Cardiac Mitochondrial Respiration and ROS Production and Consumption with Changing Temperature in: *Oncorhynchus mykiss, Cyprinus carpio,* and *Acipenser fulvescens*

By

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Dedication

This is the work of a lost Paraguayan-Brazilian woman scientist, adopted Canadian, which wants to dedicate this humble research to her family.

The faraway family, Nanni and Abuelo, I know you are proud of Marcionilo Natureza's grand-daughter, my Biology and love for animals came from that small aboriginal man from Pernambuco. To Anahi, my sister, beautiful and talented.

And the Canadian family, Ronni, well…..I will not elaborate; and my not so little mongoose, I hope my crazy journey through Science will help you see the world with wonder and excitement; never stop playing music, girls are capable of anything.

Table of Contents

Abstract

Cardiac mitochondrial metabolism of *Oncorhyncus mykiss*; *Cyprinus carpio*; and *Acipenser fulvescens*, was examined with increasing temperature. Experiments assayed mitochondrial oxygen consumption, and ROS, reactive oxygen species production and consumption. Rates of two mitochondrial enzymes were determined (citrate synthase and cytochrome c oxidase). Oxygen consumption rises with temperature for *C. carpio* and *A. fulvescens*, but not for *O. mykiss*. Respiratory control ratio decreases with increasing temperature for all but *C. carpio*. ROS production increases with temperature for *A. fulvescens*, with *O. mykiss* and *C. carpio* being more variable in their response. ROS consumption does not change with temperature in *C. carpio,* and *O. mykiss* mitochondria, with *A. fulvescens* increasing. Interspecific differences across species disappear when rates are expressed as cytochrome c oxidase activity. The ratio between ROS production and consumption, and fractional electron leak increased with temperature, which may be an indication of incumbent stress due to acute temperature warming.

List of Tables

List of Figures

Figure 12. Oxygen consumption rates with change in temperature of the assay.

Figure 13. Respiratory control ratios (RCR) with increasing temperature for the rates on the heart mitochondria of the three fish species.

Figure 14. H_2O_2 production rates with change in temperature for fish heart mitochondria.

Figure 15. H_2O_2 rates of consumption of fish heart mitochondria with change in temperature in state 2 for both substrates and background consumption.

Figure 16. Cytochrome c oxidase and citrate synthase activities for fish heart mitochondria with temperature change.

Figure 17. Rates of respiration for GMS substrate combination with change in temperature expressed as CCO activity.

Figure 18. Rates of respiration with increase temperature for PCM substrate combination expressed as CCO activity.

Figure 19. H_2O_2 production rates expressed per CCO activity with increasing temperature.

Figure 20. H_2O_2 consumption rates with temperature increase expressed as CCO activity.

Figure 21. The FEL with increasing temperature (on state 2, non-phosphorylating) for both substrates on the three species sampled.

Figure 22. Oxidative ratio graph comparing fish heart mitochondria across assay temperature change.

1. Introduction

Temperature is one the most important abiotic factor influencing the physiology and behaviour of fishes (Cossins & Bowler, 1987). Fish are ectotherms, with some exceptions (Blue Fin Tunas, Shortfin Makos, Opahs; Lamnoid Sharks) (Wiens et al., 2017); as such the body temperature the majority of fish is determined by heat sources outside the body, and runs parallel to the ambient temperature (Clarke & Johnston, 1999). Biochemical and cellular level responses to sudden changes in environmental temperature could determine survival of fishes in a changing environment. In this context studying fish cardiac mitochondrial metabolism in response to acute temperature increase can shed light on how mitochondria cope with temperature stress, especially in an important organ as the heart, the main pump of the vertebrate circulatory system. In this study the mitochondrial metabolism, including oxygen consumption, reactive oxygen species (ROS) production and consumption, was assayed over increasing temperatures in the ventricular mitochondria of three temperate fish. This was done to determine how their mitochondria respond to acute temperature increase.

1. 1. Temperature and Its Importance in Ectotherm Physiology

Temperature influences heat transfer to and from the environment, the rate of chemical reactions, the stability of chemical bonds, membrane fluidity in the cell, which in turn will determine protein function and stability, and rates of enzyme reactions; this leading to altered rates of protein, nucleotide, and fatty acid synthesis and as a consequence growth (Currie & Schulte, 2014; Farrell, 1997) Temperature is defined as a measure of the kinetic energy present in atoms and molecules (Cossins & Bowler, 1987). As a consequence in ectothermic organisms temperature influences feeding strategies, life history traits and overall physiological metabolism (Cossins & Bowler, 1987).

Climate change influence in global marine and fresh waters, will bring acute temperature changes to aquatic species (Hordoir & Meier, 2012; Jones et al., 2006). Thermoclines, which are the stratified temperature layers found across oceans, lakes and other bodies of water, will be affected with climate change and global increasing average temperature rise (National Oceanic Atmospheric Administration, 2017). This will affect and change seasonal cycles that in turn influence timing and temperature stratification and modify nutrient fluxes and as consequence pose a stress to organisms that live in those waters (Rummer et al., 2014). Daily temperature cycling will also be influenced by increases in average temperatures brought by climate change, this in turn will have an influence in the physiology of organisms living in the water column like fish, which are mostly ectotherms and their physiology runs parallel to the temperature of the waters that they swim in. Increase in average temperatures has decreased the daily temperature variation and in the long-term may be changing species life cycle events by influencing aerobic scope and other physiological traits in aquatic organisms (Wang & Dillon, 2014). Furthermore, physical factors like temperature, are known to influence distribution of biomass as zooplankton, and other microorganism which will influence patterns of species diversity across the water column in aquatic environments (Sameoto, 1986). The physical influences driven by climate change, brought as temperature challenge, can be a concomitant stress for aquatic species; sudden changes in seasonal temperature and daily thermal cycles are modified, this might negatively affect species already living close to their thermal maxima and can add stress to population survival (Rummer et al., 2014), as well migratory fish can be especially affected by increasing temperature in lakes and streams if water temperature acutely rises (Rodnick et al., 2014; Martins et al., 2012; Jones et al., 2006). Furthermore, as such relationship between mitochondrial thermal response and tolerance

and how this response is linked to cardiac function will shed light on the physiological process of acute heat stress.

1. 2. Mitochondria Structure and Function

Mitochondria are the main producers of ATP for the cells and, mitochondrial metabolism is crucial for physiological mechanisms required for fish thermal tolerance. Specifically the response of cardiac mitochondria metabolism to increasing temperature has been linked to the limits of thermal tolerance in fishes (Iftikar, et al. 2014). Therefore it is of importance to understand the thermal tolerance and response of fish mitochondria. Mitochondrial are double membrane-bound organelles responsible for the majority of ATP production in the eukaryotic cell, and are involved in calcium signalling, ROS production, nitrogen metabolism, apoptosis and necrosis of the cell (Treberg, et al. 2015; Jastroch, et al. 2010; Rich & Marechal, 2010) The mitochondrial inner membrane houses the electron transport chain (ETC) (Rich & Marechal, 2010); the ETC is a series of electron carriers, and enzyme complexes, which transport electrons that originate from the oxidation of respiratory substrates through redox exchanges that culminate with the reduction of molecular oxygen to water (Figure 1). The exergonic nature of these redox reactions is used to pump protons across the inner mitochondrial membrane into the mitochondrial intermembrane space. This disequilibrium of protons creates a protonmotive force (Δp) which can then be used to drive protons back to the mitochondrial matrix by passage through ATP synthase, or complex V of the electron transport chain (Jastroch et al., 2010).

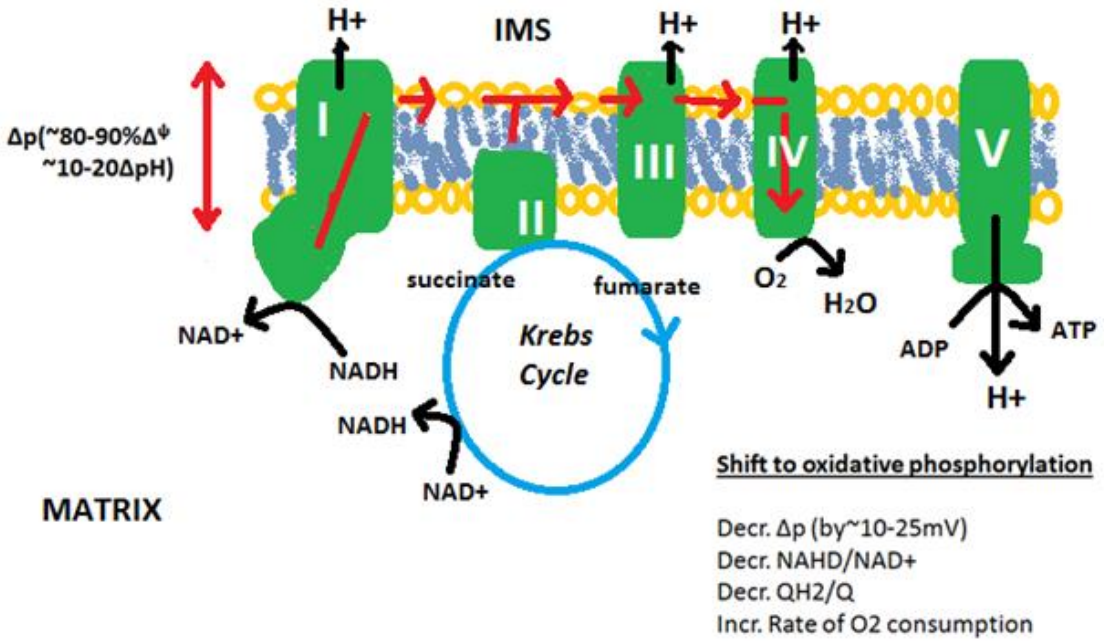

Figure 1. *Electron transport chain and oxidative phosphorylation in typical animal mitochondria*. IMS, inter membrane space; H^+, protons; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Q, ubiquinone; QH_2, ubiquinol; NAD^+, nicotinamide dinucleotide oxidized; NADH, nicotinamide dinucleotide reduced. Δp, proton motive force, which in the mitochondria is formed by 80-90% of membrane potential, and 10-20 % pH across the inner mitochondrial membrane. Shift to oxidative phosphorylation describes the change in Δp (proton motive force); NADH/NAD+, in nicotinamide dinucleotide ratio; QH_2/Q, ubiquinone pool reduction state; and the rate of oxygen consumption, during the oxidative phosphorylation process while ADP is being phosphorylated into ATP. Decr., decreasing; Incr. increasing. (After Treberg 2015, personal communication).

Mitochondrial respiration can be assayed under many conditions, respiration rates in mitochondria in this study are measured and classified as: state 2 respiration (mitochondria in the presence of substrate); which is initiated by adding of mitochondrial substrates to respiring mitochondria; and state 3 respiration is the consecutive addition of ADP to the mitochondria plus substrate present; which represents the maximum respiration rate and mitochondrial phosphorylation of ADP. Furthermore, RCRs (respiratory control ratios) which are the ratios of the slopes after and before

the addition of ADP during a respirometry reading are a way to ensure that mitochondria are indeed carrying cellular respiration. High RCR ratios are associated with good in quality mitochondria preparation (Brand & Nicholls, 2011).

1. 3. Mitochondrial Enzymes Sampled in the Project

Enzyme rates of cytochrome c oxidase and citrate synthase were used to determine the oxygen consumption, and ROS production and consumption on the protein expressed rates, as ratios, to examine possible different denominators in mitochondrial enzyme rates that may explain similarities or differences between species and patterns of influence of temperature on the mitochondrial rates measured in this study.

Complex IV (Cytochrome c Oxidase, CCO; EC 1.9.3.1): this is an important enzyme in the ETC and catalyzes the irreversible reaction that transfers electrons from cytochrome c (oxidized) to oxygen, generating proton and electrochemical gradient across the mitochondrial inner membrane, it is the last reduction step on the ETC. Furthermore, Q_{10} for the activity of cytochrome c oxidase has been shown to be similar to the Q_{10} of mitochondrial respiration in *Salvellinus fontinalis*, and they suggest a functional link between mitochondrial respiration and cytochrome c oxidase activity (Blier & Lemieux, 2001). Other workers (Guderley et al., 2005), have found that interspecific differences in mitochondrial respiration rates across three species of vertebrates (*Bufo marinus, Rattus norvegicus, Pogona vitticeps*) are diminished when respiration rates are expressed relative to the abundance of cytochrome a_1, component of cytochrome c oxidase, complex IV of the ETC.

Citrate synthase, CS, (EC 2.3.3.1): It is the enzyme which links cellular and mitochondrial glycolytic pathways to oxidative phosphorylation in the mitochondria by catalyzing the reaction of acetyl-CoA with oxaloacetate to form citrate, which corresponds to the first reaction of the

Krebs cycle (Goodsell, 2012). Furthermore, citrate synthase and cytochrome c oxidase are commonly used as markers of mitochondrial activity in cells (Lemieux et al., 2010; Thomson et al., 2007).

1. 4. Mitochondria H_2O_2 Metabolism

Mitochondria are known to produce ROS, which are molecules derived from oxygen (an electronegative element) that are chemically reactive. Some ROS possess a free radical (molecules containing an unpaired electron in the outermost electron orbital) in their structure (superoxide, $^1O^{2-\bullet}$; hydroxyl radical, $HO^\bullet$; peroxyl radicals, $ROO^\bullet$, and other oxygen species) and others like hydrogen peroxide, H_2O_2, are non-radicals, however are an activated form of oxygen and therefore reactive (Decker, 2011). ROS may react with compounds and molecules in the cell and some, like the free radicals, can be quite destructive to organic molecules. ROS molecules may be produced by enzymes in related and the electron transport chain, beta-oxidation and Krebs cycle related enzymes (Quinlan, et al. 2013). The overproduction of ROS might be detrimental to the cell and mitochondrial structures (like DNA, proteins, phospholipids and other important molecules). Mitochondrial ROS production is related to two processes happening in the mitochondria, proton leak and electron leak. Proton leak happens during the coupling of substrate oxidation with ATP synthesis in the electron transport chain (ETC), and is the process in which protons cross the mitochondrial inner membrane to the matrix independently from the ATP-synthase. Therefore, mitochondrial proton leak creates inefficiency in the proton circuit of oxidative phosphorylation. Proton leak can happen by diffusion around integral proteins in the membrane, transport mediated by the adenosine nucleotide transporter (ANT) and uncoupling proteins (UCPs) (Jastroch et al., 2010).

Electron leak occurs during the redox reactions of the mitochondrion where an electron may prematurely escape the enzyme complexes or electron carriers, but before reaching Complex IV of the ETC, and oxygen may catch it, therefore ROS molecule may be produced in the form of superoxide or H_2O_2. Furthermore, Δp influences electron leak by modifying the membrane potential across the membrane. A small production of ROS in mitochondria occurs continuously at a basal level under normal physiological conditions; however, ROS production that overrides the cellular antioxidant pathways is said to be detrimental to the animal (Jastroch et al., 2010).

The main sites of ROS production in the mitochondria are often claimed to be complex I (NADH-oxidoreductase) and complex III (cytochrome bc_1 complex) of the ETC; however, studies show that there are at least five other important sites of ROS production in the mitochondria: ubisemiquinone, UQ•; complex II, succinate dehydrogenase; the electron transferring flavoprotein system; pyruvate dehydrogenase; and α-ketoglutarate dehydrogenase (Munro & Treberg, 2017; Perevoshchikova, et al. 2013; Brand, 2000). The majority of ROS production sites in the mitochondria catalyze the formation of the superoxide ions, the superoxide ion produced in the mitochondrial matrix is a polar molecule, so does not cross the mitochondrial membrane. However, this reactive molecule can be dismutated to H_2O_2 by the enzyme superoxide dismutase; H_2O_2 is less active than the superoxide ion and also readily crosses the mitochondrial membranes and enters the cytosol of the cell (Brand, 2010). Some of the ROS produced by the mitochondria is in the form of H_2O_2 and the hydroxyl ion (Figure 2).

While mitochondria are known to produce ROS, mitochondria are also capable of consuming those molecules (Figure 2). Metabolic pathways have evolved which will detoxify and neutralize ROS produced by the mitochondria (Munro & Treberg, 2017; Starkov, 2008). An example of this is the glutathione (GSH) peroxidase pathway, which catalyzes the decomposition of H_2O_2 into water.

Other ROS scavenging fates in the cell mitochondria include the thioredoxin pathway; the superoxide dismutase enzyme; and the catalase enzyme (Kembro, et al. 2013; Quinlan, et al. 2013). As such studies which look at mitochondrial ROS metabolism should include not only the rates of ROS production, but also the mechanisms of mitochondrial antioxidant pathways and the rates of consumption of ROS such as H_2O_2. The measurement of both production and consumption processes may be particularly important due to the intramitochondrial antioxidant systems, which will lead to some underestimation of the rate of ROS production (Munro & Treberg, 2017).

