THE EFFECTS OF HYPOXIA ON KETONE BODY METABOLISM AND CARDIAC FUNCTION IN THE SHORTNOSE STURGEON (*ACIPENSER BREVIROSTRUM*)

BY

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ABSTRACT

The goal of this study was to understand the effects of chronic hypoxia on ketone body metabolism and cardiac contractile function in the shortnose sturgeon (*Acipenser brevirostrum*). Ketone bodies can be used in metabolic processes during a hypoxic exposure. They have the ability to protect tissues during hypoxia, while being energetically efficient. In particular, ketone bodies produce more ATP per mole of oxygen consumed compared to glucose, while decreasing the production of reactive oxygen species. To understand ketone body metabolism during hypoxia, sturgeon were exposed for 10 days in one of three conditions: control (9 mg/L dissolved oxygen, fed), starved (9 mg/L dissolved oxygen, fed) and hypoxia (2.5 mg/L dissolved oxygen, starved). Cardiac contractile function was measured following this exposure and blood and tissue samples were collected for glycogen and ketone body concentration analysis. We found that liver glycogen was significantly decreased following the hypoxic exposure. However, ß-hydroxybutyrate dehydrogenase (BHBD) activity in the liver, total plasma ketone body concentration and succinyl-Coa:3 ketoacid CoA transferase (SCOT) activity in the heart and skeletal muscle did not change following the hypoxic exposure. Furthermore, the maximum cardiac contractile force (Fmax) of the ventricles was not affected by substrate supplementation with glucose or ketones. Cardiac contractile function was maintained in the chronic hypoxic group but decreased in the starved group. Together, these findings do not support our hypothesis that chronic hypoxia would elicit a ketogenic response. It is likely that the energetic requirements were still being met by limited glucose throughout the 10-day hypoxic exposure, providing evidence for their innate hypoxia tolerance and ability to sustain cardiac function in harsh environments.

LIST OF FIGURES

LIST OF ABBREVIATIONS

DO……Dissolved oxygen

TCA…… Tricarboxylic acid

ATP…… Adenosine triphosphate

ADP…… Adenosine diphosphate

NADH…… Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

$FADH_2$…… Flavin adenine dinucleotide (FAD) + hydrogen (H)

NAD^+…… Nicotinamide adenine dinucleotide

FAD^+…… Flavin adenine dinucleotide

$NADP^+$…… Nicotinamide adenine dinucleotide phosphate

HEPES…… 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

CO_2…… Carbon dioxide

O_2 ……Oxygen

G6PDH ……Glucose-6-phosphate dehydrogenase

$MgSO_4$……Magnesium sulfate

ETC……Electron transport chain

COX……Cytochrome c oxidase

HIF……Hypoxia inducible factor

ROS……Reactive oxygen species

HMG-CoA synthase…… 3-hydroxymethylglutaryl-CoA synthase

MCT……Monocarboxylate transporters

SCOT……Succinyl-CoA: 3 ketoacid-CoA transferase

BHBD……ß-hydroxybutyrate dehydrogenase

BOH……ß-hydroxybutyrate

MCHC……Mean corpuscular hemoglobin concentration

Hct……Hematocrit

Hb……Hemoglobin

Fmax……Maximum cardiac contractile force

ANOVA……Analysis of variance

INTRODUCTION

The survival of an aquatic organism partly depends on its ability to detect and respond to changes in dissolved oxygen (DO) in aquatic environments (Giaccia *et al.*, 2004). Low DO environments, also known as hypoxic environments, are becoming more prevalent around the globe due to a range of factors. Global change - a combination of climate change, increased population, intense industrialization and an increase in agribusiness, is a leading cause of the increased prevalence of these hypoxic environments due to increased eutrophication in estuarine and coastal waters (Rabalais *et al.,* 2009). Eutrophication is the influx of nutrients into water systems and has many harmful effects such as reduced water quality, loss of habitat and increased hypoxia. This increase in hypoxia is due to limitations imposed on primary producers as a direct result of an increase in biological oxygen demand by those primary producers, increased cloud cover and decreased water quality (Rabalais *et al.*, 2009). In addition, there is an increase in the production of reactive nitrogen and phosphorous - mainly through agriculture and fossil fuel combustion that is changing the natural fluctuations of these gases in the environment such that amount of each compound is far greater than normal, which affects primary producers and decreases DO concentrations (Rabalais *et al.*, 2009). Aquatic animals living in these environments have evolved a variety of physiological responses to hypoxia that enable their survival.

Oxygen and Energy Production

Environmental hypoxia can lead to a decrease in oxygen concentration in tissues, resulting in a wide range of physiological changes, including but not limited to changes in energy production (Wiebe and Machulla, 1999). The ability for an organism to maintain oxygen homeostasis involves physiological systems that have evolved to ensure maximum and efficient oxygen extraction and transport throughout the body (Michiels, 2004).

Under normoxic (normal oxygen levels) and fed conditions, fatty acids and glucose are the main source of energy for ATP production. Other macromolecules, such as proteins and other carbohydrates can be used to produce energy, although glucose is usually more abundant and accessible (Bonora *et al.*, 2012). Glucose is broken down through glycolysis,

the tricarboxylic acid (TCA) cycle and oxidative phosphorylation to ultimately produce ATP, which is then used to facilitate and sustain cellular metabolism (Weber *et al.*, 2016).

ATP production under normoxic conditions begins as pyruvate from glycolysis is transported to the mitochondria, where it is converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA enters the TCA cycle as it combines with oxaloacetate to form citrate. Acetyl-CoA is an important molecule that links multiple pathways such that ATP can be produced from the catabolism of carbohydrates, lipids and proteins. Acetyl-CoA is thus the reason why many different molecules can be used in ATP production (Bonora *et al.*, 2012). Continuing through the TCA cycle, NADH and $FADH_2$ are produced and then oxidized to form NAD^+ and FAD^+ by complex I and II in the electron transport chain (ETC) (Wheaton and Chandel, 2011). The donated electrons are transported to complex III, then cytochrome oxidase (COX; complex IV) where they are transferred to molecular oxygen, which acts as the terminal electron acceptor. As the electrons move through the ETC, proton translocation occurs and produces an electrochemical gradient. The protons either run through F_1F_o-ATP synthase to produce ATP or they leak back into the mitochondrial matrix. The ATP that is produced is transported to the cytosol where it's used by enzymes such as Na^+-K^+-ATPase to regenerate ADP. The ADP is then transported back to the mitochondria and the cycle continues. The increase in mitochondrial ADP causes an increase in oxygen consumption and for ATP synthase to increase the rate of the formation of ATP (Wheaton and Chandel, 2011). Mitochondrial oxygen consumption is a combination of coupled and uncoupled respiration. Uncoupled respiration is the rate of oxygen consumption by complex IV since it is not coupled to ATP production during proton leak. Coupled respiration is the rate of oxygen consumption by complex IV when it is coupled to ATP production by complex V. Other factors that affect respiratory rate include the availability of NADH and $FADH_2$ from the TCA cycle, electron flux through the ETC, the amount of ADP available and the severity of proton leak (Wheaton and Chandel, 2011).

Acute Hypoxia

During hypoxia, there are a series of metabolic alterations that occur that depend on both the severity and duration of the hypoxic event. Acute hypoxia is when an organism is

exposed to low-oxygen environments for short periods of time (usually ~4-24 h) (Ackerly *et al.,* 2018). As oxygen levels start to decline, many fish will respond by initially increasing ventilation and heart rate (Claësson *et al.*, 2016), increasing the affinity of hemoglobin for oxygen or the concentration of hemoglobin (Powers, 1980), and increasing gill perfusion (Gattuso *et al*., 2018) to maintain oxygen extraction. If these mechanisms do not work to increase cellular oxygen delivery, ATP production must switch from aerobic to anaerobic metabolism, where ATP is produced by substrate-level phosphorylation (Mandic *et al.,* 2013). As a result, lactate, a by-product of anaerobic metabolism, accumulates as tissues are not able to use and process the molecule without oxygen (Weber *et al.*, 2016). Anaerobic glycolysis produces 2 ATP/glucose molecule while aerobic glycolysis produces approximately 32 ATP/glucose molecule, showing how energy-deprivation can occur if anaerobic glycolysis continues for too long (Melkonian and Schury, 2021). Glucose remains the substrate of choice during acute hypoxia, as long as glycogen stores in liver and muscle are available or food is abundant (Pescador *et al.*, 2010). Although, when fish are exposed to hypoxia, they normally stop eating in an effort to decrease the energetic costs associated with specific dynamic action (SDA), as SDA leads to an increase in metabolic rate (Fitzgibbon *et al.,* 2007). Glycogen stores are more extensive in small-bodied, anoxia-tolerant fish species, as they mostly rely on anaerobic glycolysis when oxygen stores are low or completely depleted (Vornanen and Haverinen, 2011). There is also evidence that hypoxia may trigger the accumulation of glycogen by hypoxia inducible factors (HIF) (Pescador *et al.*, 2010). Hypoxia-inducible factor-1 (HIF-1) in particular is important in the hypoxic response. The stabilization of HIF-1 allows for ETC activity to decrease as it decreases the “carbon-flux” into the TCA cycle, limiting the amount of NADH and $FADH_2$ available to enter the ETC to act as reducing equivalents. Cytochrome c oxidase (COX; Complex IV) is inhibited by HIF-1, ATP production decreases, and cells lower their oxygen consumption without noticeable cell injury (Wheaton and Chandel, 2011). This is only advantageous if ATP utilization also decreases. This decrease in metabolism can help to limit the production of reactive oxygen species in the mitochondria that can damage the cell.

Reactive oxygen species (ROS) are formed from the partial reduction of oxygen, resulting in either radical (hydroxyl radical) or non-radical (superoxide anion and hydrogen peroxide) oxygen species (Ray *et al.*, 2012). This occurs endogenously, similar to mitochondrial oxidative phosphorylation. The generation of ROS begins as molecular oxygen is reduced, forming an anion superoxide. This precursor molecule undergoes a dismutation reaction, which forms hydrogen peroxide (Beckhauser *et al.*, 2016). The reactive molecules that are formed from the conversion of hydrogen peroxide have the ability to remove electrons from other oxygen species, which triggers a cascade-like reaction and can produce a large-quantity of ROS, leading to oxidative stress (Beckhauser *et al.*, 2016). As ROS levels increase, it can damage structures including the mitochondria, lipids, DNA and proteins (Ray *et al.*, 2012).

Chronic Hypoxia

While the effects of acute hypoxia have been widely studied and are well known, the effects of chronic hypoxia on energy production remain in question. Currently, many studies investigating the effects of chronic hypoxia have focused on teleost fishes and have been limited to manipulating food intake, oxygen carrying capacity and reproduction (Petersen and Gamperl, 2010). The ability to survive under extreme and long-term hypoxic conditions involves downregulating metabolic demand such that production of ATP matches cellular use (Wheaton and Chandel, 2011). If the hypoxic exposure is long, glycogen stores may not be a sufficient source of glucose for ATP production, particularly in hypoxia-sensitive species. If so, other substrates may be used to produce ATP. Fatty acids in particular are an unlikely substrate to be used to produce ATP during a prolonged hypoxia exposure, as they are known to produce ROS (Murphy, 2009), they require a lot of oxygen to be oxidized and they produce less ATP per mole of oxygen consumed when compared to both glucose and ketones (Morash *et al*., 2013).

Ketone Body Metabolism

Ketone bodies can be metabolized by the heart, brain and skeletal muscle when carbohydrate stores (glucose/glycogen) have been depleted (i.e. starvation) (Cotter *et al.*,

2013). Ketogenesis is considered a “spill-over pathway” as excess acetyl-CoA produced by fatty acid oxidation (ß-oxidation) is used to make ketones in the liver. The rate of ketogenesis is dependent and proportional on the rate of ß-oxidation when carbohydrate stores are low (Cotter *et al.,* 2013). A decrease in carbohydrate stores is normally seen during periods of starvation and this is when ketone bodies may become the main substrate used for energy production (Cotter *et al.*, 2013).

Ketone bodies are synthesized mainly in the mitochondrial matrix within the liver (Fig. 1) (Cotter *et al.*, 2013). Following ß-oxidation, 3-hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to HMG-CoA. HMG-CoA lyase cleaves a molecule of acetyl-CoA from HMG-CoA, producing the ketone body acetoacetate. Acetoacetate can then undergo two different reactions: D-ß-hydroxybutyrate dehydrogenase catalyzes the production of D-ß-hydroxybutyrate or acetoacetate decarboxylase catalyzes the production of acetone (Puchalska and Crawford, 2017). These ketone bodies are then transported through the blood stream to be used in extra-hepatic tissues for ATP production.

