THE EFFECTS OF PROLONGED FASTING AND CHRONIC HYPOXIA ON SUBSTRATE MOBILIZATION, MITOCHONDRIAL FUNCTION, AND REACTIVE OXYGEN SPECIES PRODUCTION IN CARDIOMYOCYTES OF BROOK TROUT (*SALVELINUS FONTINALIS*)

BY

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Abstract

As average global temperatures rise and human practices promote increased occurrences of oxygen depleting algal blooms, we are seeing more prevalent and longer lasting aquatic hypoxic events that are predicted to worsen. Oxygen is essential for energy production via oxidative phosphorylation, the primary method of ATP synthesis in fish. A lack of oxygen can increase the production of reactive oxygen species (ROS) in the mitochondria, and slow aerobic ATP production. Acute hypoxia typically results in increased glycolysis using stored glycogen as exogenous feeding typically stops. However, we currently do not know what substrates may be used during chronic hypoxia in highly active organs like the heart and how this may impact ROS accumulation. First to understand the effects of prolonged fasting on substrate selection and glucose stores, brook trout (*Salvelinus fontinalis*) were fasted for 6 weeks. Plasma non-esterified fatty acids (NEFA) and glucose were measured every 2 weeks, and total hepatic glycogen stores were calculated. Then to explore the effects of chronic hypoxia on substrate selection and mitochondrial function, brook trout were exposed to normoxia (100% dissolved oxygen; fasted) or hypoxia (45% dissolved oxygen; fasted) for seven days. Following exposure, mitochondrial respiration (using pyruvate or palmitoylcarnitine) and ROS production were assessed in isolated cardiomyocyte mitochondria. Glycogen stores and circulating plasma glucose and NEFA were also measured to understand whole animal substrate use. After fasting we found no significant change in circulating NEFA, and significantly lower circulating glucose and total hepatic glucose stores. In addition, we found no significant difference in circulating plasma glucose, plasma free fatty acids, and total liver glucose after chronic hypoxia exposure. Chronic hypoxia had no effect on mitochondrial function or ROS production, however the use of palmitoylcarnitine showed decreased mitochondrial function and increased ROS production. These results suggest that after chronic hypoxia brook trout glycogen stores are not depleted, and it is unlikely that they would switch to relying on lipids in the heart because of diminished mitochondrial function and increased oxidative stress. This work can be used to predict the physiological and metabolic effects of hypoxic events on brook trout populations as they increase in prevalence and duration due to the effects of anthropogenic climate change.

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List of Abbreviations

CO_2…….. Carbon dioxide

ATP……. Adenosine triphosphate

ADP……. Adenosine diphosphate

FA…… Fatty acid

OXPHOS……. Oxidative phosphorylation

ETS…….. Electron transport system

TCA …… Tri-carboxylic acid cycle

CPT-I …….. Carnitine palmitoyltransferase

CACT …….. Carnitine:acylcarnitine translocase

ETF …….. Electron transferring flavoprotein

ROS ……. Reactive oxygen species

H_2O_2 ……. Hydrogen peroxide

CI ……. Complex 1

CoQ ……. Coenzyme Q

CII …… Complex 2

CIII …….. Complex 3

CytC …… Cytochrome C

CIV …….. Complex 4

CV …… Complex 5

LN_2 …… Liquid nitrogen

LDH …….. Lactate dehydrogenase

SUIT ……. Substrate uncoupler inhibitor titration

PC ……. Palmitoyl carnitine

DTPA …… Diethylenetriaminepentaacetic acid

SOD …….. Superoxide dismutase

HRP …….. Horseradish peroxidase

FCCP …… Carbonyl cyanide-p-(trifluoro-methoxy) phenyl-hydrazone

AMA ……. Antimycin A

TMPD …… tetramethyl-*p*-phenylenediamine dihydrochloride

ANOVA …… Analysis of variance

MANOVA ……. Multivariate analysis of variance

LEAK …… Non-phosphorylating respiration

OXP-I …… CI fueled OXPHOS

RCR …….. Respiratory control ratio

ET ……. Electron transport capacity

OXP-II …….. CII fueled OXPHOS

UCR …….. Uncoupling control ratio

CCO …….. CIV only oxygen consumption

Introduction

Climate Change and Hypoxia

As the effects of climate change continue to worsen, natural environments face potential threats that could impact many animal species populations and the essential ecological interactions between these species and their abiotic surroundings. The warming of earth as a result of anthropogenic climate change is now a heavily studied and accepted phenomenon; global surface temperatures have risen ~0.2ºC each decade between the 1980s and 2010s (Hansen et al., 2006). As of 2020, average global surface temperatures have increased by 1ºC since the preindustrial era (1880-1900) (Wuebbles et al., 2017), with an increase of 1.5ºC being the accepted temperature increase that has the potential to cause wide scale rapid ecological damage (Michaelowa et al., 2018). Fossil fuel combustion causing the continuous introduction of carbon dioxide (CO_2) into the atmosphere is the main anthropogenic driver of climate warming (Höök and Tang, 2013). If little action is taken, climate models predict that by the year 2100 average global atmospheric temperatures will increase by 4.8ºC since the preindustrial era (Meehl et al., 2012). This warming and the loss of biodiversity caused by the overexploitation of 70-75% of ice-free land for human practices, has led to changing and worsening conditions for many species (Arneth et al., 2020, p. 202).

Aquatic environments are not exempt from warming due to atmospheric temperature change. Freshwater species are particularly susceptible to damage from warming due to the important role that thermal regimes play in their distribution and ecological interactions (van Vliet et al., 2013). Water temperatures influence water quality, and this can affect the development of aquatic organisms (van Vliet et al., 2013), and can greatly reduce habitable zones(Eaton and Scheller, 1996). Oxygen solubility into water decreases as the temperature increases, meaning that during extended periods of warm weather, less oxygen can be transferred from the atmosphere to a body of water leading to a lower dissolved oxygen content (Schmidtko et al., 2017). Eutrophication is also a threat to freshwater oxygen content. During the process of eutrophication, excess limiting nutrients such as nitrogen and phosphorus are introduced to a body of water where they promote the excessive growth of algae and cyanobacteria (Zhang et al., 2021), causing a substantial drop in the dissolved oxygen content as the overabundance of plant life dies

and is decomposed by oxygen dependant decomposers (Jenny et al., 2016; Khan and Mohammad, 2014). Instances of eutrophication have been increasing since the 1960s (Y. Zhang et al., 2021), and can be caused by runoff from increased fertilizer use, improper sewage management, and industrial waste (Khan and Mohammad, 2014). Physical heating and eutrophication limit the mixing of surface water with water at lower depths by creating thermoclines and chemoclines, respectively. These differences in temperature and chemical content can limit the natural mixing cycles of freshwater bodies that disperse nutrients essential for the base of food webs, and oxygen dissolved from the atmosphere above. This can lead to areas of hypoxia where the dissolved oxygen content is greatly decreased and poses a threat to life living in these areas (Read et al., 2011).

Both the rise in global atmospheric temperature and increased fertilizer use causing eutrophication are responsible for oxygen depletion of freshwater and coastal environments (Diaz and Breitburg, 2009; Jenny et al., 2016). The compounding effects of these human-induced phenomena have led to the increased occurrence of hypoxic freshwater environments (Jenny et al., 2016). Hypoxic events occur naturally without the influence of human activities especially in freshwater environments with little water movement (Diaz and Breitburg, 2009). Through metabolic acclimation and behavioural changes, fish can endure these acute hypoxic events until normal oxygen conditions return. However, little is known about the physiological and metabolic responses of fish to chronic hypoxia lasting several days to weeks. The future duration and frequency of occurrence of hypoxia are reliant on the rate of anthropogenic climate change, responsible management of industrial and sewage waste, and safe agricultural practices (Diaz and Breitburg, 2009). With the continual warming of the atmosphere and surface waters, along with the unchecked addition of limiting nutrients into aquatic environments, it is expected that hypoxic events will become more severe and last longer (Jenny et al., 2016). Prolonged hypoxia results in many issues for fish development, hormone activity, mate choice, sexual organ development, and most notably for this study, decreased food intake, the downregulation of metabolism, and/or stress on their oxygen dependant ATP production (Iftikar et al., 2010).

Teleost Fish Metabolism

Teleost fish are classified as bony fishes or ray finned fishes and make up a large portion of not only freshwater and marine fish species, but vertebrates in general, with nearly half of the total living vertebrate species belonging to this infraclass (Volff, 2005). The clade Actinopterygii is comprised of over 99% teleosts and makes up more than 95% of all extant fish species, however, they share a closer phylogenetic relation to tetrapods than elasmobranchs and agnathans (Volff, 2005). The incredible genetic, morphologic, and ecologic diversity seen in this group has allowed them to expand their area of occupancy to environments all over the world (Volff, 2005). Because of their wide distribution, they are likely to experience many effects of climate change and human actions including hypoxic conditions that have the potential to become more frequent and last longer.

Brook trout (*Salvelinus fontinalis*) are a species of carnivorous teleosts that live in freshwater environments across North and South America, Africa, Eurasia and New Zealand. They are native to eastern North America but have spread globally through periods of immigration and human introduction since the 1800s (MacCrimmon and Campbell, 1969). Their diet consists of a wide range of aquatic and terrestrial invertebrates as well as several smaller fish species depending on their location (Tiberti et al., 2016). Their optimal water temperature is between 10 and 19ºC, and they require clean, well oxygenated water, and good mixing regimes of rivers, streams and lakes for the best growth and development (Q. Zhang et al., 2021). They are very sensitive to the effects of temperature and habitat change; the survival rate of brook trout at all ages is greatly decreased with an increase in temperature during the summer months (Xu et al., 2010). Populations have seen a decline in viable habitats as a result of anthropogenic climate change and ecosystem alteration (Weltman-Fahs and Taylor, 2013). It has been shown that acute hypoxia tolerance is significantly lower in higher water temperatures but improves through higher temperature acclimation, showing their ability to alter physiological processes to cope with hypoxic conditions (Jensen and Benfey, 2022).

Dietary requirements for teleosts vary, but carbohydrates are not an essential part of their diets. They mainly rely on protein and lipid catabolism for their metabolic needs (Wilson, 1994), with protein being the most important substrate for energy production in a fed state (Amin et al., 2014). Carbohydrates can, however, be an essential energy source

depending on environmental conditions and allow them to reduce their reliance on protein catabolism and improve growth rates (Zhang et al., 2021). The necessary metabolic pathways for carbohydrate digestion and anabolic processes like glycolysis, the tricarboxylic acid cycle, gluconeogenesis, and glyconeogenesis are present in teleosts (Wilson, 1994). Carbohydrates consumed in their diets can be metabolized to generate energy or converted into glycogen for storage and later use (Davison and Goldspink, 1984). An excess of carbohydrate consumption (>200 g Kg^{-1}) can slow growth, and cause issues with antioxidant ability and both lipid and glucose metabolism (Zhang et al., 2021).

Proteins are an important part of brook trout diets as an energy source and to be broken down for use in growth and development. Often, a large percentage of their energy production from exogenous food sources comes from protein catabolism (Sedgwick, 1979). Many aspects of growth are highly dependent on temperature including optimal protein consumption. At 15 and 19ºC, the dietary protein percentages for best growth in brook trout are 39 and 35% respectively (Amin et al., 2014). An appropriate protein to non-protein energy source consumption ratio is important for proper growth as limited protein consumption can lead to the catabolism of proteins rather than allocating them towards development (Amin et al., 2014). It has been shown in several species including brook trout that the use of lipids as an energy source during difficult conditions allows them to save protein from their diet for growth (Sedgwick, 1979). Brook trout have also shown the ability to substitute proteins with carbohydrates to generate energy, as they do with lipids, to save consumed protein for growth (Sedgwick, 1979). During starvation, protein stores for energy production are generally saved until lipid and glucose stores are limited. Protein sources from the liver, spleen, kidneys, and intestines are used during earlier stages of fasting, and as the duration of starvation increases proteolysis for energy production moves primarily to white muscle (Navarro and Gutiérrez, 1995).

Lipids, along with proteins, make up the largest part of the teleost diet for energy production (Henderson and Tocher, 1987), and are necessary for cell maintenance and membrane production (Cowey and Sargent, 1977). These lipids include fatty acids (FA) from dietary sources and endogenously synthesized lipids from their fat stores. In most freshwater fish including brook trout, triacylglycerols are the main source of fats found in their diet (Cowey and Sargent, 1977), and their primary source of calories (Bilinski and

Gardner, 1968). There is a wide range of FA and esters consumed in teleost diets that are stored in their neutral lipid stores; most of which are long-chain polyunsaturated fatty acids (Crockett and Sidell, 1993). Lipid oxidation is favoured over glucose oxidation as an energy source. During migration, internal lipid stores will typically drop as they are metabolized for energy while liver glycogen will only slightly fluctuate under ideal migration conditions (Cowey and Sargent, 1977). Trout have low digestibility of unsaturated fats, and wax esters generally aren't a part of freshwater diets, contrary to the intake of marine teleosts (Cowey and Sargent, 1977). Consumed FA are esterified into triacylglycerol and phospholipids by the glycerol-3-phosphate pathway for storage until required for energy production (Henderson and Tocher, 1987). After a short period of starvation, the concentration of free FA is increased in the blood as they are mobilized from fat stores and moved to active tissues where they are metabolized, but the circulating FA concentration depends on activity level, species, and duration of the fasted state (Bilinski and Gardner, 1968). Heavy reliance on lipids for energy is not ubiquitous across all tissues; teleosts favour the catabolism of carbohydrates in their gills and produce ATP yields 3 to 30 times higher than when using lipids which differs from other aerobic tissues where lipids are favoured during normoxia (Crockett et al., 1999).

White and red muscle types are found in brook trout and most teleosts. White muscle is generally used for short duration high speed swimming often when escaping a potential threat or capturing prey and can generate energy anaerobically using glucose from glycogen stores when oxygen delivery doesn't allow for aerobic energy production (Davison and Goldspink, 1984). The highly vascularized red muscle is used for long distance exercise and uses lipids when swimming at prolonged low speeds when oxygen is readily available (Davison and Goldspink, 1984). When swimming to fatigue, trout use around 70% of their muscle glycogen stores indicating that glucose from glycogen is an important substrate for energy production when oxygen availability to muscle tissue is reduced (Hochachka, 1961). These different dietary substrates are metabolized in the mitochondria to produce ATP. However, changing environmental conditions regulate internal physiological alterations in substrate mobilization and energy production in the mitochondria.

