DTNB) and glutathione reductase (3.33U/mL KPE), of which 125µL is added to 20µL of standard/sample. 60µL of 0.8mM β-NADPH was added to start the reaction, and the rate of TNB formation was monitored at 412nm for 5min. GSH was calculated as the difference between total glutathione and GSSG. The redox status in (mV) was calculated using a simplified Nernst equation for GSH:GSSG: \( E_{\text{rc}} = E_o + 30 \log\left(\frac{[\text{GSSG}]}{[\text{GSH}]^2}\right) \) where \( E_o \) is -264mV at pH 7.4 (Garcia et al., 2010; Jones, 2002).

4.2.7 Thiobarbuturic acid reactive substances (TBARS)
TBARS was assessed using a commercially available kit (TBARS Parameter™ kit; R&D systems). Tissues were homogenized in homogenization buffer (in mM at pH7.75: 100 TrisHCl, 2 EDTA, and 5 MgCl₂·6H₂O) after which the suspension was centrifuged at 1600g for 10min at 4°C. The
supernatant was divided into aliquots for the total scavenging oxidative capacity assay (TOSC; described below), for the determination of protein content using the Bradford’s method (Bradford, 1976), and the remaining supernatant was used for the determination of TBARS. Briefly, equal volumes of sample and acid reagent were combined and incubated for 15 min at room temperature. The sample was then centrifuged twice at 12000 g for 4 min at 4°C, and the supernatant was used for the assay. TBARS standards were prepared according to manufacturer instructions. The samples and standards were then incubated with TBA reagent (2:1 v/v) for 3 hrs at 50°C, and absorbance was measured at 532 nm. TBARS levels were normalized to tissue protein concentration.

4.2.8 Total oxidative scavenging capacity (TOSC)
TOSC was determined as the overall activity of H$_2$O$_2$ removal, where H$_2$O$_2$ in the sample was monitored with Amplex Ultrared (Invitrogen). 100 µL of assay buffer (with 0.1 mM Amplex Ultrared, 1 U/mL horseradish peroxidase in 50 mM sodium citrate at pH 6.0) was added to 50 µL catalase standard or sample (homogenate prepared for the TBARS assay) in a spectrophotometer plate and pre-read at 565 nm. 50 µL of 160 µM H$_2$O$_2$ was then added to each well and the endpoint absorbance was measured after 5 min. The final TOSC was expressed as units of catalase activity (catalase standard curve was generated (from 0 to 45 units of activity) with an exponential fit). TOSC was normalized to tissue protein concentration.

4.2.9 Statistical analyses
In order to account for MitoP and MitoB decay in tissues over time due to excretion, each timepoint had to be compared to a control sampled at the same time. The normoxic controls sampled at 6 hrs and after an additional 1 hr of recovery were not significantly different, and thus were grouped into a single normoxic sample to compare with O$_2$ treatment and recovery samples.

In order to compare interspecies response to the different O$_2$ treatments, two-way ANOVA with Holm-Sidak’s multiple comparison correction was performed to compare the effect of species (O. maculosus and S. marmoratus), treatment (normoxia, 6 hrs hypoxia/hyperoxia treatment, or recovery from hypoxia/hyperoxia) for each measure (MitoP/MitoB, GSH:GSSG, TBARS, and TOSC). TBARS and TOSC values were normalized to normoxic control values.
4.3 Results

4.3.1 Validation of MitoB and MitoP in marine sculpins

As this is the first study to use MitoB in marine fish, we first validated its use in *O. maculosus* by examining tissue distribution and changes in concentrations of MitoB and the product MitoP over 72hr following injection of MitoB, which is similar to the validation approach used by Salin et al. (2017) for work in brown trout *Salmo trutta*. MitoB was reliably detected within 30min of injection in liver, brain, and gill, but the extent of uptake varied between tissues (in pmol/mg tissue: gill 1.42 ± 0.23, brain 0.38 ± 0.08, liver 13.71 ± 3.78; Supplementary Fig.4.2). MitoP was also detected after 30 min in the liver, brain and gill, but at lower concentrations (in pmol/mg tissue: gill 0.51 ± 0.07, brain 0.13 ± 0.06, liver 4.64 ± 1.75). In white muscle, MitoB and MitoP levels peaked at 1hr after injection, but tissue concentrations were much lower compared with the other tissues sampled (highest levels at 1hr of 0.09 MitoB and 0.06 MitoP pmol/mg tissue), so white muscle was not analysed for this study. Both MitoB and MitoP decreased exponentially over the 72hr time course in liver, brain, and gill, but we were able to accurately determine the MitoP/MitoB for the full 8hr required for treatment and normoxia exposures for our study.

4.3.2 Hypoxia and hypoxia-recovery at a common PO₂ (3.5kPa)

**Brain**

Two-way ANOVA revealed no significant effect of normoxia, 6hr hypoxia (3.5kPa), or hypoxia-recovery (treatment effect, F= 3.26, p= 0.058), no significant effect of species (F= 0.091, p= 0.77), and no significant treatment x species interaction (F=0.47, p=0.63) on brain GSH:GSSG (Fig.4.1A). Similarly, there was no effect of treatment (F=2.43, p=0.10), species (f=1.74, p=0.20), or treatment x species interaction (F=1.34, p=0.27) on MitoP/MitoB (Fig.4.1B). There were, however, significant effects of treatment (F=5.34, p= 0.012), species (F=34.66, p<0.0001), and a significant treatment x species interaction (F=10.12, p=0.0006) on brain TBARS. Post-hoc analysis revealed that *O. maculosus* increased TBARS after 6hrs exposure to hypoxia compared with both normoxia and hypoxia-recovery treatments, whereas *S. marmoratus* showed no changes (Fig.4.1C). Although there were no effects of treatment (F=2.56, p=0.094), there were significant effects of species (F=7.60, p=0.01), and treatment x species interaction (F=4.28, p=0.024) on brain TOSC. Post-hoc analysis shows that *O. maculosus* increased TOSC after 6hrs exposure to hypoxia compared with both normoxia and hypoxia-recovery treatments, whereas *S. marmoratus* showed no changes (Fig.4.1D).
Liver

Two-way ANOVA revealed a significant species effect (F=18.39, p=0.0002), but no treatment effect (F=1.72, p=0.20), and of treatment x species interaction (F=0.17, p=0.85) on liver GSH:GSSG. Post-hoc analysis showed that *O. maculosus* had a more oxidized GSH:GSSG compared to *S. marmoratus* during normoxia (Fig.4.2A). Similarly, there was a significant species effect (F=13.45, p=0.0007), but no effect of treatment (F=1.303, p=0.28) or treatment x species interaction (F=0.31, p=0.74) on MitoP/MitoB. Post-hoc analysis revealed a significantly higher MitoP/MitoB in *O. maculosus* than in *S. marmoratus* in hypoxia-recovery (Fig.4.2B). There was a significant effect of species (F=8.77, p=0.0062), but no effect of treatment (F=3.27, p=0.053) or treatment x species interaction (F=2.17, p=0.13) on TBARS. Post-hoc analysis showed that *O. maculosus* had significantly higher TBARS level with 3.5kPa hypoxia exposure compared to levels during normoxia and hypoxia-recovery, whereas *S. marmoratus* showed no changes (Fig.4.2C). There was a significant effect of treatment (F=4.19, p=0.023) and species (F=6.39, p=0.016), but not treatment x species interaction (F=2.18, p=0.13) on liver TOSC levels. Post-hoc analysis revealed that *S. marmoratus* had significantly higher TOSC levels during hypoxia-recovery compared to levels during normoxia and hypoxia exposure, whereas *O. maculosus* showed no changes (Fig.4.2D).

Gill

There was a significant effect of treatment (F=3.84, p=0.04), but no effect of species (F=2.35, p=0.14) or treatment x species interaction (F=2.88, p=0.073) on gill GSH:GSSG. From the post-hoc analysis, hypoxia exposure resulted in an oxidized GSH:GSSG in *S. marmoratus* compared with normoxia and hypoxia-recovery, whereas *O. maculosus* showed no changes in GSH:GSSG (Fig.4.3A). There were no significant effects of treatment (F=1.90, p=0.16), species (F=0.036, p=0.85), or treatment x species interaction (F=0.44, p=0.64) on MitoP/MitoB. There were also no effects of treatment (F=0.13, p=0.88), species (F=3.46, p=0.074), or treatment x species interaction (F=0.86, p=0.43) on TBARS (Fig.4.3C). Similarly, there were no effects of treatment (F=0.76, p=0.47), species (F=1.59, p=0.21), or treatment x species (F=0.40, p=0.67) interaction on TOS (Fig.4.3D).

