THE EFFECTS OF FASTING AND CHRONIC HYPOXIA ON THE LIPID METABOLISM OF BROOK TROUT (*SALVELINUS FONTINALIS*)

BY

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Table of Contents

Acknowledgements iii

Abstract v

List of Figures and Tables vi

List of Abbreviations ix

Introduction 1

Hypoxia 1

Teleost Metabolism During Normoxia 1

Lipid Metabolism 2

Teleost Metabolism During Hypoxia 6

Objectives 8

Methods 9

Experimental Animals 9

Experimental Design 9

Tagging and pre-experimental body condition 9

Experimental treatments 9

Tissue Sampling 10

Tissue Permeabilization 11

Mitochondrial Respiration Assays 11

LCFA SUIT 12

SCFA SUIT 12

Enzyme Assays 13

β-hydroxy-acyl-CoA dehydrogenase (HOAD) 13

Citrate Synthase (CS) 13

Calculations 13

Body condition 13

Mitochondrial respiration 14

Enzymes 14

Statistical Analysis 15

Results 16

Body Condition and Oxygen Transport 16
Mitochondrial Respiration 17
Fatty Acid Oxidation Enzyme Activity 24
Discussion 26
Body Condition and Oxygen Transport 26
CPT I and Malonyl-CoA Inhibition 27
Fatty Acid Oxidation Enzyme Activity 29
Mitochondrial Respiration 30
Effects of treatment 30
Effects of substrate 31
Limitations and future directions 32
Conclusion 33
References 35

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Much love,
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Abstract

Due to increasing impacts of climate change, hypoxic events are becoming far more frequent, severe, and long lasting. To cope with periods of hypoxia fish can decrease their oxygen requirements by reducing energetically costly activities such as locomotion, growth, and feeding. One way fish may alter their feeding during hypoxia is by undergoing periods of fasting. During normal oxygen conditions, fasting causes an increase in lipid metabolism, as lipids are stored in large quantities and yield high ATP per molecule. However, during acute hypoxia, lipid metabolism is typically down regulated as it requires large amounts of oxygen and can produce harmful levels of reaction oxygen species. Instead, individuals will opt to breakdown carbohydrates which require less oxygen to breakdown but yield less ATP. However, carbohydrate storage is quite limited, especially in comparison to lipid storage. While the metabolic response to acute hypoxia is well understood, the response during chronic hypoxia is still largely unclear. Over prolonged periods of hypoxia, the carbohydrate stores used during acute hypoxia may become depleted resulting in fish having to potentially use lipids to fuel their energy demands, which may result in oxidative damage to the cells.

To determine the effects of chronic hypoxia and fasting on lipid metabolism, we exposed brook trout (*Salvelinus fontinalis*) to 14 days of fasting and normoxia or fasting and hypoxia at 45% dissolved oxygen. We measured the sensitivity of carnitine palmitoyltransferase I (CPT I) - a key enzyme involved in long chain fatty acid transport into the mitochondria - to its endogenous inhibitor malonyl-CoA (M-CoA). We also analysed the maximal activity of 3-hydroxyacyl CoA dehydrogenase (HOAD), a key β-oxidation enzyme, as well as citrate synthase, a TCA cycle enzyme and marker of mitochondrial density. Finally, we measured mitochondrial respiration in ventricular cardiac tissue using both a short-chain (sodium butyrate) and long-chain (palmitoyl-CoA) fatty acid. We found that fish exhibited a significant decrease in the sensitivity of CPT I to M-CoA in the heart following chronic hypoxia, signifying an increased capacity for long-chain fatty acid uptake. In addition, fish exposed to chronic hypoxia had increased oxygen consumption in the heart during complex I fueled respiration. There was no change in enzyme activity or in oxygen consumption when using the short-chain fatty acid. These findings suggest that chronic hypoxia may induce long-chain fatty acid oxidation to support cardiac function despite the potential negative side effects and provides key insights into the impacts of chronic hypoxia on fish physiology and metabolism.

List of Figures and Tables

Figures

Fig 1. The carnitine cycle. Figure created with BioRender.com

Fig 2. The electron transfer system. Reprinted from "Electron Transport Chain", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

Fig 3. Hemoglobin (g/L) of *S. fontinalis* after two weeks of exposure to either control (n = 6), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7) conditions. Values were corrected for teleost fish according to Clark et al. (2008). Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 4. LEAK state respiration (pmol O_2/s/mg) after the addition of glutamate, malate, and palmitoyl-CoA (LCFA) or sodium butyrate (SCFA) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n= 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 5. Oxygen consumption (pmol O_2/s/mg) during CI fueled OXPHOS (OXP-I) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 7), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 6. Respiratory control ratio (RCR) (OXP-I/LEAK) indicative of the mitochondrial efficiency in coupling proton movement to the phosphorylation of ADP to ATP in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by an asterisk. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 7. Average oxygen consumption (pmol O_2/s/mg) following the addition of increasing concentrations of malonyl-CoA (0.05-50µM) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (n = 8), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7). The lines for each treatment group were fit using a four-parameter logistic sigmoidal curve.

Fig 8. Oxygen consumption (pmol O_2/s/mg) during CII fueled OXPHOS (OXP-II) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 7) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 9. Oxygen consumption (pmol O_2/s/mg) due to electron donation directly to CIV in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 7), fasting (LCFA n = 7, SCFA n =7) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 10. 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity (mmol/min/g of tissue) in *S. fontinalis* cardiac muscle, liver, and red muscle (RM) after two weeks of exposure to either control conditions (n = 7), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fig 11. Citrate synthase (CS) activity (mmol/min/g of tissue) in *S. fontinalis* cardiac muscle, liver, and red muscle (RM) after two weeks of exposure to either control conditions (n = 7), fasting (n = 7) or fasting and hypoxia (45% DO; n = 8). Statistically significant differences ($p < 0.05$) within each tissue type are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Tables

Table 1. Average mass (g) of *S. fontinalis* before and after two weeks of exposure to either control (n = 6), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7) conditions. Values are means ± SD. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by different symbols, while differences between before and after within a treatment group are indicated by different letters.

Table 2. The concentration of malonyl-CoA (μM) needed to inhibit carnitine palmitoyltransferase I (CPT I) activity during complex I (CI) fueled mitochondrial oxidative phosphorylation (OXPHOS) by 50% (IC50) in *S. fontinalis* cardiac muscle. Values were determined by fitting the average O_2 consumption for each treatment group across a range of M-CoA concentrations (0.05-50μM) with a four-parameter logistic sigmoidal curve.

List of Abbreviations

DO…………. Dissolved oxygen

ATP………… Adenosine triphosphate

ADP………... Adenosine diphosphate

SCFA………. Short-chain fatty acid

MCFA……… Medium-chain fatty acid

LCFA………. Long-chain fatty acid

ROS…………Reactive oxygen species

TG………….. Triglyceride

FFA………… Free fatty acid

FA…………... Fatty acid

CoA………… Coenzyme A

FA-CoA…….. Fatty-acyl CoA

CPT I……….. Carnitine palmitoyltransferase I

CACT………. Carnitine-acylcarnitine translocase

CPT II………. Carnitine palmitoyltransferase II

M-CoA……… Malonyl-CoA

HOAD………. 3-hydroxyacyl-CoA dehydrogenase

ETS………….. Electron transfer system

TCA…………. Tricarboxylic acid cycle

CI……………. Complex 1

CII…………… Complex 2

CIII………….. Complex 3

CIV………….. Complex 4

CV……………Complex 5

OXPHOS……. Oxidative phosphorylation

LN_2………….. Liquid nitrogen

BIOPS………. Biopsy preservation solution

SUIT………… Substrate-uncoupler-inhibitor-titration

LEAK……….. Non-phosphorylating respiration

P-CoA………. Palmitoyl CoA

RCR………… Respiratory control ratio

IC50………… Half-maximal inhibitory concentration

CS…………… Citrate synthase

V_{max}…………. Maximum velocity

OXP-I……….. CI fueled OXPHOS

OXP-II………. CII fueled OXPHOS

EPHOC……… Excess post-hypoxia oxygen consumption

Introduction

Hypoxia

Oxygen is required by nearly all forms of life to ensure survival. For aquatic animals, periods of low dissolved oxygen (DO) or hypoxia are especially difficult, as obtaining oxygen already poses a challenge, with water holding a mere 3% of the oxygen that can be found in the same volume of air (Hsia *et al.*, 2013; Rubalcaba *et al.*, 2020). Two key drivers of aquatic hypoxia, eutrophication and increasing temperatures, have co-evolved over the last century due to a rise in human activity and impact (Jenny *et al.*, 2016). These anthropogenic impacts can result in changes in water pH, salinity, nutrient composition, and availability, as well as hypoxic events. The resulting hypoxic events are becoming longer lasting and occurring more frequently, which poses severe challenges for fish (Diaz, 2001; Prakash, 2021).

To cope with hypoxic conditions fish can employ several different behavioural, physiological, and anatomical modifications including; 1) relocating to oxygen rich waters, 2) attempting to maintain oxygen delivery, 3) decreasing energy expenditure, and 4) enhancing the efficiency of anaerobic metabolism (Wu, 2002). When relocating to more oxygen rich waters is not possible, fish must make physiological changes to increase or optimize oxygen uptake. These changes can include 1) gill remodeling to increase gill surface area and improve their capacity to take up oxygen from the environment (Laurent and Perry, 1991), 2) increase the production of erythrocytes or 3) increase the oxygen binding capacity of hemoglobin to allow them to carry more oxygen in the blood to be delivered to the tissues (Mandic *et al.*, 2009; Léger *et al.*, 2021). Alternatively, they can also decrease the oxygen demand of tissues to match what is available to them by reducing energetically costly activities such as growth, protein synthesis, reproduction, locomotion, and feeding (Chabot and Claireaux, 2008; Richards, 2010). Lastly, fish can also upregulate anaerobic metabolic pathways such as glycolysis to continue to produce small amounts of ATP without the need for oxygen (Regan *et al.*, 2016).

Teleost Metabolism During Normoxia

In a fed and normoxic state, teleost fish have a variable diet consisting primarily of proteins and lipids, with a limited use of carbohydrates (Tocher, 2003). While proteins and lipids can be used by a whole host of tissues, carbohydrates are almost exclusively used to provide

glucose to the central nervous system and gills (Crockett *et al.*, 1999; Polakof *et al.*, 2012). Lipids are typically avoided by neural cells in the generation of energy under all metabolic states as they can lead to high levels of oxidative stress, which neural tissues are highly susceptible to (Schönfeld and Reiser, 2013). In contrast, proteins are broken down in a wide range of tissues to generate energy and essential amino acids (Andersen *et al.*, 2016). In salmonoid species, proteins make up 35-55% of their diets, with the highest proportions occurring during early life stages when proteins are required for growth (Andersen *et al.*, 2016). Lipids can also be used by numerous tissues in fish, and alongside being a substrate that can generate ATP, they are important in reproduction, membrane formation, and general cell maintenance (Rainuzzo *et al.*, 1997; Tocher, 2003).

Lipid Metabolism

Short-chain fatty acids (SCFAs) such as butyrate ($C_4H_8O_2$) are primarily produced in the digestive system by the breakdown and fermentation of dietary fibers (Zhang *et al.*, 2020). Like medium-chain and long-chain fatty acids (MCFAs, LCFAs), they can act as sources of energy through their breakdown during β-oxidation (Schönfeld and Wojtczak, 2016). While SCFAs and MCFAs produce less energy than LCFAs, they are advantageous since they do not require proteins for binding, transport, or translocation as well as markedly decrease reactive oxygen species (ROS) production (Schönfeld and Wojtczak, 2016). It has been shown that in fasted mice, SCFAs can become important energy sources, potentially accounting for up to 50% of the ATP produced (Schönfeld and Wojtczak, 2016). LCFAs such as palmitoyl carnitine ($C_{23}H_{45}NO_4$) are more abundant than their smaller counterparts but are also acquired primarily from the diet (Tocher, 2003).