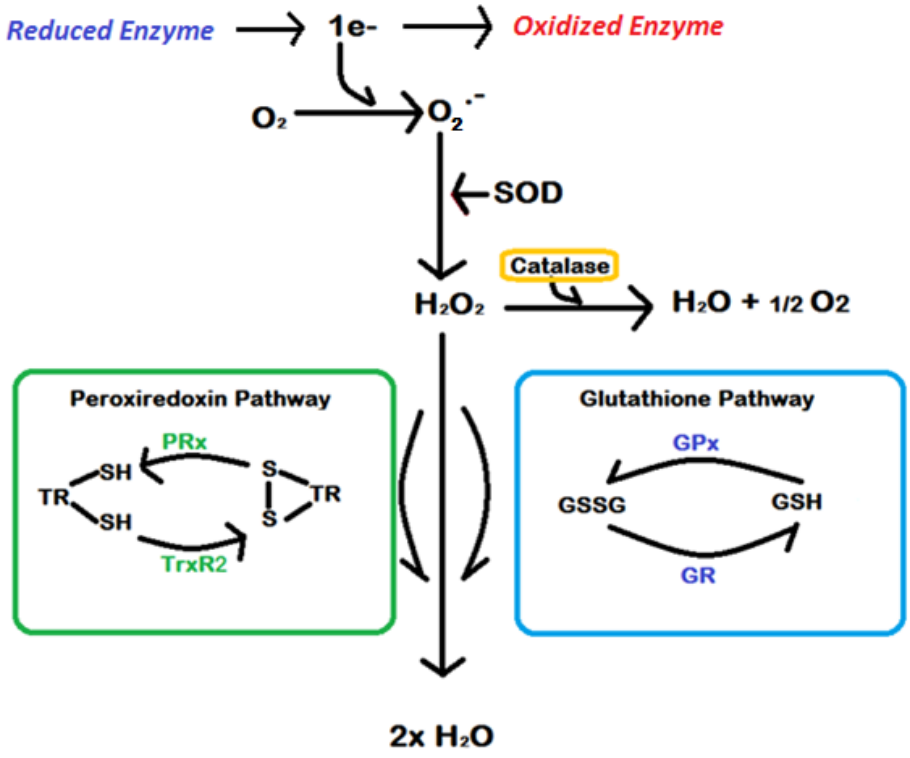

Figure 2. *General view of mitochondrial ROS production and corresponding consumption detoxification reactions*. These antioxidant pathways, peroxyredoxin and glutathione, need a supply of electrons provided by NADPH (nicotinamide dinucleotive phosphate) for their redox reaction continuation. e-, electron; SOD, superoxide dismutase; O_2•-, superoxide; GSH, glutathione reduced; GSSG, glutathione oxidized; GSH, glutathione reduced; GR glutathione reductase; GPx, glutathione peroxidase; PRx, peroxiredoxin; TrxR2, thioredoxin reductase; TR, thioredoxin (figure simplified and redrawn after Munro, et al. 2016).

1. 5. The Fish Model Used and Importance of the Fish Heart

Fish (with some exceptions, e.g. Bluefin Tuna, Shortfin Mako Shark, Lamnoid Sharks, Opahs, which use counter-current heat exchangers in musculature and other regions to warm parts or the whole organism (Opahs) (Wegner, et al. 2015), are ectothermic species, as such their body temperature parallels their aquatic environment. Water has a high specific heat capacity and low thermal conductivity in comparison with terrestrial environments; as such, aquatic environments will have much less thermal change and provide fewer possibilities for microclimate as terrestrial environments (Cossins & Bowler, 1987). However, in aquatic habitats in near shore areas in non-riverine water systems those show temperature stratification. Furthermore, fish migrate and move to different waters for foraging, reproduction and to escape predators, and these water environments may not be at suitable temperatures for the organisms, some might be approaching the upper lethal temperature for their species (Guzzo et al., 2017; Moyano et al., 2017; Zhang & Kieffer, 2014).

As temperature changes fish may behaviorally thermoregulate by swimming to preferred waters to stay away from extreme temperatures. This will be an adaptive temporal behavioral response as fish may encounter seasonal changes in water temperatures, and temperature differences across thermoclines found in the water columns which they might not be able to avoid (Cossins & Bowler, 1987). However, as variations in temperature may pose a stress to the physiology of the fish, the animal may have the ability to adjust to some extent its phenotype to changes in temperature (acclimatization). These changes in the phenotype will be limited by the animal's genotype, and will consist of changes to physiological responses at the organismal and cellular level. One system that is involved in thermal tolerance in fishes is the cardiac system as well thermal tolerance is said to be influenced by the animal's aerobic scope, the difference between maximum routine oxygen consumption for the organism, (Healy & Schulte, 2012; Pörtner et al., 2001; Fry & Hart, 1948).

The temperature at which the fish heart will fail may be one of the factors for setting the maximum thermal tolerance for the animal (Portner & Farrel, 2008). Acute warming then can potentially impact in a negative way fish physiology, which would put constraints in species distributions, and population dynamics in the long term.

1. 5. 1 Lake Sturgeon

Lake Sturgeon, *Acipenser fulvescens*, conserves many primitive characteristics that put them between the ancient Selachians and more derived Teleosts (LeBreton et al., 2004). Lake Sturgeon is a North-American species; it is one of the largest freshwater fish in North America. Their populations had experienced historically huge declines in numbers due to anthropogenic causes, such as overfishing, and dam building (Thiem et al., 2013). Lake Sturgeon can tolerate an environmental temperature range between 0 to 23.5^{o}C in southern Lake Huron (Briggs, et al. 2016). No studies have been done about Lake Sturgeon mitochondrial respiratory metabolism; this project can clarify and compare some of their mitochondrial metabolic characteristics against two other more evolutionary derived fish species. Furthermore, as mitochondria metabolism has been related to aging in some organisms by some workers (Munro et al, 2013; Barja, 2004) will be of interest to compare the Lake Sturgeon, a long lived fish to the Rainbow Trout and Common Carp, which have a different life history trait and longevity.

1. 5. 2 Common Carp

The Common Carp, *Cyprinus carpio*, is a hypoxia tolerant fish species (De Boeck et al., 2000).They are hardy and capable of living in polluted, saline (14 ppt), (Crivelli, 1981) and cold or warm waters (Moyle & Cech, 2004), hypoxia tolerant species (De Boeck, et al. 2000), as such investigating Common Carp mitochondrial metabolism and its relationship with resilience against temperature change can be informative. Their hypoxia resistance sets them apart from the lake sturgeon and the rainbow trout, and will be fruitful to compare this highly adaptable eurythermic

species against the Lake Sturgeon and Rainbow Trout. Furthermore, thermal resilience is known from this species, with lethal upper temperatures (temperature at which 50% of fish survive) recorded to be between 31-35.7^{o}C, (Okanagan lakes region) depending on the acclimation temperature (Black, 1953).

1. 5. 3 Rainbow Trout

The Rainbow Trout *(Oncorhyncus mykiss*) is an extensively studied fish and they are easily kept under laboratory conditions. As well Rainbow Trout swimming capacity, toxicity tolerance and other aspects of their physiology are well documented (Moyle & Cech, 2004). As well upper lethal temperatures have also been reported recorded, trout from British Columbia water acclimated where 50% of their population do not survive at 24^{o}C (Black, 1953).

1. 6. Relevance of the Heart

Thermal tolerance in fish is thought to be influenced by the aerobic scope, blood oxygen transport and other physiological constrains which will include metabolic controls of mitochondria function, circulating hormones, like catecholamines and other cell responses (Lurman & Gamperl, 2012). Fluctuations in temperature may influence the mitochondrial rate of oxygen consumption in the fish heart; and this may have an influence on the rate of production of ROS. The heart has a crucial role in supplying cardiovascular output for both gas and metabolite exchange throughout the body and maintaining cardiac performance is vital for proper function in fish tissue.

Increasing temperature brings increasing heart rate in fish, which in turn decreases cardiac filling time and stroke volume (Graham & Farrell, 1989). *O mykiss* cardiac performance peaks at optimal temperature; however, this optimal temperature for *O. mykiss* occurs well below the upper thermal limit for this species (Farrell et al., 1996) implicating detrimental effects of elevated temperature on cardiac performance as a putative limit to thermal tolerance. Furthermore, maximum cardiac performance and the optimal temperature will vary by species and it will limited

by the oxygen supply to the heart (Olson & Farrel, 2006). The temperature at which the heart fails is very close to the upper thermal tolerance of fish (Iftikar et al., 2015; Iftikar et al., 2014; Iftikar & Hickey, 2013; Pörtner & Knust, 2007). Importantly, the hemoglobin oxygen saturation in fish heart does not decline as the fish reaches the temperature at which heart failure occurs, indicating that organs systems periphery to the heart or involved with oxygen extraction and delivery are not responsible for the decline in heart performance at high temperature (Iftikar & Hickey, 2013). Instead, it might be the upper thermal limits for fish hearts are determined by mitochondrial capacity, which may in turn be important influence in ectotherm thermal tolerance (Iftikar & Hickey, 2013). For example, in the temperate to sub-Arctic *Gadus morhua* myocardial oxidative function decreased with a 10 °C increase in temperature (Rodnick et al., 2014). Acute increase in temperature may induce decline or levelling of oxygen consumption at temperatures approaching the upper thermal limit at the mitochondrial and cell level; mitochondrial dysfunction with increasing temperature maybe caused by ATP production deficiency, overproduction of ROS molecules, dysfunction in respiratory capacity or a combination of the three. Mitochondria dysfunction then can be brought by the possibility of mitochondria uncoupling, which can lead to declining oxidative phosphorylation efficiency, due to proton leak; furthermore, redox reactions in the ETC will keep pumping protons, but less of them will contribute to ATP synthesis when uncoupling increases due to elevated proton leak. ATP demand by the cell, the organ and the whole organism will be increasing, as temperature rises, creating a mismatch in the supply and demand for energy (Rodnick et al., 2014).

1. 7. Rationale

The metabolic capacity of cardiac mitochondria has been suggested to limit performance capacity in fish, specifically under thermal stress; in skeletal muscle (Banh et al., 2016); and the heart

(Iftikar & Hickey, 2013); and other organ systems (Currie & Schulte, 2014; Pörtner, 2002a). Furthermore, acute temperature stress is known to induce oxidative stress in cells (Abele, 2002). As well, the temperature at which the fish heart will fail may set the maximum thermal tolerance for the animal (Iftikar & Hickey, 2013; Portner, 2002b). Therefore, mitochondria might show to be the organelle that influences the cardiomyocyte thermal capacity threshold in fish. In the current study, cardiac mitochondrial metabolism of trout, carp and sturgeon was examined, by recording mitochondria respiration and ROS (H_2O_2) production and consumption by mitochondria isolated from the respective fish ventricles. The experimental design of this study was based on an *ex vivo* mimicry of acute warming with isolated mitochondria collecting data with increasing temperature. Assays were performed at three temperatures 15^{o}C, 20^{o}C, and 25^{o}C, on mitochondria from fish that where acclimated to 15^{o}C. The experiments used two mixtures of respiratory substrates: glutamate, succinate and malate (GMS), (Figure 3); the second combination of substrates used palmitoylcarnitine plus malate (PCM), (Figure 4). This was done to examine any differences in: patterns for the combinations of two mitochondrial substrates, which involve different relevant mitochondrial pathways; and within and across species differences, with temperature change. The rates of cytochrome c oxidase and citrate synthase mitochondrial enzymes were also sampled. To evaluate if alternative denominators to mg of mitochondrial protein may influence the outcomes of comparing mitochondrial rates between species the rates of these two central mitochondrial enzymes were used as alternative means for expressing the mitochondrial oxygen consumption and H_2O_2 production and H_2O_2 consumption.

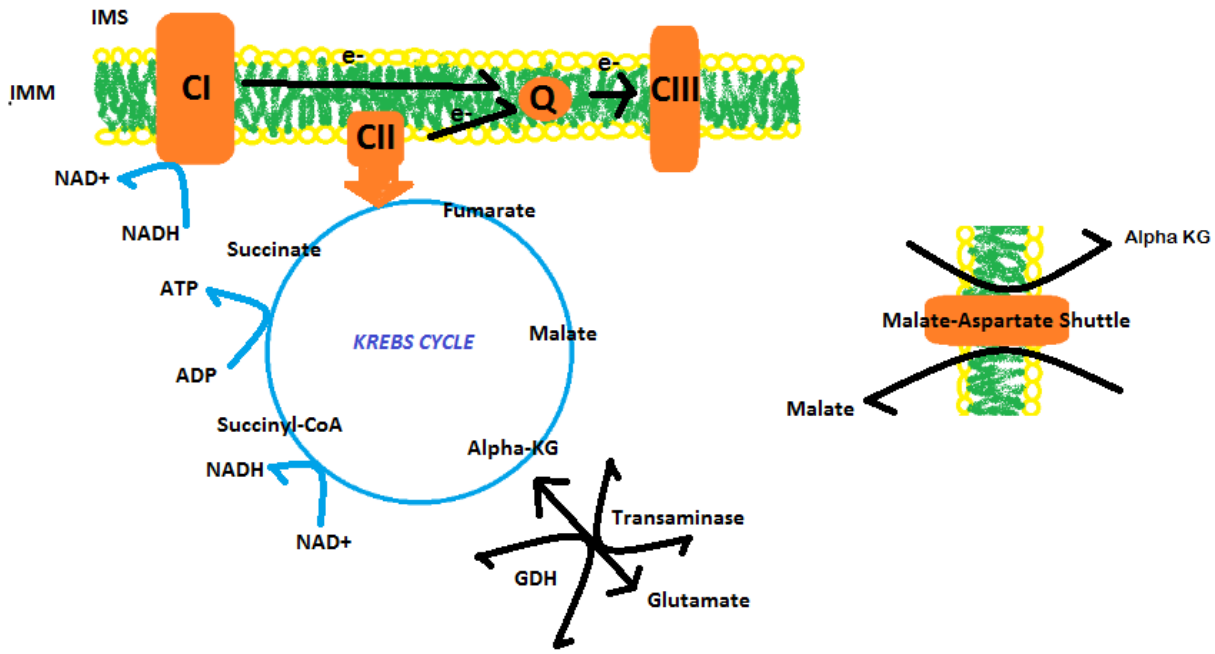

Figure 3. *Simplified cartoon sketch depicting the general pathways in the mitochondria that will be involved in the combination of substrates for GMS used in this study*. IMS, Inner membrane space; IMM, inner mitochondrial membrane; GDH, glutamate dehydrogenase; Q, ubiquinone; CI, complex I; CII, complex II; CIII, complex III; e-, electron, Alpha KG, α-ketoglutarate, modified from (Gnaiger, 2014).

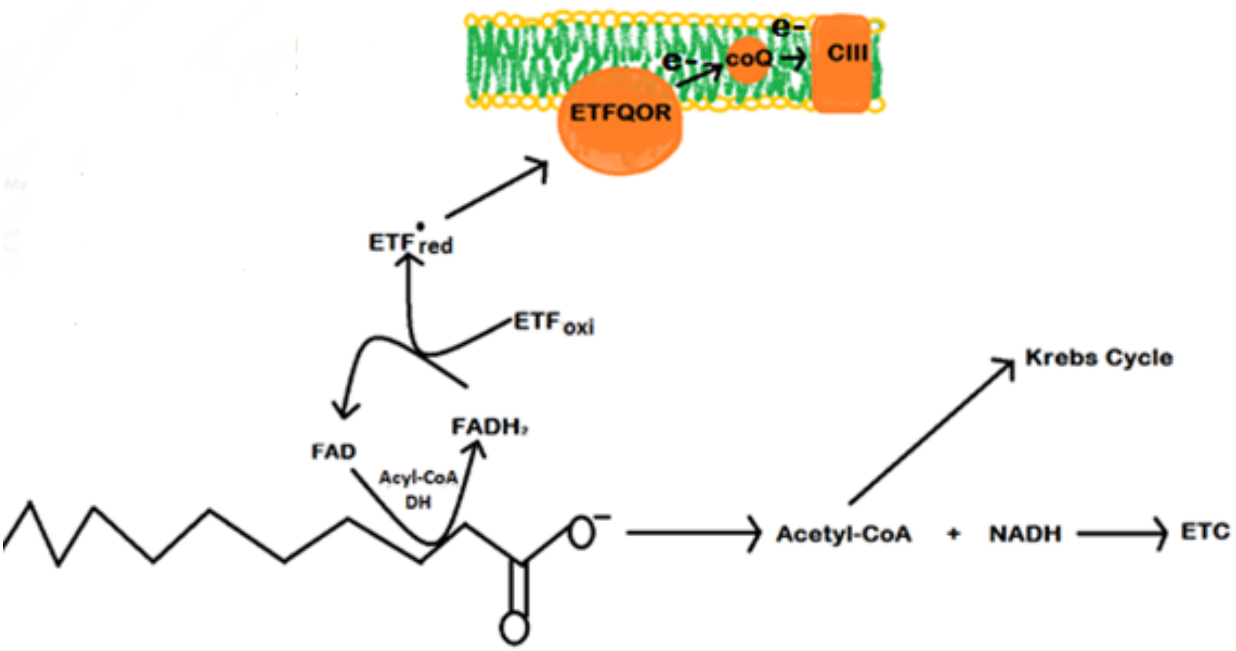

Figure 4. *Simplified cartoon sketch depicting the general pathways in the mitochondria that will be involved in the combination of substrates for PCM used in this study*. This specific pathway will represent the fatty acid β-oxidation in the mitochondria. ETF_{red}, electron transferring flavoprotein reduced; ETF_{oxi}, electron transferring flavoprotein oxidized; ETFQOR, electron transferring flavoprotein ubiquinone oxidoreductase, CoQ, ubiquinone; Acyl-CoA DH, acyl co-A dehydrogenase; e-, electron, (Gnaiger, 2014).

1. 8. Objective

In vitro test of acute stress: To determine and compare respiratory rates (mitochondrial capacity); H_2O_2 production rates; and H_2O_2 consumption rates of fish cardiac muscle at three different temperatures (15ºC, 20ºC and 25ºC), to mimic acute heat stress, of fish acclimated at 15ºC. The fish model includes: *O. mykiss, C. carpio* and *A. fulvescens*. This is done to reflect an acute challenge comparison of mitochondrial performance in an *ex vivo* heat challenge.

1. 9. Statistical Hypotheses

In vitro acute temperature change influence which mimics acute heat stress

Ho: The mitochondrial respiration and ROS production and consumption rates will not change with change of temperature of the assay

Ha: The mitochondrial respiration and, ROS production and consumption rates of the mitochondria on the fish species will increase with temperature

On the Species difference in mitochondrial rates

Ho: There will be no difference in the mitochondrial oxygen consumption and hydrogen oxide rates between the three species in this study.

Ha: There will be a difference in the mitochondrial oxygen consumption and hydrogen oxide rates between the three species in this study.

On the species difference in mitochondrial Oxidative ratio and fractional electron leak

Ho: There will be not found differences on the mitochondrial oxidative ratio and fractional electron leak between the three species while the temperature increases.

Ha: There will be differences on the mitochondrial oxidative ratio and fractional electron leak between the three species while the temperature increases.

1. 10. Prediction

Cardiomyocyte mitochondria respiratory capacity and ROS production and consumption of *O. mykiss*, *C. carpio* and *A. fulvescens* will increase exponentially with increasing temperature.

2. Methods

2. 1. Chemicals

The chemicals used in the study were purchased from these suppliers: Sigma-Aldrich, ThermoFisher-Scientific and Acros.

