Characterization of darter (*Etheostoma* spp.) interspecific energetic responses to climate change induced temperature changes

by

Allison Veronica Weber

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Shallow freshwater ecosystems are predicted to experience increases in temperature variability as the occurrence and severity of heat waves continues to rise. Ectothermic organisms like fish are especially vulnerable to these acute temperature increases as their physiological functioning is directly regulated by environmental conditions. Thus, understanding their capabilities of responding to thermal stress is critical to predict how these species will be affected by climate change.

Here, we characterized the elevated temperature responses of three closely related darter species: Fantail *(Etheostoma flabellare;* FTD), Rainbow (*Etheostoma caeruleum:* RBD), and Johnny darter (*Etheostoma nigrum;* JD) native to the Grand River of Southern Ontario, via three experiments: Experiment #1: assessment of thermal tolerance limits and energetic enzymatic activity, Experiment #2: determination of thermal preference, and Experiment #3: characterization of metabolic responses to elevated temperatures. Specifically, Experiment #1 determined each darter's CT_{max} and quantified activity and gene expression of enzymes involved in glycolysis, the Krebs cycle, and the electron transport chain in brain and heart tissue at 15°C baseline and at thermal tolerance limits. Experiment #2 determined differences in thermal preference and mobility between species, and Experiment #3 compared darter aerobic scope (AS) during exposure to four different heat ramp exposures designed to mimic previously recorded heat waves.

Significant differences were observed in the thermal tolerance limits of each species. For brain tissue, FTD had higher baseline enzymatic activity compared to JD and RBD, however at CT_{max}, this difference was lost, as both JD and FTD had similarly high enzyme activity relative to RBD. Intraspecifically, JD demonstrated a superior plastic ability, often having a significantly higher enzyme activity at CT_{max} compared to its baseline counterparts, while RBD activity declined at CT_{max} with respect to its baseline levels. Heart tissue exhibited no interspecific differences in activity levels at

baseline. At CT_{max}, however, JD had greater activity than RBD for all heart enzymes, although neither JD or RBD were different from FTD. Similar intraspecific trends as brain were observed, with FTD and JD increasing activity at CT_{max}, and RBD decreasing. No differences were observed in thermal preference between species, although FTD demonstrated significantly higher mobility than JD. Metabolically, FTD AS was significantly greater than JD and RBD at both 25°C and 30°C, however no differences were observed at 15 or 20°C.

These results suggest that FTD may be the best equipped at responding to temperature-induced increased metabolic demands due to their higher baseline enzymatic activity and broader aerobic scope. This FTD advantage, and the interspecific differences observed throughout this study, may a be a result of prior adaptation and acclimatization to each species' respective microhabitat conditions, as it is expected that FTD reside in warm, high flow, and thermally variable regions, JD in warm, moderately thermally variable, low flow environments, and RBD in cold, fast flow, and thermally stable habitats. Exposure to warm and fluctuating habitats, and high mobility levels, have been shown to broaden metabolic function, potentially explaining the high enzyme activity and aerobic scope seen in FTD, defining their superior $CT_{max.}$

Collectively, these findings provide insight to predict how climate change will affect local species, and may have conservation applications for determining which species may be most at risk with increased occurrence of extreme heat events.

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Dedication

To the Rainbow, Johnny, and Fantail darters who were sacrificed for the sake of the science conducted in this thesis. It is my hope that the conclusions made here may lead to a greater understanding of these species and inform future research, initiatives, and decisions on their protection.

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List of Abbreviations

ANOVA – Analysis of Variance

ANCOVA – Analysis of Covariance

AS – Aerobic Scope

COX – Cytochrome C Oxidase

CS – Citrate Synthase

CT_{max} – Critical Thermal Maximum

GRCA – Grand River Conservation Authority

FTD – Fantail Darter

FTP – Final Thermal Preference

JD – Johnny Darter

LDH – Lactate Dehydrogenase

MDH – Malate Dehydrogenase

MO_2 – Oxygen Consumption Rate

PK – Pyruvate Kinase

RBD – Rainbow Darter

RT-qPCR – Reverse Transcriptase Quantitative Polymerase Chain Reaction

UHI – urban heat islands

Chapter 1

Introduction

1.1 General Introduction

As climate change progresses, aquatic environments are predicted to experience elevations in temperature variability as mean temperatures, occurrence and severity of extreme heat waves, and daily temperature maximums continue to rise (Geerts, 2003; Meehl and Tebaldi, 2004; Wang and Dillon, 2014; Stillman, 2019; Bernhardt *et al.*, 2020; Kefford *et al.*, 2022). Additional anthropogenic effects like clear cutting, loss of shade cover, and warm water effluent from urban heat islands (UHI) and thermal power plants introduce further temperature variation in to freshwater ecosystems (Lynch *et al.*, 1984; Somers *et al.*, 2013; Coulter *et al.*, 2014; Kalny *et al.*, 2017; Mohajerani *et al.*, 2017). These factors culminate in adverse impacts on aquatic species, particularly those that reside in shallow habitats that lack temperature buffering capacity (Morash *et al.*, 2021). Ectothermic organisms like fish are especially vulnerable to temperature changes as they possess no internal mechanisms of maintaining body temperature, making their physiological conditions directly regulated by environmental temperatures (Fry, 1947; Schulte *et al.*, 2011). Consequently, temperature is known as the "master factor" due to its integral role in fish physiological functioning particularly through its influence on biochemical reaction rates involved in metabolism (Fry, 1947; Schulte *et al.*, 2011; Schulte, 2015). Thermal composition of fish habitats therefore is directly involved in metabolic functioning, with larger implications on species abundance, population shifts and geographical distributions (Shaklee *et al.*, 1977; Burel *et al.*, 1996; Somero, 2005; Sunday *et al.*, 2012). To date, the majority of studies examining the impacts of climate change on fish metabolic responses have focused on chronic temperature increases. However, heightened temperature variability and daily temperature spikes will likely pose a greater threat to species than gradual temperature increases over time (Paaijmans *et al.*, 2013; Vasseur *et al.*, 2014; Kingsolver *et al.*, 2016). Thus, metabolic function and plasticity play integral roles in fish capacity to respond to thermally variable habitats and is worth investigating further. This thesis aims to

elucidate the impacts of thermal profiles of microhabitats on the metabolic responses of three closely related species of darters (*Etheostoma* spp.) and utilizes environmentally-relevant temperature stressor timescales to characterize how each species will respond to acute heat waves that will likely become more common with the progression of climate change.

1.2 Metabolism

Simply defined, metabolism is the sum of all energy production-related chemical reactions that take place within an organism (Norin and Speers-Roesch, 2021). As such, metabolic rate is the amount of energy expended over a certain period a time, with the rate depending on the energetic demands of the organism (Norin and Speers-Roesch, 2021). Since metabolic rate is based on the organism's energy requirements, it determines, and potentially limits, the organism's capacity for both physical and physiological activity, and therefore its' ability to respond to different environmental conditions or changes (Brown *et al.*, 2004, 2012). In ectothermic fish, metabolic rate can be broken in to three different types, depending on the level of energy requirement. When energy demands are minimum, directed mainly towards sustaining homeostasis, and the fish is resting in a post-absorptive and non-reproductive state, the fish is said to be at standard metabolic rate (SMR; Chabot *et al.*, 2016). For situations of spontaneous, voluntary, or "routine" activity, routine metabolic rate (RMR) is reached, which tends to be more environmentally relevant than SMR considering wild species are rarely at basal and quiescent levels (Norin and Speers-Roesch, 2021). At the uppermost limits of energetic demand, usually following exhaustive exercise, maximum metabolic rate (MMR) occurs (Norin and Clark, 2016).

Comparing the respective values of each metabolic rate can elucidate invaluable information on how fish interact with their environment. Specifically, the difference between maximum and standard metabolic rate, termed aerobic scope (AS), depicts how much energy or metabolic capacity is available beyond the energy requirements needed for basic physiological maintenance (Pörtner, 2001; Clark *et al.*, 2013). AS can therefore provide a useful metric for determining the energy leftover for responding to environmental changes or challenges, and is often utilized in fish physiological analysis for drawing larger conclusions and predictions on species performance, abundance, and distributions (Pörtner and Knust, 2007; Farrell *et al.*, 2008; Schulte, 2015). For instance, previous work has shown elevations in AS to be associated with fish capable of successfully completing challenging spawning migrations, thus increasing the overall fitness of these individuals; conversely, those that did not reach spawning grounds, and were unable to handle the energetic demand required to complete their spawning migrations, were predicted to have a collapse in AS values (Farrell *et al.*, 2008; Eliason *et al.*, 2011).

1.2.1 Metabolism and Thermal Tolerance

Considering metabolic rate is so responsive, it can vary significantly both across and within different species due to a variety of biotic factors like activity, trophic level, and body mass, and abiotic factors like temperature and hypoxia (Norin and Speers-Roesch, 2021). Temperature in particular plays a major role in determination of metabolic rate, as the speed of the biochemical reactions that underlie metabolism exhibit a positive relationship with temperature, increasing as temperatures rise and decreasing as they fall (Schulte, 2015). The impact of temperature on metabolism, and physiological performance more generally, is often depicted via thermal performance curve (Fig. 1.1), which illustrates how performance, or essentially the AS of the organism, scales with temperature, increasing

steadily as temperatures rise, but dropping off suddenly at extremes (Schulte *et al.*, 2011). As temperatures surpass the organism's thermal optimum (T_{opt}) and approach temperature limits, a range of sublethal effects occur, with any deviations past temperature thresholds having lethal consequences (Ficke *et al.*, 2007).

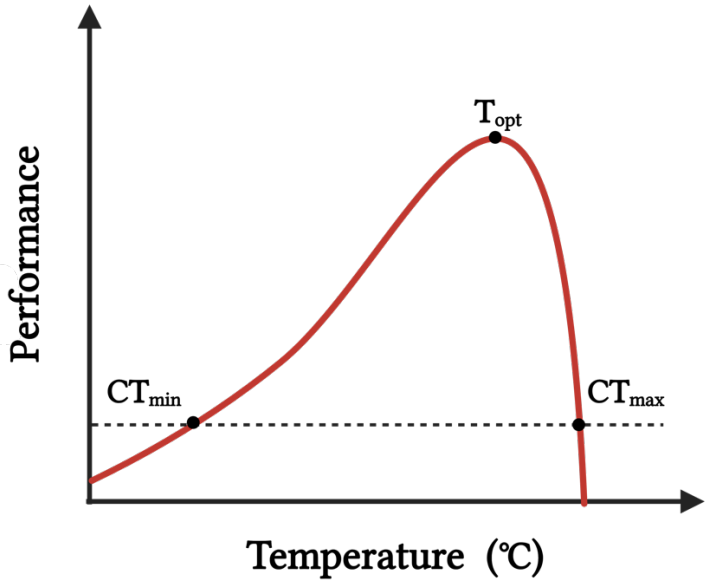


Figure 1.1: Thermal performance curve. Utilized to depict how physiological responses of ectotherms scale with temperature. Adapted from Schulte et al., 2011. CT_{min} represents the critical thermal minimum, T_{opt} the temperature optimum, and CT_{max} the critical thermal maximum.

The upper threshold at which physiological functioning is no longer possible, is known as Critical Thermal Maximum (CT_{max}), and is understood as the thermal tolerance limits of the respective organism. In fish, increases in environmental temperature increase metabolic rate, increasing oxygen demand to meet the elevated requirements of aerobic metabolism (Schulte, 2015). As temperatures continue to rise, it is hypothesized that eventually the cardiorespiratory system is unable to accommodate the increased oxygen requirement of tissues leading to insufficient oxygen delivery

(Pörtner, 2001; Pörtner and Knust, 2007). Under these circumstances, tissues may transition to anaerobic methods of energy production, however, these are less efficient and cannot meet the progressively increasing energetic demands, especially for aerobic dependent tissues like the brain and heart, resulting in a decline in AS and eventual physiological failure, thus defining CT_{max} (Pörtner and Knust, 2007; Pörtner, 2010).

Failure of brain and heart tissue specifically, are believed to be implicated in determining thermal tolerance limits (Ern *et al.*, 2023). Previous studies in fish have reported crashes in heart function when nearing thermal tolerance limits, and determined it often is one of the first organs to fail at temperature extremes (Farrell, 2002; Mendonça and Gamperl, 2010; Ern *et al.*, 2023). With the heart playing such a leading role in the cardiorespiratory system, its dysfunction causes oxygen limitation in all other downstream tissues, notably the brain, which cannot withstand hypoxia for extended periods of time. Recent developments from Andreassen et al., (2022) concluded that lack of oxygen delivery to the brain, which has high energy requirements but low energy reserves, may be responsible for determining thermal tolerance limits, explaining the locomotor dysfunction, known as loss of equilibrium (LOE), that occurs at CT_{max} in fish (Becker and Genoway, 1979; Soengas and Aldegunde, 2002; Andreassen *et al.*, 2022).

1.2.2 Metabolic Plasticity and Temperature Change

To mitigate the impacts of changes in environmental temperatures on metabolic reaction rates, physiological adjustments can be made to maintain energetic homeostasis. Adjustments that occur evolutionarily over many generations within a given species are known as metabolic compensation, which usually result in these species being well-adapted to the thermal profiles of their respective environments (Norin and Speers-Roesch, 2021). When metabolic alterations occur within an individual

fish's lifetime in response to temperature changes in their environments, metabolic thermal plasticity is said to have occurred, in which the organism, through reversible changes, becomes acclimatized to the environmental conditions of its habitat over the course of several weeks to months (Seebacher *et al.*, 2015). This plasticity can involve numerous alterations in metabolic functioning at molecular, cellular, and whole body levels in order to sustain the energy requirements needed to match the metabolic changes associated with environmental conditions (Angilletta, 2009; Seebacher *et al.*, 2015). Physiological responses to deviations in temperature can include changes to enzyme activity, through regulatory responses like changes in substrate concentration and relative enzyme quantities, alterations in mitochondrial abundance and density, or metabolic reorganization through shifts in pathway importance and reliance (Shaklee *et al.*, 1977; Somero, 1978; Angilletta, 2009).

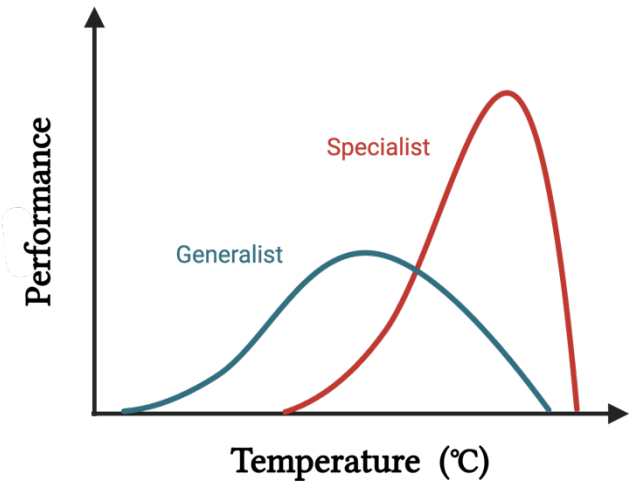


Figure 1.2: Generalist and specialist thermal performance curves. Adapted from Boyles *et al.*, 2011. Temperature generalist species typically exhibit broad tolerances across a wide range of temperatures but often at the expense of lower performance. Conversely, temperature specialists have high performance over a very narrow range of temperatures.

Consequently, metabolic variation is commonly observed both between and within species depending on thermal compensation, or on previous thermal acclimatization and life histories of individual fish, presenting broader implications on overall performance and fitness (Pörtner and Knust, 2007; Schulte, 2015). These adjustments can be observed through shifts and changes to different properties of their respective thermal performance curves. Fish adapted to thermally variable habitats, known as generalists, tend to have broader thermal performance curves as they are capable of handling a wide range of environmental temperatures, however this often comes at the expense of lower metabolic performance (Angilletta, 2009; Schulte *et al.*, 2011). Specialists, or species that prefer a small range of temperatures, have a thermal performance curve with a narrow base but tall peak, indicating that although they are confined to a limited range of temperatures, they have high metabolic capacity within those environmental conditions (Fig. 1.2; Angilletta, 2009). On a more acute scale, species that are acclimatized to warmer temperatures will have a curve that is shifted right, resulting in a higher thermal tolerance limit, and vice versa for those that are cold-acclimatized, which possess a left-ward shifted curve, and consequent lower CT_{max} (Angilletta, 2009; Schulte *et al.*, 2011; Norin and Speers-Roesch, 2021).

