

Seasonal and environmental effects on physiological tolerances and gill adaptations of imperilled Eastern Sand Darter (*Ammocrypta pellucida*)

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of

Contributions included in the 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.

Statement of Contribution

Britney Firth was the sole author for Chapters 1 and 6 which were written under the supervision of Dr. Michael Power and were not written for publication.

This thesis consists in part of four manuscripts written for publication. Exceptions to sole authorship of material are as follows:

Chapter 2: Seasonal and environmental effects on upper thermal limits of eastern sand darter (Ammocrypta pellucida). B.L. Firth, D.A.R. Drake, M. Power.

While the research was my own, all authors contributed to the final product of this chapter. BLF conducted field work, data analyses, and wrote the paper. BLF, DARD, and MP conceptualized and designed the study. DARD and MP provided financial support and editorial comments on the manuscript. This chapter was published as:

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Chapter 3: Seasonal, environmental and individual effects on hypoxia tolerance of eastern sand darter (Ammocrypta pellucida). B.L. Firth, P.M. Craig, D.A.R. Drake, M. Power.

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Chapter 4: Impact of turbidity on the gill morphology and hypoxia tolerance of eastern sand darter (Ammocrypta pellucida). B.L. Firth, P.M. Craig, D.A.R. Drake, M. Power.

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Chapter 5: Impacts of temperature and turbidity on the gill physiology of darter species.

B.L. Firth, P.M. Craig, D.A.R. Drake, M. Power.

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Abstract

In the absence of accurate biological information, conservation and recovery strategies carry the risk of failing to meet their objectives. Understanding species-specific physiological tolerances is important for several aspects of conservation, such as better understanding habitat requirements, determining the significance of future environmental change, and evaluating the suitability of recovery activities such as reintroduction. Currently, there is relatively little scientific information on the physiological tolerances of Eastern Sand Darter (*Ammocrypta pellucida*), a small benthic fish listed as threatened under the *Species at Risk Act* in Canada. The goal of this thesis was to define temperature and oxygen tolerances of Eastern Sand Darter and determine the role of temperature and turbidity, two common environmental stressors, on these tolerances. I also aimed to determine the response of three co-occurring darter species to the same environmental stressors. All objectives were conducted to evaluate physiological thresholds, thereby improving our understanding of species habitat, which allows us to identify restoration priorities and evaluate the suitability of candidate sites for reintroduction.

To achieve this goal, field trials were conducted between June and November 2019 in the Grand River, Ontario, to encompass a range of ambient water temperatures (7-25°C) and August 2020 in the comparatively more turbid Thames River, Ontario. Critical thermal maximum (CT_{max}), agitation temperature (T_{ag}), hypoxia tolerance (loss of equilibrium, LOE; and critical oxygen tension), and metabolic rate were measured to test the effect of seasonal temperature change and turbidity. Gills were collected from incidental mortalities of Eastern Sand Darter, and from three co-occurring darter species (Blackside Darter, *Percina maculata*;

Greenside Darter, *Etheostoma blennioides*; and Johnny Darter, *Etheostoma nigrum*) to assess gill morphometrics.

Chapter 2 demonstrated that T_{ag} and CT_{max} significantly increased linearly with water temperature but there was no significant influence of turbidity. However, there was a significant interaction between turbidity, mass, and CT_{max} , indicating that turbidity has a hypothesized, indirect effect on CT_{max} by reducing growth. Overall, study results better define the sensitivity of Eastern Sand Darter to temperature and turbidity fluctuations and provide a better understanding of suitable thermal habitat. In Chapter 3, I demonstrated that temperature alone had no impact on metabolism but significantly and positively impacted hypoxia tolerance metrics (Loss of equilibrium and critical oxygen tensions). However, temperature explained very little of the variability seen in the response of metabolism and both hypoxia tolerance metrics across seasons, while environmental and fish-specific factors (reproduction and condition) explained more of the variation. Overall, temperature may be a weak predictor for Eastern Sand Darter metabolism and hypoxia tolerance, but the identification of a relatively high hypoxia tolerance helps to explain why the species persists in sand substrates that may have low availability of oxygen. In Chapter 4, I determined that turbidity significantly increased gill interlamellar space, filament width, and loss of equilibrium (i.e. decreased hypoxia tolerance) but had no significant influence on any other gill morphometric measurements, or on species metabolism. The findings suggest that turbidity has limited impact on Eastern Sand Darter gill physiology and that current turbidity levels do not surpass most physiological thresholds of Eastern Sand Darter. Chapter 5 demonstrated that temperature and turbidity have limited impact on the gill morphology of three common darter

species, only impacting ionocyte number, lamellae width, and hematocrit. Findings suggest that the three darter species have limited plasticity in gill morphology under the range of temperature and turbidity tested. Having data on other darter species lets one put Eastern Sand Darter in context of other members of the ecosystem that are phylogenetically related.

Overall, my thesis provides information on the physiological tolerances of Eastern Sand Darter that can be used to inform species recovery under Canada's *Species at Risk Act*, such as refining the description of critical habitat, identifying habitat restoration objectives, and evaluating the suitability of candidate sites for reintroduction. Additionally, the discovery that current turbidity levels have limited impact on Eastern Sand Darter physiology allows for a refined understanding of how siltation impacts the species survival.

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Chapter 1: General Introduction

1.1 General context

In recent years, species extinctions have increased due to habitat degradation, pollution, and invasive species (Swan *et al.*, 2018). Climate change, urbanization, and intensified land use, such as agriculture, are predicted to further accelerate extinctions (Swan *et al.*, 2018).

Freshwater organisms, particularly small-bodied fishes, face heightened extinction risk because they are susceptible to modification of both terrestrial and aquatic landscapes, resulting in a higher number of stressors acting concurrently within aquatic habitat (Ricciardi and Rasmussen, 1999; Jenkins, 2003; Olden *et al.*, 2007). Yet, for many small-bodied fishes there is a lack of detailed knowledge of their biological and habitat requirements, leading to increased uncertainty surrounding the development and implementation of appropriate species-specific conservation measures.

In Canada, the *Species at Risk Act (SARA)* is the primary mechanism used to identify, protect, and recover species at risk of extinction. Strategies to protect SARA-listed fishes include the identification and protection of critical habitat, reduction of threats, habitat restoration, and other restoration actions, such as species reintroduction (via translocation or captive breeding). The implementation of these approaches requires knowledge of an organism's biology and life history to create and effectively implement effective recovery measures. Without adequate biological information, conservation measures carry the risk of causing unintended harm.

Understanding a species' ecological niche is crucial for implementing recovery strategies and identifying habitats that should receive legal protection under *SARA*. Hutchinson's niche

concept is characterized as a combination of constraining factors within which a population can persist and is defined principally by two axes: scenopoetic (conditions such as temperature) and bionomic (resources such as food; Hutchinson 1957, 1978). Physiological limits (e.g., temperature and oxygen tolerance) can define the bounds of a species' scenopoetic niche axis and can help to inform the critical habitat necessary for a species' survival. Additionally, understanding physiological limits is imperative for discerning climate threats facing species (e.g., increased temperature and reduced dissolved oxygen) and predicting reintroduction success. For example, Turko et al. (2020) found that Redside Dace (*Clinostomus elongatus*) thermal limits were lower than the predicted peak water temperatures likely to result from climate change. Since physiological limits are easily measured, they are an obvious tool for assessing and defining critical habitat and for ensuring that habitat restoration and reintroduction sites satisfy species' requirements.

1.2 Environmental stressors

Anthropogenic changes, such as urbanization and deforestation, in conjunction with climate change, are increasing water temperatures (Webb and Nobilis, 1994; Moatar and Gailhard, 2006; van Vliet *et al.*, 2013; IPCC, 2021), which in turn can reduce dissolved oxygen as a result of the correlation between temperature and oxygen availability (Fry, 1971; Ficke *et al.*, 2007). Fishes are particularly vulnerable to rising water temperature because as ectotherms, temperature controls their key physiological processes, e.g., metabolism and growth (McNab, 2002). Consequently, thermal thresholds and the temperature-dependent nature of numerous metabolic-based performance traits (e.g., swim speed) can contribute to defining a species' niche (Schulte *et al.*, 2011). Changes in environmental conditions; therefore, can restrict,

expand, or shift species' distributions to align with their niche requirements (Sunday *et al.*, 2012; Freeman *et al.*, 2018; Shifting habitats, 2020). Shifts in distribution are generally towards higher latitudes or elevations as species attempt to maintain their preferred ecological thermal niche (Sunday *et al.* 2012; Lenoir *et al.* 2020; Antão *et al.* 2022; Rubenstein *et al.* 2023). However, in many instances freshwater species may have a limited capacity to shift their distribution to suitable habitat due to the fragmentation of river networks and lack of connections to higher latitudes or elevations (Schmutz and Sendzimir 2018). If freshwater fishes cannot shift habitats to maintain their preferred niche, they may find themselves living in suboptimal conditions, leading to a reduction in growth, reproduction and overall fitness that could result in population declines (Fry, 1971; Ficke *et al.*, 2007). Alternatively, freshwater fishes may seek out refugia that meet existing niche requirements. For example, when temperature warms at the range boundary (southern edge for northern hemisphere and vice versa) fishes may seek areas of groundwater upwelling to maintain occupancy of thermally preferred habitat and avoid suboptimal temperatures. The resulting restrictions in local distribution are likely to increase the risk of population extirpation during a catastrophic event (Finch *et al.*, 2018; Rubenstein *et al.*, 2023).

In the short-term, fishes can withstand small changes in the environment, such as increases in temperature, by acclimatizing and shifting their thermal limits (Angilletta, 2009; Schulte *et al.*, 2011). Thermal tolerance, hypoxia tolerance, and metabolic rate increase with increasing water temperature, as do both routine metabolic rate (RMR), describing the metabolic rate when the species is at rest, and maximum metabolic rate (MMR), describing the metabolic rate associated with the maximum level of energy available (Hlohowskyj and Wissing, 1985;

Beitinger *et al.*, 2000; McDonnell and Chapman, 2015; Nyboer and Chapman, 2017).

Acclimatization and shifting thermal extremes allows ectotherms to withstand hotter temperatures (Angilletta, 2009; Schulte *et al.*, 2011). However, the acclimatization of maximum metabolic rate and thermal tolerance has limitations (Sandblom *et al.*, 2016). For example, as water temperature increases, MMR either plateaus before RMR or increases in parallel with RMR until plateauing close to the maximum tolerable temperature (Schulte, 2015). As water temperature increases past where MMR plateaus (theoretically past the fish's temperature optimum into suboptimal temperatures), fishes may demonstrate behavioural avoidance (denoted as agitation temperature, T_{ag}) and search for suitable thermal refuge (Petersen and Petersen, 1990; Herbert and Steffensen, 2006; McDonnell and Chapman, 2015). If fishes cannot find a thermal refuge, aerobic scope (MMR-RMR), the energy available for additional life processes, declines leading to a reduction in growth and reproduction that reduces fitness and can result in abundance declines (Fry, 1971; McNab, 2002; Ficke *et al.*, 2007). If temperature continues to increase, the thermal threshold (CT_{max}) is reached and ultimately death occurs if environmental conditions persist beyond CT_{max} (Cowles and Bogert, 1944; Hutchison, 1961). The ongoing and sustained accumulation of individual mortalities will inevitably lead to population extirpation. Thus, determining how key physiological parameters (i.e. metabolic rate, agitation temperature, thermal tolerance, hypoxia tolerance) change with temperature can contribute to better defining a species thermal niche. Determination of key parameters can also provide an improved understanding of how species' distributions may shift in the face of climate change, help determine the probability of reintroduction successes, and help determine the ability of species to adapt to climate-driven alterations in their environment.

Plasticity in gill morphology may allow species to implement some short-term defensive adaptations to increases in temperature and decreases in dissolved oxygen. Gills control the oxygen uptake rate and have been observed to increase in surface area to improve gas exchange with increases in temperature and decreases in dissolved oxygen (Sollid *et al.*, 2003, 2005; Sollid and Nilsson, 2006). Therefore, assessing if gill morphometry can change under different environmental conditions is important for understanding if plasticity in gill morphology can improve short-term chances of survival (i.e. on generational time scales).

However, gill remodeling may not increase oxygen uptake in the presence of multiple stressors, such as in intensive agricultural zones where turbidity can exacerbate the stress of increased water temperature and decreased dissolved oxygen (Paaijmans *et al.*, 2008). Turbidity has been observed to cause gill lesions in fishes, such as the orange-spotted grouper (*Epinephelus coioides*; Wong *et al.* 2013), and hyperplasia and epithelial lifting in the sparid fish (*Pagrus auratus*; Cumming and Herbert 2016) and green grouper (*Epinephelus coioides*; Au *et al.* 2004). Thus, it is important to assess multiple stressors concurrently given that species may withstand one stressor but suffer limitations when exposed to the potential additive or interactive effects of combined stressors.

1.3 Study species

Eastern Sand Darter (*Ammocrypta pellucida*) is a small, benthic fish that is threatened by multiple stressors and exists in disjunct, localized ranges in Canada with limited dispersal opportunity (DFO, 2011). Eastern Sand Darter has recently been assessed as Threatened (Southwestern Ontario populations; West Lake population) and Special Concern (Quebec population) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2022)

and has been listed as Threatened under SARA since 2003. Additionally, Eastern Sand Darter is listed as globally vulnerable (G3) by NatureServe and the IUCN and is considered imperilled in eight of the nine American states in which it resides (Grandmaison *et al.*, 2004; COSEWIC, 2022).

The historical range of Eastern Sand Darter has declined as a result of: invasive species, baitfish harvest, impoundments and habitat deterioration from siltation, agricultural runoff, and water contamination (Scott and Crossman, 1973; Trautman, 1981; Smith, 1986; Daniels, 1993; Holm and Mandrak, 1996). Siltation is believed to be responsible for the majority of the population declines to date, likely because of its negative impacts on habitat quality, prey availability, hatching success, and respiration (i.e. increased physiological demand; Spreitzer 1979; Holm and Mandrak 1996). In Ontario, populations of Eastern Sand Darter are located in agriculturally intensive zones where siltation is common, with the populations in Big Otter Creek, Catfish Creek, and the Ausable River (all of which have longstanding and intensive agricultural activity) now considered extirpated (Fisheries and Oceans Canada, 2012). Population trajectory and status is unknown and poor in the Sydenham River but stable and good in the Grand and Thames rivers where agriculture is dominant (COSEWIC, 2022).

Studies on the biology and life-history of Eastern Sand Darter indicate that the species is a habitat specialist, preferring clean sand and fine gravel substrate (Smith, 1986; Daniels, 1993; O'Brien and Facey, 2008). Preference appears to be for the depositional zone of lotic systems with a mean water velocity and depth of < 20 cm/sec and < 0.5 m, respectively (Daniels, 1993). Eastern Sand Darter are believed to selectively feed on chironomids (Spreitzer, 1979). However, Burbank et al. (2019) indicated that Eastern Sand Darter may be more generalist feeders with

only a slight preference for chironomids. Population viability models have shown that environmental stochasticity poses the greatest risk for reducing the population persistence of Eastern Sand Darter, particularly when the survival of age-0 fish is reduced (Finch *et al.*, 2018). Drake et al. (2008) noted that siltation and channel velocity were the most important factors affecting age-0 growth.

While there are numerous studies focused on Eastern Sand Darter life-history, growth, and feeding habitats (e.g., Spreitzer 1979; Daniels 1993; Drake et al. 2008), there is little scientific information about the species' physiological tolerances (e.g., thermal and oxygen tolerance) and therefore a lack of understanding about their physiological niche and the constraints that may be placed on the species as a result of climate change and thermal habitat loss (COSEWIC, 2009). The *Species at Risk Act* requires that recovery strategies for extirpated, endangered, and threatened species be developed using scientific biological and life-history information. In the absence of information on the physiological tolerances of Eastern Sand Darter, this project aims to estimate Eastern Sand Darter thermal and oxygen tolerances to better understand the species' physiological thresholds, inform habitat suitability, and determine their potential future susceptibility to threats.

Describing the physiological thresholds of Eastern Sand Darter will help recovery biologists identify: i) aquatic habitat within the species' range best suited to the species, ii) stressors (natural) and threats (human-induced) with the potential to surpass important physiological thresholds, and iii) water quality targets for future habitat restoration. Recovery biologists will further be able to use the knowledge to update the description of Eastern Sand Darter critical habitat and to make more informed conservation decisions. Knowledge of

physiological tolerances may also help inform the successful translocation of Eastern Sand Darter by determining the suitability of thermal and oxygen conditions at candidate reintroduction sites.

Blackside Darter (*Percina maculata*), Johnny Darter (*Etheostoma nigrum*), and Greenside Darter (*Etheostoma blennioides*) were used as phylogenetically related species that co-occur in the Grand and Thames rivers with Eastern Sand Darter and may have a similar reaction to temperature and turbidity change. Using these co-occurring darter species may allow a better understanding on the impacts of temperature and turbidity on Eastern Sand Darter physiology.

1.4 Study location

This study focuses on Eastern Sand Darter from the Grand and Thames river (Ontario, Canada). The Grand and Thames rivers are the two largest watersheds in Southwestern Ontario (6,800 km² and 5,825 km², respectively) and are ecologically similar being in the same ecozone (Wilcox *et al.*, 1998; Lake Erie Source Protection Region Technical Team, 2008). Both rivers are located in intensive agricultural zones with similar fish communities and thermal regimes (Wilcox *et al.*, 1998; Lake Erie Source Protection Region Technical Team, 2008). The main difference between the rivers is turbidity, with the Thames River having significantly higher turbidity likely due to its underlying geology and greater degree of agriculture in the surrounding watershed (Stone and Saunderson, 1992; Nürnberg and Lazerte, 2015). The Grand River was chosen because Eastern Sand Darter is known to be stable and relatively abundant in the river compared to other occupied systems (Fisheries and Oceans Canada, 2012; Dextrase *et al.*, 2014) and the Grand River has low turbidity levels at our site (Lake Erie Source Protection Region Technical Team,

2008). Conversely, the Thames River was chosen because of its high turbidity, allowing the impacts of sedimentation on Eastern Sand Darter populations to be described.

1.5 Research objectives

Given the lack of current physiological information, this PhD thesis aims to describe the thermal and oxygen tolerances of Eastern Sand Darter to gain a better understanding of how these tolerances may interact with known stressors such as changes in temperature and turbidity. Specifically, the objectives are to: 1) examine seasonal changes in thermal tolerances between habitats with different turbidity levels; 2) determine whether seasonal changes in critical oxygen tolerances occur, and if so, identify relevant predictors; 3) examine the potential effects of turbidity on gill morphology and oxygen tolerance; and, 4) examine the potential effect of temperature and turbidity on gill morphology.

Chapter 2: Seasonal and environmental effects on upper thermal limits of eastern sand darter (*Ammocrypta pellucida*).

The first study objective was to determine the upper thermal limits of Eastern Sand Darter across seasons (spring, summer, fall) and between high and low turbidity to determine seasonal and habitat-specific temperature thresholds for Eastern Sand Darter. Specifically, I tested the hypotheses that: 1) Eastern Sand Darter thermal tolerance (CT_{max}), agitation temperature, and the thermal safety margin ($CT_{max} - \text{ambient temperature}$) are temperature-dependent and positively correlated with seasonal changes in water temperature; 2) CT_{max} and T_{ag} would decrease as turbidity levels rise based on Oxygen Capacity Limiting Thermal Tolerance (OCLTT) theory; and, 3) from among several stream temperature descriptors identified in the literature (minimum, maximum, mean and variance; Arismendi *et al.*, 2013), models for predicting CT_{max}

that include temperature variance would be superior to those that do not include temperature variance.

Chapter 3: Seasonal, environmental, and individual effects on hypoxia tolerance of Eastern Sand Darter (*Ammocrypta pellucida*)

Like many imperilled fishes, there is uncertainty about the sensitivity in the likely physiological response of Eastern Sand Darter to oxygen impairment. The second study objective, therefore, was to determine the hypoxia tolerance and metabolic rate of Eastern Sand Darter across seasonal temperature changes while accounting for the role of environmental and fish-specific factors (e.g., condition, reproductive season). Specifically, I tested the hypotheses that: 1) Eastern Sand Darter hypoxia tolerance and metabolic rate are influenced by seasonal temperatures; 2) the ability to explain residual variation in hypoxia tolerance and metabolic rate is significantly improved by the inclusion of environmental turbidity, fish condition, and reproductive season as explanatory variables; and, 3) among-individual variation in hypoxia tolerance and metabolic rate is influenced by seasonal temperature.

Chapter 4: Impact of turbidity on the gill morphology and hypoxia tolerance of eastern sand darter (*Ammocrypta pellucida*).

The third study objective was to assess differences in gill morphometric, metabolic rate, and hypoxia tolerance of Eastern Sand Darter between systems with low and high turbidity. Specifically, I tested the hypotheses that: 1) increases in turbidity would cause gill structural changes impacting gas exchange and osmoregulation; and, 2) damaged gills would result in higher metabolic rate and decreased hypoxia tolerances due to inefficient gas exchange.

Chapter 5: Impacts of temperature and turbidity on the gill morphologies of three darter species

The fourth study objective was to assess the differences in gill morphometrics of three darter species (Blackside Darter, *Percina maculata*; Greenside Darter, *Etheostoma blennioides*; Johnny Darter, *Etheostoma nigrum*) in systems with low and high turbidity and between warm (summer) and cool (Fall) temperature regimes. Specifically, we tested the hypotheses that: 1) gill respiratory surface area and hematocrit percentage were greater at warmer temperatures and higher turbidity levels; and, 2) ionocyte surface area and Na⁺/K⁺ ATPase activity were greater at warmer temperatures and higher turbidity levels.

Chapter 2: Seasonal and environmental effects on upper thermal limits of eastern sand darter (*Ammocrypta pellucida*).

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2.1 Introduction

An important change occurring in the habitats of stream fishes is the increase in water temperature associated with the cumulative effects of reduced riparian vegetation, loss of watershed forest cover (particularly in headwater areas) and climate change (IPCC, 2013; Sun *et al.*, 2015; Kalny *et al.*, 2017). Water temperature is a key abiotic variable for ectotherms since temperature governs physiological performance (Fry, 1947; Magnuson *et al.*, 1979; McNab, 2002; Schulte *et al.*, 2011), thereby influencing individual and population processes, including growth and survival and, ultimately, species abundance and distribution (Somero, 2005; McCullough *et al.*, 2009; Sunday *et al.*, 2012). Given the link between temperature and physiological performance, fishes are particularly vulnerable to rising water temperatures that may restrict the thermal performance windows within which their fitness is optimized (Fry, 1947; Huey and Stevenson, 1979; Schulte *et al.*, 2011). For example, when temperatures exceed the thermal performance window, physiological function declines rapidly, leading to a range of sub-lethal and lethal effects (Cowles and Bogert, 1944; Hutchison, 1961; Bear *et al.*, 2007; Ficke *et al.*, 2007). If severe, changes in temperature can cause mortality and lead to changes in abundance and (or) the distribution of species (Perry *et al.*, 2005; Comte *et al.*, 2013, 2014). Sub-lethal chronic thermal stress may also occur, including behavioural avoidance, (i.e.

habitat shifts; (Thomas, 2010) and (or) decreases in growth and reproduction that reduce fitness and (or) survival (Bear *et al.*, 2007; Ficke *et al.*, 2007).

Small-bodied stream fishes with small geographic ranges often have limited dispersal capability and may not be able to shift their distribution in the absence of adequate dispersal corridors (Clavel *et al.*, 2011; Blanchet *et al.*, 2013; Kneitel, 2018; LeMoine *et al.*, 2020). In this situation, stream fishes may be forced to rely on acclimation potential to compensate for increasing ambient temperatures and to decrease sub-lethal stress (Ficke *et al.*, 2007; Seebacher *et al.*, 2015). However, acclimation potential is limited, particularly with respect to temperature increases (Beitinger and Bennett, 2000), and for most freshwater species acclimation potential and thermal tolerances remain unknown. Accordingly, to improve the conservation of stream fishes, an understanding is needed of acclimation potential and thermal tolerances to identify thermal risks and possible mitigation measures that might help to reduce temperature effects.

Thermal tolerance is usually measured in the laboratory using fish collected during a single season and acclimated to constant temperatures (Hutchison, 1961; Ziegeweid *et al.*, 2008; Dülger *et al.*, 2012; Dai and Suski, 2019). However, given the importance of diel and seasonal temperature cycles in the environment (Hlohowskyj and Wissing, 1985; Farless and Brewer, 2017), there is evidence that temperature variability, particularly temperature extremes, can increase thermal stress and exacerbate declines in ectothermic species (e.g. (Rohr and Raffel, 2010). Thus, field-based studies conducted across multiple seasons may provide a more biologically realistic assessment of thermal tolerance as they incorporate the effects of diel temperature fluctuations and seasonal cycles in determining the physiological plasticity and

adaptation potential to temperature changes. There remains, however, contrasting evidence for the importance of temperature fluctuations and debate about whether minimum temperature (Carveth *et al.*, 2007; Farless and Brewer, 2017), maximum temperature (Hutchison and Maness, 1979; Lutterschmidt and Hutchison, 1997a) or temperature variation (Rohr and Raffel, 2010; Clusella-Trullas *et al.*, 2011) are most important for determining thermal tolerance. The uncertainty is particularly important when attempting to assess the climate-related risks of local extirpation given that climate change will affect both temperature mean and variance (Räisänen, 2002). Additionally, field-based studies can simultaneously assess the role of temperature in relation to other habitat variables (e.g., turbidity) that might influence physiological tolerance.

High turbidity, which is caused by the underlying geology of a watershed and (or) is a consequence of anthropogenic activity in and near aquatic ecosystems, can exacerbate the stress of increased water temperatures for fishes (Paaijmans *et al.*, 2008) directly by damaging gills (Sutherland and Meyer, 2007; Cumming and Herbert, 2016) and indirectly through reduced feeding (Kjelland *et al.*, 2015), growth (Sutherland and Meyer, 2007) and (or) metabolic rate (Hess *et al.*, 2017). Although no studies of which we are aware of have assessed the influence of turbidity on thermal tolerance, oxygen capacity limiting thermal tolerance (OCLTT) theory (Ern *et al.*, 2016) would predict that turbidity induced altering of gill morphology has the potential to limit oxygen uptake and affect the thermal tolerances of oxygen-dependent fishes. For example, OCLTT theory has shown that a decrease in available oxygen can decrease CT_{max} in oxygen-dependent species (Pörtner, 2001, 2010; Ern *et al.*, 2016), making it particularly

important to understand if turbidity can act synergistically with changes in thermal tolerance to decrease the survival of a fish species.

Eastern Sand Darter (*Ammocrypta pellucida*) is a small, benthic fish that is sensitive to multiple habitat stressors, including climate-induced changes in thermal regimes and turbidity caused by agricultural and urban effects (DFO, 2011). Eastern Sand Darter exists in disjunct, localized ranges with limited natural dispersal opportunities (DFO, 2011). In Canada, Eastern Sand Darter is listed as Threatened under the *Species at Risk Act* (SARA), and in the United States it is considered imperilled in eight of the nine states in which it resides (Grandmaison *et al.*, 2004; COSEWIC, 2009). Siltation is believed to have been responsible for the majority of population declines, likely because of its negative effects on habitat quality, prey availability, and reproductive success (Spreitzer, 1979; Holm and Mandrak, 1996). Within Ontario, Eastern Sand Darter is located in agriculturally intensive zones where siltation of its preferred habitat is common. Studies conducted on the life history and ecology of Eastern Sand Darter suggest it is a habitat specialist, preferring clean sand and fine gravel substrates that facilitate fossorial behaviour (Dextrase *et al.*, 2014), and as such siltation may reduce growth of young-of-year (Drake *et al.*, 2008).

Little is known about the thermal tolerance or temperature-dependent physiological thresholds of Eastern Sand Darter, but these factors are important for understanding potential climate-related threats. Here, our objective was to determine the influence of seasonal temperature patterns and turbidity on the upper thermal limits of Eastern Sand Darter. Specifically, we tested the hypotheses that: 1) Eastern Sand Darter CT_{max} (the point at which locomotory control is lost, preventing escape from temperatures likely leading to death e.g.,

(Cox, 1974), T_{ag} (the point at which locomotory agitation in response to temperature increase is observed), and the thermal safety margin (CT_{max} – ambient temperature) are temperature-dependent and positively correlated with seasonal changes in water temperature; 2) CT_{max} and T_{ag} would decrease as turbidity levels rise based on OCLTT theory; and, 3) from among the stream temperature descriptors identified in the literature (minimum, maximum, mean and variance as (Arismendi *et al.*, 2013), models for predicting CT_{max} that include temperature variance would be superior to those that do not include temperature variance.

2.2 Materials and Methods

2.2.1 Field Sampling

Eastern Sand Darter for this study was obtained from the Grand and Thames rivers, which are the two largest watersheds in Southwestern Ontario (Wilcox *et al.*, 1998; Lake Erie Source Protection Region Technical Team, 2008). Within the Grand watershed (6,800km²), approximately 70% of the land is used for intensive agriculture, whereas in the Thames watershed (5,825km²) intensive agriculture accounts for greater than 75% of land use (Wilcox *et al.*, 1998; Lake Erie Source Protection Region Technical Team, 2008). The two rivers are generally similar, being lowland meandering main branch rivers within the same ecozone that have generally similar fish species assemblages and thermal regimes. The Thames River has a higher load of suspended sediment due to its underlying geology, although much of the turbidity is assumed to be from intensive land-use changes associated with agriculture (Stone and Saunderson, 1992; Nürnberg and Lazerte, 2015).

In the Grand River, one river reach was sampled monthly from June to November 2019 at Cockshutt Bridge, Brantford, Ontario, while in the Thames River, a river reach in the Big Bend Conservation Area, Wardsville, Ontario was sampled once in August 2020. Eastern Sand

Darter was captured using a 9.14 m bag seine with wing dimensions of 1.8 m in height x 3.7 m in width (3.0 mm mesh) and bag dimensions of 1.8 m height x 1.8 m width x 1.8 m depth (3.0 mm mesh). Seine hauls were conducted in the sand-dominated depositional zones of the Grand River. In November, and in the Thames river, water depths (>1.5 m) necessitated the use of a Gerken Siamese trawl that consisted of two 50 x 30 cm otter doors attached to a 2.4 m footrope with an 18 mm mesh inner cod end and a 3 mm mesh outer cod end. Although CT_{max} has been reported to be invariant with respect to nutritional condition (e.g., juvenile Common Carp, *Cyprinus carpio*, (Filippov and Golovanova, 2020), fish collections occurred in the morning in an effort to minimize the possible confounding effects of specific dynamic action associated with feeding. Diurnal feeding dominates among darters (e.g., (Adamson and Wissing, 1977; Schenck and Whiteside, 1977). Further, most darters greatly reduce prey consumption after sunset, with detailed feeding chronologies for Blackbanded Darter, *Percina nigojascia* (Mathur, 1973), Least Darter, *Etheostoma microperca* (Cordes and Page, 1980) and Fantail Darter, *Etheostoma flaballere* (Chalupnicki and Johnson, 2016) indicating that fish had the emptiest stomachs in the morning. Once captured, collected Eastern Sand Darter were held in a flow-through bin (56.5 cm x 39.3 cm x 30.4 cm) in the river to maintain field-related temperature acclimatization until they were used in a temperature trial.

2.2.2 Habitat Measurements

Habitat measurements were taken immediately after fish sampling at the midpoint of the capture area. A YSI EX02 multiparameter sonde was used to obtain water temperature (°C), turbidity (NTU), pH, conductivity (µS/cm) and dissolved oxygen (mg/L) approximately 0.2 m below the water surface. Additionally, two temperature loggers (Onset Water Temp Pro v2, HOBO, $\pm 0.2^{\circ}\text{C}$) were placed at the sampling site in the Grand River, which recorded

temperature data at 30-minute intervals from June to November 2019 (Figure 1). In addition, dissolved oxygen time-series data from the Brant station (12138042) were obtained from the Grand River Conservation Authority (GRCA, unpublished data; Figure 1).

2.2.3 Critical Thermal Maximum

Critical thermal maximum trials for Eastern Sand Darter were conducted with a streamside experimental unit following (Leclair *et al.*, 2020; Turko *et al.*, 2020). Water was taken directly from the river to ensure acclimatization to natural conditions at the time of sampling, with differences in thermal conditions between sampling dates effectively meaning fish were acclimatized to seasonally determined temperature regimes (June-November, October sampled twice). For each trial, a single Eastern Sand Darter was removed from the holding bin and transferred to a mesh breeder box placed within a 37.8 L glass aquarium filled with river water. The fish was left to acclimate for 5-min to eliminate the effect of any handling induced increase in metabolic rate on CT_{max} determinations. We chose a 5-min acclimation time as a preliminary study showed no significant difference in CT_{max} when 5- or 15-min acclimation times were used for Eastern Sand Darter (34.6 ± 0.5 vs. 34.8 ± 0.2 , Mann-Whitney-Wilcoxon Test, $p > 0.5$). Also, Turko *et al.* (2020) conducted a preliminary study demonstrating that there was no significant difference in CT_{max} between 15-min and 60-min acclimation periods for adult Redside Dace (*Clinostomus elongatus*) in similar streamside experiments. After the settling period, the water temperature was increased by 0.3°C per minute using a Julabo portable immersion circulator (Julabo, Seelbach, Germany). The temperature increase is recognized as fast enough that fish do not gain temperature tolerance and slow enough that fish body temperature adjusts with water temperature (Becker and Genoway, 1979). The fish was visually monitored in the breeder box for avoidance behaviour, which was defined as quick darting and other agitated swimming

behaviours (Petersen and Petersen, 1990; Herbert and Steffensen, 2006; McDonnell and Chapman, 2015). The temperature at which agitation occurred was recorded and denoted as the agitation temperature (T_{ag}). Temperature increases continued until loss of equilibrium was observed, which denoted the trial CT_{max} value. After loss of equilibrium, the fish was immediately transferred to a recovery bin with half river and half trial water to reduce thermal shock. Following recovery, mass (g) and total length (TL; mm) were measured (Table 1) and a visible implant elastomer tag (Northwest Marine Technology, Inc.) was inserted. Tagging was used to eliminate re-use of fish within each seasonal period, thereby removing the confounding effects of heat hardening on experimental results (Bilyk *et al.*, 2012). All trials were recorded using a GoPro HERO4 Black model and re-evaluated for purposes of quality control in the lab to verify the accuracy and consistency of initial T_{ag} and CT_{max} determinations in the field.