2. 2. Animal Housing, Collection and Dissection

In this study three fish species *O. mykiss*, Rainbow Trout; *C. carpio*, Common Carp; and *A. fulvescens*, Lake Sturgeon were housed in the Duff Roblin Animal Facility in flow through aquaria

held at 15ºC, supplied with the same water. The fishes were fed chow *ad libitum* three times per week and were kept in a light and dark period of 12:12 hours per day. The animals were purchased from commercial fish suppliers with exception of the Lake Sturgeon: which were raised from egg stage at The University of Manitoba Animal Care Committee animal holding facility.

For sampling, fish were caught by net and euthanized with a blow in the head, followed by severing of the gills or destruction of brain. The fish of mixed (or indeterminant in the case of Lake Sturgeon) sex were weighed: Lake Sturgeon (n=9), 1334.8g ± 114.25g; Common Carp (n=5), 1923.76g ± 269.95g; and, Rainbow Trout (n=13), 2014g ± 244.18g (values are mean ± SE, standard error of the mean); followed by their ventricles being dissected out and placed in ice cold isolation medium (below) prior to processing for mitochondrial isolation.

2. 3. Isolation of Mitochondria

The isolation method was based on: (Banh et al., 2016; Affourtit, et al. 2012). The isolation procedure used a Chappel Perry buffer for isolation, CPI (100mM KCl_2, 50mM Tris, 2mM EGTA, at 25ºC and pH 7.1). The ventricles of recently dissected hearts were diced on an ice cold cutting board and washed (3 times) with CPI buffer. The washed tissue, was transferred to a beaker containing Chappel Perry II buffer, CPII (CPI buffer plus 5mM $MgCl_2$, 1mM ATP, 0.5% (w/v) BSA, bovine serum albumin, at 4ºC at pH 7.65 and continued by the addition of 2.5 IU/ml of subtilisin A (Protease Type VIII from *Bacillus licheniformes*)), in a range of 8-10ml per gram of tissue ratio, mixed for 5 minutes and followed by 3 short bursts of homogenizing with an Ultra-Turrax Digital Homogenizer (Daigger Scientific) pestle rotor stator, at the lowest speed. The beaker was gently mixed on ice for 5 minutes.

The resulting homogenate was then put through a series of centrifugation steps, all of them at 3ºC. The first step consisted of centrifugation at 500 × g, for 10 minutes after which the supernatant

was filtered through several layers of Mini WP cheese cloth. The filtered supernatant was again centrifuged for 10 minutes at 10,000 × g and the pellet was re-suspended in CPI medium; this last step was repeated and followed by a 500 × g centrifugation step for 5 minutes. This final supernatant was then centrifuged one last time at 4,000 × g for 10 minutes. The final pellet (mitochondrial fraction) was re-suspended with CPI buffer and protein concentration was determined by Biuret Assay (with 2% (w/v) deoxycholate) and BSA used as the standard for the concentration.

2. 4. Mitochondrial Respirometry

Oxygen consumption was performed using an O2k respirometer (Innsbruck, Austria), containing two 2ml chambers. The isolated heart mitochondria samples (0.5mg/ml of sample Rainbow Trout; 0.3-0.4 mg/ml for Common Carp and Lake Sturgeon) from the three fish species, acclimated at 15^{o}C, were placed on the O2k respirometer chambers with a respiration medium containing: 140mM KCl, 20mM Hepes, 1mM EGTA, 5mM K_2HPO_4, plus 0.3% BSA. The rate of oxygen consumption and H_2O_2 efflux was measured with two substrate combinations: 5 mM each of glutamate, malate, and succinate (GMS) or a combination of palmitoyl-carnitine 50µM, and malate 5mM (PCM).

Assays were performed at three temperatures 15^{o}C, 20^{o}C, and 25^{o}C, within a time range of 30 to 45 minutes, for each sample of fish of this study. The O2k chambers were filled with respiration medium plus hexokinase 2.5IU/ml and glucose 10mM, as an ADP regenerating system, to control for ADP consumption so rates of respiration under phosphorylating conditions are not limiting and ADP availability remains stable. The chambers were closed and sequentially state 2 respiration (mitochondria in the presence of substrate) was initiated by adding of substrates either GMS or PCM. State 3 respiration (mitochondria in the presence of substrate and ADP) was started by the

addition of 800µM ADP, this rate represents mitochondrial phosphorylation of ADP. All rates in each step of the process were allowed to stabilize until at least 2 minutes of steady rate was reached.

2. 5. H_2O_2 Production

O2k fluorometry measuring H_2O_2 production were performed simultaneously to oxygen consumption. Therefore the temperature changes and substrate combinations are the same as that for the respirometer experiment. The assay for the production of H_2O_2 uses Amplex Ultra red (AUR), which is used to detect H_2O_2 in the presence of HorseRadish Peroxidase, HPx; and superoxide Dismutase, SOD, which dismutates extramitochondrial superoxide into H_2O_2. AUR reacts with H_2O_2 in the presence of HPx to produce a red fluorescent compound, resorufin. The fluorescence was monitored with O2k settings as 525nm excitation, 620nm emission, gain for sensor 1000 and polarization voltage 200mV. Steps of mitochondrial respiration were followed concurrently with the oxygen consumption, and data on H_2O_2 accumulation in the chamber was detected. At the end of the assay a known amount (0.5µM) of exogenous H_2O_2 was added in each chamber for calibration and consecutive calculation of the H_2O_2 efflux rates, which were calculated after subtracting the background rates taken of rates of H_2O_2 production with mitochondria present only.

2. 6. H_2O_2 Consumption

The assay used the same substrates as the oxygen consumption procedure and is based on (Munro et al. 2016), 5 mM each of glutamate, malate, and succinate, GMS or 50µM palmitoyl-carnitine, 5mM malate, PCM; which were added to an eppendorf tube (resting in a water bath at the proper specific temperature of 15^{o}C, 20^{o}C, or 25^{o}C) and made up to 1ml using respiration medium (140 mM KCl, 20 mM Hepes, 1 mM EGTA, 5 mM K_2HPO_4, plus 0.3% w/v BSA). This was followed by the addition of a bolus of 2.5µM H_2O_2 and then finally mitochondria are added to the tube. The

reaction was started by mixing with the pipette, while transfering of 180µl of the reaction mixture to a 96 well plate where 20µl of quenching solution (5 IU ml^{-1} HPx, 25 IU ml^{-1} SOD and 50µM AUR in respiration medium), was present. Aliquots were continually sampled every 3.0 minute intervals until the completion of 12 minutes of metabolism, (Figure 5). The 96 plate well was then immediately read on a platereader (FLUOstar Omega-BMG LABTECH) for 10 minutes (necessary for fluorescence correction of assumed linear drift), and slopes increasing fluorescence intensity were monitored at 560 nm and 590 nm emission and excitation wavelengths respectively. The chemical background was specifically made for each mitochondrial concentration sampled; background fluorescence was obtained as the by measuring the fluorescence of only medium and mitochondria on the absence of exogenous H_2O_2. The H_2O_2 consumption rates were calculated and expressed as nmol $min^{-1}mg^{-1}$ of protein.

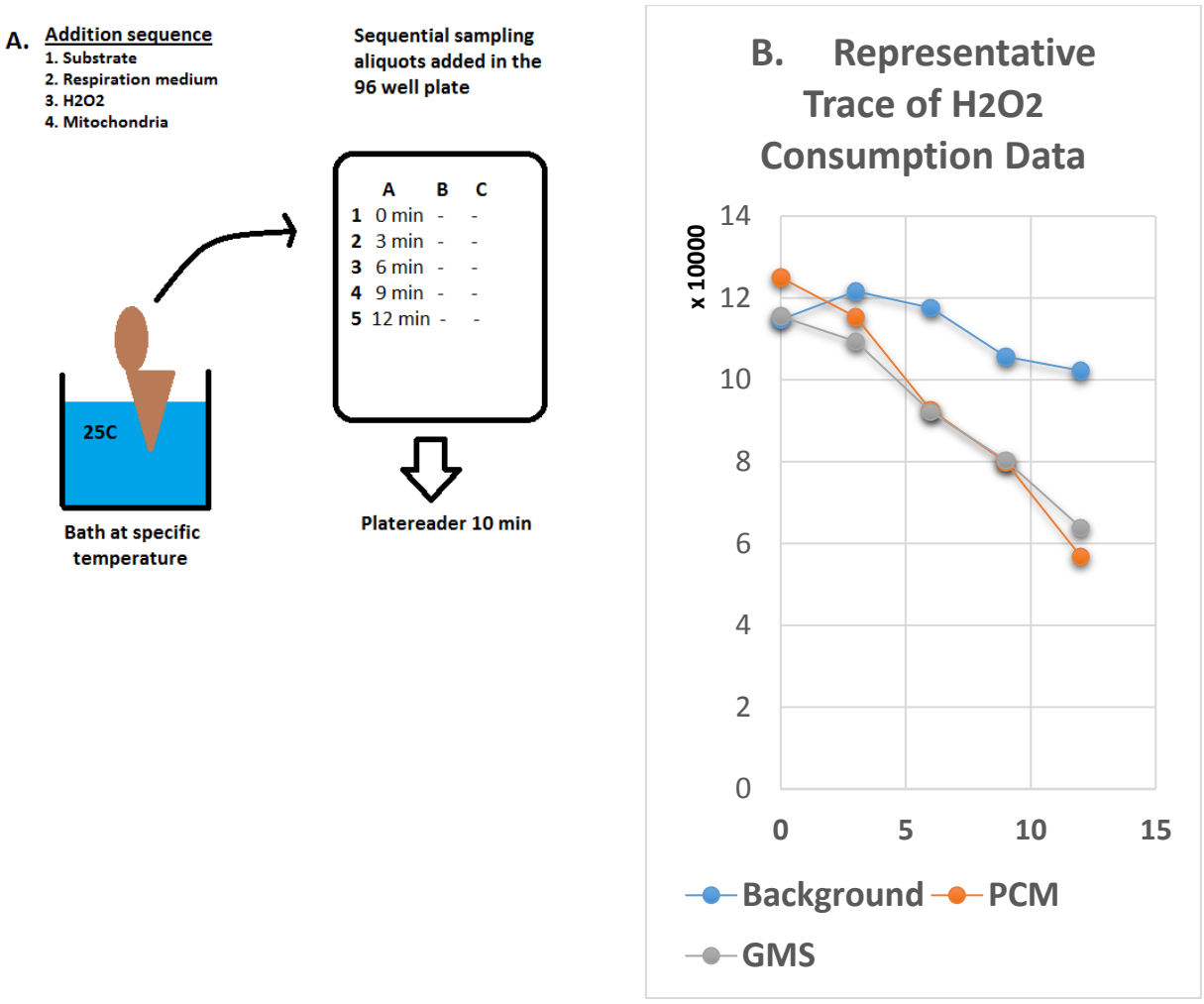

Figure 5. *Demonstration of the of end point assay for H_2O_2 consumption rate.* As seen in **A.** It is the addition sequence of chemicals in the Eppendorf filled to 1ml, the reaction was started by adding fresh mitochondria to the Eppendorf and transferring sequentially aliquots to the plate reader, which had the wells pre filled with quenching solution containing 5 IU ml^{-1} HPx, 25 IU ml^{-1} SOD and 50µM AUR in respiration medium. **B.** The plate reader was immediately taken to the spectrophotometer where the fluorescence was captured for 10 minutes, here is seen a representative trace of the readings for sturgeon mitochondria at 25°C. Redrawn from: (Daniel Munro et al., 2016)**.**

2. 7. Enzyme Activity

In some studies, the denominator for mitochondrial rates expression has been debated specifically when comparing mitochondria from within species and different taxa: *O. mykiss*; *Rattus norvegicus*, Laboratory Rat; *Pogona vitticeps*, Bearded Dragon; and *Bufo marinus*, Cane Toad

(Hulbert, et al. 2006; Guderley, et al. 2005; Leary et al., 2003). The rates of enzyme activity for cytochrome c oxidase, the enzyme complex which transfers protons to oxygen in the last step of oxidation-reduction on the electron transport chain; and citrate synthase, Krebs cycle enzyme that catalyzes the first step in this cycle, were determined. These enzyme rates were not only used to determine the specific activity of the individual enzymes in the mitochondria of these fish, but also to determine oxygen consumption, and ROS production and consumption on the protein expressed rates, to examine possible different denominators in mitochondrial rates that might explain similarities or differences between species and patterns of influence of temperature on the mitochondrial rates measured.

The frozen isolated mitochondria were kept at -80^{o}C prior to performing the assay. Prior to analysis mitochondria were thawed and kept on ice during the enzyme measurement procedure. Enzyme activity was measured by spectrofluorometric assays in a Cary 100 Series UV/VIS spectrophotometer. The enzyme assays were based on (Spinazzi et al., 2012)and were performed at three temperatures 15^{o}C, 20^{o}C, and 25^{o}C for three independent samples for each species, and at 15^{o}C and 25^{o}C for all remaining samples. All concentrations listed are final concentrations in the assay cuvette. The enzyme rates were calculated, CCO activity and CS activity represent cytochrome c oxidase and citrate synthase respectively, and the rates were expressed as: nmol * min^{-1} mg of protein^{-1}.

2. 7. 1 Cytochrome c Oxidase (CCO)

Complex VI of the ETC (EC.1.9.3.1), the enzyme is measured by following the oxidation of reduced cytochrome c at a wavelength of 550nm. Reduction of cytochrome c was carried out by adding a few crystals of sodium dithionite to a microcentrifuge tube containing 1mM cytochrome

c stock solution (1ml volume). The dithionite excess was removed by blowing oxygen gas through this stock solution.

Cuvettes were filled with 50mM potassium phosphate buffer (500µl), pH 7.0 at 25^{o}C, plus lauryl maltoside 0.05%; 0.5-1µg of thawed mitochondrial protein and water was added to fill cuvette to 1ml volume. The reaction was started by the addition of 60µM cytochrome c stock solution. The activity on the cuvettes was followed for 3 minutes for continuous and linear rates. Parallel cuvettes with 300 µM KCN were run to correct for CCO independent changes in absorbance (background); rates of background absorbance change were subtracted from the rates of the cuvettes without this CCO inhibitor. Final rates were transformed to nmol of enzyme activity* min^{-1} mg^{-1} of protein calculated with the extinction coefficient of ε = 18.5 mM^{-1} cm^{-1}.

2. 7. 2 Citrate Synthase (CS)

Citrate synthase (EC 2.3.3.1) was assayed by filling cuvettes with 100mM of Tris solution (500µl), pH 8.0, with triton-X100 (0.2% v/v); 100 µMof DTNB (5, 5-dithio-bis-(2-nitrobenzoic acid)); 300 µM of acetyl coenzyme A; and 10 µg of frozen mitochondrial protein, water was added to fill cuvette to 1ml volume. The contents of the cuvettes were mixed and the background non-CS related rate of increasing absorbance was monitored at wavelength of 412 nm for 2 minutes. The CS activity was started by the addition of 500µM of oxaloacetic acid (50µl) and the reaction rate was followed for 3 minutes at 412 nm. Final rates were transformed to nmol of enzyme activity* min^{-1}mg of protein^{-1} using ε=13.6 mM^{-1} cm^{-1}.

2. 8. Statistical Analysis

Analysis by one way ANOVA and Student's t-test were performed in R, version 3.3.0 (2016-05-03) -- "Supposedly Educational". Repeated measures ANOVA were performed on Sigma Plot 13.0. Results are showed as mean ±standard error of the mean, SE; significance was based on an

alpha of 0.05, for all tests, after a Tukey HSD multiple comparison tests were performed when appropriate. Welch's test was performed for the oxidative ratios using ©2017 GraphPad Software, Inc. All rights reserved, found at: https://www.graphpad.com/quickcalcs.

2. 9. Oxidative Ratio

The oxidative ratio was calculated as:

$$\boldsymbol{Oxidative\ Ratio} = \frac{\boldsymbol{State\ 2\ H_2O_2\ Production}}{\boldsymbol{State\ 2\ H_2O_2\ Consumption}}$$

With the increase in the value representing an increase in the overall production of H_2O_2 parallel to the consumption of ROS.

Due to the nature of the data for the Rainbow Trout in the current study, as H_2O_2 production and consumption was assayed on different experiment days for this species, the parameter for variance used for the oxidative ratio is the standard error of the mean determined by error propagation technique. This was done to deal with the independent uncertainty of samples taken independently from each other. The Lake Sturgeon and the Common Carp data were not considered as independent points, as they were assayed the same days for H_2O_2 production and consumption; however, the same calculation (error propagation) was conducted for comparison with the Rainbow Trout data. The parameter for variance was calculated using this formula for error propagation: Error propagation, with $mean_1$=H_2O_2 production, and $mean_2$=H_2O_2 consumption, and corresponding *se* (standard error):

$$\text{Combined SEM} = \sqrt{\left(\frac{se_1}{mean_1}\right)^2 / \left(\frac{se_2}{mean_2}\right)^2}$$

2. 10. Fractional Electron Leak, FEL

FEL, was calculated with the state 2 oxygen consumption and state 2 ROS production. FEL is the value which assesses ROS production in relation to the total mitochondrial electron flux (as oxygen consumption)(Wiens et al., 2017). Using SigmaPlot 13.0 an ANOVA RM with TukeyHSD was done in this data, followed with a *pos hoc,* on the FEL ratios, when heteroscedacity and non-normality where present on the data, a rank ANOVA was performed followed with a Dunn's multi-comp. No error propagation was performed. The FEL, was calculated as:

$$FEL = 100\left[\frac{State\ 2\ H_2O_2\ Production}{(Oxygen\ Consumption\ per\ O + State\ 2\ H_2\ O_2\ Production)}\right]$$

A two tailed t -test was performed in SigmaPlot 13.0; a Mann-Whitney Rank Sum Test was done for the rainbow trout and lake sturgeon FEL ratios due to non-normality and heteroscedasticity of the data for analysis of substrate combination.

3. Results

3. 1. Acclimation Temperature (15ºC)

3. 1. 1 Oxygen Consumption

When mitochondrial rates were assayed at the acclimation temperature of 15°C we found significant differences between substrate combinations in all three species where the GMS combination was higher than the PCM rates; furthermore, when comparing rates across species differences were found for both substrate combinations (Figure 6 A-B). For state 3 respiration in mitochondria, the Lake Sturgeon oxygen consumption rates are lower than those of the trout, for

both substrates (Figure 6-D). Rates in the Common Carp are intermediate for both PCM and GMS substrate combinations.