Dietary LCFAs absorbed by fish are primarily transported in the form of chylomicrons, which consist of triacylglycerides (TGs) bound to proteins (Van Den Thillart *et al.*, 2002). Absorbed lipids are cleared from the plasma into the liver where they are repackaged and passed onto other tissues to be stored or utilized in various processes (Sheridan, 1988). Before entering cells, TGs are hydrolyzed by extracellular lipoprotein lipase, generating free fatty acids (FFAs) and 2-acyl mono glycerol (Van Den Thillart *et al.*, 2002). These compounds can then be taken up into the cell and either repackaged into TGs for storage or continue to be broken down (Van Den

Thillart *et al.*, 2002). Free fatty acids can also originate via *de novo* synthesis from acetyl-CoA, or be released from the hydrolysis of acylated proteins, phospholipids, or TGs (Longo *et al.*, 2016). The primary lipid storage site can vary between species but the largest deposits in teleost fish are typically found in the liver, muscle myosepta, adipose tissue, and mesenteric membranes (Sheridan, 1988; Van Den Thillart *et al.*, 2002). Once activated from storage sites, lipids must enter the cells. While glycerol, a small lipid soluble molecule, can enter the cell via simple diffusion, FFAs require the aid of a fatty acid transporter and fatty acid binding proteins to cross the lipid bilayer (Tocher, 2003; Schwenk *et al.*, 2010). Once inside the cell, FFAs are still relatively inert and need to be activated via the addition of coenzyme A (CoA) by fatty acyl-CoA synthase (Yan *et al.*, 2015). This generates fatty acyl CoA (FA-CoA), which can then be transported into the mitochondrial matrix.

Although SCFAs and MCFAs containing carbon chains of up to 8 carbons long can freely pass through the inner mitochondrial membrane in their existing form, LCFAs must go through a modification process via the carnitine cycle before crossing into the matrix (Fig. 1) (Schönfeld and Wojtczak, 2016). Once FA-CoA crosses into the intermembrane space, carnitine is added by carnitine-palmitoyltransferase I (CPT I). CPT I is a transmembrane protein, with its catalytic site located on the inside of the outer mitochondrial membrane (Murthy and Pande, 1987; Kerner and Hoppel, 2000). Once the molecule of carnitine is added, the newly formed FA- carnitine is transported across the inner membrane by carnitine acylcarnitine translocase (CACT) (Longo *et al.*, 2016). Upon entry into the mitochondrial matrix, carnitine is removed by carnitine palmitoyltransferase II (CPT II) and FA-CoA is regenerated (Longo *et al.*, 2016). The free carnitine is then cycled back by CACT to be re-used by CPT I, while FA-CoA enters into the β-oxidation pathway (Longo *et al.*, 2016).

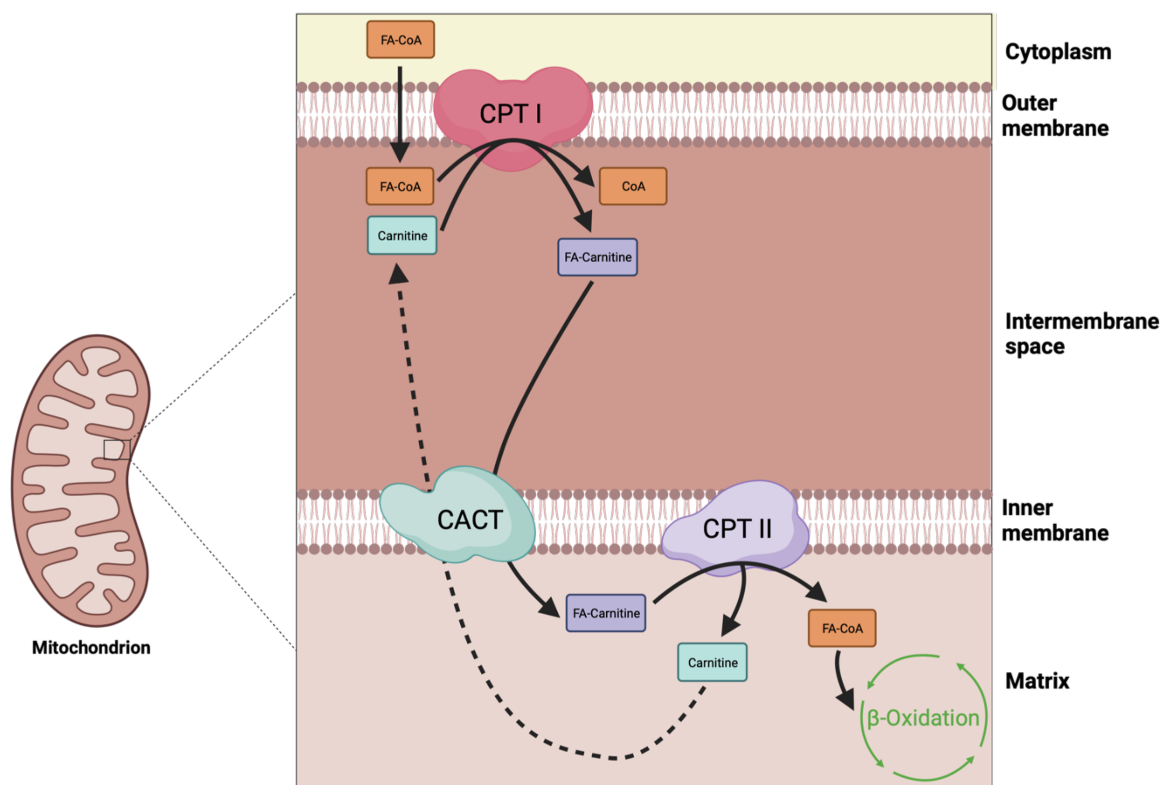

Figure 1. The carnitine cycle. Figure created with BioRender.com.

CPT I is believed to be the rate-limiting enzyme in the carnitine-dependent transport of LCFAs into the mitochondria (Holloway *et al.*, 2006). This enzyme is inhibited as a result of allosteric modulation by the endogenous metabolite malonyl-CoA (M-CoA) (McGarry *et al.*, 1977). M-CoA is generated during the first steps of fatty acid synthesis in the liver, via the carboxylation of acetyl-CoA, and helps to prevent the uptake and oxidation of newly formed lipids (Folmes and Lopaschuk, 2007). The sensitivity of CPT I to M-CoA is highly influenced by variation in mitochondrial membrane fluidity which can occur through changes in temperature, diet, and exercise (Starritt *et al.*, 2000; Morash and McClelland, 2011). CPT I also displays differential sensitivity to M-CoA in different tissues, with CPT I in fish muscle being less sensitive than the liver (Morash and McClelland, 2011). While fasting has been shown to decrease the sensitivity of CPT I to M-CoA (Morash and McClelland, 2011), the effects of hypoxia on this relationship are still unknown.

Once FA-CoA has been successfully transported inside the mitochondria, the fatty acids undergo β-oxidation. β-oxidation is a repetitive process, involving a series of enzymes, including the rate determining 3-hydroxyacyl-CoA dehydrogenase (HOAD), wherein two carbons are cleaved off acyl-CoA generating acetyl-CoA, as well as one NADH, and one $FADH_2$ which can later be used in the electron transfer system (ETS) as electron donors (Houten and Wanders, 2010). The acetyl-CoA generated by this process enters the tricarboxylic acid cycle (TCA cycle). In the TCA cycle, acetyl-CoA gets sequentially reduced, generating 2 CO_2, 3 NADH, 1 $FADH_2$, and 1 ATP (Martínez-Reyes and Chandel, 2020). The NADH and $FADH_2$ produced in the TCA cycle, like those generated in β-oxidation, can then be used as electron donors in the ETS.

The ETS is a series of closely linked protein complexes embedded in the inner mitochondrial membrane near the location of the TCA cycle in the matrix (Fig. 2) (Nolfi-Donegan *et al.*, 2020). The primary role of the ETS is to accept and pass along electrons from various donor molecules to eventually generate energy in the form of ATP. The ETS is composed of four key protein complexes, complex I (CI, NADH oxidoreductase), complex II (CII, succinate oxidoreductase), complex III (CIII, cytochrome C oxidoreductase), and complex IV (CIV, cytochrome c oxidase) (Kuznetsov *et al.*, 2008). Alongside these complexes, cytochrome c (cyt c) and coenzyme Q (CoQ, ubiquinone) also play key roles. The NADH molecules generated during β-oxidation and the TCA cycle are donated to CI, while $FADH_2$ molecules donate their electrons to CII (Gnaiger, 2020). The electrons are passed along to CoQ, then to CIII, shuttled along by cytochrome C, and finally to CIV. Once at CIV, the electrons are used in combination with H^+ to reduce exogenous oxygen into 2 molecules of water (Gnaiger, 2020). In most cells, greater than 95% of the oxygen is consumed in this step as the terminal electron acceptor (Wilson *et al.*, 2012; Kühlbrandt, 2015). This passing of electrons increases their reduction potential and causes a release of energy, which is primarily used to pump protons into the intermembrane space and create a proton gradient (Ahmad *et al.*, 2023). At the end of the ETS, the protons pumped into the intermembrane space return down their concentration gradient and re-enter the matrix through the ATP synthase complex (CV). As they pass through this complex, the movement powers an internal, rotating, motor-like structure generating energy which allows for the phosphorylation of ADP into ATP (Nolfi-Donegan *et al.*, 2020).

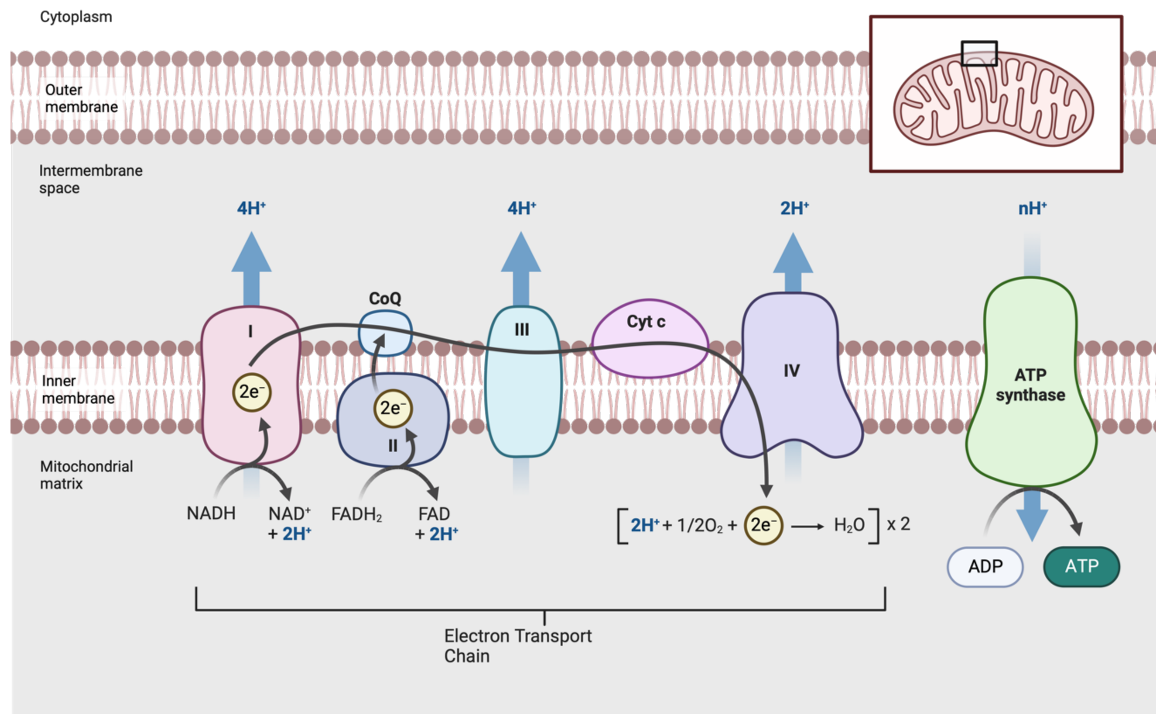

Figure 2. The electron transfer system. Reprinted from "Electron Transport Chain", by Biorender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

Lipid metabolism, while an important source of energy in a basal, non-stressed state for teleost fish, becomes increasingly important during periods of fasting (Bar and Volkoff, 2012; Weil et al., 2013; Ntantali et al., 2023). Under normal oxygen conditions, fasting causes an increase in lipid metabolism since fats generate large amounts of energy, are stored in large quantities, and can be relatively easily mobilized (Morash and McClelland, 2011). This preference for lipid oxidation by many of the tissues allows carbohydrates, such as glucose, to be saved to act as a fuel source for the brain and gills (Crockett et al., 1999; Neumann-Haefelin et al., 2004).