2.2.4 Statistical Analysis

Analysis of variance (ANOVA) was used to test for significant differences in acclimatization temperatures between Grand River sampling periods (months). Analysis of covariance (ANCOVA) was used to test for significant differences among the estimated upper thermal limits (CT_{max} and T_{ag}) for each Grand River sampling period (month), with fish mass used as a covariate (Hlohowskyj and Wissing, 1985; Nyboer and Chapman, 2017; Kamalam *et al.*, 2019; Andy J Turko *et al.*, 2020). All analyses were followed by a post-hoc Tukey's HSD test to assess pairwise differences between sampling periods. Additionally, linear regression was used to estimate the acclimation response ratio (ARR or the slope of the regression line) by regressing CT_{max} against water temperature at the start of each trial for all 2019 Grand River data (Claussen, 1977; Morley *et al.*, 2019). In the Grand River, linear regression was used to assess the relationship between CT_{max} and T_{ag} , and among variable correlations of the thermal safety

margin (CT_{max} - acclimatization temperature), the agitation safety margin (T_{ag} - acclimatization temperature), and the agitation window ($CT_{max} - T_{ag}$). Linear regression was also used to assess the relationship between agitation window and seasonal acclimation temperature in the Grand River.

To determine whether turbidity differed between the Grand and Thames rivers, a Mann-Whitney-Wilcoxon test was conducted on turbidity measurements from both rivers collected during the August sampling ($n=7$ Grand and $n=8$ Thames) and on a long-term data set collected by Fisheries and Oceans Canada over the past 10 years ($n=53$ measurements Grand and $n=113$ measurements Thames). Welch's two-sample t-test was used to determine whether acclimatization temperatures differed between the Thames and Grand River during the August trial. Significant differences among the estimated upper thermal limits (CT_{max} and T_{ag}) between two turbidity levels (Grand and Thames river) were then assessed using regression-based ANCOVA (Zar, 2018) with fish mass as a covariate.

For all data undergoing parametric statistical testing (e.g., t-test, ANOVA), normality and homogeneity of variance were checked, respectively, using the Shapiro-Wilks W and Levene's test of homogeneity (Zar, 2018). In addition, for all linear regressions, residuals were inspected to verify normality and homogeneity of variance (Gotelli and Ellison, 2013). All analyses were performed using R version 3.5.3 (R Core Team 2019) and results are displayed as mean $\pm$ standard error.

The importance of different temperature measures on CT_{max} was determined using general linear models and assessed with information theory, specifically the Akaike Information Criterion corrected for small sample sizes, AIC_c (Symonds and Moussalli, 2011). Temperature

measures considered in models used to explain the variation in CT_{max} included the mean, minimum, maximum, and variance of river water temperature, calculated for the 24-hour period preceding the trial. We chose to use temperatures from the 24 hours prior to testing as (Spigarelli *et al.*, 1977) demonstrated that body temperatures stabilized (i.e. acclimated) to instantaneous changes in temperature after 2-min to 6-hr depending on fish size. Mass (g) was included as a predictor variable since mass influences the time required for fish to reach thermal equilibrium (i.e. small-bodied fish reach thermal equilibrium quicker than larger bodied fish), thus smaller fish have a lower CT_{max} (Becker and Genoway, 1979; Zhang and Kieffer, 2014). As mean, minimum, and maximum temperature are colinear ($\rho \geq 0.984$; tested using the R package *olsrr* (Hebbali, 2020)) models used to explain variation in CT_{max} only included one of the potential predictor variables at a time so as to avoid issues with multicollinearity and the confounding effects of estimator variance inflation on significance testing (Draper and Smith, 1981). Models with $\Delta AIC_c < 2$ were considered equally plausible. Models with $\Delta AIC_c < 6$, that fell within the 95% model confidence set (Burnham and Anderson, 2002), were used in model averaging to rank predictor variables in terms of their importance for explaining CT_{max} (Symonds and Moussalli, 2011), with the Akaike model weights used to determine the model confidence set, i.e. the set of models whose cumulative Akaike weights equalled 95% (Symonds and Moussalli, 2011).

2.3 Results

Acclimatization temperature differed significantly across sample periods in the Grand River (one-way ANOVA, $F_{6,181}=820.50$, $p<0.01$; Table 1). The interaction terms between CT_{max} and sampling period (ANCOVA, $F_{6,174}=0.34$, $p>0.50$) and mass ($F_{1,174}=0.97$, $p>0.10$) were not

significant. Similarly, the interaction terms for T_{ag} and sampling period ($F_{6,174}=0.52$, $p>0.50$) and T_{ag} and mass ($F_{1,174}=0.23$, $p>0.50$) were not significant. Thus, the interaction term and the variable mass were removed from consideration in final model development. Eastern Sand Darter had the lowest mean CT_{max} and T_{ag} in November (27.93 ± 0.23 and 23.00 ± 0.52 , respectively) and the highest mean CT_{max} in July (37.01 ± 0.19) and T_{ag} in August (33.36 ± 0.27), with both CT_{max} (one-way ANOVA, $F_{6,181}=378.61$, $p<0.01$) and T_{ag} (one-way ANOVA, $F_{6,181}=104.55$, $p<0.01$) differing among months (Table 1). CT_{max} was positively correlated with seasonal acclimation temperature (linear regression $F_{1,186}=1359.0$, $r^2_{adj}=0.88$, $p<0.01$), with ARR as estimated by slope equal to 0.49 (Figure 2) over the range of acclimation temperatures tested (7 - 25°C). CT_{max} and T_{ag} correlated significantly (regression $F_{1,186}=499$, $r^2_{adj}=0.73$, $p<0.001$; Figure 3). Thermal safety margin and agitation safety margin were also significantly correlated (regression $F_{1,186}=252.4$, $r^2_{adj}=0.57$, $p<0.001$; Figure 4A) with the smallest margins occurring in the hotter months (July and August) and the largest margins occurring in the colder months (October and November). Agitation window was significantly correlated with both thermal safety margin (regression $F_{1,186}=20.93$, $r^2_{adj}=0.10$, $p<0.001$), agitation safety margin (regression $F_{1,186}=30.68$, $r^2_{adj}=0.14$, $p<0.001$), and seasonal acclimation temperature (regression $F_{1,186}=17.62$, $r^2_{adj}=0.08$, $p<0.001$; Figure 4B).

Turbidity measured during the August sampling differed significantly between the Grand (8.24 ± 1.94 NTU) and Thames (94.40 ± 8.27) rivers (Mann-Whitney-Wilcoxon Test, $W=0$, $p<0.01$), with differences being reflective of the 10 year long-term difference between the rivers (Grand, 10.02 ± 0.88 NTU and Thames, 150.78 ± 8.36 NTU; Mann-Whitney-Wilcoxon Test, $W=5.0$, $p<0.01$). The August acclimatization temperatures did not differ significantly (T-test,

$p=0.12$) between the Grand (24.09 ± 0.19 °C) and Thames (24.71 ± 0.34 °C), permitting the effect of turbidity on CT_{max} between river trials to be tested. The interaction term between CT_{max} and mass was not significantly different between turbidity levels (ANCOVA, $F_{1,52}=0.387$, $p > 0.5$), thus the interaction term was removed from the final model. Similarly, for the T_{ag} model, the mass interaction term (ANCOVA, $F_{1,52}=0.114$, $p > 0.5$) and mass (ANCOVA, $F_{1,53}=3.139$, $p > 0.05$) were not significant. Thus, the interaction term and mass were removed from the T_{ag} final model. Mean CT_{max} did not differ significantly between turbidity levels (ANCOVA, $F_{1,53}=0.06$, $p > 0.50$), but the overall effect of mass on CT_{max} was significant (ANCOVA, $F_{1,53}=7.46$, $p<0.01$; Figure 5, Panel A) and positively related to body mass in both rivers. While mean T_{ag} did not differ significantly between turbidity levels (ANOVA, $F_{1,54}=2.93$, $p > 0.05$) a significant interaction with mass yielded higher T_{ag} in the Grand for a given mass (Figure 5, Panel B).

From the set of variables considered for explaining observed variation in CT_{max} , three models yielded $\Delta AICc$ values < 2 (Table 2) and were considered essentially equivalent. The confidence set of candidate models whose cumulative Akaike weights were $\geq 95\%$ included an additional four models (Table 2) that could not be discounted. Model averaging of the parameters in the confidence set of candidate models yielded the following composite model for predicting Eastern Sand Darter CT_{max} :

$$CT_{max} = 24.63 + 0.51 \cdot \text{fish mass} + 0.49 \cdot \text{mean temp} + 0.49 \cdot \text{max temp} - 0.56 \cdot \text{temp variance} + \text{error}$$

Of the parameters included in the confidence set of candidate models, mean temperature had the highest relative importance weight (0.85), followed by the variance of water temperature calculated for the 24-hour period preceding the trial (0.69), fish mass (0.64), and the maximum temperature (0.12) in the 24-hour period preceding the trial.

2.4 Discussion

We provide the first study to assess key physiological endpoints for Eastern Sand Darter, a species of conservation concern. We showed that Eastern Sand Darter displayed physiological plasticity in CT_{max} and T_{ag} , with both measures differing significantly between months across the growing season and changing with seasonal environmental temperatures. Thermal tolerance (CT_{max}) and agitation temperature (T_{ag}) were highest in the summer months (July and August) and decreased thereafter with declines in environmental temperature. Contrary to expectations, turbidity had no measurable effect on CT_{max} and T_{ag} within the range of values tested (~8 NTU Grand River and ~94 NTU Thames River). No single combination of the predictor variables best explained the variation in CT_{max} across seasons, although model averaging indicated high relative importance (≥ 0.64) for measures based on the previous 24-hr thermal history that included temperature mean and variance, and fish mass. Maximum temperature was found to have low relative importance (< 0.12).

Eastern Sand Darter displayed physiological plasticity given the observed changes in CT_{max} with ambient water temperatures during the growing season. The linear change in Eastern Sand Darter thermal tolerance with ambient temperature observed in the field study is consistent with the generally strong linear relationship between CT_{max} and acclimation temperature for nearly all fish species tested in the lab (Beitinger *et al.*, 2000) and other imperilled species such as Redside Dace similarly tested in the field (Andy J Turko *et al.*, 2020; Leclair *et al.*, 2020). Observed seasonal changes in thermal tolerance were generally comparable with those reported for field-acclimatized Fantail Darter, Greenside Darter, and Rainbow Darter, although thermal tolerances for those darter species were 2-4°C higher in October and November (and ambient temperature ~ 5°C warmer) when compared to Eastern

Sand Darter (Hlohowskyj and Wissing, 1985). Seasonal differences in CT_{max} have also been reported for other species including: crayfish, (*Orconectes rusticus*; (Layne Jr. *et al.*, 1987), amphipods (*Paramelita nigroculus*), and mayflies (*Lestagella penicillate*) ((Dallas and Rivers-Moore, 2018). While studies that examine the influence of season on the thermal tolerance of fishes are scarce owing to the difficulties of laboratory acclimation (Golovanov, 2012), those that do exist typically show patterns similar to those reported here, with summer values exceeding winter values (Golovanov, 2012). The changes in CT_{max} were smaller than the magnitude of changes observed in ambient temperatures (Gunderson and Stillman, 2015), implying that thermal safety margins generally become smaller as temperatures warm (Sandblom *et al.*, 2016). Thus species, including Eastern Sand Darter, that live closer to their thermal limits in summer (Shultz *et al.*, 2016; Andy J Turko *et al.*, 2020) are more susceptible to temperature spikes that might occur in summer due to short-term heat waves. Conservation strategies should, therefore, aim to mitigate anthropogenic stressors that are most prominent during the summer period as they may further reduce the already small thermal safety margins.

The success of conservation approaches for many species will rely on a strong understanding of temperature effects on their physiology. While our field study provided findings that parallel lab studies, it is important to note that comparative studies have observed differences in lab- and field-based thermal tolerances for a number of species, including: Zebrafish (*Danio Rerio*), Brown Trout (*Salmo Trutta*), Rainbow Trout (*Oncorhynchus mykiss*), and Brook Trout (*Salvelinus fontinalis*) (Carline and Machung, 2001; Morgan *et al.*, 2019). Field studies that consider diurnal and seasonal variation in temperature, and possible temperature interactions with other environmental variables (i.e. the inverse relationship with DO), may be

more relevant for conservation and management purposes insofar as they better replicate the environment in which fish reside.

The ongoing survival of Eastern Sand Darter may depend on the ability of the species to acclimate (exhibit plasticity) to increasing temperatures, particularly during summer extremes. Eastern Sand Darter displayed physiological plasticity in thermal tolerance as indicated by the significant differences in monthly estimates of CT_{max} and the high ARR estimate (0.49).

Acclimation Response Ratio has been used to assess species acclimation capacity, with a higher ARR indicating a greater capacity to increase thermal tolerance after exposure to increasing acclimation temperatures (Kelley, 2014; Morley *et al.*, 2019). The ARR of Eastern Sand Darter exceeded that of closely related Fantail, Greenside, and Rainbow Darter (0.22, 0.25, and 0.31, respectively) and the estimated mean of numerous mid-latitude (0.34 for > 25- 55°) fishes ((Morley *et al.*, 2019), but lies at the mid-point of the range (0.07- 0.91) for a set of widely distributed freshwater fishes ((Comte and Olden, 2017). For species like Eastern Sand Darter that have higher ARR and shorter generation times, there tends to be a longer temporal buffer (~100 + years) before the loss of thermal safety (Morley *et al.*, 2019), with a longer thermal buffer implying both decreased vulnerability to climate change and increased possibility of evolutionary rescue (Bell, 2013).

While CT_{max} is more often reported and used in meta-analysis (Comte and Olden, 2017), T_{ag} , which is correlated with CT_{max} , may be a more useful thermal endpoint for conservation and management purposes. In our study, and a study on African Cichlids (*Pseudocrenilabrus multicolor*) and Pugnose Shiner (*Notropis anogenus*) under long-term acclimation (30 days and 103-150 days, respectively), T_{ag} exhibited physiological plasticity and was highly positively

correlated with ambient temperature (McDonnell *et al.*, 2019; Potts *et al.*, 2021). Agitation temperature is a relatively new endpoint in thermal tolerance studies and marks the onset of the search for thermal refugia (McDonnell and Chapman, 2015; McDonnell *et al.*, 2019). In nature, species will tend to avoid hotter temperatures that approach CT_{max} , making agitation temperature a more ecologically relevant endpoint for determining habitat suitability (McDonnell *et al.*, 2019). For example, (Mundahl, 1990) observed that 14 species, including Fantail Darter, Greenside Darter, and Johnny Darter (*Etheostoma nigrum*), used shaded areas and (or) cavities under rocks as thermal refugia to avoid temperatures that surpassed their laboratory estimated CT_{max} . In our experiments, Eastern Sand Darter demonstrated behavioural avoidance at the estimated agitation temperature by swimming away from the bottom of the breeding box, suggesting an attempt to locate cooler water. Although it is unknown if sufficient thermal refugia are commonly available to Eastern Sand Darter in the wild, groundwater discharge is present upstream of our study site and could provide thermal refugia that might improve species persistence in the face of thermal stress (Lake Erie Source Protection Region Technical Team, 2008; Brewer, 2013; Snyder *et al.*, 2015).

For species without adequate thermal refugia, temperatures persisting above T_{ag} have the potential to elicit sub-lethal effects that could lead to population declines before CT_{max} and thermal safety margins are reached (Bear *et al.*, 2007; Ficke *et al.*, 2007). Thus, we propose the use of agitation safety margins, which are correlated with thermal safety margins, as a promising metric to serve as an “early warning” indicator for the onset of sub-lethal effects. The range of temperatures eliciting sub-lethal effects has been termed the agitation window (Wells *et al.*, 2016; McDonnell *et al.*, 2019), with our study being among the first to assess agitation

window across a range of temperatures and to demonstrate that a smaller agitation window corresponds with hotter seasonal temperatures. The sensitivity of the agitation window as a response metric may, however, vary by species. For example, a similar study of African Cichlids tested between 21 and 29°C found no change in agitation window. Therefore, further research is warranted to understand the generality of the relationship between agitation window and ambient temperature.

Although gill impairment has been observed at turbidity levels as low as 40 NTU ((Cumming and Herbert, 2016) and the turbidity difference (~86 NTU) between the Grand and Thames rivers was more than double that threshold, we observed no turbidity effect on either CT_{max} or T_{ag} . As a fossorial species, Eastern Sand Darter may have adapted to passing sediment over the gills in ways that minimize gill surface damage and do not impair oxygen uptake. For example, there may have been local adaptation to the chronic, long-standing turbidity in the Thames River by Eastern Sand Darter (Drake *et al.*, 2008). Alternatively, increased turbidity may act indirectly to reduce growth, either as a result of reduced feeding (Rowe and Dean, 1998) or the increased metabolic costs of extracting oxygen from turbid waters (Horkel and Pearson, 1976). While the relationship between mass and thermal tolerance was not observed in the Grand River, it was observed in the Thames where fish were both notably smaller and more variable in size (mean mass of 0.56 g, coefficient of variation = 66.5%) than the Grand (mean mass of 1.42 g, coefficient of variation = 17.7%). If turbidity impacts growth, as noted by Drake *et al.* (2008), turbidity may act indirectly to affect thermal tolerance as a result of the reduction in size associated with the higher metabolic costs of increased gill ventilation rates due to long-term exposure to turbid environments (Lowe *et al.*, 2015).

Based on model averaging, important variables for explaining variation in CT_{max} included two of those previously highlighted in the literature, mean temperature (e.g., (Fry, 1947) and temperature variance (e.g., (Clusella-Trullas *et al.*, 2011), with the importance of the mean foreshadowing the significance of the seasonal patterns in CT_{max} and T_{ag} . The importance of mean temperature has been recognized for some time (Fry, 1947) and underpins the rationale of thermal acclimation in laboratory trials. Indeed, as (Elliott, 1981) noted, most studies now agree that acclimation temperatures and the period of exposure to such temperatures prior to experimentation are critical for determining CT_{max} and, as shown here, T_{ag} . Findings by (Rodgers *et al.*, 2018) and (Díaz and Bückle, 1999) demonstrated that Green Sturgeon (*Acipenser medirostris*) and Channel Catfish (*Ictalurus punctatus*) exposed to thermally stable and variable environments having the same mean temperature showed no differences in CT_{max} , indicating the fishes acclimated on the basis of the mean temperature. While temperature variation is considered less often, evidence for its importance may be traced back to the seasonally-dependent CT_{max} field investigations of (Brett, 1944) and the lab experiments of (Heath, 1963), which showed that equivalent CT_{max} values could be obtained from cyclical exposure to 10 and 20°C temperatures (mean 15°C) as from constant, pre-experimental exposure to 20°C. Although early investigators suggested the importance of variance was linked to a response to extremes (Brett, 1944; Heath, 1963), maximum temperatures did not effectively predict CT_{max} for Eastern Sand Darter. Fluctuating temperatures have also been shown to increase CT_{max} in Chinese Bream (*Parabramis pekinensis*; (Peng *et al.*, 2014) and to facilitate short-term survival by Spinedace (*Meda fulgida*; Carveth *et al.*, 2007) and Bonneville Cutthroat Trout (*Oncorhynchus clarkitah*; Schrank *et al.*, 2003) at temperatures considered lethal under stable

conditions, with respite from lethal temperatures thought to be crucial for facilitating physiological repair (Iwama *et al.*, 1999). With changing climates now expected to affect both the mean and variation of temperature (Räisänen, 2002; IPCC, 2013), consideration of the effects of temperature variation will be necessary to assess climate-related temperature risks of local extirpation in threatened fish species such as Eastern Sand Darter.

Our field-based experiments indicate that Eastern Sand Darter CT_{max} and T_{ag} vary seasonally, and that the species has a high acclimation response ratio. The high ARR combined with a short generational time of Eastern Sand Darter suggest the species is not in the same immediate danger from warming temperatures as some other freshwater species (e.g., Redside Dace, (Leclair *et al.*, 2020). Although our research indicates that Eastern Sand Darter may have a degree of resilience to future temperature increases, that capacity is limited. Given that the seasonal changes in thermal tolerance show Eastern Sand Darter have the lowest buffering capacity in the summer and an increase in thermal extremes is expected in temperate climates in the summer, steps to mitigate the effects of thermal extremes on Eastern Sand Darter habitats are warranted. Mitigating thermal extremes will require considerable conservation effort since landscape changes and climate change are now the leading causes of summer temperature extremes (IPCC, 2013). In that regard, both CT_{max} and T_{ag} should be combined with predictions of future temperature regimes to better understand the ecological impact of anthropogenically driven increases in water temperatures on Eastern Sand Darter.

Table 2.1: Mean $\pm$ standard error of sample size (n), mass (g), total length (mm), dissolved oxygen (mg/L), water (acclimatization temperature °C) at start of trial, T_{ag} (°C), and CT_{max} (°C) of Eastern Sand Darter for each sampling period for the Grand and Thames (bottom row) rivers. Common letters indicate no significant differences between months (ANOVA, Tukey's HSD post hoc test $p > 0.05$) in the Grand River.

Month	n	Mass (g)	Total Length (mm)	Dissolved Oxygen (mg/L)	Acclimatization Temperature (°C)	T_{ag} Temperature (°C)	CT_{max} Temperature (°C)
June	26	1.35 $\pm$ 0.078	62.42 $\pm$ 1.02	8.88 $\pm$ 0.04	17.11 $\pm$ 0.15 ^a	26.29 $\pm$ 0.21 ^a	32.21 $\pm$ 0.18 ^a
July	25	1.25 $\pm$ 0.058	59.68 $\pm$ 0.87	6.75 $\pm$ 0.11	25.02 $\pm$ 0.17 ^b	32.56 $\pm$ 0.36 ^b	37.01 $\pm$ 0.12 ^b
August	27	1.42 $\pm$ 0.048	63.00 $\pm$ 0.69	6.95 $\pm$ 0.14	24.09 $\pm$ 0.19 ^c	33.36 $\pm$ 0.26 ^b	36.37 $\pm$ 0.14 ^b
September	29	1.49 $\pm$ 0.033	62.97 $\pm$ 0.42	8.50 $\pm$ 0.12	19.88 $\pm$ 0.24 ^d	30.72 $\pm$ 0.34 ^c	34.85 $\pm$ 0.14 ^c
Sept/Oct	29	1.43 $\pm$ 0.057	62.93 $\pm$ 0.78	8.39 $\pm$ 0.07	18.24 $\pm$ 0.28 ^e	30.10 $\pm$ 0.34 ^c	34.40 $\pm$ 0.15 ^c
October	29	1.33 $\pm$ 0.052	60.83 $\pm$ 0.87	11.12 $\pm$ 0.35	12.48 $\pm$ 0.23 ^f	24.85 $\pm$ 0.53 ^a	30.91 $\pm$ 0.15 ^d
November	23	1.45 $\pm$ 0.044	62.48 $\pm$ 0.58	12.25 $\pm$ 0.11	7.01 $\pm$ 0.16 ^g	23.00 $\pm$ 0.52 ^d	27.93 $\pm$ 0.23 ^e
August (Thames)	29	0.54 $\pm$ 0.069	45.55 $\pm$ 1.79	6.92 $\pm$ 0.34	24.71 $\pm$ 0.34	32.71 $\pm$ 0.27	35.60 $\pm$ 0.19

Table 2.2: Sample size corrected Akaike information criterion (AIC_c) rankings of the models that best described variation in CT_{max} ($^{\circ}C$). Models shown in bold text have $\Delta AIC_c < 6$ and were included within the 95% confidence interval candidate model set. Also given is the Akaike weight (w_i) for each model that assesses the probability of it being the correct model. K defines the number of model estimate parameters.

Model	k	AIC_c	ΔAIC_c	w_i
mean + mass + var	5	421.438	0.000	0.353
mean + var	4	422.465	1.026	0.211
mean + mass	4	422.612	1.174	0.196
max + mass + var	5	424.095	2.657	0.094
mean	3	424.223	2.785	0.088
max + var	4	426.516	5.078	0.028
min + mass	4	427.871	6.433	0.014
min	3	429.281	7.843	0.007
min + mass + var	5	430.003	8.565	0.005
min + var	4	431.340	9.902	0.002
max + mass	4	433.670	12.232	0.001
max	3	437.000	15.562	0.000
var	3	773.786	352.348	0.000
var + mass	4	775.724	354.286	0.000

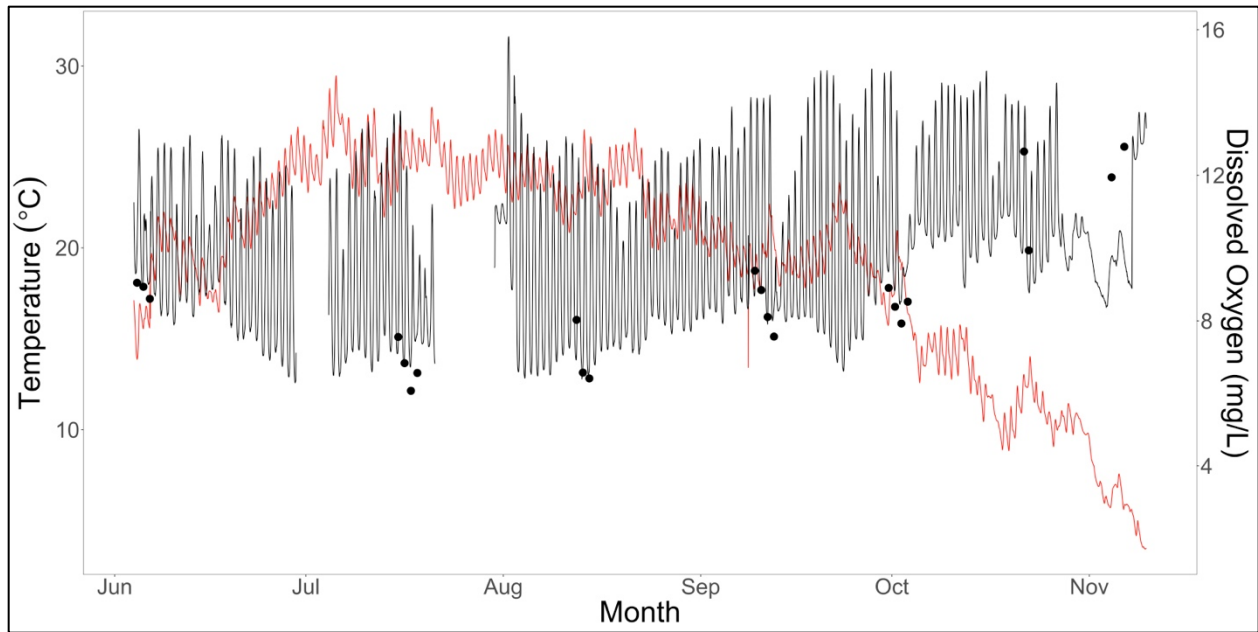


Figure 2.1: Water temperature (°C; red, measured at study site) and dissolved oxygen (mg/L; black, measured from a nearby Grand River Conservation Authority monitoring station) taken every 30 minutes from June 5th to November 10th, 2019. Black dots represent in-stream, point-based measurements of dissolved oxygen at the study site.

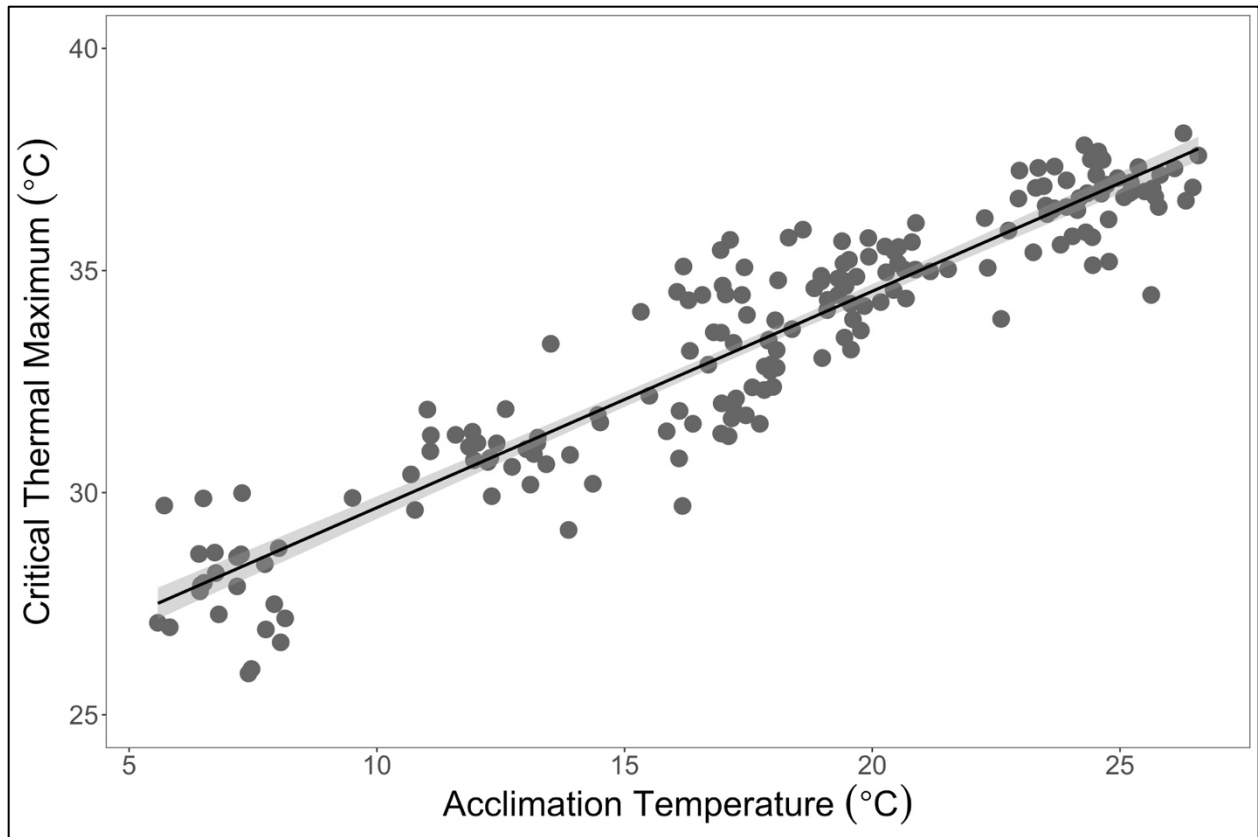


Figure 2.2: Critical thermal maximum versus acclimatization temperature for Eastern Sand Darter captured from the Grand River ($r^2_{\text{adj}}=0.88$, $p<0.01$, slope=0.49). Shaded areas represent the 95% confidence intervals.

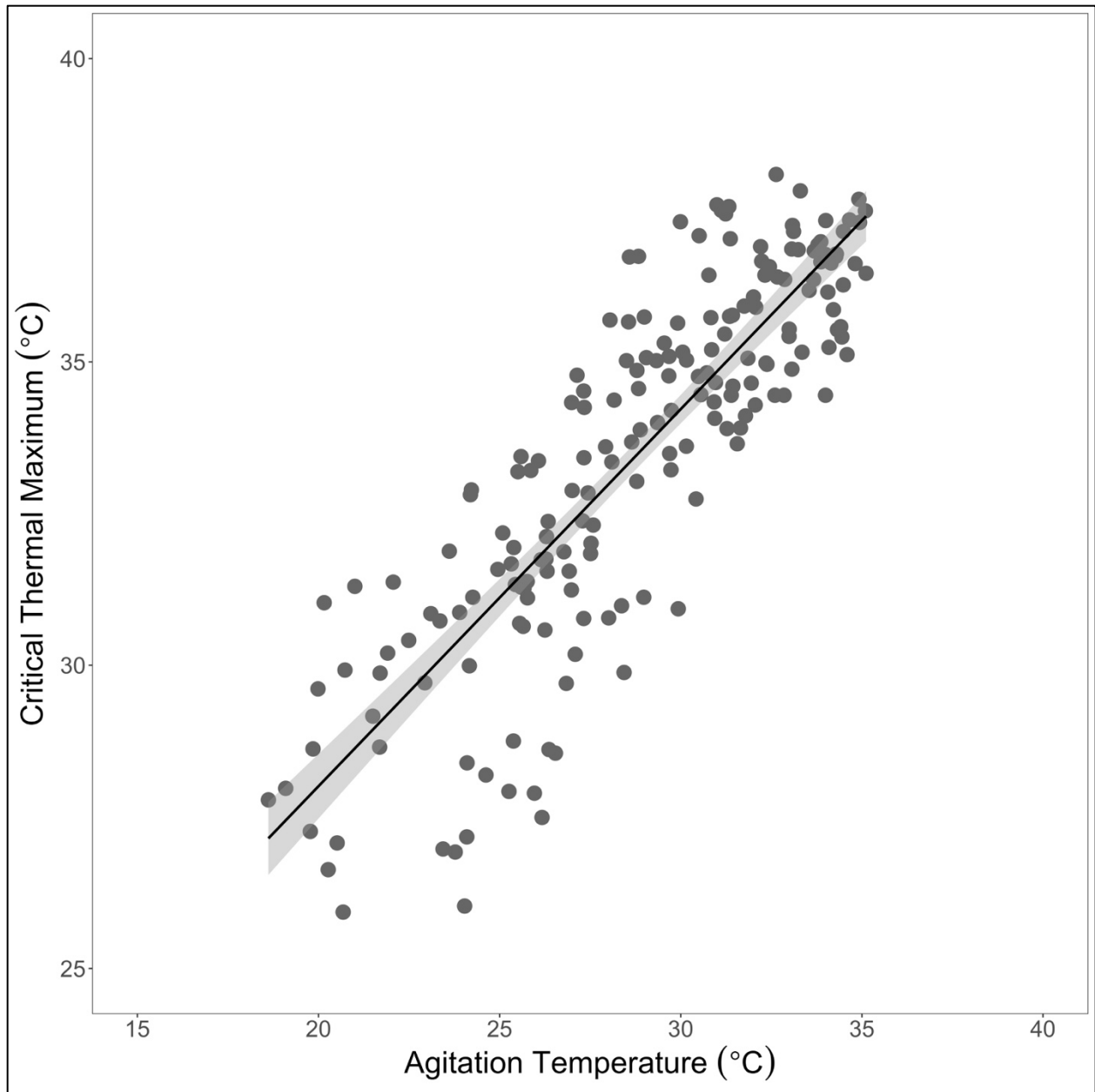


Figure 2.3: Critical thermal maximum versus agitation temperature for Eastern Sand Darter captured from the Grand River ($r^2_{\text{adj}}=0.73$, $p<0.001$, slope=0.62). Shaded areas represent the 95% confidence intervals.