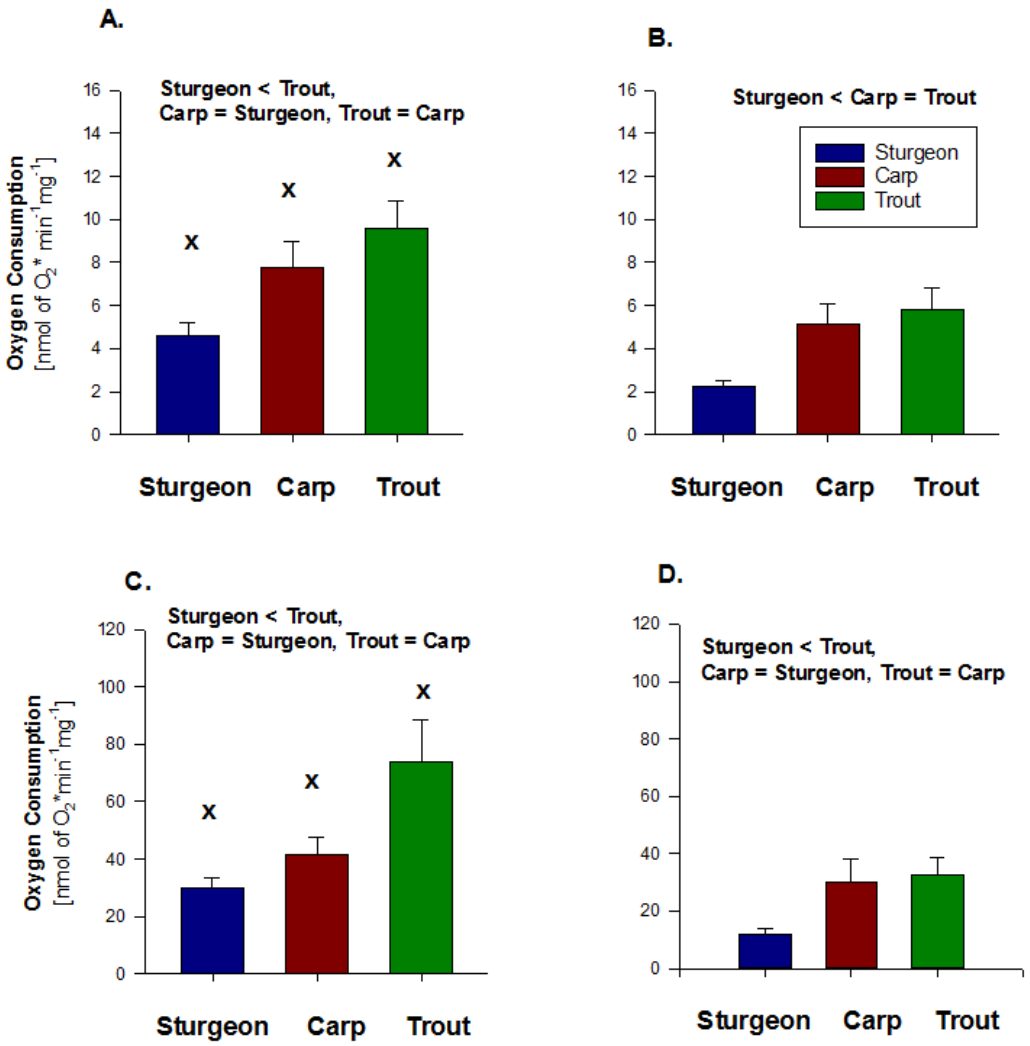

Figure 6. *Respiration rates for the heart mitochondria isolated from Lake Sturgeon, Common Carp and Rainbow Trout at acclimation temperature* (15°C). **A.** GMS rates in state 2, non-phosphorylating mitochondria. **B.** PCM rates in state 2. **C.** GMS rates of respiration in state 3, in the presence of ADP, phosphorylating mitochondria. **D.** PCM, state 3, respiration rates. Rates are shown as mean ± SE, in nmol O_{2*} $min^{-1}ml^{-1}$ of protein, α=0.05. x shows significant greater rates between substrates, with GMS rates being higher for the three species than PCM rates. Bold text represents differences across species. (N=7-8, GMS and PCM respectively on Lake Sturgeon; N= 5, Common Carp, N= 7, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout Rainbow Trout.

3. 1. 2 Respiratory Control Ratios

The respiratory control ratios (RCR), there were no difference in the RCR between substrates with the exception of Lake Sturgeon (Figure 7). There were no interspecific differences for RCR indicating that at least for the acclimation temperature the relative coupling efficiency of mitochondria from the three fish is likely similar.

3. 1. 3 H_2O_2 Metabolism

3. 1. 3. 1. H_2O_2 Production

Rates for H_2O_2 production for state 2 respiration were higher in the mitochondria from Common Carp than those from Rainbow Trout and Lake Sturgeon, (Figure 8). Rates of H_2O_2 production are not different between the Rainbow Trout and the Lake Sturgeon. For all the three species the GMS combination of substrates gave greater rates of H_2O_2 efflux than PCM (Figure 8). State 3 for H_2O_2 production data are not shown, the values decreased dramatically after the addition of ADP to the respiration chamber, but the H_2O_2 efflux rates were not proportional to the concentration of mitochondrial protein added to the chamber; therefore, the rates of H_2O_2 efflux in state 3 were deemed too low to quantify.

3. 1. 3. 2. H_2O_2 Consumption

When comparing the rate of H_2O_2 consumption across assay condition (substrate free background or the substrate mixes PCM and GMS) the GMS combination was not different from the PCM rates within a species; however, the background rates are lower than the rates in the presence of both substrates, for all species, (Figure 9-A). Comparing across species, when expressed per milligram of protein, the rates of H_2O_2 consumption for the background rate for mitochondria from Common Carp was higher than the Rainbow Trout and Lake Sturgeon. In the presence of

PCM the trout mitochondria had higher rates of H_2O_2 consumption than the Common Carp and Lake Sturgeon mitochondria (Figure 9-A).

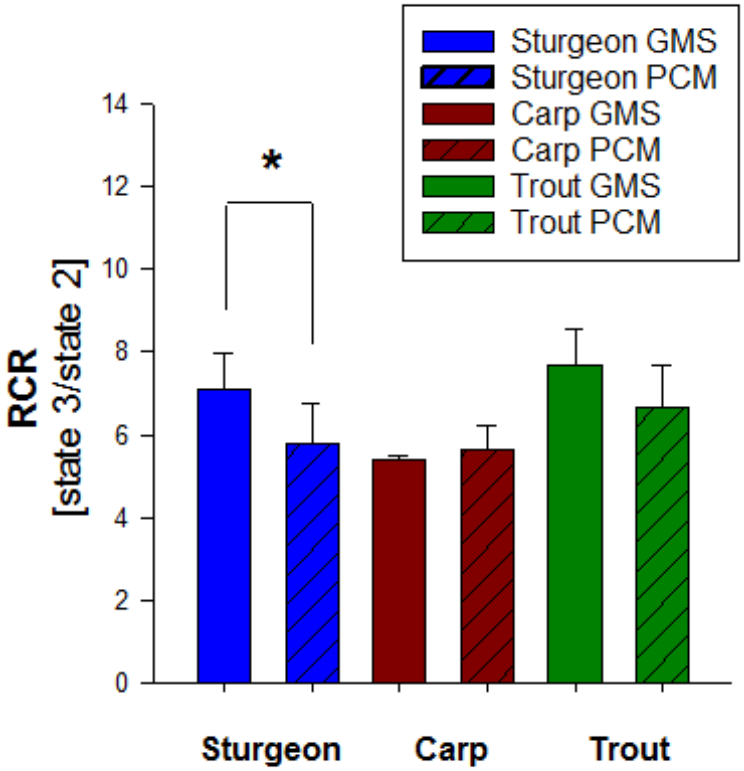

Figure 7. *Respiratory control ratios for isolated fish heart mitochondria at acclimation temperature.* These values represent an estimate of the coupling efficiency of the mitochondrial rates for the isolated mitochondria with higher rates interpreted as better mitochondria quality. As ratios values have no units, data are mean ± SE, α=0.05. ***** Represents significance within lake sturgeon across substrate used. There are no interspecific differences across species. (N=7-8, GMS and PCM respectively on Lake Sturgeon; N= 5, Common Carp, N= 7, Rainbow Trout). Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

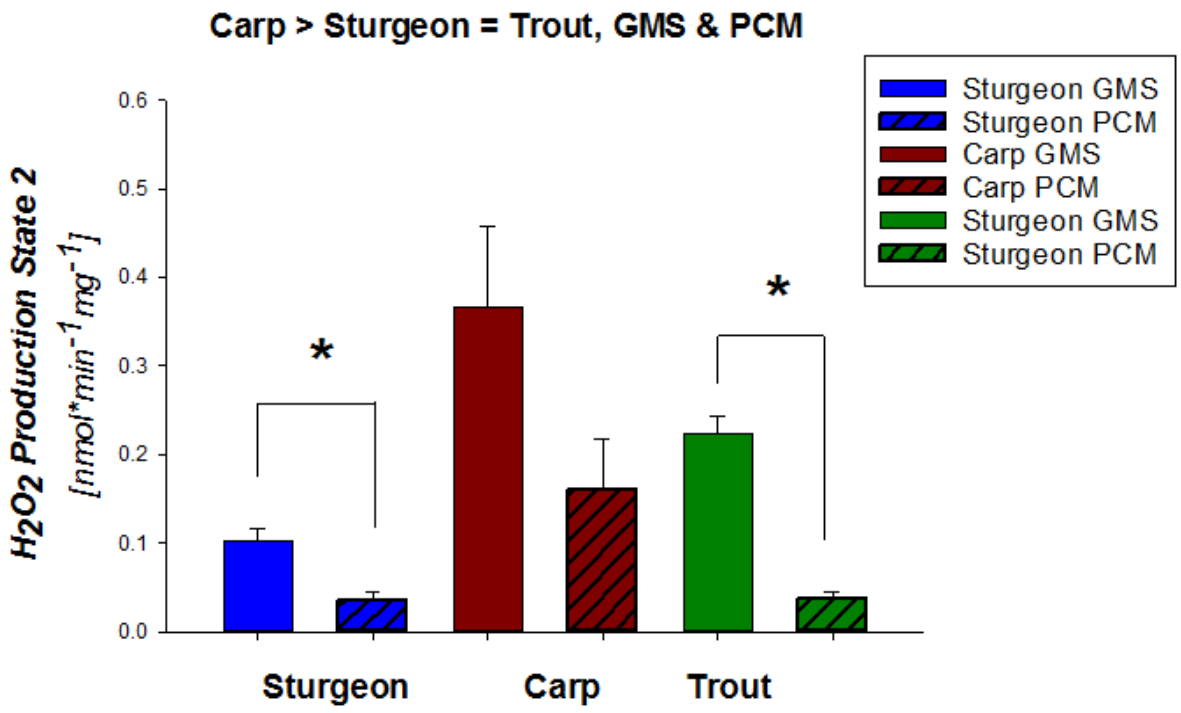

Figure 8. *Rates of H_2O_2 production by isolated fish heart mitochondria at acclimation temperature*, for mitochondria in state 2 respiration. Values are in nmol of H_2O_2*$min^{-1}mg^{-1}$ and are shown as mean ± SE, α=0.05. * Shows significance across substrates. Bold text represents differences across species. (N= 7, Lake Sturgeon, N=5 Common Carp, N=8-6 GMS and PCM respectively on Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

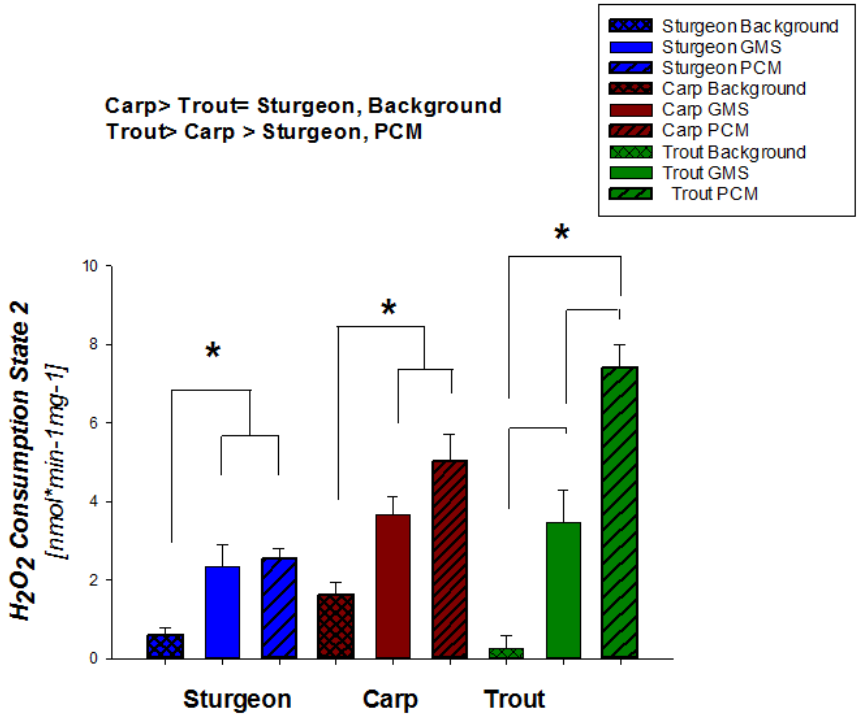

Figure 9. *Graphs showing the H_2O_2 consumption rates, assayed for sturgeon, carp and trout, for state 2, mitochondria in the presence of substrates only, at acclimation temperature*. Rates are in nmol of H_2O_2*$min^{-1}mg^{-1}$, and are shown as mean± SE, α=0.05. ***** and lines represent significance across substrates, bold writing shows significance across species. (N= 6-7, GMS and PCM for the Lake Sturgeon; N=5 Common Carp; N= 5 Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout Rainbow Trout.

3. 1. 4 Respiration Rates Expressed by Enzyme Activity

3. 1. 4. 1. Respiration Rates

Cytochrome c oxidase, CCO, normalized rates of respiration rates did not differ among species whereas CS normalized rates of respiration results paralleled the patterns seen when oxygen consumption was expressed to mg mitochondrial protein (Table 1).

3. 1. 4. 2. Enzyme Expressed Rates of H_2O_2 Production:

Rates of H_2O_2 efflux expressed per unit CCO activity do not show species differences during state 2 respiration. H_2O_2 efflux rates normalized to CS activity show a parallel pattern of significance between species as seen with the protein expressed rates, with the Common Carp rates of H_2O_2 production higher than the Rainbow Trout and the Lake Sturgeon, (Figure 10).

3. 1. 4. 3. Enzyme Expressed Rates of H_2O_2 Consumption:

When H_2O_2 consumption is express per unit CCO activity then there were no differences across species; however, differences across substrate conditions are still found. CS rates of H_2O_2 consumption had a similar pattern as the data using mg of protein as the denominator (Figure 11).

Table 1. *Respiration rates expressed per enzyme activity for the three species at the acclimation temperature (15°C),* states with CCO on the name, are rates expressed by cytochrome c oxidase activity, the rates of respiration (pmol*min^{-1} mg^{-1}) over the enzyme activity (nmol*min^{-1} mg^{-1}); as for stages with CS on name are the rates expressed per citrate synthase activity; * show significance across substrate combination for that specific rate within species; [1] shows significant value across species. Values are mean ± SE, α=0.05. (N= 6, GMS and PCM for Lake Sturgeon; N=4, GMS and PCM for Common Carp; N= 7-6, GMS and PCM respectively on Rainbow Trout).

	Lake Sturgeon		**Common Carp**		**Rainbow Trout**	
	GMS	**PCM**	**GMS**	**PCM**	**GMS**	**PCM**
State 2 CCO	1.7±0.6	0.8±0.3	2.4±1.1	1.4±0.6	2.0±0.4	1.5±0.3
State 3 CCO	12.3±4.3	5.1±2.3	12.5±6.0	6.8±2.1	14.7±2.3	9.6±2.8
State 2 CS	12.1±2.4*[1]	6.0±1.1*[1]	23.3±2.1*	15.1±2.3*	29.1±6.2	18.7±3.1
State 3 CS	84.6±11.7*[1]	31.5±6.1*[1]	123.2±9.8	80.6±18.2	240.7±74.4	120.1±23.1

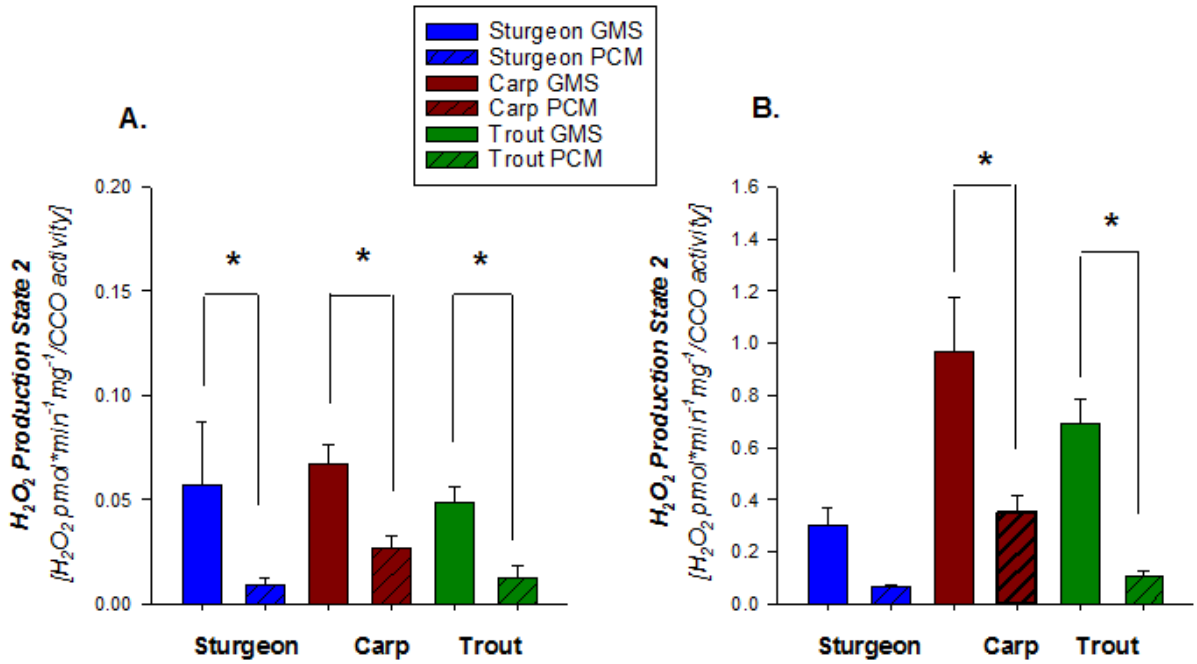

Figure 10. *Graph of the H_2O_2 production in the heart mitochondria of sturgeon, carp and trout, rates are expressed as enzyme activity, CCO rates and CS rates, and at acclimation temperature*. **A.** State 2 H_2O_2 production, CCO expressed. **B.** State 2 rates of H_2O_2 production, CS expressed. Values are in pmol H_2O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and are shown as mean± SE, α=0.05. ***** Represents significance between substrates. Species difference is marked as bold writing on the graph. (N=7-6, GMS and PCM respectively on the Lake Sturgeon; N=4, Common Carp; N=6 Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout Rainbow Trout.