Teleost Metabolism During Hypoxia

One notable response of fish to hypoxia is that they will often undergo periods of fasting as a way to conserve energy, as digestion is energetically costly (Wang *et al.*, 2009). Fasting is a common part of fish life history strategies and individuals may undergo periods of fasting in response to competition, seasonal changes in prey availability, migration, and various physiological stressors such as fluctuations in temperature, salinity, or DO (Xia *et al.*, 2014;

Furne and Sanz, 2017). In contrast to normoxia, lipid oxidation is typically inhibited during periods of acute hypoxia as their breakdown requires high levels of oxygen that is unavailable (Léger *et al.*, 2021). Because of the low oxygen availability, animals will instead opt for anaerobic mechanisms for generating ATP such as substate-level phosphorylation through glycolysis (Leverve *et al.*, 2007; Galli and Richards, 2014). While substrate-level phosphorylation only produces about 1/10 of the ATP of aerobic metabolism, it limits the generation of potentially harmful ROS that can result from oxidative phosphorylation (OXPHOS)(Galli and Richards, 2014). While ROS are generated by, and can be beneficial for numerous physiological processes including cell signaling and protein modification, an excess in their production can be extremely damaging to DNA, RNA, proteins, and membranes, as they are highly reactive molecules (Nemoto *et al.*, 2000; Li *et al.*, 2013). ROS are primarily produced in the ETS via the premature leakage of electrons from complexes I and III causing the reduction of endogenous oxygen to superoxide (O_2^-), which can then be dismutated to form hydrogen peroxide (H_2O_2) (Nolfi-Donegan *et al.*, 2020). The production of ROS has been shown to increase during hypoxia exposure likely due to the lack of oxygen at the end of the ETS to accept donor electrons (Najafi *et al.*, 2014).

Although glycogen acts as an important fuel source during periods of acute hypoxia, carbohydrate storage capacity is limited, especially in contrast with lipid storage (Flatt, 1995). It has been shown that two weeks of fasting causes a roughly 75% decrease in liver glycogen stores in brook trout (Lea, unpublished thesis). While the metabolic response and substrate selection of fish during acute hypoxia is well understood, our understanding of the impacts of chronic hypoxia is extremely limited. Acute hypoxia exposure which typically downregulates lipid metabolism, is often accompanied by periods of fasting which typically upregulates lipid metabolism, so when exposed to both simultaneously fish are likely met with conflicting metabolic signals. Over prolonged periods of hypoxia, the glucose which is used by fish during acute hypoxia may become highly depleted, forcing them to use alternative substrates such as lipids to generate ATP. Alongside a limited knowledge regarding substrate selection in freshwater teleost during chronic hypoxia, it is also unknown if or how the function of CPT I, its sensitivity to M-CoA, and its affinity for FAs, changes during chronic hypoxia exposure. In addition, it is not known if fish may rely on SCFAs or MCFAs instead of LCFAs as a way to produce ATP but

limit ROS production.

Objectives

To understand the response of CPT I and the use of both short and long-chain fatty acids during chronic hypoxia, we exposed brook trout (*Salvelinus fontinalis*), a hypoxia sensitive freshwater teleost fish, to 14 days of hypoxia and fasting, as well as a fasting only group to account for the impacts of fasting alone on lipid metabolism. Following this we measured the sensitivity of CPT I to M-CoA, mitochondrial respiration using both SCFAs and LCFAs, along with various other enzymes important in lipid metabolism. We hypothesized that the sensitivity of CPT I to M-CoA and fish's capacity for lipid breakdown would change with chronic hypoxia exposure, impacting mitochondrial respiration and enzyme activity. We predicted that if fish were breaking down lipids during periods of chronic hypoxia, we would see a decrease in the sensitivity of CPT I to M-CoA, an increase in oxygen consumption during lipid-fueled respiration, and an increase in the activity of lipid breakdown related enzymes. We expected these changes as they would facilitate an increase in lipid metabolism, which we expected to occur after glycogen stores became depleted over time.

Methods

Experimental Animals

Brook trout (*Salvelinus fontinalis*) from Fraser's Mills Fish Hatchery (St. Andrews, NS) were held in 300L holding tanks with circulating well water maintained between 14 and 16°C and small black tarps partially covering the tops of the tanks for shade. Fish were fed once daily except for Sundays, with a 50:50 mixture of Nutra RC 3mm and 4mm sinking pellets (Skretting, St. Andrews, NB, Canada) at a rate of 1% body weight per day and were kept on a 12L:12D light cycle. Our experimental protocol (#102827) was approved by the Animal Care and Use Committee at Mount Allison University, in accordance with the Canadian Council on Animal Care.

Experimental Design

Tagging and pre-experimental body condition

Fish were netted at random and placed in a 5L anaesthetic bath consisting of tricaine mesylate (MS-222, 70mg/L) and a buffer of sodium bicarbonate (140mg/L) for about 1 minute until loss of equilibrium was reached and muscle contraction ceased but regular opercular movements were maintained. Each fish was then injected, in the translucent skin just posterior to the left eye, with unique colour combinations of visible implant elastomer (Northwest Marine Technology, Anacortes, WA, USA). Colour tagged fish were then placed into a recovery bucket with aerated water from their original tank. Once individuals regained equilibrium and normal movements, they were placed into 100L holding tanks, again with circulating well water between 14 and 16°C, for a 10-day acclimation period prior to initiating treatment conditions. Fourteen days prior to sampling, fish were netted at random from their holding tanks and anaesthetized as above. Fish were weighed (±0.1g) and measured (± 1mm). Individuals were then moved into a recovery bucket until equilibrium and normal movements were regained and then placed into their 100L treatment tanks.

Experimental treatments

Treatment 1 – Control. Control fish (n=8) were held in the original acclimation conditions as outlined above for a period of 14 days to establish a baseline of lipid metabolism as well as CPT I's sensitivity to M-CoA, and enzyme activity.

Treatment 2 – Fasting control. Fasted fish (n=8) were held in the conditions outlined above but had food withheld for the duration of the 14 days. The fasted group was used to account for the effects of fasting alone as our hypoxia treatment fish were hypoxic and fasted to mimic the hypoxic response in the wild.

Treatment 3 – Chronic hypoxia. Hypoxic fish (n=8) were held in a 100L tank and were fasted for the 14-day experimental period. A dissolved oxygen concentration of 45 ± 1% was maintained via monitoring and control by various of Loligo® systems instruments. Oxygen data were collected by the Wiltrox 1 oxygen meter and sent to the WiltroxCTRL software program. This information was then relayed on to the DAQ-M which controlled the release of either compressed air or nitrogen into the water to maintain the desired oxygen level. The tank did not have circulating ground water, as this would become too challenging to regulate oxygen levels. Due to the lack of influx of fresh water, the tank was drained to approximately half its volume and then refilled 1-3 times per day to prevent the build-up of toxic ammonia and nitrite. Water was cooled to 15 ± 1°C using a chiller and a water pump was placed in the tank to allow for a constant circulation of water to ensure uniform dissolved oxygen concentration throughout.

Tissue Sampling

After 14 days of exposure to either control, fasted, or hypoxic conditions, fish were placed in a 5L euthanasia bath composed of tricaine mesylate (MS-222; 500mg/L) and sodium bicarbonate (1000mg/L) until reaching total loss of equilibrium with cessation of muscle contraction and opercular movements. The mass (±0.1g) and length (±1mm) of each fish was recorded and a 1.0mL blood sample was taken ventrally from the caudal vein using a heparinized (50 units/mL) 23-gauge needle and 1.0mL syringe. Following retrieval of the blood sample, fish were euthanized via severing of the spinal cord and blood and tissue samples were processed.

Hemoglobin (g/dL) was immediately measured using a Hemocue® Hb 201+ system and microcuvettes. The remaining blood was then placed into a 2.0mL Eppendorf tube. A subsequent small amount was transferred into a heparinized micro-hematocrit capillary tube (Fisher Scientific, Hampton, New Hampshire, USA), and then both samples were placed on ice. The 2.0mL Eppendorf tube was spun at 1300rcf for 4 mins, then plasma was extracted and pipetted

into a 2mL cryovial which was placed in liquid nitrogen (LN_2). Once flash frozen, samples were transferred to a -80°C freezer for later analysis. The micro-hematocrit capillary tube was spun at 12 000rpm for 1 min, and then the ratio of red blood cells to plasma was measured (±1mm). The heart was extracted and the atria and bulbous removed. Half of the heart was sealed in tin foil and flash frozen in LN_2, while the other half was placed in ice cold biopsy preservation solution (BIOPS; 10mM Ca-EGTA buffer, 0.1uM free calcium, 20mM imidazole, 20mM taurine, 50mM K-MES, 0.5mM DTT, 6.56mM MgCl2, 5.77mM ATP, 15mM phosphocreatine, pH 7.1). Samples of both liver and red muscle were extracted, sealed in tin foil, flash frozen in LN_2, and then transferred to a -80°C freezer along with the heart sample for later analysis.

Tissue Permeabilization

The ventricular cardiac tissue in BIOPS was gently teased into small muscle bundles (<1mm) on ice using chilled tweezers. Pieces were transferred to one well of a 6 well plate, on ice with 2mL of BIOPS and saponin solution (20mM). Saponin is a steroid-containing compound which due to its high affinity for cholesterol, can bind to the cholesterol found in the plasma membrane causing it to clump thus creating pores allowing for the diffusion of small molecules into the cell (Kuznetsov *et al.*, 2008). The samples were then placed on ice and onto a nutating mixer (Fisher Scientific, Hampton, New Hampshire, USA) at 8 degrees, 35 speed, for 30 minutes. Following this, tissue pieces were transferred to a clean well with 2mL of BIOPS to remove excess saponin and placed back on the mixer at 8 degrees, 35 speed, for 10 minutes.

Mitochondrial Respiration Assays

To measure the oxygen consumption of the mitochondria in our permeabilized tissue we performed two different substrate-uncoupler-inhibitor-titration (SUIT) protocols using an Oroboros O2K-Fluorespirometer and DatLab software (Oroboros Instruments, Innsbruck, Austria). The 2mL chambers were filled with MiR05 respiration medium (110mM Sucrose, 0.5 mM EGTA, 3mM MgCl2, 60 mM lactobionic acid, 10mM KH2PO4, 20mM Taurine, 20mM HEPES and 0.25g/L BSA, pH 7.4) and kept at 16ºC for all trials. Oxygen consumption was measured in pmol $O_2 \bullet s^{-1} mg^{-1}$. Chambers were calibrated before each assay to 100% air-saturation with MiR05 before adding 4-6mg of permeabilized heart tissue and sealing the chambers closed.

