The Effects of Metabolic Depression Induced by Food Deprivation on Hypoxia Tolerance of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

by

Scott T.D. MacIntyre

A thesis submitted to the Department of Biology in conformity with the requirements for the degree of Master of Science

Queen’s University

Kingston, Ontario, Canada

(September, 2011)

Copyright © Scott T.D. MacIntyre, 2011
Abstract

Hypoxic condition is a naturally occurring environmental stressor in aquatic ecosystems. However, due to modern anthropocentric activities, hypoxia has been increasing in prevalence and severity. Rainbow trout, a keystone species in many North American lakes, is hypoxia intolerant. As a result, this species is of particular concern when studying the effects of hypoxia on an organism’s physiological functioning. Chronic starvation was used as a tool to induce metabolic depression to determine the effect that depressed metabolic rate had on hypoxia tolerance. Juvenile rainbow trout were deprived of food for five weeks at 15°C. Each week, routine metabolic rate (RMR) and critical oxygen tension ($P_{crit}$) were measured. Concomitantly, resting and post-hypoxia fish (8 h at ~50% air saturation) were sampled to measure metabolites in blood, liver and muscle, as well as enzyme activities in select tissues. Food deprivation resulted in a decrease in routine metabolic rate (RMR) and shift towards an increased reliance on aerobic metabolism. $P_{crit}$ decreased significantly following four weeks of food deprivation respectively, indicating that metabolic depression induced by food deprivation may confer an increased tolerance to low environmental oxygen concentration ([O$_2$]). However, marginal metabolic scope (MMS), another indicator of hypoxia tolerance, did not change in response to metabolic depression. Furthermore, subjecting trout to O$_2$ limitation resulted in mobilization of carbohydrates from the liver subsequently leading to hyperglycemia. This was likely a survival technique ensuring that if severe hypoxia ensues, anaerobic substrates are ready for transport to the necessary tissues.
Co-Authorship

Dr. Yuxiang Wang contributed to the experimental design, data interpretation, writing and editing of all chapters. Dr. Yiping Luo assisted with experimental design and setup, as well as, data collection and analysis.
Acknowledgements

First and foremost, I would like to thank my supervisor, Yuxiang Wang, for his guidance and assistance, as well as his patience and encouragement throughout the last 3 years. This opportunity to learn has broadened the scope of my scientific understanding and challenged me to grow in ways I would have never imagined. Further, I would like to thank my committee members, Chris Moyes and Peter Hodson, for their help and constructive criticisms.

A very special thanks goes out to Dan Lim, Yiping Luo, Sarah Wyness, and Rhiannon Davies who made it possible for me to continue with my studies following my ankle/leg surgery last September. Their continued support enabled me to complete this project. I would also like to thank all of the wonderful friends I have made in both the Wang and Moyes Lab who assisted me immensely throughout the course of my undergrad thesis and Masters project. These are friendships I will cherish for my life.

Finally, I would like to think my family (Mom, Dad, Emily) for their unconditional love, support and encouragement.
# Table of Contents

Abstract ......................................................................................................................... ii  
Co-Authorship................................................................................................................ iii  
Acknowledgements......................................................................................................... iv  
Table of Contents........................................................................................................... v  
List of Figures ................................................................................................................ vii  
List of Tables .................................................................................................................. viii  
List of Abbreviations ..................................................................................................... ix  
Chapter 1 Introduction & Literature Review ................................................................ 1  
  1.1 Metabolism............................................................................................................. 1  
  1.2 Metabolic Depression............................................................................................ 2  
  1.3 Physiological Responses to Food Deprivation.................................................... 4  
  1.4 Hypoxia................................................................................................................... 7  
    1.4.1 Functional Hypoxia.......................................................................................... 7  
    1.4.2 Environmental Hypoxia.................................................................................. 8  
  1.5 Physiological Responses to Hypoxia.................................................................... 9  
  1.6 Rainbow Trout as the Model Organism............................................................... 12  
  1.7 Research Questions and Hypothesis...................................................................... 14  
Chapter 2 The Effects of Metabolic Depression Induced by Food Deprivation on Hypoxia Tolerance of Juvenile Rainbow Trout (Oncorhynchus mykiss) ......................................................................... 17  
  2.1 Introduction............................................................................................................ 17  
  2.2 Methods................................................................................................................ 19  
    2.2.1 Animals.......................................................................................................... 19  
    2.2.2 Live Animal Experimental Protocol............................................................. 19  
    2.2.3 Analytical Techniques .................................................................................. 22  
    2.2.4 Intracellular pH Analysis.............................................................................. 24  
    2.2.5 Calculations and Statistical Analysis............................................................ 24  
  2.3 Results.................................................................................................................... 26  
    2.3.1 Animal Mortalities....................................................................................... 26  
    2.3.2 Metabolic Oxygen Consumption................................................................... 26  
    2.3.3 Critical Oxygen Tensions.............................................................................. 26  
    2.3.4 Enzyme Activities....................................................................................... 27  
    2.3.5 Enzyme Activity Ratios............................................................................... 28
2.3.6 Alterations in Metabolite Concentrations in Response to Food Deprivation.........29
2.3.7 Alterations in Metabolite Concentrations in Response to Food Deprivation and Hypoxia................................................................................................................30
2.4 Discussion........................................................................................................31
2.4.1 Food Deprivation and Metabolism........................................................................................................31
2.4.2 White Muscle Physiological State Under Food Deprivation.................................32
2.4.3 Liver Physiological State Under Food Deprivation................................................39
2.4.4 Metabolic Depression and Hypoxic Response.........................................................41
2.4.5 Metabolic Depression and Physiological Response to O₂ limitation.....................44
2.4.6 Conclusions........................................................................................................47
Chapter 3 General Discussion........................................................................59
3.1 Overview............................................................................................................59
3.2 Physiological Responses of the White Muscle to Food Deprivation.......................60
3.3 Physiological Responses of the Liver to Food Deprivation.....................................62
3.4 Metabolic Depression Induced by Food Deprivation and Oxygen Consumption.......62
3.5 Physiological Responses of the White Muscle to Hypoxia.....................................64
3.6 Physiological Responses of the Liver to Hypoxia...................................................64
3.7 Conclusions.......................................................................................................65
References............................................................................................................66
List of Figures

Figure 1. Metabolic oxygen consumption patterns in response to food deprivation in *O. mykiss* (page 16)

Figure 2. Enzyme activities in the white muscle of *O. mykiss* in response to food deprivation (page 48)

Figure 3. Relative enzymatic ratios of the white muscle of *O. mykiss* in response to food deprivation (page 49)

Figure 4. Enzyme activities in the liver of *O. mykiss* in response to food deprivation (page 50)

Figure 5. Relative enzymatic ratios of the liver of *O. mykiss* in response to food deprivation (page 51)

Figure 6. White muscle and liver ATP concentrations of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 52)

Figure 7. White muscle glucose and glycogen concentrations of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 53)

Figure 8. White muscle pyruvate and lactate concentrations of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 54)

Figure 9. Redox status and intracellular cytosolic pH in the white muscle of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 55)

Figure 10. Plasma glucose and lactate concentrations of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 56)

Figure 11. Liver glucose, glycogen and lactate concentrations of *O. mykiss* in response to food deprivation and food deprivation + hypoxia (page 57)
List of Tables

Table 1. Metabolic oxygen consumption patterns in response to food deprivation in *Oncorhynchus mykiss* (page 48)
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine-5'-triphosphate synthase</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental protection agency</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glu</td>
<td>Glucose</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycogen</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H^+</td>
<td>Protons</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HOAD</td>
<td>3-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>ILCM</td>
<td>Inter-lamellar cellular mass</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>KF</td>
<td>Potassium fluoride</td>
</tr>
<tr>
<td>Lac</td>
<td>Lactate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MMR</td>
<td>Maximal metabolic rate</td>
</tr>
<tr>
<td>MMS</td>
<td>Marginal metabolic scope</td>
</tr>
<tr>
<td>( \dot{MO}_2 )</td>
<td>Metabolic oxygen consumption</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS-222</td>
<td>Tricaine methanesulfonate</td>
</tr>
<tr>
<td>Na^+</td>
<td>Sodium</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>Na_2NTA</td>
<td>Nitrilotriacetic acid disodium salt</td>
</tr>
<tr>
<td>O_2^-</td>
<td>Oxygen</td>
</tr>
<tr>
<td>P_{AMET}</td>
<td>Critical oxygen pressure where there is an alteration in routine O_2 consumption</td>
</tr>
<tr>
<td>P_{crit}</td>
<td>Critical oxygen pressure</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
</tbody>
</table>
PCr- Phosphocreatine
pHₐ-_ intracellular pH
PK- Pyruvate kinase
PO₂-_ partial pressure of oxygen
Pyr-_ Pyruvate
RMR-_ Routine metabolic rate
ROS-_ Reactive oxygen species
SMR-_ Standard metabolic rate
TEA-HCl-_ Triethylamine hydrochloride
UN-_ United Nations
[]-_ Concentration
Chapter 1
Introduction and Literature Review

1.1 Metabolism

The chemical processes that occur within a living organism, enabling them to grow and reproduce, maintain and regenerate biological structures, and interact with their environment, are collectively referred to as metabolism. Despite life’s amazing diversity and complexity, these processes have been highly conserved throughout evolutionary time, and virtually all life utilizes similar strategies for energy production. Metabolic processes use relatively few components; electron and hydrogen carriers, membranes to separate charges, and enzymes to catalyze redox reactions and convert membrane potentials into useful chemical energy (Nealson and Rye, 2003). Physiologists and biochemists have long been fascinated by the observation that slight variations in these components and processes allow organisms to respond and adapt to a diversity of environmental conditions. As a result, there has been a proliferation of published literature detailing the intricacies of metabolic flux in response to a variety of environmental changes, across broad phylogenetic groups.

Fry (1971) classified three levels of metabolism, standard metabolic rate (SMR), routine metabolic rate (RMR), and maximal active metabolic rate (MMR). SMR is defined as the minimum metabolic rate in the normoxic, normothermic, postabsorptive state, observed in an intact, resting, unstressed, adult animal. Due to the difficulties with satisfying the assumptions for determining SMR under laboratory settings, physiologists typically employ RMR as the basal measurement of metabolism. RMR is defined as the metabolic rate observed in an organism whose movements are restricted under laboratory conditions, but whose metabolic rate is subjected to variations largely due to uncontrollable random activity. In contrast, MMR is the
maximum sustainable ATP turnover for an active organism, and the metabolic spectrum that exists between SMR and MMR is referred to as the metabolic scope of the animal.

Under most metabolic states, ATP utilization is closely matched to production, however, numerous environmental stressors such as, temperature fluctuations, hypoxia/anoxia, hyper-salinity, and food deprivation, can cause perturbations to cellular energy homeostasis (Richards, 2010). Furthermore, these variations in environmental conditions can occur on predictable, temporal or spatial scales, as well as being irregular or unpredictable, making the maintenance of cellular energy balance a difficult physiological challenge (Richards, 2010). Ultimately, if these conditions persist, ATP demand can rise drastically, or ATP production becomes increasingly challenging, both leading to an energetic deficit. However, even under energetically stressful conditions, ATP supply and demand must remain in equilibrium (Staples and Buck, 2009), which is commonly achieved through the up and down regulation of various metabolic components.

1.2 Metabolic Depression

A reduction in metabolic rate below SMR is referred to as metabolic depression, and has been proposed as a fundamental mechanism to ensure survival during adverse conditions. Metabolic depression has received considerable attention in the scientific literature (See Guppy and Withers, 1999; Hochachka and Lutz, 2001; and Richards, 2010 for reviews) and has been documented in virtually all-animal taxa. In contrast to the organism’s metabolic scope, metabolic rate suppression is commonly thought of as the metabolic scope of survival. This marked decrease in metabolism is achieved through a coordinated, hierarchical decrease in ATP consuming pathways, to minimize the ATP demand during energetically stressful conditions. Reductions in metabolic rate can occur within 20-30 minutes following energetic stress (Ginneken and Thillart, 2009) and can last for periods of hours to days, to even seasons and years
in extreme cases (Richards, 2010). The extent of reduction has been observed to be flexibly
dependent and related to the severity and time course of the stressor. Typically in vertebrates,
metabolic rate is depressed to approximately 5-20% of basal rates; however, reductions of up to
85% have been reported in many species of mammals, anurans, and fish (Guppy and Withers,
1999; Richards, 2010).

Metabolic rate is depressed via a variety of behavioral, physiological and biochemical
rearrangements. Initial responses include reductions in muscular activity, reproductive and
courtship behaviors, and feeding. While quantifying metabolic savings associated with
alterations in behavioral activity has received little attention, it seems plausible that reductions in
activities such as swimming could translate into significant energetic savings. Nilsson et al.
(1993) observed that the crucian carp, an anoxia-tolerant species, responded to anoxia by rapidly
decreasing their locomotor activity from 1.82 m min\(^{-1}\) during normoxia, to 0.82 m min\(^{-1}\). This was
estimated to translate into a 35-40% reduction in muscle energy consumption, primarily due to a
reduction in the activity of actin-myosin ATPase and Ca\(^{2+}\) ATPase. In addition, reducing skeletal
energy demands enables the organisms to shift metabolic resources from muscle to other organ
systems that are more crucial to survival (Nilsson et al. 1993).

