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Seasonal Variation in Mitochondrial Bioenergetics of the Bluegill Sunfish, Lepomis macrochirus, from a Shallow Midwest River

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SEASONAL VARIATION IN MITOCHONDRIAL BIOENERGETICS OF THE BLUEGILL SUNFISH, *LEPOMIS MACROCHIRUS*, FROM A SHALLOW MIDWEST RIVER

By

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TABLE OF CONTENTS

ABSTRACT

As average global temperature increase, the frequency and magnitude of extreme temperatures in shallow aquatic ecosystems are more ubiquitous. In order to understand how these changing thermal regimes affect aquatic ectotherms, it is essential to develop studies evaluating the response of ectotherms to seasonal fluctuating thermal regimes. Previous studies on fluctuating temperature regimes have reported an increased physiological stress leading to morphological, behavioral and biochemical adaptations. From the latter, the adaptive capacity and seasonal performance associated with optimal function of the oxidative phosphorylation system (OXPHOS) are key for species persistence. However, studies on this matter are scarce. This study explores the seasonal changes and thermal sensitivity of the OXPHOS system in liver mitochondria of the bluegill sunfish species *Lepomis macrochirus,* inhabiting a shallow riverine system. Our study on liver mitochondria from *L. macrochirus* show significantly higher uncoupled proton conductance (LEAK) and cytochrome c oxidase (COX) activity in individuals captured in the fall compared to specimens investigated in summer and spring seasons. Flux control ratios such as coupling control ratio (CCR) and respiratory control ratio (RCR) were significantly reduced in the fall compared to warm-acclimated individuals in the summer and spring. These findings suggest that mechanisms regulating COX activity are in place to fine-tune mitochondrial function, and consequentially increase fitness in ectotherms inhabiting shallow, aquatic habitats with highly fluctuating temperatures.

Keywords: *Lepomis macrochirus*, Physiology, Mitochondria, Bioenergetics, COX, ETS, OXPHOS, Temperature, Centrarchidae

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LIST OF FIGURES AND TABLES

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Environmental temperature and ectotherm physiology

Environmental temperature is considered, by far, one of the most pervasive drivers of ectotherm physiology. In aquatic environments, environmental temperature exhibit widely disparate regimes; relatively constant warm temperatures are found throughout tropical regions, while constant frigid environments are found through Antarctic marine ecosystems. Constancy in environmental temperature pose a reduced need to adapt to changing thermal regimes, to a point where adaptive strategies to alterations in the environmental temperatures have been lost through evolutionary time (Sidell and O'Brien, 2006).

Aquatic environments with highly fluctuating thermal regimes are ubiquitously found through subtropical and temperate regions, where fluctuations in temperature of over 30°C during the course of a 24-h period can be observed in the most extreme environments (e.g. shallow flats, intertidal zones). In these systems, organisms must be able to cope with the environmental insult by seeking thermal refuges where available, often compromising feeding or reproduction time to escape physiologically challenging environments. Furthermore, the frequency and magnitude of these challenging thermal regimes are on the rise, and the consequences of these new thermal regimes remain obscure to many keystone species (Somero et. al., 2017).

Environmental warming has profound effects on the physiology and ecology of aquatic ectotherms, largely due to the influence on the catalytic function of key enzymes and the overall viscosity of biological membranes. Gradually increasing thermal regimes, whether by nature or by anthropogenic causes, leads to aquatic ectotherms to either remaining in local habitats, coping with the increasing temperatures, or to displace population centers to more thermally comfortable habitats (Rosset and Oertli 2011). The latter is rather unlikely for fishes inhabiting inland ecosystems, as these locally-confined populations have reduced thermal refuges available, thus

in situ physiological responses to rising temperature are obligate (Clark, Jeffries et al. 2011, Clark, Sandblom et al. 2013).

Several adaptive strategies are often associated with thermal acclimatization: morphological, behavioral and physiological alterations to better suit the changing environment (Angilletta Jr and Angilletta 2009, Sandblom, Gräns et al. 2014). Cellular and biochemical adjustments, particularly regulation of mitochondrial function in response to changes in the energy balance and the structure to biological membranes, collectively contribute to decreased basal and maximum metabolic rates in acclimated fishes (Pörtner 2001, Pörtner 2002, Nyboer and Chapman 2017). Acclimated specimens display reduced basal energy expenditures, and consequentially a *surplus* of cellular energy used in biosynthetic pathways associated with somatic and reproductive growth (Sandblom, Gräns et al. 2014).

Several studies have investigated the temperature responses on the catalytic properties and regulation of enzymes in aquatic ectotherms (Long, Li et al. 2012, Zhang, Loughery et al. 2017). This impact varies according to the magnitude and the duration of exposure to thermal stress. Studies involving freshwater fish species have shown a partial temperature compensation of ATP-generating capacity, achieved through changes on maximal citrate synthase and lactate dehydrogenase activities (McClelland, Craig et al. 2006, Jesus, Rosa et al. 2018). Another known response is the use of heat-shock chaperones to mitigate stressful conditions (Wegele, Muller et al. 2004). Heat shock proteins stabilize and refold denatured proteins in response to increasing temperature (Yamashita, Hirayoshi et al. 2004, Fangue, Hofmeister et al. 2006, Dong, Miller et al. 2008, Tomanek 2010). Tissue-specific responses were documented by Somero and colleagues, who found a compensatory response in succinate dehydrogenase activity of the cold-adapted *Trematomus bernachii*, with concomitant effects in brain oxygen consumption rate (Somero, Giese et al. 1968). Many enzymes are associated with biological membranes through hydrophobic interactions (as transmembrane or imbedded proteins), and the viscous properties

of these membranes are key for optimal functioning of the associated catalysts which requires homeoviscous adaptations to temperature changes in ectotherms (Pörtner 2002, Pörtner 2010).

The fluidity of biological lipid membranes in ectotherms are largely dependent on environmental temperature (Farkas, Fodor et al. 2001, Seebacher, Murray et al. 2009). A series of biochemical mechanisms collectively referred as homeoviscous adaptation (HVA) provides a counteracting strategy to preserve constancy in membrane fluidity, and biological processes that depends on it (Seebacher, Murray et al. 2009, Ernst, Ejsing et al. 2016). Studies show an increased proportion of unsaturated fatty acids in membranes of freshwater fish, which is reported as an evolutionary adaptive mechanism of maintaining optimal membrane fluidity (Skalli, Robin et al. 2006, Snyder, Schregel et al. 2012). Marine ectotherms display negligible change in membrane composition over seasons which explains their vulnerability to changing climate and their limitations in adapting to varying temperature (Somero 2010, Aronson, Thatje et al. 2011). HVA has protective roles under thermal stress conditions and must occur at multiple timescales – both seasonally and diurnally – to ensure optimal physiological function. HVA, therefore, is essential to the survival of fish in changing conditions, such as increased or decreased environmental temperature. As habitat temperature change, HVA provides a mechanism to sustain the fluidity of vital membranes involved in ATP production, such as the inner membrane of the mitochondrion. As it might be expected, the integrity of the inner membrane and the functionality of key proteins associated with the Electron Transport System (ETS) are found not to only to be tissue-specific, but also species-specific (Chung, Bryant et al. 2017).

The remarkable variability in thermal sensitivities of mitochondrial ATP production have been reported at multiple scales. Interspecific variability of the thermal performance of liver mitochondria have been reported for multiple tropical, subtropical and polar fishes. Sub-species variability in the thermal tolerance have been reported for the killifish *Fundulus heteroclitus* (Chung, Bryant et al. 2017). In tissue-specific mitochondrial function studies performed on northern and southern *F. heteroclitus*, Chung and colleagues (2017) demonstrated a significant

decline in brain citrate synthase activity at 5 and 33°C compared to 15°C controls and suggestively, no decline in cardiac citrate synthase activity following acclimation to temperatures extremes. The high diversity of thermal sensitivity among bony fishes raises a question regarding the linkages between mitochondrial function and sub-lethal effects on energy-dependent processes in response to the habitat temperature and seasonal variance.

Surprisingly, most studies on fish mitochondria have focused on fishes inhabiting saltwater ecosystems, with only a handful of studies on rainbow trout and carp species (Guderley 2004, Kraffe, Marty et al. 2007) and one study on the bluegill sunfish *Lepomis macrochirus* (Du et al, 2018). Moreover, these studies do not contemplate thermal sensitivity of the ETS, nor the seasonality, as a factor influencing mitochondrial performance.

Belonging to the centrachidae family, bluegill sunfish – *L. macrochirus* – is a naturally abundant freshwater fish species in lakes and rivers of North America. These animals are generalists that feed on zooplankton in the open water (Robinson, Wilson et al. 1993). They are usually the most dominant population in heated lakes and powerplant cooling reservoirs. This is more likely due to the well-documented ability to thrive in elevated temperatures as established by Holland et al.'s findings that *L. macrochirus* have a thermally robust physiology and are capable of adjusting to extreme temperatures, ranging from 25 – 35°C (Holland, Smith et al. 1974). In shallow riverine habitats of the Midwestern US, *L. macrochirus* are exposed to natural occurring extreme seasonal temperature fluctuations. Biochemical adaptations associated with acclimatization have a direct impact on the energy balance of the organism. More specifically, energy expenditures associate with biochemical up or down regulation pose as an energy tradeoff in order to endure the new thermal regime. Thermal acclimation capacity studies on this species report a critical thermal maximum (CTM) as high as 42.8°C (Holland, Smith et al. 1974, Pierce and Wissing 1974, Martinez, Porreca et al. 2016), demonstrating its thermal resilience and ideal choice for this field-laboratory studies.

The objective of this study was to assess the seasonal changes in the overall function of the ETS, and evaluate the thermal sensitivity of the OXPHOS system in bluegill sunfish using high-resolution respirometry (HRR). We focused on the thermal sensitivity of ETS complexes, aiming to describe the contributions of NADH-UQ oxidoreductase (Complex I) and succinate dehydrogenase (Complex II) to the OXPHOS system. We hypothesized that the coupling efficiency between the phosphorylation system and the ETS will be optimal at the corresponding habitat temperature for the season. Supraoptimal (i.e. warmer than optimal) temperatures will reduce energy transduction efficiency, reflected by a lower coupling of O_2 consumption to ATP production. In addition, mitochondrial coupling efficiency was also assessed by linking oxygen consumption due to proton conductance over the maximal ETS capacity (E). We calculated respiratory control ratios (RCR) at each assay temperature as LEAK control ratio (L/E) and coupling control ratio (OXPHOS/E). We also compared the individual contributions of complexes I and II to E, as well as the electron transfer capacity of cytochrome c oxidase (COX) as a function of assay temperature. All the indices above will serve as indicators of the efficiency of both ETS and OXPHOS, along with the thermal sensitivity and maximum electron carrying capacity of the ETS under various thermal regimes.