There are three main fates of ketones after they have been synthesized: they either undergo terminal oxidation in the mitochondria of extrahepatic tissue, are used in the lipogenesis pathway or excreted in urine/exhaled as acetone.

Ketone bodies are thought to be transported across the mammalian mitochondrial membrane by monocarboxylate transporters (MCT 1, 2 and 7). The succinyl-CoA: 3 ketoacid CoA transferase (SCOT) enzyme is the rate-limiting enzyme of ketolysis. SCOT is found in all cells that have mitochondria and it is responsible for the formation of acetoacetyl-CoA by the transfer of CoA from succinyl-CoA to acetoacetate. If SCOT were to be damaged, ketone bodies would not be used for ATP production (Fig.1) (Puchalska and Crawford, 2017).

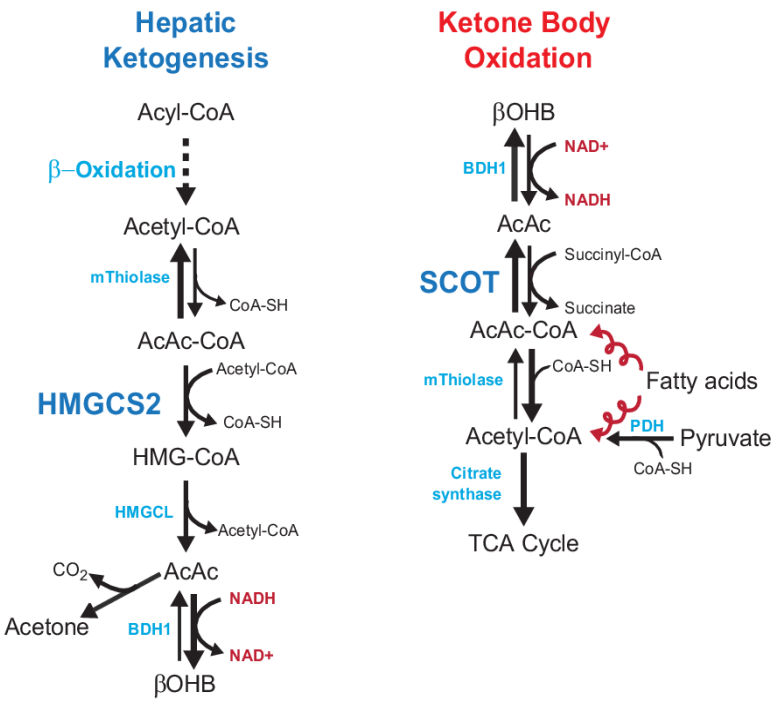

Figure 1. Hepatic ketone body production and extrahepatic ketone body oxidation pathways. Diagram adapted from Cotter *et al.* (2013).

Ketone bodies can be used in metabolic processes during a hypoxic exposure. They have the ability to protect tissues during hypoxia, while being energetically efficient. In particular, ketone bodies produce more ATP per mole of oxygen consumed compared to glucose (Veech *et al.*, 2001) while decreasing the production of ROS (Cotter *et al.*, 2013). This is important when considering how the heart cannot simply reduce its metabolic rate when exposed to hypoxia – it must keep beating in order to sustain life. In order for the heart to keep beating, it must use a more efficient metabolic fuel, which in this case, is the ketone body. Ketone bodies have been shown to be used in the heart, where the myocardium is the highest ketone body oxidizer per unit mass in mammals (Puchalska and Crawford, 2017). Ketone bodies also increase the production and stabilization of hypoxia inducible factor 1α (HIF1α), which allows for a greater hypoxic response (Puchowicz *et al.*, 2008).

Ketone Body Use

Much of what we know about ketone body metabolism comes from mammals, but the use of ketone bodies varies across taxa; elasmobranchs have a high reliance on ketones for energy (Speers-Roesch *et al.*, 2006), mammals use ketones as an alternate form of energy only when carbohydrate and fatty acid stores are limited (Puchalska and Crawford, 2017) and ketone body metabolism in teleosts is limited compared to both mammals and elasmobranchs (Soengas *et al.,* 1996). In a study conducted by Zammit & Newsholme (1979), the activities of enzymes in fat and ketone body metabolism were measured in teleost fish and elasmobranchs to determine whether fats or ketones were the main substrate for energy production. Elasmobranchs showed an increase in enzyme activity for SCOT (referred to as 3-oxo acid CoA-transferase) and 3-D-hydroxybutyrate dehydrogenase (two main enzymes involved in formation and breakdown of ketones), while teleosts showed an increase in enzyme activity for carnitine palmitoyltransferase (a main enzyme in fatty acid metabolism). Also, both acetoacetate and 3-hydroxybutyrate were found in elasmobranch blood whereas only acetoacetate was found in teleost blood. Teleost blood showed a higher concentration of non-esterified fatty acids. Together, this implies that ketone bodies are the important energy source in elasmobranchs while non-esterified fatty acids are the main energy source in teleosts (Zammit and Newsholme, 1979). The sturgeon is an evolutionary intermediate species between elasmobranchs and teleosts and are known to use ketone bodies to a certain extent (Singer *et al.*, 2011). Given the differences in the use of ketone bodies across taxa, and their potential for protecting against hypoxia, we want to examine if hypoxia will elicit a ketogenic response that may benefit cardiac metabolism. We chose sturgeon as a model as they are hypoxia tolerant and have been shown to use ketone bodies to support ATP production under certain conditions (Kieffer *et al.*, 2011).

Research Goals and Hypothesis

The goal of this study is to understand the effects of chronic hypoxia on ketone body metabolism and cardiac function in shortnose sturgeon (*Acipenser brevirostrum*) by comparing three different treatment groups: control, chronic hypoxia and starved. Sturgeon will be exposed to either normoxic conditions, DO saturation of 2.75 mg/L, or will be

deprived of food as a positive control for ketogenesis (Furné *et al*., 2012) for 10 days (Secor and Gunderson, 1998). In all 3 treatment groups, ketone body production in the liver, circulating ketones in plasma, and ketone oxidation in cardiac and skeletal muscle will be measured to determine if there is a ketogenic response. In addition, cardiac muscle force production will be measured using either ketone bodies or glucose as a supplemental fuel to determine if ketone bodies can support force production in hearts from each treatment group.

I hypothesize that chronic hypoxia will elicit a ketogenic response in shortnose sturgeon. I predict that (1) ketone production in the liver will increase after being exposed to the hypoxic environment for 10 days in comparison to the starved group and (2) that there will be an increase ketolytic enzyme activity in the heart after the hypoxic exposure, which will help maintain cardiac contractility. The results obtained from this experiment will help lead to an understanding as to how vertebrates respond to environmental stress through changes in substrate use and mitochondrial function.

METHODS

Experimental Animals

We obtained 30 commercially sourced shortnose sturgeon ((*Acipenser bervirostrum);* ~182.88 ± 44.76 g) (Acadian Sturgeon) from the UNBSJ Aqualab (Dr. James Keifer). We transported the sturgeon to the Crabtree Aqualab at Mount Allison University using a 750 L tank filled with water and oxygenated with an air stone. We kept the sturgeon in a 300 L tank with recirculating water at a temperature of 20 °C for one month to acclimate to the lab. During this month-long acclimation period and prior to experimentation, they were fed standard sturgeon feed (Ewos 1.5 mm) at least once per day until satiation. Once acclimated, each sturgeon was anesthetized in a 20 L solution of 0.1 g/L tricaine mesylate (MS-222) and 0.2 g/L sodium bicarbonate, tagged with visible implant elastomer (VIE) (Northwest Marine Technology, Shaw Island, WA, USA-NMT) under their snout according to Kozlowski *et al.* (2017) and their weight (g) was recorded.

This experimental protocol (#102827) was approved by the Animal Care and Use Committee at Mount Allison University, in accordance with the Canadian Council of Animal Care.

Experimental Design

Experimental Groups

We determined the effects of hypoxia on ketone body metabolism and cardiac function by comparing three different experimental groups; control, hypoxia and starved. The control group (n=7) was exposed to normoxic water (9 mg/L dissolved oxygen (DO)), at 19.5 °C) and fed as indicated above.

The chronic hypoxia (n=7) group was starved and exposed to 19.5 °C (± 2 °C) and a DO saturation of 2.75 mg/L (~30% DO). Hypoxic conditions were achieved by bubbling nitrogen gas into the tank until the set DO was reached. We regulated the dissolved oxygen using a Witrox dissolved oxygen meter (Loligo Systems) and a data acquisition module that controlled gas solenoids connected to compressed air (Air, Compressed, Alphagaz, UN 1002)

or compressed nitrogen (Air Liquide, Nitrogen, Compressed, 1066). Using this software, we maintained oxygen saturation at 2.75 ± 0.18 mg/L DO.

The starved group (n=7) acted as a positive control for the ketogenic response (Furné e*t al.*, 2012). Sturgeon were exposed to normoxic water (as indicated above) and deprived of food for 10 days.

Blood Sampling

Prior to any experimentation, each sturgeon was anesthetized as above and we took a 0.3ml blood sample in a heparinized saline (143.0 mM NaCl, 0.900 mM $MgSO_4$, 3.35 mM KCl, 2.30 mM NaH_2PO_4, 5.50 mM $NaHCO_3$, 10.0 mM HEPES, 2.93 mM $CaCl_2$, pH adjusted to 7.8 using 0.1 M NaOH, 100 U/mL Heparin) syringe from the caudal vasculature. Each fish was placed in a 300 L recovery tank, where pressure was applied to the site of the blood extraction until bleeding stopped. We measured blood glucose using a handheld glucose meter (One Touch Verio Reflect Blood Glucose Monitoring System), hemoglobin (HemoCue Hb 201), and hematocrit from whole blood. Hematocrit was obtained by centrifuging a capillary tube (SafeCrit Heparinized Plastic Microhematocrit Tubes, Iris Sample Processing, 40 mm, sodium heparin, HP8H, Lot. No. 9088) filled with blood at 5000 rpm for 3 min, followed by measuring the length of the red blood cells (mm) and the total length of the red blood cells and the plasma (mm). The remaining blood was placed in a 2 mL cryotube and centrifuged at 5000 rpm for 3 min. We separated plasma from the red blood cells and both samples were kept at - 80°C for further analysis.

Isometrically Contracting Ventricular Strips

We prepared ventricular strips as described in MacCormack and Driedzic (2002). We euthanized the sturgeon with an overdose of anesthetic (in 10 L of tank water: 3 g tricaine mesylate (MS-222) and 6 g sodium bicarbonate) and severed the spinal cord. We sampled approximately 1 mL of blood and completed blood analysis as above. We extracted the heart and placed in a bathing medium consisting of cold, oxygenated saline and included (in mM): 143 NaCl, 0.9 $MgSO_4$, 3.35 KCl, 2.3 NaH_2PO_4, 5.5 $NaHCO_3$, 10.0 HEPES, 2.93 $CaCl_2$, with

pH set to 7.8 with 0.1 M NaOH. We added glucose (5.0 mM) as a metabolic fuel and we added adrenaline (3.0 μM, 10 μL) every 30 minutes to the bathing solution to maintain cardiac contractility (Shiels and Farrell, 1997). We separated the atrium and bulbus arteriosus from the ventricle. We bisected the ventricle longitudinally and cut and separated two ventricle strips (<1 mm thick). We sampled liver, skeletal muscle, scute, brain and heart tissues and placed in -80 °C freezer.

We placed the two ventricle strips in their respective plexiglass clamps and connected them to an isometric force transducer (Harvard Apparatus, South Natick, Ma, USA) using surgical silk. We placed the strips between two electrodes and lowered them into their respective chambers, containing approximately 20 mL of glucose bathing solution held at 16°C. We oxygenated the chambers using a compressed gas mixture of 0.500% CO_2 balanced with O_2. We ran each strip in parallel to one another to account for any discrepancies during the experiment. We stimulated the strips using the GRASS SD9 stimulator, stretched the strips
for maximum force production and left them to stabilize for 20 min at a pacing frequency of 0.5 Hz. The resting heart rate for sturgeon cardiac muscle is 0.7 Hz under normoxia (Gräns *et al*, 2010). Since the ventricle strips were contracting isometrically at their maximum force, we lowered the pacing frequency (0.5 Hz) to account for any extra energy demands.