4.3.3 Effects of hypoxic PO₂ on responses in *O. maculosus* (2.3 and 3.5kPa)

Brain
In order to account for the fact that the *O. maculosus* and *S. marmoratus* differ in hypoxia tolerance and *P*\(_{\text{crit}}\), and exposure of both species to a common hypoxia *P*\(_{\text{O}_2}\) of 3.5kPa represents a more severe stress for *S. marmoratus* (3.5kPa represents 65% *P*\(_{\text{crit}}\) for this species and 3.5kPa which is at *P*\(_{\text{crit}}\) for this species), and hypoxia with 1hr normoxia recovery, two-way ANOVA revealed a significant treatment effect (normoxia, hypoxia, hypoxia/recovery; F=6.41, p=0.0059), but no effect of PO\(_{\text{O}_2}\) (2.3 and 3.5 kPa; F=0.40, p=0.53) and treatment x PO\(_{\text{O}_2}\) interaction (F=1.77, p=0.19) on GSH:GSSG. Post-hoc analysis revealed that *O. maculosus* exposed to hypoxia-recovery had significantly reduced GSH:GSSG compared with 3.5 kPa hypoxia, whereas *O. maculosus* from the 2.3kPa hypoxia treatments showed no changes (Fig.4A). There were no significant effects of treatment (F=1.51, p=0.23), PO\(_{\text{O}_2}\) (F=3.12, p=0.086), or treatment x PO\(_{\text{O}_2}\) interaction (F=0.79, p=0.46) on brain MitoP/MitoB (Fig.4.4B). There was a significant effect of treatment (F=15.45, p<0.001), but no effect of PO\(_{\text{O}_2}\) (F=3.977, p=0.056) or treatment x PO\(_{\text{O}_2}\) interaction (F=1.244, p=0.30) on brain TBARS. Post-hoc analysis showed a significant increase in TBARS in *O. maculosus* exposed to both 3.5kPa hypoxia and 2.3kPa hypoxia, which was significantly reduced in hypoxia-recovery after exposure to 3.5 kPa, but not 2.3kPa (Fig.4.4C). There was a significant effect of treatment (F=4.30, p=0.023), but no effect of PO\(_{\text{O}_2}\) (F=0.016, p=0.90) or treatment x PO\(_{\text{O}_2}\) interaction (F=2.16, p=0.13) on TOSC. Post-hoc analysis showed that *O. maculosus* after exposure to 3.5kPa hypoxia had significantly higher TOSC levels than during normoxia and hypoxia-recovery, whereas there were no changes in *O. maculosus* exposed to 2.3kPa hypoxia and recovered (Fig.4.4D).

**Liver**

Comparing *O. maculosus* exposed to 2.3kPa and 3.5kPa hypoxia, there were no significant effects of treatment (F=0.35, p=0.71), PO\(_{\text{O}_2}\) (F=1.93, p=0.16), or treatment x PO\(_{\text{O}_2}\) interaction (F=0.47, p=0.63; Fig.4.5A) on liver GSH:GSSG. There were also no significant effects of treatment (F=1.93, p=0.16), PO\(_{\text{O}_2}\) (F=0.086, p=0.77), or treatment x PO\(_{\text{O}_2}\) interaction (F=0.38, p=0.69; Fig.4.5B) on MitoP/MitoB. There were significant effects of PO\(_{\text{O}_2}\) (F=7.79, p=0.0094), but no effects of treatment (F=2.068, p=0.15) or treatment x PO\(_{\text{O}_2}\) interaction (F=2.58, p=0.093) on TBARS. Post-hoc analysis revealed that *O. maculosus* exposed to 3.5kPa hypoxia had significantly higher TBARS levels compared to in normoxia and hypoxia/recovery, whereas those exposed to 2.3kPa showed no
changes (Fig.4.5C). There were significant effects of PO$_2$ (F=7.55, p=0.0092), but no effects of treatment (F=2.55, p=0.092) and treatment x PO$_2$ interaction (F=4.89, p=0.013) on TOSC levels. Post-hoc analysis revealed that *O. maculosus* exposed to 3.5kPa hypoxia had significantly lower TOSC levels compared to normoxia and hypoxia-recovery, whereas *O. maculosus* exposed to 2.3kPa showed no changes (Fig.4.5D).

In light of the significant effects of PO$_2$ in liver TBARS and TOSC in *O. maculosus* (Fig.4.5C&D), we compared *O. maculosus* and *S. marmoratus* at their respective 65% P$_{crit}$ exposures, i.e. *O. maculosus* at 2.3kPa and *S. marmoratus* at 3.5kPa. There were no significant effects of treatment (F=0.019, p=0.39), species (F=0.16, p=0.86), and treatment x species interaction (F=0.23, p=0.79) on TBARS (Fig.4.7A). There were no effects of treatment (F=0.59, p=0.56) or species (F=0.46, p=0.50), but a significant treatment x species interaction (F=3.65, p=0.036) for TOSC levels. Specifically, *S. marmoratus* had significantly higher TOSC levels with hypoxia-recovery compared to levels during hypoxia, but not different from levels during normoxia, whereas *O. maculosus* showed no changes (Fig.4.7B).

**Gill**

There were no significant effects of treatment (F=1.71, p=0.20), PO$_2$ (F=2.95, p=0.095), or treatment x species interactions (F=0.74, p=0.48) on gill GSH:GSSG (Fig.4.6A). There were also no significant effects of treatment (F=3.06, p=0.058), PO$_2$ (F=1.87, p=0.18), and treatment x PO$_2$ interaction (F=1.04, p=0.36) on MitoP/MitoB. However, post-hoc analysis revealed that *O. maculosus* in hypoxia-recovery from 3.5kPa exposure had significantly lower MitoP/MitoB than in normoxia, whereas *O. maculosus* exposed to 2.3kPa and recovered show no changes (Fig.4.6B). There were no significant effects of treatment (F=0.32, p=0.73), PO$_2$ (F=0.0056, p=0.94), and treatment x PO$_2$ interaction (F=0.036, p=0.96) on TBARS (Fig.4.6C). Also, there were no significant effects of treatment (F=0.14, p=0.87), PO$_2$ (F=0.0026, p=0.96), and treatment x PO$_2$ interaction (F=0.080, p=0.92) on TOSC (Fig.4.6D).

4.3.4 Hyperoxia and hyperoxia-recovery at a common PO$_2$(64kPa)

**Brain**

Two-way ANOVA revealed no significant effect of 6hr hyperoxia (64kPa) or hyperoxia-recovery (treatment effect, F= 0.27, p= 0.76) on brain GSH:GSSG. However, there was a significant effect of
species on brain GSH:GSSG (F= 6.86, p= 0.0.16) with no treatment x species interaction (F=3.05, p=0.068). Post-hoc analysis showed that *O. maculosus* had a significantly more oxidized GSH:GSSG with hyperoxia-recovery compared to *S. marmoratus* (Fig.4.1E). Similarly, there was a significant species effect (F=10.07, p=0.0031), but no effect of treatment (F=0.27, p=0.76) and treatment x species interaction (F=0.305, p=0.068) of MitoP/MitoB. Specifically, *O. maculosus* had a significantly higher MitoP/MitoB during 6hr hyperoxia compared to *S. marmoratus* (Fig.4.1F). There was no effect of treatment (F=0.21, p=0.81), but significant effects of species (F=20.78, p<0.0001), and treatment x species interaction (F=5.25, p=0.012) on TBARS. Post-hoc analysis showed significantly higher TBARS level in *O. maculosus* with hyperoxia-recovery compared to normoxia and during hyperoxia, whereas *S. marmoratus* showed no changes (Fig.4.1G). There was no effect of treatment (F=0.98, p=0.39), but there were significant effects of species (F=23.65, p<0.0001), and treatment x species interaction (F=7.10, p=0.0031) on TOSC. Specifically, *O. maculosus* had significantly higher TOSC levels during hyperoxia and hyperoxia-recovery compared to normoxic levels, whereas *S. marmoratus* showed no changes (Fig.4.1H).

**Liver**

There was a significant effect of treatment (F=3.48, p=0.045), and species (F=11.35, p=0.0022), but no effect of treatment x species interaction (F=1.89, p=0.17) on liver GSH:GSSG. Post-hoc analyses showed that *O. maculosus* had significantly more oxidized GSH:GSSG in hyperoxia-recovery compared to during hyperoxia, which was not different from normoxic levels. *S. marmoratus* showed no changes in GSH:GSSG in hyperoxia-recovery (Fig.4.2E). There were significant effects of treatment (F=3.43, p=0.042), and species (F=20.55, p<0.0001), but no effect of treatment x species interaction (F=0.013, p=0.99) on MitoP/MitoB. Post-hoc analyses revealed that *O. maculosus* had significantly higher MitoP/MitoB than *S. marmoratus* in normoxia, hyperoxia, and hyperoxia-recovery (Fig.4.2F). There was no effect of treatment (F=1.69, p=0.20), but there was a significant effect of species (F=7.93, p=0.0091) and treatment x species interaction (F=3.54, p=0.044) on TBARS. Post-hoc analyses revealed significantly higher TBARS during hyperoxia in *O. maculosus* relative to normoxia and hyperoxia-recovery levels, whereas *S. marmoratus* showed no changes (Fig.4.1G). There was significant effect of treatment (F=4.30, p=0.022), species (F=26.51, p<0.0001) and treatment x species interaction (F=6.78, p=0.0035) on TOSC. Post-hoc analyses showed
significantly higher TOSC levels in *S. marmoratus* during treatment and hyperoxia-recovery compared to normoxic levels, whereas *O. maculosus* showed no changes (Fig.4.2H).

**Gill**

There was a significant species effect (F=6.95, p=0.013), but no effects of treatment (F=0.14, p=0.87) and treatment x species interaction (F=0.14, p=0.87) on GSH:GSSG. There was a significant effect of treatment (F=3.44, p=0.042), but no effects of species (F=1.42, p=0.24) and treatment x species interaction (F=0.10, p=0.91) of MitoP/MitoB (Fig.4.3F). There were no effects of treatment (F=0.26, p=0.77), species (F=0.40, p=0.53), and treatment x species interaction (F=0.12, p=0.89) on TBARS (Fig.4.3G). There were also no effects of treatment (F=0.40, p=0.67), species (F=0.35, p=0.56), or treatment x species interaction (F=0.23, p=0.79) on TOSC (Fig.4.3H).