CHAPTER TWO: MATERIALS AND METHODS

Collection of specimens

Experiments were conducted following IACUC approved animal care protocol #18-001. Adult bluegill sunfish (*L. macrochirus*) were collected from Sangamon River, IL in May, July and November of 2019. Specimens were transferred in aerated 50L coolers with river water and brought to the laboratory, where they were kept in 40-liter aquaria at a density lower than 1 specimen per 10 liters. Specimens were assayed within 72h of capture, and held at collection temperature until assayed.

Isolation of liver mitochondria

Fresh livers were excised, weighed to the nearest 0.1 mg, transferred into a separate ice-cold petri dish containing ice-cold Isolation Medium B1 (IMB1: 250 mM sucrose, 0.5 mM Na_2EDTA, 10 mM Tris, 1 g/L bovine serum albumin (BSA); (Eigentler, Fontana-Ayoub et al. 2012) and processed according to Martinez et. al, 2013 with minor modifications. Liver tissue (1.0 g approx.) were separately minced in ice-cold petri dish and then homogenized in a 4:1 volume of ice-cold IMB1 using an ice-cold 7 mL Dounce homogenizer (Kontes, Vineland, NJ). Homogenization involved 5 passes with an ice-cold loose-fitting pestle A, followed by 2 passes with an ice-cold tighter-fitting pestle B.

The resulting homogenate was transferred into 1.5 mL centrifuge tubes and first centrifuged at 650 g for 10 min at 4°C and supernatant was transferred to an ice-cold, 1.5 mL centrifuge tube. For two consecutive times, pellets were resuspended and washed with 1 mL of isolation medium then recollected by centrifugation at 9,600 g for 15 min at 4 °C. The final washed pellet, containing the purified isolated mitochondria, was resuspended in 200 µL of isolation medium and stored on ice until respiration assays and protein content analysis.

Substrate uncoupler inhibitor titration (SUIT) protocol

To assess the thermal tolerance of *L. macrochirus* liver mitochondria, oxygen flux was monitored employing a SUIT protocol and a high-resolution respirometry system (O2k Analyzer; Oroboros Instruments, Innsbruck, Austria). Polarographic O_2 electrodes were calibrated for each assay temperature (T_{assay} = 10, 20, 28 and 30°C) across a range of O_2 tensions (350 nmol•mL^{-1} to O_2 depleted), in the presence of 2 mL of MiRO5 respiration medium (0.5 mM EGTA, 3 Mm $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM Sucrose, 1 g/L BSA; pH 7.1 at 20°C), to account for temperature effects and background O_2 consumption by the probes. In addition, zero calibration of the probes at each T_{assay} was achieved by injecting 20 µL to each chamber, using a freshly prepared 40 mg ml^{-1} solution of sodium dithionite. The background flux was recorded prior to mitochondrial injection and subtracted in each run. Oxygen tension was maintained between 350 and 200 nmol•mL^{-1} by gently opening each respirometric chamber (2 in total) to gradually fill the gas phase above the respiration medium if needed. Respiration rates were normalized to mitochondrial protein content.

For each assay temperature run, 3 – 30 mg/mL of purified mitochondria was injected into each chamber containing 2 mL of MiRO5. To assess the temperature sensitivity of the OXPHOS system the following substrates were added and respiratory rates were assessed.

Respiration in the absence of ADP (LEAK) under complex I activation (LEAK-I) was achieved through sequential addition of 5 mM pyruvate (P), 2 mM malate (M), and 10 mM glutamate (G) to the chamber containing isolated mitochondria. After LEAK-I respiration rates stabilized, ADP (D) was added to the chamber for complex I-linked respiration in the presence of ADP (OXPHOS-I), to a final concentration of 10 mM in each chamber. Respiration under complexes I and II in the presence of ADP (OXPHOS-I-II) was measured after the addition of 10 mM succinate (S). Respiration fueled through Complex I and II without ATPase activity (LEAK-I-II) was estimated by adding 5 µM carboxyatractyloside (Cat). Cat was used as an inhibitor in place of oligomycin due

to the latter's suppressive effects on the ETS activity in similar experiments (Baris, Crawford et al. 2015, Chung, Bryant et al. 2017). Maximal electron transfer capacity (ETS-I, II, contributed by complexes I and II) was estimated through repeated stepwise addition of 0.5 µM carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP, 1µL) until maximal uncoupling of mitochondria was observed. Selective contributions to the ETS by each complex was obtained by sequentially inhibiting complexes I, II and III through the addition of rotenone, malonate and antimycin A to a final concentration of 0.1 µM, 5mM and 2.5 µM, respectively. Apparent cytochrome c-oxidase (ETS complex IV, COX) capacity was measured by the addition of 10 µL of N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) and ascorbate, for a final concentration of 0.5 m*M* and 10m*M*, respectively.

Respiratory control ratios

We estimated the limitation of OXPHOS capacity by the phosphorylation system as the coupling control ratio (CCR); that is the ratio of OXPHOS capacity (P; coupled) to ETS capacity (E; uncoupled). Leak control ratio (L/E) serves as an index of proton conductance (LEAK) relative to the total electron transfer capacity of the ETS. We determined the overall impact of temperature by calculating the respiratory control ratio (RCR) as the quotient of O_2 flux while ADP is being phosphorylated (OXPHOS) over the flux associated with proton conductance (LEAK) in the presence of the ATPase inhibitor carboxyactractyloside (Gnaiger 2011).

Each substrate, uncoupler, or inhibitor was added to the chamber and oxygen flux was monitored for 5 min or until the rate had stabilized. The chamber and cannula of stopper were rinsed with distilled water three times, the chamber was filled with 70% ethanol for three times (5 minutes each) and then filled with absolute ethanol for 15 – 20 minutes. This was followed by rinsing the chamber and cannula with distilled water three times. This cleaning protocol was done before and after using the chambers.

Protein content analysis

Mitochondrial total protein content was quantified using a Bradford Coomassie Stain Assay (Thermo Scientific, Rockford, IL). Samples were diluted hundred-fold and their absorption values were measured using a Thermo Spectronic Genesys 20 spectrophotometer at λ = 595 nm following 10 minutes of incubation at room temperature. Resulting protein content concentration was corrected by subtracting BSA concentration in the isolation medium.

Statistical analyses

Liver mitochondrial function was analyzed by looking at the responses of mitochondrial parameters with assay temperatures as factor. LEAK, OXPHOS and ETS complex-specific response to the different temperature assays effects were assessed using a two-way ANOVA, with pairwise comparison where applicable (Holm-Sidak Method).

All data are presented as mean $\pm$ SEM. Sample size (*n*) is indicated in relevant figure captions and table headings. Statistica analysis were carried using Sigmaplot (Version 12), with $\alpha = 0.05$.

CHAPTER THREE: RESULTS

Habitat water temperature

The average aquatic habitat temperature for the Sangamon River (Decatur, IL) increased by 10.14°C and 9.65°C from the late winter to spring and from spring to summer, respectively. A significant reduction of 21.16°C was reported from summer to fall (Figure 1).

LEAK in presence of complex I and II substrates

Within the fall season, LEAK contributed by both complexes I and II showed significant differences between rates measured at 30° and 10°C, 30° and 20°C and 30° and 28°C assay temperature (Figure 2; $p<0.001$, $p<0.001$ and $p=0.007$, respectively). At an assay temperature of 30°C there were significant differences across seasons (Figure 2, $p<0.001$), with fall collected specimens showing the highest LEAK (247.6 pmol s^{-1} mg $protein^{-1}$ $\pm$ 72.4).

LEAK in the presence of only complex I substrates

Within the fall season, there was significant difference between across various assay temperatures with the highest LEAK being recorded at 30°C (Figure 3; $p<0.001$). Fall and summer seasons showed a significant difference between oxygen (O_2) flux within the 28°C assay temperature (Figure 3, $p=0.023$). Again, a significant difference in O_2 fluxes was found at 30°C across seasons, with complex-I LEAK contribution in the summer being only 18% of that found in fall (Figure 3, $p<0.001$).

COX activity

For the summer, COX activity at 28°C (432.4 pmol s^{-1} mg $protein^{-1}$ $\pm$ 41.0) was significantly higher than rates at 10°C (165.3 pmol s^{-1} mg $protein^{-1}$ $\pm$ 15.1) (Figure 4, $p=0.015$), as COX activity at 30°C (445.0 pmol s^{-1} mg $protein^{-1}$ $\pm$ 67.0) was also found to significantly higher than rates ats10°C (165.3 pmol s^{-1} mg $protein^{-1}$ $\pm$ 15.1) (Figure 4, $p=0.016$). There were significant differences found between various T_{assay} pairs within the fall season except between 30°C and 28°C assay

temperature (Figure 4, $p<0.001$). At 20°C assay temperature, COX activity in the fall was significantly higher (688.6 pmol s^{-1} mg $protein^{-1}$ ± 48.6) than in summer (330 pmol s^{-1} mg $protein^{-1}$ ± 23.4), and also significantly higher than the spring (367.4 pmol s^{-1} mg $protein^{-1}$ ± 85.9) ($p<0.001$). Significant differences were found at 28°C assay temperature with fall season (1092.3 pmol s^{-1} mg $protein^{-1}$ ± 51.1) being the highest ($p<0.001$). At 30°C assay temperature, there were significant differences across seasons, with fall being the highest (1246.0 pmol s^{-1} mg $protein^{-1}$ ± 57.3) and spring being the lowest measured rate (264.6 pmol s^{-1} mg $protein^{-1}$ ± 97.7) (Figure 4).

OXPHOS contributed by both complex I and II

At an assay temperature of 30°C, there were significant differences across seasons; rates collected for specimens captured in spring and summer were only 41% and 45% of rates measured for individuals captured in fall (Figure 5, $p=0.06$ and $p=0.014$, respectively). Also, within the fall season, OXPHOS activity by complex I and II was found to have significant differences across assay temperatures with the highest rate (394.0 pmol s^{-1} mg $protein^{-1}$ ± 55.9) measured at 30°C.

OXPHOS contributed by complex I

Within the fall season, there were significant difference in across 30°C assay temperature (Figure 6, $p<0.001$). Again, a significant difference in O_2 fluxes was found at 30°C across seasons (Figure 6, $p<0.001$), with complex-I OXPHOS contribution in the fall (473.6 pmol s^{-1} mg $protein^{-1}$ ± 107.4) being significantly higher than both, the summer (82.6 ± 26.6 pmol s^{-1} mg $protein^{-1}$) and spring (46.5 pmol s^{-1} mg $protein^{-1}$ ± 6.2) seasons.

ETS activity fueled by complex I and II

At 30°C assay temperature, ETS activity (contributed by both complex I and II) in the spring and summer were only 22% and 27.8% of that of fall (766.4 pmol s^{-1} mg $protein^{-1}$ ± 65.2), respectively.