As the severity of stress increases, or the time exposed to environmental stress persists,
physiological reductions in gonad growth and development, growth and repair, digestive
functioning, and cardio-respiratory functioning occur. These reductions in physiological
functioning are accompanied by a marked decrease in heart rate and blood flow (Coulson et al.,
1977; Ginneken and Thillart, 2009). Blood flow is crucial for the transport of oxygen, metabolic
fuels, hormones, and waste products. As a result, it has been suggested that a reduction in blood
flow may be a proximate cause of metabolic depression, and may be a dominant control of
metabolic rate (Coulson et al., 1977). While the energy savings associated with these reductions
have not been quantified to a great extent in the literature, it seems likely that the down-regulation of these systems would translate into significant metabolic savings.

During more severe or chronic stressors, ATP consuming biochemical pathways are shut down in a hierarchical coordinated fashion. The biochemical underpinnings of metabolic depression have been studied to the greatest extent. It has been observed that the cellular mechanisms involved in metabolic depression are largely conserved across wide phylogenetic groups (Hochachka et al., 1996). Reductions in cellular metabolism are largely achieved through reductions in ion pumping (channel arrest), $\text{Na}^+/$$\text{K}^+$ ATPase and $\text{Ca}^{2+}$ ATPases, macromolecule synthesis (protein, urea, mRNA), and pH; increases in latent mRNA; and changes in protein phosphorylation state (Guppy and Withers, 1999; Hochachka et al., 1996; Hochachka and Lutz, 2001; Staples and Buck, 2009; Richards, 2010). However, the combination and extent in which energetic stressors affect ATP consuming processes is largely dependent on the specific environmental condition, and not all conditions will elicit the same behavioral, physiological, and biochemical responses.

1.3 Physiological Responses to Food Deprivation

Due to the spatial and temporal distribution of food in nature, food deprivation is a common environmental stressor for many organisms. When food supplies are scarce, animals must rely upon endogenous metabolic resources to fulfill energetic requirements and maintain ATP homeostasis, which can commonly lead to a reduction in swimming ability and stamina (Czesny et al., 2003). Fish have evolved a diversity of different strategies to cope with periods of food limitation, and the particular physiological pattern employed by fish is largely dependent on life history. Furthermore, responses can be significantly influenced by other environmental
factors such as temperature, salinity, season, and sex, and as a result, the response to food-deprivation is highly variable, even within particular species (Sheridan and Mommsen, 1991).

Tolerant species typically employ a temporal and spatial hierarchy in metabolite mobilization, and selectively reduce metabolic processes. While the intricacies of individual species starvation coping strategies may alter, one commonality can be observed across multiple species. During starvation there is a change in the ratio of contribution of aerobic to anaerobic processes to energy production. A common starvation response in many vertebrates is to decrease oxygen consumption while increasing the importance and efficiency of oxidative processes and decreasing the relative contribution of anaerobic processes to energy production. This leads to the accumulation of reactive oxygen species (ROS) and, as a result, an increase in oxidative stress (Bayir et al., 2011). Oxidative stress has been observed to damage DNA, proteins, steroid components, as well as unsaturated lipids in the cell membranes. To avoid these physiological perturbations, antioxidant defenses increase during prolonged starvation and these metabolic alterations persist for even three weeks following the reintroduction of food (Bayir et al., 2011).

Alternate patterns of nutrient storage and mobilization enable some fish species to tolerate prolonged periods of food deprivation and maintain limited activity, while other species are highly intolerant of starvation (Sheridan and Mommsen, 1991). During acute phases of food deprivation, most fish species will mobilize liver and/or muscle glycogen, however, the extent to which glycogen is depleted ranges. For example, species such as the snakehead (Ophiocephalus maculatus) generally employ complete glycogenolysis (Woo and Cheung, 1980), whereas salmonids will partially or almost completely protect endogenous carbohydrates, while mobilizing lipids (Jezierska et al., 1982; Sheridan and Mommsen, 1991; Raaij et al., 1996). The American eel (Anguilla anguilla) will conserve glycogen, relying heavily on protein and/or lipid catabolism to maintain metabolic energy equilibrium (Larsson and Lewander, 1973). Despite an initial depletion of ~ 50% of the endogenous muscle glycogen, many species tend to conserve
white muscle burst energy reserves, in particular high-energy phosphates (ATP and PCr), which are necessary for activities such as predator prey interactions (Beaulieu and Guderley, 1998; Kieffer and Tufts, 1998). The specific physiological changes that enable organisms to tolerate periods of chronic food-deprivation are closely linked to metabolism and patterns of energy storage and mobilization (Woo and Cheung, 1980). As a result, the diversity in metabolic strategies of selectively sparing different metabolites represents a survival advantage adapted to the particular ecological niche that species holds.

As food deprivation is prolonged it begins to have a more significant affect on organ systems. The white muscle is one of the first tissues to respond to food deprivation, experiencing a reduction in routine metabolic activities (Loughna and Goldspink, 1984; Beauliey and Guderley, 1998; Guderley et al., 2003; Tripathi and Verma, 2003). In contrast to the white muscle, red muscle metabolic integrity is maintained for much longer following the cessation of feeding (Loughna and Goldspink, 1984). In many teleosts the white muscle makes up the majority of the total body mass, is primarily glycolytic and maintains much higher rates of protein turnover relative to other tissues (Loughna and Goldspink, 1984). The white muscle plays a dual role during periods of food deprivation, as it must maintain structural and functional integrity, while also acting as a fuel reserve (Guderley et al., 2003). As the fish enters a catabolic state, muscle protein becomes an increasingly important metabolic fuel. The breakdown of white muscle macromolecules, leads to an increase in intracellular solutes, and the subsequent accumulation of water, which assists in maintaining muscle structure (Guderley et al., 2003). One of the most important elements of surviving periods of starvation, however, is the ability to return to normal resting conditions following the reintroduction of food. Therefore, the extent to which muscle proteins can be catabolized is under strict regulatory control (Beaulieu and Guderley, 1998). To maintain ATP equilibrium under energetically stressful situations the rate of protein turnover in the white muscle decreases, and the tissue enters a metabolically depressed state (Loughna and Goldspink, 1984).
1.4 Hypoxia

Hypoxia, defined as low concentrations of oxygen, has been a topic of much interest in the fields of comparative and environmental physiology, as well as biomedical research and biochemistry. Hypoxia can be divided into two general categories, functional and environmental. Functional hypoxia is a condition in which a particular tissue or tissues is deprived of adequate oxygen supply. In comparison, environmental hypoxia is a phenomenon that occurs when oxygen concentrations within a particular environment or microenvironment become limiting to the organisms inhabiting that ecosystem.

1.4.1 Functional Hypoxia

A common physiological condition that leads to functional hypoxia is exhaustive exercise. During high intensity exercise, energetic demands of the white muscle exceed its oxidative capacity, largely due to the intrinsic limitations of oxygen transport to the muscle, leading to perturbations in ATP equilibrium. In response, animals rely on anaerobic glycolysis to make up the energetic deficit. However, anaerobic processes are less efficient and are ultimately limited by endogenous anaerobic substrates. If high intensity exercise is prolonged, it leads to exhaustion (Wang et al., 1994).

Medically, functional hypoxia has important implications during organ transplants, strokes, as well as cancer research. During a stroke, blood transport to the brain is reduced or cut off, causing ischemia. The brain is an organ particularly sensitive to hypoxia and the ROS accumulation that follows reperfusion (Piantadosi and Zhang, 1996). Further, cancer is a mass of rapidly proliferating tumour cells that develops more quickly than the vascularized structures can sustain, resulting in deficiencies in oxygen transport to the cells (Brahimi-Horn et al., 2007). Such conditions trigger a molecular response, up-regulating the hypoxia-inducible transcription factor (HIF), which coordinates a large panel of genes exploited by tumour cells for survival. As
a result, HIF has been acknowledged as a major contributor to cancer progression and treatment failure, and is receiving more attention in cancer research (Semenza, 2003; Brahimi-Horn et al., 2007). Therefore, gaining insight into the cellular mechanisms underlying hypoxic responses could assist in developing treatments for these medical conditions.

1.4.2 Environmental Hypoxia

Since the appearance of molecular oxygen roughly 2.2 billion years ago, the Earth has experienced considerable fluxes in environmental oxygen concentrations. Oxygen levels can change in response to seasons, tides, altitude, and ice-cover, and have become common occurrences for many terrestrial and aquatic organisms. In regards to terrestrial species, birds, such as the bar-headed goose (Anser indicus), can climb to altitudes of 9000 meters, and have evolved metabolic mechanisms that enable them to cope with oxygen limitations associated with these heights. Adaptations in gas exchange efficiencies across the respiratory surface, oxygen-binding affinities of hemoglobin, strict regulations in cardiac output and regional oxygen delivery, as well as, many adaptations at the level of individual tissues, enable these animals to maintain energy balance during periods of hypoxia (Faraci, 1991).

Environmental hypoxia, however, is much more common in aquatic ecosystems where dissolved oxygen (DO) concentrations are in constant flux. Water contains 1/30th the amount of oxygen and diffuses at a rate 1/10 000th relative to air. Further, oxygen concentrations decrease with increasing salinity and temperature and can fluctuate on a diurnal and seasonal basis. Therefore, hypoxia occurs intermittently throughout aquatic environments, commonly encompassing 20-50% of the water column. However, depending on water depth and the presence of pycnoclines, hypoxic environments can be present in anywhere from 10-80% of the entire water column.
Nitrogen and phosphorus runoff into aquatic ecosystems, largely from human activities such as industrialized farming, and sewage treatment has led to massive increases in the abundance of phytoplankton. The Millennium Ecosystem Assessment (2005) found that human activities have doubled nitrogen and tripled phosphorus release into the environment in comparison to natural levels. While phytoplankton, through the process of photosynthesis, produces oxygen during the day, at night these plants consume oxygen during cellular respiration. In addition, as the algae die and sinks to the water’s bottom, it is broken down by bacteria, a process that further reduces DO. As a result, according to the United Nations, roughly 50% of the Earth’s freshwater ecosystems are considered to be eutrophic, and the World Resource Institute has identified 375 hypoxic marine coastal zones (UN environment program, 1988). Thus, in light of the current species extinction rate, and the rapid transformation of ecosystems, understanding physiological responses to hypoxia in a diversity of species is necessary when attempting to set guidelines for conservation and policy.

1.5 Physiological Responses to Hypoxia

Due to the common occurrence of oxygen limitations in aquatic environments, many species have evolved a diversity of unique behavioral, physiological and biochemical adaptations to cope with periods of oxygen limitation. Species such as the arapaima (Arapaima gigas), which inhabits the freshwaters of the Amazon, have evolved air-breathing organs, enabling them to escape hypoxic environments (Brauner et al., 2004). Other species will avoid hypoxia by skimming the upper surface of the water where rapid diffusion raises oxygen concentrations above the rest of the water column (Kramer and McClure, 1982). However, most aquatic organisms do not have the option of escape. For these species, the major metabolic challenge is the decrease in the capacity for ATP production via oxidative phosphorylation, leaving animals to
rly on anaerobic glycolysis and/or fermentation to maintain energy equilibrium (referred to as the Pasteur effect) (Boutilier, 2001). This, however, is substrate limited, and thus sets temporal constraints on survival, making it a temporary solution at best (Boutilier et al., 1987).

Increasing ventilatory rate and lamella perfusion, to extract more oxygen from the surrounding environment, is another hypoxia-survival technique. This strategy includes a whole host of processes aimed at increasing oxygen extraction and delivery to critical tissues while maintaining or increasing metabolic rate (Boutilier et al. 1987). The gill, however, is a multi-function organ, and while the gill has exceptional gas exchange capabilities, the characteristics that give these qualities do not come without tradeoffs (Evans et al., 2005). Enhancing gas exchange exacerbates water and ion fluxes that occur due to gradients between the fish’s extracellular fluids and the external environment, a concept referred to as, the osmo-respiratory comprise (Evans et al., 2005). Therefore, increasing lamellar perfusion and ventilatory rate concomitantly increases the energetic demands of maintaining osmotic and ionic balance (Matey et al., 2011). This increase in energy demand at the gill is partially augmented by the release of stress-related hormones, such as, catecholamine, adrenaline and epinephrine, which play a role in increasing oxygen uptake across the respiratory surface (Boutilier et al., 1988). These hormones stimulate beta-receptors, which have been observed to increase water permeability at the fish gill, in addition to its diffusional conductance (Perry et al., 1985). Further, catecholamines cause plasma acidosis, which increases the hemoglobin-O₂ affinity, in turn favoring oxygen loading at the respiratory surface (Perry et al., 1985).

Some hypoxia tolerant species have evolved intriguing adaptations to overcome the problem of oxygen extraction in oxygen-limited environments. Many carp species, such as the crucian carp (Carassius carassius) and goldfish (Carassius auratus) inhabit lakes and ponds that commonly experience hypoxia and/or anoxia. Under normoxic, normothermic conditions these species appear to lack protruding lamella, a trait that leads to a very small respiratory surface.
Under hypoxic conditions, however, there is a large reduction in the inter lamella cellular mass (ILCM), which is achieved through an increase apoptosis, in concert with a reduction in cellular proliferation (SolIid et al., 2003). This leads to an exposed surface for gas exchange that is ~7.5 larger relative to the normoxic condition, significantly increasing the capacity for oxygen uptake at low oxygen levels (SolIid et al., 2003). However, similarly to increasing the rate of water flow over the gills, as well as increasing lamella perfusion, it seems likely that this would also perturb the maintenance of osmolyte and ion balance (Mitrovic et al., 2009). Surprisingly, however, despite an increase in functional surface area, hypoxia-tolerant species, such as the Amazonian oscar (*Astronotus ocellatus*), are able to limit ion loss over the respiratory surface. This is achieved largely through a hypoxia-mediated decrease in paracellular permeability, a processes that involves the migration and shrinking of ion transporters, referred to as ionocytes. In addition, partially covering these ion-exchanging cells with pavement cells and increasing mucous production at the gill surface further reduces ionocyte exposure to the external environment (Matey et al., 2011). These responses, in turn, reduce the ATP-consuming processes involved in ion pumping across the gill membrane during exposure to hypoxia (Mitrovic et al., 2009).