Mitochondria

Mitochondria are the primary site for ATP production in most eukaryotic cells and are responsible for meeting the metabolic needs of the organism as energy requirements fluctuate with changing external and internal conditions (Brown, 2008). In teleost fish their structure is ovular to circular depending on the type of cell, made of an outer and inner lipid membrane with an intermembrane space in between, and a larger interior space called the mitochondrial matrix (Lahnsteiner et al., 2011). These membrane layers house enzymes and enzyme complexes that produce ATP, mediate the transport of substrates for energy production, and provide the ability to hold an ion imbalance in the intermembrane space to generate the electrochemical gradient necessary for ATP production through oxidative phosphorylation (OXPHOS) (Brown, 2008). The inner mitochondrial membrane has a larger surface area than the outer membrane causing it to fold into projections that reach out into the matrix called cristae (Brown, 2008). The enzyme complexes involved in the electron transport system (ETS) that generate ATP through OXPHOS are transmembrane proteins located in the inner mitochondrial membrane along the cristae facing the matrix. The tri-carboxylic acid (TCA) cycle occurs inside the matrix producing electron donors NADH and $FADH_2$ from dietary substrates that are then used in ATP production. The mitochondrial matrix is also the site of fatty acid catabolism to acetyl Co A through the β-oxidation pathway (Brown, 2008).

β-oxidation

The process of lipid catabolism by the β-oxidation pathway, alternately called the β-oxidation spiral, is used in periods of fasting and by organs with high energy requirements (Van Nieuwenhoven et al., 1996). Unlike mammals, fish lack subcutaneous adipose tissue to store fats, instead they use mesenteric tissue, liver, and red muscle as their sites of large-scale fat storage (Van Raaij, 1994). When energy is required, lipids from mesenteric, intramuscular or hepatic storage will be mobilized in the blood in the form of non-esterified fatty acids (NEFA) after they are separated into free FA chains from their triacylglycerol storage state by lipase enzymes (Nguyen et al., 2008). This movement through the blood from areas of fat storage to other tissues is required as most tissues don't have high enough lipid storage capacities to fuel their energy requirements (Kerner and Hoppel, 2000). Because FA are hydrophobic, transport through the blood requires them to be bound to albumin, a protein made by the liver that increases their solubility in the

aqueous environment of the blood or interstitial fluid (Van Nieuwenhoven et al., 1996). When FA in the blood reach target cells, transport proteins in the membrane mediate their movement into the cell (Van Nieuwenhoven et al., 1996), where they attach to cytosolic lipid binding proteins that allow for their movement through the cytosol to the mitochondria for energy production, or to the endoplasmic reticulum for use in membrane production (Ockner et al., 1972).

β-oxidation, occurs in the mitochondrial matrix or peroxisomes. Mitochondrial β-oxidation is the primary method of energy production from lipids as it is closely connected to the ETS, however a number of FA such as very long chain FA and polyunsaturated FA that are poorly metabolized by mitochondrial β-oxidation are catabolized through peroxisomal oxidation (Poirier et al., 2006). Instead of entering the mitochondria for β-oxidation, FA enter small membrane-bound organelles in the cytoplasm called peroxisomes, where they can either be shortened through several cycles of the β-oxidation pathway which generates shorter chain FA that can enter mitochondrial β-oxidation, or completely oxidised to the end products of β-oxidation (Osmundsen et al., 1991). Three of the four enzymes involved in peroxisomal β-oxidation are identical in mitochondrial β-oxidation (Poirier et al., 2006). Because of the high dependence of lipids seen in teleosts, there is evidence that peroxisomal β-oxidation is important in their energy production and can help them deal with the variety of FA they encounter in their diets that aren't able to be metabolized by mitochondria (Crockett and Sidell, 1993). Mitochondrial β-oxidation is more selective for the types of FA that it can process because of the two mitochondrial membrane layers that FA must be transported across before they can enter the pathway. In addition to aiding the mitochondria digest lipids, they also catabolize and remove certain dietary FA that would accumulate due to their inability to be digested by mitochondrial β-oxidation (Crockett et al., 1999). Peroxisomal β-oxidation in teleosts can be initiated by the consumption of different dietary lipids and in cardiac cells it can be induced by partially hydrogenated fish oil (Norseth and Thomassen, 1983), meaning that peroxisomal β-oxidation could play an important role in the digestion of lipids in cardiac cells of carnivorous brook trout that feed on smaller fish species and invertebrates containing these oils.

The movement of long chain FA that are to be metabolized through mitochondrial β-oxidation is facilitated by a series of enzymes located in the mitochondrial membranes as they cannot freely cross these membranes like short and medium chain FA (Nguyen et al., 2008). FA are transferred from lipid storage through the body via the blood as NEFA then are taken up by cells (Frayn, 1998). They are then transported through the cytosol to mitochondria via intracellular lipid binding proteins, and long chain FA are activated into fatty acyl-CoA thioesters by long-chain acyl-CoA synthetases that sit in the outer mitochondrial membrane (Kerner and Hoppel, 2000). Following activation, acyl-CoA binds to free carnitine catalysed by carnitine palmitoyltransferase I (CPT-I) forming long-chain acylcarnitine and allowing it to cross the outer mitochondrial membrane (Kerner and Hoppel, 2000). After crossing the outer membrane into the intermembrane space, carnitine:acylcarnitine translocase (CACT) translocates the long-chain acylcarnitine into the mitochondrial matrix where CPT-II converts them back into acyl-CoA and they can enter the β-oxidation pathway (Kerner and Hoppel, 2000). CPT-I is a key rate determining enzyme for β-oxidation because it controls the amount of FA that can move into the mitochondria. CPT-I is supressed by malonyl-CoA, an intermediate of lipogenesis, which stops the catabolism of free FA while the opposing pathway of FA synthesis is taking place, and with reduced mitochondrial uptake free, FA will be re-esterified into triacylglycerols and transported back to adipose tissue or the liver for storage (McGarry and Brown, 1997).

Once FA enter the mitochondrial matrix, the β-oxidation pathway involves four reactions that generate reducing equivalents $FADH_2$, and an NADH, and shorten medium and long chain acyl-CoAs into two-carbon acetyl-CoA residues that enter into the TCA cycle (Kunau et al., 1995). The first enzyme in this set of reactions is from the family of acyl-CoA dehydrogenase enzymes and the specific member of this family used is related to the FA length (Ghisla and Thorpe, 2004). Acyl-CoA dehydrogenases catalyze the desaturation of acyl-CoA to trans-2-enoyl-CoA by desaturating the α and β carbons producing two electrons that are accepted by the electron transferring flavoprotein (ETF) (Ghisla and Thorpe, 2004). ETF is an electron transfer protein containing FAD that gets reduced to $FADH_2$ by the desaturation of acyl-CoA and carries the electrons to ETF dehydrogenase that moves the electrons into the ETS (Ghisla and Thorpe, 2004). The movement of electrons to ETF makes this a potential site of reactive oxygen species (ROS)

production as superoxide or hydrogen peroxide (H_2O_2) if electrons are released by acyl-CoA dehydrogenase and not accepted by ETF (Quinlan et al., 2013). The second enzyme in the pathway is from the family of enoyl-CoA hydratases that catalyze the hydration of the β carbon of trans-2-enoyl-CoA to 3-hydroxyacyl-CoA, and similar to the first step, different enzymes in this family are specified to different length trans-2-enoyl-CoAs (Agnihotri and Liu, 2003). The third step is catalyzed by L-3-hydroxyacyl-CoA dehydrogenase that produces 3-ketoacyl-CoA from 3-hydroxyacyl-CoA in an NAD^+ dependent reaction where a hydride ion is removed from the substrate and accepted by NAD^+ making NADH (Schulz et al., 2011). The final reaction in β oxidation is the cleaving of a two carbon acetyl-CoA from 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase, a member of the family of thiolase enzymes that can cleave and add to carbon chains (Kunau et al., 1995). This final step leaves the original FA two carbons shorter as the acetyl-CoA is cleaved, this acetyl-CoA enters the TCA cycle to generate NADH that moves electrons into the ETS for ATP production along with the $FADH_2$ and NADH produced during steps one and three of β-oxidation. This cycle continues until all of the carbon tail has been oxidized (Fig 1).

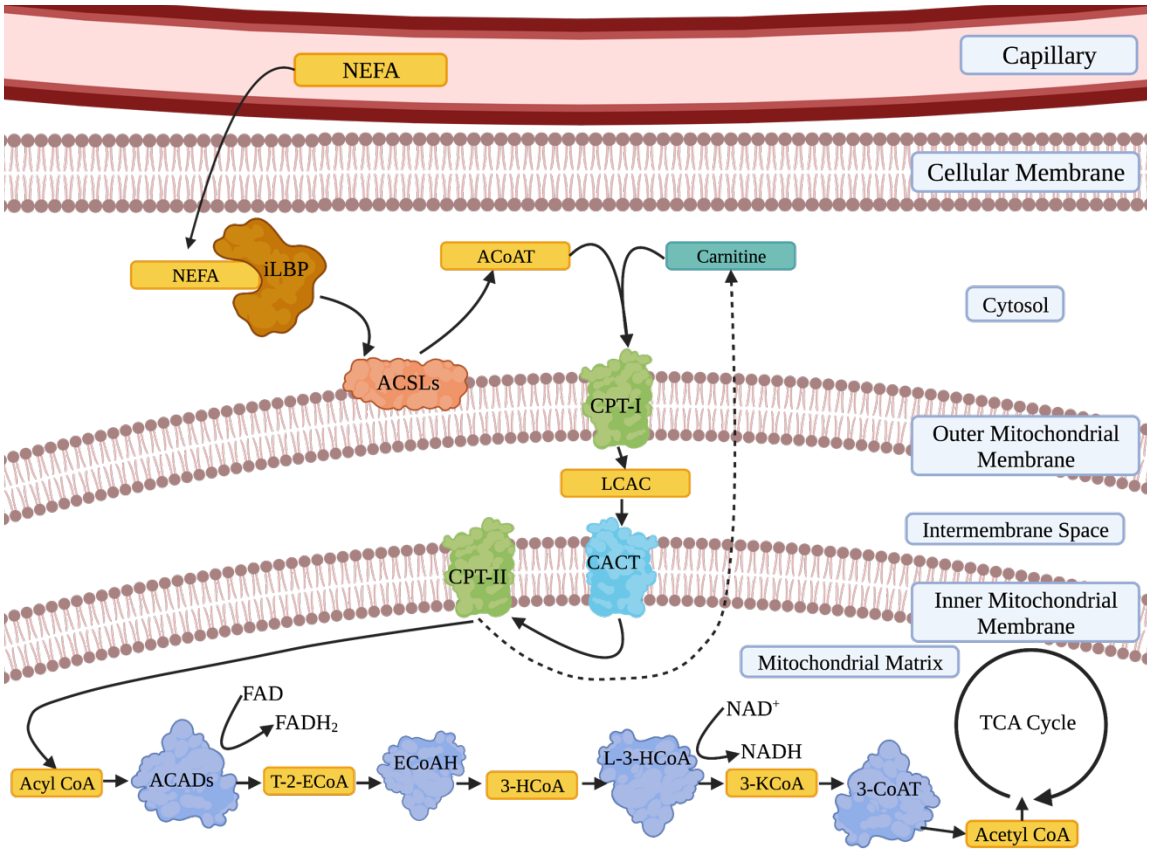

Fig 1. Fatty acid transport from the blood to the mitochondrial matrix, and the beta oxidation pathway oxidizing long chain fatty acids to Acetyl-CoA to enter the TCA cycle. NEFA: non-esterified fatty acids, iLBP: intracellular lipid binding proteins, ACSLs: long-chain acyl-CoA synthetases, ACoAT: fatty acyl-CoA thioesters, CPT-I: carnitine palmitoyltransferase I, LCAC: long-chain acylcarnitine, CACT: carnitine:acylcarnitine translocase, CPT-II: carnitine palmitoyltransferase II, ACADs: acyl-CoA dehydrogenases, T-2-ECoA: trans-2-enoyl-CoA, ECoAH: enoyl-CoA hydratases, 3-HCoA: 3-hydroxyacyl-CoA, L-3HCoA: L-3-hydroxyacyl-CoA dehydrogenase, 3-KCoA: 3-ketoacyl-CoA, 3-CoAT: 3-ketoacyl-CoA thiolase, TCA cycle: tricarboxylic acid cycle. Created with BioRender.com.

Electron Transport System

The ETS is a series of four transmembrane multisubunit protein complexes and free moving electron carriers located in the inner mitochondrial membrane. Electrons are transferred through the complexes facilitating the pumping of protons from the mitochondrial matrix into the mitochondrial intermembrane space (Mitchell, 1961). This process forms an electrochemical proton gradient used to generate ATP from a final protein complex, sometimes considered complex V of the ETS, called F_1F_0 ATP synthase through OXPHOS (Zhao et al., 2019).

Complex I (CI), called NADH-ubiquinone oxidoreductase is the first complex in the ETS. It accepts electrons from NADH generated in the TCA cycle and transfers them

to the mobile electron carrier coenzyme Q (CoQ) that moves in the hydrophobic space of the lipid bilayer of the inner mitochondrial membrane, for every two electrons donated by NADH four protons are pumped through to the intermembrane space (Wikström, 1984). Electrons move through CI to CoQ along a series of iron-sulfur clusters. Rotenone is an inhibitor of CI and acts by blocking the iron-sulfur clusters stopping the flow of electrons and the pumping of protons (Singer and Ramsay, 1994).

Complex II (CI), succinate:ubiquinone oxidoreductase, acts similar to CI as it passes electrons from a reducing equivalent to CoQ, however there are some notable differences. CII is a direct tie to the TCA cycle and catalyzes the reaction of succinate to fumarate coupled with the reduction of an internal FAD prosthetic group to $FADH_2$ which then donates two electrons to CoQ (Sun et al., 2005). While CI and CII both donate electrons to CoQ, the movement of electrons through CII is not complemented by the pumping of protons into the intermembrane space (Sun et al., 2005).