**4.4. Discussion**

Our previous *in vitro* work on sculpins has shown that interspecific variation in hypoxia tolerance was associated with variation in mitochondrial ROS generation, with mitochondria from more hypoxia tolerant species generating more ROS/O\(_2\) under reducing conditions than those from hypoxia intolerant species. In addition, mitochondria from hypoxia tolerant sculpins were more sensitive to changes in the mitochondrial redox environment, and did not recover state III respiration after an *in vitro* anoxia-reoxygenation exposure to the same extent as the less hypoxia tolerant sculpins (Chapter 3). These counterintuitive results, however, were based on experiments using mitochondria isolated from the brain which may not accurately reflect *in vivo* conditions and responses. Thus, the primary goal of the present study was to determine if the observed results from *in vitro* studies (Chapter 3) were also observed *in vivo* and whether there were tissue specific differences in ROS metabolism in response to hypoxia, hypoxia-recovery, hyperoxia, hyperoxia-recovery exposures. Indeed, the more hypoxia tolerant *O. maculosus* generally exhibited greater responses in ROS metabolism, *i.e.* changes in glutathione redox status, TBARS, and TOSC, to both hypoxia and hyperoxia exposures followed by normoxia recovery, and there were tissue specific differences. However, the changes in oxidative damage and scavenging capacity in response to hypoxia and hyperoxia do not appear to be caused by ROS accumulated in the mitochondrial matrix as indicated by no changes in MitoP/MitoB, pointing to other possible sources of ROS.
4.4.1 MitoB for the detection of \textit{in vivo} mitochondrial ROS generation in marine sculpins

The recent development of the mitochondrially-targeted MitoB has proven to be a valuable tool in the assessment of \textit{in vivo} mitochondrial ROS production and has contributed greatly to our understanding of mammalian ROS production in response to cardiac ischemic-reperfusion (Chouchani et al., 2014). Although MitoB is well used in the study of human-related pathologies in murine models, far fewer studies have employed MitoB in the comparative or environmentally-oriented context. Salin et al. (2017) used MitoB and showed that brown trout with high metabolic rates had lower liver ROS generation. MitoB was also used in drosophila to show that ageing was related to increase in H$_2$O$_2$ accumulation (Cochemé et al., 2011), and also that hyperoxia led to an increase in H$_2$O$_2$ (Cochemé et al., 2012). MitoB has not yet been used in an interspecies comparison, in animals that inhabit different O$_2$ environments.

Even though MitoB has already been used in teleost fish (Salin et al., 2015), the use of the probe must first be validated in any study organism to account for species-specific excretion rates and tissue-specific uptake. Our validation study in sculpins shows that for the most part MitoB accumulated in various tissues to a similar extent as in other animal systems (Cochemé et al., 2012; Logan et al., 2014; Salin et al., 2017). MitoB quickly accumulated in brain, gill and liver after injection (within 30min), after which there was an exponential decay with reliable detection for up to 8hrs post-injection (Supplementary Fig.4.2). MitoB was also quickly converted to MitoP in brain, gill, and liver tissues, which similarly showed an exponential decay over the 72hrs. The concentrations of MitoP and MitoB result in a stable MitoP/MitoB over the first 8hrs post-injection (Supplementary Fig.4.2). The timeline of accumulation, however, differed from that of the freshwater brown trout where MitoB was still at high levels in liver and white muscle at 72hrs post-injection (Salin et al. 2017). There were also differences in tissue MitoP/MitoB between sculpins and brown trout, where brown trout white muscle (0.27) showed a much higher ratio than in liver (0.073), whereas in sculpins we were unable to reliably detect MitoP/MitoB in white muscle due to the slow uptake of MitoB (Supplementary Fig.4.2; Salin et al. 2017). These species-specific differences in tissue MitoB uptake and excretion could be due to comparison between freshwater and seawater fish and due to the difference in osmoregulatory strategies. It is also important to note that we observed MitoB uptake in the sculpin brain, which has not been previously observed in mammals (personal observation), likely due to the presence of a tight blood brain barrier. Teleost fish are also believed to have a tight endothelial-based blood brain barrier analogous to that of other vertebrates, but the
uptake of MitoB in sculpins brain suggest that there are functional differences in the blood brain barrier between teleosts and mammals that allowed for uptake of this charged molecule (Kniesel and Wolburg, 2000; Wolburg et al., 1983). These differences in MitoB uptake across species emphasize the importance of validating its use in a new species before undertaking experiments.

4.4.2 Hypoxia and hyperoxia exposure had greater effects on ROS metabolism in *O. maculosus* than *S. marmoratus*

In addition to using MitoB to measure mitochondrial H$_2$O$_2$, we chose to measure representative indices of tissue redox status, oxidative damage, and scavenging capacity to study different aspects of ROS metabolism. Tissue GSH:GSSG represent the dominant redox pair within the cell and is easily assessed by measuring the concentrations of GSH and GSSG (Rahman et al., 2006). Changes in glutathione redox status have been used to describe changes in the cellular redox environment, which can drive changes in ROS generation rate (Aon et al., 2010; Jones, 2002; Schafer and Buettner, 2001). To assess oxidative damage, we quantified TBARS levels as an indicator of lipid peroxidation, although proteins, lipids, and DNA are also susceptible to oxidative damage, which may occur over different time scales (Cadet, 2003; Reznick and Packer, 1994). Redox imbalance and oxidative damage could signal an increase in ROS scavenging capacity. In lieu of measuring only a few select antioxidant enzymes, we assessed the overall ability of the tissue to metabolize H$_2$O$_2$ with the TOSC assay. However, there are different types of antioxidants, enzymatic and non-enzymatic, each with different chemical properties and reactivity to different ROS species that would not be encompassed by this TOSC measure. Overall, the indices that we chose provide us with an overview of how the two sculpin species respond to changes in environmental PO$_2$.

Hypoxia- and hyperoxia-recovery generally had a greater impact on ROS metabolism in *O. maculosus* than *S. marmoratus*. *O. maculosus* showed signs of increased oxidative damage with a simultaneous increase in TOSC levels during hypoxia, hyperoxia, and hyperoxia-recovery, whereas *S. marmoratus* showed fewer responses.

In *O. maculosus*, hypoxia at a common PO$_2$ and recovery yielded no effects on redox balance and H$_2$O$_2$ in the brain, liver, and gill. Although there were no signs of redox imbalance and H$_2$O$_2$ accumulation in this hypoxia tolerant species, there were significant effects of ROS in brain and
liver, but not the gills. In the brain, the increase in TBARS was concomitant with an increase in TOSC, suggesting that the increased scavenging capacity was not enough to prevent oxidative damage. These increased levels of brain TBARS and TOSC capacity, for the most part, recovered after 1hr at normoxia, which is within the timeframe of a typical tidal cycle. There was a similar increase of TBARS in the liver, however, it was not accompanied by an increase in TOSC. In contrast, *S. marmoratus* showed relatively few changes in hypoxia-recovery. There was no indication of redox imbalance, changes in H$_2$O$_2$, oxidative damage, or scavenging capacity in the brain to hypoxia-recovery. The liver also did not show indications of redox imbalance, changes in H$_2$O$_2$, or oxidative damage. However, there was an increase in TOSC levels in recovery from hypoxia, suggesting that the upregulation of scavenging capacity may have prevented any oxidative damage (*i.e.* increase in TBARS). In the gills, there was oxidation of glutathione redox status to hypoxia that was recovered in normoxia but this sign of redox imbalance did not yield changes in H$_2$O$_2$ and there was also no evidence of oxidative damage.

In *O. maculosus*, hyperoxia did not lead to redox imbalance or changes in H$_2$O$_2$ in the brain. However, there were increases in TBARS and TOSC that remained high in recovery. In the liver, GSH:GSSG was significantly reduced in hyperoxia but was recovered in normoxia. This did not cause any changes in H$_2$O$_2$. Similar to the hypoxia response, increase in liver TBARS was not mirrored by TOSC levels. Finally in the gills, GSH:GSSG was reduced in hyperoxia and recovered in normoxia, but no changes in H$_2$O$_2$ and no signs of oxidative damage were observed. *S. marmoratus* did not show any response to hyperoxia-recovery, except for a significant increase in liver TOSC in hyperoxia that remained high in recovery.

Our results showed few changes in MitoP/MitoB in response to hypoxia and hyperoxia in either species, suggesting that very little mitochondrial H$_2$O$_2$ was generated even though there was evidence of ROS effects with changes in TBARS and TOSC. There are several possible explanations that may contribute to this discrepancy. First, the oxidative damage could have been caused by ROS released by ETS complexes sites orientated toward the cytoplasmic side (complex III and glycerol-3-phosphate dehydrogenase (GPDH)) and not detected in the matrix by MitoB. Second, ROS other than H$_2$O$_2$ (*e.g.* superoxide and hydroxyl radicals which are normally quickly scavenged and converted to less reactive H$_2$O$_2$), and also reactive species other than ROS (*e.g.* reactive sulfur and nitrogen species; DeLeon et al., 2016) may be causing oxidative damage in response to O$_2$ stress.
rather than $\text{H}_2\text{O}_2$. Third, non-mitochondrial sites have been shown to have the capacity to generate a significant amount of ROS (Brown and Borutaite, 2012). It was estimated in the rat liver that mitochondria contribute 18% of total ROS, but microsomes and peroxisomes can generate 45% and 35% respectively of total ROS (Chance et al., 1979). Lastly, although less likely, is that the MitoB probe is not sensitive to mitochondrial ROS production in marine teleosts. Although it is unlikely that the chemistry of the probe itself would differ in a different animal model (and our validation shows that MitoB works similarly in sculpins), it is conceivable that there are details that require further validation with a positive control using a teleost study model (e.g. using MitoB to detect superoxide generation stimulated by mitochondrial-targeted paraquat, a redox cycler).

4.4.3 Tissue specific responses of ROS metabolism to $\text{O}_2$ stress

The gills in both species were generally more resistant to both hypoxia and hyperoxia, showing no changes in mitochondrial $\text{H}_2\text{O}_2$, TBARS or TOSC, whereas the brain and liver tissues showed greater responses in ROS metabolism to $\text{O}_2$ variability. No changes in TBARS level would suggest that no ROS was generated in response to hypoxia and hyperoxia, and thus it was not necessary to elicit any TOSC responses. Given its functional role as the gas exchange surface, exposed gills would presumably be more susceptible to oxidative stress. Thus, it was surprising that we did not observe large responses in ROS metabolism in the gills that would act to buffer changes in $\text{O}_2$ levels to minimize the impact on internal organs. Gills in fish represent a unique respiratory surface compared to other organisms in that they not only act as the site of gas exchange, but also for ion exchange. It is possible that there are adaptive mechanisms to protect the gills in order to sustain both of these essential functions. Curiously, the resistance of gills to oxidative damage was not due to constitutively higher levels of TOSC that were at similar levels to the brain (Supplementary Table 4.1). However, our assessment of TOSC (i.e. ability to metabolize $\text{H}_2\text{O}_2$) does not encompass all of the antioxidant mechanisms within the cell, and thus there could be other ROS scavenging processes that give the gills higher buffering capacity to any changes in ROS metabolism. Also, gills in anoxia and hypoxia tolerant animals have been shown to have incredible morphological plasticity and can be rapidly modified in response to changes to environmental $\text{O}_2$ (Sollid and Nilsson 2006).