It is well documented that acclimation to warmer temperatures improves the CT_{max} and metabolic capacity of fishes (Comte and Olden, 2017), however less is known on the impact of thermal variability within habitats on thermal tolerance limits. It has been hypothesized that increased habitat temperature variability increases plasticity and acclimation capacity of fishes, in turn improving their ability to respond to climate change (Addo-Bediako *et al.*, 2000; Bozinovic *et al.*, 2011; Morgan *et al.*, 2022). However, others have observed no relationship between the level of habitat thermal variability and the ability to acclimate or respond to acute temperature changes in the environment, in addition to no impacts on CT_{max} (Sunday *et al.*, 2012; Comte and Olden, 2017; Gunderson *et al.*, 2017; Campos *et*

al., 2018) As such, differences in metabolic rate and CT_{max} between species due to prior acclimatization to their respective habitat temperature conditions, is worth investigating further as it may dictate which are most capable of tolerating rapidly changing environments.

1.3 Study Location and Species

The Grand River watershed constitutes a significant component of Southern Ontario's freshwater ecosystems, comprising of ~6,800 km^2 and housing more than one million people including the Six Nations of the Grand River Territory and the Mississaugas of the Credit First Nation (Grand River Conservation Authority, 2022). The watershed encompasses all land drained by the Grand River and its tributaries, including four rivers: the Conestogo, Nith, Speed, and Eramosa, resulting in a combined river length of approximately 11,000 km (Grand River Conservation Authority, 2022). More than 90 species of fish have been recorded within Grand River watershed, including numerous species of darters *(Etheostoma* spp.), small perches common to North American freshwater streams and rivers (Kuehne and Barbour, 1983; Grand River Conservation Authority, 2022). Benthic in nature, darters feed on a variety of insect larvae, midges, and tiny crustacea, and serve as a potential food source for other fish or birds within the watershed (Kuehne and Barbour, 1983). *Etheostoma* is the largest and most diverse genus of North American fishes, with seven different species residing in Canada specifically (Nelson, 2004; Dextrase, 2016). Here, three species native to the Grand River: Rainbow darter (*Etheostoma caeruleum:* RBD), Fantail darter (*Etheostoma flabellare;* FTD), and Johnny darter (*Etheostoma nigrum;* JD), are of particular interest as, although closely related, each has a distinct microhabitat and environmental preference.

JD have low mobility, and prefer stagnant and occasionally deep water, often residing in sandy, silty pools or shorelines (Kuehne and Barbour, 1983; Ingersoll and Claussen, 1984). If given a choice, JD will select warmer temperatures compared to cooler waters, and are recorded to have a temperature preference of around 22.8° C (Ingersoll and Claussen, 1984; Coker *et al.*, 2001). JD are additionally reported as having broad environmental tolerances, capable of withstanding turbidity, and organic and inorganic pollutants (Becker, 1983; Kuehne and Barbour, 1983). Conversely, RBD are often very abundant, inhabiting rocky, fast-flowing riffles, (Kuehne and Barbour, 1983), and preferring temperatures near 19.8° C (Wichert and Lin, 1996; Coker *et al.*, 2001). RBD are noted as being intolerant, and more sensitive to pollution and siltation than JD (Eakins, 2023). They have also been termed moderately mobile (Hicks and Servos, 2017). FTD can be found in shallow, gravelly riffles (Ingersoll and Claussen, 1984), and are described as the most variable of all the darters (Kuehne and Barbour, 1983). Similar in abundance as RBD, they prefer temperatures near 22.4° C but in comparison to JD, will choose cooler, thermally-variable waters over warm (Ingersoll and Claussen, 1984; Coker *et al.*, 2001). Although deemed an intolerant species, they can withstand some siltation (Eakins, 2023), and have also been labeled as more mobile than other darters (Mundahl and Ingersoll, 1983). Collectively, each of these species have been recorded to demonstrate high site fidelity, remaining within its distinct microhabitat throughout the majority of their lifetime, ensuring each species experiences its respective, habitat-specific, thermal profile. (Roberts and Angermeier, 2007; Krause *et al.*, 2010; Hicks and Servos, 2017). As a result, JD, RBD, and FTD are an effective group of species to analyze the impacts of thermal profiles on interspecific variation in physiological responses.

Although highly common, a relatively small amount of physiological and thermal tolerance research has been done on darters, and even less on determining how climate change will impact these species. While previous studies have examined impacts of temperature increases on darters in terms of

habitat elevation (Troia and Giam, 2019), or compared darter AS values relative to invasive species (White *et al.*, 2017), virtually no studies have examined how darters may respond metabolically to acute temperature spikes associated with climate change, aside from recent work done by Borowiec et al., which reported darters having rapid metabolic recovery to hypoxia, acute temperature stress, and exhaustive exercise (Borowiec *et al.*, 2024). Consequently, little is known on the metabolic capacity of darters to handle temperature increases, presenting broader questions on which species may be most capable of tolerating climate change.

1.4 Study Objectives and Hypothesis

From 1951 to 2013, the Grand River watershed has observed increases in the frequency and duration of extreme heat waves, decreases in low temperature events, and increases in the frequency, intensity, and duration of extreme precipitation (Deen *et al.*, 2021), suggesting that the Grand River and its tributaries are already experiencing increases in temperature variation. This variability is likely to increase further as current projections for Southern Ontario predict increased duration and occurrence of droughts and extreme heat events as summer month precipitation declines while overall temperatures rise (Deen *et al.*, 2021). Understanding how these three species of darters native to the Grand River will respond to climate and anthropogenic induced temperature changes is therefore of paramount importance in order to predict how our local watershed and freshwater ecosystem will be affected.

This thesis sought to characterize the CT_{max}, thermal preference, and respective metabolic responses of three closely related species, RBD, JD, and FTD, of varying microhabitats to assess and predict their resilience to future climate induced temperature changes. **I hypothesized that darters from different habitats of varying thermal profiles will have different thermal tolerance limits**

and preferences, as a result of interspecific differences in metabolic function. This was analyzed through completion of the following objectives:

Objective 1: Determine thermal tolerance limits between species, and characterize differences in enzymatic activity both within and between species at river temperatures and at thermal tolerance limits.

Objective 2: Determine the thermal preference of RBD, JD, and FTD.

Objective 3: Analyze the metabolic responses each species following environmentally-relevant temperature increases.

These were addressed through the completion of three experiments, each corresponding to their respective objective. Specifically, the first measured CT_{max} and quantified energetic enzyme activity and gene expression at baseline and CT_{max} temperatures. The second determined differences in thermal preference and mobility between species, and the third compared darter AS during exposure to four different temperature ramp profiles.

Chapter 2

Materials and Methods

2.1 Study Design

This study was divided into three separate experiments, Experiment 1: Assessment of thermal tolerance limits and energetic enzymatic activity, Experiment 2: Determination of thermal preference, and Experiment 3: Characterization of metabolic responses to elevated temperatures. For all experiments, RBD, JD, and FTD of mixed sex were collected from the Grand River at West Montrose (43.58574, -80.48150) via backpack electrofishing. Darters were brought back to the Waterloo Aquatic Threats in Environmental Research (WATER) facility at the University of Waterloo and housed in a 385 L Z-HAB rack system (Pentair Aquatic Eco-Systems Inc., Apopka, Florida, USA) with approximately 12 fish per 10 L acrylic tank. Tanks were supplied with de-chlorinated, reverse-osmosis, softened city water, and maintained at a temperature of 15°C and pH of 8.0 to match conditions recorded from the catch site. Fish were acclimated for two weeks, under which they were fed frozen bloodworms to satiety daily, and kept under 12 hour light:dark cycles, with 0.5 hour ramp transitions. Considering darters are benthic species, housing structures were placed in each tank for enrichment. All experiments performed in this study were in accordance with the Canadian Council of Animal Care guidelines as reviewed by the University of Waterloo Animal Care Committee (AUP #44638).

2.2 Experiment #1: Assessment of Thermal Tolerance Limits and Energetic Enzymatic Activity

This experiment determined the CT_{max} of each species, along with their enzyme activity and gene expression, at a baseline temperature of 15°C and at their respective thermal tolerance limits. RBD, JD, and FTD were collected as outlined in section 2.1 in the Fall of October 2022. After acclimating in lab for two weeks, 20 fish per species were humanely euthanized and sampled to serve

as the 15°C baseline. In the following two days, CT_{max} trials were conducted on 20 fish per species, 4 fish at a time. These fish were immediately euthanized following loss of equilibrium (LOE). Brain and heart tissues were extracted, flash frozen on dry ice, and stored at -80°C until use. Enzyme activity and gene expression were analyzed via enzyme activity assay and quantitative PCR on key metabolic enzymes: pyruvate kinase (PK), lactate dehydrogenase (LDH), involved in glycolytic reactions, citrate synthase (CS), malate dehydrogenase (MDH), involved in the citric acid cycle, and cytochrome C oxidase (COX), involved in the electron transport chain (Fig. 2.1). Expression of corticotropin releasing hormone (CRH) was also quantified, solely in brain tissue, to provide insight into relative stress responses of each species.

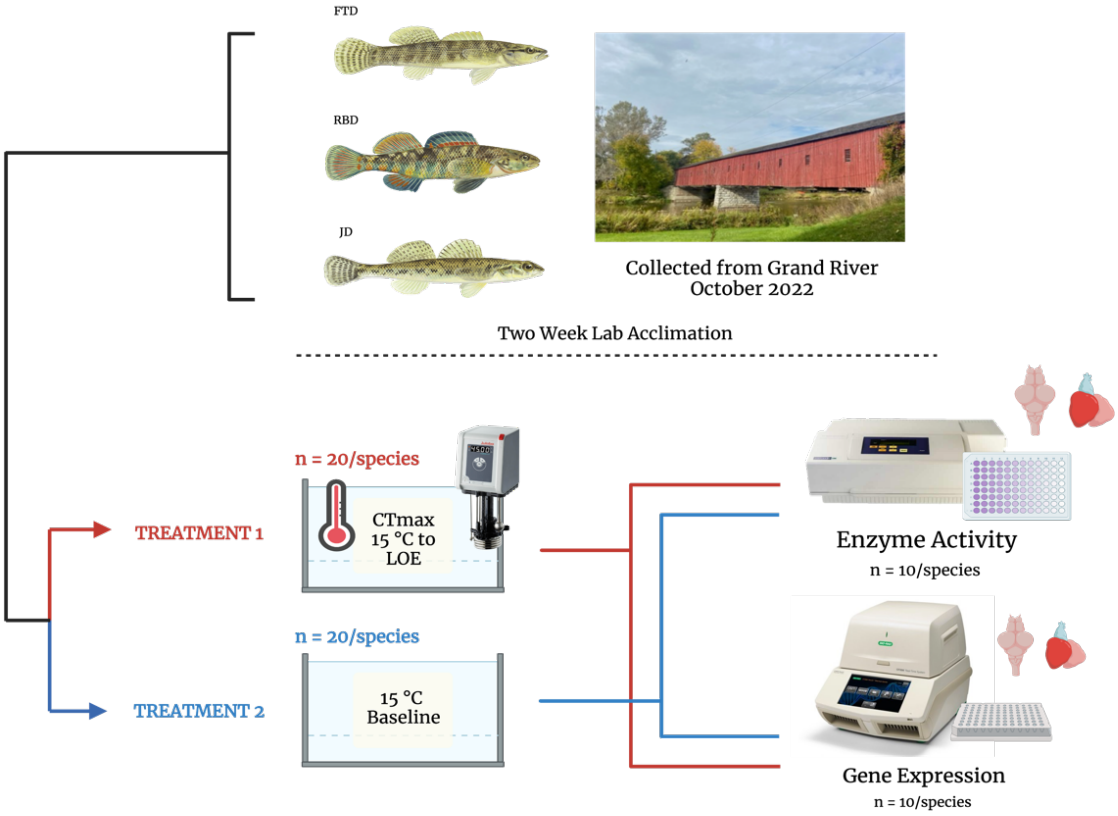


Figure 2.1: Experiment #1 schematic. Assessment of thermal tolerance limits and energetic enzymatic responses. Darters were collected from the Grand River in October 2022, brought back to lab, and acclimated for two weeks. 20 fish per species either served as a temperature baseline treatment group, or a CT_{max} treatment group, with enzyme activity and gene expression quantified in brain and heart tissue of both treatment groups.

Table 2.1: mRNA primers of genes of interest used for RT-qPCR. All primer sequences are listed in the 5' to 3' direction with F representing forward and R reverse primers. All efficiencies fell between 90 and 110%. All reference genes were deemed stable (M score < 0.5).

Target	Amplicon	Accession #	Primer Sequence (5' - 3')
PK	137 BP	XM_034868611.1	F: CTCACTGGCTCTGGAAGGTC R: TTGGTGTAGAGGACGGGGAA
LDH	144 BP	XM_034878773.1	F: TACAGTGTGACGGCCAACTC R: GTTGGGGCTGTACTTGACGA
MDH	132 BP	XM_034875172.1	F: GGAGTGCGGTTATGTCAGGT R: GCGACCAGCTTCTCCTCAAA
CS	97 BP	XM_034877304.1	F: CTGAGGGTGTCCACAAGTCC R: CGGTAGATCTTGGCAGCGAT
COX1	100 BP	NC_042248.1	F: AACACCGTCTCATCCATCGG R: CAAGAACTTCCCGTTTGGCG
CRF	167 BP	XM_034862630.1	F: GCGGAACACCTGCAATCTAC R: ATCCAGGGATATAGGCGGGT
β-actin	114 BP	XM_032527833	F: GTACCCCGGCATCGCA R: CCTCCGATCCAGACAGAGTATT
ef1α	106 BP	XM_034897828	F: TGGTGACAACGTGGGCTTTA R: GTTGGCAGCTTCTTGTGGTG

2.2.1 Assessment of Thermal Tolerance

Thermal tolerance limits were determined via CT_{max} thermal challenge, with all fish fasted 24 hours prior to the CT_{max} procedure. Five trials were conducted per species, each trial consisting of 10 fish total with 2 fish of a single species placed into two, 16.5 x 12.5 x 13 cm mesh breeding boxes,

resulting in n=20 per species. Breeding boxes were fitted side by side in a 30L glass aquarium water bath and left to acclimate for 15 minutes at 15°C. Temperature of the water bath was then increased incrementally by 0.33°C per minute through a Julabo portable immersion circulator (Seelbach, Baden-Württemberg, Germany). Significant mixing of warmed water was performed by a water pump on the Julabo circulator, with warm water diffusively entering the mesh box. Fish were visually monitored, and occasionally prodded with a probe to ensure LOE, the point at which the fish became unable to maintain its position in the water column, going belly up. The temperature at which LOE occurred was recorded, after which the fish were removed from the mesh box and immediately euthanized using buffered 0.5 g MS-222. Brain and heart tissue dissected and stored at -80°C till further use. No unexpected mortalities occurred during any of the CT_{max} trials.

2.2.2 Enzymatic Assays

Frozen brain and heart tissue were homogenized on ice in 20 mL of buffer per 1 mg of tissue (20 mM Hepes, 1 mM EDTA, 0.1% Triton X-100, pH 7.4, 1 anti-protease pill/10 mL of buffer) via OMNI TH handheld tissue homogenizer (Kennesaw, Georgia, USA). Homogenates were then centrifuged (12,000 g, 10 min, 4°C) and supernatants removed for use in enzyme activity assays. All assays were performed in 96-well microplates and measured for ten minutes at 25°C using a Molecular Devices Spectramax 190 spectrophotometer (San Jose, California, USA) in tandem with the SoftMax Pro 6.3 software. Cytosolic enzymes, PK and LDH, were measured first after one freeze thaw cycle; mitochondrial enzymes, CS, MDH, and COX, were measured after two freeze thaw cycles to allow for sufficient opening of mitochondria. All freeze thaws were consistent across all samples within each enzyme. PK, LDH, and MDH were all measured at 340 nm, CS at 412 nm, and COX at 550 nm. Enzyme

reaction buffers were made according to the protocols outlined in Mehdi *et al.*, 2018 and Dawson *et al.*, 2016. The remaining sample was used in Bicinchoninic acid (BCA) assays to determine protein quantity of each sample and used to normalize enzyme activity to protein content. For all enzyme assays conducted, a sample size of n = 10 per group was used.

2.2.3 Gene Expression

Frozen brain and heart tissue were homogenized in Qiagen RLT Lysis Buffer, again via OMNI tissue homogenizer, and extracted using a Qiagen RNeasy extraction kit (Hilden, North-Rhine Westphalia, Germany) following the manufacturer's instructions. Samples were reconstituted in Qiagen DNAse/RNAse free-water and RNA concentration was determined using a Life Technologies Qubit 3.0 Fluorometer (Carlsbad, California, USA). cDNA was synthesized from 150 ng of RNA via Qiagen QuantiTect Reverse Transcription kit (Hilden, North-Rhine Westphalia, Germany) and stored at -20°C until gene expression analysis. The relative expression levels of genes*: ldh, pk, cs, mdh*, *cox and crh* (Table 2.1) were determined by RT-qPCR on a BioRad CFX96 Touch Thermal Cycler (Hercules, California, USA) with a sample size of n = 6 per enzyme transcript. A 7-point standard curve ranging from a dilution of 12x to 768x was constructed for all targets using the pooled samples to ensure that the primers used had an efficiency between 90 to 110% and a R^2 value greater than 0.98 for tissues. Each reaction consisted of 2 uL diluted cDNA, 5 uL of BioRad SYBR® Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), 1 uL of 5 uM forward and reverse primers and 1 uL of water. cDNA was incubated at 95°C for 30 s, denatured at 95°C for 10 s, and annealed at 60°C for 20 s. Fluorescence was detected and followed by 39 more cycles. Gene expression was normalized to several housekeeping genes, specifically, *β-actin* and *ef1α*, for brain and heart tissue of all species,

which were deemed stable across all groups (M score < 0.5). Primers were specifically designed for darter species based off prior species sequencing performed in lab.