CoQ reduced from CI and CII moves to complex III (CIII), CoQ-cytochrome c reductase, where electrons are accepted by the cytochrome b subunit of CIII and carried to a different mobile electron carrier, cytochrome c (CytC), through a complex electron moving process called the Q cycle (Yang and Trumpower, 1986). Just as in CI, four protons are pumped into the intermembrane space coupled with the movement of two electrons through CIII (Alexandre et al., 1978). Antimycin A is a strong inhibitor of CIII and acts by binding to the cytochrome b subunit blocking the transfer of electrons to CytC causing proton pumping by CIII and electron movement to CytC to halt (Hatefi et al., 1962).

CytC is a mobile electron carrier that moves in the intermembrane space loosely connected to the inner mitochondrial membrane carrying four electrons from CIII to complex 4 (CIV) cytochrome c oxidase (Ferguson-Miller et al., 1976). When the electrons reach CIV, they are rapidly transferred to the site of oxygen reduction where molecular O_2 free in the mitochondrial matrix acts as the terminal electron acceptor in the ETS and is reduced to two H_2O (Ferguson-Miller et al., 1976). When CIV reduces O_2 to H_2O, it interacts with a total of six protons, using four of them to form the two water molecules with the electrons from CytC, and simultaneously pumping the other two protons into the intermembrane space (Wikstrom, 1977). The action of CIV is important in the generation of the electrochemical gradient necessary for the process of OXPHOS and the activity of

ATP synthase as it moves four protons into the intermembrane space, but also uses four protons to form two water molecules in the matrix, which increases the proton gradient between the intermembrane space and the matrix more than any other complex. Sodium azide is an inhibitor of CIV that prevents molecular oxygen from binding to the oxygen reduction site leading to a cessation of the production of water, an increase in the release of free electrons, and a reduction in O_2 usage resulting in mitochondria damage and cell death (Bennett et al., 1996).

The pumping of protons into the intermembrane space by CI, CIII and CIV create the proton gradient necessary for the action of complex V (CV) F_1F_0 ATP synthase that is the site of the majority of ATP synthesis through OXPHOS (Capaldi et al., 1994). CV is a large multisubunit membrane bound protein that extends into the mitochondrial matrix connecting to the intermembrane space to allow for the flow of protons. The inner and outer mitochondrial membranes block the leakage of protons causing the movement of protons through CV down their concentration gradient causing conformational changes to the rotating protein subunits that catalyse the endergonic ADP + P_i to ATP reaction (Capaldi et al., 1994).

Reactive Oxygen Species

When issues with electron flow arise in the ETS, ROS can be produced. ROS are free oxygen molecules containing an unpaired electron that increases their reactivity and are involved in the effects of metabolic stress and essential signalling pathways inside the cell (Alhayaza et al., 2020). While damaging to many components inside the cell, they play an important role in cell signaling pathways regulating apoptosis (Nemoto et al., 2000). However, when ROS production and accumulation increases, they can oxidize components of the cell they come in contact with and lead to damage of many cellular components like membranes, DNA, and proteins and have been linked to the development of many diseases (Alhayaza et al., 2020). Their production during metabolism first described by (Jensen, 1966), occurs predominantly in the mitochondria during the process of oxidative phosphorylation at complexes of the ETS where electrons will occasionally leak out of the system and come in contact with molecular oxygen in the cell producing superoxide free radicals or hydrogen peroxide (Turrens, 2003). The negatively charged superoxide ion is produced by the reduction of oxygen by one free electron and is quickly converted to H_2O_2

either spontaneously or by the antioxidant enzyme superoxide dismutase found in the cytoplasm and mitochondria (Zelko et al., 2002). H_2O_2 can then be partially reduced to the negatively charged free radical hydroxyl radical, one of the most damaging forms of ROS (Turrens, 2003).

CI is the main source of ROS production in the ETS; occasionally small amounts are also produced at CIII if electrons passed from CoQ leak into the matrix (Kowaltowski et al., 2009). Under normoxic conditions, ROS are very rarely generated at CIV where electrons will reduce O_2 to water and generally not be released free into the matrix (Muramoto et al., 2010). Under hypoxic conditions, oxidative damage as a result of increased ROS production can occur when oxygen is not available at CIV and more electrons leak out of the system and reduce molecular oxygen, creating free radicals (Lundby et al., 2003). While it does seem counter intuitive that ROS production increases during periods of lower oxygen concentration, it is used as an alert to hypoxia inside the cell and potentially hypoxic external conditions (Lundby et al., 2003). High levels of lipid catabolism can also increase ROS production in mitochondria by uncoupling the respiration chain, creating many electron donors to the ETS from the breakdown of the large energy rich carbon chains, and limiting ROS scavenging leading to free radical formation and escape into the cell (Cortassa et al., 2017).

Hearts and Hypoxia

The metabolic requirements of different tissues vary and are typically met by aerobic ATP production. However, during exposure to hypoxia, aerobic ATP production is limited and does not meet the demands of the tissue. To balance the supply and demand mismatch during hypoxic conditions, trout can decrease their metabolic demand by limiting energetically costly functions like protein synthesis, searching for food, and digestion which can greatly decrease their oxygen demand (Dunn, 1985). This typically results in the fish limiting food consumption or completely ceasing to feed as the process of digestion is very energy intensive (Magnoni et al., 2018). Even when oxygen may be limiting, the heart is a highly active organ that must maintain normal function for survival. In trout, like all vertebrates, cardiac contractility is the main factor controlling cardiac output and the movement of oxygen, hormones, and nutrients to all cells in the body to maintain homeostasis and life. Hypoxic conditions pose whole organism threats such as

lower metabolic and heart rates to decrease energy demand; however, even in hypoxia with increased bradycardia, teleost hearts still need a constant energy supply and rely heavily on aerobic energy production. Fish ventricles can often become hypoxic due to changing environmental conditions leading to decreased contractile force, making it necessary for them to alter the primary substate used in ATP production from higher energy yielding, but oxygen consuming lipid catabolism, to less oxygen dependant means (Gesser, 2002). Under acute hypoxic conditions (1-3 hours) trout, like most other vertebrates, can switch to the use of phosphocreatine and anaerobic glycolysis to generate some of the ATP required when there isn't sufficient oxygen available for OXPHOS to meet ATP requirements (Dunn and Hochachka, 1986).

Glycolytically produced ATP allows for teleost heart contraction and relaxation during hypoxia (Bailey et al., 2000). In acute hypoxia, it has been shown that trout heart glycogen levels drop significantly, and lactate levels increase indicating that anaerobic glycolysis was present (Dunn and Hochachka, 1986). Even with the activation of anaerobic glycolysis, they found that the concentrations of high energy phosphate compounds used for rapid anaerobic ATP production dropped implying that the cardiac cells were not tolerating the low oxygen environment well and were not relying on anaerobic glycolysis as much as expected. Circulating lactate, the end product of anaerobic glycolysis, increases in rainbow trout during acute hypoxia (1.5 hours) at $2.6 mgL^{-1}$ dissolved oxygen along with an increase in circulating plasma glucose, indicating a heavier reliance on anaerobic glycolysis in extreme hypoxia during short periods of time (Omlin and Weber, 2010). However, this does not show the full picture as even in normoxia, the rate of lactate turnover is quite fast, and hypoxia and exercise increase the rate of utilization and depletion from the plasma (Omlin and Weber, 2010). Rainbow trout are capable of gluconeogenesis and can oxidize lactate in the liver, kidney, red muscle, gills, and heart indicating that they can rebuild their glucose stores for high energy tissues that require glucose during acute hypoxia (Bilinski and Jonas, 1972). However, after exhaustive exercise, the majority of plasma glucose is used to rebuild hepatic glycogen stores rather than muscle glycogen (Pagnotta and Milligan, 1991). While this shows they do have the capacity to rebuild glycogen stores, it is not indicative of their metabolic, or fuel selective response to chronic hypoxia. Other fish species like the crucian carp and goldfish both have excellent strategies

of hypoxic energy production where they produce ATP through anaerobic glycolysis converting lactate to ethanol and CO_2 that can be diffused across the gills out of the body (Shoubridge and Hochachka, 1980). This adaptation is incredibly beneficial because increased lactate buildup through prolonged anaerobic glycolysis without sufficient time to be metabolized can lead to blood acidosis (Shoubridge and Hochachka, 1980). Trout are generally quite sensitive to changes in dissolved oxygen during acute hypoxia through chemoreception in the gills that elicit physiological responses to slow heart rate while increasing stroke volume and opercular movement to increase water flow over gills (Poulsen et al., 2011). Because of the heavy reliance on lipids and low carbohydrate consumption during normoxia, the efficiency of carbohydrate metabolism in these fish is quite low and they will build large glycogen stores in their livers when ingesting carbohydrates (Hilton and Dixon, 1982). These larger hepatic glycogen stores could be beneficial to brook trout during extended hypoxia and provide them with a longer lasting supply of glucose for anaerobic glycolysis during long hypoxic events.

As hypoxic events are becoming more frequent, lasting longer, and covering larger areas around the globe resulting from human activities, it is important to understand the physiological and metabolic responses of freshwater fish exposed to these environments. If in chronic hypoxia these fish are in a fasted state to reduce oxygen and ATP demand and relying more heavily on their relatively limited glucose stores for anaerobic glycolysis, it is possible that without dietary glucose intake, their glucose stores could become limited or depleted. Fasting along with other behavioural and physiological adaptations, can significantly decrease their oxygen demand, but some highly active tissues like the heart still require a significant ATP supply to function properly for survival. Because of this, they may need to switch or supplement their use of glucose with an alternate energy substrate which would likely be their abundant lipid stores; however, this comes with two major issues. First, the requirement for oxygen, as lipid oxidation produces ATP through OXPHOS by generating electron donors through the TCA cycle for the ETS which requires oxygen. Second, the increase in ROS production associated with hypoxia and lipid oxidation that could lead to a rise in cell damage and whole organism effects. The acclimation to acute hypoxia and the associated metabolic and behavioural effects have been more heavily studied (Dunn, 1985; Dunn and Hochachka, 1986; Farhat et al., 2021;

Gattuso et al., 2018; Iftikar et al., 2010; Jensen and Benfey, 2022; Omlin and Weber, 2010; Poulsen et al., 2011; Q. Zhang et al., 2021); however, there is a lack of information on the metabolic effects and responses of freshwater fish to chronic hypoxia. If alternate substrates can be efficiently used and proper mitochondrial function can be maintained throughout chronic hypoxia, we will have a better understanding of how freshwater fish physiologically respond to chronic hypoxia and provide information to predict how specific species and aquatic environments will react to the increasing threat of chronic hypoxic events.

Objectives

The overall objective of this thesis is to examine the effects of chronic hypoxia on fatty acid use in cardiomyocytes of brook trout. However, since fish normally fast when exposed to severe hypoxia, we first wanted to determine the changes in glycogen stores and substrate mobilization elicited by prolonged fasting alone. Brook trout were fasted for six weeks, and blood and liver samples were taken to determine the mobilization of energy substrates throughout the body, and total hepatic glucose stores. After six weeks of fasting, we predicted that if total liver glucose stores became limited or depleted, then we would see an increase in circulating NEFA to meet energy requirements.

Then knowing the impact of fasting alone, we wanted to see if chronic hypoxia and fasting induce an increase in lipid metabolism and a change in circulating metabolites. In addition, during the hypoxia exposure we also measured plasma lactate levels that can be metabolized in hypoxic conditions and acts as an indicator of anaerobic glycolysis. We predicted that after one week of chronic hypoxia, there would be an increase in circulating glucose, no change in circulating NEFA, an increase in blood lactate, and a decrease in total hepatic glycogen stores as they rely more heavily on anaerobic glycolysis in response to the low oxygen environment.

Additionally, if NEFAs were being relied on during hypoxia, we wanted to explore the impact of lipid use on ATP and ROS production and how this could change from exposure to chronic hypoxia. We predicted that chronic hypoxia would have a negative influence on mitochondrial function and induce increased ROS production. We also expected to see an increase in ATP and ROS production when using lipids. The results from these experiments will give us insight into the metabolic responses caused by hypoxia

and help us predict how these fish might cope physiologically with the increasing risk of chronic hypoxia exposure in the natural environment.

Methods

Experimental Fish

Brook trout (*Salvelinus fontinalis*) were obtained from The University of New Brunswick Fredericton breeding program and held in indoor circular 300L holding tanks with recirculating ground water cooled between 14 and 16ºC. Strips of black plastic partially covered the tank opening to provide shade. A period of one month was given to acclimate fish to lab conditions. Fish were fed once daily with Nutra RC 3mm sinking pellets (Skretting, St. Andrews NB) at a rate of 1.5% body weight per day and held under a 12L:12D light-dark cycle. Our experimental protocol (#102827) was approved by the Animal Care and Use Committee at Mount Allison University, in accordance with the Canadian Council of Animal Care.

Experimental Design

We conducted two separate experiments to understand the effects of fasting alone on substrate mobilization, and the effects of combined hypoxia/fasting on substrate use and mitochondrial function. In both experiments, before separation into exposure groups, fish were randomly netted and anesthetized for ~ 45s in a 5L bath containing tricaine mesylate (MS-222) anesthetic (0.1g/L) and sodium bicarbonate buffer (0.2g/L) until cessation of movement and loss of equilibrium. Fish were weighed (g), measured (mm), and then injected just below the skin in translucent tissue directly posterior to each eye with Visible Implant Elastomer (Northwest Marine Technology) in colour patterns unique to each fish. Tagged fish were placed in a recovery tank of fresh oxygenated water to regain equilibrium and normal swimming patterns, then placed into their respective exposure tanks.

Experiment I – Effects of fasting

Control fish (n = 10) were held in standard holding conditions described above and sampled after two (n = 5) and six (n = 5) weeks. Fasted fish (n = 20) were held in standard holding conditions without feeding and were sampled after two (n = 6), four (n = 6), and six (n = 8) weeks of fasting.

Experiment II – Effects of Chronic Hypoxia and Fasting

Control fish for hypoxia exposure (n = 8) were held in standard holding conditions with no access to food and were sampled after one week. Hypoxic fish (n = 8) were held

in standard holding conditions as described previously, however the dissolved oxygen concentration in the water was kept steady between 45 and 47% air saturation by bubbling in air or nitrogen to displace oxygen, controlled using a Loligo Systems (Viborg, Denmark) Witrox 4 optic oxygen meter. They were also unfed to mimic the eating habits of hypoxic fish. Fish were sampled after one week of chronic hypoxia.