Tissue redox status of liver from $O. \text{maculosus}$ was generally more oxidized (concomitant with a higher MitoP/MitoB) than liver from $S. \text{marmoratus}$. Under hypoxic conditions, lowered ETS flux would increase protonmotive force and drive ETS complexes into a more reduced state, resulting in
an overall reduced redox environment. Under hyperoxic conditions, high $O_2$ availability leads to a relatively more oxidized redox environment. In fact, liver and gill of hyperoxia exposed $O.\ maculosus$ had more reduced GSH:GSSG which was recovered in normoxia (Fig.4.2E & 4.3E), and $S.\ marmoratus$ hypoxic gill had more oxidized GSH:GSSG which was recovered in normoxia (Fig.4.3A). These changes in GSH:GSSG are contrary to what would be predicted (as $O_2$ limitation would typically lead to more reduced redox status), potentially indicating regulation of tissue GSH:GSSG in response to the $O_2$ exposure in both species. In fact, tissues and cells have been observed to export GSSG in attempts to preserve GSH:GSSG, and it appears that this ability to regulate GSH:GSSG is correlated with protection of the tissue from oxidative stress (Ishikawa and Sies, 1984; Sies and Akerboom, 1984). Thus, $O.\ maculosus$ may be actively regulating GSH:GSSG in liver and gills in response to hyperoxia exposure. Of note, the pattern of changes in GSH:GSSG in gills and liver in both species exposed to hyperoxia-recovery were similar, but the response in ROS, TBARS and TOSC differed in both of these tissues.

The TOSC response to hypoxia and hyperoxia differed between tissues in both species. Although the brain and liver had similar patterns of change in TBARS (i.e. where $S.\ marmoratus$ showed no changes, and $O.\ maculosus$ showed an increase in TBARS during $O_2$ treatment), the TOSC response differed. In the brain, the increase in TBARS in $O.\ maculosus$ was concomitant with an increase in TOSC, which indicates that the increased scavenging capacity was likely overwhelmed and excess ROS likely caused TBARS to increase. This is in contrast to what was observed in the liver, where TOSC did not increase with the increase in TBARS in $O.\ maculosus$ in hypoxia and hyperoxia, and $S.\ marmoratus$ showed a significant increase in TOSC that appeared to mitigate oxidative damage.

4.4.4 Effect of $P_{O_2}$ on ROS metabolism in $O.\ maculosus$

As we are comparing responses in two species that differ in hypoxia tolerance, exposure to a common $P_{O_2}$ represents a more severe hypoxic stress for the less hypoxia tolerant species than the more tolerant species (e.g. 3.5kPa is 65% $P_{crit}$ for $S.\ marmoratus$ and at $P_{crit}$ for $O.\ maculosus$). In order to address this issue, we also exposed $O.\ maculosus$ to a $P_{O_2}$ equivalent to 65% $P_{crit}$ (2.3kPa) and compared the responses to those of $O.\ maculosus$ exposed to a $P_{O_2}$ at $P_{crit}$ (3.5kPa). These differences in $P_{O_2}$ had little effects on ROS metabolism across tissues and in fact, there were only significant effects of this lower $P_{O_2}$ on liver TBARS and TOSC. $O.\ maculosus$ at 3.5kPa had significantly higher TBARS than in fish exposed to 2.3kPa, which was recovered in normoxia, but at the same time
TOSC levels were significantly reduced during exposure to 3.5kPa whereas those under 2.3kPa showed no changes in TOSC (Fig. 4.5C & D). The response of *O. maculosus* to 65% *P*<sub>crit</sub> (2.3kPa) hypoxia is not unlike *S. marmoratus*, which would indicate similar responses at similar relative levels of PO2, although *S. marmoratus* showed an increase in TOSC to hypoxia-recovery (Fig.4.2D).

It is interesting to consider what occurs at *P*<sub>crit</sub> to explain how hypoxia exposure at *P*<sub>crit</sub> (3.5kPa for *O. maculosus*) induced more oxidative damage than exposure to the lower 65% *P*<sub>crit</sub> (2.3kPa for *O. maculosus*) exposure in *O. maculosus*. *P*<sub>crit</sub> is the PO2 at which animals switch from being oxy-regulating to oxy-conforming as environmental PO2 continues to decline, and it reflects the ability of an animal to extract sufficient O2 from the environment to sustain a constant rate of metabolism. Below *P*<sub>crit</sub>, the dependency upon anaerobic metabolism increases, and ultimately results in a significant accumulation of lactate in anoxia-tolerant goldfish and tilapia (Regan et al., 2017; Speers-Roesch et al., 2010). At *P*<sub>crit</sub> (3.5kPa), reducing equivalents may still be available to mitochondria to support ETS flux, but the change in ETS flux caused by changes in PO2 still led to an increase in oxidative damage, whereas below *P*<sub>crit</sub> a downregulation of ETS flux (regulated by pyruvate dehydrogenase; Papandreou et al., 2006b; Richards et al., 2008) may reduce ROS generation which would explain the lack of TBARS accumulation observed with 2.3kPa hypoxia exposure.

As there were significant effects of PO2 on *O. maculosus* liver TBARS and TOSC (Fig.4.5C & D), we also compared *O. maculosus* and *S. marmoratus* both at 65% *P*<sub>crit</sub> hypoxia exposure (Fig.4.7). While there were no interspecies differences in TBARS (Fig.4.7A), *S. marmoratus* significantly increased TOSC levels during hypoxia-recovery whereas *O. maculosus* showed no changes in TOSC (Fig.4.7B). Assuming that the relative hypoxia exposure led to the same arterial PO2 (Speers-Roesch et al., 2013), this indicates that the only difference in response to the same level of hypoxemia is in liver scavenging capacity with normoxia-recovery. This signifies inherent differences in the O2 sensitivity of tissue scavenging capacity in liver.

### 4.4.5 Greater response in ROS metabolism as an adaptive response to O2 variability
Our results show that ROS metabolism in the more hypoxia tolerant *O. maculosus* were more perturbed by O2 variability when compared to the less tolerant *S. marmoratus* in the timeframe that mimics a typical tidal cycle. Similarly, *O. maculosus* that were exposed to temperature changes exhibited a more sensitive response in heat shock protein expression (Todgham et al. 2006; Chapter 3). These studies show that quick responses mirroring changes in abiotic factors in the environment
may confer survival advantage in the higher intertidal. However, it appears that the timing of responses depends on which level of biological organization is in question. At the transcript level, the less hypoxia tolerant sculpin *Blepsias cirrhosus* showed changes in mRNA expression between 3-24hrs in response to hypoxia whereas *O. maculosus* showed changes later between 24-72hrs, indicating that upper intertidal *O. maculosus* adopted a more generalist approach to more prolonged exposure to hypoxia (Mandic et al., 2014). Perhaps the preferred strategy for higher intertidal living is dependent upon sensitive detection of environmental changes and initiating plastic responses to quickly modify existing proteins such as antioxidant enzymes (e.g. via post-translational modifications) and slower changes in mRNA transcription. The coordination of a global transcriptional response would be energetically expensive and is likely reserved for coordinating acclimation response to chronic O₂ stress.