2.2.4 Statistical Analysis

All statistical analyses, unless stated otherwise, were performed by GraphPad Prism 8.1.2 using an alpha of 0.05 (GraphPad, San Diego). All data was tested for normality and lognormality, and normality of residuals was determined with each parametric test conducted. Datasets that failed to pass normality but suggested to have lognormal distributions were log transformed and confirmed to have normal residuals following completion of their respective parametric analyses. For brain tissue, all enzyme activity data was log transformed; heart enzymatic data was normally distributed except for COX, which was log transformed. A One-Way Analysis of Variance (ANOVA) with a Tukey post-hoc test was performed to determine significant differences in CT_{max} values between RBD, JD, and FTD species, followed by an Analysis of Covariance (ANCOVA) performed on RStudio 4.3.3 to assess for any impacts of body weight on CT_{max}. Outliers were detected by 1.5*IQR, resulting in the removal of two datapoints, RBD 27.9°C and FTD 30.59°C, which had no impact on the statistical differences reported between species. Differences in enzyme activity between temperature treatment groups and species were assessed by a Two-Way ANOVA with Tukey post hoc test. When a significant interaction between the CT_{max} treatment (henceforth referred to as heat treatment) and species was reported, data was split individually by variable and re-analyzed to assess for differences between species at each heat treatment, determined by t-test, or differences within species determined via One Way ANOVA. Differences in enzyme transcript gene expression between baseline and CT_{max} within species was determined via t-test. All values are presented as mean ± standard error.

2.3 Experiment #2: Determination of Thermal Preference

In May 2023, RBD, JD, and FTD were collected from the Grand River at West Montrose for completion of Experiment #2, which focused on elucidating thermal preference differences between the three species. Following their two-week acclimation to lab housing conditions, described in section 2.1, each species was cycled through filmed trials, in which they were placed in a long, temperature gradient chamber, and analyzed for preference to any sections within the gradient (Fig. 2.2).

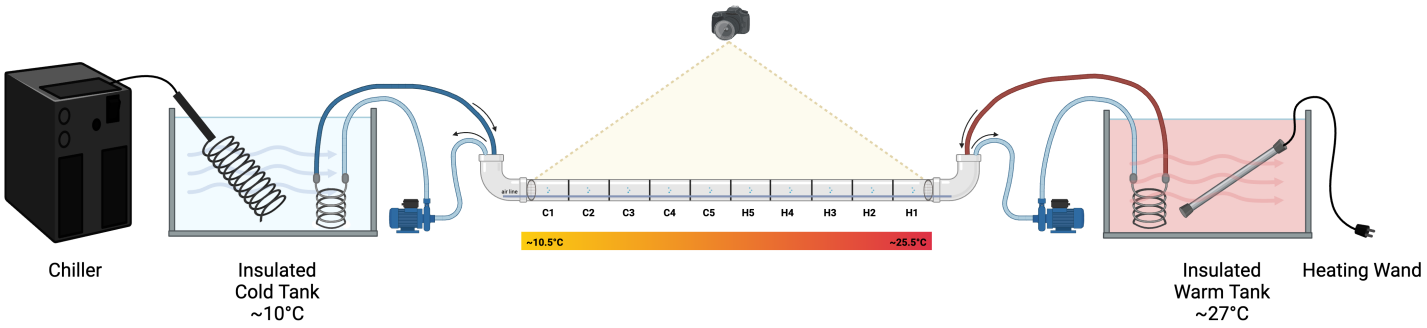


Figure 2.2: Experiment #2 schematic. Eight-foot trough gradient, ranging from approximately 10° to 25° C, divided in 10, equally aerated sections. Fish were placed in the middle of the gradient and left for approximately 5 hours while being filmed.

2.3.1 Creation of Thermal Gradient

A thermal preference chamber was designed and built by trimming the top 30% (lengthwise) of a 10 foot long, 4-inch diameter, insulated PVC pipe to create a trough, with elbow joints connected at the ends. Influx and efflux water lines were placed at each end, with the efflux lines pumping water through a metal coil placed in either a cold or hot 30 L water bath. Heated or chilled water returned to

the ends of the pipe via the influx lines, which had a filter sponge placed at the end of the line to disrupt flow upon re-entry. The warm water reservoir was heated to approximately 25°C, and the cold water to approximately 10°C, creating a thermal gradient spanning the length of the pipe. Grates were placed in front of the influx and efflux lines to prevent fish from interacting with the lines, resulting in total gradient length of approximately 8 feet, which was then divided up in to 10, 9.5 inch sections, named C1 – C5 for the fiver colder portions of the trough, with section C1 coldest being closest to the cold reservoir influx, and likewise with the five warmer sections, H5 – H1, with H1 being hottest (Fig. 2.2). To ensure each section had equal aeration, an airline ran along the bottom of the pipe trough, with holes in the line placed in the center of each of the ten individual sections. This line was a closed loop to prevent air level from decreasing as it continued down the line. Additionally, water-proofed (via vacuum sealing) Thermochron iButton (DS1925L) temperature loggers were placed into each of the 10 sections to determine the temperature of each of the portions of the gradient. The entire thermal preference set up, trough and water reservoirs included, was surrounded by shower curtains to minimize external stimuli and/or disruptions.

2.3.2 Thermal Preference Trials

To determine the thermal preference of each of the darter species, fish were individually placed into the trough for 10 hours and filmed using a GoPro camera mounted to the ceiling above the trough. All fish were fasted 24 hours prior to the start of the trial. At 7 AM, after allowing >30 minutes for the trough temperatures to stabilize, fish were released directly into the middle of the gradient, between sections C5 and H5. At 5 PM the fish was removed and placed back in its holding tank. 10 fish per species were cycled through the thermal preference trial in this manner, with the order of species structured such that each species spent the same amount of time housed in the lab to mitigate acclimation effects. Considering that 10 fish per species were assessed, and fish were placed in the

chamber one at time, the trial took approximately 30 days. Two FTD mortalities occurred during the trial, due to jumping out of the trough, resulting in an n of 10 for JD and RBD, and 8 for FTD.

2.3.3 Film and Temperature Analysis

Film was analyzed using Animal Tracking and Analysis (AnimalTA) software, on only the last ~5 hours (17,000 seconds) to provide ample time for the fish to acclimate and orient itself in the gradient without confounding analysis. Within the AnimalTA software, each of the 10 sections C1:C5,H5:H1 were defined to determine the percentage of time the fish spent in each respective section. Distance traveled and moving time were also analyzed. iButtons were removed weekly and their temperature data recorded. The percentage of time spent in each section was then compared with the iButton temperature of that section. Thermal preference was determined by taking a weighted average of all of the sections, wherein the percentage of time for a section was multiplied by the temperature of that section; this was calculated for all 10 sections and then averaged to determine one average temperature point for an individual fish.

2.3.4 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 8.1.2 utilizing an alpha of 0.05. A One Way ANOVA with Tukey post hoc test was used to determine significant differences in the temperature preference of each species in addition to differences in distance traveled and time moving. Acclimation effects were analyzed via simple linear regression to determine if there were any impacts of acclimation to lab conditions on thermal preference, distance traveled, or time moving over the

course of the 30 days that the experiment was conducted. For all analyses, normality tests were conducted, with all datasets deemed normal. All values are presented as mean ± standard error.

2.4 Experiment #3: Characterization of Metabolic Responses to Elevated Temperatures

The following fall, RBD, JD, and FTD were again collected from the Grand River at West Montrose in October 2023, with all capture and housing conditions directly matching those delineated in 2.1. All species were acclimated to lab conditions for two weeks. 14 fish per species were then exposed to five different consecutive, environmentally relevant temperature profiles, specifically, 15-30°C over 8 hours, 15°C held over 5 hours, 15-20°C over 5 hours, 15-25°C over 5 hours, and 15-30°C over 5 hours, under which their oxygen consumption (MO_2) was measured via intermittent flow respirometry. The 15-30°C 8 hour ramp was used for determination of routine MO_2 at 15, 20, 25, and 30°C, while the following four, 5 hour trials were used for determination of maximum MO_2 at 15, 20, 25, and 30°C. Each species was divided in two sets of seven fish which cycled through each of the temperature profiles working through Set 1 of each species first followed by Set 2. For example, the order would proceed as follows: RBD set 1 (n=7) exposed to 15-30°C over 8 hours, then FTD set 1 (n=7), JD set 1 (n=7), RBD set 2 (n=7), FTD set 2 (n=7) and lastly JD set 2 (n=7), with each species set taking one full day per temperature profile (Fig. 2.3). This set up also allowed for each set to have one week of rest and feeding after each respirometry trial. As such, each temperature profile took roughly one week to complete, resulting in a total experimental duration of 5 weeks.

2.4.1 Intermittent Flow Respirometry

The rate of oxygen consumption used in this study was determined via intermittent flow respirometry, which alternates between periods of active and inactive water flow, through pump connections controlled by Loligo AutoResp v3 software (Vborg, Jutland, Denmark). During the activated water flow cycle, known as the flush, fresh water is pumped from the surrounding water of the trough through the chamber and expelled back to the trough water. The deactivated water flow period, known as the measurement cycle, pumps water in a closed loop from the chamber across a fibre-optic O_2 sensor (PreSens, Regensburg, Germany), effectively determining the MO_2 by measuring the decline in PO_2 of the closed loop water over time. In between the flush and measurement cycles is a short wait period wherein the flush pumps are turned off and measurements are turned on, priming the closed loop for measurement without data collection. Here, we employed a 3 minute flush period, 30 second wait, and 3 minute measurement.

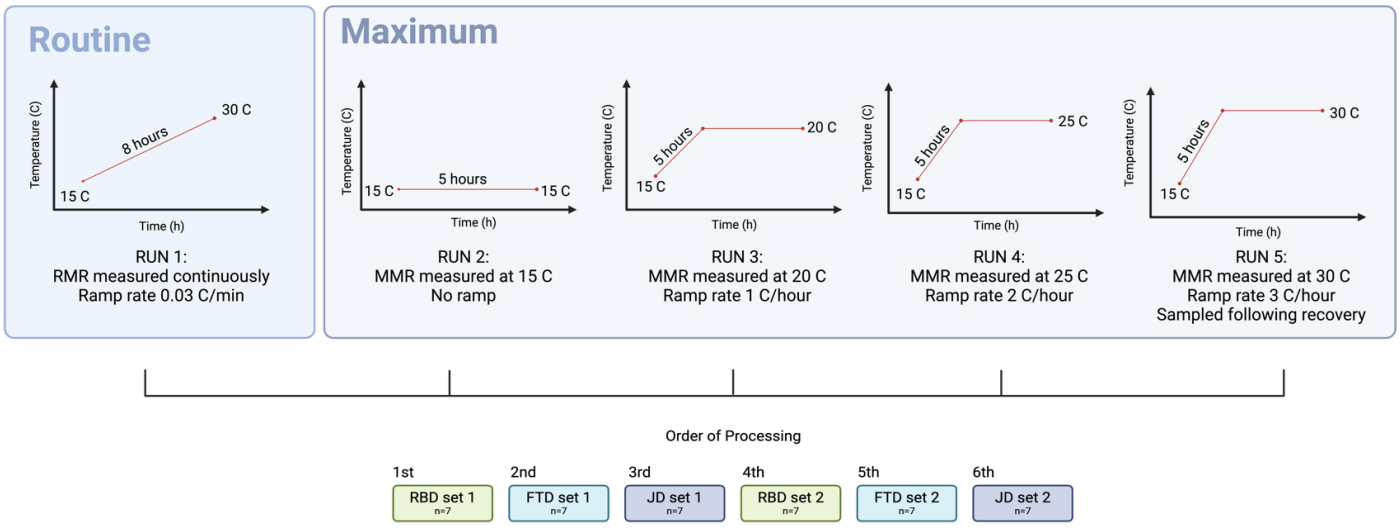


Figure 2.3: Experiment #3 schematic. Intermittent flow respirometry was utilized for determination of oxygen consumption (MO_2) in Johnny, Rainbow and Fantail darters, following 5 different temperature ramps. All fish were placed into chambers at 15°C overnight prior to measurement. Each species set rested for one week following trial.

At 5 PM, after fasting for 24 hours, seven fish of one species were removed from their holding tanks, weighed, and placed into 150 mL cylindrical glass chambers immersed in a ~50 L plastic trough, with one chamber left empty to determine background respiration. Once in their respective chambers, a privacy shade was placed around the perimeter of the trough to eliminate external disruptions, and fish were left to acclimate overnight at 15°C. Without removing the privacy shade, temperature ramp profiles were started at 8:30 AM the following morning, in which the 50 L water bath was heated via Julabo portable immersion circulator (Seelbach, Baden-Württemberg, Germany) specifically programmed for each thermal profile through the EasyTemp software. After completion of the 5-hour temperature ramps, water bath temperature was held at the upper limit of the ramp as fish were individually removed from their chambers for a two minute air exposure to determine maximum metabolic rate. Air exposure was used in lieu of the typical chase protocol due to previous reports of darter inactivity in response to a chase stimulus (Roche *et al.*, 2013; Hodgson *et al.*, 2020).

2.4.2 Calculations

Absolute AS was calculated for each of the four temperature points, 15, 20, 25, and 30°C, by subtracting the lowest routine MO_2 value for a given temperature from the maximum MO_2 value of the same temperature, with maximum MO_2 deemed the rate of oxygen consumption measured immediately upon return to the respirometry chamber (Fig. 3.9).

2.4.3 Statistical Analysis

All statistical analyses were conducted using either GraphPad prism 8.1.2 or RStudio 4.3.3, with normality assessed on all datasets. AS data was analyzed via repeated measures Two-Way ANCOVA to determine the effect of weight on oxygen consumption. Unsurprisingly, weight was a significant covariate (Appendix Table C.1); consequently, AS data was analyzed in three different ways: the first method, referred to henceforth as weight covariate AS, analyzed AS between species and 15, 20, 25 and 30°C by performing a Two Way ANCOVA that included weight as a covariate. This test saw a significant interaction between species and heat treatment. As a result, data was separated based on variable, and analyzed via One Way ANCOVA, with weight still included as a covariate, and followed by a Tukey post hoc test. In the second method, termed size adjusted AS, differences were assessed on size adjusted MO_2, in which data points were corrected to a common body mass, utilizing the slope of whole animal MO_2 x Body Mass, and analyzed via mixed effects model with Tukey post hoc test. The last analysis, referred to as mass corrected AS, performed a mixed effects model with Tukey post hoc test on AS data relative to individual weight, in which data points were simply divided by the weight of the fish. Mixed effects models were performed in GraphPad Prism; ANCOVAs were performed on RStudio, note that both analyses accounted for the repeated measures nature of the data. Additionally, weight and Fulton's condition factor (K) were compared between species by One Way ANOVA with Tukey post hoc test, to gain insight on the effect of mass on MO_2 for each species.

Chapter 3

Results

3.1 Experiment #1:

3.1.1 Critical Thermal Maximum

Thermal tolerance limits (CT_{max}) were significantly different between all three darter species (One-Way ANOVA, $F_{2,55}$ =82.59, p <0.0001) with FTD expressing the highest CT_{max} (33.3°C ± 0.18), JD second (31.9°C ± 0.14), and RBD the lowest (30.7 ± 0.21; Fig. 3.1). Weight had no impact on the CT_{max} of any species (Appendix Table A.1).

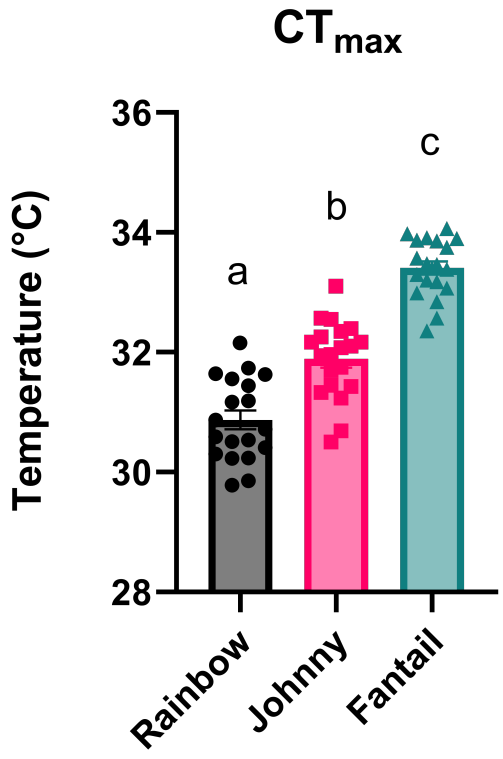


Figure 3.1: Rainbow, Johnny, and Fantail darter Critical Thermal Maximum differences. Different letters denote significant differences between groups ($p < 0.05$; n = 20; 2 CT_{max} trials with n = 10 per trial) as determined via One-Way ANOVA and Tukey post hoc test. Dots represent individual fish. Bars represent mean ± standard error.