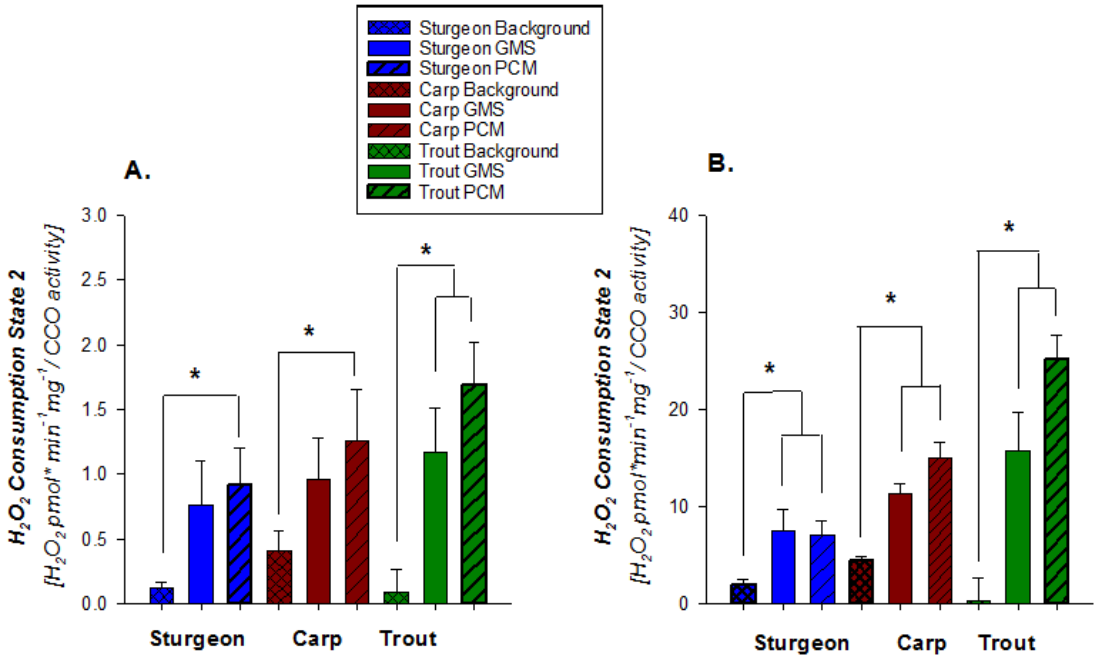

Figure 11. *Graphs showing the H_2O_2 consumption rates for state 2, mitochondria in the presence of substrates only, at acclimation temperature.* **A.** Consumption for H_2O_2 rates as CCO normalized. **B.** Consumption of H_2O_2 in the mitochondria, as CS normalized. Values are in pmol H_2O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and are shown as mean± SE, α=0.05. *****Represents significance across substrates. Species difference is marked as bold writing on the graph. (N= 5-6, GMS and PCM respectively for the Lake Sturgeon; N= 4, Common Carp; N= 5-6, GMS and PCM respectively on the Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout Rainbow Trout.

3. 2. Comparing acute ex vivo heat challenge on mitochondrial performance.

3. 2. 1 Oxygen Consumption

Lake Sturgeon: State 2 GMS on the Lake Sturgeon significantly increased between 15 and 20^{o}C and 25^{o}C, (Figure 12-A). Using the PCM substrate combination state 2 respiration in Lake Sturgeon was significantly higher at 25^{o}C compared to 15^{o}C, (Figure 12-B). State 3, GMS phosphorylating rate, oxygen consumption rates on the Lake Sturgeon increased across all the three temperature combinations, (Figure 12-C). State 3 the PCM substrate combination with the sturgeon increase with temperature. At 25^{o}C there are found substrate differences on Lake Sturgeon respirometry rates, for state 2 and state 3 respiration.

Common Carp: There was no difference across oxygen consumption rates for GMS substrate combination for the Common Carp on all states of mitochondrial respiration. As for state 2 PCM had no difference for the Common Carp; however, state 3 PCM at 25^{o}C Common Carp increased with temperature. State 2 GMS respiration for Rainbow Trout heart mitochondria had an increase between the range of 15 to 25^{o}C (Figure 12-A). Furthermore, the Common Carp respiration rates are not different across substrates.

Rainbow Trout: No differences were found in the Rainbow Trout for state 3 GMS, (Figure 12-C). Furthermore, for PCM substrate combination respiration by the Rainbow Trout heart mitochondria did not display temperature sensitivity.

As stated before at 15^{o}C there are found interspecific differences across species; however, respiration rates at 20^{o}C and 25^{o}C were found not to be different across species.

Respiratory Control Ratios, RCR: The RCR for Lake Sturgeon and Common Carp had no difference with increase temperature for GMS. The Rainbow Trout on the other hand has significant decrease in RCR as temperature increases for the GMS substrate combination, (Figure

13-A). There were found interspecific differences across the species for the RCR at 25ºC RCR on GMS between the Common Carp versus the Rainbow Trout. For the PCM substrate combination the RCR displayed no differences across species and temperatures (Figure 13-B).

3. 2. 2 H_2O_2 Production

To maximize rates, the H_2O_2 production was only measured for state 2 where, respiratory substrate is present but not ADP. With GMS these rates increase with increasing temperature in all three species, (Figure 14-A). The PCM substrate combination rates of H_2O_2 production increase but follow a different pattern for the three species, (Figure 14-B).

Interspecific species differences on H_2O_2 production, GMS at 20ºC we find differences with the Common Carp having a higher capacity of H_2O_2 production than the Rainbow Trout, the Lake Sturgeon sits in the middle of the two other species, (Figure 14-A). The Lake Sturgeon has a higher H_2O_2 production at 25ºC in comparison with the other two species; furthermore, we find no substrate differences between the two substrate combinations for the Common Carp and the Rainbow Trout at 20ºC and 25ºC. As stated in the first part of the results, there are substrate differences for the Lake Sturgeon and the Rainbow Trout at 15ºC.

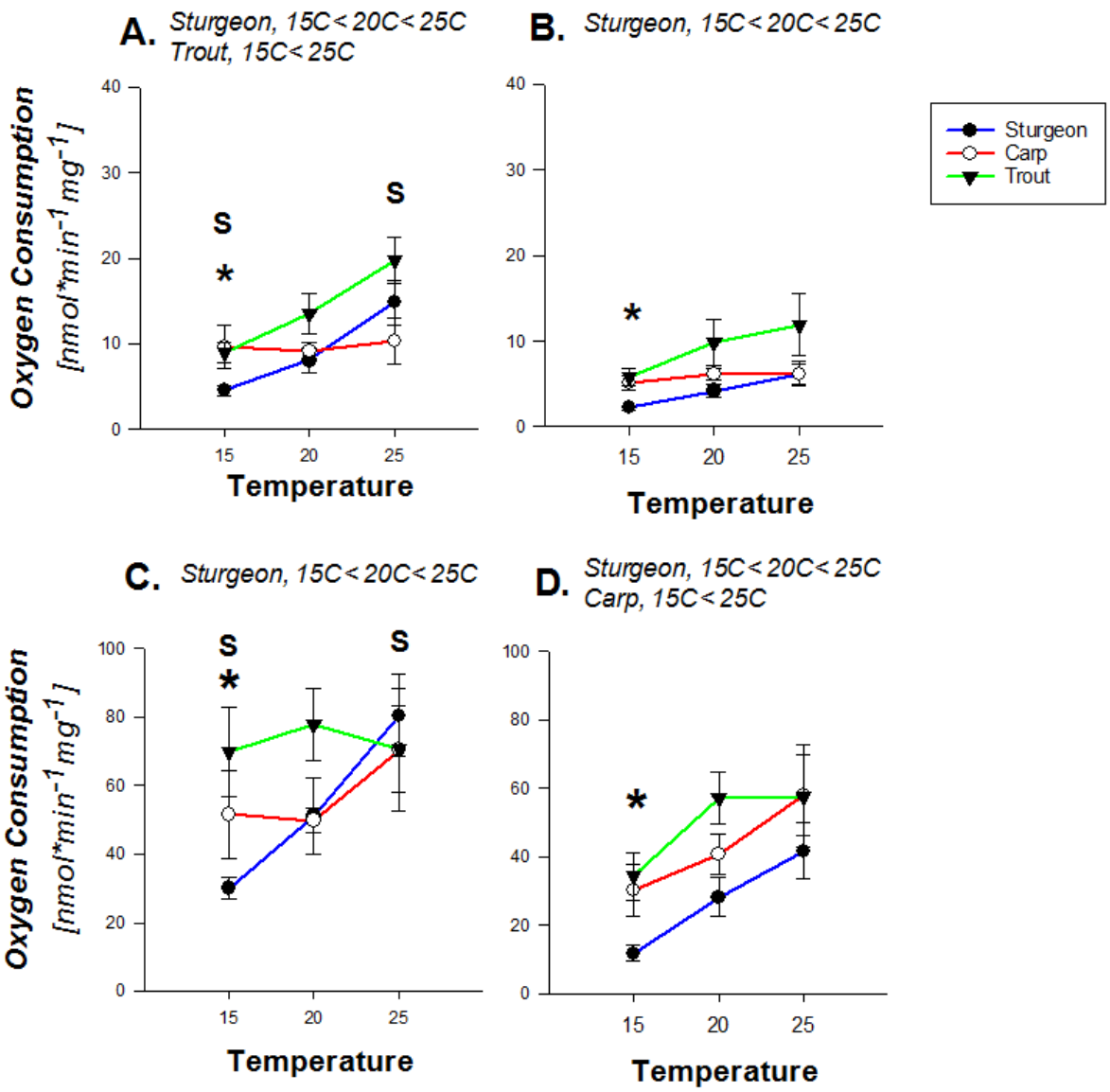

Figure 12. *Oxygen consumption rates with change in temperature of the assay.* **A.** State 2 with GMS as substrate combination. **B.** Rates for state 2 with PCM as substrate combination. **C.** State 3 (phosphorylating stage) respiration on GMS substrate combination. **D.** State 3 respiration on PCM substrates. Values are in nmol of O_2*$min^{-1}mg^{-1}$, and shown as mean± SE, α=0.05. *show significance across species combination for that specific rate within species. ^{S} Shows significance across substrates, which only applies for the fish at 15ºC and the Lake Sturgeon only at 25ºC, GMS rates being higher. Bold writing shows significance within species with temperature change. (N=7-8, GMS and PCM respectively on Lake Sturgeon; N= 5, Common Carp, N= 7, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

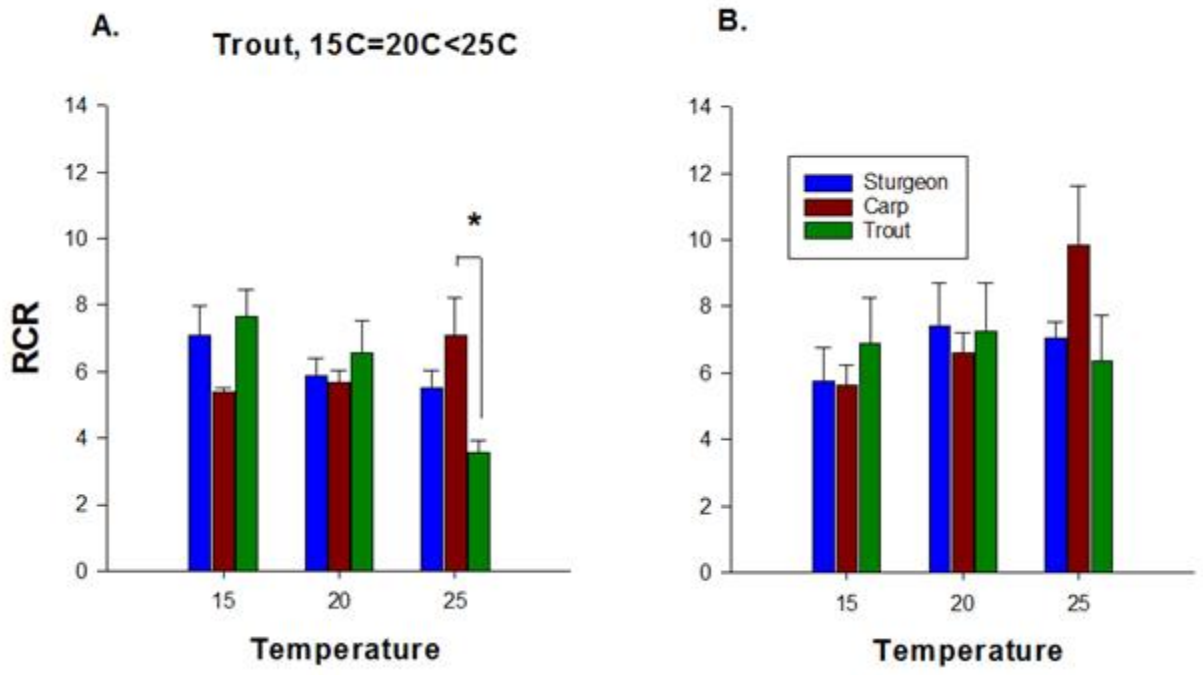

Figure 13. *Respiratory control ratios (RCR) for the rates of respiration on the heart mitochondria of the three fish species.* **A.** GMS substrate combination RCR, here we see only significant difference across species at 25^{o}C where the trout has markedly lower RCR than the Common Carp. The Lake Sturgeon is the middle ground. **B.** RCR for the PCM substrate combination, no significant differences found across species. Values are ratios have no units and are shown as mean± SE, α=0.05. *show significance across species for that specific temperature. However not marked on the graph there are substrate differences in the Lake Sturgeon at 25^{o}C, PCM RCR being higher. (N=7-8, GMS and PCM respectively on Lake Sturgeon; N= 5, Common Carp, N= 7, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

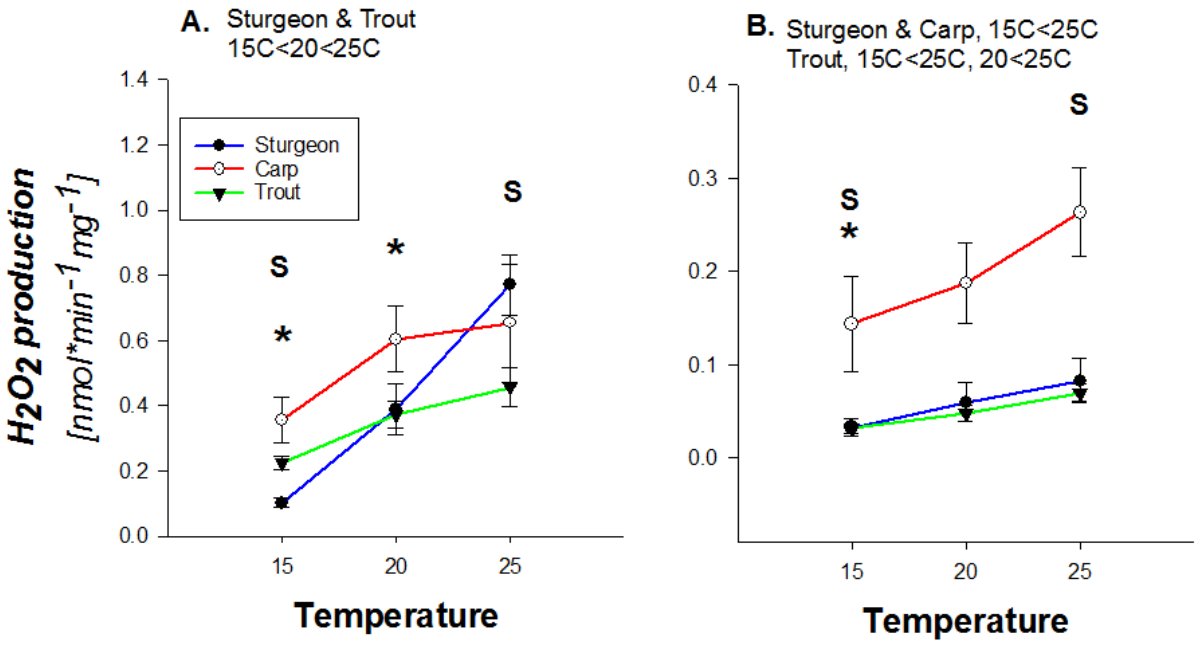

Figure 14. *H_2O_2 production rates for fish heart mitochondria*. **A.** production of H_2O_2 in state 2 with GMS as substrate combination. **B.** State 2 production of H_2O_2 with PCM as substrate. Values are in nmol of O_2*$min^{-1}mg^{-1}$, and shown as mean± SE, α=0.05. *show significant interspecific differences for that specific rate and temperature. ^{S} Shows significance across substrates, Rainbow Trout and Lake Sturgeon at 15ºC and the Lake Sturgeon only in 25ºC, GMS rates being greater. Bold writing shows significance within species with temperature change. (N=7-6, GMS and PCM respectively on the Lake Sturgeon; N=4, Common Carp; N=6 Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

3. 2. 3 Consumption of H_2O_2

Background consumption rates of H_2O_2 consumption in the presence of both GMS and PCM, substrates increased with temperature, (Figure 15); further, background consumption rates differed between substrate types. Common Carp do not showed for the three states of H_2O_2 consumption background, GMS, and PCM substrate combinations increase with temperature, (Figure 15). Furthermore, the rates of H_2O_2 consumption are higher for PCM substrate combination versus the background H_2O_2 consumption, and at 25^{o}C with P=0.05.