To assess cardiac contractile function, we measured the maximum force of contraction (Fmax), post-rest potentiation and the force-frequency relationship. We measured Fmax at 0.5 Hz directly following the 20 min rest period. During post-rest potentiation, we stopped stimulations for 3 min and analyzed the contractions after turning the stimulations back on. We then measured the force-frequency relationship by increasing the frequency by 0.2 Hz every 30 s until contractions became asymmetrical.

We drained the chambers and them filled with a second bathing solution, where we added lithium acetoacetate (Sigma Aldrich, Lot No. SLBX9935) as the metabolic fuel (5.0 mM) instead of glucose. Disturbance to the ventricle strips was minimal during the switch from a glucose bathing solution to a lithium acetoacetate bathing solution. The strips were left to stabilize for 20 min at a pacing frequency of 0.5 Hz. Following the exposure

of this second substrate, iodoacetic acid (0.5 M) was added to the lithium acetoacetate bathing solution to inhibit glyceraldehyde 3-phosphate dehydrogenase, thus inhibiting glycolysis. This was to ensure that the ventricle strips were only using ketone bodies to sustain cardiac contractions. The strips were left to stabilize for 20 min at a pacing frequency of 0.5 Hz, and the experiments for Fmax, post-rest potentiation and force-frequency relationships were repeated.

Tissue Glycogen Extraction

We aliquoted approximately 50mg of frozen heart, liver and skeletal muscle tissue under liquid nitrogen using a mortar and pestle. We added 300 µL KOH (30%) to each sample and then placed them in the 100 °C dry bath for 10 min. We added 100 µL of 2% and 800 µL of 95% ethanol to the samples and centrifuged at 4800 rpm for 10 min. We discarded the supernatant, washed the pellet with 1 mL 66% ethanol, vortexed and centrifuged at 4800 rpm for 10 min. We discarded the supernatant and added 750 µL HCl (1M) and 750 µl reverse osmosis water before leaving the samples in the 100 °C dry bath for 2 hours. We placed the samples in the -80 °C freezer until further use.

Glucose Assay

We measured the glucose concentration using a standard curve of D-glucose. We removed the samples from the -80 °C freezer and thawed on ice. We made a 7-point standard curve with D-glucose (2 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM, 0.00 mM) and prepared the assay buffer (250 mM imidazole, 5.0 mM $MgSO_4$, 10.0 mM ATP, 0.8 mM $NADP^+$, pH=7.8). We diluted the liver samples by 20X and assayed all standards and tissue samples (liver, heart, skeletal muscle) in duplicate on a 96-well plate. Following the addition of G6PHD (10 U/mL), we waited 10 min, then read the plate at 340 nm using a SpectraMax 190 Plate Reader. We then added hexokinase (10U/mL), waited 45 min and then read the plate at 340 nm using a SpectraMax 190 Plate Reader.

Enzyme Assays

We homogenized approximately 50 mg of frozen tissue (liver, heart, skeletal muscle) with 1000 µL of enzyme extraction buffer (0.1% Triton X-100, 1 mM EDTA, 2 0mM HEPES) using a PowerGen tissue homogenizer. We centrifuged the samples at 3000 rpm for 30 s and used the supernatant to measure the enzyme activities of BHBD in the liver and SCOT in the heart and muscle. All assays were performed in duplicate with one well acting as a blank, on a 96-well plate. Assays were read using a SpectraMax 190 Plate Reader and the SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

a. *ß-Hydroxybutyrate Dehydrogenase Assay*

The assay buffer solution contained 50 mM imidazole and 0.1 mM NADH and the reaction was started with 2 mM of lithium acetoacetate, except in the blanks. The plate was read at 340 nm for 3 min.

b. *Succinyl-CoA:3 Ketoacid CoA Transferase (SCOT) Assay*

The assay buffer contained 50 mM TRIS-HCl (pH 8.5), 5 mM $MgCl_2$ and 0.2 mM succinyl-CoA and the reaction was started with 50 mM of lithium acetoacetate, except in the blanks. The plate was read at 310 nm for 3 min.

Plasma Ketone Body Assay

We used the Sigma-Aldrich Ketone Body Assay Kit (MAK134) and assayed both acetoacetate and ß-hydroxybutyrate according to the kit instructions. We prepared reactions mixes for both ketone bodies, one for the sample and standards (assay buffer, ketone body reagent, HBHD enzyme) and one for the sample blanks (assay buffer, ketone body reagent). The standard solution contained 8mM of the ketone body standard (either acetoacetate or BOH) and the blank solution contained ddH_2O. We incubated the plate in the dark for 5 min and then read plate at 340nm in the SpectraMax 190 Plate Reader.

Data Collection and Analyses

I corrected hemoglobin values to y=0.815x - 2.198 (Clark *et al.*, 2008) and used these corrected values in the MCHC calculations, as the HemoCue hemoglobin monitor is known to overestimate hemoglobin concentrations.

We connected the muscle force transducers to a PowerLab 8/SP computerized unit and data collected data on a PC computer using LabChart 8 programming. To calculate the force of a contraction, the maximum force of the contraction is subtracted from the resting potential. We analyzed three contractions for each data point and we performed statistical analyses using data from these three contractions at each testing interval.

Statistical Analyses

We used RStudio (version 1.4.1103) for all statistical analyses ($\alpha = 0.05$). To compare mass, blood glucose, hemoglobin, hematocrit, MCHC, BHBD activity in the liver, SCOT activity in the heart and skeletal muscle and glycogen concentration in the liver, heart and skeletal muscle between experimental groups, we performed 1-way ANOVAs with post hoc tests (Tukey HSD). We log transformed blood glucose data to meet the assumption of homogeneity of variances, as well as BHBD activity in the liver, SCOT activity in the heart, glycogen concentration in the liver, heart and skeletal muscle to meet the assumption of normality. To compare total plasma ketone body concentration among the experimental groups, we performed a Wilcoxon signed-rank test with Kruskal-Wallis post hoc testing, as the data were not normally distributed.

To compare the Fmax between groups, as well as comparing the Fmax following different substrate supplementation, we first performed a linear mixed model, but due to problems with normality, we performed a nonparametric analysis of longitudinal data in factorial experiments. Significant differences were analyzed non-parametrically using confidence intervals (CI), such that if the CI from two groups did not overlap, a significant difference can be concluded.

To compare the maximum pacing frequency between groups, as well as following different substrate supplementation, we performed a linear mixed model after log transformation due to the data not being normally distributed.

To compare the force frequency curve, we performed a repeated measures linear mixed effects model after rank transformation due to the data not being normally distributed.

Box and whisker plots presented in the results were used to visualize the findings. The horizontal line indicates the median, whiskers are the minimum and maximum values. The boxes represent the 25^{th} and 75^{th} percentiles and potential outliers are shown as black dots.

RESULTS

Mass

The mass (g) of each individual sturgeon was measured after the 10-day experimental treatments (Table 1). There were no significant differences in mass between any of the experimental groups: control *vs* starved (p=0.98), control *vs* hypoxia (p=0.62), starved *vs* hypoxia (0.73).

Table 1. The mean mass (g) of the fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) after experimental group exposure for 10 days.

Experimental Group	Mean mass (g)
Control	146.54 ± 49.12
Starved	173.77 ± 72.58
Hypoxia	151.15 ± 28.01

Blood Analyses

Hemoglobin and Hematocrit

We measured hematocrit values and hemoglobin concentration after treatment exposure and calculated the mean corpuscular hemoglobin concentration (MCHC) to understand if there were any changes in oxygen carrying capacity (Fig. 2). There was a significant decrease in hematocrit of the starved compared to the hypoxia group (p=0.028), but neither were significantly different from the control group. There were no significant differences between experimental groups for hemoglobin or for MCHC.

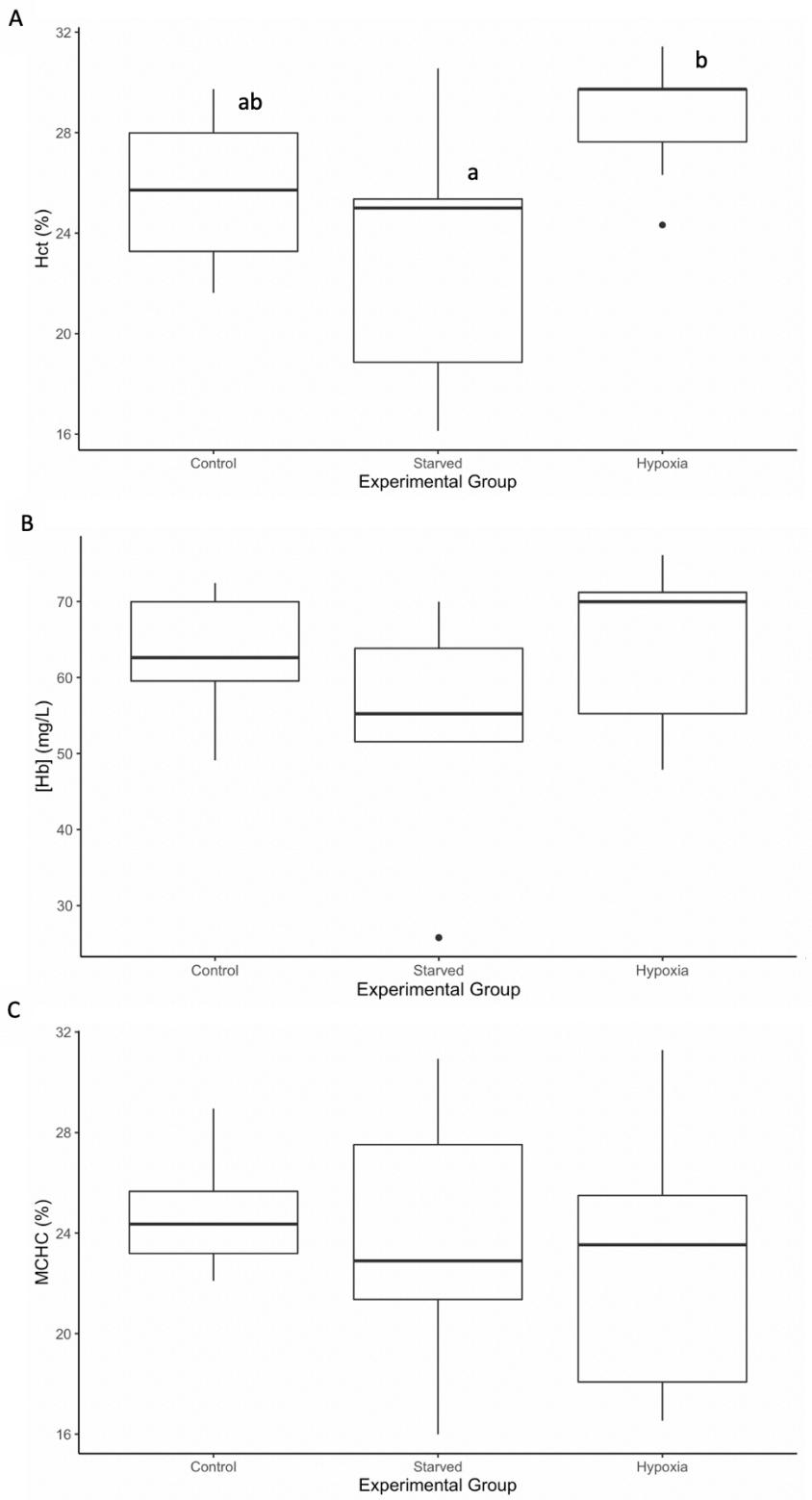

Figure 2. Blood analyses including A) hematocrit (%), B) hemoglobin concentration (mg/L) and C) mean corpuscular hemoglobin concentration (MCHC) (%) of the fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)), after treatment exposure. Different letters denote significant differences ($p<0.05$).

Blood Glucose

Blood glucose concentration of individual sturgeon was measured after the 10-day experimental treatment exposure (Fig. 3). There was a significant difference in blood glucose concentrations between each of the experimental groups (p=3.69E-06). Both the starved and hypoxic fish had significantly lower glucose than the control (p=2.3E-06 and p=0.002, respectively) and the starved fish had approximately half as much blood glucose than the hypoxic treatment (p=0.008).