3.1.2 Energetic Enzyme Activity

In brain tissue, all enzymes but COX expressed a significant interactive effect between species and heat treatment (Appendix Table A.2). Similarly, all enzymes had a species effect, while only MDH had a significant effect of heat treatment. Of the brain cytosolic enzymes (Fig. 3.2 A), PK and LDH, FTD appeared to have a baseline advantage, having 141% and 71% higher baseline enzymatic activity than RBD and JD for PK (Tukey, JD vs FTD, $p<0.0001$; RBD vs FTD $p = 0.0003$) and 122% higher than JD for LDH (Tukey, FTD vs JD, $p<0.0001$). Conversely, JD consistently had the lowest LDH and PK activity at baseline, with RBD and FTD activity significantly higher than JD for both (Tukey, PK: RBD vs JD, $p = 0.0147$; LDH: RBD vs JD, $p=0.0142$; PK: FTD vs JD, $p<0.0001$; LDH: FTD vs JD, $p<0.0001$). At CT_{max}, RBD LDH activity was ~26% lower compared to both JD and FTD (Tukey, JD vs RBD, $p=0.0027$; FTD vs RBD, $p=0.002$). For PK, all species were different in their CT_{max} activity level with FTD the highest, and RBD the lowest (Tukey, RBD vs JD, $p = 0.0301$; FTD vs RBD, $p<0.0001$; JD vs FTD, $p<0.0001$). Interestingly, JD PK and LDH activity increased by 39% and 74% respectively, at CT_{max} compared to baseline JD activity levels (t-test, PK: $t_9 = 3.087$, $p = 0.0064$; LDH: $t_9 = 4.258$, $p=0.0005$), while RBD PK activity decreased by 19% at CT_{max} relative to its baseline counter parts (t-test, $t_9 = 2.240$, $p=0.0379$). FTD saw no intraspecific differences across heat treatment. Mitochondrial enzymes appeared to express similar trends as the cytosolic enzymes (Fig. 3.2 B). For MDH and CS, FTD tended to have higher baseline activity compared to JD and RBD (Tukey, MDH: FTD vs JD, $p = 0.0001$; FTD vs RBD, $p<0.0001$; CS: FTD vs JD, Tukey, $p = 0.0002$; FTD vs RBD, $p = 0.0354$) and higher than JD for COX (Tukey, $p = 0.0005$) Although, FTD still showed higher MDH and CS activity levels than RBD at CT_{max} (Tukey, $p<0.0001$ for both MDH and CS), the difference over JD was lost, due to a JD-specific increase in MDH (+72%) and CS (+47%) activity at CT_{max} (t-test, MDH: $t_9 = 5.242$, $p<0.001$; CS: $t_9=3.659$, $p = 0.0018$), as seen in cytosolic enzymes. RBD saw a 20% intraspecific decrease in CS activity at CT_{max} compared with baseline levels (t-test, $t_9 = 2.469$, $p =$

0.0018). COX CT_{max} activity level had no differences between species, nor any differences within species between treatment groups.

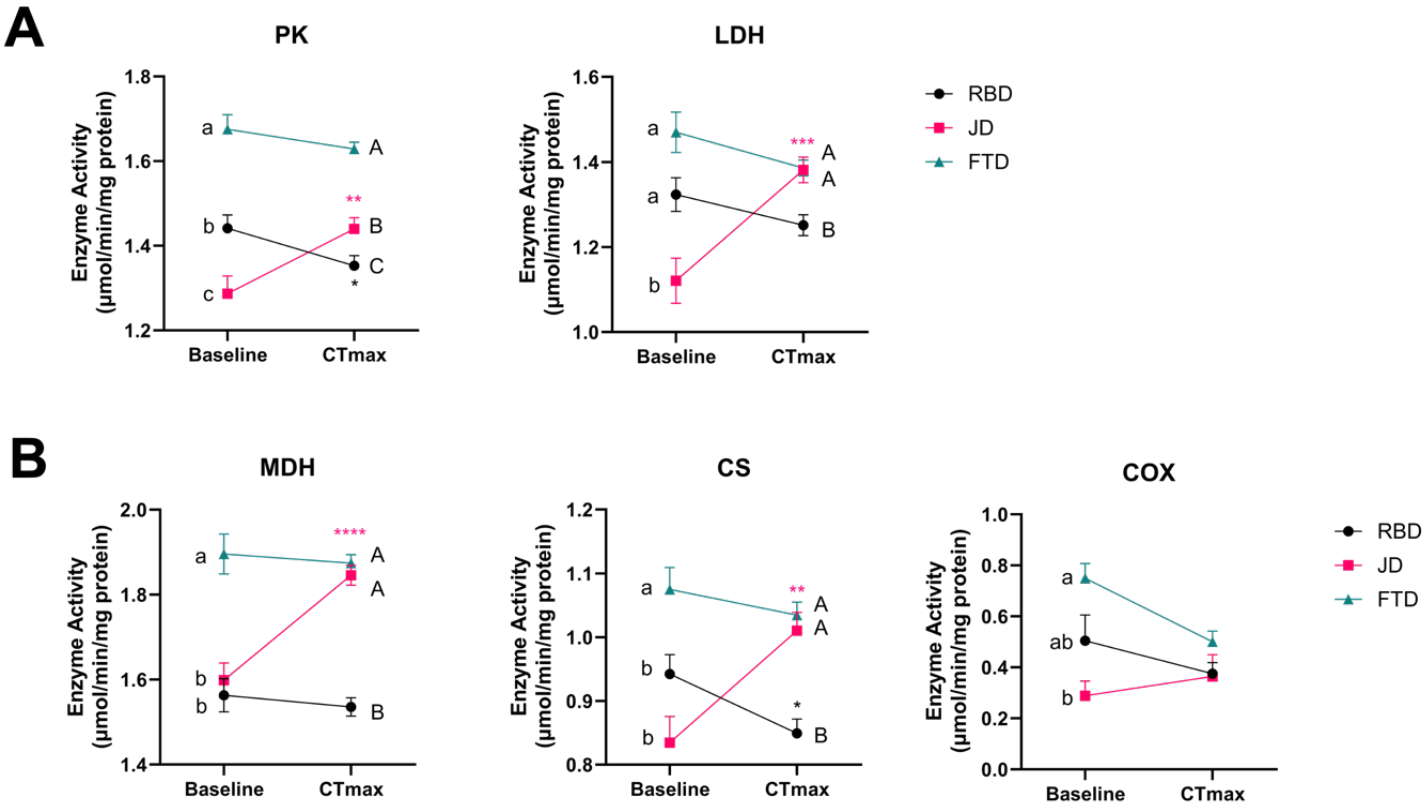


Figure 3.2: Normalized enzyme activity of Rainbow, Johnny and Fantail brain tissue at temperature baseline (15°C) and at CT_{max} in cytosolic (A) and mitochondrial (B) enzymes. Interspecies differences in activity at baseline denoted by different lowercase letters, interspecies differences in activity at CT_{max} denoted by different uppercase letter. Intraspecies differences between Baseline and CT_{max} denoted by stars ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****). All differences were determined by a Two-Way ANOVA with Tukey's post hoc test ($p < 0.05$; $n = 10$) presented as mean ± SEM. All data was log transformed.

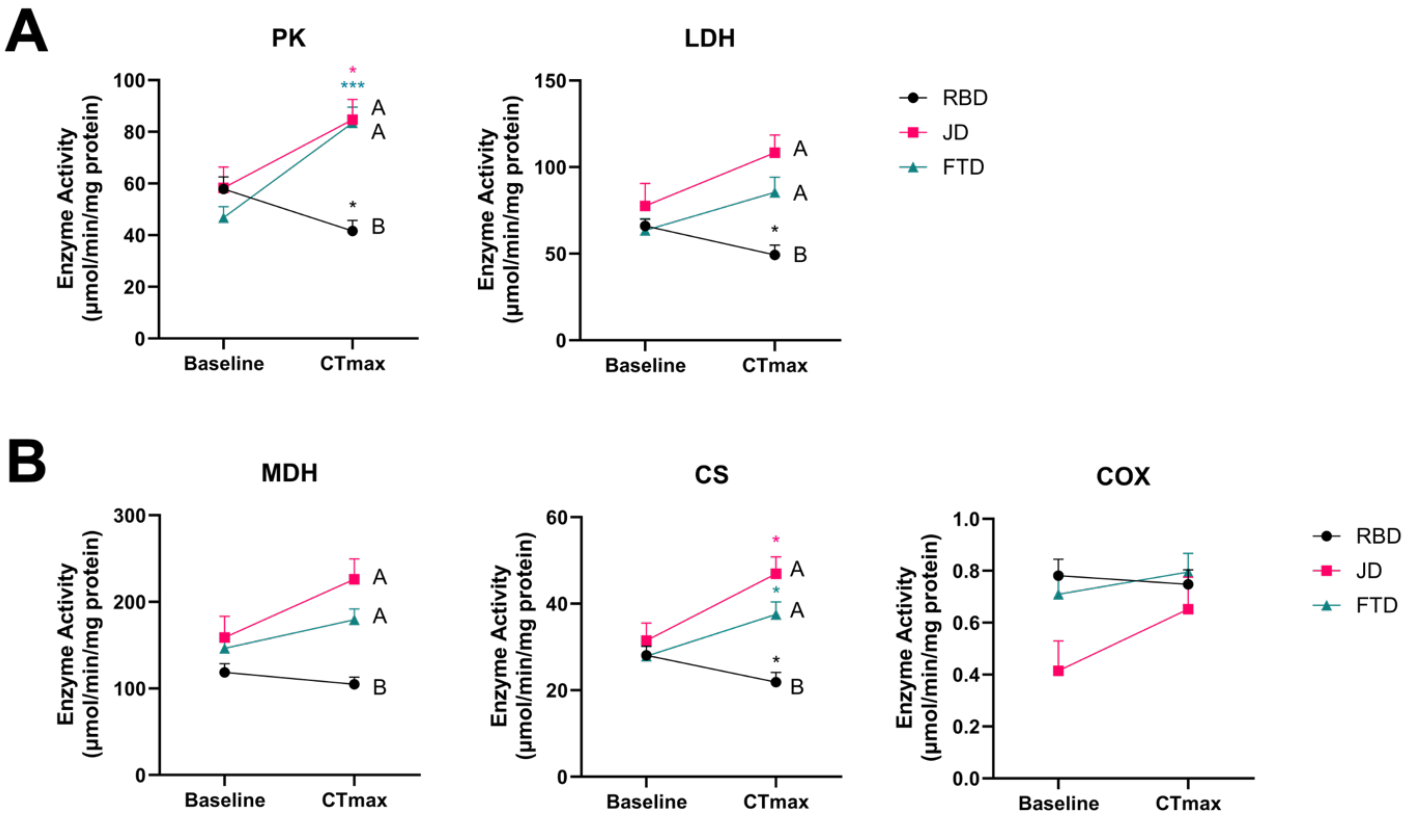


Figure 3.3: Normalized enzyme activity of Rainbow, Johnny and Fantail heart tissue at temperature baseline (15°C) and at CT_{max} in cytosolic (A) and mitochondrial (B) enzymes. Interspecies differences in activity at baseline denoted by different lowercase letters, interspecies differences in activity at CT_{max} denoted by different uppercase letters. Intraspecies differences between Baseline and CT_{max} denoted by stars ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****). All differences were determined by a Two-Way ANOVA with Tukey's post hoc test ($p < 0.05$; $n = 10$) presented as mean ± SEM. COX data was log transformed to achieve normality.

Within heart tissue, all enzymes had a significant species effect (Appendix Table A.3; Fig. 3.3 A & B). LDH and COX did not have a significant heat treatment effect, and MDH and COX did not express any interactive effect of the two variables. Heart enzymatic activity across all enzymes saw no

differences between species at baseline temperature levels. However, there did appear to be a consistent trend, excluding COX, wherein FTD and JD had significantly higher enzymatic activity than RBD at CT_{max} (Tukey, PK: FTD vs RBD, $p = 0.0002$; LDH: $p = 0.0147$; MDH: $p = 0.008$; CS: $p = 0.0037$; PK: JD vs RBD, $p = 0.0001$; LDH $p = 0.0001$; MDH: $p<0.0001$; CS: $p<0.0001$). Intraspecifically, JD seemed to have a tendency of increasing activity at CT_{max} compared to baseline across all enzymes, although this was only significantly different in PK (+45%) and CS (+39%; t-test, $t_9 = 2.354$, $p = 0.031$; t-test, $t_9 = 2.765$, $p = 0.0127$ respectively). Additionally, RBD activity levels decreased 28% in PK, 25% in LDH, and 22% in CS at CT_{max} (t-test, PK: $t_9 = 2.618$, $p = 0.0174$; LDH: $t_9 = 2.793$, $p=0.0120$; CS: $t_9 = 2.383$, $p = 0.0284$). Unlike brain tissue, FTD expressed intraspecific differences, having 78% higher PK and 24% higher CS activity at CT_{max} relative to baseline (t-test, PK: $t_9 = 4.950$, $p = 0.0001$; CS: $t_9 = 2.602$, $p = 0.0180$). COX showed no differences, neither species nor treatment related.

3.1.3 Gene Expression

Expression levels of all enzyme transcripts saw no differences between baseline or CT_{max} within heart tissue (Appendix Table A.4; Fig. 3.4). Within brain tissue, PK and LDH relative expression were 1.6% and 0.5% higher at CT_{max} in RBD (t-test, PK: $t_4 = 2.890$, $p = 0.0161$; LDH: $t_4 = 3.708$, $p = 0.0041$) which also saw a 27% decrease in CRH compared to baseline (t-test, $t_4 = 4.510$, $p = 0.0011$; Fig. 3.4B). Similarly, JD observed a 43% decrease CRH expression at thermal tolerance limits (t-test, $t_5 = 3.699$, $p = 0.0041$) along with a 5% increase in COX transcripts (t-test, $t_5 = 2.411$, $p = 0.0366$). FTD expressed only a 28% decrease in COX expression at CT_{max} (Appendix Table A.5; t-test, $t_5 = 2.263$, $p = 0.047$).