LCFA SUIT

Once the oxygen concentration in the chamber stabilized, we assessed LEAK state respiration by adding the first set of TCA cycle intermediates, glutamate (10mM) and malate (2mM), as well as our 16-carbon fatty acid palmitoyl-CoA (P-CoA ,10µM) and carnitine (0.5mM), which must be added to P-CoA by CPT I in order for the LCFA to pass through the mitochondrial membrane. ADP (5mM) was added to assess OXPHOS I (OXP-I). We then titrated in increasing concentrations of M-CoA (0.05-50µM) to inhibit CPT I activity and generate an inhibition curve. Once inhibition was maximized, we added palmitoyl-carnitine (40µM). Since the carnitine is already bound to P-CoA it can bypass the inhibited CPT I and enter directly into the mitochondrial matrix. Succinate (10mM) was then added to stimulate complex II, followed by cytochrome c (10µM) to assess mitochondrial membrane integrity. Repeated injections of FCCP (5µM) were added until no increase in O_2 flux was observed marking the noncoupled state wherein the mitochondrial membrane potential is notably reduced and the respiratory capacity of the ETS can be evaluated without being limited by the phosphorylation of ADP at CV. To measure the activity of complex II on its own, we added rotenone (0.5µM) to inhibit complex I, followed by antimycin A (2.5µM) to inhibit complex III. To assess complex IV function, we added TMPD (0.5mM) and ascorbate (2mM) which donate electrons directly to CIV, bypassing complexes I – III. Finally, sodium azide (100mM) was added which inhibited all mitochondrial respiration and allowed for the determination of background oxygen consumption in the chambers.

SCFA SUIT

To assess the ability of the mitochondria to use SCFAs we performed a similar SUIT protocol using sodium butyrate, a common 4-carbon SCFA, in a separate chamber. After stabilization of the oxygen concentration, we once again assessed LEAK state respiration by adding glutamate (10mM), malate (2mM) and sodium butyrate (0.1mM). To assess OXPHOS we added ADP (5mM). Since SCFAs do not require the addition of carnitine by CPT I we did not perform the M-CoA inhibition titration and instead proceeded with the rest of the SUIT in the exact same manner as for the LCFA assay.

Enzymes Assays

To extract enzymes for analysis, frozen tissues were ground into a fine powder using a LN_2 cooled mortar and pestle. Approximately 50mg of ground tissue was weighed out and homogenized in 20 volumes of extraction buffer (20mM HEPES, 1mM EDTA, 0.1% Triton X-100, pH 7.4) using a PowerGen 125 tissue homogenizer. (Fisher Scientific, Hampton, New Hampshire, USA). Both enzyme assays were run in duplicates at 25°C, using a SpectraMAX 190 spectrophotometer (Molecular Devices, San Jose CA, USA) and clear 96-well plates.

β-hydroxy-acyl-CoA dehydrogenase (HOAD)

This assay was adapted from McClelland et al. (2005) and contained 50mM imidazole (pH 7.4), 0.1mM acetoacetyl-CoA, 0.15mM NADH, and 0.1% Triton X-100. NADH absorbance was measured at 340nm for 5 minutes. Controls without acetoacetyl-CoA were used to account for any background activity.

Citrate Synthase (CS)

This assay was also adapted from McClelland et al. (2005) and contained 20mM TRIS (pH 8.0), 0.3mM acetyl-CoA, 0.1mM DTNB, and 0.5mM oxaloacetate. The increase in absorbance due to the formation of TNB, was measured at 412nm for 3 minutes. Controls lacking acetyl-CoA were used to account for the background absorbance of each sample.

Calculations

Hemoglobin

Because the Hemocue® Hb 201+ is designed for use on humans, the values given by the system need to be adjusted to accurately represent the hemoglobin concentration in fish. The following formula, where x is the value given by the Hemocue® and y is the corrected value, was determined by Clark et. al (2008) and allows for such a conversion ($r^2 = 0.967$).

$$y = 0.815x - 2.198$$

Mitochondrial respiration

DatLab software was used to calculate the average rate of oxygen consumption over time once the rate stabilized after the addition of each of the substrates, uncouplers, and inhibitors outlined above. To assess the intactness of the mitochondrial membrane we calculated the fractional change in O_2 flux after the addition of cytochrome using the following equation;

$$Fractional\ increase\ in\ O2\ flux = \frac{(oxygen\ consumption\ after\ addition\ of\ cytochrome\ c)}{(OXP\ I/II)} - 1$$

Cytochrome c should not be able to freely enter the matrix and increase oxygen consumption thus an increase greater than 10% would suggest there is damage to the outer mitochondrial membrane.

The respiratory control ratio (RCR) was calculated to evaluate the efficiency of ATP production relative to O_2 consumption. The coupling of the system was calculated using the following equation;

$$RCR = \frac{(OXP\ I)}{(LEAK)}$$

The function of CIV was calculated by measuring the rate of O_2 flux after the addition of TMPD and Ascorbate which donate their electrons directly to CIV, bypassing all other complexes in the ETS. CIV function was calculated as follows;

$$CIV = (oxygen\ consumption\ after\ addition\ of\ TMPD + Asc) - background\ oxygen\ consumption$$

Enzymes

The activity of both HOAD and CS were calculated from absorbance data using the Beer-Lambert law;

$$A = \varepsilon bC$$

Where A is the absorbance of the sample, ε is extinction coefficient, b is the path length, and C is the concentration of the enzyme in the sample. For both enzymes, the path length was 0.596cm. In the HOAD assay we used an extinction coefficient 6220 L/M/cm for NADH, while, in the CS assay the extinction coefficient of DNTB used was 14140 L/M/cm.

Statistical Analysis

All statistical analyses were performed using R (v.4.3.2, R Core Team, 2023), unless otherwise indicated. An alpha significance level of 0.05 was determined *a priori* for all tests. Summary statistics were assessed using "*summarize*" function from the "*tidyverse*" package (Wickham *et al.*, 2019), and plots were generated using the "*ggplot2*" package (Wickham, 2016). The assumption of normality was tested with a Shapiro test as well as visually with "*qqplots*", while homogeneity of variance was assessed with a Levene test (Fox and Weisberg, 2019), as well as visually by plotting standardized residuals. Mass data were analyzed with a repeated measures mixed effects model using the "*afex*" package (Singmann *et al.*, 2024). All measures of mitochondrial respiration (LEAK, OXP-I, RCR, OXP-II, CIV), along with hemoglobin and enzyme data were analyzed using linear mixed effects models from the "*nlme*" package (Pinheiro and Bates, 2000), followed by post-hoc testing using "*emmeans*" (Lenth, 2024). Mass was included as a covariate in the linear mixed effects models due a significant difference in starting masses between treatment groups. Each individual fish ID was also included as a random factor in the mixed models to help account for individual variability. The M-CoA data were plotted, and each treatment group was fitted with a four-parameter logistic sigmoidal curve in GraphPad Prism (v.10.0.0, GraphPad Software, Boston, MA, USA).

Results

Body Condition and Oxygen Transport

We measured the mass of fish both before and after treatment and found that fish's masses were significantly lower after treatment than before ($p = 0.006$), with hypoxic fish showing the largest decrease in mass after treatment (Table 1; $p = 0.053$). We also found that control fish had a significantly lower starting mass than the hypoxic fish (Table 1; $p = 0.022$). Mass was therefore included as a covariate in all subsequent models.

Table 1. Average mass (g) of *S. fontinalis* before and after two weeks of exposure to either control (n = 6), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7) conditions. Values are means ± SD. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by different symbols, while differences between before and after within a treatment group are indicated by different letters.

Treatment Group (n)	Mean Mass Before Treatment (g)	Mean Mass After Treatment (g)	Mean Change in Mass (g)
Control (7)	$144.91 \pm 36.92^{*}$	144.21 ± 50.12	-0.700 ± 24.19
Fasted (7)	$195.96 \pm 57.87^{*+}$	183.74 ± 50.40	-12.21 ± 23.14
Hypoxic (8)	$217.18 \pm 42.82^{+a}$	188.15 ± 34.82^{b}	-29.03 ± 20.03

Hemoglobin concentration (g/L) was measured in fish following two weeks in their respective treatments groups to determine if chronic hypoxia prompted an increase in hemoglobin to facilitate a higher oxygen-carrying capacity. We found that there was no significant difference between the hemoglobin concentration of any of the treatment groups (Figure 3; $p = 0.446$).

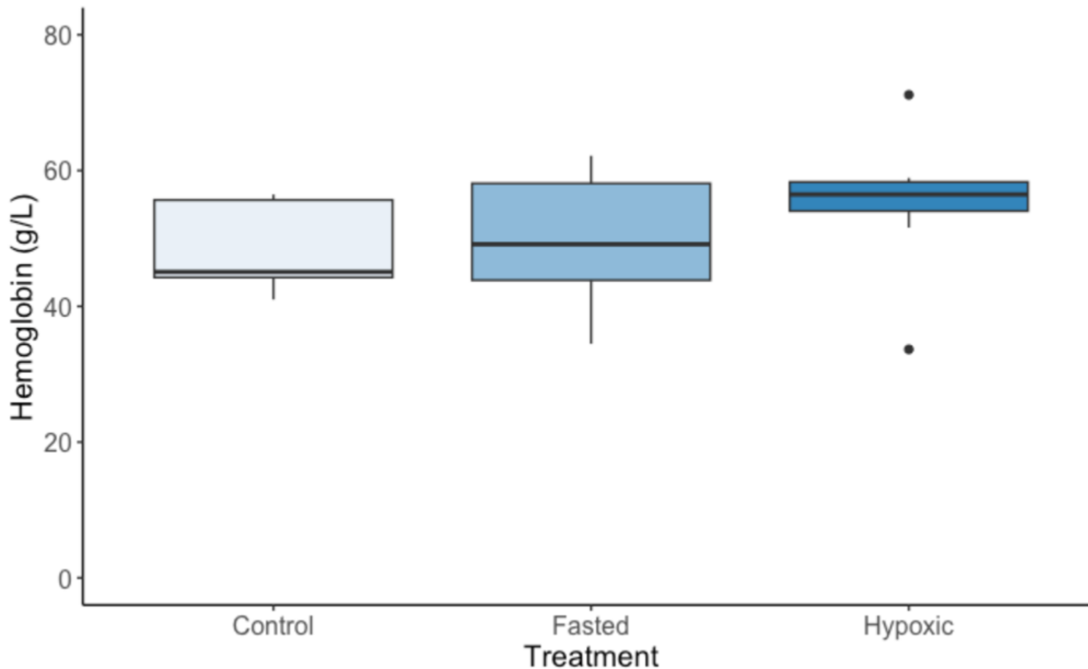

Figure 3. Hemoglobin (g/L) of *S. fontinalis* after two weeks of exposure to either control (n = 6), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7) conditions. Values were corrected for teleost fish according to Clark et al. (2008). Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Mitochondrial Respiration

To assess the effect of chronic hypoxia on lipid-fueled respiration, we provided mitochondria from permeabilized cardiac muscle either a LCFA (palmitoyl-CoA) or a SCFA (sodium butyrate) and measured O_2 consumption. First, we measured LEAK state respiration wherein mitochondria are supplied with TCA cycle intermediates (glutamate, malate) as well as either the LCFA or SCFA. During LEAK, any O_2 consumption observed is due to proton movement across the membrane that is not coupled to ATP synthesis. We found no significant effect of treatment ($p = 0.094$), but we did find a significant effect of substrate (Figure 4; $p = 0.016$).