Sample Collection

Following exposure to control, fasted, or hypoxic conditions, fish were anesthetized by submersion in a 5L – 20L bath of an overdose of MS-222 (Syncaine) anesthetic (0.2g/L) and sodium bicarbonate buffer (0.4g/L) until loss of equilibrium and opercular flap movement. They were then weighed (g) and measured (mm) before removal of a 10mL blood sample from the caudal vein using a heparinized (100units/mL) syringe for circulating metabolite assays (plasma NEFA and glucose). Fish were then euthanized by severing of the spinal cord using surgical scissors. Blood was rapidly transferred to a 1.5mL Eppendorf tube and centrifuged at 5.0xg for 5 minutes to separate blood cells from plasma. The plasma was pipetted into a 1.5mL twist cap cryo-tube and immediately flash frozen in liquid nitrogen (LN_2) and stored at -80ºC (fasting trial) or placed on ice then moved to -80ºC storage after collection of tissues (hypoxic trial). Whole livers were excised, weighed, then flash frozen in LN_2 and stored at -80ºC for glucose assays. The heart was excised and the atria and bulbous arteriosus were removed to isolate the ventricle, which was placed in ice-cold heart mitochondrial isolation buffer (0.5M mannitol, 0.5M sucrose, and 0.1M EGTA) before quickly proceeding to the mitochondrial assays. Heart samples from hypoxic and control fish for mitochondrial respiration and ROS assays were collected during the morning (~9-10:30am) or afternoon (~1:30-3pm).

Metabolite Assays

Plasma NEFA

Plasma NEFA levels were measured using a colorimetric Free Fatty Acid Quantification Kit (Sigma Aldrich). To undiluted plasma samples, we added Acyl CoA Synthetase catalyzing the addition of coenzyme A onto fatty acids in the sample which is then oxidized by Acyl CoA Oxidase resulting in the production of H_2O_2 and 2,3-trans-Enoyl-CoA. This H_2O_2 then reacts with a colorimetric probe generating a compound with a maximum absorbance at 570nm, which was then read using a SpectraMAX 190 spectrophotometer (Molecular Devices, San Jose CA, USA) at 570nm.

Glucose assay

Glycogen extraction

Glycogen was extracted from liver and split into single glucose molecules for the glucose assay following a modified procedure from (Clow et al., 2008). Frozen liver samples were crushed in a LN_2 cooled cryo-mortar and pestle and weighed to ~50mg in labelled twist cap cryo-tubes and kept frozen in LN2. Samples were then removed from LN_2 and 30% weight per volume KOH solution was added to each tube. Samples were vortexed, then heated in a dry bath at 100ºC for 10 minutes. After heating, 2% weight per volume Na_2SO_4 solution and 95% ethanol were added, the samples were vortexed, then centrifuged at 4ºC for 10 minutes at 4700 rpm using an Eppendorf 5430R centrifuge (Eppendorf, Hamburg Germany). The supernatant was removed, and the pellet was washed with 1mL 66% ethanol, vortexed, and centrifuged again as before. After the second spin, the supernatant was removed and 1.0M HCl and H_2O were added. The sample was vortexed ensuring the pellet was resuspended, then placed back in dry bath at 100ºC for two hours to dissolve the pellet. Samples were then flash frozen in LN_2 and stored at -80ºC to be used for glucose analysis.

Glucose Assay

Glucose concentrations were determined using a modified procedure from (Bergmeyer, 2012). Assay media was prepared using 250mM imidazole, 5.0mM $MgSO_4$, 10.0mM ATP, 0.8mM $NADP^+$, and adjusted pH to 7.8 using an electronic pH meter. A glucose standard curve (2mM-0.005mM), plasma (1:10 dilution), or extracted glucose from liver glycogen (1:30 dilution), G6PDH solution (10 units/mL), and 175µL assay media were pipetted into a 96 well plate and incubated in the dark for 30 minutes at 37ºC. After incubation absorbance was read at 340nm, hexokinase (10 units/mL) was added to each well and incubated again in the dark for 45 minutes at 37ºC, then read again at 340nm. All wells were repeated in duplicate. Initial absorbance values were subtracted from final absorbances, then plasma glucose concentration was calculated using the slope of the linear fit of the absorbance of the standard curve. Total liver glucose was calculated using glucose concentration per ~50mg and total liver weight.

Lactate Assay

Thawed plasma samples were prepared in a 1:4 solution with 6% perchloric acid, repeated in triplicate. Two assay media solutions were prepared: one containing NAD^+,

H_2O, and glycine buffer (Sigma G-5418), and one containing only H_2O and glycine buffer. The lactate dehydrogenase (LDH) enzyme solution (100 u/mL) was prepared using assay media with no NAD^+ as LDH catalyses the reaction of lactate to pyruvate, with the oxidation of cofactor NAD^+ to NADH. Lactic acid standards (0-2mM) were prepared by dilution in 6% perchloric acid.

To each well, prepared plasma samples or lactate standards were added with NAD^+ containing assay media. Background absorbance was read after incubation at 25ºC for 15 minutes at 340nm which is absorbed strongly by NADH produced from the lactate to pyruvate reaction. Following the initial read, LDH solution was added to each well and the final absorbance was recorded after incubation at 25ºC for 90 minutes to allow for completion of the LDH reaction and production of NADH. Corrected absorbances were calculated by subtracting initial from final absorbances. Plasma lactate concentrations were determined using the equation of the slope of the standard curve absorbances.

Mitochondrial Respiration and ROS Production

Mitochondria Isolation

To homogenize heart tissue, the heart was shredded using sharp forceps into ~1mm diameter pieces in a shallow bath of heart mitochondria isolation buffer. The shredded heart tissue was homogenized in a chilled 10ml glass tube with a tight-fitting Teflon homogenizing pestle attached to a drill with ~6 slow up-and-downs and a slow rotation speed. This process lyses the cells but does not damage the mitochondria as they are small enough to pass between the glass walls of the tube and the Teflon pestle. The resulting homogenized heart and isolation buffer solution was then placed in a chilled centrifuge tube and spun at 1000x g at 4ºC in a Sorvall Legend RT centrifuge (Thermo Fisher Scientific, Waltham USA) to remove cellular debris from the solution. The supernatant was kept and placed in a fresh chilled centrifuge tube and spun again as before to further remove fat and unwanted cell components. The supernatant was placed in a fresh chilled centrifuge tube and centrifuged at 10 000x g to pellet the mitochondria. The supernatant was poured off and the mitochondria pellet was carefully resuspended in 1mL heart isolation buffer and placed on ice.

Protein Assay

A Bradford protein assay (Bradford, 1976) was performed on all isolated mitochondria solutions prior to respiration and ROS assays to determine the protein

concentration (mg/mL) of mitochondria. The standard curve was made from serial dilutions of bovine serum albumin (Sigma Aldrich) from 0.5 to 0.03125 mg/mL. The standard curve and isolated mitochondria (1:10 dilution) were pipetted into a 96 well plate in duplicate, and Bio-Rad Protein Assay Reagent Dye was added to each well, incubated at room temperature for 5 minutes, and absorbance was read at 595nm. Protein concentration was calculated using the slope of the linear fit of the absorbance of the standard curve.

Mitochondrial Respiration and ROS Assays

Two substrate-uncoupler-inhibitor-titration (SUIT) protocols were used to assess respiration rate and ROS production in mitochondria from the heart using an Oroboros O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, Austria). Unlit chambers were filled with MiR05 respiration media at 16ºC for all trials, and data was collected using DatLab software (Oroboros Instruments). Oxygen consumption (pmol $O_2^{-1}s^{-1}mg$) indicating the rate of respiration, and ROS (pmol*s/mL) production were compared between two energy substrates: pyruvate or palmitoyl carnitine (PC), in hypoxic and control heart mitochondria.

ROS Assay

After stabilization of oxygen flux in the chamber, we began the ROS assay by adding 5mM diethylenetriaminepentaacetic acid (DTPA), 5u/mL superoxide dismutase (SOD), 10µM Amplex UltraRed fluorophore (Thermo Fisher Scientific), and 1u/mL horseradish peroxidase (HRP). HRP catalyzes the production of resorufin from Amplex UltraRed and H_2O_2 which fluoresces and is detected by the Oroboros to quantify the amount of H_2O_2 (ROS) produced before and after the addition of ADP (Fig. 2).

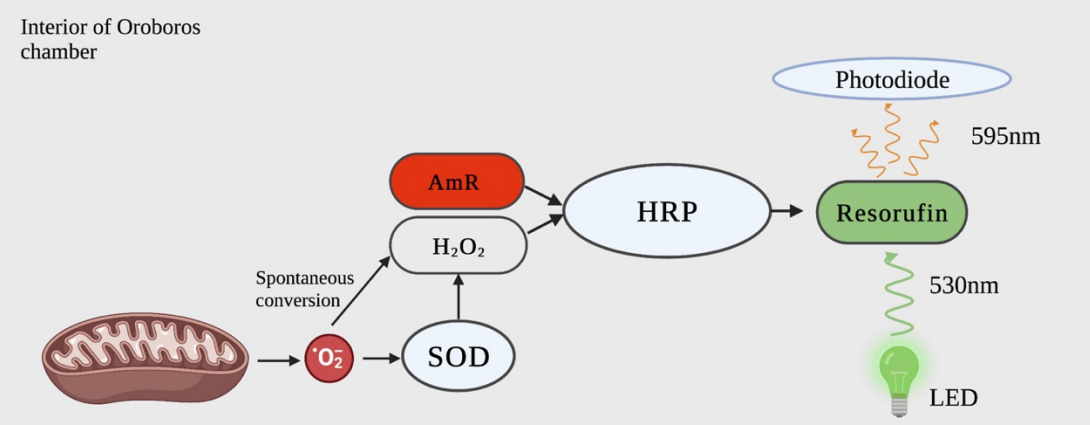

Fig 2. Schematic diagram of the Amplex UltraRed assay. Superoxide (O^{-}_2) produced from the ETS converted to H_2O_2 by SOD and through spontaneous conversion reacts with Amplex UltraRed (AmR) to resorufin catalysed by horseradish peroxidase (HRP). The fluorescence of resorufin at 595nm from excitation at 530nm is detected by a photodiode to quantify ROS production. Created with BioRender.com.

Respiration Assay

Following the addition of ROS assay components, we began the respiration assay starting with TCA cycle intermediates: 2mM malate, mM glutamate and 20µM succinate, and TCA cycle precursors: 2M pyruvate or 10 mM PC. Next, 500µM ADP was added to allow for OXPHOS, followed by 4mM cytochrome c, then repeated admission of 1mM carbonyl cyanide-p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) until no increase in oxygen flux was observed. Following this, we added 1mM rotenone, 5mM antimycin A (AMA), 200mM tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), 800mM ascorbate, and finally 4M sodium azide to inhibit CIV and show background oxygen consumption. Stabilized oxygen consumption was measured following the addition of each component of the respiration assay to determine the activity of the ETS as a whole, and specific complex activity (Fig. 3).

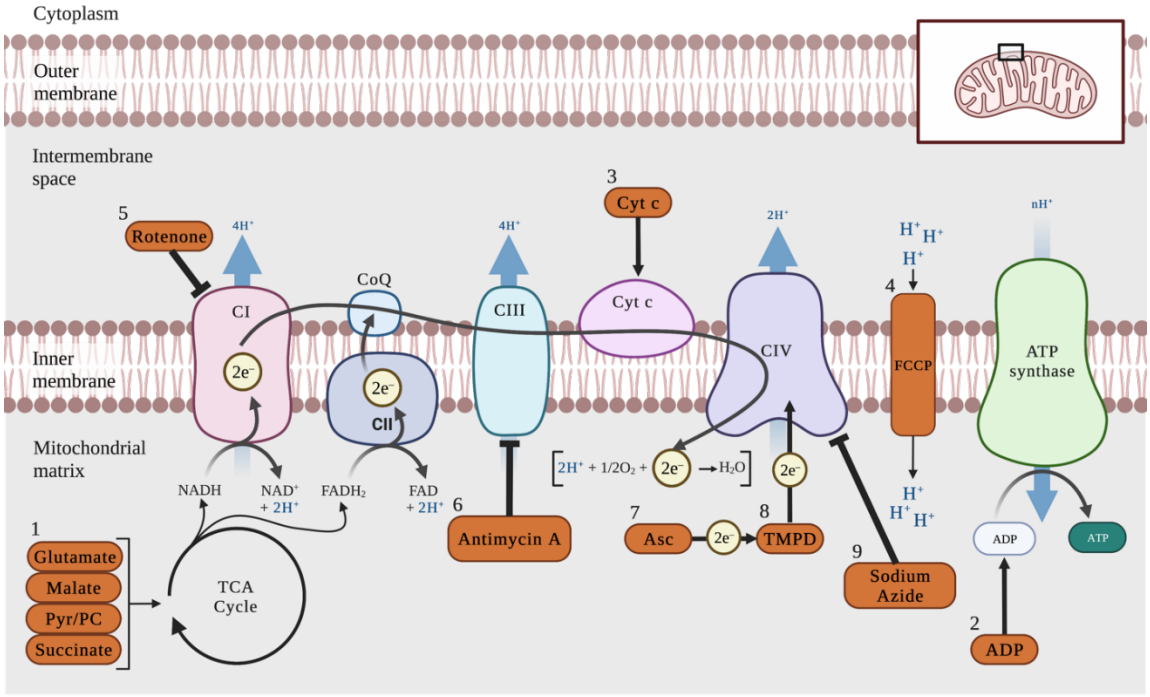

Fig 3. Schematic diagram of all components of the SUIT protocol and their function in the ETS. The order of addition to the Oroboros chambers is indicated by number. (1) TCA cycle intermediates; glutamate, malate, succinate, and precursors pyruvate and palmitoyl carnitine (PC) producing NADH and $FADH_2$ electron (e^-) donors. (2) ADP for phosphorylation to ATP at ATP synthase. (3) Mobile electron carrier cytochrome C (Cyt C) that moves electrons between CIII and CIV to indicate damage to the outer mitochondrial membrane. (4) FCCP transports protons (H^+) from the intermembrane space to the mitochondrial matrix uncoupling the rate of proton pumping from the rate of phosphorylation of ADP. (5) Rotenone, an inhibitor of CI to show CII e^- transport fueled OXPHOS. (6) aAntimycin A, an inhibitor of CIII stopping electron transport to CIV via Cyt C. (7) Ascorbate (Asc) is added to ensure the constant reduction of TMPD (8) to donate electrons to CIV. (9) Sodium azide binds to the oxygen reduction site on CIV to stop oxygen consumption and show background oxygen consumption in the chamber. Created with BioRender.com.