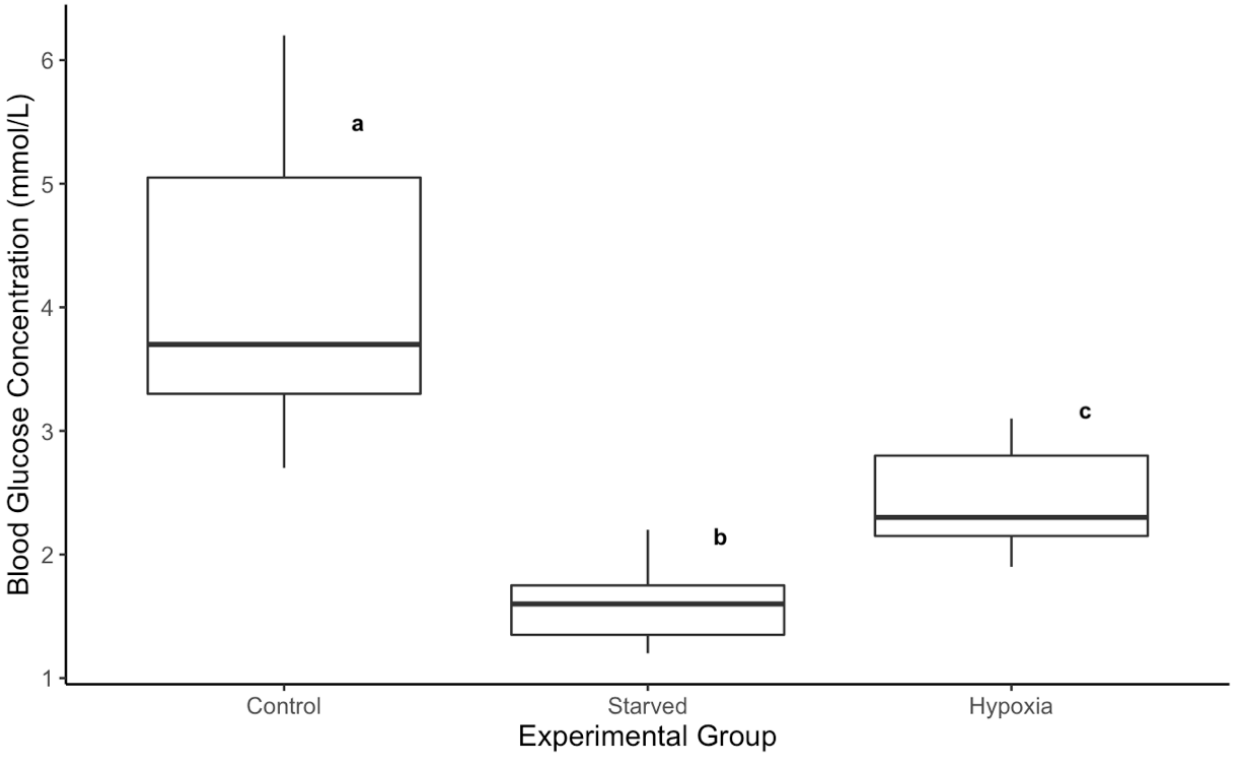

Figure 3. Blood glucose concentration (mmol/L) of the fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) after experimental group exposure for 10 days. Different letters denote significant differences (p<0.05).

Glucose Metabolism

Glycogen concentration in the heart, skeletal muscle and liver tissues for each fish was measured and analyzed following the 10-day experimental group exposure (Fig. 4). There were no significant differences in glycogen concentration in the heart tissue between the experimental groups. However, in skeletal muscle tissue, there was a significant increase in glycogen concentration from the control group to the starved group (p=0.003). In liver

tissue, there was a significant decrease in glycogen concentration in both the starved group ($p=0.0006$) and the hypoxia group ($p=0.001$) compared to controls.

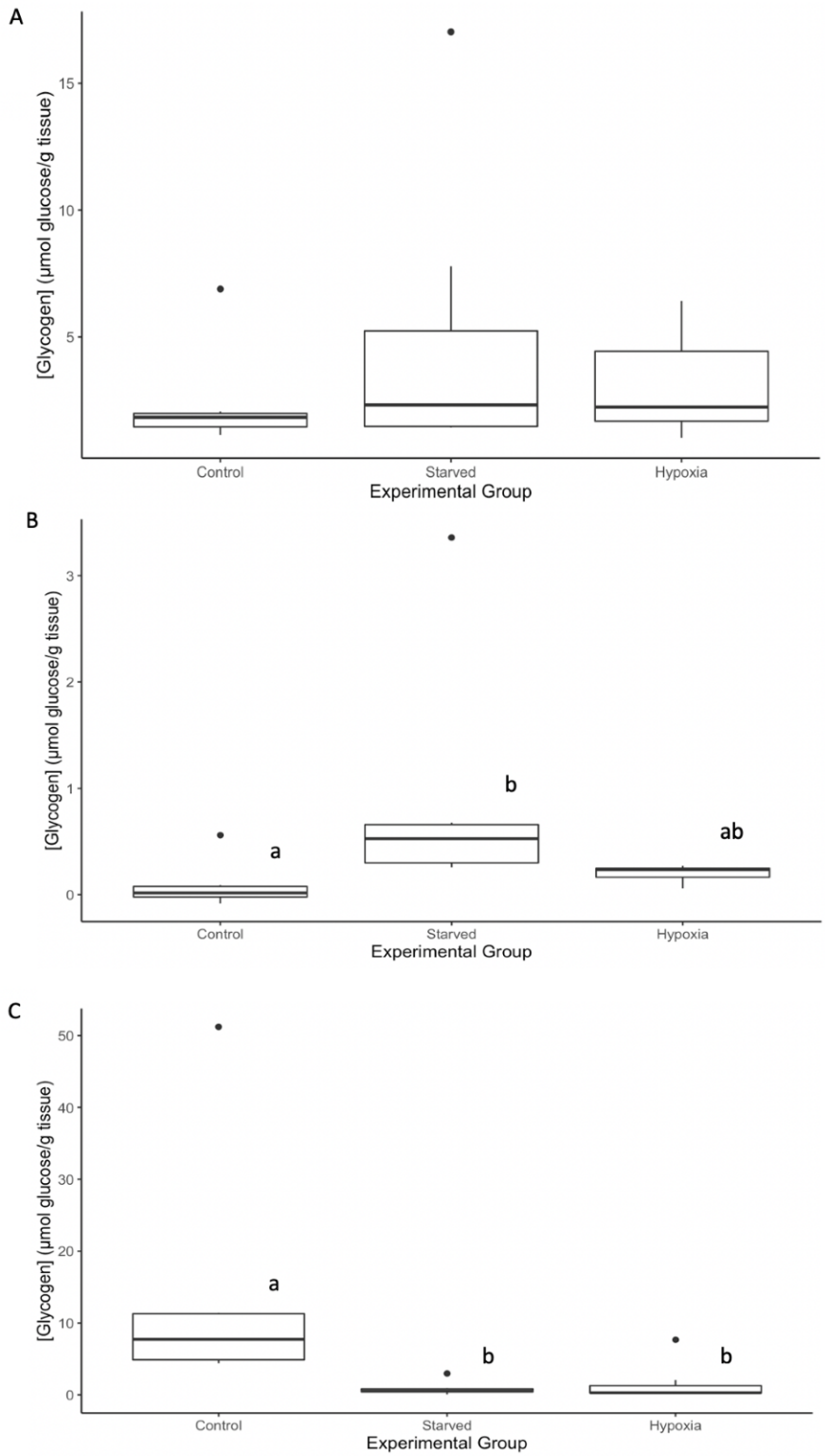

Figure 4. Glycogen concentration (µmol glucose/g tissue) in A) heart, B) skeletal muscle and C) liver of each fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) following the 10-day experimental group exposure. Different letters denote significant differences ($p<0.05$).

Ketone Body Metabolism

ß-Hydroxybutyrate Dehydrogenase Activity in the Liver

To understand ketone body production in the liver, we measured ß-hydroxybutyrate dehydrogenase (BHBD) activity following experimental group exposure (Fig. 5). There were no significant changes in BHBD activity between any of the experimental groups.

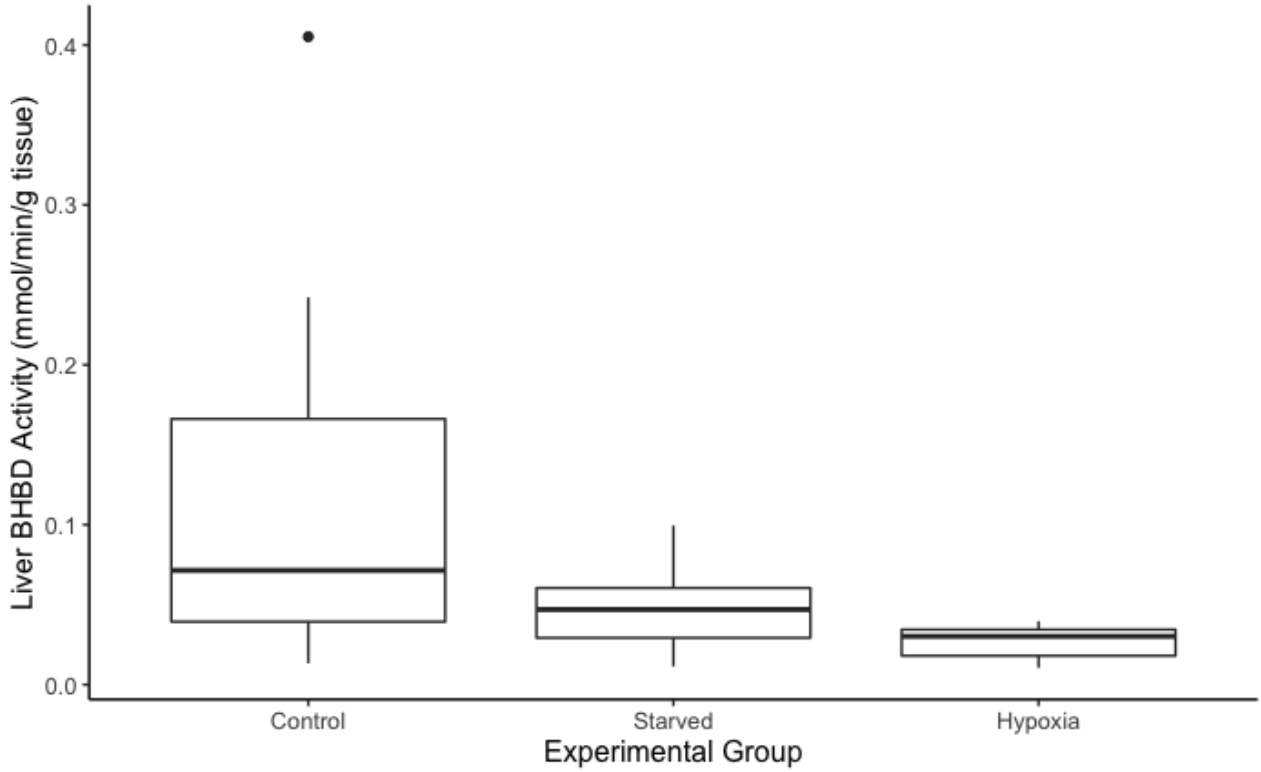

Figure 5. Liver enzyme activity of ß-hydroxybutyrate (mmol/min/g tissue) for fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) following the 10-day experimental group exposure.

Plasma Ketone Body

Ketone body concentration of both BOH and acetoacetate in the plasma for each fish was measured following the 10-day experimental group exposure and analyzed as total ketone body concentration (Fig. 6). There were no significant differences in total ketone body concentration between any of the experimental groups.