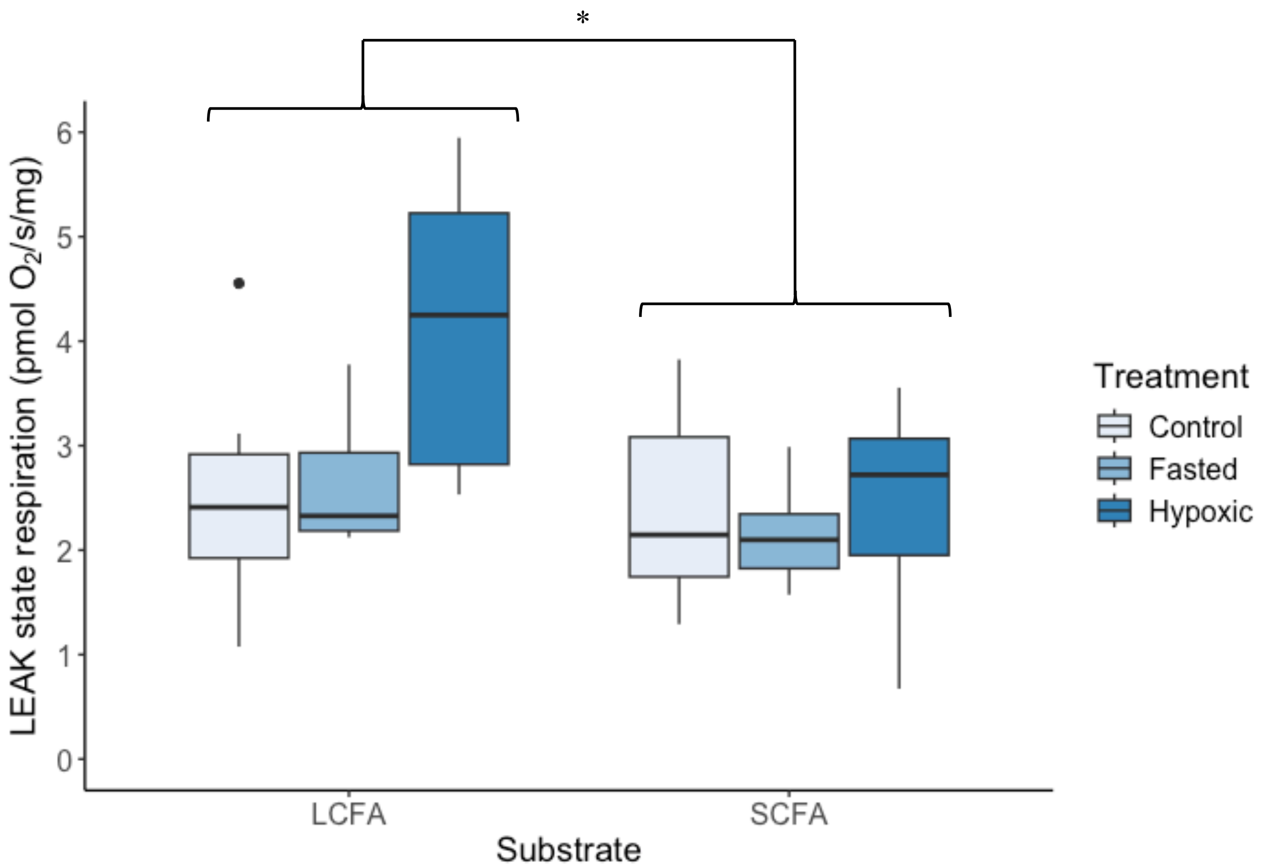

Figure 4. LEAK state respiration (pmol O_2/s/mg) after the addition of glutamate, malate, and palmitoyl-CoA (LCFA) or sodium butyrate (SCFA) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n= 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by an asterisk. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

We next looked at CI fueled OXPHOS (OXP-I), by adding ADP. We found a significant interaction between treatment and substrate ($p = 0.001$). Within the LCFA treatment, hypoxic fish had a nearly two-fold significantly higher O_2 consumption than control fish (Figure 6; $p < 0.001$), however neither differed from fasted fish. In addition, control fish had a significantly higher respiration rate when using the SCFA as opposed to the LCFA ($p = 0.005$).

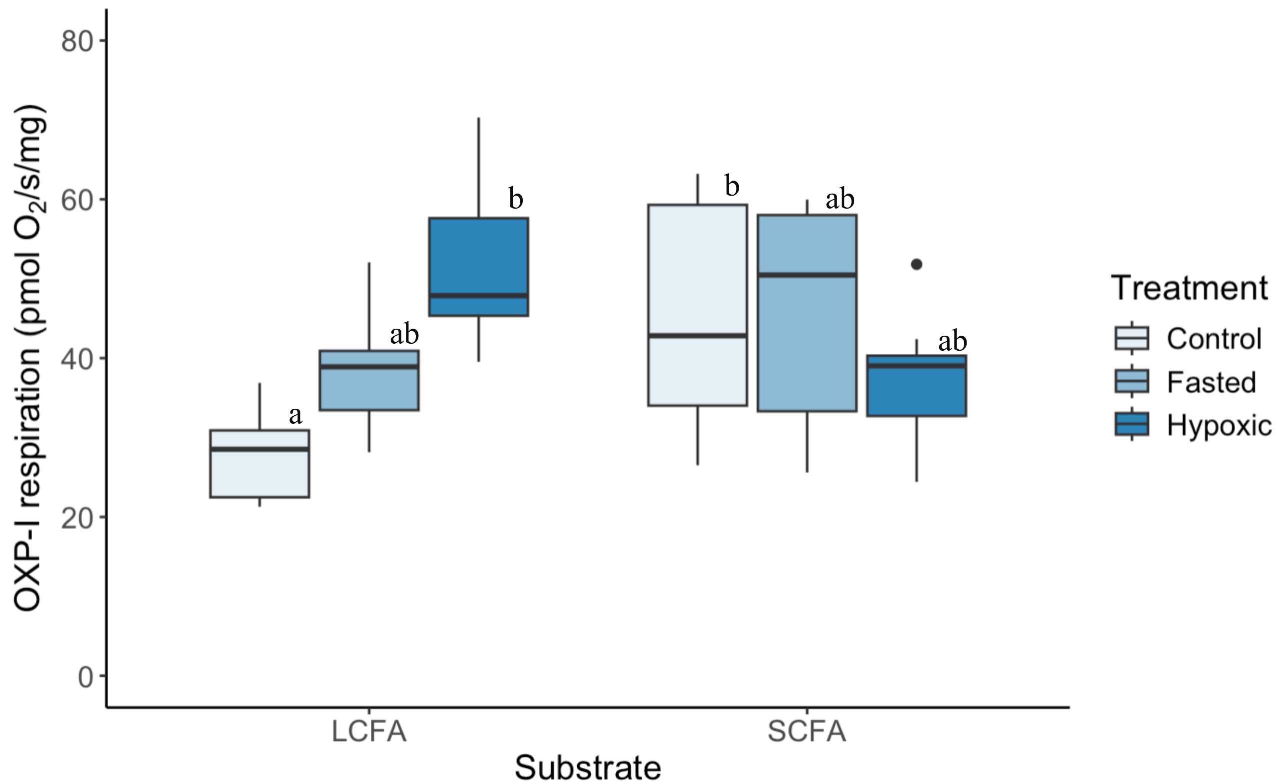

Figure 5. Oxygen consumption (pmol O_2/s/mg) during CI fueled OXPHOS (OXP-I) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 7), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Values for LEAK and OXP-I were then used to calculate the respiratory control ratio (RCR). RCR is a measure of how coupled the movement of protons across the inner mitochondrial membrane is to the phosphorylation of ADP. We found that there was no significant effect of treatment ($p = 0.376$), but that there was a significant effect of substrate type with SCFAs being more highly coupled than LCFAs (Figure 7; $p = 0.002$).

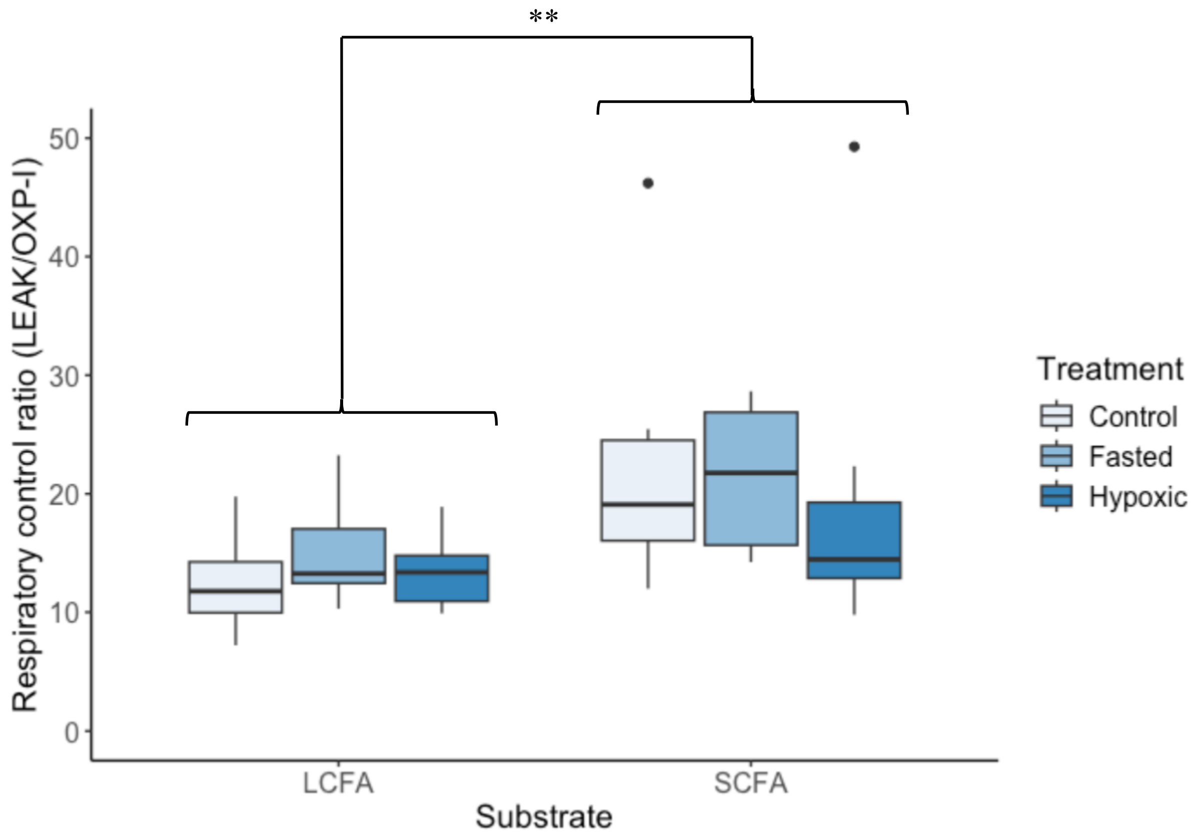

Figure 6. Respiratory control ratio (RCR) (OXP-I/LEAK) indicative of the mitochondrial efficiency in coupling proton movement to the phosphorylation of ADP to ATP in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 6) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by an asterisk. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Then, to determine if the sensitivity of CPT I to M-CoA changed with chronic hypoxia exposure we titrated in increasing concentrations of M-CoA and measured the decrease in O_2 consumption during OXP-I fueled by P-CoA (Figure 7).