However, organisms are required to rely on endogenous energy reserves during chronic bouts of hypoxia, and maintaining metabolic rate would seemingly lead to some degree of substrate limitation, and therefore set a temporal constraint on the individual’s survival (Boutilier et al., 1988).

The other potential strategy, and perhaps the most effective, is to minimize the hypoxic insult by conforming to environmental oxygen concentrations through a significant reduction in metabolic rate (Boutilier et al., 1988). Depressing metabolic rate during hypoxia is a key mechanism for the conservation of endogenous substrates thereby extending the amount of time that can be spent under oxygen limiting conditions. This process, however, requires coordinated metabolic reorganization aimed at decreasing ATP demands to match the capacity for production
(Jibb and Richards, 2008). The point at which the organism is required to switch to a metabolically depressed state has received considerable attention in the literature. Recently, it has been observed that AMP-activated protein kinase (AMPK) plays a significant role in this process. Jibb and Richards (2008) found that in the goldfish liver, AMPK activity increases five-fold within 30 minutes of hypoxic exposure, and was temporally associated with an eleven-fold increase in [AMP\textsubscript{free}]/[ATP] (Jibb and Richards, 2008). This disruption in cellular energy status appeared to be critical to the activation of AMPK. An increase in AMPK activity inhibits anabolic processes, increases carbohydrate transportation at the cellular level, and increases the capacity for anaerobic energy production (Jibb and Richards, 2010). As a result, AMPK has been suggested as the cellular switch coordinating the down-regulation of metabolic processes, enabling organisms to maintain energy balance during energetically stressful conditions, increasing their scope of survival (Jibb and Richards, 2008).

1.6 Rainbow Trout as the Model Organism

Rainbow trout are a member of the Salmonidae family, native to the Pacific coast of North America and Russia, and have been widely introduced to many cold water regions of the world. Rainbow trout have become one of the most extensively studied fish as natural populations are available and easily obtained, and are easy to hold, culture and manipulate under laboratory conditions (Thorgaard et al., 2002). Furthermore, extensive cultivation as a food and sport fish has led to the accumulation of a large amount of basic knowledge on the species (Thorgaard et al., 2002).

Rainbow trout are closely related to species within the Oncorhynchus, Salmo, and Salvelinus genera making them an ideal surrogate for research needed on economically and ecologically important species, such as the Atlantic and Pacific salmon species, which has led to
an extensive characterization of their genome (Thorgaard et al., 2002). The ability of rainbow trout to undergo extensive bursts of exercise, coupled with their innate capacity for sustained swimming has made them an attractive organism for researchers interested in studying exercise physiology (See Kieffer, 2000 for review). In addition, rainbow trout are a keystone species in many North American lakes and an important bio-indicator of ecosystem health. The environmental protection agency (EPA) currently uses this species as a regulatory indicator for acute freshwater toxicity. The extensive use of rainbow trout as a model organism has resulted in a detailed understanding of their genetics, physiology, and ecology, which has been applied to a wide variety of research fields including carcinogenesis, toxicology, comparative immunology, disease ecology, physiology, and nutrition (Thorgaard et al., 2002).

For the present study, rainbow trout were an attractive model for a couple of reasons. First, the hypoxic-response of rainbow trout has been well characterized. Rainbow trout respond to hypoxia by attempting to maintain metabolic rate via hyperventilation, tachycardia, increasing hemoglobin’s affinity to oxygen, and redistributing of blood flow (Dunn and Hochachka, 1982; Boutillier et al., 1988; Omlin and Weber, 2010; Barnes et al., 2011). To produce ATP in the absence of an adequate oxygen supply, flux through anaerobic glycolysis increases. This response, however, sets temporal constraints on survival, as it leads to the exhaustion of endogenous carbohydrates and the accumulation of metabolic end products. As a result, rainbow trout are thought to be highly intolerant of hypoxia, and oxygen begins to become physiologically limiting at ~6mg dO₂ L⁻¹ (Davis, 1975). Therefore, rainbow trout are seemingly of heightened concern when considering the increased prevalence and severity of aquatic hypoxic regions.

This study aimed to determine if a hypoxia-intolerant species could be made more tolerant via a reduction in metabolic rate induced by food deprivation, which included two important considerations. First, rainbow trout have been observed previously to enter a metabolically depressed state in response to food deprivation (Salem et al., 2007), instilling
confidence that the organism’s metabolic rate could be reduced via a food deprivation protocol. The second, and perhaps more important consideration, is that rainbow trout are quite sensitive to environmental hypoxia. When trying to increase tolerance to environmental hypoxia, using a species that is tolerant to low-oxygen levels may pose difficulties in detecting further increases in hypoxia tolerance. This is because many of these species can withstand periods of anoxia, making it difficult to quantify further tolerance to hypoxia. Therefore, using an organism whose metabolic rate is flexibly responsive to food deprivation, in addition to being a relatively hypoxia-intolerant species, was necessary to answering our specific research questions.

1.7 Research Questions and Hypotheses

The specific research questions of this study were three-fold. The first aim was to observe the effect of chronic food deprivation on routine metabolic rate. To determine this, oxygen consumption in concert with key glycolytic and oxidative enzymes and/or metabolites in the white muscle, liver, and plasma were measured. Secondly, if chronic food deprivation causes a significant reduction in metabolic rate, how would this metabolic depression influence the trout’s ability to cope with hypoxia, as indicated by an alteration in $P_{crit}$. The final aim was to determine what physiological strategy would be employed to cope with moderate hypoxia under a metabolically depressed state. To answer these questions metabolites involved in glycolysis in both the liver and white muscle were measured, in addition to white muscle pH, and redox status, and plasma glucose and lactate.

Based on previous findings the metabolic response of rainbow trout’s to food deprivation (Aslop and Wood, 1997), I speculate that chronic food deprivation, will significantly decrease routine oxygen consumption and there will be a metabolic shift to increasing the efficiency of aerobic processes. I expect this to be exemplified by an increase in the contribution of aerobic
enzyme activities citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) relative to the anaerobic enzyme activities, pyruvate kinase (PK) and lactate dehydrogenase (LDH) (See Figure 1).

The conventional wisdom is that as metabolic rate decreases the environmental oxygen concentration limiting metabolic processes decreases concurrently. Routley et al. (2002) observed that the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*) enters a phase of metabolic and ventilatory depression, and that pre-conditioning the shark to hypoxia and subsequently lowering metabolic rate increased acute hypoxia tolerance. Rainbow trout’s physiological response to hypoxia is designed to maintain metabolic rate, a strategy that infers poor tolerance. Therefore, decreasing the trout’s metabolic rate prior to hypoxic exposure should increase tolerance to low oxygen levels.

Food deprivation is speculated to decrease metabolic rate, largely through a reduction in carbohydrate metabolism. Subjecting juvenile trout to a moderate bout of oxygen limitation should have an opposing effect resulting in the mobilization of anaerobic substrates. More specifically liver carbohydrate synthesis is expected to increase, which may result in hyperglycemia. Due to oxygen limitation, it is proposed that the mitochondrial oxidative phosphorylation will not be able to meet ATP demand. Therefore, food deprivation in concert with O₂ limitation should result in an increase in carbohydrate metabolism, reflected by an increase in glucose and lactate in the blood plasma, and a subsequent decrease in white muscle and liver glucose and glycogen. Further, due to an increase in anaerobic energy production we expect to observe the onset of acidosis and a reduction in redox state in the white muscle.
Figure 1 Schematic of biochemical pathways assayed in this experiment. Pyruvate kinase (PK) is involved in both aerobic and anaerobic glycolysis. Lactate dehydrogenase (LDH) is involved primarily in anaerobic glycolysis. Citrate synthase (CS) controls entrance into the citric acid cycle. 3-hydroxyacyl-CoA dehydrogenase is involved in amino acid and fatty acid catabolism. Bold indicates that the metabolites or enzymes were measured in this experiment.
Chapter 2

The Effects of Metabolic Depression Induced by Food Deprivation on Hypoxia Tolerance of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

2.1 Introduction

Food-deprivation and hypoxia are common occurrences in aquatic ecosystems, and fish may have to cope with these environmental stressors simultaneously. Hypoxia is a common natural phenomenon and occurs due to changes in photoperiod and temperature, or from haloclines and thermoclines. However, hypoxia is becoming more commonly associated with eutrophication, as a result of anthropogenic inputs of nitrogen and phosphorus into the environment (Pollock *et al.*, 2007). Hypoxia is considered to be one of the most pressing concerns in regards to aquatic ecosystem health (Goldberg, 1995), and has caused mass fish kills and local extirpation as well as alterations in population dynamics leading to ecological disruptions (Pollack *et al.*, 2007).

Rainbow trout (*Oncorhynchus mykiss*) sustain a high routine metabolic rate (RMR) and have a hypoxic-response strategy designed to maintain RMR while supplementing falling mitochondrial ATP production with anaerobic glycolysis (Dunn and Hochachka, 1982; Boutillier *et al.*, 1988; Omlin and Weber, 2010; Barnes *et al.*, 2011). As a result, they are a relatively hypoxia intolerant species. Considering that rainbow trout are of economic and ecological importance, and are commonly used as a bio-indicator in freshwater aquatic ecosystems, it is important to understand how this species may respond to the increasing prevalence and severity of hypoxia.
During hypoxia the sequence leading to cell death is very similar in tolerant and intolerant organisms. The difference is the time course until loss of cellular equilibriums and subsequently death (Boutilier and St-Pierre, 2000). Cell death due to hypoxia begins when anaerobic ATP production can no longer sustain the energetic demands of maintaining ionic and osmotic equilibrium, causing a decline of high-energy phosphates. This results in the failure of ionoregulatory ATPases, membrane depolarization, and uncontrollable cell swelling leading to necrosis (Hochachka et al., 1996). Therefore, hypoxia tolerance at the cellular level does not appear to be due to an increased capacity to cope with ionic disequilibrium or to maintain a sustained energy deficit. Instead, hypoxia tolerance appears to lie in the organism’s ability to suppress ATP demands to match production under energetically stressful situations (Hochachka et al., 1996; Boutilier and St-Pierre, 2000; Hochachka and Lutz, 2001).

Preliminary evidence suggests that inducing metabolic depression prior to hypoxic exposure may increase tolerance. Studies on both the hypoxia tolerant zebrafish (Danio rerio) (Rees et al., 2001) and the epaulette shark (Hemiscyllium ocellatum) (Routley et al., 2002) demonstrated that exposure to non-lethal hypoxia increased acute hypoxia tolerance and survival time to subsequent bouts of more severe hypoxia. Rees et al. (2001) suggested that pre-exposing these fish to hypoxia may have led to a decrease in routine metabolic rate, a common hypoxic response of tolerant species that reduces ATP demand during periods of limited aerobic ATP-production. Rainbow trout depress metabolic rate in response to food deprivation (Aslop and Wood, 1997; Salem et al., 2007) but they strive to maintain routine metabolic processes during hypoxia (Richards et al., 2009). As such, the underlying goal of this present study was to determine if a hypoxia intolerant species could become increasingly tolerant by preconditioning into a state of metabolic depression via food deprivation.
2.2 Methods

2.2.1 Animals

Four hundred juvenile rainbow trout (~ 4 g average biomass) were obtained from Rainbow Springs Hatchery in Thamesford, Ontario and kept in the aquatic animal care facility at Queen's University, Kingston, Ontario. Fish were stored in an 1800 L flow-through tank, and supplied with fresh, dechlorinated Kingston tap water at a rate of 3 L min\(^{-1}\) for at least two weeks prior to experimentation. The water had a neutral pH, was saturated with air, and temperature was maintained at 15±1°C throughout the acclimation period and experimentation. The photoperiod was 12:12 light:darkness. The trout were fed commercial floating fish pellets (Corey Salmonid Feed) on a maintenance ration diet of 1% average body mass per day. Animal handling and experiments were in accordance with Queen’s University Animal Care guidelines under protocol #wang-2009-021-R1.

2.2.2 Live Animal Experimental Protocol

Following the two-week acclimation period, feeding was stopped and the fish were divided into three different experiments. Prior to any experimentation, a brief two-day period of suspension of feed was employed to minimize physiological responses associated with feeding.

2.2.2.1 Protocol One – Respirometry

First, to determine the effect of food deprivation had on routine oxygen consumption, after being subjected to 0, 1, 2, 3, 4, and 5 weeks of food deprivation (N=8-12) trout were first placed in 1.1L respirometers prior to any measurements the fish underwent a 12 h acclimatization period in dechlorinated, aerated water. Water temperature in the system was maintained at 15°C using two Neslab RTE-111 water chillers (Neslab Inc. Portsmouth, New Hampshire, USA). To
avoid accumulation of nitrogenous waste from fish, water was filtered using a Fluval 205 external filtration system equipped with a particle biofilter (Rolf C. Hagen Inc. International).