Within the fall season, significant differences were found between assay temperatures with 30°C being the highest (766.4 pmol s^{-1} mg $protein^{-1}$ ± 65.2) (Figure 7).

ETS activity involving complex II

Within the fall season, significant differences were found in oxygen flux among assay temperatures with 30°C stimulating the highest rate measured (569.0 pmol s^{-1} mg $protein^{-1}$ ± 75.5). There was also a significant difference between seasons at an assay temperature of 30°C (Figure 8, $p<0.001$).

Flux Control Ratios

The calculated coupling control ratios (P/E) within the spring season showed significant differences between assay temperatures of 30° and 10°C, 28° and 10°C and 20° and 10°C (Figure 9, $p<0.001$). Within the summer, significant differences were found between in P/E ratios at 28° and 10°C ($p=0.003$) and 30° and 10°C ($p=0.005$). There was significant difference in this parameter for individual captured in spring vs. fall, summer vs. fall as well as between spring and summer at an assay temperature of 20°C (Figure 9, $p<0.001$, $p=0.015$ and $p=0.017$, respectively). At 28°C there was a significant difference in ratios obtained for spring and fall, as well as summer and fall (Figure 9, $p<0.001$). At 30°C assay temperature, there was significant difference between spring and fall, as well as summer and fall ratios (Figure 9, $p<0.001$ and $p=0.001$, respectively).

For the LEAK control ratio (L/E), there were no significant differences among various T_{assay} as well as among seasons. There was also no effect of interaction between T_{assay} and season on LEAK control ratio (Figure 10)

For L/P, there were no significant differences found except between the fall and summer season captured individuals (Figure 11, $p=0.012$).

With RCR, there was significant difference between spring and fall samples (Figure 12, $p=0.024$) as well as between summer and fall samples (Figure 12, $p=0.045$) within 20°C assay

temperature. Additionally, significant difference was found in RCR between the summer and fall captured individual if measured an assay temperature of 28˚C (Figure 12, p=0.047).

CHAPTER FOUR: DISCUSSION

This study represents a comprehensive examination of the effects of environmental and habitat temperature acclimation and adaptation on the mitochondrial electron transport system. With the activation of both complex I (CI, NADH dehydrogenase) and complex II (CII, Succinate dehydrogenase), our goal was to describe the thermal sensitivity of the ETS and phosphorylation systems, and assess whether compensatory mechanisms are in place over seasons. The data presented in this study suggest compensatory mechanisms occurring to the ETS complexes are potentially contributing to the physiological plasticity in *L. macrochirus* over seasonal temperature variations.

Oxygen flux associated with LEAK in presence of NADH- and $FADH_2$-generating substrates was found to be thermosensitive across seasons. From our study, we found a higher LEAK in fall samples (Figure 2). The observed thermal sensitivity of LEAK in our study can be at least partly be explained by changes in the membrane fluidity with changes in environmental temperature. As seen in previous studies, acclimation temperatures (both supraoptimal and suboptimal) cause modification of fatty acid composition and saturation of phospholipid membrane leading to concomitant alteration in proton permeability (Brookes, Buckingham et al. 1998, Harper, Dickinson et al. 2001, Bryant, Chung et al. 2018). Also, studies involving warm-acclimated trout (*Oncorhynchus mykiss*) reported restructured phospholipid membrane classes (Kraffe, Marty et al. 2007) and the loss of membrane integrity under supraoptimal habitat temperature (Hazel 1995, Hochachka, Somero et al. 2002). These membrane changes, leading to reduced performance (as a result of incomplete compensation through HVA), contribute to increase in proton conductance (LEAK) across supraoptimal temperatures. A similar increased proton conductance emerged if we compare the LEAK thermal sensitivity of *L. macrochirus* with the Nothothenioid *N. coriiceps,* who have evolved in the constantly frigid waters of Antarctica (Mark, Lucassen et al. 2012). In addition, our results are in agreement with other studies evaluating mitochondrial

energetics in cold acclimated fishes, which suggest that not only HVA to cold habitat temperature increase membrane *leakiness* if exposed to warmer temperatures, but also the upregulation of ETS complexes lead to increase of the electron transfer capacity of the ETS (Bryant, Chung et al. 2018). Moreover, when we integrate the measured activity of cytochrome *c* oxidase (complex IV; COX) across seasons, we found further supporting evidence suggesting that COX regulation is a key modulator of the ETS under *in vitro* conditions.

COX is an important transmembrane, multi-subunit enzyme of the mitochondrial ETS. It catalyzes the final step of electron transfer from cytochrome *c* to oxygen and hydrogen protons to form water. In addition, it also acts as a proton pump and contributes to the protonmotive force across the inner membrane of the mitochondrion (Nicholls and Ferguson, 2002). Although COX function is linked with oxygen availability its kinetics are largely insensitive to changes in either [ADP] or [O_2] available (Gnaiger et. al, 1998). In turn, COX activity is modulated by other compounds including nitric oxide (NO), which alters the affinity of COX to O_2 (Sarti, et. al, 2012). Therefore, both intrinsic modulators like NO and external factors such as temperature can exert changes in the catalytic properties of COX and consequentially, the overall function of the ETS as a rate liming step (Pannala, et. al, 2016).

COX activity in this study was higher at supraoptimal assay temperature for the cold-acclimated individuals in the fall season (Figure 4). This significant increase in COX activity can be attributed to increase in overall expression of the enzyme, driven by lower habitat temperature during acclimatization in the fall (Lucassen, Koschnick et al. 2006, LeMoine, Genge et al. 2008). Due to the presence of radical scavengers in the respiration media, it is unlikely that the differences observed in COX activity were due to the presence of NO during the trials. Modulation of COX activity at low temperatures therefore represents an adaptive strategy to sustain higher electron transfer capacity at low habitat temperatures, and potentially contributes energy to biosynthetic pathways and energy storage in the liver, which was indicated by increased

hepatosomatic indices during the cold months as compared to specimens collected in summer and spring.

Oxygen flux in the presence of ADP was also thermosensitive, increasing with assay temperature for each season (Figure 5). Comparing OXPHOS within season, across assay temperatures, the rates can be explained largely by the thermodynamic response of enzymes to the temperature regime, where a respiratory quotient of close to 2 is observed in each season. However, at assay temperatures between 20-38°C, the rate of increase for both spring and summer were negligible. These are striking results, as during both spring and summer seasons higher ATP demands due to increased metabolism are expected (Johnston 2003, Mark, Hirse et al. 2005). During the fall, individuals can likely utilize ATP to increase swimming performance and other ecologically important processes depending upon active metabolism, as their basal energetic demands are at minimum (Johnston and Dunn 1987, Killen 2014).

Evaluating the level of uncoupling of *L. macrochirus* mitochondrial across assay temperatures and season, we found no significant differences among groups (Figure 10). This can be due to adjustments in the ETS complexes in response to changes in the metabolic demands, as seen in a number of ectotherms (Brookes, Buckingham et al. 1998, Hardewig, Pörtner et al. 1999, Bishop and Brand 2000, Boutilier and St-Pierre 2002, Brown, Gerson et al. 2007). Previous studies report a significant contribution of mitochondrial proton leakage (about 20%) in ectothermic vertebrates (Brand, Couture et al. 1991, Bishop and Brand 2000). For example, in the toad *B. marinus*, Trzcionka and colleagues reported that adjustments in proton leak is achieved not by a reduction in membrane permeability but by a decrease in ETS activity (Trzcionka, Withers et al. 2008). Previous studies also report that increased proton leakage due to increasing temperature reduces ATP production per total oxygen consumed (Brand 2000, Brand and Esteves 2005), thus regulatory mechanisms are key for optimal ETS function. While the capacity for thermal adjustments on proton leak is likely tissue- and species-specific (Bilyk and DeVries 2011, Enzor,

Zippay et al. 2013, Strobel, Graeve et al. 2013), further work on the underlying mechanisms supporting metabolic shifts and the trade-offs at whole organism level will be highly valuable.

Generally, the electron transfer capacity of the ETS for spring and summer seasons were similar at each assay temperature (Figure 8). Moreover, the thermal sensitivity of the ETS capacity across assay temperatures displayed a Q_{10} of less than 2, which suggest that a limiting step, likely COX activity, is restricting the electron transfer capabilities of the system at the warmer assay temperatures. Gnaiger and colleagues (1998) describe the concepts underlying the excess electron transfer capacity of COX in mammalian mitochondria, and over two decades later the thermal sensitivity of this excess capacity remains largely unexplored in ectotherms. Furthermore, available studies on fish liver COX activity show either thermal insensitivity (Chung, Bryant et al. 2017) or inversely proportional effects of temperature to brain COX activity (i.e. low activity at warmer acclimation temperature) in the same species (Chung, Sparagna et al. 2018). It is noteworthy that the aforementioned studies were carried under acclimation regimes to a fixed laboratory regime where seasonal temperature, diet and photoperiod were not included in the experimental design. Further work on this area will yield insights with regards of the advantages of increased COX activity and potential advantages of increasing COX activity in highly fluctuating thermal environments.

Interestingly, coupling control ratios (CCR) obtained for the summer and spring seasons were found to be close to maximum ETS capacity (Figure 9). For fall samples, CCR were found to be only around 40% of the total ETS capacity. One plausible explanation for these differences might be attributed to the increase in COX activity previously discussed, as this change leads to increase ETS capacity and thus a reduction in CCR. As a result, our results suggest that individuals in the fall season are likely to have better response to ATP phosphorylation and less limited by ETS compared to individuals in the summer and spring.

RCR was used as an index to complement our evaluation of the coupling efficiency of O_2 to ATP production (Figure 12). The seasonal differences in RCR values also corresponds to

adaptive strategies in place to enhance the electron transfer capacity of the ETS. Interestingly, thermally stable RCR values found in the fall across assay temperature are due to thermosensitive LEAK and OXPHOS rates. As these two respiratory states displayed a similar Q_{10} across assay temperatures, RCR's remained stable. Other studies also reported lowered RCR (Dos Santos, Galina et al. 2013, Chung and Schulte 2015, Chung, Sparagna et al. 2018), suggesting a link between low OXPHOS rates with acclimation temperature. This represents a partial compensation for the effects of suboptimal temperature on enzyme activity and the fluid state of the inner mitochondrial membrane (Guderley 1990, Chung and Schulte 2015).

This study evaluates the thermal sensitivity of liver mitochondria of *L. macrochirus* exposed to seasonal changes in a riverine system. Our results suggest that biochemical changes, particularly related to the activity of the gatepoint enzyme COX, are key to ensure optimal functioning of the ETS and OXPHOS. Although we measured mitochondrial performance under *in vitro* conditions, the patterns emerged suggest that *L. macrochirus* mitochondria are capable of improved performance, even at drastically fluctuating environments such as this shallow, riverine system. Further work on evaluating the significance of this adaptive strategy under laboratory controlled fluctuating environments are warranted and are part of ongoing efforts to elucidate how freshwater species cope with the increasing extreme temperature fluctuations associated with global climate change.