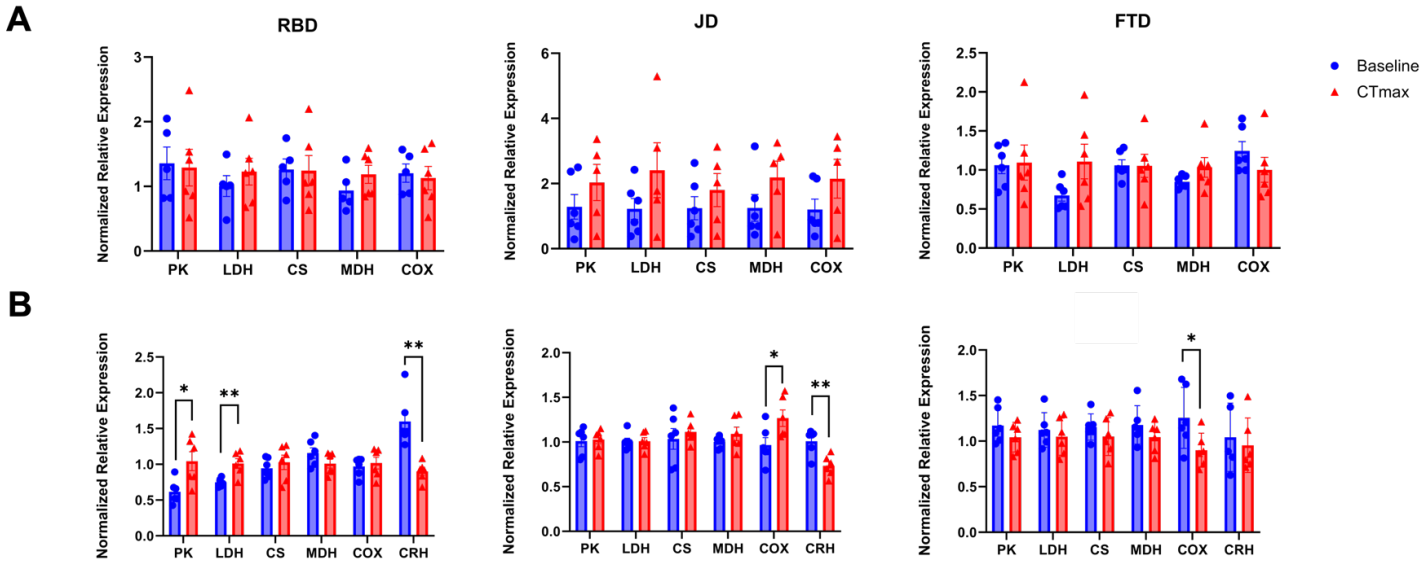


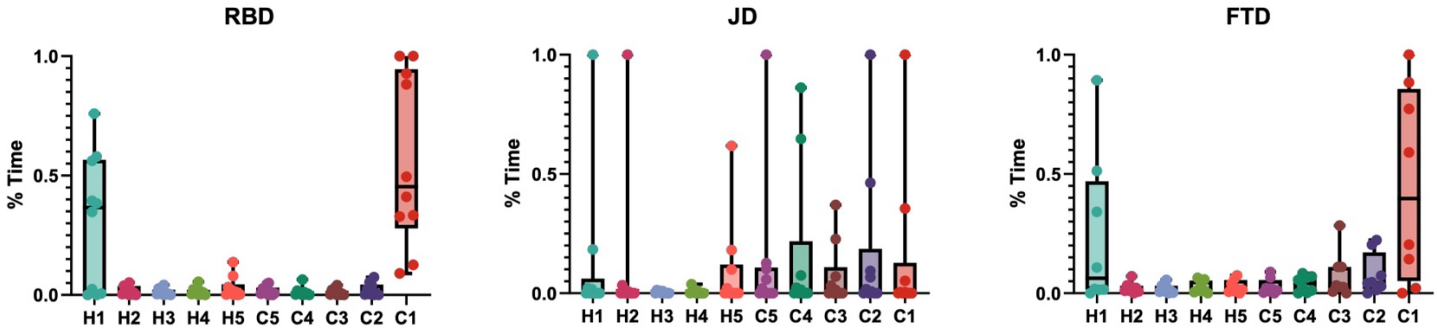
Figure 3.4: Enzyme gene expression of brain and heart tissue. The relative gene expression (mean ± SEM) of *pk, ldh, cs, mdh, crh,* and *cox* in darter heart (A) and brain (B) in either baseline or CT_{max} fish followed. Bars with differing symbols show significant differences between them as determined by a Two-Way ANOVA with Tukey's post hoc test ($p < 0.05$; n = 5-6). Dots represent individual fish.

3.2 Experiment #2

3.2.1 Thermal Preference

No differences were observed between RBD (16.40°C ± 1.47), JD (16.79°C ± 1.45), and FTD (16.08°C ± 1.85) thermal preferences (Appendix Table B.1; Fig. 3.5 & 3.6), however, both distance traveled and time moving were significant (One Way ANOVA, $F_{2,25} = 6.913, 6.432$, $p = 0.0041, 0.0056$, respectively; Fig. 3.7), with FTD being significantly higher than JD in both metrics (Tukey, $p = 0.0040$ time moving; $p = 0.0034$ distance). Additionally, none of the linear regressions conducted were

significant indicating no acclimatory effects on the distance traveled, average temperature preference, or the time moving of the darters over the course of the experiment (Appendix Table B.2).



RBD	Temperature Range (°C)
C1	10.76 – 11.48
C2	12.55 – 13.56
C3	14.05 – 15.18
C4	15.44 – 16.27
C5	16.93 – 18.09
H5	18.11 – 19.60
H4	19.51 – 20.59
H3	21.25 – 22.40
H2	22.60 – 24.36
H1	22.60 – 24.36

JD	Temperature Range (°C)
C1	10.54 – 11.39
C2	12.27 – 13.07
C3	13.99 – 14.90
C4	15.01 – 16.33
C5	15.96 – 17.45
H5	18.10 – 19.17
H4	19.40 – 20.80
H3	21.20 – 22.62
H2	22.60 – 24.16
H1	23.55 – 26.29

FTD	Temperature Range (°C)
C1	10.54 – 11.52
C2	11.96 – 13.57
C3	14.01 – 14.89
C4	15.06 – 16.09
C5	16.57 – 17.66
H5	17.87 – 18.82
H4	19.26 – 21.36
H3	21.00 – 22.39
H2	22.44 – 24.18
H1	24.46 – 25.98

Figure 3.5: Distribution of proportion of time spent in each thermal section of Rainbow, Johnny, and Fantail darters. Temperature ranges of each section over 4 weeks recorded in tables below. Dots represent individual data points. n = 10 for JD, RBD, n = 8 for FTD.

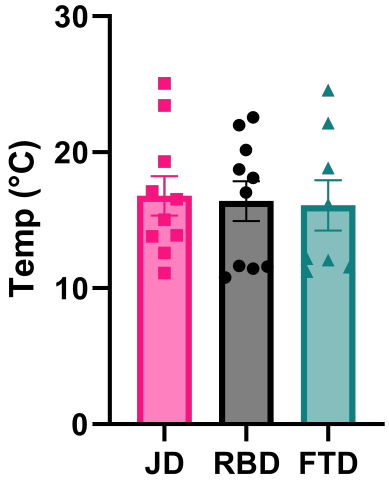


Figure 3.6: Rainbow, Johnny, and Fantail temperature preference. Determined via weighted average, wherein the proportion of time spent in a given section was multiplied by the temperature of that given section, the product for each section was then averaged to determine a single temperature. Differences were determined via One-Way ANOVA ($p < 0.05$, $n = 10$ for JD, RBD, $n = 8$ for FTD). Dots represent individual fish.

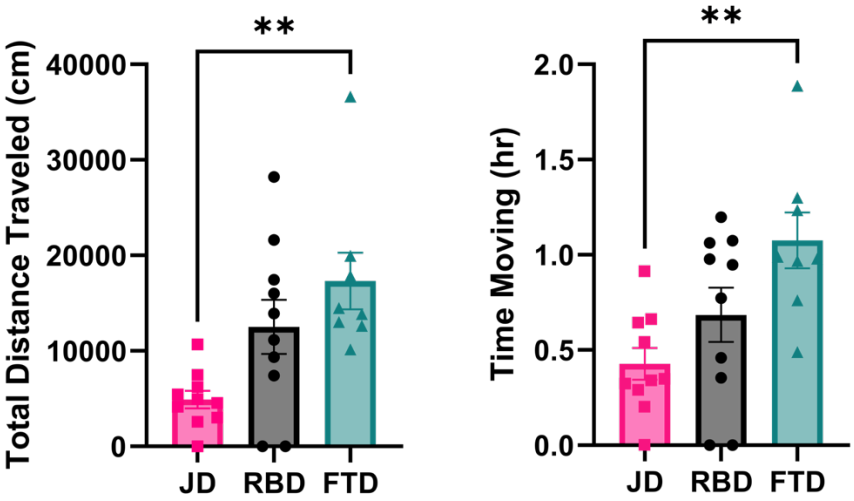


Figure 3.7: Rainbow, Johnny, and Fantail distance traveled and time moving per thermal preference trial. Determined via Animal TA tracking software. Differences were determined via One Way ANOVA ($p < 0.05$, $n = 10$ for JD, RBD, $n = 8$ for FTD). Dots represent individual data fish.

3.3 Experiment #3

3.3.1 Respirometry

Considering that weight was a significant covariate, it is no surprise that weight and condition factor (K) were significantly different between species (Appendix Table C.1; One Way ANOVA; $F_{2,35} = 18.40$, $p<0.0001$; $F_{2,33} = 41.48$, $p<0.0001$; Fig. 3.8). RBD had the highest condition, compared to JD and FTD (Tukey, $p<0.0001$ for both) which were not significantly different from each other, even though JD had a significantly lower weight than FTD (Tukey, $p = 0.0023$). RBD had the largest weight, followed by FTD, and JD last (Tukey, RBD vs JD, $p<0.0001$; RBD vs FTD, $p = 0.035$; JD vs FTD, $p = 0.0023$).

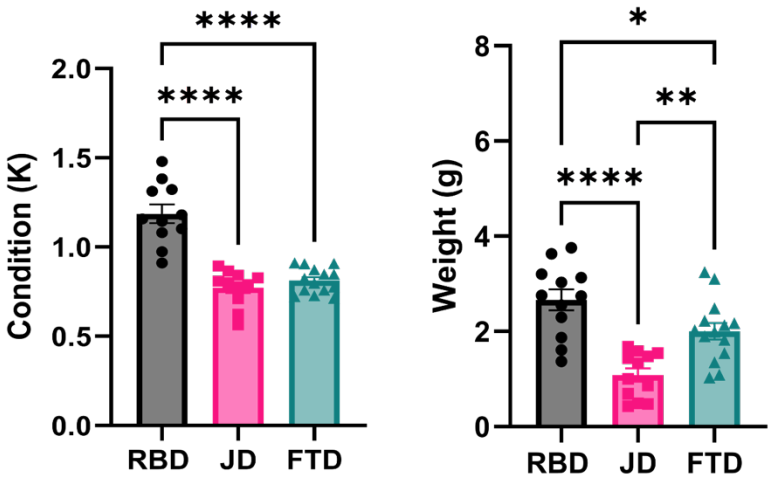


Figure 3.8: Rainbow, Johnny, and Fantail body size metrics for fish used in respirometry trials. Differences were determined via One-Way ANOVA ($p < 0.05$, $n = 10$ for JD, RBD, $n = 8$ for FTD). Dots represent individual data points.

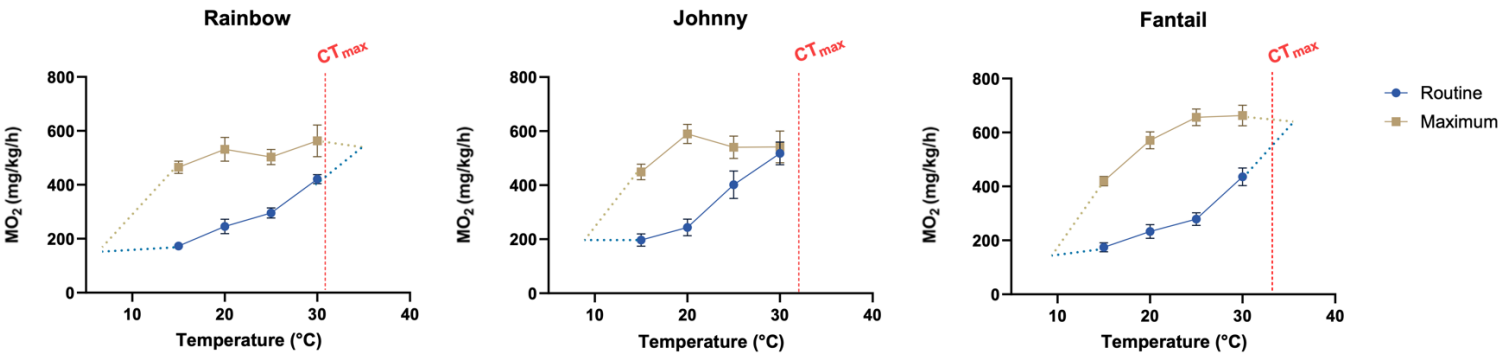


Figure 3.9: Comparison of maximum and routine MO_2 of Rainbow, Johnny, and Fantail darters across 15, 20, 25, and 30°C. Lowest routine and highest maximum values plotted for each given temperature, used in AS calculation. Previously determined CT_{max} values overlayed for comparison. Dotted lines represent hypothetical extrapolations of routine and maximum oxygen consumption.

3.3.2 Weight Covariate Aerobic Scope analysis

For data split by variable and assessed via One Way ANCOVA (Appendix Table C.2; Fig. 3.10), termed weight covariate AS, both 15 and 20°C saw no differences in AS among species (Appendix Table C.2.1). Alternatively, at elevated temperatures, 25 and 30°C, FTD had the highest AS compared to JD, (Tukey, 25°C: $p = 0.0243$; 30°C: $p = 0.0009$) and RBD (Tukey, 25°C: $p = 0.0008$; 30°C: $p = 0.0071$), having a 252% higher AS than JD, and 46% higher than RBD at 25°C, and a 425% and 148% higher AS than JD and RBD at 30°C. Analyzing across temperatures (Appendix Table C.2.2), RBD and JD had their highest AS values at 20°C, with RBD decreasing by 76% between 20 and 30°C (Tukey, $p = 0.0011$), JD by 41% between 20 and 25°C (Tukey, $p = 0.0111$), and 76% from 20 and 30°C (Tukey, $p<0.0001$). Additionally, FTD AS peaked at 25°C, having no differences between 20 and 25°C (Tukey, $p = 0.8309$) but a significant 35% decrease between 20 and 30°C (Tukey, $p = 0.0004$) and a 40% decrease from 25 to 30°C (Tukey, $p = 0.0004$).

3.3.3 Size Adjusted Aerobic Scope Analysis

For size adjusted AS (Fig. 3.10), all variables were significant, temperature, species and their interaction (Appendix Table C.3). For interspecific differences within each temperature (Appendix Table C.3.1), at 15°C JD had significantly lower AS values compared to RBD (Tukey, $p = 0.0057$), for all the other remaining temperatures JD consistently had the lowest AS out of the three species (Tukey, 20°C: RBD vs JD, $p<0.0001$; JD vs FTD, $p = 0.0006$; 25°C: RBD vs JD, $p = 0.0008$; JD vs FTD, $p<0.0001$; 30°C: RBD vs JD, $p = 0.0434$; JD vs FTD, $p<0.0001$). Unlike the weight covariate analysis, FTD did not have significantly different AS than RBD at any of the temperatures, and instead ended up only being higher than JD at 20 (+92%), 25 (+254%), and 30°C (+1678%). Within species across temperatures (Appendix Table C.3.2), RBD appeared to have its highest absolute AS at 20°C, at which it peaked and promptly crashed as temperatures continued to rise, seen as in a 31% drop in AS between 20 and 25°C (Tukey, $p = 0.0261$), a 54% decrease between 25 and 30°C (Tukey, $p = 0.0072$), and 68% for 20 to 30°C on the whole (Tukey, $p<0.0001$). Similar responses were observed in JD, peaking at 20°C, and decreasing in AS by 92% between 20 and 30°C (Tukey, $p=0.0006$). FTD were the only species to peak beyond 25°C, with no differences between 20 and 25°C, but both temperature points significantly different than 30°C, suggesting that FTD crashed following 25°C (20-30°C -34%; 25-30°C -39%; Tukey, 20 vs 30°C, $p = 0.0311$; 25 vs 30°C, $p = 0.0047$).

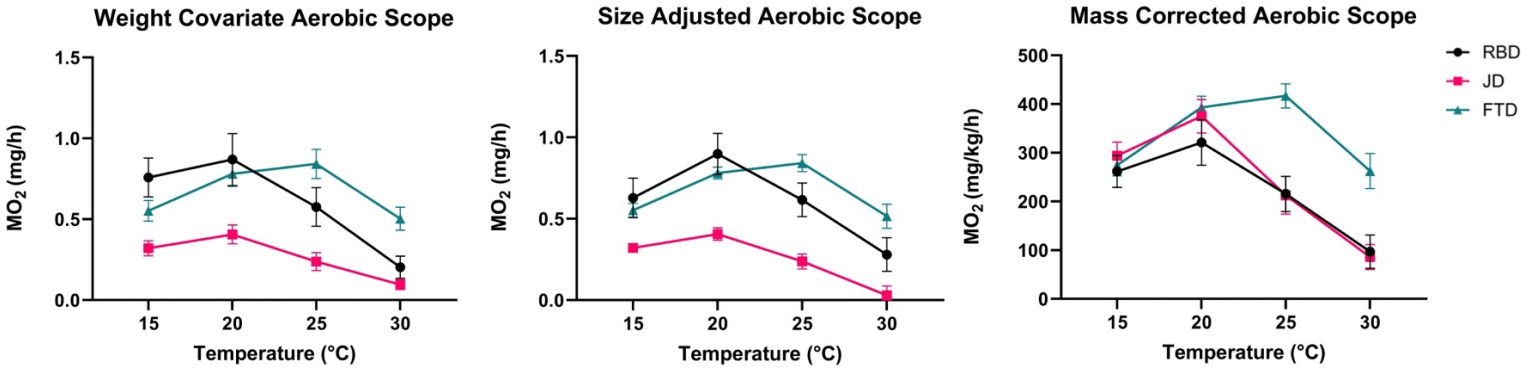


Figure 3.10: Rainbow, Johnny, and Fantail absolute aerobic scope across 15, 20, 25, and 30°C. Aerobic Scope Weight Covariate data accommodated for the impact of weight on MO_2 and was analyzed via ANCOVA, Size Adjusted Aerobic Scope corrected data using a slope (of MO_2 and body mass) conversion and was analyzed via Mixed Effects Model, and Mass Corrected Aerobic Scope divided MO_2 by each individual fish weight, analyzed also by Mixed Effects Model. Post hoc comparisons can be found in Appendix C. Individual points represent mean ± SEM.