4.4.6 Summary
Overall, we found that the more hypoxia tolerant sculpin *O. maculosus* generally showed more effects of ROS metabolism (changes in levels of TBARS and TOSC) compared to the less hypoxia tolerant sculpin *S. marmoratus*, indicating a more sensitive response to hypoxia and hyperoxia under O₂ conditions frequently encountered in the intertidal environment. These results from whole animal exposures are generally consistent with what has been observed in *in vitro* work on isolated brain mitochondria (Chapter 3), whereby more hypoxia tolerant sculpins generated more ROS under resting conditions and were more sensitive to changes in the extramitochondrial redox environment. The overall greater responses in more hypoxia tolerant sculpins may be part of a sensitive detection of changes in environmental O₂ to coordinate quick cellular responses.
Figure 4.1. The effect of hypoxia (3.5kPa)-recovery (A-D) and hyperoxia (64.0kPa)-recovery (E-H) in brain of *Oligocottus maculosus* (hollow squares) and *Scorpaenichthys marmoratus* (solid squares) on ROS metabolism as assessed by (A, E) tissue GSH:GSSG redox status, (B, F) MitoP/MitoB, (C, G) TBARS (normalized to normoxia control value), (D, H) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O$_2$ treatments (normoxia, hypoxia or hyperoxia, recovery) within a species are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between species within an O$_2$ treatment. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.1.
Figure 4.2. The effect of hypoxia (3.5kPa)-recovery (A-D) and hyperoxia (64.0kPa)-recovery (E-H) in liver of *Oligocottus maculosus* (hollow squares) and *Scorpaenichthys marmoratus* (solid squares) on ROS metabolism as assessed by (A, E) tissue GSH:GSSG redox status, (B, F) MitoP/MitoB, (C, G) TBARS (normalized to normoxia control value), (D, H) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O₂ treatments (normoxia, hypoxia or hyperoxia, recovery) within a species are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between species within an O₂ treatment. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.2.
**Figure 4.3.** The effect of hypoxia (3.5kPa)-recovery (A-D) and hyperoxia (64.0kPa)-recovery (E-H) in gill of *Oligocottus maculosus* (hollow squares) and *Scorpaenichthys marmoratus* (solid squares) on ROS metabolism as assessed by (A, E) tissue GSH:GSSG redox status, (B, F) MitoP/MitoB, (C, G) TBARS (normalized to normoxia control value), (D, H) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O\(_2\) treatments (normoxia, hypoxia or hyperoxia, recovery) within a species are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between species within an O\(_2\) treatment. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.3
Figure 4.4. The effect of 2.3kPa (solid triangle) and 3.5kPa (clear square) hypoxia-recovery in brain of *O. maculosus* on ROS metabolism assessed by (A) tissue GSH:GSSG redox state, (B) MitoP/MitoB, (C) TBARS (normalized to normoxia control value), (D) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O$_2$ treatments (normoxia, hypoxia, and recovery) are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between 2.3 and 3.5kPa hypoxia exposures. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.4
Figure 4.5. The effect of 2.3kPa (solid triangle) and 3.5kPa (clear square) hypoxia-recover in liver of *O. maculosus* on ROS metabolism assessed by (A) tissue GSH:GSSG redox state, (B) MitoP/MitoB, (C) TBARS (normalized to normoxia control value), (D) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O₂ treatments (normoxia, hypoxia, and recovery) are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between 2.3 and 3.5kPa hypoxia exposures. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.5
**Figure 4.6.** The effect of 2.3kPa (solid triangle) and 3.5kPa (clear square) hypoxia-recovery in gill of *O. maculosus* on ROS metabolism assessed by (A) tissue GSH:GSSG redox state, (B) MitoP/MitoB, (C) TBARS (normalized to normoxia control value), (D) TOSC (normalized to normoxia control value). Data are means ± standard error of mean. The effects of O\(_2\) treatments (normoxia, hypoxia, and recovery) are shown with letters, where data points with different letters are significantly different. Asterisks indicate significant differences between 2.3 and 3.5kPa hypoxia exposures. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
Figure 4.6
Figure 4.7. Effect of relative hypoxia exposure and recovery in liver (A) TBARS and (B) TOSC of *O. maculosus* and *S. marmoratus* (2.3kPa for *O. maculosus* (hollow triangles) and 3.5kPa for *S. marmoratus* (solid squares)). The effects of O$_2$ treatments (normoxia, hypoxia, and recovery) are shown with letters, where data points with different letters are significantly different. There were no significant differences between species within an O$_2$ treatment. No significant differences in datasets are indicated as ‘n.s.’ in the figure.
The overall objective of my PhD thesis was to determine if there is evidence of adaptive variation in mitochondrial function across species that inhabit O₂ variable environments. Specifically, I aimed to investigate the interplay between aerobic metabolism and mitochondrial ROS emission, which are both processes that environmental O₂ variability could exert strong selective pressure on. To do so, I used multiple species of sculpin that live along the marine intertidal zone as my study model since these species have previously been shown to vary in their hypoxia tolerance (Mandic et al., 2009; Mandic et al., 2013). In chapter 2, I demonstrated that the adaptive variation in the O₂ transport cascade previously described in sculpins (Mandic et al. 2009) extends to the level of mitochondria and COX. I then explored the in vitro relationship between oxidative phosphorylation and ROS metabolism in isolated mitochondria from sculpins in chapter 3. Finally, I examined ROS metabolism in vivo by investigating whether there were species differences to whole animal exposure to hypoxia, hyperoxia and normoxic recovery in Chapter 4.

In this discussion, I will first summarize the major findings from my thesis and develop a revised model of the O₂ transport cascade that distinguishes between hypoxia tolerant and intolerant sculpins. I will then describe how my research fits with and extends what is currently known about the responses of organisms to O₂ fluctuations, and finish with some considerations for current and futures studies on ROS metabolism.

5.1 Major Findings

5.1.1 Adaptive variation in oxygen binding of mitochondria and COX (Chapter 2)
Using intertidal sculpins, I provide the first evidence of adaptive variation in mitochondrial and COX O₂ binding across species that vary in hypoxia tolerance. In order to investigate the mechanism underlying the variation in COX Kₘ,app,O₂, I performed in silico protein analyses on the catalytic core and showed a strong relationship between COX Kₘ,app,O₂ and COX3 subunit stability, which suggests a significant role of COX3 in determining the O₂ kinetics of the overall protein. Even though the COX3 subunit is not thought to directly interact with O₂, my results from comparative protein modeling revealed residue differences in the COX3 v-cleft structure which
could influence interactions with the mitochondrial membrane lipid, cardiolipin, and affect O₂ diffusion into the COX protein en route to the COX1 catalytic site.

Previous studies in animals that have evolved to inhabit low O₂ environments have shown evidence of adaptive variation at multiple steps of the O₂ transport cascade down to the level of O₂ diffusion at the cellular level (Mahalingam et al., 2017; Scott et al., 2011; Mandic et al., 2009; Mandic et al., 2013). My thesis extends this general phenomenon to the site of O₂ use at the subcellular level in intertidal sculpins. Indeed, sculpins show parallel interspecific relationships between P₉₀, whole red blood cell P₅₀, stripped hemoglobin-O₂ P₅₀, and also at the subcellular level between mitochondrial Pₙ₀ and COX Kₘ,app O₂ (Fig.5.1) such that hypoxia tolerance is associated with improved or sustained O₂ diffusion and extraction from the environment to mitochondria. The parallel relationships between the different traits indicate that the maintenance of the gradients of O₂ diffusion is important for environments that are or frequently become hypoxic, presumably to improve O₂ supply to mitochondria.

Additionally, my results demonstrate that there is selective pressure on both COX Vₘₐₓ and COX respiration as there were significant relationships of both to mitochondrial P₅₀. The opposite relationships of mitochondrial O₂ kinetics, and COX Vₘₐₓ and respiration are intriguing; hypoxia tolerant sculpins have lower COX enzyme (Vₘₐₓ = kₗ₁ x [E]), but higher COX respiration rate in uncoupled mitochondria altogether indicating that hypoxia tolerant sculpins have a higher functioning COX enzyme relative to that in hypoxia intolerant sculpins.

Influences of protein stability on enzyme function

Variation in protein stability and net protein surface charge can have a number of effects, direct or indirect, on protein function. For instance, variation in myoglobin surface charge was demonstrated to be associated with the maximum concentration of myoglobin and used to infer mammalian dive times (Mirceta et al., 2013). Mechanistically, increased electrostatic repulsion between myoglobin molecules is thought to prevent aggregation at high myoglobin concentrations, which would maintain high O₂ binding affinity and support longer dive times (Mirceta et al. 2013). In the case of COX in the sculpins, the variation in COX3 protein stability may reflect differences in how COX3 interacts with its immediate environment, including interactions with other COX subunits or the mitochondrial lipid bilayer. Changes in protein stability can also exert a more indirect effect; a more
stable protein is an indication that the protein is more compact, which can affect catalytic rate by bringing catalytically important residues within the active site closer together. Additionally, an increase in overall protein stability has been hypothesized to increase the evolvability of a protein by stabilizing mutations that would otherwise destabilize the protein and cause unfolding (Bloom et al., 2006). With increasingly more powerful protein modeling methods that are easier to use, functional analyses can continue to be combined with in silico analyses to investigate how the environment has shaped protein adaptation in both direct and indirect ways.

The power (and limitations) of comparative protein modeling

Online tools that are available to assess protein function rely on a well-resolved crystal structure (which include relevant ligands, such as the high-affinity bound cardiolipin in the case of my study on sculpin COX). Using the bovine protein structure, I was able to isolate the mitochondrially-encoded catalytic core of COX for further in silico investigation of possible interspecific differences in the sculpin proteins that would underlie variation in COX O₂ kinetics. Using modeling programs, I generated predicted sculpin COX protein structures to estimate protein stability of the three COX subunits in isolation, study protein subunit interaction, and perform in silico alanine-mutagenesis to investigate differences in cardiolipin and COX3 interactions. The available tools to perform in silico protein analyses will provide increasingly powerful methods to illuminate underlying mechanisms of protein function for empirical testing. Following the discovery of the relationship between COX Kₘ,app O₂ and COX3 protein stability, I hypothesized that there would be variation in the second functional role of COX3 which is to protect the microenvironment of the proton acceptor of the proton-pumping D-pathway. I thus designed a protocol to test whether there were differences in proton pumping rate between sculpin species (Fig.5.1). Although there was no evidence of a relationship between COX Kₘ,app O₂ and proton pumping rate, it does appear to be interspecies differences in proton pumping rate that would warrant further detailed study (Supplementary Fig.2.2).

It is important, however, to keep in mind the limitations of comparative protein modeling. Although COX is a highly-conserved protein, it is possible that there are minute differences that are lost when using a mammalian protein template to estimate a protein structure. Also, there could be influences of the nuclear-encoded COX subunits on overall COX function that needs to be further
investigated. Thus, while I have isolated a potential mechanism that may underlie interspecies variation in COX $K_{m,app} O_2$, there is still a lot more to learn about adaptive variation in COX function (discussed in Section 5.3.1 under Future Directions).