REFERENCES

Angilletta Jr, M. J. and M. J. Angilletta (2009). Thermal adaptation: a theoretical and empirical synthesis, Oxford University Press.

Aronson, R. B., S. Thatje, J. B. McClintock and K. A. Hughes (2011). "Anthropogenic impacts on marine ecosystems in Antarctica." Annals of the New York Academy of Sciences **1223**(1): 82-107.

Baris, T. Z., D. L. Crawford and M. F. Oleksiak (2015). "Acclimation and acute temperature effects on population differences in oxidative phosphorylation." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **310**(2): R185-R196.

Bilyk, K. T. and A. L. DeVries (2011). "Heat tolerance and its plasticity in Antarctic fishes." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **158**(4): 382-390.

Bishop, T. and M. Brand (2000). "Processes contributing to metabolic depression in hepatopancreas cells from the snail Helix aspersa." Journal of Experimental Biology **203**(23): 3603-3612.

Boutilier, R. G. and J. St-Pierre (2002). "Adaptive plasticity of skeletal muscle energetics in hibernating frogs: mitochondrial proton leak during metabolic depression." Journal of Experimental Biology **205**(15): 2287-2296.

Brand, M. (2000). "Uncoupling to survive? The role of mitochondrial inefficiency in ageing." Experimental gerontology **35**(6-7): 811-820.

Brand, M. D., P. Couture, P. L. Else, K. W. Withers and A. Hulbert (1991). "Evolution of energy metabolism. Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile." Biochemical Journal **275**(1): 81-86.

Brand, M. D. and T. C. Esteves (2005). "Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3." Cell metabolism **2**(2): 85-93.

Brookes, P. S., J. A. Buckingham, A. M. Tenreiro, A. Hulbert and M. D. Brand (1998). "The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **119**(2): 325-334.

Brown, J. C., A. R. Gerson and J. F. Staples (2007). "Mitochondrial metabolism during daily torpor in the dwarf Siberian hamster: role of active regulated changes and passive thermal effects." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **293**(5): R1833-R1845.

Bryant, H. J., D. J. Chung and P. M. Schulte (2018). "Subspecies differences in thermal acclimation of mitochondrial function and the role of uncoupling proteins in killifish." Journal of Experimental Biology **221**(24).

Chung, D. J., H. J. Bryant and P. M. Schulte (2017). "Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (Fundulus heteroclitus)." Journal of Experimental Biology: jeb. 151217.

Chung, D. J. and P. M. Schulte (2015). "Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (Fundulus heteroclitus)." Journal of Experimental Biology **218**(11): 1621-1631.

Chung, D. J., G. C. Sparagna, A. J. Chicco and P. M. Schulte (2018). "Patterns of mitochondrial membrane remodeling parallel functional adaptations to thermal stress." Journal of Experimental Biology **221**(7): jeb174458.

Clark, T. D., K. M. Jeffries, S. G. Hinch and A. P. Farrell (2011). "Exceptional aerobic scope and cardiovascular performance of pink salmon (Oncorhynchus gorbuscha) may underlie resilience in a warming climate." Journal of Experimental Biology **214**(18): 3074-3081.

Clark, T. D., E. Sandblom and F. Jutfelt (2013). "Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations." Journal of Experimental Biology **216**(15): 2771-2782.

Dong, Y., L. P. Miller, J. G. Sanders and G. N. Somero (2008). "Heat-shock protein 70 (Hsp70) expression in four limpets of the genus Lottia: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress." Biol Bull **215**(2): 173-181.

Dos Santos, R. S., A. Galina and W. S. Da-Silva (2013). "Cold acclimation increases mitochondrial oxidative capacity without inducing mitochondrial uncoupling in goldfish white skeletal muscle." Biology open **2**(1): 82-87.

Eigentler, A., M. Fontana-Ayoub, M. Fasching, B. Lassnig, S. Stadlmann, G. Rieger, B. Haffner, H. Lemieux and E. Gnaiger (2012). "Selected media and chemicals for respirometry with mitochondria and permeabilized cells." Mitochondrial Physiology Network **3**(9): 1-9.

Enzor, L. A., M. L. Zippay and S. P. Place (2013). "High latitude fish in a high CO2 world: synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **164**(1): 154-161.

Ernst, R., C. S. Ejsing and B. Antonny (2016). "Homeoviscous adaptation and the regulation of membrane lipids." Journal of molecular biology **428**(24): 4776-4791.

Fangue, N. A., M. Hofmeister and P. M. Schulte (2006). "Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus." J Exp Biol **209**(Pt 15): 2859-2872.

Farkas, T., E. Fodor, K. Kitajka and J. Halver (2001). "Response of fish membranes to environmental temperature." Aquaculture Research **32**(8): 645-655.

Gnaiger, E. (2011). "MitoPathways: Respiratory states and coupling control ratios." Mitochondr. Physiol. Netw **12**.

Guderley, H. (1990). "Functional significance of metabolic responses to thermal acclimation in fish muscle." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **259**(2): R245-R252.

Guderley, H. (2004). "Metabolic responses to low temperature in fish muscle." Biological reviews **79**(2): 409-427.

Hardewig, I., H. Pörtner and L. Peck (1999). "Thermal sensitivity of mitochondrial function in the Antarctic Notothenioid Lepidonotothen nudifrons." Journal of Comparative Physiology B **169**(8): 597-604.

Harper, J., K. Dickinson and M. Brand (2001). "Mitochondrial uncoupling as a target for drug development for the treatment of obesity." Obesity Reviews **2**(4): 255-265.

Hazel, J. R. (1995). "Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?" Annual review of physiology **57**(1): 19-42.

Hochachka, P., G. Somero, P. Hochachka and G. Somero (2002). Biochemical adaptation: Mechanism and process in physiological evolution. , Oxford University Press: New York.

Holland, W. E., M. H. Smith, J. W. Gibbons and D. H. Brown (1974). "Thermal tolerances of fish from a reservoir receiving heated effluent from a nuclear reactor." Physiological Zoology **47**(2): 110-118.

Jesus, T. F., I. C. Rosa, T. Repolho, A. R. Lopes, M. S. Pimentel, V. M. F. Almeida-Val, M. M. Coelho and R. Rosa (2018). "Different ecophysiological responses of freshwater fish to warming and acidification." Comp Biochem Physiol A Mol Integr Physiol **216**: 34-41.

Johnston, I. and J. Dunn (1987). Temperature acclimation and metabolism in ectotherms with particular reference to teleost fish. Symp Soc Exp Biol.

Johnston, I. A. (2003). "Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **136**(4): 701-713.

Killen, S. S. (2014). "Growth trajectory influences temperature preference in fish through an effect on metabolic rate." Journal of Animal Ecology **83**(6): 1513-1522.

Kraffe, E., Y. Marty and H. Guderley (2007). "Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout Oncorhynchus mykiss: roles of membrane proteins, phospholipids and their fatty acid compositions." Journal of Experimental Biology **210**(1): 149-165.

LeMoine, C. M., C. E. Genge and C. D. Moyes (2008). "Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature." Journal of Experimental Biology **211**(9): 1448-1455.

Long, Y., L. Li, Q. Li, X. He and Z. Cui (2012). "Transcriptomic characterization of temperature stress responses in larval zebrafish." PloS one **7**(5): e37209.

Lucassen, M., N. Koschnick, L. Eckerle and H.-O. Pörtner (2006). "Mitochondrial mechanisms of cold adaptation in cod (Gadus morhua L.) populations from different climatic zones." Journal of Experimental Biology **209**(13): 2462-2471.

Mark, F. C., T. Hirse and H.-O. Pörtner (2005). "Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes." Polar biology **28**(11): 805-814.

Mark, F. C., M. Lucassen, A. Strobel, E. Barrera-Oro, N. Koschnick, L. Zane, T. Patarnello, H. O. Pörtner and C. Papetti (2012). "Mitochondrial function in Antarctic nototheniids with ND6 translocation." PloS one **7**(2): e31860.

Martinez, E., A. P. Porreca, R. E. Colombo and M. A. Menze (2016). "Tradeoffs of warm adaptation in aquatic ectotherms: Live fast, die young?" Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **191**: 209-215.

McClelland, G. B., P. M. Craig, K. Dhekney and S. Dipardo (2006). "Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (Danio rerio)." J Physiol **577**(Pt 2): 739-751.

Nyboer, E. A. and L. J. Chapman (2017). "Elevated temperature and acclimation time affect metabolic performance in the heavily exploited Nile perch of Lake Victoria." Journal of Experimental Biology: jeb. 163022.

Pierce, R. J. and T. E. Wissing (1974). "Energy cost of food utilization in the bluegill (Lepomis macrochirus)." Transactions of the American Fisheries Society **103**(1): 38-45.

Pörtner, H.-O. (2002). "Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **132**(4): 739-761.

Pörtner, H.-O. (2010). "Oxygen-and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems." Journal of Experimental Biology **213**(6): 881-893.

Pörtner, H. O. (2001). "Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals." Naturwissenschaften **88**(4): 137-146.

Robinson, B. W., D. S. Wilson, A. S. Margosian and P. T. Lotito (1993). "Ecological and morphological differentiation of pumpkinseed sunfish in lakes without bluegill sunfish." Evolutionary Ecology **7**(5): 451-464.

Rosset, V. and B. Oertli (2011). "Freshwater biodiversity under climate warming pressure: identifying the winners and losers in temperate standing waterbodies." Biological Conservation **144**(9): 2311-2319.

Sandblom, E., A. Gräns, M. Axelsson and H. Seth (2014). "Temperature acclimation rate of aerobic scope and feeding metabolism in fishes: implications in a thermally extreme future." Proceedings of the Royal Society of London B: Biological Sciences **281**(1794): 20141490.

Seebacher, F., S. Murray and P. Else (2009). "Thermal acclimation and regulation of metabolism in a reptile (Crocodylus porosus): the importance of transcriptional mechanisms and membrane composition." Physiological and Biochemical Zoology **82**(6): 766-775.

Skalli, A., J. Robin, N. Le Bayon, H. Le Delliou and J. Person-Le Ruyet (2006). "Impact of essential fatty acid deficiency and temperature on tissues' fatty acid composition of European sea bass (Dicentrarchus labrax)." Aquaculture **255**(1-4): 223-232.

Snyder, R. J., W. D. Schregel and Y. Wei (2012). "Effects of thermal acclimation on tissue fatty acid composition of freshwater alewives (Alosa pseudoharengus)." Fish Physiology and Biochemistry **38**(2): 363-373.

Somero, G. (2010). "The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'." Journal of Experimental Biology **213**(6): 912-920.

Somero, G. N., A. C. Giese and D. E. Wohlschlag (1968). "Cold adaptation of the Antarctic fish Trematomus bernacchii." Comparative Biochemistry and Physiology **26**(1): 223-233.