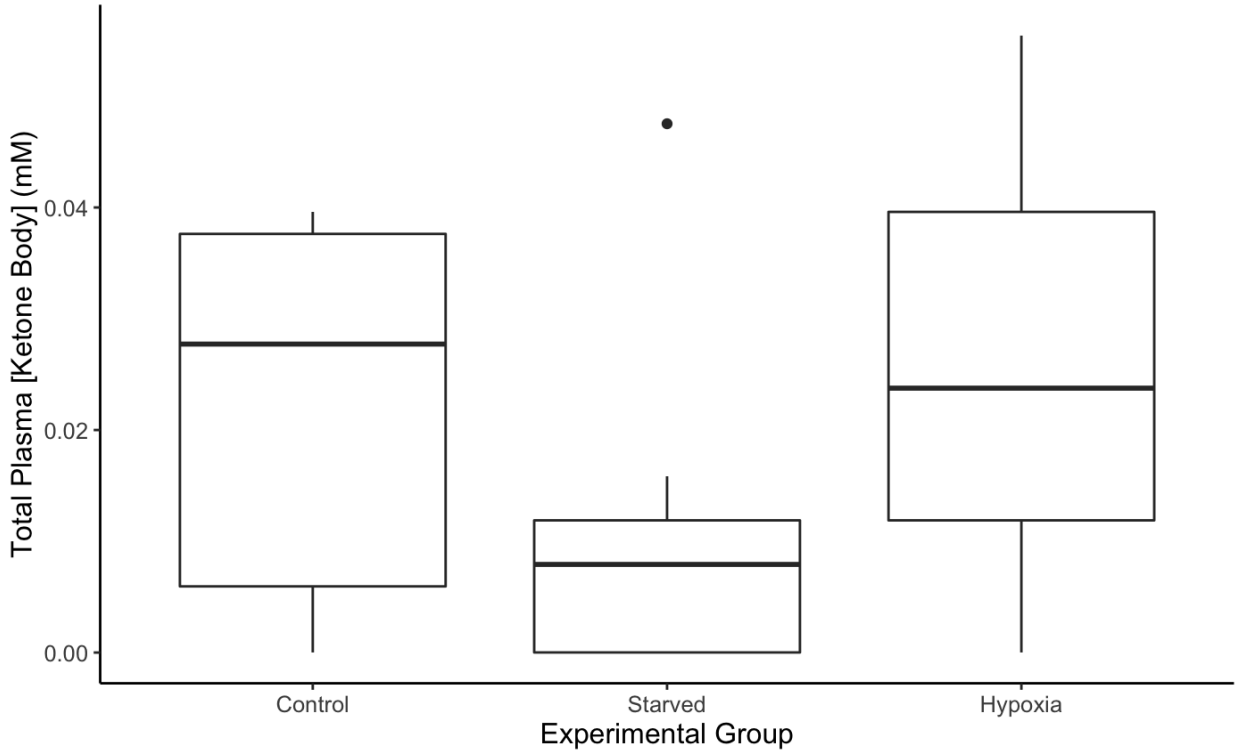

Figure 6. Total ketone body concentration in plasma for fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) following the 10-day experimental group exposure.

Succinyl-CoA:3 Ketoacid CoA Transferase (SCOT) Activity in Heart and Skeletal Muscle Tissues

To understand ketone body oxidation in heart and skeletal muscle tissues, SCOT activity was measured following experimental group exposure (Fig. 7). There was no significant difference in SCOT activity in the heart or skeletal muscle between any of the experimental groups.

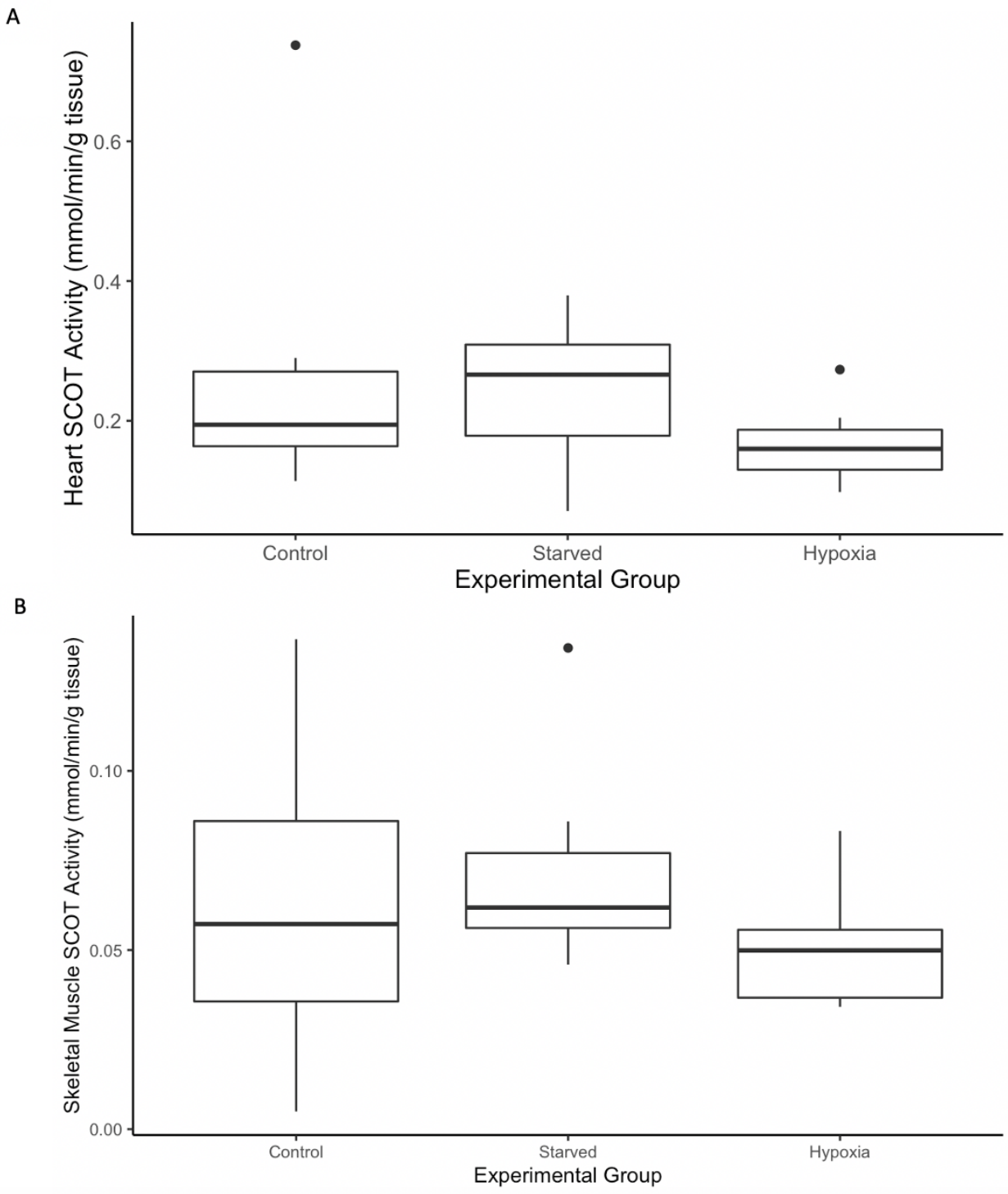

Figure 7. Succinyl-CoA:3-ketoacid CoA transferase (SCOT) activity (mmol/min/g tissue) in A) the heart and B) skeletal muscle for each fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) following the 10-day experimental group exposure.

Cardiac Function

Fmax

The maximum contraction force (Fmax) of the ventricles from fish in each experimental group was measured and analyzed while either being supplemented with glucose or ketone bodies (Fig. 8). There were no significant differences between the substrates within each experimental group, however there was a significant effect of group on the Fmax (p=0.49). There were significant differences between the groups depending on the substrate used. There was a significant decrease in the Fmax between the control group with glucose supplementation (CI=0.48 to 0.74) and the starved group with ketone supplementation (CI=0.18 to 0.43). There was also a significant decrease between the hypoxia group with glucose supplementation (CI=0.44 to 0.78) and the starved group with ketone supplementation (CI=0.18 to 0.43).

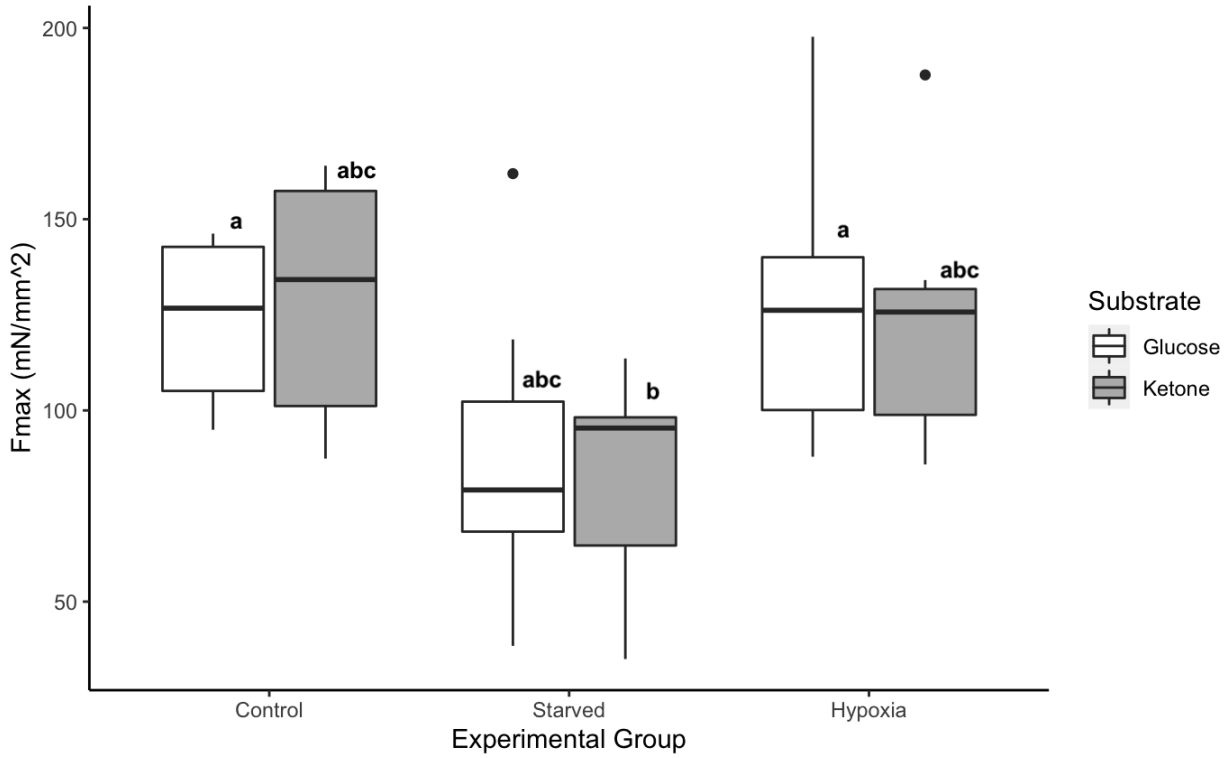

Figure 8. The maximum force production of the ventricle (Fmax, mN/mm^2) for fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) while in the presence of glucose and acetoacetate (ketone). Different letters denote significant differences ($p<0.05$).

Maximum Pacing Frequency

The maximum pacing frequency of the ventricles from fish in each experimental group was measured while being supplemented with either glucose or ketone bodies (Fig. 9). This was done to understand how well the cardiac muscle could transport Ca^{2+} in and out of the sarcoplasmic reticulum. There was no significant effect of overall group on the maximum pacing frequency but there was a significant decrease in the maximum pacing frequency from the starved group to the hypoxia groups (0.045). Although, there was no significant effect of substrate supplementation with glucose or ketones on the maximum pacing frequency across experimental groups (0.43).