3.3.4 Mass Corrected Aerobic Scope analysis

For the mass corrected analysis that did not include weight as a covariate, but instead had data points divided by weight of the individual, the results were largely the same (Fig. 3.10; Appendix Table C.4), with no differences between species at neither 15 nor 20°C, however FTD did have significantly elevated AS compared to RBD and JD at 25 and 30°C, specifically, ~95% higher than both JD and RBD at 25°C, and 171% and 205% higher than RBD and JD at 30°C (Appendix Table C.4.1; Tukey, 25°C: $p<0.0001$ for both; 30°C: RBD vs FTD, $p = 0.0026$; JD vs FTD, $p = 0.0007$). For RBD and JD, again their AS values peaked at 20°C (Appendix Table C.4.2; Tukey, RBD: 20 vs 30°C, $p<0.0001$; 25 vs 30°C, $p = 0.0444$; JD: 20 vs 25°C, $p = 0.0008$; 20 vs 30°C, $p<0.000$; 25 vs 30°C, $p = 0.016$) with both species declining at temperatures beyond this; RBD decreased by 70% and 55% between 20 to 30°C and 25 to 30°C, JD dropped by 77% from 20 to 30°C, and 43% between 20 to 25°C, and 60%

between 25 to 30°C. FTD peaked at 25°C, as before, (20 to 30°C -33%; 25 to 30°C -37%; Tukey, 20 vs 30°C, $p = 0.0105$; 25 vs 30°C, $p = 0.0017$) although there were now significant increases in AS leading up to 25°C (15 to 20°C +43%; 15 to 25°C +51%; Tukey, 15 vs 20°C, $p = 0.0199$; 15 vs 25°C, $p = 0.0032$).

Chapter 4

Discussion

In fish, analysis of metabolic function under temperature elevations is essential for predicting how species will respond to extreme heat events and determining the future implications of climate change on species abundance and distributions (Schulte, 2024). This study characterized the metabolic ability of Rainbow, Johnny, and Fantail darters to cope with temperature increases through examination of their thermal tolerance limits, thermal preferences, and alterations in enzymatic function and AS following exposure to acute temperature stressors. In all experiments conducted, each species responded differently, suggesting that although closely related, some may be more resilient than others, providing insight into potential alterations that may be observed in local ecosystems with the progression of climate change.

4.1 Experiment #1: Assessment of Thermal Tolerance Limits and Energetic Enzymatic Activity

4.1.1 Determination of Thermal Tolerance Limits

Significant differences in thermal tolerance limits between darter species were observed, with FTD demonstrating the highest $CT_{max,}$ JD moderate, and RBD lowest. These results align with trends observed by Ingersoll and Claussen, who found FTD to have higher thermal tolerance limits than JD, and with Borowiec et al., who observed nearly identical RBD CT_{max} values as those demonstrated here (Ingersoll and Claussen, 1984; Borowiec *et al.*, 2024). It is possible the differences in darter thermal tolerance limits may be a reflection of the expected thermal profiles of each species' microhabitats, of which it was assumed that FTD, preferring shallow riffles, experience the most thermal variability (Kuehne and Barbour, 1983; Dallas and Rivers-Moore, 2011), RBD, residing in deep waters with fast flow, occupy the most thermally stable habitats, and JD, which select stagnant pools and shorelines, inhabit environments with intermediate temperature variability (Pratt and Lauer, 2013). Lower thermal

tolerances have been shown to be associated with species from more thermally stable habitats (Farless and Brewer, 2017), and similar trends have been reported in Orangethroat darters, where populations from fluctuating thermal environments tended to have a higher CT_{max} compared to those from more fixed conditions (Feminella and Matthews, 1984). Furthermore, Ingersoll and Claussen believed that their recorded differences between JD and FTD thermal tolerance limits were a result of habitat temperature fluctuation differences (Ingersoll and Claussen, 1984), collectively suggesting that acclimatization to variable habitats may be involved in determining RBD, JD, and FTD responses to thermal stress.

However, the impact of fluctuating habitat temperatures on thermal tolerance limits is still debated as some studies have found no correlation between temperature variability and CT_{max} or acclimation capacity (Gunderson *et al.*, 2017; Sandersfeld *et al.*, 2017; Campos *et al.*, 2018). Therefore, it may be the trends observed in this study could be attributed to microhabitat temperature alone, rather than variability, as it is well established that warm acclimated fishes have higher thermal tolerances (Comte and Olden, 2017), and prior research has reported a significant effect of acclimation temperature on CT_{max} in Fantail and Rainbow darters (Hlohowskyj and Wissing, 1985). Here, the respective depths of RBD, JD, and FTD microhabitats would be expected to result in average temperatures of low to high, coinciding with their recorded CT_{max} values, potentially alluding to the influence of acclimatization to different habitat temperatures, although this cannot be conclusively determined as no data on microhabitat depth was collected.

4.1.2 Enzyme Activity and Thermal Tolerance

RBD, JD, and FTD enzyme activity levels at baseline (15°C) and CT_{max} matched their thermal tolerance limits, with FTD demonstrating elevated enzymatic activity, and RBD low or unchanging

activity. These findings suggest that RBD may not be capable of responding or adjusting to the increased metabolic demands associated with acute temperature exposure, as their lower enzyme activity that did not increase with temperature elevations could potentially hinder the metabolic increases needed to maintain energy demand in the face of elevated temperatures. Conversely, the higher enzymatic activity in FTD at both baseline and CT_{max} may provide an advantage at handling acute temperature increases, as they are already primed to respond to increased metabolic demands, translating to high thermal tolerance limits. When interpreting these results in the context of habitat thermal profiles, these responses appear to be the opposite of what would be expected. If FTD are acclimatized to the warmest, and/or most thermally variable microhabitats, and RBD the coldest and most thermally stable, RBD would be expected to exhibit high baseline enzyme activity levels, and FTD low, as previous studies have demonstrated acclimation to warmer temperatures decrease mitochondrial enzymatic levels to accommodate the biochemical increase in metabolic rate with temperature (Shaklee *et al.*, 1977; Kraffe *et al.*, 2007; Chung and Schulte, 2015). Warm acclimation, and resultant lower enzyme activity levels, would be presumed to allow ample room to increase enzymatic activity in order to meet the energetic demands required at elevated temperatures without reaching a metabolic ceiling, resulting in a high CT_{max}. Thus, considering the unexpected results observed, it is possible that these differences in enzymatic activity at baseline and CT_{max} may not be a result of prior acclimatization, but rather support for evolutionary differences between species more generally.

Regardless, it does appear that the enzymatic activity levels observed within each species seem to correlate or play a role in their respective thermal tolerance limits. This is corroborated by recent work that observed similar responses when comparing the aerobic enzymatic activity levels of two Antarctic notothenioid fishes, white-blooded (hemoglobinless) *Chaenocephalus aceratus* and red-

blooded *Notothenia coriiceps*, measured at ambient baseline temperatures and at CT_{max}. *N. coriiceps,* which had the higher CT_{max}, tended to have higher CS and COX activities levels, compared to *C. aceratus,* in addition to possessing higher ATP levels (O'Brien *et al.*, 2018). While it is known that white-blooded fishes have lower thermal tolerance limits compared to red-blooded, this has been shown to not be a result of differences in oxygen carrying capacity (Devor *et al.*, 2016). Rather, this work illustrated cardiac mitochondrial function, particularly through increases in aerobic enzymes, may contribute to elevated thermal tolerance limits, suggesting that for both the present study, and the study mentioned, higher enzymatic activities may be responsible for elevated thermal tolerance limits particularly through improved metabolic performance (O'Brien *et al.*, 2018).

Furthermore, it was expected that, when comparing baseline and CT_{max} enzymatic activity levels intra-specifically, activity should increase with acute heat exposure due to inherent biochemical increases in reaction rates (Fry, 1947; Pichaud *et al.*, 2017). Interestingly, this was most consistently observed in JD, occasionally in some enzymes of FTD, and never in RBD. It is uncertain why JD appeared to be more plastic than the other two species, capable of increasing their enzymatic activity significantly on such acute time scales, however this again may be a result of the habitat conditions JD populations experience, as it has been shown that fish from more variable microhabitats can exhibit greater plasticity than those from more stable environments (Morgan *et al.*, 2022). Residing in sandy, stagnant pools and shorelines, often with little riparian cover, could result in these regions of the river heating up several degrees relatively quickly compared to the shallow, but fast flowing regions that FTD inhabit (Kuehne and Barbour, 1983; Ingersoll and Claussen, 1984). While FTD microhabitats would be assumed to have more temperature variability due to their shallowness, they may experience fluctuations over longer periods of time compared to JD habitats as a result of increased water flow. Although differences in timescales of acute temperature increases between JD and FTD microhabitats

were not collected here, these trends were reflected in the more moderate FTD intraspecific increases in activity: some enzymes showed significant increases in CT_{max} compared to baseline, but were not as pronounced as those observed in JD. Physiologically, it's unclear what mechanisms specifically are responsible for the striking increases in enzyme activity between CT_{max} and baseline in JD, although it could be related to increases in isozymes, enzyme co-factors, or enzyme conformational changes, all of which have been shown to increase with acclimation to warmer environments, and translate to elevations in enzymatic activity (Somero and Hochachka, 1968; Somero, 1975; Shaklee *et al.*, 1977). Alternatively, RBD likely do not experience acute temperature increases in their deep and fast flowing habitats, potentially accounting for their inability to induce alterations in activity at elevated temperatures. It's possible that acclimatization to colder, more stable conditions results in enzyme denaturation occurring at lower temperatures compared to JD and FTD, contributing to the overall lower enzymatic activity levels in RBD at elevated temperatures (Reynolds and Casterlin, 1980; Fields *et al.*, 2015), however pinpointing the exact physiological reasoning behind this is beyond the scope of this study.

From an organ-level perspective, both brain and heart enzymatic activities expressed similar trends, with the exception of interspecific differences in baseline activity levels, demonstrating no notable differences between aerobic and anaerobic enzymes measured. This may due to the reliance of brain and heart tissue almost entirely on aerobic processes (Soengas & Aldegude, 2002; Rodnick & Gesser, 2017), resulting in relatively minor shifts to anaerobic metabolism that simply were not detected, or due to the short timescale of the CT_{max} trial, which may not have provided enough time to allow a full initiation of anaerobic pathways. Elevations in anaerobic activity and decreases in aerobic enzyme activity levels may be more apparent in tissue like muscle, that is more capable of sustaining

hypoxic conditions, and thus would clearly depict the shift from aerobic to anaerobic energy production due to low tissue oxygen as a result of acute temperature increases.

4.1.3 Enzyme Gene Expression and Thermal Tolerance

Enzymatic gene expression changed minimally between baseline and CT_{max} levels in both brain and heart tissue, likely due to the acute nature of the heat stressor (~1hr), which may not have been a sufficient amount of time to elicit a transcriptional response, as evidenced by recent studies (Wang *et al.*, 2023). However, of the changes that did occur in brain tissue, differences in CRH seem to correlate somewhat with the thermal tolerance limits of the species. With both JD and RBD having greater than 27% decreases in CRH expression at CT_{max} compared to baseline, and both having lower thermal tolerance limits, it's possible that the nature of the CT_{max} stressor, being an acute and intense heat exposure, may have quickly depleted CRH stores or suppressed CRH release via negative feedback, potentially resulting in a decline in CRH transcripts. (Huising *et al.*, 2004; Doyon *et al.*, 2005). The lack of CRH changes observed in FTD could indicate that these darters were less stressed at higher temperatures compared to RBD and JD, supporting their high CT_{max}, however these conclusions remain speculative as further analysis of cortisol levels would provide greater insight into the relative stress responses of each species.

While RBD did demonstrate increases in PK and LDH enzyme expression, potentially indicative of a switch to anaerobic energy production methods at thermal tolerance limits, these differences were ~1%, and thus likely not biologically significant. Furthermore, the changes in relative transcript expression of COX at $CT_{max,}$ increasing in JD, and decreasing in FTD, are puzzling. The 5% elevation in JD may be an attempt to increase aerobic capacity, although similar responses were not observed in any of the other aerobic enzymes measured. Likewise, the 28% decrease in COX expression

at CT_{max} in FTD, is the opposite of what would be expected. It could be that energy was allocated elsewhere, potentially toward expression of transcripts of higher need like heat shock proteins that would help mitigate protein misfolding occurring near thermal tolerance limits (Somero, 2020), however this remains uncertain. Either way, the results observed here are inconclusive; to better understand transcriptional changes in enzyme gene expression, extended heat stressor timescales may be more appropriate.

4.1.4 Experiment #1 Conclusions

The significant differences in RBD, JD, and FTD thermal tolerance limits and enzyme activity appear to share a common thread tracing back to microhabitat temperatures and variability. While it does seem like there is an influence of habitat conditions on enzymatic activity and thus thermal tolerance limits, this relationship cannot be fully proven, as the direct impact of acclimatization to warm and/or fluctuating temperatures was not compared between the three study species. Despite this, it may be possible that the differences in microhabitats could be increasing darter resilience to temperature extremes potentially through alterations in enzyme activity, although further study is needed to fully tease apart the role of microhabitat thermal profile from other species-specific differences. Ultimately, based on their microhabitat or not, FTD are likely to have an advantage over RBD and JD when tolerating increased heat waves, due their high CT_{max} and energetic enzyme activity levels.

4.2 Experiment #2: Determination of Thermal Preference

With differences in CT_{max} and enzymatic activity appearing to match what was expected of microhabitat temperature conditions, a thermal preference trial was conducted to observe if habitat

preferences could be further validated via differences in temperature selection. Final temperature preference (FTP), which is believed to not be impacted by lab acclimation, was determined by analyzing temperature selection after five hours of acclimation to a thermal gradient (Haro, 1991; Tabin *et al.*, 2018). Surprisingly, no differences in preference were observed between each of the species, however there were significant differences in mobility metrics, with FTD traveling longer distances, and spending longer periods of time moving. These results were consistent with observations made throughout the trials, as there were a number of behavioral trends within species. Most FTD tended to swim the length of the pipe the majority of the time, while JD would often pick a specific region and stay there for the duration of the trial, although the temperature of their chosen region was never consistent. RBD preferred to sit at either end of the gradient, wedging themselves under the airline tube that exited the gradient at each end. These behavioral responses are easily observable in Fig. 3.5, where JD trials are spread across all sections, often with each individual spending a large percentage of time within a specific region, and RBD and FTD spending lots of time at either end, at which FTD would occasionally sit before turning to pace the length of the pipe again.

4.2.1 Experiment #2 Conclusions

Considering the lack of a singular temperature preference and the prominence of the behavioral responses, it was concluded that temperature of habitat conditions mattered less compared to other abiotic habitat factors that darters might select for. Previous research on habitat selection in darters have found substrate to matter most for RBD, JD and FTD over water depth and flow velocity, with RBD preferring large boulders for hiding, JD preferring sand, and FTD preferring large cobble and bedrock (Pratt and Lauer, 2013). These preferences seem to agree with what was observed, with RBD and FTD preferring the ends in an attempt to have a space to hide, reflecting their selection of larger substrates, and JD having no consistent preference as they are likely comfortable with open environments with

fewer hiding places. Additionally, the mobility levels observed between FTD and JD match with previously recorded differences between the two (Mundahl and Ingersoll, 1983), and correspond with what is known of the flow conditions of each species respective microhabitats (Kuehne and Barbour, 1983). Within FTD and RBD, higher mobility may be a result of residing in habitats with high flow velocity. In contrast, JD are presumably used to sitting in one place due to their preference for stagnant pools and shorelines (Kuehne and Barbour, 1983; Pratt and Lauer, 2013).

Although unsuccessful in determination of the thermal optimum of these species, these trials revealed that thermal profiles of microhabitats may be of less importance to these species, in comparison to other abiotic factors like substrate or water flow. To effectively determine RBD JD and FTD thermal preference, a gradient that incorporates substrate preferences may be more useful for narrowing down a singular temperature range per species.