Strobel, A., M. Graeve, H. O. Poertner and F. C. Mark (2013). "Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, Notothenia rossii and Lepidonotothen squamifrons." PloS one **8**(7).

Tomanek, L. (2010). "Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs." J Exp Biol **213**(6): 971-979.

Trzcionka, M., K. W. Withers, M. Klingenspor and M. Jastroch (2008). "The effects of fasting and cold exposure on metabolic rate and mitochondrial proton leak in liver and skeletal muscle of an amphibian, the cane toad Bufo marinus." Journal of Experimental Biology **211**(12): 1911-1918.

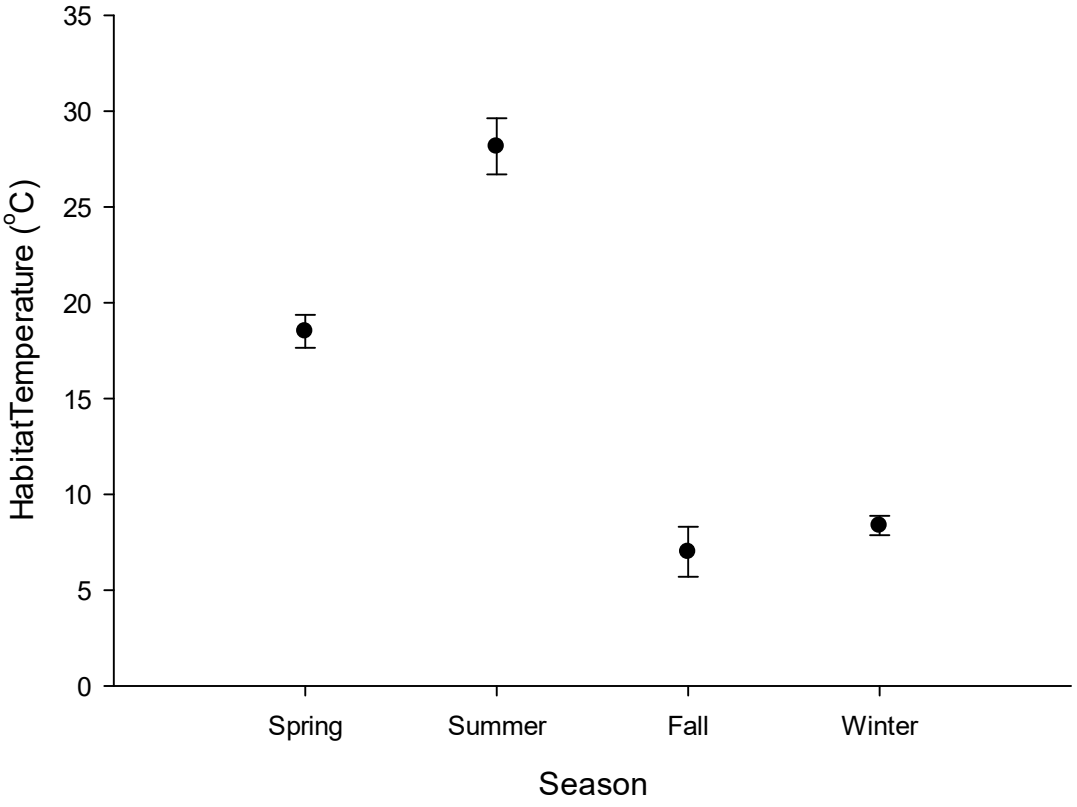
Valiela, I., J. Wright, J. M. Teal and S. Volkmann (1977). "Growth, production and energy transformations in the salt-marsh killifish Fundulus heteroclitus." Marine Biology **40**(2): 135-144.

Wegele, H., L. Muller and J. Buchner (2004). "Hsp70 and Hsp90--a relay team for protein folding." Rev Physiol Biochem Pharmacol **151**: 1-44.

Yamashita, M., K. Hirayoshi and K. Nagata (2004). "Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates." Gene **336**(2): 207-218.

Zhang, Y., J. R. Loughery, C. J. Martyniuk and J. D. Kieffer (2017). "Physiological and molecular responses of juvenile shortnose sturgeon (Acipenser brevirostrum) to thermal stress." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **203**: 314-321.

APPENDIX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30
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20
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HabitatTemperature (°C)
Spring
Summer
Fall
Winter
Season


Sangamon River
The Reserve At Lakeview Apartment Homes
First Mid Bank & Trust Decatur S. Franklin
Equipment Relocation Services
Storage Masters South Shores
Stop & Wash
South Shores Auto Service
Dairy Queen Grill & Chill
Monical's Pizza
Hickory Point Bank & Trust
The Wharf
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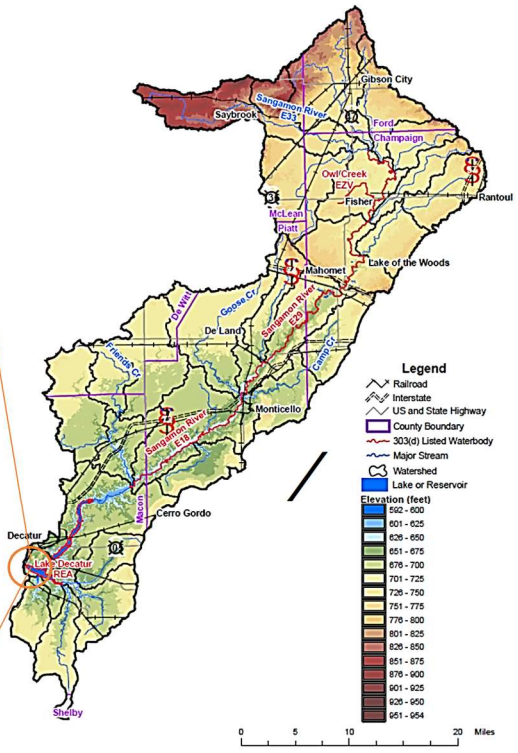
Gibson City
Saybrook
Ford
Champaign
Owl Creek
Fisher
Rantoul
McLean
Piatt
Mahomet
Lake of the Woods
De Land
Monticello
Cerro Gordo
Decatur
Lake Decatur
Shelby
Legend
Railroad
Interstate
US and State Highway
County Boundary
303(d) Listed Waterbody
Major Stream
Watershed
Lake or Reservoir
Elevation (feet)
592 - 600
601 - 625
626 - 650
651 - 675
676 - 700
701 - 725
726 - 750
751 - 775
776 - 800
801 - 825
826 - 850
851 - 875
876 - 900
901 - 925
926 - 950
951 - 954
0
5
10
20
Miles

Figure 1**.** Site Map and average daily habitat temperature at the collection site (Sangamon River; Decatur, IL) across seasons in 2019. Data points obtained as an average of 7 days per each season using HOBO Pendant temperature logger (Model #UA-001-08). Significant differences are shown (One-way ANOVA, Holm-Sidak test; mean ± SD).

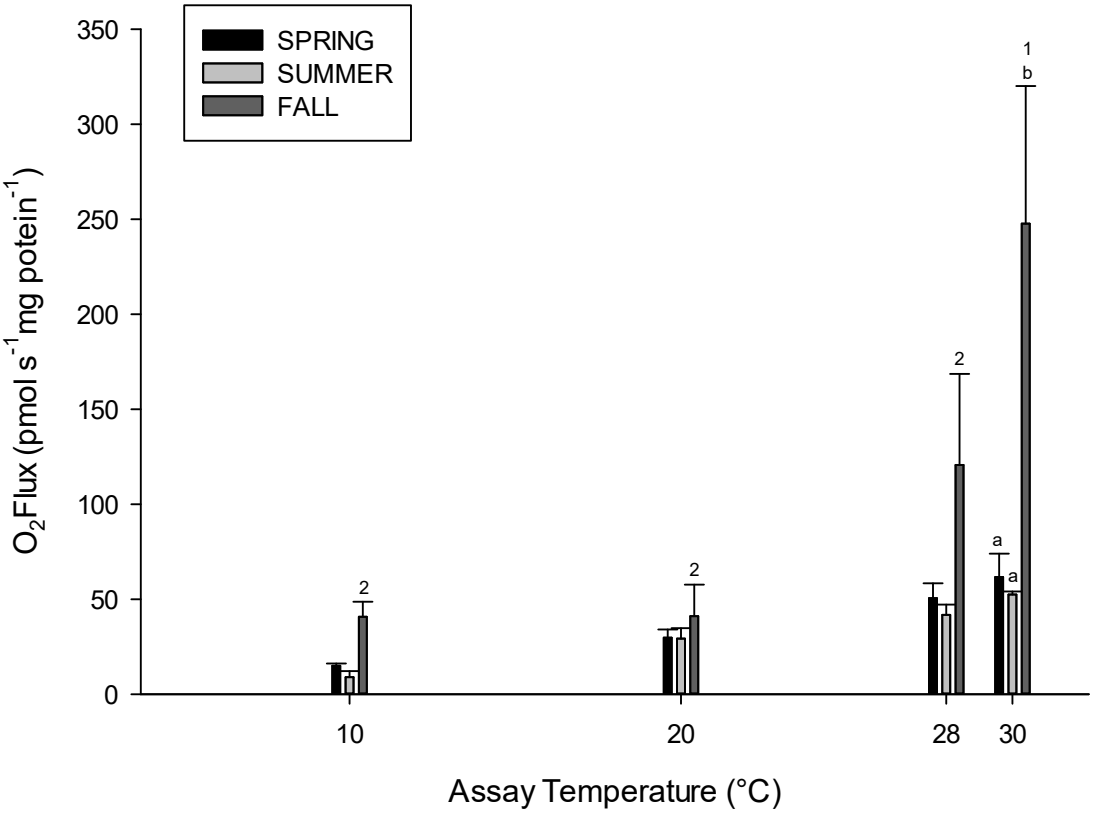

Figure 2. LEAK oxygen flux of *Lepomis macrochirus* liver mitochondria across seasons and assay temperature. Respiratory flux was determined in the presence of both complex I activating substrates (pyruvate, malate and glutamate) and complex II activating substrate (succinate). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