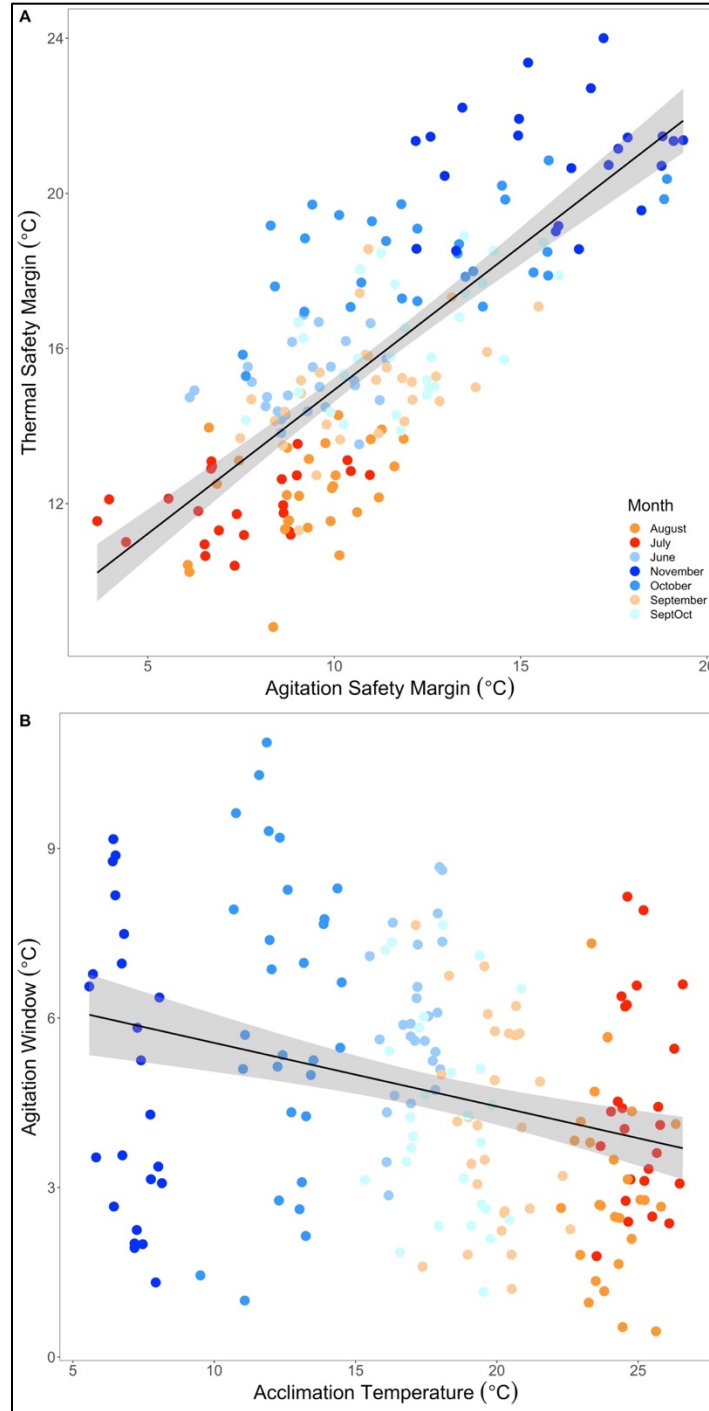


Figure 2.4: A) Thermal safety margin versus agitation safety margin ($r^2_{\text{adj}}=0.57$, $p<0.001$), and B) agitation window versus acclimation temperature ($r^2_{\text{adj}}=0.08$, $p<0.001$) of Eastern Sand Darter from the Grand River. The colour gradient represents the months of testing from hottest (July; red) to coldest (November; dark blue). Shaded areas represent the 95% confidence intervals.

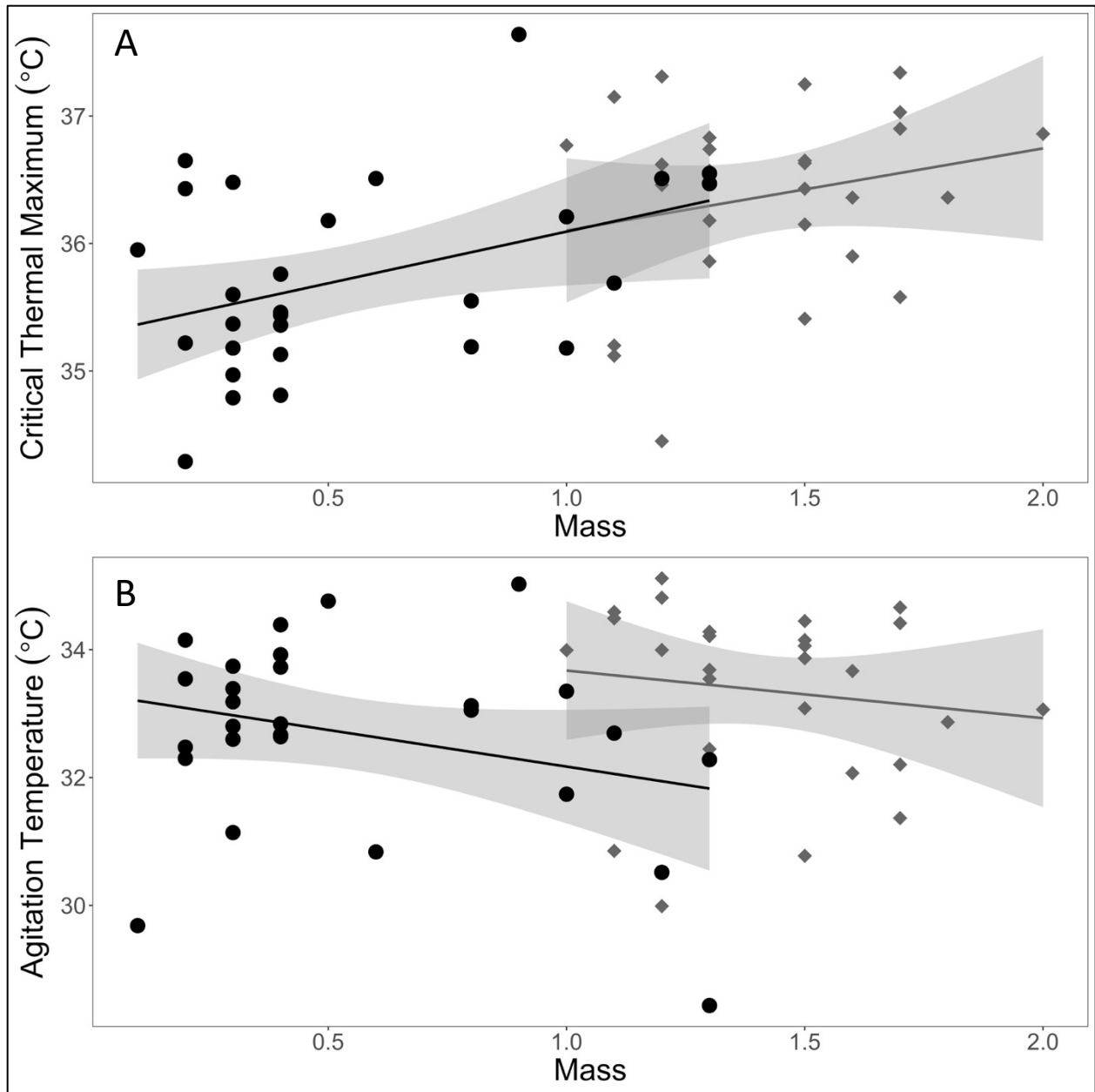


Figure 2.5: Eastern Sand Darter: A) August critical thermal maximum for the Thames (black circles) and Grand (grey diamonds) rivers, and B) August agitation temperatures for the Thames and Grand rivers plotted versus the covariate mass for statistically similar acclimatization temperatures. Shaded areas represent the 95% confidence intervals.

Chapter 3: Seasonal, environmental and individual effects on hypoxia tolerance of Eastern Sand Darter (*Ammocrypta pellucida*).

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3.1 Introduction

Species extinctions have increased in recent years due to anthropogenic effects and are predicted to further increase with climate change (Burkhead, 2012; Swan et al., 2018). In particular, freshwater organisms face heightened extinction risks because they are susceptible to modification of both terrestrial and aquatic landscapes (Ricciardi and Rasmussen, 1999; Jenkins, 2003; Olden et al., 2007). Anthropogenic factors such as deforestation, agricultural-driven nutrient and sediment loading, urbanization and climate change (Donohue and Garcia Molinos, 2009; IPCC, 2021) can all lead to increased water temperature fluctuations and the prevalence of hypoxic zones (low oxygen), both of which are known habitat stressors in aquatic ecosystems (Diaz, 2001; Diaz and Rosenberg, 2008; IPCC, 2013). Increases in temperature may induce behavioural changes that help avoid unfavourable conditions and can trigger population-level changes in abundance caused by temperature-dependent changes in mortality, growth and/or reproduction (Pörtner and Peck, 2010). Similarly, dissolved oxygen reductions can result in aquatic organisms having an oxygen deficiency in their tissues, which can lead to changes in locomotory activity, growth, reproduction and alter the dynamics of predator–prey interactions due to physiological and behavioural compensation (Robb and Abrahams, 2003; Landry et al., 2007; Pollock et al., 2007; Oldham et al., 2019). Therefore,

understanding an organism's metabolic rate and hypoxia tolerance in relation to changing temperature is necessary for determining the consequences of hypoxia on species survival.

Because fishes are ectotherms, temperature is the key factor controlling metabolic rate (Fry, 1947; McNab, 2002; Schulte et al., 2011). Measures of metabolic rate and hypoxia tolerance (critical oxygen tolerance and LOE) are correlated and generally increase with environmental temperature (Fry, 1947; Hlohowskyj and Wissing, 1987; McBryan et al., 2013; Rogers et al., 2016). However, some species have demonstrated metabolic compensation, allowing them to completely or partially maintain metabolic rate over a range of temperatures (Precht, 1958; Vetter, 1982; Sandblom et al., 2014; Abe et al., 2019). Metabolic compensation could be beneficial in the wild where individuals are exposed to fluctuating temperatures and would allow species to conserve energy and/or remain active in situations when temperatures impact locomotion, growth and predator–prey interactions (Fry, 1971; Vetter, 1982; Evans, 1990; Abe et al., 2019). Despite the importance of these principles, there remains a lack of information about metabolic rate and hypoxia tolerance in the wild where species face temperature changes on diurnal and seasonal cycles (Treberg et al., 2016).

Metabolic rate and hypoxia tolerances are also influenced by factors other than temperature (Metcalf et al., 2016; Rogers et al., 2016), including food availability (Auer et al., 2015), salinity (Allan et al., 2006), turbidity (Hess et al., 2017) and fish mass (Clarke and Johnston, 1999). Under constant environmental conditions, and for fish of similar mass and sex, both routine metabolic rate and hypoxia tolerance show a large degree of individual variability (Burton et al., 2011; Metcalf et al., 2016). Some of the among-individual variability can be explained by liver enzyme activity (Norin and Malte, 2012), social behaviour (Metcalf et al.,

1995) and cardiac performance (Joyce et al., 2016). Norin et al. (2016) showed a change in among-individual variability of standard metabolic rate (SMR) in barramundi (*Lates calcarifer*) in response to low salinity, high temperature and hypoxia. Low SMR-control individuals displayed a larger increase in metabolic rate at a higher temperature than high SMR-control individuals, leading to a decrease in among-individual variability at hotter temperatures (Norin et al., 2016). Such findings suggest that even within a species, fish are not uniformly impacted by changes in temperature for a given set of environmental conditions. At the population-level, among-individual variation in metabolic responses to temperature change implies some intrinsic buffering capacity, at least to low or moderate changes in environmental conditions. However, few studies have assessed among-individual variability in metabolic rate and hypoxia tolerance under natural conditions, raising questions regarding how fishes of conservation concern may respond to hypoxia and thermal stress.

Despite knowledge of how temperature and other abiotic and biotic variables influence metabolic rate and hypoxia tolerance for some freshwater fish species (e.g. Fry, 1947; Clarke and Johnston, 1999; Metcalfe et al., 2016; Rogers et al., 2016), there is a paucity of information about physiological responses under natural conditions where fish experience dynamically changing environments (Treberg et al., 2016). In the wild, fish experience changes in reproduction, food availability, and abiotic conditions (e.g., temperature, dissolved oxygen) simultaneously across the seasons. Additionally, transgenerational impacts (e.g., grandparent and maternal/paternal effects) and developmental plasticity can also influence physiological metrics (Schaefer and Walters, 2010; Le Roy et al., 2017). As both the mean and variance of water temperatures are expected to increase with climate change (Räisänen, 2002),

investigating the effect of temperature-driven changes in oxygen availability and related fish responses in the wild will be important for understanding physiological plasticity, variation and determining local extinction risk.

To date, few studies have investigated the metabolic rate and hypoxia tolerance for fishes of conservation concern, yet knowledge of individual and species-specific responses will have important implications for evaluating threats and habitat suitability. We investigate these concepts for eastern sand darter (*Ammocrypta pellucida*), which is a small, benthic fish listed as threatened in Canada under the Species at Risk Act and is imperilled throughout its North American range (Grandmaison et al., 2004; Fisheries and Oceans Canada, 2012). Eastern sand darter is a habitat specialist that prefers clean sand and fine gravel substrate in rivers (Dextrase et al., 2014) and some lake areas. Numerous anthropogenic stressors have been associated with the decline of eastern sand darter, with siltation of preferred habitat suggested as a major source of population decline (COSEWIC, 2009). Siltation is thought to be linked to decreased oxygen availability in the substrate, which may lead to reduced burying behaviour and increased energy expenditure (Daniels, 1989; Drake et al., 2008; COSEWIC, 2009). However, like many imperilled fishes, there is uncertainty about the physiological response of eastern sand darter to habitat stressors, including its sensitivity to oxygen impairment. To overcome this limitation, the overall objective of this study was to determine how the hypoxia tolerance of eastern sand darter varied in response to seasonal temperature changes while accounting for the role of environmental and fish-specific factors (e.g., condition, reproductive season). Specifically, the study objectives were to determine if: (i) seasonal temperature influenced hypoxia tolerance (PO_2 at LOE and P_{crit}) and metabolic rate in eastern sand darter; (ii) the

inclusion of environmental turbidity, fish condition and reproductive season variables significantly improved the ability to explain residual variation in hypoxia tolerance and metabolic rate; and, (iii) seasonal temperature influenced among-individual variation in hypoxia tolerance (PO₂ at LOE and P_{crit}) and metabolic rate.

3.2 Material and Methods

3.2.1 Field Sampling

Fish capture methods followed those described in detail in Firth et al. (2021). Briefly, eastern sand darter were collected monthly in the Grand River at Cockshutt Bridge, Brantford, Ontario (43.110°N, 80.245°W) from June to October 2019 (for a detailed description, see Gáspárdy and Drake, 2021) using an 9.14 m bag seine (1.8 x 1.8 x 1.8 m bag with 3.0 mm mesh) hauled downstream in sand-dominated depositional areas. All animal use and collection were conducted under a Species at Risk Act permit (#19-PCAA-00021) and approved by the University of Waterloo (AUP #40909) and Fisheries and Oceans Canada (AUP #1968/2068) Animal Care Committees. Field collections focused on adults, with all tested individuals measuring >50 mm in total length. As digestion impacts metabolism (i.e. specific dynamic action; SDA), fish are commonly fasted before testing as a means of allowing them to attain their routine/SMR (Jobling, 1993; Secor, 2009). Field-based testing, however, precluded the use of fasting. To reduce the effects of SDA on metabolic calculations in this study, all fish were collected before 10 am (except for three days in October when fish were collected until 12 pm). Darter species generally display diurnal feeding (e.g., Schenck and Whiteside, 1977; Chalupnicki and Johnson, 2016), with studies on least darter (*Etheostoma microperca*), banded darter (*Etheostoma zonale*; Cordes and Page, 1980), rainbow darter (*Etheostoma caeruleum*), fantail darter

(*Etheostoma flabellare*; Adamson and Wissing, 1977) and blackbanded darter (*Percina nigrofasciata*; Mathur, 1973) demonstrating increased feeding activity during the day and decreased feeding after sunset. Studies have also reported the stomachs of darter species to be mostly empty in the morning (Mathur, 1973; Adamson and Wissing, 1977; Cordes and Page, 1980; Chalupnicki and Johnson, 2016).

Following capture, fish were held for 30 minutes to 4 hours prior to experimentation in a flow-through bin (56.5 × 39.3 × 30.4 cm), which was supplied with river-sourced water to ensure that field-related temperature acclimation remained consistent with natural river conditions.

3.2.2 Habitat Measurements

At the center of each sampling site, water temperature (°C), dissolved oxygen (mg/L), conductivity (μS/cm), turbidity (NTU) and pH were collected ~0.2 m below the water surface using a YSI EX02 multiparameter sonde following Firth et al. (2021) and Gáspárdy and Drake (2021) (Figure 1).

3.2.3 Respirometry Protocol

There were three measurement endpoints in this study: field metabolic rate (FMR), critical oxygen tension (P_{crit}) and oxygen tension at loss of equilibrium (PO₂ at LOE). FMR was defined as the baseline oxygen requirement for living of a post-absorptive, inactive fish plus the additional cost of maintaining posture and position in the water column and any additional unknown cost of specific dynamic action (Treberg et al., 2016) or postprandial increase in oxygen uptake. FMR was considered as the energy expenditure of a wild fish in natural conditions (Treberg et al., 2016). Thus, FMR can provide an improved understanding of natural environmental disturbances (e.g., thermal fluctuations, seasons, oxygen availability) on fish

performance (Treberg et al., 2016). The P_{crit} and PO₂ at LOE endpoints represent different aspects of an organism's response to oxygen availability (Ultsch et al., 1978; Rogers et al., 2016). Critical oxygen tension being the point at which a species can no longer sustain oxyregulation (i.e. maintain oxygen consumption to support metabolic rate) in the face of decreasing environmental oxygen levels, and switches to oxyconforming where oxygen consumption is decreased in concert with decreasing environmental oxygen levels (Pörtner and Grieshaber, 1993). A lower P_{crit} suggests greater hypoxia tolerance as fish can maintain aerobic metabolism, and thus routine function, for a longer period in environments with decreased oxygen availability (Regan and Richards, 2017). In contrast, PO₂ at LOE signifies the oxygen concentration at which delivery of oxygen to aerobic tissues can no longer be sustained. At PO₂ at LOE a fish loses the ability to maintain its position in the water column and is likely to suffer mortality unless dissolved oxygen in the water increases (Fry, 1971; McBryan et al., 2013; Franklin, 2014; Rees and Matute, 2018).

Metabolic rate and hypoxia tolerance were measured streamside using closed respirometry. Each respirometry trial consisted of housing fish in an apparatus that included four chambers in a bin separated by barriers to prevent fish from seeing each other and to isolate them from external disturbances. Individual fish were placed in 60 ml cylindrical Plexi-glass chambers (length, 70 mm; diameter, 32 mm) with the ratio of fish mass to water volume averaging 1:42 (minimum 1:31 to maximum 1:85). Plexi-glass chambers were carefully inspected pre- and post-experimentation to ensure there was no leak or extra off gassing of oxygen (Stevens, 1992). A continuous flow of river water was provided to the bin and pumped through vinyl tubing to each chamber to maintain water temperature and oxygen levels

throughout a ninety-minute acclimation period. Tests using rainbow darter have shown that a 90-minute acclimation period is sufficient to restore metabolic rate to routine levels after initial capture and handling (Mehdi et al., 2018; Hodgson et al., 2020). After acclimation, the respirometers were closed, and the oxygen was depleted in the chamber by fish respiration. Trial duration lasted 93 minutes on average (range: 27–201 minutes). Following Ultsch et al. (1978), Hlohowskyj and Wissing (1987), De Boeck et al. (2013) and Craig et al. (2014) chamber mixing during respirometry was achieved by fish movement and ventilation. During the entire trial, river water was continuously pumped into the holding bin to maintain ambient river water temperature. In all trials, temperature stayed within 1.5°C of the measured start temperature.

The rate of oxygen depletion was measured using fiber-optic oxygen sensors placed in the center of each respirometer (FireStingO2, Pyro Science, Aachen, Germany), with one chamber left empty during every trial to measure background rates of biological oxygen demand. Oxygen concentration in the chambers was measured every second using Pyro Oxygen Logger Software (Pyro Science, Aachen, Germany) and a temperature probe in the bin allowed for software temperature compensation during recording. Recalibration of both oxygen (0 and 100%) and temperature probes occurred between each monthly set of trials. The rate of oxygen consumption was measured until LOE occurred, which was denoted by inverted swimming and operculum muscle spasms (Lutterschmidt and Hutchison, 1997). After LOE, the fish was immediately placed in a recovery bin with 100% oxygen saturation. Once the fish recovered, fish total length (mm) and mass (g) were measured (Table 1) and the fish was tagged with a visible implant elastomer tag (Northwest Marine Technology, Inc.) to prevent re-use in subsequent trials.

The oxygen consumption rate was calculated over 5-minute intervals with the R package 'respirometry' (Birk, 2019) using the equation provided in (Rogers et al., 2016):

$$MO_2 = [(\Delta V \times \Delta O_2) / (\Delta t \times w)]$$

where ΔV is the respirometer volume (ml) minus the fish volume (ml; assumed to equal w), ΔO_2 is the measured change in oxygen content (mg/L), Δt is the time interval (minutes) that aligns with the measured ΔO_2 and w is fish mass (g). Similar to Regan and Richards (2017), to standardize FMR we calculated the mean oxygen consumption of each fish using the calculated 5-minute intervals that fell between 110% and 80% partial oxygen pressure in water. Fish ($n=19$) with a partial oxygen pressure < 80% in the first 5-minute interval were selected as potential outliers ($n=1$ excluded). Measured oxygen consumption with slopes having low r^2 (< 0.5) were also considered outliers and were removed from the data set ($n=5$, 5% of the data). The average r^2 of all metabolic rate calculations, including those used for calculation of P_{crit} , was 0.87.

The critical oxygen level (P_{crit}) was calculated with the R package 'Segmented' (Muggeo, 2003) using the curve of the 5-minute MO_2 intervals with the corresponding PO_2 for individual fish (Supplementary Fig. S1). During P_{crit} calculations, 12-34 MO_2 intervals were used per P_{crit} calculation depending on the length of the trial. The segmented procedure allows for the identification of multiple break points (Muggeo, 2003; Borowiec et al., 2020). When individuals demonstrated only 2 break points ($n=6$), the lower PO_2 breakpoint was designated as P_{crit} following Borowiec et al. (2020). Fish ($n=10$) for which there was no break point were removed from the P_{crit} data set used for analysis.

3.2.4 Statistical Analysis

All data used in parametric statistical analyses were tested for normality and homogeneity of variance using the Shapiro-Wilk W and Levene's homogeneity tests, respectively (Zar, 2018).

General linear models were used to estimate the relationships between seasonal temperature and all dependent variables (FMR, PO₂ at LOE, P_{crit}).

Multivariate linear models were developed and evaluated using the Akaike Information Criterion (AIC) adjusted for small sample sizes (AICc; Symonds and Moussalli, 2011) with turbidity, body condition, and reproductive season considered as potential explanatory variables. Turbidity (NTU) has been shown to increase metabolic rate in cinnamon clownfish (*Amphiprion melanopus*; Hess et al., 2017) and during our study turbidity increased during rain events (i.e. from 2.1 to 19.5 NTU). Fulton's K, an index of body condition, was included as a predictor variable and used as a proxy for food availability since low ration corresponds to low body condition (Turko et al., 2020) and low food availability has been observed to decrease metabolic rate in brown trout (*Salmo trutta*; Auer et al., 2015, Archer et al., 2020).

Reproductive season was included as a predictor variable since reproduction is energetically costly and can increase metabolic demand when individuals are reproductively active (Angilletta and Sears, 2000; Pollock et al., 2007; Holt and Jørgensen, 2015). Sex is difficult to distinguish in eastern sand darter with non-lethal methods and reproductive status is not clear based on visual inspection alone. Finch et al. (2012), however, have noted that in the Thames River, Ontario, the reproductive season occurs between late April and late June. The geographic proximity of the Thames and Grand Rivers (Wilcox et al., 1998; Lake Erie Source Protection Region Technical Team, 2008) suggests that reproductive season in the Grand River will be similar. Thus, here the reproductive season for experimental fish was defined as occurring from

May 1st to June 30th using a binary variable; i.e. 1= the period when eastern sand darter reproduction typically occurs and 0 = the reproductively inactive period. Models contained in the 95% confidence interval set, i.e. the set of models whose cumulative Akaike weights best approximate 0.95 (Burnham and Anderson, 2002; Symonds and Moussalli, 2011), were used to rank the importance of predictor variables in explaining each dependent variable (Symonds and Moussalli, 2011). All statistical analyses were performed in R Studio version 1.4.1717 (RStudio Team, 2021).

To test the effect of variation, all data were averaged for a given experimental testing day and the corresponding coefficient of variation for all dependent variables was regressed against seasonal temperature to determine the significance of relationships between seasonal temperature and the among- individual variation of each response variable.

3.3 Results

Eastern sand darter had the lowest mean $\pm$ standard error PO₂ at LOE and FMR in October (0.50 ± 0.07 and 10.67 ± 0.89 , respectively), P_{crit} in June (8.39 ± 0.93), the highest mean PO₂ at LOE and P_{crit} in July (1.14 ± 0.04 , 13.31 ± 1.52 , respectively) and FMR in June (20.49 ± 1.15 ; Table 1). Both PO₂ at LOE (regression $F_{1,88} = 41.15$, $r^2_{adj} = 0.31$, $P < 0.001$; Figure 2A) and P_{crit} (regression $F_{1,73} = 6.74$, $r^2_{adj} = 0.07$, $P < 0.05$; Figure 3A) were significantly and positively related to seasonal temperature, but FMR was not significantly related to seasonal temperature (regression $F_{1,82} = 1.85$, $r^2_{adj} = 0.01$, $P = 0.18$; Figure 4A). Temperature alone explained (r^2_{adj}) $\leq 31\%$ of the variation observed in any of the dependent variables modelled.

For PO₂ at LOE the single variable temperature model was ranked as the best model for explaining variation in the dependent variable. For P_{crit}, temperature combined with turbidity and reproductive season best explained variation in the dependent variable. For FMR,

temperature combined with reproductive season and fish condition best explained variation in the dependent variable. In all cases there were equally plausible multivariate models (i.e. $\Delta AIC_c < 2$, Table 2–4) for explaining variation in the dependent variable. Within the 95% confidence interval set of models, all of the considered predictor variables appeared in at least one model. Within the PO2 at LOE model confidence interval set, seasonal temperature was the only parameter with a high relative importance weight (0.942), being greater than 2× relatively more important than turbidity (0.433) and condition (0.412), and 4.6× relatively more important than reproductive season (0.203) for predicting PO2 at LOE. For PO2 at LOE, the top model was the same as the simple linear regression described above, with $r^2_{adj} = 0.31$. Parameter importance for PO2 at LOE combined with the model analysis demonstrated that consideration of other variables did not improve explained variation. Within the P_{crit} model confidence interval set, the best model contained other variables. However, seasonal temperature was the only parameter with a high relative importance weight (0.937), being 1.8×, 2.2× and 4.3× relatively more important than turbidity (0.523), reproduction (0.433) and condition (0.218), respectively. The top model for P_{crit} (regression $F_{3,71} = 3.94$, $r^2_{adj} = 0.11$, $P < 0.05$) was significantly related to seasonal temperature (0.29, $P < 0.05$), but not turbidity (−0.29, $P = 0.10$) or reproductive season (−2.06, $P = 0.12$) and explained 1.48× more of the variation compared to the single temperature model (Figure 3B). The P_{crit} model analysis demonstrated that consideration of other variables did improve explained variation. However, the parameter importance for the variables was low and the variables in the top model were not significant, although both variables are important for adding predictive power to the model from an information theoretic point of view (Burnham and Anderson, 2002). When estimating FMR, however, the best model contained

other variables and the relative importance weight for parameters in the FMR confidence set indicated that temperature (0.958) and reproductive season (0.958) were equally important for determining FMR, with condition (0.839) also being important. Turbidity was of marginal importance, with the top three variables being 3.1× more important than turbidity (importance weight=0.229). For FMR, consideration of other variables helped improve the ability to explain variation in the data. The top model for FMR (regression $F_{3,80}=9.65$, $r^2_{\text{adj}}=0.24$, $P<0.001$) was significantly related to seasonal temperature (0.51, $P<0.01$), reproductive season (7.39, $P<0.001$) and condition (-36.28 , $P<0.05$) and explained 24× more of the variation compared to the single temperature model. Being within the reproductive season was associated with a significant ($P<0.001$) average increase of 7.39 $\mu\text{mol/hr}\cdot\text{g}$ in FMR compared to not being within the reproductive season (Figure 4B). Further, as Fulton's K condition factor increased by a unit of 1, FMR decreased by -36.28 $\mu\text{mol/hr}\cdot\text{g}$.

The coefficient of variation (CV) for PO₂ at LOE and P_{crit} at each testing day did not vary significantly with temperature (PO₂ at LOE: regression $F_{1,20}=0.32$, $r^2_{\text{adj}}=-0.03$, $P=0.58$; P_{crit} : regression $F_{1,18}=0.07$, $r^2_{\text{adj}}=0.02$, $P=0.26$; Figures 2B and 3C). For the FMR regression, the CV was significantly and positively related to seasonal temperature (regression $F_{1,20}=8.03$, $r^2_{\text{adj}}=0.25$, $P<0.05$; Figure 5).

3.4 Discussion

We provide the first comparison of metabolic rate and hypoxia tolerance for eastern sand darter across seasonal temperature changes in a field setting. Eastern sand darter hypoxia tolerance metrics (P_{crit} and PO₂ at LOE) were positively correlated with seasonal temperature over the study period. Temperature was also the best overall predictor of both PO₂ at LOE and P_{crit} , although turbidity and reproductive season helped improve explanation of among-

individual variation in P_{crit} . In contrast, for FMR temperature alone was not the best predictor, with more of the variation in FMR being explained when temperature, reproductive season, and condition were used jointly in a regression model. Furthermore, temperature did not provide strong explanatory power for either hypoxia tolerance or metabolic rate, suggesting that (1) eastern sand darter may be able to biochemically, molecularly and/or physiologically compensate across temperatures; and/or (2) the effect of temperature may be masked due to interactions with abiotic and biotic environmental factors (e.g. dissolved oxygen, ground water, species interactions). Among-individual variation in FMR did increase with temperature, whereas temperature did not influence among-individual variability in either PO₂ at LOE and P_{crit} , possibly as a result of different individual thermal sensitivities. The result implies some intrinsic buffering capacity in metabolic rate to increased temperature changes under natural conditions. Overall, findings suggest that temperature may be a weak predictor of metabolic rate and hypoxia tolerance in a field setting where biotic and abiotic factors can act concurrently on physiological variables to affect tolerances.