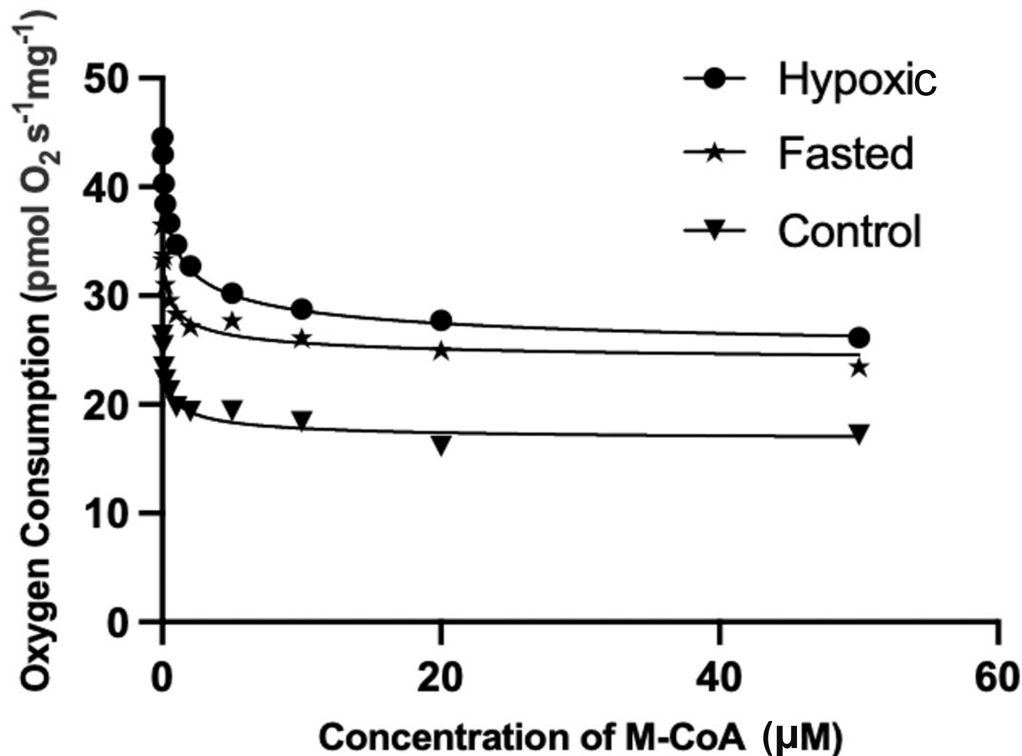

Figure 7. Average oxygen consumption (pmol O_2/s/mg) following the addition of increasing concentrations of malonyl-CoA (0.05-50μM) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (n = 8), fasting (n = 7) or fasting and hypoxia (45% DO; n = 7). The lines for each treatment group were fit using a four-parameter logistic sigmoidal curve.

Using the curves fitted to the values for each treatment group we found that the concentration of M-CoA needed to inhibit CPT I activity by 50% (IC50), was nearly two-fold higher in the hypoxic treatment compared to both control and fasted treatments (Table 2).

Table 2. The concentration of malonyl-CoA (μM) needed to inhibit carnitine palmitoyltransferase I (CPT I) activity during complex I (CI) fueled mitochondrial oxidative phosphorylation (OXPHOS) by 50% (IC50) in *S. fontinalis* cardiac muscle. Values were determined by fitting the average O_2 consumption for each treatment group across a range of M-CoA concentrations (0.05-50μM) with a four-parameter logistic sigmoidal curve.

	Treatment		
	Control (n = 8)	Fasted (n = 7)	Hypoxic (n = 7)
IC50	0.3280	0.3369	0.6522

Following this we assessed CII fueled OXPHOS (OXP-II) by adding palmitoyl-carnitine to bypass the CPT I inhibition, as well as succinate to donate electrons to CII, rotenone to inhibit the flow of electrons through CI. This allowed us to see only the movement of electrons as they are donated to CII. Similar to OXP-I, we found a significant interaction between the effects of treatment and substrate (Figure 8; $p = 0.029$). Interestingly when using the SCFA, control fish exhibited a significantly higher respiration rate than the hypoxic fish, with an increase of about 30% ($p = 0.003$).

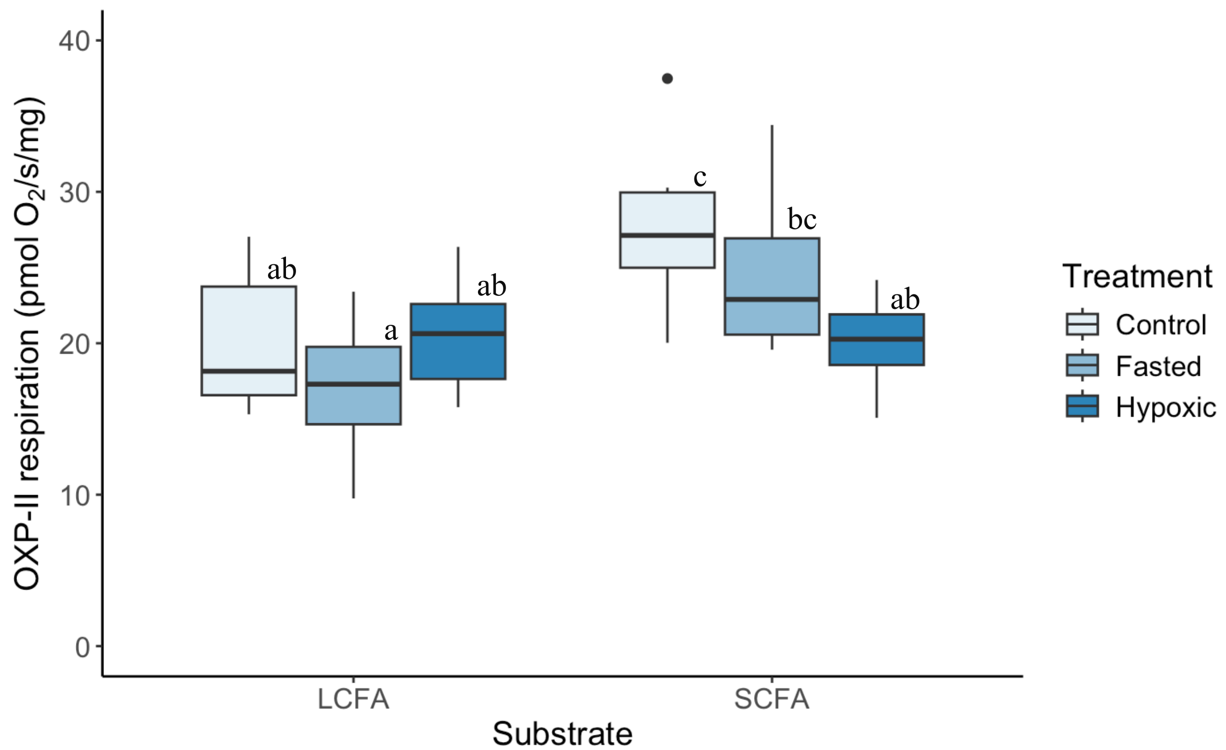

Figure 8. Oxygen consumption (pmol O_2/s/mg) during CII fueled OXPHOS (OXP-II) in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 6), fasting (LCFA n = 7, SCFA n = 7) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

The final mitochondrial respiration measure we assessed was CIV activity. We inhibited the flow of electron through CIII by adding antimycin A, and then donated electrons directly to CIV via the addition of TMPD and ascorbate. Once again, we found a significant interaction between treatment and substrate (Figure 9; p = 0.003). Like in OXP-II there were no significant differences between any of the treatment groups when using the LCFA, but control fish had an approximately 2.5 times significantly higher respiration when using the SCFA in comparison to the LCFA ($p < 0.001$).

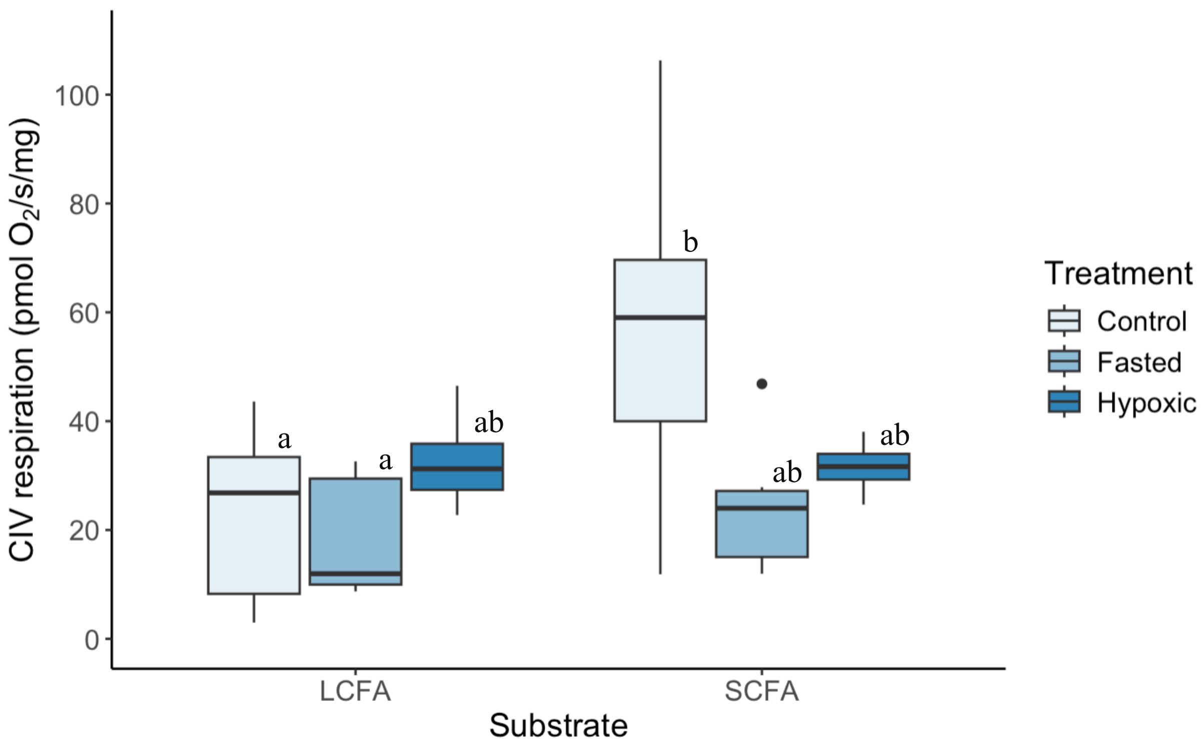

Figure 9. Oxygen consumption (pmol O_2/s/mg) due to electron donation directly to CIV in *S. fontinalis* cardiac muscle after two weeks of exposure to either control conditions (LCFA n = 8, SCFA n = 7), fasting (LCFA n = 7, SCFA n =7) or fasting and hypoxia (45% DO; LCFA n = 7, SCFA n = 8). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Fatty Acid Oxidation Enzyme Activity

To determine if enzyme activity changed in response to fasting or chronic hypoxia exposure and contributed to differences found in mitochondria respiration, we measured a key enzyme in ß-oxidation, 3-hydroxyacyl-CoA dehydrogenase (HOAD). We found that there was a significant interaction between treatment and tissue type (Figure 10; $p = 0.0261$). The difference appears to be driven by higher activity in red muscle, particularly between fasted red muscle and fasted heart ($p < 0.001$), an increase which is not significant within control or hypoxic groups.