Statistical Analysis

Statistical analysis was performed in R Studio (version 1.3.1093). An alpha significance level of 0.05 was determined for all tests *a priori*. Assumptions for normally distributed data were tested using Shapiro Wilk tests, and homogeneity of variance was tested using Bartlett or Levene's tests. For fasting metabolite data, one-way Analysis of Variances (ANOVA) were performed to determine differences in plasma glucose, NEFA, and total liver glucose between control, two-week, and six-week fasted fish. We used unpaired t tests to compare plasma glucose, NEFA, lactate, and total liver glucose in control and hypoxic fish. For respiration data, a multivariate analysis of variance (MANOVA) was performed to check for a significant effect of interaction between treatment and substrate

independent variables. All multivariate normality and covariance passed, and no interaction was detected so we could interpret univariate results. Univariate testing was performed using two-way ANOVAs or a non-parametric alternative (aligned rank test) when transformations didn't allow the data to pass assumptions, for all measures of mitochondrial function (LEAK, OXP-I, RCR, ET, OXP-II, URC, CCO), and ROS production. Tukey HSD post hoc tests were used to determine differences between groups in one and two-way ANOVAs.

Results

Fish Size

After two and six-weeks of fasting, we saw no significant change in total body mass compared to the control group ($F = 1.961$, $p = 0.145$), and significantly lower hepatosomatic indexes (HSI) in four ($p = 0.002$) and six-week ($p < 0.001$) fasted fish. We also found no significant change in average fish mass after one week of hypoxia ($p = 0.265$), and a significantly higher average HSI in hypoxic fish ($p = 0.031$) (Table 1).

Table 1. Mean mass (g), standard deviation, and hepatosomatic index of fish in all treatment groups used for fasting and hypoxia experiments.

Treatment Group (n)	Mean Mass (g)	Hepatosomatic Index
Fasting Control (10)	270.68 ± 60.14	1.43
2 Week Fasted (6)	227.83 ± 14.42	0.85
4 Week Fasted (6)	246.50 ± 21.51	0.65
6 Week Fasted (10)	231.71 ± 22.23	0.57
Hypoxia Control (8)	747.36 ± 204.56	0.32
Hypoxia (8)	818.46 ± 44.71	0.4

Experiment I – Effects of fasting

Circulating metabolites and glycogen stores

Concentrations of plasma NEFA and glucose, and total liver glucose were measured to assess how substrate selection and hepatic glycogen stores changed over six weeks of fasting and standard holding (control) conditions. We found significantly higher circulating NEFA concentrations in the two-week fasted fish compared to six weeks of fasting ($p = 0.0231$) and control ($p = 0.0053$) conditions. Interestingly, we observed very similar NEFA concentrations between 6 week fasted and control fish (Fig. 4). There were no significant changes in circulating plasma glucose after 2 weeks of fasting (Fig. 5).

However, there was a significant decrease in plasma glucose after 6 weeks of fasting (p = 0.0147) compared to the control fish, but not compared to 2 weeks of fasting (p = 0.0655). Finally, we observed lower total hepatic glucose stores in two-week fasted fish averaging over four times less than control ($p = 3.07 \times 10^{-4}$), and six week fasted fish over ten times less than control ($p = 1.0 \times 10^{-7}$) (Fig. 6). Further, there was a significant decrease in hepatic glucose between two and six weeks of fasting (p = 0.0335).

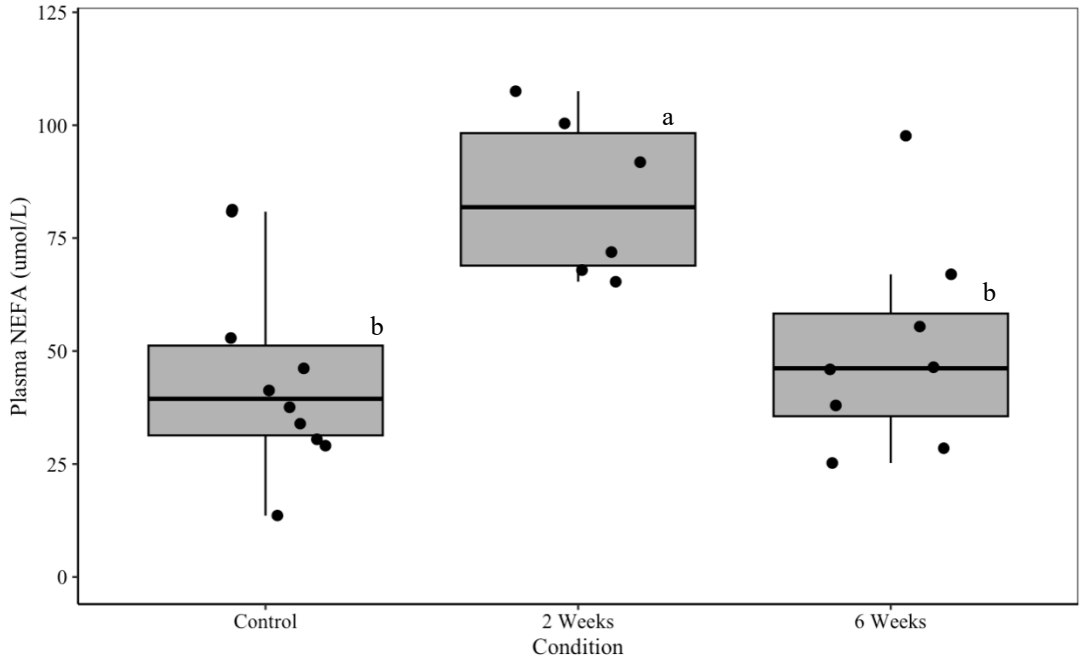

Fig. 4. Circulating plasma non-esterified fatty acids (NEFA) (µmol/L plasma) of *S. fontinalis* plasma after two (n=6) and six (n=8) weeks of fasting, and 6 weeks of standard control conditions (n=10) measured using fatty acid quantification assay. Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

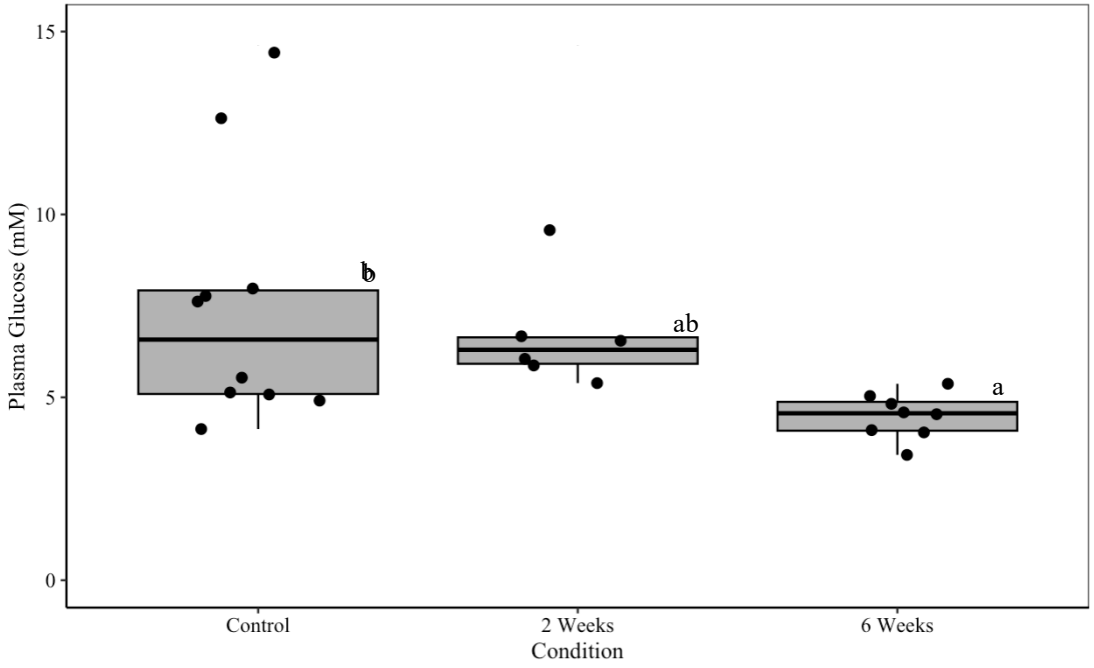

Fig 5. Circulating plasma glucose (mM) of *S. fontinalis* after two (n = 6) and six (n = 8) weeks of fasting and six weeks in control (n = 10) conditions measured using a glucose assay. Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

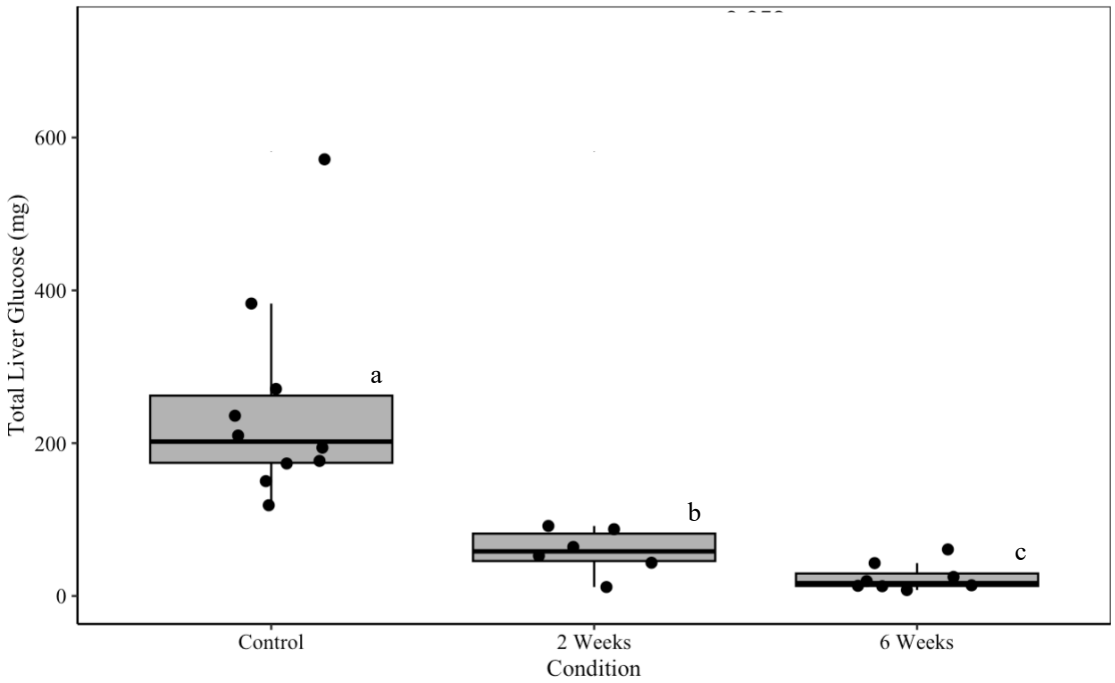

Fig 6. Total hepatic glucose (mg in liver) of *S. fontinalis* after two (n = 6) and six (n = 8) weeks of fasting and six weeks in control (n = 10) conditions. Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

Experiment II – Effects of Chronic Hypoxia and Fasting

Next, we wanted to assess the changes in circulating NEFA, glucose, lactate, and hepatic glucose stores after one week of chronic hypoxia to look at the changes in energy substrate mobilization compared to fed normoxic fish. There was no significant effect of chronic hypoxia on plasma NEFA concentration ($p = 0.354$), plasma glucose ($p = 0.7213$), total liver glucose ($p = 0.1571$), or plasma lactate ($p = 0.224$) (Fig. 7).

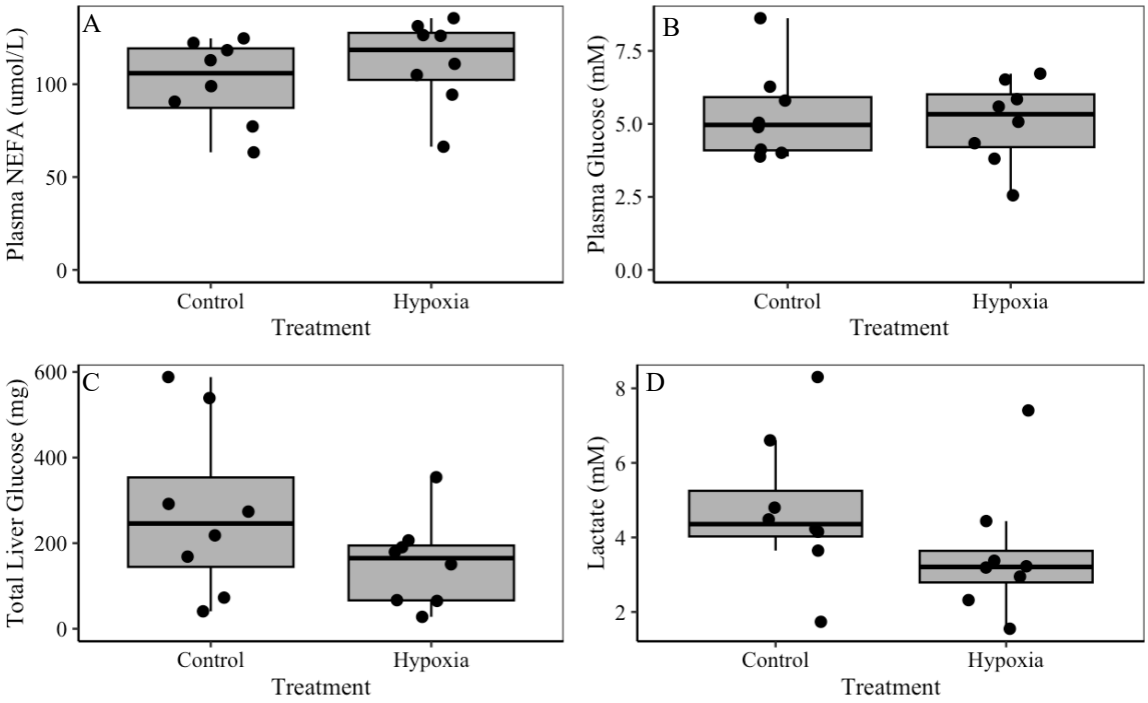

Fig 7. (A)Plasma non-esterified fatty acids (NEFA) (μmol/L plasma), (B) plasma glucose (mM), (C) total liver glucose stores (mg in liver), (D) and plasma lactate concentration (mM) of *S. fontinalis* after one week of no feeding and standard control conditions (100% DO_2) (7 days n = 8) and chronic hypoxia (45-47% DO_2) (7 days n = 8). Boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

Mitochondrial Function

We next wanted to assess the effect of chronic hypoxia on mitochondrial function and ROS production using either fatty acids or pyruvate as a substrate in the mitochondria of cardiomyocytes. TCA cycle intermediates glutamate, malate, succinate, and precursors pyruvate or PC were added to chambers to generate electron donors for CI to look at the non-phosphorylating (LEAK) state where ATP synthase is not active because of the absence of ADP causing natural uncoupling, and the proton gradient can increase to its maximum. We found no significant effect of hypoxia exposure ($p = 0.101$), but a significant effect of substrate ($p = 4.76 \times 10^{-4}$, Fig. 8). In hypoxic fish, mitochondria using PC as a substrate had significantly higher LEAK respiration ($p = 0.004$) an effect not observed in control fish.