Following the 12 h acclimation period the respirometer chambers were sealed and oxygen concentration ([O$_2$]) was monitored continuously using an Ocean-Optics Fiber-Optic Oxygen Sensor probe connected to a multifrequency phase fluorometer (TauTheta Instruments LLC, Boulder, Colorado, USA). OOI Sensors Oxygen Measurement Software (Ocean Optics Inc., Dunedin, Florida, USA) was used for digital data acquisition and recording. Fish were allowed to consume oxygen to deplete the [O$_2$] in the respirometer until they lost equilibrium, at which point they were moved to black perspex boxes with a constant supply of aerated water to recover. Routine metabolic rate ($\mu$mol·min$^{-1}$·g$^{-1}$) was determined by calculating the rate of O$_2$ decline within the first 30 min of closing the respirometer. The $P_{crit}$ (torr) was determined using a critical points calculator developed in our lab based on the BASIC program for the determination of critical points (Yeager and Ultsch, 1989). Briefly, the program identifies the critical partial pressure of oxygen ($pO_2$) where the shift from metabolic regulation of O$_2$ consumption to metabolic O$_2$ conformation occurs. At $pO_2$’s above $P_{crit}$ the $MO_2$ is constant and the slope of the regression line of $O_2$ consumption against $pO_2$ has a value not statistically different from zero. At $pO_2$’s below the $P_{crit}$ the regression line has a significant positive slope. To determine where this point occurs, the program first sorts the data into ascending order and computes a regression line for all data, and tests for the significance of the slope. The program then completes a two-segment fit on the sorted data. The point where the intersection of the two best fit regression lines that divides the data points into two significantly different data sets is defined as the critical point (Yeager and Ultsch, 1989). It has been suggested that marginal metabolic scope (MMS) can provide another index of the capacity for an organism’s metabolic performance (Neill and Bryan, 1991; Del Toro-Silva et al, 2008). Marginal metabolic scope (MMS) was determined based on the calculations of Neill and Bryan (1991) where MMS=RMR/$P_{crit}$.
Protocol Two – Weekly Tissue Sampling

On a weekly basis of over 5 weeks of food-deprivation, trout (N=8 for each treatment) were sacrificed using 0.5 mg L⁻¹ tricaine methanesulfonate (MS-222 Syndel International Inc., Vancouver, BC), buffered to a neutral pH with Na₂CO₃ (Bioshop, Canada). Immediately after the fish stopped respiring, the craniocervical was severed and blood was collected via a caudal puncture using a heparinised syringe, and plasma and red blood cells were separated immediately by a 2-min centrifugation at 7,500 g. White muscle and liver were then excised and subsequently flash-frozen in liquid nitrogen. For each fish process from removing the fish from the tank, to having all tissues frozen in liquid nitrogen was completed in less than five minutes. Tissues were stored at -80°C before metabolite and enzymatic analysis was completed.

Protocol Three - Hypoxic Exposure

To demonstrate how a single bout of acute moderate hypoxia was affected by metabolic depression induced by starvation, fish after being subjected to various period of food deprivation (0, 1, 2, 3, 4 and 5 weeks, N=8 for each treatment) were acclimated for a minimum of 12 h in 4 L covered glass jars (two fish/jar) receiving a supply of fresh oxygenated water. Following the acclimation period, [O₂] was steadily decreased from 100% air saturation (~156 torr) to 50% saturation (~78 torr) within 30 min, and maintained for 8 h. Oxygen levels were controlled using a mixture of compressed nitrogen and air (Praxair) using a GF-2 gas mixing flowmeter (Cameron Instruments USA). Oxygen concentrations were continuously monitored using the multifrequency phase fluorometer. Following the hypoxic exposure fish were sampled terminally following the same protocol as Protocol One. The only alteration to the protocol was fish were sacrificed in the hypoxic chambers to avoid any physiological responses associated with handling. The process from administering the MS-222 to having all tissues excised took under five minutes.
2.2.3 Analytical Techniques

Enzyme Activity Analysis

Frozen white muscle and liver tissues were powdered in liquid nitrogen using a mortar and pestle. Roughly 50 mg of powdered tissue were homogenized using a glass homogenizer in 1:20, wt:vol 50 mM HEPES buffer containing 1 mM ethylene-diaminetetraacetic acid (EDTA) and 0.1% Triton-X 100 detergent. Cytosolic enzymes, PK and LDH, were assayed immediately following extraction, and mitochondrial enzymes, CS and HOAD, were assayed following a freezing period at –20°C.

The enzyme kinetic assays were performed based on Bergmeyer (1986) using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, California, USA). Specific conditions for the assays were as follows: LDH (EC 1.1.1.27): 50 mM HEPES, 0.2 mM NADH, 2 mM pyruvate, pH 7.4, read at 340 nm; PK (EC 2.7.1.40): 50 mM HEPES, 100 mM KCl, 10 mM MgCl₂, 0.2 mM NADH, 5 mM ADP, 1x10⁻⁵ M fructose-1,6-biphosphate, 5 mM phosphoenolpyruvate, 10 IU/mL LDH (EC 1.1.1.27, Sigma-Aldrich), pH 7.4; The CS assay was observed to have background activity meaning that without adding the substrate oxaloacetate, there was still an observed Vmax. Therefore, one of the four quadruplicates did not receive oxaloacetate and the Vmax of this well was subtracted from the average Vmax of the other three triplicates. The specific conditions for the CS assay are as follows. CS (EC 4.1.3.7): 50 mM triethanolamine HCl (TEA-HCl), 0.2 mM acetyl-CoA, 0.1mM DTNB, 0.5 mM oxaloacetate (omitted in background), pH 8.1; HOAD (E.C. 1.1.1.35): 50 mM HEPES, 0.2 mM NADH, and 2 mM acetoacetyl-CoA, pH 7.4. All enzyme kinetic assays were run in quadruplicate at 25°C and the activities were expressed in µmol of substrate converted to product per minute per gram wet tissue weight.

Metabolite Analysis
White muscle ATP, glucose, glycogen, pyruvate, and lactate; liver ATP, glucose, glycogen and lactate; and plasma glucose and lactate were determined enzymatically using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, California, USA). Approximately 50 mg white muscle and liver, and 50 µl plasma were deprotenized using 350 µl 6% perchloric acid (PCA) and homogenized. A 100 µl aliquot for glycogen was removed, neutralized with 2.5 M K$_2$CO$_3$ and frozen at -80°C until analysis could be completed. The remaining homogenate was centrifuged at 4°C for 10 min and neutralized with 2.5 M K$_2$CO$_3$. Aliquots of the resulting supernatant were removed for glucose, lactate and ATP analysis. Analysis of [ATP] and [Pyr] was completed immediately to avoid the possibility of degradation during the freeze-thaw process, and the remaining metabolite aliquots were frozen at -80°C until analysis.

Analysis of [ATP] was completed using spectrophotometry as described by Milligan and Wood (1986). More specifically, the assay buffer (pH 7.5) contained 0.1 M TEA-HCl buffer, 0.6 mM NAD$^+$ and G-6-PDH (EC 1.1.1.49; Sigma-Aldrich) was added to the sample. Following a background reading at 340 nm, hexokinase (HK) (EC 2.7.1.1, Roche Diagnostics) was added to all wells, allowed to incubate for 20 min at 25°C and subsequently re-read. Glucose, glycogen, lactate, and pyruvate concentrations were assayed using spectrophotometry following the protocol of Bergmeyer (1986). More specifically, the 1 ml glucose/glycogen aliquot was divided into two samples one to assay for free [Glu] and the other to assay for [Gly]. The samples designated for assaying [Gly] were digested into glucose using 2 µl amylloglucosidase (EC 3.2.1.3; Sigma-Aldrich) at 37°C for 180 min. The sample designated for free [Glu] and equivalent glycosyl units digested from glycogen were subsequently assayed in parallel in a 0.3 M triethylamine hydrochloride (TEA-HCl) buffer (pH 7.5) containing 8 mM ATP, 2 mM NAD$^+$ and glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1.1.1.49; Sigma-Aldrich). Following a background reading at 340 nm, hexokinase (EC 2.7.1.1, Roche Diagnostics) was administered to all wells, allowed to incubate for 15 min at 25°C, and re-read. The free [Glu] was then subtracted
from the digested [Glu] to calculate the [Gly]. [Lac] analysis was conducted using a 0.6 M hydrazine-glycine assay buffer (pH 9.2) containing 2 mM NAD$^+$ and LDH. The assay buffer was added to the samples and allowed to incubate for 60 min at 37°C and then read at 340 nm. [Pyr] assays were completed by adding TEA-HCl buffer (pH 7) containing 0.2 mM NAD$^+$ to the samples and taking a reading at 340nm. Lactate dehydrogenase was then added, and the samples were re-read.

2.2.4 Intracellular pH (pH$_i$) Analysis

Approximately 100 mg of frozen white muscle tissue were weighed out and powdered under liquid nitrogen using a mortar and pestle. The powered tissue was then added to a 1.5 ml-bullet tube containing 150 mM potassium fluoride (KF) and 6 mM nitrilotriacetic acid disodium salt (Na$_2$NTA) (Pörtner et al. 1990) and the pH of the solution was determined using an Accumet 1.5” microelectrode (Fisher Scientific) connected to a PHM82 standard radiometer (Copenhagen Instruments).

2.2.5 Calculations and Statistical Analysis

Enzyme Activity Ratios

Enzyme activity ratios were calculated according to Hochachka et al. (1982). Calculated ratios were log transformed to meet the assumptions required for statistical analysis, and normalized to 1 for graphical representation, using the fed normoxic fish as the control-value of 1.

Redox Status
Redox status was calculated in accordance with Wang et al. (1994) based on the calculations of Williamson et al. (1967).

\[
\frac{[NAD^+]}{[NADH]} = \frac{[Pyr][H^+]}{[Lac] \cdot K}
\]

Where:

\[H^+ = 10^{-\frac{\varnothing}{\varnothing}}\]

and K= 2.106x10^{12}, which is the equilibrium constant for lactate dehydrogenase reported by Williamson et al. (1967) and corrected to 15°C according to the Van’t Hoff equation (Williams and Williams, 1973). Redox data were first calculated and then log-transformed to meet the assumptions of the statistical analysis.

**Statistical Analysis**

The data are presented as mean ± standard error of mean and JMP 9.0 was used to conduct all of the statistical analyses. All data were first analyzed for normality using a Shapiro-Wilk test. If the data were not normally distributed, they were log-transformed to meet the assumptions of an ANOVA.

To determine the effects of food deprivation over the five-week period, a one-way ANOVA was conducted on all the data simultaneously. Subsequently, a Tukey-Kramer honestly significant difference *post-hoc* test was completed to compare differences amongst the means of the five weeks. Similarly, to determine the effects of O₂ deprivation in concert with food deprivation over the five-week starvation period, a one-way ANOVA was conducted on all the data simultaneously. A Tukey-Kramer honestly significant difference *post-hoc* test was completed to compare the differences amongst the means of the five weeks.
To understand whether different periods of food deprivation had effect on how trout respond to single bout of low O$_2$ (50% Air Sat), the food deprived hypoxic fish were compared to the corresponding weekly food deprived normoxic fish. When data were non-parametric a Wilcoxon test was completed and if parametric a students t-test was completed. In all cases $\alpha=0.05$ and a p-value $<0.05$.

2.3 Results

2.3.1 Animal Mortalities

Animal mortalities during the acclimation period were $<10\%$. The food-deprivation protocol adopted by this study resulted in $<1\%$ mortality in juvenile rainbow trout. When fish were subjected to 8 h of moderate hypoxia, only 1 death each was recorded in the four and five-week food deprivation group. These fish were excluded from analysis.

2.3.2 Metabolic Oxygen Consumption ($\dot{M}O_2$)

Starvation had a significant effect on the rainbow trout’s routine $\dot{M}O_2$. A significant reduction in routine oxygen consumption (27-34%) was recorded at all weekly time intervals following two weeks of food-deprivation (Table 1; Figure 2).

2.3.3 Critical Oxygen Tensions

Metabolic depression induced by food deprivation resulted in a significant reduction in $P_{cri}$ and following four weeks of food deprivation, the $P_{cri}$ was depressed by $\sim 20\%$ (Table 1; Figure 2). Interestingly, the $\dot{M}O_2$ at $P_{cri}$ was consistently $\sim 7.5$ $\mu$mol min$^{-1}$ g$^{-1}$ at every weekly
interval following the cessation of feeding (Table 1; Figure 2). As a result, as the $\dot{M}O_2$ decreased following 2 weeks of food deprivation, the appearance of another critical point ($P_{\Delta MET}$) became apparent, at $\sim$75 torr (Table 1, Figure 2). At $P_{\Delta MET}$ $\dot{M}O_2$ began to steadily increase until reaching $P_{\text{crit}}$ at which point $\dot{M}O_2$ began to subsequently fall, and following four weeks of starvation all fish illustrated this pattern (Table 1; Figure 2).

2.3.4 Enzyme Activities

White Muscle

Long-term starvation caused significant reductions in glycolytic enzyme activity (LDH and PK) of the white muscle, but mitochondrial enzyme activities (CS and HOAD) were less perturbed (Figure 3). During the first two weeks following the cessation of feeding, LDH activity remained at values that were similar to fed fish (Figure 3). Three weeks of food deprivation reduced the activity of LDH by $>50\%$ from 407 to 181 $\mu$mol min$^{-1}$ g$^{-1}$ wet wt, at which point, no further significant reductions were observed at the subsequent weekly intervals (Figure 3). PK followed a very similar pattern to LDH, and after three weeks of no feeding, PK activity fell significantly from 157 to 82 $\mu$mol min$^{-1}$ g$^{-1}$ wet wt where it remained for the following two weeks (Figure 3). In regards to the mitochondrial enzymes, both enzymes illustrated a declining trend, however, neither CS nor HOAD differed significantly from the fed fish at any point during food-deprivation (Figure 3). However, both CS and HOAD were significantly lower after 4 weeks of food deprivation relative to the activities after one week (Figure 3).