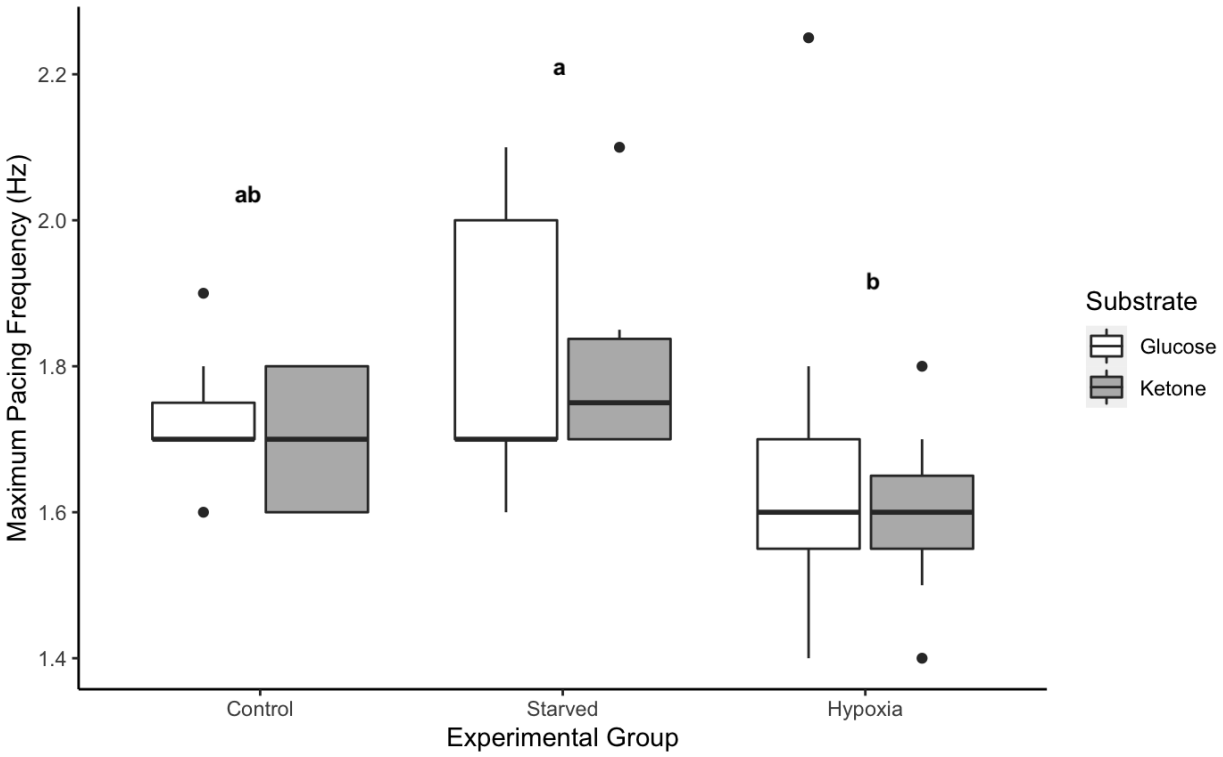

Figure 9. The maximum pacing frequency of the ventricles (Hz) for fish in each experimental group (control (n=7), starved (n=7), hypoxia (n=7)) while in the presence of glucose and acetoacetate (ketone). Different letters denote significant differences ($p<0.05$).

Force Frequency

The force frequency curve of the ventricles from fish in each experimental group was measured and analyzed while being supplemented with either glucose or ketone bodies and with increasing frequency (Fig. 10). When the ventricles were supplemented with glucose, there was a significant effect of experimental group on the force frequency at

0.5Hz, 0.7Hz and 0.9Hz, between the control and starved groups ($p=0.03$, $p=0.028$, $p=0.031$, respectively). There was no significant effect of experimental group on the force frequency when ventricles were supplemented with ketones.

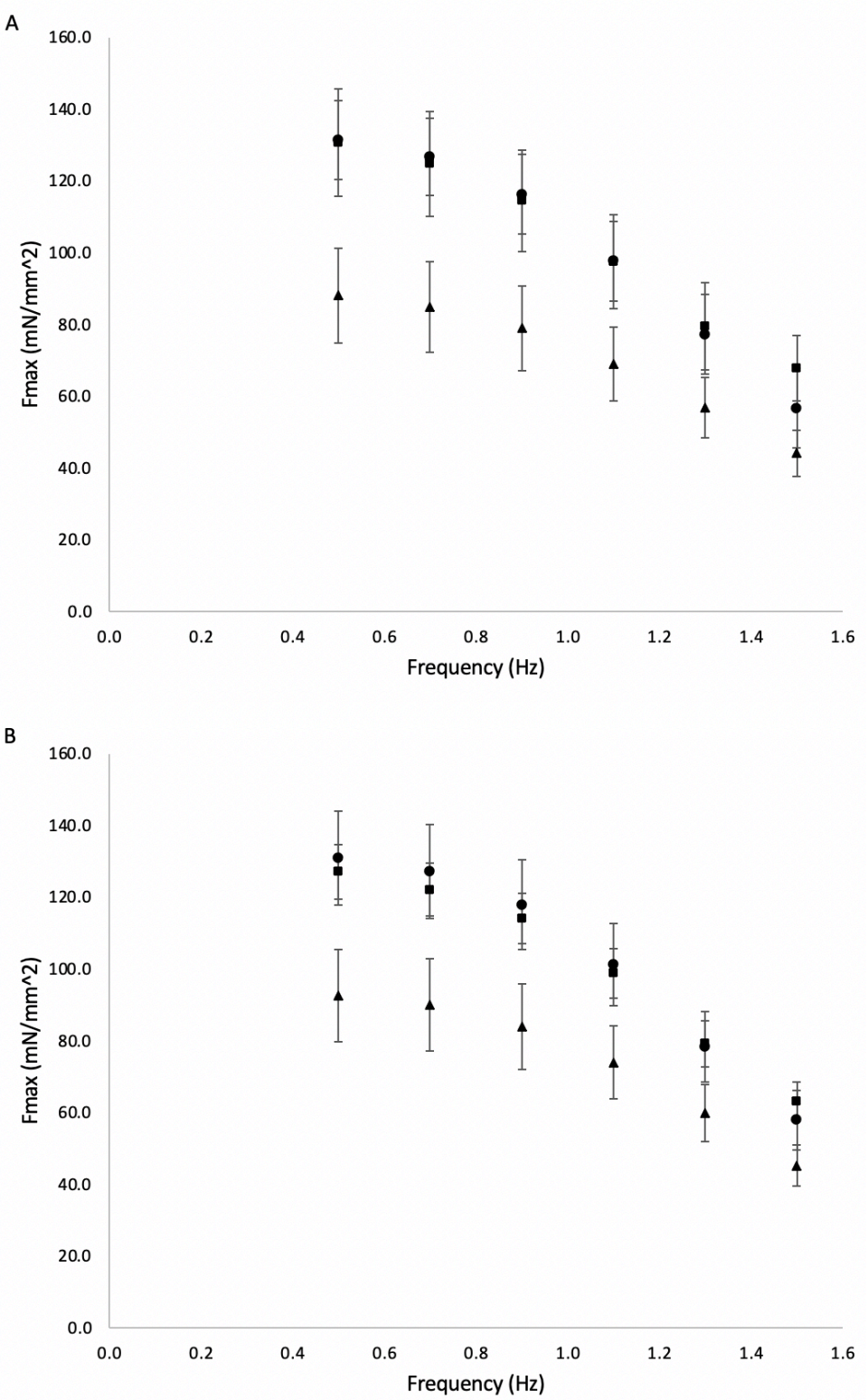

Figure 10. The maximum force production (Fmax) of the heart ventricle (mN/mm^2) for fish in each experimental group (control (n=7) (●), starved (n=7) (▲), hypoxia (n=7) (■) while in the presence of A) glucose or B) ketones (acetoacetate) with increasing frequency (Hz). The error bars represent the standard error of the mean.

DISCUSSION

The goal of this study was to understand the effects of chronic hypoxia on ketone body metabolism and cardiac contractile function in the sturgeon. The results indicate that the 10-day chronic hypoxia exposure did not elicit a ketogenic response in the sturgeon. Glucose and liver glycogen concentrations decreased following the hypoxic exposure and there were no significant changes in heart and skeletal muscle glycogen concentrations following experimental group exposure. BHBD activity in the liver and SCOT activity in heart and skeletal muscle decreased following the hypoxic exposure. Cardiac contractile function was not affected by substrate (glucose or ketones) and did not change following the hypoxic exposure. Although, there was a significant decrease in cardiac contractile function in the starved group. Knowing that the sturgeon in the hypoxia group were also starved, the hypoxic exposure essentially reversed the effects of starvation on cardiac function. We also found that oxygen carrying mechanisms were not enhanced, suggesting that oxygen transport was not affected. Overall, it is likely that the sturgeon were using either glucose or fats to support ATP production throughout the 10-day hypoxic exposure and did not require a ketogenic response.

Glucose Metabolism

Following the hypoxic exposure, we predicted there would be a decrease in glucose/glycogen that would force the sturgeon to synthesize ketone bodies to support energy production.

During hypoxia, if ATP use is greater than ATP production, ATP will be synthesized anaerobically instead of aerobically as a result of insufficient oxygen availability (Wheaton and Chandel, 2011). In order for anaerobic respiration to maintain adequate ATP production, the use of glucose and glycogen must significantly increase. This results in a decrease in liver glycogen concentration as the glycogen is broken down (glycogenolysis) to glucose and shuttled to the blood where it can be taken up by multiple tissues to make ATP (Abdel-Tawwab *et al*., 2019). Here, we found a similar result in that glycogen concentration in liver tissue was significantly decreased following the hypoxic exposure

when compared to the control group. Blood glucose concentration also decreased significantly following both the starved and hypoxic exposures when compared to the control group, although blood glucose was significantly increased following the hypoxic exposure when compared to the starved group.

Through glycogenolysis, the liver is able to supply glucose to extrahepatic tissues for energy production (Chang *et al.*, 2007). No other tissue is able to support ATP production like this, such that glycogen stores in skeletal muscle or heart can only be used by those tissues. When comparing glycogen concentrations between tissues, the liver had the greatest concentration of glycogen, while skeletal muscle had the lowest concentration of glycogen. It is likely that the skeletal muscle was using its own glycogen stores to support ATP production. In an experiment completed by Williams *et al.* (2019), they found control liver glycogen concentrations to be approximately 200 µmol/g tissue in rainbow trout, whereas the highest concentration of liver glycogen in the sturgeon in our experiment was approximately 11 µmol/g tissue (control group). Although, in an experiment completed by Kieffer *et al.* (2001), they found control liver glycogen concentrations to be approximately 15 µmol/g tissue in shortnose sturgeon. This suggests that it is normal for glycogen concentrations to be low in sturgeon. While these fish may rely on glycogen to support ATP production, they must also rely on other substrates for energy production since the overall concentration of glycogen is low compared to other species.

Glycogen concentration did not change significantly in any of the experimental groups in the muscle or heart tissue. Since the heart must always function and contract to sustain life, it requires a constant supply of energy in order to sustain contractions and to keep blood circulating around the body, delivering oxygen to various tissues (Giordano, 2005). The heart was likely using glucose that originated from glycogen in the liver tissue, ultimately conserving heart glycogen for later use.

The significant decrease in blood glucose following the starved exposure is likely due to the fish being starved, but also because of the relatively low amount of glycogen present in the liver. The significant increase in muscle glycogen following the starved exposure suggests that these glycogen stores were not being used compared to those in the liver,

possibly as a result of the fish decreasing their mobility. Although, the muscle glycogen concentration in the fish following the starved exposure is very low (~0.5 µmol/g tissue), suggesting that sturgeon may not be using glucose to support energy production.

Ketone Body Metabolism

With this decrease in liver glycogen and plasma glucose, we expect there to be an increase in ketone body production in the liver to support extra-hepatic ATP synthesis.

Ketone body production occurs in the liver while oxidation of ketone bodies occurs in extrahepatic tissues (Harrison and Long, 1940). Ketone body formation is expected to occur as glycogen stores decrease, as they can act as an alternative fuel source to maintain energy production. BHBD in particular is an enzyme that is found in the liver that catalyzes the reversible reaction between the formation of BOH from acetoacetate.

BHBD activity in sturgeon liver was not significantly different following any of the three treatment groups, suggesting that ketone body production was minimal following starvation and hypoxic exposures. Although, in an experiment by Singer *et al*. (1990), they found regular BHBD activity in sturgeon liver to be 0.028 µmol/min/g tissue. The lowest activity measured in sturgeon liver in this experiment, between all treatment groups, was 10.0 µmol/min/g tissue. This activity is 357X greater, which suggests that ketone bodies are being produced in the liver on a regular basis. Interestingly, the variance in the BHBD activity data decreases after each experimental group exposure, such that the control group has the highest variance and the hypoxia group has the lowest variance. BHBD activity converges following the hypoxic exposure, suggesting that the majority of the sturgeon are conforming to a similar metabolic profile. This may be the result of further decreasing metabolic demand in an effort to conserve oxygen. It is important to remember that sturgeon in the hypoxic group were also starved, which shows how the potential decrease in metabolic demand is enhanced during hypoxia.

The total plasma ketone body concentration in the sturgeon provided insight as to whether the sturgeon were circulating ketones around the body following their production

in the liver. This concentration included both acetoacetate and BOH, both of which were very low in the sturgeon following experimental group exposures. The lack of any significant changes in ketone body concentration following the hypoxic exposure suggests that the sturgeon may have been producing and using these ketones at the same rate. It could also be that they were not transporting an increased number of ketone bodies to extrahepatic tissues, like the heart or skeletal muscle, to aid in energy production. This is similar to findings in teleost fish, such that BOH was not detectable in plasma following a 150 day starvation period and the average concentration of acetoacetate was approximately 0.04 µM following this same 150-day starvation period (Zammit and Newsholme, 1979). In this same study, elasmobranchs showed a baseline total ketone body concentration of 0.1mM, which increased to 2.0mM following the starvation period (Zammit and Newsholme, 1979). Although our study focused on the response to the hypoxic exposure while under starved conditions, this helps compare ketone body circulation in sturgeon, teleosts and elasmobranchs, such that sturgeon seem to be following teleost trends in ketone body circulation. This may provide evidence as to why the hypoxic exposure did not elicit a ketogenic response.