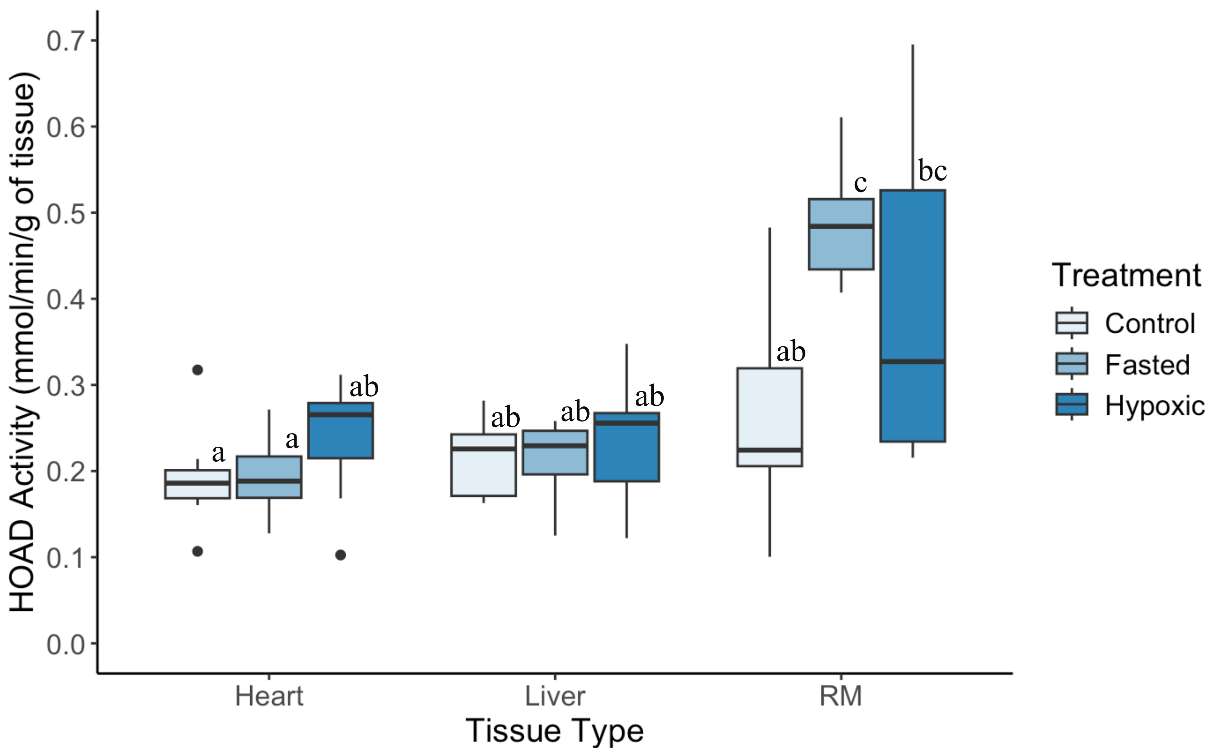

Figure 10. 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity (mmol/min/g of tissue) in *S. fontinalis* cardiac muscle, liver, and red muscle (RM) after two weeks of exposure to either control conditions ($n = 7$), fasting ($n = 7$) or fasting and hypoxia (45% DO; $n = 7$). Statistically significant differences ($p < 0.05$) are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

We also measured the activity of citrate synthase (CS), an important enzyme in the TCA cycle. In addition, CS is often used to assess mitochondria density and can highlight if our treatments were causing changes in the number of mitochondria (Larsen *et al.*, 2012). We again, found a significant interaction between treatment and tissue type (Figure 11; p = 0.0270). There were no significant differences between any of the treatment groups within a tissue type. However, red muscle had a significantly higher activity in all treatment groups than heart and liver.

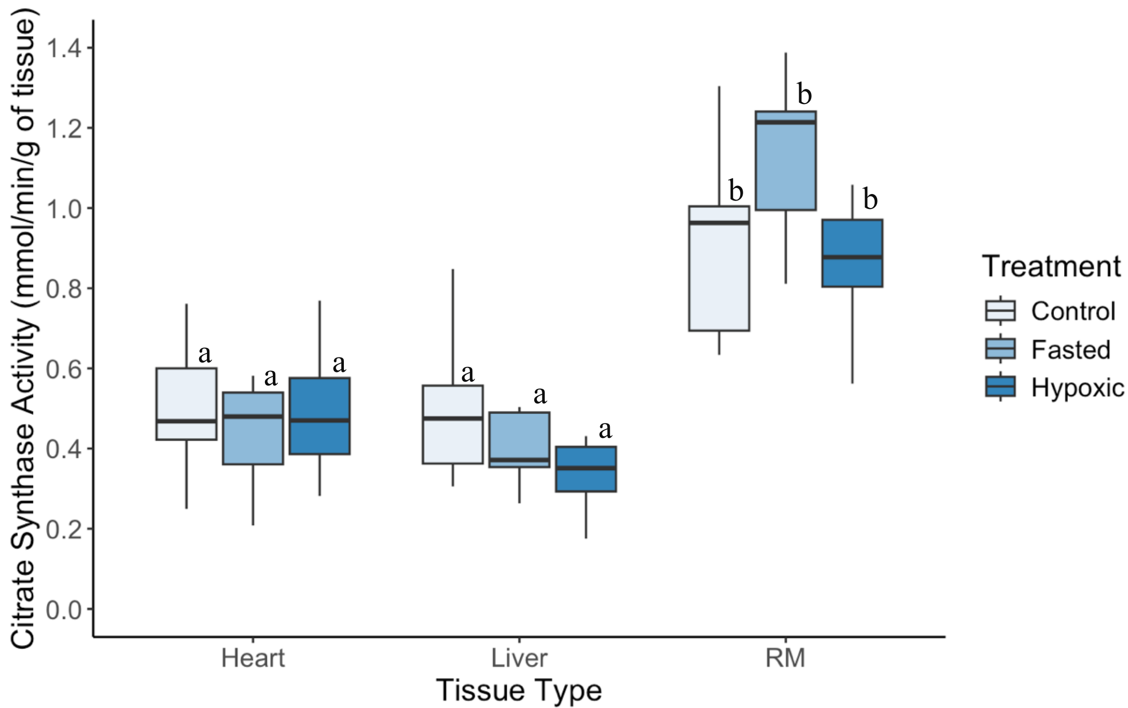

Figure 11. Citrate synthase (CS) activity (mmol/min/g of tissue) in *S. fontinalis* cardiac muscle, liver, and red muscle (RM) after two weeks of exposure to either control conditions (n = 7), fasting (n = 7) or fasting and hypoxia (45% DO; n = 8). Statistically significant differences (p < 0.05) within each tissue type are indicated by different letters. Boxes represent the interquartile range, with the horizontal line as the median value, and whiskers denoting the upper and lower quartiles of the data. Outliers are denoted by black circles.

Discussion

Our study aimed to determine the effects of chronic hypoxia and fasting on the lipid metabolism of brook trout. During periods of fasting in normoxia, fish typically upregulate lipid metabolism as fats are stored in large amounts and have a high energy yield when broken down (Morash and McClelland, 2011). But, during acute hypoxia, lipid metabolism is downregulated to protect from cellular damage, and instead anaerobic glycolysis is used to meet energy requirements (Leverve *et al.*, 2007; Galli and Richards, 2014). During chronic hypoxia, glucose stores likely become depleted and thus fish may be forced to upregulate lipid oxidation despite the potential negative side effects. To examine the response to chronic hypoxia, fish were exposed to fasting, or fasting and chronic hypoxia for 14 days at 45% DO. We found that following hypoxia exposure the sensitivity of CPT I to M-CoA decreased, as well as saw a significant increase in O_2 respiration fueled by CI substrates. Overall, this suggests that chronic hypoxia may induce fat oxidation to support metabolic functions in the heart despite the potential negative side effects.

Body Condition and Oxygen Transport

Fishes often respond to hypoxia in a stepwise fashion wherein they will first avoid hypoxic waters, then attempt to maintain oxygen delivery, and finally as last resorts decrease energy expenditure and increase the efficiency of anaerobic metabolic pathways (Wu, 2002; Wang *et al.*, 2023). While increasing hemoglobin levels is a common physiological response to hypoxia in fishes as a mechanism to maintain oxygen delivery, we did not see this in our study (Mandic *et al.*, 2009; Léger *et al.*, 2021). However, other studies have also found hemoglobin levels remained unchanged after prolonged hypoxia exposure (Cook *et al.*, 2012). It may be possible that under certain conditions, upregulating oxygen delivery processes such as the production of hemoglobin is energetically unfavorable, where the cost of producing the hemoglobin outweighs the overall improvement in oxygen carrying capacity. Under these conditions fish may instead prioritize metabolic rate depression so that their oxygen requirements match what is available in the environment, and then, if necessary, increase mechanisms for oxygen delivery. Substantial decreases in metabolic rate have been observed in trout as a response to acute hypoxia and this likely continues during chronic exposure (Boutilier *et al.*, 1988). This decrease in metabolic rate is often achieved through the downregulation of genes for

protein synthesis, which could potentially account for as much as 41% of the resting metabolic rate in aquatic ectotherms, as well as through decreasing locomotion activity (Wu, 2002; Fraser and Rogers, 2007; Chapman and McKenzie, 2009; Wang *et al.*, 2023). A decrease in locomotion activity, while not assessed using formal behavioural tests, was noted in the chronic hypoxia group in our study, which may suggest this mechanism was being used.

Following the two-week experimental period, we found a significant decrease in the mass of all three treatment groups. All but one of the fasted fish experienced a decrease in mass, likely due to the mobilization and breakdown of lipids being used for energy (Jezierska et al., 1982; Morales et al., 2011; Araújo et al., 2022; Lea, unpublished thesis). This finding also aligns with previous work in a similar freshwater species, largemouth bass, where after 16 days of fasting fish lost an average of 16g (Gingerich *et al.*, 2010). The larger loss in the hypoxic fish, although not significantly more than the fasted fish, may be explained by the use of glycogen during early stages of hypoxia. Each gram of glycogen is accompanied by 3-4 grams of water due to it hydrophilic nature, and therefore when stores are utilised the loss of mass can be highly noticeable (Fernández-Elías *et al.*, 2015; Shiose *et al.*, 2022). This significant loss of mass during hypoxia is consistent with previous work which found that hypoxia not only slowed, but resulted in negative growth at DO levels below 5.0mg/L, which is slightly above our DO content of approximately 4.5mg/L (45% saturation in freshwater at 15°C, 1 atm) (Soderberg *et al.*, 1983; Rosenfeld and Lee, 2022). The difference in starting masses is due to the fact that we could only carry out one treatment at a time, and therefore the hypoxic fish had nearly a month longer to grow prior to the start of the experiment.

CPT I and Malonyl-CoA Inhibition

To determine if fish were modifying their lipid metabolism during hypoxia exposure by altering the sensitivity of CPT I to M-CoA, we supplied the mitochondria with increasing concentrations of M-CoA, measured the change in oxygen consumption, and used it to determine the concentration needed to inhibit respiration by 50%. We predicted that CPT I would be less sensitive to M-CoA in hypoxic fish, as this would support the increased need for an alternate substrate to fuel ATP production as glycogen stores became depleted. We did in fact support our prediction and found that hypoxic fish required nearly twice as much M-CoA to inhibit their

respiration by 50% than either control or fasted fish. A decrease in the sensitivity of CPT I to M-CoA means that more LCFAs can be shuttled across the inner mitochondrial membrane to be broken down in the matrix via β-oxidation. This finding also aligns with previous work on tench (*Tinca tinca*), which found that 6-weeks of exposure to hypoxia resulted in a significant increase in CPT activity, indicating an increased capacity for fatty acid oxidation (Johnston and Bernard, 1982).

The decrease in CPT I sensitivity to M-CoA was not observed in the fasted group, indicating that the change in sensitivity is not driven solely by the effects of fasting. The lack of change in sensitivity in the fasted group is somewhat surprising given previous findings that detected a decrease in sensitivity following fasting in rainbow trout (Morash and McClelland, 2011). However, in their study fasting was over a five-week period, which is 2.5 times longer than fish were fasted in our work. They measured the change in sensitivity only in red muscle and liver but not cardiac tissue, so it is possible that cardiac CPT I sensitivity is regulated differently in this tissue. It has been previously established that different tissues exhibit differential sensitivity of CPT I to M-CoA (Morash *et al.*, 2008), which may also explain why we did not observe this difference in our experiment.

The sensitivity of CPT I to M-CoA is suspected to be influenced by changes in mitochondrial membrane fluidity (Morash *et al.*, 2008). Increases in membrane fluidity may potentially lead to a decrease in the sensitivity of CPT I to M-CoA due to conformational changes that occur in terminal amino groups of CPT I (Jackson *et al.*, 2000). Previous work has established that ischemia, accompanied by hypoxia, can lead to such an increase in mitochondrial membrane fluidity (Jašová *et al.*, 2017). This change, particularly in the heart, may help to facilitate the transport of ATP from the mitochondrial matrix to the cytoplasm to be used by transporters and in other metabolic processes, which would prevent the heart from becoming energy deficient (Jašová *et al.*, 2017). Changes in pH may also impact the sensitivity of CPT I to M-CoA (Starritt *et al.*, 2000), which during periods of hypoxia could be caused by high levels of lactate formed during anaerobic metabolism (Swenson, 2016). Ultimately however, the exact mechanisms which drives the change in the sensitivity of CPT I to M-CoA are still largely unknown, and further work should consider these mechanisms.