Liver

The liver enzyme responses to food deprivation were considerably different from those observed in the white muscle. One week following the cessation of feeding, liver LDH activity
increased significantly from 237 to 387 \(\mu\)mol min\(^{-1}\) g\(^{-1}\) wet wt (Figure 4). LDH activity returned to fed-fish values for week two through four, and subsequently increased again at week five to values statistically similar to week one (Figure 4). Liver PK activity was maintained for a week following the start of food deprivation, however, after two weeks, PK activity declined significantly from 6.1 to 4.2 \(\mu\)mol min\(^{-1}\) g\(^{-1}\) wet wt, and after 5 weeks of starvation PK activity was \(~50\%\) of the values for fed fish (Figure 4). In regards to the liver mitochondrial enzymes, both CS and HOAD activity increased \(~30\%\) and \(~50\%\) respectively, and were maintained at statistically similar activities for the remainder of the starvation period (Figure 4).

### 2.3.5 Enzyme Activity Ratios

**White Muscle**

Enzyme activity ratios were calculated to assess the relative contribution of glycolytic and oxidative enzymes in both the white muscle and the liver during starvation. Following three weeks of food-deprivation there was an increased reliance on oxidative metabolic processes for the remainder of the starvation period as indicated by a significant decrease of \(~15\%-20\%\) LDH:CS (Figure 5). Furthermore, \(\beta\)-oxidation and the Krebs Cycle maximum capacities increased concurrently based on the observation that HOAD:CS showed no statistical differences at any point (Figure 5).

**Liver**

The ratio of LDH:CS, an indicator of relative capacity of anaerobic glycolysis was similar to that of the fed fish at all points of time during starvation (data not shown). When LDH:CS activity ratios are similar, PK:CS activity ratios yield a measure of the relative capacity for aerobic glycolysis (Hochachka et al. 1982). The relative capacity of aerobic glycolysis
indicated by the ratio of PK:CS significantly decreased by ~20% after a week of food deprivation, and was maintained at this level for the remainder of the starvation period (Figure 6). Similar to the white muscle, the ratio of HOAD:CS was unaltered by starvation at all intervals of time (Figure 6).

2.3.6 Alterations in Metabolite Concentrations in Response to Food Deprivation

White Muscle

Five weeks of starvation resulted in the reduction of the endogenous metabolites in white muscle. White muscle [ATP] decreased significantly from 5.9 to 3.6 µmol·g⁻¹ after five weeks of starvation (Figure 7). White muscle [Glu], [Gly] and [Lac] decreased from 1.1 to 0.6 µmol·g⁻¹, 4.1 to 1.5 µmol·g⁻¹, and 7.2 to 3.4 µmol·g⁻¹ respectively, however, these reductions were insignificant (Figure 8 and 9 respectively). [Pyr] was unaffected by starvation at all weekly time intervals (Figure 9). Both white muscle pHᵢ and redox status increased significantly following four and five weeks of food-deprivation (Figure 10).

Plasma

Chronic food-deprivation caused perturbations in both plasma [Glu] and [Lac] in rainbow trout (Figure 11). One week of starvation caused [Glu] to drop significantly from 1.92 to 1.34 µmol·ml⁻¹, and then subsequently recover to fed normoxic concentrations for the remainder of the experiment (Figure 11). Four and five weeks of starvation resulted in the significant reductions of plasma [Lac] from 1.56 to 0.41 and 0.6 µmol·ml⁻¹ respectively (Figure 11).

Liver
Chronic starvation had relatively little effect on the liver metabolites measured in this experiment. [Glu], [Lac], and [ATP] remained statistically representative of the fed fish at all weekly time intervals (Figure 12 and Figure 7 respectively). Furthermore, [Gly] was only significantly depressed from 112.5 to 39.2 µmol ml\(^{-1}\) at one week of starvation, and returned to fed normoxic concentrations at all subsequent weeks (Figure 12).

### 2.3.7 Alterations in Metabolite Concentrations in Response to Food Deprivation and Hypoxia

**White Muscle**

An eight-hour bout of moderate hypoxic exposure (Po\(_2\) = 75 torr) caused white muscle [ATP] to decline significantly from 5.91 µmol g\(^{-1}\) in normoxic fish, to 2.24 µmol g\(^{-1}\) (Figure 7). Rainbow trout subjected to hypoxic condition responded with significantly elevated [Glu] after one, three, four and five weeks of food deprivation (Figure 8). Interestingly, however, [Gly] was only significantly depressed following hypoxic exposure at one-week starvation (Figure 8). White muscle [Pyr] was relatively unaffected by the hypoxia protocol used in this experiment (Figure 9). Only after two weeks of starvation did hypoxia have any significant effect on [Pyr], resulting in elevated concentrations (Figure 9). White muscle [Lac] following hypoxia was not significantly different at any of the weekly intervals, however, acidosis following hypoxia was observed at one, two, three and four weeks of starvation (Figure 9). The redox status increased significantly by ~30% in hypoxic fed fish, relative to the fed normoxic group (Figure 10). At all time points during starvation, however, there was no significant difference between hypoxic and normoxic fish.

**Plasma**
In response to hypoxia, rainbow trout experienced significant increases in plasma [Glu] at all weekly intervals throughout the five-week starvation (Figure 11). Furthermore, plasma [Lac] was elevated in response to hypoxia by roughly two-fold at all time intervals following starvation, with the exception of five weeks (Figure 11).

Liver

The liver [ATP] of hypoxic fish was maintained at normoxic concentrations at all time points during the starvation protocol (Figure 12). Following hypoxia [Glu] was significantly higher at weeks one, two and five (Figure 12). Liver [Gly] was significantly higher at all weekly sampling intervals (Figure 12). Liver [Lac] illustrated significant accumulation following hypoxia at all weekly time intervals except week two (Figure 12).

2.4 Discussion

2.4.1 Food Deprivation and Metabolism

To our knowledge, this is the first study to examine the interactions between metabolic depression induced by food deprivation and hypoxia tolerance. Both food-deprivation and hypoxia are common occurrences in aquatic ecosystems, and therefore, it is seemingly possible that fish may have to cope with these environmental stressors simultaneously.

The first aim of this study was to determine if food-deprivation resulted in metabolic depression. Two weeks following the cessation of feeding, juvenile rainbow trout significantly decreased $\dot{M}O_2$, which stabilized for the remainder of the five-week protocol (Table 1, Figure 1). It is important to note that this study did not have a corresponding weekly fed normoxic control group. In previous studies examining the influence of starvation on the $\dot{M}O_2$ of juvenile rainbow trout $\dot{M}O_2$ of fed fish never changed during a 17-day period (Aslop and Wood 1997). Based on
these findings, the alterations $\dot{MO}_2$ observed in this study are most likely a result of food deprivation. However, further examination including a fed normoxic group would be required to conclusively rule out all other factors that may affect $\dot{MO}_2$ such as changes in seasons, or growth and maturity. The observation that food deprivation reduced $\dot{MO}_2$ is consistent with other studies (Alsop and Wood, 1997). However, many of these studies overlooked the contribution of anaerobic energy production, using metabolic oxygen consumption as the sole indicator of metabolic rate (Richards, 2010). Anaerobic pathways can play a significant role in energy production, especially during conditions such as exhaustive exercise or hypoxia, and as a result, should be included when examining overall metabolic rate. This limitation can be circumvented through direct calorimetry, quantified by heat dissipation, or by observing key indicators of anaerobic metabolism (Richards, 2010).

In rainbow trout, the white muscle, a predominantly glycolytic tissue, composes upwards of 90% of the musculature and ~60% of the entire mass of the fish (Milligan, 1996). As a result, valuable insight into metabolic processes at the whole organism level can be obtained by analyzing the metabolic profile of this particular tissue. Further, LDH is involved in the conversion of pyruvate to lactate and is a commonly used indicator of anaerobic glycolysis (Hochachka et al., 1982). Following three weeks of food deprivation, LDH activity significantly decreased to <50% fed activity (Figure 2). Considering the decrease in $\dot{MO}_2$, and white muscle LDH activity, it seems likely that chronic food deprivation resulted in both aerobic and anaerobic metabolic depression in juvenile rainbow trout.

### 2.4.2 White Muscle Physiological State Under Food Deprivation

In addition to a reduction in LDH activity, the other cytosolic enzyme, PK, also experienced significant decreases in activity following three weeks of food deprivation (Figure 2). PK is a rate-limiting enzyme involved in both aerobic and anaerobic glycolysis (Wright et al.,
Therefore, the decrease in PK activity may represent a decreased contribution of glycogenolysis to overall energy production. Unlike PK and LDH, the activity of the mitochondrial enzymes, CS and HOAD, did not decrease (Figure 2). These enzymes are common indicators of aerobic metabolism, with CS activity representative of overall oxidative capacity, and, HOAD activity is more specifically used as an index for fatty acid oxidation (Hochachka et al., 1982). However, HOAD is also involved in other metabolic processes, such as the catabolism of amino acids. Therefore, the reliability of HOAD as a sole indicator for fatty-acid degradation may be over simplistic. It seems more likely that HOAD may be an indicator of non-carbohydrate based oxidative capacity. Starvation in salmonids has been observed to elicit a metabolic shift towards oxidative processes (Jezienska et al. 1982; Loughna and Goldspink, 1984; Guderley et al., 2003; Salem et al., 2007). The relative conservation of both CS and HOAD activities in the white muscle may therefore be a consequence of increased reliance on aerobic energy production. Past research has suggested that protein becomes an increasingly important fuel as starvation time is prolonged (Salem et al., 2007), especially in the white muscle (Loughna and Goldspink, 1984; Guderley et al., 2003). In teleosts, the white muscle is particularly well adapted for rapid protein mobilization due to high quantities of catheptic enzymes (Loughna and Goldspink, 1984). During periods of food deprivation white muscle protein becomes an important fuel reserve, as well as, a source of metabolic precursors (Loughna and Goldspink, 1984). As a result of white muscle macromolecule degradation the muscle experiences an increase in osmolytes, causing the muscle to retain water. As a result, an increase in white muscle H$_2$O content has been used as an indicator of protein degradation. Under prolonged starvation conditions, both rainbow trout (Loughna and Goldspink, 1984) and Atlantic cod (Black and Love 1986; Guderley et al., 2003) white muscle has been observed to significantly increase water content. Therefore, it appears that starvation commonly results in the mobilization of teleost white muscle protein. Considering this, it is possible that the conservation of mitochondrial enzyme activity is involved in the oxidative process of white muscle protein.
catabolism. This, however, is speculative based on indirect evidence. In order to determine the specific contributions of proteins and fatty acids to overall energy metabolism, future work should focus on parameters such as nitrogenous waste excretion (indicator of protein catabolism), and the presence of triglycerides in the muscle (indicator of fatty acid metabolism). Another possibility is to look at instantaneous fuel usage by observing nitrogen and carbon dioxide excretion, as well as, oxygen consumption all relative to one another, a technique described by Lauff and Wood (1996). Further, it is important to note that this study measured enzyme activity in relation to the tissues wet weight. It is possible that the decreased activity of some enzymes observed in this study may be a consequence of reductions in overall protein or tissue edema with excessive water retention in the intracellular space. Therefore, future work should acknowledge this and analyze enzyme activity relative to protein concentration.

The metabolic shift towards aerobic processes was also reflected in the enzymatic ratios of the white muscle. Examining the ratios of key aerobic and anaerobic enzymes was originally proposed by Hochachka et al. (1982) as a means to yield insight into the relative contribution of aerobic and anaerobic processes to overall energy production. Juvenile rainbow trout deprived of food for three weeks experienced significant reductions in the ratio of LDH/CS (Figure 4). The ratio of LDH/CS has been used to assess the relative capacity for anaerobic glycolysis versus overall aerobic metabolism (Hochachka et al., 1982). On the other hand, HOAD/CS ratios are an index of the relative capacity for non-carbohydrate based oxidative capacity versus overall aerobic capacity (Hochachka, 1982). HOAD/CS ratios remained unchanged at any time interval following the cessation of feeding (Figure 4). Therefore, it appears that chronic food deprivation caused a significant shift away from anaerobic metabolism to aerobic energy production. Further, the relative contribution of non-carbohydrate based substrates to aerobic energy production appeared to be directly reliant upon the overall oxidative capacity of the white muscle.

The shift away from anaerobic energy production in the white muscle was also reflected
in the tissues cytosolic redox state. The four and five weeks of food deprivation resulted in the increase in the white muscle’s redox state (Figure 9). Redox state represents the availability of NAD$^+$, relative to its reduced form, NADH. Redox state is an important cellular signaling mechanism reflecting the concentration of oxidizable and reducible substrates, coordinating the catabolic processes of the cell (Williamson et al., 1966). When [NAD$^+$] are high, this favors the transformation of pyruvate to acetyl-CoA and subsequent entry into the Krebs Cycle. This is because NAD$^+$ is a coenzyme of the rate limiting enzyme pyruvate dehydrogenase (PDH). Therefore, an increased concentration of this specific coenzyme stimulates PDH’s activity. In contrast, the anaerobic enzyme LDH converts pyruvate and NADH to NAD$^+$ and lactate. The accumulation of NAD$^+$ thereby inhibits anaerobic glycolysis through a negative product feedback mechanism. Therefore, an increase in the ratio of [NAD$^+$]/[NADH] is representative of a shift towards an increased reliance on oxidative energy production.