As predicted, eastern sand darter PO₂ at LOE and P_{crit} increased with increasing ambient temperature. The increase in PO₂ at LOE and P_{crit} with increasing temperature has been observed in numerous fish species (He et al., 2015; McDonnell and Chapman, 2015; Borowiec et al., 2016; Rogers et al., 2016). However, closely related darter species have demonstrated varying hypoxia tolerance responses to changes in temperature. For example, with an increase in field temperature fantail darter PO₂ at LOE did not change, rainbow darter PO₂ at LOE decreased and greenside darter (*E. blennioides*) PO₂ at LOE increased (Hlohowskyj and Wissing, 1987). Similarly, P_{crit} and oxygen concentration at death (the point past PO₂ at LOE) increased in

E. squamiceps and decreased in *E. rufilineatum*, *E. flabellare*, *E. duryi*, *E. boschungii* and *E. fusiforme* (Ultsch et al., 1978). The different relationships observed between temperature and hypoxia tolerance among darter species could be explained by the modifying effects of microhabitat preferences, as darters occupy a wide range of microhabitats (Ultsch et al., 1978; Chipps et al., 1994; Stauffer et al., 1996). For example, darters have been observed to segregate based on substrate, velocity and depth, which can impact the degree of temperature change darters experience on a daily and seasonal basis (Chipps et al., 1994; Stauffer et al., 1996). Ultsch et al. (1978) compared hypoxia tolerances with habitat preferences in six darters and found species with low tolerances (3.5–5.3 mg/L) were restricted to fast moving water while highly tolerant (0.76–1.8 mg/L) species occupied small and shallow streams dominated by still water. Eastern sand darter typically occupies low velocity microhabitats, and showed a similar hypoxia tolerance to the highly hypoxia tolerant *E. fusiforme* that similarly occupies slow-moving waters (Ultsch et al., 1978). Eastern sand darter may have a high hypoxia tolerance compared to other darters because of their fossorial behaviour and preference for sand substrates (Daniels, 1989), which typically contain less than 2 mg/L of dissolved oxygen within the sediment (Barnucz et al., 2022).

Although many studies have reported a strong correlation between metabolic rate and temperature, or significant shifts in metabolic rate as temperature increases (Fry, 1947; Clarke and Johnston, 1999; Gillooly et al., 2001; Allen and Gillooly, 2007; Borowiec et al., 2016), the relationship is by no means ubiquitous, with some species demonstrating biochemical, molecular, and/or physiological compensation of metabolic rate across temperatures (Bullock, 1955). Rosyside dace (*Clinostomus funduloides*) and mottled sculpin (*Cottus bairdi*) tested

across seasons demonstrated higher metabolic rates in spring (10°C) compared to summer (15°C) due to gonadal recrudescence (Facey and Grossman, 1990). Similarly, with eastern sand darter, the FMR model including reproductive season better explained changes in FMR as temperature increased, although the overall explanatory power of the relationship remained weak ($r^2_{\text{adj}} = 0.24$). The weak temperature-metabolic relationship observed in eastern sand darter may reflect partial metabolic compensation, which has been observed in a number of species. Metabolic compensation can be categorized into: complete compensation, where metabolic rate is maintained with changing temperature; no compensation, where metabolic rate increases with increasing temperature; and, partial compensation, between complete and no compensation where fish show some temperature effect (Precht, 1958). Partial metabolic compensation across natural, species-specific temperature ranges has been demonstrated in snow trout (*Schizothorax richardsonii*; Kamalam et al., 2019), African cichlid (*Pseudocrenilabrus multicolor*; McDonnell and Chapman, 2015) and rainbow trout (*Oncorhynchus mykiss*; Facey and Grossman, 1990) tested close to field conditions. Additionally, a longer acclimation period has been shown to lead to greater metabolic compensation in spiny chromis (*Acanthochromis polyacanthus*; Donelson and Munday, 2012), Nile perch (*Lates niloticus*; Nyboer and Chapman, 2017) and shorthorn sculpin (*Myoxocephalus Scorpius*; Sandblom et al., 2014). Wild zebrafish (*Danio reio*) appear to have greater metabolic compensation than lab fish, likely as a result of the need to maintain metabolic rate over a fluctuating range of temperatures in the wild (Morgan et al., 2022).

Eastern sand darter are exposed to diurnal temperature fluctuations of ~ 0.02–4.08°C from June to October, which on a monthly basis show average fluctuations of ~ 10°C, implying

mean river temperatures change gradually across each month (Figure 1). Such gradual changes in mean temperature across the month from shifts in diurnal temperature fluctuations may result in wild fish undergoing longer acclimation, which, in turn, may lead to increased metabolic compensation in the wild. Partial compensation in metabolism would allow eastern sand darter to conserve energy and increase usable energy to maintain growth, locomotion and avoid predators across periods of diurnal and seasonal temperature changes (Evans, 1990).

The inclusion of turbidity and reproductive season, and fish condition and reproductive season, increased explanatory power in the P_{crit} and FMR models respectively, but not the PO₂ at LOE model. Although turbidity and reproductive season increased explanatory power in the P_{crit} model, they were not significant in the regressions. Turbidity may have had only limited importance in the eastern sand darter models because its observed range was small (2.1–19.5 NTU) over the study, with only two days having NTU above 7. Turbidity has been shown to have no significant effect on routine metabolic rate in cinnamon clownfish until at/above 7.0 NTU (Hess et al., 2017). In contrast, turbidity has been shown to increase P_{crit} in imperilled pugnose shiner (*Notropis anogenus*) and bridle shiner (*Notropis bifrenatus*) at 7.31 ± 0.2 NTU (Gray et al., 2016).

Body condition contributed significantly to explaining among-individual variation in eastern sand darter FMR, despite the low variability (5% coefficient of variation) among experimental individuals, with FMR decreasing with increasing body condition. In contrast, blue catfish (*Ictalurus furcatus*) demonstrated no relationship between metabolic rate and body condition (Nepal et al., 2021). In our study, condition alone explained only 7% of the variation in FMR and interactive effects between fish weight, sex, seasonal changes in condition, and/or

reproductive season could explain the significance observed (Weatherley, 1963; Hoeinghaus et al., 2006; Nepal et al., 2021).

Reproductive season significantly contributed to explaining among-individual variation in FMR of eastern sand darter, with metabolic rate increasing during the reproductive season. The overall influence of reproductive season on metabolic rate of oviparous fish species is poorly understood, particularly for eastern sand darter (Spreitzer, 1979; Johnston, 1989), but has been investigated in some fish species. Facey and Grossman (1990) attributed higher SMR in the spring and fall to reproduction in oviparous rosieside dace and mottled sculpin as most individuals showed signs of gonadal maturation during spring and fall. Similarly, two oviparous lizards, *Sceloporus undulates* and *Sceloporus aeneus aeneus*, demonstrated a 122% and 221% increase, respectively, in metabolic rate compared to non-gravid females (Guillette, 1982; Angilletta and Sears, 2000). Viviparous fishes have also shown significant increases in metabolic rate when reproductively active (Boehlert et al., 1991; Masonjones, 2001; Timmerman and Chapman, 2003). Well-known trade-offs between metabolic and reproductive activity may, however, limit the capacity of species to simultaneously adjust metabolic rate and maintain reproductive output under increased temperatures and/or hypoxic conditions (e.g., Fry, 1947; Healy and Schulte, 2012; Dupont-Prinet et al., 2013; Sandblom et al., 2014; Gilmore et al., 2019). For example, prolonged hypoxia has been observed to significantly reduce the gonadosomatic index of gulf killifish (*Fundulus grandis*; Landry et al., 2007) and common carp (*Cyprinus carpio*; Wu et al., 2003). If fish species, including eastern sand darter, are unable to sufficiently increase their metabolic rate during reproduction because of increased temperature and/or hypoxia, reduced energy allocation for reproduction could translate to reduced fitness

and have negative consequences for population abundance and, ultimately, species conservation.

Among-individual variation increased as a function of temperature for FMR only. A linear change in among-individual variation across temperature implies that individuals have different thermal sensitivities, as has been observed previously for metabolic rate but not for PO₂ at LOE and *P_{crit}* (Nespolo et al., 2003; Careau et al., 2014; Norin et al., 2016; Drown et al., 2021). Our field study showed an increase in variation with increasing temperature, whereas lab studies often observe a decrease in variation with increasing temperature, e.g., juvenile barramundi (Norin et al., 2016) and mummichog (*Fundulus heteroclitus*; Drown et al., 2021). Similar to our study, wild zebrafish and wild-caught slimy salamander (*Plethodon albagula*) have both shown increased variation with increasing temperature (Careau et al., 2014; Morgan et al., 2022). In the wild, fish experience simultaneous seasonal and diurnal changes in environmental variables with temperature fluctuations tending to being greater in the summer (i.e. thermally heterogeneous) than in the winter (i.e. thermally homogeneous; Łaszewski, 2018). As thermal heterogeneity increases, variation in metabolic rate may have occurred for two reasons. First, individual fish may inhabit different micro-habitats with differing thermal characteristics (Kita et al., 1996; Schulte et al., 2011; Goller et al., 2014). Those inhabiting thermal refugia influenced by ground water may experience less immediate impacts of changes in water temperature, with the differences in among micro-habitat thermal characteristics becoming larger as temperatures rise (Snyder et al., 2015). In this case, fish would have larger variability in initial acclimation temperatures in the summer relative to the point measurements that were taken in the field to characterize the thermal status of the ecosystem. Second,

individual fish may have different metabolic thermal sensitivities. Highly sensitive individuals would have a larger thermal response to any given increase in temperature and likely acclimate more quickly to the hotter temperature of diurnal fluctuations in summer (Norin et al., 2016). Understanding field-based variation is difficult as there is a lack of control on the specific conditions an individual may have experienced prior to testing. Nonetheless, field-based studies can provide a practical assessment of physiological tolerances and related in-stream fish responses to environmental stressors, which may help to inform conservation decisions.

A large degree of individual variation was observed for all three metrics (FMR, PO₂ at LOE, and *P_{crit}*) across the range of test temperatures. High among-individual variation in traits provides a portfolio effect by buffering a species from the potential negative consequences of environmental change, leading to a better overall chance of individual survival (Bolnick et al., 2011). If individuals survive the initial environmental change, and the environment continues to be habitable, a shift in the metabolic rate and hypoxia tolerance trait distributions beneficial for the species in the changed environment may occur in the next generation (e.g., Bolnick et al., 2011). For example, metabolic rate has been shown to be heritable in birds (Mathot et al., 2013) and mammals (Zub et al., 2012; Boratynski et al., 2013). A large degree of among-individual variation, in conjunction with the short generation time of eastern sand darter, therefore, could have positive ecological and evolutionary significance for the survival of this species based on the portfolio effect. However, it is important to note that heritability could be limited since the influences of developmental plasticity and transgenerational effects (i.e. the environment that previous generations were exposed to) may also influence the observed

variation in adult eastern sand darter metabolic rate (Schaefer and Walters, 2010; Le Roy et al., 2017).

Our findings demonstrate the complexity of conducting physiological studies in the field and the effect that the lack of control on biotic and abiotic covariates can have on physiological metrics. Fry (1947) noted that temperature is the key controlling metabolic factor and subsequent authors have shown that temperature is a controlling factor in general for ectotherm physiology (Magnuson et al., 1979; McNab, 2002; Schulte et al., 2011). In contrast, our study shows that in a field setting, temperature is a weak controlling factor, with the reduced apparent importance of temperature being attributed to its interactions with other environmental variables that mask its effect. Although laboratory studies may facilitate accurate understanding of the effects of temperature alone on physiological metrics, as predictors of climate change impacts, they are less reliable because of their failure to account for the simultaneously occurring action of those variables which field experiments cannot control (e.g. reproductive period, condition, turbidity) and which we have shown can influence key physiological metrics. Thus, while the conduct of field studies may be ‘messy’, they nevertheless provide a more realistic understanding of how changes in the environment will interact to affect the prospects for species survival.

Our field-based experiments indicated that eastern sand darter metabolic rate and hypoxia tolerance varied with season, with temperature being the most important but weak predictor of the traits overall. In the wild, diurnal and seasonal fluctuating temperatures may act in concert with other biotic and abiotic factors to determine fish physiological changes in metabolic rate and hypoxia tolerance. Our results demonstrate a large degree of individual

variation across seasons, which could indicate that eastern sand darter has a greater chance of evolutionary adaptation to persistent increases in temperature and decreases in oxygen.

However, a better understanding of the factors that influence this variation in the wild and determination of whether the underlying differences have a genetic basis is required to fully understand the ecological implications of our findings.

Table 3.1: Sample size (n), mean $\pm$ standard error of fish mass (g), total length (mm), river temperature ($^{\circ}\text{C}$), and dissolved oxygen concentrations (mg/L) and measures of field metabolic rate (FMR; $\mu\text{mol/hr-g}$), oxygen pressure at loss of equilibrium (PO_2 at LOE; mg/L), and critical oxygen tension (P_{crit} ; kpa) for Eastern Sand Darter sampled in the Grand River, summarized by sample month.

Month (# days sampled)	n	Mass (g)	Total Length (mm)	Temperature ($^{\circ}\text{C}$)	Dissolved Oxygen (mg/L)	FMR ($\mu\text{mol/h-g}$)	PO_2 at LOE (mg/L)	P_{crit} (kpa)
June (4)	18	1.38 ± 0.054	63.06 ± 0.63	16.43 ± 0.50	8.88 ± 0.04	20.49 ± 1.15	0.64 ± 0.056	8.39 ± 0.93
July (4)	15	1.19 ± 0.096	58.40 ± 1.44	25.28 ± 0.26	6.75 ± 0.11	16.42 ± 1.57	1.14 ± 0.042	13.31 ± 1.52
August (3)	15	1.48 ± 0.048	63.73 ± 0.59	24.45 ± 0.24	6.95 ± 0.14	18.50 ± 3.04	0.89 ± 0.091	11.09 ± 1.59
September (4)	15	1.51 ± 0.046	62.40 ± 0.69	19.64 ± 0.28	8.50 ± 0.12	12.79 ± 1.57	0.81 ± 0.082	10.61 ± 1.61
Sept/Oct (3)	15	1.41 ± 0.054	63.47 ± 0.87	18.90 ± 0.32	8.39 ± 0.07	12.45 ± 1.55	0.63 ± 0.045	11.34 ± 1.05
October (4)	12	1.35 ± 0.053	61.83 ± 0.78	13.01 ± 0.29	11.12 ± 0.35	10.67 ± 0.89	0.50 ± 0.067	9.64 ± 1.06

Table 3.2: AIC_c and rankings of variables used to explain among-individual variation in Eastern Sand Darter field metabolic rate (FMR). Bolded models are in the 95% confidence interval set. K defines the number of model parameters. w_i is the model Akaike weight that defines the probability that a given model is the best approximating model. Variables considered included: test water temperature (Temp), fish condition as defined by Fulton's K (Condition), measured river turbidity in NTU (Turb), and reproductive season (Repro).

Model	k	AIC _c	Δ AIC _c	w_i
Temp + Repro + Condition	5	550.452	0.000	0.610
Temp + Repro + Turb + Condition	6	552.417	1.965	0.229
Temp + Repro	4	553.725	3.273	0.119
Temp + Repro + Turb	5	555.851	5.399	0.041
Temp + Condition	4	564.109	13.656	0.001
Temp + Turb + Condition	5	565.488	15.036	0.000
Temp	3	570.300	19.847	0.000
Temp + Turb	4	572.217	21.764	0.000

Table 3.3: AIC_c and rankings of variables used to explain among-individual variation in Eastern Sand Darter oxygen pressure at loss of equilibrium (LOE). Bolded models are in the 95% confidence interval set. K defines the number of model parameters. w_i is the model Akaike weight that defines the probability that a given model is the best approximating model. Variables considered included: test water temperature (Temp), fish condition as defined by Fulton's K (Condition), measured river turbidity in NTU (Turb), and reproductive season (Repro).

Model	k	AIC _c	ΔAIC _c	w_i
Temp	3	22.411	0.000	0.197
Temp + Turb	4	22.422	0.011	0.196
Temp + Condition	4	22.621	0.210	0.177
Temp + Turb + Condition	5	22.723	0.312	0.169
Temp + Repro	4	24.511	2.100	0.069
Temp + Repro + Turb	5	24.538	2.127	0.068
Temp + Repro + Condition	5	24.612	2.201	0.066
Temp + Turb + Condition + Repro	6	24.837	2.426	0.059

Table 3.4: AIC_c and rankings of variables used to explain among-individual variation in Eastern Sand Darter critical oxygen tension (P_{crit}). Bolded models are in the 95% confidence interval set. K defines the number of model parameters. w_i is the model Akaike weight that defines the probability that a given model is the best approximating model. Variables considered included: test water temperature (Temp), fish condition as defined by Fulton's K (Condition), measured river turbidity in NTU (Turb), and reproductive season (Repro).

Model	k	AIC _c	Δ AIC _c	w_i
Temp + Repro + Turb	5	440.545	0.000	0.209
Temp + Turb	4	440.847	0.302	0.180
Temp	3	440.938	0.393	0.172
Temp + Repro	4	441.116	0.571	0.157
Temp + Condition	4	442.367	1.822	0.084
Temp + Turb + Condition	5	442.814	2.269	0.067
Temp + Repro + Turb + Condition	6	442.833	2.288	0.067
Temp + Repro + Condition	6	442.952	2.407	0.063

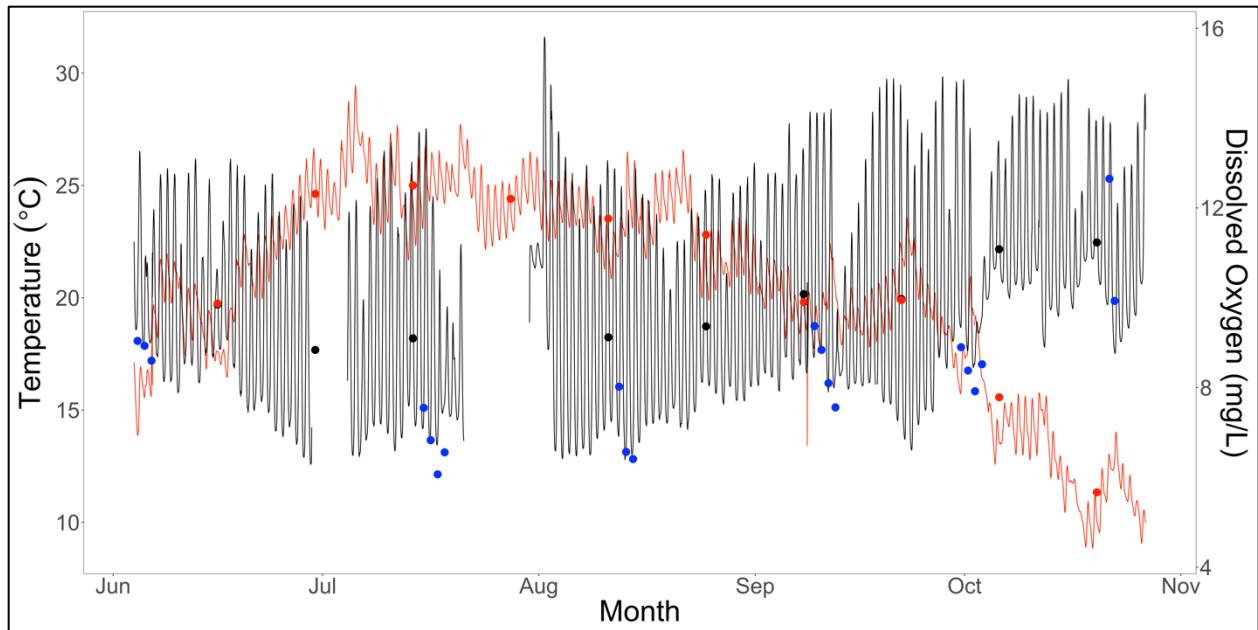


Figure 3.1: Figure based on Firth et al. (2021) showing water temperature ($^{\circ}\text{C}$; red) and dissolved oxygen (mg/L; black, data from Grand River Conservation Authority) taken over the sampling season in 2019. Red and Black dots represent a bi-weekly mean of the corresponding environmental variables. Blue dots represent single measurements of dissolved oxygen taken at the study site.

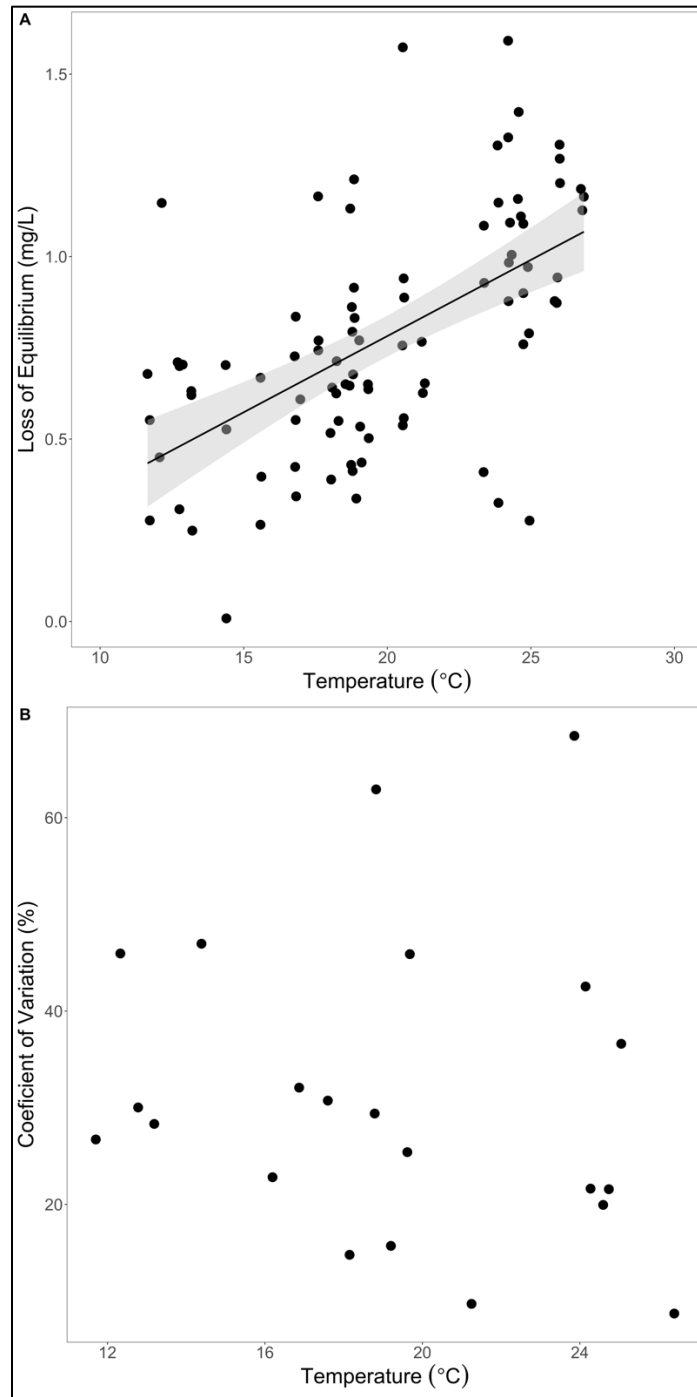


Figure 3.2: Relationship between oxygen pressure at loss of equilibrium (PO₂ at LOE) and acclimation temperature (°C) of Eastern sand darter (A) and the coefficient of variation for PO₂ at LOE measured for each sample day at a given acclimation temperature (°C; B). Significant regressions are shown as solid lines with associated 95% confidence intervals (grey shaded).

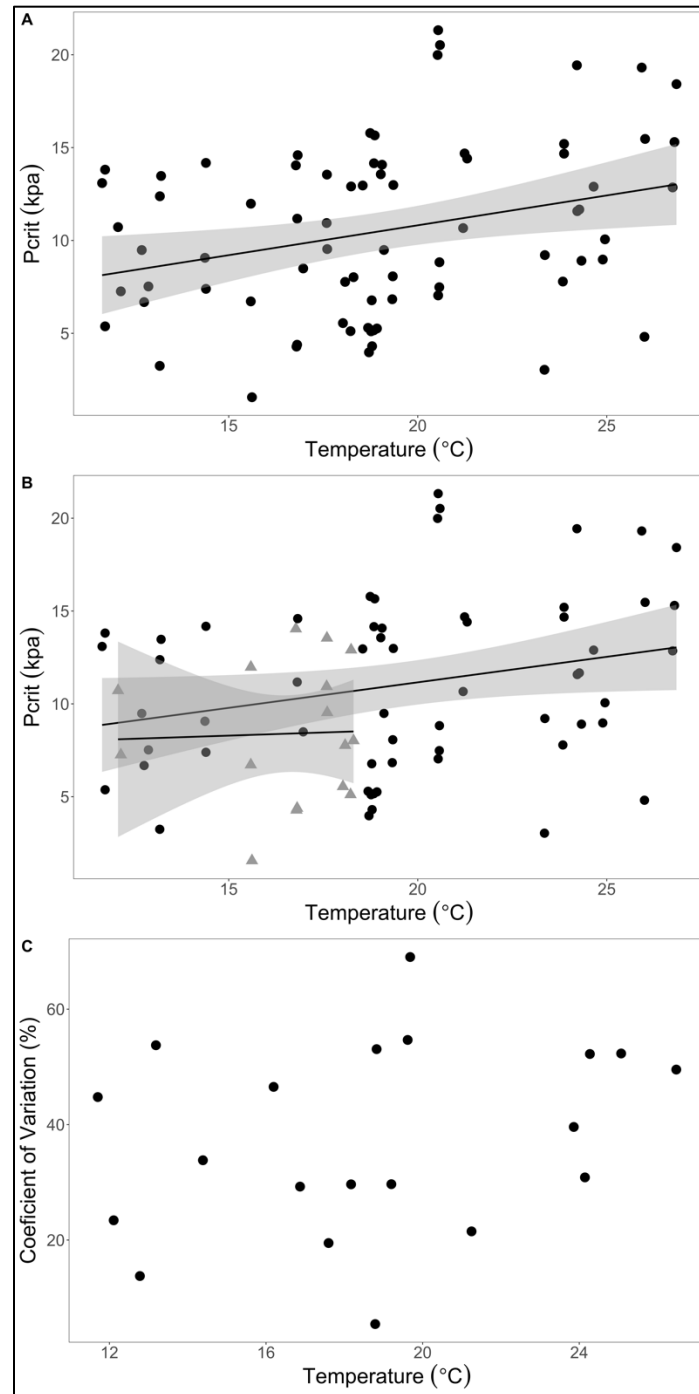


Figure 3.3: Relationship between critical oxygen tension (Pcrit) and acclimation temperature (°C) of Eastern sand darter (A) and the coefficient of variation for Pcrit measured for each sample day at a given acclimation temperature (°C; B). Significant regressions are shown as solid lines with associated 95% confidence intervals (grey shaded).

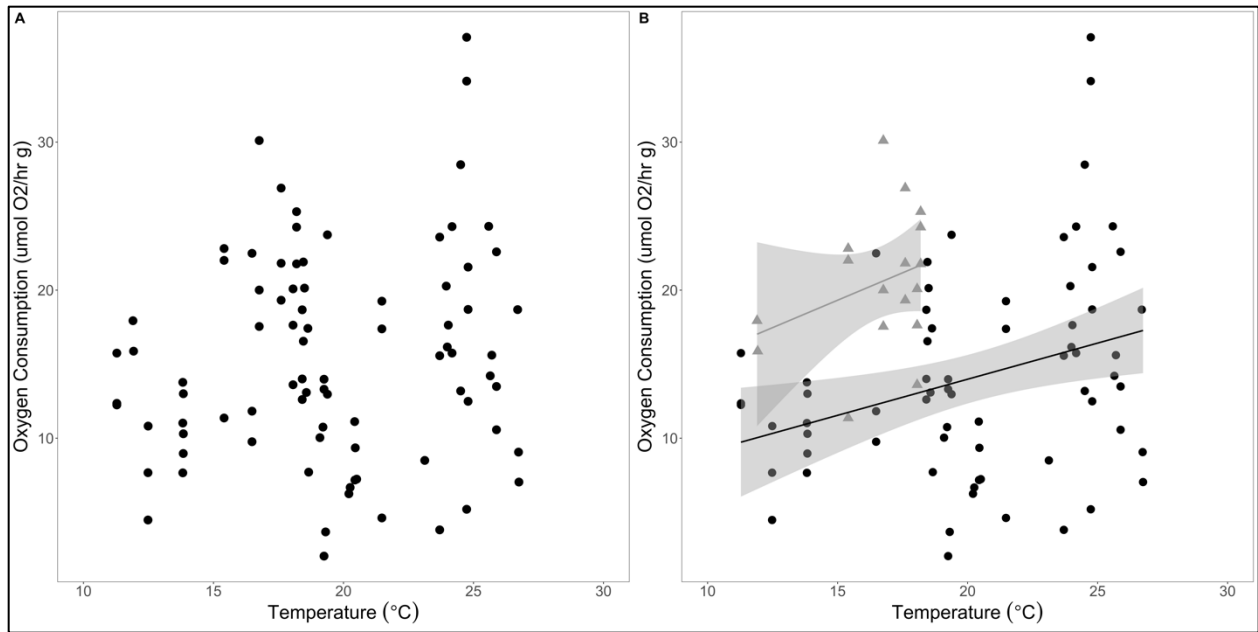


Figure 3.4: Relationships for eastern sand darter between (A) FMR (oxygen consumption, umol O₂/hr g) and acclimation temperature (°C), and (B) and FMR and acclimation temperature when distinguishing between reproductive (grey triangles) and non-reproductive (black circles) periods. Significant regressions are shown as solid lines with associated 95% confidence intervals (grey shaded).

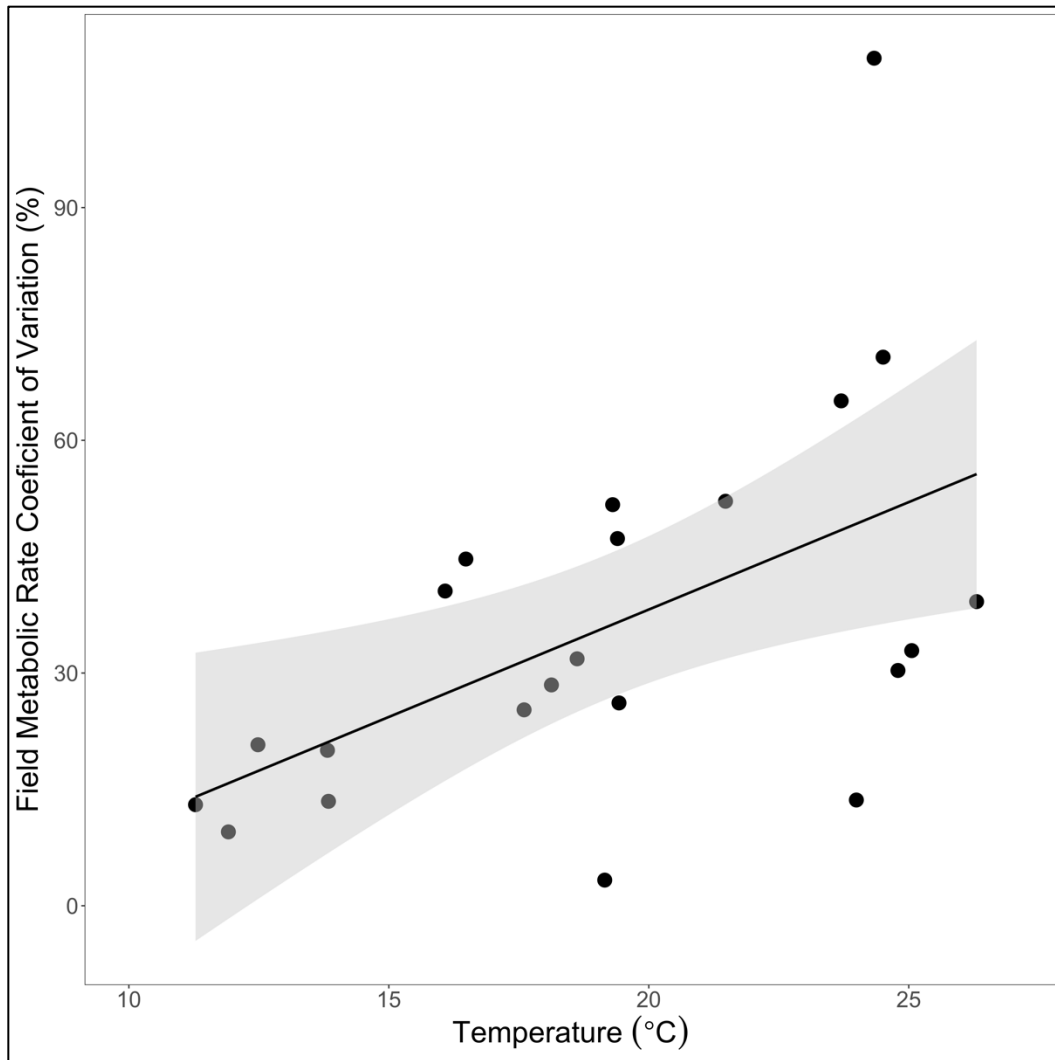


Figure 3.5: The linear regression (solid line) and associated 95% confidence intervals (grey shading) for explaining the significant relationship between coefficient of variation (%) computed from the FMR data measured for individual eastern sand darter and temperature given as acclimation temperature (°C).

Chapter 4: Impact of turbidity on the gill morphology and hypoxia tolerance of Eastern Sand Darter (*Ammocrypta pellucida*)

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4.1 Introduction

Anthropogenic activities such as deforestation, agriculture, and urbanization are increasing sediment loading (i.e. turbidity) in aquatic ecosystems (Collins *et al.*, 2009; Nelson *et al.*, 2009; Restrepo *et al.*, 2015). Turbidity can negatively affect fish health through direct behavioural and physiological changes as well as through indirect impacts on the environment (for review see Kemp *et al.*, 2011). Increased turbidity has been shown to hinder visual-based behaviours, including foraging (i.e. reduced feeding; Rowe & Dean, 1998; Swanbrow Becker *et al.*, 2016) and mate selection (Aspbury *et al.*, 2019; Sundin *et al.*, 2010), and to reduce egg survivorship (Gray *et al.*, 2012) and growth (Shaw & Richardson, 2001; Sutherland & Meyer, 2007), increase susceptibility to disease (Hess *et al.*, 2015; Redding *et al.*, 1987), and result in gill damage that could impact gas exchange and osmoregulation (Au *et al.*, 2004; Cumming & Herbert, 2016; Lowe *et al.*, 2015). Turbidity can also decrease oxygen within the benthic layer (Bruton, 1985), with fine sediment clogging substrate interstices and decreasing oxygen availability for benthic organisms (Chutter, 1969; Donohue & Garcia Molinos, 2009; Heywood & Walling, 2007). Moreover, increases in organic matter settling on streambeds can result in increased sediment oxygen demand, leading to bottom-up hypoxia (Gilbert *et al.*, 2005; Jaiswal & Pandey, 2019).