5.1.2 Hypoxia tolerance in sculpins is not associated with efficient mitochondrial $O_2$ use (Chapter 3) The finding that hypoxia tolerance in sculpins is associated with a higher mitochondrial and COX $O_2$ binding affinity led me to hypothesize that the intact ETS would be more $O_2$ efficient, which would be reflected in increased mitochondrial coupling and phosphorylation efficiency. My *in vitro* analysis using mitochondria isolated from the brain of various species of sculpin however, did not yield evidence to support my hypothesis. In fact, there was no evidence for an association between interspecific variation in hypoxia tolerance and more efficient $O_2$ use in sculpin brain mitochondria including no relationship between $P_{crit}$ and mitochondrial coupling (respiratory control ratio; RCR), leak respiration (state IV respiration), or phosphorylation efficiency (ADP/O; Fig.5.2). Intriguingly, there were significant correlations between complex I and II flux capacities and $P_{crit}$, an indicator of hypoxia tolerance, where species with lower $P_{crit}$ had low complex I flux capacity and high complex II flux capacity and the opposite was observed in hypoxia intolerant sculpins. Complex I is a proton pump that contributes directly to the generation of the proton motive force whereas complex II is not a proton pump, thus reduced dependence on complex I and increased dependence on complex II in hypoxia tolerant sculpins, would presumably reduce the magnitude of the protonmotive force and reduce phosphorylation efficiency (Fig.5.2). However, this did not appear to be associated with any differences in phosphorylation efficiency (ADP/O) or mitochondrial coupling (respiratory control ratio; RCR). Complex I is also a major site of mitochondrial ROS generation, thus it is possible that the lower dependency on complex I in hypoxia tolerance sculpins compared with intolerance sculpins could be associated with the need to reduce ROS generation. To investigate whether the variation in complex I was associated with mitochondrial ROS emission, I continued

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*4 Certain nuclear subunits show tissue-specific expression, such as COX4 in Nile tilapia (Porplycia et al. 2017), COX6a in rats and cattle (Schlerf et al. 1988; Schillace et al. 1994). Also, different isoforms of the COX subunits can be expressed under certain situations, such as the COX4-1 to 4-2 switch observed in mammals under hypoxic conditions (Semenza 2007), although this switch was not observed in hypoxia-exposed Nile tilapia, goldfish, or turtles (Porplycia et al. 2017). The exact functions of the nuclear-encoded COX subunits are still relatively unclear in the literature.*
with *in vitro* analysis and also combined it with *in vivo* experiments to characterize whole animal responses to O$_2$ variability.

5.1.3 Hypoxia tolerant sculpins generate more ROS (Chapters 3&4)

Contrary to my expectation that more hypoxia tolerant sculpin that inhabit the more O$_2$ variable environment would generate less ROS, my *in vitro* and *in vivo* analyses demonstrate that more hypoxia tolerant sculpins, in fact, generate more ROS compared to less tolerant species. Lower ROS generation would reduce oxidative damage, especially in sculpins that live in O$_2$ variable upper intertidal environments. However, mitochondria from the brain of hypoxia tolerant sculpins generated more ROS/O$_2$ (under state IV conditions) than those from less tolerant sculpins. As the redox environment has been shown to have a profound effect on ROS emission which also likely varies with O$_2$ variability, I also assessed the responses of mitochondria to changes in the extramitochondrial redox environment. While hypoxia tolerant sculpins appeared to buffer matrix glutathione redox status (GSH:GSSG) better, they were generally more sensitive to changes in extramitochondrial GSH:GSSG and had higher ROS/O$_2$ than less tolerant species (Fig.5.2). Also, when challenged with 20min *in vitro* anoxia-recovery, brain mitochondria from hypoxia tolerant sculpins showed a significant 25% decrease in state III respiration rate, whereas that from the less tolerant species did not show any reduction in state III respiration upon recovery. Interestingly, the post-anoxic reduction in state III respiration rate observed in the hypoxia tolerant sculpin is similar to that observed in typical hypoxia-sensitive mammalian systems. Isolated rat heart mitochondria recovered only 50% of state III respiration after 30min of *in vitro* anoxia exposure (Shiva et al., 2007) and liver mitochondria recovered 75% after 1 or 10min anoxia exposure (Du et al., 1998). While the anoxia-induced reduction in state III respiration rate in mammals is often attributed to oxidative damage, it may not be the case in sculpin brain mitochondria. It would appear that all three sculpin species reduced ROS/mg protein (in state IV; although not significant in the mid-tolerance species *Artedius lateralis*), but there was interspecific variation in how this was achieved. In hypoxia tolerant *Oligocephalus maculosus* this may have been due to a general reduction in ETS capacity, as indicated by the reduction in state III respiration rate and state IV ROS/mg protein, whereas in the less tolerant *Scorpaenichthys marmoratus* there was a change in the ‘efficiency’ of ROS emission, as there was no change in respiration rate but a reduction in ROS/O$_2$. Whether there are advantages to either strategy in response to anoxia-recovery remains to be determined.
Although the results from my *in vitro* work are counterintuitive, this is the first study to compare these responses in a system with closely-related species with the goal of understanding adaptive variation in mitochondrial ROS metabolism. Thus, it sets new directions for further investigation to determine how these variations would give certain species an evolutionary advantage to inhabit O$_2$-variable environments.

Building on results from isolated brain mitochondria in chapter 3, I hypothesized that there would be consistent responses *in vivo* with whole animal exposures to hypoxia and hyperoxia, and hypoxia tolerant sculpins would show more oxidative damage (estimated as lipid peroxidation levels with TBARS measurements) and generally greater responses in ROS metabolism (GSH redox status, H$_2$O$_2$, and TOSC). I also predicted that there would be tissue-specific responses as tissue vary in metabolic activity and O$_2$ requirements. As ROS generation rates are heavily dependent on mitochondrial energy status, tissues with high metabolic activity, more O$_2$-sensitive, and generally function closer to state III conditions would presumably generate ROS at lower rates than tissues that function closer to state IV resting conditions.

To address these hypotheses, I focused on two species that differed in hypoxia tolerance and showed differences in ROS emission (difference in ROS/O$_2$ and response with GSH manipulations; Chapter 3) and exposed both species to hypoxia (at a common PO$_2$ and also relative PO$_2$ exposure to each species’ $P_{crit}$), hyperoxia, and normoxia-recovery and monitored the response in ROS metabolism. The interspecies differences to whole animal exposure to hypoxia and hyperoxia were consistent with what was observed at the mitochondrial level (Chapter 3). In general, *O. maculosus* were more perturbed by O$_2$ variability than *S. marmoratus*. Although both species showed changes in GSH:GSSG (though the directionality would suggest regulation of GSH:GSSG), there were no changes in MitoP/MitoB which indicates no accumulation of matrix H$_2$O$_2$ with hypoxia or hyperoxia with normoxic recovery. However, the more hypoxia tolerant *O. maculosus* showed more ROS effects, showing increases in TBARS and generally similar patterns of TOSC increase in both the brain and liver. Further, there were tissue specific differences in ROS metabolism, where the gills were more resistant to O$_2$ variability, where there were only significant changes in GSH:GSSG but no changes in H$_2$O$_2$, TBARS, and TOSC. Similar patterns of GSH:GSSG change were associated with similar changes in TBARS and TOSC in the brain, but not the liver. This difference in TOSC response to O$_2$ variability suggests tissue difference in regulation of scavenging capacity.
Collectively, the results from my in vitro and in vivo studies revealed that hypoxia tolerance in sculpins is associated with more sensitive responses of ROS metabolism. Though the response patterns were tissue specific, changes in GSH:GSSG, TBARS, and TOSC were for the most part quickly reversed with 1hr normoxic recovery. These quick responses were possibly coordinated by modifications of existing proteins in order to scavenge ROS emitted and repair lipid peroxidation.

5.1.4 Summary model: hypoxia tolerant vs. hypoxia intolerant sculpin
I have summarized the major findings of this thesis in Figure 5.3 to compare the responses of hypoxia tolerant vs. hypoxia intolerant sculpins. There was evidence of adaptive variation in mitochondrial function in sculpins that inhabit environments that vary in O$_2$ levels. Previous studies determined that hypoxia tolerance was associated with a number of changes in various levels of the O$_2$ transport cascade (at the respiratory surface, in circulation, and capillary diffusion distance; Lui et al., 2015; Mandic et al., 2009; Scott et al., 2011), but we now know that this variation in O$_2$ transport and binding extends to the mitochondrial and COX levels. The differences in COX O$_2$ kinetics were likely associated with interspecific variation in COX3 subunit interaction with cardiolipin, which may subsequently affect the path of O$_2$ travel to the catalytic site. These variations in O$_2$ kinetics however were not related to differences in mitochondrial phosphorylation efficiency and coupling, in fact, the relationship between P$_{ox}$ and complex I (lower in hypoxia tolerant sculpin) and complex II (higher in hypoxia tolerant sculpin) was not consistent with increased contribution of protons to the proton gradient. It appears that more O$_2$ in hypoxia tolerant sculpins is used for higher generation of ROS compared to less tolerant sculpins, and overall more sensitive responses to in vitro redox challenge (GSH:GSSG manipulation) and anoxia-recovery challenge. These in vitro responses were consistent with in vivo responses where more hypoxia tolerant species showed higher levels of lipid peroxidation (increase TBARS levels) and increased scavenging capacity (increase in TOSC) compared to the less tolerant species. Given the emerging role of ROS in cell signaling, it is possible that the sensitive responses observed in hypoxia tolerant sculpins may be part of the adaptive strategy of living in the more O$_2$ variable higher intertidal.

5.2 Study Considerations

5.2.1 Interacting abiotic factors in the intertidal
Although I have focused my thesis on O$_2$ variability and the intertidal sculpin model was chosen because they show variability in hypoxia tolerance, sculpins experience changes in other abiotic
factors in their environment that may also impose powerful selective pressures and have interacting effects with the putative adaptations to hypoxia. For instance, the fluctuations in temperature on top of variation in PO$_2$ may have an additional impact on the level of available O$_2$. Daytime emergence of tidepools occurs with both warm temperatures and hyperoxia (up to 400% air saturation; Richards, 2011). As discussed in chapter 2, warmer temperatures and the alpha-stat effect may cause a general increase in enzyme K$_m$ values and reduce substrate binding, including COX, but hyperoxic conditions may be able to compensate by increasing O$_2$ supply to tissues in order to maintain aerobic metabolism. In order to fully understand the selective pressures of life in the intertidal, the physiological effects of these multiple stressors that mimic the naturally fluctuating patterns in the natural environment need to be characterized.