An increase in intracellular pH, as observed in this study following five weeks of food deprivation (Figure 9), also shifted the LDH equilibrium away from lactate production and favoured entry of substrate into the citric acid cycle (Robergs et al., 2004). When considering acid/base equilibrium, physiological processes associated with H$^+$ production or removal must be considered. The process of glycolysis is a net proton producer through the hydrolysis of ATP in the hexokinase (HK) and phosphofructokinase (PFK) reactions. Enzymatic analysis suggests that there may be a decreased reliance on glycolysis, especially anaerobic glycolysis, which may be one of the factors contributing to the increase in cytosolic pH.

Historically, lactate accumulation has been postulated to be a significant source of H$^+$, however, recent evidence suggested that the opposite might be the case. The process of removing lactate from the muscle involves the use of a monocarboxylate transporter (MCT) (Robergs et al., 2004). MCTs are also proton symporters, transporting H$^+$ out of the muscle along with lactate. Under other energetically stressful situations, such as during acute hypoxia, lactate disposal out of
the rainbow trout muscle increases (Omlin and Weber, 2010). It is possible that a similar scenario is occurring during chronic starvation. Lactate being produced in the white muscle is deposited in the blood, and transported to other more oxidative tissues such as the heart or red muscle for metabolism. If this were occurring, it would also result in alkalinization in the white muscle cell. Further investigative analysis into lactate flux during starvation, however, would need to be completed to verify this speculation.

Another source of H\(^+\) is from the accumulation of NADH + H\(^+\), as would be the case with a reduction in the cellular redox state (Robergs et al., 2004). As previously discussed, the redox state of the trout’s white muscle increased as the starvation period was prolonged. This is likely another contributing factor to the increased intracellular pH observed in this study.

The dominant source of H\(^+\) production has been determined to be the result of increased ATP hydrolysis (Robergs et al., 2004). During conditions where ATP demand exceeds supply from mitochondrial respiration, there is an increased reliance on cytosolic ATP. Under these conditions there is an increase in ATP hydrolysis resulting in the accumulation of H\(^+\). This study observed significant decreases in [ATP] following four and five weeks of food deprivation (Figure 6), however, this was not accompanied by cellular acidosis. Therefore, it seems unlikely that this decrease in [ATP] was a result of increased hydrolysis. It is possible that the white muscle may be entering a new dynamic ATP equilibrium. Chronic starvation has been observed to result in metabolic depression of trout’s white muscle as indicated by decreases in protein turnover (Loughna and Goldspink, 1984). Under these conditions there would be a decrease in ATP demand, and presumably a decrease in hydrolysis. It is likely that this decrease in hydrolysis could raise the cellular pH of the muscle, which may be occurring in this study. It would be informative to observe ATP turnover in response to chronic food deprivation to gain further insight into metabolic reorganization in white muscle under starvation conditions.
The dominant consumer of H\(^+\) is the mitochondria for use in oxidative phosphorylation. Starvation results in an increased reliance on aerobic pathways (Jezierska et al. 1982; Loughna and Goldspink, 1984; Guderley et al., 2003). During periods of increased aerobic energy production the mitochondria essentially functions as a depository for H\(^+\) (Robergs et al., 2004). This could potentially be leading to an increase in alkalinity as observed in this study.

An increase in redox state coinciding with an increase in pH, favours aerobic processes, and subsequently inhibits anaerobic glycolysis (Williamson et al., 1966). Considering this observation in conjunction with the decrease in the ratio of LDH/CS, it appears that chronic food deprivation results in a decrease in anaerobic glycolysis and a shift towards aerobic energy production. The decrease in anaerobic glycolysis would explain the decreasing trend in [Lac] (Figure 8). This response, however, was not significant, which can likely be attributed to the large variation in [Lac].

In regards to white muscle carbohydrates, neither glucose or glycogen showed significant differences at any weekly time interval following the cessation of feeding (Figure 7). It is important to note, however, that [Gly] in this study was observed to be quite low relative to other studies on juvenile rainbow trout. Endogenous white muscle [Gly] has been previously reported to be \(\sim 25 \mu\text{mol g}^{-1}\) wet weight (Kieffer and Tufts, 1998), which is drastically higher relative to the \(\sim 5 \mu\text{mol g}^{-1}\) wet weight observed in this study. One possible explanation is differences in mass with the fish in this study being approximately half the weight relative to Kieffer and Tufts (1998). White muscle glycogen content been observed to be relatively constant at approximately 1% of muscle weight (Goolish, 1991). Therefore, [Gly] will scale with an increase in muscle mass. This discrepancy, however, is unlikely to account entirely for the dramatic differences in [Gly].

Further, the feeding regimes utilized between the two studies differed, which may offer an explanation to the low [Gly]. Kieffer and Tufts (1998) fed fish daily to satiation, where as,
this study employed a daily 1% ration diet. The organization of hierarchies has been well documented in this species, and is especially apparent when food rations are limited. Under a rationed diet dominant fish consume a significantly greater share of the group meal (McCarthy et al. 1992). As food availability increases, these hierarchies begin to become less apparent (McCarthy et al. 1992). The fed normoxic [Gly] reported in this study is more representative of fish that had been deprived of food for 7 days (Kieffer and Tufts, 1998), and was highly variable between individual fish. Therefore, it is possible that many fish were not gaining access to food in quantities large enough to support white muscle glycogen synthesis, which translated into lower [Gly] in white muscle.

Another possible explanation may be related to the method in which fish were sampled for this experiment. Trout were continuously removed from the same tank over the course of the experiment. As a result, the stocking density of the tank decreased over time. It has been suggested that higher stocking densities may result in increased stress levels in fish (Procarione et al., 2011). The only partial indicator of stress measured in this study was [Lac]. Resting [Lac] reported in this study was slightly elevated relative to other reports on juvenile rainbow trout (Scarabello et al., 1991), by ~20-25%. This may indicate that the rearing conditions at the beginning of the experiment were more stressful relative to the end of the starvation period, which would influence the results of this study. The stocking density in this experiment, however, never exceeded 2 g L⁻¹ and never fell below 0.8 g L⁻¹. This range is well below 5.6 g L⁻¹ which was the stocking density where juvenile rainbow trout begin to show physiological perturbations associated with density (Procarione et al., 2011). If anything, at lower densities, netting fish may have been increasingly difficult of catching fish, which would result in an increase in [Lac] at latter weekly intervals. To avoid any complications associated with chasing and netting, trout were allowed to acclimate for 12 h prior to experimentation and subsequent sampling. A period of 12 h has been observed to be long enough for juvenile rainbow trout to recover from a bout of
exhaustive exercise (Scarabello et al., 1991; Wang et al., 1994). Therefore, if there was any increased stress associated with the netting process at lower fish densities, the fish should have been able to recover during the acclimation period allotted.

2.4.3 Liver Physiological State Under Food Deprivation

The liver responded to starvation considerably differently and did not show the same physiological perturbations to chronic food deprivation in comparison to the white muscle. Liver [ATP] showed no alterations at any time interval following the cessation of feeding (Figure 6), suggesting the homeostasis of liver physiology during chronic food deprivation. Further, there were not the same reductions in enzymatic activity in the liver relative to the white muscle.

One week of food deprivation resulted in a significant increase in liver LDH activity and the maintenance of PK activity, which was mirrored by significant decreases in [Gly] (Figure 4). These initial responses suggest that carbohydrate metabolism in the liver increased during the first 7 days following the last feed. Following 2 weeks of food deprivation, however, the livers response changed dramatically. LDH returned to activities that were statistically similar to non-starved level, PK activity significantly decreased, and [Gly] stabilized to non-starved levels (Figure 4 and Figure 11 respectively). Furthermore, the PK/LDH decreased following one week of food deprivation, and subsequently stabilized for the remainder of the 5 weeks (Figure 7). This suggests that flux through glycolysis may be decreasing and/or occurring in the reverse direction, which is associated with gluconeogenesis.

PK has been suggested to be an important rate-limiting enzyme for glycolysis and gluconeogenesis in the liver (Wright et al., 1989). In this experiment PK was assayed in the forward direction. In vivo a decrease in PK activity in this direction has been associated with an
increase in gluconeogenesis (Wright et al., 1989). Although PK is not directly involved in
gluconeogenesis, it controls flux through glycolysis. The lower activity of the enzyme, the more
decreased flux through glycolysis, subsequently resulting in an increase in gluconeogenesis
(Wright et al., 1989; Sheridan and Mommsen, 1991). Therefore, it appears that following two
weeks of food deprivation, the liver of juvenile trout decreased carbohydrate catabolism and
shifted towards gluconeogenesis.

In regards to the liver mitochondrial enzymes, both CS and HOAD significantly
increased following one week of food deprivation and stabilized for the remainder of the
starvation protocol (Figure 4). This indicates an increase in oxidative capacity of the liver in
response to food deprivation. Further, the stoichiometric 1:1 relationship between liver CS and
HOAD (Figure 5) suggests that non-carbohydrate substrate catabolism increases with overall
increases in oxidative capacity.

An increase in gluconeogenesis has been observed in the liver of other species of the
Oncorhynchus genus as a response to food deprivation (Wright et al., 1989; Sheridan and
Mommsen, 1991). An increase in glucogneogensis is likely what is enabling the trout to maintain
endogenous [Glu] and [Gly] (Figure 7), as well as, plasma [Glu] despite the lack of exogenous
sources of carbohydrates supplied through the diet (Figure 11). Gluconeogenesis is an
oxidatively demanding process. Therefore, an increase in the mitochondria’s oxidative capacity
comes as little surprise. The question arises is what is the dominant aerobic substrate involved in
energy production? It appears that carbohydrate metabolism in the liver is decreasing as
indicated by PK, and non-carbohydrate catabolism is increasing as indicated by the increase in
CS and HOAD. As previously discussed HOAD has been used as an indicator of fatty acid β-
oxidation, however, it is also involved in amino acid catabolism as well. As a result, it is difficult
to determine whether the liver is catabolising proteins or fats for energy. A previous study
documented that coho salmon (Oncorhynchus kisutch) subjected to food deprivation, had an
increase in liver lipolysis by ~40% (Sheridan and Mommsen, 1991). Therefore, it is likely that this study is witnessing an increase in fatty acid catabolism in the liver during starvation. Our data can only, however, provide indirect evidence to support this claim.

Regardless of the specific substrate that is being catabolised, it appears the starvation increased mitochondrial capacity in the liver, possibly as a consequence of mitochondrial biogenesis. Increasing mitochondrial biogenesis is a critical adaptation to chronic periods of energy stress (Zong et al., 2002). It would be interesting to look at some common indicators of mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha), to gain a better understanding of how starvation may be influencing mitochondrial capacity.

2.4.4 Metabolic Depression and Hypoxic Response

In fish, there are two general metabolic responses to decreasing environmental [O₂], conform or regulate. Oxygen conformers consistently decrease metabolic rate as environmental [O₂] decreases. In comparison, oxygen regulators maintain metabolic rate over a wide range of [O₂] until a critical O₂ tension (P₉₅) is reached, at which point metabolic rate becomes responsive to environmental [O₂] (Barnes et al. 2011). In other words, this point dictates how well an organism is able to extract O₂ from their environment. Above the P₉₅, organisms retain routine physiological functioning, but once this critical O₂ threshold has been reached, there is a cascade of physiological mechanisms aimed to meet the increasing energetic challenges of hypoxia. As a result, P₉₅ has been commonly used as an indicator of hypoxia tolerance, with tolerant species having a lower P₉₅ (Barnes et al., 2011).
Salmonids are highly active species, generally considered to be a hypoxia sensitive group of fishes. Historically, it has been theorized that these species would conform to decreasing [O$_2$], as they require high levels of oxygen to meet their routine metabolic demands (Barnes et al., 2011). However, to our knowledge, there is little evidence to support this speculation, and studies suggest that salmonids may regulate $\dot{M}$O$_2$ until certain critical [O$_2$] threshold, albeit at a rather high concentration, is met (Stevens et al., 1998; Barnes et al., 2011).

Juvenile trout in this study regulated their $\dot{M}$O$_2$ until a partial pressure of O$_2$ of 62.8±3.6 torr at which point significant reductions in $\dot{M}$O$_2$ were observed (Table 1, Figure 1). Four and five weeks of food deprivation resulted in a significant decrease in the $P_{crit}$ (Table 1, Figure 1), initially suggesting that hypoxia tolerance of this species was enhanced by starvation-induced metabolic depression. It is possible that preconditioning the organism to a metabolically depressed state increases hypoxia tolerance, and there is some experimental evidence to support this claim. Rees et al. (2001) observed that zebrafish exposed to 48 hours of moderate non-lethal hypoxia significantly increased survival time when subsequently exposed to severe hypoxia. However, similar experiments were carried out on rainbow trout, and preconditioning rainbow trout to hypoxic conditions didn’t translate into an increased hypoxia tolerance (Gamperl et al., 2004). One possible explanation to this discrepancy is that zebrafish are known to be a hypoxia tolerant species, and depress metabolic rate following hypoxic exposure (Rees et al., 2001). In comparison, rainbow trout respond to hypoxia by attempting to maintain metabolic rate through hyperventilation and tachycardia, while stimulating anaerobic glycolysis to produce ATP in the absence of an adequate oxygen supply (Omlin and Weber, 2010). Therefore, preconditioning trout to hypoxia likely does not depress their metabolic rate, and if anything, may deplete endogenous anaerobic fuels. This would ultimately lead to a decrease in tolerance to more severe or longer bouts of hypoxia.
Food deprivation results in metabolic depression in rainbow trout (Aslop and Wood, 1997; Salem et al., 2007), which is consistent with the findings from this study. The ability to depress metabolic rate has emerged as the unifying adaptive strategy for hypoxia tolerant species as it enables the organism to balance cellular supply and demand during conditions when ATP production is largely through limited anaerobic processes (Hochachka et al., 1996). Therefore, it is possible that metabolic-depression induced by food deprivation reduces cellular ATP demand, assisting the fish to maintain cellular energy balance under oxygen-limited conditions.