The SCOT enzyme is found in extrahepatic tissue, including the heart and skeletal muscle and is the rate-limiting enzyme in the process of oxidizing ketone bodies (Cotter *et al*., 2013). An increase in SCOT activity suggests an increased capacity to oxidize ketone bodies in these tissues. SCOT is the only transferase enzyme that catalyzes a "near equilibrium" reaction between acetoacetate and acetoacetyl-CoA (Cotter *et al*., 2013). In order for SCOT to oxidize ketones, there needs to be an abundance of acetoacetate, since the reaction thermodynamically favours the formation of acetoacetate (Cotter *et al.*, 2013). With respect to circulating ketone bodies in plasma, results from this experiment found that concentrations of acetoacetate were undetectable in the control and starved groups. Since SCOT requires an abundance of acetoacetate to carry out the reaction, the activity of SCOT would be entirely dependent on the concentration of BOH and the ability for BHBD to convert BOH to acetoacetate.

In a study using Lake sturgeon in the fed state, SCOT activities in the heart were 0.43 µmol/min/g tissue and in red muscle were 0.40 µmol/min/g tissue (Singer *et al.*, 1990). In our study, SCOT enzyme activities in the control group for both the heart (0.27 mmol/min/g tissue) and skeletal muscle (0.06 mmol/min/g tissue) were higher than these reported values, suggesting that ketone bodies are being oxidized to a greater extent in the shortnose sturgeon. Average SCOT activities were lowest in sturgeon following the hypoxic exposure in both the heart (0.17 mmol/min/g tissue) and skeletal muscle (0.05 mmol/min/g tissue), suggesting that ketones were not being used to support energy production. It is therefore possible that instead of using ketone bodies to support energy demands during chronic hypoxia, sturgeon were using other substrates to support ATP production.

When comparing ketone body oxidation in the heart and skeletal muscle, SCOT enzyme activities are always greater in the heart following exposure to each of the experimental groups. Since the heart is an aerobic tissue and requires a constant input of ATP to sustain cardiac function (Speers-Roesch *et al.,* 2009), this only makes sense that the heart is oxidizing more ketones compared to the skeletal muscle. In response to chronic hypoxia, fish typically decrease their heart rates to conserve energy, but they also decrease their movement (Pollock *et al.*, 2007). When exposed to chronic hypoxia, we observed the sturgeon decreased their movement and remained at the bottom of the tank for the majority of the 10-day hypoxia exposure, although we did not quantify this behavioural response. This may be the reason as to why skeletal muscle SCOT activities were lower, such that their skeletal muscles were using very little ATP so did not require an input of energy to support movement.

Whole Animal Response to Chronic Hypoxia

For an animal to survive, the heart in particular must continually contract and circulate oxygen, fuel and waste around the body. It is flexible in the sources of energy that it can use to produce ATP, such that it can oxidize glucose, ketones, lactate and amino acids (Speers-Roesch *et al.*, 2009; Weber *et al.*, 2016). When the availability of oxygen decreases for longer periods of time (i.e. chronic hypoxia), fish can use anaerobic metabolism to

produce ATP and sustain cardiac function. ATP is produced by substrate-level phosphorylation mainly through the oxidation of glucose, but it cannot always sustain contractile function without experiencing energy deprivation (Melkonian and Schury, 2021). This leads to detrimental effects on the individual, unless the fish have some other way to maintain ATP production. We proposed that this occurred by the oxidation of ketone bodies, but our data do not support this theory.

The maximum cardiac contractile force (Fmax) provides information on the efficiency of substrate use within the heart to produce a cardiac contraction. We found that there was no effect of substrate supplementation (glucose of ketones) on the Fmax value in any of the experimental groups, suggesting that the sturgeon ventricles oxidized glucose and ketones equally as efficiently to produce ATP and therefore a cardiac contraction. It could also be that the ventricles were using substrates already present in the heart, such as lipids, to maintain the contraction, since these ventricle strips were fully oxygenated. While there was no difference in substrate use, there was a significant effect of the treatment on the Fmax value, such that the starved group produced a lower Fmax value when compared to both the control and hypoxia groups. It could be that the starvation treatment caused the breakdown of cardiac muscle in an effort to liberate amino acids for ATP synthesis (Cassidy *et al.,* 2016). Since protein synthesis decreases under starvation conditions in an effort to conserve energy (Cassidy *et al.*, 2016), it is possible that the cardiac muscle was used to maintain cardiac energy production. It could also be that lipids stored in the cardiac muscle were still available to support ATP production following the hypoxic exposure. Lipids are oxidized much more readily under starved conditions, although under chronic hypoxic conditions, they are considered a main energy reserve and are only mobilized when absolutely needed (Gracey *et al.,* 2011). Since the sturgeon in the hypoxic group were also starved, it suggests that the hypoxic exposure might have led to the conservation of lipid stores to support ATP production and cardiac function, ultimately leading to the increased Fmax value in the hypoxic group.

Another reason for the decrease in Fmax in the starved group may be explained by the damaging effects of ROS on cardiac muscle. On a molecular level, the heart sustains

contractions by regulating calcium ion flux. ROS have been shown to damage L-type calcium channels, sodium channels and potassium channels located in the heart muscle (Giordano, 2005). The loss of function of these channels makes it impossible to maintain cardiac function, especially under stress (Giordano, 2005). Superoxide is a ROS that is produced in response to starvation, especially starvation that involves depleting glucose (Chen *et al*., 2009). While there is evidence that chronic hypoxia may elicit a ketogenic response in sturgeon (Furné *et al.*, 2012; Kieffer *et al.*, 2011), and that ketone bodies can protect tissues like the heart from the negative effects of ROS (Ray *et al*., 2012), the decreased Fmax value in the starved group may be due to the production of, and the damage by ROS. This also suggests that the cardiac muscle from fish in the hypoxia group may have been protected by the few ketones that were produced in the liver and oxidized in the heart. Since the fish in the hypoxia group were also starved, and if ketones were not available to protect the heart against ROS, then there would have been the same decrease in Fmax as in the starved group. This leads one to believe that ketones were in fact present and helping decrease oxidative stress (Poljsak *et al.*, 2013), allowing the maintenance of Fmax.

Another explanation as to why the ventricles from the starved group produced a lower Fmax may be explained by the differences in glucose concentration and the total plasma ketone body concentration in the heart between the starved and hypoxia groups. The hypoxia group had a greater blood glucose concentration than the starved group, which could suggest that there was enough glucose available in the heart tissue to support the cardiac contraction. There is evidence that HIFs may cause the accumulation of glycogen under chronic hypoxic conditions (Pescador *et al.*, 2010), which could explain why blood glucose is elevated in fish following the hypoxic exposure. This HIF-induced glycogen accumulation is only seen in chronic hypoxic exposures, as it takes time to express HIF genes and to then replenish lost glycogen stores (Pescador *et al.,* 2010). Due to the relatively similar glycogen concentrations in heart tissue between the starved and hypoxia groups, it could be that the 10-day hypoxic exposure was beginning to cause the expression of these genes and the regeneration of glycogen. This could be the reason why there was an increase in the Fmax value in the hypoxia group compared to the starved group.

Although we don't have hard evidence supporting the breakdown of the cardiac muscle to liberate amino acids for ATP synthesis, or that ROS damaged ion channels needed for proper cardiac contractions, or that HIFs were causing the accumulation of heart glycogen, they are nonetheless possible reasons as to why Fmax decreased in the starved group and was maintained in the hypoxia group (compared to the control group).

The maximum pacing frequency of cardiac muscle is an indicator as to how well the cardiac muscle can transport Ca^{2+} out from, and in to the sarcoplasmic reticulum (Haverinen and Vornanen, 2009). This connects with how well the ventricles can use glucose or ketones to sustain cardiac contractions, such that the ventricles will have a higher maximum pacing frequency if they can produce and use ATP more efficiently. There was no significant effect of substrate supplementation with either glucose or ketones, so the ventricles used these substrates equally as well to produce ATP. There was a significant effect of experimental group on the pacing frequency such that the starved group had a higher pacing frequency when compared to the hypoxia group. When compared to the Fmax, the ventricles from fish exposed to the hypoxia group were able to maintain a greater force production at the expense of pacing frequency, whereas the ventricles from fish exposed to the starved group were able to contract at greater frequencies but produced a lower Fmax. This inverse relationship may be explained by how chronic hypoxia decreases cardiac pumping capacity, which is more severe when fish are "swum to exhaustion" (Carnevale *et al.*, 2019). This relates to our study as during exercise, the heart must increase the speed at which it contracts to supply tissues with sufficient oxygen and fuel. This increase in contraction rate is paralleled to increasing the frequency on the stimulator. Perhaps the chronic hypoxic exposure did decrease cardiac pumping capacity, which would explain the decrease in pacing frequency in the hypoxic group compared to the starved group. Furthermore, research has shown that when hypoxia-tolerant fish are acclimated to hypoxic conditions, the ability for the ventricle muscle fibers to shorten is impaired compared to fish that are acclimated to normoxic conditions (Carnevale *et al*., 2019). This could explain why the ventricles from fish exposed to chronic hypoxia seem to be under-performing compared to those in the starved group.

The force frequency relationship states that as the frequency of a stimulation increases, the contractile force of the cardiac muscle usually decreases (Driedzic and Gesser, 1988). Analyzing the force frequency of ventricle strips helps understand the "contractile state" of the cardiac muscle, such that it can provide insight on the uptake and release of calcium ions from the sarcoplasmic reticulum (SR) (Shiels *et al*., 2002). Results showed that there was a significant effect of experimental group when ventricles were supplemented with glucose, such that the starved group produced a lower Fmax when compared to the control and hypoxia groups at 0.5Hz, 0.7Hz and 0.9Hz. This difference decreased in its significance as the frequency increased, such that the Fmax curves converged towards the end (1.5Hz). The same trend is seen when ventricles were supplemented with ketones, although no significant effects were seen following force frequency analysis. These findings suggest that the ventricles in the control and hypoxia groups, when supplemented with either glucose or ketones, were able to better control the movement of Ca^{2+} through L-type Ca^{2+} channels into the cytoplasm and then back in to the SR compared to the ventricles in the starved group (Shiels *et al.*, 2002). This may be the resulting effect of the damaging effects of ROS on ion channels in the SR in the starved group (Giordano, 2005). Similarly, as in the maximum cardiac contractile force data, the cardiac muscle of the fish in the hypoxia group may have been protected by ketone bodies that were produced and oxidized in the heart.

Based on the cardiac responses of the sturgeon, as well as the non-significant ketone body production, circulation and oxidation, the 10-day hypoxic exposure was not stressful enough to elicit any kind of ketogenic response. This is further supported by the limited changes in blood composition following the hypoxic exposure. There were no changes in hemoglobin nor MCHC levels following experimental group exposure, although there was an increase in hematocrit levels following the hypoxic exposure. This indicates that the concentration of red blood cells (RBCs) increased in the sturgeon, but that oxygen carrying capacity of these RBCs may not have changed in response to being starved or to being exposed to chronic hypoxic conditions. Normally, to ensure that ATP production matches

cellular use under hypoxic conditions, oxygen transport is enhanced by increasing the resting respiration rate, hematocrit levels and the concentration of hemoglobin in circulating blood (Horscroft and Murray, 2014). The lack of any significant change in hemoglobin suggests that even though the number of RBCs increased (hematocrit), the ability for each individual RBC to carry oxygen throughout the body did not change. Since the sturgeon are hypoxia-tolerant (Kieffer *et al*., 2011), it is possible that the sturgeon were not stressed adequately to elicit a full change in blood composition, which could explain the lack of differences in hemoglobin or MCHC levels among experimental groups.

Conclusion

The findings from this study suggest that the 10-day exposure to hypoxia did not elicit a ketogenic response in the shortnose sturgeon. It is believed that the sturgeon were using glucose liberated from glycogen stores to support energetic requirements, or that there was enough oxygen available to support the use of fats or amino acids in ATP synthesis. In relation to climate change, chronic hypoxic environments are becoming more severe and longer in duration. Even so, sturgeon seem to be able to withstand a chronic hypoxic exposure of 10 days with minimal changes to their metabolic machinery. This supports evidence regarding their hypoxia-tolerance and overall resilience.