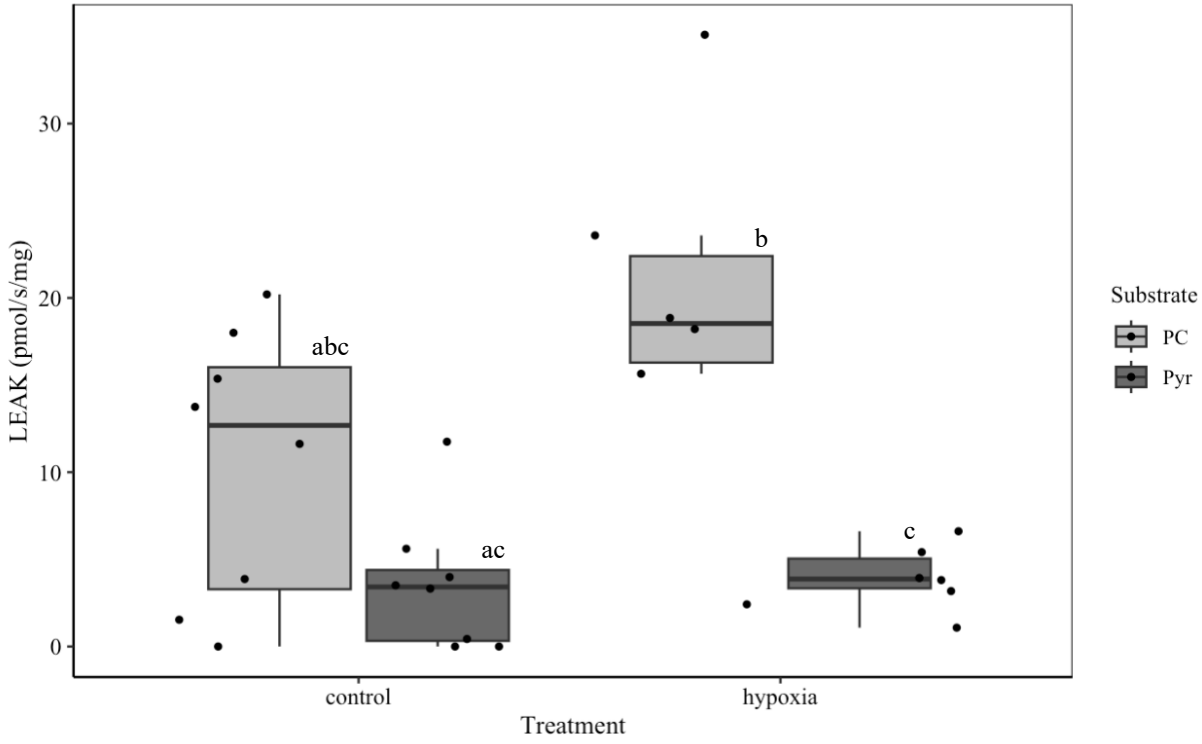

Fig 8. Non phosphorylating (LEAK) state oxygen consumption (pmol O2/s/mg) after the addition of glutamate, malate, succinate and pyruvate or palmitoyl carnitine (PC) in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

With the addition of ADP to the chamber, we then looked at normal CI fueled OXPHOS (OXP-I) (Fig. 9). We found no significant difference ($p = 0.147$) in oxygen

consumption between control and hypoxic groups, but there was again a significant effect of substrate (p = 2.29x10^{-4}). Control pyruvate showed significantly higher oxygen consumption (p = 9.43x10^{-3}) than PC; however, the same response was not seen in the hypoxic group with slightly increased but non-significant (p = 0.072) oxygen consumption.

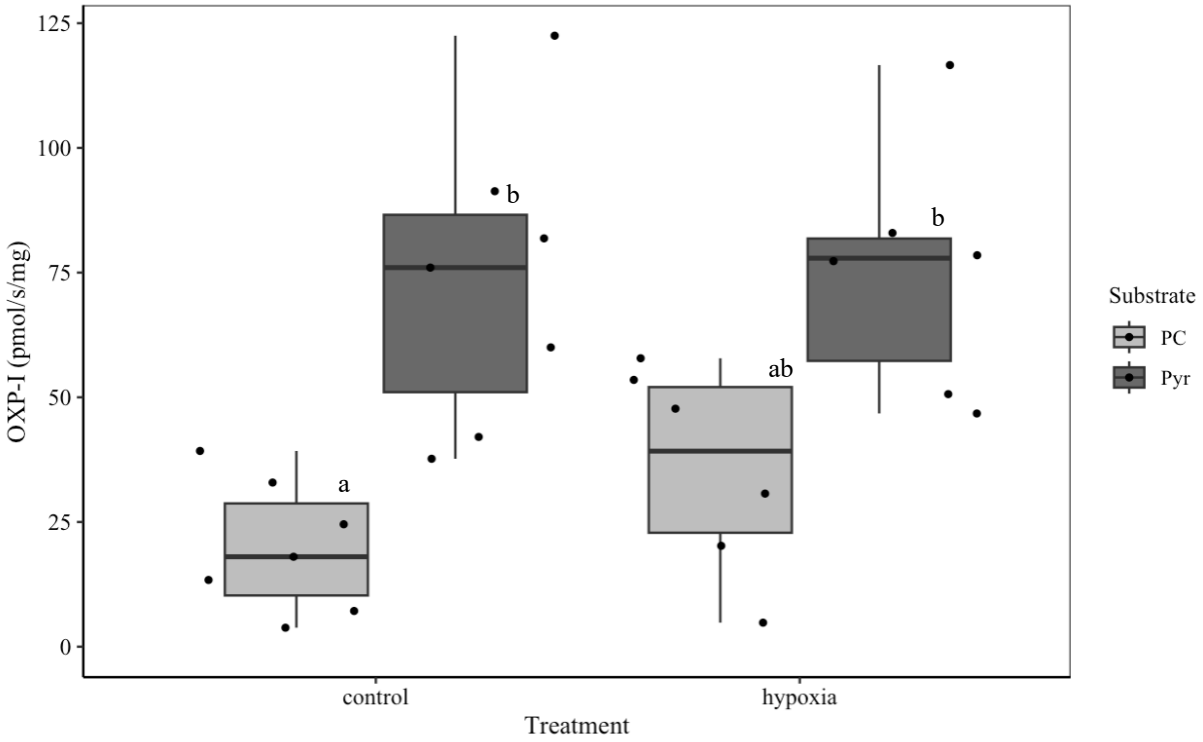

Fig 9. Oxygen consumption (pmol O2/s/mg) during CI fueled OXPHOS (OXP-I) after the addition of ADP in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

Using the results from LEAK state and OXP-I respiration, we can calculate the respiratory control ratio (RCR) which is the ratio of oxygen consumption after and before the addition of ADP. This value acts as an indicator of the efficiency of the mitochondria and how coupled the movement of electrons and consumption of oxygen is to the phosphorylation of ADP to ATP in the ETS (Fig. 10). We found no effects of hypoxia on the RCR (p = 0.611), however there was a significant effect of substrate (p = 1.18x10^{-7}). There was a significantly higher RCR value when using pyruvate in both control (p = 2.02x10^{-4}) and hypoxia (p = 4.17x10^{-5}) showing increased efficiency and tighter coupling when using pyruvate compared to PC.

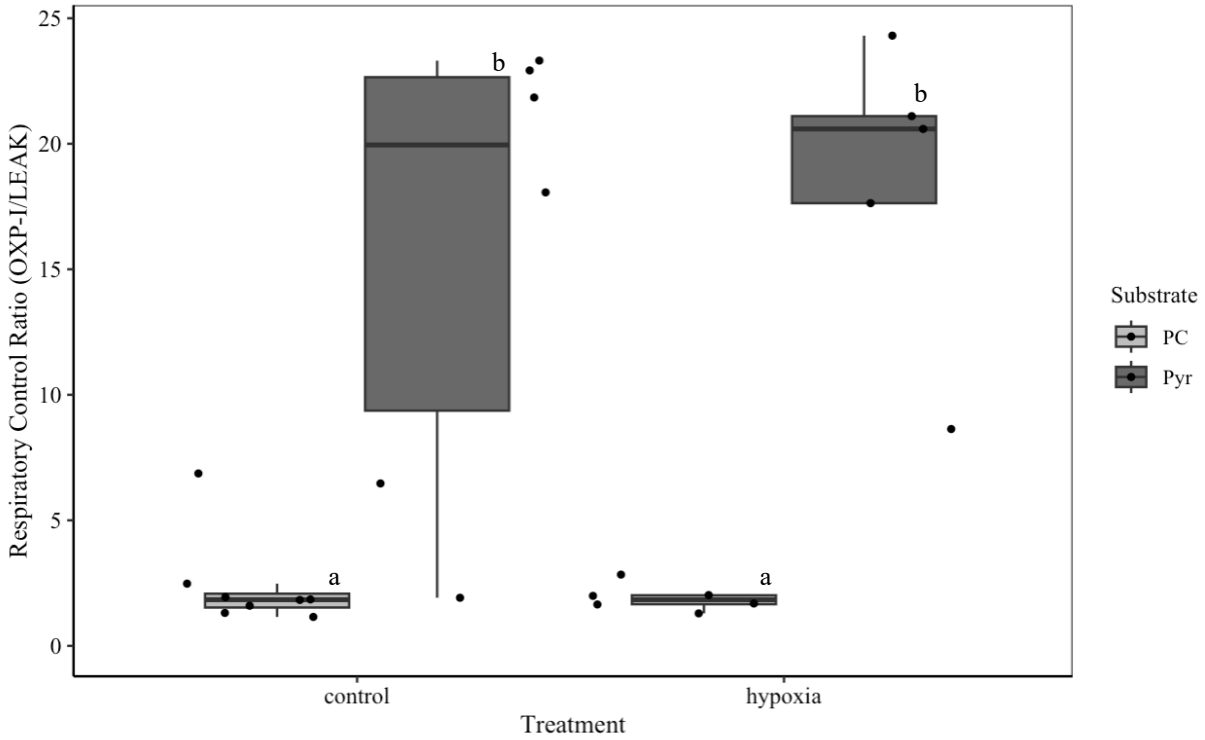

Fig 10. Respiratory control ratio (RCR) (OXP-I O_2 consumption/LEAK O_2 consumption) indicating the efficiency of mitochondrial function and coupling of electron flow to ADP phosphorylation in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

Next, we looked at the electron transport capacity (ET) of the mitochondria. The ETS uncoupler FCCP was added which transports protons across the inner mitochondrial membrane from the intermembrane space to the mitochondrial matrix (Fig. 11). This allows protons to be pumped through the ETS without being impacted by the proton gradient, and shows the maximum rate that electrons can be moved through the ETS by monitoring oxygen consumption as electrons moved through the ETS reduce oxygen to water at CIV. From this, we saw that there was a significant effect of substrate ($F = 22.37$, $p < 0.0001$) and no effect of treatment ($F = 2.93$, $p = 0.099$). Within substrate, there was significantly higher ET in the presence of pyruvate in both control ($p = 0.007$) and hypoxic fish ($p = 0.028$).