Another indicator of hypoxia tolerance is marginal metabolic scope (MMS), which is the slope of the line following $P_{\text{crit}}$ (Del Toro-Silva et al., 2008). It has recently been suggested that MMS is a good indicator of an organism’s metabolic capacity as well as hypoxia tolerance (Del Toro-Silva et al., 2008). Interestingly, in this study, while the $P_{\text{crit}}$ decreased, experiencing a left shift, the MMS did not change (Table 1, Figure 1). Physiologically this indicates that at the same $P_{\text{O}_2}$ below $P_{\text{crit}}$ trout that had been starved had a higher aerobic capacity. This, however, does not necessarily infer an increased hypoxia tolerance. Organisms adapted to low oxygen environments typically exhibit relatively high MMS relative to hypoxia sensitive organisms (Del Toro-Silva et al. 2008). In other words, hypoxia tolerant species rapidly decrease the aerobic contribution to energy production at $[O_2]$ below $P_{\text{crit}}$. Goldfish (*Carassius auratus*), a species known for their hypoxia/anoxia tolerance, for instance, entirely eliminates the aerobic component of metabolism during severe $O_2$ limitations (Waversveld et al., 1989). Instead, goldfish significantly reduce metabolic rate and rely on carbohydrate fermentation utilizing large hepatic glycogen stores for energy production (Waversveld et al., 1989). This is reflected in another important consideration of hypoxia tolerance, which is the theoretical y-intercept of the line following $P_{\text{crit}}$. Since goldfish completely eliminate aerobic metabolism, the line following the $P_{\text{crit}}$ has a negative y-intercept. In comparison the trout in this study depicted a positive y-intercept (Figure 1). This indicates that at an environmental $[O_2]$ of zero, trout would
theoretically still be attempting to carry on some aerobic processes. It appears, therefore, that trout are unable to maintain energy equilibrium when forced to rely almost entirely on anaerobic processes, a requirement of severe hypoxia tolerance. Since the $P_{crit}$ is shifting left on the graph, and the MMS is not changing, the $y$-intercept is shifting up over the course of starvation (Figure 1). This may indicate that while starvation has resulted in an increase in aerobic capacities, this has come at a reduction in their scope for anaerobic metabolism. This could be speculated to be detrimental to the physiological response of trout to severe hypoxia. Further investigation is required before it can be conclusively determined that food-deprivation does in fact increase hypoxia tolerance or just decreases the [O$_2$] required to maintain routine physiological processes at a metabolically depressed state.

2.4.5 Metabolic Depression and Physiological Response to O$_2$ limitation

Metabolic depression induced by food deprivation altered the trout’s physiological responses to a diminishing environmental [O$_2$]. As the trout entered a state of metabolic depression caused by food deprivation, there was an appearance of a second critical O$_2$ threshold ($P_{\Delta MET}$) at ~70-75 torr, which was observed in all fish following four and five weeks of food deprivation (Table 1, Figure 1). Once this [O$_2$] was reached, $\dot{M}$O$_2$ significantly increased until reaching $P_{crit}$, at which point $\dot{M}$O$_2$ subsequently fell. To our knowledge this is the first study to report this observation in trout.

As discussed previously, during periods of food-deprivation, aerobic metabolism becomes increasingly important for maintaining energy homeostasis. However, under hypoxic environments the mitochondria is limited in its capacity to generate ATP due to the lack of oxygen available to act as the terminal electron acceptor in the electron transport chain. Therefore, as the trout deplete O$_2$ in the respirometers, a [O$_2$] is reached, which we have termed $P_{\Delta MET}$, where relying on aerobic energy production to maintain routine metabolism becomes
increasingly difficult. At this point, we speculate that there is some cellular O₂ signaling mechanism that occurs resulting in the mobilization of anaerobic substrates. This would seemingly be a good defense mechanism to ensure that if [O₂] continues to decrease, appropriate substrates are mobile and available to crucial tissues.

To determine if this speculation was correct, and to reveal to what physiological processes may be occurring at P_{AMET}, this study employed an 8 h oxygen limited exposure (78.01±6.24 torr, ~50% O₂ saturation), and subsequently analyzed metabolite concentrations. At most weekly intervals subjecting the fish to this [O₂] resulted in a decrease in white muscle pHᵢ relative to the corresponding weekly normoxic fish (Figure 9). It is likely that this acidosis may be due to increased adenylate catabolism, which would explain the significant decreases in ATP following exposure to an O₂ limited environment (Figure 6). This would suggest that the white muscle was undergoing energetic stress under these environmental conditions. However, after five weeks of food deprivation, there was no difference in pHᵢ between normoxic and hypoxic fish despite significantly decreased [ATP] (Figure 9 and Figure 6 respectively). This may potentially suggest that the white muscle entered a state of metabolic depression and was not energetically perturbed by the addition of O₂ limitation.

In regards to the carbohydrate metabolism of the white muscle [Glu] was the only carbohydrate to change in response to food deprivation, decreasing significantly in the majority of the weekly exposures to O₂ limitation (Figure 7). This suggests one of two things, either glucose is being catabolised to satisfy the energetic demands of the muscle, or mobilized and transported through the blood to glucose dependent tissues. Interestingly, [Lac] did not change in response to O₂ limitation (Figure 8). Plasma lactate, however, was significantly elevated at all weekly intervals, with the exception of 5 weeks (Figure 7 and Figure 8 respectively). White muscle is a predominant producer of lactate, and the majority of the lactate in the tissue as the substrate for in situ gluconeogenesis (Dunn and Hochachka 1982; Milligan, 1996). However, under hypoxic
conditions, the white muscle will release lactate into the blood (Omlin and Weber, 2010). Therefore, it seems likely that the increase in plasma [Lac] is a consequence of increased carbohydrate catabolism in the white muscle, and subsequent disposal of lactate into the blood plasma to be transported and oxidized by other tissues such as the red muscle and liver.

In the fed fish the redox state of the white muscle in fish subjected to O\textsubscript{2} limitation increased becoming more oxidized (Figure 9). It was hypothesized that hypoxia would result in a decrease in redox state as a result of increased flux through anaerobic pathways. This made us question whether the [O\textsubscript{2}] concentration of ~50 O\textsubscript{2} saturation was actually to be considered hypoxic. Even though at this [O\textsubscript{2}] we observed perturbations in routine metabolism, it appeared that trout were entering a metabolic transition state where they are preparing for an ensuing hypoxic insult.

Exposure to the [O\textsubscript{2}] near P\textsubscript{\text{A\text{\text{\tiny{net}}}}\text{\text{)}}} was observed to result in the mobilization of carbohydrates. After 8 h at 50% O\textsubscript{2} saturation, both [Glu] and [Gly] increased significantly in the liver (Figure 11) and was mirrored by a significant increase in plasma glucose (Figure 10). This suggests that the liver was synthesizing carbohydrates and shuttling glucose into the blood stream to critical tissues. Gluconeogenesis and subsequent hyperglycemia is likely a survival mechanism to diminishing environmental [O\textsubscript{2}] ensuring that if a hypoxic insult pursues, appropriate anaerobic substrates are mobile. As previously discussed, gluconeogenesis is an oxidative process. Therefore, the increase in redox state in fish subjected to O\textsubscript{2} limitation may be a consequence of the increased aerobic demand associated with gluconeogenesis. Further, liver [ATP] did not fluctuate when fish were subjected to O\textsubscript{2} limitation (Figure 5). Despite the probable increase in energetic demand associated with carbohydrate mobilization, it appears the liver was not experiencing energetic stress. Surprisingly, even after 5 weeks of food deprivation, and being subjected to acute bout of O\textsubscript{2} limitation, the liver not only maintained, but may have increased its metabolic capacity.
2.4.6 Conclusions

To our knowledge this is the first study to examine the interactive effects of food deprivation induced metabolic depression and hypoxia tolerance in trout. A period of three weeks without feeding resulted in metabolic depression, with an increased reliance on oxidative ATP-producing pathways, and an increased capacity for gluconeogenesis in the liver. Further, inducing metabolic depression may increase hypoxia tolerance. However, starvation induced metabolic depression may decrease anaerobic capacity, which would likely be problematic during severe bouts of hypoxia. Finally, once \([O_2]\) are reduced below 75 torr, there are physiological adjustments aimed at mobilizing anaerobic substrates predominantly from the liver in rainbow trout.
Table 1 $\dot{M}O_2$, metabolic oxygen consumption; $P_{\text{crit}}$, critical oxygen tension; $\dot{M}O_2$ at $P_{\text{crit}}$, metabolic oxygen consumption at critical oxygen tension; $P_{\Delta \text{MET}}$, partial pressure of $O_2$ that elicits an alteration in routine metabolism; Ratio of $P_{\Delta \text{MET}}$, percentage of fish showing this oxygen consumption pattern; MMS, marginal metabolic scope; in response to food deprivation in *Oncorhynchus mykiss*. Treatments sharing the same letter are not significantly different (P<0.05). n= 8-12. Data are presented ± SEM.

<table>
<thead>
<tr>
<th>Food Deprivation (Weeks)</th>
<th>$\dot{M}O_2$ ($\mu$mol min$^{-1}$ g$^{-1}$)</th>
<th>$P_{\text{Crit}}$ (torr)</th>
<th>$\dot{M}O_2$ at $P_{\text{Crit}}$ (torr)</th>
<th>$P_{\Delta \text{MET}}$ (torr)</th>
<th>Prevalence of $P_{\Delta \text{MET}}$</th>
<th>MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.31±0.47 a</td>
<td>62.81±3.55 a</td>
<td>7.60±0.34</td>
<td>NA</td>
<td>0</td>
<td>0.12±0.013</td>
</tr>
<tr>
<td>1</td>
<td>7.10±0.53 ab</td>
<td>57.07±2.66 ab</td>
<td>8.1±0.42</td>
<td>88.07</td>
<td>0.14</td>
<td>0.125±0.008</td>
</tr>
<tr>
<td>2</td>
<td>6.06±0.45 b</td>
<td>54.35±3.45 ab</td>
<td>7.41±0.39</td>
<td>74.94±3.67</td>
<td>0.88</td>
<td>0.115±0.009</td>
</tr>
<tr>
<td>3</td>
<td>5.91±0.54 b</td>
<td>51.59±3.29 ab</td>
<td>6.94±0.17</td>
<td>70.62±2.57</td>
<td>0.88</td>
<td>0.118±0.008</td>
</tr>
<tr>
<td>4</td>
<td>6.32±0.44 b</td>
<td>49.01±4.16 b</td>
<td>7.04±0.46</td>
<td>72.01±5.19</td>
<td>1</td>
<td>0.138±0.008</td>
</tr>
<tr>
<td>5</td>
<td>5.40±0.33 b</td>
<td>49.52±0.78 b</td>
<td>6.84±0.62</td>
<td>74.20±1.66</td>
<td>1</td>
<td>0.106±0.004</td>
</tr>
</tbody>
</table>

Figure 2 Conceptual schematic of $\dot{M}O_2$, metabolic oxygen consumption, in response to food deprivation in *Oncorhynchus mykiss* based on Table 1. The dotted line following $P_{\text{crit}}$ is where trout began to loss equilibrium and roll over, at which point the respirometry was stopped.
Figure 3 Enzyme activities in the white muscle of *Oncorhynchus mykiss* in response to food deprivation. (A) LDH, lactate dehydrogenase; (B) PK, pyruvate kinase; (C) CS, citrate synthase; (D) HOAD, 3-hydroxyacyl-CoA dehydrogenase. Treatments sharing the same letter are not significantly different (P<0.05). n=8 for all groups. Data are presented ± SEM.
Figure 4 Enzyme activities in the liver of *Oncorhynchus mykiss* in response to food deprivation. (A) LDH, lactate dehydrogenase; (B) PK, pyruvate kinase; (C) CS, citrate synthase; (D) HOAD, 3-hydroxyacyl-CoA dehydrogenase. Treatments sharing the same letter are not significantly different (P<0.05). n=8 for all groups. Data are presented ± SEM.
**Figure 5** Relative log enzyme activity ratios of the white muscle of *Oncorhynchus mykiss* in response to food deprivation. (A) LDH/CS, lactate dehydrogenase/citrate synthase; (B) HOAD/CS, 3-hydroxyacyl-CoA dehydrogenase/citrate synthase. Treatments sharing the same letter are not significantly different (P<0.05). n=8 for all groups. Data has been log transformed and scaled to 1. All data are presented ± SEM.
Figure 6 Relative log enzyme ratios of the liver of *Oncorhynchus mykiss* in response to food deprivation. (A) PK/LDH, pyruvate kinase/lactate dehydrogenase (B) HOAD/CS, 3-hydroxyacyl-CoA dehydrogenase/citrate synthase. Treatments sharing the same letter are not significantly different (P<0.05). n=8 for all groups. Data has been log transformed and scaled to 1. All data are presented ± SEM.
Figure 7 Adenosine-5'-triphosphate in the white muscle (A) and liver (B) of Oncorhynchus mykiss in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Figure 8 Metabolite concentrations in the white muscle of *Oncorhynchus mykiss* in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). (A) Glu, glucose; (B) Gly, glycogen. Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Figure 9 Metabolite concentrations in the white muscle of *Oncorhynchus mykiss* in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). (A) Pyr, pyruvate; (B) Lac, lactate. Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Figure 10 (A) Redox status and (B) intracellular cytosolic pH in the white muscle of *Oncorhynchus mykiss* in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Figure 11 Metabolite concentrations in the blood plasma of *Oncorhynchus mykiss* in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). (A) Glu, glucose; (B) Lac, lactate. Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Figure 12 Metabolite concentrations in the liver of *Oncorhynchus mykiss* in response to food deprivation (line graph) and food deprivation + hypoxia (bar graphs). (A) Glu, glucose; (B) Gly, glycogen (C) Lac, lactate. Dotted line is an extension of the fed normoxic fish metabolic concentration for conceptual purposes. Treatments sharing the same letter are not significantly different (P<0.05). (*) indicates significant differences (P<0.05) between normoxic and hypoxic groups. n=8 for all groups. Data are presented ± SEM.
Chapter 3

General Discussion

3.1 Overview

It has become apparent that the physiological responses to environmental stressors are highly coordinated amongst different tissues, and metabolic adjustments are aimed at balancing ATP demand and supply. Under fully aerobic, non-stressed conditions, ~95% of the ATP synthesized is derived from oxidative phosphorylation. However, when ATP demand outweighs supply, or when adequate O\textsubscript{2} is not available to act as the final electron acceptor in the electron transport chain, anaerobic energy production must supplement overall ATP production to maintain energetic equilibrium. Oxidative ATP synthesis generates ATP at a rate greater than that of anaerobic processes. However, on a mol-to-mol basis, anaerobic processes are ~15-30 fold less efficient (Hockachka and Lutz, 2001; Wang and Richards, in press). Therefore, the relative contribution of different ATP-producing pathways is directly influenced by the metabolic demands of the cell.