5.2.2 Technical challenges of assessing ROS metabolism *in vivo* and *in vitro*
The assessment of ROS formation and accumulation remains challenging. For my *in vitro* analysis, I chose to use Amplex Ultrared, which is a commonly used fluorophore that reacts with H$_2$O$_2$ in a reaction catalyzed by horseradish peroxidase. Amplex Ultrared, and another commonly used ROS detection method, 2',7'-dihydrodichlorofluorescein, however, have also been shown to be sensitive to reactive sulfur species and possibly reactive nitrogen species (DeLeon et al., 2016). The nonspecificity of the current *in vitro* tools we have to investigate ROS metabolism makes it challenging to assign causal relationships to ROS specifically. Thus, it is important with existing reactive species detection tools to be aware of the limitations of the technology and to make efforts to confirm the actual role of ROS. For instance, to confirm that H$_2$O$_2$ is the major contributor to the Amplex Ultrared signal by using catalase (which catalyzes the reaction of H$_2$O$_2$ to O$_2$) to remove H$_2$O$_2$ from solution, or by assessing known specific ROS targets, e.g. aconitase activity is reversibly inhibited by superoxide and often also measured to demonstrate the presence of ROS (Armstrong et al., 2004). With their limitations in mind, fluorophores like Amplex Ultrared are useful *in vitro* tools to monitor ROS emission from isolated mitochondria and cells in real-time. However, they are not ideal for whole animal studies as tissue level responses would be impossible to view in real-time and ROS generation from sampled tissue is hard to assess due to the reactive nature of ROS.

Chemical probes like MitoB offers an effective solution to some of the limitations of the fluorometric ROS detection probes and enable the study of ROS metabolism *in vivo*. This probe is targeted to the mitochondrial matrix (as it is conjugated to a tetraphenylphosphonium ion; TPP$^+$)
and it forms a stable product upon reaction with H₂O₂. The product MitoP is stable throughout the sampling and processing procedures and thus one can gain an accurate estimation of in vivo ROS accumulation. As previously mentioned, in both brain and liver there were no changes in MitoP/MitoB indicating no accumulation of matrix H₂O₂ but there were changes in both GSH:GSSG, TBARS and TOSC in response to hypoxia and hyperoxia exposure and normoxic recovery. In other words, there was evidence of ROS effects, but no evidence of the presence of mitochondrial ROS. There are three possible explanations for this observation: (1) that the ROS effects were not caused by ROS generated by ETS sites within the mitochondrial matrix, but instead by sites that face the cytoplasmic side (complex III and GPDH), (2) that the ROS effects were caused by non-mitochondrial sources (e.g. ER, peroxisomes), and/or (3) that MitoB does not work in this teleost study model. The second explanation would be exciting since mitochondria have been presumed to be the major site of ROS generation in response to O₂ variability. This possibility is also supported by recent studies that have identified non-mitochondrial sites of ROS generation that can function at high rates (Brown and Borutiate 2012). Whether these non-mitochondrial sites of ROS generation are at play in the sculpins requires further study with careful analysis to isolate which cellular compartments contribute to ROS generation in vivo. The less likely option is the latter one, that MitoB did not work in the sculpin model. As with any new technique, it is important to carefully validate its use in a new animal model. From our validation of MitoB, it appears that MitoB works similarly in sculpins as in murine and drosophila studies (Cochemé et al., 2012; Logan et al., 2014). Although MitoB has been previously used in brown trout, there has yet to be a positive control of MitoB generated in a teleost study model. This positive control can be generated in sculpins using both MitoB and MitoParaquat (a mitochondrial-targeted redox cycler; Mulvey et al., 2017) simultaneously, which would stimulate superoxide generation that is quickly converted to H₂O₂ in the matrix via superoxide dismutase activity and thus confirm that MitoB can indeed detect H₂O₂ in the matrix in sculpins.

5.2.3 Multiple factors affect ROS metabolism
As illustrated by Aon et al. (2012), multiple factors must be considered when studying ROS emission, including energy status, redox environment, and O₂ levels. Depending on the species and tissue type, the relationship of mitochondrial ROS generation and each of these factors may differ (Chapter 3&4; Aon et al., 2010; Munro and Treberg, 2017). Rather than only assessing amounts of ROS emitted under a single condition, I think a more insightful approach for future interspecies
comparison of ROS metabolism would be to compare ROS emission kinetics with manipulation of a factor of interest. For instance, I manipulated extramitochondrial GSH:GSSG to investigate the potential differences in the response to an *in vitro* redox challenge (Chapter 3). Similarly, monitoring ROS emission kinetics simultaneously with O$_2$ kinetics may illuminate potential O$_2$ dependency of ROS emission (Hoffman and Brookes, 2009), and ROS generation under different energy states of mitochondria monitored with state III to state IV transitions (Munro and Treberg 2017).

Moreover, we should also aim to combine observations at multiple levels of biological organization as we continue to understand the subtleties of the interaction between aerobic and ROS metabolism. For example, elasmobranchs generally show reduced antioxidant levels when compared to teleosts (Leveelahti et al., 2014). Thus, by comparing observations from *in vitro* and *in vivo* studies in elasmobranchs and teleosts, we can potentially gain further insight into the regulation of antioxidant responses to O$_2$ variability.

### 5.3 Future Studies

5.3.1 Are there other underlying mechanisms contributing to interspecies variation in COX function?

Other mechanisms beyond those examined in this thesis could potentially contribute to interspecies variation in COX function, one of which is the variation in membrane phospholipids which are intimately linked to membrane proteins and affect protein function. Particularly, cardiolipin differences can have direct effects on mitochondrial enzyme function (such as the effects I described on COX in Chapter 2) and also in the formation of supercomplexes (Claypool, 2009; Zhang et al., 2005). The composition of phospholipids can vary with diet, changing the acyl chain composition of mitochondrial phospholipids, and influence mitochondrial oxidative capacities (shown in rainbow trout *Oncorhynchus mykiss*; Guderley et al., 2008). Thus, it is important to compare the phospholipid composition between sculpins that vary in hypoxia tolerance. Alternatively, COX from different species can be purified and reconstituted into phospholipid vesicles such that the enzymes would be within a common membrane background and any differences in function (*e.g.* $K_{m,app}$ O$_2$) can be attributed to the COX enzyme itself.

It would also be interesting to perform more detailed studies on the cardiolipin that binds to the COX3 v-cleft discussed in Chapter 2. The variation in COX3 amino acid residues between species
that interact with the cardiolipin would presumably affect the affinity of the cardiolipin to its ligand binding site. However, this is challenging as it only takes one cardiolipin molecule to fill the binding site, and due to its high affinity would be difficult to chemically remove from the protein structure without causing damage to the rest of the enzyme. It would also be interesting to investigate whether the chemical species of that particular cardiolipin molecule differ between sculpins and has functional consequences on COX.

A number of other regulatory factors on COX function require further investigation. The nuclear-encoded subunits of COX are thought to have regulatory roles on overall COX function, so that COX can be regulated under different physiological conditions (e.g. changes in ADP/ATP, or O2) but the specific effects on COX function are unclear (Kocha et al., 2014; Little et al., 2010). Thus, it is important to determine whether sculpins differ in the composition of nuclear-encoded subunits in the multisubunit COX. Also, there are gases that regulate COX function which could play an important role when O2 levels vary. For instance, nitric oxide generated by nitric oxide synthase has been shown to inhibit COX in vivo, which could be a quick method of reversible modification of ETS flux when O2 is scarce (Cooper, 2002). Hydrogen sulfide has been shown to act as a substrate and inhibitor to COX, and accumulate in vivo during myocardial ischemia (Arndt et al. 2017). Investigation in hypoxia tolerant animals is necessary to establish the role of these modulators in response to O2 variability.

5.3.2 What are the functional consequences of the difference in ETS complex flux capacities when comparing sculpins of varying hypoxia tolerance?

Hypoxia tolerant sculpins had higher complex I flux capacity and lower complex II flux capacity compared with less hypoxia tolerant species (Chapter 3). I also observed a relationship between P_crit and COX activity where more hypoxia tolerant sculpins had more powerful COX, i.e. COX V_max (which is the product of k_cat and enzyme concentration) was lower but COX respiration was higher, compared to less tolerant species (Chapter 2). None of these variations in complex flux capacities appeared to be related to more efficient O2 use in isolated brain mitochondria, which would have been reflected in increased ADP/O and/or reduced ROS generation. To further investigate the role of varying the ETS complex flux capacities on mitochondrial function, it would be interesting to inhibit each of these complexes (I, II, and IV) by titrating low doses of specific inhibitors and assess the subsequent impact on phosphorylation efficiency, membrane potential, O2 kinetics and ROS.
generation. In particular, I would be interested in the role of COX, given it is well known to be present in excess capacity relative to the other ETS capacities. This excess COX capacity has been thought to be beneficial when O₂ supply varies to enhance mitochondrial P₅₀, lower the COX flux control ratio to maintain mitochondrial function under O₂ limiting conditions, and potentially alleviate oxidative damage to hyperoxic conditions (discussed in Chapter 2; Campian et al., 2007; Gnaiger et al., 1998; Suarez et al., 1996). The functional consequence of more powerful COX in more hypoxia tolerant sculpins can be investigated with titration of potassium cyanide in order to relate variation to its possible role in whole animal tolerance to environmental O₂ variability.