Fatty Acid Oxidation Enzyme Activity

If CPT I sensitivity is decreased and more LCFAs are entering the mitochondria, we predicted that the activity of HOAD would also increase to enhance the breakdown of fats in ß-oxidation. However, hypoxia exposure did not lead to any change in HOAD activity relative to control fish. The only differences in HOAD activity were tissue-related and driven by a significantly higher activity in fasted red muscle, which aligns with the fact that fasting often results in an increase in lipid metabolism, which allows glucose to be saved for ROS-sensitive tissue like the brain and gills (Crockett *et al.*, 1999; Neumann-Haefelin *et al.*, 2004; Morash and McClelland, 2011). While hypoxia did not lead to a significant increase in HOAD activity, it also did not lead to a significant decrease in HOAD activity, which has been seen following chronic hypoxia exposure (summarized in Farhat and Weber, 2021). ß-oxidation is often decreased during hypoxia, likely as a way to adjust the ATP supply to match lower energy demands following metabolic rate depression (Farhat and Weber, 2021). It is important to note that for HOAD we measured the maximal activity (V_{max}) *in vitro*. It is possible that the V_{max} of this enzyme is high enough in baseline conditions to support an increased demand for fat breakdown *in vivo* during chronic hypoxia exposure. However, we cannot say that the actual flux through this pathway did not vary under different treatment conditions. To determine whether flux was changing, a labelled fatty acid would need to be followed throughout its breakdown (Crown and Antoniewicz, 2013). Our finding of unchanged HOAD activity following chronic hypoxia exposure indicates that fish are not increasing the maximal activity of ß-oxidation to fuel their energy demands.

We also measured the activity of citrate synthase, an enzyme in the TCA cycle and a marker of mitochondrial density (Larsen *et al.*, 2012), to determine if two weeks of hypoxia caused a change in the number of mitochondria present in the tissues. Once again, we found that chronic hypoxia exposure did not result in significant change in the number of mitochondria. The only difference observed was higher activity in the red muscle in comparison to the heart and liver, a finding consistent with previous work which also found CS to be higher in the red muscle than in other tissues (Morash *et al.*, 2008). This finding is interesting given that most animals have been shown to decrease CS activity during chronic hypoxia exposure to slow flux through the TCA cycle and thus match the decreased demand for ATP production (summarized in Farhat

and Weber, 2021). Chronic hypoxia exposure has also been shown to cause a decrease in the number of mitochondria via autophagy, potentially as a way to reduce the risk of oxidative damage caused by OXPHOS (Spronk and Addink, 1982; Fuhrmann *et al.*, 2013; Farhat and Weber, 2021). The absence of this change may again indicate that the need for the ability to break down a non-glucose substrate during prolonged periods of hypoxia outweighs the potentially harmful effects of ROS.

Mitochondrial Respiration

Effects of treatment

LCFA. We found that two weeks of hypoxia had no significant effect on LEAK state respiration or RCR. This is promising as it indicates that there is not an increase in proton leakage across the membrane, or a decrease in the coupling of this proton movement to the generation of ATP. Overall, these two findings demonstrate that cardiac mitochondrial efficiency is not impaired when using LCFAs during chronic hypoxia exposure in brook trout. We also observed that the activity of both CII and CIV, when using the LCFA, remained largely unchanged after hypoxia exposure. This goes against the typical response of ectothermic animals to hypoxia, which is to downregulate mitochondrial respiration as a protective mechanism to prevent oxidative damage due to ROS formation (Galli and Richards, 2014; Fuhrmann and Brüne, 2017). The lack of changes seen in LEAK, RCR, CII, and CIV when using the LCFA, following chronic hypoxia exposure (1 week up to 6 months) is however, consistent with other findings in sablefish (Gerber *et al.*, 2019), killifish (Du *et al.*, 2016), red drum (Ackerly *et al.*, 2023), snapper (Cook *et al.*, 2012), and goldfish (Farhat *et al.*, 2021), wherein OXPHOS when using pyruvate, remained largely unchanged.

Despite not observing any substantial changes throughout much of the OXPHOS states and complexes, we did find that at CI, hypoxic fish exhibited significantly higher respiration than control fish when using the LCFA. This indicates that hypoxic fish have increased their ability to breakdown LCFAs. Most alterations to the ETS occur at CI, as it is the dominant acceptor of electrons, and therefore changes at this complex provides the most control over electron flow through the rest of the system (Fuhrmann and Brüne, 2017). Changing just one complex also provides the advantage of only expending the energy to modifying a single complex and permits

the rapid return to standard conformation and function, upon the return to normoxic conditions (Fuhrmann and Brüne, 2017). It is also important to note that fasted fish did not exhibit this significant increase and as such the change in respiration at CI during hypoxia cannot be explained by the effects of fasting alone.

SCFA. If fish were not upregulating their lipid metabolism with LCFAs, we hypothesized that they may instead increase the breakdown of SCFAs to fuel their energy requirements. However, like when using the LCFA, two-weeks of hypoxia did not result in any changes in LEAK or RCR. Again, this indicates that the use of a SCFA following two-weeks of hypoxia does not result in excess proton leak or an uncoupling of the OXPHOS system. We also did not observe any statistically significant changes in respiration during OXP-I or at CIV. This further supports that the mechanism causing the increase in O_2 consumption during OXP-I with the LCFA, is unlikely to be processes shared by the two substrates, such as the upregulation of ß-oxidation. Interestingly, we did find that during OXP-II, when using the SCFA, the O_2 consumption of hypoxic fish was significantly decreased in comparison to control fish. This indicates that following two-weeks of hypoxia the fish are not using SCFAs as much to fuel CII respiration. Given that the use of SCFAs has been shown to have protective effects under physiological stress such as cardiac failure, as well as decrease ROS production in comparison to LCFA, this finding is somewhat surprising (Schönfeld and Wojtczak, 2016; Lkhagva *et al.*, 2018). However, some work has shown that SCFAs can cause an increase in O_2 uptake and lower the ATP per O_2 ratio meaning that it may decrease energy generation efficiency, which could explain the downregulation during hypoxia (Schönfeld and Wojtczak, 2016).

Effects of substrate

While treatment did not have an effect, LEAK state respiration was significantly increased and the coupling of proton movement to ATP synthesis was significantly decreased, when fish were given a LCFA in comparison to a SCFA. This may be due to the fact that LCFAs, being larger molecules, are donating more electrons to the ETS and thus there is more proton conductance (Schönfeld *et al.*, 1989). It could also be a result of increased ROS production and subsequent lipid peroxidation, wherein lipid molecules in surrounding membranes have their structure altered by ROS molecules, resulting in membrane damage, which may increase proton

leakage (Brookes *et al.*, 1998; Ayala *et al.*, 2014; Fuchs *et al.*, 2014). In addition, the respiration rate of control fish at CII was significantly higher when using the SCFA in contrast to the LCFA, an effect also seen at CI and CIV. This indicates that in a fed and normoxic state, brook trout may prefer to breakdown SCFAs over LCFAs. This may be linked to our finding with respect to LEAK state and RCR, in that mitochondrial efficiency may be higher with SCFA due to slightly lower proton conductance through the ETS, which could result in less electron backup or reverse electron flow, as well as a decrease in ROS production and lipid peroxidation.

To summarize, chronic hypoxia appears only to alter mitochondria respiration with LCFAs at CI. This may indicate that over prolonged exposure, the need for a substrate to fuel ATP generation outweighs the risk of potential cellular damage during LCFA-fueled OXPHOS. Additionally, hypoxic fish seem to be able to use both SCFAs and LCFAs equally as efficiently. However, control fish may more efficiently fuel OXPHOS with SCFAs over LCFAs, and in baseline conditions SCFAs may provide a more highly coupled ETS than LCFAs.

Limitations and future directions

While not the only cause of hypoxia, there is a well-established relationship between the co-occurrence of high-temperatures and aquatic hypoxia, and therefore there is an increasingly urgent need to understand the confounding impacts of chronic hypoxia and high temperatures (Earhart *et al.*, 2022). High temperatures increase an animal's metabolic rate, while simultaneously decreasing oxygen's solubility in water, leading to an insufficient amount of available O_2 to match the increase in demand (Earhart *et al.*, 2022). Our study was performed at roughly 15°C, which while near the thermal optima for brook trout (Durhack *et al.*, 2021), is unlikely to represent a temperature these fish frequently encounter during hypoxic events. Alongside studying hypoxia at elevated temperatures, understanding the impacts of diverse forms of hypoxia is also important. In freshwater aquatic systems hypoxia typically presents itself in one of four forms a) hypolimnetic, oxygen concentration is stratified and deeper waters are more hypoxic; b) over-winter, cover from ice and snow reduces primary production and blocks the diffusion of air into the water; c) diel, cessation of photosynthesis overnight halts O_2 production which resumes during the day; and d) episodic, rapid nutrient increase from storm runoff/precipitation, or rapid upwelling driven by strong winds or currents (Tellier *et al.*, 2022).

Each form of hypoxia presents unique challenges, including things such as substrate scarcity or excess post-hypoxic oxygen consumption (EPHOC), which could alter the physiological responses observed (Bergstedt *et al.*, 2021).

Also of note, is that in our study, during hypoxia exposure in particular, fish became idle near the bottom of the tank, likely to conserve energy. However, this behavior is unlikely to be feasible in the wild, where they face pressures from predation as well as competition, which would likely cause an increase in metabolism and a more rapid depletion of energy stores. In addition, another factor which may differ in wild fishes, is their diet prior to periods of fasting and hypoxia. Our fish were fed a consistent diet throughout their lifetime leading up to fasting and hypoxia. Availability of food in their natural habitat may vary by season and therefore alter what they have built up in terms of internal substrate storage which could be mobilized during periods of prolonged stress. A final thing to note as a difference between our captive fish and those in the wild, is previous exposure to hypoxic events. Until exposure in our study, the fish had never experienced hypoxic events. However, prior exposure can play an important role in the response to and survival during hypoxia and this could have impacted the response we observed (Fu *et al.*, 2011; Del Rio *et al.*, 2019; Gilmore *et al.*, 2019; Williams *et al.*, 2019).

Future work in this area could explore if other tissues also increase the use of lipids during chronic hypoxia, as well as potentially determine the impacts that this prolonged lipid use may have on ROS production and cellular damage, as we know that ROS levels do not significantly increase following 7 days of hypoxia exposure (Lea, unpublished thesis). Measuring whole animal metabolic rate during hypoxia exposure could also be important as it would help to determine if trout may elect for metabolic rate depression prior to other physiological adaptations such as altering red blood cells or using alternative substrates.

Conclusion

In conclusion, we have demonstrated that chronic hypoxia exposure does impact lipid metabolism in brook trout. Two weeks of hypoxia led to an increase in mitochondrial respiration at CI, likely supported by the decreased sensitivity of CPT I to M-CoA. While we observed no change in HOAD activity, it is possible that *in vivo* flux may have changed even though V_{max} did

not. Overall, it appears that in cardiac muscle that must continue to contract, the need to fuel ATP production during prolonged exposure to fasting and hypoxia may outweigh the risk of increased ROS production and cellular damage that is associated with lipid breakdown. These findings provide key insights into the impacts of chronic hypoxia on metabolism and substrate selection in freshwater teleost fish. If fish continue to experience repeated or prolonged periods of hypoxia, we are likely to see large population declines as a result of direct mortality due to energy imbalance and cellular damage, impaired reproduction, as well as shifts in species composition and diversity due to variation in species' tolerances to hypoxic conditions. Being able to quantify the impacts of hypoxia on fish populations is important for prioritizing high-risk species and areas, as well as developing cost-effective and efficient management strategies regarding the conservation of aquatic species.

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