The main physiological challenge associated with food deprivation is maintaining ATP demand and supply while conserving endogenous metabolic substrates. This study observed a generalized decline in metabolic rate and an increased reliance on non carbohydrate-based aerobic energy production. This observation is consistent with numerous studies examining the metabolic response to food deprivation (Jezierska et al., 1982; Aslop and Wood, 1997; Salem et al., 2007; Bayır et al., 2011; Kullgren et al., 2011). Rainbow trout, a carnivorous fish, has limited stores of endogenous carbohydrates, which can be depleted relatively quickly (Sheridan and Mommsen, 1991). Therefore, relying on carbohydrate-based metabolism during starvation is likely not an effective strategy for trout, as it sets temporal constraints on survival. However, trout have significant stores of visceral fats (Jezierska et al., 1982) and muscle protein (Loughna
and Goldspink, 1994). Therefore, during periods of food deprivation, fatty acids and proteins are likely more suitable substrates for ATP-production.

Under O\textsubscript{2} limited conditions, however, oxidative capacities are considerably limited and anaerobic processes must supplement ATP production to maintain an energy balance. The critical [O\textsubscript{2}] at which an oxygen regulator will begin to conform to decreasing environmental [O\textsubscript{2}] is referred to as the \( P_{\text{crit}} \) and is used as an indicator of hypoxia tolerance (Ultsch \textit{et al.}, 1981; Barnes \textit{et al.} 2011). Hypoxia tolerant species, such as the crucian carp (\textit{Carassius carassius}), can maintain their SMR to <10 torr O\textsubscript{2} (Sollid \textit{et al.}, 2003), which is in stark contrast to the 62.8±3.5 torr O\textsubscript{2} observed in rainbow trout in this study. As reviewed by Nilsson and Renshaw (2004), the species with the highest hypoxia tolerance respond rapidly to decreasing [O\textsubscript{2}] by reorganizing their metabolism to limit ATP-consumption while increasing the contribution of anaerobic glycolysis to energy production. The ability to undergo metabolic depression has emerged as the unifying adaptive strategy for hypoxia tolerant species. Metabolic depression enables cellular supply and demand to remain in equilibrium during conditions when ATP production is largely limited to anaerobic processes (Hochachka \textit{et al.}, 1996). Rainbow trout have a relatively high routine metabolic rate (Barnes \textit{et al.}, 2011) and maintain metabolic rate in response to hypoxia through behavioral and physiological mechanisms aimed at increasing O\textsubscript{2} transport to tissues (Richards \textit{et al.}, 2009; Omlin and Weber, 2010). As a result, rainbow trout have been categorized as a hypoxia intolerant species.

3.2 Physiological Responses of the White Muscle to Food Deprivation

Individual organ response patterns to food deprivation were variable and coordinated in a hierarchical fashion in the white muscle. Following three weeks of no feeding the activity of the cytosolic, glycolytic enzymes, LDH and PK, both decreased. In comparison, the mitochondrial enzymes, CS and HOAD activity did not respond to food deprivation. This suggests that
anaerobic energy production was contributing significantly less during starvation. This was exemplified by shift towards an increased reliance on aerobic metabolism as indicated by the ratios of LDH/CS. Further, the ratio of HOAD/CS did not change at any time during the five-week starvation period, suggesting that non-carbohydrate catabolism is directly proportional to overall oxidative capacity.

This metabolic shift towards aerobic energy production was further observed in the redox state of the white muscle. Following four weeks of food deprivation white muscle redox state increased significantly. Redox state is important in the coordination of substrate catabolism (Robergs et al., 2004). An increase in redox state typically favours aerobic processes, and subsequently inhibits anaerobic glycolysis (Williamson et al., 1966). Therefore, it appears that food deprivation results in an increased reliance on aerobic energy production.

Following five-weeks of food deprivation there was a significant decline in [ATP]. It appears unlikely that this decrease in [ATP] is caused by a decrease in hydrolysis, as this would be accompanied by a decrease in pH, (Robergs et al., 2004). Another possibility is that the decrease in [ATP] indicates a decrease in ATP production associated with a decrease in the metabolism of this particular tissue. Compared to other tissues teleost white muscle has the highest glycolytic potential, (Moon and Johnson, 1980), and high rates of protein turnover (Loughna and Goldpsink, 1984). Considering that maintaining protein turnover accounts for ~25% of the overall cellular energetic budget (Salem et al. 2007), depressing white muscle metabolism likely translates into significant energy savings and substrate conservation. This, however, is just a speculation and future investigations into energy charge and the rate of ATP turnover are required to conclusively satisfy these speculations.
3.3 Physiological Responses of the Liver to Food Deprivation

The liver’s response to starvation was markedly different relative to the white muscle. Initial responses to starvation (one week) appeared to be involved in metabolizing carbohydrates. This was indicated by an increase in LDH, the maintenance of PK, and the decrease in liver [Gly]. However, following two weeks of food deprivation livers physiological response changed. Liver PK activity decreased significantly, and LDH activity and [Gly] returned to concentrations similar to those of the fed normoxic fish. In addition, there was a shift towards oxidative ATP synthesis as indicated by the ratio of PK/LDH. This increase in aerobic metabolism was likely supported by non-carbohydrate substrates in the liver. This is because as the overall oxidative capacity increased, as indicated by an increase in CS activity, catabolism of non-carbohydrate substrates increased concurrently as indicated by the increase in HOAD activity. This resulted in the ratio of HOAD/CS to remain 1:1 throughout the course of starvation. Further, the decrease in PK activity potentially suggests a decreased flux in the forward direction through glycolysis, which has been associated with an increase in gluconeogenesis (Wright et al., 1989; Sheridan and Moon, 1990). [ATP] did not fluctuate, potentially suggesting that food deprivation did not result in energetic stress, or metabolic depression of this tissue. Considering that the liver a high capacity to undergo gluconeogenesis (Loughna and Goldpsink, 1984), maintaining the metabolic functioning of the liver seems necessary to maintain adequate supplies of necessary substrates to glucose-dependent tissues.

3.4 Metabolic Depression Induced by Food Deprivation and O$_2$ Consumption

Initial observations suggest that preconditioning the trout into a metabolically depressed state subsequently increased hypoxia tolerance as evidenced by a decreasing $P_{crit}$. A similar relationship between metabolic rate and $P_{crit}$ has been observed in toadfish (Opsanus tau), which led the authors to conclude that $P_{crit}$ may be dependent primarily upon the absolute level of the
organism’s metabolic rate (Ultsch et al., 1981). As a result, it seems that by depressing metabolic rate, the ambient \([O_2]\) where mitochondrial ATP-synthesis is no longer able to meet routine metabolic ATP-demands subsequently decreases.

Another indicator of hypoxia tolerance is the organism’s marginal metabolic scope (MMS). Hypoxia-tolerant species typically exhibit a higher MMS relative to hypoxia sensitive organisms (Del Toro-Silva et al. 2008). Therefore, these species will rapidly decrease the contribution of aerobic metabolism to overall energy production at \([O_2]\) below \(P_{\text{crit}}\). Typically, during environmental hypoxia, these species will rely on anaerobic metabolism utilizing large endogenous liver carbohydrate stores to maintain ATP equilibrium (Waversveld et al., 1989). In this study there was no alteration in the trout’s MMS in response to preconditioned metabolic depression. This means that at a specific PO\(_2\) below \(P_{\text{crit}}\) starved rainbow trout have a higher aerobic capacity. This does not necessarily translate into an increased hypoxia tolerance. Since \(P_{\text{crit}}\) is decreasing, and MMS is not changing in response to starvation, the theoretical \(y\)-intercept is increasing. This suggests that during complete anoxia rainbow trout are unable to completely abandon aerobic metabolism relying entirely on anaerobic metabolism to maintain ATP equilibrium. Therefore, it appears that food deprivation while it increases aerobic capacity may be decreasing anaerobic capacity. This would likely translate into a decrease in hypoxia tolerance. In order to conclude whether or not preconditioning metabolic depression increases tolerance to \(O_2\)-limited environments further research is required. One possible approach may be to observe both the acute and chronic tolerance to hypoxia in metabolically depressed rainbow trout, by observing the time and/or effective concentration until 50% of the trout lose equilibrium and roll over.

This study observed that metabolic depression affected the trout’s oxygen consumption pattern. After two weeks of starvation the appearance of a second critical \([O_2]\) was observed in 88% of the trout, which was observed in all fish following four weeks. This point occurred at
~70-75 torr O₂ and was termed P⁰MET, the partial pressure of O₂ that elicits an alteration in routine metabolism. Following P⁰MET, \( \dot{M}O₂ \) increased until reaching \( P_{\text{crit}} \) which was consistently at a \( \dot{M}O₂ \) of \( \sim 7.5\mu \text{mol min}^{-1}\text{g}^{-1} \). To explore the physiological mechanisms that may be causing the appearance of P⁰MET and the subsequent increase in \( \dot{M}O₂ \) until reaching \( P_{\text{crit}} \), trout were subjected weekly to an 8 h hypoxic exposure of \( \sim 78 \text{ torr} \).

3.5 Physiological Responses of the White Muscle to Hypoxia

During the 8 h exposure to an \([O₂]\) of \( \sim 78 \) torr the white muscle was energetically stressed as evidenced by the decrease in [ATP] and subsequent acidosis. However, following five weeks of starvation there was no acidosis associated with the decreased [ATP], suggesting that the decrease [ATP] was not associated with an increased in hydrolysis. It is possible that following five weeks of food deprivation the metabolic rate of the white muscle had been depressed to such a point that subjecting the fish to O₂ limitations did not result in further energetic stress. Further investigations into ATP-turnover would be required to determine if the white muscle was metabolically depressed. Hypoxia was also observed to increase anaerobic ATP production and during most weekly intervals [Glu] was lower relative to normoxic fish. Interestingly, [Lac] in the white muscle did not change following 8 h of O₂ limitation. However, it has been observed that during hypoxia the white muscle releases lactate into the blood, to be oxidized by other tissues such as the red muscle (Omlin and Weber, 2010). Plasma [Lac] was significantly elevated, and therefore, it is likely that the origin of the lactate was from anaerobic processes in the white muscle.

3.6 Physiological Responses of the Liver to Hypoxia

An 8 h exposure to moderate hypoxia did not affect the [ATP] of the liver, but it did cause a significant increase in liver [Glu] and [Gly]. As a result, it appears that the liver maintained metabolic homeostasis while increasing gluconeogenesis. The increase in liver
gluconeogenesis is likely to be responsible for the increase in plasma [Glu]. This is likely a survival mechanism ensuring that if O$_2$ becomes increasingly limited, appropriate anaerobic substrates are in circulation for quick delivery to dependent tissues, such as the nervous system (Wright et al., 1989).

3.7 Conclusions

To our knowledge, this is the first study to examine how preconditioning fish into a metabolically depressed state via food deprivation influences hypoxia tolerance and subsequent physiological responses to O$_2$-limitation. Three weeks of food deprivation caused metabolic depression, and after four weeks of starvation there was significant decrease in the trout’s $P_{crit}$. This suggests that metabolic depression may increase hypoxia tolerance. However, the observation that food deprivation did not alter MMS may suggest otherwise. Furthermore, as routine metabolic rate decreased, there was the appearance of a second critical [O$_2$], which was associated with an increase in carbohydrate mobilization via gluconeogenesis in the liver leading to hyperglycemia. Overall, this study indicates that food deprivation resulted in metabolic depression and a shift towards oxidative ATP-production in rainbow trout, influencing the physiological responses to hypoxia.
References


Guppy M., and Withers P. 1999. Metabolic depression in animals, physiological


Kieffer J.D., and Tufts B.L. 1998. Effects of food deprivation on white muscle energy reserves in rainbow trout (Oncorhynchus mykiss): the relationships with body size and temperature. Fish Physiology and Biochemistry. 19: 239-245.