5.3.3 Is there a role of ROS in the adaptive response to environmental O₂ variability?

It seems counterintuitive that while hypoxia tolerant sculpins have more efficient O₂ transport to mitochondria, and also mitochondria that are more prone to higher ROS emission compared to less tolerant sculpins. Perhaps this is because maintaining aerobic metabolism during O₂ variability is more important than ROS metabolism, and having mechanisms to quickly repair oxidative damage (Chapter 4) is the strategy to life in the higher intertidal. It is possible that the increase in ROS emission plays a large part in coordinating cellular responses to O₂ variability. ROS has emerged as an important signaling molecule with specific targets (D’Autréaux and Toledano, 2007). For instance, superoxide generated from complex III is thought to stabilize transcription factor HIF-1α (Klimova and Chandel, 2008) and initiate transcription of hypoxia-protective genes. Increases in ROS can also serve as a mechanism to signal changes in cellular O₂ levels (Guzy and Schumacker, 2006). Thus, further work is needed to determine whether this increase in ROS emission plays a role in this increased hypoxia tolerance in sculpins. This could potentially be investigated by increasing dietary antioxidants (e.g. vitamin C) in sculpins and observing in vivo responses in ROS metabolism with whole animal exposure to hypoxia and hyperoxia. In vitro studies can also be performed by removing H₂O₂ that is generated by mitochondria (e.g. by providing an antioxidant in the mitochondrial suspension buffer) and observing performance following O₂ insults such as anoxia-recovery exposure (e.g. recovery of state III respiration rate and also changes in ROS emission rate).

5.4 Conclusion

The relationship between mitochondria and O₂ is not straightforward. Animals that are frequently exposed to O₂ variation face constant pressure to balance both sides of a “double-edged sword”, where on one side O₂ is essential to aerobic metabolism and maintenance of cellular energy balance.
and on the other O₂ generates potentially harmful ROS. Although we know that hypoxia tolerant animals or animals that live in O₂ variable conditions show adaptive traits to increase O₂ movement in the O₂ transport cascade and increase O₂ delivery to tissues, I have demonstrated in this thesis that this O₂ may not be used any more efficiently at mitochondria in hypoxia tolerant sculpins compared to less tolerant sculpins (from the perspective of ADP phosphorylation). In fact, this thesis shows that hypoxia tolerance in intertidal fish is associated with higher ROS generation and generally more sensitive response of ROS metabolism at both the mitochondrial and tissue level.
Figure 5.1. Relationship of the various levels of the sculpin oxygen transport cascade ($P_{\text{crit}}$, Hemoglobin ($Hb$) $P_{50}$, stripped $Hb$ $P_{50}$, mitochondrial $P_{50}$ and $COX K_{m,\text{app}}O_2$ (Mandic et al. 2009 and 2013) to whole-organism time to loss of equilibrium (LOE). The equations for the OLS regressions are $y= -0.0044x +6.03$ for $P_{\text{crit}}$ (circle), $y= -0.0078x +7.77$ for $Hb P_{50}$ (square), $y= -0.002x +1.34$ for stripped $Hb$ $P_{50}$ (triangle), $y= -0.00075x +0.079$ for mitochondrial $P_{50}$ (inverted triangle), and $y= -0.000051x +0.047$ for $COX K_{m,\text{app}}O_2$ (diamond). This figure is also Supplementary Fig 2.1.
Figure 5.2. Revised model of brain mitochondria electron transport system associated with hypoxia tolerance in sculpins. More hypoxia tolerant sculpins compared to less hypoxia tolerant sculpins showed (1) increased mitochondrial and COX O₂ binding, (2) reduced complex I and increased complex II dependency, which was not associated with any differences in phosphorylation efficiency (3) or proton leak (4). Finally, more hypoxia tolerant sculpins showed increased ROS generation and redox sensitivity compared to less hypoxia tolerant sculpins (5).
**Figure 5.3.** Revised model comparing the oxygen transport cascade between hypoxia tolerant and intolerant sculpins. Detailed description in Section 5.1.4.
Bibliography


Brown, G. C. and Borutaite, V. (2012). There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion 12, 1–4.


Munro, D., Banh, S., Sotiri, E., Tamanna, N. and Treberg, J. R. (2016). The thioredoxin and glutathione-dependent H\textsubscript{2}O\textsubscript{2} consumption pathways in muscle mitochondria: Involvement in H\textsubscript{2}O\textsubscript{2} metabolism and consequence to H\textsubscript{2}O\textsubscript{2} efflux assays. Free Radic. Biol. Med. 96, 334–346.


ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J. Exp. Med. 204, 2089–2102.


Appendix

Supplementary Figure 2.1. Relationship of the various levels of the sculpin oxygen transport cascade ($P_{\text{crit}}$, Hemoglobin (Hb) $P_{50}$, stripped Hb $P_{50}$, mitochondrial $P_{50}$ and COX $K_{m,\text{app}} \, O_2$ (Mandic et al. 2009 and 2013). The equations for the OLS regressions are $y= -0.0044x +6.03$ for $P_{\text{crit}}$ (circle), $y= -0.0078x +7.77$ for Hb $P_{50}$ (square), $y= -0.002x +1.34$ for stripped Hb $P_{50}$ (triangle), $y= -0.000075x +0.079$ for mitochondrial $P_{50}$ (inverted triangle), and $y= -0.000051x +0.047$ for COX $K_{m,\text{app}} \, O_2$ (diamond).
Supplementary Figure 2.2. Measurement of post-anoxic recovery rate of membrane potential in isolated brain mitochondria of five species of sculpins (data are means ± s.e.m.). No significant differences between means with one-way ANOVA.
**Supplementary Figure 2.3.** Phylogenetic transitions of the important amino acid residues 55 and 224 on COX3 in sculpins (shown with $P_{\text{crit}}$ (Mandic et al., 2009), mitochondrial $P_{50}$ (mito $P_{50}$), and COX $K_{m,\text{app}} O_2$ values that show that upper intertidal species have functionally different amino acid residues when compared to lower intertidal species (data are means ± s.e.m.); expanded from Figure 2.3 in the main manuscript.

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**Supplementary Table 2.1.** COX1 interspecies residue differences that show changes in amino acid functional groups (chain F= subunit 5b, chain D= subunit 4-1, chain M= subunit 8b); Species abbreviated as follows: *O. maculosus* = OLMA, *A. fenestralis* = ARFE, *A. lateralis* = ARLA, *M. polyacanthocephalus* = MYPO, *B. cirrhosus* = BLCI, *L. armatus* = LEAR
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**Supplementary Table 2.2.** COX3 interspecies residue differences that show changes in amino acid functional groups (chain J= subunit 7a, chain G= subunit 6a, chain H= subunit 6b, chain F= subunit 5b); Species abbreviated as follows: *O. maculosus*= OLMA, *A. fenestralis*= ARFE, *A. lateralis*= ARLA, *M. polyacanthocephalus*= MYPO, *B. cirrhosus*= BLCI, *L. armatus*= LEAR
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### Supplementary Table 2.3

Degenerate and specific primers designed (using GeneTool) to sequence \( \text{cox1} \) and \( \text{cox3} \) genes from six cottid species.

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Supplementary Material Figure 3.1. Brain mitochondrial respiration rate (expressed to mg mitochondrial protein) at various steps of substrate-utilization inhibitor titration (SUIT) protocol for Part I in six species of sculpins.
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<td>0.39 ± 0.068</td>
<td>1.88 ± 0.48</td>
<td>0.40 ± 0.083</td>
<td>1.58 ± 0.12</td>
<td>1.17 ± 0.11</td>
</tr>
<tr>
<td><em>S. marmoratus</em></td>
<td>0.47 ± 0.069</td>
<td>2.29 ± 0.34</td>
<td>0.39 ± 0.052</td>
<td>1.69 ± 0.19</td>
<td>1.51 ± 0.31</td>
</tr>
</tbody>
</table>

**Supplementary Table 3.1.** ROS/mg protein values from Experiment Part II in Figure 3.2.
### Supplementary Table 3.2

ROS/mg protein values from Experiment Part IV in Figure 3.4.

<table>
<thead>
<tr>
<th>Species</th>
<th>State III</th>
<th>State IV</th>
<th>Rot</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. maculosus</em></td>
<td>0.99 ± 0.29</td>
<td>0.56 ± 0.08</td>
<td>0.77 ± 0.12</td>
</tr>
<tr>
<td><em>A. lateralis</em></td>
<td>0.89 ± 0.18</td>
<td>0.67 ± 0.12</td>
<td>0.64 ± 0.13</td>
</tr>
<tr>
<td><em>S. marmoratus</em></td>
<td>1.02 ± 0.18</td>
<td>0.89 ± 0.34</td>
<td>0.67 ± 0.16</td>
</tr>
</tbody>
</table>
Supplementary Figure 3.2. Relationship between mitochondrial pellet GSH:GSSG and ROS/mg protein data in *O. maculosus* (species 1; squares and solid line) and *S. marmoratus* (species 5; circles and dotted line) from Experiment Part III in Figure 3.3A.
Supplementary Figure 4.1. MitoB (top; $R^2 = 1.00$) and MitoP (bottom; $R^2 = 0.99$) standard curves
Supplementary Figure 4.2. Normoxic timecourse over 72hrs for MitoP (top), MitoB (middle), and MitoP/MitoB (bottom) to determine excretion rates; Gill in circle, brain in square, liver in triangle, and muscle in hollow square.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Oligocottus maculosus</th>
<th>Scorpaenichthys marmoratus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td><strong>TBARS</strong></td>
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<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.31 ± 0.020</td>
<td>0.68 ± 0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>0.24 ± 0.019</td>
<td>0.38 ± 0.045</td>
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<tr>
<td>Gill</td>
<td>1.017 ± 0.26</td>
<td>0.80 ± 0.081</td>
</tr>
<tr>
<td><strong>TOSC</strong></td>
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<tr>
<td>Brain</td>
<td>1.21 ± 0.046</td>
<td>2.02 ± 0.57</td>
</tr>
<tr>
<td>Liver</td>
<td>8.83 ± 0.94</td>
<td>3.12 ± 0.94</td>
</tr>
<tr>
<td>Gill</td>
<td>2.23 ± 0.23</td>
<td>2.10 ± 0.16</td>
</tr>
</tbody>
</table>

Supplementary Table 4.1. TBARS (in µM) and TOSC levels (in units of catalase activity) to 3.5kPa hypoxia-recovery and 64.0kPa hyperoxia-recovery