Background and GMS H_2O_2 consumption rates for Rainbow Trout increased with temperature; however, mitochondria H_2O_2 consumption rates did not increased with temperature for the PCM substrate combination. As well, for the Rainbow Trout rates of H_2O_2 consumption in the presence of substrate are much higher when they are compared to the H_2O_2 consumption rate.

Interspecific species difference: Noted is the higher consumption of the Rainbow Trout at all temperatures assayed, for the heart mitochondria H_2O_2 consumption rates in the presence of substrate; however, for the background state of respiration, the Common Carp was the species with the highest H_2O_2 consumption.

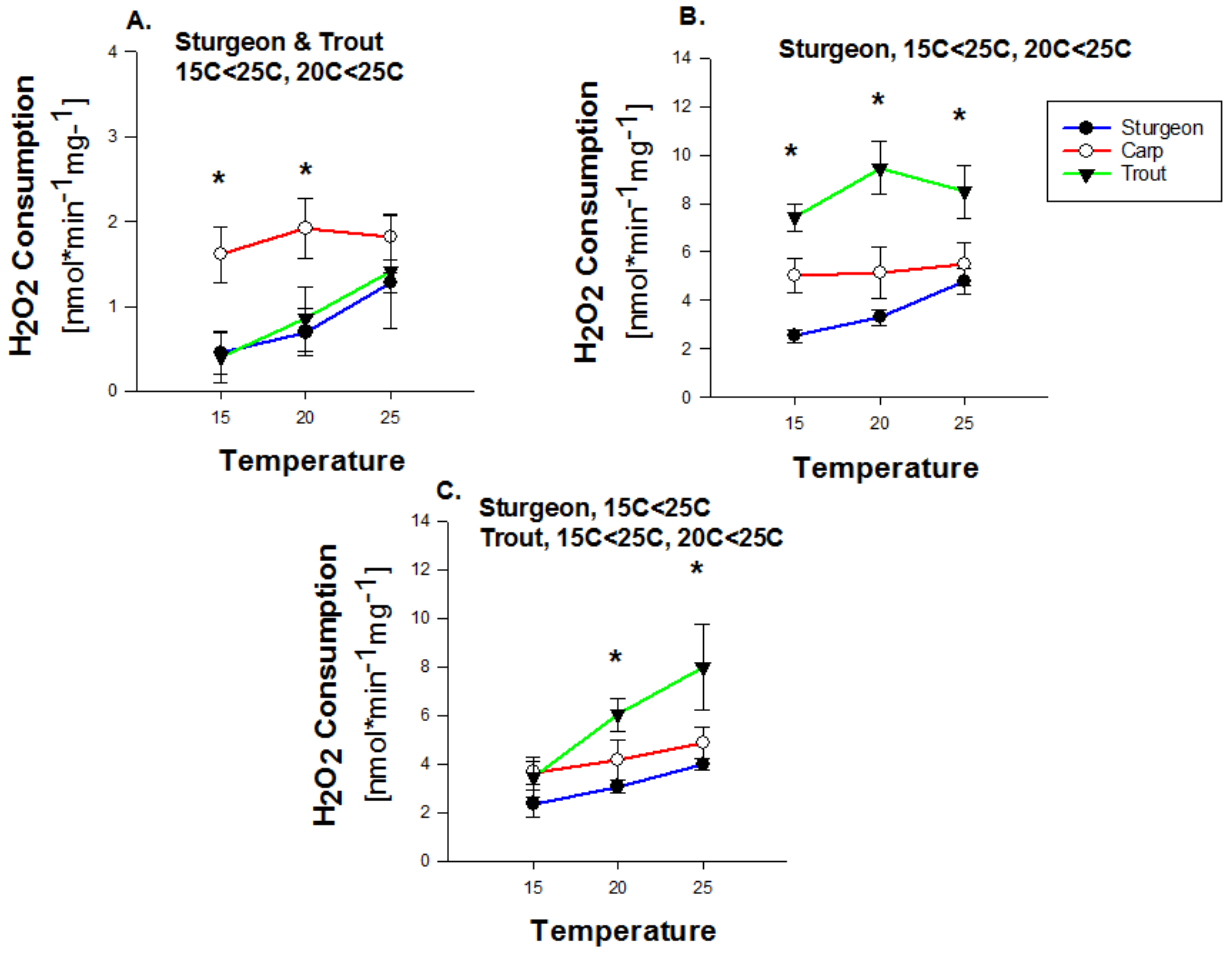

Figure 15. *H_2O_2 rates of consumption of fish heart mitochondria in state 2 for both substrates and background consumption.* **A.** Background H_2O_2 consumption. There is species differences with the Common Carp having higher consumption at 15ºC and 20ºC. **B.** H_2O_2 consumption with PCM substrates, rates on the Rainbow Trout is higher. **C.** Consumption of H_2O_2 with GMS as substrates. Here we find as well the Rainbow Trout presenting higher rates of consumption with temperature increase. Values are in nmol of H_2O_2*$min^{-1}mg^{-1}$, and shown as mean± SE, α=0.05. *show significant interspecific differences for that specific rate and temperature. However not marked on the graph, the background rates are significantly lower than rates with substrate present, for the 3 species and temperatures. Bold writing shows significance within species with temperature change. (N= 6-7, GMS and PCM respectively for the Lake Sturgeon; N=5, Common Carp; N= 5 Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

3. 2. 4 Enzyme Rates Change with Increased Temperature

All enzyme activity rates increased with temperature; however, the effect was species dependent. The activity rates of both enzymes increased exponentially for the Lake Sturgeon and Common Carp. However, in the Rainbow Trout the enzyme rates increase exponentially for citrate synthase only, (Figure 16-A). Activity rates for citrate synthase were found to be highest in Lake Sturgeon (Figure 16-B). However, there was no variation in cytochrome c oxidase activity across species at any temperature (Figure 16-A).

3. 2. 4. 1. Mitochondrial Rates with Temperature Increase, CCO Normalized Values

Respiration Rates: The rates of respiration for the mitochondria for the three fish are not different across temperature for GMS during state 2 (Figure 17-A) and state 3 (Figure 17-B) when respiration rates are normalized by CCO activity; this is also true for PCM rates on state 2 (Figure 18-A) and state 3 respiration (Figure 18-B). However, there are interspecies differences however with CCO normalized rates of respiration for the PCM substrate combination for state 3 where the Rainbow Trout is higher than the rate the Lake Sturgeon at 25^{o}C, (Figure 18-B).

H_2O_2 Production: Normalization of rates using CCO activity leads to the rates of H_2O_2 production also being not different across temperatures for the three species and both substrates, (Figure 18).

H_2O_2 consumption: Normalizing H_2O_2 consumption rates by expressing them relative to CCO activity also removes differences found among species with increasing temperature. . Noted is that there are interspecies differences at 15^{o}C on the background stage and PCM substrate combination where the Rainbow Trout is higher than the other species when normalizing rates relative to the CCO activity present in the isolated mitochondria (Figure 20).

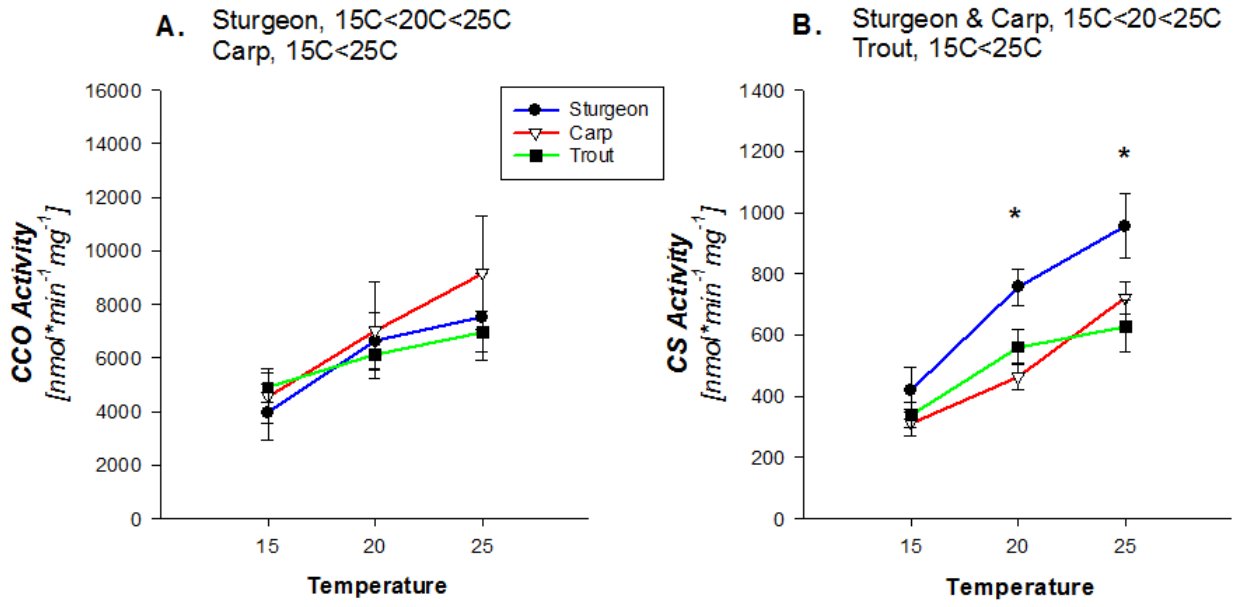

Figure 16. *Cytochrome c oxidase and citrate synthase activities for fish heart mitochondria with temperature change.* **A.** Cytochrome c oxidase activity rates with temperature increase, noted that there are no interspecific differences across species for this enzyme. **B.** Citrate synthase activity rates with temperature increase. Here we see a marked difference across species as the temperature increases, with the Lake Sturgeon having the higher rates in the activity of this enzyme, Rates are in nmol of enzyme *$min^{-1}mg^{-1}$, mean± SE, α=0.05. * show significance for that specific temperature within species. Bold writing shows significance within species with temperature change. (N=7, 15°C-25°C and N=5, 20°C for the Lake Sturgeon; N=4 Common Carp; N=13, 15°C-25°C and N=8, 20°C for the Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

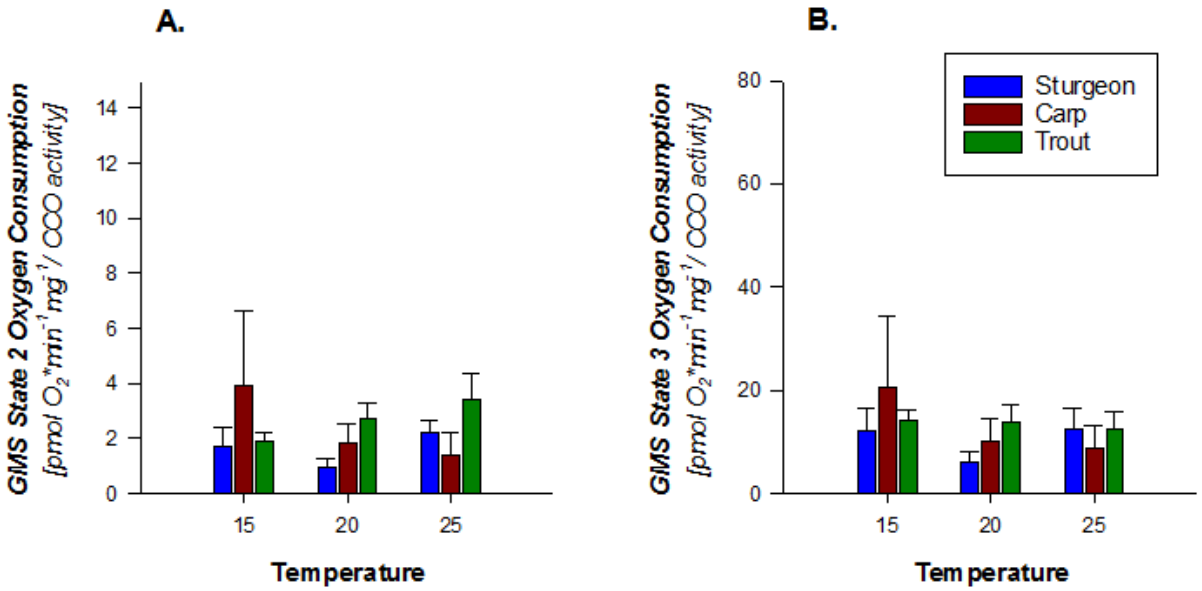

Figure 17. *Rates of respiration for GMS substrate combination expressed as CCO activity*. **A.** Rate of respiration in state 2. **B.** Rate of respiration in state 3. Values are in pmol O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and as mean± SE, α=0.05. (N=5, 15ºC-25ºC and N=3, 20ºC, Rainbow Sturgeon; N=4, Common Carp; N=7, 15ºC-25ºC and N=4, 20ºC, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

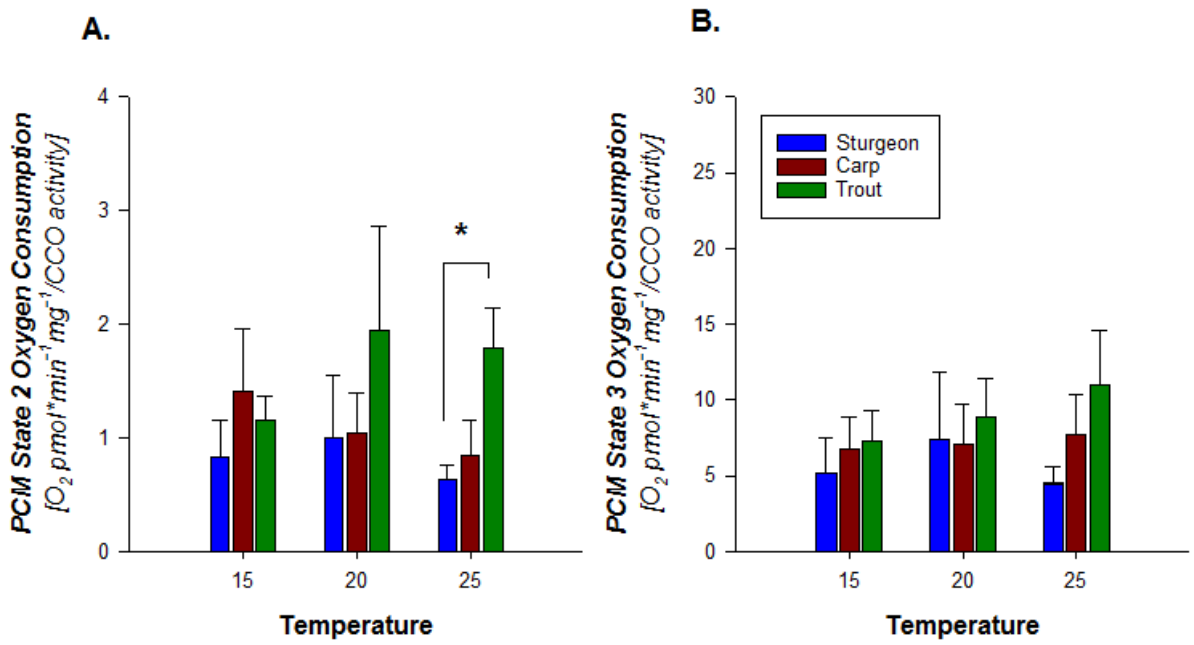

Figure 18. *Rates of respiration for PCM substrate combination expressed as CCO activity.* For this substrate combination there are differences for the 25°C on state 2, with the Rainbow Trout higher than the Lake Sturgeon. **A.** Rate of respiration in state 2. **B.** Rate of respiration in state 3. * Shows differences across species. Values are in pmol O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and as mean± SE, α=0.05, (N=6-4, Lake Sturgeon; N=4, Common Carp; N=6-3, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

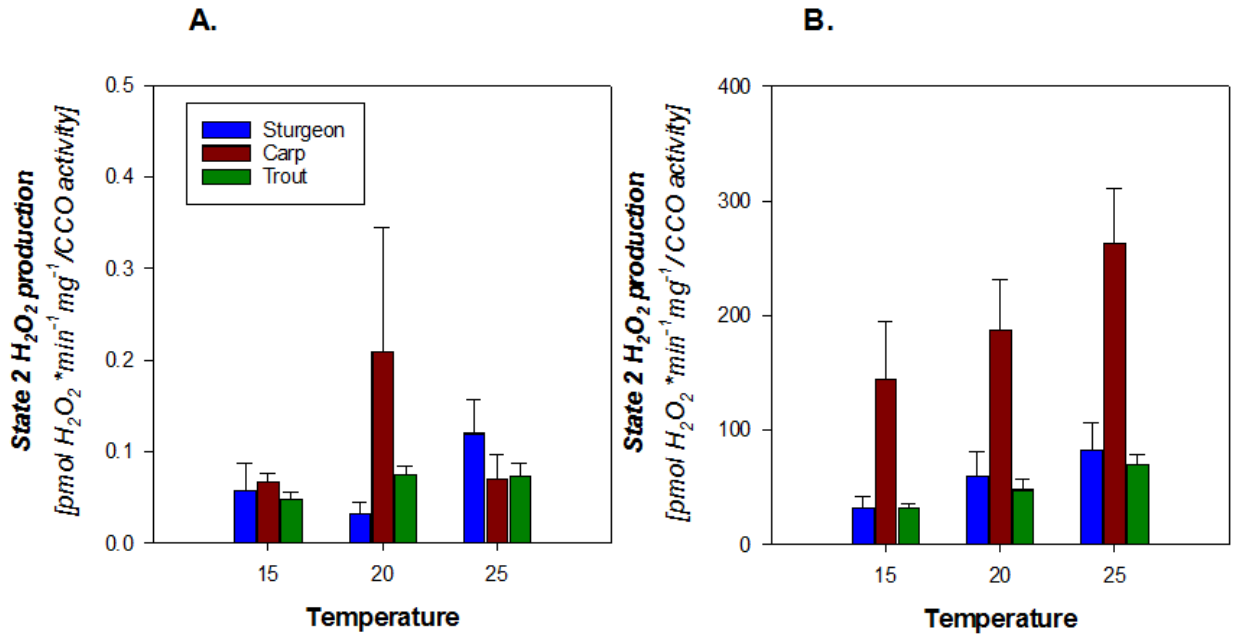

Figure 19. *H_2O_2 production rates expressed per CCO activity.* **A.** State 2 respiration, there are not differences across species or with temperature increase for this stage. Results for the 20°C should be taken with caution as the data for the Common Carp shows high variance. **B.** State 3 respiration. Values are in pmol H_2O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and are shown as mean± SE, α=0.05. (N=6-4 GMS and N=5-4 PCM, Rainbow Sturgeon; N=4, Common Carp; N=8-5 GMS and N=5-4 PCM, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