4.3 Experiment #3: Characterization of Metabolic Responses to Elevated Temperatures

4.3.1 Analytical Methods

Despite the different approaches, each of the methods utilized to accommodate size effects when determining AS differences between darters produced similar results, with all demonstrating a decline in RBD and JD AS after 20°C, and FTD after 25°C. Of the three, size adjusted AS generated the most different results, which is unsurprising considering the data was mathematically altered to convert all points to a common body mass, as opposed to the other two methods which simply accommodated individual mass into their analysis. Specifically, size adjusted data found JD to have the lowest AS compared to both RBD and FTD across three out of the four of the temperatures measured, a response not observed in the covariate nor mass corrected analyses. This is likely a result of JD having

a lower AS compared to FTD in the mass-corrected analysis of AS data, despite having the smallest body mass. Typically, smaller mass would result in a larger mass-corrected AS, due to scaling of body mass and metabolic rate (Clarke and Johnston, 1999; Peck *et al.*, 2009; Kraskura *et al.*, 2023). However, JD unexpectedly had similar AS values to RBD, the largest species. Since JD did not have the highest AS when mass corrected, the slope conversion to a common body mass, which was likely larger than the average JD body size, resulted in further decreases in AS values, causing JD to display the lowest AS for all temperatures in the size adjusted analysis. Additionally, size adjusted was the only method to report FTD AS as higher only than JD at 20 and 25°C. In the weight covariate and mass corrected analyses, FTD AS was higher than both RBD and JD at elevated temperatures. It's possible that the slope conversion adjusted RBD values to a common body mass that was smaller than their species average mass, resulting in an increase in their metabolic rates, and thus AS. Consequently, the difference between FTD and RBD may have decreased, making FTD AS values at elevated temperatures higher than JD.

Even with these differences, FTD, despite not having the smallest body mass, consistently exhibited higher and sustained AS values at elevated temperatures compared to JD and RBD, in weight covariate, size adjusted, and mass corrected analyses, suggesting a true metabolic advantage over the other two species, independent of body size.

4.3.2 Analysis of Aerobic Scope

The intra- and interspecific differences in AS amongst darters, especially the responses observed in FTD, further substantiate the patterns observed in the CT_{max}, enzyme activity, and thermal preference experiments. FTD's superior AS and their ability to sustain it at higher temperatures before metabolic failure, matches their high CT_{max} and enzymatic responses, developing a complete story where FTD's enzymatic activity and high AS at extreme temperatures culminate in an exceptional

energetic capacity to handle temperature stressors, resulting in high thermal tolerance limits. Although FTD metabolic responses didn't scale as expected with their intermediate body mass, their increased AS may again be a reflection of their warm and variable habitat conditions. Previous studies have found broader and higher AS in fish from variable conditions compared to those kept in stable conditions in a lab, further supporting the potential influence of microhabitat on resilience to temperature increases (Morgan *et al.*, 2022). Additionally, FTD mobility may play a significant role in their high AS. More mobile species, particularly those from faster flowing environments, are known to have elevated AS, potentially due to their ability to maintain higher levels of energy expenditure (Peck *et al.*, 2009; Fu *et al.*, 2022). Although a benthic species, FTD's mobility may also result in these species experiencing a variety of habitat temperatures, as evidenced by their constant movement through the temperature gradient in the thermal preference trials, thus resulting in a high CT_{max} and AS values at elevated temperatures (Farless and Brewer, 2017; Morgan *et al.*, 2022).

The metabolic responses of RBD to elevated temperatures and the associated decline in AS were expected given their body mass, as larger fish are known to decline earlier in AS with temperature elevations (Kraskura *et al.*, 2023). Like FTD, RBD's responses correspond with their expected microhabitat, cold and thermally stable, which may also be partly responsible for their lower AS at temperature extremes, considering their adaptation and acclimatization to colder environments may render these species metabolically incapable of responding to temperature elevations, compared to other species from warmer habitats. Additionally, given their expected preference for colder environments, exposure temperatures used in the AS trials may have exceeded the optimal range at which RBD achieve their highest AS.

Conversely, the low AS of JD was surprising, as it was assumed that smaller fish from more thermally variable habitats would exhibit high AS, capable of withstanding acute temperature increases.

This may indicate that mobility, or other lifestyle differences, have a greater impact than habitat temperature. In the thermal preference trial, JD had the lowest distance traveled and time moving, and their selection of stagnant waters (Kuehne and Barbour, 1983; Ingersoll and Claussen, 1984), further suggests that they move very little. RBD may have exhibited a greater AS than JD potentially due to differences in water flow experienced in RBD and JD microhabitats; fishes from regions of faster flow have been recorded to have higher AS (Fu *et al.*, 2022), and thus the stagnant pools that JD are found in likely exert little energetic influence. Consequently, JD limited movement may result in lower metabolic functions, making them incapable of handling increased energetic demands associated with acute temperature elevations.

4.3.3 Experiment #3 Conclusions

In summary, while the differences in AS observed between species did not follow expected metabolic body size relationships, they did support the established trends consistent throughout this study. FTD's superior AS at high temperatures matches their elevated enzymatic activity and high thermal tolerance limits, potentially implying their excess aerobic capacity may be behind their comparatively high CT_{max}. Although the demonstrated differences in AS do correlate with RBD, JD, and FTD temperature conditions, the exact role of microhabitat cannot be conclusively determined, as other interspecific factors, like differences in mobility, may also have impacted darter AS differences at each of the temperatures studied.

4.4 General Discussion and Future Directions

Overall, the combination of the three experiments conducted in this study present a comprehensive narrative on the capabilities of RBD, JD, FTD to handle acute temperature increases, and broader climate change. While thermal tolerance limits, enzyme activity, and AS of each species appear to align well with what would be expected of the temperature conditions and variability of their respective microhabitats, it is likely that habitat acclimatization is not solely responsible for the interspecific differences observed between RBD, JD, and FTD. Lifestyle and activity, as seen in the mobility differences between species, can greatly impact metabolism and thus responses to environmental temperature increases in fish, along with differences in the evolutionary history of species (Farless and Brewer, 2017). While it is known that RBD, JD, and FTD are closely related, all belonging to the genus *Etheostoma*, phylogenetic analyses revealed FTD and JD to be more closely related than RBD (Near *et al.*, 2011). In light of this, and the interspecific differences observed in this study, it was theorized that FTD and JD could be temperature generalists, given their occupancy in shallow or stagnant, thermally variable habitats, ability to handle acute temperature elevations, and lack of thermal preference, observed when sitting in any section of a temperature gradient in the case of JD, or swimming continuously without selecting a specific temperature in FTD. Conversely, RBD may be more of a temperature specialist, preferring cooler and thermally stable waters, and performing poorly (i.e. low thermal tolerance limits, enzymatic activity, and AS) whenever temperatures are elevated. Although it might be expected that temperature specialists have a distinct and narrow thermal optimum or preference, this was not observed within the scope of this study, potentially due to the lack of incorporation of substrate in the thermal preference trial, which may be more important than temperature for habitat selection in RBD. Additionally, it is possible that the metabolic AS analyses conducted in this study did not provide a low enough temperature to exhibit optimum RBD performance

and high AS values, often associated with specialists. Consequently, the distinctions of specialist to generalist between RBD, JD, and FTD may fall along a spectrum, of which it was hypothesized RBD to have more specialist tendencies, and FTD more generalist, with JD falling somewhere in the middle.

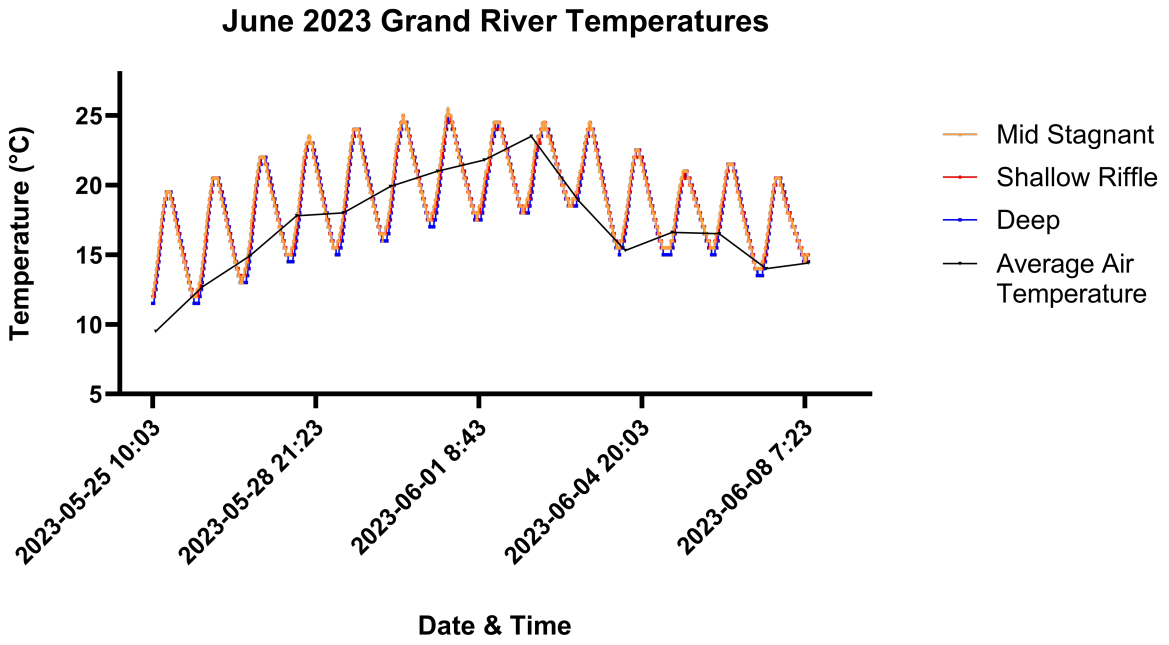


Figure 4.1: Summer 2023 West Montrose temperatures. Thermochron iButtons were placed at different regions of the river and recorded temperature every 10 minutes over the course of two weeks.

Unfortunately, the role of microhabitat thermal conditions in determining thermal tolerance limits cannot be conclusively determined here, as we did not have definitive results on differences in temperature conditions between RBD, JD, and FTD microhabitats. Although temperature probes were placed at three different sites of different depths for two weeks (Fig. 4.1), no significant differences were observed between the temperature conditions of each site over that time. This may have been too short of a period to effectively capture habitat temperature differences, or, considering these probes

were placed at the beginning of this study, the exact species location sites had not be fully determined at that time, and therefore, the probes may not have been placed in the exact location of each species' specific microhabitats. Regardless, it is still expected that the three darter species experience different thermal profiles, based on historical reports of RBD, JD, and FTD habitats, and previous research done on RBD, JD, and FTD habitat temperatures, all of which claim habitat temperature and variability differences (Becker, 1983; Kuehne and Barbour, 1983; Ingersoll and Claussen, 1984; Pratt and Lauer, 2013). To fully determine the exact impact of habitat temperature profiles on each species ability to handle acute temperature stress, lab acclimation to warm, cold, or variable conditions may be better suited.

Additionally, it should be noted that although exposure to warm habitats and temperature variability may improve thermal tolerance limits, this may not necessarily imply an improved ability of responding to long term climate change. Recent studies have found that fishes from more thermally variable habitats did not have elevated acclimation capacities to warmer temperatures, suggesting that, although their CT_{max} may be high, they have limited acclimatory ability, potentially resulting in an inability to respond to more chronic habitat temperature changes (Gunderson and Stillman, 2015; Campos *et al.*, 2018). Furthermore, while acclimatization to warmer habitats may translate to a higher CT_{max} in fish, this often comes at the cost of a lower thermal safety margin, the difference between acclimation temperature and their thermal tolerance limits, making these species more vulnerable to acute environmental warming (Campos *et al.*, 2018). Similarly, previous studies on barramundi (*Lates calcarifer*) have shown that maximal AS values can occur at temperatures within a few degrees of lethal limits, suggesting that an optimum AS at a high temperature may not imply elevated ability to handle further temperature extremes (Norin *et al.*, 2013). Consequently, this study cannot comment fully on

darter capabilities at handling acute thermal stress following long term changes in habitat temperatures with climate change, as their differences in acclimation capacity were not studied.

Nonetheless, high CT_{max} and elevated AS have been shown to translate to an enhanced ability to handle acute temperature stress in the wild (Farrell *et al.*, 2008; Eliason *et al.*, 2011), as such FTD will likely handle these stressors best of out the three darters studied. However, if microhabitat temperature conditions are indeed partially responsible for the interspecific differences observed here, then broader conclusions on RBD, JD, and FTD responses to acute thermal stress may be most applicable to populations found near the capture site, within the Grand River, as other populations may be acclimatized to different habitat temperature conditions. Even so, it is suspected that differences in capabilities between darters are influenced, in part, by non-acclimatory impacts like lifestyle, habitat selection, and evolutionary history (i.e. the effects of previous adaptations), that are likely consistently exhibited within species across populations. Although these specific species differences were not directly examined in current study, the observed results align closely with previous research on other RBD, JD, and FTD populations' thermal tolerance, thermal preference or habitat selection, suggesting that many of the findings here may be applicable species-wide (Ingersoll and Claussen, 1984; Borowiec *et al.*, 2024).

Ultimately, FTD may be the best equipped at responding to temperature-driven increased metabolic demands given their elevated baseline enzymatic activity and broader AS, with this advantage supported by their superior thermal tolerance limits. In contrast, RBD are likely the most vulnerable to acute heat stress due to their low $CT_{max,}$ and reduced energetic enzymatic activity and AS at elevated temperatures. Although JD exhibited a similarly low AS as RBD, their elevations in enzyme activity at temperature extremes and higher thermal tolerance limits suggest that they may be more resilient than RBD, but less so than FTD. Regardless of their interspecific differences, all species' AS

began declining significantly at temperatures well before their thermal tolerance limits, suggesting that even at temperatures as low as 20 to 25°C, RBD, JD, and FTD experience metabolic challenges, and may have to allocate more energy toward accommodating these temperature increases. iButton probes placed at West Montrose recorded temperatures as high as 25°C in the Grand River in June of 2023 (Fig. 4.1), and live, publicly-available water temperature monitoring by Grand River Conservation Authority (GRCA) logged a high temperature of 29.2°C in July of 2024. These temperatures surpass the levels demonstrated to induce aerobic decline, and are dangerously close to the thermal tolerance limits of RBD, JD, and FTD, indicating that these darters are already at a serious risk of being adversely impacted by climate change, and may be vulnerable to extirpation if extreme heat events continue to rise without human intervention to reduce water temperatures. Additionally, contaminants like pharmaceuticals and herbicides (Table 4.1) are being detected at biologically relevant concentrations within the Grand River. These chemicals are known to have a number of adverse physiological effects on exposed fishes, including alterations in metabolic function (Schultz and Furlong, 2008; Bisesi *et al.*, 2014; Mehdi *et al.*, 2018; Weber *et al.*, 2023), resulting in further metabolic stress and decline in AS. Given the combination of these chemical and temperature stressors in the wild, RBD, JD, and FTD may need to make metabolic tradeoffs to maintain physiological homeostasis, and consequently, less energy may be available for processes like growth and reproduction, potentially resulting in decreases in population sizes.

Going forward, there are a number of different routes of study that could build upon the data demonstrated within this thesis. To further understand how RBD, JD, and FTD will be impacted by climate change, future studies focused on characterizing darter metabolic responses like aerobic scope to acute temperature increases in tandem with exposure to biologically-relevant concentrations of contaminants may provide a more environmentally-reflective picture of the relative abilities of these

species to handle multiple stressor interactions. Analyzing AS at a wider range of temperatures, in addition to the four measured here, could also be used to develop thermal performance curves for each species, providing additional metrics, like thermal breadth and thermal optimum, that could further characterize darter responses to climate change. Moreover, analysis of microhabitat temperature profiles and the impact of acclimation to different degrees of habitat temperature variability may shed more light on the role of microhabitat temperature conditions on darter resilience to temperature increases.

Table 4.1: Contaminants recorded in the Grand River at West Montrose Summer 2023. Grab samples were taken from the middle and shorelines of the Grand River, and quantified via solid phase extraction followed by liquid chromatography and mass spectrometry for determination of chemical contaminants present.