The fish gill is a complex organ comprised of fragile lamellae membranes that are directly exposed to the environment for gas exchange (Perry & Gilmour, 2002; Piiper, 1982; Piipper & Schumann, 1967) and ion regulation (Evans, 1980; Goss *et al.*, 1992; Zimmer & Perry,

2022). Thus the gill plays an important role in determining metabolic rate, hypoxia tolerance, and osmoregulation (Dhillon *et al.*, 2013; Evans *et al.*, 2005). Suspended and dissolved substances (i.e. sediment) in water can induce lamellae structural changes that impact gill function (Au *et al.*, 2004; Cumming & Herbert, 2016; Hess *et al.*, 2015; Hodgson *et al.*, 2020; Li & Shen, 2012; Lowe *et al.*, 2015; Sutherland & Meyer, 2007). Chronic exposures to low turbidity (~ 20 NTU) have been shown to have little impact on gill function and structure in most fishes (Angeles-Escobar *et al.*, 2021; Becke *et al.*, 2017, 2018; Ljubobratović *et al.*, 2019); however, chronic exposure to high turbidity can trigger structural gill changes that effect oxygen diffusion distance, including: basal hyperplasia, epithelial lifting, lamellar thickness and height, lamellar fusion, and increased goblet cells, i.e. increased mucous production (Au *et al.*, 2004; Cumming & Herbert, 2016; Lowe *et al.*, 2015; Wong *et al.*, 2013). Additionally, mitochondrial-rich ionocyte cells, located on the gill filaments and (or) lamellae that are important for maintaining osmoregulation, have been shown to increase in density with turbidity, indicating that fish could be combatting osmoregulatory stress (Au *et al.*, 2004; Li & Shen, 2012; Wong *et al.*, 2013). Gill structural changes are believed to protect the gills from abrasion but are also likely to impact gas exchange efficiency (i.e. reduced oxygen uptake) and ion regulation loss to the environment, thereby impacting osmoregulation.

Reduced gas exchange efficiency should impact aerobic performance as reflected in physiological performance measures such as metabolic rate and hypoxia tolerance. Routine metabolic rate (RMR) reflects the oxygen requirements for an at-rest fish holding position in the water column and is an important component of field metabolic rate (FMR), which defines the energy expenditure of a fish in its natural environment (Treberg *et al.*, 2016). Hypoxia tolerance

can be characterized using critical oxygen tension (P_{crit}), the point where RMR, or FMR in the field, switches from oxyregulation to oxyconforming, and / or oxygen partial pressure at loss of equilibrium (P_{O_2} at LOE), the oxygen concentration where fish lose the ability to right themselves in the water column (Franklin, 2014; Fry, 1971; Pörtner & Grieshaber, 1993). The impact of turbidity on RMR and the hypoxia tolerance metric P_{crit} is species-specific, with most species being unaffected (Cumming & Herbert, 2016; Gray *et al.*, 2016; Hess *et al.*, 2017). Exceptions include the imperilled pugnose shiner (*Notropis anogenus*) and bridle shiner (*Notropis bifrenatus*), which are threatened by siltation and display increases in P_{crit} (i.e. a decrease in hypoxia tolerance) in response to increased turbidity (Gray *et al.*, 2016). To our knowledge, no one has similarly investigated the impact of turbidity on the hypoxia metric loss of equilibrium.

The negative effects of turbidity on fish health, including reduced foraging, reduced growth, and damaged gills (Cumming & Herbert, 2016; Kemp *et al.*, 2011; Sutherland & Meyer, 2007; Swanbrow Becker *et al.*, 2016), have been described in the literature. Nevertheless, there is limited information available on how fish species in general persist within and adapt to turbid conditions in the wild. Eastern sand darter, a small-bodied benthic fish listed as threatened within Canada, is one species where improved understanding of physiological responses to siltation would improve species conservation (COSEWIC, 2022). Eastern sand darter is an ideal species to examine turbidity impacts because of its fossorial behaviour, limited home range in turbid and clear environments, and because turbidity has been implicated as the overarching threat to its survival (COSEWIC, 2022; Daniels, 1989). Our objectives were to determine the impact of turbidity on eastern sand darter gill morphometrics, FMR, and hypoxia tolerance

measures (P_{O_2} at LOE and P_{crit}) in turbid and non-turbid systems. Specifically, we hypothesized that (1) turbidity would cause gill structural changes in lamellae width, leading to increased oxygen diffusion distance and an increase in ionocyte number and 2-dimensional area to maintain gas exchange and osmoregulation; and, (2) damaged gills with increased oxygen diffusion distance would result in higher FMR and decreased hypoxia tolerance due to inefficient gas exchange.

4.2 Material and Methods

4.2.1 Specimen Collection

Animal use and collection was conducted under Species at Risk permits (#19-PCAA-00021, #20-PCAA-00029) and approved by Fisheries and Oceans Canada (AUP #1968/2068) and the University of Waterloo (AUP #40909) Animal Care committees. Eastern sand darter was collected from the Grand (August 2019) and Thames (August 2020) rivers in southern Ontario. Both study rivers are ecologically similar, being meandering lowland rivers in watersheds with large agricultural and urban influences (Lake Erie Source Protection Region Technical Team, 2008; Wilcox *et al.*, 1998). The rivers differ in turbidity levels, with the Thames River having higher turbidity due to its underlying geology and greater agriculture land-use (Nürnberg & Lazerte, 2015; Stone & Saunderson, 1992). Over the past 10-years the Thames River was consistently and significantly (Mann-Whitney-Wilcoxon test, $P < 0.01$) more turbid (150.78 ± 8.36 NTU), on average, than the Grand River (10.02 ± 0.88 NTU), which was reflected in the turbidity measures obtained during August sampling in the Grand (8.24 ± 1.94 NTU) and the Thames (94.40 ± 8.27 NTU; Firth *et al.*, 2021).

Fish used in the respirometry trials were obtained for a parallel set of studies (Firth *et al.*, 2021, 2023) following methods described in Firth *et al.* (2021), Gáspárdy & Drake (2021),

and Barnucz *et al.* (2022). The Grand River respirometry data used here (FMR, LOE, and P_{crit}) were previously published in Firth *et al.* (2023). Briefly, fish were captured using a 9.14 m bag seine (1.8 m height x 3.7 m width with 1.8 m x 1.8 m x 1.8 m bag with 3.0 mm mesh) in the Grand River and a Gerken Siamese trawl (inner cod end mesh of 18 mm and outer cod end mesh of 3 mm) in the Thames River. During the period of respirometry testing, river water temperatures did not differ significantly (*t*-test, $P = 0.12$) between the two rivers (mean 24.09 ± 0.19 °C in the Grand River and 24.71 ± 0.34 °C in the Thames River). Fish were held in a 56.5 cm x 39.3 cm x 30.4 cm flow-through bin to maintain field-acclimatization until respirometry trials. The respirometry and thermal tolerance (Firth *et al.*, 2021) experimental procedures were designed to be non-lethal; however, some fishes experienced mortality during capture, holding, and at the end of trials and were subsequently retained for gill analysis.

4.2.2 Habitat Measurements

After fish collection, a YSI EX02 multiparameter sonde was used to collect water temperature (°C), dissolved oxygen (mg/L), turbidity (NTU), conductivity ($\mu\text{S}/\text{cm}$), and pH at the midpoint of the site (Table 1).

4.2.3 Gill Histology

Gill histology was completed using the first gill arch from the left gill basket obtained from incidental mortalities ($n = 11$ Grand; $n = 9$ Thames). Given the species conservation status in Canada, histology sample sizes were supplemented with the left gill arches collected from ten preserved specimens from the Royal Ontario Museum ($n = 5$ each from the Grand and Thames rivers; collection ID and collection dates ROM 62039 Grand R. 22 July 2020 and ROM 62087 Thames R. 25 July 1991). Museum specimens were likely fixed in formalin and then stored in 70% ethanol; however, it should be noted that records of specimen preservation were not

available. Our collected gill tissue was fixed in Davidson's solution for 24 – 36 hrs at 4°C and then transferred to 70% ethanol for storage. Gills were dehydrated through a series of ethanol concentrations (70%, 95%, and 100% 3x), cleared with 100% xylene (2x), and infiltrated with paraffin (2x) using a Citdael 1000 tissue processor (Thermo Fisher, Wilmington, DE, USA) to prepare tissues for embedding. Processed gills were embedded in paraffin and sectioned into 5 µm increments using a microtome (Leica RM2235, Concord, ON, Canada). For each fish, half the sections were mounted on glass slides for hematoxylin and eosin (H&E) staining and the other half were mounted on (3-Aminoprpyl)triethoxysilane coated glass slides for immunofluorescence.

Sections used for H&E were de-paraffinized then rehydrated using 100% xylene (2 x 5 min), 100% ethanol (1min), 95% ethanol (1 min), 70% ethanol (1 min), and running water (5 min). Sections were subsequently stained using hematoxylin (1 min then 5 min water wash) and eosin (5 min then 5 min water wash) then dehydrated with 100% ethanol (2 x 2 and 3 min) and 100% xylene (2 x 2 and 3 min) before a coverslip was applied with DPX mounting medium. For each fish, three gill filaments were randomly chosen, and images were taken using a Leica microscope (DM 1000 LED) and camera (MC170 HD) at 40x magnification in conjunction with the Leica Application Suite (LAS version 10) software (Nußloch, Stuttgart, Germany). Images were randomized to ensure blind measurements and analyzed by a single reader using ImageJ (Schneider *et al.*, 2012) to determine filament length and width as well as lamellae height, lamellar width, and inter-lamellar space width on six randomly chosen lamellae (Figure 1A). Filament length was multiplied by 1.05 to correct for any curvature occurring during fixation and sectioning (Hughes, 1984; Rees & Matute, 2018). All gill measures were expressed relative

to fish body mass following Audzijonyte *et al.* (2019), Lefevre *et al.* (2017), and Pauly (1981) to account for differences in size. Additionally, any gill pathologies such as hypertrophy, edema, fusion, and epithelial lifting were noted and gills coded based on the presence (1) or absence (0) of any pathologies using descriptions from (Becke *et al.*, 2018).

Sections used for immunofluorescence were deparaffinized then rehydrated using 100% xylene (3 x 5 min), 100% ethanol (rinse then 2 x 5 min), 70% ethanol (3 min) and deionized water (5 min). Slides were boiled in 0.05% Citraconic Anhydride (30 minutes) for antigen-retrieval then washed using deionized water (5 min), 1% Sodium dodecyl sulphate (5 min then rinsed under tap water), deionized water (3 x 5 min), and 0.05% Tween[®] 20 in Phosphate-Buffered Saline (TPBS, 5 min). Sections were circled with a DAKO hydrophobic pen then incubated with blocking agent, 5% normal goat serum in Immobilon[®] Block Fluorescent Blocker (BLOK) solution, in a humidity chamber for twenty minutes at room temperature. Slides were incubated in a humidity chamber overnight at 4°C with primary antibody $\alpha 5$ against the Na⁺K⁺-ATPase alpha subunit (mouse monoclonal antibody developed by Fambrough, D.M. was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242), diluted with 5% normal goat serum in BLOK (1:100). Slides were washed in TPBS (5, 10, 15 min) then incubated with goat anti-mouse IgG (Alexa Fluor 488; Invitrogen, USA) diluted in BLOK (1:500) in a humidity chamber for 1 hour at 37°C. Slides were then washed in TPBS (5 min), 0.008% DAPI in TPBS (10 min), and TPBS (15 min) before coverslips were applied with mounting medium (1:1 glycerol:PBS + 0.1% sodium azide). For each fish, three gill filaments were randomly chosen, and fluorescent images were taken at the midpoint of each filament to

standardize the location of analysis using an EVOS M5000 Imaging System (ThermoFisher Scientific) at 40x magnification. Images were randomized to ensure blind measurements and analyzed using ImageJ (Schneider *et al.*, 2012) to determine number and 2-dimensional area of ionocytes on six randomly chosen lamellae and the associated filament section (Figure 1B).

4.2.5 Respirometry Protocol

As described in Firth *et al.* (2023), closed chamber respirometry using four 60 ml cylindrical Plexi-glass chambers was employed to determine metabolic rate and hypoxia tolerance streamside. Water was pumped from the river to maintain temperature and oxygen levels in the chambers during a ninety-minute acclimation, which has been shown to be an adequate time period to restore routine metabolic rate in Rainbow Darter (*Etheostoma caeruleum*; Hodgson *et al.*, 2020; Mehdi *et al.*, 2018). After acclimation, the chambers were sealed and oxygen depletion from fish respiration was measured every second using fiber-optic oxygen sensors (FireStingO2) connected to Pyro Oxygen Logger Software (Pyro Science, Aachen, Germany). The rate of oxygen depletion was logged until fish reached loss of equilibrium, denoted by inverted swimming (Lutterschmidt & Hutchison, 1997), after which fish were placed in a bin with 100% oxygenated water for recovery. After recovery, fish total length (mm) and weight (g) were measured.

In each trial one chamber was left empty to measure background respiration from biological oxygen demand. Background respiration was found to be negligible for each chamber ($P \geq 0.20$) and thus was not accounted for in the metabolic rate calculations. The R package 'respirometry' (Birk, 2019) was used to calculate metabolic rate as follows:

$$MO_2 = [(\Delta V \times \Delta O_2) / (\Delta t \times w)]$$

where ΔV is the volume (ml) of water in the respirometer (respirometer volume minus the fish volume; assumed to be the same as w), ΔO_2 is the measured change in oxygen content (mg/L) that aligns with Δt , Δt is the time interval (5-minutes), and w is the fish mass (g). To standardize our metabolic rate estimates, the 5-minute intervals in the range of 110-80% partial pressures of oxygen in water were averaged (similar to Regan & Richards, 2017). Grand River data were obtained from Firth *et al.* (2023). In the Thames River, 12 fish were run through trials but only seven reached the LOE endpoint ($n_{LOE} = 7$). Critical oxygen tension was only calculated on fish that obtained LOE to ensure chamber oxygen levels were low enough for P_{crit} to have occurred. Of the seven fish P_{crit} was calculated on, two fish had measured oxygen consumption slopes with $r^2 < 0.5$ and were removed from the FMR and P_{crit} data sets ($n_{FMR} = 10$, $n_{P_{crit}} = 5$).

The R package 'Segmented' (Muggeo, 2003) was used to calculate the critical oxygen level (P_{crit}) for each fish by identifying break point(s) over the calculated 5-minute metabolic rate intervals with the related partial pressure of oxygen in water.

4.2.5 Statistical Analysis

A two-sample t-test with equal variance was used to test for significant differences in fish mass and condition between the Grand and Thames rivers. Fish body condition was calculated using Fulton's condition factor: $K = W/L^3 \times 100000$, where W is the mass of the fish and L is the length of the fish (Fulton, 1911). To account for fish size differences, all gill H&E morphometries were expressed relative to fish body mass. As mass was not available for Thames River fish gill samples, so Thames River gills were scaled using the average mass from the respirometry experiments. An independent two-sample t-test was used to test for significant differences in fresh collected and museum samples for all gill metrics (Supplementary Material Table B1).

Ionocyte 2-dimensional area in the Thames museum samples ($0.74 \pm 0.32 \mu m^2$) was significantly

smaller (t -test, $P = 0.02$) than the Thames fresh samples ($2.01 \pm 0.29 \mu\text{m}^2$). Thus, museum samples were not included in immunohistochemistry analysis. The difference in all gill morphometrics between the Grand and Thames fish samples was then tested using a two-sample t -test with equal variance. Between-river differences in the prevalence of pathologies were evaluated using a Fisher's exact test.

Analysis of covariance (ANCOVA) was used to test for significant differences between the Grand and Thames fish for FMR, P_{crit} , and P_{O_2} at LOE, using fish mass (g) as a covariate. Results are displayed as mean $\pm$ standard error of the mean. All statistical analyses were performed using RStudio version 4.04 (RStudio Team, 2021).

4.3 Results

Based on the respirometry fish, eastern sand darter was significantly smaller (t -test, $P < 0.001$) in the Thames River ($0.63 \pm 0.10 \text{ g}$) compared to the Grand River ($1.48 \text{ g} \pm 0.05 \text{ g}$). Additionally, eastern sand darter in the Thames River ($0.51 \pm 0.02 \text{ g/mm}^3$) had significantly lower body condition (t -test, $P < 0.05$) than in the Grand River ($0.57 \pm 0.01 \text{ g/mm}^3$). Mean standardized filament lengths in the Grand River ($1931.96 \pm 181.09 \mu\text{m/g}$) and the Thames River ($2367.53 \pm 244.77 \mu\text{m/g}$) were not significantly different (t -test, $P = 0.16$; Figure 2A). Mean filament widths were significantly (t -test, $P = 0.04$; Figure 2B) smaller in the Grand River ($91.47 \pm 9.11 \mu\text{m/g}$) compared to the Thames River ($123.35 \pm 12.02 \mu\text{m/g}$). Mean lamellae heights in the Grand River ($266.40 \pm 27.01 \mu\text{m/g}$) and the Thames River ($320.02 \pm 22.45 \mu\text{m/g}$) were not significantly different (t -test, $P = 0.14$; Figure 2C). Similarly, mean lamellae width was not significantly different (t -test, $P = 0.055$; Figure 2D) in fish from the Grand River ($34.83 \pm 2.83 \mu\text{m/g}$) and the Thames River ($43.90 \pm 3.61 \mu\text{m/g}$). Lastly, the mean interlamellar space in the Grand River ($40.44 \pm 5.48 \mu\text{m/g}$) was significantly smaller (t -test, $P = 0.02$; Figure 2E) than in the Thames

River ($59.23 \pm 4.82 \mu\text{m/g}$). There were no statistically significant differences in the proportional occurrence of pathologies in the Grand and Thames rivers (Fisher's exact, $P = 0.068$; Figure 2F). Mild pathological alterations were observed in most fish, demonstrating a background level of gill damage in fish from both populations in the wild.

Ionocyte number per gram of fish in the Grand (17.89 ± 1.72) and Thames rivers (19.33 ± 3.04) were not significantly different (t -test, $P = 0.67$; Figure 3A). Similarly, ionocyte 2-dimensional areas were not significantly different (t -test, $P = 0.89$; Figure 3B) between the Grand ($1.93 \pm 0.35 \mu\text{m}^2$) and Thames fish ($2.01 \pm 0.29 \mu\text{m}^2$).

The FMR and sampling period (ANCOVA, $F_{1,18} = 1325.16$, $P > 0.50$) and FMR and mass ($F_{1,19} = 1546.32$, $P > 0.50$) interaction terms were not significant. Similarly, the interaction terms between P_{O_2} at LOE and sampling period ($F_{1,18} = 3.40$, $P > 0.50$) and P_{O_2} at LOE and mass ($F_{1,19} = 3.45$, $P > 0.50$) were not significant. Lastly, the interaction terms for P_{crit} and sampling period ($F_{1,10} = 223.42$, $P > 0.50$) and P_{crit} and mass ($F_{1,11} = 224.12$, $P > 0.50$) were not significant. Thus, the interaction terms and mass were removed from final model consideration and a two-sample t -test with equal variance was used to assess between-sampling period differences for FMR, P_{crit} , and P_{O_2} at LOE. The FMR estimates for Grand ($18.50 \pm 3.04 \mu\text{mol/g-hr}$) and Thames ($19.16 \pm 1.91 \mu\text{mol/g-hr}$) fish were not significantly different (t -test, $P=0.86$; Figure 4). Eastern sand darter loss of equilibrium differed significantly between the study rivers (t -test, $P=0.049$), occurring at higher oxygen concentrations in the Thames River ($1.29 \pm 0.20 \text{ mg/L}$) than the Grand River ($0.89 \pm 0.09 \text{ mg/L}$; Figure 5A). Whereas, P_{crit} of eastern sand darter was not significantly different (t -test, $P=0.103$) in the Thames River ($6.83 \pm 1.44 \text{ kPa}$) than in the Grand River ($11.09 \pm 1.59 \text{ kPa}$; Figure 5B).

4.4 Discussion

Consistent exposure of eastern sand darter in the wild to differences in turbidity over the range of 8-94 NTU had minimal consequences for gill structure. Filament width and inter-lamellae space were significantly larger under turbid conditions. No between-river differences in filament lengths, lamellae heights and widths, or the occurrence of abnormal gill pathologies were noted. Additionally, neither ionocyte number nor 2-dimensional area and FMR and the P_{crit} hypoxia tolerance metric differed between fish sampled from the differing turbidity environments. Nevertheless, the P_{O_2} at LOE hypoxia tolerance metric differed significantly between fish from the two environments, with fish from the higher-turbidity Thames River being less tolerant to hypoxia.

The observable structural changes in the gills exposed to high turbidity were minimal, with only the inter lamellae spaces and filament widths being significantly different. An increase in inter lamellae space in turbid conditions may be an adaptation to decrease abrasion and (or) clogging of gill lamellae. Turbid water typically has particles sizes ranging from < 1 to almost $100\text{ }\mu\text{m}$, with particles $< 63\text{ }\mu\text{m}$ being predominant (Walling & Moorehead, 1989; Zhu *et al.*, 2022). An increase in inter lamellae space by an average of $18.79\text{ }\mu\text{m}$ per gram of fish mass in the Thames fish as compared to the Grand fish would allow larger, and likely more, particles to pass through the gills, thereby decreasing clogging. However, an increase in inter lamellae space decreases the amount of oxygen uptake by the gills as the oxygen transfer rate is decreased as the distance between lamella increases (Hughes, 1966; Park *et al.*, 2014). Generally, when fish are exposed to decreased oxygen conditions (i.e. increased temperature, hypoxia, turbidity) ventilation is increased to maintain oxygen uptake (Lowe *et al.*, 2015). With increased ventilation, adjacent gill filaments can be separated allowing for water to ‘escape’

between the filaments and for oxygenated water to pass through the gill without being diffused into the bloodstream (Hughes, 1966; Strother, 2013). Turko *et al.* (2020) demonstrated that *Kryptolebias marmoratus* gills exposed to decreased oxygen conditions (i.e. hypoxia, increased temperature, and air exposure) had an increase in calcium deposition on the filaments, which is believed to provide additional structural support for the filaments with increased ventilation. We suggest that an increase in filament width could provide a similar structural support function during increased ventilation to reduce the amount of oxygenated water ‘lost’ between the filaments. Increased filament width could also provide structural support under increased physical tension if particles become clogged in the gills.

The most common gill changes observed under increased turbidity (epithelial lifting, epithelial thickening, hyperplasia, and mucous production) typically increase lamellae width, increasing the oxygen diffusion distance, which is presumed to protect the gills from abrasion and irritants reaching the bloodstream (Mallatt, 1985). Eastern sand darter may not have been exposed to sufficiently high turbidity levels at our study sites to have elicited significant changes in lamellae width, gill pathologies, or ionocytes. A lack of observable gill structural changes until turbidity thresholds have been surpassed has been reported in other species. For example, arctic grayling (*Thymallus arcticus*) tested at ≤ 1000 mg/L of suspended sediment (~292 NTU) demonstrated no significant gill structural change or notable gill damage (McLeay *et al.*, 1987). However, arctic grayling exposed to 1205 mg/L (~352 NTU) and 1388 mg/L (~405 NTU) suspended sediment levels showed moderate gill damage (Reynolds *et al.*, 1989). An increase in chloride cell (ionocyte) density was not seen in juvenile black sea bream (*Acanthopagrus schlegelii*) or juvenile green grouper (*Epinephelus coioides*) until exposed to turbidity

concentrations in the range of 1000 mg/l and 2000 mg/L, respectively (Au *et al.*, 2004; Li & Shen, 2012). Juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to increasing concentrations of inert kaolin clays similarly displayed no appreciable histological changes in the gill at low to moderate exposure levels (Goldes *et al.*, 1988), with changes apparent only after lengthy exposure (16-32 days) to the highest concentrations (4887 mg/l). However, gills demonstrated no histological change by 64 days with return to control states, implying gills may have adapted to the turbid conditions (Goldes *et al.*, 1988). Eastern sand darter has been exposed to high levels of turbidity in the Thames River for generations. As a fossorial species that buries in the sand (Daniels, 1989), the species may have similarly adapted protections from sediment abrasion.

Eastern sand darter was significantly smaller with lower body condition in the Thames River as compared to the Grand River. Three other darter species, blackside darter (*Percina maculata*), greenside darter (*Etheostoma blennioides*), and johnny darter (*Etheostoma nigrum*) that reside in both rivers are also significantly smaller in the Thames River (Firth *et al.*, 2024). Increasing turbidity has been shown to decrease individual growth rate in a number of fish species (Cumming & Herbert, 2016; Lowe *et al.*, 2015). The decreased growth has been hypothesized to result from gill structural changes that impact oxygen uptake (Cumming & Herbert, 2016; Lowe *et al.*, 2015; Wong *et al.*, 2013). However, three out of four fish that demonstrated gill structural changes under increasing turbidity levels showed no associated impacts on metabolic rate (Cumming & Herbert, 2016; Hess *et al.*, 2017). Similarly, we also observed no significant change in the metabolic rates of eastern sand darter between turbidity levels. Although not assessed here, metabolic rate could be maintained by increased

ventilation, which would lead to decreased growth (Horkel & Pearson, 1976). Gray *et al.* (2016) also did not observe a change in metabolic rate in three *Notropis* spp. exposed to increased turbidity. To our knowledge, only one study has previously assessed all three metrics concurrently (gill structural change, metabolic rate, and fish growth/size), making it difficult to interpret how the variables are associated.

Our study is the second to assess gill structural changes, metabolic rate, and fish size simultaneously at different turbidity levels. Similar to juvenile sparid fish (*Pagrus auratus*) in Cumming & Herbert (2016), eastern sand darter was significantly smaller under turbid conditions and showed no significant difference in metabolic rate. No apparent difference in metabolic rate suggests eastern sand darter should be able to maintain similar growth in both turbid and clear environments provided energy uptake does not change (e.g., Elliot, 1976). Being smaller under turbid conditions implies lower energy intake in the Thames River, such that once metabolic cost is met there is less energy surplus available for growth (Hess *et al.*, 2017; Oldham *et al.*, 2019; Pollock *et al.*, 2007; Robb & Abrahams, 2003). As the feeding rate of visual predators is known to decline as turbidity increases (Hecht and van der Lingen, 1992; Henley *et al.*, 2000), changes in growth at higher turbidity levels may relate to hindered visual and olfactory performance and (or) lowered food availability that reduces feeding, rather than as a result of respiratory distress (Drake *et al.*, 2008; Rowe & Dean, 1998; Swanbrow Becker & Gabor, 2012; Swanbrow Becker *et al.*, 2016).

Eastern sand darter demonstrated no change in P_{crit} but there was a significant decrease in hypoxia tolerance based on P_{O_2} at LOE in turbid water. Each metric represents a different aspect of hypoxia tolerance, with P_{crit} representing the switch from oxy-regulating (i.e.,

maintaining aerobic respiration), to oxy-conforming, where anaerobic glycolysis is relied on for metabolism, and P_{O_2} at LOE representing the physical response and being a precursor to mortality unless dissolved oxygen levels increase (Fry, 1971; Pörtner & Grieshaber, 1993; Ultsch & Regan, 2019). Similar to our study, P_{crit} of juvenile sparid fish and blacknose shiner (*Notropis heterolepis*) was not impacted by turbidity (Cumming & Herbert, 2016; Gray *et al.*, 2016). In contrast, two imperilled fishes, pugnose shiner and bridled shiner, demonstrated increased P_{crit} (i.e. decreased hypoxia tolerance) when exposed to turbidity (Gray *et al.*, 2016). No observed change in P_{crit} in eastern sand darter from the Thames River suggests fish are capable of regulating their metabolic rate, which is expected since we only observed minimal gill structural change and there was no evidence for respiratory limitation.

To our knowledge, our study is the first to assess hypoxia tolerance using P_{O_2} at LOE under turbid conditions. An increased P_{O_2} at LOE under turbid conditions could result from the smaller size and lower body condition of fish in the Thames River. Smaller fish have been shown to reach P_{O_2} at LOE sooner than larger fish, as they have decreased energy reserves for anaerobic metabolism and reduced capacity to buffer lactate buildup (Nilsson & Östlund-Nilsson, 2008; Urbina & Glover, 2013). An increased P_{O_2} at LOE due to turbidity could be detrimental to eastern sand darter in the face of co-occurring stressors such as bottom-up hypoxia. However, eastern sand darter appears more tolerant of hypoxia in the turbid Thames River (1.29 ± 0.20 mg/L) than greenside darter (3.39 ± 1.48 mg/L), fantail darter (*Etheostoma flabellare*; 2.36 ± 0.77 mg/L) and rainbow darter (1.64 ± 0.38 mg/L) are in non-turbid waters (Hlohowskyj & Wissing, 1985). Thus, while eastern sand darter may have decreased hypoxia

tolerance under turbid conditions, it appears to be more hypoxia-tolerant compared to similar species.

4.4.1 Conclusions

Our field-based study demonstrates that turbidity has minimal detectable impact on eastern sand darter respiration and osmoregulation based on gill morphology, metabolic rate, and hypoxia tolerance over the range of turbidity values tested (8 - 94 NTU). Observed changes in eastern sand darter hypoxia tolerance (P_{O_2} at LOE) under turbid conditions were small, with results indicating superior hypoxia tolerance compared to similar darter species. Although we did not observe an overall change in gill morphology and metabolic rate, efforts should be undertaken to ensure turbidity does not surpass current levels as the threshold for eastern sand darter effects is unknown, and because of the effects of turbidity on other life history processes (e.g., reproduction, feeding).

Table 4.1: Average water temperature (C°), turbidity (NTU), dissolved oxygen (mg/L), pH, and conductivity ($\mu\text{s}/\text{cm}$) measured during this study and average historical turbidity (NTU) at eastern sand darter sites collected by Fisheries and Oceans Canada over the past 10 years in the Grand and Thames rivers.

Location	Temperature (C°)	Study Turbidity (NTU)	Historical Turbidity (NTU)	Dissolved Oxygen (mg/L)	pH	Conductivity ($\mu\text{s}/\text{cm}$)
Grand River	24.09 $\pm$ 0.19	8.24 $\pm$ 1.94	10.02 $\pm$ 0.88	6.95 $\pm$ 0.14	8.48 $\pm$ 0.06	831.33 $\pm$ 1.33
Thames River	24.71 $\pm$ 0.34	94.40 $\pm$ 8.27	150.78 $\pm$ 8.36	6.92 $\pm$ 0.34	8.28 $\pm$ 0.09	669.75 $\pm$ 33.74

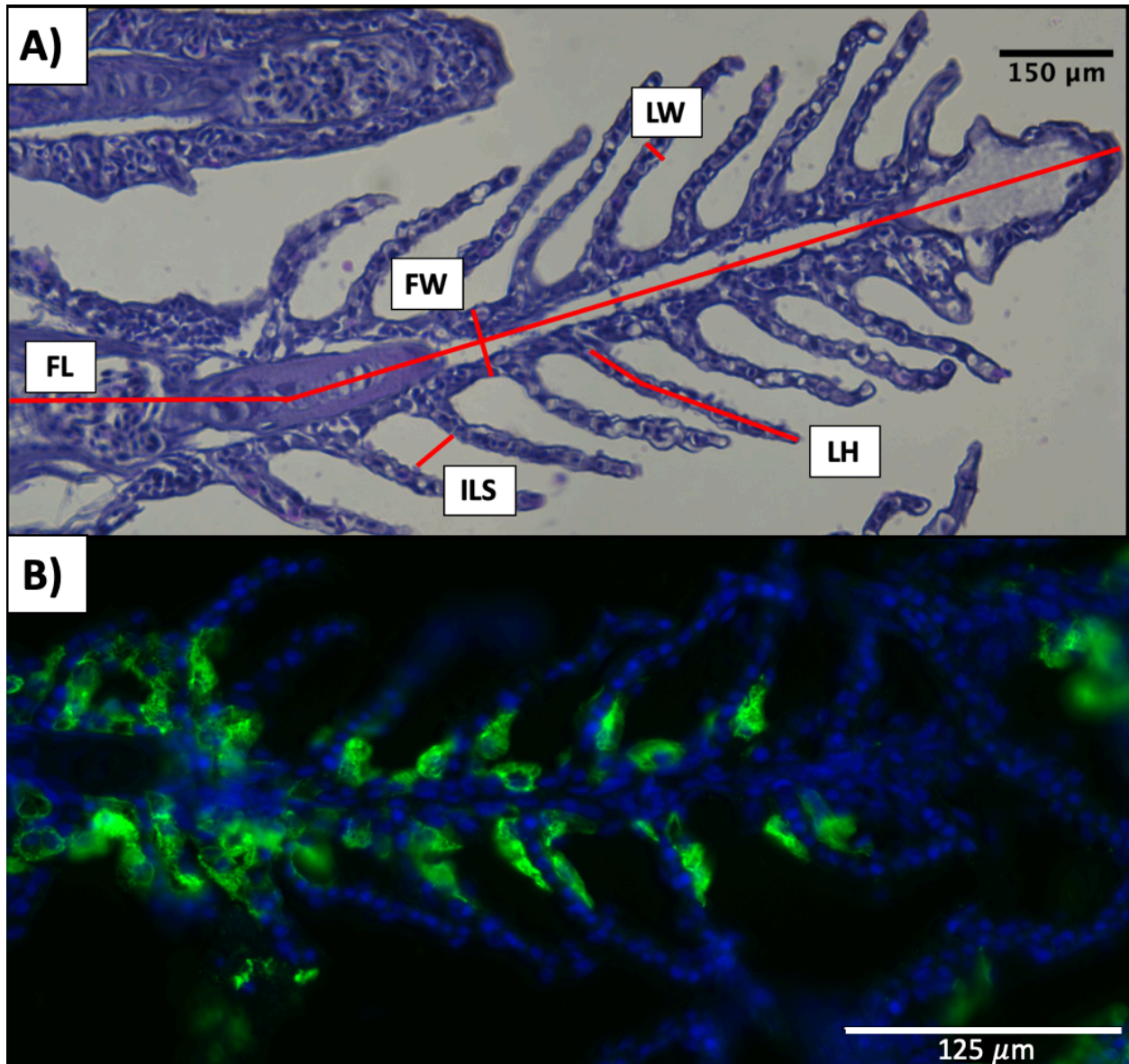


Figure 4.1: Example of an eastern sand darter: (A) H&E (hematoxylin and eosin)-stained gill and the corresponding morphometric measures taken: filament length (FL), filament width (FW), lamellae height (LH), lamellae width (LW), and inter-lamellae space (ILS) and (B) immunofluorescence-stained gill with Na⁺K⁺-ATPase ionocytes (green) and nuclei (blue).