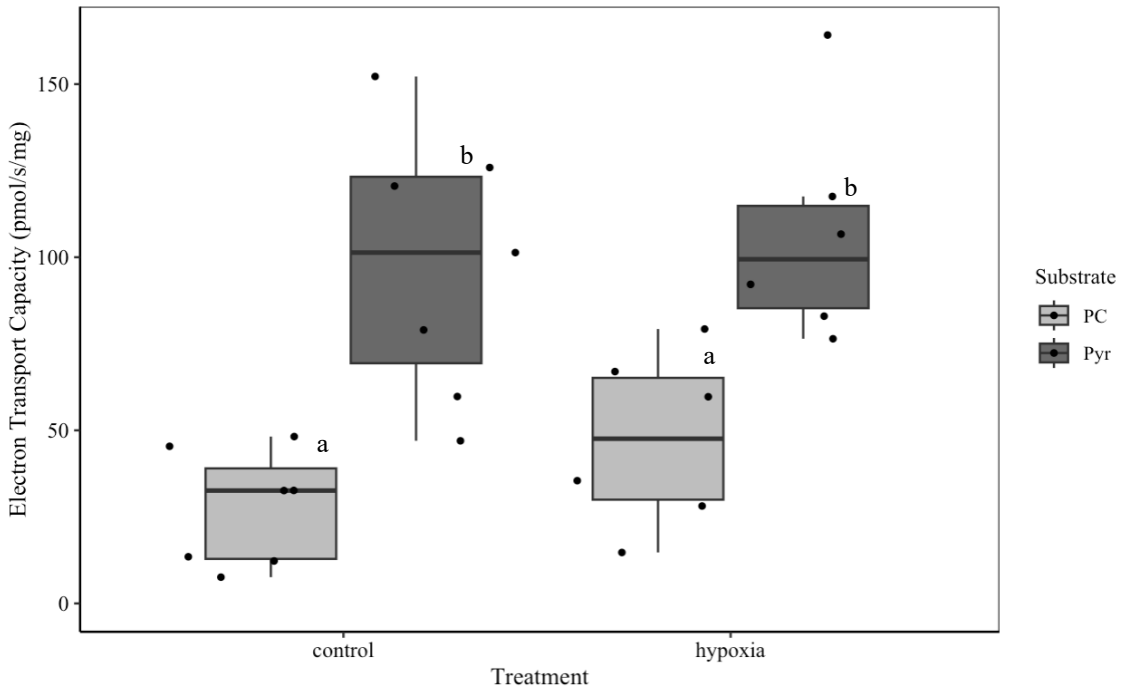

Fig 11. Electron transport capacity (ET) (pmol O_2/s/mg) in *S. fontinalis* heart mitochondria in the presence of FCCP after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

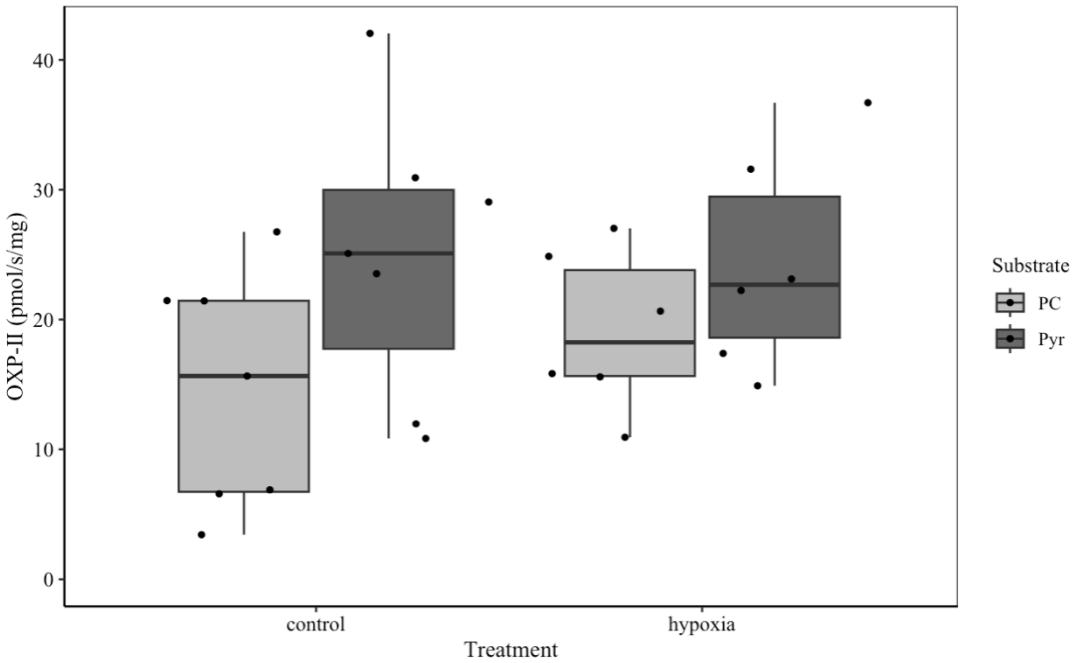

Fig 12. Oxygen consumption (pmol O_2/s/mg) during CII fueled OXPHOS (OXP-II) in *S. fontinalis* heart mitochondria in the presence of rotenone after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

To assess the activity of CII, we administered rotenone, an inhibitor of CI, to stop the flow of electrons into the ETS through CI. By monitoring oxygen consumption with CI inhibited, we can selectively look at the activity of CII through its movement of electrons into the ETS (OXP-II). We found no significant effect of substrate ($F = 3.579$, $p = 0.071$) or treatment ($F = 1.403$, $p = 0.248$) (Fig. 12). However, there was a slightly elevated oxygen consumption in both control and hypoxic groups when using pyruvate.

To measure the coupling of electron transport to ADP phosphorylation we calculated the uncoupling control ratio (UCR) by dividing the oxygen consumption in an uncoupled system (ET), and a fully functioning ETS with saturating ADP levels (OXP-I) (Fig.13). We found no difference between treatment groups ($F = 0.731$, $p = 0.403$) and a significant effect of substrate within treatments ($F = 33.13$, $p < 0.001$) and an increased UCR when using pyruvate in control ($p < 0.01$) and hypoxic treatments ($p < 0.001$).

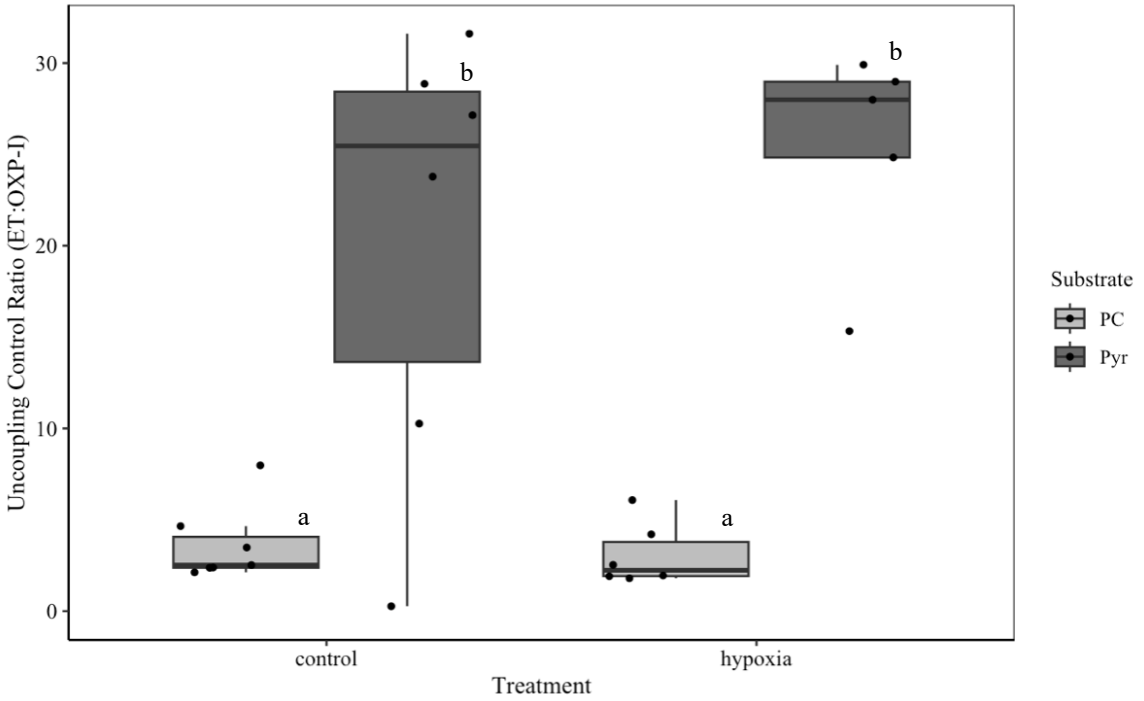

Fig 13. Uncoupling control ratio (UCR) indicating capacity for uncoupling of the system in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

With electron transport from CI and CII previously inhibited by rotenone and antimycin A, we cut off electron transport through the system and could measure the specific function of CIV (CCO). We recorded oxygen consumption with the addition of TMPD, a CIV specific electron donor, and ascorbate to ensure that TMPD remained reduced to constantly supply CIV with electrons (Fig. 14). We found no significant difference in response between control and hypoxic groups (F = 1.551, p = 0.225), or between substrates (F = 2.463, p = 0.130).

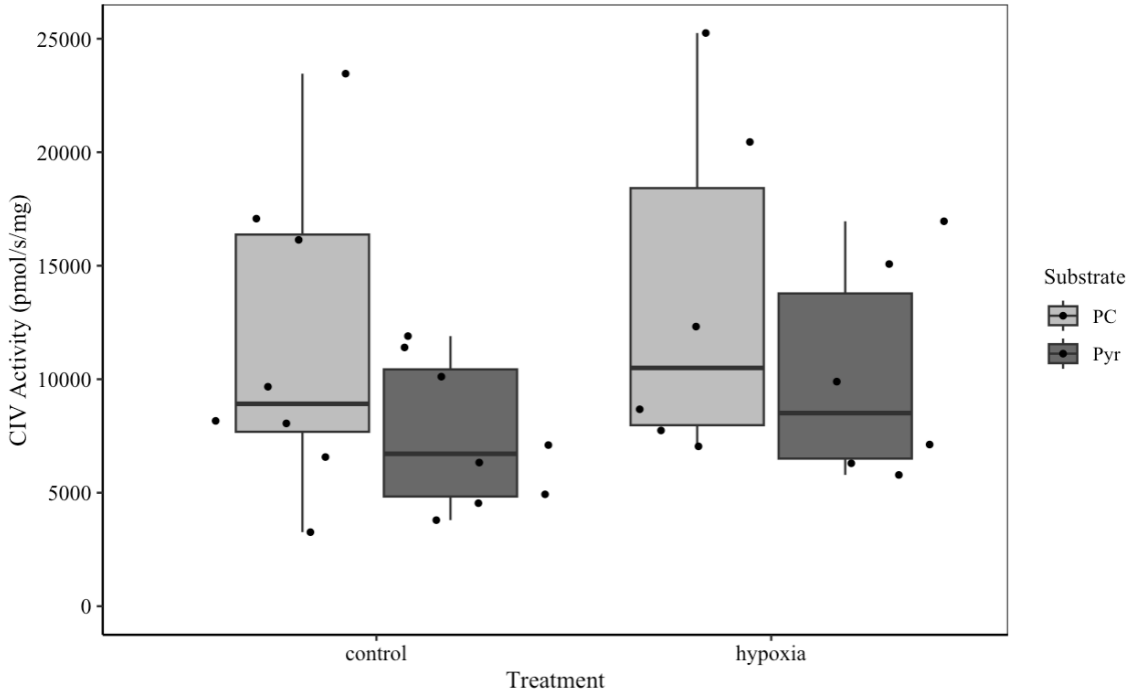

Fig 14. Oxygen consumption (pmol/s/mg) during CIV specific proton pumping indicating CIV specific function in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

ROS Production

Using an Amplex UltraRed assay (Fig. 2) we determined ROS production before in LEAK state respiration (Fig. 15), and after the addition of ADP (OXP-I) (Fig. 15) to compare ROS production in fully functioning ETS between treatment and substrate. We found no significant difference in ROS produced between control and hypoxia (F = 1.359, p = 0.255), but there was a significant effect of substrate (F = 9.916, p = 0.004). However, this was only shown in the control group where ROS production was significantly higher in mitochondria given PC (p = 0.008).

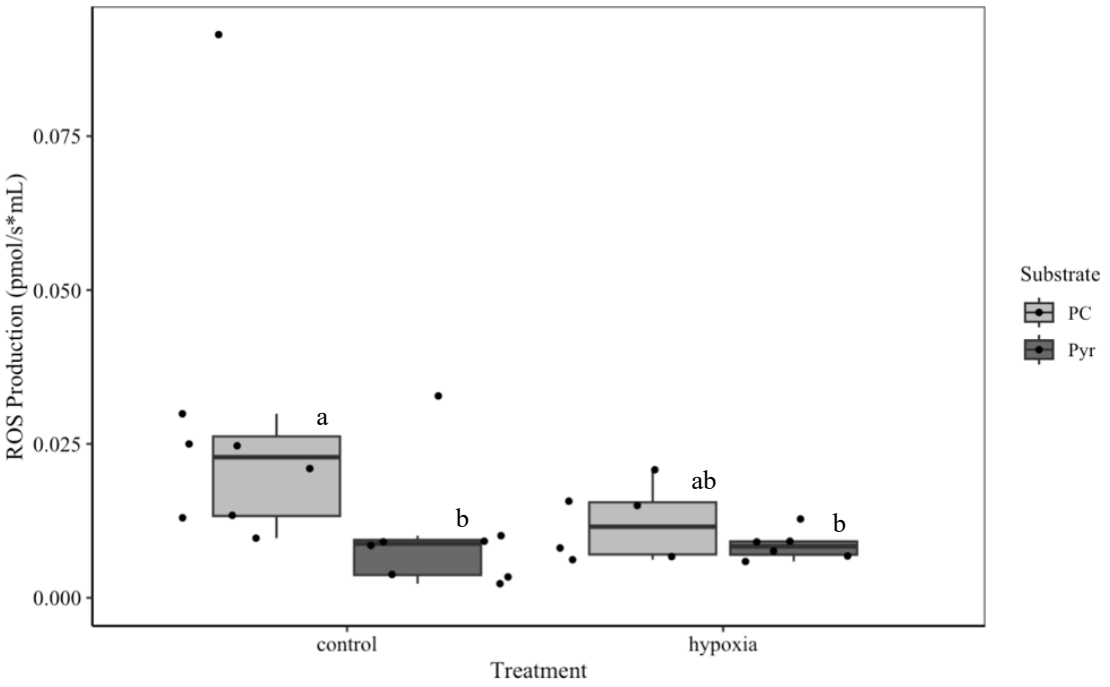

Fig 15. ROS production (pmol/s*mL) after the addition of ADP (OXP-I) measured with an Amplex UltraRed fluorometric assay in *S. fontinalis* heart mitochondria after one week of control (n = 8), and chronic hypoxia (45-47% DO_2) exposure (n = 8). Significant differences ($p < 0.05$) indicated by different letters, boxes indicate interquartile level, horizontal lines indicate median values, and whiskers indicate lower and upper quartile of data distribution.

Collectively, these data suggest that six weeks of fasting causes a significant decrease but does not deplete hepatic glucose stores. Additionally fasting does not alter substrate mobilization. One week of chronic hypoxia did not induce an increase in glucose circulation and did not significantly lower glucose stores as predicted. We also report that one week of hypoxic exposure does not change overall mitochondrial function or ROS production in cardiomyocytes. Finally, we show that using lipids in cardiac cells significantly decreases mitochondrial function and provide evidence indicating that these cells would likely favour the use of glucose.