Classification	Pharmaceutical	[ng/L]
NSAIDs	Naproxen	1.9
	Diclofenac	7.2
	Ibuprofen	3.1
Fibrates	Gemfibrozil	0.3
Antiepileptics	11,12-epoxide carbamazepine	0.4
	Carbamazepine	5.3
Antibacterials	Triclosan	3.3
	Triclocarban	0.2
	Sulfamethazine	9.6
	Sulfamethoxazole	0.0
	Trimethoprim	2.7
Pain relievers	Acetaminophen	0.0
Stimulant	Caffeine	10.5
SNRI	Desmethyl-venlafaxine	24.5
	Venlafaxine	6.0
SSRI	Fluoxetine	0.6
	Norfluoxetine	0.0
Antibiotic	Lincomycin	0.0
	Monensin	0.0
Statins	p-hydroxy atorvastatin	0.0
	o-hydroxy atorvastatin	0.0
	Atorvastatin	0.0
Herbicide	Atrazine	11.9

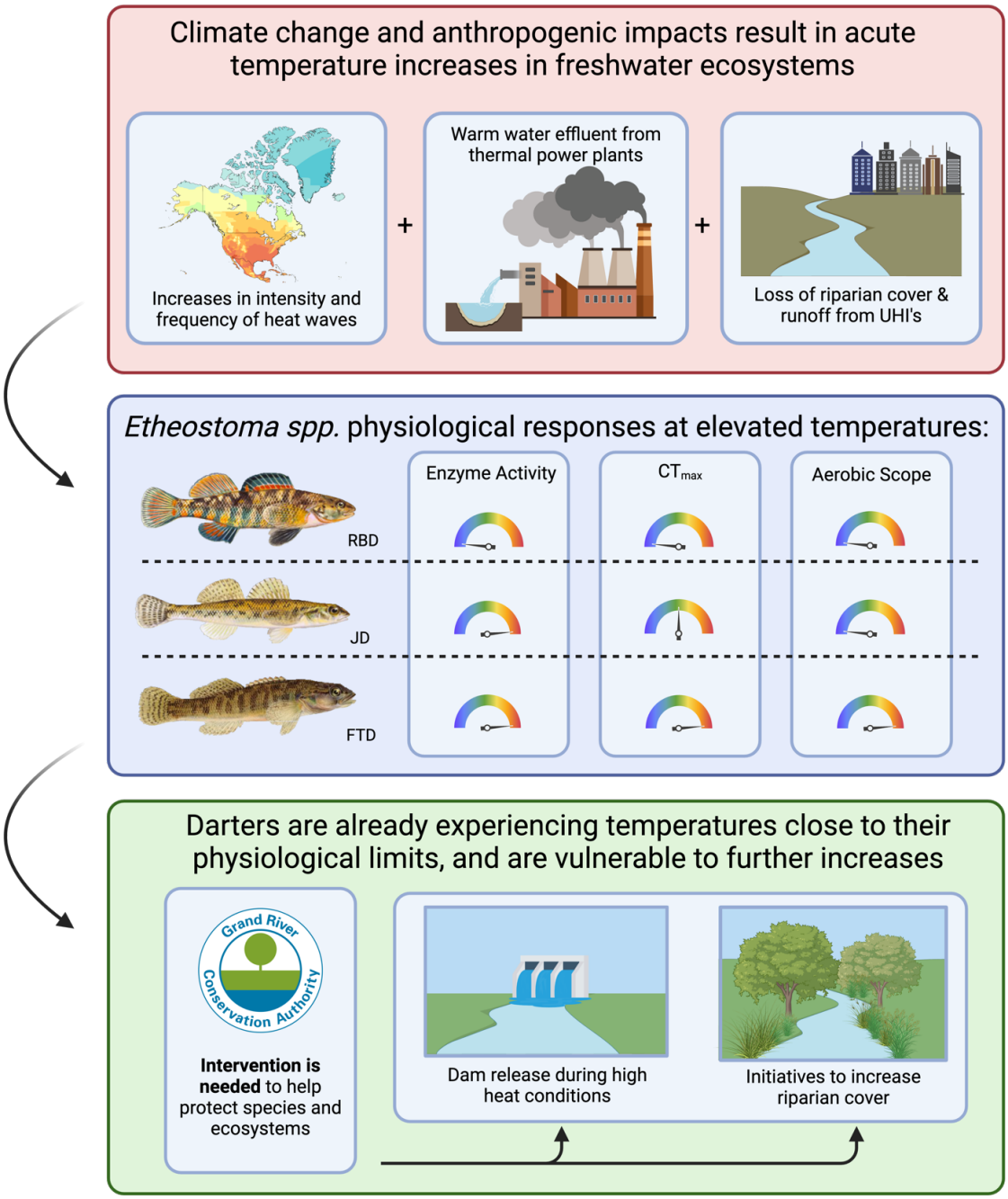


Figure 4.2: Summary of results, takeaways, and suggestions. Considering that freshwater ecosystems are already experiencing acute temperature increases, and given the sensitivities of darters to temperatures over 25°C demonstrated in this study, immediate action is recommended to mediate potential adverse effects on these species from climate change.

4.4.1 Conclusions and Suggestions

Climate change will continue to increase the occurrence and severity of extreme heat events around the globe, introducing significant thermal stress to shallow freshwater ecosystems . Given their proximity to human activity, additional anthropogenic effects like clear cutting, and loss of shade cover (Lynch *et al.*, 1984; Kalny *et al.*, 2017), along with contaminants like herbicides from agricultural run-off and pharmaceuticals from waste water effluent (Metcalfe *et al.*, 2010; Hodgson *et al.*, 2020; Gauvreau *et al.*, 2022), present further temperature fluctuations and physiological stress on exposed organisms. Since 1940, the magnitude of mass mortality events (MME) have been increasing in intensity for fishes, with the largest magnitude events caused by multiple stressor interactions (Fey *et al.*, 2015). As such, elucidating the physiological responses of freshwater species to environmental stressors is of paramount importance not only for future predictions on the impact of climate change, but for current conservation purposes as well.

The Grand River watershed of Southern Ontario has already reported increases in the frequency of heat waves, with temperature variability projected to progress further as two different models predict increases in drought occurrence and extreme heat events (Deen *et al.*, 2021). Temperature mediation and fish monitoring strategies are thus necessary to ensure the protection of these species against adverse outcomes associated with acute thermal stress from climate change. I recommend the GRCA, and other waterbody-specific conservation authorities across Southern Ontario, take action to determine potential methods that may be employed to lower water temperatures during extreme heat conditions, and develop initiatives centered on monitoring darter populations (Fig. 4.2).

The Grand River contains seven large, multi-purpose dams, built for flood control, and often released to support municipal drinking and waste water systems (Grand River Conservation Authority, 2022). Given how close RBD, JD, and FTD thermal tolerance limits are to the elevated temperatures

being recorded in the Grand River, and considering the GRCA's live reporting of river temperatures, I would advise the GRCA to release dams on particularly warm days in an attempt to cool shallow river water temperatures located downstream. Additionally, development of programs focused on increasing riparian cover over the Grand may help in keeping stagnant, shallow shorelines cool. These initiatives, in tandem with yearly darter population monitoring, may help to mitigate some of the deleterious effects ecosystems will likely experience with the expected increase in water temperatures from climate change.

Overall, it is known that physiological responses to climate change will vary across species based on their sensitivity and resilience (Williams *et al.*, 2008). However, there is a lack of research determining this in North American freshwater species that are not salmonids (Whitney *et al.*, 2016), with virtually no climate change-focused studies on *Etheostomatinae* specifically, which make up a large percentage of biomass in rivers and streams (Small, 1975), and play and integral role in the ecosystem as a food source for larger fish and birds (Kuehne and Barbour, 1983). It is therefore imperative to understand the physiological resilience of darter species to environmental stressors for protection of freshwater ecosystems, predictions on population abundance and distributions, and for informing future conservation and restoration interventions, such as potential habitat relocations. (Cooke and Suski, 2008). This thesis attempted to fill in these gaps, providing extensive results on Rainbow, Johnny, and Fantail darter physiological responses to acute temperature increases across several levels of biological organization, from biochemical to whole body, and suggests that conservation management should attempt to take the necessary measures to protect these species, in addition to North American freshwater ecosystems as a whole.

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Appendix A

Experiment #1 Supplementary Data

Table A.1 : CTmax vs Weight ANCOVA statistical results.

Metric	F	df	p
Weight	2.3533	2,54	0.1047

Table A.2 : Brain enzyme activity Two-Way ANOVA statistical results.

Enzyme	Variable	F	df	p
PK	Interaction	9.115	2,54	0.0004
	Species	54.91	2,54	<0.0001
	CT_{max}	0.0595	1,54	0.8082
LDH	Interaction	13.35	2,54	<0.0001
	Species	12.23	2,54	<0.0001
	CT_{max}	1.275	1,54	0.2639
MDH	Interaction	10.79	2,54	0.0001
	Species	49.42	2,54	<0.0001
	CT_{max}	5.728	1,54	0.0202
CS	Interaction	11.05	2,54	<0.001
	Species	15.75	2,54	<0.001
	CT_{max}	0.324	1,54	0.5716
COX	Interaction	2.943	2,54	0.0612
	Species	9.874	2,54	0.0002
	CT_{max}	3.305	1,54	0.0746

Table A.3 : Heart enzyme activity Two-Way ANOVA statistical results.

Enzyme		F	df	p
PK	Interaction	10.78	2,54	0.0001
	Species	6.826	2,54	0.0023
	CT_{max}	9.989	1,54	0.0026
LDH	Interaction	4.33	2,54	0.0180
	Species	8.433	2,54	0.0006
	CT_{max}	2.917	1,54	0.0934
MDH	Interaction	3.077	2,54	0.0542
	Species	12.36	2,54	<0.0001
	CT_{max}	4,639	1,54	0.0357
CS	Interaction	6.951	2,54	0.0021
	Species	11.18	2,54	<0.0001
	CT_{max}	6.587	1,54	0.0131
COX	Interaction	1.171	2,54	0.3177
	Species	4.321	2,54	0.0182
	CT_{max}	1.784	1,54	0.1872

Table A.4: Heart enzyme gene expression independent t-test statistical results.

Endpoint	t, F	df	p
RBD PK	0.1674, 1.508	5,4	0.8707
RBD LDH	0.8244, 1.965	5,4	0.4310
RBD CS	0.06334, 2.490	5,4	0.9509
RBD MDH	1.252, 1.201	5,4	0.2422
RBD COX	0.3217, 1.953	5,4	0.7551
FTD PK	0.1313, 4.218	5,5	0.8982
FTD LDH	1.865, 9.561	5,5	0.0918
FTD CS	0.04321, 4.376	5,5	0.9664
FTD MDH	1.426, 14.79	5,5	0.1842
FTD COX	1.226, 1.833	5,5	0.2482
JD PK	1.143, 1.794	5,5	0.2825
JD LDH	1.417, 5.755	5,5	0.1902
JD CS	0.9286, 1.703	5,5	0.3773
JD MDH	1.463, 1.241	5,5	0.1775
JD COX	1.466, 2.892	5,5	0.1767

Table A.5: Brain enzyme gene expression independent t-test statistical results.

Endpoint	t, F	df	p
RBD PK	2.890, 3.828	5,5	0.0161
RBD LDH	3.708, 8.202	5,5	0.0041
RBD CS	0.7058, 3.139	5,5	0.4964
RBD MDH	1.555, 1.433	5,5	0.1511
RBD COX	0.4863, 2.380	5,5	0.6372
RBD CRH	4.510, 6.975	5,5	0.0011
JD PK	0.2085, 1.885	5,5	0.8391
JD LDH	0.09575, 1.047	5,5	0.9256
JD CS	0.6073, 5.006	5,5	0.5572
JD MDH	1.110, 6.755	5,5	0.2929
JD COX	2.411, 1.205	5,5	0.0366
JD CRH	3.699, 1.465	5,5	0.0041
FTD PK	1.239, 1.458	5,5	0.2437
FTD LDH	0.6475, 1.140	5,5	0.5319
FTD CS	1.010, 2.086	5,5	0.3362
FTD MDH	1.244, 1.674	5,5	0.2419
FTD COX	2.263, 3.210	5,5	0.0471
FTD CRH	1.107, 12.12	5,5	0.2941

Appendix B

Experiment #2 Supplementary Data

Table B.1: Thermal preference metrics One Way ANOVA statistical results.

Metric	F	df	p
Thermal Preference	0.04911	2,25	0.9522
Distance Traveled (cm)	6.913	2,25	0.0041
Time Moving (h)	6.432	2,25	0.0056

Table B.2: Thermal preference acclimation linear regression statistical results.

Metric	Species	F	df	p
Time Moving (h)	FTD	0.06126	1,6	0.8128
	RBD	0.2015	1,8	0.6654
	JD	1.410	1,8	0.2691
Traveled Distance (cm)	FTD	0.03497	1,6	0.8578
	RBD	1.216	1,8	0.3023
	JD	1.167	1,8	0.3115
Temperature Avg	FTD	0.8827	1,6	0.3837
	JD	0.001960	1,8	0.9658
	RBD	0.1359	1,8	0.7219

Appendix C

Experiment #3 Supplementary Data

Table C.1: Repeated measures Two Way ANCOVA to assess weight for respirometry data analysis. Weight proved to be a significant covariate.

Metric	F	df	p
Weight	50.5788	1,38	<0.0001

Table C.2: ANCOVA and Tukey post hoc test statistics on Aerobic Scope Weight Covariate data

	F	df	p
Temperature	19.76	3,106	<0.0001
Species	8.76	2,36	0.0008
Interaction	4.067	6,106	0.001

Table C.2.1: ANCOVA Tukey post hoc test within temperatures on Aerobic Scope Weight Covariate data

Temperature	Comparison	Stars	p
15°C	RBD vs JD		0.4551
	RBD vs FTD		0.7546
	JD vs FTD		0.6242
20°C	RBD vs JD		0.2835
	RBD vs FTD		0.1854
	JD vs FTD		0.9670
25°C	RBD vs JD		0.6817
	RBD vs FTD	***	0.0008
	JD vs FTD	*	0.0243
30°C	RBD vs JD		0.735
	RBD vs FTD	**	0.0071
	JD vs FTD	***	0.0009

Table C.2.2: ANCOVA Tukey post hoc test within species on Aerobic Scope Weight Covariate data

Species	Comparison (°C)	Stars	p
RBD	15 vs 20		0.7909
	15 vs 25		0.7850
	15 vs 30	*	0.0152
	20 vs 25		0.2571
	20 vs 30	**	0.0011
	25 vs 30		0.1272
JD	15 vs 20		0.3816
	15 vs 25		0.3529
	15 vs 30	***	0.0008
	20 vs 25	*	0.0111
	20 vs 30	****	<0.0001
	25 vs 30		0.0821
FTD	15 vs 20	*	0.0135
	15 vs 25	**	0.0011
	15 vs 30		0.9611
	20 vs 25		0.8309
	20 vs 30	**	0.0048
	25 vs 30	***	0.0004

Table C.3: Mixed effects model and Tukey post hoc test statistics on Size Adjusted Aerobic Scope data

	F	df	p
Temperature	19.58	3,105	<0.0001
Species	29.95	2,39	<0.0001
Interaction	2.678	6,105	0.0185

Table C.3.1: Mixed effects model Tukey post hoc test within temperatures on Size Adjusted Aerobic Scope data

Temperature	Comparison	Stars	p
15°C	RBD vs JD	**	0.0057
	RBD vs FTD		0.6532
	JD vs FTD		0.0519
20°C	RBD vs JD	****	<0.0001
	RBD vs FTD		0.4674
	JD vs FTD	***	0.0006
25°C	RBD vs JD	***	0.0008
	RBD vs FTD		0.0793
	JD vs FTD	****	<0.0001
30°C	RBD vs JD	*	0.0434
	RBD vs FTD		0.0928
	JD vs FTD	****	<0.0001

Table C.3.2: Mixed effects model Tukey post hoc test within species on Size Adjusted Aerobic Scope data

Species	Comparison (°C)	Stars	p
RBD	15 vs 20		0.0513
	15 vs 25		0.9959
	15 vs 30	**	0.004
	20 vs 25	*	0.0261
	20 vs 30	****	<0.0001
	25 vs 30	**	0.0072
JD	15 vs 20		0.787
	15 vs 25		0.798
	15 vs 30	*	0.0119
	20 vs 25		0.2581
	20 vs 30	***	0.0006
	25 vs 30		0.1195
FTD	15 vs 20		0.0651
	15 vs 25	*	0.0105
	15 vs 30		0.9787
	20 vs 25		0.9082
	20 vs 30	*	0.0311
	25 vs 30	**	0.00474

Table C.4: Mixed effects model and Tukey post hoc test statistics on Mass Corrected Aerobic Scope data

	F	df	p
Temperature	25.84	3,103	<0.0001
Species	9.942	2,39	0.0003
Interaction	4.164	6,103	0.0009

Table C.4.1: Mixed effects model Tukey post hoc test within temperatures on Mass Corrected Aerobic Scope data

Temperature	Comparison	Stars	p
15°C	RBD vs JD		0.8258
	RBD vs FTD		0.9876
	JD vs FTD		0.8915
20°C	RBD vs JD		0.4627
	RBD vs FTD		0.2566
	JD vs FTD		0.9113
25°C	RBD vs JD		0.9971
	RBD vs FTD	****	<0.0001
	JD vs FTD	****	<0.0001
30°C	RBD vs JD		0.9511
	RBD vs FTD	**	0.0026
	JD vs FTD	***	0.0007

Table C.4.2: Mixed effects model Tukey post hoc test within species on Mass Corrected Aerobic Scope data

Species	Comparison (°C)	Stars	p
RBD	15 vs 20		0.608
	15 vs 25		0.6452
	15 vs 30	**	0.0015
	20 vs 25		0.0775
	20 vs 30	****	<0.0001
	25 vs 30	*	0.0444
JD	15 vs 20		0.188
	15 vs 25		0.2005
	15 vs 30	****	<0.0001
	20 vs 25	***	0.0008
	20 vs 30	****	<0.0001
	25 vs 30	*	0.016
FTD	15 vs 20	*	0.0199
	15 vs 25	**	0.0032
	15 vs 30		0.9856
	20 vs 25		0.9345
	20 vs 30	*	0.0105
	25 vs 30	**	0.0017