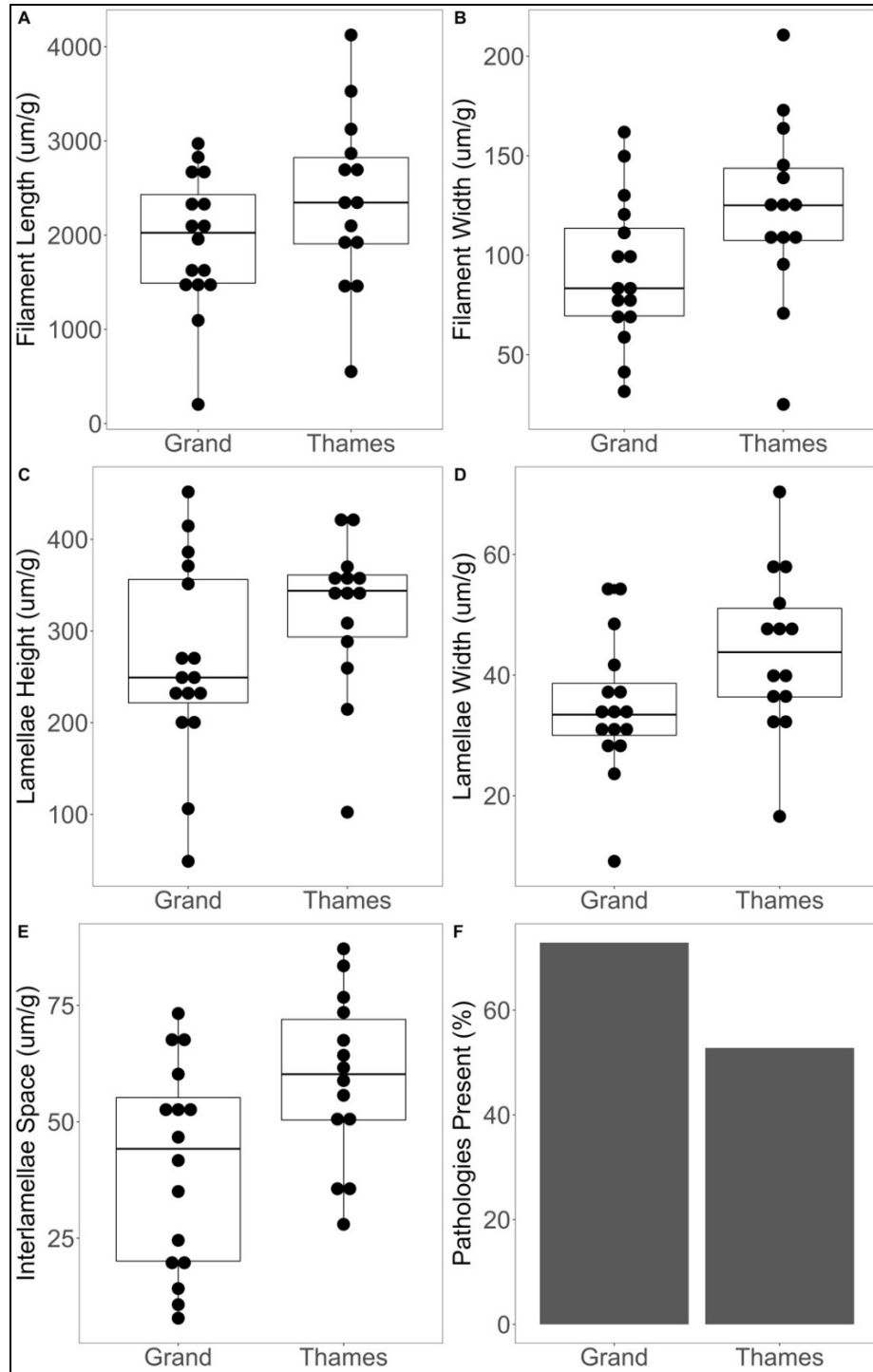


Figure 4.2: The effect of river turbidity level on (A) lamellae width, (B) lamellae height, (C) filament width, (D) filament length, (E) interlamellae space, and (F) the presence (grey) and absence (black) of gill pathologies in eastern sand darter. Raw data points are overlaid on the boxplots.

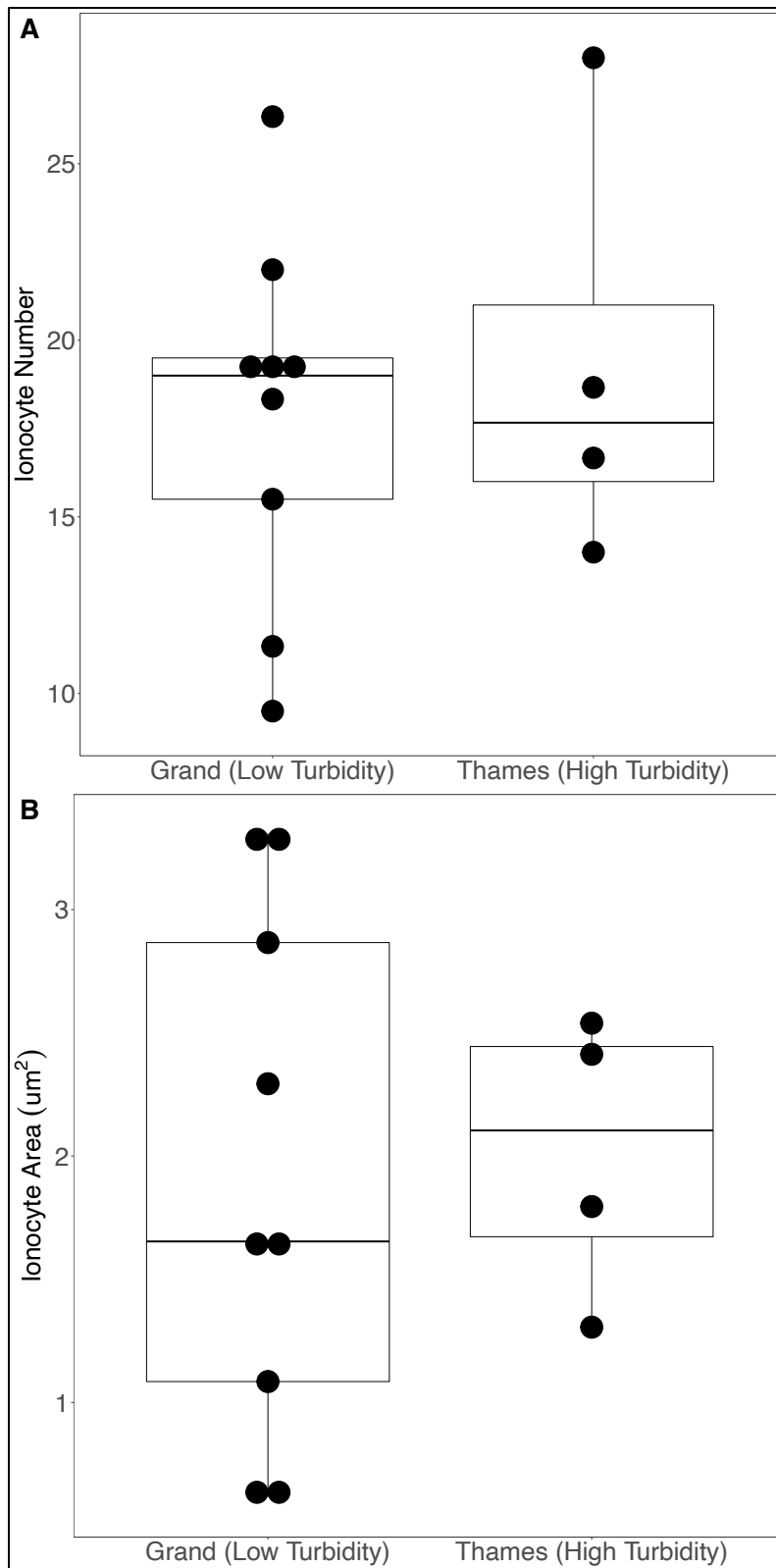


Figure 4.3: The effect of river turbidity level on (A) ionocyte area and (B) ionocyte number in eastern sand darter. Raw data points are overlaid on the boxplots.

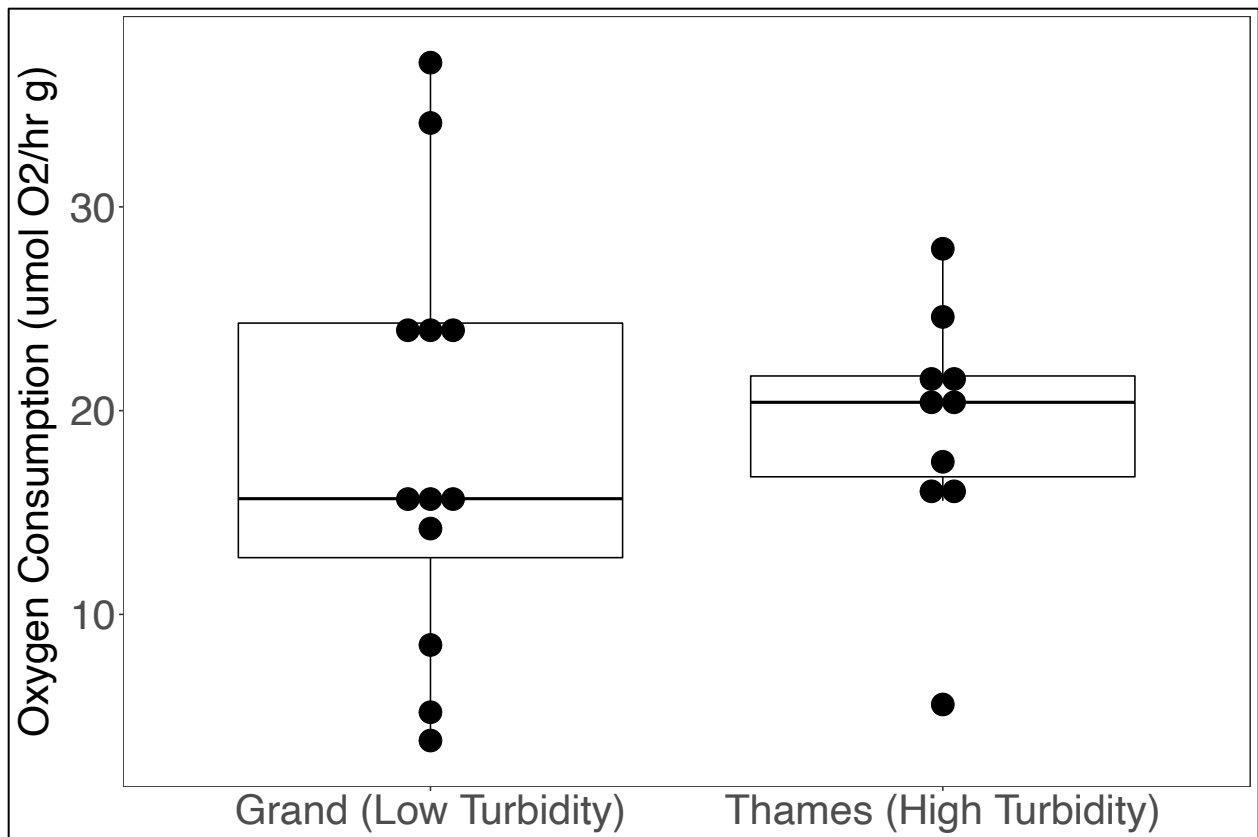


Figure 4.4: The effect of river turbidity level on field metabolic rate (FMR) in eastern sand darter. Raw data points are overlaid on the boxplots.

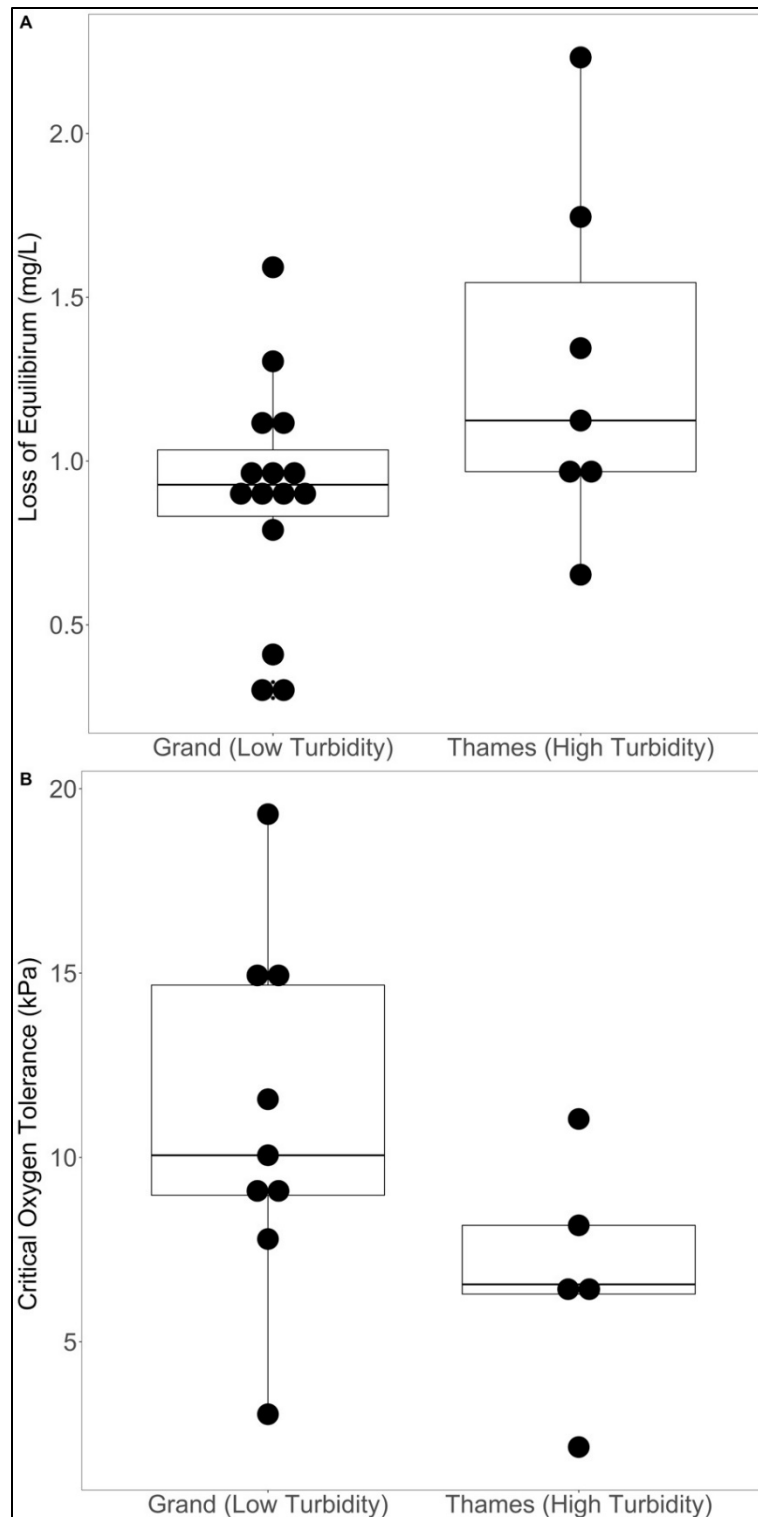


Figure 4.5: The effect of river turbidity level on hypoxia metrics, (A) oxygen pressure at loss of equilibrium (P_{O_2} at LOE) and (B) critical oxygen tension (P_{crit}), in eastern sand darter. Raw data points are overlaid on the boxplots.

Chapter 5: Impacts of temperature and turbidity on the gill physiology of darter species

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5.1 Introduction

The fish gill (Evans et al., 2005) is a complex organ and the site of gas exchange (Perry and Gilmour, 2002; Piiper, 1982; Piipper and Schumann, 1967), ion regulation (Evans, 1980; Goss et al., 1992; Zimmer and Perry, 2022), acid-base regulation (Claiborne et al., 2002; Goss et al., 1992; Zimmer and Perry, 2022), nitrogenous waste excretion (Mommensen and Walsh, 1992; Wright et al., 1995), chemoreception (Burleson et al., 2006; Burleson and Milsom, 1995; Milsom, 2002), and immune function (Dawe et al., 2024). These functions are all affected by the surface area of the gill epithelium and (or) the number and surface area of specialized cells, such as mitochondrial-rich cells (i.e. ionocytes; Evans et al., 2005; Wilson and Laurent, 2002). The epithelial surface area varies in fish species due to the size and number of lamellae that protrude from the gill filaments, with highly active and hypoxia-tolerant species having a larger surface area to improve oxygen uptake compared to sedentary and hypoxia-intolerant species (Chapman et al., 2000; Dhillon et al., 2013; Gray, 1954; Wilson and Laurent, 2002).

To meet oxygen and ion demands in changing environments, numerous fish species have been shown to remodel their gills (Gibbons et al., 2018; Ma et al., 2022) in response to temperature (Chen et al., 2019; Mitrovic and Perry, 2009; Sollid et al., 2005), oxygen (de Assis Porto et al., 2021; Sollid et al., 2003), and salinity levels, and to protect gills from turbidity (Au et al., 2004; Becke et al., 2018; Cumming and Herbert, 2016), contaminants (Hodgson et al.,

2020; Schjolden et al., 2007), and pathogens (Nilsson et al., 2012). Gill remodelling to maintain oxygen demands with changing temperature and turbidity commonly occurs via modification of the lamellae surface area by altering the lamellae length and width (Bowden et al., 2014) and (or) developing/removing an inter lamellar cell mass (Sollid et al., 2003). Remodelling may also occur via changes in the diffusion distance (Hess et al., 2015), and (or) increasing the number of goblet cells (Becke et al., 2018). Gill remodelling to increase gas exchange in freshwater species leads to an increase in passive ion loss to the environment, which can result in osmoregulatory stress due to the energetic cost associated with actively absorbing salts (Gonzalez and McDonald, 1994; Randall et al., 1972; Sollid and Nilsson, 2006). The trade-off between gas exchange and ion regulation has been termed the osmo-respiratory compromise (Gonzalez and McDonald, 1994; Nilsson, 1986; Randall et al., 1972). To maintain osmo-respiratory homeostasis when gills are remodelled for gas exchange and (or) protection, mitochondrial rich cells that maintain ionic balance have been shown to relocate along the gill filament, altering their surface area and (or) number (Bradshaw et al., 2012; Mitrovic and Perry, 2009).

Gill remodelling has been predominantly demonstrated in lab settings where fish are held in constant abiotic conditions. It is poorly understood how gills may remodel in the wild where fish are exposed to varying environmental conditions such as diurnal and seasonal temperature fluctuations and variable turbidity. To our knowledge, only one study has assessed gill remodelling in the wild, and found that gills do not undergo the same method or extent of remodelling in the wild as has been observed in the lab (A.J. Turko, University of Guelph; personal communication). Thus, gill remodelling may be too energetically costly to occur in response to short term environmental change in the wild (e.g., diurnal cycles), and in those

situations fish may alternatively rely on behavioural responses, adjustments in the circulatory system (i.e. heart rate and hematocrit), and gill enzymatic activity (Na^+/K^+ ATPase activity; Sollid and Nilsson 2006). Hematocrit levels and Na^+/K^+ ATPase activity (NKA) have been shown to have species-specific directional adjustments in response to variation in temperature and turbidity (Au et al., 2004; Gibbons et al., 2018; Li and Shen, 2012; Mitrovic and Perry, 2009; Rodgers et al., 2019), thereby complicating predictions about how any individual species may respond to changing environmental conditions.

Adjustments of the circulatory system, such as increased hematocrit and gill enzymatic activity, could improve gas exchange and ion regulation for fish exposed to chronically high turbidity and high temperature conditions that can have implications for gill structural damage. Structural damage can include: increased basal hyperplasia, epithelial lifting, lesions, and epithelial thickening that can all result in an inefficient osmo-respiratory compromise with gills not efficiently taking up oxygen or, conversely, becoming leakier and having to work harder to maintain osmoregulation (Angeles-Escobar et al., 2021; Au et al., 2004; Becke et al., 2017; Cumming and Herbert, 2016; Hess et al., 2015; Li and Shen, 2012). Some species, however, have occupied chronically turbid areas for generations and presumably their gills have adapted and ultimately have reduced structural damage to maintain osmo-respiratory homeostasis.

Darters make up a large part of the family Percidae and occupy a variety of macro and microhabitats. The greenside darter (*Etheostoma blennioides*), blackside darter (*Percina maculata*), and johnny darter (*Etheostoma nigrum*) have all occupied clear and turbid riverine ecosystems at the macrohabitat scale for generations and may show adaptations to chronic turbidity. At the microhabitat scale, the greenside darter prefers fast to moderate flow with

cobble and gravel substrate, blackside darter prefers slow to moderate current with cobble and gravel substrate, while johnny darter prefers slow flow and has no substrate preference occupying a variety of substrates from sand to gravel (Scott and Crossman, 1973; Smith, P.W., 1979). Differences in occupied water velocity have been shown to be related to different phenotypic responses in gill morphology in cyprinids (Dhillon et al., 2013; Fu et al., 2014). For example, slow-flow habitats typically exhibit large daily fluctuations in dissolved oxygen and hypoxia occurrence is greater, thus species that in habitat slow-flow habitats have increased gill plasticity (Dhillon et al., 2013; Fu et al., 2014). Substrate preferences could also lead to differential phenotypic responses as sand dwelling species may be more accustomed to gill irritation than species preferring rock substrates.

The objectives of this study were to determine if temperature and turbidity alter the respiratory surface area and osmoregulation of three co-occurring darter species (greenside darter, blackside darter, and johnny darter). Specifically, we hypothesize that: 1) gill respiratory surface area and hematocrit percentage will be greater at warmer temperatures and higher turbidity levels; and, 2) ionocyte surface area and Na^+/K^+ ATPase activity will be increased at warmer temperatures and higher turbidity.

5.2 Material and Methods.

5.2.1 Specimen Collection

Greenside darter, blackside darter, and johnny darter were collected in August and October 2020 from the Grand River and in August 2020 from the Thames River, Ontario (Figure 1).

October acclimatization temperature ($11.87\text{ }^{\circ}\text{C} \pm 0.70$) in the Grand River was significantly colder (ANOVA, $F_{2,6} = 168.4$, $P < 0.001$) than August acclimatization temperatures in either the Grand ($22.36\text{ }^{\circ}\text{C} \pm 0.31$) or the Thames ($22.59\text{ }^{\circ}\text{C} \pm 0.29$), which did not differ significantly ($P >$

0.05). The Grand and Thames rivers are the two largest watersheds in southern Ontario and are generally considered to be ecologically similar, both being main branch meandering rivers surrounded by intensive agricultural land use with somewhat similar thermal regimes and species assemblages (Lake Erie Source Protection Region Technical Team, 2008; Wilcox et al., 1998). However, the Thames River has significantly (Mann-Whitney-Wilcoxon test, $P < 0.01$; Firth et al. 2021) higher suspended sediment (150.78 ± 8.36 NTU) resulting from its underlying physiography and greater extent of agriculture in the watershed (Nürnberg and Lazerte, 2015; Stone and Saunderson, 1992) than the Grand River (10.02 ± 0.88 NTU).

In the Grand River, fishes were captured near Brantford, Ontario (43.11001, -80.24494) using a 9.14 m bag seine with 3.0 mm mesh, 1.8 m height x 3.7 m width wings and 1.8 m x 1.8 m x 1.8 m bag. In the Thames River, fishes were captured near Wardsville, Ontario (42.64190, -81.70792) using a Gerken Siamese trawl with two 50 x 30 cm otter doors, an 18 mm inner cod end and a 3 mm outer cod end mesh (Barnucz et al., 2022). Different sampling methods were used in each river due to differences in bank morphology, water depth, and flow during sampling that precluded the use of certain sampling techniques.

All animal use and collection was approved by the Environment and Climate Change Canada and Fisheries and Oceans Canada Animal Care Committee (AUP 2068). Upon capture fishes were euthanized with an overdose of clove oil and total length (TL) and mass (g) were measured (Table 1). The caudal peduncle was severed to collect blood from each fish using a hematocrit tube. Hematocrit tubes were immediately centrifuged, and hematocrit values determined using digital calipers. The left gill arches were collected for gill histology and the right gill arches were collected for Na^+/K^+ ATPase activity.

5.2.2 Gill Histology

The first and second gill arch from the left gill basket were removed and fixed in Davidson's solution for 24 – 36 hrs before being placed in 70% ethanol for storage. Gill tissue was routinely processed (Citadel 1000 tissue processor, Thermo Fisher, Wilmington, DE, USA), embedded in paraffin, and sectioned at 5 µm thickness using a microtome (Leica RM2235, Concord, ON, Canada) until multiple sections with the whole arches visible were obtained. The gill sections for each fish were then divided in half and half the gill sections were mounted on glass slides for haematoxylin and eosin (H&E) staining and the other half were mounted on (3-Aminopropyl)triethoxysilane (APS) coated glass slides for immunofluorescence staining.

H&E sections were deparaffinized, rehydrated and routinely stained using hematoxylin and eosin. Stained slides were viewed at 40x magnification using a Leica microscope (DM 1000 LED) and camera (MC170 HD) connected to the Leica Application Suite (LAS version 10) software (Nußloch, Stuttgart, Germany). Images were taken of three random filaments per fish, then images were randomized to reduce bias. Randomized images were analyzed by one person using ImageJ (Schneider et al., 2012) to assess filament and lamellae length and width as well as the width of the interlamellar space on six random lamellae per filament (Figure 2A). To correct for curvature during the fixation process filament length was multiplied by 1.05 (Hughes, 1984; Rees and Matute, 2018). Further, the two-dimensional lamellae surface area (lamellae width x length) was calculated. Gill pathologies were observed but were not analyzed on the advice of a qualified veterinary pathologist (Karlee Craig personal communication). Photomicrographs are provided in the supplementary materials (Figure S1).

Immunofluorescent sections were deparaffinized, rehydrated, and blocked using 5% normal Goat Serum in Immobilon® Block Fluorescent Blocker (BLOK). Primary antibody staining

consisted of overnight incubation of $\alpha 5$ (mouse monoclonal antibody against the Na^+K^+ -ATPase alpha subunit developed by Fambrough, D.M. was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242) diluted (1:100) in 5% normal goat serum and BLOK at 4°C in a humidity chamber. Secondary antibody staining consisted of 1-hour incubation of diluted 1:500 goat anti-mouse IgG (Alexa Fluor 488; Invitrogen, USA) in BLOK at 37°C in a humidity chamber. Then slides were washed in Tween[®] 20 Phosphate Buffered Saline (TPBS) and counterstained with DAPI. Slides were viewed at 40x magnification using an EVOS M5000 Imaging System (ThermoFisher Scientific) and images of three randomly chosen gill filaments were taken at the midpoint of the filament for each fish and randomized to reduce bias. Randomized images were analyzed to determine average ionocyte 2-dimensional area and number using ImageJ (Schneider et al., 2012) for six random lamellae per filament and the associated filament section (Figure 2B).

5.2.4 Na^+/K^+ ATPase Activity

Na^+/K^+ ATPase activity was determined following the methods of McCormick (1993). Gill tissue was immersed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.5) and immediately frozen using dry-ice and stored at -80°C for no longer than three months. Gill tissue was homogenized with 25 μl of SEID (100 ml SEI buffer plus 0.5 g sodium deoxycholate) and centrifuged to remove insoluble material. The microplate was kept cold and 10 μl of sample homogenate and 200 μl of solution (see McCormick, 1993) was added to each of four wells. Two wells included ouabain, the Na^+/K^+ ATPase inhibitor, and two wells did not. The microplate was read at 340 nm for ten minutes at 25°C using a SpectraMax 190

Microplate Reader and the Na^+/K^+ ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain and expressed as $\mu\text{mol}/\text{mg}$ protein/hour. The remaining homogenate was used to calculate protein levels using the Bradford Assay (Bradford, 1976).

5.2.5 Statistical Analysis

All analyses were performed using RStudio version 4.0.4 (RStudio Team, 2021). All values are presented as mean $\pm$ standard error. All data were assessed for normality and homogeneity of variance using Shapiro-Wilk's W and Levene's homogeneity test, respectively (Zar, 2018). In addition, residuals for parametric tests were visually assessed to validate normality and homogeneity of variance (Gotelli and Ellison, 2013). Sampling periods were not pooled, as conducting two statistical tests facilitated examination of the singular effects of temperature (Grand October and Grand August) and turbidity (Thames and Grand August).

Two-way analysis of covariance (ANCOVA) was used to determine the effect of sampling period (i.e. temperature, Grand October and Grand August; turbidity, Thames and Grand August) and species (greenside darter, blackside darter, and johnny darter) on hematocrit percentage and Na^+/K^+ ATPase activity, with fish mass used as a covariate. To assess pairwise differences, analyses used Tukey's honestly significant difference (HSD) post-hoc test.

All gill measurements were standardized to fish total length (mm) due to the observed differences in fish size between rivers and species (Table 1). Two-way ANOVA was then used to determine the effect of sampling period and species on all gill measurements. The R package emmeans was used to assess post-hoc differences using Tukey's HSD (Lenth et al., 2020). The chosen statistical tests met the required assumptions as described in Gotelli and Ellison (2013) and were applied following the Hodgson et al. (2020) study of darter gill physiology.

5.3 Results

The interaction terms between mass and sampling period (i.e. temperature or turbidity), mass and species, and mass and dependent variable (i.e. hematocrit or NKA) were not significant for any of the ANCOVAs (Supplementary Table 2). Thus, mass and all mass-associated interaction terms were removed from the final models for describing the effects of changes in temperature and turbidity and type III two-way ANOVAs were used.

Tests with significant results are presented in Tables 2 and 3, with the relevant Tukey's post-hoc HSD test results provided in Table 4. All non-significant test results are provided in supplementary Table 3 and Figure 2. Significant results are discussed below.

5.3.1 Respiratory

5.3.1.1 *Temperature*

Temperature alone did not significantly influence any of the metrics, whereas significant results for the effect of species were found for mean standardized filament width ($p = 0.039$), lamellae length ($p = 0.015$), lamellae width ($p < 0.001$), and two-dimensional lamellae surface area ($p = 0.006$; Table 2, Figure 4). Overall, greenside darter mean standardized filament width was 1.15% thicker than blackside darter ($p = 0.045$; Table 4), but did not differ significantly from johnny darter ($p = 0.940$). Johnny darter and blackside darter filament widths did not differ significantly ($p = 0.080$). Greenside darter mean standardized lamellae length was 1.15% shorter ($p \leq 0.013$) than blackside darter and johnny darter. Greenside darter mean standardized lamellae width was 0.87% thicker ($p \leq 0.020$) than blackside darter and 1.13% thinner than johnny darter. Johnny darter mean standardized lamellae width was 1.31% thicker than blackside darter ($p < 0.001$). Greenside darter two-dimensional lamellae surface area was 1.19% smaller ($p \leq 0.023$) than blackside darter and johnny darter.

In most instances the interaction term was insignificant ($p \geq 0.05$), an exception being hematocrit percentage where the species-temperature interaction term was significant ($p = 0.02$; Table 2, Figure 3). Blackside darter hematocrit percentage did not differ significantly (Tukey HSD, $p = 1.00$) between the August (47.78 ± 2.27) and October (46.57 ± 2.83) Grand sampling periods. Johnny darter hematocrit percentage was significantly lower in the October Grand ($p < 0.002$; 40.59 ± 1.47) than in the August Grand (52.68 ± 2.95) sampling period. Similarly, greenside darter hematocrit percentage was significantly lower in the October Grand ($p < 0.001$; 37.79 ± 1.77) than in the Grand August (51.512 ± 1.49) sampling period.