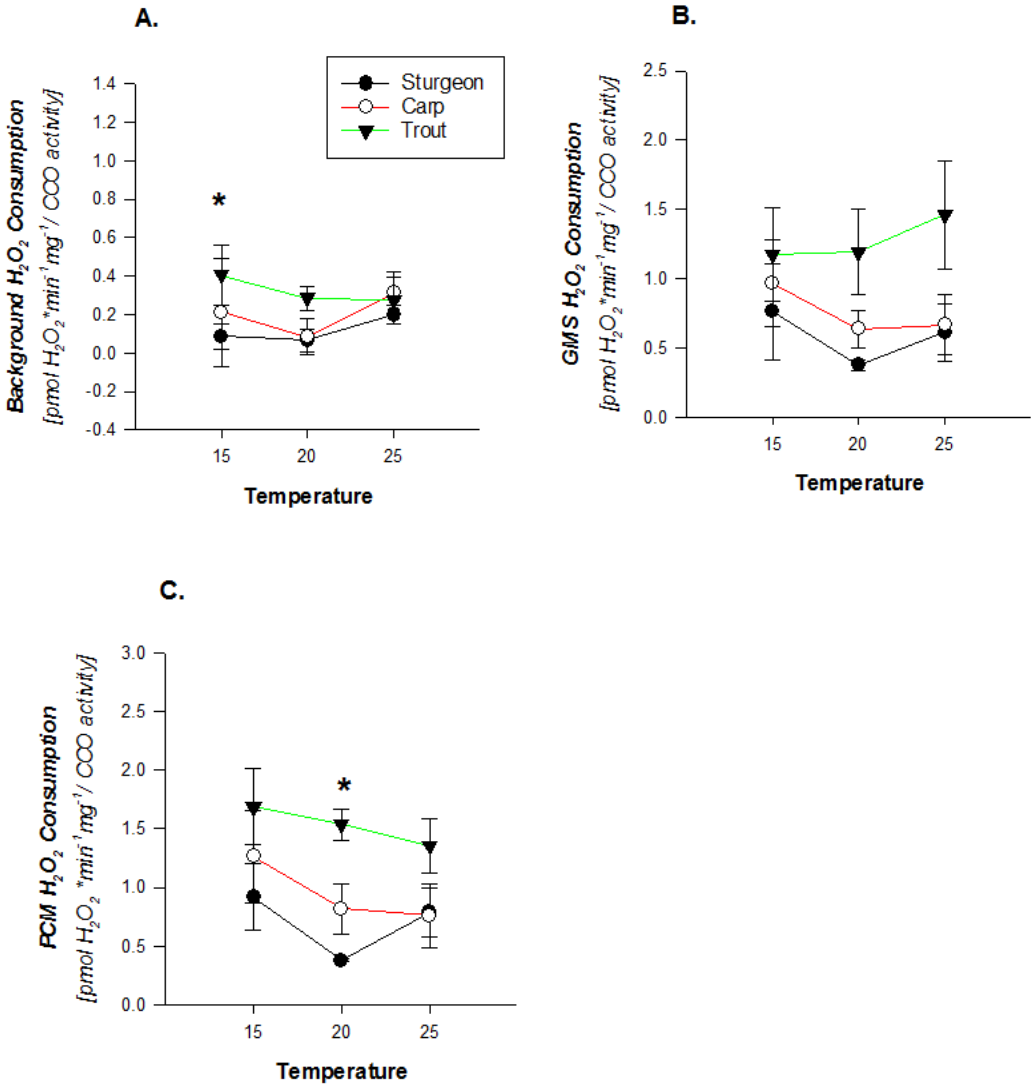

Figure 20. *H_2O_2 consumption rates expressed as CCO activity.* **A.** Background H_2O_2 consumption rates, no difference found across rates for temperature increase, however Common Carp rates are higher than the Rainbow Trout and the Lake Sturgeon, at 15ºC. **B.** H_2O_2 consumption rates for GMS substrate combination, no differences across species or temperature. **C.** H_2O_2 consumption rates for PCM substrate combination, no differences across rates due to temperature, but rates of the Rainbow Trout are higher at 20ºC. C. Values are in pmol H_2O_2 *min^{-1} mg^{-1}/CCO activity in nmol *min^{-1} mg^{-1}, and are mean± SE, α=0.05. (N= 6-4 Background, N=6-4 GMS and N=5-4 PCM, Rainbow Sturgeon; N=4, Common Carp; N= 5-4 Background, N=4-3 GMS and N=5-4 PCM, Rainbow Trout), Sturgeon, Lake Sturgeon; Carp, Common Carp; Trout, Rainbow Trout.

3. 2. 5 Oxidative Ratio

The oxidative ratio in the Lake Sturgeon GMS substrate combination showed as marked increase with temperature, suggesting a shift to a more pro-oxidant status with increasing temperature, as this ratio increases this represents either a relative increase in H_2O_2 production or decrease H_2O_2 consumption capacity. However, for PCM substrate combination we found no difference across temperature for oxidative ratio on the Lake Sturgeon. The Common Carp had no significant difference within temperature for oxidative ratios for both substrates. Furthermore, the Rainbow Trout GMS substrate combination has no increase across temperatures, for the PCM combination we find however that the oxidative ratio increased with temperature.

Oxidative ratio interspecific comparisons revealed that at 15^{o}C on the GMS substrate combination there are differences between the Lake Sturgeon and the Common Carp, with the Lake Sturgeon having the lowest ratios, Common Carp the highest, with the Rainbow Trout being intermediate (Figure 22 A). At 20^{o}C, using the GMS substrate combination the Common Carp continuous to be high in comparison with the Rainbow Trout; as for the 25^{o}C with GMS as substrate combination there is a significant difference between the Rainbow Trout and Lake Sturgeon, which shows a marked increase in oxidative ratio at this temperature for the Lake Sturgeon, (Figure 22-A). On 15^{o}C PCM substrate combination the Common Carp shows significant higher oxidative ratios in comparison with the Lake Sturgeon and the Rainbow Trout (with error propagation very large on the Rainbow Trout); there are no differences between the Rainbow Trout and the Lake Sturgeon. At 20^{o}C for PCM the Common Carp has the highest oxidative ratio in comparison to the Lake Sturgeon and the Rainbow Trout; as for 25^{o}C on PCM substrate combination there are no difference between Rainbow Trout and Lake Sturgeon, but the Common Carp is significantly higher than the rest of the fish; with markedly increase on oxidative ratio, (Figure 22-B).

Furthermore, substrate differences are found across all temperatures for the three species, with GMS producing the highest values.

3. 2. 6 FEL, Fractional Electron Leak

Results for the GMS substrate combination showed that FEL increases with temperature in both Lake Sturgeon and Rainbow Trout (Figure 21 A); conversely, in Common Carp heart mitochondria had no differences in FEL across temperatures using the GMS substrate combination, (Figure 21-A). With the PCM substrate combination we found that heart mitochondrial FEL increased with increasing assay temperature for the three fish (Figure 21 B).

Interspecific differences for GMS FEL ratios have the Common Carp higher than the Lake Sturgeon, with no differences between the Rainbow Trout and the Common Carp at 15^{o}C. FEL on GMS is also higher on the Common Carp versus the Lake Sturgeon and the Rainbow Trout. The Lake Sturgeon FEL ratios at 25^{o}C are much higher than those of the Rainbow Trout (Figure 21-B**)**. Interspecific difference for PCM at 15^{o}C showed the Common Carp FEL significantly higher than the Lake Sturgeon and the Rainbow Trout. We find as well differences at 20^{o}C reflecting the same pattern as 15^{o}C; as well, there are not significant differences in FEL across species at 25^{o}C for PCM substrate combinations. There were found differences between the two substrate combinations for FEL, between all temperatures sampled; although it should be noted that the GMS substrate combination is the one that gives the highest FEL ratios for all three species

4. Discussion

Mitochondrial respiratory capacity and H_2O_2 metabolism shows a differential response for Rainbow Trout, Common Carp and Lake Sturgeon at acclimation temperature. As the temperature increases in the respirometry assay, cardiac mitochondrial respiration rates increases with temperature on the state 2, substrate only, and in the phosphorylating state, rates of respiration did

not increase in the Rainbow Trout using GMS substrate combination. H_2O_2 production indeed increased with temperature for all three species of fish. The current study shows as well that, at least for the three *Actinopterygii* species used in this study, mitochondria are well capable to consume H_2O_2; however, the species show a differential response to temperature as well for the rates of H_2O_2 consumption. Interestingly, when the respiration rates are expressed per cytochrome c oxidase activity the mitochondrial rates do not change as temperature increases, this is not found when the mitochondrial rates are expressed as citrate synthase activity. Finally, the fractional electron leak and the oxidative ratio on the cardiac mitochondria of the three fish tend to increase with temperature warming, however differentially across species.

4. 1. Temperature Range Chosen in This Project

The temperature of 25^{o}C may be considered at or close to the upper thermal limit for the Rainbow Trout (Black, 1953). For the Lake Sturgeon, adult individuals are said to decrease their activity when temperatures rise to 19 and over (LeBreton, et al. 2004); furthermore, the sampled Lake Sturgeon used in this study are from Manitoba populations from Nelson and Winnipeg rivers originally in where the average high temperature in the Summer will reach 21.5^{o}C to 20.0^{o}C, respectively. Also, populations on the Lake Huron of Lake Sturgeon that were archive tagged at this lake are said to range from 0 to up to 23.5^{o}C (Briggs et al., 2016). Noted is that these temperature ranges are related to the thermal maxima for the species in this study; however, they will be not considered the highest environmental temperature for the species studied.

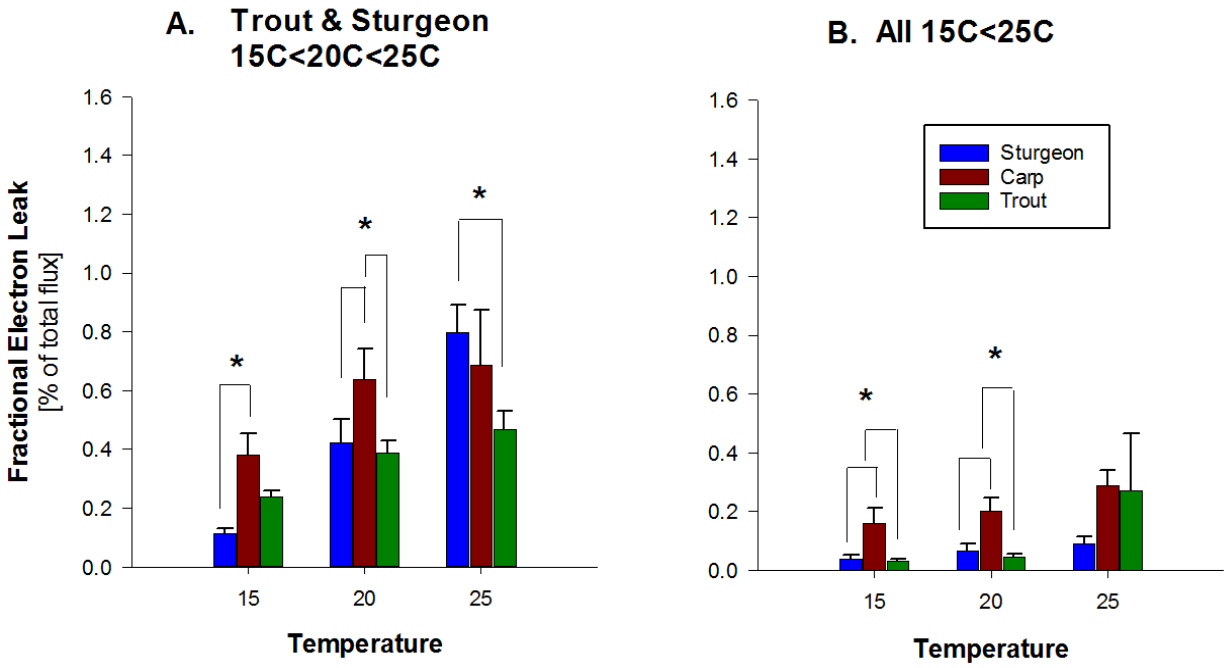

Figure 21. *Graph showing the FEL (on state 2, non-phosphorylating) for both substrates on the three species sampled.* **A.** GMS ratios of FEL, here we see a marked difference across species. As well the FEL ratios increase significantly on the three species with the only exception of Common Carp in GMS substrate combination. **B**. PCM FEL ratios. However not marked on the graph FEL increases with temperature for the three fish for PCM. Also there are substrate differences across all species and temperatures, with GMS FELs being higher than PCM FELs. Values represent ratios so have no units and are shown as mean± SE, α=0.05.*****show significance for that specific temperature within species. (N= 6-7, GMS and PCM respectively for the Lake Sturgeon; N=5 Common Carp; N= 8-6 GMS and PCM respectively for the Rainbow Trout).

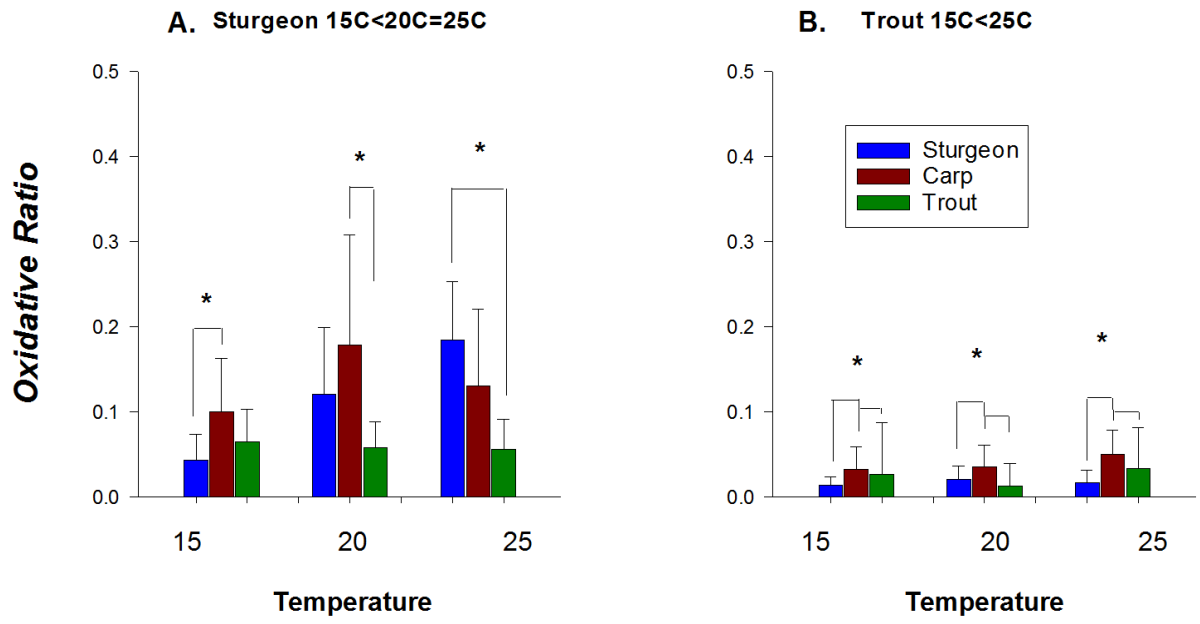

Figure 22. *Oxidative ratio graph comparing fish heart mitochondria across assay temperature change.* **A.** GMS ratios, we find the Common Carp showing higher values than the Lake Sturgeon at 15ºC and 20ºC, with 25ºC having markedly increases of FEL on the Lake Sturgeon. **B.** PCM oxidative ratios show species differences across the three temperature, with the Common Carp being the highest. However these results may be tainted with the high variability of the error propagation used for the statistics and should be taken with caution. In general the Common Carp has no differences with increase temperature and the Lake Sturgeon having marked increase with GMS as substrate on the ratios, no increase of oxidative ratio with temperature for the Rainbow Trout. Values represent ratios so have no units, and are shown as mean ± SE, α=0.05. * shows differences across species. (N= 6-7, GMS and PCM respectively for the Lake Sturgeon; N=5 Common Carp; N= 5-6 GMS and PCM respectively for the Rainbow Trout).

4. 2. Study Deficiencies

One of the drawbacks in this study is the fact that each species has a different range of possible temperature adaptation strategies, aerobic scope and rate-temperature curves in their biological processes, and this study recorded the mitochondrial rates between the temperatures of 15ºC and 25ºC, for the three species included. However, deciphering their mitochondrial heart metabolism with increasing temperature, starting with their acclimation temperature 15ºC, can clarify how fish of different taxonomy and adaptations are compared in their metabolic responses, as the three species are indeed adapted to the same initial temperature and the three are northern temperate

species. Even with the limitations of this study, the mitochondrial rates assayed will shed light on how mitochondrial on the heart of fish change with temperature, this can be of interest and utility to better understand comparative mitochondrial metabolism across vertebrate taxa. This current project is an *in vitro* study, the main model is the mitochondria of the ventricle in the fish. However, as this can only look closely at the responses to temperature of the metabolic rates in a separate mitochondrial system, not at the cellular level, this study tells nothing on the possible interactions that would happen between the cell and the mitochondria while the temperature changes, which maybe a future avenue for further studies.

4. 3. Temperature Similarities and Differences at Acclimation Temperature

4. 3. 1 Respiration Rates

In all states of oxygen consumption the respiration rates of the Rainbow Trout tend to double the rates of the Lake Sturgeon, the Common Carp rates are intermediate. This trend of higher mitochondria respiration for the Salmonid mitochondria may reflect and parallel the characteristic of the Rainbow Trout as an active fish; the ATP requirement for high activity may reflect the higher oxygen consumption in metabolically active organ such as the heart. The lower rates of oxygen consumption are found in the Lake Sturgeon mitochondrion, which is a benthic fish, furthermore, it is known to be a long lived species, and as such there is some understanding that long lived species have a tendency of lower metabolic rates, specifically standard metabolic rate, SMR or basal metabolic rate, BMR (LeBreton, et al. 2004).