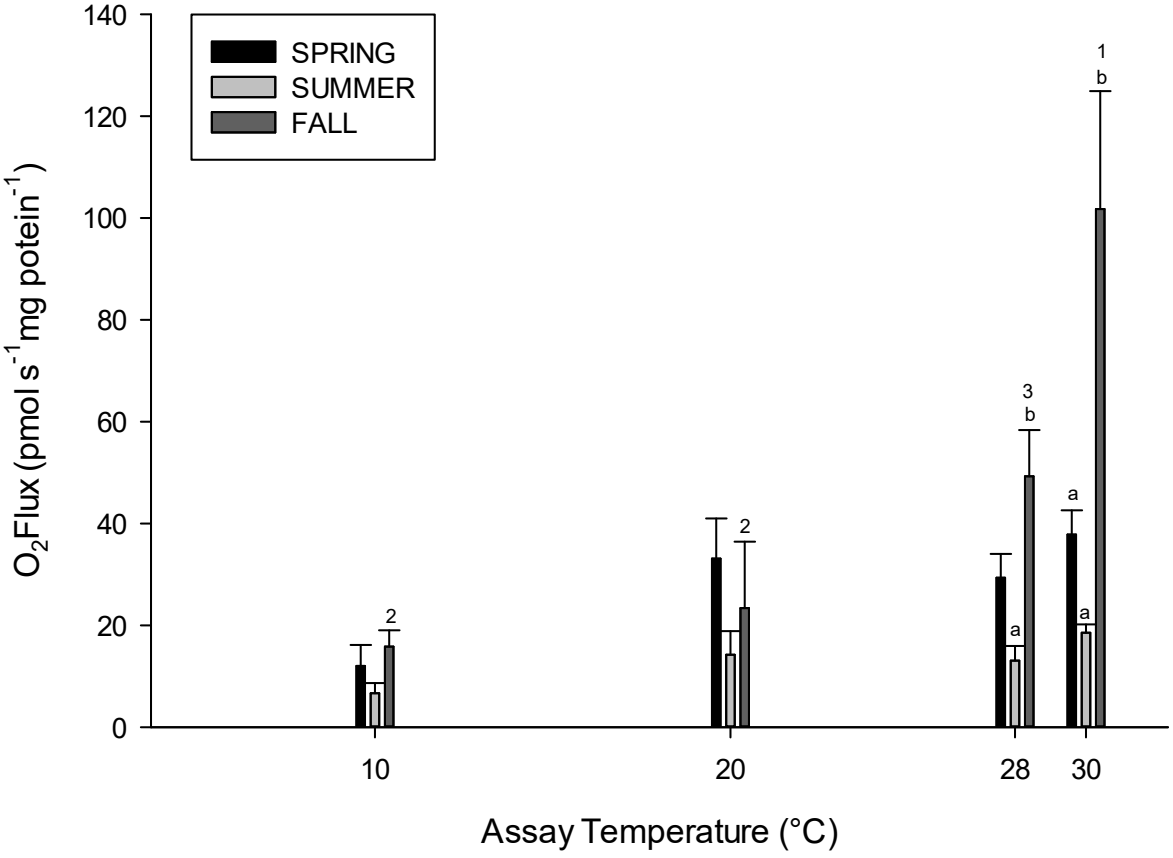

Figure 3. LEAK oxygen flux of *Lepomis macrochirus* liver mitochondria across seasons and assay temperature. Respiratory flux was determined in the presence of complex I activating substrates (pyruvate, malate and glutamate). Significant difference are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

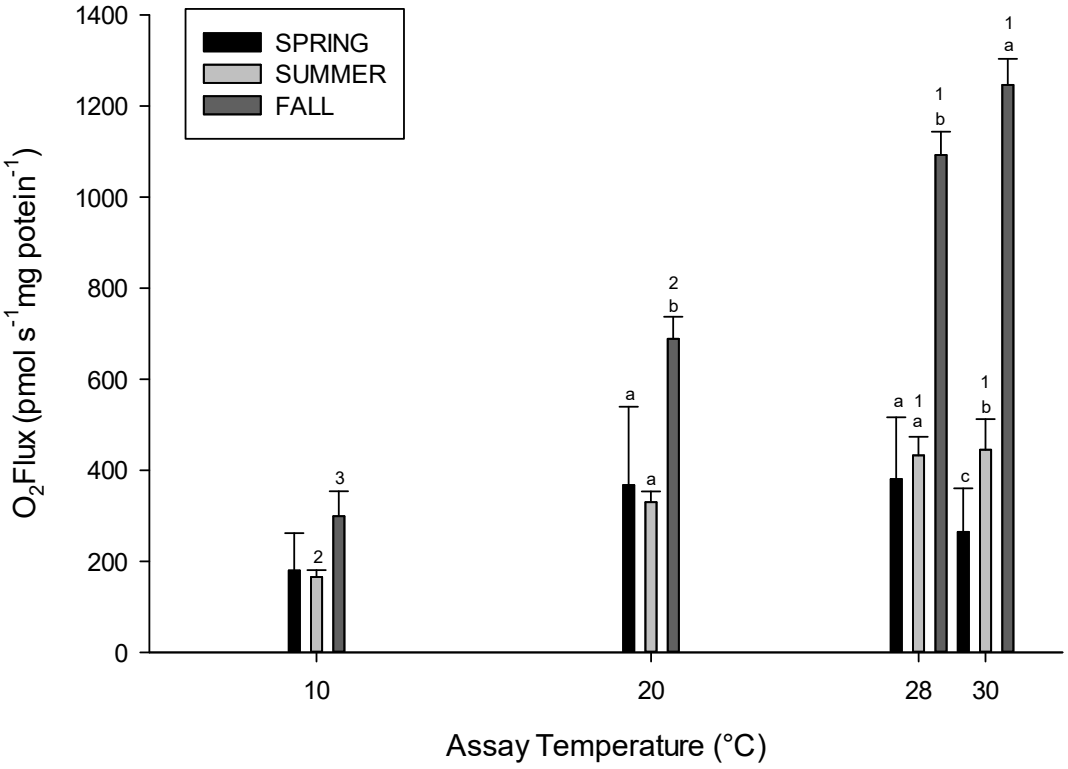

Figure 4. The oxygen flux cytochrome c oxidase (COX) in the presence of TMPD and ascorbate in *Lepomis macrochirus* liver mitochondria across seasons and assay temperature. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

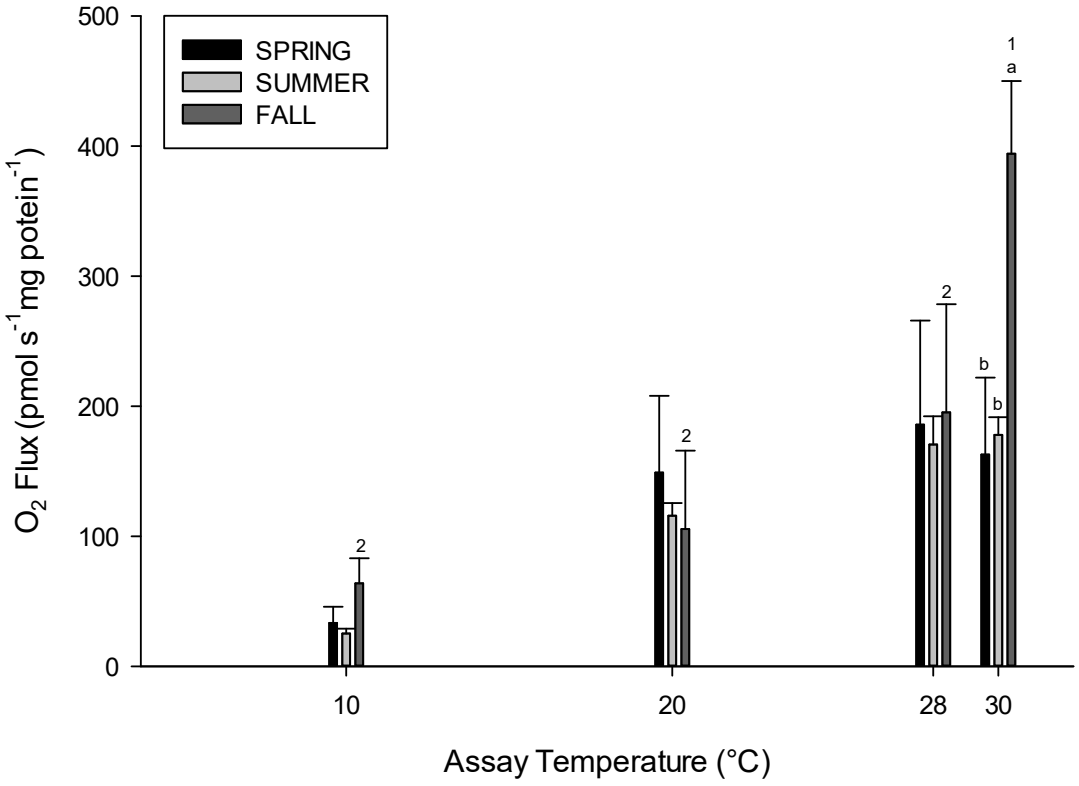

Figure 5. ADP-induced oxygen flux (OXPHOS) as a function of season and assay temperature for *Lepomis macrochirus* liver mitochondria. OXPHOS fluxes obtained in the presence of both complex I activating substrates (pyruvate, malate and glutamate) and Complex II activating substrate (succinate). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

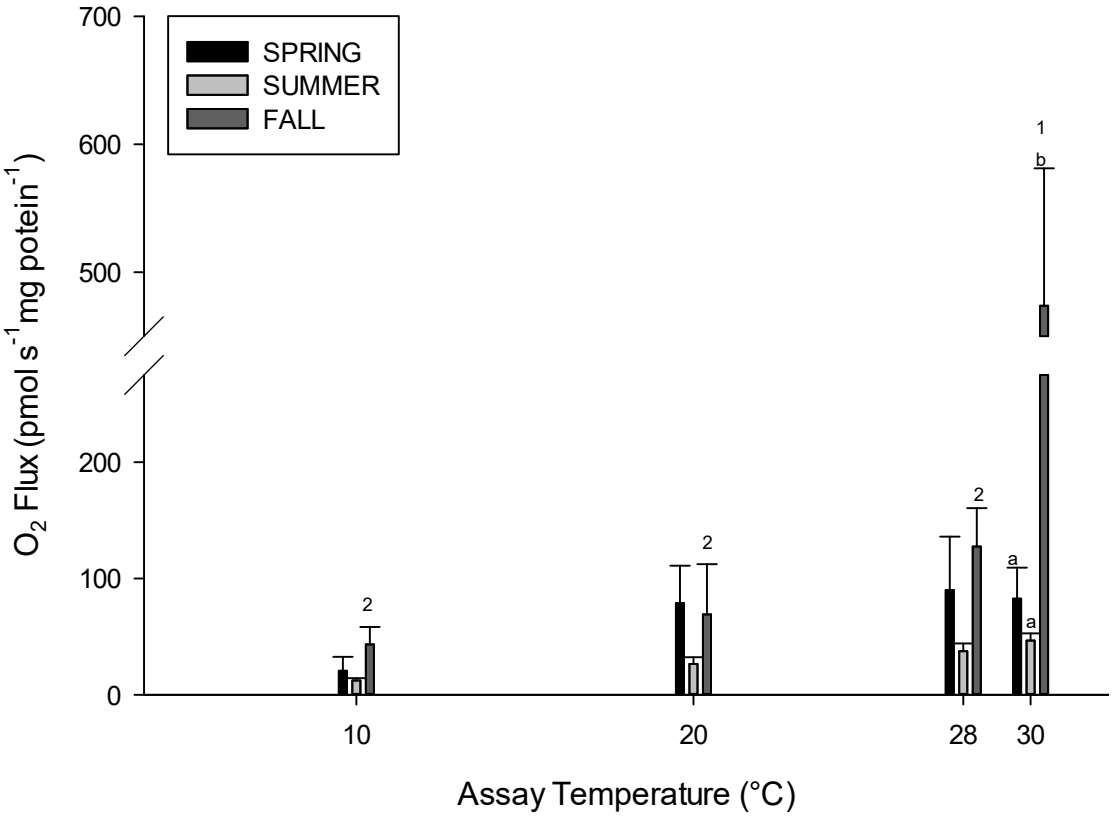

Figure 6. ADP-induced oxygen flux (OXPHOS) as a function of season and assay temperature for *Lepomis macrochirus* liver mitochondria. OXPHOS fluxes obtained in the presence of complex I activating substrates (pyruvate, malate and glutamate). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