5.3.1.2 Turbidity

Turbidity significantly influenced mean standardized lamellae width ($p = 0.045$) with lamellae width being significantly thicker in the Thames River ($0.92 \mu\text{m}/\text{mm} \pm 0.03$) than the Grand River ($0.81 \mu\text{m}/\text{mm} \pm 0.03$). Significant results (Table 2, Figure 4) for the effect of species were found for mean standardized lamellae length ($p < 0.001$), mean standardized lamellae width ($p = 0.030$), and two-dimensional surface area ($p = 0.015$). Greenside darter lamellae length was 1.14% shorter ($p \leq 0.012$; Table 4) than for blackside darter and johnny darter. Johnny darter lamellae width was 0.82% thicker ($p \leq 0.003$) than for blackside darter and greenside darter. Greenside darter two-dimensional lamellae surface area was 1.19% smaller ($p = 0.020$) than for blackside darter and johnny darter.

In most instances the interaction term was insignificant ($p > 0.05$), with the exception of hematocrit percentage where the species-turbidity interaction term was significant ($p = 0.04$; Table 2, Figure 3). Blackside darter hematocrit percentage did not differ ($p = 1.00$) between the Grand (47.78 ± 2.27) and Thames (47.18 ± 2.25) rivers. Similarly, johnny darter hematocrit percentage did not differ ($p = 0.14$) between the Thames (41.72 ± 1.23) and Grand ($52.68 \pm$

2.95) rivers. Greenside darter hematocrit percentage was significantly lower in the Thames River ($p < 0.01$; 41.40 ± 1.61) than in the Grand River (51.512 ± 1.49).

5.3.2 Osmoregulation

5.3.2.1 Temperature

A significant result for the effect of temperature on osmoregulation metrics was only found for ionocyte number, with ionocyte number being significantly greater ($p = 0.04$) in the Grand October ($0.66 / \text{mm} \pm 0.03$) than the Grand August ($0.51 / \text{mm} \pm 0.01$; Table 3, Figure 5) sampling period. Na^+/K^+ ATPase activity was significantly different among species ($p < 0.001$), with greenside darter NKA being significantly higher ($p \leq 0.005$) than blackside and johnny darter (Table 4, Figure 5).

5.3.2.2 Turbidity

Turbidity did not significantly impact any of the measured osmoregulation metrics while NKA differed significantly ($p = 0.02$) among species (Table 3, Figure 5). Overall, greenside darter NKA was significantly higher ($p \leq 0.001$) than blackside and johnny darter (Table 4, Figure 5).

5.4 Discussion

Minimal alteration of gill measures for respiration and osmoregulation were observed in the three darter species in relation to differences in seasonal temperatures (10-25°C) or turbidity levels (8-94 NTU). Across all the metrics measured, only three metrics (ionocyte number, lamellae width, and hematocrit) were significantly impacted by either temperature and/or turbidity. An increase in temperature decreased ionocyte number, while an increase in turbidity increased lamellae width. Temperature and turbidity both impacted hematocrit, although the observed response was species-specific.

There was no observable remodelling in darter gill measures for improving gas exchange with increased temperature. Similarly, many species exhibit little to no plasticity in gill

remodelling across a 5-10°C temperature range (Bowden et al., 2014; Lefevre et al., 2014; Mitrovic and Perry, 2009; Sollid et al., 2005; Uliano et al., 2010). The ability to remodel gills with temperature appears to be species-specific and partially related to the occupancy of slow-flow habitats, where large fluctuations in dissolved oxygen and hypoxia may be more common, and there is a concomitant need to improve oxygen uptake (Chen et al., 2019; Dhillon et al., 2013; Fu et al., 2014; Sollid et al., 2005). However, not all slow flow habitats have low oxygen. Two of our study darters, blackside and johnny darter, occupy slow-flow habitats but oxygen content remained high (> 6 mg/l) throughout the period of seasonal temperature change. As a result, individual darters may not require gill remodelling to maintain sufficient oxygen uptake. In fact, eastern sand darter (*Ammocrypta pellucida*) resides in slow-flow habitats at the same study site and demonstrated minimal changes in metabolism (i.e. oxygen uptake) over a similar period of seasonal change (Firth et al., 2023). As we did not observe a decrease in oxygen availability across seasons, future work should be conducted to understand if individuals are able to remodel gills in response to hypoxia, thereby helping to understand their ability to cope with future increases in hypoxic episodes. If oxygen uptake ability becomes compromised, there are consequences for overall population status and survival reflected in decreased growth (Chabot and Dutil, 1999) and reduced reproduction (Landry et al., 2007; Wu et al., 2003).

Although there was no observed gill structural remodelling for adjusting gas exchange, there was a significant increase in ionocyte number at colder temperatures. Similar increases in ionocyte number have been observed in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) exposed to colder temperatures (Chou et al., 2008; Mitrovic and Perry, 2009). In zebrafish, similar gill metrics to those measured here were not simultaneously assessed, but there was an

upregulation of gene expression of two Na⁺/K⁺-ATPase transporters (Chou et al., 2008). In goldfish the increase in ionocyte number occurred with a decrease in gill surface area and a decrease in NKA activity (Mitrovic and Perry, 2009). Enzymatic activity, including NKA, typically decreases with cold temperatures (Kang et al., 2015; Mitrovic and Perry, 2009; Sardella et al., 2008). Here we did not observe a change in NKA; however, our samples were assayed at the same temperature (25°C). When 'cold collected samples' were assayed at a warmer temperature they were observed to have significantly greater activity than when assayed at their cold collection temperature (Kang et al., 2015; Metz et al., 2003). Thus, our results may over-estimate the NKA activity of the 'cold collected samples' and the increase in ionocyte number may have been necessary to compensate for a decrease in activity to maintain ionic homeostasis.

Here an increase in turbidity was associated with an increase in lamellae width in all three darter species. Exposure to contaminants downstream of a wastewater treatment plant comparably increased lamellae width for greenside darter and rainbow darter (*Etheostoma caeruleum*). Similarly, other studies have shown the most common gill pathologies (epithelial lifting, epithelial thickening, hyperplasia, and mucous production) typically increase lamellae width so as to increase diffusion distance and, presumably, protect the gills from abrasion and contaminants entering the bloodstream (Cumming and Herbert, 2016; Li and Shen, 2012; Mallatt, 1985; Wong et al., 2013). Although an increase in lamellae width likely increased diffusion distance, which would impact gas exchange and ion-transport capacity, there was no observed change in hematocrit and NKA or ionocyte values to maintain homeostasis. Similar to our findings, red pacu fingerlings (*Piaractus brachipomus*), juvenile green grouper (*Epinephelus*

coioides), and rainbow trout (*Oncorhynchus mykiss*) exposed to turbidity have demonstrated no change in hematocrit (Angeles-Escobar et al., 2021; Au et al., 2004; Becke et al., 2017).

However, in contrast to our findings, an increase in ionocyte number and decrease in NKA was observed with turbidity exposure in juvenile green grouper, juvenile black seabream (*Acanthopagrus schlegelii*), and juvenile orange-spotted grouper (*Epinephelus coioides*) (Au et al., 2004; Li and Shen, 2012; Wong et al., 2013). The changes observed in osmoregulation metrics appear to occur at higher turbidity concentrations (1000-2000 mg/l; 292-584 NTU) after gill structural change (~40-200 mg/l; ~12-58 NTU) is detected. Thus, turbidity levels in our study (~94 NTU; ~321 mg/l) may not have been high enough to elicit osmoregulation changes in these species. Moreover, our species were taken directly from the wild where the Thames River fish have lived in turbid conditions for generations. Shifts in the lamellae width in the Thames River fish, therefore, may represent the minimum adaptation necessary to protect the gill without triggering changes in underlying fish physiology (i.e. gas exchange and ion transport). The minimal changes observed in the darter species studied here that have experienced chronic turbidity thus suggests that it is possible for some species to live in turbid environments with minimal physiological consequences (Becke et al., 2018).

We only observed species-specific differences in hematocrit results in response to temperature and turbidity. Hematocrit significantly increased in greenside and johnny darter with hotter temperatures and decreased in greenside darter with higher turbidity. As our fish were collected in the wild where many factors can influence hematocrit, such as stress (Fazio et al., 2015), diet (Lu et al., 2023), malnutrition, infection, and/or environmental toxins (Seibel et al., 2021; Witeska, 2015) we cannot ascertain whether the hematocrit results are solely related

to changes in temperature and turbidity; therefore, it is difficult to interpret what may have had an influence on the species-specific responses observed.

We observed species differences in gill metrics and NKA. Blackside darter and johnny darter gills and NKA are more similar compared to greenside darter, which ultimately has a smaller surface area. There are two main reasons why greenside darter may have different gill morphology than blackside and johnny darter. First, greenside darter occupies riffles with fast flow compared to blackside and johnny darter. Fast flow environments such as riffles tend to have higher oxygen content due to turbulence causing greater contact between air and water, allowing more oxygen to dissolve into the water. Thus, a smaller surface area for greenside darter may be sufficient to meet oxygen uptake requirements. Second, the clade containing greenside darter has morphologically distinguishable head and jaw characteristics compared to other darter species that could influence gill shape (Carlson and Wainwright, 2010).

Our study demonstrated that temperature and turbidity had limited impact on the wild blackside darter, greenside darter, and johnny darter respiration and osmoregulation metrics between the temperature (15-25°C) and turbidity (8-94 NTU) values tested. The differences observed were minimal and may not impact the overall health and fitness of the fish as they have lived in these conditions for generations and there have been no observed changes to their conservation status (COSEWIC, 2006; NatureServe, 2024). Our results demonstrate that these field-collected darters that have experienced seasonal temperature change and turbid conditions in the Thames River for generations have limited ability to remodel their gills under the conditions tested, and there were no compensatory changes in hematocrit or NKA to maintain homeostasis. Nonetheless, management effort should be taken to decrease turbidity

even in the absence of physiological effects in our study, as turbidity can negatively influence other factors such as hypoxia tolerance and food intake. Future work should assess the species with replicated samples over multiple time periods to better understand the potential for plastic responses to variation in temperature and turbidity.

Table 5.1: Sample size and mean mass (g) and total length (mm) of greenside darter (GSD), johnny darter (JD), and blackside darter (BSD) from each sampling period. Sample size varies for each measured metric due to sample preparation; see supplementary table S1 for sample size for each metric.

Metric	Species	Thames River	Grand August	Grand October
Sample Size	GSD	20	20	20
	JD	14	20	20
	BSD	20	18	7
Mass (g)	GSD	0.50 ± 0.04	0.89 ± 0.05	1.13 ± 0.08
	JD	0.41 ± 0.04	0.68 ± 0.09	0.82 ± 0.07
	BSD	0.77 ± 0.04	1.45 ± 0.22	2.14 ± 0.32
Total Length (mm)	GSD	42.58 ± 0.89	49.00 ± 0.95	52.35 ± 1.02
	JD	39.36 ± 1.17	44.68 ± 2.01	48.60 ± 1.34
	BSD	50.90 ± 0.87	56.89 ± 1.95	66.57 ± 2.49

Table 5.2: Degrees of freedom (df), F-statistics (F) and P-values (p) for the two-way ANOVAs used for testing for significant differences among the temperature and turbidity respiratory metrics. Bolded values indicate significance at $\alpha = 0.05$. All non-significant results are reported in the supplementary file (Table S3).

	Temperature			Turbidity		
	df	F	<i>p</i>	df	F	<i>p</i>
<i>Hematocrit</i>						
Treatment	1,81	0.12	0.73	1,83	0.05	0.83
Species	2,81	1.47	0.24	2,83	1.31	0.27
Treatment x Species	2,81	4.12	< 0.05	2,83	3.37	< 0.05
<i>Filament Width</i>						
Treatment	1,79	3.70	0.06	1,82	0.28	0.60
Species	2,79	3.37	< 0.05	2,82	1.18	0.31
Treatment x Species	2,79	1.79	0.17	2,82	2.56	0.08
<i>Lamellae Length</i>						
Treatment	1,79	0.07	0.79	1,82	0.55	0.46
Species	2,79	4.45	< 0.05	2,82	11.17	< 0.01
Treatment x Species	2,79	0.96	0.39	2,82	1.18	0.31
<i>Lamellae Width</i>						
Treatment	1,79	0.26	0.61	1,82	4.14	< 0.05
Species	2,79	8.56	< 0.01	2,82	3.67	< 0.05
Treatment x Species	2,79	1.02	0.37	2,82	1.39	0.26
<i>Two-dimensional Lamellae Surface Area</i>						
Treatment	1,79	1.40	0.24	1,82	0.75	0.39
Species	2,79	5.48	< 0.01	2,82	4.40	< 0.05
Treatment x Species	2,79	0.11	0.89	2,82	0.01	0.99

Table 5.3: Degrees of freedom (df), F-statistics (F) and P-values (*p*) for the two-way ANOVAs used for testing for significant differences among the temperature and turbidity osmoregulation metrics. Bolded values indicate significance at $\alpha = 0.05$. All non-significant results are reported in the supplementary file (Table S3).

	Temperature			Turbidity		
	df	F	<i>p</i>	df	F	<i>p</i>
<i>Na⁺/K⁺ ATPase Activity</i>						
Treatment	1,74	0.51	0.48	1,90	2.68	0.10
Species	2,74	11.01	< 0.01	2,90	4.30	< 0.05
Treatment x Species	2,74	1.05	0.35	2,90	1.47	0.23
<i>Ionocyte Number</i>						
Treatment	1,70	4.39	< 0.05	1,72	0.003	0.96
Species	2,70	2.07	0.13	2,72	0.39	0.68
Treatment x Species	2,70	0.71	0.49	2,72	0.90	0.41

Table 5.4: Tukey's HSD test results for testing of pairwise significant species differences based on the two-way ANOVAs for temperature and turbidity analyses. For each dependent variable and temperature or turbidity grouping, species sharing the same superscript do not differ significantly at $\alpha = 0.05$ level of significance. GSD, JD, and BSD indicate greenside darter, johnny darter, and blackside darter, respectively.

Dependent variable	Species	Temperature Mean	Turbidity Mean
Filament Width $\mu\text{m}/\text{mm}$	GSD	3.58 ± 0.17^a	
	JD	3.51 ± 0.12^{ab}	
	BSD	3.10 ± 0.18^b	
Lamellae Length $\mu\text{m}/\text{mm}$	GSD	4.54 ± 0.15^a	4.78 ± 0.13^a
	JD	5.65 ± 0.14^b	5.43 ± 0.13^b
	BSD	5.26 ± 0.19^b	5.86 ± 0.20^b
Lamellae Width $\mu\text{m}/\text{mm}$	GSD	0.82 ± 0.02^a	0.86 ± 0.03^a
	JD	0.93 ± 0.03^b	0.96 ± 0.04^b
	BSD	0.71 ± 0.03^c	0.79 ± 0.03^a
2-dimensional Lamellae Surface Area $\mu\text{m}^3/\text{mm}$	GSD	187.97 ± 8.10^a	189.44 ± 8.09^a
	BSD	224.54 ± 13.29^b	223.37 ± 8.43^b
	JD	241.12 ± 8.80^b	233.98 ± 9.90^b
Na^+/K^+ ATPase Activity $\mu\text{mol}/\text{min}/\text{mg}$ protein	GSD	$6.39\text{e-}04 \pm 4.30\text{e-}05^a$	$6.06\text{e-}04 \pm 4.94\text{e-}05^a$
	BSD	$4.20\text{e-}04 \pm 4.42\text{e-}05^b$	$3.20\text{e-}04 \pm 2.98\text{e-}05^b$
	JD	$3.73\text{e-}04 \pm 4.30\text{e-}05^b$	$3.72\text{e-}04 \pm 3.15\text{e-}05^b$

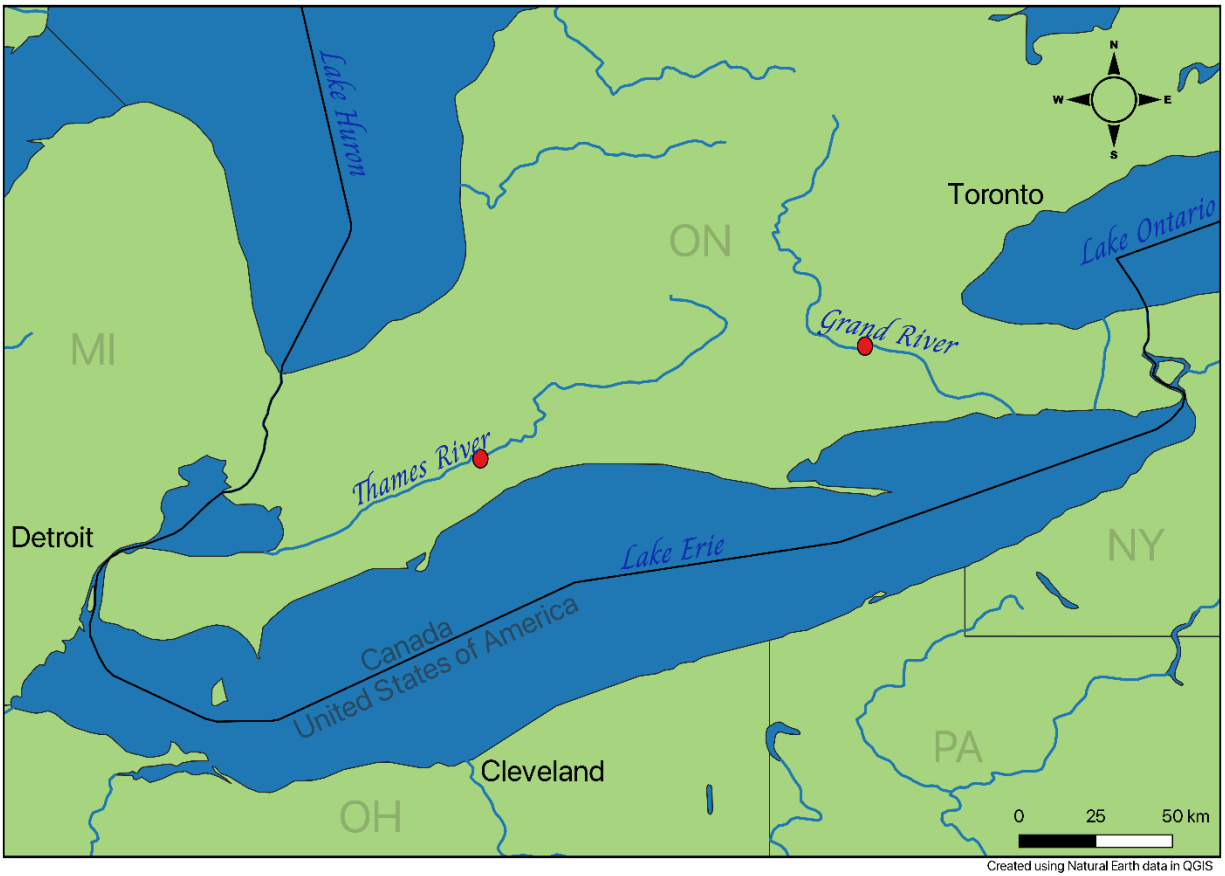


Figure 5.1: Sampled locations in the Thames and Grand rivers, Ontario, Canada shown in red.

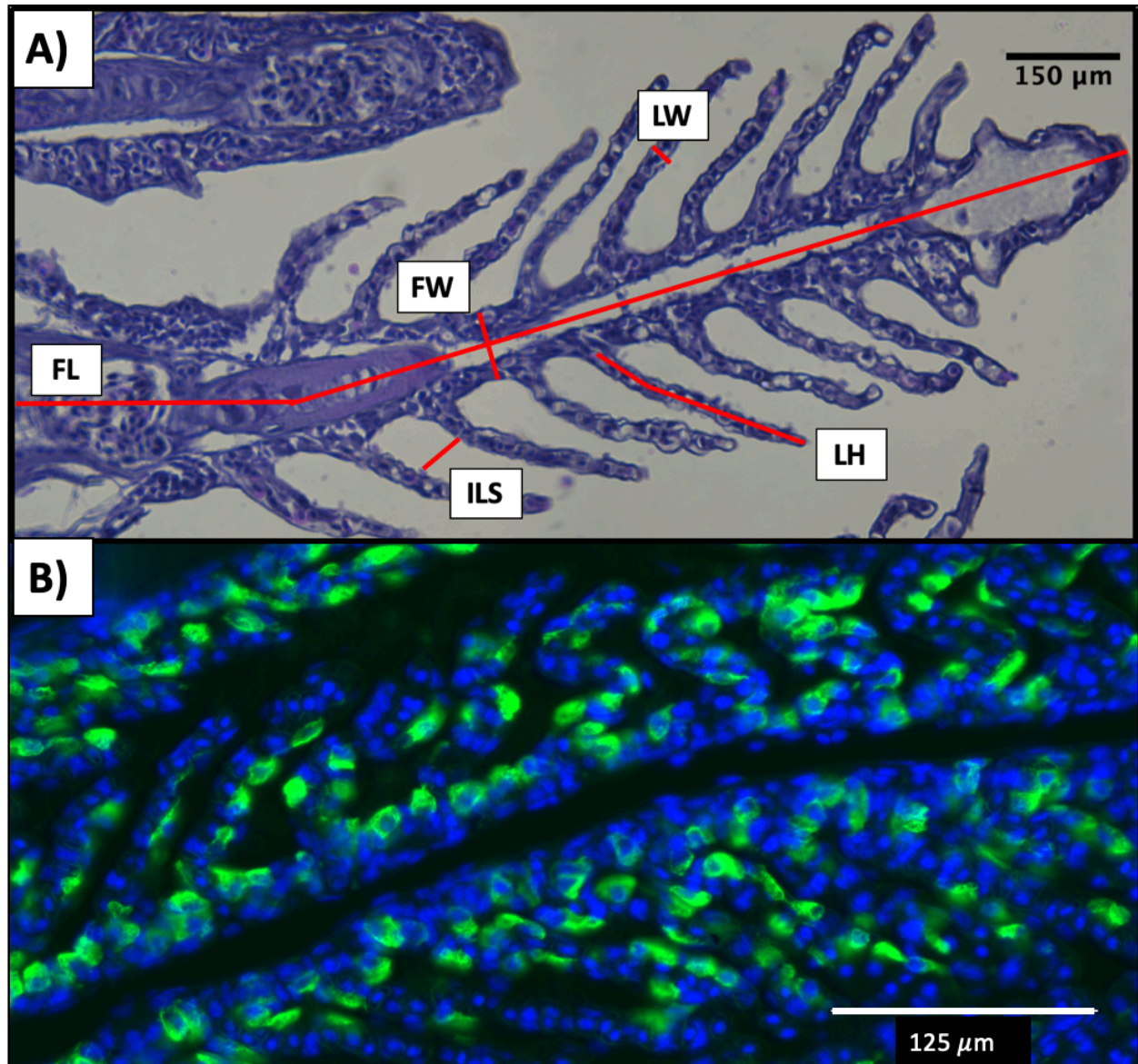


Figure 5.2: Example of an A) H&E stained gill and the corresponding morphometric measures taken: filament length (FL), filament width (FW), lamellae height (LH), lamellae width (LW), and inter-lamellae space (ILS) and B) immunofluorescence stained gill with Na⁺K⁺-ATPase ionocytes (green) and nuclei (blue). Reproduced and modified from Firth et al. (2024).

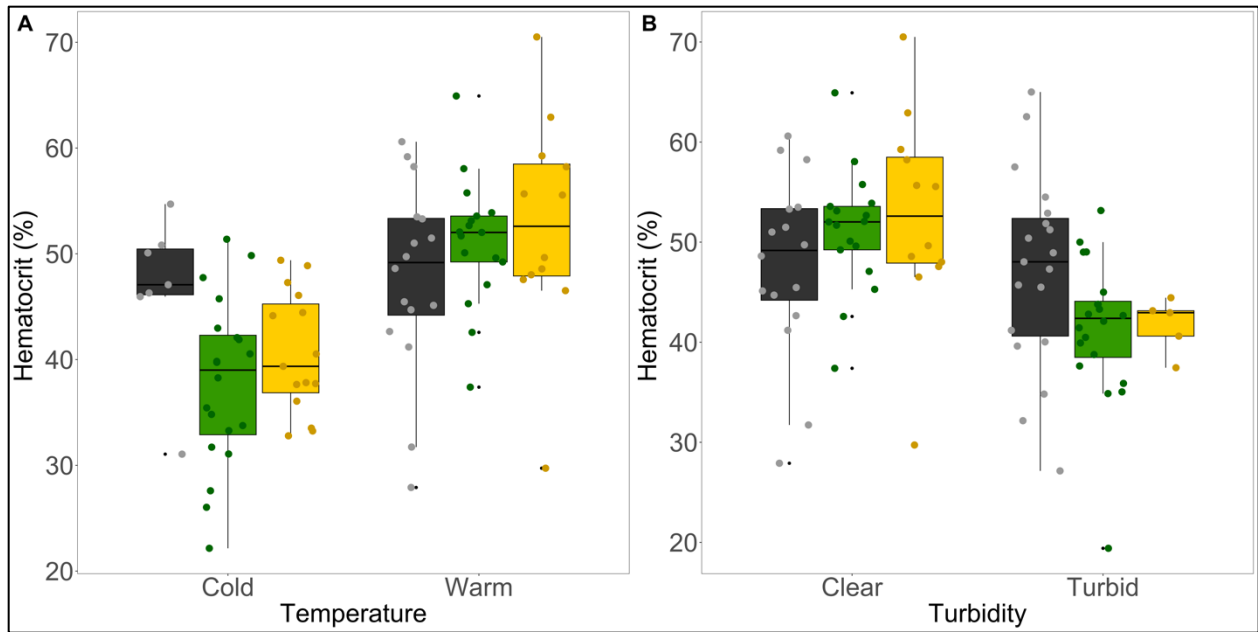


Figure 5.3: Hematocrit percentage for blackside darter (Black), greenside darter (Green), and johnny darter (Yellow) between temperature (A; Cold, Grand October, and Warm, Grand August) and between turbidity (B; Clear, Grand August, and Turbid, Thames River).

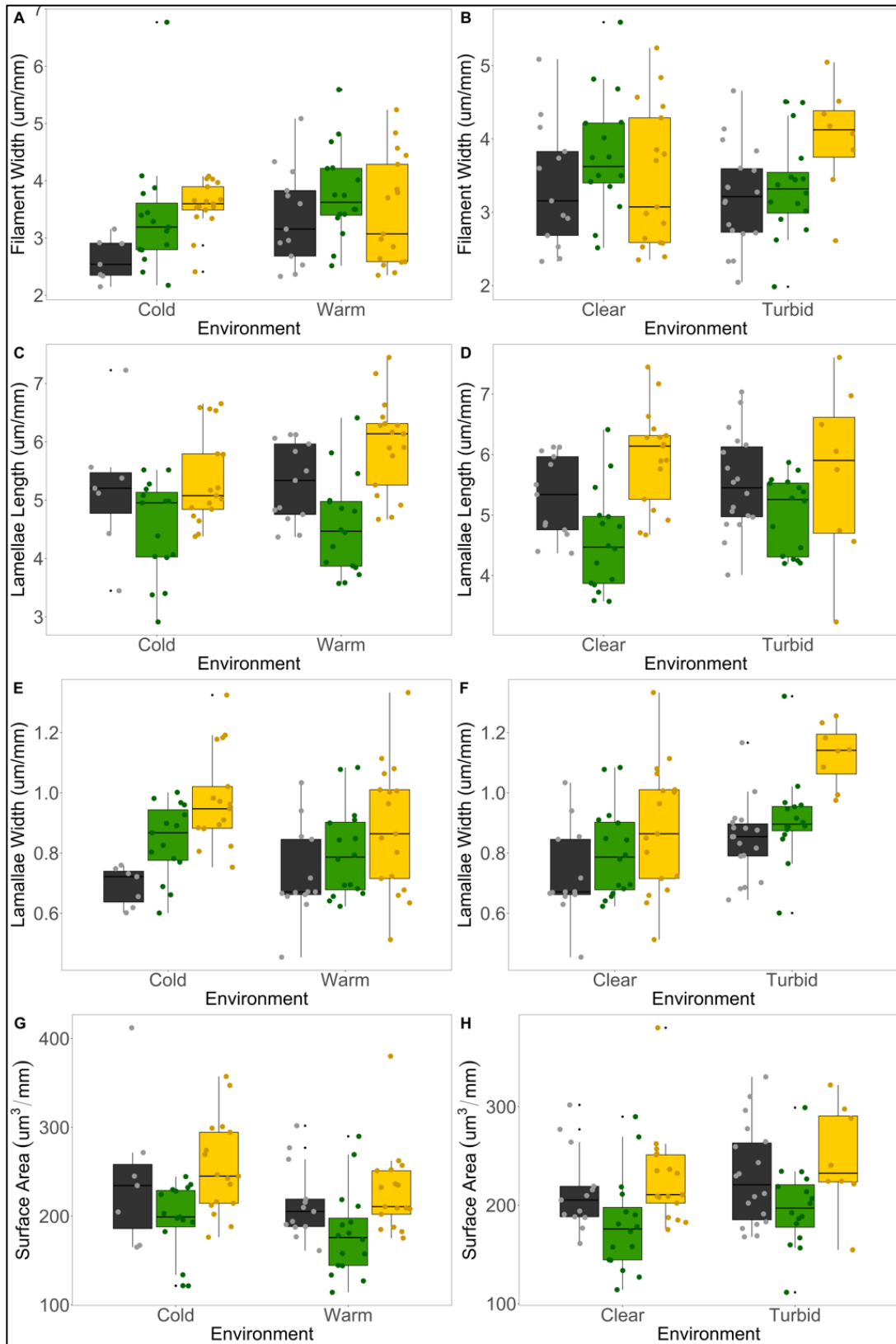


Figure 5.4: Effects of temperature (A, C, E, G; Cold, Grand October, and Warm, Grand August) and turbidity (B, D, F, H; Clear, Grand August, and Turbid, Thames River) on blackside darter (Black), greenside darter (Green), and johnny darter (Yellow) filament width ($\mu\text{m}/\text{mm}$; A, B), lamellae length ($\mu\text{m}/\text{mm}$; C, D), lamellae width ($\mu\text{m}/\text{mm}$; E, F), and 2-dimensional surface area (SA; $\mu\text{m}^3/\text{mm}$; G, H).

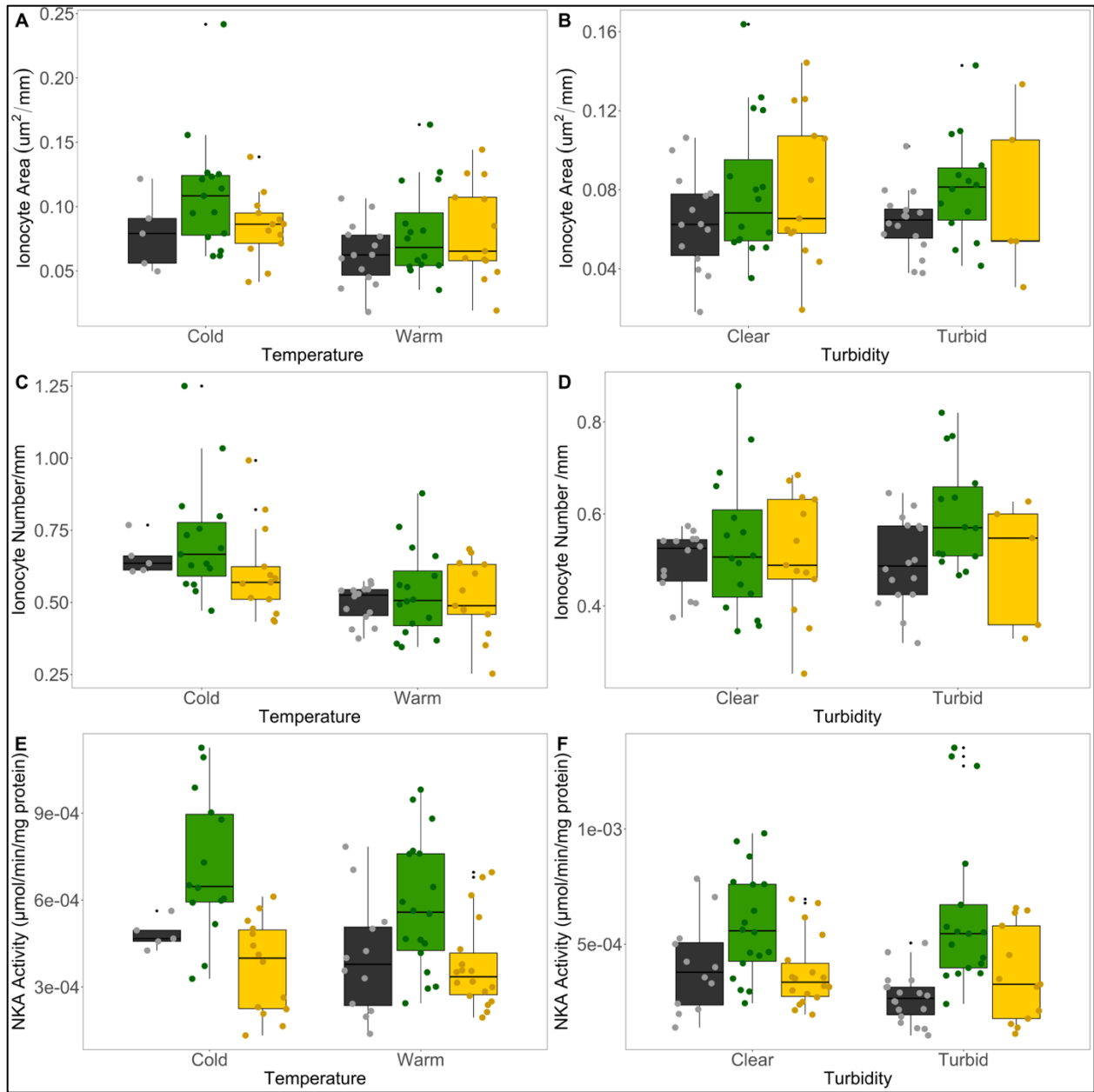


Figure 5.5: Effects of temperature (A, C, E; Cold, Grand October, and Warm, Grand August) and turbidity (B, D, F; Clear, Grand August, and Turbid, Thames River) on blackside darter (Black), greenside darter (Green), and johnny darter (Yellow) ionocyte area ($\mu\text{m}^2/\text{mm}$; A, B), ionocyte number (C, D), and Na^+/K^+ ATPase activity ($\mu\text{mol}/\text{min}/\text{mg protein}$; E, F).