The RCRs calculated between the phosphorylating and non-phosphorylating rates of oxygen consumption at acclimation temperature, only found differences within Lake Sturgeon, between PCM and GMS, the GMS ratios being higher. This suggest that although the rates of oxygen consumption for the Rainbow Trout are higher in absolute values than the Lake Sturgeon and the

Common Carp rates, the capacity of the mitochondria to couple the oxidative phosphorylation may be similar for the species at acclimation temperature.

4. 3. 2 Production of H_2O_2

At acclimation temperature we also find differences across species on the rates of H_2O_2 production; however those differences do not follow a parallel pattern to the oxygen consumption. It is noted that for the H_2O_2 production rates, the Common Carp rates versus the other two species are much higher, this applies for both substrate combinations. This shows that at least for the acclimation temperature the Common Carp heart mitochondria produces double the ROS than the Rainbow Trout and the Lake Sturgeon, and is the fish which shows the highest capacity for H_2O_2 production at 15^{o}C.

4. 3. 3 Consumption of H_2O_2

In recent years researchers' views on mitochondria have evolved from not only being the main ATP producers of the cell to be at the forefront of metabolic studies that extended their function to be producers and consumers of ROS. The concept of mitochondria as only ROS producers has been challenged by studies that look at the mitochondrial ROS consumption pathways (Banh & Treberg, 2013; Brown & Borutaite, 2012). However, as mitochondria are capable of producing ROS, more recent studies have been focusing into the pathways in the mitochondria that are responsible for detoxification of ROS molecules, the ROS consumption pathways.

In this study the consumption of H_2O_2, shows markedly differences depending on the state of respiration and respiration substrate taken into consideration. For the background state of H_2O_2 consumption where no respiratory substrate is present, the Common Carp is the species with the highest consumption. It is worth noting that the rates of H_2O_2 background consumption are most likely due to catalase activity in the tissue (Munro & Treberg, 2017). Although catalase appears to

have a small influence in heart mitochondria, it is however an important antioxidant pathway in some organs like the liver (Salvi et al., 2007). However, Rainbow Trout was the highest consumer of H_2O_2 in the presence of PCM substrate combination. The GMS substrate combination was intermediate in that we found no species differences and the consumption was at the middle of the range, between 2-4 nmol of H_2O_2*$min^{-1}mg^{-1}$. This study then corroborates that as GMS is the substrate combination that tends to produce more H_2O_2 at 15ºC. The Lake Sturgeon finds not differences between substrate combinations for the ROS consumption, and it shows tendency for low ROS production as well.

4. 4. Comparing Acute Temperature Increase Influence on Mitochondrial Rates

4. 4. 1 Oxygen Consumption

The results for respiration rates on the heart mitochondria show oxygen consumption increase differentially across the three species in this study. It was noted that at acclimation temperature the starting rates are not similar, and each species basic starting point is different. This differential capacity of the mitochondria across species may be a general reflection of the activity of the fish to the environment in which each species lives. However, the Lake Sturgeon is the species whose rates follow closely a rise with increasing temperature. The Common Carp rates of oxygen consumption are temperature independent with exception of state 3 PCM. Furthermore, Common Carp are known to have a high threshold for high temperatures as well (Black, 1953). Rainbow Trout mitochondrial rates of oxygen consumption rise with temperature for the non-phosphorylating states of oxygen consumption, yet the trout respiration rates do not increase on the phosphorylating state 3 condition. This may show a decrease of phosphorylating capacity on the heart of the Rainbow Trout with increasing temperature, which may be of importance for Rainbow Trout performance under conditions of high environmental temperatures. Furthermore,

although we do not find differences in RCR at acclimation temperature, at 25^{o}C the ratios of the trout are markedly lower for GMS. The Common Carp RCRs tend to increase with temperature, showing that the Common Carp fairs well as the temperature increases, the mitochondria from the Common Carp hearts are still capable of coupling effectively while temperature increases. The Lake Sturgeon RCR shows that coupling of isolated mitochondria from the heart of the Lake Sturgeon seem to be independent of temperature, as they do not show significant change with increasing temperature, reflecting a capacity of this species to couple mitochondria even under the influence of acute warming.

4. 4. 2 Production of H_2O_2

All the species rates of H_2O_2 production show temperature dependent increases with acute warming; it is noted however that the Common Carp is the species that has the highest rates of H_2O_2 production in general. However, there are subtle differences across substrate conditions on for the state 2 H_2O_2 production we see differences for the Common Carp and the Lake Sturgeon as temperature increases, specifically the GMS substrate combination shows the Lake Sturgeon with a great increase on ROS production, but much less production for PCM substrate combination. The Rainbow Trout show the lowest rates of production, yet the rates double with a 10^{o}C increase. The differential responses to ROS production may suggest that the production of H_2O_2 comes from different sites or enzymes and that those may be influenced differently by change in temperature (Brand, 2010; Quinlan, et al. 2012; Perevoshchikova, et al. 2013).

4. 4. 3 Consumption of H_2O_2

To better understand the ROS overall metabolism of the mitochondria in these three species of fish, the current study also assayed the consumption rates of H_2O_2. Although most comparative

studies focus on the ROS production mitochondrial metabolism does include the pathways which have evolved to deal with the damage inflicted by the possible over production of reactive species. The consumption rates assayed in this study show that, as with the respirometry and H_2O_2 production rates, we find differential responses to the acute temperature increase across species and substrate present. The Common Carp, interestingly their H_2O_2 consumption is much higher at the background stage, this stage which may be attributed to the enzyme catalase and has been assayed in the experiment. The Common Carp shows independence of temperature on the H_2O_2 consumption rates; this follows the pattern of respiratory rates in this species. The Lake Sturgeon is the species which has the lowest consumption of H_2O_2 rates; however, the consumption of H_2O_2 tends to increase as temperature increases. The Rainbow Trout is the species that shows a more differential response on the H_2O_2 consumption with very low background rates, however with substrate present, specifically with the GMS substrate combination the H_2O_2 consumption rates increased with acute warming. The PCM substrate combination is independent of temperature for H_2O_2 consumption on the Rainbow Trout.

4. 5. Cytochrome c Oxidase Expressed Rates

Another step for the analysis of the mitochondrial rates in this study was done by expressing the metabolic rates by the enzyme activity assayed; this is dividing the mitochondrial rates by cytochrome c oxidase and citrate synthase activities. This was done to investigate if the rates expressed by enzyme activity might explain to some extent the differences across species and the influence of temperature on the rates of respiration and ROS metabolism for these three species.

As stated before the mitochondrial respiration rates expressed as milligram of protein do show a differential response to the temperature increase, and they were also differences across species; however, when we express the oxygen consumption rates by as a function of cytochrome c oxidase

activity, the differences across species are greatly decreased and the influence of temperature on the rates disappears, with the exception of PCM state 2 of respiration.

4. 6. Citrate Synthase Expressed Rates

For the citrate synthase expressed rates, we do not see the same effect as cytochrome c oxidase expressed rates. The pattern of citrate synthase expressed mitochondrial rates follow a similar pattern with the milligram of protein expressed mitochondrial rates for the three species of fish. This similar response between milligram of protein and citrate synthase expressed mitochondrial rates may be perhaps due to problems related rates of electron leak with differing respiratory capacities across the three fish species mitochondria, as well as the function of citrate synthase in the Krebs cycle might influence this.

4. 7. Fractional Electron Leak, FEL

FEL values, which represent the fraction of electrons that may leak during redox reactions in the ETC, while the mitochondria is in active phosphorylation state. These electron leaks may form ROS under both substrate conditions. This fraction or ratio may be used, together with other mitochondrial denominators, to gain some insight if acute temperature stress may induce the mitochondria of the heart to be an agent or influence in possible oxidative stress. Both Rainbow Trout and markedly the Lake Sturgeon had an increase of FEL ratios, while temperature increased; the Common Carp also shows an increase on FEL ratios with increasing temperature, however less marked than the Lake Sturgeon and Rainbow Trout. We see the same pattern reflected on the PCM rates; however, the differences across species across species are not evident at 25^{o}C. In general there is a trend as temperature increases that the mitochondria of these fish might have a higher capacity of producing H_2O_2, as conditions for this, possible electrons leaking to form ROS, are shown to be an influence in the mitochondria in the current study.

4. 8. Oxidative Ratio

Another specific ratio defined as the oxidative ratio in was the ratio of H_2O_2 production over the consumption of H_2O_2, expressed by milligram of protein. If the oxidative ratio increases the capacity of the heart mitochondria to produce ROS increases disproportionally, relatively to the antioxidant capacity of the mitochondria, which is estimated by H_2O_2 consumption.

The mitochondrial oxidative ratios for the Lake Sturgeon respiring on GMS substrate combination increased with temperature, while the Common Carp and the Rainbow Trout oxidative ratios appear to be independent from temperature. In general the Rainbow Trout had lower oxidative ratios than the other species. When the oxidative ratio was calculated with the PCM rates of H_2O_2 metabolism, we found that the Common Carp is higher than the Rainbow Trout and the Lake Sturgeon. Noted is the fact that the PCM oxidative ratios are independent from the influence of acute temperature stress. Furthermore, the oxidative ratios were calculated by independent rates across species, and the statistical value of variation was calculated by error propagation, noted is that the variation in the data is fairly high, so these results should be taken with caution.

4. 9. Conclusions

This study had the objective of characterizing the mitochondrial metabolism of three species of *Actinopterygii* fish at the oxygen consumption level and the ROS production and consumption levels, with temperature. Empirical knowledge will imply that mitochondrial rates on the heart of the three fish species sampled in this study will follow an exponential like increase; the rates will increase with temperature until hitting the range of the species critical maximum temperature. However, the results for the acute temperature increase assays paints a diverse picture for the three species mitochondrial metabolism with temperature increase. Respiration rates and H_2O_2 metabolism changes differentially with temperature; rate patterns differences seem to depend on

the substrate used on the assays, the specific species sampled, and the state of respiration that is been sampled on the assay.

Respirometry rates show that the Rainbow Trout mitochondria indeed have a higher rate of oxygen consumption than the Common Carp and the Lake Sturgeon at 15^{o}C; however, when the temperature increases the phosphorylation rate specifically in the Rainbow Trout do not follow an increase and the RCR values as well tend to decrease. Noted is that the Rainbow Trout mitochondria might be getting near to the to the upper thermal limit for this species (Bidgood & Berst, 1969; Black, 1953), this might be throwing their heart mitochondria to uncouple. Furthermore, cardiac peak function occurs well below the upper thermal limit in the intact heart (Farrell, et al. 1996), it also has been suggested that heart failure under heat stress might be incumbent with mitochondria failure (Iftikar & Hickey, 2013), this might be happening at least for the Rainbow Trout. Common Carp mitochondria seems to fair well with temperature as well for the Lake Sturgeon.

Previously (Banh, et al. 2016), fish muscle mitochondria, were found to have a high capacity of ROS production with increase in temperature. The production of H_2O_2 in the heart of the fishes in my study, found that the production of H_2O_2 indeed increases with temperature, under both substrate conditions and differentially across species. As stated before the highest producer of ROS was the Common Carp, with the Rainbow Trout being the less "oxidative" *per se*, the Lake Sturgeon has a differential response to the different substrates. Looking at the different substrate responses in H_2O_2 efflux on the fish, for all three species the GMS substrate combination is the highest in absolute value for the three species. The reason for this difference might accrue to the different possible sites of ROS production that might be involved in both substrate combinations during mitochondria respiration, specifically the GMS substrate combination may have more

possible redox carriers on its pathway that are known to be possible sites of ROS production. The GMS substrate combinations will have possible native sites of ROS production being C1, CII, and CIII plus Krebs cycle possible sites like α-ketoglutarate dehydrogenase, which with the non-phosphorylating state 2 might be able to support high membrane potential required and low oxygen consumption which will entice these redox carriers to produce ROS. According to (Perevoshchikova, et al. 2013) possible native sites of ROS production during fatty acid oxidation will contrive CI, CII, CIII and ETFQOR (electron transferring flavoprotein: ubiquinone oxido reductase), plus Krebs cycle enzymes as well like α-ketoglutarate dehydrogenase, plus β-oxidation sites like ETF, electron transferring flavoprotein, and acyl-CoA dehydrogenase. This substrate mixture shows a complete different pathway for passing of electrons through electron carriers and which they will be differently influenced by membrane potential, and phosphorylating rate.

Cytochrome c oxidase is the last enzyme in the ETC which delivers electrons to oxygen the final acceptor in the process of oxidative phosphorylation, while translocating protons from the matrix to the intra membrane space, increasing the proton gradient across the mitochondrial membrane (Wikstrom, 1977). The relationship of cytochrome c oxidase with respiration in mitochondria is constant with a study (Guderley, et al. 2005), in which there is found a relationship between respiration rates and the quantity of cytochrome a_1, subunit of cytochrome c oxidase, in *B. marinus*, *P. vitticeps* and *R. norvegicus*. Furthermore, older studies have suggested that CCO is a one possible member of a mitochondrial control system over respiration, which is formed by CCO plus the dicarboxylate carrier and the adenine dinucleotide carrier (Blier & Lemieux, 2001). According to Blier and Lemieux 2001, CCO might be an important factor in ectotherm thermal sensibility. CCO may be a potential site for allosteric regulation and that CCO activity in human cells is close to the electron transfer which is present in state 3 respiration in those cells (Villani, et al. 1998).

According to (Blier & Lemieux, 2001) they may be a functional or structural link between respirometry rates and cytochrome c oxidase activity, this is at least for red muscle mitochondria.

4. 10. Summary

This project investigated the heart mitochondrial rates of three temperate *Actinopterygii* fish (Rainbow Trout, Common Carp and Lake Sturgeon), under the influence of acute temperature increase. Acute *ex vivo* warming differently affected the three species sampled in this project. Rates of respiration rise with temperature for the Lake Sturgeon; the Rainbow Trout respiration rises only for the PCM, β-oxidation representative substrate in the study; however, for the GMS combination the Rainbow Trout falls short in comparison with the other two species. Interestingly, the Common Carp heart mitochondria are independent of temperature for the respiration rates. The RCRs confirm this pattern for the species, with the trout RCR decreasing with temperature, for GMS substrate combination. Production of ROS increases with temperature, as well differently for each fish; this reflects a possibility for acute warming to be an important stressor for these species. It is important to note, that this study has looked at the H_2O_2 consumption of the heart mitochondria as well for these fish species. Antioxidant capacity also increases with temperature, and every species of fish shows a different pattern under the effect of temperature increase. The species differential response showed that the Rainbow Trout is the highest consumer of ROS. This is reflected in the oxidative ratios calculated in the study for GMS substrate combination, and show that the Lake Sturgeon and the Common Carp indeed produce more ROS than they are able to consume. Interesting GMS substrate combination shows the highest capacity for ROS production, for the species sampled in this study. Furthermore, FEL ratios, which represent the percent flux of electrons that could escape to produce ROS, also increase with temperature warming, for the

Common Carp, the Rainbow Trout and the Lake Sturgeon. Therefore, this study has shown that acute warming can indeed affect the performance for fish heart mitochondria.

Furthermore, we can speculate that when we express the mitochondrial rates by cytochrome c activity, we might be equalizing those rates by a possible control, which is present in mitochondrial metabolism, and it might be a good denominator for interspecies comparative mitochondrial studies. As when the mitochondrial rates for respiration and ROS production and to some extent the ROS consumption are expressed by cytochrome c oxidase activity, the influence of temperature stops being significant. This is not relevant when the mitochondrial rates are expressed per citrate synthase activity**.**

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6. Appendix I. Abbreviations

Alpha KG, α-ketoglutarate.

ANT, adenosine nucleotide transporter.

AUR, amplex ultra red.

Acyl-CoA DH, acyl co-A dehydrogenase.

CI, complex I, NADH-oxidoreductase.

CII, complex II, succinate dehydrogenase.

CIII, complex III, cytochrome bc_1 complex.

CCO, and CIV, complex IV, cytochrome c oxidase.

CoQ, ubiquinone.

CPI, Chapell Perry I

Decr., decreasing.

DNA, deoxyribonucleic acid.

e-, electron.

ETF_{red}, electron transferring flavoprotein reduced.

ETF_{oxi}, electron transferring flavoprotein oxidized.

ETFQOR, electron transferring flavoprotein ubiquinone oxidoreductase.

ETF, electron transferring flavoprotein.

ETFQOR, electron transferring flavoprotein ubiquinone oxidoreductase.

FAD, Flavin adenine dinucleotide oxidized.

$FADH_2$, flavin adenine dinucleotide reduced.

GSSG, glutathione oxidized.

GSH, and GSH2, glutathione reduced.

GR, glutathione reductase.

GPx, glutathione peroxidase.

GDH, glutamate dehydrogenase.

GMS, glutamate malate succinate.

H_2O_2, hydrogen peroxide.

H^+, protons.

HPx; horse radish peroxidase.

$HO^{\bullet}$, hydroxyl radical.

IMS, Inner membrane space.

IMM, inner mitochondrial membrane.

Incr., increasing.

NAD^+, nicotinamide dinucleotide oxidized.

NADH, nicotinamide dinucleotide reduced.

NADH/NAD+, nicotinamide dinucleotide ratio.

$O_2^{\cdot -}$, superoxide.

PC, palmitoyl carnitine.

PCM, palmitoyl carnitine malate.

PRx, peroxiredoxin.

Q, ubiquinone.

QH_2, ubiquinol.

QH_2/Q, ubiquinone pool reduction state.

$ROO^{\bullet}$, peroxyl radical.

SOD, superoxide dismutase.

State 2 CCO, rate on state 2 expressed by cytochrome c oxidase activity.

State 3 CCO, rate on state 3 expressed by cytochrome c oxidase activity.

State 2 CS, rate on state 2 expressed by citrate synthase activity.

State 3 CS, rate on state 3 expressed by citrate synthase activity.

SE, standard error of the mean.

TrxR2, mitochondrial specific thioredoxin reductase

TR, thioredoxin.

UCPs, uncoupling proteins.