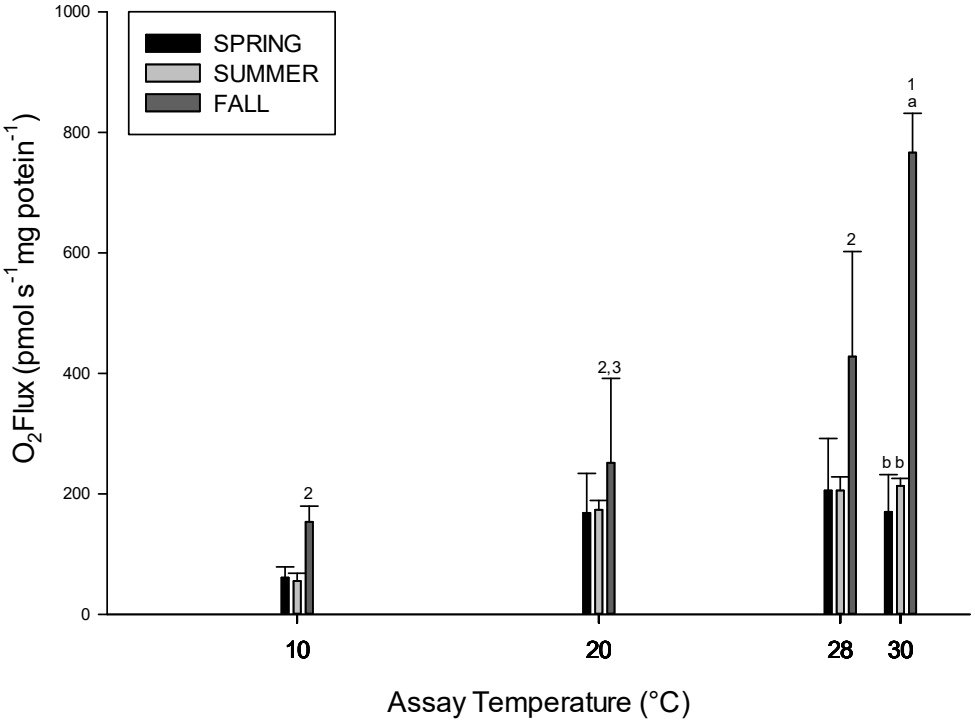

Figure 7**.** Oxygen flux of *L macrochirus* liver mitochondria under maximal electron transfer capacity of the ETS, in the presence of the protonophore FCCP and complex I activating substrates (pyruvate, malate and glutamate) and complex II activating substrate (succinate). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

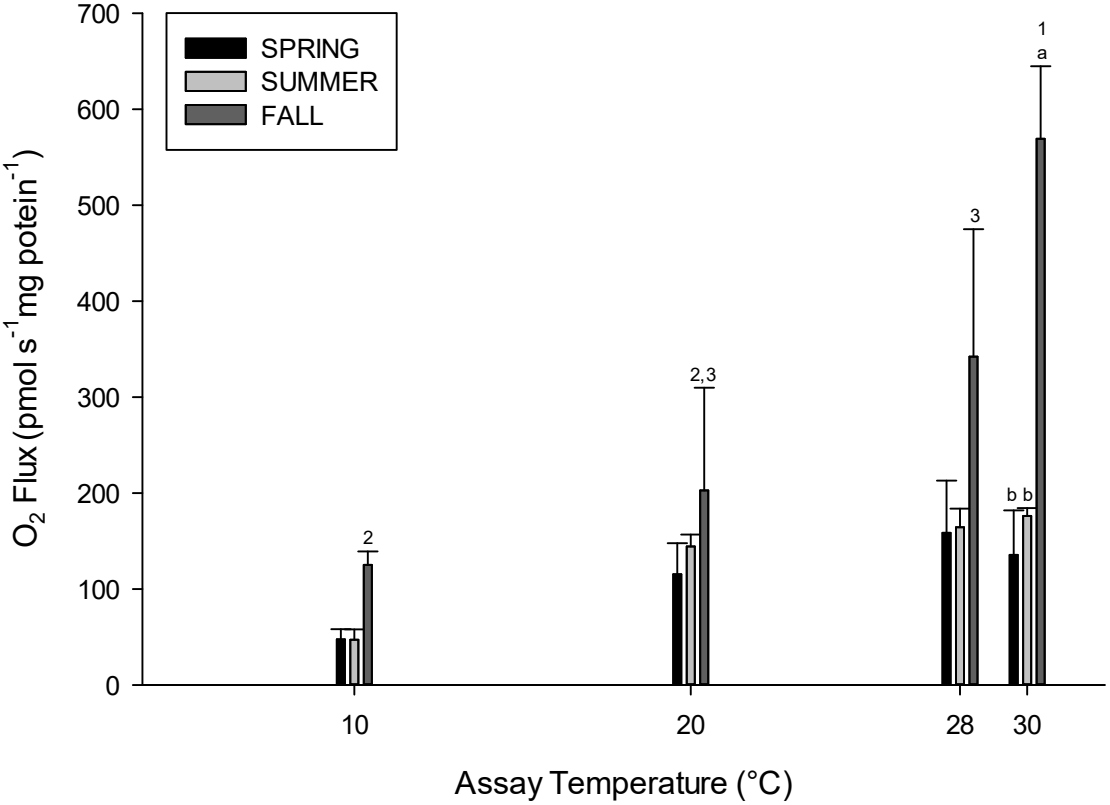

Figure 8. Oxygen flux of *L macrochirus* liver mitochondria under maximal electron transfer capacity of the ETS, in the presence of FCCP and complex I inhibitor rotenone, as well as complex II activating substrate (succinate). Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

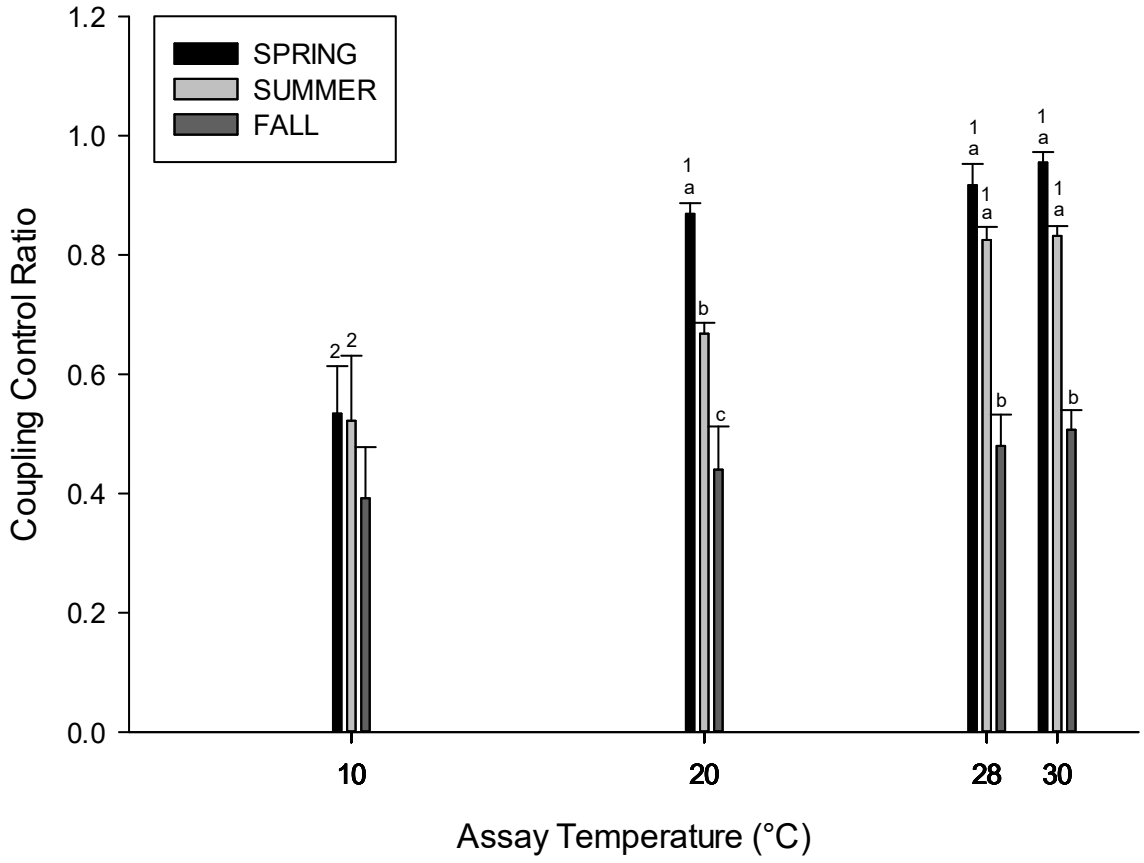

Figure 9. Coupling control ratio (OXPHOS/ETS; P/E) in the presence of both complex I activating substrates (pyruvate, malate and glutamate) and complex II activating substrate (succinate) in liver mitochondria of *Lepomis macrochirus*. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons

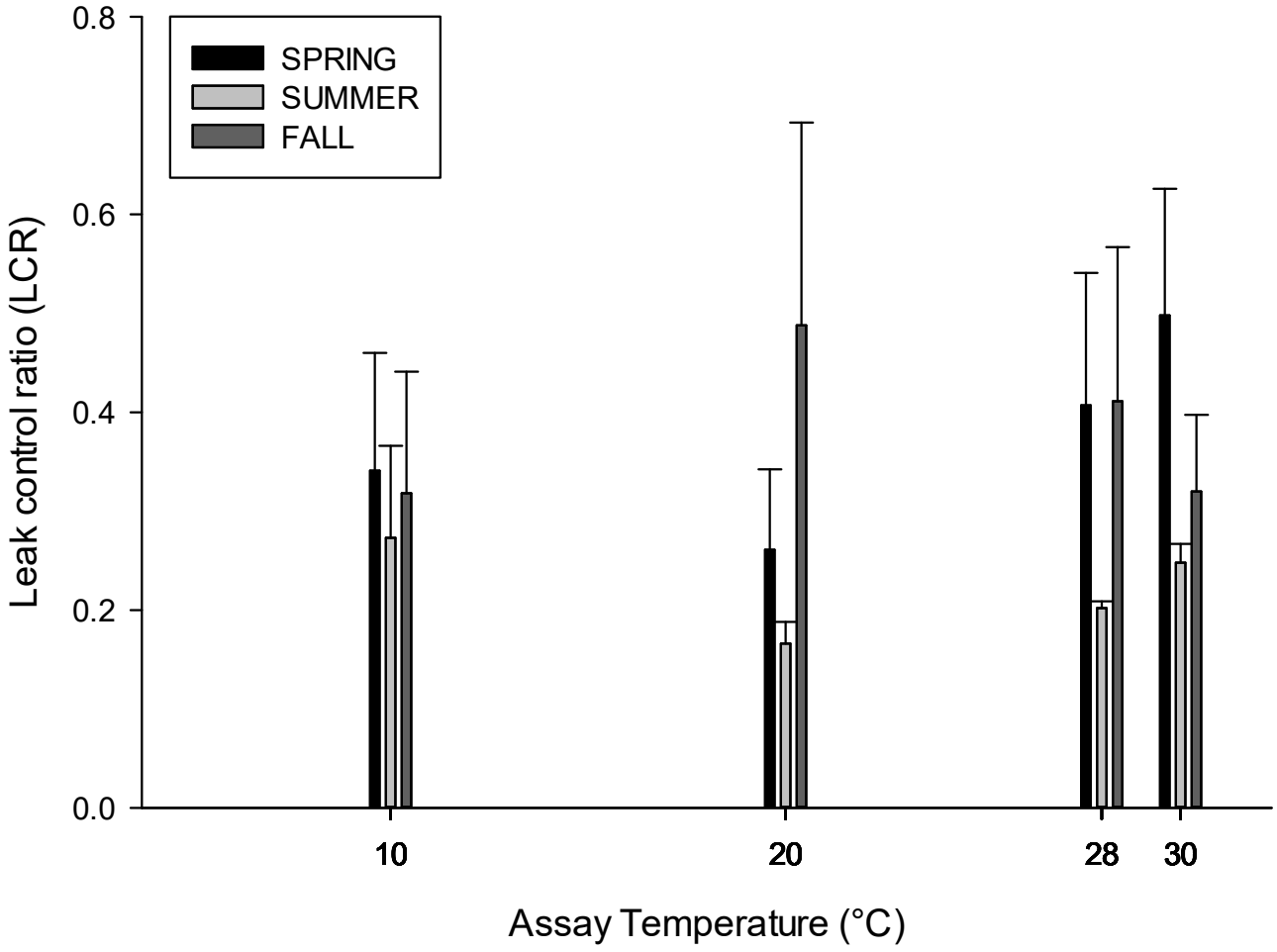

Figure 10. Proton conductance control ratio (LEAK/ETS; L/E) in liver mitochondria of *Lepomis macrochirus* as a function of season and assay temperature. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

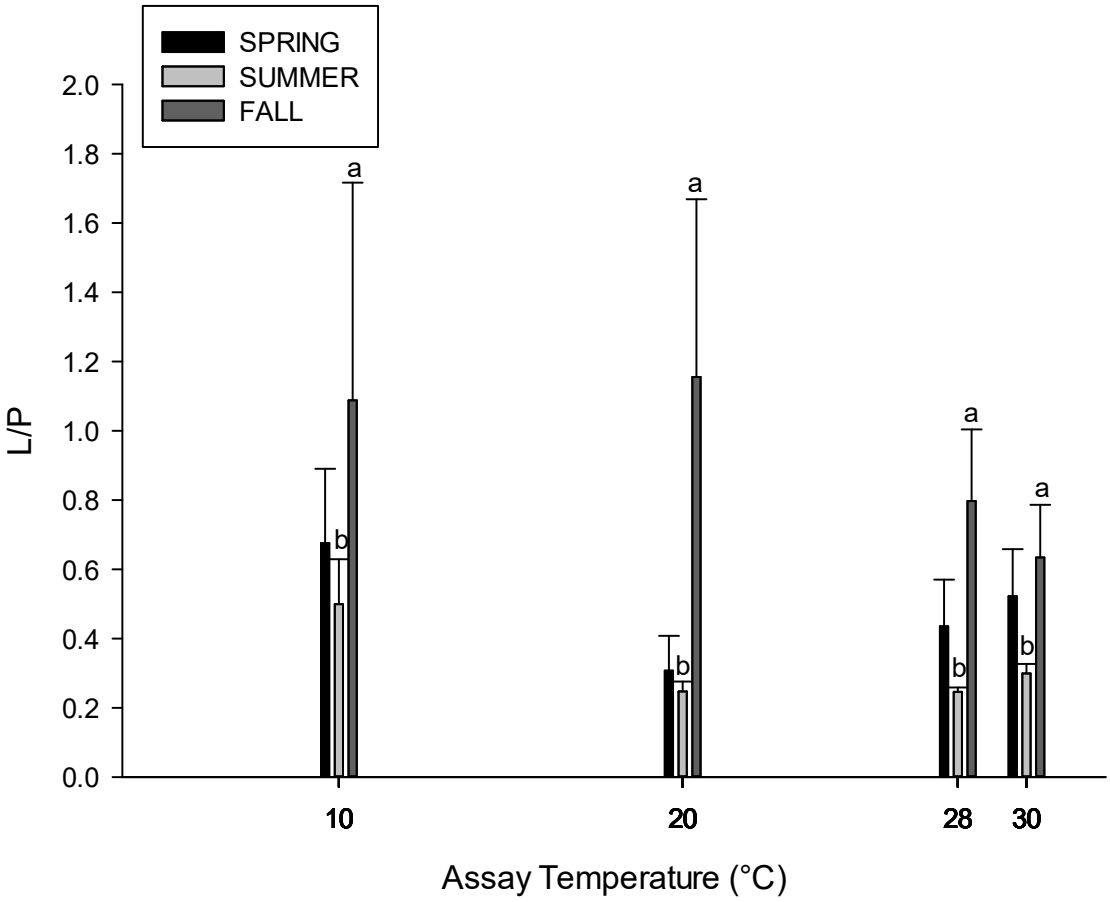

Figure 11. Control ratio resulting from comparing the relation between LEAK flux and OXPHOS flux observed across treatments (LEAK/OXPHOS; L/P; 1/RCR) in liver mitochondria of *Lepomis macrochirus*. Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.

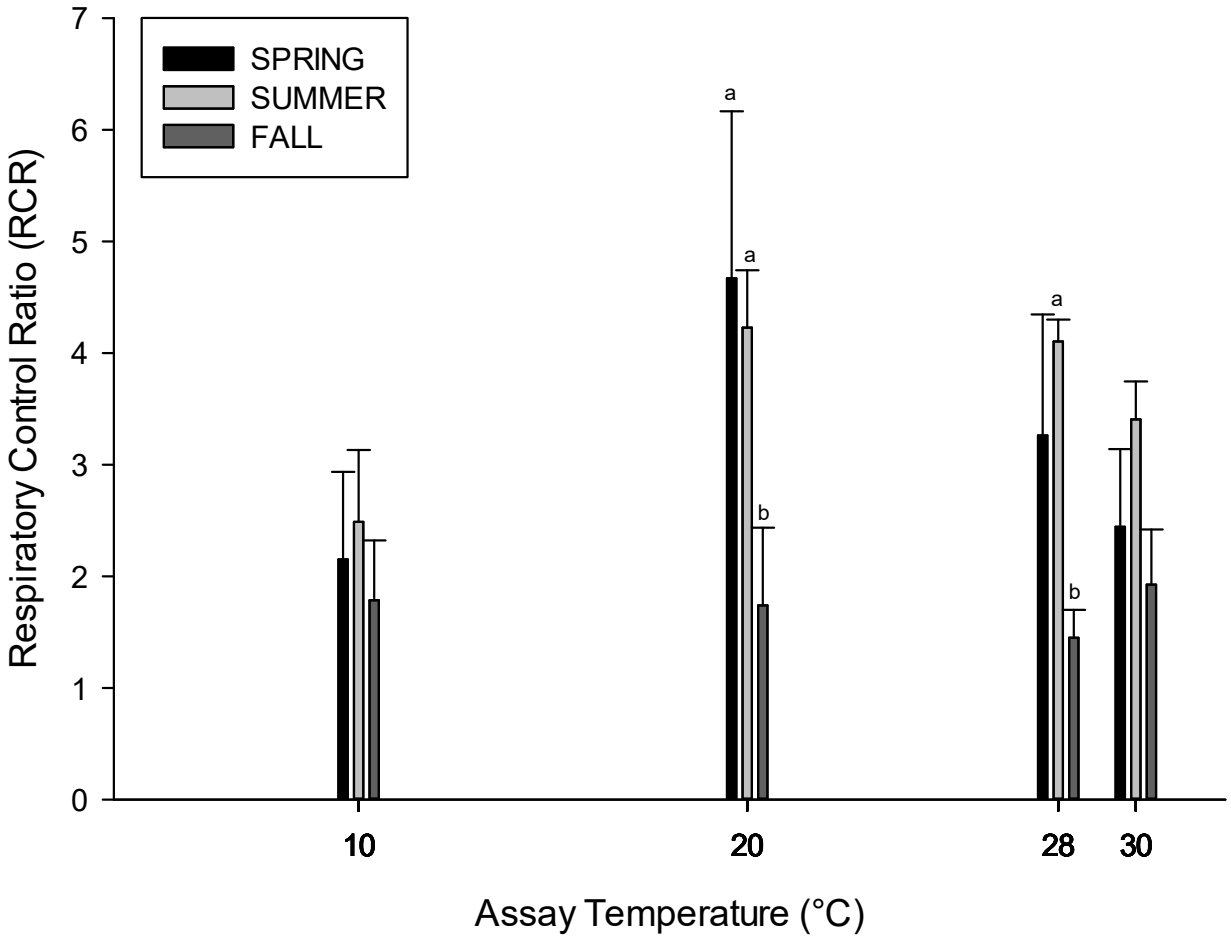

Figure 12. Respiratory control ratio (RCR) as a function of season and assay temperature, calculated from OXPHOS and LEAK fluxes (OXPHOS/LEAK; P/L) in liver mitochondria of *Lepomis macrochirus.* Significant differences are shown (Two-way ANOVA, Holm-Sidak pairwise comparisons; mean ±SEM; n = 4). Numbers represent significant difference in the mean across assay temperature, while letters represent significant difference in the mean within assay temperature, among seasons.