REFERENCES

Abdel-Tawwab, M., Monier, M.N., Hoseinifar, S.H., Faggio, C., 2019. Fish response to hypoxia stress: growth, physiological, and immunological biomarkers. Fish Physiol Biochem 45, 997–1013. https://doi.org/10.1007/s10695-019-00614-9

Ackerly, K.L., Krahe, R., Sanford, C.P., Chapman, L.J., 2018. Effects of hypoxia on swimming and sensing in a weakly electric fish. Journal of Experimental Biology 221. https://doi.org/10.1242/jeb.172130

Beckhauser, T.F., Francis-Oliveira, J., De Pasquale, R., 2016. Reactive oxygen species: physiological and physiopathological effects on synaptic plasticity. J Exp Neurosci 10, 23–48. https://doi.org/10.4137/JEN.S39887

Bonora, M., Patergnani, S., Rimessi, A., De Marchi, E., Suski, J.M., Bononi, A., Giorgi, C., Marchi, S., Missiroli, S., Poletti, F., Wieckowski, M.R., Pinton, P., 2012. ATP synthesis and storage. Purinergic Signal 8, 343–357. https://doi.org/10.1007/s11302-012-9305-8

Carnevale, C., Roberts, J.C., Syme, D.A., Gamperl, A.K., 2019. Hypoxic acclimation negatively impacts the contractility of steelhead trout (*Oncorhynchus mykiss*) spongy myocardium. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 318, R214–R226. https://doi.org/10.1152/ajpregu.00107.2019

Cassidy, A.A., Saulnier, R.J., Lamarre, S.G., 2016. Adjustments of protein metabolism in fasting arctic charr, *Salvelinus alpinus.* PLoS One 11. https://doi.org/10.1371/journal.pone.0153364

Chang, J.C.-H., Wu, S.-M., Tseng, Y.-C., Lee, Y.-C., Baba, O., Hwang, P.-P., 2007. Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus)* during acclimation to seawater. Journal of Experimental Biology 210, 3494–3504. https://doi.org/10.1242/jeb.007146

Chen, Y., Azad, M.B., Gibson, S.B., 2009. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death & Differentiation 16, 1040–1052. https://doi.org/10.1038/cdd.2009.49

Claësson, D., Wang, T., Malte, H., 2016. Maximal oxygen consumption increases with temperature in the European eel (*Anguilla anguilla*) through increased heart rate and arteriovenous extraction. Conserv Physiol 4. https://doi.org/10.1093/conphys/cow027

Clark, T.D., Eliason, E.J., Sandblom, E., Hinch, S.G., Farrell, A.P., 2008. Calibration of a hand-held haemoglobin analyser for use on fish blood. Journal of Fish Biology 73, 2587–2595. https://doi.org/10.1111/j.1095-8649.2008.02109.x

Cotter, D.G., Schugar, R.C., Crawford, P.A., 2013. Ketone body metabolism and cardiovascular disease. Am J Physiol Heart Circ Physiol 304, H1060–H1076. https://doi.org/10.1152/ajpheart.00646.2012

Driedzic, R., Gesser, H., 1988. Differences in force-frequency relationships and calcium dependency between elasmobranch and teleost hearts.

Fitzgibbon, Q.P., Seymour, R.S., Ellis, D., Buchanan, J., 2007. The energetic consequence of specific dynamic action in southern bluefin tuna *Thunnus maccoyii*. Journal of Experimental Biology 210, 290–298. https://doi.org/10.1242/jeb.02641

Furné, M., Morales, A.E., Trenzado, C.E., García-Gallego, M., Carmen Hidalgo, M., Domezain, A., Sanz Rus, A., 2012. The metabolic effects of prolonged starvation and refeeding in sturgeon and rainbow trout. J Comp Physiol B 182, 63–76. https://doi.org/10.1007/s00360-011-0596-9

Gattuso, A., Garofalo, F., Cerra, M.C., Imbrogno, S., 2018. Hypoxia tolerance in teleosts: implications of cardiac nitrosative signals. Front Physiol 9. https://doi.org/10.3389/fphys.2018.00366

Giaccia, A.J., Simon, M.C., Johnson, R., 2004. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev 18, 2183–2194. https://doi.org/10.1101/gad.1243304

Giordano, F.J., 2005. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest 115, 500–508. https://doi.org/10.1172/JCI200524408

Gracey, A.Y., Lee, T.-H., Higashi, R.M., Fan, T., 2011. Hypoxia-induced mobilization of stored triglycerides in the euryoxic goby *Gillichthys mirabilis*. Journal of Experimental Biology 214, 3005–3012. https://doi.org/10.1242/jeb.059907

Gräns, A., Olsson, C., Pitsillides, K., Nelson, H.E., Cech, J.J., Axelsson, M., 2010. Effects of feeding on thermoregulatory behaviours and gut blood flow in white sturgeon (*Acipenser transmontanus*) using biotelemetry in combination with standard techniques. Journal of Experimental Biology 213, 3198–3206. https://doi.org/10.1242/jeb.043570

Harrison, H.C., Long, C.N.H., 1940. The distribution of ketone bodies in tissues. Journal of Biological Chemistry 133, 209–218.

Haverinen, J., Vornanen, M., 2009. Comparison of sarcoplasmic reticulum calcium content in atrial and ventricular myocytes of three fish species. American journal of physiology. Regulatory, integrative and comparative physiology 297, R1180-7. https://doi.org/10.1152/ajpregu.00022.2009

Horscroft, J.A., Murray, A.J., 2014. Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. Extrem Physiol Med 3. https://doi.org/10.1186/2046-7648-3-19

Kieffer, J.D., Baker, D.W., Wood, A.M., Papadopoulos, C.N., 2011. The effects of temperature on the physiological response to low oxygen in Atlantic sturgeon. Fish Physiol Biochem 37, 809–819. https://doi.org/10.1007/s10695-011-9479-y

Kieffer, J.D., Wakefield, A.M., Litvak, M.K., 2001. Juvenile sturgeon exhibit reduced physiological responses to exercise. Journal of Experimental Biology 204, 4281–4289.

Mandic, M., Speers-Roesch, B., Richards, J.G., 2013. Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches 86, 92–105. https://doi.org/10.1086/667938

Melkonian, E.A., Schury, M.P., 2021. Biochemistry, Anaerobic Glycolysis, in: StatPearls. StatPearls Publishing, Treasure Island (FL).

Michiels, C., 2004. Physiological and Pathological Responses to Hypoxia. Am J Pathol 164, 1875–1882.

Morash, A.J., Kotwica, A.O., Murray, A.J., 2013. Tissue-specific changes in fatty acid oxidation in hypoxic heart and skeletal muscle. Am J Physiol Regul Integr Comp Physiol 305, R534–R541. https://doi.org/10.1152/ajpregu.00510.2012

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochem J 417, 1–13. https://doi.org/10.1042/BJ20081386

Pescador, N., Villar, D., Cifuentes, D., Garcia-Rocha, M., Ortiz-Barahona, A., Vazquez, S., Ordoñez, A., Cuevas, Y., Saez-Morales, D., Garcia-Bermejo, M.L., Landazuri, M.O., Guinovart, J., del Peso, L., 2010. Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. PLoS One 5. https://doi.org/10.1371/journal.pone.0009644

Petersen, L.H., Gamperl, A.K., 2010. Effect of acute and chronic hypoxia on the swimming performance, metabolic capacity and cardiac function of Atlantic cod (*Gadus morhua*). Journal of Experimental Biology 213, 808–819. https://doi.org/10.1242/jeb.033746

Poljsak, B., Šuput, D., Milisav, I., 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxidative Medicine and Cellular Longevity 2013, e956792. https://doi.org/10.1155/2013/956792

Pollock, M.S.P.S., Clarke, L.M.J.C.M.J., Dubé, M.G.D.G., 2007. The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environmental Reviews. https://doi.org/10.1139/a06-006

Powers, D.A., 1980. Molecular ecology of teleost fish hemoglobins: strategies for adapting to changing environments. American Zoologist 20, 139–162.

Puchalska, P., Crawford, P.A., 2017. Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. Cell Metab 25, 262–284. https://doi.org/10.1016/j.cmet.2016.12.022

Puchowicz, M.A., Zechel, J., Valerio, J., Emancipator, D., Xu, K., Pundik, S., LaManna, J.C., Lust, W.D., 2008. Neuroprotection in diet induced ketotic rat brain following focal ischemia. J Cereb Blood Flow Metab 28, 1907–1916. https://doi.org/10.1038/jcbfm.2008.79

Rabalais, N.N., Turner, R.E., Díaz, R.J., Justić, D., 2009. Global change and eutrophication of coastal waters. ICES J Mar Sci 66, 1528–1537. https://doi.org/10.1093/icesjms/fsp047

Ray, P.D., Huang, B.-W., Tsuji, Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 24, 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008

Secor, D.H., Gunderson, T., 1998. Effects of hypoxia and temperature on survival, growth, and respiration of juvenile Atlantic sturgeon, *Acipenser oxyrinchus*. Fishery Bulletin-National Oceanic and Atmospheric Administration 96, 603–613.

Shiels, null, Farrell, null, 1997. The effect of temperature and adrenaline on the relative importance of the sarcoplasmic reticulum in contributing Ca2+ to force development in isolated ventricular trabeculae from rainbow trout. J Exp Biol 200, 1607–1621.

Shiels, H., Vornanen, M., Farrell, A., 2002. The force-frequency relationship in fish hearts - A review. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology 132, 811–26. https://doi.org/10.1016/S1095-6433(02)00050-8

Singer, T.D., Mahadevappa, V.G., Ballantyne, J.S., 2011. Aspects of the energy metabolism of lake sturgeon, *Acipenser fulvescens*, with special emphasis on lipid and ketone body metabolism. Canadian Journal of Fisheries and Aquatic Sciences. https://doi.org/10.1139/f90-100

Soengas, J.L., Strong, E.F., Fuentes, J., Veira, J. a. R., Andrés, M.D., 1996. Food deprivation and refeeding in Atlantic salmon, *Salmo salar* : effects on brain and liver carbohydrate and ketone bodies metabolism. Fish Physiol Biochem 15, 491–511. https://doi.org/10.1007/BF01874923

Speers-Roesch, B., Ip, Y.K., Ballantyne, J.S., 2006. Metabolic organization of freshwater, euryhaline, and marine elasmobranchs: implications for the evolution of energy metabolism in sharks and rays. Journal of Experimental Biology 209, 2495–2508. https://doi.org/10.1242/jeb.02294

Speers-Roesch, B., Sandblom, E., Lau, G.Y., Farrell, A.P., Richards, J.G., 2009. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 298, R104–R119. https://doi.org/10.1152/ajpregu.00418.2009

Veech, R.L., Chance, B., Kashiwaya, Y., Lardy, H.A., Cahill, G.F., 2001. Ketone bodies, potential therapeutic uses. IUBMB Life 51, 241–247. https://doi.org/10.1080/152165401753311780

Vornanen, M., Haverinen, J., 2011. Seasonality of glycogen phosphorylase activity in crucian carp (*Carassius carassius L.*). Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology 181, 917–26. https://doi.org/10.1007/s00360-011-0580-4

Weber, J.-M., Choi, K., Gonzalez, A., Omlin, T., 2016. Metabolic fuel kinetics in fish: swimming, hypoxia and muscle membranes. Journal of Experimental Biology 219, 250–258. https://doi.org/10.1242/jeb.125294

Wheaton, W.W., Chandel, N.S., 2011. Hypoxia. 2. Hypoxia regulates cellular metabolism. Am J Physiol Cell Physiol 300, C385–C393. https://doi.org/10.1152/ajpcell.00485.2010

Wiebe, L.I., Machulla, H.-J., 1999. Hypoxia: an Introduction, in: Machulla, H.-J. (Ed.), Imaging of Hypoxia: Tracer Developments, Developments in Nuclear Medicine. Springer Netherlands, Dordrecht, pp. 1–18. https://doi.org/10.1007/978-94-017-1828-8_1

Williams, K.J., Cassidy, A.A., Verhille, C.E., Lamarre, S.G., MacCormack, T.J., 2019. Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): evidence of physiological and metabolic plasticity. Journal of Experimental Biology 222. https://doi.org/10.1242/jeb.206045

Zammit, V.A., Newsholme, E.A., 1979. Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. Biochem J 184, 313–322.