Chapter 6: General Conclusions

6.1 Summary

Effective conservation requires accurate biological information to protect and recover species at-risk. Understanding physiological tolerances is important for informing several aspects of the conservation process, such as understanding threat mechanisms, defining habitat requirements, and developing approaches for species reintroduction (Castañeda *et al.*, 2021). Yet, many small-bodied species, including Eastern Sand Darter, lack a detailed assessment of their physiological tolerances. Research presented in this thesis addresses knowledge gaps in the physiological tolerances of Eastern Sand Darter, covering thermal and hypoxia tolerance, metabolism, and gill morphology.

Chapter 2 provides a detailed assessment of the upper thermal limits of Eastern Sand Darter across seasons and between turbidity levels. Results demonstrated that critical thermal maximum and agitation temperature significantly increase with ambient temperature, with a high acclimation response (0.49). The mean temperature 24-hours prior to testing was the most important factor in predicting critical thermal maximum, but temperature variance, maximum temperature, and fish mass also had an effect. The thermal safety margin of Eastern Sand Darter was smallest in the summer. There was no significant impact of turbidity on critical thermal maximum or agitation temperature; however, there was an interaction between turbidity, mass, and thermal tolerance.

Chapter 3 examines the effect of seasonal temperature change and environmental and fish-specific factors on Eastern Sand Darter metabolism and hypoxia tolerance. Findings demonstrated that ambient water temperature positively and significantly affected both Eastern Sand Darter hypoxia tolerance metrics (i.e. hypoxia tolerance was lower in the summer)

but not metabolism until reproductive season and body condition were taken into account. Temperature alone explained 1-31% of the variation observed in the data and reproductive season and body condition explained some of the residual variation in all three metrics. Reproductive season increased metabolic rate significantly by 159-176%. Lastly, the among-individual variation observed in the data increased significantly with temperature for metabolism but not either of the hypoxia tolerance metrics.

The results in chapter 4 sought to enhance understanding about how turbidity could effect the gill morphology, metabolism, and hypoxia tolerance of Eastern Sand Darter. Findings demonstrated that turbidity had minimal impact on gill morphology, with only interlamellar space and filament width noted to increase significantly with turbidity. Turbidity had no impact on metabolism and critical oxygen tension (a hypoxia tolerance metric), but significantly increased loss of equilibrium (i.e. hypoxia tolerance was worse under turbid conditions).

Chapter 5 evaluates the effect of seasonal temperature change and turbidity on the gill morphology of three co-occurring darter species. Temperature and turbidity were found to have minimal impact on gill morphology, with an increase in temperature decreasing ionocyte number and an increase in turbidity increasing lamellae width. Hematocrit showed a significant interaction between temperature/turbidity and species. Temperature and turbidity may not have had a large impact on physiology, but numerous differences in gill morphology were observed between the co-occurring darter species.

6.2 Implications for the Conservation of Eastern Sand Darter

This thesis assesses the physiology of Eastern Sand Darter, and provides insight into how temperature and turbidity, two common anthropogenic stressors, influence the thermal and oxygen physiology of the species. Chapters 2 and 3 provided the first assessment of how

temperature effects Eastern Sand Darter thermal and oxygen physiology, including seasonal periods when the species is most sensitive to fluctuations in these variables. Findings from both chapters will improve conservation and recovery of the species in several ways. First, they define the thermal and hypoxia tolerance of Eastern Sand Darter across seasons and establish what the suitable thermal and oxygen habitat requirements for the species are throughout the bulk of the growing season. Establishing suitable thermal and oxygen habitat requirements will allow recovery biologists to understand the suitability of currently occupied habitats, including those that require restoration to maintain species viability, as well as the suitability of thermal and oxygen conditions at potential reintroduction sites. Additionally, the defined thermal and oxygen tolerances can be employed in species distribution models to predict if the species distributional range will shift, expand, or shrink under future climate change scenarios (Evans *et al.*, 2015; Gamliel *et al.*, 2020; Gong *et al.*, 2023).

Defining the thermal and hypoxia tolerances for Eastern Sand Darter also demonstrated that the species has similar thermal tolerance and higher hypoxia tolerance to other darter species that occur in the Grand River (Hlohowskyj and Wissing, 1985, 1987). Thus, increasing temperature and decreasing oxygen conditions resulting from environmental change may be less stressful for Eastern Sand Darter than other co-occurring darter species, assuming all else is equal. Moreover, Eastern Sand Darter demonstrated a high acclimation response ratio for thermal tolerance and a large degree of variation in hypoxia tolerance and metabolism. Both findings, in conjunction with the short generation time of the species, should buffer them from future environmental change of increased temperature and decreased oxygen and may lead to an increased chance of evolutionary rescue and species survival (Bolnick *et al.*, 2011; Bell,

2013). However, the results of chapters 2 and 3 also demonstrate that Eastern Sand Darter has a smaller thermal safety margin and lower hypoxia tolerance in July and August. The results suggest the species is living closer to its thermal and oxygen limits in the summer and is more susceptible to short-term environmental changes, such as heat waves. Thus, conservation approaches should aim to mitigate increased temperatures (i.e. riparian vegetation planting) in the summer that could surpass the physiological limits of the species.

Chapters 2 and 4 provided insight into how turbidity effects the thermal and oxygen physiology of Eastern Sand Darter. Turbidity was used as a surrogate measure for siltation, the main threat believed to be responsible for reducing habitat quality and leading to species decline (COSEWIC, 2009, 2022). However, it is important to note that turbidity measurements encompass several environmental variables, such as clay, silt, and algae. The chapters demonstrated that turbidity had minimal impact on the physiological metrics of Eastern Sand Darter and results refined understanding of how siltation impacts species survival and conservation approaches for the species. Establishing thermal and hypoxia tolerance between turbidity levels improved understanding of the potential interactive effects between turbidity and environmental change by showing that individuals in different habitats may be impacted differently from the dominant effects of climate change. Turbidity had no direct effect on thermal limits and minimal effects on oxygen physiology. However, turbid environments were associated with lower growth and smaller adult body size for Eastern Sand Darter and all three co-occurring darters. Thus there was an indirect effect of turbidity on thermal limits and hypoxia tolerance as smaller fish were shown to have lower tolerances. Thus, individuals in the turbid Thames River are anticipated to be more sensitive to the thermal and oxygen

consequences of climate change since they have smaller adult body size. Accordingly, the Thames population will likely need to be managed differently with respect to climate change impacts to ensure temperature and dissolved oxygen levels do not surpass their physiological limits.

Defining hypoxia tolerance of Eastern Sand Darter under turbid conditions demonstrated that Eastern Sand Darter had lower hypoxia tolerance in a turbid environment. However, the marginally statistically significant difference in hypoxia tolerance may not be biologically significant as the tolerance was higher than other darter species living in “clean” environments. One of the hypothesized mechanisms of siltation on Eastern Sand Darter decline is reduced substrate oxygen availability necessary for fossorial behaviour (COSEWIC, 2009, 2022). Eastern Sand Darter hypoxia tolerance of 1.29 mg/l under turbid conditions falls below the measured available dissolved oxygen 1 cm below the substrate surface (1.34 mg/l) and at the substrate surface interface (9.57 mg/l). Eastern Sand Darter has been observed to bury with their snout poking out (i.e. have access to substrate surface interface oxygen) and fully bury (Spreitzer, 1979; Daniels, 1989). However, even fully buried the species snout is likely close to the substrate surface interface and not 1cm below the surface due to the size of the species. Thus, oxygen availability is likely not impacting fossorial behaviour and consequently our findings suggest that reduced oxygen substrate availability decreasing fossorial behaviour may not be the siltation threat mechanism impacting the species. Although turbidity did not have an observed effect on Eastern Sand Darter physiology, efforts should be taken to protect and restore turbid habitat to decrease the indirect effect of turbidity on thermal and hypoxia

tolerance so as to ensure that other life history processes (e.g., egg and larval survival, food supply) are not influenced by turbidity.

Chapter 5 provided the first assessment of the gill morphology of darters as it relates to temperature and turbidity levels and demonstrated that the Blackside, Johnny, and Greenside darter had limited gill plasticity. Although this study did not assess Eastern Sand Darter, having data on the three co-occurring darter species lets one put Eastern Sand Darter in context with other members of the ecosystem that are phylogenetically related. This study further provided information that turbidity is likely not a large threat for Blackside, Johnny, and Greenside darter as well as Eastern Sand Darter in the Thames River when it comes to oxygen physiology and homeostasis as species have minimal changes in measured physiological metrics and likely have adapted coping mechanisms from many generations of exposure. However, conservation approaches such as increased riparian vegetation should be implemented to try and minimize temperature and turbidity increases as there remains uncertainty about whether 1) darters have the ability to remodel gills with decreases in oxygen and 2) what turbidity levels may negatively impact homeostasis. If darter species do not have the ability to remodel their gills, oxygen uptake may become compromised during future increases in hypoxic episodes from climate change, which can have negative consequences for overall population survival.

Altogether, this thesis provided insight into threat mechanisms and means of assessing habitat suitability of Eastern Sand Darter that can be directly used to inform the development of species recovery strategies under Canada's *Species at Risk Act*. Federal recovery strategies and COSEWIC assessments require information about species physiology, which can now be accomplished with these results. Overall, Eastern Sand Darter appears to have the ability to

cope with certain aspects of probable future environmental change (i.e. temperature change) and provided physiological thresholds can be used to monitor habitat and indicate when habitat restoration is needed to ensure conditions remain within the documented physiological thresholds provided here.

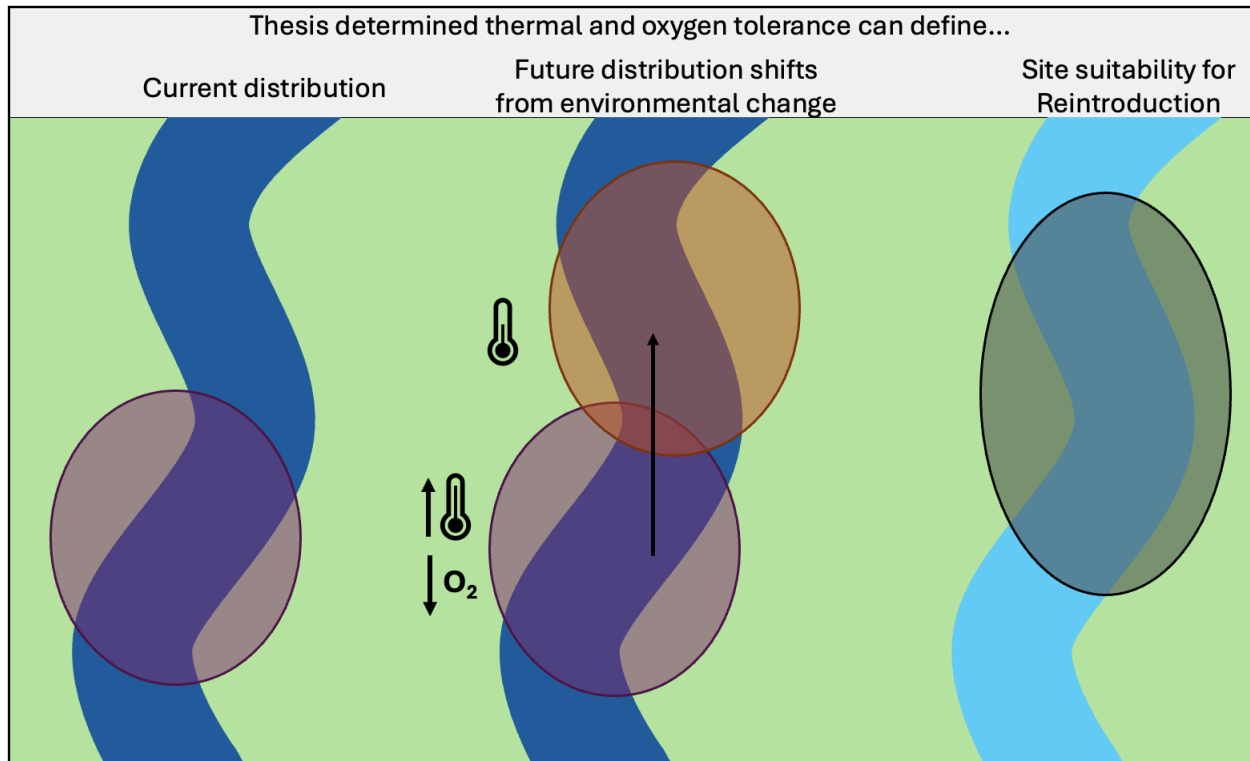


Figure 6.1: A conceptual diagram summarizing thesis implications for the conservation of eastern sand darter. Thesis determined thermal and oxygen tolerances can define 1) current distribution (purple), 2) future distribution shifts resulting from environmental change (purple to orange), and 3) site suitability for reintroduction (lighter river and black circle).

6.3 Implications for Conservation Physiology

Although this thesis was designed to address existing knowledge gaps regarding Eastern Sand Darter physiology, it also addressed some general knowledge gaps in fish physiology. Chapter 2 provided insight into which aspects of temperature (i.e. mean, maximum, minimum and variance) may be most important for predicting thermal tolerance, which may help in understanding how changes in water temperature may impact measured upper thermal limits for many fish species. Chapter 3 was among the first to study metabolism and hypoxia tolerances of a fish across multiple seasons in the field. Findings showed that temperature was not a large controlling factor for Eastern Sand Darter, indicating that temperature may not be as important for determining metabolism and hypoxia tolerance in the wild where other environmental variables can interact with temperature to mitigate or amplify its effect. Chapter 4 is one of the first studies to assess the relationship between fish size, gill morphology, metabolism, and hypoxia tolerance concurrently between turbidity levels. Findings provided further support for the notion that turbidity affects fish size through a mechanism other than oxygen uptake.

6.4 Future Directions

6.4.1 Eastern Sand Darter Conservation

Although this thesis has advanced understanding of the thermal and oxygen physiology of Eastern Sand Darter, its findings have raised additional questions, which if answered may help to further advance our understanding of the species' physiology and provide information likely to help in the conservation of the species. Below I highlight those questions:

- 1) Physiological tolerances were only assessed at ambient stream temperatures. Given that thermal tolerance has less flexibility to increase (i.e. concrete ceilings; Sandblom *et al.*,

2016) and climate change is increasing mean temperature and extreme weather events (Räisänen, 2002), finding physiological tolerances above ambient conditions similar to McDonnell and Chapman (2015) and Dressler *et al.* (2023) is imperative for improved understanding of how the species may cope with climate change. Additionally, findings will demonstrate if Eastern Sand Darter is at the edge of their thermal window.

- 2) When sampling Eastern Sand Darter, collection methods differed in the cooler months as the species shifted to deeper water. Future research should assess the winter ecology of Eastern Sand Darter to determine seasonal movement patterns and better document species over-wintering strategies including the occupied thermal and experienced oxygen regimes during winter to provide insight into how climate change may impact energy stores, which could impact growth and reproduction in the spring.
- 3) Physiological tolerances were only assessed from June-November, which omitted winter. Winter months are currently experiencing higher mean temperatures and greater variance due to extreme weather events (Liu *et al.*, 2006; Gubler *et al.*, 2023), both of which could impact the thermal and oxygen physiology of Eastern Sand Darter. Thus, future research should conduct thermal and oxygen physiology tests in the winter to better understand how environmental change may impact Eastern Sand Darter in all seasons.
- 4) Siltation was identified as the main threat to Eastern Sand Darter survival (COSEWIC, 2009), but turbidity was found to not have a strong impact on thermal and oxygen physiology. The range over which turbidity impacts were measured, however, were limited. Thus, tests conducted over a wider range of turbidity's are needed.

Furthermore, additional research should build on the findings here and attempt to determine the key mechanism behind turbidity's impact on fish size and other population vital rates. There are many ways turbidity could be impacting Eastern Sand Darters. Three of the key possible ways that should be assessed to improve conservation management decisions are given below:

- a. Understanding the mechanisms behind why fish are smaller in turbid environments, i.e., reductions in food supply and/or reduced feeding efficiency resulting from reduced visibility, could help lead to conservation management decisions that may reduce the indirect effect turbidity has on physiology through fish size.
- b. While reduced substrate oxygen may not impact adult Eastern Sand Darter due to their high hypoxia tolerance, further investigation is needed to understand how reduced substrate oxygen from siltation might affect egg and hatch survival. If eggs cannot uptake sufficient oxygen, egg survivorship and overall Eastern Sand Darter recruitment will be negatively affected.
- c. As found here, hypoxia tolerance was only marginally but significantly impacted by turbidity. However, it is unclear if the response is biologically significant. If the decreased hypoxia tolerance is biologically significant, and reduces burying behaviour, Eastern Sand Darter could face increased predation threats. Further, station holding on substrate instead of burying might raise metabolic costs and reduce energy stores critical for over-winter survival and through changes in condition may impact physiological tolerances.

- 5) Findings in chapter 5 suggested that many darter species evidence limited gill plasticity over a range of temperature and turbidity levels commonly found in natural environments. Not all fish have the capability to remodel their gills (Uliano *et al.*, 2010; Bowden *et al.*, 2014; Lefevre *et al.*, 2014) and from the findings here it is unclear if darters cannot remodel their gills in response to environmental change or whether remodelling was unnecessary due to the high oxygen levels and fluctuating abiotic conditions found in the wild. Thus, future research should investigate whether darters have the ability to remodel gills with changes in oxygen levels by experimentally exposing fish to a wider range of temperature and turbidity conditions to determine if adaptations in gill morphology occur and could help the species survive changing environmental conditions.

6.4.2 Thermal and Oxygen Physiology

Two additional questions that can benefit the field of thermal and oxygen physiology also became apparent during the course of completing the thesis research and are highlighted below:

- 1) Chapter 3 demonstrated that metabolism and hypoxia showed a lot of variation in the field and that ambient water temperature explained very little of the variation. To the author's knowledge this thesis was the first to examine metabolism under field conditions across seasons. Consequently, future research is needed to determine if the large degree of variation and limited effect of temperature in explaining the variation observed here is an over-arching effect of field conditions resulting from the interaction of environmental variables or a species-specific outcome.

2) Chapter 3 also showed that metabolism increased by 159-176% during the reproductive season. Although metabolism is known to increase during reproduction (Boehlert *et al.*, 1991; Masonjones, 2001; Timmerman and Chapman, 2003), it is unclear how future environmental change may impact species' abilities to increase metabolic rate for reproductive activity. Future research should assess routine and maximum metabolic rate in reproductively active species under different temperatures to better understand if routine metabolic rate approaches maximum metabolic rate as temperatures increase, implying species will eventually not be able to sufficiently increase metabolic rate to accommodate reproductive activity.

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Appendix A: Supplementary data for Chapter 3

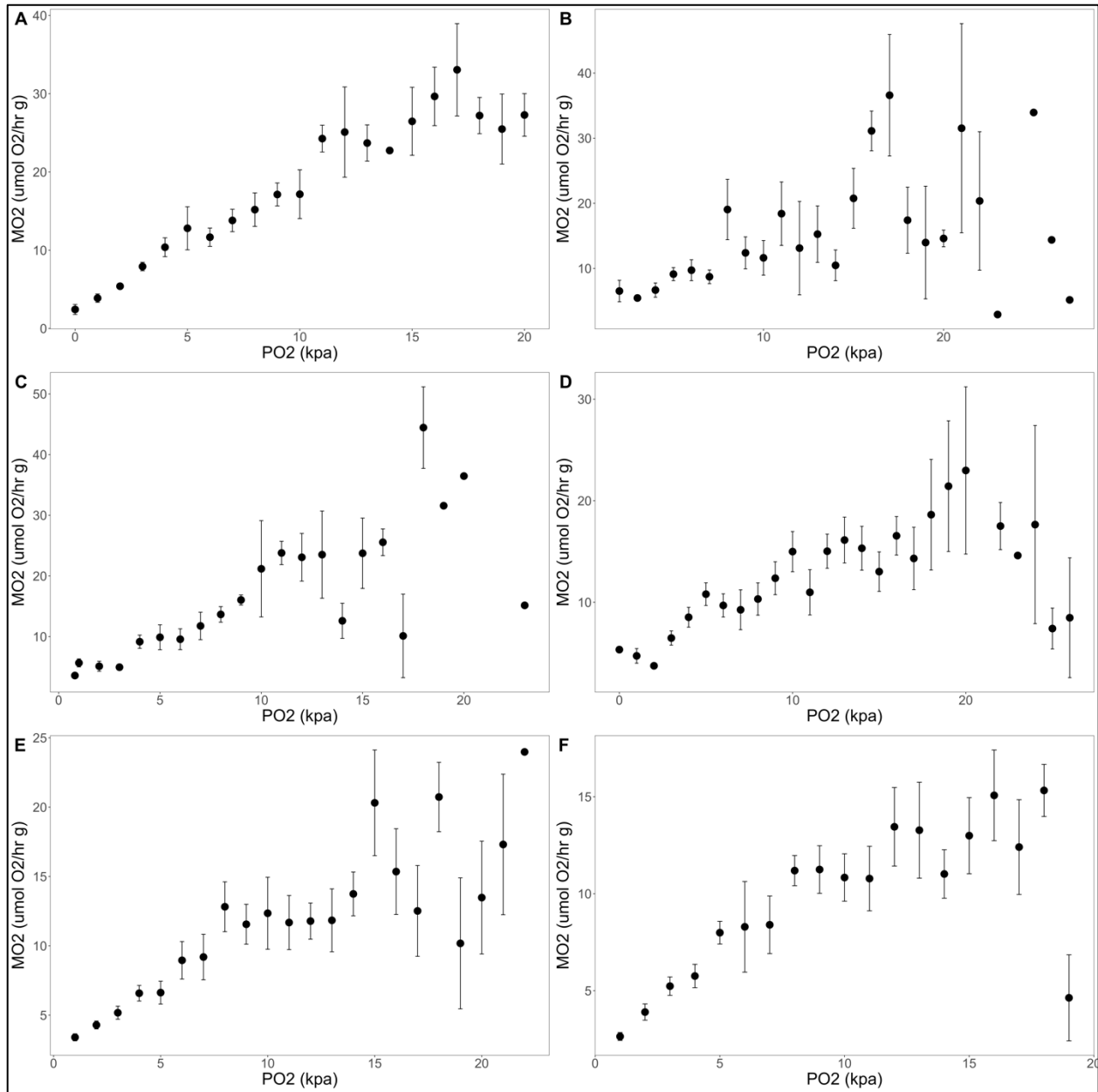


Figure A.1: Average response of field metabolic rate (MO₂; μmol O₂/hr g) across declining water oxygen tensions (PO₂; kPa) for each testing month (A) June, (B) July, (C) August, (D) September, (E) September / October, and (F) October. Data are presented as mean ± standard error for each PO₂.

Appendix B: Supplementary data for Chapter 4

Table B.1: Independent two sample t-test results for testing of significant differences between fresh collect and museum preserved for all gill morphometrics in the Grand and Thames River. Significant values with an alpha level of 0.5 are denoted in bold.

Parameter	Thames			Grand		
	t	Df	p	t	Df	p
Filament Length	-1.15	12	0.27	-1.89	14	0.08
Filament Widths	-0.64	12	0.53	-1.84	14	0.09
Lamellae Length	-0.79	12	0.44	-1.77	14	0.10
Lamellae Width	1.73	12	0.11	0.64	14	0.53
Interlamellae Space	0.89	12	0.39	-1.73	14	0.11
Ionocyte Area	2.91	7	0.02	1.26	12	0.23
Ionocyte Count	2.27	7	0.06	0.63	12	0.54

Appendix C: Supplementary data for Chapter 5

Table C.1: Sample size of greenside darter (GSD), johnny darter (JD), and blackside darter (BSD)

for each gill metric from each sampling period.

Metric	Species	Thames River	Grand August	Grand October
Hematocrit	GSD	20	17	20
	JD	5	12	15
	BSD	19	16	7
Na ⁺ /K ⁺ ATPase	GSD	17	18	14
Activity	JD	13	18	14
	BSD	18	12	5
H&E	GSD	17	16	15
Stained Gills	JD	8	18	17
	BSD	18	13	7
Immunofluorescent	GSD	15	16	15
Stained Gills	JD	5	13	13
	BSD	16	14	5

Table C.2: Two-way ANCOVA results for testing of significant differences in hematocrit and Na^+/K^+ ATPase Activity for temperature and turbidity analyses with mass as a covariate.

	Temperature			Turbidity		
	df	F	<i>p</i>	df	F	<i>p</i>
<i>Hematocrit</i>						
Treatment	1,77	1.21	0.28	1,78	2.51	0.11
Species	2,77	2.47	0.09	2,78	1.44	0.24
Mass	1,77	0.67	0.41	1,78	0.70	0.40
Treatment x Species	2,77	1.42	0.25	2,78	1.24	0.29
Treatment x Mass	1,77	0.72	0.40	1,78	3.72	0.06
Species x Mass	2,77	1.49	0.23	2,78	0.42	0.65
<i>Na^+/K^+ ATPase Activity</i>						
Treatment	1,69	3.34	0.07	1,84	2.81	0.10
Species	2,69	1.22	0.30	2,84	0.74	0.48
Mass	1,69	3.87	0.05	1,84	0.02	0.89
Treatment x Species	2,69	1.28	0.28	2,84	1.01	0.37
Treatment x Mass	1,69	3.97	0.05	1,84	1.42	0.24
Species x Mass	2,69	0.71	0.49	2,84	0.32	0.73

Table C.3: Two-way ANOVA results for testing of significant differences among filament length, interlamellar space, and ionocyte area for temperature and turbidity analyses.

	Temperature			Turbidity		
	df	F	<i>p</i>	df	F	<i>p</i>
<i>Filament Length</i>						
Treatment	1,79	0.07	0.79	1,82	0.03	0.85
Species	2,79	1.52	0.22	2,82	2.85	0.06
Treatment x Species	2,79	0.19	0.82	2,82	0.42	0.66
<i>Interlamellae Space</i>						
Treatment	1,79	2.44	0.12	1,82	1.17	0.28
Species	2,79	0.98	0.38	2,82	2.53	0.08
Treatment x Species	2,79	0.31	0.73	2,82	0.42	0.66
<i>Ionocyte Area</i>						
Treatment	1,70	0.76	0.39	1,72	0.00	0.99
Species	2,70	2.51	0.09	2,72	1.42	0.25
Treatment x Species	2,70	1.04	0.36	2,72	0.06	0.94

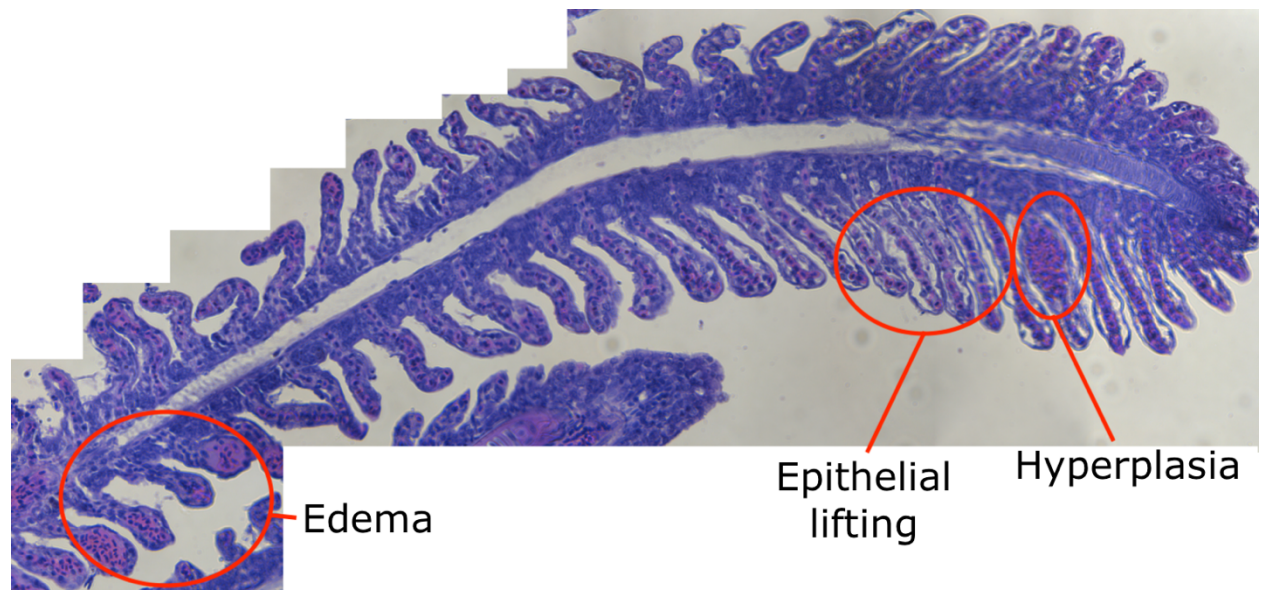


Figure C.1: Examples of the observed gill pathologies

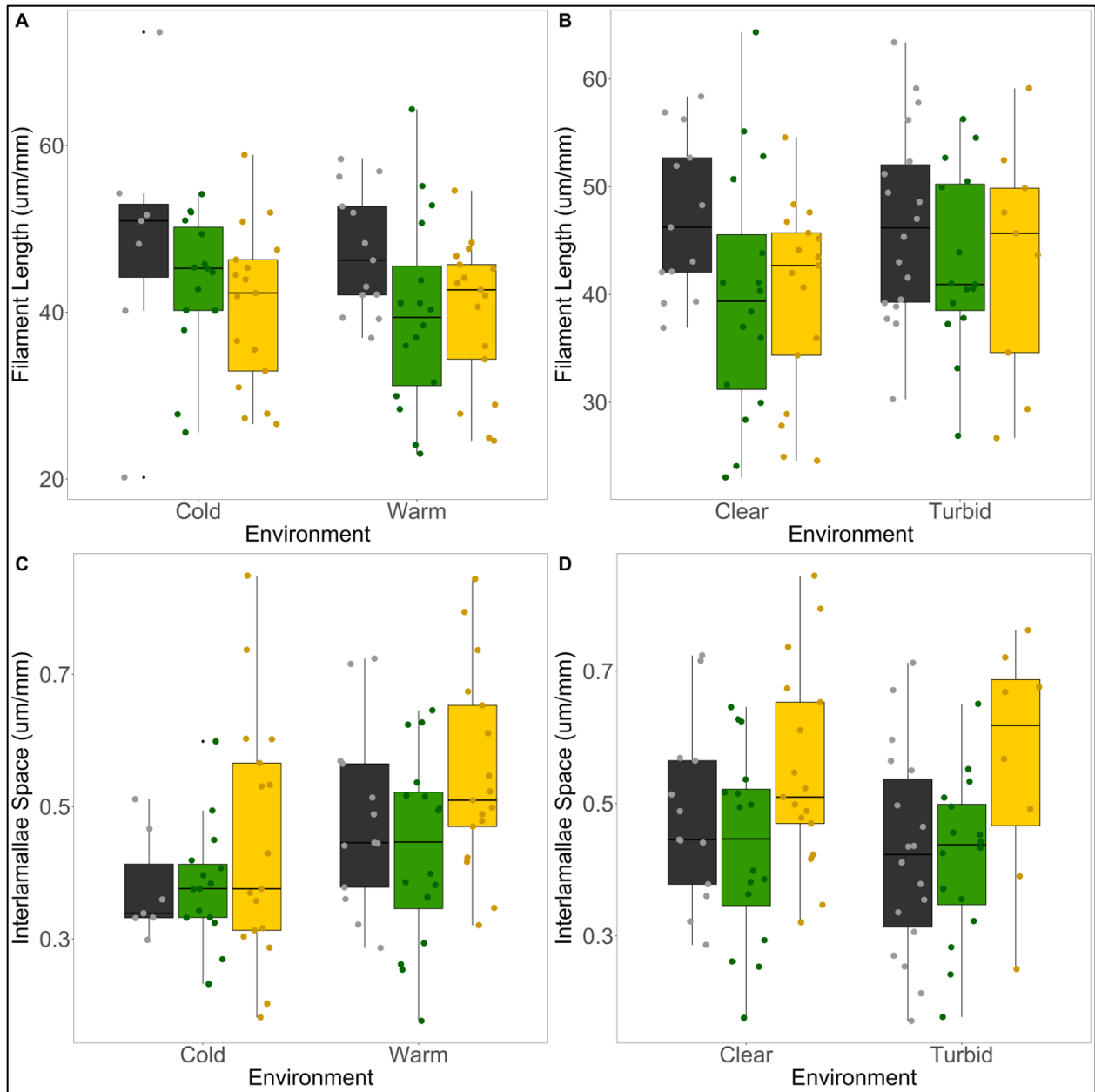


Figure C.2: Effects of temperature (A, C; Cold, Grand October, and Warm, Grand August) and turbidity (B, D; Clear, Grand August, and Turbid, Thames River) on blackside darter (Black), greenside darter (Green), and johnny darter (Yellow) filament length (um/mm; A, B) and interlamellae space (um/mm; C, D).