Discussion

In this study, we aimed to investigate the effects of prolonged fasting and chronic hypoxia on brook trout substrate mobilization, and mitochondrial function. To explore these effects, we designed two experiments: (I) Explore the effects of fasting throughout a six-week period on circulating metabolites and energy stores, and (II) determine the circulating metabolite concentrations and energy stores, and mitochondrial function and ROS production in cardiomyocyte mitochondria after one week of chronic hypoxia and fasting combined. Here, we provide insight into the physiological and metabolic responses of brook trout to prolonged fasting and chronic hypoxia. We found that after six weeks of fasting there was a decrease in hepatic glycogen stores, but after one week of chronic hypoxia, brook trout did not deplete their hepatic glycogen stores or increase NEFA circulation. We also showed that hypoxia had minimal effects on mitochondrial function and ROS production, however the use of lipids caused diminished mitochondrial function and increased ROS production.

Experiment I – Effects of fasting

After two weeks of fasting, we found no significant change in overall body mass which aligns with similar studies that report no change in rainbow trout body mass after three weeks of fasting (Raine et al., 2005). Six weeks of fasting surprisingly resulted in no significant change in overall body mass in contrast to a similar study where seven weeks of fasting caused significant loss of body mass (Navarro et al., 1992). Other salmonid species such as the sock eye salmon that use the majority of their lipid stores during their long migrations will replace lost lipid mass with an equal weight of water (Idler and Bitners, 1958), resulting in no significant change in their body mass. While we did not measure changes in fat store size in these fish, this could be why we observed no significant change in mass during prolonged fasting.

We reported significantly lower average HSI values after four and six weeks of fasting, which is likely due to a decrease in hepatic glycogen and lipid stores from glucose and lipid mobilization. In other salmonid species, lower HSI values are associated with decreased glycogen stores during fasting as the fish mobilises glucose to tissues in the body (Barton et al., 1988). In a similar study on rainbow trout, Simpkins et al., 2003 found no change in average HSI after 105 days of fasting. This is indicative of their capacity for gluconeogenesis in the liver that can produce glucose from non-carbohydrate precursors to

maintain blood glucose levels during fasting where glucogenic enzyme activity is increased (Morata et al., 1982). While we did see a significant decrease in total liver glucose after six weeks of fasting, the decrease in plasma glucose after two weeks of fasting was insignificant. There was a significant decrease in plasma glucose after six weeks of fasting, however this response was relatively small, and could provide further evidence supporting the gluconeogenic abilities of these fish to maintain plasma glucose during prolonged fasting.

We did not observe the expected increase in plasma NEFA after six weeks as we had originally predicted. Instead, we saw a significant increase after two weeks of fasting and a return to concentrations similar to control after six weeks. This more closely reflects the physiological responses of these fish to fasting where they will lower their overall metabolic rate to decrease energy demand (Beamish, 1964; Karatas et al., 2021) thus decreasing circulating NEFA levels back to pre-fasting levels. A very similar response was reported by Bilinski and Gardner, 1968 where after two weeks of fasting they saw a significant increase in circulating NEFA followed by a decrease back to within the range of no significant change after six weeks. Further, Bilinski and Gardner, 1968 continued fasting their fish for ten weeks where they still reported no significant increase in NEFA circulation as they likely maintained a decreased metabolic rate for a much longer period of time to conserve energy stores during prolonged fasting. During an even more severe fasting period, rainbow trout NEFA levels were kept constant over 107 days of fasting using 80-90% of their lipid reserves (Simpkins et al., 2003). Our data show that even after six weeks of fasting, we don't see the depletion of liver glycogen or an increase in the circulation of NEFA and heavier reliance on lipids. This data from fasting allows us to compare to the effects of chronic hypoxia on glucose store depletion and determine that the changes we see during hypoxia are due to hypoxic exposure and not just from fasting during hypoxia.

Experiment II – Effects of chronic hypoxia and fasting

One week of chronic hypoxia caused no significant decrease in body mass, consistent with our findings after two weeks of fasting alone. Trout have the capacity to rapidly lower their metabolic rate in response to hypoxic conditions to reduce energy and oxygen demand, while conserving their energy reserves and maintaining weight (Boutilier

et al., 1988). This could also explain why we saw no change in circulating NEFA, glucose, or lactate as the decrease in metabolic rate slowed the mobilization of substrates throughout the body. Most notably however, we saw no increase in circulating glucose, contrary to what was expected as we predicted that an increase on the reliance of anaerobic glycolysis would increase glucose circulation from hepatic stores. It is possible that there was an increase in mobilization of glucose from the liver, but a decrease in metabolic rate and demand for energy in response to low oxygen could have negated the expected increase in plasma glucose in comparison to the control group.

While we did not report a statistically significant decrease in hepatic glycogen, this result should not be viewed as no change between control and hypoxic groups as there is a decreasing trend. Trout store glycogen in their liver and white muscle, but glucose stored in the musculature is mainly metabolized anaerobically within those cells to fuel movement and is not mobilized (Bilinski and Jonas, 1972; Davison and Goldspink, 1984). A decrease in total liver glucose could be indicative of an increase in reliance on glucose in other tissues as these fish have characteristically large hepatic glycogen stores that they will mobilize throughout the body and could be beneficial in hypoxia (Hilton and Dixon, 1982). The stress response could also be involved in maintaining proper glucose circulation during hypoxia. Catecholamines released in response to the stress of acute hypoxia in trout activate β-adrenergic receptors in the liver inducing glycogenolysis and releasing glucose into the blood, while decreasing hepatic glycolysis (Wright et al., 1989). This increase in glucose release, however, may not cause an increase in circulating glucose, if there is increased glucose uptake into tissues to be used in anaerobic glycolysis (Wright et al., 1989). This effect of the stress response on metabolic changes could be a potential mechanism behind why we saw a decrease in liver glycogen stores, but no increase in glucose circulation as would be expected with increased anaerobic glycolysis. Our plasma lactate levels did not reflect a large increase in anaerobic glycolysis, however there could be lactate buildup in tissues from anaerobic glycolysis rather than release into the blood. This could be a factor contributing towards the decrease in hepatic glucose stores and no change in circulating lactate.

Lactate produced as the end product of anaerobic glycolysis can be oxidized to pyruvate through the activity of LDH, which can then enter the TCA cycle to produce

electron donors for OXPHOS. Once converted to acetyl-CoA it can also be involved in the synthesis of lipids in the liver which could help maintain hepatic fat stores (Bilinski and Jonas, 1972). This would not be particularly beneficial during hypoxia as lipid stores would likely not be at risk of depletion, but this could explain the unexpected higher HSI we reported in hypoxic fish which would be expected to decrease in chronic hypoxia with the mobilization of hepatic glycogen. After one week of chronic hypoxia, we saw no increase in plasma lactate. In contrast, acute hypoxia of less than one hour has been shown to induce significantly higher circulating plasma lactate (Wright et al., 1989). With more time in hypoxia, it is possible that increases in plasma lactate levels have subsided as it is taken up by tissues to be oxidized. In trout, the liver is an important site of lactate oxidation to pyruvate, however the activity of complete oxidation is low, meaning that it could be involved in biosynthetic pathways rather than energy production (Bilinski and Jonas, 1972). The heart also shows a significant capacity for lactate oxidation (Bilinski and Jonas, 1972), which aligns with our results where heart mitochondria showed more efficient respiration when using pyruvate in comparison to lipids. Circulating lactate could also play an important role in gill function and oxygen diffusion during hypoxia. Decreased environmental oxygen induces changes in gill morphology where they will increase the surface area of cells that are rich in mitochondria through cell expansion (Iftikar et al., 2010). This facilitates higher oxygen diffusion into circulation and provides oxygen necessary for the complete oxidation of lactate (Bilinski and Jonas, 1972) and glucose which is strongly favoured in gills (Crockett et al., 1999).

Impact of chronic hypoxia and substrate use on mitochondrial function and ROS production

After assessing the changes in circulating metabolites and glucose stores, we found no change in response to one week of hypoxia contrary to what we had predicted. We then moved on to look at the metabolic impacts in cardiomyocyte mitochondria from chronic hypoxia exposure and how using different substrates in this condition might affect their function. Across all measures of mitochondrial function and ROS production we found no significant effect of exposure to one week of hypoxia. This result is promising as we expected to see decreased mitochondrial function from hypoxic exposure due to an increase in oxidative stress that could damage mitochondria and limit their function.

Hypoxia can have detrimental effects on energy allocation and development especially with prolonged exposure (Iftikar et al., 2010), however we have shown here that mitochondrial function and energy production is not impaired by one week of prolonged chronic hypoxia. We have also shown that similar to the gills, the heart seems to favor the use of glucose over lipids, contrary to many other tissues (Crockett et al., 1999). Using lipids in cardiomyocytes may decrease mitochondrial function and is likely not a viable option for brook trout in hypoxia.

During LEAK state respiration, we observed higher oxygen consumption in mitochondria given lipids. Higher LEAK respiration here when using lipids indicates a more leaky mitochondrial membrane. This is less efficient as energy being used to build the proton gradient is being lost through the leak of protons. It is possible that this is due to the amount of energy stored in lipids as their long carbon chains can generate many electron donors, increasing the movement of electrons through the system resulting in more protons pumped. However, the ET respiration does not support this explanation as we saw an increase in ET oxygen consumption in the presence of pyruvate. ET is a measure of the maximum rate at which the ETS can move electrons through the system. By using the uncoupler FCCP the limitation of reaching the maximum proton gradient can be avoided which would slow the movement of electrons and the pumping of protons. We see significantly higher ET respiration when using pyruvate indicating that the ETS can transport more electrons and pump more protons when using pyruvate. This does not indicate more electron donors produced from lipids are causing an increase in proton pumping leading to higher leak rates.

While indicative of decreased mitochondrial efficiency, increased proton leak is potentially an important defensive mechanism against the oxidative damage of ROS. An increase in mitochondrial ROS production induces proton leak *via* uncoupling proteins which acts as a protective measure against oxidative stress (Cheng et al., 2017). Proton leak protects against increased ROS accumulation by lowering the mitochondrial membrane potential. This decreases the supply of electrons through the system which lowers the risk of oversupply and leak of electrons out of the system that can generate ROS (Papa and Skulachev, 1997). Our results showed significantly higher ROS production when using lipids in the control but not hypoxia treatment. Coinciding with

this, we saw significantly higher LEAK respiration in the hypoxia treatment but not in control. The increase in proton leak could be a protective strategy against increased oxidative stress induced from hypoxia as hypoxic conditions cause an increase in mitochondrial ROS production (Lundby et al., 2003).

We also see a higher oxygen consumption using pyruvate during OXP-I respiration. This shows more oxygen being consumed and protons pumped, providing evidence supporting higher ATP production when using pyruvate. This is further supported by our RCR values where we see that oxygen consumption is more tightly coupled to the activity of ATP synthase in mitochondria given pyruvate. Further, the UCR values indicate that there is an increased ability to quickly upregulate ATP production with changes in energy demand when using pyruvate, compared to much lower values when using lipids. This tells us that mitochondria in cardiac tissue significantly favour the use of glucose over lipids. This could be due to the quick energy provided by glucose or reduced oxygen availability that can make hearts become hypoxic (Gesser, 2002).

In summary, the negative effects on mitochondrial function that we have shown when using lipids in cardiomyocytes shows that it is unlikely that brook trout will switch to using fatty acids if their glycogen stores are limited or depleted as a result of prolonged hypoxia. Promisingly however, we show that one week of chronic hypoxia does not deplete or significantly decrease total hepatic glucose stores. We also show evidence that glucose is likely favoured over lipids for energy production in the heart of brook trout. Additionally, there appears to be no effect of one week of chronic hypoxia on mitochondrial function or ROS production indicating no serious negative metabolic consequences in the heart in response to chronic hypoxia. This could be due to their ability to decrease overall metabolic rate and reduce energy demand and could be why we saw no increase in circulating glucose in response to hypoxia and increased anaerobic metabolism.

Limitations and future directions

Where our results show that brook trout cardiomyocytes favour the use of glucose in cardiomyocytes, it could be beneficial in the future to look at lactate levels in cardiac tissue to assess anaerobic metabolism in the heart. Measuring plasma lactate levels may

not be an accurate measurement of substrate selection in hypoxia as tissues such as the heart have the capacity for lactate oxidation rather than the conversion to glucose in the liver (Milligan and Girard, 1993). This could mean that lactate produced from anaerobic glycolysis can remain in cells and be oxidized to produce energy aerobically.

It is also important to note that in the natural setting it would be rare that fish will experience only one hypoxic event especially as they have become more frequent (Jenny et al., 2016). Future studies should explore the effects of repeated chronic hypoxic exposure on glycogen stores and mitochondrial function to better replicate conditions in the wild and better understand long term effects of hypoxia. Eutrophication events are also strong drivers of hypoxia which often occur during warmer months in temperate regions (Zhang et al., 2021). The confounding effects of warmer water temperatures and hypoxia should be assessed to mimic the natural environment more closely as the fish used in this study were held in water maintained at 16ºC. The resting metabolic rate of fish exposed to one week of hypoxia could also be measured to see if overall metabolic rate is diminished to better explain changes in circulating metabolite levels. The fish used in this study were held in captivity without the influence on behaviour and energy expenditure by the conditions of their natural habitats. These fish became very docile and rested near the bottom of their tanks in order to conserve energy. This behaviour would likely not be possible in the wild as they would need to remain alert for predators and compete with other fish for access to cooler patches of calm water. Movement is a very energy intensive activity that would not be avoidable as it was in their holding conditions and would contribute significantly to the more rapid depletion of their on-board energy reserves. The combined effects of differences in living conditions in the natural environment could lead to unaccounted for responses in regard to energy storage usage and depletion.

Conclusion

Overall, we show that brook trout do not deplete glycogen stores or increase the circulation of NEFA in response to prolonged fasting. We also show no significant change in glycogen stores in chronic hypoxia which is likely due to a decrease in overall energy demand through behavioural and physiological changes. Finally, chronic hypoxia does not affect overall mitochondrial function in the heart, and our evidence suggests that glucose

is the favoured substrate in cardiomyocytes regardless of external oxygen content. This work provides important insights into the physiological changes of brook trout in response to chronic hypoxia, and aids in predicting future ecological damage associated with anthropogenic climate change and alteration of habitats.

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